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Early embryo development is dependent on maternally derived RNA and protein synthesized during oogenesis. In the bovine embryo this dependence lasts until the 8-16 cell stage, at which time embryonic transcripts become essential for continued development. One proposed mechanism for this transition from maternal to zygotic control of development (maternal to zygotic genome activation; MZGA) is the appearance of transcription factors that activate specific genes in the embryonic genome. The E26 transforming specific (Ets) family of transcription factors is involved in development, differentiation and protease regulation, and is a logical candidate gene to be involved in MZGA. Determining the role of Ets-1 in early embryo development may provide useful insight into mechanisms contributing to early embryonic death and pregnancy failure.

Ets-1 transcripts were detected in bovine primary and secondary oocytes, primary and secondary cumulus oocyte complexes (COC), presumed zygotes, and 2- to 16-cell *in vitro*-derived embryos. Ets-1 transcripts were also detected in Days 10 and 12 porcine *in vivo*-derived embryos.

Blocking Ets-1 expression with antisense oligonucleotides inhibited development in *in vitro*-derived bovine embryos. Urokinase plasminogen activator (uPA) production by *in vivo*-derived bovine embryos was reduced with antisense oligonucleotides.

These results suggest that Ets-1 is likely constitutively expressed in early cleavage stage bovine embryos. Although Ets-1 is probably not involved in MZGA, blocking translation of this transcript reduced bovine embryo development and protease production.

Involvement of the Ets-1 Transcription Factor in Early Bovine and Porcine Embryo Development

by

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Leah Stauber, Author

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Introduction

Early mammalian embryonic development is dependent on a complex and detailed pattern of gene expression. Regulation of gene expression is controlled by transcription factors that activate or repress gene transcription. Early cleavage stage embryos depend on maternally-derived mRNA and protein. In the bovine embryo, this dependence lasts until the 8-16 cell stage (Frei et al., 1989; Kopecny et al., 1989; Telford et al., 1990) after which time the embryo begins transcribing its own genes. This wave of new transcription is referred to as maternal to zygotic genome activation (MZGA).

The Ets family of transcription factors may be among those genes transcribed during this time. The E26 transforming specific (Ets) family is one of the largest transcription factor families with over 30 members. These genes were first discovered while studying the avian erythroblastosis virus, E26 (Leprince et al., 1983; Nunn et al., 1983), and have been found to be regulated by specific growth factors and hormones (Turque et al., 1997; Watabe et al., 1998; Raouf and Seth, 2000). Ets transcription factors play a vital role in regulating the expression of various proteases during development (Yordy and Muise-Helmericks, 2000). Ets transcription factors regulate the production of matrix metalloproteinases (MMP), tissue inhibitors of MMP (TIMP), and urokinase-type plasminogen

activator (uPA) (Oikawa and Yamada, 2003). These proteases are essential for numerous reproductive processes such as oocyte maturation (Moor et al., 1998), fertilization (Huarte et al., 1993), proper embryo development (Kaaekuahiwi and Menino, 1990; Birkedal-Hansen et al., 1993; Huarte et al., 1993; Salamonsen, 1994), hatching (Lee et al., 1997; Menino and Williams, 1987), and endometrial remodeling during implantation, placentation and menstruation (Salamonsen, 1994; Duc-Goiran et al., 1999). In early embryos, uPA production is positively correlated with embryo size, developmental stage, and cell number (Kaaekuahiwi and Menino, 1990; Bartlett and Menino, 1995).

Little conclusive research has been performed identifying the genes expressed during MZGA. Evaluation of the Ets family of transcription factors in the early embryo may provide insight into genes involved in MZGA, factors affecting embryo development and regulation of uPA production. The objectives of this research were to: 1) identify the expression pattern of Ets-1 in preimplantation bovine and porcine embryos, 2) determine if blocking Ets-1 expression affects early bovine embryo development, and 3) determine if uPA expression in early bovine embryos is dependent on Ets-1.

Review of the Literature

Bovine and Porcine Embryo Development

Early embryo development involves a complex set of processes beginning in the oocyte and ending with the embryo implanting into the endometrium. The oocyte is surrounded by an acellular glycoprotein matrix known as the zona pellucida. The zona pellucida provides a protective coating for the embryo and a sperm recognition site during fertilization. After fertilization the embryo undergoes a series of cleavage divisions that results in totipotent blastomeres. The early cleavage stage embryo depends on maternally derived mRNA and protein for development. Embryonic differentiation is characterized by the polarization of individual cells within the embryo. These cells sequentially develop cytoplasmic and plasma membrane polarity with distinct apical and basolateral regions. Further cell divisions result in the formation of the morula and reallocation of polarized cells to produce two distinct cell populations at the blastocyst stage. Polar cells on the periphery develop into the trophectoderm and apolar cells on the inside become the inner cell mass (ICM). Trophectoderm will give rise to fetal membranes and the ICM will become the fetus. The blastocyst stage is also characterized by the presence of a fluid-filled cavity called the blastocoel. As development progresses, the blastocoel continues to expand, and in concert with proteolysis, weakens the zona pellucida allowing the embryo to escape in the hatching process. After hatching, porcine and bovine blastocysts begin

elongating into a filamentous type morphology. After a period of elongation, the embryo adheres and attaches to the endometrium to establish fetal-maternal contact and the placenta.

Bovine blastocyst formation occurs on Day 7 (where Day 0=onset of estrus) and hatching occurs on Day 10. On Day 8, endodermal cells leave the ICM and completely surround the blastocoelic side of the trophectoderm by Day 10. Bovine blastocysts develop from a 3 mm sphere on Day 11 to a 25 cm filament on Day 17. On Day 14, mesodermal cells from the embryonic disc migrate between the extraembryonic endoderm and the trophectoderm to form a continuous layer of extraembryonic mesoderm by Day 18 (Betteridge and Flechon, 1988).

Porcine blastocyst formation occurs on Days 4-5 and hatching occurs on Day 6. Primitive endodermal cells appear in the ICM on Day 7 and migrate over the blastocoelic surface of the trophectoderm to form extraembryonic endoderm by Day 10 (Stroband et al., 1984). Mesodermal cells leave the embryonic disc on Day 12 and migrate between newly formed endoderm and trophectoderm to form a continuous layer of extraembryonic mesoderm by Day 16 (Richoux et al., 1989). Porcine blastocysts increase steadily in size from a 150 μm sphere on Day 6 to a 9 mm sphere on Day 11. Following a rapid elongation phase on Day 12 the filamentous embryo reaches 100 cm in length Day 14 (Geisert et al., 1982). The rapid expansion and elongation of livestock embryos after

hatching and before implantation have been speculated to require protease involvement during extracellular matrix (ECM) remodeling, cellular migration and differentiation (Menino et al., 1997).

The Ets Family of Transcription Factors

The Ets family of transcription factors was first discovered while studying the avian erythroblastosis virus E26. The original viral Ets (v-ets) sequence was isolated in the genome of the E26 virus and was found capable of inducing acute transformation in normal genes, namely erythroblastosis (presence of erythroblasts in the peripheral blood) in infected chickens (Leprince et al., 1983; Nunn et al., 1983). The v-ets sequence differs from vertebrate Ets-1 by coding for a two amino acid substitution in the DNA binding domain (Leprince et al., 1992). To date, over thirty proteins have been identified in the Ets family, all of which are related through a highly conserved DNA binding domain.

The Ets proteins are divided into three classes based on the location of the DNA binding domain. In the first class, of which the majority of the proteins belong, the Ets domain is located at the carboxy terminus. The next two classes are significantly smaller; three proteins have a central Ets domain and two have an Ets domain at the amino terminus (Macleod et al., 1992). This structural classification relates to function as the flanking sequences determine Ets protein affinity and specificity to target sequences (Wasylyk et al., 1992).

The Ets Transcription Factor Binding Domain

Ets transcription factors are related through a novel Ets DNA binding domain (EBD), covering approximately 85 amino acids and consisting of two or three conserved tryptophan residues (Watson et al., 1988; Nye et al., 1992). The tryptophan residues appear to be functionally important for the binding of the transcription factor to the DNA binding site. The Ets EBD is unique in that it shares no structural similarity to other known DNA binding motifs, and has remained highly conserved across species. Identification of the EBD has demonstrated these transcription factors bind as monomers or complexes with other proteins to purine-rich portions of the DNA (Karim et al., 1990; Nye et al., 1992).

Amino acid sequences flanking the core GGA(A/T) binding site of the EBD determine the specificity and affinity of the Ets proteins to the target sequences (Wasylyk et al., 1992; Dhulipal, 1997). Specificity is further achieved by partitioning between cell compartments, cell specificity, interactions with other proteins, and covalent modification (Nerlov et al., 1991; Wasylyk et al., 1992). Transactivation of promoters by certain Ets proteins occurs in conjunction with AP-1 and PEA3 binding elements, serum response factors (SRF), and between multiple Ets binding sites (EBS) on certain promoters (Nerlov et al., 1991; Wasylyk et al., 1991; Macleod et al., 1992; Buttice et al., 1996). These protein-protein interactions can confer preferential binding of one distinct Ets protein to a

specific target sequence. Formation of these ternary-complex factors (TCF) requires protein-DNA interactions. The transactivation domain of the TCF contains multiple serine and threonine residues that can be phosphorylated by MAPK (Wasylyk et al., 1998; Sharrocks, 2001). Ets transcription factors have also been shown to recruit adapter proteins, such as the p300/CBP complex, during transcriptional activation of certain promoters (Jayaraman et al., 1999).

The EBS has been found in many viral and cellular gene regulatory sequences. Some of these include the long terminal repeats of the human T-cell leukemia virus, Moloney Sarcoma virus, the interleukin-2 enhancer, and the human immunodeficiency virus-2. The Ras-responsive element (RRE) contains an EBS and is involved in Jun family proteins and MAPK signaling (Imler et al., 1988).

Regulation of The Ets Family of Transcription Factors

Many of the Ets family members contain a pointed domain and/or a transactivation domain. Binding of regulatory proteins to an Ets transactivation domain activates promoters controlling transcription (Dhulipal, 1997). The pointed domain is located at the N-terminal region, and forms a helix-loop-helix structure (Kim et al., 2001). The pointed and transactivation domains promote protein-protein interactions and phosphorylation blocks inhibitory domains (Oettgen, 2006). Ets proteins are also known to control other transcription factor genes such as c-fos,

Jun B, GATA-1, and even other Ets genes (Macleod et al., 1992). This property suggests a possible relationship between expression of Ets genes and MZGA during early embryo development. Genes involved in ECM degradation, such as MMP (stromelysins and collegenases), TIMP, and uPA either contain EBS in their regulatory sequences or are activated by Ets proteins (Wasylyk et al., 1991; Nerlov et al., 1991; Tymms and Kola, 1994; Higashino et al., 1995; Buttice et al., 1996; Iwasaka et al., 1996; Logan et al., 1996; Westermarck et al., 1997; Naito et al., 2002). Thus Ets proteins may be important factors involved in regulating proteolysis during embryonic growth and differentiation.

Ets-1 was first to be identified among the Ets gene family and was associated with pathological angiogenesis. Ets-1 expression increased in cultured endothelial cells and in endothelial cells of new vessels during adult tumor angiogenesis (Watanabe et al., 2004). During early dermal cell migration, treating cells with hydrocortisone down-regulated Ets-1 expression and was concomitant with decreased MMP-2 mRNA levels (Turque et al., 1997). MMP-2 is known to be involved in ECM degradation and cell migration (Werb et al., 1992). Retinoic acid also regulates Ets transcription factors. This hormone induces cellular differentiation and regulates apoptosis in many different cell types. Retinoic acid induces Ets-1 expression in differentiating cells and research has suggested this regulation is at the transcriptional level (Raouf and Seth, 2000; Vary et al., 2000). Proper migration and differentiation of mouse cardiac neural

crest in the formation of interventricular septum require Ets-1. Hepatocyte growth factor (HGF) induces Ets-1 expression in epithelial cells (Gao et al., 2010). Ets-1 expression was coincident with increases in uPA and collagenase expression, as well as cell dispersion (Fafeur et al., 1997; Jiang et al., 2001; Ozaki et al., 2002). Invasive tumor cells treated with Ets-1 antisense oligonucleotides have suppressed uPA expression and reduced tumor cell invasive ability (Li and Jiang, 2010).

Expression Pattern of The Ets Family of Transcription Factors

The Ets family of transcription factors has been found to play a pivotal role in early embryonic development. Ets proteins are expressed ubiquitously throughout the life of an animal (Sementchenko and Watson, 2000), although predominantly during embryonic development (Maroulakou and Bowe, 2000). Research in mammalian, avian, amphibian, and echinoderm embryology has led to increased knowledge of the roles of the Ets genes. In Xenopus laevis, Ets-1 expression is constant during cleavage, decreases at gastrulation, and increases at neurulation (Meyer et al., 1997). Antisense Ets-2 oligonucleotides inhibit meiotic maturation in Xenopus oocytes (Dhulipal, 1997). Ets-2 is structurally similar to Ets-1 and is expressed in mouse trophectodermal cells (Yamamoto et al., 1998). Mouse embryos lacking the Ets-2 DNA binding domain die before 8.5 days of embryonic development. In ruminants, Ets-2 is responsible for the upregulation of interferon-tau (IFN-tau). IFN-tau

is expressed by the trophoblast cells of Day 14 bovine embryos and is the maternal recognition of pregnancy signal (Ezashi et al., 1998). Ets-1 deficient mice have defects in natural killer cell, T-cell, and B-cell development, as well as vascular inflammation and remodeling (Bories et al., 1995; Barton et al., 1998; Eyquem et al., 2004a; Eyquem et al., 2004b; Wang et al., 2005; Zhan et al., 2005; Clements et al., 2006). Human decidual fibroblasts treated with antisense Ets-1 oligonucleotides have significantly less mRNA for decidualization markers compared to controls (Kessler et al., 2006). In chick embryos, intravenous injection of a retrovirus encoding antisense RNA directed against Ets-1 and Ets-2 resulted in embryos with a thin epicardium, disorganized coronary arteries and ventricular septal defects (Lie-Venema et al., 2003). There is evidence that Ets-1 and Ets-2 play a redundant role in endothelial cell survival during embryonic angiogenesis (Wei et al., 2009). Ets expression has been detected in Day 52 human (Tymms and Kola, 1994), Day 9 mouse (Kola et al., 1993) and Day 2 chicken embryos (Fafeur et al., 1997). Morphogenic and invasive processes, such as organ formation, tissue remodeling and tumor invasion, seem to be common factors in expression of Ets transcription factors across species. Ets-1 expression levels in tumors correlate well with grade of invasiveness and metastasis and indicate poor prognosis in cancer patients (Behrens et al., 2001; Nakayama et al., 2001).

In the developing fetus, Ets-1 expression is found in the vascular and nervous systems, lymphoid tissue, lungs, kidneys, and skin and is involved in hematopoeisis (Bartel et al., 2000; Maroulakou and Bowe, 2000; Dittmer, 2003). Ets-1 transcripts have been found in adult tissues such as spleen, thymus, mammary glands, endometrium, ovaries, gastrointestinal tract, and central nervous system.

Plasminogen Activator and Its Role in Early Embryo Development

Plasminogen activators (PA) are serine proteases that convert the pro-enzyme plasminogen into plasmin. Plasminogen activators exist in two forms; tissue-type PA (tPA) and uPA. The two forms are categorically based on molecular weight, fibrin affinity, and immunological reactivity. Tissue-type PA is active in fibrinolysis and has a molecular mass of 70kD. Urokinase-type PA is 55kD and was first discovered in human urine. Urokinase-type PA is associated with inflammation, tissue remodeling and cellular migration (Dano et al., 1985; Gudewicz and Gilboa, 1987; Saksela and Rifkin, 1988). Urokinase-type PA is a glycoprotein synthesized as a single-chain proenzyme (55kD) and, upon proteolytic cleavage, is converted into a disulfide-linked two-chained molecule (Gunzler et al., 1982). The high molecular mass form of uPA consists of non-catalytic (A-chain, 25kD) and catalytic subunits (B-chain, 33kD). The A-chain or light chain contains one kringle domain which contributes to stabilization of uPA with its receptor (Bdeir et al., 2003). The A-chain also contains an

epidermal growth factor-like domain at the amino terminus and uPA binds its receptor through this domain (Dano et al., 1985). The B-chain is homologous to the catalytic subunit of other serine proteases (Dano et al., 1985; Vassalli et al., 1985; Hart and Rehemtulla, 1988). Bovine uPA is encoded by a six kb gene composed of ten exons and nine introns located on chromosome nine (Zimin et al., 2009). Urokinase-type PA receptor (uPAR) is a heavily glycosylated, 3-domain, glycosyl-phosphatidylinositol (GPI) anchored protein that transduces signals to the cell's interior (Behrendt et al., 1995; Koshelnick et al., 1999). Urokinase-type PAR possesses four N-linked glycosylation sites and 14 disulfide bonds (Llinas et al., 2005). Urokinase-type PA up-regulates its own expression by binding uPAR (Li et al., 2001).

Plasmin, the product of PA, is involved primarily in fibrin breakdown. Plasmin can proteolytically cleave growth factors (Gray and Ellis, 2008), growth factor binding proteins (Marcinkiewicz and Gordon, 2008), ECM proteins (vitronectin, laminin, and collagen) and zymogens of other proteases (PA and pro-MMP). Plasma with reduced levels of PA have been correlated with increased risk of stroke (Lindgren et al., 1996) and sub-fertility (Ebisch et al., 2008).

Plasminogen activators have a well-documented involvement during ovulation. Knockout mice lacking both uPA and tPA, although fertile, have severely impaired ovulation rates, reduced growth rates and poor cardiovascular health (Carmeliet et al., 1994; Carmeliet and Collen, 1995;

Liu et al., 2006; Wahlberg et al., 2007). Gonadotropins stimulate the release of PA from ovarian granulosa cells in a time and dose-dependent manner (Beers, 1975). FSH is more effective than LH in inducing PA expression, and prostaglandins and cAMP also stimulate PA production by granulosa cells (Strickland and Beers, 1976). Both tPA and uPA are expressed in cumulus oocyte complexes (COC), and granulosa, thecal and Sertoli cells (Hettle et al., 1986; Liu and Hsueh, 1987; Liu et al., 1987). Mammary epithelial cells treated i*n vitro* with progesterone, hydrocortisone or dexamethasone have decreased PA production, but treatment with human chorionic gonadotropin and prolactin increased PA production (Sasaki et al., 1999). Prolactin and growth hormone increase PA by suppressing production of insulin-like growth factor-binding protein-5 and increasing synthesis of insulin-like growth factor-1, respectively (Tonner et al., 2000). Transforming growth factor (Wileman et al., 2000), fibroblast growth factor (LaPolt et al., 1990; Flaumenhaft et al., 1992), insulin-like growth factor-1 (Dunn et al., 2001) and epidermal growth factor (Watabe et al., 1998) increase PA production in various tissues. The over-expression of uPA is found in many malignant tumors, including advanced ovarian cancer (Cai et al., 2007).

Embryonic PA production has been identified in several species including mice (Strickland et al., 1976), swine (Mullins et al., 1980), cattle (Menino and Williams, 1987) and sheep (Menino et al., 1989). Plasminogen activator expression is associated with hatching, endodermal

cell migration, implantation and mesodermal cell migration during embryo development. In developmental processes where PA has been observed, uPA is prominent. Mouse embryos cultured in medium supplemented with either plasminogen or plasmin, had greater rates of blastocyst formation and hatching compared to embryos cultured in medium supplemented with pronase or trypsin (Menino and O'Claray, 1986). Bovine embryos express PA during blastocoelic expansion and hatching (Menino and Williams, 1987). In sheep, PA levels were low until the morula stage, increased during the morula-blastocyst transition and remain elevated throughout blastocoelic expansion and hatching (Menino et al., 1989). Trophoblast cells, not ICM, are responsible for PA production by bovine embryos (Dyk and Menino, 1991). Day 12 to 14 bovine embryos treated with anti-uPA antibodies lose PA activity whereas treatment with anti-tPA antibodies had no effect (Berg and Menino, 1992), suggesting that uPA is the predominant PA in early bovine embryo development.

Maternal to Zygotic Genome Activation

Early embryo development is dependent on maternally derived RNA and protein synthesized during oogenesis. Early embryos rely on these pre-packaged transcripts from the oocyte to control development through the first few cleavage stages. During MZGA the embryo starts to synthesize its own mRNA, and subsequently protein, to develop beyond the early cleavage stages (De Sousa et al., 1998). In the mouse, MZGA

occurs at the end of the one-cell stage (Schultz, 1993) and at the four to eight-cell stage in humans (Telford et al., 1990). In the bovine, MZGA occurs at the 8 to 16-cell stage or Day 4 of gestation (Camous et al., 1986; King et al., 1988; Frei et al., 1989; Telford et al., 1990).

Maternal stockpiles of mRNA are masked in messenger ribonucleoprotein complexes (mRNPs) that protect mRNA from premature translation and degradation (Weston and Sommerville, 2006). Y-box protein 2 (YBX2) is a major component of mRNPs found in Xenopus oocytes (Bouvet and Wolffe, 1994; Yu et al., 2001) and is essential for storage of maternal mRNA in mice (Yu et al., 2004). In the bovine, YBX2 mRNA levels decrease steadily from the oocyte to the 16-cell stage (Vigneault et al., 2009).

For transcription to begin, the basic transcription initiation complex, consisting of transcriptional activators, DNA, and RNA polymerase II, must be present (Memili and First, 1998). RNA polymerase II is the polymerase responsible for transcription of mRNA and small nuclear RNA. The hypophosphorylated form of RNA polymerase II, the active form, is present at higher levels in 2-cell embryos compared to 4- and 8-cell bovine embryos. This suggests that bovine 2-cell embryos are transcriptionally competent (Memili and First, 1999). This is consistent with other studies showing MZGA occurs gradually in the bovine, with a minor activation of the embryonic genome in 1 to 4-cell stage embryos, and a major activation of the embryonic genome at the 8-cell stage

(Barnes and First, 1991; Plante et al., 1994; Hyttel et al., 1996; Viuff et al., 1996; Lavoir et al., 1997; Natale et al., 2000; Jakobsen et al., 2006). Functional organization of the nucleolus in bovine embryos is not completely gained before the 8-cell or even the 16-cell stage (Lequarre et al., 2003). MZGA occurs in a stepwise manner, with sequential changes in nuclear and chromatin structure regulating the process (Memili and First, 1999). The chromatin needs to be modified in order for the transcriptional machinery to bind and for transcription to occur. Acetylation of histone tails from the nucleosome results in relaxed chromatin structure (Hassig and Schreiber, 1997). Major activation of the embryonic genome is accompanied by a transcriptionally repressive environment, defined by the requirement for enhancer sequence elements to promote transcription (De Sousa et al., 1998). This may explain why embryos in the presence of alpha-amanitin, a RNA polymerase II inhibitor, are developmentally accelerated compared to controls, or simply the cells are not slowed by transcription in these embryos (De Sousa et al., 1998; Lequarre et al., 2003).

Activation of the embryonic genome must also be accompanied by gradual degradation of maternally inherited mRNA and proteins. As embryonic transcripts are being transcribed, maternally inherited mRNA molecules decay (Bachvarova and De Leon, 1980). A rapid decrease in maternal mRNA levels of stockpiled genes occurs in the first cleavage stages in bovine embryos (Vigneault et al., 2004; McGraw et al., 2007).

The accumulation of embryonic transcripts initiates reprogramming of the embryonic genome. This prepares the embryo for later developmental events and is essential for embryo survival (Vigneault et al., 2009).

Maternal factors in the ooplasm may induce certain genes in the embryonic genome to become activated first (Memili and First, 1999). These genes may have a specific role within the embryo, perhaps as an activator for further transcription of genes essential for subsequent development. Considering its role in induction of downstream transcription factors, Ets-1 is a logical candidate to participate in MZGA. It is important to identify genes involved in MZGA for clarification of the basic mechanisms controlling cell proliferation and differentiation during early embryo development. This knowledge could lead to improvements in embryo culture systems, transgenics and cloning and potentially provide a marker for viable embryos for transfer or freezing.

Chapter 2: Ets-1 Expression in Preimplantation Bovine and Porcine Embryos

Abstract

The expression patterns of Ets-1 in bovine and porcine oocytes and preimplantation embryos were identified. RNA was extracted and reverse transcribed from bovine oocytes, *in vitro*-derived presumed zygotes, 2-, 4-, 8- and 16-cell stage embryos and *in vivo*-derived Days 6, 8, 10, 12 and 14 embryos. cDNA was amplified by PCR using primers designed for Ets-1. Ets-1 transcripts were present in primary and secondary oocytes, primary and secondary cumulus oocyte complexes (COC), *in vitro*-derived presumed zygotes, 2- to 16-cell embryos and bovine ovary (positive control). Expression was not observed in Days 6, 8, 10, 12 and 14 *in vivo*-derived bovine embryos. Porcine oocytes and embryos were collected at slaughter and similarly analyzed for Ets-1. Ets-1 expression was observed in Days 10 and 12 porcine embryos. Expression was not observed in primary and secondary porcine oocytes, COC, and Days 4, 6 and 8 porcine embryos. These results suggest that Ets-1 expression is associated with early cleavage stage bovine embryos and elongating porcine embryos.

Introduction

Early embryo development involves a complex set of processes beginning in the oocyte and ending with implantation into the endometrium. The early cleavage stage embryo depends on maternally derived mRNA and protein for development. In the bovine embryo, this dependence lasts until the 8-16 cell stage (Frei et al., 1989; Kopecny et al., 1989; Telford et al., 1990), after which time, the embryo begins transcribing its own genes. This wave of new transcription is referred to as maternal to zygotic genome activation (MZGA). The E26 transforming specific (Ets) family of transcription factors may be among the genes transcribed during this time. The Ets family is one of the largest transcription factor families with over 30 members and plays a pivotal role in early embryonic development. Research in mammalian, avian, amphibian, and echinoderm embryology has led to increased knowledge of the effects of Ets genes. In Xenopus embryos, Ets-1 expression is constant during cleavage, decreases at gastrulation, and increases at neurulation (Meyer et al., 1997). Antisense Ets-2 oligonucleotides inhibit meiotic maturation in Xenopus oocytes (Dhulipal, 1997). Ets transcription factors also play a vital role in regulating the expression of various proteases during development (Yordy and Muise-Helmericks, 2000). These proteases are essential for numerous reproductive processes such as oocyte maturation (Moor et al., 1998), fertilization (Huarte et al., 1993), proper embryo development (Kaaekuahiwi and Menino, 1990;

Birkedal-Hansen et al., 1993; Huarte et al., 1993; Salamonsen, 1994), hatching (Lee et al., 1997; Menino and Williams, 1987), and endometrial remodeling during implantation, placentation and menstruation (Salamonsen, 1994; Duc-Goiran et al., 1999). Evaluation of the Ets family of transcription factors in the early embryo may provide insight into factors affecting MZGA, development and differentiation and protease production. The objectives of this study were to identify the expression patterns of Ets-1 in bovine and porcine oocytes and preimplantation embryos.

Materials and Methods

Embryo and Oocyte Collection

Bovine Oocytes and *In Vitro*-Derived Embryos –To provide oocytes and generate cleavage stage bovine embryos, bovine ovaries were collected from a local abattoir and transported to the laboratory in 20 to 30°C Dulbecco's phosphate buffered saline (DPBS). Cumulus oocyte complexes (COC) were recovered from ovaries utilizing a slashing and rinsing with DPBS technique that allowed the surrounding cumulus cells to remain intact. Cumulus oocyte complexes were recovered from the rinse medium by aspiration and washed three times in M2 medium (Watson et al., 1992). Replicates of 20 to 30 primary COC were recovered immediately, washed in DPBS containing 0.1% bovine serum albumin (BSA; Sigma, St. Louis, MO) and snap frozen in liquid nitrogen or ethanol and dry ice and stored for RT-PCR at -80°C. Denuded primary oocytes were obtained by vortexing primary COC for 2 min to remove cumulus cells. Denuded primary oocytes were washed in DPBS with 0.1% BSA, snap frozen in liquid nitrogen or ethanol and dry ice and stored for RT-PCR at -80°C.

Twenty to thirty COC with three or more layers of cumulus cells were matured in 50 µl microdrops of maturation medium [(TCM 199; Invitrogen, Carlsbad, CA) containing pyruvate (14 mg/ml; Sigma), 10% heat treated fetal calf serum (HTFCS; Invitrogen), 1 µg/ml luteinizing hormone (LH; Sigma), 0.5 µl follicle stimulating hormone (FSH; Sioux

Biochemicals, Sioux City, IA), and 1 µg/ml estradiol (Sigma)] covered with paraffin oil for 24 h at 39°C in a humidified atmosphere of 5% CO_2 in air. Secondary COC were recovered from the microdrops, washed in DPBS containing 0.1% BSA, and snap frozen in liquid nitrogen or ethanol and dry ice and stored for RT-PCR at -80°C. Denuded secondary oocytes were prepared by vortexing secondary COC for 2 min to remove cumulus cells. Denuded secondary oocytes were washed in DPBS with 0.1% BSA, snap frozen in liquid nitrogen or ethanol and dry ice and stored for RT-PCR at -80°C.

Secondary COC were washed twice in sperm TALP (Watson et al., 1992), and once in fertilization medium (Watson et al., 1992). Washed COC were transferred to culture plates containing 50 µl microdrops of fertilization medium under paraffin oil. Frozen sperm from more than one bull was used for *in vitro* fertilization. Following the sperm "swim up" procedure (Watson et al., 1992), where only the most motile sperm are selected for *in vitro* fertilization, 50,000 sperm were added to each drop containing 20 to 25 COC. Insemination time in the present study was considered as 0 h. COC and sperm were co-cultured at 39°C for 18 to 20 h in a humidified atmosphere of 5% CO_2 in air. Presumed zygotes were aspirated from the fertilization drops and placed in microcentrifuge tubes with 1 ml of warm M2 medium and vortexed for 2 minutes to remove cumulus cells. Thirty cumulus cell-free presumptive zygotes were washed three times and cultured in 50 µl microdrops of synthetic oviductal fluid

(SOF) culture medium (Sagirkaya et al., 2006). At 96 h of culture, 10% HTFCS was added to each microdrop.

Presumed zygotes and 2-, 4-, 8- and 16-cell embryos were recovered at 18 to 20 h, 20 to 36 h, 36 to 48 h, 48 to 72h and 72 to 96 h post insemination, respectively. Embryos were snap frozen in liquid nitrogen or ethanol and dry ice and stored for RT-PCR at -80°C.

In Vivo-Derived Bovine Embryos – Cross-bred beef cows were estrous synchronized using prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse, The Upjohn Co., Kalamazoo, MI) and superovulated with porcine follicle stimulating hormone (pFSH; Sioux Biochemicals, Sioux City, IA). The initial intramuscular injection of 25 mg Lutalyse (Day 0=first injection of Lutalyse) was followed by twice daily injections of FSH on days 9-12. Respective dosages of FSH were 5, 4, 2, and 1 mg. Evening injections of FSH on Day 11 and morning injections on Day 12 were accompanied by an additional injection of 25 and 12.5 mg Lutalyse, respectively. Estrus detection was initiated 24 h after the second injection of Lutalyse. Cows found to be in standing estrus were artificially inseminated at 0, 12 and 24 h after onset. The first insemination was accompanied with an intramuscular injection of 100 µg of gonadotropin releasing hormone (GnRH; Sigma). All cows not found in estrus were artificially inseminated at 48, 60 and 72 h after the second Lutalyse injection. Embryos were recovered on Days 6, 8, 10, 12 and 14 (Day 0= Day of estrus onset) by non-surgically flushing the uterus with DPBS containing 2 ml/L heat-

treated cow serum (HTCS) and 10 ml/L antibiotic/antimycotic solution (Sigma). Embryos were recovered from the flushes by aspiration and washed three times in microdrops of DPBS containing 0.1% BSA (Sigma) and evaluated for normal morphology. Replicates of 20 Days 6 and 8 embryos, and 15 Days 10, 12 and 14 embryos were snap frozen in liquid nitrogen or ethanol and dry ice and stored for RT-PCR at -80°C.

Porcine Oocytes and COC- Porcine ovaries were collected from a local abattoir and transported to the laboratory in 20 to 30°C DPBS. Primary COC were aspirated from 3 to 6 mm follicles using an 18-gauge needle attached to a 10 ml disposable syringe. Denuded primary oocytes were prepared by vortexing COC for 2 min to remove cumulus cells. Replicates of 30 to 40 primary COC and primary denuded oocytes were washed in DPBS containing 0.1 % polyvinylpyrrolidone (PVP; Sigma), and snap frozen in liquid nitrogen or ethanol and dry ice and stored for RT-PCR at -80°C. COC with three or more layers of cumulus cells were cultured in 50-µl microdrops of maturation medium [(TCM 199; Invitrogen) supplemented with 10 ng/ml epidermal growth factor (EGF; Sigma), 4 IU/ml pregnant mare serum gonadotropin (PMSG;Cal-Biochem, La Jolla, CA), 4 IU/ml human chorionic gonadotropin, (Chorulon, hCG; Intervet, Millsboro,DE), and 10% porcine follicular fluid (pFF)] were covered with paraffin oil for at 39°C in a humidified atmosphere of 5% CO_2 in air. The pFF was aspirated from 3 to 7 mm follicles. After 22 h of culture, COC were washed three times and cultured in PMSG and hCG-free TCM-199

medium for an additional 22 h. Secondary COC were recovered from the microdrops and denuded secondary oocytes were prepared by vortexing for 2 min to remove cumulus cells. Replicates of 30 to 40 secondary COC and secondary denuded oocytes were washed in DPBS containing 0.1% PVP, snap frozen in liquid nitrogen or ethanol and dry ice and stored for RT-PCR at -80°C.

In Vivo-Derived Porcine Embryos- Twenty five cross-bred gilts and sows were checked daily for estrus and handmated to one of three boars. The reproductive tracts were recovered at slaughter 2-12 days after estrus (Day 0=onset of estrus) and transported to the laboratory where embryos were collected by flushing the oviducts or uterus with DPBS containing 0.1% PVP. Embryos were evaluated for normal morphology and snap frozen in liquid nitrogen or ethanol and dry ice and stored for RT-PCR at -80°C.

RNA Extraction

Oocytes and embryos were pooled by cell stage and mRNA was extracted using the RNAqueous micro kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Briefly, each pooled sample was lysed with lysis buffer and vortexed, and ethanol was added before a final vortexing was performed. Samples were placed in a filter cartridge and centrifuged. The filter was washed three times and eluted twice with 8 µl and 5 µl of RNA elution solution, respectively.

Reverse Transcription Polymerase Chain Reaction

Primer pairs for Ets-1 were designed from published human, mouse, rat, and chicken cDNA sequences (Table 1). RT-PCR was performed on oocytes and embryos following the protocol of Arcellana-Panlilio and Schultz (1993). Extracted RNA was incubated with 0.5 mg of oligodeoxythymidine (oligo dT) 12-18 primers (Invitrogen) for 10 minutes at 70°C in a total volume of 12 µl with sterile distilled water (dH_20) and quick chilled on ice. Four microliters of 5X first strand buffer (Invitrogen), 2 µl 0.1 M dithiothreitol (Invitrogen), 1 µl (10 µM) dNTPs (Invitrogen), and 1 µl (200U) Superscript II reverse transcriptase (Invitrogen) were added and the mixture was incubated at 42°C for 120 min, followed by a 7 min soak at 95°C. The reaction mixture was diluted to 50 µl with dH_2O and 5 µl aliquots were used for PCR. Reaction volumes for PCR contained the following in a total volume of 50 µl: 5 µl 10X PCR buffer (Invitrogen), 5 µl 25 mM $MgCl_2$ (Invitrogen), 1 µl (10 µM) dNTP's, 1 µl (100 µM) 3' primer (Invitrogen), 1 µl (100 µM) 5' primer (Invitrogen), 0.2 µl (5 U/µl) Taq DNA polymerase (Invitrogen), and 5 µl RT product. The reaction mixture was overlaid with paraffin oil to prevent desiccation and amplified in a thermal cycler. PCR program conditions were: a 4 min soak at 94°C, 40 cycles of denaturation for 1 min at 94°C, 2 min of annealing, and extension for 2 min at 72°C, followed by a 10 min soak at 72°C. PCR products were resolved on 2% agarose gels, stained in SYBR Green (Invitrogen), and

photographed under ultraviolet light using the Kodak 1D Image Analysis software. Bovine ovary cDNA and water replaced the RT product and were used as the positive and negative controls, respectively. Amplification of β-actin was used as a control for genomic DNA contamination because primers for β-actin span an intron (Tokunaga et al., 1986). Genomic DNA would generate a 381 bp product whereas cDNA would produce a 243 bp fragment.

Following PCR, selected products were purified using a QIAquick PCR Purification Kit (Qaigen; Valencia, CA) and sequenced at the Oregon State University Center for Genome Research and Biocomputing. Product identity was confirmed using the online sequencing program Blast (Altschul et al., 1997).

Figure 1. PCR primers for Ets-1 and β-actin.

Primer Name	Primer Sequence	PCR Fragment Size (bp)	Citation
Ets-1	5′ primer- TTCGGGACTGGGTGATGTG (19nt) 3′ primer- TAGGACTCTGTGATGAAGC TGG (22nt)	325	Watson et al., 1988 Human nt 230-554
β-actin	5′ primer- TTGGCCTTAGGGTTCAGGG GGG (22nt) 3′ primer- CGTGGGCCGCCCTAGGCAC CA (21nt)	243	Tokunaga et al., 1986 Mouse nt 182-424

Results

Ets-1 Transcription Factor Expression

RT-PCR was used to amplify a 325 bp fragment of Ets-1 (Table 2). Bovine ovary Ets-1 cDNA was 83.8%, 81.8% and 81.2% homologous to published human, rat and mouse sequences, respectively. Ets-1 was expressed in bovine primary and secondary oocytes and COC, presumed zygotes and 2-, 4-, 8- and 16-cell *in vitro*-derived embryos (Figure 1a). Expression was not observed in Days 6, 8, 10, 12 and 14 *in vivo*-derived bovine embryos. The Ets-1 PCR product was successfully sequenced from bovine ovary and confirmed in primary bovine oocytes. Bovine primary and secondary bovine oocytes and COC exhibited two bands, one at 325 bp and a smaller PCR product when PCR was performed with an annealing temperature of 55°C (Figure 1a). Increasing the annealing temperature to 59°C eliminated the smaller PCR product (Figure 1b). Ets-1 was expressed in Days 10 and 12 porcine *in vivo*-derived embryos (Figure 1c). Expression was not observed in porcine primary and secondary oocytes and COC and Days 4, 6 and 8 *in vivo*-derived embryos.

β-actin Expression

A PCR product of the expected size for β-actin, 243 bp, was observed in all bovine and porcine oocyte and embryo stages evaluated (Figures 2 and 3). No genomic contamination was observed.

1 40
TTCGGGACTG GGTGATGTGG GCTGTCAACG AGTTCAGCCT
41 80
GAAGGGTGTG GACTTCCAGA AGTTCTGTAT GAACGGGGCA
81 120
GCCCTCTGCG CACTGGGGAA AGACTGCTTT CTGGAGCTGG
121 160
CCCCAGACTT CGTTGGGGAC ATCTTGTGGG AACACCTGGA
161 200
GATCCTGCAG AAAGAGGATG TGAAGCCATA CCAAGTTAAT
200 240
GGAGTCAACC CTCCCTACCC AGAGTCCCGC TATACCTCGG
240 280
ATTACTTCAT TAGCTACGGT ATCGAGCATG CCCAGTGCGT
281 320
CCCTCCCTCG GAGTTCTCCG AGCCCAGCTT CATCACAGAG
321
TCCTA

Figure 1. Bovine Ets-1 cDNA sequence.

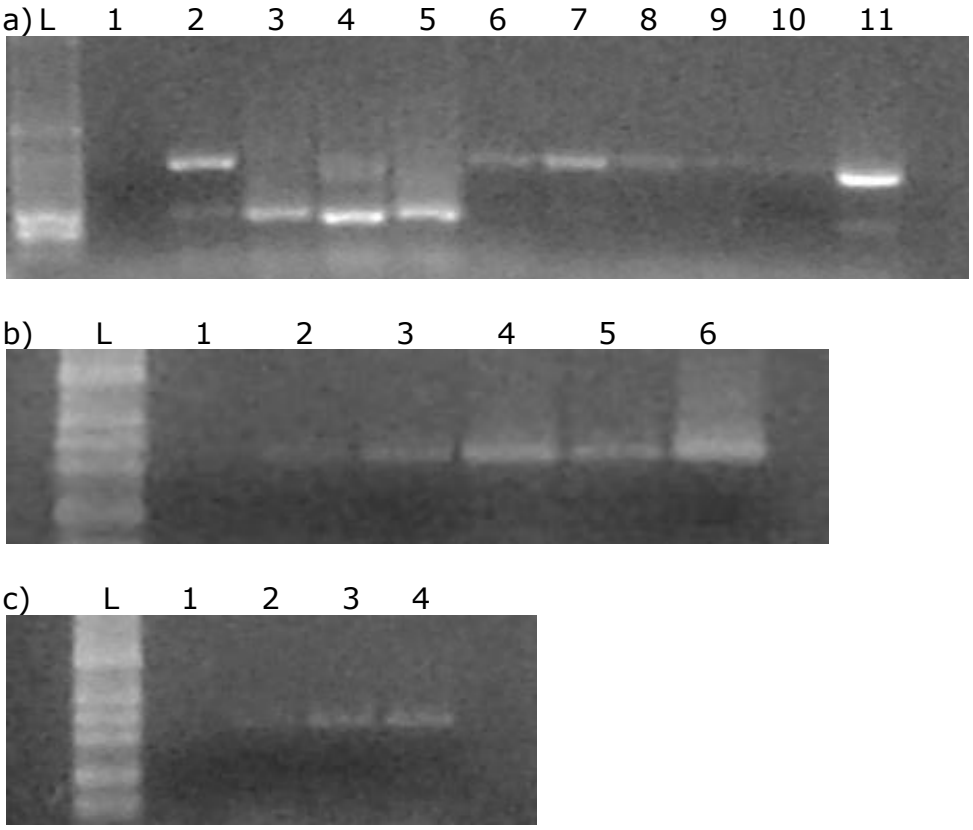

Figure 2. Ets-1 expression in a) bovine oocytes and embryos, b) bovine oocytes and COC and c) porcine embryos. PCR products for a) Ets-1 in bovine oocytes and embryos. Lanes are: L) DNA ladder, 1) water, 2) primary oocytes, 3) primary COC, 4) secondary oocytes, 5) secondary COC, 6) presumed zygotes, and 7) 2-cell, 8) 4-cell, 9) 8-cell, and 10) 16-cell embryos, and 11) bovine ovary. PCR products for b) Ets-1 in bovine oocytes and COC. Lanes are: L) DNA ladder, 1) water, 2) primary oocytes, 3) primary COC, 4) secondary oocytes, 5) secondary COC, and 6) bovine ovary. PCR products for c) Ets-1 in porcine embryos. Lanes are: L) DNA ladder, 1) water, 2) Day 10, and 3) Day 12 porcine embryos, and 4) bovine ovary.

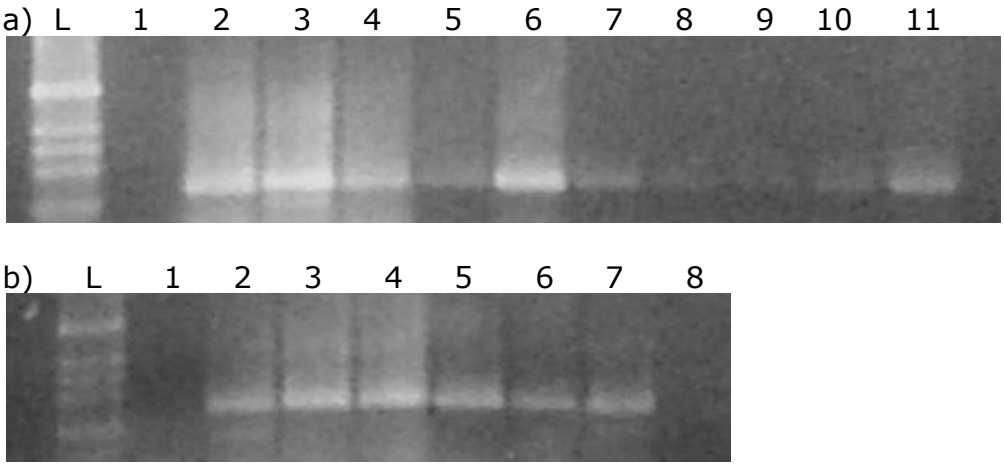

Figure 3. β-actin expression in bovine a) oocytes and *in vitro*–derived embryos and b) *in vivo*-derived embryos. PCR products for a) β-actin in oocytes and *in vitro*–derived embryos. Lanes are: L) DNA ladder, 1) water, 2) primary oocytes, 3) primary COC, 4) secondary oocytes, 5) secondary COC, 6) presumed zygotes, and 7) 2-cell, 8) 4-cell, 9) 8-cell and 10) 16-cell embryos, and 11) bovine ovary. PCR products for b) β-actin in *in vivo*-derived embryos. Lanes are: L) DNA ladder, 1) water, 2) Day 6, 3) Day 8, 4) Day 10, 5) Day 12 and 6) Day 14 bovine embryos and 7) bovine ovary.

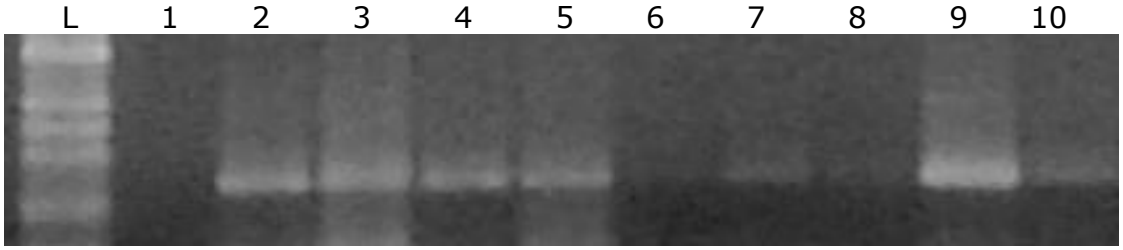

Figure 4. β-actin expression in porcine oocytes and embryos. Lanes are: L) DNA ladder, 1) water, 2) primary oocytes, 3) primary COC, 4) secondary oocytes, 5) secondary COC, and 6) Day 4, 7) Day 6, 8) Day 8, 9) Day 10 and 10) Day 12 porcine embryos.

Discussion

The objective of this study was to identify the expression patterns of Ets-1 in bovine and porcine oocytes and preimplantation embryos. Ets proteins are expressed ubiquitously throughout the life of an animal (Sementchenko and Watson, 2000), although predominantly during embryonic development (Maroulakou and Bowe, 2000). In the developing fetus, Ets-1 expression is found in the vascular and nervous systems, lymphoid tissue, lungs, kidneys, and skin and is involved in hematopoeisis (Bartel et al., 2000; Maroulakou and Bowe, 2000; Dittmer, 2003). Human and avian embryos express Ets-1 extensively throughout development, especially in tissues undergoing cellular differentiation and migration, such as the lymphatic and vascular systems, during lung, kidney and heart formation, and limb bud development (Macias et al., 1998; Vandenbunder et al., 1989; Maroulakou and Bowe, 2000; Gao et al., 2010). Ets-1 expression was observed in bovine primary and secondary oocytes, COC, presumed zygotes, and 2-, 4-, 8- and 16-cell *in vitro*-derived embryos. This observation suggests Ets-1 may be involved in regulation of cell growth and differentiation during the early cleavage stages of the bovine embryo. Ets-1 expression in primary and secondary oocytes and COC suggests Ets-1 may be involved in cell cycle regulation during the resumption of meiosis. Ets-2 transactivates the cdc2 (Wen et al., 1995) and cyclin D1 promoters (Albanese et al., 1995). Cdk 10, a Cdc2-related kinase regulating G2/M phase of the cell cycle, interacts with the pointed

domain of Ets-2 to inhibit Ets-2-mediated transactivation (Kasten and Giordano, 2001). There is evidence supporting a redundant role of Ets-1 and Ets-2 in endothelial cell survival during embryonic angiogenesis (Wei et al., 2009) and Ets-1 may have a role similar to Ets-2 in cell cycle regulation during meiotic resumption. It is doubtful that Ets-1 is responsible for MZGA because Ets-1 expression was not observed in *in vivo*-derived bovine embryos in spite of consistent actin expression. However, it is possible that the number of *in vivo*-derived embryos used for RT-PCR was insufficient to provide detection.

Ets-1 expression was not observed in porcine embryos until Days 10 and 12. Insufficient amounts of embryo RNA may also be the explanation for the lack of detection in porcine oocytes and embryos earlier than Day 8 of development if the Ets-1 transcript is of low copy number. Porcine embryos increase steadily in size from a 150 μm sphere on Day 6 to a 9 mm sphere on Day 11 (Geisert et al., 1982). Ets-1 may be involved in this rapid expansion and elongation after hatching and before implantation in porcine embryos. Expansion and elongation would involve changes in cellular association with the extracellular matrix (ECM) and may require protease involvement. Plasminogen activator production has been detected in Days 10 to 16 porcine embryos and coincides with the transformation from spherical to filamentous morphology (Mullins et al., 1980). Days 8 to 15 porcine embryos also produce matrix metalloproteinases 2 and 9 (Chamberlin and Menino, 1995; Menino et al.,

1997). Ets transcription factors regulate the production of MMP, tissue inhibitors of MMP (TIMP), and urokinase-type plasminogen activator (Oikawa and Yamada, 2003). Genes involved in ECM degredation, such as MMP (stromelysins and collegenases), TIMP, and uPA either contain EBS in their regulatory sequences or are activated by Ets proteins (Wasylyk et al., 1991; Nerlov et al., 1991; Tymms and Kola, 1994; Higashino et al., 1995; Buttice et al., 1996; Iwasaka et al., 1996; Logan et al., 1996; Westermarck et al., 1997; Naito et al., 2002). Porcine embryos may require Ets-1 to regulate proteases involved during elongation to facilitate ECM remodeling and cellular migration.

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Chapter 3: Effects of Ets-1 Oligonucleotides on Development of *In Vitro*-Derived Bovine Embryos

Abstract

Effects of Ets-1 oligonucleotides on development of *in vitro*-derived bovine embryos beyond the 2-, 4- and 8-cell stages were determined. *In vitro*-derived bovine embryos were cultured in medium containing 0 (Control), 2.5 and 5 μM sense and antisense Ets-1 oligonucleotides and observed for development at 24-h intervals. Fewer ($P<0.05$) 2-cell embryos treated with 2.5 μM antisense Ets-1 developed to the 4-cell stage compared to sense Ets-1 treated and Control embryos. Fewer ($P<0.05$) 4-cell embryos treated with 2.5 μM antisense Ets-1 developed to the 8-cell, morula and blastocyst stages. Treatment with 5.0 μM antisense decreased ($P<0.05$) development of 4-cell embryos to the 8-cell stage compared to sense Ets-1 and Control embryos. Although fewer antisense Ets-1 treated 8-cell embryos developed to subsequent stages of development when compared to sense Ets-1 and Control embryos, no significant differences were observed. No differences ($P>0.10$) in development between sense Ets-1 and Control embryos were observed at any of the developmental stages.

Treatment with antisense Ets-1 delayed ($P<0.05$) cleavage of 2-cell embryos to the 8- and 16-cell stages compared to sense Ets-1 and Control embryos. Delays ($P<0.05$) were also observed in 4-cell embryos treated with 2.5 and 5 μM antisense Ets-1 developing to the 16-cell and

morula stages, respectively. Delays in development due to antisense Ets-1 treatment were observed throughout the 4-cell stages but were not statistically significant. Retarded development was observed in all 8-cell antisense Ets-1 treated embryos but no significant differences were observed. No differences ($P>0.10$) in times required to develop to subsequent cell stages were observed between sense Ets-1 and Control embryos. These data suggest that blocking Ets-1 translation at the 2- and 4-cell stages decreases and delays subsequent development.

Introduction

Embryonic development is dependent on a complex and detailed pattern of gene expression. Regulation of gene expression is controlled by transcription factors that activate or repress gene transcription. Early embryo development is dependent on maternally derived RNA and protein synthesized during oogenesis. The early cleavage stage embryo relies on these pre-packaged transcripts from the oocyte to control development through the first few cleavage stages. In the bovine embryo, this dependence lasts until the 8-16 cell stage (Frei et al., 1989; Kopecny et al., 1989; Telford et al., 1990) after which time the embryo begins transcribing its own genes. This wave of new transcription is referred to as maternal to zygotic genome activation (MZGA). During MZGA the embryo starts to synthesize its own mRNA, and subsequently protein, to develop beyond the early cleavage stages (De Sousa et al., 1998).

The Ets family of transcription factors may be among those genes transcribed during MZGA. The E26 transforming specific (Ets) family is one of the largest transcription factor families with over 30 members. These genes were first discovered while studying the avian erythroblastosis virus, E26 (Leprince et al., 1983; Nunn et al., 1983) and have been found to be regulated by specific growth factors and hormones (Turque et al., 1997; Watabe et al., 1998; Raouf and Seth, 2000). Ets proteins are known to control other transcription factor genes, such as c-fos, Jun B, GATA-1, and

even other Ets genes (Macleod et al., 1992). This property suggests a logical relationship between expression of Ets genes and MZGA.

Little research has been performed identifying a role for the Ets family of transcription factors in early development. Evaluation of Ets-1 may provide insight into factors affecting MZGA and contributing to early embryonic mortality. Therefore the objective of the study was to determine if blocking Ets-1 expression affected early bovine embryo development.

Materials and Methods

Embryo and Oocyte Collection

In Vitro-Derived Bovine Embryos –To generate cleavage stage bovine embryos, bovine ovaries were collected from a local abattoir and transported to the laboratory in 20 to 30°C Dulbecco's phosphate buffered saline (DPBS). Cumulus oocyte complexes (COC) were recovered from ovaries utilizing a slashing and rinsing with DPBS technique that allowed the surrounding cumulus cells to remain intact. Cumulus oocyte complexes were recovered from the rinse medium by aspiration and washed three times in M2 medium (Watson et al., 1992). Twenty to thirty COC with three or more layers of cumulus cells were matured in 50 µl microdrops of maturation medium [(TCM 199; Invitrogen, Carlsbad, CA) containing pyruvate (14 mg/ml; Sigma), 10% heat treated fetal calf serum (HTFCS; Invitrogen), 1 µg/ml luteinizing hormone (LH; Sigma), 0.5 µl follicle stimulating hormone (FSH; Sioux Biochemicals, Sioux City, IA), and 1 µg/ml estradiol (Sigma)] covered with paraffin oil for 24 h at 39°C in a humidified atmosphere of 5% CO_2 in air.

Secondary COC were washed twice in sperm TALP (Watson et al., 1992) and once in fertilization medium (Watson et al., 1992). Washed COC were transferred to culture plates containing 50 µl microdrops of fertilization medium under paraffin oil. Frozen sperm from more than one bull was used for *in vitro* fertilization. Following the sperm "swim up" procedure (Watson et al., 1992), where only the most motile sperm are

selected for *in vitro* fertilization, 50,000 sperm were added to each drop containing 20 to 25 COC. Insemination time in the present study was considered as 0 h. COC and sperm were co-cultured at 39°C for 18 to 20 h in a humidified atmosphere of 5% CO_2 in air. Presumed zygotes were aspirated from the fertilization drops and placed in microcentrifuge tubes with 1 ml of warm M2 medium and vortexed for 2 minutes to remove cumulus cells. Thirty cumulus cell-free presumptive zygotes were washed three times and cultured in 50 µl microdrops of synthetic oviductal fluid (SOF) culture medium (Sagirkaya et al., 2006). At 96 h of culture, 10% HTFCS was added to each microdrop.

Presumed zygotes were aspirated from the fertilization drops and placed into a microcentrifuge tube with 1 ml of warm M2 medium and vortexed for two minutes to remove cumulus cells. Thirty cumulus free presumptive zygotes were washed three times and cultured in 50 µl microdrops of synthetic oviductal fluid (SOF) culture medium (Sagirkaya et al., 2006). At 96 h of culture, where insemination time was considered as 0 h, 10% HTFCS was added to each microdrop. Two-, four- and eight-cell embryos were recovered at 20 to 36 h, 36 to 48 h and 48 to 72h post insemination, respectively, and cultured in medium containing oligonucleotides.

Antisense Experiments

Antisense Ets-1 experiments were performed to determine the role of Ets-1 in development of *in vitro*-derived bovine embryos. Embryos were cultured in 50 µl microdrops of SOF containing 1.5% BSA and 0 (Control), 2.5 or 5 µM of Ets-1 sense (S) or antisense (AS) oligonucleotides. Sense and AS oligonucleotides spanned from 230 to 248 nt and 533 to 554 nt of the Ets-1 sequence, respectively (Watson et al., 1988). Microdrops containing 20 to 30 embryos were covered with paraffin oil and cultured for up to 192 h at 39°C in a humidified atmosphere of 5% CO_2 in air. Embryo development was observed at 24-h intervals (Figure 5).

Statistical Analysis

Differences due to treatment in the percentage of embryos developing to a particular cell stage were evaluated by Chi-square procedures. Differences due to treatment in the times required for embryos to develop to subsequent cell stages were evaluated by one-way analysis of variance (ANOVA). If significant effects were observed in the ANOVA, differences between means were determined using Duncan's multiple comparisons test. All analyses were conducted using the NCSS 2000 software program (Number Cruncher Statistical System; Hintze, J.C.; Kaysville, UT).

Results

Effects of Ets-1 Antisense Oligonucleotides on Embryo Development

One hundred four, 106 and 106 2-cell embryos were cultured in medium containing 0 or 2.5 μM S or AS oligonucleotides, respectively. Fewer ($P<0.05$) 2-cell embryos treated with 2.5 μM AS developed to the 4-cell stage compared to S and Control embryos (Figure 6a). Reductions in development due to AS treatment were observed throughout subsequent stages but were not statistically significant. One hundred one, 94 and 96 2-cell embryos were cultured in medium containing 0 or 5 μM S or AS oligonucleotides, respectively. Fewer ($P<0.05$) 2-cell embryos treated with 5 μM AS developed to morulae compared to Control embryos.

Sixty-eight, 68 and 69 4-cell embryos were cultured in medium containing 0 or 2.5 μM S or AS oligonucleotides, respectively. Treatment with 2.5 μM AS decreased ($P<0.05$) development of 4-cell embryos to the 8-cell, morula and blastocyst stages of development. Seventy-one, 71 and 74 4-cell embryos were cultured in medium containing 0 or 5 μM S or AS oligonucleotides, respectively. Fewer ($P<0.05$) 4-cell embryos treated with 5.0 μM AS developed to the 8-cell stage compared to S and Control embryos (Figure 7b). No differences ($P>0.10$) between S and Control embryos were observed at any of the developmental stages.

Sixty-nine, 70 and 70 8-cell embryos were cultured in medium containing 0 or 2.5 μM S or AS oligonucleotides, respectively. Sixty, 60 and 61 8-cell embryos were cultured in medium containing 0 or 5 μM S or

AS oligonucleotides, respectively (Figure 8b). Although fewer AS treated 8-cell embryos developed to subsequent stages compared to S and Control embryos (Figure 8a and 8b), no significant differences were observed for either oligonucleotide concentration. No differences (P>0.10) between S and Control embryos were observed at any of the developmental stages.

Effects of Ets-1 Antisense Oligonucleotides Timing of Development

Two-cell embryos treated with 2.5 μM AS were delayed (P<0.05) in cleaving to the 8- (Figure 9) and 16-cell (Figure 10) stages compared to S and Control embryos. Developmental delays were not observed in 2-cell embryos cleaving to the morula (Figure 11) and blastocyst (Figure 12) stages compared to S and Control embryos. No differences (P>0.10) between S and Control embryos were observed at any of the developmental stages.

Developmental delays (P<0.05) were observed in 4-cell embryos treated with 2.5 μM AS attaining the 16-cell (Figure 13) and 5 μM AS attaining the morula (Figure 14) stages compared to S and Control embryos. Developmental delays due to AS treatment were observed throughout the 4-cell stages but were not statistically significant (Figure 15). No differences (P>0.10) between S and Control embryos were observed at any of the developmental stages.

Developmental delays were observed in all 8-cell AS embryos cleaving to the morula (Figure 16) and blastocyst (Figure 17) stages, but were not statistically different from S and Control embryos.

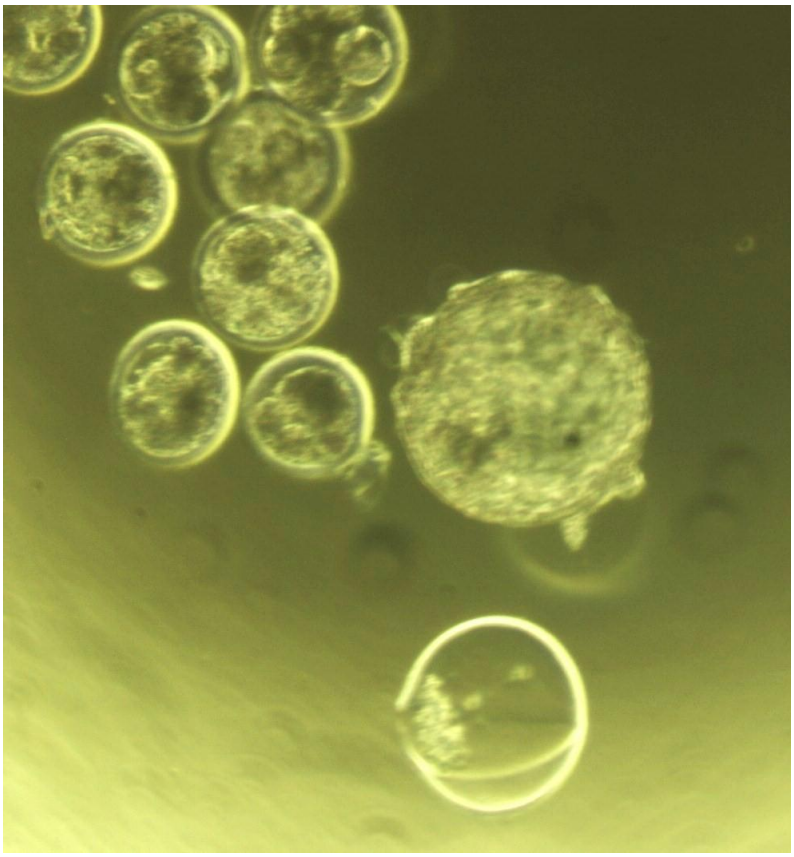


Figure 5. *In vitro*-derived bovine embryos. Hatched blastocyst with shed zona pellucida.

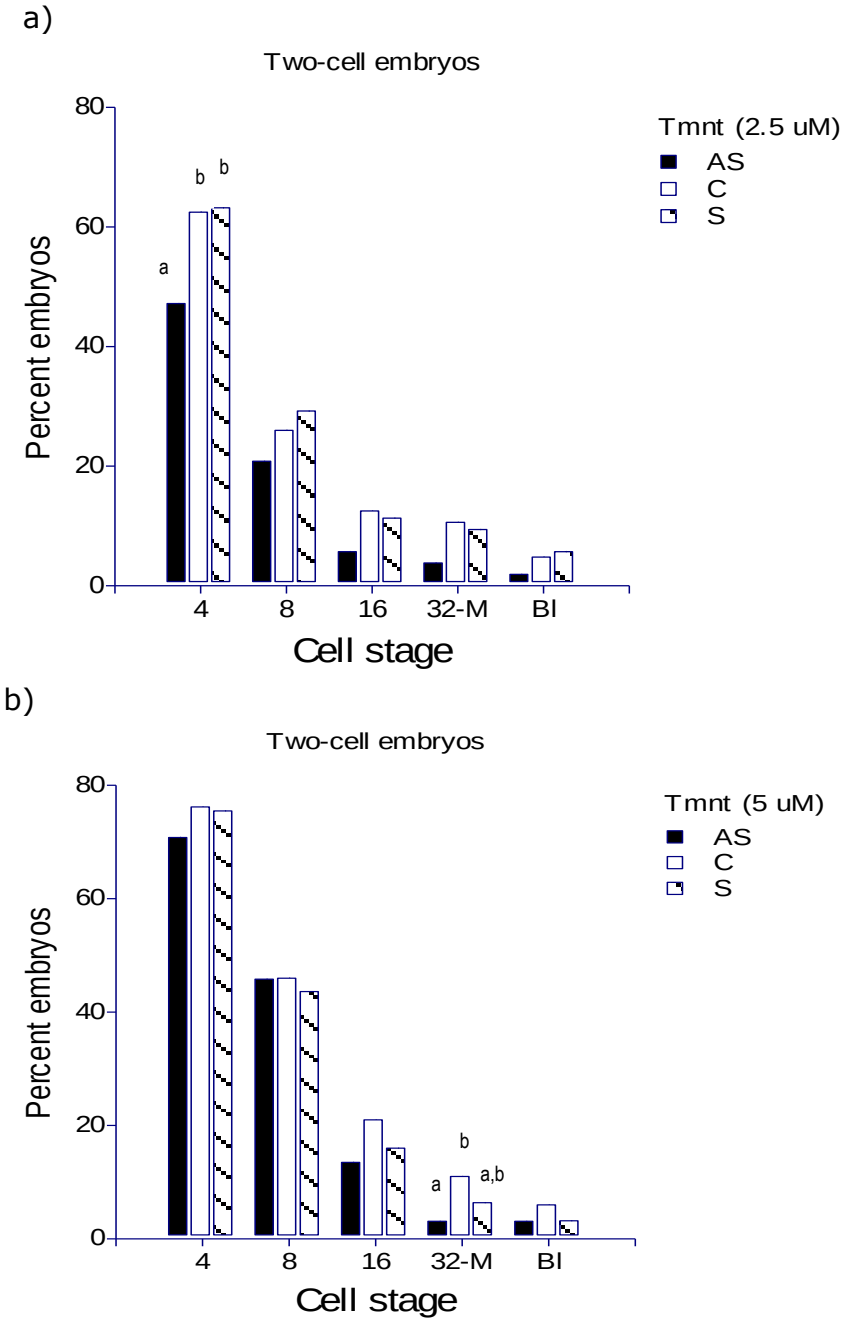
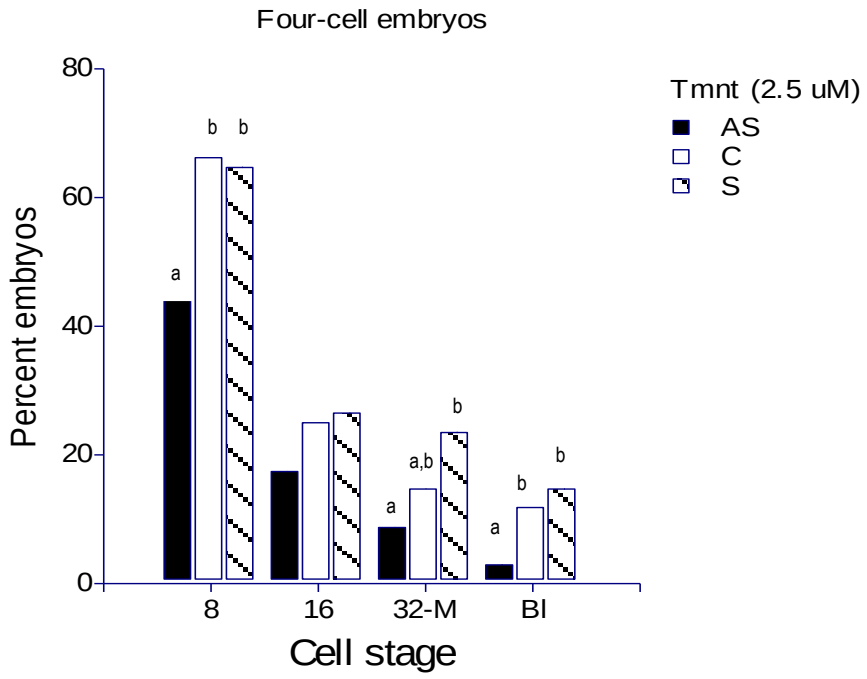


Figure 6. Development of *in vitro*-derived embryos treated with 0 (C), a) 2.5 µM or b) 5 µM sense (S) and antisense (AS) Ets-1 oligonucleotides at the 2-cell stage. [a,b] Percentages with different superscripts within a cell stage differ ($P<0.05$). M=morula and Bl=blastocyst

a)



b)

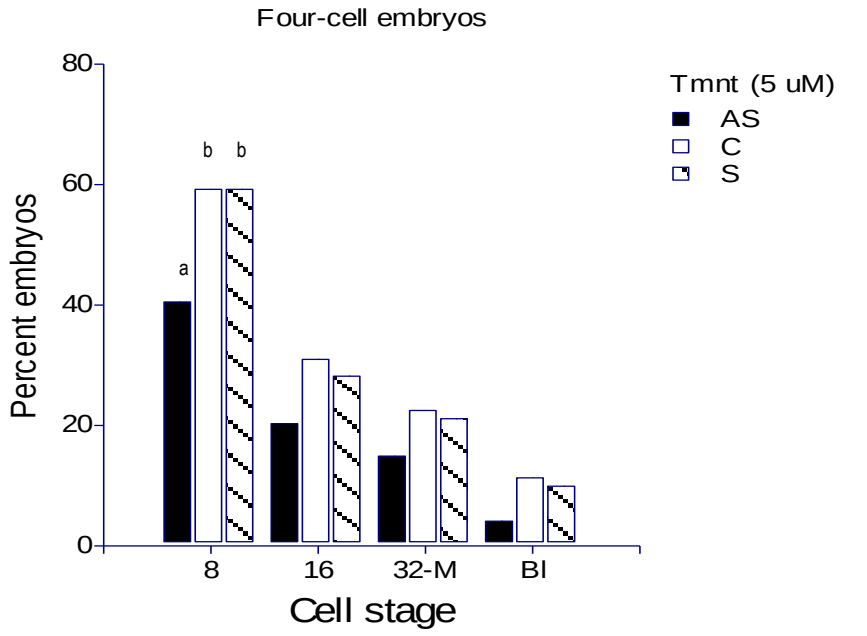
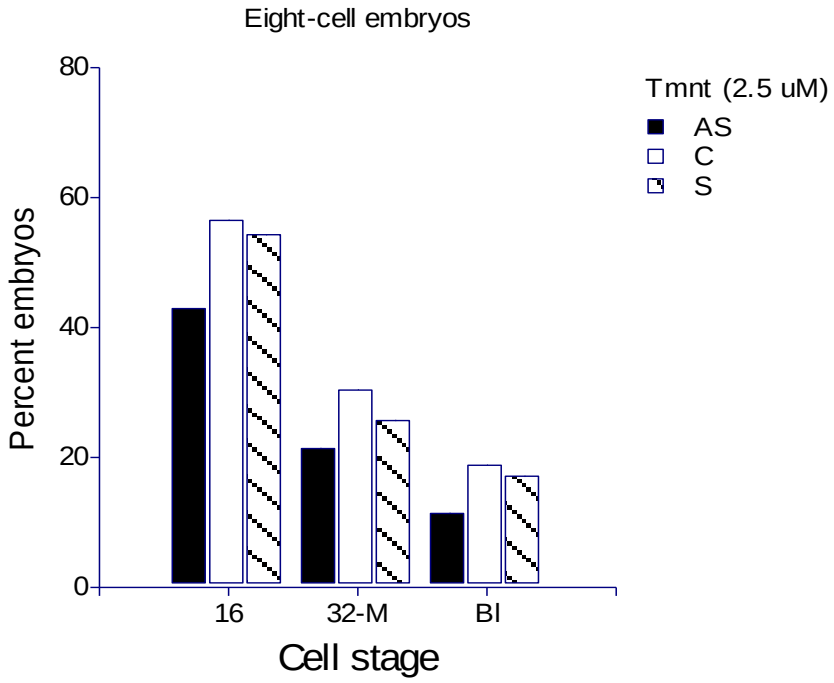


Figure 7. Development of *in vitro*-derived embryos treated with 0 (C), a) 2.5 µM or b) 5 µM S and AS Ets-1 oligonucleotides at the 4-cell stage. [a,b] Percentages with different superscripts within a cell stage differ (P<0.05). M=morula and Bl=blastocyst

a)



b)

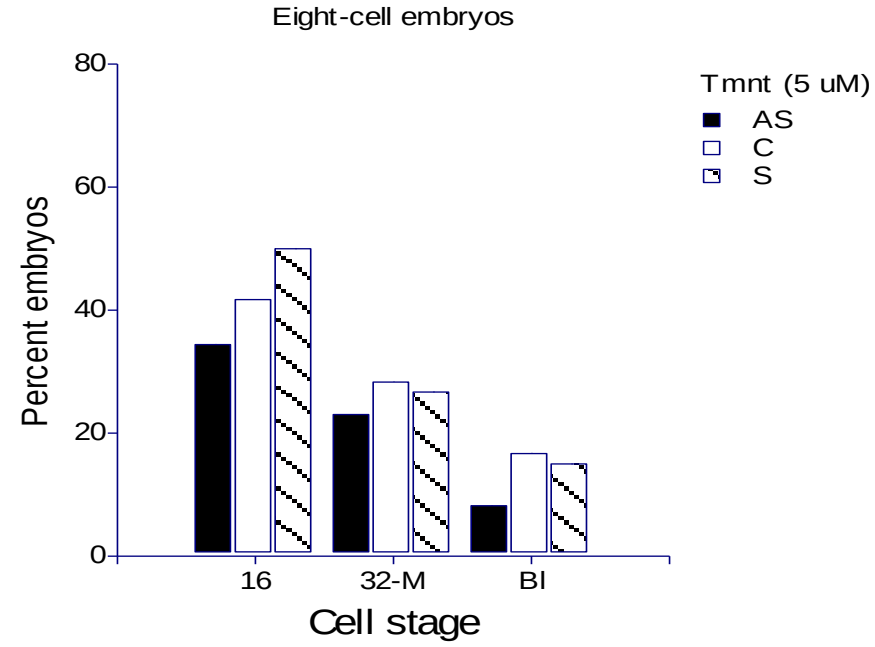


Figure 8. Development of *in vitro*-derived embryos treated with 0 (C), a) 2.5 µM or b) 5 µM S and AS Ets-1 oligonucleotides at the 8-cell stage. M=morula and Bl=blastocyst

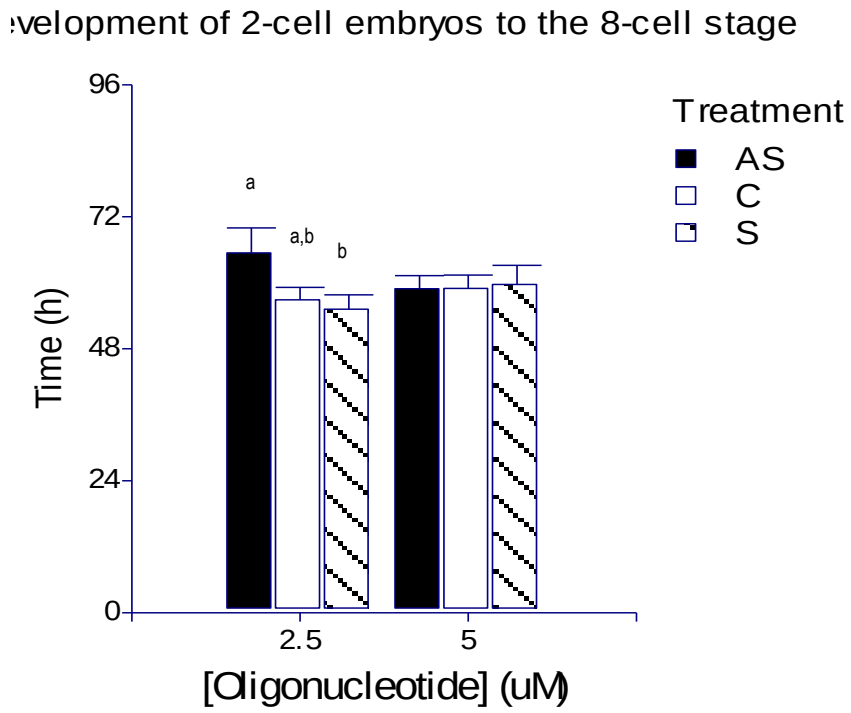


Figure 9. Development of bovine 2-cell embryos treated with 0 (C), a) 2.5 µM or b) 5 µM S and AS Ets-1 oligonucleotides to the 8-cell stage. [a,b] Means with different superscripts within a concentration differ (P<0.05).

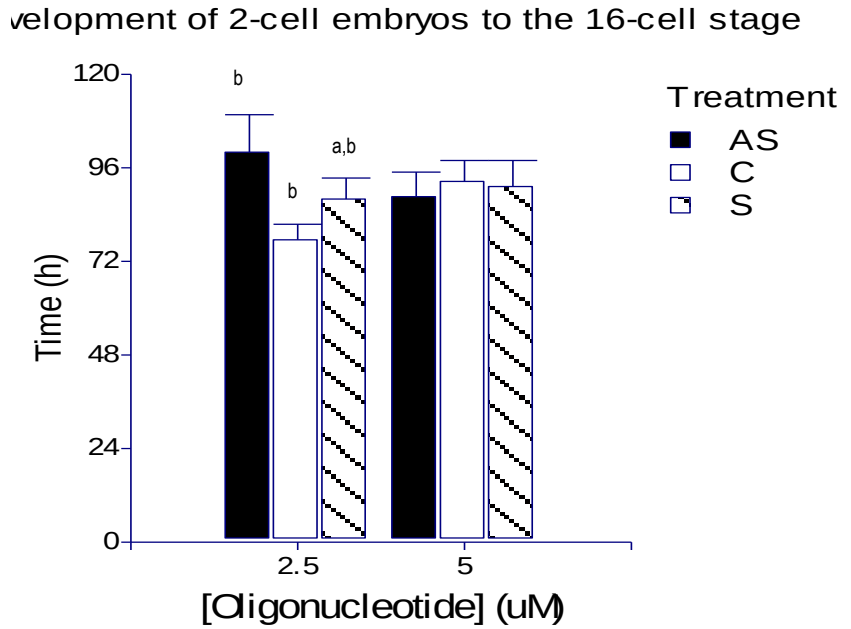


Figure 10. Development of bovine 2-cell embryos treated with 0 (C), a) 2.5 µM or b) 5 µM S and AS Ets-1 oligonucleotides to the 16-cell stage. [a,b] Means with different superscripts within a concentration differ (P<0.05).

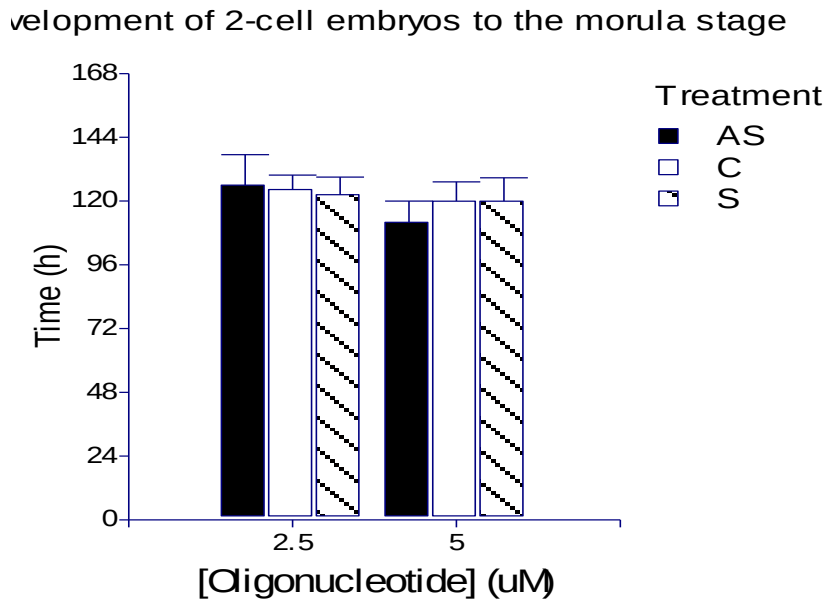


Figure 11. Development of bovine 2-cell embryos treated with 0 (C), a) 2.5 µM or b) 5 µM S and AS Ets-1 oligonucleotides to the morula stage.

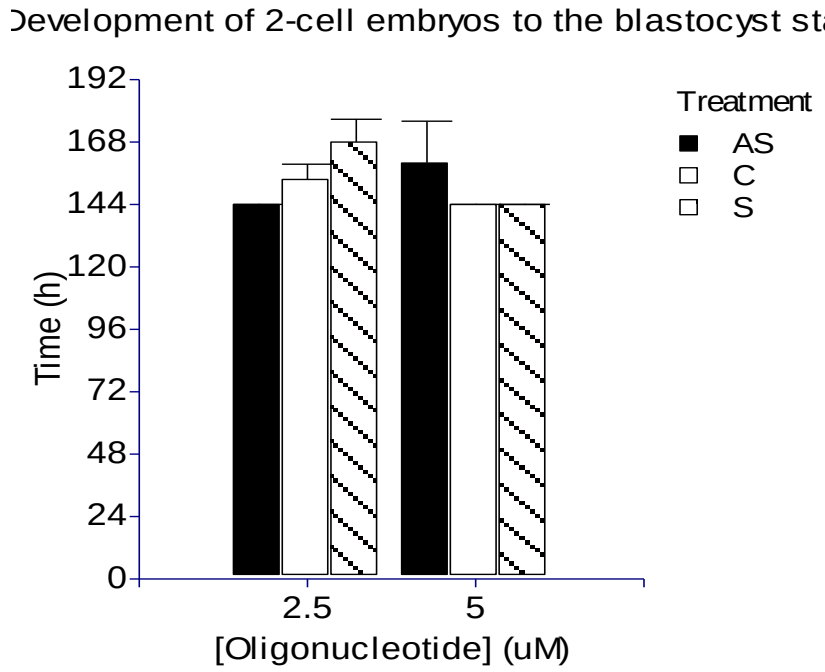


Figure 12. Development of bovine 2-cell embryos treated with 0 (C), a) 2.5 µM or b) 5 µM S and AS Ets-1 oligonucleotides to the blastocyst stage.

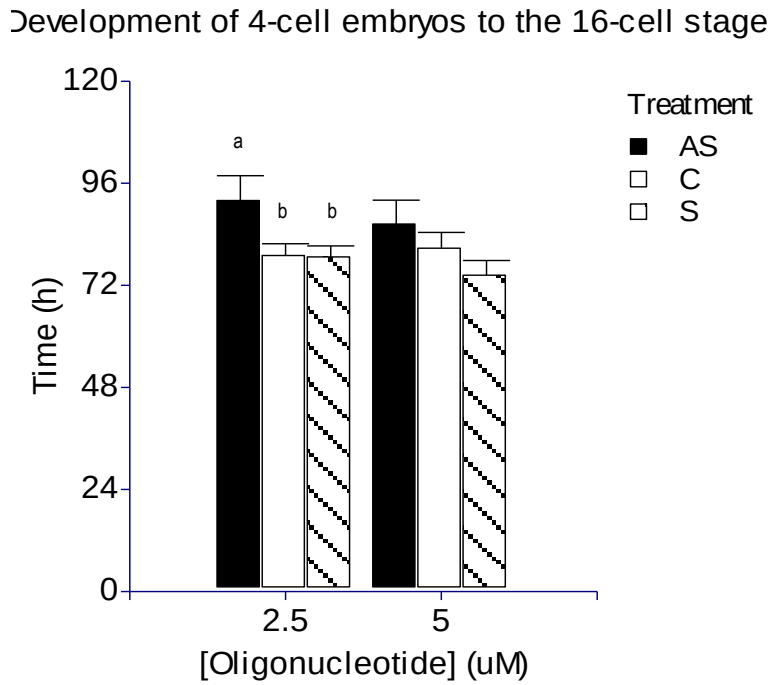


Figure 13. Development of bovine 4-cell embryos treated with 0 (C), a) 2.5 µM or b) 5 µM S and AS Ets-1 oligonucleotides to the 16-cell stage. [a,b] Means with different superscripts within a concentration differ (P<0.05).

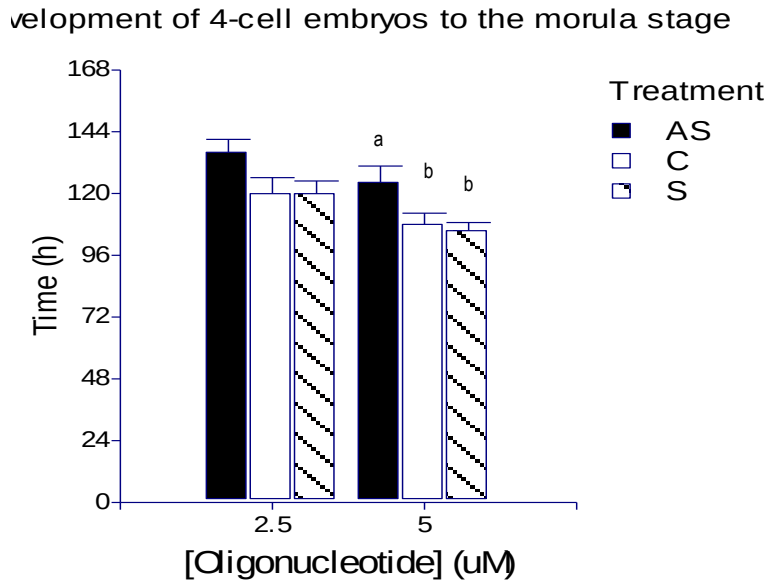


Figure 14. Development of bovine 4-cell embryos treated with 0 (C), a) 2.5 µM or b) 5 µM S and AS Ets-1 oligonucleotides to the morula stage. [a,b] Means with different superscripts within a concentration differ (P<0.05).

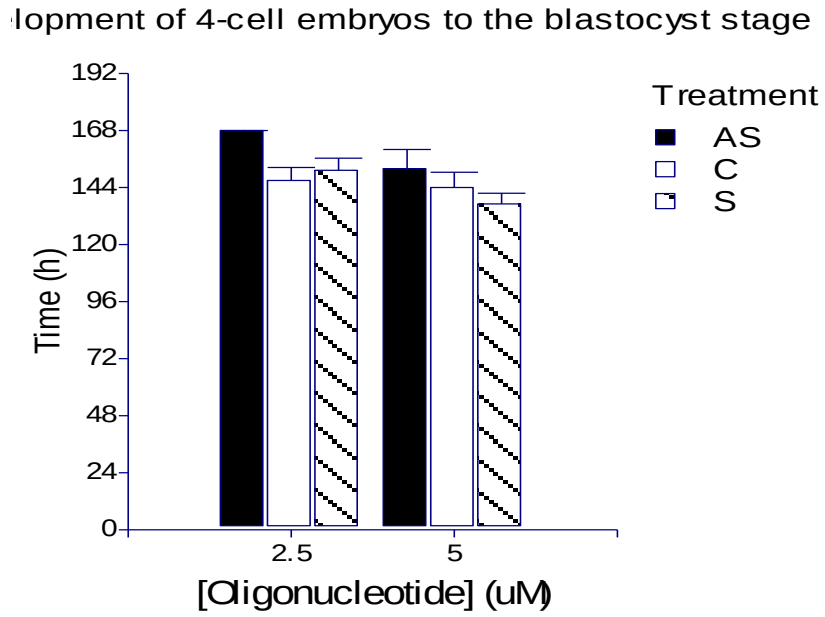


Figure 15. Development of bovine 4-cell embryos treated with C, a) 2.5 µM or b) 5 µM S and AS Ets-1 oligonucleotides to the blastocyst stage.

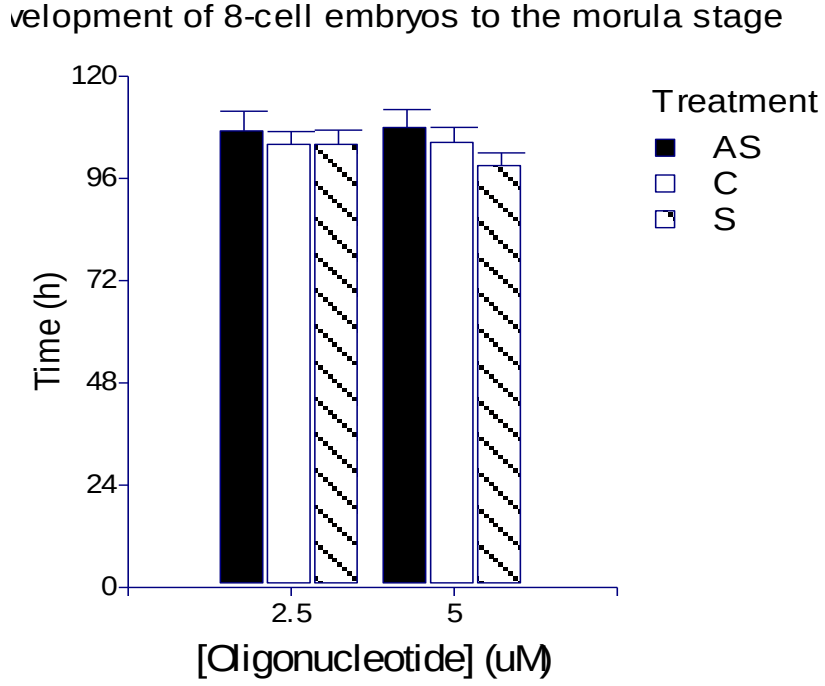


Figure 16. Development of bovine 8-cell embryos treated with 0 (C), a) 2.5 µM or b) 5 µM S and AS Ets-1 oligonucleotides to the morula stage.

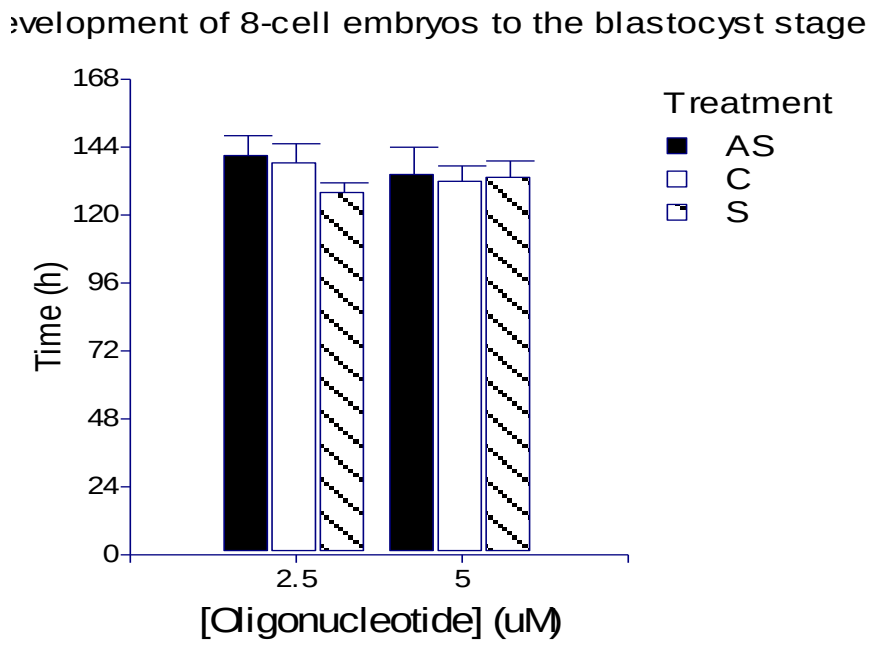


Figure 17. Development of bovine 8-cell embryos treated with 0 (C), a) 2.5 µM or b) 5 µM S and AS Ets-1 oligonucleotides to the blastocyst stage.

Discussion

The objective of the study was to determine if blocking Ets-1 expression affected early bovine embryo development. The Ets family of transcription factors has been found to play a pivotal role in early embryonic development (Maroulakou and Bowe, 2000). Our results showed fewer embryos reached subsequent stages of development when treated with AS Ets-1 at the 2- and 4-cell stages. Reductions in development due to AS treatment were observed in 8-cell embryos throughout subsequent stages but were not statistically significant.

Ets proteins are expressed ubiquitously throughout the life of an animal (Sementchenko and Watson, 2000), although predominantly during embryonic development (Kola et al., 1993; Tymms and Kola, 1994; Fafeur et al., 1997; Maroulakou and Bowe, 2000). In Xenopus, Ets-1 expression is constant during cleavage, decreases at gastrulation, and increases at neurulation (Meyer et al., 1997). Antisense Ets-2 oligonucleotides inhibit meiotic maturation in Xenopus oocytes (Dhulipal, 1997). Ets-2 is structurally similar to Ets-1 and is expressed in trophectoderm cells (Yamamoto et al., 1998). Mouse embryos lacking the Ets-2 DNA binding domain die before 8.5 days of embryonic development. Ets-1 deficient mice have defects in natural killer cell, T-cell, and B-cell development, as well as vascular inflammation and remodeling (Bories et al., 1995; Barton et al., 1998; Eyquem et al., 2004a; Eyquem et al., 2004b; Wang et al., 2005; Zhan et al., 2005; Clements et al., 2006).

Human decidual fibroblasts treated with antisense Ets- 1 oligonucleotides have significantly less mRNA for decidualization markers compared to controls (Kessler et al., 2006). In chick embryos, intravenous injection of a retrovirus encoding an antisense RNA directed against Ets-1 and Ets-2 resulted in embryos with a thin epicardium, disorganized coronary arteries and ventrical septal defects (Lie-Venema et al., 2003).

Developmental delays due to AS Ets-1 treatment occurred at the 2- and 4-cell stages. Two-cell embryos were delayed cleaving to 8- and 16-cell stages and 4-cell embryos were delayed cleaving to 16-cell and morula stages. Developmental delays were observed in all 8-cell antisense treated embryos but were not statistically significant. This could be due to the embryo activating its own genome while degrading stored maternal transcripts. Maternal stockpiles of mRNA are masked in messenger ribonucleoprotein complexes (mRNPs) that protect from premature translation and degradation (Weston and Sommerville, 2006). Activation of the embryonic genome must be accompanied by gradual degradation of maternally inherited mRNA and proteins. A rapid decrease in maternal mRNA levels of stockpiled genes occurs in the first cleavage stages in bovine embryos (Vigneault et al., 2004; McGraw et al., 2007). As embryonic transcripts are being transcribed, maternally inherited mRNA molecules decay (Bachvarova and De Leon, 1980). This prepares the embryo for later developmental events and is essential for embryo survival (Vigneault et al., 2009). Ets-1 appears to be primarily a maternal

transcript in the bovine embryo according to our results. A significant effect was not seen in 8-cell embryos treated with AS oligonucleotides and this could be due to 8-cell embryos being able to use an alternative transcription factor to facilitate development. Insufficient numbers of 8-cell embryos in each treatment could also have had an impact in this study. It is doubtful that Ets-1 is responsible for MZGA although it may be involved in the cell cycle or contribute to other transcription factors responsible for MZGA. Ets-2, which is structurally similar to Ets-1, transactivates the cdc2 (Wen et al., 1995) and cyclin D1 promoters (Albanese et al., 1995). Cdk 10, a Cdc2-related kinase regulating G2/M phase of the cell cycle, interacts with the pointed domain of Ets-2 to inhibit Ets-2-mediated transactivation (Kasten and Giordano, 2001). There is evidence that Ets-1 and Ets-2 play a redundant role in endothelial cell survival during embryonic angiogenesis (Wei et al., 2009) and possibly Ets-1 could have a similar role to Ets-2, and be involved in cell cycle regulation. Ets-1 AS oligonucleotides did have a significant effect on development and time required to reach subsequent cell stages in 2- and 4-cell embryos, indicating that Ets-1 is required for normal early embryonic development in the bovine.

It is important to identify the roles of genes involved in early embryo development for clarification of the basic mechanisms controlling cell proliferation and differentiation. This knowledge could lead to

advancements in embryo culture systems, transgenics and cloning and potentially provide a marker for viable embryos for transfer or freezing.

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Chapter 4: Effects of Ets-1 Oligonucleotides on Development and Urokinase Type Plasminogen Activator Production in *In Vivo*-Derived Bovine Embryos

Abstract

The effects of Ets-1 oligonucleotides on plasminogen activator (PA) production and development in Days 5, 6 and 7 bovine embryos were evaluated. Embryos were non-surgically collected from superovulated cows and cultured in medium containing 0 (Control), 2.5 or 5 μM Ets-1 sense (S) and antisense (AS) oligonucleotides. No differences (P>0.10) in development to the blastocyst stage were observed in Days 5 and 6 embryos treated with 2.5 or 5 μM AS and S oligonucleotides compared to Controls. However, more (P<0.10) Day 5 embryos treated with 2.5 μM AS oligonucleotides expanded compared to S and Control embryos. Similar (P>0.10) numbers of Day 5 embryos treated with 5 μM oligonucleotides developed to the expanded blastocyst stage compared to Controls. More (P<0.05) Day 7 embryos treated with 2.5 μM AS developed to the hatching and hatched blastocyst stages compared to Controls. No differences (P>0.10) in development were observed in Day 7 embryos treated with 5 μM oligonucleotides compared to Controls. Mean PA production by Day 5 embryos treated with 2.5 μM or 5 μM AS oligonucleotides was lower compared to Controls; however, no significant differences were detected. Plasminogen activator production by Day 5 embryos was greatest (P<0.05) at 168 h of culture compared to all earlier intervals of embryos treated with 2.5 μM oligonucleotides. Day 6 embryos

treated with 2.5 or 5 μM AS oligonucleotides produced less mean PA than Control embryos; however, no significant differences were detected. Plasminogen activator production by Day 6 embryos treated with 2.5 μM oligonucleotides was greater ($P<0.05$) at 96 h compared to 24, 48, 144 and 168 h of culture. Orthogonal comparison between Day 7 embryos treated with 2.5 μM AS versus S and Control embryos revealed suppressed ($P<0.10$) PA production by AS embryos. Mean PA production by Day 7 embryos treated with 5 μM AS oligonucleotides was lower compared to S-treated embryos and Controls, but no significant differences were detected. Plasminogen activator production by Day 7 embryos treated with 2.5 μM oligonucleotides was greater ($P<0.05$) at 48 h compared to 24, 96, 120, 144 and 168 h of culture. Plasminogen activator production by embryos developing in medium containing 5 μM oligonucleotides was greater ($P<0.05$) at 72 and 96 h compared to 24, 144 and 168 h of culture. These data suggest that treatment with Ets-1 AS reduces PA production in Day 7 bovine embryos but has no deleterious effects on development.

Introduction

The E26 transforming specific (Ets) family is one of the largest transcription factor families with over 30 members. Ets transcription factors play a vital role in regulating expression of various proteases during development (Yordy and Muise-Helmericks, 2000). Ets transcription factors regulate the production of matrix metalloproteinases (MMP), tissue inhibitors of MMP (TIMP), and urokinase-type plasminogen activator (uPA) (Oikawa and Yamada, 2003). These proteases are essential for numerous reproductive processes such as oocyte maturation (Moor et al., 1998), fertilization (Huarte et al., 1993), proper embryo development (Kaaekuahiwi and Menino, 1990; Birkedal-Hansen et al., 1993; Huarte et al., 1993; Salamonsen, 1994), hatching (Lee et al., 1997; Menino and Williams, 1987), and endometrial remodeling during implantation, placentation and menstruation (Salamonsen, 1994; Duc-Goiran et al., 1999). Genes involved in extracellular degradation, such as MMP (stromelysins and collegenases), TIMP, and uPA either contain Ets binding sites in their regulatory sequences or are activated by Ets proteins (Wasylyk et al., 1991; Nerlov et al., 1991; Tymms and Kola, 1994; Higashino et al., 1995; Buttice et al., 1996; Iwasaka et al., 1996; Logan et al., 1996; Westermarck et al., 1997; Naito et al., 2002). Two Ets binding sites exist in the uPA gene and Ets-1 or Ets-2 can bind to promote transcription (Nagamine et al., 2005).

Several species of embryos produce PA. In developmental processes where PA has been observed, uPA is the prominent form. Embryonic PA production has been identified in mice (Strickland et al., 1976), swine (Mullins et al., 1980), cattle (Menino and Williams, 1987) and sheep (Menino et al., 1989). Mouse embryos cultured in medium supplemented with either plasminogen or plasmin, had greater rates of blastocyst formation and hatching compared to embryos cultured in medium supplemented with pronase or trypsin (Menino and O'Claray, 1986). In sheep, PA levels were low until the morula stage, increased during the morula-blastocyst transition and remained elevated through blastocoelic expansion and hatching (Menino et al., 1989). In early bovine embryos, PA production is positively correlated with embryo size, developmental stage, and cell number (Kaaekuahiwi and Menino, 1990; Bartlett and Menino, 1995). Trophoblast cells, not ICM, are responsible for PA production by bovine embryos (Dyk and Menino, 1991). Day 12 to 14 bovine embryos treated with anti-uPA antibodies lose PA activity whereas treatment with anti-tPA antibodies had no effect confirming the presence of uPA (Berg and Menino, 1992). Knockout mice lacking both uPA and tPA, although fertile, have severely impaired ovulation rates, reduced growth rates and poor cardiovascular health (Carmeliet et al., 1994; Carmeliet and Collen, 1995; Liu et al., 2006; Wahlberg et al., 2007).

Little is known regarding how embryonic PA production is regulated. Attempts to perturb embryonic PA production by hormones or mediators of

cell-signaling mechanisms were unsuccessful (al-Hozab and Menino, 1992). Because of the link between measures of embryo viability and PA production (Kaaekuahiwi and Menino, 1990), identifying regulation of this enzyme may provide insights into pregnancy failure. Therefore, the objective of this research was to establish if PA expression in early bovine embryos is dependent on Ets-1.

Materials and Methods

Embryo Collection

Cross-bred beef cows were estrous synchronized using prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse, The Upjohn Co., Kalamazoo, MI) and superovulated with porcine follicle stimulating hormone (pFSH; Sioux Biochemicals, Sioux City, IA). The initial 25 mg intramuscular injection of Lutalyse (Day 0=first injection of Lutalyse) was followed by twice daily injections of FSH on days 9-12. Respective dosages of FSH were 5, 4, 2, and 1 mg. Evening injections of FSH on Day 11 and morning injections on Day 12 were accompanied by an additional injection of 25 and 12.5 mg Lutalyse, respectively. Estrus detection was initiated 24 h after the second injection of Lutalyse. Cows found to be in standing estrus were artificially inseminated at 0, 12 and 24 h after onset. The first insemination was accompanied with an intramuscular injection of 100 µg of gonadotropin releasing hormone (GnRH; Sigma, St. Louis, MO). All cows not found in estrus at the first observation were artificially inseminated at 48, 60 and 72 h after the second Lutalyse injection. Embryos were recovered on days 5, 6 and 7 (Day 0= Day of estrus onset) by non-surgically flushing the uterus with Dulbecco's phosphate buffered saline (DPBS) containing 2 ml/L heat-treated cows serum (HTCS) and 10 ml/L antibiotic/antimycotic solution (Sigma). Embryos were recovered from the flush medium by aspiration and washed three times in microdrops of Ham's F-12 with 1.5%

BSA (Ham's F-12+BSA; Sigma) under paraffin oil (Fisher Scientific Co., Tustin, CA).

Antisense Experiments

Antisense Ets-1 experiments were performed to determine the effects of Ets-1 on embryonic PA production and development. Embryos were cultured in 25 µl microdrops of Ham's F-12 containing 1.5% BSA and 0 (control), 2.5 or 5 µM concentrations of Ets-1 sense (S) and antisense (AS) oligonucleotides. Sense and AS oligonucleotides spanned from 230 to 248 nt and 533 to 554 nt of the Ets-1 sequence, respectively (Watson et al, 1988). Each microdrop contained one Day 5, 6 or 7 embryo and embryos were cultured for up to 168 h. Microdrops were covered with paraffin oil and cultured at 39°C in a humidified atmosphere of 5% CO_2 in air. At 24-h intervals, embryo development was observed and 10 µl of conditioned medium were recovered and frozen at -20°C for PA analysis.

Plasminogen Activator Assay

Plasminogen activator activity in embryo conditioned medium was determined as described by (Kaaekuahiwi and Menino, 1990) using a caseinolytic agar gel assay with urokinase (E.C.3.4.21.73, America Diagnostica Inc, Greenwich, CT) as the standard. Ten microliters of conditioned medium or urokinase standard were combined with 15 µl of 120 µg/ml human plasminogen (Sigma) and incubated for 30 min at 39°C.

Twenty microliters of this solution were pipetted into pre-cut wells in a casein-agar gel plate. Plates were incubated at room temperature for 24 h and fixed with 3% acetic acid. Plasminogen activator activity was determined by measuring the diameter of the lytic zone using a digital caliper. Correlation-regression analysis was used to determine PA activities in the conditioned medium from plots of lytic ring diameters versus log urokinase concentrations. Intra- and interassay coefficients of variation for PA using this assay were 9.7 and 11.2%, respectively.

Statistical Analysis

Differences due to treatment in PA conditioned medium were analyzed using repeated measures ANOVA. Sources of variation in the ANOVA for PA activity were Treatment (control, sense and antisense), Time, and the Treatment x Time interaction. If significant effects were observed in the ANOVA, differences between means were determined using Duncan's multiple comparisons test. Differences in the number of embryos achieving a particular cell stage were determined by Chi-square procedures. All analyses were conducted using the NCSS 2000 software program (Number Cruncher Statistical System; Hintze, J.C.; Kaysville, UT).

Results

No differences (P>0.10) in development to the blastocyst stage were observed among Day 5 embryos treated with 2.5 μM or 5 μM AS and S oligonucleotides compared to Controls (Table 2). However, more (P<0.10) Day 5 embryos treated with 2.5 μM AS oligonucleotides expanded compared to embryos treated with S oligonucleotides or Controls (Table 2). Similar (P>0.10) numbers of embryos treated with 5 μM oligonucleotides developed to the expanded blastocyst stage compared to Controls (Table 2).

Although mean PA production by Day 5 embryos treated with 2.5 or 5 μM AS oligonucleotides was lower compared to Controls, no significant differences were detected (Table 3). Time in culture was a significant effect for PA production by Day 5 embryos treated with 2.5 μM oligonucleotides (Figure 18). Plasminogen activator production was greater (P<0.05) at 168 h of culture compared to all earlier intervals for embryos treated with 2.5 μM oligonucleotides (Figure 18). Time in culture was not a significant effect for PA production by Day 5 embryos treated with 5 μM oligonucleotides (Figure 19). The Treatment x Time interaction was not significant for Day 5 embryos treated with either 2.5 or 5 μM oligonucleotides.

Treatment with 2.5 or 5 μM oligonucleotides had no effect (P>0.10) on development of Day 6 embryos to subsequent blastocyst stages compared to Controls (Table 4). Similar to Day 5 embryos, Day 6

embryos treated with 2.5 or 5 μM AS oligonucleotides produced less mean PA than Control embryos; however, no significant differences were detected (Table 5). Time in culture was a significant effect for PA production by Day 6 embryos treated with 2.5 μM oligonucleotides (Figure 20). Plasminogen activator production by Day 6 embryos treated with 2.5 μM oligonucleotides was greater ($P<0.05$) at 96 h compared to 24, 48, 144 and 168 h of culture (Figure 20). Time in culture was not a significant effect for PA production by Day 6 embryos treated with 5 μM oligonucleotides (Figure 21). The Treatment x Time interaction was not significant for Day 6 embryos treated with either 2.5 or 5 μM oligonucleotides.

More ($P<0.05$) Day 7 embryos treated with 2.5 μM AS oligonucleotides developed to the hatching and hatched blastocyst stages compared to Controls (Table 6). No differences ($P>0.10$) in development were observed in Day 7 embryos treated with 5 μM oligonucleotides compared to Controls (Table 6). Although mean PA production by Day 7 embryos treated with 2.5 or 5 μM AS oligonucleotides was lower compared to S-treated embryos and Controls, no significant differences were detected (Table 7). However, the orthogonal comparison between Day 7 embryos treated with 2.5 μM AS oligonucleotides versus S-treated and Control embryos revealed suppressed ($P<0.10$) PA production by AS-treated embryos. Time in culture was a significant effect for PA production by Day 7 embryos treated with 2.5 or 5 μM oligonucleotides (Figures 22

and 23). Plasminogen activator production by embryos treated with 2.5 μM oligonucleotides was greater ($P<0.05$) at 48 h compared to 24, 96, 120, 144 and 168 h of culture (Figure 22). Plasminogen activator production by embryos developing in medium containing 5 μM oligonucleotides was greater ($P<0.05$) at 72 and 96 h compared to 24, 144 and 168 h of culture (Figure 23). The Treatment x Time interaction was not significant for embryos treated with either 2.5 or 5 μM oligonucleotides. Examples of embryos encountered in this study are provided in Figure 24.

Table 2. Development of Day 5 bovine embryos to the blastocyst (Bl) and expanded blastocyst (XBl) stages in medium containing Ets-1 oligonucleotides.

Oligonucleotides (μM)						
	2.5			5		
Treatment	n	Bl	XBl	n	Bl	XBl
Control	4	50(2)*	0(0)[b]	5	80(4)	0(0)
Sense	4	75(3)	0(0)[b]	6	50(3)	17(1)
Antisense	4	75(3)	50(2)[a]	5	60(3)	20(1)

* Values presented are the percentages (numbers) of embryos developing to a particular cell stage. [a,b] Percentages in a column without common superscripts differ ($P<0.10$).

Table 3. Plasminogen activator production (U/ml) by Day 5 bovine embryos cultured in medium containing 2.5 μM or 5 μM sense or antisense Ets-1 oligonucleotides.

	2.5 μM		5 μM	
Treatment	n	Mean ± SE	n	Mean ± SE
Control	4	0.015 ± 0.005	5	0.022 ± 0.004
Sense	4	0.017 ± 0.005	5	0.014 ± 0.004
Antisense	4	0.010 ± 0.005	5	0.014 ± 0.005

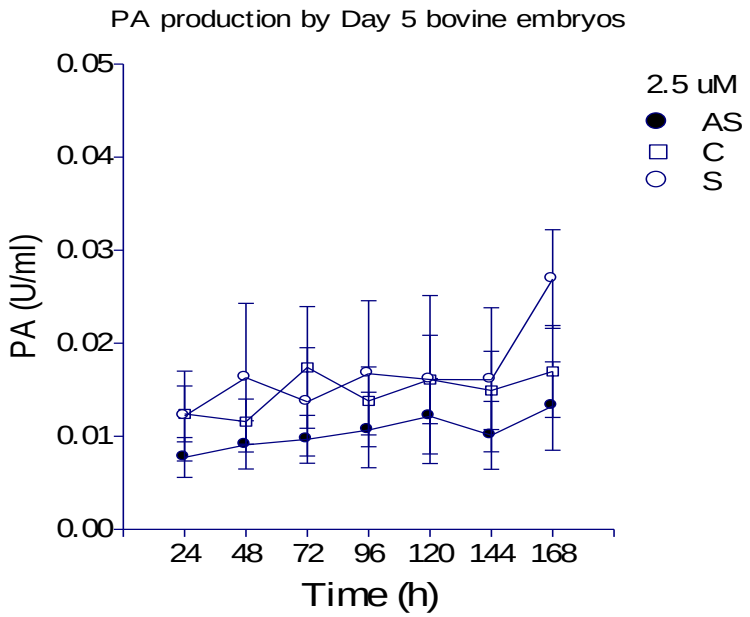


Figure 18. Plasminogen activator production by Day 5 embryos treated with 0 (C) or 2.5 µM sense (S) or antisense (AS) Ets-1 oligonucleotides.

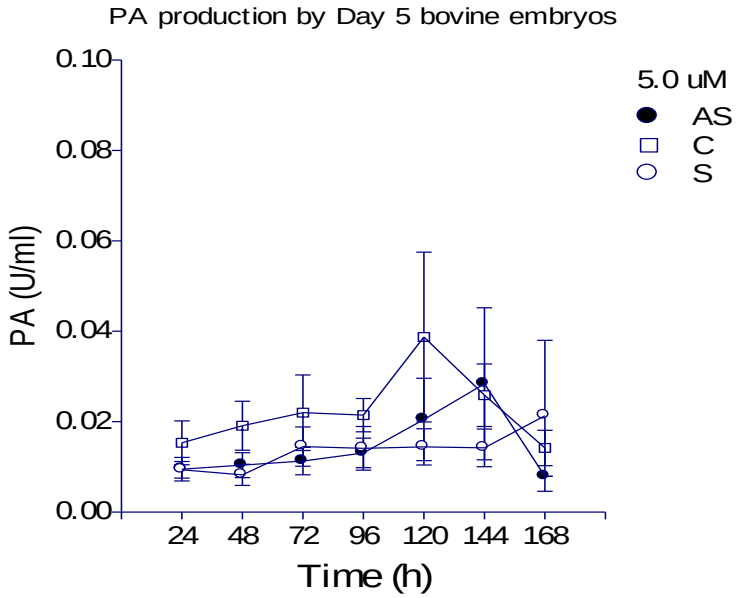


Figure 19. Plasminogen activator production by Day 5 embryos treated with 0 (C) or 5 µM sense (S) or antisense (AS) Ets-1 oligonucleotides.

Table 4. Development of Day 6 bovine embryos to the expanded (XBl), initiating hatching (IHBl) and hatched (HBl) blastocyst stages in medium containing Ets-1 oligonucleotides.

Oligonucleotides (μM)							
	2.5			5			
Treatment	n	XBl	IHBl	n	XBl	IHBl	HBl
Control	4	75(3)*	0(0)	5	60(3)	40(2)	20(1)
Sense	3	67(2)	33(1)	3	67(2)	0(0)	0(0)
Antisense	3	100(3)	0(0)	3	67(2)	0(0)	0(0)

* Values presented are the percentages (numbers) of embryos developing to a particular cell stage.

Table 5. Plasminogen activator production (U/ml) by Day 6 bovine embryos cultured in medium containing 2.5 μM or 5 μM sense or antisense Ets-1 oligonucleotides.

	2.5 μM		5 μM	
Treatment	n	Mean ± SE	n	Mean ± SE
Control	4	0.034 ± 0.009	5	0.031 ± 0.007
Sense	3	0.024 ± 0.010	3	0.021 ± 0.009
Antisense	3	0.024 ± 0.010	3	0.014 ± 0.009

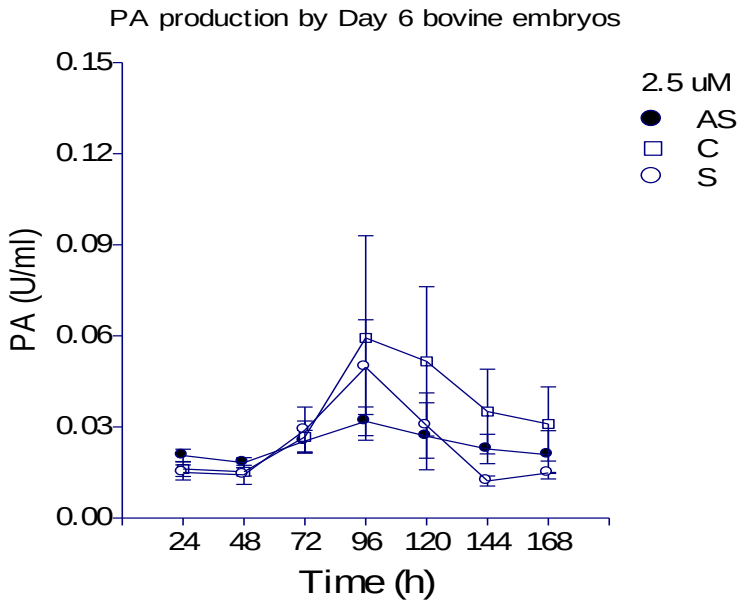


Figure 20. Plasminogen activator production by Day 6 embryos treated with 0 (C) or 2.5 µM sense (S) or antisense (AS) Ets-1 oligonucleotides.

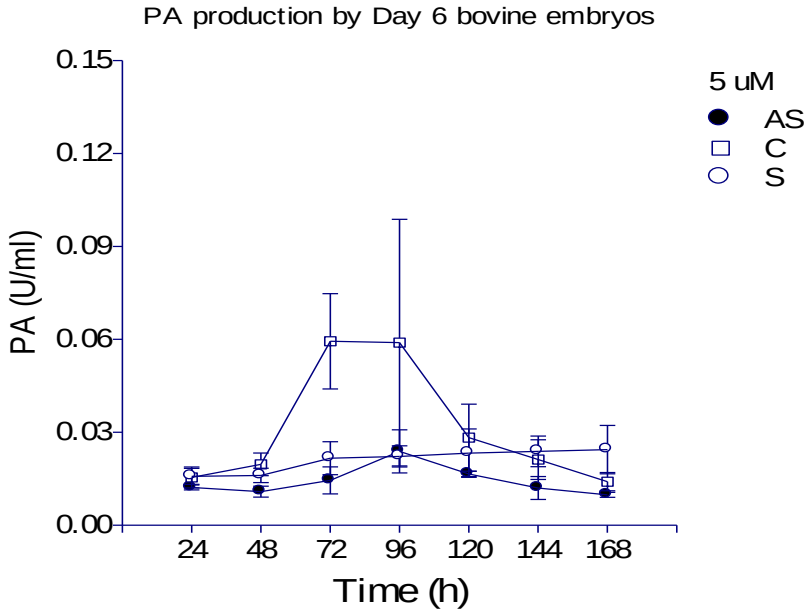


Figure 21. Plasminogen activator production by Day 6 embryos treated with 0 (C) or 5 µM sense (S) or antisense (AS) Ets-1 oligonucleotides.

Table 6. Development of Day 7 bovine embryos to the expanded (XBl), initiating hatching (IHBl) and hatched (HBl) blastocyst stages in medium containing Ets-1 oligonucleotides.

Oligonucleotides (μM)								
	2.5				5			
Treatment	N	XBl	IHBl	HBl	n	XBl	IHBl	HBl
Control	5	100(5)*	0(0)[b]	0(0)[b]	6	83(5)	67(4)	50(3)
Sense	4	100(4)	50(2)[a,b]	50(2)[a,b]	6	100(6)	67(4)	67(4)
Antisense	3	100(3)	100(3)[a]	100(3)[a]	6	100(6)	83(5)	67(4)

* Values presented are the percentages (numbers) of embryos developing to a particular cell stage. [a,b] Percentages in a column without common superscripts differ ($P<0.05$).

Table 7. Plasminogen activator production (U/ml) by Day 7 bovine embryos cultured in medium containing 2.5 μM or 5 μM sense or antisense Ets-1 oligonucleotides.

	2.5 μM		5 μM	
Treatment	n	Mean ± SE	n	Mean ± SE
Control	5	0.035 ± 0.009	6	0.038 ± 0.013
Sense	4	0.031 ± 0.001	6	0.034 ± 0.014
Antisense	3	0.008 ± 0.011	6	0.028 ± 0.014

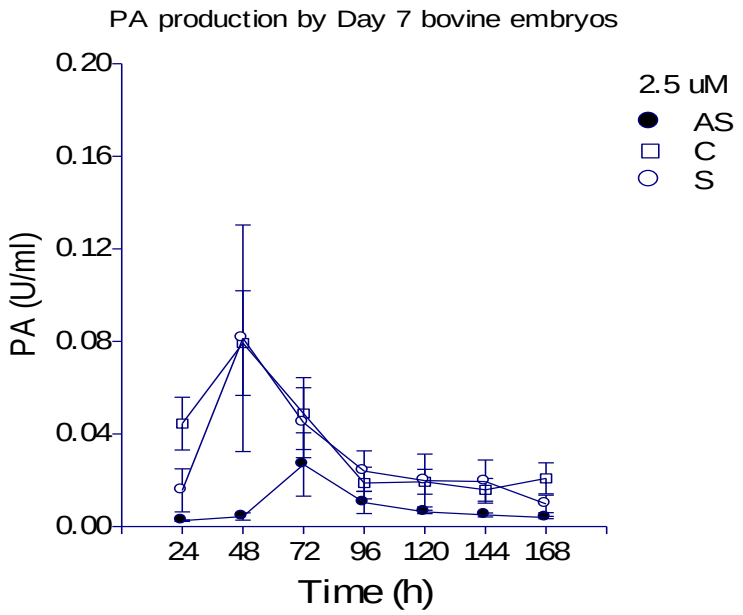


Figure 22. Plasminogen activator production by Day 7 embryos treated with 0 (C) or 2.5 µM sense (S) or antisense (AS) Ets-1 oligonucleotides.

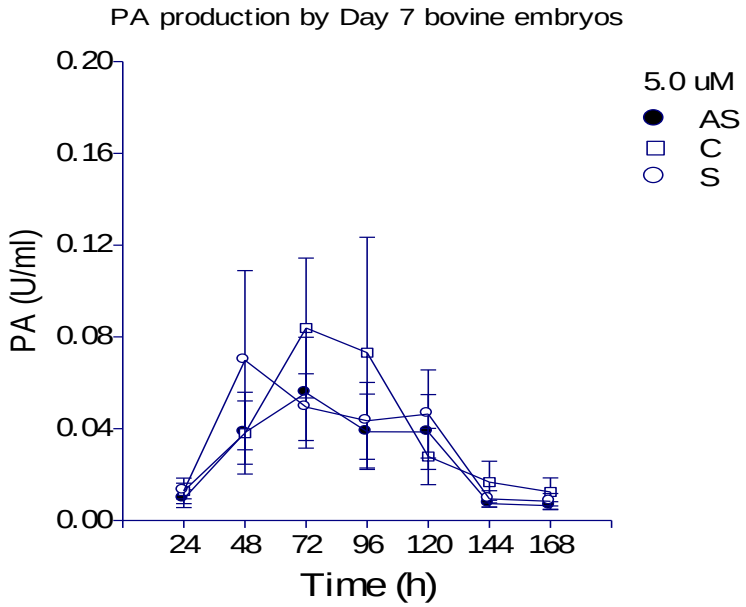
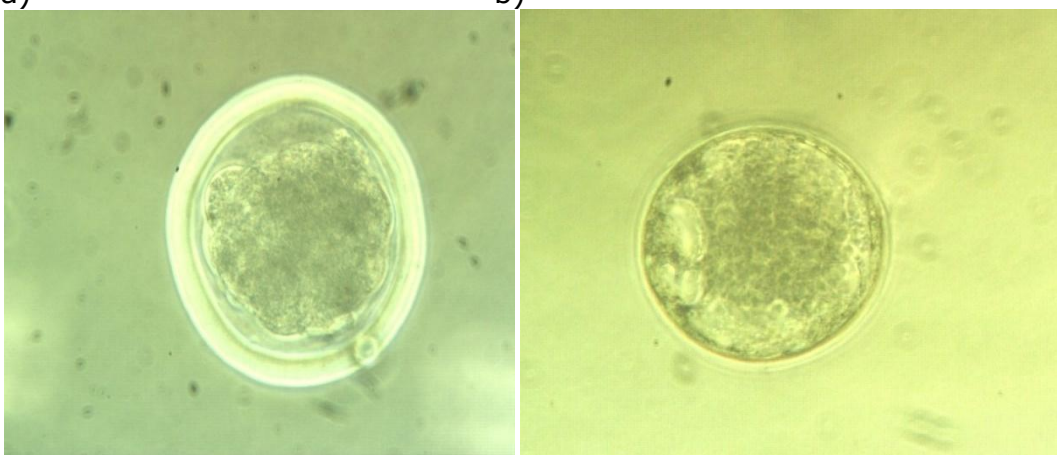


Figure 23. Plasminogen activator production by Day 7 embryos treated with 0 (C) or 5 µM sense (S) or antisense (AS) Ets-1 oligonucleotides.

a) b)



c)

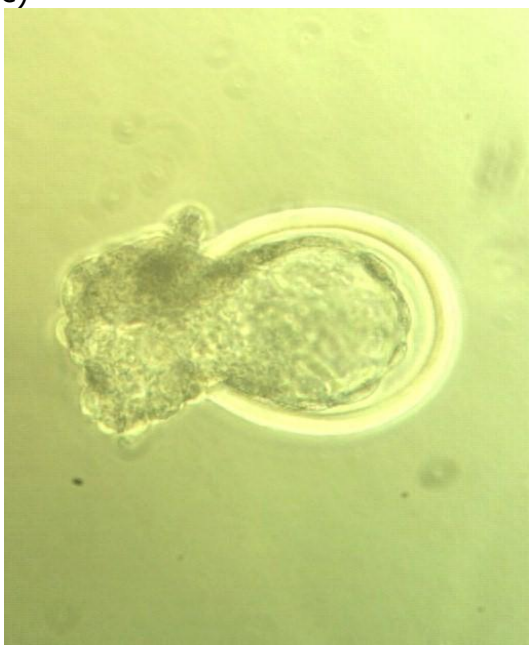


Figure 24. *In vivo*-derived bovine embryos: a) morula, b) expanded blastocyst and c) initiating hatching blastocyst.

Discussion

The aim of this research was to determine the effects of Ets-1 oligonucleotides on development and PA production in Days 5, 6 and 7 bovine embryos. No detrimental effects of the AS or S oligonucleotides at the concentrations used in this study were observed on embryo development. In fact, in some cultures more AS-treated embryos developed to later blastocyst stages compared to S and Control embryos. Previous work in our laboratory reported deleterious effects of 10 μM S and AS Ets-1 oligonucleotides on bovine embryo development (Collins, 2002).

Bovine embryos express PA during blastocoelic expansion and hatching *in vitro* (Menino and Williams, 1987). Plasminogen activator does not appear to have a direct lytic effect on the bovine zona pellucida *in vitro* (Cannon and Menino, 1998). However, embryonic PA can activate plasminogen and induce a sublytic effect through plasmin proteolysis where the zona pellicida becomes more soluble in acidified PBS or SDS (Menino et al., 1989; Cannon and Menino, 1998). Although PA production was reduced in medium containing AS oligonucleotides, a significant effect was only detected in Day 7 embryos treated with 2.5 μM oligonucleotides. Two, neither exclusive, explanations exist for this observation. First, it is possible earlier stage embryos use alternative transcription factors to regulate PA production. Second, numbers of embryos dedicated to the

treatments were low which would contribute to the lack of statistical significance in the PA analysis.

Interestingly, Day 7 2.5 μM Control embryos failed to hatch, yet remained viable and produced PA. By chance, this group of embryos may have had slightly fewer cells and formed a smaller trophectoderm thereby limiting blastocoelic expansion and hatching. Variation in the peak of PA production between the two concentrations of AS oligonucleotides in Day 7 embryos is likely a result of either variability between donor cows or subtle differences in embryo age when staged on the basis of days post-onset of estrus.

Nevertheless, Ets-1 does appear to be involved in regulating PA production in Day 7 bovine embryos yet has little effect on cell division, blastocoel formation and development of the Na/K ATPase. Future research is warranted to determine the mechanisms eliciting Ets-1 transcription and initiating PA expression and to identify other transcription factors participating in PA production.

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Conclusion

This dissertation investigated the expression pattern of Ets-1 in bovine and porcine oocytes and preimplantation embryos, determined if blocking Ets-1 expression affected early bovine embryo development and determined if uPA expression in early bovine embryos is dependent on Ets-1. Ets-1 expression was associated with early cleavage stage bovine embryos, suggesting that Ets-1 may be involved in regulation of cell growth and differentiation during the early cleavage stages of the bovine embryo. Ets-1 may be involved in cell cycle regulation during the resumption of meiosis since Ets-1 expression was found in bovine primary and secondary oocytes and COC. Ets-1 was detected in Days 10 and 12 porcine embryos and may be regulating proteases during elongation to facilitate extracellular matrix remodeling and cellular migration.

Treatment with Ets-1 AS oligonucleotides delayed development and decreased the number of bovine embryos attaining subsequent stages in in vitro-derived embryos. This observation suggests Ets-1 plays a role in normal early embryonic development in the bovine. Ets-1 AS treatment reduced PA production in Day 7 bovine embryos but had no deleterious effects on development. These data suggest Ets-1 is likely a regulator of embryonic PA production but is not associated with development through the blastocyst stages and hatching.

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