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Blastocysts derived from adult fibroblasts of a rhesus monkey (*Macaca mulatta*) using interspecies somatic cell nuclear transfer

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Summary

In non-human primates, it is difficult to collect sufficient numbers of oocytes for producing identical embryos by somatic cell nuclear transfer (SCNT). Because of this factor, inter-species SCNT (iSCNT) using heterospecific oocytes is an attractive alternative approach. The objective of this study was to produce iSCNT-derived blastocysts using enucleated cow (*Bos taurus*) metaphase II oocytes and adult rhesus monkey (*Macaca mulatta*) fibroblasts. Ear skin tissue from a 6-year-old male rhesus monkey was collected by biopsy and fibroblasts were isolated. Immature cumulus–oocyte complexes from cow ovaries were collected and matured *in vitro* in Medium 199. The enucleated oocytes were reconstructed with rhesus monkey fibroblasts and iSCNT embryos were cultured in modified synthetic oviduct fluid in an atmosphere of 5–5.5% CO_2 under various conditions (37–39 °C and 5–20% O_2) to examine the effects of *in vitro* culture conditions. Most embryos were arrested at the 8- or 16-cell stage and only three blastocysts were derived in this way using iSCNT from a total of 1153 cultured activated embryos (0.26% production rate). Two of the three blastocysts were used for counting nuclear numbers using bisbenzimide staining, which were 51 and 24. The other iSCNT-derived blastocyst was used to analyse mitochondrial DNA (mtDNA) by PCR, and both rhesus monkey and cow mtDNA were detected. Although the development rate was extremely low, this study established that iSCNT using two phylogenetically distant species, including a primate, could produce blastocysts. With improvements in the development rate, it may be possible to produce rhesus monkey iSCNT-derived embryonic stem cell lines for studies on primate nucleus and cow mitochondria interaction mechanisms.

Keywords: Blastocysts, Cow, Interspecies somatic cell nuclear transfer, Mitochondria, Rhesus monkey

Introduction

Interspecies somatic cell nuclear transfer (iSCNT) is a useful tool for the conservation of endangered species by cloning and for preclinical or basic studies using iSCNT-derived embryonic stem (iSCNTES) cell lines (Tecirlioglu *et al*., 2006; Beyhan *et al*., 2007). At present, it is much more difficult to support full-term development with iSCNT than with intraspecies somatic cell nuclear transfer (SCNT), therefore the main focus of iSCNT is to produce blastocysts and establish iSCNTES cell lines (Tecirlioglu *et al*., 2006; Beyhan *et al*., 2007). Because specific cell types like neurons, pancreatic beta cells and cardiomyocytes obtained from patients' biopsies may be abnormal, differentiated cell types derived from iSCNTES cell lines could be useful for preclinical testing of new drugs (Minger, 2007). Furthermore, the use of iSCNTES cell lines could be a valuable tool for studies on nucleus–mitochondrion (mt) interaction mechanisms during early embryonic development (Tecirlioglu *et al*., 2006; Beyhan *et al*., 2007; St John *et al*., 2008).

Thus far, only a few phylogenetically close interspecies offspring have been born (Tecirlioglu *et al*., 2006; Beyhan *et al*., 2007). Interspecies to interclass

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blastocysts were produced by iSCNT mainly using cow or rabbit oocytes (Tecirlioglu *et al*., 2006; Beyhan *et al*., 2007). Although human with rabbit iSCNTES cell lines have been established, the technique was not reproducible until recently (Chen *et al*., 2003). Especially for primates, there have been only a few reports of iSCNT blastocyst production: human with cow (Chang *et al*., 2003; Illmensee *et al*., 2006; Li *et al*., 2008), human with rabbit (Chen *et al*., 2003), cynomolgus monkey with cow (Dominko *et al*., 1999) and rhesus monkey with rabbit (Yang *et al*., 2003). Although cow oocytes fused with cynomolgus monkey somatic cells supported iSCNT blastocyst development in one report (Dominko *et al*., 1999), other groups have had contradictory results (Lorthongpanich *et al*., 2008).

Cow and rabbit oocytes have mainly been used for iSCNT (Tecirlioglu *et al*., 2006; Beyhan *et al*., 2007). Although rabbit oocytes show higher developmental competence than cow oocytes when used in iSCNT (Beyhan *et al*., 2007), the oocyte retrieval procedure from rabbits is more complicated than that with cows (Yang *et al*., 2003; Kwon *et al*., 2009). Rabbit oocytes have to be retrieved from oviducts by a complex surgical procedure following ovarian hyperstimulation treatment (Yang *et al*., 2003); moreover, only a few oocytes can be obtained from each animal. In contrast, immature cow oocytes are readily available from slaughterhouse ovaries and can be matured *in vitro* up to the metaphase II (MII) stage; they have been successfully used for interspecies to interclass SCNT as with rabbit oocytes (Tecirlioglu *et al*., 2006; Beyhan *et al*., 2007). Using this well established *in vitro* maturation (IVM) system, large numbers of cow oocytes can be made available for iSCNT more easily and economically than rabbit oocytes, and without using living animals. The objective of the present study was to confirm whether cow enucleated ooplasm could support blastocyst development of rhesus monkey somatic nucleus.

Materials and methods

Preparation of rhesus monkey somatic cells

The ear skin biopsy of a male 6-year-old rhesus monkey was approved by the Seoul Zoo (Gyeonggido, Korea). Primary culture of ear skin tissue was performed following the previous work of Kwon *et al.* (2009). Briefly, pieces of ear skin tissue (approximately 0.5 cm^2) were minced and treated overnight with collagenase type I (Invitrogen) in a 37 °C incubator under 5% CO_2 in air to isolate growing cells. One day later, isolated cells were washed and reseeded with Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) at 37 °C under 5% CO_2 in air. After culture to confluency, the cells were cryopreserved in liquid nitrogen. At 3–4 days before iSCNT, cells were thawed and used as the donor somatic cells.

Preparation of recipient cow oocytes

Preparation of recipient cow oocytes was carried out using following the methodology of Kwon *et al.* (2009) with minor modifications. Basically, the IVM medium used was Medium-199 (Invitrogen) supplemented with pyruvate (Sigma-Aldrich), kanamycin (Sigma-Aldrich), 10% (v/v) FBS, 0.005 IU/ml follicle stimulating hormone (FSH; Sigma-Aldrich) and 1 μg/ml 17β-estradiol (Sigma-Aldrich). Incubation conditions were 39 °C with 5% CO_2. Ovaries were transported from a local slaughterhouse to the laboratory in 0.9% NaCl solution at around 30 °C. After aspiration of cumulus–oocyte complexes (COCs) from the ovaries using an 18-gauge needle attached to a syringe, COCs were washed four times and cultured at 20–30 oocytes per well in a 4-well dish (SPL).

Interspecies SCNT and *in vitro* culture (IVC)

A total of 58 iSCNT experiments were done once or twice a week. The same batch of medium was used for IVM–IVC consecutively in two experiments per week. Methodology from Kwon *et al*. (2009) was employed in setting up iSCNT and IVC with minor modifications. Briefly, cow COCs were denuded by repeated pipetting in 0.1% hyaluronidase (Sigma-Aldrich) after 21–24 h of IVM culture. Squish enucleation was done in the presence of 5 μg/ml bisbenzimide (Hoechst 33342; Sigma-Aldrich) and 5 μg/ml cytochalasin B (Sigma-Aldrich) under UV light. After enucleation, a single rhesus monkey somatic cell was injected into the perivitelline space of an enucleated oocyte through the slit in the zona pellucida made during enucleation. Subsequently, the rhesus monkey somatic cell and cow oocyte membranes were electrically fused using an Electro-Cell Fusion apparatus (NEPA GENE, Chiba, Japan). Fusion conditions were two pulses of direct current of 34–38 V for 15 μs duration with 0.26 M calcium-free mannitol (Sigma-Aldrich) solution. Subsequently, the non-activated interspecies cloned couplets were cultured for 2h in IVM medium without hormones to allow reprogramming to occur. Then, couplets were activated using a two-step protocol consisting of treatment with 5 μM ionomycin (Sigma-Aldrich) for 4–5 min and subsequently with 2 mM 6-dimethylaminopurine (6-DMAP; Sigma-Aldrich) for 4 h. After washing without 6-DMAP in modified synthetic oviduct fluid (mSOF), activated interspecies cloned embryos ($n = 1153$) were cultured

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Table 1 Fusion, blastocyst development rate and blastocyst nuclear number of rhesus monkey iSCNT embryos

No. of oocytes (%)			No. of iSCNT embryo to develop (%)					
Enucleated	Injected (% per enucleated)	Fused (% per injected)	IVC (% per fused)	Blastocyst (% per IVC)	Hatching blastocyst (% per blastocyst)	ID	Hoechst staining day[a]	Nuclear number[b]
2090	2065 (98.80)	1159 (56.13)	1153 (99.48)	3 (0.26)	1 (33.33)	A	–[c]	–[c]
						B	7.0	51
						C	7.5	24

These experiments were replicated 58 times.
Average number of IVC embryos per replicate was 19.9.
[a]Days after IVC.
[b]Nuclear number of rhesus monkey iSCNT blastocyst was counted three times and the average number was calculated.
[c]Not determined.
iSCNT, inter-species somatic cell nuclear transfer; IVC, *in vitro* culture.

for 10 days in mSOF at 5–5.5% CO_2 with various conditions (at 37–39 °C and 5–20% O_2) to examine the effects of IVC conditions (see Table 2).

Counting nuclear number of blastocyst

iSCNT blastocysts were used for nuclear number analysis. After staining in bisbenzimide solution for 5–10 min at 37 °C, the blastocysts were examined under a UV microscope (excitation wavelength 351–364 nm). Nuclear counting was carried out three times on each iSCNT blastocyst and the average number was calculated.

Polymerase chain reaction (PCR) of rhesus monkey and cow mtDNA

DNA preparation was conducted in accordance with the manufacturer's protocol (iNtRON). Final elution volume was 50 µl per single blastocyst. For amplification of the D-loop region of mtDNA from cow and rhesus monkey, KNCF1 (RhDF2) and KNCR2000 (RhDR) primers were used (Do *et al*., 2002; Byrne *et al*., 2007). The PCR reaction of cow mtDNA was carried out for one cycle with denaturation at 94 °C for 5 min (2 min for rhesus monkey) and 55 subsequent cycles with denaturation at 94 °C for 1 min (30 s), annealing at 52 °C (55 °C) for 1 min (30 s), extension at 74 °C (72 °C) for 1 min (1 min 30 s) and a final extension at 74 °C (72 °C) for 7 min (3 min). The PCR product size for cow and rhesus monkey was 960 and 544 bps, respectively.

Table 2 Effect of temperature and oxygen concentration during IVC of rhesus monkey iSCNT embryos

ID	Temperature (°C)/ Oxygen (%)	No. of embryos (IVC)	No. of blastocysts (% per IVC)
A	38.5–39.0/20.0	275	1 (0.36)
B	38.5–39.0/5.0	864	1 (0.12)
C	37.0/5.0	14	1 (7.14)

iSCNT, inter-species somatic cell nuclear transfer; IVC, *in vitro* culture.

Results

Development of rhesus monkey-cow iSCNT blastocysts

The fusion rate of rhesus monkey somatic cells and cow enucleated oocytes was 56.13% from the total injected oocytes (Table 1). Of IVC embryos ($n = 1153$), three blastocysts developed *in vitro* (Table 1, Fig. 1*A*, *B*,*D*). One is a hatching blastocyst (ID: B, Fig. 1*B*) and the other two are early blastocysts (ID: A and C, Fig. 1*A*,*D*). One iSCNT blastocyst (ID: A, Fig. 1*A*) shrank quickly after forming a prominent blastocoele. Figure 1*A* shows a pre-expansion compacted morula (CM) and it is morphologically similar to a rhesus monkey SCNT morula in a previous report (Simerly *et al*., 2004). The iSCNT blastocyst development rate was 0.26% of the total IVC iSCNT embryos (Table 1). The nuclear number of two iSCNT blastocysts was 51 and 24 (Table 1, Fig. 1*C*,*E*). The hatching iSCNT blastocyst shrank after exposure to bisbenzimide for nuclear counting (Fig. 1*C*). The use of two different oxygen concentrations (5% and 20%) and two temperatures (37 °C and 38.5–39.0 °C) during IVC of iSCNT embryos showed that all three tested conditions support iSCNT blastocyst development (Table 2).

Analysis of mixed mtDNA in iSCNT blastocysts

One iSCNT blastocyst had both cow and rhesus monkey mtDNA (Fig. 2). The band intensity was more dominant for cow mtDNA than rhesus monkey mtDNA (Fig. 2).

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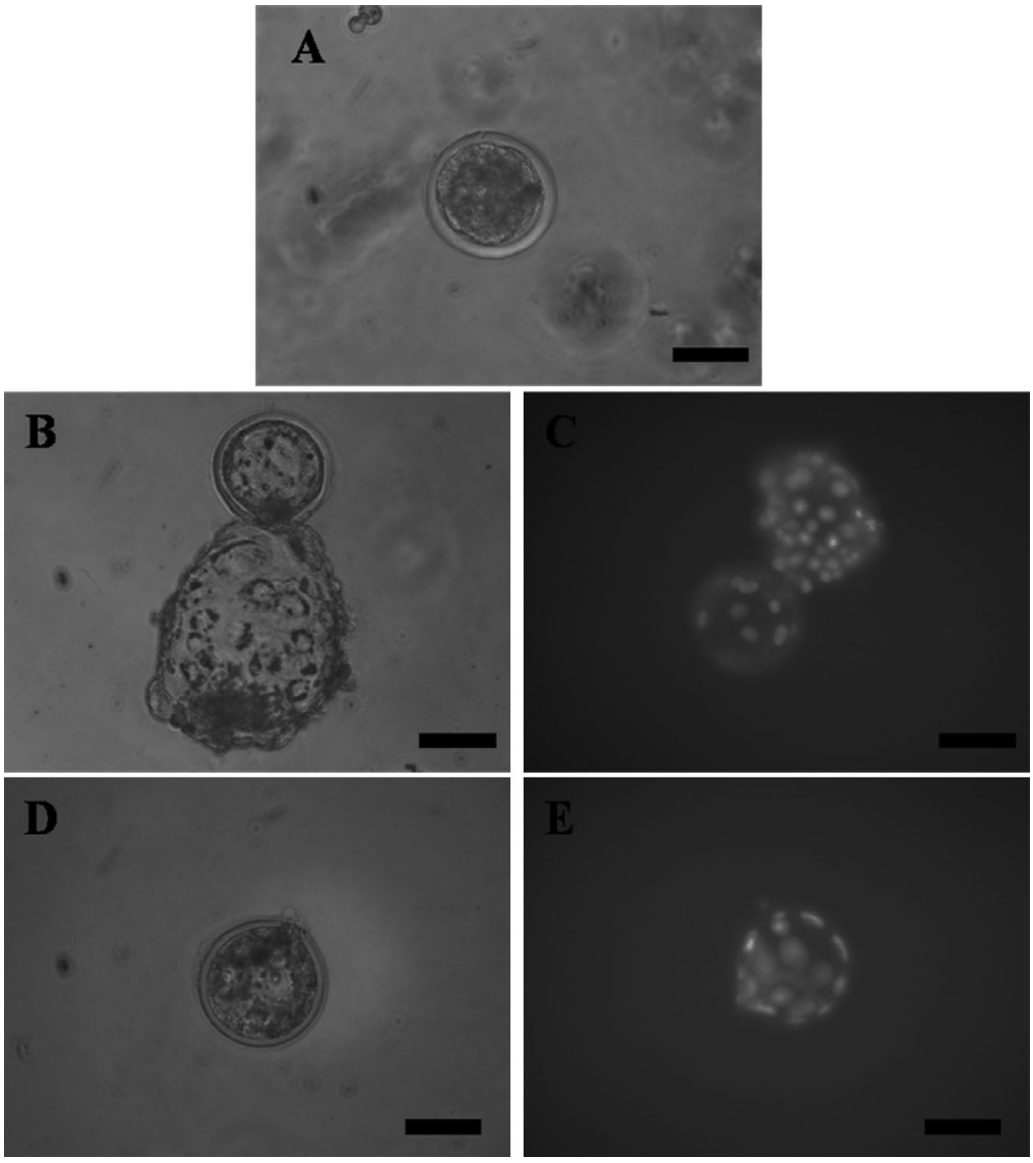

Figure 1 Development of rhesus monkey and cow inter-species somatic cell nuclear transfer (iSCNT)-derived blastocysts. Phase contrast and bisbenzimide staining image of rhesus monkey iSCNT compacted morula (*A*), hatching blastocysts (*B*,*C*) and early blastocyst (*D*,*E*). The compacted morula (*A*) later expanded to the early blastocyst stage. (*B*/*C*) and (*D*/*E*) are not merged images. Scale bar = 100 μm

Discussion

The aim of this study was to confirm whether cow enucleated ooplasm could support blastocyst development of rhesus monkey somatic nucleus. This report is the first to show rhesus monkey iSCNT blastocyst development *in vitro*, showing prominent blastocoele cavity formation. In this experiment, we generated an iSCNT blastocyst developmental rate of 0.26% from the total IVC iSCNT embryos, and the nuclear numbers of two iSCNT blastocysts were 51 and 24. Because a hatching blastocyst appeared at around 5.5 days of IVC, it seems that iSCNT embryos follow the donor cell-specific developmental programme (Dominko *et al*., 1999) wherein monkey SCNT blastocysts appeared around days 4.5–6.0 (Simerly *et al*., 2004; Zhou *et al*., 2006) and cow SCNT blastocysts appeared around days 6.0–7.0 after activation (Dominko *et al*., 1999). Only one iSCNT blastocyst was subjected to mtDNA analysis. The mixed mtDNA data from this blastocyst showed that it originated from the fusion of an enucleated cow oocyte and a rhesus monkey somatic cell. As in previous reports (Yang *et al*., 2003, 2004), the PCR band intensity of both species' mtDNA indirectly indicates that the oocyte origin cow mtDNA was

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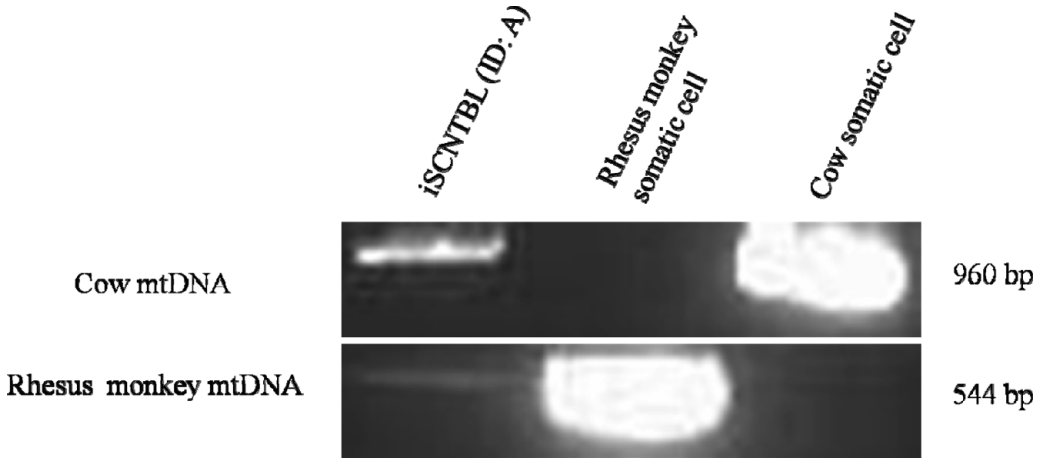

Figure 2 Mixed mtDNA of rhesus monkey inter-species somatic cell nuclear transfer (iSCNT) blastocyst. PCR analysis confirmed that this iSCNT blastocyst has both cow (960 bp) and rhesus monkey mtDNA (544 bp).

dominant over the somatic cell origin rhesus monkey mtDNA. Two iSCNT blastocysts failed mtDNA analysis in the DNA preparation step. Although this report showed an extremely low blastocyst development rate, it proves that cow enucleated ooplasm can support blastocyst development driven by adult rhesus monkey somatic nucleus. This result is indirect evidence that extraordinarily phylogenetically distant species such as cow and rhesus monkey have relatively common mechanisms of nuclear reprogramming, DNA replication and gene expression in preimplantation stage embryos (Li *et al*., 2008). For direct evidence, it would be necessary to produce viable iSCNT offspring but that was beyond the aims of the present study.

In this study, we succeeded in iSCNT blastocyst development only in three experiments out of 58 iSCNT experiments. Interspecies SCNT blastocyst (ID: B and C) were produced in the consecutive one day term iSCNT experiments and were used in the same batch medium from IVM to IVC. These two iSCNT experiments showed the iSCNT blastocyst developmental rate to be 5.71% (2/35), and it is about 21 times higher than the total iSCNT blastocyst developmental rate (0.26%, 3/1153). As the micromanipulation was done by same person and the conditions of each step of iSCNT were similar in 58 experiments, this situation reflects that the medium batch used in iSCNT experiments could be a critical factor. Otherwise, oxygen concentration and temperature during IVC might be non-critical factors for the development to the blastocyst stage.

Although several data indicate that there is no clear answer about the optimal conditions of rhesus monkey iSCNT blastocyst development *in vitro*, it is reasonable to speculate that medium (IVM–IVC) batch could be critical whereas oxygen concentration and temperature during IVC are not. In spite of the extremely low blastocyst developmental rate, the fact that three blastocysts produced by three independent iSCNT experiments reflect that this experiment could be fairly reproducible following optimization of conditions.

In this iSCNT study, it was shown that rhesus monkey iSCNT blastocyst development is possible *in vitro*. However, conditions have to be adjusted to improve the blastocyst development rate and quality for future rhesus monkey iSCNTES cell line isolation. As with the consecutive establishment of monkey and human ES cell lines (Thomson *et al*., 1995, 1998), monkey SCNT research could be a very useful preliminary tool for human SCNT research. Because the general methodology of human SCNT blastocyst development is similar to this iSCNT study (Stojkovic *et al*., 2005; French *et al*., 2008), this report could also be used as a model for producing high quality human SCNT blastocysts used to establish human SCNTES cell lines.

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