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Follistatins have potential functional role in Porcine Embryogenesis

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Abstract

In animal reproduction, the quality of oocytes and embryos has been evaluated by the expression of specific molecules. Follistatin (FST), which was isolated from follicular fluid, binds and bio-neutralizes the TGF-β superfamily members. Previous studies using the bovine model showed FST could be an important molecular determinant of embryo developmental competence. However, the effect of FST treatment on porcine embryo developmental competence has not been established. In this study, the effect of exogenous FST on porcine embryo developmental competence was investigated during in vitro culture. FST (10 ng/ml) treatment induced a significant decrease in the rate of cell arrest at the 4-cell stage. The expression levels of DNA-methyltransferase 1 (DNMT1), histone deacetylase 1 (HDAC1), and histone deacetylase 2 (HDAC2) were decreased in 4-cell stage embryos. FST treatment also resulted in significant improvements in developmental competence of embryos in terms of blastocyst formation rate and OCT-4 mRNA levels, the latter being related to pluripotency. In conclusion, during in vitro culture, FST treatment significantly ameliorated 4-cell block during embryonic development and improved embryo developmental competence. Therefore, FST treatment may potentially have a functional role in porcine embryogenesis that is broadly applicable to enhance in vitro embryo development.

Keywords: embryo developmental competence, follistatin, maternal to zygotic transition, porcine embryo

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Introduction

The developmental ability of porcine oocytes to develop into blastocyst stage embryos after in vitro maturation (IVM) and in vitro fertilization (IVF) is generally inferior to *in vivo* produced embryos (Kashiwazaki et al., 2001). Initialembryonic development is associated to the switch of developmental control from the maternal mRNA to zygotic mRNA. This maternal to zygotic transition (MZT) occurs during the 4-cell stage in porcine embryos (Hyttel et al., 2000). MZT is considered to be a critical event for the viability of embryos during early development (Meirelles et al., 2004) because the oocytes need the recruitment

of large amounts of stored mRNAs to support maturation, fertilization, and early development before MZT (Kakourou et al., 2013). In addition, the transition is crucial in epigenetic modification to regulate gene expressions and play an important role in controlling reprogramming events during early embryogenesis (Baroux et al., 2009).

The quality of oocytes and the fate of embryos are determined by the maternal legacy (Bettegowda et al., 2008; Lechniak et al., 2008). It has been considered that some of the specific molecules, which are not well known, determine oocyte quality. The lack of such information is a major hurdle for predicting the oocyte quality or developmental potency. Previous studies reported RNA transcript profiles were related to oocyte developmental competence of poor and good oocytes by microarray analysis in bovine (Patel et al., 2007). The expression level of FST was 4-fold higher in good quality oocytes, and higher in 2-cell bovine embryos which were cleaved earlier than other oocytes. The rate of blastocyst formation was relatively higher in embryos which were cleaved earlier than those of embryos which were cleaved later. From that, it was implied that low FST mRNA levels would be related to the quality of embryos and would affect further embryonic development.

FST is one of the binding and bio-neutralizing members of the TGF-β superfamily (Balemans and Van Hul, 2002; Otsuka et al., 2001). FST was isolated from porcine follicular fluid. FST is also known as a high-affinity activin binding protein (Nakamura et al., 1990) that prevents activin-mediated action on Type I and II serine threonine kinase receptors for Smad activation (Thompson et al., 2005). It is also an activin antagonist that regulates the follicle-stimulating hormone (FSH) (Robertson et al., 1987).

Activin, inhibin, and the FST systems present in the follicular cells of swine, as well as in those of other mammalian species, such as the cow (Knight and Glister, 2006), goat (Silva et al., 2004), rat (Findlay et al., 2002), and mouse (Albano and Smith, 1994), may have effects on oocyte development. Previous studies investigated the effect of supplementation of exogenous activin, inhibin, and FST in vitro during early embryonic development (Silva and Knight, 1998; Stock et al., 1997; Yoshioka and Kamomae, 1996; Yoshioka et al., 1998). When embryos were exposed to FST, the development of bovine embryos were arrested at 9-cell to 16-cell stage (Yoshioka et al., 1998). In contrast, a non-human primate study, showed a significant increase in the number of embryos that were cleaved earlier and reached the blastocyst stage in the FST-treated group than in the control group (VandeVoort et al., 2009).

The function of FST in follicular development is related with the mechanism of maternal gene expression in oocytes that is highly preserved in non-rodent species (Sidis et al., 1998). With that, FST could have a potential and functional role in embryonic development that affects embryo quality (Patel et al., 2007; Bettegowda et al., 2008). Therefore, it was hypothesized that FST supplementation would enhance the embryonic development when it was treated in culture medium during in vitro culture of porcine embryos after IVF.

Materials and Methods

Chemicals and reagents

Unless specified otherwise, all chemicals for in vitro maturation and embryo culture were purchased from Sigma-Aldrich (St. Louis, MO, USA)

Recovery and in vitro maturation

Ovaries were collected from a local slaughterhouse and, within 3 h of collection, transported to the laboratory in physiological saline supplemented with 1% penicillin/streptomycin sulphate at 25°C. Cumulus-oocyte complexes (COCs) from follicles 3 to 8 mm in diameter were aspirated using an 18-gauge needle attached to a 10 mL disposable syringe. Compact COCs were selected and cultured in tissue culture medium TCM-199, supplemented with 2.5 mM fructose, 0.4 mM L-cystein, 1 mM sodium pytuvate, 0.13 mM Kanamycin, 10 ng/ml epidermal growth factor, and 500 IU/mL gonadrotropin hormone. After culturing for 22 h, COCs were washed three times and cultured in FSH and hCG-free IVM medium for another 22 h. Each group of COCs was cultured in 500 μL IVM medium incubated at 39℃ in a humidified atmosphere of 5% CO_2 in air.

In vitro fertilization and in vitro culture

After the completion of IVM, COCs with expanded cumulus cells were treated with 0.1% hyaluronidase in NCSU-W and gently agitated in a pipette until denudation was completed. Oocytes with visible first polar bodies were used for all experiments. 45 μL modified tris-buffered medium (mTBM) was placed on dishes that had been pre-equilibrated before IVF and 15-20 oocytes were placed in each 45 μL mTBM at 39℃ under 5% CO_2 in air. The dishes were kept in the incubator until spermatozoa were added for fertilization.

Fresh semen was supplied weekly by the Darby Pig Artificial Insemination Center (Anseong, Korea). Semen was washed one time by centrifugation with Phosphate buffered saline (PBS). After washing, the sperm pellet was suspended in mTBM to give a concentration of 1.5×10^6 sperm/mL. The oocytes were co-incubated with the spermatozoa for 6 h. After co-incubation, the attached sperms were removed from the zona pellucida by gentle pipetting. Fertilized oocytes were then transferred into 40 μL droplets of PZM-3 medium in a dish, covered by mineral oil, and cultured at 39℃ and 5% CO_2 in air for 7 days.

FST treatment during in vitro culture

The maximal effective concentration of FST observed in studies of embryotrophic effects in the bovine model system was reported by Lee et al. (2009). In this study, FST at 10 ng/mL was used. To determine the effect of FST on porcine embryo development, embryos were cultured with or without FST in PZM-3 medium for 7 days after post-insemination.

Evaluation of embryo development and the total cell number

The day on which IVF was performed was designated as Day 0. The rate of blastocyst formation was assessed and the total number of cells in each blastocyst was evaluated as indicators of embryo quality in each group. To determine the total cell number, blastocysts at Day 7 were collected, and the zona pellucida of blastocysts was dissolved with 0.5% protease. The zona-free blastocysts were washed in NCSU-W, stained with 10 mg/mL Hoechst staining for 5 minutes, and then were mounted on glass slides. The blastocysts were gently squashed under a cover slip, and observed by fluorescence microscopy.

Quantitative real-time RT-PCR

After harvesting, total RNA was isolated from frozen samples using the RNeasy MicroRNA Isolation Kit

(Qiagen) as recommended by the manufacturer. Reverse transcription was performed directly after RNA isolation using the QuantiTect Reverse Transcription Kit (Qiagen) as recommended by the manufacturer. Reverse-transcribed cDNA was diluted 1:5 with nuclease-free water and stored at -80℃ until quantitative real-time PCR was performed. The relative amount of target gene expression for each sample was conducted using a Bio-Rad real-time PCR instrument. Relative level of mRNAs was normalized to the abundance of a control housekeeping transcript, Glyceraldehyde 3-phosphate dehydrogenase (Gapdh), and internal reference genes are presented in Table 1.

Table 1. Primers used for real time RT-PCR.

Genes	Primer sequences (5'→3')	Accession number
Follistatin	F : GGG CTG GAT GGG AAA ACC TA	NM_001003662.1
	R : CAG TTC CGG CTG CTC TTT ACA	
DNMT3A	F : CTG AGA AGC CCA AGG TCA AG	CJ026384
	R : CAG CAG ATG GTG CAG TAG GA	
DNMT1	F : GTG AGG ACA TGC AGC TTT CA	NM_001032355
	R : AAC TTG TTG TCC TCC GTT GG	
HDAC2	F : TGG GAG GAG GTG GAT ACA CAA	EW621236.2
	R : AGC TTG AAG TCG GGT CCA AA	
HDAC1	F : CGC ATG ACT CAC AAT TTG CT	BC108371
	R : AGC CAT CAA ATA CCG GAC AG	
OCT-4	F : CTG GAC AAG GAG AAG CTG	NM_001113060.1
	R : GAT GGT CGT TTG GCT GAA	
Gapdh	F : TTC CAC GGC ACA CTC AAG GC	NM_001206359
	R : CAT GGT CGT GAA GAC ACC AG	

F: Forward primer, R: Reverse primer

Statistical analysis

Results were the means ± SEM from at least three sets of replicated experiments. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests if the overall p values were significant at $p<0.05$ using IBM SPSS Statistics 22.

Results and Discussion

The effect of FST on in vitro development of IVF embryos

To determine whether FST exerts a positive effect on early embryogenesis in porcine, embryos were transferred to PZM-3 medium with or without FST, and embryo development in vitro was monitored (Table 2 and Fig. 1). The outcome of fertilization was checked based on the cleavage status at 30 h after post-insemination. The number of 4-cell embryos at Day 2 and the number of 8-cell embryos at Day 3 were counted and compared with those of control groups. The percentage of 2-cell (78.4 ± 8.12 and 84.1 ± 7.61) and 4-cell embryos (69.1 ± 8.41 and 79.7 ± 9.41) were not significantly different between the control and FST treatment groups, respectively. When embryos were examined at Day 3, the proportion of 8-cell embryos was significantly increased in FST treatment groups (72.5%) compared with the control group (48.4%) ($p<0.05$).

Table 2. The effect of FST treatment on in vitro development of porcine embryos.

Groups	No. of Oocytes	No. of Embryos (%)		
		2-Cell (%)	4-Cell (%)	8-Cell (%)
Con				
1	40	25 (62.5)	24 (60)	18 (45)
2	40	38 (95)	34 (85)	21 (52.5)
3	30	20 (66.7)	15 (50)	12 (40)
4	48	43 (89.5)	39 (81.3)	27 (56.3)
Total	158	126 (78.4 ± 8.12)a[z]	112 (71.6 ± 8.41)a	78 (48.4 ± 3.66)b
FST				
1	40	33 (82.5)	33 (82.5)	33 (82.5)
2	40	37 (92.5)	34 (85)	29 (72.5)
3	30	19 (63)	16 (53.3)	13 (43.3)
4	48	47 (97.9)	47 (97.9)	44 (91.6)
Total	158	136 (84.1 ± 7.61)a	130 (80 ± 9.41)a	119 (72.5 ± 10.48)a

Four replicates were performed. FST; Follistatin
[z]Values with different superscripts are significantly different ($p<0.05$)

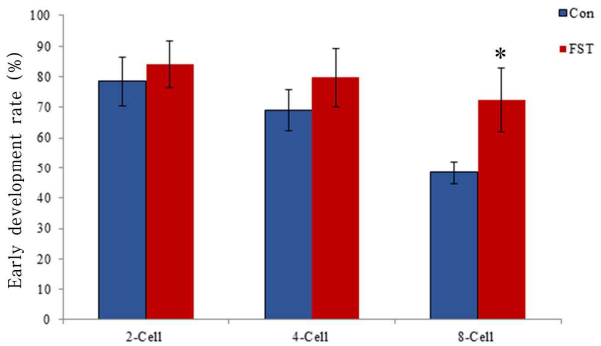

Fig. 1. Early development of porcine IVF embryos with FST treatment during in vitro culture. Data are expressed as mean ± SEM from four replicates (n=158 embryos). * $p<0.05$.

The effect of FST on epigenetic modification related genes

In a previous bovine IVF embryo study, the highest intensity of acetylation was observed at the 8-cell stage, and the level of DNA methylation was the lowest at the 8-cell stage that coincided with zygotic genome activation (Maalouf et al., 2008). In this study, four epigenetic modification related genes were investigated to determine whether FST treatment affected the expression of those genes at 4-cell embryos during MZT. As shown in Fig. 2, the expression levels of HDAC2 and HDAC1 of 4-cell stage embryos treated with FST was lower than in the untreated control group ($p<0.05$). The level of DNMT1 expression was decreased in the FST treatment group ($p<0.05$). However, the level of DNMT3A expression was not significantly different in 4-cell embryos between two groups.

MZT occurs at 4-cell stage during porcine embryonic development. During MZT maternal factors including proteins and transcripts are replaced with embryonic factors by embryonic genome activation for leading to embryo development (Schultz, 2002). Methylation and acetylation are highly involved in the MZT process. Bovine SCNT embryos (low-grade embryos) showed significantly increased DNMT1 mRNA levels, and the

acetylation of H4 lysine peaked at the 8-cell stage compared with embryos produced in vivo (Zhu, 2004). Porcine SCNT embryos expressed significantly higher DNMTs mRNA levels than IVF embryos, indicating relatively high levels of DNMTs and HDACs activities during the period of MZT on SCNT embryo development (Suteevun et al., 2006). Therefore, it was concluded that incomplete embryonic genome activation was affected by different expression pattern of DNA methylation and histone modification related genes during MZT.

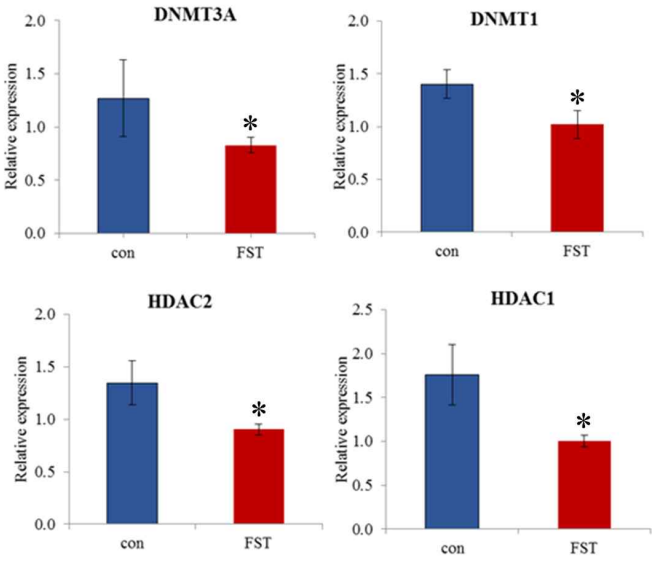

Fig. 2. Relative abundance of DNMT3A, DNMT1, HDAC1, HDAC2 mRNA in 4-cell stage embryo non-treated and treated with FST. Data are expressed as mean ± SEM from three replicates (n=220-225 embryos per treatment). * $p<0.05$.

Relative abundance of FST transcript during early embryogenesis in vitro

As shown in Fig. 3, the expression level of FST in 2-cell stage embryos with FST treatment was up-regulated compared to the non-FST treatment control group ($p<0.05$). The result is similar to the findings from the previous study in bovine model (Patel et al., 2007), and indicates that FST may play a role in porcine embryonic development and that FST mRNA may be used to predict oocyte competence.

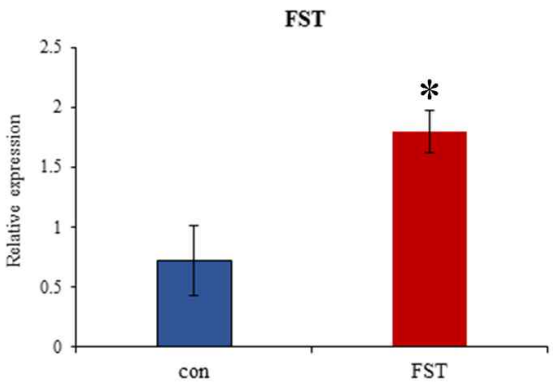

Fig. 3. Quantitative real-time RT-PCR analysis of FST mRNA level in 2-cell stage embryo treated with FST during in vitro culture. Data are expressed as mean ± SEM from three replicates (n=400-450 embryos per treatment). * $p<0.05$.

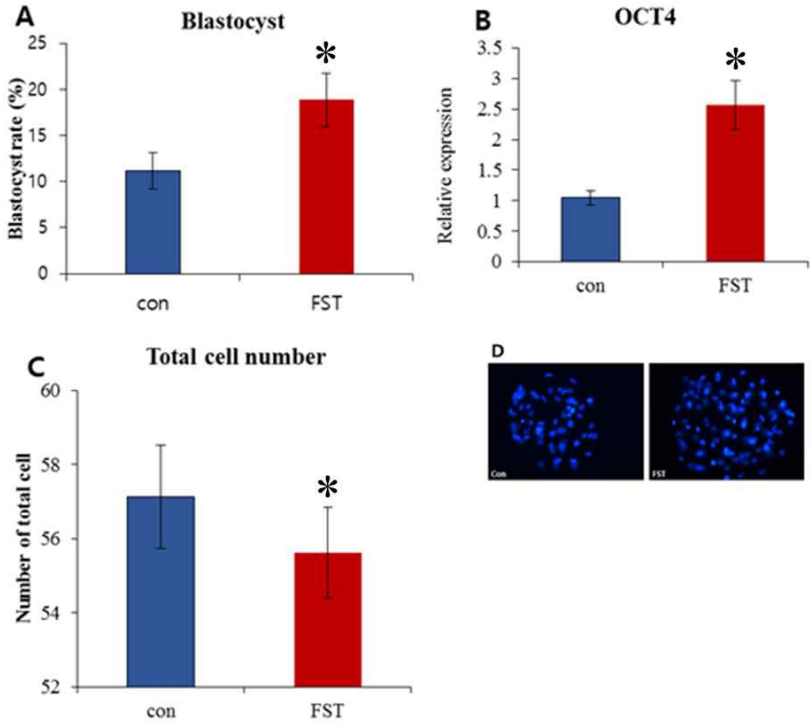

Fig. 4. Effect of FST on blastocyst formation and quality during in vitro culture in porcine embryo (at day 7). Effects of exogenous FST treatment on proportion of embryos developing to the blastocyst stage (mean ± SEM from four replicates and 50 embryos per treatment in each replicate) (A). Expression of OCT-4 mRNA levels in blastocysts (mean ± SEM from three replicates and 20 embryos per treatment in each replicate) (B). Number of total cell number in 7 day blastocyst in control group and treatment group (mean ± SEM from three replicates and 15 embryos per group per treatment in each replicate) (C). Hoechst 33342 staining of the blastocysts (left: control group, right: FST treatment group) (D). * $p<0.05$.

Developmental competence of porcine embryos on the treatment with FST

As shown in Fig. 4A, FST treatment improved embryo development to blastocysts in a dose-dependent manner, respectively (11.2 ± 2 and 18.9 ± 2.9) ($p<0.05$).

To study the expression patterns of pluripotency related genes at the pre-implantation stage, the quantitative real-time PCR analysis was performed on blastocysts. The levels of OCT-4 expression in blastocysts treated with FST was increased compared with those of non-treated group ($p<0.05$) (Fig. 4B). OCT-4 is an important factor in the pluripotent cell populations in developing embryos. Embryos with decreased OCT-4 mRNA levels lose their pluripotency (Nichols et al., 1998). With FST treatment, OCT-4 expression and total cell number increased in blastocysts. The total cell number of blastocysts was determined (shown in Fig. 4C) by staining blastocysts with Hoechst 33342 and counting the total number of nuclei, (Fig. 4D). The total cell number was counted with 45 blastocysts, and there was no statistically significant difference between treated and non-treated groups (57.14 ± 1.39 and 55.63 ± 1.23), respectively. The total cell number in pre-implantation embryos has been demonstrated to be important for the progression of normal development (Machaty, 1998). FST-treatment in the culture medium was sufficient to improve the developmental competence of porcine IVF embryos in vitro.

Conclusion

In this study, the effect of FST was investigated to determine whether it could significantly improve porcine embryo development. The exogenous FST improved porcine IVF embryo development into blastocysts. Especially, the proportion of embryos developed from 4-cell stage to 8-cell stage was increased. Supplementation of FST could improve porcine embryo development by ameliorating 4 cell block in MZT process directly or indirectly. The results reveal a positive effect of FST on embryogenesis that may be broadly applicable to enhance in vitro embryo development.

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