Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Cumulus-specific genes are transcriptionally silent following somatic cell nuclear transfer in a mouse model*

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Received Apr. 28, 2007; revision accepted May 31, 2007

Abstract: This study investigated whether four cumulus-specific genes: follicular stimulating hormone receptor (FSHr), hyaluronan synthase 2 (Has2), prostaglandin synthase 2 (Ptgs2) and steroidogenic acute regulator protein (Star), were correctly reprogrammed to be transcriptionally silent following somatic cell nuclear transfer (SCNT) in a murine model. Cumulus cells of C57×CBA F1 female mouse were injected into enucleated oocytes, followed by activation in 10 μmol/L strontium chloride for 5 h and subsequent in vitro culture up to the blastocyst stage. Expression of cumulus-specific genes in SCNT-derived embryos at 2-cell, 4-cell and day 4.5 blastocyst stages was compared with corresponding in vivo fertilized embryos by real-time PCR. It was demonstrated that immediately after the first cell cycle, SCNT-derived 2-cell stage embryos did not express all four cumulus-specific genes, which continually remained silent at the 4-cell and blastocyst stages. It is therefore concluded that all four cumulus-specific genes were correctly reprogrammed to be silent following nuclear transfer with cumulus donor cells in the mouse model. This would imply that the poor preimplantation developmental competence of SCNT embryos derived from cumulus cells is due to incomplete reprogramming of other embryonic genes, rather than cumulus-specific genes.

Key words: Somatic cell nuclear transfer (SCNT), Nuclear reprogramming, Embryo, Development
doi:10.1631/jzus.2007.B0533 **Document code:** A **CLC number:** Q322

INTRODUCTION

Somatic cell nuclear transfer (SCNT) has successfully produced live-born animals in several mammalian species to date. These include sheep (Wilmut *et al*., 1997), goat (Baguisi *et al*., 1999), mouse (Hosaka *et al*., 2000), cattle (Kato *et al*., 2000), pig (Polejaeva *et al*., 2000), rabbit (Challah-Jacques *et al*., 2003), cat (Gómez *et al*., 2004), horse (Hinrichs *et al*., 2006; Galli *et al*., 2003), rat (Zhou *et al*., 2003), dog (Lee *et al*., 2005) and ferret (Li *et al*., 2006). Even endangered domestic animal sub-species such as gaur (Lanza *et al*., 2000), mouflon (Loi *et al*., 2001) and banteng (Sansinena *et al*., 2005) have been cloned. Besides reproductive cloning which aims to produce cloned live-born animals, SCNT has also been applied to create embryonic stem cell lines in mouse (Wakayama, 2003), cattle (Cibelli *et al*., 1998) and rabbit (Fang *et al*., 2006), in what is commonly referred to as therapeutic cloning. However, the efficiency of both reproductive and therapeutic cloning remains extremely low. Generally, less than 3% of reconstructed embryos survive to term regardless of species or nuclear transfer techniques being utilized (Thibault, 2003). The efficiency of generating isogenic embryonic stem cells through therapeutic cloning is equally low. For instance, only 5.6% of reconstructed embryos gave rise to embryonic stem cell

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* Project (No. R-174-000-065-112/303) supported by the National University of Singapore

lines in the mouse model (Wakayama, 2003). Additionally, live-born cloned animals frequently exhibit various types of phenotypic, metabolic and epigenetic abnormalities (Thibault, 2003; Valenzuela, 2005; Han *et al*., 2003).

It is believed that incomplete reprogramming of embryonic genes, particularly pluripotency markers (Pou5F1, Sox2, Nanog) is primarily responsible for the developmental abnormalities observed with SCNT embryos. Previous studies suggested that failure to reactivate the full spectrum of Oct4-related genes may contribute to embryonic lethality in somatic-cell clones (Bortvin *et al*., 2003). Embryonic genes were found aberrantly expressed in cloned embryos at the 2-cell, 4-cell and blastocyst stages when cumulus donor cells were utilized (Tong *et al*., 2006).

It was also demonstrated that somatic markers were aberrantly expressed in cloned mouse embryos derived from donor myoblast nuclei. The profoundly altered gene-expression profile of mouse cloned embryos appears to be donor cell-type specific (Gao *et al*., 2003). Hence, exposure of cloned embryos to standard embryo culture conditions may lead to disruption in basic homeostasis and inhibition of a range of essential processes including further nuclear reprogramming, which might contribute to their demise (Gao *et al*., 2003).

In this study, the expression of four cumulus-specific genes: follicular stimulating hormone receptor (FSHr), hyaluronan synthase 2 (Has2), prostaglandin synthase 2 (Ptgs2) and steroidogenic acute regulator protein (Star), is to be investigated in cloned mouse embryos at the 2-cell, 4-cell and blastocyst stages, in comparison with in vivo fertilized embryos. FSHr is expressed in mammalian cumulus and is a developmental competence marker for bovine oocyte-cumulus complex (Calder *et al*., 2003). Has2 and Star are enzymes secreted by granulosa cells that are involved in cumulus expansion and maintenance of an optimal oocyte microenvironment, processes that are essential for normal ovulation, fertilization, and female reproduction (Elvin *et al*., 1999). Ptgs2 interacts with gonadotropins to modulate cumulus expansion for successful fertilization (Shimada *et al*., 2006; Liu and Sirois, 1998).

Has2, Ptgs2 and Star are expressed in metaphase II oocytes (Elvin *et al*., 1999; Shimada *et al*., 2006; Liu and Sirois *et al*., 1998), but not FSHr. However, all of these four genes are subsequently switched-off at the cleavage and blastocyst stages of wild-type in vivo fertilized embryos. Unexpectedly, our study showed that these four genes were immediately silenced after the first cell cycle at the 2-cell stage of cloned embryos, and continually remained non-expressed at the 4-cell and blastocyst stages. Hence, this would imply that poor preimplantation developmental competence of cloned mouse embryos derived from donor cumulus nuclei is not due to incomplete reprogramming of cumulus-specific genes, but can instead be attributed to incomplete reprogramming of other key developmental genes.

MATERIALS AND METHODS

Reagents, chemicals, culture media and labware consumables

Unless otherwise stated, all reagents and chemicals used in this study were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). For handling and culture of mouse oocytes and embryos, CZB (Chatot, Ziomet and Bavister) medium was prepared according to the formulation of Chatot *et al*.(1989), while KSOM (potassium (K) simplex optimized medium) was prepared according to the formulation of Erbach *et al*.(1994). All labware consumables were purchased from Becton Dickinson Inc. (Franklin Lakes, NJ, USA).

Laboratory animals and ethics governing their use in research

C57×CBA F1 female mice (6~8 weeks old) and Swiss Albino male mice (12~16 weeks old) were obtained from the Animal Holding Unit (AHU) of the National University of Singapore. All experiments involving live animals and animal-derived tissues were in accordance with the International Guiding Principle for Biomedical Research Involving Animals, and were approved by the Ethics Committee for Experimental Animals of the National University of Singapore. All animals were acclimatized under controlled temperature (25 °C), lighting (lights on from 07:00 to 19:00) and humidity (50%~70%) for at least 1-week prior to use.

In vivo embryo production

C57×CBA F1 female mice were injected intraperitoneally with 10 IU pregnant mare serum gonadotrophin (PMSG, Intervet Pty. Ltd., Bendigo East, Victoria, Australia) followed by 10 IU human chorionic gonadotrophin (hCG, Intervet Pty. Ltd., Bendigo East, Victoria, Australia) 48 h later, and were subsequently mated with Swiss Albino male mice overnight. Female mice with positive vaginal semen plug were sacrificed by cervical dislocation to harvest the in vivo fertilized embryos. Oocyte cumulus complexes (OCCs) were denuded with 80 IU/ml hyaluronidase in 20 mmol/L HEPES-buffered CZB medium, and washed through three droplets of CZB medium. In vivo fertilized mouse embryos were identified by the presence of two pronuclei, and subsequently cultured in modified KSOM medium supplemented with 1% (v/v) essential and 1% (v/v) non-essential amino acid, until up to the blastocyst-stage on day 4.

Somatic cell nuclear transfer

C57×CBA F1 female mice were hyperstimulated with gonadotrophins, as described previously, and were sacrificed for oocyte collection by cervical dislocation, 13 h after intraperitoneal injection of hCG. Enucleation and cell microinjection were carried out on a 37 °C warm stage under an inverted microscope (Olympus Inc., Tokyo, Japan) with two Narashige micromanipulators (Narashige Scientific Instruments Inc., Tokyo, Japan). Self-fabricated holding pipettes (80~100 μm in diameter), enucleation pipettes (10~15 μm opening in diameter) and injection pipettes (6~7 μm opening in diameter) were sterilized by exposure to UV light in a bio-safety class II laminar hood (Nuaire Inc., Plymouth, MN, USA).

Oocytes were enucleated in 5 μl droplets of 20 mmol/L HEPES-buffered CZB medium supplemented with 0.1% polyvinyl alcohol, 5.5 mmol/L glucose and 5 μg/ml cytochalasin B under equilibrated mineral oil. Metaphase II oocytes were positioned by positive and negative suction pressure from the holding pipette, with the metaphase II spindle being stabilized at 3 o'clock, as clearly seen under the inverted microscope. The sharply spiked enucleation pipette was directly inserted through the zona pellucida and the metaphase II spindle was gently aspirated. Twenty-five to thirty oocytes were enucleated within 1 h and the cytochalasin B was washed off, before culture in CZB medium for at least half an hour before cell injection. Discarded oocyte chromosomal structures were stained with 10 μmol/L Hoechst 33342 and exposed to UV light to verify complete enucleation. When the discarded spindle failed to give a bright blue signal, the corresponding oocytes were considered to be enucleation failure and were not used for nuclear injection.

Fresh cumulus cells from mouse OCCs were utilized as donor nuclei for nuclear transfer. Nuclear transfer was performed in 5 μl droplets of CZB medium supplemented with 20% (v/v) fetal bovine serum (FBS, Gibco-BRL Inc., Long Island, NY, USA) and 5.5 mmol/L glucose. Cumulus cells were picked up from the cell suspension and were ruptured by gentle aspiration in and out of the injection pipette, in a 10% (v/v) polyvinyl pyrollidone (PVP) droplet. Ten ruptured cumuli were then loaded within a single injection pipette for 10 subsequent nuclear injections. Holding pipette stabilized oocytes were directly punctured by a spiked microinjection pipette and the cumulus nucleus was securely deposited within the ooplasm. Twenty-five to thirty oocytes were successively injected with cumulus nuclei within one and a half hours, and then washed through 3 droplets of CZB medium, before culture in CZB medium for one hour prior to activation. Subsequently, reconstituted embryos were activated by 5 h exposure to 10 mmol/L strontium chloride in calcium-free CZB medium supplemented with 5 μg/ml cytochalasin B. Activated embryos with clear pseudopronuclei were washed and cultured in KSOM medium (supplemented with 1% (v/v) essential amino acid and 1% (v/v) non-essential amino acid) within a 5% CO_2 incubator set at 37 °C. Development of cleavage-stage embryos was monitored daily until day 4.

Real time polymerase chain reaction (RT-PCR)

For RT-PCR analysis of gene expression, groups of 10 embryos were collected at three cleavage stages (2-cell, 4-cell and blastocyst), for both the SCNT and corresponding control (in vivo fertilized) groups. The embryos were washed in PBS, placed in 5 μl PBS within sterile Eppendorf tubes, and cryopreserved at –80 °C until RNA extraction.

Picopure RNA extraction kit (Arcturus Inc., Sunnyvale, CA, USA) was utilized for all RNA ex-

tractions in this study. Briefly, 50 μl of extraction buffer was added to sterile Eppendorf tubes (200 μl) containing 10 embryos. The embryos were ruptured gently by repeated pipetting, and incubated at 42 °C for 30 min, before being put through binding and elution columns with the corresponding reagents. Eluted RNA was kept at –80 °C until reverse transcription.

Reverse transcription of the extracted RNA was achieved with the high-capacity RNA archive kit (Applied Biosystems Inc., Foster City, CA, USA). RT-PCR was performed by the Applied Biosystems 7900HT system, utilizing the micro fluidic card (Applied Biosystems Inc., Foster City, CA, USA) platform. Probes and primers (Table 1) of the four genes (FSHr, Has2, Ptgs2 and Star) were primed individually on a micro fluidic card plate, which was custom-fabricated (Applied Biosystems Inc., Foster City, CA, USA) for this study at our request. Thawed cDNA samples were gently vortexed and then centrifuged briefly for 5 s. The following components were added to the labeled 1.0 ml microcentrifuge tubes: cDNA sample (40 μl), RNA-free water (160 μl) and TaqMan universal PCR master mix (200 μl). The micro fluidic card was loaded with 100 μl of sample-specific PCR mix per reservoir pool. After the fill reservoir of a micro fluidic card has been loaded with the sample-specific PCR mix, the card was spun at 1200 r/min for 1 min for two times by centrifuge (Sorvall Legend RT, Sorvall GmbH, Hanau, Germany) with specific rotors and adaptors (Applied Biosystems Inc., Foster City, CA, USA). The micro fluidic card was then sealed and the fill reservoirs were trimmed off. The micro fluidic card template was created for relative quantification using the SDS (sequence detector software) set up file under ABI PRISM® 7900HT sequence detection system (Applied Biosystems Inc., Foster City, CA, USA). The card was run with default thermal cycling conditions (AmpErase UNG activation at 50 °C for 2 min, AmpliTaq Gold DNA polymerase activation at 94.5 °C for 10 min, melt, anneal and extension for 40 cycles). All together, two replicates (pooled mRNA of 10 embryos or oocytes) were performed for each experimental group, and the mean *Ct* (threshold cycle) values obtained are presented in Tables 2 and 3. One *Ct* value equals 2 times difference of gene expression level. Expression of individual genes among different embryo stages was compared and analyzed, using 18SrRNA (housekeeping gene) as the endogenous control for normalization.

Table 1 List of PCR primer sequences used for amplification of the gene transcripts of FSHr, Has2, Ptgs2 and Star

Mouse gene	Primer sequences for PCR amplification of gene transcript
FSHr	Forward=5′ TCCCCCGGAACGCCATTGAA 3′
	Reverse=5′ GCCTTAAAATAGACTTGTTGCA 3′
Has2	Forward=5′-CTGTGAAAAGGCTGACCTAC-3′
	Reverse=5′-TCAGTAAGGCACTTGGACCG-3′
Ptgs2	Forward=5′-AAGCCCTCTACAGTGACATCGA-3′
	Reverse=5′-ATGGTCTCCCCAAAGATAGCAT-3′
Star	Forward=5′-GACCTTGAAAGGCTCAGGAAGAAC-3′
	Reverse=5′-TAGCTGAAGATGGACAGACTTGC-3′

RESULTS

As seen in Table 2, Has2, Ptgs2 and Star were expressed in metaphase II oocytes, but not FSHr. The RT-PCR data obtained from 10 pooled denuded oocytes showed that Has2, Ptgs2 and Star were expressed with *Ct* values of 33.40, 33.12 and 35.70

Table 2 Expression of cumulus-specific genes in metaphase II (MII) oocyte and in vivo fertilized embryos

	Mean *Ct* values from RT-PCR (*n*=2)					
	MII oocyte	In vivo fertilized 2-cell embryo	In vivo fertilized 4-cell embryo	In vivo fertilized 8-cell embryo	In vivo fertilized morula	In vivo fertilized blastocyst
FSHr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Has2	33.40	n.d.	n.d.	n.d.	n.d.	n.d.
Ptgs2	33.12	n.d.	n.d.	n.d.	n.d.	n.d.
Star	35.70	n.d.	n.d.	n.d.	n.d.	n.d.
18SrRNA	16.78	17.75	17.82	16.61	15.21	15.12

Note: Has2, Ptgs2 and Star, but not FSHr were expressed in metaphase II (MII) oocytes. However all four cumulus-specific genes were non-expressed throughout preimplantation development of in vivo fertilized embryos. The gene expression levels detected by RT-PCR are represented by *Ct* values. Expression levels of 18SrRNA (housekeeping gene) served as the endogenous control. All data are presented as mean values of two technical replicates. All four cumulus-specific genes were previously conformed to be expressed in mouse cumulus cells with the same RT-PCR system used in this study (data not shown). n.d.: Non-detected

respectively, while expression of FSHr was non-detected (Table 2). However all of these four genes were non-expressed throughout preimplantation development of both in vivo fertilized (Table 2) and cloned (Table 3) embryos.

Table 3 Expression of cumulus-specific genes in cloned embryos

	Mean *Ct* values from RT-PCR (*n*=2)		
	Cloned 2-cell embryo	Cloned 4-cell embryo	Cloned blastocyst
FSHr	n.d.	n.d.	n.d.
Has2	n.d.	n.d.	n.d.
Ptgs2	n.d.	n.d.	n.d.
Star	n.d.	n.d.	n.d.
18SrRNA	18.78	18.51	15.07

Note: All four cumulus-specific genes (Has2, Ptgs2, Star, and FSHr) were found to be non-expressed in cloned embryos derived from donor cumulus nuclei, at the 2-cell, 4-cell and blastocyst stages. The gene expression levels detected by RT-PCR are represented by *Ct* values. Expression levels of 18SrRNA (housekeeping gene) served as the endogenous control. All data are presented as mean values of two technical replicates. All four cumulus-specific genes were previously conformed to be expressed in mouse cumulus cells with the same RT-PCR system used in this study (data not shown). n.d.: Non-detected

DISCUSSION

It is well established that the poor developmental competence of cloned mouse embryos is due to their failure in expressing several key "pluripotency genes". Similar findings were also reported for SCNT-derived monkey and cattle embryos (Bortvin *et al*., 2003; Yamazaki *et al*., 2006). Previously, it was demonstrated that Pou5F1 was abnormally expressed in trophectoderm, or expressed in both trophectoderm and inner cell masses, or completely absent in cloned blastocysts (Tong *et al*., 2006). Abnormal expression of trophectoderm lineage marker such as Cdx2 was also reported. Other transcriptional factors, cell cycle regulators, protein structure modifiers, cellular adhesion markers and chromatin modifiers were also abnormally expressed in cloned embryos at various preimplantation stages (Tong *et al*., 2006). Failure to activate embryonic genes was believed to be the main cause of early embryo demise (Lee *et al*., 2006; Armstrong *et al*., 2006).

Upon injection of somatic cell nucleus into the enucleated oocyte, many profound biological processes take place. Molecules, proteins and enzymes are exchanged between cytoplast and karyoplast. Donor nucleus is prematurely condensed, in what is commonly termed premature chromatin condensation (PCC) (Prather *et al*., 2004). Although the exact molecular reprogramming mechanism still remains largely unelucidated, it is believed that this is an epigenetic process, which actively involves alteration of DNA methylation, histone methylation and histone acetylation (Santos and Dean, 2004; Smillie *et al*., 2004; Meehan, 2003). Failure to demethylate the promoter region of "pluripotency marker" genes probably impairs viability of SCNT-derived embryos.

Additionally, failure to switch off somatic donor cell specific genes has also been demonstrated in cloned mouse embryos derived from donor myoblast nuclei. These embryos express myoblast-specific genes that might favor culture conditions more appropriate for somatic cells (Gao *et al*., 2003). Hence, it is no surprise that developmental competence was impaired under culture conditions appropriate for preimplantation embryos. For the first time, this study demonstrated that cumulus-specific genes are successfully reprogrammed to be silent immediately after SCNT. This could indicate that the resulting poor developmental competence is more associated with failure to restore embryonic gene expression, rather than the failure to silence somatic genes.

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