Development of Reversing the Usual Order of Somatic Cell Nuclear Transfer in Mice

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ABSTRACT

Somatic cell nuclear transfer (SCNT) is a useful tool for reproducing genetically identical animals or producing transgenic animals. Many reports have demonstrated that the efficiency of animal cloning by SCNT requires reprogramming of the somatic nucleus to a totipotent like-state. The SCNT-related reprogramming might mimic the natural reprogramming process that occurs during normal mammalian development. However, recent evidence indicates that the reprogramming event by SCNT is incomplete. In this study, the traditional SCNT procedure (TNT) was modified by injecting donor nuclei into recipient cytoplasm prior to the enucleation process to expose the donor nucleus before removing the karyoplast containing the chromosomes of the oocytes which might possess additional reprogramming factors, and this modified technique was named as reversing the usual order of SCNT (RONT). Other procedures including activation and *in vitro* culture were the same as TNT. Contrary to expectations, the rate of blastocyst development was not different significantly between RONT and TNT (8.6% and 7.9%, respectively). However, duration of micromanipulation performed by the same technician and equipments was remarkably reduced because the ruptured oocytes after nuclear injection were excluded from the enucleation process. This study suggests that RONT, a simplified SCNT protocol, shortens the duration of SCNT procedure and this less time-costing protocol may enable the researchers to perform murine SCNT easier.

(Key words : mouse, somatic cell nuclear transfer, maternal chromosome associated factor)

INTRODUCTION

Somatic cell nuclear transfer (SCNT) has advanced since the birth of the first mammalian clone, Dolly the sheep, in 1997 (Wilmut *et al.*, 1997). This technology can be applied to autologous stem cell therapy and preservation of endangered animal species, pet or domestic animal cloning (Kato *et al.*, 2000; Wakayama *et al.*, 2001; Shin *et al.*, 2002; Kim *et al.*, 2007). Like other species, mouse cloning by SCNT has demonstrated since its first report in 1998 although the efficiency is very low (Wakayama *et al.*, 1998). In general, for the success of murine SCNT program, skilled technical experts and special device such as piezo-actuated micromanipulator are required. It is known that there are many factors affecting the success of SCNT such as oocyte activation (Kishikawa *et al.*, 1999), timing of enucleation and the injection of the somatic cell nucleus (Wakayama *et al.*, 2003), donor cell pre-treatment before nuclear transfer (Wade *et al.*, 2002) and supplementation of various factors in culture medium (Kishigami *et al.*, 2006; Rybouchkin *et al.*, 2006). Molecular analyses of cloned embryos also have shown irregular gene expression in placenta, kidney and liver caused by abnormal epigenetic modifications such as DNA methylation and histone modification (Ohgane *et al.*, 2004). It is believed that these epigenetic errors are due to insufficient reprogramming of donor cell nuclei. Although the level of donor cell reprogramming can be partially improved by the addition of the factors such as Trichostatin A, a histone deacetylase inhibitor (Enright *et al.*, 2003; Kishigami *et al.*, 2006), SCNT program is still need to be improved for better outcome.

The metaphase II (MII) arrested oocytes is activated by sperm penetration or artificial activation stimulus, and developed into the embryo. In the fertilization process in mammals, the fertilized oocyte expels half of their chromosomes known as polar body extrusion. SCNT embryos may possess relatively lesser

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reprogramming factors due to enucleation process prior to donor cell injection. Therefore, loss of a number of unknown critical factors in the perinuclear area may occur in the enucleation step which is commonly performed prior to nuclear injection in SCNT program. It is already known that reprogramming related maternal chromatin associated factors are located around the oocyte nuclei (Szollosi *et al.*, 1986; Latham 1999). Hence, removal of the karyoplast containing the chromosomes of the oocyte should affect the reprogramming of donor cell nucleus in SCNT experiment. In these regards, we postulate that the exposure of donor cell nuclei to intact oocyte support more complete reprogramming by increased opportunity for recruitment of somatic cell and some chromatin associated factors.

In this study, we hypothesized that donor cell injection prior to enucleation would influence the developmental competence of the SCNT murine embryo. Therefore to evaluate this hypothesis, traditional SCNT procedure (TNT) was modified by injecting the donor nucleus into the recipient oocyte prior to enucleation. This modified technique was named as reversing the usual order of SCNT (RONT).

MATERIALS AND METHODS

1. Animals and Chemicals

Six-week-old C57BL6 X DBA2 F1-hybrid (B6D2F1) female mice were used as sources for the recipient oocytes and donor cumulus cells. All inorganic and organic compounds were obtained from Sigma-Aldrich Korea (Yong-in, Korea) unless otherwise stated. All culture and handling media used were based on CZB and KSOM (Chatot *et al.*, 1989; Erbach *et al.*, 1994)

2. Collection of Oocytes and Preparation of Donor Cells

Female B6D2F1 mice were superovulated by intra-peritoneal injections of 5 IU equine chorionic gonadotropin (eCG, Intervet., Boxmeer, Holland) and 5 IU human chorionic gonadotropin (hCG, Intervet., Boxmeer, Holland), given 48 h apart. Oocytes were recovered 13~15 h after hCG injection, and the oviducts were removed and transferred to Hepes-CZB medium (HCZB) supplemented with 300 IU/ml hyaluronidase. The cumulus-oocytes complexes were released. After 5 min exposure to hyaluronidase-containing HCZB, the cumulus-free oocytes were washed three times in HCZB before micromanipulation. The cumulus cell suspension in a 5 μl HCZB droplet was mixed with HCZB supplemented with 12% (w/v) polyvinylpyrrolidone (PVP) and the dish was covered with mineral oil. The donor cell and PVP solution mixture (PVP-HCZB) was kept at room temperature before nuclear injection (Wakayama *et al.*, 2001).

3. Enucleation

Ten MII oocytes (in TNT) or 10 donor cell-injected oocytes (in RONT) were transferred to a 10 μl droplet of HCZB containing 5 μg/ml cytochalasin B (CB), which had previously been placed under mineral oil in the operation heat chamber on the microscope stage. The zona pellucida of the oocyte was 'drilled' by applying several piezo pulses to the tip of an enucleation pipette with a 7- to 10-μm diameter using piezo-actuated micromanipulator (PMM-150FU, Prime Tech Ltd., Ibaraki, Japan). MII chromosome-spindle complex was drawn into the pipette with a small amount of accompanying ooplasm and removed from the oocytes. In RONT, MII chromosome spindle complex protruded after nuclear injection and this made easier for enucleation (Fig. 1). After enucleation, the oocytes were transferred into KSOM and kept for up to 30 min before nuclear injection (TNT) or activation (RONT).

4. Nuclear Injection

Donor cell nuclear injection in TNT group was carried out within 30 min after enucleation whereas the injection in RONT group was carried out within 10 min after collection of MII oocytes. In a droplet of PVP-HCZB, a cumulus cell was drawn into an injection pipette with a 5-μm diameter and the cell was moved in and out with operating piezo pulses until the plasma membrane was broken. Then, the pipette moved to a CB and BSA-free HCZB droplet where the enucleated oocytes were placed. The zona pellucida of the oocyte was 'drilled' by applying several piezo pulses. The pipette containing the donor cell was directly injected either into the enucleated oocytes in TNT group or into the MII intact oocytes in RONT group through cytoplasmic membrane by applying one or two piezo pulses. After the injection, the reconstructed embryos remained in the medium before activation (TNT) and stabilized in the medium before enucleation (RONT). All micromanipulation processes were performed at room temperature.

5. Activation and *In Vitro* Culture

Reconstructed murine oocytes in both experimental groups were exposed to an activation medium consisting of 10 mM $SrCl_2$ with μg/ml CB in calcium-free CZB for 6 h and then cultured for 120 h in KSOM at 37℃ in a humidified 5% CO_2

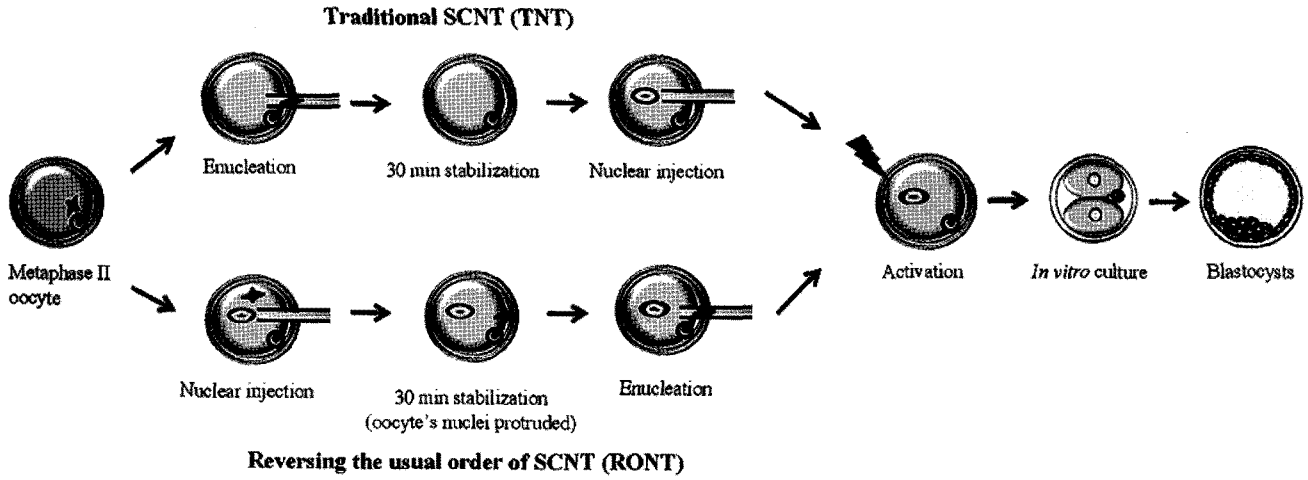

Fig. 1. Experimental scheme of reversing the usual order of somatic cell nuclear transfer (SCNT) named RONT.

in air. The medium was pre-incubated for 2 h in a CO_2 incubator before culturing activated oocytes. Success rate of enucleation, nuclear injection and development to the blastocyst stage were examined.

6. Experiment Design

Detailed description of RONT and TNT was shown in Fig. 1.

7. Statistical Analysis

Each experiment was replicated at least three times. Success rates of enucleation and injection, the rates of cleavage and further embryonic development after *in vitro* culture between two groups were compared by student *t*-test using SPSS software package (Version 12.0; SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant for P values <0.05.

8. Animal Ethics

All animal experiments were approved and performed under the guidelines of the Institutional Animal Care and Use Committee of Seoul National University.

RESULTS

In vitro developmental rate to the blastocyst stage was not significantly different between RONT and TNT (8.6% and 7.9%, respectively, Table 1). Average duration for whole SCNT micromanipulation processes for 40 oocytes in each experiment was 1.5 h (RONT) versus 2.5 h (TNT) and total duration for micromanipulation was remarkably reduced in RONT group (Table 1) because destructed SCNT oocytes after injection were excluded from the enucleation process.

DISCUSSION

In the present study, we modified SCNT technique by injecting donor nuclei into recipient oocytes prior to the removal of karyoplast containing the chromosomes of the oocytes and

Table 1. Comparison of TNT and RONT on the development to the blastocyst stage in murine SCNT program*

Group	Total oocytes for micromanipulation**	Survived after injection in RONT	Survived after enucleation	Survived after injection in TNT	Blastocysts	Duration (h)***
TNT	115	N/A	103 (89.6%)	51 (44.3%)	9 (7.9%)	2.5
RONT	115	62 (53.9%)	54 (46.9%)	N/A	10 (8.6%)	1.5

* SCNT: somatic cell nuclear transfer; TNT: traditional SCNT; RONT: reversing the usual order of SCNT.

** Three replicates; 40 oocytes per experiment (40 × 3) and 5 oocytes from total three experiments were excluded from the micromanipulation process due to their poor quality.

*** Average duration of micromanipulation for 40 oocytes per experiment performed by the same technician and equipments.

named RONT. One of the benefits of RONT is that it retains the volume of the oocyte's cytoplasm when foreign nucleus is introduced like in normal fertilization. However, it is known that compensated or increased volume of the cytoplasm of enucleated oocytes does not enhance reprogramming potential (Wakayama *et al.,* 2008). The most significant difference between RONT and TNT other than the volume of cytoplasm during nuclear injection is the exposure of donor nuclei to reprogramming related maternal chromatin associated factors which are located around the nucleus of the oocytes. Various development and reprogramming related factors in the MII cytoplasm have been reported to support reprogramming of sperm and donor somatic cell nuclei, and such factors are lost after fertilization or activation within 1 h (Szollosi *et al.,* 1986; Latham 1999; Kim *et al.,* 2002). In TNT procedure, some of maternal chromatin-associated reprogramming factors may have been removed during enucleation process as well. Donor cell injection is followed by this enucleation step. Hence, introduced somatic donor nucleus may have no chance to encounter some maternal chromatin associated reprogramming factors which might be important for complete reprogramming. On the other hand, RONT technique enables somatic donor nucleus to contact with the maternal chromatin associated reprogramming factors for more than 30 min before enucleation. This contact may be important for further development of SCNT embryos. It has been reported that numbers of important cytoplasmic and maternal chromatin associated factors in oocytes, such as AKAP95 and active MPF, are localized near metaphase chromosomes (Casas *et al.,* 1999; Collas *et al.,* 1999). So, loss of the maternal chromatin associated reprogramming factors can be minimized when RONT is applied for murine cloning instead of TNT.

In the present study, however, differing from our expectation, developmental rate to the blastocyst stage was not significantly different between RONT and TNT. Although the rate of *in vitro* blastocyst development is not improved by RONT application, average duration for whole SCNT micromanipulation processes was shortened by 40% in RONT when compared with TNT. This is because the ruptured SCNT oocytes after nuclear injection have been excluded from the enucleation process. In general, many oocytes are ruptured after nuclear injection and discarded, which is especially common in murine SCNT process. In addition, protruded MII plate can be found more easily in RONT than in TNT (Fig. 1). Hence, further studies are needed for the evaluation of the benefit of RONT application including embryo transfer and molecular analysis.

Although *in vitro* data did not show significant improvement on the production of SCNT murine embryos, this study suggests that RONT, a simplified SCNT protocol, shortens the duration of SCNT procedure and can be applied as an alternative way to traditional murine SCNT program. Because the enucleation step is the most time-consuming process in murine SCNT, this less time-costing protocol may enable the researcher to perform murine SCNT more productively with the same fruitful effort.

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