MATRIX DEGRADING PROTEASES IN THE OVARY:
Expression and function

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TABLE OF CONTENTS

ABBREVIATIONS 5
ABSTRACT 6
PUBLICATION LIST 7
INTRODUCTION 8

1. THE EXTRACELLULAR MATRIX 9
   1.1 The components of the ECM 9
   1.2 Biological functions of the ECM 9
   1.3 ECM remodeling 10

2. THE PLASMINOGEN ACTIVATOR SYSTEM 10
   2.1 Proteolytic enzymes of the PA system 11
      2.1.1 Plasmin/plasminogen 11
      2.1.2 Plasminogen activators 12
   2.2 Inhibitors of the PA system 13
      2.2.1 PA inhibitors 13
      2.2.2 Other physiological plasmin/PA inhibitors 14

3. THE MATRIX METALLOPROTEINASE SYSTEM 15
   3.1 The MMP family 15
   3.2 Regulation of MMPs 18
   3.3 Inhibition of MMP activity 20
      3.3.1 Physiological MMP inhibitors 20
      3.3.2 Synthetic MMP inhibitors 20

4. THE PA AND MMP SYSTEMS IN PHYSIOLOGICAL AND
   PATHOLOGICAL PROCESSES 21
   4.1 Cooperation and redundancy between the PA and MMP systems 21
   4.2 Tumor invasion and metastasis 22
   4.3 Angiogenesis 22
   4.4 Embryo implantation 23
   4.5 Protease and protease inhibitor deficient mice 24

5. THE OVARY 27
   5.1 Follicular development and atresia 27
   5.2 Ovulation 28
   5.3 Corpus luteum formation 30
   5.4 Corpus luteum function 30
   5.5 Corpus luteum regression 31
   5.6 The PA and MMP systems in the ovary 31
   5.7 Animal models for the study of ovary function 33

6. SUMMARY OF THE PRESENT STUDY 34
   6.1 Coordinated and cell-specific regulation of membrane-type matrix
       metalloproteinase 1 (MT1-MMP) and its substrate matrix metalloproteinase
       2 (MMP-2) by physiological signals during follicular development and
       ovulation (Paper I). 34
   6.2 Distinct expression of gelatinase A (MMP-2), collagenase-3 (MMP-13),
       3
membrane-type MMP 1 (MT1-MMP), and tissue inhibitor of MMPs type 1 (TIMP-1) mediated by physiological signals during formation and regression of the rat corpus luteum (Paper II).

6.3 Expression pattern and functional studies of matrix degrading proteases and their inhibitors in the mouse corpus luteum (Paper III).

6.4 A synchronized gonadotropin-induced corpus luteum model in the mouse (Paper IV).

6.5 Plasminogen is required for normal progesterone production in the mouse (Paper V).

CONCLUSIONS

ACKNOWLEDGEMENTS

REFERENCES

PAPERS I-V
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>$\alpha_2$-AP</td>
<td>$\alpha_2$-antiplasmin</td>
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<tr>
<td>ADAM</td>
<td>A disintegrin and metalloprotease domain</td>
</tr>
<tr>
<td>ADAM-TS</td>
<td>ADAM with thrombospondin motifs</td>
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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<tr>
<td>CL</td>
<td>corpus luteum</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
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<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<tr>
<td>IGFBP</td>
<td>IGF-binding protein</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>LH</td>
<td>luteinizing hormone</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MT-MMP</td>
<td>membrane-type MMP</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PA</td>
<td>plasminogen activator</td>
</tr>
<tr>
<td>PAI</td>
<td>PA inhibitor</td>
</tr>
<tr>
<td>PMSG</td>
<td>pregnant mare serum gonadotropin</td>
</tr>
<tr>
<td>PN-1</td>
<td>protease nexin-1</td>
</tr>
<tr>
<td>psp</td>
<td>pseudopregnant</td>
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<tr>
<td>sc-tPA</td>
<td>single-chain tPA</td>
</tr>
<tr>
<td>sc-uPA</td>
<td>single-chain uPA</td>
</tr>
<tr>
<td>serpin</td>
<td>serine protease inhibitor</td>
</tr>
<tr>
<td>StAR</td>
<td>steroidogenic acute regulatory protein</td>
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<tr>
<td>tc-tPA</td>
<td>two-chain tPA</td>
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<tr>
<td>tc-uPA</td>
<td>two-chain uPA</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
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<tr>
<td>tPA</td>
<td>tissue-type PA</td>
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<td>uPA</td>
<td>urokinase-type PA</td>
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<tr>
<td>uPAR</td>
<td>uPA receptor</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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ABSTRACT

MATRIX DEGRADING PROTEASES IN THE OVARY:
Expression and function

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Extracellular matrix degrading proteases from the plasminogen (plg) activator (PA) and the matrix metalloproteinase (MMP) systems have been implicated as important mediators of ovulation and corpus luteum (CL) formation and regression. The aim of this thesis was to investigate the expression and regulation of PAs and MMPs in the ovary and to examine their functional roles in CL formation and function.

The expression of membrane-type MMP-1 (MT1-MMP) and its substrate gelatinase A (MMP-2) mRNAs was studied during pregnant mare serum gonadotropin (PMSG)/human chorionic gonadotropin (hCG)-induced ovulation in immature rats. These proteases were coordinately regulated so that both were highly expressed in the theca cells of large preovulatory follicles. This suggests that MT1-MMP activates gelatinase A in preovulatory follicles to degrade the follicular wall during ovulation.

In pseudopregnant (psp) rats, MT1-MMP mRNA was expressed in the CL throughout the luteal phase. Tissue inhibitor of metalloproteases type-1 (TIMP-1) mRNA was expressed during CL formation and regression. MMP-2 and collagenase-3 mRNAs were expressed during CL formation and regression, respectively. When the luteal phase was artificially prolonged or shortened, TIMP-1 and collagenase-3 mRNAs were induced only after the serum progesterone levels had decreased, indicating a close association with luteolysis in the rat.

In psp mice, the expression of mRNAs coding for both PAs, seven MMPs, and five protease inhibitors was studied. Most of the molecules studied were coordinately expressed during formation or regression of the CL. However, uPA, MT1-MMP, and TIMP-3 mRNAs were expressed throughout the luteal phase. The role of uPA was examined in psp uPA deficient mice. These mice displayed no abnormalities in luteal function or vascularity. The role of uPA is thus either not essential, or its absence can be compensated for by other proteases.

In order to control the timing of CL formation, a mouse model for PMSG/hCG-induced CL formation was developed. Five different protocols were evaluated. One of them provided CL that were stable for six days. In that protocol, the mice were treated with prolactin (PRL) twice daily from day 2 of CL life onward. The expression of the steroid acute regulatory protein (StAR) mRNA in the psp CL was also characterized to assess its use as a molecular marker for CL development and regression. It was highly expressed in the forming and functional CL, and was downregulated at a late stage of CL regression.

The functional role of plg and MMPs in CL formation and function was investigated in plg deficient mice treated with the MMP inhibitor galardin (GM6001). Both psp mice and PMSG/hCG +PRL-induced CL formation were used. Several molecular markers for CL development and regression were used to evaluate the health status of the CL. Our data showed that healthy and vascularized CL formed even in plg deficient mice treated with the inhibitor. However, serum progesterone levels were significantly reduced in these mice, an effect that was mainly attributable to the plg deficiency. In conclusion, neither plg nor MMPs, alone or in combination, appear to be essential for the development of a functional CL.

Key words: ovary / ovulation / corpus luteum / plasminogen / PA / MMP / rat / mouse.

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INTRODUCTION

Proteolytic activity generated by the plasminogen activator (PA) and matrix metalloproteinase (MMP) systems has been implicated in a large number of physiological and pathological tissue remodeling processes. These include cancer invasion and metastasis, angiogenesis, wound healing, embryo implantation, ovulation and many more processes (Dano et al., 1985; Lund et al., 1999; Mignatti and Rifkin, 1996; Strickland and Richards, 1992; Tsafriri and Reich, 1999).

The main player in the PA system is the serine protease plasmin, which is activated by either of the two PAs, tissue-type PA (tPA) or urokinase-type PA (uPA). The active protease plasmin can then degrade a number of extracellular matrix (ECM) components as well as activate members of the MMP system. The PAs are synthesized and released by different cell types in response to hormones, growth factors, and other signals. The PAs can also be inhibited by the physiological PA inhibitors (PAIs) to prevent unrestrained proteolysis by this system.

The MMPs are a large and growing family of metalloproteases consisting of more than 20 proteases, some of which are membrane bound (membrane-type MMPs; MT-MMPs). Together, the MMPs have activity against virtually all of the ECM components. MMP activity can be inhibited by the physiological tissue inhibitors of metalloproteases, TIMPs, or by synthetic agents that chelate the Zn$^{2+}$ at the active site (Visse and Nagase, 2003; Vu and Werb, 2000).

The PA and MMP systems are often regulated in a coordinated fashion during tissue remodeling, which can create both a proteolytic cascade and a functional redundancy to ensure efficient ECM remodeling. Also, the PAIs and TIMPs are often coordinately regulated in order to prevent unrestrained tissue destruction by the PA and MMP systems (Lund et al., 1999; Mignatti, 1995; Solberg et al., 2003).

The main function of the mammalian ovary is to produce fertilizable ova. During each estrus cycle, several follicles develop to finally release the ovum. Both follicular development and the rupture of the follicular wall at ovulation require extensive tissue remodeling (Tsafriri and Reich, 1999). After ovulation, the ovulated follicle develops into a corpus luteum (CL), which produces progesterone to prepare the uterus for embryo implantation. This process involves tissue remodeling, cell migration and rapid angiogenesis as the previously avascular follicle transforms into the highly vascularized CL (Findlay, 1986). If successful implantation does not occur, the CL stops producing progesterone (functional luteolysis) and goes into structural regression (structural luteolysis) whereby the nonfunctional CL tissue is rapidly degraded (Michael et al., 1994).

A large number of studies have implicated the PA and MMP systems as being important factors in all of these ovarian tissue remodeling processes (Curry, Jr. and Osteen, 2003; Ny et al., 2002; Tsafriri and Reich, 1999).

The aim of this thesis was to investigate the involvement of the PA and MMP systems in ovulation and in the formation, function, and regression of the CL. The functional relevance of these protease systems for CL development and function has also been studied by using mice deficient in uPA or plasminogen and by treating mice with the broad-spectrum, synthetic MMP inhibitor, galardin (GM6001).
1. THE EXTRACELLULAR MATRIX

The extracellular matrix (ECM) is crucial for tissue architecture and stability. It consists of protein fibers, ground substance, and tissue fluid. The ECM is not only important for tissue structure but can also play an active role in many processes such as cell migration, cell differentiation, and cell-cell signaling (Alberts et al., 1994; Junqueira et al., 1995).

1.1 The components of the extracellular matrix

The ECM consists of various types of collagen fibers/fibrils, proteoglycans such as heparan sulphate, glycoproteins such as fibronectin, and bound water (Alberts et al., 1994; Junqueira et al., 1995). The exact composition depends on the needs of a specific tissue. For example, bone tissue is rich in hydroxyapatite crystals associated with type I collagen fibers, to give it its hardness and toughness. In basement membranes, a dense network of non-fibrillar collagen type IV, heparan sulphate, laminin and other substances gives the membrane its flexible yet resilient properties (Junqueira et al., 1995).

The collagens deserve special mention, as they make up roughly 25% of the protein mass in mammals. There are a large number of genes encoding collagen subunits, which form the collagen by association into triple-stranded helices (Alberts et al., 1994). Some collagens, such as collagen type I, form long fibers while others, such as collagen type II, form short fibrils. Collagen type IV is a collagen that does not form fibers or fibrils at all. Different types of collagen dominate different tissues, depending on the needs of the tissue (Junqueira et al., 1995).

1.2 Biological functions of the ECM

A major function of the ECM is to give tissues mechanical support. The ECM also has many other functions. For example, many growth factors bind to components of the ECM, which can both inhibit and facilitate the signaling under different circumstances. The binding of growth factors to the ECM can create a pool of growth factors that may be released later by proteolytic activation or by controlled degradation of the ECM (Bunn and Fowlkes, 2003; Visse and Nagase, 2003; Vu, 2001; Yu and Stamenkovic, 2000). The ECM also plays an important role in cell survival, growth, and differentiation (Adams and Watt, 1993). Without proper binding to the ECM, a cell may commit apoptosis, which in this context is referred to as anoikis (Frisch and Sreeton, 2001). Migrating cells depend on ECM attachment for forward movement. They also receive signals from the ECM that guide the cells to their proper destinations.

Basement membranes, upon which epithelial cells rest, have additional functions. One is to give polarity to the cells that rest upon the membrane. Another is to act as a molecular sieve, which is especially important in the glomerulus of the kidney (Junqueira et al., 1995).
1.3 ECM remodeling

The ECM is constantly being both synthesized and degraded. The turnover rate varies a great deal between different tissues. In bone tissue, a collagen molecule has a life of about ten years whereas in the uterus dramatic remodeling of the ECM takes place every menstrual cycle (Alberts et al., 1994; Junqueira et al., 1995). The ECM is mainly synthesized by fibroblasts, which also organize the components of the ECM to fit the needs of the tissue. The degradation of ECM is mainly a function of extracellular proteases, but secreted enzymes that hydrolyze the carbohydrate parts of ECM molecules may also be important. Imbalance between ECM synthesis and degradation is often seen in disease states. The most famous example is scurvy, where a deficiency of ascorbic acid (vitamin C), which is essential for proper collagen synthesis, leads to the inevitable degradation of the connective tissues of the body (Junqueira et al., 1995).

The PA and MMP systems appear to play central roles in the degradation of ECM components during many different tissue remodeling events (Birkedal-Hansen, 1995; Mignatti, 1995). These proteases are synthesized and released in response to various hormones and growth factors. The ability of these proteases to activate other proteases may lead to a proteolytic cascade with activity against most or all of the ECM proteins. Migrating cells can make use of the membrane-type MMPs (MT-MMPs) to create a local proteolytic activity at the leading edge of the cell (Seiki, 2002; Werb, 1997). The proteolytic activities are balanced both by the secretion of physiological protease inhibitors and by the synthesis of new ECM components, mainly by fibroblasts. This creates a dynamic yet stable extracellular environment, in which the ECM is degraded locally according to the needs of the tissue (Birkedal-Hansen, 1995; Mignatti, 1995; Price et al., 1997).

The degradation of ECM may release bound growth factors to allow them to interact with their cell-surface receptors on nearby cells. Degradation of certain ECM components can also give rise to protein fragments with signaling properties. As discussed further in section 4.3, this appears to be especially important in the regulation of angiogenesis.

2. THE PLASMINOGEN ACTIVATOR SYSTEM

The PA system is a versatile protease system, which is involved not only in fibrinolysis but also in a wide range of tissue remodeling processes. The main component is the proenzyme plasminogen (plg), which can be activated by either of the two PAs, tissue-type PA (tPA) or urokinase-type PA (uPA), to the broad-spectrum protease plasmin. Plasmin degrades fibrin clots and also has activity against several components of the ECM. It can also activate some members of the MMP system (Fig. 1). Plasminogen is synthesized in large quantities by the liver and released into the bloodstream, which carries it to most tissues. Thus, this system is mainly regulated by the local synthesis of PAs. To prevent excessive tissue degradation, the activity of the PA system is balanced by inhibitors such as PA inhibitors (PAIs), α₂-antiplasmin (α₂-AP) and protease nexin-1 (PN-1) (Saksela and Rifkin, 1988; Vassalli et al., 1991).
Figure 1. Schematic representation of the PA system and its regulation. Synthesis of tPA and uPA by specific cells is regulated by hormones, growth factors, and cytokines. In the extracellular space, PA and plasmin activities are controlled by specific inhibitors, PAI-1, PAI-2 and $\alpha_2$-AP. Binding of PAs and plasmin to cellular binding sites (R) can result in localized proteolytic activity on the cell surface.

2.1 Proteolytic enzymes of the PA system

Plasmin, tPA, and uPA belong to the serine protease family which is characterized by the presence of the catalytic triad (histidine, asparagine, and serine) in the catalytic site.

2.1.1 Plasmin/plasminogen

Plasminogen is a 92-kDa glycoprotein of 790 amino acids. It is mainly synthesized in the liver and reaches a plasma concentration of about 200 $\mu$g/ml (~2 $\mu$M) (Raum et al., 1980; Wallen, 1980). Plasminogen can be found in most extracellular fluids. It is also expressed locally in some tissues where it cannot penetrate from plasma (Sharon et al., 2002; Zhang et al., 2002). Plasminogen is synthesized as a native single-chain, 790-amino acid protein. It is found in two different forms. The native uncleaved plasminogen has an amino-terminal glutamic acid residue and is referred to as Glu-plasminogen. Plasmin can cleave the Lys76-Lys77 bond, releasing a 76-amino acid peptide and giving rise to Lys-plasminogen with an amino-terminal lysine residue (Wallen and Wiman, 1970; Wallen and Wiman, 1972; Wiman and Wallen, 1975). Lys-plasminogen has higher affinity than Glu-plasminogen for fibrin, due to the exposure of a fibrin binding site after the conversion (Wallen, 1980). However, it has a shorter half-life in plasma (0.8 days as compared to 2.2 days for Glu-plasminogen) (Bachmann, 1987).
Both Glu-plasminogen and Lys-plasminogen can be activated by tPA or uPA. The PA cuts the Arg\textsubscript{560}-Val\textsubscript{561} bond, giving rise to the two-chain plasmin, where the two chains are connected through a disulfide bond (Sottrup-Jensen et al., 1975). The heavy A-chain is glycosylated and contains five “kringle” domains. The kringle domains contain lysine binding sites that can bind fibrin, α\textsubscript{2}-antiplasmin, and other proteins (Wiman et al., 1979). Kringle 1 has a lysine-binding site with very high affinity for fibrin, which is exposed after conversion from Glu-plasminogen to Lys-plasminogen (Lerch et al., 1980; Winn et al., 1980). The light B-chain contains the catalytic triad (histidine, asparagine, and serine). It has a broad-spectrum catalytic activity and hydrolyzes peptide bonds after lysine (preferred) or arginine residues (Bachmann, 1987; Wallen, 1980).

One of the main functions of plasmin is vascular fibrinolysis, to prevent pathological clot formation. Plasmin binds to fibrin through its lysine binding sites. tPA also binds to fibrin, which dramatically enhances both its affinity for plasminogen and the rate at which it activates plasminogen (Hoylaerts et al., 1982; Ranby et al., 1982). Localization of both the zymogen and its activator to the fibrin surface leads to specific and efficient fibrinolysis. Furthermore, the binding of plasmin to the inhibitor α\textsubscript{2}-antiplasmin depends on the same lysine binding sites that bind to fibrin. The fibrin-attached plasmin is thus protected against inhibition until it has dissolved the fibrin (Longstaff and Gaffney, 1991).

Plasmin not only has proteolytic activity against fibrin, but also against several components of the ECM such as different collagens, laminin, fibronectin and proteoglycans (Alexander and Werb, 1991). Plasmin can also activate members of the MMP family and some growth factors; it may also degrade growth factor binding proteins to release the sequestered growth factors (Bunn and Fowlkes, 2003; Vu, 2001). With its multitude of actions, plasmin has been implicated in a wide range of tissue remodeling events, such as ovulation, tumor invasion, angiogenesis and wound healing (Carmeliet and Collen, 1998; Dano et al., 1985; Ny et al., 2002; Saksela and Rifkin, 1988). The role of plasmin in these processes will be described in more detail in Chapters 4 and 5.

2.1.2 Plasminogen activators

There are two known physiological plasminogen activators: tPA and uPA. They have similar domain structures, containing kringle domains and EGF-like domains. Traditionally, tPA was viewed as a fibrinolysis-specific PA while uPA, which does not bind to fibrin, appeared to have separate functions. It has turned out that both PAs are expressed in a diversity of tissues in response to various signals such as growth factors, cytokines, and peptide and steroid hormones. During tissue remodeling events, it is common that both PAs are expressed, creating functional redundancy. This is discussed further in Chapter 4; the single PA gene deficient mice display rather weak phenotypes, while mice that lack both PAs have more severe phenotypes, resembling those seen in the plasminogen deficient mouse.

**tPA** is synthesized as a 530-amino acid single-chain 68-kDa glycoprotein (sc-tPA) (Pennica et al., 1983; Pohl et al., 1984; Rijken and Collen, 1981). Its concentration in normal plasma is about 5 ng/ml, and it is mainly produced by vascular endothelial cells. Most of the tPA in plasma is bound to the PA inhibitor type 1 (PAI-1), but release of tPA from endothelial cells can create high local concentrations of free tPA (Bachmann, 1987). Plasmin, plasma kallikrein and other proteases can cleave sc-tPA to form the disulfide linked two-chain tc-tPA (Bachmann, 1987; Wallen et al., 1983). Both forms of tPA are active proteases (Rijken et al.,
1982). The tc-tPA has a similar structure to that of plasmin. It has a heavy chain, containing a fibrin binding finger domain, and two kringle domains with lysine binding sites that also bind to fibrin. The light chain contains the catalytic serine protease domain. Interestingly, each tPA domain is encoded by a separate exon (Ny et al., 1984), which suggests that tPA has been assembled according to the exon shuffling model (Patthy, 1985). As mentioned previously, binding of tPA to fibrin enhances its plasminogen activation rate by about 200-400 fold. Besides activating plasminogen during fibrinolysis, tPA also has other important functions, both dependent and independent of plasminogen, in many tissues - such as the brain, where it is involved in excitotoxic damage and long-term potentiation in the hippocampus (Huang et al., 1996; Wu et al., 2000).

**uPA** is synthesized as a proteolytically inactive, single-chain 54-kDa glycoprotein (sc-uPA). Its concentration in plasma is about 2-20 ng/ml (Nielsen et al., 1982; Petersen et al., 1988; Wun et al., 1982). Plasmin and other proteases such as glandular kallikrein can cleave the Lys^{158}-Ile^{159} bond to create the proteolytically active, disulfide linked two-chain uPA (tc-uPA) (Bachmann, 1987; List et al., 2000). The heavy chain contains an EGF-like domain and a kringle domain. The light chain contains the serine protease active site (Ichinose et al., 1986). Since uPA does not bind to fibrin and is expressed in many different tissues during tissue remodeling, it is believed that its main function is to stimulate plasmin-mediated tissue remodeling (Dano et al., 1985; Vassalli et al., 1991). A 55-kDa receptor for uPA (uPAR) is expressed on the surface of many different cell types. The binding of uPA to uPAR leads to local activation of plasminogen at the cell surface, which may be of importance to cells actively migrating into or invading tissues (Blasi et al., 1994; Saksel and Rifkin, 1988). The importance of the uPAR remains uncertain, however, since the uPAR deficient mice display no distinct phenotypes (Bugge et al., 1995b; Dewerchin et al., 1996).

### 2.2 Inhibitors of the PA system

By itself, the PA system has a high degree of destructive potential as plasmin can degrade several protein components of the ECM. Furthermore, plasmin can activate some MMPs (Mignatti and Rifkin, 1993). To prevent the PA system from wreaking havoc, it is crucial to restrict its proteolytic activities both spatially and temporally. There are a number of protease inhibitors, which are active against the PA system. Most of these, including α_{2}-antiplasmin (α_{2}-AP), PAI-1, PAI-2, and protease nexin-1 (PN-1) belong to the serine protease inhibitor (serpin) superfamily. Serpins have similar structures and act as suicide inhibitors, where they function as substrates for the protease which becomes trapped in an inactive complex with the inhibitor (Pike et al., 2002; Silverman et al., 2001).

#### 2.2.1 PA inhibitors

Two major physiological PA inhibitors (PAIs) have been described: PAI-1 and PAI-2.

**PAI-1** is a 52-kDa glycoprotein, which is synthesized and secreted by a variety of cell types (Ny et al., 1986; van Mourik et al., 1984; Wiman et al., 1984). Its plasma concentration is low, but sufficient to inhibit most of the circulating tPA (Bachmann, 1987). PAI-1 can inhibit sc-tPA, tc-tPA, and tc-uPA (Loskutoff et al., 1989). It is secreted from cells in an active conformation but is quickly converted into an inactive, latent conformation. PAI-1 has high affinity for vitronectin, which is found both in plasma and in the ECM. The binding of PAI-1
to vitronectin stabilizes its active conformation (Declerck et al., 1988; Mimuro and Loskutoff, 1989; Seiffert et al., 1990; Wiman et al., 1988). The binding of a PA to the vitronectin-bound PAI-1 reduces its affinity for vitronectin. This is important for cell migration events, especially during angiogenesis. When PAI-1 is bound to vitronectin, the binding of αvβ3 integrin to vitronectin is inhibited (Zhou et al., 2003). When uPA binds to PAI-1, however, it is released from the vitronectin and the integrin binding site is exposed. In this way, a cycle of attachment-detachment-reattachment could promote cell migration (Stefansson and Lawrence, 2003).

As discussed in Chapter 4, these and other findings have clearly shown that PAI-1 has other important functions besides regulating vascular fibrinolysis.

**PAI-2** belongs to the subgroup of ovalbumin-like serpins (ov-serpins) (Silverman et al., 2001). PAI-2 can be secreted as a glycosylated 60-kDa protein which has high inhibitory activity against uPA, and less so for tPA (Kruithof et al., 1986; Åstedt et al., 1987). However, its secretion is inefficient and most of the PAI-2 protein is found as an intracellular 47-kDa protein (von Heijne et al., 1991). Expression of PAI-2 is normally restricted to a few cell types such as macrophages and its plasma concentration is below detectable levels. However, during the third trimester of pregnancy, there is a dramatic increase in PAI-2 levels in plasma, and it is also highly expressed in the placenta (Jensen, 1997; Kruithof et al., 1986; Åstedt et al., 1987).

The physiological functions of PAI-2 have not been clearly determined. PAI-2 deficient mice are fertile and lack obvious phenotypes (Dougherty et al., 1999). Some studies have suggested that the intracellular form protects against TNF-α mediated apoptosis (Dickinson et al., 1995; Kumar and Baglioni, 1991). Others have shown that PAI-2 can interact with the Rb protein in vivo, suggesting a role in cell cycle regulation (Darnell et al., 2003). PAI-2 can be found in two conformations: a stable, monomeric form and a conformation that can form polymers under physiological conditions (Mikus and Ny, 1996). The redox status of the environment determines the formation of an intramolecular disulfide bond, which in turn determines whether PAI-2 will form stable monomers or polymerize. By this mechanism, the PAI-2 that is secreted will be in the polymerogenic form while the intracellular PAI-2 is in the monomeric form (Wilczynska et al., 2003). These forms are fully interconvertible through formation and reduction of the intramolecular disulfide bond (Lobov et al., 2004, unpublished). The functional relevance of the different forms of PAI-2 is so far unknown.

### 2.2.2 Other physiological plasmin/PA inhibitors

**α2-antiplasmin (α2-AP)** is a 70-kDa glycoprotein produced by the liver. Its concentration in plasma is roughly half of that of plasminogen (1 µM; 70 µg/ml) (Bachmann, 1987; Collen and Wiman, 1979; Moroi and Aoki, 1976; Wiman and Collen, 1977). α2-AP inhibits plasmin rapidly and also inhibits the binding of plasmin to fibrin. The binding of plasmin to fibrin involves the same lysine binding sites as those involved in the binding to α2-AP (Collen and Wiman, 1979; Sasaki et al., 1986). This means that the fibrin-bound plasmin is protected from inhibition until the fibrin has been dissolved. On the other hand, free plasmin is rapidly inhibited by α2-AP in order to restrict the plasmin activity to the fibrin surface (Bachmann, 1987; Longstaff and Gaffney, 1991; Wallen, 1980).
**Protease nexin-1 (PN-1)** is a 45-kDa glycoprotein, which is produced by many different cell types, but it is found only at very low levels in plasma (Eaton and Baker, 1983; Scott and Baker, 1983; Scott et al., 1985). The very low levels of PN-1 in plasma suggest that its main function is not to regulate vascular fibrinolysis. It appears to play an important role in normal male fertility, however, as PN-1 deficient male mice have dysfunctional semen which renders these mice less fertile than their wild-type counterparts (Murer et al., 2001). PN-1 has activity against plasmin, uPA, and other proteases (Scott et al., 1985). The PN-1/protease complex binds to cell-surface receptors and is internalized and degraded in lysosomes (Saksela and Rifkin, 1988).

\( \alpha_2 \)-macroglobulin is a complex of four disulfide linked protein chains with a total mass of 725 kDa. \( \alpha_2 \)-macroglobulin is synthesized by the liver and has a concentration in plasma of about 2.5 mg/ml (Barrett and Starkey, 1973; Travis and Salvesen, 1983). Its large size prevents it from gaining access to some tissues. \( \alpha_2 \)-macroglobulin is a very broad protease inhibitor, containing a bait region that acts as a substrate for most protease classes. When a protease cuts the bait peptide a stable, covalent complex with the inhibitor is formed (Sottrup-Jensen and Birkedal-Hansen, 1989). This inhibition is rather slow compared to other protease inhibitors. \( \alpha_2 \)-macroglobulin is therefore viewed as a “second line of defense”, capturing the excess of plasmin and other proteases that has not been neutralized by other inhibitors. The \( \alpha_2 \)-macroglobulin/protease complexes bind to cell-surface receptors and are internalized and degraded in lysosomes (Bachmann, 1987).

### 3 THE MATRIX METALLOPROTEINASE SYSTEM

The matrix metalloproteinases (MMPs) constitute a large and still growing protease family. MMPs depend on a Zn\(^{2+}\) ion in the active site and are usually secreted as inactive proforms that can be activated by limited cleavage by serine proteases or other MMPs. The MMPs have diverse substrate specificities and together have activity against virtually all protein components of the ECM (Visse and Nagase, 2003; Vu and Werb, 2000). MMPs have been shown to be involved in a wide range of physiological and pathological tissue remodeling processes. Recent findings indicate that MMPs are not limited to ECM degradation, but may also be important in regulating growth factors and adhesion molecules and other non-ECM molecules (McCawley and Matrisian, 2001; Vu and Werb, 2000).

### 3.1 The MMP family

25 MMPs have been characterized so far, and still more may have yet to be discovered. The known MMPs and relevant substrates are summarized in Table 1. The MMP family can be divided into different groups, based on structural similarities and substrate specificities (Visse and Nagase, 2003). Some of the MMPs do not fit very well into these categories and are sometimes referred to as “other MMPs”.

**The collagenases** include collagenase-1 (interstitial collagenase; MMP-1), collagenase-2 (neutrophil collagenase; MMP-8), and collagenase-3 (MMP-13). The main function of collagenases is to cleave fibrillar collagen, which is resistant to cleavage by most other MMPs (Shingleton et al., 1996). After the initial cleavage, the collagens become denatured and form gelatin, which can be further degraded by other MMPs and also by other proteases such as plasmin (Murphy and Docherty, 1992).
The gelatinases (gelatinase A; MMP-2 and gelatinase B; MMP-9) are highly active against gelatin (denatured collagen), native collagen type IV and other components of basement membranes. Gelatinases are often expressed and are seen as important mediators of basement membrane degradation during processes such as angiogenesis, tumor invasion and embryo implantation (Giannelli and Antonaci, 2002; Van den Steen et al., 2002).

The stromelysins include stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), and stromelysin-3 (MMP-11). Stromelysins generally have rather broad substrate specificities against gelatin and components of the basement membrane (Visse and Nagase, 2003). Some of them can also activate other MMPs (Murphy et al., 1987; Nagase, 1997).

The matrilysins are matrilysin-1 (MMP-7; PUMP-1) and matrilysin-2 (MMP-26; endometase). Matrilysins lack the hemopexin domain. Matrilysin-1 is the most studied of these MMPs (Wilson and Matrisian, 1996). It has activity against many ECM components but has also been shown to process Fas-ligand, E-cadherin, and other cell-surface molecules (Noe et al., 2001; Powell et al., 1999).

The membrane-type MMPs include MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-17), MT5-MMP (MMP-24), and MT6-MMP (MMP-25). Four of these have transmembrane domains, while the remaining two (MT4-MMP and MT6-MMP) are anchored to the cell membrane by glycosyl phosphatidylinositol anchors. All of the MT-MMPs are activated by furin in the Golgi apparatus and are thus secreted as active proteases at the cell surface. The MT-MMPs cleave a variety of ECM molecules but are also important activators of gelatinase A and collagenase-3. The MT-MMPs can therefore produce a high local proteolytic activity at the cell surface, which may be especially important for cell migration processes (Seiki, 2002).

Table 1. List of the currently known MMPs and their known substrates.
(MMPs 4, 5, and 6 have been deleted due to duplication.)

<table>
<thead>
<tr>
<th>MMP</th>
<th>Matrix substrates</th>
<th>Non-matrix substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-3/stromelysin-1</td>
<td>Collagen III, IV, V, VII, IX, X, XI, elastin, laminin, fibronectin, gelatin, aggrecan, entactin, decorin, tenasin, vitronectin, perlecan</td>
<td>HB-EGF, IL-1β, plasminogen, E-cadherin, IGFBP-3, TNF-α, α1-AC, α2-MG, α1-PI</td>
</tr>
<tr>
<td>MMP-7/matrilysin</td>
<td>Collagen I, IV, aggrecan, laminin, fibronectin, gelatin, entactin, decorin, elastin, tenascin, vitronectin</td>
<td>FAS-L, β3-integrin, E-cadherin, HB-EGF, plasminogen, TNF-α</td>
</tr>
<tr>
<td>MMP-8/collagenase-2</td>
<td>Collagen I, II, III, aggrecan</td>
<td>α2-MG, α1-PI</td>
</tr>
<tr>
<td>MMP-9/gelatinase B</td>
<td>Collagen IV, V, XI, XIV, decorin, gelatin, elastin, laminin, aggrecan, vitronectin</td>
<td>TGF-β2, IL-1β, TNF-α, IL-2Ra, plasminogen, α1-AC, α2-MG, α1-PI</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>MMP-10/stromelysin-2</td>
<td>Collagen III, IV, V, aggrecan, elastin, laminin, fibronectin, gelatin</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-11/stromelysin-3</td>
<td>ND</td>
<td>IGFBP-1, α2-MG, α1-PI</td>
</tr>
<tr>
<td>MMP-12/metalloelastase</td>
<td>Collagen I, IV, aggrecan, decorin, gelatin, elastin, fibronectin, laminin, vitronectin, entactin</td>
<td>Plasminogen, α2-MG, α1-PI</td>
</tr>
<tr>
<td>MMP-13/collagenase-3</td>
<td>Collagen I, II, III, VI, IX, X, XIV, gelatin, fibronectin, aggrecan</td>
<td>α2-MG</td>
</tr>
<tr>
<td>MMP-14/MT1-MMP</td>
<td>Collagen I, II, III, gelatin, fibronectin, laminin, entactin, vitronectin, aggrecan</td>
<td>CD44, transglutaminase, α2-MG, α1-PI</td>
</tr>
<tr>
<td>MMP-15/MT2-MMP</td>
<td>Aggrecan, entactin, fibronectin, laminin, tenascin</td>
<td>Transglutaminase</td>
</tr>
<tr>
<td>MMP-16/MT3-MMP</td>
<td>Collagen III, fibronectin, gelatin</td>
<td>Transglutaminase</td>
</tr>
<tr>
<td>MMP-17/MT4-MMP</td>
<td>Gelatin</td>
<td>α2-MG, TNF-α</td>
</tr>
<tr>
<td>MMP-18/collagenase-4 (Xenopus)</td>
<td>Collagen I</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-19/RASI</td>
<td>Collagen I, IV, fibronectin, gelatin, tenascin, laminin, aggrecan, entactin, COMP</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-20/enamelysin</td>
<td>Collagen XVIII, aggrecan, amelogenin, COMP</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-21/XMMP (Xenopus)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-22/C MMP (chicken)</td>
<td>Gelatin</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-23/cysteine array MMP (CA-MMP)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-24/MT5-MMP</td>
<td>Collagen I, gelatin, fibronectin, laminin</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-25/MT6-MMP</td>
<td>Collagen IV, gelatin, fibronectin</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-26/matrilysin-2/endometase</td>
<td>Collagen IV, gelatin, fibronectin</td>
<td>α1-PI</td>
</tr>
<tr>
<td>MMP-27</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-28/epilysin</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

IGFBP = insulin-like growth factor binding protein, TGF-β = transforming growth factor-β, HB-EGF = heparin-bound epidermal growth factor, IL = interleukin, α1-AC = α1-antichymotrypsin, TNF-α = tumor necrosis factor-α, α2-MG = α2-macroglobulin, α1-PI = α1-proteinase inhibitor, COMP = cartilage oligomeric matrix protein, FGF = fibroblast growth factor, MCP-3 = monocyte chemotactic protein-3, SDF-1 = stromal derived factor-1, ND = not determined/unknown. The table was adapted from Lynch and Matrisian (2002).
The MMPs share many structural features, but also have many differences in their domain structure (Fig. 2). Common to all MMPs is the highly conserved catalytic domain, which contains the HEXXHXXGXXH sequence that binds the Zn$^{2+}$ ion in the active site. MMPs are synthesized with a C-terminal propeptide that is cleaved off during activation. The MT-MMPs, stromelysin-3, MMP-21, and MMP-22 have a furin-cleavage site in the junction between the C-terminal propeptide and the catalytic domain. These MMPs are processed by furin in the Golgi apparatus and are thus released as active proteases (Nagase, 1997; Visse and Nagase, 2003).

**Figure 2.** Domain structure of the MMPs.

The specificity of the MMPs is mainly determined by the amino acids facing the substrate binding pocket. The carboxy-terminal hemopexin domain found in most MMPs binds substrates and inhibitors, and is therefore also an important determinant of specificity (Brinckerhoff and Matrisian, 2002; Visse and Nagase, 2003). The fibronectin type II domain found in the gelatinases is believed to be important for the binding and processing of collagens (Van den Steen et al., 2002).

### 3.2 Regulation of MMPs

The MMPs constitute a potent proteolytic system with the ability to degrade most protein components of the ECM. Thus, in order to maintain a healthy tissue, the activity of MMPs must be tightly controlled. The cells use three main mechanisms to control the extent of MMP activity: gene transcription, proenzyme activation, and protease inhibition. The tissue inhibitors of MMPs (TIMPs) are the main physiological MMP inhibitors and will be discussed in more detail in section 3.3.
MMP genes are actively transcribed in response to a wide range of signals in healthy and diseased tissues. There is no clear consensus as to which factors will induce which MMP gene. The main theme is rather that the factors that induce tissue remodeling, cell migration and similar processes usually induce at least some MMPs. Which genes that are activated will be different in different processes and will reflect the tissue elements that must be degraded. TIMP genes are commonly expressed together with MMPs during tissue remodeling processes, probably to prevent excessive tissue degradation (Borden and Heller, 1997; Denhardt et al., 1993).

The critical regulatory event of MMPs is the proteolytic activation of the latent proenzyme. This activation is different between individual MMPs, but a general model, the “cysteine switch”, is used to describe it (Fig. 3). The propeptide of the latent MMP has a conserved cysteine that interacts with the Zn\(^{2+}\) of the active site. The propeptide can be cut by serine proteases such as plasmin, trypsin, and kallikreins, or by other MMPs. This causes a conformational change that dislodges the cysteine from the active site and results in a partially active MMP. This MMP can then be fully activated, either by autolytic cleavage of the remainder of the prodomain or by cleavage by other active MMPs (Nagase, 1997; Visse and Nagase, 2003).

![Figure 3](image)

Figure 3. The cysteine switch model of MMP activation. Disruption of the interaction between the propeptide cysteine and the active site Zn\(^{2+}\) leads to the formation of an active intermediate MMP form. This intermediate can be cleaved further to generate the active enzyme. Modified from Woessner (1991).

MMPs can also be artificially activated by chemicals that react with the thiol group of the cysteine. These chemicals include 4-aminophenylmercuric acetate (APMA), HgCl\(_2\), and SDS. This reaction causes the cysteine to leave the active pocket and the prodomain can then be cleaved intramoleculary (Nagase, 1997; Visse and Nagase, 2003). Interestingly, it has
recently been shown that this mode of activation can also take place in vivo. During cerebral ischemia, nitric oxide (NO) can activate MMP-9 by nitrosylating the thiol group of the cysteine switch (Gu et al., 2002).

3.3 Inhibition of MMP activity

In vivo, MMP activity is mainly inhibited by the tissue inhibitors of metalloproteinases (TIMPs) and α2-macroglobulin. The involvement of MMPs in a great number of disease processes has stimulated pharmaceutical companies to develop small, synthetic MMP inhibitors - some of which are currently undergoing clinical trials.

3.3.1 Physiological MMP inhibitors

In plasma, MMP activity is mainly inhibited by α2-macroglobulin. Many MMPs have higher affinity for α2-macroglobulin than collagen and will thus be efficiently inhibited in plasma. In the extracellular tissue environment, however, the TIMPs are the main MMP inhibitors (Gomez et al., 1997). To date, four TIMPs have been cloned: TIMP-1 to TIMP-4. TIMPs form a 1:1 noncovalent complex with the MMPs by binding like a wedge into the active site and chelating the Zn$^{2+}$. As a group, TIMPs can inhibit all MMPs. However, TIMP-3 appears to have higher specificity for the related protease families ADAMs and ADAM-TSs (a disintegrin and metalloprotease domain; ADAM with thrombospondin type-1 domains).

Besides inhibiting MMPs, TIMPs can also regulate cell growth, differentiation, and apoptosis by as yet unknown mechanisms (Gomez et al., 1997).

A membrane-bound glycoprotein, RECK (reversion-inducing cysteine-rich protein with Kazal motifs), has recently been found to be an inhibitor of some MMPs (Rhee and Coussens, 2002). Its importance was first demonstrated by Oh and coworkers (Oh et al., 2001) who found that RECK deficient mice died in utero at day 10.5. These mice displayed defects in the ECM and the vasculature.

3.3.2 Synthetic MMP inhibitors

Several small, synthetic MMP inhibitors have been produced and some of them are now being used in clinical trials against various diseases, with cancer being the most prominent. They fall into three categories: collagen peptidomimetics (usually hydroxamic acid derivatives), tetracycline derivatives, and bisphosphonates (Pavlaki and Zucker, 2003). Most MMP inhibitors used in clinical trials to date have been hydroxamic acid-based. They resemble the collagen substrate and chelate the Zn$^{2+}$ in the active site of the MMP. In paper V, we used the hydroxamic acid-based MMP inhibitor galardin (GM6001) (Fig. 4). At the concentrations used in this study, it has inhibitory activity against most MMPs (Grobelny et al., 1992).
Figure 4. Inhibitory mechanism for galardin (GM6001). The inhibitor binds to the substrate pocket and chelates the Zn$^{2+}$ in the active site (Schultz et al., 1992).

So far, clinical trials using MMP inhibitors have had poor outcome and many side effects, such as joint and muscle pain and nausea (Pavlaki and Zucker, 2003). There are several problems associated with these trials. One is the low selectivity of the inhibitors. Although MMPs are often involved in and important for tumor progression, any given MMP does not necessarily play a promotive role. As discussed in section 4.3, some MMPs may also have inhibitory functions, which would be negated by inhibitor treatment. For example, Krüger and coworkers (Krüger et al., 2001) showed that batimastat (hydroxamic acid-based) actually promoted liver metastasis in a mouse model. Inhibitors which are more selective towards those MMPs that play a promoting role in a certain tumor should be more effective and have fewer side effects (Pavlaki and Zucker, 2003). Another major problem is that the trials carried out so far have typically involved late-stage cancer patients, where the tumors have already formed metastases and the role of MMPs may have been relatively small. Experiments in mice have shown that MMP inhibitors may be more beneficial if used at earlier stages of tumor development (Bergers et al., 1999).

4 THE PA AND MMP SYSTEMS IN PHYSIOLOGICAL AND PATHOLOGICAL PROCESSES

4.1 Cooperation and redundancy between the PA and MMP systems

Together, the proteolytic activities generated by the PA and MMP systems can degrade virtually all the protein components of the ECM. There is, however, a significant overlap in the substrate specificities of plasmin and the MMPs (Alexander and Werb, 1991). Recently, it has also been shown that plasmin is not the only important fibrinolytic enzyme, but that certain MT-MMPs have the ability to degrade fibrin in the absence of plasminogen (Hiraoka et al., 1998; Hotary et al., 2002).

In many events involving ECM degradation, the PA and MMP systems are expressed in a coordinated fashion. Many signals in these processes induce parallel expression of both PAs and MMPs. Likewise, some factors downregulate both PAs and MMPs while at the same time inducing the expression of PAIs and TIMPs. These similarities suggest that not only do these
systems generate a cascade of proteolytic activity, but that they may also supplement each other; that one system can compensate for the absence of the other to ensure sufficient degradation of the ECM (Mignatti and Rifkin, 1993; Saksela and Rifkin, 1988). There is a study by Lund and coworkers which supports this hypothesis (Lund et al., 1999). Skin wound healing was investigated in plasminogen deficient mice treated with the MMP inhibitor galardin (GM6001). It was found that while the wound healing was retarded in plasminogen deficient mice or wild-type mice treated with the inhibitor, it was totally blocked in plasminogen deficient mice treated with the inhibitor. This kind of functional redundancy may explain the fact that most mice that are deficient in a single protease gene either have no aberrant phenotype, or have surprisingly mild phenotypes (Ny et al., 2002).

4.2 Tumor invasion and metastasis

There are certain steps that a tumor cell must go through in order to form a metastasis. First, it must escape from its original site. This is usually achieved by gaining access to a lymphatic vessel or blood vessel (Carmeliet and Jain, 2000; Mandriota et al., 2001; Pepper, 2001). As small tumors do not have their own vascular network, they first need to attract these vessels. When the contact with a blood or lymphatic vessel is established, the tumor cell must detach from its current location and pass through the vessel wall into the lumen. When the cell has found a suitable destination, it must be able to escape from the vessel through the basement membrane and invade the surrounding tissue. To grow at the new location, the metastasis must then attract blood vessels for oxygen and nutrition. Many studies have implicated both the PA and the MMP systems in all of these processes (Coussens and Werb, 1996; Price et al., 1997). The proposed function of these proteases is mainly to degrade basement membranes and the ECM as the tumor and endothelial cells migrate through different tissues.

Studies using protease gene deficient mice, or mice treated with synthetic protease inhibitors, have indicated that the PA and MMP systems may be used as targets for anti-tumor therapy (Carmeliet and Collen, 1998; Pavlaki and Zucker, 2003). For example, when MMP-2 deficient mice were implanted or injected with melanoma cells or lung carcinoma cells, the tumor growth and number of tumor colonies formed in the lungs were significantly reduced as compared to wild-type mice (Itoh et al., 1998).

4.3 Angiogenesis

Angiogenesis is the process of formation and growth of new blood vessels (Carmeliet, 2000). It is very intense during embryonic development, but is almost non-existent in the adult organism. In the healthy male, wound healing is the predominant angiogenesis process, while in the female there is also considerable angiogenesis in the ovaries and the uterus throughout the menstrual cycle (Findlay, 1986). On the other hand, there are several pathological states that involve angiogenesis, a prime example being tumor growth. A tumor needs to be vascularized to get nutrients to grow, and also to gain access to the blood and lymph streams - to which it sends out cells that form metastases in other parts of the body (Carmeliet and Jain, 2000; Pepper, 2001). Starving tumors by inhibiting angiogenesis appears to be an efficient strategy to combat tumor growth and metastasis (Parangi et al., 1996; Voest, 1996).

Several growth factors and other secreted molecules have been shown to stimulate angiogenesis, the most studied being the members of the vascular endothelial growth factor
(VEGF) family and basic fibroblast growth factor (bFGF). VEGF is often induced as a result of hypoxia, and its production leads to the attraction of blood vessels carrying oxygen-rich blood to the hypoxic region (Choi et al., 2003).

During angiogenesis, the endothelial cells form sprouts that degrade the surrounding basement membrane before invading the surrounding tissue. It is conceivable that proteolytic activity generated by the PA and MMP systems is important in this process. Accordingly, many studies have implicated the PA and MMP systems in angiogenesis (Coussens and Werb, 1996; Mignatti and Rifkin, 1996). However, more precise roles for individual proteases belonging to these families have been difficult to establish. Mice deficient in these proteases or their inhibitors do not usually show any major defects in angiogenesis. Also, depending on the model system, the same molecule may have either stimulatory or inhibitory roles. For example, mice deficient in PAI-1 show reduced vascularization in implanted tumors (Bajou et al., 1998). When wild-type mice are implanted with matrigel, angiogenesis into the gel can be stimulated by small doses of PAI-1 but it is inhibited by high doses (McMahon et al., 2001).

Adding to the complexity of angiogenesis regulation, it has recently been shown that proteolytic fragments of plasminogen and certain collagen molecules can act as inhibitors of angiogenesis (O’Reilly et al., 1994; O’Reilly et al., 1997). Several MMPs, including MMP-12 (Dong et al., 1997), MMP-9, and matrilysin (Patterson and Sang, 1997) can cleave plasminogen to form angiostatin, which inhibits angiogenesis in vitro by blocking endothelial cell migration, reducing endothelial cell proliferation, and inducing apoptosis in endothelial cells (Claesson-Welsh et al., 1998; Ji et al., 1998; Lucas et al., 1998). Elastases (Wen et al., 1999), cathepsin L (Felbor et al., 2000), matrilysin (Lin et al., 2001), and other proteases can cleave collagen type XVIII to release the anti-angiogenic fragment endostatin, which acts by inhibiting endothelial cell migration (Sudhakar et al., 2003). It also appears that certain proteases can degrade endostatin, thus creating yet another level of regulation (Ferreras et al., 2000). Finally, cleavage of basement membrane collagen type IV yields the tumstatin fragment which blocks protein synthesis in endothelial cells (Maeshima et al., 2002; Sudhakar et al., 2003). Interestingly, mice deficient in the integrin α1 chain have been shown to display reduced tumor vascularization (Pozzi et al., 2000). This was caused by an elevated level of MMP activity which in turn generated an increased amount of angiostatin. Effects like this indicate that targeting proteases to inhibit angiogenesis requires selective inhibitors and good timing of the treatment.

### 4.4 Embryo implantation

There are certain requirements for successful implantation to take place. Firstly, the uterus has to be prepared for embryo implantation. Progesterone secreted by the corpus luteum gives the uterus its secretory phenotype, which can nourish the implanted embryo. Secondly, the trophoblast must be able to invade the endometrial tissue. This trophoblast invasion may require proteolytic activity generated by PAs, MMPs, and other proteases (Strickland and Richards, 1992). Supporting this hypothesis, trophoblast cells have been shown to express uPA (Sappino et al., 1989; Teesalu et al., 1996) and certain cathepsins (Afonso et al., 1997; Afonso et al., 1999). The cathepsins have been shown to be critical for successful implantation (Afonso et al., 1997). However, both uPA and plasminogen deficient mice have successful implantation (Bugge et al., 1995a; Carmeliet et al., 1994; Ploplis et al., 1995).
Several MMPs are also expressed during trophoblast invasion (Alexander et al., 1996; Das et al., 1997; Salamonsen and Nie, 2002). However, overexpression of TIMP-1 or treatment with a hydroxamic acid MMP inhibitor led to a decreased decidual size, but did not block implantation (Alexander et al., 1996). Also, as discussed in section 4.5, most single MMP gene deficient mice are viable and fertile and thus have successful implantation.

A recent study has shown that there is a functional overlap between the PA and MMP systems during placental formation and embryo survival (Solberg et al., 2003). Plasminogen deficient mice or wild-type mice treated with an MMP inhibitor had only minor defects in implantation and placentation. However, plasminogen deficient mice treated with the MMP inhibitor had defective formation of decidua. Growth of maternal vessels into the decidua was also reduced. This led to significant embryonic lethality (Solberg et al., 2003).

Besides degradation of the ECM, the PA and MMP systems may play other important roles during embryo implantation. For example, both plasmin and MMPs can release bioactive molecules from the ECM or from cell membranes by limited proteolysis. Examples of this are release of insulin-like growth factor (IGF) by proteolysis of IGF-binding proteins (IGFBPs), and release of FGF or transforming growth factor \( \beta \) (TGF\( \beta \)) by proteolysis of ECM molecules that have sequestered these growth factors (Salamonsen and Nie, 2002).

### 4.5 Protease and protease inhibitor deficient mice

The last decade has witnessed an explosion in the use of gene deficient, “knockout” mice. These constitute a useful tool for revealing the functional roles of individual genes. Gene deficient mice are especially important for studies of the MMPs, where a lack of selective inhibitors has hampered the studies of the functional roles of individual MMPs. Interestingly, most mice deficient in components of the PA or MMP systems survive embryonic development, display no aberrant or only mild phenotypes, and are fertile (Carmeliet and Collen, 1998; Ny et al., 2002). This is believed to reflect the functional redundancy in these protease classes (see 4.1). Nevertheless, as shown below, protease gene and protease inhibitor gene deficient mice have revealed a plethora of functions.

**tPA deficient mice** are generally healthy and fertile, although they display a reduced capacity for clot lysis and an increased thrombotic tendency after endotoxin treatment (Carmeliet et al., 1994). This has been shown to be important during cerebral ischemia, where tPA deficient mice have a significantly increased infarct size, which correlates with fibrin depositions (Tabrizi et al., 1999). tPA appears to have a protective role in rheumatoid arthritis, as tPA deficient mice displayed a more severe phenotype in two different arthritis models (Cook et al., 2002; Yang et al., 2001). Several studies using tPA deficient mice have shown that tPA is important during several physiological and pathological neurological events. For example, tPA mediates excitotoxic damage through plasmin-mediated cleavage of laminin (Chen and Strickland, 1997; Tsirka et al., 1997b). It can also play a protective role during peripheral nerve damage by promoting fibrin dissolution (Akassoglou et al., 2000). Finally, tPA is an important mediator of long-term potentiation in the hippocampus (Huang et al., 1996).

**uPA deficient mice** are generally healthy and fertile but, like tPA deficient mice, they display an increased thrombotic tendency after endotoxin treatment (Carmeliet et al., 1994). A few uPA deficient mice also develop rectal prolapse and fibrin deposition in various tissues (Carmeliet et al., 1994). uPA deficient mice have displayed both milder and more severe
rheumatoid arthritis, depending on which arthritis model was used (Busso et al., 1998; Cook et al., 2002; Yang et al., 2001). uPA deficient mice have compromised defense against *Cryptococcus neoformans* infection, apparently due to reduced recruitment of macrophages, neutrophils, and lymphocytes to the infection site (Gyetko et al., 1996). Also, uPA generated plasmin activity has been shown to be important for smooth cell migration during neointima formation after vessel wall injury (Carmeliet et al., 1997).

**tPA/uPA double deficient mice and plasminogen deficient mice** show similar phenotypes. Their growth is retarded, they develop rectal prolapse, they have impaired thrombolytic capacity, develop fibrin depositions in several internal organs, and die prematurely (Bugge et al., 1995a; Carmeliet et al., 1994; Ploplis et al., 1995). The more severe phenotypes seen in the uPA/tPA double deficient mice as compared to the single PA deficient mice show that the PAs act in a complimentary fashion in many processes. The fertility of female plasminogen deficient mice will be discussed in section 5.6. Plasminogen deficient mice have retarded skin wound healing (Romer et al., 1996). Several of the phenotypes described for uPA and tPA single deficient mice have been replicated in plasminogen deficient mice; for example, neointima formation in these mice is impaired, as in uPA deficient mice (Carmeliet et al., 1997).

**Plasminogen/fibrinogen double deficient mice** have a phenotype that is indistinguishable from fibrinogen deficient mice (Bugge et al., 1996). Although this suggests that fibrinolysis may be the only essential function for plasmin, it was later shown that activity of plasmin on other substrates may be important. One example is plasmin-mediated degradation of laminin, which is essential for excitotoxic neurodegeneration (Chen and Strickland, 1997; Tsirka et al., 1997b; Tsirka et al., 1997a).

**uPA receptor deficient mice** do not display any obvious aberrant phenotypes (Bugge et al., 1995b; Dewerchin et al., 1996). uPAR has, however, been shown to be important for leukocyte recruitment in various models of infection and inflammation (Gyetko et al., 2000; May et al., 1998; Rijneveld et al., 2002).

**PAI-1 deficient mice** (Carmeliet et al., 1993a) and **PAI-2 deficient mice** (Dougherty et al., 1999) have no major health problems. In fact, PAI-2 deficient mice are indistinguishable from their wild-type littermates (Dougherty et al., 1999). However, PAI-1 deficient mice have an increased thrombotic activity (Carmeliet et al., 1993b). PAI-1/PAI-2 double deficient mice have not revealed any functional overlap between these two serpins (Dougherty et al., 1999).

**α₂-antiplasmin deficient mice** are healthy and fertile (Lijnen et al., 1999). They have an increased fibrinolytic capacity but do not show overt bleeding. Mice that are double deficient for both α₂-antiplasmin and PAI-1 show a similar fibrinolytic capacity (Dewerchin et al., 2001).

**Gelatinase A deficient mice** have a slightly delayed growth but are otherwise healthy and fertile (Itoh et al., 1997). Different tumor models using MMP-2 deficient mice have shown that host MMP-2 is important for tumor growth and angiogenesis (Itoh et al., 1998; Takahashi et al., 2002). Recent studies using MMP-2 deficient mice (Kato et al., 2001) and MMP-2/MMP-9 double deficient mice (Lambert et al., 2003) have confirmed its important role during angiogenesis.
Gelatinase B deficient mice have distorted apoptosis, vascularization, and ossification in the skeletal growth plates (Vu et al., 1998). However, these defects mostly compensate for each other to give the mice an almost normal phenotype. Recently, there have been a large number of studies of MMP-9 deficient mice in many different pathological conditions; for a review, see Van den Steen et al. (2002). Using MMP-9 deficient mice, it has been shown that host-produced MMP-9 is important for metastasis formation (Itoh et al., 1999) and that MMP-9 can release VEGF to trigger an "angiogenic switch" (Bergers et al., 2000). MMP-9 mediated proteolysis leads to the exposure of a cryptic type IV collagen site that is important for endothelial cell migration during angiogenesis (Hangai et al., 2002). MMP-9 can also cleave type IV collagen to release the tumstatin fragment, which suppresses angiogenesis and tumor growth (Hamano et al., 2003).

Stromelysin-1 deficient mice are healthy and fertile (Mudgett et al., 1998); however, wound contraction has been shown to be impaired in these mice (Bullard et al., 1999a). This appeared to be a result of defective fibroblast contraction (Bullard et al., 1999b).

Stromelysin-3 deficient mice are fertile and normal, but are resistant to chemical-induced tumorigenesis (Masson et al., 1998). Fibroblasts from these mice cannot promote implantation of breast cancer cells into nude mice, a paracrine action that is dependent on extracellular growth factors.

Matrilysin-1 deficient mice showed a reduced number and size of tumors in a model for intestinal tumorigenicity (Wilson et al., 1997). These mice have reduced protection against orally administered bacteria, which is a result of deficient processing of α-defensin in the small intestine (Wilson et al., 1999). Furthermore, matrilysin deficient mice have defective apoptosis during prostate involution due to deficient release of soluble Fas ligand, which has a pro-apoptotic function (Powell et al., 1999).

MMP-12 deficient mice are healthy and fertile, although the litter sizes are reduced by about 40%. MMP-12 deficient macrophages were shown to be unable to invade through basement membranes (Shipley et al., 1996). In a model of acute alveolitis, lung damage was significantly reduced in MMP-12 deficient mice, which appeared to be due in part to reduced influx of neutrophils into the alveolar space (Warner et al., 2001).

Collagenase-2 deficient mice are indistinguishable from wild-type mice. However, collagenase-2 deficient male mice are more susceptible to chemical-induced cancer (Balbin et al., 2003).

MT1-MMP deficient mice have severe defects in skeletal and other connective tissues, and die a few weeks after birth (Holmbeck et al., 1999; Zhou et al., 2000). Impaired angiogenesis in forming bones appeared to be a major contributor to the condition. Also, MT1-MMP deficient mice lack angiogenic response to FGF-2 and have impaired activation of MMP-2 (Zhou et al., 2000).

TIMP-1 deficient mice appear normal and healthy (Soloway et al., 1996). However, female TIMP-1 deficient mice display some reproductive abnormalities such as shortened estrus period (Nothnick, 2000), reduced reproductive lifespan (Nothnick, 2001), and reduced progesterone production during corpus luteum development (Nothnick, 2003).
TIMP-2 deficient mice are healthy and fertile (Wang et al., 2000). However, these mice have a dramatically reduced capacity to activate MMP-2 \textit{in vivo}. Cells from these mice failed to activate MMP-2 \textit{in vitro}.

TIMP-3 deficient mice develop alveolar enlargements in the lungs, apparently due to an abnormal MMP/TIMP ratio (Leco et al., 2001). This condition becomes more severe over time and the mice die after about one year. These mice also have earlier apoptosis during mammary gland involution, indicating an important role for TIMP-3 as an epithelial cell survival factor (Fata et al., 2001).

5 THE OVARY

The primary function of the ovary is to produce fertilizable ova. Every estrus cycle, premature follicles are recruited into growth and development, mainly in response to the pituitary hormone FSH (follicle stimulating hormone). However, most follicles do not reach an advanced stage of development but will regress instead, a process called atresia. The developed follicles respond to the pituitary hormone LH (luteinizing hormone) by further growth, which culminates in the rupture of the follicular wall and expulsion of the mature oocyte (Erickson, 1986; Hsueh et al., 1984; Richards, 1980; Richards et al., 1987). The ovulated follicle will thereafter develop into the corpus luteum (CL). The CL produces progesterone, which prepares the uterus for embryo implantation. If no successful implantation occurs, the CL stops producing progesterone (functional luteolysis) and goes into structural regression (Niswender and Nett, 1994). These processes are characterized by intense tissue remodeling and many studies have implicated important roles for the PAs and MMPs (Curry, Jr. and Osteen, 2003; Ny et al., 1993; Ny et al., 2002; Tsafriri, 1995).

5.1 Follicular development and atresia

The mammalian ovary consists of follicles at many different developmental stages (Fig. 5). Most of these follicles are primordial and consist of a small oocyte surrounded by a single layer of granulosa cells. The human ovary contains around two million follicles at birth. Only a few hundred of these will reach ovulation; most of them will instead go into the degenerative process called atresia (Erickson, 1986; Kaipia and Hsueh, 1997). Every menstrual cycle, a number of primordial follicles will be selected for growth and development. Of these, one or more will reach ovulation while the remainder will go into atresia. During follicular growth, the oocyte grows in volume and the granulosa cells surrounding it proliferate to form several layers of cells around it (Erickson, 1986). A rich vascular network develops around the growing follicle. There is a continuous growth of these vessels to maintain proper nutrient and oxygen supply to the growing follicle (Bassett, 1943; Findlay, 1986). During follicular development, the fibroblasts surrounding the primordial follicle develop into the endocrine theca cells. These cells are in close contact with the vascular network and produce androgens and collagens (Erickson, 1986). At a later stage in follicular development, an antrum cavity is formed within the granulosa cells (Fig. 5). This antrum keeps growing until ovulation takes place. At this stage, the granulosa cells surrounding the oocyte become separated from other granulosa cells and form the cumulus complex, which communicates with the oocyte through gap junctions. The cumulus cells provide the oocyte with nutrients and protect it until the time of fertilization. Shortly before
At the time of ovulation, the wall of the preovulatory follicle ruptures and the mature oocyte is released into the oviduct. However, there is a significant mechanical hindrance that must be overcome in order for a successful ovulation to occur. In the follicular wall, there are two basement membranes with the collagen-rich theca tissue in-between (Fig. 6). Also, the adhesion between cells in the follicle wall must be overcome. The exact mechanism of follicular rupture is still not clear, but a number of possibilities have been investigated.

Some rhythmical contractions in the follicles have been observed, due to what appears to be smooth muscle cells in the theca tissue (Motta et al., 1992). This may lead to an increased follicular pressure that would facilitate ovulation. It may also be important to contract the
ovulated follicle during corpus luteum formation. However, the physiological roles of these contractions remain speculative (Espey and Lipner, 1994).

The possibility that increased follicular pressure facilitates ovulation has been recognized for centuries. Although several studies have addressed this possibility, the issue has still not been fully resolved. Early studies could not detect any increased follicular pressure during ovulation (Espey and Lipner, 1963). However, recent studies using more precise methods have shown such an increase in follicular pressure during ovulation, which seems to be associated with the intraovarian NO system as well as the systemic blood pressure (Matousek et al., 2001a; Matousek et al., 2001b). Interestingly, rats treated with the NO synthase inhibitor L-NAME (Bonello et al., 1996) and mice deficient in the endothelial NO synthase, eNOS, display dramatically reduced ovulation rates (Jablonka-Shariff and Olson, 1998). The effects of NO in the ovary are many, and the relative importance of the effect on follicular pressure remains to be investigated.

![Figure 6. The follicular wall. From Espey and Lipner (1994).](image)

A third mechanism, which was postulated as early as 1916 and which has attracted the greatest attention during the last couple of decades, is the proteolytic degradation of the follicular wall (Curry, Jr. and Osteen, 2003; Ny et al., 2002; Schochet, 1916; Tsafriri and Reich, 1999). A close inspection of the components of the follicular wall shows that proteases that can degrade collagens and basement membrane components are likely to be important mediators of wall rupture (Fig. 6). The mechanism that has been proposed is that cells in the follicular wall produce proteases from the PA, MMP, and other systems, that act together to degrade the ECM of the follicular wall, thereby reducing its tensile strength. Collagen fibers
and other components of the follicular wall appear to be degraded during ovulation (Bjersing and Cajander, 1974; Parr, 1974). This should reduce the tensile strength of the follicular wall, which facilitates rupture at the protruding part of the follicle. As discussed further in section 5.6, the importance of different protease systems in ovulation remains obscure.

5.3 Corpus luteum formation

The LH surge induces ovulation and the differentiation of the ovulated follicle into the corpus luteum (CL). This process is referred to as luteinization. During luteinization, theca and granulosa cells invade the ovulated follicle and differentiate into luteal cells. Moreover, the vascular network around the follicle rapidly invades the forming CL, a process which transforms the avascular follicle into one of the most vascularized tissues in the body (Bassett, 1943).

The invasion of theca and granulosa cells into the forming CL requires degradation of the follicular basement membrane, which is believed to be achieved mainly by extracellular proteases from the PA and MMP systems (Curry, Jr. and Osteen, 2003; Ny et al., 2002). The angiogenesis during CL formation requires special mention, as it is one of the most rapid angiogenesis processes in the body (Findlay, 1986). When labeling cells with \( ^{3} \)H-thymidine, no less than about 30% of the endothelial cells in the forming CL show active DNA synthesis (Gaede et al., 1985). Several factors, such as VEGF and bFGF, have been implicated to be regulators of this angiogenesis process. To date, however, VEGF is the only factor that has been demonstrated to play an important role in vivo. In a gonadotropin-induced ovulation model in the rat, a soluble VEGF receptor was administered before gonadotropin treatment (Ferrara et al., 1998). The CL that formed were small and had necrotic cores. They also displayed a severely impaired vascularization and reduced progesterone production. Unfortunately, this experiment did not differentiate between the role of VEGF during follicular development and its role during CL formation. However, it is clear that VEGF is a crucial angiogenesis factor in the ovary.

5.4 Corpus luteum function

The main function of the CL is to secrete progesterone, which acts on the uterus to prepare it for embryo implantation. In primates, the CL also secretes estradiol, which has a negative feedback effect on the pituitary gland to inhibit FSH release (Knobil, 1980). This prevents new ovulations during pregnancy. If no successful fertilization and implantation occurs, progesterone production will cease, the CL goes into regression, and the ovary enters a new estrus cycle (Michael et al., 1994; Niswender and Nett, 1994).

The substrate for progesterone synthesis is cholesterol. The luteal cells aquire cholesterol by sequestering cholesterol-containing LDL particles via LDL receptors on their surface. The rate of progesterone synthesis in the CL is very high. Therefore, each luteal cell must be in close contact with a capillary to get a good supply of LDL particles. The cholesterol is released from the LDL particle and transported into the mitochondrion, where it is cleaved to pregnenolone by the side-chain cleavage enzyme (Niswender, 2002). The transport of cholesterol into the mitochondrion appears to be the rate-limiting step in progesterone synthesis. The steroid acute regulatory protein (StAR) is a protein located in the outer mitochondrial membrane which facilitates the transport of cholesterol across the membrane (Stocco, 2001). StAR is regulated both transcriptionally and post-translationally. It appears
that degradation of the StAR protein is the main mechanism for fast regulation of its activity (Granot et al., 2002). In the rat, StAR mRNA is highly expressed in the functional CL, when progesterone production is high (Chen et al., 1999). In Paper IV, we investigated the expression of StAR mRNA in the psp mouse CL and found a similar pattern.

5.5 Corpus luteum regression (luteolysis)

The CL is a transient endocrine organ. If fertilization or embryo implantation is unsuccessful, the CL will lose its ability to produce progesterone (functional luteolysis) and will go into structural regression, during which the luteal tissue is degraded (Michael et al., 1994; Niswender and Nett, 1994).

There are several morphological features associated with luteolysis. The luteal cells accumulate lipid droplets and become reduced in size. At a later stage, these cells undergo apoptosis and the vascular network degenerates. An influx of macrophages into the regressing CL has been observed (Gaytan et al., 1997; Paavola, 1979). These macrophages may participate in the destruction of luteal tissue and phagocytosis of dead luteal cells. As discussed further in the next section, extracellular proteases such as the PAs and MMPs, are believed to play important roles in the degradation of the nonfunctional luteal tissue.

In many species, the uterus plays a central role in the regulation of CL regression. This has been demonstrated repeatedly by performing hysterectomy on psp animals, which significantly prolongs the luteal life span (Niswender and Nett, 1994). The prostaglandin PGF$_{2\alpha}$ is the main regulatory factor in several species. It is produced in the uterus and transported by the blood to the ovary (Pharriss and Wyngarden, 1969). The luteal cells have a cell-surface receptor to PGF$_{2\alpha}$ (Olofsson and Leung, 1996), which has several downstream effects that lead to luteolysis: it inhibits progesterone synthesis; it induces the expression of 20α-hydroxysteroid dehydrogenase, which catabolises progesterone; it decreases the luteal blood flow; it decreases the number of LH receptors; it uncouples the LH receptors from adenylate cyclase; and it induces apoptosis (Niswender and Nett, 1994). Reactive oxygen species that are generated during CL regression appear to be important mediators of some of these effects (Behrman et al., 2001).

5.6 The PA and MMP systems in the ovary

In the ovary, extensive tissue remodeling takes place during follicular growth and atresia, ovulation, corpus luteum formation and regression. A large number of studies conducted in many species have implicated important roles for both the PAs and the MMPs in these processes. These are reviewed in (Curry, Jr. and Osteen, 2003; Espey and Lipner, 1994; Hulboy et al., 1997; Ny et al., 1993; Ny et al., 2002; Tsafiriri and Reich, 1999).

During follicular growth, the PA system appears to play a minor role, as the expression of its components remains rather low until shortly before ovulation. In the rat, some tPA expression can be detected in the theca tissue of growing follicles (Peng et al., 1993; Sappino et al., 1989). In the PMSG-primed mouse, uPA is expressed in granulosa cells in large follicles (Hagglund et al., 1996). Several MMPs and their inhibitors are expressed during follicular growth (Curry, Jr. and Osteen, 2003; Ny et al., 2002). The gelatinases (MMP-2 and MMP-9) are upregulated in larger follicles in the rat (Bagavandoss, 1998). Hagglund et al. (1999)
showed that several MMPs, including MT1-MMP and its substrate MMP-2, are expressed during follicular development in the mouse. The TIMPs are also expressed during follicular development and show individual regulation patterns, which may reflect different roles. TIMP-1 is upregulated during follicular growth, whereas TIMP-2 expression is unchanged, and TIMP-3 is downregulated as follicular development proceeds (Chun et al., 1992; Kennedy III et al., 1996). So far, no clear functional role has been determined for any of these proteases during follicular development (Ny et al., 2002).

The possible role of proteases during rupture of the follicular wall was recognized as early as 1916 (Schochet, 1916). Accordingly, it was later shown that the granulosa cell basement membrane is degraded and that the collagenous matrix in the apex of the follicle is fragmented (Bjersing and Cajander, 1974). Also, a preovulatory increased release of hydroxyproline fragments, a product of collagenolysis, implied increased collagenolysis during ovulation (Morales et al., 1983). Many studies have implicated that the PA system has important functional roles during ovulation (Ny et al., 1993; Tsafriri and Reich, 1999). For example, intrabursal injections of α2-AP or anti-tPA antibodies reduce the gonadotropin-induced ovulation rate in rats (Reich et al., 1985; Tsafriri et al., 1989). Furthermore, treatment of in vitro perfused rabbit ovaries with streptokinase, a bacterial plasminogen activator, induces ovulation in the absence of gonadotropins (Yoshimura et al., 1987). This suggests that PA activity is sufficient to generate proteolytic activity that degrades the follicular wall. However, studies with single or double PA gene deficient mice and with plasminogen deficient mice did not show any major defects in fertility or ovulation efficiency (Bugge et al., 1995a; Carmeliet et al., 1994; Leonardsson et al., 1995; Ny et al., 1999; Ploplis et al., 1995). Only a small effect on ovulation was seen in the plasminogen deficient mice, which appeared to be secondary to a lower body weight in these mice (Ny et al., 1999). Like the PA system, many studies have suggested that the MMP system has important roles during ovulation (Curry, Jr. and Osteen, 2003; Hulboy et al., 1997; Ny et al., 2002). In an early study by Espey and Lipner (1965), it was shown that injections of bacterial collagenase into rabbit follicles was sufficient to induce ovulation only a few minutes later. When treating in vitro perfused ovaries with various metalloprotease inhibitors, ovulation was partly or completely blocked, suggesting an essential role for MMPs in ovulation (Brannstrom et al., 1988; Butler et al., 1991; Ichikawa et al., 1983). However, like the PA deficient mice, most of the MMP deficient mouse strains studied to date have displayed no or mild defects in fertility, except the MT1-MMP deficient mice which had such severely compromised health that they could not breed (Holmbeck et al., 1999; Zhou et al., 2000). In our laboratory, we have recently found that the ovulation rate is only reduced by 28% in plasminogen deficient mice treated with the MMP inhibitor galardin (GM6001) (Leonardsson et al., 2004, manuscript in preparation). In vitro experiments performed in that study confirmed that galardin is distributed to the ovary in sufficient amounts to fully inhibit gelatinase activity. This indicates that the functional importance of these proteases in ovulation may be less than previously appreciated. It is possible that proteases from other families can compensate for the lack of PA and MMP activity, to ensure proper reproductive function.

During CL formation, there is a massive invasion of blood vessels and luteinizing granulosa and theca cells into the ruptured follicle. Extracellular proteases from the PA and MMP systems are likely to have important roles, as this dramatic tissue remodeling involves intense angiogenesis, invasion through basement membranes and cell migration (Curry, Jr. and Osteen, 2003; Findlay, 1986; Niswender and Nett, 1994; Ny et al., 2002). Likewise, during CL regression, PAs and MMPs could be important for the degradation of nonfunctional luteal tissue and may be used by macrophages that invade the regressing CL to remove dead luteal...
cells by phagocytosis (Michael et al., 1994; Paavola, 1979). On the contrary, the PA and MMP systems are not believed to be essential during the functional phase as the luteal tissue is stable during this period. Several studies in various species have confirmed that PAs and MMPs are mainly expressed during the formation and regression of the CL, and not during the functional phase (Curry, Jr. and Osteen, 2003; Ny et al., 2002). For example, in the forming rat CL, uPA is expressed in a pattern that resembles invading blood vessels (Bacharach et al., 1992) and tPA is highly and evenly expressed (Liu et al., 1996). Collagenase, gelatinases, and TIMPs are also expressed at this time point (Bagavandoss, 1998; Nothnick et al., 1995; Nothnick et al., 1996). However, there has been a general lack of studies regarding the functional role of the PA and MMP systems in the CL, but as before it should be pointed out that most of the protease deficient mice studied to date have had no obvious defects in female fertility and should thus have a relatively normal CL function (Ny et al., 2002). An interesting exception is the MMP-12 (macrophage metalloelastase) deficient mice. These mice have litters that are only 60% of the normal size (Shipley et al., 1996). As MMP-12 is highly expressed in the placenta (Belaouaj et al., 1995), abnormal placental function may explain this effect. Another possibility could be that the macrophages that invade the regressing CL have an impaired ability to clear nonfunctional tissue in these mice. This would then cause an accumulation of dead luteal tissue in the ovaries, which may interfere with future estrus cycles.

5.7 Animal models for the study of ovary function

In our laboratory, we are using different animal models to study follicular development, ovulation, and corpus luteum formation, function, and regression. Each model has its own advantages and disadvantages.

For studies of follicular development and ovulation, we mainly use a gonadotropin-induced ovulation model in mice. In this model, the gonadotropins PMSG (pregnant mare serum gonadotropin; mainly FSH activity) and hCG (human chorionic gonadotropin; mainly LH activity) are administered intraperitoneally (i.p.) to immature females. The PMSG treatment stimulates follicular growth and development and the hCG treatment induces ovulation, which takes place about 10-12 hours after hCG treatment (Wilson and Zarrow, 1962; Zarrow and Wilson, 1961). With this model, the animals have not yet entered their own estrus cycle, which simplifies analysis of the tissue. Also, timing of the ovarian events are well known in this model, which allows tissue sampling and pharmacological treatments at defined time points during follicular development and ovulation.

For studies of the CL, we use female adult psp mice or rats (Finn, 1965; Selstam et al., 1985). Pseudopregnancy is induced by housing an adult female with a vasectomized adult male. The appearance of a vaginal sperm plug in the morning indicates mating during the night and is defined as day 1 of CL development. The CL formed in this way are functional for about 8-10 days. This model has the advantage of being fully physiological and requires no exogenous stimuli. However, the timing of mating is difficult to predict, which makes pharmacological treatment - in our case with protease inhibitors - difficult to perform with this model. Another drawback is that these animals have entered estrus cycles. Their ovaries may therefore contain luteal tissue from previous cycles, which may complicate the analysis of the tissue. In Paper IV, we developed a model for PMSG/hCG-induced CL formation in immature mice. This model was then used in Paper V to study the formation and function of the CL in plasminogen deficient mice treated with an MMP inhibitor.
6 SUMMARY OF THE PRESENT STUDY

This section is a summary of the five papers (numbered I-V) that constitute this thesis. The figures and tables are referred to by their numbers as given in the original paper, followed by the corresponding Roman numerals.

6.1 Coordinated and cell-specific regulation of membrane-type matrix metalloproteinase 1 (MT1-MMP) and its substrate matrix metalloproteinase 2 (MMP-2) by physiological signals during follicular development and ovulation (Paper I)

Extensive tissue remodeling takes place during both follicular development and the rupture of the follicular wall during ovulation. Proteases belonging to the PA and MMP systems are believed to play crucial roles during these processes. In this study, in situ hybridization was used to follow the expression and localization of MT1-MMP and MMP-2 mRNAs during gonadotropin-induced ovulation in the rat. MT1-MMP is a membrane-bound MMP that is not only capable of degrading components of the ECM directly, but it is also an important activator of MMP-2.

In ovaries from untreated immature (23-day-old) rats, the expression of both MT1-MMP and MMP-2 mRNAs was low (Figs. 1B and C, Paper I). MT1-MMP was expressed in granulosa cells and theca-interstitial cells while MMP-2 was mainly expressed in theca-interstitial cells. Stimulation with PMSG induced a higher expression of both MT1-MMP and MMP-2 (Figs. 1E and F, Paper I). After an ovulatory dose of hCG, the expression of MT1-MMP was dramatically downregulated in the granulosa cells of large, preovulatory follicles while both MT1-MMP and MMP-2 appeared to be further upregulated in the theca-interstitial cells (Figs. 1H-L, Paper I). These data suggest a dual role for MT1-MMP in ovulation. Initially, it may directly degrade ECM components inside the developing follicle. Later, at the time of ovulation, it may activate MMP-2. The localization of these transcripts in the preovulatory follicles suggests that these proteases have an important role in degradation of the follicular wall.

6.2 Distinct expression of gelatinase A (MMP-2), collagenase-3 (MMP-13), membrane-type MMP 1 (MT1-MMP), and tissue inhibitor of MMPs type 1 (TIMP-1) mediated by physiological signals during formation and regression of the rat corpus luteum (Paper II)

The CL is formed from an ovulated follicle. During this process, capillaries from the surrounding theca tissue invade the forming CL in a rapid angiogenesis process. This transforms the follicle into one of the most vascularized tissues in the body. CL formation is also characterized by intense tissue remodeling as cells from the theca and granulosa layers invade the forming CL and differentiate to become luteal cells. Once formed, the CL produces progesterone, which prepares the uterus for embryo implantation. However, if no implantation takes place, the CL first loses its ability to produce progesterone (functional luteolysis) and will later go into structural regression, where the nonfunctional CL tissue is rapidly degraded. Both the formation and regression of the CL are believed to be mediated by matrix degrading proteases from the PA and MMP systems.

To increase our understanding of the roles of MMPs in the CL, the expression of MT1-MMP, MMP-2, MMP-13, and TIMP-1 mRNAs in the adult psp rat was studied using in situ hybridization and Northern blot. MMP-2 was expressed at a high level during CL formation and at a low level in the other stages of the CL life cycle (Figs. 1B and 4D-F, Paper II). MT1-
MMP was expressed throughout the entire CL life cycle (Figs. 1D and 4J-L, Paper II). MMP-13, which like MMP-2 can be activated by MT1-MMP, was expressed exclusively during CL regression (Figs. 1C and 4G-I, Paper II). TIMP-1 was expressed at high levels during formation and regression of the CL (Figs. 1E and 4M-O, Paper II). These expression patterns suggest that MT1-MMP may activate MMP-2 during CL formation and MMP-13 during CL regression. It may also play a role in the maintenance and function of the CL. TIMP-1 may have an important role during the formation and regression of the CL, by limiting the extent of MMP activity in order to prevent unrestrained tissue degradation. To further study the regulation of MMP-13 and TIMP-1 during CL regression, we both prolonged CL lifespan by performing hysterectomies and shortened it by treating psp rats with the PGF\(_2\alpha\) analogue cloprostenol, which induces premature regression. Hysterectomy prolonged CL life from 13 days to 19 days (Fig. 2, Paper II). After hysterectomy, the expression of both MMP-13 and TIMP-1 mRNAs was delayed until D19, when the serum progesterone levels had decreased to baseline levels (Fig. 2, Paper II). The cloprostenol treatment resulted in a sharp drop in serum progesterone levels within a few hours (Fig. 3A, Paper II). After cloprostenol treatment, MMP-13 was transiently induced after 3 hours but was expressed below detection levels from 6 hours to 48 hours after cloprostenol treatment (Fig. 3B, Paper II). TIMP-1 was induced 1 hour after the cloprostenol treatment and was then highly expressed from 6 hours to 48 hours (Fig. 3C, Paper II). Together, the data show that the expression of TIMP-1 and MMP-13 is closely related to CL regression. The finding that these mRNAs are not expressed until the progesterone levels drop suggests that progesterone may be an important regulator of these genes.

6.3 Expression pattern and functional studies of matrix degrading proteases and their inhibitors in the mouse corpus luteum (Paper III)

In this study, *in situ* hybridization was used to follow the expression and distribution of tPA, uPA, PAI-1, protease nexin-1 (PN-1), seven MMPs, TIMP-1, TIMP-2, and TIMP-3 mRNAs during the CL life cycle in the adult psp mouse. The expression of MMP-9 (gelatinase B), MMP-13, and MMP-19 was below the detection level. During CL formation, expression of both PAs, MT1-MMP, MMP-2, stromelysin-3 (MMP-11), and all inhibitors was detected (Table 1; Figs. 2A-I, Paper III). This suggests a role for these proteases during tissue remodeling and angiogenesis associated with CL formation. It also shows how the proteolytic activities may be balanced by the simultaneous expression of several protease inhibitors in order to prevent excessive tissue degradation. In the functional CL, only MT1-MMP, uPA, and TIMP-3 mRNAs were detected (Table 1; Figs. 2J-L, Paper III). As tissue remodeling in the functional CL is modest, these molecules may have other functions that support the maintenance of CL function. During CL regression, a battery of proteases and inhibitors was expressed, including both PAs, PN-1, MT1-MMP, MMP-2, stromelysin-3, MMP-12 (macrophage metalloelastase), and all TIMPs (Table 1; Figs. 2M-U, Paper III), suggesting that these proteases and inhibitors act together to create a balanced and coordinated proteolytic activity that degrades the regressing CL. MMP-12 was expressed exclusively in the regressing CL (Fig. 2P, Paper III), which suggests that it may take part in the degradation of nonfunctional luteal tissue. It may also be a good molecular marker for CL regression in the mouse. The expression of uPA in the functional CL suggested that it may have an important role in CL maintenance (Figs. 2B, J, M, Paper III). We therefore examined the luteal conditions in uPA deficient mice. Serum progesterone levels, ovarian weights, and the luteal vascularity were recorded. There was no difference in ovarian weights (Fig. 3B, Paper III). The serum progesterone levels were generally normal but were slightly reduced at D10 in
the uPA deficient mice (Fig. 3A, Paper III), suggesting a somewhat shorter CL lifespan in these mice. Also, CL from uPA deficient mice were fully vascularized (Fig. 4, Paper III). This shows that uPA is either not crucial for normal CL development or that in its absence, its function can be efficiently compensated for by other proteases.

6.4 A synchronized gonadotropin-induced corpus luteum model in the mouse (Paper IV)

In this study, a gonadotropin-induced model for CL development in immature mice was developed. While the adult psp mouse model is a useful physiological model, it does have certain limitations. The main drawback is that it does not allow control of the time of ovulation. The lack of clearly defined mouse models for gonadotropin-induced CL formation encouraged us to evaluate five different conditions, one of which provided CL that were viable until day 6, when the study was terminated. In that protocol, 23-day-old mice were treated with 1 IU PMSG followed by 5 IU hCG 46 h later, which was denoted day 0. Starting at day 2, the mice were subjected to two daily treatments with 50 µg ovine prolactin. As tools for following CL formation and regression, a battery of markers was used, including expression of CD31 protein, StAR, LH receptor, MMP-12 mRNAs, and TUNEL staining (Fig. 3; Table 1, Paper IV). Since StAR mRNA expression in the mouse CL had not been characterized before, it was investigated in the psp mouse CL (Figs. 1G-I, Paper IV). It was highly expressed in the forming and functional CL in a pattern resembling that of LH receptor mRNA. In the regressing CL, the transcript lingered until a late stage, after LH receptor had been downregulated. The expression of StAR mRNA may thus be a useful molecular marker for following the development and regression of the CL. It could also be used in parallel with expression of LH receptor mRNA to differentiate between early and late stages of CL regression.

6.5 Plasminogen is required for normal progesterone production in the mouse (Paper V)

Several studies have indicated important roles for plasminogen and the MMPs during CL formation. In this study, the formation and function of the CL were studied in plasminogen deficient mice treated with the MMP inhibitor galardin (GM6001). Both adult psp mice and immature gonadotropin-primed mice were used. To follow the development and evaluate the viability of the CL, we measured the serum progesterone levels, counted the CL, weighed the ovaries and stained tissue sections for the molecular markers characterized in Paper IV. In plasminogen deficient mice, a normal number of CL formed (Tables 1-3, Paper V). These CL were fully vascularized and appeared healthy (Fig. 2, Paper V). However, the serum progesterone levels were significantly reduced in the plasminogen deficient mice (Tables 1 and 2 and Fig. 1, Paper V). In contrast to what has previously been observed in other tissue remodeling events, treatment of plasminogen deficient mice with galardin had no significant additional effect on any of the parameters measured in these mice (Tables 1-3, Paper V). Surprisingly, vascularized and healthy CL formed in plasminogen deficient mice treated with galardin (Fig. 2, Paper V). Together, these data suggest that neither plasminogen nor MMPs, alone or in combination, are essential for the formation of a viable CL. The reduced serum progesterone levels observed in the plasminogen deficient mice appeared not to be a result of defective CL formation, as the expression pattern of several molecular markers was normal in CL from these mice. It appears that plasmin may instead have a novel role in the maintenance
of the luteal function, possibly through proteolytic activation of growth factors and other paracrine factors.
**CONCLUSIONS**

* MT1-MMP and MMP-2 are coordinately regulated during ovulation in the rat. This suggests that MT1-MMP activates MMP-2 to facilitate the rupture of the follicular wall at the time of ovulation. MT1-MMP may also have a separate role inside the developing follicle.

* The regulation of MT1-MMP, MMP-2, MMP-13, and TIMP-1 in the rat CL suggests that MT1-MMP may activate MMP-2 and MMP-13 during CL formation and regression, respectively. These MMPs could then generate proteolytic activity, balanced by TIMP-1, which mediates tissue remodeling, angiogenesis, and tissue destruction during these processes.

* The expression pattern of PAs, MMPs, and their physiological inhibitors in the mouse CL suggest that these protease classes play important roles throughout the entire lifespan of the CL, particularly during its formation and regression. However, viable and functional CL do form in uPA deficient mice, plasminogen deficient mice, and in plasminogen deficient mice treated with the MMP inhibitor galardin (GM6001). The importance of these proteases may thus be less than what was previously thought.

* Although it is not required for the development of a healthy CL, plasmin appears to have an important role for normal progesterone production.

* The expression pattern of various molecular markers for CL development and regression has been characterized and used as a set of tools for following the development of the CL in these studies.
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Reference List


47


