Expression Patterns of TGF-β1, TGF-β Receptor Type I, II and Substrate Proteins Smad 2, 3, 4 and 7 in Bovine Oocytes and Embryos

Hak-Jae Chung[1,†], Bong-Ki Kim[1], Jong-Mu Kim[2], Hyun-Gi Lee[1], Joo-Hee Han[1], Nam-Hyung Kim[2], Jin-Ki Park[1], Hwan-Hoo Seong[1], Boh Suk Yang[1], Won-Kyong Chang[1] and Yeoung Gyu Ko[1,†]

[1]*Molecular Endocrinology Laboratory, Department of Animal Biotechnology, National Livestock Research Institute, RDA, Suwon 441-706, Korea*
[2]*Department of Animal Science, Chungbuk National University, Cheongju 361-763, Korea*

ABSTRACT

Transforming growth factor-β (TGF-β) has been shown to have a positive effect on *in vitro* fertilization (IVF) and has been reported to stimulate meiosis at follicular level in variety of species. The study was designed to determine the expression patterns of TGF-β1, TGF-β receptors type I, II and Smads gene in bovine oocytes and embryos. TGF-β1 and their receptors were observed in the unfertilized oocytes. TGF-β1 and type II receptor were not expressed at the blastocyst stage, however, only type I receptor was exclusively observed at the same stage. The blastocyst stage, in particular, showed high levels of mRNA expression patterns containing a TGF-β type I receptor. The mRNA expression pattern of Smad 2 at all stages of embryonic development was similar in all respect with TGF-β1 type I receptor. On the contrary, Smad 3 and 4 were expressed with high and low level mRNA at the blastocyst stage. In conclusion, it is suggested that TGF-β signaling may be regarded as an important entity during the preimplantation embryo development.

(Key words : TGF-β1, Smads, Gene expression, Preimplantation embryo, Bovine)

INTRODUCTION

In spite of several efforts during past decades concerning bovine *in vitro* fertilization (IVF), implantation rates have invariably remained low and reaching only 64.3% per embryo transferred in large clinics (Seike *et al.*, 1989). Causes of implantation failure have been attributed either to arrested development of embryos due to chromosomal defects (Papadalos *et al.*, 1989; Jamieson *et al.*, 1994), suboptimal conditions or alternatively transferred embryos to a non receptive endometrium. The success of oocyte donation programs (Rozenwaks, 1987; Navot *et al.*, 1991) has allowed the implantation window to be opened up and helped further in transferring oocytes to an appropriately primed endometrium.

Growth factors, such as epidermal growth factor (EGF), transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF), insulin and insulin like growth factor-I (IGF-I) have been demonstrated to act as mitogens and stimulate differentiation in murine and bovine preimplantation embryonic development (Wood and Kaye, 1989; Paria and Dey, 1990; Dardik and Schultz, 1991; Harvey and Kaye, 1990, 1991; Larson *et al.*, 1992). In addition, the study of stage specific expression of growth factors and their receptors in bovine preimplantation embryos forms a rational basic for formulating improved culture media for *in vitro* development.

The growth of embryos is regulated by oviduct and uteri factors; i.e., various processes of embryonic growth are influenced by cytokines and growth factors, among which TGF-β superfamily groups have been reported to play an important role in embryonic event (Schimd *et al.*, 1991; Watson *et al.*, 1992; Fischer *et al.*, 1994; Kliem and Fischer, 1995; Das *et al.*, 1997; Rappolle *et al.*, 1998). TGF-β superfamily is a large group of extracellular growth factors that play key roles in various biological processes, including cell growth, proliferation and differentiation, angiogenesis, apoptosis and extracellular matrix remodeling (Massagu, 1998; Zwijsen *et al.*, 2001; Attisano and Wrana, 2002). TGF-β signals are conveyed through serine-threonine kinase receptors at the cell surface to specific intracellular mediators such as Smad proteins (Moustakas *et al.*, 2001). Activation of Smad proteins results in their translocation to the nucleus and subsequent activation of gene expression (Wrana and Attisano, 2000).

Smad proteins have been identified as intracellular mediators for members of the TGF-β superfamily. Currently, eight vertebrate Smads have been reported (Derynck *et al.*, 1996; Roberts, 1999; Wrana, 2000). Based on structural and functional features, Smads can be classified into three subfamilies. The first is receptor-regu-

*This work was supported in part by the grant from BioGreen 21.

† Corresponding author : Phone: +82-31-290-1631, E-mail: hakjaena@rda.go.kr (Dr. Chung) and kog4556@rda.go.kr (Dr. Ko)

lated Smads (R-Smads) which interact transiently with activated type I receptors and become phosphorylated at their C-terminus. Smad 2 and 3 have been shown to be specific mediators of the TGF-β and activin pathway, whereas, Smad 1, 5 and 8 are involved in bone morphogenetic protein (BMP) signaling (Roberts, 1999; Massagu and Wotton, 2000; Wrana, 2000). The second group of Smads associates with R-Smads and are referred to as common Smads (Co-Smad). For example, Smad 4 forms hetero-oligomers with the R-Smads and is a common mediator of TGF-β, activin and BMP signaling (Derynck *et al.*, 1996; Lagna *et al.*, 1996; Roberts, 1999; Massagu and Wotton, 2000; Wrana, 2000). The last group comprises the inhibitory Smads (I-Smads) and includes Smad 6 and 7. They inhibit the signaling function of the first two groups by interfering with phosphorylation of R-Smads or by competitive inhibition of complex formation between R-Smad (Roberts, 1999; Massagu and Wotton, 2000). Smad 6 preferentially inhibits BMP (Hata *et al.*, 1998), while Smad 7 inhibits activin, TGF-β and BMP signaling (Nakao *et al.*, 1997).

The functional roles of EGF receptor and its ligands, in particular, have been well demonstrated in rodent embryos. In mice, EGF and its ligands increased protein synthesis, cell number, rate of cavitation and blastocyst expansion (Wood and Kaye, 1989; Paria and Dey, 1990; Dardik and Schultz, 1991). Moreover, the role of EGF and EGF receptor has been implicated in the initiation of implantation of both rat and mouse embryos, respectively (Johnson and Chatterjee, 1993; Das *et al.*, 1994).

This study was conducted to investigate the expression patterns of TGF-β1 and TGF-β receptors type I and II in bovine oocyte and early embryonic development, and the regulation patterns of specific genes of Smad family (Smad 2, 3, 4 and 7) as well as TGF-β and their respective type I and II receptors.

MATERIALS AND METHODS

In Vitro Maturation (IVM) and *In Vitro* Fertilization (IVF)

Bovine cumulus-oocyte complexes (COCs) were obtained by aspiration of 2 to 6 mm follicles of ovaries from slaughtered cows. Oocytes surrounded by multiple layers of compact follicular cells were washed twice in PBS supplemented with 36 mg/l pyruvate, streptomycin and 0.5 mg/ml BSA (fraction V, Sigma, St. Louis, MO, USA), and then once in the maturation medium. The maturation medium was composed by TCM-199 (Gibco-BRL, Grand Island, NY, USA) with Earle's salts supplemented with 10% fetal cow serum (FCS), 1 μg/ml estradiol, 35 μg/ml FSH (Folltropin, Bioniche Animal Health), 10 IU/ml hCG, 0.4 mM glutamine, 0.2 mM sodium pyruvate, and 50 μg/ml streptomycin. The oocytes were matured in 400 μl of the maturation medium, overlaid with mineral oil, in 4-well culture dishes (NUNCLON, Cat #. 176740; 4000 Roskilde, Denmark) at 38.5℃, 5% CO_2 in air with 100% humidity for 24 hr. After maturation, the oocytes were washed four times in modified Tyrode's solution (TALP-HEPES) and once in fertilization medium (TALP-F) containing hypotaurin (10 mM), penicillamine (20 mM), epinephrine (1.0 mM), heparin (10 μg/ml), and 0.6% BSA before being transferred to fertilization wells. A discontinuous Percoll density gradient (45 and 90% Percoll) was used to prepare spermatozoa for IVF. Briefly, frozen-thawed pooled semen from six commercial bulls was added on the top of 45% Percoll gradient in a 15 ml centrifuge tube. The tube was centrifuged at 700 ×g for 25 min at room temperature. The sperm pellet was removed from the bottom of the 90% Percoll gradient and washed once by centrifugation in 1 ml of TALP-F, then resuspended in TALP-F. Fertilization of oocytes was carried out with approximately 1 × 10^6/ml of motile sperm cells at 38.5℃ in 5% CO_2 in air. At then 18 hr of insemination, cumulus cells were removed from the oocytes by vortexing for 2 min in 1 ml of PBS. Subsequently, presumptive zygotes were washed in PBS medium before being transferred to 500 μl of modified synthetic oviductal fluid (SOF) and incubated for 7 days under mineral oil at 38.5℃ in 5% CO_2, 5% O_2 and 90% N_2 (Holm *et al.*, 1999). The numbers of embryos cleaved and developed to the morula or blastocyst stage were examined.

Immunohistochemistry

Immunohistochemical localization of TGF-β1, receptor I and II was performed as described previously (Das *et al.*, 1992; Slayden *et al.*, 1993). TGF-β1, TGF-β1 receptors from human expressed in sf 21 cells was purchased from Sigma Chemical Company. Ovary tissues at the follicle growth stage were fixed in 4% cold paraformaldehyde in PBS for 1 hr, serially dehydrated in ethanol and paraffin embedded in gelatin capsules. Serial sections (4~6 μm) were retrieved individually on silane-coated slide prepared by method of Senior *et al.* (1988) taking care not to miss the first section. Immunohistochemistry was performed using mouse monoclonal anti-human TGF-β1 and anti-human TGF-β receptor type I and II antibodies on individual dewaxed sections of ovaries, which was located under bright-field microscopy. All antibodies were obtained from Sigma Chemical Company and used at a dilution of 10 μl/ml. Slides were incubated with 10 μg of either the TGF-β 1mAb or isotype control (IgG1; BD Pharmingen) for 2 hr at room temperature. Biotinylated secondary Abs and streptavidin-HRP from the Vectastain ABC (avidin-biotin complex) kit (Vector Laboratories) was added for 45 min at room temperature. Slides were developed with diamino benzidine (DakoCytomation), counter stained with haematoxylin (Sigma), and mounted with Vecta-

shield (Vector Laboratories). For visualization of the TGF-β1, receptor I and II, ovaries and COCs were fixed for 30 min in 3.7% paraformaldehyde. They were subsequently washed several times in PBS plus 0.1% Triton X-100 and preincubated with 5% BSA for 1 hr to block nonspecific binding of IgG, then incubated with the 1st antibody (each dilution 1:100) for overnight at 4℃. After several washes as described above, ovary and oocytes were incubated with the 2nd antibody (goat-anti rabbit IgG, FITC; Sigma) for 1 hr (each dilution 1:100). The secondary incubation took place at ambient temperature. As a negative control, ovary and oocytes were incubated in non-immuno bovine serum. After monitoring, slides were stored in the dark at 4℃ until observation.

Real-Time PCR

Isolated trophoblasts were lysed in TRIzol reagent (Invitrogen Life Technologies), and total RNA was collected according to the manufacturer's instructions (Invitrogen Life Technologies). One microgram (μ g) of total RNA was reverse transcribed into cDNA. Real-time PCR was performed on RocheLight Cycler using the FastStart SYBR Green kit (Roche), and accession numbers were as follows: TGF-β1, 5'-atacgtcagacattcgggaagcagtg-3' and 5'-aatagttggtatccagggctctccg-3', 447 bp; TGF-β1RI, 5'-cgttacagtgtttctgccacct-3', and agacgaagcacactggtccagc-3', 315 bp; TGF-β1RII, 5'-gctctacgtgcgccaacaacatca-3' and 5'-actgcttgcccatctccgtcttcc-3', 279 bp; Smad 2, 5'-ctcggcacacagagattc-3', and 5'-cgactgagccagaagagc-3', 650 bp; Smad 3, 5'-gttggacgagctggagaag-3' and 5'-gtagtaggagatggagcac-3', 526 bp; Smad 4, 5'-gagagcaaggttgcacatag-3' and 5'-aatctcaatccagcacgg-3', 451 bp; Smad 7, 5'-tcctgctgtgcaaagtgtttc-3' and 5'-agtaaggaggagggggagac-3', 484 bp. PCR amplification was performed under the following conditions: each cycle involved 1) 95℃ for 10 min (enzyme activation); 2) 95℃ for 5 sec (denaturation); 3) 63℃ for 5 sec for the first 10 cycles, a 0.5℃ temperature decrease over the next 16 cycles to a final annealing temperature of 55℃ for 26 additional cycles (a total of 55 cycles); and 4) 72℃ for 14 sec (extension). Samples were run in duplicate. Quantitation was performed by second derivative maximum analysis. PCR product size was confirmed by gel electrophoresis.

RESULTS

Immunostaining of TGF-β1 and Its Receptors

To examine whether TGF-β1 mRNA is translated in the bovine ovaries, cellular distribution of TGF-β1 proteins was examined by immunohistochemistry using a mouse or human antibodies raised against recombinant TGF-β1. A similar distribution patterns was observed in TGF-β1 and its receptors for both antibodies. At pre-antrum stage, immunoreactive TGF-β1, TGF-β receptor I and II were detected in the granulosa cells surrounding the oocyte and along the pre-antrum chamber (Fig. 1B-D). Preneutralization of the antibody with an excess of antigen resulted in loss of specific staining in similar pre-antrum sections (Fig. 1A). The immunostaining patterns of TGF-β1 and its receptors were consistent with the distribution of TGF-β1 mRNA as described above. Little of immunostaining was observed in the theca cell site (data not shown).

Immunofluorescence of Preimplantation Stage Embryos

Micrographs of sectioned ovaries illustrating the observed pattern of staining with immunofluorescence antibodies to TGF-β1, TGF-β receptor I and II shown in Fig. 2. In most of oocytes and embryos, zonae dissolved during the 90% dehydration step, so almost all the specimens had no zonae. In general, intense cytoplasmic stainings peripherally were observed in metaphase II (MII) oocyte, 4- and 8-cell embryos (Fig. 2). Similarly, with the all antibodies, variable intensities of staining observed within the same embryo even though same concentrations of antibodies were used. These could reflect the variable

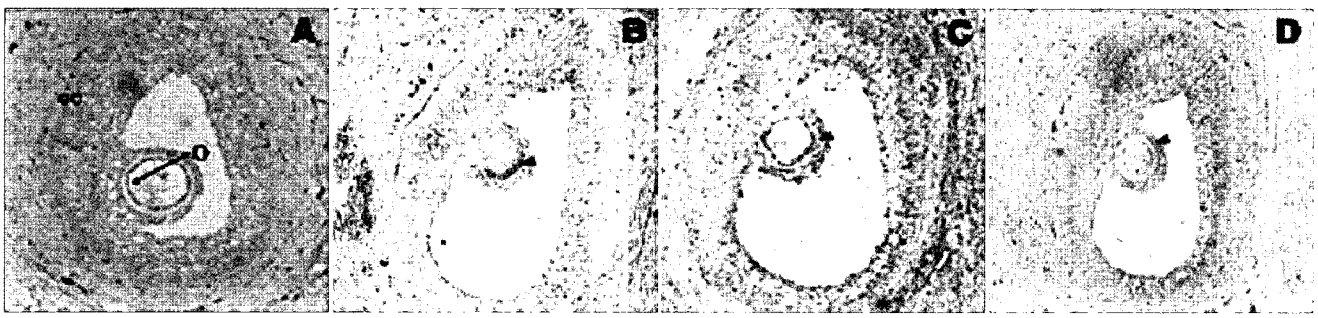

Fig. 1. Immunohistochemistry of TGF-β1, TGF-β1 receptor I and II in the bovine pre-ovulation ovary. Note distribution of TGF-β1 (B) and TGF-β1 receptor I (C) and II (D) in granulosa cells surrounding the maturing oocyte in the pre-antrum stage (Red deposits, arrow heads). No immunostaining was noted when similar sections were incubated with the 1st antibody prenutralized with excess antigen (A). All magnifications are ×280. GC, granulosa cell; O, oocyte.

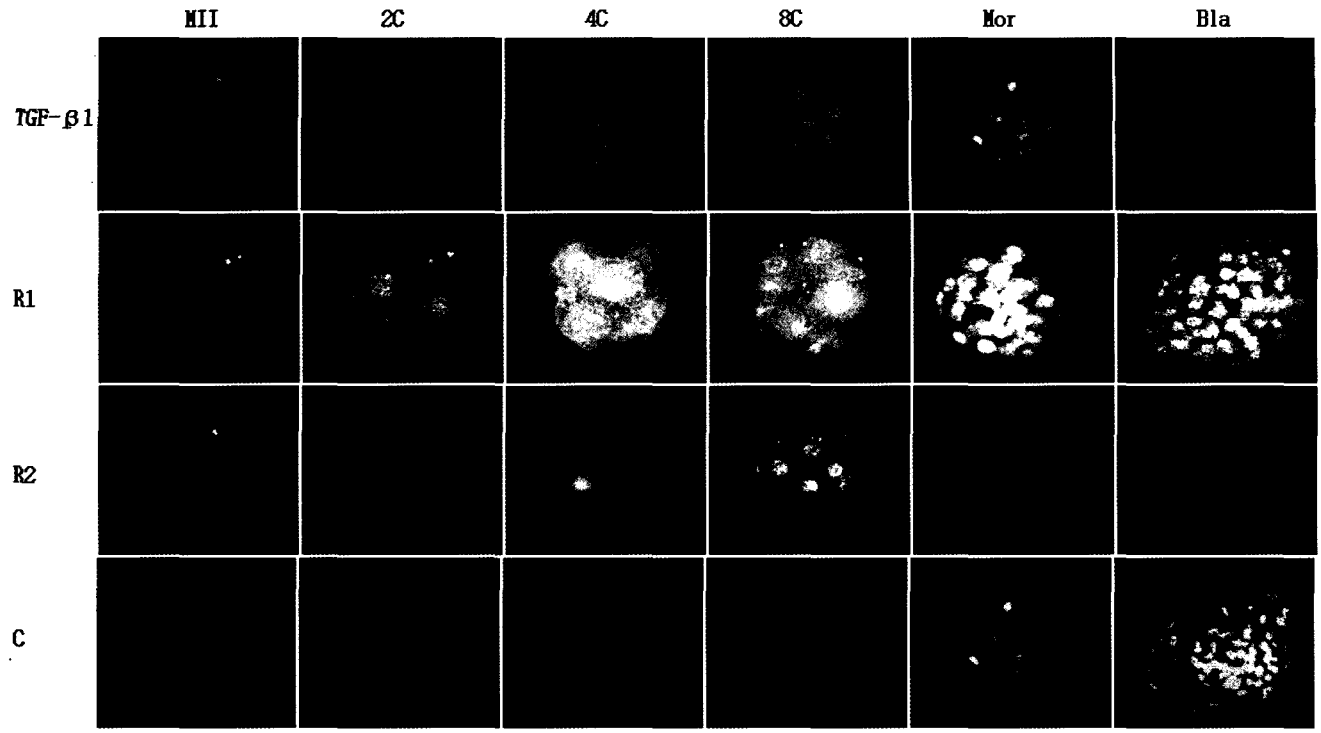

Fig. 2. Expression patterns of TGF-β1, TGF-β1 receptor I and II in perimplantation bovine oocytes and embryos. Negative controls (line c) showing greatly reduced immunostaining were included by incubation with either non-immuno bovine serum or 1st antibody preneutralized with an excess of blocking peptide. Green: TGF-β1, TGF-β1 receptor I and II immunofluorescence positive reaction; Red: Propidium Iodide. All magnifications are ×400.

amount of growth factors and between difference embryos.

Real-Time PCR

To determine the temporal sequence of TGF-β1 and its receptors (type I, II), and Smads (Smad 2, 3, 4 and 7) expression, RNA was extracted from oocytes and embryos at various stages during preimplantation development using real time PCR. TGF-β1, receptor type I and II, and Smad 4 and 7 were detected at morula and blastocyst stages, whereas, TGF-β1 receptor type II and Smad 2 and 3 were highly detected at the MII stage. The Smad 3 was detected at all stage, especially MII to 8-cell stages, except the morula stage (Fig. 3 and Table 1).

DISCUSSION

We demonstrated the concurrent expression of the TGF-β1, TGF-β1 receptors and its related Smad genes during bovine preimplantation development by immunoreaction and real time PCR. In the present study, expression of TGF-β1 and receptors type I and II were observed in pre-antrum stage bovine ovaries (see Fig. 1), Co-expression of TGF-β1 in serial sections of individual embryos was also observed from MII oocytes up to 16-cell stage embryos, but not observed in the morula and blastocyst stage embryos (see Fig. 2). The combination of these techniques provided a powerful approach to study of growth factors in bovine preimplantation em-

Table. 1. Gene expression patterns by preimplantation stages of bovine MII oocytes and embryos*

	Stages of oocytes and embryos						
	MII	2-Cell	4-Cell	8-Cell	Morula	Blastocyst (BL)	Hatched BL
TGF-β1	-	-	-	-	+	++	+++
TGFR-I	+	+-	+-	+-	+	++	+++
TGFR-II	+++	+-	+-	+-	+-	+	++++
Smad 2	++++	+-	+-	+-	-	+-	+-
Smad 3	++++	+++	+++	+++	+-	++	+
Smad 4	+	+-	+-	-	+	+	+++
Smad 7	-	-	-	-	+	++	+++

*MII oocytes and preimplantation embryos were analyzed by real time PCR for the expression of TGF-β1, TGF-β1 receptor (TGFR) I and II, and Smad 2, 3, 4 and 7. Expression levels: -, not detected; +, weak; ++, mid; +++, strong

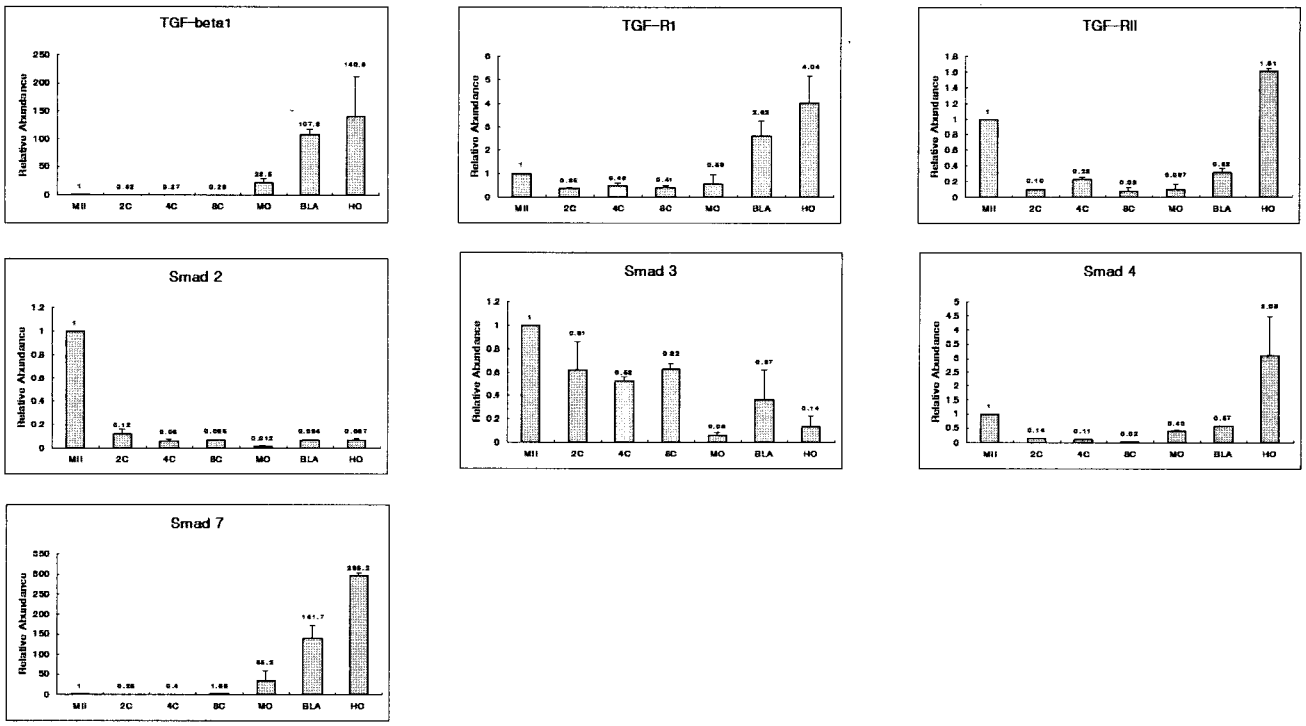

Fig. 3. Relative expression levels of TGF-β1, TGF-β1 receptor I and II and substrate protein Smad 2, 3, 4 and 7 in bovine embryos by real time PCR. H2a gene expression was used as an internal standard. Results are the means±SE from three individual experiments and are represented as a percentage of the control value.

bryos as it possible to co-amplify TGF-β1, TGF-β receptors and Smad consistently from cDNA aliquots, which are equivalent to 2/5 or 1/2 of an oocyte and embryo. Furthermore, 10 (MII oocyte and cleavage stages) and 10~14 (blastocysts) serial sections from individual oocytes or embryos were available for analysis with different antibodies. The presence of transcripts in unfertilized oocytes and the cleavage and blastocyst stages suggests that maternal transcription occurs in the oocyte and that these transcripts persist at the cleavage stages before activation of the embryonic genome. Autoradiographic studies have revealed that usually zygotic transcription begins at the 4~8-cell stage in human embryos due to inhibition of the synthesis of polypeptides with transcriptional inhibitor, α-amantin (Braude *et al.*, 1988), and absence of uridine labeling of nascent RNA transcripts in the nucleoli prior to the 6- to 8-cells (Tesarik *et al.*, 1986). However, in the mouse, there is an evidence of persistence of maternal mRNA and proteins which are functional throughout preimplantation development, e.g. gap junction protein connexon 32 (Barron *et al.*, 1989) and glucose phosphate isomerase (Kidder, 1993).

The concurrent expression of TGF-β1 and other growth factors in bovine oocytes varies in their expressed pattern compared to other species. In the mouse, sheep and pig, TGF-β1 and EGF are absent throughout preimplantation development (Watson *et al.*, 1994 Mummery, 2001; Wei *et al.*, 2001). Preimplantation mouse embryos produce TGF-β1; in turn, the embryos and reproductive tract are responsive to TGF-β1 during the preimplantation period (Chow *et al.*, 2001). In the sheep, paracrine growth factor circuit may exist in the oviduct epithelium and the early ovine embryo. Transcripts for insulin, epidermal growth factor (EGF) and nerve growth factor (NGF) were not detected in any stage of the ovine preimplantation embryo or within the oviduct cell preparations (Watson *et al.*, 1994). In the pig, mRNA expression of EGF receptor in compact morulae and blastocysts were influenced by exogenous EGF *in vitro* (Wei *et al.*, 2001). TGF-β1 and receptors has been suggested to play an important role in regulating the development and functions of the cattle preimplantation embryos. However, the expression of TGF-β1 and its receptors in the cattle preimplantation embryos have not been demonstrated.

Recent studies have identified Smads as molecules that transducer TGF-β signals from the cell surface to the nucleus. In the present study, we examined Smads expression in the cattle MII stage oocytes and the expression pattern of Smads in the TGF-β1-mediated transcriptional response (see Fig. 3). We demonstrated, for the first time, that the Smads expressing patterns are functional in preimplantation cattle embryos. On balance,

the real time PCR analysis showed that the expression patterns of various developmentally regulated genes in the Smads are mediated by some of their TGF-β1 and its receptors genes. However, our analysis was limited by the semiquantitative character of the PCR analysis on culture conditions of embryos, and a conclusion can not be drawn as to whether TGF-β1 and its receptors, which appeared to show little difference between Smad 2, 3, 4 and 7, might show some up-down regulation upon development in the cattle embryos or not. In the present study, the mRNA expression of Smad 2 appeared low at all embyronic stages, whereas, that of Smad 4 and 7 were expressed after the morula stage, especially at the hatched blastocyst stage. These findings are consistent with previous reports on several human cell lines in which an induction of Smad 7 by TGF-β1 (Nakao *et al.*, 1997; Afrakhte *et al.*, 1998). Smad 7 is known to the inhibit TGF-β action by interrupting the Smad signaling cascade (Nakao *et al.*, 1997; Roberts, 1999; Massagu and Wotton, 2000; Wrana, 2000). Therefore, it is possible that Smad 4 and 7 are involved in a negative feedback loop that regulates TGF-β signaling. TGF-β may initiate Smad 4 and 7 transcriptions via TGF-β receptor/Smad pathway and up-regulation of Smad 4 and 7, which in turn inhibits TGF-β signaling. This study provides the first evidence of TGF-β1 and its receptors/Smads genes are expressed in cattle MII oocytes and preimplantation embryos.

Conclusively, TGF-β and Smads signaling are therefore, assumed to play important roles during the maturation of the oocyte and the preimplantation embryo development in cattle.

REFERENCES

1. Afrakhte M, Moren A, Jossan S, Itoh S, Sampath K, Westermark B, Heldin CH, HeldinNE, ten Dijke P (1998): Induction of inhibitory Smad 6 and Smad 7 mRNA by TGF-β family members. Biochem Biophy Res Commun 249:505-511.
2. Attisano L, Wrana JL (2002): Signal transduction by the TGF-β superfamily. Science 296:1646-1647.
3. Barron DJ, Valdimarsson G, Paul DL, Kidder GK (1989): Connexin 32 a gap junction protein, is a persistent oogenetic product through preimplantation development of the mouse. Dev Genet 10:318-323.
4. Braude P, Bolton V, Moore S (1988): Human gene expression first occurs between the four and eight cell stages of preimplantation development. Nature 332: 459-461.
5. Chow JFC, Lee KF, Chan STH, Yeung WSB (2001): Quantification of transforming growth factor β1 (TGF-β1) mRNA expression in mouse preimplantation embryos and determination of TGF-β receptor (type I and type II) expression in mouse embryos and reproductive tract. Mol Human Reprod 7:1047-1056.
6. Dardik A, Schultz RM (1991): Blatocoel expansion in the preimplantation mouse embryo: stimulatory effect of TGF-β and EGF. Development 113 919-930.
7. Das SK, Flanders KC, Andrews GK, Dey SK (1992): Expression of transforming growth factor-β isoforms (2 and 3) in the mouse uterus: Analysis of the preimplantation period and effects of ovarian steroids. Endocrinology 130:3459-3466.
8. Das SK, Lim H, Wang J, Paria BC, BazDresch M, Dey SK (1997): In appropiate expression of human transforming growth factor (TGF-β) in the uterus of transgenic mouse causes downregulation of TGF-β receptors and delays the blastocyst-attachment reaction. J Mol Endocrinol 18:243-257.
9. Das SK, Wang XN, Paria BC, Damm D, Abraham JA, Klagsbrun M, Andrews GK, Dey SK (1994): Heparin-binding EGF-like growth factor gene is involved in the mouse uterus temporally by the blastocyst solely at the sites of its apposition: A possible ligand for interaction with blastocyst EGF receptor in implantation. Development 120:1071-1083.
10. Derynck R, Gelbart WM, Harland RM, Heldin CH, Kern SE, Massagu J, Melton DA, Mlodzik M, Padgett RW, Roberts AB, Smith J, Thomsen GH, Vogelstein B, Wang XF (1996): Nomenclature: vertebrate mediators of TGF-β family signals. Cell 87:173.
11. Schimd P, Cox D, Bilbe G, Maier R, McMaster GK (1991): Differential expression of TGF-β1, 2 and 3 genes during mouse embryogenesis. Development 111: 117-130.
12. Fischer B, Rose-Hellekan TA, Sheffield LG, Bertics PJ, Bavister BD (1994): Binding of epidermal growth factor and transforming growth factor-β in mammalian preimplantation embryos. Theriogenology 41: 879-887.
13. Harvey MB, Kaye PL (1990): Insulin increase the cell number of the inner cell mass and stimulates morphological development of mouse blastocysts *in vitro*. Development 110:963-967.
14. Harvey MB, Kaye PL (1991): Mouse blastocysts respond metabolically to short-term stimulation by insulin and IGF-I through the insulin receptor. Mor Reprod Dev 29:253-258.
15. Hata A, Lagna G, Massagu J, Hemmati-Brivanlou A (1998): Smad 6 inhibits BMP/SmadI signaling by specifically competing with the Smad 4 tumor suppressor. Genes Dev 12:186-197.
16. Holm P, Booth PJ, Schmidt MH, Greve T, Callesen H (1999): High bovine blastocyst development in a vitro production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology 52:683-700.
17. Jamieson ME, Coutts JRT, Connor JM (1994): The

chromosome constitution of human preimplantation embryos fertilized *in vitro*. Human Reprod 9:709-715.
18. Johnson DC, Chatterjee S (1993): Epidermal growth factor (EGF) replaces estradiol for the initiation of embryo implantation in the hypophysectomized rat. Placenta 14:429-438.
19. Kidder GM (1993): Genes involved in cleavage, compaction and blastocyst formation. In Genes in Mammalian Development. R. B. L. Gwatkin ed. New York: Wiley Liss. pp 45-71.
20. Kliem A, Fischer B (1995): Differential expression of the erb B2 gene in the perimplantation embryos of cattle and pig. Reprod Dom Anim 30:269(Abstr).
21. Lagna G, Hata A, Hemmati-Brivanlou A, Massagu J (1996): Partnership between DPC4 and SMAD proteins in TGF-β signaling pathways. Nature 383:832-836.
22. Larson RC, Ignotz GG, Currie WB (1992): Platelet derived growth factor (PDGF) stimulates development of bovine embryos during the fourth cell cycle. Development 115:821-826.
23. Wood SA, Kaye PL (1989): Effects of epidermal growth factor on preimplantation mouse embryos. J Reprod Fertil 85:575-582.
24. Massagu J (1998): TGF-β signal transduction. Annu Rev Biochem 67:753-791.
25. Massagu J, Wotton D (2000): Transcriptional control by the TGF-β/Smad signaling system. EMBO J 19: 1745-1754.
26. Moustakas A, Souchelnytskyi S, Heldin CH (2001): Smad regulation in TGF-β signal transduction. J Cell Sci 114:4359-4369.
27. Mummery CL (2001): Transforming growth factor beta and mouse development. Microsc Res Tech 15:374-386.
28. Nakao A, Afrakhte M, Moren A, Nakayama T, Christian JL, Heuchel R (1997): Identification of Smad 7, a TGF-β-inducible antagonist of TGF-β signaling. Nature 389:631-635.
29. Navot D, Scott RT, Droesch K, Veeck LL, Liu HC, Rosenwaks Z (1991): The window of embryo transfer and the efficiency of human conception *in vitro*. Fertil Steril 55:114-118.
30. Papadalos G, Templeton A, Fisk N, Randall J (1989): The frequency of chromosome anomalies in human preimplantation embryos after IVF. Human Reprod 4:91-98.
31. Paria BC, Dey SK (1990): Preimplantation embryo development *in vitro*: Cooperative interactions among embryos and role of growth factors. Proc Natl Acad Sci USA 87:4756-4760.
32. Rappolle DA, Brenner CA, Schulz R, Mark D, Werb Z (1988): Developmental expression of PDGF, TGF-β and TGF-β genes in preimplantation mouse embryos. Science 242:1823-1825.
33. Roberts AB (1999): TGF-β signaling from receptors to the nucleus. Microbes Infect 1:1265-1273.
34. Seike N, Teranishi M, Yamada S, Takakura R, Nagao Y, Kanagawa H (1989): Incerase in calf production by the transfer of bisected bovine embryos. Nippon Juigaku Zasshi 51:1193-1199.
35. Senior PV, Critchley DR, Beck F, Walker RA, Varley JM (1988): The localization of laminin mRNA and protein in the postimplantation embryo and placenta of the mouse: An *in situ* hybridization and immunohistochemical study. Development 104:431-446.
36. Slayden OD, Hirst JJ, Brenner RM (1993): Estrogen action in the reproduction tract of Rhesus monkey during antiprogestin treatment. Endocrinology 132: 1845-1856.
37. Tesarik J, Kopecny V, Plachot M, Mandelbaun J (1986): Activation of nucleolar and extranucleolar RNA synthesis and changes in the ribosomal content of human embryos developing *in vitro*. J Reprod Fertil 78:463-470.
38. Watson AJ, Hogan A, Hahnel A, Weimer KE, Schultz GA (1992): Expression of growth factor ligand and receptor genes in the preimpplantation bovine embryo. Mol Reprod Dev 31:87-95.
39. Watson AJ, Watson PH, Arcellana-Panlilio M, Warnes D, Walker SK (1994): A growth factor phenotype map for ovine preimplantation. Biol Reprod 50: 725-733.
40. Wei Z, Park WK, Day BN, Prather (2001): Effects of epidermal growth factor on preimplantation development and its receptor expression in porcine embryos. Mol Reprod Dev 60:457-462.
41. Wrana JL (2000): Regulation of Smad activity. Cell 100:189-192.
42. Wrana JL, Attisano L (2000): The Smad pathway. Cytokine Growth Factor Rev 11:5-13.
43. Zwijsen A, van Grunsven LA, Bosman EA, Collart C, Nelles L, Umans L (2001): Trasforming growth factor β signaling *in vitro* and *in vivo*: Activin ligand-receptor interaction, Smad5 in vasculogenesis, and repression of target genes by the dEF1/ZEB-related SIP1 in the vertebrate embryo. Mol Cell Endocrinol 180:13-24.

(Received: 16 July 2006/ Accepted: 30 October 2006)