The first cell cycle after transfer of somatic cell nuclei in a non-human primate

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Summary

Production of genetically identical non-human primates through somatic cell nuclear transfer (SCNT) can provide diseased genotypes for research and clarify embryonic stem cell potentials. Understanding the cellular and molecular changes in SCNT is crucial to its success. Thus the changes in the first cell cycle of reconstructed zygotes after nuclear transfer (NT) of somatic cells in the Long-tailed Macaque (*Macaca fascicularis*) were studied. Embryos were reconstructed by injecting cumulus and fibroblasts from *M. fascicularis* and *M. silenus*, into enucleated *M. fascicularis* oocytes. A spindle of unduplicated premature condensed chromosome (PCC spindle) from the donor somatic cell was formed at 2 hours after NT. Following activation, the chromosomes segregated and moved towards the two PCC spindle poles, then formed two nuclei. Twenty-four hours after activation, the first cell division occurred. A schematic of the first cell cycle changes following injection of a somatic cell into an enucleated oocyte is proposed. Ninety-three reconstructed embryos were transferred into 31 recipients, resulting in 7 pregnancies that were confirmed by ultrasound; unfortunately none progressed beyond 60 days.

Key words: Somatic cell nuclear transfer, Cell cycle, Spindle formation, Non-human primate, Embryo transfer

Introduction

Somatic cell nuclear transfer (SCNT) is a powerful technique for multiplication of unique animal genotypes and preservation of endangered animals (Wells et al., 1998). Its application is being further expanded to the areas of transgenics (Schnieke et al., 1997), knock-in (McCreath et al., 2000) or knock-out livestock (Lai et al., 2002). Basic understanding of cell dynamics in cancer can also be aided by SCNT research (Li et al., 2003). In non-human primates, production of genetically identical non-human primates would reduce the number of animals required for biomedical research, and would dramatically impact on studies pertaining to immune system function and early development of specific genetic diseases. Further application of SCNT research will also help clarify embryonic stem cell potentials (Thomson et al., 1998).

Although successful production of animal clones from somatic cells has been achieved in many species, including sheep (Wilmut et al., 1997), cattle (Cibelli et al., 1998), mice (Wakayama et al., 1998), goat (Baguisi et al., 1999), pig (Onishi et al., 2000), cat (Shin et al., 2002b), rabbit (Chesne et al., 2002), mule (Woods et al., 2003) horse (Galli et al., 2003) and rat (Zhou et al., 2003), there has been no success in non-human primates. In addition, although blastocysts have been developed from rhesus embryos after SCNT (Mitalipov et al., 2002), only nuclear transfer of embryonic cells in non-human primates has resulted in live-births (Meng et al., 1997).

The success of SCNT depends on several parameters that impact on the ability of the cytoplast to reprogram the nucleus of the donor cell, or to reverse the epigenetic changes that occur during development (Wilmut et al., 2002). Quiescent G0 donor cells were used during initial SCNT experiments (Wilmut et al., 1997). However, SCNT has also been achieved with donor cells in G1 (Cibelli et al., 1998), G2 (Wakayama et al., 1999) and G2/M (Ono et al., 2001). In mice (Wakayama et al., 1998; Gao et al., 2002), bovine (Shin et al., 2002a) and rabbit (Chesne et al., 2002), it had been reported that there was spindle formation after somatic donor cell introduction into the enucleated oocyte, and that misaligned metaphase plates were also observed. By contrast, it was reported that primates were different from other animals, as disarrayed abnormal mitotic spindles with misaligned chromosomes were formed in all SCNT embryos, and no pregnancies resulted from SCNT embryos transferred into surrogates (Simerly et al., 2003). It was suggested that meiotic spindle removal may be the source of primate SCNT anomalies, and primate NT appears to be challenged by stricter molecular requirements [Nuclear Mitotic Apparatus (NuMA) and Kinesin-related protein HSET (human spleen, embryo, testes)] for mitotic spindle assembly than does NT in other mammals (Simerly et al., 2003). Although cell cycle co-ordination in cloned embryos by NT was reviewed previously (Campbell et al., 1996), there has been no data about spindle and nuclear formation published in non-human primates, and there are also no reports on the events in the first cell cycle of SCNT embryos.

In this study, we describe the first cell cycle changes of SCNT embryos in non-human primates for the first time. Our data demonstrate that SCNT embryos of the non-human primate are similar to other animals in that they can form a normal PCC spindle. We also report early pregnancy failures after embryo transfers of such reconstructed embryos.

Materials and methods

Animals

Mature female Long-tailed Macaques (*M. fascicularis*) weighing between 2.0 and 2.5 kg were used. Monkeys were kept housed in individual cages during experiments and were fed as previously described (Ng et al., 2002), at other times they were housed in large enclosures in their troops (all females). All procedures were approved by the Animal Ethics and Animal Welfare Committees, Animal Holding Unit, NUS. This species offers several advantages over the Rhesus monkey (*M. mulatta*), including its easier availability and non-seasonal breeding cycles, with many ovarian stimulations occuring within a year.

Establishment and culture of donor cell

Tissue sources

Skin biopsy specimens were derived from a 180-day-old male *M. fascicularis* fetus and an adult male Lion-tailed Macaque (*M. silenus*). Fresh cumulus cells were obtained from the follicles of the macaques that had oocyte recoveries (ORs).

Establishment and culture of fibroblast cells

Skin biopsy specimens were washed in Ca^{2+}- and Mg^{2+}-free Dulbecco PBS (Invitrogen) and minced into pieces. Tissue pieces were cultured in DMEM (Invitrogen) supplemented with penicillin, streptomycin and 10% (v/v) FBS (Invitrogen), and cultured at 37°C in 5% CO_2. Tissue pieces were removed using a 30G needle when cells with a fibroblast-like morphology started to migrate out of the tissues. After reaching 100% confluency, cell monolayers were disaggregated using PBS containing 0.15% (w/v) trypsin and 1.8 mM EDTA, then were passaged twice more before being frozen in DMEM with 20% FBS and 10% (w/v) DMSO (Sigma), and stored in liquid nitrogen.

Fibroblast treatments and flow cytometric analysis of the cell cycle

Cell culture flasks (75 cm^3) were plated with frozen/thawed fibroblasts at 1-3×10^6 cells/flask. At 70-80% confluency, cells were fixed in ethanol. Other cells were grown to 100% confluency and then treated as follows before being fixed: (1) serum starved by culturing in DMEM + 0.5% FBS for another 2 or 5 days; or (2) cultured in regular growth medium that was changed every 2-3 days for another 2 or 5 days to promote contact inhibition. For fixation, cells from each treatment were disaggregated, pelleted by centrifugation, resuspended and then slowly mixed with 4.5 ml of cold 70% (v/v) ethanol. After 12 hours of ethanol fixation at 4°C, cells were stained in PBS containing 0.1% (v/v) Triton X-100, 0.2 mg/ml of RNase A and 20 mg/ml of propidium iodide (Sigma), for 15 minutes at 37°C. Cells were analyzed using an Epics-Elite flow-analyzer (Coulter, USA). Cell cycle phases were calculated using Winmdi version 2.8, based on the PMT4 histogram.

Ovarian stimulation and macaque oocytes recovery

Procedures for superovulation of the Long-tailed Macaque (LoTM, *M. fascicularis*) and collection of their oocytes have been described previously (Ng et al., 2002). The female monkeys were hyperstimulated with a fixed regimen comprised initially of downregulation with a GnRH agonist, triptorelin (Decapeptyl, Ferring, Kiel, Germany). A dose of 1.8 mg active triptorelin per 2 kg body weight was administered intramuscularly. Two weeks later, human recombinant follicle stimulating hormone (rFSH; Gonal-F, 75 IU, Serono, Geneva) was administered at 37.5 IU per 2 kg body weight daily for 12 days. On the last day of FSH treatment, 1000 IU human chorionic gonadotropin (hCG; Profasi, Serono, Geneva) was administered intramuscularly to each monkey. Oocyte recovery was then performed 34-36 hours after hCG treatment, During the OR procedure monkeys were sedated with Zoletil 100 (Virbac, Peakhurst, Australia) at 8 mg per kg body weight. Laparoscopic recovery of the oocytes was performed with a 4 mm Storz laparoscope attached to a video system. The oocytes were aspirated with a double-lumen needle (FAS Set C2, Gynetics Medical Products NV, Hamont-Achel, Belgium) attached to the Cook aspiration and flushing systems (V-MAR 5100 and V-MAR 4000, respectively; Cook Australia, 12 Electronics Street, Brisbane Technology Park, Eight Mile Plains, Queensland 4113, Australia). The cumulus-oocyte complexes (COCs) were collected in HEPES-buffered Ferticult Flushing Medium (FFM), washed, and then cultured in Ferticult IVF medium (FertiPro NV, 8730 Beemem, Belgium) in 5% CO_2 in air. Oocytes stripped of cumulus cells by exposure to hyaluronidase (80 IU/ml; Type IV-S bovine testes, Sigma-Aldrich, St Louis, MO, USA) were placed in medium IVF-20 (Vitrolife), at 37°C in 5% CO_2 until further use.

SCNT procedures

Enucleation

Recipient MII oocytes were loaded individually into 5 μl droplets of HEPES-buffered IVF medium (Ferticult, Belgium) containing 10 μg/ml cytochalasin B (Sigma). A small opening on the zona pellucida was made by acidic Tyrode solution (pH 1.8) and then the second meiotic spindle was aspirated with a small volume cytoplasm (<1% of oocyte) under polarized microscopy (SpindleView, CRI, MA). After enucleation, the karyoplast was stained with 1 μg/ml Hoechst 33342. Maternal chromosome removal was confirmed twice by DNA epifluorescence imaging.

Nuclear transfer

LoTM fresh cumulus and starved fetal skin fibroblast cells, as well as starved LiTM adult skin fibroblast cells, were used as donor cells for nuclear transfer. I.D. spiked pipettes, 8 μm and 5 μm, were used for fibroblast and cumulus cells, respectively. Single donor cells, collected from the 10% PVP droplet, were ruptured by gentle aspiration out of the injection needle, and then directly microinjected into the enucleated oocyte.

Activation

Cells were induced 2 hours after microinjection by electric pulses, and then after a further 2 hours combined with 5 μM ionomycin (Sigma) or 7% ethanol for 5 minutes. Two consecutive direct current pulses (1.5 kV/cm, 50 μseconds) were delivered by a BTX Cell Manipulator 2001 (Genentronics, San Diego, CA).

SCNT embryo culture

All SCNT embryos after manipulation were cultured in IVF-20 (Vitrolife) at 37°C with 5% CO_2, 5% O_2 and 90% N_2. After activation, SCNT embryos were cultured in IVF-20 containing 5 μM cytochalasin B and 10 μg/ml Cycloheximide for 5 hours, then in IVF-20. 14-16 hours after activation, nucleus formation was checked before transfer to G1.2 (Vitrolife). 24 hours later, all SCNT embryos were transferred to G2.2 (Vitrolife). After culture for another 28-30 hours, 4- to 8-cell SCNT embryos were selected for replacement.

Embryo transfer and pregnancy monitoring

The procedure for embryo transfer of the reconstructed embryos has been described previously (Ng et al., 2002). Three days after SCNT, selected cleaved embryos were replaced into the fallopian tube of the monkey from whom oocytes were recovered earlier. Laparoscopic tubal embryo transfer (TET) was performed using a homemade

Table 1. Efficiency of SCNT in Macaques*

Donor cell		Cell injection				% Reconstruced nuclear formation					% First cell division			
Macaque	Cell type	Number of nucleated oocytes	Number of injected oocytes[A]	% Success	Number of injected oocytes*	With RN	0 RN	1 RN	2 RN	⩾3RN	Normal	Abnormal	One cell	Fragmented
LoTM	Cumulus	224	219	97.8	171	32.7	67.3	11.1[a]	14.0[a]	7.6	24.0	14.6[a]	42.7[a]	18.7
	Fetal skin fibroblast	54	52	96.3	44	38.6	61.4	15.9[ab]	15.9[a]	6.8	22.7	22.7[ab]	40.9[ab]	13.6
LiTM	Adult skin fibroblast	259	239	92.3	171	39.8	60.2	22.8[b]	5.3[b]	11.7	24.6	34.5[b]	29.8[b]	11.1
Total		537	510	95.0	386	36.5	63.5	16.8	10.4	9.3	24.1	24.4	36.8	14.7

*The numbers of injected oocytes shown here are less than those given in the previous column because some were used for other experiments; for example, in PCC spindle formation checks.

[a,b]Letters indicate that figures within the column show a significant difference, i.e. a is significantly different from b ($P<0.05$), but ab is not significantly different from a or b ($P<0.05$).

RN, reconstructed nucleus.

Table 2. Spindle formation in SCNT embryos at 2 hours after cell injection*

PCC spindle formation	DNA condensed to PCC	Microtubule assembly	Number of oocytes*	% oocytes	Figure
Normal PCC spindle	Condensation	Normal assembly with two poles	8	14.8	2A
Abnormal PCC Spindle	Condensation	Normal assembly but improper chromosome capture	5	9.3	2B
	Condensation	Abnormal assembly	17	31.5	2C
	Condensation	No assembly	8	14.8	2D
	Almost no change	Abnormal assembly	7	13.0	2E
No PCC spindle	No change	No assembly	9	16.7	2F

*Total number of oocytes, n=54.

embryo transfer catheter, a flexible polythene tube (o.d., 1.09 mm; i.d., 0.38 mm), threaded through a 25 g hypodermic needle. Luteal phase support was provided by 10 mg progesterone administered intramuscularly for 14 days starting on the day of OR. Pregnancies were ascertained by fetal ultrasound, with the presence of a viable gestational sac and heart beat.

Imaging

Periodically, reconstructed oocytes were stained immunocytochemically to observe cytoskeletal organization and DNA configuration (Shin et al., 2002a). Controls included non-immune and secondary antibodies alone. β-Tubulin antibody was used as a primary antibody to detect microtubules. Laser-scanning confocal microscopy was performed using a Zeiss LSM500, equipped with Argon and Helium-Neon lasers for the simultaneous excitation of FITC-conjugated secondary antibodies (Sigma) and propidium iodide DNA stain.

Statistical analysis

Results were analyzed using the Pearson's χ^2 test.

Results

Nuclear formation and first cell division of SCNT with three types of donor cells

A total of 1268 oocytes were retrieved from 32 Cynomolgus monkeys, or Long-tailed Macaques (LoTMs, *Macaca fascicularis*), in 77 cycles by laparoscopy. 61.3% (777/1268) of these oocytes were matured (MII) oocytes. 95.9% of the MII oocytes (538/561; the remaining 216 MII oocytes were used for other experiments) were successfully enucleated for SCNT under polarized microscopy (SpindleView, CRI, Woburn MA). 95.0% of enucleated oocytes were successfully microinjected with three types of somatic cells, i.e. LoTM cumulus, LoTM fetal skin fibroblasts and Lion-tailed macaque (LiTM, *Macaca silenus*) adult skin fibroblasts. After 2, 5 and 8 days of serum starvation, 66%, 77% and 74% of the fibroblasts were in G1/G0, respectively. Table 1 shows the results of nuclear formation and first cleavage after activation using three types of donor cells. Nuclear formation and normal division of SCNT embryos was not markedly affected by donor cell types, as it was similar among the three different types of donor cell: cumulus, fetal fibroblast and cross-species adult fibroblast. Interestingly, the two-nuclei formation rate was significantly higher in isospecies NT (cumulus and fetal skin fibroblast) than in heterospecies NT (adult skin fibroblast), and abnormal divisions were significantly lower in isospecies NT than in heterospecies NT.

PCC spindle formation in NT embryos

Microtubule assembly and DNA changes in reconstructed oocytes were examined by fixing the oocytes at different time-points after the somatic cells were introduced into enucleated oocytes. Minimal change in somatic DNA and no microtubule assembly was observed within the first 30 minutes after cell injection. Within 1-2 hours, the chromosomes condensed prematurely (PCC), microtubules were assembled and spindle-like structures (PCC spindles) were formed (Fig. 1A). The prematurely condensed chromosomes then segregated and moved towards the two PCC spindle poles (Fig. 1G), thus forming two nuclei. Fifty-four reconstructed oocytes were fixed at 2 hours after cell injection; 70.4% (38/54) of somatic cell DNA underwent PCC, microtubule assembly occurred in 68.5% (37/54) of the reconstructed embryos, and 14.8% (8/54)

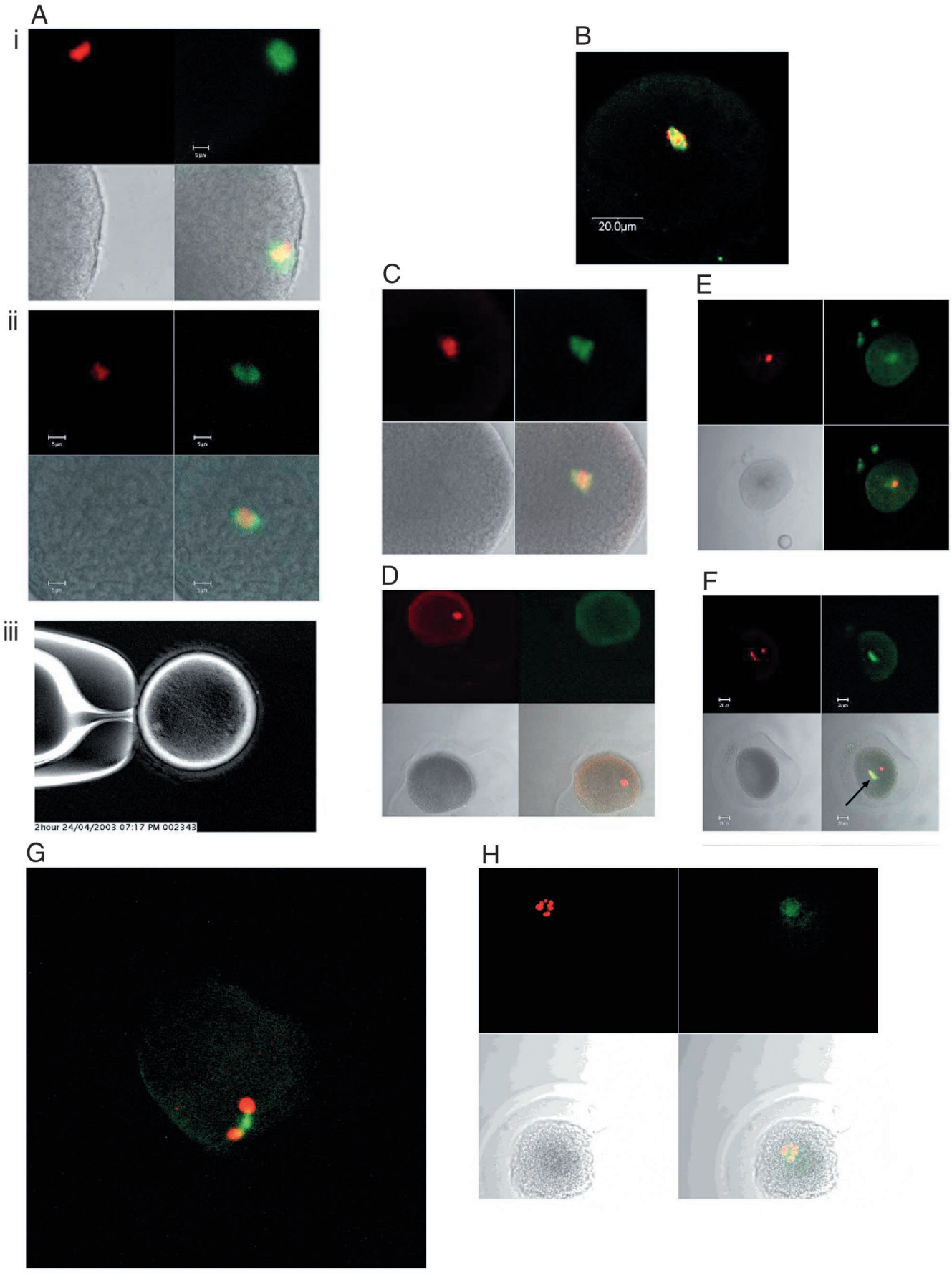
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Fig. 1. PCC spindle formation in a SCNT-reconstructed LoTM zygote, 2 hours after injection of the donor LoTM cumulus cell. Red, DNA; green, α-tubulin in confocal images. (A) Chromosome condensation and microtubule assembly into a normal PCC spindle. (i) Chromosomes in the middle of the PCC spindle. (ii) Another reconstructed zygote with chromosomes in the middle of the PCC spindle. (iii) Polarized microscopy view of a normal PCC spindle. (B) Chromosome condensation but improper capture onto normally assembled microtubules. (C) Chromosome condensation and microtubule assembly into an abnormal PCC spindle. (D) Chromosome condensation without microtubule assembly. (E) Non-condensed chromosomes and microtubule assembly into an abnormal PCC spindle. (F) No chromosome change and no microtubule assembly (arrow shows first polar body). (G) Normal separation in anaphase of a SCNT-reconstructed LoTM zygote, 2 hours after activation. (H) Abnormal separation of chromosomes in multiple directions on a diffused microtubule assembly, 2 hours after activation.

formed the PCC spindle normally with two poles (see Table 2). Control somatic cells injected into non-enucleated oocytes also formed normal spindles (data not shown).

Pregnancy outcomes after embryo transfer

Three days after SCNT, 93 reconstructed embryos (4- to 10-cell stage) were transferred to 31 LoTMs (the same macaques from which the oocytes were collected). The results of SCNT-embryo transfer are shown in Table 3. The pregnancy rate was not markedly different amongst different donor cell types. The pregnancies were confirmed by ultrasound (Fig. 2C). Unfortunately, none proceeded beyond 60 days of gestation (the average gestation period of the Long-tailed Macaque is 157-165 days). We were unable to obtain tissues from hysterotomy to confirm genetically that the early pregnancy was identical to the donor, as the macaques were not trained, and hence the need to catch and anesthetize them was kept to a minimum. Details of the pregnancies are shown in Table 4.

The first cell cycle of SCNT-reconstructed embryos

Based on our data, we believe that the G1/G0 donor nucleus (diploid, 2n) needs to undergo chromosomal changes, as does any other cell undergoing mitotic division. Hence, the chromosomes of the somatic cell undergo condensation

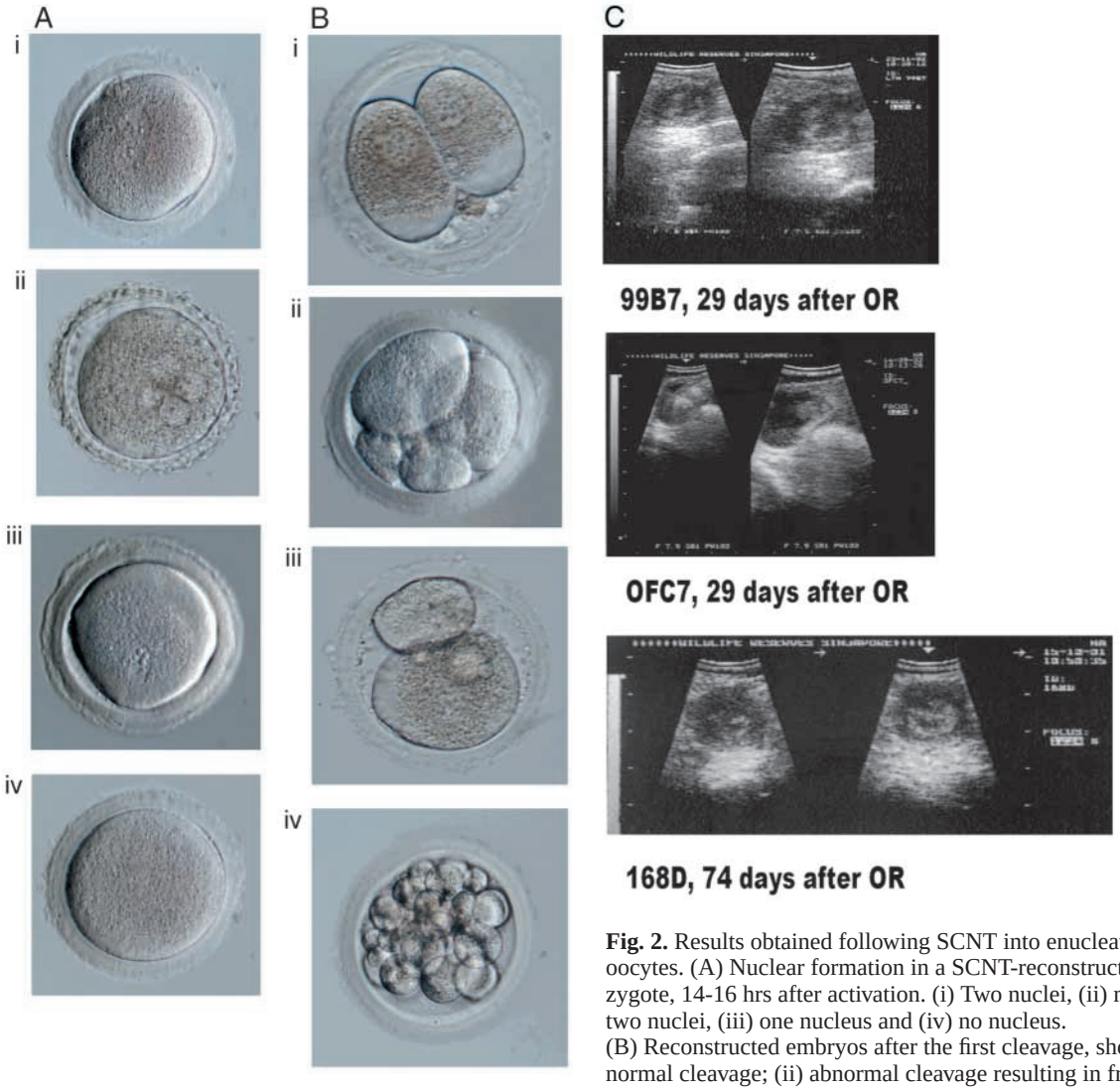
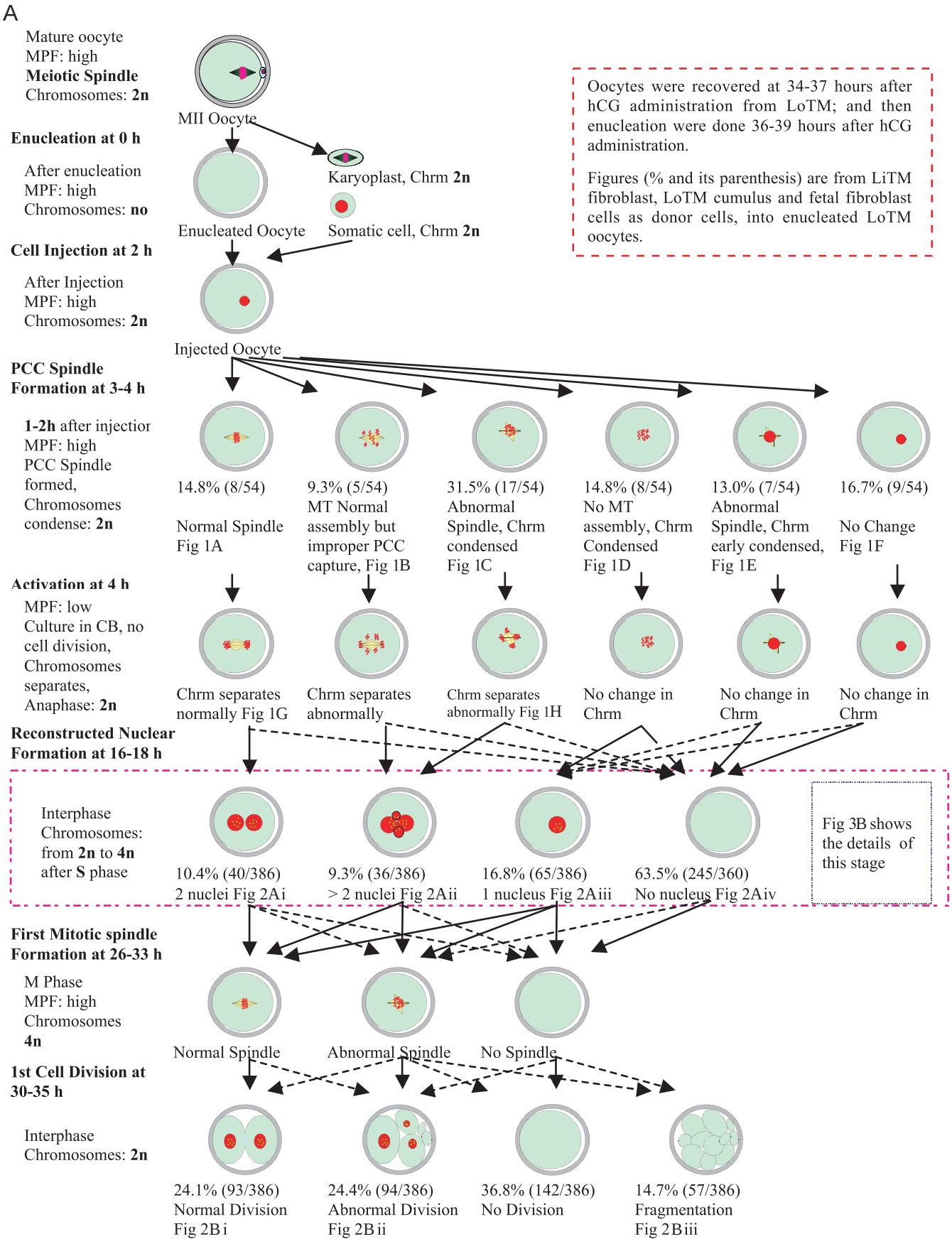
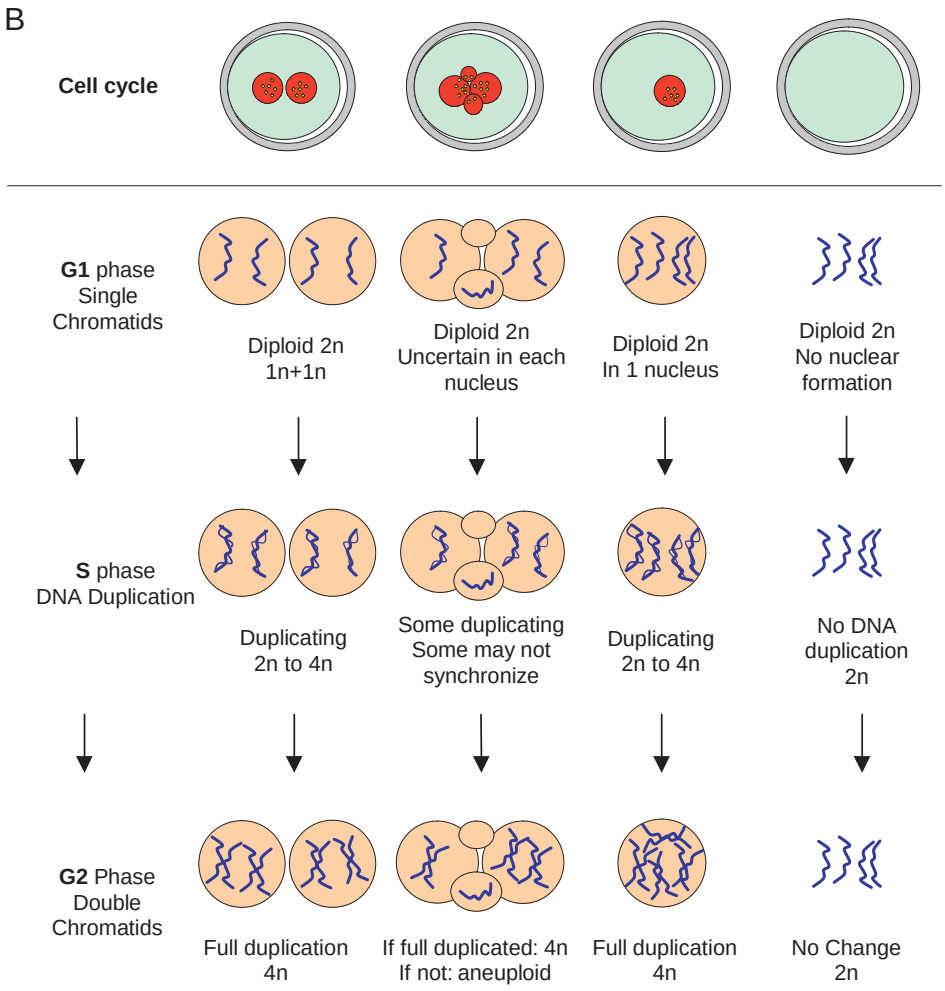


Fig. 2. Results obtained following SCNT into enucleated LoTM oocytes. (A) Nuclear formation in a SCNT-reconstructed LoTM zygote, 14-16 hrs after activation. (i) Two nuclei, (ii) more than two nuclei, (iii) one nucleus and (iv) no nucleus. (B) Reconstructed embryos after the first cleavage, showing: (i) normal cleavage; (ii) abnormal cleavage resulting in fragmentation (iia) and/or unequal blastomeres (iib); and (iii) highly fragmented nuclei. (C) Pregnancy sacs on ultrasound for 99B7 on the 15th day (29 days after OR), OFC7 on the 15th day (29 days after OR), and 168D the 60th day (75 days after OR) after completion of the luteal phase.


A
Mature oocyte
MPF: high
Meiotic Spindle
Chromosomes: 2n
MII Oocyte
Enucleation at 0 h
After enucleation
MPF: high
Chromosomes: no
Karyoplast, Chrm 2n
Enucleated Oocyte
Somatic cell, Chrm 2n
Cell Injection at 2 h
After Injection
MPF: high
Chromosomes: 2n
Injected Oocyte
PCC Spindle Formation at 3-4 h
1-2h after injection
MPF: high
PCC Spindle formed,
Chromosomes condense: 2n
14.8% (8/54)
Normal Spindle
Fig 1A
9.3% (5/54)
MT Normal assembly but improper PCC capture, Fig 1B
31.5% (17/54)
Abnormal Spindle, Chrm condensed
Fig 1C
14.8% (8/54)
No MT assembly, Chrm Condensed
Fig 1D
13.0% (7/54)
Abnormal Spindle, Chrm early condensed,
Fig 1E
16.7% (9/54)
No Change
Fig 1F
Activation at 4 h
MPF: low
Culture in CB, no cell division,
Chromosomes separates,
Anaphase: 2n
Chrm separates normally Fig 1G
Chrm separates abnormally
Chrm separates abnormally Fig 1H
No change in Chrm
No change in Chrm
No change in Chrm
Reconstructed Nuclear Formation at 16-18 h
Interphase
Chromosomes: from 2n to 4n
after S phase
10.4% (40/386)
2 nuclei Fig 2Ai
9.3% (36/386)
> 2 nuclei Fig 2Aii
16.8% (65/386)
1 nucleus Fig 2Aiii
63.5% (245/360)
No nucleus Fig 2Aiv
Fig 3B shows the details of this stage
First Mitotic spindle Formation at 26-33 h
M Phase
MPF: high
Chromosomes
4n
Normal Spindle
Abnormal Spindle
No Spindle
1st Cell Division at 30-35 h
Interphase
Chromosomes: 2n
24.1% (93/386)
Normal Division
Fig 2B i
24.4% (94/386)
Abnormal Division
Fig 2B ii
36.8% (142/386)
No Division
14.7% (57/386)
Fragmentation
Fig 2B iii
Oocytes were recovered at 34-37 hours after hCG administration from LoTM; and then enucleation were done 36-39 hours after hCG administration.
Figures (% and its parenthesis) are from LiTM fibroblast, LoTM cumulus and fetal fibroblast cells as donor cells, into enucleated LoTM oocytes.



* DNA duplication in nucleus is shown schematically within nucleus.

Fig. 3. Schematic of cell cycle changes following SCNT. (A) First cell cycle in reconstructed embryos following injection of somatic cells, with various possible pathways, with the number of embryos observed in parenthesis. (B) Chromosomes changes after nuclear reformation during the first cell cycle.

prematurely (PCC, 2n), within 2 hours of being introduced into the oocyte, with the formation of a normal PCC spindle (Fig. 1A) in about 14.8%. The majority undergo various abnormal changes, including: formation of normal microtubules but with improper capture of condensed chromosomes (Fig. 1B); formation of an abnormal PCC spindle (Fig. 1C); absence of microtubule assembly (Fig. 1D); absence of chromosome condensation with an abnormal PCC spindle (Fig. 1E); or even absence of change (Fig. 1F) (see Fig. 3A). Following activation, with a decline in maturation/meiosis/mitosis-promoting factor (MPF) amongst other signals, mitosis resumes and the chromosomes separate either normally (Fig. 1G) or abnormally (Fig. 1H). After this, the PCC (2n) decondense to chromatin, the nuclear membrane reforms, the cell goes into G1 and S phase, and the chromosomes start to duplicate from 2n to 4n. The details of chromosome changes after nuclear formation are shown in Fig. 1. Culture in Cytochalasin B for a few hours after activation is critical as it prevents cleavage to a haploid state (1n), thus resulting in two nuclei (Fig. 2A, part i) about 14-16 hours after activation, as is seen in 10.4% of our reconstructed embryos. Multiple and single nuclei are also seen in 9.3% and 16.8% of reconstructed embryos, respectively; the former probably arise from an abnormal PCC spindle, whereas the latter probably arise from an absence of chromosome separation or an absence of microtubule formation (Fig. 1E). After completing S phase, the reconstructed embryo goes into M phase again, in which a normal mitotic spindle reforms, the chromosomes (4n) then re-separate into normal diploid states (2n, Fig. 1G), or abnormal aneuploid states (Fig. 2H), sometimes resulting in severe fragmentation (Fig. 2B, part iii).

Discussion

Our findings demonstrate that 14.8% of somatic cell chromosomes will condense into a normal PCC spindle within 2 hours of injection into enucleated oocytes, and that these reconstructed SCNT embryos can result in implantation and pregnancy after transfer into recipient hosts. Nuclear formation, normal division of SCNT embryos and pregnancy after SCNT-embryo transfer were not markedly affected by the donor cell, as they were similar for the three different type of donor cell used: cumulus, fetal fibroblast and adult fibroblast.

Understanding the first cell cycle changes is crucial in designing strategies for successful SCNT in different species. The co-ordination of nuclear and cytoplasmic events during embryo reconstruction was reviewed previously (Campbell et al., 1996). Our data suggests the following sequence: the donor cell nuclear membrane breaks down because of the direct and indirect effects of MPF upon the transferred nucleus; the chromatin in G1/G0 condenses into a premature condensed chromosomal state; and then microtubule assembly occurs to form a PCC spindle from oocyte signals that would have formed the second meiotic spindle if the maternal chromosomes were still present. It also confirms that somatic cell DNA in non-human primates can form a normal PCC spindle after the somatic cell is introduced into a enucleated oocyte, as is seen in mice (Gao et al., 2002) and bovine (Shin et al., 2002a).

In this study, 70.4% of transferred somatic cells underwent chromosome condensation following their introduction into an enucleated MII oocyte, microtubule assembly occurred in 68.5% of reconstructed embryos, and 14.8% of them had

Table 3. Results of SCNT-embryo transfer*

Donor cell type	Number of SCNT embryos transferred	Number of recipients	Number of pregnancies	% Pregnancy rate
LiTM adult fibroblast	57	18	4	22.2
LoTM cumulus	25	9	2	22.2
LiTM adult fibroblast	3	2	0	0
LoTM cumulus	2			
LoTM fetal fibroblast	4	2	1	50.0
LoTM cumulus	2			
Total	93	31	7	22.6

*Pregnancy was confirmed by ultrasound.

normal PCC spindles with 2 poles. PCC spindle formation is influenced both by the donor cell and by the oocyte. Poor quality oocytes may not be able to initiate nuclear membrane breakdown of the somatic cell, chromosome condensation or microtubule assembly. Our data is suggestive of problems in both chromosome condensation and microtubule assembly in SCNT embryos. Misaligned metaphase plates were found in 9.3% of reconstructed embryos in which microtubules were normally assembled with 2 poles, but the prematurely condensed chromosomes were improperly captured. In mice (Wakayama et al., 1998), and rabbit (Chesne et al., 2002), such misaligned metaphase plates were also observed. This may due to lack of, or inadequate levels of, certain factors, such as HSET (Simerly et al., 2003).

The age of the oocytes could be a very important factor that affects results. Aging oocytes may not be able to reprogram the somatic cell. The majority of oocytes used in this study were collected at 34-37 hours after hCG administration, and were then enucleated 2 hours later. 21% (143/684) of oocytes used for enucleation had just reached the MII stage (extruded the first polar body) before enucleation. In some oocytes that were enucleated 4-5 hours later, unusual changes were observed, including fusion of 2-cell embryos back to 1-cell embryos with two nuclei. This suggests that cytoskeleton changes in aging oocytes may affect cell dynamics after reconstruction.

Nuclear formation rate in this study was only 36.5%, although chromosome condensation and microtubule assembly occurred in 70% of reconstructed embryos. This suggests that nuclear reformation is a problem in a large number of such embryos. Nuclear formation in fertilization following nuclear transfer is controlled by the oocyte cytoplasm (Collas, 1998). However, the sequence of events leading to nuclear reformation in cloned embryos is unclear, as are the factors influencing nuclear reformation. We have observed a one-day delay in nuclear formation in this study. Interestingly, there were some embryos without nuclear formation after cell injection that cleaved or became fragmented. It is possible that there was no chromosome duplication, which thus resulted in fragmentation; another possibility is that the S phase was too short and that they were missed. Further studies are needed.

The chromosome and cell cycle changes postulated on the basis of our data are for the injection technique. These changes may be different when using the cell fusion method (electrofusion), as the direct current used for fusion may partially activate the oocyte. Although cell injection may provide a mechanical stimulus, a gentle technique will minimize that. Reprogramming factors, as yet unknown, in the MII oocyte are capable of remodeling the fully differentiated somatic nucleus, although time is needed. Hence, activation 2 hours after the introduction of the somatic nucleus may be essential for donor cell reprogramming (Wilmut et al., 2002).

To date, there has been no successful live birth from SCNT in non-human primates. This lack of success was recently postulated to be due to the removal of molecular signals during enucleation, especially of NuMA and HSET (Simerly et al., 2003). Primate NT appears to be challenged by stricter molecular requirements for mitotic spindle assembly than are needed in other mammals. Our data suggest that this may not necessarily be the case.

NuMA (Nuclear Mitotic Apparatus) is an intranuclear matrix protein (Zeng et al., 2000) that has kinase-recognition motifs, including sites for cAMP-dependent kinase, PKC, CDC2 (MPF) and Ca^{2+}/calmodulin kinase, in the C-terminal domain of the protein (Yang and Snyder, 1992) and that is present in the nucleus during interphase (Compton et al., 1992). During mitosis, NuMA is essential for the terminal phases of chromosome separation and/or nuclear reassembly (Compton et al., 1992; Price et al., 1986). It is abundant in the cell (Compton et al., 1992), and hence in donor cells too. After the cell is introduced into an enucleated oocyte, NuMA from the donor cell should assist in forming a normal spindle. Simerly et al. reported that, in the non-human primate, all reconstructed SCNTs and ECNTs examined displayed aberrant spindles, and that NuMA was not detected in abnormal

Table 4. Details of pregnant *M. fasicularis*

	NT-embryo transfer			Ultrasound results					
				First ultrasound		Second ultrasound		Third ultrasound	
Surrogate ID	Date	Number of embryos	Donor cell	Day*	GS/FH	Day*	GS/FH	Day*	GS/FH
168D	16-11-01	4	LiTM fibroblast	60	+/+	85	–/–		
9FC0	21-12-01	4	LiTM fibroblast	19	+/+	50	–/–		
9F41	18-01-02	1	LoTM cumulus	18	+/±	50	–/–		
6245	08-03-02	3	LoTM cumulus	15	+/±	47	–/–		
E22E	12-07-02	2	LiTM fibroblast	18	+/±	25	–/–		
OFC7	30-08-02	4	LiTm fibroblast	15	+/±	42	–/–		
99B7	08-11-02	2	LoTM fetal fibroblast	15	+/+	34	+/±	55	–/–
		1	LoTM cumulus						

*Day after completion of luteal phase support, i.e. an additional 14 days after oocyte recovery (OR).

spindles (Simerly et al., 2003). We believe this may be due in part to technical problems, such as excessive aspiration of the cytoplasm (we remove less than 2% of the cytoplasm with the Spindle View System). Species difference may partially explain the difference, and culture environment may also be a contributing factor, as an optimal medium for SCNT has not been reported. In fact, our data supports the conventional belief that incomplete nuclear re-programming is likely to be the reason for the lack of live births in primates.

From our experiments, successful implantation implies the ability to develop embryonic stem cells and undergo early gastrulation. However, there are many possible reasons for failure to develop to term in SCNT, as have been reviewed previously (Wilmut et al., 2002). Developmental manipulations (including SCNT) or non-physiological culture environments may result in inappropriate epigenetic modification of imprinted genes during early embryogenesis when many allele-specific imprints were established or maintained (Young and Fairburn, 2000), including defective fetal reprogramming in livestock (Young et al., 2001). Data has been conflicting, reflecting the state of uncertainty regarding gene expression following SCNT. In SCNT bovine embryos, normally expressed metabolic enzyme genes have been reported (Winger et al., 2000), as well as the abnormal expression of genes essential for early embryonic development (Daniels et al., 2000). Such abnormal expression has also been reported in cloned mouse blastocysts or ES cells (Boiani et al., 2002), and genes important to early development frequently fail to be activated in mouse embryos cloned from adult cells (Bortvin et al., 2003), suggesting that aberrant transcription patterns detected in cloned embryos may lead to abnormalities at various embryonic stages.

As it is likely that there are many differences among species, we need to optimize the protocol for primates. The success of ECNT (Meng et al., 1997) has confirmed that enucleation is not a problem for NT. Meng et al. used embryos generated by in vitro fertilization as donor cells, cryo-preserved the reconstructed embryos and then transferred them into synchronized surrogates; they obtained two live births from 29 reconstituted embryos. However, it is still possible that spindle-associated factors may play a role, and, if so, a minimal enucleation procedure will be crucial. Thus, new strategies are needed. An approach whereby the donor cell is introduced before the oocyte DNA is removed (‘reverse-order’ cloning method) has been described by Peura et al., and results in significantly higher blastocyst rates in the sheep (Peura et al., 2003). However, Wakayama recently reported that this reverse-order cloning method had no effect on cloned-embryo development in mouse (Wakayama et al., 2003); these findings suggest that neither oocyte chromosome depletion per se, nor the potential removal of ‘reprogramming’ factors during enucleation, explain the low efficiency of NT cloning.

In conclusion, there is a crucial need to understand the molecular and cellular events that occur after the introduction of a somatic cell into an enucleated oocyte. It is only with this understanding that it will be possible to use this technique to its maximal potential.

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