AN ABSTRACT OF THE THESIS OF

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Title: Membrane-Type Matrix Metalloproteinase and Inhibitor Expression in Sheep Embryos and Uterus.

Approved: Redacted for privacy

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Expression of membrane-type matrix metalloproteinases (MT) and tissue inhibitors of matrix metalloproteinases (TIMP) was evaluated in sheep embryos and uterus during the pre- and peri-implantation periods. Embryos and uterine samples were surgically collected from ewes on days 9, 11, 13, and 15 of pregnancy (n = 3 ewes/day) and of the estrous cycle (n = 2 ewes/day). Total RNA was extracted and RT-PCR were performed using primers specifically designed from published human, mouse, and bovine complete cDNA sequences for MT-1, -2, -3, and -5, and TIMP-1, -2 and -3. Multiplex PCR were performed on uterine samples for each gene at optimal cycles and temperatures with 18S rRNA as the internal standard. For embryos, PCR were conducted for 40 cycles at optimal temperatures. MT-1, -2, -3, and –5 were observed in pregnant and nonpregnant uterus during all days of collection. No difference ($P > 0.10$) was observed in MT-1 or –2 expression due to day of collection. However, pregnant uterus expressed more ($P = 0.096$) MT-1 than nonpregnant uterus, whereas expression of MT-2 was greater ($P < 0.05$) in nonpregnant compared to pregnant uterus. No differences ($P > 0.10$) in MT-3 expression were observed due to pregnancy status, however Day 9 and 11 expressed more MT-3 than Day 15. Uterine MT-5 expression was not different ($P > 0.10$) between pregnant and nonpregnant females, however Day 15 uterus expressed less ($P < 0.05$) MT-5 then Day 11 and 13 uteri. TIMP-1 expression was greater ($P < 0.05$) in pregnant compared to nonpregnant uterus, but did not differ ($P > 0.10$) by day of

collection. TIMP-2 did not differ ($P > 0.10$) by pregnancy status or day of collection but the interaction was significant ($P < 0.05$). TIMP-2 expression was greatest in Day 9 pregnant uterus and least in Day 9 nonpregnant uterus. No difference ($P > 0.10$) was observed in expression of TIMP-3 due to day of collection or pregnancy status. Embryos expressed MT-3 and –5 during Days 9-15 of development, however, MT-1 and -2 were not detected. The presence of MT and TIMP in the endometrium suggests these proteins may play important roles in regulating extracellular matrix degradation and activating other matrix metalloproteinases for endometrial remodeling and preparation for implantation. Embryonic MT may participate in the processes of embryonic expansion, elongation and attachment.

Membrane-Type Matrix Metalloproteinase and Inhibitor Expression in Sheep Embryos and Uterus

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Membrane-Type Matrix Metalloproteinase and Inhibitor Expression in Sheep Embryos and Uterus

INTRODUCTION

Embryonic and uterine factors facilitating the implantation process are critical to a successful pregnancy. Uterine receptivity to the invading embryo at the appropriate time is dependent upon cellular apoptosis and replication (Duc Goiran et al., 1999). For the embryo to develop from a one-cell zygote to a blastocyst poised to attach to the endometrium a broad spectrum of cellular replication, migration, and reassociations must occur. Cellular changes in the uterus and embryo in preparation for implantation require extensive regulation of extracellular matrix (ECM) degradation. The major proteins involved in regulating ECM breakdown are the matrix metalloproteinases (MMP) and the tissue inhibitors of MMP (TIMP).

Matrix metalloproteinases are zinc endopeptidases that degrade various components of the ECM. There are several classes of MMP; however the gelatinases are the most abundant in reproductive tissues and embryos. In the rodent, MMP have been found in both the embryo (Werb et al., 1992; Bany et al., 2000) and uterus (Alexander et al., 1996; Das et al., 1997; Nuttall et al., 1999; Bany et al., 2000). Rodents have a highly invasive type of implantation, which occurs when the embryo reaches the uterus. Ruminants have a less invasive type of implantation, and the embryo undergoes a period of expansion and elongation prior to uterine attachment. Salamonsen et al. (1993) and Vognoni et al. (1998) detected MMP-2 and MMP-9 in the pregnant and non-pregnant uteri of the ewe. While the sheep embryo doesn't express any proteases, besides uPA, until Day 13 of gestation (Menino et al., 1999).

The newest class of MMP, the membrane-type MMP (MT), have not been studied in the reproductive tissues of the livestock species. Membrane-type matrix metalloproteinases are involved in ECM degradation (Ohuchi et al., 1997: D'ortho et al., 1997; D'ortho et al., 1998; Knauper and Murphy et al., 1998; Koshikawa et al., 2000) and

MMP-2 (gelatinase A) activation (Lichte et al., 1996; Knauper et al., 1996; Lehti et al., 2000), and they may participate in pre- and peri- implantation events.

Tissue inhibitors of matrix metalloproteinases are important in regulating MMP activity and inhibit excessive ECM degradation. The TIMP have also been found to link latent MMP-2 to MT-1 in the MMP-2 activation cascade. They have also been found in the uteri of the livestock species (Buhi et al., 1997; Hampton et al., 1995; Salamonsen et al., 1995), and the embryo (Menino et al., 1997,1999). Hampton et al. (1995) observed that TIMP-1 and –2 were both expressed in the cyclic and pregnant sheep uterus. In the cyclic ewe, TIMP-1 and –2 levels increased during the cycle but in the pregnant ewe levels increased from Day 4-14 and remained high until Day 20. TIMP-1, -2 and –3 have also been detected in pig and sheep embryos (Menino et al., 1997,1999).

Membrane-type matrix metalloproteinases and TIMP-3 expression has not been evaluated in sheep uterus. Furthermore, MT expression has not been investigated in the sheep embryo. Our hypothesis is that MT and TIMP are expressed in the sheep uterus and embryo during the period of embryonic expansion, elongation and uterine attachment. The goals of this study were to 1) generate a complete phenotypic map of MT and TIMP in the pregnant and non-pregnant sheep uterus during the pre and peri implantation period, and 2) evaluate expression of MT in the pre- and peri-implantation sheep embryo.

LITERATURE REVIEW

The Implantation Process

The preimplantation period of embryo development is the most critical period in determining embryo survival (Carnegie et al., 1985). However, pre-implantation and implantation events differ between species. For example, human implantation begins almost immediately upon the embryo's arrival in the uterus, and is extremely invasive. The ruminant embryo, on the other hand, has a less invasive type of implantation and undergoes a prolonged period of elongation and expansion before beginning attachment. In all species implantation revolves around the coordination of two events: blastocyst formation and the uterine "window of receptivity" (Duc-Goiran et al., 1999). Several days after fertilization (Day 0) the zygote has reached the multicellular morula stage. Coincident with an increase in Na/K ATPase activity, colony-stimulating factor-1 (CSF-1), and E-cadherin, the morula compacts and a fluid filled cavity develops giving rise to the blastocyst. The developing embryo secretes a variety of factors including: cytokines, early pregnancy factor, pre-implantation factor, growth factors, PGE_2, platelet activating factor, hCG, and INFτ, and upregulates receptors for CSF-1, EGF, and LIF (Duc Goiran et al., 1999). At the same time, glandular epithelium of the uterus, under the influence of estrogen and progesterone, secretes LIF, placental protein 14, and prolactin, and up-regulates EGF receptors and some integrins. In the human uterus, expression of the vitronectin receptor, $\alpha v\beta 4$, marks the beginning of the "window of receptivity" and the disappearance of the $\alpha 4\beta 1$ integrin indicates the end of this "window" (Duc-Goiran et al., 1999).

The human embryo reaches the uterus on Day 5-6 and begins the process of implantation almost immediately. Implantation begins first with apposition, where the embryo orients itself with the inner cell mass apposed to the endometrium at the site of implantation (Bentin-Ley et al., 2000). Once in this position, adhesion begins and desmosome-like connections form between trophectoderm and luminal epithelium. The uterus displays an inflammatory response to the invading embryo and clamps down around the embryo. At the site of attachment in the human and rodent, endometrial

decidual cells develop (Cross et al., 1994) and secrete TIMP, TGF-β, and LIF (Duc-Goiran et al., 1999; Vogiagis & Salamonsen, 1999). In a coordinate fashion, the implanting embryo also secretes many proteinases to facilitate uterine penetration. At the implantation site, uterine apoptosis is apparent and trophoblastic cytoplasmic protrusions interdigitate between uterine epithelial cells. The trophoblast cytoplasmic protrusions spread along the basement membrane and invade the uterine stroma, giving rise to the formation of intercommunicating lacunae (Bloom & Faccett 1975). By Day 11, the embryo has penetrated the endometrium and reached the myometrium constituting the heamochorial type of placentation (Kimber, 2000).

The ruminant has a less invasive type of placentation and is classified as epitheliochorial (Cross et al., 1994), where fetal trophoblast attaches to maternal endometrium with little penetration of the epithelium. In sheep, the embryo attains the morula stage by Day 4 and arrives in the uterus on Day 4-5. On Day 6.5, the morula develops into a blastocyst and by Day 8 endodermal cells differentiate and migrate away from the ICM to form the bilayered trophoblast. The Day 8 sheep embryo has a squamous monolayer of trophoblast with microvilli and cytoplasmic projections. The embryo loses the zona pellucida on Day 8-9, and undergoes a period of elongation and expansion. On Day 11, elongation begins and continues to Day 14, where the once spherical embryo becomes filamentous in shape and is approximately 10 cm in length (Wintenberger-Torres & Flechon, 1974). Apposition begins on Day 15 and the embryo orients itself with the abembryonic trophectoderm closest to the uterine epithelium. Microvillious projections from the trophectoderm penetrate uterine glands and signal onset of adhesion (King et al., 1982). As trophectodermal cells continue to differentiate and secrete placental lactogen and IFNτ on Day 14 and 15, respectively, (King et al., 1985; Salamonsen et al., 1994; Vogiagis & Salamonsen 1999), chorion and amnion develop (Guillomot et al., 1982). At this time, endometrial cells produce MMP (Salamonsen et al., 1994), and neutral endopeptidase (Riley et al., 1995), and express EGF receptors (Gharib-Hamrouche et al., 1993), osteopontin, integrins for fibronectin, and down regulate mucin expression (Bowen et al., 2000). At attachment, uterine LIF expression peaks at the caruncular and intercaruncular regions (Vogiagis et al., 1997; Vogiagis & Salamonsen, 1999). Expression of integrins and osteopontin allow

trophectodermal cells to attach and migrate on the uterine epithelium. During implantation, the embryo continues to grow and by Day 16, the trophectodermal cells have differentiated into uninucleate and binucleate cells (Wooding et al., 1982; King et al., 1982; Carnegie et al., 1985; Guilomot et al., 1995). Binucleate cells begin migrating on Day 18 and continue migrating through the placentomes, forming a synctium, that persists throughout gestation. In the ewe, there is more extensive uterine degeneration during the first month of gestation and attachment is diffuse. The placentomes become more developed after the first month of gestation and possess interlocking chorionic villi which remain the main sites of attachment throughout gestation (King et al., 1982). Attachment is complete when there is adhesion of interdigitating uterine microvilli and trophoblastic plasma membrane in the placentome. The events in early gestation are highly regulated and proteinases such as MMP are proposed to be extensively involved.

Matrix Metalloproteinases

Matrix metalloproteinases (MMP) are a family of endopeptidases that degrade the extracellular matrix (ECM). They have high sequence homology and are structurally related with common multidomain organization (Figure 1). Matrix metalloproteinases are latent proenzymes, which usually begin with a 20 amino acid (a.a.) signal peptide, followed by an ~80 a.a. prodomain, and an ~170 a.a. catalytic domain, linked by a proline rich hinge region to the ~195 a.a. C-terminal haemopexin-like domain. The catalytic domain contains two calcium-binding domains and a zinc-binding region. Zinc and calcium ion binding is required for structural stability and activity of the enzyme (Birkedal-Hansen et al., 1993). The hemopexin domain is the site of substrate recognition (Woessner, 1998). A recently discovered group of MMP, the membrane-type MMP (MT), are different from the other MMP because they are fixed to the membrane. Membrane-type matrix metalloproteinases lack a signal peptide and have a 75-100 a.a. extension on the C-terminus that forms the transmembrane domain and cytoplasmic tail (Bode et al., 1999).

Matrix metalloproteinases activity is highly regulated by several processes: transcription, activation, and regulation by tissue inhibitors of MMP (TIMP). Transcriptional control is affected by several proteins: interleukin-1 (IL-1), tumor

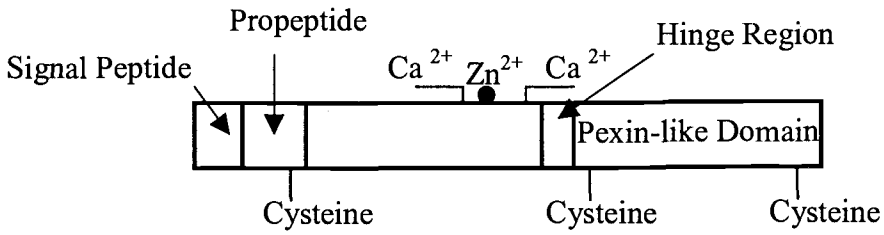

Figure 1. Core structure of matrix metalloproteinase (Birkedal-Hansen et al., 1993)

necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), integrin linked kinase (ILK) (Troussard et al., 2000), epidermal growth factor (EGF) and several other growth factors, cytokines, and hormones (Birkedal-Hansen et al., 1993) acting at the AP-1 binding site on the gene. Growth factors and cytokines, in conjunction with transcription factors Fos and Jun, bind to the AP-1 binding site and induce transcription of MMP. The AP-1 binding site can also have inhibitory action, when glucocorticoids and retinoic acid receptors bind and inhibit transcription, however MMP-2, -10, and -11 lack the AP-1 binding site (Borden & Heller, 1997; Zhang et al., 2000). The Ets binding site (PEA-3) is another important DNA binding region that controls transcription of MMP. Ets family proteins bind to DNA and promote transcription of MMP, however human MMP-2 and MMP-9 lack the ets binding sites. Some MMP have TGF-inhibitory elements that are putative sites for the direct binding of TGF-β, which inhibits the production of MMP. There are also several other minor binding sites that can control transcription of MMP (Borden & Heller, 1997).

The process of activation cleaves the "pro" segment of the zymogen and produces the active enzyme. In vitro organomercurials or trypsin can activate MMP by cleaving an 8 kDa peptide downstream from the preserved cysteine residue. Nagase (1997) identified three types of activation processes: 1) stepwise, 2) activation on the cell surface, and 3) intracellular activation. The stepwise method starts with the prohormone that is converted into several intermediates via proteinase activity or treatment with reagents such as mercurial compounds. Corcoran et al (1996) divided the stepwise extracellular activation into two groups, activation via non-MMP proteinases and MMP. One of the proposed pathways for MMP activation in vivo is via the plasminogen/plasmin pathway. Lijen and coworkers (1998) studied the relationship between the plasminogen/plasmin pathway and activation of proMMP-2 and –9. ProMMP-2 and –9 activation is enhanced by the presence of plasmin, but proMMP-2 and –9 were activated in plasmin(ogen) null mice fibroblasts. Plasmin(ogen) null mice lacked active MMP-9 in muscle, therefore activation of proMMP-9 in muscle cells may occur through a plasmin-dependent pathway. Plasmin has also been found to convert MMP-1, -8, -10, and –11 into intermediates. The intermediates are converted to the active MMP by activated MMP in the extracellular space. Matrix metalloproteinases can also be activated in the

extracellular space by other MMP. For example, MMP-7 can activate MMP-3 and –9 (Nagase 1997). The cell surface activation is mediated via the membrane-type (MT) MMP. Lastly MMP can be activated intracellulary by furin, a golgi-associated subtilisin-like proteinase.

Matrix metalloproteinases are divided into classes depending on their structure and proteinase substrate. Matrix metalloproteinase-1, -8, and –13 make up the collagenase family, which degrade collagens I, II, III, VII, VIII, and X, gelatin, and proteoglycan core proteins. Collagenases have been found in human periodontal tissue, bone, skin, synovial fluid, and uterus (Jeffery, 1998). Specifically, MMP-1 is expressed in reproductive tissues of the rodent (Hurst et al., 1999) and the ewe (Salamonsen et al., 1994; Salamonsen et al., 1995).

There are three stromelysins, MMP-3, -10, and –11. Stromelysins degrade ECM components such as proteoglycan core proteins, fibronectin, laminin, collagenases IV, V, IX, and X, and elastin. These enzymes have been found to play a role in embryo development, morphogenesis, tissue resorption, reproduction and angiogenesis (Nagase, 1998). Matrix metalloproteinase-3 is weakly expressed in placenta and uterus of the ewe (Zhang et al., 1998) and uterus of the rodent (Hurst et al., 1999).

Matrix metalloproteinase -7, the only matrilysin, degrades fibronectin, laminin, collagen IV, gelatin, and proteoglycan core proteins. It has been found in reproductive tissues such as uterus, epididymis, and efferent ducts, however MMP-7 null mice have normal reproduction and embryonic development (Wilson & Matrisian, 1998).

Gelatinases (MMP-2 and –9) are expressed at high levels in normal tissue remodeling and cell invasion involved in reproduction. Gelatinases breakdown gelatin, collagen IV, V, VII, X, and XI, elastin, fibronectin, and proteoglycan core proteins (Birkedal-Hansen et al., 1993;Yu et al., 1998; Woessner & Nagase, 2000). In mouse uteri, MMP-9 is the most abundant gelatinase found in decidual cells (Nuttall et al., 1999). Matrix metalloproteinase-2 is also present in the rodent uterus, however it has low expression early in gestation, peaks between Days 4-8 and remains low in the decidualized zone (Alexander et al., 1996; Das et al., 1997; Bany et al., 2000). In the rat, MMP-2 and –9 are expressed slightly and diffusely throughout the uterus (Hurst et al., 1999). Using RT-PCR, MMP-2 and –9 have been found in rodent embryos (Werb et al.,

1992; Bany et al., 2000), however immunocyotochemical analysis indicates only slight expression of MMP-2. Matrix metalloproteinase -9 is abundantly expressed in parietal endoderm (Behrendtsen & Werb, 1997) and primary trophoblast giant cells surrounding the implantation site (Alexander et al., 1996, Das et al., 1997; Hurst et al., 1999; Bany et al., 2000). Interestingly MMP-9 null mice are fertile, and display normal implantation, embryonic and fetal survival to term, and normal post-natal development. The only stark phenotypic difference in the MMP-9 null mice was shorter long bones than wild-type mice (Vu et al., 1998). Latent MMP-9 has been identified in the sheep uterus (Salamonsen et al., 1993; Vagnoni et al., 1998; Riley et al., 2000), however MMP-2 appears to be the major MMP expressed in the uterus of pregnant and nonpregnant ewes (Salamonsen et al., 1993; Salamonsen et al., 1994; Salamonsen et al., 1995; Vagnoni et al., 1998). Abundant MMP-2 is also observed in amniotic fluid and cervical and placental homogenates during the last trimester of gestation, whereas MMP-9 expression is low in amniotic fluid and placental and cervical homogenates (Riley et al., 2000). MMP-2 and –9 have also been found in the sheep embryo. Matrix metalloproteinase-9 was expressed in Day 13 and 15 embryos, whereas MMP-2 was only expressed in Day 15 embryos (Menino et al., 1999). Day 17 sheep trophoblasts produced MMP-2, -3 and –9, and MMP-9 was the most abundant of the three studied (Salamonsen et al., 1995)

Membrane-type MMP (MT) are the newest class of MMP. To date there are six enzymes in this family: MT-1 (Sato et al., 1994), MT-2 (Will et al., 1995), MT-3 (Matsumoto et al., 1997; Takino et al., 1995), MT-4 (Kajita et al., 1999), MT-5 (Llano et al., 1999; Pei, 1999 a) and MT-6 (Pei, 1999 b). The MT retain the same basic structure as the MMP including two Ca^{2+} and Zn^{2+} binding regions (Ferndez-Catalan et al., 1998). However the C-terminal domain includes the transmembrane domain and cytoplasmic tail and the propeptide end contains a potential furin/prohormone convertase cleavage site (Seiki, 1999; Knauper and Murphy 1998). The furin/prohormone convertase cleavage site found on the prodomain allows furin and other intracellular prohormone convertases to activate MT (Sato et al., 1996; Knauper and Murphy, 1998; Seiki, 1999). Contradictory reports also exist demonstrating furin is not required to activate MT-1 (Seiki, 1999), and other investigators have described plasmin activation of MT (Okumura et al., 1997; Knauper and Murphy, 1998). Current evidence suggests that MT must be

activated before transport to the membrane, and although furin can activate MT intracellulary, it is not the sole intracellular mediator for MT activation (Knauper and Murphy, 1998).

Transcriptional control of MT is primarily determined by the type of cell expressing the gene. The MT gene lacks the TATA box and transcription factor binding sites (Seiki, 1999), however MT-1 expression increases with GnRH and is inhibited by dexamethasone, indicating that an AP-1 binding site is involved (Lohi et al., 1996; Goto et al., 1999). TNFα also stimulates MT-1 mRNA expression (Migita et al., 1996) whereas IL-1β, EGF, bFGF, and TGFβ have no effect. For the most part, transcriptional control of MT is unknown.

The main function of MT is ECM degradation either via activation of MMP-2 or direct degradation of ECM components. Membrane-type matrix metalloproteinase-1 protoelytic activities include degrading fibronectin, tenascin C, collagen I, II, and III, nidogen, gelatin I, vitronectin, and laminin-1 and –5 (Ohuchi et al., 1997; D'ortho et al., 1997, 1998; Knauper and Murphy et al., 1998; Koshikawa et al., 2000). Membrane-type matrix metalloproteinase -2 degrades fibronectin, collagen I and III, laminin, and perlecan (D'ortho et al., 1997). The proteolytic capabilities of the rest of the MT are not completely known. The MT are best known for activating MMP-2. To understand the mechanism of MT-1 activation of MMP-2, several studies were conducted with MT-1 mutants. Early studies revealed MT-1 transmembrane deletions resulted in reduced activation of MMP-2 (Cao et al., 1995). Later studies with truncated MT-1 showed that MT-1 activated MMP-2 but exogenous TIMP-2 inhibited activation (Lichte et al., 1996; Knauper et al., 1996; Lehti et al., 2000). Results from several studies concur that MT-1 activates MMP-2 but disagree on the role of TIMP-2 (Strongin et al., 1995; Knauper et al., 1996; Kinoh et al., 1996; Lichte et al., 1996; Bjorn et al., 1997; Butler et al., 1997;Yu et al., 1997; Cowell et al., 1998; Nagase, 1998; Zucker et al., 1998; Murphy et al., 1999; Zhang et al., 2000). Recent studies suggest that TIMP-2 is required for MMP-2 activation via MT-1. In TIMP-2 null mice, MMP-2 activation does not occur, but can be restored with addition of exogenous TIMP-2. However, high levels of TIMP-2 inhibit MMP-2 activation via MT-1 (Wang et al., 2000; Caterina et al., 2000). Membrane-type matrix metalloproteinase -1 activates MMP-13 via the MMP activation cascade described

by Cowell et al. (1998) and Knauper et al. (1996). Matrix metalloproteinase-2 can also be activated by MT-2 and –5 (Butler et al., 1997; Kolkenbrock et al., 1997; Llano et al., 1999; Miyamori et al., 2000), which would explain MMP-2 activation in culture systems with truncated MT-1. Two models have been proposed for the MT mediated activation of MMP-2. Strogin et al. (1995), Corcoran et al. (1996) and Kinoh et al. (1996) have proposed a model for the cooperation of MT-1 and TIMP-2 in activating proMMP-2. ProMMP-2 binds TIMP-2 and becomes a proMMP-2*TIMP-2 complex. This complex binds to MT-1 and the "pro" portion of the enzyme is cleaved and the active complex is released. Seiki (1999) questioned the Corcoran model regarding how MMP-2 is cleaved and proposed a model including 2 MT and 1 TIMP to bind and activate MMP-2 (Figure 2). It is not known how MT-3, participate in MMP activation, but MT-4 and –6 don't activate MMP (English et al., 2000, 2001).

Membrane-type matrix metalloproteinases have been found in variety of tissues including tumors, embryos, smooth muscle cells, microvasculature, mammary gland epithelia, osteoclasts, kidney, ovary, uterus, and placenta (Kinoh et al., 1996; Puente et al., 1996; Sato et al., 1997; Shofuda et al., 1997; Bjorn et al., 1997;Tanaka et al., 1997; Sato et al., 1997; Ota et al., 1998; Haas et al., 1998; Kanwar et al., 1999; Goto et al., 1999; Maatta et al., 2000; Zhang et al., 2000; Hotary et al., 2000; Nakada et al., 2001). In tumor cells, MT facilitate migration and cellular invasion by breaking down collagen, activating MMP-2 (Hotary et al., 2000; Nakada et al., 2001), and regulating migration over laminin (Koshikawa et al., 2000). Other studies have reported similar results with normal microvascular remodeling demonstrating up-regulation of MMP-2 and MT-1 (Haas et al., 1998). Membrane-type matrix metalloproteinase-1 null mice have the greatest problems with bone development and wasting (Holmbeck et al., 1999). Null embryos developed normally to term, but post-natal growth was impaired. Apporximately 33% die before weaning, and the phenotype includes dwarfism, osteopenia, fibrosis, arthritis, skeletal dysplasia, and absence of sexual maturity. In normal reproductive tissues, MT have been found throughout gestation. In human placenta, MT-1 is present throughout the trophoblastic columns, intermediate trophoblast and decidual cells (Hurskainen et al., 1998; Nawrocki et al., 1996) and colocalizes with MMP-2 mRNA in cytotrophoblasts (Kinoh et al., 1996). Membrane-type matrix

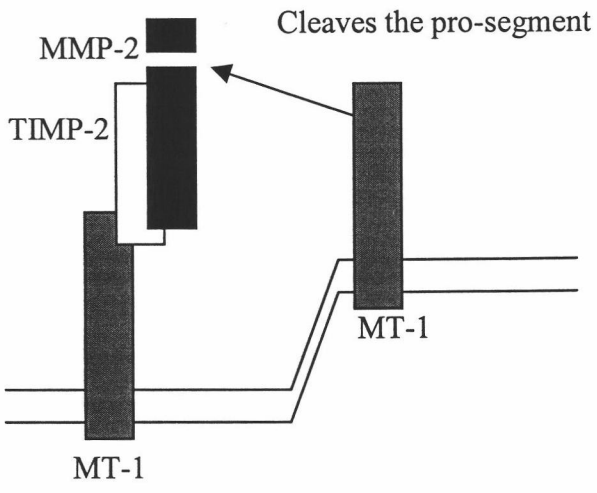

Figure 2. Model for MMP-2 activation (Seiki, 1999).

metalloproteinase -2 has been found in cytotrophoblasts and decidual cells (Bjorn et al., 2000), while MT-3 has been observed in human placenta complexed with TIMP-1 (Takino et al., 1995). In cyclic endometrium, MT-1 mRNA is weakly expressed in the stromal cells (Maatta et al., 2000). Zhang et al (2000) observed MT-1 activation of MMP-2 in human endometrium. In rodents, MT-1 has been detected in embryos, uterus, and placenta. In mouse embryos the MT-1 mRNA is first detected at the 4-cell stage and increases to term coexpressed with MMP-2 and TIMP-2 (Kinoh et al., 1996; Apte et al., 1997; Tanaka et al., 1998). In early embryos, MT-1 expression is localized to the ICM, ectoplacental cone and trophoblast cells (Apte et al., 1997). Furthermore, MT-1 expression is high in the embryonic kidney during late gestation and the proper balance of TIMP-2 and MMP-2 is required for kidney development (Ota et al., 1998; Kanwar et al., 1999). In the uterus, MT-1 is observed at Day 7.5, 9.5 and 10.5 with expression localized to endometrial glands and myometrium. On Day 12.5, MT-1 is expressed in umbilicus, decidua and spongiotrophoblasts (Apte et al., 1997). Membrane-type matrix metalloproteinase-5 expression has been found in a pattern consistent with ets, and is expressed in mouse embryos from Day 11 to 15 and decreases before birth (Pei, 1999). Both rodent and humans have an invasive type of implantation and the expression of MT in species with non-invasive implantation, such as the sheep, has not yet been evaluated until this report.

Tissue Inhibitors of Matrix Metalloproteinases

Tissue inhibitors of matrix metalloproteinases (TIMP) are specific proteins that bind irreversibly to MMP in a 1:1 stoichiometric complex (Maata et al 2000). The basic structure of TIMP begins with a 29 a.a. leader sequence, which is cleaved to leave the mature protein, followed by 12 disulfide-bonded cysteines. The arrangement of bonds allows for the N-terminal domain to bind to the MMP active site with high affinity and inhibit proteolytic activity (Woessner and Nagase, 2000). Tissue inhibitor of matrix metalloproteinase-1, 184 a.a., is extensively glycosylated whereas,TIMP-2 (21kDa), TIMP-3 (21kDa), and TIMP-4 (24kDa) are unglycosylated.TIMP-2 is 40% homologous to TIMP-1, TIMP-3 is 30% homologus to TIMP-1 and –2, and TIMP-4 is 37% homologus to TIMP-1 and 51% homologus to TIMP-2 and –3 (Gomez et al., 1997).

Regulatory factors that stimulate TIMP expression included: IL-1β, TGF-β1, retinoids, EGF, IL-6, oncostatin, and LIF. Concanavalin A and dexamethasone suppress TIMP activity. The TIMP-1 gene has a TATA-less promoter and the promoter region contains a TPA-response element and AP-1, SP-1, and ets sites. Tissue inhibitor of matrix metalloproteinase-2 has a TATA-like element, SP-1, and AP-1 sites. These regions on the TIMP genes allow for transcriptional control by hormones and growth factors.

The main function of TIMP is to inhibit MMP activity, however several reports have described growth promoting effects (Hayakawa et al., 1992) and erythriod-potentiating activities. Specifically TIMP-1 can bind proMMP-9 and inhibit enzyme activity, yet it can bind MMP-2 and facilitate activation (Woessner and Nagase, 2000). TIMP-1 does not inhibit MMP activity when bound to MT-1 or MT-5 (Llano et al., 1999; Woessner and Nagase, 2000). Tissue inhibitor of matrix metalloproteinase -2 is part of a complex with MT-1 and proMMP-2 involved in activating MMP-2 (Corcoran et al., 1996), and it also inhibits other MMP (Woessner and Nagase, 2000). Tissue inhibitor of matrix metalloproteinase -3 and –4 are the most recently discovered TIMP, and their functions beyond inhibiting MMP activity are not known.

Tissue inhibitors of matrix metalloproteinases have been found in a variety of tissues: skin, lung, mucosal membranes, synovial tissues, ovaries (Berkdal-Hansen et al., 1993), embryos, uterus, and oviducts (Buhi et al., 1997). Tissue inhibitors of matrix metalloproteinases have been implicated in several biological functions including: erythroid potentiating activity, endometrial remodeling, implantation, steriodogenesis, and growth (Buhi et al., 1997). In human endometrium, TIMP have been found throughout the menstrual cycle and during placentation (Hampton et al., 1994; Fata et al., 2000; Maata et al., 2000). In humans, TIMP-1 and –2 are up regulated by progesterone and are not detected until the late secretory phase, whereas TIMP-3 is differentially expressed throughout the cycle (Maata et al., 2000).

In rodent uterus and ovary, TIMP are expressed throughout the estrous cycle and gestation. Early studies of TIMP in the pregnant uterus found TIMP-1 high on Day 6 and declining to Day 8 (Waterhouse et al., 1993). However, later studies evaluating TIMP-1 in myometrium found expression to be low on Day 1-3, increase on Day 4 and peak on

Day 7-8. TIMP-1 expression was also localized to decidual cells (Alexander et al., 1996; Das et al., 1997). Tissue inhibitor of matrix metalloproteinase-1 expression was correlated with the most invasive period of embryo attachment to the uterus in the rodent. Tissue inhibitor of matrix metalloproteinase-1 null mice, exhibited variable reproductive phenotypes, including: estrous cycle of normal length, constant estrus, constant diestrus, or cycles with a long proestrus and short estrus. Tissue inhibitor of matrix metalloproteinase-1 null mice also have altered uterine morphology with excessive luminal folds and branching. During estrus, uteri were heavier, and serum progesterone was lower and estrogen was higher (Nothnick, 2000). Tissue inhibitor of matrix metalloproteinase-2 was found to gradually increase throughout gestation (Waterhouse et al., 1993), however more recent reports have indicated constant expression of TIMP-2 throughout gestation (Alexander et al., 1996; Das et al., 1997). Tissue inhibitor of matrix metalloproteinase -2 null mice have normal development and fertility but decreased MMP-2 activity (Caterina et al., 2000; Wang et al., 2000). In the amnion, TIMP-1 increases to Day 18 and remains high throughout gestation, whereas TIMP-2 gradually increases throughout gestation reaching maximal levels at term in the mouse (Waterhouse et al., 1993). Waterhouse et al. (1993) also observed that TIMP levels where similar between induced decidual tissue and unmanipulated decidual tissue. However, in amniotic fluid, TIMP-3 has the greatest expression from mid-gestation to term, while Riley et al. (2000) observed TIMP-2 and –4 were expressed in extremely small quantities. Tissue inhibitor of matrix metalloproteinase-3 is differentially expressed in the uterus throughout gestation. Alexander et al. (1996) observed that TIMP-3 levels peaked by Day 6.5 and decreased to nondetectable levels by Day 8. In contrast, Das et al. (1997) and Leco et al. (1996) observed TIMP-3 to be low from Day 1-6 and dramatically increase in decidual cells on Day 7-8. In the pre-pubertal ovary, TIMP-1 expression is high during the estrous cycle and low during gestation, however TIMP-2 increases slightly throughout gestation (Waterhouse et al., 1993).

Embryos undergo extensive cell growth and development, and TIMP may participate in these processes (Reponen et al., 1995; Leco et al., 1997; Bany and Schultz, 2001). In early in vitro developing embryos, secreted or supplemented TIMP has important growth effects (Behrendtsen and Werb, 1997). In preimplantation embryos

exogenous TIMP-1 increases the number of cells and the distance cells migrate out of the inner cell mass of the embryo (Werb et al., 1992). However in midgestation embryos, exogenous TIMP-1 and –2 retard growth and development (Del Bigio et al., 1999).

Tissue inhibitor of matrix metalloproteinase-1 and –2 are expressed in female reproductive tissues throughout the estrous cycle and gestation in the livestock species. In gilts, TIMP-1 is expressed in the oviduct throughout pregnancy and differentially throughout the estrous cycle. In OVX steroid-treated gilts, de novo TIMP-1 synthesis increased with progesterone and testosterone treatment (Buhi et al., 1997). In ewes, TIMP-1 and –2 expression is high during the luteal phase. In OVX ewes, TIMP-1 decreases with estrogen and progesterone treatment while TIMP-2 increases with estrogen treatment. However, during pregnancy, TIMP-1 increases from Day 9-14 and remains high until Day 20, with greatest expression in the inter-caruncular regions (Hampton et al., 1995; Salamonsen et al., 1995). In late gestation sheep, Day 70 to term, TIMP-3 is expressed throughout the uninucleate trophoblasts and the fetal components of the placentome (Riley et al., 2000). Similar to the rodent embryo, TIMP-1 has a positive effect on growth in the bovine embryo (Satoh et al., 1994). Swine and sheep embryos express TIMP during pre- and peri-implantation development. Menino et al. (1997) observed expression of TIMP-1, -2 and –3 in day 16 swine embryos. In sheep embryos, TIMP-1 and –3 are expressed during Days 9-15 and TIMP-2 is expressed during Day 11-15 of development (Menino et al., 1999). The expression of TIMP by the uterus and embryo during the period of implantation suggests that TIMP may be important in regulating growth, attachment, and uterine penetration during gestation.

Membrane-type matrix metalloproteinases have not been studied in sheep uterus or embryos. Furthermore, TIMP-3 has only been studied in cultured endometrial cells. This study was undertaken to define an expression pattern for MT and TIMP in the pre- and peri-implantation uterus and to observe expression of MT in embryos collected during the same time period.

MATERIALS & METHODS

Tissue collection

Twenty cross-bred ewes were used in this study. Ewes in the pregnant group (n = 12) were estrous synchronized and superovluated, whereas ewes in the nonpregnant group (n = 8) were only estrous synchronized. For estrous synchronization, ewes received two i.m. injections of 100μg cloprostenol sodium (Estrumate; Haver, Shawnee, KS) on Days 0 and 9. For superovulation, porcine follicle stimulating hormone (pFSH; Sioux Biochemical, Sioux Center, Iowa) injections were administered i.m. at dosages of 4, 3, and 2 mg twice daily on Days 8, 9, and 10. Estrus detection began 24 h following the second injection of Estrumate. Ewes in the pregnant group were hand-mated to one of three rams at the onset of estrus and at 12-h intervals thereafter until the ewe no longer accepted the ram or for a total of 3 matings. Heat was detected in ewes in the nonpregnant group using a vasectomized ram. On Days 9, 11, 13 and 15 following the onset of estrus, uterine tissues and embryos were surgically recovered. Anesthesia was induced with a 10 ml injection of pentothal (Abbott Laboratories, North Chicago, IL) into the jugular vein and maintained during surgery via inhalation of halothane (Halocarbon Laboratories, River Edge, NJ) and oxygen. The reproductive tract was exteriorized through a ventral midline laparotomy and uteri from mated ewes were flushed with Ham's F-12 (Ham's F-12; Sigma Chemical Co., St. Louis, MO), buffered with 25 mM HEPES (HF-12+H; Sigma). Embryos were washed in 5-10 ml of HF-12+H with 15 mg/ml BSA (HF-12+H+BSA; Sigma), examined for morphology, aliquoted into tubes, frozen in dry ice and ethanol, and stored at -80°C. One-cm square section of uterus from pregnant and nonpregnant ewes were surgically removed, blotted, frozen in dry ice and ethanol, and stored at -80°C.

Reverse Transcription-Polymerase Chain Reaction

Membrane-Type Matrix Metalloproteinases in Uterine Tissue - Primer pairs were designed from published human, mouse, and rat cDNA sequences for MT-1, -2, -3, -4, and –5 (Table 1). RNA was extracted from uterine tissues using procedures described by

Table 1. Membrane-type matrix metalloproteinases (MT) PCR primers and fragment size.

Primer Name	Primer Sequence	PCR Fragment Size (bp)	Citation	Optimal Cycles	Optimal Temp.
MT-1	3' primer = TCGTGCACAGCCACC AGG (18nt) 5' primer = GAGGGCCATGAGAA GCAGG (19nt)	215	Lohi et al., 1996 nt 617-831 of human cDNA	27	64°C
MT-2	3' primer = TGCACAGCCACCAGG AAGAG (20nt) 5' primer = CTCGACGAAGAGAC CAAGGAG (21nt)	356	Will & Hinzman, 1995 nt 469-824 of human cDNA	37	62°C
MT-3	3' primer = CTCCAATCCCAGAGC ATGTC (20nt) 5' primer = GCAAACGTGATGTGG ATATAACC (23nt)	233	Shofuda et al., 1997 nt 568-800 of human cDNA	29	62°C
MT-4	3' primer = CCACTGCAAACAGGT CCATC (20nt) 5' primer = GACATTCCCACGGGA CTCAC (20nt)	306	Kajita et al., 1999 nt 537-842 of human cDNA	30	57°C
MT-5	3' primer = TGCACAGCCACCAGG AAGAG (20nt) 5' primer = ACCACGAGATCAAA AGTGACCG (22nt)	223	Llano et al., 1999 nt 623-845 of human cDNA	33	60°C

Chomczynski and Sacchi (1987). RNA was quantified by UV-spectrophotometry and dissolved in 50 µl autoclaved water. Multiplex relative quantitative reverse transcription-polymerase chain reactions (RT-PCR) were performed following the procedure described by Arcellana-Panlilio and Schultz (1993) and Ambion's Quantum RNA18S Internal Standards Kit (Ambion; Austin, Texas). For RT, one µg of RNA was incubated with 2 µl random decamer (Ambion; Austin, Texas) for 10 min at 70°C in a total volume of 12µl and quick chilled on ice. Four microliters 5X first-strand buffer (250 mM Tris HCl, pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$), 2 µl 0.1 M dithiothreitol, 1 µl (10µM) dNTPs, 1µl (200 U) Superscript II reverse transcriptase (Gibco BRL; Rockville, MD) was added to the mixture and incubated at 42° C for 90 min followed by a 10-min soak at 95° C. The reaction mixture was diluted to 50 µl with sterile distilled water and 5 µl aliquots were used for PCR. PCR was conducted in a 50 µl reaction volume containing 5 µl Promega 10X PCR buffer, 5 µl Promega $MgCl_2$, 1µl (10 µM) dNTPs (Amersham Pharmacia Biotech; Piscataway, New Jersey), 1 µl (100 µM) 3' primer, 1 µl (100 µM) 5' primer, 0.2 µl (5 u/µl) Promega Taq DNA polymerase, 0.4 µl 18S primer pair, 3.6 µl 18S competimer pair (primer: competimer ratio of 1:9), and 5 µl RT product or water. The reaction mixtures were overlaid with mineral oil and amplified in a DNA thermal cycler. PCR conditions consisted of 1) 4-min soak at 94°C; 2) 27-37 cycles of denaturation for 1 min at 94°C, 2 min annealing at the optimal temperature for the gene of interest (Table 1) and extension for 2 min at 72°C and 3) incubation at 72°C for 7 min. PCR products were resolved on 2-4% agarose gels, and visualized using SYBR Green stain (Molecular Probes; Eugene, Oregon). β-actin was used as check for contamination with genomic DNA. Primers for β-actin span an intron (Tokunaga et al., 1986), hence genomic DNA would generate a 381 bp product, whereas cDNA would produce a 243 bp fragment (Table 2). Water in place of the RT product was used as the negative control.

Following PCR, products were purified using a QIAquick PCR Purification Kit (Qaigen; Valencia, California) and sequenced at the Oregon State University Center for Gene Research Central Services Laboratory. Product identity was confirmed using Fasta (Pearson and Lipman, 1988).

Tissue Inhibitors of Matrix Metalloproteinase in Endometrial Tissue - Primers were designed from published porcine, bovine and human sequences (Table 2). RNA extraction and reverse transcription was carried out as described. PCR reaction volumes contained the same reagents listed above, however, the 18S primer pair: 18S competimer pair was added at a ratio of 2:8. Reaction mixtures were overlaid with mineral oil and amplified in a DNA thermal cycler. PCR conditions consisted of 1) 4-min soak at 94°C, 2) 25 cycles of denaturation for 1 min at 94°C, 2 min annealing at optimal temperature for the target gene (Table 2) and extension for 2 min at 72°C and 3) incubation at 72°C for 7 min. PCR products were resolved on 4% agarose gels for TIMP-1 and 2% agarose gels for TIMP-2 and –3 and visualized following staining with SYBR Green for 10-30 minutes (Molecular Probes; Eugene, Oregon).

Membrane-type Matrix Metalloproteinases in the Embryo - The same primers used for evaluating MT expression in endometrial samples were used for the embryos. RNA extraction was carried out as described by Arcellana-Panlilio and Schultz (1993). For RT, 1 μl oligo (dT) 12-18 primers (Gibco BRL, Grand Island, New York) was added to 11 μl of embryonic RNA consisting of 5.5, 2, 1, and 0.25 embryos from Day 9, 11, 13, and 15, respectively. Annealing was carried out at 70°C for 10 minutes and the reaction mixture was chilled at 4°C for 5 minutes. The RT mixture was the same for all embryo samples: 4 μl 5x first-strand buffer (250 mM Tris HCl, pH8.3, 375 mM KCl, and 15 mM $MgCl_2$), 2 μl 0.1 M dithiothreitol, 1 μl (10 μM) dNTPs, and 1 μl (200 U) Superscript II reverse transcriptase (Gibco BRL; Rockville, MD). The RT mixture was added to the annealing mixture and incubated at 42°C for 90 minutes followed by a 10 minute soak at 95°C. The RT mixture was then diluted with 30 μl of autoclaved distilled water and 5 μl aliquots were used in PCR. PCR were carried out as described above with 40 cycles at the optimal temperature for each gene (Table 1). PCR products were visualized on 2% agarose gels stained with SYBR Green for 10-20 minutes.

Table 2. Tissue inhibitor of matrix metalloproteinases (TIMP) PCR primers and fragment size.

Primer Name	Primer Sequence	PCR Fragement Size (bp)	Citation	Optimal Cycles	Optimal Temp.
TIMP-1	3' primer = AGTTTGCAGGGGATG GATG (19 nt) 5' primer = CCAGCAGTTATGAGA TCAAGATG (22 nt)	312	Tanaka et al., 1992; Freundenst ein et al., 1990 nt 180-491 of bovine cDNA	25	60°C
TIMP-2	3' primer = CACAGGAGCCGTCAC TTCTCTTG (23 nt) 5' primer = GGCGTTTTGCAATGC AGATGTAG (23 nt)	497	Stetler-Stevenson et al., 1989; Boone et al., 1990 nt 398-894 of bovine cDNA	25	55°C
TIMP-3	3' primer = TGCCGGATGCAGGCG TAGTGTTT (23 nt) 5' primer = CTTCTGCAACTCCGA CATCGTG (22 nt)	459	Wilde et al., 1994 nt 378-836 of human cDNA	25	55°C
Actin	3' primer = CGTGGGCCGCCCTAG GCACCA (21nt) 5' primer = TTGGCCTTAGGGTTC AGGGGGG (22nt)	243	Tokunaga et al., 1986 nt 182-424 of mouse cDNA	25	55°C

Statistics

Densitometric analysis of band intensity was performed using the Kodak 1D Image Analysis software. Expression of target genes in the uterus was measured as a ratio of band intensities of the target gene to18S rRNA. Target gene expression was analyzed using multi-way ANOVA where reproductive status (pregnant and nonpregnant) and day of collection (9, 11, 13, and 15) were the main effects. Where appropriate, differences in target gene expression were determined using Fishers LSD Multiple Comparison test. All analysis was conducted using the NCSS analysis software (Number Cruncher Statistical System, Version 2000; Hintze 1998, Kaysville, Utah).

Presence or absence of a band in embryonic cDNA was used as the criteria for expression of the target gene.

RESULTS

Membrane-Type Matrix Metalloproteinases in Uterine Tissue

MT-1 Expression- Relative quantitative RT-PCR were used to amplify a 216 bp fragment of MT-1 (Fig. 3a). Ovine MT-1 was 97.9%, 94.1%, and 94.1% homologous to goat, human and pig sequences, respectively. MT-1 expression was similar ($P > 0.10$) over the four days of collection, however pregnant uterus expressed more MT-1 than nonpregnant uterus ($P = 0.096$) (Fig. 4a). The pregnancy status by day interaction was not significant ($P > 0.10$).

MT-2 Expression- A 355 bp fragment of MT-2 was amplified using RT-PCR (Fig. 3b). Ovine MT-2 was 93.0%, 86.5% and 78.6% homologous to human, mouse and chicken sequences, respectively. Nonpregnant sheep uterus expressed more ($P < 0.05$) MT-2 than pregnant sheep uterus (Fig. 4b). Similar to MT-1, MT-2 expression was similar ($P > 0.10$) over the four days of collection and the pregnancy status by day interaction was not significant ($P > 0.10$).

MT-3 Expression- A 232 bp fragment of MT-3 was amplified by RT-PCR (Fig. 3c). Ovine MT-3 was 93.7%, 88.4%, and 87.8% homologous with published sequences for human, chicken and rat MT-3, respectively. Expression of uterine MT-3 did not differ ($P > 0.10$) between pregnant and nonpregnant females. MT-3 expression in the pregnant uterus decreased over the four days of collection (Fig. 4c), with Day 15 having less ($P < 0.05$) MT-3 than Days 9 and 11. MT-3 expression did not differ ($P > 0.10$) by day of collection in nonpregnant uterus.

MT-4 Expression- MT-4 was not detected in the sheep uterus. PCR products for MT-4 were amplified in the positive control tissue, human colon (data not shown).

MT-5 Expression- Relative quantitative RT-PCR amplified a 222 bp fragment from sheep uterus (Fig. 3d, lanes 1-9). Ovine MT-5 was 92.0%, 89.0% and 79.8% homologous to the sequences for human, mouse, and chicken MT-5, respectively. MT-5

expression was similar ($P > 0.10$) in pregnant and nonpregnant uterus. Expression of MT-5 on Day 11, and 13 was greater ($P < 0.05$) than expression on Day 15 of collection. This expression pattern was strongest in pregnant tissues where Day 11 and 13 uteri expressed more ($P < 0.05$) MT-5 than Day 15 uterus (Fig. 4d). Expression of MT-5 in nonpregnant uterus did not differ ($P > 0.10$) due to day of collection.

Tissue Inhibitors of Matrix Metalloproteinase in Endometrial Tissue

TIMP-1 Expression- The 312 bp fragment of TIMP-1 amplified in this report was 99.6% homologus to the published sequence for ovine TIMP-1 (Smith et al., 1994). TIMP-1 expression in pregnant uterus was greater ($P < 0.05$) than in nonpregnant uterus (Figs. 5a and 6a), however expression did not vary ($P > 0.10$) over the 4 days of collection. The interaction between day of collection and pregnancy status was significant ($P = 0.07$). Day 9 nonpregnant uterus expressed less TIMP-1 than Day 9, 11, 13 and 15 pregnant uteri, and Day 13 and 15 nonpregnant uteri. Furthermore Day 11 nonpregnant uterus expressed less TIMP-1 than Day 11 pregnant uterus.

TIMP-2 Expression- The ovine TIMP-2 fragment amplified by RT-PCR was 497 bp (Fig 5b) and was 96.4%, 92.5% and 92.1% homologous with bovine, human, and dog sequences, respectively. No difference ($P > 0.10$) was observed in TIMP-2 expression due to pregnancy status or day of collection, however the interaction was significant ($P < 0.05$) (Fig. 6b). Day 9 nonpregnant uterus expressed less ($P < 0.05$) TIMP-2 than Day 11 and 13 nonpregnant uteri, and Day 9 and 13 pregnant uteri. Day 9 pregnant uterus expressed more ($P < 0.05$) TIMP-2 than Day 11 and 15 pregnant and Day 15 nonpregnant uteri.

TIMP-3 Expression- The ovine TIMP-3 fragment amplified by RT-PCR was 459 bp and had high homology with bovine (95.9%), horse (94.9%), and pig (95.1%) TIMP-3. No difference ($P > 0.10$) in TIMP-3 was observed due to day of collection or pregnancy status (Figs. 5c and 6c) and the pregnancy status by day interaction was not significant ($P > 0.10$).

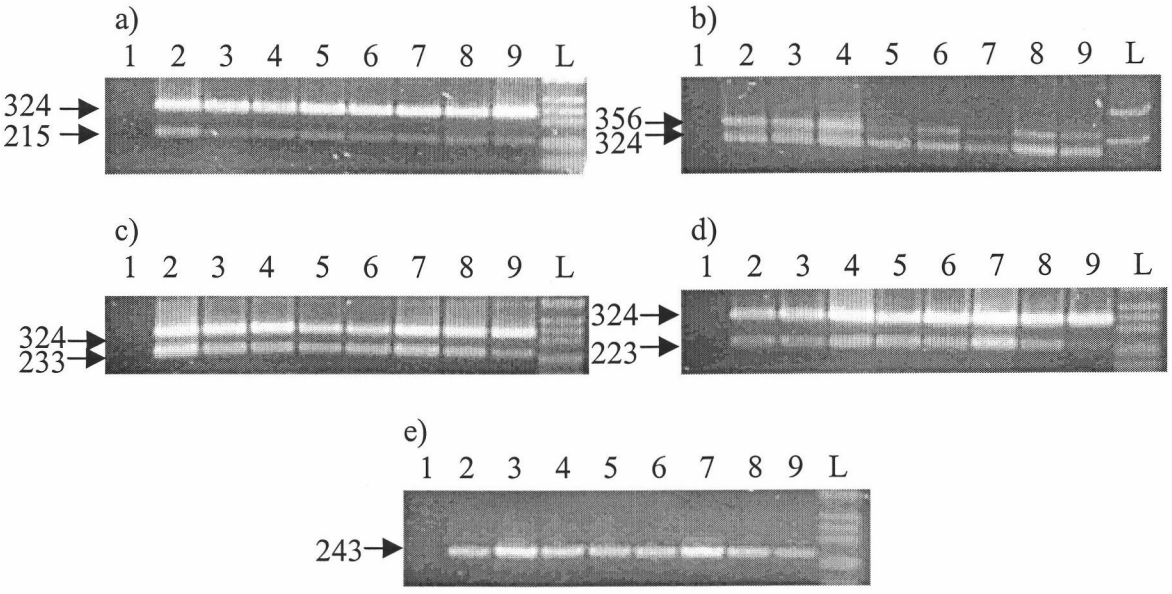

Figure 3. MT and 18S rRNA expression in sheep uterus. PCR products for a) MT-1, b) MT-2, c) MT-3, d) MT-5, and e) β-actin in sheep uterus. Lane 1 is water, lanes 2, 3, 4, and 5 are nonpregnant sheep uteri on Days 9, 11, 13, and 15, respectively, lanes 6, 7, 8, and 9 are pregnant uteri from Days 9, 11, 13, and 15, respectively and lane L is the DNA Ladder. In panels a, c, and d, 18S rRNA is the top band and the target gene is the bottom band. In panel b the top band is the target gene and the bottom band is the 18S rRNA.

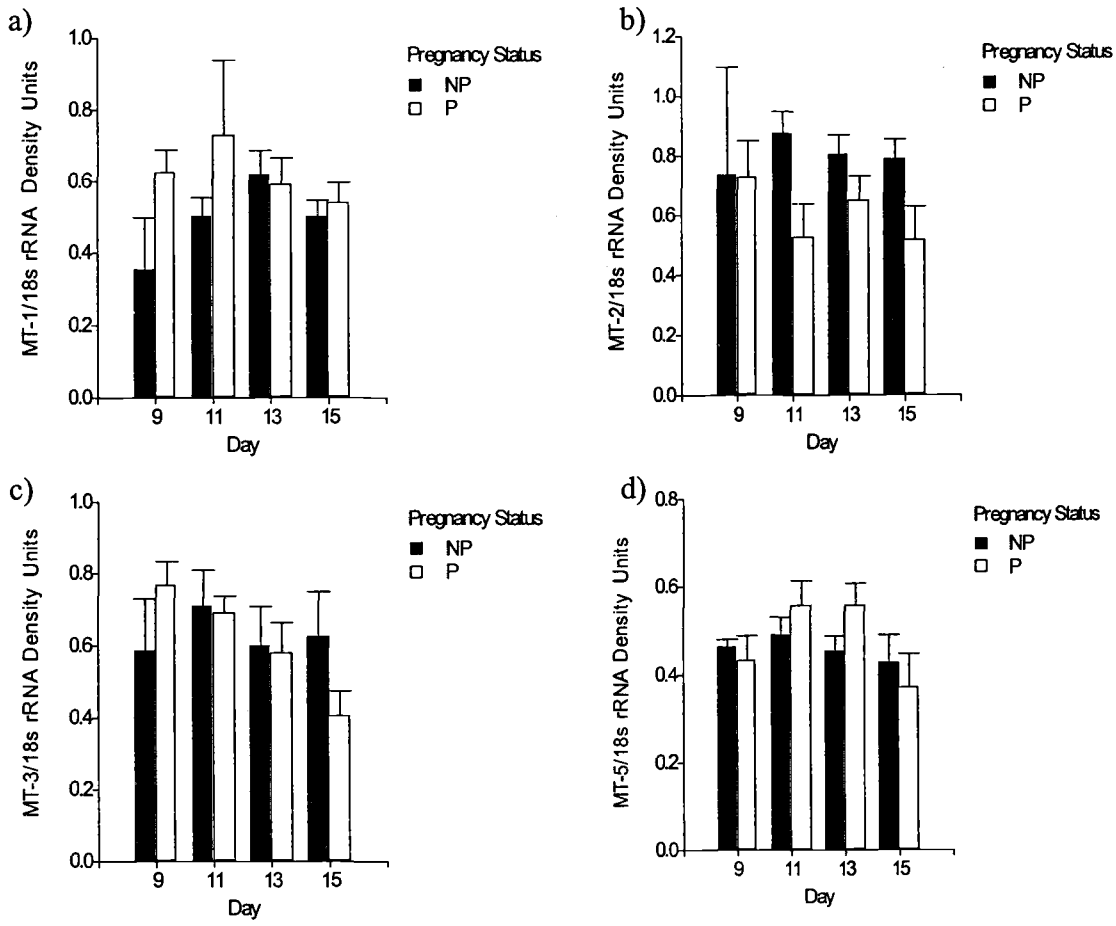

Figure 4. Expression of a) MT-1, b) MT-2, c) MT-3, and d) MT-5 in pregnant and nonpregnant sheep uteri 9, 11, 13, and 15 days after the onset of estrus.

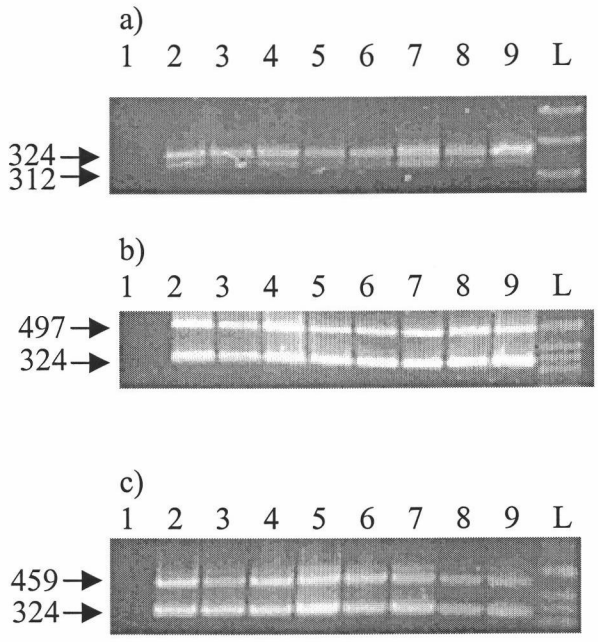

Figure 5. TIMP and 18S rRNA expression in sheep uterus. PCR products for a) TIMP-1, b) TIMP-2, and c) TIMP-3. Lane 1 is water, lanes 2, 3, 4 and 5 are nonpregnant uteri Day 9, 11, 13, and 15, respectively, lanes 6, 7, 8 and 9 are pregnant uteri Day 9, 11, 13 and 15, respectively and lane L is DNA Ladder. In panel a, the 18S rRNA is the top band and the target gene is the bottom band. Panels b and c the top band is the target gene and the bottom band is the 18S rRNA.

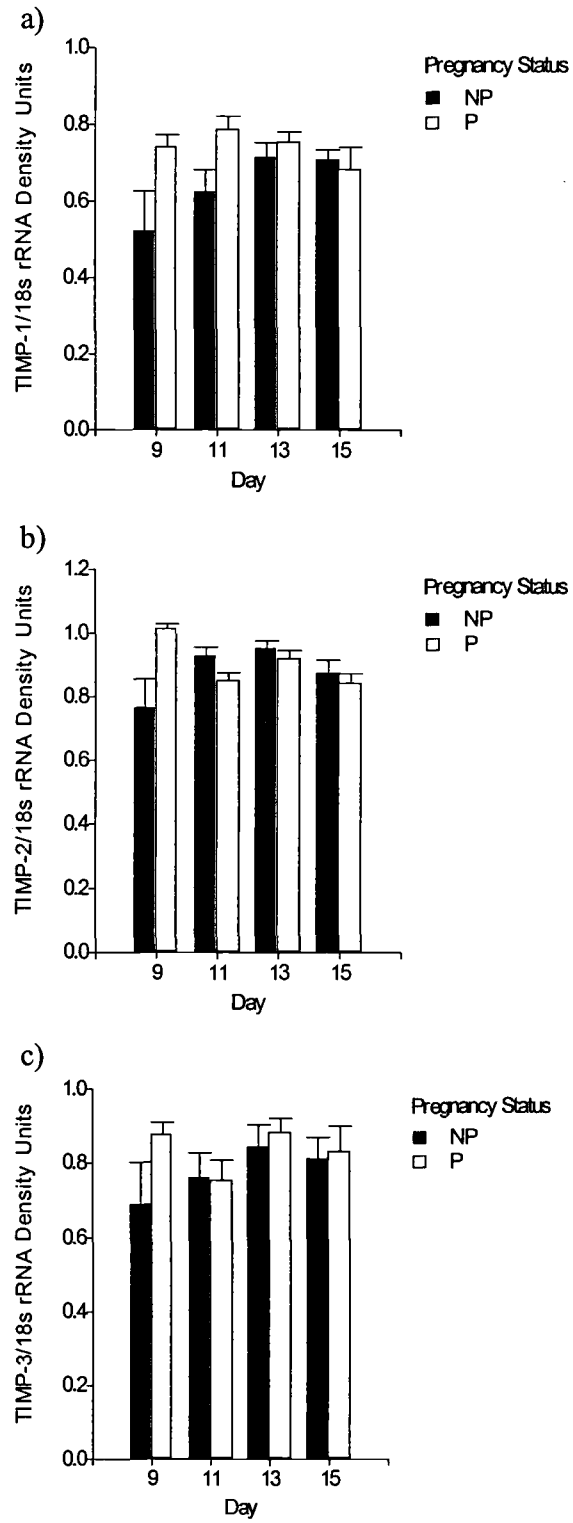

Figure 6. Expression of TIMP in pregnant and nonpregnant sheep uteri over the four days of collection, a) TIMP-1, b) TIMP-2, and c) TIMP-3.

Membrane-type Matrix Metalloproteinases in the Embryo

Thirty-three Day 9, 22 Day 11, 10 Day 13, and six Day 15 embryos were collected from estrous synchronized and superovulated ewes. RT-PCR revealed MT-3 (Fig. 7a) and –5 (Fig. 7b) expression in sheep embryos recovered on Days 9, 11, 13, and 15 of gestation. MT-1 and –2 were not detected in embryos during the period of collection despite positive signals in uterine tissues run as positive controls (data not shown). Embryo MT-3 PCR products were 94.8%, 87.8%, and 88.8% homologous with human, rat, and chicken published sequences. Embryonic MT-5 was 90.3%, 88.3% and 87.3% homologous to human, mouse, and rat sequences, respectively.

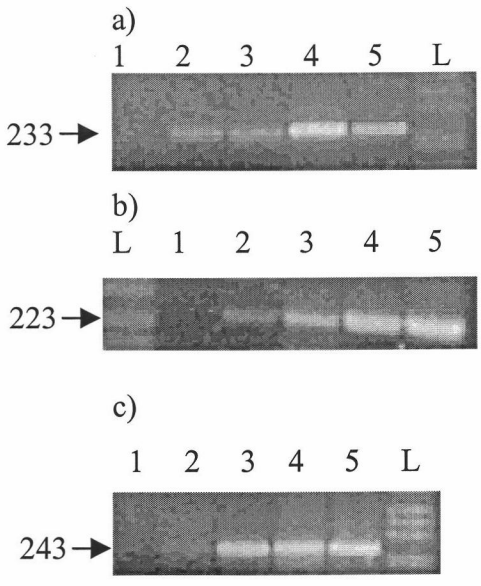

Figure 7. Embryonic expression of a) MT-3, b) MT-5, and c) β-actin. Lane 1 is water, lanes 2, 3, 4, and 5 are cDNA from Days 9, 11, 13, and 15 embryos, respectively, and lane L is the DNA ladder.

DISCUSSION

Pregnant and nonpregnant sheep uterus expressed MT-1, -2, -3 and –5 during the pre- and peri-implantation period. The role of MT in degrading components of the ECM and activating MMP-2 make them prime targets of study in events which require extensive cellular reassociations and migration, such as the menstrual or estrous cycles and the pre- and peri-implantation period. The primary role of MT is ECM degradation. Membrane-type matrix metalloproteinase-1 and -2 are able to degrade several uterine ECM components such as laminin and fibronectin (Ohuchi et al., 1997; D'ortho et al., 1997, 1998; Knauper and Murphy, 1998; Duc Gorian et al., 1999; Koshikawa et al., 2000). Membrane-type matrix metalloproteinase-1 and -2 expression in the uterus may indicate that these enzymes are important in cellular events in the uterus. Furthermore, since MT-1 has been found to regulate epithelial cell migration over laminin (Koshikawa et al., 2000), uterine MT-1 may participate in endometrial remodeling during the peri-implantation period, especially since expression was greater during pregnancy.

Zhang et al. (2000) evaluated expression and co-localization of MMP-2 with MT-1 and -2 in the human endometrium during the menstrual cycle. Matrix metalloproteinase-2 was expressed throughout the menstrual cycle with widespread distribution in the endometrium. Membrane-type matrix metalloproteinase-1 was also observed throughout the cycle, but mainly localized to leukocytes. Membrane-type matrix metalloproteinase-2, which had intense staining in the glandular epithelium, was more widespread through uterine cellular compartments than MT-1. Furthermore, MMP-2 and MT-1 expression in human endometrial cell cultures was attenuated under the influence of progesterone. In studies evaluating MMP expression in sheep endometrium, Salamonsen et al. (1993) established that proMMP-2 was produced in large quantities by endometrial cells and both latent and active MMP-2 were produced by endometrial stromal cells in culture. In a later review, Salamonsen et al. (1995) reported MMP-1, -2, -3, and –9 were present in uterine flushates from pregnant ewes. It is appropriate that MT-1, -2, and –5 are present in pregnant and nonpregnant sheep uterus to activate MMP-2, because the uterus undergoes many cellular changes during the estrous cycle and early gestation. Zhang et al. (2000) reported decreased MT-1 expression in human

endometrium treated with progesterone. Results in the present study suggest there is a different trend in the sheep uterus. Membrane-type matrix metalloproteinase-1 is greater in the pregnant uterus, which is under the influence of progesterone, and increases through the luteal phase of the cycle. While MT-2 expression is reduced in the pregnant sheep uterus compared to the nonpregnant uterus. Expression of MT-5 did not differ between pregnant and nonpregnant females. It would be interesting to determine active MMP-2 expression in pregnant and nonpregnant ewes, and correlate it to MT expression. MT-3 has also been found in reproductive tissues. In human placenta, Takino et al. (1995) found MT-3 complexed with TIMP-1. Membrane-type matrix metalloproteinase-3 and TIMP-1 were both expressed in the sheep uterus, however the expression patterns of these two genes were not similar.

Similar to Hampton et al. (1995), TIMP-1 expression in cyclic ewes in the present study increased during the luteal phase. Expression of TIMP-1 dramatically increased from Day 9 to Day 13 and stabilized on Day 15. Pregnant uterus expressed more TIMP-1 than nonpregnant uterus. The trend of TIMP-1 expression in the pregnant uterus indicated a slight increase from Day 9 to Day 11, and steady decrease to Day 15. No difference in levels of TIMP-1 was observed in the pregnant uterus across the days of collection, however Hampton et al. (1995) observed up regulation of TIMP-1 in the endometrium. This deviation in results can probably be attributed to uterine RNA used in the present study compared to only endometrial RNA in the report by Hampton et al. (1995).

Hampton et al. (1995) also evaluated TIMP-2 expression in cyclic and pregnant ewes. Similar to Hampton et al. (1995), TIMP-2 expression in the cyclic ewe in this report increased dramatically between Day 9 and Day 13 and declined slightly on Day 15. In the pregnant uterus, greatest expression of TIMP-2 was on Day 9 of pregnancy, and dropped to Day 11, increased slightly to Day 13, and dropped again on Day 15. In endometrium, Hampton et al. (1995) observed a peak in TIMP-2 expression between days 12-14. Besides the role of TIMP-2 in inhibiting MMP activity, TIMP-2 participates in the MT activation cascades of MMP-2. It is interesting that MT-1 is more highly expressed in the pregnant ewe than the nonpregnant ewe, however the expression pattern of MT-1 and TIMP-2 differed over the days of collection. On the other hand, MT-2 was

more widely expressed in the nonpregnant ewe. Furthermore, the amount of TIMP-2 expressed from the uterus may be enough to inhibit MMP-2 activation instead of promoting MMP-2 activation via the MT activation cascades. Since high levels of TIMP-2 have been found to inhibit MMP-2 activation (Wang et al., 2000; Caterina et al., 2000).

Putative TIMP-3 has been found through reverse zymography of culture media collected from sheep endometrial cells (Hampton et al., 1995). However there are no reports describing the expression pattern in the pregnant and nonpregnant ewe. Our study found similar TIMP-3 expression between pregnant and nonpregnant ewes, however Day 9 pregnant ewes expressed significantly greater TIMP-3 than Day 9 nonpregnant ewes. Overall, there was no difference in expression of TIMP-3 due to pregnancy status or day of collection.

Membrane-type matrix metalloproteinase-3 and –5 were expressed in the pre- and peri-implantation sheep embryos, but MT-1 and –2 were not detected. Membrane-type matrix metalloproteinase-1 and -5 are the only MT found in embryos, and they were both observed in the mouse embryo (Kinoh et al., 1996; Apte et al., 1997; Tanaka et al., 1998; Pie, 1999). Membrane-type matrix metalloprotienase-1 was found in mouse embryos collected during early and late gestation (Kinoh et al., 1996; Apte et al., 1997; Tanaka et al., 1998), whereas MT-5 has only been found in mouse embryos collected during late gestation (Pei, 1999). Sheep embryos expressed MT-3 and -5 during the period of endodermal cell migration, embryonic expansion and elongation, mesoderm formation and uterine attachment. With exception of uPA, no other ECM degrading proteases have been detected until Day 13 (Menino et al., 1999). Hence, MT-3 and –5 may be the enzymes responsible for ECM degradation, cellular activity, and tissue remodeling in the early embryo. The absence of MT-1 and –2 may also serve as a possible mechanism to regulate MMP-2 activation, thereby limiting invasiveness of the sheep embryo.

The process of implantation is highly regulated and differs between species. Some species differences can be related to the expression patterns of MMP and TIMP. In the rodent MMP-2 is the most abundant MMP in the uterus (Alexander et al., 1996), while the main TIMP expressed in the uterus is TIMP-3 (Bany et al., 2001). The invading embryo expresses low levels of TIMP-3 and large amounts of MMP-9 (Brenner et al.,

1989; Werb et al., 1992) hence the invading embryo can penetrate the uterus with little inhibition from TIMP. Sheep embryos and uteri, which have a much less invasive type of placentation, expresses large amounts of TIMP (Menino et al., 1999). Sheep embryos express TIMP-1 and –3 throughout the pre- and peri-implantation period and begin expressing TIMP-2 on Day 11(Menino et al., 1999). Uterine expression of TIMP seems to peak at implantation (Hampton et al., 1995). The lack of MT-1 and –2 expression observed in this study may explain why the sheep embryo is not invasive. Uterine expression of MT probably plays a role in ECM degradation and cellular reassociations while TIMP controls embryo invasiveness. The results of this study have provided some evidence defining biochemical characteristics of the sheep embryo and uterus that may contribute to its less invasive type of embryo attachment.

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APPENDIX

```
            1                                                   50
sheep MT-1  TATGAGGGCC ATGAGAAGCA GGCCGACATC ATGATCTTCT TTGCTGAGGG
 goat MT-1  CGTGAGGGCC ACGAGAAGCA GGCCGACGTC ATGATCTTCT TTGCTGAGGG
human MT-1  CGTGAGGGCC ATGAGAAGCA GGCCGACATC ATGATCTTCT TTGCCGAGGG
  pig MT-1  CGTGAGGGCC ACGAGAAGCA GGCGGACATC ATGATCTTCT TTGCTGAAGG

            51                                                  100
sheep MT-1  CTTCCATGGT GACAGCACGC CTTTTGATGG CGAGGGCGGC TTCCTGGCCC
 goat MT-1  CTTCCATGGT GACAGCACGC CTTTCGATGG CGAGGGCGGC TTCCTGGCCC
human MT-1  CTTCCATGGC GACAGCACGC CCTTCGATGG TGAGGGCGGC TTCCTGGCCC
  pig MT-1  TTTCCATGGT GACAGCACGC CTTTCGATGG CGAGGGTGGC TTCCTGGCCC

            101                                                 150
sheep MT-1  ATGCCTACTT CCCAGGCCCC AACATTGGAG GGGACACCCA CTTTGAGTCT
 goat MT-1  ATGCCTACTT CCCAGGCCCC AACATTGGAG GGGACACCCA CTTTGACTCT
human MT-1  ATGCCTACTT CCCAGGCCCC AACATTGGAG GAGACACCCA CTTTGACTCT
  pig MT-1  ATGCCTACTT CCCAGGCCCC AACATTGGAG GAGACACGCA CTTTGACTCT

            151
sheep MT-1  GCCGAGCCCT GGACTGTCCG GAATGAGGAT CTAAATGGGA GANTCT
 goat MT-1  GCCGAGCCCT GGACTGTCCG GAATGAGGAT CTAAATGGGA ATGACA
human MT-1  GCCGAGCCTT GGACTGTCAG GAATGAGGAT CTGAATGGAA ATGACA
  pig MT-1  GCCGAACCCT GGACTGTCCG GAATGAGGAT CTGAATGGGA ATGACA

Goat MT-1 accession number AB010921
Human MT-1 accession number Z48481
Pig MT-1 accession number AF067419
```

Appendix Fig 1. MT-1 multiple species sequence alignment.

```
                1                                                    50
  sheep MT-2    TTTACACANN ACNGTNNAAG CTAGGACTGG TACCACTCGC TGGAGGCGGT
  human MT-2    ATCCAGAACT ACACGGAGAA GTTGGGCTGG TACCACTCGA TGGAGGCGGT
  mouse MT-2    TCCAGAACTA CACTGAGAAG CT.GGGCTGG TACAACTCCA TGGAGGCGGT
chicken MT-2    TCCAGAACTA CACGGAGAAG CT.GGGCCGC TACCACTCGT ACGAGGCCAT

               51                                                   100
  sheep MT-2    GCGCCGGGCC TTCCGTGTGT GGGAGCAGGC CACGCCCCTG GTCTTCCAGG
  human MT-2    GCGCAGGGCC TTCCGCGTGT GGGAGCAGGC CACGCCCCTG GTCTTCCAGG
  mouse MT-2    GCGCAGGGCT TTCCAAGTGT GGGAGCAGGT CACACCATTG GTCTTCCAGG
chicken MT-2    CCGCCAGGCG TTCCGCGTGT GGGAGCGGGC CACGCCGCTG GCCTTCCAGG

              101                                                   150
  sheep MT-2    AGGTGCCTTA TGAGGACATC CGACTGCGGC GGCAGAAGGA GGCAGACATC
  human MT-2    AGGTGCCCTA TGAGGACATC CGGCTGCGGC GACAGAAGGA GGCCGACATC
  mouse MT-2    AAGTATCCTA TGATGACATT CGGCTACGAA GGCGAGCGGA GGCTGACATC
chicken MT-2    AGGTGCCCTA TGAAGACATC CGCCAGAAGC GGAAGAAGGA GGCTGACATC

              151                                                   200
  sheep MT-2    ATGGTACTCT TTGCCTCTGG TTTCCATGGC GACAGCTCGC CATTTGATGG
  human MT-2    ATGGTACTCT TTGCCTCTGG CTTCCACGGC GACAGCTCGC CGTTTGATGG
  mouse MT-2    ATGGTACTCT TTGCCTCTGG CTTCCATGGC GACAGCTCAC CGTTTGATGG
chicken MT-2    ATGGTGCTCT TCGCCTCGGG CTTCCACGGC GACAGCTCCC CTTTCGACGG

             201                                                    250
  sheep MT-2    CACGGGTGGC TTTCTAGCCC ACGCCTATTT CCCTGGCCCT GGTTTGGGTG
  human MT-2    CACCGGTGGC TTTCTGGCCC ACGCCTATTT CCCTGGCCCC GGCCTAGGCG
  mouse MT-2    CGTGGGTGGC TTTCTGGCCC ACGCTTATTT CCCCGGCCCT GGTCTGGGTG
chicken MT-2    CGTCGGGGGG TTCTTGGCTC ACGCTTATTT CCCCGGCCCG GGGATGGGGG

              251                                                   300
  sheep MT-2    GGGACACCCA TTTTGATGCA GATGAGCCCT GGACCTTCTC CAGCACTGAC
  human MT-2    GGGACACCCA TTTTGACGCA GATGAGCCCT GGACCTTCTC CAGCACTGAC
  mouse MT-2    GGGACACCCA TTTCGACGCA GATGAACCCT GGACCTTCTC CAGCACTGAC
chicken MT-2    GGGACACACA CTTTGACTCG GACGAGCCCT GGACGCTGGA AAACACGGAC

              301
  sheep MT-2    CTGCATGGAA ACAGCCTCTT CCTGGTGGCT GTGCAAAA
  human MT-2    CTGCATGGAA ACAACCTCTT CCTGGTGGCA GTGCATGA
  mouse MT-2    CTGCATGGAA TCAGCCTCTT TCTGGTGGCC GTGCATGA
chicken MT-2    GTGTCGGGGA ACAACCTCTT CCTGGTGGCT GTGCACGA

Human MT-2 accession number Z48482
Mouse MT-2 accession number D86332
Chicken MT-2 accession number AL588301
```

Appendix Fig 2. MT-2 multiple species sequences alignment.

```
                1                                                   50
  sheep MT-3    TGGAGACAGT TCTCCTTTTG ATGGAGAGGG AGGGTTTTTG GCACATGCCT
  human MT-3    TGGGGACAGC TCTCCCTTTG ATGGAGAGGG AGGATTTTTG GCACATGCCT
chicken MT-3    TGGAGACAGT TCTCCCTTTG ATGGGGAGGG AGGATTTTTG GCCCATGCAT
    rat MT-3    TGGAGACAGA TCCCCCTTTG ATGGGGAGGG AGGATTTTTG GCCCATGCTT

                51                                                  100
  sheep MT-3    ATTTCCCTGG ACCAGGAATT GGGGGAGATA CTCATTTTGA CTCAGATGAG
  human MT-3    ACTTCCCTGG ACCAGGAATT GGAGGAGATA CCCATTTTGA CTCAGATGAG
chicken MT-3    ATTTCCCTGG GCCAGGAATT GGGGGAGACA CTCATTTTGA CTCAGATGAA
    rat MT-3    ATTTCCCTGG GCCAGGAATT GGAGGCGATA CTCATTTTGA TTCTGATGAG

                101                                                 150
  sheep MT-3    CCATGGACAC TAGGAAATCC TAATCATGAT GGAAATGACT TATTTCTTGT
  human MT-3    CCATGGACAC TAGGAAATCC TAATCATGAT GGAAATGACT TATTTCTTGT
chicken MT-3    CCATGGACTT TGGGAAATCC TAATCATGAT GGAAATGATC TGTTTCTAGT
    rat MT-3    CCATGGACAC TGGGAAATCC CAATCATGAT GGAAATGATT TATTTCTTGT

                151
  sheep MT-3    GGCAGTTCAT GAACTGGGAC ATGCTCTGGG AATGAAGGA
  human MT-3    AGCAGTCCAT GAACTGGGAC ATGCTCTGGG ATTGGAGCA
chicken MT-3    GGCTGTGCAC GAACTGGGAC ATGCTCTGGG CTTGGAGCA
    rat MT-3    AGCAGTCCAT GAGCTGGGAC ATGCTCTAGG GCTGGAGCA

Human MT-3 accession number D83646
Chicken MT-3 accession number U66463
Rat MT-3 accession number D63886
```

Appendix Fig 3. MT-3 multiple species sequence alignment.

```
                1                                                   50
  sheep MT-5    AAGAAGAGGC GGACATCATG ATCTTCTTTG CCTCTGGTTT CCATGGCGAC
  human MT-5    GGAAGGAGGC AGACATCATG ATCTTTTTTG CTTCTGGTTT CCATGGCGAC
  mouse MT-5    GGAAGGAGGC AGACATCATG ATCTTCTTTG CTTCTGGTTT CCATGGTGAC
chicken MT-5    AGAGAGACGT GGATATTACG ATCATTTTTG CATCAGGTTT TCATGGAGAC

                51                                                  100
  sheep MT-5    AGCTCCCCAT TTGATGGAGA AGGGGGATTC CTGGCTCATG CCTATTTCCC
  human MT-5    AGCTCCCCAT TTGATGGAGA AGGGGGATTC CTGGCCCATG CCTACTTCCC
  mouse MT-5    AGCTCCCCAT TTGATGGGGA AGGGGGATTC CTAGCCCATG CCTACTTTCC
chicken MT-5    AGTTCTCCCT TTGATGGGGA GGGAGGATTT TTGGCCCATG CATATTTCCC

                101                                                 150
  sheep MT-5    TGGTCCAGGG ATTGGTGGAG ACACTCACTT CGACTCAGAC GAGCCGTGGA
  human MT-5    TGGCCCAGGG ATTGGAGGAG ACACCCACTT TGACTCCGAT GAGCCATGGA
  mouse MT-5    TGGCCCAGGG ATCGGAGGAG ACACTCACTT TGATTCAGAT GAACCCTGGA
chicken MT-5    TGGGCCAGGA ATTGGGGGAG ACACTCATTT TGACTCAGAT GAACCATGGA

                151                                                 200
  sheep MT-5    CATTAGGAAA TGCCAACCAT GATGGGAACG ACCTCTTCCT GGTGGCTGTG
  human MT-5    CGCTAGGAAA TGCCAACCAT GACGGGAACG ACCTCTTCCT GGTGGCTGTG
  mouse MT-5    CGCTAGGAAA TGCCAACCAT GATGGCAATG ACCTCTTCCT GGTGGCCGTG
chicken MT-5    CTTTGGGAAA TCCTAATCAT GATGGAAATG ATCTGTTTCT AGTGGCTGTG

                201
  sheep MT-5    CAA
  human MT-5    CAT
  mouse MT-5    CAT
chicken MT-5    CAC

Human MT-5 accession number AF131284
Mouse MT-5 accession number AB021226
Chicken MT-5 accession number U66463
```

Appendix Fig 4. MT-5 multiple species sequences alignment.

Appendix Fig 5. TIMP-2 multiple species sequence alignment.

```
                1                                                   50
 sheep TIMP-2   AAGGAGGTGG ACTCTGGCAA CGACATCTAT GGCAACCCCA TCAAGCGGAT
bovine TIMP-2   AAGGAGGTGG ACTCTGGCAA CGACATCTAC GGCAACCCCA TCAAGCGGAT
 human TIMP-2   AAGGAAGTGG ACTCTGGAAA CGACATTTAT GGCAACCCTA TCAAGAGGAT
   dog TIMP-2   AAGGAGGTGG ACTCTGGGAA TGACATCTAC GGCAACCCCA TCAAGCGGAT

               51                                                  100
 sheep TIMP-2   TCAGTATGAG ATCAAGCNNA TAAAGATGTT CAAGGGACCT GACCAGGACA
bovine TIMP-2   TCAGTATGAG ATCAAGCAGA TAAAGATGTT CAAGGGACCT GATCAGGACA
 human TIMP-2   CCAGTATGAG ATCAAGCAGA TAAAGATGTT CAAAGGGCCT GAGAAGGATA
   dog TIMP-2   TCAGTATGAG ATCAAGCAGA TAAAGATGTT CAAAGGACCA GACAAGGACA

               101                                                 150
 sheep TIMP-2   TAGAGTTTAT CTACACAGCC CCCTCCTCTG CCGTGTGCGG GGTCTCGCTG
bovine TIMP-2   TAGAGTTTAT CTACACAGCC CCCGCCGCTG CCGTGTGTGG GGTCTCGCTG
 human TIMP-2   TAGAGTTTAT CTACACGGCC CCCTCCTCGG CAGTGTGTGG GGTCTCGCTG
   dog TIMP-2   TAGAGTTTAT CTACACGGCT CCTTCCTCCG CCGTATGCGG GGTCTCCCTG

               151                                                 200
 sheep TIMP-2   GACATTGGAG GAAAGAAGGA GTATCTCATT GCAGGGAAGG CCGAGGGNAA
bovine TIMP-2   GACATTGGAG GAAAGAAGGA GTATCTCATT GCAGGGAAGG CCGAGGGGAA
 human TIMP-2   GACGTTGGAG GAAAGAAGGA ATATCTCATT GCAGGAAAGG CCGAGGGGGA
   dog TIMP-2   GACATCGGAG GAAAGAAGGA GTATCTCATT GCGGGAAAGG CCGAGGGGAA

               201                                                 250
 sheep TIMP-2   TGGCAATATG CATATCACCC TCTGTGACTT CATCGTGCCC TGGGACACCC
bovine TIMP-2   TGGCAATATG CATATCACCC TCTGTGACTT CATCGTGCCC TGGGACACCC
 human TIMP-2   CGGCAAGATG CACATCACCC TCTGTGACTT CATCGTGCCC TGGGACACCC
   dog TIMP-2   CGGCAAGATG CACATCACCC TTTGTGACTT CATCGTGCCC TGGGACACCC

               251                                                 300
 sheep TIMP-2   TGAGCGCCAC CCAGAAGAAG AGCCTGAACC ACAGGTACCA GATGGGCTGT
bovine TIMP-2   TGAGTGCCAC CCAGAAGAAG AGCCTGAACC ACAGGTACCA GATGGGCTGT
 human TIMP-2   TGAGCACCAC CCAGAAGAAG AGCCTGAACC ACAGGTACCA GATGGGCTGC
   dog TIMP-2   TGAGCAGCAC CCAGAAGAAG AGTCTGAACC ATAGGTACCA GATGGGCTGT

               301                                                 350
 sheep TIMP-2   GAGTGCAAGA TCACGCGCTG CCCCATGATC CCATGCTACA TCTCCTCCCC
bovine TIMP-2   GAGTGCAAGA TCACTCGATG CCCCATGATC CCATGCTACA TCTCCTCTCC
 human TIMP-2   GAGTGCAAGA TCACGCGCTG CCCCATGATC CCGTGCTACA TCTCCTCCCC
   dog TIMP-2   GAGTGCAAGA TCACGCGCTG CCCCATGATC CCGTGCTATA TCTCGTCTCC

               351                                                 400
 sheep TIMP-2   GGACGAGTGC CTTCTGGATG GACTGGGTCA CGGAGAAGAA CATCAACGGA
bovine TIMP-2   GGACGAGTGC C.TCTGGATG GACTGGGTCA CGGAGAAGAA CATCAACGGA
 human TIMP-2   GGACGAGTGC C.TCTGGATG GACTGGGTCA CAGAGAAGAA CATCAACGGG
   dog TIMP-2   GGATGAGTGC C.TCTGGATG GACTGGGTCA CGGAGAAGAG CATCAACGGG

               401                                                 450
 sheep TIMP-2   CATCAGGCCA AGTTCTTCGC CTGCATCAAG AGAAGTGACG GCTCCTGTGA
bovine TIMP-2   CACCAGGCCA AGTTCTTCGC CTGCATCAAG AGAAGCGACG GCTCCTGCGC
 human TIMP-2   CACCAGGCCA AGTTCTTCGC CTGCATCAAG AGAAGTGACG GCTCCTGTGC
   dog TIMP-2   CATCAGGCCA AGTTCTTCGC CTGCATCAAG AGAAGCGACG GCTCCTGTGC
```

Bovine TIMP-2 accession number M32303
Human TIMP-2 accession number S48568
Dog TIMP-2 accession number AF112115

Appendix Fig 6. TIMP-3 multiple species sequence alignments.

```
                1                                                   50
 sheep TIMP-3   CACCGGGCCA AGGTGGTTGG GAAGAAACTG GTGAAGGAGG GGCCCTTTGG
bovine TIMP-3   ATCCGAGCCA AGGTGGTAGG GAAGAAACTG CTGAAGGAGG GGCCCTTTGG
 horse TIMP-3   ATCCGGGCCA AGGTGGTGGG GAAGAAGCTG GTGAAGGAGG GGCCCTTTGG
   pig TIMP-3   ATCCGGGCCA AGGTGGTGGG GAAGAAGCTG GTGAAGGAGG GGCCCTTTGG

                51                                                  100
 sheep TIMP-3   CACACTGGTC TACACCATCA AGCAGATGAA GATGTACCGN NGGTTCACCA
bovine TIMP-3   CACGATGGTC TACACCATCA AGCAGATGAA GATGTACCGA GGATTCACCA
 horse TIMP-3   CACACTGGTC TACACCATCA AGCAGATGAA GATGTACCGA GGCTTCACCA
   pig TIMP-3   CACACTGGTC TACACCATCA AGCAGATGAA GATGTACCGA GGCTTCACCA

                101                                                 150
 sheep TIMP-3   AGATGCCCCA TGTGCAGTAC ATCCACACGG AAGCCTCTGA AAGTCTCTGT
bovine TIMP-3   AGATGCCCCA TGTGCAGTAC ATCCACACAG AAGCTTCTGA AAGTCTCTGT
 horse TIMP-3   AGATGCCCCA TGTGCAGTAC ATCCACACGG AAGCTTCCGA AAGTCTCTGT
   pig TIMP-3   AGATGCCCCA TGTGCAGTAT ATCCACACAG AAGCTTCTGA AAGTCTCTGT

                151                                                 200
 sheep TIMP-3   GGCCTTAAGC TTGAGGTCAA CAAGTACCAG TACCTGCTGA CAGGCCGTGT
bovine TIMP-3   GGCCTTAAGC TTGAGGTCAA CAAGTACCAG TACCTGCTGA CAGGCCGAGT
 horse TIMP-3   GGCCTTAAGC TGGAGGTCAA CAAGTACCAG TACCTGCTGA CAGGCCGTGT
   pig TIMP-3   GGCCTTAAGC TAGAAGTCAA CAAGTACCAG TACCTGCTGA CAGGCCGTGT

                201                                                 250
 sheep TIMP-3   CTATGATGGC AAGATGTACA CAGGACTGTG TAACTTCGTG GAGAGGTGGG
bovine TIMP-3   CTATGATGGC AAGATGTACA CAGGACTGTG TAACTTTGTA GAGAGGTGGG
 horse TIMP-3   CTACGACGGA AAGATGTACA CAGGACTCTG CAACTTCGTG GAGAGGTGGG
   pig TIMP-3   CTATGATGGC AAGATGTACA CAGGATTGTG TAACTTTGTG GAGAGATGGG

                251                                                 300
 sheep TIMP-3   ACCAGCTCAC CCTCTCCCAG CGCAAGGGGT TGAACTATCG ATATCACCTG
bovine TIMP-3   ACCAGCTCAC CCTCTCCCAG CGCAAGGGGC TGAACTATCG ATATCACCTG
 horse TIMP-3   ACCAGCTCAC CCTCTCCCAG CGCAAGGGGC TGAACTATCG GTATCACCTG
   pig TIMP-3   ACCAGCTCAC CCTCTCCCAG CGCAAGGGGC TGAACTATCG ATATCATCTG

                301                                                 350
 sheep TIMP-3   GGCTGTAACT GCAAGATCAA ATCCTGCTAC TACCTGCCTT GCTTTGTAAC
bovine TIMP-3   GGCTGTAACT GCAAGATCAA ATCCTGCTAC TACCTGCCTT GCTTTGTAAC
 horse TIMP-3   GGTTGTAACT GCAAGATCAA ATCCTGCTAC TACCTGCCTT GCTACGTGAC
   pig TIMP-3   GGTTGTAACT GCAAGATTAA ATCCTGCTAC TACCTGCCTT GCTTTGTGAC

                351                                                 400
 sheep TIMP-3   CTCCAAGAAT GAGTGTCTTT GGACCGACAT GCTCTCCAAT TTCGGCTACC
bovine TIMP-3   CTCCAAGAAC GAGTGTCTCT GGACCGACAT GCTCTCCAAT TTCGGCTACC
 horse TIMP-3   CTCCAAGAAC GAGTGTCTCT GGACCGACAT GCTCTCCAAT TTCGGGTACC
   pig TIMP-3   CTCCAAGAAT GAGTGTCTCT GGACAGACAT GCTCTCCAAT TTCGGCTACC

                401
 sheep TIMP-3   CTGGCTACCA GTCCAAACAC TACGCCTGCA TCCGGCAA
bovine TIMP-3   CTGGCTACCA GTCCAAACAC TACGCTTGCA TCCGGCAG
 horse TIMP-3   CTGGCTACCA GTCCAAACAC TACGCCTGCA TCCGGCAG
   pig TIMP-3   CGGGCTACCA GTCCAAACAC TACGCCTGCA TCCG....
```

Bovine TIMP-3 accession number U77588
Horse TIMP-3 accession number AJ243283
Pig TIMP-3 accession number AF156031

```
                1                                                    50
 embryo MT-3    AGGACATGCN CTGGGATTGN AGAGATGGAG AGGGAGGGTT TTTTGGCACA
  human MT-3    TCCATGGGGA CAGCTCTCCC TTTGATGGAG AGGGA.GGAT TTTTGGCACA
    rat MT-3    TCCATGGAGA CAGATCCCCC TTTGATGGGG AGGGA.GGAT TTTTGGCCCA
chicken MT-3    TTCATGGAGA CAGTTCTCCC TTTGATGGGG AGGGA.GGAT TTTTGGCCCA

                51                                                  100
 embryo MT-3    TGCCTATTTC CCTGGACCAG GAATTGGGGG AGATACTCAT TTTGACTCAG
  human MT-3    TGCCTACTTC CCTGGACCAG GAATTGGAGG AGATACCCAT TTTGACTCAG
    rat MT-3    TGCTTATTTC CCTGGGCCAG GAATTGGAGG CGATACTCAT TTTGATTCTG
chicken MT-3    TGCATATTTC CCTGGGCCAG GAATTGGGGG AGACACTCAT TTTGACTCAG

                101                                                 150
 embryo MT-3    ATGAGCCATG GACACTAGGA AATCCTAATC ATGATGGAAA TGACTTATTT
  human MT-3    ATGAGCCATG GACACTAGGA AATCCTAATC ATGATGGAAA TGACTTATTT
    rat MT-3    ATGAGCCATG GACACTGGGA AATCCCAATC ATGATGGAAA TGATTTATTT
chicken MT-3    ATGAACCATG GACTTTGGGA AATCCTAATC ATGATGGAAA TGATCTGTTT

                151                                                 200
 embryo MT-3    CTTGTGGCAG TTCATGAACT GGGACATGCT CTGGGATTGG AGATTTTTTT
  human MT-3    CTTGTAGCAG TCCATGAACT GGGACATGCT CTGGGATTGG AGCATTCCAA
    rat MT-3    CTTGTAGCAG TCCATGAGCT GGGACATGCT CTAGGGCTGG AGCATTCCAA
chicken MT-3    CTAGTGGCTG TGCACGAACT GGGACATGCT CTGGGCTTGG AGCACTCTAA


Human MT-3 accession number D83646
Rat MT-3 accession number D63886
Chicken MT-3 accession number U66463
```

Appendix Fig 7. Sheep embryo MT-3 multiple species sequence alignment.

```
            1                                                        50
sheep MT-5  CTACCACGAG ATCAAAAGTG ACCGGAAGGA GGCGGACATC ATGATCTTCT
human MT-5  ATACCATGAG ATCAAAAGTG ACCGGAAGGA GGCAGACATC ATGATCTTTT
mouse MT-5  ATACCATGAG ATCAAAAGTG ACCGGAAGGA GGCAGACATC ATGATCTTCT
  rat MT-5  ATACCATGAG ATCAAAAGTG ACCGGAAGGA GGCAGACATC ATGATCTTCT

            51                                                      100
sheep MT-5  TTGCCTCTGG TTTCCATGGC GACAGCTCCC CATTTGATGG AGAAGGGGGA
human MT-5  TTGCTTCTGG TTTCCATGGC GACAGCTCCC CATTTGATGG AGAAGGGGGA
mouse MT-5  TTGCTTCTGG TTTCCATGGT GACAGCTCCC CATTTGATGG GGAAGGGGGA
  rat MT-5  TTGCTTCTGG TTTCCATGGT GACAGCTCTC CTTTTGATGG GGAAGGGGGC

            101                                                     150
sheep MT-5  TTCCTGGCTC ATGCCTATTT CCCTGGTCCA GGGATTGGTG GAGACACTCA
human MT-5  TTCCTGGCCC ATGCCTACTT CCCTGGCCCA GGGATTGGAG GAGACACCCA
mouse MT-5  TTCCTAGCCC ATGCCTACTT TCCTGGCCCA GGGATCGGAG GAGACACTCA
  rat MT-5  TTCCTAGCCC ATGCCTACTT TCCTGGCCCA GGAATCGGAG GAGACACTCA

            151
sheep MT-5  CTTCGACTCA GACGAGCCGT G
human MT-5  CTTTGACTCC GATGAGCCAT G
mouse MT-5  CTTTGATTCA GATGAACCCT G
  rat MT-5  CTTTGATTCA GATGAGCCCT G

Human MT-5 accession number AL121753
Mouse MT-5 accession number AB021226
Rat MT-5 accession number AB023659
```

Appendix Fig 8. Sheep embryo MT-5 multiple species sequence alignment.