Recombinant human activin A promotes development of bovine somatic cell nuclear transfer embryos matured *in vitro*

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ABSTRACT: To improve the culture system of bovine somatic cell nuclear transfer (SCNT) embryos, we studied the effects of activin A on developmental competence of bovine SCNT embryos during the early development stage based on the traditional culture method, and analyzed the expression level of the genes related to blastocyst hatching (Na/K-ATPase, Glut-1) and related to activin A signalling pathway (ActRII and Smad2). We generated the bovine SCNT embryo using a Holstein cow oocyte as recipient cytoplasm and a foetal ear fibroblast (Holstein cow, 120 days) as donor cell. The embryos were cultured as follows: experiment 1, the addition of activin A at the concentrations of 0 (control), 20 (M1–20), 40 (M1–40) or 80 ng/ml (M1–80) to the media during the first three days and no addition during the subsequent 5 days; experiment 2, no addition of activin A to the media during the first 3 days and the addition of activin A at the concentrations of 0 (control), 20 (M2–20), 40 (M2–40) or 80 ng/ml (M2–80) during the subsequent 5 days. The results indicated that the blastocyst formation rate and hatching rate, and total blastomere numbers as well as ICM/TE obtained in experiment 1 were not significantly different from the control group ($P > 0.05$). In contrast, these values obtained in experiment 2 were significantly higher than in the control group ($P < 0.05$). In addition, the relative abundance (ratio to GAPDH mRNA) of each gene (Glut-1, ActR II and Smad2) was not significantly different among the treatments in the experiment. The expression levels of 4 genes (Na/K-ATPase, Glut-1, ActR II and Smad2) in blastocysts obtained in experiment 2 were higher than those obtained in experiment 1. In conclusion, the present study suggests that the addition of activin A to the culture media from day 4 to day 8 can enhance the developmental competence of bovine SCNT embryos.

Keywords: activin A; SCNT; embryo development; bovine

Animal somatic cell cloning technology has developed rapidly in recent years. With a variety of animals having been cloned successfully (Hossein et al., 2009), the application of somatic cell cloning technology in life sciences, basic research, human medical research, and to save rare and endangered wild animals, has been receiving an increasing attention.

A certain period of culture *in vitro* is essential for *in vitro* production of embryos, and only good quality embryos are selected for transplant into the womb. Therefore, the culture *in vitro* of bovine somatic cell cloned embryos is a pivotal step for successful cloning. Although a lot of work has be done to improve *in vitro* culture conditions of bovine embryos, the culture *in vitro* is still less efficient than *in vivo* (Enright et al., 2000; Rizos et al., 2002; Khatir et al., 2007). The main reason is that the development of embryos *in vivo* is controlled by unknown cytokines derived from the maternal

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reproductive tract that influence the embryo development in a spatial and temporal manner (Jones et al., 2006). However, these components must be complemented artificially during *in vitro* culture.

Activin A, a member of the TGF-β superfamily, is a functional cytokine of maternal-foetal interaction, mainly produced in uterine epithelial cells and endometrial cells (Manova et al., 1992; Orimo et al., 1996; Park et al., 2008). Activin subunits and activin receptor mRNAs are expressed throughout embryo development, which play important roles not only for embryonic development but also for implantation (Yoshioka et al., 1998). Activin A is produced mainly by oviduct epithelial cells in bovines, so the addition of activin A to embryo culture medium *in vitro* may imitate the physiological environment in the oviduct *in vivo* (Gandolfi et al., 1995). Activin A is presently also known for its activity on the growth and differentiation of various cell types (Barnes and Eyestone, 1990; Hirai et al., 2007), is present in mammalian oviducts and may physiologically regulate the development of preimplantation embryos (Albano et al., 1993).

Furthermore, the vectorial transport of Na^+ and Cl^- from the medium into the blastocoelic cavity is very important for blastocyst formation, and the transport of Na^+ is likely to be controlled by Na/K-ATPase (Watson and Kidder, 1988; Vorbrodt et al., 1997). It has also been reported that bovine embryos acquire the capacity to utilize glucose after the morula stage. The gene Glut-1 is a developmentally important gene involved in the transport and metabolism of glucose, and Rieger et al. (1992) reported that glucose transport is upregulated with the formation of the blastocyst, and downregulation results in decreased blastocyst cell numbers and increased apoptosis. Furthermore, activin receptors, type I (ActRI and ActRI B) and type II (ActRII and ActRIIB), have been characterized and the formation of a complex of type I and type II receptors is required to initiate intracellular second messenger signalling. Activin activates signalling molecules known as receptor-associated Smads, and the activated Smad complexes in turn translocate into the nucleus to mediate the expression of target genes (Coss et al., 2005). So, the comparison of the expression patterns of ActRII and Smad2 is required to evaluate the effect of activin during *in vitro* culture.

Yoshioka and Kamomae (1996) reported that the addition of activin A to bovine embryo culture media had increased the number of 1-cell embryos reaching the morula or blastocyst stage. Another study showed that the development-enhancing effects of activin A were observed in bovine IVF embryos until the fourth cell cycle (Yoshioka et al., 1998). Although many studies have reported the roles for activin A in embryo development (Stock et al., 1997; Silva and Knight, 1998; Yoshioka et al., 2000; Park et al., 2008), results are inconsistent. Moreover, no studies on activin A added to the culture media of bovine SCNT embryos have been reported. In order to promote the development of bovine SCNT embryos, based on the conventional technology of bovine SCNT embryo culture, we analyzed the effects of activin A on the development of bovine SCNT embryos and compared the expression levels of Na/K-ATPase, Glut-1, ActRII and Smad2.

MATERIAL AND METHODS

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Company (USA). Disposable, sterile plasticware was purchased from Nunclon (Roskilde, Denmark).

In vitro maturation of oocytes

Cumulus-oocyte complexes (COCs) were obtained by aspiration from the ovaries of a slaughtered Holstein cow within 3–4 hours immediately after slaughter. After washing twice in modified PBS supplemented with 0.31 mmol/l pyruvate and 5% (v/v) foetal bovine serum (FBS, Gibco), oocytes surrounded by at least three layers of cumulus cells were transferred into an oocyte maturation medium and cultured for 22 h at 38.5°C under an atmosphere of 5% CO_2 in the air with maximum humidity. Cumulus-oocyte complexes were stripped from cumulus cells by pipetting with a finely-drawn glass pipette in 0.3% hyaluronidase dissolved in HEPES-buffered TCM199. Oocytes with visible polar bodies were selected for use in the procedures described below.

Preparation of donor cells

A small piece of foetal (Holstein cow, 120 days) ear skin tissue was collected from the abattoir. The tissue was washed three times in PBS and finely cut into 1 mm^2 pieces, then digested in 0.25% trypsin–

EDTA solution for 15 min. After washing twice in PBS by centrifugation at 1 000 rpm, dissociated cells were washed three times by centrifugation to obtain a cell pellet. The pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS, and placed in a plastic culture dish. After the cells had formed a confluent monolayer, the culture was continued for three to five passages, and those cells with normal karyotype in the previous generation by karyotype analysis were used as donor cells. Before the nuclear transfer, they were made quiescent by culturing in DMEM with 0.5% FBS. Specific methods were described previously by Hua et al. (2007).

Nuclear transfer and embryo culture

Denuded oocytes selected previously were enucleated by aspirating the first polar body and the MII plate. Donor cells were placed in a drop of TCM199 supplemented with 5% FBS and 5 μg/ml cytochalasin B, and the enucleated oocytes (cytoplasts) were placed in the same drop. Individual donor cells were injected into the perivitelline space using a fine bore pipette. After injection, the reconstructed embryos were transferred into an electrical fusion chamber. Cell fusion was induced with two direct current pulses. Embryos were then activated by 5 μm ionomycin in TCM199 for 5 min, followed by a 4-hour incubation in 2mM 6-DMAP. Activated embryos were transferred to modified synthetic oviduct fluid (mSOF) (Takahashi and First, 1992), supplemented with or without activin A. Embryo development was monitored daily for 8 days.

Blastomere counting

Differential staining of ICM and TE cells of blastocysts (day 8) was performed as described by Koo et al. (2002). The zona pellucida of each blastocyst was removed by a brief exposure to acidified PBS (pH 3.0). After rinsing in PBS (pH 7.0) containing 1 mg/ml polyvinyl alcohol, denuded embryos were exposed to a 1:5 dilution of rabbit anti-rat whole serum for 1 h, rinsed three times for 5 min each in PBS (pH 7.0), and placed into a 1:5 dilution of guinea rat complement containing 10 mg/ml propidium iodide and 10 mg per ml Hoechst 33342 for 1 h. Then the embryos were placed onto microscope slides in a small volume of solution and mounted under glass coverslip. The embryos were examined under ultraviolet light using an epifluorescent microscope. Blue and red colours were designated as ICM and TE cells, respectively.

Relative quantification by RT-PCR

Poly(A)RNA was isolated from single embryos using the High Pure Viral RNA Kit (Roch, Germany) in accordance with instructions. The method used for quantification of expression was a relative standard curve method. The quantification was normalized to an endogenous control (GAPDH). The standard was prepared with bovine GAPDH mRNA, reverse transcribed, purified, am-

Table 1. Primer sequences

Gene	Primer sequence	Tm (°C)	Fragment size (bp)
GAPDH	F-5'-CATCA CCATC TTCCA GGAGC GAGA	55	573
	R-5'-CCTGC TTCAC CACCT TCTTG ATGT		
Na/K ATPase	F-5'-AATCC ATCGC CTACA CCC	55	330
	R-5'-GAAGC CGCGA AGGTA GGA		
Glut-1	F-5'- AGGAG CTGTT CCACC CCCTG GGAGC	55	327
ActRII	R-5'-TGTGG GTGAA GGAGA CTCTG GCTGA	53	287
	F-5'-AGATA GAACG AATCG CACTG		
	R-5'-GAGGC TTAGG AGTTA CTGGA TT		
Smad2	F-5'-TTGCC GAGTG CCTAA GTG	55	366
	R-5'-TGAGC AACGC ACTGA AGG		

Table 2. The development of cloned embryos cultured in the medium with activin A for the first 3 days and without activin A for the subsequent 5 days (in M1 media)

Activin A	*n*	Percent of cleavage	Percent of 8/16 cell embryos	Percent of blastocysts	Rates of hatched blastocysts
0	219	150 (72.1)	92 (42.3)	47 (21.5)	34 (15.6)
20	208	166 (79.8)	79 (37.8)	42 (20.2)	31 (14.9)
40	213	153 (71.8)	92 (43.2)	49 (23.2)	34 (15.7)
80	220	158 (71.8)	75 (34.1)	59 (26.6)	39 (17.8)

values within each column are not significantly different ($P > 0.05$)

plified by PCR, extracted from the gel and cloned. Reverse transcription was performed using the High Fidelity PrimeScript^{RT} RT-PCR Kit (Takara Biotech) following the manufacturer's protocol to produce cDNA. The plasmid DNA was quantified and serially diluted. cDNA templates for individual embryos were used for the amplification of GAPDH, Na/K ATPase, Glut-1 and Smad2 (primers are listed in Table 1). PCR reactions were set up in 25 µl reaction mixtures containing 12.5 µl SYBR Premix Ex TaqTM (Takara, Biotech Co. Ltd.), 0.5 µl each primer, 1.5 µl distilled water, and 10 µl template. The reaction conditions were as follows: 94°C for 30 s, followed by 40 cycles at 95°C for 15 s, and 55°C for 20 s. The experiments were carried out on a SmartCycler (Cepheid, USA).

Experiment designs

M1 and M2 are the same medium formations, they are both mSOF, differing in the time of activin A addition (M1 = early addition, M2 = late addition). Experiment 1 and experiment 2 were conducted at the same time.

Experiment 1: Reconstructed embryos were cultured in the M1 medium with different concentrations of activin A (0, 20 ng/ml, 40 ng/ml, 80 ng/ml) for the first 3 days, then the embryos were cultured without activin A during the subsequent 5 days.

Experiment 2: Reconstructed embryos were cultured without activin A for the first 3 days, and then the embryos were cultured in the M2 medium with different concentrations of activin A (0, 20 ng per ml, 40 ng/ml, 80 ng/ml) during the subsequent 5 days.

Experiment 3: Based on experiments 1 and 2, day-8 blastocysts were selected randomly to analyze the relative expression levels of Na/K-ATPase, Glut-1, ActRII and Smad2.

Data statistics

Each experiment was repeated at least three times. The developmental potential, measured as

Table 3. The development of cloned embryos cultured in the media without activin A for the first 3 days and with activin A for the subsequent 5 days (in M2 media)

Activin A	*n*	Percent of cleavage	Percent of 8/16 cell embryos	Percent of blastocysts	Rates of hatched blastocysts
0	256	185 (72.3)	82 (32.1)^{a}	49 (19.4)^{a}	39 (15.5)^{a}
20	263	198 (75.3)	115 (43.5)^{b}	70 (26.4)^{b}	58 (22.8)^{b}
40	245	191 (78.0)	126 (51.6)^{b}	78 (31.8)^{b}	62 (25.2)^{b}
80	251	179 (71.3)	122 (48.7)^{b}	67 (26.7)^{b}	56 (22.3)^{b}

values within each column with different superscripts differ significantly ($P < 0.05$)

Table 4. Numbers of ICM, TE and total blastomeres in blastocysts obtained with activin A for the first 3 days and without activin A for the subsequent 5 days (in M1 media)

Activin A	*n*	ICM	TE	Total	ICM/TE
0	19	21.23 ± 5.65	48.23 ± 4.85	67.37 ± 5.32	0.43 ± 0.23
20	21	25.14 ± 3.43	50.63 ± 4.78	80.20 ± 4.56	0.44 ± 0.21
40	21	27.16 ± 3.65	54.12 ± 5.92	79.23 ± 8.29	0.50 ± 0.18
80	20	28.80 ± 6.82	56.43 ± 6.41	81.14 ± 6.57	0.51 ± 0.24

values within each column are not significantly different ($P > 0.05$)

Table 5. Numbers of ICM, TE and total blastomeres in blastocysts obtained without activin A for the first 3 days and with activin A for the subsequent 5 days (in M2 media)

Activin A	*n*	ICM	TE	Total	ICM/TE
0	19	20.12 ± 4.11	41.23 ± 2.15	64.17 ± 5.64^a	0.46 ± 0.22^a
20	19	26.81 ± 4.43	56.38 ± 4.63	82.21 ± 9.44^b	0.51 ± 0.13^b
40	19	32.16 ± 3.74	54.42 ± 7.87	85.23 ± 5.25^b	0.58 ± 0.24^b
80	20	30.33 ± 5.56	50.31 ± 5.33	84.67 ± 7.55^b	0.55 ± 0.15^b

values within each column with different superscripts differ significantly ($P < 0.05$)

cleavage rates, 8/16-cell formation rates, blastocyst and hatched blastocyst rates, was analyzed by the Chi-square test. The expression of 4 genes and the numbers of ICM, TE and the ratios of ICM to TE were compared by ANOVA. The software package SPSS 10.0 was used to analyze the data. *P*-value < 0.05 was considered statistically significant.

RESULTS

Embryo development

There was no significant difference in the percentages of embryos that developed at each stage after the reconstructed embryos were cultured

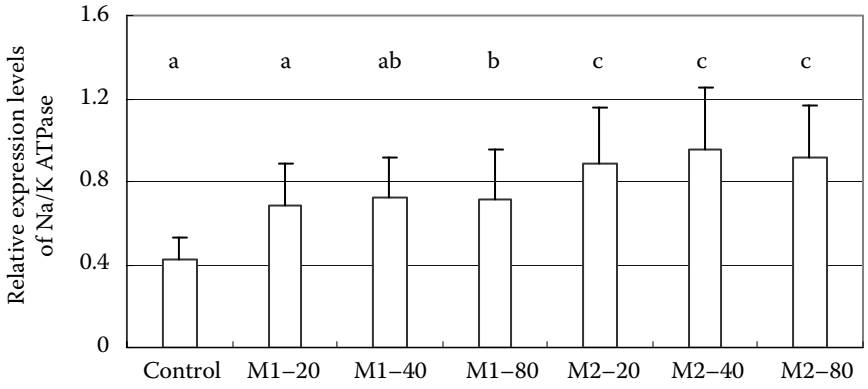

Figure 1. Relative expression amount of Na/K-ATPase in blastocysts cultured in M1 and M2 media; the y axis represents the relative amount of Na/K-ATPase mRNA present in different blastocysts compared to that of GAPDH mRNA present in one embryo; the *x* axis represents the culture media with different concentrations of activin A; expression levels of Na/K-ATPase were significantly higher in M1 media with 80 ng/ml activin A than those with 20 ng/ml or control group; furthermore, expression levels of Na/K-ATPase mRNA in blastocysts obtained in M2 media were higher than those obtained in M1 media

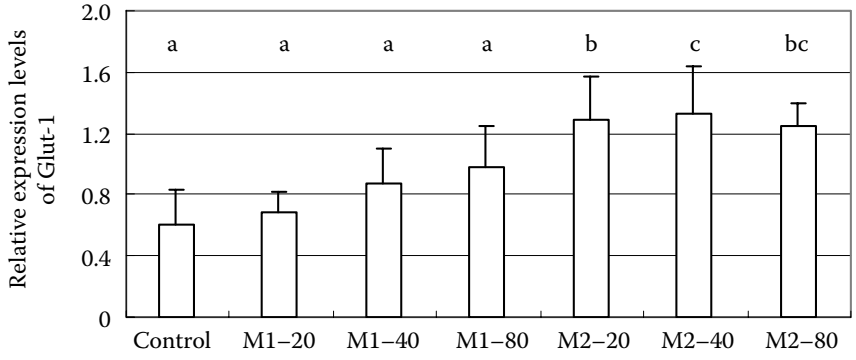

Figure 2. The transcript levels of Glut-1 were remarkably higher in M2 media than those in M1 media; however, no significant difference was observed in the amounts of Glut-1 mRNA in M2 media with 40 ng/ml and 80 ng/ml activin A, Glut-1 expression level was the highest in the numerical in M2 media; the experiment was performed in triplicate using three pools of embryos and the mean ± SEM values are expressed; different lowercase letters indicate significant differences ($P < 0.05$)

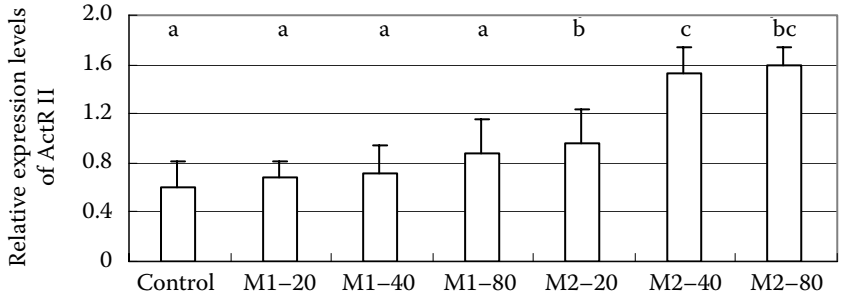

Figure 3. The expression patterns of ActRII were similar to those of Glut-1; the experiment was performed in triplicate using three pools of embryos and the mean ± SEM values are expressed; different lowercase letters indicate significant differences ($P < 0.05$)

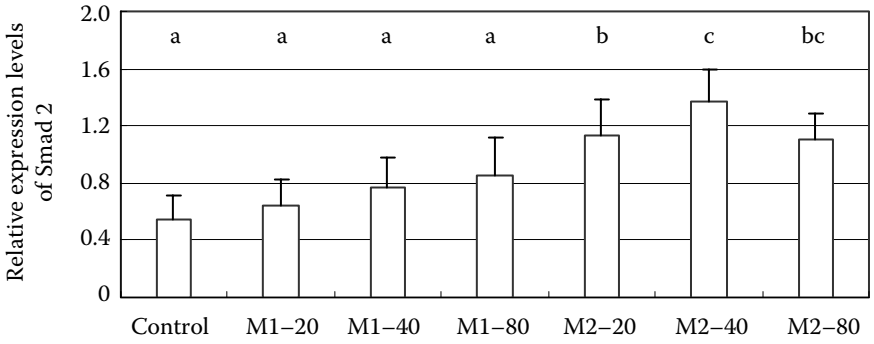

Figure 4. The expression patterns of Smad2 were completely similar to those of Glut-1; the experiment was performed in triplicate using three pools of embryos and the mean ± SEM values are expressed; different lowercase letters indicate significant differences ($P < 0.05$)

in M1 media with different concentrations of activin A (Table 2) during the first 3 days, followed by culturing without activin A ($P > 0.05$) during the subsequent 5 days. However, no addition of activin A to the M2 media during the first 3 days and supplementation with activin A during the subsequent 5 days improved the percentages of cleavage, 8/16-cells, blastocysts and hatched blastocysts ($P < 0.05$). After the addition of 40 ng/ml activin A to the culture medium, the formation

rates of 8/16-cells and blastocysts as well as the rate of hatched blastocysts were the highest in the numerical expression (Table 3).

Blastocyst cell count

Blastocysts that developed in each treatment were collected on day 8 post culture and their total cell numbers were counted (Table 4 and Table 5). The total cell numbers and ICM/TE obtained in M1 media were not significantly different among different treatments ($P > 0.05$). On the contrary, the total cell numbers and ICM/TE of blastocysts obtained in M2 media were significantly higher than those obtained in the control ($P < 0.05$). In addition, the concentration of activin A had no significant effect on the total cell numbers and ICM/TE of blastocysts. Yet, after the addition of 40 ng/ml activin A to the culture medium, the total cell numbers and ICM/TE of blastocysts were higher in the numerical expression than those of other concentrations.

Analysis of gene expression

The expression of GAPDH gene was used as an internal control, and real-time fluorescent RT-PCR technology was used to detect the expression of Na/K-ATPase (Figure 1), Glut-1(Figure 2), ActR II (Figure 3), Smad2 (Figure 4). The results indicated that the expression levels of 4 genes from day-8 blastocysts obtained in M2 media were significantly higher than those obtained in M1 media. Furthermore, the expression levels of genes on day 8 blastocysts obtained in M2 media with activin A at a concentration of 40 ng/ml were higher in the numerical than those obtained with activin A at a concentration of 20 ng/ml or 80 ng/ml.

DISCUSSION

In the present study, the effects of activin A on the development of bovine preimplantation embryos derived from SCNT and partial mechanism of the effect were investigated.

We found that the time of activin A supplementation on the development of bovine SCNT was significant. The addition of activin A to the media during the earlier (3 days, M1 media) stage had no significant effect on the percentages of blastocyst development and the total number of blastomeres or ICM/TE. However, when activin A was added to M2 media for the subsequent 5 days, the embryo development was significantly improved compared to the control.

Bovine IVF embryos cultured *in vitro* commonly arrest during the 8-cell stage. It means the transition from a program controlled by proteins synthesized from maternal mRNAs stored in the oocyte to a program controlled by proteins synthesized from the embryonic genome (Barnes and Eyestone, 1990). This transition normally occurs from the third to the fourth day as the embryo grows from 8 to 16 cells in the bovines. It is accompanied by major changes in protein synthesis and new RNA production (Barnes and First, 1991). The developmental block that occurs from the 8-cell to the 16-cell stage will inhibit the transition of *in vitro* cultured bovine embryos (Camous et al., 1984). The results suggest that maternal components stored in the oocyte seem sufficient to meet the development of bovine SCNT at the previous 8-cell stage, yet, from the 8-cell stage onwards the exogenous addition of activin A to the culture medium is essential. Furthermore, *in vitro* studies with mouse and bovine embryos indicate that exogenous activin A may promote the morula and blastocyst development (Orimo et al., 1996; Yoshioka et al., 1998; Mtango et al., 2003), basically supporting the results of the present studies.

However, in contrast to our results, Yoshioka et al. (1998) reported that activin A should be added to the medium during the first 3 days to improve the number of cells in bovine morula stage embryos via IVF. Different results may be due to different basic culture media and culture methods. In the present study, we used a two-step culture system which has different conditions for the first 3 days and the subsequent 5 days based on the basic medium mSOF. Whereas Yoshioka et al. (1998) used mSOF-PVA media throughout the culture period.

During the formation of blastocyst cavitation, the transport of Na^+ and Cl^- from the medium into the blastocoelic cavity is mediated by a Na/K-ATPase (Watson and Kidder, 1988; Vorbrodt et al., 1997). In addition, bovine IVF embryos do not utilize glucose until the morula and blastocyst stages (Rieger et al., 1992). So, in our study we selected the two genes (Na/K-ATPase, Glut-1) which are very closely related to blastocyst expansion and hatching (Wrenzycki et al., 2003). And Glut-1 is

also an important gene related to the transport and metabolism of glucose and the very high expression levels of Glut-1 may be associated with high quality embryos (Rizos et al., 2002; Wrenzycki et al., 2003; Balasubramanian et al., 2007). We found that the expression levels of Na/K-ATPase and Glut-1 obtained in M2 media were significantly higher than those obtained in M1 media.

Furthermore, in order to evaluate the action mechanism of activin A, we analyzed the expression levels of ActIIR and Smad2 related to the activin A signalling pathway. Coss et al. (2005) reported that ActIIR and Smad2 play a positive regulation role in the bovine embryo development. In the study, we observed that the expression levels of ActIIR and Smad2 were significantly higher in blastocysts cultured in the M2 media compared to the M1 media, suggesting that activin A may improve the quality of SCNT during the subsequent 5 days. Park et al. (2008) also reported that during the later stage of *in vitro* bovine IVF embryo development activin A can enhance the *in vitro* development of embryos by increasing hatching rates and affecting expression levels of genes related to the embryo development. These findings are in part consistent with the results in the present study.

In conclusion, the results suggest that activin A may play important roles in the regulation of the developmental kinetics of bovine SCNT embryos, especially from the fourth cell cycle onwards. However, the effects of activin A post implantation need to be further studied.

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