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THE ROLE OF MATRIX METALLOPROTEINASES AND TISSUE INHIBITORS OF METALLOPROTEINASES IN OVULATION

By

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ABSTRACT

THE ROLE OF MATRIX METALLOPROTEINASES AND TISSUE INHIBITORS OF METALLOPROTEINASES IN OVULATION

By

Leanne Joy Bakke

Extensive extracellular matrix (ECM) remodeling occurs in many physiological processes such as angiogenesis, ovulation, and bone development and many pathological processes such as tumor growth and metastasis, arthritis, and wound healing. The matrix metalloproteinases (MMPs) are a family of metal dependent enzymes that are largely responsible for degradation and restructuring of the ECM. Activity of the MMPs in the extracellular milieu is regulated by the tissue inhibitors of metalloproteinases (TIMPs). However, little is known regarding the intracellular and extracellular signaling that regulates the MMPs and TIMPs. The ovulatory process is an ideal model for studying the MMPs and their inhibitors because it involves extensive tissue remodeling. The preovulatory LH surge is the endocrine signal that triggers ovulation. My hypothesis is that MMP and TIMP mRNA expression and activity are increased following a gonadotropin-induced LH surge. Specifically, an increase in gelatinase-A (MMP-2), collagenase (MMP-1 and MMP-13) and membrane-type MMP (MMP-14) is anticipated that results in the ultimate dissolution of the ECM resulting in follicle rupture. Likewise, an increase in TIMP expression and activity is anticipated to provide a homeostasis in the extracellular milieu that prevents overproduction and unrestrained activity of the MMPs. To test my hypothesis, I investigated the effect of a gonadotropin releasing hormone (GnRH)-induced LH surge on localization, expression, and activity of

selected MMPs (MMP-1, 2, 13 and 14) and TIMPs (TIMPs 1-3). Following synchronization, bovine ovulatory follicles or new corpora lutea (CL) were collected by colpotomy at 0, 6, 12, 18, 24, and 48 h (CL) after a GnRH injection resulting in the LH surge (n = 5-8 per time point). MMP-14 expression, as well as TIMP-2 expression and activity, were increased following the LH surge, while MMP-2 mRNA expression and activity were unchanged. In addition, the collagenases MMP-1 and MMP-13 demonstrated increased mRNA expression following the LH surge. Of the two MMP-1 mRNA species detected, a 2.4 kb transcript was primarily expressed in the follicle, and a 1.8 kb transcript was predominant in the new CL. While TIMP-1 mRNA and activity were increased, TIMP-3 mRNA declined following the LH surge. Expression of MMP-14, MMP-2, and TIMP-3 mRNA was primarily localized to the theca cells, while TIMP-1 and TIMP-2 expression was localized to the granulosa cells. Based on their substrate specificity, intrafollicular localization, and increased mRNA expression following the LH surge, I conclude that MMP-1, MMP-13, and MMP-14 may mediate degradation of the type I and III collagen rich ECM in the theca layer and tunica albuginea. While MMP-2 mRNA remained unchanged, localization of MMP-2 activity to the cell surface may be important for basement membrane breakdown. The physiological significance of increased TIMP 1 and 2 following the LH surge is not yet known. TIMPs are multifunctional molecules that have been implicated in several functions within the ovary in addition to MMP inhibition. Finally, decreased expression of TIMP-3 following the LH surge may facilitate a shift in the MMP/TIMP ratio in the theca layer in favor of the MMP and thus promote ECM breakdown.

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Introduction

The extracellular matrix (ECM) provides both a scaffolding to which cells can attach and take shape and a specialized microenvironment that sequesters growth factors, contains ligands that interact with cell surface receptors, and directs cell motility [1, 2]. Extensive ECM remodeling occurs in many physiological and pathological processes. ECM remodeling is largely mediated by two families of proteinases, the matrix metalloproteinase family and the plasminogen activator/plasmin family, and their inhibitors [3]. The matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) play a role in ECM reorganization during normal physiological processes such as angiogenesis, embryo development, ovulation, mammary growth and involution, and bone development [4]. They also play a role during pathological processes such as tumor growth, rheumatoid arthritis, tumor cell metastasis, wound healing, and osteoarthritis [2, 5]. However, little is known regarding the intracellular and extracellular signaling that regulates the MMPs and their inhibitors.

Because the ovulatory process involves extensive tissue remodeling, it is an ideal model for studying the MMPs and TIMPs. Ovulation involves controlled degradation of both the epithelial and stromal layers of the follicle. Following follicle rupture, further cellular remodeling occurs during corpus luteum (CL) formation. Degradation of the ECM liberates growth factors and binding proteins, which regulate cell proliferation and migration [6]. Angiogenesis is also a prerequisite for CL development as the theca and granulosa layers become vascularized and begin to luteinize. Cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) are released by granulosa cells and/or macrophages. Both factors are potential regulators of MMP expression.

While much is known concerning the endocrinology of ovulation, little is known about the cellular events involved in follicle rupture. A better understanding of the mechanism of ovulation may lead to improved treatments for reproductive anomalies in humans and other species. A classic example is the luteinized unruptured follicle syndrome in humans. In this condition, mature follicles fail to rupture, and the luteinizing granulosa cells trap the ovum. This syndrome is often seen in women treated with prostaglandin synthase inhibitors [7, 8].

Additionally, MMPs, specifically gelatinase A (MMP-2), gelatinase B (MMP-9), and membrane type-1 MMP (MMP-14), have been implicated in ovarian cancer and metastasis. The majority of ovarian cancers develop after malignant transformation of the surface epithelium. Following follicle rupture, the ovarian surface repairs itself through rapid epithelial cell proliferation and remodeling. Ovarian epithelial cancers are believed to result from disruption of this normal tissue repair process resulting in uncontrolled degradation and invasion of the ovarian surface layer following follicle rupture [9].

Finally, an increased understanding of the mechanism of ovulation may ultimately be applicable to animal agriculture. For example, synchroniziation of ovulation in lactating dairy cows provides more control in artificial insemination (AI) programs and removes dependence on the cow to exhibit estrus or on the manager to detect/observe estrus before AI. Development of simpler and less costly methods of ovulation control than are currently available would have a major impact on reproductive management of dairy cows.

Chapter I

Literature Review

The mechanism of ovulation. Successful ovulation is necessary for fertilization and reproductive success (Figure 1). While much is known about the endocrinology of ovulation, little is known about the cellular mechanisms involved in follicle rupture. Scientists have been interested in ovulation since 1670 when de Graaf first recognized that the egg comes from the ovary but incorrectly concluded that the entire follicle was the egg [10]. Since then, many theories concerning the mechanisms of ovulation have arisen. At the beginning of the 20th century, investigators began examining the smooth muscle theory. It was brought about by persistent reports that the dominant cell type in the theca externa layer of the follicle wall was a smooth-muscle cell [11]. However, investigators were never able to chemically or electrically stimulate the follicle to rupture. The pressure theory was also popular at the beginning of the 20th century based on observations that follicle rupture was truly "an explosive phenomenon". However, intrafollicular pressure does not increase prior to ovulation [12]. The current and accepted theory is the proteolytic enzyme theory. The follicle wall becomes progressively weaker prior to follicle rupture. Initial evidence for this theory showed that treatment of follicle wall strips with proteolytic enzymes mimics preovulatory degradation of the follicular connective tissue [13].

The LH surge. The preovulatory LH surge is the endocrine signal that triggers ovulation. The follicular phase begins with regression of the corpus luteum. The

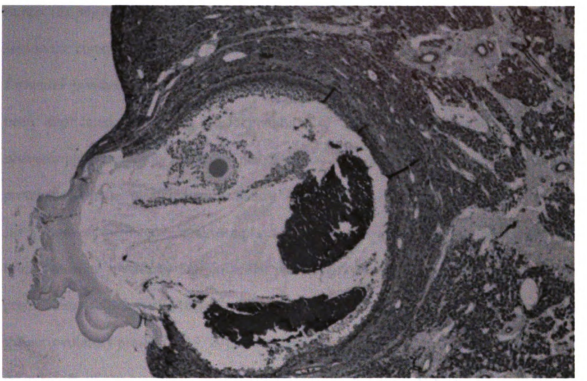


Figure 1: The mechanism of ovulation.

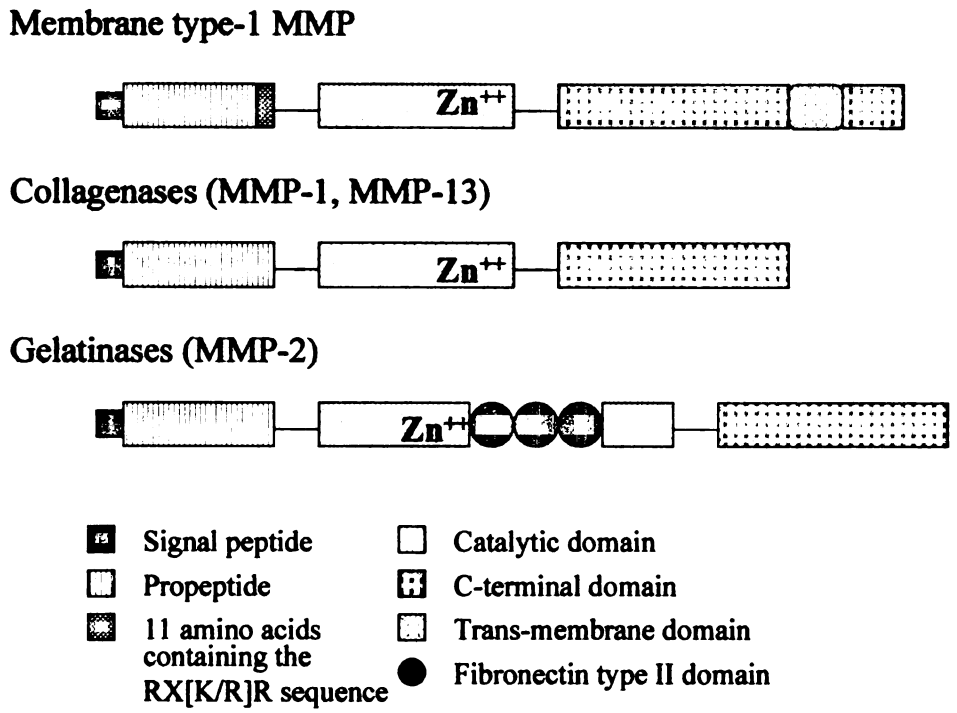
subsequent decrease in progesterone leads to an increase in LH pulse frequency, which induces further maturation of the ovulatory follicle. An increase in estradiol production from the dominant follicle results in increased gonadotropin releasing hormone (GnRH) culminating in the LH surge. Increasing evidence indicates that proteolytic extracellular matrix (ECM) degradation at the apex of ovulatory follicles prior to ovulation is a crucial step in the complex cascade of events initiated by the LH surge [14-17].

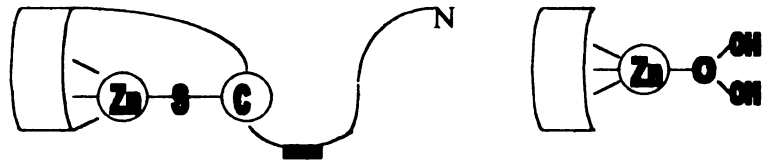
Enzymes involved in proteolysis of the follicle wall. Two families of enzymes have been implicated in causing the degradation of the follicle wall: the plasminogen activator/plasmin family and the matrix metalloproteinase family [3]. The serine proteinases tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) convert the inactive zymogen plasminogen into its active form plasmin, which has broad substrate specificity. Intrafollicular plasmin can decrease the tensile strength of the preovulatory follicle wall [18]. Concentrations of tPA, uPA, and plasmin increase during the periovulatory period [3, 19, 20]. Several studies suggest a role for plasminogen in the early stages of the ovulatory process such as in the activation of interstitial collagenase (MMP-1) and stromelysin-1 [21].

Mice with targeted mutations in the tPA and uPA genes have been examined to determine the absolute requirement of these enzymes in ovulation. Ovulation rates are normal in mice with a homozygous null mutation in either the tPA or the uPA gene [22]. However, mice carrying homozygous null mutations in both genes exhibit a 26% reduction in ovulation rate. Therefore, the plasminogen activator/plasmin enzyme family does contribute to the ovulatory process. However, these enzymes do not directly cleave all collagenous components of the ECM. Consequently, the activity of these enzymes

alone cannot account for the proteolytic degradation of the follicle wall that occurs prior to ovulation.

Structural and biochemical properties of the MMPs. The MMP family consists of >26 members to date; however, this number is rapidly increasing. The family is divided into four subclasses based on structural and biochemical properties. The subclasses are the collagenases, gelatinases, stromelysins, and the membrane type [23]. All MMPs are synthesized as preproenzymes. With the exception of the membrane type 1-MMP (MMP-14) and stromelysin-3, MMPs are secreted from cells as a latent proenzyme [23]. The full length MMP is made up of a ~ 20 amino acid signal peptide, an ~ 80 residue N-terminal pro-domain followed by the ~ 170 residue catalytic domain (Figure 2). The catalytic domain (except for matrilysin) is covalently connected through a 10 to 70 residue proline-rich linker to a ~ 195 residue C-terminal hemopexin-like domain [24]. In the membrane type-MMPs, the polypeptide chain contains an additional 75 to 100 residue extension which is thought to form a transmembrane helix and a small cytoplasmic domain. In the gelatinases, an additional 175 amino acids are inserted into the catalytic domain, which consists of three fibronectin-related type II modules that bind gelatin and collagen [24]. MMPs are further characterized by the unique sequence motif, PRCG[V/N]PD, in the propeptide, and the zinc binding motif, HEXGHX[L/M]G[L/M]XH, in the catalytic domain in which three histidines bind to the catalytic zinc atom [23].





Pro-MMP Active MMP

Figure 2: Structure of MMP Domains. The propeptide contains the cysteine switch, which interacts with Zn in the pro-MMP. The Zn/cys interaction is disrupted during the activation process. The pro-region is cleaved off resulting in the active MMP. (Modified from Nagase, 1997, reference 23).

Control of MMP activity. Upon secretion, control of MMP activity is achieved through three basic mechanisms: activation, inhibition, and localization (Figure 3). MMPs can be activated by a number of different mechanisms including intracellular processing by furin (MT-MMPs) or extracellular processing by soluble activators such as mast cell proteinases, serine proteinases (plasmin and kallikriens), and other proteolytically active MMPs [2]. Activation of proMMPs occurs in a stepwise process. The inactive MMP contains a cysteine in the pro-region whose sulfur interacts with the zinc that is bound to the catalytic domain. An initial cleavage of a proteinase susceptible "bait" region of the N-terminal domain disrupts this Cys-Zinc interaction. A second cleavage removes the remaining pro-region allowing the zinc to react with a water molecule and the MMP to become active [23]. This second cleavage is usually catalyzed by an MMP, but not by the activator proteinase that carries out the initial cleavage.

Inhibition of MMPs in the extracellular milieu is mediated by a family of proteins called the tissue inhibitors of metalloproteinases (TIMPs) as well as α_2- macroglobulin. Currently, four members of the TIMP family (TIMPs 1-4) have been identified [25]. TIMPs form a reversible noncovalent bond with MMPs with a 1:1 stoichiometry and high affinity, thereby inhibiting their activity [26]. The ratio of MMPs to TIMPs may be an important factor in ECM degradation since agents that stimulate MMP expression will also increase TIMP expression.

Localization of MMPs also regulates their activity. For example, MMP-2 can be localized to the cell surface through interactions with MMP-14 [27]. MMPs can also bind to integrins through their hemopexin-like domain, resulting in localization at the cell surface [28].

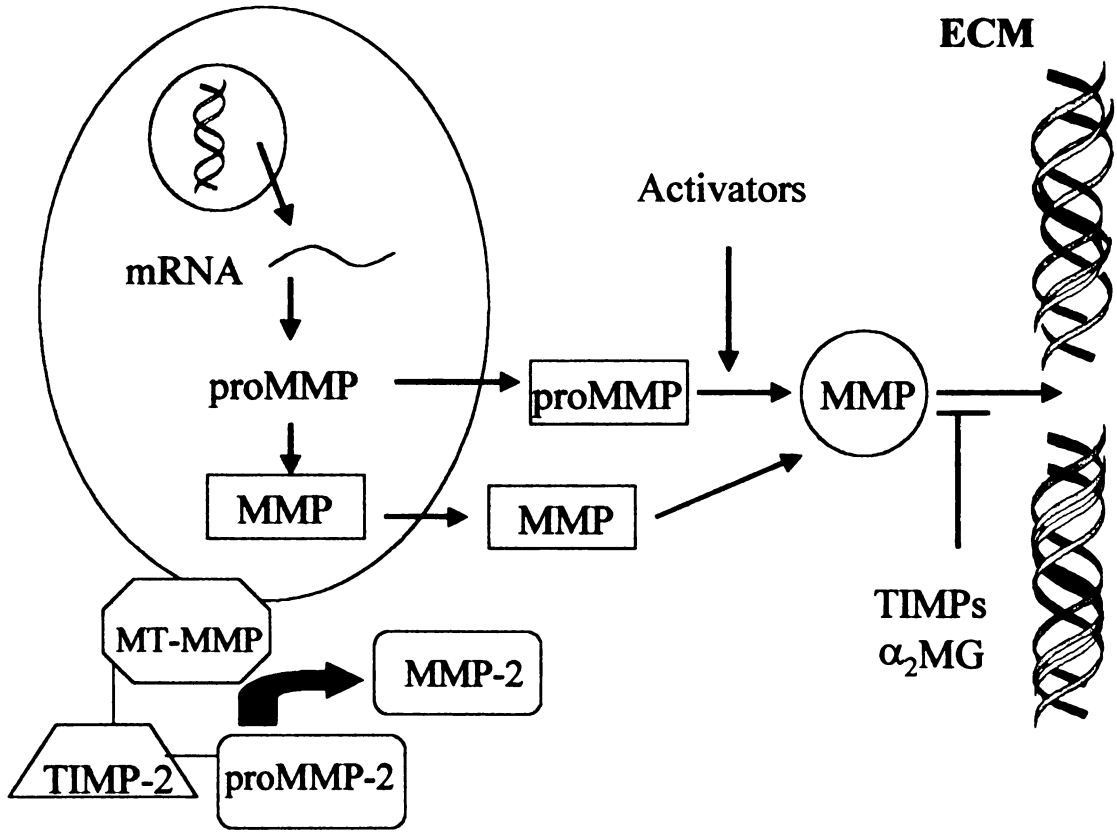


Figure 3: Control of MMP activity. A schematic representation of the pathways for matrix metalloproteinase (MMP) production, activation, and inhibition.

The Extracellular Matrix Components of an Ovarian Follicle.

The follicle wall. In order for an oocyte to be released from a follicle, it must pass through the many layers of the follicle wall (Figure 4). The first layer it encounters is the granulosa cell layer whose primary ECM component is fibronectin [15]. Next, it reaches the basement membrane, which separates the granulosa cells from the theca cells and contains collagen IV as well as laminin, fibronectin, and proteoglycan. The theca layer follows which consists of collagens I and III succeeded by the tunica albuginea which also consists primarily of collagens I and III [15]. Finally it reaches the surface epithelium which is a single layer of cuboidal cells which are loosely attached to a thin basement membrane at the surface of the tunica albuginea. The surface epithelium contains collagens I, III, and V as well as keratin and laminin.

The mechanism whereby the structural organization of the follicle promotes selective degradation at the apex of the follicle versus the base is not understood. Homogeneous layers of theca and granulosa cells surround the follicle. At the time of ovulation, granulosa and theca cells at the apex and base contain LH receptors and can respond to the LH surge. Electron microscopy of sheep follicles showed a decrease in theca collagen fibrils at the apex versus the base 24 hours after a GnRH-induced LH surge [17]. Furthermore, Murdoch detected increased collagenolytic activity at the apex versus the base of the follicle [17]. Further examination of enzyme activity at the apex versus the base is needed to understand the spatial regulation of the ovulatory process.

The surface epithelium. The most notable distinction between the follicle apex and base is the presence of the surface epithelium. During the ovulatory process, the surface

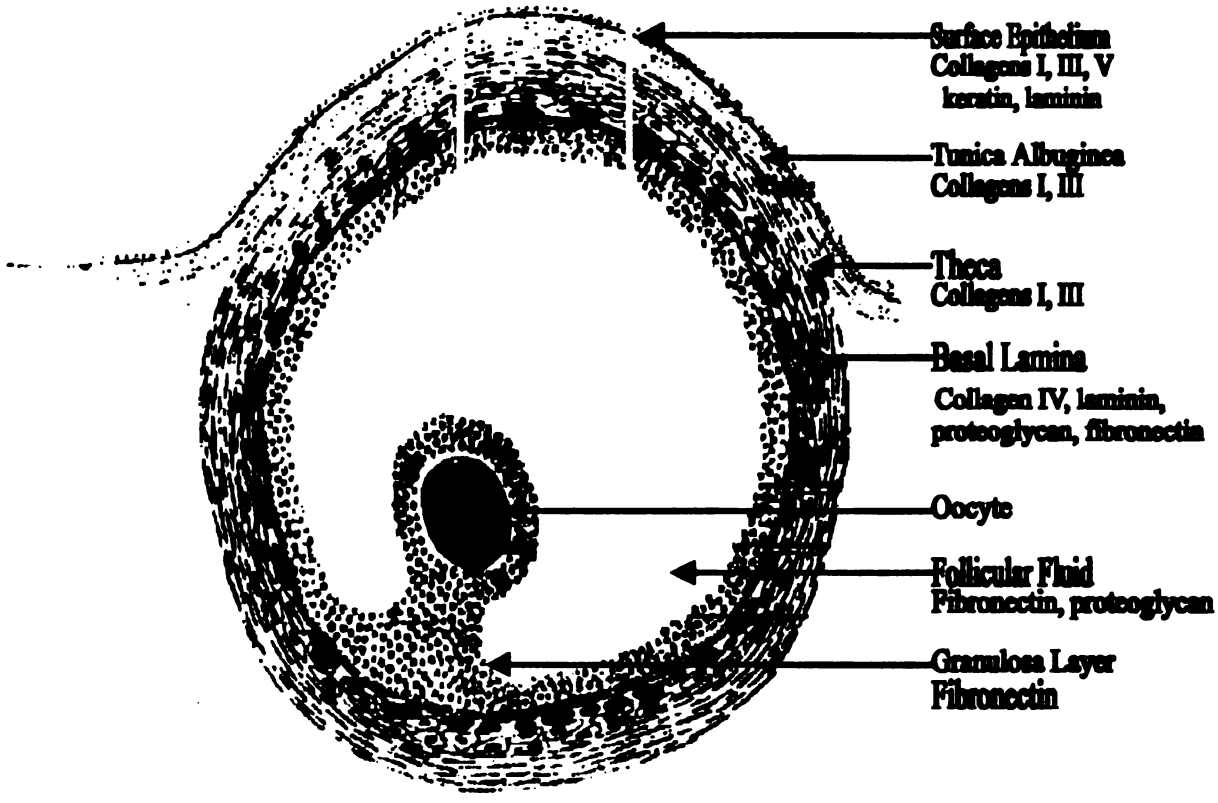


Figure 4: The extracellular matrix components of an ovarian follicle. (Modified from Espey and Lipner, 1994, reference 15).

epithelium undergoes apoptosis, and it is thought that proteolytic enzymes released from these dying cells could preferentially degrade the collagen at the apex of the follicle [29]. Several biochemical mediators have been implicated in the induction of apoptosis during ovulation such as tumor necrosis factor-α (TNF-α) and prostaglandin E_2 (PGE_2). Prostaglandin E_2 stimulated apoptosis in primary cultures of sheep ovarian surface epithelium [30]. TNF-α is a known apoptotic agent [31] that is secreted by bovine preovulatory follicles [32] and has enhanced ovulation rates [33]. Furthermore, TNF-α antibodies inhibited ovulation [34]. TNF-α is also thought to contribute to the ovulatory process by stimulating the expression of collagenase 1 (MMP-1) [35].

Scientists have long debated whether the ovulatory degradation of the follicular apex starts at the outside and successively progresses to the interior of the follicle wall, or whether the initiation of degradation lies in secretion of proteolytic enzymes originating from cells in the interior of the follicle such as granulosa or theca cells. Several studies point to the surface epithelial cells as the origin of proteolytic enzymes and follicle wall degeneration [29, 36, 37]. Furthermore, in certain species ovulation is restricted to an area (fossa) of the ovary covered by surface epithelium [38]. However, this hypothesis has not been supported by other studies showing that the surface epithelium is no longer a component of the follicle wall at the site where rupture occurs [39, 40]. In addition, previous studies have shown that if the surface epithelium is scraped from mature follicles, some of the scraped follicles will still ovulate after gonadotropin stimulation [41]. Finally, the fate of the ovarian surface epithelium may be related to the presence or absence of a basement membrane. At the time of hCG-induced ovulation, some ovarian surface epithelial cells undergo mitosis, while others undergo apoptosis [42, 43]. A series

of studies examining the role of potential growth factors within the ovary have shown that in the absence of extracellular matrix, hepatocyte growth factor (HGF) decreases cell contact, promotes cell migration, and induces apoptosis [44]. The dissolution of the basement membrane may result in apoptosis of the surface epithelial cells at the site of rupture, while the ovarian surface epithelial cells adjacent to the apex undergo mitosis and eventually fill in the stigma [42, 43, 45]. Further studies evaluating the presence of proteolytic enzymes at the apex of the follicle are necessary in order to understand the spatial regulation of follicle rupture.

Proteoglycans. The fibrous proteins of the extracellular matrix are imbedded in a gel-like "ground substance" which consists of glycosaminoglycan and proteoglycan molecules. Glycosaminoglycans (GAGs) are unbranched repeating unit polysaccharide chains that are usually sulfated. Glycosaminoglycans are divided into four groups based on the type and linkage of their sugar residues and the number and location of sulfate groups: heparin sulfate (HS) and heparin, chondroitin/dermatan sulfate (CS/DS), hyaluronic acid, and keratan sulfate. Except for hyaluronic acid, all GAGs are found covalently attached to a protein core in the form of proteoglycans. Several functions have been proposed for the proteoglycans in follicle development and ovarian function. The predominant GAGs in bovine follicular fluid are chondroitin/dermatan sulfates [46]. Chondroitin/dermatan sulfate was also localized to the basement membrane as well as the theca and granulosa cell layers within bovine antral follicles [47]. The CS/DS containing proteoglycans versican and decorin, as well as the HS-containing proteoglycan perlecan, were also observed in bovine antral follicles [47]. Versican is considered to be important in stabilizing extracellular matrix components [48], and decorin is known to regulate

collagen formation and ECM structure development [49]. Perlecan is found in the basement membrane and is thought to be involved in serum filtration and follicular fluid formation. Proteoglycans have also been proposed to influence the availability of lipoprotein as a steroidogenic substrate for granulosa cells [50]. In addition, theca proteoglycans stabilize the connective tissue matrix through interactions with collagen fibers, and breakdown of proteoglycans precedes follicle rupture [51].

The Role of MMPs in Ovulation

Although several MMPs likely play a role in ovulation, we have chosen to initially examine the roles of membrane type 1-MMP (MMP-14), gelatinase A (MMP-2), interstitial collagenase (MMP-1), and collagenase 3 (MMP-13) based on their substrate specificity and biochemical properties (Figure 5). A summary of available information on intrafollicular localization and periovulatory regulation of such MMPs in other species is outlined in Figure 6.

Membrane type 1-MMP. Membrane type MMPs (MT-MMPs) have been termed as possible "master switches" that control ECM remodeling [52]. Membrane type 1-MMP (MMP-14) is most noted for its ability to localize MMP activity to the cell surface [53]. MMP-14 can also activate MMPs, hence localizing proteolytic activity [54]. MMP-14 has been shown to activate gelatinase A (MMP-2) and collagenase 3 (MMP-13) [53, 55]. Activation of MMP-2, for example, occurs in several steps. First, TIMP-2 binds to MMP-14 at the cell surface. Next, pro-MMP-2 binds to TIMP-2 through its carboxy-terminal hemopexin domain. Binding to TIMP-2 localizes pro-MMP-2 at the cell surface and brings it in close proximity to MMP-14. A nearby MMP-14 molecule cleaves the propiece of MMP-2 yielding active MMP-2 in a trimolecular complex at the cell surface

Matrix Metalloproteinases of Interest

Enzyme	MMP No.	Matrix Substrate or Functions
Collagenases		
Interstitial Collagenase	MMP-1	Collagens I, II, III, IV, X
Collagenase 3	MMP-13	Collagens I, II, III
Gelatinases		
Gelatinase A	MMP-2	Collagens I, IV, V, gelatins
Membrane Type MMPs		
Membrane Type 1-MMP	MMP-14	Activates pro-MMP-2 Activates pro-MMP-13 Collagens I, III Fibronectin, laminin, proteoglycans

Figure 5: Matrix metalloproteinases of interest [23, 55].

[56]. MMP-14 may also play a direct role in degradation of the preovulatory follicle wall via its ability to cleave type I and III collagen as well as fibronectin, laminin, and proteoglycans. Espey and Lipner [15] postulated that proteinases localized on the cell surface promote cell dissociation and breakdown of the surrounding collagen fibers within the theca externa and tunica albuginea during ovulation. In addition, MMP-14 can indirectly promote degradation of types I and III collagen by its ability to activate procollagenase 3 (MMP-13) [55]. Furthermore, MMP-14 is not inhibited by TIMP-1, allowing degradation to occur in the presence of high concentrations of inhibitor [57]. This may be especially significant, given TIMP-1 is dramatically increased in preovulatory follicles of several species in response to the gonadotropin surge [58-62].

The role of MMP-14 in the ovulatory process is not understood (Figure 6). In mouse and rat preovulatory follicles, MMP-14 mRNA expression is evident in the granulosa cells and theca cells [58, 63]. In situ mRNA localization in mouse [58] and rat [63] preovulatory follicles revealed a decrease in MMP-14 mRNA expression in the granulosa layer with a simultaneous increase in MMP-14 expression in the theca-interstitial cells in response to hCG injection. However, previous studies of MMP-14 mRNA expression in mice (by Northern analysis) demonstrated no change in expression in the ovary following an ovulatory stimulus (hCG injection) [58].

Gelatinase A. The activity of gelatinase A may also contribute to the ovulatory process. Gelatinase A (MMP-2) is most noted for its ability to cleave the denatured helix of collagen (gelatin) and type IV collagen, a major component of the basement membrane. During the ovulatory process, it is thought that MMP-2 plays a role in degradation of the

Regulation of MMPs in Response to the LH Surge

	MT-1 MMP	Gelatinase A	Interstitial Collagenase	Collagenase 3
RNA	No change (mice)	↑ (rats) ↑ (macaque) No change (mice)	↑ (rats) ↑ (macaque)	?
In Situ	↓ GC (mice/rats) ↑ theca (mice/rats)	theca (mouse/rat)	GC/theca preov (gilt) theca after LH surge (gilt) GC (macaque, rabbit, human)	theca (rats)
Activity	?	↑ (rats) No change (mice) ↑ (sheep)	↑ (rats) ↑ (sheep)	?

Figure 6: Summary of intrafollicular localization and periovulatory regulation of MT1-MMP (MMP-14), gelatinase A (MMP-2), interstitial collagenase (MMP-1), and collagenase 3 (MMP-13) [35, 58, 63-68, 71, 74, 78].

basement membrane that separates the granulosa from the theca cells. It also further hydrolyzes the denatured fibrils of collagen following their initial cleavage by a collagenase. The effect of an ovulatory stimulus on MMP-2 expression during the periovulatory period appears to be species specific. MMP-2 mRNA expression [64] and activity [65] are increased following injection of surge levels of LH. Matrix metalloproteinase-2 mRNA is also increased in preovulatory macaque granulosa cells in response to hCG administration in vivo [66]. These results are in contrast to previous studies in the mouse which report that expression of MMP-2 mRNA and activity is constitutive during the periovulatory period [58]. In situ localization studies in the mouse and rat have localized MMP-2 mRNA to the theca layer [63, 64]. Further evidence of a role of MMP-2 in ovulation is seen in a study by Russel, et al, who immunized ewes against the N-terminal peptide of the 43-kDa subunit of α-N inhibin [67]. Following hCG administration, MMP-2 activity increased in follicular fluid of control ewes. However, MMP-2 activity decreased in the follicular fluid of immunized animals, and the ovulatory process was impaired. In addition, the LH surge increases MMP-2 activity in sheep follicular extracts [68] and intrafollicular injection of MMP-2 antibodies can block ovulation [69]. The precise function of MMP-2 in bovine preovulatory follicles awaits the localization of its expression and the regulation of its activity following the LH surge.

Collagenases. It has long been accepted that collagenolysis is a prerequisite for ovulation considering the dense meshwork of collagen fibers that make up the ECM of the follicle. The oocyte must penetrate three collagen rich layers of the follicle in order to be released. The collagenases are best noted for their ability to degrade types I, II, and III collagen. While much redundancy is seen among MMPs and their substrates, the

collagenases are unique in their ability to cleave the triple helical collagen molecule. Following this initial site-specific cleavage, the fragmented collagen becomes less stable and more soluble as it uncoils [70]. Following denaturation, the collagen products become susceptible to further degradation by other proteinases. In 1965, Espey and Lipner provided the first evidence for a role of collagenase in follicle rupture [13]. They injected minute amounts of bacterial collagenase into rabbit follicles and observed rupture minutes later. Reich first correlated collagenase activity with ovulation. He labeled follicular collagen and observed a 40% decrease in follicle collagen content following a hCG-induced LH surge [71]. Blocking the LH surge abolished this decrease. In the rat and sheep, an increase in preovulatory collagenolytic activity is seen following the LH surge [17, 71]. Furthermore, agents that inhibit collagenase activity inhibit ovulation [72]. Finally, mice with a targeted mutation in the type I collagen gene, resulting in a molecule that is resistant to collagenase, show markedly reduced fertility, potentially due to an impairment of the ovulatory process [73].

Preovulatory follicle collagenase activity is also regulated by the LH surge. Collagenolytic activity is increased in rat ovaries [71, 74] and sheep preovulatory follicles [35] in response to an ovulatory stimulus. Furthermore, there is evidence that selective collagen degradation occurs at the apex of the follicle, which could be attributable to collagenase activity. Espey first showed that as ovulation approaches, the follicle wall becomes thinner and the concentration of collagen fibrils is decreased at the apex [75]. Electron microscopy of sheep follicles showed a decrease in collagen fibrils at the apex versus the base 24 hours after a GnRH-induced LH surge [17]. Furthermore,

Murdoch detected increased collagenolytic activity at the apex versus the base of the follicle [17].

Interstitial collagenase. Interstitial collagenase (MMP-1) is most likely responsible for the initial degradation and unwinding of the triple helical fibers of collagen within the follicular apex prior to ovulation. The expression and localization of interstitial collagenase has been examined in several species (Figure 6). Interstitial collagenase mRNA increases 25-fold following the LH surge in rats [64]. MMP-1 mRNA is also increased in macaque granulosa cells following an ovulatory stimulus [66]. In gilts, in situ hybridization studies localized interstitial collagenase mRNA to the granulosa and theca interna cells of preovulatory follicles, but only to the theca interna following LH/hCG stimulation [76]. Immunolocalization of interstitial collagenase in rabbits demonstrated it is present within the theca and granulosa cells and increases within these cells after follicle rupture [77]. In monkeys, MMP-1 mRNA is expressed by granulosa cells following the LH surge, and human granulosa cells have also been shown to secrete MMP-1 [16, 66]. Previous studies have also shown increased proMMP-1 at the apex of rabbit follicles following gonadotropin stimulation [77].

Collagenase 3. Like interstitial collagenase, collagenase 3 (MMP-13) cleaves collagens I, II, and III. Unlike many of the other members of the MMP family that are expressed in several tissues throughout the body, in rats MMP-13 is highly expressed in the ovary [78]. The effect of the LH surge on MMP-13 expression is unclear. In rats, MMP-13 mRNA is highly expressed by theca/stroma cells of antral follicles [78]. Furthermore, while MMP-13 is not induced in immature rats following an ovulatory dose of hCG, it is expressed at high levels during proestrus and estrus in mature rats. These studies showed

MMP-13 to be at a minimum at metestrus and at a gradual incline from diestrus to proestrus [78]. It was postulated that an element required for induction of MMP-13 expression is not present in an immature rat. MMP-13 mRNA is undetectable by Northern analysis in the mouse ovary during the periovulatory period [58]. MMP-13 activity may also be localized to the cell surface by MMP-14 since MMP-14 activates pro-MMP-13 [23]. Furthermore, MMP-14 activation of MMP-13 is enhanced in the presence of gelatinase A [55].

Other MMPs. Limited information is available regarding the intrafollicular localization and periovulatory regulation of other newly described MMPs with relatively uncharacterized substrate specificity. In mice, MMP-19 is increased 5-10 fold following hCG treatment and is localized to the granulosa and theca cells of large preovulatory follicles [58]. Furthermore, MMP-23 is predominantly expressed in reproductive tissues [79] and could play a role in ovulation. However, increased expression of MMP-23 mRNA is not required for follicle rupture in cattle [80]. Further investigation will be required to elucidate the temporal and spatial regulation of expression of individual MMP family members and their precise contribution to the ovarian ECM degradation characteristic of the ovulatory process.

Matrix metalloproteinase-9 (gelatinase B) has also been implicated in the ovulatory process. In rats [56] and macaques [66], MMP-9 mRNA is increased in response to an ovulatory stimulus. Matrix metalloproteinase-9 activity is also increased in ovarian homogenates following an ovulatory dose of hCG in the mouse [81]. In rats, MMP-9, mRNA is localized to the theca cells of preovulatory follicles [82], and preliminary data in bovine show a similar result. In the bovine, MMP-9 may be the primary mediator of

follicular basement membrane breakdown prior to ovulation. Messenger RNA for MMP-9 is increased in bovine preovulatory follicles in response to the gonadotropin surge (Cassar, Bakke, and Smith, unpublished data).

The Role of MMP Inhibitors in Ovulation

Tissue Inhibitors of Metalloproteinases. The primary inhibitors of MMP activity in the ECM are a family of proteins called the tissue inhibitors of metalloproteinases (TIMPs 1-4). TIMPs play a critical role in controlling MMP-mediated ECM remodeling. TIMPs are thought to provide a homeostasis that prevents overproduction and unrestrained activity of MMPs. The MMP/TIMP ratio is an important regulator of the amount of ECM degradation. If the ratio favors MMP activity, degradation occurs. When the ratio favors the TIMPs, ECM deposition may occur [2]. Likewise, overproduction and unrestrained activity of MMPs is involved in tumor metastasis and osteoarthritis [83].

Tissue inhibitors of metalloproteinases inhibit MMP activity by binding noncovalently with a 1:1 stoichiometry and high affinity [26]. Although they share structural similarities, each TIMP is a separate gene product containing 12 cysteine residues that form six disulfide bonds and contribute to the stability of TIMP molecules [84]. TIMPs differ in their degree of glycosylation, molecular weight, as well as their solubility [85, 86]. While TIMPs 1, 2, and 4 are soluble within the extracellular milieu, TIMP-3 is secreted and then bound to the extracellular matrix [87, 88]. The least is known about TIMP-4, although preliminary studies suggest that it is most similar to TIMP-2 [85, 89]. TIMPs are multifunctional molecules that have been implicated in several functions within the ovary in addition to MMP inhibition. For example, TIMPs have been noted to promote steroidogenesis [90], stimulate cell growth in a variety of

tissues [91], help promote activation of MMPs [53], inhibit angiogenesis [92], and influence apoptosis [93], all processes that may contribute to ovulation and subsequent corpus luteum formation.

The specific functions of TIMPs during the periovulatory period are unclear. Interestingly, MMPs and TIMPs are often secreted in parallel as many factors that stimulate expression of MMPs also increase expression of the inhibitors [94]. The LH-mediated regulation of TIMPs 1, 2, and 3 appears to be species specific. While TIMP-1 mRNA is increased in the mouse [58, 95], rat [61, 64, 96], sheep [60], and macaque [66] following gonadotropin stimulation, the spatial regulation of TIMP-1 is distinct in these species. In the mouse, TIMP-1 expression is localized to granulosa and theca-interstitial cells of preovulatory follicles [58]. In mice that were not hormone primed, TIMP-1 expression was not observed in granulosa or theca cells at any stage of the cycle [95]. TIMP-1 expression in the rat is observed in the theca interna and to a lesser extent in the granulosa cells following hCG administration [97]. Other studies in the rat report the presence of TIMP-1 mRNA in both the granulosa cells and the residual tissue, and expression is increased after hCG stimulation [64]. In sheep, the granulosa cells are the primary source of TIMP-1 [60].

While TIMPs were once thought to be purely inhibitory to MMP function and degradation, it appears TIMP-2 can actually aid in the activation of proMMP-2 by localizing it with MMP-14 at the cell surface [23]. Following an ovulatory stimulus, TIMP-2 expression is constitutive in rat [98] and mouse [58, 95] ovaries and sheep preovulatory follicles [99], but is increased in macaque granulosa cells [66]. Previous studies observed increased expression of TIMP-2 mRNA in preovulatory follicular and

luteal tissue collected from beef heifers within 8 and 48 h (CL) of the preovulatory gonadotropin surge [100], but the localization of TIMP-2 mRNA and changes in TIMP-2 activity were not determined. Messenger RNA for TIMP-2 was localized exclusively to the theca layer of ovine follicles [99], and a similar localization has been observed in rats and mice [58, 101].

Previous reports examining the regulation of TIMP-3 are conflicting [58, 95]. In cycling mice, TIMP-3 is increased at early proestrus [95], while in eCG-primed mice, TIMP-3 expression is unchanged following hCG administration [58]. Furthermore, in mice [95] and rats [97], TIMP-3 expression is observed in both granulosa and theca cells as well as interstitial tissue following gonadotropin stimulation.

In the sheep, TIMP-1 mRNA is localized predominantly within the granulosa layer of ovine follicles collected 12 hours after the LH surge [102]. In contrast, TIMP-2 mRNA is localized exclusively within the theca layer of ovine follicles collected at the same time point, suggesting complementary yet distinct roles for each inhibitor during the preovulatory period [99].

α_2-Macroglobulin. The original nomenclature of MMP inhibitors consisted of two major classes of inhibitors: serum-borne, or fluid phase inhibitors, and tissue-derived [85]. The traditional serum-borne inhibitors are α_1-macroglobulin, α_2-macroglobulin, and α_1-inhibitor$_3$. TIMPs were considered to be the key inhibitors in the tissue. However, the terminology is now changing due to the detection of α_2-macroglobulin in the ovary [103] and follicular fluid [104], and the detection of TIMPs in serum. Previous studies have detected α_2-macroglobulin in theca cells, luteinized granulosa cells and CL

[103]. While α_2-macroglobulin has been shown to account for about 95% of collagenase inhibition in serum, its large molecular size (M_r=720,000) as well as its broad range of specificity appears to limit its physiological significance in the ovarian extracellular connective tissue matrix [104, 105]. Yet, as ovulation approaches, increased permeability of follicular capillaries and basement membrane breakdown may permit access to the extracellular matrix and follicular fluid. It has been postulated that α_2-macroglobulin might function in ovulation to restrict collagenolytic activity to the vicinity of the mature follicles that are destined to rupture [15, 106]. However, TIMPs remain the main focus for study of MMP inhibitory regulation due to their high abundance and specificity, as well as dynamic temporal and cell-specific regulation during the periovulatory period.

Pathways for Regulation of MMP Expression.

Ultimately, it will be of great importance to determine the cellular signaling that occurs between the LH surge and the time of MMP and TIMP induction. Several mechanisms have been proposed. There is evidence that the LH surge stimulates production of both eicosanoids (prostaglandins, leukotrienes, and thromboxanes) and the progesterone receptor, ultimately leading to stimulation of MMPs (Figure 7). The proposed pathway is as follows. LH works through a G-protein coupled receptor to activate adenylate cyclase followed by increased cAMP and protein kinase A (PKA) activity. The increased PKA activity changes the steroidogenic capacity of the granulosa and theca cells from production of primarily estrogen to production of large quantities of progesterone. This pathway also leads to induction of progesterone receptor gene expression. Stimulation of

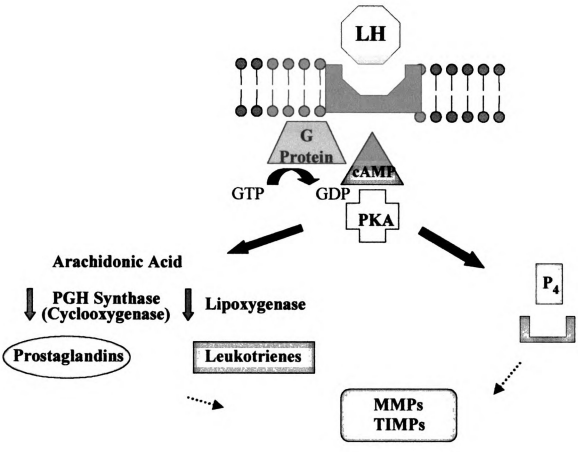


Figure 7: Model of cellular signaling that occurs between the LH surge and the time of MMP and TIMP induction.

the cyclooxygenase pathway leads to prostaglandin production, while stimulation of the lipoxygenase pathway leads to leukotriene production.

Transcriptional regulation of MMPs may be controlled on a number of different levels. The human MMP-1 promoter contains several regulatory sequences including five activator protein-1 (AP-1), five activator protein-2 (AP-2), five glucocorticoid-response elements and multiple ets/polyoma enhancer-binding 3 elements [107]. While some MMP-1 promoter AP-1 sites contribute to constitutive expression or upregulation by phorbol esters [108], no AP-1 sites have been found in the promoter region of the human MMP-2 gene, and MMP-2 expression is not induced by 12-O-tetradecanoyl-phorbol-13-acetate in most cell types [109]. Furthermore, the transcription factors Sp1, Sp3, and AP-2 are required for MMP-2 gene expression in certain cell types [110]. In contrast the human MMP-14 gene appears to have distinctive structural and functional features compared with other MMP genes. The locations of two exon-intron splicing sites are distinct from their conserved positions in other MMPs [111]. In addition, the MMP-14 promoter contains several putative regulatory elements, including one crucial Sp-1 site and four CCAAT-boxes, with no TATA-box [111]. Further investigation of MMP promoter regulatory sites and their interaction with transcriptional regulators such as NF-kappaB [112], p53 [113], transforming growth factor β [114], immediate early genes [108], and several other growth factors [107] will lead to a better understanding of the potential intra and intercellular mechanisms involved in MMP transcriptional regulation.

Eicosanoids. A considerable amount of data supports the role of eicosanoids in ovulation and MMP regulation. Inhibitors of arachidonic acid metabolism prevent

ovulation [115]. In rats, inhibitors of eicosanoid synthesis also suppress LH-induced interstitial collagenase mRNA expression [64]. Furthermore, indomethacin, an inhibitor of cyclooxygenase, and NDGA (nordihydroguaiaretic acid), an inhibitor of the lipoxygenase pathway, prevent ovarian collagenolysis and ovulation in rats [71]. Likewise, a specific inhibitor of 5-lipoxygenase, MK-886, suppressed collagenase activity as well as ovulation [116]. While a direct relationship between arachidonic acid derivatives and MMP activity in the bovine is still unknown, previous studies have shown that the cyclooxygenase-2 enzyme (COX-2; prostaglandin synthase enzyme) is induced within the granulosa layer of bovine preovulatory follicles following the LH surge [117, 118]. Follicular fluid PGE_2 levels are increased within 18 h after hCG treatment in cattle [117, 119]. Furthermore, preliminary results indicate that bovine preovulatory follicles express the PGE_2 receptor subtype EP2 (Cassar/Smith, unpublished). Mice with targeted mutations in the COX-2 gene fail to ovulate even when stimulated with exogenous gonadotropins [120, 121], and PGE_2 supplementation restores ovulation [122]. Furthermore, the ovulation rate is reduced in rats following administration of the COX-2 inhibitor NS-398 [123]. Likewise, LH-mediated increases in COX-2 mRNA and protein are seen in the rat, cow, and mare [124].

When discussing the role of LH-induced prostaglandin synthase and prostaglandin production in ovulation it is important to clarify the prostaglandin synthase being described. The LH surge was first shown to stimulate immunoreactive prostaglandin endoperoxide synthase (PSG), also called cyclooxygenase (COX), in preovulatory rat follicles in vivo in 1987 [125]. Initially, it was assumed that this was the same PGS (COX) that was originally isolated from ovine seminal vesicles [126].

However, affinity-purified antibodies indicated that there were two forms of PGS. One was inducible by LH in granulosa cells of preovulatory follicles (now called PGS-2/COX-2); the other was constitutively expressed in theca cells of developing follicles, corpora lutea, and uterus, but is not present in granulosa cells [127, 128]. In the cow, no detectable PGS-1 mRNA and protein are observed in preovulatory follicles indicating that PGS-2/COX-2 is the exclusive and critical source of prostaglandins during the ovulatory process [117]. Furthermore, mice with a targeted disruption of COX-2, but not COX-1, fail to ovulate in response to gonadotropin stimulation [120, 121].

Progesterone Of further interest is the role of progesterone in the signaling pathway between the LH surge and MMP expression. One of the first experiments to establish progesterone as an important mediator of the ovulatory process showed that ovulation could be inhibited by epostane [129]. Epostane is a competitive inhibitor of 3β-hydroxysteroid dehydrogenase, and most likely exerts its antiovulatory effects by blocking the conversion of pregnenolone to progesterone. In these experiments, treatment with exogenous progesterone could overcome the epostane-mediated inhibition of ovulation. Furthermore, progesterone antiserum administration also inhibited ovulation in rats [130].

A critical characteristic of granulosa cells exposed to the preovulatory LH surge is the ability to produce large amounts of progesterone. Prior to the LH surge, progesterone production by preovulatory follicles is low. The progesterone receptor is also a key participant in the ovulatory process. The LH surge induces progesterone receptor gene expression in rat [131] and mouse [132] preovulatory follicles in a time course similar to that of COX-2. Targeted mutation of the progesterone receptor revealed a critical role of

progesterone receptor signaling in the ovulatory process, as mice deficient in the progesterone receptor fail to ovulate [133]. Further evaluation of progesterone receptor-deficient mice revealed that following hCG administration, the ovaries contained unruptured preovulatory follicles, containing intact healthy mature oocytes, that had undergone all the necessary stages of follicular development. It was postulated that the progesterone receptor may be required for the final stages of ovulation, including the breakdown of the follicle wall by proteolytic enzymes [134].

Results in the bovine also support a potential role for progesterone receptor signaling pathways in the control of bovine follicle rupture. Progesterone receptor mRNA is expressed by bovine preovulatory follicles and is transiently increased following the LH surge. Progesterone receptor mRNA in the granulosa layer of bovine follicles was dramatically increased within 6 h following GnRH injection [135]. Therefore, the progesterone receptor signaling pathway which is required for ovulation in rodents may also play a role in the regulation of MMPs/TIMPs and ovulation in the bovine.

Structural changes. Structural changes in the extracellular matrix (ECM) can also regulate MMP expression. Degradation of the ECM liberates binding proteins and growth factors that are bound to ECM components, such as fibroblast growth factor (FGF), platelet derived growth factor (PDGF), and transforming growth factor-β (TGF-β), to name a few. PDGF, which is present in the capillary lumina at the apices of preovulatory follicles just before ovulation, has been shown to stimulate proMMP-1 production by aortic smooth muscle cells and skin fibroblasts [136, 137]. Furthermore, it is possible that the integrity of the basement membrane may regulate collagenase expression. Keratinocytes, *in vivo*, only express MMP-1 when they are not in contact

with an intact basement membrane [138]. Findings of Sudbeck et al suggest that re-establishment of the basement membrane at the completion of wound closure mediates down-regulation of MMP-1 expression [139]. Thus, ECM component interactions mediate functionally distinct cellular activities.

Summary

Extensive extracellular matrix remodeling occurs in many physiological and pathological processes and is largely mediated by the matrix metalloproteinases. Ovulation and subsequent corpus luteum formation involve the cyclic process of follicle rupture and controlled tissue degradation followed by further cellular remodeling and tissue repair. A delicate balance exists between the MMPs and their ability to digest specific ECM components and the TIMPs and their capacity to provide a homeostasis that prevents overproduction and unrestrained activity of MMPs.

Early studies of ovulation focused primarily on the endocrine control of periovulatory follicle maturation and the hormonal stimulation involved in ovulation. In more recent years, the cellular mechanisms and biochemical signals involved in the ovulatory process have gained increased attention. Specifically, the proteolysis theory of ovulation focusing on the morphological changes occurring at the follicle apex as the result of enzymatic degradation has become the accepted theory of ovulation. A growing body of evidence indicates that MMPs, regulated by TIMPs, play a key role in degradation of the follicle wall and subsequent follicle rupture.

A further knowledge of the mechanisms whereby the LH surge regulates specific MMPs and TIMPs may increase our understanding of the ovulatory process and the control of ECM remodeling. The experiments presented in the following chapters were

designed to contribute new information regarding the temporal and spatial localization and regulation of mRNA and activity for specific MMPs (MMP-1, -2, -13 and -14) and TIMPs (TIMP 1-3) during the ovulatory process in a model system (bovine) relevant to both animal agriculture and human health and infertility.

Chapter II

Materials and Methods

Materials and Methods

Animal Care

All procedures described where animals were used were approved by the All University Committee on Animal Use and Care at Michigan State University (Protocol # 04/98-056-00).

Experimental Model

Follicle development and timing of the preovulatory gonadotropin surge were synchronized in lactating Holstein cows at greater than 60 days postpartum using the Ovsynch procedure (GnRH-7d-$PGF_{2\alpha}$-36h-GnRH; Figure 8) [140]. Briefly, GnRH is injected to start a new wave of follicle growth and thus a new dominant follicle. Seven days later, $PGF_{2\alpha}$ is given to regress corpora lutea. A second GnRH injection is given 36 h later to induce a LH surge resulting in ovulation of the dominant follicle an average of 28 hours later [141]. Daily ultrasound analyses were performed after the first GnRH injection until the time of follicle collection to verify follicle synchrony and to exclude animals that turned over a new follicular wave prior to the second GnRH injection. Ovaries containing ovulatory follicles or new CL were collected by colpotomy at 0, 6, 12, 18, 24 and 48 h (CL) after the second GnRH injection (n = 5-8 per timepoint). Blood samples were collected at the time of $PGF_{2\alpha}$ injection and at the time of the second GnRH injection (Figure 9). Serum progesterone concentrations in these samples were measured by RIA (Diagnostic Products Corporation, Los Angeles, CA) to ensure that all

EXPERIMENTAL MODEL

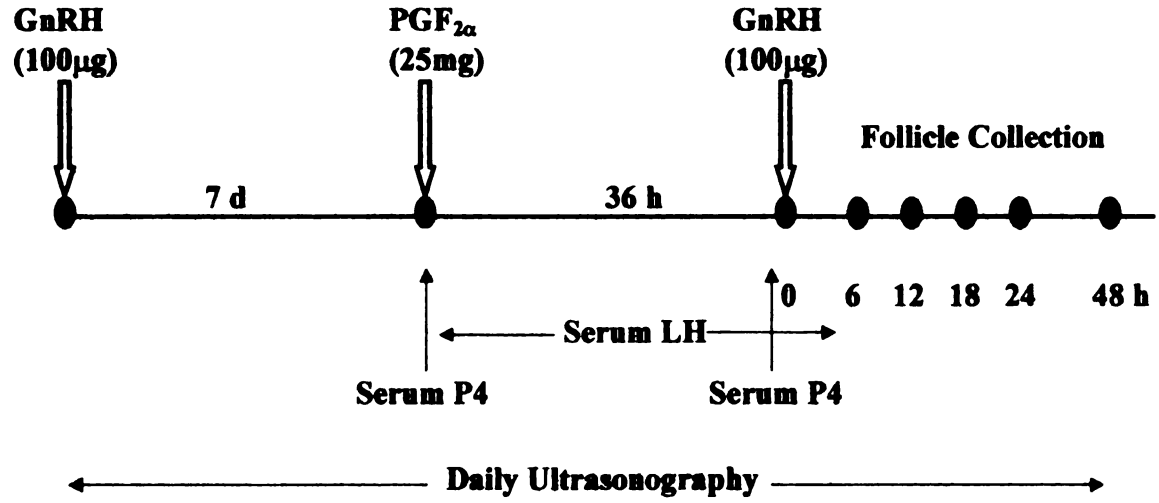


Figure 8: Experimental model used for collection of preovulatory follicles (0, 6, 12, 18, and 24 h) and CL (48 h) at specific time points relative to a GnRH-induced LH surge.

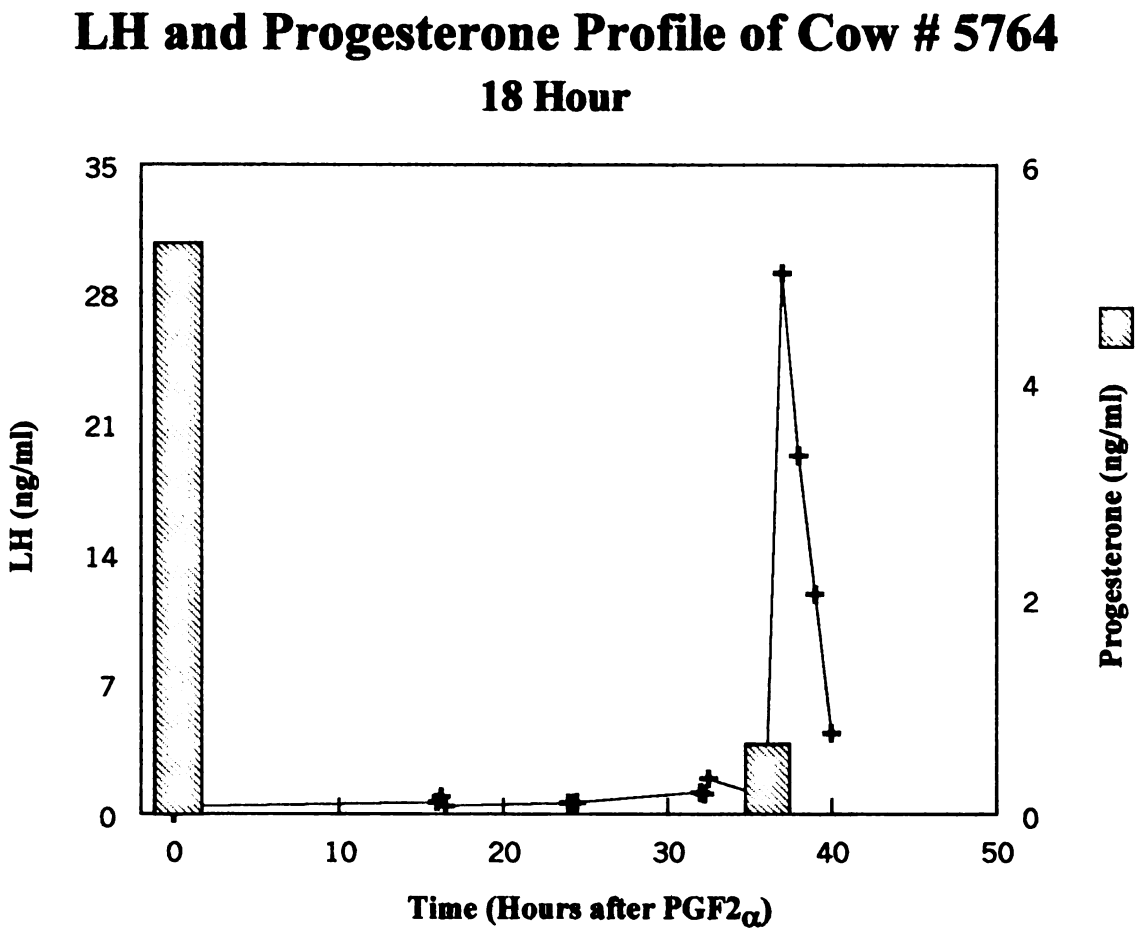


Figure 9: Representative serum LH and progesterone profile for a cow that was synchronized for follicle collection 18 h following a GnRH-induced LH surge.

animals responded to $PGF_{2\alpha}$ injection. Assay of serum progesterone levels at the time of $PGF_{2\alpha}$ injection revealed levels indicative of a functional corpus luteum. At the time of the second GnRH injection, progesterone levels were below 1 ng/ml in all animals included in the study, indicative of a regressing corpus luteum. Intraassay and interassay coefficients of variation for the progesterone radioimmunoassay were 5.6 and 9.1% respectively. To exclude any animals that exhibited a preovulatory gonadotropin surge prior to the second GnRH injection, three blood samples at 15-min intervals were collected every 8 h beginning 16 h after $PGF_{2\alpha}$ injection until the time of ovariectomy or GnRH injection. In order to confirm that a LH surge was elicited by the second GnRH injection, blood samples were also collected every hour for 4 h after the second GnRH injection. Concentrations of serum LH in the above samples were measured by RIA [142, 143]. For all animals included in the study, a premature LH surge was not detected in any of the animals in the 0 h (pre-gonadotropin surge) group. A LH surge was detected 1-2 h after GnRH injection for all animals in the post-surge groups (6, 12, 18, 24 and 48 h), verifying control of timing of the gonadotropin surge in our model system (Figure 9). Intraassay and interassay coefficients of variation for the LH radioimmunoassay were 5.8 and 15.6%.

Tissue Collection

For mRNA quantification and enzyme activity assays, ovaries containing the ovulatory follicle or new CL were collected at 0, 6, 12, 18, 24 and 48 h (n = 5-8 each) following the second GnRH injection. Following ovariectomy, the ovulatory follicle or new CL was isolated by cutting away all remaining ovarian stroma and small follicles such that the

ultrastructure at the apex of the follicle remained intact. Follicular fluid was aspirated, centrifuged, aliquoted and stored at -20°C until activity assays. Follicles were then sagitally cut in half so that each half contained the apex and base. One half was used for total RNA isolation. For protein analysis, the remaining half was cut transversely in two equal pieces, one containing the follicle apex and one the base. New CL collected 48 h post GnRH injection were only used for mRNA analyses. Samples were frozen at –80°C within 15 min of ovariectomy. For in situ hybridization, ovaries containing the ovulatory follicles were collected at 0, 12, and 24 h (n = 3 each) following GnRH injection. Ovulatory follicles were dissected from the ovary, immediately immersed in embedding medium, frozen over liquid nitrogen vapors, and stored at –80°C until sectioned. Twelve micrometer frozen sections were cut at -20 °C and mounted onto positively charged slides (Superfrost/Plus; Fisher Scientific, Pittsburgh, PA). Slides were then stored in an airtight box at -80 °C until hybridization.

Generation of cDNAs

Bovine complementary DNAs (cDNA) were generated by the reverse transcription polymerase chain reaction (RT-PCR) using bovine corpus luteum RNA and nucleotide primers (~20 nucleotides in length) corresponding to 100% conserved sequences present in the nucleotide sequence of analogous human and rat cDNAs. A 416 bp partial cDNA encoding bovine MMP-2 [corresponding to nucleotides 1317 to 1733 of human MMP-2 cDNA (Genbank accession #AJ243311.1)] and a 360 bp partial cDNA encoding bovine MMP-14 [corresponding to nucleotides 599 to 959 of human MMP-14 cDNA (Genbank accession #XM033440.1)] were ligated into pBluescript plasmids (Stratagene, La Jolla, CA) and subjected to fluorescent dye terminator sequencing to verify identity and

determine orientation. A 289 bp fragment of an ovine TIMP-2 cDNA [99], a 309 bp fragment of an ovine TIMP-1 cDNA [60], and a 340 bp fragment of an ovine TIMP-3 cDNA [144] were used for detection of bovine TIMP-1, TIMP-2, and TIMP-3 mRNA in the present studies.

To generate bovine MMP-1 and MMP-13 cDNAs, a 356 bp partial cDNA encoding MMP-1 [corresponding to nucleotides 632 to 988 of human MMP-1 cDNA (Genbank accession #XM 040735.1)] and a 376 bp partial cDNA encoding MMP-13 [corresponding to nucleotides 900 to 1275 of human MMP-13 cDNA (Genbank accession #XM 040746.1)] were ligated into pBluescript plasmids (Strategene, La Jolla, CA) and subjected to fluorescent dye terminator sequencing to verify identity and determine orientation. A second 334 bp MMP-1 partial cDNA encoding a portion of MMP-1 upstream of the previously mentioned MMP-1 cDNA [corresponding to nucleotides 175 to 508 of human MMP-1 cDNA (Genbank accession #NM 002421.2)] was also generated for use in subsequent experiments.

Characterization of mRNA Abundance

Total RNA was isolated using the Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. Poly A^+ RNA was isolated from the total RNA according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN) to increase sensitivity for MMP-13 Northern blots. To determine transcript size and number and to optimize specificity of hybridization conditions, approximately 15 μg pooled total RNA or 6 μg poly A^+ RNA (MMP-13) from each sample per time point was subjected to electrophoresis through 1% agarose-formaldehyde gels. RNA was then capillary transferred to nylon membranes (BioRad, Richmond, CA)

and UV crosslinked. For quantification of mRNA abundance, 5-6 μg total RNA (6 μg MMP-1, 5 μg all others) isolated from each sample were spotted in duplicate onto nylon membranes (Zetaprobe, BioRad, Richmond, CA) using a dot blot apparatus (BioRad, Richmond, CA). Membranes were allowed to air dry and then UV crosslinked.

Specific ^{32}P-labeled MMP-2, MMP-14, TIMP-2, MMP-1, MMP-13, TIMP-1, TIMP-3, and ribosomal protein L-19 (RPL-19) cDNA probes were prepared by PCR with α-[^{32}P] dCTP (3000Ci/mMol; Dupont/New England Nuclear, Wilmington, DE) [145]. RPL-19 was used for normalization purposes. Each PCR mix contained 1X PCR buffer, 2.5 mM $MgCl_2$, 1.55 μM dNTPs (minus dCTP), 1.5 units of Taq polymerase (Life Technologies, Inc., Gaithersburg, MD), 0.25 μM of each primer, 5 μl α-[^{32}P] dCTP and 100 pg of template. PCR conditions were as follows: 95°C 5 min, 40 cycles of [94°C 30s, 54°C 1 min, 72°C 1.5 min], 72°C 10 min. After amplification, the unincorporated ^{32}P was removed by spun column chromatography through G-50 Sephadex (Sigma Chemical Co., St. Louis, MO) minicolumns [146].

Membranes were prehybridized at 42°C overnight in either 50% formamide, 5X SSC (Saline-sodium citrate buffer; 1X is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5X Denhardt's (1X is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 50 mM $NaPO_4$, 0.1% SDS, and 250 μg herring sperm DNA/ml prehybridization buffer or Ambion Ultrahyb buffer (Ambion, Austin, TX) containing 250 μg herring sperm DNA/ml (MMP-13). Subsequent hybridizations took place at 42°C for 18 h in 50%

formamide, 5X SSC, 1X Denhardt's, 20 mM $NaPO_4$, 0.1% SDS, 10% dextran sulfate, or Ambion Ultrahyb (MMP-13) with the addition of 100 μg herring sperm DNA/ml hybridization buffer and > 1 x 10^6 cpm/ml ^{32}P-labeled cDNA. Membranes were washed in 1X SSC, 0.1% SDS, 0.1% sodium pyrophosphate for 20 min at 42°C followed by one 20 min wash in 0.1X SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 42°C, one 20 min wash at 47°C and one 20 min wash at 50°C. Following washing, filters were exposed to a phosphoimager cassette. After exposure (2-24 h) the cassette was scanned using a phosphoimager (BioRad, Hercules, CA). After Northern analyses, size of RNA transcripts was determined based on relative migration of RNA molecular weight markers (Roche, Indianapolis, IN). After hybridizations for each specific MMP or TIMP, the membranes were then stripped and reprobed with the ^{32}P RPL-19 cDNA. Preliminary experiments demonstrated that RPL-19 mRNA abundance in bovine preovulatory follicles and new CL is not regulated by the gonadotropin surge ($P > 0.05$). Relative densitometric units for MMP-2, MMP-14, TIMP-2, MMP-1, TIMP-1, and TIMP-3, were quantitated and adjusted relative to RPL-19 mRNA expression using Molecular Analyst Version 1.5 software (BioRad, Hercules, CA). Preliminary Northern blot experiments demonstrated that hybridization and washing conditions used in subsequent dot blot analyses were specific and yielded hybridization to single transcripts of the expected size for each mRNA of interest. Preliminary experiments also demonstrated that an increase in hybridization intensity was detected following hybridization of each cDNA to increasing amounts of sample RNA (1-10 μg).

Semi-quantitative multiplex RT-PCR

Relative levels of MMP-13 mRNA in periovulatory follicular and luteal RNA samples were determined by semiquantitative multiplex RT-PCR analysis as described by Kurebayashi, et al [147]. A 1 μg aliquot of each RNA sample was treated with RNase free DNase I (Life Technologies, Gaithersburg, MD) and a 91 ng fraction was utilized in first strand cDNA synthesis with Superscript II reverse transcriptase according to the manufacturer's instructions. Synthesized cDNA was then used as a template for co-amplification of a 245 bp partial cDNA for bovine MMP-13 and a 360 bp cDNA encoding for a housekeeping control gene (bovine RPL-19). Negative control cDNA synthesis reactions were conducted in the absence of reverse transcriptase and used as template in PCR to verify absence of genomic DNA contamination. Ratios of primer sets between MMP-13 and RPL-19 and number of cycles were determined to amplify both products logarithmically at relatively similar efficiencies without competition. PCR reactions for each sample were carried out in duplicate. A portion of each reaction was run on an agarose gel and following ethidium bromide staining, intensity of amplified products was determined with image analysis software (Quantity One, BioRad Laboratories, Hercules, CA). Preliminary experiments were conducted to verify a linear relationship between amount of product loaded on gel and density detected with the image analysis program. Relative expression levels are reported as the density of the amplification product for MMP-13 divided by that of the housekeeping gene from the same cDNA. Data are reported as % increase in expression levels over 0 h (pre gonadotropin surge) samples. Intra and interassay coefficients of variation were < 6%.

In Situ Hybridization

Messenger RNAs for MMP-2, MMP-14, TIMP-1, TIMP-2, and TIMP-3 were localized by in situ hybridization in follicles collected at 0, 12, and 24 h following GnRH injection (n = 3 per time point). Before hybridization, mounted sections were fixed in 4% formalin for 5 min, acetylated in 0.25% acetic anhydride in 0.1M triethanolamine (pH 8) for 20 min, and dehydrated in ethanol before hybridization. Hybridizations were carried out on three serial sections for each probe on each tissue sample and done in triplicate.

Both antisense and sense [^{35}S] (MMP-2, MMP-14, TIMP-1, TIMP-2) or [^{33}P] (TIMP-3) UTP-labeled cRNA probes were transcribed from linearized cDNA templates using a transcription kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The cRNA probes were purified by centrifugation on a Sephadex G-50 column (Sigma Chemical Co., St. Louis, MO) and used for hybridization within 24 h. For hybridization, the labeled probes were diluted in hybridization buffer [50% formamide, 0.3 M NaCl, 10 mM Tris (pH 8), 1 mM EDTA, 1X Denhardt's solution, 10 mM dithiothreitol, 500 μg/ml yeast transfer RNA, and 10% dextran sulfate] to 12,500 cpm/μl. Hybridization was performed using 80 μl (1 million cpm) diluted probe per slide in a humidified oven at 55°C for 18 h. After hybridization, slides were washed twice in 2X SSC for 15 min each at room temperature and treated with ribonuclease-A (50 μg/ml) for 30 min at 37°C. Slides were then washed at 55°C in 2X SSC/ 0.1% β-mercaptoethanol (β-me) for 15 min, 1X SSC/0.1% β-me for 15 min, 1X SSC/50% formamide/ 0.1% β-me for 30 min, and 0.1X SSC/0.1% β-me for 30 min. The slides were then dehydrated, air dried, dipped in Kodak NTB-2 emulsion (Eastman Kodak,

Rochester, NY) and exposed for 4 days (MMP-2), two days (TIMP-1 and TIMP-2), or two weeks (MMP-14 and TIMP-3) at 4°C. The slides were then developed, lightly counterstained with hematoxylin and eosin, and coverslipped for microscopic examination.

Preparation of Follicle Homogenates

Follicles were homogenized using procedures previously described by Murdoch [17]. Briefly, the apical or basal sections of follicles were homogenized using a polytron homogenizer (Fisher Scientific, Chicago, IL) in 800 µl of homogenization buffer [10 mM $CaCl_2$, 0.25% Triton X-100], then centrifuged at 9,000 x g for 30 min at 4°C. The supernatants were removed and hereafter referred to as Triton extracts. Each pellet was resuspended in 200 µl of resuspension buffer [50 mM Tris (pH 7.6), 100 mM $CaCl_2$, 0.15 M NaCl] and heated for 10 min at 60°C followed by centrifugation at 27,000 x g for 30 min at 4°C. Supernatants were removed and hereafter referred to as heat extracts.

Gelatin Zymography/Reverse Zymography

Gelatin zymography was performed for semi-quantitative analysis of gelatinolytic activity in follicular fluid and follicle extracts. Follicular fluid (0.25 µl) as well as proteins in triton (80 µg/lane) and heat (20 µg/lane) extracts from the apex and base of each follicle were subjected to electrophoresis in duplicate on one-dimensional SDS-polyacrylamide gels containing 0.25% (w/v) gelatin. All samples in a given experimental group (i.e. triton apex or heat base) were run together on a given day. Protein concentration for each sample was determined using a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Follicular fluid was treated as a bodily fluid and

loaded by volume; however, follicular fluid protein concentrations in each sample were measured, and variation in protein concentration was < 10%. Following electrophoresis, gels were incubated in 2.5% (v/v) Triton X-100 to remove SDS. Gels were rinsed and incubated overnight in 50 mM Tris (pH 7.5), 5 mM $CaCl_2$, and 0.02% NaN_3 with or without 1,10 phenanthroline (final concentration 0.05 mM) at 37°C. Gels were then stained with Coomassie Brilliant Blue for 1 h to visualize clear bands of digested gelatin representing gelatinolytic activity. Gels were photographed using a Gel Documentation System (BioRad, Hercules, CA). Area of degradation was measured using Molecular Analyst Version 1.5 software (BioRad, Hercules, CA) to derive individual estimates of MMP-2 activity. A pooled follicular fluid standard was run on each gel and used to calculate intrassay coefficients of variation (gel to gel variation) for analysis of each set of samples. Intraassay coefficients of variation were all $\leq$ to 1.5 %. All gelatinolytic activity was inhibited when 1,10 phenanthroline was included in the incubation buffer. To confirm that gelatinolytic activity detected corresponded primarily to MMP-2, recombinant proMMP-2 as well as a combined MMP-2/MMP-9 standard (Oncogene Research Products, Cambridge, MA) were run with each group of samples analyzed. Activity for MMP-2 co-migrated with appropriate standards. To further confirm that the majority of activity detected represented the latent form of MMP-2, selected samples and MMP-2 standards were subjected to organomercurial activation by incubation in 50 mM Tris (pH 7.5), 5 mM $CaCl_2$, 1 mM aminophenylmercuric acetate for 30 min at 37°C prior to gelatin zymographic analysis. Preliminary experiments established optimal incubation

time and demonstrated that increasing activity was detected when increasing amounts of sample protein were loaded on gels.

Reverse zymography was performed to characterize effects of the preovulatory gonadotropin surge on TIMP-1, TIMP-2, and TIMP-3 activity. Triton extracts (80 μg/lane), heat extracts (15 μg/lane), and follicular fluid (2 μl/lane) samples were subjected to electrophoresis in one dimensional SDS polyacrylamide gels containing 0.6 % gelatin and 400 μl of BHK cell conditioned media (source of gelatinases). Standards for TIMP-1, TIMP-2, and TIMP-3 (University Technologies International, Calgary, Alberta, Canada) were run with each set of samples analyzed. Activity corresponding to TIMP-1 and TIMP-2 migrated similarly to the respective standards. Preliminary experiments established optimal incubation times and demonstrated that increasing activity was detected when increasing amounts of sample protein were loaded on reverse zymography gels.

Statistical Analyses

Differences in mRNA abundance and enzyme activity were determined by one-way analysis of variance using the General Linear Models procedure of the Statistical Analysis System (SAS; Version 8.0). Semiquanitiative RT-PCR values were evaluated after being subjected to arcsin transformation. Individual comparisons of mean RNA concentrations were performed using Fisher's Protected Least Significant Differences test and results are reported as mean ± SEM.

Chapter III

Effect of the Preovulatory Gonadotropin Surge on Matrix Metalloproteinase-14, Matrix Metalloproteinase-2, and Tissue Inhibitor of Metalloproteinases-2 Expression Within Bovine Periovulatory Follicular and Luteal Tissue

Abstract

The matrix metalloproteinases (MMPs) have been implicated in the ovulatory process, but the specific roles of individual MMPs are unclear. This study examined the effect of the preovulatory gonadotropin surge on localization and regulation of MMP-2, MMP-14 and tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA and MMP-2 and TIMP-2 activity in bovine preovulatory follicles and new corpora lutea (CL). Samples were collected at approximately 0, 6, 12, 18, 24 and 48 h (CL) after a GnRH-induced gonadotropin surge. Messenger RNA for TIMP-2 and MMP-14 increased within 6 and 24 h of the gonadotropin surge respectively, while MMP-2 mRNA was constitutively expressed. Activity for MMP-2 in follicular fluid and follicle homogenates was not changed, but follicular fluid TIMP-2 activity increased in response to the gonadotropin surge. Messenger RNA for MMP-2 was localized to the theca layer of bovine preovulatory follicles, whereas MMP-14 mRNA was localized primarily to the theca layer and adjacent ovarian stroma. Expression of MMP-14 was also observed in the granulosa layer after the gonadotropin surge. In contrast, TIMP-2 mRNA was localized predominantly to the granulosa layer with intense expression in the antral portion of the granulosa layer in response to the gonadotropin surge. These data support the hypothesis that increased expression of MMP-14 and TIMP-2 may help regulate follicle rupture and (or) the ovulatory follicle/corpus luteum transition in cattle.

Introduction

Rupture of a mature ovarian follicle and subsequent release of a viable oocyte are prerequisites for reproductive success. Numerous studies have implicated the matrix metalloproteinases (MMPs) as important mediators of ovulation and subsequent corpus luteum (CL) formation [56, 148]. The MMPs are a large gene family of > 26 metal dependent enzymes that digest specific components (collagens, laminin, fibronectin, and proteoglycans) of the extracellular matrix (ECM) and are noted for their role in cell remodeling, extracellular matrix degradation, and tissue repair [149, 150]. The collagenous layers in the theca externa, tunica albuginea, and surface epithelium at the follicle apex must be degraded for a follicle to rupture. Furthermore, breakdown of the basement membrane, which separates the granulosa and theca cells and contains primarily type IV collagen, is required for release of the oocyte. Administration of synthetic MMP inhibitors blocks ovulation [72, 151]. However, the specific MMPs required for follicle rupture are not known.

The membrane type-MMPs have been termed as possible "master switches" that control ECM remodeling [52]. Membrane type 1-MMP (MMP-14) is noted both for its ability to activate other MMPs (MMP-2, MMP-13) and focalize their activity to the cell surface and for its own capacity to degrade numerous ECM substrates [53-55]. Matrix metalloproteinase-14 has been shown to activate progelatinase A (MMP-2) and localize its activity in a complex with tissue inhibitor of metalloproteinases-2 (TIMP-2) at the cell surface [53]. MMP-14 may also play a direct role in degradation of the preovulatory follicle wall via its ability to cleave type I and III collagen, fibronectin, laminin, and

proteoglycans [54]. However, the specific role of MMP-14 in mediating follicle rupture is unknown.

The specific functions of MMP-2 and TIMP-2 during the periovulatory period are also unclear. Matrix metalloproteinase-2 has been proposed to play an important role in degradation of the basement membrane that separates the granulosa cells from the theca cells, and it also likely further hydrolyzes the denatured collagen fibrils in the follicle wall following their initial cleavage by a collagenase. In addition to its potential role in proMMP-2 activation and focalization of MMP-2 activity at the cell surface, TIMP-2 is most known for its ability to bind MMPs in a 1:1 stoichiometry and inhibit their activity.

The regulation of MMP-2 and TIMP-2 during the periovulatory period is also species specific. Matrix metalloproteinase-2 mRNA and enzyme activity are increased in rat ovaries following an ovulatory dose of hCG [64, 152], whereas expression in mouse ovaries in response to an ovulatory stimulus is constitutive [58]. TIMP-2 expression is constitutive in rat [97] and mouse [58, 95] ovaries and sheep preovulatory follicles [99] following an ovulatory stimulus, but is increased in macaque granulosa cells [66].

As an initial step toward elucidating the physiological roles of MMP-14, MMP-2, and TIMP-2 in the ovulatory process, we characterized the effect of the preovulatory gonadotropin surge on localization and regulation of MMP-2, MMP-14 and TIMP-2 mRNA and MMP-2 and TIMP-2 activity in bovine preovulatory follicles and new CL. Results presented here support the hypothesis that increased expression of MMP-14 and TIMP-2 may help regulate follicle rupture and (or) the ovulatory follicle/corpus luteum transition in cattle.

Results

Effect of the preovulatory gonadotropin surge on MMP-14 mRNA abundance and localization

Relative abundance of MMP-14 mRNA in bovine periovulatory follicular and luteal tissue was regulated by the preovulatory gonadotropin surge. The MMP-14 cDNA hybridized specifically to a 3.1 kb transcript present within bovine periovulatory follicles and new CL (Figure 10). Expression of MMP-14 mRNA was increased in preovulatory follicles within 24 h of the gonadotropin surge and remained elevated in new CL (24 and 48 h versus 0 h; $P < 0.01$). Matrix metalloproteinase-14 mRNA was localized primarily to the theca layer and adjacent ovarian stroma of bovine preovulatory follicles. However, significant hybridization was also observed in the granulosa layer at the 12 and 24 h time points (Figure 11).

Effect of the preovulatory gonadotropin surge on bovine periovulatory follicular MMP-2 mRNA and enzyme activity

In contrast to the ontogeny of MMP-14 mRNA observed above, expression of MMP-2 mRNA was not regulated by the preovulatory gonadotropin surge. The bovine MMP-2 cDNA hybridized specifically to a 2.7 kb transcript (Figure 12). Expression of MMP-2 mRNA was not different at any of the time points evaluated ($P > 0.05$). MMP-2 mRNA was localized primarily to the surrounding theca layer of bovine preovulatory follicles (Figure 13). Likewise, bovine follicular MMP-2 activity was not regulated by the preovulatory gonadotropin surge. Relative levels of MMP-2 activity in follicular fluid (Figure 14) and follicle extracts (triton and heat extracts, apex and base; Figures 15-18) were not different at any of the time points examined (0, 6, 12, 18, 24 h after GnRH

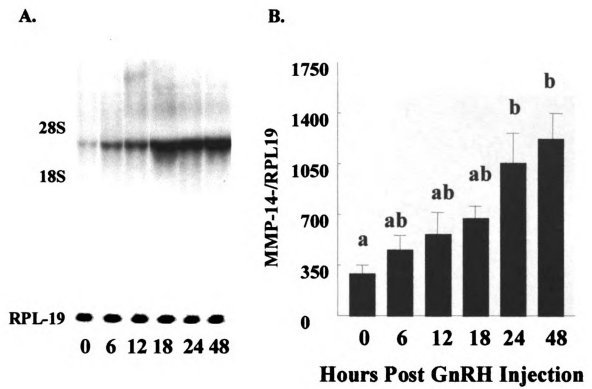


Figure 10: Effect of a GnRH-induced gonadotropin surge on MMP-14 mRNA abundance in bovine periovulatory follicular and luteal tissue A) Northern analysis of MMP-14 mRNA expression: Note hybridization to single 3.1 kb transcript. B) Effect of the preovulatory gonadotropin surge on relative levels of MMP-14 mRNA in bovine periovulatory follicles and new CL. Data are expressed as relative units MMP-14 mRNA per unit RPL-19 mRNA. Data shown as mean ± SE. Time points without a common superscript are different at $P < 0.05$.

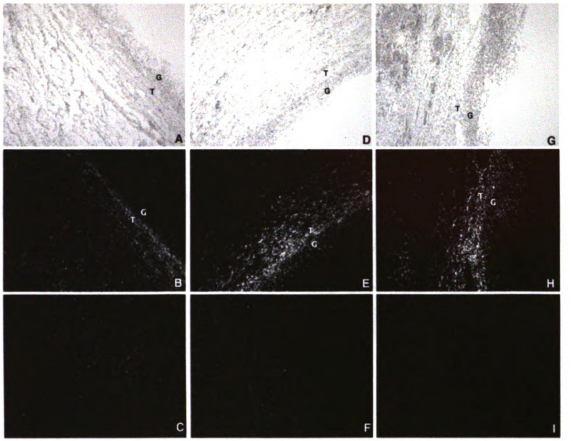


Figure 11: In situ localization of MMP-14 mRNA within bovine periovulatory follicles collected at 0, 12 and 24 h after GnRH injection (Magnification 120X). A) Bright-field micrograph of a preovulatory follicle collected at the 0 h timepoint and stained with hematoxylin and eosin. B) Dark-field micrograph of the same section hybridized with a ^{35}S antisense MMP-14 cRNA C) Dark-field micrograph of adjacent serial section of the same follicle hybridized with a ^{35}S sense MMP-14 cRNA. D, G) Similar to A except preovulatory follicle collected at 12 and 24 h, respectively. E, H) Similar to B except preovulatory follicle collected at 12 and 24 h, respectively. F, I) Similar to C except preovulatory follicle collected at 12 and 24 h, respectively (n = 3 follicles per timepoint). Note highest expression of MMP-14 mRNA in the theca layer, with additional localization in granulosa layer of follicles collected at 12 h and 24 h post GnRH injection.

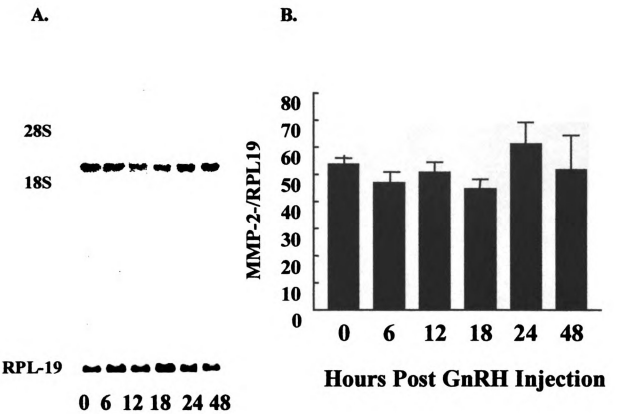


Figure 12: Effect of a GnRH-induced gonadotropin surge on MMP-2 mRNA abundance in bovine periovulatory follicular and luteal tissue A) Northern analysis of MMP-2 mRNA expression: Note hybridization to single 2.7 kb transcript. B) Effect of the preovulatory gonadotropin surge on relative levels of MMP-2 mRNA in bovine periovulatory follicles and new CL. Data are expressed as relative units MMP-2 mRNA per unit RPL-19 mRNA (n = 6 per timepoint). Data shown as mean ± SE. Expression of MMP-2 mRNA in bovine periovulatory follicles was not increased following the gonadotropin surge ($P > 0.05$).

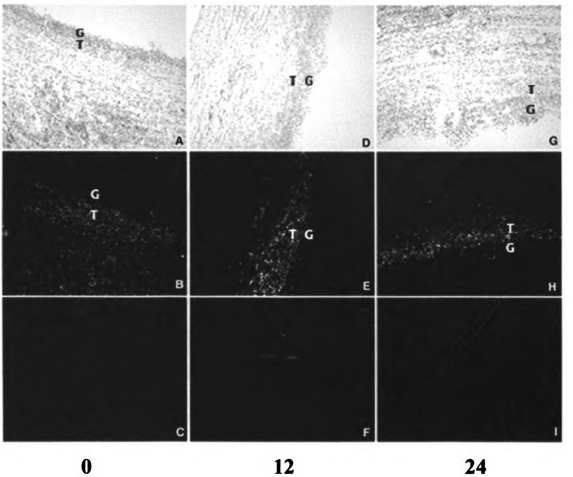


Figure 13: In situ localization of MMP-2 mRNA within bovine periovulatory follicles collected at 0, 12 and 24 h after GnRH injection (Magnification 120X). A) Bright-field micrograph of a preovulatory follicle collected at the 0 h timepoint and stained with hematoxylin and eosin. B) Dark-field micrograph of the same section hybridized with a ^{35}S antisense MMP-2 cRNA C) Dark-field micrograph of adjacent serial section of the same follicle hybridized with a ^{35}S sense MMP-2 cRNA. D, G) Similar to A except preovulatory follicle collected at 12 and 24 h, respectively. E, H) Similar to B except preovulatory follicle collected at 12 and 24 h, respectively. F, I) Similar to C except preovulatory follicle collected at 12 and 24 h, respectively ($n = 3$ follicles per timepoint). Note highest expression of MMP-2 mRNA in the theca layer.

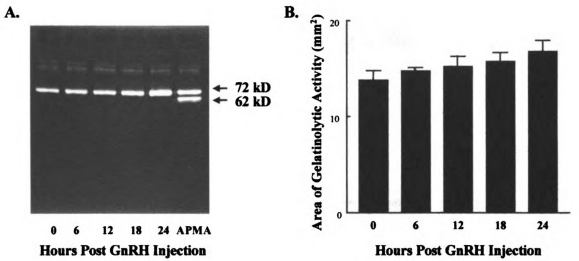


Figure 14: Gelatin zymographic analysis of MMP-2 activity in bovine periovulatory follicular fluid. A) Representative zymogram demonstrating MMP-2 activity in follicular fluid of bovine periovulatory follicles. B) Semiquantitative analysis of MMP-2 activity in follicular fluid of bovine periovulatory follicles collected at 0, 6, 12, 18, and 24 h after GnRH injection. Note prominent activity of approximately 72,000 Mr corresponding to the proform of MMP-2. Data depicted as mean ± SE (n = 6 per timepoint). Addition of 1,10 phenanthroline to incubation buffer inhibited all gelatinase activity. Matrix metalloproteinase-2 activity in periovulatory follicular fluid was not increased following the gonadotropin surge ($P > 0.05$).

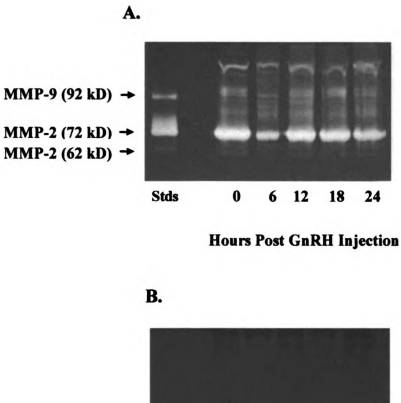


Figure 15: Gelatin zymographic analysis of MMP-2 activity in bovine follicle apex heat extracts. A) Representative zymogram demonstrating MMP-2 activity in pooled follicle apex heat extracts collected at 0, 6, 12, 18, and 24 h after GnRH injection. (n = 6 per timepoint) Note prominent activity of approximately 72,000 Mr corresponding to the proform of MMP-2. B) Representative zymogram demonstrating inhibition of MMP-2 activity in the presence of 1, 10 phenanthroline. Combined MMP-2/MMP-9 standards were included on each gel (Stds). Matrix metalloproteinase-2 activity in apex heat extracts was not increased following the gonadotropin surge ($P > 0.05$).

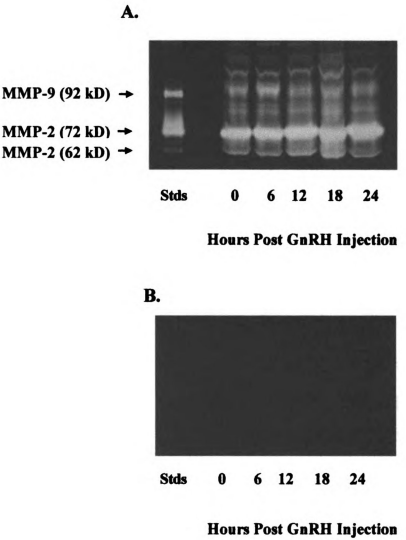


Figure 16: Gelatin zymographic analysis of MMP-2 activity in bovine follicle base heat extracts. A) Representative zymogram demonstrating MMP-2 activity in pooled follicle base heat extracts collected at 0, 6, 12, 18, and 24 h after GnRH injection (n = 6 per timepoint). Note prominent activity of approximately 72,000 Mr corresponding to the proform of MMP-2. B) Representative zymogram demonstrating inhibition of MMP-2 activity in the presence of 1, 10 phenanthroline. Combined MMP-2/MMP-9 standards were included on each gel (Stds). Matrix metalloproteinase-2 activity in base heat extracts was not increased following the gonadotropin surge ($P > 0.05$).

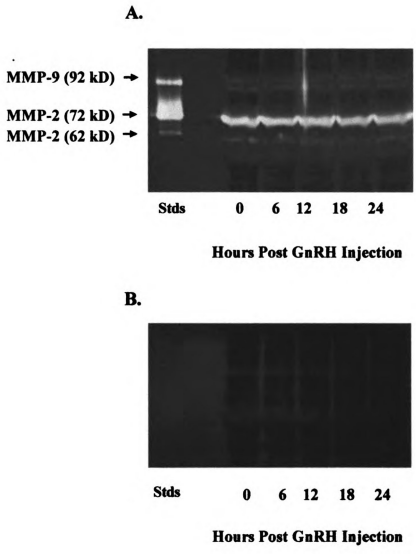


Figure 17: Gelatin zymographic analysis of MMP-2 activity in bovine follicle apex triton extracts. A) Representative zymogram demonstrating MMP-2 activity in pooled follicle apex triton extracts collected at 0, 6, 12, 18, and 24 h after GnRH injection. (n = 6 per timepoint) Note prominent activity of approximately 72,000 Mr corresponding to the proform of MMP-2. B) Representative zymogram demonstrating inhibition of MMP-2 activity in the presence of 1, 10 phenanthroline. Combined MMP-2/MMP-9 standards were included on each gel (Stds). Matrix metalloproteinase-2 activity in apex triton extracts was not increased following the gonadotropin surge ($P > 0.05$).

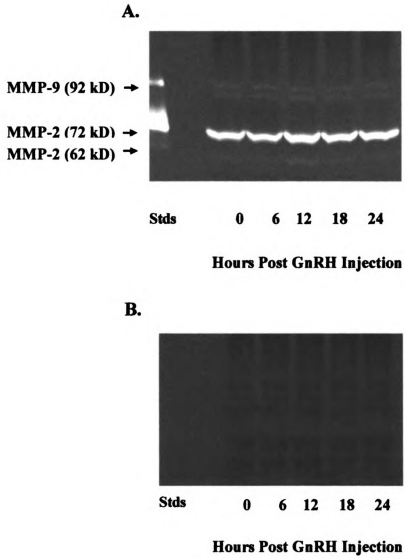


Figure 18: Gelatin zymographic analysis of MMP-2 activity in bovine follicle base triton extracts. A) Representative zymogram demonstrating MMP-2 activity in pooled follicle base triton extracts collected at 0, 6, 12, 18, and 24 h after GnRH injection. (n = 6 per timepoint) Note prominent activity of approximately 72,000 Mr corresponding to the proform of MMP-2. B) Representative zymogram demonstrating inhibition of MMP-2 activity in the presence of 1, 10 phenanthroline. Combined MMP-2/MMP-9 standards were included on each gel (Stds). Matrix metalloproteinase-2 activity in base triton extracts was not increased following the gonadotropin surge ($P > 0.05$).

injection; $P > 0.05$). The latent form of MMP-2 was the predominant form detected in both follicular fluid (Figure 14) and follicle homogenates (Figures 15-18). Addition of 1,10 phenanthroline to incubation buffer inhibited all gelatinase activity.

Effect of the preovulatory gonadotropin surge on TIMP-2 mRNA and activity

Localization and abundance of TIMP-2 mRNA and TIMP-2 activity were regulated by the preovulatory gonadotropin surge. The TIMP-2 cDNA hybridized specifically to a 1.1 kb transcript (Figure 19). Messenger RNA abundance for TIMP-2 was increased within 6 h of the preovulatory gonadotropin surge and remained elevated throughout the entire periovulatory period (6-48 h; $P < 0.05$). Messenger RNA for TIMP-2 was localized specifically to the granulosa layer at all time points examined (0, 12 and 24). In follicles collected prior to the gonadotropin surge, expression of TIMP-2 mRNA was prominent in the portion of the granulosa layer proximal to the basement membrane. At subsequent time points, expression was increased in the distal (antral) portion of the granulosa layer. Expression in the theca layer was nondetectable in comparison to the high level of expression detected in the granulosa layer (Figure 20). No obvious differences in TIMP-2 activity were detected in follicle homogenates (triton and heat extracts, apex and base; Figure 21, heat apex depicted) collected before versus after the gonadotropin surge. However, TIMP-2 activity was clearly increased in periovulatory follicular fluid samples collected after the gonadotropin surge (Figure 22).

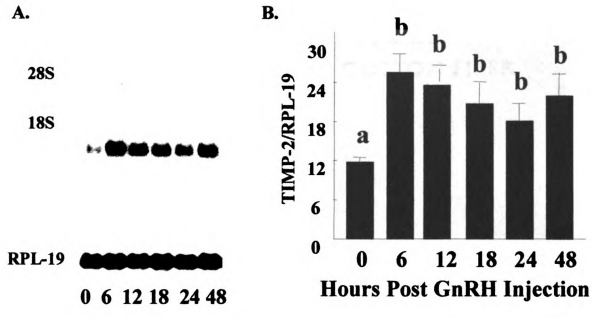


Figure 19: Effect of a GnRH-induced gonadotropin surge on TIMP-2 mRNA abundance in bovine periovulatory follicular and luteal tissue A) Northern analysis of TIMP-2 mRNA expression: Note hybridization to single 1.1 kb transcript. B) Effect of the preovulatory gonadotropin surge on relative levels of TIMP-2 mRNA in bovine periovulatory follicles and new CL. Data are expressed as relative units TIMP-2 mRNA per unit RPL-19 mRNA. Data shown as mean ± SE (n = 6 per time point). Time points without a common superscript are different at $P < 0.05$.

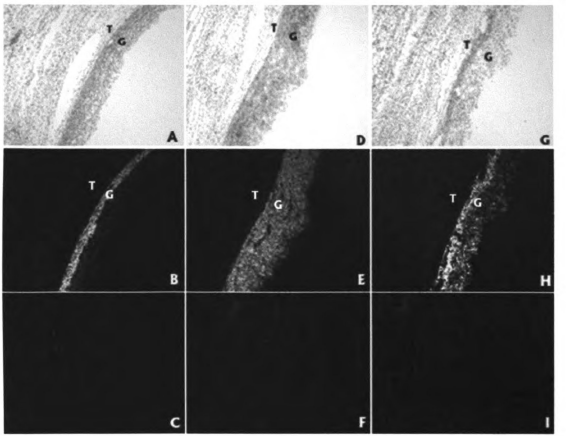


Figure 20: In situ localization of TIMP-2 mRNA within bovine periovulatory follicles collected at 0, 12 and 24 h after GnRH injection (Magnification 120X). A) Bright-field micrograph of a preovulatory follicle collected at the 0 h timepoint and stained with hematoxylin and eosin. B) Dark-field micrograph of the same section hybridized with a ^{35}S antisense TIMP-2 cRNA C) Dark-field micrograph of adjacent serial section of the same follicle hybridized with a ^{35}S sense TIMP-2 cRNA. D, G) Similar to A except preovulatory follicle collected at 12 and 24 h, respectively. E, H) Similar to B except preovulatory follicle collected at 12 and 24 h, respectively. F, I) Similar to C except preovulatory follicle collected at 12 and 24 h, respectively (n = 3 follicles per time point). Note localization of TIMP-2 mRNA specifically to the granulosa layer at above time points, with the highest expression in the granulosa cells adjacent to the basement membrane at 0 h and additional expression in the antral portion of the granulosa layer at 12 and 24 h after GnRH injection.

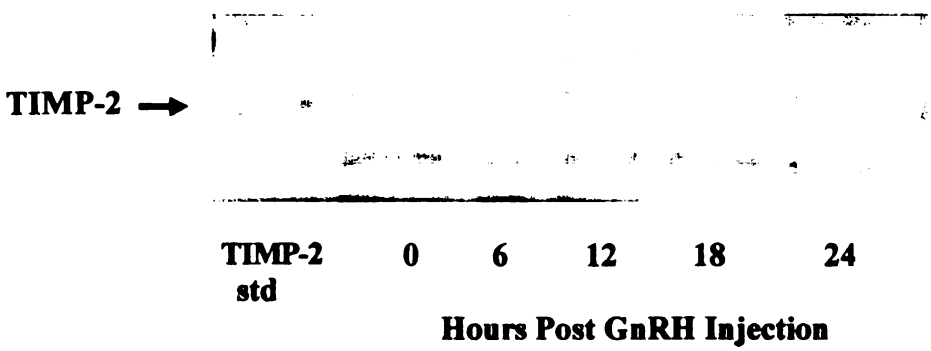


Figure 21: Representative reverse zymographic analysis of TIMP-2 activity in bovine follicle homogenates collected at 0, 6, 12, 18, and 24 h after GnRH injection (apex heat depicted; n = 6 per timepoint).

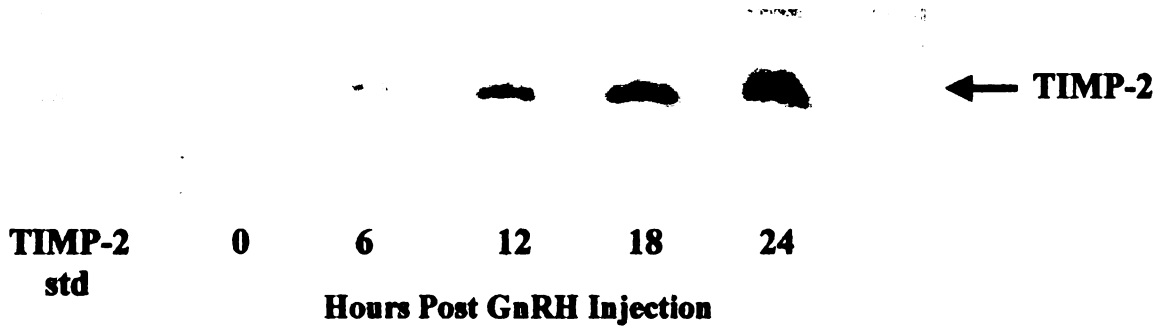


Figure 22: Detection of TIMP-2 activity in bovine periovulatory follicular fluid. Representative reverse zymogram of TIMP-2 activity in follicular fluid of bovine periovulatory follicles collected at 0, 6, 12, 18, and 24 h after GnRH injection (2 μl follicular fluid per lane, from pooled samples). Note observed increase in follicular fluid TIMP-2 activity in response to the gonadotropin surge.

Discussion

Successful ovulation is necessary for fertilization and reproductive success to occur. The MMPs have been implicated as important mediators of the ovulatory process due to their ability to degrade specific components (collagens, laminin, fibronectin, and proteoglycans) of the follicle wall and regulate ECM remodeling. However, the effect of the preovulatory gonadotropin surge on the intrafollicular localization and regulation of mRNA expression for individual MMPs and their specific requirements for follicle rupture are not well understood.

In the present study, MMP-14 mRNA was increased in bovine preovulatory follicular and luteal tissue following exposure to a gonadotropin surge. Previous studies of MMP-14 mRNA expression in mice (by Northern analysis) demonstrated no change in expression in the ovary following an ovulatory stimulus (hCG injection) [58]. However, these studies in mice were likely compromised by examination of periovulatory regulation of MMP-14 mRNA abundance in the ovary as a whole (including stroma and other nonovulatory follicles). In our studies, periovulatory regulation of MMP-14 mRNA expression was examined specifically in the preovulatory follicle. The spatial regulation of MMP-14 expression observed in bovine preovulatory follicles following the LH surge also was distinct. Expression of MMP-14 mRNA was detected primarily in the theca layer and adjacent ovarian stroma, with expression also detected in the granulosa cells at the 12 and 24 h time points. In rats [63] and mice [58], MMP-14 mRNA is also localized to the theca layer of preovulatory follicles. In mouse and rat preovulatory follicles, abundant MMP-14 mRNA expression is also evident in the granulosa cells, but

expression is down regulated in the granulosa layer and increased in the theca layer in response to hCG injection [58, 63].

Although MMP-14 is most often noted for its ability to localize and activate MMP-2, it may contribute to follicular ECM degradation through other mechanisms. Matrix metalloproteinase-14 may be directly involved in degradation of types I and III collagen as well as fibronectin, laminin and proteoglycans [54, 153]. Espey and Lipner [15] postulated that proteinases localized on the cell surface promote cell dissociation and breakdown of the surrounding collagen fibers within the theca externa and tunica albuginea during ovulation. In addition, MMP-14 can indirectly promote degradation of types I and III collagen by its ability to activate procollagenase 3 (MMP-13) [55]. Matrix metalloproteinase-13 mRNA is upregulated in bovine preovulatory follicles following the gonadotropin surge (Chapter IV). Furthermore, MMP-14 is not inhibited by TIMP-1, allowing degradation to occur in the presence of high concentrations of inhibitor [57]. This may be especially significant, given TIMP-1 is dramatically increased in preovulatory follicles of several species in response to the gonadotropin surge [Chapter V; 58, 60-62, 66].

The localization and regulation of TIMP-2 mRNA observed in the present studies in cattle are markedly different than what has been previously reported for other species. In the present study, TIMP-2 mRNA and activity were increased following the preovulatory gonadotropin surge. In contrast, TIMP-2 mRNA is constitutively expressed in ovine, mouse, and rat follicles following an ovulatory stimulus [60, 95, 98]. We previously observed increased expression of TIMP-2 mRNA in preovulatory follicular and luteal tissue collected from beef heifers within 8 and 48 h (CL) of the preovulatory

gonadotropin surge [100], but the localization of TIMP-2 mRNA and changes in TIMP-2 activity were not determined. Furthermore, the localization of TIMP-2 mRNA observed in bovine follicles also was unique. Messenger RNA for TIMP-2 was localized exclusively to the theca layer of ovine follicles [99], and a similar localization has been observed in rats and mice [58, 101]. In contrast, TIMP-2 mRNA was localized specifically to the granulosa layer of bovine preovulatory follicles. The physiological significance of the observed increase in TIMP-2 mRNA expression following the gonadotropin surge in the antral portion of the granulosa layer is not known. Differences in gene expression in specific components of the granulosa layer have been observed previously [154-156].

In the present study, MMP-2 mRNA expression and enzyme activity were unchanged following exposure of bovine follicles to a gonadotropin surge. The effect of an ovulatory stimulus on MMP-2 expression during the periovulatory period appears species specific. Like the cow, expression of MMP-2 mRNA and activity is constitutive during the periovulatory period in the mouse ovary [58]. These results are in contrast to previous studies which report an increase in MMP-2 mRNA and gelatinolytic activity within rat ovaries following exposure to an ovulatory stimulus [64, 152]. While MMP-2 mRNA and activity are reportedly increased in rat ovaries following hCG injection, localization studies have attributed this increase to small and large luteinizing follicles and to CL represented in the whole ovary mRNA preparation [97]. Matrix metalloproteinase-2 mRNA is also increased in preovulatory macaque granulosa cells in response to hCG administration, however, this increase was seen only in two out of the three monkeys evaluated [66]. Furthermore, critical evaluation of each model is

necessary when comparing the single bovine ovulatory follicle to hormonally stimulated multiple ovulatory follicles collected from a species that is naturally monotocous.

The localization of MMP-2 mRNA to the theca layer observed in the present studies is consistent with results of previously published studies in rats and mice [63, 64]. Consistent, significant expression of MMP-2 mRNA in the granulosa layer was not detected in the present studies.

The potential absolute requirement of MMP-2 for follicle rupture and subsequent reproductive success may also be species specific. Gene targeting experiments revealed that a functional MMP-2 gene is not required for successful pregnancy [157]. Potential direct effects of the MMP-2 null mutation on ovulation rate in the MMP-2 knockout mice have not been reported. In contrast, results in sheep indicate that MMP-2 may play a key role in the ovulatory process. The LH surge increases MMP-2 activity in sheep follicular extracts [68], and intrafollicular injection of MMP-2 antibodies can block ovulation [69]. In addition, ewes immunized against the N-terminal peptide of the 43-kDA subunit of α-N inhibin show decreased MMP-2 activity in follicular fluid and an impairment of the ovulatory process [67]. In the bovine, MMP-9 may be the primary mediator of follicular basement membrane breakdown prior to ovulation. Messenger RNA for MMP-9 is increased in bovine preovulatory follicles in response to the gonadotropin surge (Cassar, Bakke and Smith, unpublished data).

While several of the results presented in the current studies appear to be species specific, when comparing the results of any group of studies, it is important to closely examine the model that each investigator uses. When studying possible regulators of the ovulatory process and corpus luteum formation, it is standard procedure to synchronize

an animal's reproductive cycle. While the standard reproductive rodent models use immature, non-cycling animals injected with PMSG and hCG, superstimulation of follicle growth with exogenous gonadotropins is not commonly utilized in models for the study of the ovulatory process in farm animals such as sheep and cows. With the expectation that the ovine model would be more similar to a cow than a rodent model, the distinct regulation of TIMP-2 and MMP-2 in the cow versus the sheep was unanticipated. While sheep are polytocous, it is unclear if this contributes to the differences seen in TIMP-2 or MMP-2 regulation. It is possible that redundancies among MMPs and TIMPs have resulted in species specificity. As was stated previously, MMP-9 (also a gelatinase) may primarily mediate follicular basement membrane breakdown in the bovine, while MMP-2 may play a more dominant role in the sheep. Yet, differences between the bovine and ovine models further substantiate the need for investigation in the specific model system of interest.

In this study we have examined three potential regulators of follicle wall degradation following the LH surge. Considerable species differences were found in terms of localization and regulation of MMP-2, MMP-14 and TIMP-2 in cattle versus previously reported results for other species. Thus, the potential role or absolute requirement of above regulators in the ovulatory process and (or) the ovulatory follicle/corpus luteum transition may be distinct.

In summary, we have shown that the gonadotropin surge results in increased MMP-14 mRNA and TIMP-2 mRNA and activity in bovine preovulatory follicles while MMP-2 mRNA and enzyme activity are constitutively expressed. The intrafollicular localization of MMP-14, MMP-2, and TIMP-2 mRNA expression in bovine preovulatory

follicles was distinct and does not strongly support a potential direct role for TIMP-2 in proMMP-2 activation and localization of MMP-2 activity at the cell surface. However, a role of TIMP-2 of granulosa cell origin in proMMP-2 activation and localization at the cell surface in the theca layer cannot totally be discounted. Our results support the hypothesis that increased expression of MMP-14 and TIMP-2, but not MMP-2 may help regulate follicle rupture and (or) the ovulatory follicle/corpus luteum transition in cattle.

Chapter IV

Differential Upregulation of Interstitial Collagenase (MMP-1) and Collagenase-3 (MMP-13) mRNA Transcripts in Bovine Periovulatory Follicular and Luteal Tissue Following the LH Surge.

Abstract

Matrix metalloproteinases (MMPs) are a large family of metal dependent enzymes that play a role in cell remodeling, extracellular matrix degradation and tissue repair. Numerous studies have implicated MMPs as important mediators of the ovulatory process. Collagenolysis is a prerequisite for ovulation and subsequent corpus luteum (CL) formation considering the oocyte must penetrate three collagen I rich layers of the follicle wall in order to be released. Collagenases are most likely responsible for the initial degradation and unwinding of the triple helical collagen fibers within the follicular apex prior to ovulation. Here we test the hypothesis that the LH surge upregulates collagenase 1 (MMP-1) and collagenase 3 (MMP-13) mRNA expression in bovine periovulatory follicular and luteal tissue. Ovulation was synchronized in dairy cows using Ovsynch (GnRH-7d-PGF2$_\alpha$-36h-GnRH). Periovulatory follicular and luteal tissue were collected at 0, 6, 12, 18, 24, and 48 h after the 2nd GnRH injection (n = 5-8 per timepoint) and RNA was subjected to Northern analysis and either dot blot or semiquantitative RT-PCR analysis for MMP-1 and MMP-13. Two MMP-1 mRNA species were detected (2.4 and 1.8 kb). Relative levels of MMP-1 mRNA were greater at 6, 12, and 48 h relative to the 0 h (pre-LH surge) timepoint ($P < 0.05$). Differential upregulation of the 2.4 and 1.8 kb transcripts was observed. Expression of the 2.4 kb transcript was increased at the 6 and 12 h timepoints. In contrast, the 1.8 kb transcript

was dramatically upregulated and predominant at 48 h (new CL). Upregulation of the larger transcript was predominant in follicular tissue and may be associated with the ovulatory process, while upregulation of the smaller transcript accompanied CL formation. A single 3.0 kb MMP-13 mRNA transcript was detected in bovine periovulatory follicular tissue. Furthermore, MMP-13 expression was increased at 6 h and then again at 24 h following the LH surge. These results indicate that the LH surge increases expression of MMP-1 and MMP-13 mRNA within bovine periovulatory follicular and luteal tissue. Increased expression of MMP-1 and MMP-13 mRNA following the LH surge is supportive of a potential role of these enzymes in the ovulatory process and luteal development.

Introduction

Scientists have been interested in ovulation since 1670 when de Graf first identified ovarian follicles [10]. In 1916, Schochet first suggested that ovulation occurs through extracellular matrix degradation by proteinases [158]. Although the proteolytic enzyme theory of ovulation was firmly established in the 1970s, the identification and subsequent biochemical and functional characterization of the enzymes involved in degradation of the follicle wall is incomplete.

In order for an oocyte to be released from a follicle, it must pass through the many layers of the follicle wall. It first encounters the granulosa cells, which are separated from the theca interna by the type IV collagen rich basement membrane. Next it confronts the theca externa and tunica albuginea, whose dense collagen network (types I and III) provides the tensile strength of the follicle wall. Finally it must pass through the surface epithelium. For a follicle to ovulate, the collagenous layers of the theca externa,

tunica albuginea, and surface epithelium at the follicle apex must be degraded. Immediately prior to ovulation, the surface epithelium detaches from the underlying basement membrane, the concentration of collagen fibrils is decreased, and the follicle wall becomes thinner at the apex [15]. This decrease in collagen is presumed to result from an increase in proteolytic activity with a potentially important role for the collagenases.

Matrix metalloproteinases (MMPs) are a large family of > 26 metal dependent enzymes that play a role in cell remodeling, extracellular matrix degradation, and tissue repair [149, 150]. The collagenases MMP-1 (interstitial collagenase) and MMP-13 (collagenase 3) are best noted for their ability to degrade types I, II, and III collagen. In the rat and sheep, an increase in preovulatory collagenolytic activity is seen following the LH surge [17, 159]. While much redundancy is seen among MMPs and their substrates, the collagenases are unique in their ability to cleave the triple helical collagen molecule. Following this initial site-specific cleavage, the fragmented collagen becomes less stable and more soluble as it uncoils [70]. Following denaturation, the collagen products become susceptible to further degradation by other proteinases. Therefore, hormonal regulation of specific members of the collagenase subfamily of MMPs may play a key role in mediating follicle wall degradation prior to ovulation. As an initial step towards understanding the potential role of specific collagenases in follicle rupture, we determined the effect of the preovulatory gonadotropin surge on MMP-1 and MMP-13 mRNA expression in bovine preovulatory follicles.

Results

Expression of MMP-1 mRNA during the periovulatory period

Relative abundance of MMP-1 mRNA in bovine periovulatory follicular and luteal tissue was regulated by the preovulatory gonadotropin surge. The MMP-1 cDNA hybridized specifically to two MMP-1 mRNA species of 2.4 and 1.8 Kb (Figure 23). Relative levels of MMP-1 mRNA were greater at 6, 12, and 48 h relative to the 0 h (pre-LH surge) time point ($P < 0.05$). Differential upregulation of the 2.4 and 1.8 Kb transcripts was observed. Expression of the 2.4 Kb transcript was increased at the 6 and 12 h time points. In contrast, the 1.8 Kb transcript was dramatically upregulated and was predominant at 48 h (new CL). To confirm that both transcripts encoded for MMP-1 and not a related or undescribed MMP, a second upstream MMP-1 cDNA was generated and utilized in Northern analysis. The upstream cDNA also recognized both MMP-1 transcripts (Figure 24).

Expression of MMP-13 mRNA during the periovulatory period

Expression of MMP-13 mRNA was also regulated by the preovulatory LH surge. The MMP-13 cDNA hybridized specifically to a 3.0 Kb transcript (Figure 25). Relative levels of MMP-13 mRNA were transiently increased at 6 h relative to the 0 h (pre-gonadotropin surge) timepoint. MMP-13 mRNA expression then decreased at 12 and 18 h followed by an increase at 24 h and a second decline at 48 h (Figure 26).

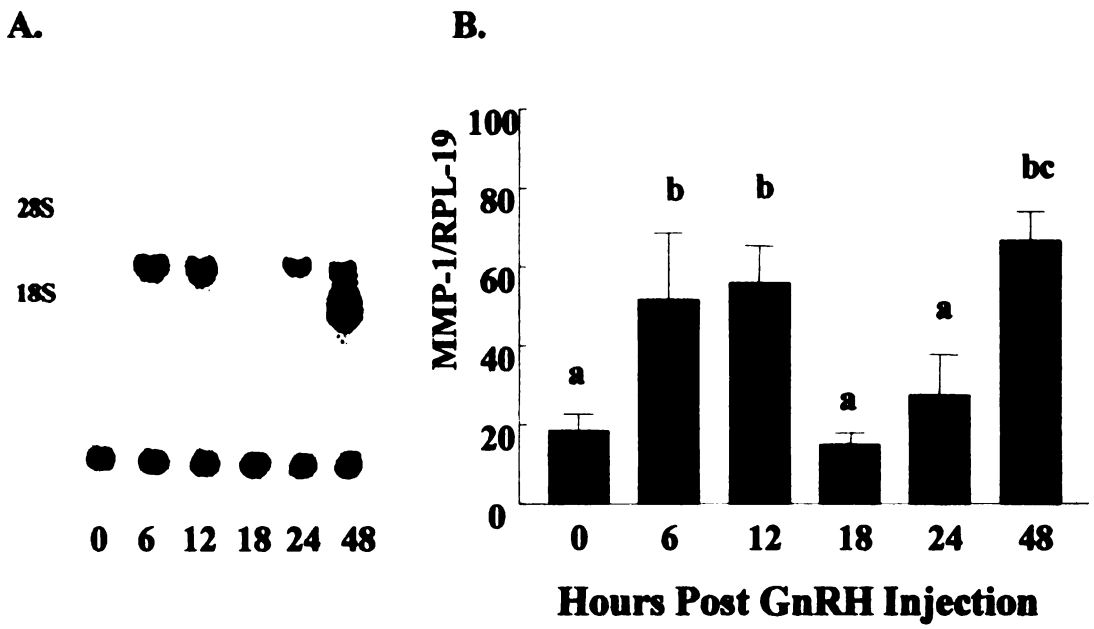


Figure 23: Effect of a GnRH-induced gonadotropin surge on MMP-1 mRNA abundance in bovine periovulatory follicular and luteal tissue A) Northern analysis of MMP-1 mRNA expression: Note hybridization of the MMP-1 cDNA to a 2.4 kb transcript at 6, 12, and 24 h and appearance of a second 1.8 kb transcript at 48 h. B) Effect of the preovulatory gonadotropin surge on relative levels of MMP-1 mRNA in bovine periovulatory follicles and new CL. Data (B) are expressed as relative units MMP-1 mRNA per unit RPL-19 mRNA. Data shown as mean ± SE. Time points without a common superscript are different at $P < 0.05$.

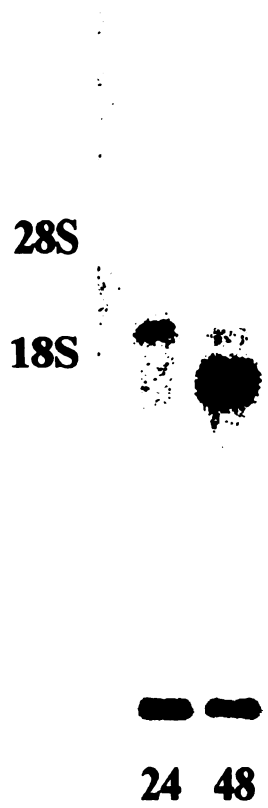


Figure 24: Effect of a GnRH-induced gonadotropin surge on MMP-1 mRNA abundance in bovine periovulatory follicular and luteal tissue. Northern analysis of MMP-1 mRNA expression using a ^{32}P cDNA encoding for a region of the MMP-1 mRNA located 5' to that used in Figure 23. Note hybridization of the MMP-1 cDNA to a 2.4 kb transcript at 24 h and a 1.8 kb transcript at 48 h.

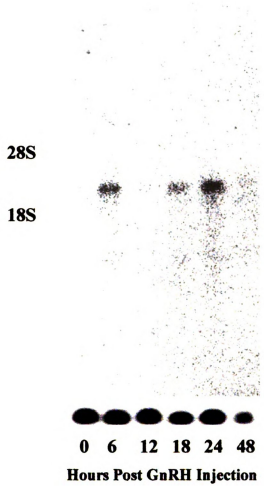


Figure 25: Effect of a GnRH-induced gonadotropin surge on MMP-13 mRNA abundance in bovine periovulatory follicular and luteal tissue. Northern analysis of MMP-13 mRNA expression: Note hybridization of the MMP-13 cDNA to a single 3.0 kb transcript.

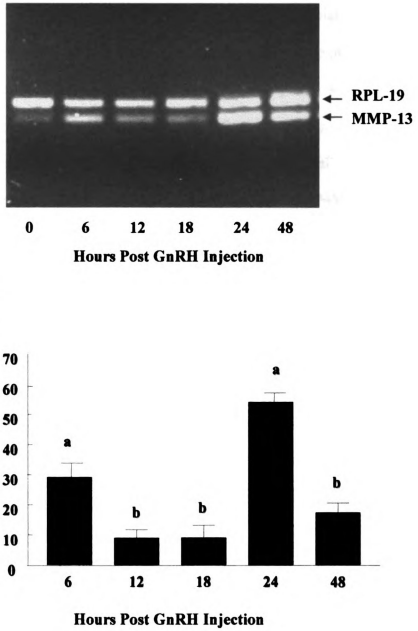


Figure 26: Effect of a GnRH induced gonadotropin surge on MMP-13 mRNA abundance in bovine periovulatory follicular and luteal tissue A) Semi-quantitative RT-PCR analysis of MMP-13 mRNA expression. B) Effect of the preovulatory gonadotropin surge on relative levels of MMP-13 mRNA in bovine periovulatory follicles and new CL. Data are expressed as % increase in expression levels over 0 h (pre gonadotropin surge) samples (n = 6 per time point). Data shown as mean ± SE. Time points without a common superscript are different at $P < 0.05$.

Discussion

Rupture of a mature ovarian follicle and subsequent release of a viable oocyte is an absolute requirement for reproductive success. Numerous studies have implicated MMPs as important mediators of the ovulatory process. Collagenolysis is a prerequisite for ovulation and subsequent corpus luteum (CL) formation considering the oocyte must penetrate three collagen rich layers of the follicle in order to be released. In this study we examined the effect of the preovulatory LH surge on expression of MMP-1 (interstitial collagenase) and MMP-13 (collagenase-3) mRNAs.

In the present study, two MMP-1 transcripts were detected that were differentially regulated. The 2.4 kb transcript increased substantially at 6 and 12 h compared to the 0 h time point. At 18 h it decreased to baseline levels and began to increase again at 24 and 48 h. The 1.8 kb transcript was undetectable at all preovulatory time points examined with a dramatic increase at 48 h (new CL) following the LH surge. To be confidant that both transcripts encoded for MMP-1 and not a related or undescribed MMP, a second Northen analysis was performed using a MMP-1 cDNA encoding for a region 5' to the original MMP-1 probe that was generated, confirming the presence of two MMP-1 transcripts. While upregulation of MMP-1 mRNA is seen during the ovulatory process in several other species, two MMP-1 mRNA transcripts are unique to the bovine ovary. Upregulation of the larger transcript is predominant in follicular tissue and may be associated with the ovulatory process, while upregulation of the smaller transcript accompanies CL formation. The appearance of two MMP-1 transcripts in the bovine was unanticipated. However, it is important to note that different MMP-1 transcript sizes have been reported in different tissues in the rat. In the current study, we report a 1.8 kb

transcript and a 2.4 kb transcript. Previous studies in the rat ovary have reported a 1.7 or 1.8 kb MMP-1 transcript [64, 78]. In contrast, a study examining MMP-1 mRNA expression in different rat cell types, reported a 2.9 kb MMP-1 transcript in osteoblast cells with a slightly larger MMP-1 mRNA in uterine smooth muscle cells [160]. Post-transcriptional mechanisms have also been shown to regulate collagenase mRNA [161]. The human collagenase mRNA contains three AUUUA motifs in the 3' end [162] that help regulate mRNA stability. Previous studies have shown that the AUUUA motifs present in the collagenase transcript enhance mRNA degradation, an effect that is antagonized in response to cytokines such as Interleukin-1β (IL-1β) [162]. However, the ability of IL-1β to prevent mRNA decay occurs only in specific cell types. Therefore, it is possible that cell specific post-transcriptional modification could alter the size of the collagenase mRNA in response to the microenvironment of that cell type.

Like the cow, expression of MMP-1 mRNA is increased in rat ovaries [64] and macaque granulosa cells [66] following an ovulatory stimulus. Preliminary data evaluating bovine MMP-1 mRNA expression by RT-PCR revealed expression by both granulosa cells and theca cells. This is consistent with what is seen in rats and rabbits following the LH surge [64, 77]. In gilts, MMP-1 mRNA is localized to the granulosa and theca interna cells of preovulatory follicles, but an increase in expression is only seen in the theca interna following LH/hCG stimulation [76]. In monkeys, MMP-1 mRNA is expressed by granulosa cells following the LH surge, and human granulosa cells have also been shown to secrete MMP-1 [16, 66].

Matrix metalloproteinase-13 mRNA expression is also increased in bovine preovulatory follicles following the LH surge. Considerably less information is known

regarding the regulation and localization of MMP-13 mRNA following the LH surge. Unlike many other MMP family members that are expressed in several tissues throughout the body, MMP-13 is highly expressed in the ovary in rats [78]. Furthermore, while MMP-13 is not induced in immature rat ovaries following an ovulatory dose of hCG, it is expressed at high levels during proestrus and estrus in mature rats. These studies showed MMP-13 to be at a minimum at metestrus and gradually increase from diestrus to proestrus [78]. It was postulated that an element required for induction of MMP-13 expression is not present in the immature rat ovary. Several investigators have questioned the results obtained from immature, gonadotropin-primed animals when trying to relate the results to mature, cycling animals [78, 176]. In contrast, MMP-13 mRNA is undetectable by Northern analysis in the mouse ovary during the periovulatory period [58]. However, these studies in mice and rats were likely compromised by examination of periovulatory regulation of MMP-13 mRNA abundance in the ovary as a whole (including stroma and other nonovulatory follicles). Preliminary studies evaluating MMP-13 mRNA expression in the bovine preovulatory follicular cell types indicate MMP-13 is expressed primarily by the theca layer and tunica albuginea. Similar results were reported in the rat [78]. However, the bovine model is a more appropriate model for studying the ovulatory process because it allows one to examine the regulatory events occurring in a single ovulatory follicle (versus an entire ovary containing multiple ovulatory follicles used in rodent models) of a mature, cycling animal (versus an animal that has not reached reproductive maturity).

Preovulatory follicle collagenase activity is also regulated by the LH surge. Collagenolytic activity is increased in rat ovaries [74, 159] and sheep preovulatory

follicles [35] in response to an ovulatory stimulus. Furthermore, there is evidence that selective collagen degradation occurs at the apex of the follicle, which could be attributable to collagenase activity. Espey first showed that as ovulation approaches, the follicle wall becomes thinner and the concentration of collagen fibrils is decreased at the apex [75]. Electron microscopy of sheep follicles showed a decrease in collagen fibrils at the apex versus the base 24 h after a GnRH-induced LH surge [17]. Furthermore, Murdoch detected increased collagenolytic activity at the apex versus the base of the follicle [17]. Previous studies have also shown increased proMMP-1 at the apex of rabbit follicles following gonadotropin stimulation [77]. Further studies will be required to determine the effect of the preovulatory gonadotropin surge on individual enzyme activity for MMP-1 and MMP-13.

Of particular interest are the mechanisms or factors downstream from the LH surge that are regulating mRNA expression for the collagenases. While a considerable amount of data supports the role of eicosanoids in ovulation, some evidence also supports a direct role of eicosanoids in regulation of ovarian collagenase expression. In rats, inhibitors of eicosanoid synthesis suppress LH-induced MMP-1 mRNA expression [64]. Furthermore, indomethacin, an inhibitor of cyclooxygenase, and NDGA (nordihydroguaiaretic acid), an inhibitor of the lipoxygenase pathway, prevent ovarian collagenolysis and ovulation in rats [159]. Indomethacin was also shown to inhibit collagenase activity in the rabbit and the sheep [163]. Likewise, a specific inhibitor of 5-lipoxygenase, MK 886, suppressed collagenase activity as well as ovulation [116]. While a direct relationship between arachidonic acid derivatives and collagenase activity in the bovine is still unknown, previous studies have shown the COX-2 enzyme is induced

within the granulosa layer of bovine preovulatory follicles following the LH surge [117, 118]. Follicular fluid PGE_2 levels are increased within 18 h after hCG treatment in cattle [117, 119]. Furthermore, preliminary results indicate that bovine preovulatory follicles express the PGE_2 receptor subtype EP2 (Cassar/Smith, unpublished). Mice with targeted mutations in the COX-2 gene fail to ovulate [121], and PGE_2 supplementation restores ovulation [122]. Thus, PGE_2 may play a role in regulation of MMP-1 and MMP-13 expression in bovine preovulatory follicles, but further investigation will be required.

Progesterone is another possible regulator of collagenase expression. The antiprogesterone RU486 prevented the hCG-induced rise in collagenase activity in PMSG-primed rat ovaries [164]. Furthermore, the LH surge initiated a rapid induction of the progesterone receptor mRNA in bovine preovulatory follicles [135]. Both MMP-1 and MMP-13 mRNA show an initial increase in mRNA expression followed by a decline and then a second increase in expression. It is possible that the LH surge is directly responsible for the initial increase in collagenase expression, while downstream effects of the LH surge such as the increase in cyclooxygenase-2 (PGE_2) and upregulation of the progesterone receptor, may be necessary for the subsequent increase in collagenase mRNA.

While substrate redundancy seems to be prevalent among the MMP family, it is believed that MMP-1 and MMP-13 have evolved as specialized enzymes [55]. Due to structural differences on account of peptide bond formations, MMP-1 is both more difficult to activate and more resilient to degradation than MMP-13 [55]. Due to an amino acid substitution in its active site, MMP-13 also appears to have a broader range of

substrates in its ability to cleave gelatin [55]. Therefore, MMP-1 and MMP-13 may have complimentary but distinct roles in the degradation of collagen during the ovulatory process.

In summary, we have shown the preovulatory gonadotropin surge results in increased MMP-1 and MMP-13 mRNA expression. Increased collagenase expression may play a critical role in the obligatory degradation of the collagenous layers of the follicle wall during the ovulatory process. Further studies will be required to determine the effect of the preovulatory gonadotropin surge on MMP-1 and MMP-13 activity and to determine the specific intrafollicular signaling pathways that mediate the gonadotropin surge-induced increases in MMP-1 and MMP-13 mRNA. Our results support the hypothesis that increased expression of MMP-1 and MMP-13 mRNA may help regulate follicle rupture and (or) the ovulatory follicle/corpus luteum transition in cattle.

Chapter V

Differential Regulation of Tissue Inhibitors of Metalloproteinases (TIMP) 1 and 3 in Bovine Preovulatory Follicles and Luteal Tissue Following the LH surge: Implications for Regulation of the Ovulatory Process

Abstract

Matrix metalloproteinases (MMPs) are metal-dependent enzymes that degrade various extracellular matrix (ECM) components. Numerous studies have implicated MMPs as important mediators of the follicular ECM degradation required for ovulation. MMP activity is regulated via specific MMP inhibitors, such as the tissue inhibitors of metalloproteinases (TIMP). Regulation of TIMPs may be an important factor controlling the ovulatory process. Here we test the hypothesis that localization and expression of TIMP 1 and 3 are differentially regulated in bovine periovulatory follicles following a GnRH-induced LH surge. Follicle growth and timing of the LH surge were synchronized in dairy cows and ovaries containing preovulatory follicles or new corpora lutea (CL) collected at 0, 6, 12, 18, 24 and 48 h (CL) after GnRH injection (n = 5-8 each). Relative levels of TIMP-1 mRNA increased within 6 h following the LH surge and remained elevated through the 48 h timepoint ($P < 0.05$). Follicular fluid TIMP-1 activity also increased in response to the gonadotropin surge. In contrast, TIMP-3 mRNA was not upregulated following the LH surge and decreased at 18 and 48 h ($P < 0.05$) relative to the 0 h (pre LH surge) timepoint. The intrafollicular localization of TIMP 1 and 3 mRNA was also differentially regulated during the periovulatory period. TIMP-1 mRNA was undetectable prior to the LH surge. Following gonadotropin stimulation, TIMP-1 was localized primarily to the granulosa layer of bovine preovulatory follicles. In contrast,

TIMP-3 mRNA was localized specifically to the theca layers and adjacent ovarian stroma. Our results indicate the TIMPs are regulated in bovine preovulatory follicles following the LH surge in a temporally and spatially specific fashion. Constitutive and (or) decreased expression of TIMP-3 in the theca layers/tunica albuginea may favor a net increase in the MMP/TIMP ratio and subsequent degradation of the follicle wall prior to ovulation.

Introduction

Ovulation and subsequent corpus luteum (CL) formation involve the cyclic process of follicle rupture and tissue degradation followed by tissue repair. A growing body of evidence indicates that proteolytic degradation of the extracellular matrix (ECM) at the apex of ovulatory follicles prior to ovulation is a crucial step in the complex cascade of events initiated by the LH surge. The matrix metalloproteinases (MMPs) digest specific components (collagens, laminin, fibronectin, and proteoglycans) of the ECM and are noted for their role in ECM remodeling. Tissue inhibitors of metalloproteinases (TIMPs) are presently a family of four proteins (TIMPs 1-4) that have been implicated in multiple roles in the ovary, including inhibition of MMPs. Degradation of the ECM may be governed by the MMP/TIMP ratio. TIMPs are thought to provide a homeostasis that prevents overproduction and unrestrained activity of MMPs.

Tissue inhibitors of metalloproteinases inhibit MMP activity by binding noncovalently with a 1:1 stoichiometry and high affinity [26]. Although they share structural similarities, each TIMP is a separate gene product containing 12 cysteine residues that form six disulfide bonds and contribute to the stability of TIMP molecules [84]. TIMPs differ in their degree of glycosylation, molecular weight, as well as their

solubility [85, 86]. While TIMPs 1, 2, and 4 are soluble within the extracellular milieu, TIMP-3 is secreted and then bound to the extracellular matrix [87, 88]. The least is known about TIMP-4, although preliminary studies suggest that it is most similar to TIMP-2 [85, 89]. TIMPs are multifunctional molecules that have been implicated in several functions within the ovary in addition to MMP inhibition. For example, TIMPs have been noted to promote steroidogenesis [90], stimulate cell growth in a variety of tissues [91], help promote activation of MMPs [53], inhibit angiogenesis [92], and influence apoptosis [93], all processes that may contribute to ovulation and subsequent CL formation.

The specific functions of TIMPs during the periovulatory period are unclear. Interestingly, MMPs and TIMPs are often secreted in parallel as many factors that stimulate expression of MMPs also increase expression of the inhibitors [94]. The regulation of TIMPs 1 and 3 appears to be species specific. While TIMP-1 mRNA is increased in the mouse [58, 95], rat [61, 64, 96], sheep [60], and macaque [66] following gonadotropin stimulation, the cell specific regulation of TIMP-1 is distinct in these species. Furthermore, previous reports examining the regulation of TIMP-3 are conflicting [58, 95].

To examine the potential role of TIMPs in the ovulatory process, we examined the temporal and spatial regulation of TIMPs 1 and 3. These molecules cooperatively regulate ECM remodeling in other systems. However, their role in the ovulatory process in cattle is not well understood.

Results

Effect of the preovulatory gonadotropin surge on TIMP-1 mRNA abundance, localization and enzyme activity

Relative abundance of TIMP-1 mRNA in bovine periovulatory follicular and luteal tissue was regulated by the preovulatory gonadotropin surge. The TIMP-1 cDNA hybridized specifically to a 0.9 kb transcript present within bovine periovulatory follicles and new CL (Figure 27). Relative to the 0 h timepoint, expression of TIMP-1 mRNA was significantly increased in preovulatory follicles at all timepoints examined following the gonadotropin surge. TIMP-1 mRNA was increased within 6 h of the gonadotropin surge. Levels of expression declined slightly at 24 h and then were dramatically elevated in new CL at 48 h ($P < 0.05$). TIMP-1 mRNA was nearly undetectable by in situ hybridization prior to the LH surge and was localized primarily to the granulosa layer of bovine preovulatory follicles following the LH surge (Figure 28). No obvious differences in TIMP-1 activity were detected in follicle homogenates (triton and heat extracts, apex and base; Figure 29, apex heat depicted) collected before versus after the gonadotropin surge. However, TIMP-1 activity was clearly increased in periovulatory follicular fluid samples collected after the gonadotropin surge (Figure 30).

Effect of the preovulatory gonadotropin surge on bovine periovulatory follicular TIMP-3 mRNA and enzyme activity

In contrast to the ontogeny of TIMP-1 mRNA observed above, expression of TIMP-3 mRNA was elevated prior to and at 6 and 12 h following the preovulatory gonadotropin surge (Figure 31). Levels of TIMP-3 mRNA declined at 18 h and substantially declined again at 48 h following the LH surge ($P < 0.05$). The bovine TIMP-3 cDNA hybridized

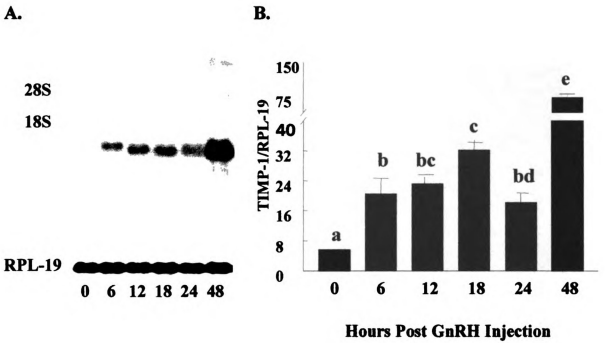


Figure 27: Effect of a GnRH-induced gonadotropin surge on TIMP-1 mRNA abundance in bovine periovulatory follicular and luteal tissue. A) Northern analysis of TIMP-1 mRNA expression: Note hybridization to a single 0.9 kb transcript. B) Effect of the preovulatory gonadotropin surge on relative levels of TIMP-1 mRNA in bovine periovulatory follicles and new CL. Data (B) are expressed as relative units TIMP-1 mRNA per unit RPL-19 mRNA. Data shown as mean ± SE (n = 6 per time point). Time points without a common superscript are different at $P < 0.05$.

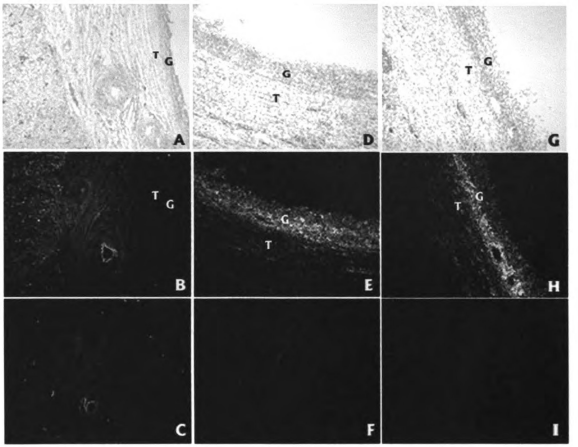


Figure 28: In situ localization of TIMP-1 mRNA within bovine periovulatory follicles collected at 0, 12 and 24 h after GnRH injection (Magnification 120X). A) Bright-field micrograph of a preovulatory follicle collected at the 0 h timepoint and stained with hematoxylin and eosin. B) Dark-field micrograph of the same section hybridized with a ^{35}S antisense TIMP-1 cRNA C) Dark-field micrograph of adjacent serial section of the same follicle hybridized with a ^{35}S sense TIMP-1 cRNA. D, G) Similar to A except preovulatory follicle collected at 12 and 24 h, respectively. E, H) Similar to B except preovulatory follicle collected at 12 and 24 h, respectively. F, I) Similar to C except preovulatory follicle collected at 12 and 24 h, respectively. Note highest expression of TIMP-1 mRNA in the granulosa layer following the LH surge.

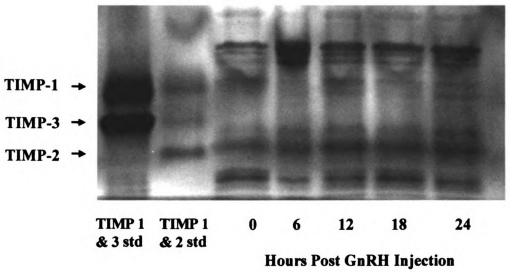


Figure 29: Representative reverse zymographic analysis of TIMP-1, TIMP-2, and TIMP-3 activity in bovine follicle extracts collected at 0, 6, 12, 18, and 24 h after GnRH injection (apex heat depicted; n = 6 per time point).

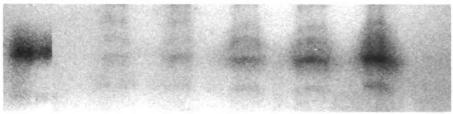


Figure 30: Effect of preovulatory gonadotropin surge on TIMP-1 activity in bovine follicular fluid. Representative zymogram depicting TIMP-1 activity in follicular fluid of bovine periovulatory follicles collected at 0, 6, 12, 18, and 24 h after GnRH injection (2 μl pooled sample per lane).

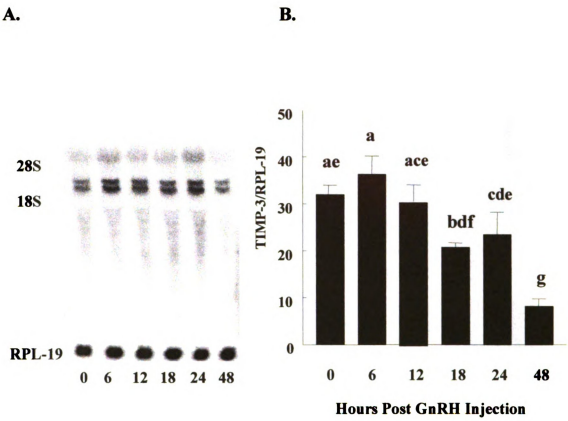


Figure 31: Effect of a GnRH induced gonadotropin surge on TIMP-3 mRNA abundance in bovine periovulatory follicular and luteal tissue. A) Northern analysis of TIMP-3 mRNA expression: Note hybridization to multiple transcripts (5, 2.8, and 2.4 kb). B) Effect of the preovulatory gonadotropin surge on relative levels of TIMP-3 mRNA in bovine periovulatory follicles and new CL. Data (B) are expressed as relative units TIMP-3 mRNA per unit RPL-19 mRNA. Data shown as mean ± SE (n = 6 per time point). Time points without a common superscript are different at $P < 0.05$.

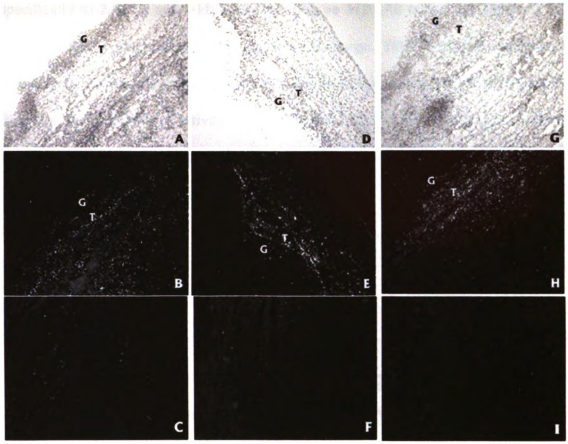


Figure 32: In situ localization of TIMP-3 mRNA within bovine periovulatory follicles collected at 0, 12 and 24 h after GnRH injection (Magnification 120X). A) Bright-field micrograph of a preovulatory follicle collected at the 0 h timepoint and stained with hematoxylin and eosin. B) Dark-field micrograph of the same section hybridized with a ^{33}P antisense TIMP-3 cRNA C) Dark-field micrograph of adjacent serial section of the same follicle hybridized with a ^{33}P sense TIMP-3 cRNA. D, G) Similar to A except preovulatory follicle collected at 12 and 24 h, respectively. E, H) Similar to B except preovulatory follicle collected at 12 and 24 h, respectively. F, I) Similar to C except preovulatory follicle collected at 12 and 24 h, respectively. Note highest expression of TIMP-3 mRNA in the theca layer.

specifically to 2.4, 2.8 and 5 kb transcripts (Figure 31). All transcripts appeared to be coordinately regulated following the gonadotropin surge. TIMP-3 mRNA was localized primarily to the theca layer/tunica albuginea of bovine preovulatory follicles (Figure 32). Bands of MMP inhibitory activity that co-migrated with the TIMP-3 standard were not detected by reverse zymographic analysis of follicle homogenates and follicular fluid (Figure 29).

Discussion

Follicle rupture is dependent upon localized degradation of the ECM at the apex of the preovulatory follicle wall. A growing body of evidence indicates that MMPs are important mediators of the ovulatory process. However, of equal importance is the controlled regulation of MMPs throughout the cyclic process of degradation and tissue repair that occurs during ovulation and subsequent corpus luteum (CL) formation. Tissue inhibitors of metalloproteinases (TIMPs) have been implicated in multiple roles in the ovary, including inhibition of MMPs.

In the present study, TIMP-1 mRNA was increased in bovine preovulatory follicular and luteal tissue following exposure to a gonadotropin surge. This is in agreement with previous studies in the mouse [58, 95], rat [61, 64, 96], sheep [60], and macaque [66]. Expression of TIMP-1 mRNA was detected primarily in the granulosa cells. The spatial regulation of TIMP-1 appears to be species specific. In the mouse, TIMP-1 expression is localized to granulosa and theca-interstitial cells of preovulatory follicles [58]. In mice that were not hormone primed, TIMP-1 expression was not observed in granulosa or theca cells at any stage of the cycle [95]. However, several investigators have questioned the results obtained from immature, gonadotropin-primed

animals when trying to relate the results to mature, cycling animals [78, 176]. TIMP-1 expression in the rat is observed in the theca interna and to a lesser extent in the granulosa cells following hCG administration [97]. Other studies in the rat reported the presence of TIMP-1 mRNA in both the granulosa cells and the residual ovarian tissue and levels were increased after hCG stimulation. However, in these studies mRNA localization was determined by Northern analysis of granulosa cells collected from ovulatory follicular fluid and the remaining theca/residual tissue versus in situ hybridization studies [64]. In sheep, the granulosa cells are the primary source of TIMP-1 [60].

Unlike in other species where TIMP-1 and TIMP-2 appear to be differentially regulated, TIMP-1 and TIMP-2 show similar spatial and temporal expression patterns in the bovine [86]. TIMP-2 was also increased in granulosa cells at all timepoints examined following the LH surge (discussed in chapter III). Like TIMP-1, TIMP-2 has also been shown to possess growth factor-like activity and the ability to influence cell morphology [86]. While it is possible that TIMPs 1 and 2 play a coordinated role in the ovulatory process, they are proposed to act selectively on different MMPs. For example, TIMP-1 preferentially binds the collagenases and MMP-9, whereas TIMP-2 has a high affinity for MMP-2 and may play a role in activating and localizing MMP-2 activity at the cell surface [85, 86, 165]. Additionally current evidence indicates that all four TIMPs described to date are expressed in the bovine ovary. TIMP-4 mRNA is expressed in bovine periovulatory follicles at similar time points as examined in the current experiments [166]. Studies suggest that TIMP-4 has many traits similar to those of

TIMP-2 [85, 89]. The potential specific role of individual TIMPs during the periovulatory period is not known.

In contrast to TIMP-1, TIMP-3 mRNA expression is not upregulated in bovine periovulatory follicles following the gonadotropin surge. Levels of TIMP-3 mRNA were reduced at 18 h and substantially decreased at 48 h versus 0 h following the LH surge. Conflicting reports exist in the mouse regarding the hormonal regulation of ovarian TIMP-3 expression. In cycling mice, TIMP-3 mRNA is increased at early proestrus [95], while in eCG-primed mice, TIMP-3 expression is unchanged following hCG administration [58]. As discussed previously, while the standard reproductive rodent model uses immature, non-cycling animals injected with PMSG and hCG, studies using mature, cycling rodents have produced differing results [78, 95, 176]. TIMP-3 mRNA was present primarily in the theca layer and adjacent ovarian stroma of bovine preovulatory follicles. In mice [95] and rats [97] TIMP-3 expression is observed in both granulosa and theca cells as well as interstitial tissue following gonadotropin stimulation. However, these results reflected the TIMP-3 localization in all growing follicles, not specifically the ovulatory follicles.

In the present study we have examined two potential regulators of ECM remodeling during the ovulatory process. When TIMPs were initially discovered, MMP inhibition was thought to be their primary role. However, many recent studies have shown numerous potential roles for TIMPs both in the ovulatory process as well as other physiological and pathological processes. In general, TIMPs consist of two domains. The N-terminal domain, which contains a highly conserved CXC motif, is responsible for inhibiting MMP action through interaction with the active site on the catalytic domain

and the substrate-binding groove [56, 167]. The C-terminal domain gives specific biochemical and physiological attributes to individual TIMPs, such as the ability of TIMP-3 to bind the extracellular matrix [167]. Furthermore, the C-terminal domain is proposed to be responsible for MMP-independent functions of TIMPs such as regulation of cell proliferation and apoptosis. Regulation of such processes is believed crucial to the ovulatory process and CL formation [86].

Throughout the ovulatory process and CL formation the ECM is in a constant state of degradation and deposition that is in part regulated by the MMPs and the TIMPs. Degradation of the ECM may be governed by the MMP/TIMP ratio. A series of experiments in transgenic pregnant mice elegantly illustrated the effects of altering the MMP/TIMP ratio [168, 169]. Overexpression of MMP-3 resulted in dedifferentiation of mammary epithelial cells, apoptosis, and premature mammary gland involution. Crossing mice that overexpress MMP-3 with mice that overexpress TIMP-1 resulted in normal glandular differentiation and delayed mammary gland involution. Accordingly, the LH surge-induced increase in TIMP-1 mRNA concomitant with the increase in other MMPs such as the collagenases is not contradictory to follicle rupture. TIMPs may allow focalized degradation to occur while limiting excessive proteolysis. The LH surge may upregulate TIMP and MMP expression through different pathways. Inhibitors of eicosanoid synthesis that blocked collagenase (MMP-1) expression and ovulation in the rat had no effect on the LH surge–induced increase in TIMP-1 expression [64]. Furthermore, constitutive or declined expression of TIMP-3 in the theca layer/adjacent ovarian stroma during the ovulatory process in the face of increased MMP expression may favor ECM degradation.

While the ability of TIMPs to provide focalized yet unrestrained activity of MMPs may be critical to the ovulatory process, TIMPs have many MMP-independent functions that may play an equally important role in ovulation and CL formation. For example, TIMP-1 has been shown to promote cell growth in a variety of cell types or cells lines [91, 170]. TIMP-1 has also been implicated in gene regulation. It has been reported to stimulate collagenase secretion in fibroblasts, suggesting that TIMP-1 may regulate MMP production [171]. TIMP-1 deficient mice express lower levels of TIMP-2 and –3. Furthermore, TIMP-1 has been detected in the nuclei of fibroblasts, further implicating it in gene regulation [172]. In addition, TIMP-1 has been shown to stimulate the growth of endothelial cells but inhibit their migration, which may be important in CL formation or control of angiogenesis during the ovulatory process [91, 170, 173].

Of controversy is the role of TIMP-1 in steroid regulation. Several studies have documented the ability of TIMP-1 to stimulate steroidogenesis in vitro [90, 174, 175]. However, initial in vivo studies involving mice lacking a functional TIMP-1 gene found no significant changes in either systemic estradiol or progesterone content using immature, gonadotropin-primed mice [174]. More recent studies involving mature cycling TIMP-1 null mice provide in vivo evidence that TIMP-1 regulates steroidogenesis [176]. In these mice, serum progesterone levels were decreased during estrus and serum estradiol levels were elevated at estrus and diestrus compared to wild-type. Therefore, TIMP-1 is potentially capable of modulating steroidogenesis of the newly developing corpus luteum. Furthermore, TIMP-1 has been reported to share a 124-base-pair sequence homology with StAR, a protein which controls the transport of

cholesterol to the inner mitochondrial membrane [177]. Thus, TIMP-1 may function as a coregulator of steroidogenesis in vivo along with several other factors.

Unlike TIMPs 1 and 2 which are soluble within the extracellular milieu, TIMP-3 is bound to the ECM, potentially allowing it to directly interact with enzymes involved in ECM component proteolysis [87, 88]. TIMP-3 also appears to be multi-functional, although many reports are conflicting. For example, several studies have shown that TIMP-3 promotes apoptosis [178, 179], and further studies have shown this effect to be independent of MMP inhibition [180]. It is possible that TIMP-3 promotes apoptosis by stabilizing tumor necrosis factor-α (TNF-α) receptors [178] and inhibiting TNF-α converting enzyme (TACE) [181]. The most notable distinction between the ovulatory follicle apex and base is the presence of the surface epithelium. During the ovulatory process, the surface epithelium undergoes apoptosis, and it is thought that proteolytic enzymes are released from these dying cells that could preferentially degrade the collagen at the apex of the follicle [29]. In contrast, other reports examining TIMP-3 in rat ovaries found TIMP-3 to be more abundant in granulosa cells of healthy follicles compared to adjacent atretic follicles [101]. It was suggested that TIMP-3 might actually reflect whether a follicle remains healthy or becomes apoptotic. Furthermore, TIMP-3 was regulated during G_1 progression during the cell cycle as well as differentiation [182]. Therefore, it may play a role in the differentiation of follicular cells prior to follicle maturation or in the follicle to CL transition.

In summary, we have shown that the gonadotropin surge results in increased TIMP-1 mRNA and activity while TIMP-3 mRNA is slightly declined. Upregulated expression of TIMP-1 in the granulosa cells may play an important role in regulation of

collagenolytic activity and steroidogenesis. The dramatic increase in TIMP-1 expression in the newly formed CL supports a potential role for TIMP-1 in the follicle to CL transition. Constitutive and decreased expression of TIMP-3 in the theca layers/tunica albuginea may favor a net increase in the MMP/TIMP ratio and subsequent degradation of the follicle wall prior to ovulation.

Chapter VI

General Conclusions and Limitations

Current Findings

In the present study the question was asked, do MMPs and TIMPs play a role in the ovulatory process? As an initial step in the exploration of this question, I have examined the mRNA expression, localization, and activity of selected MMPs and TIMPs following a GnRH-induced LH surge. The preovulatory LH surge is the endocrine signal that triggers ovulation. Our model allows us to examine changes in mRNA and protein/activity for specific MMPs and TIMPs at known time points following the LH surge. Based on their substrate specificity, intrafollicular localization, and increased mRNA expression following the LH surge, MMP-1, MMP-13, and MMP-14 may mediate degradation of the type I and III collagen rich ECM in the theca layer and tunica albuginea. While MMP-2 mRNA, localized in the theca cells, and MMP-2 activity remained unchanged during the periovulatory period, localization of MMP-2 activity to the cell surface may be important for basement membrane breakdown. The physiological significance of increased TIMP 1 and 2 mRNA expression and activity in the granulosa layer following the LH surge is not yet known. TIMPs are multifunctional molecules that have been implicated in several functions within the ovary in addition to MMP inhibition [90-93]. Finally, decreased expression of TIMP-3 following the LH surge may facilitate a shift in the MMP/TIMP ratio in the theca layers and tunica albuginea in favor of the MMP and thus promote ECM breakdown.

What is occurring at the protein level for these specific MMPs and TIMPs is not completely clear. While zymography and reverse zymography for MMP-2, TIMP-1, and

TIMP-2 revealed no change in the activity of these proteins in follicle homogenates, limitations of the assays employed leave room for further evaluation (see below). Zymographic analysis of MMP-2 activity showed no change in MMP-2 activity in follicle homogenates when separated on a SDS-PAGE gel. However, future development of an assay that is sensitive enough to evaluate MMP-2 activity (in the follicle homogenate) in a test tube, would allow the evaluation of the net MMP-2 activity (MMP-2 activity in the presence of inhibitors and other MMP-2:protein interactions, i.e. proteins that inhibit, activate or localize MMP-2 activity) present at the specific time points examined. Likewise, while TIMP-1 and TIMP-2 activity were increased in follicular fluid, it is unclear why follicle homogenate TIMP-1 and TIMP-2 activity were not increased corresponding to the increase in RNA abundance. Once again, the TIMP activity that is detected on a reverse zymography gel evaluates TIMP molecules that have been separated from other protein:protein interactions. Furthermore, detection of TIMP-3 by reverse zymographic analysis was compromised by the abundance of protein molecules of similar Mr in the follicle homogenate preparations. Further studies will be required to gain a more accurate representation of TIMP activity in vivo.

Animal models/Species specificity

When comparing the results of any group of studies, it is important to closely examine the model that each investigator uses. In the field of ovulation and reproduction, careful evaluation of the investigator's model and the species that is being used in the study is especially important. When investigating possible regulators of the ovulatory process and corpus luteum formation, it is standard procedure to synchronize an animal's reproductive cycle. In the literature, it has become commonplace to simply compare one

investigator's results to another's without truly evaluating the models being used. Critical evaluation of each model is necessary when considering experiments that produce differing results.

The standard reproductive mouse and rat model uses immature, non-cycling animals. The mice/rats are injected with PMSG (pregnant mare serum gonadotropin; hCG) to stimulate follicle growth, followed 48 h later by hCG to induce ovulation. While this model has become the standard for studying the ovulatory process in mice and rats, several investigators have begun to question some of the results obtained from immature, gonadotropin-primed animals when trying to relate the results to mature, cycling animals. In several instances, different results for preovulatory regulation of MMP and TIMP expression and localization were obtained using immature, gonadotropin-primed mice versus mature, cycling mice [58, 95, 98, 176].

In contrast, a different method of synchronization is used in farm animals such as sheep and cows [17, 140]. Generally in sheep, ewes are observed for estrus behavior with vasectomized rams. On day 14 of the estrous cycle (day 0 is the 1st day of estrus) animals are injected with $PGF_{2\alpha}$ to synchronize luteal regression. An ovulatory dose of GnRH is then injected 36 h later to produce a preovulatory LH surge. The largest diameter follicle in the pair of ovaries will ovulate ~ 24 h after GnRH injection. Similarly, in the Ovsynch model described in the present studies (See Chapter 2), follicle growth is synchronized through a series of GnRH and $PGF_{2\alpha}$ injections. An ovulatory dose of GnRH is administered to produce a LH surge that results in ovulation of the dominant follicle ~28 h later. Assay of serum LH is used with this model to confirm that

an LH surge was elicited by the second GnRH injection and that a premature LH surge did not occur. Therefore, the typical sheep model is more similar to the cow model presented in the current studies, compared with standard rodent models reported in the literature that stimulate growth of multiple follicles with exogenous gonadotropins.

In contrast, the rhesus monkey is synchronized using yet another model [66]. Adult rhesus monkeys exhibiting normal menstrual cycles of approximately 28 days are stimulated with recombinant human gonadotropins (rhFSH for 8 days and rhLH on days 7 and 8) beginning 1-3 days from the onset of menses to promote the development of multiple preovulatory follicles. A GnRH antagonist is also injected to prevent an endogenous LH surge. An ovulatory stimulus of rhCG is injected on day 8 to initiate the periovulatory process.

Models are a necessary and integral part of any experimental design. Critical evaluation is necessary to reveal the strengths and weaknesses of each model. For example, in the mouse model, the follicle is too small to collect a single ovulatory follicle, and therefore the entire ovary is evaluated instead of the ovulatory follicle. Evaluation of mRNA or activity in the ovary as a whole, rather than just in preovulatory follicles, results in a greater contribution of stroma and other follicles at various stages of development to the sample being evaluated. Furthermore, the mouse and rat produce multiple ovulatory follicles and therefore may require signaling mechanisms that are dissimilar to those in the human and the cow which typically ovulates a single follicle per cycle. Likewise, models that use adult cycling animals are more comparable to a human model than models that use immature animals. Therefore, the cow model is a more appropriate model for studying the ovulatory process because it allows one to examine

the events occurring in the single ovulatory follicle following the ovulatory endocrine signal, the LH surge, and it utilizes adult, cycling animals versus animals that have not reached reproductive maturity.

Limitations of the Present Studies

Protein assays. The ultimate objective when studying MMPs and TIMPs is to understand what is occurring at the activity level. While RNA data is usually an excellent indicator of regulation occurring at the protein level, protein data is a more accurate indicator of the protein interactions occurring in the tissue at the time points being examined. Studying an enzyme includes an additional limitation because the essential question is not the amount of protein present, but the amount of activity present. Standard protein determination assays such as Westerns, radioimmunoassays, and ELISAs therefore do not provide all of the needed information about activity, and the investigator is left to use a substrate assay or an assay that measures a byproduct of the enzymatic reaction.

In addition to the successful approaches outlined in Chapter II, I made numerous attempts with several different assays to evaluate the activity of MMP-2 (gelatinase), and MMP-1 and MMP-13 (collagenases). These attempts were unsuccessful. The nature of the MMP molecule and its regulation make it particularly difficult to design an assay or pick a commercially available assay that most accurately shows how much activity is present *in vivo*. The full length MMP molecule consists of a pro-domain that is cleaved resulting in the active MMP. Many assays are designed to "capture" the MMP with an antibody, and then evaluate how much activity exists in the proteins that were captured. However, an antibody that captures both the full length (proform) and cleaved MMP

(active form) will result in a higher activity than if just the shorter "active" form were captured. Activity is detected for the proform because of chemical modifications in the incubation buffers included with the assays. Secondly, most assays cannot delineate whether the activity measured corresponds to that of active MMP, active plus proform of MMP, and (or) MMP in a complex with TIMPs. Therefore, if activity is present in a sample, it is often difficult to extrapolate that activity to what is occurring *in vivo*. Likewise, even the MMP activity seen on zymography gels represents activity present only after the proteins have been separated from MMP/TIMP complexes that may be occurring *in vivo*.

My initial objective was to find a quantitative assay that most closely addressed the question of what was occurring in each sample at the time point collected *in vivo*. The first assay attempted was the EnzChek Gelatinase/Collagenase assay from Molecular Probes (Eugene, OR). In this assay, each sample is incubated with a fluorescently labeled gelatin substrate in a 96 well plate in an incubation buffer containing the appropriate amount of $CaCl_2$ for optimal MMP activity. An increase in fluorescence is proportional to proteolytic activity and can be monitored with a fluorescence microplate reader. Collagenase purified from *Clostridium histolyticum* is provided with the kit to serve as a positive control. While the positive control worked with each plate attempted, the samples assayed did not produce a linear response with increasing time or with increasing amount of sample. Furthermore, 1,10-phenanthroline (an effective MMP inhibitor included with the kit) did not inhibit the limited amount of fluorescence seen in the samples. After many attempts to resolve these difficulties, I decided to try another commercially available assay for the following reasons. First, in some situations,

fluorescent assays result in a high signal to noise ratio that cannot be overcome. Second, phenanthroline has been shown to produce a fluorescent signal in some scenarios. Finally, it is possible that the $CaCl_2$ concentration present in the homogenization buffer was interfering with the optimum $CaCl_2$ concentration for this assay.

Next I decided to try the MMP Gelatinase Activity assay kit from Chemicon (Temecula, CA). The principle of this assay is based upon cleavage of a biotinylated gelatinase substrate by active MMP-2. The biotinylated byproduct is then added to a streptavidin-enzyme complex in a 96 well plate. Addition of enzyme substrate results in a colored product that is read by a plate reader at 450 nm. This assay looked promising in theory because all of the original sample is washed away except the gelatinase byproduct; the signal is amplified by the streptavidin detection system; and the colorometric detection system eliminates the complications of fluorescent detection. However, like the first assay, I could not show this assay to be linear and parallel to the standard curve.

Because I could obtain semiquantitative information on gelatinase (MMP-2) activity from gelatin zymography, I shifted my efforts to measurement of collagenase activity. I began with a type I collagenase assay kit from Chemicon. In this assay, fluorescently labeled collagen is incubated with the sample in a test tube. Fluorescently labeled cleavage products are ethanol extracted and the fluorescence in each sample correlates with collagenase activity. The principles of the assay looked promising again, because the fluorescent product was precipitated from the original sample eliminating any fluorescent interference. However, similar problems were encountered as with above assays. Finally, I attempted to measure collagenolytic activity by incubation of the

samples with ^{3}H type I collagen. As a measure of collagenolytic activity, radiolabeled digested collagen fragments are collected in the supernatant after appropriate incubation and centrifugation and activity counted (in scintillation cocktail) in a beta counter. Unfortunately the assay did not have the needed sensitivity, the ^{3}H type I collagen proved very unstable, and once again there was a high signal to noise ratio. With the success of the gelatin zymography technique, collagen zymography was also tried. However, because of the density of collagen, samples did not migrate through the impregnated acrylamide gel with the same clarity as the gelatin gels, and I was unable to detect clear bands representing collagenolytic activity. Measurement of collagenase activity in these samples awaits the development of a sensitive assay that can amplify the activity in the sample without producing a high signal to noise ratio.

Sample preparation. Sample preparation can also prove challenging when attempting to measure MMP activity. MMPs require $CaCl_2$ to remain stable, and therefore $CaCl_2$ is a key component of MMP homogenization or extraction buffers. However, most activity assays also require a specific $CaCl_2$ concentration for assay optimization. Although I tried to adjust the $CaCl_2$ concentration in the assay buffers to account for the amounts present in the homogenization buffers, optimum $CaCl_2$ concentration was still a concern. Secondly, there is debate over the stability of MMPs in a frozen sample. Each tissue sample was frozen in liquid nitrogen when collected and then thawed once at the time of homogenization. After homogenization, heating, and centrifugation, each sample was then frozen again until assayed. Because active MMPs are further broken down into

smaller byproducts after activation, it is possible that the active MMPs are even more difficult to detect after freezing and thawing. Gelatin zymography of the samples showed nearly 100% of the activity detected was the proform (vs the active form).

The presence of endogenous MMP inhibitors in samples also makes measurement of MMP activity difficult. In previous studies, MMP activity was detectable in ovine preovulatory follicles only after reduction and alkylation to inactivate the TIMPs [17]. When attempted, inactivation of TIMPs by reduction and alkylation did not alleviate problems encountered with above assays.

Several attempts were also made to "clean up" the homogenized samples in order to remove excess salt or large proteins that may be interfering with the assays. Initially I tried to dialyze the samples into a buffer containing 50 mM Tris, 5 mM $CaCl_2$, 0.05% Brig 35 or into water through dialysis membranes. I also attempted to dialyze and concentrate the samples using Centricon (Millipore, Bedford, MA) centrifugal filter devices. Lastly, I tried to concentrate the MMPs in the samples by running each sample over a gelatin sepharose or heparin sepharose column with or without dialysis following affinity purification. All of the assays mentioned in the above sections and in the work presented here were attempted with all of these combinations of treated samples. Described partial purification procedures did not alleviate described technical impediments to quantitation of gelatinolytic and collagenolytic activity.

Ultimately, it will also be important to understand the periovulatory regulation of MMP-14 at the protein/activity level. Preliminary experiments were conducted using published procedures [183] to attempt to solubilize membrane bound MMP-14 from follicular membranes to measure its activity. The procedure involved homogenization of

the tissue in a biphasic detergent (Triton X-114) and centrifugation. The detergent phase contained the membrane proteins (potentially MMP-14) and the aqueous phase contained the cellular proteins. However, limitations on the amount of tissue available made this extra extraction procedure impractical at this time, and reliable assays for measuring MMP-14 activity have not been developed.

In situ hybridization.

Delineation of the intrafollicular localization of MMP-1 and MMP-13 mRNA (using in situ hybridization) proved difficult. Several attempts were made to modify the in situ procedure in order to increase sensitivity. Both ^{35}S and ^{33}P labeled probes were utilized. Sometimes an RNA probe used for in situ hybridization will have internal complementation that will decrease the amount of probe available for binding to the mRNA of interest. Upstream cDNAs were generated for both MMP-1 and MMP-13 with hopes of finding more effective probes for efficient detection of RNAs of interest. Shorter cDNAs were also generated with the hopes of decreasing internal complementation and increasing the ease with which labeled cRNA could penetrate the tissue. In addition, slides were hybridized with both the upstream probe and the original probe at the same time to increase the amount of signal that could be obtained for each target RNA molecule. A different emulsion was also tried that was advertised to have increased sensitivity, but unfortunately it also had a much higher signal to noise ratio. The reasons why in situ localization of MMP-1 and MMP-13 mRNA proved unsuccessful are unclear as MMP-2 and MMP-14 mRNAs were successfully localized using the same samples. In addition, MMP-1 and MMP-13 mRNA were detectable using Northern analysis.

Chapter VII

Future Directions

The work presented here poses two intertwined yet distinct questions: 1) What are the intrafollicular mechanisms whereby the LH surge causes ovulation? and 2) How are MMPs and TIMPs regulated, and are they involved in the ovulatory process? While the nature of these two questions is similar, and many of the future experiments would be the same in each case, it is still important to clearly define the long-term goal of one's research.

Firstly, there are several aspects of the current studies that require further investigation. As was previously discussed, protein data is critical in understanding the events occurring in the follicle extracellular matrix at the time of ovulation. While developing an assay that allows one to accurately quantify the net proteinase activity (the amount of activity occurring in each sample in the presence of TIMPs and other protein: protein interactions) itself would be timely, the information obtained using this assay would also represent a significant contribution to the understanding of regulation of MMP activity during the ovulatory process. Immunodetection assays such as Westerns would also be helpful in understanding MMP and TIMP protein regulation. Currently, there are several antibodies available that were made to various regions of the MMPs and TIMPs of interest. Likewise, further studies confirming the source of the collagenases need to be completed. While preliminary studies determined their localization, further studies are needed to increase the number of samples evaluated to confirm the precise localization of MMP-1 and MMP-13.

To direct the research focus towards understanding the key regulators of the ovulatory process, a functional genomics approach should be used, namely microarrays. Using microarrays created from normalized bovine cDNA libraries and non-redundant expressed sequence tags (EST), gene expression patterns of known and unidentified potential regulatory molecules can be studied. While this approach may result in further investigation of MMPs and TIMPs, other new candidate genes or potential regulators of the ovulatory process may become the focus of future experiments.

In contrast, the experiments presented here have identified six candidate MMPs and TIMPs that are regulated (5 increased and 1 decreased) following the preovulatory LH surge and may help control follicle rupture. Two experimental approaches would provide further insight into their role in the ovulatory process. First, because numerous MMP knockout models [184, 185, 186, 187] have pointed to the possibility of substantial redundancies among MMPs, it has been difficult to truly identify the absolute requirement and unique roles of individual MMPs. For example, while MMP-2 may contribute to follicle rupture through breakdown of the follicular basement membrane and may play an important role in the ovulatory process in sheep [67, 68, 69], MMP-2 deficient mice are fertile [184]. Likewise, previous studies have reported TIMP-2-independent activation of MMP-2 through MMP-15 (MT2-MMP) [188]. A series of transgenic experiments overexpressing individual MMPs and/or TIMPs in the ovary would offer insight to the individual contributions of the MMPs and TIMPs to the ovulatory process *in vivo*. For example, overexpression of MMP-14 in the ovary would allow examination of increased proteolytic activity of other MMPs, such as MMP-2 and MMP-13, due to increased activation or localization to the cell surface or to MMP

activators, as well as examination of increased degradation of ECM due to MMP-14 collagenolytic activity. Previous studies showed that pro-MMP-2 activation is impaired in MMP-14 deficient mice [185]. Overexpression of TIMP-1 in the ovary may lend further insight to its multifunctional, MMP-independent roles in the ovulatory process such as steroid regulation and gene regulation [56]. Crossing MMP and TIMP ovary specific transgenic mice would also provide further understanding of the coordinated regulation that specific MMPs and TIMPs contribute to the homeostasis necessary for successful ovulation and subsequent tissue repair by determining whether a TIMP overexpressor would rescue the phenotype of a MMP transgenic mouse. Furthermore, the use of inducible overexpression techniques would allow the specific transgene to be expressed at a specific point in time, namely after the mouse has reached sexual maturity [189].

As was stated earlier, measuring MMP activity that is comparable to what is occurring *in vivo* is difficult for numerous reasons. Extraction procedures are complicated when trying to maintain the integrity of the MMPs with appropriate $CaCl_2$ concentrations without interfering with assay conditions, and when trying to obtain preparations to measure both soluble and membrane bound MMPs. In addition, MMPs are often complexed with TIMPs. These MMP:TIMP interactions may result in activation, inhibition, or sequestration, and may involve an active MMP or the full-length MMP. Therefore, a transgenic approach targeted to the ovary would offer insight at the systems level and at the cellular level as to the unique contributions of individual MMPs and TIMPs.

Finally, further investigation of the intrafollicular signaling pathways downstream of the LH surge would identify key regulators of both MMPs and TIMPs and follicle rupture. As was discussed previously, the progesterone receptor (P_4) [135, 164] and prostaglandin E_2 (PGE_2) [117, 122] receptor signaling pathways are potential mediators of MMP and TIMP regulation in the ovulatory process. A series of experiments designed to inhibit each of these pathways in order to evaluate their effects on MMP/TIMP regulation and follicle rupture would provide insight as to the basic mechanisms involved in follicle rupture and the ovulatory process. For example, injection of a selective P_4 receptor antagonist or COX-2 inhibitor into bovine preovulatory follicles after GnRH injection (to inhibit the progesterone receptor or prostaglandin signaling pathways, respectively) would allow for the evaluation of the MMP and TIMP response to the inhibition of these pathways. Both MMP-1 and MMP-13 mRNA in bovine preovulatory follicles show an initial increase in mRNA followed by a decline and then a second increase in expression (Chapter IV). The LH surge may be directly responsible for the initial increase in collagenase expression, while downstream effects of the LH surge, such as the increase in COX-2 (and subsequent increase in PGE_2) and the upregulation of the progesterone receptor, may be necessary for the subsequent increase in collagenase mRNA. These experiments would allow us to further determine if the P_4 receptor and PGE_2 receptor signaling pathways play a key role in control of follicle rupture and regulation of MMPs and their inhibitors in bovine preovulatory follicles. In addition,

these experiments would provide insight as to which MMPs and TIMPs are truly obligatory for follicle rupture and lead to a better understanding of the ovulatory process.

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