Exposure of mouse cumulus cell nuclei to porcine ooplasmic extract eliminates TATA box protein binding to chromatin, but has no effect on DNA methylation

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Received: 9 October 2006 / Accepted: 6 November 2006 / Published online: 7 December 2006
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Abstract *Purpose:* The low cloning efficiency with SCNT is due to incomplete or partial reprogramming of the donor somatic cell nuclei after microinjection into the enucleated oocyte. A possible solution may be to initiate nuclear reprogramming prior to SCNT.

Methods: Pre-exposure of donor somatic cell nuclei to a novel porcine ooplasmic extract prior to microinjection could possibly extend the duration of exposure to ooplasmic nuclear reprogramming factors. The effects of the porcine ooplasmic extract on two major markers of nuclear reprogramming: (1) TATA box protein binding to chromatin and (2) DNA methylation was investigated.

Results: The results showed that pre-exposure of mouse cumulus cell nuclei to porcine ooplasmic extract drastically reduced TATA box protein binding to chromatin, but had no effect on DNA methylation.

Conclusions: Pre-exposure to the porcine ooplasmic extract had some limited effects on nuclear reprogramming. Whether this can lead to enhanced cloning efficiency needs to be further investigated.

Keywords Methylation · Nuclear reprogramming · Ooplasm · Porcine · TATA box

Introduction

In recent years, mammalian cloning through somatic cell nuclear transfer (SCNT) into enucleated oocytes has emerged

as intensive field of research, which holds much promise for numerous research and therapeutic applications. These includes the multiplication of unique animal genotypes and preservation of endangered animals [1], therapeutic cloning for the production of isogenic embryonic stem cells [2] and the production of transgenic knock-in or knock-out domestic livestock and laboratory animals [3–5]. Additionally, SCNT is also emerging as a powerful tool in basic scientific research that may aid our understanding of early developmental events [6] and the cell dynamics of cancer [7].

However, a major bottleneck of this technology is the relatively low cloning efficiency that has been reported by virtually all studies to date [8]. The success of SCNT depends on several parameters that impact on the ability of the ooplasm to reprogram the nucleus of the donor cell, and to reverse the epigenetic changes that occur during development [9]. One of these parameters has been proposed to be the limited duration of exposure of the donor nuclei to reprogramming factors present within the ooplasm [10, 11], which could in turn lead to incomplete or partial nuclear reprogramming, and hence compromised developmental competence of the cloned embryos.

Hence, this study investigated a possible novel strategy to enhance the cloning efficiency of SCNT – through pre-exposure of donor somatic cell nuclei to a novel porcine ooplasmic extract prior to microinjection. This would extend the duration of exposure of the donor nuclei to ooplasmic reprogramming factors which may lead to more complete nuclear reprogramming, and hence possibly enhance the cloning efficiency of SCNT. The effects of the porcine ooplasmic extract on two major markers of nuclear programming: (1) TATA box protein (TBP) binding to chromatin [12] and (2) DNA methylation [13] would be investigated in this study. Mouse cumulus cells would be utilized for pre-exposure to the porcine ooplasmic extract, since these

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are the most commonly used donor cell-type in SCNT research.

Materials and methods

Ethical approval for the use of animals in scientific research

All experiments using animals were in accordance with the International Guiding Principle for Biomedical Research Involving Animals and all experimental protocols were approved by the Ethics Committee for Experimental Animals of the National University of Singapore.

Culture media, reagents, chemicals and labware

Unless otherwise stated, all culture media, reagents and chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Human recombinant follicular stimulating hormone (FSH, Gonadotropin-Releasing HormoneTM) and human chorionic gonadotrophin (hCG, ProfasiTM) were obtained from Serono Inc. (Aubonne, Switzerland). Tissue culture medium 199 (M199) was obtained from Gibco BRL, Inc. (Gaithersburg, MD, USA). The four-well dishes used in all experiments were purchased from Nunc Inc. (Roskilde, Denmark).

Collection of porcine germinal vesicle stage oocytes and subsequent in vitro maturation

Collection of porcine germinal vesicle (GV) stage oocytes and subsequent maturation to the metaphase II (MII) stage was carried out as described previously [14]. Ovaries were collected from slaughtered pig carcasses (Landrace × Large White × Duroc, 6 to 8 month of age, 75 to 115 kg of body weight) in a local abattoir and washed in PBS supplemented with 50 g/L of penicillin and 75 g/L of streptomycin. The ovaries were maintained at 30 to 35°C in phosphate buffered saline (PBS) during transport to the laboratory. Cumulus oocyte complexes (COC) were aspirated from antral follicles (3 to 7 mm diameter) using a beveled 16-gauge needle fixed to a 10-mL syringe. Only COC with at least three layers of granulosa cells were selected. These were washed four times in M199 supplemented with 20 mM HEPES, 1 g/L polyvinylalcohol, 50 g/L of penicillin, and 75 g/L of streptomycin, to remove all debris and blood. Oocyte maturation in vitro was carried out in four-well Nunclon dishes containing 0.5 mL of equilibrated culture medium overlaid with mineral oil (embryo tested). The culture medium used was M199 supplemented with 10% (vol/vol) follicular fluid, 1 mM glutamine, 0.03 mM sodium pyruvate, 0.1 IU/mL of FSH, 0.5 IU/mL of hCG, 0.57 M cysteine, 50 g/L of penicillin, and 75 g/L of streptomycin. After 36 h of in vitro

culture in a 5% CO₂ incubator set at 39°C, the COC were denuded by repeated pipetting with 80 IU/mL of hyaluronidase in 20 mM HEPES buffered M199.

Assessment of nuclear maturation

Completely denuded oocytes were stained with 10 g/mL of bisbenzimidazole (Hoechst 33342) for 5 min and viewed under UV light with a Hoffman-modulation contrast microscope at 100× magnification. Two distinct spots of fluorescence would be observed for mature metaphase II (MII) stage oocytes. These corresponded to the nucleus of the first polar body and the chromosomes of the MII spindle of the mature oocyte.

Preparation of porcine oocyte extract (POE)

400 to 600 in vitro matured cumulus-denuded mature MII porcine oocytes were washed through three droplets of PBS medium and finally suspended in 5 µL of energy regeneration system (ERS, 1mM ATP, 10 mM creatine phosphate, 25 µg/mL creatine kinase, 100 µM GTP). These were briefly spun down and excessive solution was discarded, followed by resuspension in 1.5 µL of ERS. Rupturing of the oocytes was achieved through the use of a fine glass pipette, and the porcine oocyte extract (POE) was kept on ice until permeabilized mouse cumulus cells were ready for co-incubation.

Collection of mouse cumulus cells

Mature MII oocytes were collected from gonadotrophin-primed 4 to 6 week-old C57CBA F1 female mice, which were acclimatized under controlled temperature (25°C), lighting (lights on from 7 am to 7 pm) and humidity (50–70%) for at least one week before use. Gonadotrophin priming was achieved by intraperitoneal injection of 10 IU pregnant mare's serum gonadotrophin (FolligonTM; Intervet Inc., Australia) followed by 10 IU human chorionic gonadotrophin (ChorulonTM; Intervet Inc., Australia) intraperitoneal injection 48 h later to induce ovulation. The stimulated female mice were then sacrificed by neck dislocation 12 to 14 h after human chorionic gonadotrophin administration. Fallopian tube enclosed cumulus oocyte complexes were dissected and brought to the laboratory in 25 mM pre-warmed Hepes buffered CZB medium. After going through one wash in 1.5 ml Hepes buffered CZB medium the oocyte cumulus complexes were released from fallopian tubes by puncture with a 22 gauge injection needle. The cumulus cells were stripped off by incubation of the oocyte cumulus complexes in 80 IU/mL hyaluronidase for 4–5 min. The released cumulus cells were then collected, spun down by centrifugation (1500 rpm for 2 mins) and resuspended in PBS.

Mouse cumulus cell permeabilization and treatment with porcine oocyte extract

Mouse cumulus cells were washed in 1.0 ml of Ca^{2+} -free PBS medium and then spun down at 1500 rpm for 2 min. Supernatant was discarded and 100 μL of Ca^{2+} -free PBS medium containing 4000 IU/mL Streptolysin O (Sigma-Aldrich Inc., St. Louis, MO, USA) was added to the cumulus pellet. Cumulus cells in streptolysin O were incubated at 38.5°C for 45 min to achieve permeabilization. Permeabilized cumulus cells were spun down at 1500 rpm for 2 min and the supernatant was discarded. 1.5 μL POE in ERS was then added and co-incubated for 45 min before fixing the cells for immunocytochemical analysis.

Immunocytochemical staining to detect TATA box protein binding to chromatin after pre-exposure of mouse cumulus cells to the porcine oocyte extract

POE-treated and non-treated cumulus cells were washed in PBS, fixed for 15 min in 4% paraformaldehyde in PBS, and permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature. For immunocytochemical staining, the cells were incubated with the first antibody (mouse monoclonal anti-TATA box protein antibody) for 1 h at 37°C , before being washed in PBS and incubated in blocking medium (0.1 M glycine, 1% goat serum, 0.01% Triton X-100, 1% skim milk, 0.05% BSA, 0.02% sodium azide, and PBS) for 1 h at 37°C . Subsequently, the cells were incubated with the second antibody (FITC-conjugated rabbit anti-mouse antibody, 1:200 dilution) at 37°C for 1 h and then washed in PBS. Nuclear DNA was stained with propidium iodide.

Immunocytochemical staining to determine the level of DNA methylation after pre-exposure of mouse cumulus cells to the porcine oocyte extract

POE-treated and non-treated cumulus cells were washed in PBS, fixed for 15 min in 4% paraformaldehyde in PBS, and permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature. This was followed by treatment with 2 M HCl at room temperature for 30 min and subsequent neutralization with 100 mM Tris/HCl buffer (pH 8.5) for 10 min. After extensive washing with 0.05% Tween 20 in PBS, the cells were blocked overnight at 4°C in 1% BSA, 0.05% Tween 20 in PBS, to prevent non-specific binding. Specific binding of Anti-5-methyl-cytosine (5-MeC) antibodies [15] to the fixed cells were detected by a secondary antibody coupled to FITC (Jackson Immuno-research Inc., USA), while DNA was stained with propidium iodide (PI).

Results

Effects of pre-exposure to porcine oocyte extract on TATA box protein binding to chromatin

After incubation with POE for one hour, the permeabilized cumulus cells were subjected to immunocytochemical staining to determine whether TBP in cumulus nuclei were removed by reprogramming factors present within the POE. It was observed that TBP was completely removed from the pre-exposed cumulus nuclei, as seen in Fig. 1d to f. Figure 1a to c showed permeabilized cumulus cells which were not exposed to POE that served as the negative control. The non POE-treated cells were positively stained with anti-TBP antibody (bright green fluorescence) thereby indicating the presence of TBP binding to chromatin (Fig. 1a). Figure 1b showed the non-treated cumulus nuclei being stained with PI (red fluorescence), while figure 1c showed the corresponding cells observed under bright-field microscopy. Figure 1d to f showed permeabilized cumulus cells that were pre-exposed to POE. TBP was absent from POE-treated cumulus nuclei (Fig. 1d) and were consequently unstained by the anti-TBP antibodies. Figure 1e showed the POE-treated cumulus nuclei being stained with PI (red fluorescence, while figure 1f showed the corresponding bright-field microscopy image.

Effects of pre-exposure to porcine oocyte extract on DNA methylation

POE treatment of permeabilized cumulus cells was not able to demethylate 5-methyl cytosine on nuclear DNA, as seen in Fig. 2. Figures 2a to c presented images of cells not exposed to POE that served as negative controls. Figure 2a showed bright-field microscopy images of non-treated cumulus cells, Fig. 2b showed cells being positively stained with FITC-conjugated 5-M-C antibody (green fluorescence), while Fig. 2c showed PI stained nuclei (red fluorescence). Figure 2d to f showed images of cumulus cells which were exposed to POE treatment. In Fig. 2f, it was clearly seen that 5-methyl cytosine was not demethylated by POE treatment. Hence, it can be concluded that POE treatment had no significant effect on DNA methylation.

Discussion

Although there have been numerous reported successes of SCNT in the cloning of several mammalian species such as sheep, goat, mouse, cattle, pig, rabbit, cat, mule and horse, the efficiency of SCNT is universally low. Generally, less than 3% of reconstituted embryos develop to term to yield viable offsprings, regardless of the species or the technology being applied [8, 9]. The overwhelming majority of cloned

Fig. 1 TATA box protein binding to chromatin was removed by pre-treatment of mouse cumulus cell nuclei with porcine oocyte extract. **a–c:** negative control – cumulus nuclei not exposed to porcine oocyte extract treatment showed FITC-conjugated TBP antibody staining. **d–f:**

cumulus nuclei exposed to porcine oocyte extract treatment showed absence of FITC-conjugated TBP antibody staining. Green fluorescence: FITC-conjugated TBP antibody staining. Red fluorescence: Nuclear DNA stained with propidium iodide

Fig. 2 DNA methylation was not significantly affected by pre-treatment of mouse cumulus cell nuclei with porcine oocyte extract. **a–c:** negative control – cumulus nuclei not exposed to porcine oocyte extract treatment showed FITC-conjugated 5-methylcytosine antibody staining. **d–f:** cumulus nuclei exposed to porcine oocyte extract treatment

also showed similar levels of FITC-conjugated 5-methylcytosine antibody staining. Green fluorescence: FITC-conjugated 5-methylcytosine antibody staining. Red fluorescence: Nuclear DNA stained with propidium iodide

embryos arrest at the cleavage stage before implantation or abort soon after implantation. Even those pregnancies that do survive to term, very often yield phenotypically or genetically defective live-born that die soon after birth [8, 9].

The low efficiency of cloned animal production with SCNT has been postulated to be the result of an incomplete reprogramming of the donor somatic cell nucleus within the enucleated oocyte [9, 10]. This would in turn lead to a lack of, or aberrant expression of developmentally important genes. Indeed, this has been validated by several studies. Daniels et al. [16] found that a number of morula- and blastocyst-stage embryos derived from bovine SCNT showed abnormal transcription of IL6, FGF4, and FGF2. Bortvin et al. [17] analysed expression of Oct4 and 10 Oct4-related genes in individual cumulus cell-derived cloned mouse blastocysts, and found that only 62% of cloned embryos correctly expressed all tested genes. In contrast to this incomplete reactivation of Oct4-related genes in somatic clones, ES cell-derived cloned blastocysts and normal control embryos displayed normal expression of these genes. Notably, the contrast between expression patterns of the Oct4-related genes appear to correlate with the discrepancy in developmental competence of somatic and ES cell-derived cloned blastocysts to term.

The extremely low efficiency of SCNT [8] poses a major obstacle for further application either to reproductive or therapeutic cloning. Hence, it is imperative to develop new techniques for improving the efficiency of SCNT. A possible novel approach would be to look at pre-treating somatic donor nuclei with ooplasmic extracts, prior to transfer into the enucleated oocyte. The rationale is that this would extend the duration of exposure of the somatic donor nuclei to ooplasmic reprogramming factors, leading to more complete nuclear reprogramming, which could in turn enhance the cloning efficiency of SCNT.

Indeed, a number of previous studies would give credence to such a novel approach. It was reported that serial nuclear transplantation, which extends the duration of exposure of the somatic cell nuclei to ooplasmic reprogramming factors, significantly enhanced the degree of nuclear reprogramming, as evidenced by the improved developmental competence of cloned embryos [10, 11]. Hansis et al. [18] demonstrated that extracts from *Xenopus laevis* eggs and early embryos upregulated the expression of both Oct4 and germ cell alkaline phosphatase (GCAP) in 293T cells and human primary leukocytes. However, the 'reprogrammed' leukocytes was found to have a limited life span and failed to express surface antigens characteristic of pluripotent cells, indicating incomplete nuclear reprogramming [18]. In another study, Kikyo et al. [19] reported active remodeling of mammalian somatic nuclei in egg cytoplasm by the nucleosomal ATPase ISWI. It was found that ISWI actively erases TATA box protein association with the nuclear matrix. Cytoplasmic extracts of mammalian somatic cells have also been reported to possess

the capacity for nuclear reprogramming. Hakelien et al. [20, 21] demonstrated functional reprogramming of both a somatic cell line (293T fibroblasts) and primary skin fibroblasts through the use of cytoplasmic extracts derived from T cells and neuronal precursors. Healthy calves were produced by chromatin transfer after re-programming in a mitotic cell extract to promote the removal of nuclear factors solubilized during chromosomal condensation [22]. Hence, it would be conceivable to utilize extracts of mammalian oocytes to pre-program donor somatic cell nuclei prior to nuclear transfer, so as to improve the efficiency of SCNT. Indeed, this has been proposed for some time. Nevertheless, there has not yet been any successful study that has been reported to date.

In this study, in vitro matured porcine oocytes were utilized to produce the cytosolic extract for reprogramming donor somatic cell nuclei. This is because immature porcine oocytes at the GV stage are readily available in large numbers from the ovaries of post-slaughtered sow carcasses in a local abattoir [14]. Upon maturation in vitro, these would then provide an abundant source of ooplasm. Compared to oocytes obtained from gonadotrophin-stimulated laboratory animals i.e. mice, hamsters; this would certainly be a much more economical and practical alternative, given the time and resource constraints of this study.

To assay the effects of the porcine oocyte extract on nuclear reprogramming of mouse cumulus nuclei, two major markers of nuclear programming: would be assayed: (1) TATA box protein (TBP) binding to chromatin [12] and (2) DNA methylation [13].

TBP is a fundamental component of the general transcription machinery required to correctly initiate the transcription of ribosomal, messenger, small nuclear and transfer RNAs, by all three RNA polymerases [23]. Hence, at the zygotic and early embryonic stages when the genome is transcriptionally quiescent, the chromatin would be expected to be free of association with TBP [12]. Indeed, it was reported by Worrad and Schultz [12] in the mouse model that transcripts for TBP decreases during oocyte maturation and reaches a minimum level at the two-cell stage, after which time the abundance of these transcripts increases progressively to the blastocyst stage. This concurs with transcriptional activation of the murine embryonic genome at the two-cell stage [24].

DNA methylation is another useful marker of nuclear reprogramming [13]. It is a major epigenetic modification of the genome that regulates crucial aspects of its function. Genomic DNA methylation patterns in differentiated somatic cells are generally stable and heritable. However, in mammals there are at least two developmental stages: (1) germ cells and (2) preimplantation embryos, in which DNA methylation patterns are reprogrammed globally, for the purpose of generating cells with a broad developmental potential. It is thought that reprogramming through global changes in DNA methylation re-establishes nuclear totipotency through

the erasure of acquired epigenetic information. Typically, a substantial part of the genome is demethylated during early development, and after some time remethylated, in a cell- or tissue-specific pattern [25], as development progresses. A stepwise passive loss of DNA methylation in the embryonic nucleus has been observed as DNA replicates between the two-cell and morula stages, with somatic levels of methylation being re-established by, or after the blastocyst stage when differentiated lineages are formed [26]. During SCNT, demethylation of DNA is absolutely necessary for the epigenetic reprogramming of somatic cell nuclei [13].

The results of this study showed that pre-exposure of mouse cumulus cell nuclei to the porcine oocyte extract drastically reduced TBP binding to chromatin, implying that enzymes responsible for TBP removal in porcine oocytes retain their biochemical activity, despite the relatively harsh procedures used in extract preparation. This is consistent with the study of Kikyo et al. [19], which demonstrated that cytoplasmic extracts of *Xenopus* eggs possessed the ability to erase TBP association with the nuclear matrix of mammalian somatic cells. Nevertheless, their study was based on non-mammalian egg cytoplasm. Our findings therefore complement their data, by demonstrating that mammalian ooplasmic extract possesses a similar ability to eliminate TBP association with chromatin.

By contrast to TBP binding, our results showed that pre-exposure of mouse cumulus cell nuclei to the porcine oocyte extract had no significant effect on DNA methylation. Nevertheless, a number of SCNT studies had previously demonstrated a high degree of demethylation of genomic DNA, upon microinjection of the donor somatic nuclei into the enucleated oocyte [13, 27, 28]. This would indicate that demethylating enzymes present within the porcine oocyte are probably damaged during preparation and processing of the ooplasmic extract.

Future studies would investigate whether pre-exposure of donor somatic nuclei to the porcine oocyte extract could translate to enhanced developmental competence of the generated cloned embryos, upon subsequent nuclear transfer into enucleated oocytes. If this is the case, then pre-exposure of donor somatic nuclei to mammalian ooplasmic extracts prior to nuclear transfer, could represent a novel strategy for improving SCNT efficiency.

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