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# Production of rhesus monkey cloned embryos expressing monomeric red fluorescent protein by interspecies somatic cell nuclear transfer

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ABSTRACT

Interspecies somatic cell nuclear transfer (iSCNT) is a promising method to clone endangered animals from which oocytes are difficult to obtain. Monomeric red fluorescent protein 1 (mRFP1) is an excellent selection marker for transgenically modified cloned embryos during somatic cell nuclear transfer (SCNT). In this study, mRFP-expressing rhesus monkey cells or porcine cells were transferred into enucleated porcine oocytes to generate iSCNT and SCNT embryos, respectively. The development of these embryos was studied *in vitro*. The percentage of embryos that underwent cleavage did not significantly differ between iSCNT and SCNT embryos ($P > 0.05$; 71.53% vs. 80.30%). However, significantly fewer iSCNT embryos than SCNT embryos reached the blastocyst stage (2.04% vs. 10.19%, $P < 0.05$). Valproic acid was used in an attempt to increase the percentage of iSCNT embryos that developed to the blastocyst stage. However, the percentages of embryos that underwent cleavage and reached the blastocyst stage were similar between untreated iSCNT embryos and iSCNT embryos treated with 2 mM valproic acid for 24 h (72.12% vs. 70.83% and 2.67% vs. 2.35%, respectively). These data suggest that porcine-rhesus monkey interspecies embryos can be generated that efficiently express mRFP1. However, a significantly lower proportion of iSCNT embryos than SCNT embryos reach the blastocyst stage. Valproic acid does not increase the percentage of porcine-rhesus monkey iSCNT embryos that reach the blastocyst stage. The mechanisms underling nuclear reprogramming and epigenetic modifications in iSCNT need to be investigated further.



## 1. Introduction

Interspecies somatic cell nuclear transfer (iSCNT) is a promising method for therapeutic cloning [1,2], for cloning of endangered animals from which oocytes are difficult to obtain [3,4], and to research nucleo-cytoplasmic interactions [5]. In previous studies in which iSCNT was performed, recipient animals became pregnant [6–9] and offspring were born [10,11]. The rhesus monkey is a primate that is closely related to humans and is a useful experimental model for somatic cell nuclear transfer (SCNT) research and therapeutic cloning. Only a few studies have generated blastocysts by performing iSCNT with monkey cytoplasts or karyoplasts; these studies generated interspecies embryos of cynomolgus monkey with cow [12], rhesus monkey with rabbit [13], and cow with rhesus monkey [14]. However, the percentage of iSCNT embryos that reach the blastocyst stage is extremely low.

The newly generated fluorescent protein marker monomeric red fluorescent protein 1 (mRFP1) is particularly attractive because of its rapid maturation and minimal interference with green fluorescent protein (GFP) and GFP-derived markers. High and ubiquitous expression of mRFP1 does not affect the development, general physiology, or reproduction of transgenic mice produced by SCNT. In transgenic mice of an albino background, mRFP1 can be readily detected in daylight by the unaided eye. Therefore, mRFP1 is an attractive marker for many applications in transgenic research [15]. The *mRFP1* gene can be efficiently transduced into ear fibroblasts of miniature pigs using electroporation, enabling transgenic miniature pigs to be generated that ubiquitously express mRFP1 [16]. *mRFP1* is an excellent selection marker for transgenically modified cloned embryos. Whether an embryo expresses the transgene can be simply determined according to whether it exhibits red fluorescence, which can be ascertained without compromising embryo viability. However, iSCNT has not been reported using mRFP1-expressing rhesus monkey donor cells.

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The efficiency with which embryos are produced by iSCNT is extremely low [17]. Valproic acid (VPA) is a cell-permeable short-chain fatty acid that inhibits histone deacetylases (HDACs). VPA can induce reprogramming of differentiated cells and improves the efficiency of mouse embryonic fibroblast reprogramming [18]. Costa-Borges et al. [19] reported that VPA treatment improves the *in vitro* and full-term development of cloned mouse embryos. VPA treatment increases the proportion of SCNT miniature pig embryos that develop to the blastocyst stage [20,21]. However, the effects of VPA on iSCNT embryos have not been reported.

In this study, rhesus monkey cells were established and electroporated with a plasmid harboring mRFP1, and an mRFP1-expressing rhesus monkey cell line was generated. mRFP1-expressing rhesus monkey cells or porcine cells were transferred into enucleated pig oocytes to generate iSCNT and SCNT embryos, respectively. The development of these embryos was studied *in vitro*. We also determined the effect of VPA on the development of iSCNT embryos.

## 2. Materials and methods

### 2.1. Animals

This research was approved by the ethics committee of Yanbian University.

### 2.2. Chemicals

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise indicated.

### 2.3. Establishment of rhesus monkey cells

Abdominal tissue was obtained from an adult rhesus monkey during trauma treatment at Yanji Zoo (Yanji, China). The tissue was cut into small pieces and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 20% (v/v) fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY), 100 μg/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere of 5% $CO_2$ at 38.5 °C. Cells were observed around the pieces of tissue 3–5 days later, after which the medium was replaced every 2 days until a fibroblast layer was established (7–10 days). The cells were then passaged 3–4 times.

### 2.4. Transfection of pCX-mRFP1-pgk-neoR into rhesus monkey cells

The pCX-mRFP1-pgk-neoR vector was created using the linearized pCX-mRFP1 vector (kindly provided by Dr. Xiaohui Wu, Institute of Developmental Biology and Molecular Medicine, Fudan University, Shanghai, PR China) [15], as we previously described [16].

Electroporation was performed using the Lonza Nucleofector system (Lonza Biologics, Cologne, Germany). Ear fibroblasts (approximately $1.36 \times 10^6$ cells) were electroporated with pCX-mRFP1-pgk-neoR (3 μg linearized DNA) using the AmaxaTM Basic Nucleofector™ for Primary Fibroblasts Kit (VPI-1002, Lonza), program V-026. After electroporation, cells were resuspended in 2 ml of cell culture medium and cultured in 5% $CO_2$ at 38 °C. After 48 h, 200 μg/ml G418 was added to the medium and cells were cultured for a further 12 days to select transfected cells. Dishes were observed under ultraviolet light. Colonies with a high level of uniform fluorescence were picked and transferred to 96-well plates. Cells were cultured in DMEM containing 10% (v/v) FBS in a humidified atmosphere of 5% $CO_2$ at 38.5 °C.

### 2.5. Karyotyping of mRFP1-expressing rhesus monkey cells

mRFP1-expressing rhesus monkey fibroblasts were cultured in DMEM containing 20% (v/v) FBS until they reached 70–80% confluency, after which they were treated with 0.5 μg/ml colcemid for 4 h in 5% $CO_2$ at 38 °C to arrest cell division at metaphase. Arrested cells were treated with a hypotonic solution of 75 μM KCl for 20 min at 37 °C. Swollen cells were fixed in a methanol/acetic acid (3:1) for 30 min at 4 °C, and then centrifuged for 10 min at 1800 rpm. The fixation procedure was repeated three times. In the last repeat, cells were kept in the fixative and placed onto pre-chilled glass slides. Chromosome spreads were kept at 25 °C for 2 days, baked at 65 °C for 4 h, stained with 1% Giemsa solution for 15 min, and imaged.

### 2.6. Preparation of donor cells

mRFP-expressing rhesus monkey fibroblasts used for iSCNT were prepared as described above. mRFP1-expressing porcine fibroblasts were previously generated in this laboratory [16]. After thawing, these porcine cells were cultured in DMEM containing 15% (v/v) FBS until approximately 80% confluent (passage 4), after which SCNT was performed.

### 2.7. In vitro maturation of porcine oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory at 25–35 °C. Antral follicles (2–6 mm in diameter) were aspirated using an 18-gauge needle. Aspirated oocytes that had a uniformly granulated cytoplasm and were surrounded by at least three uniform layers of compact cumulus cells were selected and washed three times in Hepes-buffered NCSU-37 medium containing 0.1% polyvinyl alcohol (PVA). Oocytes were cultured in 4-well plates (Nunc) for 20 h, with each well containing 500 μl of NCSU-37 medium supplemented with 10% porcine follicular fluid, 0.6 mM cysteine, 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP), and 0.1 IU/mL human menopausal gonadotropin (hMG, Teikokuzoki, Tokyo, Japan), followed by culture in the absence of dbcAMP and hMG for a further 18–24 h.

### 2.8. Nuclear transfer

Nuclear transfer was performed as described previously [22]. Briefly, mature eggs that had formed the first polar body were cultured in medium supplemented with 0.4 mg/mL demecolcine and 0.05 M sucrose for 1 h. Sucrose was used to enlarge the perivitelline space. Oocytes with a protruding membrane were moved to medium supplemented with 5 mg/mL cytochalasin B (CB) and 0.4 mg/mL demecolcine, and the protrusion was removed using a beveled pipette. A single donor cell was injected into the perivitelline space of each oocyte, which was then electrically fused using two direct current pulses of 150 V/mm for 50 μs in 0.28 M mannitol supplemented with 0.1 mM $MgSO_4$ and 0.01% PVA. Fused oocytes were cultured in NCSU-37 medium for 1 h, electroactivated, and then cultured in 5 mg/mL of CB-supplemented medium for 4 h. Reconstructed oocytes were activated by two direct current pulses of 100 V/mm for 20 μs in 0.28 M mannitol supplemented with 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$. Activated eggs were cultured in this medium for 6 days in 5% $CO_2$ and 95% air at 39 °C. Finally, blastocysts were placed onto a drop of glycerol/PBS (9:1) containing 20 μg/mL Hoechst 33342 on a microscope slide. A coverslip was placed on top of the blastocysts and the edge was sealed with nail polish. The nuclei were counted under ultraviolet light.

### 2.9. Culture of iSCNT and SCNT embryos

iSCNT embryos were cultured in hamster embryo culture medium with amino acids (HECM-9aa) until they reached the 4–8-cell stage (Day 1). For culture to the blastocyst stage, embryos at the 4–8-cell stage (end of Day 2) were transferred to HECM-9aa medium supplemented with 5% (v/v) FBS, after which the medium was replaced every 2 days.

Activated SCNT embryos were cultured in NCSU-37 medium for 6 days.

### 2.10. Analysis of the level of acetylation on the lysine 9 residue of histone H3 (AcH3/K9) in iSCNT embryos

iSCNT embryos were washed three times with PBS, fixed with PBS containing 4% paraformaldehyde for 30 min, permeabilized with 1% Triton X-100 at 37 °C for 30 min, and blocked in PBS containing 2% bovine serum albumin for 1 h at 37 °C. Embryos were incubated with a primary anti-acetylated lysine antibody (1:200; Upstate Biotechnology, Lake Placid, NY) at 4 °C overnight. Embryos were then incubated with a goat-anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (1:200; Jackson Immuno Research Laboratories Inc., West Grove, PA) for 1 h at room temperature. Stained embryos were mounted beneath a coverslip using antifade mounting medium to retard photo-bleaching. Each experiment was repeated at least three times, and at least five randomly selected embryos were examined per experiment. Slides were imaged using an epifluorescence microscope (IX71 Olympus, Tokyo, Japan) and an appropriate filter to detect fluorescein isothiocyanate.

### 2.11. Experimental design

In Experiment 1, the developmental capacity and mRFP1 expression of iSCNT and SCNT embryos were examined. The proportions of these embryos that underwent cleavage and developed to the blastocyst stage were determined.

In Experiment 2, the effect of VPA on the development of iSCNT embryos was determined. A stock solution of VPA solution was prepared in MilliQ water, which was then diluted in culture medium to a final concentration of 2 mM, as previously described [20]. After activation for 4 h, an equal number of randomly selected iSCNT embryos were incubated in HECM-9aa medium that contained or lacked 2 mM VPA for 24 h. The proportions of embryos that underwent cleavage and developed to the blastocyst stage were determined. Alternatively, to determine the effect of VPA on the level of AcH3/K9, iSCNT embryos were cultured in the absence or presence of 2 mM VPA for 6 h.

### 2.12. Statistical analysis

Each experiment was repeated at least three times. Data expressed as percentages were analyzed using the chi-square test. Numbers of nuclei were analyzed by performing an ANOVA using SAS 6.12 (SAS Institute, Cary, NC, USA).

## 3. Results

### 3.1. Establishment of rhesus monkey cells and generation of an mRFP1-expressing cell line

Pieces of abdominal tissue from a rhesus monkey were cultured *in vitro*. Three days after the tissue had adhered to the culture dish, cells began to migrate away from the tissue. The number of cells that migrated away from the tissue increased over time. These primary cells rapidly proliferated after the medium was changed. The cells were sub-cultured when they reached 90% confluency, and were transfected between passages 4 and 8 (Fig. 1A and B).

Transgenic fibroblasts were generated by transfection of the linearized pCX-mRFP1-pgk-neoR plasmid using electroporation. Stable transfectants were selected using G418 (Fig. 2A and B).

Karyotype analysis demonstrated that 78% of mRFP1-expressing rhesus monkey cells at passage 4 were diploid and had a normal chromosome complement (42 chromosomes) (Table 1, Fig. 2C).

### 3.2. In vitro development of mRFP1-expressing iSCNT and SCNT embryos

mRFP1-expressing rhesus monkey cells or porcine cells were fused with enucleated porcine oocytes to create iSCNT and SCNT embryos, respectively. The percentages of iSCNT and SCNT that underwent cleavage did not significantly differ ($P > 0.05$; 71.53% vs. 80.30%). However, a significantly lower percentage of iSCNT embryos than SCNT embryos developed to the blastocyst stage ($P < 0.05$; 2.04% vs. 10.19%). Most iSCNT embryos completed the first two cleavages normally and became compact at the 8–16-cell stage, at which point development arrested, although some embryos reached the blastocyst stage (Fig. 3, Table 2).

### 3.3. Effect of VPA on the development of mRFP1-expressing iSCNT embryos into blastocysts

The percentages of embryos that underwent cleavage (Day 2) and reached the blastocyst stage (Day 7) did not significantly differ between untreated iSCNT embryos and iSCNT embryos treated with 2 mM VPA for 24 h (72.12% vs. 70.83% and 2.67% vs. 2.35%,

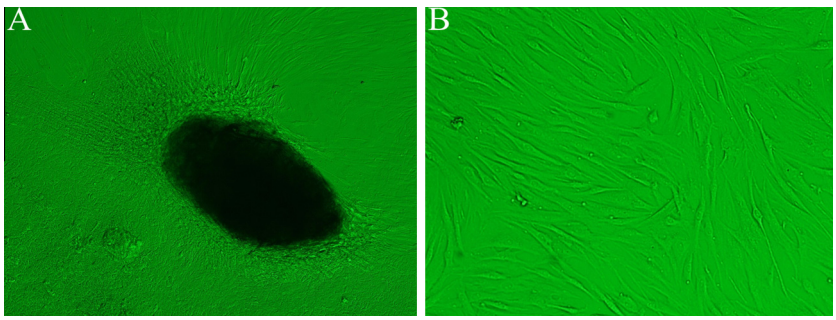


**Fig. 1.** Establishment of rhesus monkey cells. (A) When abdominal tissue derived from a rhesus monkey was cultured *in vitro*, fibroblasts migrated away from the tissue (×40). (B) Primary cells at 90% confluency (×100).

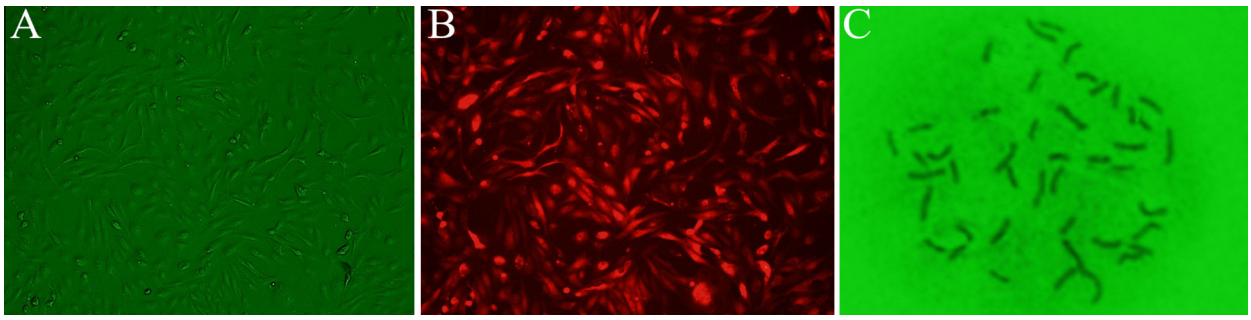


**Fig. 2.** Selection of mRFP1-expressing rhesus monkey cells using G418. (A) Bright-field image of mRFP1-expressing rhesus monkey cells (×100). (B) Red fluorescence in mRFP1-expressing rhesus monkey cells (×100). (C) Karyotype analysis of a rhesus monkey cell showing a diploid number of chromosomes (×600).

**Table 1**
Karyotype analysis of mRFP1-expressing rhesus monkey cells.

| Total number of cells scored | number of cells | | |
|---|---|---|---|
| | Hypodiploid (%) | Diploid (%) | Hyperdiploid (%) |
| 53 | 7 (13.5) | 41 (78.8) | 5 (9.6) |

respectively) (Table 3). The blastocysts produced from VPA-treated and untreated iSCNT embryos contained a similar number of cells.

### *3.4. Effect of VPA on the level of AcH3/K9 in mRFP1-expressing iSCNT embryos*

The level of AcH3/K9 did not markedly differ between iSCNT embryos treated with 2 mM VPA for 6 h and untreated embryos (Fig. 4).

## 4. Discussion

iSCNT is a promising new technology to preserve species and for therapeutic cloning in humans. This study compared the *in vitro* developmental potential of iSCNT and SCNT embryos that were

**Table 2**
*In vitro* development of mRFP1-expressing porcine-rhesus monkey iSCNT embryos and porcine–porcine SCNT embryos.

| Embryo type | No. of embryos cultured | No. of embryos that reached the 2–4-cell stage (%) | No. of embryos that reached the blastocyst stage (%) |
|---|---|---|---|
| iSCNT[c] | 274 | 196 (71.53)[a] | 4 (2.04)[a] |
| SCNT[d] | 269 | 216 (80.30)[a] | 22 (10.19)[b] |

[a,b] Values with different superscripts in the same column are significantly different ($p < 0.05$).
[c] iSCNT, mRFP1-expressing porcine-rhesus monkey iSCNT embryos.
[d] SCNT, mRFP1-expressing porcine–porcine SCNT embryos.

**Table 3**
Effect of VPA on the development of porcine-rhesus monkey iSCNT embryos.

| Treatment | No. of embryos cultured | No. of embryos that reached the 2–4-cell stage (%) | No. of embryos that reached the blastocyst stage (%) |
|---|---|---|---|
| Untreated | 104 | 75 (72.12) | 2 (2.67) |
| VPA[a] | 120 | 85 (70.83) | 2 (2.35) |

These experiments were repeated at least six times.
[a] VPA, Valproic acid.

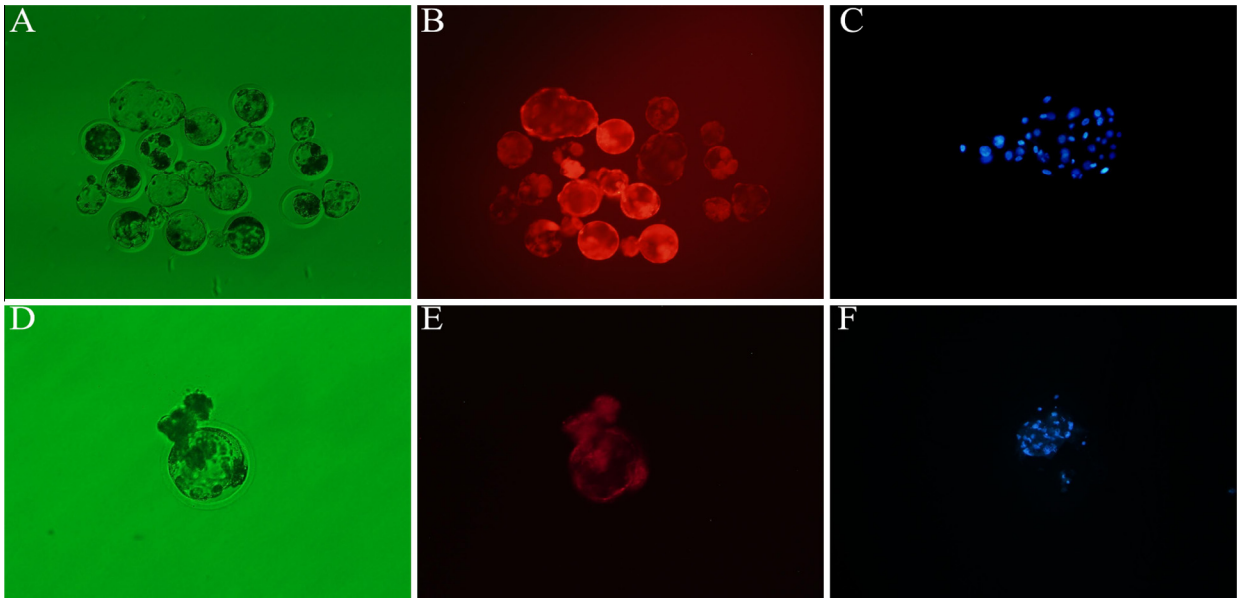


**Fig. 3.** mRFP1-expressing porcine-rhesus monkey iSCNT blastocysts and porcine–porcine SCNT blastocysts. Bright-field images of mRFP1-expressing SCNT (A) and iSCNT (D) blastocysts. Red fluorescence in mRFP1-expressing SCNT (B) and iSCNT (E) blastocysts. mRFP1-expressing SCNT (C) and iSCNT (F) blastocysts stained with Hoechst 33342 and imaged with ultraviolet light (A and B ×100, C–F ×200).

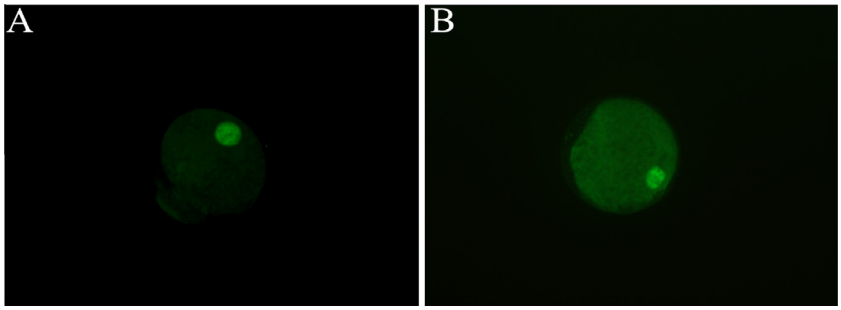


**Fig. 4.** Pattern of AcH3/K9 in porcine-rhesus monkey iSCNT embryos. AcH3K9 in untreated iSCNT embryos (A) and in iSCNT embryos treated with 2 mM VPA for 6 h after activation (B) (×400).

generated by transferring mRFP1-expressing rhesus monkey cells or porcine cells, respectively, into enucleated porcine oocytes. The effect of VPA on the development of iSCNT embryos was also examined.

Rhesus monkey cells were established (Fig. 1) and electroporated with the pCX-mRFP1-pgk-neoR plasmid. The transfection efficiency is higher using electroporation than using a transfection reagent [16]. All cells that survived following culture with 200 μg/ml G418 exhibited red fluorescence (Fig. 2A and B). mRFP1-expressing transgenic intraspecies SCNT embryos have been produced from mice and pigs. When these embryos were transferred into surrogates, the resulting offspring exhibited ubiquitous red fluorescence [16]. This indicates that mRFP1 is a valuable marker to select transgenic embryos. By simply observing red fluorescence, we confirmed that all embryos generated using mRFP1-expressing cells contained cytoplasmic mRFP1 at the 2-cell, 4-cell, and blastocyst stages, and did not exhibit mosaicism (Table 2, Fig. 3D and E). These data indicate that mRFP1 can be used as a marker to select transgenic iSCNT embryos prior to their transfer into surrogates.

Our results also indicate that xenogeneic oocytes can reprogram somatic cells from phylogenetically distant species, and that the resulting embryos can develop to the blastocyst stage *in vitro* (Table 2, Fig. 3D–F). The percentages of embryos that underwent cleavage and developed to the 2–4-cell stage were comparable between iSCNT and SCNT embryos. However, a significantly lower percentage of iSCNT embryos than SCNT embryos developed to the blastocyst stage (Table 2, Fig. 3). The transfer of monkey cells into porcine cytoplasts has not been previously reported. The results of this study are consistent with those showing that enhanced green fluorescent protein (EGFP)-expressing iSCNT embryos have lower development rates than EGFP-expressing intraspecies SCNT embryos [23]. Our data support the hypothesis that mechanisms regulating early embryonic development are conserved among mammalian species [24].

Incomplete donor nuclei reprogramming and abnormal epigenetic reprogramming are thought to be related to the low efficiency of the development of iSCNT cloned embryos [25]. The failure of cloned embryos to develop has been suggested to be linked to abnormal patterns of histone acetylation [26]. VPA treatment improves the *in vitro* developmental rate of cloned intraspecies SCNT embryos of mice and miniature pigs [20–22]. In a study by Kang et al. [20], treatment with 2 mM VPA for 24 h increased the percentage of porcine SCNT embryos that developed to the blastocyst stage. However, this treatment did not affect the development of porcine-rhesus monkey iSCNT embryos in the present study (Table 3, Fig. 4). It is possible that porcine HDACs did not deacetylate rhesus monkey chromatin within the time period studied. Nuclear reprogramming is time-constrained, and genome remodeling must be completed before the embryo genome is activated for successful full-term development [27]. The HDAC inhibitor trichostatin A (TSA) might be able to modify patterns of deacetylation and reacetylation on histone lysine residues in iSCNT cat–cow embryos during a limited period of development [28]. However, in contrast to this previous study, TSA treatment does not improve the *in vitro* development of iSCNT embryos generated using human somatic cells and enucleated rabbit oocytes [29]. Furthermore, TSA does not have any beneficial effects on the full-term development of gaur–bovine iSCNT embryos [11]. These differences in the effects elicited by TSA might be owing to the conditions of TSA treatment (concentration, duration, and experimental design), species-specific effects, and the phylogenetic distance between the enucleated oocyte and the somatic cell donor [28]. Therefore, for VPA to improve the development of iSCNT embryos, treatment conditions might need to be optimized. The low percentage of iSCNT embryos that reach the blastocyst stage indicates that the mechanism underlying nuclear reprogramming in iSCNT needs to be investigated further. Perturbation of this mechanism might underlie the inefficiency of iSCNT cloning.

In conclusion, our results show that porcine-rhesus monkey interspecies cloned embryos can be generated that efficiently express mRFP1. However, a significantly lower percentage of these iSCNT embryos than intraspecies SCNT embryos reach the blastocyst stage. In the future, it will be important to track various fluorescent transgenes and to study interspecies nuclear reprogram mechanisms. VPA cannot improve the developmental rate of porcine-rhesus monkey iSCNT embryos. To improve the efficiency with which iSCNT embryos develop, nuclear reprogramming in iSCNT needs to be studied further.

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