In Vitro Development of Interspecies Nuclear Transfer Embryos using Porcine Oocytes with Goat and Rabbit Somatic Cells

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ABSTRACT

Interspecies somatic cell nuclear transfer (iSCNT) is a valuable tool for studying the interactions between an oocyte and somatic nucleus. The object of this study was to investigate the developmental competence of *in vitro*-matured porcine oocytes after transfer of the somatic cell nuclei of 2 different species (goat and rabbit). Porcine cumulus oocytes were obtained from the follicles of ovaries and matured in TCM-199. The reconstructed embryos were electrically fused with 2 DC pulses of 1.1 kV/cm for 30 μs in 0.3 M mannitol medium. The activated cloned embryos were cultured in porcine zygote medium-3 (PZM-3), mSOF or RDH medium for 7 days. The blastocyst formation rate of the embryos reconstructed from goat or rabbit fetal fibroblasts was significantly lower than that of the embryos reconstructed from porcine fetal fibroblast cells. However, a significantly higher number of embryos reconstructed from goat or rabbit fetal fibroblasts cultured in mSOF or RDH, respectively, developed to the morular stage than those cultured in PZM-3. These results suggest that goat and bovine fetal fibroblasts were less efficacious than porcine-porcine cloned embryos and that culture condition could be an important factor in iSCNT. The lower developmental potential of goat-porcine and porcine-bovine cloned embryos may be due to incompatibility between the porcine oocyte cytoplasm and goat and bovine somatic nuclei.

(Key words : Interspecies nuclear transfer, Porcine oocytes, Rabbit fetal fibroblast, Goat fetal fibroblast)

INTRODUCTION

Somatic cell nuclear transfer (SCNT) is a valuable tool for producing genetically identical cloned animals and unveiling fundamental biological features such as the reversibility of cell differentiation.

As SCNT techniques have improved, there have been some efforts to preserve endangered species (Loi *et al.*, 2001), investigate the interactions between recipient oocytes and donor cells (Dominko *et al.*, 1999), and overcome the shortage of recipient oocytes (Kitiyanant *et al.*, 2001) by using interspecies nuclear transfer.

One of the first attempts at interspecies nuclear transfer was by Dominko *et al* (1999) who used enucleated bovine oocytes as the recipient cytoplast. They used monkey, sheep, pig and rat fibroblasts as donor cells, and their experiments achieved various degrees of early *in vitro* development. Other attempts at interspecies nuclear transfer have been conducted with bovine oocytes as the recipients and donor cells from other species such as guar (Lanza *et al.*, 2000), pig (Yoon *et al.*, 2001), buffalo (Kitiyanant *et al.*, 2001), and mouse (Arat *et al.*, 2003). There have been several reports of interspecies nuclear transfer using rabbit oocytes as the recipient and donor somatic cells from giant panda (Chen *et al.*, 2002), bovine (Techakumphu *et al.*, 2005), elephant (Numchaisrika *et al.*, 2004), and cat (Wen *et al.*, 2003) cloned embryos. Further, porcine oocytes have been used for interspecies nuclear transfer with somatic cells from rabbit (Chen *et al.*, 2006) and tiger (Hashem *et al.*, 2007) embryos. Oocyte cytoplasm could dedifferentiate somatic cells from different species and supported the early development of these interspecies nuclear transferred embryos to the blastocyst stage. In addition, offspring of 4 endangered species were successfully produced by interspecies somatic cell nuclear transfer: gaur (Lanza *et al.*, 2000), mouflon (Loi *et al.*, 2001), banteng (Janssen *et al.*, 2004), and African wild cat (Gomez *et al.*, 2004). However, at

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present, the interspecies-nuclear transfer technique is not as efficient as interspecies-nuclear transfer. Universal oocytes using swine oocytes as laboratory animal's oocytes may contribute to further applications of nuclear transfer.

The present study aimed to understand if porcine oocytes used for iSCNT could reprogram the nuclei from goat and rabbit fetal fibroblasts.

MATERIALS AND METHODS

Culture of Donor Cells from Various Species

A porcine fetus was obtained from a pregnant sow on day 35 after insemination, and the tissue was cut into small pieces with fine scissors. A goat fetus was obtained from a pregnant sow on day 50 after insemination. A rabbit fetus was obtained from a pregnant sow on day 17 after insemination. The tissues were washed 3 times and incubated for 10 min at 37℃ in phosphate-buffered saline (PBS) (Sigma, P-3288) containing 0.05% trypsin and 0.5 mM ethylenediaminetetraacetic acid (EDTA). This suspension was centrifuged at 500 g for 10 min. The cell pellet was resuspended in Dulbecco's modified Eagle medium (DMEM) (Gibco, 16000-044) supplemented with 75 μg/ml penicillin G, 50 μg/ml streptomycin, 5% (v/v) fetal bovine serum (FBS), and 5% (v/v) fetal calf serum (FCS, Gibco, 26010-074) and cultured at 38.5℃. Before nuclear transfer, the cells were washed twice in PBS and treated with 0.25% trypsin and 0.5 mM EDTA for single cell isolation at 2 min in a 38.5℃ incubator.

Oocyte Collection and *In Vitro* Maturation

Porcine ovaries were collected from prepubertal gilts at a local abattoir. The ovaries were excised within 30 min of slaughter and transported to the laboratory within 3 h in a thermos containing PBS at 35~37℃ and transported to a laboratory at 25℃. The ovaries were warmed in PBS containing 100 IU/ml penicillin and 50 μg/ml streptomycin. Cumulus-oocyte complexes (COCs) were aspirated from ovarian follicles that were 2~6 mm in diameter by using a 10 ml syringe fixed with an 18 gauge needle. Only oocytes possessing a compact complete cumulus oophorus and evenly granulated cytoplasm were selected for *in vitro* maturation (IVM). Next, the COCs were washed twice in TL-Hepes containing 0.1% (w/v) polyvinyl alcohol (PVA, Sigma, P-8136). The oocytes were transferred into 500 ml of maturation medium, which had been covered with mineral oil, in a 4-well multidish (Nunc, Roskide, Denmark) and incubated for 42~44 h at 38.5℃ in an atmosphere of 5% CO_2 with maximum humidity. The medium used for the IVM of oocytes was TCM-199 (Sigma, M-4530) supplemented with 0.57 mM l-cysteine, 0.5 μg/ml LH (L-5269, Sigma Chemical Co, St. Louis, MO), 0.5 μg/ml FSH (Sigma, F-2292), 10 ng/ml epidermal growth factor (Sigma, E-4127), 100 U/ml penicillin, and 50 μg/ml streptomycin. After 22 h of maturation culture, the oocytes were washed 3 times and transferred into 500 μl of basic medium without hormones for an additional 22 h of culture.

Enucleation of Recipient Oocytes Nuclear Transfer

Following *in vitro* maturation, cumulus cells were removed by repeated pipetting in TL-Hepes supplemented with 0.1% PVA and 0.3% hyaluronidase (Sigma, H-3506). Cumulus-free oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm with a fine glass pipette in TCM 199 supplemented with Hepes, 0.3% BSA (Sigma, A-8022), and 7.5 μg/ml cytochalasin B (Sigma, C-6762). A single donor cell was placed in the perivitelline space of the oocyte so that it could be in contact with the oocyte membrane. Injected oocytes were placed between two 0.2 mm diameter platinum electrodes (1 mm apart) of a fusion chamber slide in fusion and activation medium. The medium comprised 0.3 mM mannitol (Sigma, M-9647), 1.0 mM $CaCl_2 \cdot H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$, and 0.5 mM HEPES. For fusion and activation, 2 DC pulses of 1.1 kV/cm for 30 μs were used from a BTX Elector-Cell Manipulator 2001 (BTX, San Diego, CA).

Experimental Design and Embryo Culture

Two experiments were conducted.

In experiment 1, embryos reconstructed by the nuclear transfer of porcine cells into enucleated porcine oocytes were electrically activated and cultured as controls. Reconstructed embryos that were formed by the nuclear transfer of goat cells into enucleated porcine oocytes were electrically activated and cultured as the experimental group. The *in vitro* culture (IVC) medium was prepared using porcine zygote medium-3 (PZM-3) or mSOF.

In experiment 2, embryos reconstructed by the nuclear transfer of porcine cells into enucleated porcine oocytes were electrically activated and cultured as controls. Reconstructed embryos that were formed by the nuclear transfer of rabbit cells into enucleated porcine oocytes were electrically activated and cultured as the experimental group. The IVC medium for the control was contained PZM-3. The IVC medium of the experimental group contained PZM-3 and RDH. The RDH medium was prepared by mixing RPMI1640 (Gibco, 22400-089), DMEM (Gibco, 11995-065), and Ham's F10 (Gibco, 11550-043) in a 1:1:1 concentration (Jin *et al.*, 2000).

Assessment of the Developmental Potential of the Activated Oocytes

After activation treatment, the nuclear transferred embryos were examined at 48 h or 72 h to check if cleavage had occurred. They were examined under a stereomicroscope. Blastocyst formation was examined on day 7. The nuclear transferred embryos were washed twice and stained with Hoechst 33342 (2 mg/ml in 2.3 % sodium citrate) at 37℃. The cells were counted under a fluorescent microscope (Olympus, Japan).

Statistical Analysis

Each experiment was repeated at least 4 times. Differences among the treatment groups were determined as significant or not by using analysis of variance (ANOVA). Duncan's multiple range test was used to determine the significance between treatments. A P value less than 0.05 denoted a statistically significant difference.

RESULTS

In Vitro Development of Reconstructed Embryos Prepared by the Nuclear Transfer of Porcine and Goat Fetal Fibroblasts into Enucleated Porcine Oocytes

Embryos reconstructed by the nuclear transfer of porcine fetal fibroblast cells and goat fetal fibroblast cells into enucleated porcine oocytes (porcine-porcine cloned embryos and goat-porcine cloned embryos, respectively) were cultured in PZM-3 or mSOF medium. The cleavage rates of the goat-porcine cloned embryos were similar to those of the porcine-porcine cloned embryos. However, the morular formation rates of the goat-porcine cloned embryos cultured in mSOF (14.0%) were significantly higher than that of those cultured in PZM-3 (6.11%). the blastocyst formation rate of the goat-porcine cloned embryos was significantly lower than that of the porcine-porcine cloned embryos (0.74 and 1.4%, vs. 11.5%, respectively) (Table 1, Fig. 1).

In Vitro Development of Reconstructed Embryos with Porcine and Rabbit Fetal Fibroblasts into Porcine Oocytes

Embryos reconstructed by the nuclear transfer of porcine fetal fibroblast cells into porcine oocytes were allocated and cultured in PZM-3 medium. Embryos reconstructed by the nuclear transfer of rabbit fetal fibroblast cells into porcine oocytes (rabbit-porcine cloned embryos) were cultured in PZM-3 and RDH media. The RDH medium was prepared by mixing RPMI 1640, DMEM and Ham's F10 in a 1:1:1 concen-

Table 1. ***In vitro*** **development of the embryos prepared by interspecies nuclear transfer of porcine and goat somatic cells into porcine oocytes**

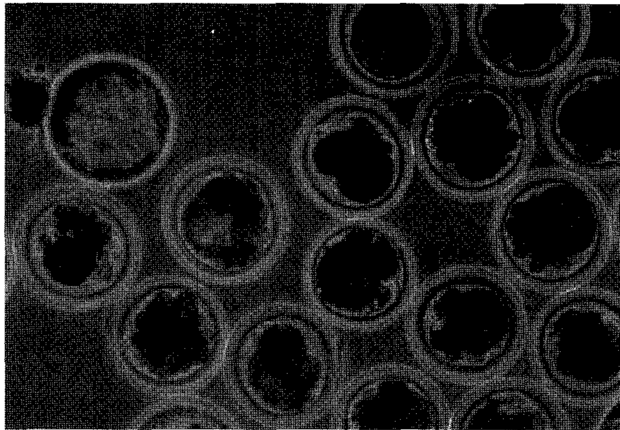
Donor cell (medium)	No. of oocytes	Cleavage rates (mean ± SE)	Morula rates (mean ± SE)	Blastocyst rates (mean ± SE)	Cell No. (mean ± SE)
Porcine FF[1] (PZM-3)	148	74.3 ± 2.31	11.6 ± 2.90[a]	11.5 ± 0.76[a]	28 ± 1.59
Goat FF[2] (PZM-3)	150	67.4 ± 3.37	6.11 ± 1.28[b]	0.74 ± 0.66[b]	16 ± 5.50
Goat FF[3] (mSOF)	143	68.4 ± 6.14	14.0 ± 1.66 [a]	1.40 ± 1.79[b]	17 ± 3.11

[a,b] Within a column, values with different superscripts are significantly different (p<0.05).
[1] Porcine-porcine cloned embryos were cultured in PZM-3 medium.
[2] Goat-porcine cloned embryos were cultured in PZM-3 medium.
[3] Goat-porcine cloned embryos were cultured in mSOF medium.

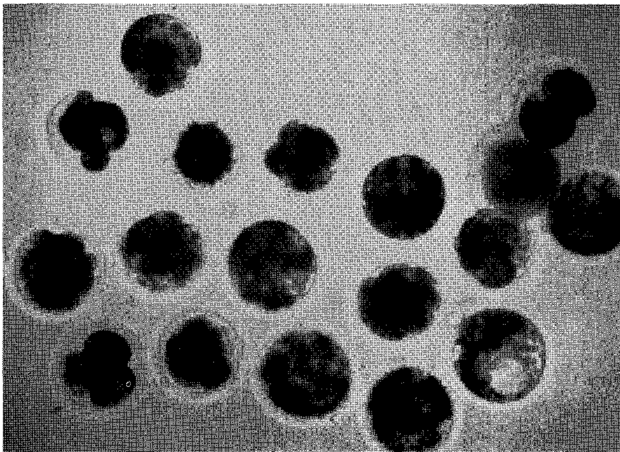
Table 2. ***In vitro*** **development of embryos prepared by interspecies nuclear transfer of porcine and rabbit somatic cells into porcine oocytes**

Donor cell (medium)	No. of oocytes	Cleavage rates (mean ± SE)	Morula rates (mean ± SE)	Blastocyst rates (mean ± SE)	Cell No. (mean ± SE)
Porcine FF (PZM-3)	116	71.6 ± 1.25[ab]	12.9 ± 3.62[b]	11.2 ± 0.75[a]	25 ± 3.50
Rabbit FF (PZM-3)	112	67.1 ± 1.41[b]	6.84 ± 3.10[b]	4.42 ± 0.70[b]	20 ± 6.36
Rabbit FF (RDH)[1]	116	80.4 ± 2.94[a]	38.7 ± 2.92[a]	5.26 ± 1.13[b]	22 ± 2.65

[a,b] Within a column, values with different superscripts are significantly different (p<0.05).
[1] RDH medium was prepared by mixing RPMI1640, DMEM, and Ham's F10 in a 1:1:1 concentration.



(A)



(B)

Fig. 1. Interspecies nuclear transferred embryos. (A) Goat-porcine cloned embryos at day 7 of culture. (B) Rabbit-porcine cloned embryos at day 7 of culture (blastocyst stage).

tration. The percentage cleavage rate of the rabbit-porcine cloned embryos did not differ significantly from that of the porcine-porcine cloned embryos. However, the morular formation rates of the rabbit-porcine cloned embryos cultured in RDH (38.7%) were significantly higher than that of those cultured in PZM-3 (6.84%) and the controls (12.9%). The blastocyst formation rate of the rabbit-porcine cloned embryos was significantly lower than that of the porcine-porcine cloned embryos (4.42 and 5.26% vs. 11.2%, respectively) (Table 2, Fig. 1).

DISCUSSION

Many factors affect the development of cloned embryos, such as oocyte culture periods (Skrzyszowska *et al.*, 2002), cell type, the stages of the donor cell cycle (Miyoshi *et al.*, 2002), and the gender of the donor cell lines (Sansinena *et al.*, 2005). An advantage of using porcine oocytes as the recipient cytoplasm in interspecies cloning is that these oocytes can be easily obtained. In this study, we demonstrated the development of preimplantation goat-porcine, rabbit-porcine embryos prepared by the interspecies nuclear transfer of fetal fibroblast cells (goat and rabbit, respectively) into enucleated porcine oocytes.

In this experiment the goat and rabbit fetal fibroblast cells were less efficacious than the porcine fetal fibroblast cells in supporting the development of embryos reconstructed by their (fibroblasts) nuclear transfer into porcine oocytes. The lower developmental potential of the goat-porcine cloned embryos may be due to their poor quality. This poor quality of the reconstructed embryos could be because the porcine oocyte cytoplasm and goat somatic nucleus are incompatible. However, the development of the reconstructed embryos into morular stage when grown in the culture medium of the donor cell species was higher than if the reconstructed embryos were grown in the culture medium of the recipient species. Further, the percentage of rabbit-porcine cloned embryos grown in RDH medium that developed to the morular stage was higher than that of porcine-porcine and rabbit-porcine cloned embryos grown in PZM-3 medium. This is unlike the results previously reported with iSCNT embryos; iSCNT embryos mostly depend on their recipient cytoplasm during *in vitro* culture (Roh, 2004). Rabbit-porcine cultured embryos cultured in RDH medium may be favorable to development into morular stage and blocked from progressing to the morular stage from the blastocyst stage (Zhao *et al.*, 2006).

Another explanation may be that chromosome number similarity and interactions between the donor and recipient play important roles in determining the timing of maternal-zygotic transition (MZT). The M & Bl (sum of morula and blastocyst) formation rates of rabbit-porcine cloned embryos were higher than those of goat-porcine cloned embryos. It is speculated that chromosome number similarity and interactions between the donor cells and recipient cytoplasm can explain the higher developmental potential of rabbit-porcine cloned embryos. Compared to other species, pig (2n=38) and rabbit (2n=44) have similar chromosome numbers. However, it was not clear whether this chromosome similarity could contribute to the developmental potential of interspecies cloned embryos.

In conclusion, the developmental potentials of interspecies cloned embryos were lower than those of corresponding SCNT embryos. Our data demonstrated that goat-porcine and rabbit-porcine iSCNT embryos can be produced. Further, a higher number of rabbit-porcine cloned embryos developed into morular stage when grown in the culture medium of the donor cell species than when grown in the culture medium of the recipient species. Therefore, it can be speculated that the

developmental potential of reconstructed embryos is affected more by donor cell type than by the enucleated recipient oocyte cytoplasm.

REFERENCES

1. Arat S, Rzucidlo SJ, Stice SL (2003): Gene expression and *in vitro* development of inter-species nuclear transfer embryos. Mol Reprod Dev 66:334-342.
2. Chen D, Wen D, Zhang Y, Sun Q, Han Z, Lui Z, Shi P, Li J, Xiangyu J, Lian L, Kou Z, Wu Y, Chen Y, Wang P, Zhang H (2002): Interspecies implantation and mitochondria fate of panda-rabbit cloned embryos. Biol Reprod 67:637-642.
3. Chen T, Zhang YL, Jiang Y, Liu JH, Schatten H, Chen DY, Sun QY (2006): Interspecies nuclear transfer reveals that demethylation of specific repetitive sequences is determined by recipient ooplasm but not by donor intrinsic property in cloned embryos. Mol Reprod Dev 73:313-317.
4. Dominko T, Mitalipova M, Haley B, Beyhan Z, Memili E, McKusick B, First NL (1999): Bovine oocyte cytoplasm supports development of embryos produced by nuclear transfer of somatic cell nuclei from various mammalian species. Biol Reprod 60: 1496-1502.
5. Gomez MC, Pope CE, Giraldo AM, Lyons L, Harris RF, King A, Cole A, Godke RA, Dresser BL (2004): Birth of African wild cat cloned kittens born from domestic cat. Cloning Stem Cells 6:247-258.
6. Hashem A, Bhandari DP, Kang SK, Lee BC (2007): Cell cycle analysis and interspecies nuclear transfer of *in vitro* cultured skin fibroblasts of the Siberian tiger (*Panthera tigris* Altaica). Mol Reprod Dev 74: 403-411.
7. Janssen DL, Edward ML, Koster JA, Lanza RP, Ryder OA (2004): Postnatal management of cryptordich banteng calves cloned by nuclear transfer utilizing frozen fibroblast cultures and enucleated cow ova. Reprod Fert Dev 16:224 (Abstract).
8. Jin DI, Kim DK, Im KS, Choi WS (2000): Successful pregnancy after transfer of rabbit blastocysts grown *in vitro* from single-cell zygotes. Theriogenology 54:1109-1116.
9. Kitiyanant Y, Saikhun J, Chaisalee B, White KL, Pavasuthipaisit K (2001): Somatic cell cloning in buffalo (*Bubalus bubalis*): Effects of interspecies cytoplasmic recipients and activation procedures. Cloning Stem Cells 3:97-104.
10. Lanza RP, Cibelli JB, Diaz F, Moraes CT, Farin PW, Farin CE, Hammer CJ, West MD, Damiani P (2000): Cloning of an endangered species (*Bos gaurus*) using interspecies nuclear transfer. Cloning 2:79-90.
11. Loi P, Ptak G, Barboni B, Fulka, Jr J, Cappai P, Clinton M (2001): Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. Nat Biotechnol 19:962-964.
12. Miyoshi K, Rzucidlo SJ, Pratt SL, Stice S (2002): Improvements in cloning efficiencies may be possible by increasing uniformity in recipient oocytes and donor cells. Biol Reprod 68:1079-1086.
13. Numchaisrika P, Rungsiwiwut R, Thongphakdee A, Techakumphu M (2004): A preliminary study of the *in vitro* development of asian elephant cloned embryos, reconstructed using a rabbit recipient oocyte. Reprod Fert Dev 16:179-180 (Abstract).
14. Roh S (2004): *In vitro* development of interspecies nuclear transfer embryos: Effects of culture systems. Reprod Dev Biol 28:253-256.
15. Sansinena MJ, Hylan D, Hebert K, Denniston RS, Godke RA (2005): Banteng (*Bos javanicus*) embryos and pregnancies produced by interspecies nuclear transfer. Theriogenology 63:1081-1091.
16. Skrzyszowska M, Katska L, Rynska B, Kania G, Smorag Z, Pienkowski M (2002): *In vitro* developmental competence of domestic cat embryos after somatic cloning: A preliminary report. Theriogenology 58:1615-1621.
17. Techakumphu M, Numchaisrika P, Thongphakdee A, Omsongkram S (2005): Cloned embryos production using bovine adult fibroblast transferred into rabbit oocyte recipient. Thai J Vet Med Suppl 35: 111.
18. Wen D, Yang C, Cheng Y, Li JS, Liu Z, Sun Q, Zang J, Lei L, Wu Y, Kou Z, Chen D (2003): Comparison of developmental capacity for intra- and interspecies cloning cat (*Felis catus*) embryos. Mol Reprod Dev 66:38-45.
19. Yoon JT, Cho EJ, Han KY, Shim H, Roh S (2001): *In vitro* development of embryos produced by nuclear transfer of porcine somatic cell nuclei into bovine oocytes using three different culture systems. Theriogenology 55:298.
20. Zhao ZJ, Ouyang YC, Nan CL, Lei ZL, Song XF, Sun QY, Chen DY (2006): Rabbit oocyte cytoplasm supports development of nuclear transfer embryos derived from the somatic cells of the camel and tibetan antelope. J Reprod Dev 52:449-459.

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