The Roles of the Plasminogen Activator and Matrix Metalloproteinase Systems in Ovulation and Corpus Luteum Formation

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Till lillebror Pontus,
du var för bra för den här världen...
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<tbody>
<tr>
<td>α2-AP</td>
<td>α2-antiplasmin</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>CL</td>
<td>corpus luteum</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cells</td>
</tr>
<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>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenously</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin growth factor</td>
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<tr>
<td>IU</td>
<td>international units</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</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>PA</td>
<td>plasminogen activator</td>
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<tr>
<td>PAI</td>
<td>PA inhibitor</td>
</tr>
<tr>
<td>plg&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>plasminogen-deficient homozygous mice</td>
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<tr>
<td>plg&lt;sup&gt;/−&lt;/sup&gt; mice</td>
<td>plasminogen-deficient heterozygous mice</td>
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<tr>
<td>plg&lt;sup&gt;+/+&lt;/sup&gt; mice</td>
<td>wild-type mice concerning plasminogen deficiency</td>
</tr>
<tr>
<td>PMSG</td>
<td>pregnant mare serum gonadotropin</td>
</tr>
<tr>
<td>PN-1</td>
<td>protease nexin-1</td>
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<tr>
<td>proMMPs</td>
<td>pro-matrix metalloproteinases</td>
</tr>
<tr>
<td>psp</td>
<td>pseudopregnant</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneously</td>
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<tr>
<td>sc-tPA</td>
<td>single-chain tPA</td>
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<tr>
<td>sc-uPA</td>
<td>single-chain uPA</td>
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<tr>
<td>SERPIN</td>
<td>serine protease inhibitor</td>
</tr>
<tr>
<td>StAR</td>
<td>steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>tc-tPA</td>
<td>two-chain tPA</td>
</tr>
<tr>
<td>tc-uPA</td>
<td>two-chain uPA</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitors of metalloproteinase</td>
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<tr>
<td>TM</td>
<td>tympanic membrane</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue-type PA</td>
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<tr>
<td>uPA</td>
<td>urokinase-type PA</td>
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<tr>
<td>uPAR</td>
<td>uPA receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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ABSTRACT

The Roles of the Plasminogen Activator and Matrix Metalloproteinase Systems in Ovulation and Corpus Luteum Formation

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Proteases of the plasminogen activator (PA) and the matrix metalloproteinase (MMP) enzyme systems are expressed in the ovulatory follicle and in the developing corpus luteum (CL). However, the functional role of these extracellular degrading protease systems in the ovulatory and CL development processes remains elusive. The first aim of this thesis was to develop a mouse model to study gonadotropin-induced CL formation. The second aim was to study the involvement of the PA and the MMP systems in gonadotropin-induced ovulation, and in CL formation and function.

A mouse model for gonadotropin-induced CL formation was developed in order to control the timing of CL formation. In this model, immature mice were induced to ovulate by administering gonadotropins and the endogenous prolactin surges were mimicked by administration of prolactin twice daily from day 2 of CL development. We observed that steroidogenic acute regulatory protein (StAR) mRNA was highly expressed at days 3 and day 6 of CL development and the levels remained high until late stages of CL regression.

Since mice lacking plasminogen (plg−/−) only have a 14% reduction of ovulation efficiency, our hypothesis was that the MMP system could compensate for the loss of plasminogen. When administrating the MMP-inhibitor galardin to gonadotropin-primed ovulating mice, we found that wild-type mice (plg+/+ and C67BL/J6) and heterozygous mice (plg+/−) had an 18-20% reduction in ovulation efficiency as compared to untreated mice.

Two models for CL formation, the adult pseudopregnant (psp) mouse model and a model whereby immature gonadotropin-primed mice were treated with prolactin, were used to study the formation and function of the CL in plg−/− mice treated with galardin. At day 3 of CL development, we found no alterations other than a slightly lower number of CL in plg−/− mice. This is most likely a secondary effect of the lower ovulation efficiency found in these mice. On the other hand, we found a 54% reduction in serum progesterone levels in plg−/− mice and a 37% reduction in the plg+/− mice as compared to wild type mice. At day 6 of CL development we saw a 45% reduction of serum progesterone level in the plg−/− mice and a 22% reduction in the plg+/− mice. A similar trend was observed at day 3 of CL development in immature gonadotropin-primed mice treated with prolactin. Galardin treatment did not alter the results significantly and the CLs were healthy and viable in these mice.

In conclusion, our data suggest that both plasminogen and MMPs, alone or in combination, are dispensable for ovulation and for the formation of a viable CL under the conditions used in this study. The reduced serum progesterone levels observed in the plg−/− mice did not appear to be a result of defective CL formation. Instead, plasmin may have a novel role in the maintenance of luteal function. StAR expression may also be a good marker for CL development and regression in mice.

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

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INTRODUCTION

During the mammalian female estrus cycle, the ovary produces fertilizable ova. Follicles in the ovary develop and at a certain point the follicle ruptures and a mature ovum is released. If the ovum becomes fertilized, the ovulated follicle develops into a corpus luteum (CL), which produces progesterone to prepare the uterus for embryo implantation. If implantation does not occur, the CL stops producing progesterone and goes into structural regression whereby the nonfunctional CL tissue is rapidly degraded (Findlay, 1986; Michael et al., 1994; Tsafriri and Reich, 1999).

Both follicular development and ovulation require extensive tissue remodeling (Tsafriri and Reich, 1999). Tissue remodeling is also important for CL development, which in addition involves cell migration and rapid angiogenesis. The degradation of the CL also requires tissue remodeling. A large number of studies have implicated the plasminogen activator (PA) and matrix metalloproteinase (MMP) systems as being important factors in all of these ovarian tissue remodeling processes (Tsafriri and Reich, 1999; Ny et al., 2002; Curry and Osteen, 2003).

The main component of the PA system is the proenzyme plasminogen, which can be activated by tissue-type PA (tPA) or urokinase-type PA (uPA). When it becomes activated, plasminogen turns into the protease plasmin. Plasmin degrades fibrin and several components of the extracellular matrix (ECM). Plasmin also activates some members of the MMP system. To prevent excessive tissue degradation, the activity of the PA system is regulated by PA inhibitor-1 (PAI-1), PA inhibitor-2 (PAI-2), α2-antiplasmin (α2-AP), and protease nexin-1 (PN-1) (Saksela and Rifkin, 1988; Vassalli et al., 1991).

The MMPs constitute a large protease family with diverse substrate specificities. Together, they have enzymatic activities against virtually all components of the ECM. The MMPs are not limited to ECM degradation, however, but may also be important in regulation of growth factors and other non-ECM molecules. All MMPs share a similar domain, with Zn2+ in the catalytic site. MMP activity can be inhibited by the physiological tissue inhibitors of metalloproteases (TIMPs) or by synthetic agents that chelate the Zn2+ at the active site (Visse and Nagase, 2003; McCawely and Matrisian, 2001; Vu and Werb, 2000).

The first aim of this thesis was to develop a mouse model to study gonadotropin-induced CL formation. The second aim was to study the involvement of the PA and the MMP systems in gonadotropin-induced ovulation, and in CL formation and function. The functional relevance of these protease systems for ovulation and CL formation and function was studied by using mice deficient in plasminogen and by treating mice with the broad-spectrum synthetic MMP inhibitor galardin (GM6001).

1. THE PLASMINOGEN ACTIVATOR (PA) SYSTEM

The PA system is a versatile, temporally controlled enzymatic system. The key component of the PA system is plasminogen, a proenzyme that can be activated to the broad-spectrum serine protease plasmin by either of two PAs, tPA or uPA. Activation of the PA system is initiated by
the release of PAs by specific cells in response to external signals and results in local extracellular proteolytic activity (Saksela and Rifkin, 1988; Vassalli et al., 1991). The PA system is also regulated by specific inhibitors such as PAI-1, PAI-2, α₂-AP, and PN-1 that are directed against PAs and plasmin (Bachmann, 1987; Saksela and Rifkin, 1988; Vassalli et al., 1991 and Ny et al., 1993).

**Figure 1.** Schematic representation of the PA system and its regulation. The synthesis of tPA and uPA by specific cells is regulated by hormones, growth factors, and cytokines. In the extracellular space, the activities of PA and plasmin are controlled by the specific inhibitors PAI-1, PAI-2 and α₂-AP. Binding of PAs and plasmin to cellular binding sites (R) can result in localized proteolytic activity at the cell surface.

### 1.1. The role of Plasminogen and Plasmin

Plasminogen is mainly produced in the liver and it is present in most body fluids at a concentration of about 200 µg/ml (Castellino, 1984). Plasminogen exists in two forms. The so-called Glu-plasminogen is a 750 aa-long single chain protein with a molecular weight of 92 kDa (Robbins et al., 1967; Wallen, 1980; Saksela and Rifkin, 1988), and it has an amino-terminal glutamic acid residue (Wallen and Wiman, 1970; Wallen and Wiman, 1972; Wiman and Wallen, 1975). If Glu-plasminogen is cleaved at Lys76 – Lys77, the second form, called Lys-plasminogen, is formed. Lys-plasminogen has a higher affinity for fibrin than Glu-plasminogen, but Glu-plasminogen has a longer half-life (2.2 days) than Lys-plasminogen (0.8 days) (Wallen, 1980; Bachmann, 1987).

If any of the plasminogen proteins are cleaved by tPA or uPA at the single peptide bond between Arg560 and Val561, the inactive plasminogen is converted into the active enzyme plasmin (Robbins et al., 1967; Sottrup-Jensen et al., 1975). Plasmin is a disulphide-linked two-chain molecule. The larger of the two chains, the A-chain, has a molecular weight of 38 kDa and consists of five
domains called “kringles”. The different kringle domains contain lysine binding sites that can bind fibrin, $\alpha_2$-AP and other proteins (Wiman, 1980). Kringle 1 has a lysine binding site with a 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 smaller B-chain of plasmin has a molecular weight of 30 kDa and contains the catalytic center composed of the typical serine protease triad (His, Asp and Ser) (Wallen, 1980; Castellino, 1984; Bachmann, 1987).

Plasmin is known to play an important role in different tissue remodeling processes such as angiogenesis and ECM remodeling (Saksela and Rifkin, 1988; Vassalli et al., 1991; Mignatti and Rifkin, 1993). It also degrades fibrin and thereby prevents pathological blood clot formation (Collen and Lijnen, 1991; Lijnen, 1996). Furthermore, plasmin activates pro-matrix metalloproteinases (proMMPs) and latent forms of certain growth factors, suggesting that it may have other roles in addition to being involved in the degradation events (Rifkin et al., 1990; Woessner, 1991; Matrisian, 1992; Imai et al., 1995).

1.2. Plasminogen activators

tPA and uPA are two serine proteases which convert plasminogen to plasmin by single proteolytic cleavage.

tPA is synthesized as a 530 aa-long single-chain glycoprotein (sc-tPA) with a molecular weight of 68 kDa. It is mainly produced by vascular endothelial cells and its concentration in the blood is about 5 ng/ml (Pennica et al., 1983). Different proteases such as plasmin and plasma kallikrein cleave tPA at Arg257 – Ile276 to a disulfide-linked two-chain (tc-) tPA (Wallen et al., 1983). This increases the catalytic effect of tPA about 10 fold. tc-tPA has a structure similar to plasmin, with a heavy A-chain and a lighter B-chain (Ny et al., 1984). The A-chain contains a fibrin-binding finger domain and two kringle domains with lysine binding sites. Binding of tPA to fibrin activates the enzyme 300-400 fold (Hoylaerts et al., 1982; Ranby et al., 1982). The B-chain contains the serine protease domain (Ny et al., 1984). Both forms of tPA are active and able to convert plasminogen into plasmin (Rijken et al., 1982).

uPA is a 411 aa-long single-chain protease with a molecular weight of 54 kDa (Bachmann, 1987). uPA is not active in its single-chain form, but becomes activated when cleaved between Lys128 and Ile159 by plasmin, kallikrein, factor XIIa or cathepsin B (Bachmann, 1987; Vassalli et al., 1991; List et al., 2000). Active uPA is a two-chain disulfide-linked protease. The heavy chain contains an epidermal growth factor (EGF)-like domain and a kringle domain. The light chain contains the serine protease active site (Ichinose et al., 1986).

Both tPA and uPA are able to activate plasminogen to plasmin, but they differ in tissue/organ localization. uPA is found in urine, plasma, extracellular matrix, and also on the surface of many tumor cells (Williams, 1951; Dano et al., 1985; Andreasen et al., 1997), while tPA is also found in the ovary, brain, heart, kidney, and uterus (Bykowska et al., 1981; Rijken and Collen, 1981; Ny et al., 1985; Canipari et al., 1987; Saksela and Rifkin, 1988). The intravenous localization, and the high-affinity binding of tPA to fibrin, makes tPA the major vascular PA when plasmin is needed for dissolving blood clots (Bachmann, 1987; Collen and Lijnen, 1991). In the brain, tPA is involved in excitotoxic damage and long-term potentiation in the hippocampus (Huang et al., 1996; Wu et al., 2000). uPA has a specific cell-surface receptor (uPAR) that binds uPA and
directs its activity to the cell surface (Stoppelli et al., 1985; Vassalli et al., 1985; Blasi et al., 1986; Nielsen et al., 1988). uPAR has been found on the surface of many migrating cells such as spermatozoa, trophoblasts and several neoplastic cell types. Thus, uPA is believed to be the major PA during tissue remodeling and ECM degrading processes, such as embryo implantation and angiogenesis (Huarte et al., 1987; Nielsen et al., 1988; Zini et al., 1992; Andreasen et al., 1997).

1.3. The inhibitors of the PA system

Inhibitors of PAs and plasmin have an important function in controlling the proteolytic function of the PA system. These inhibitors are PAI-1 and PAI-2, α2-AP and PN-1. They all belong to the SERine Protease INhibitor (SERPIN) superfamily. Serpins inhibit proteases by mimicking their natural substrates, but after being cleaved they become trapped in an inactive 1:1 complex with the protease. Serpins are therefore called suicide inhibitors. All Serpins are metastable and can exist in one of three forms: active, cleaved or latent. (Carell and Travis, 1985; Pike et al., 2002).

**PAI-1** is secreted as a 379 aa-long single-chain glycoprotein which lacks cysteines. It has a molecular weight of approximately 50 kDa and inhibits both tPA and uPA efficiently (Ginsburg et al., 1986; Ny et al., 1986; Pannekoek et al., 1986; Lindahl et al., 1990). PAI-1 is regulated by two cofactors: vitronectin, which stabilizes PAI-1 in its active form, and heparin, which increases the activity of PAI-1 towards thrombin. Interactions between PAI-1 and vitronectin in the ECM, and uPA bound to uPAR at the cell surface, have been shown to regulate cell adhesion and thereby angiogenesis (Declerck et al., 1988; Gils and Declerck, 1998; Mimuro and Loskutoff, 1989). It has also been suggested that the inhibition of uPA by PAI-1 promotes cell migration (Stefansson and Lawrence, 2003) and that deficiency of PAI-1 inhibits uPA/uPAR-dependent polarization of integrin adhesion to vitronectin. This inhibits the directed cell migration that is necessary for angiogenesis (McMahon et al., 2001).

**PAI-2** is a 415 aa-long protein that is characterized as an inhibitor of uPA in human placenta, but it is also found in keratinocytes, macrophages, monocytes, microglia and other cell types (Kruithof et al., 1995; Jensen, 1997). PAI-2 exists in two molecular and topological forms: an intracellular, non-glycosylated form with a molecular weight of 45 kDa, and an extracellular, glycosylated form with a molecular weight of 60 kDa. The biological role of PAI-2 still remains speculative. PAI-2 deficient mice show no deficiencies in monocyte function, wound healing and fertility (Dougherty et al., 1999). In addition to inhibition of uPA, PAI-2 can also inhibit tc-tPA, but not sc-tPA (Thorsen et al., 1988). However, sc-tPA is the predominant form in the blood and fibrin-bound tPA is protected from PAI-2. Therefore, PAI-2 may play only a minor role in controlling tPA-mediated fibrinolysis and may function primarily in inhibiting uPA in processes such as pregnancy, cancer and inflammation (Bachmann, 1987; Dear and Medcalf, 1995; Kruithof et al., 1995).

**α2-AP** is a 452 aa-long single-chain glycoprotein with a molecular weight of 67 kDa (Moroi and Aoki, 1976; Wiman and Collen, 1977; Holmes et al., 1987). α2-AP is the major physiological plasmin inhibitor in human plasma (Collen, 1976; Moroi and Aoku 1976; Saksela and Rifkin, 1988). Like plasmin, α2-AP is mainly produced in the liver and is most active in the blood circulation – since the ability of α2-AP to bind plasmin is decreased when plasmin is bound to fibrin (Collen and Wiman, 1979; Sasaki et al., 1986; Longstaff and Gaffney, 1991). Thus, the activity of free plasmin is quickly inhibited by α2-AP in order to restrict its activity to the fibrin
surface, and the activity of fibrin-bound plasmin is protected from inhibition until the fibrin has been dissolved (Wallen, 1980; Bachman, 1987; Longstaff and Gaffney, 1991).

**PN-1** is comprises 392 aa and has a molecular weight of 45 kDa (Scott and Baker, 1983; Scott et al., 1985). PN-1 is a broad-specificity inhibitor which inhibits tPA, uPA and plasmin as well as other proteases (Scott et al., 1985). Since PN-1 is detected only at low levels in plasma, it is unlikely that it plays an important role in the vascular system (Eaton and Baker, 1983; Hiramoto and Cunningham, 1988). However, PN-1 has activity against plasmin, uPA and other proteases (Scott et al., 1985). It is expressed in mouse tissues such as the ovary, bone, and brain, which suggests that it may play a role in extracellular proteolysis (Hägglund et al., 1996). PN-1 deficient male mice have dysfunctional semen, which renders these mice less fertile than their wild-type counterparts (Murer et al., 2001).

2. **THE MATRIX METALLOPROTEINASE (MMP) SYSTEM**

Like the PA system, the MMP system is thought to play an important role in several physiological and pathological processes that require proteolytic activity i.e. embryo implantation, inflammation, wound healing, ovulation, arthritis, tumor invasion and metastasis (Liotta et al., 1991; Woessner, 1991; Birkedal Hansen, 1995).

The MMP family is a large and still growing proteinase family. To date, 28 MMPs have been characterized. All MMPs have a Zn$^{2+}$ ion at their active site and they have been divided into different subgroups according to their structural similarities and substrate specificities (Visse and Nagase, 2003). These are: collagenases, gelatinases, stromalysins, matrilysins, membrane-type MMPs (MT-MMPs), and those which do not fit into any of these subgroups, which are commonly referred to as “other MMPs” (Shingelton et al., 1996; Wilson and Matrisian, 1996; Gianelli and Antonaci, 2002; Seiki, 2002; van den Steen et al., 2002; Visse and Nagase, 2003). Together, the MMPs have enzymatic activity against virtually all components of the ECM (Vu and Werb, 2000; Visse and Nagase, 2003). The activities of MMPs are not just limited to ECM degradation, however; they also regulate growth factors, adhesion molecules and other non-ECM molecules (Vu and Werb, 2000; McCawley and Matrisian, 2001). Some of the substrates of the MMPs are very specific. As an example, the main function of collagenases is to cleave fibrillar collagen, which is resistant to cleavage by most other MMPs (Shingelton et al., 1996). After the initial cleavage, the collagens become denatured and form gelatin – which can be degraded further by other MMPs, and also by other proteases such as plasmin (Murphy and Docherty, 1992).

2.1. **Regulation and inhibition of MMP activity**

The mechanisms controlling the activation of MMPs under physiological conditions *in vivo* are poorly understood. However, the activities of MMPs are tightly controlled at the level of gene transcription, activation of proenzymes and by inhibition of active MMPs by different inhibitors (Sternlicht and Werb, 2001; Chakraborti et al., 2003).

The activity of MMPs is mostly controlled at the transcriptional level. However, this regulation is rather complex and highly individualized patterns of expression of the various members of the
MMP family are apparent. Examples of cell-type and tissue-specific regulation, inducible and constitutive expression, discrepancies between \textit{in vitro} and \textit{in vivo} patterns of expression add to the complexity (Matrisian, 1994; Chakraborti et al., 2003). However, several molecules such as growth factors, hormones and cytokines are involved in the transcriptional regulation of MMPs (Arthur, 1992; Ray and Stetler Stevenson, 1994; Mignatti, 1995).

Most of the MMPs are formed in an inactive form and activation requires that a highly conserved propeptide region (PRCGXPD) is cleaved off. This causes the Zn$^{2+}$ ion at the active site to interact with H$_2$O, which is required for catalysis (Springman et al., 1990). Serine proteases such as plasmin can cleave the propeptide of some proMMPs and thereby initiate their activation (Coussens and Werb, 1996). Other proMMPs, including proMMP-2 and proMMP-13, can be activated through a membrane-associated mechanism by membrane-type MMP 1 (MT1-MMP) (Sato et al., 1994; Strongin et al., 1995; Coussens and Werb, 1996; Knauper et al., 1996; Nagase, 1997; Okada et al., 1997).

MMP activity can be inhibited by physiological inhibitors (denoted TIMPs), chelating agents and synthetic inhibitors (Birkedal Hansen 1995; Mignatti, 1995; Coussens and Werb, 1996).

2.1.1. Physiological MMP inhibitors
The specific TIMPs and the broad-spectrum inhibitor $\alpha_2$-macroglobulin are the major physiological inhibitors of the MMP system.

TIMPs form a family of relatively small proteins of 20-30 kDa which consists of four members: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. TIMPs are expressed in a variety of cells, and are found in most tissues and body fluids. At least TIMP-1, TIMP-2 and TIMP-3 inhibit MMPs by binding to the active sites in a 1:1 stoichiometric, non-covalent complex (Brew et al., 2000). Initially TIMPs were identified by their ability to inhibit matrix metalloproteinases, but numerous studies have described a wide variety of other functions. Some of them are attributed to MMP inhibition, but TIMPs also exhibit cellular activities that appear to be independent of MMP inhibition, such as cell growth-promoting activity, anti-apoptotic activity, steroidogenic activity, anti-angiogenic activity and embryogenic activity (for a review, see Lambert et al., 2004).

$\alpha_2$-macroglobulin is a large (750 kDa) and prominent protease inhibitor. It is produced in the liver and secreted to the plasma (Barrett and Starkey, 1973). The inhibitory function of $\alpha_2$-macroglobulin works by sterically blocking access of proteases to their substrates (Sotrup-Jensen and Birkedahl-Hansen, 1989). Since some MMPs have a higher affinity for $\alpha_2$-macroglobulin than for their natural substrate, these MMPs are highly inhibited in plasma (Wojtowicz-Praga et al., 1997). Compared to other protease inhibitors, $\alpha_2$-macroglobulin is a rather slow inhibitor and is therefore viewed as a “second line of defense”, capturing the excess of MMPs, plasmin and other proteases that have not been neutralized by other proteases (Bachman, 1987).

2.1.2. Synthetic MMP inhibitors
Many of the synthetic MMP inhibitors are short peptides with a collagen mimicking structure. Some also have a metal binding group, such as a hydroxamic acid group, which chelates the Zn$^{2+}$ ion at the active site of the MMP (see Fig. 2). Marimastat (BB-2516), batimastat (BB-94) and
galardin (ilomastat, GM6001) are examples of hydroxamic acid chelating inhibitors that inhibit a broad spectrum of MMPs (Grobelny et al., 1992; Rasmussen and McCann, 1997; Levy et al., 1998). Oxamflatin, a novel hydroxamic acid derivative, inhibits uPA mRNA expression and proteolytic activity while simultaneously upregulating the expression of the natural inhibitor of uPA and PAI-2 in metastatic cancer cells (Cakarovski et al., 2004). Galardin inhibits gelatinase A (MMP-2), gelatinase B (MMP-9), interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8) and stromelysin-1 (MMP-3). Since all the MMPs are similar in their function, and the inhibitory mechanism of galardin is general for MMPs (see Fig. 2), the degree of inhibition may be similar for other MMPs (Grobelny et al., 1992; Saghatelian et al., 2004). We used galardin to inhibit the MMP system during ovulation and corpus luteum formation in mice in Papers II and III. Galardin and other synthetic MMP inhibitors have been used in clinical trials as anti-cancer drugs (Pavlaki and Zucker, 2003). However, the lack of positive clinical effects of MMP inhibitors in patients with advanced cancer is a drawback. The positive trends seen with marimastat in the treatment of gastric and pancreatic cancer provides the only hope for the use of non-selective hydroxamic acid derivatives in advanced cancer (Pavlaki and Zucker, 2003).

Figure 2. Inhibitory mechanism of galardin. The inhibitor binds to the substrate pockets (S₁', S₂') and chelates the Zn²⁺ ion in the active site. (From Saghatelian et al., 2004).

3. THE ROLES OF THE PA AND MMP SYSTEMS IN THE OVARY

The mammalian ovaries are heterogeneous organs which contain follicles at different developmental stages – including primordial, primary, secondary, tertiary and Graafian or preovulatory follicles, as well as regressing atretic follicles and corpora lutea (CL) (see Fig. 3) (Richardson, 1967). During the processes of follicular development, atresia, ovulation, CL formation, CL function and CL regression, extensive tissue remodeling takes place. Matrix remodeling proteases belonging to the PA and the MMP systems are thought to play important roles in these processes (Ny et al., 1993; Tsafriri, 1995; Ny et al., 2002; Curry and Osteen, 2003).
In every estrus cycle, premature follicles are recruited into growth and development, which culminates in the rupture of the follicular wall and expulsion of the mature oocyte – the ovulation. The mammalian ovary consists of several hundred follicles. However, just a few of these will reach ovulation; most of them will instead go into a degenerative process called atresia (Richards, 1980; Hsueh et al., 1984; Erickson, 1986; Richards et al., 1987).

3.1. Ovulation

The follicular wall surrounding the Graafian follicles consists of two layers of basal membranes, with a collagen-rich theca tissue in-between. In order for ovulation to occur, the follicular wall must rupture (fig. 4). Since 1916, there has been evidence indicating that a protease activity is essential during this event, and it has been shown that the different layers in the follicular wall are degraded and fragmented at the time of ovulation (Schochet, 1916). The proposed mechanism is that cells in the follicular wall produce proteases from the PA system, MMP system and other systems, which then act together to degrade the ECM of the follicular wall (Morales et al., 1983; Murdoch and McCormick, 1992; Espey and Lipner, 1994; Curry and Osteen, 2001; Curry et al., 2001, Curry and Osteen, 2003; Goldman and Shalev, 2004; Liu et al., 2004).
Shortly before ovulation, several components of the PA system are expressed in the cells surrounding the follicle, which suggests a relevant role for them during the degradation of the follicular wall. Liu et al. (1991) found that PAI-1 activity, tPA activity and mRNA levels, were coordinately regulated by gonadotropins in a time-dependent and cell-specific manner, such that a surge of PA activity happened just prior to ovulation in the rat ovary. Both theca-interstitial and granulosa cells synthesized PAI-1, but their maximal PAI-1 expression occurred at different times during the periovulatory period, ensuring inhibition of proteolytic activity in ovarian extracellular compartments both before and after ovulation. The coordinated regulation of tPA and PAI-1 in the rat ovary may fine-tune the peak of PA activity, which may be important for regulation of the ovulatory process. Intrabursal injections of α2-AP or anti-tPA antibodies reduce the gonadotropin-induced ovulation rate in rats (Reich et al., 1985; Tsafiriri et al., 1989). Furthermore, treatment of *in vitro* perfused rabbit ovaries with streptokinase, a bacterial PA, induces ovulation in the absence of gonadotropins (Yoshimura et al., 1987). This suggests that PA activity is sufficient to generate enough proteolytic activity to degrade the follicular wall (Ny et al., 2002; Liu, 2004).

However, studies have shown that mice deficient in parts of the PA system do not show any major defects in fertility or ovulation efficiency (Carmeliet et al., 1994; Bugge et al., 1995a; Leonardsson et al., 1995; Ploplis et al., 1995; Ny et al., 1999). Leonardsson et al. (1995) found that ovulation efficiency is normal in mice deficient in either tPA or uPA, but reduced by 26% in mice lacking both physiological PAs. This result suggests that plasminogen activation may play a
role in the ovulatory response, although neither tPA nor uPA, individually or in combination, is obligatory for ovulation. The loss of an individual PA seems to be functionally compensated for by the remaining PA, but this compensation does not appear to involve any compensatory upregulation. These data imply that a functionally redundant mechanism for plasmin formation operates during gonadotropin-induced ovulation and that PAs together with other proteases generate the proteolytic activity required for follicular wall degradation. However, Ny et al. (1999) showed that during physiological ovulation, adult plg−/− mice had a normal ovulation efficiency compared to wild-type mice. There was, however, a trend towards slightly reduced ovulation efficiency in the plg−/− mice. Taken together, these results indicate that under the conditions used in this study, plasmin is not required for efficient follicular rupture or for activation of other proteases involved in this process. Alternatively, the role of plasmin may be effectively compensated for by other mechanisms in the absence of plasmin (Ny et al., 1999).

Several studies have suggested that the MMP system has important roles during ovulation, and that the MMPs might compensate for the absence of plasmin (Hulboy et al., 1997; Ny et al., 2002; Curry and Osteen, 2003). In an early study by Espey and Lipner (1965), it was found that injections of bacterial collagenase into rabbit follicles were sufficient to induce ovulation. When treating in vitro perfused ovaries with various metalloproteinase inhibitors, it was possible to block ovulation, suggesting an essential role for MMPs in ovulation (Ichikawa et al., 1983; Brannstrom et al., 1988; Butler et al., 1991). Hägglund and co-workers (1999) examined the regulation of 11 MMPs and three TIMPs during gonadotropin-induced ovulation in the mouse. The majority of the MMP mRNAs examined were below the detection levels (MMP-3, -7, -8, -9, -10, -12, and -13), while other mRNAs (MMP-2, -11 and -14, TIMP-2 and TIMP-3) were expressed during the periovulatory period at a constant level. However, MMP-19 and TIMP-1 were both induced and upregulated 5-10 fold by human chorionic gonadotropin (hCG), and both reached their maximum levels at 12 h after hCG treatment. At this time point, corresponding to the time of ovulation, MMP-19 and TIMP-1 mRNA were localized to the granulosa and thecal-interstitial cells of large preovulatory and ovulating follicles. This regulation pattern suggests that MMP-19 may be involved in the tissue degradation that occurs during follicular rupture, and that TIMP-1 could have a role in termination of MMP activity after ovulation (Smith et al., 1994; Hägglund et al., 1999). Liu and co-workers (1998) used in situ hybridization to study the regulation and distribution of mRNAs coding for MMP-2 and its cell surface activator: membrane-type MMP-1 (MT1-MMP) during gonadotropin-induced ovulation in the rat. The levels of both MT1-MMP and MMP-2 mRNAs were low in the ovaries of untreated immature rats. MMP-2 mRNA found in thecal-interstitial cells and MT1-MMP mRNA found in granulosa and thecal-interstitial cells were both induced after stimulation with PMSG. Following hCG stimulation, the expression of MMP-2 and MT1-MMP remained and appeared to be upregulated together in the thecal-interstitial cells surrounding the large preovulatory follicles. However, the expression of MT1-MMP was extensively downregulated in the granulosa cell layers of these large preovulatory follicles. The kinetic of expression and tissue distribution support the notion that MT1-MMP may have dual functions in the ovary. MT1-MMP may act initially as a matrix-degrading protease inside the follicle during follicular development and later, just prior to ovulation, as an activator of proMMP-2 in thecal-interstitial cells surrounding preovulatory follicles (Liu et al., 1998; Brew et al., 2000). Most of the MMP-deficient mouse strains have displayed no or only mild defects in fertility, except the MT1-MMP deficient mice which had such severely compromised health that they could not breed at all (Holmbeck et al., 1999; Zhou et al., 2000).
We have recently found that the ovulation rate is reduced by only 28% in plg$^{-/-}$ mice treated with the MMP inhibitor galardin (Paper II). *In vitro* experiments performed in that study confirmed that galardin is distributed in the ovary in sufficient amounts to fully inhibit gelatinase activity. This indicates that the functional importance of plasmin and MMPs in ovulation may be less than previously appreciated. It is possible that proteases from other families can compensate for the lack of plasmin and MMP activity, to ensure proper reproductive function.

3.2. Corpus luteum formation, function and regression

The corpus luteum (CL) is formed from the ovulated follicle in a process that involves rapid tissue remodeling and angiogenesis, and converts the ruptured follicle into a transient endocrine organ (Basset, 1943; Michael et al., 1994; Niswender and Nett, 1994; Findlay, 1986). The formation process involves invasion of a capillary network from the theca tissue into the granulosa layers, and is accompanied by differentiation of the theca and granulosa cells into luteal cells. Once formed, the CL is one of the most vascularized organs in the body and it secretes progesterone to prepare the uterus for implantation (Basset, 1943; Damber et al., 1981; Janson et al., 1981). In both rodents and primates, the CL development is a rapid process with very high cellular turnover (Niswender and Nett, 1994; Duncan et al., 1998). This process is thought to be PA and MMP dependent (Ny et al., 2002; Goldman and Shalev, 2004; Liu, 2004). The regulatory and functional roles of the PA and MMP systems in angiogenesis and tissue remodeling in the developing and degrading CL have been widely studied over the last decade (Feng et al., 1993; Liu et al., 1994; Liu et al., 1995; Liu et al., 1996; Liu et al., 1997; Liu et al., 1998; Liu et al., 1999; Chen et al., 1999; Smith et al., 1999; Duncan, 2000; Ny et al., 2002; Liu et al., 2003a; Liu et al., 2003b). The roles of the PA and MMP systems in angiogenesis are further discussed in section 4.2.

Liu and co-workers (2003a) have shown that the tPA, uPA, MMP-2, MMP-11, MT1-MMP, PAI-1, PN-1 and also the TIMP-1, -2 and -3 mRNAs are expressed in the CL basement membrane area and/or in cells localized inside the CL in the forming (1-day-old) mouse. In both the rat and in the rhesus monkey, the expression pattern of tPA and PAI-1 mRNA suggests that proteolytic activities that are mediated by tPA and regulated by PAI-1 are important for CL formation (Liu et al., 1991; Liu, 2004). uPA is also expressed in a pattern that resembles invading blood vessels (Bacharach et al., 1992), as are collagenases, gelatinases and TIMPs (Nothnick et al., 1995; Nothnick et al., 1996; Bagavandoss, 1998). On the contrary, the PA and MMP systems are not believed to be essential during the functional phase since the luteal tissue is stable during this period (Ny et al., 2002; Curry and Osteen, 2003). During the functional luteotrophic phase of the mouse CL, the production of progesterone is high – as revealed by high levels of serum progesterone. During this phase (6-day-old CL), the expression of most mRNAs was below the detection level (Liu et al., 2003a). However, the mRNAs coding for uPA, MT1-MMP, and TIMP-3 were expressed and showed a dotted expression pattern, with uPA mRNA being the most abundant of the three (Liu et al., 2003a).

If fertilization of the released ovum does not occur, the functional phase of the CL is terminated and luteolysis is initiated. This involves a rapid loss of progesterone production, followed by degradation of the luteal tissue into small fibrous remnants within a few days (Auletta and Flint,
During CL regression, PAs and MMPs secreted by macrophages that invade the regressing CL can be used to remove dead luteal cells by phagocytosis (Paavola, 1979; Michael et al., 1994). In the regressing (16-day-old) mouse CL, both uPA and macrophage metalloelastase (MMP-12) revealed a dotted expression pattern. Also, tPA showed a dotted expression pattern in the regressing CL, but the expression level was lower than that of uPA. Several other proteases and protease inhibitors including MMP-2, MMP-11, MT1-MMP, PN-1, TIMP-1, TIMP-2, and TIMP-3 showed a rather evenly distributed expression during the regression phase (Liu et al., 2003a). Since MMP-12 is expressed exclusively in the regressing mouse CL, it might be used as a marker of regressing CLs (Liu et al., 2003a). MMP-13 is expressed in the regressing rat CL (Liu et al., 1999) but not at all in the mouse CL (Liu et al., 2003a).

The temporal and spatial expression pattern of proteases and protease inhibitors during the life span of the CL suggests that members of the PA and MMP systems may play important roles in the angiogenesis and tissue remodeling processes during CL formation, as well as in the tissue degradation during luteal regression (Liu et al., 2003a). However, there have been no studies regarding the functional role of the PA and MMP systems in the CL. The absence of reproductive phenotypes in mice lacking uPA and several other matrix-degrading proteases indicates that there are redundancies among different matrix-degrading proteases, or that tissue remodeling in the ovary may involve other additional unique elements (Ny et al., 2002; Liu et al., 2003a). In Paper III, we investigated the formation and function of CL in mice with compromised PA and MMP systems. In these mice, the CL development was normal compared to wild-type mice. These data suggest that neither plasmin nor the MMPs, alone or in combination, are essential for CL formation. However, in this study the serum progesterone levels were reduced to about 50% compared to carrier-treated plg⁻/ mice and to plg⁻/ mice treated with the MMP inhibitor galardin. The reduced serum progesterone levels suggest that plasmin may play a novel role in the maintenance of luteal function, possibly through proteolytic activation of growth factors and other paracrine factors (Paper III). An increase in tPA and PAI-1 activity is found in the regressive rat and monkey CL. The increase in tPA and PAI-1 is closely related to a dramatic decrease in the production of steroidogenic acute regulatory protein (StAR) and progesterone. This suggests that the coordinated expression of tPA and PAI-1 in the CL may be involved in luteal regression (Chen et al., 1999; Liu et al., 2003b). The level of StAR in luteal tissue correlates well with progesterone production (Pescador et al., 1996; Townson et al., 1996), indicating that StAR is a key regulator of CL function and can be used as a marker of steroidogenesis in rat, monkey, human and mouse (Chen et al., 1993; Chen et al., 1999; Liu et al., 2003b; Paper I).

### 3.3. Murine models for the study of ovarian function

When studying follicular development and ovulation, we mainly used a gonadotropin-induced ovulation model. In this model, immature 25-day-old female mice were injected intraperitoneally (i.p.) with 1.5 IU of pregnant mare serum gonadotropin (PMSG) to stimulate follicle growth and, 48 h later, with 5 IU hCG to induce ovulation, which takes place 10-12 h after hCG treatment (Zarrow and Wilson, 1961; Wilson and Zarrow, 1962). These immature mice have not yet entered their own estrus cycle, which simplifies the analysis of the tissue since the mouse’s own ovulation will not interfere with the induced ovulation.
When studying the formation, function and regression of CL, we used an adult pseudopregnant (psp) mouse model. This model has been described previously (Finn, 1965; Selstam et al., 1985). Briefly, one female mouse, 8-12 weeks old, was housed with one vasectomized male mouse until a vaginal sperm plug was detected, which indicated day 1 of pseudopregnancy/CL development. In this way, the CLs stay functional for 8–10 days and are fully physiological without any exogenous stimuli. However, even though the adult female mice ovulate every three days, the time for mating is difficult to predict. Also, remnants of CLs from earlier cycles may interfere with the analysis of the newly formed CL. In Paper I, we therefore developed a model of gonadotropin-induced corpus luteum formation in immature mice. Immature, 25-day-old female mice were treated with 1 IU PMSG i.p. to stimulate follicular development. About 46 hours later, the mice were treated with 5 IU hCG i.p. to induce ovulation. At day 2, the mice were treated twice with 50 µg ovine prolactin subcutaneously (s.c.). In this new model there were no CL remnants from earlier cycles, but the CL did not stay fully functional as long as in the psp model. This model was then used in Paper III to study the formation and function of the CL in plg−/− mice treated with an MMP inhibitor.

4. THE PA AND MMP SYSTEMS IN OTHER PHYSIOLOGICAL AND PATHOLOGICAL PROCESSES

Proteases belonging to the PA and the MMP systems are thought to play important roles in many physiological and pathological processes that involve tissue remodeling and ECM degradation. In addition to the proteolytic processes in the ovary, the PA and MMP systems play roles in processes such as fibrinolysis, angiogenesis, tumor invasion, metastasis, wound healing, embryo implantation, and inflammation (Danø et al., 1985; Saksela and Rifkin, 1988; Alexander and Werb, 1991; Ny et al., 1993; Mignatti and Rifkin, 1993; Mignatti, 1995; Birkedal Hansen, 1995; Liu et al., 1996; Andreasen et al., 1997; Collen, 1999; Liu et al., 1999; Tsafriri and Reich, 1999).

4.1. Vascular fibrinolysis

Vascular fibrinolysis is the process responsible for clearing the vascular system from fibrin clots (Ranby and Brandstrom, 1988; Thorsen, 1992; Angles-Cano, 1994; Collen, 1999; Nieuwenhuizen, 2001; Rijken and Sakharov, 2001). tPA is thought to be the functionally important PA in vascular fibrinolysis, due to its pronounced activation by fibrin (Bachman, 1987). Even if uPA lacks affinity for fibrin, it has been suggested that uPA has a compensatory role in the fibrinolytic process (Carmeliet et al., 1994). Clinical studies of patients with deficiency in fibrinolytic activity have revealed the importance of a strict balance between PAs and PA inhibitors in vascular fibrinolysis (Schneiderman et al., 1991; Malmberg et al., 1994). Reduced PAI-1 levels in plasma make patients suffer from bleeding problems (Dieval et al., 1991; Lee et al., 1993), whereas exceedingly high levels of PAI-1 may cause myocardial infarction, deep-vein thrombosis, pulmonary embolism, diabetes, and arteriosclerosis (Hamsten et al., 1985; Nilsson et al., 1985; Juhan-Vague et al., 1987; Gray et al., 1993; Wallberg-Jonsson et al., 1993; Lang et al., 1998).
4.2. Angiogenesis

Angiogenesis is the process of new capillary formation from pre-existing vessels (Carmeliet, 2000). This mechanism of vascular development is distinguished from vasculogenesis, which has been defined as the development of new blood vessels from angioblasts which are undergoing differentiation. Both angiogenesis and vasculogenesis can occur during embryonic development, but in contrast to vasculogenesis, angiogenesis can occur throughout adult life. Angiogenesis is a tightly regulated event which occurs rarely under normal physiological conditions in adults. In males, the predominant angiogenesis process is wound healing, but in the female there is also considerable angiogenesis in the ovaries and in the uterus throughout the menstrual cycle (Findlay, 1986).

The PA and MMP systems may play a significant role in the angiogenesis process (Coussens and Werb, 1996; Mignatti and Rifkin, 1996). For instance, PAI-1 is thought to contribute to angiogenesis by regulating plasmin-mediated proteolysis (Bajou et al., 2001; Devy et al., 2002) and/or by modulating cell migration in a dose-dependent manner (Deng et al., 1996; Kjoller et al., 1997; Isogai et al., 2001; McMahon et al., 2001; Czekay et al., 2003; Bajo et al., 2004). When new blood vessels are formed, capillary endothelial cells (EC) start to migrate out through the pericapillary membrane that surrounds the vessels, in a proteolysis-dependent manner. Then they can proliferate and eventually elongate into a new capillary. The proteolysis is not only needed for the migration of EC, but also in the subsequent migration through the ECM towards the angiogenic stimulus. Remodeling of ECM has been shown to be necessary for the migration, proliferation and capillary tube formation that is required for the formation of new capillaries (Moses, 1997). The growth of these new vessels is controlled by angiogenic factors (Anderson and Little, 1985; Niswender and Nett, 1994). Although several molecules have been implicated as mediators of angiogenesis, there is at present no direct evidence for the involvement of any other factors except the vascular endothelial growth factor (VEGF) (Ferrara et al., 1998; Ferrara, 1999; Choi et al., 2003). It has, however, also been suggested 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). For example, MMP-12, MMP-9 and MMP-7 (matrilysin) can cleave plasminogen to form angiostatin, which inhibits angiogenesis in vitro (Dong et al., 1997; Patterson and Sang, 1997).

4.3. Tumor invasion and metastasis

Pathological angiogenesis is a common theme in processes such as tumor growth and metastasis formation (Mignatti and Rifkin, 1996). In order to obtain nutrition for the proliferation process cancer tumors need a vascular system which is produced by pathological angiogenesis. Cancer metastasis is also dependent on the level of vascularization of the tumor. Metastasis is the result of several interdependent processes which include: (i) detachment of cancer cells from their original locations, (ii) cancer cell migration, (iii) access of cancer cells to blood and lymphatic vessels, (iv) adhesion to and invasion through the endothelium, allowing colonization at distant sites in the organism, and (v) invasion of cancer cells into the surrounding tissue, a process that involves both adhesion to and degradation of ECM components (Carmeliet and Jain, 2000; Mandriota et al., 2001; Pepper, 2001). The PA and MMP systems have been shown to be involved in these processes and are possible targets for antitumor therapy (Coussens and Werb,
Kim and co-workers (1998) have shown that mice treated with the MMP inhibitor marimastat reduced intravasation by more than 90% and also that uPA/uPAR and MMP-9 are required to break the vascular wall in order for tumors to metastasize. Itoh and co-workers (1998) investigated the specific role of MMP-2 in these events using MMP-2 deficient mice. In these mice, tumor-induced angiogenesis was suppressed according to a dorsal air sac assay. When B16-BL6 melanoma cells or Lewis lung carcinoma cells were implanted intradermally, the tumor volumes decreased by 39% for B16-BL6 melanoma and by 24% for Lewis lung carcinoma at 3 weeks after implantation in the MMP-2 deficient mice. The number of lung colonies of i.v. injections fell by 54% for B16-BL6 melanoma and 77% for Lewis lung carcinoma. These results indicated that host-derived MMP-2 plays an important role in angiogenesis and tumor progression, suggesting that MMP-2 inhibitors would be useful for anticancer chemotherapy.

Since metastases spread through the vascular system, highly vascularized tumors are more prone to form metastases (for review, see Sivridis et al., 2003). A study by Bajou et al. (1998) demonstrated that both local tumor invasion and vascularization of transplanted malignant keratinocytes are prevented in PAI-1 deficient mice. Recently, Bajou and co-workers (2004) have shown that physiological levels of PAI-1 promote in vivo tumor invasion and angiogenesis. In sharp contrast, inhibition of tumor vascularization was observed when PAI-1 was produced at supraphysiological levels, either by host cells (transgenic mice overexpressing PAI-1) or by tumor cells (after transfection with murine PAI-1 cDNA). This study provides evidence for a dose-dependent effect of PAI-1 on tumor angiogenesis. It was therefore concluded from these investigations that host-produced PAI is essential for cancer cell invasion and angiogenesis.

4.4. Wound healing

Proteolytic activity is required during wound healing in many processes including inflammation, provisional ECM removal, formation of granulose tissue, ECM formation, and also migration of keratinocytes from the wound edges towards the centre of the wound (Planus et al., 1999; Murphy and Gavrilovic, 1999; Giannelli et al., 2003). The involvement of the PA system in wound healing has been well established (Vassalli and Saurat, 1996; Li et al., 2003). The importance of plasmin was demonstrated in a skin wound healing study in which plg−/− mice were challenged with incision wounds (Romer et al., 1996). It was found that the wound healing of plg−/− mice was strikingly delayed, with impaired keratinocyte migration. It was concluded that plasminogen function is eventually taken over by other factors, resulting in a delay in the healing process (Romer et al., 1996). When a similar study was performed on plasminogen/fibrinogen doubly deficient mice, the healing time was normal (Bugge et al., 1996). In an as yet unpublished study performed by Li and co-workers, on the healing of mouse tympanic membrane (TM) perforations, it was found that the healing was permanently impaired in plg−/− mice. Several of the dynamic interactive processes associated with a successful TM healing were disturbed in the plg−/− mice (Li et al., 2004, submitted). In another study by Lund and co-workers (1999), a retarded wound healing was found in plg−/− mice, and also in wild-type mice treated with galardin, but in both cases wound closure was ultimately completed in all mice within 60 days. The expression of several MMPs in keratinocytes migrating to cover the wound was strongly enhanced by galardin treatment. However, when plg−/− mice were treated with galardin, healing
was completely arrested and wound closure was not seen during an observation period of 100 days. This indicates that there is a functional overlap between the PA and MMP systems, probably in the dissection of the fibrin-rich provisional matrix by migrating keratinocytes (Lund et al., 1999).

4.5. Embryo implantation

Several MMPs, uPA, uPAR and other proteases are expressed during the time of embryo implantation. These proteases are responsible for releasing different factors that are necessary for implantation, such as the insulin growth factor (IGF). The MMP and PA systems are also necessary for the trophoblasts to invade endometrial tissue (Strickland and Richards, 1992). In support of this, there are several lines of evidence suggesting that trophoblasts can express uPA, cathepsins and several MMPs (Sappino et al., 1989; Alexander et al., 1996; Teesalu et al., 1996; Afonso et al., 1997; Das et al., 1997; Salamonsen and Nie, 2002). However, most MMP-deficient mice are perfectly fertile, as are uPA- and uPAR-deficient mice (Carmeliet et al., 1994; Bugge et al., 1995a; Ploplis et al., 1995).

Solberg and co-workers (2003) have found a functional overlap between the PA and MMP systems in the events of embryo implantation and placenta development, whereby plg−/− mice treated with the synthetic MMP inhibitor galardin were shown to be unable to deliver healthy young due to a failure in proper placental vascularization. Galardin-treated wild-type mice and untreated plg−/− mice were able to deliver healthy young.

4.6. Studies of plasminogen-gene deficient mice

plg−/− mice were generated by two research groups, utilizing two separate strategies to inactivate the plasminogen gene (Bugge et al., 1995a; Ploplis et al., 1995). The plasminogen gene deficiency is not embryonically lethal and the plg−/− mice survive well into adulthood. Due to fibrin depositions in a number of organs, spontaneous phenotypes of plg−/− mice occur, such as disturbed thrombolysis and improper physical development (Ploplis et al., 1995). Reconstitution with murine plasminogen normalized the thrombolytic potential, indicating that plasminogen plays a critical role in in vivo dissolution of fibrin clots (Lijnen et al., 1996). Differences in physical development were observed after only 4 weeks of age, with less weight gain and delayed vaginal patency in plg−/− mice (Hoover-Plow et al., 1999). Ovulation efficiency in young, age-matched wild-type, plg+/− and plg−/− female mice was studied after stimulation with gonadotropin. These studies indicated a slight, but statistically insignificant, reduction in ovulation efficiency (Ny et al., 1999). However, other studies have demonstrated a critical, dose-dependent requirement for plasminogen in lactational differentiation and mammary gland remodeling during involution (Lund et al., 2000). Even so, mice deficient in both uPA and tPA suffer form severe anemia and the plasminogen deficiency has no significant influence on hematological parameters (Carmeliet et al., 1994). Induced phenotypes of plg−/− mice include increased inflammation and infection, altered vascular remodeling, disrupted wound healing, neurodegeneration, and also decreased cancer growth and metastasis (Romer et al., 1996; Bugge et al., 1997; Carmeliet et al., 1997; Kitching et al., 1997; Tsirka et al., 1997a; Tsirka et al., 1997b; Xiao et al., 1997; Bugge et al., 1998; Kao et al., 1998; Moons et al., 1998; Ploplis et al., 1998; Bezerra et al., 1999; Gebbia et al., 1999; Nagai et al., 1999; Shi et al., 1999; Akassoglou et al., 2000; Busuttil et al., 2000; Creemers et al., 2000; Drew et al., 2000a; Drew et al., 2000b; Goguen et al., 2000). Most of them
indicate reduced recruitment of inflammatory cells and disturbed tissue remodeling processes. A strong relationship between plasminogen and fibrin(ogen) has been suggested to contribute to these processes (Ploplis, 2001).

5. SUMMARY OF THE PRESENT STUDY

5.1. A synchronized gonadotropin-induced corpus luteum model in the mouse (Paper I)

The CL is formed from the ovulated follicle in a process that involves rapid tissue remodeling and angiogenesis. It is possible to study function of the CL using a pseudopregnant mouse model. However, a drawback with the psp model is that it is hard to predict the exact time when the ovulation will take place. Also, in the ovaries of mature mice there might be residues of old, degraded CL that interfere when studying the formation of new CL. One common way of restricting the time of ovulation is to treat immature mice with the gonadotropins PMSG and hCG, which leads to ovulation about 10-12 h after hCG treatment. In rats, this model is highly efficient but in mice the formation of the CL is interrupted and the CL degrades rapidly. Therefore, in this study we developed a new mouse model for studying CL formation and function in mice. Using the well-established psp model as a control, we compared the formation and function of the CL in mice treated according to five different protocols. To determine the development and health status of the CL, ovaries were sectioned and stained for a battery of markers of CL development and regression. As markers for the development of a functional CL, we used expression of the CD31 protein, which is specific for endothelial cells in blood vessels, and expression of LH receptor mRNA. The expression of StAR mRNA was followed during CL development in four of the models tested. As markers of luteal regression, we used TUNEL staining to detect apoptotic cells and expression of MMP-12 mRNA as a marker for macrophages that may participate in degradation of the regressing CL tissue. In addition, as a marker for function of CL, the levels of progesterone in blood plasma were measured.

In the first protocol, 25-day-old female mice were treated with 1 IU PMSG and with 5 IU hCG 46 hours later (day 0 of CL development). At days 1 and 2, the CL developed normally but quickly degenerated at day 4 of CL development. In the second protocol, 31-day-old mice were treated in the same way as the mice in the first protocol, but some of these mice had already ovulated and thus the timing of ovulation in these mice was unreliable. Also, the CL formation and function followed the first protocol. In the third protocol, 25-day-old mice were treated with 4 IU PMSG and 5 IU hCG. In these mice, a large number of CL started to develop but at day 3 of CL development they had degenerated.

In mice, the formation and function of CL is regulated by luteinizing hormone (LH) and prolactin. Cervical stimulation during mating induces prolactin release in two daily surges. Thus, in the fourth protocol, 25-day-old mice were treated as in the first protocol, but the mice were then housed with vasectomized males and checked for a vaginal plug the morning after (day 1 of CL development). Unfortunately, the CLs appeared to be healthy only until day 3, when they started to regress. Finally, in the fifth and most preferable protocol, the mice were induced to ovulate as in protocol 1, but then we mimicked the endogenous prolactin surges by administration of prolactin twice daily from day 2 of CL development. In contrast to the previously used
protocols, the CL appeared healthy until day 6 of CL development but the function of the CL was not as efficient as in the control psp mice.

Since StAR-mediated transport of cholesterol across the mitochondrial membrane is the rate-limiting step in progesterone synthesis, we characterized the expression of StAR mRNA during the different life stages of the CL in the adult psp mouse. StAR mRNA was highly expressed at day 3 and day 6 of CL development, and the levels remained high until the late stages of CL regression. StAR expression may therefore be a good marker of CL development and regression.

5.2. Successful ovulation in plasminogen-deficient mice treated with the broad-spectrum matrix metalloproteinase inhibitor galardin. (Paper II)

Extensive ECM degradation must take place in order for a mature ovum to be released from the preovulatory follicle in the ovary. There are indications have been made that the PA system and the MMP system provide the proteolytic activity necessary for follicular wall degradation at the time of ovulation. These systems have been liked previously tissue remodeling processes such as fibrinolysis, ovulation, embryo implantation, wound healing, angiogenesis, etc.

Earlier studies have, however, indicated that ovulation is rather normal in the absence of a functional PA system. Since adult cycling mice deficient in a functional plasminogen gene have a normal ovulation efficiency, we assumed that the MMP system could compensate for the loss of plasminogen.

To test this hypothesis, we first showed in vitro and in vivo that the active MMPs in the ovary could be inhibited by the synthetic MMP inhibitor galardin (GM6001) (Figs 1 and 2, Paper II). Subsequently, 25-day-old female plg+/+, plg-/+ and plg-/- mice were injected i.p. with 1.5 IU of PMSG to stimulate follicle growth and maturation. 48 hours later, the mice were treated with 5 IU hCG to induce ovulation, which takes place 10-12 hours after hCG treatment. The mice were sacrificed 20 hours after hCG treatment and the number of ova was recorded. When injecting the MMP inhibitor (100 mg/kg body weight) at the same time as hCG, or 6 hours before, we found that wild-type mice (plg+/+ and C67BL/J6) and heterozygous mice (plg-/+ ) had an 18-20% reduction in ovulation efficiency when treated with galardin, compared to vehicle-treated mice. plg-/- mice had only a 14% reduction in ovulation efficiency (Tables 1 and 2, Paper II). The MMP inhibitor treatment did not delay the onset of follicular wall rupture. Taken together, the data suggest that neither MMPs nor plasmin are obligatory for ovulation under the conditions used in this study.

We also tested whether the MMP system and the PA system cooperate, or if they can replace each other during follicular development and ovulation. Wild-type, plg+/+, and plg-/- mice were treated as described before, but this time with twice as much of the MMP inhibitor during the whole follicular development (at the same time as PMSG, 24 hours after PMSG, and at the same time as hCG). We found that these mice showed a similar reduction in ovulation efficiency as in mice treated with the inhibitor only once, at the same time as hCG. The similarity in results between the two studies indicates that there are no obvious synergistic effects on ovulation efficiency when MMPs are suppressed in plg-/- mice during follicular development.
5.3. Plasminogen is required for normal progesterone production in the mouse (Paper III)

After ovulation, the ruptured follicle transforms into the CL, which is one of the most vascularized organs in the body, in a process that involves extensive tissue remodeling and angiogenesis. This includes granulosa and theca cell invasion of the forming CL and their conversion into steroid-producing luteal cells.

Earlier studies have shown that different components of the PA system and the MMP system are expressed during CL formation. The present study was performed in order to assess the functional role of the PA and MMP systems in CL formation. Paper II showed that the MMP and PA systems are not obligatory for ovulation efficiency in mice. This, however, does not exclude the possibility that the PA and/or MMP systems are important for CL formation and function.

Using two models for CL formation, adult psp mice and immature gonadotropin-primed mice treated with prolactin (methods described in Paper I), the formation and function of the CL were studied in plg−/− mice treated with the MMP inhibitor galardin. 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 I.

Psp mice were sacrificed at day 3 and at day 6 of CL formation, and any alterations in CL development in plg−/−, and plg+/− mice were compared to their wild-type counterparts (plg+/+ mice). Surprisingly, at day 3 we did not find any alterations – other than a slightly lower number of CL in plg−/− mice, which can be explained as a secondary effect of the lower ovulation efficiency found in these mice (Paper II). On the other hand, we found a 54% reduction in serum progesterone in plg−/− mice and a 37% reduction in the plg+/− mice compared to wild-type mice. At day 6, the reduction in serum progesterone levels was slightly lower (a 45% reduction in serum progesterone in the plg−/− mice and a 22% reduction in the plg+/− mice). For immature gonadotropin-primed mice treated with prolactin, we observed the same trend at day 3 of CL development as for psp mice. All the CL seemed healthy and indistinguishable from the wild-type CL when stained with markers for function, regression and development.

Since there might be a functional overlap between the PA system and the MMP system, plg−/−, plg+/− and plg+/+ mice were treated with galardin, or vehicle as control. The mice were injected i.p. with the MMP inhibitor (100 mg/kg body weight) once daily, starting at day 1 of CL development for psp mice, and at day 0 (the same time as hCG) for gonadotropin-primed mice. This ensured the presence of galardin in the ovaries by the time of initiation of CL formation. Galardin treatment did not alter the results significantly. All the CLs were healthy and viable, even if treated with galardin.

Together, these data suggest that neither plasminogen nor MMPs, alone or in combination, are essential for the formation of viable CL. The reduced serum progesterone levels observed in the plg−/− mice did not appear 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 luteal function, possibly through proteolytic activation of growth factors and other paracrine factors.
CONCLUSIONS

⇒ In plg\textsuperscript{-/-} mice, suppression of MMP activity resulted in 14% reduced ovulation efficiency, suggesting that there is no obvious cooperation between the PA and MMP systems during ovulation and that plasmin and MMPs do not complement each other functionally during this process.

⇒ Mimicking the endogenous prolactin surges constitutes a good mouse model for the study of gonadotropin-induced CL formation

⇒ The expression pattern of StAR as a molecular marker for CL function has been characterized and used in this study.

⇒ Viable and functional CL form in plg\textsuperscript{-/-} mice, and in plg\textsuperscript{-/-} 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 in normal progesterone production in mice.
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