To the Graduate Council:

I am submitting herewith a dissertation written by Jules Joseph E. Doré, Jr. entitled "Changes in mRNA expression of ovine transforming growth factor-ß isoforms in late estrous cycle and early gestational endometrium and early gestation embryonic membranes". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

James D. Godkin, Major Professor

J. Erby Wilkinson, Major Professor

We have read this dissertation
and recommend its acceptance:

George A. Baumbach

James P. Chen

Accepted for the Council:

C. W. Minkel
Associate Vice Chancellor
and Dean of The Graduate School.

# CHANGES IN mRNA EXPRESSION OF OVINE TRANSFORMING GROWTH FACTOR-ß ISOFORMS IN LATE ESTROUS CYCLE AND EARLY GESTATIONAL ENDOMETRIUM AND EARLY GESTATION EMBRYONIC MEMBRANES.

A DISSERTATION

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Jules Joseph Eugene Doré, Jr.

August 1993

**ACKNOWLEDGEMENTS**

I would like to express my heartfelt thanks to both my Supervisors, Dr. Jim Godkin and Dr. Erby Wilkinson for their faith in me and guidance throughout my doctoral program. Thanks are also due to Dr. George Baumbach, you are wholly responsible for my coming to Tennessee to continue my education. I would also like to express my appreciation to Dr. James Chen for his constructive input as a member of my graduate committee.

I would like to extend my gratitude to my fellow graduate students, though some have moved on as Post-Doctoral fellows, Scott E. Smith (recent PhD), Dr. Kaung (Arthur) H. Liu and Weirong Shang all helped uncounted times with surgery and animal handling.

Kudos are also due to my parents Jules and Geraldine Doré for their seemingly infinite patience and frequent encouragement to strive for my best effort. Thanks to Teresa Herring and Nikki Bell for their unfailing humor and clerical assistance. Drs. J. Grizzel and T. Rowles also deserve honorable mention for keeping life from getting to sedate.

**ABSTRACT**

Utilizing cDNA clones of bovine transforming growth factor-ß1, ß2 and ß3, (TGF-ß) three DNA probes were constructed so as to be isotype specific. Total RNA was isolated from ovine endometrium from days 13 and 16 of the estrous cycle and days 13, 16, 23, 27 and 30 of gestation. RNA was also isolated from day 13 and 16 expanding blastocysts and days 23, 27 and 30 chorion and allantoic membranes. Using Northern blot methodology the sizes of each TGF-ß isoforms transcripts was determined. In both embryonic and endometrial tissues the transcript sizes were similar for each isoform. Transcript sizes for TGF-ß1 was 2.6 kb in length, the TGF-ß2 five transcripts lengths of 6.2, 5.2, 4.7, 4.2 and 2.7 kb and TGF-ß3 transcript of 3.8 kb in length. Using slot blot methodology, changes in relative mRNA expression levels of each isoform were determined in both endometrial and embryonic tissues. All three TGF-ß isoforms were found in the endometrium at all days examined, regardless of status. In embryonic tissues only levels of TGF-ß1 and ß2 were described, since TGF-ß3 levels were at or below detection limits. TGF-ß1 was primarily expressed by the developing allantois, with both placental membranes expressing TGF-ß2. Temporal changes in both maternal and conceptus TGF-ß expression corresponds to major morphological changes in the developing placenta, suggesting a link to placental development, as well as endometrial modifications during the late estrous cycle.

TABLE OF CONTENTS

## LIST OF FIGURES

Figure Page

**PART 2:**

**EARLY GESTATIONAL EXPRESSION OF TRANSFORMING GROWTH FACTOR BETA ISOFORMS BY THE OVINE PLACENTA**

Figure Page

**PART 3:**

**CHANGES IN OVINE ENDOMETRIAL mRNA EXPRESSION OF TRANSFORMING GROWTH FACTOR BETA ISOFORMS DURING THE PERI-IMPLANTATION PERIOD**

# PART 1: INTRODUCTION AND LITERATURE REVIEW

# CHAPTER 1

## INTRODUCTION

Infertility in domestic ruminants have major economic impacts and are a primary source of production inefficiency. The basis of infertility problems is multifunctional, but can be separated into failure of fertilization, failure of embryo signalling, failure of implantation, early embryo expulsion and birth of young with poor viability. While numerous studies have examined the endocrine and morphological changes during early embryonic development (Wimsatt, 1975; King et al., 1982; Bazer and First, 1983; Thatcher et al., 1986; Gluckman, 1986) and mechanisms of maternal recognition of pregnancy (Godkin et al., 1982, 1984; Lifsey et al., 1989; Roberts, 1989; Bazer et al., 1991), recent studies have begun to examine the role of endogenous growth and differentiation factors in the growth of the preimplantation and early implantation embryo, development of the extraembryonic membranes and modifications of the endometrium during early pregnancy (Heine et al., 1987; Huet-Hudson et al., 1990; Simmen et al., 1990; Larson et al., 1992).

As summarized by Gluckman et al. (1986) "little is known of the regulation of placental growth" despite the importance of the placenta in fetal development. With the exception of fertilization, the placenta plays a key role in all aspects of development. Complex embryogenesis and rapid fetal growth rely on an adequate placenta. Although the placenta is

recognized as the critical "link" of pregnancy, virtually nothing is known about the specific factors which control development of this essential organ or the molecular and cellular interactions occurring between the fetus, the placenta and the uterus.

The overall purpose of this project was to describe the relative transcription levels of the three common forms of transforming growth factor beta (TGF-ß), TGF-ß1, ß2 and ß3 during early placental formation in sheep. Utilizing isoform specific probes, the relative quantity of messenger ribonucleic acid (mRNA) for each TGF-ß was determined in endometrium and conceptus membranes. The period studied spanned the time of initiation of maternal recognition of pregnancy and initiation of blastocyst elongation (day 13) through initial interdigitation of the chorionic villi to form the placentome (day 30).

The objectives of this study were to:

1. Identify the mRNA transcripts for each isoform of TGF-ß and determine their size(s) by Northern blot analysis,
2. Determine the distribution of each TGF-ß isoform expression, with respect to maternal or fetal expression, as well as relative expression levels between the chorion and allantois,
3. Determine and quantify any differences in endometrial TGF-ß mRNA expression levels between

tissues of the same day from late estrous cycle and early pregnancy using slot blot methodology, and

4. Identify any changes in early conceptus and extraembryonic membrane TGF-ß isoform mRNA expression levels during blastocyst expansion, fusion of the chorion and allantois and placentome formation.

## CHAPTER 2

## LITERATURE REVIEW

Among the many factors that appear to be important in early gestation are the autocrine and paracrine growth factors. These may function to promote the remarkable growth and development of the preimplantation embryo, alter the morphology and physiology of the endometrium and promote the growth and neovascularization of the uterus. Currently little is known about the nature, source, developmental expression and function of such endogenous embryonic growth factors. Localization of TGF-ß production in the mouse and human placenta has indicated tissue and temporal specific expression of each TGF-ß (Schmid et al., 1991; Tamada et al., 1991; Das et al., 1992; Graham et al., 1992). Studies done previously in our laboratories have shown that TGF-ß1 and ß2 are expressed by bovine and ovine endometrium and extraembryonic membranes (Doré et al., 1991; Gao et al., 1992; Doré, Gao and Godkin, unpublished data). It is unlikely that TGF-ßs are the only growth factor involved in the complex processes of embryonic, placental and uterine development during pregnancy. Numerous growth factors are known to act in synergistic, complimentary and/or competing ways to modulate cellular growth and differentiation and tissue induction and morphogenesis (Knöchel and Tiedemann, 1989; Paria and Dey, 1990; Munson et al., 1992). As described previously for the insulin-like growth factors (Mondschein et

al., 1988, 1989), TGF-ßs have a bipotential role that depends in part on the simultaneous presence of other growth and differentiation factors (Assoian et al., 1984; von Zoelen et al., 1986; Kimelman and Kirschner, 1987; Torti et al., 1989; Sporn and Roberts, 1991).

*Early Ovine Embryonic Development*

After fertilization the embryo spends 3-4 days in the oviduct before entering the uterus. During this time, cleavage occurs and the embryo develops to the morula stage (Rowson and Moor, 1966). From days 6 to 9 the zona pellucida fragments (Rowson and Moor, 1966) and the blastocyst, composed of an inner cell mass and a single layer of trophectoderm, is said to have "hatched". Extraembryonic endoderm then progressively extends from the inner cell mass to line the inner surface of the trophectoderm. Mesoderm then grows between the trophectoderm and the endoderm, again extending from the inner cell mass. The mesoderm and trophectoderm comprise the presumptive chorion, while mesoderm and endoderm comprise the presumptive yolk sac. The yolk sac forms the primordial circulatory system and by day 25 regresses to a vestigial remnant (Assheton, 1906).

On days 8-9, the blastocyst is spherical and approximately 0.4 mm in diameter. By days 12-13 the blastocyst begins to elongate (1 x 3.3 mm). Over the next 5 days the blastocyst undergoes tremendous growth, attaining a

length of 1 x 68 mm on day 14 and 1 x 150 mm on day 16. By day 17, the blastocyst fills the gravid horn and extends well into the contralateral horn (Assheton, 1906; Green and Winters, 1945).

Contact between the endometrium and trophectoderm is initiated on days 13-14 as a tenuous interdigitation of trophectoderm membrane microvilli with caruncular epithelium (Boshier, 1969; Guillomot et al., 1981). This microvillus interdigitation becomes more prominent by day 16 (Guillomot et al., 1981). Interdigitation of intercaruncular epithelium and trophectoderm has also been described and may function to anchor the expanding blastocyst (Steven et al., 1981; Guillomot et al., 1981).

During the fourth week of gestation (days 21-28) the chorion apposing the caruncular endometrium forms a series of parallel folds (Wimsatt, 1950; Boshier, 1969). From the apex of these folds, primary villi extend into the caruncle. By day 42 of gestation, rudimentary placentomes can be seen in the vicinity of the fetus complete with chorionic villi and crypt structures (Steven et al., 1981). Over the next 30 to 40 days the chorionic villi and caruncular endometrium form the complex, interdigitating mature placentome (Assheton, 1906; Barcroft and Barron, 1946). This remarkable metamorphosis from a simple spherical blastocyst to the complex structure of villous cotyledonary and intercotyledonary chorion would require extensive cellular

proliferation and differentiation.

Differentiation of some trophoblastic cells into binucleate cells, which have both endocrine and migratory capabilities occurs throughout gestation (Wimsatt, 1951; King et al., 1982). The appearance of giant cells is first seen around day 16 of gestation (Boshier, 1969). These giant cells do not participate in the initial microvillus attachment, but may be involved in anchoring the placenta later as they migrate to the uterine epithelium (Wimsatt, 1951; King et al., 1981, 1982). Within the caruncle, the binucleate cells are interspersed with epithelial syncytium. However, the "principle" or mononucleate cells of the chorion form the main microvillous attachments to the syncytial cells (Steven et al., 1981). The proportion of mononucleate to multinucleate cells in the cotyledonary chorion are similar to that in the intercotyledonary area. Differences in the intercotyledonary area include a loose microvillus attachment of principle cells to the uterine epithelium and elaborations of apical surface of the chorionic cells apposing the opening of uterine glands, referred to as areolae (Wimsatt,1950; Steven et al., 1981).

The allantois, an outgrowth of the hind gut, consists of an inner layer of endoderm and an outer layer of vascularized mesoderm. Allantois is detectable at approximately day 17 of gestation (Assheton, 1906; Fléchon, 1978) and by day 23 is a prominent structure, filling most of the extraembryonic coelome (Assheton, 1906). Between days 17 and 25 the

allantois undergoes a dramatic expansion with progressive attachment to the chorion, beginning at the embryo and radiating outward (Assheton, 1906; Steven et al., 1981). By day 25 the allantois has filled the extraembryonic coelome, except for the necrotic tips, and has established extensive contact with the chorion forming the chorioallantois (Assheton, 1906).

The remarkable growth and development of the preimplantation embryo and fetal membranes likely requires both endogenous growth factors, as well as growth factors obtained from uterine secretions. Treating *in vitro* fertilized embryos with growth factors in defined medium suggests that certain exogenously supplied growth factors may be necessary for development of the blastocyst *in vivo* (Larson et al., 1992). This is further supported by the ability to culture bovine embryos through the 8-cell block by adding medium conditioned by oviduct cells (Eyestone and First, 1989; Ellington et al., 1990). Detection of mRNA of several growth factors and their receptors in the early developing mouse (Rappolee et al., 1988, 1989) and bovine (Watson et al., 1992) embryo has indicated that these transcripts are present. The necessity of maternal growth factors suggests that transcripts for the growth factors are available but may not be utilized.

The major processes involved in establishing an adequate placenta and the formation of placentomes (cellular proliferation and differentiation) are the two functions best

demonstrated by the transforming growth factor beta family of polypeptides (TGF-ß; Massagué, 1990; Jessell and Melton, 1992; Sporn and Roberts, 1991). The possible role of the TGF-ßs in early embryonic growth and placental development is the focus of the proposed studies.

*Uterine Changes in Early Pregnancy*

Communication between the developing conceptus and maternal tissues initiates prior to implantation, continues throughout pregnancy and terminates at parturition. Prior to attachment of the conceptus, there is increased edema, increased vascularization and glandular hypertrophy of the uterus (Amoroso,1952). While considerable work has been done on the endocrinology of the fetal-maternal interaction (Bazer and First, 1983; Thatcher et al., 1986; Gluckman, 1986), little is known about the nature of the signals that initiate cell proliferation, tissue differentiation and neovascularization of the pregnant uterus (Lingham et al., 1988; Huet-Hudson et al., 1990; Geisert et al., 1991). Uterine secretions (histotroph) are thought to be important in the growth and development of the early conceptus (Amoroso, 1952) but direct evidence for this is lacking.

Prior to day 13, no detectable difference between the pregnant and nonpregnant endometrium has been identified (Guillomot et al., 1981; Smith et al., 1990). During day 14, the pregnant caruncular surface appears folded and concave.

Nonpregnant caruncles did not show these morphological changes. There was no detected difference up to day 16 in the intercaruncular areas between pregnant and nonpregnant endometrium. Day 16 pregnant endometrium is lined by uniformly columnar epithelium (King et al., 1982). By day 18 the endometrial epithelium has a flat cuboidal appearance and demonstrates areas of syncytial formation and degeneration, with the presence of chorionic binucleate and giant cells. Over the next six days extensive syncytial transformation and loss of uterine epithelium was noted to occur in caruncular areas (King et al., 1982; Wooding, 1984). Reports of syncytial formation in the intercaruncular epithelium are contradictory (Lawn et al, 1969; King et al., 1982; Wooding, 1984). The cellular origin of the syncytium is unclear. Binucleate cell migration and their subsequent fusion to the maternal endometrium has been suggested as the source of the syncytium (Wooding, 1984). Rather than a fetal-maternal hybrid, King et al. (1981) suggest the syncytium is formed from fusion of migrating chorionic binucleate cells and the uterine epithelium degenerates providing space for the migrating cells, while Steven et al. (1981) discuss a uterine epithelial syncytium. From the syncytium, projections into the basal surface extend through the uterine epithelial basement membrane (Lawn et al., 1969) by day 42 of pregnancy. At this time the caruncle contains rudimentary crypts (Steven et al., 1981). Crypt and chorionic villi growth and branching

continues until approximately 90 days of gestation when formation of the mature placentome is complete (Assheton, 1906; Barcroft and Barron, 1946). During this time the intercaruncular endometrium appears to maintain its columnar appearance and intercellular junctions (Lawn et al, 1969). Long microvilli maintain a fragile contact with the chorion. Unlike the caruncular capillary endothelium, which thickens markedly, the intercaruncular capillaries have a thin, fenestrated appearance to their endothelium (Lawn et al., 1969).

*TGF-ß Regulation*

Transforming growth factor beta was the name originally given to an activity which supported the transient induction of a transformed phenotype in normal indicator cells (DeLarco and Todaro, 1978; Anzano et al., 1982). This phenotype, colony formation in soft agar, demonstrates anchorage independent growth. Since then a group of structurally similar proteins have been described as the TGF-ß superfamily (Reviewed by Massagué, 1990). Included in this superfamily are TGF-ßs (ß1-5), activins and inhibins, Müllerian inhibiting substance, as well as bone morphogenic proteins (BMP1-7) and their close relatives the Vegetal-related/decapeptapelegic proteins (Vgr/DPP). Although the receptors for each of these ligands has not been defined, the recent cloning of the TGF-ß and activin type II receptors suggests that a common mechanism

of signal transduction may be utilized by this superfamily of proteins. Expression cloning of the type II receptors for TGF-ß and activin produced the first two members of a new family of serine/threonine kinase transmembrane receptors (Mathews and Vale, 1991; Attisano et al., 1992; Lin et al., 1992).

In mammals the most common forms of TGF-ß are TGF-ß1, ß2 and ß3. One or more of these isoforms appears to be secreted by virtually all cells as a biologically inactive precursor form (Derynck et al., 1986; Moses et al., 1987; Miyazono et al., 1988). There are reports of some breast cancer cell lines secreting active TGF-ß (Knabbe et al., 1987), however, it is more likely that these cells secrete the precursor form, as well as an activating factor. *In vitro* activation of the latent forms of TGF-ß can be accomplished by transient acidification or proteolytic cleavage by serine proteases, such as plasmin and cathepsin D (Lawrence et al., 1985; Olridge and D'Amore, 1987; Lyons et al., 1988). *In vivo* activation may also utilize plasmin cleavage in some instances (Sato and Rifkin, 1989; Antonelli-Olridge et al., 1989), while binding to the IGF-II/mannose-6-phosphate receptor and cycling through the acidic endosome may be utilized in other instances (Purchio et al., 1988; Kovacina et al., 1989; Dennis and Rifkin, 1991).

Since most mammalian cells secrete one or more isoforms of TGF-ß and TGF-ß receptors are likewise ubiquitously

expressed (Wakefield et al., 1987), strict regulation of production and/or activation is necessary. The regulation of TGF-ß production is poorly understood. In mammary tumor cells, antiestrogens stimulate secretion of TGF-ß (Knabbe et al., 1987), while *in vivo* treatment of ovariectomized mice with estrogen causes a transient (within 12 hours post injection) increase in uterine TGF-ß2, but not ß3, mRNA levels (Das et al., 1992). This apparent discrepancy in regulation, within two steroid responsive tissues, may be related to differences in tissues or that mammary tumor epithelial cell lines may use altered signal transduction pathways not normally found *in vivo*.

Other modulators of TGF-ß production include the TGF-ßs themselves, retinoic acid and extracellular matrix proteins. The TGF-ß1 isoform has been found to be autoinductive and feedback to stimulate its own production (van Obberghen-Schelling et al., 1988). Glick et al. (1989) demonstrated that retinoic acid stimulates keratinocytes to dramatically increase TGF-ß2, but not TGF-ß1 secretion. These results may reflect the later finding that cells grown on plastic constitutively express high levels of TGF-ß1, while TGF-ß2 expression is limited by other factors (Streuli et al., 1993). However, when cells are grown on an appropriate extracellular matrix (ECM), TGF-ß1 production is suppressed. Extracellular matrix proteins have been shown to bind latent TGF-ßs, thus blocking biological activity (Paralkar et al., 1991), as well

as downregulating TGF-ß1 transcription (Streuli et al., 1993). The mechanism by which the ECM alters gene expression through effects on the cytoskeleton is unknown.

To facilitate identification of potential regulators of TGF-ß expression, investigators have cloned the 5' flanking promoter regions of all three TGF-ß isoforms (Kim et al., 1989a; Malipiero et al., 1990; Lafyatis et al., 1990). There are both similarities and extensive differences in the promoter regions of the three isoforms of TGF-ß. In contrast to the extremely high conservation within the coding regions of the mature proteins of the three isoforms (Massagué, 1990; Sporn and Roberts, 1990), the respective promoter regions show vast divergence. The promoter region of TGF-ß1 and ß2 were found to contain two distinct transcriptional start sites, compared to a single site for ß3 (Kim et al., 1989a; Lafyatis et al., 1990; O'Reilly et al., 1992). Although a single 2.7 kb transcript has been identified for TGF-ß1, five distinct transcripts have been identified for human TGF-ß2 which are 5.8, 5.1, 4.0, 3.8 and 2.8 kb in length (O'Reilly et al., 1992). Likewise, five transcripts of slightly different sizes have been described for murine TGF-ß2 (Miller et al., 1989). The 5.8, 4.0 and 3.8 kb transcripts are products of the 5' most initiation site (O'Reilly et al., 1992). All three promoters contained AP-2 binding sites, however only TGF-ß1 and ß2 contain AP-1 sites for the Fos/Jun oncogenes (Kim et al., 1989a; Lafyatis et al., 1990; Malipiero et al., 1990).

The AP-1 sites in the TGF-ß1 promoter region have been implicated in activating transcription of the TGF-ß1 gene by phorbol esters or TGF-ß1 autoinduction (Kim et al. 1989b, 1989c, 1990). The retinoblastoma gene product (Rb-1) activates ß1 through an Rb-1 specific element and TGF-ß2 through an ATF-2 binding sequence (Kim et al., 1992). Both TGF-ß2 and ß3 have cAMP response elements, however only TGF-ß3 appears to be cAMP responsive (Malipiero et al., 1990; Lafyatis et al., 1990). Both ß2 and ß3 also have a motif common to the cytokine gene promoters, named CK-1, which is absent in the TGF-ß1 promoter (Malipiero et al., 1990). The finding of a complex group of enhancer and silencer regions within each promoter has identified common and divergent molecular mechanisms by which each TGF-ß may be controlled in the different tissues. None of the reviewed literature reports the presence of any of the steroid response elements. Despite finding specific binding sites within the promoter regions of the TGF-ß genes, it is still unclear what the actual external ques are or how they are utilized in the body for appropriate TGF-ß expression.

*TGF-ß Physiology*

The actions of TGF-ßs are contradictory at times and involve a variety of cell types. As a general rule it is thought that TGF-ßs stimulate mitosis of cells of mesenchymal origin and inhibit mitosis in all others (Reviewed by Sporn

and Roberts, 1990; Massagué, 1990). Although the mechanism of TGF-ß actions on gene regulation is clouded with many unknowns, there are clues indicating how TGF-ßs may be able to control cellular functions. Both epithelial and mesenchymal cells have been shown to express the c-sis oncogene mRNA (platelet-derived growth factor-B) when treated with TGF-ß (Loef et al., 1986; Gerwin et al., 1987; Bronzert et al., 1990). TGF-ß has also been shown to stimulate expression of the EGF receptor (Assoian et al., 1984; Valverius et al., 1989). It has been speculated that through this indirect mechanism of stimulating growth factors and/or their receptors, TGF-ßs regulate cellular hyperplasia (Massagué, 1990).

Other possible methods TGF-ßs may use to control cell replication and differentiation are demonstrated by their effects on myoblasts and rat kidney cells (Massagué,1990). TGF-ß appears to inhibit myoblast differentiation by two distinct mechanisms. TGF-ß1 causes myoblast cultures to reduce transcription of myogenin and MyoD1 genes (Vaija et al., 1989; Heino and Massagué, 1990). Both of these gene products are necessary for myoblast differentiation. In contrast, Fos and Jun proto-oncogene transcription is increased, promoting myoblast replication which is likewise incompatible with myoblast differentiation (Lassar et al., 1989; Massagué, 1990). TGF-ß also may act by increasing synthesis and deposition of type I collagen. Collagen rich

matrices have been found to inhibit L6 myoblast differentiation, despite elevated levels of myogenin mRNA (Heino and Massagué, 1990). The effects of TGF-ß on NRK rat kidney cells demonstrates the effects of matrix on cell replication. In monolayer cultures TGF-ß treatment inhibits cell replication (Nugent and Newman, 1989). However, cells treated with TGF-ß and serum replicate in soft agar (DeLarco and Todaro, 1978; Moses et al., 1981; Anzano et al., 1982). Through altering transcription of genes necessary for differentiation and/or modifying the extracellular matrix, TGF-ßs appear to regulate cellular responsiveness to other factors present in the extracellular environment.

The functions of TGF-ßs in early germ layer induction and axis formation have been described only in lower vertebrate embryos (Kimelman and Kirschner, 1987; Weeks and Melton, 1987; Jessel and Melton, 1992). In the *Xenopus* blastocyst, TGF-ß2 but not ß1, has been shown to stimulate mesoderm formation (Rosa et al., 1988). However, it is likely that the more potent mesoderm inductive agents of the TGF-ß superfamily, the activins and Vgr proteins, are the physiological regulators. In higher vertebrates, TGF-ß1 and ß2 mRNA have been identified in early mouse (Rappolee et al, 1988) and bovine embryos (Watson et al., 1992), respectively. Despite the presence of these mRNA transcripts in early embryos, exogenous TGF-ßs are required by the bovine embryo to overcome the 8-cell blockage during *in vitro* culture (Larson et al, 1992). *In vivo* the

source of the TGF-ß would presumably be histotroph produced by the endometrium.

The roles of TGF-ßs in embryogenesis have been deduced mainly by association. Areas of intense development and morphogenesis express high levels of TGF-ßs (Pelton et al., 1989, 1991; Gatherer et al., 1990; Schmid et al., 1991). Immunolocalization and *in situ* hybridization studies have demonstrated that areas of synthesis are not always areas of protein localization (Schmid et al., 1991; Pelton et al., 1991), suggesting TGF-ßs act in an autocrine, paracrine and/or endocrine manner. Localization studies have also shown that each isoform has distinct but overlapping tissue distributions (Gatherer et al., 1990; Schmid et al., 1991; Pelton et al., 1991), indicating each TGF-ß has a distinct function in organogenesis, but there is biological redundancy in TGF-ß functions. This is best demonstrated by the TGF-ß1 "knock-out" mouse (Shull et al., 1992). The lack of TGF-ß1 was not lethal to 75% of the embryos during development. However, the complete neonate lethality observed illustrates that TGF-ß1 serves an indispensable physiological role postnatally.

Studies describing cellular localization of the TGF-ß proteins and mRNA transcripts and their potential roles during embryonic development are expansive in the literature. However, few studies have examined the role of TGF-ßs in placental development despite the absolute requirement of an adequate placenta for pregnancy to proceed (Pelton et al.,

1989; Stickland and Richards, 1992; Graham et al., 1992). Reports on the contribution of TGF-ß regulation of morphological changes in the uterus during the estrous cycle and early pregnancy are equally scarce (Tamada et al., 1990; Das et al., 1992). Information on the possible contribution of TGF-ßs to uterine glandular hypertrophy, neovascularization and endometrial remodelling at the end of an estrous or menstrual cycle is nonexistent. The work described in this dissertation was undertaken to provide information regarding the relationship between TGF-ßs and fetal-maternal interactions involved in placental development.

## LIST OF REFERENCES


Amoroso, E.C. (1952). Placentation. In: *Marshall's Physiology of Reproduction, 3rd ed. vol. 2,* (edited by A.S. Parkes) Longman, Green and Co., London, 127-311.

Antonelli-Olridge, A., Saunders, K.B., Smith, S.R. and D'Amore, P.A. (1989). An activated form of transforming growth factor-ß is produced by cocultures of endothelial cells and pericytes. Proc. Natl. Acad. Sci. USA 86, 4544-4588.

Anzano, M.A., Roberts, A.B., Meyers, C.A., Komoriya, A., Lamb, L.C., Smith, J.M. and Sporn, M.B. (1982). Synergistic interaction of two classes of transforming growth factors from murine sarcoma cells. Cancer Res. 42, 4776-4778.

Assheton, R. (1906). The morphology of the ungulate placenta, particularly the development of that organ in the sheep, and notes upon the placenta of the elephant and Hyrax. Phil. Trans. Royal Soc. London 198 (Series B), 143-225.

Assoian, R.K., Frolik, C.A., Roberts, A.B., Miller, D.M. and Sporn, M.B. (1984). Transforming growth factor-ß controls receptor levels for epidermal growth factor in NRK fibroblasts. Cell 36, 35-41.

Attisano, L., Wrana, J.L., Chieifetz, S. and Massagué, J. (1992). Novel activin receptors: distinct genes and

alternative mRNA splicing generate a repertoire of serine/threonine receptors. Cell 68, 97-108.

Barcroft, J. and Barron, D.H. (1946). Observations upon the form and relations of the maternal and fetal vessels in the placenta of sheep. Anat. Rec. 94, 569-595.

Bazer, F.W. and First, N.L. (1983). Pregnancy and parturition. J. Anim. Sci. 57, 425-460.

Bazer, F.W., Thatcher, W.W., Hansen, P.J., Mirando, M.A., Ott, T.L. and Plante, C. (1991) Physiological mechanisms of pregnancy recognition in ruminants. J. Reprod. Fert. 43 (suppl.), 39-47.

Boshier D.P. (1969). A histological and histochemical examination of implantation and early placentome formation in sheep. J. Reprod. Fert. 19, 51-61.

Bronzert, D.A., Bates, S.E., Sheridan, J.P., Lindsey, R., Valverius, E.M., Stampfer, M.R., Lippman, M.E. and Dickson, R.B. (1990). Transforming growth factor-ß induces platelet-derived growth factor (PDGF) messenger RNA and PDGF secretion while inhibiting growth in normal human mammary epithelial cells. Mol. Endocrinol. 4, 981-989.

Das, S.K., Flanders, K.C., Andrews, G.K. and Dey S.K. (1992). Expression of transforming growth factor-ß isoforms (ß2 and ß3) in the mouse uterus: analysis of the peri-implantation period and effects of ovarian steroids. Endocrinol. 130, 3459-3466.

DeLarco, J. and Todaro, G. (1978). Growth factors from murine sarcoma virus-transformed cells. Proc. Natl. Acad. Sci. USA 75, 4001-4005.

Dennis, P.A. and Rifkin, D.B. (1991). Cellular activation of latent transforming growth factor ß requires binding to the cation-independent mannose-6-phosphate/insulin-like growth factor type II receptor. Proc. Natl. Acad. Sci. USA 88, 580-584.

Derynck, R., Jarrett, J.A., Chen, E.Y. and Goeddel, D.V. (1986). The murine transforming growth factor-ß precursor. J. Biol. Chem. 261, 4377-4379.

Doré, J.J.E. Jr, Gao, K., Bouley, D.M., Wilkinson J.E. and Godkin, J.D. (1991). Immunohistochemical localization of TGF-α and TGF-ß1 in bovine endometrium. Biol. Reprod. 44 (suppl.1), 97 (Abst. 180).

Ellington, J.E., Carney, E.W. Farrell, P.B., Simkin, M.E. and Foote, R.H. (1990). Bovine 1-2-cell embryo development using a simple medium in three oviduct epithelial cell coculture systems. Biol. Reprod. 43, 97-104.

Eyestone, W.H. and First, N.L. (1989). Co-culture of early cattle embryos to the blastocyst stage with oviductal tissue or in conditioned medium . J. Reprod. Fert. 85, 715-720.

Fléchon, J.E. (1978). Morphological aspects of embryonic disc at the time of its appearance in the blastocyst of

farm mammals. Scanning Electron Microscopy 2, 541-547.

Gao, K., Doré, J.J.E. Jr., Wilkinson, J.E. and Godkin, J.D. (1991). PAP immunostaining of TGF-α and TGF-ß1 in the sheep uterine endometrium using PEG sections transferred onto Digene silicanated slides. J. Cell Biol. 115, 268a (Abst. 1551).

Gatherer, D., ten Dijke, P., Baird, D.T. and Akhurst, R.J. (1990). Expression of TGF-ß isoforms during first trimester human embryogenesis. Development 110,445-460.

Geisert, R.D., Lee, C-Y., Simmen, F.A., Zavy, M.T., Fliss, A.E., Bazer, F.W. and Simmen, R.C.M. (1991). Expression of messenger RNAs encoding insulin-like growth factor-I, II and insulin-like growth factor binding protein-2 in bovine endometrium during the estrous cycle and early pregnancy. Biol. Reprod. 45, 975-983.

Gerwin, B.I., Lechner, J.F., Reddel, R.R., Roberts, A.B., Robbins, K.C., Gabrielson, E.W. and Harris, C.C. (1987). Comparison of production of transforming growth factor-ß and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. Cancer Res. 47, 6180-6184.

Glick, A.B., Flanders, K.C., Danielpour, D., Yuspa, S.H. and Sporn, M.B. (1989). Retinoic acid induces transforming growth factor-ß2 in cultured keratinocytes and mouse epidermis. Cell Regul. 1, 87-97.

Gluckman P.D. (1986). The role of pituitary hormones, growth

factors and insulin in the regulation of fetal growth. In: *Oxford Reviews of Reproductive Biology*. Edited by J.R. Clark, Oxford, Clarendon Press, 1-59.

Godkin, J.D., Bazer, F.W., Moffat, J., Sessions, F. and Roberts, R.M. (1982). Purification and properties of a major low molecular weight protein released by the trophoblast of sheep blastocysts at day 13-21. J. Reprod. Fert. 65, 141-150.

Godkin, J.D., Bazer, F.W. and Roberts, R.M. (1984). Ovine trophoblast protein 1, an early secreted blastocyst protein, binds to uterine endometrium and affects protein synthesis. Endocrinol. 114, 120-130.

Graham, C.H., Lysiak, J.J., McCrae, K.R. and Lala, P.K. (1992). Localization of transforming growth factor-ß at the human fetal-maternal interface: role in trophoblast growth and differentiation. Biol. Reprod. 46, 561-572.

Green, W. and Winters, M. (1945). Prenatal development of the sheep. Tech. Bull., Univ. Minnesota, Agric. Exper. Stat., 169, 1-35.

Guillomot, M., Fléchon, J.E. and Wintenberger-Torres, S. (1981). Conceptus attachment in the ewe: an ultrastructural study. Placenta 2, 169-182.

Heine, U.I., Munoz, E.F., Flanders, K.C., Ellingsworth, L.R., Lam, H-Y.P., Thompson, N.L., Roberts, A.B. and Sporn, M.B. (1987). Role of transforming growth factor-ß

in the development of the mouse embryo. J. Biol. Chem. 105, 2861-2876.

Heino, J. and Massagué (1990). Cell adhesion to collagen and decreased myogenic gene expression implicated in the control of myogenesis by TGF-ß. J. Biol. Chem. 265, 10181-10184.

Huet-Hudson, Y.M., Chakraborty, C., De, S.K., Suzuki, Y., Andrews, G.K. and Dey, S.K. (1990). Estrogen regulates the synthesis of EGF in mouse uterine epithelial cells. Mol. Endocrinol. 4, 510-523.

Jessel, T.M. and Melton, D.A. (1992). Diffusible factors in vertebrate embryonic induction. Cell 68, 257-270.

Kim, S-J., Glick, A., Sporn M.B. and Roberts, A.B. (1989a). Characterization of the promoter region of the human transforming growth factor-ß1 gene. J. Biol. Chem. 264, 402-408.

Kim, S-J., Jeang, K-T., Glick, A.B., Sporn, M.B. and Roberts, A.B. (1989b). Promoter sequences of the human transforming growth factor-ß1 gene responsive to transforming growth factor-ß1 autoinduction. J. Biol. Chem. 264, 7041-7045.

Kim, S-J., Denhez, F., Kim, K.Y., Holt, J.T., Sporn, M.B. and Robert, A.B. (1989c). Activation of the second promoter of the transforming growth factor-ß 1 gene by transforming growth factor-ß1 and phorbol ester occurs

through the same target sequences. J. Biol. Chem. 264, 19373-19378.

Kim, S-J., Angel, P., Lafyatis, R., Hattori, K., Kim, K.Y., Sporn, M.B., Karin, M. and Roberts, A.B. (1990). Autoinduction of TGF-ß1 is mediated by the AP-1 complex. Mol. Cell. Biol. 10, 1492-1497.

Kim, S-J., Lee, H.D., Robbins, P.D., Busam, K., Sporn, M.B. and Roberts, A.B. (1991). Regulation of transforming growth factor-ß1 gene expression by the products of the retinoblastoma-susceptibility gene. Proc. Natl. Acad. Sci. USA 88, 3052-3056.

Kim, S-J., Wagner, S., Liu, F., O'Reilly, M.A., Robbins, P.D. and Green, M.R. (1992). Retinoblastoma gene product activates expression of the human TGF-ß2 gene through transcription factor ATF-2. Nature 358, 331-334.

Kimelman, D. and Kirschner, M. (1987). Synergistic induction of mesoderm by FGF and TGF-ß and the identification of an mRNA coding for FGF in the early Xenopus embryo. Cell 51, 869-877.

King, G.J., Atkinson, B.A. and Robertson, H.A. (1981). Development of the intercaruncular areas during early gestation and establishment of the bovine placenta. J. Reprod. Fert. 61, 469-474.

King, G.J., Atkinson, B.A. and Robertson, H.A. (1982). Implantation and early placentation in domestic ungulates. J. Reprod. Fert. (suppl.31), 17-30.

Knabbe, C., Lippman, M.E., Wakefield, L.M., Flanders, K.C., Kasid, A., Derynck, R. and Dickson, R.B. (1987). Evidence that transforming growth factor-ß is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48, 417-428.

Knöchel, W. and Tiedemann, H., (1989). Embryonic inducers, growth factors, transcription factors and oncogenes. Cell Differ. Devel. 26, 163-171.

Kovacina, K.S., Steele-Perkins, G., Purchio, A.F., Lioubin, M.N., Miyazono, K., Heldin, C-H. and Roth, R.A. (1989). Interactions of recombinant and platelet transforming growth factor-ß1 precursor with the insulin-like growth factor/mannose 6-phosphate receptor. Biochem. Biophys. Res. Commun. 160, 393-403.

Lafyatis, R., Lechleider, R., Kim, S-J., Jakowlew, S., Roberts, A.B. and Sporn, M.B. (1990). Structural and functional characterization of the transforming growth factor ß3 promoter. J. Biol. Chem. 265, 19128-19136.

Larson, R.C., Ignotz, G.G. and Currie, W.B. (1992). Transforming growth factor ß and fibroblast growth factor synergistically promote early bovine embryo development during the fourth cell cycle. Mol. Reprod. Dev. 33, 432-435.

Lassar, A.B., Thayer, M.J., Overell, R.W. and Weintraub, H. (1989). Transformation by activated ras or fos prevents

myogenesis by inhibiting expression of MyoD1. Cell 58, 659-667.

Lawn, A.M., Chiquoine, A.D. and Amoroso, E.C. (1969). The development of the placenta in the sheep and goat: an electron microscope study. J. Anat. 105, 557-578.

Lawrence, D., Pircher, R. and Jullien, P. (1985). Conversion of a high molecular weight latent beat-TGF from chicken embryo fibroblasts to a low molecular weight active beta-TGF under acidic conditions. Biochem. Biophys. Res. Commun. 133, 1026-1034.

Lifsey, B.J. Jr., Baumbach, G.A. and Godkin, J.D. (1989). Isolation, characterization and immunocytochemical localization of bovine trophoblast protein-1. Biol. Reprod. 40, 343-352.

Lin, H.Y., Wang, X-F., Ng-Eaton, E., Weinberg, R.A. and Lodish, H.F. (1992). Expression cloning of the TGF-ß type II receptor, a functional transmembrane serine/threonine kinase. Cell 68, 775-785.

Lingham, R.B. and Stancell, G.M. (1988). Estrogen induction of the epidermal growth factor receptor mRNA. Mol. Endocrinol. 2, 230-235.

Liu, K.H., Gao, K., Baumbach, G.A. and Godkin, J.D. (1992). Purification and immunolocalization of ovine placental retinol-binding protein. Biol. Reprod. 46, 23-29.

Liu, K.H., Doré, J.J.E. Jr., Roberts, M.P., Rajesh, K., Hopkins, F.M. and Godkin, J.D. (1993). Expression and

cellular localization of retinol-binding protein mRNA in bovine blastocysts and extraembryonic membranes. Biol. Reprod. 49, in press.

Loef, E.B., Proper, J.A., Goustin, A.S., Shipley, G.D., DiCorleto, P.E. and Moses, H.L. (1986). Induction of c-sis mRNA and activity similar to platelet-derived growth factor by transforming growth factor-ß: a proposed model for indirect mitogenesis involving autocrine activity. Proc. Natl. Acad. Sci. USA 83, 2453-2457.

Lyons, R.M., Keski-Oja, J. and Moses, H.L. (1988). Proteolytic activation of transforming growth factor-ß from fibroblast-conditioned medium. J. Cell Biol. 106, 1659-1665.

Malipiero, U., Höller, M., Werner, U. and Fontana, A. (1990). Sequence analysis of the promoter region of the glioblastoma derived cell suppressor factor/transforming growth factor (TGF)-ß2 gene reveals striking differences to the TGF-ß1 and -ß3 genes. Biochem. Biophys. Res. Commun. 171, 1145-1151.

Massagué, J. (1990). The transforming growth factor-ß family. Annu. Rev. Cell Biol. 6, 597-641.

Massagué, J. (1992). Receptors for the TGF-ß family. Cell 69, 1067-1070.

Mathews, L.S. and Vale, W.W. (1991). Expression cloning of an activin receptor, a predicted transmembrane serine kinase. Cell 65, 973-982.

Miller, D.A., Lee, A., Pelton, R.W., Chen, E.Y., Moses, H.L. and Derynck, R. (1989). Murine transforming growth factor-ß2 cDNA sequence and expression in adult tissues and embryos. Mol. Endocrinol. 3, 1108-1114.

Miyazono, K., Hellman, U., Wernstedt, C. and Heldin, C-H. (1988). Latent high molecular weight complex of transforming growth factor ß1. J. Biol. Chem. 263, 6407-6415.

Mondschein, J.S., Canning, S.F. and Hammond, J.M. (1988). Effects of transforming growth factor-beta on the production of immunoreactive insulin-like growth factor I and progesterone and $^{3}$H-thymidine incorporation in porcine granulosa cell cultures. Endocrinol. 123, 1970-1976.

Mondschein, J.S., Canning, S.F., Miller, D.Q. and Hammond, J.M. (1989). Insulin-like growth factors (IGFs) as autocrine/paracrine regulators of granulosa cell differentiation and growth: studies with a neutralizing monoclonal antibody to IGF-I. Biol. Reprod. 40, 79-85.

Moses, H.L., Branum, E.L., Proper, J.A. and Robinson, R.A. (1981). Transforming growth factor production by chemically transformed cells. Cancer Res. 41, 2842-2848.

Moses, H.L., Coffey, R.J. Jr., Leof, E.B., Lyons, R.M. and Keski-Oja, J. (1987). Transforming growth factor beta regulation of cell proliferation. J. Cell Physiol. (suppl. 5), 1-7.

Munson, L., Wilkinson, J.E. and Bechtel, M.K. (1992). Transforming growth factor-ß and platelet-derived growth factor are synergistic mitogens for bovine trophoblastic and endometrial epithelial cells. Biol. Reprod. 46 (suppl. 1), 67 (Abst.66).

Nugent, M.A. and Newman, M.J. (1989). Inhibition of normal rat kidney cell growth by transforming growth factor-ß is mediated by collagen. J. Biol. Chem. 264, 18060-18067.

Olridge, A. and D'Amore. P.A. (1987). Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. J. Cell Biol. 105, 1455-1462.

O'Reilly, M.A., Geiser, A.G., Kim, S-J., Bruggeman, L.A., Luu, A.X., Roberts, A.B. and Sporn, M.B. (1992). Identification of an activating transcription factor (ATF) binding site in the human transforming growth factor-ß2 promoter. J. Biol. Chem. 267, 19938-19943.

Paralkar, V.M., Vukicevic, S. and Reddi, A.H. (1991). Transforming growth factor ß type 1 binds to collagen IV of basement membrane matrix: Implications for development. Devel. Biol. 143, 303-308.

Paria, B.C. and Dey, S.K. (1990). Preimplantation embryo development *in vitro*: Comparative interaction among embryos and role of growth factors. Proc. Natl. Acad. Sci. USA 87, 4756-4760.

Pelton, R.W., Nomura, S., Moses, H.L. and Hogan, B.L.M. (1989). Expression of transforming growth factor ß2 RNA

during murine embryogenesis. Development, 106, 759-767.

Pelton, R.W., Saxena, B., Jones, M., Moses H.L. and Gold, L.I. (1991). Immunohistochemical localization of TGF-ß1, TGF-ß2 and TGF-ß3 in the mouse embryo: expression patterns suggest multiple roles during embryonic development. J. Cell Biol. 115, 1091-1105.

Purchio, A.F., Cooper, J.A., Brunner, A.M., Lioubin, M.N., Gentry, L.E., Kovacina, K.S., Roth, R.A. and Marquardt, H. (1988). Identification of mannose 6-phosphate in two asparagine-linked sugar chains on recombinant transforming growth factor-ß1 precursor. J. Biol. Chem. 263, 14211-14215.

Rappolee, D.A., Brenner, C.A., Schultz, R., Mark, D. and Werb, Z. (1988). Developmental expression of PDGF, TGF-α and TGF-ß genes in preimplantation mouse embryo. Science, 241, 1823-1825.

Rappolee, D.A., Wang, A., Mark, D. and Werb, Z. (1989). Novel method for studying mRNA phenotypes in single or small numbers of cells. J. Cell. Biochem. 39, 1-11.

Roberts, R.M. (1989). Minireview: Conceptus interferons and maternal recognition of pregnancy. Biol. Reprod. 40, 449-452.

Rosa, F., Roberts, A.B., Danielpour, D., Dart, L.L., Sporn, M.B. and Dawid, I.B. (1988). Mesoderm induction in amphibians: the role of TGF-ß2-like factors. Science 239, 783-785.

Rowson, L.E.A. and Moor, R.M. (1966). Development of the sheep conceptus during the first fourteen days. J. Anat. 100, 777-785.

Sato, Y. and Rifken, D.B. (1989). Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-ß1-like molecule by plasmin during co-culture. J. Cell Biol. 109, 309-315.

Schmid, P., Cox, D., Bilbe, G., Maier, R. and McMaster, G.K. (1991). Differential expression of TGF ß1, ß2 and ß3 genes during mouse embryogenesis. Development 111, 117-130.

Shull, M.M., Ormsby, I., Kier, A.B., Pawlowski, S., Diebold, R.J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., Annunziata, N. and Doetschman, T. (1992). Targeted disruption of the mouse transforming growth factor-ß1 gene results in multifocal inflammatory disease. Nature 359, 693-699.

Simmen, R.C.M., Simmen, F.A., Hofig, A., Farmer, S.J. and Bazer, F.W. (1990). Hormonal regulation of insulin-like growth factor gene expression in the pig uterus. Endocrinol. 127, 2166-2174.

Smith, S.E., Cullin, W.C. and Godkin, J.D. (1990). Ultrastructural morphometric analysis of the uterine epithelium during early pregnancy in the sheep. J. Reprod. Fert. 89, 517-525.

Sporn M.B. and Roberts, A.B. (1990). TGF-ß: problems and prospects. Cell Regul. 1, 875-882.

Sporn, M.B. and Roberts, A.B. (1991). Interactions of retinoids and transforming growth factor-ß in regulation of cell differentiation and proliferation. Mol. Endocrinol. 5, 3-7.

Steven, D.H., Burton, G.J. and Samuel, C.A. (1981). Histological and electron microscopy of sheep placental membranes. Placenta (suppl. 2), 11-34.

Streuli, C.H., Schmidhauser, C., Korbin, M., Bissell, M.J. and Derynck, R. (1993). Extracellular matrix regulates expression of the TGF-ß1 gene. J. Cell Biol. 120, 253-260.

Strickland, S. and Richards W.G. (1992). Invasion of the trophoblasts. Cell 71, 355-357.

Tamada, H., McMaster, T., Flanders, K.C., Andrews, G.K. and Dey, S.K. (1990). Cell type-specific expression of transforming growth factor-ß1 in the mouse uterus during the peri-implantation period. Mol. Endocrinol. 4, 965-972.

Thatcher, W.W., Bazer, F.W., Sharp. D.C. and Roberts, R.M. (1986). Interrelationships between uterus and conceptus to maintain corpus luteum function in early pregnancy: sheep, cattle, pigs and horses. J. Anim. Sci. 62 (suppl.2), 25-46.

Torti, F.M., Torti, S.V., Larrick, J.W. and Ringold, G.M. (1989). Modulation of adipocyte differentiation by tumor necrosis factor and transforming growth factor beta. J. Cell Biol. 108, 1105-1113.

Vaija, T.B., Rhodes, S.J., Taparowsky, E.J. and Konieczny, S.F. (1989). Fibroblast growth factor and transforming growth factor ß repress transcription of myogenic regulatory gene MyoD1. Mol. Cell Biol. 9, 3576-3579.

Valverius E.M., Bates, S.E., Stampfer, M.R., Clark, R., McCormick, F., Salomon, D.S., Lippman, M.E. and Dickson, R.B. (1989). Transforming growth factor α production and epidermal growth factor receptor expression in normal and oncogene transformed human mammary epithelial cells. Mol. Endocrinol. 3, 203-214.

van Obberghen-Schelling, E., Roche, N.S., Flanders, K.C., Sporn, M.B. and Baker, C.C. (1988). Transforming growth factor-ß1 positively regulates its own expression in normal and transformed cells. J. Biol. Chem. 263, 7741-7746.

von Zoelen, E.J.J., van Oostwaard, M.J. and de Laat, S.W. (1986). Transforming growth factor-ß and retinoic acid modulate phenotypic transformation of normal rat kidney cells induced by epidermal growth factor and platelet-derived growth factor. J. Biol. Chem. 261, 5003-5009.

Wakefield, L.M., Smith, D.M., Masui, T., Harris, C.C. and Sporn, M.B. (1987). Distribution and modulation of the

cellular receptor for transforming growth factor-beta. J. Cell Biol. 105, 965-975.

Watson, A.J., Hogan, A., Hahnel, A., Wiemer, K.E. and Schultz, G.A. (1992). Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. Mol. Reprod. Dev. 31, 87-95.

Webb, N.R., Madisen, L., Rose, T.M. and Purchio, A.F. (1988). Structural and sequence analysis of TGF-ß2 cDNA clones predicts two different precursor proteins produced by alternative mRNA splicing. DNA 7, 493-497.

Weeks, D.L. and Melton, D.A. (1987). A maternal mRNA localized to the vegetal hemisphere in Xenopus eggs codes for a growth factor related to TGF-ß. Cell 51, 861-867.

Wimsatt, W.A. (1950). New histological observations on the placenta of the sheep. Amer. J. Anat. 87, 391-457.

Wimsatt, W.A. (1951). Observations on the morphogenesis, cytochemistry and significance of the binucleate giant cells of the placenta of ruminants. Amer. J. Anat. 89, 233-282.

Wimsatt, W.A. (1975). Some comparative aspects of implantation. Biol. Reprod. 12, 1-40.

Wooding, F.B.P. (1984). Role of binucleate cells in fetomaternal cell fusion at implantation in the sheep. Amer. J. Anat. 170, 233-250.

# PART 2: EARLY GESTATIONAL EXPRESSION OF TRANSFORMING GROWTH FACTOR BETA ISOFORMS BY THE OVINE PLACENTA

## CHAPTER 1

## ABSTRACT

Compared to humans and rodents, ungulate species form a diffuse placenta which does not implant or invade the uterine endometrium. To form the diffuse placenta, the developing blastocyst must go through an expansion stage, filling the entire uterine lumen. The expansion of the ovine blastocyst from a 1 mm sphere to a 1 x 190 mm thread takes place between days 12 to 15 of gestation. The mechanisms involved in signaling this explosive growth are unknown. Possible regulators include the polypeptide growth factors. Because of their ability to regulate cellular hyperplasia and differentiation, as well as modulate extracellular matrix formation, the transforming growth factor beta (TGF-ß) family would appear to be likely candidates to regulate embryonic expansion. During mammalian development three isoforms of TGF-ß, TGF-ß1, ß2 and ß3, are most commonly expressed in embryonic tissues. Using partial cDNA clones of the three isoforms isolated from a bovine conceptus library, transcript sizes of ovine TGF-ß1, ß2 and ß3 and relative changes in expression of each isoform were described in the expanding blastocyst and peri-implantation placental membranes by Slot and Northern blot methodologies. TGF-ß1 was expressed as a single transcript of 2.6 kb in length. Expression was present in early expansion blastocysts (day 13) through early stages of extraembryonic membrane differentiation and placental

formation (day 30). Levels increased by 14.8-fold from day 13, when levels were barely detectable, to day 27, when they were highest (P=0.0001). Separating the day 23 placenta into chorionic and allantoic membrane portions demonstrated that the allantoic membrane contained 4.4-fold greater levels (P=0.003) of TGF-ß1 message than the chorion. Five transcripts of TGF-ß2 were identified in the extraembryonic membranes of 6.2, 5.2, 4.8, 4.0 and 2.7 kb in length. TGF-ß2 was expressed in conceptus tissues from the time of early expansion through initial placental formation. From day 13 to day 23, there was a 6.2-fold increase (P=0.0005) in TGF-ß2 expression, thereafter, expression declined 80% by day 30 (P=0.03). Expression of TGF-ß3 was not detectable by Northern blots using 1.5µg of mRNA or 20µg of total RNA. However, Slot blot analysis detected trace quantities of TGF-ß3 transcript in samples from days 13 through 27. The different expression patterns of each TGF-ß suggests a different role for each in early placental development. In the ovine placenta, TGF-ß1 and ß2 appear to be the most prominent isoforms. The coincident increase in expression of these two isoforms with expansion suggests they contribute to the initiation and regulation of blastocyst expansion and early placental development in sheep.

# CHAPTER 2

## INTRODUCTION

There are a variety of different types of placenta formed in eutherian mammals. Within the ungulate species the placenta are referred to as diffuse, with the fetal membranes forming contacts with the majority of the uterine endometrial surface with little erosion of the endometrium occurring (Amoroso, 1952; King et al., 1982). The formation of the placenta creates distinct maternal and fetal portions in apposition to one another (Amoroso, 1952; Björkman, 1973), making the term placentation rather than implantation appropriate. To form the fetal portion of the placenta the blastocyst goes through a stage of elongation. In the sheep, the day 12-13, 1 mm spherical blastocyst elongates to a 1 x 190 mm thread by day 15 (Rowson and Moor, 1966; Bindon, 1971). In the pig, the initial stages of expansion are associated with redistribution of the trophoblast cells, rather than cellular division (Geisert et al., 1982). Once fully expanded, the trophectoderm and underlying avascular mesoderm differentiate into the chorionic membrane, forming the fetal-maternal placental interface. In sheep, by day 25 the allantoic membrane, formed from endoderm and vascular mesoderm derived from the primitive hindgut, has grown to fill the extraembryonic coelome (Assheton, 1906). As it extends from the embryo the allantois fuses with the chorion forming the fetal placental membrane, the chorioallantois.

The family of transforming growth factor (TGF) proteins have been identified in the early embryo (Reviewed by Massagué, 1990, 1992; Jessell and Melton, 1992). These proteins include TGF-ßs, bone morphogenic proteins, activins and the vegetal-related group (Vgr) of proteins. The induction of mesoderm in early vertebrates (Kimelman and Kischner, 1987; Rosa et al., 1988) and mesoderm derivatives in higher vertebrates (Torti et al., 1989; Joyce et al,1990; Hayamizu et al., 1991; D'Angelo and Greene, 1991), are functions ascribed to the TGF-ß family of proteins. Both TGF-ß1 (Rappolee et al., 1988) and TGF-ß2 (Watson et al., 1992) mRNA have been detected during early cell division stages in mammalian embryos. The *in vitro* block of bovine embryonic growth at the 8 cell stage can be overcome by including TGF-ß1 in defined culture medium (Larson et al., 1992). The requirement of TGF-ßs for blastocyst development and their role in induction of mesoderm derived cells, suggests they are necessary for early embryonic development. Their ability to induce cellular proliferation (Rizzino, 1988) and differentiation of placental cell lines (Graham et al., 1992), as well as their expression by trophoblast (Paria et al., 1992) and syncytiotrophoblast cells (Schmid et al., 1991) indicates TGF-ßs are likely involved in establishing a viable placenta.

In the developing embryo of both humans and mice there has also been described differential tissue and temporal

expression of the isoforms of TGF-ß (Gatherer et al., 1990; Schmid et al.,1991; Pelton et al., 1991; Das et al., 1992). The distinct cellular expression within a single tissue in the embryo and placenta suggests specific roles of each TGF-ß isoform during development. Information on placental TGF-ß distribution in species which do not form invasive placenta would assist in elucidating basic mechanisms involved in placental development. This investigation was undertaken to describe the presence and relative transcription levels of the three isoforms of TGF-ß prior to and during early placental formation in the sheep.

## CHAPTER 3

## MATERIALS AND METHODS

Guanidine isothiocyanate solution (GTC) was prepared by the method of Chirgwin (1980). Briefly, 4M guanidine isothiocyanate was dissolved in a solution of 50mM Tris-HCl (pH 7.6), 5mM EDTA (pH 7.6), 2% (w/v) N-lauryl sarcosine and 1% (v/v) ß-mercaptoethanol all purchased from Sigma Chemical Co. (St. Louis, MO). Cesium trifluoroacetate (CsTFA) solution was purchased from Pharmacia (Piscataway, NJ) and prepared according to manufacturer's instructions. Agarose was acquired from Baxter Scientific Products (McGraw Park, IL), while all buffer reagents were Molecular Biology grade and purchased from Sigma Chemical Co. Formamide, formaldehyde and Random Prime labeling kits were purchased from United States Biochemicals (Cleveland, OH). Radionucleotide $\alpha^{32}$P-dCTP (3,000 Ci/mmole) was purchased from ICN Biomedicals (Irvine, CA). RNA molecular weight standards were purchased from IBI (New Haven, CT). Nytran, positively charged nylon membrane (0.22$\mu$m pore size), was obtained from Schleicher and Schull (Keene, NH). Low melting point agarose (Seaplaque GTG) was purchased from FMC Bioproducts (Rockland, MA) and Gelase was obtained from Epicentre Technologies (Madison, WI). Restriction endonuclease BssHII was obtained from Stratagene (La Jolla, CA).

*Sample Collection*

Mixed breed ewes were observed daily for estrus utilizing teaser rams, with the day of estrus designated day 0. Ewes were bred with fertile rams to obtain conceptus tissue. Ewes were randomly allotted to days 13, 16, 23, 27 or 30 of gestation. Uteri were collected surgically by hysterectomy and transported to the laboratory on ice.

Day 13 and 16 blastocysts were flushed from the uterus with phosphate buffered saline (PBS; 0.1 M Na Phosphate, pH 7.4, 0.15 M NaCl). Day 13 blastocysts were sorted by stage of elongation into two groups of <3 cm and >3 cm in length. Blastocysts were then dissociated by vortexing in GTC. Day 16 blastocysts were washed with PBS and Dounce homogenized in GTC. Day 23, 27 and 30 chorion and allantoic membranes were dissected from the endometrium collected, washed and Dounce homogenized in GTC.

Conceptus homogenates were layered over a cushion of equal volume of CsTFA and subjected to isopycnic ultracentrifugation for 24 h at 117,000 x g in a Beckman SW41Ti rotor and Beckman L5-65 ultracentrifuge (Fullerton, CA). Solutions were pretreated with 0.1% (v/v) diethylpyrocarbonate (Sigma Chemical Co.; St. Louis, MO) to reduce RNase contamination. Pelleted total RNA was resuspended in a solution of 30mM sodium citrate (pH 7.4), 0.1% (w/v) sodium lauryl sulfate (SDS) and 1% (v/v) ß-mercaptoethanol and residual cesium removed by precipitation

with 0.3M sodium acetate (pH 5.2) and 2.5 volumes of ethanol at -20°C overnight. Samples were centrifuged at 14,000 x g for 30 min., the supernatant discarded and the pellet resuspended in a small volume of a solution of 1x MOPS (40mM Morpholinopropanesulfonate, pH 7.0, 10mM sodium acetate and 1mM EDTA, pH 8.0; Sambrook et al., 1989), 0.1% SDS and 1% ß-mercaptoethanol. RNA was quantified by absorbance at 260 nm and stored at -80°C.

*TGF-ß1 Northern and Slot Blot*

Expression of mRNA was analyzed by both Northern and Slot blot methodologies. Northern blots were prepared by electrophoresing 20µg of total RNA in a 1.2% (w/v) agarose formaldehyde/MOPS gel (Sambrook et al., 1989). The gel was stained with 0.2µg/ml ethidium bromide to confirm integrity of RNA prior to capillary transfer to Nytran nylon membrane with 10x SSPE (1x= 0.15M NaCl, 10mM $NaPO_4$, pH 7.4, 1mM EDTA) for 24 h. RNA was crosslinked to the membranes by UV irradiation with 0.12 Joules (Hoeffer;San Francisco, CA) and the location of 18S and 28S rRNA marked for reference. Molecular weight markers (0.36-9.5 kb range) were included in each gel and their migration distances used to construct a standard curve used to determine the size of the ovine TGF-ß1 transcript. Slot blots were prepared by adsorbing 20µg of total RNA to nylon membranes, using a Minifold slot blot apparatus, as described by the manufacturer (Schleicher and Schull; Keene,

NH) and crosslinked to the membrane by UV irradiation. Membranes were prehybridized at 65°C in a solution of 50% (v/v) formamide, 5x SSPE, 5x Denhardt's reagent (1x= 0.1% w/v of each bovine serum albumin, Ficoll and polyvinylpyrrolidone), 5% (v/v) dextran sulphate, 0.1% (w/v) SDS and 0.1 mg/ml heat denatured, sheared salmon sperm DNA for 4 h in a rotary hybridization oven (Hybaid;Teddington, UK). This solution was discarded and replaced with an identical solution containing $4x10^6$ cpm/ml heat denatured probe and hybridized for 24 h at 42°C.

The probe consisted of a 258 bp TGF-ß1 specific fragment of our partial cDNA bovine TGF-ß1 clone, bpTGF-ß1.4, which was random prime labeled with $\alpha^{32}$P-dCTP to specific activities of 1.5-2.5 x $10^9$ cpm/$\mu$g DNA (see Appendix). The probe was constructed from the clone by polymerase chain reaction (PCR) amplification of a 5' segment of the coding region of the precursor protein of TGF-ß1. Primers used to amplify the specific segment were; 5' end GCTCTAGAACTAGTGGATC and 3' end TCTGTGGAGCTGAAGCAG. This amplimer was then subcloned into the multiple cloning site of pBluescript $SK^+$II (Stratagene, La Jolla, CA). Probe was obtained by excising the precursor insert from the vector by restriction endonuclease BssHII treatment. The insert was then purified by electrophoresis in SeaPlaque agarose, cutting the specific band from the gel, digesting the agarose with Gelase and precipitating the DNA with ethanol.

Following hybridization, membranes were then washed twice in 5x SSPE/0.1% (w/v) SDS once in 1x SSPE/0.1% (w/v) SDS, at 42°C, then twice in 0.1x SSPE/0.1% (w/v) SDS at 65°C for the final high stringency washes. Multiple autoradiographs were obtained for each blot by exposing Kodak XAR film at -80°C using Cronex intensifying screens (Dupont;Wilmington, DE). Multiple exposures were obtained to ensure data was collected within the linear response range of the film. After exposure, the membranes were stripped of probe by placing membranes in a boiling water bath. Preliminary studies demonstrated probe could be effectively removed from membranes, to a maximum of three stripping procedures, without detectable loss of bound RNA. Membranes were then prehybridized, hybridized and washed using identical conditions as previously described but substituting a 600 bp rat cDNA ß-actin clone as the probe. The probe was a PstI fragment, random prime labelled with $\alpha^{32}$P-dCTP to specific activities of 1-2 x $10^9$ cpm/$\mu$g DNA. Autoradiographs were obtained as previously described.

Slot blot signal intensities and Northern blot migration distances of detected bands were determined by computer assisted laser scanning densitometry (Ultroscan; LKB; Piscataway, NJ). Slot blot sample signal intensities were integrated and the areas calculated in arbitrary absorbance units (GSXL ver. 2 software; LKB). For each TGF-ß1 sample the corresponding area from the ß-actin autoradiograph was used to correct sample areas for differences in total RNA loaded onto

the membrane. The integrated area for ß-actin from one of the samples, which was approximately the median area of all samples and was similar to those of the same day of gestation, was chosen. Dividing this median area by the areas of each sample produced a correction value for each sample. Each correction value was then multiplied by the corresponding TGF-ß1 sample area to obtain the TGF-ß1 corrected area used for evaluation.

*TGF-ß2 and ß3 Northern and Slot blot*

Due to distortion of the mRNA transcripts in proximity of the rRNA bands, accurate molecular weight determination of TGF-ß2 and ß3 was not possible from Northern blots utilizing total RNA. For this reason poly-A enriched RNA was prepared from representative samples of day 23, 27 and 30 placental membranes, as well as control tissues, utilizing oligo-dT paramagnetic beads (PolyATtract;Promega;Madison, WI). Poly-A enriched RNA was electrophoresed in a 1.2% agarose formaldehyde/MOPS gel and transferred to Nytran as previously described. Molecular weight standards were included to generate a standard curve. Slot blots were prepared as previous described by adsorbing 20$\mu$g of total RNA to nylon membrane. Prehybridization, hybridization and wash stringency conditions were identical to those described previously for TGF-ß1.

The TGF-ß2 probe consisted of a 415 bp fragment of our

bovine TGF-ß2 partial cDNA clone, pbTGF-ß2.1, which corresponds to a portion of the coding region for the ß2 precursor protein (see Appendix). The TGF-ß3 probe was a 350 bp fragment of our bovine TGF-ß3 partial cDNA clone, pbTGF-ß3.6, containing 13 bp of the 3' end of the coding region and a portion of the 3' untranslated region (see Appendix). Both probes were generated by PCR amplification of the chosen region and subcloned into the multiple cloning site of pBluescript $SK^{+}II$ plasmid. The TGF-ß2 amplimer was produced using the two primers, 5' end GCTCTAGAACTAGTGGATC and 3' end CAGCTCGAGCCGTTGTTCA, while the TGF-ß3 amplimer was produced using the primers, 5' end CCTCGAAGTGCAGCTGA and 3' end GTAAGTCGACTCACTATAGGG. Probes used consisted of insert DNA excised from the vector by restriction endonuclease BssHII and electrophoretic purification of the insert as previously described for bTGF-ß1.

Membranes were hybridized with random prime labelled probes of 1-1.5 x $10^9$ cpm/$\mu$g DNA and 0.7-1.2 x $10^9$cpm/$\mu$g DNA, for TGF-ß2 and TGF-ß3 respectively. Since the quantity of total RNA obtained from conceptus tissues was limited, the same Northern blot was used for both TGF-ß2 and ß3 transcript evaluation. After probing for TGF-ß2 the probe was striped from the membrane in boiling water and reprobed for TGF-ß3.

*Statistical Analysis*

Changes in the relative quantity of specific transcript

for each of the TGF-ßs throughout early gestation were analyzed by least squares analysis of variance using the General Linear Models procedure of the Statistical Analysis System (Spector et al., 1985). Sample areas used in the analysis were corrected prior to analysis using ß-actin as previously described. Standard error of the mean were calculated from the sample areas and were not those generated by the least squares analysis.

## CHAPTER 4

## RESULTS

*TGF-ß1*

Northern blot analysis of ovine conceptus RNA identified the presence of a 2.6 kb transcript at all stages examined (fig. 1). The presence of a single transcript demonstrated the conditions and probe were specific to TGF-ß1. Differences in the relative quantity of TGF-ß1 message were apparent between days. There was no difference in transcript size detected between the conceptus tissues and the adult bovine spleen positive control.

To quantify relative changes in the steady state levels of TGF-ß1 mRNA expression, slot blot analysis was performed (fig. 2A,B). Graphic representation of the mean transcription levels of TGF-ß1 throughout early gestation are shown in figure 3. TGF-ß1 transcript was barely detectable in day 13 blastocyst samples, with no detectable differences between sample pools of differing length. The sample pools marked (*) were embryos <3cm, while unmarked samples were pools of embryos >3cm. The level increased 6.2 fold (P=0.015) in day 16 expanding blastocysts and peaked around days 23 to 27 at 14 (P=0.0001) and 14.8 fold (P=0.0001), relative to day 13 levels. There was a slight decrease (P=0.052) detected in the levels of day 30 chorioallantois relative to day 27. At day 23 the chorion-allantois fusion process has just begun and the two membranes can be separated to obtain an enriched

Figure 1. Northern blot of ovine embryonic total RNA probed for TGF-ß1. The 2.6 kb major transcript is indicated on the left. Samples in each lane are; 1, day 13 early expansion blastocyst, 2, day 16 expanded blastocyst, 3, day 23 chorion and allantois, 4, day 27 chorioallantois, 5, day 30 chorioallantois, 6, adult bovine spleen as the positive control, 7, adult bovine liver as the negative control. The same blot probed for ß-actin is shown to demonstrate similar sample loading. Length time of exposure of film was 24 h for TGF-ß1 and 4 h for ß-actin.

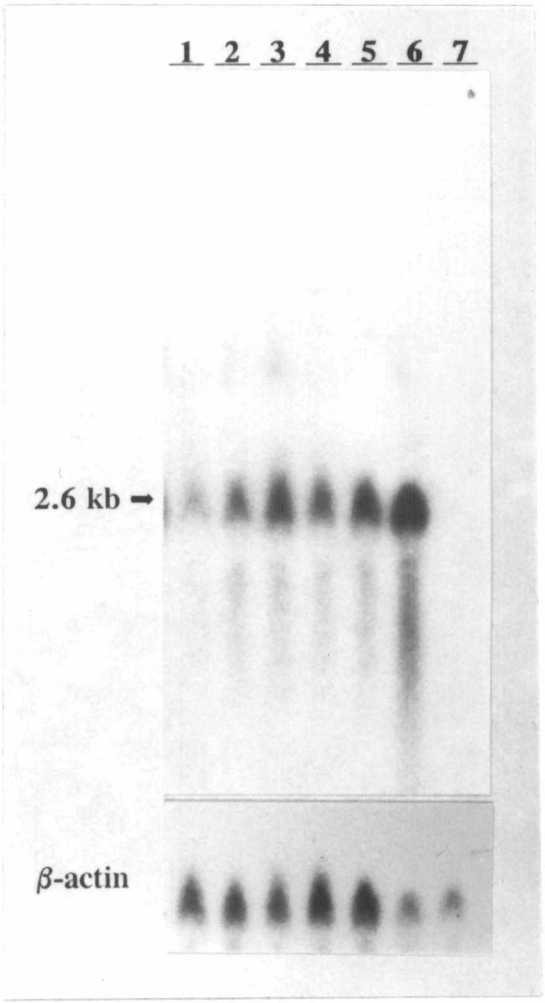
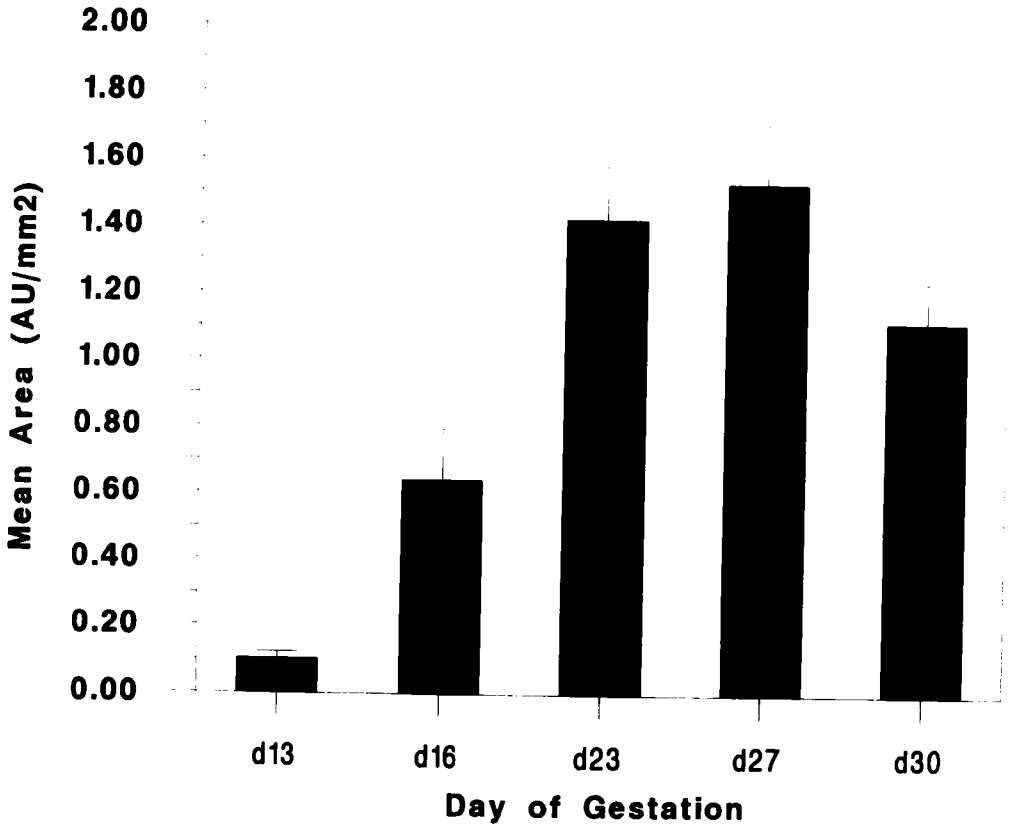

1 2 3 4 5 6 7
2.6 kb
β-actin

Figure 2. Slot blot of ovine embryonic total RNA probed for TGF-ß1 (A) and ß-actin (B). Day of gestation from which the samples were taken is indicated above each group of samples (n=4). In the day 13 samples, the pools of embryos obtained from those <3cm are indicated by *. Length of time of exposure of film was 48 h for TGF-ß1 and 9 h for ß-actin.


d13
d16
d23
d27
d30
A
B

Figure 3. Graphic representation of the mean relative expression levels of conceptus and extraembryonic membrane TGF-ß1 during early gestation. Mean areas were calculated from the computer integrated densitometric scan of the slot blots shown in figure 2. Error bars are SEM calculated from the corrected sample means to indicate sample variation within each day of gestation.


2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Mean Area (AU/mm2)
d13
d16
d23
d27
d30
Day of Gestation

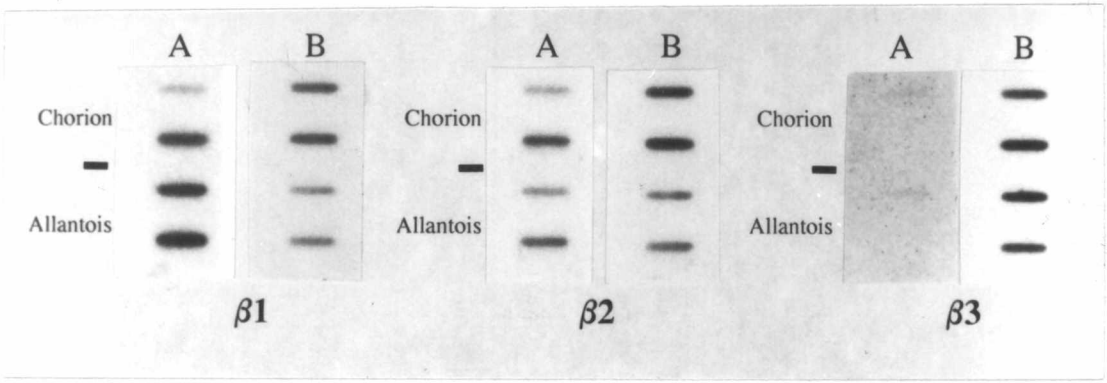
preparations of each membrane. Enriched preparations of day 23 chorion and allantois were assayed by slot blot (fig. 4). The allantois membranes contained 4.4 fold (P=0.003) greater levels of TGF-ß1 mRNA than the chorion.

*TGF-ß2*

Northern blot analysis of poly-A enriched RNA was performed on samples of ovine extraembryonic membranes (fig. 5). There were 5 distinct transcripts identified of 6.2, 5.2, 4.8, 4.0 and 2.7 kb in length. There did not appear to be any change in the intensity of any one band relative to another between the samples examined. The transcript sizes were slightly different from those of the murine placenta positive control (6.0, 5.2, 4.2, 3.1 and 2.8 kb), however the same number of transcripts were detected, indicating possible species differences in specific RNA transcripts. Preliminary Northern blot studies using total RNA demonstrated the probe bound to neither 28S or 18S rRNA bands (data not shown). Together with the similarity in transcript sizes and number to the murine placental control and those previously reported for murine and human TGF-ß2 (Miller et al., 1989; O'Reilly et al., 1992), this demonstrates the specific recognition of TGF-ß2 transcripts by the probe.

Slot blot analysis was used to obtain relative quantitative changes in TGF-ß2 mRNA production during early pregnancy (fig. 6A,B). Mean expression levels of ovine conceptus and extraembryonic membrane TGF-ß2 during early

Figure 4. Slot blot (top panel) of TGF-ß1, ß2 and ß3 expression and graphic representation (bottom panel) of TGF-ß1 and ß2 expression by samples (n=2) of day 23 ovine chorion and allantois. Mean sample areas were calculated from computer integration of densitometric scans of the slot blot after correction for loading variation using ß-actin.


A
B
Chorion
Allantois
β1
A
B
Chorion
Allantois
β2
A
B
Chorion
Allantois
β3

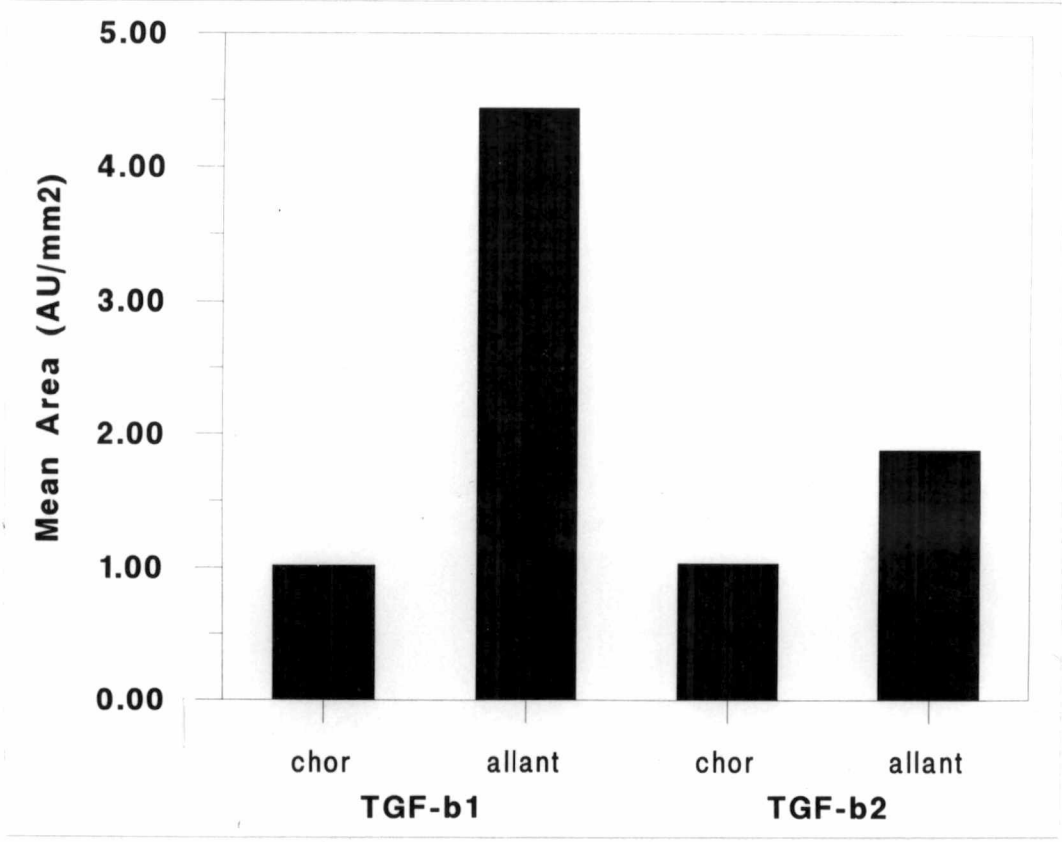

5.00
4.00
3.00
2.00
1.00
0.00
Mean Area (AU/mm2)
chor
allant
chor
allant
TGF-b1
TGF-b2

Figure 5. Northern blot of ovine embryonic poly-A enriched RNA probed for TGF-ß2. The 5 major transcripts of 6.2, 5.2, 4.8, 4.0 and 2.7 kb are indicated on the left. Samples in each lane are; 1, day 23 chorion and allantois, 2, day 27 chorioallantois, 3, day 30 chorioallantois, 4, adult bovine liver poly-A RNA as the negative control, 5, murine placenta poly-A RNA as the positive control. Length of time of exposure of film was 72 h.

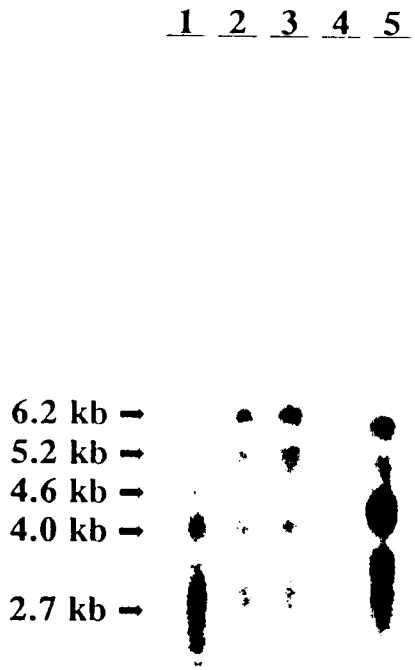
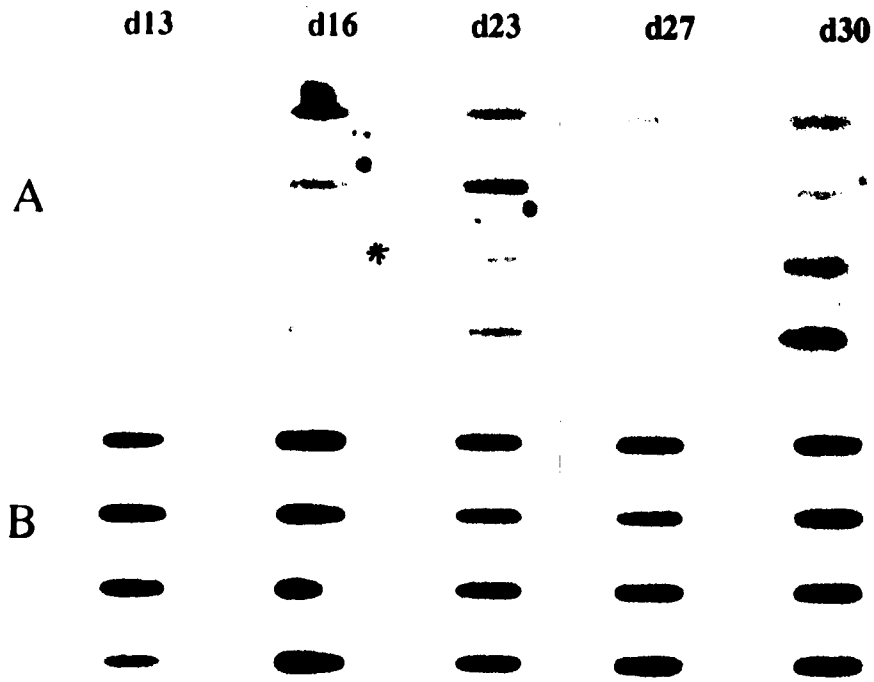

1 2 3 4 5
6.2 kb
5.2 kb
4.6 kb
4.0 kb
2.7 kb

Figure 6. Slot blot of ovine embryonic total RNA probed for TGF-ß2 (A) and ß-actin (B). Day of gestation from which the samples were taken is indicated above each group of samples (n=4). Day 16 (n=3) sample indicated (*) was not included in the analysis due to degradation. Length of time of exposure of film was 12 days for TGF-ß2 and 18 h for ß-actin.


d13
d16
d23
d27
d30
A
B

gestation are shown in figure 7. Although present in detectable quantities in day 13 early expanding blastocysts, the levels increased 6.2 fold (P=0.0005) to peak on day 23. Subsequent levels of expression declined by 1.8 fold (P=0.03) by day 30. Preparations of day 23 placental membranes indicated the allantois had slightly greater expression (P=0.17) of TGF-ß2 mRNA (fig. 4) than the chorion.

*TGF-ß3*

Expression of TGF-ß3 transcripts could not be identified by Northern blot analysis of either total RNA or poly-A enriched RNA (fig. 8). Slot blot analysis detected trace amounts of TGF-ß3 message, but the amount was at the minimum level of detection with our probe (fig 9). Message was detected in samples from day 13 and 16 blastocysts, as well as day 23 and 27 extraembryonic membranes but not day 30 membrane samples. TGF-ß3 was detected in samples of both day 23 chorion and allantois. Other tissue's RNA included in the Northern blot demonstrated the TGF-ß3 probe was specific and the lack of signal was due to small quantities of TGF-ß3 message.

Figure 7. Graphic representation of the mean relative expression levels of conceptus and extraembryonic membrane TGF-ß2 during early gestation. Mean areas were calculated from the computer integrated densitometric scan of the slot blots shown in figure 6. Error bars are SEM calculated from the corrected sample means to indicate sample variation within each day of gestation.

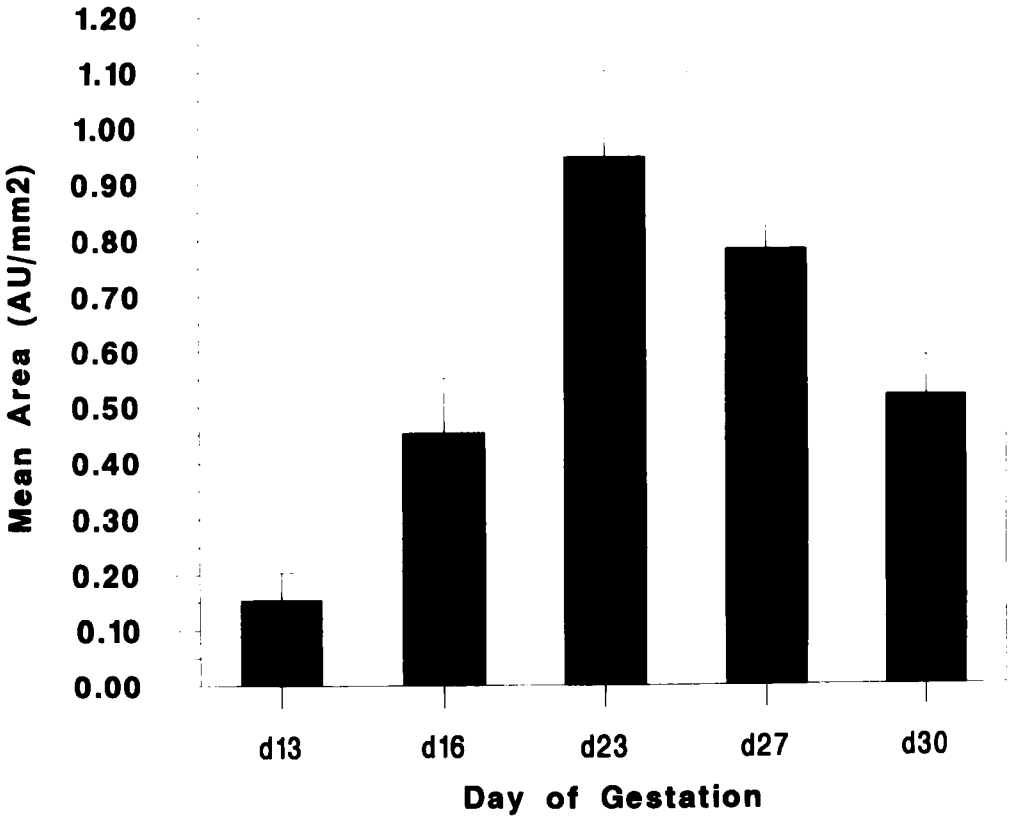
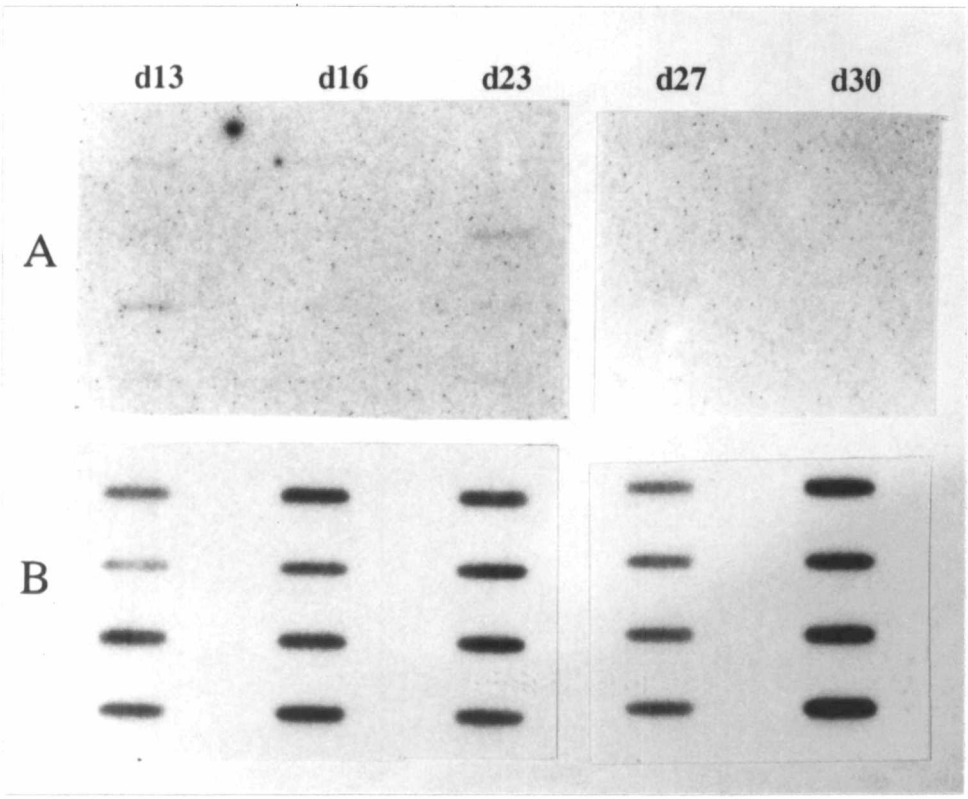

1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Mean Area (AU/mm2)
d13
d16
d23
d27
d30
Day of Gestation

Figure 8. Northern blot of ovine poly-A enriched RNA probed for TGF-ß3. The major transcript of 3.8 kb is indicated on the left. Samples in each lane are; 1, day 16 nonpregnant ovine endometrium, 2, day 16 pregnant ovine endometrium, 3, day 23 pregnant ovine endometrium, 4, day 23 chorion and allantois, 5, day 27 chorioallantois, 6, day 30 chorioallantois. Length of time of exposure of film was 19 h.

1 2 3 4 5 6

Figure 9. Slot blot of ovine embryonic total RNA probed for TGF-ß3 (A) and ß-actin (B). Day of gestation from which the samples were taken is indicated above each group of samples (n=4). Length of time of exposure of film was 18 days for TGF-ß3 and 18 h for ß-actin.


d13
d16
d23
d27
d30
A
B

# CHAPTER 5

# DISCUSSION

In ruminants, placentation is of the noninvasive epitheliochorial type, in which the cellular barriers to physiological exchange between fetal and maternal consists of six layers (Amoroso, 1952). To overcome problems associated with diffusion of gases and nutrients through multiple cell layers the noninvasive placenta develops elaborate structures to increase the absorptive surface area. The chorion contacts a large portion of the luminal surface of the endometrium in order to have the nutrient transport capacity necessary to support the growing fetus. In contrast to the murine and primate blastocyst invading into the endometrium, the ungulate blastocyst expands longitudinally within the uterine lumen at a rate of several millimeters per hour (Bindon, 1971). The signals controlling this explosive growth are unknown.

Early developmental expression of several growth factors has been described in cattle (Watson et al., 1992) and mice (Rappolee et al., 1988, 1992). The presence of transcripts for TGF-ß1 and ß2 has been shown, but temporal changes in expression, after formation of the blastocyst, have not been previously quantified. In this study TGF-ß1, ß2 and ß3 were detectable in day 13 early expansion ovine blastocysts. *In vitro* maturation of bovine blastocysts has demonstrated the requirement for TGF-ß and fibroblast growth factor in the culture medium to circumvent the developmental blockage

associated with activation of the embryonic genome (Barnes, 1988; Larson et al., 1992). These data suggest that during early cell division, the embryo must rely on external TGF-ß to support development, despite the apparent ability to produce it endogenously. This link to the maternal endometrium may help explain the requirement of maternal-embryonic synchrony demonstrated in embryo transfer studies (Moor and Rowson, 1966; Rowson and Moor,1966).

In this study, expression of TGF-ß1 and ß2 increased dramatically during elongation of the blastocyst. It is unknown whether the increased expression levels cause or are caused by expansion of the blastocyst. The expansion process has previously been found to initiate expression of other embryonic proteins, including the trophoblast interferon-τ (Godkin at al., 1982; Farin et al., 1989, 1990) and retinol-binding protein (Liu et al., 1993), indicating expansion activates a number of different genes.

During expansion of the blastocyst the yolk sac develops, serving as the initial source of the embryonic hematopoietic system. The cells associated with the developing embryonic circulation have been shown to express high levels of TGF-ß1 (Schmid et al., 1991; Millan et al., 1991). Since yolk sac was not separated from trophectoderm in this study, the relative contribution of each membrane could not be obtained. However, the allantoic and chorionic membranes were separated on day 23 and the allantoic membrane demonstrated

significantly greater TGF-ß1 levels. With it the developing allantois brings the placental blood vessels and is therefore actively angiogenic (Steven et al., 1981). In the chick chorioallantoic membrane, TGF-ß1 was found to be a potent angiogenic agent (Yang and Moses, 1990). Peak expression levels of TGF-ß1 during the time of allantoic growth and neovascularization, as well as the predominant expression in the allantois, relative to chorion, suggests that TGF-ß1 plays a role in the establishment of the placental vasculature in the ovine placenta.

The differential expression pattern of TGF-ß2 during early ovine development suggests it has a different function from TGF-ß1. Both TGF-ß1 and ß2 have been found in the developing mouse placenta, however TGF-ß3 was not (Tamada et al., 1990; Das et al., 1992). This information is consistent with the present study in the sheep. The increase in TGF-ß2 expression was not as dramatic as for TGF-ß1, however it did coincide with blastocyst elongation. The similar expression levels in both allantois and chorion indicates both membranes produce this growth factor. As the allantois grows out from the hindgut, it contacts and fuses with the chorion, producing the mature chorioallantoic placental membrane. The pattern of proteins produced by the extraembryonic membranes changed after fusion of the two membranes, based on 2-dimensional electrophoretic analysis (Bartol et al., 1985; Godkin et al., 1988). The proteins produced resemble those of the allantois,

suggesting fusion inhibited or altered chorionic protein production. By day 23 the allantois is near maximal size and fusion of the chorion and allantois membranes has begun. The peak expression of TGF-ß2 on day 23 and the slightly higher allantoic expression suggests the allantois may influence the chorion through TGF-ß2. This influence may result in alteration of the number and type of proteins secreted.

Peak expression of TGF-ß2 corresponds to early stages of chorion allantois fusion. Stimulation and control of extracellular matrix formation is one of the function previously described for TGF-ßs (Rizzino, 1988; Borsi et al., 1990; D'Angelo and Green, 1991; Delannet and Duband, 1992). Histologically, after fusion the chorion and allantois are separated by a stroma, containing few cells and extensive extracellular matrix (Assheton, 1906; see figure 6C of Liu et al., 1992). Placental extracellular matrix has been shown to contain collagen types I, III, IV,V and VI (Glanville et al., 1979; Jander et al., 1981; Ayad et al., 1985; Sharpe et al., 1990). Recently we have described the synthesis and secretion of collagen α1(III) by the bovine chorion and allantois, as well as by the fused membranes (Shang, Doré and Godkin, unpublished data). TGF-ßs have been shown to stimulate the secretion of several types of collagen (Penttinen et al., 1988; Rizzino, 1988), suggesting that TGF-ß2 may be acting in an autocrine/paracrine manner regulating the fusion of the placental membranes through stimulating deposition of

extracellular matrix.

The finding that TGF-ß3 is transcribed at extremely low levels suggests that this growth factor plays a minor role in early placental formation. The possibility exists that transcription of TGF-ß3 is limited to a small time span between time points in this study or to a subset of cells within the tissues examined. This latter possibility is most intriguing. Since RNA was prepared from the entire extraembryonic membranes, if only a small portion of the cells expressed TGF-ß3, their contribution to the total RNA pool would be small, thus providing barely detectable level of transcription.

Although the suggested roles of each TGF-ß in development of the ovine placenta are speculative, this study does demonstrate the temporal expression patterns for each TGF-ß differs during early placental development. The timing of maximal expression is highly suggestive to possible roles of both ß1 and ß2 in the formation of the placenta. Further work is therefore necessary to determine the functions of the TGF-ßs in the formation of the ovine placenta.

## LIST OF REFERENCES


Amoroso, E.C. (1952). Placentation. In: *Marshall's Physiology of Reproduction, 3rd ed. vol. 2,* (edited by A.S. Parkes) Longman, Green and Co., London, 127-311.

Assheton, R. (1906). The morphology of the ungulate placenta, particularly the development of that organ in the sheep, and notes upon the placenta of the elephant and Hyrax. Phil. Trans. Royal Soc. London 198 (Series B), 143-225.

Ayad, S., Chambers, C.A., Shuttleworth, C.A. and Grant, M.E. (1985). Isolation from bovine elastic tissues of collagen type VI and characterization of its form in vivo. Biochem. J. 230, 465-474.

Barnes, F.L. (1988). Characterization of the onset of embryonic control and early development in bovine embryos. Ph.D. Thesis, University of Madison, WI.

Bartol, F.F., Roberts, R.M., Bazer, F.W., Lewis, G.S., Godkin, J.D. and Thatcher, W.W. (1985). Characterization of proteins produced in vitro by periattachment bovine conceptuses. Biol. Reprod. 32, 681-693.

Bindon, B.M. (1971). Systematic study of preimplantation stages of pregnancy in the sheep. Aust. J. Biol. Sci. 24, 131-147.

Björkman, N. (1973). Fine structure of the fetal-maternal area of exchange in the epitheliochorial and

endotheliochorial types of placentation. Acta Anat. 61 (suppl.), 1-22.

Borsi, L. Castellani, P., Risso, A.M., Leprini, A. and Luciano, Z. (1990). Transforming growth factor-ß regulates the splicing pattern of fibronectin messenger RNA precursor. FEBS, 261, 175-178.

Chirgwin, J.M. Przybyla, A.E., MacDonald R.J. and Rutler W.J. (1980). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294-5298.

D'Angelo, M. and Greene, R.M. (1991). Transforming growth factor-ß modulation of glycosaminoglycan production by mesenchymal cells of the developing murine secondary palate. Dev. Biol. 145, 374-378.

Das, S.K., Flanders, K.C., Andrews, G.K. and Dey S.K. (1992). Expression of transforming growth factor-ß isoforms (ß2 and ß3) in the mouse uterus: analysis of the peri-implantation period and effects of ovarian steroids. Endocrinol. 130, 3459-3466.

Delannet, M. and Duband, J-L. (1992). Transforming growth factor-ß control of cell-substratum adhesion during avian neural crest cell emigration in vitro. Development, 116, 275-287.

Farin, C.E., Imakawa, K. and Roberts, M.R. (1989). In situ localization of mRNA for the interferons, ovine

trophoblast protein-1, during early embryonic development in sheep. Mol. Endocrinol. 3, 1099-1107.

Farin, C.E., Imakawa, K., Hansen, T.R., McDonnell, J.J., Murphy, C.N., Farin, P.W. and Roberts, R.M. (1990). Expression on trophoblastic interferon genes is sheep and cattle. Biol. Reprod. 43, 210-218.

Gatherer, D., ten Dijke, P., Baird, D.T. and Akhurst, R.J. (1990). Expression of TGF-ß isoforms during first trimester human embryogenesis. Development 110,445-460.

Geisert, R.D., Brookbank, J.W., Roberts, M.R. and Bazer, F.W. (1982). Establishment of pregnancy in the pig: II cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biol. Reprod. 27. 941-955.

Glanville, R.W., Rauter, A. and Fietzek, P.P. (1979). Isolation and characterization of a native placental basement membrane collagen and its component alpha chains. Eur. J. Biochem. 95, 383-389.

Godkin, J.D., Bazer, F.W., Moffat, J., Sessions, F. and Roberts, R.M. (1982). Purification and properties of a major low molecular weight protein released by the trophoblast of sheep blastocysts at day 13-21. J. Reprod. Fert. 65, 141-150.

Godkin, J.D., Lifsey, B.J. Jr. and Baumbach, G.A. (1988). Characterization of protein production by bovine

chorionic and allantoic membranes. Biol. Reprod. 39, 195-204.

Graham, C.H., Lysiak, J.J., McCrae, K.R. and Lala, P.K. (1992). Localization of transforming growth factor-ß at the human fetal-maternal interface: role in trophoblast growth and differentiation. Biol. Reprod. 46, 561-572.

Hayamizu, T.F., Sessions, S.K., Wanek, N. and Bryant, S.V. (1991). Effects of localized application of transforming growth factor ß1 on developing chick limbs. Devel. Biol. 145, 164-173.

Jander, R., Rauterberg, J., Voss, B. and von Bassewitz, D.B. (1981). A cysteine-rich collagenous protein from bovine placenta: Isolation of its constituent polypeptide chains and some properties of the non-denatured protein. Eur. J. Biochem. 114, 17-25.

Joyce, M.E., Roberts, A.B., Sporn, M.B. and Bolander, M.E. (1990). Transforming growth factor-ß and the initiation of chondrogenesis and osteogenesis in the rat femur. J. Cell Biol. 110, 2195-2207.

Kimelman, D. and Kirschner, M. (1987). Synergistic induction of mesoderm by FGF and TGF-ß and the identification of an mRNA coding for FGF in the early Xenopus embryo. Cell 51, 869-877.

King, G.J., Atkinson, B.A. and Robertson, H.A. (1982). Implantation and early placentation in domestic ungulates. J. Reprod. Fert. (suppl.31), 17-30.

Larson, R.C., Ignotz, G.G. and Currie, W.B. (1992). Transforming growth factor ß and fibroblast growth factor synergistically promote early bovine embryo development during the fourth cell cycle. Mol. Reprod. Dev. 33, 432-435.

Liu, K.H., Gao, K., Baumbach, G.A. and Godkin, J.D. (1992). Purification and immunolocalization of ovine placental retinol-binding protein. Biol. Reprod. 46, 23-29.

Liu, K.H., Doré, J.J.E. Jr., Roberts, M.P., Rajesh, K., Hopkins, F.M. and Godkin, J.D. (1993). Expression and cellular localization of retinol-binding protein mRNA in bovine blastocysts and extraembryonic membranes. Biol. Reprod. 49, in press.

Massagué, J. (1990). The transforming growth factor-ß family. Annu. Rev. Cell Biol. 6, 597-641.

Massagué, J. (1992). Receptors for the TGF-ß family. Cell 69, 1067-1070.

Millan, F.A., Denhez, F., Kondaiah, P. and Akhurst, R.J. (1991). Embryonic gene expression patterns of TGF-ß1, ß2 and ß3 suggest different developmental functions *in vivo*. Development 111, 131-141.

Miller, D.A., Lee, A., Pelton, R.W., Chen, E.Y., Moses, H.L. and Derynck, R. (1989). Murine transforming growth factor-ß2 cDNA sequence and expression in adult tissues and embryos. Mol. Endocrinol. 3, 1108-1114.

Moor, R.M. and Rowson, L.E.A. (1966). The corpus luteum of

the sheep: functional relationships between the embryo and corpus luteum. J. Endocrinol. 34, 233-239.

O'Reilly, M.A., Geiser, A.G., Kim, S-J., Bruggeman, L.A., Luu, A.X., Roberts, A.B. and Sporn, M.B. (1992). Identification of an activating transcription factor (ATF) binding site in the human transforming growth factor-ß2 promoter. J. Biol. Chem. 267, 19938-19943.

Paria, B.C., Jones, K.L., Flanders, K.C. and Dey, S.K. (1992). Localization and binding of transforming growth factor-ß isoforms in mouse preimplantation embryos and in delayed and activated blastocysts. Dev. Biol. 151, 91-104.

Pelton, R.W., Saxena, B., Jones, M., Moses H.L. and Gold, L.I. (1991). Immunohistochemical localization of TGF-ß1, TGF-ß2 and TGF-ß3 in the mouse embryo: expression patterns suggest multiple roles during embryonic development. J. Cell Biol. 115, 1091-1105.

Penttinen, R.P., Kobayashi, S. and Bornstein, P. (1988). Transforming growth factor ß increases mRNA for matrix proteins both in the presence and absence of changes in mRNA stability. Proc. Natl. Acad. Sci. USA 85, 1105-1108.

Rappolee, D.A., Brenner, C.A., Schultz, R., Mark, D. and Werb, Z. (1988). Developmental expression of PDGF, TGF-α and TGF-ß genes in preimplantation mouse embryo. Science, 241, 1823-1825.

Rappolee, D.A. (1992). Endogenous insulin-like growth factor

II mediates growth in preimplantation mouse embryos through the insulin-like growth factor I receptor: A case study on the role of growth factors in mammalian development. J. Anim. Sci. 70 (suppl.2), 42-50.

Rizzino, A. (1988). Transforming growth factor-ß: Multiple effects on cell differentiation and extracellular matrices. Dev. Biol. 130, 411-422.

Rowson, L.E.A. and Moor, R.M. (1966). Development of the sheep conceptus during the first fourteen days. J. Anat. 100, 777-785.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor.

Schmid, P., Cox, D., Bilbe, G., Maier, R. and McMaster, G.K. (1991). Differential expression of TGF ß1, ß2 and ß3 genes during mouse embryogenesis. Development 111, 117-130.

Sharpe, K.L., Eiler, H. and Hopkins, F.M. (1990). Changes in the proportion of type I and type III collagen in the developing and retained bovine placentome. Biol. Reprod. 43, 229-235.

Spector, P.C., Goodnight, J.H., Sall, J.P. and Sarle, W.S. (1985). The GLM procedures. In: *SAS User's Guide; Statistics*, ver. 5. SAS Institute, Cary, NC.

Steven, D.H., Burton, G.J. and Samuel, C.A. (1981). Histological and electron microscopy of sheep placental

membranes. Placenta (suppl. 2), 11-34.

Tamada, H., McMaster, T., Flanders, K.C., Andrews, G.K. and Dey, S.K. (1990). Cell type-specific expression of transforming growth factor-ß1 in the mouse uterus during the peri-implantation period. Mol. Endocrinol. 4, 965-972.

Torti, F.M., Torti, S.V., Larrick, J.W. and Ringold, G.M. (1989). Modulation of adipocyte differentiation by tumor necrosis factor and transforming growth factor beta. J. Cell Biol. 108, 1105-1113.

Watson, A.J., Hogan, A., Hahnel, A., Wiemer, K.E. and Schultz, G.A. (1992). Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. Mol. Reprod. Dev. 31, 87-95.

Yang, E.Y. and Moses, H.L. (1990). Transforming growth factor ß1-induced changes in cell migration, proliferation and angiogenesis in the chicken chorioallantoic membrane. J. Cell Biol. 111, 731-741.

# PART 3: CHANGES IN OVINE ENDOMETRIAL mRNA EXPRESSION OF TRANSFORMING GROWTH FACTOR BETA ISOFORMS DURING THE PERI-IMPLANTATION PERIOD

## CHAPTER 1

## ABSTRACT

During the estrous cycle and early pregnancy the uterus undergoes a variety of morphological changes. Unlike primates, the endometrium of the domestic ruminants is not lost but does go through extensive remodelling and cellular replacement. If pregnancy is established, this restructuring does not occur. The uterine caruncles differentiate into extensively vascularized placentomes, the main gas and nutrient exchange unit of the ruminant placenta. Because of their powerful effects on angiogenesis, extracellular matrix modification, as well as cellular proliferation and differentiation, the transforming growth factor beta (TGF-ß) family of polypeptides may be involved in the regulation of endometrial modification. In this study uterine endometrial expression of the most common isoforms, TGF-ß1, ß2 and ß3, was quantified during the later part of the estrous cycle (day 13) through the initial stages of placentome formation (day 30) in the sheep. All three TGF-ß isoforms were expressed by the ovine endometrium throughout the period examined. TGF-ß1 was expressed as a single transcript of 2.6 kb in length. Expression levels were 2.0-fold higher on day 16 of the estrous cycle than on day 13 (P=0.002) of the estrous cycle or day 16 (P=0.008) of gestation. In pregnant endometrium, peak TGF-ß1 expression occurred on day 27, being 2.5-fold higher (P=0.0001) than on day 16 of pregnancy. TGF-ß2 was expressed

as five distinct transcripts of 6.2, 5.2, 4.6, 4.0 and 2.7 kb in length. TGF-ß2 expression levels were lowest on day 16 of pregnancy. Thereafter the expression level increased steadily through day 30 (P=0.0003). TGF-ß3 was expressed as a single transcript of 3.8 kb. Like TGF-ß1, there was a dramatic difference in TGF-ß3 levels between day 16 of pregnancy and day 16 of the estrous cycle (3.8-fold,P=0.0001). In pregnant ewes endometrial TGF-ß3 levels increased 1.9-fold (P=0.13) between day 16 and 23 and remained relatively constant through day 30. Both TGF-ß1 and ß3 dramatically increased transcription levels during proestrus, suggesting they may play a role in the reorganization of the endometrium for the next estrous cycle. The consistent finding that all TGF-ß levels were depressed on day 16 of pregnancy suggests that an embryonic factor inhibits TGF-ß transcription. The increase in all TGF-ß transcription levels at day 23 indicates that production of this conceptus-derived suppressor activity is limited temporally. These results demonstrate that the ovine endometrium expresses all three isoforms of TGF-ß and that expression of each isoform differs during both the estrous cycle and early pregnancy.

## CHAPTER 2

## INTRODUCTION

The transforming growth factor ß (TGF-ß) family of polypeptides have been implicated in modulating a variety of physiological functions. Although there have been several members identified (Reviewed by Massagué, 1990), in mammals there appears to be three isoforms, TGF-ß1, ß2, and ß3, which are commonly expressed. The exact nature of the roles of the different members of the TGF-ß family of peptides in early development of the placenta and morphological changes in the endometrium during the estrous cycle is unclear. However, in other tissues they have been found to stimulate cellular proliferation and differentiation (Rizzino, 1988; Sporn and Roberts, 1991), both positively and negatively effect immunological responses (Kehrl et al., 1986; Rook et al., 1986; Wahl et al., 1987; Wiseman et al., 1988; Tsunawaki et al., 1988) and regulate extracellular matrix deposition and degradation (Rizzino, 1988). All of these events are involved in normal cyclicity and/or during establishment of pregnancy. Regulation of immune response varies from chemotaxis (Wahl et al., 1987; Wiseman et al., 1988), to suppression of macrophage and T-cell activity (de Martin et al., 1987; Wrann et al., 1987; Tsunawaki et al., 1988). The presence of an embryo in the uterus is similar to an allograft, yet no immunological rejection occurs. Through the course of a single estrous cycle the uterine endometrium morphology is extensively

altered (Boshier, 1969; Guillomot et al., 1981). The extensive changes in the endometrium during the estrous cycle and pregnancy, either through growth or tissue remodeling, suggests that the tissue is undergoing dramatic matrix modifications.

Unlike primate and rodent placentae, the ungulate species do not form either decidualized endometrium or invasive syncytial trophoblast tissue. Limited syncytial formation in the ungulate placenta has been described (Amoroso, 1952; Wooding, 1984). However, the endometrium is thought to be protected from extensive trophoblast invasion by secretion of protease inhibitors during pregnancy (Mullins et al.,1980; Fazleabas et al., 1982), although this theory has not been well established. The porcine embryo has been shown to aggressively invade tissues when placed at ectopic sites, demonstrating an inherent capacity of embryonic invasion (Samuel, 1971; Samuel and Perry, 1972). It has been suggested that decidualizing tissue limits the extent of fetal tissue invasion by loading the uterine stroma with latent TGF-ß (Strickland and Richards, 1992). Latent TGF-ß precursor forms have been shown to bind to extracellular matrix collagen (Paralkar et al., 1991). TGF-ß has been demonstrated to stimulate protease inhibitor production (Laiho and Keski-Oja, 1989), secretion of extracellular matrix proteins (Rizzo, 1988) and regulate transcription of specific integrin subunits (Ignotz et al., 1989; Heino et al., 1989; Sheppard et al.,

1992). Through these mechanisms the ungulate uterus may prevent the developing conceptus from embedding in the uterine endometrium.

In the mouse the temporal and tissue distribution of the three TGF-ßs has been described in the uterus and early placenta (Tamada et al., 1990; Das et al., 1992). Decidualizing tissue was found to contain both TGF-ß1 and ß2, but not ß3. The sheep placenta forms a limited syncytium but does not invade the endometrial stroma. Expression of the TGF-ß isoforms by the ovine placenta has not been previously described. Studying the methods used by different species in the formation of a viable placenta may provide incite into the mechanisms tissues use to invade or prevent invasion of neighboring tissues. In this study we demonstrate that endometrial expression of the three TGF-ßs differs between pregnancy and cyclic tissues, as well as at different times during early pregnancy.

## CHAPTER 3

## MATERIALS AND METHODS

Guanidine isothiocyanate solution (GTC) was prepared by the method of Chirgwin (1980). Briefly, 4M guanidine isothiocyanate was dissolved in a solution of 50mM Tris-HCl (pH 7.6), 5mM EDTA (pH 7.6), 2% (w/v) N-lauryl sarcosine and 1% (v/v) ß-mercaptoethanol all purchased from Sigma Chemical Co. (St. Louis, MO). Cesium trifluoroacetate (CsTFA) solution was purchased from Pharmacia (Piscataway, NJ) and prepared according to manufacturers instructions. Agarose was acquired from Baxter Scientific Products (McGraw Park, IL), while all buffer reagents were of Molecular Biology grade quality and purchased from Sigma Chemical Co. Formamide, formaldehyde and Random Prime labeling kits were purchased from United States Biochemicals (Cleveland, OH). Radionucleotide $\alpha^{32}$P-dCTP (3,000 Ci/mmole) was purchased from ICN Biomedicals (Irvine, CA). RNA molecular weight standards were purchased from IBI (New Haven, CT). Nytran, positively charged nylon membrane (0.22$\mu$m pore size), was obtained from Schleicher and Schull (Keene, NH). Low melting point agarose (Seaplaque GTG) was purchased from FMC Bioproducts (Rockland, MA) and Gelase was obtained from Epicentre Technologies (Madison, WI). Restriction endonuclease BssHII was obtained from Stratagene (La Jolla, CA).

*Sample Collection*

Mixed breed ewes were observed daily for estrus utilizing teaser rams, with day of estrus designated day 0. Ewes were bred with fertile rams and randomly allotted to groups for surgery on days 13, 16, 23, 27 or 30 of gestation. Cyclic ewes were not exposed to rams and allotted to groups of day 13 or 16 of the estrous cycle. Uteri were collected surgically by hysterectomy and transported to the laboratory on ice.

Pregnancy was confirmed by the presence of a conceptus in the uterus and endometrium isolated by dissection, avoiding myometrial contamination. Endometrium in contact with embryonic membranes was collected to ensure detection of proximal embryonic effects. Endometrium from cyclic ewes was taken from the uterine horn ipsilateral to the corpus luteum. Endometrium was washed twice in PBS to reduce blood contamination before homogenization in GTC solution using a Tissuemizer (Tekmar;Cincinnati,OH). Tissue debris was removed by centrifugation for 30 min. at 16,000 x g and the supernatant decanted.

Cleared endometrial homogenates were loaded onto an equal volume cushion of CsTFA and subjected to isopycnic ultracentrifugation for 24 h at 117,000 x g (26,000 RPM) in a Beckman SW41 Ti rotor and Beckman L5-65 ultracentrifuge (Fullerton, CA). Solutions were pretreated with 0.1% (v/v) diethylpyrocarbonate (Sigma Chemical Co., St. Louis, MO) to reduce RNase contamination. Pelleted RNA was resuspended in

a solution of 30mM sodium citrate, pH 7.4, 0.1% (w/v) SDS, 1% (v/v) ß-mercaptoethanol. Residual cesium was removed by precipitation with sodium acetate, pH 5.2, and ethanol at -20 °C. Samples were centrifuged at 14,000 x g for 30 min., the supernatant was discarded and the pellet resuspended in a small volume of a solution of 1x MOPS (40mM Morpholino-propanesulfonate, pH 7.0, 10mM sodium acetate and 1mM EDTA, pH 8.0; Sambrook et al., 1989), 0.1% (w/v) SDS and 1% (v/v) ß-mercaptoethanol. RNA was quantified by absorbance at 260 nm and stored at -80°C.

*TGF-ß1 Northern and Slot Blot*

Expression of TGF-ß1 mRNA was analyzed by both Northern and slot blot methodologies. For Northern blots 20µg of total RNA was electrophoresed in a 1.2% (w/v) agarose formaldehyde/MOPS gel (Sambrook et al., 1989). The gel was stained with 0.2µg/ml ethidium bromide to confirm integrity of RNA prior to capillary transfer to nylon membrane with 10x SSPE (1x= 0.15M NaCl, 10mM $NaPO_4$, pH 7.4, 1mM EDTA) for 24 h. RNA was crosslinked to the membranes by UV irradiation with 0.12 Joules (Hoeffer;San Francisco, CA) and the location of 18S and 28S rRNA marked for reference. Molecular weight markers (0.36-9.5 kb range) were included with each gel in order to construct the standard curve used to determine the size of ovine TGF-ß1 transcript. Slot blots were prepared by adsorbing 20µg of total RNA to nylon membranes, using a

Minifold slot blot apparatus, as described by the manufacturer (Schleicher and Schull;Keene, NH). RNA was crosslinked to the membrane by UV irradiation. Membranes were prehybridized at 65°C in a solution of 50% formamide (v/v), 5x SSPE, 5x Denhardt's reagent (1x= 0.1% w/v of each: bovine serum albumin, Ficoll and polyvinylpyrrolidone), 5% (v/v) dextran sulphate, 0.1% (w/v) SDS and 0.1 mg/ml heat denatured, sheared salmon sperm DNA for 4 h in a rotary hybridization oven (Hybaid;Teddington, UK). This solution was discarded and replaced with an identical solution containing $4x10^6$ cpm/ml heat denatured probe and hybridized for 24 h at 42°C.

The probe consisted of a 258 bp TGF-ß1 specific fragment of our partial cDNA bovine TGF-ß1 clone, pbTGF-ß1.4 clone, which was random prime labeled with $\alpha^{32}$P-dCTP to specific activities of 1.5-2.5 x $10^9$ cpm/$\mu$g DNA. The probe was constructed from the clone by polymerase chain reaction (PCR) amplification of a 5' segment of the coding region of the precursor protein of bovine TGF-ß1. Primers used to amplify the specific segment consisted of, 5' end GCTCTAGAACTAGTGGATC and 3' end TCTGTGGAGCTGAAGCAG. This amplimer was then subcloned into the multiple cloning site of pBluescript $SK^+II$ (Stratagene;La Jolla, CA). Probe was obtained by excising the precursor insert from the vector using restriction endonuclease BssHII. The fragment was separated from the vector by electrophoresis in SeaPlaque agarose. The fragment was then cut out, the gel digested with Gelase and DNA

precipitated with ethanol.

Following hybridization, membranes were washed twice in 5x SSPE/0.1% (w/v) SDS once in 1x SSPE/0.1% (w/v) SDS, at 42°C, then twice in 0.1x SSPE/0.1% (w/v) SDS at 65°C for the final high stringency washes. Multiple autoradiographs were obtained, to ensure data within the film's linear response range, by exposing Kodak XAR film at -80°C using Cronex intensifying screens (Dupont;Wilmington, DE). After exposure, the membranes were stripped of probe by placing membranes in boiling water bath. Preliminary studies demonstrated probe could be effectively removed from membranes, to a maximum of three stripping procedures, without detectable loss of signal occurring. Membranes were then prehybridized, hybridized and washed using identicle conditions as previously described but substituting rat cDNA ß-actin as the probe. The probe, a 600 bp PstI fragment of rat ß-actin cDNA, was random prime labelled with $\alpha^{32}$P-dCTP to specific activities of 1-2 x $10^9$ cpm/$\mu$g DNA. Autoradiographs were obtained as previously described.

Slot blot signal intensities and Northern blot migration distance of detected bands were determined by computer assisted laser scanning densitometry (Ultroscan;LKB: Piscataway, NJ). Slot blot sample signal intensity was integrated and the area for each sample calculated in arbitrary absorbance units (GSXL ver. 2 software; LKB). For each TGF-ß1 sample area the corresponding area from the ß-

actin autoradiograph was used as a measure of total RNA present in the sample. Sample areas were normalized using the corresponding ß-actin area to correct for differences in signal intensity due to differences in amount of RNA loaded. Using the corrected TGF-ß1 sample areas, the mean area for each day and status (cyclic or pregnant) was calculated.

*TGF-ß2 and ß3 Northern and Slot blot*

Due to distortion of the mRNA transcripts in the proximity of the rRNA bands, accurate molecular weight determinations of TGF-ß2 and ß3 were not possible from Northern blots utilizing total RNA. For this reason poly-A enriched RNA was prepared from representative samples of endometrium from day 16 of the estrous cycle and days 16 and 23 of pregnancy, as well as control tissues, utilizing oligo-dT paramagnetic beads (PolyATract;Promega;Madison, WI). Poly-A enriched RNA was electrophoresed in a 1.2% agarose formaldehyde/MOPS gel and transferred to Nytran as previously described. Molecular weight standards were included to generate a standard curve. Slot blots were prepared as previous described by adsorbing 20$\mu$g of total RNA to nylon membrane. Northern blots of total RNA demonstrated the bovine TGF-ß2 and ß3 probes recognized specific transcripts and did not bind rRNA (data not shown). Prehybridization, hybridization and wash stringency conditions were identical to those described previously for TGF-ß1.

The TGF-ß2 probe consisted of a 415 bp fragment, corresponding to a portion of the precursor protein, of our bovine TGF-ß2 partial cDNA clone, pbTGF-ß2.1. The TGF-ß3 probe consisted of a 350 bp fragment of our bovine TGF-ß3 partial cDNA clone, pbTGF-ß3.6, containing 13 bp of the 3' end of the mature protein coding region and a portion of the 3' untranslated region. Both probes were generated by PCR amplification of the chosen region and subcloned into the multiple cloning site of pBluescript $SK^{+}II$ plasmid. Primers used to produce the amplification products of the specific regions were; ß2 5' end GCTCTAGAACTAGTGGAATC and 3' end CAGCTCGAGCCGTTGTTCA, ß3 5' end CCTCGAAGTGCAGCTGA and 3' end GTAAGTCGACTCACTATAGGG. Probes used consisted of insert DNA excised from the plasmid by restriction endonuclease BssHII and electrophoretic purification of the insert as previously described for bTGF-ß1.

Membranes were hybridized with random prime labelled probes of 1-1.5 x $10^9$ cpm/$\mu$g DNA and 0.7-1.2 x $10^9$ cpm/$\mu$g DNA, for bTGF-ß2 and bTGF-ß3 respectively. Due to the quantity of total RNA needed to extract poly-A enriched RNA, the same Northern blot was used for both TGF-ß2 and ß3 transcript size evaluation. After probing for TGF-ß2 the membrane was striped in boiling water and reprobed for TGF-ß3. Different slot blots were prepared for independent evaluation of TGF-ß2 and ß3 transcript levels.

*Statistical Analysis*

Changes in the relative quantity of specific transcript for each of the TGF-ßs throughout early pregnancy and late estrous cycle were analyzed by least squares analysis of variance using the General Linear Models procedure of the Statistical Analysis System (Spector et al., 1985). Sample areas used to generate the mean for each day and status were corrected prior to analysis using ß-actin, as previously described.

## CHAPTER 4

## RESULTS

*TGF-ß1*

Northern blot analysis of ovine endometrial total RNA demonstrated a single transcript of 2.6 kb in length in all tissues examined (fig. 1). There was no difference between the size of the ovine endometrial transcript and that of the bovine spleen positive control. The lack of nonspecific background demonstrated the probe and conditions to be specific for detection of TGF-ß1.

Relative changes in the steady-state levels of TGF-ß1 mRNA expression in endometrium of cyclic and early pregnant endometrium were determined by slot blot analysis (fig. 2A,B). Graphic representation of the mean transcription levels of TGF-ß1 during early pregnancy and late estrous cycle are shown in figure 3. No differences were observed between samples on day 13 regardless of status (pregnant vs. cyclic). However, there was a 2.2-fold (P=0.002) increase in endometrial TGF-ß1 transcript levels from day 13 to day 16 of the estrous cycle. In addition, levels of TGF-ß1 transcription were 2.0-fold (P=0.008) greater in endometrium from cyclic ewes than pregnant ewes on day 16. TGF-ß1 mRNA levels increase from day 16 of pregnancy 2.0-fold by day 23 (P=0.0035) and continue increasing to peak on day 27 at 2.5-fold (P=0.0001).

*TGF-ß2*

Northern blot analysis of poly-A enriched RNA

Figure 1. Northern blot of ovine endometrial total RNA probed for TGF-ß1. The 2.6 kb major transcript is indicated on the left. Endometrial samples in each lane are; 1, day 13 nonpregnant, 2, day 13 pregnant, 3, day 16 nonpregnant, 4, day 16 pregnant, 5, day 23 pregnant, 6, day 27 pregnant, 7, day 30 pregnant, 8, adult bovine spleen as the positive control, 9, adult bovine liver as the negative control. The same blot probed for ß-actin is shown to demonstrate similar sample loading. Length of time of exposure of the film was 24 h for TGF-ß1 and 6 h for ß-actin.

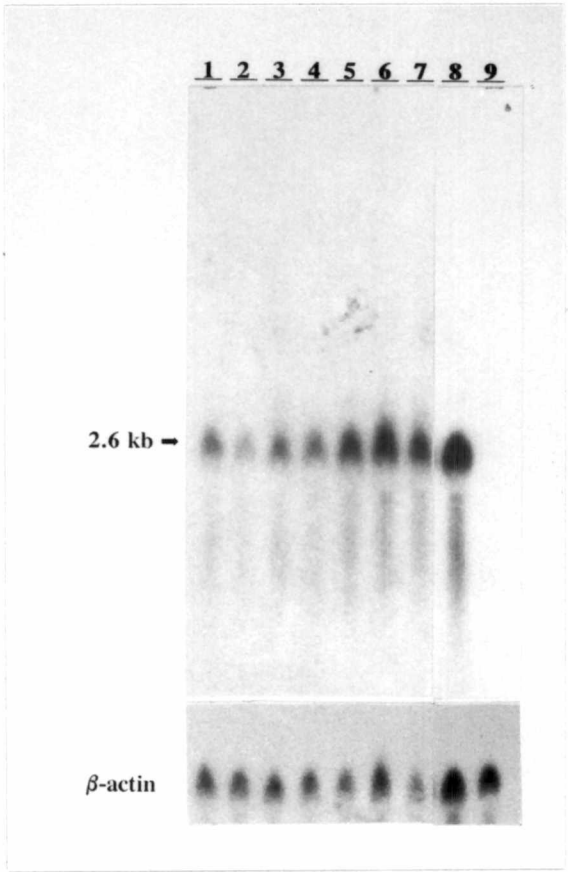

1 2 3 4 5 6 7 8 9
2.6 kb
β-actin

Figure 2. Slot blot of ovine endometrial total RNA probed for TGF-ß1 (A) and ß-actin (B). Day of gestation (P) or cycle (N) from which the samples were taken is indicated beside each group of samples (n=4; day 16 n=3). Length of time of exposure of film was 44 h for TGF-ß1 and 14 h for ß-actin.

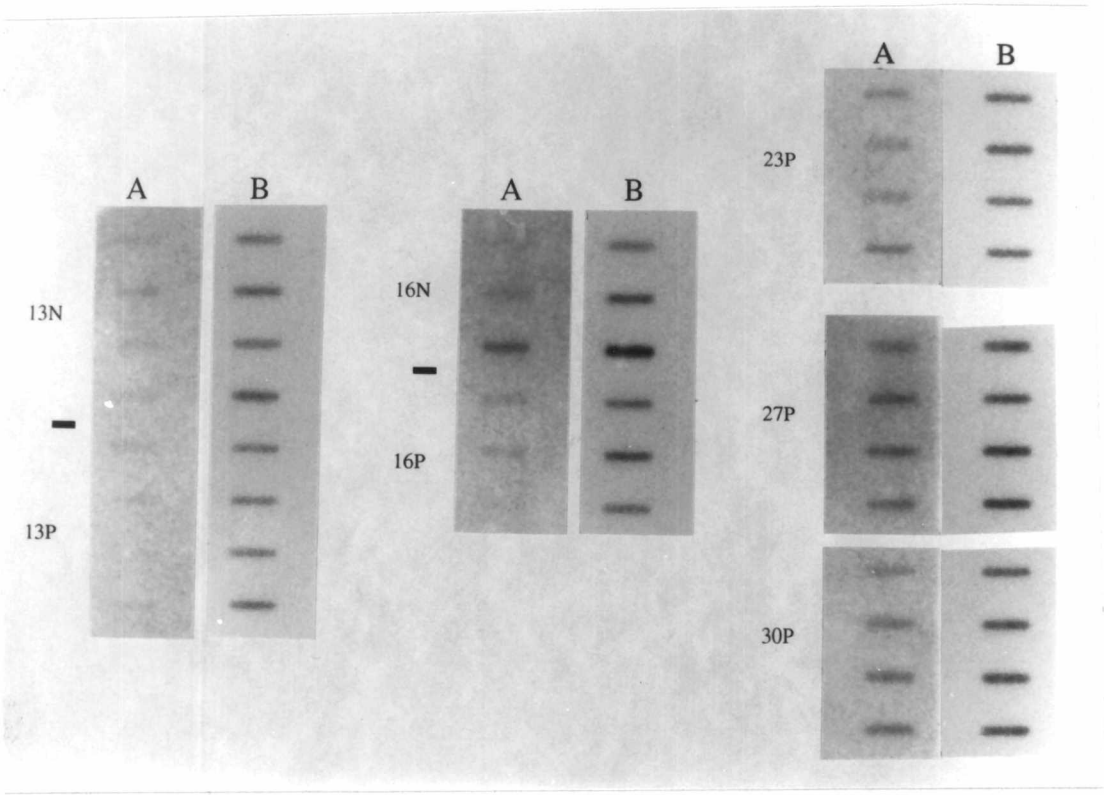
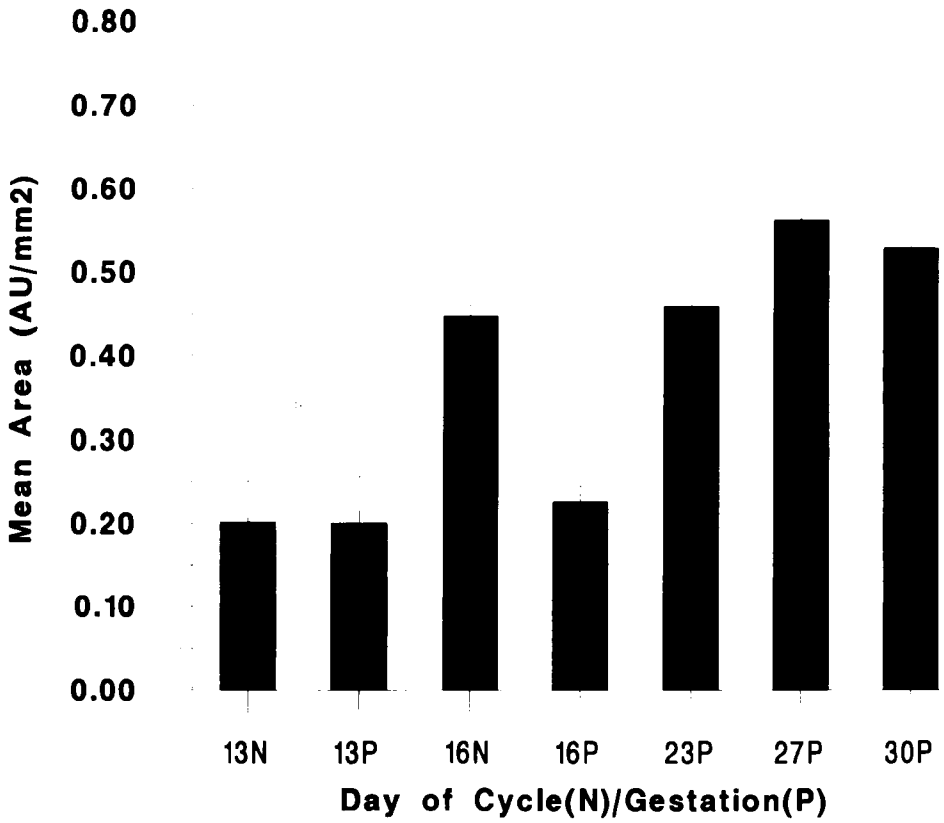

A
B
13N
13P
A
B
16N
16P
A
B
23P
27P
30P

Figure 3. Graphic representation of the mean relative expression levels of endometrial TGF-ß1 during late estrous cycle (N) and early gestation (P). Mean areas were calculated from the computer integrated densitometric scan of the slot blots shown in figure 2. Error bars are SEM calculated from the corrected sample means to indicate sample variation within each day and status.


0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Mean Area (AU/mm2)
13N
13P
16N
16P
23P
27P
30P
Day of Cycle(N)/Gestation(P)

demonstrated that ovine endometrium expressed 5 distinct transcripts of TGF-ß2 (fig. 4). In the ovine endometrial samples, the probe recognized 5 distinct transcripts of 6.2, 5.2, 4.6, 4.0 and 2.7 kb in length. The similar transcript sizes between ovine endometrium, those of the murine positive control and those previously reported for murine and human TGF-ß2 (Miller et al., 1989; O'Rielly et al., 1992) demonstrate the specificity of the probe. Although some of the transcripts differed slightly in size from the mouse placental mRNA control transcripts, the number of transcripts was identical, indicating species differences in specific transcript sizes. Preliminary studies using total RNA indicated the probe bound neither the 28S or 18S rRNA bands (data not shown). There was no detectable change in the relative composition of the transcripts within a sample, between any of the days examined.

Relative quantitation of TGF-ß2 transcripts was achieved by slot blot analysis (fig.5A,B). Correction for differences between samples due to loading variation was as previously described for TGF-ß1 slot blots. Mean areas of each day of cycle or pregnancy were calculated and shown in figure 6. There was no difference between endometrial samples of pregnant or cyclic ewes on day 13. There was, however, a 2.0-fold decrease (P=0.014) in TGF-ß2 message levels from day 13 to 16 of pregnancy. This decline was related to a total decrease in TGF-ß2 transcription rather than a drop in any one

Figure 4. Northern blot of ovine endometrium poly-A enriched RNA probed for TGF-ß2. The 5 major transcripts of 6.2, 5.2, 4.8, 4.0 and 2.7 kb are indicated on the left. Endometrium samples in each lane are; 1, day 16 nonpregnant, 2, day 16 pregnant, 3, day 23 pregnant, 4, adult bovine liver poly-A RNA as the negative control, 5, murine placenta poly-A RNA as the positive control. Length of time of exposure of film was 72 h.

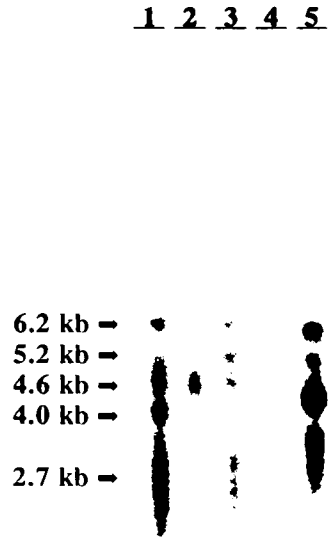

1 2 3 4 5
6.2 kb
5.2 kb
4.6 kb
4.0 kb
2.7 kb

Figure 5. Slot blot of ovine endometrial total RNA probed for TGF-ß2 (A) and ß-actin (B). Day of gestation (P) and cycle (N) from which the samples were taken is indicated above each group (n=4). Length of time of exposure of film was 12 days for TGF-ß2 and 24 h for ß-actin.

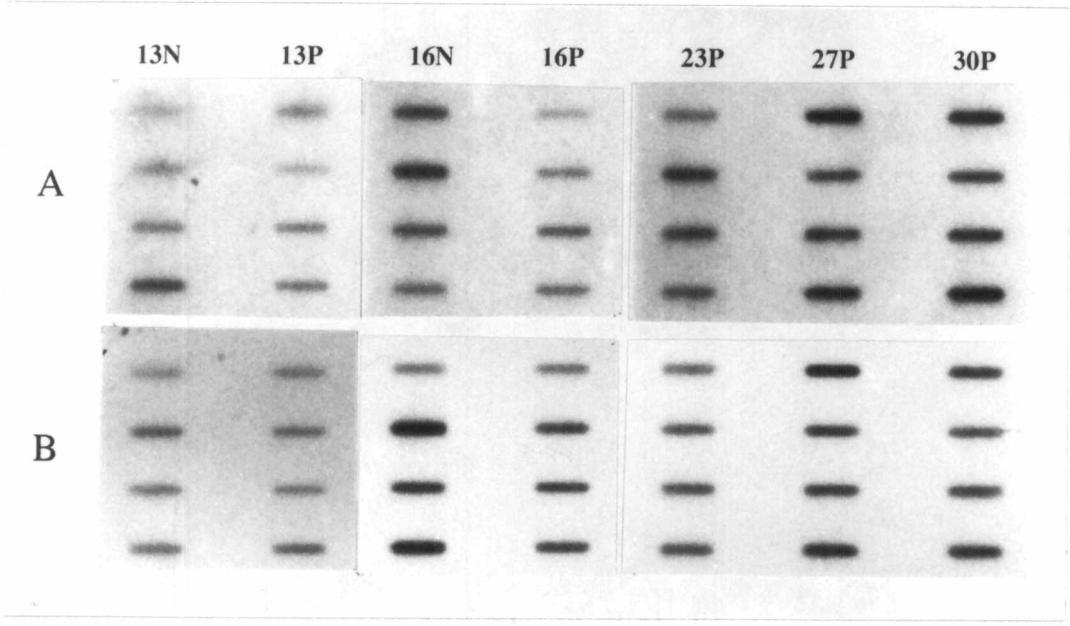
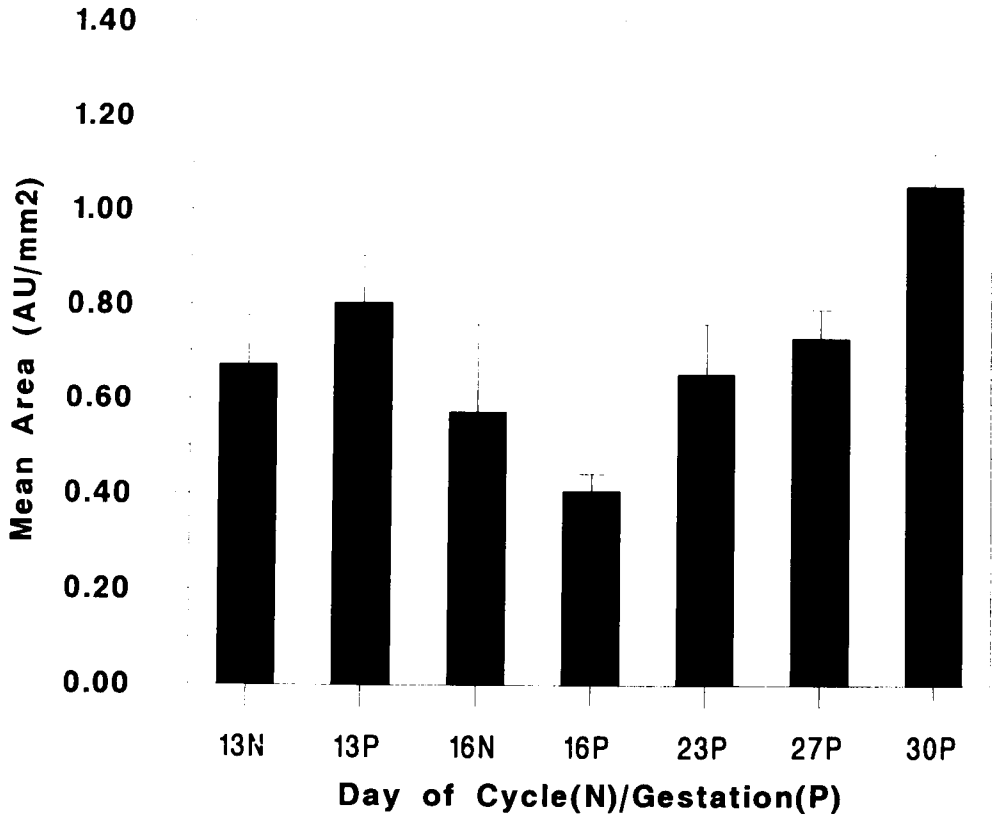

13N
13P
16N
16P
23P
27P
30P
A
B

Figure 6. Graphic representation of the mean relative expression levels of endometrial TGF-ß2 during early gestation (P) and late estrous cycle (N). Mean areas were calculated from the computer integrated densitometric scan of the slot blots shown in figure 5. Error bars are SEM calculated from the corrected sample means to indicate sample variation within each day of gestation or cycle.


1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Mean Area (AU/mm2)
13N
13P
16N
16P
23P
27P
30P
Day of Cycle(N)/Gestation(P)

transcript. Although day 16 levels were the lowest detected, the variability within the cyclic samples negated any detectable differences due to status on this day. Relative to the levels on day 16 of pregnancy, expression of TGF-ß2 increased by 1.6-fold (P=0.11) by day 23, 1.8-fold (P=0.04) by day 27 and 2.6-fold (0.0003) by day 30.

*TGF-ß3*

A single transcript was identified by Northern blot of representative samples of ovine endometrial, poly-A enriched RNA (fig. 7). The ovine endometrial TGF-ß3 transcript was found to be 3.8 kb in length and was present in all days examined. The lack of any other size transcripts demonstrates the specificity of the probe for TGF-ß3, under the high stringency conditions used.

Quantitative changes in TGF-ß3 expression in endometrium of cyclic and early pregnant ewes were determined by slot blot analysis of total RNA (fig. 8A,B). Graphic representation of the mean areas for each day and status is shown in figure 9.

The transcription levels of TGF-ß3 did not differ between pregnant and cyclic day 13 endometrial samples. There was however, a 2.4-fold (P=0.0009) increase in transcript levels from day 13 to day 16 in cyclic samples. This increase was not found in pregnant endometrium, with the levels actually decreasing slightly. A 3.8-fold (P=0.0001) difference was found between tissue from pregnant and cyclic animals on day

Figure 7. Northern blot of ovine endometrial and embryonic poly-A enriched RNA probed for TGF-ß3. The major transcript of 3.8 kb is indicated on the left. Samples in each lane are; 1, day 16 nonpregnant ovine endometrium, 2, day 16 pregnant ovine endometrium, 3, day 23 pregnant ovine endometrium, 4, day 23 chorion and allantois, 5, day 27 chorioallantois, 6, day 30 chorioallantois. Length of time of exposure of film was 19 h.

1 2 3 4 5 6

Figure 8. Slot blot of ovine endometrial total RNA probed for TGF-ß3 (A) and ß-actin (B). Day of gestation (P) and estrous cycle (N) from which the samples were taken is indicated above each group of samples (n=4). Length of time of exposure of film was 16 days for TGF-ß3 and 18 h for ß-actin.

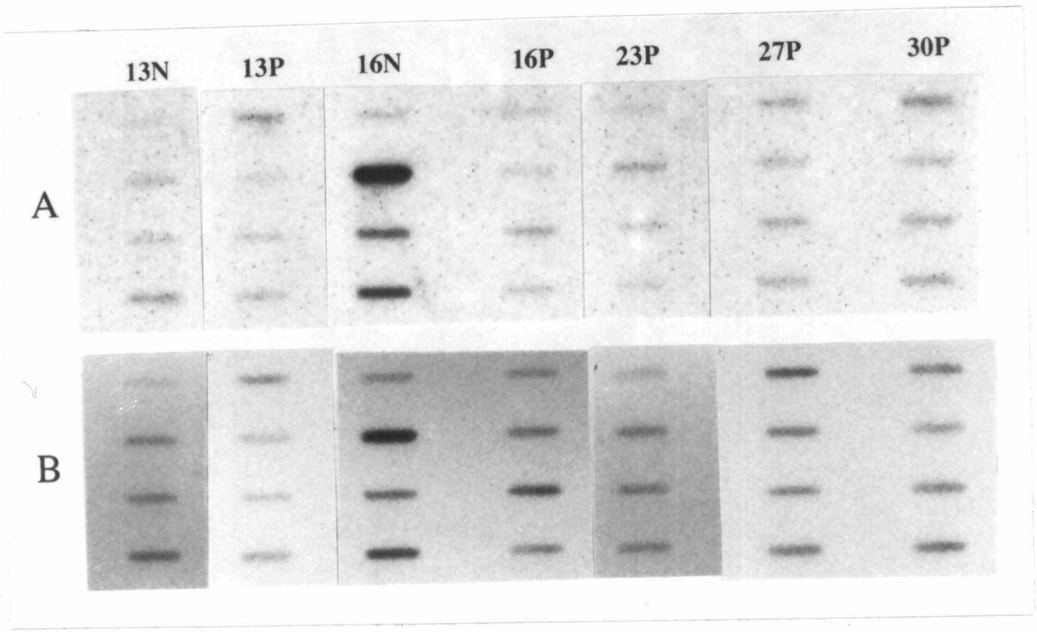
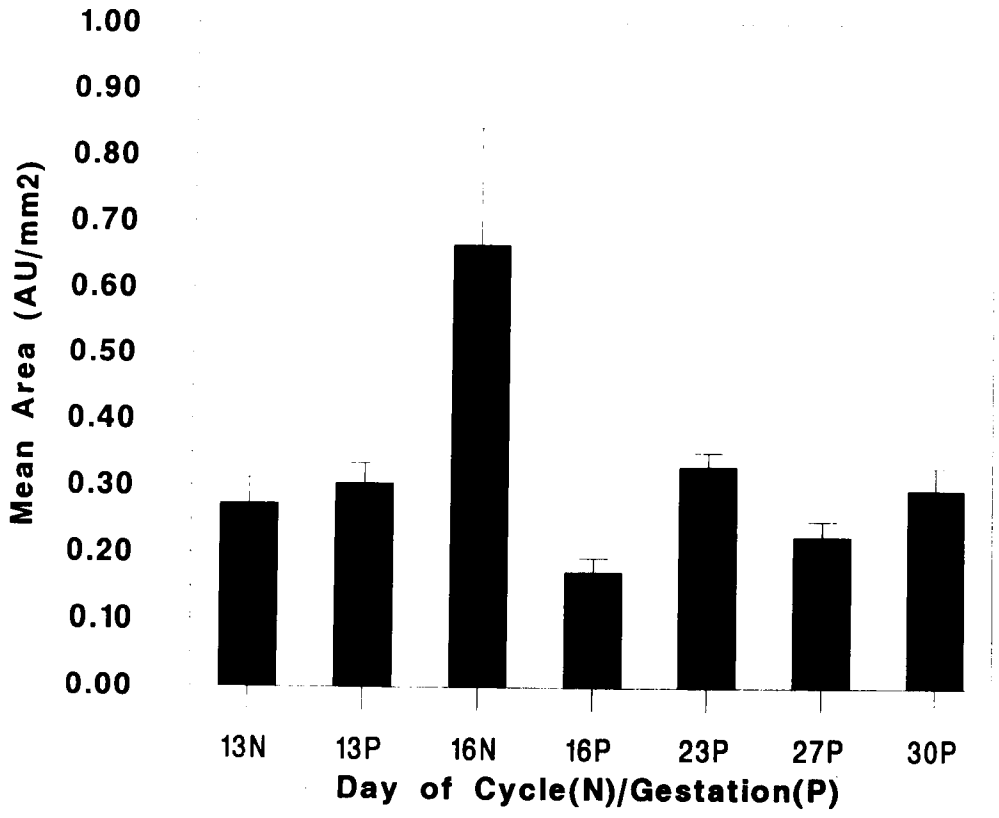

13N
13P
16N
16P
23P
27P
30P
A
B

Figure 9. Graphic representation of the mean relative expression levels of endometrial TGF-ß2 during early gestation (P) and late estrous cycle (N). Mean areas were calculated from the computer integrated densitometric scan of the slot blots shown in figure 8. Error bars are SEM calculated from the corrected sample means to indicate sample variation within each day of gestation or cycle.


1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Mean Area (AU/mm2)
13N
13P
16N
16P
23P
27P
30P
Day of Cycle(N)/Gestation(P)

16. By day 23 TGF-ß3 levels increased to approximately those of day 13 endometrial samples. Levels of TGF-ß3 appeared to decrease slightly in day 27 samples but rebounded by day 30.

# CHAPTER 5

## DISCUSSION

In the ruminant there are no detectable morphological differences between the pregnant and nonpregnant uterus prior to day 14 (Guillomot et al., 1981). During this time the endometrium undergoes extensive glandular development and vascularization in both caruncular and intercaruncular areas (Amoroso, 1952). In this study we found that there was no difference between pregnant or nonpregnant endometrial TGF-ß RNA expression prior to day 16. Since TGF-ßs have been described to stimulate tissue remodelling, this data is consistent with the lack of morphological differences seen between the pregnant and nonpregnant uterus prior to day 16.

At day 16 of pregnancy, expression of all three TGF-ß isoforms were less than cyclic endometrium. Endometrial transition from one estrous cycle to the next requires drastic restructuring. In primates, the majority of the endometrial lining is lost during menses, while in ruminants the luminal epithelium is replaced and the stroma is restructured (Amoroso, 1952). TGF-ßs have been previously shown to stimulate extracellular matrix formation (Rizzino, 1988), cellular matrix binding receptors (Ignotz et al., 1989; Heino et al., 1989; Sheppard et al., 1992) and have been implicated as factors involved in tissue remodelling. In this study, both TGF-ß1 and ß3 expression were found to be greatly increased during diestrus. This increase suggests that these

two TGF-ßs play a part in regulating the restructuring of the endometrium to begin a new estrous cycle.

The transition between each successive estrous cycle involves the loss of corpus luteum progesterone production and increased estrogen production by the newly maturing follicle. In ovariectomized mice, uterine TGF-ß2, but not TGF-ß3, expression was transiently increased by estrogen injection. Progesterone injection had no effect on expression of either TGF-ß isoform (Das et al., 1992). Since estrogen stimulation of TGF-ß2 transcription was limited to less than 12 hours in duration, it is possible that the variation observed in day 16 nonpregnant endometrial TGF-ß2 levels reflects animal variation with respect to levels of circulating estrogen. In the sheep, *in vivo* steady state levels of TGF-ß2 were not found to differ between day 13 and 16 cyclic endometrium. Day 13 would be an environment of high progesterone/low estrogen, while day 16 is low progesterone/increasing estrogen. This inconsistency in observed transcription levels of the TGF-ßs at different stages of the estrous cycle, combined with the lack of reported steroid receptor binding motifs in the promoter regions of any of the TGF-ßs (Kim et al., 1989a; Lafyatis et al., 1990; Malipiero et al., 1990), suggests that the TGF-ßs are not regulated directly by steroid hormones.

In the mouse, TGF-ß3 was found exclusively in the myometrium (Das et al., 1992). In this study, carefully dissected endometrium contained significant quantity of TGF-ß3

mRNA. This may reflect species differences in endometrial TGF-ß isoform expression. Alternatively, TGF-ß3 expression in sheep endometrium was found to be low in early pregnant endometrium and increased dramatically only during late estrous cycle at the transition between estrous cycles. Thus, induction of TGF-ß3 in the endometrium may be temporally limited to a short period during the termination of the estrous cycle which was not included in the mouse studies.

During the time of estrus transition, all TGF-ß expression levels were found to be lower in pregnant than cyclic endometrium. For TGF-ß1, levels did not differ from day 13, however, for both TGF-ß2 and ß3 the levels were the lowest of all days examined. By day 23 of pregnancy the expression levels of all three TGF-ß isoforms increased to levels similar or greater than those prior to day 16. TGF-ß mRNA levels were diminished in pregnant endometrium relative to nonpregnant endometrial levels, indicating the presence of an embryo in the uterus on day 16 suppressed TGF-ß gene expression. The ovine embryo begins to elongate and express several proteins early on day 13 of gestation (Godkin et al., 1984; Farin et al., 1989; Liu et al., 1992). The most abundant of these proteins is an embryonic interferon-$\tau$ (INF-$\tau$), previously called ovine trophoblast protein 1 (Godkin et al., 1984; Farin et al., 1989). Interferons have been shown to have antagonistic effects on TGF-ß stimulation of collagen gene expression (Varga et al., 1990). Conversely, TGF-ß has

been found to inhibit interferon responsiveness of natural killer cells (Rook et al., 1986). However, there are no studies demonstrating any inhibition of interferon and TGF-ß on either's gene transcription.

The time of pregnancy recognition in sheep is approximately day 13 through 16 (Rowson and Moor, 1966; Moor and Rowson, 1966). During this time, secretion of the embryonic INF-$\tau$ alters endometrial release of prostaglandins such that the corpus luteum is maintained for the subsequent pregnancy (Salamonsen et al., 1988; Vallet et al., 1989). In addition, INF-$\tau$ has been shown to alter secretion of several proteins produced by the endometrium (Godkin et al., 1984; Salamonsen et al., 1988). Production of INF-$\tau$ is temporally limited to between days 13 and 21 of gestation. The suppression of TGF-ß expression is limited to between days 13 and sometime after day 16 but before day 23, since samples were obtained early on days 13 and 23. Although the expanding blastocyst secretes a number of products during expansion and extraembryonic membrane formation, the coincidental timing of embryonic INF-$\tau$ production, day 13 through 21, and the alterations of endometrial TGF-ß expression during a similar time, strongly suggest INF-$\tau$ inhibition of endometrial TGF-ß transcription. A number of other products of the conceptus also demonstrate similar temporal expression, therefore further study is required to determine if INF-$\tau$ or some other embryonic factor suppresses endometrial TGF-ß production.

After the establishment of pregnancy, the transcription of each TGF-ß differs greatly, suggesting individual roles for each. Expression of TGF-ß1 increases 2.5 fold from day 16 to peak levels on day 27. The enhanced transcription of TGF-ß1 between days 23 through 30 corresponds to initial stages of placentome development in sheep. During this period endometrial caruncles become extensively vascularized (Amoroso, 1952) and the placenta forms a limited syncytium at the fetal maternal interface (Amoroso, 1952; Wooding, 1984). Angiogenesis, stimulation of placental neovascularization (Pepper et al., 1990; Yang and Moses, 1990), as well as being localized in the placenta (Frolik et al., 1983; Tamada et al., 1989; Graham et al., 1992; Das et al., 1992), are all properties of TGF-ßs and suggest their involvement formation of the placental vasculature. Differentiation of the chorionic cotyledon is localized to the area in contact with the caruncle. On day 27 "reddened patches" on the chorionic membrane become visible in areas in apposition to caruncles. Peak expression of endometrium TGF-ß1 on day 27 corresponds to the initiation of cotyledon differentiation, as well as vascularization of the caruncle.

The role of TGF-ß3 in placental development appears to be minor since induction above a low baseline level only occurs when the ewe is returning to estrus. This observation suggests that the role of TGF-ß3 may be to restructure the endometrium during the transition from one estrous cycle to

the next rather than in placental formation. In contrast to the other two isoforms, TGF-ß2 levels increase significantly between days 27 and 30 of gestation, suggesting its role may be in endometrial modifications during pregnancy rather than endometrial restructuring during the estrous cycle. During this time the caruncle is undergoing structural modifications allowing the chorionic villi to interdigitate and form the crypts of the mature placentome (Steven et al., 1981). Determination of TGF-ß2 mRNA levels at times later in gestation and its cell specific localization, would help clarify the possible role of caruncular TGF-ß2 in placentome formation.

The ovine endometrium was found to express all three isoforms of TGF-ß at the earliest time point examined. During early expansion the change in the porcine blastocyst from spherical to elongated thread is accomplished primarily through cell migration and morphological changes (Geisert et al., 1982). Both of these effects have been previously described for cells treated with TGF-ß (Montesano and Orci, 1988; Delannet and Duband, 1992). Bovine blastocysts cultured *in vitro* are stimulated to progress through the 8 cell block by a mixture of TGF-ß and basic fibroblast growth factor (bFGF), while bFGF or TGF-ß alone were insufficient (Larson et al., 1992). The expression of TGF-ßs by the endometrium may act on the early blastocyst to stimulate activation of the embryonic genome at the 8 cell stage. It has long been

suspected that histotroph produced by the uterus influences the embryo (Amoroso, 1952), and that synchrony of the uterus with the developmental stage of the embryo is important for successful embryo transfer (Rowson and Moor, 1966; Moor and Rowson, 1966). As a component of histotroph, the TGF-ßs may be one of the important signals in the determination of synchrony.

In summary, this study demonstrates that the ovine endometrium expresses all three isoforms of TGF-ß during the estrous cycle and early pregnancy. Species differences and placental structure may explain the differences between this study and previous studies in the mouse (Tamada et al.,1990; Das et al., 1992). Differences in TGF-ß isoform expression between cyclic and pregnant sheep endometrium suggest that TGF-ßs may be involved in normal estrous cycle functions. The expanding blastocyst appears to secrete a factor capable of suppressing transcription of all three isoforms of TGF-ß, identifying three uterine gene products modulated by the developing conceptus. Regulation of expression levels of each isoform differ during early pregnancy, further supporting the concept of each isoform having a distinct function during placental development.

## LIST OF REFERENCES


Amoroso, E.C. (1952). Placentation. In: *Marshall's Physiology of Reproduction, 3rd ed. vol. 2,* (edited by A.S. Parkes) Longman, Green and Co., London, 127-311.

Boshier D.P. (1969). A histological and histochemical examination of implantation and early placentome formation in sheep. J. Reprod. Fert. 19, 51-61.

Chirgwin, J.M. Przybyla, A.E., MacDonald R.J. and Rutler W.J. (1980). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294-5298.

Das, S.K., Flanders, K.C., Andrews, G.K. and Dey S.K. (1992). Expression of transforming growth factor-ß isoforms (ß2 and ß3) in the mouse uterus: analysis of the peri-implantation period and effects of ovarian steroids. Endocrinol. 130, 3459-3466.

de Martin, R., Haendler, B., Hofer-Warbinek, R., Gaugitsch, H., Wrann,M., Schüsener, H., Seifert. J.M., Bodmer, S., Fontana, A. and Hofer, E. (1987). Complimentary DNA for human glioblastoma-derived T cell suppressor factor, a novel member of the transforming growth factor-ß gene family. EMBO J. 6, 3673-3677.

Delannet, M. and Duband, J-L. (1992). Transforming growth factor-ß control of cell-substratum adhesion during avian neural crest cell emigration in vitro. Development, 116, 275-287.

Farin, C.E., Imakawa, K. and Roberts M.R. (1989). In situ localization of mRNA for the interferons, ovine trophoblast protein-1, during early embryonic development in sheep. Mol. Endocrinol. 3, 1099-1107.

Fazleabas, A.T., Bazer, F.W. and Roberts, R.M. (1982). Purification and properties of a progesterone-induced plasmin/trypsin inhibitor from uterine secretions of pigs and its immunolocalization in the pregnant uterus. J. Biol. Chem. 257, 6886-6897.

Frolik, C.A., Dart, L.L., Meyers, C.A., Smith, D.M. and Sporn, M.B. (1983). Purification and initial characterization of a type ß transforming growth factor from human placenta. Proc. Natl. Acad. Sci. USA 80, 3676-3680.

Geisert, R.D., Brookbank, J.W., Roberts, M.R. and Bazer, F.W. (1982). Establishment of pregnancy in the pig: II cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biol. Reprod. 27. 941-955.

Godkin, J.D., Bazer, F.W. and Roberts, R.M. (1984). Ovine trophoblast protein 1, an early secreted blastocyst protein, binds to uterine endometrium and affects protein synthesis. Endocrinol. 114, 120-130.

Guillomot, M., Fléchon, J.E. and Wintenberger-Torres, S. (1981). Conceptus attachment in the ewe: an ultrastructural study. Placenta 2, 169-182.

Heino, J., Ignotz, R.A., Hemler, M.E., Crouse, C. and Massagué, J. (1989). Regulation of cell adhesion receptors by transforming growth factor-ß: concomitant regulation of integrins that share a common $ß_1$ subunit. J. Biol. Chem. 264, 380-388.

Ignotz, R.A., Heino, J. and Massagué, J. (1989). Regulation of cell adhesion receptors by transforming growth factor-ß: regulation of vitronectin receptor and LFA-1. J. Biol. Chem. 264, 389-392.

Kehrl, J.H., Roberts, A.B., Wakefield, L.M., Jakowlew, S., Sporn, M.B. and Fauci, A.S. (1986). Transforming growth factor-ß is an important immunomodulatory protein for human B lymphocytes. J. Immunol. 137, 3855-3860.

Kim, S-J., Glick, A., Sporn M.B. and Roberts, A.B. (1989a). Characterization of the promoter region of the human transforming growth factor-ß1 gene. J. Biol. Chem. 264, 402-408.

Lafyatis, R., Lechleider, R., Kim, S-J., Jakowlew, S., Roberts, A.B. and Sporn, M.B. (1990). Structural and functional characterization of the transforming growth factor ß3 promoter. J. Biol. Chem. 265, 19128-19136.

Laiho, M. and Keski-Oja, J. (1989). Growth factors in the regulation of pericellular proteolysis: a review. Cancer Res. 49, 2533-2553.

Larson, R.C., Ignotz, G.G. and Currie, W.B. (1992). Transforming growth factor ß and fibroblast growth factor

synergistically promote early bovine embryo development during the fourth cell cycle. Mol. Reprod. Dev. 33, 432-435.

Liu, K.H., Gao, K., Baumbach, G.A. and Godkin, J.D. (1992). Purification and immunolocalization of ovine placental retinol-binding protein. Biol. Reprod. 46, 23-29.

Malipiero, U., Höller, M., Werner, U. and Fontana, A. (1990). Sequence analysis of the promoter region of the glioblastoma derived cell suppressor factor/ transforming growth factor (TGF)-ß2 gene reveals striking differences to the TGF-ß1 and -ß3 genes. Biochem. Biophys. Res. Commun. 171, 1145-1151.

Massagué, J. (1990). The transforming growth factor-ß family. Annu. Rev. Cell Biol. 6, 597-641.

Miller, D.A., Lee, A., Pelton, R.W., Chen, E.Y., Moses, H.L. and Derynck, R. (1989). Murine transforming growth factor-ß2 cDNA sequence and expression in adult tissues and embryos. Mol. Endocrinol. 3, 1108-1114.

Montesano, R. and Orci, L. (1988). Transforming growth factor ß stimulates collagen-matrix contraction by fibroblasts: implications for wound healing. Proc. Natl. Acad. Sci. USA 85, 4894-4897.

Moor, R.M. and Rowson, L.E.A. (1966). The corpus luteum of the sheep: functional relationships between the embryo and corpus luteum. J. Endocrinol. 34, 233-239.

Mullins, D.E., Bazer, F.W. and Roberts, R.M. (1980).

Secretion of a progesterone-induced inhibitor of plasminogen activator by the porcine uterus. Cell 20, 865-872.

O'Reilly, M.A., Geiser, A.G., Kim, S-J., Bruggeman, L.A., Luu, A.X., Roberts, A.B. and Sporn, M.B. (1992). Identification of an activating transcription factor (ATF) binding site in the human transforming growth factor-ß2 promoter. J. Biol. Chem. 267, 19938-19943.

Paralkar, V.M., Vukicevic, S. and Reddi, A.H. (1991). Transforming growth factor ß type 1 binds to collagen IV of basement membrane matrix: Implications for development. Devel. Biol. 143, 303-308.

Pepper, M.S., Belin, D., Montesano, R., Orci, L. Vassalli, J-D. (1990). Transforming growth factor-beta 1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells *in vitro*. J. Cell Biol. 111, 743-755.

Rizzino, A. (1988). Transforming growth factor-ß: Multiple effects on cell differentiation and extracellular matrices. Dev. Biol. 130, 411-422.

Rook, A.H., Kehrl, J.H., Wakefield, L.M., Roberts, A.B., Sporn, M.B. (1986). Effects of transforming growth factor ß on the functions of natural killer cells: depressed cytolytic activity and blunting of interferon responsiveness. J. Immunol. 136, 3916-3920.

Rowson, L.E.A. and Moor, R.M. (1966). Development of the

sheep conceptus during the first fourteen days. J. Anat. 100, 777-785.

Salamonsen, L.A., Stuchbery, S.J., O'Grady, C.M., Godkin, J.D. and Findlay, J.K. (1988). Interferon-α mimics effects of ovine trophoblast protein 1 on prostaglandin and protein secretion by ovine endometrial cells *in vitro*. J. Endocrinol. 117, R1-R4.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor.

Samuel, C.A. and Perry, J.S. (1972). The ultrastructure of the pig trophoblast transplanted to an ectopic site in the uterine wall. J. Anat. 113, 139-149.

Samuel, C.A. (1971). The development of pig trophoblast in ectopic sites. J. Reprod. Fert. 27, 494-495.

Sheppard, D., Cohen, D.S., Wang, A. and Busk, M. (1992). Transforming growth factor ß differentially regulates expression of integrin subunits in guinea pig airway epithelial cells. J. Biol. Chem. 267, 17409-17414.

Spector, P.C., Goodnight, J.H., Sall, J.P. and Sarle, W.S. (1985). The GLM procedures. In: *SAS User's Guide; Statistics*, ver. 5. SAS Institute, Cary, NC.

Sporn, M.B. and Roberts, A.B. (1991). Interactions of retinoids and transforming growth factor-ß in regulation of cell differentiation and proliferation. Mol. Endocrinol. 5, 3-7.

Steven, D.H., Burton, G.J. and Samuel, C.A. (1981). Histological and electron microscopy of sheep placental membranes. Placenta (suppl. 2), 11-34.

Tamada, H., McMaster, T., Flanders, K.C., Andrews, G.K. and Dey, S.K. (1990). Cell type-specific expression of transforming growth factor-ß1 in the mouse uterus during the peri-implantation period. Mol. Endocrinol. 4, 965-972.

Tsunawaki, S., Sporn, M.B., Ding, A. and Nathan, C. (1988). Deactivation of macrophages by transforming growth factor-ß. Nature 334, 260-262.

Vallet, J.L., Gross, T.S., Fliss, M.F.V. and Bazer, F.W. (1989). Effects of pregnancy, oxytocin, ovine trophoblast protein-1 and their interactions on endometrial production of prostaglandin $F_{2\alpha}$ *in vitro* in perfusion chambers. Prostaglandins 38, 113-125.

Varga, J., Olsen, A., Herhal, J., Constantine, G., Rosenbloom, J. and Jimenez, S.A. (1990). Interferon-$\gamma$ reverses the stimulation of collagen but not fibronectin gene expression by transforming growth factor-ß in normal human fibroblasts. J. Clin. Invest. 20, 487-493.

Wahl, S.M., Hunt, D.A., Wakefield , L.M., McCartney-Francis, N., Wahl, L.M., Roberts, A.B. and Sporn M.B. (1987). Transforming growth factor ß induces monocyte chemotaxis and growth factor production. Proc. Natl. Acad. Sci. USA 84, 5788-5792.

Wiseman, D.M., Polverini, P.J., Kamp, D.W. and Leibovich, S.J. (1988). Transforming growth factor-beta (TGFß) is chemotactic for human monocytes and induces their expression of angiogenic activity. Biochem. Biophys. Res. Comm. 157, 793-800.

Wooding, F.B.P. (1984). Role of binucleate cells in fetomaternal cell fusion at implantation in the sheep. Amer. J. Anat. 170, 233-250.

Wrann, M., Bodmer,S., de Mártin, R., Siepl, C., Hofer-Warbinek, R., (1987). T cell suppressor factor from human glioblastoma cells is a 12.5 kd protein closely related to transforming growth factor-ß. EMBO J. 6, 1633-1636.

Yang, E.Y. and Moses, H.L. (1990). Transforming growth factor ß1-induced changes in cell migration, proliferation and angiogenesis in the chicken chorioallantoic membrane. J. Cell Biol. 111, 731-741.

# PART 4: GENERAL DISCUSSION

## CHAPTER 1

## GENERAL DISCUSSION OF PARTS 2 AND 3

The purpose for undertaking this study was to obtain information about changes in expression of the three isoforms of TGF-ß (TGF-ß1, ß2 and ß3) during the time of placental formation in the sheep. The main gas and nutrient exchange portion of the sheep placenta is made up of two distinct tissues, the cotyledon and the caruncle (Amoroso, 1952). Unlike the primate or rodent placenta, which form a large syncytium of maternal and fetal tissues, the ruminant placenta forms a limited syncytium of fetal and maternal tissues (Wooding, 1984), although the evidence for this is not conclusive, thereby allowing determination of the source of a particular factor of interest. Because of the physical structure of the sheep placenta it was possible to describe expression of each TGF-ß isoform in both the maternal and fetal portions of the placenta.

Steroids from the ovary regulate many effects on the uterus during the estrous cycle. They stimulate expression of growth factors as well as their receptors (Lingham et al., 1988; Huet-Hudson et al., 1990; Simmen et al., 1990; Geisert et al., 1991), which in turn would cause proliferation and differentiation of uterine cells. The data regarding the effects of steroids on TGF-ß synthesis are not clear. Estrogens are reported to have no effect on transcription rates of TGF-ß1 or ß3 (Das et al., 1992). Antiestrogens have

been reported to stimulate TGF-ß1 protein secretion with no increase in transcription (Knabbe et al., 1987), while conflicting data demonstrated estrogen stimulation of TGF-ß2 transcription (Das et al.,1992). In this study there is no consistent correlation between steroid levels and regulation of any TGF-ß. Progesterone can not be said to be either stimulatory or inhibitory, since all TGF-ß day 16 pregnant endometrium transcription levels are depressed, compared to all subsequent days of pregnancy. Estrogen may have a stimulatory effect on TGF-ß1 and ß3 since day 16 cyclic endometrium mRNA levels are elevated. This elevation may correspond to the high estrogen levels preceding ovulation. However, this contradicts previous reports regarding estrogen effects on these two TGF-ß isoforms.

Placental development in the sheep requires the coordinated growth and differentiation of both the endometrium and the chorion. In this study we found that both the endometrium and the chorioallantois express TGF-ß1 mRNA. The temporal changes in expression level in each of the tissues are similar, i.e. peak expression occurred on day 27 and declined by day 30. From the parallel pattern of expression it would appear that the factor(s) regulating TGF-ß1 expression effect both maternal and fetal components of the early developing placenta in the same way. In contrast, the expression levels of TGF-ß2 in the two placental tissues are mirror images of each other. Endometrial expression

dramatically increases between days 27 and 30 as chorioallantoic expression levels steadily decline from day 23. This difference indicates the regulation of TGF-ß2 is tissue specific, relative to the two sources of placental tissue. Any difference in regulation of TGF-ß3 between the two tissues could not be determined, since chorioallantoic expression was negligible.

The function of TGF-ßs in the immune response appears to be both positive and negative. Because of their immunosuppressive capability, TGF-ßs have been suggested to be involved with blocking the maternal immune response (Clark et al., 1990). In contrast, TGF-ß has also been shown to stimulate the inflammatory response by acting as a chemotactic agent to leukocytes (Wahl et al., 1987; Wiseman et al., 1988; Riebman et al., 1991). Although the embryo could be considered an allograft, no immune rejection occurs. In this study we have shown that all TGF-ß mRNA isoforms are expressed by both the early conceptus and endometrium. However, during embryonic expansion and maternal recognition of pregnancy embryonic TGF-ß expression is slowly increasing while maternal expression is depressed. Teleologically, it would seem to be detrimental for the day 16 embryo to block endometrial TGF-ß production, thus the suppression of the maternal immune response, before the capacity to perform this function has developed in the embryo. Perhaps the function of the inhibition of endometrial TGF-ß expression is not to change

the responsiveness of the existing endometrial immune cell population, but to inhibit recruitment of new immune cells into the endometrium.

In conclusion, this study found:

1. The ovine endometrium does express TGF-ß1, ß2 and ß3 during the late estrous cycle and peri-implantation period.
2. Expression does not differ in day 13 cyclic or pregnant endometrial samples, but is significantly decreased in day 16 pregnant samples relative to cyclic endometrium.
3. The apparent embryonic inhibition of endometrial TGF-ß expression was transient, temporally limited to a time between days 13 and 22, including day 16.
4. Each TGF-ß isoform had a unique pattern of expression throughout the span of this study, suggesting each isoform has different regulatory mechanisms.
5. The ovine blastocyst expresses all three common TGF-ß isoforms, but predominantly TGF-ß1 and ß2.
6. Although present in the pre-expansion blastocyst, both TGF-ß1 and ß2 isoform transcript levels increase during blastocyst expansion, either through increases in gene transcription rates or number of cells expressing TGF-ß.
7. During growth and development of the extraembryonic membranes, the growing allantois expresses more ß1 and ß2 than the fully developed chorion.
8. Like in the endometrium, temporal expression of each TGF-

ß isoform differs in the chorioallantois, suggesting unique roles for each during early placental development.

## LIST OF REFERENCES

Amoroso, E.C. (1952). Placentation. In: *Marshall's Physiology of Reproduction, 3rd ed. vol. 2,* (edited by A.S. Parkes) Longman, Green and Co., London, 127-311.

Clark, D.A., Flanders, K.C., Banwatt, D., Millar-Book, W., Manuel, J., Stedronska-Clark, J. and Rowley, B. (1990). Murine pregnancy decidua produces a unique immuno-suppressive molecule related to transforming growth factor ß2. J. Immunol. 144, 3008-3014.

Das, S.K., Flanders, K.C., Andrews, G.K. and Dey S.K. (1992). Expression of transforming growth factor-ß isoforms (ß2 and ß3) in the mouse uterus: analysis of the peri-implantation period and effects of ovarian steroids. Endocrinol. 130, 3459-3466.

Geisert, R.D., Lee, C-Y., Simmen, F.A., Zavy, M.T., Fliss, A.E., Bazer, F.W. and Simmen, R.C.M. (1991). Expression of messenger RNAs encoding insulin-like growth factor-I, II and insulin-like growth factor binding protein-2 in bovine endometrium during the estrous cycle and early pregnancy. Biol. Reprod. 45, 975-983.

Huet-Hudson, Y.M., Chakraborty, C., De, S.K., Suzuki, Y., Andrews, G.K. and Dey, S.K. (1990). Estrogen regulates the synthesis of EGF in mouse uterine epithelial cells. Mol. Endocrinol. 4, 510-523.

Knabbe, C., Lippman, M.E., Wakefield, L.M., Flanders, K.C., Kasid, A., Derynck, R. and Dickson, R.B. (1987). Evidence

that transforming growth factor-ß is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48, 417-428.

Lingham, R.B. and Stancell, G.M. (1988). Estrogen induction of the epidermal growth factor receptor mRNA. Mol. Endocrinol. 2, 230-235.

Reibman, J., Meixler, S., Lee, T.L. Gold, L.I., Cronstein, B.N., Haines, K.A., Kolasinski, S.L. and Weissmann, G. (1991). Transforming growth factor ß1, a potent chemoattractant for human neutrophils, bypasses classical signal-transduction pathway. Proc. Natl. Acad. Sci. USA 88, 6805-6809.

Simmen, R.C.M., Simmen, F.A., Hofig, A., Farmer, S.J. and Bazer, F.W. (1990). Hormonal regulation of insulin-like growth factor gene expression in the pig uterus. Endocrinol. 127, 2166-2174.

Wahl, S.M., Hunt, D.A., Wakefield , L.M., McCartney-Francis, N., Wahl, L.M., Roberts, A.B. and Sporn M.B. (1987). Transforming growth factor ß induces monocyte chemotaxis and growth factor production. Proc. Natl. Acad. Sci. USA 84, 5788-5792.

Wiseman, D.M., Polverini, P.J., Kamp, D.W. and Leibovich, S.J. (1988). Transforming growth factor-beta (TGF-ß) is chemotactic for human monocytes and induces their expression of angiogenic activity. Biochem. Biophys. Res. Comm. 157, 793-800.

Wooding, F.B.P. (1984). Role of binucleate cells in fetomaternal cell fusion at implantation in the sheep. Amer. J. Anat. 170, 233-250.

# APPENDICES

**APPENDIX A**

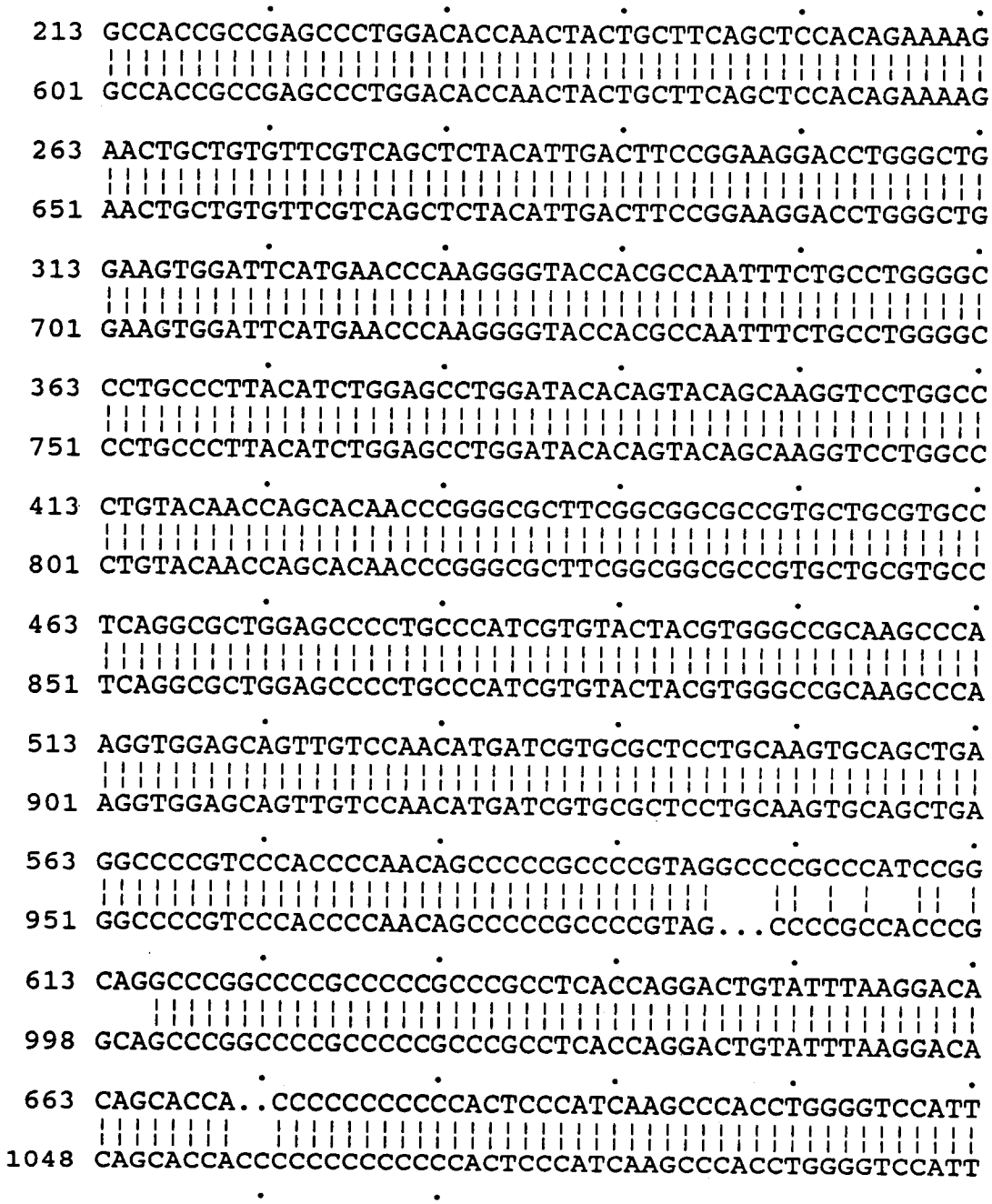
Sequence and Homology Analysis of
cDNA Clone pbTGF-ß1.4

LOCUS BTGFB14 726 BP ENTERED 07/26/92
COMMENT Sequence of bovine TGF-ß1.4 partial cDNA clone. The cDNA clone was isolated from an early bovine conceptus library, constructed using Lambda XR vector. Primer site for generation of TGF-ß1 specific probe is shown in bold italics, while the TGF-ß1 probe is shown in italics. Poly-adenylation site is underlined.

```
1   GCGGCAGTGG CTGACCCGCA GAGAGGAAAT AGAGGGCTTT CGCCTCAGTG
51  CCCACTGTTC CTGTGACAGT AAAGATAACA CGCTTCAAGT GGACATTAAC
101 GGGTTCAGTT CCGGCCGCCG GGGTGACCTC GCCACCATTC ACGGCATGAA
151 CCGGCCCTTC CTGCTCCTCA TGGCCACCCC TCTGGAGAGG GCCCAGCACC
201 TGCACAGCTC CCGCCACCGC CGAGCCCTGG ACACCAACTA CTGCTTCAGC
251 TCCACAGAAA AGAACTGCTG TGTTCGTCAG CTCTACATTG ACTTCCGGAA
301 GGACCTGGGC TGGAAGTGGA TTCATGAACC CAAGGGGTAC CACGCCAATT
351 TCTGCCTGGG GCCCTGCCCT TACATCTGGA GCCTGGATAC ACAGTACAGC
401 AAGGTCCTGG CCCTGTACAA CCAGCACAAC CCGGGCGCTT CGGCGGCGCC
451 GTGCTGCGTG CCTCAGGCGC TGGAGCCCCT GCCCATCGTG TACTACGTGG
501 GCCGCAAGCC CAAGGTGGAG CAGTTGTCCA ACATGATCGT GCGCTCCTGC
551 AAGTGCAGCT GAGGCCCCGT CCCACCCCAA CAGCCCCCGC CCCGTAGGCC
601 CCGCCCATCC GGCAGGCCCG GCCCCGCCCC CGCCCGCCTC ACCAGGACTG
651 TATTTAAGGA CACAGCACCA CCCCCCCCCC CACTCCCATC AAGCCCACCT
701 GGGGTCCATT AAAGGTGGCG AGAGGA
```

Comparison of pbTGF-ß1.4 to previously sequenced bovine TGF-ß1 (Genbank access. M36271).
Percent Identity: 98.894

```
  1 .....................................GCGGCAGTGGCT
                                         ||||||||||||
351 TCACCGGAGTGGCTGTCCTTTGACGTCACTGGAGTTGTGCGGCAGTGGCT
     .         .         .         .         .
 13 GACCCGCAGAGAGGAAATAGAGGGCTTTCGCCTCAGTGCCCACTGTTCCT
    ||||||||||||||||||||||||||||||||||||||||||||||||||
401 GACCCGCAGAGAGGAAATAGAGGGCTTTCGCCTCAGTGCCCACTGTTCCT
     .         .         .         .         .
 63 GTGACAGTAAAGATAACACGCTTCAAGTGGACATTAACGGGTTCAGTTCC
    ||||||||||||||||||||||||||||||||||||||||||||||||||
451 GTGACAGTAAAGATAACACGCTTCAAGTGGACATTAACGGGTTCAGTTCC
     .         .         .         .         .
113 GGCCGCCGGGGTGACCTCGCCACCATTCACGGCATGAACCGGCCCTTCCT
    ||||||||||||||||||||||||||||||||||||||||||||||||||
501 GGCCGCCGGGGTGACCTCGCCACCATTCACGGCATGAACCGGCCCTTCCT
     .         .         .         .         .
163 GCTCCTCATGGCCACCCCTCTGGAGAGGGCCCAGCACCTGCACAGCTCCC
    ||||||||||||||||||||||||||||||||||||||||||||||||||
551 GCTCCTCATGGCCACCCCTCTGGAGAGGGCCCAGCACCTGCACAGCTCCC
```


213 GCCACCGCCGAGCCCTGGACACCAACTACTGCTTCAGCTCCACAGAAAAG
601 GCCACCGCCGAGCCCTGGACACCAACTACTGCTTCAGCTCCACAGAAAAG
263 AACTGCTGTGTTCGTCAGCTCTACATTGACTTCCGGAAGGACCTGGGCTG
651 AACTGCTGTGTTCGTCAGCTCTACATTGACTTCCGGAAGGACCTGGGCTG
313 GAAGTGGATTCATGAACCCAAGGGGTACCACGCCAATTTCTGCCTGGGGC
701 GAAGTGGATTCATGAACCCAAGGGGTACCACGCCAATTTCTGCCTGGGGC
363 CCTGCCCTTACATCTGGAGCCTGGATACACAGTACAGCAAGGTCCTGGCC
751 CCTGCCCTTACATCTGGAGCCTGGATACACAGTACAGCAAGGTCCTGGCC
413 CTGTACAACCAGCACAACCCGGGCGCTTCGGCGGCGCCGTGCTGCGTGCC
801 CTGTACAACCAGCACAACCCGGGCGCTTCGGCGGCGCCGTGCTGCGTGCC
463 TCAGGCGCTGGAGCCCCTGCCCATCGTGTACTACGTGGGCCGCAAGCCCA
851 TCAGGCGCTGGAGCCCCTGCCCATCGTGTACTACGTGGGCCGCAAGCCCA
513 AGGTGGAGCAGTTGTCCAACATGATCGTGCGCTCCTGCAAGTGCAGCTGA
901 AGGTGGAGCAGTTGTCCAACATGATCGTGCGCTCCTGCAAGTGCAGCTGA
563 GGCCCCGTCCCACCCCAACAGCCCCCGCCCCGTAGGCCCCGCCCATCCGG
951 GGCCCCGTCCCACCCCAACAGCCCCCGCCCCGTAG...CCCCGCCACCCG
613 CAGGCCCGGCCCCGCCCCCGCCCGCCTCACCAGGACTGTATTTAAGGACA
998 GCAGCCCGGCCCCGCCCCCGCCCGCCTCACCAGGACTGTATTTAAGGACA
663 CAGCACCA..CCCCCCCCCCCACTCCCATCAAGCCCACCTGGGGTCCATT
1048 CAGCACCACCCCCCCCCCCCCACTCCCATCAAGCCCACCTGGGGTCCATT
711 AAAGGTGGCGAGAGGA.... 726
1098 AAAGGTGGCGAGAGGAAAAA 1117

**APPENDIX B**

Sequence and Homology Analysis of
cDNA Clone pbTGF-ß2.1

LOCUS BTGF-ß2.1 1422 BP DS-DNA ENTERED 12/20/92
Comment Bovine TGF-ß2 partial cDNA clone incomplete sequence. Although this represents the entire length of the clone the sequence has not been confirmed and as such is preliminary. The probe for generating the specific TGF-ß2 probe is indicated in italics. The primer used to generate that probe is indicated in bold, as is the PstI site that produced the 5' end of the probe.

```
1    TCGAGTTTTT TTTTTTTTTT TTTCTTACTT TTTACTTAAA AGAAAAAAAA
51   ATTTTTTTTC CACCTTTAAA AAAAATGCAC TACTGTGTGC TGAGCGCGTT
101  TCTGCTCCTG CATCTGGTCA CGGTCGCGCT CAGCCTGTCT ACCTGCAGCA
151  CGCTCGACAT GGACCAGTTC ATGCGCAAGA GGATCGAGGC GATCCGCGGG
201  CAGATCCTGA GCAAGCTGAA GCTCACCAGC CCCCCAGAAG ACTACCCCGA
251  ACCCGAGGAG GTCCCCCCGG AGGTGATCTC CATCTACAAC AGCACCAGGG
301  ACTTGCTCCA GGAGAAGGCG AGCCGGAGGC GCCGCCTGCG AGCGCGAGCA
351  GCACGAGGAA TACTACGCCA AGGAGGTTTA CAAAATAGAC ATGCCGTCCT
401  TCTTACCCTC GGAAAATGCC ATCCCGCCCA CTTTCTACAG ACCGTACTTT
451  AGAATTGTCC GATTGACGTC TCCTCGATGG AGAAGAATGC TTCCAATTTG
501  GTGAAGGCCG AGTTCAGAGT CTTTCGTTTA CAGAACCCAA AAGCCAGAGT
551  GCCTGAACAA CGGATCGAGC TGTATCAGAT TCTCAAATCC AAAGATTTAA
601  CATCTCCAAC CCAGCGCTAC ATTGACAGCA AACTCGTGAA AACCAGAGCA
651  GAGGGTGAAT GGCTCTCCTT TGACGTCACT GATGCTGTTC ACGAATGGCT
701  CCACCATAAA GACAGGAACC TGGGATTTAA AATAAGTTTA CACTGTCCCT
751  GCTGCACCTT TGTACCATCG AATAATTACA TCATCCCCCA ATAAAAGTGA
801  AGAACTAGAA GCACGCTTTG CAGGTATTCA TGGCACCTCT ACCTATACCA
851  GTGGTGATCA GAAAACAATA AAGTCCACTA GGAAAAAAAA ACAGTGGGAA
901  GAGCCCACAT CTCCTGCTAA TGTTGTTGCC CTCCTACAGA CTTGAGTCCC
951  AACAGTCCAA CCGGCGGAAG AAGCGCGCTC TGGATGCAGC CTATTGCTTT
1001 AGAAATGTGC AGGATAATTG TTGCCTACGC CCACTTTACA TTGATTTCAA
1051 GAGGGATCTT GGGTGGAAAT GGATTCATGA GCCTAAAGGG TACAATGCCA
1101 ACTTCTGTGC TGGAGCATGC CCATATCTGT GGAGCTCAGA CACGTCAGCN
1151 CAGTAGGGTT CTCAGCTTAT ATAATACCAT AAATCCAGAA GCGTCTGCTT
1201 CCCCTTGCTG CGTGTCCACA AAGATTTAGA GACCGCTCAA CCATTCTCTA
1251 CTACATTGGC AAAACACCCA AGATCGAACA GCTTTCTAAT ATGATTGTCA
1301 AGTCTTGCAA ATGCAGCTAA GATTCTCGGA AAAGTGACAA GATGATAATC
1351 GCACGATGAT GATGGTGATG ATGGTGACAA AGGCAATGTT TGTAACAAGA
1401 AAACACAAGA GAGCCTTGCT TCATCAGTGT TA
```

Comparison of the bovine TGF-ß2 region used as the isoform specific probe to murine TGF-ß2 (Genbank access. X57413).
Percent Identity: 88.140

```
          .         .         .         .         .
1251 CTGCATCTGGTCCCGGTGGCGCTCAGTCTGTCTACCTGCAGCACCCTCGA
                                         ||||||||| |||||
 143 .....................................CTGCAGCACGCTCGA
```

```
1301 CATGGATCAGTTTATGCGCAAGAGGATCGAGGCCATCCGCGGGCAGATCC
     |||||| ||||| |||||||||||||||||||| ||||||||||||||||
 157 CATGGACCAGTTCATGCGCAAGAGGATCGAGGCGATCCGCGGGCAGATCC

1351 TGAGCAAGCTGAAGCTCACCAGCCCCCCGGAAGACTATCCGGAGCCGGAT
     |||||||||||||||||||||||||||||| |||||||| || || || ||
 207 TGAGCAAGCTGAAGCTCACCAGCCCCCCAGAAGACTACCCCGAACCCGAG

1401 GAGGTCCCCCCGGAGGTGATTTCCATCTACAACAGTACCAGGGACTTACT
     |||||||||||||||||||| |||||||||||||| |||||||||||  ||
 257 GAGGTCCCCCCGGAGGTGATCTCCATCTACAACAGCACCAGGGACTTGCT

1451 GCAGGAGAAGGCAAGCCGGAGGGCAGCCGCCTGCGAGCGCGAGCGGAGCG
      |||||||||||| |||||||||   ||||||||||||||||||| |  ||
 307 CCAGGAGAAGGCGAGCCGGAGG..CGCCGCCTGCGAGCGCGAGCAGCACG

1501 AGCAGGAGTACTACGCCAAGGAGGTTTATAAAATCGACATGCCGTCCCAC
        |||| |||||||||||||||||||| ||||| ||||||||||||  |
 355 ...AGGAATACTACGCCAAGGAGGTTTACAAAATAGACATGCCGTCCTTC

1551 CTCCCCTCCGAAAATGCCATCCCGCCCACTTTCTACAGACCCTACTTCAG
      | ||||| |||||||||||||||||||||||||||||||||||| ||||| ||
 402 TTACCCTCGGAAAATGCCATCCCGCCCACTTTCTACAGACCGTACTTTAG

1601 AATCGTCCGCTTTGATGTCTCAACAATGGAGAAAAATGCTTCGAATCTGG
     ||| || || ||||| |||||  | |||||||| |||||||| ||| |||
 452 AATTGT.CGATTTGACGTCTCCTCGATGGAGAAGAATGCTTCCAATTTGG

1651 TGAAGGCAGAGTTCAGGGTCTTCCGCTTGCAAAACCCCAAAGCCAGAGTG
     ||||||| |||||||| ||||| || || || |||||| ||||||||||||
 502 TGAAGGCCGAGTTCAGAGTCTTTCGTTTACAGAACCCAAAAGCCAGAGTG

1701 GCCGAGCAGCGGATTGAACTGTATCAGATCCTTAAATCCAAAGACTTAAC
      | || || ||||| || |||
 552 CCTGAACAACGGATCGAGCTG.........................
```

**APPENDIX C**
Sequence and Homology Analysis of
the cDNA Clone pbTGF-ß3.6


LOCUS BTGF-ß3.6 518 BP DS-DNA ENTERED 06/28/91
Comment Bovine TGF-ß3 partial cDNA clone sequence of the 5' most end. Sequence corresponds to region "A" on the above diagram of the clone.

```
1    GTTGGCTTGT CACCCCCTTG GTTATGGTCA CATAAGCGTA ACGAAGACTG
51   CTAGAGCTCT CGGCATCTTG TCTGTGCTCA AGAACAAGAG GAGGAGGGAA
101  GTAGCAGAAC CAGCAGGTTT CCTTTTCCAT CTGTGACTTT TTAAAAAATA
151  CATTTATATA TTTATTTGGC TATACCAGGT CTTAGTTGCA GCACACGGGA
201  TCTTTGATCT TCCTTGCACC ATGTGAGATC TTTAGTTGTG GCATGTGGAT
251  TTAGTTCCCT TACCAGGGAT TGAACCTGTG TCCCCTGCAT TGGGAGTGTG
301  GAGTCTTAGC CACTGAACCA CGAAGGAGGT CCCTCATCTT TGACTTTTAT
351  AAAGAATCAA ATATCTGCCA GAACTTGCCC TTGCCCACTA CATACTTCTG
401  CTTCTGTCTG ATTGATCTAT GCAGGGTTGT ATGACAATCT CTACCTGCAA
451  GGAGGCCAGG AAAACAAATA TCTGGTGTTC CAGACTGTAA TGGGAGGTGG
501  CAAGAGAGAA GAGGAGTA
```

LOCUS BTGF-ß3.6 289 BP DS-DNA ENTERED 03/23/91
Comment Bovine TGF-ß3 partial cDNA clone sequence continues from within the clone beginning at a unique PstI site in the region "B" on the above diagram.

```
1    TGCAGGCAAC AGAACTAAAG TGGCCTGAGA GTCACACTGG GGGTAATCTA
51   GGAATCATGA GTCTAATGGA AGGATGTGTC TCATTTTGAT TCAATAGACT
101  TTAGAATTGG ACCGAGCAGT CATTTACTAT AGCGTGTAAG TGCCGGAGCT
151  TAGAGTTCAT TTCTGGTTGC GTGCACATTA GGGAGGTAAC AGTAAAACCA
201  GCTCAACAAA GTTGAGTTGG CTAATAACCC TTAGGCTTGG TTTTAGGTGG
251  AACACTTCAA GTCTTTATTT TGATTCATGA TGTAAAGGG
```

LOCUS BTGF-ß3.6 1291 BP DS-DNA ENTERED 05/20/91
Comment Bovine TGF-ß3 partail cDNA clone sequence continues within the clone. Sequence corresponds to region "C" in the above diagram and includes the coding region of the mature active protein region and intron/exon boundries similar to human TGF-ß3 at exon 6.

```
1    TCTGGATCTG TCTCACAGAG AAATGCTTAT GTTAATACAG CTCAGTGCAC
51   TTGAACCTGG GTCCCACTGA CCAGAGCCAC CTCTTATGAT GACCAGAAGA
101  CTCCAGTGCC CAGATGAGGC CACTTGTGGA GTTTTTATGT TAGTGATTTC
151  CTTGTCAGTT TTTTTTTTTC TTTTTTTAGT AATTTATTTT TCAATTGTTG
201  CAGCAATTTG GAGGAGAACT GCTGTGTGCG TCCTCTCTAC ATTGACTTCC
```

```
251  GACAGGATCT GGGCTGGAAA TGGGTCCATG AACCTAAGGG CTACTATGCC
301  AACTTCTGCT CAGGCCCATG CCCATACCTC CGCAGCTCAG AACAGCACGG
351  TATGGTACAG GGTCATGCCT TATGGGGTGC TGGGGCTGAG CTGACCAGGC
401  CACTTGTTTA AAAAGGATGC ATGATGGGCT GCATGTTAAA TGAGTGGATG
451  GATGATGATC TGGGGTGTAA AATCCCTTAT GGTGAAGGTT CTGTATTTGG
501  GCCCAACTCC AGTGGGTNGA CATTCTACCC TTTATAACTT TCTTGCATAG
551  TTTCAACCAG ACTTTTAAGT TGTTTATTTG CTCTTGTACT ATGGGAATTA
601  CTTAGCTGTC ACATGCCACT CCATATGTCA GAGAGTTTGG GGGCCAAAAG
651  ACTTCTATGA TCAGATGCTG CTGCTGCTGC TAAATCGCTT CAGTTGTGTC
701  CGACTCTGTG CAACCCCATA GAAGGCAGCC CACCAGGCTC CTCTGTCCCT
751  GGGATTCTCC AGGCAAGAAT ACTGGAGTGG GTTGCCATTT CCCTCTCCAT
801  CTACGATCAG ATAAAGAACT CTAATAATTC TTTTCTCTTG AAAGTAGAAG
851  TTATTTTAAA AGATAACGTA AAACACACAG AAGAAGCCAA CATGAAACAA
901  AGGGAAGAGA CAAGAGAGAG GGAAGTCGAC GTCTGAAGCT AGCTTGTCTT
951  TCTCTCCAGG GGAATCCGAG AGAGGGCCGT AACTTGTGAA GCATATTTCC
1001 TCATAGCTCC TCACCAAAGT GACCAGTTAC AGCTAGGAGG AACCAACATA
1051 GGCATGTTTC TTCCCAGCCA AGAGAATTTC ATGATCAGTT CCAAGTCCCT
1101 TGGTTGTTGT GGGGGTCATG CTTAAACCAG TAATGCTCAG ACCAGTGCCG
1151 TATGTTGCAT TCCTGTGAGC AGCAACCTGC CACTCACCCA AACTGGGTTC
1201 CGCCAGTCCT CCTGAAATAA CATCAGTGGC CTATTTTGGT GGCTCAGACG
1251 GTAAAGAATT TGCTGGCAAT GCAGGAGACC CGGATTTGAT C
```

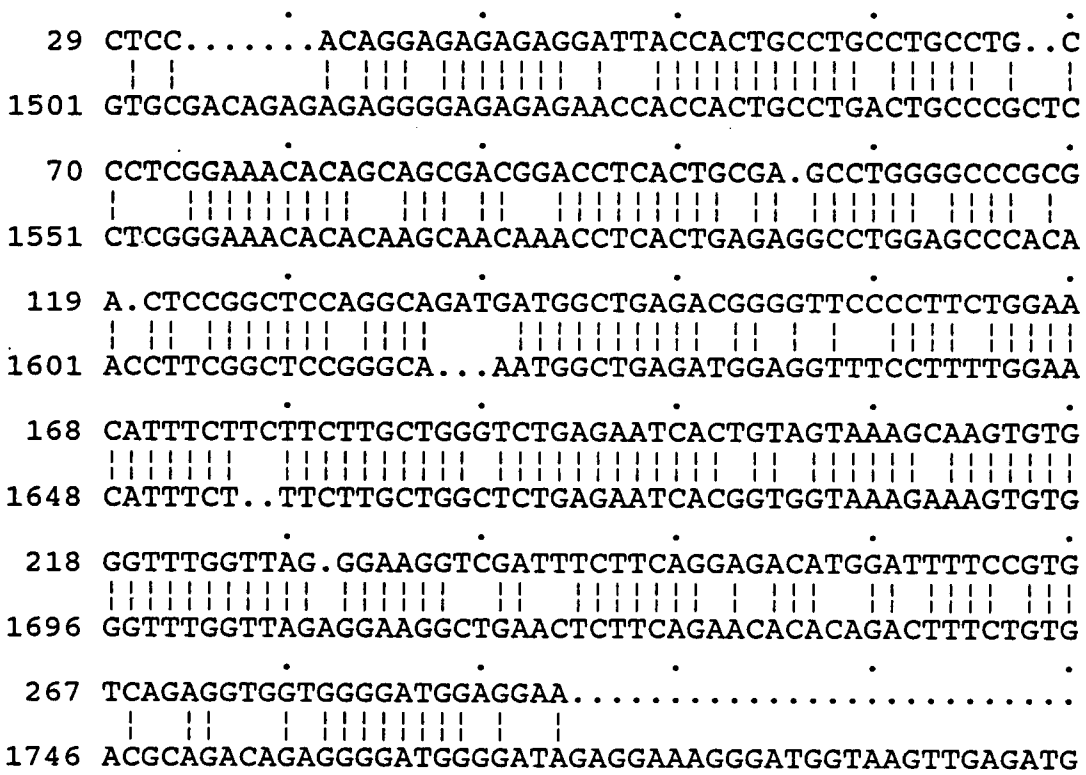
LOCUS BTGF-ß3.6 291 BP DS-DNA ENTERED 05/20/91

Comment Bovine TGF-ß3 partial cDNA clone sequence continues within the clone corresponding to region "D" of the above diagram. The sequence is near, but not inclusive of the complete 5' end of the clone and includes the 3' end of the mature protein coding region as well as a portion of the 3' untranslated region. This area corresponds to the human exon 7 region. The TGF-ß3 specific probe was generated from this sequence and is indicated in italics, although the probe contains sequence not included here. The primer used to generate the probe is shown in bold.

```
1    AAGTCCTCGA AGTGCAGCTG AGGCCCGACC TCCACAGGAG AGAGAGGATT
51   ACCACTGCCT GCCTGCCTGC CCTCGGAAAC ACAGCAGCGA CGGACCTCAC
101  TGCGAGCCTG GGGCCCGCGA CTCCGGCTCC AGGCAGATGA TGGCTGAGAC
151  GGGGTTCCCC TTCTGGAACA TTTCTTCTTC TTGCTGGGTC TGAGAATCAC
201  TGTAGTAAAG CAAGTGTGGG TTTGGTTAGG GAAGGTCGAT TTCTTCAGGA
251  GACATGGATT TTCCGTGTCA GAGGTGGTGG GGATGGAGGA A
```

Comparison of bovine TGF-ß3 region isolated to be used as the specific probe to human TGF-ß3 mRNA (Genbank access. X14149).
Percent Identity: 78.671

```
                .         .         .         .         .
      1 .....................AAGTCCTCGAAGTGCAGCTGAGGCCCGAC
                              ||||| |  || || |||||||  ||| ||
   1451 CAGCTCTCCAACATGGTGGTGAAGTCTTGTAAATGTAGCTGAGACCCCAC
```


29 CTCC.....ACAGGAGAGAGAGGATTACCACTGCCTGCCTGCCTG..C
1501 GTGCGACAGAGAGAGGGGAGAGAGAACCACCACTGCCTGACTGCCCGCTC
70 CCTCGGAAACACAGCAGCGACGGACCTCACTGCGA.GCCTGGGGCCCGCG
1551 CTCGGGAAACACACAAGCAACAAACCTCACTGAGAGGCCTGGAGCCCACA
119 A.CTCCGGCTCCAGGCAGATGATGGCTGAGACGGGGTTCCCCTTCTGGAA
1601 ACCTTCGGCTCCGGGCA...AATGGCTGAGATGGAGGTTTCCTTTTGGAA
168 CATTTCTTCTTCTTGCTGGGTCTGAGAATCACTGTAGTAAAGCAAGTGTG
1648 CATTTCT..TTCTTGCTGGCTCTGAGAATCACGGTGGTAAAGAAAGTGTG
218 GGTTTGGTTAG.GGAAGGTCGATTTCTTCAGGAGACATGGATTTTCCGTG
1696 GGTTTGGTTAGAGGAAGGCTGAACTCTTCAGAACACACAGACTTTCTGTG
267 TCAGAGGTGGTGGGGATGGAGGAA.....
1746 ACGCAGACAGAGGGGATGGGGATAGAGGAAAGGGATGGTAAGTTGAGATG

# VITA

Jules Joseph E. Doré, Jr. was born November 9, 1962 in Calgary, Alberta, Canada. He is the third son of Jules and Geraldine Doré, the fourth of six children. He graduated from the University of British Columbia in 1984 with an honors degree in Zoology. After graduating, he entered the graduate program at The University of Florida where in 1987 he received a Master of Science degree in Animal Science. After a two year hiatus, in August of 1989 he was admitted to the Graduate School at The University of Tennessee, Knoxville in the College of Veterinary Medicine's program of Comparative and Experimental Medicine. In August of 1993 he was awarded the degree of Doctor of Philosophy.