# Effect of Osmolarity of Culture Medium on Imprinting and Apoptotic Gene Expression in Miniature Pig Nuclear Transfer Embryos

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## ABSTRACT

**This study was conducted to investigate the development and gene expression in miniature pig nuclear transfer (mNT) embryos produced under different osmolarity culture conditions. Control group of mNT embryos was cultured in PZM-3 for 6 days. Treatment group of mNT embryos was cultured in modified PZM-3 with NaCl (mPZM-3, 320 mOsmol) for 2 days, and then cultured in PZM-3 (270 mOsmol) for 4 days. Blastocyst formation rate of the treatment group was significantly higher than the control and the apoptosis rate was significantly lower in treatment group. Bax-α and caspase-3 mRNA expression were significantly higher in the control than the treatment group. Also, the majority of imprinting genes were expressed aberrantly in *in vitro* produced mNT blastocysts compared to *in vivo* derived blastocyst. H19 and Xist mRNA expression were significantly lower in the control than the treatment group or *in vivo*. IGF2 mRNA expression was significantly higher in the control than the treatment group or *in vivo*. IGF2r mRNA expression was significantly lower in the control. Methylation profiles of individual DNA strands in H19 upstream T-DMR sequences showed a similar methylation status between treatment group and *in vivo*. These results indicate that the modification of osmolarity in culture medium at early culture stage could provide more beneficial culture environments for mNT embryos.**

**(Key words : Miniature pig, NT embryos, Osmolarity, Gene expression)**

## INTRODUCTION

It is generally accepted that mammalian pre-implantation embryos are sensitive to their environment and culture conditions can affect future growth and developmental potential both pre and postnatally (Bavister, 1995; Kruip and Den Daas, 1997). Studies have been performed to improve the developmental competence of porcine *in vitro* produced embryos (Abeydeera *et al.*, 1998; Kano *et al.*, 1998) and have demonstrated that many factors are involved in *in vitro* embryo development and viability after transfer. It is expected that components in culture medium are one of the important factors affecting embryo viability of porcine nuclear transfer (NT) embryos. Osmolarity of culture media is also considered to be one of the important factors affecting *in vitro* development of pre-implantation mammalian embryos to the blastocyst stage. However, the optimal osmolarity of the medium for early embryonic development is species-specific and depends on the developmental stage of the embryos (Naglee *et al.*, 1969; Dawson *et al.*, 1998; Nguyen *et al.*, 2003). We have previously shown that the modification of the osmolarity in culture medium can improve porcine NT blastocyst quality (Im *et al.*, 2005; Hwang *et al.*, 2007).

Differences in the relative abundance of some developmentally important gene transcripts have been reported between *in vivo* - and *in vitro* - produced porcine embryos. It is known that the conditions of *in vitro* culture can alter gene expression in the embryo (Doherty *et al.*, 2000; Wrenzychi *et al.*, 2001). The percentage of cells undergoing apoptosis in porcine somatic cell NT blastocysts was higher than that of the IVF embryos and increased with time *in vitro* (Hao *et al.*, 2003). Imprinted genes may be particularly susceptible to methylation changes that occur during pre-implantation development. Fetal abnormalities observed as a consequence of pre-implantation *in vitro* culture have been proposed to result from aberrant changes in the methylation status of imprinted genes (Nagy *et al.*, 1993; Reik *et al.*, 1993; Dean *et al.*, 1998).

It is clear that any improvement in the quality of blastocysts produced *in vitro* is likely to derive from the modification of the culture conditions in which the manipulated embryos are cultured. The objective of this study was to examine the effect of the osmolarity during *in vitro* culture of mNT embryos on 1) develop-

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ment rate, 2) the expression of imprinting genes and apoptosis and 3) methylation pattern in T-DMR of H19 in blastocysts.

## MATERIALS AND METHODS

### Oocyte Recovery and Maturation

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All procedures used in this experiment were approved by the animal care and use committee of National Institute of Animal Science (Suwon, Korea). Ovaries were obtained from prepubertal crossbred gilts at a local slaughterhouse and transported to the laboratory at 30 ~35℃. Cumulus-oocyte complexes (COCs) were collected by the aspiration of ovary antral follicles (3~6 mm diameter) with 18 gauge needle fixed to a 10 ml disposable syringe. COCs were matured for 40 to 44 h at 38.5℃ under 5% $CO_2$ in air. The maturation medium was TCM-199 supplemented with 0.1% (w/v) polyvinyl alcohol (PVA), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 μg/ml LH (Sigma, from ovine pituitary), 0.5 μg/ml FSH (Sigma, from porcine pituitary), 75 μg/ml penicillin G and 50 μg/ml streptomycin.

### Preparation of Nuclear Donor Cells

Ear skin tissue of 8-month-old miniature pig, which was imported from Immerge Bio Therapeutics Inc. (Cambridge, MA, USA), was collected. The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco-BRL, Grand Island, NY, USA) supplemented with 15% (v/v) fetal bovine serum and 75 μg/ml antibiotics. The cells were passaged two times, and then frozen with DMEM supplemented with 10% dimethylsulfoxide. To be used as donor cells, cells were thawed and cultured until they reached confluence. The ear fibroblast cells were cultured, passaged (3~8 passages) and used as donor cells for NT.

### Production of Nuclear Transfer Embryos

The matured oocytes were denuded by vortexing the COCs in PBS supplemented with 0.1% (w/v) PVA and 0.1% (w/v) hyaluronidase for 4 min. Oocytes were enucleated by the aspiration of the first polar body and metaphase-II plate in a small amount of surrounding cytoplasm with a glass pipette. All micromanipulation procedures were performed in TCM-199 supplemented with 3 mg/ml BSA and 5 μg/ml cytochalasin B. Enucleation was confirmed by staining the oocytes with 10 μg/ml Hoechst 33342 for 15~20 min at 39℃. After enucleation, the oocytes were held in TCM-199 supplemented with 3 mg/ml BSA until injection of donor cells. Reconstructed oocytes were then placed between 0.2 mm diameter wire electrodes(1 mm apart) of a fusion chamber overlaid with 0.3 M mannitol solution supplemented with 0.1 mM $MgSO_4$, 1.0 mM $CaCl_2$, and 0.5 mM Hepes. For fusion, two DC pulses of 1.2 kV/cm were applied for 30 μs using a BTX Electro Cell Manipulator 2001 (BTX, San Diago, CA, USA). After fusion, the reconstructed oocytes were divided into two groups and the fusion was determined after 1 h. Basic culture medium was porcine zygote medium 3 (Yoshioka *et al.*, 2002). Control group of mNT embryos was cultured in PZM-3 (270 mOsmol) for 6 days. Treatment group of mNT embryos was cultured in modified PZM-3 (mPZM-3) for 2 days, and then cultured in PZM-3 for 4 days. The osmolarity of treatment group was modified to 320 mOsmol by increasing NaCl to 138 mM.

### Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Assay

Day 6 NT blastocysts were washed twice in PBS supplemented with 0.1% (w/v) polyvinylpyrolidone (PBS/PVP) and fixed in 4% (v/v) paraformaldehyde solution for 24 h at 4℃. Membranes were permeabilized in 0.5% (v/v) Triton X-100 for 30 min at room temperature. The embryos were then washed twice in PBS/PVP and incubated with fluorescein-conjugated d-UTP and the terminal deoxynucleotidyl transferase enzyme (*in situ* cell death detection kit, Roche, Mannheim, Germany) in the dark for 1 h at 38.5℃. The embryos were washed and transferred into 10 μg/ml Hoechest 33342 for 30 min at room temperature in the dark. The embryos were washed three times and mounted on slides with mounting medium (Vectashield, Burlingame, CA, USA). The slides were stored at 20℃. The numbers of apoptotic nuclei and total numbers of nuclei were determined from optical images of whole-mount embryos under an epifluorescent microscope (Nikon, Tokyo, Japan).

### Collection of *In Vivo* Blastocysts

Embryos were collected from synchronized 7 to 11-month-old Landrace gilts. Gilts were artificially inseminated 24 and 36 h after hCG injection. Immediately after stunning and bleeding of the animals, approximately 168 h after hCG (120 h after estimated ovulation), the genital tract was removed and flushed with PBS for embryo collection. Embryos were transported to the laboratory in Tyrode's lactate-Hepes containing 0.1% (w/v) PVA at 37℃ within 30 min after collection.

### Realtime PCR Quantification

Total RNA was prepared from NT-and *in vivo*-derived blastocysts (n=2 blastocysts/treatment). The blastocysts were washed in DEPC-treated water and stored

at 70℃. In all experiments, β-actin mRNA was used as an internal standard. First strand cDNA synthesis was achieved by reverse transcription of the RNA by using the Oligo (dT) 15 primer and the 1st strand c-DNA synthesis kit (Roche, Mannheim, Germany). The mRNAs of Bax-α, p53, Caspase-3, H19, IGF2, IGF2r, Xist and β-actin were then detected by Realtime PCR with specific primer pairs (Table 1). PCR reactions were performed according to the instructions of the Real-time PCR machine manufacturer (LightCycler, Roche, Indianapolis, IN, USA) and detected with SYBR Green, a double-stranded DNA-specific fluorescent dye included in the SYBR Green PCR premix. Each PCR run 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 samples were measured in triplicate. The following amplification program was employed: preincubation for HotStart 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℃. The relative quantification of gene expression was analyzed by the 2-ddCt method (Livak and Schmittgen, 2001).

**Bisulfite DNA Methylation Analysis**

Genomic DNA with methylation CpG was processed using the EZ DNA Methylation Gold kit™ (Zymo Research, Orange, CA, USA). The whole sample was treated with the CT conversion reagent, and purified with the affinity column (Zymo research). DNA was eluted in 10 ul of elution buffer, and used for two successive rounds of PCR, which are specific to the top strand of mutagenized DNA (Table 2). All PCR reactions were preformed using immolase™ DNA polymerase Mix (Bioline, Taunton, MA, USA). The condition of the first PCR was as follows: The thermocycling program was 43 cycles of 94℃ for 30 sec, 55℃ for 30 sec, and 72℃ for 1 min, preceded and followed by 10 min of incubation at 94℃ and 72℃, respectively. The first PCR product were eluted in 100 ul of water, and used for the second round of PCR reactions. The entire PCR products were run on a 2 % agarose gel to verify the amplification of specific bands, which were then excised from the gel for purification with the affinity column. The electrophoresed PCR fragments were cloned into TOPO TA cloning kits (invitrogen, Carlsbad, CA, USA) and sequenced for each sample.

**Statistical Analysis**

The generalized linear model procedure (PROC-GLM) of the Statistical Analysis System (SAS User's Guide, Statistical Analysis System, Inc., Cary, NC, USA) was used to analyze data from all experiments. Differences among treatment means were determined by using the Duncan's multiple range-test and *P*-values of <0.05 were considered significant.

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

| Gene | Primer sequence | Annealing temp.(℃) | Gene bank accession no. |
|---|---|---|---|
| β-Actin | F: CAC TGG CAT TGT CAT GGA CT<br>R: GAG AAG AGC TAC GAG CTG CC | 60 | U07786 |
| Bax-α | F: ACT GGA CAG TAA CAT GGA GC<br>R: GTC CCA AAG TAG GAG AGG AG | 63 | AJ606301 |
| p53 | F: AGC AAA AGA AGA AAC CAC TG<br>R: CCC CTT CTT AGA CTT CAG GT | 63 | AF124298 |
| Caspase-3 | F: GAA AAT ACC AGT TGA GGC AG<br>R: CAT GGA CAC AAT ACA TGG AA | 63 | AB029345 |
| H19 | F: AAA GAG CAT CTC AAG CGA GTC T<br>R: GCT CCT GTA CCT GCT ACT AAA TGA A | 60 | AY044827 |
| IGF2 | F: CTC GTG CTG CTA TGC TGC TTA C<br>R: CAG GTG TCA TAG CGG AAG AAC T | 62 | NM213883 |
| IGF2r | F: ATA AAC ACC AAT ATA ACA CT<br>R: GCA CAC GTT AAT ATA AAA CT | 62 | AF339885 |
| Xist | F: ACT AGT GAT GGT TAT GAA AA<br>R: GTA AGA GGA AAG AAA TGA AG | 62 | AJ429140 |

**Table 2. Primer sequences for bisulfite PCR**

| Gene | Primer sequence | Fragment size (bp) |
|---|---|---|
| H19 - Outer | F: GTT GTT TAG AGT GGA TTT TA<br>R: ACC CAA AAC CCT ATA CCA CC | 600 |
| H19 - Inner | F: AGG ATT TTA GCG GTA TTT GT<br>R: CCG AAA ATA AAA ATC CCT CCC | 356 |

## RESULTS

### Effects of High Osmolarity at Early Culture Stage on Development of NT Embryos

As shown in Table 3, there was no difference in the cleavage rate of mNT embryos cultured in the control and treatment group (88.4 vs. 75.9%, respectively). However, the developmental rate to the blastocyst stage was significantly higher in the treatment group compared to the control (22.6 vs. 18.9%, $p<0.05$). However, the fusion rate was lower ($p<0.05$) in the treatment group than the control.

### Apoptosis and Total Cells in Different Culture Systems

The apoptosis rate was significantly lower ($p<0.05$) in treatment group than the control (1.85 vs. 3.76 %, respectively, Table 4). However, the total cell number in the blastocysts was not significantly different between groups (30.7 vs. 29.8%).

### Analysis of Apoptosis Gene Expression in Blastocysts by Realtime PCR

In the control, Bax-α and caspase-3 mRNA expression were higher than that of treatment group ($p<0.05$, Fig. 1). There were no differences between groups in p53 mRNA expression.

### Imprinting Gene Expression Pattern in Different Culture Systems

Expression of imprinting genes was examined in blastocysts obtained from different culture systems. The analysis of transcript abundance revealed a large degree of variability in NT blastocysts compared to *in vivo*-derived blastocysts. As shown in Fig. 2, H19 mRNA expression of the control was significantly lower than the treatment or *in vivo* group ($p<0.05$). IGF2 mRNA expression was significantly higher in the control than the treatment group ($p<0.05$), and *in vivo* blastocysts showed a significant difference compared to *in vitro* culture systems. IGF2r mRNA expression of the treatment group was significantly higher than the control, but lower than *in vivo* group. In case of Xist, *in vivo* blastocysts showed a significantly higher expression level than that of mNT blastocysts produced by different *in vitro* culture conditions ($p<0.05$), also there was significant difference between the treatment group and control. Although the majority of imprinting genes were expressed aberrantly in *in vitro* produced NT blastocysts compared to *in vivo* blastosysts, the control showed more aberrant expression patterns than the treatment group.

**Table 3. Development of porcine oocytes reconstructed with miniature pig ear fibroblast cells in different culture conditions**

| Culture conditions (mOsmol)* | Fused (Mean±SE) | No. of cleaved (Mean±SE) | No. of blastocysts (Mean±SE) |
|---|---|---|---|
| PZM-3 (270) | 232/272(85.3±0.9)$^{a}$ | 205(88.4±1.1) | 44(18.9±1.3)$^{b}$ |
| mPZM-3 (320) | 199/264(75.4±0.9)$^{b}$ | 151(75.9±4.3) | 45(22.6±1.5)$^{a}$ |

$^{a,b}$ Values with different superscripts within a column are significantly different ($p<0.05$).

**Table 4. Effects of osmolarity on the apoptosis in miniature pig NT blastocyst**

| Culture conditions (mOsmol) | No. of blastocysts | No. of total cells (Mean±SE) | Apoptotic cells | % TUNEL (Mean±SE) |
|---|---|---|---|---|
| PZM-3 (270) | 26 | 799(30.7±1.8) | 30 | 3.76±0.01$^{a}$ |
| mPZM-3 (320) | 20 | 596(29.8±2.3) | 11 | 1.85±0.00$^{b}$ |

$^{a,b}$ Values with different superscripts within a column are significantly different ($p<0.05$).

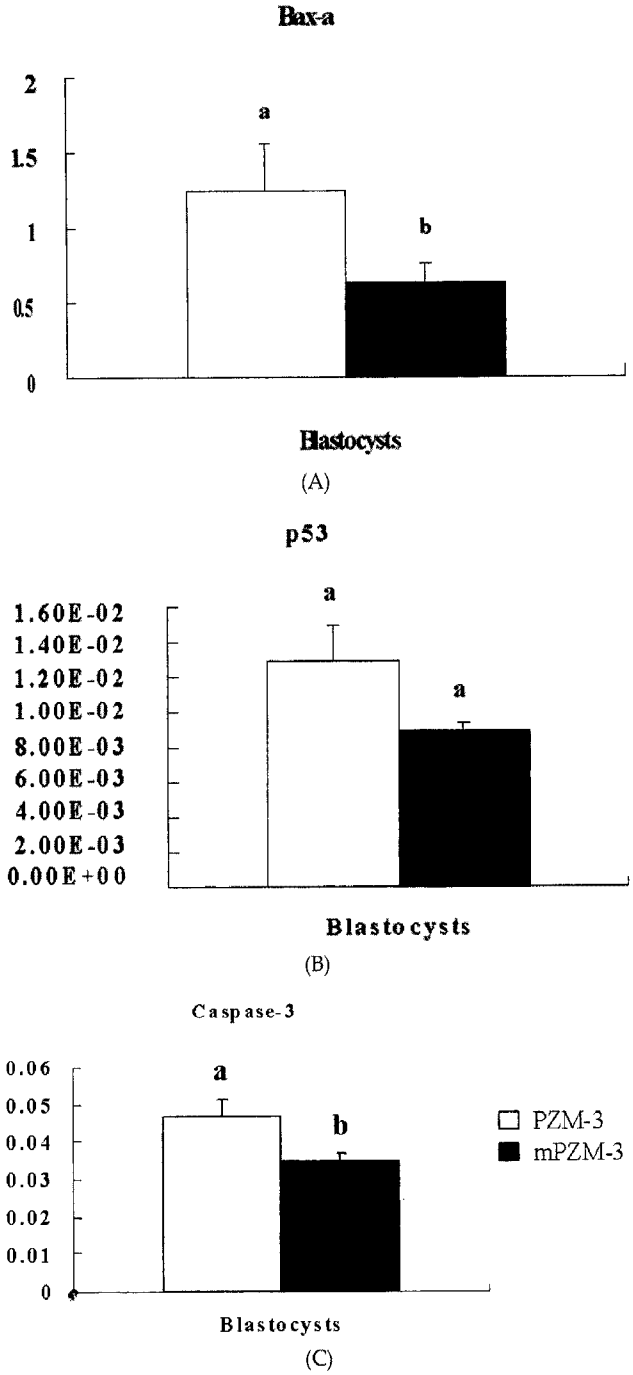


**Fig. 1. Relative apoptosis-related gene expression levels (means±SE) of miniature pig NT blastocysts produced in different culture conditions.** Bars with different superscripts are significantly different ($p<0.05$).

### Methylation Pattern in Different Culture Systems

We designed PCR primers that amplify the upstream sequences of H19 (Table 2), and examined the methylation status in mNT blastocysts produced by different culture conditions. As shown in Fig. 3, the methylation status of the blastocysts produced in the control was significantly higher than that of the blastocysts produced from the treatment group or *in vivo* (87.9% vs. 42.2 and 45.2%, $p<0.05$).

## DISCUSSION

The *in vitro* culture system is one of the most useful strategies for understanding the mechanisms of early development and establishing the optimum culture conditions. The results of the present study indicate that osmolarity affects the development of mNT embryos to the blastocyst stage. The culture of embryos in a high osmolarity for the first 2 days of culture period can improve membrane stabilization resulting in improved development rate. High osmolarity was not effective for the development of early cleaving stages of NT embryos, but was effective for their later stages. Im *et al.* (2005) reported that porcine embryos cultured in PZM-3, of which osmolarity was increased with sorbitol or sucrose to above 315 mOsmol, showed a significantly lower blastocyst rate than that of 269 mOsmol. Only embryos transferred into 269 mOsmol from 315 mOsmol after 48 h showed a significantly higher developmental rate to the blastocyst stage. It is similar to the result of the previous report that the optimal osmolarity of culture medium was 280~320 mOsmol for the first 2 days and 250~270 mOsmol for the remaining period (Nguyen *et al.*, 2003). These similar results clearly show that the osmolarity of culture medium also affects embryonic development, and that hypertonic medium is effective in promoting blastosyst formation in mNT embryos.

In the present study, we found that PZM-3 medium in the presence of 138 mM NaCl significantly reduced the expression of apoptosis and apoptosis-related genes in blastocysts. Environmental stresses, such as those imposed by *in vitro* culture, can induce unscheduled apoptosis in cultured embryos, which may lead to arrest or abnormal development and lower viability of embryos (Jurisicova *et al.*, 1996; Hardy, 1999). The incidence of apoptosis is higher in blastocysts produced by NT than in embryos produced *in vivo* and IVF (Hao *et al.*, 2003). Proteins of the Bcl-2 family, which contain both anti-apoptotic (e.g., Bcl-2 and Bcl-xl) and pro-apoptotic (e.g., Bax) members, are known as key regulators of apoptosis in development, autoimmunity and disease (Adams and Cory, 1998; Korsmeyer, 1999). Caspase-3 has been reported to be one of the main effecter caspases. Thus, activation of caspase-3 was recently observed in embryos developing *in vitro* that responded to numerous chemical teratogens, heat shock and high glucose (Mirkes and Little, 1998; Hinck *et al.*, 2001). The expression levels of p53 were slightly lower in the treatment group than the control, but there was no significant difference. There are different opinions on the relevance of p53 during embryonic develop-

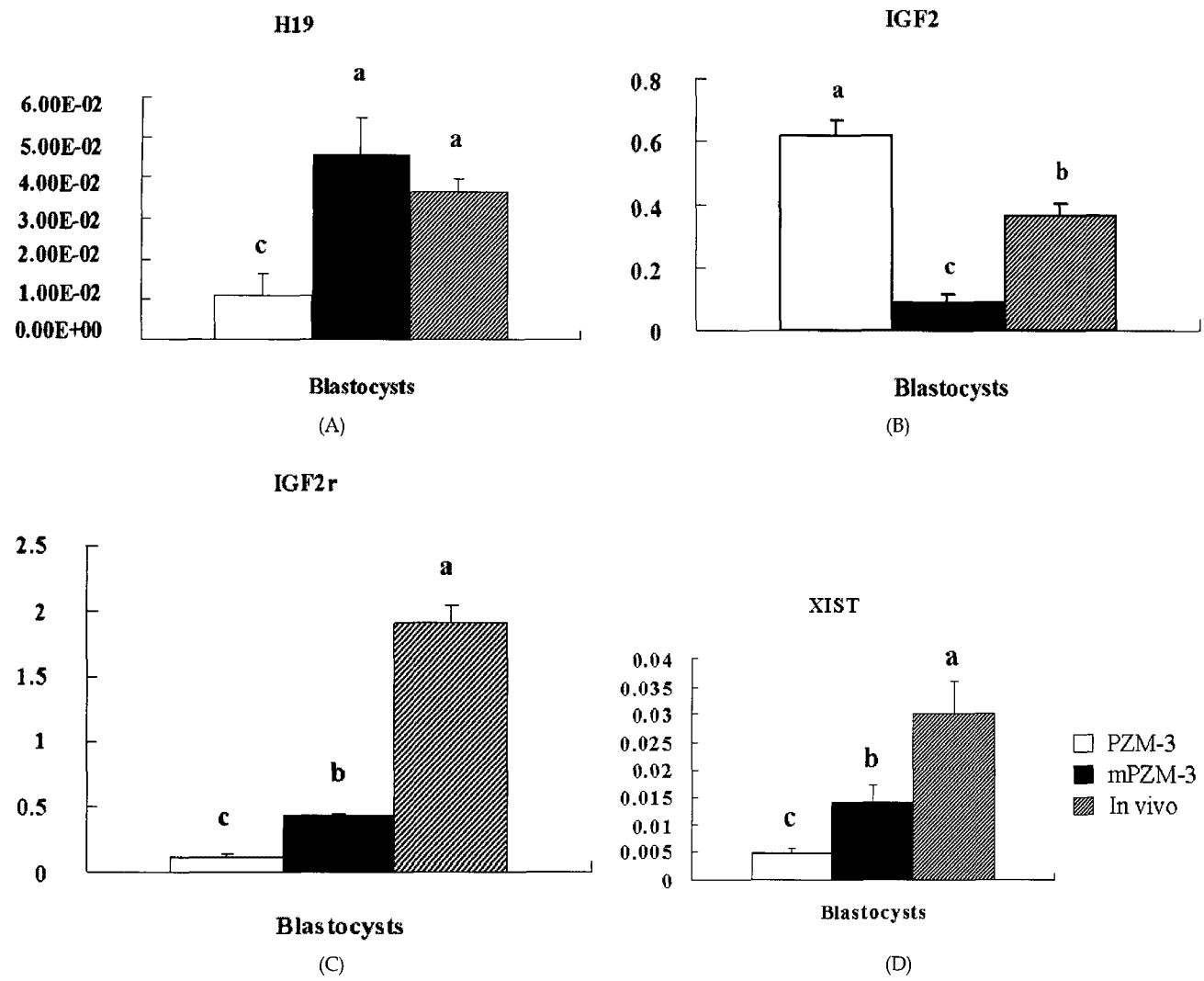


**Fig. 2. Relative imprinting gene expression levels (means±SE) of miniature pig NT blastocysts produced in different culture conditions and *in vivo* blastocysts.** Bars with different superscripts are significantly different($p<0.05$).

ment. For instance, Jurisicova *et al.* (1998) detected high p53 levels in the inner cell mass of mouse blastocysts, suggesting that p53 is necessary for apoptosis to occur. Conversely, other group (Frenkel *et al.*, 1999) suggested that apoptosis could occur in the mouse embryo, in either a p53-dependent or independent way. In bovine preimplantation embryos, a lack of p53 nuclear localization was detected, suggesting that p53 might not be active during early bovine embryo development (Matwee *et al.*, 2000). These observations suggest that the relative importance and/or function of p53 during early embryonic development have not been fully elucidated yet.

Many imprinted genes have significant roles in fetal and placental growth and differentiation. Previous studies in mice have suggested that *in vitro* culture of embryos and embryonic stem cells can lead to reduced viability and growth, developmental abnormalities and aberrant imprinted gene expression (Nagy *et al.*, 1993; Reik *et al.*, 1993; Dean *et al.*, 1998; Doherty *et al.*, 2000). In the current study, mRNA expression patterns of four imprinted genes were analyzed quantitatively to understand mechanisms underlying the different culture system of mNT embryos. The results showed that H19, IGF2r and Xist gene were completely repressed in control. Of the maternally imprinted genes, H19 is imprinted in domestic cattle and imprinting disruption of H19 can be present in developmentally abnormal animals produced by nuclear transfer (Zhang *et al.*, 2004). In H19-null mice, however, in which elevated expression of IGF2 is responsible for the fetal overgrowth, IGF2 concentration is not elevated in the serum, although it is increased in the tissue (Eggenschwiler *et al.*, 1997). Paternally imprinted genes include Xist and IGF2. Xist is required for X inactivation (Norris *et al.*, 1994) and is also expressed strongly in the blastocyst stage (Hartshorn *et al.*, 2002; Peippo *et al.*, 2002). In addition, transcriptional defect of Xist is lethal in the early developmental stage (Takagi and Abe, 1990; Migean *et al.*, 1993). The IGF2 and IGF2r genes are am-

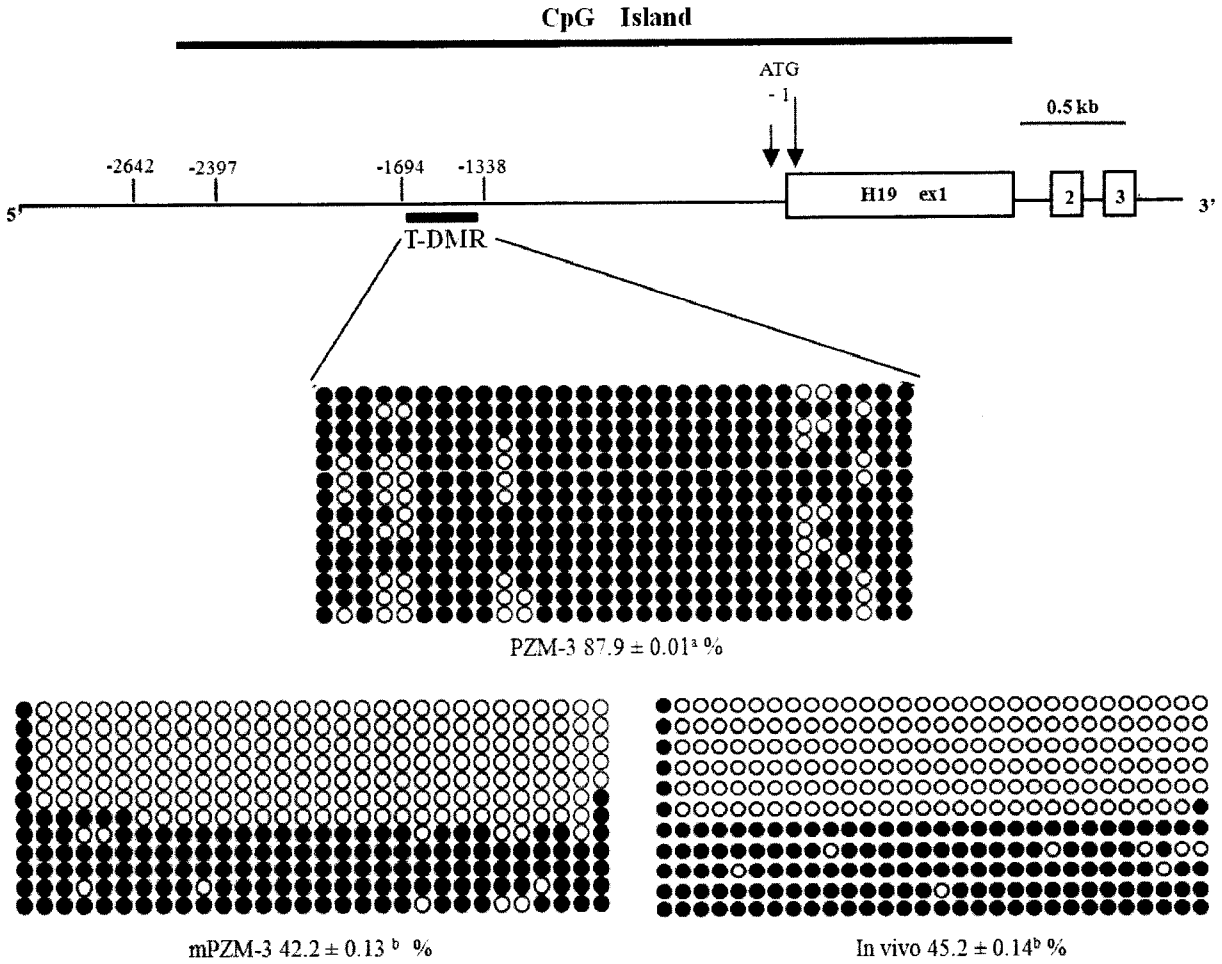


**Fig. 3. Methylation profiles of individual DNA strands in the H19 upstream T-DMR sequences in miniature pig NT and *in vivo* embryos (pools of 20 blastocysts).** Unmethylated CpGs are represented as empty circles; methylated CpGs are depicted as filled circles. Each line denotes an individual strand of DNA. Numbers indicate the proportion of methylated CpG sites relative to the whole CpG sites counted.

ong the best studied imprinted genes involved in fetal growth regulation, and are essential for normal development (Stoger *et al.*, 1993; Latham *et al.*, 1994). Previous studies have shown donor cell dependent expression patterns of IGF2 and IGF2r genes in NT and/or *in vivo* derived embryos (Kumar *et al.*, 2007). The expression of these transcripts showed a significant difference in the control compared to treatment group and *in vivo* derived embryos. The present study analysed DNA methylation of the H19 DMR (differentially methylated region), and found that methylation status differed depending on the different culture condition. DNA methylation status of a DMR located on upstream of the H19 gene is essential for the imprinted expression of both H19 and IGF2 (Tremblay *et al.*, 1997; Thorvaldsen *et al.*, 1998). It is reported that H19 is hypersensitive to environmental stress (Doherty *et al.*, 2000). For DNA methylation level in T-DMR of H19 gene, the NT blastocysts cultured in the treatment group and control were 42.2% and 87.9%, respectively. However, *in vivo* and treatment group showed similar levels. This finding supports the fact that the hypermethylated H19 T-DMR causes complete repression of the gene throughout development in the control.

In conclusion, our observations show that the higher osmolarity (treatment) than isotonic medium (control) at the early embryonic culture stage around the first and second cell cycles can increase the *in vitro* development of mNT embryos to the blastocyst stage. It can be concluded that use of a sequential culture method with different osmolarities could increase *in vitro* developmental ability of mNT embryos.

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