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Reprogramming donor cells with oocyte extracts improves in vitro development of nuclear transfer embryos

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ABSTRACT

This study investigated the effects of donor cells pretreated with oocyte extracts on in vitro development of cloned embryos. Bovine fibroblasts were exposed to immature, mature and parthenogenetic oocyte extracts respectively before nuclear transfer. The detectable expression of Oct4 and global deacetylation in the treated cells showed that extracts could reprogram fibroblasts. Although all three groups of extracts exhibited reprogramming capacity, embryo development was not compliant with reprogramming effect. Improved quality and development of blastocysts were observed only in the mature extract treated group. We demonstrated that pretreatment of donor cells with mature oocyte extract improved in vitro development of cloned embryos. Our results suggested that reprogramming donor nuclei to a state synchronized with recipient cytoplasm before nuclear transfer would be beneficial for the development of cloned embryos.

1. Introduction

During development, cells undergo a process of covalent modification of histone or DNA and differentiate to specific cell types. The differentiation state of certain somatic cells can be stably inherited through cell division and proliferation, which lead to epigenetic memory of somatic cells (Jouneau and Renard, 2003). This does not mean that cells have lost their developmental totipotency forever. The historic work by Wilmut et al. (1997) proved that, by somatic cell nuclear transfer (SCNT), highly

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differentiated nucleus could be reprogrammed to a totipotent state and develop into viable offspring. Whereafter, most farm livestock and lab animals have been cloned successfully (Kato et al., 1998; Wakayama et al., 1998; Cibelli et al., 1998; Baguisi et al., 1999; Polejaeva et al., 2000). However, following SCNT, the donor nuclei often fail to express early embryonic and related genes, as also fail to establish a normal embryonic pattern of chromatin modifications (Bird, 2002; Bortvin et al., 2003; Santos and Dean, 2004; Eilertsen et al., 2007). Typically, less than 3% of reconstructed embryos developed into adult animals (Rideout et al., 2001; Wilmut et al., 2002). Relative studies have provided accumulated evidence that this low efficiency was due to incomplete reprogramming of donor nuclei (Bourc'his et al., 2001; Dean et al., 2001; Santos et al., 2003).

The reprogramming mainly comprises the reversal of developmental progress. Therefore, some researches focused on the correlation of different sources and differential stages of donor cells with reprogramming efficiency. Usually, the developmental competence of cloned embryos receiving well-differentiated donor nuclei is inferior to that of those receiving lower differentiated donors. It is shown that embryonic stem cells and the cells in early developmental stage are easier to clone and global hypomethylation of a differentiated cell genome improves cloning efficiency (Wakayama et al., 1999; Humpherys et al., 2001; Wakayama and Yanagimachi, 2001; Hochedlinger and Jaenisch, 2002; Blelloch et al., 2006). Thus, it will be essential to select differentiated cell types and donor populations that are intrinsically more reprogrammable by the oocyte cytoplasm (Solter, 2000). These results reveal that the differentiation and epigenetic state of the donor nuclei can influence reprogramming efficiency.

Some reports suggested that somatic cells could be reprogrammed to a specific state by cytoplasm extracted from various cell populations. Fibroblasts were functionally reprogrammed when exposed to cell extracts from T cells and neuronal cells, which induced expression of hematopoietic and neural specific genes (Hakelien et al., 2002). Recent studies have reported that *Xenopus laevis* egg extracts had the ability to reprogram mammalian somatic cells to express stem cell gene Oct4, and the cells could be partially reprogrammed to an embryonic state. The remodeled cells acquired the capacity of dedifferentiation through cell culture (Byrne et al., 2003; Hansis et al., 2004; Alberio et al., 2005; Miyamoto et al., 2006).

Based on previous work, we now ask whether pretreatment of donor cells with oocyte extracts to a lower differentiated state could improve the quality of reconstructed embryos. If so, what degree to which the donor nuclei are reprogrammed is more suitable for nuclear transfer? To answer the question, we here first incubated donor cells in bovine immature, mature and parthenogenetic oocyte extracts respectively, and then performed the normal nuclear transfer (NT) procedures. We compared Oct4 expression and deacetylation in the treated cells to assess the reprogramming capacity of these extracts. Oct4 (the octamer-binding transcription factor 4) is a key pluripotency specific gene of which the accurate expression is crucial for preimplantation embryo development (Nichols et al., 1998; Pesce and Scholer, 2001). Reactivation of Oct4 expression is a marker of nuclear reprogramming (Daniels et al., 2000; Byrne et al., 2003; Hansis et al., 2004; Miyamoto et al., 2006). Histone acetylation patterns are generally stable and heritable in differentiated cells. High level of histone acetylation induces loose chromatin structure and formation of a transcriptionally activated state (Hong et al., 1993; Lee et al., 1993; Strahl and Allis, 2000). Then we analyzed the correlation between reprogramming effects and the quality and development of blastocysts among three groups of NT embryos from pretreated donor cells compared with normal NT embryos.

2. Materials and methods

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

2.1. Collection and maturation of bovine oocytes

Bovine ovaries were obtained from local slaughterhouses and transported to the laboratory within 4 h after slaughter in sterile 0.9% NaCl saline at 25–30 °C in a thermos bottle. Cumulus oocyte complexes (COCs) were aspired from 2 mm to 8 mm follicles using a 12-gauge needle into phosphate buffered saline (PBS) plus 0.5 IU/ml heparin and 5% (v/v) fetal bovine serum (FBS). COCs with even cytoplasm and surrounded by compact cumulus cells were collected from the follicular fluid and PBS mixture.

The collected COCs were first washed twice and then incubated in TCM-199 (Gibco) supplemented with 10% (v/v) FBS, 1 μg/ml 17β-estradiol and 0.075 IU/ml human menopausal gonadotropin for 20 h at 38.5 °C in 5% CO_2 in air.

2.2. Bovine oocyte extracts preparation

To obtain immature oocytes, COCs were treated with 0.1% bovine testicular hyaluronidase in PBS. COCs were pipetted until the cumulus cells were dispersed. The most oocytes were at germinal vesicle (GV) stage. Respectively, about 400–800 immature, mature metaphase II and parthenogenetic (5 μM ionomycin for 4 min) oocytes were washed three times in serum-free PBS and then digested with 0.5% pronase in serum-free PBS for 3–4 min to remove zona pellucida. Once the zona pellucida disappeared, oocytes were transferred into PBS containing 20% FBS to counteract the effect of pronase. The zona-free oocytes were washed twice in energy regeneration system (ERS: 1 mM ATP, 10 mM phosphocreatine, 25 μg/ml creatine kinase) and transferred into 5 μl ERS in a 0.2 ml Eppendorf tube and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was used as extracts, and stored at −80 °C. The procedure was repeated to acquire sufficient extracts.

2.3. Donor cells preparation

Bovine fibroblasts primary cultures were derived from the ear biopsy of a fetus Holstein cow by mincing the tissues with sterile scissors in a 35 mm Petri dish (Nunc, Denmark). Explants (approximately 1 mm in diameter) were cultured in DMEM (Gibco) containing 20% FBS, 1 mM sodium pyruvate, 100 IU/ml penicillin and 100 mg/ml streptomycin under 5% CO_2 in air at 38 °C. Once cells reached 90% confluence, they were trypsinized and reconstituted at a concentration of 1×10^6 cells/ml. The second to fifth passage of cell line were utilized as nuclei donors.

2.4. Donor cells pretreatment with bovine oocyte extracts

Donor cells were harvested and washed twice in PBS (Ca^{2+}, Mg^{2+} free), spun down at 1000 rpm for 5 min and supernatant was discarded carefully. Cells were suspended in 500 μl PBS (Ca^{2+}, Mg^{2+} free) containing 1.5 U/ml streptolysin-O at 38 °C for 30 min (Walev et al., 2001). Three aliquots of permeable cells (about 1×10^4 cells/aliquot) were respectively resuspended in 10 μl different oocyte extracts and incubated at 38 °C for 30 min. Then the mixture was transferred into culture medium droplets for 24 h under 5% CO_2 in air at 38 °C.

To assess the integrity of chromosomal DNA of donor cells after streptolysin-O treatment, about 1×10^6 treated cells were cultured for 24 h separately. Then donor cell DNA was extracted using Apoptotic DNA Fragment Extraction Kit (Takara) according to user's manual. 5 μl of DNA fragment and genomic DNA were run on 1% agarose gels.

2.5. RT-PCR analysis

First-strand cDNA synthesis of cells was carried out using Cellsdirect cDNA Synthesis System (Invitrogen) according to user's manual. PCR was performed with the primers listed in Table 1 and amplification cycles were as follows: 94 °C × 30 s, 55 °C × 30 s, 72 °C × 2 min by 30 cycles for β-actin

Table 1
Primers used in PCR amplification.

Gene	Primer sequence	Accession no.	Product
β-Actin	Actb1-CGGGAAATCGTCCGTGAC Actb2-CCGTGTTGGCGTAGAGGT	BT030480	277 bp
Oct4	Oct41-CAATTTGCCAAGCTCCTAAAGC Oct42-TCTCACTCGGTTCTCGATACTCG	NM174580	285 bp

and 94 °C × 30 s, 52 °C × 30 s, 72 °C × 2 min by 30 cycles for Oct4. 5 μl of products were then run on 1% agarose gels.

To identify the amplification product, 20 μl of products were recovered from the gel and ligated to pGEM-T-easy (Promega) vector. Sequencing was performed using ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequencing result was aligned with Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The relative quantification of Oct4 expression in three groups of treated cells was determined using gels analysis function in ImageJ software from the National Institutes of Health (http://rsb.info.nih.gov/ij/) by the ratio of Oct4 to β-actin signal intensity.

2.6. Immunofluorescence confocal microscopy

All reagents for fixation, wash and blocking were purchased from Beyotime (Beyotime Institute of Biotechnology, China). Cells were grown on poly-lysine-coated coverslips. Samples were fixed for 1 h at room temperature and permeablized in 0.2% Triton X-100 in PBS for 4 min. Then they were blocked overnight at room temperature. Cells were incubated in acetyl histone H3 (K18) primary antibody (Abcam) overnight. Cells were then washed extensively and incubated in FITC-labeled secondary antibody (Beyotime) for 3 h. Chromatin was stained with 10 μg/ml propidium iodide (PI) for 15 min at room temperature in the dark. Cells were mounted on slides and observed under a confocal laser scanning microscope (Carl Zeiss, LSM 510).

For quantitative analysis of deacetylation of donor cells, fluorescence images were subjected to densitometric analysis using ImageJ software. The value for the cytoplasm region was subtracted as background, and then the pixel value of fluorescence was measured using region of interest (ROI) function. Five different regions were measured, and the relative intensity of fluorescence of donor nuclei acetylation was calculated as a percentage of the fluorescence intensity of PI.

2.7. Nuclear transfer

After maturation, the cumulus cells were dispersed by treatment with 0.1% bovine testicular hyaluronidase in PBS. The mature oocytes were transferred into droplets of PBS supplemented with 7.5 μg/ml cytochalasin B and 10% FBS. Enucleation was performed with a 20 μm (internal diameter) glass pipette by aspirating the first polar body and a small amount of surrounding cytoplasm. The expelled cytoplasm was stained with 10 μg/ml of Hoechst 33342 to confirm that the nuclear material had been removed.

The enucleated oocytes were divided into four groups of recipients. The donor cells and treated donor cells were transferred into perivitelline space of enucleated oocytes. The oocyte–cell couplets were vertically sandwiched by a pair of platinum microelectrodes connected to the micromanipulator in fusion medium (0.27 M mannitol, 0.1 mM $MgSO_4$, 0.5 mM HEPES and 1 mg/ml BSA) droplet. A double electrical pulse of 35 V for 10 μs was applied to conduct fusion. Successfully reconstructed embryos were kept in SOFaa containing 5 μg/ml cytochalasin B for 2 h until activation.

All fused embryos were further activated in 5 μM ionomycin for 4 min followed by 4 h exposure to 1.9 mM 6-dimethylaminopurine in SOFaa. Activated embryos were cultured in SOFaa with 8 mg/ml BSA for 72 h and then transferred to SOFaa supplemented with 10% FBS under 5% CO_2 in air at 38.5 °C for 96 h (7 days from activation).

2.8. Differential staining of blastocysts

The quality of blastocysts was assessed by differential staining of the inner cell mass (ICM) and the trophectoderm (TE) cells according to the procedure of Thouas et al. (2001). Blastocysts (Day 7) were incubated in 500 μl of BSA-free, HEPES-buffered TCM-199 with 1% Triton X-100 and 100 μg/ml PI for 30 s. Then blastocysts were fixed overnight in 500 μl absolute ethanol with 25 μg/ml of Hoechst 33342 at 4 °C. The blastocysts were mounted on glass slides and observed using epifluorescence microscopy. TE cells labeled with PI and Hoechst 33342 were identified by their pink fluorescence and ICM cells labeled with Hoechst 33342 appeared blue.

2.9. Statistical analysis

The nuclear transfer experiments were repeated 3 times. For each group, more than 20 blastocysts were assessed by differential staining of ICM and TE cells. Any blastocysts without dual staining and/or with less than 64 total cell nuclei were excluded from data analysis. All data were analyzed by one-way ANOVA using Sigmastat3.5 (Systat Software, Inc., USA). Differences between experimental groups were considered significant at $P < 0.05$.

3. Results

3.1. Integrity of donor nuclei after streptolysin-O treatment

After streptolysin-O treatment, chromosomal DNA of donor cells was extracted. No sheared DNA fragments were detected in streptolysin-O treated cells as well as in control donor cells (Fig. 1). It showed that streptolysin-O treatment had no damage to chromosomal DNA of donor cells.

3.2. Reprogramming of donor cells

After bovine fibroblast cells were exposed to immature, mature and parthenogenetic oocyte extracts respectively, we analyzed the expression of pluripotent marker gene Oct4 and the global level of histone acetylation for assessing the changes in epigenetic state of the treated cells. After culture for 24 h, detectable Oct4 expression while faint was observed in the treated cells (Fig. 2). The alignment of sequencing result has a complete matching in BLAST database. Deacetylation of histone H3 at lysine 18 was also detected in extracts treated cells, suggesting that reprogramming could be induced by oocyte extracts (Fig. 3).

3.3. In vitro development of NT embryos and three groups of NT embryos from pretreated donor cells

Pretreatment of donor cells with immature, mature and parthenogenetic oocyte extracts affected in vitro development of embryos derived from these cells (Table 2). Donor cells pretreated with mature oocyte extracts had the best effect on improvement of blastocyst rate in three treated groups compared with NT control. It is surprising to find that the parthenogenetic oocyte extract treated

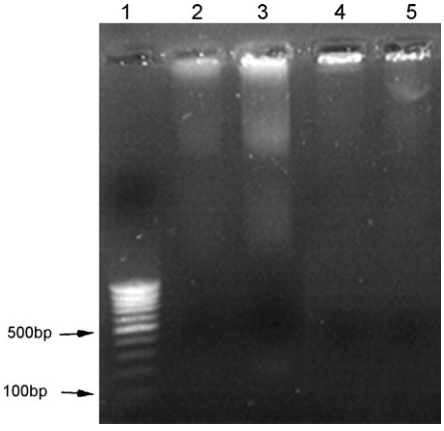

Fig. 1. Integrity of donor nuclei after streptolysin-O treatment. DNA fragments and genomic DNA extracted from untreated and streptolysin-O treated donor cells were assessed by agarose gel electrophoresis. Lane 1 was 100 bp marker. Lane 2 and lane 4 were DNA fragments and genomic DNA extracted from untreated donor cells, respectively. Lanes 3 and 5 represented DNA fragments and genomic DNA from the treated cells, respectively.

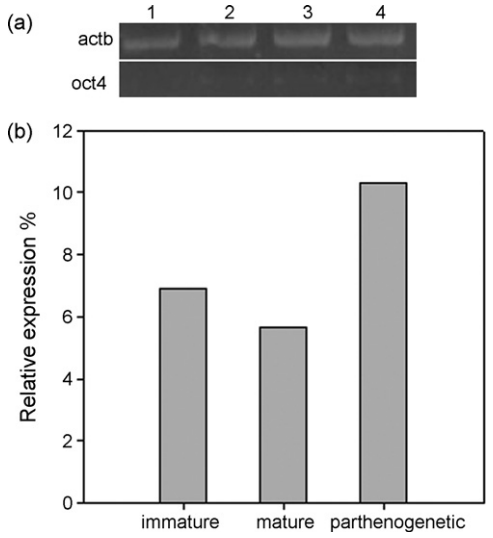

Fig. 2. Oct4 reactivation by oocyte extracts in donor cells. (a) RT-PCR amplified β-actin and Oct4 from untreated and oocyte extracts treated donor cells. Lane 1 represented the cDNA from untreated donor cells. Lanes 2–4 represented the cDNA from donor cells treated with immature, mature and parthenogenetic oocyte extracts, respectively. (b) Relative quantification of Oct4 expression by the ratio of Oct4 to β-actin signal intensity in donor cells treated with immature, mature and parthenogenetic oocyte extracts.

group, despite the efficient reprogramming effect, showed only a slight increase in development into blastocysts.

3.4. Differential counting of ICM and TE cells

The number of ICM and TE cells in Day 7 blastocysts was counted on by differential staining. As shown in Table 3, a significant increase in total and ICM cell number was observed in blastocysts reconstructed with mature oocyte extract treated cells compared to the other groups. No significant differences were observed in TE cell number and the ratio of ICM to total cells.

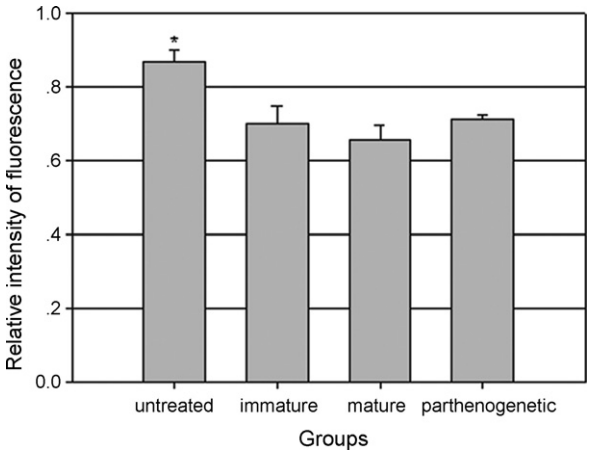

Fig. 3. Deacetylation in oocyte extracts treated donor cells. *Significant difference ($P < 0.05$) of the acetylation levels between untreated donor cells and oocyte extracts treated donor cells. Bars indicate standard error.

Table 2
In vitro development of NT embryos and three groups of NT embryos derived from donor cells pretreated with immature, mature and parthenogenetic oocyte extracts.

Origin of embryos	No. (% of oocytes)		No. (% of fused) of embryos developed to		
	Oocytes	Oocytes fused	2-cell	Morula	Blastocyst
NT	144	116 (80.6)	91 (78.4)	55 (47.4)	33 (28.4)[a]
Immature-treated	142	113 (79.6)	85 (75.2)	54 (47.8)	32 (28.3)[a]
Mature-treated	150	118 (78.7)	96 (81.4)	64 (54.2)	48 (40.7)[b]
Parthenogenetic-treated	141	112 (79.4)	86 (76.8)	55 (49.1)	33 (29.5)[a]

The development of reconstructed embryos to 2-cell, morula and blastocyst stage was respectively evaluated at 48 h, 120 h and 168 h after activation. Within the same column, values with different superscripts are significantly different ($P < 0.05$).

Table 3
Total cell number, ICM, TE cell number, and the ratio of ICM to total cell (mean ± S.E.M.) in Day 7 blastocysts.

Origin of embryos	Total	ICM	TE	ICM:total
NT	94.5 ± 3.0[a]	27.5 ± 1.4[a]	67.0 ± 2.4	0.291 ± 0.011
Immature-treated	96.0 ± 2.3[a]	29.0 ± 1.5[a]	67.0 ± 1.8	0.301 ± 0.012
Mature-treated	110.1 ± 3.3[b]	35.5 ± 1.5[b]	74.7 ± 2.6	0.323 ± 0.011
Parthenogenetic-treated	98.8 ± 2.4[a]	28.5 ± 1.4[a]	70.3 ± 1.7	0.287 ± 0.010

Within the same column, values with different superscripts are significantly different ($P < 0.05$).

4. Discussion

In the present study, increased blastocyst formation rates were observed in three treated groups of cloned embryos. It is virtually unknown about the mechanisms underlying the improved efficiency and quality of NT embryos from pretreated donor cells. It is now commonly accepted that epigenetic mechanism plays an important role in developmental competence of reconstructed embryos (Solter, 2000; Rideout et al., 2001; Bird, 2002; Wade and Kikyo, 2002; Dean et al., 2003; Simonsson and Gurdon, 2004). The possible reason for our results may be as follows: (1) Oocyte extracts have changed chromatin structure of donor nuclei so that recipient cytoplasm was easier to reprogram them. It was pointed out that partial erasure of preexisting epigenetic marks of donor cells by trichostatin A, an inhibitor of histone deacetylase, could improve subsequent in vitro development of cloned embryos (Enright et al., 2003). (2) The prolonged exposure of donor nuclei to reprogramming factors provided sufficient time for reprogramming. In our laboratory, we have obtained five generations of cloned embryo goats by serial nuclear transfer, a strategy that can extend the duration of donor nuclei exposure to ooplasm (Zhang and Li, 1998). In mouse cloning, developmental competence of reconstructed embryos was also significantly improved by serial nuclear transfer (Heindryckx et al., 2002).

In some ways, the above explanations are right but not complete. Does reprogramming of donor nuclei to a lower state surely enhance developmental potential of reconstructed embryos? We observed that all immature, mature and parthenogenetic oocyte extracts had the ability to reprogram donor cells. However, immature and parthenogenetic oocyte extracts had only limited capacity in improvement of in vitro developmental competence of cloned embryos and the quality of blastocysts compared to mature oocyte extract. This might result from nuclear–cytoplasmic interaction. Nuclear transfer in sheep embryos showed that cell cycle coordination between nucleus and cytoplasm resulted in the birth of lambs (Liu et al., 1997). In vitro developmental rates of bovine nuclear transfer embryos to the blastocyst stage were dependent on the stage of the cell cycle of donor cells and recipient cytoplasts (Kurosaka et al., 2002). However, some reports showed that cell cycle had limited effect on dedifferentiation and reprogramming of donor nuclei (Wakayama et al., 1999; Kasinathan et al., 2001). The previous reports focused on cell cycle synchronization between nucleus and cytoplasm. The present study suggests that it is synchronization between the epigenetic state of donor nuclei and recipient cytoplasm that plays a more important role in nuclear–cytoplasmic interaction. Therefore, either erasure of preexisting epigenetic marks or prolonged duration of exposure to reprogramming factors is only one aspect of the problem. Our results imply that, rather than reprogramming

donor nuclei to a lower differentiation state, reprogramming them to a state coordinate with recipient cytoplasm would be beneficial for in vitro development of cloned embryos.

5. Conclusion

Our results here provide evidence that pretreatment of donor cells with oocyte extracts can improve in vitro developmental potential of reconstructed embryos. In three groups of extracts, mature oocyte extract makes the epigenetic state of donor nuclei conform to the environment of recipient cytoplasm, and thus has a favorable effect on the development of nuclear transfer embryos.

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