

## Murine somatic cell nuclear transfer using reprogrammed donor cells expressing male germ cell-specific genes

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**ABSTRACT.** *In vivo*-matured mouse oocytes were enucleated, and a single murine embryonic fibroblast (control or reprogrammed by introducing extracts from murine testis tissue, which showed expression of male germ cell-specific genes) was injected into the cytoplasm of the oocytes. The rate of blastocyst development and expression levels of Oct-4, Eomes and Cdx-2 were not significantly different in both experimental groups. However, the expression levels of Nanog, Sox9 and Glut-1 were significantly increased when reprogrammed cells were used as donor nuclei. Increased expression of Nanog can be supportive of complete reprogramming of somatic cell nuclear transfer murine embryos. The present study suggested that donor cells expressing male germ cell-specific genes can be reconstructed and can develop into embryos with normal high expression of developmentally essential genes.

**KEY WORDS:** mouse, reprogramming, somatic cell nuclear transfer, testis extract

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Offspring from somatic cell nuclear transfer (SCNT) have been reported in several mammalian species, but the overall efficiency has remained low even though advances in birth rate have been reported [17, 30]. There have been a lot of studies on the SCNT processes, including oocyte activation [12], enucleation of oocytes [28], reprogramming of donor cells [27] and culture conditions for reconstructed embryos [11, 21]. With regard to improvement of cloning efficiency, the reprogramming status of the donor cells recently has been considered to have a key role influencing the overall efficiency of SCNT [17, 30].

A number of reports have established that successful cloning by SCNT requires reprogramming of the somatic donor cell to a totipotent state resembling the status of the germ cell [26]. Several studies have reported reprogramming somatic cells to a germ-cell like state using injection of egg extracts from mammals or amphibians [9, 15, 16, 22, 25]. However, the efficiency and method of using oocyte extracts are in need of improvement despite these encouraging results [15]. Meanwhile, it has been suggested that mammalian sperm has unique features allowing it to be easily reprogrammed in oocytes at the time of fertilization [26]. Although their results regarding effectiveness have been controversial, several trials have been performed to improve the reprogramming efficiency in SCNT using sperm features in murine, equine and bovine species, such as activation of cloned oocyte by sperm or sperm extract injection [3, 4, 10, 12]

and use of zygotic cytoplasm treated with sperm contents [7, 23]. Recently, somatic donor cells dedifferentiated by treatment with a testis extract were shown to improve the developmental rate of reconstructed embryos derived from porcine SCNT [19]. In the present study, we investigated the efficiency of murine SCNT and the gene expression patterns of reconstructed embryos using donor cells reprogrammed by treatment with a testis extract (TE) expressing male-specific genes.

Female *B6D2F1* (*C57BL6* X *DBA2* F1-hybrid) mice were superovulated for collection of recipient oocytes by intraperitoneal injections of 5 IU equine chorionic gonadotropin (eCG, Intervet., Boxmeer, Netherlands) and 5 IU human chorionic gonadotropin (hCG, Intervet.), given 48 hr apart. Oocytes were recovered from oviducts 15 hr after hCG injection and placed in 3 ml Hepes-CZB medium (HCZB) supplemented with 300 IU/ml hyaluronidase. After 5 min exposure to the hyaluronidase-containing HCZB, the cumulus-free oocytes were washed three times in HCZB before micromanipulation. Primary murine embryonic fibroblasts (MEFs) were cultured in humidified air with 5% CO<sub>2</sub> at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS, Hyclone, Logan, UT, U.S.A.). Reprogrammed MEFs were provided by the Department of Animal Biotechnology, Konkuk University. Cell characterization and a method of reprogramming cells using a TE were described in detail in our previous paper [19]. Only cells expressing murine testis-specific marker genes, such as PRO1, SHG and ACR, were used as reprogrammed donor cells, as described in our previous paper [19]. The normal MEFs and reprogrammed MEFs were kept under mineral oil at room temperature for up to 2 hr before nuclear injection in a 5 µl HCZB droplet supplemented with 12% (w/v) polyvinylpyrrolidone (PVP) [29].

Ten to 15 oocytes were transferred to a 10 µl droplet of

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HCZB containing 5  $\mu\text{g/ml}$  cytochalasin B (CB), and the zona pellucidae of the oocytes were “drilled out” by applying several piezo pulses to the tip of an enucleation pipette with a 7- to 10- $\mu\text{m}$  diameter using a piezo-actuated micromanipulator (PMM-150FU, Prime Tech Ltd., Tsuchiura, Japan). The metaphase II chromosome–spindle complex was drawn into the pipette and removed from the oocytes. After enucleation, the oocytes were washed 3 times into HCZB, transferred into KSOM and kept for up to 30 min before nuclear injection. Nuclear injection was carried out in a droplet of PVP-HCZB. The enucleated oocytes were placed in a CB- and BSA-free HCZB droplet, and then, a normal fibroblast and reprogrammed fibroblast were drawn into an injection pipette, which was moved subsequently to the droplet. The pipette containing the donor cells was directly injected into the enucleated oocytes (Fig. 1). The reconstructed oocytes were exposed to an activation medium consisting of 10 mM  $\text{SrCl}_2$  with 5  $\mu\text{g/ml}$  CB in calcium-free CZB for 6 hr. After activation, the reconstructed oocytes were moved to KSOM for *in vitro* culture at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  in air for 4 days.

The pluripotency-related genes (OCT4 and NANOG), embryo metabolism-related gene (GLUT1), male germ cell-related gene (SOX9) and trophectoderm differentiation-related genes (CDX2 and EOMES) were analyzed by Q-PCR. Amplification of target genes was performed with the specific primers listed in Table 1. The primer sequences were obtained from PrimerBank (<http://pga.mgh.harvard.edu/primerbank/>). First, RNA was extracted from pooled blastocysts in the control and experimental groups using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.), and then, the RNA was reverse transcribed to synthesize cDNA. Q-PCR was performed using an ABI PRISM 7500 system and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, U.S.A.). Extraction of mRNAs and synthesis of cDNA were performed twice, and all samples were run in triplicate to obtain technical replicates. Gene expression was always related to the expression of murine *Gapdh*, a housekeeping gene, known to be a good reference gene for normalization of the expression levels of target genes. Quantification was performed using the  $\Delta\Delta\text{CT}$  method. Each experiment was replicated at least three times. Cleavage and blastocyst rates were analyzed by the  $\chi^2$  test. Data regarding the expression of specific genes in blastocyst embryos were analyzed by one-way ANOVA using the SPSS software (Version 12.0; SPSS Inc., Chicago, IL, U.S.A.).  $P < 0.05$  was considered significant. All animal experiments were approved and performed under the guidelines of the Institutional Animal Care and Use Committee of Seoul National University (SNU-061023-1). All inorganic and organic compounds were obtained from Sigma-Aldrich Korea (Yong-in, Republic of Korea) unless otherwise stated. All culture and handling media used were based on CZB [1] and KSOM [5].

A total of 273 SCNT embryos (3 replicates for each control and experimental groups) were scored for developmental rate (%) at the 2-cell (cleavage) (42.7 vs. 41.8) and blastocyst stage ( $19.7 \pm 2.4$  vs.  $21.2 \pm 2.9$ ). There was no significant difference in developmental rate between groups

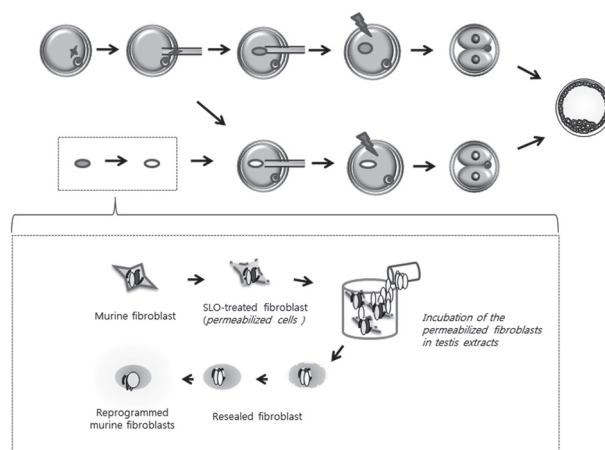


Fig. 1. The experimental scheme of this study. The procedural steps were the same until the enucleation process. For donor cell injection, intact murine fibroblasts (control, pink) or fibroblasts reprogrammed using the testis extract (experimental group, yellow) were used. The reprogramming process is briefly described in the dotted rectangle. After activation, embryos were cultured in standard *in vitro* culture medium for up to 120 hr.

(Table 2).

Pretreatment of donor cells brought about changes in the expression of specific genes during embryonic development. Gene expressions of reconstructed blastocysts are described in Fig. 2. Expression of the OCT4, CDX2 and EOMES genes did not vary significantly between the experimental groups, whereas expression of the NANOG, SOX9 and GLUT1 genes increased significantly (up to 2-fold) compared with the control group.

In the present study, the rate of development to the blastocyst stage did not show a significant difference between the experimental groups, unlike a previous report in pigs [19]. The expression levels of developmentally important genes were also investigated in cloned embryos with donor cells reprogrammed using the testis extract. The expression levels of the Oct-4, Cdx2 and Eomes genes did not show significant differences between experimental groups. However, the expression levels of Nanog, Sox9 and Glut1 genes in blastocysts reconstructed with cells treated with the testis extract were significantly higher than in the control. Oct-4 and Nanog are important regulators for pluripotency of the inner cell mass (ICM) [24]. The higher expression level of Nanog in embryos reconstructed with cells treated with the testis extract corresponded to that in previous results [19]. The expression of both Oct-4 and Nanog was reported to be higher in *in vivo*-derived embryos than in SCNT-derived counterparts [13]. Based on a recent study reporting that the ICM of Oct-4-null embryos showed unexpected higher expressions of Nanog than the ICM of heterozygous embryos [14], the expression levels of Oct-4 and Nanog in the present study may suggest the presence of some complementary relationship between the two genes to maintain the specificity of the ICM [14]. The caudal-type homeodomain protein

Table 1. Primer sequences used in quantitative PCR analysis

Gene	Forward sequence	Reverse sequence
OCT4	CGGTGTGAGGTGGAGTCTGG	GCGATGTGAGTGATCTGCTG
NANOG	GAAATCCCTTCCCTCGCCAT	CTCAGTAGCAGACCCCTTGTA
SOX9	CGGAACAGACTCACATCTCTCC	GCTTGCACGTCGGTTTTGG
GLUT1	TCTCGGCTTAGGGCATGGAT	TCTATGACGCCGTGATAGCAG
CDX2	TGGACGCTGCGAGAATCC	TGTCCTCAATCTCCATCAGTAGATG
EOMES	GGCCCTATGGCTCAAATTC	GAACCACTTCCACGAAAACATTG

Table 2. *In vitro* development of murine embryos reconstructed using reprogrammed donor cells

	No. of cultured	Cleavage rate (no.)	Blastocyst rate (no.)
Control	132	42.7 (56)	19.7 ± 2.4 (26)
Reprogrammed donor cell*	141	41.8 (59)	21.2 ± 2.9 (30)

\*Mouse embryonic fibroblasts reprogrammed with a mouse testis extract.

Cdx2 and Eomes are important genes required at a distinct stage during murine trophectoderm formation [2, 20]. Several studies have reconstructed embryos with aberrant reprogramming that showed an abnormal expression pattern of Cdx2 and Eomes [8, 18]. It can be suggested that the donor cells treated with testis extract in the present study could be successfully reprogrammed to express the genes related to trophectoderm differentiation in the reconstructed embryos as in the control embryos. Higher expression of Sox9 indicates that treatment of somatic donor cells with the testis extract may allow male germ cell-specific features to remain in reconstructed embryos after SCNT. Glut-1 also showed higher expression in embryos reconstructed with cells treated with the testis extract in the present study. It was reported that SCNT-derived mouse embryos showed somatic cell-like features with precocious Glut1 and Glut4 expression [6]. Taking into consideration the results regarding Sox9 expression, augmented expression of Glut1 may reflect altered reprogramming of somatic donor cells after SCNT or remnants of a testis-derived factor in the cytoplasm of the reconstructed embryos. However, expression of Glut-1 is known as the indicator of cell growth, glucose metabolism and transcriptional activity, and strong and stable expression of Glut-1 in the reconstructed embryos might have had a beneficial effect on embryonic development compared with the control embryos in the present study. Taken together, although the developmental rate of reconstructed embryos did not show significant improvement in the present study, the somatic donor cells treated with the testis extract could change the state of the cloned embryos, and they may support early embryonic development after SCNT in mice.

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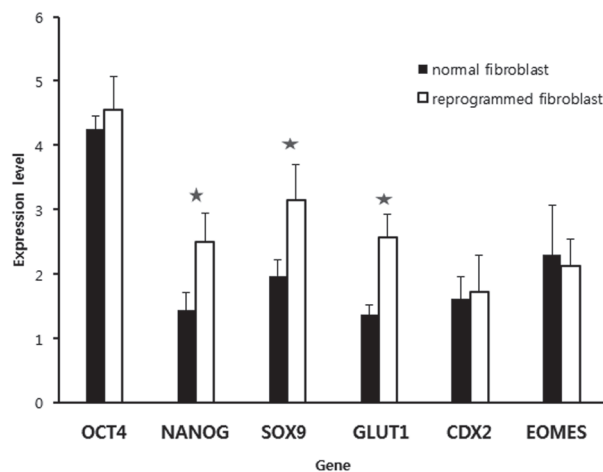


Fig. 2. The expression level of genes related to early embryonic development in cloned blastocysts derived from reprogrammed donor cells. Values with different superscripts (★) in the same column are significantly different ( $P < 0.05$ ). Extraction of mRNAs and synthesis of cDNAs were performed triplicate, and all samples were run in triplicate to obtain technical replicates. The SOX9, GLUT1 and NANOG gene expression levels were significantly higher in cloned embryos derived from reprogrammed donor cells than in the control.

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