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Somatic Cell Nuclear Transfer in Rodents, the Little Big Animals

Sangho Roh*

Cellular Reprogramming and Embryo Biotechnology Laboratory, Dental Research Institute and CLS21, Seoul National University School of Dentistry, Seoul 110-749, Korea

ABSTRACT

Transgenic rats and mice are useful experimental animal models for medical research including human disease model studies. Somatic cell nuclear transfer (SCNT) technology is successfully applied in most mammalian species including cattle, sheep, pig and mouse. SCNT is also considered to increase the efficacy of transgenic/knockout mouse and rat production. However, in the area of reproductive biotechnology, the rodent model is inadequate because of technical obstacles in manipulating the oocytes including intracytoplasmic sperm injection and SCNT. In particular, success of rat SCNT is very limited so far. In this review, the history of rodent cloning is described.

(Key words : mouse, rat, SCNT, cloning)

PIEZO-DRIVEN NUCLEAR INJECTION FOR MURINE NUCLEAR TRANSFER

Although success of cloned sheep following somatic cell nuclear transfer (SCNT) was reported in 1997 by British scientists (Wilmut *et al*., 1997), at that time the scientists of reproductive biotechnology research thought that only two-cell blastomeres could be used as recipient cytoplasm for the production of nuclear transfer (NT)-derived mice (Ogura *et al*., 2001), and it had been limited to blastomeric cell NT technology. In addition, the karyoplast of the two-cell blastomere was the only donor nucleus which can be reprogrammed in enucleated matured oocytes after one-step simple NT (Kono *et al*., 1991). Although Two Japanese groups achieved full-term development of blastomeric cell NT murine embryos independently using enucleated matured oocytes as recipient cytoplasts (Kwon and Kono, 1996; Tsunoda and Kato, 1997, 1998), both research groups used these cytoplasts for the first round of NT. According to their protocols, blastomeres of four-cells or later-stage embryos must be transferred twice: first into enucleated oocytes and then into enucleated embryos (Ogura *et al*., 2001). The breakthrough for murine SCNT was achieved by another Japanese research group (Wakayama *et al*., 1998) and the novel method for murine SCNT developed by Dr. Wakayama and his colleagues is also widely known as 'Honolulu technique'. This SCNT protocol has a unique process for the transfer of donor cells. Instead of electrofusion of a donor cell and an enucleated oocyte, the donor nucleus is directly inserted into the cytoplasm of an enucleated oocyte following the puncture of the plasma membrane of the oocyte by nano-scale movement of the donor nucleus containing microinjection pipette attached to piezo-actuated micromanipulator. Although oocyte activation method (strontium treatment) in Honolulu protocol is also different from conventional protocols for ungulate and pig SCNT, such as electric stimulus and addition of Ca-ionophore/iomomycin (Wilmut *et al*., 1997; Wells *et al*., 1999; Roh and Hwang, 2002), other reports clearly demonstrated that strontium activation is not essential for a success of murine SCNT (Kishikawa *et al*., 1999; Wakayama *et al*., 1999).

ALTERNATE METHODS FOR MURINE SCNT

After the first report of mouse cloning by Wakayama *et al*. (1998), many research groups have attempted to reproduce their results with original Honolulu technique. However, reproducing microinjection technique using Piezo-actuated micromanipulator is not easy to learn for unskilled technicians. In addition, the fragility of the recipient oocytes after enucleation compounds the technical problems (Ogura *et al*., 2001). Different from Japanese groups' preference of Honolulu SCNT

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* Correspondence : E-mail : sangho@snu.ac.kr

method, many other scientists claim that conventional method of nuclear injection using beveled pipette (Rybouchkin and Dhont, 2000; Zhou *et al*., 2000) or electrofusion of donor cell and enucleated oocytes (Gao *et al*., 2003; Ribas *et al*., 2005) can be applied to murine SCNT. According to their reports, adding serum to the medium during injection (Rybouchkin and Dhont, 2000) or careful micromanipulation of the nuclear injection pipette (Zhou *et al*., 2000) enable complete murine SCNT without Piezo-actuated micromanipulator. To avoid use of expensive equipment and requirement of great micromanipulation skills, zona-free NT method was attempted to perform murine SCNT (Ribas *et al*., 2005), which is also known as 'hand-made cloning', had been applied for pig and cattle cloning (Booth *et al*., 2001; Vajta *et al*., 2001; Oback *et al*., 2003). The zona-free SCNT showed similar efficiency in the process of mouse cloning when compared with Honolulu method in their research articles. However, up to date, Honolulu technique is still considered as the standard and most efficient murine SCNT protocol, and commonly used in this research field worldwide.

It is known that reprogramming related maternal chromatin associated factors are located around the oocyte nuclei (Szollosi *et al*., 1986; Latham 1999). With the hypothesis that the exposure of donor nucleus to intact oocyte may support reprogramming by increased opportunities for recruitment of somatic cell and chromatin associated reprogramming factors, donor nucleus injection prior to enucleation method was suggested (Kang *et al*., 2011), which is similar to Honolulu technique except the order of enucleation and nuclear injection was reversed. According to their report, the developmental rate to the blastocyst was not significantly improved but less time-costing protocol because ruptured oocytes by nuclear injection could be excluded prior to enucleation.

TYPES OF DONOR NUCLEI AND RECIPIENT CYTOPLASM

In general, cumulus cells are the choice of donor cells for SCNT in mice when Honolulu method is applied because of their small cell size and soft cytoplasm. The donor cell source of world's first cloned mice was also cumulus cells (Wakayama *et al*., 1998). In addition, those cells are naturally arrested at G0/G1 stage in the cell cycle. However, another factor of donor cell choice is the strain of the mouse. When cumulus cells from F1-hybrid mice (B6D2F1; most recommended strain for both donor cells and recipient oocytes) are used, more than 50% of reconstructed oocytes developed to blastocysts, while almost none of the SCNT embryos developed to term when cumulus cells are collected from inbred mice (B6, DBA/2, 129/ SvJ; Wakayama and Yanagimachi, 2001). Immature Sertoli cells which are the male counterpart of cumulus cells are also used in the murine SCNT program (Ogura *et al*., 2000). According to their report, full-term success rate is slightly higher than those obtained from cumulus cells.

When applying Honolulu technique in murine SCNT program, injecting larger cells such as fibroblasts is more difficult because larger diameter microinjection pipette is required. Rigid cytoplasm of fibroblast often leave plasma membrane of injected cell intact within oocytes, and thus their nuclei failed to intermingle with the ooplasm (Ogura *et al*., 2001). Although Honolulu method is considered as the standard protocol of murine SCNT, electrofusion is recommended if the large size donor cell is used for SCNT in mice such as fibroblasts or myoblasts (Ogura *et al*., 2000; Gao *et al*., 2003). It is claimed that calcium ion should be removed from fusion medium to allow exposure of the donor chromosomes to the metaphase II ooplasm containing reprogramming factors (Ogura *et al*., 2000) whereas the other group added calcium ion in electrofusion medium and obtained comparable rate of blastocysts (Gao *et al*., 2003).

Embryonic stem cells (ESC) are also used in murine SCNT program. The primary aim of using ESC is generating cloned mice with targeted genetic modification (Rideout *et al*., 2000; Eggan *et al*., 2001). Interestingly, the embryos cloned from ESC show poorer initial embryonic development during the pre-implantation stages and higher post-implantation development, an opposite pattern that seen in SCNT embryos from cumulus cells (Wakayama and Yanagimachi, 1999). The poor initial embryonic development is, in part, from cell cycle asynchrony of the donor and recipient cells because, unlike other cell types, actively cycling ESC cannot be induced to enter the G0/G1 phase, not by confluency nor by serum-deprivation (Ogura *et al*., 2001). The unstable character of the epigenetic status in ESC also resulted in inconsistency of imprinting gene expression even the cells are derived from the same ESC line or subclones (Humpherys *et al*., 2001).

The cloned mouse embryos produced using myoblast nuclei fail to develop in standard embryo culture medium while thrive in myoblast culture medium favored by the donor cells themselves, forming blastocysts at a significant rate, with high total

cell number, and a normal allocation of cells to the inner cell mass in the embryos (Gao *et al*., 2003), and the results imply that providing an *in vitro* culture condition optimized for donor cell type may be critical for maintaining homeostasis and supporting the reprogramming events in cloned embryos.

In general, mouse cloning by SCNT relies on introducing a nucleus into a meiotic oocyte at metaphase II stage. In the experiments using fertilized or artificially activated oocytes as recipients, developmental potential after SCNT decreased rapidly after fertilization or parthenogenetic activation (Wakayama *et al*., 2000). Unlike interphase zygotes, however, when the metaphase-arrested donor chromosome was introduced into the chromosome-extracted mitotic zygote which was also arrested at metaphase, developmental reprogramming of the donor nucleus was successful (Egli *et al*., 2007). This SCNT model suggests that the oocyte-free SCNT program may enable to generate autologous human ESC although recent novel induced pluripotent stem cell (iPSC) technology (Takahashi and Yamanaka, 2006) make this model less interesting.

INHIBITION OF ABNORMAL EPIGENETIC CHANGES

The low success rate of mammalian cloning by SCNT is largely associated with epigenetic errors such as abnormal DNA hypermethylation (Kang *et al*., 2001). In mice, molecular analyses of cloned embryos have shown irregular gene expression in the placenta, kidney and liver caused by abnormal epigenetic modification (Ohgane *et al*., 2004). These epigenetic irregularities in cloned mice result in low success rates of fulltem development. Therefore, inhibition of abnormal epigenetic changes including DNA hypermethylation and hypo-acetylation can improve SCNT efficiency. It is reported that 5-aza-20-deoxycytidine, an inhibitor of DNA methylation, resulted in an improvement of cloning efficiency in mice (Enlight *et al*., 2003; Rybouchkin *et al*., 2006). Other research groups have suggested that treatment with trichostatin A (TSA), which enhances the pool of acetylated histones and DNA demethylation, leads to 4～5 times higher blastocyst rates after SCNT in mice (Kishigami *et al*., 2006), and the higher developmental rate is caused by upregulation of genes for pluripotency and embryonic growth/trophectoderm formation as well as downregulation of DNA methylation and histone deacetylation (Kang and Roh, 2011). Currently, TSA treatment during the peri- and post-activation period of reconstructed murine oocytes is widely used in many laboratories of this field.

NUCLEAR TRANSFER IN THE RAT

Transgenic rat is useful experimental animal model for medical research along with transgenic mice. However, most transgenic rats have been produced by DNA microinjection with fewer than 20 transgenic rat lines generated (Charreau *et al*., 1996) and application of SCNT technology in this species is poorly successful so far. There is only one report on the production of SCNT rats (Zhou *et al*., 2003), however the protocol they used has not been reproduced by other scientists yet and other attempts to produce cloned rat show very limited success without cloned rat production (Hayes *et al*., 2001; Iannaccone *et al*., 2001; Du *et al*., 2002; Jiang *et al*., 2002; Roh, 2005, 2007) although a couple of scientists claimed the post-implantation evidence of SCNT rat embryos (Du *et al*., 2002; Roh, 2007). In particular, different from the mouse and other species, even nuclear exchange at the same embryonic stage is rarely reported (Kono *et al*., 1988; Roh *et al*., 2003a).

In rats, a high rate of spontaneous activation occurs in ovulated oocytes during *in vitro* culture. Auto activated oocytes extrude the second polar body within 60 to 90 min of culture and show scattered chromosomes, a state termed metaphase III. After reaching this MIII state, oocytes exhibit very low rates of normal cleavage following induced activation (Keefer and Schuetz, 1982). This represents a major obstacle for SCNT in the rat, as control of activation is a crucial step for successful SCNT. To overcome this incomplete and abortive activation, Zhou *et al*. (2003) used a protease inhibitor that reversibly stabilizes most oocyte metaphases II for up to 3 h and obtained two cloned pups after SCNT. The other group suggested that oocytes must be activated within 2 h after collection from donor animals (Roh *et al*., 2003b), and the reconstructed embryos should be activated immediately after nuclear injection in the rat SCNT program (Roh, 2005).

As described above, very limited research groups have involved in rat SCNT field. However, recent success of rat ESC (Kawamata and Ochiya, 2010) and iPSC (Liskovykh *et. al*., 2011) can support improving the SCNT technology in this valuable experimental animal species for biomedical research.

REFERENCES

Booth PJ, Tan SJ, Holm P and Callesen H. 2001. Application

of the zona-free manipulation technique to porcine somatic nuclear transfer. Cloning Stem Cells 3:191-197.

Charreau B, Tesson L, Soulillou JP, Pourcel C and Anegon I. 1996. Transgenesis in rats: technical aspects and models. Transgenic Res. 5:223-234.

Du ZT, Roh S, Fida S, Handford C, Malakooti N, Morrison J and Trounson A. 2002. Postimplantation development of rat embryos cloned with neural stem cells. Theriogenology 57:410.

Eggan K, Akutsu H, Loring J, Jackson-Grusby L, Klemm M, Rideout WM 3rd, Yanagimachi R and Jaenisch R. 2001. Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. Proc. Natl. Acad. Sci. U.S.A. 98:6209-6214.

Egli D, Rosains J, Birkhoff G and Eggan K. 2007. Developmental reprogramming after chromosome transfer into mitotic mouse zygotes. Nature 447:679-685.

Enright BP, Kubota C, Yang X and Tian XC. 2003. Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin A or 5-aza-2'-deoxycytidine. Biol. Reprod. 69:896-901.

Gao S, Chung YG, Williams JW, Riley J, Moley K and Latham KE. 2003. Somatic cell-like features of cloned mouse embryos prepared with cultured myoblast nuclei. Biol. Reprod. 69:48-56.

Hayes E, Galea S, Verkuylen A, Pera M, Morrison J, Lacham-Kaplan O and Trounson AO. 2001. Nuclear transfer of adult and genetically modified fetal cells of the rat. Physiol. Genomics 5:193-203.

Humpherys D, Eggan K, Akutsu H, Hochedlinger K, Rideout WM 3rd, Biniszkiewicz D, Yanagimachi R and Jaenisch R. 2001. Epigenetic instability in ES cells and cloned mice. Science 293:95-97.

Iannaccone P, Taborn G and Garton R. 2001. Preimplantation and postimplantation development of rat embryos cloned with cumulus cells and fibroblasts. Zygote 9:135-143.

Jiang JY, Mizuno S, Mizutani E, Miyoshi K, Kimura N, Sasada H and Sato E. 2002. Nuclear transfer in rats using an established embryonic cell line and cumulus cells. J. Reprod. Dev. 48:505-511.

Kang H and Roh S. 2011. Extended exposure to trichostatin-A after activation improves *in vitro* development of somatic cell nuclear transfer murine embryos. J. Vet. Med. Sci. 73: 623-631.

Kang H, Sung J and Roh S. 2011. Development of reversing the usual order of somatic cell nuclear transfer in mice. J. Emb. Trans. 26:85-89.

Kang YK, Koo DB, Park JS, Choi YH, Chung AS, Lee KK and Han YM. 2001. Aberrant methylation of donor genome in cloned bovine embryos. Nat. Genet. 28:173-177.

Kawamata M and Ochiya T. 2010. Generation of genetically modified rats from embryonic stem cells. Proc. Natl. Acad. Sci. U.S.A. 107:14223-14228.

Keefer CL and Schuetz AW. 1982. Spontaneous activation of ovulated rat oocytes during *in vitro* culture. J. Exp. Zool. 224:371-377.

Kishigami S, Mizutani E, Ohta H, Hikichi T, Thuan NV, Wakayama S, Bui HT and Wakayama T. 2006. Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. Biochem. Biophys. Res. Commun. 340:183-189.

Kishigawa H, Wakayama T and Yanagimachi R. 1999. Comparison of oocyte-activating agents for mouse cloning. Cloning 1:153-159.

Kono T, Shinoda Y and Tsunoda Y. 1988. Nuclear transplantation of rat embryos. J. Exp. Zool. 248:303-305.

Kono T, Kwon OY and Nakahara T. 1991. Development of enucleated mouse oocytes reconstituted with embryonic nuclei. J. Reprod. Fertil. 93:165-172.

Kwon OY and Kono T. 1996. Production of identical sextuplet mice by transferring metaphase nuclei from four-cell embryos. Proc. Natl. Acad. Sci. U.S.A. 93:13010-13013.

Latham K E. 1999. Mechanisms and control of embryonic genome activation in mammalian embryos. Int. Rev. Cytol. 193:71-124.

Liskovykh M, Chuykin I, Ranjan A, Safina D, Popova E, Tolkunova E, Mosienko V, Minina JM, Zhdanova NS, Mullins JJ, Bader M, Alenina N and Tomilin A. 2011. Derivation, characterization, and stable transfection of induced pluripotent stem cells from Fischer344 rats. PLoS One 6:e27345.

Oback B, Wiersema AT, Gaynor P, Laible G, Tucker FC, Oliver JE, Miller AL, Troskie HE, Wilson KL, Forsyth JT, Berg MC, Cockrem K, McMillan V, Tervit HR and Wells DN. 2003. Cloned cattle derived from a novel zona-free embryo reconstruction system. Cloning Stem Cells 5:3-12.

Ohgane J, Wakayama T, Senda S, Yamazaki Y, Inoue K, Ogura A, Marh J, Tanaka S, Yanagimachi R and Shiota K. 2004. The Sall3 locus is an epigenetic hotspot of aberrant DNA methylation associated with placentomegaly of cloned mice. Genes Cells 9:253-260.

Ogura A, Inoue K, Ogonuki N, Noguchi A, Takano K, Nagano R, Suzuki O, Lee J, Ishino F and Matsuda J. 2000. Production of male cloned mice from fresh, cultured, and cryopreserved immature Sertoli cells. Biol. Reprod. 62:1579-1584.

Ogura A, Ogonuki N, Takano K and Inoue K. 2001. Microinsemination, nuclear transfer, and cytoplasmic transfer: the application of new reproductive engineering techniques to mouse genetics. Mamm. Genome 12:803-812.

Ribas R, Oback B, Ritchie W, Chebotareva T, Ferrier P, Clarke C, Taylor J, Gallagher EJ, Mauricio AC, Sousa M and Wilmut I. 2005. Development of a zona-free method of nuclear transfer in the mouse. Cloning Stem Cells 7:126-138.

Rideout WM 3rd, Wakayama T, Wutz A, Eggan K, Jackson-Grusby L, Dausman J, Yanagimachi R and Jaenisch R. 2000. Generation of mice from wild-type and targeted ES cells by nuclear cloning. Nat. Genet. 24:109-110.

Roh S. 2005. Effect of the timing of oocyte activation on development of rat somatic cell nuclear transfer embryos. Reprod. Dev. Biol. 29:229-234.

Roh S. 2007. Effect of cytochalasin B in activation medium on the development of rat somatic cell nuclear transfer embryos. Reprod. Dev. Biol. 31:109-113.

Roh S and Hwang WS. 2002. *In vitro* development of porcine parthenogenetic and cloned embryos: comparison of oocyte-activating techniques, various culture systems and nuclear transfer methods. Reprod. Fertil. Dev. 14:93-99.

Roh S, Guo J, Malakooti N, Morrison JR, Trounson AO and Du ZT. 2003a. Birth of rats following nuclear exchange at the 2-cell stage. Zygote 11:317-321.

Roh S, Malakooti N, Morrison JR, Trounson AO and Du ZT. 2003b. Parthenogenetic activation of rat oocytes and their development *in vitro*. Reprod. Fertil. Dev. 15:135-140.

Rybouchkim A and Dhont. 2000. Nuclear transfer into mouse oocytes by a conventional method of injection. Theriogenology 53:241.

Rybouchkin A, Kato Y and Tsunoda Y. 2006. Role of histone acetylation in reprogramming of somatic nuclei following nuclear transfer. Biol. Reprod. 74:1083-1089.

Szollosi D, Czolowska R, Soltynska MS and Tarkowski AK. 1986. Remodelling of thymocyte nuclei in activated mouse oocytes: An ultrastructural study. Eur. J. Cell. Biol. 42: 140-151.

Takahashi K and Yamanaka S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663-676.

Tsunoda Y and Kato Y. 1997. Full-term development after transfer of nuclei from 4-cell and compacted morula stage embryos to enucleated oocytes in the mouse. J. Exp. Zool. 278:250-254.

Tsunoda Y and Kato Y. 1998. Not only inner cell mass cell nuclei but also trophectoderm nuclei of mouse blastocysts have a developmental totipotency. J. Reprod. Fertil. 113: 181-184.

Vajta G, Lewis IM, Thouas GA and Trounson AO. 2001. Somatic cell cloning without micromanipulators. Cloning 3:89-95.

Wakayama T and Yanagimachi R. 1999. Cloning the laboratory mouse. Semin. Cell Dev. Biol. 10:253-258.

Wakayama T and Yanagimachi R. 2001. Mouse cloning with nucleus donor cells of different age and type. Mol. Reprod. Dev. 58:376-383.

Wakayama T, Perry ACF, Zuccotti M, Johnson KR and Yanagimachi R. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394:369-374.

Wakayama T, Mombaerts P, Rodriguez I, Perry ACF and Yanagimachi R. 1999. Mice cloned from embryonic stem cells. Proc. Natl. Acad. Sci. U.S.A. 96:14984-14989.

Wakayama T, Shinkai Y, Tamashiro KL, Niida H, Blanchard DC, Blanchard RJ, Ogura A, Tanemura K, Tachibana M, Perry AC, Colgan DF, Mombaerts P and Yanagimachi R. 2000. Cloning of mice to six generations. Nature 407:318-319.

Wells DN, Misica PM and Tervit HR. 1999. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. Biol. Reprod. 60:996-1005.

Wilmut I, Schieke AE, McWhir J, Kind AJ and Campbell KHS. 2007. Viable offspring derived from fetal and adult mammalian cells. Nature 385:810-813.

Zhou Q, Boulanger L and Renard JP. 2000. A simplified method for the reconstruction of fully competent mouse zygotes from adult somatic donor nuclei. Cloning 2:35-44.

Zhou Q, Renard J-P, Le Friec G, Brochard V, Beaujean N, Cherifi Y, Fraichard A and Cozzi J. 2003. Generation of fertile cloned rats by regulating oocyte activation. Science 302:1179.

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