Generation of Chimeric Rhesus Monkeys

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SUMMARY

Totipotent cells in early embryos are progenitors of all stem cells and are capable of developing into a whole organism, including extraembryonic tissues such as placenta. Pluripotent cells in the inner cell mass (ICM) are the descendants of totipotent cells and can differentiate into any cell type of a body except extraembryonic tissues. The ability to contribute to chimeric animals upon reintroduction into host embryos is the key feature of murine totipotent and pluripotent cells. Here, we demonstrate that rhesus monkey embryonic stem cells (ESCs) and isolated ICMs fail to incorporate into host embryos and develop into chimeras. However, chimeric offspring were produced following aggregation of totipotent cells of the four-cell embryos. These results provide insights into the species-specific nature of primate embryos and suggest that a chimera assay using pluripotent cells may not be feasible.

INTRODUCTION

Embryonic stem cells (ESCs) are the in vitro counterparts of pluripotent cells residing in the inner cell mass (ICM) of blastocysts (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1995, 1998). Whereas natural pluripotent cells in the developing embryo exist transiently, ESCs can be maintained in vitro indefinitely, providing an unlimited source of undifferentiated cells. When reintroduced into blastocysts, mouse ESCs engraft into the ICMs and participate, in concert with host embryonic cells, in the development of chimeric fetuses and offspring (Bradley et al., 1984). Furthermore, in ICM-deficient, tetraploid host embryos, injected mouse ESCs can rescue the embryo proper, resulting in exclusively ESC-derived offspring (Nagy et al., 1990). This unique feature of ESCs has been greatly exploited in the creation of knockout mice and studies of mammalian gene function (Capecchi, 1989).

The first chimera studies of Tarkowski (1961) and Mintz (1962) independently demonstrated that two or more cleaving mouse embryos when aggregated together could produce a single chimeric mouse of normal size. The organs and tissues of such animals consist of a mixture of genetically divergent cells derived

from the parental embryos. A modified technique was developed by Gardner (1968), whereby cells injected into blastocysts were incorporated into the host ICM to form chimeras. A variety of donor cell types support mouse chimera production, including ICM (Gardner, 1968), teratocarcinoma cells (Mintz and Illmensee, 1975), ESCs (Bradley et al., 1984), embryonic germ cells (Matsui et al., 1992), as well as pluripotent cells experimentally generated by somatic cell nuclear transfer (SCNT) (Wakayama et al., 2001) or direct reprogramming (induced pluripotent stem cells [iPSCs]) (Okita et al., 2007). Chimeric animals have also been produced in several other mammals including rats (Mayer and Fritz, 1974), rabbits (Gardner and Munro, 1974), sheep (Tucker et al., 1974), and cattle (Brem et al., 1984). Moreover, live chimeras have been produced by aggregating preimplantation embryos of different species (Fehilly et al., 1984). The ability of mouse cultured pluripotent cells, including those derived experimentally, to contribute to chimeric tissues of the embryo proper after introduction into preimplantation host embryos has become an ultimate test for pluripotency. However, such a stringent chimera-based pluripotency assay has not been developed for primates, in large part, due to the limited availability of animals and the lack of relevant technological and genotyping expertise.

RESULTS

Potential of Monkey ESCs to Form Chimeras

We initially evaluated the ability of rhesus monkey ESCs to contribute to chimeric fetuses upon injection into in vitro fertilization (IVF)-derived host blastocysts. To aid in the tracking of injected cells, we transduced ESCs with a lentiviral vector carrying GFP and selected pure populations of cells highly expressing the transgene. Approximately 20–30 disaggregated ESCs were injected into the host blastocyst and placed next to the ICM (Figure S1; Movie S1, ESC injection). To eliminate risks that ESC disaggregation may affect cell survival, some blastocysts were injected with mechanically dispersed cell clumps. To exclude the possibility that GFP-expressing ESCs may have compromised developmental potential, we also injected nontransgenic ESCs. We evaluated several previously characterized rhesus ESC lines including IVF-derived ORMES-22 (XX) and -23 (XY) as well as SCNT-derived CRES-2 (Byrne et al., 2007).

A total of 26 ESC-injected blastocysts were immediately transplanted into seven synchronized recipients. The details of this

Table 1. Summary of In Vivo Chimera Studies with Rhesus Monkey ESCs and Embryos							
Tested Cells	Host Embryo Stage	Total Offspring Generated	Developed to Separate Embryo	Developed to Embryo Proper Chimera			
ORMES 22	blastocyst	4	no	no			
ORMES 23	blastocyst	2	no	no			
CRES 2 GFP	blastocyst	1	no	no			
Whole ICM	blastocyst	3	yes	restricted ^a (hematopoietic)			
Four cell embryo	four cell embryo	10	no	yes			
ORMES 22 GFP	four cell embryo	1	no	no			

ORMES 22 (XX) and 23 (XY) are IVF derived rhesus monkey ESC lines. CRES 2 (XY) is a SCNT derived ESC line (Byrne et al., 2007). See also Tables S1, S2, S3, S4, S5, and S6, Figures S1, S2, S3, S4, S5, and S6, and Movie S1. ^aChimerism was restricted to livers, spleens, and placentas.

experiment including host embryo stage, ESC type, and embryo transfer outcomes are presented in Table S1. Four females became pregnant-one carrying quadruplets and three carrying singletons. In addition, three recipients contained gestational sacs without fetuses. The overall pregnancy and implantation rates were 57% (4/7) per recipient animal and 27% (7/26) per embryos transferred, respectively. All pregnancies were terminated at midgestation, and seven fetuses were recovered by caesarean section. Multiple tissues and organs from each fetus were analyzed for the presence of ESC progeny by (1) microsatellite parentage analysis of genomic DNA employing 41 short tandem repeats (STR); (2) mitochondrial (mt)DNA parentage analysis using restriction fragment length polymorphism (RFLP); and (3) direct GFP fluorescence analysis followed by GFPspecific PCR. None of these assays showed a contribution of ESCs in analyzed fetuses (Table 1).

Potency Determination in Monkey ICMs

The failure to generate chimeras may indicate either limited developmental potential of primate ESCs or inability of host blastocysts to incorporate foreign embryonic cells. To address these questions, we tested whether noncultured pluripotent cells residing in the ICM can incorporate into host embryos and form chimeras. Our attempts to enzymatically disaggregate monkey ICMs into single cells prior to injections resulted in poor survival and cell death. Therefore, whole ICMs were immunosurgically isolated from IVF-produced expanded blastocysts and immediately injected into host blastocysts from unrelated monkeys (Figure S1; Movie S1, ICM injection). A total of 44 ICM-injected blastocysts were generated and transferred into 11 recipients (Table S1). Three females were confirmed pregnant based on blood progesterone profiles. However, one recipient carried a single gestational sac without a viable fetus. The second recipient (recipient #10) carried two sacs; one was empty, whereas the second contained two fetuses separated by a thin membrane, indicative of a monochorionic di-amniotic twin pregnancy (Figures 1A and 1B). The third recipient (recipient #18) carried a single gestational sac with one fetus. The pregnancy and implantation rates with ICM-injected blastocysts were 18% (2/11) and 7% (3/44), respectively (Table S1). All three fetuses were recovered by caesarean section as described above and analyzed for the contribution of transplanted ICMs.

Naturally occurring, monochorionic twin pregnancies usually carry monozygotic, genetically identical fetuses due to spontaneous duplication of the embryo during early stages of development (Cunningham and Williams, 2005). Unexpectedly, our initial morphological examinations of the monochorionic twin pregnancy revealed that the fetuses were of different genders (ICM-f1-female and ICM-f2-male). Moreover, detailed STR analysis demonstrated that one fetus originated from the host blastocyst, whereas the second fetus was derived from the injected ICMs (Figure 1 and Table S2). Interestingly, we detected chimerism in the livers and spleens of both fetuses, but not in other organs and tissues. For example, analysis of the STR locus for D11S2002 in livers indicated the presence of three different alleles representing both the host blastocyst and injected ICM (Figure 1). In addition, the gender-specific STR marker (AME) confirmed the presence of male cells within the liver of the female fetus ICM-f1. To further validate these results, we analyzed gender using PCR-based size differences in the amplicons of the X- and Y-linked zinc finger protein genes (ZFX and ZFY) (Mitalipov et al., 2007) and confirmed chimerism in livers and spleens. We also genotyped fetal mtDNAs based on the G/A single-nucleotide polymorphism (SNP) within the rhDHV1 region (Tachibana et al., 2009) and the ability of the SphI (Pael) enzyme to digest the G but not the A allele. MtDNA analysis confirmed that ICM-f2 originated from transplanted ICM and chimerism in livers and spleens (Figure 1). The placental sample containing a mixture of several extraembryonic membranes (including chorion and amnion) was contributed by both the host embryo and injected ICM.

The fetus associated with the singleton pregnancy (ICM-f3) was male and originated solely from the injected ICM, whereas the placental (trophectoderm) component was female and mainly contributed by the host blastocyst (Figure 2 and Table S2).

These results demonstrate that contrary to the mouse and some other species, monkey blastocysts do not readily incorporate ESCs or foreign ICMs and form embryo proper chimeras. However, transplanted ICMs were capable of forming separate viable fetuses while sharing the trophectodermal compartment of the host embryo. The chimerism detected exclusively in livers and spleens of twin fetuses could result from the exchange of blood and hematopoietic progenitors through placental perfusions. We also observed contribution of transplanted ICMs to extraembryonic membranes. This is an expected outcome based on the evidence that mouse ICMs contribute to several vital extraembryonic tissues including amnion and extraembryonic mesoderm of the chorion (Nagy et al., 1990).

Production of Monkey Chimeras by Aggregation of Four-Cell Embryos

We reasoned that the totipotent blastomeres of cleaving embryos should be capable of incorporating foreign blastomeres and forming chimeras. We focused on the four-cell stage based on the evidence that an isolated single blastomere from this stage embryo can implant and develop into a viable rhesus offspring (Chan et al., 2000). To investigate this, we initially attempted to generate chimeric embryos by replacing two blastomeres in the four-cell stage embryo with two blastomeres isolated from different developmentally comparable embryos (Figure S1). We generated 29 chimeric four-cell embryos of which 19 reached the blastocyst stage. Analysis revealed that only 10 of these blastocysts contained total cell counts similar to nonmanipulated controls, indicative of successful aggregation (Table S3). However, remaining embryos either failed to aggregate and formed two separate blastocysts or developed into a single blastocyst with significantly reduced cell numbers, indicating that either transplanted or host blastomeres arrested and failed to contribute to chimeric blastocysts (Figure S2). We also observed blastocysts with two distinct cavities or two ICMs within a single trophectoderm vesicle (Figure S2). Although we did not incorporate markers that would distinguish between donor and host blastomeres, this phenomenon was not seen in control intact embryos or in prior studies of preimplantation embryogenesis (Wolf et al., 2004). Nevertheless, these observations motivated us to carry out further studies whereby we aggregated together whole embryos in order to increase the yield of chimeric blastocysts and eventually offspring.

We reasoned that aggregating of three or more whole cleaving embryos together would allow better contact between blastomeres and ensure that at least two of these embryos would develop to blastocysts and contribute to chimeric ICMs (Figure S1). We created a total of 29 aggregates using between three and six individual four-cell stage embryos and cultured to the blastocyst stage (Table S4). Remarkably, all 29 aggregates developed to blastocysts, and cell count analysis suggested that 26 blastocysts (90%) consisted of at least twice the normal cell counts, indicating successful aggregation (Table S5 and Figure S3). To corroborate these observations, we injected GFP-RNA construct into parental oocytes and generated GFPexpressing cleaving embryos. GFP signal was always confined to the RNA-injected oocytes and daughter blastomeres but was not found in aggregated blastomeres from noninjected embryos (Figure S3). We aggregated GFP-tagged embryos with noninjected controls and confirmed successful aggregation of parental embryos into a singe blastocyst. Finally, we selected 14 chimeric blastocysts consisting of high cell counts and transplanted these into five recipient females (Table S1). All five recipients became pregnant, including two with singletons, two with twins, and one female carrying quadruplets. The pregnancy and implantation rates with chimeric blastocysts were 100% (5/5) and 71% (10/14), respectively. These remarkably high pregnancy outcomes were not seen among other treatments in this study or in our prior studies (Wolf et al., 2004). On average, pregnancy and implantation rates with nonmanipulated rhesus embryos do not exceed 36% and 17%, respectively (Wolf et al., 2004). High pregnancy and implantation results observed

with chimeric blastocysts suggest that higher cell numbers in embryos are critical for pregnancy initiation.

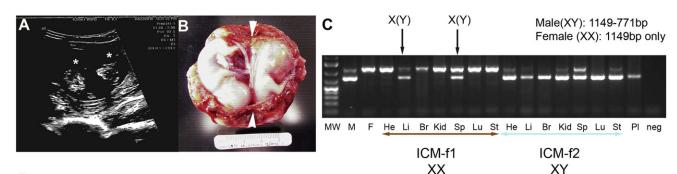
Three pregnancies with chimeric embryos were terminated, and seven fetuses recovered for genetic analysis. Remarkably, all fetuses were of normal size and had no obvious defects or congenital abnormalities. As expected, STR analysis confirmed that all seven fetuses were indeed chimeras (Table S6). Moreover, chimerism was found in all sampled organs and tissues of all fetuses. Some chimeras displayed up to five different alleles within informative individual STR loci, indicating that at least three separate genotypes (embryos) contributed to chimeric tissues (Figure S4; Table S6). Further, the AME marker allows us to determine the gender of fetuses, and three chimeras (EA-f1, 5, and 7) were identified as sex chimeras, indicating that contributing parental embryos were of different genders. Because several chimeras were generated by aggregating embryos from two unrelated females, we also used mtDNA genotyping to confirm presence of two different mtDNA haplotypes in offspring (Figures S4 and S5).

The remaining two recipients (#25 and #27) were allowed to carry pregnancies to term and delivered healthy twin (named Roku and Hex) and singleton (Chimero) infants, respectively (Figure 3). All three infants were phenotypic males with no obvious congenital abnormalities. We genotyped cord blood, cord (representing yolk sac and allantois), fetal membrane (representing amnion, chorion, and decidua), and placental (chorio-amniotic placenta) samples and confirmed that all three infants were indeed chimeras (Figure 3). To our knowledge, these infants are the world's first primate chimeras. Although all three offspring were phenotypic males, we reasoned that these infants could also be sex chimeras due to the high probability that some contributing embryos were genetically females (XX). To investigate this, we carried out detailed cytogenetic analyses of blood by G-banding and fluorescence in situ hybridization (FISH). Indeed, results confirmed that blood samples from Roku contained both male and female cells. Molecular cytogenetic studies revealed the presence of two signals for the rhesus monkey X chromosome in approximately 4% of analyzed cells, whereas remaining cells showed one signal for the X chromosome and one signal for the Y chromosome (Figure S6).

Lineage Segregation in Primate Blastocysts

We next revisited the question of why monkey blastocysts are unable to incorporate transplanted ICMs or ESCs and form embryo proper chimeras. Mouse chimeras with ESCs are routinely generated using embryonic day (E) 3.5 blastocysts as host embryos. However, the ability to form chimeras sharply declines when more advanced stage host blastocysts are used (Ohta et al., 2008). Although underlying mechanisms remain unclear, differentiation of host ICMs into epiblast (EPI) and extraembryonic progenitors is believed to restrict homing of injected ESCs into an ICM (Ohta et al., 2008). For example, in peri-implantation mouse blastocysts (E4.5), the ICM differentiates into two restricted lineages, EPI and the primitive endoderm (PE) (Chazaud et al., 2006; Morrisey et al., 1998). Developmental studies indicated that although the ICMs of E3.5 mouse blastocysts can contribute to all tissues except those of trophectodermal origin, chimeric contributions of EPI or PE cells are restricted

Cell



D

Animal	DNA Origin	AME	D11S2002	↓ ICM-f1 Liver ↓ D11S2002=256/260/264/268	Normal AME profile for male
Sperm donor Male #3	Blood	XY	260/264		
Egg donor Female #5 (host blastocyst)	Blood	xx	256/256		
Egg donor Female #6 (ICM donor)	Blood	XX	244/268	/- ICM-f2 Liver D11S2002=256/260/264/268	als do ats do els do
ICM-f1	Heart	XX	256/200		AME profile for ICM-f1 Liver
ICM-f1	Brain	XX	256/2/60/		
ICM-f1	Liver	X(Y)	256/260/264/20	Twin placenta	
ICM-f2	Heart	XY	264/268//	- D11S2002=256/260/264/268	
ICM-f2	Brain	XY	264/288/		Low Y peak
ICM-f2	Liver	X(Y)	256/260/264/26	8_	
ICM Twin	Placenta	X(Y)	256/260/264/268	8 Maria	to obs sta obs sta

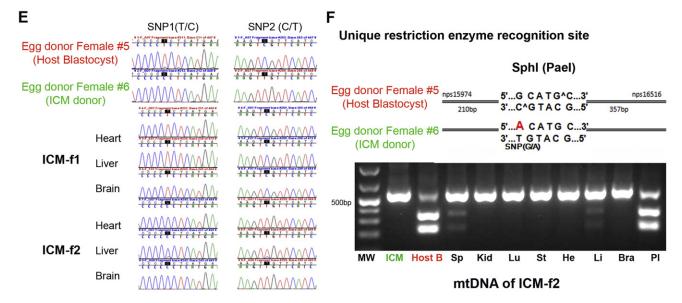


Figure 1. Monochorionic Twin Fetuses Produced by Injection of an ICM into a Blastocyst

(A) Ultrasonography image of a twin pregnancy at 30 days of gestation. Asterisks depict individual fetuses.

(B) Morphological analysis of fetuses recovered on day 51 of gestation. Note that whereas two fetuses share a single placenta, a thin septum (arrowheads) separates each fetal cavity, indicating monochorionic but di amniotic pregnancy.

(C) PCR amplification of ZFX and ZFY regions produced two DNA fragments (male and female). Detection of 771 bp fragment in liver and spleen samples of ICM f1 indicates presence of male cells in female organs.

(D) Analysis of D11S2002 and AME microsatellite loci detected the presence of three different alleles in livers and a placenta of fetuses.

A 1149 771	-	1	_	Ξ	_	Ξ	(,	19-771bp 1149bp only
	Male XY	Female XX	Brain XY	Heart XY	Liver XY	Spleen XY	Stomach XY	Skin XY	Placenta XX(Y)
в							с		
A	nimal	DNA Or	igin	AME	9F	P 06	_	¥ 21114-P 1 T C	2010-10-08,010 Fragment base #330, Base 330 of 4 C T C T C T C A C C A C G G C T C C T C R C C R C G G
Sperm de	onor Male #2	Blood		XX	175	/191	Egg donor Female #3	$\Delta \Delta$	
	or Female #3 blastocyst)	Blood		xx	183	/189	(host blasto	;yst) ⁱ i c	
Egg don	or Female #7 VI donor)	Blood		XY	187	/187	Egg donor Female #7 (ICM donor)	5 T f	
Reci	pient #18	Blood		XX	179	/185	Recipient #1		
IC	CM-f3	Heart		XY	187	/191			00000000000000000000000000000000000000
10	CM-f3	Liver		XY	187	/191	ICM-f3 Brain		
IC	CM-f3	Brain		XY	187	/191	ICM-f3 Heart	$\sqrt{\Lambda}$	
10	CM-f3	Spleer	ı	XY	187	/191		1 C	1026,000 Program base RIDE. Base C T C T C A C T C T C R
IC	CM-f3	Stomac	:h	XY	187	/191	ICM-f3 Liver	\mathbf{M}	$MMM \land \land$
10	CM-f3	Skin		XY	187	/191		- <u>+</u> -200	
IC	CM-f3	Placent	a >	(X(small Y)	179/185	/ <u>189</u> / <u>191</u>	ICM-f3 Place	nta M	Matmana

Figure 2. Parentage Analysis of Offspring Derived from the ICM

(A) Gender specific PCR analysis demonstrating normal profiles for rhesus male (two fragments of 1149 bp and 771 bp size) and female (one 1149 bp fragment) DNA samples. Analysis of tissues and organs from ICM f3 fetus produced by ICM injection demonstrated that the fetus is male whereas the placenta is female. In addition, the placenta showed a faint 771 bp fragment (arrowhead) indicating presence of male cells at low levels.

(B) Microsatellite genotyping within the STR locus 9P06 clearly demonstrating that the fetus originated from the injected ICM, whereas placenta was mainly from the host blastocyst. AME STR locus showed a limited presence of male cells in the female placenta possibly indicating amniotic contribution from the trans planted ICM.

(C) Chromatogram of mtDNA DHV1 region demonstrating informative SNPs that can distinguish mitochondrial contribution in tissues. A mtDNA profile of the ICM f3 fetus matched to the transplanted ICM, whereas the placental mitochondrial genome was a mixture of the host blastocyst and ICM f3. Limited mtDNA contribution from the fetus was also evident in the placental tissues.

See also Table S2 for detailed STR data.

to their own lineages (Gardner, 1982, 1984; Gardner and Rossant, 1979). We reasoned that such differentiation and segregation may already be initiated in the ICMs of monkey blastocysts, thus restricting their chimeric potential. To investigate this possibility, we immunolabeled whole monkey blastocysts or isolated ICMs with markers for EPI (NANOG) and PE (GATA-6). The results indicated that blastocysts indeed contain a layer of GATA-6-positive cells overlaying NANOG-positive EPI cells within ICMs (Figure S7). Even early monkey blastocysts con-

tained spatially segregated GATA-6-positive cells within ICMs. Thus, it is reasonable to speculate that primate ICMs in preimplantation blastocysts consist of at least two lineage-restricted cell types resulting in limited ability to incorporate foreign pluripotent cells.

ESC Integration into Four-Cell Embryos

Based on our results suggesting that monkey cleaving four-cell embryos are capable of incorporating foreign embryonic cells

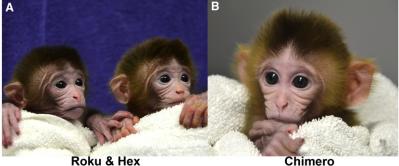
See also Table S2 for detailed STR data.

⁽E) Chromatogram of the rhesus mtDNA DHV1 region showing informative SNPs. Fetus ICM f1 originated from a host blastocyst, whereas ICM f2 developed from the injected ICM.

⁽F) mtDNA RFLP analysis. G allele in mtDNA of the host blastocyst egg donor female #5 is recognized and digested by SphI (Pael), whereas an A allele in the injected ICM egg donor female #6 precludes restriction. mtDNA haplotype of egg donor female #5 was detected in the liver and spleen of the ICM f2 fetus derived from the ICM egg donor female #6.

Abbreviations in (C): MW, M, F, He, Li, Br, Kid, Sp, Lu, St, Pl, and neg indicate molecular weight, male, female, heart, liver, brain, kidney, spleen, lung, stomach, placenta, and negative control, respectively.

С



Roku & Hex

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Animal	DNA origin	AME	D22S685	162B17B
Sperm donor male #6	blood	XY	315/327	295/309
Egg donor Female #15	blood	XX	311/319	309/317
Egg donor Female #16	blood	XX	287/359	289/315
Recipient #25	blood	XX	323/331	293/309
EA-f8 (ROKU)	cord blood	XY	287/315	289/295/309/315
EA-f8 (ROKU)	cord	XY	287/315	289/295/309/315
EA-f8 (ROKU)	Membrane	XY	287/315/323/331	289/293/295/309
EA-f8 (ROKU)	Placental body	XY	287/315/ <u>323</u> / <u>331</u>	289/295/ <u>309</u> / <u>315</u>
EA-f9 (HEX)	cord blood	XY	287/327	309/315
EA-f9 (HEX)	cord	XY	287/319/327	309/315/317
EA-f8 (HEX)	Membrane	XY	287/319/323/327/331	293/309/315/317
EA-f8 (HEX)	Placental body	XY	<u>287/311/315/319/327</u>	309/315
D				
Animal	DNA origin	AME	D7S513	D9S921
Sperm donor Male #1	blood	XY	197/201	167/199
Egg donor Female #17	blood	XX	189/205	179/195
Egg donor Female #18	blood	XX	191/235	187/195
Recipient #27	blood	XX	199/215	195/207
EA-f10 (CHIMERO)	cord blood	XY	191/197/201/235	167/187/195/199
EA-f10 (CHIMERO)	cord	XY	191/197/201/235	167/187/195/199
EA-f10 (CHIMERO)	Membrane	XY	191/197/201/235	167/187/195/199
EA-f10 (CHIMERO)	Placental body	XY	191/199/201/215/235	195/199/207

and forming chimeras, we reasoned that injection of ESCs into four-cell embryos might support generation of embryo proper chimeras. GFP-expressing monkey ESCs were injected under the zona pellucida and placed between blastomeres of fourcell embryos and resulting aggregates cultured to the blastocyst stage. We examined blastocysts for aggregation with ESCs based on GFP expression and transferred six GFP-positive embryos into two recipients (Figure 4 and Table S1). One became pregnant carrying a singleton fetus that was recovered at midgestation for the analysis. However, again, we found no contribution of ESCs in tested tissues and organs.

Because ESCs are pluripotent and are not developmentally equivalent to host totipotent blastomeres of the four-cell embryo, we reasoned that injected ESCs may prematurely differentiate prior to blastocyst formation, thus precluding their contribution to the ICM. To test this assumption, we injected GFPexpressing ESCs into four-cell embryos and analyzed resulting blastocysts by immunocytochemistry. Based on GFP expression, the majority of blastocysts contained embedded ESCs within TE or ICM (Figure 4). In some blastocysts, ESCs detached from host embryos and formed free-floating embryoid bodies. We selected and labeled blastocysts expressing GFP specifically in the ICM area with NANOG, and subsequent analysis indicated that GFP-expressing ESCs are NANOG negative, whereas

Figure 3. Chimeric Infants Generated by Whole **Embryo Aggregation**

(A and B) Live chimeric offspring (Roku, Hex [indicating "six" in Japanese and Greek], and Chimero) each pro duced by aggregating of six individual embryos. The pictures were taken at 7 days after birth.

(C and D) Genetic analysis of blood and extraembryonic tissues based on microsatellite examination demon strating presence of more than two alleles for each locus. See also Tables S4 and S6 and Figure S6.

remaining host embryo ICM cells strongly express NANOG. Thus, these results support the notion that although cleaving host embryos can incorporate ESCs, the environment does not support undifferentiated growth of ESCs. During the 4-5 day window that is required for injected embryos to reach the blastocyst stage, aggregated ESCs undergo differentiation and lose pluripotency. This phenomenon is likely to preclude contribution of ESCs to the fetal tissues and organs.

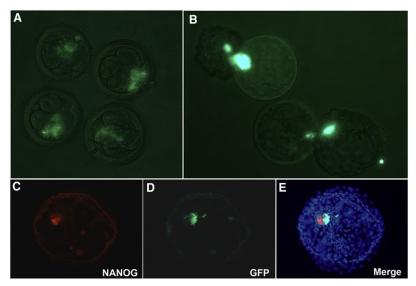
DISCUSSION

Based on the comprehensive analysis of the developmental potency in vivo, we demonstrate that monkey blastocysts do not readily aggregate with transplanted ICMs or ESCs and form embryo proper chimeras (Figure 5). Our results suggest that cells within monkey ICMs, even in early preimplantation blastocysts, are not homogeneous. We detected two cell types

within monkey ICMs where a cluster of NANOG-positive EPI cells is covered by GATA-6-positive PE cells. Mouse studies have shown that segregation of ICMs into EPI and PE becomes apparent in mouse peri-implantation stage blastocysts (E4.5) (Cockburn and Rossant, 2010). Mouse chimeras are routinely generated using E3.5 host blastocysts, where the ICM is not visibly differentiated into EPI and PE fates yet. However, differentiation and lineage commitment in later-stage blastocysts significantly inhibit integration of injected ESCs into EPI and formation of embryo proper chimeras (Ohta et al., 2008). It is possible that segregation of ICMs seen in monkey blastocysts may have diminished their ability to incorporate foreign cells and development of embryo proper chimeras.

Remarkably, transplanted monkey ICMs developed into viable fetuses with a TE support from a host blastocyst. This demonstrates an extraordinary developmental potential of primate ICMs compared to that of established ESCs.

Mouse studies showed that in addition to the embryo proper, the EPI gives rise to several extraembryonic derivatives including amniotic ectoderm, extraembryonic mesoderm of the amnion, allantois, visceral yolk sac, and chorion (Gardner, 1983; Mackay and West, 2005). The PE contributes to the endoderm layer of the visceral yolk sac and the parietal endoderm associated with Reichert's membrane. The TE part underlying the ICM, known



as the polar trophectoderm, forms the chorionic ectoderm and placental trophoblast, whereas the TE part surrounding the blastocyst cavity, known as the mural trophectoderm, contributes to the primary trophoblast giant cells (Mackay and West, 2005). Thus, whereas the embryo proper develops exclusively from the ICM, both the ICM and TE contribute to the extraembryonic lineages. In this study, we did not incorporate easily traceable markers that would allow examination of what specific extraembryonic cells and layers were contributed by the host embryo or developed from the transplanted ICMs. However, DNA analysis of mixed extraembryonic samples containing presumably chorion and amnion revealed that both host embryo and injected ICMs contributed to these tissues.

Chimerism in the body of ICM twins produced in this study was limited to organs rich in blood, suggesting that placental vascular anastomoses and blood mixture could be involved rather than true chimerism in solid tissues. Blood chimerism has been reported in human twin pregnancies (van Dijk et al., 1996). Interestingly, in marmosets and other callitrichid primates, even fraternal twins exchange blood through chorionic fusions, and up to 95% of pregnancies result in the birth of hematopoietic chimeras (Gengozian et al., 1964). As mentioned above, naturally occurring human monochorionic twins are predominantly monozygotic, but rare cases of monochorionic dizygotic twin pregnancies have been reported in association with clinical IVF procedures (Quintero et al., 2003; Souter et al., 2003). Monochorionic pregnancies carry increased risk of abortions or premature birth due to aberrant vascularizations leading to twin-to-twin transfusion syndrome and/or vascular disruptions. In the case of our monkey twin fetuses, we did not find any apparent malformations or abnormalities, and examination of the placenta showed no obvious defects. Our experimental approach for generating offspring from transplanted ICMs may represent an important nonhuman primate model for studying the development of genetically different fetuses in a monochorionic environment.

In contrast to ICMs, monkey ESCs did not form completely stem cell-derived fetuses, most likely due to inability of host

Figure 4. Detection of ESCs in Blastocysts Devel oped from Four Cell Embryos Injected with GFP Positive ESCs

(A) GFP expressing ESCs were injected into four cell embryos and placed between blastomeres.

(B) Blastocysts with GFP embedded cells.

(C E) Immune staining for NANOG demonstrated that GFP expressing ESCs within an ICM were NANOG negative, indicating premature differentiation. Original magnifications: (A E), ×200.

embryos to support with vital extraembryonic tissues. Comparative studies between mouse ICMs and ESCs suggested that the latter are restricted to the embryo proper and extraembryonic mesoderm lineages, whereas ICMs contribute to the embryo proper and a wide range of extraembryonic lineages (Beddington and Robertson, 1989; Tam and Rossant, 2003). Particularly, it is well known that mouse ESCs

are not capable of contributing to the PE lineage that gives rise to yolk sac, a critical structure for normal fetal development (Beddington and Robertson, 1989; Rossant, 2007). Chimera studies indicated that this lineage is preserved in isolated mouse ICMs, and when transplanted into host embryo, ICMs can contribute to the PE (Tam and Rossant, 2003). Interestingly, although mouse ICMs injected into host diploid (2N) blastocysts form chimeras, their ability to develop into separate fetuses has not been reported.

Mouse ESCs can develop into whole stem cell-derived offspring when aggregated with tetraploid (4N) host embryos (Nagy et al., 1990, 1993). Chimera studies with 4N-2N embryos have shown that tetraploid cells can contribute to functional trophectoderm and PE lineages but not to the EPI (James et al., 1995). Thus, in mouse ESC-4N chimeras, two embryonic cell types complement each other, with ESCs forming the embryo proper, and 4N embryos developing into extraembryonic lineages. It is important to note that tetraploid complementation is considered to be the most stringent but extremely inefficient pluripotency test for mouse ESCs. Although the majority of mouse ESC lines do contribute to conventional chimeras, only selected lines are able to produce whole ESC-derived live offspring after aggregation with tetraploid host embryos (Nagy et al., 1990, 1993).

We also demonstrate here that totipotent, cleaving monkey embryos cannot serve as a host for testing pluripotency of ESCs. Unlike mouse, where two- to eight-cell stage embryos engraft ESCs and form chimeras (Wood et al., 1993), cleaving primate embryos do not seem to provide a niche for undifferentiated maintenance of ESCs until the host ICM is formed. Although we did not test directly in this study, it is likely that ICMs injected into four-cell embryos would also prematurely differentiate. Based on these results, it is reasonable to speculate that a chimera assay is not a feasible pluripotency assay with the approaches employed here.

The ability of mouse pluripotent cells to generate chimeras or completely ESC-derived offspring incited ethical concerns that

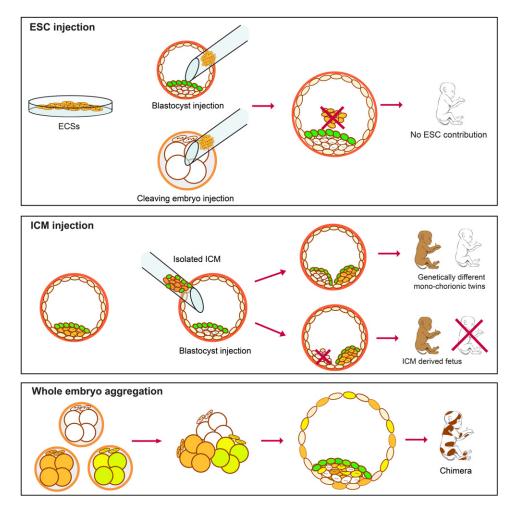


Figure 5. Summary of Chimera Studies with Monkey Embryos and Embryonic Cells

Rhesus monkey ESCs as well as isolated ICMs, blastomeres, or whole embryos were tested for their ability to incorporate into host embryos and generate chimeric offspring. Established ESCs and freshly isolated ICMs failed to produce chimeras when injected into host blastocysts. However, ICMs developed into separate fetuses with placental support from the host embryo. Aggregating of several four cell embryos efficiently produced live chimeric offspring. See also Figure S7 and Movie S1.

human ESCs and iPSCs could be used to clone humans or create chimeras (Lanza, 2007). However, based on our study, these concerns seem to be unattainable or more challenging, at least when using monkey cells and embryos.

On the other hand, although monkey ESCs did not contribute to fetuses in our study, we cannot rule out their broad pluripotency and ability to differentiate to many if not all cell types of the embryo proper. As we previously described, monkey ESCs can form teratomas in vivo or differentiate in vitro into a broad range of cell and tissue types representing all three germ layers (Mitalipov et al., 2006). It will be important to develop additional in vivo assays defining potency of primate ESCs and determining their potential in regenerative medicine. In addition, further studies with other human or monkey experimental pluripotent stem cells may be warranted.

On a related issue, monkey and human ESCs are considered to be more similar to mouse epiblast stem cells (EpiSCs) derived from postimplantation stage embryos than to mouse ESCs (Brons et al., 2007; Rossant, 2008; Tesar et al., 2007). Notably, although mouse EpiSCs can differentiate into teratomas, they display limited capacity to form chimeras. Therefore, failure of monkey ESCs to contribute to chimeras may be due to their epiblast stem cell-like nature. Interestingly, recent studies have shown that mouse EpiSCs can be converted to more potent "naive" ESC state capable of forming chimeras (Guo et al., 2009). It has been speculated that human and monkey ESCs can be also directed toward a similar naive state (Hanna et al., 2010). If such cells become available, our chimera assay would be critical for ultimate testing of their potential.

We demonstrate here that aggregating of three or more cleaving monkey embryos results in chimeric offspring with extensive contribution to the embryo proper and extraembryonic lineages. In fetuses, chimerism was present in all tissues and organs including gonads, suggesting a broad developmental potential of parental blastomeres. Moreover, because we aggregated up to six individual embryos per chimera, we observed that both female (XX) and male (XY) parental embryos contributed to a chimera. Gender ratio in mouse chimeras produced by injection of male ESCs is skewed toward males due to conversion of some sex (XY-XX) chimeras into phenotypic males (Delhaise et al., 1993). All three live infants in our study were phenotypically male, suggesting that presence of XY cells may also convert chimeras to males. Based on the cytogenetic analysis of blood, sex chimerism was confirmed in one infant. It would be critical to investigate further chimerism in various tissues including gonads and reproductive capacity in these animals.

Remarkable in vivo developmental rates were obtained when several four-cell embryos were aggregated together. It would be interesting to investigate whether such "enhanced" host embryos can improve chimera outcomes with ESCs. Another potential use of this chimera assay is to enhance or complement development of cloned monkey embryos. We previously demonstrated that monkey SCNT embryos are capable of developing efficiently into ESCs (Byrne et al., 2007; Sparman et al., 2009). However, their in vivo potential after transfer into recipients remains limited. We have been able to establish several early pregnancies, but none of them progressed to term (Sparman et al., 2010). Aggregation chimeras of SCNT embryos with fertilized counterparts would be extremely useful to determine whether developmental failure of SCNT embryos is due to inefficient reprogramming into specific extraembryonic or embryo proper lineages.

Currently, there is little known about human and nonhuman primate embryo development and lineage specification and how closely the mouse development reflects primates. Our study presents an indication of the similarities and differences between mouse and primate preimplantation embryo development and offers an important experimental model to investigate lineage commitment and interactions.

EXPERIMENTAL PROCEDURES

All animal procedures were approved by the Institutional Animal Care and Use Committee at the ONPRC/OHSU.

ESC Culture and Preparation for Injections

ESCs were cultured on a feeder layer consisting of mouse embryonic fibroblasts (MEFs) in DMEM/F12 medium with high glucose but without sodium pyruvate and supplemented with 1% nonessential amino acids, 2 mM I glutamine, 0.1 mM β mercaptoethanol, and 15% FBS at 37°C, 3% CO2, 5% O2, and 92% N2. Established rhesus monkey ESCs were fully characterized and tested by in vitro and in vivo differentiation as previously described (Byrne et al., 2007; Mitalipov et al., 2006). To generate GFP expressing cells, ESCs were transduced with replication deficient lentiviral vector carrying a GFP reporter gene down stream of the pSin EF2 Puromycin sequence (Addgene, Inc). Twenty four hours after transduction, ESCs were split onto new puromycin resistant MEF feeders and cultured in a medium with 2 μ g/ml puromycin for 2 3 days. Culture medium was changed daily, and ESC colonies were typically split every 5 7 days by manual dissociation and replating collected clumps onto fresh MEFs.

For disaggregation prior to injections, ESCs were treated with nonenzymatic TRYPLE (Invitrogen), dissociated by pipetting into single cells, and resus pended with TH3 medium. Alternatively, ESC colonies were manually picked up and mechanically dispersed to smaller clumps before injections.

Injection of ESCs

Embryos were generated by intracytoplasmic sperm injection (ICSI) as described in the Extended Experimental Procedures. Host embryos at the

four cell or blastocyst stages were transferred to a 30 μ l manipulation droplet of TH3 placed on the center of glass bottom manipulation dishes (http://www. wpiinc.com) covered with paraffin oil (Zander IVF). ESCs were placed into a separate 5 μ l droplet next to the manipulation drop containing host embryos. A host blastocyst was held with holding pipette with an ICM positioned at 12 o'clock (see Movie S1). Approximately 20 30 disaggregated ESCs or clumps of cells were drawn into an injection pipette. A single laser pulse (http://www. hamiltonthorne.com) was fired to ablate the zona pellucida and underlying tro phectodermal layer, and an injection pipette was immediately pierced through the hole. Next, ESCs were expelled and placed close to the host ICM (see Movie S1). Injected blastocysts were immediately transferred into oviducts of synchronized recipient females. For four cell stage host embryos, ESCs were injected between blastomeres, and embryos were cultured to the blasto cyst stage. When GFP expressing ESCs were used, GFP expression was regularly monitored with an epifluorescent microscope. Only blastocysts with visible GFP expressing cells were transferred into recipients.

Injection of ICMs

Intact ICMs were isolated by immunosurgery. In brief, the zona pellucida from each blastocyst was removed by brief (10 s) treatment with 0.5% protease (pronase, Sigma, P 8811). Blastocysts were then incubated in anti monkey whole serum (Sigma, M 0403) for 30 min at 37°C, washed three times with TH3, and transferred into Guinea pig complement (Sigma, S 1639) for 30 min. Blastocysts were gently pipetted with a small bore pipette to disperse lysed trophectodermal cells, and isolated ICMs were washed and immediately injected into host blastocysts as described above (See Movie S1).

Blastomere Replacement

Four cell stage (D2) embryos were generated by ICSI from unrelated females and treated with 0.5% pronase to remove zona pellucida. Embryos were incubated in Ca and Mg free medium (Invitrogen) for 15 min and transferred to 30 µl manipulation drop of TH3 medium containing 10 µg/ml cytochalasin B. Embryos were further incubated at 37°C for an additional 10 15 min before manipulation. Blastomeres from a four cell embryo were split in half using an injection pipette (45 50 outer diameter, polished), and each half (two blasto meres) was transferred inside of two separate empty zona pellucida. Then, another half set of blastomeres from an unrelated female was added under each zona. Aggregated embryos were cultured to the blastocyst stage and transferred into oviducts of synchronized recipients.

Whole-Embryo Aggregation

Four cell stage (D2) embryos were treated with 0.5% pronase to remove zona pellucida. Three to six zona free embryos were aggregated together by mechanically pushing against each other. Aggregates were transferred into 30 μ l culture drop containing HECM 9 medium and cultured individually at 37°C in 6% CO2, 5% O2, and 89% N2.

Embryo Transfers

Beginning 8 days after menses during a spontaneous menstrual cycle, blood samples were collected daily from the saphenous vein of rhesus females for estradiol level analysis by radioimmunoassay. The day after the serum estradiol peak was considered the day of ovulation (day 0). This peak occurred on average on day 11 post menses, with a range from 8 to 17 days. Two to six days after the estradiol peak, embryos were transferred surgically into the oviduct ipsilateral to the ovary bearing the ovulatory stigma, a described previ ously (Wolf et al., 2004).

Fetal Recovery and Sample Collection

Fetuses were recovered by cesarean section at midgestation, and fetuses were euthanized with transumbilical cord injection of pentobarbital. Autopsies were conducted to collect tissues from each organ separately.

Parentage Analysis

DNA was extracted from blood or tissues using commercial kits (Gentra). STR microsatellite parentage analysis was conducted by the Veterinary Genetics Laboratory at University of California, Davis as described previously (Byrne et al., 2007; Tachibana et al., 2009). In brief, six multiplexed PCR reactions

were set up for the amplification of 39 markers representing 25 autosomal loci and 14 autosomal, MHC linked loci. On the basis of the published rhesus macaque linkage map (Rogers et al., 2006), these markers are distributed in 19 chromosomes. Two of the markers included in the panel, MFGT21 and MFGT22 (Domingo Roura et al., 1997), were developed from Macaca fuscata and do not have chromosome assignment.

For gender determination, X and Y linked zinc finger protein genes (ZFX and ZFY) were amplified as previously described (Mitalipov et al., 2007). MtDNA analysis was performed as previously described (Tachibana et al., 2009). In brief, the rhesus macaque mitochondrial D loop hypervariable region 1 (RhDHV1) sequence was amplified using forward (5' CCAACACCCA AAGCTGGCATTCTA 3') and reverse (5' ATGGCCCTGAGGTAAGAACCA GAT 3') primers. PCR analysis was performed using PCR super mix high fidelity DNA polymerase (Invitrogen) containing 0.5 µM of each primer (final volume 50 μ l). Reaction conditions were initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s; annealing at 60°C for 30 s; extension at 68°C for 90 s; and a final extension at 68°C for 3 min, generating 547 bp of sequence covering the RhDHV1 region. PCR products were sequenced, and informative SNPs encompassing Macaca mulatta mtDNA nucleotide positions 15974 16516 (GenBank NC 005943) were identified using Sequencher v. 4.7 software (GeneCodes). For restriction fragment length polymorphism (RFLP) analysis, PCR products were amplified as described above. Unique restriction digestion sites were identified with Sequencher v. 4.7. Restriction enzymes were from Fermentas. For reactions, guantity of PCR products was adjusted to 500 ng and digested by appropriate enzymes. Samples were analyzed using 3% agarose gel.

For PCR based detection of ESC progeny carrying GFP, eGFP sequence was amplified using forward (5' GCACAAGCTGGAGTACAACTACAACAGC 3') and reverse (5' TCACGAACTCCAGCAGGACCAT 3') primers as previously de scribed (Sasaki et al., 2009). PCR reaction was performed as follows: initial dena turation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s; annealing at 58°C for 30 s; extension at 68°C for 90 s; and final extension at 68°C for 3 min.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, six tables, and one movie and can be found with this article online at doi:10.1016/j.cell.2011.12.007.

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REFERENCES

Beddington, R.S., and Robertson, E.J. (1989). An assessment of the develop mental potential of embryonic stem cells in the midgestation mouse embryo. Development *105*, 733–737.

Bradley, A., Evans, M., Kaufman, M.H., and Robertson, E. (1984). Formation of germ line chimaeras from embryo derived teratocarcinoma cell lines. Nature *309*, 255 256.

Brem, G., Tenhumberg, H., and Kräußlich, H. (1984). Chimerism in cattle through microsurgical aggregation of morulae. Theriogenology 22, 609 613.

Brons, I.G., Smithers, L.E., Trotter, M.W., Rugg Gunn, P., Sun, B., Chuva de Sousa Lopes, S.M., Howlett, S.K., Clarkson, A., Ahrlund Richter, L., Pedersen, R.A., and Vallier, L. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 448, 191 195.

Byrne, J.A., Pedersen, D.A., Clepper, L.L., Nelson, M., Sanger, W.G., Gokhale, S., Wolf, D.P., and Mitalipov, S.M. (2007). Producing primate embryonic stem cells by somatic cell nuclear transfer. Nature *450*, 497 502.

Capecchi, M.R. (1989). Altering the genome by homologous recombination. Science 244, 1288 1292.

Chan, A.W., Dominko, T., Luetjens, C.M., Neuber, E., Martinovich, C., Hewit son, L., Simerly, C.R., and Schatten, G.P. (2000). Clonal propagation of primate offspring by embryo splitting. Science 287, 317 319.

Chazaud, C., Yamanaka, Y., Pawson, T., and Rossant, J. (2006). Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2 MAPK pathway. Dev. Cell *10*, 615–624.

Cockburn, K., and Rossant, J. (2010). Making the blastocyst: lessons from the mouse. J. Clin. Invest. *120*, 995 1003.

Cunningham, F.G., and Williams, J.W. (2005). Williams Obstetrics, Twenty Second Edition (New York: McGraw Hill Professional).

Delhaise, F., Zhao, X., Bralion, V., Dessy, F., and Georges, M. (1993). Quanti tative estimation of chimerism in mice using microsatellite markers. Mol. Reprod. Dev. *34*, 127 132.

Domingo Roura, X., López Giráldez, T., Shinohara, M., and Takenaka, O. (1997). Hypervariable microsatellite loci in the Japanese macaque (Macaca fuscata) conserved in related species. Am. J. Primatol. *43*, 357 360.

Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripoten tial cells from mouse embryos. Nature *292*, 154 156.

Fehilly, C.B., Willadsen, S.M., and Tucker, E.M. (1984). Interspecific chimae rism between sheep and goat. Nature *307*, 634 636.

Gardner, R.L. (1968). Mouse chimeras obtained by the injection of cells into the blastocyst. Nature 220, 596 597.

Gardner, R.L. (1982). Investigation of cell lineage and differentiation in the extraembryonic endoderm of the mouse embryo. J. Embryol. Exp. Morphol. *68*, 175–198.

Gardner, R.L. (1983). Origin and differentiation of extraembryonic tissues in the mouse. Int. Rev. Exp. Pathol. 24, 63 133.

Gardner, R.L. (1984). An in situ cell marker for clonal analysis of development of the extraembryonic endoderm in the mouse. J. Embryol. Exp. Morphol. *80*, 251 288.

Gardner, R.L., and Munro, A.J. (1974). Successful construction of chimaeric rabbit. Nature 250, 146 147.

Gardner, R.L., and Rossant, J. (1979). Investigation of the fate of 4 5 day post coitum mouse inner cell mass cells by blastocyst injection. J. Embryol. Exp. Morphol. *52*, 141 152.

Gengozian, N., Batson, J.S., and Eide, P. (1964). Hematologic and cytogenetic evidence for hematopoietic chimerism in the marmoset, tamarinus nigricollis. Cytogenetics *10*, 384 393.

Guo, G., Yang, J., Nichols, J., Hall, J.S., Eyres, I., Mansfield, W., and Smith, A. (2009). Klf4 reverts developmentally programmed restriction of ground state pluripotency. Development *136*, 1063–1069.

Hanna, J., Cheng, A.W., Saha, K., Kim, J., Lengner, C.J., Soldner, F., Cassady, J.P., Muffat, J., Carey, B.W., and Jaenisch, R. (2010). Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. Proc. Natl. Acad. Sci. USA *107*, 9222 9227.

James, R.M., Klerkx, A.H., Keighren, M., Flockhart, J.H., and West, J.D. (1995). Restricted distribution of tetraploid cells in mouse tetraploid<==>diploid chimaeras. Dev. Biol. *167*, 213 226.

Lanza, R. (2007). Stem cell breakthrough: don't forget ethics. Science 318, 1865.

Mackay, G.E., and West, J.D. (2005). Fate of tetraploid cells in 4n< $\,$ >2n chimeric mouse blastocysts. Mech. Dev. 122, 1266 1281.

Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. USA 78, 7634 7638.

Matsui, Y., Zsebo, K., and Hogan, B.L. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell *70*, 841 847.

Mayer, J.F., Jr., and Fritz, H.I. (1974). The culture of preimplantation rat embryos and the production of allophenic rats. J. Reprod. Fertil. 39, 1 9.

Mintz, B. (1962). Experimental study of the developing mammalian egg: Removal of the zona pellucida. Science *138*, 594 595.

Mintz, B., and Illmensee, K. (1975). Normal genetically mosaic mice produced from malignant teratocarcinoma cells. Proc. Natl. Acad. Sci. USA *72*, 3585 3589.

Mitalipov, S., Kuo, H.C., Byrne, J., Clepper, L., Meisner, L., Johnson, J., Zeier, R., and Wolf, D. (2006). Isolation and characterization of novel rhesus monkey embryonic stem cell lines. Stem Cells *24*, 2177 2186.

Mitalipov, S.M., Zhou, Q., Byrne, J.A., Ji, W.Z., Norgren, R.B., and Wolf, D.P. (2007). Reprogramming following somatic cell nuclear transfer in primates is dependent upon nuclear remodeling. Hum. Reprod. *22*, 2232 2242.

Morrisey, E.E., Tang, Z., Sigrist, K., Lu, M.M., Jiang, F., Ip, H.S., and Parmacek, M.S. (1998). GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. Genes Dev. *12*, 3579 3590.

Nagy, A., Gócza, E., Diaz, E.M., Prideaux, V.R., Iványi, E., Markkula, M., and Rossant, J. (1990). Embryonic stem cells alone are able to support fetal devel opment in the mouse. Development *110*, 815 821.

Nagy, A., Rossant, J., Nagy, R., Abramow Newerly, W., and Roder, J.C. (1993). Derivation of completely cell culture derived mice from early passage embryonic stem cells. Proc. Natl. Acad. Sci. USA *90*, 8424 8428.

Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germline competent induced pluripotent stem cells. Nature *448*, 313 317.

Ohta, H., Sakaide, Y., and Wakayama, T. (2008). Generation of mice derived from embryonic stem cells using blastocysts of different developmental ages. Reproduction *136*, 581 587.

Quintero, R.A., Mueller, O.T., Martínez, J.M., Arroyo, J., Gilbert Barness, E., Hilbelink, D., Papenhausen, P., and Sutcliffe, M. (2003). Twin twin transfusion syndrome in a dizygotic monochorionic diamniotic twin pregnancy. J. Matern. Fetal Neonatal Med. *14*, 279–281.

Rogers, J., Garcia, R., Shelledy, W., Kaplan, J., Arya, A., Johnson, Z., Berg strom, M., Novakowski, L., Nair, P., Vinson, A., et al. (2006). An initial genetic linkage map of the rhesus macaque (Macaca mulatta) genome using human microsatellite loci. Genomics *87*, 30 38.

Rossant, J. (2007). Stem cells and lineage development in the mammalian blastocyst. Reprod. Fertil. Dev. 19, 111 118.

Rossant, J. (2008). Stem cells and early lineage development. Cell 132, 527 531.

Sasaki, E., Suemizu, H., Shimada, A., Hanazawa, K., Oiwa, R., Kamioka, M., Tomioka, I., Sotomaru, Y., Hirakawa, R., Eto, T., et al. (2009). Generation of transgenic non human primates with germline transmission. Nature *459*, 523 527.

Souter, V.L., Kapur, R.P., Nyholt, D.R., Skogerboe, K., Myerson, D., Ton, C.C., Opheim, K.E., Easterling, T.R., Shields, L.E., Montgomery, G.W., and Glass, I.A. (2003). A report of dizygous monochorionic twins. N. Engl. J. Med. *349*, 154 158.

Sparman, M., Dighe, V., Sritanaudomchai, H., Ma, H., Ramsey, C., Pedersen, D., Clepper, L., Nighot, P., Wolf, D., Hennebold, J., and Mitalipov, S. (2009). Epigenetic reprogramming by somatic cell nuclear transfer in primates. Stem Cells *27*, 1255 1264.

Sparman, M.L., Tachibana, M., and Mitalipov, S.M. (2010). Cloning of non human primates: the road "less traveled by". Int. J. Dev. Biol. 54, 1671 1678.

Tachibana, M., Sparman, M., Sritanaudomchai, H., Ma, H., Clepper, L., Wood ward, J., Li, Y., Ramsey, C., Kolotushkina, O., and Mitalipov, S. (2009). Mito chondrial gene replacement in primate offspring and embryonic stem cells. Nature *461*, 367–372.

Tam, P.P., and Rossant, J. (2003). Mouse embryonic chimeras: tools for studying mammalian development. Development *130*, 6155 6163.

Tarkowski, A.K. (1961). Mouse chimaeras developed from fused eggs. Nature 190, 857 860.

Tesar, P.J., Chenoweth, J.G., Brook, F.A., Davies, T.J., Evans, E.P., Mack, D.L., Gardner, R.L., and McKay, R.D. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature *448*, 196–199.

Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A., and Hearn, J.P. (1995). Isolation of a primate embryonic stem cell line. Proc. Natl. Acad. Sci. USA *92*, 7844–7848.

Thomson, J.A., Itskovitz Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145–1147.

Tucker, E.M., Moor, R.M., and Rowson, L.E. (1974). Tetraparental sheep chimaeras induced by blastomere transplantation. Changes in blood type with age. Immunology *26*, 613 621.

van Dijk, B.A., Boomsma, D.I., and de Man, A.J. (1996). Blood group chime rism in human multiple births is not rare. Am. J. Med. Genet. *61*, 264 268.

Wakayama, T., Tabar, V., Rodriguez, I., Perry, A.C., Studer, L., and Mom baerts, P. (2001). Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science *292*, 740–743.

Wolf, D.P., Thormahlen, S., Ramsey, C., Yeoman, R.R., Fanton, J., and Mita lipov, S. (2004). Use of assisted reproductive technologies in the propagation of rhesus macaque offspring. Biol. Reprod. *71*, 486–493.

Wood, S.A., Pascoe, W.S., Schmidt, C., Kemler, R., Evans, M.J., and Allen, N.D. (1993). Simple and efficient production of embryonic stem cell embryo chimeras by coculture. Proc. Natl. Acad. Sci. USA *90*, 4582–4585.