Asian-Aust. J. Anim. Sci.
Vol. 21, No. 11 : 1665 - 1672
November 2008

www.ajas.info

In vitro Development of Interspecies Somatic Cell Nuclear Transfer Embryos Derived from Murine Embryonic Fibroblasts and Bovine Oocytes

J. I. Yun[a], B. S. Koo[a], S. W. Yun and C. K. Lee*

Department of Agricultural Biotechnology, Animal Biotechnology Major,
and Research Institute for Agriculture and Life Science, Seoul National University, Seoul 151-921, Korea

ABSTRACT : Interspecies somatic cell nuclear transfer (iSCNT) is a useful method to preserve endangered species and to study the reprogramming event of a nuclear donor cell by the oocyte. Although several studies of iSCNT using murine cells and bovine oocytes have been reported, the development of murine-bovine iSCNT embryos beyond the 8-cell stage has not been successful. In this paper, we examined the developmental potential of embryos reconstructed with a murine embryonic fibroblast as the nuclear donor and a bovine oocyte as the cytoplasm recipient. The reconstructed embryos were cultured in CZB (murine medium) or CR1aa (bovine medium). In addition, for the development of a murine-bovine iSCNT blastocyst, the antioxidant β-mercaptoethanol (βME) was supplemented to CR1aa medium. Furthermore, to verify the mouse genome activation in murine-bovine iSCNT embryos, RT-PCR analysis of murine *Xist* was performed. The development of the murine-bovine iSCNT embryos cultured in CR1aa was significantly higher than that in CZB ($p<0.05$). With respect to the effect of BME on the development of the murine-bovine iSCNT blastocyst, addition of BME produced a significant increase in blastocyst development ($p<0.05$). Karyotype analysis confirmed that the reconstructed embryos were derived from murine cells (40XX). The *Xist* gene was gradually increased from the 8-cell stage to the blastocyst stage. This is the first report of blastocyst development of iSCNT embryos derived from murine somatic cells and bovine oocytes. These results demonstrate that bovine cytoplasm can support the development of later stages of a preimplantation embryo from murine-bovine iSCNT. (**Key Words :** Murine, Bovine, Interspecies Somatic Cell Nuclear Transfer, β-Mercaptoethanol, *Xist*)

INTRODUCTION

Somatic cell nuclear transfer (SCNT) is a technology to create an exact genetic match of the donor by transferring the nucleus moved from donor into the enucleated recipient oocyte. Since the Dolly the sheep was born in 1997 (Wilmut et al., 1997), the SCNT has been attempted in many other species (Kato et al., 1998; Wakayama et al., 1998; Baguisi et al., 1999; Polejaeva et al., 2000; Chesne et al., 2002; Shin et al., 2002; Constance et al., 2003; Galli et al., 2003) and expanded its application in basic science as well as in applied science. It was suggested that SCNT could be adopted to increase the population of endangered mammals and preserve them, or even to restore the extinct species (Kenneth, 2002). However, in exotic or endangered species, the lack of oocytes and recipients precludes the use of traditional SCNT. Therefore, an approach such as interspecies SCNT (iSCNT), nuclear transfer using the somatic cells from the one species and the oocytes from other species, may be the only alternative to produce embryos and offspring (Saninena et al., 2005).

In several studies, it was reported that oocyte cytoplasm from bovines, rabbits, and sheep can support early development of embryos produced by nuclear transfer of somatic cell nuclei from various mammalian species (Chen et al., 1999; Domingo et al., 1999). Dominko et al. (1999) reported that bovine oocyte cytoplasm could support the blastocyst development of embryos produced by the NT of somatic cell nuclear material from sheep, pigs and monkeys. Other studies insisted that iSCNT experiments using giant panda (Chen et al., 1999), water buffalo (Kitiyanant et al., 2001), and argali (White et al., 1999) have had been successful in the production of the blastocyst. In addition, interspecies somatic cloned gaur (Lanza et al., 2000) and mouflon (Loi et al., 2001) have also been successfully produced. However, in spite of these successes, iSCNT, even between closely related species often fail to produce viable offspring (White et al., 1999; Lanza et al., 2000). In

* Corresponding Author: Chang-Kyu Lee. Tel: +82-2-880-4805, Fax: +82-2-873-4805, E-mail: leeck@snu.ac.kr
[a] These authors are equally contributed to this work.
Received October 27, 2007; Accepted July 26, 2008

addition, the iSCNT embryos using distant species, such as inter-genus or inter-family, were capable of only limited embryonic development (Chen et al., 1999; Dominko et al., 1999). Recently, by the iSCNT using dog oocytes, cloned gray wolves were successfully born and alive in good health (Kim et al., 2007).

Mice were successfully cloned first by Wakayama et al (Wakayama et al., 1998) and others (Oguara et al., 2000). Since the mouse is one of the best well-established experimental animals, the SCNT using mouse system could provide valuable information on the fundamental mechanism of the nuclear transfer. Moreover, the iSCNT using mouse system could offer a good model for the preservation of the endangered species by iSCNT as well as exploiting the production of embryonic stem cells by therapeutic cloning.

Unfortunately, until now, a few papers about iSCNT experiments using mouse somatic cells and bovine oocytes were reported (Arat et al., 2003; Park et al., 2004). Arat et al. (2003) and Park et al. (2004) attempted murine-bovine iSCNT by transferring mouse embryonic fibroblast cell into bovine cytoplasm using electrofusion method. However, they had failed to produce the murine-bovine iSCNT blastocyst except all cloned embryos were arrested at 8-cell stage. They suggested that it is associated with abnormal reprogramming around the time of the embryonic genome activation rather than chromatin remodeling. In addition, a sub-optimal culture conditions for the murine-bovine iSCNT embryos could result in the developmental failure (Arat et al., 2003).

In this study, we observed the developmental potential of iSCNT embryos reconstructed with murine embryonic fibroblasts and bovine oocytes. In particular, the effect of an antioxidant, β-mercaptoethnoal, on the development of *in vitro*-cultured murine-bovine iSCNT embryos was assessed. We also examined murine *Xist* gene expression during the embryonic genome activation in the murine-bovine iSCNT embryos. In conclusion, a bovine cytoplasm can support the development of later stage of a preimplantation embryo and the expression of murine gene during embryonic genome activation in the murine-bovine iSCNT embryos. This is the first report of the blastocyst development of iSCNT embryos derived from the murine somatic cells and the bovine oocytes.

MATERIALS AND METHODS

Bovine oocyte collection and *in vitro* maturation

Cumulus-oocyte complexes (COCs) were aspirated from antral follicles (2-8 mm) on ovaries obtained from a slaughterhouse. Groups of 35 COCs were incubated and matured in TCM-199 medium (Gibco, Grand Island, NY) with 10% FBS (Gemini, St. Woodland, CA), 1% penicillin-streptomycin (Giboco), LH (0.5 μg/ml; Sigma-Aldrich, St Louis, MO) and FSH (0.5 μg/ml; Sigma-Aldrich) at 39°C in 5% CO_2 in humidified air for 20-24 h.

Preparation of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were isolated by removed organs from each fetus at the 14th d.p.c. and dispersed by exposure to 10 ml 0.25% trypsin-EDTA solution (Gibco). MEFs were collected by centrifugation and cultured on culture medium; Dullecco modified eagle medium (DMEM; Gibco) supplemented with 10% FBS (Gemini) and 1% penicillin-streptomycin (Gibco) at 37°C, 5% CO_2 in humidified air. The MEFs isolated from each fetus were karyotyped, and female cells were used as the donor. After culturing for several passages, cells were arrested at quiescent (G_0) stage by culturing in the serum starvation medium with 0.5% FBS (Gemini) for 3-7 days.

Murine-bovine interspecies somatic cell nuclear transfer (iSCNT)

Matured COCs were vortexed in 1.5 ml eppendorf tubes with 0.1% hyaluronidase (Sigma-Aldrich) for 5 min to remove cumulus cells. The zona pellucida of matured oocytes was slit by a cutting pipette, and the polar body was removed with parts of the cytoplasm, possibly containing bovine nucleus, by squeezing the oocyte. A single donor cell was deposited into periviteline space of each enucleated. Fusion of mouse cell and bovine enucleated oocyte was performed in fusion medium (280 mM mannitol) with two DC pulses of 1.90 kV/cm for 15 μsec duration with 1 sec interval (ECM2001; BTX, San Diego, CA). After fusion, the reconstructed embryos were treated with 5 μg/ml ionomycin (Sigma-Aldrich) for 4 min and subsequently with 2.0 mM 6-dimethylaminopurine (DMAP) (Sigma-Aldrich) under a humidified atmosphere of 5% CO_2 at 39°C for 3 h. The developmental potential of the murine-bovine iSCNT embryos were investigated under either the CR1aa or the CZB. In addition, the murine-bovine iSCNT embryos were cultured either in the CR1aa only or in the CR1aa until 72 h and then in the CZB henceforth (sequential medium). Moreover, either 5.5 μM or 11 μM β-mercaptoethanol (BME; Gibco) was supplemented into CR1aa to improve the developmental competence of the murine-bovine iSCNT embryos. The development of embryos was observed daily under an inverted microscope (Nikon, Japan).

Karyotype analysis and differential staining of murine-bovine iSCNT embryos

Murine-bovine iSCNT embryos were incubated in CR1aa containing 0.08 μg/ml of colchicine (Sigma-Aldrich) for 7-9 h to stop mitosis at metaphase, and then treated with hypotonic solution of 1% sodium citrate (Sigma-Aldrich) for 10 min and placed on a clean grease-free glass slide.

Table 1. *In vitro* development of murine-bovine interspecies somatic cell nuclear transfer embryos cultured in CZB or CR1aa medium

Medium	No. of reconstructed embryos	No. (%)[a] of embryos fused	No. (%)[b] of embryos developed to				
			2-cell	4-cell	8-cell	Morula	Blastocyst
CZB	106	68 (64)	51 (75)	19 (28)	1 (2)[c]	0 (0)[c]	0 (0)
CR1aa	144	91 (63)	61 (67)	21 (23)	16 (18)[d]	5 (5)[d]	1 (1)

Fusion was induced with two DC pulses of 1.90 kV/cm for 15 μsec with 1 sec interval. After fusion, the reconstructed embryos were treated with 5 μg/ml ionomycin for 4 min and subsequently with 2.0 mM 6-dimethylaminopurine for 3 h.

[a] Percentage of reconstructed embryos. [b] Percentage of fused embryos.

[c, d] Means with different superscripts within the same column are significantly different at $p<0.05$.

The slide was treated with a fixative mixture of methanol:acetic acid in a ratio of 1:1, followed by staining with 5% Giemsa (Gibco) for 10 min. The spread chromosomes were counted under a microscope (Olympus, Japan) at 1,000 fold magnification. To observe the nuclear number of inner cell mass (ICM) and trophectoderm (TE) cells of the murine-bovine iSCNT blastocysts, the differential staining (DS) method was performed. Briefly, TE cells were stained with 100 μg/ml of the fluorochrome propidium iodide after treatment with permeabilizing solution containing 1% (v/v) of the non-ionic detergent Triton X-100. The blastocysts were then incubated in a second solution containing 100% ethanol for fixation at 4-8°C and bisbenzimide for staining the ICM. Fixed and stained whole blastocysts were mounted and photographed under UV illumination using an inverted microscope (Nikon) with differential interference contrast optics.

RT-PCR analysis

RNA in iSCNT embryo was isolated from the Dynalbeads® mRNA kit (Dynal Biotech ASA, Oslo, Norway) and RT-PCR analysis was carried out using and i-StarTaq kit (iNtRON Biotechnology, Seoul, Korea). In brief, the RT-PCR reactions were initiated by reverse transcriptase reaction at 45°C for 30 min, and denaturation of the RNA:cDNA hybrid at 94°C for 15 min. PCR reactions were initiated by denaturing at 94°C for 30 sec, followed by 35 cycles of: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. After the last cycle, PCR products were allowed to extend at 72°C for 5 min. The murine *β-actin* gene was amplified from total cDNA with the forward primer, 5'-AGGTTTTGTCAAAGAAAGGG-3', and the reverse primer, 5'-AGAGTGCTGTCTGGTGGTAC-3'. The murine *Xist* gene was amplified from that with the forward primer, 5'-ACTGCCAGCAGCCTATACAG-3', and reverse primer, 5'-GTTGATCCTCGGGTCATTTA-3'. The PCR products were separated on a 2% agarose gel and visualized by staining with ethidium bromide.

Statistical analysis

All data were analyzed using a one-way ANOVA and generalized linear model (PROC-GLM) in the Statistical Analysis System (SAS) program was employed for evaluation of the all the treatment effects. Treatment differences were considered significant when the p value was less than 0.05.

RESULTS AND DISCUSSION

Effect of culture medium on the development of murine-bovine interspecies somatic cell nuclear transfer embryos

To determine the optimal *in vitro* culture medium for the murine-bovine interspecies somatic cell nuclear transfer (iSCNT) embryos, the generally used bovine embryo culture medium, CR1aa, and the murine embryo culture medium, CZB, were compared. The rate of blastocyst development in normal bovine-bovine SCNT using same condition and CR1aa medium was in the range of 20-25% (data not shown). The murine-bovine iSCNT embryos cultured in CR1aa medium developed to 8-cell and morula embryo stages, while those cultured in CZB were all arrested at 8-cell stage (Table 1). Even though there was no considerable development to blastocyst stage, one blastocyst was formed from the murine-bovine iSCNT embryos cultured in CR1aa (Table 1). Interestingly, no developmental block was observed at 2-cell stage embryos even with using the cells from the mouse strain (ICR) having 2-cell block. The overall developmental velocity of the murine-bovine iSCNT embryos was similar to that of the bovine embryos (data not shown).

Since the murine-bovine iSCNT embryos cultured in the CZB medium were all arrested at 8-cell stage, which is the stage of developmental block in bovine embryos, the sequential culture of the murine-bovine iSCNT embryos were attempted. The reconstructed embryos were cultured in CR1aa medium until 72 h (8-cell stage) and then cultured in the CZB medium henceforth. However, none of the embryos was developed over 8-cell stage (Table 2). The gross morphology of the normal murine-bovine iSCNT embryos showed similar to that of the bovine preimplantation stage embryos rather than the mouse (Figure 1). However, there were no detectable differences in the morphology of the murine-bovine iSCNT embryos cultured in two different media (data not shown).

In a previous study, Arat et al. (2003) and Park et al. (2004) attempted the murine-bovine iSCNT by transferring mouse embryonic fibroblast cell into bovine cytoplasm

Table 2. *In vitro* development of the murine-bovine interspecies somatic cell nuclear transfer embryos cultured in CR1aa only or in sequential medium

Medium	No. of reconstructed embryos	No. (%)[b] of embryos fused	No. (%)[c] of embryos developed to				
			2-cell	4-cell	8-cell	Morula	Blastocyst
CR1aa only	47	28 (60)	25 (89)	12 (43)	7 (25)	5 (18)[d]	0 (0)
Sequential medium[a]	59	36 (61)	32 (89)	14 (39)	5 (14)	0 (0)[e]	0 (0)

Fusion was induced with two DC pulses of 1.90 kV/cm for 15 μsec with 1 sec interval. After fusion, the reconstructed embryos were treated with 5 μg/ml ionomycin for 4 min and subsequently with 2.0 mM 6-dimethylaminopurine for 3 h.

[a] Embryos were cultured in the CR1aa medium until 72 h, and then cultured in CZB medium henceforth.

[b] Percentage of reconstructed embryos. [c] Percentage of fused embryos.

[d, e] Means with different superscripts within the same column are significantly different at $p<0.05$.

using electro-fusion method. Park et al. (2004) examined the nuclear and microtubule dynamics in murine-bovine iSCNT embryos and Arat et al. (2003) also investigated the nuclear structure of murine-bovine iSCNT embryos after activation. They reported that the bovine oocyte cytoplasm could support the nuclear remodeling in mouse-bovine iSCNT embryos. However, all of the murine-bovine iSCNT embryos were arrested at the 8-cell stage and no blastocysts were produced. They explained that the sub-optimal culture conditions might be related to the arrest in the development at the 8-cell stage. Therefore, we attempted to develop the *in vitro* culture medium for improving the blastocyst formation of mouse-bovine iSCNT embryo.

Laibfried-Rutledge et al. (1997) suggested that it is known that there are species-specific requirements for embryo culture media. In the iSCNT experiments using bovine oocyte cytoplasm, the bovine culture medium was utilized as a basal *in vitro* culture medium (Dominko et la., 1999; Dindot et al., 2004; Yanxin et al., 2006). Dominko et al. (1999) cultured the iSCNT embryos generated by the NT of somatic cell nuclear material from sheep, pigs, monkeys and rats with CR1aa medium, and they produced the blastocyst from sheep, pigs and monkeys (rat excepted). They proposed that the high proportions of cleaving embryos, regardless of the species of donor nucleus, developed to advanced stages in a culture medium designed for bovine embryo (Dominko et al., 1999). Sansinena et al. (2005) also cultured banteng-bovine iSCNT embryo with the CR1aa medium and they produced the pregnancies. In experiments, utilizing bovine oocytes and the TCM199 as a culture medium, the iSCNT blastocysts and fetuses were produced (Dindot et al., 2004; Yanxin et al., 2006).

In the case of murine-bovine iSCNT, Arat et al. (2003) used the TCM199 and Park et al. (2004) utilized the CR1aa

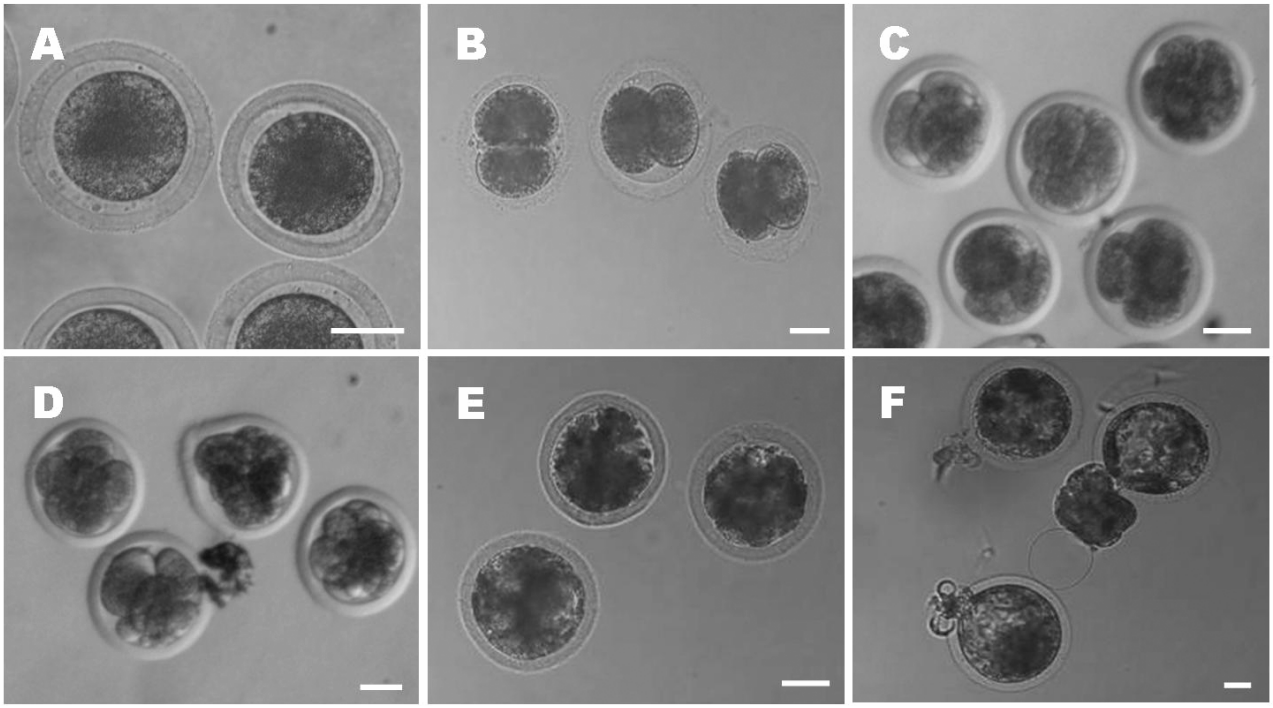

Figure 1. *In vitro* development of the murine-bovine interspecies somatic cell nuclear transfer embryos. (A) 1-cell stage embryos, (B) 2-cell stage embryos, (C) 4-cell stage embryos, (D) 8-cell stage embryos, (E) Morula stage embryos, (F) A blastocyst stage embryo. Scale bars = 50 μm.

Table 3. Effect of β-mercaptoethanol on *in vitro* development of murine-bovine interspecies nuclear transfer embryos cultured in CR1aa medium

Concentration of β-mercaptoethanol (μM)	No. of reconstructed embryos	No. (%)[a] of embryos fused	No. (%)[b] of embryos developed to				
			2-cell	4-cell	8-cell	Morula	Blastocyst
0	198	119 (60)	86 (72)	33 (27)	23 (19)	5 (4)	0 (0)[c]
5.5	154	91 (59)	74 (81)	32 (35)	14 (15)	5 (5)	2 (2)[cd]
11	166	103 (62)	85 (82)	39 (38)	19 (18)	6 (6)	4 (4)[d]

Fusion was induced with two DC pulses of 1.90 kV/cm for 15 μsec with 1 sec interval. After fusion, the reconstructed embryos were treated with 5 μg/ml ionomycin for 4 min and subsequently with 2.0 mM 6-dimethylaminopurine for 3 h.

[a] Percentage of total oocytes. [b] Percentage of reconstructed embryos.

[c, d] Means with different superscripts within the same column are significantly different at $p<0.05$.

as a basal culture medium. Arat et al. (2003) insisted that the ability of these bovine specific media to support cleavage development of inter-species embryos might be due to the fact that the initial development of embryos is generally driven by recipient cytoplasm, and thus any media supporting bovine embryo development might also support the initial development of interspecies NT embryos. However, all of the murine-bovine iSCNT embryos were arrested at 8-cell stage (Arat et al., 2003; Park et al., 2004). It is supposed that the distant species iSCNT embryos were capable of only limited embryonic development (Chen et al., 1999; Dominko et al., 1999; Arat et al., 2003; Park et al., 2004). Though CR1aa medium was designed for bovine embryo culture (Rosenkrans et al., 1993), Dominko et al. (1999) explained that CR1aa might be supporting the development of iSCNT embryos as successfully as it supports bovine embryos. Results in this study also present empirical evidence to support that CR1aa might be a more effective medium than CZB (mouse medium) in the murine-bovine iSCNT.

Effects of β-mercaptoethanol on the development of murine-bovine interspecies somatic cell nuclear transfer embryos

Although a single blastocyst development was observed in the murine-bovine iSCNT embryos cultured in CR1aa medium, the majority of the reconstructed embryos showed abnormal developments, such as fragmentation and irregular division of the blastomeres. To increase the development rate and the blastocyst formation rate in the murine-bovine iSCNT embryos, β-mercaptoethanol (BME), an antioxidant, was supplemented to CR1aa medium. Addition of BME (both 5.5 μM and 11.0 μM) increased the development rate of 2- and 4-cell embryo compared to the control, although there was no significant difference (Table 3). Although no significant differences in the embryonic development of 8-cell and morula stage among the control and treatment groups were founded, the addition of BME (both 5.5 μM and 11.0 μM) significantly increased the development of blastocyst compared to the control ($p<0.05$) (Table 3). The number of the murine-bovine iSCNT blastocysts in culture with 11.0 μM BME was slightly higher than in culture with 5.5 μM BME, even though there was no significant difference in the blastocyst formation rate (Table 3).

To characterize the murine-bovine iSCNT blastocysts, the differential staining and the karyotype analysis were performed. Differential staining showed the morphology of the blastocyst with good quality and well-developed inner cell mass (Figure 2A). The number of cells in the inner cell mass and trophectoderm of was 22 and 44, respectively. The murine-bovine iSCNT blastocysts showed normal 40 XX chromosomes by karyotype analysis, indicating that the blastocyst was derived from the mouse embryonic

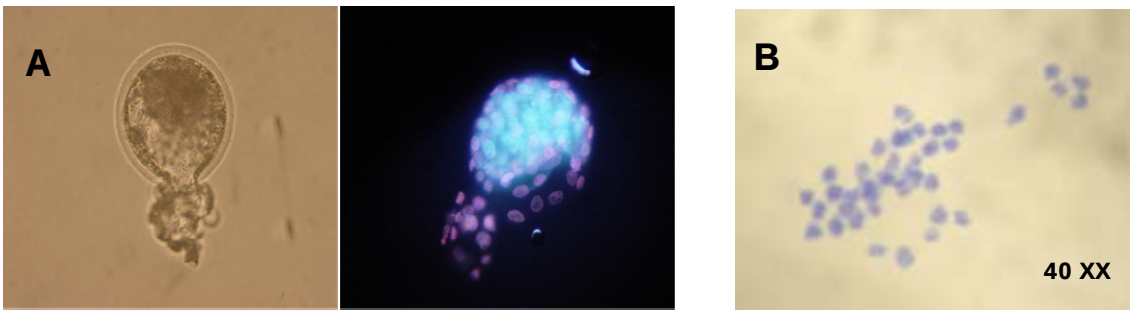

Figure 2. Differential staining and karyotype analysis of murine-bovine interspecies nuclear transfer blastocyst. (A) Differential staining of a murine-bovine interspecies nuclear transfer blastocyst, (B) Karyotype analysis of a murine-bovine interspecies nuclear transfer blastocyst (40XX).

fibroblast (Figure 2B).

Tedford et al. (1990) reviewed that the earliest stages of embryogenesis in normal embryos are regulated by maternally inherited gene products stored within the oocyte cytoplasm and progression of development becomes dependent in embryonic gene activation at a species-specific developmental stage. Therefore, we tested the effect of sequential medium, using bovine culture medium until 8-cell stage embryos and then mouse culture medium afterwards, for enhancing the *in vitro* development of murine-bovine iSCNT embryos. Nevertheless, in counterpoint to the hypothesis, the sequential medium might not be more effective than the CR1aa medium only.

In order to increase the blastocyst formation rate in the iSCNT embryo, we examined the effects of antioxidant-supplemented media. Oxidative stress due to the formation of reactive oxygen species (ROS) is also well known to influence the development of early mammalian embryos *in vitro* (Quinn et al., 1978; Noda et al., 1991). Toxic effects of oxygen tension on embryo development have been reported in mouse (Quinn et al., 1978; Pabon et al., 1989). Culturing mouse embryos under low oxygen conditions increases the blastocyst rate (Umaoka et al., 1992). Antioxidants play an important role in reducing stress-related damages in embryo development (Takahashi et al., 1993; Biggers et al., 2000). It is known that, in mouse, addition of BME, a strong antioxidant, in culture medium improves embryo development (Pabon et al., 1989). Takahashi et al. (1993) demonstrated that the addition of low molecular weight thiols, such as cysteamine and BME, to culture medium enhanced cysteine mediated GSH synthesis and improved the production of bovine embryos *in vitro*. This increased intracellular GSH content in oocytes and embryos of varied developmental stages improves embryonic development and embryo quality, resulting in higher blastocyst yield (Biggers et al., 2000). They investigated to determine the effect of 10 μM and 50 μM BME on the development of bovine embryo (Takahashi et al., 1993) and Cammano et al. (1996) also attempted to determine the effects of 0 μM and 100 μM BME on the development of bovine embryo. In cloned bovine embryos, Park et al. (2004) reported that antioxidants improved the embryonic development by decreasing the apoptosis in blastomeres. In mouse, Bagis et al. (2004) examined the effects of 10 μM BME on the development of mouse embryos obtained from *in vivo* and *in vitro*, and obtained increased blastocyst formation rate by addition of BME.

Since the supplementation of the antioxidants in culture medium could increase the developmental potential of *in vitro* produced embryos (Takahashi et al., 1993; Caamano et al., 1996; Bagis et al., 2004; Park et al., 2004), we expected that the addition of an antioxidant to the media for iSCNT embryos would have a similar beneficial effect. We

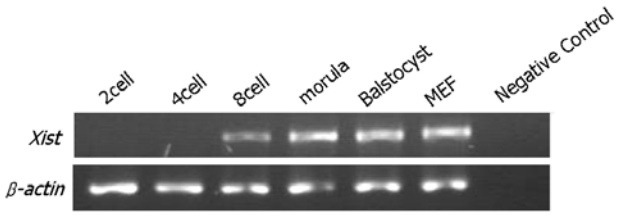

Figure 3. RT-PCR analysis of murine *Xist* gene expression in the murine-bovine interspecies somatic cell nuclear transfer embryos. Murine *Xist* gene was expressed gradually from 8-cell to blastocyst stage embryo.

observed that non-BME treated murine-bovine iSCNT embryos resulted in fewer 8-cell stage embryos and, although a single blastocyst was formed, no blastocysts were obtained afterward. However, BME-treated embryos developed into blastocysts at a rate of 2% and 4%, for 5.5 μM and 11.0 μM BME, respectively. In this study, the optimal concentration (11.0 μM) of BME for the murine-bovine iSCNT embryos was different from the result (50 μM) reported in bovine by Takahashi et al. (1993). In this study, the concentration of BME was followed that of used in mouse experiment (Bagis et al., 2004) since the iSCNT embryos were derived from mouse donor cell. Therefore, we propose that the improvement in blastocyst formation rate in the presence of BME the murine-bovine iSCNT embryos might be due to the beneficial effect of BME on embryo development *in vitro*, possibly reducing the oxidative stress.

RT-PCR analysis of the murine *Xist* gene expression pattern in the murine-bovine interspecies somatic cell nuclear transfer embryos

The zygotic genome activation in preimplantation stage embryo usually occurs at 2-cell stage embryos in the murine embryos, while at 8-cell stage embryos in the bovine embryos. To assess the mouse genome activation in the murine-bovine iSCNT embryos, the murine *Xist* gene expression was analyzed by the RT-PCR assay. Murine *Xist* gene was initially expressed at the 8-cell embryos and the expression continued up to the blastocysts (Figure 3). This result indicated that the expression of mouse genome activated not at 2-cell stage but at 8-cell stage murine-bovine iSCNT embryos, which might controlled by the bovine cytoplasm.

The gene expression patterns in SCNT embryos have been studied by comparing to the *in vitro* fertilized embryos, and a wide-range of the irregular gene expression was observed (Daniels et al., 2000; Winger et al., 2000; Rideout et al., 2001; Boiani et al., 2002; Niemann et al., 2002). Researchers suggested that the abnormal gene expression might result in the debased the development on

preimplantation embryos. Arat et al. (1993) and Park et al. (2004) investigated how the bovine ooplasm affected gene expression in mouse fibroblast nuclei at the 8-cell stage of development to develop a base understanding of gene expression in the murine-bovine iSCNT embryos. As a result, abnormal gene expression profiles were observed in several genes, such as housekeeping mouse genes (*hsp70, hprt, bax,* and *glu-1*), developmental related gene (*Oct-4* and *E-cad*) and zygotic genome activation related gene (*eAP*) in mouse-bovine iSCNT embryos (Arat et al., 1993; Park et al., 2004). They described that these results are related to the arrest in the development at the 8-cell stage of murine-bovine iSCNT embryos.

One of the differentially expressed genes by sequential time in development between murine and bovine preimplantation embryos is the *Xist*, an embryonic zygotic genome activation related gene. *Xist* is one of a considerable fraction of genes expressed during the early phase of mammalian development. In female mammals, the *Xist* gene is located at the X-inactivation center (XIC) (Maurizio et al., 2002) and is expressed beginning at the 1-cell stage in human (Daniels et al., 1997) the 4-cell stage in murine (Kay et al., 1993; Kay et al., 1994) and the 8-cell stage in bovine embryos (De La Fuente et al., 1999). Therefore, the *Xist* gene is appropriate for detecting differences between the murine and the murine-bovine iSCNT embryos because the *Xist* gene is not expressed until the developmental block and it is known to affect several developmental functions after the block. In this study, the *Xist* gene was expressed at the 8-cell stage in the murine-bovine iSCNT embryos, showing that the expressed gene related with zygotic activation is similar to that of bovine.

In this study, we have produced the murine-bovine iSCNT blastocyst for the first time using the bovine medium, CR1aa, supplemented with an antioxidant, BME. Though the research on iSCNT, in spatially distance species, is still in its early stage as the brevity of the bibliography attests, we tentatively conclude that these results might be provide the useful information and technology for better understanding of the potential and mechanisms for the development of iSCNT embryos. Further studies regarding the detailed molecular mechanisms and the behavior of mitochondria in iSCNT embryos are needed to expand its practical application.

REFERENCES

Arat, S., S. J. Rzucidlo and S. L. Stice. 2003. Gene expression and *in vitro* development of inter-species nuclear transfer embryos. Mol. Reprod. Dev. 66:334-342.

Bagis, H. and H. Odaman Mercan. 2004. Effect of chemically defined culture medium supplemented with β-mercaptoethanol and amino acids on implantation and development of different stage *in vivo*- or *in vitro*-derived mouse embryos. Mol. Reprod. Dev. 69:52-59.

Baguisi, A., E. Behboodi, D. T. Melican, J. S. Pollock, M. M. Destrempes, C. Cammuso, J. L. Williams, S. D. Nims, C. A. Porter, P. Midura, M. J. Palacios, S. L. Ayres, R. S. Denniston, M. L. Hayes, C. A. Ziomek, H. M. Meade, R. A. Godke, W. G. Gavin, Y. Overstrom and Y. Echelard. 1999. Production of goats by somatic cell nuclear transfer. Nat. Biotechnol. 17:456-461.

Biggers, J. D., L. K. McGinnis and M. Raffin. 2000. Aminoacids and preimplantation development of the mouse in protein-free potassium simplex optimized medium. Biol. Reprod. 63:281-293.

Boiani, M., S. Eckardt, H. R. Scholer and K. J. McLaughlin. 2002. Oct4 distribution and level in mouse clones: Consequences for pluripotency. Genes Dev. 16:1209-1219.

Caamano, J. N., Z. A. Ryoo, J. A. Thomas and C. R. Youngs. 1996. β-mercaptoethanol enhances blastocyst formation rate of bovine *in vitro*-matured/*in vitro* fertilized embryos. Bio. Reprod. 55:1179-1184.

Chen, D. Y., Q. Y. Sun, G. P. Liu, G. P. Li, L. Lian, M. K. Wang, Z. M. Han, X. F. Song, J. S. Li, Q. Sun, Y. C. Chen, Y. P. Chang and B. Ding. 1999. The giant panda (Ailuroda melanoleuca) somatic nucleus can dedifferentiate in rabbit ooplasm and support early development the reconstructed egg. Sci. China (Series C) 29:324-330.

Chesne, P., P. G. Adenot, C. Viglietta, M. Baratte, L. Boulanger and J. P. Renard. 2002. Cloned rabbits produced by nuclear transfer from adult somatic cells. Nat. Biotechnol. 20:366-369.

Constance, H. 2003. First cloned mule races to finish line. Sci. 300:1354.

Daniels, R., S. Lowell, V. Bolton and M. Monk. 1997. Transcription of tissue-specific genes in human preimplantation embryos. Human Reprod. 12:2251-2256.

Daniels, R., V. Hall and A. O. Trounson. 2000. Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. Biol. Reprod. 63:1034-1040.

De La Fuente, R., A. Hahnel, P. R. Rasrur and W. A. King. 1999. X inactive-specific transcript (*Xist*) expression and X chromosome inactivation in the preattachment bovine embryo. Biol. Reprod. 60:769-775.

Dindot, S. V., P. W. Farin, C. E. Rarin, J. Romano, S. Walker, C. Long and J. A. Piedrahita. 2004. Epigenetic and genomic imprinting analysis in nuclear transfer derived Bos gaurus/Bos Taurus hybrid fetuses. Biol. Reprod. 71:470-478.

Dominko, T., M. Mitalipova, B. Haley, Z. Beyhan, E. Memili, B. Mckusick and N. L. First. 1999. Bovine oocyte cytoplasm supports development of embryos produced by nuclear transfer of somatic cell nuclei from various mammalian species. Biol. Reprod. 60:1496-1502.

Galli, C., I. Laguina, G. Crotti, S. Colleoni, P. Turini, N. Ponderato, R. Duchi and G. Lazzari. 2003. A cloned horse born to its dam twin. Nature 424:635.

Kato, Y., T. Tani, Y. Sotomaru, K. Kurokawa, J. Kato, H. Doguchi, H. Yasue and Y. Tsunoda. 1998. Eight calves cloned from somatic cells of a single adult. Sci. 282:2095-2098.

Kay, G. F., G. D. Penny, D. Patel, A. Ashworth, N. Brockdorff and S. Rastan. 1993. Expression of *Xist* during mouse development suggests a role in the initiation of X Chromosome inactivation. Cell 72:171-182.

Kay, G. F., S. C. Barton, M. A. Surani and S. Rastan. 1994. Imprinting and X chromosome counting mechanisms determine *Xist* expression in early mouse development. Cell 77:639-650.

Kenneth, L. 2001. Can cloning wave endangered species?. Curr. Biol. 11:245-246.

Kin, M. K., G. Jang, H. J. Oh, F. Yuda, H. J. Kim, W. S. Whang, M. S. Hossein, J. J. Kim, N. S. Shin, S. K. Kang and B. C. Lee. 2007. Endangered wolves cloned from adult somatic cells. Cloning Stem Cells 9:130-137.

Kitiyanant, Y., J. Saikhun, B. Chaisalee, K. L. White and K. Pavasuthipaisit. 2001. Somatic cell cloning in buffalo (*Bubalus bubalis*): Effects of interspecies cytoplasmic recipients and activation procedures. Cloning Stem Cells 3:97-104.

Laibrfied-Retledge, M. L., T. Dominko, E. S. Critser and J. K. Crister. 1999. Tissue maturation *in vivo* and *in vitro*: Gamete and early embryos ontogeny, In: Reproductive tissue banking (Ed. A. M. Karow and J. M. Crister). New York Press 22-137.

Lanza, R. P., J. B. Cibelli, F. Diaz, C. T. Moraes, P. W. Farin, C. E. Farin, C. J. Hammer, M. D. West and P. Damiani. 2000. Cloning of an endangered species (*Bos gaurus*) using interspecies nuclear transfer. Cloning 2:79-90.

Loi, P., G. Ptak, B. Barboni, J. Fulka, P. Cappai and M. Clinton. 2001. Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. Nat. Biotechnol. 19:962-964.

Maurizio, Z. and B. Michele. 2002. Mouse xist express begins at zygotic genome activation and timed by z zygotic clock. Mol. Rep. Dev. 61:14-20.

Niemann, H., C. Wrenzycki, A. Lucas-Hahn, T. Brambrink, W. A. Kues and J. W. Carnwath. 2002. Gene expression patterns in bovine *in vitro* produced and nuclear transfer-derived embryos and their implications for early development. Clon. Stem Cell 4:29-38.

Noda, Y., H. Matsumoto, Y. Umaoka, K. Tatsumi and T. Mori. 1991. Improvement of superoxide radicals in mouse two-cell block phenomenon. Mol. Reprod. Dev. 28:356-360.

Oguara, A., K. Inoue, K. Takano, T. Wakayama and R. Yanagimachi. 2000. Birth of mice after nuclear transfer by electrofusion using tail tip cells. Mol. Reprod. Dev. 57:55-59.

Pabon, J. E. J., W. E. Findley and W. E. Gibbons. 1989. The toxic effect of short exposure to the atmospheric oxygen concentration on early mouse embryonic development. Fertil. Steril. 51:896-900.

Park, S. H., M. R. Shin and N. H. Kim. 2004. Bovine oocyte cytoplasm supports nuclear remodeling but not reprogramming of murine fibroblast cells. Mol. Reprod. Dev. 68:25-34.

Park, E. S., W. S. Hwang, S. K. Kang, B. C. Lee, J. Y. Han and J. M. Lim. 2004. Improved embryo development with decreased apoptosis in blastomeres after the treatment of cloned bovine embryos with β-mercaptoethanol and Hemoglobin. Mol. Reprod. Dev. 67:200-206.

Polejaeva, I. A., S. H. Chen, T. D. Vaught, R. L. Page, J. Mullins, S. Ball, Y. Dai, J. Boone, S. Walker, D. L. Ayares, A. Colman and K. H. Campbell. 2000. Cloned pigs produced by nuclear transferring adult somatic cells. Nature 407:86-90.

Quinn, P. and G. M. Harlow. 1978. The effect of oxygen on the development of preimplantation mouse embryos *in vitro*. J. Exp. Zool. 206:73-80.

Rideout, W. M., K. Eggan and R. Jaenish. 2001. Nuclear cloning and epigenetic reprogramming of the genome. Sci. 293:1093-1098.

Rosenkrans, C. F., G. Q. Zeng, G. T. McNamara, P. K. Schoff and N. L. First. 1993. Development of bovine embryos *in vitro* is affected by energy substrate. Biol. Reprod. 49:459-462.

Sansinena, M. J., D. Hylan, K. Hebert, R. S. Denniston and R. A. Godke. 2005. Banteng (*Bos javanicus*) embryos and pregnancies produced by interspecies nuclear transfer. Theriogenol. 63:1081-1091.

Shin, T., D. Kraemer, J. Pryor, L. Liu, J. Rugila, L. Howe, S. Buck, K. Murphy, L. Lyons and M. Westhusin. 2002. A cat cloned by nuclear transplantation. Nature 415:859.

Relford, N.A., A. J. Watson and G. A. Schulz. 1990. Transition from the maternal to embryonic control in early mammalian development: a comparison of several species. Mol. Reprod. Dev. 26:90-100.

Takahashi, M., T. Nagai, S. Hamano, M. Kuwayama, N. Okamura and A. Okano. 1993. Effect of thiol compounds on *in vitro* development and intracellular glutathione content of bovine embryos. Biol. Reprod. 49:228-232.

Umaoka, Y., Y. Noda, K. Narimoto and T. Mori. 1992. Effects of oxygentoxicity on early development of mouse embryos. Mol. Reprod. Dev. 31:28-33.

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

White, K. L., T. D. Bunch, S. Mitalipov and W. A. Reed. 1999. Establishment of pregnancy after the transfer of nuclear transfer embryos produced from the fusion of Argali (*Ovis ammon*) nuclei into domestic sheep (*Ovis aries*) enucleated oocytes. Cloning 1:47-54.

Wilmut, I., A. E. Schnieke, J. McWhir, A. J. Kind and K. H. S. Campbell. 1997. Viable offspring derived from fet al and adult mammalian cells. Nature 385:810-813.

Winger, Q., J. A. Hill, T. Shin, A. J. Watson, D. C. Kraemer and M. E. Westhusin. 2000. Genetic reprogramming of lactate dehydrogenase, citrate synthase and phosphofructokinase mRNA in bovine nuclear transfer embryos produced using bovine fibroblast cell nuclei. Mol. Reprod.Dev. 56:458-464.

Yanxin, L., D. Yunping, D. Weihua, Z. Chunjiang, W. Haiping, W. Lili, L. Rong, L. Ying, W. Rong and L. Ning. 2006. Cloned endangered species takin (*Budorcas taxicolor*) by interspecies nuclear transfer and comparison of the blastocyst development with yak (*Bos grunnuens*) and bovine. Mol. Reprod. Dev. 73:189-195.