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Gene Expression of the *In Vitro* Fertilized or Somatic Cell Nuclear Transfer Embryos Cultured in Medium Supplemented with Different Proteins or Energy Substrates

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ABSTRACT

Several cloned animals have been produced using somatic cell nuclear transfer (SCNT) and have interested in producing the transgenic cloned animals to date. But still its efficiency was low due to a number of reasons, such as sub-optimal culture condition, aberrant gene expression and nuclear reprogramming. The purpose of this study was to analyze gene expression pattern in *in vitro* fertilized (IVF) or SCNT pre-implantation embryos. IVF- or SCNT-embryos were cultured in media supplemented with different proteins (FBS and BSA) or energy sources (glucose or fructose). Blastocysts from IVF or SCNT were analyzed using semi-quantitative RT-PCR in terms of development- or metabolic-related genes. Culture medium supplemented different proteins or energy sources had affected on the expression of developmental or metabolic genes in the SCNT blastocysts.

(Key words : bovine embryos, SCNT, IVF, gene expression, culture condition)

INTRODUCTION

Assisted reproductive technologies (ARTs) such as IVM/IVF/IVC, cryopreservation, and somatic cell nuclear transfer (SCNT) was so fast improved in bovine species. Recently, transgenic and cloned calves was born (Cibelli *et al.*, 1998; Brophy *et al.*, 2003) following the first live calf from IVF was born (Brackett *et al.*, 1982). Although advances of ARTs was done with related to the several factors including the embryo manipulation skills, various *in vitro* culture media i.e. synthetic oviduct fluid (SOF) media for cattle and sheep (Walker *et al.*, 1992; Matsuyama *et al.*, 1993), NCSU23 (North Carolina State University) for pigs (Machaty *et al.*, 1998) and CZB or KSOM for mice (Chatot *et al.*, 1989) have played a key role to produce the *in vitro* produced (IVP) animals. Embryos in cattle and sheep are generally cultured *in vitro* to the preimplantation stage before transfer and so this approach allows embryos quality to be assessed.

A number of *in vitro* culture media have been developed for individual species, these have included co-culture systems utilizing primary oviductal cell mono-layers or established cell lines (Thompson, 2000; Menezo and Herubel, 2002). Traditionally fetal bovine serum (FBS) and bovine serum albumin (BSA) were used to media supplement; however, more recently defined culture media have been developed (Oyamada and Fukui, 2004; Nedambale *et al.*, 2006).

According to previous our researches, embryonic developmental response to protein supplementation in KSOM were different between bovine IVF, non-transgenic and transgenic cloned embryos (Bhuiyan *et al.*, 2004) and effect of energy substrates (glucose & fructose) was evaluated with regard to developmental competence between IVF and cloned embryos (Kwun *et al.*, 2003).

Thus, the purpose of this study is to analyze the embryos in order to know whether embryo cultured in different proteins and energy sources affect the gene expression or not. The genes include *Bos taurus* apoptosis regulator box-α (Bax), E-cadherin (E-cad), interferon tau (IFN-τ), Lactate Dehydrogenase A (LDHA), insulin-like growth factor 2 receptor (Igf2r), Glucose-6Phosphate Dehydrogenase (G6PD), mammalian achaete-scute homologue (Mash) 2, Phosphogycerate Kinase (PGK), Na/K-ATPase, Glut-1 Glucose Transporter-1 (Glut-1) and gly-

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ceraldehyde-3-phosphate dehydrogenase (GAPDH).

MATERIALS AND METHODS

1. *In Vitro* Maturation

Bovine ovaries collected from a local slaughterhouse were transported to the laboratory within 2 hr in a 0.9% (v/v) NaCl solution at 35℃. The COCs were retrieved from small antral follicles 3 to 8 mm in diameter by aspiration with an 18 gauge hypodermic needle attached to a 10-ml syringe and washed several times in Hepes-buffered tissue culture medium (TCM)-199 (cat. no. 11150-059; Invitrogen) supplemented with 10% (v/v) FBS, 2 mM $NaHCO_3$, 5 mg/ml BSA (Invitrogen), and a 1% (v/v) mixture of penicillin and streptomycin (Sigma-Aldrich Corp., St. Louis, MO, USA). The COCs with evenly granulated cytoplasm and enclosed by more than 3 layers of compact cumulus cells were selected. A group of 30 to 40 COCs were cultured for maturation in one well of a multi-well dish containing 0.5 ml of bicarbonate-buffered TCM-199 supplemented with 10% (v/v) FBS, 0.005 IU/ml FSH (Antrin, Teikoku Seiyaku, Denka, Kanagawa, Japan) and 1 μg/mL 17β-estradiol (cat. no. E-8875, Sigma-Aldrich Corp.) at 39℃ in a humidified atmosphere of 5% CO_2.

2. Donor Cell Preparation for SCNT

Fetal fibroblasts were isolated from bovine fetuses on Day 40 of gestation. The head of the fetus was removed using iris scissors, and soft tissues such as liver and intestine were discarded by scooping out with two watchmaker's forceps. After washing three times with DPBS (cat. no. 14190-144, Life Technologies, Rockville, MD), the carcass was minced with a surgical blade in a 100-mm culture dish (Becton Dickinson, Lincoln Park, NJ). The minced fetal were dissociated in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 0.25% (w/v) trypsin and 1 mM EDTA (Invitrogen) for 1 hr at 37℃. Trypsinized cells were washed once in Ca^{2+}- and Mg^{2+}-free DPBS by centrifugation at 43×g for 2 min, and subsequently seeded into 100-mm plastic culture dishes. Seeded cells were subsequently cultured for 6 to 8 days in DMEM supplemented with 10% (v/v) FBS (Life Technologies), 1 mM glutamine (Invitrogen), 25 mM $NaHCO_3$ (Sigma-Aldrich Corp) and 1% (v/v) minimal essential medium (MEM) nonessential amino acid solution (Life Technologies) at 39℃ in a humidified atmosphere of 5% CO_2 and 95% air. After removal of unattached clumps of cells or explants, attached cells were further cultured until confluent, subcultured at intervals of 4 to 6 days by trypsinization for 5 min using 0.1% trypsin and 0.02% EDTA, allocated to new three dishes for further passaging and then stored in freezing medium in liquid nitrogen at −196℃. The freezing medium consisted of 80% (v/v) DMEM, 10% (v/v) DMSO (Sigma-Aldrich Corp.) and 10% (v/v) FBS. Fresh cells at passages 4 to 6 were used for SCNT. Prior to SCNT, cells were thawed, cultured for 3 to 4 days until 100% confluency for contact inhibition, and retrieved from the monolayer by trypsinization for 30 sec.

3. *In Vitro* Fertilizaiton and Reconstruction of Embryo and *In Vitro* Culture

Motile spermatozoa were selected by the swim-up technique (Parrish *et al.* 1986). At 22 hr of IVM, oocytes were then inseminated with 1×10^6 spermatozoa/ml for 18 hr in 50 μl/well of Tyrode's Albumin-Lactate-Pyruvate (TALP)-IVF medium on a 4 well plate.

At the end of maturation culture, COCs were transferred to Hepes-buffered calcium-free CRaa medium with amino acids (hCR2aa, Rosenkrans *et al.*, 1991) containing 0.1% (w/v) hyaluronidase (from bovine testis, Sigma-Aldrich Corp.) for 1 min and the cumulus cells were subsequently removed by gentle pipetting. After denuding, oocytes were placed in hCR2aa supplemented with 10% FBS and 7.5 μl/ml cytochalasin B (Sigma-Aldrich Corp.) overlaid with mineral oil in a micromanipulation chamber (Falcon). Enucleation was carried out according to the methods of Shin *et al.* (2001). At 22 hr of IVM, a cumulus-free oocyte was held with a holding micropipette and the zona pellucida was partially dissected with a fine glass needle to make a slit near the first polar body. The first polar body and adjacent cytoplasm presumably containing the metaphase-II chromosomes were extruded by squeezing with the same glass needle. After enucleation, oocytes were stained with 5 μg/mL bisbenzimide (Hoechst 33342, Sigma-Aldrich Corp.) for 5 min and observed under an inverted microscope equipped with epifluorescence. Oocytes still containing DNA materials were excluded from experiments. The enucleated cytoplasts were washed thoroughly in Hepes-TCM 199+10% FBS and incubated in TCM 199+10% FBS for 1 to 2 hr until injection of donor cells. Enucleated oocytes were placed in a 4 μl drop of Hepes-TCM199 containing 10% FBS and 100 μg/ml phytohemagglutinin (Sigma-Aldrich Corp.). A 25 μm diameter pipette containing the donor cell was introduced through the same slit in the zona pellucida made during enucleation. Recons-

tructed embryos were electrically fused in a buffer comprising 0.28 M mannitol (Sigma-Aldrich Corp.), 0.5 mM Hepes (Sigma-Aldrich Corp.), and 0.05% fatty acid-free BSA (Sigma-Aldrich Corp.) with 0.1 mM $MgCl_2$ (Sigma-Aldrich Corp.). Fusion was performed at room temperature in a chamber with two stainless steel electrodes 3.4 mm apart that were overlaid with fusion buffer. Cell fusion was induced with two DC pulses of 1.75 kV/cm for 15 μsec/each using a BTX Electro-cell Manipulator 2001 (BTX, San Diego, CA). After fusion, reconstructed embryos were cultured for 4 hr in mSOF (Takahashi and First, 1992) containing 8 mg/ml BSA (control mSOF). Activation was performed as described previously (Shin *et al.*, 2001). Briefly, chemical activation was induced by incubating reconstructed embryos in 30 μl drops of Hepes-TCM199 containing 5 μM ionomycin (Sigma-Aldrich Corp.) for 4 min at 39℃. Embryos were then thoroughly washed in Hepes-TCM 199 for 5 min and further incubated for 4 hr in the control mSOF supplemented with 1.9 mM 6-dimethylaminopurine (6-DMAP, Sigma-Aldrich Corp.).

Reconstructed couplets or IVF embryos were cultured in 25 μl microdrops of the control mSOF overlaid with mineral oil (Sigma-Aldrich Corp.). Groups of 6 or 7 embryos were cultured together for 7 days at 39℃ in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. Embryo cleavage and blastocyst development were examined at 2 and 7 days of culture, respectively.

4. Relative Abundance of Gene Expression

Six blastocysts were washed in three changes of PBS and transferred into 0.2 ml of 4 M guanidium isothiocyanate lysis solution containing 1% β-mercaptoethanol. Total RNA was extracted by thiocyanate extraction and dissolved in 10 μl RNase-free water as described by Szafranska *et al.* (1995) and was subjected to reverse transcription-polymerase chain reaction (RT-PCR). Reverse transcription was carried out at 37℃ for 60 min. Individual RT reactions (15 μl each) consisted of 5 mM $MgCl_2$, 1×RT buffer, 2.5 μM oligo (dT), 1 mM dNTP, and 50 IU murine leukemia virus reverse transcriptase (Amersham Pharmacia Biotechnologies, Oakville, ON, Canada). Primer sequences, annealing temperature and the approximate sizes of the amplified fragments are listed in Table 1. All primers were selected based on the bovine sequence of the respective genes available in the gene database at the National Center for Biotechnology Information. The cDNA (1 μl) was amplified in a 50-μl PCR reaction containing 1.25 units hot start *Taq* polymerase (Qiuagen, Hilden, Germany) and its buffer, 1.5 mM $MgCl_2$, 2 mM dNTP, and 25 pmol specific primers. The PCR amplification was carried out for one cycle with denaturing at 95℃ for 15 min, and 35 subsequent cycles with denaturing at 95℃ for 30 sec, annealing for 30 sec, extension at 72℃ for 30 sec and a final extension at 72℃ for 10 min. Ten microliters of PCR products were fractionated on a 1.5% agarose gel, and stained with ethidium bromide. For each pair of gene-specific primers, semilog plots of the fragment intensity as a function of cycle number were used to determine the range of cycle number over which linear amplification occurred, and the number of PCR cycles was kept within this range. The expression level for each gene was determined by densitometric analysis using a Gel Doc software (Bio-Rad, Hercules, CA). Relative expression levels of each gene were represented as the ratio of each gene to GAPDH gene.

5. Experiment Designs

1) Experiment 1

Embryos from IVF or SCNT cultured in control mSOF and KSOM with different protein supplementation (0.8 % BSA, 10% FBS and 0. 01% PVA) was collected and analyzed with the gene expression (Table 1).

2) Experiment 2

Bovine embryos derived from IVF or SCNT were cultured in mSOF with glucose or fructose. Pre-implantation stage embryos were analyzed with relevant glucose metabolic gene expression levels (Table 1).

6. Statistical Analysis

Data were analyzed using a statistical analysis system (SAS) program. Each value of experimental parameter was subjected to analysis of variance (ANOVA) and protected least significant different (LSD) test using general linear models to determine differences among experimental groups. When a significant treatment effect was found in each experimental parameter, data were compared by the least squares method. Statistical significance was determined where the *P* value was less than 0.05.

RESULTS

1. Experiment 1. Effect of Protein Sources (BSA, FBS, and PVA) on the IVF and SCNT Embryos

Representative gel photographs of a semi-quantitative RT-

Table 1. The list of amplified genes and their primer sequences and PCR conditions

Gene	Primer sequences	Annealing temp. (℃)	Cycle number	Fragment size (bp)
LDHA	5'-TTGCTCTTGTTGATGTCATGGAAG 3'-CACTCCATACAGGCACACTAGAGT	50	30	465
G6PD	5'-CGCTGGGACGGGGTGCCCTTCATC 3'-CGCCAGGCCTCCCGCAGTTCATCA	64	31	347
PGF	5'-TTATTGGTGGTGGAATGGCTTTTA 3'-GGACCATTCCACACAATCTGCTTA	59	36	358
Na/K-ATpase	5'-ACCTGTTGGGCATCCGAGAGAC 3'-AGGGGAAGGCACAGAACCACCA	58	31	336
Glut-1	5'-AGGAGCTGTTCCACCCCCTGGGAG 3'-TGTGGGTGAAGGAGACTCTGGCTG	59	32	327
Bax	5'-TGCAGAGGATGATCGCAGCTGTG 3'-CCAATGTCCAGCCCATCATGGTC	60	30	198
IFN-tau	5'-GCTATCTCTGTGCTCCATGAGATG 3'- AGTGAGTTCAGATCTCCACCCATC	55	30	359
Igf2r	5'-CGCCTACAGCGAGAAGGGGTTAGTC 3'-AGAAAAGCGTGCACGTGCGCTTGTC	60	50	293
Mash2	5'-CGCTGCGCTCGGCGGTGGAGTA 3'-GGGACCCGGGCTCCGAGCTGTG	65	50	210
GAPDH	5'-CATCACCATCTTCCAGGAGCGAGA 3'-CCTGCTTCACCACCTTCTTGATGT	55	30	573

PCR assay of the analyzed gene transcripts in Day 8 blastocysts were shown in Fig. 1. The expression of LADA in SCNT-KSOM-PVA medium was decreased. The G6PD transcripts level was increased in IVF-mSOF-BSA, KOSM-FBS and SCNT-KSOM-PVA. While expression level of GLUT-1 in all SCNT groups was higher than IVF ones, expression pattern of NA/K-ATPase and E-cad in the all IVF and SCNT groups showed similarly. SCNT-mSOF-BSA embryos showed higher expression in PGF than any other group and If-tau expression was increased in IVF-KSOM-FBS and SCNT-KSOM-PVA. The expression of Igf2r in IVF-KSOM-BSA and SCNT-KSOM-FBS was decreased and expression level of Mash2 in IVF-KSOM-BSA, -FBS and SCNT-KSOM-BSA, -FBS was also decreased.

2. Experiment 2. Effect on the Gene Expression of Energy Substrate (Glucose and Fructose) in IVF and SCNT Embryos

Representative gel photographs of a semi-quantitative RT-PCR assay of the analyzed gene transcripts in Day 8 blastocysts were shown in Fig. 2. The expression of G6PD, PGF, Na/K-ATPase, E-cad, Igf2r and GLUT-1 in IVF/SCNT-Glucose or Fructose embryos did not showed different pattern. While the LADA transcripts level in the blastocysts from IVF-Glucose medium was increased, embryos derived from IVF-Fructose medium showed the higher expression level of Bax than any other group. In case of Mash2 gene expression, blastocysts from IVF-Glucose, -Fructose was higher than SCNT ones, and this gene expression of IVF-Fructose medium was significantly higher than IVF-Glucose one.

DISCUSSION

In this study, it was demonstrated that various gene expressions on the pre-implantation stage embryos derived from IVF and SCNT in term of protein supplementation and different energy sources showed the different pattern (Figs. 1 and 2). In cattle embryos, means to evaluate the viability of embryos were morphological observation, cell number and tolerance of cryopreservation. Recently, gene expression level was used to assess the embryo quality and showed the different

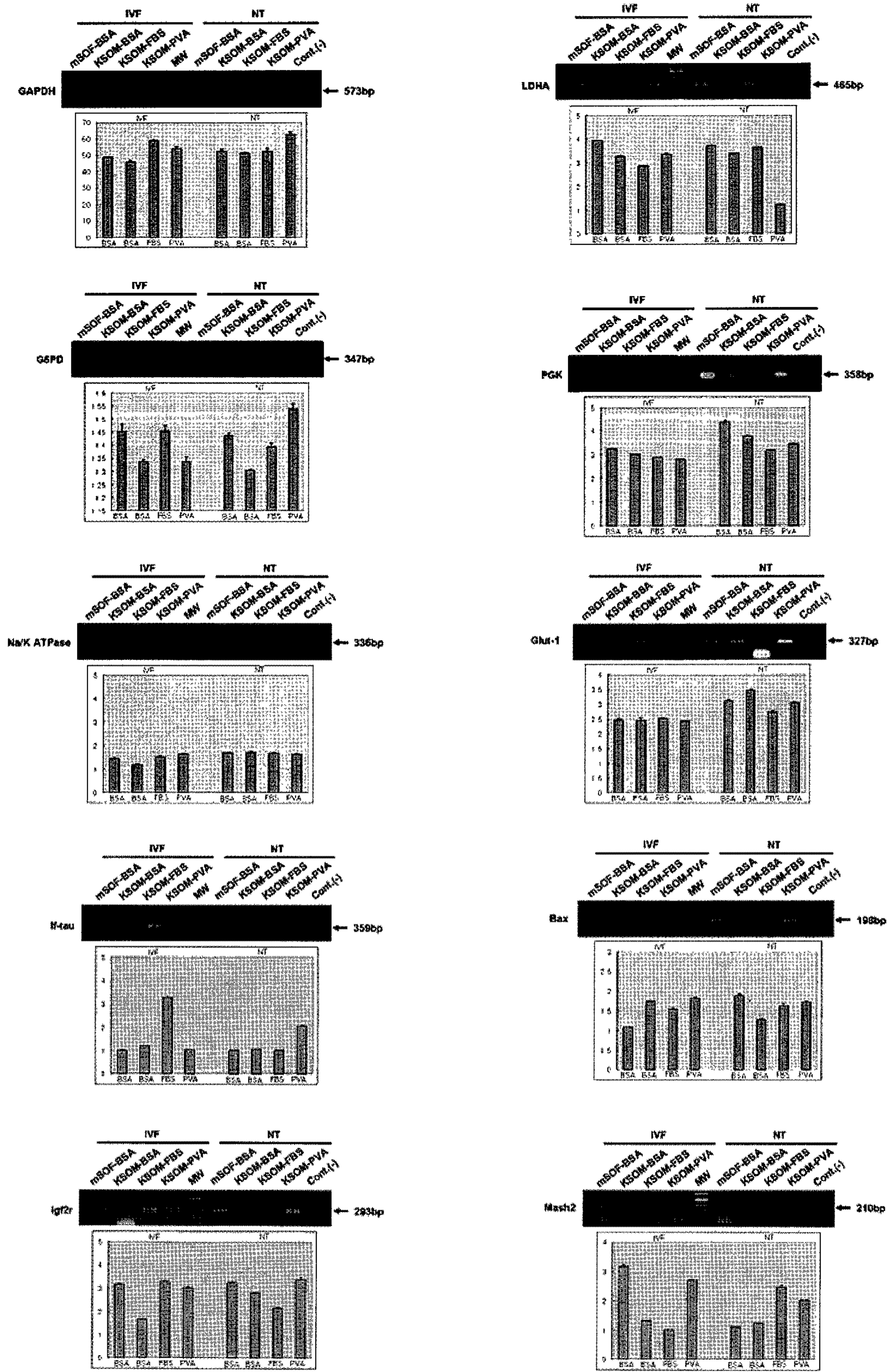

Fig. 1. Electrophoresis figure and relative abundance of transcripts of ten genes important to development and implantation as detected by semi-quantitative RT-PCR.

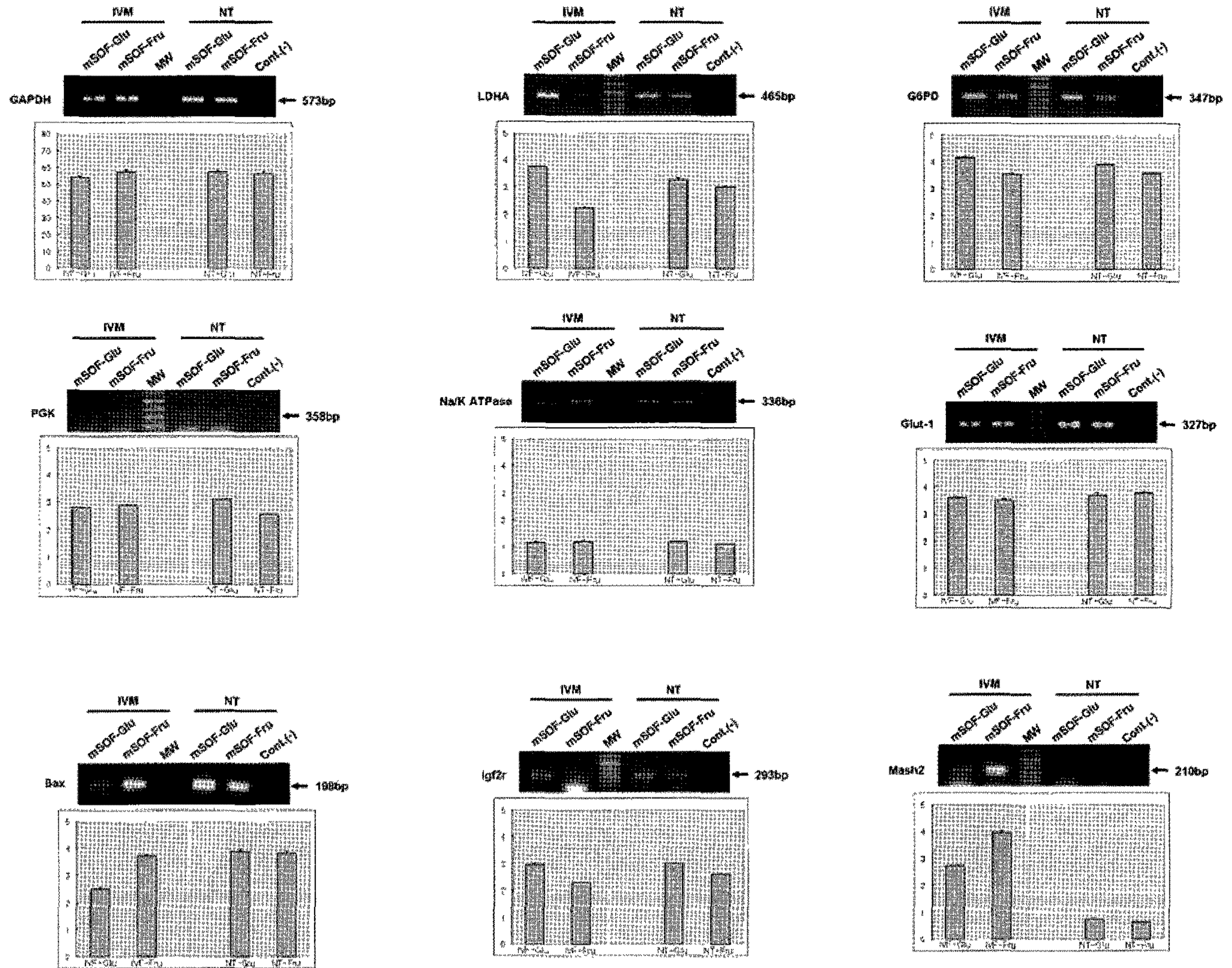

Fig. 2. Electrophoresis figures and relative abundance of transcripts of ten genes important to development and implantation as detected by semi-quantitative RT-PCR.m

pattern under the culture condition and experimental protocols.

In many research groups, cattle embryos were cultured in mSOF containing BSA was widely used. However, BSA and FBS might be related to incidence of large offspring syndrome. So, defined culture media without BSA and FBS was used and showed the same developmental competence with culture media containing BSA and FBS. And KSOM containing BSA was effective in supporting development of bovine cloned embryos. In over the world, research team for producing the cattle embryo derived from IVF or SCNT has the favorite media such as, mSOF containing BSA or FBS, CR, KSOM, and defined culture media. These culture media supported the developmental competence in *in vitro* and also guarantee to produce the live calves. Here, we compared the several gene expressions in mSOF-BSA and KSOM supplemented BSA, FBS, or PVA. According to previous reports, mSOF-BSA and KSOM-BSA showed the similar effect on the developmental competence in bovine embryos and we found the different the gene expression pattern in blastocysts derived from different culture media (Figs. 1 and 2).

The temporal or spatial and qualitative or quantitative shifts of the well-orchestrated expression patterns of developmental important genes have been investigated in pre-implantation bovine embryos following *in vitro* embryo manipulations. Gene expression varies according to the respective IVP system and NT protocols (Niemann and Wrenzycki, 2000; Niemann *et al.*, 2002; Wrenzycki *et al.*, 2002; Lonergan *et al.*, 2003). Imprinted genes appear to be more susceptible to alterations in epigenetic

modifications (Moore, 2001), especially after IVC of ovine (Young *et al.*, 2001) or bovine (Blondin *et al.*, 2000) embryos.

It was demonstrated that expression of G6PD that is important role to detoxify oxygen radicals were increased in IVF-mSOF-BSA and IVF-KSOM-FBS compared with other groups. PGK expression, a key enzyme in glycosis, is encoded from X choromosme showed similar pattern in blastocysts derived from IVF-mSOF or KSOM media. Also, blastocysts derived from IVF-mSOF-BSA, or KSOM containing BSA, FBS, or PVA was not found the difference in metabolic related genes, LDHA, Glut-1, E-cad and Na/K ATPase. In SCNT embryos derived from SOF-BSA, KSOM-BSA, -FBS, or -PVA, we did not find the difference of transcripts of Na/K ATPase, Glut-1, Bax and E-cad. The expression of LDHA; enzyme that change the pyruvate into lactate, was significantly decreased compared to other SCNT groups. In case of G6PD gene, blastocysts derived from SCNT-KSOM-PVA expressed the higher transcripts. It could be thought that early stage embryo, in general used lactate and pyruvate as the energy source, be affected and then decrease the viability of blastocysts.

Compared to mSOF-BSA, expression of bax showed significantly higher level in KSOM containing BSA, FBS, or PVA. Bax, known as pro-apoptotic of bcl-2 family is involved in programmed cell death. Apoptosis is part of the normal developmental program of embryos (Pierce *et al.*, 1989; Hardy, 1997) and is developmentally regulated (Matwee *et al.*, 2000). However, the percentage of apoptotic cells is significantly higher in blastocysts produced *in vitro* compared to blastocysts *in vivo* (Jurisicova *et al.*, 1998). We suggest that the increased expression of bax in KSOM media may show the detrimental effect for developing the embryonic stage and fetal growth.

In IVF embryos derived from KSOM-BSA, expression of Igf2r and Mash2 was reduced compared to mSOF-BSA. Igf2r expression in SCNT-KSOM-FBS was decreased and transcript level of Mash was also decreased in SCNT-mSOF-BSA or -KSOM-BSA. It was thought as the same results that transcripts level of these gene in IVP system was increased compared to their *in vivo* counterparts (Wrenzycki *et al.*, 2001b; Bertolini *et al.*, 2002).

IF-tau expression level was increased not only in IVF-KSOM-FBS embryos, but also in SCNT-KSOM-PVA embryos. IF-tau is secreted by trophectoderm and is related to the implantation of bovine blastocysts. This gene product inhibits Cox to assist implantation of the embryo. Inhibition and deficiency of Cox, which generates prostaglandin via the cyclooxygenase pathway, causes abnormalities in ovulation, fertilization, implantation and decidualization (Langenbach *et al.*, 1995; Lim *et al.*, 1997).

In the composition of embryo culture media, glucose has broadly been used as a major energy substrate. The role glucose in pre-implantation development has been demonstrated in various species, but the rationale for glucose supplementation is still ambivalent. Exposure to high concentrations of glucose during early embryonic stages caused developmental retardation in many species including hamsters (Schini and Bavister, 1988; Barnett and Bavister, 1996; Barnett *et al.*, 1997), mice (Chatot *et al.*, 1989; Lawitts and Biggers, 1991; Scott and Whittingham, 1996), rats (Kishi *et al.*, 1991; Miyoshi *et al.*, 1994), cattle (Kim *et al.*, 1993), sheep (Thompson *et al.*, 1992), and human (Conaghan *et al.*, 1993; Quinn, 1995). In our previous result, fructose might be more efficient energy substrate for producing the large number of transferable blastocysts derived from SCNT (Kwun *et al.*, 2003). In spite of like beneficial effect on the embryonic developmental competence, up to date, fructose was not broadly used in culture media than using the glucose. In this study, we demonstrated that analysis of expression relevant metabolic gene on the IVF/SCNT embryos derived from mSOF containing glucose or fructose was done with semi-quantitative RT-PCR. While we did not find the difference of transcript level in SCNT embryos derived from culture media containing glucose or fructose, in IVF embryos, expression showed the different pattern in LDHA, Bax, and Mash2 genes. Blastocysts from IVF-mSOF-fructose expressed the higher transcripts in Bax and Mash2. And expression of LDHA in the IVF-mSOF-glucose was increased. These different gene expression may be possible to detrimental effect on the embryonic growth because the low pregnancy rate was observed compared to transfer the embryo derived from culture media containing glucose (not shown data).

In summarized, this study demonstrated that the expression pattern of developmental and metabolic related genes in IVF/SCNT pre-implantation embryos derived from different proteins could be changed. And, IVF embryos from culture media containing the fructose instead of glucose showed the a little different transcripts levels in metabolic related genes. Further more study regarding for *in vivo* counterparts and fetal growth may help to choose the optimized culture media.

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