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On the Development of Parthenogenetic Oocytes by Cytochalasin B and Production of Cloned Mice by SCNT

Bo-Woong Sim[1,2] and Kwan-Sik Min[1,†]

[1]*Animal Biotechnology, Graduate School of Bio. and Information Technology, Institute of Genetic Engineering, Hankyong National University, Ansung 456-749, Korea*
[2]*National Primate Research Center, Korea Research Institute of Bioscience and Biotechnology, Ochang 363-883, Korea*

ABSTRACT

This study was conducted to optimize the efficiency of cloning and to produce cloned mice. The majority of cloned mammals derived by nuclear transfer (NT) die during gestation and have enlarged and dysfunctional placentas. In this study, the optimized conditions were established to produce clone mice. The parthenogenetic oocytes were activated after 6 h regardless of cytochalasin B (CB) concentration. CB treatment (2 μg/ml) was found second polar body. Lower concentration of CB was decreased the activation rate, but the second polar body was the best highly increased during 6 h incubation. The small fragments were exhibited in the 5 μg/ml treatment of CB, but it was not found in lower concentration groups (> 2.5 μg/ml). To examine effects of $SrCl_2$ on the adult cumulus cells, somatic cell NT oocytes were exposed during 0.5, 1 and 6 hrs. The second polar body was significantly greater in 0.5 h exposure group (6.6%) than 1, 6 hrs. Developmental rate from 2-cell to 4-cell was the lowest in 7.5 mM Strontium chloride ($SrCl_2$) groups (84.1% and 64.3%) than 5, 10 m $MSrCl_2$. The implantation rate was not significantly difference among 5, 7.5 and 10 m $MSrCl_2$ group. Three live fetuses were produced by SCNT. SCNT placentas were remarkably heavier than IVF group (8 fetuses) (0.34, 0.34, 0.33 vs 0.14 g) compared with the placenta weight of IVF and SCNT clones.
(Key words : parthenogenetic oocytes, cytochalasin B, cloned mice)

INTRODUCTION

The breakthrough in the study of cloning was the birth of the first clone from a somatic cell (Wilmut *et al.*, 1997). Successful somatic cell cloning has been reported in various animal, such as cows (Kato *et al.*, 1998; Cibelli *et al.*, 1998; Wells *et al.*, 1999), goats (Baguisi *et al.*, 1999), pigs (Onishi *et al.*, 2000; Polejaeva *et al.*, 2000), cats (Shin *et al.*, 2002) and mice (Wakayama *et al.*, 1998). Mice have been cloned using a piezo-actuated nucleus transfer (NT) method with cumulus cells (Wakayama *et al.*, 1998), tail-tip cells (Wakayama and Yanagimachi, 1999), sertoli cells (Ogura *et al.*, 2000), embryonic stem cells (Wakayama *et al.*, 1999b) and germ cells (Miki *et al.*, 2005) as nucleus donors.

To improve development of SCNT embryos, cytochalasin B should be applied at a concentration that varies with the degree of spindle formation after SCNT. Cytochalasin D, like CB, is a microfilament-disrupting reagent that has been used to induce polyploidy in pre-implantation embryos (Snow, 1973; Siracusa *et al.*, 1980; Bos-Mikich *et al.*, 1997). Mammalian cells adapted to *in vitro* culture generally undergo mutations with time, including gross karyotypic alterations, and such genomic changes are unlikely to be compatible with normal embryonic development (Wakayama *et al.*, 1999). Molecular analysis of cloned embryos reveals abnormal epigenetic modification, such as DNA methylation and histone modification (Dean *et al.*, 1998, 2001; Kang *et al.*, 2001; Ohgane *et al.*, 2001; Rybouchkin *et al.*, 2006). Cloning mice from embryonic stem (ES) cells also has practical implications for manipulating the genome. Gene targeting in ES cells has been widely used to create strains of mice with targeted mutations (Capecchi, 1989; Ramirez-Solis *et al.*, 1993).

Here, we report that the parthenogenetic oocytes were activated with varied parameters (CB, $SrCl_2$, exposure time). We cloned three live-born offspring from the cumulus cell.

MATERIALS AND METHODS

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† Correspondence : ksmin@hknu.ac.kr

1. Animals

B6D2F1 mice (C57BL/6 × DBA/2) were used to prepare oocytes and cumulus cells. Surrogate females were ICR females mated with vasectomized males of the same strain. All experi- ments were conducted according to the Guidelines for the Care and Use of Animals, Hankyong National University. The pro- tocol was approved by the Committee on the Ethics of Animal Experiments of the Hankyong National University (Permit Number : 2012. 10).

2. Collection of Oocytes and Preparation of Donor Cells

Briefly, MII oocytes were collected from the oviducts of 6～12-week-old females that had been induced to superovulate by injection of pregnant mare serum gonadotropin (PMSG) (7.5 IU) followed by human chorionic gonadotropin (hCG) (7.5 IU) 48 h later. Oocytes were collected from oviducts 13～14 h after hCG injection, placed in HEPES-buffered CZB medium, and treated with 300 unit/ml hyaluronidase until the cumulus cells dispersed. The oocytes were transferred to fresh droplets of HEPES-buffered CZB medium and were denuded of almost all cumulus cells by gentle pipetting. The oocytes were then placed in synthetic oviductal medium enriched with potassium medium (KSOM) (Summers *et al.*, 1995), covered with paraffin oil (Nacalai Tesque, Kyoto, Japan), and stored at 37℃ in a 5% CO_2 at mosphere until use.

Cumulus cells of B6D2F1 mice were removed from oocytes using hyaluronidase as described above. Cumulus cells were transferred and maintained in HEPES-buffered CZB medium at 4℃. Immediately prior to use, cumulus cells were resuspended in 10% polyvinylpyrrolidine (PVPD) in HEPES-buffered CZB medium.

3. Production of Parthenogenetic Activation Embryos

MII oocytes were collected from superovulated B6D2F1 female mice 14 h after hCG injection. The oocytes were incuba- ted in KSOM at 37℃ under 5% CO_2 for 3～4 h prior to PA by $SrCl_2$ in Ca^{2+}-free CZB supplemented with 2, 3, 4 or 5 μg/ml CB for 6 h. After activation, the PA embryos were cul- tured in KSOM at 37℃ under 5% CO_2 until the morula and blastocyst stage.

4. Enucleation of MII Oocytes and Activation and Culture of Injected Oocytes

Enucleation was performed in HEPES-buffered CZB medium supplemented with 3 or 5 μg/ml CB. The zona pellucida was “drilled” by applying several piezo-pulses at the tip of an enu- cleation pipette (~12 μm outside diameter). The chromosome- meiotic spindle complex was then gently pulled away from the oocyte until the cytoplasmic bridge was severed (Kono *et al.*, 1993). Enucleated oocytes were washed three times in CB-free CZB medium, transferred into CB-free KSOM, and stored for up to 1 h at 37 ℃ under 5% CO_2 until nucleus transfer.

The injected oocytes were activated by 5, 7.5 or 10 mM $SrCl_2$ in Ca^{2+}-free CZB at 37℃ under 5% CO_2. Oocytes activated for 0.5 or 1 h was cultured for an additional 5.5 or 5 h in the presence of 2.5 μg/ml CB (for a total of 6 h) to prevent the extrusion of polar bodies that contained donor chromosomes. Oocytes with at least one pseudo-pronucleus and without a second polar body were considered normally activated and were washed and cultured in KSOM at 37℃, 5% CO_2 until they reached the 2-cell or morula/blastocyst stage.

5. Nucleus Transfer

SCNT embryos were produced by injecting a cumulus cell nucleus into an enucleated B6D2F mature oocyte. Single cu- mulus cells in 10% PVP medium were drawn in and out of an injection pipette (6～7 μm inner diameter) until the plasma membranes were broke. After a nucleus was drawn deep into the pipette, the pipette was then passed through the zona pellu- cida of an enucleated oocyte by applying piezo pulses. The nucleus was then injected into an enucleated oocyte at room temperature using the piezo-driven pipette, as described pre- viously (Wakayama *et al.*, 1998). Injected oocytes were kept in KSOM for 1～2 h before activation treatment at 37℃ under 5% CO_2 in air and transferred.

6. Embryo Transfer and Examination of Placentas

Mice induced to superovulate by injection of PMSG (7.5 IU) followed by hCG (7.5 IU) 48 h later. Oocytes were collected from oviducts 13～14 h after hCG injection. After maturation, a detailed procedure has described previously (Fukuda *et al.*, 1987). Some of two-cell embryos (24 h after the onset of activation) were transferred to the oviducts of foster mothers (ICR, albino) that had been mated with vasectomized ICR males 1 day previously. SCNT embryos were transferred to the uteri of foster mothers (ICR, albino) that had been mated with vasec- tomized ICR males 3 days previously. Each recipient surrogate female received 10～20 SCNT embryos. Some of the recipient

females were euthanized at 9.5 dpc and examined for the presence or absence of fetuses and implantation sites. Live fetuses were raised by lactating foster mothers (ICR). Offspring and placenta obtained by Caesarean section on day 19 of pregnancy were individually weighed.

7. Statistical Analysis

Statistical analyses were performed using Minitab software. Data were analyzed using one-way ANOVA. Differences were considered significant (P<0.05, P<0.01).

RESULTS

1. Development Rate of PA Oocyte and Embryos by CB Concentration

Four different CB concentration (2, 3, 4 and 5 μg/ml) to activate MⅡ oocytes was assessed to optimize in activation medium. The majority (83.1～94.4%) of PA oocytes was activated after 6 h regardless of the used CB concentration. The second polar body (10.2%) was found in 2 μg/ml CB treatment group (Table 1). The developmental rate of activated PA embryos was analyzed to the expanded blastocyst. PA embryos were developed to 2-cell (97.3～100%), 4-cell (97.3～100%) and blastocyst (89.7～95.9%) in different CB concentrations. Finally, the expended blastocyst rate was not significant differences as 89.7～95.9% (Table 2).

2. Development Rate of SCNT Embryos by CB Concentration

To accurately evaluate the optimal concentration of CB in SCNT embryo's activation, the activation and development rates of reconstructed embryo were determined in five different CB concentrations (2～5 μg/ml). SCNT oocytes were activated over 84.5% at CB concentration (>2.5 μg/ml). And there was observed two pseudo-pronucleus after 6 h (Fig. 1B). But, activation

Table 1. Activation rate of PA oocytes activated with CB concentrations for 6 h

Conc. CB (μg/ml)	No. used oocyte	No. >2PP* (%)	No. not activated (%)	No. 1pp and 2pb* (%)	No. lysis (%)
5	72	68(94.4)	0(0.0)	0(0.0)	4(5.6)
4	79	73(92.4)	0(0.0)	0(0.0)	6(7.6)
3	76	67(88.2)	0(0.0)	0 (0.0)	9(11.8)
2	59	49(83.1)	0(0.0)	6(10.2)	4(6.8)

* PP: pseudo-pronucleus, 2pb : second polar body.
There was no significantly different between each value in the same column.

rate was decreased to 72.6% in CB 2 μg/ml group. Activation rate in CB 2 μg/ml group was significantly lower than that of CB 5 μg/ml group. Especially, 12.9% of SCNT embryos activated with CB 2 μg/ml was extruded the second polar body (Table 3).

SCNT embryos of all groups were developed to the 2-cell stage (83.3～94.0%) in the developmental rate in different CB concentrations. However, the SCNT embryos activated with

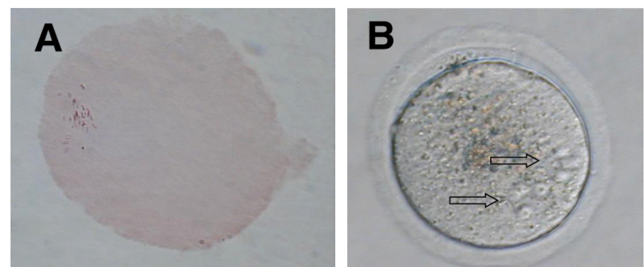

Fig. 1. Reconstruction of the somatic nuclei. (A) The nucleus of cumulus cell as donor cell were transformated into disarrayed chromosome at 3 hours after nucleus transfer. (B) Two pseudo- pronucleus (allow) was formed 6 hours after activation in SCNT embryos.

Table 2. Development of PA embryos activated with CB concentration

Conc. CB (μg/ml)	No. use oocytes	No. activated (%)	No. embryos development to (%)			
			2-cell	4-cell	Blastocyst	Expanded blastocyst
5	72	68(94.4)	68(100.0)	68(100.0)	61(89.7)	61(89.7)
4	79	73(92.4)	71(97.3)	71(97.3)	70(95.9)	70(95.9)
3	76	67(88.2)	67(100.0)	67(100.0)	64(95.5)	64(95.5)
2	59	49(83.1)	48(98.0)	48(98.0)	47(95.9)	47(95.9)

There was no significantly different between each value in the same column.

Table 3. Activation rate of SCNT embryos activated with CB concentration for 6 h

Conc. CB (μg/ml)	No. injected oocytes	No. >2 PP* (%)	No. not activated (%)	No. 1pp and 2pb* (%)	Lysis (%)
5	163	150(92.0)[a]	6(3.7)	0 (0.0)[b]	7(4.3)
4	105	91(86.7)[a]	5(4.8)	0(0.0)[b]	9(8.6)
3	142	120(84.5)[a]	8(5.6)	1 (0.7)[b]	14(9.9)
2.5	162	140(86.4)[a]	20(12.3)	0(0.0)[b]	2(1.2)
2	62	45(72.6)[b]	8(12.9)	8(12.9)[a]	1(1.6)

* PP : pseudo-pronucleus, 2pb : second polar body.
Values with different superscripts were significantly different (P<0.05).

CB 4 or 5 μg/ml were resulted in a significant decrease in the development rate from 4 cell to expended blastocyst. Especially, it was greatly decreased in the 4-cell stage of CB 4 or 5 μg/ml groups (P<0.01). The rate was significantly different in the expended blastocyst. It was remarkably increased in CB 2, 3 and 2.5 μg/ml treated groups (48.9%, 52.9% and 46.7%, respectively, P<0.01) (Table 4). The two-cell stage embryos was found small fragments between blastomeres in SCNT embryos activated with CB 5 μg/ml. However, it was not detected in CB 2.5 μg/ml group (Fig. 2).

Thus, results suggest that the highest frequency of preimplantation development is SCNT oocytes activated with CB 2.5 μg/ml (expended blastocyst rate; 52.9%) (Table 4).

3. Activation Rate of SCNT Embryos by $SrCl_2$ Treatment and Production of Cloned Mice by SCNT

Three different exposure time (0.5, 1 and 6 h) to activate MⅡ oocytes was assessed to optimize in activation time. The majority (86.2～88.0%) of SCNT embryos was activated in exposure time during 1 and 6 h. It was significantly decreased in the 0.5 h exposure group (63.3%) (P<0.01). However, the second polar body was significantly greater in 0.5 h exposure group (6.6%) (Table 5).

To examine effects of $SrCl_2$ concentration on adult cumulus cell clone, SCNT oocytes were activated by $SrCl_2$ (5, 7.5 and 10 mM) treatment for 6 h. Developmental rate from 2-cell to 4-cell was the lowest in 7.5 mM $SrCl_2$ groups (84.1% and 64.3%, respectively). And then, the morula/blastocyst stage was also the lowest (45.2%). The morula/blastocyst stage SCNT embryos were transferred to surrogate mothers. Recipient females were examined the implantation rate at 9.5 day of pregnancy. There was nosignificantly difference in implantation rate among 5, 7.5 and 10 mM $SrCl_2$ group (data not shown). As shown in Fig. 3, three live fetuses were produced by SCNT. To compare the placenta weight of IVF and SCNT clones, SCNT placentas were remarkably heavier than IVF group (8 fetus) (0.34, 0.34, 0.33 vs 0.14g, respectively).

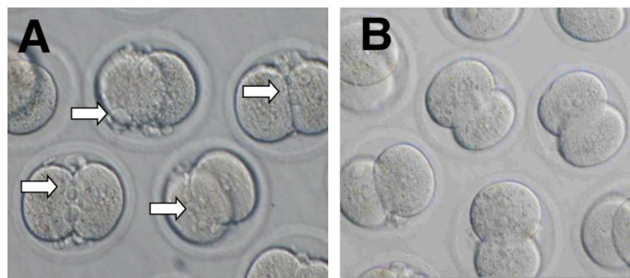

Fig. 2. Two-cell stage of SCNT embryos. (A) SCNT embryos were activated with 5 μg/ml CB, almost embryos show fragments between blastomeres (arrows). (B) SCNT embryos were activated with 2.5 μg/ml CB.

Table 4. Development of SCNT embryos activated with CB concentrations

Conc. CB (μg/ml)	No. injected oocytes	No. activated embryos (%)	No. embryos development to (%)				
			2-cell	4-cell	Morula	Blastocyst	Expanded blastocyst (/activated), (/injected)
5	163	150(92.0)[a]	138(92.0)	62(41.3)[df]	47(31.3)[df]	40(26.7)[df]	34(22.7)[bdf],(20.9)[d]
4	105	91(86.7)[a]	86(94.5)	42(46.2)[bf]	36(39.5)[bf]	32(35.2)[bf]	29(31.9)[d],(27.6)[b]
3	142	120(84.5)[a]	100(83.3)	88(73.3)[e]	70(58.3)[e]	66(55.0)[ac]	56(46.7)[e],(39.4)[c]
2.5	162	140(86.4)[a]	128(91.4)	102(72.9)[e]	89(63.6)[e]	80(57.1)[e]	74(52.9)[c],(45.7)[ac]
2	62	45(72.6)[b]	41(91.9)	33(73.3)[e]	30(66.7)[ac]	26(57.8)[c]	22(48.9)[a],(35.5)

Values with different superscripts were significantly different (a vs. b (P<0.05), c vs. d and e vs. f (P<0.01)).

Table 5. Activation rate of SCNT embryos activated by $SrCl_2$ treatment

Time for exposure	No. of treated embryo	No. >2PP*(%)	No. not activated(%)	No. 1pp and 2pb*(%)	No. lysis(%)
0.5h	316	200(63.3)[c]	81(25.6)[d]	21(6.6)[f]	14(4.4)
1h	181	156(86.2)[a]	15(8.3)[e]	3(1.7)[g]	7(3.9)
6h	209	184(88.0)[a]	11(5.3)[e]	0(0.0)[g]	14(6.7)

* PP : pseudo-pronucleus, 2pb : second polar body.
Values with different superscripts were significantly different ($P<0.01$).

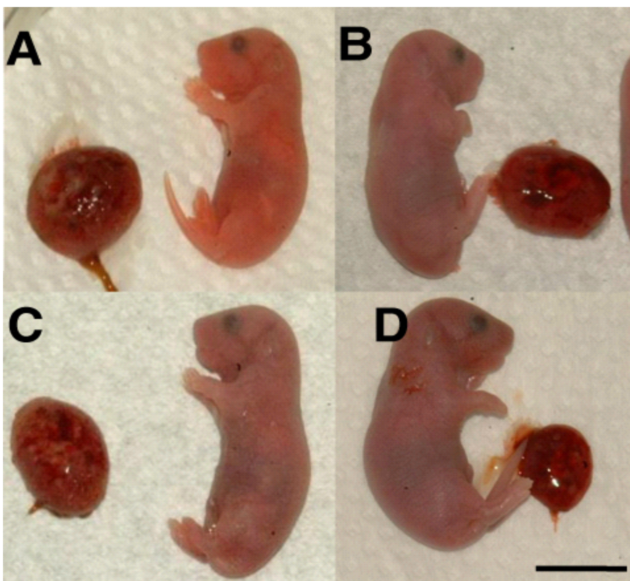

Fig. 3. The first 3 cloned pups born after nuclear transfer with adult cumulus cell (A-C). A pups born after In vitro fertilization as control (D). Gross abnormalities were not found in the pups. The first surviving cloned mouse, Rogen (a). The placenta was larger than of an *in vivo* fertilized pups (0.34, 0.34. 0.33 and 0.14g, respectively), Bar=1cm.

DISCUSSION

We produced three live-born clones from cumulus cell lines. Placentas weight in SCNT clones was greater heavier than IVF group. In the present study, most of the 2-cell stage embryos treated with CB (5 μg/ml) during activation had a small degree of fragmentation between blastomere. The most commonly used reagents for activation are CB and CD (Bos-Mikich, 1997), which disrupt actin filaments. Similarly, reconstructed embryos derived from aged fertilization-failure oocytes had small fragments between blastomeres (Wakayama *et al.*, 2007). These fragmentations may be especially harmful to the subsequent development of SCNT embryos.

For cloning, 5 μg/ml CB is usually included in strontium-containing activation media (Ono *et al.*, 2001; Inoue *et al.*, 2002). In the present study, a small percentage of the SCNT embryos exposed to 5 μg/ml CB developed into morula/blastocysts. The developmental rate to expended blastocyst resulted less than did SCNT embryos treated with CB 3 μg/ml. In contrast to reconstructed zygotes activated in the presence of CB, reconstructed zygotes activated with nocodazole have many small pseudo- pronuclei (Wakayama *et al.*, 2001). Thus, we suggest that 5 μg/ml CB is useful for reducing physical damage from micromanipulation during the enucleation.

$SrCl_2$ has been used as are agent for mouse oocyte activation in both physiological (Cuthbertson *et al.*, 1981; O'Neill *et al.*, 1991) and cloning studies (Wakayama *et al.*, 1998; Ono *et al.*, 2001; Kono *et al.*, 2004). $SrCl_2$ concentrations and the duration of treatment vary greatly between studies; ranging from 1～100 mM and from 10 min to 6 h (Ono *et al.*, 2001; Kono *et al.*, 2004; Otaegui *et al.*, 1999; Marcus *et al.*, 1990). Usually, 10 mM $SrCl_2$ is included in activation media used for cloning (Klishigami *et al.*, 2006; Boiani *et al.*, 2003).

In the present study, placenta overgrowth was only observed in SCNT clones and not in IVF. High placental weights have been reported in cloned farm animals and mice (Young *et al.*, 1998; Wakayama *et al.*, 1998; Ono *et al.*, 2001). In bovine, SCNT clones commonly have a high rate of placental defects (Yang *et al.*, 2007). In the present study, the placental weight of SCNT embryos was significantly increased in comparison to that of IVF. Previous reports also indicate that clone fetuses from ES cells have a mean placental weight of 0.32 g: significantly heavier than the placentas of ES cell-tetraploid pups and pups derived from normal embryos cultured *in vitro* to the blastocyst stage (Eggan *et al.*, 2001).

Our results demonstrate that cloning using cumulus cells have been observed a larger placental weight. Thus, to increase the rate of cloning in mice SCNT, placental weight should be

reduce. Researchers will be required to evaluate the specific function of the differentially expressed molecules in cloned placentae and regulatory mechanisms. It is thus important to determine when and in what part of the developing SCNT embryo critical genes for normal development of the placenta are differentially expressed.

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