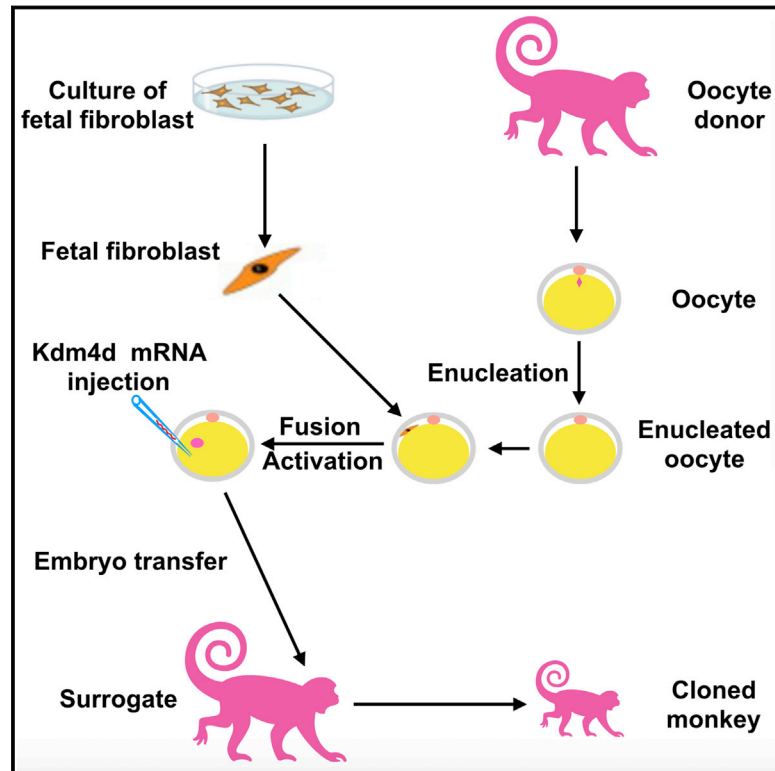


Cloning of Macaque Monkeys by Somatic Cell Nuclear Transfer

Graphical Abstract



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In Brief

Generation of cloned cynomolgus monkeys by somatic cell nuclear transfer using fetal monkey fibroblasts.

Highlights

- Somatic cell nuclear transfer (SCNT) using fetal fibroblasts yielded two live monkeys
- Epigenetic modulators promoted development and pregnancy rate of SCNT embryos
- SCNT using adult cumulus cells yielded live births of monkeys that were short-lived
- Genetic analysis confirmed the clonal origin of the SCNT monkey offspring



Cloning of Macaque Monkeys by Somatic Cell Nuclear Transfer

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SUMMARY

Generation of genetically uniform non-human primates may help to establish animal models for primate biology and biomedical research. In this study, we have successfully cloned cynomolgus monkeys (*Macaca fascicularis*) by somatic cell nuclear transfer (SCNT). We found that injection of H3K9me3 demethylase *Kdm4d* mRNA and treatment with histone deacetylase inhibitor trichostatin A at one-cell stage following SCNT greatly improved blastocyst development and pregnancy rate of transplanted SCNT embryos in surrogate monkeys. For SCNT using fetal monkey fibroblasts, 6 pregnancies were confirmed in 21 surrogates and yielded 2 healthy babies. For SCNT using adult monkey cumulus cells, 22 pregnancies were confirmed in 42 surrogates and yielded 2 babies that were short-lived. In both cases, genetic analyses confirmed that the nuclear DNA and mitochondria DNA of the monkey offspring originated from the nucleus donor cell and the oocyte donor monkey, respectively. Thus, cloning macaque monkeys by SCNT is feasible using fetal fibroblasts.

INTRODUCTION

Cloning of animals by somatic cell nuclear transfer (SCNT) has been achieved in 23 mammalian species (see review Rodriguez-Osorio et al., 2012), including sheep (Wilmut et al., 2007), mouse (Wakayama et al., 1998), cattle (Kato et al., 1998), pig (Polejaeva et al., 2000), cat (Shin et al., 2002), rat (Zhou et al., 2003), and dog (Lee et al., 2005). As species closer to humans, non-human primates are ideal animal models for studying physiological functions unique to primates and for developing therapeutic treatments of human diseases (Izpisua Belmonte et al., 2015; Jennings et al., 2016). Animal models with genetic uniformity are often desirable (Schramm and Paprocki, 2004), but an inbreeding approach as used in generating rodent models is not practical for non-human primates because of their long generation time (Smedley et al., 2002), although the latter could be shortened by speeding sperm maturation with testicular

xenografting (Liu et al., 2016b). Cloning of non-human primates by SCNT has failed to generate live offspring so far (Mitalipov et al., 2002; Mitalipov et al., 2007; Ng et al., 2004; Simerly et al., 2003; Sparman et al., 2010). In view of the fact that live monkeys could be generated using nuclear transfer of early blastomeres (Meng et al., 1997), previous failure of SCNT appears to be caused by inappropriate reprogramming of the somatic nucleus for supporting the development of transplanted embryos.

Histone deacetylase inhibitors such as trichostatin A and scriptaid have been used to improve the efficiency of mammalian SCNT in several species, including mouse, bovine, pig, monkey, and human (Akagi et al., 2011; Kishigami et al., 2006; Sparman et al., 2010; Tachibana et al., 2013; Zhao et al., 2009). Furthermore, reprogramming-resistant regions (RRRs) that are enriched for histone 3 lysine 9 trimethylation (H3K9me3) modification were identified in SCNT embryos. Expression of human H3K9me3 demethylase *Kdm4d/4a* could reduce the H3K9me3 level and significantly improve the efficiency of both mouse and human SCNT (Antony et al., 2013; Chung et al., 2015; Liu et al., 2016a; Matoba et al., 2014), suggesting that RRRs are conserved among mammalian species. In this study, we applied both histone demethylase *Kdm4d* mRNA and histone deacetylase inhibitor trichostatin A (TSA) to the cloning of macaque monkeys. We performed SCNT using both fetal monkey fibroblasts and adult monkey cumulus cells and successfully produced live birth of monkey offspring carrying nuclear DNA of the donor cell and mitochondria DNA of the oocyte donor monkey. Monkey neonates generated using fetal fibroblasts were healthy, whereas those generated using adult cumulus cells survived only briefly after birth. Since fetal fibroblasts could be genetically modified efficiently *in vitro* and properly screened for precise gene editing (Gao et al., 2017; Lai et al., 2016; Rogers, 2016) prior to SCNT, our results pave the way for the generation of genetically uniform monkey models for basic research and biomedical applications.

RESULTS

Optimization of SCNT Methods

We first optimized the SCNT protocol, including polarized-light imaging for the removal of spindle-chromosome complex from the oocyte, incubation of the nuclear donor cell with viral envelope isolated from the Hemagglutinin Virus of Japan (HVJ-E,



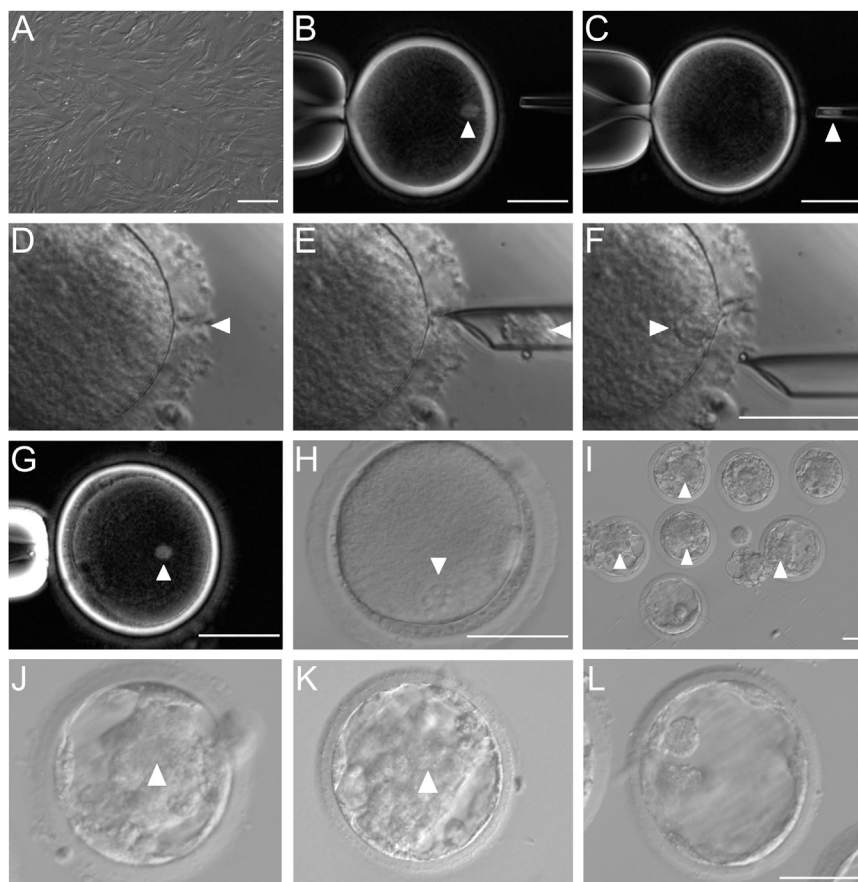


Figure 1. Procedure for Monkey SCNT Using Fetal Monkey Fibroblasts

(A) Fetal monkey fibroblasts in primary culture. (B and C) Spindle-chromosome complex (arrowhead) in a monkey MII oocyte before (B) and after (C) removal. (D–F) An oocyte with a slit in the zona pellucida (D, arrowhead) induced by laser irradiation, and the HVJ-E-incubated fibroblast (arrowhead) before (E) and after (F) injection into the perivitelline space. (G) Spindle-like structure formed by the donor fibroblast nucleus after fibroblast-cytoplasm fusion. (H) Single nucleus formed after embryo activation with ionomycin and DMAP. (I) Blastocysts developed from *Kdm4d* mRNA-injected embryos, which were produced by SCNT using fetal fibroblasts (arrowheads: ICM). (J–L) Example images of blastocysts with and without normal development of ICM shown at a higher resolution. Blastocysts with a prominent ICM (arrowhead) obtained by SCNT (J), blastocysts with a prominent ICM (arrowhead) obtained by ICSI (K), and poor-quality blastocysts without clear ICM obtained by SCNT (L). All scale bars, 60 μ m.

Tachibana et al., 2010), and facilitation of donor cell insertion into the perivitelline space with laser lesion of zona pellucida (Figure 1A–1F). Clear spindle-like structure was detected after the fusion of the fetal monkey fibroblast with the enucleated oocyte (Figures 1G and S1A). About 1–2 hr after the fusion, “reconstructed” oocytes were activated with ionomycin and 6-dimethylaminopurine (I/D). All activated embryos showed a single pronucleus (Figures 1H and S1B) and were further cultured for *in vitro* development. A small percentage (4/30, or 13.8%) of I/D-activated SCNT embryos developed to the blastocyst stage. To improve the developmental potential of SCNT embryos, we treated another group of SCNT embryos (I/D/T) with 10 nM of the histone deacetylase inhibitor trichostatin A (TSA) (Kishigami et al., 2006) for 10 hr during and after I/D activation. We found a similarly low blastocyst rate (5/31, or 16.7%), but the quality of blastocysts was improved. For the I/D-activated group, we found no or little inner cell mass (ICM), but prominent ICM was observed in 2/5 of the blastocysts in the I/D/T group (Figures 2A, 2B, S1C, and S1D).

Based on the recent progress in studying epigenetic modification of transferred nuclear DNA in mammalian cloning by SCNT (Matoba et al., 2014), we injected exogenous human *Kdm4d* mRNA to the SCNT embryos after the activation under I/D/T condition (I/D/T/K). We found that a much higher percentage (17/38, or 44.7%) of *Kdm4d* mRNA-injected SCNT embryos derived from fetal monkey fibroblasts were able to develop into

blastocysts, among which a large fraction (11/17, or 64.7%) showed prominent ICM similar to the ICM in embryos obtained by intracytoplasmic sperm injection (Figures 1I–1L, 2A, and 2B). We also tested the effect of *Kdm4d* mRNA injection on SCNT embryos derived from cumulus

cells of adult female monkeys (from which the oocytes were obtained) and found that all SCNT embryos showed a single pronucleus after activation under I/D/T condition (Figures S2A–S2D). The majority of them (24/33, or 72.7%) developed into blastocysts, most of which (15/24, or 62.5%) showed prominent ICM. By contrast, in the absence of *Kdm4d* mRNA injection, only 5% (1/20) of SCNT embryos derived from cumulus cells developed into blastocysts, none of which showed prominent ICM (Figures 2C, 2D, S2E, and S2F).

To further understand the mechanism by which *Kdm4d* mRNA improved the development of monkey SCNT embryos, RNA sequencing (RNA-seq) was performed on cumulus cells, 4- and 8-cell embryos obtained by ICSI, and 8-cell SCNT embryos (derived from cumulus cells) with and without *Kdm4d* mRNA injection. Analysis of RNA-seq datasets of monkey ICSI embryos at 4- and 8-cell stage ($n = 4$ embryos each) led to identification of 3,997 regions (20–160 kb) that were expressed at least 5-fold higher ($FC > 5$) in 8-cell embryos as compared to 4-cell embryos (Data S1), indicating massive up-regulation of genes during early embryonic development. Comparison of these regions between 8-cell ICSI embryos and 8-cell SCNT embryos without *Kdm4d* mRNA injection showed 2,465 RRRs ($FC > 5$) in SCNT embryos (Data S1). By contrast, 2,178/2,465 RRRs were markedly upregulated ($FC > 2$) in *Kdm4d* mRNA-injected SCNT embryos (Figure 2E, Data S1). The effect of *Kdm4d* mRNA expression in removing the histone

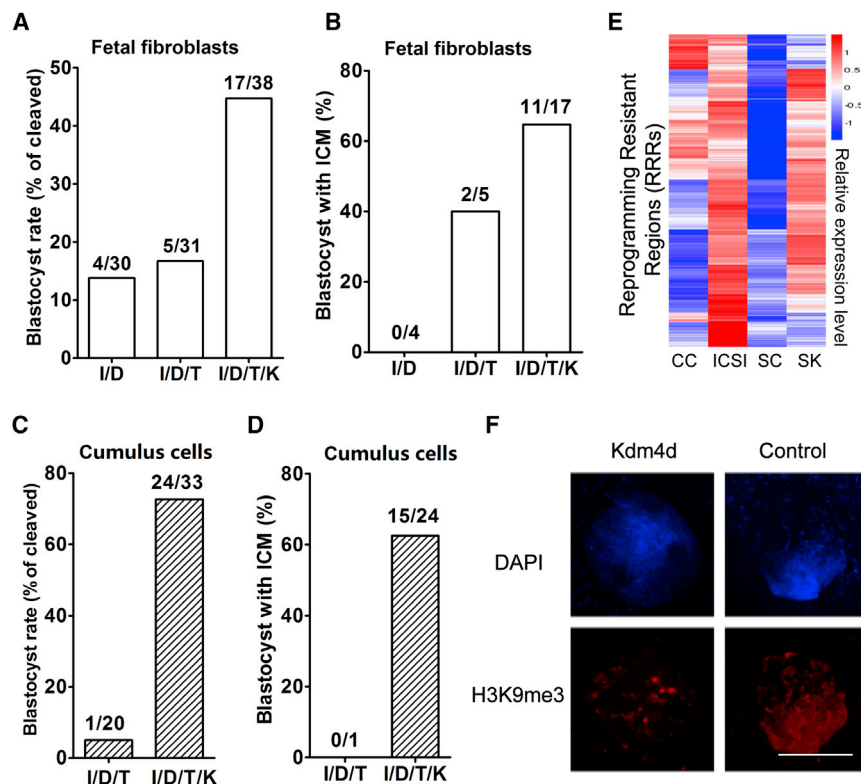


Figure 2. Blastocyst Development of SCNT Monkey Embryos

(A and C) Percentages of cleaved embryos that developed into blastocysts (blastocyst rate) of SCNT embryos using fetal fibroblasts (A) and adult cumulus cells (C) under different conditions. I: ionomycin, D: 6-dimethylaminopurine, T: TSA, K: *Kdm4d* mRNA.

(B and D) Percentages of blastocysts showing prominent ICM in SCNT embryos using fetal fibroblasts (B) and adult cumulus cells (D).

(E) Upregulation of RRRs resulting from *Kdm4d* modification in SCNT embryos. CC: Expression levels of RRRs in native donor cumulus cells. ICSI: Expression levels of RRRs in eight cell-stage monkey embryos obtained by intracytoplasmic sperm injection (ICSI), indicating normal developmental upregulation of RRRs. SC: expression levels of RRRs in cumulus cells derived eight cell-stage control SCNT embryos in the absence of *Kdm4d* mRNA injection, showing low-level expression of RRRs. SK: expression levels of RRRs in cumulus cells derived eight cell-stage SCNT embryos injected with *Kdm4d* mRNA, showing elevated expression of many RRRs, some of which correspond to those of ICSI eight cell-stage embryos.

(F) Representative nuclear images of cumulus cells derived one-cell stage SCNT embryos stained with anti-H3K9me3 and DAPI at 5 hr after *Kdm4d* mRNA injection. Scale bar, 30 μ m.

methylation site H3K9me3 of SCNT embryos was further confirmed by immunostaining of H3K9me3 (Figure 2F), showing that the level of H3K9me3 was high in control one-cell SCNT embryos but greatly reduced in those injected with *Kdm4d* mRNA.

To identify candidate genes that were repressed by H3K9me3 and may be responsible for the poor development of monkey SCNT embryos, we further examined the RNA-seq data and found that some developmental pluripotency-associated genes such as *Dppa2*, *Dppa4*, and *Myc* were repressed by H3K9me3 in monkey SCNT embryos. Other repressed genes found in mouse SCNT embryos, such as *Zscan4* and *Polr3h*, were also identified in monkey SCNT embryos. A more complete list of genes identified is shown in Data S1. Further studies on directly manipulating these genes in SCNT embryos are needed to confirm their roles in facilitating embryonic development (Matoba et al., 2014). Taken together, the expression of H3K9me3 demethylase *Kdm4d* significantly improved epigenetic reprogramming in monkey SCNT embryos similar to that found in other species (Chung et al., 2015; Matoba et al., 2014). Thus, *Kdm4d* mRNA injection was used in all subsequent experiments in the present study.

SCNT Using Fetal Monkey Fibroblasts

Fetal fibroblasts in primary culture derived from an aborted female cynomolgus monkey fetus were prepared by standard methods (see STAR Methods) and used for SCNT. These cells were chosen for their potential in obtaining a large number of nuclei with uniform genetic background. Using the SCNT protocol described above, a total of 109 *Kdm4d* mRNA-injected SCNT embryos under I/D/T condition were obtained using 127 MII-stage oocytes, and 79 of them (between 2-cell to blastocyst stage) were transferred to 21 cynomolgus female surrogates (Sun et al., 2008) (Table 1 and Data S2). The choice of the embryo transfer time was based on the number of SCNT embryos prepared and that of surrogates available at the time. Pregnancy was confirmed in 6 surrogates by ultrasound examination one month later, 4 of them carried 5 fetuses (one twin), and the other 2 carried only gestational sacs (GS, Figures 3C and 3D). Among the 4 pregnancies, 2 aborted at the early gestation stage (within two months), and 2 developed beyond 140 days (Figure 3E and Data S2). Two live babies were obtained at full term (155 and 141 days) by caesarean section. The newborn baby monkeys, named Zhong Zhong (ZZ) and Hua Hua (HH) (Figures 4A and

Table 1. Statistics on the Development of SCNT Embryos

Donor cells	Oocytes	SCNT embryos	Embryos transferred	Surrogates	Pregnancies	Live birth	Survived offspring
Fetal fibroblasts	127	109	79	21	6	2	2
Cumulus cells	290	192	181	42	22	2	0

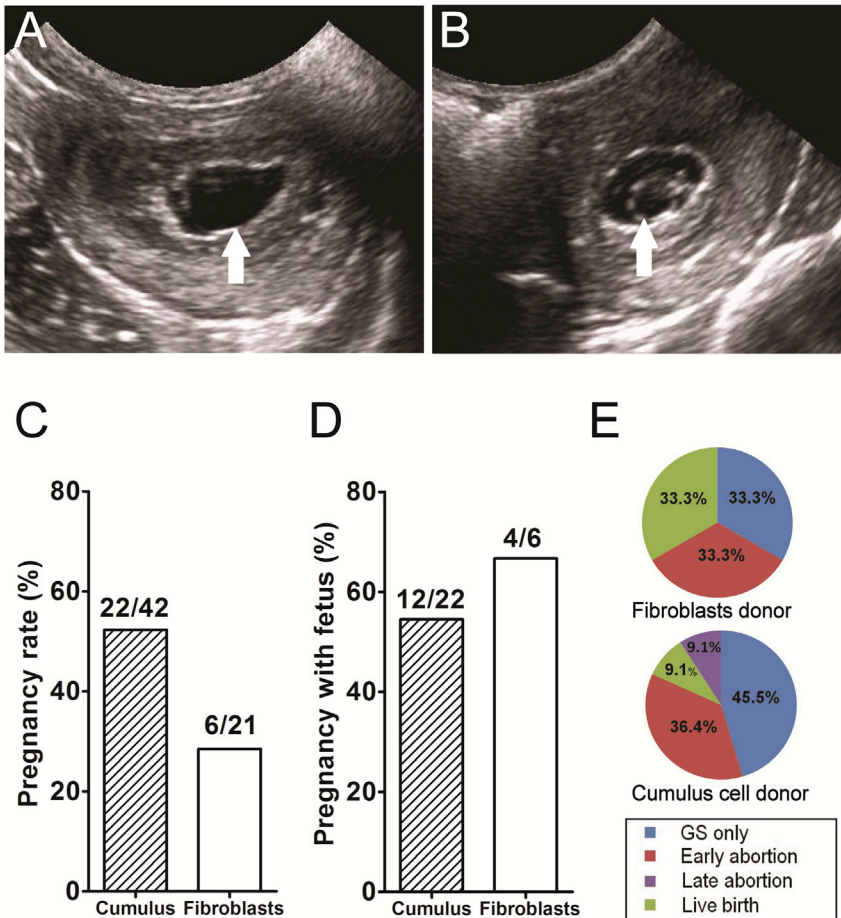


Figure 3. Pregnancy and Fetal Development of SCNT Embryos

(A and B) Ultrasound imaging of the pregnancy status from SCNT with cumulus cells, showing example images of a surrogate uterus containing a gestational sac (GS, arrow) without a fetus (A) and with a fetus (B, arrow).

(C and D) Pregnancy rates (C) and percentage of pregnancies with a fetus (D) for two groups of SCNT embryos using fetal fibroblasts and adult cumulus cells, respectively.

(E) Overall percentages of pregnancies that yielded gestational sacs (GS) without a fetus, pregnancies with fetuses that led to early abortion (within two months) without a fetus, late abortion with a fetus, and live births (by caesarian section) for SCNT using fetal fibroblasts (in healthy condition) and adult cumulus cells (lived for a short time), respectively.

4B), survived in good conditions under human feeding and care for 50 and 40 days, respectively, at the time of manuscript resubmission. In comparison with newborn monkeys obtained by natural fertilization, they showed no hypertrophy of umbilical cord at birth, normal postnatal temperature regulation, and exhibited sucking reflex during feeding, as well as normal growth rate in body weight and head circumference (Movie S1).

SCNT Using Adult Monkey Cumulus Cells

We also used adult cumulus cells of female monkeys (from which the oocytes were obtained) as donor cells for SCNT. A total of 192 SCNT embryos obtained from 290 MII stage oocytes under I/D/T activation condition, were injected with *Kdm4d* mRNA at the pronuclear stage, and 181 of them (at 2-cell to blastocyst stage) were transferred to 42 monkey female surrogates (Table 1 and Data S2). Pregnancy was confirmed in 22 surrogates, 12 of them carried 17 live fetuses, and the rest yielded only GS (Figures 3A–3D). Among the 12 pregnant surrogates with fetuses, 8 aborted at early gestation stage (within 2 months) and 2 aborted on day 84 and 94. The last two pregnancies developed beyond 130 days and yielded two live births (infants A and B) via caesarean section on day 135 and 137, respectively (Figure 3E, Data S2). Infant A

showed normal head circumference but impaired body development at birth and died 3 hr later due to apparent respiratory failure. Infant B had apparent normal head and body development and showed normal breathing and food and water intake but died 30 hr later with respiratory failure (see Data S4).

Genetic Analyses of Monkeys Generated by SCNT

Single-nucleotide polymorphism (SNP) and short-tandem repeat (STR) analyses

(Penedo et al., 2005; Tachibana et al., 2009) were performed to examine the mitochondria and nuclear DNAs of monkey babies obtained by SCNT. The STR analysis of 27 loci demonstrated that the nuclear DNAs of ZZ and HH ear tissues completely matched that of the fetal fibroblast donor, and three example loci are shown in Figure 4C (complete information on 27 loci are shown in Data S3). The SNPs of the *ND3* gene of mitochondria DNA for the ear tissue obtained from ZZ and HH were identical to that of their respective oocyte donor monkeys (examples shown in Figures 4D and 4E; more examples are shown in Figure S3) due to the overwhelming abundance of oocyte mitochondria in comparison to that of somatic cell used for SCNT. Similar SNP and STR analyses performed on the ear tissues of deceased infants A and B obtained by SCNT using monkey cumulus cells also confirmed the origin of the mitochondria and nuclear DNAs from their respective oocyte donor monkey and cumulus cells obtained from the latter (Figure S4 and Data S3). These genetic analyses fully confirmed the clonal origin of the monkeys generated by our SCNT method.

DISCUSSION

In this study, we have generated two healthy cynomolgus monkeys by the SCNT method using fetal monkey fibroblasts. We

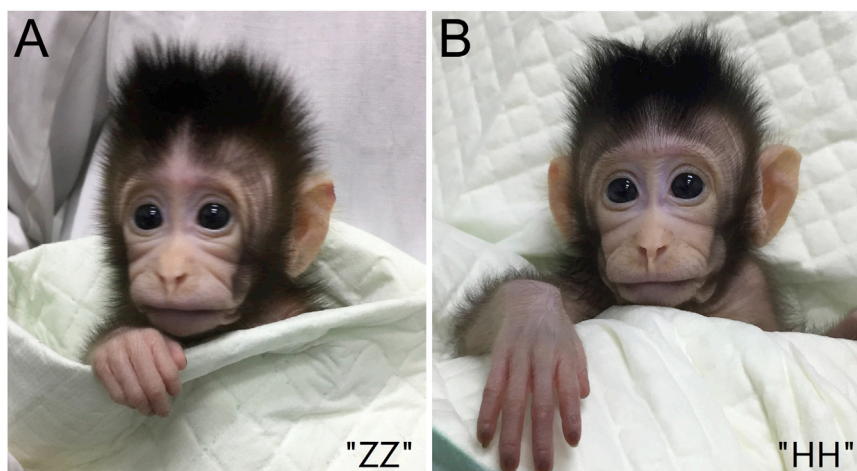


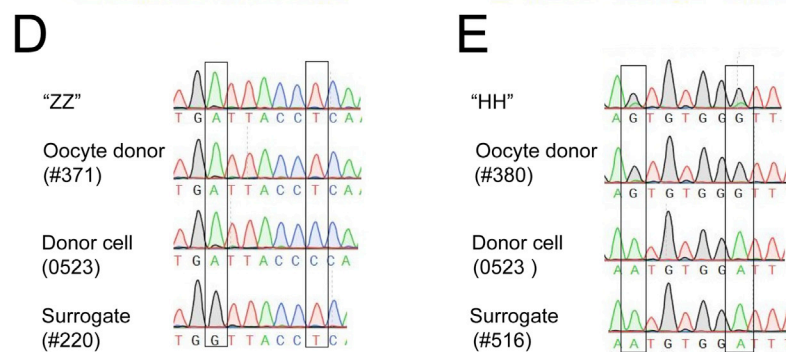
Figure 4. Analysis of Cloned Cynomolgus Monkeys

(A and B) Images of Zhong Zhong (ZZ) and Hua Hua (HH), two cynomolgus monkeys generated by SCNT using fetal monkey fibroblasts, at 20 and 34 days after birth, respectively.

(C) Three examples of short tandem repeats (STRs) from ear tissues of “ZZ” and “HH,” showing that their nuclear DNA was identical to that of the donor fibroblast but different from those of their oocyte donor and surrogate monkeys. More extensive list of STRs are shown in [Data S3](#). (D and E) Examples of single-nucleotide polymorphisms (SNPs) for “ZZ” and “HH,” respectively, showing mitochondria DNA SNPs that were identical to those of the oocyte donor monkey but different from those of surrogate monkey and donor fibroblast. More examples are shown in [Figure S3](#).

C

Monkey/Donor cell	D6S2741	D16S409	D18S72
Cloned monkey “ZZ”	250/277	135/148	200/217
Cloned monkey “HH”	250/277	135/148	200/217
Fibroblast donor cell (0523)	250/277	135/148	200/217
Oocyte donor for “ZZ”(#371)	274/278	255/255	202/223
Surrogate for “ZZ”(#220)	268/268	254/256	202/208
Oocyte donor for “HH”(#380)	271/271	133/148	198/204
Surrogate for “HH”(#516)	262/262	131/131	207/210



attributed the success of cloning of a non-human primate by SCNT to the optimization of the nuclear transfer protocol, the use of fetal cell nuclei, and epigenetic modifications by *Kdm4d* and TSA, and all of them together greatly improved the quality of blastocyst development and pregnancy rate. Genetic analysis fully confirmed the clonal origin of the monkeys generated by SCNT. This study demonstrated that cloning of non-human primates is feasible by SCNT using fetal somatic cells, which could be efficiently modified by genetic editing and screening *in vitro* (Gao et al., 2017; Lai et al., 2016; Rogers, 2016). Such cloning allows the production of genetically uniform monkeys as animal models for basic research in primate biology and for studying human disease mechanisms and therapeutic treatments.

Previous studies on monkey SCNT using adult fibroblasts (Sparman et al., 2010) and cumulus cells (Ng et al., 2004) have yielded pregnancies up to 80 days. The main obstacle to the live birth appeared to be poor reprogramming of transferred nuclei for supporting embryonic development. In this study, we

demonstrated the effectiveness of H3K9me3 demethylase *Kdm4d* and histone deacetylase inhibitor TSA in promoting normal blastocyst formation, pregnancy rate, and fetal development. Immunostaining of *Kdm4d* mRNA-injected one-cell embryos showed that H3K9me3 sites were greatly reduced (Figure 2F), and transcriptome analysis further confirmed that *Kdm4d* treatment indeed promoted the upregulation of RRRs of the embryo's genome at the early blastomere stage (Figure 2E). In addition to the use of H3K9me3 demethylase and histone deacetylase inhibitor, several other approaches such as impeding *Xist* gene expression of the active X chromosome (Inoue et al., 2010), embryo aggregation (Boiani et al., 2003), trophectoderm replacement (Lin et al., 2011), and H3K4me3 demethylase injection (Liu et al., 2016a) were also found to improve the development of mouse SCNT embryos. Previous studies showed that caffeine, electric stimulation (Tachibana et al., 2013), and translation inhibitor puromycin (Yamada et al., 2014) could facilitate the production of human embryonic stem cells via SCNT methods. All these additional approaches could be used for further improving the efficiency of monkey SCNT. Finally, we noted that the lower efficiency in SCNT using adult cumulus cells as compared to fetal fibroblasts could be attributed to the fact that reprogramming of adult nuclei is less efficient than fetal nuclei or, alternatively, to the difference in the somatic cell type. More extensive studies of reprogramming adult somatic nuclei are required for the future use of SCNT on adult somatic cells.

Our choice of using fetal fibroblasts as donor cells for SCNT is based on the consideration of not only reprogramming potential of fetal cells, but also their potential application for genetic

editing. Although recent advances in gene editing methods, e.g., CRISPR/Cas9 technology, could achieve efficient gene knockout or knockin monkey embryos (Niu et al., 2014; Cui et al., 2018; Yao et al., 2018), mosaicism in gene editing and off-target effects remain problematic for direct gene editing of monkey embryos. *In vitro* screening of gene-edited fibroblasts prior to SCNT could circumvent some of these problems and allow the generation of a large number of cloned monkeys with uniform genetic background. This is particularly relevant for developing monkey models for human diseases with defined genetic defects that could be used for studying disease mechanisms and testing potential therapeutic treatments. Basic studies on the genetic basis of primate-specific traits will find genetically uniform clones of non-human primates a useful laboratory animal model.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- [EXPERIMENTAL MODEL AND SUBJECT DETAILS](#)
 - Animal Ethics Statements
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 - Preparation of Nuclear Donor Cells
 - Superovulation and Oocyte Collection
 - Monkey SCNT and ICSI Procedures
 - Culture of Monkey Embryos and Embryos Transfer
 - Genetic Analysis of Cloned Monkeys
 - Kdm4d In Vitro Transcription
 - Immunostaining
 - RNA-Sequencing and Data Analysis

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, four data files, and one movie and can be found with this article online at <https://doi.org/10.1016/j.cell.2018.01.020>.

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AUTHOR CONTRIBUTION

Q.S. and Z.L. designed the experiments. Q.S. and M.P. supervised the project. Z.L. performed the somatic cell nuclear transfer procedure. Y.C. performed *Kdm4d* mRNA *in vitro* transcription and fibroblasts culturing. Z.L., Y.W., Y.N., C.Z., and Y.X. performed oocyte collection, embryo transfer, pregnancy examination, and infant care. Z.L. and Z.W. performed immunofluorescence study and STR/SNP analyses. Z.L., Q.S., and M.P. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-H3K9me3	Abcam	ab8898; RRID: AB_306848
Cy3 AffiniPure-conjugated donkey anti-Rabbit IgG	Jackson	711-165-152; RRID: AB_2307443
Chemicals, Peptides, and Recombinant Proteins		
Ionomycin	Beyotime	S1672
6-dimethylaminopurine	Sigma	D2629
Trichostatin A	Sigma	T1952
Cytochalasin B	Sigma	C6762
HVJ-E	COSMO BIO CO. LTD	808920
Chorionic Gonadotrophin Human	Sigma	CG10-10VL
Recombinant Human Follitropin	Merck Serono	S20130055 (75IU)
Hamster embryo culture medium 9 (HECM-9)	Liu et al., 2016b	N/A
HEPES-buffered Tyrode's lactate medium (TH3)	Liu et al., 2016b	N/A
Experimental Models: Organisms/Strains		
Cynomolgus monkeys (<i>Macaca fascicularis</i>)	Suzhou Xishan	N/A
Oligonucleotides		
Primer:ND3 Forward:CCACTTCACATCAAACCATCACTT	This paper	N/A
Primer:ND3 Reverse:CAAGCAGCGAATACCAGCAAAA	This paper	N/A
Primer: <i>Kdm4d</i> <i>in vitro</i> transcription Forward:TTAATACGACTCACTATAGGGATGGAAACT ATGAAGTCTAAGGCAACT	This paper	N/A
Primer: <i>Kdm4d</i> <i>in vitro</i> transcription Reverse:ATATAAA GACAGCCCGTGGACTTAGG	This paper	N/A
Software and Algorithms		
ABI PRISM 3730 genetic analyzer	ABI	N/A
Other		
rTaq	Takara	R500Z
Piezo impact drive system	Prime Tech	N/A
Oosight imaging system	CRi	N/A
Laser system	Hamilton Thorne	N/A
P97 micropipet puller	Sutter Instrument	N/A
Inverted microscope	Olympus	IM-73
Syringe IM 9B	Narishige	N/A
Syringe CellTram vario	Eppendorf	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Qiang Sun (qsun@ion.ac.cn).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal Ethics Statements

Healthy female cynomolgus monkeys (*Macaca fascicularis*), ranging in age from 5 to 12 years, were selected for this study. All animals were housed in the sunny room. The use and care of animals complied with the guideline of the Animal Advisory Committee at the

Shanghai Institute of Biological Science, Chinese Academy of Sciences. The ethics application entitled “Reproductive physiology of cynomolgus monkeys and establishment transgenic monkeys” (#ER-SIBS-221 106P) was approved by Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences.

METHOD DETAILS

Preparation of Nuclear Donor Cells

Primary fibroblasts were isolated from a 61 day-old freshly aborted cynomolgus fetus. The fetal tissue were cut into small pieces after removing the head, tail, limbs, and viscera and digested with DNase (1 mg/ml) and collagenase IV (0.5 mg/ml) in cell culture medium containing DMEM supplemented with 100 IU/ml penicillin and streptomycin, 10% FBS, non-essential amino acids, and glutamine at 37°C in 5% CO₂ for 4 h. Isolated monkey fibroblasts were cultured in 10 cm dishes for 10–20 h until they reached confluency, disaggregated by trypsin, and stored in liquid nitrogen in cell culture medium containing 10% dimethylsulfoxide. Frozen fibroblasts (P0–P5) were thawed and cultured until confluency, and used for SCNT 3–7 days later. For preparation of adult monkey cumulus cells, fresh cumulus cells were collected from the follicular aspirates of oocyte donors and washed twice with TH3 medium. Re-suspended cumulus cells were used directly for SCNT.

Superovulation and Oocyte Collection

Healthy female cynomolgus monkeys with regular menstrual cycles were chosen for superovulation and laparoscopy was used for oocyte collection. From day 3 of menstrual cycle, 25 IU recombinant human follitropin was injected intramuscularly twice daily for 7–8 days. On day 11, 1000 IU of human chorionic gonadotrophin (hCG) was also injected, oocytes were aspirated from follicles 2–8 mm in diameter 36 h later. The collected oocytes were cultured in the pre-equilibrated hamster embryo culture medium 9 (HECM-9) medium. Metaphase II-arrested oocytes were selected for manipulation.

Monkey SCNT and ICSI Procedures

For monkey SCNT, a group of 15–20 monkey MII oocytes were transferred to manipulation drops containing HEPES-buffered Tyrode's lactate medium (TH3) with 5 μg/ml cytochalasin B in a glass bottom dish. The spindle–chromosome complex was removed rapidly by a piezo-driven pipette under a spindle imaging microscopic system (Oosight) within 10 min for the entire group of oocytes. The enucleated oocytes were transferred to pre-equilibrated HECM-9 at 37°C under 5% CO₂. After all oocytes were enucleated, a group of 15–20 enucleated oocytes were re-transferred to manipulation drops with TH3 and 5 μg/ml cytochalasin B. Fibroblasts or cumulus cells were briefly incubated in a medium containing fusogenic viral envelop HVJ-E and introduced to the perivitelline space of the enucleated oocytes by a micropipette through a slit in the zona pellucida that was created by laser irradiation. At 1–2 h after cell fusion, reconstructed embryos were activated in TH3 medium with 5 μM ionomycin for 5 min and transferred to HECM-9 medium containing 2 mM 6-dimethylaminopurine for 5 h. TSA (10 nM) were applied for 10 h during and after activation. For *Kdm4d* mRNA injection, 10 pl of 1000 ng/μl *Kdm4d* mRNA were used for injection at 6 h after activation with a piezo-driven micromanipulator (Primetech). It is possible that the *Kdm4d* mRNA concentration we used was not optimal and further optimization of the concentration is needed.

For monkey intracytoplasmic sperm injection (ICSI), previously published procedure was used (Liu et al., 2016b). Briefly, a single picked sperm was injected into the ooplasm using a piezo-driven micromanipulator and fertilization was confirmed 6 h later by the presence of two pronuclei. Embryos were cultured in pre-equilibrated HECM-9 at 37°C under 5% CO₂.

Culture of Monkey Embryos and Embryos Transfer

Monkey ICSI and SCNT embryos were cultured in HECM-9 medium at 37°C under 5% CO₂. The embryos were transferred to HECM-9+5% FBS medium after reaching 8-cell stage and the medium was changed every other day until the embryo reached the blastocyst stage. For embryo transfer, females with synchronous menstrual cycle whose ovaries had a stigma or fresh corpus luteum were used as surrogates. Embryos (at 2-cell to blastocyst stage) were transferred to the oviduct.

Genetic Analysis of Cloned Monkeys

For short tandem repeats (STR) analysis, ear tissue sample collected from monkeys were used to extract DNA. Locus-specific primers each containing a fluorescent dye (FAM/HEX/TMR) were used for PCR amplification in batches. FAM, HEX or TMR-labeled STR amplicons were diluted and mixed with internal size standard ROX500 and deionized formamide, followed by capillary electrophoresis on ABI PRISM 3730 genetic analyzer to obtain the raw data. Sequencer-generated raw data were analyzed with the program Gene Marker 2.2.0, which produces wave plots, Excel documents (including information such as size and genotype), and DNA profiles.

For single nucleotide polymorphism (SNP) analysis, ear tissue sample collected from monkeys were used to extract DNA. PCR with specific primers (F: CCACTTCACATCAAACCATCACTT, R: CAAGCAGCGAATACCAGCAAAA) in mtDNA were performed. DNA was amplified with 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a 5-min extension at 72°C. The PCR products were used for sequencing and the result were used for the SNP analysis.

Kdm4d In Vitro Transcription

For *Kdm4d* *in vitro* transcription, CDS of human *Kdm4d* gene was cloned from the cDNA plasmid provided by Dr. J.H. Han of Xiamen University. The DNA template was amplified by the T7 promoter containing primer (F: TTAATACGACTCACTATAGGGATGGAAAC TATGAAGTCTAAGGCCAACT, R: ATATAAAGACAGCCCGTGGACTTAGG). T7-*Kdm4d* PCR product was purified and used as the template for *in vitro* transcription (IVT) using mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies, AM1345). The RNA products were purified using MEGA clear kit (Life Technologies, AM1908) and eluted in RNase-free water.

Immunostaining

Monkey SCNT embryos (one-cell stage) were fixed in PBS with 4% paraformaldehyde (PFA) for 20 min. After being washed three times with PBS, embryos were permeabilized with 0.5% Triton X-100 in PBS (permeabilization buffer) for 30 min. After being blocked in a permeabilization buffer with 10% donkey serum for 1h, embryos were incubated for overnight at 4°C with anti-H3K9me3 (1/200, Abcam, ab8898), which were diluted in a permeabilization buffer containing 10% donkey serum. After being washed with PBS for 3 times every 10 min, Cy3 AffiniPure-conjugated donkey anti-Rabbit IgG (1:500, Jackson: 711-165-152) for 2 hr at 37°C. After being washed with PBS and counterstain of nucleus chromosome (nuclear DNA) with DAPI (Sigma), embryos were transfer into a spinning disk for confocal microscopic analysis (Olympus Microsystems).

RNA-Sequencing and Data Analysis

Cumulus cells and cumulus cell-derived SCNT embryos (8-cell stage with and without *Kdm4d* mRNA injection, 4/group), ICSI embryos (4-cell and 8-cell stage, 4/group), and cumulus cells were collected for lysed and cDNA synthesis using SMARTer Ultra Low Input RNA cDNA preparation kit (Clontech 634936). Sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Library preparations were sequenced on an Illumina Hiseq platform and 125 bp/150 bp paired-end reads were generated. Expression level of each gene was quantified with normalized FPKM (fragments per kilobase of exon per million mapped fragments). A sliding window (size 20 kb, step size 10 kb) was used to assess the genome-wide expression level of monkey embryos. For each window, the expression level was quantified with normalized RPM (reads per millions of uniquely mapped reads).

A sliding window (size 100 kb, step size 20 kb) was used to assess the genome-wide expression level of embryos following the previous reported method ([Matoba et al., 2014](#)). For each window, the expression level was quantified with normalized RPM (reads per millions of uniquely mapped reads) and used for identification of reprogramming resistant regions.

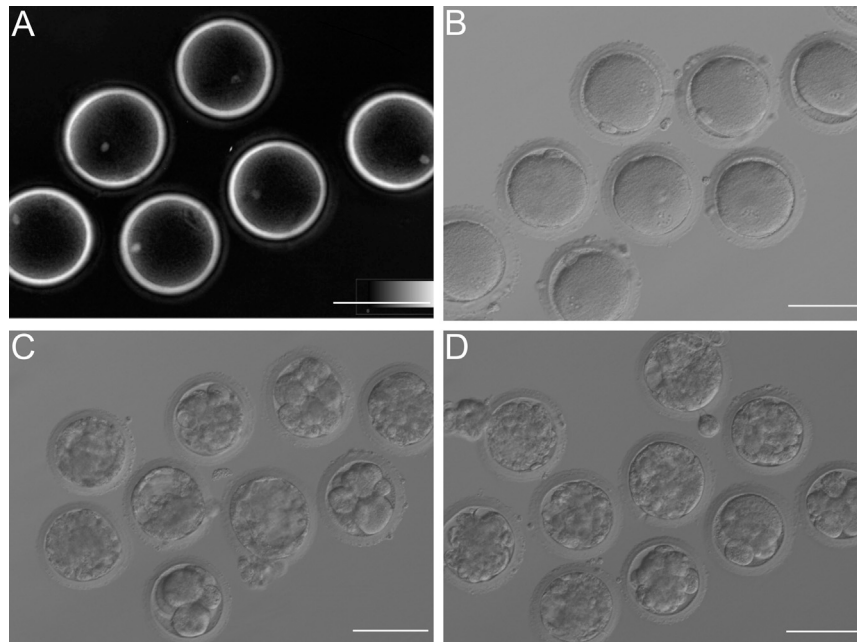


Figure S1. SCNT Monkey Embryos Using Fetal Fibroblasts as Donors, Related to Figure 1 and 2

(A) Similar to Figure 1G, but showing a group of embryos exhibiting spindle-like structures after fusion of fetal fibroblasts with enucleated oocytes. (Red rectangle on the lower right side is inherent form of the software and it does not have any relationship with the figure).

(B) Similar to Figure 1H, but showing a group of embryos exhibiting single well-formed nucleus after embryo activation.

(C and D) Blastocysts developed after fibroblast nuclear transfer under I/D condition (C) and I/D/T condition (D), showing poor quality of embryo development, with no or very few embryo with inner cell mass (ICM).

All scale bars, 120 μ m.

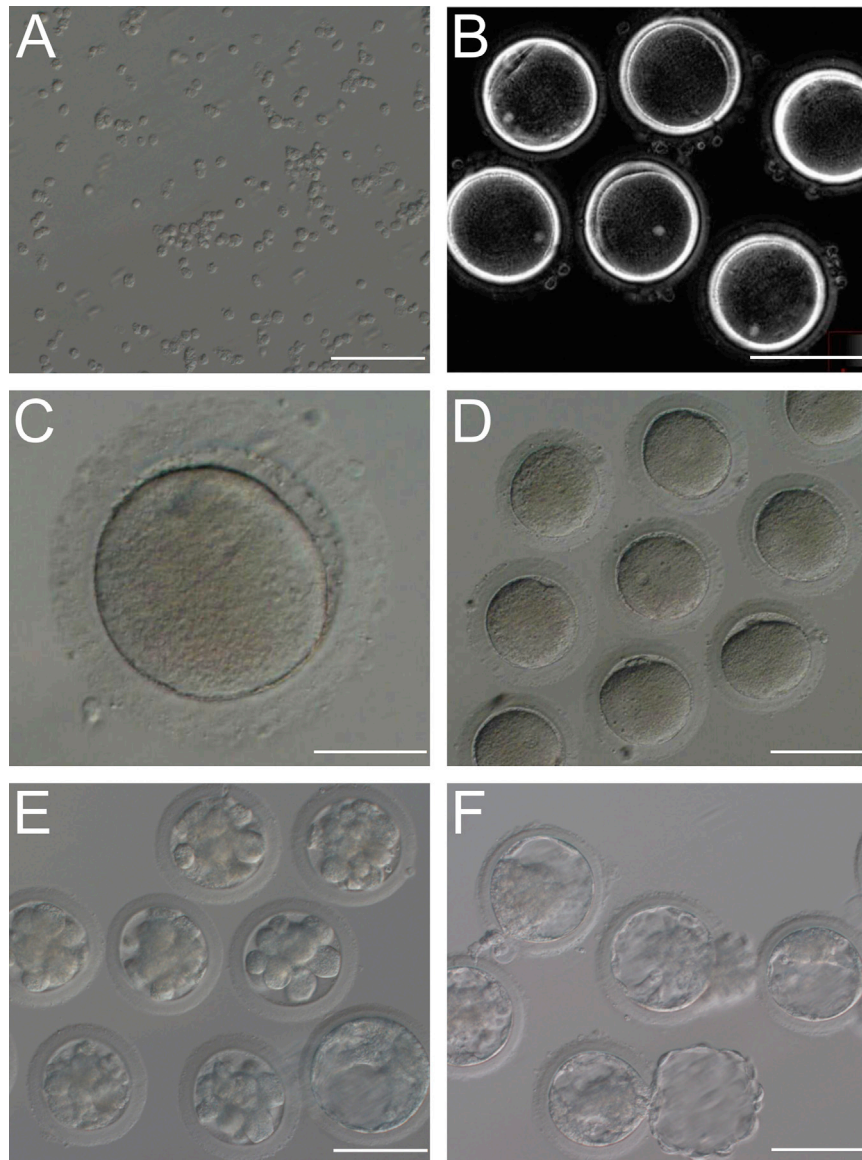


Figure S2. SCNT Monkey Embryos Using Cumulus Cells as Donors, Related to Figure 2

(A) Adult monkey cumulus cells isolated from a female monkey used for obtaining the oocytes for SCNT. Scale bar, 100 μm .

(B–F) Similar to those presented for SCNT using fetal fibroblasts, showing spindle-like structures formed by cumulus cell nuclei (B, Scale bar, 120 μm), single nucleus formed after embryo activation (C, scale bar, 50 μm ; D, scale bar, 120 μm), blastocysts developed from SCNT embryos with cumulus cells under I/D/T condition (E, scale bar, 120 μm) and I/D/T/K condition (F, scale bar, 120 μm).

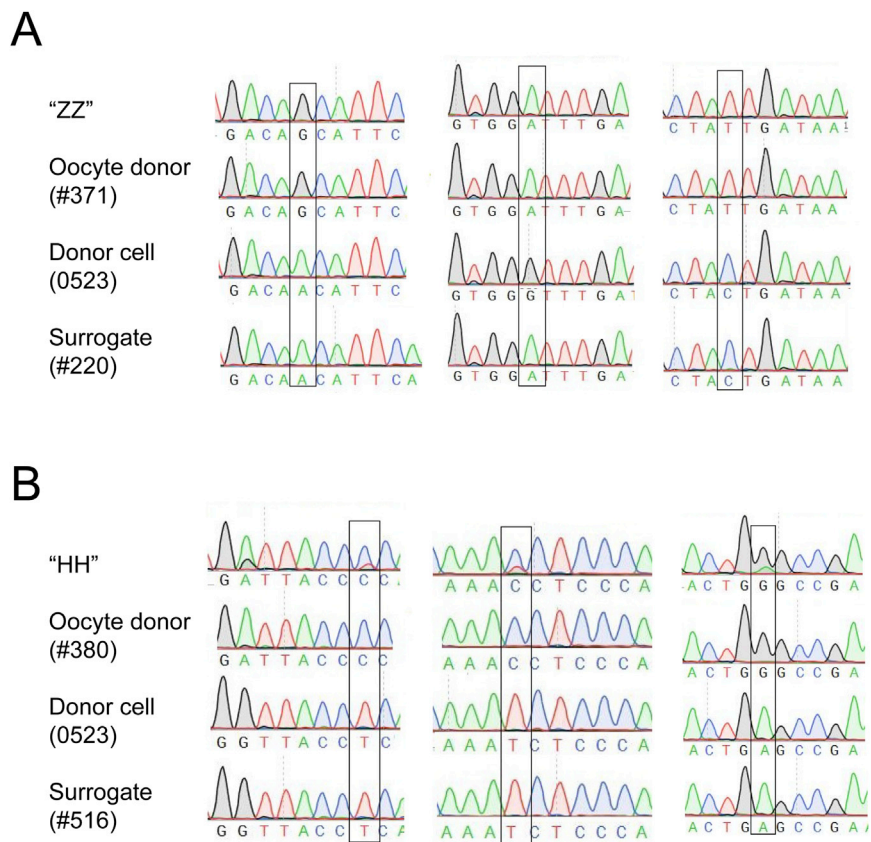


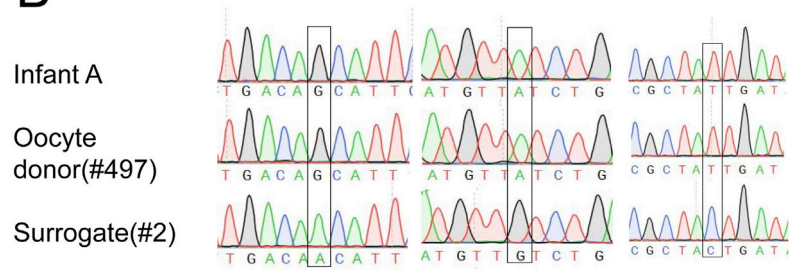
Figure S3. SNP Analysis of "ZZ" and "HH" Generated by SCNT Using Fetal Fibroblasts, Related to Figure 4

(A and B) Three examples of single nucleotide polymorphism (SNP) for "ZZ" and "HH," showing that their mitochondria DNA SNPs were identical to those of the oocyte donor monkey, but different from those of the surrogate monkey and donor cell.

A

Monkey/Donor cell	D2S1333	D4S2365	D12S364
Deceased cloned monkey infant A	289/296	273/281	126/130
Oocyte and cumulus donor of A (#497)	289/296	273/281	126/130
Surrogate monkey of A (#2)	313/313	277/277	113/124
Deceased cloned monkey infant B	296/296	265/285	120/122
Oocyte and cumulus donor of B (#358)	296/296	265/285	120/122
Surrogate monkey of B (#128)	288/292	277/281	103/103

B



C

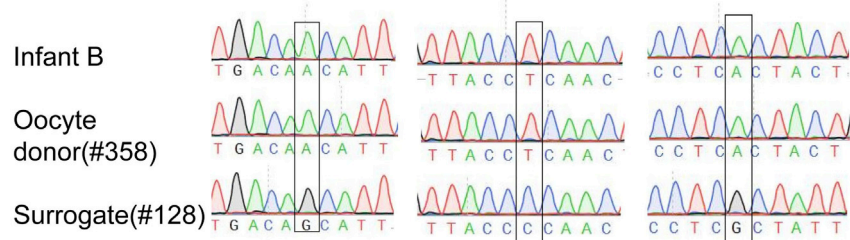


Figure S4. Genetic Analysis of Deceased Monkeys Neonates Generated by SCNT Using Adult Cumulus Cells, Related to Table 1 and Figure 3
 (A) Three examples of short tandem repeats (STRs) in tissues from deceased monkey infants "A" and "B," showing that their nuclear DNA was identical to those of the donor cumulus cell and their oocyte donor monkey, but different from that of the surrogate monkey. More extensive list of STRs are shown in [Data S3](#).
 (B and C) An example of single nucleotide polymorphism (SNP) for two deceased cloned monkey infants "A" and "B," showing that their mitochondria DNA SNPs were identical to those of the oocyte donor monkey, but different from those of the surrogate monkey.