Polscope-Assisted Enucleation for Nuclear Transfer in Mice

Won, Ji Young, Jee Hyun Kang and Hosup Shim*

Department of Physiology, College of Medicine, Dankook University

ABSTRACT

Efficiency of somatic cell nuclear transfer was investigated in mice. First, oocyte activation was induced by $SrCl_2$, and the rate of development was compared with embryos from normal fertilization. Although more than one half of $SrCl_2$-treated oocytes developed to blastocysts (146/262, 55.7%), the rate of blastocyst formation was significantly lower than normal fertilization controls (59/79, 74.6%). Second, enucleation of oocytes was performed using Polscope that enables non-invasive visualization of metaphase spindles. Such approach could not only avoid damage of oocytes during an exposure to UV light often employed in conventional enucleation procedures, but could also assure the removal of nuclei from all oocytes operated because of monitoring the location of spindles during an entire process of enucleation. Morphologically normal blastocysts were obtained from the transfer of cumulus cell nuclei into enucleated oocytes. However, the rate of development into the blastocyst stage was still low (4/93, 4.3%). This reflects that the nuclear transfer procedure used in this study was not sufficiently optimized, and other factors may also impact greatly the efficiency of nuclear transfer. Including an induction of oocyte activation and method of enucleation tested in this study, a lot more elements are remained to be optimized to improve the efficiency of somatic cell nuclear transfer in mice.

(Key words: Polscope, Enucleation, Nuclear transfer, Mouse)

INTRODUCTION

Cloning by somatic cell nuclear transfer has been achieved in several mammalian species including sheep (Willmut et al., 1999), cattle (Cibelli et al., 1997), goat (Baguisi et al., 1999), mice (Wakayama et al., 1998) and pigs (Polejaeva et al., 2000). However, the rates of generating live offspring from nuclear-transferred oocytes have been approximately 2 to 10% with variation among species, indicating an overall efficiency of cloning still remains disappointingly low.

Majority of studies related to cloning has been done in farm animals perhaps because of their economic values. However, cloning in laboratory animals such as mice would also be useful because they are not only relatively inexpensive but also their genetic backgrounds are defined much closely than large animals. Cloning of laboratory mouse will provide valuable tools in expanding our scientific knowledges in mammalian development and accelerate researches in nuclear transfer.

Cloning mice from cumulus cells (Wakayama et al., 1998), tail-tip cells of adults animal (Wakayama and Yanagimachi, 1999), embryonic stem cells (Wakayama et al., 1999) and Sertoli cells (Ogura et al., 2000) has been reported. However only a handful of researchers has succeeded in births of cloned mice, and yet the nuclear transfer procedures varies greatly among laboratories. The variation includes enucleation of oocytes to prepare cytoplasts that accept somatic nuclei and induction of oocyte activation to initiate embryonic development of nuclear-transferred oocytes. To increase an efficiency of cloning mice, such variations in the nuclear transfer procedure needs to be minimized.

Aiming an efficient production of nuclear transfer embryos, we investigated an effectiveness of oocyte activation protocol using strontium by comparing with *in vivo* fertilization. Moreover, a new method of enucleation was tested. The enucleation was assisted by Polscope that visualizes spindles without staining of oocytes and eliminates a requirement of DNA-specific dye and subsequent exposure to UV light to identify the second metaphase chromosomes.

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† Corresponding author : Dr. Hosup Shim, Department of Physiology, College of Medicine, Dankook University, Cheonan, Chungnam 330-714, Korea, E-mail: shim@dku.edu

MATERIALS AND METHODS

Collection of Oocytes

Female B6D2F1 (C57BL/6 × DBA/2) mice (8-10 weeks old) were used to collect oocytes. Forty eight hours apart, 5 IU each of PMSG (pregnant mare serum gonadotropin; Intervet, The Netherlands) and hCG (human chorionic gonadotropin; Intervet) were injected into the abdominal cavity of the mouse. Thirteen hours after the hCG injection, matured oocytes were surgically harvested from the oviduct.

Media

Parthenogenetic embryos and reconstructed oocytes/embryos were cultured in CZB-glucose (CZBG) and MEMα (minimum essential medium Eagle alpha modification, Sigma Chemical Co., USA), respectively at 37℃ under 5% CO_2 in air (Chung et al., 2002). Oocyte/embryo manipulations prior to culture were carried out in Hepes-CZB-glucose (HCZBG) at room temperature (Chung et al., 2002).

Preparation of Donor Cells

Cumulus cells were allowed to disperse in HCZBG containing 0.1% (w/v) bovine testicular hyaluronidase for 5 minutes. Scattered cells were collected and cultured in CZBG at 37℃ under 5% CO_2 in air until injection into oocytes.

Nuclear Transfer

Maturated oocytes were transferred into a drop of HCZBG containing 2.5 μg/ml cytochalasin B. Oocyte was immobilized by a holding pipette, and spindles presumably stretched from the chromosomes of the second metaphase oocyte were visualized by Polscope (SpindleView, Cambridge Research & Instrumentation, USA). Chromosome spindle complex was removed using a blunt enucleation pipette with inner diameter of 10 μm attached to piezo-driven micromanipulator (PrimeTech, Japan). The procedure of Polscope-assisted enucleation was represented in Fig. 1.

Prior to injection into oocytes, cumulus cells were transferred into a drop of HCZBG containing 5% PVP (polyvinylpyrolidone, v/v). Enucleated oocytes were cultured in CZBG at 37℃ under 5% CO_2 for 1 hour and transferred into HCZBG. An individual cumulus cells were aspirated into an injection pipette with inner diameter of 6~8 μm. The cells was drawn in and out of the pipette to break the cell membrane. With several piezo pulses a single cumulus cell was gently injected into the oocyte. The procedure of nuclear transfer was represented in Fig. 2.

Oocyte Activation

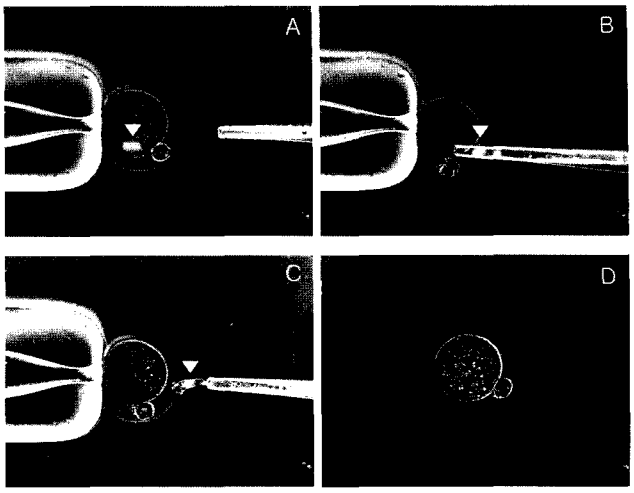

Fig. 1. Enucleation procedures. Removal of metaphase using a holding (left) and an enucleation (right) pipette. A spindle visualized under Polscope (arrowhead). (A) Immobilized oocyte using a holding pipette prior to enucleation. (B and C) Removal of chromosome spindle complex using an enucleation pipette. (D) Oocyte after the removal of the nucleus.

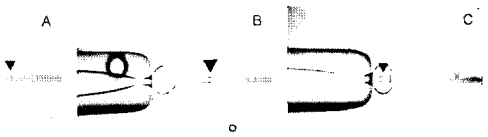

Fig. 2. Nuclear transfer procedures. (A) Aspiration of a cumulus cell (arrowhead) into an injection pipette. (B and C) Injection of a cumulus cell nucleus (arrowhead) into an enucleated oocyte.

To produce parthenogenetic embryos, oocytes were activated by an incubation in Ca^{2+}-free CZBG containing 5 μg/ml cytochalasin B and 10 mM $SrCl_2$ for 6 hours. Development of activated oocytes to the blastocyst stage was compared with embryos obtained from normal fertilization, and the results were analysed using Student's *t*-test. Reconstructed oocytes were placed in CZBG for 2 hours and activated by the same procedure used for the production of parthenotes. Nuclear- transferred embryos were cultured in MEMα, and the development into the blastocyst stage was monitored.

RESULTS

Induction of Oocyte Activation

In vitro development of oocytes from normal fertilization and parthenogenetic activation was presented in Table 1. Although oocytes activated by $SrCl_2$ treatment developed to the blastocyst stage, the rate of development was yet inferior to the embryos from normal

Table 1. *In vitro* development of activated oocytes

Embryos from	No. of oocytes	No. (%) of embryos developed to	
		2 cell	Blastocyst
Normal fertilization	79	69 (87.3)	59 (74.6)[a]
$SrCl_2$ treatment	262	241 (91.9)	146 (55.7)[b]

[a,b] Values with different superscripts within the same column are significantly different ($P<0.01$).

Table 2. *In vitro* development of nuclear-transferred embryos

No. of reconstructed oocytes	No. (%) of nuclear-transferred embryos developed to				
	2 cell	4 cell	8 cell	Morula	Blastocyst
93	14 (15.0)	12 (12.9)	10 (10.7)	7 (7.5)	4 (4.3)

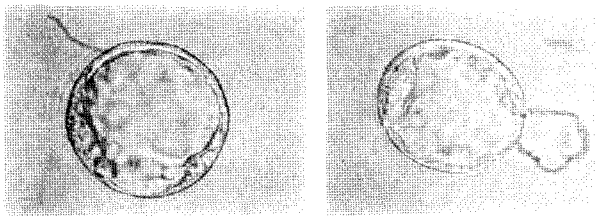

Fig. 3. Nuclear-transferred embryos. The transfer of cumulus cell nuclei into enucleated oocytes gave rise to expanded blastocyst (left) and hatching blastocyst (right) stage embryos.

fertilization.

Somatic Cell Nuclear Transfer

The results from the transfer of nuclei from cumulus cells into enucleated oocytes was summarized in Table 2. Despite of obtaining normal blastocysts based on their morphology (Fig. 3), the rate of development to the blastocyst stage was extremely low (4.3%).

DISCUSSION

The present study aimed for an improvement of nuclear transfer procedures in mice. One way of achieving such improvement may be an optimization of oocyte activation. We tested the $SrCl_2$ treatment which is often used for the activation of mouse oocytes. As summarized in Table 1, approximately a half of the strontium-treated oocytes developed into morphologically normal blastocysts. Comparing to normal fertilization, however, the rate of blastocyst development was not sufficient, indicating that the discrepancy was close to 20% (74.6% vs 55.7%) in blastocyst formation. This suggests that activation protocols of wide use still leave room for an improvement. In mice, strontium has been shown to induce multiple intracellular spikes in a fashion similar to that of normal fertilization (Bos-Mikich et al., 1995) and has efficiently been used to induce activation of oocytes (Cuthbertson et al., 1981). However, other treatments should also be in consideration to enhance the effectiveness of oocyte activation in mice, including ethanol (Cuthbertson, 1983), calcium ionophore (Nakasaka et al., 2000), puromycin (Nakasaka et al., 2000), propofol (Janssenswillen et al., 1997), 1,2-propanol (Shaw and Trounson, 1989) and electric pulses (Onodera and Tsunoda, 1989; Henery and Kaufman, 1993). These treatments may be used either alone or in combination with protein synthesis inhibitor (Siracusa et al., 1978; Hagemann et al., 1995). Optimization of procedures inducing oocyte activation in mice still remains for further development.

Enucleation of oocyte is a prerequisite step in nuclear transfer. Conventional procedure of enucleation involves the staining of nuclear material with DNA-specific dye such as Hoechst 33342 and subsequent exposure to UV light to locate the second metaphase chromosomes. However UV light may cause a damage of both genomic and mitochondrial DNA. To avoid such damage we attempted enucleation of oocytes with an assistance of Polscope in the present study. Polscope provides non-invasive imaging of intracellular structure such as metaphase spindles (Liu, 2000), so that it was able to locate a metaphase plate in oocyte without using of DNA-specific dye and UV. Since we could assure the removal of metaphase chromosomes in all oocytes that the enucleation was performed due to a visualization of metaphase spindle during an entire procedure of enucleation. This may not just only be advantages that the procedure is more convenient to operator and less harmful to oocytes, but may also be beneficial in long-term development of nuclear-transferred embryos.

The results of nuclear transfer carried out in the present study fell short of expectation. The rate of blastocyst formation that ultimately reflects an efficiency of nuclear transfer was disappointingly low (4.3%). However, this may not necessarily imply that the factors tested in this study to optimize the nuclear transfer procedure were not important. Rather there would be lot more elements that need to be optimized by further studies since the nuclear transfer is a complicated procedure, and an optimization of a single factor may not drastically improve experimental results.

Other factors that should be closely investigated in the future include the strains of the mouse permissive to the nuclear transfer procedure, media for embryo micromanipulation and culture, treatment of nuclear donor cells, coordination of cell cycles between the karyoplast and cytoplast, embryo micromanipulation equipments

and techniques, mitochondrial contribution in nuclear transfer, electrofusion between enucleated oocytes and donor cells, embyro culture method, embryo transfer, pregnancy maintenance, expertness of experimentor, and so on. This would be only a part of the list that active researches are currently in progress. With outcome of such researches, an efficiency of nuclear transfer will be markedly improved. It has been only a few years since the first success of somatic cell nuclear transfer in mammal was acknowledged by the birth of cloned lamb 'Dolly' (Wilmut et al., 1997), and perhaps we are still standing at the starting line of this exciting new field of science.

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