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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Origin and Progress of Nuclear Transfer in Nonmammalian Animals

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Summary

This chapter traces the orgin and progress of nuclear transfer that later became the paradigm for cloning animals. Classic studies in cytology, embryology, or genetics spanning more than five centuries that led to nuclear transfers in unicellular animals and to those in oocytes of insects, fish and amphibians are reviewed. The impetus for the development of successful nuclear transfers in amphibian oocytes in 1952 was to determine whether or not differentiated somatic cell nuclei are developmentally equivalent to zygote nuclei. Experiments in amphibians demonstrated several important results: (1) specialized somatic cell nuclei are extensively multipotent; (2) fertile adult amphibians can be cloned from embryonic and larval nuclei; (3) serial cloning expands the number of clones; (4) transplanting nuclei into oocyte cytoplasm induces reprogramming of their gene function; and (5) amphibian cloning became the model for cloning mammals. Subsequent studies in mice, a more technically favorable species, revealed that specialized cell nuclei are equivalent to zygote nuclei.

Key Words: Nuclear transfer; cloning; origin and history; unicellular animals; amphibians; insects; fish.

1. Introduction

It is most appropriate that this volume is published now. Just more than 50 yr ago, the first successful cloning of a metazoan animal via nuclear transfer was reported by Robert Briggs (1911–1983) and Thomas J. King (1921–2000). In their now classic paper, they produced frog tadpoles by transplanting blastula nuclei into enucleated eggs ***(1)***. In addition to the timeliness of this volume, so many scientific advances stemming from nuclear transfer have emanated over the decades that need to be compiled. Beyond mammalian cloning and related issues, this volume deals with two especially important subjects aided by nuclear transfer, namely, cell reprogramming and transgenesis.

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This chapter focuses on early scientific questions in cytology, embryology, and genetics that pointed the way for nuclear transplantation (**Table 1** and **refs.** *1–20*) and nuclear transfers in unicellular organisms, insects, fish, and amphibians.

2. Origin of the Problem

The formation of a newborn has always fascinated scientists and nonscientists alike. As early as the second century BCE, Aristotle observed chick development and concluded their structures emerged from a formless mass *(2)*. However, during the 17th and 18th centuries, many embryologists rejected this view and proposed the preformation theory, which states that the embryo is preformed in either the egg or the spermatozoan. Charles Bonnet (1745) of Switzerland refined this theory into one of emboitment or encasement, that is, embryos are encased within embryos, like boxes within boxes, to provide for all future generations. When only 26 yr old, Bonnet made his famous discovery, parthenogenesis in the insect, aphid, although he used parthenogenesis to support his erroneous theory. He carefully raised a female spindle-tree aphid and observed that she produced 95 offspring without mating, proof for him that the female ovum contained preformed individuals *(2,3)*. Other scientists claimed to have seen a tiny creature, homunculus, curled up in the spermatozoan head and favored sperm as vehicles of preformed individuals.

Some contemporaries rejected the theories of preformation. For example, Caspar Friedrich Wolff (1759) at St. Petersburg Academy of Sciences observed new structures forming progressively during chick development and supported the theory of epigenesis in which the adult gradually emerges from a somewhat-formless egg, as originally proposed by Aristotle *(2–4)*. However, not until the early part of the 19th century did most biologists accept epigenesis. Preformation and epigenesis are antithetical concepts and, in retrospect, arose because early scientists, unaware of the gene, thought that preformation explained the constancy of the species and epigenesis accounted for the external structures emerging during embryogenesis. Today, epigenetics is applied to stable alterations in gene expression not involving changes in gene content.

Although epigenesis became the accepted mechanism for the origin of external structures, the nature of germ cells, the process of fertilization, and the role of somatic cells during embryogenesis developed only gradually during the 19th century in a rudimentary fashion. Actually, the explanations of these processes depended on discoveries made at least as early as 1590, when the first compound microscope was invented by two Dutch manufacturers of spectacles, followed by the first description of "cells" in a piece of cork by Hooke in 1667 *(2,3)*. During the early years of the 19th century, as more and more cytologists held that organisms are composed of cells, the cell theory was formulated.

Table 1
Some Contributions to the Origin and Advances in Animal Cloning

Year	Contribution	Discovered by	Ref.
300s BCE	Structures emerge from a formless mass in chick embryos	Aristotle	*2*
1590	First compound microscope	Dutch makers of spectacles	*2*
1600–1700s	Preformation theories popular		*2,3*
1667	Concept of a "cell" in cork	Hooke	*2,3*
1745	Ovist preformation theory	Bonnet	*2,3*
1759	Epigenesis	Wolff and others	*2–4*
1800s	Preformation replaced by Epigenesis		*2–4*
1831	Nucleus identified	Brown	*2,3*
1839	Organism composed of cells	Schwann and others	*2,3*
1855	Embryos develop from cell divisions	Remak	*2,3*
1855	*Omnis cellula e cellula*	Virchow	*2–4*
1865	Sperm considered cells	Schweigger-Seidel and St. George	*2–4*
1876	Fertilization results from union of egg and spermatozoan; germ cell nuclei transmit heredity	Hertwig	*2–4*
1883	Egg and spermatozoa contribute the same number of chromosomes	Van Benenden	*2,3*
1880s	Chromosomes transmit heredity	Hertwig; Kolloker; Strasburger; Weismann	*2–4*
1883	Cell type determined by genetic losses—theory	Roux	*2,4,5*
1892	Cell type determined by genetic losses—theory more detailed	Weismann	*2–5*
1892	Blastomeres from two-cell stage of sea urchin each develop normally	Driesch	*2,4,5*
1894	Cleavage nuclei of sea urchin are equipotential	Loeb	*2,4*
1895	Blastomeres of two-cell stage of salamander are equipotential	Endres	*2,5*
1900	Rediscovery and understanding of Mendel's laws of heredity		*2,3*
1902	Micromanipulator and micropipets made	Barber	*6*

(continued)

1904	Blastomeres of two-cell stage of frog are equipotential	Brachet, A.	***5***
1914	Nuclei of 8–16 cell stage from salamander are equipotential	Spemann	***2,5,7***
1932	Cleavage nuclei of insect are equipotential	Seidel	***2,5***
1934	Recombination of nucleus and cytoplasm in *Acetabularia*	Hämmerling	***5***
1939	Nuclear transfer between *Amebae*	Commandon and de Fronbrune	***5***
1942	Blastomeres of two-cell stage in rat are equipotential	Nicholas	***5***
1952	Nuclear transfer of blastula nuclei in *Rana* oocytes produce tadpoles	Briggs and King	***1***
1960s	Fish cloned from blastula nuclei	Tung; Yan	***8***
1961/2	Nuclear totipotency of frog blastula nuclei demonstrated	Gurdon; McKinnell	***9,10***
1966	Totipotency of larval nuclei from intestine cells	Gurdon and Uehlinger	***11***
1973	Insects cloned from blastula and early gastrula nuclei	Illmensee; Zalokar	***12,13***
1986	Feeding tadpoles cloned from erythrocyte nuclei	Di Berardino et al.	***14***
1986	Lambs cloned from eight-stage blastomeres	Willadsen	***15***
1997	Dolly (fertile sheep) cloned from an adult cell	Wilmut et al	***16***
1997	Transgenic cloned sheep produce human clotting factor IX in milk; gene randomly inserted into donor cells	Schnieke et al.	***17***
1998	Fertile mice cloned from cumulus cells	Wakayama et al.	***18***
2000	Transgenic cloned sheep produce human α-1-antitrypsin in milk; gene inserted via gene targeting in donor cells	McCreath et al.	***19***
2002	Fertile mice cloned from fully differentiated lymphocytes	Hoechedlinger and Jaesisch	***20***

Those researchers generally associated with this theory were Dutrochet, Schleiden, and Schwann who, in the latter case, published his treatise on the microscopic structure of organisms in 1839, when he was only 29 yr old ***(2,3)***. Schwann considered a structure to be a cell if it contained a nucleus that was first described by Robert Brown in 1831, but Schwann did not recognize its importance in heredity ***(2,3)***. Gradually more and more cytologists, such as Remak and Naegeli, concluded that cells reproduce by cell division. In fact, in 1855 Remak published a treatise on embryology that described the formation of the embryo from cells produced by divisions of the ovum and, in the same year, Virchow made his famous proclamation *omnis cellula e cellula* (all cells from cells ***[2,3]***). From this time onward, the cell acquired critical significance, for it provided an explanation for the continuity of life. This idea was further supported by Schwann's belief that the ovum found in the ovary was a cell. Although spermatozoa were considered important in fertilization as early as 1824, it was not until 1865 when Schweigger-Seidel and St. George recognized them as cells. Finally, critical studies of Oscar Hertwig in 1876 established that fertilization results from the union of the egg and spermatozoa. Furthermore, he concluded that germ-cell nuclei, and not the cytoplasm, transmit heredity ***(2–4)***. His studies launched a new period of investigation into the role of the nucleus in fertilization and development.

The latter part of the 19th century also was the period when cytologists focused on the chromosomes, the chief structures in the nucleus. For example, in 1883 Van Benenden observed in fertilized eggs of *Ascaris megalocephala* that the egg and spermatozoan each contributed the same number of chromosomes, which subsequently divided equally and were distributed to opposite poles prior to cell division ***(2,3)***. Confirmation of his observations by others in various animal and plant species led Hertwig, Strasburger, Kolliker, and Weismann each to conclude that the chromosomes were the structures transmitting heredity ***(2–4)***. Those cytological observations and others aided scientists in interpreting Gregor Mendel's laws of heredity around 1900 ***(2,3)***.

The stage was now set to determine the role of chromosomes in embryogenesis and cell specialization. In 1883, Wilhelm Roux proposed all hereditary material is present at fertilization in the form of determinants, which are aligned on the chromosomes and then distributed unequally to daughter cells with the final portion directing cell fate ***(2,4,5)***. August Weismann in 1892 expanded Roux's theory, proposing the primary heredity units to be biophores that merged to form determinants; the determinants then combined to produce ids that united to form idants, considered equal to chromosomes. He then proposed a series of programmed genetic losses during embryogenesis: the id gradually fragmented into smaller units of determinants, becoming isolated in different daughter cells until only one type of determinant remained in a specific group of cells; finally, the determinant split into biophores, the particles instructing

each cell to acquire specific characteristics. The second part of his theory stated that the germ plasm, isolated early in development, is transmitted into the next generation without hereditary losses *(2–5)*. Thus, both Roux and Weismann predicted genetic deletions controlled somatic cell specialization. Although the Roux-Weismann hypothesis was refuted in classical and modern studies in many animal species, certain species of protozoans, invertebrates, and vertebrates do suffer genetic losses during certain stages of cell differentiation, but these cases are by far exceptions (*see* Chapter 12 in **ref.** *5*). Their contribution was important as it stimulated others to test their theory, thus paving the way for experimental embryology. What followed were experiments to determine whether or not genetic deletions control somatic cell specialization.

Roux performed in 1888 the first test that is one of the most-cited experiments with an erroneous conclusion *(5)*. He killed one of the blastomeres of two-cell embryos of *Rana esculenta* (the European edible frog) by inserting a hot needle into the cell. The normal blastomeres remained attached to the injured one and developed into right- or left half-embryos, a result that Roux claimed supported his theory.

Hans Driesch in 1892 used a more direct approach by isolating blastomeres and observing their developmental potential. He separated two-cell embryos of sea urchins by vigorously pipetting or exposing them to calcium-free seawater. In some cases each of the two-cell blastomeres developed into undersized-but-complete larva *(2,4,5)*. Later studies demonstrated the equipotentiality of two-cell blastomeres from urodelen (Endres, 1895; Herlitzka, 1897; Spemann, 1901; Ruud, 1925) and anuran (Brachet, 1904; McClendon, 1910; Schmidt, 1933) amphibian embryos *(2–5)*. Here, too, the blastomeres were separated, usually by constricting the embryo along the cleavage plane with a hair loop. When each of the isolated blastomeres contained sufficient gray crescent material, two complete-but-diminutive larvae developed. Other investigators showed that blastomeres of the two-cell stage in hydroids (Zoja, 1895), the nemertean *Cerebratulus* (Wilson, 1893), the chordate amphioxus (Wilson, 1893), and vertebrate fishes (Morgan, 1895) formed normal-but-tiny larvae *(3)*. Many more examples of regulative development were summarized by Wilson *(3)* and include some nonmammalian species with complete but diminutive development from blastomeres of four- and eight-cell embryos. Considering the sophisticated equipment available today, it is remarkable how scientists more than a century ago used simple tools and obtained important answers.

It should be noted, although beyond the focus of this article, other tests of the Roux-Weismann theory revealed that in some invertebrate species the early blastomeres were not regulative and equipotential but rather mosaic and restricted *(3–5)*. Those animal groups involving ctenophores, rotifers, annelids, nematodes, ascidians, and others have eggs containing cytoplasmic localizations that

are distributed after fertilization unequally to the blastomeres. The substances contain various maternal RNAs and proteins that confer on the embryo its mosaic character because they act as cell type and morphogenetic determinants. Blastomeres containing such substances are restricted to a specific path of cell differentiation and, when isolated, cannot self-regulate and form only part of an embryo.

The distinction between regulative and mosaic embryos was recognized by investigators as they continued their quest of the developmental potential of early-stage blastomeres from regulative embryos. However, they soon realized that blastomere isolation in nonmammalian species had its limitations, even in regulative embryos, when performed on later cleavage stages, because the volume of cytoplasm in the blastomeres was so reduced that negative results would be equivocal and uninformative. However, in mammals, the analysis of blastomere potential from later cleavage stages has been quite informative, including not only the technique of blastomere isolation but also the procedures of embryo fusion, injection of embryonic cells into recipient blastocysts, and splitting of embryos. Initially, the fate of isolated mammalian blastomeres was determined in small species. Individual two-cell blastomeres of rat (Chapter 1 in **ref.** ***5***), mouse ***(21)***, and rabbit ***(22)*** developed into live offspring. More recently, isolated blastomeres from two-cell embryos of larger species resulted in monozygotic twins in sheep, cattle, goats, pigs, and horses ***(23)***. Even some individual blastomeres from eight-cell embryos of the rabbit ***(24)*** and sheep ***(23)*** have developed into normal offspring, but this stage appears to be the upper limit for obtaining normal development from isolated blastomeres as the resulting blastocysts contained fewer cells than normal.

To avoid the problem of reduced blastomere size at the eight-cell stage and beyond, investigators fused mouse embryos. After removal of the zona pellucida, they fused pairs of cleaving embryos. Although cells in their original location underwent considerable displacement, the reorganized giant embryos after transfer to surrogate mothers developed into normal chimeric offspring ***(25–27)***. Notably, Markert and Petters ***(28)*** fused three different genetically marked eight-cell embryos and produced hexaparental mice in which the genotypes from six parents were expressed in the pigment patterns of the adults. Chimeric lambs stemming from eight-cell embryos were also produced by the fusion technique ***(23)***.

Microinjection of genetically marked donor cells from the inner cell mass of blastocysts into recipient blastocysts revealed those cells to be totipotent. In an initial study, the injected cells proliferated and contributed to the skin, hair, and retinal pigmentation of the chimeric mice ***(27)***, demonstrating their pluripotency and, in a later study, formed functional germ cells ***(29)*** showing their totipotency. Finally, the fate of bisected embryos also revealed the regulative

ability of mammalian embryos. Embryos of bovines, sheep, swine, horses, rabbits, and mice have been bisected at the morula or blastocysts stages to produce twin offspring (e.g., **refs.** *30* and *31*).

3. Role of the Nucleus

The successful tests of whole cells described here indicated that both the cytoplasm and the nucleus maintained developmental potential and, in the case of mammals, at least into the blastocyst stage. But long before these studies, some investigators focused on nuclear potential because the chromosomes in the nucleus were considered to be the carriers of heredity. Jacques Loeb in 1894 performed the first primitive cloning experiment. He placed sea urchin zygotes in hypotonic saline solution that produced ruptures in their vitelline membranes through which a portion of the zygote herniated. Initially, the herniated part lacked a nucleus, but later a daughter nucleus sometimes moved into the herniated part. If this occurred, two whole and separate embryos or a double embryo developed, one from the nonherniated part and one from the herniated portion (Chapter 3 in **ref.** *5*). In 1894, Delage proposed that the "egg nucleus could be replaced by the nucleus of an ordinary embryonic cell" *(31a)*.

Nineteen years later, Hans Spemann *(7)* in 1914 extended Loeb's experiment to newt eggs. Before the first division, he constricted newt zygotes with ligatures made from flexible baby hair. The constriction produced a dumbbell-shaped egg with its two segments connected by a narrow bridge. Although he could not see through the opaque egg, he assumed one portion only would contain the zygote nucleus. Retrospectively, he concluded that the nucleated part cleaved whereas the non-nucleated part did not. When the cleaved part had formed 8–16 cells, he loosened the ligature expecting a nucleus to pass through the bridge into the previously non-enucleated portion. Then, applying a strong constriction, he severed the two halves. In some cases, each half developed into a larva, indicating that some nuclei from the 8- to 16-cell stages could direct larval development. Later, in 1930, Fankhauser duplicated Spemann's experiment and demonstrated in histological sections that Spemann's interpretation of the internal events was correct *(32)*. Similar constriction experiments performed on dragonfly embryos indicated their nuclei from early cleavage stages remain identical in developmental potential Seidel *(2,5)*.

Spemann, a 1933 Nobel laureate, later discussed the question of nuclear potential in his book *Embryonic Development and Induction*, which was complied from his Silliman Lectures delivered at Yale University (pp. 210–211 *[7]*). Although he could not technically propose how to test nuclei from older embryonic stages, he did speculate on "an experiment which appears, at first sight, to be somewhat fantastical," the isolation of a nucleus from a morula and the insertion of such a nucleus into an egg or an egg fragment without an egg nucleus, but he "saw no way

at the moment." He then stated, "This experiment might possibly show that even nuclei of differentiated cells can initiate normal development in the egg protoplasm." Fourteen years would elapse before the first metazoan nucleus from late cleavage stages was successfully transplanted into an enucleated egg ***(1)***, but first let us reflect briefly on two events that paved the way for nuclear transplantation in frogs: micromanipulation and nuclear transfer in unicellular forms.

As early as 1902, Marshall Barber made micropipets from capillary tubing and constructed a micromanipulator to isolate yeast and bacterial cells ***(6)***. Others, including Robert Chambers, contributed revisions, and it was Chambers who at New York University instructed Tom King, the first successful frog cloner, how to construct pipets and inject biological material.

The first successful recombination of nucleus and cytoplasm, although not a direct nuclear transfer, was performed by Hämmerling in the giant unicellular green alga plant, *Acetabularia* (Chapter 2 in **ref.** ***5***), whereas the first direct nuclear transfers were performed in the unicellular animal, *Ameba*. In 1911, Barber succeeded in removing their nuclei with a micropipet ***(6)***, and in 1939 Comandon and de Fronbrune (Chapter 2 in **ref.** ***5***) successfully transplanted nuclei between amebae. Later, other investigators transferred nuclei in the unicellular animals *Stentor* ***(33,34)*** and *Paramecium* ***(35)***, and even in the plant *Neurospora* ***(36)***. These and subsequent nuclear transfers were performed to analyze nuclear–cytoplasmic interactions and organelle development. Their results, particularly in *Acetabularia, Ameba*, and *Stentor*, predicted several important cellular principles, for example, information in DNA is transferred via RNA to the cytoplasm, where proteins are synthesized, and many protein species are translocated to the nucleus, where they function in the cell cycle, mitosis, and control of gene expression (Chapter 2 in **ref.** ***5***).

Rauber in 1886 probably was the first person to attempt nuclear transfer in a metazoan form ***(5)***. He wanted to determine whether a frog egg would develop into a toad and vice versa when he exchanged their nuclei 1 h after fertilization. He stated, "To my surprise, however, the eggs developed no further." Recall, during this period, investigators were using different approaches to determine whether the nucleus alone contained the hereditary functions. Thus, the concept of nuclear transfer in eggs existed more than a century ago.

4. The "Fantastical" Experiment Accomplished in *R. pipiens*

During the early 1940s Robert Briggs, then head of Embryology at the Institute for Cancer Research (now part of Fox Chase Cancer Center in Philadelphia), was discussing with Jack Schultz, a colleague and geneticist, "ways of testing for functional equivalence (or lack of it) in nuclei of somatic [not germ] cells" ***(37)***. Schultz suggested the nuclear transplantation approach. Briggs was aware that "actual attempts to transplant nuclei (into amphibian

eggs) had been made by Rostand and Lopashov." Rostand *(38)* had pricked frog eggs with embryonic brei and assumed that he would transfer nuclei, whereas Lopashov *(39)* had transplanted blastula nuclei into non-nucleated fragments of *Triton* eggs, but both were unsuccessful.

By 1949, shortly after I joined Bob Briggs, he was formulating definite plans to develop nuclear transplantation in the northern leopard frog *R. pipiens* and had begun searching for a research fellow to join him. Roberts Rugh, a prominent embryologist at Columbia University, suggested Thomas J. King, a doctoral student at New York University. King had training in microsurgery from Michael J. Kopac, a well-known microsurgeon and cell physiologist, and Robert Chambers, one of the pioneers in microsurgical techniques. To support the research, Briggs applied to The National Institutes of Health, where his application was assigned to the National Cancer Institute (NCI). The review group immediately rejected the proposal on the basis that it was a "hare-brained scheme" with little chance of success. A resubmission was successful, but only after a site visit and the intellectual support of a NCI administrator. Finally, the project was funded and King began his collaboration with Briggs in 1950.

Before testing the developmental potential of embryonic nuclei, the pioneers analyzed the development of activated eggs lacking a functional nucleus so that future nuclear transplant experiments could be properly interpreted. *R. pipiens* eggs were inseminated with previously irradiated *R. pipiens* sperm that inactivated the chromatin of the spermatozoan but left the centriole competent to provide a cleavage center for the egg. Approximately 10–15 min later (18°C), as the eggs were rotating, a pit resembling a black dot under reflected light appeared in the animal pole. The pit was situated directing over the internal egg nucleus that was arrested at the second meiotic metaphase. A glass microneedle was inserted adjacent to and directly under the pit and then pulled upward through the cortex resulting in an exovate containing the egg's spindle, chromosomes, and some cytoplasm; such eggs, fertilized by inactivated sperm, cleaved and developed at best into partially cleaved blastulae *(40)*. This information established that tests of nuclear potential via their transplantation into enucleated amphibian eggs would be meaningful if the nuclear transplants developed beyond the blastula stage.

The pioneers initially tested the developmental potential of nuclei from blastula cells of the animal hemisphere, an undetermined region. The removal of the egg nucleus was already an established procedure *(41)*, but how to transplant a *living* nucleus into an egg without harming it was unknown. After many trials and errors, King succeeded, and the team began their experiments with Briggs as the enucleator and King as the injector.

Their procedure was the following (**Fig. 1** and **refs.** *109* and *110*). Mature oocytes arrested at metaphase II were activated by pricking with a glass microneedle. During an interval of approx 15–30 min after activation, the eggs

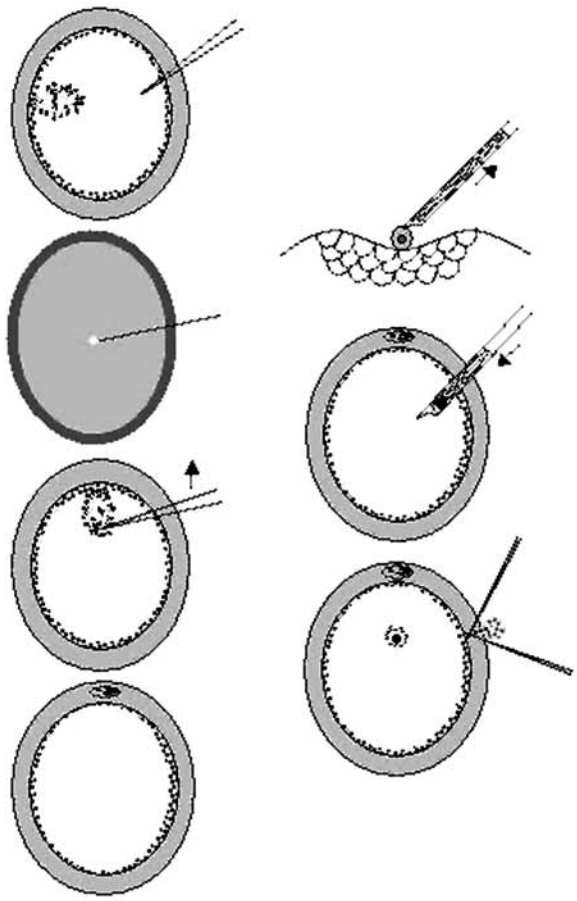

Fig. 1. Nuclear transplantation in *Rana pipiens*. **Left,** activation of a metaphase II oocyte by pricking with a glass needle. Within 10 min, the oocyte rotates and a pit situated over the interior spindle and chromosomes appears on the surface of the animal hemisphere. The spindle, chromosomes, and some cytoplasm are removed with a glass needle resulting in an exovate located outside the oocyte. **Right,** a donor cell, aspirated into a glass micropipet, is broken gently and transplanted into the animal hemisphere of a previously enucleated egg. After removal of the pipet, a canal forms that is severed from the oocyte with a pair of glass needles to prevent further leakage. Figure prepared by Barbara Engle after King ***(109)*** and Di Berardino ***(110)***.

were enucleated, and their excess jelly was removed with scissors to prevent clogging the glass micropipet. Next, a single blastula cell was aspirated into a micropipet, the inner diameter of which was slightly less than that of the donor cell, causing a slight deformation and rupture of the cell. Cell breakage was necessary, permitting the nucleus to interact directly with the host's cytoplasm,

but had to be performed carefully to prevent harming the donor nucleus. Next, the micropipet was inserted in the animal hemisphere of the enucleated egg and moved approximately one-third of the way down where the broken cell and its components were injected into the host via positive pressure with a syringe. During removal of the micropipet, a small canal formed that was severed with a pair of glass needles to prevent leakage. On the following day, the majority of injected eggs cleaved into blastulae, and within the next 4–11 d, the majority of completely cleaved blastulae developed into normal postneurulae or feeding tadpoles, respectively ***(1)***.

Thus, their research was not a "harebrained scheme" but was, in the words of Spemann, "fantastical," demonstrating that nuclei from embryos containing 8000–16,000 cells had the potential to direct enucleated eggs to develop into feeding tadpoles. What is now a notable understatement, Briggs and King concluded, "Although the method of nuclear transplantation should be valuable principally for the study of nuclear differentiation, it may have other uses." Some of the uses included the study of haploidy ***(42)***, hybrid incompatibility ***(43–45)***, cancer ***(46)***, cellular aging ***(47,48)***, nuclear reprogramming ***(5,49)***, and genomic imprinting ***(50,51)***. But, notably, *Rana* nuclear transplantation became the prototype for cloning metazoan animals, including those from insects, fish, amphibians and mammals (**Fig. 2**). Although the diverse applications of somatic nuclear transfer to the agricultural, biomedical, and basic sciences were not immediately obvious, its significance certainly was appreciated by many and in 1972, stemming from the initial recommendation of Louis Gallien (University of Paris), Briggs and King were awarded the Charles Leopold Mayer Prize of the Académie de Sciences, Institut de France for their pioneering studies and were the first Americans to be so honored.

Soon after the initial success in *R. pipiens*, the nuclear transfer procedure was extended to several anuran species (*Rana nigromaculata brevipoda* ***[52]***, *Rana sylvatica* ***[53]***, *Xenopus laevis* ***[54]*** and, in later years, to many others [summarized in **ref.** ***5***, p. 52]). Soon metamorphic and adult frogs bearing genetic markers were obtained in *R. nigromaculata brevipoda* ***(52)*** and *Xenopus* ***(55)***, respectively. The main difference in *Xenopus* compared with other anurans is that the egg nucleus is destroyed by ultraviolet radiation that also activates the egg.

The proper conditions for enucleation, egg activation, and the timing of nuclear transfer for urodelen eggs were first solved in *Ambystoma mexicanum*, the Mexican axolotl ***(56)*** and then similar conditions sufficed for the eggs of *Pleurodeles waltlii* ***(57)***. Eggs of axolotl were activated by heat shock and enucleated by ultraviolet irradiation; then, blastula nuclei were transferred singly 2–4 h after heat treatment. Later, electric shock was reported to be better than heat shock ***(58)***. Because valuable mutants were available in axolotl, they used one for a genetic marker. Donor blastula nuclei carrying the allele for black

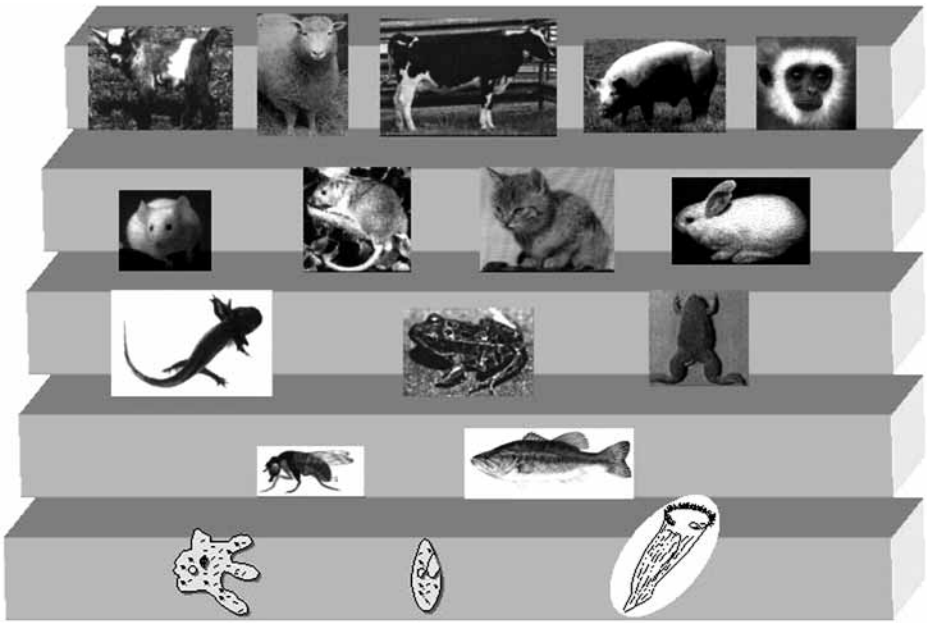

Fig. 2. Animal forms that have been cloned via nuclear transfer. Left to right: **Stair 1**, Unicellular *Ameba, Paramecium*, and *Stentor*; **Stair 2**, insect (*Drosophila*) and fish (carp, medaka); **Stair 3**, amphibian urodeles (*Ambystoma mexicanum* and *Pleurodeles waltlii*) and anurans (*Rana pipiens* and *Xenopus laevis*, etc.*)*; **Stair 4,** mouse, rat, cat, rabbit; **Stair 5**, goat, sheep, cattle, pig, and monkey. Animals cloned from **adult** cells are mouse, cat, rabbit, sheep, cattle, and pig. After this review was completed, a mule was cloned from a fetal cell ***(111)***, and a horse was cloned from an adult cell ***(112).*** Figure prepared by Barbara Engle.

pigment were transplanted singly into enucleated eggs from a recessive white female, and all successful nuclear transplants were black.

Nuclear totipotency is demonstrated in apparently normal adult clones when they produce normal offspring. Such results signify the original test nucleus had the gene pool to specify all normal somatic cell types, as well as normal germ cells, that is, it is equivalent to a zygote nucleus. Totipotency was shown for nuclei from undetermined regions of embryos in diverse amphibian species: initial examples expressing genetic markers of the test nuclei were *Xenopus laevis victorianus* ***(9)***, *R. pipiens* ***(10)***, *Rana japonica* ***(59)***, *A. mexicanum* ***(60)***, and *P. waltlii* ***(61)***.

5. Nuclear Potential of Amphibian Cells From Advanced Embryonic Stages

Successful nuclear transfer, even with nuclei from undetermined regions of embryos, was not consistent. Several technical problems were obvious: improper

placement of nuclei in the oocytes could result in partially cleaved blastulae; nuclei could be harmed in the pipet; withdrawal of the pipet could result in loss of the injected nucleus; injected nuclei could be asynchronous with the cell cycle of the recipient egg,. These factors could not be controlled then and some still exist in mammalian studies, rendering nuclear transfer in part a roulette game. To circumvent the problems, the results from frog nuclei of undetermined regions were used as a basis for comparison with those from more advanced embryonic stages.

A multitude of nuclear transfers were made with embryonic nuclei from determined regions of the gastrula up to the hatching larval stage containing primitive tissues and organ systems with cells in various differentiating states (Tables 4.2–4.4 in **ref.** *5*). Extensive analysis initially in *R. pipiens* revealed a decrease in the percentage of nuclei from ectodermal, mesodermal, and endodermal cell lineages that were capable of promoting normal development of the egg compared with nuclei from undetermined regions *(62)*. In the main, the decrease was progressive as nuclei were taken from progressively older embryonic stages. Other investigators using different anuran species as well as urodelen ones reported similar results (Chapter 4 in **ref.** *5*). Nevertheless, despite the significant decrease, a few nuclei retained extensive pluripotency and, in fewer cases, totipotency. For example, a neurula nucleus from a *Xenopus* presumptive brain cell supported adult formation *(63)*, 4 neurula nuclei from mesoderm of *Xenopus* promoted metamorphosed frogs *(55)*, and 14 tailbud nuclei from endoderm of *Pleurodeles* directed the formation of adults, one of which was fertile *(61)*. Whether the few successful donor nuclei from advanced embryonic stages originated from stem or differentiated cells was unresolved. This dilemma led to a series of nuclear transfers from larval and adult donor cells displaying some characteristic of the differentiated state (next section).

6. Nuclear Potential of Amphibian Cells From Larvae and Adults

Only a few larval nuclei directed the formation of fertile adults: in *Pleurodeles,* one from a gut cell of a prehatching larva *(61)*; in *Xenopus*-hatching larvae, 1 from each of two epidermal cells *(63,64)*, and 20 from gut cells *(65)*; and in *Xenopus* swimming larvae two from intestinal cells *(11)*. A recent study using intestinal donor nuclei equivalent to those in the latter study reported one apparently normal tadpole *(66)*. A few *Xenopus* clones developed as far as metamorphosis: one from larval intestine *(67)* and three from cultures of minced tadpoles *(68)*. However, it is unknown whether the adult and metamorphosed clones resulted from differentiated or stem cells.

The most informative tests used donor cells displaying some type of differentiation. Identification through the stereomicroscope provided direct proof. When visual identification of the donor cells was not possible, the percentage of undifferentiated cells in the population or its equivalent had to be so small

Table 2
Pre- and Posthatching Tadpoles Cloned From Differentiated Somatic Frog Cells

Donor	Oocyte host	Prehatching (no.)	Posthatching (no.)	%	Reference (no.)
Melanophore	Metaphase II	2	–	0.8	***64***
Skin	Metaphase II	2	4	4.7	***69***
Lymphocyte	Metaphase II	–	6	6.0	***70***
Erythroblast	Metaphase II	8	–	1.8	***71***
Erythrocyte	Diplotene	4	3	4.7	***72***
Erythrocyte	Metaphase I	5	6	8.5[a]	***73***
Erythrocyte	Metaphase I	–	4	7.8[a,b]	***14***
Erythrocyte	Metaphase I	3	3	4.8[a]	***72***
Erythroblast	Metaphase I	1	1	1.9[a]	***72***
Leukocyte	Metaphase I	3	3	10.0[a]	***72***

[a]Some tadpoles advanced beyond the feeding stage.
[b]Some survived for 1 mo.

that the percentage of transplants developing had to significantly exceed the percentage of undifferentiated cells. Those cases, including one of the criteria, are listed in **Table 2** ***(14,64,69–73)*** and described briefly here.

The donor cell types directly identified under the stereomicroscope at the time of nuclear transfer were melanophores, erythrocytes, erythroblasts, and leukocytes. The unique properties of melanophores and erythroid cells, melanin pigment, and hemoglobin, respectively, permitted direct identification. The leukocytes, separated from erythroid cells via centrifugation of whole blood, were taken from the buffy coat layer and verified microscopically to lack hemoglobin at the time of nuclear transfer. In two other studies using skin cells and lymphocytes, the donor cells were derived from a nearly homogeneous population. Correlative studies on skin cultures revealed that more than 99% of the cells were producing immunoreactive keratin and correlative studies on lymphocytes showed that 96.1–98.7% of the cells were producing immunoglobulins.

Nuclei from six differentiated somatic cell types directed the formation of prehatching tadpoles and nuclei from five specialized cell types programmed for the development of posthatching tadpoles. Tadpole development was most extensive when *Rana* erythrocyte, erythroblast, and leukocyte nuclei were injected into metaphase I oocytes than when nuclei were injected into diplotene or metaphase II oocytes. The greatest percentage and most advanced tadpoles emerged from *Rana* erythrocyte nuclei of juvenile frogs that were tested in oocytes at first meiotic metaphase ***(14)***. In that study, 7.8% attained the feeding

stage or beyond. Three original nuclei after recloning gave rise to 19 clonal tadpoles. All 19 tadpoles fed, differentiated hind limb buds, and in the best cases, survived as independent organisms up to 1 mo. Metaphase I oocytes were used for two reasons: to induce breakdown of the nuclear membrane so that essential cytoplasmic molecules could access the chromatin and to provide more time for nuclear reprogramming of the G_0 erythrocyte nucleus. After injection of the nucleus, the nuclear envelope disappeared and the interphase nucleus converted into metaphase chromosomes aligned on a newly induced spindle. Approximately 24 h later, the oocyte matured, was activated, and the egg nucleus was removed. However, when erythrocyte nuclei were tested in metaphase II oocytes, they were forced into the first mitotic metaphase about three hours after injection, had little time to under reprogramming, and developed no further than the early gastrula stage ***(73)***. Recently, nuclear envelope permeabilization was shown to optimize chromatin remodeling in one-cell stage mammalian clones ***(74)***. Although the use of *Rana* metaphase I hosts led to enhanced expression of developmental potential in amphibians, at least for erythroid and leukocytic nuclei, conditions were not found in amphibian cloning to reveal totipotency in adult cells. We had to wait for progress in mammalian cloning where "the long cell cycles of cleavage stages in the mammalian embryo may permit more extensive molecular reprogramming of adult nuclei and better integration into the host's cell cycle than that achieved with amphibian nuclear transplants, in which the cell cycles of the host are very rapid" (p. 213 ***[5]***).

7. Clarifications From Mouse Cloning

Recent murine studies clarified two unresolved questions in frog cloning: the totipotency of fully differentiated somatic cells and the phenotypic restrictions of some transplants from endodermal and neural nuclei.

Throughout the decades of frog cloning, there was only one claim for the totipotency of nuclei from fully differentiated somatic cells, namely from intestinal cells of *Xenopus* larvae ***(11)***. In that study, 4 (0.6%) adults, two of whom were fertile, developed from four original nuclei. Recently, because of the regular presence of less differentiated stem cells intermingled within differentiated tissues, Hochedlinger and Jaenisch ***(20,75)*** tested the nuclear potential of murine B- and T-lymphocytes under conditions that would yield unequivocal results. Mature lymphocyte nuclei carrying fully rearranged immunoglobulin or T-cell receptor alleles from cell lines derived from the peripheral lymph node were transferred into enucleated oocytes and embryonic stem (ES) cell lines were derived from the subsequent blastocysts. ES cells were then injected into tetraploid blastocysts, a procedure in which the embryo develops from the ES cells and the extraembryonic tissues from the tetraploid host ***(76)***. Sixteen fertile adults cloned from B-cell nuclei carried the fully rearranged immunoglobulin alleles in all tissues, and a

nonviable neonate cloned from a T-cell nucleus carried the rearranged T-cell receptor genes in all tissues. The production of monoclonal mice with the genetic markers of the donor nuclei demonstrated unequivocally that terminally differentiated somatic cells could be reprogrammed to produce clones. The authors critically point out that the two-step procedure does not show that nuclei of B- and T-cell lymphocytes can be directly reprogrammed to direct the formation of extraembryonic cell lineages, but in the past, ES cell nuclei have been shown to specify the development of both embryonic and extra-embryonic tissues ***(77)***.

Five decades ago, a characteristic syndrome of morphological and cellular abnormalities was reported in some *Rana* nuclear transplants derived from endodermal nuclei of late gastrulae and neurulae ***(62)***. During postneurula and early tadpole stages, some clones displayed deficiencies and degenerative changes primarily in the ectodermal and mesodermal derivatives, but good differentiation in the endodermal derivatives, a phenotype termed the endoderm syndrome. A second group of abnormal clones displayed generalized deficiencies, whereas the third group was normal. Both the endoderm syndrome and the generalized deficiencies were stable as neither was reversed by serial transplantation ***(62,78)***. Furthermore, the restrictions were intrinsic; they could not be reversed by parabiosis of nuclear transplant embryos with normal ones ***(79)***, nor were they corrected when the haploid nucleus of the egg was combined with the diploid endodermal nucleus ***(80)***.

Clarification of the three different phenotypes was provided primarily from chromosomes studies of the nuclear transplants ***(81)***. Those with generalized deficiencies displayed extensive and variable aneuploidy, with severe structural alterations in the chromosomes, whereas the group displaying the endoderm syndrome (40%) was euploid or predominantly euploid. The third group that developed normally showed no chromosomal abnormalities. These studies were confirmed and extended to include chromosomal analysis of clonal donors used in serial nuclear transplantation ***(78)***. Seventy-five percent of the abnormal clones that were euploid or predominantly euploid exhibited the endoderm syndrome, whereas the remaining abnormal ones displayed generalized cellular deficiencies and were extensively and variably aneuploid with structural alterations in the chromosomes.

An analogous study on clones from nuclei of the presumptive medullary plate region of late gastrulae and the definitive medullary plate of early and mid-neurulae from the ectodermal lineage revealed different but complementary restrictions ***(81)***. In addition to normal larvae, there were two groups of abnormal clones during postneurula and larval stage stages: 13% displayed cellular deficiencies mainly in mesodermal and endodermal derivatives but good differentiation in the organs and tissues of ectodermal origin. The chromosomes in half of this group were examined during larval stages and found to be euploid with an apparent normal karyotype. The other group comprising abnormal postneurulae

and larvae (87%) exhibited generalized cellular deficiencies and the chromosomes analyzed from a sample were abnormal in number and structure.

The two distinct but complementary phenotypes suggested that some endodermal and neural nuclei acquire stable properties for specific pathways of differentiation; however, the data were limited because clones with numerical and structural chromosome abnormalities had to be eliminated and, furthermore, submicroscopic aberrations, if present, could not have been detected. Later, analysis of nuclear transplants during the first cell cycle revealed the chromosome abnormalities were traceable to an asynchrony in the cell cycles of the donor nuclei and recipient oocytes *(82,83)*.

Although these phenotypes were described long ago, the genetic mechanism responsible for them remained elusive, although we considered different patterns of gene expression probably accounted for the phenotypes. The restrictions in *Rana* nuclear transplants have now been clarified in murine clones *(75,84)*. Global gene expression of more than 10,000 genes was assessed by microarray analysis of RNA from the placentas and livers of neonatal cloned mice derived from either cultured embryonic stem cells or freshly isolated cumulus cells. Although most abnormally expressed genes were common to both types of clones, a smaller set of abnormally expressed genes differed between the ES and cumulus cell-derived clones, reflecting the particular donor nucleus. Other murine studies have revealed altered phenotypes in clones that are donor specific as early as preimplantation stages *(85)*.

The problem of the endoderm/neural syndromes in frog clones is a good paradigm for young scientists: record what you observe and eventually its significance will be revealed. The lengthy cell cycle (approx 1 d) in mammalian one-cell embryos offers a distinct advantage over amphibian cloning where the first cell cycle is quite short (approx 1 h in *Xenopus* and 3 h in *Rana*), causing an asynchrony between the cell cycle of the donor nucleus and the oocyte that induces chromosome abnormalities. Although chromosome aberrations can occur in mammalian cloning (e.g., **ref.** *86*), they are less frequent than that observed in amphibian clones. However, synchrony of the cell cycle is not sufficient. Another major problem in both mammalian and frog cloning is the incomplete reprogramming of gene expression in donor nuclei. Perhaps this problem may be solved in mammals for the nonimprinted genes by exposure of donor nuclei in vitro to appropriate inducers before nuclear transfer. But how to reset the correct pattern of genetic imprinting that is set over a protracted period from germ cell differentiation through embryogenesis will be a more formidable task.

8. Nuclear Reprogramming

Nuclear reprogramming, a phrase used early in amphibian cloning, denoted the morphological and molecular changes that nuclei undergo after transplantation

into oocyte or egg cytoplasm and, later, included changes in chromatin and gene expression. Probably one of the most significant byproducts of nuclear transfer will be the understanding of the molecular mechanisms controlling the reversible expression of chromatin and genes in transplanted nuclei. In the immediate future, such knowledge should improve cloning efficiency. In the more distant future, it might permit us to convert differentiated adult cells into immature cells and then redirect the latter into specific cell types required for tissue repair. Here, I consider briefly some examples of nuclear reprogramming in transplanted amphibian nuclei, but first I cite two pioneering studies demonstrating the cytoplasmic control of nuclear and chromosomal behavior. More than 80 yr ago, Brachet ***(87)*** observed that, when sperm prematurely entered sea urchin oocytes still undergoing maturation divisions, the sperm chromatin rapidly condensed into chromosomes similar to those of the oocytes; the same phenomenon was later observed in precociously inseminated oocytes of amphibians by Battaillon and Tchou-Su ***(88)***.

Initial observations of amphibian embryonic nuclei transplanted to metaphase II oocytes revealed morphological changes similar to those the egg and sperm nuclei undergo following fertilization: nuclear enlargement, chromatin decondensation, nuclear migration toward the equator, and spindle formation for the first mitosis ***(89,90)***. Numerous studies ensued in which nuclei from various cells types were injected into oocytes or eggs of *Buffo, Pleurodeles, Rana*, and *Xenopus* that firmly established the cytoplasmic control of nuclear, chromosomal, and gene function ***(5,49***).

In the diplotene host, transplanted nuclei enlarged, transcribed RNA, and synthesized new proteins. Liver nuclei of *A. mexicanum* directing the synthesis of alcohol dehydrogenase, a liver-specific enzyme, and lactate dehydrogenase, common to many cell types, were injected into diplotene oocytes of *A. mexicanum*. Starch gel electrophoresis differentiated the enzyme forms between the two species and revealed the oocytes ceased synthesizing alcohol dehydrogenase but continued making lactate dehydrogenase ***(91)***. Likewise, when *Xenopus* nuclei from a kidney cell line were injected into *Pleurodeles* oocytes, two-dimensional gel electrophoresis revealed cessation of *Xenopus* kidney-specific proteins and expression of *Xenopus* oocyte-specific proteins ***(92)***. Also, when nuclei from *Xenopus* erythrocytes and cultured kidney cells were injected into diplotene oocytes, oocyte-specific 5s ribosomal genes were activated ***(93)***.

After germinal vesicle breakdown, transplanted nuclei from various cell types converted into metaphase chromosomes aligned on newly induced spindles in concert with the oocyte's nucleus (p. 97 ***[5]***). When nuclei were injected into diplotene or maturing oocytes and, subsequently, the oocytes were matured and activated, the nuclei transformed into pronuclei ***(94,95)***.

In activated eggs transplanted nuclei were induced to synthesize DNA and enter mitosis *(96)*. During the first cell cycle, a bidirectional exchange of proteins occurred between injected nuclei and the host cytoplasm. Transplanted nuclei accumulated radioactive labeled proteins of the cytoplasm *(97,98)* that were composed of histone and nonhistone proteins *(99)*. On the other hand, when labeled nuclei were injected into activated eggs, histone proteins were primarily retained whereas a significant amount of nonhistone proteins were lost *(100,101)*. Two decades later, the remodeling of chromatin was directly analyzed. After transcriptionally inactive sperm nuclei were incubated in extracts from activated amphibian eggs, sperm-specific histone proteins were replaced by somatic histones H2A and H2B via the molecular chaperon nucleoplasmin *(102)*. Similarly, erythrocyte chromatin was remodeled when their nuclei were incubated in extracts of activated amphibian eggs: somatic histones H1 and H10 were released from chromatin into egg cytoplasm, oocyte-specific linker B4 and high-mobility group 1 were incorporated into remodeled chromatin, and somatic histones H2A and H4 were phosphorylated *(103)*.

The switching of gene expression was examined in *Xenopus* eggs and embryos. For example, nuclei synthesizing 28s and 18s ribosomal RNA or muscle actin RNA ceased to do so after nuclear transfer, but began at the gastrula stage when controls did *(49)*.

Many of the aforementioned studies have been confirmed in mammalian clones and significantly extended, especially deciphering various molecular, biochemical and physiological changes occurring during nuclear reprogramming (*see* **Section 3.**).

9. Nuclear Transfers in Insects, Ascidians, and Fish

In addition to the cloning of various mammalian species, the only other metazoan animal groups to be cloned have been insects and fish. In the case of ascidians, nuclear transplantation was used to study the effect of cytoplasm on nuclear function but mature clones were not produced.

Cloning studies in *Drosophila* were launched during the 1960s. Because enucleation of the fragile host eggs was not feasible, investigators injected several genetically marked nuclei into the posterior region of each unfertilized *(12)* or fertilized *(13)* egg at the site where pole cells, the progenitors of the germ cells, would later form. They assumed correctly that, in some nuclear transplants, host as well as injected nuclei would populate the pole cells and, subsequently, the fertility of adult gametes from both host and injected nuclei could be assessed. In many cases, the fertilized egg hosts developed directly into normal adults. In the case of the unfertilized but activated egg hosts, they formed defective nuclear transplants but were rescued by transplanting the pole cells of embryos or gonads of larvae into normal hosts. When the adults successfully mated, they produced

normal progeny from both the host and donor nuclei, demonstrating the totipotency of the preblastoderm ***(13)*** and early gastrula ***(12)*** donor nuclei. The impetus for nuclear transfers in *Drosophila* in the 1960s was the expectation that the valuable genetics available in this species would be helpful in analyzing problems of nuclear differentiation but, unfortunately, cloning in this species has not continued. Perhaps important problems can still be pursued. For example, insect clones derived from donor cells containing modified genomes might alleviate the negative effects of insects on fauna and flora in general and especially on humans.

In the protochordate ascidian, *Ciona intestinalis,* no clones were produced, but the effects of cytoplasm from mosaic eggs and embryos on transplanted nuclei were studied in intraspecies ***(104)*** and interphylum ***(105)*** combinations. Interspecific combinations comprised nuclei from ectodermal, mesodermal, or endodermal cells of gastrula or tailbud embryos of one species that were injected into non-nucleated egg fragments from another species. Cytological examination of the resultant embryos revealed that nuclei from all sources participated in the formation of cells in all three germ layers. They concluded that cell differentiation was principally determined by the components of the mature egg cytoplasm, and nuclear function was influenced by components in the surrounding cytoplasm ***(104)***.

Interphylum nucleocytoplasmic hybrids, containing a blastula nucleus from the sand dollar *Echinarachnius parma* and an activated and anucleated egg fragment of *C. intestinalis*, were analyzed to determine whether cytoplasmic determinants from Chordata would still affect a nucleus from the phylum Echinodermata. Despite the distance between the phyla, the ascidian cytoplasm influenced the supermolecular assemblage of the components of extracellular matrix and neural cells ***(105)***.

Nuclear transplantation in fish was initiated by the late T. C. Tung (Tong Dizhou) in the early 1960s in China and extensively investigated by Yan ***(8)***. One of Yan's goals was the production of fish clones for agricultural and commercial purposes. To this end, he constructed nucleocytoplasmic hybrids by transplanting a blastula nucleus of one species into an enucleated oocyte from a different species. Combinations of nuclei and cytoplasm from different genera and even different subfamilies resulted in fertile adults, demonstrating the totipotency of fish blastula nuclei and their compatibility with the cytoplasm from another genus and even from another subfamily. The intergenera constructs exhibited higher growth rates, increased protein content, and lower fat content than the original species, suggesting the feasibility of producing commercially valuable fish by cloning.

Recently, Wakamatsu et al. ***(106)*** in Japan initiated a cloning program with embryos of the small laboratory medaka fish, *Oryzias latipes* to study nuclear differentiation and gene function. Nuclei from blastula cells containing the gene for green fluorescent protein (GFP) were injected in unfertilized eggs enucleated by

X-ray irradiation. Six fertile adults resulted expressing GFP that they transmitted to the F_1 and F_2 offspring in Mendelian fashion. Also, fertile zebrafish expressing and transmitting GFP to their progeny were produced from embryonic fibroblast nuclei *(107)*. The fish model should be valuable for cell lineage studies, especially in those species whose internal development is somewhat transparent.

Throughout this review my use the of the word clone(s) has conformed to the current meaning adopted by scientists and nonscientists alike, namely an organism derived from an oocyte injected with any cell nucleus. Because a metazoan clone may not share in its oocyte the same mitochondrial DNA and maternal RNAs originally endowed to the donor cell, classifying a nuclear transplant as a clone is not scientifically correct, but this appellation has gained extensive use. Now that the word animal cloning has become ubiquitous for one meaning, it is appropriate that a recent scholarly report on the origin and evolution of the many meanings of clone has been published *(108)*.

10. Concluding Comments

Just more than 50 yr ago, the first tadpoles were cloned from frog blastula nuclei *(1)*. It is a challenge in this small review to capture fully the significant events that occurred during the past 50 yr, but I have attempted to highlight the historical roots of nuclear transfer, as well as the progress made in cloning non-mammalian species. In succeeding chapters experts in mammalian species will discuss advances in mammalian cloning, cell reprogramming, and transgenesis. As one of the early frog cloners and an enthusiastic observer of mammalian cloning, I would like to briefly cite four advances in mammalian cloning that I consider highly significant: (1) successful cloning of adult cells; (2) unequivocal demonstration that nuclei from fully differentiated cells can direct the formation of fertile adults; (3) important advancements in our knowledge of nuclear reprogramming; and (4) the construction of transgenic clones, especially via gene targeting of donor cells. I look forward to continued advances: the production of animal clones with modified genomes for commercial improvements and resistance to diseases as well as for medical applications to humans and, on the cellular level, further advances in nuclear reprogramming and, hopefully, to the extent that we may someday control the state of cell differentiation in vitro for applications in vivo.

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Cell Reprogramming and Transgenesis

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
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- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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In Vitro Maturation and Embryo Production in Cattle

Marc-André Sirard and Karine Coenen

Summary

When immature bovine oocytes are released from their follicles and are cultured in standard maturation medium, they resume the first meiotic division. The alteration of basic maturation conditions can affect oocyte competence significantly, as reflected by the morula and blastocyst yield after in vitro fertilization. The conditions used from the beginning of maturation up to the blastocyst stage have been shown to influence not only the developmental competence but also, potentially, the normal epigenetic make-up of the embryo. The methods described in this chapter outline the different steps of in vitro production of bovine embryos up to the blastocyst stage in semidefined conditions: (1) oocyte maturation, (2) in vitro fertilization, and (3) in vitro development. The first section explains procedures of ovary collection and oocyte aspiration and selection for in vitro maturation. The second section involves methods for the preparation of semen and oocytes for fertilization. The last section explains the best conditions to obtain blastocysts after 8 d of in vitro culture.

Key Words: Bovine; oocyte maturation; culture; developmental competence.

1. Introduction

When immature bovine oocytes are released from their follicles and are cultured in standard maturation medium, they resume the first meiotic division *(1)*. Although it may appear difficult to influence the quality of an oocyte during the maturational period, the alteration of basic maturation conditions can affect oocyte competence significantly, as reflected by the morula and blastocyst yield after in vitro fertilization (IVF *[2]*). The conditions used from the beginning of maturation up to the blastocyst stage have been shown to influence not only the developmental competence but potentially the normal epigenetic make-up of the embryo *(3)*.

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During in vitro maturation (IVM), oocytes undergo a series of cytoplasmic changes before the resumption of nuclear maturation, leading to variable competence of the resulting embryo *(4,5)*. In addition, the synthesis and storage of certain forms of messenger RNA and protein during IVM and early embryonic development are thought to be necessary for further development *(6,7)*.

The limited developmental competence of bovine oocytes after IVM can be used to understand the factors involved in the acquisition of such ability. For this reason and to understand the requirements for development of immature oocytes through IVM, all products with undefined components should be eliminated from culture conditions. Although serum or bovine serum albumin (BSA) typically are added to the medium as a protein supplement to improve culture efficiency *(8)*, different lots of this protein with differing purity levels can produce highly variable effects during the period of culture in hamsters, ranging from highly stimulatory to highly inhibitory *(9)*.

2. Materials

Unless otherwise stated, all chemicals used are purchased from Sigma-Aldrich (St. Louis, MO) and are cell culture tested or embryo tested. Every new lot of a chemical is tested individually before using. Every solution is prepared in Milli-Q water (18.2 mΩ.cm; total organic carbon< 5 ppb) produced by a Milli-Q Synthesis/A10 system (Millipore, Bedford, MA) and sterile filtered (0.22 µm) with a MF-Millipore membrane filter GSWP (Millipore). To remove all soap solution residue, glassware is soaked in reverse osmosis water for 1 d and then rinsed two times with Milli-Q water. When filtering small volumes of solution (≤10 mL), the first 0.5 mL is rejected to avoid filter cytotoxicity.

1. Saline.
2. Antibiotic antimycotic powder.
3. HEPES-buffered tyrode's medium: 114 m*M* NaCl, 3.2 m*M* KCl, 2 m*M* $NaHCO_3$, 0.34 m*M* NaH_2PO_4, 10 m*M* Na lactate, 2.0 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 10 m*M* HEPES, and 10 mg/L phenol red in Milli-Q water. Adjust pH to 7.4. The osmolarity should be 255 to 265 mosmol. Keep at 4°C for up to 15 d. Can be frozen. Supplement appropriately (*see* medium compositions in **step 29**) on day of use.
4. BSA.
5. Sodium pyruvate: Prepare a 40-m*M* solution in saline and freeze at –20°C.
6. Gentamicin: Prepare a 50-mg/mL solution in saline and keep at 4°C for 1 mo. Protect from light. The solution can become cytotoxic if powder is too old. A dark, detached, and unexpanded cumulus after oocyte maturation is a sign of cytotoxicity.
7. Mineral oil: Oil is filtered on an analytical filter funnel of 0.45 µm (Nalgene), blended with phosphate-buffered saline (PBS; 5:1 oil:PBS) to remove toxic hydrosoluble metallic ions (Fe^{2+}, Cu^{2+}), and left to stand for 24 h to separate the

PBS from oil before use. PBS can be replaced by Hank's or any other cell culture medium without phenol red. Possible contamination of oil would be visible at the interface of oil and PBS. Protect from light.

8. Synthetic oviduct fluid (SOF): 107.70 m*M* NaCl, 7.16 m*M* KCl, 1.19 m*M* KH_2PO_4, 1.71 m*M* $CaCl_2$, 0.49 m*M* $MgCl_2$, 25.07 m*M* $NaHCO_3$, 3.30 m*M* Na lactate, and 1.50 m*M* glucose ***(10)*** in Milli-Q water. The osmolarity should be 270 mosmol and the pH 7.55. Keep at 4°C for 15 d. Do not freeze. Supplement appropriately (*see* medium compositions in **steps 30, 33,** and **34**), on day of use.
9. Modified Eagle's medium (MEM) nonessential amino acids (Invitrogen, Burlington, Ontario, Canada): Keep at 4°C and protect from light.
10. MEM essential amino acids (Invitrogen): Keep at 4°C and protect from light.
11. Glutamine: Prepare a 50 m*M* solution in SOF and use fresh.
12. Follicle-stimulating hormone (FSH; National hormone and peptide program): Dissolve in 0.5% BSA in Milli-Q water, aliquot, lyophilize, and freeze at –20°C.
13. Luteinizing hormone (LH; National hormone and peptide program): Dissolve in 0.5% BSA in Milli-Q water, aliquot, lyophilize, and freeze at –20°C.
14. Estradiol-17β: Prepare a 1 mg/mL solution in 95% ethanol and keep at –20°C for 1 mo. Use and put back rapidly in freezer. Protect from light.
15. Modified Tyrode's solution: 114 m*M* NaCl, 3.2 m*M* KCl, 25 m*M* $NaHCO_3$, 0.34 m*M* NaH_2PO_4, 10 m*M* Na lactate, 2.0 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$ and 10 mg/L phenol red ***(11)*** in Milli-Q water. The osmolarity should be 280–300 mosmol. Keep at 4°C for 15 d. Do not freeze. Supplement appropriately (*see* medium composition in **step 31**) on day of use.
16. Fatty acid-free BSA.
17. Heparin: dissolve in saline and freeze at –20°C.
18. 2 m*M* Penicillamine, 1 m*M* hypotaurine, 250 µ*M* epinephrine (PHE): The PHE mixture is prepared using the procedure described by Miller et al. ***(12)***. A penicillamine stock solution is prepared by adding 3 mg of penicillamine to 10 mL physiological saline. A hypotaurine stock solution is prepared by adding 1.09 mg of hypotaurine to 10 mL of physiological saline. The epinephrine stock solution is prepared by adding 165 mg of sodium lactate (syrup, 60%) and 50 mg of sodium meta-bisulfite to 50 mL of Milli-Q water. After adjusting the pH to 4.0 with 1 *N* HCl, 1.83 mg of epinephrine is added to 40 mL of this solution. The PHE stock solution is prepared by adding 250 µL of penicillamine stock, 250 µL of hypotaurine stock, and 200 µL of epinephrine stock to 400 µL of physiological saline. The PHE stock solution is placed into vials, wrapped in aluminum fold and stored at –20°C.
19. Percoll.
20. Modified Tyrode's medium for sperm (Sp-TALP): 100 m*M* NaCl, 3.1 m*M* KCl, 25 m*M* $NaHCO_3$, 0.29 m*M* NaH_2PO_4, 21.6 m*M* Na lactate, 2.0 m*M* $CaCl_2$, 1.5 m*M* $MgCl_2$, 10 m*M* HEPES, and 10 mg/L phenol red ***(13)*** in Milli-Q water. Adjust pH to 7.4. The osmolarity should be 280–300 mosmol. Keep at 4°C for 15 d. The medium can be frozen. Supplement with 0.6% BSA, 1 m*M* sodium pyruvate, and 50 µg/mL gentamicin.

21. PBS: 136.89 m*M* NaCl, 2.68 m*M* KCl, 8.03 m*M* Na_2HPO_4, 0.66 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 1.47 m*M* KH_2PO_4, 5.55 m*M* glucose, and 1 m*M* sodium pyruvate in Milli-Q water. Adjust pH to 7.1. Keep at 4°C for 15 d. Supplement appropriately (*see* medium compositions in **step 32**), on day of use.
22. Ethylene diamine tetraacetic acid: Prepare a 10 m*M* solution in SOF and keep at 4°C. Protect from light.
23. Propidium iodide.
24. Hoechst 33354.
25. Formalin solution (HT50-1-1). A general histological fixative for in vitro diagnostic.
26. Triton X-100.
27. Mowiol gelatin.
28. Transportation medium: 0.9% NaCl aqueous solution (saline) containing 100 000 IU/L penicillin, 100 mg/L streptomycin, and 250 µg/L amphotericin B (antibiotic antimycotic powder).
29. Oocyte wash medium: HEPES-buffered Tyrode's medium supplemented with 0.3% BSA, 0.2 m*M* sodium pyruvate, and 50 µg/mL gentamicin.
30. Maturation medium: SOF supplemented with 0.8% BSA, 0.33 m*M* sodium pyruvate, 1X MEM nonessential amino acids, 1X MEM essential amino acids, 1 m*M* glutamine, 0.5 µg/mL FSH, 5 µg/mL LH, 1 µg/mL estradiol-17β, and 50 µg/mL gentamicin.
31. Fertilization medium: Modified Tyrode's solution , supplemented with 0.6% fatty acid-free BSA, 0.2 m*M* sodium pyruvate, 2 µg/mL heparin, and 50 µg/mL gentamicin (*see* **Note 1**).
32. Decumulation medium: Modified PBS without Ca^{2+} and Mg^{2+} supplemented with 0.3% BSA and 50 µg/mL gentamicin.
33. Development medium 1 (used for the first 72 h of culture): SOF supplemented with 0.8% BSA, 0.33 m*M* sodium pyruvate, 1X MEM nonessential amino acids, 1 m*M* glutamine, 10 µ*M* ethyelene diamine tetraacetic acid, and 50 µg/mL gentamicin.
34. Development medium 2: SOF supplemented with 0.8% BSA, 0.33 m*M* sodium pyruvate, 1X MEM essential amino acids, 1X MEM nonessential amino acids, 1 m*M* glutamine, and 50 µg/mL gentamicin.

3. Methods

The methods described below outline (1) oocyte maturation in vitro, (2) in vitro fertilization, and (3) culture of embryo up to the blastocyst stage in semi-defined conditions (*see* **Note 2**).

3.1. Oocyte Maturation

1. Collect ovaries from cycling heifers or cows within 30 min after slaughter and transport to the laboratory at 30–35°C in transportation medium (*see* **Note 3**).
2. Wash the ovaries with transportation medium (*see* **Note 4**).

3. Aspirate follicular fluid from 3- to 6-mm follicles using an 18-gage needle (Becton-Dickinson) on a 10-mL syringe (Becton-Dickinson; *see* **Note 5**).
4. Stand tubes containing follicular fluid at room temperature for 10–15 min and aspirate precipitate (*see* **Note 6**). Transfer the precipitate into a Petri dish (Fisher, 100 × 15 mm).
5. Centrifuge supernatant at 3000*g* during 10 min at room temperature to obtain clear follicular fluid. Using a stereomicroscope, select oocytes with at least five layers of compact cumulus cells and belonged to classes 1–3 (**Table 1** and **ref.** ***14***), and wash them in follicular fluid.
6. Rapidly wash oocytes three times in oocyte wash medium in Petri dishes (Nunc, 35 × 10 mm).
7. Transfer selected oocytes into 50-µL droplets (10 oocytes/drop) of maturation medium overlaid with 9 mL of mineral oil in Nunc Petri dishes, 60 × 15 mm (*see* **Note 7**).
8. Culture oocytes for 24 h at 38.5°C under 5% CO_2 and 95% air atmosphere with saturated humidity.

3.2. In Vitro Fertilization

IVF is performed using frozen semen from selected bulls (*see* **Note 8**).

1. Prepare a discontinous Percoll gradient by adding 2 mL of 45% Percoll over 2 mL of 90% Percoll in a 15-mL Falcon centrifuge tube ***(15)***.
2. Let the tube stand until the Percoll solution has reached room temperature.
3. Thaw one or two straws of spermatozoa (100×10^6 spermatozoa) in a 35°C water bath for 1 min.
4. Blend the semen sample and add it on top of the Percoll gradient.
5. Centrifuge at 700*g* for 30 min at 26°C.
6. Remove rapidly the supernatant and resuspend the pellet in 1 mL of Sp-TALP.
7. Centrifuge the resuspended pellet at 250*g* for 5 min at 26°C.
8. Remove rapidly the supernatant and blend the pellet.
9. Use aliquots of 5 µL of sperm pellet diluted in fertilization medium to evaluate the concentration and motility of spermatozoa with an hemocytometer chamber.
10. Resuspend the sperm pellet in fertilization medium to obtain a concentration of 25×10^6 cells/mL.
11. After maturation, wash cumulus–oocyte complexes (COCs) twice for 5 min in 2 mL of oocyte wash medium in Nunc Petri dishes (35 × 10 mm).
12. Transfer washed COCs in 48-µL droplets (5 COCs/drop) of fertilization medium overlaid with 9 mL of mineral oil (*see* **Note 9**).
13. Following the transfer of oocytes and just before adding spermatozoa, add 2 µL of PHE to each droplet.
14. Add 2 µL of sperm suspension into each fertilization droplet containing COCs (final concentration = 1×10^6 cells/mL). Coincubate COCs and spermatozoa for 15–18 h at 38.5°C under 5% CO_2 in air with saturated humidity.

Table 1
Classification of Bovine Oocytes[a]

Class	No. of cumulus layers	Expansion of cumulus
1 and 2	≥5	Compact
3	≥5	Slight expansion in outer layers
4	≥5	Full expansion with dark clumps
5	1	Only corona radiata
6	0	No cumulus

[a]All oocytes, not including the cumulus, have diameters ≥120 µm.
(Adapted from Blondin and Sirard *[14]*).

3.3. In Vitro Development

1. Between 15 and 18 h after insemination, strip presumptive zygotes of all surrounding cumulus cells by repeated pipetting in decumulation medium and wash them three times in the same medium in order to avoid transfer of any cumulus cell to the development medium (development medium 1).
2. Transfer presumptive zygotes in 50-µL droplets (20–30 zygotes/drop) of development medium 1, overlaid with 9 mL of mineral oil and culture them at 38.5°C under 5% CO_2, 7% O_2, 88% N_2 with saturated humidity.
3. After 72 h of culture, assess cleavage stage and transfer all the embryos into development medium 2. Transfer embryos to new drops of medium every 72 h.
4. On day 8 of culture, assess the number and quality of embryos developing to the morula and blastocyst stage.

Determination of inner cell mass (ICM) and trophectoderm cell numbers can also be done in blastocysts (*see* **Note 10**).

4. Notes

1. Heparin concentration must be adjusted for each lot of semen used. A heparin concentration of 1, 2, 5, and 10 µg/mL should be tested and the penetration/polyspermy rate verified.
2. Multiple factors are critical to the outcome of IVM–in vitro production: exposure to cytotoxic materials (Petri dish, syringe, needle, surgical gloves), water quality, chemical quality, gas composition, temperature, pH, osmolality, light exposure, and assay techniques. For a review of the general topic of quality control during mammalian IVF and embryo culture, *see* **refs.** *16* and *17*. Each new brand or lot of plastic material should be tested. Each new lot of a chemical should also be tested. Every protocol must contain a control treatment to assess the reproducibility of results obtained week after week.

3. The time interval that the oocytes remained in the postmortem ovarian follicles has a significant effect on the subsequent embryonic development. The optimal time for oocyte recovery after the ovaries are collected is 4 h ***(18)***.
4. To avoid contamination, ovary washing and aspiration are performed outside the culture room.
5. To remove toxicity of plastic syringe and needle, aspirate follicular fluid of 8- to 10-mm follicles to rinse the needle and syringe before to use them for aspiration of 3- to 6-mm follicles. The needle must be changed frequently to keep a sharp cutting tip.
6. The culture room can be maintained at 27°C or warmer.
7. The droplets, covered with mineral oil, should be preincubated under the culture conditions for a minimum of 2 h before transfer of oocytes or embryos.
8. Frozen bovine semen used can come from an individual bull or a pool of bulls (usually five). Final sperm concentration must be adjusted for every lot of semen used. Sometimes 500,000 spermatozoa/mL give better results than 1×10^6 cells/mL.
9. To limit the contact surface of droplets with Petri dish, droplets are made in two steps in Fisher Petri dishes (60 × 15 mm). Drops of 18 µL of fertilization medium are overlaid with 9 mL of mineral oil and then another 30 µL of fertilization medium is added to each drop.
10. Nuclei of ICM and trophectoderm are differentially labeled with propidium iodide and Hoescht, respectively, with the protocol described by Van Soom et al. ***(19)*** with two modifications. Oocytes are washed in HEPES-buffered Tyrode's medium instead of TCM-199 HEPES. For the coloration of the ICM by Hoescht, blastocysts are fixed in 100 µL of formalin solution for 15 min, then rinsed in 200 µL of 0.5% Triton X-100 before 100 µL being mounted over a drop of Mowiol gelatin containing Hoescht 33354 (5 mg/mL ***[20]***).

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The authors provide readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer. Of immense benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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3

Activation of Oocytes After Nuclear Transfer

Zoltán Macháty

Summary

After nuclear transfer, the recipient oocyte must be stimulated to initiate development. This stimulation is achieved by inducing changes in the oocyte cytoplasm that normally are triggered by the sperm during fertilization. In most cases, such changes include a transient increase in the intracellular-free calcium concentration induced by an electrical pulse or alternatively, by chemical agents. Many times, particularly in aged oocytes, this calcium signal is sufficient to stimulate the oocyte developmental program. Other activation protocols were designed to target pathways downstream of the initial calcium signal to affect the activity of regulatory proteins that play central roles in maintaining developmental arrest. This is achieved by the application of protein kinase or protein synthesis inhibitors; combined with a calcium stimulus such inhibitors are widely used for oocyte activation after nuclear transfer and are able to support embryonic development to term.

Key Words: Oocyte; meiotic arrest; calcium; oocyte activation; nuclear transfer; NT; electroporation; calcium ionophore; strontium; ethanol; protein kinase inhibitors; 6-dimethylaminopurine; butyrolactone; protein synthesis inhibitors; cycloheximide.

1. Introduction

At the time of ovulation, the oocyte is developmentally arrested at a point of the cell cycle that varies with the species; mammalian oocytes typically are arrested at the metaphase of the second meiotic division *(1)*. The fertilizing sperm induces an elevation in the oocyte cytoplasmic-free calcium concentration that triggers a series of cellular events generally referred to as activation *(2)*. As a result, the oocyte will resume meiosis and initiate development. During nuclear transfer (NT), a donor nucleus is introduced into the cytoplasm of an "enucleated," unfertilized oocyte. In this case, the complete chromosomal material necessary for normal development is provided by the donor cell nucleus with no contribution from the sperm. Thus, in the absence of fertilization, the oocyte must be exposed to artificial stimuli that are expected

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to mimic one or more of the sperm-induced changes to activate its developmental program. Activation can be induced artificially by using a variety of physical and chemical agents (for reviews, *see* **refs**. *3* and *4*). Most activation protocols involve the generation of a calcium signal either by itself, or in combination with the downregulation of certain cell cycle-related regulatory proteins. This downregulation can be achieved directly by inhibiting protein phosphorylation or by blocking protein synthesis. Here, the most effective methods currently available that have been used successfully for oocyte activation to produce live offspring via NT will be described.

2. Materials

1. Electroporation system with chamber (e.g., BTX 2001, chamber size 0.5 mm).
2. Cytochalasin B: Protect from light; dilute in dimethylsulfoxide (DMSO), prepare 10 µg/µL stock, make 5-µL aliquots, and store at −20°C. Add 1 aliquot to 10 mL of medium to make 5 µg/mL working solution.
3. Calcium ionophore A23187 (free acid): Protect from light, dissolve in DMSO. Prepare 5 m*M* stock solution, make 10-µL aliquots, and store at −20°C. Add 1 aliquot to 10 mL of culture medium with rapid pipetting to make a 5 µ*M* working solution. Rapid mixing is necessary because A23187 is very insoluble in water and precipitates easily.
4. Ionomycin (calcium salt): Protect from light, prepare stock (typical concentration 5 µ*M*) by dissolving ionomycin in DMSO, make aliquots, and store at −20°C.
5. Strontium chloride ($SrCl_2$): Dissolve in culture medium (typical concentration 10 m*M*).
6. Ethanol: Mix with culture medium before use, avoid ethanol evaporation.
7. 6-Dimethylaminopurine (6-DMAP): Protect from light, dilute in culture medium (typical concentration 2 m*M*), aliquot, and store aliquots at −20°C.
8. Butyrolactone I: Dissolve in DMSO (typical concentration 100 µ*M*), aliquot, and store at −20°C.
9. Cycloheximide: Dissolve in ethanol (typical concentration 5 – 10 µg/mL), aliquot, and store at −20°C.

3. Methods

A number of methods are available that are able to induce embryonic development in oocytes. The method of choice would depend on the species as well as on the particular experimental conditions (*see* **Note 1**). The various activation protocols used in conjunction with NT are described in this section along with the mechanisms by which they trigger development. The methods are categorized according to which pathway/cellular mechanism they target to stimulate activation.

3.1. Elevating Intracellular Calcium Levels

At fertilization, the universal signal for oocyte activation is a transient increase in the intracellular free calcium concentration. The fertilizing sperm

triggers the release of calcium from the intracellular stores via a mechanism that is not fully understood. In mammalian oocytes, the first calcium transient is followed by a low frequency calcium oscillation over a period of several hours *(5)*. Several lines of evidence support the notion that the calcium signal is necessary and sufficient for the activation of the oocyte developmental program. Because of this fundamental role, several protocols have been developed that induce activation by eliciting a single or multiple calcium transients.

3.1.1. Electroporation

Electroporation is the formation of pores in the plasma membrane induced by an electrical pulse. Short, high-voltage, direct-current (DC) pulses cause reversible electric breakdown of the plasma membranes, resulting in the formation of temporary pores and allowing the flow of ions along their concentration gradients through the plasma membrane into the cytosol. The ionic influx is influenced by several parameters, including the electric pulse-induced change in the membrane potential, duration of the pulse, ionic strength of the medium, and cell type *(6)*. In the presence of a very low concentration of ions in the external medium, the oocytes are resistant to high-voltage electric field pulses if the electrical current during pulses is sufficiently low *(7)*. The low ionic strength medium also serves to reduce the number of ionic components that pass into the oocyte and helps to diminish deleterious Joule heating that occurs during pulse application. The electrical pulse itself is not believed to induce activation. However, in the presence of extracellular calcium it was shown to generate a transmembrane calcium influx that triggered oocyte activation in several species *(3)*.

An activation protocol using electroporation would generally include the following steps:

1. Rinse reconstructed oocytes in the electroporation medium (*see* **Note 2**).
2. Equilibrate oocytes in the electroporation medium for approx 5 min.
3. Transfer equilibrated oocytes between the electrodes of an electroporation chamber (*see* **Note 3**) filled with electroporation medium.
4. Apply DC pulse, which may be preceded by an alternating-current (AC) pulse (*see* **Note 4**).
5. After the electrical pulse, the oocytes are kept in the electroporation medium for approx 2 min, which would allow sufficient time for the membrane pores to close.
6. Transfer electroporated oocytes into culture medium.

The parameters used for the ACs and DCs vary depending on several factors. Typically, for orientation before fusion an AC of 3–10 V/mm is applied for

5–10 s followed by one or two DC pulses of approx 20–300 V/mm with 30- to 100-µs duration. Electroporation has been successfully applied for oocyte activation after NT to produce live offspring in a number of species. Listed are a few examples of the parameters used.

3.1.1.1. Applications

Cattle: Three DC pulses, each 20 V/mm for 20 µs, pulses 22 min apart *(8)*.

Sheep: Two consecutive DC pulses, each 200 V/mm for 75 µs *(9)*.

Pig: AC current of 5 V for 5 s, followed by two DC pulses, 150 V/mm for 60 µs *(10)*.

Goat: AC current of 5–10 V for 7 s, then a DC pulse of 140–180 V/mm for 70 µs *(11)*.

Rabbit: One DC pulse of 160 V/mm for 60 µs *(12)*.

Mouse: Two DC pulses; 150 V/mm for 50 µs, followed by 50 V/mm for 50 µs. A second set of pulses with the same parameters was applied 20 min later *(13)*.

3.1.2. Calcium Ionophores

Ionophores are compounds capable of forming complexes with ions and transporting them through biological membranes. The compound A23187 is an antibiotic that is widely used as an ionophore because it forms stable complexes with divalent cations, including calcium. There is evidence that A23187 may also trigger the release of calcium ions from intracellular stores *(14)*. In mouse and porcine oocytes, the induced calcium transient was significantly smaller in the absence of calcium in the culture medium *(15,16)*, suggesting that its major role is that of a calcium transporter. The calcium transient induced by incubation with A23187 stimulates varying degrees of activation in oocytes from different species (reviewed in **ref.** *3*).

Ionomycin is more powerful as a calcium ion carrier than A23187. Moreover, in certain cell types, it was demonstrated to stimulate intracellular calcium release and induce a calcium influx via activation of endogenous entry pathways and not by plasmalemmal translocation *(17)*. Because it is very effective in promoting oocyte activation and embryonic development, it is widely used during NT in a number of species. For activation, it is generally sufficient to incubate oocytes for a short time in approximately 5 µ*M* ionomycin, although higher concentrations also have been used. Bovine oocytes activated with ionomycin were shown to develop to term after NT *(18)*. Most of the time, calcium ionophores are used in combination with protein kinase or protein synthesis inhibitors. For a detailed description of these methods, *see* **Subheadings 3.2.1.** and **3.2.2.**

3.1.2.1. Application

To trigger development in cattle, reconstructed bovine oocytes are exposed to medium containing 5 m*M* ionomycin for 45 s ***(18)***.

3.1.3. Strontium

Strontium (Sr^{2+}) is a divalent cation that in certain cell types is handled much like calcium. In muscle cells, it was able to stimulate the release of calcium from the sarcoplasmic reticulum ***(19)***, and it was shown to induce oocyte activation in a number of species, including mouse, hamster, and the rodents *Calomys musculinus* and *Calomys laucha* ***(20,21)***. Because Sr^{2+} generally is more effective in the absence of external calcium, it probably induces calcium release from intracellular stores. Incubation of mouse oocytes in the presence of Sr^{2+} in calcium- and magnesium-free medium for an extended period of time induced repetitive calcium transients ***(22)***.

3.1.3.1. Application

For activation in mice after NT, transfer mouse oocytes into calcium-free medium containing 10 m*M* $SrCl_2$ and 5 µg/mL cytochalasin B and culture for 6 h ***(23)***.

3.1.4. Ethanol

Ethanol was reported to stimulate activation in oocytes of various species, including mouse, cattle, and pig ***(3)***. It was shown to stimulate the production of the second messenger inositol 1,4,5-trisphosphate ($InsP_3$) and thus promote the discharge of calcium through the $InsP_3$ calcium release channel receptors ***(24)***. During long incubations, ethanol effectively inhibited the $InsP_3$ receptors.

Incubation of methaphase II oocytes in the presence of 7–8% (v/v) ethanol for 5 to 7 min generally is sufficient to stimulate pronuclear formation and in some cases, further embryonic development. Because in most instances it is used in combination with protein kinase or protein synthesis inhibitors, its application is described in **Subheadings 3.2.1** and **3.2.2.**

3.2. Altering the Activity of Cell Cycle Regulatory Proteins

The cell cycle is normally regulated by cyclin-dependent kinases (CDKs), a family of serine/threonine kinases activated by a family of cyclins. The first identified CDK, *cdc2* was discovered in the yeast; its human homolog, CDK1, was soon identified and confirmed to be present in all eukaryotes investigated. The 34-kDa protein homologous to the product of the *cdc2* gene of the fission yeast is also called $p34^{cdc2}$. Cyclin B was discovered in sea urchin oocytes and was demonstrated to belong to a family of several members that can be found

in a wide variety of cells. CDKs complexed with cyclins play a role not only in the regulation of the cycle of cell division but also in apoptosis, and in the control of transcription (reviewed in **ref. *25***).

As with mitosis in other cells, meiosis in oocytes is initiated by the M-phase-promoting factor (MPF), which is a heterodimer of $p34^{cdc2}$ and cyclin B. During interphase, cyclin B accumulates and binds to $p34^{cdc2}$. At this point, phosphorylation of $p34^{cdc2}$ inhibits the function of the complex, whereas subsequent dephosphorylation by the phosphatase cdc25 provides $p34^{cdc2}$ with kinase activity, leading to the generation of active MPF and entry into first metaphase. In mammals, the meiotic arrest at metaphase II after ovulation is also sustained by high MPF activity that is maintained through an equilibrium of continuous synthesis and degradation of cyclin B.

MPF is stabilized by the cytostatic factor, which is composed of at least three proteins: Mos, mitogen-activated protein (MAP) kinase, and $p90^{Rsk}$ ***(4)***. In general, these proteins are responsible for maintaining the condensed status of the chromatin during the meiotic arrest.

After sperm–egg interaction, the activity of these cell cycle-related regulatory proteins decreases remarkably. The increase in the intracellular free calcium concentration at fertilization triggers the activation of calmodulin-dependent protein kinase II, which in turn triggers the degradation of cyclin B which, together with $p34^{cdc2}$ phosphorylation, results in the inactivation of MPF. Low MPF activity will stimulate the resumption of meiosis, chromatin decondensation, and entry into interphase. The activation of calmodulin-dependent protein kinase II also induces cytostatic factor inactivation ***(26)***. MAP kinase activity also decreases after oocyte activation: in the mouse, the dephosphorylation of MAP kinase results in its inactivation that correlates with the formation of the nuclear envelope and initiation of DNA synthesis ***(27)***.

During parthenogenetic activation of young bovine ***(28)*** and rabbit ***(29)*** oocytes, MPF activity rapidly recovered after an activating stimulus, which allowed recondensation of chromosomes and re-entry of the oocytes into a new M-phase arrest known as metaphase III. This arrest can be circumvented by treatments that are able to inhibit MPF (and possibly MAP kinase) activity.

3.2.1. Inhibiting MPF Activity With Protein Kinase Inhibitors

Inhibiting protein kinases to dephosphorylate proteins in general seems to trigger oocyte activation. Nuclear lamins are proteoglycans that constitute the nuclear envelope and are thought to facilitate the overall organization of the nucleus. Their dephosphorylated state corresponds to the formation of the nuclear envelope and, when phosphorylated by the $p34^{cdc2}$/cyclin B complex at the G2/M transition, nuclear lamins are disassembled. Furthermore, a cyclic adenosine monophosphate-dependent kinase inhibitor seems to localize in the

nucleus of G2/M cells. Its inhibition results in the arrest of the cell cycle indicating that protein kinase inhibition may be a normal function for the M/G1 transition ***(30)***. Inhibition of protein kinases with the nonspecific kinase inhibitors staurosporine or H7 results in the resumption of meiosis and formation of pronuclei in mouse and porcine oocytes ***(31,32)***. Inhibition of MAP kinase activity, independently of MPF inactivation using U0126, a MAP kinase inhibitor also induced some degree of activation in porcine oocytes ***(33)***. Finally, inhibition of myosin light-chain kinase activity may partially be responsible for activation stimulated by kinase inhibition. It was demonstrated that blocking myosin light-chain kinase activity eventually releases the cyclin degradation machinery resulting in pronuclear formation in porcine oocytes ***(34)***.

As mentioned previously, dephosphorylation of the $p34^{cdc2}$-cyclin B complex generates active MPF that is involved in maintaining the meiotic arrest. The dephosphorylation is catalyzed by the phosphatase cdc25, whose activity can be blocked by inhibiting its phosphorylation by protein kinase inhibitors. In *Xenopus laevis* oocytes the compound 6-DMAP, a broad-spectrum kinase inhibitor, causes release from the meiotic arrest by inhibiting cdc25, thus preventing dephosphorylation (i.e., activation) of MPF ***(4)***. It is also thought to be important in inhibiting protein phosphorylation necessary for the spindle apparatus because, in many species, 6-DMAP inhibits the extrusion of the second polar body as well (*see* **Note 5**). Applied after an induced calcium transient, it was used effectively for oocyte activation in a number of NT experiments.

3.2.1.1. Ionomycin Plus 6-DMAP

The incubation of oocytes with ionomycin and subsequently, with 6-DMAP stimulates activation effectively ***(28)*** and, by means of this combined activation, live offspring have been produced in a number of species after NT. Recently ovulated (young) oocytes, although believed to be of better quality, have proved difficult to activate because of consistently high MPF activity. By preventing reactivation of MPF, 6-DMAP allows the use of younger oocytes for NT, at least in cattle.

Applications:

In cattle: Incubation in 5 m*M* ionomycin for 4 min, then in 2 m*M* 6-DMAP for 3 h ***(35)***.

In sheep: 5 m*M* Ionomycin for 5 min, then 2 m*M* 6-DMAP and 7.5 mg/mL cytochalasin B for 1 h, followed by an incubation in 2 m*M* 6-DMAP for an additional 1 h ***(36)***.

In pig: 15 m*M* Ionomycin for 20 min followed by 1.9 m*M* 6-DMAP for 3 to 4 h ***(37)***.

In goat: 5-min Incubation in 5 m*M* ionomycin then 3 h in 2 m*M* 6-DMAP *(38)*.

In Rhesus monkey: 5 m*M* Ionomycin for 2 min followed by 4 h in 2 m*M* 6-DMAP and 5 mg/mL cytochalasin B *(39)*.

Oocytes activated with the combined ionomycin/6-DMAP treatment reportedly have alterations in their DNA content owing to an abnormal pattern of karyokinesis during their first cell cycle *(40)*. In somatic cells arrested in S-phase, 6-DMAP also was shown to induce premature mitosis followed by reformation of the nuclear envelope around decondensed and fragmented chromatin to form numerous micronuclei *(41)*. When used to activate oocytes after NT, placental malformations and perinatal death have been reported in the resulting offspring *(35)*. These problems might indicate that protein kinase inhibitors with low selectivity are not acting on one specific kinase only but they interfere with several metabolic pathways involved in other cell functions whose inhibition may have deleterious consequences in subsequent cellular events *(4)*.

3.2.1.2. Ionomycin Plus Butyrolactone I

6-DMAP has been characterized as a broad-spectrum kinase inhibitor. The targeted inhibition of specific kinases that play crucial roles in cell cycle regulation seems to be a more attractive way to induce oocyte activation. Because naturally occurring CDK inhibitors are important in cell cycle control, artificial inhibition of these CDKs is believed to be an effective way to influence the cell cycle *(25)*. Selective compounds for the inhibition of MPF activity were developed; butyrolactone I inhibits selectively CDK1 ($p34^{cdc2}$) and CDK2, effectively arresting the cell cycle at G2/M and G1/S *(42)*. By inhibiting cdc2, the catalytic subunit of MPF, butyrolactone I was described to inhibit meiotic resumption in GV stage oocytes or to induce activation in oocytes that are arrested in the MII stage *(43)*. A combined electrical and butyrolactone I treatment resulted in high rates of pronuclear formation and cleavage in porcine oocytes *(44)*, and butyrolactone I after an ionomycin-induced calcium transient has been reported to stimulate development to term in bovine NT experiments *(45)*.

3.2.1.3. Application

In cattle, expose NT bovine oocytes to 5 μ*M* ionomycin for 4 min, and then transfer them into 100 μ*M* butyrolactone I for a 4-h culture *(45)*.

3.2.2. Inhibiting MPF Activity With Protein Synthesis Inhibitors

To maintain MPF activity, cyclin B must be synthesized continuously. Blocking the production of cyclin B with protein synthesis inhibitors was

shown to stimulate oocyte activation. Inhibitors such as puromycin or cycloheximide could induce entry into the first interphase in mouse and human but not in rat and pig oocytes (reviewed in **refs.** *3* and *4*).

3.2.2.1. Cycloheximide

Inhibition of protein synthesis is possible by exposing the oocytes to cycloheximide, an antifungal antibiotic, for an extended period of time. The treatment was reported to activate rhesus monkey and bovine oocytes after NT.

Applications:

In Rhesus monkey: Treat NT oocytes with 7.5 µg/mL cycloheximide and 7.5 µg/mL cytochalasin B for 1 h. Fuse subsequently with an electrical pulse in the presence of 100 µ*M* calcium which may provide an additional activating stimulus *(46)*.

In cattle: incubate reconstructed oocytes in 10 µg/mL cycloheximide and 5 µg/mL cytochalasin B for 1 h, then in cycloheximide alone for an additional 4 h *(47)*.

3.2.2.2. Electroporation Plus Cycloheximide

Activation and subsequent development has generally been more successful when the inhibition of protein synthesis is preceded by a stimulus that triggers a calcium transient *(48)*. As mentioned previously, the efficiency of parthenogenetic oocyte activation is age dependent with freshly ovulated oocytes being more difficult to activate. Similarly to protein kinase inhibition, blocking protein synthesis in combination with an induced calcium release caused activation of bovine oocytes that was independent of age *(49)*.

After an electric pulse, cycloheximide reportedly triggered term development of NT embryos of various species.

Applications:

In cattle: Two DC pulses of 250V/mm for 10 µs each, followed by a culture in 10 µg/mL cycloheximide for an additional 5 h *(50)*.

In goat: A single pulse of 100 V/mm for 160 µs, then culture in the presence of 7 µg/mL cycloheximide for 3 h *(51)*.

In cat: Two 100 V/mm, 50-µs pulses, 5 s apart. Subsequently, incubation for 6–7 h in 10 µg/mL cycloheximide and 5 µg/mL cytochalasin B *(52)*.

Rabbit: Three DC pulses of 320 V/mm for 20 µs each; a second set of pulses 1 h later. Then incubation in 5 µg/mL cycloheximide and 2 m*M* 6-DMAP for 1 h *(53)*.

3.2.2.3. Ethanol Plus Cycloheximide

When used in combination with ethanol, cycloheximide was successfully used after bovine NT.

Application: In cattle, incubate reconstructed bovine oocytes in 7% (v/v) ethanol for 5 min and then culture for 5 h in 10 µg/mL cycloheximide and 5 µg/mL cytochalasin B. ***(54)***.

3.2.2.4. A23187 Plus Cycloheximide

Cycloheximide also was applied in combination with the calcium ionophore A23187 for the production of cloned calves.

Application: After NT in cattle, expose the reconstructed oocytes to 5 µ*M* A23187, 5 µg/mL cytochalasin B, and 10 µg/mL cycloheximide for 10 min, then incubate in 10 µg/mL cycloheximide and 5 µg/mL cytochalasin B for 1 h. Follow by culture in the presence of 10 µg/mL cycloheximide for an additional 5 h ***(55)***.

3.2.2.5. Ionomycin Plus Cycloheximide

Cycloheximide treatment after a calcium transient induced by ionomycin was reported to induce term development of ovine and horse oocytes after NT.

Applications:

In sheep: Incubate oocytes with 5 µ*M* ionomycin for 5 min and, subsequently, in 10 µg/mL cycloheximide and 7.5 µg/mL cytochalasin B for 5 h ***(56)***.

In horse: Enucleated equine oocytes were fused with donor cells isolated from a mule fetus and cultured in 5 µ*M* ionomycin for 5 min, followed by a 5-h culture in 10 µg/mL cycloheximide. During activation, the calcium concentration in the medium was markedly higher (4.26 m*M*) than reported for other species ***(57)***.

The inhibition of protein synthesis with cycloheximide is widely used for oocyte activation after NT. It was reported, however, that besides depleting the oocytes of proteins that maintain MPF activity cycloheximide also inhibits translation of proteins responsible for the initiation of DNA replication ***(58)***. In NT embryos activated by ethanol/cycloheximide the initiation of DNA synthesis was delayed ***(59)***, and a great percentage of offspring had various health-related problems ***(54)***.

3.2.3. Reduction of MPF by Preactivation of Oocytes

In certain cases, the recipient oocytes are activated before the donor nucleus is introduced into the cytoplasm. This process is called preactivation and is

applied primarily when blastomeres are used as donor cells for NT. The rationale behind preactivation is the necessity of cell cycle synchronization between the two cells. In cattle, it was demonstrated that most blastomeres are in the S-phase of their cell cycle, and activation of the recipient cytoplasm approx 4 h before fusion will induce MPF levels to decrease and the oocyte will be in the S-phase by the time the donor nucleus is transplanted into the cytoplasm ***(60)***. During somatic cell NT, when the donor cells are generally synchronized in the G_0 or G_1 stage of their cell cycle, better development can be expected if the recipient cytoplasm is at the metaphase stage. To achieve this, the oocytes should be activated at the time of fusion or some time thereafter.

4. Notes

1. The effectiveness of an oocyte activation method can be measured by how efficiently it can stimulate the initiation of the oocyte developmental program. Before being used for activation during a NT experiment, each method should be tested; pronuclear formation and development to the blastocyst stage provide convenient end points for an initial evaluation.
2. The electroporation medium typically contains a very low amount of electrolyte; the main component is a sugar such as sucrose or mannitol. The Zimmermann fusion medium (0.28 *M* sucrose, 0.5 m*M* $Mg(CH_3CO_2)_2$, 0.1 m*M* $Ca(CH_3CO_2)_2$, 1 m*M* K_2HPO_4, 0.1 m*M* gluthatione, and 0.01 mg/mL bovine serum albumin ***[6]***) is probably the most established medium used for electroporation after NT. We found that a medium consisting of 0.3 *M* mannitol, 0.1 m*M* $CaCl_2$, 0.1 m*M* $MgSO_4$, 0.5 m*M* HEPES, and 0.01 mg/mL bovine serum albumin also works very sufficiently, at least for porcine oocytes ***(61)***.
3. Electroporation system. The equipment needed for electroporation is an electroporation/electrofusion machine with the chamber. The requirements for such an apparatus are fairly simple, and a number of systems are available on the market that would be adequate for the purposes of oocyte activation. The machines most commonly used for such purposes are those manufactured by BTX (BTX has recently become part of Harvard Apparatus; Harvard Apparatus/BTX, Holliston, MA). The electroporation system should be able to deliver an adjustable AC current for dielectrophoretic aggregation of the cells. This is believed to be helpful during membrane fusion although in cases, when the AC current causes rotation of the reconstructed oocyte, manual alignment might be preferred. Alignment is normally followed by a variable DC square pulse to induce pore formation in the plasma membranes. The electrical output necessary depends primarily on the distance between the platinum or stainless steel electrodes of the electroporation chamber which most frequently is in the range of 0.5–1.0 mm. The duration and number of pulses should also be adjustable. After the DC current, AC reapplication may be helpful to keep cells together for more efficient fusion. An optional part of the BTX system is the "Optimizer," by which the user can verify whether the parameters delivered are actually those that were selected. In most situations, however, using the Optimizer is not essential. Recently, needle-type

electrodes have also been developed *(62)*. In this system the cell couplets are sandwiched between two wires attached to micromanipulators; the role of the manipulators is to make orientation easier and hence, fusion more effective.

4. The membrane destabilization evoked by the DC pulse also induces fusion of two cell membranes that are in direct contact. Because of this reason, electrofusion is frequently used to fuse the enucleated oocyte with the nuclear donor cell. In this case the reconstructed oocytes should be transferred one by one between the electrodes and aligned—either with an AC current or manually, using a fine capillary—so that the contact surface between the cytoplast and the donor cell is parallel to the electrodes. Then fusion and activation will be triggered with the same DC pulse.
5. There are contradictory reports about the necessity to prevent second polar body extrusion after activation. It generally is accepted that if NT takes place before DNA replication in the donor cell (G_0-G_1 stage), extrusion of the second polar body after karyokinesis may result in the loss of chromosomes. Several possibilities exist for the prevention of the extrusion of the second polar body. Bovine oocytes incubated in the presence of cytochalasin B (the potent microtubule inhibitor) after a treatment with ionomycin completed the second meiotic division but did not extrude the second polar body resulting in the formation of multiple pronuclei. Alternatively, a 6-DMAP treatment after an induced calcium transient caused the second meiotic spindle to disintegrate and the oocytes progressed directly into interphase while forming only a single pronucleus *(63)*. Finally phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C was also reported to inhibit second polar body extrusion. Incubation of electroporated porcine oocytes with PMA induced activation of protein kinase C that subsequently triggered inactivation of MAP kinase. MAP kinase inactivation ultimately caused the oocytes to go directly from metaphase into interphase without extruding the second polar body *(64)*. However, inhibition of chromatin extrusion may not be beneficial if it occurs when the transferred nucleus is in G_2. When large embryonic stem cells in the presumptive G_2 stage were used to produce mice by means of NT, cytochalasin B was omitted from the culture medium following activation; it was present only when small cells (presumably prior to DNA replication) were used as nuclear donors *(65)*. There also seems to exist an endogenous control mechanism in the oocyte: chromatin extrusion was seen in 81% of porcine oocytes receiving 4N somatic cell nuclei (those in G_2 at the time of transfer) but in only 22% of oocytes receiving 2N nuclei *(66)*. A treatment with cytochalasin B dissolved in DMSO is most commonly applied to prevent the extrusion of the second polar body. However, DMSO recently has been found to affect murine embryonic development after NT. Because of this reason, the use of ethanol as a solvent for cytochalasin B might be preferred for experiments whose purpose is to investigate nuclear–cytoplasmic interactions *(67)*. With the use of ethanol special care must be taken to avoid evaporation to maintain the desired concentration in the medium.

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The authors provide readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer. Of immense benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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4

Culture Medium Preferences of Pre-Implantation Cloned Mouse Embryos

Björn Heindryckx, Josiane Van der Elst, and Marc Dhont

Summary

Somatic cloning technology involves the transfer of a somatic cell nucleus into an enucleated oocyte, followed by activation and in vitro culture. Efficiency in terms of live offspring generally remains very low. Little attention has been devoted so far to the impact of culture environment on cloned embryo development. Failure of genomic reprogramming of the donor nucleus in nuclear transfer (NT) experiments could lead to an altered phenotype in these cloned embryos that could be manifested by different medium preferences of the NT embryos. We describe here the application of sequential culture media to support preimplantation development of mouse embryos reconstructed using conventional NT techniques. Embryo-quality analysis was performed on NT blastocysts obtained. Additionally, NT embryos that arrested during development also were analyzed.

Key Words: In vitro culture; mouse cloned embryos; conventional method; sequential media; preimplantation.

1. Introduction

Current nuclear transfer (NT) technology involves the transfer of an adult or embryonic cell nucleus into an enucleated recipient oocyte, followed by artificial activation of and subsequent development of the reconstructed embryos. The success rate, in terms of live offspring, after nuclear transfer of differentiated adult somatic nuclei generally remains very low, usually not exceeding 3% in the mouse. The challenge is to understand the underlying reasons for this low efficacy. Little attention has been devoted so far to the possible effect of culture media on preimplantation development of NT embryos. Blastocyst formation rate of NT mouse embryos varies widely among different research groups and is not always described in detail *(1–7)*. Morula and blastocyst formation rates

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often have been combined as a single endpoint that makes it difficult to verify the exact percentage of blastocysts alone. Despite this, it is clear that development of NT embryos is lower than of in vivo or in vitro fertilized oocytes or parthenogenetic embryos, which can reach the morula/blastocyst stage at a rate of 83–99% when appropriate culture environment is used *(4,8–11)*.

In vitro culture of preimplantation embryos is very complex and requires strictly defined culture media to sustain both their viability and quality. The oviduct provides the mammalian embryo with a unique environment that nurtures the early embryo which is very hard to mimic in vitro. It is now generally is accepted that the in vitro culture environment exerts significant effects on embryo development. For example, certain strains of mouse embryos exhibit a two-cell stage arrest, but it can be overcome using optimised culture media. These early culture condition variations have the potential to exert long-time effects on development. Substantial progress in media development for in vitro culture of preimplantation mammalian embryos has been made since the first in vitro culture of mouse embryos was published by Whitten *(12)*, who reported the culture of eight-cell mouse embryos to blastocysts in a chemically defined medium. However, the best media currently used are still suboptimal and consequently cause imbalances and stress to the embryos. Gardner and Lane *(13)* have reported the use of sequential media for mouse in vitro culture, suggesting sequential media are more physiological and better able to address the changing metabolic needs of the developing embryo *(14,15)*.

The use of multistep medium protocols for mouse embryos was first described by Chatot et al. *(9,16)*, who reported the use of CZB medium, which lacks glucose, for the first-cycle divisions at which time glucose was supplemented to support blastocyst formation. The use of sequential media has been extensively introduced in human embryo culture systems and is now commercially available.

Preimplantation development of mouse NT embryos is dependent on the efficiency of reprogramming of the inserted donor somatic cell nucleus. Failure to switch the donor cell program into an embryonic state could lead to altered embryonic phenotypes and may result in gene expression products more typical for the introduced donor cell. Therefore, cloned embryos may require specific culture requirements. It is now clear that many genes control the development of embryos and that in vitro culture may disrupt the activity of these genes. A number of studies report that culture conditions can influence gene expressions *(17–20)* and demonstrate that the altered expression of genes is the result of suboptimal in vitro culture. Appropriate culture conditions can result in expression patterns of in vitro cultured mouse embryos that are similar to their in vivo counterparts *(21)*. Culture media also may exert a significant effect on the energy metabolism of preimplantation embryos, leading to

developmental blocks *(14)*. Another study *(22)* showed that embryos cultured in KSOM gave rise to blastocysts with a lower apoptotic cell number than M16. Apoptotic regulators may be perturbed by suboptimal culture conditions.

In our experiments *(23–25)*, on average, as much as 50% of the NT mouse embryos fail to complete the second cleavage division. This arrest may be related to the zygote-to-embryo genome activation switch, which takes place in the mouse at the early two-cell stage *(26)*. By adaptation of culture media, it may be possible to overcome this defective development analogous with overcoming the so-called two-cell block in some mouse strains. Indeed, we have shown that by proper selection of culture medium, KSOM medium facilitates this second cleavage division of NT mouse embryos *(23)*. The high rate of arrest at the two- and three-cell stage and the high incidence of binucleated blastomeres *(23)* may point to a significant difference between NT embryos and embryos from strains exhibiting the "two-cell block." According to our observations this multinucleation was the result of karyokinesis without cytokinesis, probably the result of factors specifically related to the NT embryos. We further investigated whether the use of sequential culture media such as KSOM/G_2 and G_1/G_2 were superior in supporting preimplantation development of NT mouse embryos compared with a monoculture such as CZB, which has been used successfully by many laboratories *(1,2,4,6)*. We found that the blastocyst formation rate of NT embryos can be increased by using the sequential KSOM/G2 media instead of G_1/G_2 and that both two-step culture systems performed better than CZB medium used for the whole culture. Surprisingly, both sequential media performed equally in supporting development of parthenogenetically activated control embryos. The significantly different development of NT embryos in KSOM/G_2 vs G_1/G_2 medium may refer to differential ability of both media to sustain gene expression in the reconstructed NT embryos. Strikingly, this difference is not observed in the control embryos, revealing that NT embryos are more sensitive and may require other culture conditions to obtain transcriptional stability and subsequent full development. By selection of appropriate mono- or multistep culture systems, it may be possible to improve cloned embryo development.

Recently, two studies confirmed our findings about the influence of culture media on preimplantation development of cloned mouse embryos. Chung et al. *(27)* showed the beneficial effect of glucose during the first divisions and that culture in Whitten medium or initially in Whitten medium and then changing to KSOM at the late four-cell stage is superior over the use of CZB medium alone. Gao et al. *(28)* showed that NT embryos using myoblast donor nuclei fail to thrive in standard embryo culture media. These myoblast-cloned embryos require somatic cell-like culture conditions, which differ from those required by normally fertilized or parthenogenetic control embryos as we also

demonstrated *(23)*. They also stated that the reproductive tract environment may not be optimal for some cloned embryos, which require somatic cell specific culture conditions. So, an optimized culture environment could be critical for maintaining the cloned embryo homeostasis to minimize the stress caused by suboptimal culture and to support the reprogramming process.

This chapter will present the protocol used in our laboratory to create mouse cloned embryos in specific culture media using a conventional method of NT. The manufacture of the used culture media, the conventional NT procedure and the blastocyst quality analysis will be emphasized.

2. Materials

2.1. Mice for NT and for Zygote Culture Quality Testing

1. Female B6D2 F1 (C57Bl / 6 J ×DBA / 2) hybrid mice, 8–12 wk of age, were obtained from IFFA Credo (Brussels, Belgium) and were used as a source of recipient oocytes and cumulus nuclear donor cells for NT.
2. Female B6D2 F1 hybrid mice, 8–15 wk of age, and male CD1 outbred mice, 8–50 wk of age (IFFA Credo) were used for the zygote culture quality test.
3. All mice were kept under controlled temperature (23 ± 2°C) and lighting conditions (inverted light/dark cycle regime with 14 h light, from 11:00 AM to 01:00 AM, and 10 h of dark) and were given food and water ad libitum.

2.2. Disposables, Glassware, and Microscopy

1. 30-mm Petri dish (Falcon cat. no. 353001, VWR, Leuven, Belgium).
2. 60-mm Petri dish (Falcon cat. no. 353002, VWR).
3. Four-well plates (Nunclon, Gibco cat. no. 176740, NV Invitrogen SA, Merelbeke, Belgium).
4. Stericup 1000 mL vacuum, 0.22 µm, GV Durapore Membrane (cat. no. SCGVU10RE, Millipore, Brussels, Belgium).
5. 50-mL Culture flasks (Falcon cat. no. 353014, VWR).
6. Microneedles (Drummond microcaps 30 µL, Ankersmit, Edegem, Belgium) were used to prepare micromanipulation pipets (holding pipet, partial zona dissection [PZD] pipet, and injection pipet) with a Narishige's puller PB-7 and microforge MF-9 (Omnilabo, Aartselaar, Belgium).
7. Pasteur pipets with cotton plug (Hilgenberg, Novolab cat. no. 3151101, Geraardsbergen, Belgium) for handling of cumulus oocyte complexes, oocytes, and embryos.
8. The micromanipulation setup consisted of hydraulic Narishige's micromanipulators MO-202, micro-injectors IM-6 (Omnilabo), and an Olympus IX 70 inverted microscope with Hoffman modulation contrast (Olympus, Omnilabo).
9. Axioplan 2 fluorescence microscope (Carl Zeiss, Zaventem, Belgium).
10. Stereomicroscope (Olympus SZ 60, Omnilabo).

11. Sterivex-GV Millipore 0.22-µm filter unit (cat. no. SVGV01ORS, Millipore).
12. Osmolarity meter (Osmomat 030-D Gonotec, Van Hopplynus, Brussels, Belgium).
13. Temperature regulator for microscopic stage: Techne Microscope Thermal Stage MTS-1 (Techne, Proton-Wilten, Antwerpen, Belgium).
14. Eppendorf tubes: 0.5 and 1.5 mL (for aliquots); (Sigma-Aldrich Chemie, Bornem, Belgium).
15. 5-mL test tubes, round-bottomed (VWR).

2.3. Culture Media

All the components for preparation of KSOM and KSOM–HEPES (**Table 1** and **ref.** ***29***) were purchased from Sigma (Sigma-Aldrich Chemie) unless stated otherwise (*see* **Note 1**). KSOM and KSOM–HEPES were prepared in the laboratory. During manipulation and incubation, all culture drops are overlaid with mineral oil (M-8410, Sigma).

1. G2.2. medium: commercially obtained (Vitrolife Sweden AB, Kungsbacka, Sweden) (**Table 1**).
2. Cook blastocyst medium: commercially obtained (Cook Ireland LTD, Limerick, Ireland; **Table 1**).
3. KSOM (500 mL; weights/molarity; **Table 1**): All components should be stored at room temperature (RT), unless stated otherwise: NaCl (S-7653), KCl (P-5405), KH_2PO_4 (P-5655), $CaCl_2$ $2H_2O$ (C-7902), $MgSO_4$ (M-2643), $NaHCO_3$ (S-6014), pyruvic acid (P-4582, stored at 4°C in dessicator), lactic acid (L-7900, stored at 4°C), D-glucose (G-6152), ethylene diamine tetraacetic acid (EDTA; E-6758), L-glutamine (G-5763; *see* **Note 2**), bovine serum albumin (BSA), Fraction V, Crystalline (BSA cat. no. 103700, MP Biomedicals Europe NV/SA, Asse-Relegem, Belgium) stored at 4°C; phenol red (P-3532), penicillin G (P-4687), streptomycin sulfate (S-6501, stored at 4°C in dessicator), and embryo-tested water (W-1503). All components (except for bovine serum albumin [BSA]) are dissolved in ± 300 mL of embryo-tested water. Then, the osmolarity are adjusted to 265 mosm by adding water and the medium subsequently is filtered (0.22 µm). Aliquot 10 mL in 14-mL plastic tubes (Falcon; cat. no. 352057) and freeze to –20°C. For experimental use thaw one tube, add 4 mg/mL BSA, and filter it. We use these thawed tubes for 5 d stored at 4°C. For artificial activation of the oocytes, use the same protocol as for KSOM with omission of Ca^{2+}.
4. KSOM–HEPES (500 mL; weights/molarity; **Table 1**): KSOM–HEPES is prepared using a similar procedure as for KSOM with addition of HEPES (Gibco BRL; cat. no. 15630-056, Invitrogen) stored at 4°C and without phenol red. Adjust the osmolarity to 285 mosm for KSOM–HEPES and pH to 7.2 to 7.4. Add 4 mg/mL BSA and filter-sterilize it using the Sterivex 0.22-µm filter. Aliquot this 500 mL of KSOM–HEPES into 50 mL of culture flasks (20 mL per flask) and store at 4°C. These flasks can be used for up to 2 mo.

Table 1
Composition of KSOM, KSOM–HEPES, G2.2., and Cook Blastocyst (in m*M*, Unless Stated Otherwise)

	KSOM	KSOM–HEPES	G2.2.[a]	Cook blastocyst[b]
NaCl	95	95	90.08	+
KCl	2.5	2.5	5.5	+
KH_2PO_4	0.35	0.35	–	+
NaH_2PO_4	–	–	0.25	–
$CaCl_2.2H_2O$	1.7	1.7	–	
$CaCl_2$	–	–	1.8	+
$MgSO_4$	0.2	0.2	1.0	+
$NaHCO_3$	25.0	5.0	25.0	+
Na-pyruvate	0.2	0.2	0.1	+
Na-lactate	10.0	10.0	5.87	+
Glucose	0.2	0.2	3.15	+
EDTA	0.01	0.01	–	–
Glutamine (*see* **Note 2**)	1.0	1.0	–	+
Alanyl-glutamine (*see* **Note 2**)	–	–	1.0	–
HEPES	–	20.0	–	–
BSA (mg/mL)	4.0	4.0	–	–
Phenol red (g/L)	0.01	–	0.005	–
Penicillin G (IU/mL)	100	100	–	
Penicillin (g/L)	–	–	0.06	
Streptomycin sulfate (mg/mL)	5.0	5.0	–	
Gentamycin	–	–	–	+
Human serum albumin	–	–	5 mg/mL	+

[a]Quantitative composition of G2.2 is published by Lane et al. (*29*), where the full list of all included amino acids is published.

[b]For Cook blastocyst only the qualitative composition is known, amino acids are not shown here.

BSA, bovine serum albumin.

2.4. Nuclear Transfer

1. Equine chorionic gonadotropin (eCG) and human chorionic gonadotrophins (hCG; Folligon, 1000 IU and hCG, Chorulon, 1500 IU) were purchased from Intervet, Turnhout, Belgium and were prepared in stock concentrations of 100 IU/mL (10X).

Dissolve Folligon and Chorulon in 5 mL of commercial solvent and 15 and 25 mL resp. 0.9% NaCl (S-8776, stored at 4°C), aliquot 0.4 mL in 0.5-mL tubes and store at –20°C.
2. Micropipets were filled with a highly viscous inert fluid, dimethylpolysiloxane (DMPS-12M, Sigma).
3. Fetal bovine serum (FBS; Gibco BRL cat. no. 101108-157) 20%; 100% FBS (frozen at –20°C) was diluted in KSOM–HEPES to a final concentration of 20% and kept in the refrigerator (4°C) for up to 1 mo. Aliquot 3 mL in 5-mL tubes.
4. Polyvinylpyrrolidone (PVP) solution (ICSI-100, Vitrolife Sweden AB) stored in the refrigerator.
5. Cytochalasine D (C-8373, Sigma) stored in stock concentration of 1 mg/mL (1000X) diluted in 99.5% dimethyl sulfoxide (D-5879, Sigma) with 10-µL aliquots in 0.5-mL Eppendorf tubes stored at –20°C. For each experiment, one tube was thawed, and 1 µL of the stock concentration was diluted in 1 mL of KSOM–HEPES for enucleation of oocytes (final concentration 1 µg/mL for enucleation; for activation: 2 µg/mL).
6. Strontium chloride ($SrCl_2$; S-0390, Sigma) diluted in water in a stock concentration of 1 *M* (100X) and stored at RT.

2.5. Staining

2.5.1. Nuclei Staining

Hoechst staining of blastomere nuclei: Hoechst 33528 (B-2883;10 mg/mL, 1000X) stored at 4°C.

2.5.2. Giemsa Staining

1. Fixation solution: methanol:acetic acid 3:1. Methanol (M1770, Sigma) and acetic acid (A-6283, Sigma) are mixed, stored at –20°C in 50-mL culture flasks and used for up to 3 d.
2. Hypotonic solution: 75 m*M* KCl (P-5405, Sigma; solved in embryo-tested water).
3. Stain: 3 mL of modified Giemsa staining (GS-500, Sigma; 0.4%, w/v) diluted in 60 mL of water.

2.5.3. Differential Staining

1. Acid Tyrode solution (T-1788, Sigma), stored at –20°C.
2. Trinitrobenzenesulphonic acid (TNBS; 5%, P-2297) dissolved in phosphate-buffered saline (PBS; P-4417) containing 3 mg/mL PVP, final concentration TNBS 0.5%, stored at 4°C (light-sensitive).
3. Rabbit anti-dinitrophenyl (DNP)-BSA antiserum (D9656; 2 mL): aliquot 100 µL in 0.5-mL tubes and store at –20°C.
4. Guinea-pig serum complement (S-1639) dissolved in 5 mL of deionized water (ice-cold) and aliquot 60 µLin 0.5-mL tubes and store at –20°C.
5. Propidium iodide (PI; P-4170; stock: 1 mg/mL); aliquot 30 µL in 0.5-mL tubes and store at –20°C.
6. Ethanol (99.7–100%, L530606, BDH Laboratory, Asse, Belgium) at RT.
7. Hoechst 33528 (10 mg/mL, 1000X) stored at 4°C.

3. Methods

The methods described below discuss (1) mouse zygote culture quality test, (2) NT technology, (3) activation and culture system, and (4) embryo-quality analysis.

3.1. Mouse Zygote Culture Quality Test

Before any new prepared or new batch of commercially obtained medium is used in our experiments, these media undergo the mouse zygote culture test in at least two replicate experiments with additional analysis of blastocyst quality.

1. Superovulate B6D2 females by injections of 7.5–10 IU of eCG (0.075–0.1 mL) and 7.5–10 IU of hCG at a 48-h interval.
2. Immediately after hCG injection, put one female per male (CD1 outbred) and check for a copulation plug the next morning to ensure the identification of all mated females.
3. Dissect out oviducts from the mated females, 21 h after hCG, in KSOM–HEPES medium and harvest zygotes from the ampullae of the excised oviducts.
4. After a brief exposure to 200 IU/mL hyaluronidase (maximum of 2 min is sufficient) for dispersing the cumulus cells, wash zygotes and randomly divide over the new medium (media) and a medium of proven good quality (control; *see* **Note 3**).
5. Embryo culture (*see* **Note 4**). For a monoculture system put the zygotes in 50-µL culture drops in a 60-mm Petri dish overlaid with mineral oil in a quantity of approximately 20 zygotes per culture drop. For a two-step sequential culture, put the zygotes in the first medium until 70 h after hCG and then transfer them to the sequential medium.
6. Embryos should be checked at 43 h after hCG (two-cell) and at 115 h after hCG (blastocyst). At least 90% of the cultured zygotes should form two-cell embryos, with a minimum of 85% and preferably more than 90% of these two cells developing into blastocysts.
7. To assess morphology and embryo quality, divide blastocysts into five different categories: (1) blastocysts showing less than half blastocoel cavity, (2) blastocysts showing more than half blastocoel cavity, (3) expanded blastocysts, (4) blastocysts that start hatching, and (5) completely hatched blastocysts.
8. At 116 h after hCG, place blastocysts in hypotonic solution in a 30-mm Petri dish for 8 min.
9. Transfer them into the ice-cold methanol/acetic acid fixation solution for at least 2 min (*see* **Note 5**).
10. Prepare a glass slide by marking it with a scratch (diamond pen) at the reverse side of the slide, put the blastocysts next to the scratch, and let air-dry at RT.
11. At least 2 d after fixation, blastocysts should be stained in Giemsa solution for at least 20 min and air-dried.
12. Count the total cell number. In our quality tests, blastocysts show a total cell number of at least 55 cells per blastocyst at 116 h after hCG (*see* **Fig. 1**)

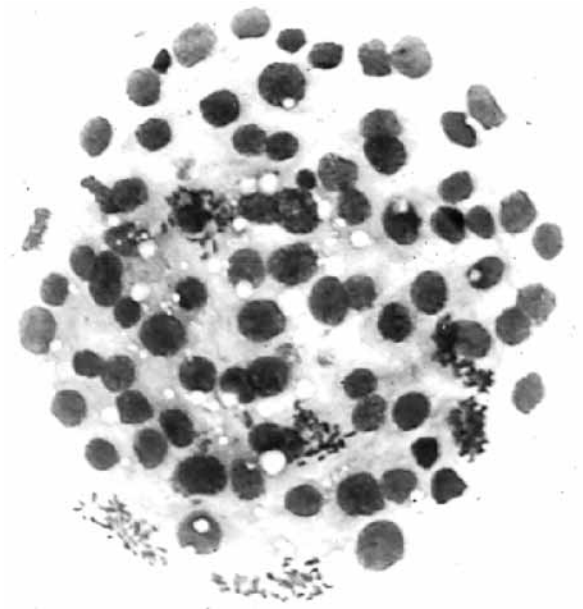

Fig. 1. Zygote culture quality test: Giemsa-stained blastocyst at 116 h after human chorinic gonadotropin.

3.2. NT Technology (see *Fig. 2*)

3.2.1. Experimental Design (see ***Note 6***)

When the influence of culture media on preimplantation development of mouse-cloned embryos is tested, at least four replicate experiments should be carried out using media from the same stock (when self prepared media are used) or from the same batch (when commercially obtained media are used). In one experiment, at least 100 oocytes should be collected (minimum four stimulated mice) and divided over:

1. NT group: partial zona-dissected and enucleated oocytes injected with cumulus cell nuclei (80 oocytes).
2. Medium control group: oocytes not subjected to micromanipulation, which serve as a control of the culture environment (parthenogenetic activated oocytes; at least 20 oocytes).

We are currently using two sequential culture media: KSOM/G2.2 and KSOM/Cook blastocyst medium for the development of NT embryos reconstructed with cumulus cell nuclei using a conventional method.

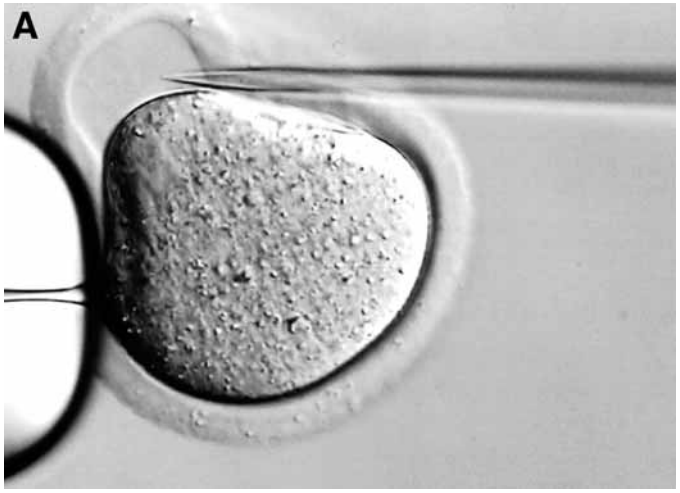

Fig. 2. Nuclear transfer protocol (conventional method): **(A)** Partial zona dissection, **(B)** removal of oocyte chromosomes, **(C)** selection of cumulus cells, and **(D)** injection of donor cumulus cell into enucleated oocyte.

3.2.2. Collection of Recipient Oocytes

1. Superovulate B6D2 F1 females by intraperitoneal injection of 7.5–10 IU eCG followed 48 h later by 7.5–10 IU hCG.
2. Collect the cumulus–oocyte complexes at 13–14 h after hCG in KSOM–HEPES.
3. Remove the cumulus cells by transferring the oocyte–cumulus complexes into 50-µL droplets of KSOM–HEPES supplemented with 200 IU/mL hyaluronidase for 5 min (*see* **Note 7**).
4. Wash the oocytes thoroughly, to ensure they are free from hyaluronidase (to avoid spontaneous activation of the oocyte), by rinsing in several droplets of KSOM–HEPES. Place approx 80 oocytes in 3- to 5-µL droplets of KSOM–HEPES (20 oocytes per drop) under mineral oil in the cover of a 60-mm Petri dish for partial zona dissection.
5. Transfer remaining oocytes to preincubated KSOM medium in a 6% CO2 incubator in air in 50-µL culture droplets (maximum 20 oocytes per droplet) to serve as medium controls.

3.2.3. Preparation of Donor Somatic Cells

1. Collect cumulus cells, which serve as donor somatic nuclei, as quickly as possible out of the hyaluronidase, and place in a 1.5-mL Eppendorf tube.
2. Add 1 mL of KSOM–HEPES to the tube.

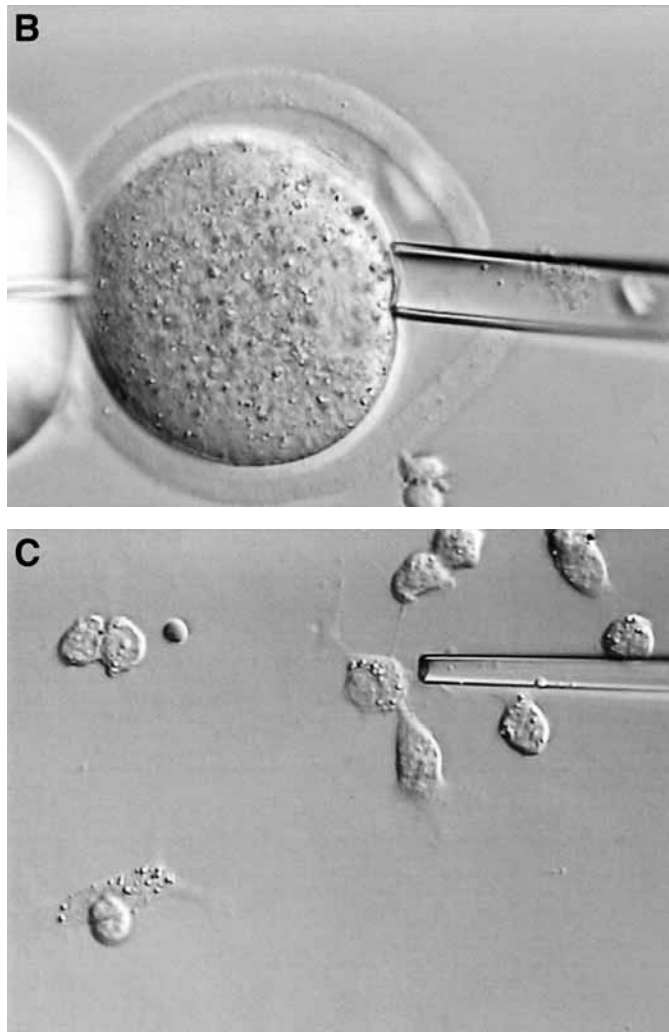

Fig. 2. (*continued*)

3. Centrifuge twice (500*g*, 10 s) and keep the formed pellet of cumulus cells.
4. Transfer the cumulus cells to a central 3- to 5-µL droplet of KSOM–HEPES under mineral oil in a manipulation dish at RT (22–25°C) for up to 3 to 5 h until injection.
5. Add 8% (w/v) PVP solution to the droplet of cumulus cells (in a 1:1 proportion of KSOM–HEPES:PVP) at 30 min to 1 h before nucleus injection. This allows the cumulus cells to spread and attach at the ground surface of the dish and avoids stickyness during injection. Use medium-sized cumulus cells (8–13 µm) for injection.

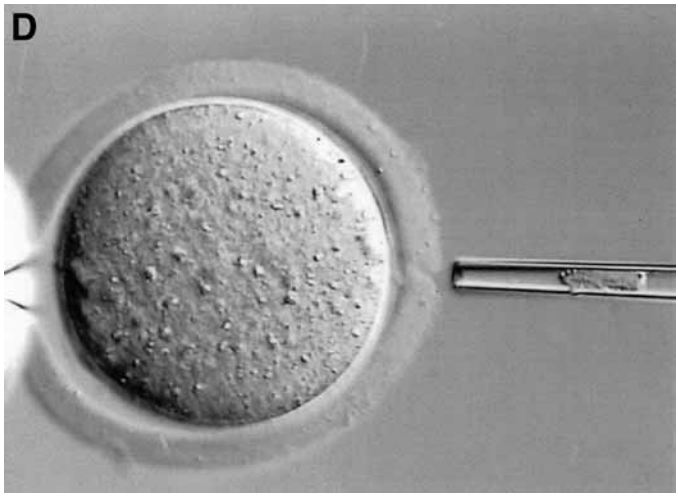

Fig. 2. (*continued*)

3.2.4. NT by Conventional Method

3.2.4.1. Partial Zona Dissection

1. Immobilize the oocyte with the holding pipet, keeping the spindle complex, visible as a translucent, nongranulated area at 12 o'clock position.
2. Pierce the sharp needle through the zona pellucida beginning at about 2 o'clock and ending at about 10 o'clock to obtain 10–15% of the circumference of the zone pellucida.
3. Release the oocyte from the holding pipet and rub the PZD pipet (on which the oocyte is impaled) along the length of the holding pipet until the zona is cut, automatically releasing the oocyte. It takes around 35 min to make this tangential slit opening in 80 oocytes.
4. Place dissected oocytes into KSOM medium in the incubator.

3.2.4.2. Enucleation

1. Place the oocytes (in groups of 20 each time) in KSOM–HEPES-supplemented with 1 µg/mL cytochalasine D.
2. After 5 min, start removing the chromosome-spindle complex, using a blunt polished pipet with inside diameter of 10–15 µm, through the slit made by PZD. Avoid aspirating excessive amount of surrounding cytoplasm.
3. Wash the oocytes free of cytochalasine D with KSOM–HEPES, in a 30-mm Petri dish and place back in KSOM medium in the incubator for up to 4 h before being used for nuclear injection.

3.2.4.3. Injection of Donor Somatic Cumulus Cell Nuclei (*see* **Note 8**)

Inject oocytes in 3- to 5-µL droplets in the manipulation chamber in the presence of 20% FBS, diluted in KSOM–HEPES.

1. Set up the micromanipulation dish with a vertical line of five 3- to 5-µL drops with: (1) KSOM–HEPES + 20% FBS, (2) KSOM–HEPES, (3) central drop: cumulus cells in KSOM + PVP (1:1), (4) KSOM–HEPES, and (5) KSOM–HEPES + 20% FBS, starting from the top.
2. Incubate the first group of oocytes (20 oocytes) in 50-µL culture droplets of KSOM–HEPES with 20% FBS for 15 min at RT and transfer them to the manipulation dish.
3. Place 10 oocytes each in drop (1) and (5) and let the oocytes cool down for 15 min before starting of injection on a pre-cooled microscope stage of 15–17°C.
4. Aspirate two to four nuclei into the injection pipet (blunt, 6- to 7-µm inner diameter) and aspirate the cumulus cells in and out repeatedly until the nucleus is freed from its cytoplasm.
5. Move the injection pipet to the droplet of oocytes and insert the needle, through the previously made slit in the zona, deep into the oocyte creating an invagination of the oolemma. At this time the oocyte membrane is still intact.
6. After bringing the cumulus cell nucleus to the very tip of the injection needle, start aspirating oocyte cytoplasm slowly until breakage of the membrane, visible by the sudden movement of the ooplasma and the lack of tension of the oolemma.
7. Inject the cumulus cell nucleus into the ooplasm with the smallest amount of accompanying injection medium possible.
8. It should take 30 min to inject a group of 20 oocytes. After injection of all oocytes in one group, leave for 15 min on the cooled stage and incubate for an additional 15 min at RT before transferring back to KSOM medium in the incubator for up to 1–3 h before artificial activation.

3.3. Oocyte Activation and Embryo Culture

3.3.1. Activation

Activate the oocytes (both NT and medium controls) in calcium-free KSOM medium containing 10 m*M* $SrCl_2$ and 2 µg/mL cytochalasine D during 6 h. Sr^{2+}- ions are used to induce artificial activation, whereas cytochalasine D prevents extrusion of a second polar body to maintain the correct diploid ploidy of the reconstructed cloned embryo. Check the number of activated oocytes, by the presence of predominantly two pseudo-pronuclei at 6 h after activation and wash through 50-µL droplets of KSOM.

3.3.2. Culture

Place activated oocytes in 50-µL droplets of KSOM medium (20 per drop at 37°C in a 6% CO_2 atmosphere). Check two-cell, three/four-cell formation 20 h and 44 h, respectively, after activation (start of activation). After 50–60 h after activation, randomly divide embryos between G2.2 and Cook blastocyst medium (both NT and medium controls) and check compacted morula and blastocyst formation rates at 68 h and 110 h, respectively, after activation (*see* **Fig. 3**).

3.4. Embryo Quality Analysis (Nuclei and Differential Staining)

3.4.1. Nuclei Staining

Place NT-reconstructed embryos that arrest, at the two- or three-cell stage at 48 h after activation, in 10 µg/mL Hoechst 33258 dissolved in KSOM–HEPES (warm plate at 37°C) for 10 min. Rinse the embryos in 50-µL drops of KSOM–HEPES and check under ultraviolet light. Make a note of the number of nuclei in each blastomere (*see* **Fig. 4**).

3.4.2. Differential Staining

The trophectoderm and inner cell mass cells of blastocysts were differentially labelled with polynucleotide-specific fluorochromes at 110–115 h after the start of activation by a differential staining technique described by Hardy et al. ***(30)***, which was slightly modified.

3.4.2.1. Preparation of Solutions

1. In a 60-mm Petri dish, prepare 3X 50-µL drops acid Tyrode and 5X 50-µL drops KSOM–HEPES and overlay with oil.
2. Add 0.5 mL TNBS solution in each well of a four-well plate and keep at 4°C.
3. Thaw anti-DNP serum and mix 90 µL of anti-DNP with 210 µL of KSOM–HEPES in a 1.5-mL Eppendorf tube. Make 50-µL culture drops of this mixture in a 60-mm Petri dish and place on the warming tray.
4. Prepare 9X 50-µL drops of KSOM–HEPES over a 60-mm Petri dish (washing medium).
5. Thaw complement and PI and mix 50 µL of complement, 5 µL of PI, and 200 µL of KSOM–HEPES in a 1.5 mL of Eppendorf. Put 50-µL drops of this mixture in a 60-mm Petri dish.
6. In two wells of a four-well plate, place 1 mL KSOM–HEPES supplemented with 10 µL of PI. Fill the other two wells with 1 mL of ethanol and store at 4°C.
7. Put 1 µL of Hoechst 33258 in 1 mL of ethanol in each well of a four-well plate and store at 4°C.

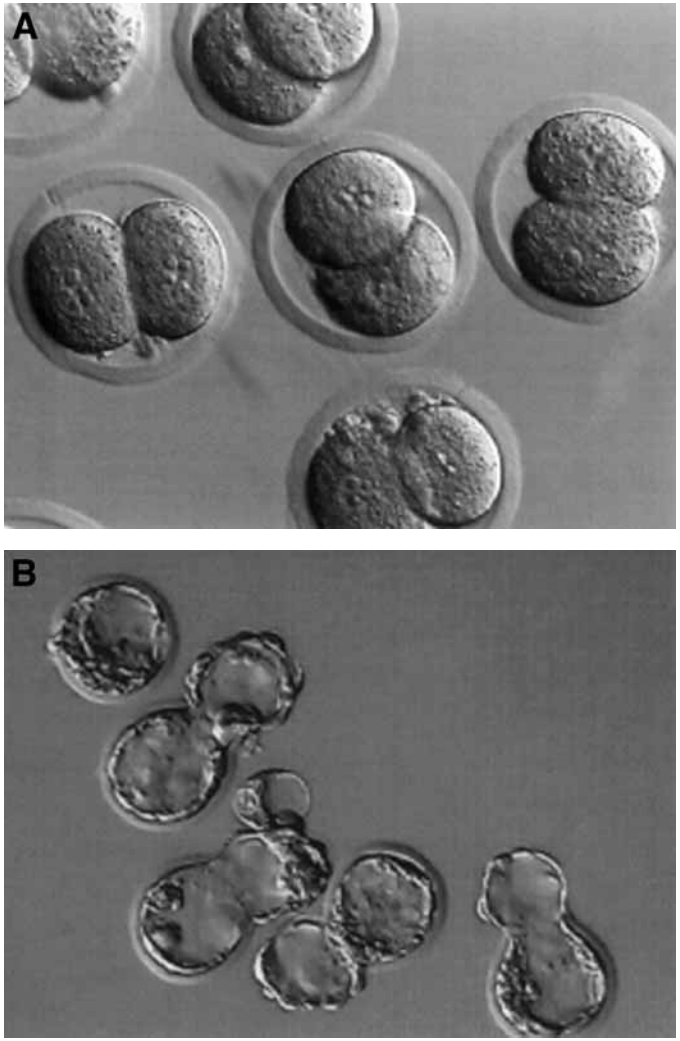

Fig. 3. Nuclear transfer embryos at 20 h **(A)** and 110 h **(B)** after activation, respectively.

3.4.2.2. Staining Procedure

1. Remove the zona pellucida by exposing the blastocysts (when still surrounded by a zona) to pre-warmed (37°C) acid Tyrode solution (pH 2.1) for a few seconds. Monitor constantly under the stereomicroscope, remove blastocysts immediately and wash away any remaining acid Tyrode through several 50 µL of KSOM–HEPES droplets. (*see* **Note 9**)

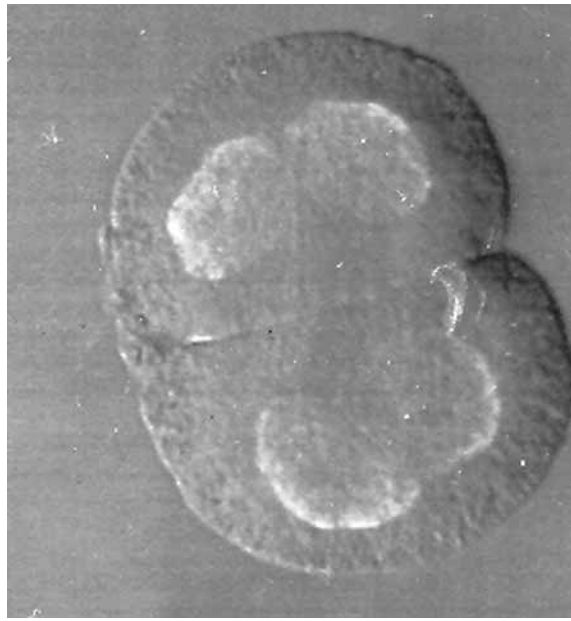

Fig. 4. Nuclear transfer-arrested two-cell embryo at 48 h after activation showing binucleation in both blastomeres.

2. Incubate the zona-free blastocysts in 0.5% TNBS in PBS containing 3 mg/mL polyvinylpyrrolidone for 10 min at 4°C. Wash away excess TNBS by washing in a 30 mm dish of KSOM–HEPES.
3. Incubate the blastocysts for 10 min in 30% rabbit anti-DNP-BSA antiserum in KSOM–HEPES (37°C), and wash briefly through several 50-µL drops of KSOM–HEPES.
4. Treat blastocysts with 20% guinea pig serum complement supplemented with 20 µg/mL PI for 10 min (37°C).
5. Wash the blastocysts in BSA-free KSOM–HEPES + 10 µg/mL PI.
6. Fix blastocysts for 5 min in ethanol (4°C).
7. Stain blastocysts overnight in absolute ethanol supplemented with 10 µg/mL Hoechst 33528 at 4°C.
8. Transfer blastocysts into 2 mL of glycerol in a watch glass and transfer to 0.5-µL droplets of glycerol on a glass slide. Place a cover slip over the blastocysts and apply pressure to induce better spreading of the cells.
9. Count inner cell mass (blue) and trophectoderm (red–pink) nuclei under a fluorescent microscope (filter set 02). Attach a monitor to the microscope for accurate counting.

4. Notes

1. All reagents used for the preparation of mouse culture media should be of the highest tissue culture grade and handled using sterile techniques. It is further recommended that reagent batch tracking is performed as part of good laboratory practice.
2. L-Glutamine may be replaced in the future by a more stable form like glycyl-glutamine or alanyl-glutamine based on the possible negative build-up of ammonium published recently ***(30)***.
3. Zygotes should be carefully selected with unfertilized oocytes, zygotes with excessive granulation or enlarged perivitelline space being discarded.
4. Before using culture medium for in vitro culture, always preincubate (for equilibration) for 24 h in the incubator. Transferring of embryos from one medium to another should be accompanied by several washings in the new medium.
5. Perform a minimal 2-min fixation, but not much longer, to prevent the blastocyst from sticking to the wall of the transfer needle.
6. It is very important to work consistently and stick to a well-defined, structured protocol.
7. After a 3-min exposure to hyaluronidase, start mechanical removal of cumulus cells. It is important to minimize the exposure time of oocytes and cumulus cells to hyaluronidase.
8. For nuclear injection: For the first group after enucleation; wait at least 1 h and 30 min before injection and increase the incubation time to 30 min on the cooled stage. The first group always gives the lowest survival rate (average survival should be 75–80%). Groups of oocytes should be injected in order of their enucleation. Injection technique: first blow out injection medium to make a "pocket," then bring the nucleus at the tip of the needle and start aspirating ooplasm until the oolemma breaks, then inject the nucleus, if again you see tension appearing on the oolemma then perform repeated aspirations until no tension is apparent on the oocyte membrane! For efficient injection, do not withdraw the injection needle during injection.
9. Differential staining: be sure to remove the zona pellucida completely. This is sometimes difficult to visualize. If the zona is not removed completely than the whole blastocyst will stain blue. Follow the exact timing and washing steps for successful staining.

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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5

Maintenance of Pregnancy in Pigs With Limited Viable Embryos

Tim King and Paul A. De Sousa

Summary

Interest is increasing in assisted reproductive technologies in the pig involving embryo cryopreservation, cloning, and genetic modification. Although inherently inefficient and variable in their outcome, the successful application of these techniques in this species is confounded by unique mechanisms for pregnancy recognition and maintenance that require a minimum number of viable embryos during a critical window of time early in gestation. These mechanisms require both local and systemic interactions between conceptuses and the maternal reproductive axis. Here, we describe a method wherein cotransfer of parthenogenetic embryos with the capacity for limited development can be used to sustain the pregnancy of fewer than four viable conceptuses to term.

Key Words: Pregnancy maintenance; parthenogenetic embryo; somatic cell nuclear transfer; cloning.

1. Introduction

Pigs are litter-bearing animals, normally delivering approx 11 piglets. Small litters are unusual, largely because of the system of early pregnancy recognition in the pig (for review, *see* **ref.** *1*). The normal reproductive cycle of a pig lasts 21 d, with ovulation occurring on day 1. Unless specific signals are received, the uterus secretes increasing amounts of prostaglandin into the blood stream from days 12 to 16 of the cycle, causing the corpora lutea, which are formed from follicles after they have ovulated, to regress, and allowing the recruitment and development of another group of follicles that will grow to a preovulatory size by day 21. These large follicles release increasing amounts of estrogen, which make the female pig receptive to the male and cause a surge of lutenizing

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hormone that triggers ovulation, starting the whole cycle over again. These cycles begin in pigs at approx 6–8 mo of age and continue until successful mating and fertilization takes place.

If the eggs released by the follicles are successfully fertilized, they will begin to develop. These developing embryos migrate around the uterus of the pig and, by day 10–11, are evenly spaced throughout the tract. The growing embryos secrete many products that give signals, some of which act locally whereas others act systemically. One such embryo-derived signal is estrogen, which redirects uterine cell secretion of prostaglandin to the lumen of the uterus (instead of into circulating blood). This redirection of prostaglandin prevents the regression of the corpora lutea. The continued presence of corpora lutea maintains increased levels of progesterone preventing the resumption of the pig's estrus cycle and so the maintenance of any pregnancy.

The significance of local embryo–uterine interactions in pregnancy recognition and maintenance was established in studies evaluating embryo migration, distribution, and spacing. A pig that by day 11 of the cycle does not have sufficient embryos to occupy more than 50% of the uterus will not maintain pregnancy. The minimum number of embryos required to occupy this area and give sufficient signal is thought to be around 4 ***(2)***. However, if sufficient embryos are present to occupy more than 50% of the tract but are restricted to only one-third of the tract, the pregnancy will not be maintained. Furthermore, if embryos (present in sufficient numbers to maintain pregnancy) are confined to one-third of the tract to day 10 of the cycle and then released, pregnancy is maintained. By contrast, the pregnancy is lost if embryos are released after day 11 ***(3)***. It has also been shown that all the embryos in one uterine horn can be removed after day 15 without compromising maintenance of pregnancy to term for the remaining conceptuses ***(4)***. Thus, the long-term maintenance of pregnancy to term in the pig is critically dependent on early local interactions between embryos and the proximate uterine environment up until day 11–15 of gestation.

There is increasing interest in the use of embryo technologies in the pig. These technologies, such as cryopreservation, cloning, and genetic modification, remain inherently inefficient and variable in outcome. Because of the unique pregnancy-recognition mechanisms in the pig, large numbers of manipulated embryos often are required to ensure successful establishment of pregnancy. However, the use of large numbers of embryos at transfer poses two major problems: (1) on any given day of transfer, there may be only a limited number of valuable embryos available for transfer; and (2) if large numbers of embryos are transferred and the viability on that day is good, then overcrowding can result in embryonic loss. Thus, there is a need for a robust method of pregnancy maintenance in the pig that will allow transfer of limited numbers of viable embryos to a pig.

1.1. Artificial Pregnancy Maintenance

There have been several articles that describe the artificial maintenance of pregnancy in pigs *(5–8)*. These methods usually rely on the injection, or feeding, of hormones either to mimic the signals released by embryos (estrogen) or to artificially maintain progesterone levels at a level that prevents the normal cycles of pigs. Our studies have shown these methods to have low efficiency *(9)*. In addition, there may be occasions when the transferred embryos develop at a slower rate than would normally be expected. Injection of hormones may alter the environment in the uterus prematurely and actually kill these slowly developing embryos *(10)*.

An alternative method of support for these transferred embryos is to transfer them into a pig that already has been mated and has sufficient normal embryos to maintain a pregnancy *(11)*. Although this method would appear to be the simplest answer to supporting pregnancy, it does have some major problems: (1) When a pregnancy is diagnosed by ultrasound, there is no way of telling whether the pregnancy contains the added high value embryos or is simply the result of embryos produced by mating the recipient; (2) The normal embryos that are of low value but are needed to maintain the pregnancy may overcrowd and prevent the added embryos from developing to term. This is highly likely as the added embryos may well already be compromised.

One of the most recent published solutions to pregnancy maintenance in the pig has been our method of co-transferring parthenogenetic embryos *(9)*. Parthenogenetic embryos are those that have been stimulated (i.e., activated) to begin development in the complete absence of a male gamete, that is, without fertilization. These can be readily produced from eggs collected from slaughterhouse-recovered ovaries. In several vertebrate species, parthenogenetic embryos have been shown to develop to postimplantation/attachment stages. In mice, parthenogenetic embryos fail at day 11 (term day 21 *[12]*). Parthenogenetic ovine embryos remain viable until day 21 of pregnancy (term, 150 d) but are dead by day 25 *(13)*. Published studies have shown that pig parthenogenetic embryos transferred into a recipient pig will develop to at least day 21 *(14)*, demonstrating that they are capable of releasing the correct signals to maintain pregnancy. Our own research has found that parthenogenetic pig embryos beginning to fail by day 30 and are dead by day 35 *(15)*, which would suggest that they are unlikely to cause overcrowding problems with any other embryos still developing within the uterus later in gestation when this would become an issue. The essential technical capacities required to achieve pregnancy maintenance by parthenogenetic embryo co-transfer should be amenable to any laboratory already attempting assisted reproductive technologies in this species. They include the ability to support (1) in vitro maturation of oocytes, (2) artificial activation of oocytes using electrical pulses or other established

means, (3) in vitro culture of embryos to at least the two-cell stage, and (4) surgical embryo transfer. In the balance of this chapter, we describe the requirements for these methods and the factors influencing their success.

2. Materials

All chemical reagents supplied by Sigma (Poole, Dorset, UK) unless otherwise stated.

2.1. Culture and Manipulation

1. Abattoir ovary collection medium: Dulbecco's phosphate-buffered saline (PBS; cat. no. BR14, Oxoid, Basingstoke, Hampshire, UK).
2. Oocyte wash: TL–HEPES–PVA (*16*).
3. Medium for oocyte maturation (modified NCSU23 [mNCSU23]): 108.73 m*M* NaCl, 4.78 m*M* KCl, 1.7 m*M* $CaCl_2$, 1.19 m*M* $MgSO_4$, 25.07 m*M* $NaHCO_3$, 1.19 m*M* KH_2PO_4, 5.55 m*M* glucose, 1 m*M* glutamine, 7 m*M* taurine, 5 m*M* hypotaurine, 100 IU/L penicillin-G, 50 mg/L streptomycin supplemented with 1 m*M* cysteine, and 5 mg/mL insulin.
4. Activation medium: 0.3 *M* mannitol, 100 µ*M* $MgCl_2$, 50 µ*M* $CaCl_2$.
5. Embryo handling (HEPES-buffered NCSU23): 131.7 m*M* NaCl, 4.78 m*M* KCl, 1.7 m*M* $CaCl_2$, 1.19 m*M* $MgSO_4$, 2 m*M* $NaHCO_3$, 10 m*M* HEPES, 1.19 m*M* KH_2PO_4, 5.55 m*M* glucose, 1 m*M* glutamine, 12 m*M* taurine, 0.4% bovine serum albumin-V, 100 U/L penicillin G, 50 mg/L streptomycin).
6. Embryo culture (NCSU23): 108.73 m*M* NaCl, 4.78 m*M* KCl, 1.7 m*M* $CaCl_2$, 1.19 m*M* $MgSO_4$, 25.07 m*M* $NaHCO_3$, 1.19 m*M* KH_2PO_4, 5.55 m*M* glucose, 1 m*M* glutamine, 7 m*M* taurine, 5 m*M* hypotaurine, 0.4% bovine serum albumin-V (A-7906, Sigma), 100 U/L penicillin-G, 50 mg/L streptomycin.

2.2. Animal Preparation and Surgery

1. Cloprostenol (Planate, Schering-Plough Animal Health).
2. Altrenogest (Regumate, Hoechst Roussel Vet., Milton Keynes, UK).
3. Equine chorionic gonadotrophin (eCG; Folligon, Intervet UK, Cambridge, UK).
4. Human chorionic gonadrotrophin (hCG; Chorulon, Intervet UK).
5. Etradiol benzoate (Estradiol benzoate, Intervet UK).
6. Ketamine (Vetalar V, Pharmacia and Upjohn, Crawley, UK).
7. Azaparone (Stresnil, Janssen Animal Health, High Wycombe, UK).
8. Midazalon (Hypnovel, Roche, Welwyn Garden City, UK).
9. Halothane (Halothane Vet, Merial Animal Health, Harlo, UK).

2.3. Laboratory Equipment

1. Fusion machine: BLS CF 150/B Electrical Impulse Generator (Budapest, Hungary) or equivalent.
2. Activation chamber: Constructed by coupling wire leads to two platinum electrodes, spaced at least 200-µm apart, and glued to the bottom of a glass Petri dish using araldite epoxy resin (*see* **Fig. 1**).

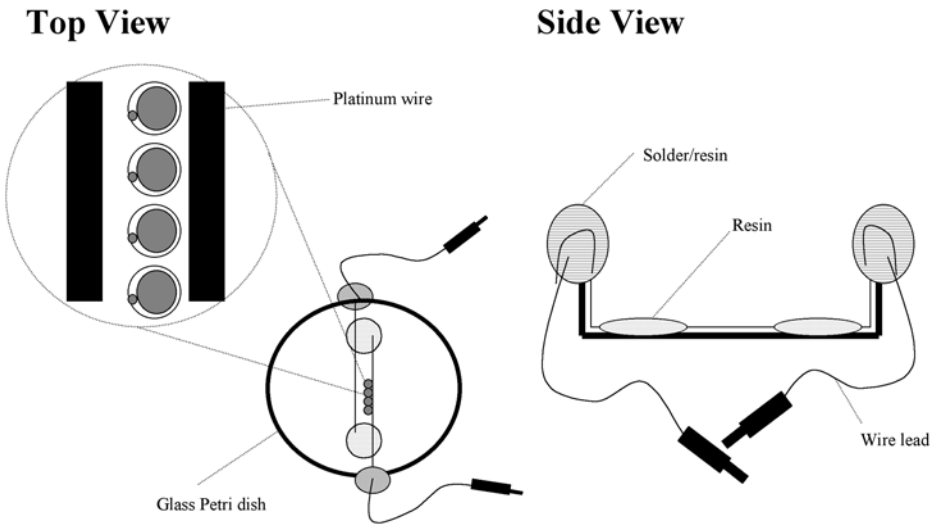

Fig. 1. Electrical activation (fusion) chamber. Two platinum wires, each approx 100- to 200-µm thick, are secured to the Petri dish using epon araldite resin. The gap between resin secured wires is kept constant, with an optimal width of approx 200 µm being sufficient to permit alignment of eggs in a single continuous row. Solder is used to connect platinum wires to clips and leads, whereas resin is used to fix all three to the side of the Petri dish.

3. Stereo dissecting microscope with a magnification range from 12 to 160X (i.e., Leica MZ 7.5–12.5 fitted with a 1X planar lens, an adjustable 0.8 to 10X magnification objective lens, and 16X oculars), fitted with a warming stage set at 38.5°C.
4. Fixed or variable gas incubator to provide 5% CO_2 in air atmosphere at 38.5°C.
5. Warming stages.

3. Methods

3.1. Oocyte Collection and In Vitro Maturation

1. Collect pig ovaries from a local abattoir, and maintain at 25–28°C in sterile PBS until returning to the laboratory within 2 h of retrieval (*see* **Note 1**).
2. Wash and maintain ovaries in Dulbecco's PBS at 37°C.
3. Aspirate follicles of 3–8 mm using an 18-gage needle attached to 10-mL syringe.
4. Wash follicular aspirant three times with 5–10 mL of TL–HEPES–PVA prewarmed to 37°C. Allow 1–2 min between washes to allow cumulus–oocyte–complexes (COCs) to settle.
5. Sort washed COCs in sterile 60-mm dishes on stages heated to 37°C.
6. Select only those COCs possessing at least three complete layers of compact cumulus for maturation.

7. Wash selected COCs three times with fresh TL–HEPES–PVA, followed by passage through 3X 500-µL drops of NCSU23 modified for oocyte maturation (mNCSU23).
8. Mature COCs in groups of 50 in 500 µL of mNCSU23 at 38°C in 5% CO_2 in air.
9. After 22 h, transfer COCs to mNCSU23 without hormones for a further 22 h of culture.
10. At end of maturation culture, strip oocytes until they are completely devoid of cumulus by manual pipetting.

3.2. Oocyte Activation and Embryo Culture

1. Activate cumulus-free oocytes 44–46 h after maturation (*see* **Note 2**).
2. Oocytes are activated electrically in activation media using three pulses of 80-µs duration at 1.0 kV/cm gap direct current (DC) after a 5-s pulse at 0.25 kV/cm gap alternating current (AC). The gap refers to the space between platinum electrodes. Thus, a 200-µm gap between electrodes would require the application of 20 V AC and 5 V DC. A larger or smaller gap would require an increase or decrease in the applied voltage, respectively (*see* **Note 3**).
3. Electrical activation is followed by three washes in HEPES-buffered NCSU23.
4. To maintain a diploid (2N) DNA content in parthenogenetically activated eggs, it is necessary to suppress the emission of a second polar body into which replicated chromosomes are segregated. This can be achieved by incubating activated eggs in NCSU23 containing 7.5 µg/mL cytochalasin B for 6 h at 38.5°C, in a 5% CO_2 in air atmosphere.
5. Activated oocytes are then washed by passage in at least 3–5 vol of NCSU23 before incubation overnight in the same.
6. If transfer takes place on the first day after activation, both uncleaved and cleaved parthenogenetic embryos should be transferred.
7. If transfer takes place on day 2 or more after activation, only cleaved embryos need be transferred.

3.3. Synchronization of Recipients

Establishment of pregnancy is reduced when transferred embryos are developmentally lagging with respect to the estrus cycle of the recipient ***(17)***. This reduction is especially marked after culture of embryos under suboptimal conditions ***(18)***. In our experience, embryo transfer requires that the development of transferred embryos be synchronous or 1–2 d in advance of the recipient gilt being in estrus (i.e., heat). To define the appropriate time to transfer embryos of interest and parthenogenetic helper embryos, one must consider when gilts would be likely to ovulate and the development of the recipient's eggs if they were fertilized by natural mating. Normally, ovulation and fertilization occurs 24–36 h after onset of estrus or if synchronized by gonadotropin injection 40–48 h after hGG. The timing of this event can be narrowed by periodic ultrasound scanning to monitor the disappearance of follicles ***[19]***). Although in

vitro developed embryos tend to develop slower than those in vivo, eggs begin to cleave to the two-cell stage by 24 h after activation or fertilization. Thus, if one-cell embryos are to be transferred they should be transferred to a gilt that is approx 24 h after estrus (synchronous). Alternatively, if embryos are to be transferred at the two-cell stage, then the recipient should be 48 h after estrus or less (asynchronous, with transferred embryos being advanced; *see* **Note 4**).

There are several methods for synchronizing pigs estrus cycles in relation to the day of embryo transfer. If enough gilts are available, selecting gilts that exhibit a natural heat on the required day is probably optimal (*see* **Note 5**). Otherwise, synchronization of their estrus cycle will be required. Two methods of synchronization are suggested (*see* **Note 6**).

3.3.1. Synchronization Treatment A

1. Cycling gilts are fed 20 mg altrenogest daily for 18 d.
2. After this treatment, estrus is detected by a boar once/twice daily.
3. Between days 11 and 16 after observed estrus, gilts are injected with 175 µg of cloprostenol and 1500 IU of eCG at 19.00 h. Eighty-three to eighty-eight hours later (11.00 h), these gilts are injected with 750 IU hCG.

3.3.2. Synchronization Treatment B

1. Gilts are estrus, detected by a boar daily.
2. Between days 11 and 15 after an observed estrus, gilts are fed 20 mg altrenogest once daily for 4 d and 20 mg altrenogest twice on the fifth day.
3. On the sixth day, gilts are injected with 1500 IU of eCG at 19.00 h.
4. Eighty-three to eighty-eight hours later, the gilts are injected with 750 IU hCG.

In both treatments, heat should be observed on the day after the hCG treatment.

3.4. Embryo Transfer

Many authors have described embryo transfer in the pig. A suitable technique would be as follows.

3.4.1. Anesthesia

1. Sedate recipient pig with and intramuscular injection of 6 mg/kg ketamine and 1 mg/kg azaparone.
2. Induce anesthesia by and intravenous injection of 2 mg/kg ketamine and 0.04 mg/kg midazolam.
3. Maintain anesthesia with 2.5% halothane in oxygen by inhalation.

3.4.2. Surgery

1. Place pig in dorsal recumbence.
2. Prepare surgical site by clipping of hair and sterilizing skin.

3. Make 10- to 15-cm midline incision of skin at level of second caudal pair of nipples.
4. Divide subcutaneous fat by blunt dissection (*see* **Note 7**).
5. Incise through muscle along the linea alba.
6. Incise peritoneum (*see* **Note 7**).
7. Expose uterine tract and exteriorize.
8. Puncture oviduct with a blunt 16-gage needle.
9. Transfer embryos into oviduct or uterus using a 3.5-French gage tom-cat catheter (*see* **Note 8**).
10. Repair peritoneum, muscle fat, and skin in separate layers using reabsorbable sutures.

3.5. Pregnancy Maintenance by Cotransfer of Parthenogenetic Embryos

Pregnancy is maintained by this method by transferring approx 60 uncleaved or 30 cleaved parthenogenetic embryos simultaneously with embryos of interest, on day 1 or 2 after activation, respectively. Synchronization and embryo transfer is as described previously.

3.6. Pregnancy Diagnosis

Early diagnosis of pregnancy by ultrasound at day 21 to 25 will not be able to distinguish between pregnancies resulting from valuable embryos of interest vs parthenogenetic cohorts. However, usually by day 30 and certainly by day 35, parthogenetic embryos will be significantly smaller (25-mm cf. 35-mm crown rump length) than normal embryos at this stage. Beyond day 35, parthenogenetic embryos are unlikely to have a visible heart beat and will not develop beyond 30 mm crown rump length. Although parthenogenetic pregnancies will still be visible up to day 50, at this stage, only fetal fluids are observed, and any fetus will either no longer be visible or will be extremely small.

4. Notes

1. Although the oocytes and embryos of all species are sensitive to temperature, this is especially true in the pig. Our experience in the pig suggests that transportation of abattoir collected ovaries to the laboratory within 2 h of their collection is best done at approx 25–28°C, with higher temperatures promoting oocyte atresia. Once in the laboratory, ovaries should be washed with saline at 37°C and kept at this temperature during follicular aspiration. Subsequent sorting and handling of COCs also should be at this temperature with variations avoided by working on heated surfaces, and with prewarmed media.
2. A broad range of physical and chemical stimuli are known to parthenogenetically activate mammalian oocytes and have been applied to pig oocytes (reviewed by ***[20]***). Those that have been used successfully in the pig for somatic cell nuclear transfer include electrical activation ***(11,19)***, and calcium ionophore followed by the serine threonine kinase inhibitor, DMAP ***(21)***. Our preferred method for pig

oocyte activation is using electrical stimulation for which we have found that oocyte age, voltage field strength, and pulse number and duration all affect developmental response ***(16)***.

3. The quality of the activation chamber is critical to reproducibility of the developmental response. This chamber can be purpose built in the laboratory or in an engineering workshop using a glass Petri dish or slide, as shown in **Fig. 1.** The optimal gap between platinum electrodes is approx 200 µm. It can range from approx 150 to 300 µm but it is essential that the gap is consistent across its entire length and should permit the deposition of a single row of eggs. The placement of these eggs need not be precisely linear, although it is important that only a single oocyte occupy the width of the gap at any given point along its length. Epoxy resin can be used to fix platinum electrodes to the glass surface. The space between beads of resin, in the centre of the dish (i.e., defining the useable length of electrodes for oocyte activation/fusion), should not exceed 1 cm. This should readily permit the deposition up to 50 oocytes aligned in a single row. A longer length than this can create variations in the gap width because of unevenness in the electrodes that may either exist initially or develop over time. When using the chamber care must be taken to avoid touching the electrodes with whatever instrument is used to deposit oocytes. Electrical activation is of course dependent on the conductivity of the medium it is performed in. We have found that 0.3 *M* mannitol 100 µ*M* $MgCl_2$, 50 µ*M* $CaCl_2$ is the optimum pig oocyte activation ***(22)***. Sugar-based media is preferred to salt-based media that are more conductive and heat generating if AC pulses are used (for discussion, *see* **ref.** ***23***). The number of eggs stimulated at any one time is dependent on the skill and experience of the manipulator, and should not exceed 50. To avoid contamination of the activation media in the chamber with the media that the oocytes were matured in, pools of oocytes should first be transferred into activation medium in a separate drop or dish. These can then be transferred either directly between electrodes, or to one of the flanking areas if serially activating smaller pools of oocytes. In total exposure to activation medium should not exceed 5 min. To minimize changes in the activation media composition resulting from evaporation and pipetting, medium should be changed frequently.
4. If embryos are transferred on the same day of activation, fertilization, or nuclear transfer, that is, before cleavage, they must be transferred into a gilt on her first day after estrus (day 1), which would be considered a synchronous transfer. Embryos should not be transferred into a gilt that is still exhibiting signs of estrus. If embryos are transferred at later stages of development some benefit may be obtained by transferring into a gilt in which estrus occurred 1 d later than the developmental stage of the embryos transferred.
5. The availability of recipients that have exhibited estrus and ovulated in synchrony with the developmental stage of the transferred embryos is critical. If there are sufficient gilts available on site, selection of those that exhibit an estrus naturally will be optimal as there is less chance of cystic ovaries because of hyperstimulation that may result from synchronization regimes. When gilts exhibiting a natural estrus are selected, it is important that this estrus should not be their first and that

the inter-estrus interval should be regular (i.e., between 18 and 23 d). If limited numbers of gilts are available, they must be synchronized using gonadotrophin treatments.

6. Of the two methods described, treatment B was found to be superior with far greater numbers of treated gilts exhibiting a synchronous estrus. Using this treatment, more gilts are found to ovulate within 48 h of the hCG injection and fewer gilts develop cystic follicles *(24)*. Using this method it is advisable to treat 10 gilts in order to obtain six recipients. To eliminate the risk of using a gilt that exhibits a synchronous estrus but has failed to ovulate, either because of a prolonged onset of estrus to ovulation interval, or the development of cystic ovaries, the ovaries may be observed using a transabdominal ultrasound examination *(25)*.
7. Surgical embryo transfer in the pig is conducted by a ventral midline approach to the uterus. As with any abdominal surgery, the length of time the peritoneal cavity is exposed, the amount of hemorrhage and the handling of any internal organs should be kept to a minimum. This is even more critical during embryo transfer if a successful pregnancy is to be obtained. To achieve this, the following should be considered:
    a. After a midline skin incision, it is important to divide the subcutaneous fat by blunt dissection. This reduces hemorrhage to a minimum and allows for easier identification of the linea alba. Incision of the muscle through the linea alba is essential to reduce hemorrhage and will simplify the suturing of the muscle layer following transfer.
    b. Before the incision of the peritoneum, the placement of stay sutures will speed up the final repair after transfer.
    c. The uterus must be handled gently and the minimum amount exteriorized before transfer. The uterus must also be kept moist while exteriorized with swabs soaked in warmed saline.
8. Embryos need only be transferred unilaterally because they will migrate and occupy both horns after transfer. Embryos are transferred using a 3.5-French gage tom-cat catheter. Embryos up to the four-cell stage should be transferred directly into the oviduct, which is best achieved by making a hole in the oviduct around the ampulla with an 18-gage blunt-ended needle then advancing the catheter proximally at least 5 cm before depositing the embryos. Embryos beyond this stage of development should be transferred into the uterus. This is best achieved by making a hole in the oviduct about 2 cm from the uterotubular junction. The catheter is then advanced through the uterotubular junction into the tip of the uterine horn before depositing the embryos. After the removal of the catheter, no suturing of the oviduct is required. The number of parthenogenetic embryos required to support a pregnancy will depend on the efficacy of activation protocols in individual laboratories. Although these can be established in advance, we suggest that for any given experiment a sample of 25 or more parthenogenetic embryos be permitted to develop to the blastocyst stage in vitro, to relate back to pregnancy maintenance outcome. The number of single-cell or cleavage staged parthenogenetic embryos co-transferred with embryos of interest, should be sufficient to produce at least 18–20 blastocysts, if cultured in vitro.

On the basis of our own studies of in vivo parthenogenetic embryo potential using our optimized method of electrical activation we believe that this number of blastocysts will ensure that sufficient are present and alive during the critical period between day 11 and day 15 of gestation ***(15)***.

Acknowledgments

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
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Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The authors provide readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer. Of immense benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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6

Health and Neonatal Care of Bovine Clones

Jacqui T. Forsyth and David N. Wells

Summary

The management of clone-bearing recipients and neonatal clones is a critical component of successful cloning of mammals by nuclear transfer. The methodology discussed in this chapter is based largely on a double corticosteroid regime to induce parturition and fetal organ maturation in bovine clones. Lung maturation, particularly, is a major factor in clone calf viability. The animal health care involved in maximizing clone survival begins at embryo transfer and continues for the life of that animal.

Key Words: Cloning; nuclear transfer; bovine; pregnancy; placentation; hydroallantois; neonatal care; parturition; neonatal respiratory distress; corticosteroids.

1. Introduction

Cloning animals from either somatic or embryonic cells using the technique of nuclear transfer increasingly is being used in the basic research, with promising future applications in agriculture and biomedicine. Although cloning is clearly possible and has been accomplished in an ever increasing list of species, the efficiencies are still very low ***(1)***. Current cloning technology is typified by higher rates of pregnancy loss throughout gestation, greater birthweight, higher rates of perinatal mortality, and greater long-term losses. These create serious animal welfare issues that limit the acceptability and applicability of the technique. Ultimately, improved reprogramming of the donor nucleus will increase cloning efficiency and lessen the welfare concerns. Nevertheless, now and in the foreseeable future numerous veterinary measures can be implemented to increase the viability of cloned offspring. Improvement in cloned calf viability may be achieved by appropriate:

- Recipient pregnancy monitoring.
- Identification of hydrops.
- Late pregnancy care.

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- Controlled calving.
- *In utero* pregnancy assessments.
- Calving assistance.
- Neonatal care.
- Postnatal care.

To date, few studies have been published about the calving management, neonatal health care, or health issues in cloned cattle, with some exceptions *(2–5)*. The intention of this chapter is to outline the methodology used in the authors' experience for calving clone-bearing recipients and to discuss some of the health issues that can occur and preventative measures that can be implemented.

The methodology outlined is used in a fully pastoral-based feed system as is common in New Zealand. This may make some procedures inappropriate in alternative systems. Variations in the type of abnormalities in neonatal clones observed between different laboratories worldwide suggest that the effectiveness of the health care protocols outlined in this chapter will vary greatly. However, these protocols have been developed to manage the major pregnancy and parturition issues encountered in our laboratory. Some of the processes discussed require the use of veterinary-only medicines and require veterinary supervision and skills.

2. Materials

Appropriate stock-handling facilities are required for all work with cattle. The requirements will vary depending on the type of farm system and the disposition of the recipient cattle.

2.1. Recipient Pregnancy Monitoring

1. Ultrasound scanner with a 5- or 7.5-MHz linear rectal probe (e.g., Piemed 200 scanner; Phillipsweg, Maastricht, The Netherlands).
2. Obstetrical lubricant.
3. Shoulder-length rectal gloves.

2.2. Identification of Hydrops

1. Ultrasound scanner (*see* **Subheading 2.1.**).
2. Obstetrical lubricant.
3. Shoulder-length rectal gloves.
4. Prostaglandin (Estroplan, Parnell Laboratories, East Tamaki, NZ). Store at temperatures less than 25°C. Protect from light.
5. Dexavet AP 25 mg/recipient (5 mg/mL dexamethasone trimethylacetate; Bomac Laboratories, Manukau City, NZ). Store at less than 25°C. Protect from light.
6. Dexone-5, 25 mg per recipient (5 mg/mL dexamethasone sodium phosphate; Sanofi Animal Health, Canada). Store at less than 30°C. Do not freeze.
7. Hypodermic needles (various sizes).
8. Syringes (various sizes).

2.3. Late Pregnancy Care

1. Rotavec K99 vaccine. (inactivated adjuvanted oil emulsion vaccine combining bovine rotavirus and *Escherichia coli* K99 antigens; Schering-Plough Animal Health, Upper Hutt, NZ). Store in a cool (2–8°C), dark place. Do not freeze.
2. Rumbul bullets (molded cylinders composed of an alloy containing 86% magnesium, 12% aluminum, and 2% copper. Contains 40 g of elemental magnesium; Elanco Animal Health, Manukau City, NZ).
3. Intraruminal bolus applicator.
4. Vacutainers: EDTA; serum/plain vacutainers (cat. no. 367643, Becton Dickinson, Plymouth, UK).
5. Vacutainer needles (cat. no.360214, Becton Dickinson).
6. Vacutainer holder.
7. Propylene glycol (Ketol or Ketol Xtra; Bomac Laboratories, Manukau City, NZ). Store at less than 30°C. Protect from light.

2.4. Controlled Calving

1. Dexavet AP 25 mg per recipient (5 mg/mL dexamethasone trimethylacetate; Bomac Laboratories). Store at less than 25°C. Protect from light.
2. Hideject 5 mL per recipient (500,000 IU vitamin D_3, 60,000 IU vitamin A, and 25 mg vitamin E per milliliter; Bomac Laboratories). Store at less than 20°C. Protect from light.
3. Dexone-5 25 mg per recipient (5 mg/mL of dexamethasone sodium phosphate; Sanofi Animal Health). Store at less than 30°C. Do not freeze.

2.5. In Utero Pregnancy Assessments

1. Shoulder-length rectal gloves.
2. Obstetrical lubricant.
3. Paper towels.
4. Record sheets, to keep all palpation and observation records together. Preferably, each cow has her own record sheet and it includes space for calving assessments and calf information.

2.6. Calving Assistance

1. Standard surgical equipment for Caesarean delivery.
2. Calving pulley system/calving jack.
3. Calving ropes/snares/chains.
4. Humidified oxygen system (**Fig. 1**). A suitable face mask can be made by cutting a 2-L plastic container with a screw lid in half. The tubing can be attached to the screw top lid. The face mask can simply be placed over the calf's nose without worrying the calf, and it is very easy to keep clean.
5. Towels.
6. Padded mat.

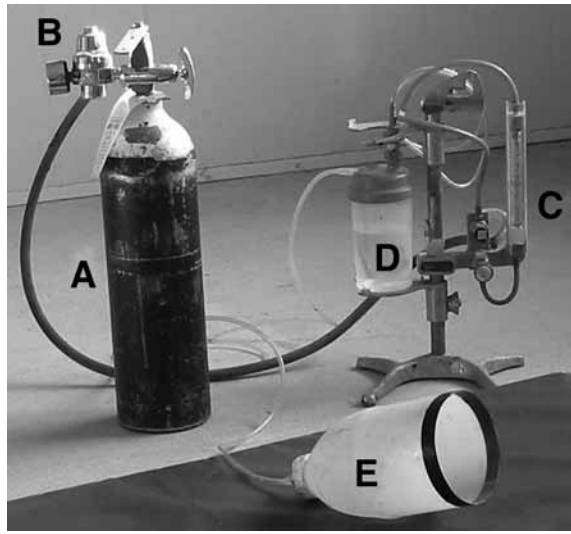

Fig. 1. A basic humidified oxygen and face mask system. An oxygen cylinder **(A)** with a regulator **(B)** is connected to an oxygen flow-meter **(C)**. The oxygen is bubbled through sterile water **(D)** before passing through tubing to the simple face mask **(E)**.

7. Obstetrical lubricant.
8. Oxytocin (e.g., Leotocin; Leo Pharmaceutical Products, Ballerup, Denmark). Store at 8–15°C.
9. Dopram (20 mg/mL doxapram hydrochloride; Elkins-Sinn, Cherry Hill, NJ). Store at controlled room temperature (15–30°C).
10. Millophyline (140 mg/mL etamyphilline camsylate; Arnolds Veterinary Products, Shrewsbury, UK). Store at less than 30°C.
11. Scales for weighing the newborn calf.
12. Identification neck band.
13. Iodine tincture spray.
14. Plastic umbilical cord clamps. They can be purchased from animal health equipment suppliers.
15. Sterile intestinal clamps. These are useful for stemming blood flow from the umbilicus before ligation or clamping can take place.
16. Energy drench containing calcium and magnesium. Aim for 300–500 mL containing approx 70–100 g calcium and 15 g of magnesium per dosage. For example, Headstart (Virbac Laboratories, Auckland, NZ).
17. Intrauterine antibiotic pessaries.
18. Prostaglandin (*see* **Subheading 2.2.**).
19. Antibiotics.
20. Excenel (50 mg/mL ceftiofur hydrochloride; Pharmacia Animal Health, Auckland, NZ). Store at 25–30°C. Protect from freezing.

2.7. Neonatal Care

1. Fresh or frozen colostrum. Freshly stripped, good-quality colostrum is ideal. If this is not available, frozen first milking colostrum can be used. Thaw at room temperature or in warm water bath. Colostrum can be pasteurized to reduce the level of contamination.
2. Colozen (150 mg of immunoglobulins, and vitamins A and B_{12}/30 mL dose; Pharmazen, Dunedin, NZ). Store unmixed for up to 2 yr in a cool (<15°C) dry place. Once mixed, refrigerate (2–8°C) for 8 wk.
3. Calf teat feeder bottle or bucket.
4. Esophageal tube feeder.
5. A suitable powdered calf milk replacer.

2.8. Postnatal Care

1. BOS leg splint (BOS Industries, Kaponga, NZ) or PVC tubing (2.5-in. diameter, split in half lengthwise), foam rubber, and tape or adhesive bandage.
2. Vacutainers and vacutainer needles (*see* Subheading **2.3.**).
3. General animal husbandry equipment.

3. Methods

Supplementary information for each respective step is provided in the **Notes** section.

3.1. Recipient Pregnancy Monitoring

Because of the potential late pregnancy complications and calving issues, it is the authors' recommendation that only a single cloned embryo is transferred to each recipient. Recipients initially are confirmed pregnant by transrectal ultrasonography at days 30–35 of gestation. For earlier pregnancy diagnosis and ongoing monitoring various biochemical measures can be determined in maternal serum, for example, progesterone and pregnancy-specific proteins ***(6–9)***.

By ultrasonography, a positive pregnancy is confirmed by the presence of a fetus and fluid in the uterine horns. The fetus is observed for the presence of a beating heart.

Those cows in which the diagnosis, fetal viability, or normality cannot be determined are rechecked a week later at 42 d of gestation.

The next planned scan is at approximately days 60–70 of pregnancy to coincide with the placentomes being obvious by both ultrasonographic imaging and rectal palpation. The day-60 scan provides good initial information on the quantity and normality of the placentomes. Comments are noted if there are no, very few, or irregularly sized placentomes observed.

Pregnant cows are then either scanned or palpated rectally as appropriate on a monthly basis for the remainder of the gestation. The palpations are performed to

record observations on pregnancy status, "normality" of the pregnancy, and to identify cases of hydroallantois.

3.2. Identification of Hydrops

Hydroallantois (hydrops) usually becomes clinically obvious during the last 3 mo of gestation when there is very marked abdominal distension. The accumulation of vast excesses of allantoic fluid tends to progress rapidly, and the recipient suffers from interference to respiration and reduction in appetite. If left unattended, the syndrome typically is fatal for the recipient cow. It is therefore critical to identify the condition of hydroallantois as early as possible by rectal palpation (or other means) and in the current absence of therapeutic alternatives, to terminate the abnormal pregnancy. Excess fluid, relative to contemporary herdmates, can be identified by rectal examination between days 120 and 180 of pregnancy and perhaps earlier with an experienced operator. Rectal examination reveals a distended tight uterus with difficulty in palpating either the fetus or placentomes owing to the fluid pressure. Suspect cases are closely monitored on a weekly basis to confirm their diagnosis. Research is underway to identify the syndrome in the first trimester to lessen the ethical issue of aborting midgestation-stage fetuses.

There are two different methods used to induce elective abortion in hydrops-affected pregnancies. For pregnancies less than 150 d of gestation, a single prostaglandin injection is used. For those animals with gestations older than 150 d, a double corticosteroid injection regime very similar to that of the controlled calvings is used (*see* **Subheading 3.4.**).

The hydrops animal is injected intramuscularly (preferably in the anterior neck region) with 25 mg of long-acting corticosteroid. Seven days later, 25 mg of short-acting corticosteroid, dexamethasone sodium phosphate, is administered intramuscularly, preferably into the anterior neck region.

The pregnancy should be terminated and fetus expelled within 3 d of the administration of prostaglandin or within 2 d of the administration of dexamethasone sodium phosphate. Treated animals need to be kept under observation and vaginally examined to ensure that expulsion of the fetus and placental membranes is complete (refer to **Subheading 3.6.4.** for retained fetal membranes). If the pregnancy is not terminated, a repeat treatment may be required.

3.3. Late Pregnancy Care

At least 1 mo (and up to 3 mo) before the planned calving date (as opposed to their full-term due date) all recipients are vaccinated with 1 mL of Rotavec K99 intramuscularly. Rotavec is an inactivated, adjuvanted vaccine for cattle combining bovine rotavirus and *E. coli* K99 antigens. The vaccine provides passive immunity for the calf against enteritis caused by

rotavirus and enterotoxigenic *E. coli* after the consumption of colostrum from the recipient cow.

On the same occasion, a serum vacutainer is obtained for purposes of assessing recipient metabolic status. As a minimum, β-hydroxybutyrate, magnesium, and calcium levels are determined.

Four to six weeks before the expected calving date, two Rumbul bullets are administered orally into the rumen of all recipients. Rumbul bullets are molded cylinders composed of an alloy, containing 86% magnesium, 12% aluminum, and 2% copper. Each bullet contains 40 g of elemental magnesium, which is released by electrolytic erosion of the bullet in the reticulum or rumen providing 1.5 g of magnesium per bullet to the cow per day. This may be repeated in 4 wk depending on the magnesium status. Magnesium is important to the late pregnancy cow for preventing periparturient hypomagnesemia and for allowing the cow to absorb increased amounts of calcium from the intestine post calving. The release of parathyroid hormone, which increases the absorption of calcium from the intestine, is dependent upon magnesium levels. In the last few weeks of pregnancy, the recipients are given access to molasses as a high-energy supplement (*see* **Note 3**).

It had been a previous policy in the clone-bearing recipients to control feed intake in the last few weeks of gestation to minimize calf bodyweight, but recent experience suggests this is not necessary. In fact, the cow is likely to calve more easily if she is fed *ad libitum* near full term.

3.4. Controlled Calving

The aim is for a vaginal delivery with assistance as required. Caesarean delivery is a last resort. Recipient cows are scheduled for "controlled calving," using corticosteroids, between days 270 and 282 of gestation. Nine days before the scheduled due date, all recipients are injected intramuscularly with 25 mg of long-acting corticosteroid (e.g., dexamethasone trimethylacetate; *see* **Note 4** for alternative corticosteroids). At the same time, 1 ml/100 kg Hideject also is administered. Hideject is an injectable solution of vitamins A, D_3, and E that is used to aid the prevention of milk fever (hypocalcemia) and increase the animals' resistance to infection at parturition. Seven days later, 25 mg of short-acting corticosteroid (e.g., dexamethasone sodium phosphate) is administered intramuscularly. Calving usually begins the next day, peaking around 43 h after the short-acting corticosteroid injection.

3.5. In Utero *Pregnancy Assessments*

Daily cervical and rectal palpations of recipients begin at the time of short-acting corticosteroid administration and are conducted each evening to assess the likelihood of that recipient calving overnight.

Vaginally, the parameters that are assessed are dilation of the cervix, the consistency and volume of cervical mucus, and the degree of cervical softening. Cervical dilation is measured on a "finger scale." The number of fingers of the practitioner able to fit inside the external os, middle cervical region, and internal os are recorded and monitored for progress at each examination.

Rectally, observations about calf viability, relative size, fetal positioning, and membrane "normality" are assessed. Mammary development is recorded on a 0–3 scale. A 0 score reflects an udder has no active mammary tissue. An udder scored at 3 is tightly bagged and premilk or milk can be expressed.

These measurements will allow judgments to be made about the progression of the calvings. From day 8 of the program, that is, the day after short-acting corticosteroid administration, the animals need to be closely observed. It is recommended that one monitor their progress every 4 h, the reason being that if a cow is not showing any signs of calving at one observation, it is unlikely she will have calved before the next observation. It is possible to increase the time period after a palpation to 6 h because of the more accurate information obtained.

3.6. Calving Assistance

3.6.1. Parturition Management

Recipients are assisted at parturition to the degree necessary to accomplish a successful birth. The ultimate aim is for the cow to be allowed to calve herself. Obviously, this philosophy needs to be tempered with the desire to produce a viable calf. Recipient cattle are given access to well grassed small paddocks neighboring the covered cattle yards. The cows are also given supplementary silage, grain, or hay if necessary. Once any sign of calving begins, the cow needs to be regularly monitored by observation and physical examination if necessary and progress recorded.

If no limbs appear within an hour of the "water bag"/allantoic sac breaking, despite the cow physically pushing, an examination of the cervix is necessary to ensure that the calf is correctly presented and of an appropriate size. If the calf is not presented correctly but is engaged in the pelvis it is necessary to correct the presentation. After correction the cow is returned to her paddock to continue labor.

If the calf is not yet positioned, that is, still turning, the cow is left to continue. If the calf is presented but not engaged the calf is manually assisted into the pelvis.

It requires discipline and confidence to stand by and observe the cow calving, but every effort is made to allow the cow to calve unassisted. If at any stage the cow is in distress or there is no obvious progress, intervention is necessary.

For future reference, a calving difficulty score is assigned immediately after parturition. The calving difficulty is a 0–2 scale, where 0 denotes no assistance and 2 reflects a difficult delivery or Caesarean delivery.

3.6.2. Immediate Postpartum Calf Care

Immediately after assisted delivery (either manual traction or caesarean delivery), the calf needs to be hung upside down for 10–30 s. This position allows for drainage of fetal fluids from the lungs.

In an area adjacent to the cattle crush, lay the calf on a padded mat in lateral recumbency. If the calf is not breathing but has a strong, regular heartbeat, a breathing stimulant is administered intravenously (either 0.25 mL of Dopram [20 mg/ml or Doxapram HCl] or 1.0 mL of millophyline V injection [140 mg/mL etamiphylline]).

The nasal and oral cavities are cleaned with a dry towel and humidified oxygen is administered via a face mask (**Fig. 1**). Oxygen is administered until the calf's mucous membranes are pink, it is breathing normally, and is posturally strong. The calf is rubbed vigorously with towels, which helps with drying, warmth, expulsion of pulmonary fluid, and stimulation of cardiorespiratory function. Tickling the calf's nostrils with a straw also can stimulate sneezing and respiratory movements. Once the calf is breathing well, it is placed in sternal recumbency to minimize the adverse effects of ventilation/perfusion inequalities. If the initial respiratory stimulant does not give an adequate response, a second dose can be administered.

The processes used in our hands are conservative relative to some other cloning laboratories. Other more invasive procedures which can be used to assist the neonate, such as surfactant, nitric oxygen, and monitoring blood biochemistry, are discussed in **Notes 4–6.**

Once the calf appears to be breathing satisfactorily and behaving appropriately (i.e., sitting up, with a suckle reflex and good demeanor) apply 1–3% iodine to the umbilicus (*see* **Note 7**).

The calf is weighed and moved to a position where it can bond with the recipient cow.

Ensure the calf has an identification neck band until permanent identification is in place. There will be occasions when the newborn calf will require immediate euthanasia because of gross abnormalities, such as severely contracted flexor tendons, scoliosis, or arthrogryposis.

3.6.3. Immediate Postpartum Cow Care

Check the cow vaginally for the presence of an (unexpected) second calf and for any uterine or vaginal tears that may need to be corrected. Administer 30–50 IU of oxytocin intramuscularly to assist uterine involution. Finally, orally

drench the cow with an energy drench containing calcium and magnesium (e.g.. Starter plus or Headstart).

3.6.4. Retained Fetal Membranes

There will be an increase in retained fetal membranes in the clone-bearing recipients because of both the abnormal placentation and the corticosteroid use in the controlled calving protocol. Recipient cows are injected intramuscularly with 30–50 IU oxytocin every 12 h for 48 h or until entirely cleaned (if before 48 h postpartum) to aid involution and remove uterine debris. This intervention also allows the opportunity to observe the recipient for metabolic problems and mastitis.

Recipients are palpated approximately every 2 d after calving. The fetal membranes are removed manually when they have detached. It often is necessary to administer antibiotics to the cow during the first week after calving to avoid septicemia while the fetal membranes are still retained. Effective antibiotics include ceftiofur or penicillin, particularly in combination with intrauterine tetracyclines. It is the authors' experience that the membranes are quite thickened and tend to not detach in one piece as normal. It helps to remove as much placental material as is possible at each palpation. Once the majority of the placenta is removed, intrauterine tetracycline pessaries (2 g) seem beneficial in reducing further complications. Prostaglandins are also recommended for use in metritis treatments. Prostaglandin has a stimulatory effect on the smooth muscle of the uterus causing contraction and concurrent cervical relaxation.

3.7. Neonatal Care

The approach outlined here is relatively conservative but forms our standard practice. The cow and calf are checked regularly (i.e., every 15 min in the first hour of life) to identify when the calf stands and suckles from the recipient.

If the calf does not stand, suckle or progress towards standing within an hour, colostrum is fed. Ideally, colostrum is stripped from the recipient but frozen stocks of first milking colostrum should be readily available in situations where mammary development is poor or the cow is showing signs of a metabolic disease, mastitis, or general malaise. Initially, 2 L should be suckle-fed via a bottle teat feeder if possible. If the calf is not exhibiting a suckle response, the volume is delivered via an osophageal tube feeder (*see* **Note 8**).

Once the first feed has been consumed, the calf is orally drenched with 30 mL of Colozen (*see* **Note 8**). Passive transfer of immunoglobulins is confirmed by elevated 24-h serum γ-glutamyltransferase levels.

It is our practice for the calves to be reared by suckling the recipient cows if at all possible. In situations where the calf fails to bond with the cow, calves are reared on a suitable powdered milk replacer according to the manufacturer's instructions.

3.8. Postnatal Care

Calves are closely monitored during the first few weeks of life for potential umbilical problems, general demeanor, and to ensure they are being adequately fed.

Routine blood samples for hematology and biochemistry are taken at 1 d, 1 wk, and 1 mo of age to aid monitoring general health status and in any situations of clinical disease. A 5-mL vacutainer of serum and blood, placed in EDTA, are collected from the jugular vein for this purpose.

Clone calves are reared in accordance with standard beef suckling farm practice as much as is possible. Particular health concerns in cloned calves include umbilical infections, flexor tendon contraction, and immune system deficiencies (*see* **Note 9**).

3.9. Longevity and Long-Term Health

The longevity of clones has always been questioned and highlighted by "Dolly's" relatively shorter telomeres ***(10)*** and the suggestion that her death may have been the result of premature aging. Telomeres are the regions of DNA at the ends of chromosomes that progressively shorten at each cell division. There are conflicting reports on the effect of cloning by nuclear transfer on telomere length, with evidence also of restoration to normal lengths ***(11)***. Indeed, there is no proven link between the telomere length and premature aging ***(11)***. That aside, there are still issues concerning the longevity and health of cloned animals. The incidence of these clone-associated phenotypes varies according to the particular species, genotype, sex, cell type, and specific aspects of the nuclear transfer and culture protocols used. The lifespan of mice cloned from Sertoli cells from one particular mouse strain were shown to be 25% shorter than their wild-type controls but were normal in other mouse strains and cell types ***(12)***. There has not been a thorough analysis of clones in livestock species with longer biological lifespan. This is certainly an area for detailed investigation and carries the expectation of a lower incidence of clone-associated health complications with the prospect of improved reprogramming of the donor cell genome in the future. This will improve the welfare of cloned animals and their recipients and increase the efficiency and the applicability of the technology. In the interim, however, procedures still need to be in place to maximize the viability of the clone animals born. Fortunately, the health problems associated with some clones do not appear to be manifest in their offspring after sexual reproduction. The epigenetic aberrations that occur during the cloning process are repaired during gametogenesis ***(13)***, providing some confidence in those eventual applications of cloning that capture the potential of breeding from clones as a means of genetic dissemination ***(14)***.

Table 1
Embryo Transfer and Nuclear Transfer Increase Birthweight in Cattle

Calf type	Number	Mean
Clone	148	47.5 kg[a]
MOET	2221	37.1 kg[b]
AI/NM	4180	34.6 kg[c]

Means with a different superscript are significantly different ($p > 0.05$).
MOET, multiple ovulation embryo transfer; AI/NM, artificial insemination/natural mating.

4. Notes

1. Recipient pregnancy monitoring: there needs to be some forethought about recipient selection. Multiparous cows are superior to heifers in their ability to calve *per vaginum*, especially with the increased birthweight associated with cloning (**Table 1** ***[15]***). It is also wise to choose larger-framed cows. Suitable cows in the authors' experience are either Friesian or Friesian crossed with Hereford. There are some drawbacks, however, with using a high milk-producing breed, such as the Friesian, if the suckle rearing system is used, for example, the risk of mastitis. It is imperative that the cows are of good temperament because a lot of handling is involved in the management of clone-bearing recipients. The recipients need to be fed suitable planes of nutrition throughout gestation to prevent metabolic complications and calving difficulties. In a pastoral-based system, it can be difficult to avoid overfeeding when the number of animals in a group is small. Local disease risks need to be taken into account when managing the recipients and appropriate husbandry preventative measures put in place. For example, in Hamilton, New Zealand we need to be aware of the facial eczema risk over the summer and treat accordingly. Pregnancy health is determined by ultrasonography or rectal palpation. Observations are made with respect to the number, regularity, and size of placentomes and fetal size relative to other fetuses of the same age, sex, and cell line. There will be opportunities in the future to assess pregnancy health/fetal viability by determining the levels of pregnancy-specific proteins in the maternal serum. Potential proteins include pregnancy-specific protein B, pregnancy serum protein 60, and pregnancy-associated glycoprotein ***(6–8)***. Research is underway to investigate monitoring fetal/uterine health by serial ultrasound guided sampling of fetal fluids with the aim of identifying diagnostic markers for predicting pregnancy failure and abnormal placental development in early gestation ***(16)***. As well as the health of the pregnancy, the general health of clone-bearing recipients also needs to be closely monitored. Routine hematology, serology, and biochemistry provide the necessary information.
2. Identification of hydrops: abnormal placentation is a major cause of pregnancy failure after nuclear cloning ***(9,17)***. The most common placental abnormality in established pregnancies is hydroallantois. Abnormal placental development also contributes to inappropriate fetal/maternal communication especially evident near term with delayed or failed parturition. Clone placentas tend to have approximately half the number of placentomes compared with artificially inseminated or in vitro fertilized

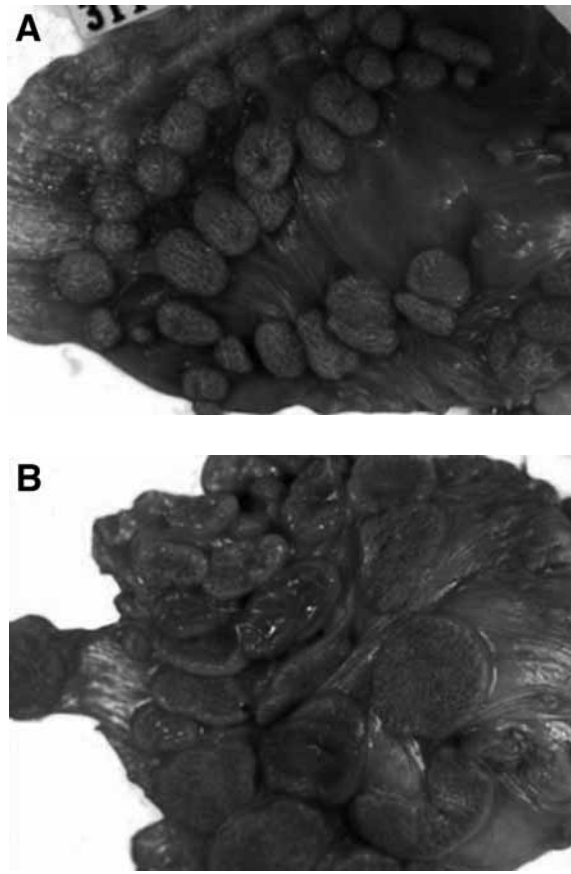

Fig. 2. Placental morphology in artificially inseminated and clone-bearing pregnancies. **(A)** Artificially inseminated caruncles and **(B)** clone caruncles.

controls *(9)*. Compensatory overgrowth occurs, resulting in clone placentomes weighing approximately twice that of normal (**Fig. 2**). On average, in the authors' experience, 42% of recipients pregnant at day 120 of gestation will fail before term and 58% of these losses are associated with hydrops. To minimize distress to the recipient cow, it is our practice to selectively abort recipients as soon as hydroallantois is diagnosed. Hydroallantoic fetuses tend to be edematous and often are accompanied by other pathology, such as hydronephrosis and enlarged hearts and livers. Only in the mildest cases are the fetuses potentially viable.

3. Late pregnancy care: it is not unusual for late pregnancy clone-bearing recipients to develop mild ketosis, particularly in instances where borderline hydroallantois may be present. Hence, maternal serum β-hydroxybutyrate levels are monitored near-term and recipients are allowed free access to molasses. In these cases, Ketol,

Ketol xtra, or a similar propylene glycol drench are used to elevate blood glucose and remove ketones. Nutrition of the clone-bearing recipient is an integral component of the system. Transition cow management (the transition of the cow from pregnancy to lactation) is important in any pastoral grazing system. The potential for clone-bearing recipients to be more prone to ketosis makes this area of nutrition critical. It is our practice to feed according to recommended requirements for each stage of pregnancy. This is sometimes difficult to achieve given the lack of seasonality in our clone research. In the times of poor quality or quantity of grass, supplementary feeds are provided.

4. Controlled calving: historically, cloned calves have been delivered by Caesarean delivery and, internationally, it remains a common trend. On the basis of animal welfare and public acceptability issues, our approach at AgResearch is to work toward delivering a high proportion of cloned calves more naturally *per vaginum*. This route carries with it some risks. It generally is accepted that the cloning process increases calf birthweights by 30% to 40% compared with artificial insemination or natural mating (**Table 1**). It also is accepted that cloned animals are prone to neonatal respiratory distress as a result of poor lung maturation before birth. Using corticosteroids to induce parturition at or near full-term, we have developed a "controlled calving" procedure to ensure fetal lung maturation, recipient mammary development, and appropriate "mimicked" communication between fetus and recipient cow. Because this management practice synchronizes parturition in a group of contemporary recipients, husbandry efforts can be focused intensively during a 2-d period, allowing all calvings to be observed. In the authors' experience, controlled parturition results in approx 10% of deliveries without any assistance whatsoever, 10% still require Caesarean section, and the remainder require only minor intervention or assistance. Alternative long-acting corticosteroids substituting dexamethasone trimethylacetate are 20 mg of dexamethasone phenylpropionate or dexamethasone 21-isonicotinate. The fetal endocrine changes that occur in late gestation not only initiate parturition and ensure lung maturation but also stimulate a variety of maturational changes in organs that enables the neonate to survive extra-uterine life. The authors' use a long-acting corticosteroid followed by a short-acting corticosteroid approach in an attempt to achieve better organ maturation than when using a single short-acting corticosteroid injection. Neonatal respiratory distress is evidenced as a progressive respiratory failure that culminates in a combined respiratory and metabolic acidosis in the course of the first hour of life. It is caused by immaturity of type II alveolar epithelial cells, leading to an inadequate pulmonary surfactant function. Surfactant is a mixture of various phospholipids and proteins that increases lung compliance, stabilizes alveoli, and protects epithelial cell surfaces. In vitro, corticosteroids stimulate the synthesis of lecithin (an integral part of surfactant) in the type II pneumocytes. In vivo corticosteroids also promote the maturation of the surfactant system ***(18)***. Surfactant administration is an option in cases of respiratory distress syndrome but has not been part of the authors' practice. Hill et al. ***(3)*** describe a technique for recovering crude pulmonary surfactant from slaughterhouse adult cattle lungs. Alternatively there is a

commercial human product available called Intrasurf (ONY, Amherst, NY). There have also been reports of metabolic acidosis in newborn clones ***(2,3)***. Most neonates are born with some degree of hypoxia and acidosis associated with the delivery. In the normal neonate there are compensatory mechanisms which counter the acidosis. Zaremba et al. ***(19)*** found in experiments using corticosteroids to induce pulmonary maturity that the degree of acidosis after birth is directly related to pulmonary maturity. This would suggest that the acidosis observed in clone neonates is purely a secondary effect and if lung maturation can be achieved, the metabolic acidosis would be removed.

5. *In utero* pregnancy assessments: calf size is related to the particular breed, sex, and cell line being cloned, but a relative indication of individuals within a particular group can be given by assessing foot size. The number of finger widths that can be laid across the foot region measures a foot size. Note, it should be recorded if the observation is on a hind limb because they tend to be larger than the front feet.
6. Parturition management: the practitioner's preference will determine the specific calving equipment and technique to be used. The author uses a three-pulley system and calving ropes that reduces the physical exertion required and allows for slow steady assistance to the calving. If traction is used, it is necessary to catch the calf as it is expelled to avoid the umbilicus tearing too closely to the abdomen. A Caesarean delivery is required if the calf is disproportionately large for the cow's pelvis, unusually presented, or the cow has not responded to corticosteroid treatment. Caesarean delivery is a last resort as the postoperative survival of the recipient cow is poor. It is thought that the immunosuppressive effect of the corticosteroids used in the controlled calving protocol make the recipient more vulnerable to infection. For deliveries via Caesarean section , it is vitally important for the future health of the calf that the umbilical vessels are torn manually rather than clamped and cut. Otherwise, there is a considerably higher likelihood of umbilical infection and subsequent complications, such as herniation. Only in situations in which umbilical vessels are so thickened (a consequence of abnormal placentation) that they prevent manual tearing should clamping and cutting be performed. In such cases, prophylactic antibiotics need to be administered. Excenel® (50 mg/mL ceftiofur hydrochloride) at a dose rate of 5 mg/kg is effective.
7. Immediate post-partum calf care: as much as 10% iodine w/v in ethanol has been used for navel disinfection but this is difficult to obtain. A 1–2% iodine tincture is satisfactory if thoroughly and regularly applied. Although the methodology outlined in **Subheading 3.6.** is our practice, many groups would recommend a more intensive or aggressive approach toward neonatal care. In the authors' experience, if pulmonary maturation is not an issue, the intervention required at birth is minimal. It is our experience that intensive treatment often delays the inevitable health complications in later life but, as mentioned previously, there is considerable variation in neonatal viability between cloning laboratories. Hill and Chavatte-Palmer ***(5)*** advocate providing ventilation support immediately after birth, initially by face mask and then either endotracheal tube or nasal cannula for at least 1 h, often up

to 24 h. To assist pulmonary arterial vasodilation 80 ppm nitric oxide is administered for the first 30 min after birth. As discussed in **Note 4**, surfactant administration may be useful for cases of neonatal respiratory distress. Arterial blood gases (O_2, CO_2, HCO_3) and pH can be monitored in the neonate to assist in treatment decisions. Other supportive treatments for consideration in neonatal care are body temperature regulation, intravenous fluids, or glucose and prophylactic antibiotics. There will be instances in which the intensive care required by the neonate clone can only be provided by a veterinary hospital.

8. Neonatal care: current recommendations for colostrum feeding are up to 4 L as a bolus as soon as possible after birth for a 40 kg calf (Smith, B. P., Australia and New Zealand Combined Dairy Vets Conference 2003, personal communication). This recommendation ensures that even if poor quality colostrum is administered, the calf should receive at least 120 mg of immunoglobulins. If colostrum quality is assured (i.e., if a colostrometer has been used) or if a supplement such as Colozen® is used then this volume is not necessary. Our practice is to aim for a volume of colostrum representing 10% of the calf's bodyweight in a 12-h period. In a newborn calf, four to six hourly feeds are ideal. For example, a 40-kg calf needs to be fed 4 L in its first 12 h. This amount would be broken down into feeds of 1.5–2 L. Colozen is a complex mixture of antibodies and vitamins A and B_{12}, with a minimum of 150 mg of immunoglobulins per dose. This adequately fulfills the recommended requirement of 120 mg of immunoglobulin as soon as possible after birth.
9. Postnatal care: the analyses included in the routine hematology and biochemistry are hemoglobin, hematocrit, red blood cells, mean corpuscular, mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, white blood cells, differential blood count (segmented neutrophils, lymphocytes, monocytes), fibrinogen, plasma protein and plasma protein/fibrinogen ratio, serum creatine kinase, aspartate aminotransferase, glutamate dehydrogenase, γ-glutamyltransferase, bilirubin, protein, albumin, globulin, albumin/globulin ratio, creatinine, urea, phosphorus, magnesium, β-hydroxybutyrate, and calcium. Umbilical infections are a major concern in cloned calves, particularly with those born by Caesarean section. Particular attention needs to be paid to umbilical length at calving, iodine dowsing and general calf health. The administration of Colozen aims to reduce this problem by ensuring all calves have optimum immunity. Umbilical infections are usually only superficial and broad spectrum antibiotics and daily iodine spraying for 5 d is normally effective. Instances of clone calves having enlarged umbilical vessels are particularly prevalent in those requiring Caesarean section. It is necessary to apply sterile surgical intestine clamps or similar to control the bleeding while the appropriate long-term hemostasis is applied, that is, ligation of the arteries or clamping with a plastic umbilical clamp. It is common for pulsations to be palpable in the umbilical arteries in these calves for at least 12 h. In these cases, umbilical infections may occasionally be chronic because of necrotic tissue being contained within the umbilicus. The best treatment is to open the umbilicus slightly and irrigate with 0.9% saline under pressure. The causative tissue is usually expelled. Antibiotics still need to be administered. Effective antibiotics include Clavulox® (clavulanic acid and amoxicillin), tetracyclines, procaine penicillin, cephalosporins, or trimethoprim–sulpha combinations, in

that order. With some cell lines, flexor tendon contraction or laxity have occurred in some calves. For best results the tendons need to be dealt with promptly. Splints adapted from PVC tubing or BOS leg splints® are usually effective. Splinted calves require rigorous monitoring and redressing (approximately every 2 d) to avoid maceration or secondary infection. It is important that the calf still exercises the splinted leg. If there is any likelihood of pain during the splinting, anti-inflammatories are recommended. It is widely postulated that cloned animals have compromised immune systems. French research ***(20)*** has reported cases of lymphoid hypoplasia in somatic cell clones. Equally, it has been stated that "cloned cattle can be healthy and normal" ***(21)***. As with all cattle, sporadic infections of various body systems occur in clones but at possibly higher frequency. In AgResearch's experience, the biggest challenges have been umbilical infections, enteritis, and respiratory infections.

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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7

Optimization of Procedures for Cloning by Somatic Cell Nuclear Transfer in Mice

Young Gie Chung, Shaorong Gao, and Keith E. Latham

Summary

Cloning by somatic cell nuclear transfer is a complex procedure that is dependent on correct interactions between oocyte and donor cell genome. These interactions require minimal insult to either the oocyte or the transplanted nucleus. Available data also indicate that reprogramming the donor cell genome may be slow, so that the cloned embryo expresses genes typical of the donor cell, and thus has different characteristics from normal embryos. Procedures that minimize damage to the donor genome and that address the unique characteristics of the cloned construct should enhance the efficacy of the method.

Key Words: Cloning; nuclear transfer; culture medium.

1. Introduction

Cloning by somatic cell nuclear transfer offers exciting new opportunities for examining the nuclear–cytoplasmic interactions that occur in early embryos and other basic questions related to gene function, differentiation, and embryogenesis, as well as for novel applications in such areas as species preservation and therapeutic procedures in humans. The inefficiency of cloning, however, has inhibited its implementation in the applied forum and also raises concerns about the validity of conclusions drawn regarding basic biological questions. The cloning methodology has been applied only in a comparatively small number of laboratories. It is clear that procedural differences likely exist between laboratories. It is equally clear that the effects of these procedural differences have not been documented. Whether additional procedural differences exist but remain unacknowledged cannot be readily gauged and, in truth, it may be difficult to judge the impact of any given aspect of a cloning protocol. The overall perspective that must be kept in mind is that cloning remains a novel,

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experimental procedure that is still in its earliest stages of technological development. The number of studies and the number of laboratories conducting such studies remains quite small, whereas the number of variables and experimental parameters remaining to be tested is quite large. In this chapter, we relate observations made in this laboratory regarding how some parameters in the cloning procedure can affect outcome.

2. Materials

2.1. Chemicals

Chemicals of high purity for preparation of culture media are purchased from either Fisher Scientific (Pittsburgh, PA) or Fluka Chemical (Milwaukee, WI).

1. 10 mg/mL Gentamycin (Gibco/Invitrogne, Carlsbad, CA).
2. Phenol red solution (0.5%, Gibco/Invitrogen).
3. Polyvinylpryrolidone (PVP; Sigma, St. Louis, MO).
4. Strontium chloride (Sigma).
5. Cytochalasin B (2.5 and 5.0 mg/mL for 1000X solutions in ethanol, prepared cold to avoid evaporation and stored –70°C; Sigma).
6. Mannitol (Sigma).
7. HEPES (Sigma).
8. Human chorionic gonadotropin (hCG; Sigma).
9. Equine chorionic gonadotropin (Calbiochem, La Jolla, CA).
10. Hyaluronidase (ICN, Aurora, OH).
11. Bovine serum albumin (BSA), Fraction V (ICN).

2.2. Instruments and Equipment

1. Modular plastic incubator (Billups-Rothenberg, Del Mar, CA).
2. ECM 2001 embryo manipulation system fusion machine (BTX/Genetronics, San Diego, CA).
3. Flaming-Brown pipet puller (Model P-87 or equivalent; Sutter Instruments, Novato, CA).
4. Olympus stereomicroscope with Gimbal mounted mirror.
5. Olympus IX71 microscope equipped with modulation contrast optics, 10, 20, and 40 objectives, with both ×10 and ×15 oculars.
6. Micromanipulation equipment: Consisting of a Nikon Diaphot inverted microscope equipped with differential interference contrast optics and objectives as mentioned previously. Narishige three-axis hanging joystick micromanipulators with coarse manipulators and Narishige IM-6 and IM-9B microinjectors.
7. Piezo pipet driver (PMM Controller, Prime Tech, Ibaraki, Japan).

2.3. Media

1. Modified Eagle's medium (MEM)-α (Sigma).
2. Ham's F10 (Gibco/Invitrogen).

3. Dulbecco's modified Eagle's media (Gibco/Invitrogen).
4. CZB medium ***(1)***.
5. CZB media augmented with glucose to 5.5 m*M* (CZB-G ***[2]***).
6. HEPES-CZB-G medium: HEPES is added to 20 m*M* and sodium bicarbonate is reduced to 5 m*M*.
7. Calcium-free CZB-G medium.
8. Whitten's medium (WM ***[3]***).
9. KSOM medium ***(4)***.
10. KSOM augmented with amino acids ***(5)***.
11. M2 medium ***(6)***.
12. Hyaluronidase stock: hyaluronidase of 600 U/mg or greater activity at a concentration of approx 600 U/mL prepared in M2 medium.
13. Electrofusion medium: 275 m*M* mannitol, 0.1 m*M* $MgSO_4$, 0.05 m*M* $CaCl_2$, and 0.3% BSA, pH 7.2–7.4.

2.3.1. Media Preparation

As with any embryological procedure, the culture medium used is of fundamental importance (*see* **Notes 1–5**). In our opinion, one important pre-requisite for maximizing yield in cloning procedures is to use only culture medium that is 2 wk or less in age, preferably no more than 1 wk since preparation (*see* **Note 6**).

Exclusive use of either disposable tissue culture ware or dedicated glassware that is thoroughly rinsed and dried between uses is recommended, to avoid any contamination with detergents or other chemicals. Dry chemicals should only be weighed with disposable utensils. The water should be of the highest possible purity. Water produced from a well-maintained MilliQ system, for example, should suffice; it should be noted that continuing improvements in the quality of water that can be produced from such systems may require periodic updating of equipment to achieve the maximum possible water quality. Any effort feasible should be taken to minimize the degree to which water-borne trace contaminants enter culture media.

Prepare CZB, WM, and KSOM media from dry powders, store at 4°C, and use for up to 2 wk. Gas the air spaces in bottles containing bicarbonate-buffered media with a 5% CO_2 mixture to avoid equilibration to an alkaline pH. The correct osmolarity of the culture medium is important, as differences in osmolarity can affect embryo development (*see* **Note 7**).

The source and manufacturing lot of BSA can also affect medium quality and should be batch tested (*see* **Note 8**). We have had success with BSA from ICN Chemicals.

2.3.2. Culture Dishes and Incubator

We prefer the use of nontissue culture plastic Petri dishes for all of our cultures (*see* **Note 9**). Embryos can be cultured using a system of droplets of

medium under light mineral oil in 35- or 60-mm Petri dishes. The source and manufacturing lot of mineral oil must be tested. We have had good reproducibility with the light mineral oil from Fisher Scientific. Maintain dishes in a CO_2 cell culture incubator while the experiment is in progress, and then transfer to a plastic modular incubator, which is then gassed and maintained at 37°C. The gas mixture that we generally use for culturing cloned embryos is 5% CO_2, 21% O_2, and a balance of N_2 (*see* **Note 10**).

3. Methods

3.1. Cloning Procedure

There is considerable heterogeneity among cloned embryo constructs. Boiani et al. *(7)* documented heterogeneity in *Oct4* expression, both between embryos and between cells within a given embryo (i.e., mosaicism). We have observed mosaic expression of Dnmt1 proteins in 100% of eight-cell stage cloned embryos examined *(8)*. Accordingly, it appears that most embryos are defective in recapitulating a truly embryonic mode of development and gene expression, so that only a minority would be expected to initiate postimplantation development, and only a very small fraction of these would be expected to develop to term, as is observed *(9,10)*. Success in cloning could likely be improved by reducing the heterogeneity between embryos and blastomeres, and by shifting the balance in favor of successful reprogramming (*see* **Note 11**). A schematic diagram of the clone procedure in mice is given in **Fig. 1**. Details are given herein.

3.1.1. Oocyte Isolation

The mouse oocyte is a very fragile cell and can easily have its competence to support long-term development compromised by careless handling, without any immediate, obvious morphological evidence of this effect. Because cloning is obviously critically dependent on the oocyte for success, care must be taken not to fail in the experiment during its first step. One consideration is the method employed for removing contaminating cumulus cells.

1. Dilute the hyaluronidase to 100–150 U/mL and prepare a large droplet in a dish with an oil overlay.
2. Isolate the oviducts in M2 medium.
3. Place the oviducts directly into the oil and drag them into the enzyme solution one at a time until all cumulus oocyte complexes are released. It is also acceptable to work without the oil overlay, provided that desiccation is avoided. Handling a few oviducts at a time minimizes the amount of time that the oocytes are exposed to room temperature, by minimizing the amount of time required to wash each batch of oocytes.
4. After brief enzymatic treatment, aspirate the cumulus–oocyte complexes with a pipet of the appropriate size (200–300 μ*M*). This step accomplishes rapid and efficient dissociation of the cumulus cells without damaging the oocytes.

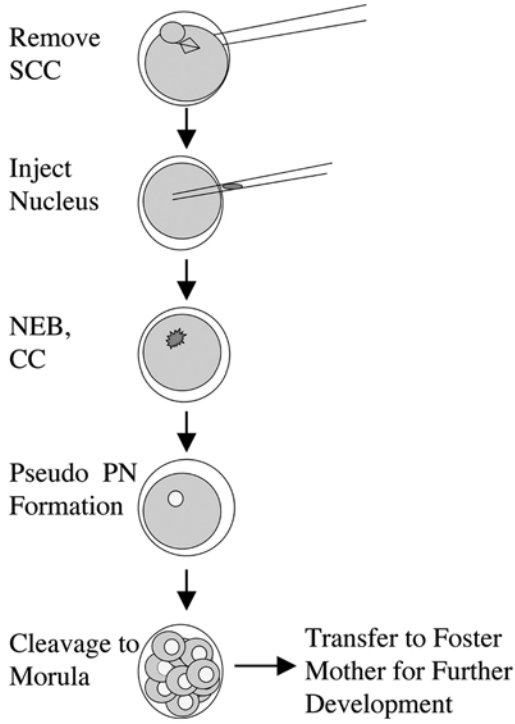

Fig. 1. Schematic outline of cloning procedure in mice. Spindle-chromosomes complex is removed by aspiration without penetrating the plasma membrane. Donor nuclei are introduced either by injection (as shown) or by electrofusion, as described in **Section 3**. The donor nuclear envelope breaks down and chromosomes condense. After oocyte activation, pseudopronuclei form, and the embryo begins to cleave. Morula stage embryos are transferred to uteri of foster mothers. SCC, spindle-chromosomes complex; NEB, nuclear envelope breaks down; CC, chromosomes condense; PN, pseudopronuclei.

5. Wash oocytes extensively to remove both cumulus cells and enzyme, then wash them through several changes of CZB-G medium and culture in CZB-G until use.

3.1.2. Removing the Spindle-Chromosome Complex

The spindle-chromosome complex (SCC) can be difficult to visualize. We have had excellent success employing either of two types of optics for this purpose (*see* **Note 12**).

1. Manipulate oocytes in HEPES-buffered CZB-G medium supplemented with 2.5–3.0 µg/mL cytochalasin B (*see* **Note 13**). It is important that the oocytes be exposed to room temperature for only approx 10 min during the removal of the

spindle. Thus, groups of oocytes must be small when an operator is first learning, but can eventually be increased to approx 20–25 oocytes.
2. Use a piezo pipet driver for the rapid penetration of the zona pelucida without disturbing the ooplasm. Insert a blunt pipet of approx 8–10 µm inside diameter through the zona pellucida using multiple piezo pulses. Speed and intensity settings are generally in the range of 2–4 for penetrating the zona and a setting of 1 for penetrating the plasma membrane (*see* **Subheading 3.1.3.**). Amount and position of mercury in the pipet will affect actual settings. We find that a 2- to 3-mm long bead of mercury inside the main bore of the pipet constitutes the correct amount of mercury. Insert the mercury into the pipet near the beginning of the taper using a 36-g spinal tap needle (*see* **Note 14**). The pipet is backfilled with water and inserted into the injection holder free of any air bubbles. Using the injector, move the mercury to the tip of the pipet after mounting on the micromanipulator and microscope.
3. Take care not to penetrate the plasma membrane during removal of the SCC. Typically, it is easiest if the SCC is positioned in the hemisphere opposite from that where the oocyte is held by the holding pipet. The tip of the pipet is positioned near the SCC and mild suction is applied to aspirate a portion of the plasma membrane, the SCC, and a minimal volume of cytoplasm. A microinjector with very fine control is preferable, with no more than 5 µL per turn.
4. Gently withdraw the pipet after the SCC is aspirated and expel the SCC. The SCC need not be entirely within the pipet to be withdrawn.

3.1.3. Introduction of Nuclei

The introduction of nuclei can be achieved by either of two methods: injection or electrofusion. The size of the donor nucleus is the determining factor. Nuclei larger than approx 7–8 µ*M* in diameter may be damaged with the injection pipet. In such cases, electrofusion is preferable.

3.1.3.1. Injection

Introducing nuclei by injection is most easily performed using a piezo-driven pipet. The size and shape of the pipet are critical. We find that a very elongated, gradually tapering pipet with an inside diameter of approx 5 µm works well.

1. Prepare dishes for injection using the lids of standard 10-cm plastic disposable Petri dishes.
2. Drops of different media are prepared on the dish and covered with oil. These include drops of 12% PVP in medium, 2–3% PVP in medium (*see* **Note 13**), and injection medium with no PVP. Injections can be performed using either HEPES-buffered CZB-G or bicarbonate-buffered CZB-G (*see* **Note 15**).
3. Oocytes for injection are placed in the medium drop with no PVP.
4. Donor cells are mixed in the drop with 2–3% PVP.
5. The drop containing 12% PVP is used for washing the pipet. Wash the tip of the pipet several times in the drop containing 12% PVP.

6. Aspirate donor cells in medium with 2–3% PVP and pipet several times to ensure lysis of the membrane and to remove the cytoplasm. Aspirate several nuclei with convenient spacing between them to allow one to be injected at a time. Draw the last nucleus aspirated up the pipet away from the tip.
7. Use several piezo pulses to penetrate the zona a second time. Advance the tip of the pipet near the opposite side of the oocyte where the oocyte is held by the holding pipet. The diameter of the opening of the holding pipet should be around 25 µm to permit a small pocket to be formed by drawing a portion of the ooplasm into the holding pipet.
8. While the tip of the injection pipet is being advanced toward the holding pipet, expel liquid from the pipet to bring the nucleus to the tip. When the nucleus reaches the tip, stop the outward flow of medium and use a single low-energy piezo pulse to penetrate the membrane.
9. Place the tip of the injection pipet at the opening of the pocket formed by the holding pipet and expel the nucleus. Withdraw the injection pipet quickly but without damaging the oocyte.

3.1.3.2 Electrofusion

Electrofusion is the preferred method for larger size donor nuclei. The removal of the SCC is performed as described previously, except that any remaining polar body or its debris is removed at the time of SCC removal.

1. Resuspend donor cells in the same drop of injection medium as the oocytes themselves.
2. Use a larger-bore (25–30 µm) injection pipet to make an opening in the zona pellucida.
3. Aspirate an intact donor cell and reinsert the pipet through this opening.
4. Expel the donor cell at a location far enough away from the opening to prevent it from exiting the perivitelline space when the pipet is withdrawn. A smaller diameter pipet may be used, but take care to avoid damage to the donor cell, so that it does not lyse before fusion can be achieved.
5. After the donor cell is inserted into the perivitelline space, electrofusion can be performed. Elecrofusion requires a fusion chamber connected to a suitable pulse generator, such as the BTX ECM 2001.
6. Use fusion chambers with an approx 1 mm distance between electrodes. This separation should be measured, because it can vary.
7. Wash oocytes and equilibrate in the electrofusion medium before placing them between the electrodes (*see* **Note 16**).
8. A low-voltage (2–10 V) alternating current can be applied to assist in aligning the cloned constructs within the electric field before fusion (membranes at point of contact between cells must be parallel to electrodes). Successful rotation should occur within a few seconds. Any debris between the electrodes, debris, or cumulus cells clinging to the oocytes, or residual polar bodies can prevent successful rotation and alignment.

9. Ooyctes are fused using a single 90-V pulse (900 V/cm) delivered for 10 µs. Wash oocytes after pulsing to remove the electrofusion medium and then incubate for approx 1 h.
10. Subject unfused constructs remaining after a 30-min incubation to a second pulse. Although additional pulses can be given, in our experience, constructs that do not fuse after two pulses generally will not be viable. The electrical pulses do not adequately activate the mouse oocyte. Activation is described in the next section.

3.1.4. Oocyte Activation

After either injection or electrofusion, the constructs are incubated for a period of time before activation. The optimum length of this delay before activation for most donor cell types tested is approx 1 h (*see* **Note 17**). Activation is performed in Ca^{2+} free CZB-G medium supplemented with 10 m*M* $SrCl_2$ and 5 µg/mL cytochalasin B (*see* **Notes 2** and **13**). The $SrCl_2$ has a tendency to precipitate, so it should be added only after the culture medium is fully equilibrated at 37°C and 5% CO_2.

1. Prepare drops under oil quickly and return to the incubator. Care must be taken to ensure that no precipitate forms before or during the activation.
2. Maintain oocytes in this medium for 5–6 h. The exact length of the activation period required may vary with donor cell type. For example, we find that a 6-h activation works well for cumulus donor nuclei, whereas a 5-h activation works well for myoblast donor nuclei.
3. After activation, examine the cloned constructs for the presence of pseudopronuclei, wash the successfully activated constructs extensively and then culture.

3.1.5. Embryo Culture Before Transfer

One important consideration is the time at which embryos are transferred to foster mothers. Cloned embryos exhibit altered preferences with respect to their culture environment, and often are decidedly nonembryo-like ***(11,12)***. Thus, transferring embryos at a very early stage (e.g., two-cell) could be counterproductive. We have found that embryo transfer to the uterus at the morula stage provides a reasonable compromise between allowing a greater time for reprogramming vs minimizing the time in culture.

4. Notes

1. We ***(11,12)*** and others ***(13)*** have used a variety of different culture media in cloning studies. These include predominantly "traditional" embryo culture media, such as WM, M16 medium, CZB or CZB-G, Gardner's media G1 and G2, and KSOM medium, and somatic cell culture formulations, such as MEM-α. Several factors affect the choice of culture medium.
2. One factor that affects the choice of culture medium is whether dimethylsulfoxide (DMSO) is used in the activation protocol. Cumulus cell-cloned constructs

develop reasonably well beyond the two-cell stage in either CZB or CZBG when DMSO is used ***(9,11)***. We find, however, that when DMSO is not used that cumulus cell-cloned embryos develop better in MEM-α, WM, or in combination systems involving medium replacement at the morula stage (e.g., WM → KSOMaa, KSOM → KSOMaa, CZB-G → KSOMaa). The effects of DMSO appear to be donor cell type specific and dependent upon other variables. We have observed that DMSO does not have the same effect in myoblast-cloned embryos as in cumulus cell-cloned embryos ***(12)***. Whereas DMSO treatment may permit cumulus cell-cloned embryos to tolerate standard embryo culture media, this is not seen with myoblast-cloned embryos. DMSO did have a positive effect on myoblast-cloned constructs when the oocyte activation procedure was delayed ***(12)***. Success can be achieved without DMSO, relying instead on correct optimization of cloned embryo culture medium for any given donor cell type.

3. We ***(11,12)*** and others ***(13)*** have reported that cloned mouse embryos can differ dramatically from normal fertilized embryos or parthenogenetic embryos with respect to culture system preference. A striking example of this is the response of myoblast-cloned embryos. Whereas appreciable development to blastocyst stage can be achieved with the WM/KSOMaa culture system for cumulus cell cloned embryos, this culture system produces variable results with myoblasts of different origins. Clones made with nuclei from the established and widely propagated myoblast cell line C2C12 develop moderately well in this system, albeit less well than cumulus cell cloned embryos. Primary myoblast lines, however, develop poorly in this medium, exhibiting a severe two-cell block ***(12)***. Other established cell lines that we have employed also exhibit this block. For primary myoblasts, the block can be overcome by employing a medium based on the same somatic cell culture medium as is used to grow the myoblasts, specifically a 1:1 mixture Ham's F10:DMEM supplemented with BSA, penicillin, streptomycin, and pyruvate ***(12)***. Clones made with primary myoblast nuclei can exhibit more than 40% formation of blastocysts with increased cell numbers and robust morphologies ***(12)***. This culture medium produces slightly less efficient development using some other established myoblast lines, a difference that may reflect an effect of genetic background or possibly specific muscle of origin. Other donor cell types (e.g., B-cells) that we have employed likewise support poor development of cloned constructs in standard embryo culture media (S. Gao, unpublished). For cumulus cell cloning, the MEM-α formulation has proven in our laboratory to be superior to standard embryo culture media.
4. Observations collectively indicate that the donor nucleus significantly alters the phenotype of cloned embryo constructs, which is most likely the result of comparatively slow and inefficient reprogramming of the donor nucleus coupled with the precocious timing of embryonic genome activation in mice ***(14)***. As the ooplasm becomes permissive for transcription by the mid-late one-cell stage, poorly reprogrammed donor nuclei would initiate expression of an at least partial somatic cell repertoire of genes, including genes that affect basic cellular functions such as metabolism and homeostasis. Cumulus cell clones exhibit a strong

preference for glucose containing media at the one-cell stage, when normal embryos do not require glucose, as well as enhanced glucose uptake relative to control embryos *(11,12)*. This is expected if the cumulus cell genome directs the expression of genes that promote glucose uptake and enhanced metabolism of glucose over other compounds, such as lactate or pyruvate. Cumulus cell cloned embryos also display a sensitivity to amino acids in the medium, suggesting that genes affecting amino acid uptake and homeostasis may also be expressed aberrantly *(11,13)*. Myoblast-cloned constructs express the glucose transporter GLUT4, which is normally expressed in myoblasts but not in preimplantation embryos *(12)*.

5. The apparent ability of donor cell characteristics to be elaborated within early cloned embryo constructs calls into question assertions that differentiated cell nuclei may be inherently less able than undifferentiated cell nuclei to be reprogrammed successfully. Although this may indeed be true in some cases, it must be appreciated that in many cases differentiated and undifferentiated cells also differ with respect to spatial location within the tissue and with respect to metabolic or physiological properties. Such differences could affect the ability of cloned embryos to develop owing to metabolic or physiological derangements in the cloned embryos, which would be distinct from effects related to, for instance, chromatin structure differences between differentiated and undifferentiated cells. Because of this, it may be necessary to optimize the culture medium not only according to cell type, but also according to differentiation state of the donor cell.
6. Different approaches can be taken to achieve this objective. One way is to prepare concentrated stock solutions of basic salts that can then be combined to prepare fresh medium, with the addition of more labile components like BSA, pyruvate, etc. Alternatively, a modest amount (50–100 mL) of medium can be prepared weekly or biweekly from dry chemicals.
7. One way to ensure this is to test each batch of medium using an osmometer. Alternatively, water can be added to volume by monitoring total mass of the medium in a preweighed vessel. In our experience, this leads to a satisfactorily consistent osmolarity. With either method, batches of media should be tested on spare embryos or oocytes to ensure correct osmolarity. Syringes used to sterilize the medium or medium components should be free of lubricants. They can be rinsed with ethanol and then allowed to dry before use. Sterilization filters should be first pretreated by filtering a small quantity of medium, which is then discarded before filtering the remainder.
8. It is recommended that several different sources and lots be tested in a well-controlled manner for suitability. This testing requires the preparation of media using the new lot and then comparison to media employing a lot previously proven to be satisfactory.
9. We have had experiences in the past in which treated tissue culture grade dishes have adversely affected the embryos in culture. Alterations in the coating process can be made by the manufacturer without the scientist's knowledge, and can have adverse consequences. The untreated dishes are also less expensive.

10. Although embryo culture conditions often incorporate a reduced amount (5%) of O_2, we have not found that for cloned embryos the reduced O_2 is beneficial, and may in some cases be detrimental.
11. Currently, it is unknown from where the heterogeneity comes. Potentially, this could result from heterogeneity among recipient oocytes, heterogeneity in how the oocytes are handled, heterogeneity among the donor cell population, and heterogeneity in effects of the cloning procedure on the donor nucleus. Any of these variables should be subject to experimental control to some degree. Care should be exercised in the timing and dose of hormonal injections for superovulation, the time of oocyte isolation, and the selection of those oocytes of greatest apparent morphological quality. Care should be taken to handle the oocytes in small groups in order to avoid differences in handling and to minimize insults to the oocytes. Correct fabrication of injection pipets, selection of cells for aspiration, and pipeting method to remove cytoplasm are likely all advantageous. It is possible that the heterogeneity could be lessened by culture medium optimization as well. The health and quality of donor cells and their nuclei is also an obvious factor that should be controlled. It may also be possible to improve cloning by prior treatment of donor cells, either to adapt them metabolically and physiologically to embryo-like culture conditions, or to initiate reprogramming before nuclear transfer.
12. Two types of optics can be employed for visualization of the SCC. One is the use of a fixed-stage microscope equipped with water immersion objectives and Hoffman Modulation Contrast optics. Excellent clarity can be achieved with this system, but the configuration can be inconvenient for some purposes, as it requires immersion of the objectives in the bath with the embryos, and thus is not compatible with separation of embryos or cells into multiple drops under oil. If a single microscope must be employed for injections as well as SCC removal, then an inverted microscope configuration will be preferable. Excellent visualization of the SCC can be achieved with Hoffman Modulation Contrast optics, and this is preferable to that achieved with differential interference contrast optics.
13. We use a cytochalasin B stock (e.g., 2.5 or 5 mg/mL) that is dissolved in ethanol. Others use a 100X stock dissolved in DMSO. We find, however, that the use of DMSO in this manner can result in unexpected and uncontrolled effects on the cloned constructs, and that such effects can be donor cell-specific ***(12)***. We keep the concentration of cytochalasin B as low as possible to permit SCC removal and to suppress polar body extrusion, typically in the range of 2.5–3 µg/mL for SCC removal and 5 µg/mL for activation. When DMSO is not used for either SCC removal or activation, we find that this alters what can be tolerated during the injection procedure. Most noticeably, we find that the use of high concentrations (e.g., 12% w/v) of PVP in the medium in which the donor cells are suspended leads to dramatic reductions in development ***(11)***. When DMSO is not used, therefore, we reduce the concentration of PVP to approx 2–3%. The lower PVP concentration reduces the amount of lubricating effect observed with the higher concentration of PVP. To counter this, one can siliconize the injection pipets by treating with a 5% solution of dimethyldichlorsilane in chloroform, followed by extensive washing with water.

14. Care should be taken handling the mercury, including protective clothing (e.g., gloves) and a chemical fume hood. All mercury should be recovered at the end of the experiment. Used mercury can be cleaned by washing with alcohol, and then re-used.
15. Using HEPES-buffered medium allows a larger number of oocytes to be injected at a single time, but may result in the injection of HEPES into the cell, which could be harmful. The use of bicarbonate buffered medium requires that multiple dishes be set up in advance and kept equilibrated with 5% CO_2 (typically in a 37°C incubator). Groups of no more than approx 10 oocytes are injected before the dish is returned to the incubator. In this way, the oocytes are injected before any significant change in medium pH occurs. This technique also minimizes the length of time spent at room temperature during the injection procedure. A modified protocol involves putting the oocytes in HEPES-buffered medium but using a bicarbonate-buffered medium for the suspension of donor cells. The donor cell drop can be refreshed periodically, and the use of HEPES-buffered medium for the oocytes permits larger groups of oocytes to be injected. We have observed an effect of the medium in which oocytes are injected on later responses of cloned embryos to culture media. Thus, for any given donor cell type it may be helpful to test combinations of media for different steps in the procedure.
16. An often-used formulation with 300 m*M* mannitol is hypertonic to the oocytes and typically results in shrinking, osmotic stress, and poor efficiency of fusion.
17. It is advantageous to initiate oocyte activation as soon as possible. We have observed that the total length of time between injection of females with hCG to induce ovulation and the oocyte activation step can affect outcome ***(12)***. To limit the duration of the delay before oocyte activation, oocytes can be isolated at approx 13 h after hCG injection and those to be used subjected to SCC removal within 1–2 h. By keeping the cohorts of embryos in numbered drops, one can ensure that the injection procedure begins with those oocytes from which the SCC was removed first. This allows adequate time for recovery from the cytochalasin B treatment, thus avoiding lysis during the injection. Injection can often be completed within 1 h. After the 1 h delay, oocytes can be activated.

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METHODS IN MOLECULAR BIOLOGY™

Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
Alan O. Trounson

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Cell Reprogramming and Transgenesis

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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8

Production of Cloned Calves Using Roscovitine-Treated Adult Somatic Cells as Donors

Kazuchika Miyoshi, Sezen Arat, and Steven L. Stice

Summary

The stage of the donor cell cycle is a major factor in the success of cloning. Quiescent cells arrested in the G_0/G_1 phases of the cell cycle by either serum starvation or growth arrest when cultured cells reach confluence have been used as donors to produce cloned animals. Recently, we have developed a novel and effective method using roscovitine to synchronize adult bovine granulosa cells in the G_0/G_1 cell cycle stage. The resulting fetal and calf survival after transfer of cloned embryos was enhanced in the roscovitine-treated group compared with serum-starved controls. The methods described in this chapter outline (1) the preparation of donor cells, (2) the preparation of recipient oocytes, and (3) the production of cloned embryos. The first section involves methods for the preparation of donor cell stocks from isolated granulosa cells and the roscovitine treatment of the cells before nuclear transfer. The second section explains procedures of in vitro maturation of recipient oocytes. The last section involves methods for the production of cell–oocyte complexes, the fusion of the complexes, and the activation, in vitro culture, and transfer into recipient females of cloned embryos.

Key Words: Bovine; cell cycle; cloning; embryos; implantation; nuclear transfer; pregnancy; roscovitine.

1. Introduction

Cloning of mammals by nuclear transfer of differentiated cells has become a tool to propagate valuable animals ***(1–5)*** and can be used as an avenue to produce genetically modified animals ***(6,7)***. The stage of the donor cell cycle is a major factor in the success of nuclear transfer in mammals ***(1,8)***. Quiescent donor cells arrested in G_0/G_1 phases of the cell cycle have been used to produce cloned mice ***(9)***, sheep ***(1,10)***, goats ***(11)***, pigs ***(12,13)***, rabbits ***(14)***, and cattle (**refs.** *2* and *15*; *see* **Note 1**). Serum starvation and growth arrest when cultured cells reach confluence are two major methods to synchronize donor cells in the

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G_0/G_1 cell cycle stage. However, pregnancy losses during the last trimester of gestation *(16,17)* and compromised postnatal health of the offspring *(18,19)* have been observed repeatedly. Currently, the widespread application of this cloning technology is limited as the result of these third-trimester fetal losses and the unacceptable first-month mortality rates for clones.

The specific cyclin-dependent kinase 2 inhibitor roscovitine has been shown to effectively arrest human fibroblasts in G_0/G_1 phases of the cell cycle *(20)* as well as maintain bovine oocytes at the germinal vesicle stage of maturation by inhibiting maturation/meiosis/mitosis-promoting factor, a member of the cyclin-dependent kinase family *(21)*. After the removal of roscovitine, cells arrested in G_0/G_1 phases resume cycling and enter the S phase as expected *(20)*, and oocytes arrested at the germinal vesicle stage progress to metaphase II *(21)*, indicating that the effects of roscovitine are fully reversible. In our recent report *(22)*, bovine granulosa cells treated with roscovitine were compared with serum-starved cells. A higher proportion of cells were synchronized in G_0/G_1 phases of the cell cycle with the roscovitine treatment relative to the control cells (cycling cells) or serum-starved cells. Although a higher percentage of blastocysts were produced using the serum-starved cells as donors, fetal survivability after embryo transfer was significantly higher in the roscovitine-treated group during the last 60 d of gestation. There were more healthy calves that survived gestation, parturition, and the first 60 d of life in the roscovitine-treated group than in the serum-starved group.

2. Materials

1. Saline.
2. Heparin (Sigma, St. Louis, MO).
3. Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Sigma).
4. Fetal calf serum (FCS; Cambrex Biosciences, Walkersville, MD).
5. Penicillin–streptomycin solution (10,000 U/mL penicillin G and 10,000 µg/mL streptomycin; Sigma).
6. Dulbecco's phosphate-buffered solution (Cambrex).
7. 0.4% Trypan blue solution (Sigma).
8. 0.25% Trypsin–EDTA solution (Sigma).
9. Dimethyl sulfoxide (Sigma).
10. Liquid nitrogen.
11. Roscovitine (Sigma).
12. Mineral oil (Sigma).
13. Tissue culture medium-199 (TCM-199; Gibco, Grand Island, NY).
14. Recombinant insulin-like growth factor (rIGF)-1 (Sigma).
15. Bovine luteinizing hormone (bLH; Sioux Biochemicals, Sioux Center, IA).
16. Bovine follicle-stimulating hormone (bFSH; Sioux Biochemicals).
17. Hyaluronidase (Sigma).
18. Hoechst 33342 (Sigma).

19. Cytochalasin B (Sigma).
20. Cycloheximide (Sigma).
21. Calcium ionophore (Sigma).
22. Cell culture medium: DMEM/F12 medium supplemented with 10% (v/v) FCS, and 1% (v/v) penicillin–streptomycin solution.
23. Maturation medium: TCM-199 supplemented with 10% FCS, 50 µg/mL sodium pyruvate, 1% penicillin–streptomycin solution, 1 ng/mL rIGF-1, 0.01 U/mL bLH, and 0.01 U/mL bFSH.
24. 2X Freezing medium: DMEM/F12 medium supplemented with 20% FCS, 1% penicillin–streptomycin solution, and 20% (v/v) dimethyl sulfoxide.
25. Roscovitine culture medium: DMEM/F12 medium supplemented with 10% FCS, 1% penicillin–streptomycin solution, and 15 µ*M* roscovitine.
26. Activation medium: TCM-199 supplemented with 1% FCS, 5 µg/mL cytochalasin B, 10 µg/mL cycloheximide, and 5 µ*M* calcium ionophore.
27. Wire mesh filter: 75 µm (Kenis, Osaka, Japan, or similar).

3. Methods

The methods described below outline (1) the preparation of donor cells, (2) the preparation of recipient oocytes, and (3) the production of cloned embryos.

3.1. Donor Cell Preparation

The next steps in this process involve the preparation of donor cell stocks from isolated granulosa cells followed by the roscovitine treatment of the cells before nuclear transfer.

3.1.1. Isolation of Granulosa Cells and Preparation of Frozen Stocks

1. Aspirate follicular fluid containing granulosa cells from ovarian follicles of a donor cow by using an ultrasound-guided approach that is used to recover oocytes for in vitro fertilization (*see* **Note 2** and **ref. *23***). Transfer follicular fluid into 50-mL centrifuge tubes containing saline supplemented with 25 U/mL heparin. Filter follicular fluid and saline through a filter with a wire mesh of approx 75 µm. Stand tubes containing filtered follicular fluid in ice for 10–15 min and pipet precipitate out. Transfer the precipitate into 1.5-mL centrifuge tubes on ice and transport them to the laboratory.
2. Transfer the precipitate from **step 1** into a 15-mL centrifuge tube containing 12 mL of cell culture medium. Centrifuge the tube at 200*g* for 5 min at room temperature. Remove supernatant and resuspend the pellet in 1 mL of the cell culture medium. Dilute 5 µL of the cell suspension in 45 µL of 0.4% Trypan blue solution and calculate the concentration of cells using a hemacytometer. Seed cells into a six-well tissue culture plate (2 mL of the cell culture medium per well) with concentrations ranging from 10^4 to 10^5 cells/mL.

3. Change the cell culture medium every 48–72 h. After reaching confluency, wash the cells with Dulbecco's phosphate-buffered solution, treat with 0.25% trypsin–EDTA solution (300 µL per well) at 37°C for 10 min, and suspend in the cell culture medium. After centrifugation as in **step 2**, resuspend the pellet in the cell culture medium, reseed into another plates, and grow to confluency.
4. Prepare a single-cell suspension by the standard trypsinization as in **step 3** and collect cells by centrifugation as in **step 2**. Resuspend the cells in 1 mL of DMEM/F12 medium supplemented with 20% FCS and 1% penicillin–streptomycin solution. Calculate the concentration of cells as in **step 2** and adjust volume of the cell suspension to get a concentration of 10^6 cells/mL. Transfer 250 µL of 2X freezing medium into 2-mL cryovials and add 250 µL of the cell suspension into each vial. Transfer the vials into a freezing container. Let it stand for 15 min at room temperature and transfer into a –80°C freezer. The next day, transfer the stocks of donor cells into liquid nitrogen.

3.1.2. Roscovitine Treatment of Donor Cells

1. After thawing the stock in a water bath, suspend the cells in 10 mL of the cell culture medium. Collect the cells by centrifugation, resuspend in the cell culture medium, and culture in a six-well plate to approx 80% confluency as in **step 2** of **Subheading 3.1.1.** (*see* **Note 3**).
2. Passage half the cells from an 80% confluent well to a 35-mm culture dish and culture for 24 h.
3. The next day change culture medium to the cell culture medium supplemented with 15 µ*M* roscovitine (roscovitine culture medium) and continue to culture for an additional 24 h before nuclear transfer.
4. Prepare a pellet of donor cells from a well by the standard trypsinization and centrifugation as in **steps 2** and **3** of **Subheading 3.1.1.** Resuspend the cells in 150 µL of roscovitine culture medium.

3.2. Recipient Oocyte Preparation

1. Collect ovaries at a local slaughterhouse and transport to the laboratory at 30 to 35°C. Wash the ovaries with saline supplemented with 1% penicillin–streptomycin solution.
2. Aspirate follicular fluid from antral follicles (3–8 mm in diameter) with an 18-gage needle using vacuum suction.
3. Stand tubes containing follicular fluid at room temperature for 10–15 min and pipet precipitate out. Transfer the precipitate into a grid dish and add TL-HEPES medium *(24)*. Select oocytes that have a homogenous cytoplasm and at least three layers of cumulus cells and wash them with TL-HEPES medium three times.
4. Transfer selected oocytes into four-well plates (approx 50 oocytes per well) containing 500 µL of maturation medium overlaid with 400 µL of mineral oil. Culture oocytes for 16–18 h at 39°C under 5% CO_2 in air.
5. After maturation, transfer oocytes with expanded cumulus cells into a 15-mL centrifuge tube containing 500 µL of TL-HEPES medium supplemented with 0.1%

(w/v) hyaluronidase. Vortex the tube for 6 min to remove cumulus cells from oocytes. Wash oocytes with TL-HEPES medium once, transfer into TCM-199 supplemented with 10% FCS, and incubate until enucleation.

3.3. Nuclear Transfer

The steps described in **Subheadings 3.3.1.–3.3.3**. outlines the procedure for producing cloned embryos from donor cells and recipient oocytes prepared as described previously.

3.3.1. Enucleation of Recipient Oocytes and Insertion of Donor Cells

1. Incubate oocytes in TL-HEPES medium supplemented with 2 µg/mL Hoechst 33342 and 7.5 µg/mL cytochalasin B for 10–15 min. Transfer stained oocytes and donor cells into 100 µL of TL-HEPES medium supplemented with 7.5 µg/mL cytochalasin B and 15 µ*M* roscovitine overlaid with mineral oil on a microscope stage.
2. Aspirate the first polar body and metaphase II plate in a small volume of surrounding cytoplasm from each oocyte with a 15-µm inner-diameter glass pipet under ultraviolet light. Insert a donor cell into the perivitelline space of each enucleated oocyte using the same glass pipet (*see* **Note 4**).
3. Wash cell–oocyte complexes with TCM-199 supplemented with 10% FCS and 15 µ*M* roscovitine and incubate them in the same medium until fusion.

3.3.2. Fusion of Cell–Oocyte Complexes

1. At 22–24 h after maturation, wash cell–oocyte complexes (approx 15–20 complexes in a group) with Zimmermann's cell fusion medium ***(25)*** and transfer them into a 100-mm dish filled with 15 mL of the medium on a microscope stage.
2. Sandwich the single cell–oocyte complex between two needle-type electrodes that are attached to micromanipulators. Orient the complex with the donor cell against the positive electrode and with the contact surface between the cytoplast and the donor cell perpendicular to the electrodes (**Fig. 1**). Apply a single direct-current pulse of 40 V for a duration of 20 µs with a prepulse of alternating-current field of 5 V, 1 MHz for 2 s to the complex (*see* **Note 5**).
3. After the fusion pulse, transfer the complexes into TCM-199 supplemented with 10% FCS and incubate until activation.

3.3.3. Activation, Culture, and Transfer of Cloned Embryos

1. At 2 h after fusion, transfer cloned embryos into activation medium and incubate for 10 min at 39°C under 5% CO_2 in air (*see* **Note 6**).
2. Transfer the embryos into TCM-199 supplemented with 10% FCS, 5 µg/mL cytochalasin B, and 10 µg/mL cycloheximide and culture for 1 h at 39°C under 5% CO_2 in air.

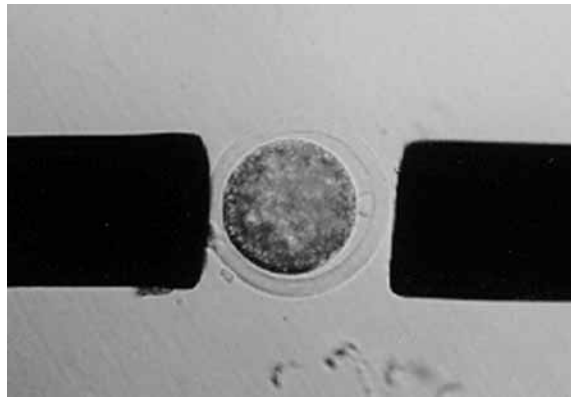

Fig. 1. Fusion of cell–oocyte complexes by the needle-like electrodes.

3. Transfer the embryos into TCM-199 supplemented with 10% FCS and 10 µg/mL cycloheximide and culture for 5 h at 39°C under 5% CO_2, 5% O_2, and 90% N_2.
4. After activation *(26)*, culture cloned embryos in BARC medium (*see* **Note 7** and **ref.** *27*) for 7 or 8 d at 39°C under 5% CO_2, 5% O_2, and 90% N_2.
5. Transfer cloned embryos that reached the blastocyst stage (either one or two embryos per recipient) nonsurgically into the uterine horn ipsilateral to the ovary containing a palpable corpus luteum in recipient cattle that are approx 7 d after a synchronized estrus.

4. Notes

1. It has now been shown that donor somatic cells not in G_0/G_1 phases also can be used to cloned offspring. Cloned mice were produced from embryonic stem cells synchronized in M phase by nocodazole treatment *(28)*. This method was applied to produce a cloned calf from cumulus cells *(29)* and cloned mice from fetal fibroblasts *(30)*. Recently, a cloned piglet was obtained by using colchicine-treated somatic cell nuclei as donors, most of which are in the G_2/M-cell cycle stage *(31)*. However, it may be difficult to obtain a homogenous population of cells in G_2/M phases because colchicine treatment synchronized 70.5% of the cells in G_2/M phases, but 18.8% of them were in G_0/G_1 phases *(31)*.
2. To produce cloned calves from a specified live cow, granulosa cells were recovered from ovarian follicles of the cow by using an ultrasound-guided approach. Alternatively, granulosa cells can be recovered during aspiration of oocytes from slaughterhouse ovaries as in **steps 1** and **2** of **Subheading 3.2.** *(32)*.
3. In vitro culture may cause genetic damage to donor cells. Therefore, fresh or short-term cultured (fewer than 10 passages) donor cells have been used for production of cloned animals *(1,2,6,9,11–15)*. However, detrimental culture effects are debatable. Higher developmental rates to the blastocyst stage were obtained with high-passage (passages 10 and 15) adult somatic bovine cells as

compared with low-passage (passage 5) cells ***(33)***. We obtained similar results by using enhanced green fluorescence protein gene-transfected and nontransfected bovine granulosa donor cells. In vitro developmental rates of cloned embryos derived from cells at passage 15 were higher than those derived from donor cells of lower passages (passages 10, 11, and 13 ***[32]***). We also observed similar result from transfected and nontransfected fetal and adult bovine fibroblast of the same genotype. Donor cells from high passage number (20 population doubling) and low passage number (passage 4) gave similar embryo development rates in vitro ***(34)***. In addition, cloned calves were obtained from embryos reconstituted with the high-passaged cells; whereas, all cloned fetuses derived from the low-passaged donor cells were aborted ***(33)***.

4. It is important to select small donor cells (<15 μm in diameter) with smooth surface. Donor cells greater than 20 μm in diameter are more likely to be beyond G_1 phase of the cell cycle ***(35)***. The small donor cells are technically more difficult to fuse because of the limited contacted area between donor cells and recipient oocytes. Selection of cells having a smooth surface increases the contacted area and may increase fusion rates when compared to the rough surfaced cells.
5. Fusion rates also vary greatly among donor cell types, that is, blastomeres, fetal fibroblasts, and adult somatic cells. Most scientists use a fusion chamber consisting of two parallel wire electrodes mounted on a glass slide or dish at 0.5- to 1-mm apart for fusion of cell–oocyte complexes. Low fusion rates occur when the orientation of the cell and enucleated oocyte to the electrodes is not correct and/or there is little or no cell-to-oocyte contact. We directly compared two systems: a chamber consisting of two parallel wire electrodes vs needle-like electrodes. Our results indicated that the needle-like electrodes improved fusion rates without reducing blastocyst formation rates when using bovine granulosa donor cells ***(36)***.
6. Recently, we have shown that this step is not necessary to activate cloned embryos; there were no significant differences in cleavage and blastocyst formation rates of cloned embryos treated with or without calcium ionophore ***(37,38)***.
7. BARC is not commercially available and its use has never been published, therefore, we have inserted the CR1aa medium that can be used interchangeably with BARC, which was published in **ref.** ***39***.

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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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9

A Double Nuclear Transfer Technique for Cloning Pigs

Irina A. Polejaeva, Shawn Walker, and Keith Campbell

Summary

The first round of double nuclear transfer (NT) procedure includes the following steps: transfer of somatic cell nuclei into enucleated recipient oocytes, fusion, activation, and culture of reconstructed oocytes. The next day, a second round of NT is performed by removing karyoplasts from 1-d-old NT embryos and transferring them into in vivo-derived zygotes from which the two pronuclei have been removed. Couplets are then fused using an electrical pulse and transferred into synchronized recipient gilts. This system, which uses fertilized oocytes as cytoplast recipients, bypasses the inefficiencies of artificial activation procedures, and may promote more successful development.

Key Words: Somatic cell nuclear transfer; cloning; pronuclear exchange; activation; reprogramming.

1. Introduction

The double somatic cell nuclear transfer (NT) procedure described in this chapter was successfully used to produce the worlds' first cloned pigs ***(1)***. Unlike most of the previously described serial NT procedures in which a blastomere of a 4- to 64-cell stage NT-derived embryo was transferred into an enucleated oocyte ***(2–4)***, this method involves a "pronuclear exchange" step, in which the pronuclear-like structure derived from a somatic cell NT zygote is placed into an enucleated naturally fertilized zygote. This step could improve reprogramming and activation processes, leading to increased developmental potential of cloned embryos, possibly as the result of exposure of the donor nucleus to zygotic factors ***(1,5)***.

In addition to the original activation procedure previously published in *Nature* ***(1)***, we have included an alternative method of activation that has been shown to be more efficient for traditional single-step NT ***(6)*** and might also help to increase the efficiency of double NT. The double NT procedure

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described here is technically more challenging than conventional NT; however, this procedure might be advantageous, especially for species in which either activation or somatic cell reprogramming after NT is inefficient and does not allow for development beyond the stage of genomic activation (*see* Chapter 11).

2. Materials

The material list only contains significant pieces of equipment. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Glass, plastic wear, and instruments for cell isolation are not included. A more complete list can be found in Freshney *(7)*.

1. Laminar flow hood.
2. Inverted Zeiss 451316 or comparable Nikon microscope with UV set-up foot pedal shutter (Zeiss, Thornwood, NY).
3. Micromanipulators: Cell Tram Vario (Zeiss-5176000.033).
4. Microsyringes: Cell Tram Air (Zeiss-5176000.017).
5. Narishige or comparable micromanipulators (Narishige Micromanipulators, McHenry, IL).
6. Antivibration table (Zeiss-910837-0093).
7. Slide warmer VWR-15160-800 (VWR International, West Chester, PA).
8. 5% CO_2 Humidified Incubator Forma Scientific 3130 (Marietta, OH).
9. Centrifuge.
10. Aspiration pumps (VWR-54908-035).
11. BTX Fusion Machine Model ECM 2001 (Genetronics, San Diego, CA).
12. BTX fusion chamber, 3.2 mm.
13. Two stereo microscopes (Stemi 2000, Zeiss 455052).
14. Water bath.
15. –80°C Freezer.
16. Liquid nitrogen or vapor phase cell storage unit.
17. In vitro-matured oocytes (BoMed, Madison, WI or TransOva, Sioux Center, IA).
18. Human chorionic gonadotropin (hCG; Intervet America, Millsboro, DE).
19. Pregnant mare serum gonadotropin (PMSG).
20. Regu-Mate (Altrenogest, Hoercst, Warren, NJ).
21. Estrumate (Schering-Plough Animal Health, Union, NJ).
22. Cell culture medium: Dulbecco's modified Eagle medium (DMEM; Gibco, Invitrogen Corporation; Carlsbad, CA), 10% fetal bovine serum (FBS; HyClone, Logan, UT), 1X nonessential amino acids (Gibco), 2 ng/mL basic fibroblast growth factor (bFGF), and 6 µL/mL gentamicin (Gibco; *see* **Note 1**). Store at 4°C for 2 wk.
23. Maturation I: Earls tissue-culture medium (TCM)-199 medium (Gibco), supplemented with 5 µg/mL insulin, 10 ng/mL epidermal growth factor, 0.6 m*M* cysteine, 0.2 m*M* sodium pyruvate, 25 µg/mL gentamycin, 5 µg/mL follicle-stimulating hormone (Sioux Biochemical, Sioux Center, IA), and 10% porcine follicular fluid. Make fresh before use.

24. Maturation II: Earls TCM-199 supplemented with 5 µg/mL insulin, 10 ng/mL epidermal growth factor, 0.6 m*M* cysteine, 0.2 m*M* sodium pyruvate, 25 µg/mL gentamicin, and 10% porcine follicular fluid. Make fresh before use.
25. NCSU23 culture medium: 108.73 m*M* NaCl, 4.78 m*M* KCl, 1.7 m*M* $CaCl_2$, 1.19 m*M* $MgSO_4$, 25.07 m*M* $NaHCO_3$, 1.19 m*M* KH_2PO_4, 5.5 m*M* D-glucose, 1 m*M* L-glutamine, 7 m*M* taurine, 5 m*M* hypotaurine, 0.4% bovine serum albumin (BSA)-V, 100 U/L penicillin-G, and 50 mg/L streptomycin. To avoid precipitation, prepare $CaCl_2$, $MgSO_4$ and $NaHCO_3$ separately and add it to the other ingredients at the end of the preparation. Osmolarity range 275–290 mosm, pH 7.4. Store at 4°C for 1 wk.
26. NCSU23 salt medium: 143.7 m*M* NaCl, 4.78 m*M* KCl, 0.85 m*M* $CaCl_2$, 1.19 m*M* $MgSO_4$, 1.19 m*M* KH_2PO_4, 5.5 m*M* D-glucose, 1 m*M* L-glutamine, 7 m*M* taurine, 5 m*M* hypotaurine, 10% FBS, 0.01 g/L phenol red, 100 U/L penicillin-G, and 50 mg/L streptomycin. Store at 4°C for 1 wk.
27. Low Ca^{2+} fusion medium: 0.297 *M* mannitol, 0.001 m*M* $CaCl_2$ and 0.05 m*M* $MgCl_2$. Store at 4°C for 1 mo.
28. Fusion/activation medium: 0.297 *M* mannitol, 0.1 m*M* $CaCl_2$, and 0.05 m*M* $MgCl_2$. Store at 4°C for 1 mo.
29. Cell freezing medium: 50% FBS, 10% dimethyl sulfoxide, and 40% DMEM.
30. Manipulation medium: TCM-199–HEPES medium (Gibco) containing 10% FBS, 50 U/mL penicillin-G, and 50 µg/mL streptomycin sulfate.
31. Sorbitol fusion medium: 0.3 *M* D-Sorbitol, 0.1 m*M* $MgSO_4$, 0.05 m*M* $CaCl_2$.

3. Methods

The methods described below outline (1) isolation of primary cells and preparation of cells for nuclear transfer, (2) oocyte and zygote collection, (3) first NT step, (4) fusion and activation, (5) second NT procedure, and (6) embryo transfer.

3.1. Preparation of Somatic Primary Cells for NT

Several different types of primary cells have been successfully used for cloning including, but not limited to, fetal fibroblasts, adult fibroblasts, cumulus cells, granulosa cells, and epithelial cells *(8)*. We describe methods in **Subheadings 3.1.1.–3.1.3.** for the isolation of granulosa cells and fetal primary fibroblasts, the most commonly used cell types for NT.

3.1.1. Isolation of Porcine Granulosa Cells

1. Animals should be prepared as described in **Subheading 3.2.2.** for oocyte collection.
2. Harvest granulosa cells from ovarian follicles by needle aspiration of follicular fluid.
3. Aspirate follicles of 2–5 mm diameter by penetrating through the cortex of the ovary and collecting follicular fluid into a 15-mL conical tube.
4. Centrifuge follicular fluid at 168*g* for 10 min, remove supernatant, and resuspend the cell pellet in 10 mL of cell culture medium.

5. Plate cells in a T-75 flask at 38.5°C in 5% CO_2 and allow for outgrowth.
6. After isolation, expand cells for several days and then cryopreserve using standard cell cryopreservation techniques as described in **Subheading 3.1.2.**

3.1.2. Cell Cryopreservation

1. Aspirate culture medium from the flask and rinse the flask with 12 mL of $Ca^{2+}Mg^{2+}$-free phosphate-buffered saline (PBS). Rock the flask so that the solution covers the entire flask.
2. Aspirate PBS from the flask. Pipet 1 mL of trypsin–EDTA solution (0.25% trypsin, 1 m*M* EDTA) into the flask. Rock the flask to let trypsin cover the entire flask.
3. Incubate the flask at 37°C for 3 min. Monitor the cells under the microscope to avoid long exposure of cells to trypsin. Trypsin treatment causes cells to detach from their culture surface. If cells are not detached, place them in an incubator for more time.
4. Once cells are detached, add 10 mL of cell culture medium to the T 75 and wash cells off the flask surface via repeat pipetting.
5. Transfer the cells to a 15-mL conical tube, take a 20-µL sample, and count cells using a hemacytometer.
6. Centrifuge cells for 3 min at 672*g*.
7. Resuspend pellet in cell freezing medium at approx 1×10^6 to 3×10^6 cells per milliliter.
8. Place 0.25–1 mL of cell suspension into each cryovial.
9. Place cryovials in Nalgene Cryo 1°C freezing container. Place the container into the –80°C freezer.
10. Between 12 and 48 h after freezing, move cryovials to liquid nitrogen tank.

3.1.3. Isolation of Primary Fetal Fibroblasts

3.1.3.1. Mechanical Isolation

1. Using sterile scissors and forceps cut open the uterine wall, remove 30- to 40-d-old fetal/placental units and transfer each into separate 10-cm culture plates containing 10 mL of Dulbecco's phosphate-buffered saline (DPBS) with 25 µg/mL gentamicin (Gibco).
2. Treat fetuses individually as follows. Using two pairs of forceps, dissect the fetus free from placental membranes. Decapitate fetus then dissect out and discard the soft tissue (e.g., heart, liver, and other viscera). Wash the carcass in DPBS containing 25 µg/mL of gentamicin.
3. Move the fetal carcass into a fresh dish containing a small amount (1–2 mL) of culture medium, to keep the fetus moist. Using curved scissors or a scalpel, finely mince the tissue.
4. Add 5 mL of culture medium to the minced tissue and split the contents between two T-75 flasks. Add additional culture medium to cover the surface of the flask; note that too much medium might cause tissue to float and inhibit cell attachment and outgrowth.

5. Leave the flask undisturbed for at least 24 h, only changing medium to remove tissue debris after initial cell growth is observed.
6. Expand cells for several days and then cryopreserve using standard cell cryopreservation techniques as described in **Subheading 3.1.2.**

3.1.3.2. Enzymatic Digestion

1. Prepare fetal carcass as described in **Subheading 3.1.3.1., steps 1** and **2.**
2. Place fetal carcass into a fresh dish containing a small amount (1–2 mL) of trypsin–EDTA solution (0.25% trypsin, 1 m*M* EDTA). Using curved scissors or a scalpel finely, mince the tissue. Add 5 mL of trypsin–EDTA to minced tissue and transfer content of the dish into a 15-mL conical tube, incubate tube for 5–10 min at 37 to 38°C.
3. Following incubation, shake the tube vigorously. Neutralize the trypsin by adding 5 mL of DMEM plus 10% FBS to the tube. Digest fetuses older than 40 d for 2 h in 1% (w/v) trypsin and 1 mg/mL collagenase type 2 in DMEM containing antibiotic–antimycotic solution.
4. Allow the large pieces of cellular debris to settle out over a period of 1 to 2 min. Place the supernatant into a fresh tube and spin down the cells (672*g*, 3 min). Aspirate the supernatant and resuspend the cell pellet in 10 mL of cell culture medium.
5. Place resuspended cells in a T-75 flask and leave undisturbed for at least 12 h. Only change medium to remove tissue debris after the initial cell attachment is observed.
6. Expand cells for several days and then cryopreserve using standard cell cryopreservation techniques as described in **Subheading 3.1.2.**

3.1.4. Preparation of Cells for NT

To maximize the number of healthy cells with a diploid DNA content, cell cycle synchronization was obtained using a cell contact inhibition method (*see* **Note 2** ***[1]***).

1. Seed donor cells at 1–5 × 10^4 cells per 35-mm dish in DMEM supplemented with 2 ng/mL bFGF and 10% FBS. Allow cells to grow to confluency prior to use in NT. Cells should be confluent for 12–48 h before to use in NT.
2. Place the 35-mm dish containing confluent donor cells into a cell culture hood and remove media via aspiration with a Pasteur pipet.
3. Rinse dish with 1 mL of warmed PBS (Ca^{2+}, Mg^{2+}-free), swirl, and remove via aspiration.
4. Add 500 µL of warmed trypsin–EDTA solution to dish and place on warm plate and monitor until cells begin to round up (usually within 1–2 min); carefully aspirate excess trypsin before the cells detach from the bottom.
5. Shake the dish to loosen cells and then resuspend in 1–2 mL of manipulation medium or in NCSU23 salt medium ***(6)***. Serum in the medium will inhibit any residual proteolytic activity.

6. Aspirate medium containing the detached cells using a 2-mL serological pipet and place into a labeled 15-mL centrifuge tube.
7. Allow cells to recover following trypsinization for at least 15 min before use.
8. Cells can be stored in suspension at 38°C for 2–3 h.

3.2. Oocyte and Zygote Collection

Either in vivo or in vitro oocytes can be used for the first NT procedure.

3.2.1. In Vitro-Matured Oocytes

1. Aspirate cumulus–oocyte complexes from ovarian follicles and mature in pre-equilibrated maturation I medium at 38.5°C in 5% CO_2 in air for 20–22 h.
2. After 20- to 22-h maturation, move oocytes into maturation II medium pre-equilibrated for 2 h at 38.5°C in 5% CO_2 in air and culture for an additional 18–20 h. As an alternative, in vitro-matured oocytes may be purchased from a commercial supplier.

3.2.2. Recovery of In Vivo-Matured Oocytes and Zygotes

1. Synchronize gilts (280–320 lbs) by oral administration of 18–20 mg Regu-Mate (Altrenogest) mixed into the feed. Feed Regu-Mate for 5–14 d, depending on gilt estrous cycle stage.
2. Administer Estrumate (250 µg) intramuscularly on the last day of Regu-Mate treatment.
3. Induce superovulation by intramuscular injection of 1600 IU of PMSG 15 to 17 h after the final Regu-Mate feeding.
4. Administer 1500 IU of hCG 82 h after PMSG injection.
5. Collect oocytes by reverse flush of the oviducts 50–52 h after hCG injection using DPBS containing 4 g/L BSA.
6. For zygotes breed naturally or artificially, inseminate gilts 24–36 h after hCG injection and collect zygotes by reverse flush of the oviducts 52–54 h after hCG injection using DBPS containing 4 g of BSA per liter.

3.3. First NT

3.3.1. Oocyte Metaphase II Plate Removal

1. Remove cumulus cells from in vitro matured oocytes at 38–40 h after onset of maturation (hpm), either by repeat pipetting or vortexing in TCM-199 medium containing 0.1–0.3% hyaluronidase.
2. After removal of cumulus cells, rinse oocytes through three drops of manipulation medium. NCSU23 salt medium may be used as an alternative to manipulation medium for the following steps.
3. Begin metaphase II plate removal (MIIPR; typically but not appropriately called enucleation) of in vitro matured oocytes between 38 and 42 hpm. When in vivo-matured oocytes are used, begin MIIPR immediately after oocyte recovery.
4. Place oocytes for MIIPR in manipulation medium containing 5 µg/mL cytochalasin B and 7.5 µg/mL Hoechst 33342 at 38.6°C.

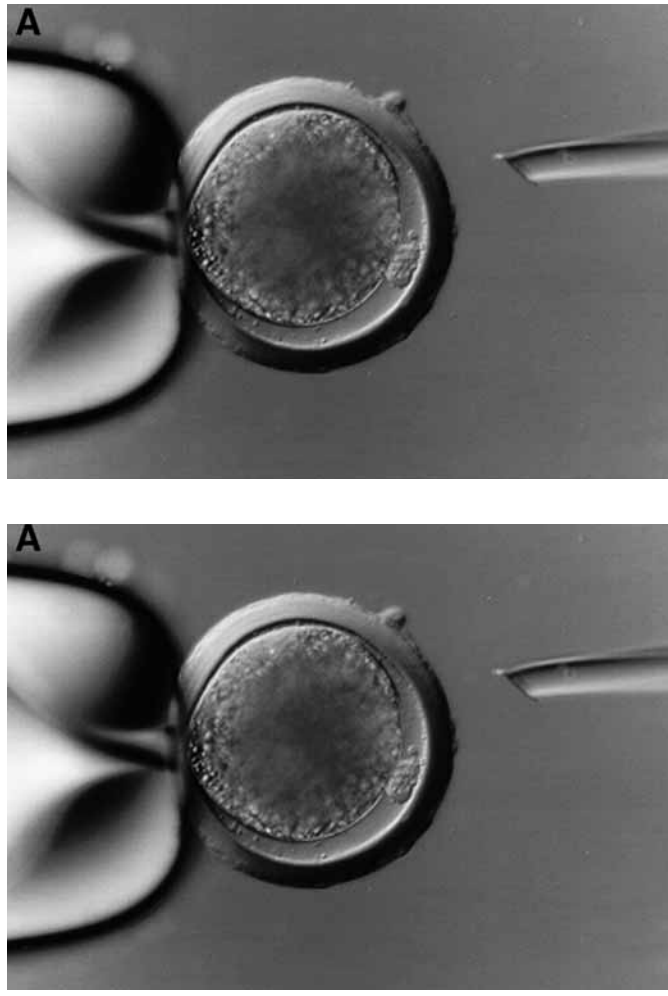

Fig. 1. First step of double NT procedure: In vivo-matured oocyte with extruded polar body. **(A)** Removal of metaphase plate and polar body. **(B)** Injection of somatic cell under zona pellucida of enucleated oocytes. (**C**,**D**) (Courtesy of Revivicor, formerly PPL Therapeutics Inc.)

5. Lower a 140- to 160-µm oocyte-holding pipet into the drop containing oocytes and anchor a single MII oocyte via suction. Once anchored, puncture the zona pellucida and then gently aspirate the first polar body and a small amount of cytoplasm from directly beneath the first polar body using a 17-µm beveled glass pipet (*see* **Fig. 1A**,**B**; *see* **Note 3**).
6. Confirm MIIPR by exposing the aspirated portion of cytoplasm to ultraviolet fluorescent light and checking for the presence of a metaphase plate.

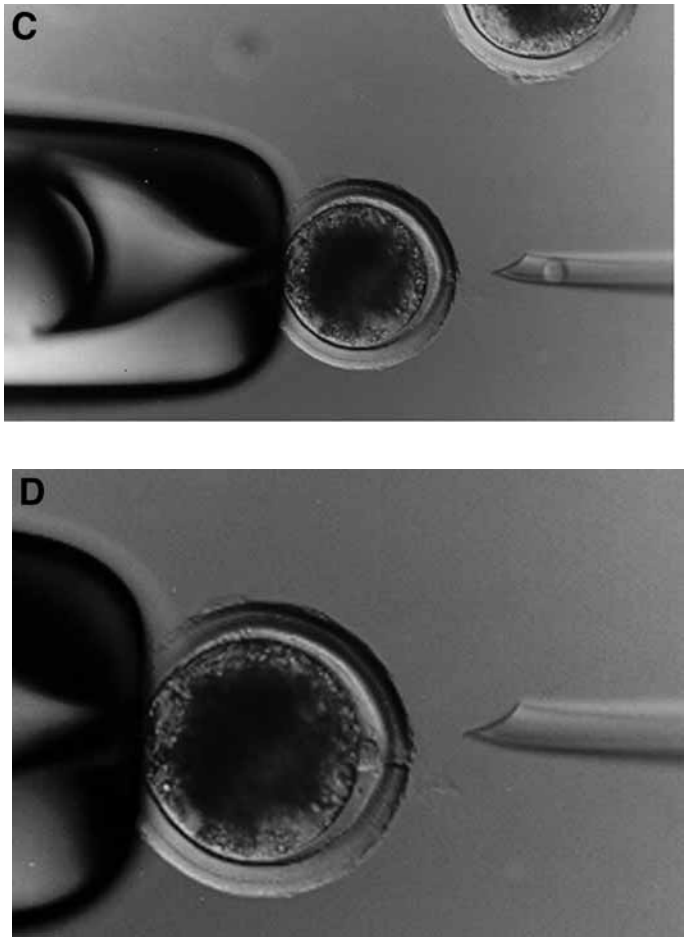

Fig. 1. (*continued*)

3.3.2. Embryo Reconstruction (Day 1)

1. Prepare cells as described in **Subheading 3.1.4.**
2. Transfer cells into a drop containing the same media in which cells were resuspended during cell preparation.
3. Transfer 20–60 MIIPR oocytes to a drop of manipulation medium in preparation for cell transfer.
4. Aspirate 15–30 cells into a 15- to 25-µm beveled micropipet in preparation for cell transfer. The size of the pipet depends on the size of the donor cells. As soon as enough cells are loaded into the micropipet, move micropipets into the drop containing MIIPR oocytes.
5. A moving stage system allows for movement of micropipets between the media drops.

6. At 53–56 h after hCG injection (in vivo oocytes) or at 40–44 hpm (in vitro oocytes), puncture the zona pellucida and transfer a single cell into the perivitelline space forming an oocyte/cell couplet (*see* **Fig. 1C,D**). When all oocytes in the chamber are injected, remove couplets and transfer them to the fusion station.
7. Alternatively, the donor nucleus may be transferred by either manual or piezo-electric-aided injection or by any other chemical or physical means of producing cell fusion.

3.4. Fusion and Activation

3.4.1. Simultaneous Fusion and Activation

1. Equilibrate the oocyte/cell couplets in the fusion/activation medium, then transfer groups of 10–25 reconstructed oocytes to the 3.2-mm fusion chamber containing 700 µL of fusion/activation medium.
2. Using BTX fusion machine, induce fusion and activation by application of an alternating-current (AC) pulse of 3 V for 5 s followed by 2 direct-current (DC) pulses of 1.5 kV/Cm for 60 µs (*see* **Note 4**).
3. Wash the couplets in NCSU23 culture medium *(9)* and incubate for 0.5–1 h at 38.6°C, 5% CO_2.
4. Give an additional activation pulse to reconstructed embryos by applying two DC pulses of 1.2 kV/Cm, for 60 µs, in fusion/activation medium. Wash activated embryos in NCSU-23 culture medium and culture overnight in four-well plate (500 µL/well) at 38.6°C, 5% CO_2.

3.4.2. Fusion and Delayed Activation

In addition to the simultaneous fusion activation protocol described previously, an alternative method termed fusion and delayed activation may be used in preparing first-step NTs. In reports postdating our initial *Nature* article, a great success was obtained using this method.

1. After reconstruction, fuse oocyte/cell couplets in a 3.2-mm fusion chamber by giving a 2-V AC pulse for 2 s followed by 2 DC pulses of 1.5 kV/Cm for 60 µsec in low Ca^{2+} fusion medium.
2. After fusion, incubate couplets in NCSU23 culture medium for 1 h at 38.6°C, 5 % CO_2 before activation.
3. After a 1-h incubation, remove fused oocytes from NCSU23 and activate by application of two 1.2 kV/Cm DC pulses for 60 µs in fusion/activation medium.
4. Wash activated embryos in NCSU23 culture medium and culture overnight in four-well plate (500 µL/well) at 38.6°C, 5% CO_2.

3.5. Second NT (Day 2)

Second NT procedure refers to the reconstruction of enucleated zygotes on day 2, using the pronuclei-like structure produced by day 1 NT.

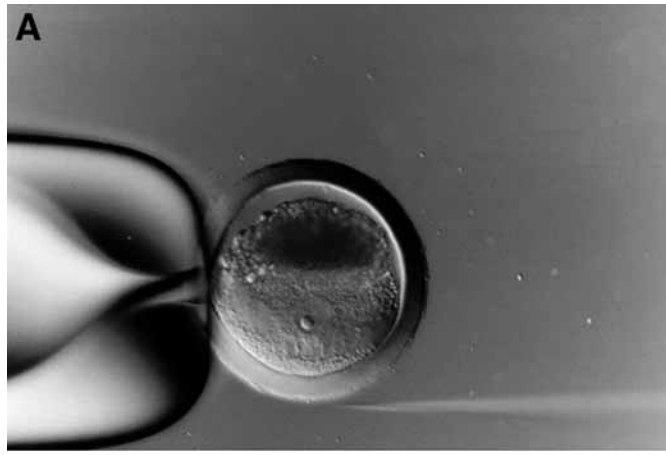

Fig. 2. Second step of double nuclear transfer procedure (Day 2). Appearance of the pronuclear-like structure in Day 1 reconstructed embryos: pronuclear membrane is almost invisible, nuceolus is well defined. **(A,B)** Preparation of karyoplast for "pronuclear exchange." **(C,D)** (Courtesy of Revivicor, formerly PPL Therapeutics Inc.)

3.5.1. Assessment of Nuclear Formation From the Previous Day of NT

1. After an overnight culture, remove reconstructed NT embryos from culture and centrifuged in a Biofuge at 15,000*g* for 15 min to separate out the lipids and enhance observation of the nuclear structure.
2. Place embryos in manipulation chamber in manipulation medium and observe nuclear formation at 200X (*see* **Fig 2A,B**).
3. Select embryos with a nuclear structure and return to a NCSU23 culture plate in the incubator at 38.6°C, with 5% CO_2 in air.

3.5.2. Zygote Enucleation

1. Centrifuge recovered zygotes (*see* **Subheading 3.2.2**) in a 1.5-mL tube containing manipulation medium at 15,000*g* for 15 min in a Biofuge 13 centrifuge.
2. Place zygotes in manipulation medium containing 5 µg/mL cytochalasin B at 38.6°C and incubate for 20 min before enucleation. The use of both microfilament and microtubule (nocodazole, 5 ng/mL) inhibitors might be beneficial for the enucleation step (*see* **Note 5**).
3. Enucleate only fertilized zygotes with two pronuclei (*see* **Fig. 3A**). Insert a pipet (25–35 µm) through the zona pelucida at the 3- to 4-o'clock position into the cytoplasm so that the opening of the enucleation pipet is close to the pronuclei (*see* **Fig. 3B**). Aspirate both the pronuclei and polar body by slowly applying negative pressure on the enucleation line.

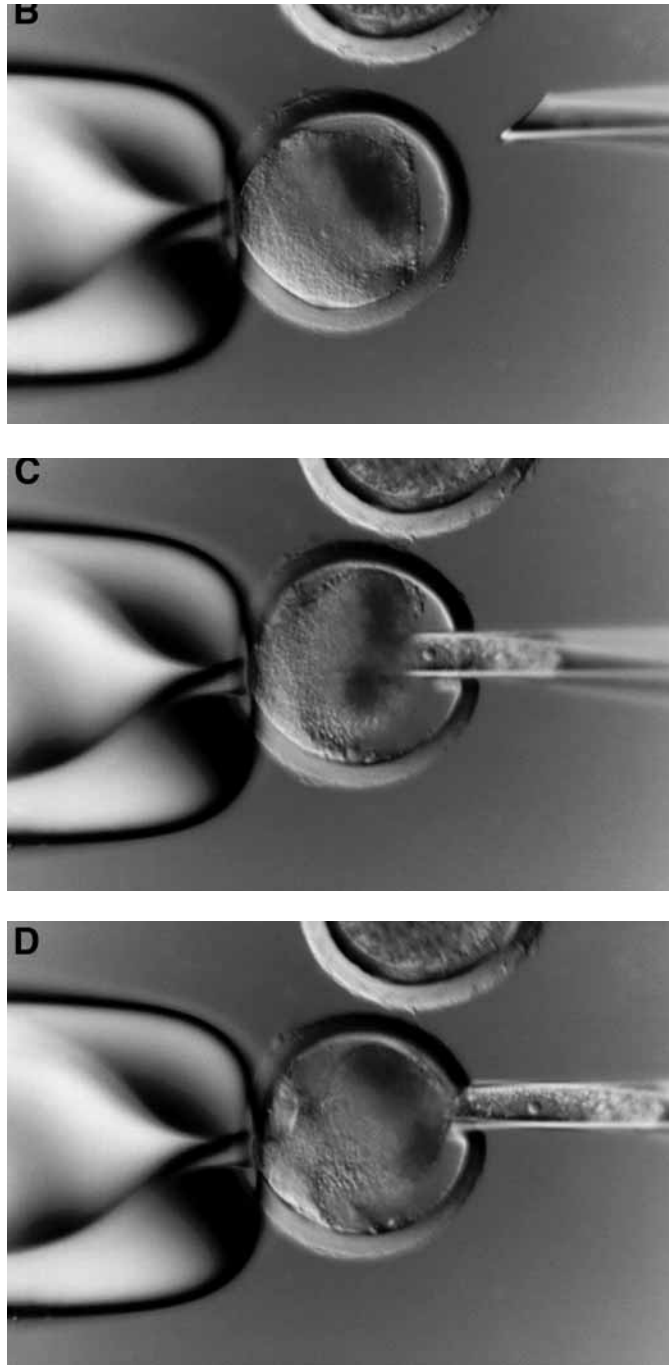

Fig. 2. (*continued*)

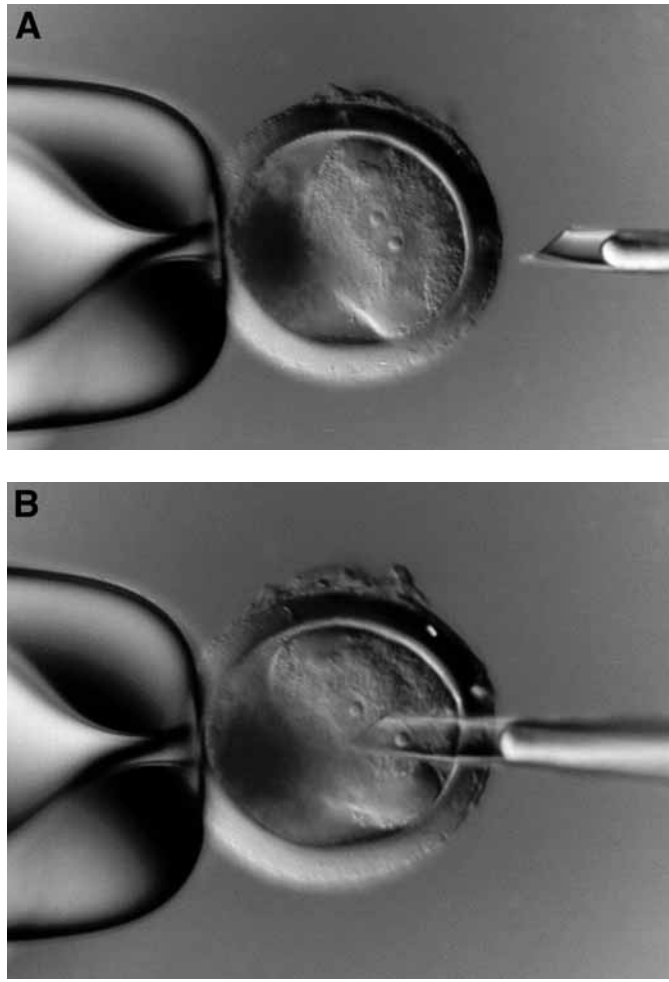

Fig. 3. Appearance of zygote after centrifugation. **(A)** Removal of male and female pronuclei from fertilized zygote. **(B)** Karyoplast preparation **(C)** (the same as **Fig. 2D**). **(D)** Appearance of day 2 reconstructed embryo prior fusion, a couplet of enucleated zygotic cytoplasm and pronuclear-like structure derived from somatic cell. (Courtesy of Revivicor, formerly PPL Therapeutics Inc.)

3.5.3. Reconstruction of Enucleated Zygotes

1. Place reconstructed day 1 embryos (18–20 h after culture) into manipulation medium containing 5 µg/mL cytochalasin B and 5 ng/mL nocodazole.
2. Use a 30- to 45-µm enucleation pipet for manipulation. Prepare karyoplasts using a similar technique to zygote enucleation (*see* **Fig. 2C**,**D**) and insert the enucleation pipet containing the encapsulated nucleus into the enucleated zygote.

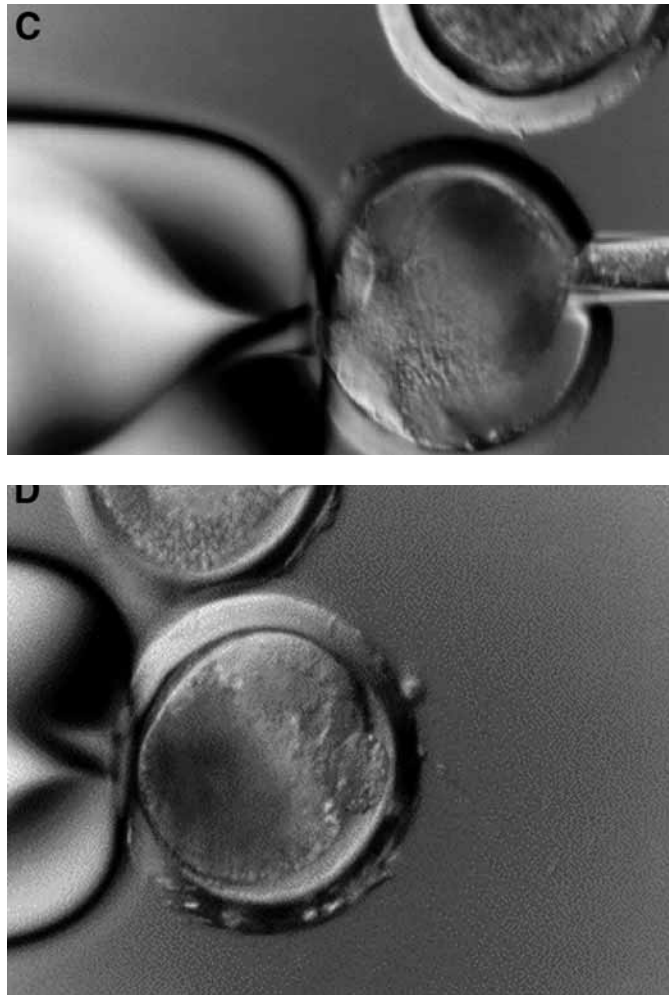

Fig. 3 (*continued*)

Slowly expel the encapsulated nucleus into the perivitelline space of the enucleated zygote (*see* **Fig. 3D**).

3. Fuse couplets in 700 µL of sorbitol fusion medium. Induce fusion by the application of an AC pulse of 3 V for 5 s followed by 2 DC pulses of 1.2 kV/Cm for 60 µs.
4. Wash couplets and place in NCSU23 culture medium for 0.5–1 h at 38.6°C, 5% CO_2 and check for fusion.
5. Transfer fused couplets as soon as possible to the oviduct of a synchronized recipient gilt.

3.6. Embryo Transfer

3.6.1. Recipient Synchronization

Synchronize recipient gilts by oral administration of 18–20 mg of Regu-Mate (Altrenogest) mixed into their feed. Feed Regu-Mate for 14 consecutive days. Administer 1000 U of hCG intramuscularly 105 h after the last Regu-Mate treatment. Transfer embryos 22–26 h after hCG injection (*see* **Note 6**).

3.6.2. Pregnancy Maintenance

Pregnancy maintenance can be aided by using a combination of PMSG and hCG. Inject PMSG (1000 IU) intramuscularly on day 10 of the estrous cycle, day 1 being the first day of estrus. Inject hCG (500 IU) intramuscularly 3–3.5 days later, on day 13 of the cycle ***(10)***.

4. Notes

1. The media given here is one recommended formulation for culture of the selected cell type. Additionally, alternative media formulations suitable for optimal cell growth may be used. This media may vary depending on cell type ***(11)***.
2. In addition to contact inhibition for synchronization of cells, serum starvation may be used. Serum starvation involves culturing cells for 4–6 d in cell culture medium with FBS reduced to 0.5%, which causes cell synchronization at G_1-G_0 ***(12,13)***.
3. During the enucleation process, the cytochalasin B concentration may need to be adjusted depending on oocyte rigidity. If manipulated oocytes are rigid and lysing, slightly increasing the cytochalasin B concentration may alleviate the problem. If oocytes appear soft and stringy, decreasing the cytochalasin B concentrations may reduce the problem.
4. Because pulse generators vary from machine to machine pulse strength recommended for fusion and activation may need to be varied to obtain acceptable levels of fusion and activation without lysis of reconstructed oocytes.
5. If lysis is present after enucleation of the zygote or day 1 reconstructed embryos, then nocodazole and cytocolasin B concentrations should be increased. Additionally, fire polishing of the enucleation tool may help decrease lysis.
6. In addition to using artificially synchronized gilts, naturally cycling gilts may be used for recipients.

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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
Alan O. Trounson

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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The authors provide readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer. Of immense benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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10

Nuclear Transfer in Nonhuman Primates

Shoukhrat M. Mitalipov and Don P. Wolf

Summary

The nonhuman primate is a highly relevant model for the study of human diseases, and currently there is a significant need for populations of animals with specific genotypes that can not be satisfied by the capture of animals from the wild or by conventional breeding. There is an even greater need for genetically identical animals in vaccine development or tissue transplantation research, where immune system function is under study. Efficient somatic cell nuclear transfer (SCNT) procedures could provide a source for genetically identical nonhuman primates for biomedical research. SCNT offers the possibility of cloning animals using cultured cells and potentially provides an alternative approach for the genetic modification of primates. The opportunity to introduce precise genetic modifications into cultured cells by gene targeting procedures, and then use these cells as nuclear donors in SCNT, has potential application in the production of loss-of-function monkey models of human diseases. We were initially successful in producing monkeys by NT using embryonic blastomeres as the source of donor nuclei and have repeated that success. However, when somatic cells are used as nuclear donor cells, the developmental potential of monkey SCNT embryos is limited, and somatic cell cloning has not yet been accomplished in primates. High rates of in vitro development to blastocysts, comparable with in vitro fertilization controls, and successful production of rhesus monkeys by NT from embryonic blastomeres suggests that basic cloning procedures, including enucleation, fusion, and activation, are consistent with the production of viable embryos. Although modifications or additional steps in SCNT are clearly warranted, the basic procedures will likely be similar to those extant for embryonic cell NT. In this chapter, we describe detailed protocols for rhesus macaque embryonic cell NT, including oocyte and embryo production, micromanipulation, and embryo transfer in nonhuman primates.

Key Words: Monkey; embryos; nuclear transfer; cloning.

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1. Introduction

The nonhuman primate is a highly relevant model for the study of human diseases, and currently there is a significant need for populations of animals with specific genotypes that can not be satisfied by capture of animals from the wild or by conventional breeding. Rhesus macaques of Indian origin, carrying the major histocompatibility complex class 1 allele, A 01 (Mamu-A*01), are particularly needed for vaccine development research because they provide a unique opportunity to understand the mechanisms of protective immunity. There is an even greater need for genetically identical animals in vaccine development or tissue transplantation research, where immune system function is under study. Efficient somatic cell cloning protocols could provide a source for genetically identical nonhuman primates for biomedical research. Nuclear transfer (NT), when combined with gene targeting technology, also would support the generation of knockout monkey models for human genetic diseases in which mouse models have been inadequate or inappropriate. Because of the considerable anatomical and physiological differences between mice and humans, particularly as they relate to nervous system function, genetically modified or knockout mouse models often have limited utility in preclinical studies. The lack of accurate animal models potentially hinders development of effective treatments for neurogenetic diseases, such as Kallmann's syndrome, Lesch-Nyhan's disease, or ataxia-telangiectasia.

Remarkable progress in mammalian cloning has been achieved in the past several years. However, somatic cell cloning has not yet been accomplished in primates. We initially were successful in producing monkeys by NT using embryonic blastomeres as the source of donor nuclei *(1)* and have recently repeated that success *(2)*. Moreover, we demonstrated a high potential, similar to sperm-fertilized controls, in vitro blastocyst formation potential of NT embryos derived from 8- to 16-cell stage blastomeres *(2)*. However, the in vivo developmental potential of such embryos, with a pregnancy rate of 9%, was significantly lower than that observed after the oviductal transfer of in vitro fertilization (IVF)-produced embryos (66%) in our laboratory *(3)*.

The developmental potential of somatic cell NT (SCNT) monkey embryos has been limited, seldom progressing beyond the eight-cell stage in vitro. When fetal fibroblasts were used as nuclear donor cells, the resultant NT embryos grew to the blastocyst stage at a frequency of only 1–2% and no pregnancies were initiated after embryo transfer. We concluded that the failure of somatic but not embryonic cell cloning in the monkey was caused by the incomplete reprogramming of the somatic cell nucleus *(2)*. Our studies demonstrating aberrant expression of Oct-4 in reconstructed embryos are consistent with this conclusion *(4)*. Recently, SCNT embryos were created in cynomolgus monkeys

after the transfer of amniotic epithelial cells, with 67% development to blastocysts (*5*). This, along with the arguments herein, suggests that refinements in donor cell selection and cloning protocols will eventually lead to success in somatic cell cloning in nonhuman primates.

High rates of in vitro development to blastocysts, comparable with IVF controls, and successful production of rhesus monkeys by NT from embryonic blastomeres suggests that basic cloning procedures, including enucleation, fusion, and activation, are consistent with the production of viable embryos. Although modifications or additional steps in SCNT are clearly warranted, the basic procedures in somatic cell cloning will likely be similar to those extant for embryonic cell NT. Protocols for rhesus macaque embryonic cell NT, including oocyte and embryo production, micromanipulation and embryo transfer are described in this chapter to illustrate the methods of NT in nonhuman primates.

2. Materials

1. Recombinant human follicle-stimulating hormone, luteinizing hormone (LH), and chorionic gonadotrophin (CG; Ares Advanced Technologies, Norwell, MA; or recombinant monkey gonadotropins when available).
2. Antide (GnRH antagonist, Ares Advanced Technologies).
3. Ketamine (Vedco, St. Joseph, MO).
4. TH3 medium: HEPES-buffered TALP medium, containing 0.3% bovine serum albumin (BSA *[6]*). Prepare medium by adding the indicated amounts of each reagent (Sigma, St. Louis, MO) to 1 L of Milli-Q water.

NaCl	6.660 g
KCl	0.239 g
$CaCl_2 \cdot 2H_2O$	0.294 g
$MgCl_2 \cdot 6H_2O$	0.102 g
Na_2HPO_4	0.048 g
Glucose	0.900 g
Na lactate	1.87 mL
Phenol red	0.010 g
$NaHCO_3$	0.168 g
Gentamicin sulfate	0.050 g
HEPES	2.603 g
Na pyruvate	0.060 g
pH	7.2–7.4
Osmolarity	282 ± 10

   Filter the medium using a 0.2-µ filter unit and store for up to one month at +4°C. Add BSA (Sigma) at 3 mg/mL before use and refilter.
5. Hamster embryo culture medium (HECM)-9 (*7*): Prepare HECM-9 base medium by adding the indicated amounts of each reagent (Sigma) to 1 L of Milli-Q water.

Polyvinyl alcohol (PVA)	0.1 g

NaCl	6.639 g
KCl	0.224 g
$CaCl_2 \cdot 2H_2O$	0.279 g
$MgCl_2 \cdot 6H_2O$	0.102
$NaHCO_3$	2.1 g
Lactic acid, Na salt, 60% syrup	632 µL
Gentamicin sulfate	0.01 g
pH	7.2–7.4
Osmolarity	277 ± 5

Filter the medium using a 0.2-µm filter unit and store for up to 1 wk at +4°C.

6. 100X Amino acid–pantothenate stock: Prepare stock by adding the indicated amounts of each reagent (Sigma) to 1 L of Milli-Q water.

Taurine	6.260 g
Asparagine	0.130 g
Cysteine	0.18 g
Histidine	0.21 g
Lysine	0.18 g
Proline	0.12 g
Serine	0.11 g
Aspartic acid	0.13 g
Glycine	0.08 g
Glutamic acid	0.17 g
Glutamine	2.92 g
Pantothenic acid	0.07 g

Filter and aliquot 500 µL per 1.5 mL tubes and store at –20°C for up to 3 mo.

7. HECM-9aa medium: Add amino acid–pantothenate stock to HECM-9 base medium at a ratio of 1:100 before use. HECM-9aa is used to hold oocytes from the time of recovery until IVF, intracytoplasmic sperm injection (ICSI), or NT, as well as to culture embryos until the four- to eight-cell stage (or day 2). For extended culture (to the blastocyst stage), embryos are transferred at the four- to eight-cell stage (end of day 2) to HECM-9aa medium supplemented with 5% fetal bovine serum (FBS; HyClone, v/v). Embryos are transferred to fresh HECM-9aa + 5% FBS every other day.
8. D-Sorbitol fusion medium *(2)*: Prepare fusion medium by adding the indicated amounts of each reagent (Sigma) to 1 L of Milli-Q water.

D-Sorbitol	46.378 g
Ca acetate	0.0158 g
Mg acetate	0.107 g
HEPES	0.119 g

Filter using a 0.2-µ filter unit and store for up to one month at +4°C. Add fatty acid-free BSA (Sigma) at 3 mg/mL before use and refilter.

9. Activation stocks: Ionomycin, Ca-salt (CalBoichem cat. no. 407952): for 5 m*M* (1000X) stock, reconstitute 1 mg ionomycin in 267 µL of dimethyl sulfoxide (Sigma D-2650), aliquot and store at –20°C.

10. TH1 medium: HEPES-buffered TALP medium, containing 1 mg/mL BSA.
11. TH30 medium: HEPES-buffered TALP medium, containing 30 mg/mL BSA
6-dimethylaminopurine (DMAP; Sigma D-2629) stock: for 200 m*M* (100X) stock reconstitute 1 g of DMAP in 30 mL Ca^{2+}- and Mg^{2+}- free Dulbecco's phosphate-buffered saline (DPBS) by heating the tube in near boiling water bath until all DMAP is dissolved. Separate into 0.5-mL aliquots and store at –20°C
12. Hyaluronidase (Sigma H-3506) stock: for 10X stock reconstitute 50 mg in 10 mL of HEPES-buffered TALP medium. Separate into 0.5-mL aliquots and store at –20°C
13. Polyvinylpyrrolidone (PVP; Irvine Scientific; Santa Ana, CA): Reconstitute with 1mL of HEPES-buffered TALP medium before use.
14. Ca^{2+}- and Mg^{2+}-free DPBS (Invitrogen, Carlsbad, CA).
15. Cytochalasin B (Sigma C-6762, 1 mg) stock: to prepare 5 mg/mL (1000X) stock, reconstitute 1 mg of cytochalasin B in 200 µL of dimethyl sulfoxide (Sigma). Aliquot at 5 µL per vial and store at –20°C.
16. Hoechst 33342 (Sigma B-2261): for 500X stock, reconstitute 1 mg in 1 mL of saline. Filter and aliquot 10 µL per vial. Store at –20°C; avoid light exposure.
17. Light paraffin oil (Zander IVF, Vero Beach, FL).
18. High-viscosity silicon oil DC 200, 375 mPa.s (Fluka; Sigma-Aldrich).
19. Pronase (Protease, Sigma P-8811): to prepare 0.5% stock (1X), reconstitute 100 mg of protease in 20 mL of HEPES-buffered TALP medium (no BSA), filter sterilize, aliquot 1 mL and store at –20°C.
20. Micropipets (Humagen, Charlottesville, VA).
21. Cell strainers (70-µm Nylon; Falcon; BD Biosciences, Bedford, MA).
22. Patton Laparoscopic Catheter Introducer Set (Cook OB/GYN, Spencer, IN).
23. Portable incubator (Minitube, Verona, WI).
24. Ultrasonography equipment (Philips).
25. Dissecting microscope (SZ-61, Olympus America).
26. Restraint chair (Primate Products, Miami, FL).
27. Electrolyte cream (Reflux Creme, Hewlett Packard, Waltham, MA).
28. S5 Square Pulse Physiological Stimulator (Grass Instruments, Quincy, MA).
29. CCU 1 Constant Current Unit (Grass Instruments).
30. Disposable electrodes made by folding 6- × 7-cm piece of ordinary light-weight aluminum foil six times length-wise to produce a strip measuring 1 × 6 cm.
31. Inverted fluorescent microscope (IX-70 Olympus America)
32. Micromanipulators (Joystick Oil Hydraulic MO-202D and Coarse MMN-1, Narishige International).
33. Inverted and dissecting microscope heating stages (ThermoPlate, TOKAI HIT CO. Japan) to provide temperature control at 37°C during oocyte and embryo manipulations.
34. Cell fusion equipment (BTX Electro Square Porator T820, BTX Instrument Division Harvard Apparatus, Holliston, MA).
35. Teflon® tubing (Inner diameter O.D. 0.9 mm, Outer diameter O.D. 2 mm, Narishige International).
36. Patton polyurethane transfer cannula (Cook OB/GYN).

3. Methods

3.1. Controlled Ovarian Stimulation

Protocols for controlled ovarian stimulation (COS) in rhesus monkeys with recombinant human gonadotropins have been developed at the Oregon National Primate Research Center *(8)*.

1. Monitor cycling females for menstruation and 1–4 d after onset, administer twice daily intramuscular injections of 30 IU recombinant human follicle-stimulating hormone (at 8 AM and 4 PM) for 8 d.
2. Administer Antide at a dose of 0.5 mg/kg, subcutaneously once a day for 8 d to suppress pituitary function and prevent spontaneous LH surges.
3. On the last 2 d of stimulation (days 7 and 8), additionally administer twice daily injections of recombinant human LH (30 IU intramuscularly [IM]).
4. On day 8, anesthetize animals with ketamine (10 mg/kg body weight, IM) and examine ovarian morphology by ultrasonography. Typically, a responsive ovary will be enlarged from 6 mm to an average diameter of 10 mm or greater and will contain at least five large follicles of 2–4 mm in diameter.
5. On the morning of day 9, inject monkeys meeting these criteria with recombinant human CG (hCG; 1000 IU IM) to induce oocyte maturation. Ovarian oocytes, which arrest at prophase I (germinal vesicle), resume meiosis in response to hCG and arrest again at metaphase II (MII). Approximately 20% of gonadotropin-treated female monkeys are discontinued at this time because of a lack of adequate response as judged by ultrasonography (*see* **Note 1**).

3.2. Laparoscopic Oocyte Recovery

Oocytes are collected by laporascopic follicular aspiration 27–33 h after hCG injection *(9)* via transabdominal needle aspiration of gravid ovarian follicles. Laparoscopy plays a prominent role in the IVF laboratory, with most surgical procedures accomplished by this methodology.

1. Anesthetize monkeys with isoflurane gas vaporized in 100% oxygen. Comprehensive physiological monitoring of animals should be conducted throughout the surgery, including electrocardiography, peripheral oxygen saturation, and end-expired carbon dioxide. Orotracheal intubation and mechanical ventilation to maintain expired CO_2 at less than 50 mmHg is mandatory.
2. Perform sterile skin preparation and draping after which the abdomen is insufflated with CO_2 at 15 mmHg pressure. Insert the viewing telescope via a small supraumbilical incision, with accessory ports placed in the paralumbar region.
3. Position the monkey in Trendeleburg, allowing the viscera to migrate in a cephalad direction exposing the reproductive organs.
4. Use a single small grasping forceps to stabilize the ovary for examination and needle aspiration. Rarely is a second accessory port and grasping forceps required for the experienced laparoscopist to perform this procedure.

5. After immobilization of the ovary, connect a 22-gage hypodermic needle to a source of continuous vacuum (–120 mmHg), and insert into individual follicles until all have been aspirated.
6. Reduce insufflation and close the incisions with interrupted absorbable suture in an intradermal pattern.
7. Place tubes containing follicular aspirates into a portable incubator (Minitube) at 37°C and transport quickly to the laboratory (*see* **Note 2**).
8. Add 10X hyaluronidase stock solution directly to the tubes containing aspirates at 1:10 ratio and incubate at 37°C for 30 s.
9. Gently agitate the contents with a serological pipet to disaggregate cumulus and granulosa masses and pour the entire aspirate onto a cell strainer.
10. Oocytes are retained in the mesh, while blood, cumulus, and granulosa cells are sifted through the filter
11. Quickly backwash the strainer with TH3 medium and collect the medium containing oocytes in a Petri dish.
12. Rinse oocytes, which are now easily identified in TH3 medium.
13. Any remaining cumulus cells can be removed by manual clean up with a small-bore pipet (approx 125 µm in inner diameter).
14. Oocytes can be observed at higher magnification for determination of their developmental stage (germinal vesicle, MI, or MII) as well as quality (granularity, shape, and color of the cytoplasm). On average, 40 oocytes are collected per stimulation, with more than 70% matured or maturing (MII and MI stages).
15. After evaluation, transfer oocytes into chemically defined, protein-free HECM-9aa medium *(7)* at 37° C in 5% CO_2, until further use. Most MI stage oocytes should mature to the MII stage within 3 to 4 h.

3.3 Collection of Spermatozoa

Penile electroejaculation provides a consisted, successful, and humane method for the collection of semen in the rhesus monkey *(10)*. Pregnancy-proven male monkeys assigned to electroejaculation must be evaluated on the basis of ease of restraint, number of attempts required to obtain a sample, and the animals' tolerance of the procedure (*see* **Note 3**).

1. Transfer animal to the restraining chair and secure by tying arms and legs with leather straps to the chair. The belly band restrainer can be useful on new animals to lessen animal movements.
2. Apply electrolyte cream to the entire shaft of the penis with the exception of the glans. Wrap one electrode around the base of the penis with the excess length folded to create a tab to which the negative stimulator lead is connected. Position the second electrode immediately behind the glans and connect to the positive stimulator lead.
3. With the electrodes attached, gently grasp the penis between the index and second finger, extend slightly, and position over a sterile 10-mL glass beaker.

4. Set the CCU 1 Constant Current unit output switch to NORMAL and the Current Adjustment dial to 0. At these settings, the animal receives approx 1 mA of current. This low current prepares the animal for the procedure, in a process called priming (*see* **Note 4**).
5. Adjust the S5 Square Pulse Stimulators Frequency setting to 17 pulses/s and a Duration setting of 17 ms with Multiply switches on both setting at ×1. Set the maximum volt levels (80) with Multiply switch at ×10.
6. Increase the Current Adjustment switch on CCU 1 gradually from 0 to a setting of 4 to 4.5 until collecting the sample. Never go beyond a setting of 5.
7. Continue to stimulate the animal until a sample is obtained, but never go beyond 20 s (*see* **Note 5**).
8. Turn off the Constant Current output by moving Output Adj I on CCU 1 Unit to the off position after obtaining a sample, or after a total stimulus time of 30–35 s (if priming time is added) per trial or less.
9. Allow the ejaculate to liquefy at room temperature for approx 15 min before processing.

3.4. Fertilization by ICSI and Embryo Culture

ICSI is a robust efficient fertilization procedure in the monkey resulting in high pronuclear formation rates (80–90%).

1. Wash collected spermatozoa twice by resuspending with TH3 medium followed by centrifugation of the liquid portion of the ejaculate for 7 min at 200*g*.
2. Take an aliquot and determine motility and concentration before the final centrifugation and resuspension step.
3. Adjust sperm concentration to 1×10^6 motile spermatozoa per milliliter in TH3 medium and store for approx 3 h at room temperature before ICSI.
4. The ICSI procedure is conducted on an inverted microscope equipped with Hoffman optics, heating stage (set at 37°C), and micromanipulators.
5. Immobilize an oocyte using a holding pipet (120–130-µ outer and 25–40-µ inner diameter) attached to a micropipet holder (Narishige) and controlled by air-filled Teflon tubing connected to a 20-mL plastic syringe (Becton Dickinson).
6. Fill approx half the holding micropipet with TH3 medium before the micromanipulation procedure.
7. Fill the ICSI micropipet completely with light paraffin oil and then attach it to a Milli-Q water filled Narishige pipet holder and Teflon tubing that extends to a 200-µL volume Hamilton microsyringe controlled by a microinjector (Narishige). The line, microsyringe, and ICSI micropipet must be completely free of air bubbles.
8. After setting up and positioning the micropipets, dilute a small aliquot of sperm with 10% PVP (1:4) and place a 5-µL drop in a micromanipulation chamber; usually the lid of a Falcon 1006 Petri dish.
9. Place a 30 µL drop of TH3 in the same micromanipulation chamber next to the sperm droplet and ensure both are covered with paraffin oil.

10. Place the oocytes into the micromanipulation drop and mount the chamber on the stage of the microscope.
11. Lower the ICSI pipet into the sperm drop and select a motile sperm, which is immobilized by striking the midpiece with the tip of the pipet, and slowly aspirated into the pipet tail first.
12. Move the injection pipet to the manipulation drop containing oocytes.
13. Lower the holding pipet into the manipulation drop and immobilize an individual oocyte with the polar body positioned at either 12- or 6 o'clock.
14. Slightly lower the holding pipet with oocyte attached until it touches the bottom of the plate to stabilize the egg during injection.
15. Bring the ICSI pipet into sharp focus at the 3-o'clock position and slowly push the sperm to the pipet tip using the Hamilton microsyringe.
16. Insert the ICSI pipet through the zona pellucida and inject the sperm into the cytoplasm of the oocyte, away from the polar body, making sure that the pipet completely breaks through the plasma membrane and that the sperm is deposited with a minimal amount of medium (*see* **Note 6**).
17. After ICSI, place injected oocytes in four-well dishes containing pre-equilibrated HECM-9aa medium and culture at 37°C in 5% CO_2, 5% O_2, and 90% N_2. Maintain cultures under paraffin oil throughout the culture period.
18. Assess fertilization 12–14 h after injection by the presence of pronuclei.
19. At the eight-cell stage, transfer embryos to fresh dishes of HECM-9aa medium supplemented with 5% FBS and culture for a maximum of 7 d with observation/scoring and medium change every other day (*see* **Note 7**).

3.5. Enucleation

Enucleation procedures for rhesus monkey oocytes are similar to those described in the bovine (*see* Chapter 11). We will focus here on specific details of handling monkey oocytes during enucleation and potential problems unique to this species.

1. Incubate MII oocytes for 5 min in Hoechst 33342 (5 µg/mL) for spindle visualization before enucleation.
2. Rinse oocytes two to three times in fresh drops of TH3 medium and place into a 30 µL of TH3 manipulation drop containing cytochalasin B (5 µg/mL) covered with paraffin oil (*see* **Note 8**).
3. The micromanipulation tool set up is similar to that described for the ICSI procedure except that a larger, beveled, enucleation pipet (25- to 27-µm outer diameter) is used. Completely fill the enucleation pipet with high-viscosity silicon oil to improve control over aspiration and injection.
4. Immobilize an individual oocyte using the holding pipet with the first polar body positioned at 2 o'clock and lower the holding pipet with attached oocyte slightly until it touches the bottom of the plate to stabilize the egg during enucleation.
5. Bring the enucleation pipet into sharp focus at the 3-o'clock position of the oocyte with its beveled bore opening positioned toward the polar body.

6. Slowly insert the pipet through the zona pellucida without piercing the plasma membrane (*see* **Note 9**).
7. Once the zona is penetrated, withdraw the pipet slightly and slowly aspirate the first polar body and approx 15% of the underlying cytoplasm into the enucleation pipet.
8. Confirm the presence of the metaphase spindle in the pipet by a short (less than 10 s) exposure to an ultraviolet 2A fluorescent light.
9. If the spindle is still in the egg, navigate the enucleation pipet to the spindle under epifluorescence, ensuring that the tip of pipet and the spindle are brought to the same focal plane.
10. After aspirating the spindle into the enucleation pipet, turn the ultraviolet light source off and withdraw the pipet slowly from the slit in the zona pellucida (*see* **Note 10**).
11. Place enucleated oocytes in pre-equilibrated, HECM-9aa medium and culture at 37°C in 5% CO_2, 5% O_2, and 90% N_2 for 30–60 min for recovery before further manipulation.

3.6. Activation

Cytoplast activation is an essential component of the NT procedure since the introduction of the donor nucleus into the cytoplast bypasses fertilization and sperm-induced egg activation (*see* **Note 11**). Developmental rates to the blastocyst stage for embryonic cell NT embryos are higher when unsynchronized blastomeres are fused with pre-activated cytoplasts ***(2)***.

1. Add 1 µL of ionomycin stock to1 mL of prewarmed TH1 medium and mix thoroughly.
2. Place cytoplasts in ionomycin-containing TH1 medium for 2 min and then wash for 5 min in TH30.
3. Add DMAP stock to pre-equilibrated HECM-9aa medium at 10 µL/mL.
4. Transfer activated cytoplasts into DMAP medium and incubate at 37°C in 5% CO_2, 5% O_2, and 90% N_2 for 4 h.
5. After treatment with DMAP, rinse cytoplasts extensively (four to five times) in TH3 becaues residual DMAP may cause developmental arrest.
6. Alternatively, DMAP treatment can be replaced with either 50 µ*M* roscovitine or 7.5 µg/mL cycloheximide combined with 5 µg/mL cytochalasin B.

3.7. Donor Cell Transfer

Blastomeres of 8- to 16-cell stage, ICSI-produced embryos are used as the source of donor nuclei.

1. Place embryos briefly (30–60 s) in 0.5% pronase to remove zonae pellucidae with constant monitoring under a dissecting scope.
2. Before the zonae disappear completely, move embryos quickly to TH3 medium and wash three times by gentle pipetting with movement from one drop of medium to another. During these washes, zonae will break up and completely disappear.
3. Incubate zona-free embryos in 0.5 m*M* EDTA in Ca^{2+}- and Mg^{2+}-free DPBS for 5–10 min to loosen cell-to-cell contacts.

4. Transfer donor embryo and recipient cytoplasts to micromanipulation medium containing 5 µg/mL cytochalasin B and incubate for 10–15 min before enucleation.
5. Mechanically disaggregate and then aspirate a single donor blastomere using a beveled transfer pipet (33- to 35-µm outer diameter).
6. Immobilize individual cytoplasts with a holding pipet so that the hole in the zona made previously by the enucleation pipet is positioned at 3 o'clock.
7. Insert the transfer pipet through the hole in the zona and eject the donor cell into the perivitelline space.
8. Once all transfers are completed, wash NT couplets three times in TH3 and culture in HECM-9aa medium at 37°C in 5% CO_2, 5% O_2, and 90% N_2 medium until fusion.

3.8. Couplet Fusion

Fusion of NT couplets is induced by two 50-µs direct current pulses of 2.7 kV/cm in D-sorbitol fusion buffer. The fusion chamber consists of two electrodes 0.5-mm apart placed into a 10-cm Petri dish taped to the stage of the dissecting microscope.

1. Set the BTX Electro Square Porator T820 controls as follows:

   Mode—(HV) 99 µs/3.0 kV
   Pulse length—50
   Voltage dial—0.13
   Number of pulses—2

2. Pour prewarmed (37°C) fusion medium into the dish until electrodes are covered.
3. Transfer three to five NT couplets at a time first to the four-well dish with TH3 medium and then to the well containing equal volumes of TH3 and fusion medium.
4. Allow the couplets to settle to the bottom and transfer them to the well containing pure fusion medium (*see* **Note 12**).
5. Position couplets between electrodes of the fusion chamber and align them so that the donor cell is positioned toward the black (negative) electrode.
6. Initiate a fusion pulse and after a 20-s pause, transfer couplets back to TH3 medium in a reverse stepwise fashion.
7. Once all NT couples are electroporated, transfer to and culture in HECM-9 medium.
8. Evaluate fusion microscopically, 30–45 min after electroporation by noting the presence or absence of donor blastomeres in the perivitelline space.
9. Refuse unfused couplets as described previously.

3.9. Embryo Transfer

Adult, multiparous female monkeys monitored for menses are used as recipients. Daily blood samples are collected beginning on day 8 of the menstrual

cycle and serum levels of estradiol are quantitated by radioimmunoassay. The day after the peak in serum estradiol is considered the day of ovulation (day 0). The pregnancy success rate depends on the synchrony between the age of the transferred embryos, as measured by culture time in vitro, and the host endometrium, relative to the predicted day of ovulation. The optimal timing for blastocyst (day 6/7) transfer is into a day 4 uterine environment, whereas cleavage-stage embryos at a culture age of 1–4 d can be optimally transferred into a day 2 recipients (*see* **Note 13**).

Recipient female monkeys within 1–4 d of ovulation are anesthetized with ketamine and prepared for laparoscopic embryo transfer using the same basic laparoscopic approach and anesthesia as described for follicular aspiration.

1. Examine the ovaries with a self-retaining microretractor inserted at a high paramedian position after insertion of the telescope and Trendeleburg positioning.
2. Transfer embryos preferentially into the oviduct with an ovulation site on the associated ovary.
3. Grasp the fimbria with a Patton retractor and place in traction.
4. Insert the Patton cannula transabdominally and advance through the fimbria into the oviduct to a distance of 1–3 cm.
5. Typically, transfer two ICSI or IVF embryos to the oviduct of the recipient. Given significantly lower developmental rates with NT embryos, the number of transferred embryos in this case has been increased to five to six.
6. Remove embryos from culture medium and transfer to a dish containing TH3 medium.
7. Connect the transfer catheter to a 1-mL syringe filled with about 0.01–0.02 mL of TH3 medium avoiding air bubbles.
8. Carefully load embryos near the catheter tip with a total volume not exceeding 0.03 mL.
9. Insert the catheter into the external orifice of the cannula and advance into the oviduct to a depth of 1–3 cm and deposit the embryos.
10. Carefully examine the catheter after transfer to ensure that all embryos have been transferred. If an embryo has been retained it can be subjected to a second transfer attempt.
11. The skin incision closure is identical to the follicle aspiration procedure described previously.

To detect pregnancy, serum levels of estrogen and progesterone are monitored every third day after embryo transfer. Pregnancy is confirmed by ultrasound approx 25 d after transfer and monitored periodically throughout gestation.

3.10. Somatic Cell Cloning Efforts in the Monkey

As noted previously, nuclear reprogramming is thought to be the limiting parameter in our monkey somatic cell cloning protocol. Obviously, the

reprogramming required after SCNT is more extensive than the process that occurs during embryonic cell NT. For normal full term development of cloned embryos, genes normally expressed during embryogenesis, but silent in the somatic donor nucleus, must be reactivated in an appropriate temporal and spatial manner. Despite the fact that even terminally differentiated donor cell nuclei can be reprogrammed in egg cytoplasm acquiring the capacity to support full-term development, only a few NT embryos develop to term and, of those, many die shortly after birth in all species where cloning has been successful. The most likely explanation for such outcome is the inability of the cytoplast to reprogram the epigenetic profile of the somatic donor nucleus to that of the fertilized zygote. Our current research on somatic cell cloning in the monkey is focused on the effect of various cell types (younger fetal fibroblasts, cumulus, oviductal epithelial, embryonic stem cells) on NT development in efforts to screen for a more "reprogrammable" source of donor nuclei. Apparently, the primate egg cytoplasm lacks or contains insufficient levels of factors responsible for somatic chromatin remodeling; therefore, another logical approach is to induce nuclear remodeling in cultured cells before nuclear transfer.

The feasibility of such an approach is supported by the recent demonstration that denatured somatic cells subjected to heat treatment can be reactivated after NT with development to blastocysts in vitro and to viable offspring in vivo ***(11)***. Such treatments destabilize high-order nucleoprotein complexes and, presumably, denatured chromatin is more readily accessible to the oocyte's reprogramming machinery. Often, fusion of such cells with recipient cytoplasts is not possible because pre-treatment of donor cells results in irreversible membrane damage. We have explored alternative methods of introducing donor chromatin involving direct injection without using piezo manipulators with results comparable to those achieved for cell fusion (*see* **Note 14**).

4. Notes

1. The percentage of "nonresponders" varies by season showing an increase during the summer months, reaching more than 35% in June and July. During summer, despite housing in controlled, constant environments, many female monkeys also become anovulatory, and it is impractical to attempt COS ***(12)***. Females can be recycled for COSs; however, the response to recombinant human gonadotropins is decreased gradually with increasing numbers of stimulations, apparently the result of an immune reaction. Practically, as many as three stimulations on average can be performed per female monkey, with the recovery of a reasonable number of high quality oocytes. The availability of monkey recombinant gonadotropins would allow the more efficient and extended use of females.

2. The time between aspiration and oocyte recovery should be minimized to avoid the detrimental effects of blood exposure, which usually contaminates the aspirates. The conventional approach of diluting aspirates with medium and searching for oocytes under a dissecting microscope is labor intensive often requiring two to three technicians. The recovery time can be minimized by sifting the aspirates through cell strainers.
3. Before assignment to the project, each animal receives a physical examination from the veterinary staff and is fitted with a special neck chain (using pole and collar method) to facilitate restraint. Only animals with sperm concentrations greater that 60 million per milliliter and with more that 70% motile cells with normal morphology are acceptable
4. Priming is used on those animals that show little or no movement. If an animal is a mover in the chair, stimulation can begin immediately, moving the Current Adjustment dial without priming.
5. It usually takes approx 15 s for priming but when the Current Adjustment dial is moved up, the total time from post prime to the end of trial should be no more that 15–20 s. All samples will be collected within this time frame. If data are available from previous stimulations, the Current Adjustment dial is slowly adjusted to a level that had been previously successful for that particular animal. Total stimulus current time is left at this level for a maximum of 15–20 s during which ejaculation almost always occurs. If no sample is obtained, the stimulation is repeated within 1–1.5 min. No more that five consecutive stimuli attempts are made per animal on each trial. Each animal is given an abstinence period at least 48 h between sample collections.
6. Piercing the plasma membrane during the ICSI procedure is critical and often difficult to assess because the needle can grossly invaginate the membrane without breaking through. For that reason, the injection pipet should be inserted through the zona pellucida and "into" the oocyte across approx one-third of the egg's diameter. Cytoplasm is slowly aspirated into the ICSI pipet (as far back as the needle junction with the zona pellucida) until the plasma membrane brakes. A "pop" or sudden movement of cytoplasm into the pipet indicates the release of membrane tension. Once the membrane is penetrated, the sperm is expelled into the cytoplasm, visualized as a "dive" of the sperm. Frozen-thawed sperm can also be used for ICSI. We have previously reported that fertilization rates by ICSI with cryopreserved sperm injected shortly after thaw are significantly lower than with fresh sperm ***(13)***. However, preincubation times of more than 3 h after thaw were associated with ICSI fertilizing capacity similar to that seen with fresh sperm ***(14,15)***.
7. Preimplantation development of the primate embryo as in other mammals includes explicit developmental stages from the formation of the zygote after fertilization to cleavage, morula formation, compaction and finally cavitation with the formation of the blastocyst (**Fig. 1**). In HECM-9aa + FBS, blastocysts containing 100 or so cells can be seen by day 6 or 7 in vitro. Admittedly, in vivo rates are probably somewhat faster than in vitro, which may reflect suboptimal culture conditions.
8. Monkey oocytes are more fragile than those of the cow and easily lyse during enucleation if the cytoskeleton organization has not been "softened" by cytochalasin

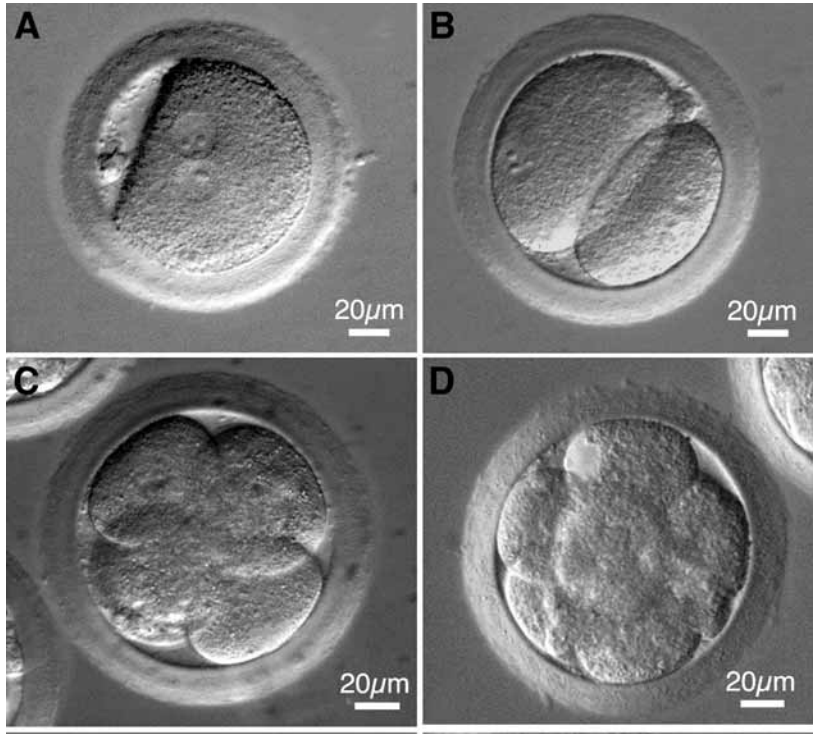

Fig. 1. Rhesus monkey preimplantation embryo development in vitro. **(A)** Pronuclear stage zygote with male and female pronuclei, 12 h after fertilization by intracytoplasmic sperm injection. **(B)** Day 1, two-cell stage embryo (day of fertilization = day zero). **(C)** Day 2, four-cell stage embryo. **(D)** Day 3, eight-cell stage embryo. **(E)** Day 4, morula stage embryo. **(F)** Day 5, compact morula. Note that individual blastomeres have maximized their intracellular contacts and compacted into a tight cell mass. **(G)** Day 6 or 7, expanded blastocyst with a single flat layer of trophectoderm surrounding a fluid filled blastocoel and the inner cell mass.

B exposure. However, prolonged incubation in cytochlasin B during enucleation may cause excessive softening and swelling of the cytoplasm.

9. Penetration through the zona during enucleation is more difficult than in the ICSI procedure because of the larger size of the enucleation pipet, and in some cases, may require extending the pipet all the way to the other side of the egg.
10. As the pipet is pulled away from the egg, the plasma membrane will stretch and form a thin bridge between the egg and the material being removed. A rapid movement of the enucleation pipet toward 6 o'clock will break this cytoplast bridge.
11. Various artificial activation treatments have been developed that mimic sperm-triggered events and induce development of NT embryos. For example, ethanol,

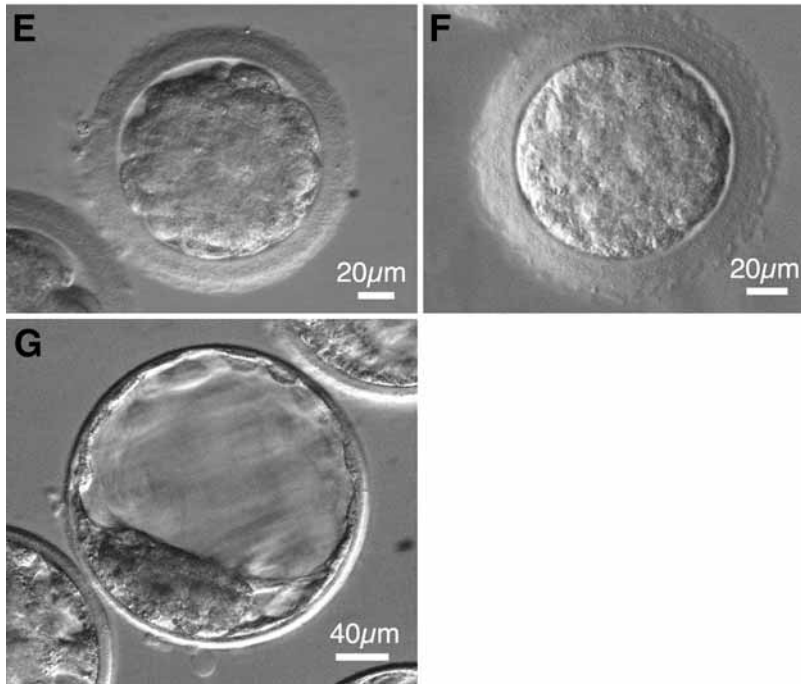

Fig. 1. (*continued*)

electroporation in calcium containing medium, ionomycin, or inositol 1,4,5-trisphosphate. However, M-phase-promoting factor activity at least in the young bovine and rabbit oocyte is quickly restored with recondensation of chromosomes and reentry of activated oocytes into a new M-phase arrest, known as MIII ***(16)***. This phase can be circumvented by additional treatments with protein synthesis (cycloheximide), protein phosphorylation (DMAP) or specific M-phase-promoting factor inhibitors (roscovitine). Thus, sequential approaches have evolved with ionomycin/DMAP, ionomycin/cycloheximide, or ionomycin /roscovitin that result in high activation rates in monkey NT embryos ***(17)***.

12. Stepwise transfer to fusion medium minimizes osmotic shock and reduces electrolyte contamination in the fusion chamber.
13. For information on optimal timing of embryo transfer, *see* **ref.** ***15***.
14. A blunt transfer pipet (5–7 μm outer diameter) is used to mechanically disrupt a single donor cell by aspiration in a 10% PVP drop. The nucleus is subsequently transferred into a conventionally prepared cytoplast via pipet passage through the hole in the zona and injection into the cytoplasm as described for the ISCI procedure. The efficiency of direct injection is comparable to fusion with 80–90% survival rate and 60–70% pronuclear formation rate.

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Cell Reprogramming and Transgenesis

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
Alan O. Trounson

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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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11

Cloning of Exotic/Endangered Species

Desert Bighorn Sheep

James "Buck" Williams, Taeyoung Shin, Ling Liu, Gabriela Flores-Foxworth, Juan Romano, Alice Blue-McClendon, Duane Kraemer, and Mark E. Westhusin

Summary

Cloning using somatic cell nuclear transfer (SCNT) may be a useful tool for conserving genetic diversity and for propagating exotic and/or endangered animal species. Somatic cells can be obtained easily, expanded in culture, cryopreserved, and thawed at a later date for use in NT. Significant challenges relevant to using SCNT for cloning wild and endangered animal species include the need for using interspecies NT and interspecies embryo transfer. Animal care and welfare issues raised that are unique to exotic and endangered species also are rasied. In this chapter, the methods used in attempts to clone the wild animal species of Desert Bighorn Sheep are described.

Key Words: Animal cloning; somatic cell nuclear transfer; SCNT; interspecies nuclear transfer; interspecies embryo transfer; genetic conservation; genetic diversity; endangered animal species; wild animal species; interspecies surrogacy; animal welfare; Desert Bighorn Sheep.

1. Introduction

Cloning animals using somatic cell nuclear transfer (SCNT) has been reported in at least 10 different mammalian species. Three of these are wild species that include Gaur ***(1)***, European Mouflon ***(2)***, and Banteng (O. Ryder, personal communication, 2003). SCNT may be a powerful tool for genetic conservation and for preserving genetic diversity in exotic and/or endangered species because it allows the reproduction of animals with genomes that otherwise may be lost or underrepresented in a population. In addition, somatic cells used for cloning often are more easily obtained and stored than are gametes and

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embryos. That the fibroblasts used for cloning the Banteng had been stored for 25 yr before use is a clear demonstration of the long-term storage potential of somatic cells. The greatest challenges confronting the use of SCNT to propagate wild and/or endangered species are the need for using interspecies NT and interspecies embryo transfer. Using these techniques is not without controversy because the resulting animals will carry mitochondrial genes derived from a different species. The methods used in attempts to clone the wild species of desert bighorn sheep using SCNT are described (*see* **Note 1**).

Desert Bighorn Sheep (*Ovis canadensis mexicana*) occupy approx 4% of their historic range. Although not endangered, decreased genetic variability and the associated loss of vigor are believed to have caused extinction or stagnation in isolated, small, free-ranging populations ***(3,4)***. The major procedures involved in cloning Desert Bighorn Sheep using SCNT are similar to those used for other animal species and include (1) the acquisition of donor cell nuclear material, (2) the acquisition of developmentally competent recipient ova, (3) transfer of the donor cell nuclei into recipient ova, (4) the preparation of surrogate female sheep, and (5) interspecies embryo transfer (*see* **Note 2**).

2. Materials

2.1. Donor Cell Collection and Cryopreservation

1. Tissue processing medium: HEPES-buffered tissue culture medium 199 with Hank's salts (TCM-199-Hank's) stored at 4°C, 10% heat inactivated fetal bovine serum (FBS) stored at −20°C, and 1% penicillin G/streptomycin (P/S) stored at −20°C.
2. Tissue culture medium: Dulbecco's modified Eagle's medium and nutrient mixture F-12 (DMEM/F12) stored at 4°C, 10% FBS stored at −20°C, and 1% P/S (1% of a solution containing 10,000 U of penicillin G and 10,000 µg streptomycin per milliliter of medium) stored at −20°C.
3. Chlorhexidine solution (diluted 1:30 in sterile water).
4. Sterile surgical equipment for skin biopsy.
5. Tissue culture apparatus and incubators.
6. Trypsin-EDTA 10X solution.
7. Micropipets with 5-, 200-, and 1000-µL pipet tips.
8. Cell counter and cuvet.
9. Cryopreservation equipment, including liquid nitrogen and liquid nitrogen storage tank.
10. Cryoprotectant: DMEM/F12 stored at 4°C, 10% dimethyl sulfoxide stored at 25°C, 10% FBS stored at −20°C, and 1% P/S stored at −20°C.

2.2. Collection of Recipient Ova

1. Oocyte aspirating unit with noncollapsing vacuum tubing, sterile embryo filter, 18-gage needle, sterile rubber stopper assembly.
2. Aspiration medium: TL-HEPES stored at 4°C, 50 U/mL heparin stored at 25°C.

3. Oocyte in vitro maturation (IVM) medium: TCM-199 with Earl's salts (TCM-199-Earl's) stored at 4°C, 10% FBS stored at −20°C, 5 µg/mL follicle-stimulating hormone (FSH) stored at −80°C, 5 µg/mL luteinizing hormone (LH) stored at −80°C, 1 µg/mL estradiol stored at −80°C, 1% P/S stored at −20°C.
4. 5% CO_2 incubator and portable incubator.

2.3. Nuclear Transfer

1. Glass capillary tube: outside diameter 1.0 mm, internal diameter 0.75 mm, length 125 mm.
2. Microforge, pipet puller, and microgrinder.
3. IVM medium: TCM-199-Earl's stored at 4°C, 10% FBS stored at −20°C, 5 µg/mL FSH stored at −80°C, 5 µg/mL LH stored at −80°C, 1 µg/mL estradiol stored at −80°C, 1% P/S stored at −20°C.
4. Manipulation medium: HEPES-buffered TCM-199 + 10% FBS.
5. Vortex and vortexing medium: TL–HEPES stored at 4°C, 0.1% hyaluronidase stored at −20°C.
6. Washing medium: TCM-199-Hank's stored at 4°C, 10% FBS stored at −20°C. Nucleus staining medium: TCM-199-Earl's stored at 4°C, 10% FBS stored at −20°C, 5 µg/mL cytochalasin B stored at −20°C, 5 µg/mL Hoechst 33342 stored at −20°C.
7. Enucleation medium: TCM-199-Hank's stored at 4°C, 10% FBS stored at −20°C.
8. Activation medium 1: TL–HEPES supplemented with 5 µ*M* ionomycin stored at −20°C.
9. Activation medium 2: TCM-199-Hank's stored at 4°C, 10% FBS stored at −20°C, 10 µg/mL cycloheximide, stored at −20°C, 5 µg/mL cytochalasin B stored at −20°C.
10. Fusion chamber 0.5-mm, stainless wire
11. Fusion medium: 0.28 *M* mannitol, 0.1 m*M* $CaCl_2$, 0.1 m*M* $MgSO_4$ stored at 25°C.
12. In vitro culture medium: G1/G2 version 3 stored at 4°C.
13. Dulbecco's phosphate-buffered saline (DPBS) stored at 25°C.
14. Mineral oil stored at 25°C.
15. 5% CO_2, 5% O_2, 90% N_2 incubator.
16. Trypsin–EDTA 0.1% solution.
17. Bovine serum albumin (BSA) stored at 4°C.
18. Stereomicroscope and fluorescent inverted microscope.
19. Micromanipulator.
20. Electrocell manipulator.
21. Warm plate.

2.4. Synchronization of Estrus

1. Intravaginal progesterone releasing device (CIDR, InterAg, Hamilton, New Zealand).
2. Inserting device (CIDR, InterAg).
3. Prostaglandin F2α (Lutalyse) stored at −20°C.

4. Equine chorionic gonadotropin (eCG) stored at –20°C.
5. Syringes and 18- and 20-gage needles.

2.5. Surgical Embryo Transfer

1. Pre-anesthesia: xylazine/ketamine mixture (2 mg xylazine to 100 mg ketamine) injected intravenously and 0.5 mg/kg atropine sulfate injected intramuscularly.
2. Anesthetic: Isoflurane; anesthetic machine, and mask.
3. Anesthetic reversal: 0.125 mg/kg yohimbine or 3 mg/kg yolazoline injected intravenously.
4. Surgical cradle and surgical equipment.
5. Embryo transport medium: TCM 199-Hank's 10% FBS at 38.5°C in hotbox.
6. Syringes with 18- and 20-gage needles.
7. Endotracheal tubes (sizes 7, 7.5, 8, 8.5, 9), laryngoscope, and stylet.
8. 5-mm, 0° laparoscope and light source equipment.
9. 5-µL Pipet.
10. Stereomicroscope.
11. 0.9% Injectable saline solution.
12. Absorbable suture material (PDS 2-0).

3. Methods

3.1. Acquisition and Preparation of Donor Cells

The methods under **Subheadings 3.1.1.–3.1.4.** describe (1) the collection of adult fibroblasts from donor Desert Bighorn ewes using skin biopsy; (2) subculture of the donor cells to increase the number available; (3) cryopreservation of the donor cells; and (4) preparation of the donor cells for NT.

3.1.1. Collection and Processing of Donor Tissue Samples

1. Prepare and anesthetize donor ewes as described in **Subheading 3.5.1.**
2. Remove a piece of abdominal skin approx 2 × 3 cm from the donor ewe using a scalpel blade and place into a solution of chlorhexidine for decontamination.
3. Dissect the tissue into pieces approx 1 mm^2 in tissue-processing medium using dissection scissors in a sterile Petri dish, then place the pieces into 25-cm^3 tissue culture flasks containing 7–10 mL of fresh culture medium and incubate at 37°C in an atmosphere of 5% CO_2 and air for 7–10 d.
4. Check the cultures daily for cell outgrowths from the tissue pieces and for contamination. Normally, one-half of the medium is replaced with fresh medium every other day because of a detectable change in pH or the presence of cell debris in the culture.

3.1.2. Expansion of Cells in Culture

1. Pour off the medium in the culture flask and add 1.0–1.5 mL of 0.05–0.1% trypsin–EDTA 10X solution. Tap the side of the flask to detach the cells. When the majority of cells are dislodged, give the flask a sharp slap on the side to remove residual cells.

2. Rapidly add approx 8 mL of tissue culture medium to make the volume in the flask up to 10 mL and then wash the remaining cells from the bottom of the flask by pipetting the cell suspension over the surface using a plastic pipet.
3. Transfer the medium containing cells to a sterile centrifuge tube. Immediately remove an aliquot of 200 µL with a micropipet and place into a cuvet for counting the cells.
4. Wash the cells remaining in the tube by centrifugation (3 min at 200*g*) and then resuspend the cells in fresh culture medium to obtain a concentration of 1 million/mL.
5. Distribute 3–5 mL of the cell suspension to new culture flasks and incubate at 37°C in an atmosphere of 5% CO_2 and air. These cells can be subpassaged five to six times (approx every 3–5 d) to expand the cell population before cryopreservation.

3.1.3. Cryopreservation of Cells

1. Collect and trypsinize the cells to be cryopreserved as described in **Subheading 3.1.2**.
2. Resuspend the cells in cryoprotectant at a concentration of 2 million per milliliter for storage or 100,000 per milliliter for use in NT.
3. Transfer the cell suspensions to cryovials with a maximum volume of 2.5 mL.
4. Place the cryovials into freezing containers in a –80°C ultra-cold freezer and freeze at a rate of –10°C/min.
5. Remove the frozen cryovials from the freezing containers on the following day, plunge into a small bucket of liquid nitrogen, and transfer the cells to storage canes contained in a tank of liquid nitrogen. It is crucial to avoid exposing these cells to air for more than 5 s prior to immersion in liquid nitrogen. Irreversible injury to the cells may occur if their temperature rises to –60°C.

3.1.4. Preparation of Cells for NT

1. Thaw cryopreserved donor cells 3 d before to use in NT.
2. Remove a cryovial containing frozen cells (100,000 cell/mL) from liquid nitrogen and place into a beaker filled with water at 38–39°C. Holding the vial and gently swirling it around in the water will rapidly thaw the cells.
3. Examine the outside of the cryovial when the cells are thawed to confirm the cells being used and decontaminate the tube using 70% alcohol spray.
4. Remove the thawed cells from the cryovial using a micropipet and resuspend in 10 mL of fresh tissue culture medium in a 15-mL polystyrene conical tube.
5. Wash the cells using centrifugation (3 min, 200*g*).
6. Remove the supernatant and place the harvested cells into two wells of a four-well culture dish. Grow the cells for 3–5 d at 37°C in an atmosphere of 5% CO_2 and air. Each well will contain about 100,000 cells in 600 µL of culture medium. Normally these cells will reach 90% confluence within 3 d of culture.
7. Remove the donor cells from the incubator, remove the medium with the aid of a pipet, and trypsinize the cells by adding 0.5 mL of 0.1% trypsin–EDTA solution by gentle pipetting for less than 1 min.

8. Transfer the cell suspension to 5 mL of enucleation medium in a 15-mL polystyrene conical tube.
9. Wash and concentrate the cells using centrifugation (3 min, 200*g*).
10. Resuspend the cells in 300 µL of enucleation medium and maintain in the conical tube in an incubator at 38.5°C in an atmosphere of 5% CO_2 and air until used in NT.

3.2. Acquisition and Preparation of Recipient Ova

Preparing the recipient domestic sheep ova for use in nuclear transplantation involved (1) collection of cumulus–oocyte complexes (COCs) by aspirating mature ovarian follicles from the ovaries of ewes obtained from an abattoir and shipping this material to the laboratory for (2) maturation of the oocytes obtained in vitro (IVM).

3.2.1. Collection of COCs

1. Set a vacuum pump at a flow rate of 30 mL/min, attach an 18-gage needle to the oocyte aspiration unit, and insert the needle into a mature ovarian follicle (>2 mm in diameter) to aspirate the contents. Repeat this process for other mature follicles.
2. Collect aspirated follicular fluid in 50-mL polystyrene tubes containing aspiration medium.
3. Pour the aspirated contents into a sterile embryo filter to isolate the oocytes in a smaller volume of medium.
4. Wash the contents by pouring fresh aspiration medium through the filter.
5. Retrieve the oocytes from the filter by inverting it in a Petri dish while rinsing the membrane and walls of the filter with a constant stream of aspiration medium pushed from a 50-mL syringe.
6. Select the oocytes for in vitro maturation based on morphology (i.e., round, uniform cytoplasm with three or more layers of cumulus cells).

3.2.2. IVM of Oocytes

1. Place IVM medium (*see* **Subheading 2.3.**) into auto-analyzer sample cups and pre-equilibrate in a humidified atmosphere at 38.5°C and 5% CO_2 and air for 3–4 h.
2. Select COCs, wash in TL–HEPES and place into auto-analyzer sample cups containing IVM medium.
3. Close the tops of the auto analyzer sample cups and wrap with Parafilm.
4. Place the oocytes into a portable incubator for overnight shipment to the laboratory. Oocytes were delivered to the laboratory in the portable incubator approx 20 h after collection.
5. Transfer the oocytes from the portable incubator to a standard incubator at 38.5°C, atmosphere of 5% CO_2, and air on delivery and remove the lids and Parafilm.
6. Keep the oocytes in the incubator until required for micromanipulation.

3.3. Nuclear Transplantation

Specially designed glass instruments are required for NT. It is essential to pay detailed attention to the preparation of these instruments to reduce any frustrations that may be encountered during the manipulation of embryos and oocytes. The methods in **Subheadings 3.3.1.–3.3.5.** describe (1) preparing the micropipets required and the various stages of SCNT; (2) enucleation of the recipient ova; (3) transfer of the donor cell nucleus into the enucleated ova; (4) activation of the cloned embryos; and (5) embryo culture of the SCNT embryos.

3.3.1. Preparation of Micropipets

Micropipets can be used several times, provided that they are thoroughly rinsed in boiling water both before and after each use and stored in a clean, dry container: 100- × 15-mm square grid dishes containing strips of modeling clay are suitable for this purpose.

1. Heat a glass capillary tube (outside diameter: 1.0 mm, inside diameter: 0.75 mm, length: 125 mm) by rotation over a microburner.
2. Manually pull the capillary tube until the ends taper as the glass begins to melt. (Alternatively, an automatic pipet puller can be used to pull micropipets).
3. Cut the ends off pipets of the appropriate length and taper to the desired outside diameter for either a holding pipet (80–150 mm) or an enucleation pipet (20–25 mm) using a microforge.
4. Position the previously pulled pipet on the microforge, parallel to the filament.
5. Lower the pipet until it sticks to a glass bead located at the end of the filament.
6. Turn off the microforge, so the filament cools and breaks the glass tube at the bonding point. The next step depends on the type of micropipet being made.
7. Fire polish the open tip of a holding pipet by carefully positioning the open/working end of the pipet above the heated filament and allowing it to melt so that a smooth end results, but without completely melting the opening shut.
8. Bevel the tip of an enucleation pipet, by positioning the pipet at a 45° angle to the grinding stone, switch on the grinder and lower the pipet until it is in contact with the grinding surface. To keep the glass dust produced during grinding from clogging the pipet, distilled water should be applied to the grinding surface while operating. When water travels up the pipet as the result of capillary action and stops, the bevel is ready.
9. Wash the pipet carefully three or four times by flushing with boiling water contained in a beaker on a hot plate. Attaching the pipet to plastic tubing connected to a syringe will do this.

3.3.2. Enucleation of Oocytes

1. Approximately 20–21 h after IVM, place expanded COCs in a 15-mL conical test tube containing approx 0.75 mL of vortexing medium for denuding (removing the cumulus cells).

2. Vortex the expanded COCs for 3 min.
3. Wash the ova in TL–HEPES and place in wells containing washing medium.
4. Transfer the oocytes into nucleus staining medium for 10 min before enucleation.
5. Wash the oocytes with manipulation medium in Petri dish wells.
6. Place 20–30 oocytes into a 200-µL drop of manipulation medium contained in a Petri dish lid and covered with mineral oil.
7. Select the oocytes to be enucleated by the presence of a first polar body and homogeneous cytoplasm. Return the oocytes not being enucleated to the incubator.
8. Aspirate a small portion of the cytoplasm, including the first polar body and metaphase II (MII) chromosome karyoplast for enucleation, using a beveled (18–20 mm outer diameter) glass pipet attached to micromanipulators and mounted on an inverted microscope.
9. For nuclear transfer use only oocytes in which the removal of both the polar body and metaphase chromosomes is confirmed by quick observation under ultraviolet light. If unsuccessful, a further attempt to remove the MII plate may be made.

3.3.3. Transfer of Donor Cell Nuclei Into Enucleated Ova

1. After trypsin–EDTA treatment (described in **Subheading 3.1.1.**), place the cultured donor cells into a separate drop of manipulation medium under oil adjacent to the drop containing enucleated ova.
2. Load 15–20 donor cells into the pipet and move to the drop containing the enucleated ova.
3. Place donor fibroblasts of median size (18–20 µm) and morphologically round into the perivitelline space of each oocyte.
4. After recombination is complete, return the oocyte–fibroblast couplets to culture-dish wells containing TCM 199-Earl's supplemented with 10% FCS in an incubator at 38.5°C, in an atmosphere of 5% CO_2 and air.
5. Transfer 10–20 oocyte–fibroblast couplets into 500 µL of fusion medium contained in culture wells on a warm plate. It is important to handle the recombined couplets with care, as it is very easy to dislodge the somatic cells from the egg plasma membrane.
6. Remove the oocyte–fibroblast couplets from the culture dish once equilibrated in fusion medium and gently place into a 0.5-mm gap PN 453 fusion chamber that contains 1 mL of fusion medium.
7. Arrange the couplets manually (using a small glass tool) so that the contact between the somatic cell membrane and the oocyte membrane is parallel to the electrodes.
8. For electrofusion, deliver two 25-µs 2.3 kV/cm direct charge pulses using an Electrocell Manipulator.
9. Return all couplets to a culture dish containing TCM 199 supplemented with 10% FCS) and 5.0 µg/mL cytochalasin B after the electropulse and culture for 1 h at 38.5°C in an atmosphere of 5% CO_2 and air.
10. Transfer the couplets to cytochalasin B-free medium for an additional hour in culture.

11. Evaluate the success of fusion using a microscope. Only successfully fused embryos are subjected to activation treatment.

3.3.4. Activation of Cloned Embryos

1. Activation of the cloned embryos occurred 2 h after electrofusion.
2. Incubate fused couplets in wells containing activation medium 1 followed by 4 min in TL–HEPES with 3 mg/mL BSA at room temperature (20–22°C). Because ionomycin is light-sensitive, this procedure is best done in a dimly lit room.
3. Wash embryos twice in TL–HEPES with 3 mg/mL BSA by gentle mouth pipetting and place into 500 µL of activation medium 2.
4. Incubate the embryos for 5 h at 38.5°C, in an atmosphere of 5% CO_2 and air.

3.3.5. Embryo Culture

1. After activation, incubate SCNT embryos overnight (at 38.5°C, in an atmosphere of 5% CO_2, 5% O_2, 90% N_2) in 500 µL of IVM in culture wells covered with mineral oil.
2. Transfer the SCNT embryos surgically into the oviducts of synchronized surrogate ewes (Armenian sheep) on day 1, where day 0 is estrus.

3.4. Synchronization of Estrus

3.4.1. Selection and Maintenance of Surrogate Female Ewes

1. Armenian ewes (*Ovis orientalis gmelini*) between the ages of 2 and 10 yr were used as SCNT embryo surrogates.
2. The ewes were housed in outdoor pens in groups of three or more and fed a diet of 14% protein creep feed and choice hay, with water provided ad lib.

3.4.2. Synchronization of Estrus in Surrogate Females

Armenian ewes were synchronized for use as surrogates using intravaginal progesterone releasing devices (CIDR®). A CIDR is an intravaginal pessary device consisting of a nylon core, surrounded by a silicone elastomer and implanted with progesterone ***(5)***. CIDRs are used to synchronize estrus in domestic and wild ruminants during any stage of the estrous cycle (*see* **Note 3** and **ref. *6***).

1. Insert CIDRs into each ewe and administer 1.5 mL of prostaglandin F2α intramuscularly (Lutalyse).
2. Leave CIDRs in the ewes for 11–12 d. Count the day of NT as day 0. Because 1–2 cell embryos are being transferred, it is important to remove the CIDR 36 h before embryo transfer, which corresponds to 12 h before the end of IVM and the time NT procedures are initiated.
3. Administer one intramuscular injection of 300 IU eCG to each Armenian ewe surrogate 12 h before CIDR removal (i.e., the day before NT, administer eCG in the morning and pull CIDRs in evening).

4. This protocol resulted in ewes exhibiting recent ovulations at the time of embryo transfer.

3.5. Embryo Transfer

3.5.1. Preparation of Surrogate Armenian Ewes for Embryo Transfer

Food and water should be withheld from surrogate ewes for 8–12 h before surgical embryo transfer.

1. Administer an injection of 1–1.5 mL of xylazine/ketamine mixture (2 mg xylazine to 100 mg ketamine) into the jugular vein (intravenous) along with an injection of 1 mL of atropine sulfate (0.5 mg/kg) intramuscularly as preanesthetic agents while a drop chute physically restrains the ewe.
2. Remove the ewe from the chute, intubate, and administer isoflurane to maintain a surgical plane of anesthesia.
3. Clip the abdominal hair from the xiphoid to the pubis, then scrub using betadine scrub followed by 70% alcohol.
4. Move the ewe to a surgical cradle that allows the head to be tilted down at a 45° angle. Tilting the ewe thus meant that insufflation of the abdomen with CO_2, a common practice in laparoscopic surgery, was not needed.

3.5.2. Surgical Embryo Transfer

1. Keep embryos in warm enucleation medium (38.5°C) in a hot box until transferred.
2. Puncture the abdomen of the ewe twice using a trocar.
3. Examine each ovary laparoscopically for a corpus luteum, indicating recent ovulation.
4. Transfer one-cell embryos to the oviduct on the side where ovulation occurred (*see* **Note 4**).
5. Exteriorize the appropriate oviduct after expanding one of the puncture wounds by grasping the tip of the uterine horn and lifting the ovary and the oviduct through the incision.
6. Grasp the fimbria with rat tooth grasping thumb forceps to expose the ostium of the oviduct (*see* **Note 5**).
7. Load the embryos into a 5-µL pipet, ensuring that the embryos are at the end of the tip.
8. Deposit the embryos into the lumen of the oviduct past the first major bend (approx 2–3 cm inside the lumen of the oviduct) with the aid of atraumatic thumb forceps. Care should be taken to avoid scraping the wall of the oviduct with the pipet. If difficulty is experienced with a glass pipet breaking, plastic capillary tubes can be used instead. Slowly withdraw the pipettor as the plunger is being depressed to avoid blocking the tip with the tubal endometrium.
9. Lubricate the exposed tissue during surgical embryo transfer with sterile saline spray.

10. Gently place back the reproductive tract into the abdomen, and suture the incision and puncture wounds using a two-layer (body wall and skin) closure with absorbable suture material.
11. Move the ewe to a recovery area, extubate, and administer the anesthetic reversal agent intravenously (0.125 mg/kg yohimbine or 3 mg/kg tolazoline). Burprenophine administered subcutaneously (3.2 mg/kg) may be used for analgesia, as needed (*see* **Note 6**).

4. Notes

1. Three separate trials were conducted using the methods described. Of 265 enucleated domestic sheep ova, 254 were successfully fused with donor Desert Bighorn Sheep cells (96%). Of these, 223 were transferred into 10 Armenian ewe surrogates. Five (50%) of these were confirmed pregnant with a single fetus by ultrasonography at day 25 of gestation. At day 35 of gestation, four surrogates (40%) remained pregnant. None of these pregnancies continued past 55 days of gestation.
2. There are currently three reports of the use of SCNT to clone wild sheep. White et al. *(7)* reported attempts to clone Argali. Skin cells were used as nucleus donors and domestic sheep were used as ovum donors and surrogates. Different from our methods, cloned embryos were cultured for 72 h in CR1aa medium, then a second round of NT was performed using the embryonic blastomeres as nucleus donors. These SCNT embryos were cultured for 72 h before transfer into recipient ewes that had been synchronized using CIDRs and one pregnancy occurred. Similar to the results reported here for Desert Bighorn Sheep, this pregnancy was lost between 49 and 59 days of gestation, and no live offspring were born *(8)*. In another study, Loi et al. *(2)* reported the birth of cloned wild European mouflon. Differences in the methods used included the following: (1) the addition of 0.3 m*M* sodium pyruvate and 100 μ*M* cysteamin to oocyte maturation medium, (2) the use of granulosa cells collected from COCs as nucleus donors, (3) direct injection of mechanically dissociated donor cells into enucleated ova rather than using electrofusion, (4) the use of synthetic oviduct medium supplemented with essential and nonessential amino acids, and (5) the use of naturally cycling ewes as surrogates, that is, no estrus synchronization was used. Similar to the methods described by White et al., domestic ewes were used both as ovum donors and embryo recipients. Seven blastocysts were transferred to four surrogates. Two surrogates were pregnant at day 35 of gestation, but one of the pregnancies was lost between days 40 and 50. The other pregnancy continued to term, with a live female mouflon lamb delivered at 155 days of gestation.
3. The synchronization of estrus in Armenian ewes was very efficient. All 10 ewes showed a recent ovulation, allowing them to be used as surrogates. This protocol may be useful when there is a limited number of embryo recipients available.
4. If one cell embryos are being transferred and a limited number of surrogates are available, those with mature, but unovulated follicles can be used, by rupturing the follicle and injecting either 500 IU human chorionic gonadotropin intramuscularly or 200 IU intravenously.

5. If cloned embryos are cultured to the blastocyst stage, as with the mouflon *(2)* embryo transfer requires only that the tip of the uterine horn is exposed and the puncture is made through the uterine wall with a blunt stylet to allow insertion of the embryos using the pipettor, which is directed toward the utero-tubal junction.
6. Working with wildlife and/or endangered species requires that special attention is given to animal care and handling. This is important not only for the welfare of the animals but also for the safety of the handlers. These animals can quickly succumb to stress leading to death and are very prone to injuring themselves in attempts to escape. Specialized facilities are required for handling wild animals to minimize stress. Special care and attention is also required when using anesthetics and/or tranquilizers. Even within the same species, different animals may respond differently. The use of a long-acting tranquilizer (LAT) has shown some success in reducing the stress in wild animal species. Dehnhard et al. *(9)* reported a reduced stress response in Roe deer after administering perphenazine enanthate and similar results were obtained in wapiti when zuclopenthixol acetate was administered *(10)*. Ebedes *(11)* reported on the history and use of LATs in a number of African wildlife species. Fluphenazine decanoate was used as a LAT on Desert Bighorn ewes during a superovulation treatment for an IVF experiment. Although the data were not collected to determine the effects of the LAT, it was clear that the ewes were calmer when the LAT was administered. In addition, during a 3-d superovulation experiment, where the ewes were handled twice a day for FSH injections, more oocytes were collected from one LAT treated Desert Bighorn ewe than from three Desert Bighorn ewes not receiving the LAT (data unpublished), suggesting that LATs have the same calming effects on Desert Bighorn ewes *(6)*. A significant concern when using SCNT to clone exotic and endangered species is acquiring suitable recipient ova and identifying suitable recipient animals to serve as surrogates for embryo transfer. Where the goal is to use the genomes from animals that are deceased to increase genetic diversity, it may be that adequate numbers of ova can be obtained from the species that is being cloned and embryo recipients of this species also are available. In this case the issue of interspecies NT and embryo transfer is not relevant. In other instances, the number of animals available is limited and closely related species must be used. This will result in cloned offspring with mitochondrial genes from the species selected as the ovum donor (e.g., a gaur with mitochondria from domestic cattle *[1]*). This can be controversial, but is it better to have Gaur containing mitochondrial genes from cattle than to have no Gaur at all? Other examples also may influence one's perspective. For instance, the great majority of live American bison carry mitochondrial genes derived from cattle because of hunting these animals to near extinction then crossbreeding with cattle. One approach to limit this problem and still use the technology for salvaging genetic diversity is to clone only male animals because mitochondria are only passed from mother to offspring. Another issue involving the use of assisted reproductive technologies for propagating wildlife is the use of domestic species as surrogates that then rear the offspring. It may be the goal that the resulting animals

remain wild and exhibit wild behavior, especially if the ultimate goal is to reintroduce these animals into a natural environment. This can be difficult when selecting domestic species as surrogates because the offspring may become tame, or at least partially domesticated. For Desert Bighorn Sheep, domestic sheep were used as egg donors, but wild sheep were used as surrogates. The goal was for the offspring to be raised by wild mothers. However, using one species as cell donors (Desert Bighorn Sheep), a second species as ovum donors (domestic sheep) and a third as surrogates (Armenian sheep) may have compromised our success in cloning Desert Bighorn.

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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Handmade Somatic Cell Cloning in Cattle

Gábor Vajta, Ian M. Lewis, and R. Tayfur Tecirlioglu

Summary

Apart from the biological and ethical problems, technical difficulties also hamper the improvement and widespread application of somatic cell nuclear transfer (NT). Recently introduced zona-free procedures may offer a solution for the latter problem. The most radical approach of these techniques is the so-called handmade cloning (HMC). It does not require micromanipulators because the manipulations required for both enucleation and nucleus transfer are performed by hand. The HMC technique includes manual bisection of zona-free oocytes, selection of cytoplasts by staining, and the simultaneous fusion of the somatic cell with two cytoplasts to produce a cloned embryo. HMC is a rapid and efficient technique that suits large-scale NT programs. It requires less expertise and time than traditional NT methods and the cost of equipment is significantly less. Production efficiency is high and embryo quality, in terms of pregnancy rates and live births, is not compromised. Although HMC has been developed particularly for bovine NT, the technique is applicable to other species. The method may become a useful tool for both experimental and commercial somatic cell cloning because it allows for standardization of procedures and provides the possibility of automation.

Key Words: Nuclear transfer; cloning; bovine; handmade; somatic cell; zona-free.

1. Introduction

The basic technical aspects of commonly used nuclear transfer (NT) methods for mammals have not changed since the original description of the first successful embryonic cell cloning *(1)*. Most embryologists regard the zona pellucida as important for appropriate embryonic development until the expanded blastocyst stage. However, preservation of an almost-intact zona during NT requires the use of micromanipulators, highly skilled labor, and expensive equipment. In addition, these factors make standardization of the procedure difficult.

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The need for an intact zona pellucida for appropriate embryo development was questioned several years ago *(2,3)* and a micromanipulator-free procedure was introduced for embryonic cell cloning *(2,4,5)*. However, the direct application of this technology for somatic cell nuclear transfer (SCNT) was unsuccessful initially *(6)*. Later, a technical modification in pairing, fusion, and the application of more appropriate culture conditions made zona-free somatic cell cloning possible in cattle and pigs *(7–10)*. Recently, other forms of the technique were described *(11–13)*. Although some of these zona-free procedures still required the use of micromanipulation for enucleation before or after zona removal *(9–11),* this step can be performed by hand as well *(7,8,12–21)*. For clear distinction, the micromanipulation-free, SCNT method has been referred to recently as handmade cloning (HMC; *see* **Fig. 1** and **ref.** *12*). A recent modification of the fusion technique has improved the efficiency and reliability of this step considerably *(14,21)*.

All publications dealing with zona-free NT techniques agree that these methods are easier to learn and apply, do not require specially skilled workforce, the productivity is high and the final embryo quality is comparable to that with traditional cloning. Pregnancy and calving data are still limited *(11,12,14–21)*; however, initial observations do not reflect any compromised developmental ability. Viability of HMC embryos also was illustrated by the fact that pregnancies and offspring were obtained after vitrification (G. Vajta, unpublished *[14,15,20,21]*).

Additionally, in the HMC procedure, no micromanipulators or related equipment are required, making the method accessible to laboratories with limited budgets. The simple and repeatable steps of the HMC method make the outcome less dependant on individual skills, meaning that a higher level of standardization of SCNT is possible. Eventually, partial or full automation of the procedure is a realistic possibility.

2. Materials

Except where otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

1. Tissue culture medium (TCM)-199 medium (M-0650): 2.5 m*M* HEPES (H-7006), 5 mM $NaHCO_3$ (S-4019), 0.2 m*M* sodium pyruvate (P-3662), 50 µg/mL gentamycine sulfate (G-1264) adjusted to pH 7.4, 280 mosm and supplemented with either 0, 2, 10, or 20% cattle serum (T0, T2, T10, and T20, respectively).
2. Phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS0; Gibco-BRL, Gaithersburg, MD; cat. no. 14190–144).
3. Fusion medium: 0.3 *M* D-mannitol (M-9546), 0.1 m*M* $MgSO_4$ (M-2393), 0.05 m*M* $CaCl_2$ (C-7902), and 1 mg/mL polyvinylalcohol (P-8136). Store in 10-mL aliquots at –20°C.
4. Mineral oil (M-8410), cell culture tested.

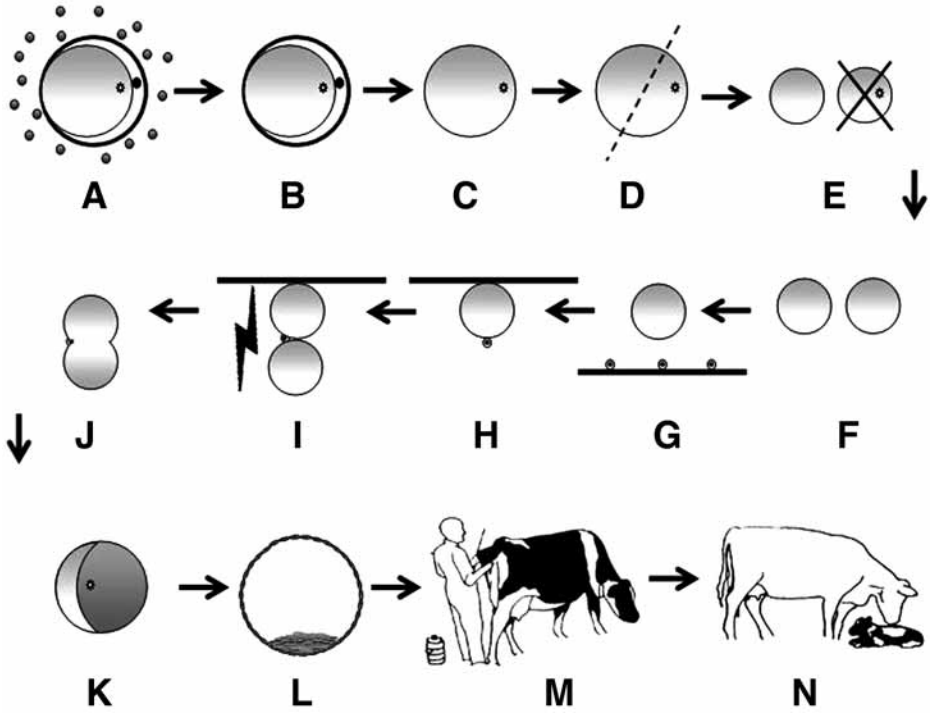

Fig. 1. Schematic illustration of handmade cloning: **(A)** cumulus intact oocytes 18–22 h after maturation; **(B)** cumulus removal by vortexing; **(C)** zonae removal; **(D)** bisection of oocytes; **(E)** Hoechst 33342 staining; **(F)** selection of cytoplasts; **(G)** attachment of somatic cell to the cytoplast; **(I)** simultaneous fusion of two cytoplasts with a somatic cell; **(J)** round-up following fusion; **(K)** activation of embryos 3 h after reconstruction; **(L)** blastocyst 7 d after reconstruction; **(M)** embryo transfer with fresh and vitrified embryos; **(N)** offspring.

5. Phytohemagglutinin (PHA; L-8754). Prepare 5 mg/mL stock solution in T0, store in 50-µL aliquots at –20°C.
6. Protease (Pronase E; P-8811). Prepare 2 mg/mL solution in T10, store in 500 µL at –20°C. Centrifuge before use.
7. Hoechst 33342 (B-2261). Prepare 1 mg/mL stock solution in ultra-pure H_2O. Store in 20-µL aliquots at –20°C in dark (light sensitive).
8. Trypsin–EDTA (Invitrogen, Carlsbad, CA; cat. no. 25300–054). Store in 100-µL aliquots at –20°C.
9. Hyaluronidase (H-4272). Prepare 0.5 mg/mL solution in T2. Store in 500-µL aliquots in 2-mL Eppendorf tubes at –20°C.
10. Calcium ionophore II (C-7522). Prepare 1 mg/mL stock in dimethyl sulfoxide (D-2650). Keep at room temperature in a small dark bottle with tightly closed cap.

11. 6-Dimethylaminopurine (DMAP; D-2629). Prepare 100 m*M* stock solution in PBS0. Use heat-durable container, heat in a hot water bath until completely dissolved. Store in 8-µL aliquots at –20°C.
12. Cytochalasin B (C-6762). Prepare 5 mg/mL stock dissolved in dimethyl sulfoxide.
13. Store in 2-µL aliquots at –20°C.
14. Two stereo microscopes with sharp image contrast, such as Olympus SZ60 (Olympus, Tokyo, Japan) or Nikon SMZ 2T (Nikon, Tokyo, Japan). One microscope was equipped with heated stage for manipulation, and the other microscope was maintained at room temperature for fusion.
15. Inverted fluorescent microscope, preferably with heated stage.
16. Fusion machine (Genaust; Bacchus Marsh, Victoria, Australia).
17. Fusion chamber. Microslide with 0.5-mm gap (BTX, San Diego, CA; cat. no., Model 450, 01–000209–01).
18. BLS aggregation needles (DN-09; BLS, Budapest, Hungary).
19. Microblade (Ultra Sharp Splitting Blades; AB Technology, Pullman, WA).
20. Four-well dishes (4WDs; Nunc, Roskilde, Denmark).

3. Methods

Methods for oocyte collection, maturation, and somatic cell preparation are described in other chapters of this book as well as in our earlier publications *(8,12)*. Briefly, 25 oocyte-cumulus cell complexes were matured in each well of a 4WD filled with 400 µL of bicarbonate buffered TCM 199 medium with additives, hormones, and 15% cattle serum, covered with 400 µL of oil. Fibroblasts were cultured in wells of a 4WD containing 400 µL of Dulbecco's modified Eagle's medium supplemented with additives and 10% cattle serum (*see* **Note 1**) and covered with 400 µL of oil for 3 to 5 d to reach almost total confluence. For one routine HMC program, 150–200 oocytes and one well of somatic cell culture were used.

This chapter deals in detail only with procedures that are specially performed in bovine HMC, that is, (1) cytoplast preparation, (2) fusion, (3) activation, and (4) embryo culture. Except where otherwise indicated, all manipulations were performed on 39°C heated stages and plates using minimum background light.

3.1. Preparation of Cytoplasts and Somatic Cell Suspension

3.1.1. Removal of the Cumulus Cells and the Zona Pellucida

1. Remove 150–200 oocytes from the maturation dish 21 h after the start of in vitro maturation (hpm) and transfer to an Eppendorf tube containing 500 µL of hyaluronidase solution.
2. Mix suspension carefully with a 1000 µL of automatic pipet until all visible cumulus clouds disappear.
3. Vortex the tube at maximum speed (8000 rpm) for 3 min.

4. Collect oocytes in a 36-mm diameter Petri dish under a stereo microscope and wash twice in T2 to remove all cumulus cells from the solution (*see* **Note 2**).
5. Fill wells of a 4WD with the following solutions:

   Well 1: Supernatant of the centrifuged pronase solution
   Well 2: 800 µL of T20
   Well 3: 800 µL of T2
   Well 4: 800 µL of T20
6. Using the smallest possible volume, transfer oocytes to well 1 and place the dish on the heated plate of a horizontal shaker and rotate at (120 rpm) for 10–15 min, then with (85 rpm) for 1 min (*see* **Note 3**).
7. Transfer zona-free oocytes collected in the middle by the slow rotation into well 2 and incubate for an additional 3 min (*see* **Note 4**).

3.1.2. Preparation of Somatic Cell Suspension

1. Remove all medium and oil from one well of the 4WD containing the fibroblast monolayer wash three times with PBS0 during pronase digestion of oocytes.
2. Incubate cells with 100 µL of trypsin–EDTA for 4 min to lift cells and then add 1 mL of of T20 neutralize trypsin–EDTA. Mix contents rigorously, transfer to an Eppendorf tube, and store at room temperature to minimize aggregation of cells.

3.1.3. Random Bisection of Oocytes

1. Prepare a bisection dish by adding 4 mL of T20 and a cytochalasin B stock tube solution into a 36-mm diameter Petri dish.
2. Transfer approx 40–50 zona-free oocytes into the bisection dish and line them up on the bottom of the dish. Facilitate the final linear arrangement with careful tapping on the side of the dish.
3. Clean the microblade with 70% ethanol and then anchor the tip of the blade to the bottom of plastic dish close to the oocytes. Lower the cutting edge of the blade close to the middle part of the oocyte to bisect each oocyte individually. After cutting three to seven oocytes, change the position of the blade tip and repeat the procedure.
4. Tap the dish carefully again to detach oocyte halves from the dish and swirl solution to collect halves in the middle.
5. Transfer all halves to the fourth well of the 4WD and repeat bisection procedure until oocytes are bisected (*see* **Note 5**).

3.1.4. Selection of Cytoplasts

1. Add 8 µL of Hoechst stock solution to well 3 of the 4WD, transfer all oocyte halves into the well, and incubate in dark for 5 min.
2. Meanwhile, prepare cytoplast sorting dish. Pipet 200-µL drops of T2 at the 12-o'clock position into the lid of a 36-mm diameter Petri dish. Place the lid on a room temperature stage to avoid excessive evaporation. Make 130–140 small droplets (approx 1–2 µL) under the stereo microscope using a thin capillary pipet and cover both the large and small droplets with mineral oil.

3. Transfer all stained halves into the large drop first and then into the small droplets in groups of three as quickly as possible to minimize light exposure. Register positions of half-oocytes without chromatin staining on a tape recorder using an inverted microscope and ultraviolet light,
4. Collect cytoplasts under a stereo microscope and transfer into well 4 of the 4WD for temporary storage.

3.2. Pairing and Fusion

1. Prepare a 4WD for cell–cytoplast pairing and fusion with following solutions:
   Well 1: 800 µL of T20
   Well 2: Supernatant of the PHA tube (mix 450 µL of T2 solution with PHA stock solution and centrifuge to remove precipitation)
   Well 3: 800 µL of T20
   Well 4: 800 µL of fusion medium.
2. Fill the middle part of the 4WD with 4 mL of T2 and keep the dish on the heated stage of stereo microscope. Transfer all cytoplasts into the well 1 of the 4WD for cell-cytoplast pairing and fusion.
3. Place the fusion chamber on the second microscope at room-temperature stage and secure it with sticky tape. Attach live (red) wire to Northern and ground (black) to the Southern platinum wire. Cover fusion chamber with 2 mL of fusion medium.
4. Turn the fusion machine on and set following parameters: alternating current (AC) = 14 V direct current (DC) = 168 V (3.36 kV/cm), pulse duration = 4 µs, number of pulses = 1, and post-AC pulse = 0 inducing a potential difference of 3 V on the somatic cell surface (*see* **Notes 6–8**).
5. Mix somatic cell suspension thoroughly with a 1000-µL automatic pipet and add 3–5 µL of the cell suspension into the middle part of the 4WD in one group avoiding extensive dispersion. Focus microscope in high magnification and adjust mirror to visualise individual cells clearly under the stereo microscope.
6. While cells are settling, remove 20–30 cytoplasts (start with 2 cytoplasts if you are inexperienced) from well 1 and transferred into well 4 containing fusion medium for equilibration. The cytoplasts were then transferred into the Southern (away from the platinum wires) part of the fusion chamber.
7. Subsequently, remove another 10–20 cytoplasts from well 1 and disperse into well 2, close to top of the PHA solution without allowing cytoplasts to touch each other or any surface of the well. Transfer all PHA coated cytoplasts within 3–4 s to the middle of the dish in a clear area away from the somatic cells.
8. Pick one cytoplast and allow cytoplast to sink over a single fibroblast that had clear/round edges. When the two cells are attached to each other, transfer cytoplast–fibroblast pair into well 4 containing fusion medium for equilibration and then to the Northern part of the fusion chamber (away from the platinum wires; *see* **Note 9**). Repeat pairing procedure with the remaining cytoplasts stored in the middle of the 4WD.

9. Turn the AC alignment on, pick the cytoplast–fibroblast pair and expel them above the platinum wires close to the Northern wire (*see* **Note 10**). Gently steer the pair with the pipet until fibroblast facing the opposite wire. While cytoplast–fibroblast pair sink in the fusion medium, the pair move toward and then attach to the Northern wire.
10. After attaching cytoplast–fibroblast pair to the wire, pick another cytoplast and expel it close to the cytoplast–fibroblast pair. This cytoplast will float toward the pair touching the fibroblast first and then cytoplast having somatic cell is sandwiched in the middle (*see* **Note 11** and **Fig. 2**).
11. Apply DC pulse, gently remove triplets and transfer into well 3 containing T20 for rounding-up. Repeat fusion steps until all cytoplasts are fused with somatic cells (*see* **Note 12**).
12. Following visible signs of fusion and round-up, transfer all reconstructed oocytes into wells of a 4WD filled with 400 µL of α-aaci medium supplemented with 5% cattle serum *(22)* and covered with 400 µL of oil in a well of a 4WD. Incubate the dish at 39.0°C in 5% CO_2 in air and maximum humidity for 3 h.

3.3. Activation

1. Activate oocytes 28 h after the start of maturation (approx 3–4 h after the fusion). Incubate reconstructed embryos first in 1 mL of T20 containing 2 µ*M* calcium ionophore (2 µL of stock solution) for 5 min at room temperature and then wash twice in T20.
2. Further incubate reconstructed embryos individually in 5-µL droplets of culture medium containing 2 m*M* DMAP (content of one stock tube dissolved in 400 µL of culture medium) and covered with oil in 5% CO_2 in air at 39°C for 6 h (*see* **Notes 13** and **14**).

3.4. Embryo Culture

1. Add 400 µL of culture medium to each well and cover with 400 µL of oil.
2. Support the bottom of the wells with a thick glass microscope filter and score 20–50 well of the wells (WOWs) in each well by applying a strong, steady vertical pressure with aggregation needle. Flush wells individually with culture medium and incubate dishes at 39°C in 5% CO_2, 5% O_2, and 90% N_2 and maximum humidity. WOWs were prepared according to the modification *(9)* of the original description *(3)*.
3. Wash reconstructed embryos three times in culture medium (*see* **Notes 15** and **16**) approx 34 hpm and culture individually in WOWs at 39°C in 5% CO_2, 5% O_2, and 90% N_2. To maintain constant conditions, do not change medium or assess the cleavage rate during the culture period (*see* **Note 17**). The number of blastocysts per reconstructed embryo is determined under a stereo microscope 7 d after reconstruction.

4. Notes

1. The use of cattle serum instead of fetal calf serum was suggested in our earlier publication *(12)*. The high protein content of cattle serum efficiently decreases

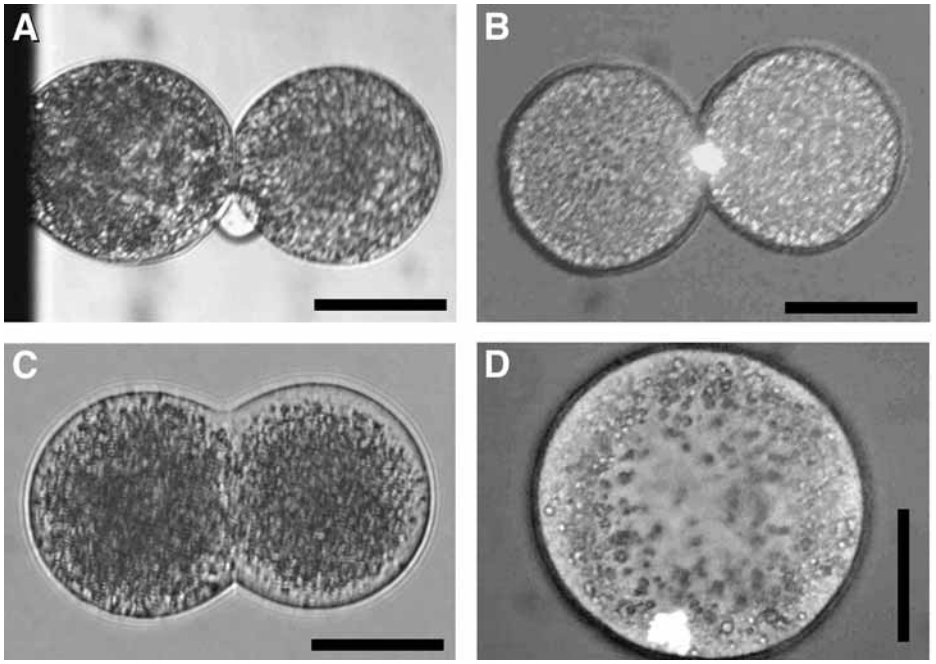

Fig. 2. One-step fusion in handmade cloning. **(A)** Simultaneous fusion of two-cytoplasts and a somatic cell before the direct current (DC) pulse; **(B)** 1 min; **(C)** 30 min; and **(D)** 6 h after the DC pulse. **(A)** and **(C)** visible light, **(B)** and **(D)** ultraviolet epifluorescence after Hoechst 33342 staining. Scale bar, 50 μm

attachment of bisected demi-oocytes to the bisection blade, improves recovery after fusion, and also may help to avoid blastomere spreading and embryo disintegration before compaction. High protein content in the medium was also suggested by Peura et al. *(4)*. Moreover, the growth factor content of cattle serum is at least twice as high as that of fetal calf serum *(12)*, which also may contribute in the high developmental rates of HMC embryos in culture.

2. In contrast to the routine procedure in traditional nuclear transfer and also to some zona-free cloning procedures *(2,4,5,11)*, selection of polar body-containing metaphase II phase oocytes is not performed in HMC, as exclusive use of polar body-containing metaphase II oocytes did not improve blastocyst rates *(7–10,12,14,20)*. For HMC, all oocytes without visible degenerative changes or signs of physical damage were processed.
3. The potential harmful effect of pronase to the oocyte membrane was minimized by the supplementation of the solution with 10% cattle serum according to the suggestion of T. Peura (personal communication). This way, as many as 300 oocytes could be digested together while the proportion of damaged embryos did not exceed 1%.

4. Proper handling and moving of oocytes and embryos with glass pipets is a key to success in HMC. Zona-free oocytes, cytoplasts, and reconstructed embryos are more fragile than zona-enclosed ones. They attach easily to surfaces and to each other and may be destroyed with even slightly increased flows of media. Moreover, HMC requires delicate movements both at enucleation, pairing, and fusion. Very finely drawn, perfectly shaped, and lightly fire-polished glass pipets should be used. Before use, pipets should be flushed with T20 to cover the walls with a thin protein layer to avoid attachment. Oil and air bubbles should be aspirated so that the handling of cells can be controlled. If manipulations cannot be controlled perfectly, a new pipet should be used or further training undertaken. For cytoplast preparation, the capillary diameter should be approx 200–250 µm (approx 1.5–2 times the size of a zona-enclosed oocyte). For fusion, the right size is approx 110–140 µm, which is about 1.5–2 times the diameter of a zona-free cytoplast (half-size of oocyte). Working with slightly polished capillaries of the appropriate size can avoid losses of 50% or more.
5. Methods for enucleation of zona-free oocytes can be divided into four groups: (1) Oriented enucleation can be performed either with Hoechst staining, ultraviolet illumination and micromanipulation ***(11)*** or positioning the polar body attached to the oocyte membrane with previous PHA incubation ***(2,4,5)***; (2) for random enucleation, high-speed centrifugation ***(23)***, micromanipulation ***(9,10)***, and manual bisection ***(8,12)*** can be used with subsequent ultraviolet selection of cytoplasts; (3) a method for chemically assisted enucleation using either micromanipulation manual bisection was established in sheep by Peura ***(13)***; (4) recently, a chemically induced enucleation was introduced in mouse by Wang and Overstrom ***(24)***. The manual bisection procedure described here is simple, rapid, efficient, and highly reliable. The fact that bisection necessitates the use of two cytoplasts for one reconstructed embryo is counterbalanced by the use of all of the oocytes, without polar body selection, and also the exceptionally high blastocyst per reconstructed embryo rates (approx 50% ***[12]***).
6. Commercially available fusion machines vary greatly in terms of pulse amplitude, pulse width, and reliability. It is recommended to test pulse parameters, preferably with a graphic pulse analyzer and an oscilloscope before initiating a cloning program. Disregard any values presented on the fusion machines and adjust pulse parameters according to readings from the analyzer.
7. Microslide fusion chambers with various gaps can be used for cell fusion, however DC amplitude (V) should be re-adjusted according to effective electrical field (E) and the gap between the wires (in centimeters: E = V/cm).
8. Effective field strength and pulse duration can be calculated for different cell types according to following formulas: $\Delta\Psi = Fg(\lambda)rE\cos\theta$ and $\tau = rC\ (\lambda_{int} + 2\ \lambda_{out}) / 2\ \lambda_{int}\ \lambda_{out}$ ***[14,20,25]***). Higher field strength is needed to cause disruption of the cell membrane of smaller cells compared with larger ones. However, when an intense external electrical field is applied, the extra energy that is needed to fuse smaller cells may be too strong for larger ones and can cause lysis if the pulse duration is not decreased.

9. Pairing of cytoplasts and donor cells in zona-free embryonic cell NT initially was performed using the AC effect in the fusion chamber *(2,4,5)*. However, the relatively small size of somatic cells does not allow this method of pairing in zona-free SCNT. The attachment facilitated by PHA was found to be an efficient way for pairing *(7,8,12)*. Subsequently, successful use of the same procedure has been reported elsewhere *(9–11,13)*.
10. The use of AC to facilitate proper alignment at fusion is a common method in zona-free NT procedures *(2,4,5,7–10,12)*. Recently, a so-called automated fusion procedure was described by Oback et al. *(11)*, where the alignment was entirely performed entirely by the AC current, using various intensities according to the requirement of the pairs. However, the reported compromised fusion rates did not fully justify this innovation.
11. The use of this sandwich type arrangement and one-step fusion *(14,20)* resulted in some unexpected benefits. The two cytoplasts stabilize the cell triplets in the correct position, allowing up to 10 or 20 triplets to be fused together. This decreases the time required for one fusion considerably. The sandwich position resulted in a uniquely increased pressure on the attaching membranes between both somatic cell and cytoplasts and between the two cytoplasts. This close physical contact might contribute considerably to the very high fusion efficiency (90–95%). Additionally, the unique location of the somatic cell does not allow contact and attachment to the bottom of the culture dish after fusion, which may be a source of fusion failure in other zona-free techniques.
12. At fusion, a careful balance between different factors should be maintained. These factors include proper equilibration in the fusion medium while keeping its toxic effects to a minimum; proper alignment of cytoplasts and somatic cells while keeping the AC effect relatively moderate; strong attachment between cytoplasts and somatic cells, but weak attachment to the wire; and having the DC pulse strong enough to cause high fusion rates but moderate enough not to cause lysis or deformities in the reconstructed embryos. *See* the following points for troubleshooting:
    a. No AC effect (i.e., no visible movement of pairs and cytoplast to the wire). First, check connections and parameters. Another possible source of the problem is that there is albumin or serum in the medium or on the surface of the wires. Flush the area of wires and between the wires rigorously with fusion medium, and replace the entire fusion medium. If no improvement is seen, remove the fusion medium, disconnect the chamber, remove it from the microscope, flush with water, clean with paper towel, flush with 70% ethanol, clean with paper towel, dry and then repeat the whole preparation procedure.
    b. The AC current pulls the pairs to the wire too aggressively. This is almost always followed with the explosion of the pairs at fusion. Common reasons include inappropriate equilibration and/or ions around the pairs. Stop the AC current, remove the pair, flush the pipet and area between wires, and start the process again.
    c. Swirling, dancing of pairs and cytoplasts between the wires at AC: again, ions in the medium, as in **item b**.

13. DMAP makes the membrane of zona-free embryos sticky; therefore incubation of embryos individually in small droplets is essential to avoid aggregation.
14. Although it may be difficult to insert into the conventional working schedule, extended incubations between fusion and activation and also in the DMAP (4 and 6 h, respectively) improve developmental rates *(12)*, in accordance with previous observations made on zona-enclosed embryos *(26–28)*.
15. Thorough and long washing, both after Ca-ionophore and DMAP incubation, are essential to prevent toxic injury during the further incubations in relatively small volumes (drops and WOWs, respectively) caused by the remnants of these chemicals.
16. At the end of DMAP incubation, the stereo microscopic view of reconstructed embryos changes significantly, including ovoid-, egg- or kidney-shaped, central dark and peripheral white areas, with well preserved, double-refracting cell membranes. Additionally, several minutes after release from DMAP, a considerable number of embryos look fragmented, some of them mimicking two- or four-cell stages. Although they appear unusual, these changes usually are correlated to efficient activation and may predict high blastocyst rates.
17. Although some zona-free NT systems still use microdrops for embryo culture *(11)*, most researchers have found microdrops inadequate and have turned to alternative ways including the glass oviduct system of Thouas et al. *(12,29)*, or microwells formed either from agar *(13)* or on the plastic bottom of the dish. The benefits of using the WOW system for single embryo culture as well as for supporting the development of zona-free embryos has been described earlier *(3)*. With the modification of Booth et al. *(9)*, the preparation of WOW has become simple, and the high blastocyst per reconstructed rates achievable in the system justifies its application *(12)*.

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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Genetic Modification of Sheep by Nuclear Transfer With Gene-Targeted Somatic Cells

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Summary

For many years the lack of germline competent embryonic stem cell lines in livestock meant that the targeted modification of endogenous genes was not possible in these species. The demonstration that livestock could be cloned by nuclear transfer from cultured somatic cells has now provided an alternative route to accomplish gene targeting. This chapter describes protocols for culturing primary sheep fibroblasts, introducing and selecting targeted modifications into them and then using these modified cells in nuclear transfer experiments.

Key Words: Gene targeting; somatic cells; primary fibrolasts; proliferative life span; nuclear transfer/sheep.

1. Introduction

Until recently, virtually all genetically modified livestock were generated using pronuclear injection, a technique first reported more than a decade and a half ago *(1)*. Although this is routine for a variety of species, there has been little improvement in the efficiency over the years, with only 2–3% of injected eggs giving rise to transgenic offspring. The multicopy nature of transgene loci, coupled with the random nature of the site of integration, gives rise to unpredictable levels of expression *(2)*. Most significantly, this technique provides no means to modify endogenous genes.

At the time pronuclear injection was being developed for livestock, embryonic stem (ES) cells were being developed and used for gene targeting in the mouse. The approach has been enormously successful and, by exploiting the capacity of these cells to contribute to the germline and undergo homologous recombination with exogenous DNA, the introduction of precise, targeted changes into the mouse germline is now routine in many laboratories *(3)*. The

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importance of this approach was realized at an early stage by those working with livestock but, despite extensive efforts, ES cells have not yet been isolated from any species of livestock *(4)*. Although some workers have described ES-like cells in pig, it is apparent that they do not contribute to the germline even though they are capable of forming somatic tissues in chimeras *(5)*. This is perhaps not so surprising when it is considered that in the mouse the ability to isolate true (i.e., germline competent) ES cells is limited to only a few inbred strains. The failure to develop ES cells has constrained a number of applications of germline manipulation technology in livestock. Therefore, the news that cloning by nuclear transfer (NT) from cultured cells had been achieved *(6,7)* was greeted with considerable interest as this opened up the possibility of developing a new cell-based route to genetically modify livestock and, in particular, the capacity to develop gene targeting in these species.

This chapter describes protocols for conducting gene targeting experiments in sheep fetal fibroblasts and preparing these cells for NT. It adapts standard methodologies for homologous recombination and the selection of gene targeted cells primarily developed in mouse ES cells to the particular problems that are encountered using primary somatic cells, which have a limited life span in culture. It describes the isolation of primary sheep fibroblast from fetal material, and the subsequent establishment and characterization of a primary cell line. It details the type of targeting vector designs that may be used, and protocols for transfection and screening of targeted populations. Finally, it describes the recovery of targeted cell populations and how they are expanded and may be used in NT experiments.

2. Materials

2.1. Tissue and Cell Culture Reagents

1. Phosphate-buffered saline (PBS) without calcium/magnesium (Gibco).
2. Glasgow minimal essential medium (GMEM): Comprises 1X GMEM supplemented with 10% fetal calf serum (FCS), 2 m*M* glutamine, 1 m*M* sodium pyruvate, 1X nonessential amino acids.
3. 1X GMEM (Gibco).
4. 200 m*M* Glutamine (Gibco).
5. 100 m*M* Sodium pyruvate (Gibco).
6. 100X MEM nonessential amino acids (Gibco).
7. FCS (Globe Farm, Guilford, or equivalent supplier).
8. Trypsin–EDTA: 1X solution (Gibco).
9. Gelatin (Sigma). Made to 0.1% solution in water and autoclaved.
10. Dimethyl sulfoxide (DMSO; Sigma).
11. Synthetic oviduct fluid (SOF; per 100 mL): 5.83 g NaCl, 0.534 g KCl, 0.162 g KH_2PO_4, 0.1 g $MgCl_2{\cdot}2H_2O$, 0.616 g Na lactate, 0.27 g glucose, 0.06 g of penicillin, 0.05 g steptomycin, 2.1 g $NaHCO_3$, 0.01 g Phenol Red, 0.36 g pyruvate,

2.52 g $CaCl_2H_2O$, 1.46 g gluthamine, 0.8 g bovine serum albumin, 1.0 mL nonessential amino acids, and 2.0 mL essential amino acids.

2.2 Antibiotics and Drug Selection Reagents

1. Gentamycin (50 mg/mL: Gibco).
2. Geneticin (G418; Sigma). Made to 250 mg/mL solution in PBS and filter sterilized.
3. Puromycin (Sigma). Made to 1 mg/mL solution in PBS and filter sterilized.

2.3. DNA Preparation

1. TNE: 50 m*M* Tris-HCl pH 8.0, 20 m*M* EDTA, 100 m*M* NaCl, 10 mg/mL proteinase K, 0.3% sodium dodecyl sulfate.
2. Isopropanol (Propanan-2-ol).
3. TE: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.5.

2.4. Fixatives

3:1 Methanol acetic acid.

3. Methods

3.1. Preparation of Fetal Fibroblasts

Primary cultures of fibroblasts are recovered from 35-d-old fetuses taken from naturally mated ewes by using the following protocol.

1. Immediately wash fetuses in PBS supplemented with gentamicin at a concentration of 100 µg/mL and transfer them to a class II tissue culture cabinet for evisceration and decapitation. Wash each carcass twice more in PBS/gentamicin.
2. From this stage on each carcass is treated separately to generate its own primary culture. Transfer individual carcasses to 5 mL of trypsin–EDTA solution. Facilitate isolation of cells by cutting to approx 1-mm cubes with scissors. This process should be completed in approx 10 min.
3. Stop action of trypsin by adding an equal volume of GMEM supplemented with 50 µg/mL gentamycin. Transfer the suspension to a tube (tube 1) and remove larger clumps of tissue by allowing them to sediment for 5 min at room temperature.
4. Transfer the single cells and small clumps contained within the supernatant to a fresh tube (tube 2) and pellet by centrifugation (200*g* for 5 min). Resuspend this pellet in GMEM/genetamicin medium and place in 175-cm^2 culture flask that has been pretreated with gelatin solution (0.1% for at least 10 min at room temperature).
5. Add 10 mL of GMEM/gentamicin medium to the sedimented pellet in tube 1. Pipet repeatedly to recover additional cells and small clumps. Sediment again and add this supernatant directly to the same flask as before (**step 4**).
6. After 24 h, and every 3–4 d thereafter, replace medium with fresh GMEM *without* gentamycin.
7. Once confluent (usually 2–7 d), release monolayers with trypsin and cryopreserve in GMEM supplemented with 10% DMSO in aliquots of approx 1×10^6 cells/vial.

Wrap vials in bubble wrap to ensure slow cooling and store at –80°C. After 24 h, transfer vials to liquid nitrogen for long-term storage.

8. Analyze cells from each fetus karyotypically to confirm sex and normalcy (*see* **Subheading 3.3.3.**).

3.2. Routine Culture of Primary Fetal Fibroblasts

1. Routinely culture cells in GMEM and passage in 25-cm^2 plastic tissue culture flasks.
2. Grow cells in 5% CO_2 in a humidified incubator at 37°C (*see* **Note 1**).
3. Routinely passage cells at subconfluency using a split ratio of between 1:5 and 1:10 before re-plating. For assessment of life span, *see* **Subheading 3.3**.

3.3. Evaluation of Primary Cultures

3.3.1. Drug Selection

Positive selection of drug resistance markers whose coding sequences are incorporated into the targeting vector is used as the basis for selection in most gene targeting experiments. In the first instance, it is important to determine the sensitivity of newly isolated primary cultures to the chosen selection marker. Both the coding sequences for neomycin phosphotransferase (*neo*), which confers resistance to a neomycin analog, G418, and puromycin-*N*-acetlaytransferase (*pac*), which confers resistance to puromycin, have been used in targeting experiments in sheep.

1. Plate primary fibroblasts (5×10^4 total, approx 5% confluency), in duplicate, into the wells of a six-well plate.
2. After 24 h, add a range of G418 concentrations (0–600 µg/mL) or puromycin concentrations (0–1.5 µg/mL). Change the selection medium every 3–4 d for 10–12 d, at which stage killing of sensitive cells should be complete. The minimum concentration required for complete cell death is noted and subsequently used for selection of targeted cells (*see* **Note 2**).

3.3.2. Proliferation and Lifespan

A key parameter to the success of gene targeting in primary somatic cells is the proliferative vigour of the culture.

1. Use primary cultures with short doubling times in the order of 24 h or less because they speed up the timing of drug selection and expansion.
2. More important is the overall proliferative capacity of the culture to be used (*see* **Note 3**). Determine this as follows: seed duplicate 25-cm^2 flasks with 2.5×10^5 cells. At subconfluence, trypsinize and count the cells. Seed a fresh flask with 2.5×10^5 cells (the aim is to achieve approx 1 in 10 split ratio). Calculate the number of population doublings (PDs; *see* **Note 4**). Continually repeat this process until the cells senesce.

3.3.3. Karyotypic Analysis

This should be done immediately after the primary culture has been established. It should also be repeated with targeted colonies before they are considered for NT experiments.

1. Split confluent cultures of cells at a ratio of 1:2 into fresh flasks. Briefly trypsinise the cells and then pellet by centrifugation (200*g* for 5 min). Resuspend in a hypotonic solution of 0.4% trisodium citrate for 30 min to induce swelling.
2. Pellet by centrifugation (200*g* for 5 min). Resuspend by adding fixative (3:1 mix of methanol:acetic acid) dropwise while vortexing.
3. Repeat **step 2** a further three times, before finally resuspending in 0.5 mL of the fixative (samples can be stored at –20°C at this stage).
4. Drop fixed cells on to cleaned (100% ethanol, 5% HCl solution) slides to ensure to an even spread of the chromosomes. After drying, stain slides with Gurr's Geimsa stain diluted 1:20 in phosphate-buffered water, pH 6.8 for 8 min. Chromosome number is routinely assessed from 30 spreads to ensure the normal chromosome complement (2n = 54 in sheep).

3.4. Targeting Vectors

1. Most gene targeting strategies are based on homologous recombination. Targeting vectors comprise two regions that are complementary to the genomic target sequence, separated by a region of non-homology, which is often a drug resistance marker (**Fig. 1**). Targeting frequencies have been shown to increase by approx 5–20-fold when isogenic (derived from the target cell population), rather than nonisogenic, DNA is used to disrupt genes in mouse ES cells (*see* **Note 5** and **refs.** ***8*** and ***9***).
2. Length of homology is important in gene targeting, in terms of absolute frequencies (targeting events on a per cell basis) and enrichment factor (targeted to non-targeted events), with exponential increases seen up to a total of approx 14 kb ***(10,11)***. Although it may be convenient to include short and long arms of homology to facilitate the screening process (*see* **Subheading 3.6.**), regions of approx 1 kb may result in reduced fidelity of recombination at the left (5′) and/or right (3′) junctions ***(12)***. In principle we would recommend targeting vectors with 1–3 kb and 3–10 kb for the short and long arms of homology; the total homology should not be less than 5 kb.
3. Different vector designs can be considered (**Fig. 1**) and these depend very much on the target gene and whether it is expressed in fetal fibroblasts. Given the range of possible vectors only general guidelines are given here (*see* **Note 6**). The promoterless strategy is likely to give the highest (approx 100-fold ***[13]***) and most reliable enrichment factor. Indeed, of 23 disruptions made in human somatic cells, all involved a promoterless strategy (*see* **Note 7** and **ref.** ***14***).

The power of this strategy arises because the selectable marker will only be active if integration occurs downstream and in-frame to transcriptionally active

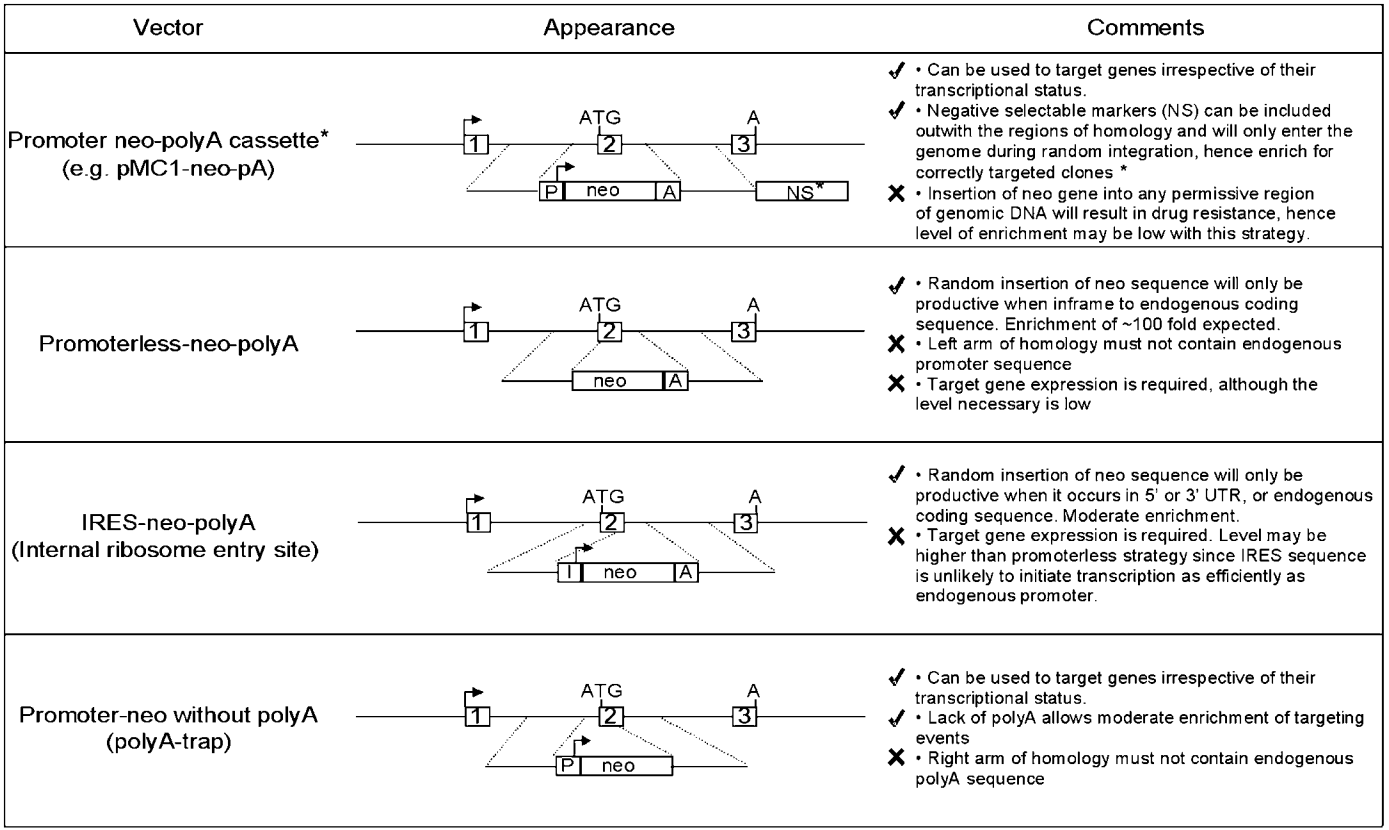

Fig. 1. Different vectors for gene targeting in somatic cells. The *neo* gene is used as an example and vector design applies equally to other resistance markers. *A negative selectable marker is shown only in the promoter-*neo*-polyA vector, but can be induced in any vector design. (Taken from **ref.** ***22***.)

coding sequences, thus limiting the number of productive integration sites within the genome. Even low levels of gene expression can support efficient targeting with this approach ***(15,16)***. Given the efficacy of the promoterless strategy, inducing expression of transcriptionally inactive target genes in the donor cell should be considered. Alternatively, given the range of cell types which are competent for NT, it may be appropriate to choose the donor cell type to express the target gene, for instance, mammary epithelial cells for milk protein genes, muscle cells for genes such as actin or myosin, although the longevity of the chosen cells must be assessed (*see* **Subheading 3.3.**).

1. When gene expression cannot be induced, other strategies such as polyA-trap or negative selection will be necessary (**Fig. 1**). With the latter approach, genes such as the HSV*tk* (sensitivity to ganciclovir ***[17]***), *gpt* (sensitivity to 6-thioguanine ***[18]***), or *dta* (diptheria toxin α-chain, directly toxic ***[19]***) are placed outside the regions of homology in the targeting construct and should only enter the genome when random integration occurs. Some reports have shown up to 2000-fold enrichment ***(17)***, but others have achieved only approximately threefold ***(11,20)***. It should be borne in mind however that the use of additional drugs for selection might reduce the viability of the cells in the NT process.

3.5. Cell Transfection

Electroporation is the favored transfection procedure for gene targeting experiments, as stably transfected clones are often the result of single integration events (*see* **Note 8**). Optimal electroporation conditions must be defined (*see* **Note 9**). The following protocol has been effective for targeting in cultures of fetal fibroblasts derived from several breeds of sheep ***(21)***. It uses a high-throughput format employing 96-well plates because gene targeting in somatic cells occurs at relatively low efficiencies.

1. Resuscitate one vial of cells from liquid nitrogen by rapidly thawing at 37°C. Remove DMSO by transferring cells to a 15-mL tube, adding 10 mL of GMEM and centrifuging (200*g* per 5 min). Resuspend the pellet in GMEM and transfer to one T175 flask. Grow cells to 80–90% confluency.
2. Approximately 3 h before transfection, aspirate the medium and replace with fresh GMEM.
3. To prepare for electroporation, wash cells once in PBS (approx 15 mL) and release them with trypsin (approx 2 mL). Once a suspension of single cells is obtained, add 8 mL of GMEM. Collect the cells by centrifugation (200*g* for 5 min), and wash twice by resuspending in 10 mL of PBS and pelleting (200*g* for 5 min). Before the final centrifugation step, determine cell numbers using a haemocytometer.
4. Mix 10–20 µg of linearized targeting vector (*see* **Note 10**) with 5×10^6 cells in a final volume of 800 µL of PBS.

5. Electroporations are performed in cuvets (0.8 mL/0.4-cm path length, Flowgen). For some sheep primary fibroblast cultures 125 μF:350 V or 250 μF:400 V is optimal (*see* **Note 9**).
6. After electroporation, allow samples to recover at room temperature for 5 min. Plate cells receiving targeting constructs immediately into twenty 96-well plates at a density of approx 2.5×10^3 cell/well (*see* **Note 11**).
7. After 24 h, replace medium with GMEM supplemented with G418 at a concentration of 300–400 μg/mL, depending on the primary cell line used (*see* **Subheading 3.3.**).
8. Change selection medium every 3–4 d. Cell death should be evident at 3–7 d, with surviving colonies becoming apparent from day 9 onward. Ideally, there should be approx 10 colonies per plate (*see* **Note 12**).
9. Trypsinize surviving clones that reach more than 80% confluency with 30 μL of trypsin: add 80 μL of of GMEM/G418 medium. Transfer 50 μL of the cell suspension in duplicate to complementary wells of two fresh 96-well plates. Cryopreserve the cells of one of the resulting 96-well plates by adding 50 μL of GMEM supplemented with 20% DMSO (resulting in 10% final concentration for freezing) and cool slowly by wrapping in bubble wrap and placing at –80°C. To the second plate, add 150 μL of GMEM/G418 medium and expand the cells for DNA analysis.

3.6. DNA Preparation

1. Continue to culture cells for DNA analysis by replacing the medium with fresh GMEM/G418 medium every 2–3 d until confluency is reached (approx 2–7 d).
2. Wash in PBS and lyse overnight in 50 μL of TNE.
3. Transfer samples to the respective location of a non-tissue culture V-bottomed 96-well plate (Greiner). The DNA in this plate will correspond with the location of viable cryopreserved cells in **Subheading 3.5.**
4. Precipitate DNA with 50 μL of isopropanol and incubate on a shaking platform for 20 min at room temperature.
5. Pellet DNA in the 96-well plates by centrifugation (3000*g* for 20 min).
6. Discard supernatant by inverting plates on paper towels. The DNA pellets should be visible at the base of the wells.
7. To wash the DNA, add 50 μL of 70% ethanol to each well and pellet DNA by centrifugation (3000*g* for 5 min).
8. Discard supernatant by inverting plates on paper towels. Air dry for 5 min. Resuspend in 50 μL of TE overnight at 4°C.
9. DNA preparations should be stored at 4°C. Alternatively, they can be stored at –20°C but freeze thaw cycles should be avoided.

3.7. Screening Strategy

1. A double polymerase chain reaction (PCR) screening strategy overcomes the problem of limiting amounts of DNA that can be isolated from targeted populations of

cells with a limited proliferative capacity. Additionally it allows a high-throughput format, which permits a relatively large number of samples to be handled (*see* **Note 13**). The precise nature of the primers depends on the specific gene being targeted. However, with PCR it is clearly important to do this analysis as rigorously and robustly as possible and, therefore, a doubling screening strategy is strongly recommended. An example of a successful strategy for the targeting of the *PrP* gene in sheep fetal fibroblasts is given in **Fig. 2** ***(21)***.

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3.8. Preparation of Targeted Cell Populations

After DNA analysis, selected targeted clones are located and resuscitated from the frozen 96-well master plate.

1. Take the frozen plate containing the targeted cells from the –80°C freezer. Use a P1000 tip to pipet a small volume of pre-warmed GMEM/G418 medium into a well to rapidly thaw the targeted cells. As the pellet thaws, transfer the cells to a well of a 24-well plate. Repeat with more GMEM/G418 medium until all the cells are transferred.
2. Once the cells have attached (3–4 h), aspirate medium and apply fresh GMEM/G418 medium to reduce any effects of DMSO.
3. If the cultures do not senesce (*see* **Note 14**), they should reach confluency in 4 to 7 d. At this stage passage them to a 6-well plate for further expansion and to prepare separate cell aliquots for storage and NT.
4. Reconfirm karyotpe and status of the cells at this stage (*see* **Subheading 3.3.**). Cell aliquots (10^4–10^5 cells, depending on yield) are stored in GMEM/10% DMSO, slow cooled in bubble wrap overnight (to –80°C) and transferred to liquid nitrogen until required.

3.9. Preparation of Targeted Cells for NT

1. Resuscitate one vial of cells from liquid nitrogen by rapidly thawing at 37°C. Remove DMSO by transferring cells to a 15-mL tube, adding 10 mL of GMEM medium, and centrifuging (200*g* for 5 min). Resuspend the cell pellet in 2.5 mL of GMEM.
2. Aliquot 0.8 mL of this suspension into each of three wells of a 24-well plate (three wells total per clone). Grow cells GMEM for 2–4 d.

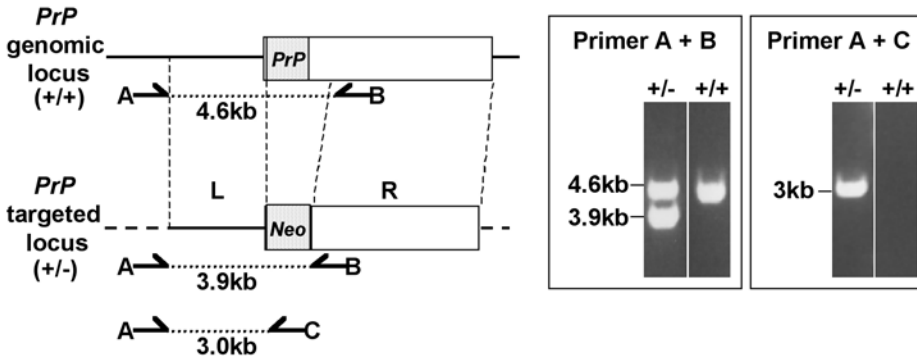

Fig. 2. The double polymerase chain reaction (PCR) screening strategy used for detecting targeting of one allele of the *PrP* gene of sheep fetal fibroblasts. Schematics of wild type (+/+) and targeted (+/-) genomic loci are shown. "L" and "R" indicate the regions that represent left and right homology in the targeting vector. Predicted PCR product sizes and their separation by agarose gel electrophoresis is illustrated. Primers A and B specifically amplify from outside the short arm of homology (in this case, the left arm) to the selectable marker (i.e., the *neo* gene). Amplified fragments of the predicted size (3 kb) are only produced when the selectable marker has correctly recombined by homologous recombination with the target locus. Primers A and C specifically amplify across short arm of homology and the selectable marker into the long arm of homology. These primers will detect not only the targeted locus (3.9 kb) but will also detect the non-targeted allele (4.6 kb). This provides a useful internal control for the PCR reactions, particularly when only relatively low amounts of DNA are recovered. It also enables a check as to whether or not the particular cell clone is likely to be pure (*see* **Note 13**). (Adapted from **ref. *21***.)

3. Five days before before the desired day of NT, rinse the cells of one well three times with GMEM containing 0.5% FCS (rather than 10%) and then incubate with 0.8 mL of this medium. Replace medium with fresh GMEM/0.5% FCS medium every 2–3 d until NT. With three wells of cells, this strategy allows 3 d of NT to be performed from one vial of targeted cells.
4. Prepare samples for NT by rinsing twice with 0.8 mL of Ca^{2+}/Mg^{2+}-free PBS.
5. Add trypsin (150 µL) and leave the cells at room temp until they float off with gentle tapping.
6. Add fresh GMEM/0.5% FCS medium (700 µL) and gently pipet five or six times across the well.
7. Transfer all the cells in to a 15-mL Falcon tube containing 10 mL of GMEM/0.5% FCS medium and collect by centrifuging (200*g* for 5 min). This gives a pellet that is often barely visible owing to the limited numbers of cells.
8. Aspirate the supernatant and resuspend the pellet gently in approx 100 µL of GMEM/0.5% FCS medium.

3.10. Nuclear Transfer

Somatic cell NT techniques are based on the method of Wilmut *(7)*.

1. Collect oocytes from superovulated Poll Dorset ewes in PBS with 1% fetal calf serum. Immediately transfer to calcium-free HEPES-buffered SOF for the removal of cumulus and enucleation.
2. Remove cumulus cells by pipetting in 600 IU/mL hyluronidase. Expose oocytes to 5 µg/mL Hoechst 33248 and 7.5 µg/mL cytochalasin B.
3. Donor serum-starved fibroblasts (from **Subheading 3.9.**) and recipient oocytes are simultaneously fused and activated by three consecutive 80-µs pulses of 1.25 kV/cm^2 in 0.3 *M* mannitol, 0.1 *M* $MgCl_2$, 0.05 m*M* $CaCl_2$.
4. Incubate reconstructed embryos for 6 d (in vitro culture) or overnight (in vivo culture) in SOF solution supplemented with bovine serum albumin in an atmosphere consisted of 5% O_2, 5% CO_2, and 90% N_2 at 38°C.
5. For in vivo culture, following the overnight culture, embed embryos in 1% agar chips in PBS and transfer into the ligated oviduct of an estrus synchronized recipient ewe for a further 6 d.
6. Recover morula and blastocyst stage embryos 7 d after activation to the uteri of estrus synchronized ewes (one to two embryos per recipient). Monitor pregnancies using transcutaneous ultrasound scanning.

4. Notes

1. If possible it is advisable to grow the cells in 2–5% O_2 to reduce oxidative damage. Similarly care should be taken in minimising exposure of the cells to any ultraviolet sources.
2. Concentrations of 300–400 µg/mL G418 and 1 and 2 µg/mL puromycin are the mimimum levels normally required for effective cell killing in sheep fetal fibroblasts.
3. We have estimated that it requires a minimum of approx 45 doubling of a primary population to introduce a targeting construct, select and expand the cells for NT *(22)*. However, different primary fibroblasts cultures exhibit a range of life spans ranging from 30 to 120 PDs *(23)*, and it is not possible isolate and expand targeted cell lines for NT from shorter lived cultures. We would recommend that the cells are capable of at least 75 PDs before attempting to use them in gene targeting experiments.
4. The number of PDs is calculated according to the formula $\log_{10}$ (split ratio)/$\log_{10}$ (2). For example, if cells are seeded at 2.5×10^5 / 25-cm^2 flask and are cultured to subconfluence to give a yield of 3×10^6, the PDs would be Log10 ($3 \times 10^6/2.5 \times 10^5$) / $\log_{10}$ (2) = 3.58.
5. In somatic cells the necessity for isogenic DNA is less clear and surveys of targeting in human cultures do not support such a strong role for isogenicity *(14,24)* nor does this appear to be the case for targeting in sheep cells *(25)*.
6. Specific examples for the construction of and utilisation of targeting vectors for the *PrP* and *GGTA1* genes for sheep are given in **refs. 22** and **25**.

7. Using a promoterless strategy to target the disruption of both *PRP* and *GGTA1* genes in sheep fetal fibroblasts gave an average a targeting frequency of approx 1 targeted cell/10^6 transfected cells *(25)*.
8. Lipofection also can be successfully used for targeting primary fetal fibroblasts *(26)*.
9. Optimal electroporation condition are commonly defined by incubating approx 3×10^5 cells with a marker expression plasmid such as pCMV-Sport-βgal (Gibco) and exposing to a range of capacitances (3–960 μF) and voltages (50–900 V) delivered by a commercial electroporater (e.g., Bio-Rad Gene Pulser II). Cells are fixed 24 to 48 h after electroporation and stained with the chromagenic agent X-gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside; Sigma). Conditions that promote high transfection efficiency (scored by number of blue stained cells indicating expression of β-gal) and high viability are selected for use in targeting experiments.
10. The targeting vector should be linearised using a restriction enzyme that cleaves in the backbone of the plasmid, beyond targeting sequences. The linear DNA is then purified by extracting twice with phenol/phenol chloroform and precipitating with ethanol by standard procedures. After washing with 70% ethanol and air-drying, the pellet is resuspended at a concentration of 10 mg/100 mL in sterile PBS.
11. The final plating density is based on the proportion of G418 resistant cells that are generated and is a balance between the requirement to screen a large number of these and the problem of generating mixed populations derived from two or more G418 progenitors.
12. An alternative method is to individually pick and expand G418 colonies from Petri dishes. This increases the chances than clonal colonies will be selected and expanded but it is difficult to maintain the necessary high throughput because only 1–10% of G418 resistant clones are targeted *(22,25)*.
13. Estimation of the intensity of the amplified fragments from the wild type vs targeted alleles can give good estimation as to whether the cell population contains a mixture of targeted and non-targeted cells. Ideally the intensity of the two fragments should be about equal, although if there are large differences in sizes between the two amplified fragments, this may bias the results from PCR.
14. Although quite reasonable targeting efficiencies can be obtained in sheep fibroblasts, the majority of the targeted cells identified senesce before they could be usefully expanded for NT *(22)*. This further emphasises the importance of selecting primary lines with the longest possible proliferative life span. One alternative has been to express telomerase in sheep fibroblasts to extend the life span *(27)*, although it is not clear if such immortalized cells are still competent for NT *(28)*.

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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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14

Transgenesis Using Nuclear Transfer in Goats

Anthoula Lazaris, Rebecca Keyston, Costas N. Karatzas, and Carol L. Keefer

Summary

Nuclear transfer (NT) using transgenic donor cells is an efficient means for generation of transgenic founder goats, especially in regard to the number of animals required to produce a transgenic founder expressing the protein of interest. Vectors can be designed for organ-specific expression and secretion of recombinant proteins within the target tissue. Furthermore, donor cells can be selected for gender, genetically modified to introduce the transgene of interest and screened for incorporation of the transgene into the genome before use in NT. This chapter describes methods for production of transgenic donor cells, subsequent NT embryo production, and transfer into recipients.

Key Words: Nuclear transfer; transgenic; goats; biopharming.

1. Introduction

Large-scale production of recombinant ***(rc)*** proteins can be achieved in a variety of systems (bacterial, fungal, plant, mammalian cells, transgenic animals, etc.). Each host system has its advantages and disadvantages. In choosing a host, considerations must be given among others to the production levels achieved, bioactivity of produced protein, post-translational modifications, and cost of production. In particular, if the manufacturing of complex proteins is required in large quantities (>50–100 kg/yr), then transgenic farm animals can provide a cost-effective alternative method of production because ***rc***- proteins can be expressed in the milk of transgenic ruminants at high concentrations ***(1)***. Although a number of methods have been used to genetically modify livestock, DNA microinjection into the zygotic pronucleus and somatic cell nuclear transfer (NT ***[2]***) are the predominant methods. NT using transgenic donor cells is an efficient means for

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generation of transgenic founder goats, especially in regard to the number of animals required to produce a transgenic founder expressing the protein of interest *(3)*. Donor cells can be selected for gender, genetically modified to introduce a transgene of interest, and screened for incorporation of the transgene into the genome, including copy number and integration site(s), before use in NT.

A number of cell types have been used as donor cells for NT *(4)*. One criterion is that the cell line used can be propagated and maintained in culture for sufficient time to allow for transfection, selection, and characterization while remaining "euploid." In mice, embryonic stem (ES) cells are the most appropriate cell type for transgenic animal production because they are pluripotent and can be cultured indefinitely allowing for a number of in vitro manipulations (i.e., gene targeting, etc.); however, in domestic species ES cell lines have not been adequately identified *(5)*. Fetal fibroblasts are generally the cell of choice for generation of transgenic cell lines; however, other cells types, including granulosa cells and skin fibroblasts cells obtained from adult animals, have been used *(6–10)*.

2. Materials

2.1. Equipment

1. Automatic pipet aid (Sarstedt; Automatic-Sarpette, cat. no. 189100).
2. Gilson pipetman set (P20, P200, P1000).
3. Laminar flow cabinet.
4. Microscope (Olympus 1X50/70 or equivalent).
5. Inverted microscope with ultraviolet fluorescence (Olympus, Leica, or equivalent).
6. Micromanipulators (Narishige or equivalent).
7. BTX EM2001 Electrocell Manipulator or equivalent.
8. Warming stages.
9. Cell culture incubators, 38.5°C, 5% CO_2.
10. Small culture chamber or tri-gas incubator (6% CO_2, 6% O_2, 88% N_2).
11. Table top centrifuge.
12. Water bath.
13. Refrigerator (4°C).
14. Freezer –80°C.
15. Freezer –20°C.
16. Liquid nitrogen tank.

2.2. Consumables

1. T175 flasks.
2. 48 Multiwell plates.
3. 6 Multiwell plates.
4. 35-mm Dish (Falcon).
5. 100-mm Dish (Falcon).

6. 100-mm Optilux dish (Falcon).
7. 15-mL Centrifuge tubes (Starstedt).
8. 2-mL Cryovials (Starstedt).
9. Cloning cylinders (Fisher Scientific).
10. Vacuum grease, silicone.
11. Fusion chamber slide.
12. 1.5-mL Microcentrifuge tubes.
13. 1000-µL Pipet tips, blue.
14. 200-µL Pipet tips, yellow.
15. 5-, 10-, and 25-mL pipets.
16. 2-mL Aspirating pipets.
17. Disposable powder-free latex examination gloves.
18. Spray bottle.
19. Permanent marker.
20. Kimwipes.

2.3. Reagents

2.3.1. Expression Vectors

Selectable marker vectors: pEF/GFP (Invitrogen) and pSV2neo (Clontech).

2.3.2. Donor Cells

1. Dulbecco's modified Eagle's medium (DMEM) high glucose (GIBCO).
2. Fibroblast isolation medium: Comprises DMEM supplemented with 20% fetal bovine serum (FBS), Australian derived (GIBCO).
3. Cell culture medium: DMEM supplemented with 10% FBS.
4. Freezing medium: 90% FBS with 10% dimethyl sulfoxide.
5. Complete medium: Consists of DMEM, 10% FBS, and appropriate antibiotic (e.g., 200–600 µg/mL Geneticin [G418]).
6. Low-serum complete medium: DMEM, 0.5% FBS, and appropriate antibiotic.
7. 20 µg/mL Gentamycin (GIBCO).
8. Ethanol 70%.
9. Phosphate-buffered saline (PBS; GIBCO).
10. Trypsin–EDTA, 0.05%
11. Trypan blue (GIBCO).
12. Lipofectamine (Canadian Life Science, Burlington, ON).

2.3.3. Oocytes/Embryos

Unless otherwise indicated all chemicals used in embryo culture were obtained from Sigma-Aldrich (St. Louis, MO).

1. EmCare (Immuno-Chemical Products, New Zealand).
2. Handling medium: Consists of EmCare supplemented with 1% FBS, Australian derived (GIBCO).

3. Tissue culture medium (TCM)-199H (GIBCO).
4. Maturation medium: TCM-199H supplemented with 0.02 U/mL bovine luteinizing hormone and 0.002 U/mL bovine follicle-stimulating hormone (Sioux Biochemicals, Sioux Center, IA), 1 μg/mL estradiol β-17, 0.2 m*M* sodium pyruvate, 50 μg/mL kanamycin, 100 μ*M* cysteamine, and 10% heat-inactivated estrus goat serum (produced at Nexia).
5. Light-weight mineral oil (Sigma M8410).
6. Hyaluronidase solution: 1 mg/mL in EmCare.
7. Bisbenzimide (H33342) stain: 5 μg/mL in handling medium.
8. Fusion medium: 0.25 *M* sorbitol, 100 μ*M* calcium acetate, 0.5 m*M* magnesium acetate, 0.1% bovine serum albumin (BSA; Sigma A6003).
9. Postfusion medium: comprises TCM-199H plus 10% fetal calf serum supplemented with 7.5 μg/mL cytochalasin B.
10. Activation medium A: EmCare containing 5 μ*M* calcium ionomycin (Sigma).
11. Activation medium B: G1.2 medium containing 10 μg/mL cycloheximide and 7.5 μg/mL cytochalasin B.
12. G1.2 medium (Vitrolife, Inc., Englewood, CO).

3. Methods

For biopharming and production of ***rc***- proteins in target tissues, the appropriate DNA expression vectors must be used. In this case, vectors are designed for organ-specific expression and secretion of ***rc***- proteins within the target tissue, the mammary gland of the transgenic animal. The regulatory controlling elements are based on milk specific protein genes. These elements include the promoter (e.g., whey acidic protein, α-lactoglobulin, β-casein, etc.), in order to direct the expression specifically to the mammary gland, and a secretion signal sequence, to direct the secretion of the protein from the cell into the milk. A second expression vector, containing a reporter or selectable marker, is required for the selection of the transgenic stable cell lines to be used as donor cells in NT. Reporter (e.g., green fluorescent protein) or selectable markers based on antibiotic resistance (e.g., neomycin resistance) are used to select transgenic from nontransgenic cells ***(11,12)***.

The method described in this chapter involves the selection of stable cell lines using an antibiotic selectable marker. In this case, the regulatory elements required include a constitutive promoter to allow expression of the marker gene during the culture period. In order to increase the efficiencies of transfection, the mammary gland specific expression vector is constructed such that the selectable marker is within a single construct. This allows the transfection of one vector, as opposed to co-transfection of two separate vectors.

The choice of the gene of interest (cDNA vs genomic), including the promoter and genetic elements such as insulators (e.g., matrix attachment regions,

locus control regions), are of critical importance in maximizing production of expressing founders (*13*). Furthermore, codon usage should be optimized for the expression system, that is, if expressing a protein in the goat, then codon usage should be optimized for the goat. Also of primary concern are the basic questions of functionality: Can a recombinant protein of interest be produced with adequate levels (>1 g/L) in the desired target tissue while minimizing the ectopic expression to ensure that the new protein does not affect the well-being of the transgenic animal.

3.1. Donor Cells

3.1.1. Isolation of Fetal Fibroblasts

1. A day-27 to -30 goat fetus is recovered surgically and placed in a 50-mL tube containing sterile PBS supplemented with 20 µg/mL gentamycin.
2. The fetus is transferred to a 100-mm dish, under a laminar flow hood (*see* **Note 1**) where the amniotic sac, head, and internal organs are removed with sterile scalpels.
3. The fetus is dipped into a dish containing 70% ethanol and then immediately transferred to a 100-mm dish containing approx 10 mL of prewarmed fibroblast isolation medium.
4. The fetus is mechanically dissociated using a set of sterile scalpels. The tissue is further dissociated by pipetting up and down using a 10-mL syringe with an 18-G 1/2-inch needle. Pipetting should be done slowly to avoid generating bubbles because vigorous pipetting will increase the shear rate and reduce the viability of the recovered cells.
5. Media and tissue/cell suspension are transferred into four T-175 flasks. A minimum volume of 50–60 mL of fibroblast isolation medium is added.
6. The flasks are incubated at 38.5°C, 5% CO_2, with media changes every 48 h.
7. When cells reach subconfluency (60–80%), they are ready to be frozen into 1-mL aliquots containing 1×10^6 cells.
8. The cells are trypsinized by first rinsing the flasks with PBS and then by adding approx 5 mL of trypsin. The flask is incubated for approx 2–3 min until the cells round up and start detaching. Fibroblast isolation medium is added immediately. A small aliquot is removed, Trypan blue is added (1/10th of the volume) and cells are counted using a hemacytometer.
9. The remaining media containing the cells is centrifuged at 450*g* for 5 min and the cell pellet resuspended in freezing medium to a final concentration of 1×10^6 cells/mL.
10. Aliquots of 1.0 mL of cells per vial are frozen at –70°C.
11. As many vials as possible are frozen back to be used as a "mini" working master cell bank for future transfection experiments.
12. Before use for transfection, the cells should be analyzed for normal chromosome count by giemsa and sexed, either by cytogenetic techniques or polymerase chain reaction (PCR).

3.1.2. Generation of Stable Transgenic Cell Lines

1. A vial of fetal fibroblasts containing 1×10^6 cells is thawed into two T-175 flasks, containing 30–50 mL of cell culture medium.
2. The cells are incubated at 38.5°C, 5% CO_2 until subconfluency (60–80%) with media changes every 48 h.
3. The cells are trypsinized, as described in **step 8**, counted using a hemacytometer and plated at $3.5–5 \times 10^5$ cells per 100-mm dish. The number of cells to plate is determined rather empirically based on their growth rate. Optimally, the cells should be between 50 and 60% confluent 24 h after plating. To ensure an ample supply of colonies for analysis at least four to eight 100-mm dishes should be transfected per construct. A negative control for the transfection (with a DNA construct not containing the selectable marker) should be included to monitor the effectiveness of the antibiotic selection. The cells are returned to the incubator overnight.
4. The next day, the medium is removed, cells are rinsed once with DMEM or PBS, and 10 mL of DMEM is added. The dishes are returned to the incubator until the lipid–DNA mix is ready to be added. The rinsing and incubation of cells in serum-free media is to ensure no serum is present because its presence will reduce the efficiency of transfection using lipids. However, this step depends on the lipids being used, for example, LipofectAMINE can be used effectively even in the presence of serum.
5. DNA–lipid mix preparation using LipofectAMINE: The quantity and ratio of DNA to lipid should be optimized for each cell line using a reporter construct, for example, pEF/green fluorescent protein. The manufacturers' recommendations for optimization should be followed in order to determine the appropriate conditions that will result in the highest percentage of green fluorescent protein-positive cells after 24–48 h.
6. The appropriate amount of DNA, assessed by using the reporter construct, is added to a 15-mL polystyrene tube and the volume is adjusted to 100 µL using DMEM for each 100-mm dish to be transfected. The amount of DNA and volume are scaled up according to the number of dishes to be transfected. If co-transfecting two constructs (i.e., transgene and selectable marker) a ratio of 10:1 or 5:1 is used to maximize the chances that the stable clones selected have integrated the transgene along with the selectable marker.
7. The appropriate amount of lipid, as determined by the reporter construct, is added to a 15-mL polystyrene tube and volume adjusted up to 100 µL with DMEM for each 100-mm dish. The amount of lipid and volume are scaled up accordingly for the number of dishes to be transfected.
8. Combine the DNA and lipid mixtures and vortex for 30 s. Incubate the solution for 30 min at room temperature.
9. Adjust the volume to 5 mL using DMEM. The amount of DMEM is scaled up accordingly for the number of dishes to be transfected. The mixture is now ready to be added to the cells.

10. The media from the cells is aspirated and 5 mL of the DNA–lipid mix is added per 100-mm dish. Then the dishes are returned to the incubator overnight.
11. The next day, the DNA–lipid mix is removed by aspiration and 10 mL of fresh prewarmed cell culture medium is added. The cells are then further incubated overnight.
12. The following day the cells are trypsinized, by first aspirating the medium, rinsing the dishes with PBS and then adding approx 2–3 mL of trypsin. When the cells start to detach, approx 10 mL of prewarmed complete media (cell culture medium with the selective antibiotic) is added to each 100-mm dish. The concentration of the selective antibiotic should be predetermined for each cell line by performing a kill curve. Typically a range of 200–600 µg of G418 per milliliter can be used for primary fetal fibroblasts. The cells are counted using a hemacytometer and approx 1×10^5 cells are replated per 100-mm dish. Prewarmed complete medium is added such that each dish contains 10 mL of medium with antibiotic. The cells are then returned to the incubator.
13. Every 2 d, the medium is removed and the cells fed with a prewarmed fresh complete medium until the colonies start to appear. It is important to maintain the selection media on the cells from this point forward.
14. After approx 10–20 d, colonies are large enough to pick. There should be at least 100 or more cells in a colony to ensure good survival and expansion. Also, colonies should be well separated to prevent cross-contamination between transfectants. The negative control plates (cells not transfected with the selectable marker) should contain no surviving cells.
15. Proceed to pick the clones using cloning cylinders (**Fig. 1**: ×50 magnification of a transfected, fetal fibroblast cell line): Before starting ensure you have a good supply of sterile cloning cylinders (approx 400; *see* **Note 2**).
16. Under the microscope, locate colonies and mark them with a permanent marker by positioning the tip of the marker under the dish. Be sure to scan the entire dish. A successful transfection should yield a minimum of approx 10–20 colonies per 100-mm dish. It is critical that the colonies are well spaced apart from each other, such that no cross-contamination occurs between them.
17. The procedure is repeated for all dishes containing colonies. Return the dishes to the incubator.
18. Place one dish in the biological hood, remove the media, and rinse once with PBS. Trace amounts of PBS should be left in the dish to prevent the cells from drying out.
19. Working with one dish at a time, place sterile cloning cylinders over all the colonies using sterile forceps. Add prewarmed trypsin to each well (approx 50 µL), return dish to incubator, and incubate for approx 2–3 min.
20. Once the cells have rounded up (check the progress of the trypsinization by viewing dish under the microscope), use a sterile tip and transfer the contents of individual cloning cylinders into a separate well of a 48-well dish. Gently rinse the same cylinder by pipetting up and down with an additional 50–100 µL of complete medium to ensure complete recovery of cells and transfer this medium to the same 48-well dish.

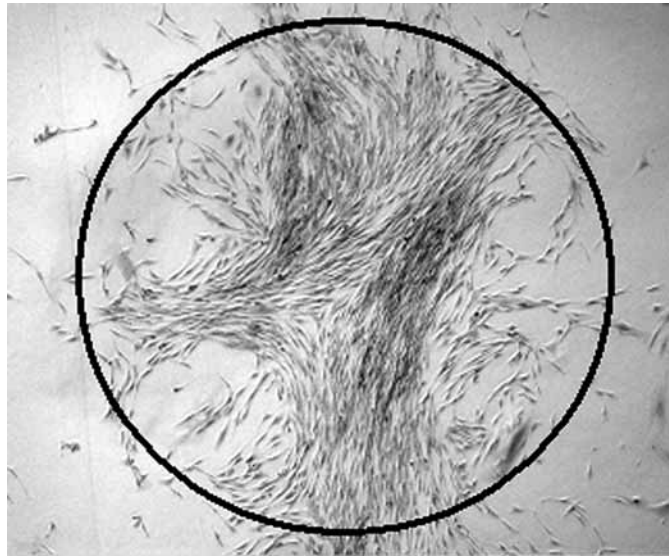

Fig. 1. Transfected fetal fibroblast cell line (×50 magnification). Circle represents a cloning cylinder placed around a colony to be picked.

21. Continue to process all the other colonies as in **step 19**.
22. Add a final volume of approx 1 mL to each well and incubate the 48-well dish at 38.5°C, 5% CO_2.
23. Once colonies from one dish have been picked, process the additional 100-mm dishes. Process one dish at a time to prevent the dishes from drying out during the cloning procedure (*see* **Note 3**).
24. Feed cells with fresh complete medium every 2 d.
25. Once the cells reach 60–70% confluency, they are ready for further expansion. Aspirate the medium, wash once with approx 1 mL trypsin and add 0.2 mL of trypsin. Incubate for approx 3 min and transfer the cell suspension into one well of a six-well dish. Rinse well with 1 mL of complete medium and transfer to the same six-well dish. Adjust the volume to approx 5 mL with DMEM containing 20% FBS and the selective antibiotic and incubate at 38.5°C, in 5% CO_2.
26. Once the cells reach 60–70% confluency, the cells are once again expanded (*see* **Note 4**). Trypsinize the wells as described in **step 16**, with 1 mL of trypsin and split the cell suspension into two wells of a six-well dish and return to the incubator.
27. Once again, when the wells have reached 60–70% confluency, trypsinize as described in **step 18** and transfer the cell suspension into a 15-mL tube.
28. Remove an aliquot of the cells and count using a hemacytometer.
29. Based on the number of cells available the following should be performed (*see* **Note 5**, **Fig. 2A**,**B**: ×250 magnification of two transfected cell lines of fetal fibroblasts):

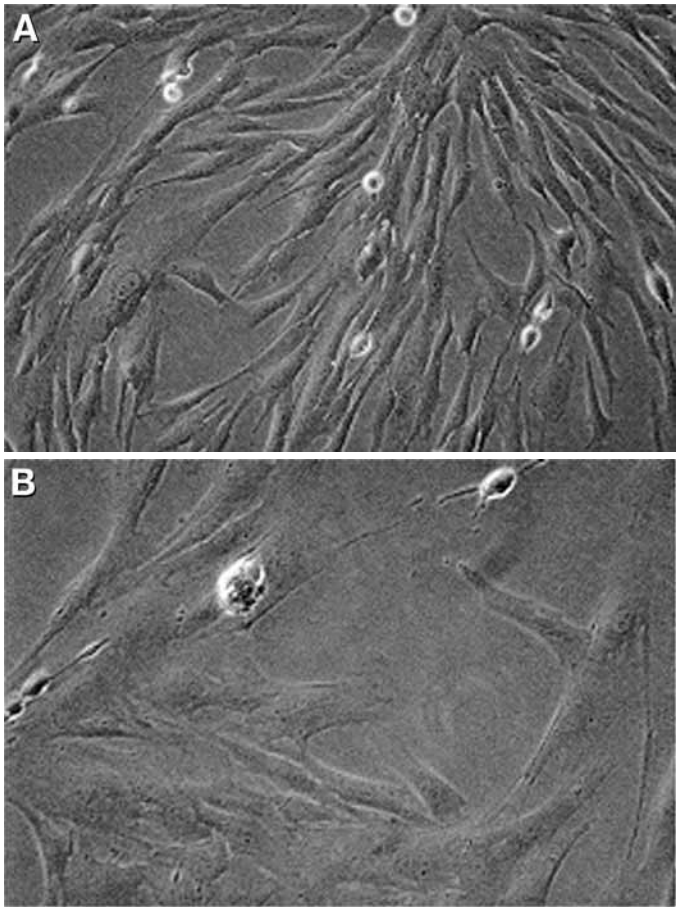

Fig. 2. Transfected fetal fibroblast cell lines. **(A)** Healthy, transfected cell line (magnification ×250); **(B)** clone with enlarged cells that did not survive expansion (magnification ×250).

a. Freezing aliquots of cells to be used as donor cells (Cell bank of transfected clones; *see* **Note 6**): a minimum of two vials with a minimum volume of 200 µL per vial must be frozen at a concentration of 5×10^5 cells/mL. Depending on the morphology of the cells (i.e., tightly compacted or loosely growing) there may or may not be enough cells. If there are not enough cells, then the cells must be further expanded.
b. Cell sample for characterization by PCR analysis: An aliquot of 1×10^4 cells is also set aside and centrifuged in a microcentrifuge tube, the supernatant removed and the cell pellet frozen at –20°C. Using standard molecular biology techniques (*see* **Note 7**) the DNA is extracted and analyzed by PCR

for the presence of the transgene. Only PCR-positive clones are analyzed further.

c. Further expansion: Any remaining cells from **step a** are replated in two six-well dishes or, if 2×10^5 cells are available, they are cultured in a 100-mm dish, grown to confluency, trypsinized, and collected as a pellet by centrifugation. The cell pellet is stored at –20°C for subsequent genomic DNA extraction and tested by Southern blotting analysis to assess the integrity and copy number of the transgene in individual clones.

3.1.3. Identification/Characterization of Stable Cell Line

After the selection of stable lines all isolated clones are screened as follows.

1. PCR: initial screening of positive lines as described in **step 29b** in **Subheading 3.1.2.**
2. Southern analysis is performed on PCR-positive cells to assess integrity of transgene and copy number. Usually anywhere from 20 to 60 clones are tested. After Southern analyses, cell lines with the intact transgene are selected. Because copy number (gene dosage) usually correlates with expression, a range of lines with intact copy number covering a range of 2–3 to 20 copies are selected.
3. Cytogenetic analysis: Fluorescent *in situ* hybridization (as described in **ref. *14***) to determine the number of integration sites and chromosomes in which the transgene has integrated should also be performed on the cell lines. Chromosome spreads should also be performed to ensure no gross chromosomal rearrangements exist.

3.1.4. Donor Cell Preparation

The donor cell should be synchronized to match the cell cycle of the recipient oocyte ***(15,16)***. In this example, donor cells are synchronized in G_0 by incubation in low serum conditions.

1. Frozen aliquots of cells are thawed approx 5 to 6 d before nuclear transfer.
2. Cells ($0.5–1 \times 10^5$) are plated into 24- or 96-well plates and cultured in cell culture medium until they reach 100% confluency.
3. The medium is replaced with low serum complete medium (20 µg/mL gentamycin) and the cells are incubated at 38°C, 5% CO_2 for 4 d until the day of the NT procedure. Alternatively, the cells may be maintained at confluency without serum starvation ***(16)***.
4. Just before cell transfer, the donor cells are collected by trypsinization using 0.05% trypsin–EDTA, they are washed twice, and resuspended in EmCare medium containing 1% BSA.

3.2. NT Procedure

3.2.1. Oocyte Collection and Maturation

1. Reproductive management is a key component in the collection of viable oocytes and successful transfer of reconstructed embryos. High standards of animal

husbandry practices must be maintained to ensure reproductive success and to satisfy regulatory guidelines and animal welfare recommendations (*see* **ref. 3**).
2. Immature oocytes in our laboratory are obtained by laparoscopic aspiration of superovulated does as previously described (*see* **ref. 3**). Oocytes are aspirated from follicles greater than 2 mm in diameter.
3. Follicular aspirants are processed immediately upon recovery from each individual animal. The oocytes and oocyte–cumulus complexes (OCCs) are removed from the aspirant and washed in TCM 199H supplemented with 10% heat-inactivated estrus goat serum.
4. Those OCCs that contained at least one layer of compact cumulus cells are placed into maturation medium.
5. OCCs are cultured in groups of 5–15 in drops (50 µL volume) of maturation medium covered with an overlay of mineral oil and incubated at 38.5–39°C in 5% CO_2.

3.2.2. Enucleation

1. After 23–24 h of maturation, the cumulus cells are removed from the matured oocytes by vortexing the OCCs for 1–2 min in a 1.5-mL cryovial containing 0.25 mL of EmCare supplemented with 1 mg/mL of hyaluronidase.
2. The denuded oocytes are washed in handling medium and are returned to maturation medium.
3. Before enucleation, the oocytes are incubated in Hoechst 33342 (Sigma; 5 µg per mL in handling medium) under an oil overlay for 15–30 min at 30–36°C in air atmosphere.
4. A small group of oocytes (5–15) is placed into manipulation drops (consisting of handling medium) covered with an overlay of mineral oil.
5. The Hoechst-stained metaphase plate is visualized by a brief exposure (few seconds) to ultraviolet light to determine its location.
6. Using a sharpened beveled micropipette to pierce the zona pellucida, but not the ooplasmic membrane, the chromosomes are removed by aspiration with a minimal volume of surrounding cytoplasm.
7. The aspirated cytoplasm should be checked for the presence of the chromosomes and polar body by examining the pipet containing the aspirated cytoplasm by ultraviolet epifluorescence.

3.2.3. Cell Transfer

1. The enucleated oocytes and dispersed donor cells are manipulated in handling medium. A group of enucleated oocytes (10–15) are placed into a manipulation drop.
2. Dispersed donor cells are placed either at the edge of the manipulation drop or in a small adjacent drop. Small (<20 µm) donor cells are picked up with a manipulation pipet and inserted into the slit made in the zona during enucleation. One donor cell is placed in the perivitelline space of the enucleated oocyte to form a couplet.
3. Upon completion of reconstructions, the group of couplets is transferred to a dish of handling medium and placed onto a warming plate until fusion. A second

person should perform the fusion procedure so that the manipulator can continue transferring cells into the remaining enucleated oocytes.

3.2.4. Fusion

1. Couplets are washed through a small dish containing EmCare supplemented with 1 mg/mL BSA and then placed into a dish containing a 1:1 mixture of EmCare: sorbitol fusion medium. The couplets are allowed to settle to the bottom of the dish.
2. The couplets are then moved into a small dish containing fusion medium and allowed to settle to the bottom of the dish.
3. A few of the couplets (two to four) are placed between the electrodes of the fusion chamber (500-µm gap fusion chamber, BTX). The couplets are manually aligned using either a closed, finely pulled pipet or a pulled open-tip pipet that is controlled by suction (mouth piece). The couplet is aligned such that the axis that runs through cell and oocyte is perpendicular to the electrodes.
4. The appropriate fusion pulse is applied. In this example, a brief fusion pulse (15 µs) at 2.39 kV/cm was administered using a BTX Electrocell Manipulator 2001.
5. After exposure to the fusion pulse the couplets are washed through a dish containing a 1:1 mixture of EmCare and sorbitol fusion medium followed by a dish of EmCare supplemented with 1 mg/mL BSA.
6. Couplets are then placed into 25-µL drops of post fusion culture medium overlaid with mineral oil.
7. After 1 h, couplets are observed for fusion. Couplets that have not fused are subjected to a second round of fusion as described in **Subheading 3.2.4.**, **step 1**.

3.2.5. Activation

1. Two to three hours after applying the first fusion pulse, the fused couplets are activated using calcium ionomycin and cycloheximide ***(17)***.
2. Briefly, couplets are incubated for 5 min in 2 mL of activation medium A.
3. They are then incubated for 5 min in 2 mL of EmCare containing 30 mg BSA per milliliter.
4. The stimulated embryos are washed through EmCare and then are cultured for 5 h in activation medium B.
5. The embryos are then washed in handling medium and placed in G1.2 medium culture drops under an oil overlay. Reconstructed embryos are incubated overnight at 38.5–39°C in 6% CO_2, 6% O_2, and 88%N_2.

3.2.6. Selection and Transport of Embryos for Transfer Into Synchronized Recipients

1. Reconstructed embryos are observed using a dissection microscope to confirm that they are intact (not lysed or fragmented). Both one-cell and cleaved (two-cell) embryos are transferred on day 1 (day 0 = day of fusion) into synchronized recipients ***(18)***.
2. Small cryovials (1.5 mL) are used to hold sufficient number of embryos for transfer into one recipient (approx 10 embryos per vial). The vials are filled with 1 mL

of pre-warmed EmCare supplemented with either 1 mg of BSA per milliliter or 1% serum.

3. The vials are then transported to the farm surgical facilities for embryo transfer into synchronized recipients.

3.3. Verification of Transgenesis of Offspring

The analyses used in the screening of NT-derived goats are similar to those performed on the cell lines. PCR, Southern blotting, and fluorescence *in situ* hybridization analysis are performed on DNA extracted from blood and/or skin tissue samples using standard molecular biology techniques ***(19)***.

4. Notes

1. All additional steps are preformed in a laminar flow hood.
2. Cloning cylinders should be cleaned with PBS and sterilized either with 70% ethanol and allowed to dry or autoclaved and kept in a sterile container prior to use.
3. To ensure the successful generation of transgenic cell lines anywhere from 200 to 400 colonies should be picked at this stage
4. It is important that the cells never reach confluence. If they reach confluence, the cells may change in morphology and the selective pressure will not work effectively, because it relies on exponentially growing cells.
5. Any wells that have not expanded should be thrown out. An average of 20–50% of clones will survive to this stage, which is why it is important to pick a large number of clones at the beginning.
6. It is important, at this point, to obtain a frozen stock of each clone in order to retain a young population of cells with a minimal number of passages.
7. Genomic DNA can be isolated from cell pellets using the DNeasy Tissue Kit (Qiagen, cat. no. 69506) and the DNA stored at 4°C until ready to use.

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The authors provide readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer. Of immense benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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Nuclear Reprogramming

An Overview

Takashi Tada

Summary

Nuclear reprogramming is an intriguing phenomenon, in which specialized somatic cells reacquire pluripotency through the global resetting of epigenetic modifications without changes occurring to their nuclear DNA information. The nuclear reprogramming activity retained by unfertilized eggs and embryonic stem cells is readily observable through the techniques of nuclear transplantation and cell fusion, respectively. Furthermore, researches involving somatic stem cells have provided evidence that spontaneous cell fusion functions, at least in part, in maintaining the homeostasis of various tissues through the generation of replacement cells in vivo. Novel approaches by application of the nuclear reprogramming activity may open new avenues producing genetically matched personalized pluripotential stem cells derived from patient somatic cells.

Key Words: Reprogramming; nuclear transfer; cell fusion; epigenetic modification; pluripotency; stem cell.

1. Introduction

In 1944, it was shown that DNA carries genetic information in its long chain composed of only four types of nucleotides containing the bases adenine (A), cytosine (C), guanine (G), and thymine (T *[1]*). Subsequently, it was found that the bases of the DNA molecule are located on the inside of a double helix built by complementary A–T and G–C pairing of two DNA strands running in opposite directions *(2)*. DNA is the substance of genes, which are precisely inherited by each daughter cell through numerous cell divisions. Considering these facts, biologists thought that fundamental issues of development and genetics would be resolved by determining the DNA sequences of genes. However, now we know that this idea is only partially true. The nucleotide sequence of a gene

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determines the amino acid sequences of a protein, but the temporal and spatial transcription pattern of each gene is regulated by epigenetic modifications of DNA, chromatin, or higher ordered chromatin structure.

One important fact is that the cells comprising a multicellular organism inherit basically the same nuclear DNA information from the fertilized egg. This was clearly proven by nuclear transplantation experiments with intestinal endoderm cells, skin cells, and blood cells derived from adult frogs *(3,4)*. Transfer of a somatic cell-derived nucleus into an egg pretreated with ultraviolet irradiation led to the successful production of normal swimming tadpoles. Therefore, cell diversification is induced not by changes of DNA sequence information but by on/off switching of gene expression during development and differentiation. Remarkably, unfertilized frogs eggs possess the potential to erase an adult somatic cell-specific gene expression pattern and to re-establish a gene expression pattern required for restarting ontogeny. We call this phenomenon "nuclear reprogramming." The nuclear reprogramming may be caused by resetting of epigenetic modifications of somatic nuclei. However, the mechanisms of the nuclear reprogramming remain largely unknown.

2. Induction of Nuclear Reprogramming

In 1997, the successful production of the cloned sheep, "Dolly," demonstrated that committed somatic cell nuclei can reacquire totipotency as a result of nuclear transplantation into enucleated unfertilized oocytes and result in subsequent embryonic development in mammals (**Fig. 1** and **ref.** *15*) Indeed, nuclear reprogramming is not an uncommon phenomenon. The nuclear reprogramming capacity of the oocyte has been shown by the production of cloned animals in various species such as the cow, mouse, pig, rabbit, monkey, and cat *(6–11)*. One of the characteristic features of cloned animals is a considerably low survival rate to adulthood (2–3% *[12]*). Most cloned embryos have an abnormally large placenta and die during various developmental stages of embryogenesis or during early postnatal stages *(13)*. Nuclear reprogramming occurs as a result of resetting of the somatic cell-specific epigenotype to the totipotential cell-specific epigenotype by exposure to factors present in the oocyte cytoplasm. It seems that the mortality of cloned embryos may be caused by imbalanced expression of genes as a result of insufficient reprogramming of somatic nuclei *(14)*.

Genomic plasticity was studied by cell fusion experiments between different types of cells nearly 20 yr ago *(15–17)*. A remarkable finding of such studies was that self-renewing and pluripotential embryonic stem (ES) cells, which are derived from the inner cell mass cells of blastocysts, have an intrinsic capacity for nuclear reprogramming of somatic cells after cell hybridization (**Fig. 1** *[18–20]*). In ES hybrid cells, acquisition of pluripotential competence by the somatic genome may be demonstrated by reactivation of the somatic cell-derived pluripotential

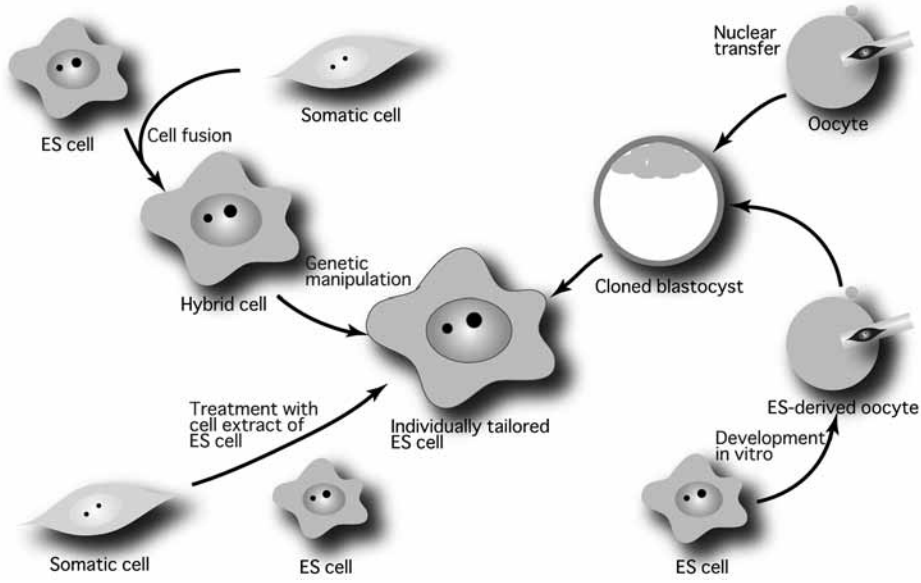

Fig. 1. Production of individually tailored ES cells via nuclear reprogramming.

cell-specific marker gene, *Oct4GFP*, by reactivation of a female somatic cell-derived inactivated X chromosome, and by proper tissue-specific gene transcription from the reprogrammed somatic genome in various types of tissues redifferentiated in vivo and in vitro. Interestingly, similar nuclear reprogramming activity is found in embryonic germ (EG) cells, which also are self-renewing and pluripotential cells derived from primordial germ cells (PGCs) of embryos ***(21,22)***. In contrast to ES cells, in the EG cells (gEG cells), which originate from PGCs soon after entry into the gonads, the memory of parental imprints is erased ***(23)***. In addition to the nuclear reprogramming for acquisition of pluripotency, the memory of parental imprints maintained on the somatic genome is erased by cell hybridization with gEG cells ***(24)***. Thus, the nuclear reprogramming activity is common to ES cells and gEG cells, whereas the activity for erasure of the parental imprints is unique to gEG cells. Key factor(s) involved in the erasure of parental imprints may be present in the gEG cells, but their nature is unknown.

An intriguing transdifferentiation phenomenon is induced by nuclear reprogramming using an in vitro cell-free system with nuclear and cytoplasmic extracts derived from different somatic cell types (*see* Chapter 18). Nuclear reprogramming of 293T and primary skin fibroblasts is shown by chromatin remodeling with binding of transcriptional factors and historic acetylation in the promoter region of the interleukin-2 gene, and also by reactivation of T-cell-specific surface molecules after treatment of the fibroblasts with an

extract from primary human T-cells or from a transformed T-cell line *(25)*. After exposure to a neuronal precursor extract, 293T fibroblasts start synthesizing a neurofilament protein and are transformed to cells characterized by the outgrowth of two neurites *(25)*. Thus, the cell-free system is a possible approach toward directly generating one type of somatic cell from another via nuclear reprogramming. Of interest would be the attempt to produce pluripotent stem cells from differentiated somatic cells using such a cell-free system with an extract from ES cells. In hybrids between ES cells and somatic cells, the somatic genome is reprogrammed and acquires pluripotential competence, indicating that ES cells possess an intrinsic capacity for nuclear reprogramming of somatic cells *(18)*. Therefore, exposure to, or stimulation by, the intrinsic factors present in an ES cell extract may allow the acquisition of pluripotential stem cell properties by differentiated somatic cells (**Fig. 1**). If this could be realized, various types of somatic cells could be made by re-differentiation of the somatic cells reprogrammed with the cell-free system. However, this has not yet been successful.

3. Nuclear Reprogramming by Spontaneous Cell Fusion

Fertilized eggs possess totipotential competence, as shown by their growth and differentiation into various types of cells, which form complex, functionally distinctive, and multicellular tissues during whole-body formation. Upon differentiation, the pluripotential competence is lost in the majority of cells in the tissues, but a subpopulation of somatic stem cells exists as multipotential cells, which maintain homeostasis in many tissues and organs by regeneration of replacement cells. In mammals, self-regenerative activity has, until recently, received less notice. However, advances in stem cell research have revealed that some types of tissue stem cells can be isolated and expanded under appropriate in vitro culture conditions. One of the well-studied somatic stem cell is the neural stem cell isolated from the ventricular system of the fore- and midbrain, hippocampus, and spinal cord *(26,27)*. Surprisingly, it was shown that adult neural stem cells retain the potential to transdifferentiate into all germ layers, including various types of somatic tissue, upon introduction into the amniotic cavity of early chick embryos and the blastocel cavity of mouse blastocysts *(28)*. This finding implies that adult stem cells have properties that are more closely related to those of ES cells than previously imagined. It is, however, reported that neural stem cells and bone marrow cells may undergo nuclear reprogramming by spontaneous cell fusion, when co-cultured with ES cells in vitro *(29,30)*. Thus, the acquisition of pluripotency by the adult neural stem cells may be mediated by spontaneous cell fusion with the inner cell mass cells following microinjection into the blastocyst. It is still under investigation whether such pluripotential cells showing multilineage contributions in chimeras are cells resulting from

fusion between the pluripotential inner cell mass cell and multipotential neural stem cell, which would be tetraploid hybrid cells.

Recent in vivo transplantation experiments with bone marrow cells have provided evidence that regenerated hepatocytes are derived from donor hematopoietic cells that undergo cell fusion with host hepatocytes, and not from the transdifferentiation of hematopoietic stem cells or hepatic cells present in bone marrow *(31,32)*. Thus, the nuclear reprogramming of one type of stem cell to produce another type of somatic cell by in vivo cell fusion is thought to play an important role in maintaining the homeostasis of some tissues by the generation of replacement cells that contribute to recuperation.

4. Applications of Nuclear Reprogramming

The epigenetic status of the nuclei from highly differentiated somatic cells can be induced to revert to that of pluripotential stem cells through the action of extrinsic transacting factors. The physiological role of the nuclear reprogramming activity found in oocytes may not be the actual reprogramming of somatic nuclei but rather equalizing epigenetic modifications between the paternal and maternal genomes for inducing cooperative zygotic expression of the parental genes after fertilization. Recent technical developments in the transformation of somatic nuclei to pluripotential nuclei via nuclear reprogramming have the potential to provide enormous benefits in the fields of agriculture, veterinary, basic science, and clinical medicine by enabling the production of animal models for human diseases, bioreactors for production of human therapeutic proteins, organ donors for transplantation into humans, and livestock, which produce valuable products with high efficiency.

Recent fascinating developments in the area of regenerative medicine have been boosted by advances of stem cell science in addition to cloning from somatic cells in mammals. Mouse ES cells are characterized by pluripotential competence and a robust capacity for proliferation. In primates, ES cells have been successfully derived from some species of monkey *(33–35)* and also from humans *(36,37)*, making ES cells a strong candidate as a source of cells for application in regenerative medicine. However, tissues generated from an ES cell source would not be appropriately matched to the majority of recipients, resulting in a high rate of immunological transplant rejection. Therefore, for successful therapeutic transplantation of replacement cells, individually tailored (autologous) ES cells would be beneficial. One approach to obtaining such cells is production of ES cells derived from blastocysts cloned from somatic cells, termed somatic cell nuclear transfer or "therapeutic cloning" *(38,39)*. The genotype of these ES cells will be identical to that of the original somatic cell; therefore, the immune system of the somatic cell donor will be completely

tolerant to the ES cells derived from the cloned blastocyst *(40)*. However, human therapeutic cloning is limited by biomedical ethical issues *(41)*. Recently it was shown that mouse ES cells are capable of developing in vitro into oogonia, which undergo meiosis and develop parthenogenetically into blastocyst-like structures *(42)*. If human ES cells possess similar developmental capacity, oocytes developed in vitro from human ES cells could be used for producing cloned blastocysts and individually tailored ES cells could be derived from the cloned blastocysts with fewer ethical problems (**Fig. 1**).

Using the cell hybridization system, individually tailored ES-like cells could be made by targeted elimination of the ES genome after cell hybridization with an individual's own somatic cells (**Fig. 1**). Previously reported data suggest that the coexistence of the ES genome may be required for the initial process of reprogramming the somatic genome in ES hybrid cells *(18)*. Thus, to eliminate the ES genome, the ES cells may have to be genetically manipulated to induce chromosomal instability after cell hybridization with somatic cells (*see* Chapter 19). Cell hybridization technology may therefore have the potential to make an important contribution to personal therapeutic applications without the need for cloning.

Ultimately, manipulation of the epigenotype may become a possibility through the use of reprogramming factors, once they have been identified. We could then envisage the production of clonal or tissue-specific stem cells from adult somatic cells, without the need for contributions from mammalian embryos. Such technology would have important implications for the production of donor cells for numerous clinical applications involving cell or tissue transplantation. The regulatory mechanisms and key factors responsible for reprogramming of somatic nuclei are only now beginning to be elucidated. Greater understanding of these mechanisms will open new and exciting fields in the areas of both basic science and clinical medicine.

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Cell Reprogramming and Transgenesis

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
Alan O. Trounson

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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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16

Expression of Imprinted Genes in Cloned Mice

Takashi Kohda, Fumitoshi Ishino, and Atsuo Ogura

Summary

Genomic imprinting is a mammalian specific epigenetic modification of the genome. Assessment of the integrity of the imprinting memory in somatic cell cloned animals is important not only for understanding of the "reprogramming" process during cloning by nuclear transfer, but also for the applications of this technique for therapeutic cloning in the future. In this chapter, we summarize the analytical methods for assessment of monoallelic expression of imprinting genes and expression analysis. From a practical point of view, the authors suggest the use of intersubspecific F1 hybrids between the laboratory mouse (*Mus musculus musculus*) and the JF1 strain (*Mus musculus molossinus*). We also list the sequence for PCR primers to detect the polymorphism of imprinted genes between *musculus* and *molossinus*.

Key Words: Somatic cell cloning; genome imprinting; intersubspecific hybrids; JF1.

1. Introduction

Somatic cloning is a powerful tool for regenerative medicine, as well as an innovative reproduction method for domestic animals. It also provides an excellent tool for basic biology in mammals, especially in studies of epigenetic control of mammalian development, because it allows the comparison of developmental processes with or without fertilization of the parental gametes. It seems likely that some of the abnormalities observed in cloned animals are genetic in origin, while others are epigenetic in origin *(1–3)*. Therefore, to improve mammalian somatic cell cloning, it is important to analyze gene regulation systems from the genetic and epigenetic aspects, to determine whether cloned animals are identical to donor animals that are derived through normal fertilization. In this chapter, we focus on the allelic expression analysis of imprinted genes and gene expression profiling of cloned mice *(3)*. Genomic

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imprinting is a mammalian-specific epigenetic memory, which is stable during the so-called "reprogramming" process. Therefore, somatic clones show normal expression profiles of imprinted genes *(3)*. However, some other epigenetic memories in the somatic cell nucleus may be erased incompletely, and this may affect the development and growth of cloned animals *(2,3)*.

To assess the allelic expression status of imprinted genes, it is necessary to distinguish the parental origin of each allele. When somatic cell clones are produced from donor cells of interspecific or intersubspecific hybrids, it is possible to distinguish the parental origin of many of the imprinted genes. It should be noted that the choice of donor cell is very important. Because cloned animals are produced from single-cell nuclei donors, it is important to select homogenous and stable donor cells. If the donor cell population is highly heterogeneous and unstable, the cloned animals may show various and unexpected phenotypes that reflect the epigenetic status of the donor cells *(2)*.

The assessment of the data quality from polymerase chain reaction (PCR) experiments is another important issue. Although it is possible to detect small quantities of template using multiple PCR cycles, the amplification often is biased. We strongly recommend that the number of PCR cycles be restricted to 30 or fewer when further analysis of the amplified fragments is required, that is, when calculating the expression ratios from two parental allelic origins. In situations in which the template concentration is too low and the number of PCR cycles exceeds 35, amplified fragments from single alleles of random origin may be observed, even in the case of nonimprinted (biallelically expressed) genes. To generate reproducible results by quantitative PCR, it is important to use sufficient amounts of the template (generally, >100 copies of template per reaction tube).

2. Materials

2.1. Production of Cloned Mice

The technical details of mouse somatic cell nuclear transfer (NT), including the requisite equipment and reagents, have been described elsewhere (Chapters 3 and 5 and **ref.** *4*).

2.2. Allelic Expression Analysis of Imprinted Genes

2.2.1. Total RNA Purification and Reverse Transcription

1. Dounce homogenizer (7 mL).
2. Isogen (Nippon Gene).
3. DNase I (RNase-free; Nippon Gene).
4. Phenol.
5. 5 *M* NaCl.

6. Ethanol.
7. SuperScript III reverse transcription (RT) kit (Invitrogen).

2.2.2. RT-PCR and Allelic Expression Analysis

1. ExTaq (Takara).
2. DNA sequencer: 3100 Genetic Analyzer (Applied Biosystems).
3. ExoSAP-IT (Amersham Biosciences).
4. BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems).
5. Appropriate primers for DNA sequencing.
6. Thermal Cycler: GeneAmp PCR System 2400 (Applied Biosystems).
7. Appropriate restriction enzymes.

2.3. Gene Expression Analysis of Cloned Mice

2.3.1. Gene Expression Profiles of Cloned Mice

1. Atlas Nylon cDNA Expression Array (Clontech).
2. [α-^{32}P]dCTP.
3. Phospho Imager: BAS2000 (Fuji Film).

2.3.2. Quantitative PCR

1. ABI Prism 7700 Sequence Detector (Applied Biosystems).
2. SYBR Green PCR Master Mix (Applied Biosystems).
3. Appropriate PCR primers.

3. Methods

3.1. Production of Cloned Mice

The technical details of mouse somatic cell NT, including the equipment, reagents, and procedure, already have been published elsewhere (Chapters 3 and 5 and **ref.** *4*). Here, we describe some technical aspects that are specific for the study of imprinted gene expression in cloned mice. The donor nuclei are transferred into enucleated oocytes by microinjection or membrane fusion (usually electrofusion). Microinjection is suitable for small cells (e.g., cumulus cells and immature Sertoli cells), whereas membrane fusion is suitable for larger, rigid cells (e.g., fibroblast cells). Embryonic stem cells and primordial germ cells can be transferred by either NT method. The microinjection and electrofusion methods require Piezo-driven micromanipulators and an electric pulse generator, respectively. In each laboratory, NT methods are selected according to the available donor cells and equipment.

3.1.1. Preparation of Donor Cells

The active parental alleles of imprinted genes can be identified by polymorphisms among the parental alleles. The most common strategy is to use the genotypes of the F1 hybrids of a laboratory strain and wild-type strain. For the

wild-type strain, the authors suggest the use of the JF1 strain (*Mus musculus molossinus* ***[5]***) because of its high reproductive performance and easy routine maintenance ***(6)***. However, these F1 donors are not always as good as F1 hybrids derived from other laboratory strains, such as B6D2F1. The cloning efficiency, particularly the developmental rate after embryo transfer, is affected significantly by the combination of donor cell type and genotype ***(7)***. We found that offspring were obtained from cumulus cells, immature Sertoli cells, and adult fibroblast cells (tail-tip cells) only when the JF1 and 129 strain genotypes were combined ***(7)***. Mid-gestation fetuses (e.g., day 9.5) were also obtained from the combination of JF1 and C57BL/6 ***(3)***.

F1 hybrid donor animals can be prepared not only by natural mating, but also by in vitro fertilization-embryo transfer. Conventional in vitro fertilization procedures for mice can be applied to JF1 spermatozoa and oocytes. Embryos are transferred into the oviducts or uteri of pseudopregnant ICR female mice, which is similar to the protocol used in embryo transfer experiments.

Fresh donor cells should be used for NT experiments because the imprinting status of the mouse genome in somatic cells is readily modified in culture. The authors have demonstrated that nuclear transfer itself does not perturb the imprinting memory, and thus the imprinting statuses of clone embryos/animals faithfully reflect those of the donor cells ***(3)***.

3.2. Expression of Imprinted Genes in Clone Embryos and Placentas

3.2.1. RNA Purification and cDNA Synthesis

Total RNA may be purified from tissue samples using several different methods. One of the most common protocols is extraction with acid phenol. The Isogen procedure is based on RNA extraction with an acid guanidinium thiocyanate–phenol–chloroform mixture, as described by Chomczynski and Sacchi ***(8)***. This method provides a pure high-yield preparation of total RNA, and allows simultaneous isolation of genomic DNA. Total RNA preparations should be treated with RNase-free DNase I to eliminate the contaminating genomic DNA.

Details of the Isogen protocol include the following:

1. Place freshly prepared or frozen stocked tissue samples (various stages of embryonic fetuses, placentas, organs, etc.) into 1 mL of Isogen solution.
2. Homogenize the tissues in a Dounce homogenizer, using 50 strokes with a loose pestle.
3. Add 200 µL of chloroform, mix well, and stand at room temperature for 3 min.
4. Centrifuge at 12,000*g* at 4°C for 15 min.
5. Carefully remove the upper water-phase layer and transfer to a clean nuclease-free tube.
6. Add 500 µL of isopropanol, mix well, and stand at room temperature for 10 min.

7. Centrifuge at 12,000*g* at 4°C for 15 min.
8. Rinse the precipitate with 1 mL of 70% ethanol.
9. Dissolve precipitate in 45 µL of nuclease-free water.
10. Add 5 µL of 10 DNase I buffer.
11. Add 1 µL of RNase-free DNase I (1 U/µL).
12. Incubate at 37°C for 30 min.
13. Phenol extract and ethanol precipitate.
14. Dissolve the precipitate in a suitable volume of nuclease-free water.

First-strand cDNA is synthesized from 1 µg of total RNA using RNase H and MMTV-RTase (SuperScript III) with the oligo(dT) primer in a 20-µL reaction mixture.

3.2.2. RT-PCR

1. Set up the PCR reaction mixture in a volume of 100 µL using the appropriate amount of cDNA (usually synthesized from 1 to 10 ng of total RNA).
2. Increase the temperature of the reaction to 85°C and add 0.3 µL of ExTaq DNA polymerase (5 U/µL).
3. Amplify the cDNA fragment; typically, 30 cycles of 96°C for 15 s, 65°C for 30 s, and 72°C for 30 s, followed by incubation at 72°C for 1 min.

The PCR primers that may be used to detect polymorphisms among the laboratory mouse strains (*Mus musculus musculus* C57BL/6, 129sv/ter, etc.) and JF1 (*M. m. molossinus*) are listed in **Table 1** ***(9–18)***. In cases where single nucleotide polymorphisms can be detected by restriction enzyme fragment-length polymorphism (RFLP), the analysis of allelic expression using RFLP with appropriate restriction enzymes is strongly recommend.

Imprinted gene-specific PCR primers that can be used to amplify fragments that contain single nucleotide polymorphisms without RFLP are also listed in Table 1. In these cases, allelic expression is determined by direct DNA sequencing of the PCR-amplified cDNA fragments.

1. Treat the PCR reaction mixture that contains the amplified fragments with *Escherichia coli* exonuclease I and shrimp alkaline phosphatase, using ExoSAP-IT to remove the PCR primers and dNTPs.
2. Sequence the ExoSAP-IT-treated PCR fragments using the BigDye Terminator v3.1 Cycle Sequencing Kit.

3.3. Gene Expression Profiles of Cloned Mice

3.3.1. Gene Expression Profiling Using DNA Macroarrays and Microarrays

It is possible to profile simultaneously the expression levels of many different genes using DNA arrays, which are available in several formats. Generally, DNA

Table 1
PCR Primers for the Detection of Polymorphism Between *M. musculus* and *M. molossinus*

Gene	Primers	Detection method	Reference
Peg1/Mest	5′-GATTCGCAACAATGACGGC-3′ 5′-ATCCAGAATCGACACTGTGG-3′	Direct sequence	9
Igf2	5′-GGAGATGTCCAGCAACCATC-3′ 5′-CTGAAGCAATGACATGCCAC-3′	RFLP with *Hpa*II	9
Peg3	5′-TGGTGCAGACATTGAAGACC-3′ 5′-TTGCTCTCTTCCTCCTCAGG-3′	RFLP with *Tth*HB8I	10
Peg5/Nnat	5′-ACTTGCCAAGGTCAGTGAGG-3′ 5′-TCATGGTAGGATCTTGTGCG-3′	RFLP with *Tth*HB8I	9
Peg9/Dlk1	5′-CGTCTTTCTCAACAAGTGCG-3′ 5′-AGATCTCCTCATCACCAGCC-3′	Direct sequence	11
Peg10	5′-GGGTAGATAATCATAAGTATTTTGGGC-3′ 5′-CAACATTCTAAACTTTATTCCAGCAAC-3′	Direct sequence	9
Peg12/Frat3	5′-GAGGAGGAAACAGGAATGGAC-3′ 5′-ACACTCAATACCAGCCACCC-3′	RFLP with *Mbo*II	11
Xist	5′-CGGGGCTTGGTGGATGGAAAT-3′ 5′-GCGTAACTGGCTCGAGAATA-3′	Direct sequence	13
Meg1/Grb10	5′-CTTGATACCACCCAGAAAGTCTG-3′ 5′-AACCCAAAGCATTTGGCAG-3′	RFLP with *Hpa*II	14
H19	5′-GGATCCAGCAAGAACAGAAGC-3′ 5′-TCTGTCCTCTCCATCACACC-3′	RFLP with *Bcl*I	9
Meg3/Gtl2	5′-TTGCACATTTCCTGTGGGAC-3′ 5′-AAGCACCATGAGCCACTAGG-3′	RFLP with *Bst*UI	15
Igf2r	5′-TTCGACCTATAAGAAGCCTT-3′ 5′-GGGTACTTTGCTTTTGGGTA-3′	Length polymorphism	10
p57Kip2	5′-GACGATGGAAGAACTCTGGG-3′	Direct sequence	9

	5′-AGCGTACTCCTTGCACATGG-3′		
Cas1[a]	5′-AGCAGAGTGTAACGAACTCCAC-3′	RFLP with *Dde*I	**16**
	5′-CACAGTGGACGGGTGAATGTGC-3′		
Neurabin	5′-ACTCTCCTGCCGAGGCTG-3′	RFLP with *Aci*I	**16**
	5′-CAGTTTCAGGGGCTCTCACT-3′		
Pon1[a]	5′-ACAAGAACCATCGGTCTTCC-3′	RFLP with *Msp*I	**16**
	5′-CCTTCTGCTACCAC CTGGAC-3′		
Pon2	5′-ACGAGCTCCTTCCAAGTGTG-3′	RFLP with *Hae*III	**16**
	5′-ACCTCTGATGCAGGAGGATG-3′		
Pon3	5′-TCAGAAGTACTACGCATCCAGG-3′	RFLP with *Ple*I	**16**
	5′-CATGGCTGAAGGTAACTGTCC-3′		
Impact	5′-ACGTTTCCCCATTTTACAAG-3′	RFLP with *Tsp*509I	**17**
	5′-CTCTACATATGATTTTCTCTAC-3		
Asb4	5′-ATGAAAACACCAAGAAAATC-3′	RFLP with *Mnl*I	**18**
	5′-TGAAGACAAAGCACGGAGTA-3′		
Ata3	5′-AAGATTGGGGCTTTGGTCTT-3′	RFLP with *Sma*I	**18**
	5′-CATCCTTCACCATTCCCAAC-3′		
Dcn	5′-CATACTCAAATAAGGCTTCACCAA-3′	RFLP with *Fnu*4HI	**18**
	5′-AAAGTTGTCTGTAGTTGTGAACTGA-3′		

[a]Preferential maternal expression in placenta.

RFLP, restriction enzyme fragment-length polymorphism.

arrays in which relatively small numbers and large amounts of probe DNA are spotted onto nylon membranes, plastic plates, or glass slides are called "macroarrays." There are two types of "microarrays;" one consists of cDNA fragments that are spotted onto glass, and the other consists of oligonucleotide probes that are synthesized *in situ* or spotted onto glass supports. In general, macroarrays contain several hundred to a thousand probes, and they are relatively more sensitive than microarrays. Microarrays make it possible to profile 10,000–30,000 genes simultaneously, albeit with less sensitivity than macroarrays.

3.3.1.1. Gene Expression Profiling Using the Atlas cDNA Expression Macroarray

In the Atlas system, 5 μg of total RNA or 1 μg of polyA(+) RNA is used to synthesize the hybridization probe. Briefly, a ^{32}P-labeled cDNA probe is synthesized, and the nylon membrane-based macroarray is hybridized with the probe. The membrane is washed in the appropriate buffers, and signals are detected by the phospho-imager. The detailed conditions are listed in the instructions provided by the manufacturer.

Gene expression profiling using DNA microarrays

Profiling with microarrays, such as the Affymetrix GeneChip system, Amersham Biosciences CodeLink system, or Agilent DNA microarray system, requires 5–20 μg of total RNA or 0.2–2 μg of polyA(+) RNA for the generation of cRNA probes. The procedures for cDNA synthesis, biotin labeling of the cRNA preparation, hybridization, and washing of the microarrays should be performed according to the manufacturers' instructions.

3.3.2. Quantitative PCR

1. Set up the PCR reaction mixture with template cDNA, appropriate primers, and SYBR Green PCR Master Mix. Usually, 1–10 ng of template cDNAs is used in each well. Each sample should be assayed in duplicate or triplicate in order to obtain reliable data.
2. Amplify the cDNA fragment and measure the fluorescence intensity of each PCR cycle. Typical PCR conditions consist of incubation at 95°C for 10 min to activate the AmpliTaq Gold, followed by 40 cycles of 96°C for 15 s, 65°C for 30 s, and 72°C for 30 s.

4. Notes

1. It is possible to use the Isogen kit to isolate genomic DNA by ethanol precipitation of the remaining organic phase. This DNA preparation may be used for genomic DNA PCR and bisulfite treatment for DNA methylation analysis ***(19,20)***, but is not suitable for Southern blot hybridization analysis.
2. It is important to use sufficient volumes of the Isogen solution. The amount of tissue to be extracted should not exceed 50 mg/l mL of Isogen.

3. Gene expression data from DNA microarrays are not always quantitative. Thus, the expression levels of the gene of interest should be confirmed by another method, such as quantitative PCR.
4. Standard copy-number DNAs are required for quantitative PCR. We routinely use copy number standards that consist of known concentrations of cDNA fragments cloned in the plasmid vector. Because diluted DNA is unstable, especially at low concentrations, the standard DNA should be freshly diluted.

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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
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Cell Reprogramming and Transgenesis

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Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The authors provide readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer. Of immense benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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17

Nuclear Remodeling Assay in *Xenopus* Egg Extract

Koichi Gonda and Nobuaki Kikyo

Summary

Xenopus egg extract is an ideal material to identify nuclear remodeling activities important for nuclear cloning. Since the protocol for egg extract preparation was established more than 20 yr ago, egg extract has been widely used as a source for the purification of factors associated with a number of physiological activities. The eggs are large, easily obtained in large quantities, and are abundant in a variety of bioactive proteins. Many aspects of somatic nuclear remodeling observed in nuclear cloning are recapitulated in somatic nuclei incubated in egg extract. Our in vitro nuclear remodeling assay has proven effective for the purification of two novel nuclear remodeling activities, ISWI, a key player in the dissociation of TATA binding protein from chromatin, and FRGY2a and FRGY2b, two proteins capable of nucleolar disassembly. Here we outline our protocol of egg extract preparation and in vitro nuclear remodeling assay, as well as the purification method for FRGY2a and FRGY2b. Our in vitro nuclear remodeling assay in combination with egg extract will serve as a powerful tool with which to biochemically uncover molecular events involved in nuclear cloning.

Key Words: Nuclear remodeling; *Xenopus* egg extract; somatic nuclei; nucleolar disassembly and protein purification.

1. Introduction

More than 50 yr of nuclear cloning has established that differentiated somatic nuclei can acquire totipotency or pluripotency when injected into unfertilized, enucleated eggs of many different vertebrate species *(1–5)*. Recent research is beginning to further our understanding of certain aspects of the nuclear remodeling process, for example, changes in DNA methylation patterns, genetic imprinting, X-chromosome inactivation, and chromatin binding proteins. However, many other facets of nuclear remodeling remain to be explored. The efficient nuclear remodeling capability of *Xenopus* egg extract,

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together with our in vitro nuclear remodeling assay, may prove to be an essential asset for the elucidation of these unexplored facets.

Xenopus laevis eggs and egg extract provide several advantages to the study of nuclear remodeling. First, *Xenopus* eggs are large, that is, approx 1.2 mm in diameter. The large size makes injection of somatic nuclei technically less demanding, eliminating the necessity of sophisticated microinjection apparatuses. Second, eggs can be easily obtained in large quantities (>50 mL per frog), producing nearly 1.5 mL of extract from a single frog. Third, *Xenopus* restores mature eggs in 4–6 mo and can be recycled for several years. Fourth, *Xenopus* eggs store large quantities of various nuclear proteins, including nuclear remodelling proteins. Finally, protocols for making egg extract have been established and are used to study a number of physiological events such as cell cycle control *(6–8)*, nucleosome assembly *(9)*, apoptosis *(10)*, and nuclear transport *(11)*. These advantages make *Xenopus* egg extract an ideal substrate, especially for the biochemical purification of nuclear remodelling activities *(12)*.

Here we introduce our in vitro nuclear remodeling assay using *Xenopus* egg extract. The fundamental strategy is to analyze the changes of various nuclear proteins via immunoblotting and immunofluorescence microscopy after incubation of *Xenopus* embryonic fibroblast cell line XL2 nuclei in egg extract for 2 h at 23°C. To date, this assay has been essential for identification of two nuclear remodeling activities. The nucleosomal ATPase ISWI was shown to be a key protein for dissociation of the TATA binding protein from somatic chromatin in egg extract *(13)*. More recently, we found that the *Xenopus* germ cell-specific proteins FRGY2a and FRGY2b can reversibly disassemble somatic nucleoli in egg extract independently of ongoing rRNA transcription *(14)*. In light of these findings, we expect that our in vitro nuclear remodeling assay will prove to be a powerful tool to analyze nuclear remodeling.

In this chapter we present a detailed description of the steps we have taken in the identification of the nucleolar disassembly activity of FRGY2a and FRGY2b. We first show the method to prepare *Xenopus* egg extract using ultracentrifugation. Then, we describe our in vitro nuclear remodeling assay, with specific regard to nucleolar disassembly as detected by the release of the protein B23 from XL2 nuclei. Finally, we present the purification methods of the nucleolar disassembly activity from egg extract by fast protein liquid chromatography (FPLC).

2. Materials

2.1. Egg Extract Preparation

1. Female *X. laevis*.
2. Two- to three-gallon plastic buckets.

3. Wide-bore plastic Pasteur pipets.
4. Beckman ultracentrifuge rotor SW41 and 12-mL tubes (14 × 89 mm, Ultraclear No.344059), or SW55 rotor and 5-mL tubes (13 × 51 mm, Ultraclear No. 344057).
5. Pregnant mare serum gonadotropin (PMSG; Calbiochem No. 367222): Stable for 2 wk at –20°C in deionized water (DW).
6. Human chorionic gonadotropin (Sigma cat. no. CG-10): Stable for 2 wk at 4°C in DW.
7. 10X Marc's modified Ringer's solution (MMR): 1 *M* NaCl, 20 m*M* KCl, 10 m*M* $MgSO_4$, 20 m*M* $CaCl_2$, 1 m*M* EDTA, 50 m*M* HEPES. Adjust to pH 7.8 with NaOH. Autoclave to sterilize.
8. 2% (w/v) L-Cysteine hydrochloride monohydrate (Sigma, cat. no. C7880): Adjust to pH 7.7 with KOH. Must be prepared fresh.
9. Cycloheximide (Sigma, cat. no. C7698): Blocks protein synthesis including cyclin B and keeps the extract in S phase.
10. Lysis buffer stock solution: 250 m*M* sucrose, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 10 m*M* HEPES (pH 7.8). Sterilize by filtration and store at 4°C.
11. Lysis buffer: Add 1 m*M* dithiothreitol (DTT) and 50 µg/mL cycloheximide to the stock solution. Must be prepared fresh.
12. Cytochalasin B (Sigma No. C6762): 5 mg/mL in dimethyl sulfoxide (DMSO). Store at –20°C.
13. DTT (Sigma, cat. no. D9779): 1 *M* in DW. Store at –20°C.
14. Aprotinin (Sigma A1153): 5 mg/mL in DW. Store at –20°C.
15. Leupeptin (Sigma L2884): 5 mg/mL in DW. Store at –20°C.

2.2. Nuclear Remodeling Assay

1. Tissue culture cells. We routinely use *Xenopus* embryonic fibroblast cells XL2.
2. Cell dissociation solution (Sigma, cat. no. C5914): Store at 4°C.
3. Digitonin (Calbiochem, cat. no. 300410): 10 mg/mL in DMSO.
4. Bovine serum albumin (BSA), fraction V, protease free (Roche 100350): 10% stock solution in DW.
5. Spermidine trihydrochloride (Sigma, cat. no. S2501): 10 m*M* in DW.
6. Spermine tetrahydrochloride (Sigma, cat. no. S2876): 10 m*M* in DW.
7. Pepstatin A (Sigma P5318): 2 mg/mL in methanol.
8. Phenylmethylsulfonyl fluoride (PMSF) (Sigma, cat. no. P7626): 100 m*M* in 2-propanol. Store at 23°C.
9. Transplantation buffer: 250 m*M* sucrose, 75 m*M* NaCl, 0.5 m*M* spermidine trihydrochloride, 0.15 m*M* spermine tetrahydrochloride, 15 m*M* HEPES (pH 7.8). Add 1 m*M* DTT, 1 µg/mL leupeptin, 2 µg/mL pepstatin A, and 0.1 m*M* PMSF just before use. Spermidine and spermine will maintain nuclear integrity. pH is 7.3 to 7.5 without adjustment.
10. Mix 0.1 *M* ATP and 0.1 *M* GTP in 10 m*M* HEPES (pH 7.8).
11. Phosphocreatine (Sigma, cat. no. P6502): 0.4 *M* in 10 m*M* HEPES (pH 7.8).
12. Creatine phosphokinase (Sigma, cat. no. C3755): 5 mg/mL in 10 m*M* HEPES (pH 7.8) and 50% glycerol.

Fig. 1. Sucrose cushion apparatus. Set the poly-L-lysine-coated cover slip on top of the white bottom piece **(left)** with the coated side upward and overlay the top piece **(middle)** on them. Insert the two pieces into a Falcon 2059 tube carefully not to break the cover slip **(right)**.

13. Energy regeneration system (ERS): Combine 1 vol of the mixture of 0.1 *M* ATP and 0.1 *M* GTP, and 5 vol of 0.4 *M* phosphocreatine. After mixing well, add 2 vol of 5 mg/mL creatine phosphokinase. Prepare fresh.
14. Apyrase grade VII (Sigma, cat. no. A6535). 125 U/mL in 10 m*M* HEPES (pH 7.8).
15. All components should be kept at –20°C unless indicated otherwise.

2.3. Sucrose Cushion Method for Immunocytochemistry

1. 12-mm Round cover slips (Fisher, cat. no.12-545-80).
2. Apparatus for sucrose cushion (Medical Specialties, Baltimore, MD; **Fig. 1**).
3. Falcon 14-mL tubes No. 2059.
4. Poly-L-lysine (Sigma cat. no. P5899): 1 mg/mL phosphate-buffered saline (PBS). Store at –20°C.
5. 4% Paraformaldehyde in PBS.
6. 30% (w/v) sucrose in PBS: Filter to sterilize and store at 4°C.
7. Histology pen (DAKO S2002).

2.4. Fractionation of Egg Extract by Column Chromatography

The following columns and buffers were used for purification of FRGY2a and FRGY2b from egg extract.

1. SP Sepharose Fast Flow (Amersham Biosciences).
2. Econo-Column, 4.91 $cm^2 \times 10$ cm (Bio-Rad).
3. HiTrap Heparin 5-mL column (Amersham Biosciences).
4. MonoQ HR 5/5 column (Amersham Biosciences).
5. Superdex 200 HR 10/30 column (Amersham Biosciences).
6. Buffer B_{75}: 10 m*M* HEPES pH 7.8, 75 m*M* NaCl, 4 m*M* $MgCl_2$, 10 m*M* β-glycerophosphate, 10% glycerol, 0.003% Triton X100. Add 1 m*M* DTT, 1 µg/mL leupeptin, 2 µg/mL pepstatin A, and 0.1 m*M* PMSF just before use. Filter with a 0.45-µm bottle top filter before use for FPLC.
7. Buffer B_{150}, B_{500} and B_{1000}: The same as B_{75} except for the NaCl concentration. They contain 150 m*M*, 500 m*M*, and 1 *M* NaCl, respectively.
8. Centricon YM10 (Millipore).

3. Methods

3.1. Egg Extract Preparation

This protocol is based on the method of Smythe and Newport *(15)*.

1. Five to ten days before egg collection, inject 100 U of PMSG into the dorsal lymph sac of each frog using a 25-gage needle (*see* **Note 1**).
2. Twelve to sixteen hours before egg collection, inject 500 U of human chorionic gonadotropin into each frog. Place one frog in each bucket containing 1.5 L of 0.1 *M* NaCl in DW (Ca^{2+}-free) and leave at 23°C. It is important to keep frogs in separate buckets because egg quality will vary.
3. Collect laid eggs into a 600-mL beaker. Remove eggs with poor quality using a wide-bore plastic Pasteur pipet (*see* **Note 2**).
4. Add 2 vol of 2% L-cysteine hydrochloride monohydrate to eggs and gently swirl for 8–10 min to remove the jelly coat from the eggs. At 4 min, change to a new cysteine solution and continue swirling. If eggs are incubated in the cysteine solution longer than 10 min, they will start to deteriorate.
5. Wash eggs with cold 0.25X MMR four times to remove cysteine. Cold MMR is essential and will prevent degradation of the eggs once they have been dejellied (*see* **Note 3**).
6. Rinse eggs with 1X vol of lysis buffer three times. Again eliminate bad eggs.
7. Gently transfer eggs to ultracentrifuge tubes using a wide-bore Pasteur plastic pipet. It may be necessary to cut the Pasteur pipet to prevent the eggs from breaking when transferring. Spin at 200*g* for 30 s using a clinical centrifuge (e.g., Beckman Allegra 3R) at 4°C to pack eggs. Remove the buffer at the top after centrifuge.
8. Add cytochalasin B, aprotinin, and leupeptin to each tube to the final concentrations of 5 µg/mL each.
9. Centrifuge at 260,000*g* with SW41 or SW55 for 4 h at 4°C.

10. Collect the clear cytoplasmic layer (under the top yellow layer of lipid) by puncturing the side of the centrifuge tube using a 21-gage needle and a 3-mL syringe. The volume of the extract is around 20% of the packed eggs. Add DTT, aprotinin, and leupeptin to the extract to the final concentration of 1 m*M*, 5 µg/mL, and 5 µg/mL, respectively. Aliquot in 0.1–1 mL and quickly freeze in liquid nitrogen. Store at –80°C (*see* **Note 4**).

3.2. In Vitro Nuclear Remodeling Assay

This protocol is a modification of Gurdon's method *(16)* and the method of Adam et al. *(17)*. The assay is performed at 29-µL scale in Eppendorf tubes, by combining 25 µL of egg extract or FPLC fractions (*see* **Note 5**), 2 µL of ERS, and 1×10^5 nuclei. If you want to deprive nuclei of ATP and GTP completely to determine their necessity in the reaction, include apyrase (apyrase possesses ATPase and ADPase activities) instead of ERS. Apyrase samples should be incubated at 23°C for 30 min before the addition of nuclei to ensure complete depletion of both ATP and GTP.

1. Determine the number of nuclei to use (1×10^5 nuclei per reaction) and calculate the required volume of transplantation buffer and ERS. Process twice as many cells as required to compensate the loss during the nuclei preparation.
2. Prepare all solutions and pre-chill them on ice. Note that digitonin takes some time to thaw and will remain frozen on ice.
3. Thaw egg extract or fractions to be assayed rapidly. Aliquot additives, ERS, and extract or fractions to each tube on ice.
4. Harvest cells by treating them with cell dissociation solution for 10 min (*see* **Note 6**).
5. Pellet cells by centrifugation at 200*g* for 5 min. Resuspend cells in 10 mL of ice-cold PBS and count cell number.
6. Spin the required number of cells again.
7. Remove the supernatant and resuspend the cells at 2×10^6 cells/mL of transplantation buffer. Add digitonin at 5 µL/mL of transplantation buffer and incubate on ice for 3 min, rocking the cell suspension every minute (*see* **Note 7**). Digitonin permeabilizes plasma membrane and nuclear envelope, eliminating effects of the regulated nuclear transport.
8. Stop the permeabilization by adding 1/10 vol of 10% BSA.
9. Spin down the permeabilized cells (called nuclei hereafter) at 200*g* for 5 min at 4°C.
10. Resuspend the nuclear pellet in 1 mL of transplantation buffer with 1% BSA. Transfer the nuclear suspension to an Eppendorf tube.
11. Spin at 500*g* for 1 min at 4°C with a microcentrifuge. Discard the supernatant.
12. Resuspend the nuclei in 1 mL of transplantation buffer with 1% BSA. Spin again and discard the supernatant.
13. Resuspend the nuclei in approx 1 vol of transplantation buffer with 1% BSA.
14. Count the number of nuclei and dilute the nuclear suspension volume to 1×10^5 nuclei/2 µL (*see* **Note 8**).

15. Add 2 µL (1×10^5 nuclei) to the reaction mixture prepared in the **step 3**, mix by pipetting and incubate at 23°C for 2 h. Tap the tubes every 20 min during the reaction to resuspend the precipitated nuclei.
16. If you are going to analyze nuclei by immunofluorescence microscopy, proceed to **Subheading 3.3**. If you are to analyze by immunoblotting, add 100 µL of the B_{75} buffer to each reaction and pellet nuclei with a microcentrifuge at 500*g* for 5 min at 4°C. Wash nuclei once with B_{75}, add sodium dodecyl sulfate sample buffer and apply to gel electrophoresis.

3.3. Sucrose Cushion Method for Immunofluorescence Microscopy

This procedure is based on the method of Madine and Coverley (*18*). Cover slips need to be pretreated with ethanol and dried before use. While waiting for the nuclear remodeling reaction, conduct **steps 1** and **2**.

1. Coat one side of round cover slips with poly-L-lysine for 15 min at 23°C. Mark the uncoated sides of the cover slips with a Histology pen. Assemble the sucrose cushion apparatus and the coated cover slips in Falcon 2059 tubes (**Fig. 1**). Make sure to set the coated side of the cover slip upward.
2. Add 1.5 mL of 30% sucrose in PBS to each tube and pre-spin at 1500*g* for 3 min with a clinical centrifuge (*see* **Note 9**). Remove approx 0.5 mL of 30% sucrose in PBS from each Falcon tube to make room for the nuclear samples in the tunnel of the cushion tube.
3. After 2 h of the nuclear remodeling reaction, fix nuclei by adding 90 µL of 4% paraformaldehyde in PBS. Leave at 23°C for 15 min.
4. Transfer the fixed nuclei into the upper space in the tunnel in sucrose cushion apparatus. Spin down the nuclei through 30% sucrose in PBS onto the cover slips at 1500*g* for 7 min at 4°C with a clinical centrifuge.
5. Transfer the cover slips to 12-well plates containing 1 mL of PBS per well. Nuclei on cover slips can be stored in PBS for up to 2 wk at 4°C. Stain nuclei on the cover slips with the antibodies of your interest and mount on glass slides for immunofluorescence microscopy.

3.4. Fractionation of Egg Extract by FPLC

Screen active fractions using the in vitro nuclear remodeling assay. To purify the nucleolar disassembly activity, we checked dispersal of B23, one of the major nucleolar components, as an indicator of nucleolar disassembly by immunofluorescence microscopy (**Fig. 2**; *see* **Note 10**). All purification procedures are performed at 4°C. Fractions are frozen quickly on dry ice and stored at –80°C. All buffers and samples are filtered through 0.22-µm filters before applying to the columns. If sample cannot be filtered because of the high protein concentration or viscosity, centrifuge at 12,000*g* for 15 min at 4°C.

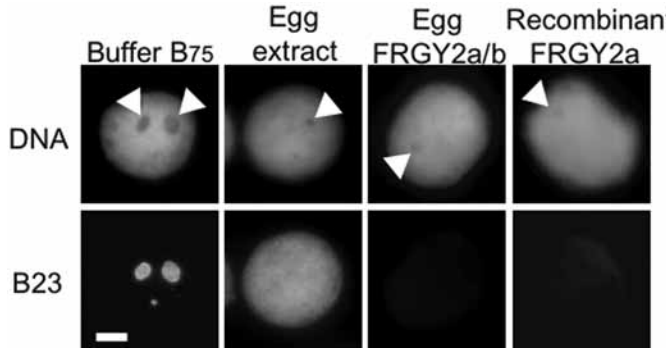

Fig. 2. Immunofluorescence micrographs of remodeled nuclei. Nuclei of *Xenopus* cell line XL2 were incubated in buffer B_{75}, egg extract, FRGY2a and FRGY2b purified from egg extract in B_{75}, and recombinant FRGY2a in B_{75} at 23°C for 2 h. After fixation with paraformaldehyde, nuclei were spun down onto cover slips and stained with the antibody against the nucleolar protein B23 and DNA dye Hoechst 33342. Nucleoli did not show significant change after incubation in B_{75} for 2 h. In contrast, B23 was dispersed from the nucleoli to the nucleoplasm after incubation of the nuclei in egg extract. B23 became undetectable when incubated with FRGY2 proteins, owing to the diffusion of B23 into the surrounding buffer ***(14)***. Nucleolar disassembly is also evident from shrinkage of the dark spots (arrowheads) corresponding to nucleoli. Bar, 5 μm.

3.4.1. SP Sepharose

1. Pack 30 mL of SP Sepharose beads into Econo-Column and equilibrate the column with Buffer B_{75}.
2. Apply 30 mL of egg extract to SP Sepharose using a peristaltic pump and wash the column with 5 column volumes (CV) of B_{75}.
3. Step-elute bound proteins with 7 CV of B_{500} and 5 CV of B_{1000}, sequentially. Collect eluate at 15 mL/fraction.

3.4.2. HiTrap Heparin

1. Dialyze the B_{1000} fractions from SP Sepharose against 100-fold volume of B_{75} for 4 h.
2. Equilibrate HiTrap Heparin with B_{75}.
3. Load the dialyzed fractions onto HiTrap Heparin. Wash the column with 5 CV B_{75} and elute the bound proteins with a 20 CV linear gradient from B_{75} to B_{1000}. Collect eluted proteins at 2.5 mL/fraction.
4. Dialyze the fractions against 100-fold volume of B_{75} for 4 hr and apply them for the nucleolar disassembly assay. The activity is eluted by 0.7 *M* NaCl.

3.4.3. MonoQ HR 5/5

1. Dialyze the active fractions from HiTrap Heparin against 100-fold volume of B_{75}.
2. Equilibrate MonoQ HR 5/5 with B_{75}.

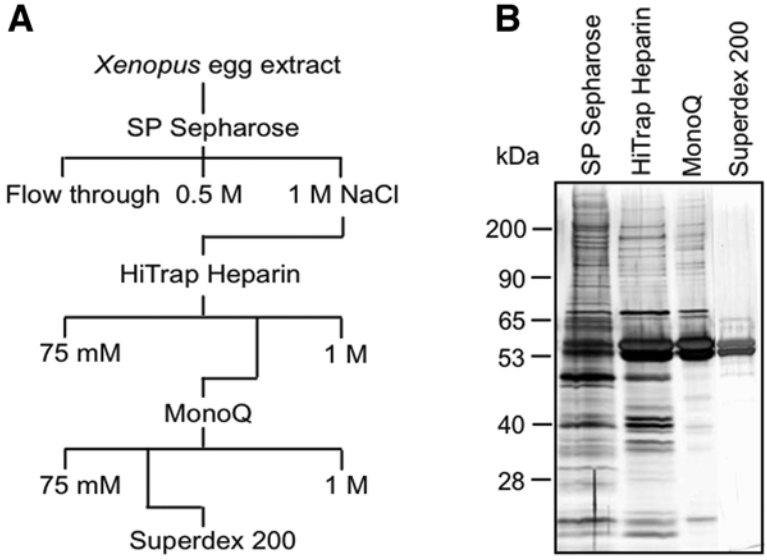

Fig. 3. Purification of FRGY2a and FRGY2b from *Xenopus* egg extract. **(A)** Flow chart showing the purification of FRGY2a and FRGY2b from egg extract. Fractions were assayed for the activity to disperse B23 from the nucleoli of XL2 nuclei. **(B)** Silver stain of the fractions that carry the nucleolar disassembly activity in each step of the purification. The doublet of 56 kDa and 54 kDa corresponds to FRGY2a and FRGY2b, respectively.

3. Apply the dialyzed active fractions of HiTrap Heparin onto MonoQ HR 5/5 and elute the bound proteins with a 10 CV linear gradient from B_{75} to B_{500}.
4. Concentrate each fraction tenfold with Centricon and assay for the nucleolar disassembly activity. The activity is in the fractions at approx 180 m*M* NaCl.

3.4.5. Superdex 200 HR 10/30

1. Equilibrate Superdex 200 HR 10/30 with B_{150}.
2. Load 200 µL of the active fractions from MonoQ and fractionate eluate at 0.5 mL/fraction.
3. Assay fractions. Nucleolar disassembly activity is eluted in the fractions corresponding to the molecular weight of 75 kDa (**Fig. 3**). Mass spectrometry showed the protein bands of 56 kDa and 54 kDa correspond to FRGY2a and FRGY2b, respectively. One of the two proteins is sufficient to disassemble nucleoli in B_{75} as confirmed using recombinant proteins (**Fig. 2**).

4. Notes

1. We generally prime 10 frogs, but only 5–8 frogs lay good eggs. Total yield of the extract is approx 1.5 mL per frog.

2. Anesthetize frogs in ice-cold water for 20 min and squeeze the back of the frog to obtain more eggs. Some frogs secrete milky exudates when squeezed. The exudate will induce egg lysis and should be washed off immediately. It is crucial to collect only good eggs because bad eggs will inhibit the nuclear remodeling activity of the extract. Good eggs are approx 1.2 mm in diameter and consist of discrete animal and vegetal regions of roughly equal size. Abnormal eggs may be grossly enlarged or non-uniformly pigmented. If abnormal eggs are more than 5% of the total eggs, this lot should be discarded. Once dejellied, extract should be prepared as promptly as possible.
3. While dejellying or washing eggs, remove bad eggs as much as possible. If you are not sure which eggs are bad, transfer eggs into a Petri dish and examine under a stereomicroscope. It is essential that all suspect eggs are removed before centrifugation to obtain functional extract. We use Falcon 3003 bacterial culture dish for this purpose. Dejellied eggs stick to a glass dish or a dish for cell culture. Do not leave eggs in a dish longer than 10 min because they tend to attach to the dish and to each other.
4. To collect as much extract as possible, we suck the extract from the centrifuge tubes in two steps. First, gently suck out only the middle portion of the clear layer of the extract and leave the border zones undisturbed. Freeze the clean extract. Then, suck out the entire remaining clear layer with some contamination of the white layer above and yellow foggy layer below and ultracentrifuge again. It is not necessary to centrifuge the "dirty" extract on the same day. Dirty extract can be frozen with the addition of protease inhibitors to be thawed and centrifuged when enough dirty extract has accumulated as to fill at least one centrifuge tube. Note that centrifuge times for the second spin may be considerably longer than the first.
5. FPLC fractions should be dialyzed against B_{75} before use in the assay. The salt concentration of egg extract is approx 75 m*M*. If necessary, concentrate the fractions with Centricon or the equivalent apparatus to detect the activity of your interest. Some proteins are lost during concentration with Centricon owing to leak through the membrane or nonspecific binding to the membrane and the plastic wall of Centricon. We routinely preblock the nonspecific binding using 0.1% BSA for 10 min followed by a brief rinse with DW.
6. We do not use trypsin to harvest cells because its potential contamination into the downstream reaction may be harmful.
7. Periodical rocking is important for efficient permeabilization. Digitonin concentration and treatment period need to be optimized for each cell line you use by checking the nuclear permeability of fluorescence-labeled IgG (150 kDa). If fluorescence-labeled IgG can enter the nuclei after digitonin treatment, the nuclear envelope is permeabilized *(18)*.
8. Usually 100-fold dilution is necessary for proper counting of the prepared nuclei.
9. Prespin the assembled sucrose cushion tube to ensure that the cover slip is sealed between the top and bottom pieces of the apparatus. This improves the recovery of the nuclei on the cover slips.

10. FRGY2a and FRGY2b are germ cell specific proteins also involved in masking of maternal mRNA and transcription of some germ-specific genes. When one of these recombinant proteins is incubated with XL2 nuclei in B_{75}, it disassembles the nucleoli, releasing various nucleolar proteins and RNA into nucleoplasm. They can also disassemble nucleoli in transfected cells ***(14)***.

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METHODS IN MOLECULAR BIOLOGY™

Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
Alan O. Trounson

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Cell Reprogramming and Transgenesis

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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In Vitro Reprogramming of Nuclei and Cells

Anne-Mari Håkelien, Thomas Küntziger, Kristine G. Gaustad, Anne Marstad, and Philippe Collas

Summary

Directly turning a somatic cell type into another would be beneficial for producing replacement cells for therapeutic purposes. To this end, novel cell reprogramming strategies are being developed. We describe here methods for functionally reprogramming a somatic cell using an extract derived from another somatic cell type. The procedure involves reversible permeabilization of 293T fibroblasts, incubation of the permeabilized cells in a nuclear and cytoplasmic extract of T-cells, resealing of the "reprogrammed" cells, and culture for assessment of reprogramming. Reprogramming has been evidenced by nuclear uptake and assembly of transcription factors, induction of activity of a chromatin remodeling complex, changes in chromatin composition, activation of lymphoid cell-specific genes, and expression of T-cell-specific surface molecules. The system is likely to constitute a powerful tool to examine the processes of nuclear reprogramming, at least as they occur in vitro.

Key Words: Cell extract; cell fractionation; cell membrane; cell culture; fibroblast; nucleus; Steptolysin O; reprogramming; T-cell; transdifferentiation.

1. Introduction

Several lines of evidence indicate that altering the fate of, or reprogramming, a differentiated cell is possible. Classical examples of nuclear reprogramming include epigenetic changes and alterations in gene expression elicited by fusion of somatic cells with less differentiated cell types *(1,2)* or the birth of clones or the production of embryonic stem cells by transplantation of somatic nuclei into oocytes *(3–6)*. Additional recent studies have shown that a somatic cell type could, at least partially, be directly turned into another somatic cell type without recapitulating development. This was achieved by exposing somatic nuclei

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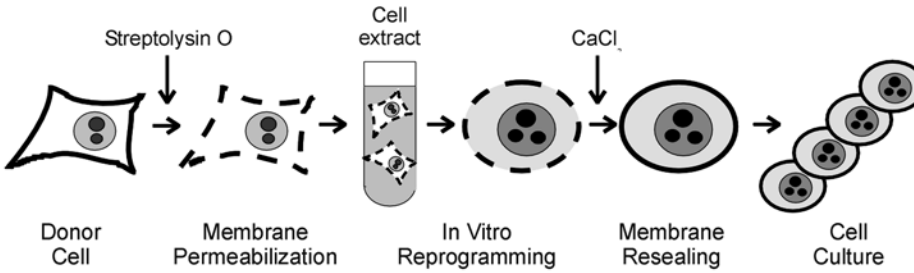

Fig. 1. In vitro cell reprogramming. Cells (for example, fibroblasts) are reversibly permeabilized with Streptolysin O, incubated for 1 h in a nuclear and cytoplasmic extract of, for example, T-cells, resealed with 2 m*M* $CaCl_2$ for 2 h and cultured for analysis of reprogramming.

or cells to an extract derived from another somatic cell type *(7,8)*. Despite these findings, the mechanisms of nuclear reprogramming remain poorly elucidated.

We describe a procedure to redirect the program of a transformed human fibroblast cell line towards that of a T-cell. The approach may in principle be applied to various cell types. The procedure (**Fig. 1**) involves incubating reversibly permeabilized fibroblasts in a nuclear and cytoplasmic extract derived from a Jurkat T-cell line. At the end of incubation, the fibroblasts are resealed and cultured to assess the expression of T-cell-specific markers and the establishment of T-cell-specific functions. For molecular assessments of reprogramming, purified nuclei, as opposed to cells, also can be incubated in the extract. As large numbers of cells or nuclei can be processed simultaneously, and considering the ability of cell extracts to be biochemically altered, the manipulation of nuclei or cells provides a means of analyzing the mechanisms of nuclear reprogramming, at least as they occur in vitro.

The methods describe the preparation of cells to be reprogrammed (referred to as the "donor" cells), the preparation of reprogramming extracts, the permeabilization of the donor cells, setting up the reprogramming reaction, the resealing of the reprogrammed cells, and examples of assessments of reprogramming. The procedures described are based on the reprogramming of human 293T fibroblasts in an extract derived from the human Jurkat TAg cell line *(7)*.

2. Materials

1. 293T fibroblasts cultured on round, 12-mm glass poly-L-lysine-coated cover slips.
2. Poly-L-lysine (cat. no. P8920, Sigma-Aldrich, St. Louis, MO).
3. Propidium iodide (cat. no. P4170, Sigma). Make a 1 mg/mL stock solution in H_2O and store at –20°C in the dark.

4. Phosphate-buffered saline (PBS).
5. RPMI-1640 medium (cat. no. R0883, Sigma) supplemented with 10% fetal calf serum.
6. Hanks' Balanced Salt Solution (HBSS; cat. no. 14170-088, Gibco-BRL; Paisley, UK).
7. Protease inhibitor cocktail (cat. no. P2714, Sigma). This is a 100X stock solution.
8. Cell lysis buffer: 50 m*M* NaCl, 5 m*M* $MgCl_2$, 20 m*M* HEPES, pH 8.2, 1 m*M* dithiothreitol, 0.1 m*M* phenylmethylsulfonyl fluoride, and the protease inhibitor cocktail; make fresh and keep on ice for up to 1 h until use.
9. Streptolysin O (SLO; cat. no. S5265, Sigma) at 100 µg/mL in H_2O, aliquoted and stored at –20°C.
10. 1 *M* $CaCl_2$ (cat. no. C4901, Sigma) in sterile H_2O.
11. ATP (cat. no. A3377, Sigma) at 200 m*M* in H_2O, aliquoted and stored at –20°C.
12. Creatine kinase (cat. no. C3755, Sigma) at 5 mg/mL in H_2O, aliquoted and stored at –20°C.
13. Phosphocreatine (cat. no. P7936, Sigma) at 2 *M* in H_2O, aliquoted and stored at –20°C.
14. GTP (cat. no. G8752, Sigma) at 10 m*M* in H_2O, aliquoted and stored at –20°C.
15. Nucleotide triphosphate (NTP) set (cat. no. 1277057, Roche, Basel, Switzerland). Prepare a stock solution by mixing 20 µL of each NTP provided in the set at a 1:1:1:1 ratio on ice. Aliquot in 10 µL and store at –20°C. This makes an NTP mix at 25 m*M* of each NTP. Prepare more stock solution as needed.
16. Sonicator fitted with a 2-mm diameter probe (Model Labsonic M, B. Braun Biotech International, Melsungen, Germany).
17. Humidified air incubator set at 37°C.
18. 50- and 15-mL conical tubes (Corning, Corning, NY).
19. 1.5-mL Centrifuge tubes.
20. 24-Well cell culture plates (Costar, cat. no. 3524, Corning).

3. Methods

3.1. Seeding 293T-Cells

Plate 293T-cells on 12-mm round, sterile, poly-L-lysine-coated glass cover slips at a density of 50,000 cells per cover slip on the day before the reprogramming reaction. Each cover slip is placed in an individual well of a 24-well culture plate. Overlay the cover slips with 500 µL of complete RPMI-1640 medium and place in culture.

3.2. Preparation of the Reprogramming Extract

3.2.1. Cell Collection

1. Transfer the Jurkat TAg cell suspension culture into 50-mL conical tubes and sediment the cells at 800*g* for 10 min at 4°C.
2. Wash the cells twice in ice-cold PBS by suspension and sedimentation at 800*g* for 10 min at 4°C. The cells can be pooled into a single tube after the first wash.

3.2.2. Swelling of the Cells

1. Resuspend the cells in 10 mL of ice-cold cell lysis buffer. It is preferable to use a graduated 15-mL conical tube to estimate cell volume after sedimentation.
2. Centrifuge at 800*g* for 10 min at 4°C.
3. Estimate the volume of the cell pellet. Resuspend the pellet into two volumes of ice-cold cell lysis buffer.
4. Hold the cells on ice for 45 min to allow swelling. This step makes it easier to lyse the cells during sonication. Keep the cells well suspended by occasional tapping of the tupe. Note that the cells can be allowed to swell for longer than 45 min. This swelling step can be omitted for Jurkat TAg of primary T-cells as these cell types lyse promptly during sonication.

3.2.3. Extract Preparation

1. Aliquot the cell suspension into 200 µL in 1.5-mL centrifuge tubes previously chilled on ice. Sonicate each tube one by one (on ice) until all cells and nuclei are lysed. Lysis is assessed by complete disruption of the cells and nuclei, as judged by the sole appearance of cell "debris" by phase contrast microscopy examination. Once lysis is achieved in a tube, keep the tube on ice and proceed with all other tubes. Power and duration of sonication vary with each cell type. For Jurkat TAg cells, sonication of each tube at 25% power and 0.5-s pulse cycle over 1 min 40 s is recommended when using the Labsonic M sonicator.
2. Pool all lysates into one (or several, if needed) chilled 1.5-mL centrifuge tubes. Sediment the lysate at 15,000*g* for 15 min at 4°C in a fixed-angled rotor. A swing-out rotor can also be used.
3. Carefully collect the supernatant with a 200-µL pipet and transfer it into a new chilled 1.5-mL tube. This is the reprogramming extract.
4. It is possible to aliquot the extract into 200-µL tubes such as those used for polymerase chain reaction, with 100 µL extract per tube. Snap-freeze each tube in liquid N_2 and store at –80°C. However, we recommend carrying out reprogramming with freshly made extract as the stability of the extract –80°C may vary with cell types and batches.
5. After sedimentation in **step 3**, remove 20 µL of extract to determine protein concentration and pH. Protein concentration should be approx 25 mg/mL. pH should be between 6.7 and 7.0 (*see* **Note 2**).

3.2.4. Extract Toxicity Assay

Each new batch of cell extract requires a cell toxicity test.

1. Add 50,000 293T-cells (or any other cell line growing in the laboratory) to 30 µL of extract on ice in a 1.5-mL centrifuge tube. The extract does not need to contain any additives (unlike for a reprogramming reaction; *see* **Subheading 3.5.1.**).
2. Incubate for 1 h at 37°C in a water bath.
3. Remove a 3-µL aliquot and assess cell morphology by phase contrast microscopy. **Figure 2** illustrates primary rat fetal fibroblasts after a 30-min exposure to

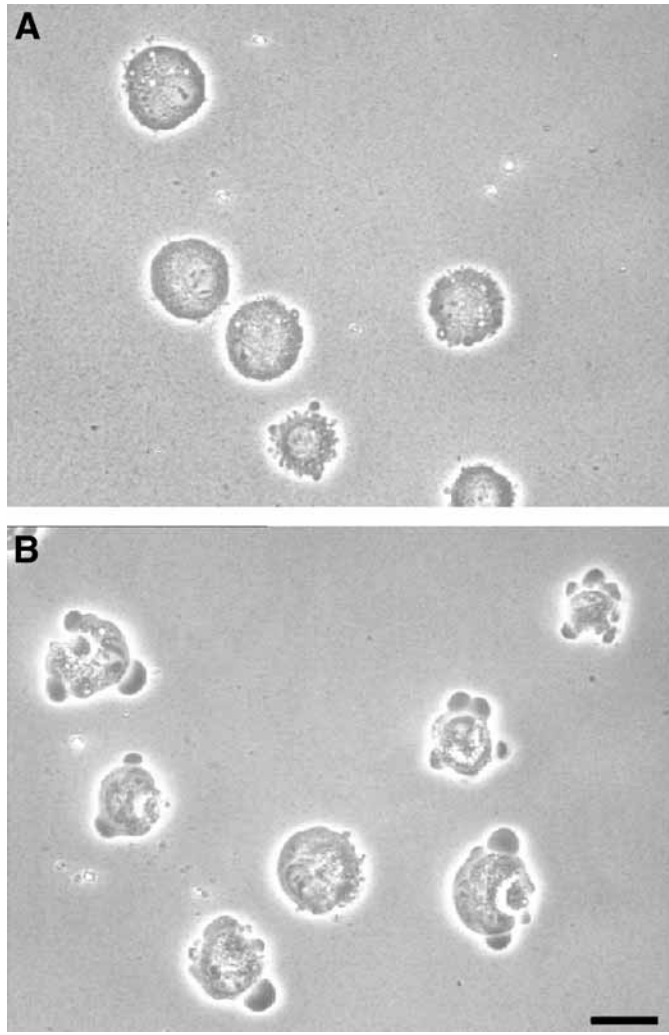

Fig. 2. Extract toxicity assay. Intact cells (here, HeLa) are incubated in each new batch of reprogramming extract for 30 min. An aliquot is removed to a slide and examined by phase contrast microscopy. Cells shown in **(A)** survive the extract exposure while cells in **(B)** will not survive in subsequent culture. Batches of extract resulting in such cells should be discarded. Bar, 20 µm.

reprogramming extracts. In our hands, the morphology of the cells after a 30-min incubation in the extract reflects their survival in culture. Cells shown in **Fig. 2A** survive the extract exposure while cells in **Fig. 2B** have been damaged by the extract and do not survive in culture. Extract batches producing such cells should be discarded.

4. If so wished, replate the cells directly from the extract in complete RPMI-1640 for an overnight culture to confirm survival. There is no need to remove the extract before replating.

3.3. Permeabilization of 293T-Cells

For components from the reprogramming extract to enter the 293T-cells, the cells must be reversibly permeabilized. Permeabilization is accomplished with the *Streptococcus pyogenes* toxin, SLO. SLO is a cholesterol-binding toxin that forms large pores in the plasma membrane of mammalian cells *(9)*.

3.3.1. SLO Stock Solution

1. Dissolve the SLO powder in sterile-filtered MilliQ H_2O at 100 µg/mL. Keep on ice while dissolving the SLO.
2. Aliquot 10 µL in 200-µL tubes and store at –20°C.
3. Discard all tubes after 1 mo of storage at –20°C and prepare a new stock. Stock aliquots should be thawed only once. Note that as commercial batches of SLO vary in specific activity, a range of SLO concentrations (100, 200, 500, 1000, 2000, and 4000 ng/mL SLO) should be tested on the cell type to be reprogrammed after a new stock is prepared. Fine-tune the SLO concentrations thereafter, as needed (*see* **Note 1**).

3.3.2. Cell Permeabilization

1. Dilute the SLO stock in ice-cold HBSS to a concentration of 230 ng/mL. This is the working solution. Note that this concentration is valid for 293T-cells and should be adjusted for other cell types using the cell permeabilization assay described in **Subheading 3.4.**
2. Keep the SLO on ice until addition to 293T-cells.
3. Remove the RPMI-1640 medium from wells containing 293T-cells grown on cover slips and wash the cells four times with PBS at room temperature to remove all Ca^{2+} from the culture medium. This step is essential as Ca^{2+} inhibits SLO activity.
4. To each well, add 250 µL of SLO working solution.
5. Incubate at 37°C in regular atmosphere for 50 min. Proceed to **Subheading 3.5.1.**

3.4. Cell Permeabilization Assay

The assay allows the evaluation of the efficiency of the SLO treatment. It is recommended to carry out this assay using four additional cover slips supporting 293T-cells as described in **Subheading 3.1.**, in addition to those used for the reprogramming reaction. The assay is based on the uptake of the fluorescent DNA stain, propidium iodide, by permeabilized cells, but not by intact cells.

1. Permeabilize cells on two cover slips with SLO as described under **Subheading 3.3.2.** However, in the first step, add propidium iodide to 0.1 µg/mL to the SLO

dilution in HBSS on one of the cover slips. Propidium iodide will be taken up as cells are being permeabilized. The other cover slip receives 250 µL of SLO dilution in HBSS without propidium iodide.
2. Two additional cover slips should also be used as controls for the absence of SLO. Add 250 µL of HBSS containing propidium iodide to one of the control cover slips as in **step 1**. The other cover slip receives 250 µL of HBSS without propidium iodide.
3. Incubate at 37°C in regular atmosphere for 50 min.
4. SLO-treated and control coverslips not containing propidium iodide: remove HBSS and immediately add 1.5 mL of preheated (37°C) complete RPMI-1640 containing 2 m*M* Ca^{2+} added from a 1 *M* stock (*see* **Subheading 2.1.**). Incubate at 37°C for 2 h to allow resealing of the plasma membrane.
5. SLO-treated and control cover slips labeled with propidium iodide: remove HBSS, rinse with PBS, and add 250 µL of PBS.
6. Assess propidium iodide labeling of the nuclei by fluorescence microscopy.
7. After the 2-h membrane resealing step described in **step 4**, remove the culture medium, rinse with PBS and add 250 µL of PBS containing 0.1 µg/mL propidium iodide; incubate for 10 min.
8. Assess propidium iodide uptake, or lack thereof, in the resealed cells as in **step 6**.

3.5. Reprogramming Reaction

3.5.1. Extract Preparation

During the SLO treatment, the extract should be prepared for reprogramming.

1. Prepare the ATP-regenerating system: mix on ice ATP:GTP:creatine kinase:phosphocreatine in a 1:1:1:1 ratio from each separate stock (*see* **Subheading 2.1.**) and keep on ice.
2. Add 5 µL of the ATP-regenerating system mix to 100 µL of extract, on ice.
3. Add 4 µL of the 25 m*M* NTP mix (*see* **Subheading 2.1.**) to 100 µL of extract, on ice.
4. Vortex briefly and replace the extract on ice.

3.5.2. Reprogramming Reaction

1. Remove SLO from the cells by careful aspiration.
2. Quickly add PBS to prevent drying of the cells.
3. Immediately transfer each cover slip into a new dry well of a 24-well plate and carefully lay 65 µL of extract (*see* **Subheading 3.2.3.**) onto each cover slip. Be careful that cells do not dry out upon transfer of the cover slip(s) to the new wells and prior to addition of the extract. It is important that the cover slip be covered by the extract during the entire incubation time. Should the extract spread out of the cover slip, transfer the cover slip into a new well and pipet the extract back onto the cells.

4. Incubate at 37°C for 1 h in regular atmosphere. Note that reprogramming was also successful upon incubation in a 5% CO_2 incubator at 37°C.

3.6. Resealing of the Reprogrammed Cells

1. At the end of incubation in reprogramming extract, directly add to each well 1.5 mL of preheated (37°C) complete RPMI-1640 containing 2 m*M* Ca^{2+} added from the 1 *M* stock (*see* **Subheading 2.1.**). The extract does not need to be removed before adding the Ca^{2+}-containing medium.
2. Incubate for 2 h in a 5% CO_2 incubator at 37°C.
3. Remove the Ca^{2+}-containing medium by gentle aspiration and replace with 250 µL of complete RPMI-1640 (Jurkat TAg cell culture medium).
4. Place the cells back into the 5% CO_2 incubator and culture until reprogramming assessments are performed.

3.7. Assessment of Nuclear Reprogramming

Various assessments of nuclear and cell reprogramming can be performed, according to the purpose of the experiment and the target cell type used to prepare the extract. Using the method described here, we have reported changes in the gene expression profile of the reprogrammed 293T-cells, using cDNA arrays from R&D Systems (Abington, UK ***[7]***). Expression of new proteins also can be monitored at regular intervals after reprogramming reaction, by immunofluorescence or flow cytometry. We have shown the expression of several antigens on the surface of the reprogrammed cells, which are specific for hematopoietic cells ***(7)***. A variety of functional assays can also be performed, such as cytokine secretion in response to stimulation of the T-cell receptor/CD3 complex in the reprogrammed cells, or expression of additional cytokine receptors on the cell surface ***(7)***.

4. Notes

1. Commercially available SLO batches vary greatly in activity. It is recommended to test a range of SLO concentration on the cell type to be reprogrammed prior to initiating reprogramming reactions. Efficiency of SLO-mediated permeabilization also varies for different cell types.
   pH of the extract: we usually observe a drop of 1–1.5 pH unit upon extract preparation, which explains the pH 8.2 of the cell lysis buffer. Notably, raising the pH of the cell lysis buffer to 8.7 with a HEPES buffer does not increase the pH of the final extract. Other buffers with greater buffering capacity have not been tested.
2. The method described here can be used with purified nuclei ***(8)*** or permeabilized cells ***(7)***. Procedures for purifying intact, membrane-enclosed, nuclei from interphase-cultured cells have been reported earlier ***(8,10)***.
3. It is currently difficult to objectively assess the extent of sonication of the Jurkat TAg cells, or of any other cell type. It is important to sonicate until all cells and

nuclei are completely lysed. Whether extended sonication after cell lysis is complete is detrimental or beneficial is at present unknown.

4. Variability in batches of reprogramming extracts is seen, even among extracts that have been rated as "non-toxic" in the toxicity assay described in **Subheading 3.2.4.** Variability is evident by the absence of markers of cell reprogramming 1 wk after reprogramming.
5. With current techniques, expression of a reprogrammed phenotype occurs for at least two months for 293T-cells reprogramming in Jurkat TAg extract. The reprogrammed phenotype may also last for shorter periods depending on the cell type reprogrammed (primary as opposed to transformed), the target cell type or the markers analyzed.

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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
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Cell Reprogramming and Transgenesis

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Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The authors provide readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer. Of immense benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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19

Techniques for Nuclear Transfer to Mouse Embryonic Stem Cells

Danièle Pralong and Paul J. Verma

Summary

In this chapter, methods are described that permit the enucleation of mouse embryonic stem (ES) cells and the transfer of donor nuclei to these cells before or after enucleation has taken place. The small size and high nucleus-to-cytoplasm volume ratio of ES cells poses a challenge to their enucleation. The first step describes the production of lines of larger, polyploid ES cells, which are more suited to enucleation than diploid ES cells. In a second step, a simple centrifugal enucleation technique is described that allows efficient bulk production of ES cell cytoplasts and karyoplasts. Finally, techniques for nuclear transfer to ES cells are described, involving either transfer of karyoplasts to cytoplasts or the formation of heterokaryons between donor and recipient cells followed by the selective elimination of the polyploid nucleus. These methods have potential applications in the generation of autologous, diploid pluripotent cells from donor somatic cells. Also, they provide a novel dynamic model for studying nucleocytoplasmic interactions in ES cells.

Key Words: Nuclear transfer; reprogramming; embryonic stem cells; enucleation; heterokaryons.

1. Introduction

Recent studies have shown that the fusion of mouse embryonic stem (ES) cells with a variety of differentiated cells results in the production of, at least partly, reprogrammed hybrids *(1–5)*, indicating that the cytoplasm of ES cells may contain reprogramming factors and that these cells could provide a practical alternative to oocytes as recipients for somatic cell nuclear transfer (SCNT). The first step of SCNT involves the enucleation of mature oocytes. Transfer of this technology to ES cells is seriously hindered by their high nucleus to cytoplasm ratio compared with oocytes. Also, the small amount of cytoplasm

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provided by single ES cells may be limiting for significant nucleocytoplasmic interactions to take place. Here a suite of techniques is presented which address these issues. The reconstruction of an ES cell is used as an example to illustrate the potential applications of these methods.

2. Materials

2.1. Cells, Plasmids, and Chemicals

1. Mouse embryonic stem cells (D3 strain, feeder-free).
2. ES cell culture medium: Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal calf serum, 1 m*M* L-glutamine, 0.1 m*M* β-mercaptoethanol, and leukemia inhibitory factor (ESGRO).
3. Antibiotic selection medium: ES cell medium containing appropriate antibiotics, for example, 1.5 µg/mL puromycin for puromycin resistance, 600 or 200 µg/mL G418 for neomycin resistance, or 55 µg/mL hygromycin B for hygromycin resistance.
4. Plasmids carrying the *puro*^{R}, *neo*^{R} or *hygro*^{R} genes (*see* **Subheading 3.1.**), under the control of a promoter expressing in mouse ES cells (e.g., phosphoglycerate kinase [PGK]).
5. pECFP-Nuc (Clontech).
6. *Escherichia coli* strain DH5.
7. Restriction enzymes, T4 DNA ligase, and calf intestinal phosphatase enzyme.
8. Agarose gel equipment and ultraviolet (UV) source.
9. Electroporation buffer: 4.77 g/L HEPES, 8.0 g/L NaCl, 0.4 g/L KCl, 0.99 g/L, Na_2HPO_4, 10 m*M* β-mercaptoethanol, 1.08 g/L-glucose, pH 7.0.
10. Puromycin (Sigma).
11. G818 (Geneticin, Gibco).
12. Hygromycin B (Sigma).
13. Polyethylene glycol (PEG) MW 6000 (BDH). Make a 50% w/v solution in 150 m*M* HEPES, pH 7.5. Filter on 0.2 µm and store at 4°C.
14. Cytochalasin B (Sigma). Prepare a 3 mg/mL stock in dimethyl sulfoxide (DMSO; Merck) and keep at −20°C.
15. Bovine plasma cellular fibronectin (Sigma).
16. Neckless polycarbonate 50 mL of Sorvall centrifuge tubes, or equivalent.
17. Hoechst 33342 (Sigma). Prepare a 5 mg/mL stock in sterile nanopure water and store away from light at −20°C.

2.2. Specialized Equipment

1. Bio-Rad Gene Pulser or equivalent.
2. Sorvall high-speed centrifuge equipped with HB-4 rotor, or equivalent.
3. FACS equipped with FITC and UV_{2A} lasers (Becton Dickinson FACStar Plus or equivalent).
4. Phase contrast/epifluorescence microscope equipped with FITC and UV_{2A} filters.

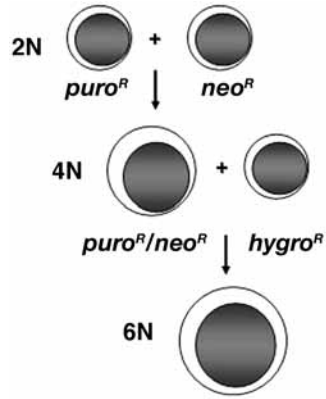

Fig. 1. Schematic representation of the generation of 4N and 6N hybrid embryonic stem cells using cells fusion and resistance to puromycin, neomycin, and hygromycin.

3. Methods

The methods below describe (1) the production of large, polyploid mouse ES cells by cell transfection, fusion, and antibiotic selection; (2) a technique for the enucleation of these cells; and (3) techniques for the transfer of novel nuclei to these cells before or after enucleation has taken place.

3.1. Production of Lines of Polyploid Mouse ES Cells

Mouse ES cells are small cells with a relatively large nucleus. In this step ES cells are prefused to each other to form larger, polyploid hybrid cells. The simple rationale behind prefusion is that larger cells will contain a larger cytoplasmic pool than normal diploid ES cells. **Subheading 3.2.** also shows that polyploid ES cells present advantages in term of enucleation. Stable lines of polyploid ES cells can be established by taking advantage of independent resistance mechanisms to the aminoglycoside antibiotics puromycin, neomycin, and hygromycin conferred by the genes *puro*^{R}, *neo*^{R}, and *hygo*^{R}, respectively. ES cells are stably transfected with plamids carrying either *puro*^{R} or *neo*^{R} (**Fig. 1**). These cells are then fused and stable 4N hybrids selected in the presence of both antibiotics. Following the same principle, 6N cells can be produced, where 4N cells resistant to puromycin and neomycin are fused with diploid ES cells transfected with a plasmid carrying resistance to *hygro*^{R} (**Fig. 1**).

3.1.1. Production of ES Cells Resistant to Neomycin, Puromycin, and Hygromycin

3.1.1.1. Generation of Antibiotic Resistance Plasmid

All cloning steps were carried out using standard molecular biology techniques *(6)*.

1. Puromycin resistance (conferred by a PGKpuro pA plasmid): Clone the *Eco*RI-TaqI fragment of the PGK promoter *(7)* in the multiple cloning site of pBluescript II KS(-), with the XbaI site at bp 677 changed to *Xho*I (pPGK). Then clone the *Hind*III-ClaI fragment of pBABEpuro *(8)* into a blunted *Pst*I/*Xba*I site of pPGK (pPGKpuro). Finally, insert the *Sac*I–*Xho*I fragment of the bovine growth hormone polyadenylation signal *(9)* into pPGKpuro.
   Neomycin resistance (conferred by an EF1α-ECFP-NLS-IRES-neo plasmid): Excise the ECFP-NLS fragment of pECFP-Nuc with a *Nhe*I/*BCl*I digest. Blunt-end the fragment and ligate into a blunt ended *Xho*I/*Not*I digest of pEF1α-IRES-neo, where the recombinant DNA and antibiotic resistance gene are transcribed as a single message under the control of the strong elongation factor 1α promoter *(10)*. In the methods described here cells transfected with this plasmid were employed solely for their resistance to neomycin.

Hygromycin resistance (conferred by a PGK-hygro plasmid): Clone the PGK promoter and the hygromycin resistance gene *(8)* into the multiple cloning site of pGEM7 (Promega).

3.1.1.2. Transfection of ES Cells

Feeder layer-independent mouse ES D3 cells *(11)* were cultured as described in *(12)*. Briefly, cells were cultured on tissue culture-grade plastic coated with 0.1% gelatin. Cells were passaged every 2–3 d and plated at a density of 500,000 cells/10-cm diameter dish. We routinely transfected ES cells by electroporation.

1. Trypsinize ES cells and perform a cell count.
2. Aliquot 5×10^6 cells in a centrifuge tube, pellet cells, and remove ES cell culture medium.
3. Resuspend cells in 2 mL of electroporation medium.
4. Repeat the centrifugation and resuspension step three times.
5. Finally resuspend cells in 0.5 mL of electroporation medium.
6. Mix 10 µg of the appropriate plasmid with the 5×10^6 ES cells in 0.5 mL of electroporation buffer.
7. Electroporate at 210 V and 960 µfd.
8. Wash electroporated cells three times with culture medium by successive steps of centrifugation and resuspension using fresh culture medium.
9. The next day, replace medium with the appropriate antibiotic selection medium and select stable transfectants over the next 7–10 d.

3.1.2. Fusion, Selection, and Characterization of Hybrid Cells

The next steps describe the production of polyploid hybrid ES cells:

1. Tryspsinize ES cells stably transfected with plasmids carrying resistance to puromycin and neomycin, respectively, and mix 2×10^6 of each cell type at a 1:1 ratio.
2. Pellet the cells and add 1 mL of 50% PEG_{6000} solution prewarmed at 37°C (*see* **Note 1**).
3. Pipet cells up and down twice, and incubate for 2 min at 37°C while gently stirring.
4. Wash cells three times with 3 mL of phosphate-buffered saline (PBS; Ca^{2+}- and Mg^{2+}-free), resuspend in ES cell medium, and put back into culture into two 100-mm tissue culture dishes.
5. Starting the next day, select cells for 6 d with 1.5 µg/mL puromycin and 600 µg/mL G418 in ES cell culture medium.
6. Stably transfected, 4N hybrid cells, which can be maintained in the presence of 1.5 µg/mL puromycin and 200 µg/mL G418, are obtained.
7. Expand hybrid cells and cryopreserve aliquots of 10^6 cells.
8. To generate 6N cells, repeat **steps 1–4** to fuse puromycin/neomycin-resistant cells (described previously) with stable hygromycin-resistant ES cells.
9. Select cells for 6 d with 600 µg/mL G418 and 55 µg/mL hygromycin B in ES cells culture medium. Stable, 6N hybrid cells are obtained that can be maintained in the presence of 200 µg/mL G418 and 22.5 µg/mL hygromycin B and cryopreserved.

Cell ploidy can be rapidly established by conventional propidium iodide or Hoechst flow cytometry, and confirmed by karyotyping. **Figure 2** illustrates some of the morphological and gene expression properties of 4N cells obtained with the method described in **Subheading 3.1.2.** (*see* **Note 2**).

3.2. Enucleation of ES Cells: Preparation of Cytoplasts and Karyoplasts

A variety of techniques exist for cell enucleation. Enucleation of oocytes for SCNT typically is conducted by aspiration of the second polar body and the associated cytoplasm (including the oocyte's chromosomes) by suction while the oocyte is immobilized with a holding pipet ***(13)***. Even in polyploid form, ES cells cannot be micromanipulated easily. Also, using micromanipulation for the production of enucleated ES cells would be time-consuming and defeat the purpose of having recipient cells accessible in large numbers. Methods have been previously developed for the bulk enucleation of adherent cultured cells such as myoblasts ***(14)***, fibroblasts ***(15)***, and HeLa cells ***(16)***. These techniques were adapted and optimized for the enucleation of polyploid ES cells and involve growing cells on small tissue culture disks, which are placed cells facing down

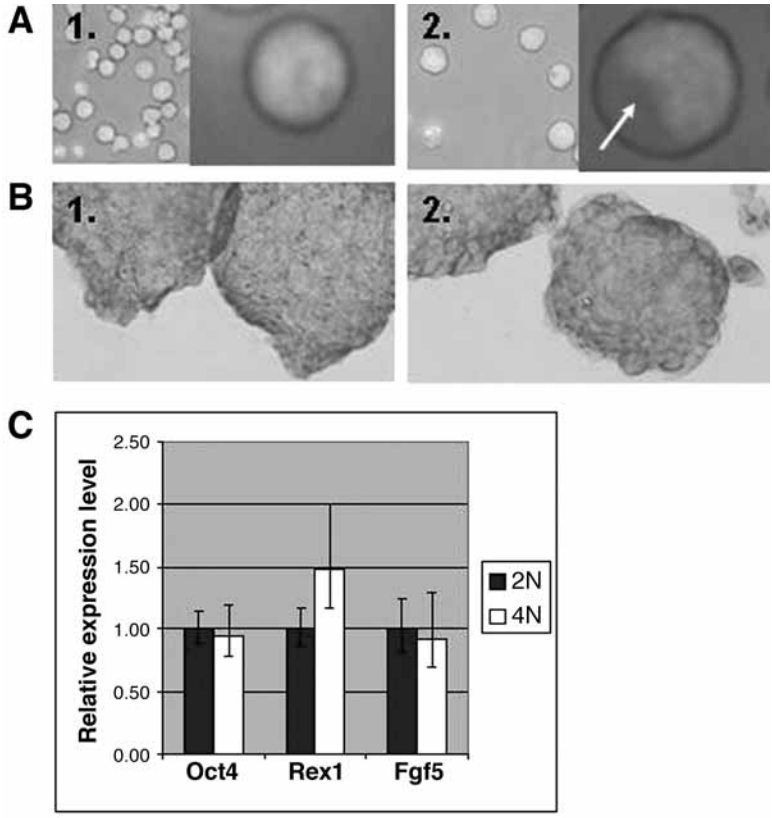

Fig. 2. Properties of 4N hybrid embryonic stem (ES) cells generated by the fusion of mouse ES cells. Cell ploidy was first determined by Hoechst 33342 flow cytometry and later confirmed by karyotyping. **(A)** Cells in suspension stained with Hoechst 33342 and viewed under phase contrast and UV_{2A}. Hybrid cells show a larger diameter **(2)** and a well visible cytoplasmic compartment (**2**, arrow) compared with diploid cells **(1)**. Pictures taken at ×400 magnification. **(B)** Hybrid ES cells **(2)** grow in compact colonies as do diploid cells **(1)** and retain the expression of the pluripotent marker alkaline phosphatase (Sigma kit 86-R). Pictures taken at ×200 magnification. **(C)** Quantitative reverse transcription-polymerase chain reaction shows that the levels of expression of the genes *Oct-4*, *Rex1*, and *Fgf5* in hybrid cells are non-significantly different compared with that of control diploid cells (Applied Biosystems SYBR Green PCR Master MIX and an ABI PRISM 5700 Real-Time Detection System). For each marker results are expressed as a percentage of the level measured in diploid cells. In diploid cells the measured level of *Fgf5* was down by 125-fold compared with pluripotent marker *Oct-4*, and 20-fold compared with pluripotent marker *Rex1*. Results are shown in quadruplicate ± SE.

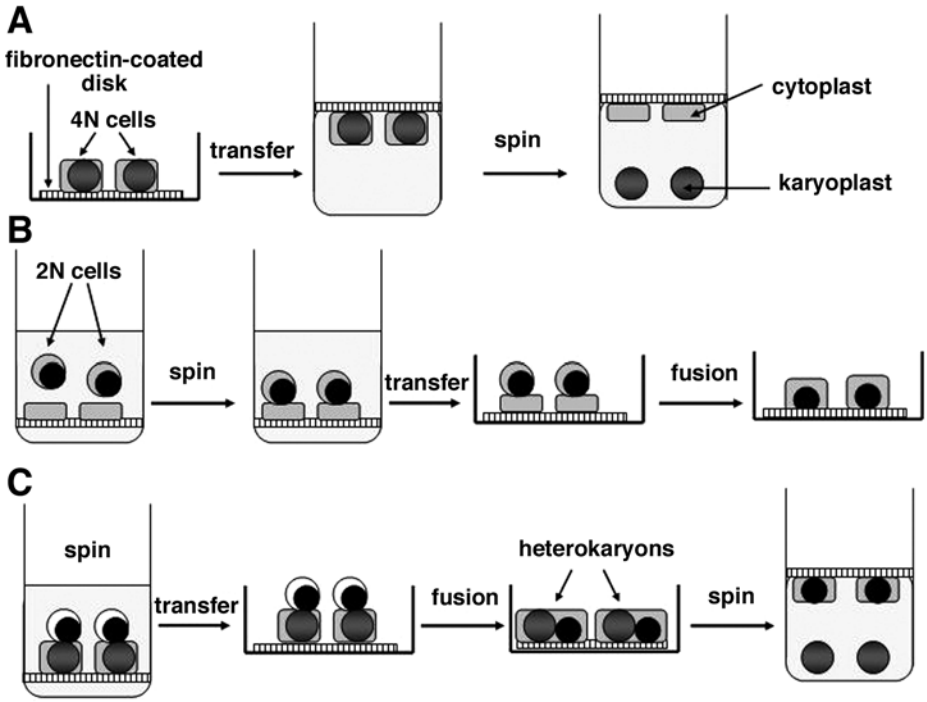

Fig. 3. Schematic representation and embryonic stem (ES) cell enucleation and nuclear transfer (NT) techniques. **(A)** Centrifugal enucleation of polyploid ES cells and production of cytoplasts and karyoplasts. **(B)** NT by fusion of karyoplasts to cytoplasts. **(C)** NT by formation of heterokaryons followed by differential centrifugal enucleation. *See* **Subheadings 3.2.** and **3.3.** for details.

in centrifuge tubes after treatment with the actin depolymerization agent cytochalasin B. Centrifugation results in the expulsion of karyoplasts (nuclei surrounded by a thin rim of cytoplasm and plasma membrane), which can be collected as a pellet at the bottom of the tube, whereas enucleated cells (cytoplasts) remain attached to the disks and are readily available for further manipulation (**Fig. 3A**; *see* **Note 3**).

1. Cut enucleation disks of 20 mm in diameter out of tissue culture dishes, using a circular heated metal device as described in Celis and Celis ***(16)***.
2. Wash disks in 70% ethanol, rinse in sterile nanopure water, and sterilize under UV light.
3. Evenly coat the tissue culture treated surface of the disks with 600 µL of cellular fibronectin, diluted at 10 µg/mL in sterile nanopure water, and let dry overnight under a laminar flow. This corresponds to 1 µg of cellular fibronectin/cm^2 (*see* **Note 4**).

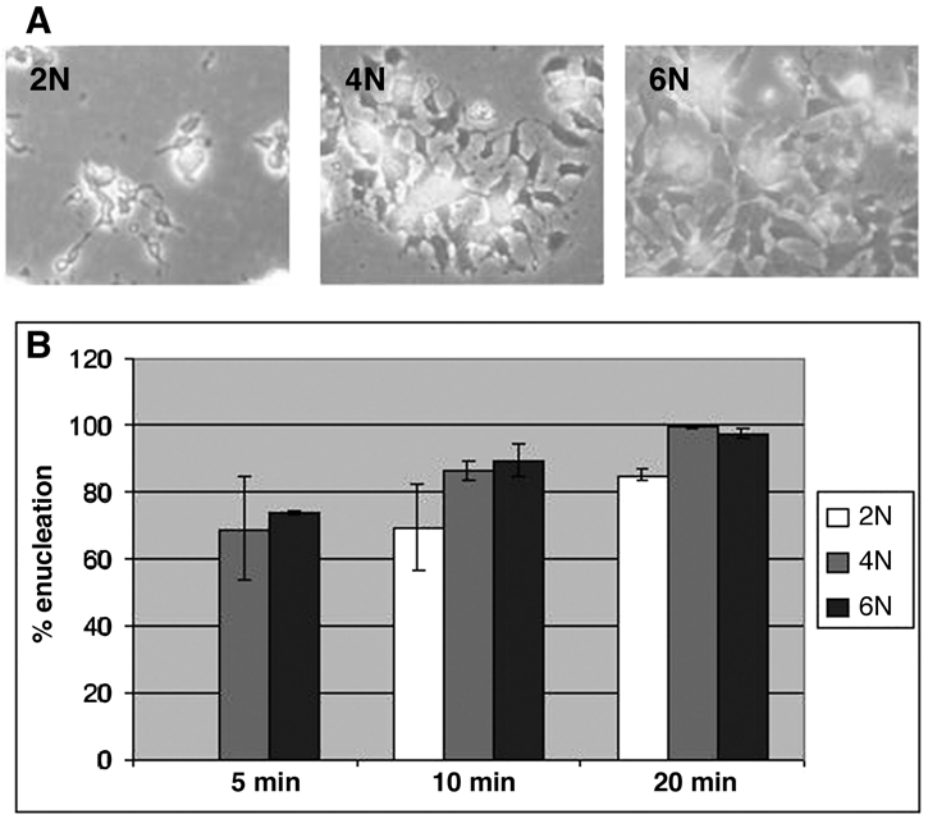

Fig. 4. Centrifugal enucleation of embryonic stem (ES) cells. **(A)** Cytoplasts and nucleated ES cells attached to enucleation disks. Cells were stained with Hoechst 33342 and are viewed under phase contrast and UV_{2A}. **(B)** Effect of ploidy and centrifugation time on ES cells's enucleation. Pictures taken at ×200 magnification. *See* **Subheading 3.2.** for details.

4. Place the disks in six-well tissue culture trays containing 4 mL of ES culture medium per well.
5. Plate 300,000 diploid or polyploid ES cells per well.
6. Culture for 48–72 h or until cells are ready to be passaged.
7. Scrape off the cells that have attached at the back of disks. Wash the disks twice with ES culture medium to eliminate loosely attached or dead cells.
8. Treat cells for 5 min with 3 µg/mL cytochalasin B in ES culture medium at 37°C.
9. Using sterile forceps, carefully place disks cells facing down in centrifuge tubes containing 3 mL of ES culture medium supplemented with 3 µg/mL cytochalasin B. Cover each tube with a piece of sterile aluminum foil.
10. Thoroughly clean the HB-4 rotor with 70% ethanol.

11. Centrifuge for 5 min or as indicated in **Fig. 4B** at 14,500*g* in the HB-4 rotor at 20°C (*see* **Note 5**).
12. Remove disks from centrifuge tube, and place with cells facing up in a fresh six-well tray containing 4 mL of ES cell culture medium per well.
13. Carefully wash the disks three times with ES culture medium.
14. Allow cells to recover for 30 min in incubator.
15. Stain cells with ES culture medium containing 5 µg/mL Hoechst 33342 for 5 min at 37°C.
16. Visualize disks under phase contrast and UV_{2A}. Cytoplasts clearly appear devoid of a fluorescent blue nucleus (**Fig. 4A**).
17. Karyoplasts are found as a pellet at the bottom of the centrifuge tube. Carefully aspirate all medium and resuspend the pellet in 2 mL of ES cell culture medium.
18. To separate karyoplasts from dead or detached cells, plate the karyoplasts suspension onto a 35-mm gelatinized tissue culture dish, and place in incubator for 2 h. Karyoplasts do not attach to the tissue culture dish and can be carefully scooped up. Karyoplasts should be used immediately for reconstruction (*see* **Subheading 3.2.**).

Figure 4A shows that applying this method to 4N and 6N ES cells results in the generation of well defined cytoplasts. By contrast, applying this method to 2N cells causes more cell detachment rather than enucleation, and the resulting cytoplasts are small and damaged. **Figure 4B** shows that enucleation rate was found to be dependent on centrifugation time and cell ploidy. For instance, after 5 min of centrifugation, there was no measurable enucleation of 2N ES cells, whereas 69% of 4N cells were enucleated. After 10 min of centrifugation, the enucleation rate had reached 70% for 2N cells and 86% for 4N cells. This property is exploited in **Subheading 3.3.2.** for the selective elimination of polyploid nuclei from heterokaryons (*see* **Note 6**).

3.3. NT to Mouse ES Cells

Two different methods are shown by which NT to polyploid mouse ES cells can be performed. **Subheading 3.3.1** shows how viable cells can be generated by fusing cytoplasts and karyoplasts prepared as described in **Subheading 3.2.** (**Fig. 3B**). **Subheading 3.3.2** describes an alternative method in which polyploid cells are first fused with diploid cells to form a heterokaryon, and selective differential centrifugation conditions are applied in which the polyploid nucleus is removed and the donor nucleus retained (**Fig. 3C**). Compared with nuclear transfer to a cytoplast, this method has the advantage of permitting two-way nucleocytoplasmic interactions between donor and recipient cells and the replenishing of cytoplasmic factors before enucleation is carried out. Previous work on has shown that significant changes in gene expression and differentiation can occur in somatic cell heterokaryons in the absence of DNA replication ***(17)***.

3.3.1. NT to Enucleated ES Cells: Fusion of ES Cell Karyoplasts to Cytoplasts

1. Prepare disks of cytoplasts from polyploid ES cells as described in **Subheading 3.2.**
2. Prepare karyoplasts from diploid ES cells as described in **Subheading 3.2.** (First stain diploid ES cells for 30 min with 5 µg/mL Hoechst 33342 at 37°C and wash three times with ES cells culture medium.)
3. Using sterile forceps, carefully place cytoplasts disks cells facing up in empty centrifuge tubes.
4. Dilute karyoplasts to a total volume of 5 mL ES cell culture medium, and gently pipet over the disks. Use 6×10^5 karyoplasts per disk.
5. Cover with a sterile piece of aluminum foil and centrifuge for 15 min at 14,500*g* at 20°C in the HB-4 rotor.
6. Carefully remove disks from the centrifuge tubes and place in an empty six-well tissue culture tray. Carefully aspirate as much remaining medium as possible from under the disks. Examine under the microscope to ensure that the karyoplasts have spun onto the cytoplasts.
7. Carefully pipet 300 µL of 50% PEG_{6000} solution prewarmed at 37°C onto the cells and incubate at 37°C for 2 min.
8. Gently wash the disks four times with 4 mL of PBS (Ca^{2+}- and Mg^{2+}-free) prewarmed at 37°C, and add 4 mL of ES cell culture medium.
9. Observe the disks for colony formation.

Figure 5 shows an example of ES cell reconstruction where karyoplasts were prepared from ES cells expressing the stabilized form of cytoplasmic green fluorescent protein (GFP; enhanced EGFP, Clontech). GFP is not detectable in these karyoplasts under epifluorescence microscopy owing to the minimal presence of cytoplasm (**Fig. 5B2**). However, cells obtained from the recontruction of these karyoplasts with ES cell cytoplasts express GFP (**Fig. 5C**). A control carried out with karyoplasts alone shows a small number of karyoplasts can regenerate without reconstruction (**Fig. 5C**). Therefore reconstruction of cells should be confirmed using cytoplasmic or plasma membrane markers.

3.3.2. Formation of Heterokaryons and Postfusion Enucleation

1. Prepare disks of 4N or 6N cells ES cells as described in under **steps 1–6** of **Subheading 3.2.** Cells must express a cytoplasmic fluorescent marker such as GFP.
2. Stain a 100-mm dish of 2N ES cells with 5 µg/mL Hoechst 33342 in ES culture medium for 30 min at 37°C.
3. Aspirate stain containing medium, and wash cells three times in PBS.
4. Trypsinize cells, and resuspend in 2 mL of ES cell culture medium.
5. Spin 6×10^5 2N ES cells onto each disk as described in **steps 4** and **5** of **Subheading 3.3.1.**
6. Conduct PEG fusion as described in **steps 7–9** of **Subheading 3.3.1**.

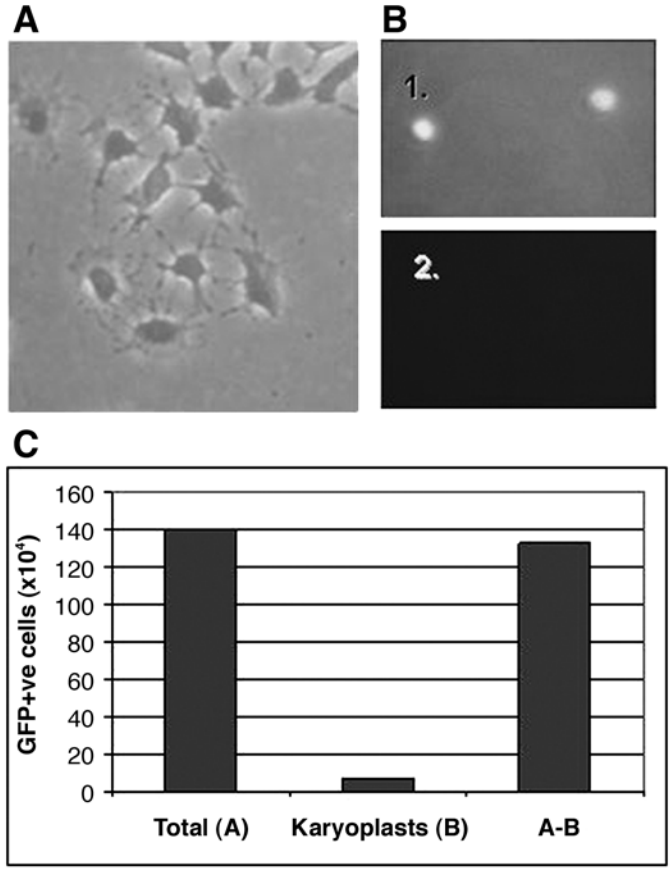

Fig. 5. Reconstruction of embryonic stem (ES) cells by fusion of karyoplasts with cytoplasts. **(A)** Cytoplasts prepared from 4N ES cells. Cytoplasts were stained with Hoechst 33342 and are viewed under phase contrast and UV_{2A}. **(B)** Karyoplasts prepared from green fluorescent protein (GFP) + ve 2N ES cells. Karyoplasts were stained with Hoechst 33342 and are viewed under phase contrast and UV_{2A} (1) or for GFP (2). **(C)** Nucleated GFP positive cells recovered per disk 5 d after reconstruction. *See* **Subheading 3.3.1.** for details.

7. Return cells to incubator for recovery overnight or for 16 h (*see* **Note 7**).
8. Submit the disks to 5 min of centrifugal enucleation by following **steps 7–11** in **Subheading 3.2**.
9. Trypsinize the cells off the disks, and resuspend in 2 mL of ES cells culture medium with 5% fetal calf serum only.
10. Sort cells for co-localization of GFP and Hoechst 33342 and for size. **Figure 6B1** shows that following differential centrifugal enucleation a population of cells can be identified that are both positive for GFP and Hoechst 33342. These double-positive cells are found in a range of sizes, some of which corresponded in size with 2N ES cells and are selected for sorting (**Fig. 6B2**).

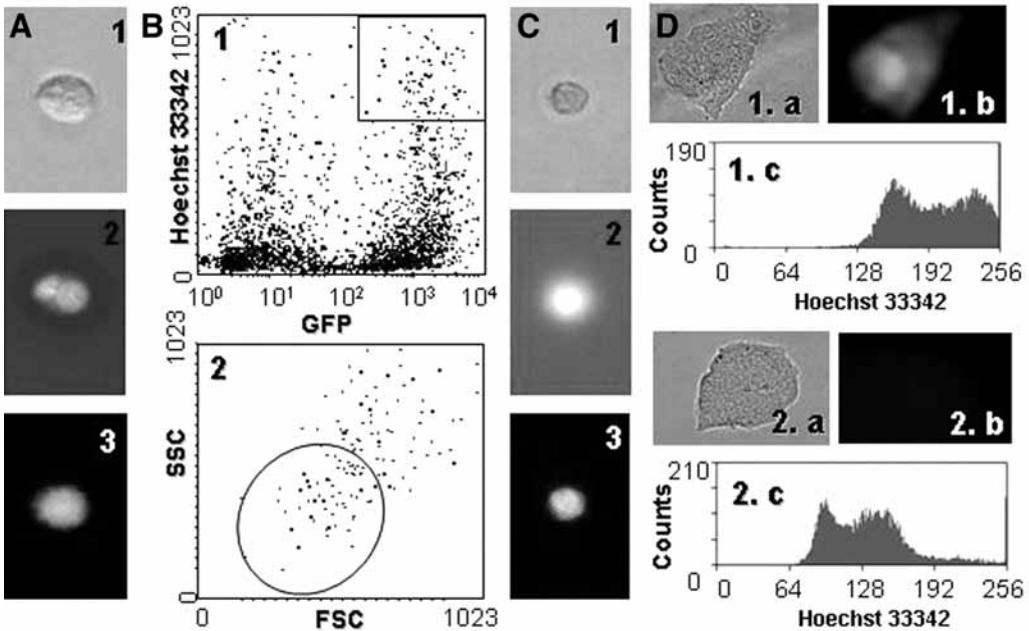

Fig. 6. Nuclear transfer to embryonic stem (ES) cells by postfusion enucleation. **(A)** Heterokaryon formed by the fusion of a Hoechst 33342-stained diploid ES cell and a green fluorescent protein (GFP)-positive tetraploid ES cell. The tetraploid nucleus was revealed following a second round of Hoechst 33342 staining. (1) Phase contrast. (2) Hoechst 33342. (3) GFP. Pictures taken at ×400 magnification. **(B)** Flow cytometry profile of cells following post-fusion enucleation. (1) GFP vs Hoechst 33342. Cells within the rectangular gate show co-localization of both markers. (2) Scattergram of cells selected for co-localization of Hoechst 33342 and GFP. Cells within the elliptic gate have a diploid ES cell profile and were selected as having undergone elimination of the tetraploid nucleus. **(C)** Hoechst 33342 and GFP-positive sorted cell showing the presence of a single small nucleus. (1) Phase contrast. (2) Hoechst 33342. (3) GFP. Pictures taken at ×400 magnification. **(D)** Properties of cells grown clonally after post-fusion enucleation. (1) Control GFP-expressing tetraploid cells. (2) Clonal population of 2N cells grown after postfusion enucleation. (a) Bright field. (b) GFP. (c) Hoechst 33342 flow cytometry. Ploidy was confirmed by karyotyping. Pictures taken at ×200 magnification.

11. Replate sorted cells at 200 cells per well of a 24-well tissue culture tray, where wells have been coated with cellular fibronectin as described under **step 3** of **Subheading 3.2**.
12. Monitor for colony formation and GFP expression. Colonies forming from heterokaryons where the polyploid nucleus has been successfully expelled will loose GFP expression and possess a 2N ploidy (**Fig. 6D**; *see* **Note 8**).

4. Notes

1. We found that mouse ES cells can be fused using a variety of methods, including electrofusion of cells in suspension and PEG fusion of monolayers of cells in coculture. PEG fusion of cells in suspension represents the most efficient way to fuse ES cells in bulk. The fusion efficiency achieved varies between 5 and 10%. Spontaneous fusion in the absence of PEG is also observed, which is consistent with the reported occurrence of spontaneous fusion between ES cells and differentiated cells *(3–5)*.
2. Hypoxanthine phosphoribosyl transferase-deficient ES cells have also been used to generate hybrids between ES cells and differentiated cells *(1,5)*, and could be used to generate ES–ES hybrids in conjunction with antibiotic resistance. Polyploid ES cells tend to revert to a normal diploid karyotype, and their polyploid karyotype needs to be enforced.
3. We found that polyploid ES cells can also be enucleated by a combination of cytochalasin B treatment and centrifugation on a Percoll density gradient as described in Tatham et al. *(18)*. However, cytoplasts appeared too light and fragile to be recovered and washed off the Percoll without major loss.
4. Coating enucleation disks with cellular fibronectin rather than gelatine improves cell attachment and favors enucleation as opposed to cell detachment.
5. The Sorvall HB-4 is a swing-out rotor, and ensures a relatively uniform enucleation over the disks. A fixed-angle rotor like the SS-34 can be used, but will result into nonuniform enucleation and localized stretching of the cytoplasts along the disks.
6. There is a trade-off between enucleation efficiency and cell detachment. Complete enucleation is accompanied by more significant cell detachment than partial enucleation.
7. At this stage, the cells could be treated with a cell cycle arresting agent in order to maintain separation of the two nuclei. However we observed that most cells do not undergo division for about 24 h after PEG fusion and sorting.
8. If donor and recipient cells from different genetic backgrounds are employed microsatellite DNA polymorphism can be used to confirm the identity of recovered cells. Because of the use of cytochalasin B, the growth of cells is slowed down for about 2 d.

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METHODS IN MOLECULAR BIOLOGY™

Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

Edited by

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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20

Gene Expression and Methylation Patterns in Cloned Embryos

Christine Wrenzycki, Doris Herrmann, Claudia Gebert, Joseph W. Carnwath, and Heiner Niemann

Summary

A considerable proportion of the offspring, in particular in ruminants and mouse, born from nuclear transfer (NT)-derived and in vitro-produced (IVP) embryos are affected by multiple abnormalities, of which a high birthweight and an extended gestation length are the predominant features; a phenomenon that has been termed "Large Offspring syndrome" (LOS). According to a current hypothesis, LOS is caused by persistent aberrations of expression patterns of developmentally important genes starting as early as at the preimplantation stages. The underlying mechanisms are widely unknown at present, but epigenetic modifications of embryonic and fetal gene expression patterns, primarily caused by alterations in DNA methylation are thought to be involved in this syndrome. Appropriate DNA methylation is essential for regular transcription during mammalian development and differentiation. Sensitive reverse transcription polymerase chain reaction assays allow the study of messenger RNA (mRNA) expression levels of specific genes in single embryos. The methylation status of a specific gene can be assessed by bisulfite sequencing. Studies to unravel mRNA expression patterns from IVP- and NT-derived embryos have revealed numerous aberrations ranging from suppression of expression to de novo overexpression or more frequently to a significant upregulation or downregulation of a specific gene. mRNA expression patterns from in vivo-derived embryos are essential as the "physiological standard" against which the findings for IVP and NT-derived embryos are to be compared. Unraveling the underlying molecular mechanisms will contribute to the production of viable embryos and aid to improve biotechnologies applied to early mammalian embryos.

Key Words: Embryo; gene expression patterns; epigenetics; in vitro production; nuclear transfer; DNA methylation.

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1. Introduction

Unusually large offspring have been born after the transfer of bovine and ovine embryos that had either been subjected to somatic nuclear transfer (NT) or exposed to an unusual in vivo or in vitro environment. This phenomenon is called "Large Offspring syndrome" (LOS) and comprises a variety of abnormal phenotypes, including significant increases in birthweight, polyhydramnios, hydrops fetalis, altered organ growth, various placental and skeletal defects, immunological defects, and increased perinatal death *(1–4)*. Unusual phenotypes also have been observed in other species, such as mice and humans *(5–7)*. Epigenetic modifications in early development have been implicated in the etiology of LOS *(8,9)*. This has prompted interest in epigenetic modulation of gene expression patterns in early embryos predominantly by using sensitive reverse transcription polymerase chain reaction (RT-PCR) assays *(10,11)*.

Epigenetics is defined as gene-regulating activity not directly related to the DNA sequence itself that can persist through one or more generations. According to current knowledge, it is primarily related to methylation of the DNA sequence, but other chromatin modifications, such as acetylation, or histone changes, are likely involved as well. DNA methylation is crucially involved in imprinting, X-chromosome inactivation, genome stability, silencing of retrotransposons, tissue-specific gene expression, and activation or inactivation of genes in cancer *(12)*. In mammalian cells, DNA methylation occurs predominantly at CpG dinucleotides and is catalyzed by two important classes of specific enzymes. The *DNA methyltransferase* 1 (Dnmt1) is responsible for maintaining methylation marks at hemi-methylated CpG dinucleotides after DNA replication *(13)*. *Dnmt3a* and *Dnmt3b* are required for *de novo* methylation in vivo and for establishing new DNA-methylation patterns during development *(14,15)*. Likewise, the oocyte-specific isoform *Dnmt1o* *(16)* is responsible for maintaining but not establishing maternal imprints. Dnmt3L, a protein that by itself has no DNMT activity, colocalizes with *Dnmt3a* and *Dnmt3b*, and is thought to be essential for establishing methylation imprints in the female germ line *(17,18)*. The most dramatic changes in the methylation level occur during gametogenesis and early embryonic development *(19,20)*.

The regular wave of demethylation and methylation has been thought to be essential for normal embryonic development *(21–23)*. The reprogramming events in the zygote via active paternal demethylation, passive demethylation in early cleavage stages followed by *de novo* methylation appear to be conserved in mammals, although their relative timing with respect to developmental stages can differ *(24)*. In mouse embryos, *de novo* methylation occurs in the inner cell mass cells of the expanded blastocyst, whereas in bovine embryos *de novo* methylation is found already from the 8- to 16-cell stage onward *(24)*.

1.1. Somatic NT and/or IVP Systems Affect Gene Expression Patterns in Generated Embryos

We have developed the hypothesis that persistent deviations from the normal gene expression pattern starting in the early embryo are causally involved in the etiology of LOS *(9–11)*. To gain insight into mechanisms causing LOS, we have initiated studies to unravel gene expression patterns in IVP and cloned embryos and have employed in vivo bovine embryos as the "physiological standard."

Extensive studies from our laboratory employing a sensitive semiquantitative RT-PCR assay (described in **Sections 2** and **3**) have shown that culture conditions affect the well-orchestrated expression pattern of developmentally important genes in bovine embryos *(25–29)*. In these studies, substantial evidence has been accumulated that bovine embryos produced in vitro differ from their in vivo-derived counterparts with regard to messenger RNA (mRNA) expression. The relative abundance of a set of developmentally important "marker genes" was analyzed in single embryos cultured in vitro under various conditions *(26,27,29,30)*. These gene transcripts are involved in compaction and cavitation ([*Cx43*], desmosomal protein plakophilin [*Plako*], desmosomal glycoproteins, desmocollin II and III [*Dc II* and *III*]), energy metabolism (*glucose transporter-1 [Glut-1]*), RNA processing (*polyA polymerase [PolyA]*), stress-response (*heatshock protein 70.1 [Hsp 70.1]*), growth factor signaling (*insulin-like growth factor family [IGF-1* and *-2, IGF-1* and *IGF-2 receptor]*), maternal recognition of pregnancy (*interferon tau [IF* **t***]*), and X-chromosome-specific expression (*phosphoglycerate kinase [PGK]*, *glucose-6-phosphate dehydrogenase [G6PD]*, *X-inactivating substance [Xist]*). Transcription of the majority of the genes was affected by serum-free conditions leading to generally increased mRNA abundances over that in embryos cultured in serum-enriched culture medium *(26)*, suggesting a stress response of the early embryo to suboptimal culture conditions. However, it was shown that the basic culture system (tissue culture medium vs synthetic oviduct fluid [SOF]), including basic medium composition, gas atmosphere, and oxygen tension, was responsible for major changes in the mRNA pattern of the developing bovine embryo and under these conditions the protein source (polyvinyl alcohol [PVA], bovine serum albumin [BSA], serum) was not a major source of variation in mRNA expression patterns *(27)*. The use of chemically defined culture conditions allowed the production of embryos with an identical mRNA expression pattern in two different laboratories, which is an important step in the production of embryos with comparable quality and developmental potential *(27)*. In an extension of the aforementioned studies, two diverging culture systems (tissue culture medium supplemented with serum vs SOF supplemented with PVA) were shown to affect the expression of genes from the IGF family (*IGF-1* and *-2*, *IGF-1* and *IGF-2 receptor [30]*). Interestingly, IGF-1 expression was not detected at any developmental stage, indicating a paracrine IGF-1 mechanism. Significant differences

were found with regard to timing and magnitude of gene expression levels of the other transcripts between the two culture systems *(30)*. In vitro culture also affected dosage compensation of X-linked genes, that is, *PGK* and *G6PD*. Dosage compensation of *PGK* was delayed or even completely lacking for *G6PD* in IVP embryos, although Xist transcripts were highly abundant in embryos of in vitro origin *(28)*.

Recently, for the first time a link between mRNA expression patterns and in vivo development of embryos derived from IVP was established and embryos with aberrant mRNA expression patterns led to a high frequency of increased birthweights *(31)*. In vitro culture of bovine embryos in the presence of serum or BSA significantly increased the relative abundance of the transcripts for *Hsp*, *Cu/Zn-SOD*, *Glut-3*, *Glut-4*, *bFGF*, and *IGFI-R* when compared with embryos from in vivo production. Birthweights of calves derived from embryos out of the IVP system were significantly higher than those of calves derived from temporary culture in sheep oviducts, superovulation, or artificial insemination (52.8 ± 9.4 kg [SOF + BSA] and 56.7 ± 12.1 kg [SOF + serum] vs 44.1 ± 5.5 kg [sheep oviduct], 41.1 ± 3.0 kg [superovulation], and 43.4 ± 4.3 kg [AI] *[31]*). These results support the hypothesis that persistent deviations from the normal expression pattern early in development are causally involved in the incidence of LOS, in particular in increased birthweights. Probably, the abnormalities observed in gene expression patterns of IVP embryos are not related to the maturation process of the oocyte. Embryos derived from in vitro- or in vivo-maturated oocytes did not display significant differences in the relative abundance of several marker genes, that is, *Glut-1*, *Dc II*, *Plako*, and *E-cadherin* transcripts *(32,33)*. Further studies involving more gene transcripts are needed to confirm this observation.

NT is not only associated with an extended in vitro culture phase of the reconstructed embryo, but its success is critically dependent on correct reprogramming of the genomic program of the transferred somatic nucleus. Few studies have investigated the mRNA expression patterns in bovine NT-derived vs control embryos. From 16 genes that were studied using a qualitative RT-PCR assay and whose expression was compared with that of in vivo- or in vitro-produced embryos, six were differentially expressed between NT, in vivo, or in vitro counterparts. In particular, mRNA for *fibroblastic growth factor (FGF)-4*, which was not found in NT-derived embryos but in their in vivo counterparts, was suggested to be a suitable marker to assess the quality of NT-reconstructed embryos *(34)*. The spatial perturbation of the expression pattern for the transcription factor *Oct-4* and an incomplete inactivation of *Oct-4*-related genes were suggested as important factors involved in the embryonic and fetal lethality of somatic cell-derived mouse clones *(35,36)*. However, these latter results cannot be directly extrapolated to other species as *Oct-4* regulation has been shown to be different in cattle

and pig embryo development *(37)*. However, recent findings for bovine blastocysts emphasize the need for a tightly regulated spatial expression in the two lineages of the bovine blastocyst *(38)*.

We have investigated changes in mRNA expression patterns related to distinct modifications of the NT protocol *(28)*. Fusion of the donor cell and cytoplasm before activation or simultaneous activation and fusion resulted in significant alterations of gene expression patterns compared with IVP and in vivo-produced "control" embryos. Aberrant expression patterns for NT embryos were related to genes thought to be critically involved in stress adaptation (*Hsp 70.1*), trophoblastic function (*interferon* **t**, *Mash2*), and *Dnmt1* during preimplantation development. Surprisingly, we did not find significant alterations for the *IGF-II receptor* gene, which is subject to imprinting in mice and humans and has been thought to be a primary candidate responsible for the pathological observations made in NT-derived fetuses and neonates.

NT also affected mRNA expression of X-chromosome linked genes *(28)*. Collectively, these findings show that type and treatment of the donor cells affect the mRNA expression pattern of the reconstituted embryos and can thus contribute to deviations from the normal mRNA pattern. Genome-wide epigenetic alterations, in particular methylation losses, have been observed in cloned bovine fetuses and were associated with developmental failures *(39)*.

A recent comparative analysis from our laboratory, including *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Xist* mRNA expression patterns in donor cells, in vitro-matured oocytes, and one-cell and eight-cell embryos derived from different NT protocols, revealed that the *Dnmt* mRNAs could not be detected in the donor cells, whereas expression of these transcripts was determined in matured oocytes and 1-cell embryos (**Fig. 1** *[11]*). At the eight-cell stage, *Dnmt1* transcripts were decreased compared with one-cell stages. *Xist* mRNA levels were detected in the donor cells but were not found in matured oocytes and one-cell embryos. At the eight-cell stage, a significant increase was observed in the experimental groups, i.e., fusion before activation and activation and fusion simultaneously, with the exception of eight-cell embryos, which had been generated by the use of preactivated cytoplasts as recipient cells. At the blastocyt stage, *Xist* (females only) and *Dnmt1* transcripts were significantly increased in in vitro-produced and NT-derived embryos compared with their in vivo-generated counterparts. However, no differences were observed for *Dnmt3a* and *Dnmt3b* transcripts in embryos of in vivo or in vitro origin *(11,27)*, whereas the relative abundance of *Dnmt3a* transcripts in NT-derived embryos was increased compared to in vivo generated, parthenogenetic and IVP blastocysts. *De novo* methylation occurs in bovine embryos at the 8- to 16-cell stage *(24)*, which is consistent with the major activation of the bovine embryonic genome *(40)* and suggests that *Dnmt3a* or *Dnmt3b* already are activated at this developmental

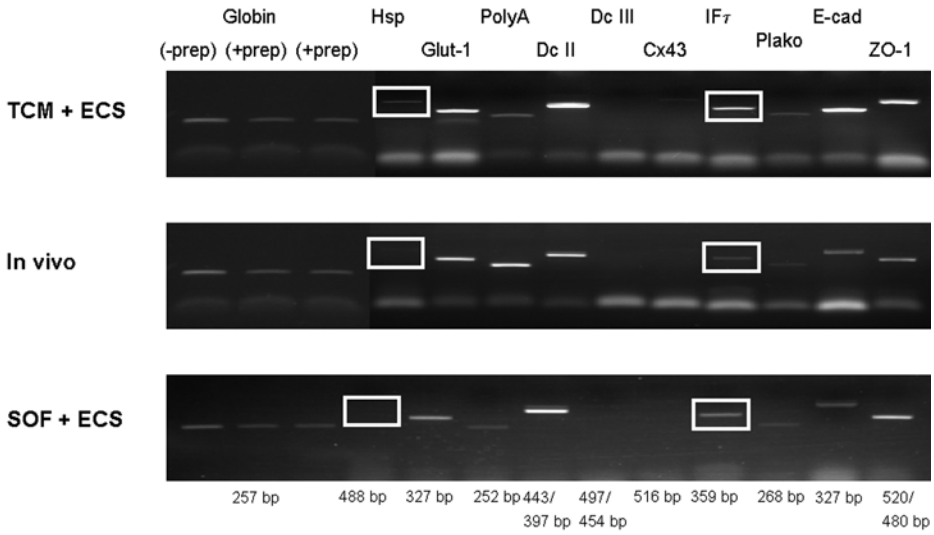

Fig. 1. Representative gel photographs showing differences in the relative amount of Hsp and interferon τ transcripts (white boxes) in bovine blastocysts generated in tissue culture medium (TCM) supplemented with serum (TCM + estrous cow serum [ECS]), in vivo, or in synthetic oviduct fluid (SOF) plus serum (SOF + ECS).

stage. In contrast, our findings showed an increased relative abundance of *Dnmt3a* and *Dnmt3b* transcripts only from the eight-cell stage to the blastocyst. The 8- to 16-cell embryo in murine development is the stage at which Dnmt1o enters the nucleus for one cell cycle to get involved in the maintenance of imprinted methylation ***(16)***. Speculatively, there is analogy with the mouse and the oocyte form of *Dnmt1* that is excluded from the nucleus, whereas the somatic form of the enzyme is introduced with the somatic donor nucleus and associates with it. Our results show that at the blastocyst stage maintenance and de novo DNA methylation are affected by the in vitro culture and/or the cloning procedure ***(38)***. In cloned mouse embryos, *Dnmt1* was aberrantly expressed both with regard to localization and timing suggesting a contribution to the developmental abnormalities ***(41)***. In conclusion, these findings show that we are at the beginning to unravel failures in early development with sensitive technologies.

2. Materials

2.1. Semiquantitative RT-PCR

1. Washing medium for embryos: Phosphate-buffered saline plus 0.1% PVA.
2. Rabbit globin RNA (BRL, Gaitersburg, MD).

3. Lysis buffer: 100 m*M* Tris-HCl, pH 8.0, 500 m*M* LiCl, 10 m*M* EDTA, 1% lithium dodecyl sulfate, 5 m*M* dithiothreitol.
4. Dynabead mRNA Direct Kit (Dynal, Oslo, Norway).
5. Buffer 1: 10 m*M* Tris-HCl, pH 8.0, 150 m*M* LiCl, 1 m*M* EDTA, 0.1% lithium dodecylsulfate.
6. Buffer 2: 10 m*M* Tris-HCl, pH 8.0, 150 m*M* LiCl, 1 m*M* EDTA.
7. Sterile water.
8. Random hexamers (Perkin-Elmer).
9. RT buffer: 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3.
10. $MgCl_2$.
11. dNTPs.
12. RNase inhibitor.
13. Moloney murine leukemia virus reverse transcriptase.
14. PCR buffer: 20 m*M* Tris-HCl, pH 8.4, 50 m*M* KCl.
15. Sequence specific primer pairs.
16. Taq DNA Polymerase.
17. Agarose.
18. TBE buffer: 90 m*M* Tris, 90 m*M* borate, 2 m*M* EDTA, pH 8.3.
19. Ethidium bromide.

2.2. Bisulfite Sequencing

1. Lysis buffer: 10 m*M* Tris-HCl, 10 m*M* EDTA, 1% sodium dodecyl sulfate.
2. Proteinase K (10 mg/mL).
3. Yeast tRNA (11 mg/mL).
4. Phenol:chlorophorm:isoamyl alcohol (25:24:1).
5. Ethanol (100%).
6. Sodium acetate (NaAc; 3 *M*).
7. Ethanol (70%).
8. Sterile water.
9. Adequate restriction enzyme and buffer.
10. Sodium hydroxide (NaOH).
11. Sodium disulphite ($Na_2S_2O_5$).
12. Hydroquinon ($C_6H_6O_2$).
13. Low-melting agarose (i.e., SeaPlaque GTG; Biozym).
14. Heavy mineral oil.
15. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.
16. PCR buffer: 20 m*M* Tris-HCl, pH 8.4, 50 m*M* KCl.
17. $MgCl_2$.
18. dNTPs.
19. Sequence-specific primer pairs.
20. Taq polymerase.
21. Agarose.
22. TBE buffer: 90 m*M* Tris, 90 m*M* borate, 2 m*M* EDTA, pH 8.3.
23. Ethidium bromide (EtBr).

- Addition of a known amount of globin RNA as internal standard to a single embryo or pools of embryos
- Preparation of RNA

- Reverse transcription (RT) of RNA in cDNA
- Polymerase chain reaction (PCR)

- Agarose gel electrophoresis and ethidium bromide staining
- Digitalisation and quantification of the gel photograph employing a computer-assisted image analysis system
- Calculation of the relative abundance of each product (ratio between the intensity of the gene specific fragment and that of the globin band)

Fig. 2. Schematic diagram of the semiquantitative reverse transcription polymerase chain reaction assay used in experiments on bovine embryonic messenger RNA expression.

3. Methods

3.1. Semiquantitative RT-PCR

The classical methods to detect gene transcripts, such as Northern blotting, require pools of hundreds of embryos. With the advent of the sensitive RT-PCR technology it has become possible to detect mRNAs of low abundance in few or even single embryos at the *quantitative* level (*see* **Note 1**). By simultaneous amplification of an internal standard it is feasible to determine the relative abundance of the desired transcript *see* **Note 2**). The key steps of this *semiquantitative* RT-PCR assay are shown in the following scheme (**Fig. 2**).

The semiquantitative RT-PCR assay can be used to compare the relative amounts of one mRNA among different samples but not the absolute level of one mRNA to that of another because the total efficiency of amplification for each set of primers during each cycle is not known ***(42)***.

3.1.1. RNA Isolation

We have developed a protocol using the Dynabeads mRNA Direct Kit to extract poly(A^+) RNA from preimplantation embryos (*see* **Note 3**):

1. Pools of oocytes/embryos or single oocytes/embryos are stored at –80°C in a minimum volume of washing medium in siliconized tubes.
2. Because a rapid lysis of oocytes/embryos is critical for obtaining undegraded mRNA, thawing of the frozen material before the lysis step is carefully avoided.

3. For semiquantitative RT-PCR, 0.1 pg per oocyte/embryo equivalent (pools) or 1 pg (single oocyte/embryo) of globin RNA is added as an internal standard.
4. Embryos or oocytes (20–50) are lysed in 150 µL (pools) or 30 µL (single embryos) of lysis buffer.
5. Samples are then centrifuged at 12,000*g* for 15 s and incubated at room temperature for 10 min.
6. Prewashed oligo dT Dynabeads (10 µL [pools] or 5 µL [single embryos]) are added to the sample. The sample is then incubated for 10 min at room temperature to allow binding of poly(A)$^+$ RNA to Dynabeads by rotating on a mixer or roller.
7. The tubes are placed in the magnetic separator for 2 min.
8. After removal of the supernatant, the beads are washed once with 100 µL (pools) or 40 µL (single embryos) of buffer 1 and three times with 100 µL of buffer 2.
9. The RNA is eluted from the beads with 11 µL of sterile water by heating at 65°C for 2 mins and is then used directly for RT.

3.1.2. A Specific Protocol for mRNA Determination in Single Bovine Embryos

Poly(A)$^+$RNA is isolated from single embryos ***(26–29)*** and is used immediately for RT, which is carried out in a total volume of 20 µL using 2.5 µ*M* random hexamers. Before RNA isolation, 1 pg of rabbit globin RNA is added as an internal standard. The reaction mixture consists of 1X RT, 5 m*M* $MgCl_2$, 1 m*M* of each dNTP, 20 IU RNase inhibitor, and 50 IU Moloney murine leukemia virus reverse transcriptase. The mixture is overlaid with mineral oil to prevent evaporation. The RT reaction is performed at 25°C for 10 min, 42°C for 1 h, followed by a denaturation step at 99°C for 5 min and flash cooling on ice.

PCR is performed with complementary DNA equivalents depending on the gene of interest from embryos of different origin as well as 50 fg of globin RNA in a final volume of 50 µL of 1X PCR buffer (20 m*M* Tris-HCI, pH 8.4, 50 m*M* KCI, Gibco BRL, Eggenstein, Germany), 1.5 m*M* $MgCl_2$, 200 µ*M* of each dNTP, 1 µ*M* of each sequence specific primer (globin: 0.5 µ*M*) using a PTC-200 thermocycler (MJ Research, Watertown, MA). To ensure specific amplification, a "hot start" PCR is employed by adding 1 IU Taq DNA polymerase (Gibco) at 72°C. PCR primers are designed from the coding regions of each gene sequence using the OLIGO™ program.

The PCR program employs an initial step of 97°C for 2 min and 72°C for 2 min (hot start) followed by different cycle numbers (depending of the different primer pairs for the different genes of interest) of 15 s each at 95°C for DNA denaturation, 15 s at different temperatures for annealing of primers, and 15 s at 72°C for primer extension. The last cycle is followed by a 5-min extension at 72°C and cooling to 4°C. As negative controls, tubes are prepared in which RNA or reverse transcriptase is omitted during the RT reaction.

The RT-PCR products are subjected to electrophoresis on a 2% agarose gel in 1X TBE buffer containing 0.2 µg/mL EtBr. EtBr is added to the running buffer to get the same concentration as in the gel. The image of each gel is recorded using a charge-coupled device camera (Quantix, Photometrics, München, Germany) and the IP Lab Spectrum program (Signal Analytics Corporation, Vienna, VA). The intensity of each band is assessed by densitometry using an image analysis program. (IP Lab Gel). The relative amount of the mRNA of interest is calculated by dividing the intensity of the band for each developmental stage by the intensity of the globin band for the corresponding embryo. A representative example illustrating different mRNA expression levels in bovine embryos derived from two different origins is given in **Fig. 3**.

3.2. Bisulfite Sequencing for Determination of Methylation Patterns in Single Embryos

Bisulfite DNA sequencing allows one to determine the methylation status of cytosine residues in a defined sequence of a single-stranded genomic DNA. The technique is based on the fact that bisulfite can specifically convert cytosine, but not 5-methylcytosine, to uracil by deamination. Bisulfite-modified DNA is amplified by the PCR to yield sufficient amounts of material for automated sequencing. During the PCR step, the uracil is itself replaced by thymine so that in the final sequence the resistant 5-methylcytosine residues appear unchanged as cytosine but non-methylated cytosine residues appear as thymine.

There are two alternative methods for undertaking this analysis (*see* **Note 4**). To date, sequence information about bovine genomic DNA available in the database is very limited. The method used in our laboratory for bovine embryos involves the production of primers from the desired bovine genomic DNA sequence at positions where CpG islands have been identified using the CpGwin computer program *(43)*. After PCR amplification and sequencing of the target regions, primers that function with both bisulfite modified and unmodified DNA are designed from relative cytosine rich regions of bovine DNA. Despite difficulties (*see* **Note 5**), there have been a number of successful applications in mice and humans, and it is expected that any procedural problems will be overcome for analysis of bovine embryos as well.

3.2.1. DNA Isolation

After sequencing of the target regions and preparation of suitable primers, the next step in bisulfite sequencing is to prepare the genomic DNA. Preimplantation embryos are lysed using a modified protocol of Lopes et al. *(44)*.

1. The embryos are boiled in a water bath for 30 min and incubated in 100 µL of lysis buffer containing 20 µL of proteinase K and 1 µL of yeast tRNA. Yeast tRNA

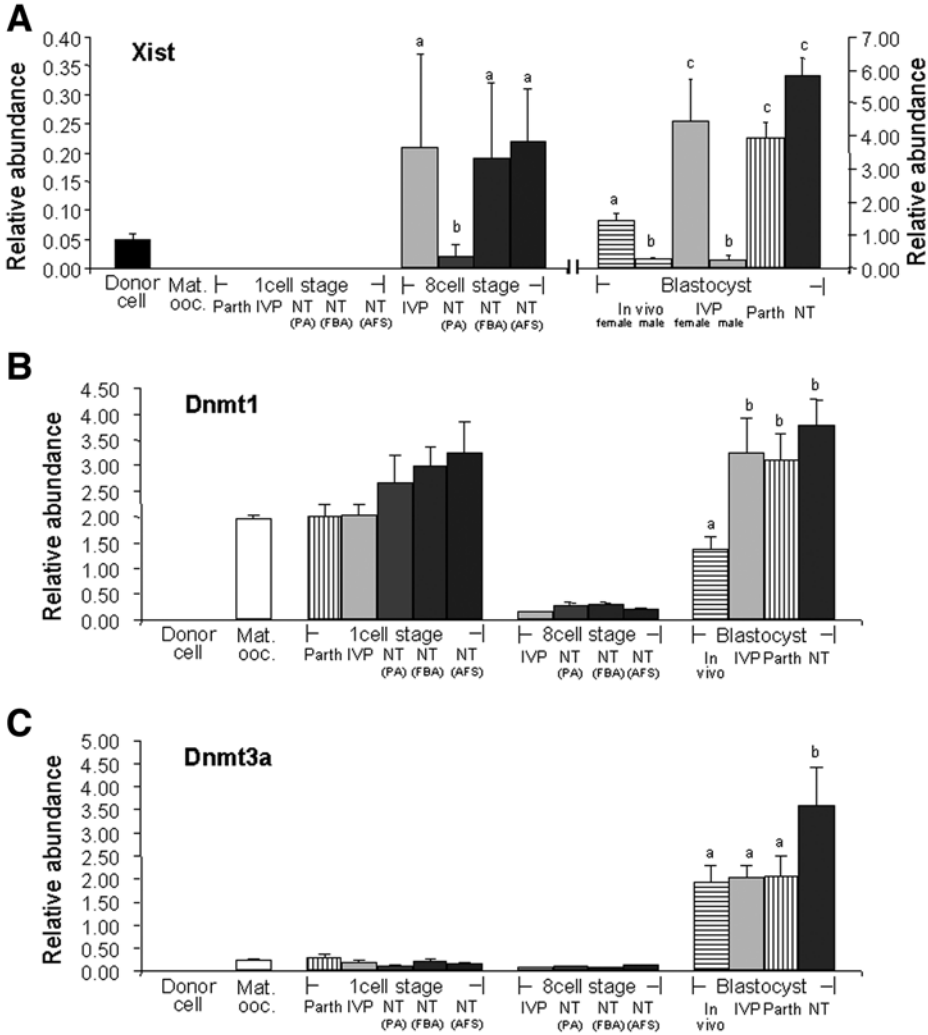

Fig. 3. Messenger RNA expression analysis of Xist and genes of the Dnmt family in donor cells, oocytes and embryos from various origins at the one-, eight-cell, and blastocyst stage.

serves as a carrier to increase the efficiency of precipitation in case of low concentrations of DNA.

2. Lysis is performed overnight in a 55°C water bath.
3. Genomic DNA is isolated by phenol chloroform extraction using the Phase Lock Gel system (Eppendorf) transferring the solution into a Phase Lock Gel tube and adding 1 vol phenol chlorophorm isoamyl alcohol followed by centrifugation for 5 min.

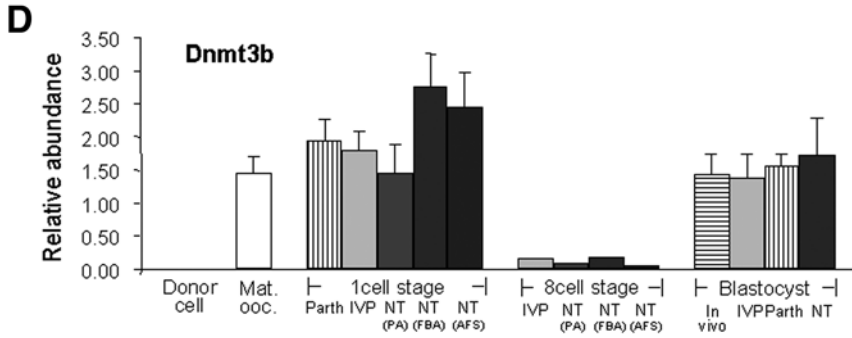

Fig. 3 (*continued)*

4. Repeat this step twice to recover the maximum amount of genomic DNA.
5. After centrifugation, the supernatant is transferred into a new tube with 2 vol of ethanol (100%), 3 *M* NaAc to get a final concentration of 0.3 *M*, and 1 µL yeast tRNA are added.
6. The DNA is precipitated for 1 to 2 h at −20°C.
7. After centrifugation for 20 min, the isolated genomic DNA appears as a white pellet on the bottom of the tube.
8. The pellet is washed twice with 500 µL of ethanol (70%) and air-dried before the DNA is eluted in 3 µL of sterile water at 4°C overnight.

3.2.2. Bisulfite Treatment

To increase the efficiency of PCR amplification of bisulfite-treated DNA, the isolated genomic DNA is first digested with a restriction enzyme that does not cut within the target region. Nondigested DNA may break because of hardening during the bisulfite treatment. Restriction enzyme digestion is performed in a total reaction volume of 3.8 µL using 0.4 µL of an adequate restriction buffer, 8 U of a specific restriction enzyme and 3 µL of isolated genomic DNA. The digestion is performed in a hot lid PTC-200 thermocycler (MJ Research, Watertown, MA) at 37°C for 30 min.

For the bisulfite treatment, a modified protocol of Hajkova et al. ***(45)*** is used in our laboratory. It is recommended to use only freshly prepared solutions and to keep these in the dark because of their sensitivity to light.

1. 2.5 mL of sterile water and 750 µL of 2 *M* sodium hydroxide (NaOH) are added to 1.9 g of sodium disulphite.
2. 500 µL of sterile water is added to 55 mg of hydroquinon.
3. Both tubes are incubated at 50°C in a water bath until the chemicals are completely dissolved.
4. Hydroquinon is mixed with the bisulfite solution and chilled on ice.

5. 20 mg of low-melting agarose is dissolved in 1 mL of sterile water by incubation in a boiling water bath and afterwards kept at 50°C until it is added to the restriction enzyme-digested DNA.
6. Bisulfite treatment is performed in 2-mL Eppendorf tubes containing 1 mL of heavy mineral oil. The tubes have to be kept under ice during preparations.
7. 7 µL of melted agarose is added to the restriction enzyme-digested DNA and quickly transferred into cold mineral oil resulting in an agarose bead.
8. Then 800 µL of the ice-cold bisulfite hydroquinon solution is directly transferred to the tubes.
9. After incubation for 30 min under ice, the tubes are kept for 4 h at 50°C in a water bath.
10. The supernatant is discarded and the beads are washed four times in 1X TE buffer (10 m*M* Tris-HCl, 1 m*M* EDTA; pH 8.0). Between each washing step, the beads were incubated for 15 min.
11. 0.4 *M* NaOH is prepared and the beads are incubated twice for 20 min before washing once in 1X TE buffer (pH 8.0) and three times in sterile water.

3.2.3. Polymerase Chain Reaction

The beads are then used for a second round of PCR amplification. The first round of PCR amplification is performed in a 100-µL vol reaction containing 1X PCR buffer (20 m*M* Tris-HCl, pH 8.4, 50 m*M* KCl; Invitrogen), 1.5 m*M* $MgCl_2$ (Invitrogen), 200 µ*M* of each dNTP (Amersham), and 0.6 µ*M* of each sequence-specific primer using a hot lid PTC-200 thermocycler (MJ Research, Watertown, MA). After a denaturation step of 95°C for 5 min, 3 IU Taq Polymerase (Invitrogen) are added at 72°C (hot start). The PCR program consists of 40 cycles of 15 s at 95°C (denaturation), 30 s at specific primer temperatures (primer annealing), and 45 s at 72°C (primer extension). To ensure a maximum of Taq Polymerase specific A at the 3′-end, the final cycle is followed by 10 min extension time at 72°C before cooling to 8°C. Two microliters of the first amplification is used for a second round of PCR in a 50-µL vol reaction after 30 cycles under the same conditions as described above.

The PCR products are purified on a 2% agarose gel in 1X TBE buffer containing 0.2 µg/mL EtBr, recovered from the gel with the GFX™ PCR DNA and Gel band purification kit (Amersham), and then either directly sequenced for the analysis of mean methylation levels or transformed into competent bacterial cells (XL10 gold; Stratagene) using the pGEM-TEasy vector system (Promega). After cloning, a minimum of 30 individual clones are sequenced from each reaction to give a representative picture of the methylation pattern. An outline of the bisulfite protocol used in our laboratory is given in **Fig. 4**.

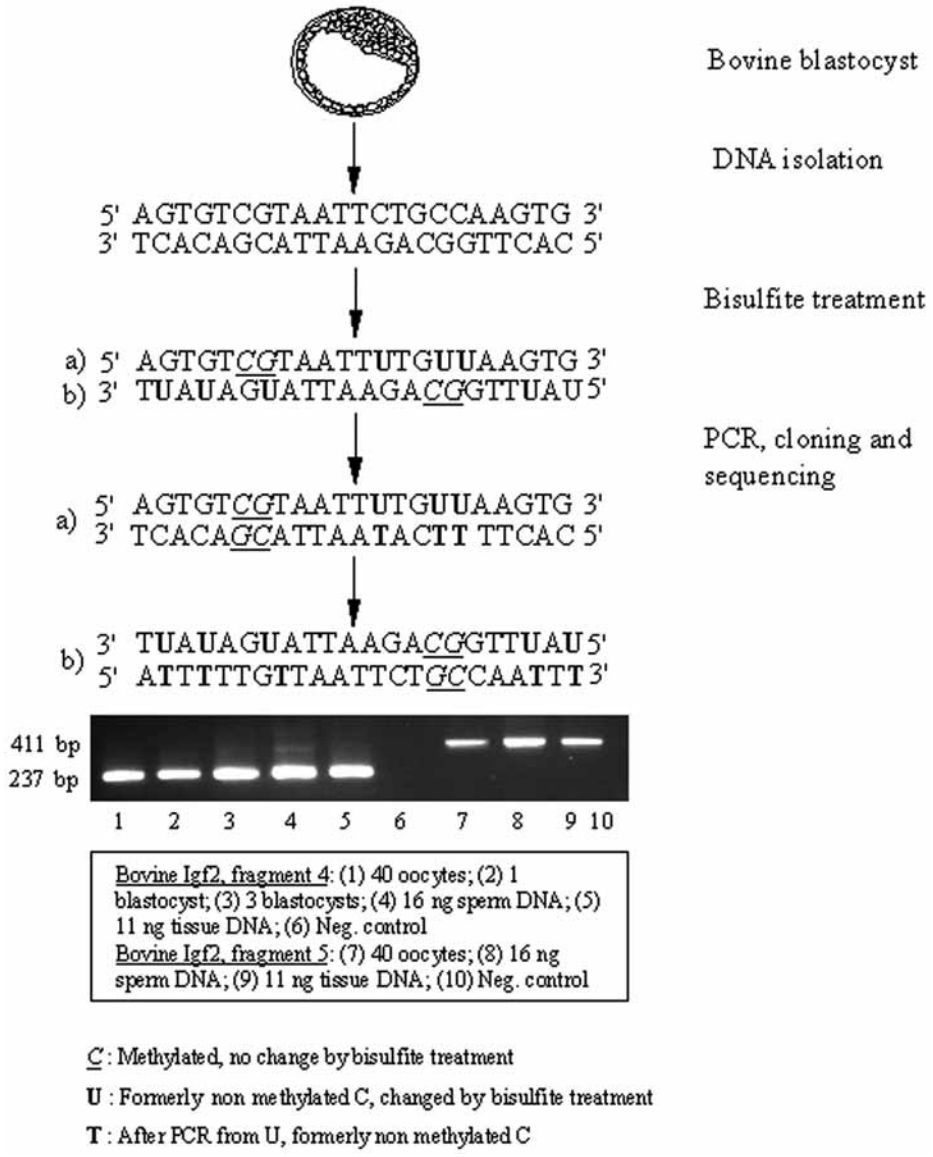

Fig. 4. Schematic graph of bisulfite sequencing to detect methylation marks in bovine embryos.

4. Notes

1. This technique has such a great sensitivity that it detects differences in mRNA expression even in small numbers or individual oocytes or embryos. However, care must be

taken to optimize RNA isolation and RT-PCR conditions. The extraction procedure of very small amounts of RNA within preimplantation embryo(s) may result in the loss of RNA. Even small losses during the extraction procedure may lead to false results. Rabbit globin RNA is added as an internal standard to overcome this problem. Priming the RT reaction with random hexamers to get the widest array of cDNAs instead of oligo-dT rules out the possibility that the increase in the amounts of a PCR product is attributed to an increased length of the poly(A) tail that results in a higher efficiency of reverse transcription employing oligo-dT ***(46)***. Furthermore, the use of random hexamer primers rather than oligo-dT minimizes the effects of sequence complexity and mRNA secondary structure. However, the highest sensitivity will be achieved when the 3′-specific primer is used during RT. The disadvantage of this approach is that only one transcript can be analyzed within one sample. PCR requires the application of specific primer pairs and thus knowledge of homologous or heterologous gene sequences. If only heterologous sequences (i.e., human or mouse sequences for bovine embryo analysis) are available, a primer pair is first designed from the given heterologous sequence. Then the product is sequenced, and the resulting bovine-specific sequence is used to create the primer pair employed to detect the bovine transcript of interest. For each pair of gene-specific primers, semi-log plots of the fragment intensity as a function of cycle number are used to determine the range of cycle number over which linear amplification occurred and the number of PCR cycles is kept within this range. To ensure specific amplification, a hot-start PCR is employed by adding the DNA polymerase at 72°C.

2. To circumvent the problem that differences in the relative abundance of the transcripts are caused by different cell numbers of the blastocysts analyzed, the number of replicates is calculated to get a high enough repeatability of the assay. The average repeatability (precision) of the assay varied from 0.60 to 0.70. A total of six to eight replicates has to be performed for each specific gene transcript from an individual embryo. This allows calculation of statistically significant differences between treatment groups for each transcript.
3. Several difficulties exist for the isolation of RNA from embryos or oocytes. The first is that the quantity of RNA is very limiting. The amount of RNA in a murine oocyte has been estimated to be 0.35–0.47 ng ***(47,48)***, and in the bovine oocyte 0.98–2.4 ng ***(48,49)***, varying with stage from 0.7 to 5.3 ng in the bovine pre-implantation embryo ***(49)***.
4. In the first method, the PCR product is sequenced directly to reveal the average methylation pattern for a target sequence as defined by specific PCR primers. In the second method, the PCR product is first cloned and single clones are sequenced so that specific methylation patterns of individual alleles and the individual strands of each allele are determined. This second method requires sequencing of 30 or more clones for each PCR product to give a representative result from the population of PCR products.
5. Primer pairs are designed such that overlapping of primers with CpG dinucleotides is avoided. A primer pair which works well with the non-methylated sequence and another working well with the methylated sequence is produced and

used in a multiplex reaction. This does not solve the problem of targets with partial methylation of both alleles and can lead to inaccuracies in the analysis of allele-specific methylation when this is masked by different efficiency levels of two competing primer pairs. There is also an increased potential for primer–primer interactions in the multiplex environment.

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METHODS IN MOLECULAR BIOLOGY™

Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
Alan O. Trounson

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Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The authors provide readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer. Of immense benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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21

Are There Any Normal Clones?

Ian Wilmut

Summary

An exhaustive study of the fidelity of a clone to its parent is prohibitive because of cost and the necessary scope of experimental design. Therefore, these data must be gathered from existing observational evidence. This in itself cannot provide a definitive accounting of the abnormalities and variation found among clones or between clones and parents because there is no standardization in the data points collected between one study and another. This literature survey shows that clone developmental abnormalities, variation among clones, and variation between clone and parent are prevalent at most stages of development (cleavage, placental, fetal, neonatal, maturity), and that occasionally the observed variation greatly exceeds that which might be expected. Some variation can be explained by differences in protocols and procedures between studies. The choice of nuclear donor cell is particularly influential of variation observed between a clone and its parent. In general, however, it appears that there is an inherent stochastic response to nuclear transfer that results in clone infidelity and variation. The survey of characteristics of clone infidelity to parent and documentation of abnormalities provided here should not be viewed as exhaustive or limiting in the recording of such data from future studies. Because controlled hypothesis testing of clone fidelity or clone health may not be possible, meticulous documentation of such observational evidence is a valuable contribution to the field.

Key Words: Clone health; clone fidelity; nuclear transfer; clone abnormality; clone development; cloned embryos; cloned mammal.

1. Introduction

Two different approaches to addressing this question can be envisaged. In one approach, direct observations could be made on the physiology of cloned animals in a prospective analysis. To be meaningful, these studies would have to be on all aspects of the lives of a very large numbers of animals. A direct comparison with similar numbers of controls produced by natural reproduction and kept under identical conditions also would be required. No published studies approach these stringent

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requirements in any species, although a small number of trials have been completed on some traits in useful numbers of animals. By contrast, the reports on some of the smaller trials that have been completed read more like promotion brochures for the companies that produced them than the results of a scientific study.

An alternative approach is to gain a general, but indirect, understanding by examination of the data that are available. Observations have been made at all stages of development from embryo to adulthood and these can be integrated to provide a general description of the likely outcome were a large controlled trial to be completed. In reality this indirect approach is the only one available to us.

The remainder of this chapter will use observations of a great variety of traits to establish such a description. The primary focus will be on cattle and mice because these two species are the most extensively studied and there are many differences in their early development and reproductive biology. However, passing mention will be made of other species when they cast a new light on clones or to provide confirmation of a conclusion drawn from observations in mice and cattle. Because a great number of different methods have been used, no attempt is made here to describe all of those methods because they are presented in the original publications that are cited. No attempt is made to record all publications on these subjects even in the selected species, but rather reports have been selected to indicate the range of observations that can be considered in analyzing the effect of a treatment upon the development of cloned embryos.

Together, the evidence that will be presented strongly suggests that the outcome of nuclear transfer (NT) is the result of interactions between several different mechanisms in a stochastic process. As a result, the product is quite variable even if the process is controlled as carefully as possible. In the mouse, both donor and recipient cells are expected to be entirely consistent in genotype because they usually are obtained from either inbred or F1 populations. Mice are kept under carefully controlled environmental conditions with a constant diet, reducing the opportunity for epigenetic effects on development. Despite this great control, the results of NT are as variable in mice as livestock. In these circumstances when planning the observations to be made on the normality of development of cloned embryos it is essential that there are no pre-conceived limitations.

Before considering the information that has been gathered after NT from somatic cells, the results will be summarized of two early studies in which nuclei were transferred from morulae or blastocysts. These reveal that even in these circumstances development is often perturbed.

In considering this summary, several limitations must be recognized. First, it is important to appreciate that by no means every dead animal is subjected to full postmortem analysis with detailed histopathology. This is true both for laboratory and farm animals. It is also quite likely that not all abnormalities that were observed have been reported in the literature. It must also be kept in mind that

these observations necessarily relate only to the present procedures and improvements are to be expected at some time in the future. An assessment of the health and normality of cloned animals should be repeated from time to time and particularly after the introduction of significant improvements to the cloning procedure.

2. NT From Morulae or Blastocysts

Evidence that present methods of NT introduce variation into offspring was the startling conclusion of the first large study of cloned calves, which is still the largest study of clones of any species that has ever been completed.

The weight of a total of 418 cloned calves was compared with that of 4687 calves obtained after embryo transfer and 8925 resulting from artificial insemination (AI) or natural mating. The same sires were used throughout the experiment as one way of reducing variation. The group of cloned calves was born after transfer of nuclei from blastomeres of morulae recovered from the donors used for embryo transfer. Hence, in this very effective design, there were groups of half sibs, full sibs, and clones, all of which were sired by the same group of bulls. It was expected that the clones would be the most uniform group and that the group of full sibs obtained after embryo transfer would be more uniform than the half sib resulting from AI or natural mating ***(1)***.

In fact, this study was the first to reveal far greater variation among clones than was expected. This greater variation was associated with a dramatic increase in the birthweight of some cloned animals. On average, cloned calves were approx 20% heavier at birth than those in the other groups after taking account of sire and dam effects. Furthermore, they were twice as variable as calves obtained after embryo transfer and four times more variable than those resulting from AI or natural meeting. The increased birthweight was associated with greater difficulties during parturition and a higher incidence of post-partum death. To a great extent, the differences found at birth were no longer apparent at 1 yr of age. By then, the clones were more uniform; however, some of this change may reflect the death of particularly large offspring.

A second report on transfer of nuclei from morulae in cattle noted an increase in weight of some calves, described difficulties at birth and the occurrence of congenital abnormalities ***(2)***. In a group of 100 pregnancies, 32 calves were delivered by Caesarean. The incidence was particularly high in those recipients that did not give birth by day 285.

These perturbations of development are now interpreted to reflect a failure to reprogram expression of genes in the transferred nucleus from those that are appropriate for a morula or blastocyst to those required for very early development (*see* below). Although such failures were anticipated when donor nuclei were taken from adult tissues, these early trials demonstrated that abnormal development occurred even when nuclei are transferred from an embryo with only 32–240 cells.

3. Abnormalities Observed in Clones From Adult or Fetal Cells

More recent studies have characterized the abnormalities obtained at different stages of development after transfer of nuclei from fetal and adult cells in many different ways and these will be considered at each stage of development, from embryo through to adult. A great variety of different abnormalities have been observed in all species in which significant numbers of clones have been produced. The abnormalities are summarized in **Tables 1–4** for each stage of development beginning with the early embryo and ending with adult clones.

3.1. Cleavage-Stage Embryo

The earliest observations of cloned embryos have been of chromatin organization in the nuclei of cleavage stage embryos (**Table 1** and **refs**. ***3–16***). At the one-cell stage, nuclei in cloned mouse embryos displayed A-type lamins that are not normally present at this stage and unusually high concentrations of nuclear matrix-associated protein ***(17)***.

The level of DNA methylation in cloned embryos of several species was found to vary greatly and often resembled that of the somatic donor nuclei rather than the lower level that is typical of early embryos ***(5, 6)***. This difference has also been associated with increased methylation of lysine residues in histone H3 in a large proportion of cloned embryos ***(7)***. Differences were noted between inner cell mass and trophectoderm ***(18)***, leading to the suggestion that hypermethylation of DNA

Table 1
Abnormalities Observed in Preimplantation Embryos

Species	Stage	Observation	Reference
Bovine	Cleavage	Present histone H1	***3***
Bovine	Blastocyst	FGF 4 FGFr2 IL-6	***4***
Bovine	Blastocyst	DNA methylation	***5–8***
Bovine	Four cell	Nucleoli	***9***
Bovine	Morulae	Xist	***10***
Bovine	Blastocyst	Chromosomal anonomly	***11, 12***
Mouse	Eight cell	DNA methylation	***13***
Mouse	Blastocyst	Expression OCT4-realted genes	***14***
Mouse	Blastocyst	Imprinted gene methylation and expression	***15***
Nonhuman primate	One-cell	Spindle organization	***16***

FGF, fibroblast growth factor, IL, interleukin.

in the trophectoderm might lead to abnormalities in placental development. Differences in DNA methylation have been described in term-cloned mice (*see* below), suggesting that in some cases differences that arise early in development may persist throughout the life of the animal. By contrast, the fact that as far as is known abnormalities are not inherited by offspring of clones suggests that epigenetic errors are corrected during subsequent gametogenesis.

In view of the fact that there are many profound changes during early development, it is surprising that so few observations have been made of cloned embryos. However, little is known even of the mechanisms that regulate the formation of pronuclei during fertilization.

Gene expression has been described in individual mouse blastocysts and found to be extremely variable. A reporter gene in which regulatory elements from Oct4 drove green fluorescence protein production revealed that expression was not re-activated in all embryos that reached the blastocyst stage and, in addition, that expression of OCT4 was mosaic in some embryos ***(19)***. Direct estimation of the level of expression of 10 genes whose pattern of expression normally follows that Oct4 was made for individual mouse blastocysts. Typical expression of all 11 genes was only observed in 62% of embryos ***(14)***.

Abnormalities of gene expression were also observed in cloned bovine embryos. Expression of X-linked genes in embryos derived from female nuclear donors was used to monitor the process of X inactivation ***(10)***. Xist expression was elevated in morulae and blastocysts derived from adult donor cells when compared with in vivo-derived embryos. By contrast, when the donor cell was derived from fetal tissue, this effect was not present.

In a study of cloned bovine blastocysts, expression of fibroblast growth factor-r2 and IL-6 was at a similar level to that in embryos produced by in vitro fertilization, whereas expression of fibroblast growth factor 4 was not detectable in a large proportion of cloned blastocysts ***(4)***. Interestingly, this effect was most evident if activation of the reconstructed embryo was induced directly after NT rather than after a 4-h delay, providing evidence of effects of protocol on gene expression.

Inappropriate expression of imprinted genes has been associated with abnormalities of development of cloned embryos of several species (*see* below for observations in fetuses and neonates).

3.2. Abnormalities of the Placenta

It is obvious to those who care for the females concerned that pregnancies with cloned embryos are more likely to develop abnormalities than those resulting from natural mating. A variety of abnormalities were observed (**Table 2** and **refs**. ***20–27***) in placental development in cattle and sheep examined around

Table 2
Abnormalities Observed in Placenta

Species	Stage	Observation	Reference
Cattle	Days 35–60	Abnormal development	*20*
Cattle	Various	Placentome: number and weight	*21,22*
Cattle	Various	Abnormal expression placental Lactogen, leptin	*23*
Mouse	Term	Expansion of spongiotrophoblast	*24*
Mouse	Term	Abnormal DNA methylation	*25*
Mouse	Term	Reduced expression imprinted genes	*26*
Sheep	Day 35	Failure of vascular development	*27*

day 35 of pregnancy. However, failure of vascular development was judged to be a critical factor in both species *(20,27)*. In cattle, the number of placentomes was reduced, but the weight of these was increased *(21)*. Several groups have independently reported a significant incidence of hydroallantois in cattle. There is less information on the weight of the placenta in this species because it is usually disrupted during natural delivery, making accurate collection of the tissues impractical. By contrast, when cloned mice are delivered by Caesarean, it is possible to weigh the placenta and several groups have noted significant increases *(29)*. In one detailed histological analysis in the mouse, the increase was the result of expansion of spongiotrophoblast *(29)*.

Analyses of gene expression in clones are characterized by variation. In the mouse, at term, it was found that the imprint had been retained accurately on several genes *(26)*. Despite this, however, the level of expression of the genes was abnormal emphasizing the point that expression of these genes is subject to several levels of regulation.

3.3. Abnormalities of the Fetus

There is no complete description of fetal development for cloned embryos of any species. However, it is evident that a variety of abnormalities (**Table 3** and **refs.** *30* and *31*) arise and contribute to the significantly increased incidence of prenatal loss. In the incomplete picture that we have at present, abnormalities were seen in several different organs that were examined at different stages of pregnancy.

Table 3
Abnormalities Observed in Fetuses

Species	Stage	Tissue	Observation	Reference
Cattle	–	Kidney	Various	*21*
Cattle	Various	Heart, kidney, liver	Weight abnormal	*30*
Cattle	Days 100–150	Various	Fetus heavier, liver and kidney heavier	*22*
Mouse	Term	Liver	Microarray	*31*
Sheep	Day 35	Liver, skin	Various in liver and skin	*27*

Specifically, abnormalities of the heart, kidney and liver were recorded in cattle at various stages of pregnancy including an increase in weight of these organs *(21,22,30)*. Similar changes were seen in the liver of sheep fetuses at day 35 of pregnancy *(27)*. No doubt these changes are associated with perturbation of gene expression of the kind described in mouse fetuses at term *(31)*.

3.4. Abnormalities Observed After Birth

The fact that parturition does not always follow a normal path was one of the earliest observations of pregnancies produced by transfer of cloned embryos (**Table 4** and **refs**. *32–47*). Frequently, the onset of parturition is delayed and the pregnant animal does not always adopt normal birthing behavior and has difficulty giving birth. Protocols were established to prevent these abnormalities threatening the health of the offspring. Mouse pups are usually delivered by Caesarean, whereas cattle are commonly given corticosteroid to induce parturition. The newborn's are given particular care and attention, especially to facilitate breathing, by clearing the respiratory tract and being given oxygen. Many of the neonates are unusually heavy at birth (e.g., in cattle *[21,22, 30,31,39]*). Similar changes have been noted in other species.

Overall, survival of cloned animals is reduced (e.g., in cattle *[21]*), and great variety of abnormalities have been reported. Many of these abnormalities are in large organs, including liver, kidney, lungs, heart, and associated major blood vessels, body wall, eyes, and ears. These have been associated with disturbances to blood cell counts and to blood biochemistry. Abnormal musculoskeletal development of limbs is described frequently in cattle.

Whereas some abnormalities occur frequently others have been described very rarely, but in some cases this may reflect difficulties in making an accurate diagnosis. One cloned calf in France became sick at 6 wk of age and finally died

Table 4
Postnatal Abnormalities Observed

Species	Age	Observation	Reference
Cattle	6 wk	Lymphoid hypoplasia	*32*
Cattle	Birth to 27 d	Increased red and white blood cell count	*33*
Cattle	Various	Hydrallantois	*34*
Cattle	Term	Leg malformation	*34*
Cattle	Birth	Shorter telomeres	*35*
Cattle	Puberty	Later in clones	*36*
Cattle	Maturity	No difference in fertility, milk yield, or composition	*37*
Cattle	Maturity	No difference in meat yield or composition	*38*
Cattle	Term	Birthweight	*21*
Cattle	Term	Reduced survival, breathing difficulties, thymic atrophy, hernia, abnormalities of kidney, liver, and pituitary. Red cell count increased, leptin elevated, T4, and cortisol lowered	*30*
Cattle	Term	Increased birthweight	*39*
Cattle	Term to 4 yr	Musculoskeletal abnormalities	*40*
Goat	Term	Abnormalities of heart, liver, and kidney	*41*
Mouse	Term	Abnormal DNA methylation	*25*
Mouse	Maturity	Obese	*28*
Pigs	Term	Eye and ear abnormalities, limb abnormalities, cleft pallet	*42*
Pig	Adult	Body weight: variable	*43*
Sheep	1 yr	Short telomeres	*44*
Sheep	Term	Kidney, liver, and brain	*45*
Sheep	Term	Loss of imprint of insulin-like growth factor 2R	*46*
Sheep	Term	Abnormality of body wall, limbs, kidney, lungs, liver, and cardiovascular system	*47*

1 wk later. Detailed analysis of blood samples that had been collected prospectively revealed that the cause of the illness was lymphoid hypoplasia associated with failure of the immune response to develop normally *(32)*. Very few groups cloning livestock have prepared for such an eventuality and detailed sampling of this kind is not possible in mice.

Inappropriate expression of genes has been described in cattle and sheep *(21,46)*. These observations included, but were not limited to imprinted genes. Telomeres were found to be short in some cloned cattle and sheep *(35,44)*. It has been suggested that this abnormality occurs only if specific cell types are used as nuclear donor.

3.5. Maturity

Abnormalities occur less frequently in those animals that survive to maturity. Indeed, reproduction, milk yield, and composition, meat yield, and quality were all normal in cloned cattle *(37,38)*. By contrast, in a different cattle experiment, puberty was delayed *(36)* and some experiments in the mouse have revealed obesity at maturity *(21)*.

4. Conclusion

All of the evidence that is available suggests that there is a stochastic response to NT, with an extremely variable outcome, although this interpretation cannot be tested. In these circumstances it is essential that anyone seeking to assess the health of their clones should not make any assumptions as to which traits might be perturbed. Account should also be taken of the fact that there is some evidence that the choice of nuclear donor cell influences the phenotype of the offspring. This might be anticipated if the abnormalities are considered to reflect a failure of the oocyte to be able to reprogram gene expression from that of the differentiated cell to that of an early embryo.

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Cell Reprogramming and Transgenesis

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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Future and Applications of Cloning

Alan O. Trounson

Summary

The birth of viable offspring from somatic cell nuclear transfer (SCNT) in mammals caused a major re-examination of the understanding of the commitment of cells to specific tissue lineages during differentiation. The questions of whether cells undergo dedifferentiation or transdifferentiation during the development of offspring and how these changes are controlled is a source of ongoing debate that is yet to be resolved. Irrespective of the outcome of this debate, it is clear that cloning using SCNT has a place and purpose in the future of research and animal breeding. The future uses of SCNT could include the production of transgenic mice, the production of transgenic livestock and assisting with the re-establishment of endangered species. Human medicine also would benefit from future use of SCNT because it would allow the production of patient-specific embryonic stem cells.

Key Words: Cloning; nuclear transfer; conservation; nuclear transfer and embryonic stem cells; future of cloning; applications of cloning; endangered species; somatic cell; nuclear transfer; livestock; differentiation; dedifferentiation; transdifferentiation; functional genomics; epigenetics; transgenesis.

1. Introduction

The report of somatic cell nuclear transfer (NT) in a mammal by Wilmut et al. *(1)* changed the understanding of development and cell commitment to specific tissue lineages during differentiation. The possibility of dedifferentiation or transdifferentiation between cells of different tissue types has become a major topic of debate that has not yet been finally resolved *(2,3)*. The ability of oocyte cytoplasm (ooplasm) to dominate somatic cell nuclei and consequently affect the reprogramming of complete development to term, albeit incomplete and inefficient, is now accepted and is beginning to be understood. The apparent dominance of embryonic stem (ES) cells over somatic cells *(4)* may enable the reprogramming of multipotentiality or even pluripotentiality without

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forming a NT embryo. These are challenging concepts that will enable future radical changes in animal breeding and human medicine.

There are a number of obvious applications for further research on the genetic and epigenetic control of development and differentiation through the production of offspring and ES cell lines. It is almost certain that transgenic mice will be produced routinely by NT *(5)* instead of by the present combination of genetically modified ES cells with embryonic cells of fertilized or tetraploid morulae or blastocysts. Adopting the use of NT will effectively reduce the time taken to produce offspring of the desired genomic type.

NT also will be used to derive transgenic domestic livestock and offspring of endangered species, simplifying the prospects of conserving genomic variability of valued species and providing a procedure to regain animals with desired genomic composition.

Human medicine also will benefit from NT through the production of ES cell lines and the analysis of functional genomics, drug screening, gene therapy and, in time, cell therapies. All of these prospects are attainable and provide a real opportunity for future research. This chapter is a discussion of these prospects.

2. The Future of NT

2.1. NT as a Platform Technology

Rudolf Jaenisch and colleagues in a series of experiments have demonstrated the application of NT in research. The method was used in one report to study the possible DNA alterations in post-mitotic olfactory sensory neurones *(6)*. In these cells, some 1500 odorant receptor genes are expressed by individual post-mitotic cells of the olfactory sensory epithelium. Offspring cloned from individual cells from this epithelium developed their own olfactory sensory epithelium expressing all the known receptors, thus indicating that no irreversible alteration to genomic DNA occurs in these cells. This study also supported the report by Hochedlinger and Jaenisch *(7)* that terminally differentiated B- and T-cell nuclei produced cloned offspring with fully rearranged immunoglobulin genes. These DNA rearrangements occur in B- and T-cells during lymphocyte maturation at the immunoglobin and T-receptor loci.

More recently, this research group showed that Ras-inducible melanoma cancer cells may be reprogrammed by NT to form ES cells and chimeric mice with contributions of these stem cells to many organs. This indicated that the melanoma phenotype is determined by epigenetic regulation of gene expression *(8)*. The genetic alterations of the melanoma, however, also were transmitted to the chimeric offspring, which subsequently all developed tumors.

The platform technology of NT may be used for research into genomics, epigenomics, functional genomics and the mechanisms involved in serious

pathologies, genetic diseases, diseases of unknown or polygenic origins, and metastatic cancers, and are extremely important applications in human medicine. These applications will derive from animal and human nuclear transfer and the production of ES cell lines *(9–11)*.

2.2. The Need to Improve Cloning

Low cloning efficiencies of 0.1–2.0% are reported for the production of offspring from somatic cell NT *(12)* and increased success rates are achieved with cloning using ES cells as nuclear donors *(13,14)*. Along with the low viability of NT embryos, there is a marked increase in embryonic wastage, pre- and perinatal losses, and unpredictable deaths thereafter *(15–17)*. Consequently, there are concerns in domestic animal industries regarding animal health and high mortality rates of cloned offspring. These complications will certainly limit applications in animal breeding and for the reconstitution of endangered animal populations and are also one of the reasons it is unethical to apply NT methods for reproductive cloning in humans *(18)*. The inefficiencies and abnormalities observed in animals would be completely unacceptable in human reproduction, but even if these problems were corrected, cloning would limit reproduction to a single biological parent, which is presently unacceptable in any society.

3. Applications of NT

3.1. Cloning Domestic Animal Species

The biomedical and agricultural applications of cloning domestic animals have been reviewed recently *(19–23)*. These applications include the production of pharmaceuticals in milk or blood from transgenic animals and alterations to animals by transgenesis for productivity, disease immunity, and biological product (e.g., milk) processing. However, this approach is not the only one to animal transgenesis, which can be achieved by pronuclear injection and viral transfection. The use of recombinase proteins to achieve homologous recombination by pronuclear injection recently was described *(24)* and already has resulted in higher birth rates of transgenic animals than NT, although most transgenic offspring resulting were mosaic.

One of the present handicaps for domestic animal transgenesis is the absence of genuine ES cell lines for targeted integration of specific gene constructs. It is presently common to attempt to transfect embryonic or adult fibroblasts of domestic animals for NT to produce transgenic offspring.

3.1.1. Identifying the Problems

The primary problem for cloned embryos is the failure to completely erase epigenetic regulation of the gene expression of the somatic cells used for

nuclear donation *(25,26)*. Consequently, there are methylation irregularities in embryos produced by NT *(27–31)*, which are apparently not corrected. These irregularities have led to attempts to erase the epigenetic signatures of somatic cells and a search for ways to expose donor cell chromatin to more efficient reprogramming by cytoplasmic factors.

There is a significant increase in developmental competence of mouse blastocysts derived by nuclear transfer of pluripotential ES cells when compared with somatic cells *(13,14,32)*. In the absence of genuine ES cells in species other than mice and primates, ES cell-like cells (ES-like cells) have been produced. In the bovine, ES-like cells were produced from preimplantation embryos by Saito et al. *(33)*. These ES-like cells appear to differentiate into neural precursor cells and when used to produce cloned calves, increased numbers of early pregnancies and reduced embryonic wastage in pregnancy was observed. Hence, there is some consistency between observations with mouse ES cells and bovine ES-like cells. It is also apparent in the mouse that cloning from hybrid strains is more successful than cloning from inbred strains *(12,34)*. It is of interest that Wakayama *(12)* has drawn attention to the increased cloning success rates with the 129/SV strain which is also a more amenable strain to conversion to ES cells and carcinomas. These cells may be more easily reprogrammed epigenetically for ES cell formation, cancer, and cloning. However, the exact mechanisms involved are presently unknown.

Placentomegaly is the major cause of embryo wastage observed in cloned embryo pregnancies *(35,36)*. In ruminants, this is associated with reduced numbers of cotyledons and is likely to be responsible for much of the developmental abnormalities observed in tissue structure and function of cloned offspring. In mice, analysis of expression profiles for mouse placentas derived from cloned embryos showed aberrant expression of imprinted genes, altered expression of regulatory genes involved in gene expression, for instance, DNA methyltransferase and histone acetyltransferase, increased expression of oncogenes and growth promoting genes, overexpression of genes involved in placental growth, for example, *Plac1*, and overexpression of many novel genes, for instance. *Pitrm1* (a metalloprotease family member) *(37)*. The large-scale dysregulation of gene expression, together with random X-chromosome inactivation in placentas of NT embryos, indicate that this should be a target for correction in future research studies. However, the reconstruction of mouse embryos using tetraploid trophectoderm was unable to correct all the developmental abnormalities observed in mice *(38,39)*, which would indicate that epigenetic abnormalities in NT embryos are present in both embryonic and extra-embryonic tissues.

3.1.2. Looking to the Future

Given the difficulties of reprogramming nuclear transfer gene expression profiles, methods that expose the chromatin to reprogramming of characteristic silenced genes and active gene expression in somatic tissues are likely to be needed. The technique known as chromatin transfer ***(40)*** involves remodeling chromatin by exposing interphase donor nuclei to cytoplasm extracts of cells in metaphase. The donor cells are permeabilized with Streptolysin-O to enable the cytoplasm to induce chromatin condensation, the release of TATA-binding proteins (a general transcription factor) and silencing of *lamin A/C* genes and reactivation of *lamin B*. This is reported to increase survival of bovine NT embryos, reducing embryonic wastage and increasing the birth of live calves ***(40)***. Further research on this method of nuclear transfer appears to confirm these observations (A. French and R. Tecirlioglu, unpublished data).

Improving the pregnancy rates and survival rates of embryos and live born offspring will encourage further applications of NT in domestic animal breeding and extend its uses to companion animals (e.g., dogs and cats), and valuable animals such as the horse ***(41)***. At the present time, these latter applications are unlikely to be strongly supported because of the extreme inefficiencies ***(15)***.

3.2. Cloning for Wildlife Conservation

3.2.1. Identifying the Problems

The use of NT for preserving the genetic variability of wildlife has been reviewed recently by Holt et al. ***(42)***. One of the major problems for reproductive cloning of rare endangered animals is the availability of sufficient numbers of ooplasts from that species, or closely related species. Although new techniques involving the production of oocytes by ovarian xenotransplantation ***(43,44)*** are being developed, which may address this difficulty, the present strategy is to use the oocytes of closely related species. A healthy Banteng bull (*Bos javanicus*) and a Gaur calf (*Bos gaurus*), which died soon after birth, were produced using domestic cattle (*Bos taurus*) ooplasts ***(45)***. Similar somatic cell cloning studies are underway with Buffalo (*Bubalus bubalis*) using cattle ooplasts, with some success ***(46)***. Cells of a deceased endangered Mouflon (*Ovis orientalis musimon*) were recovered for nuclear transfer into sheep (*Ovis aries*) to produce an apparently normal Mouflon offspring ***(47)***. However, concerns have been voiced that major placental abnormalities occur in noncloned embryos transferred to another species. Gaur offspring, for example, do not survive beyond a few hours of birth when gestated in cattle recipients ***(48)***.

3.2.2. Achieving Some Success

In more closely related species (*B. taurus* and *Bos indicus*), interspecies cloning is successful, although the donor cell mitochondrial DNA is usually reduced or lost during development *(49)*. In diverse interspecies reproductive cloning, genomic and mitochondrial transcript and protein incompatibility could be a problem for cell and tissue metabolism and embryo survival.

The maturing cloned Banteng bull on display at the San Diego Zoo is a very visible testament to the beginning of the use of interspecies reproductive cloning techniques (**Fig. 1A**). In Australia, cell lines have been derived from the majority of the remaining Northern Hairy-nosed Wombats (<100 remain in one small area) in an attempt to conserve this highly endangered animal (**Fig. 1B**). It is hoped that oocytes of the Common Wombat could be used as ooplasts for NT of the Northern Hairy-nosed cells. Many other similar projects have been proposed, but lack of finance presently limits this research.

3.3. NT for Production of ES Cells

The production of ES cells for human medicine was based on proof of concept studies in the mouse *(9–11)*, which demonstrated that NT of somatic cells into enucleated oocytes would produce embryos for which ES cell lines can be derived. Success rates for the production of such ES cells approach that achieved with embryos derived from fertilized oocytes *(12)*, and there is no apparent developmental defects in differentiating tissues or chimeric offspring reported to date using somatic cell nuclear transfer. Chimeric mice derived from ES cells produced by NT are normal and the ES cell lines differentiate into tissues in a similar manner to those derived from fertilized oocytes *(9,11,50)*. The apparent difference between the high rates of developmental abnormalities observed in reproductive cloning and absence of any obvious defects in ES cells produced by nuclear transfer may indicate that placental abnormalities contribute significantly to tissue defects in cloned embryos and offspring. It is also possible that selection occurs in ES cells for developmental competence in vitro and possibly again when ES cells are introduced into wild-type embryos. Furthermore, ES cells may be subject to additional reprogramming influences in chimeric tissues (*see* **Fig. 2**).

The potential use of NT for producing immunologically compatible ES cells for cell therapies in the human is of some interest despite the lack of human oocytes available for such applications *(51)*.

The validity of this approach has been demonstrated by Rideout et al. *(52)*, who used NT to derive mouse ES cells from mice with the *Rag2*$^{-/-}$ immune deficiency. They corrected one of the mutant alleles by homologous recombination and then introduced a *HoxB4* gene to direct differentiation into the lymphoid lineage. These

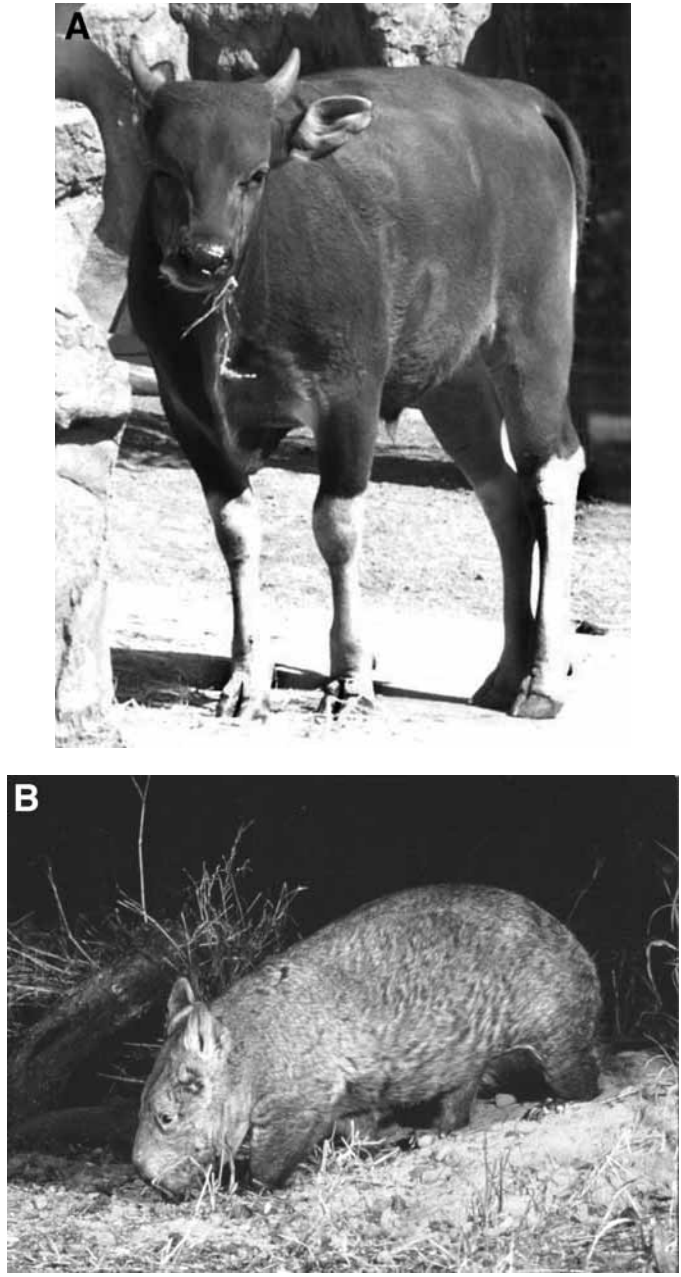

Fig. 1. **(A)** Cloned immature Banteng bull on display at the San Diego Zoo. **(B)** The endangered Northern hairy-nosed Wombat, of which less than 100 remain in one small area in the wild in Australia.

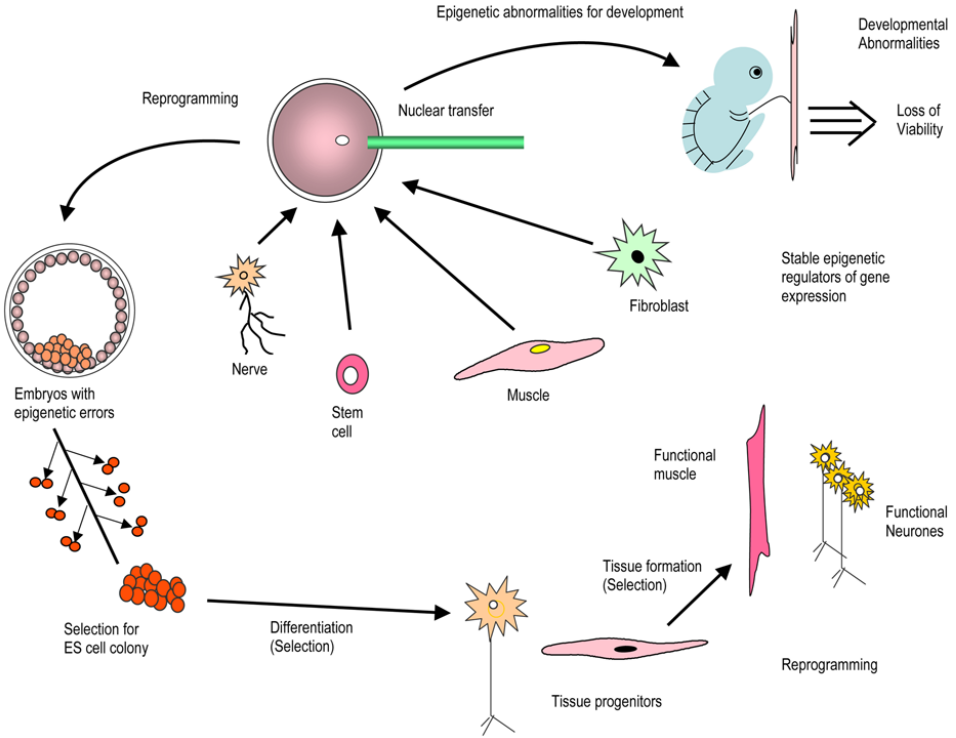

Fig. 2. Nuclear transfer and development. The reprogramming of a somatic cell nucleus by nuclear transfer will result in development to the blastocyst stage. In the case of further development of the embryo, epigenetic abnormalities that are residual from the original somatic cell impede development and result in high mortality, organ defects, and functional failure of different tissues. When the nuclear transfer blastocysts are used to derive embryonic stem (ES) cells, they appear to contribute normally to development in chimeric embryos and when combined with mature tissue. This may be either the result of selection of normal cells or to further reprogramming by the host tissues.

cells colonised the bone marrow of mutant *Rag2*$^{-/-}$ mice, producing myeloid and lymphoid cells and immunoglobulins within 3–4 wk. This is an elegant demonstration of cell and gene therapy that may be used as a model to correct genetic disease of blood cells in the human. It would probably be desirable to avoid the introduction of *HoxB4* genes, and present research is focused on differentiation of ES cells into the hematopoietic lineage without transgenic or transcription factor instruction.

It is difficult to imagine that this nuclear transfer technique would be sustainable for any clinical application for cell or gene therapy because of the requirement for large numbers of mature human oocytes that are not readily available. Even if the efficiency of this technique was to be substantially increased, this application appears unlikely. There are other sources of oocytes, including the production of oocyte-like cells from stem cell-germ cell differentiation ***(53,54)***. If this system was able to generate large numbers of oocyte-like cells with somatic cell nuclear reprogramming capacity, it may be a solution to the scarcity and unavailability mature human oocytes. Moreover, it is apparent that the ES cell phenotype is dominant in fusion hybrids with somatic cells ***(4)***, and this approach is being explored by others (P. Verma, D. Pralong, and A. Trounson).

It has also been reported ***(55)*** that enucleated rabbit oocytes reconstituted with human somatic nuclei will develop to blastocysts and these were capable of producing ES-like cells that differentiated into the three primary embryonic lineages of mesoderm, endoderm and ectoderm, as demonstrated by human cell markers. There are concerns about this report because pluripotentiality was not proven by teratoma formation. These authors reported the presence of rabbit and human mitochondria in blastocysts, but the presence of functional human mitochondria was not shown in the ES cells. Notably the nuclei of somatic mouse cells can be reprogrammed to express the POU transcription factor Oct4, which is characteristic of pluripotentiality, by amphibian (*Xenopus*) oocyte cytoplasm ***(56)***. If interspecies NT is feasible for production of human ES cells, the continuous passage of the ES cells would result in the turnover of rabbit proteins into human proteins, resulting in a virtually complete human ES cell.

3.4. Models for Human Diseases

The short-term application of NT to produce human ES cells is for the study of pathologies that affect human patients. Hence, the study of ES cells and their derivatives from patients with diseases such as cystic fibrosis, muscular dystrophies, Alzheimer's disease, Huntington's disease, motor neurone disease, and multiple sclerosis would be informative for analyses of the tissue lineage defects and for the development of drugs that may prevent the phenotypic expression of the diseases. This approach is an extremely important one

and deserves further study and the application of NT techniques. Transgenic mice have become an important source of models for human disease and will continue to be the primary resource for research to develop strategies to control and treat human pathologies. Recently it has been reported that rats can be cloned by controlling oocyte activation *(57)*. This will enable the production of genetically manipulated rats with gene knockouts and gene knock-ins that produce animals with further models of human diseases. This will almost certainly be another application of NT technologies.

4. Conclusions

NT has altered our understanding of cell type commitment during differentiation, the role of epigenetics in the determination of tissue formation and has provided the opportunity to reprogram development. Its applications presently and in the future are substantial, but require considerable research effort to determine the underlying mechanisms of differentiation and dedifferentiation. The oocyte will remain critical for reprogramming somatic cells and there is a need to determine the protein factors that are present within the ooplasm that are responsible for chromatin remodeling and the regulation of DNA transcription. The determination of these factors may enable a more complete nuclear reprogramming and reduce developmental abnormalities. The opportunities for research are substantial and compelling, and will lead to further applications of NT in animal breeding, human medicine, and bioconservation.

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METHODS IN MOLECULAR BIOLOGY™

Nuclear Transfer Protocols

Cell Reprogramming and Transgenesis

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Preface

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis is a comprehensive review of nuclear transfer technology in vertebrates, aimed at reprogramming differentiated nuclei and effecting targeted gene transfer. The emphasis here is on providing readily reproducible techniques for the generation of cloned embryos and animals in a number of key research and commercially important vertebrates. Additional chapters provide alternative cutting-edge methods for nuclear transfer, such as zona-free nuclear transfer and serial nuclear transfer. Of immense practical benefit are descriptions of procedures associated with cloning, such as in vitro maturation of oocytes, activation and culture of cloned embryos, maintenance of pregnancy, and neonatal care of clones.

Nuclear Transfer Protocols: Cell Reprogramming and Transgenesis also provides an understanding of the factors involved in nuclear reprogramming, which is imperative for the success of cloning. A section dealing with such cloning-related issues as aging and normality of clones is also included making this an essential comprehensive handbook for research and commercial laboratories involved in, or intending to work on, nuclear transfer. The volume will prove beneficial to molecular biologists, stem cell biologists, clinicians, biotechnologists, students, veterinarians, and animal care technicians involved with reprogramming, nuclear transfer, and transgenesis.

Paul J. Verma
Alan O. Trounson

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Cell Reprogramming and Transgenesis

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FEATURES

- **Offers a comprehensive review of nuclear transfer technology**
- **Provides readily reproducible techniques for the generation of cloned embryos and animals**
- **Describes techniques for cell reprogramming of donor cells for increased nuclear transfer outcomes**
- **Outlines detailed experimental layout from experts in the field**

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