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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 1.36×10^6 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.