Developmental Characteristics of SCNT Pig Embryos Knocked-out of Alpha-1,3-Galactosyltransferase Gene

Joo-Hyun Shim[1], Mi-Rung Park[1], Byoung-Chul Yang[1], Yeoung-Gyu Ko[1], Keon Bong Oh[1], Jeong-Woong Lee[2], Jae-Seok Woo[1], Eung-Woo Park[1], Soo-Bong Park[1] and Seongsoo Hwang[1,†]

[1]*National Institute of Animal Science, RDA, Suwon 441-706, Korea*

[2]*Center for Regenerative Medicine, KRIBB, Daejeon 305-809, Korea*

ABSTRACT

This study was performed to comprehend the developmental characteristics of cloned embryos knocked out (KO) of α-1,3-galactosyltransferase (GalT) gene. Immature oocytes were collected and cultured for 40 hrs (1-step) or 20hrs (with hormone) + 20hrs (without hormone) (2-step). The embryos transferred with miniature pig ear fibroblast cell were used as control. The reconstructed embryos were cultured in PZM-3 with 5% CO_2 in air at 38.5℃ for 6 days. To determine the quality of the blstocysts, TUNEL and quantitative realtime RT-PCR were performed. The embryos were transferred to a surrogate (Landrace) at an earlier stage of the estrus cycle. The maturation rate was significantly higher in 2-step method than that of 1-step ($p<0.05$). The blastocyst development of GalT KO embryos was significantly lower than that of normal cloned embryos ($p<0.05$). The total and apoptotic cell number of GalT KO blastocysts was not different statistically from control. The relative abundance of Bax-α/Bcl-xl ratio was significantly higher in both cloned blastocysts than that of *in vivo* blastocysts ($p<0.05$). Taken together, it can be postulated that the lower developmental potential and higher expression of apoptosis related genes in GalT KO SCNT embryos might be a cause of a low efficiency of GalT KO cloned miniature pig production.

(Key words : α-1,3-Galactosyltransferase, Gene knock out, Miniature pig, Embryo development, Apoptosis)

INTRODUCTION

The transplantation of organs from other species into humans is considered to be a probable solution to the shortage of human donor organs (Joziasse and Oriol, 1999). Due to similar anatomy and physiology, pig have been used in biomedical applications for decades as a model for human diseases, as a genetically defined model for surgery and xenotransplantation and as a source of human disease therapeutics (Prather *et al.*, 2003; Zhao *et al.*, 2009).

Pig-to-human organ transplantation, however, has a big problem according to immune rejection system. The presence of terminal alpha-1,3 galactose (Gal) epitopes on the surface of pig cells are the major xenoantigens causing hyperacute rejection and also involved in acute vascular rejection in pig-to-human xenotransplantation (Cooper *et al.*, 1993; Logan, 2000; Galili, 2001). Recently, the deliberate knockout of the gene encoding alpha-1,3 galactocyltransferase (GalT) in pigs has been accomplished and homozygous knock-out pigs produced (Dai *et al.*, 2002; Phelps *et al.*, 2003; Kolber-Simonds *et al.*, 2004; Nottle *et al.*, 2007).

Apoptosis may lead arrest or abnormal development and lower viability of embryos (Byrne *et al.*, 1999; Hwang *et al.*, 2008). Bcl-2 gene families are identified as apoptosis regulating genes. Off these genes, bax, bad, bak and bik promote cell death, whereas bcl-2 and bcl-XL inhibit apoptosis and promote cell survival (Yin *et al.*, 1994; Hara *et al.*, 1996). The ratio of Bax and Bcl-2 has been implicated to be a critical determinant of cell fate such that elevated Bcl-2 favors extended survival of cells and increasing levels of Bax expression accelerate cell death (Oltvai *et al.*, 1993).

This study was performed to comprehend the developmental characteristics of cloned embryos knocked out (KO) of GalT gene.

MATERIALS AND METHODS

Chemicals

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† Corresponding author : Phone: +82-31-290-1761, E-mail: hwangss@korea.kr

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).

In Vitro Maturation of Oocytes

Porcine ovaries were obtained from a local slaughterhouse and transported to the laboratory at 30~35 ℃. Cumulus-oocyte complexes (COCs) were collected and washed in Tyrode's lactate-Hepes containing 0.1% (w/v) polyvinyl alcohol (PVA). Oocytes with several layers of cumulus cells were selected and washed three times in TCM-199 (Gibco-BRL, Grand Island, NY, USA) supplemented with 0.1% PVA (w/v), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 μg/ml luteinizing hormone, 0.5 μg/ml follicle stimulating hormone, 10 ng/ml epidermal growth factor, 10% porcine follicular fluid (pFF) 75 μg/ml penicillin G and 50 μg/ml streptomycin (maturation medium). For *in vitro* culture, 50~100 of COCs were transferred into 500 μl of maturation medium in a four-well dish (Nunc, Roskilde, Denmark). The oocytes were matured for 40 hrs (1-step) or 20hrs (with hormone) + 20hrs (without hormone) (2-step) at 38.5℃ under 5% CO_2 in air.

Nuclear Transfer Procedure

After *in vitro* maturation, cumulus cells were treated with PBS supplemented with 0.1% PVA and 0.1% hyaluronidase for 4 min. The oocytes were enucleated by the aspiration of the first polar body and metaphase-II (MII) plate in a small amount of surrounding cytoplasm with a glass pipette. Enucleation was confirmed by staining the oocytes with 10 μg/ml Hoechst 33342 for 15~20 min at 39℃. Passage 2~3 of the GalT KO donor cell was transferred into a perivitelline space of an enucleated oocyte. After 1~2 h of equilibration time, the reconstructed oocytes were placed into 0.2 mm diameter wire electrodes (1 mm apart) of a fusion chamber covered with 0.3 M mannitol solution containing 0.1 mM $MgSO_4$, 1.0 mM $CaCl_2$ and 0.5 mM Hepes. For the fusion, two DC pulses (1 sec interval) of 1.2 kV/cm were applied for 30 μsec using an Electro Cell fusion (NEPA Gene, Chiba, Japan). The embryos fused were washed and transferred into PZM-3 under 5% CO_2 in air at 38.5℃. A cleavage and blastocyst development was examined on day-2 and -6 respectively.

Collection of *In Vivo* Blastocysts

In vivo blastocysts were collected from synchronized 7 to 11-month-old Landrace gilts. The gilts were artificially inseminated 24 and 36 h after hCG injection. Approximately 168 h after hCG (120 h after estimated ovulation) injection, the genital tract was removed and flushed with DPBS. The embryos found were transported to the laboratory in PVA-TL-Hepes buffered at 37℃ within 30 min after collection.

TUNEL Assay for Apoptotic Cell Death

Blastocysts on day 6 from NT were washed twice in PBS/PVP (0.1% polyvinylpyrolidone) and fixed in 4% (v/v) paraformaldehyde solution for 24 h at 4℃. After permeabilization using 0.5% Triton X-100, apoptotic cells were analyzed by *in situ* cell death detection kit (TMR red; Roche, Mannheim, USA) for 1 h at 38.5℃ in the dark. The embryos were stained with 10 μg/ml Hoechest 33342 for 30 min and mounted on slides with Prolong antifade kit (Cat. P-748, Molecular Probes, Eugene, OR, USA). The slides were stored at −20℃. The total or apoptotic cell numbers were determined from optical images of whole-mount embryos under an epifluorescent microscope (Nikon, Tokyo, Japan) (Fig. 1).

Apoptosis Related Gene Expression

cDNA synthesis of the *in vivo*, mNT and GalT blastocysts was achieved by reverse transcription of the RNA by using the 1st strand cDNA synthesis kit (Roche). The mRNAs of Bax-α, Bcl-xl and β-actin were then detected by realtime RT-PCR with specific primer pairs (Table 1). PCR reactions were performed according to the instructions of the Realtime PCR machine manufacturer (LightCycler, Roche) and detected with SYBR Green, a double-stranded DNA-specific fluorescent dye included in the SYBR Green PCR premix. Each PCR reaction was performed in 20 μl reaction buffer containing 10 μl 2× SYBR Green premix, 1 μl of forward, reverse primers (100 pmol/μl), 1 μl embryonic cDNA (0.1 blastocyst/μl equivalent). All sam-

Table 1. Primer sequences and cycling condition used in realtime RT-PCR

Gene	Primer sequences (5'-3')	Size (bp)	Realtime RT-PCR
p-β-Actin	F:CACTGGCATTGTCATGGACT R:GGCAGCTCGTAGCTCTTCTC	285	60℃, 12s 45cycles
p-Bcl-xl	F:GTTGACTTTCTCTCCTACAAGC R:GGTACCTCAGTTCAAACTCATC	277	62℃, 13s 50cycles
p-Bax-α	F:ACTGGACAGTAACATGGAGC R:GTCCCAAAGTAGGAGAGGAG	294	63℃, 13s 55cycles

ples were measured in triplicate. The following amplification program was employed: preincubation for Hot-Start polymerase activation at 95℃ for 15 min, followed by 40~55 amplification cycles of denaturation at 95℃ for 1 min (2℃/sec), annealing at 60~63℃ for 1 min (2℃/sec), elongation at 72℃ for 1 min (2℃/sec) and acquisition of fluorescence at 72 or 80℃ for 1 sec. After the end of the last cycle, the melting curve was generated by starting fluorescence acquisition at 65℃ and taking measurements every 0.2℃ until a temperature of 95℃. Product sizes were confirmed by electrophoresis on 2% agarose gel stained with ethidium bromide and visualized by ultraviolet light.

Statistical Analysis

Data were subjected to a Generalized Linear Model procedure (PROC-GLM) of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Differences among treatment means were determined by using the Duncan's multiple range tests. All data were expressed as Least Square (LS) mean ± SEM (Standard Error of the sample Mean). A probability of $p<0.05$ was considered statistically significant.

RESULTS

In Vitro Maturation of Porcine Oocytes

In vitro maturation of porcine oocytes according to different maturation methods was shown in Table 2. The maturation rate was significantly higher in 2-step method (89.8%, 2,409/2,679) compared to 1-step maturation method (79.6%, 3,235/4,057) ($p<0.01$).

Table 2. *In vitro* maturation of porcine oocytes according to different maturation methods

Method*	No. of oocytes	
	Cultured	Matured
1-step	4,057	3,235 (79.6±2.99)[b]
2-step	2,679	2,409 (89.8±0.97)[a]

* Method: 1-step; maturation medium [TCM199 + 0.1% PVA + FSH & LH (0.5 ug/ml) + EGF (10 ng/ml) + 10% porcine follicular fluid (pFF)] for 40 hr; 2-step: maturation medium for 20 hr + without hormones for 20 hr.

[ab] Values with different superscripts differ significantly ($p<0.01$).

Data were expressed as mean ± standard error (SE).

In Vitro Development of SCNT Embryos

Table 3 shows developmental rate of mNT (miniature pig ear fibroblast nuclear transfer) and GalT KO NT embryos. There was no significant difference in cleavage rate between mNT and GalT KO NT embryos. But the number of embryos developed to blastocysts stage was significantly higher in mNT (27.6±3.23) than GalT KO NT (18.0±1.74) ($p<0.05$).

Apoptosis Rate in mNT and GalT KO NT Blastocysts

The differences were not statistically significant in the number of total cell (31.4 vs. 36.1), apoptotic cell (1.1 vs. 1.55), and apoptosis rate [(apoptotic cells / total cells) * 100] (3.7 vs. 5.0) in the blastocysts from mNT and GalT KO NT (Table 4) (Fig. 1).

Table 3. *In vitro* development of porcine cloned embryos derived from mNT or GalT KO NT

Cell type	No. of embryos			
	Cultured	Lysed	≥ 2cell	Blastocyst
Control	166	23	116 (82.3±4.82)	37 (27.6±3.23)[a]
GalT KO	208	24	157 (85.3±1.72)	33 (18.0±1.74)[b]

[ab] Values with different superscripts differ significantly ($p<0.05$).

Data were expressed as mean ± SE.

Table 4. Apoptosis in porcine blastocysts derived from mNT or GalT KO NT

Source of donor cell	No. of blastocyst	No. of		Ratio* (%)
		Total cells	Apoptotic cells	
Control	20	31.4±1.0	1.1±0.23	3.68±0.86
GalT KO	22	36.1±2.4	1.55±0.3	4.99±1.03

*Apoptotic cells / Total cells * 100.

Data were expressed as mean ± SE.

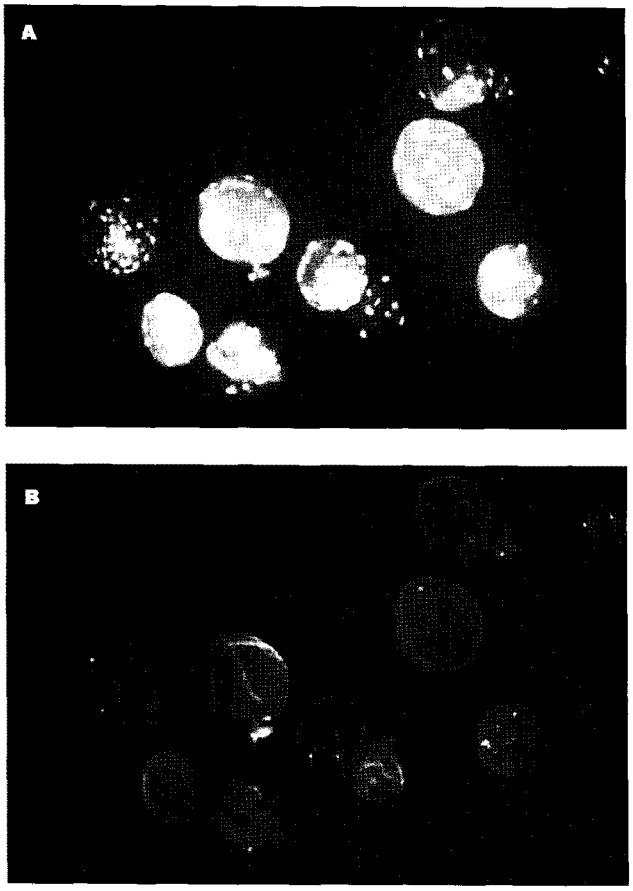

Fig. 1. Apoptotic cell in GalT KO blastocysts stained with *in situ* cell death detection kit. A: total cell, B: apoptotic cell.

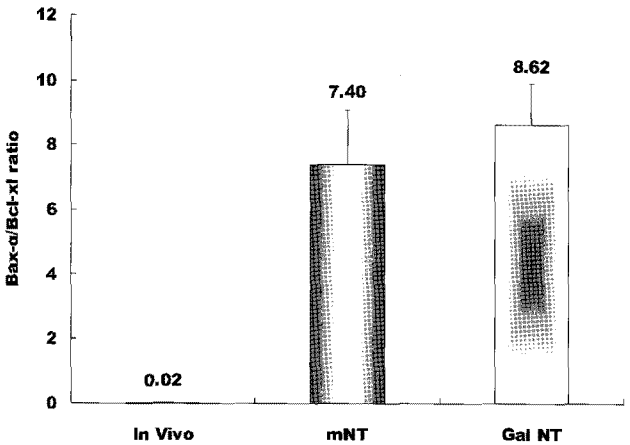

Fig. 2. Relative expression levels of Bax-α/ Bcl-xl gene in mNT and GalT KO SCNT blastocysts. [ab] Values with different superscripts differ significantly ($p<0.05$).

Analysis of Apoptosis Gene Expression in Blastocysts

As shown in Fig. 2, the relative abundance of Bax-α /Bcl-xl was clearly higher in both NT blastocysts than that of *in vivo* blastocysts ($p<0.05$).

DISCUSSION

This study was conducted to investigate the quality of the blastocyst from GalT KO miniature pig cells. The developmental capacity and apoptosis related gene expression in normal and GalT KO cloned blastocysts were examined. First, we investigated the effect of hormone supplement on *in vitro* maturation oocytes. In the present study, we obtained about 90% of maturation rate using 2-step maturation method. Recently, we reported that *in vitro* maturation rate was around 80%, when the oocytes were cultured in maturation medium with hormone for 40~44 hrs (Moon *et al.*, 2009). Unlike, removal of hormones from maturation medium at 20 hrs after the initiation of culture significantly enhanced the MII stage oocytes (>80%) (Kobayashi *et al.*, 2007; Viet Linh *et al.*, 2009).

Funahashi and Day (1993) reported that there was no difference between 1-step and 2-step methods in GVBD and MII oocyte incidence. The pronucleus formation rate, however, was significantly higher in 2-step method than 1-step after *in vitro* fertilization. Requirement of hormone supplementation was limited to the germinal vesicle breakdown phase (Lenton *et al*, 1988). So, although the maturation rate was similar between 1- and 2-step methods, *in vitro* maturation without hormone supplementation after 20 hrs may enhance a cytoplasmic maturation of pig oocytes.

When genetically modified cells are used as a donor cell, the development rate of cloned embryos is often low (Fujimura *et al*, 2008). In cattle, a genetic modification of donor cells influences the number of cloned calves born (Zakharchenko *et al.*, 2001; Forsberg *et al.*, 2002; Iguma *et al.*, 2005). In this study, developmental rate to the blastocyst stage was significantly higher in mNT than that of GalT KO NT, although the fusion and cleavage rate were not different statistically.

Apoptosis plays a pivotal role in embryo development and occurs during the pre-implantation stage in both *in vivo*- and *in vitro*- produced embryos (Levy *et al.*, 2001). Also, cytoplasmic fragmentation with developmental arrest is a model characteristic of embryos undergoing apoptosis (Hao *et al.*, 2003). It has been reported that fragmentation is frequently observed *in vitro* manipulated embryos (Kolbe and Holts, 1999; Wang *et al.*, 1999; Cheong *et al.*, 2000), especially following NT (Im *et al.*, 2005). And SCNT embryos showed different microtubule distributions (Park *et al.*, 2009).

Entry to and progression through the apoptotic pathway seem to be controlled by the balanced expression of several conserved genes that have either pro- or anti- apoptotic effects. The most important interactions are considered to lie in Bax/Bcl-2 dimerization (Xiao and Zhang, 2008). Previous reports revealed that an increase of Bax/Bcl-2 ratio may contribute partly to the

high level of apoptosis in gestation trophoblast (Qiao *et al.*, 1998) and to increase of fragmentation in the human embryo (Brenner *et al.*, 1997). However, it is not easy to find the results associated with this phenomenon in transgenic cloned embryos. The present study, both SCNT blastocysts showed significantly higher Bax-α/Bcl-xl expression compared to the *in vivo* embryos. It may affect negatively to embryo quality and fetal development.

Taken together, it can be postulated that the lower developmental potential and higher expression of apoptosis related genes in GalT KO SCNT embryos might be a cause of a low efficiency of GalT KO cloned miniature pig production.

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