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A functional overlap of plasminogen and MMPs regulates vascularization during placental development

Helene Solberg[1], **Julie Rinkenberger**[2], **Keld Danø**[1], **Zena Werb**[2], and **Leif R. Lund**[1,*]

[1] Finsen Laboratory, Rigshospitalet, Strandboulevarden 49, 2100 Copenhagen, Denmark

[2] Department of Anatomy, University of California, San Francisco, CA 94143-0452, USA

SUMMARY

Both plasminogen activators and matrix metalloproteinases (MMPs) have been implicated in a variety of developmental processes in the mouse during embryo implantation and placentation. We show here that pharmacological treatment of plasminogen-deficient mice with the broad spectrum MMP inhibitor galardin leads to a high rate of embryonic lethality. Implantation sites from plasminogen-deficient galardin-treated mice at 7.5 days post coitus (dpc) showed delay in both decidualization and invasion of maternal vessels into the decidua. At 8.5 dpc, half of the embryos were runted and still at the developmental stage of a 7.5 dpc embryo. Most embryos that escaped these initial defects eventually died, probably from defective vascularization and development of the labyrinth layer of the placenta, although a direct role on embryo development cannot be ruled out. These results demonstrate that the combination of MMPs and plasminogen is essential for the proper development of the placenta. Plasminogen deficiency alone and galardin treatment alone had much less effect and there was a pronounced synergism on both placental vascularization and embryonic lethality, indicating a functional overlap between plasminogen and MMPs.

Keywords

Invasion; Tissue remodeling; Plasminogen deficiency; Implantation; Matrix metalloproteinases

INTRODUCTION

Rodents, like humans, form a hemochorial placenta in which the embryonic tissues invade through the maternal epithelial cell layer and into the stromal tissue to make direct contact with the maternal blood sinuses (Abrahamsohn and Zorn, 1993; Rossant and Cross, 2001; Cross et al., 1994). The penetrative nature of hemochorial placentation so clearly mimics what is seen with highly invasive tumors that normal trophoblasts have been called pseudomalignant (Kirby, 1965; Axelrod, 1985). At day 4.5 of gestation in mice, the blastocyst attaches to the uterine epithelium, thus initiating implantation; the outer layer of the blastocyst, the mural trophectoderm, begins differentiating into invasive primary giant trophoblast cells. These cells breach the uterine epithelium and degrade the underlying extracellular matrix to connect with the maternal blood supply. By day 7.5, the implantation site is fully established. The terminally differentiated primary and secondary giant cells, in combination with Reichert's membrane and the parietal endoderm, form a primitive diffusion barrier to allow gas and nutrient exchange, without directly exposing the embryo to the maternal blood supply and immune system. Concurrently, the cells adjacent to the inner cell mass, the polar trophectoderm, continue to proliferate forming the ectoplacental cone (EPC). On day 6.5, the outer cells of

*Author for correspondence (lund@inet.uni2.dk).

EPC begin differentiation into invasive secondary trophoblast giant cells. Normal placentation is dependent upon the invasion of the uterine stroma by these cells. As a response to embryonic attachment to the uterine epithelia, uterine stromal cells in the immediate vicinity of the attached embryo begin to proliferate and differentiate, resulting in compression of the uterine crypt around the embryo and formation of the implantation chamber (for reviews, see Cross et al., 1994; Rinkenberger et al., 1997; Rinkenberger and Werb, 2000).

One proposed function of decidualization is to protect the mother from the invasive nature of the embryo. Transplanting the mouse embryo outside of the uterus results in extensive invasion and growth of the embryo (Kirby, 1965). It is likely that the decidua protects the uterus from inappropriate invasion of the embryo through a physical barrier as well as by the production of inhibitors of proteolytic enzymes, such as the tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitor type 1 (PAI1) that are produced by the decidual cells (Alexander et al., 1996; Das et al., 1997; Reponen et al., 1995; Teesalu et al., 1996).

From gestation days 7.5–12, fetal blood vessels form and invade the yolk sac placenta to provide nutrient and gas exchange until the true chorioallantoic placenta is formed, a process that begins at gestation day 9. By day 10 of gestation, three zones of the placenta can be visualized: the labyrinth layer, which is composed of embryonic trophoblast cell strands containing maternal blood, the junctional zone spongiotrophoblast layer, and the trophoblast giant cell zone that isolates the embryo from the mother (Rinkenberger and Werb, 2000). Embryonic vessel formation begins by day 10 in the labyrinth layer; these vessels contain nucleated fetal erythrocytes, indicating that placental circulation has begun (Adamson et al., 2002). It is in the labyrinth layer that the maternal and embryonic circulations are in closest proximity, and where gas and nutrient exchange will take place. Failure to implant or to establish a functional placenta results in death and resorption of the embryo. Malformation of the placenta results in intrauterine growth retardation and birth of runted embryos with poor survival prospects (Cross et al., 1994; Rinkenberger and Werb, 2000; Rossant and Cross, 2001).

Trophoblast invasion depends on the finely tuned balance between activated proteases and protease inhibitors, enabling the extracellular degradation and phagocytosis of maternal cells and extracellular matrix. Based on both expression and functional studies, at least three classes of proteases, matrix metalloproteinases (MMPs), serine proteases and cysteine proteases, could be involved in the extracellular matrix remodeling that facilitates trophoblast invasion (Cross et al., 1994; Alexander et al., 1996; Afonso et al., 1997). The expression of the serine protease urokinase-type plasminogen activator (uPA) (Sappino et al., 1989; Teesalu et al., 1996; Teesalu et al., 1998) and several MMPs, including MMP2, MMP9, MMP11 and mouse ColA (Alexander et al., 1996; Teesalu et al., 1999; Balbin et al., 2001) in both trophoblast giant cells and/or the decidua, indicates that they may function in the tissue remodeling and cell invasion processes that take place during implantation and placentation. However, the results of gene deletion mouse models have indicated that although protease activity is crucial for implantation success, there is considerable functional redundancy among the various classes of proteases. Deleting the plasminogen/plasmin system does not affect the viability of homozygous knockout animals (Carmeliet et al., 1994; Bugge et al., 1995a; Bugge et al., 1995b; Bugge et al., 1996; Ploplis et al., 1995; Teesalu et al., 1999). None of the MMP-null mice have so far been shown to be infertile (Itoh et al., 1998; Sternlicht and Werb, 1999; Teesalu et al., 1999; Vu and Werb, 2000). Taken together, these data suggest that the protease interactions are complex to ensure that appropriate maternal and fetal connections are made during implantation.

In the absence of plasminogen (PLG), skin wound healing, postlactational mammary gland involution and tumor metastasis are delayed but ultimately do occur (Rømer et al., 1996; Bugge

et al., 1998; Lund et al., 1999; Lund et al., 2000). In the case of wound healing, there is functional overlap between PLG and one or more MMPs, such that a complete arrest of healing requires both PLG deficiency and MMP inhibition (Lund et al., 1999). These data led us to hypothesize that there may also be a functional overlap between PLG and the MMPs during implantation and placentation. To test this hypothesis, we have studied the effect of galardin (GM6001), a hydroxamate broad-spectrum inhibitor of MMPs (Grobelny et al., 1992; Levy et al., 1998), on implantation and placentation in wild-type and *Plg*-deficient mice.

MATERIALS AND METHODS

Inhibitor

The MMP inhibitor N-[(2R)-2-(hydroxamido-carbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (galardin) was synthesized as described (Grobelny et al., 1992). Galardin inhibits the enzymatic activity of a number of MMPs, including MMP2, MMP3, MMP9 and MMP14 (Grobelny et al., 1992; Levy et al., 1998).

Animals and tissue treatment

The mice used in these experiments were *Plg* gene targeted mice of a mixed 129/Black Swiss background (Bugge et al., 1995a) backcrossed to NIHS outbred mice for 8–12 generations. For breeding of experimental animals, heterozygous $Plg^{+/-}$ mice were used as breeding pairs. In all experiments the wild-type control mice were littermates to the *Plg*-deficient mice. Stud males were littermates to the female experimental mice. Genotyping of the *Plg* alleles was performed as described (Bugge et al., 1996). To generate embryos of known genotype in mothers of the same genotype, homozygous $Plg^{-/-}$ and $Plg^{+/+}$ females were mated to homozygous $Plg^{-/-}$ and $Plg^{+/+}$ males, respectively, with proven fertility and checked for pregnancy by occurrence of vaginal plugs in the morning [midnight=0 days post-coitum (dpc)]. Galardin was administered intraperitoneally at 150 mg/kg body weight as a 30 mg/ml slurry in 4% carboxymethylcellulose (CMC) in PBS. Injections began at 3.5 dpc and were administered every 24 hours thereafter until 13.5 dpc. The mice used for histological analysis were anesthetized by injection of 0.03 ml/10 g of a 1:1 mixture of Dormicum (Midazolam, 5 mg/ml) and Hypnorm (Fluanison, 5 mg/ml and Fentanyl, 0.1 mg/ml). The mice were perfused intracardially with 10 ml ice-cold PBS followed by 10 ml 4% paraformaldehyde (PFA). The uteri were removed, the number of implantation and/or resorption sites was determined macroscopically and tissues were fixed overnight in 4% PFA. The tissue was dehydrated and embedded in paraffin wax. Animal care at the University of Copenhagen and Rigshospitalet, Copenhagen, Denmark was in accordance with national and institutional guidelines.

Antibodies for immunohistochemistry

Rabbit polyclonal antibodies (pAb) raised against the mouse receptor for urokinase-type plasminogen activator (uPAR) (Solberg et al., 2001) were used at a concentration of 10 μg/ml. Rabbit pAb against fibrin/fibrinogen were used at a dilution of 1:1000, as described previously (Bugge et al., 1995a; Suh et al., 1995). CD-34 rat monoclonal antibody (mAb) (clone MEC 14.7) (Baumhueter et al., 1993) was obtained from HyCult Biotechnology, Uden, Netherlands.

Immunohistochemistry

Paraffin wax-embedded sections were deparaffinized in xylene, hydrated through graded ethanol to aqueous solution and digested with 0.03% trypsin for 10 minutes at 37°C, blocked in 5% swine serum in TBS with 0.25% BSA (TBS-BSA) and incubated with the primary antibody in TBS-BSA. Then a biotin-conjugated swine-anti-rabbit pAb (DAKO E431) was applied, followed by AP-ABC (DAKO K 0376). Slides were developed with Fast Red (DAKO) and counterstained with Mayers Hematoxylin. As negative controls, we used pre/non-immune

rabbit IgG. As a control for the specificity of the staining obtained for uPAR we used analogous tissue from uPAR-deficient mice (Bugge et al., 1995b).

In situ hybridization

^{35}S-labeled RNA sense and antisense probes were generated by in vitro transcription from subclones of the following mouse cDNA: MMP9, MMP14, the spongiotrophoblast marker 4311, placental lactogen 1 (PL1) and PECAM, as described (Lund et al., 1996; Lund et al., 1999; Rømer et al., 1996; Baldwin et al., 1994; Colosi et al., 1987; Lescisin et al., 1988). Tissue sections were deparaffinized in xylene, hydrated through graded ethanol/water dilutions and the in situ hybridization was carried out as described (Lund et al., 1996). For each mRNA, expression was detected with two non-overlapping probes with identical results. Sense probes were included in all experiments and in all cases gave negative results.

Statistical analysis

Statistical evaluation of differences between groups of mice was done with the Wilcoxon rank sum test.

RESULTS

Embryonic and perinatal lethality in plasminogen-deficient mice treated with MMP inhibitor

To test if there is a functional overlap between plasmin and the MMPs during implantation and placentation, as has been shown during wound healing (Lund et al., 1999), *Plg*-deficient mice were treated with galardin (150 mg/kg) from 3.5 dpc until 13.5 dpc. Initial experiments showed that treatment of wild-type mice with galardin until 18.5 dpc led to severe parturition problems, where the mothers were unable to deliver the pups (L.R.L., unpublished). Therefore the galardin treatment in all experiments was stopped at day 13.5 dpc. As controls, littermate wild-type mice were treated with galardin and mice of each genotype were also mock treated with the CMC vehicle alone. The number of pups was counted immediately after birth and 24 hours thereafter. The number of mice that delivered pups was significantly lower in galardin treated *Plg*-deficient mice (2/8) than in mock-treated wild-type mice (7/7), mock-treated *Plg*-deficient mice (7/7) and galardin treated wild-type mice (5/6) (Table 1). In addition, the number of pups in each litter from the galardin treated *Plg*-deficient mice was only about one third the number of the pups in each litter from the other three groups of mice (Table 1). Furthermore, the 24 hour survival of the pups born by either wild-type or *Plg*-deficient mice treated with galardin was much lower (app 40%) than the survival of pups born by mice of either genotype that were mock treated (100%). No milk was observed in the stomachs of any of the pups born to mice treated with galardin, suggesting that an inability to lactate might be the cause of the high death rate of the newborn pups in these two groups of mice. Indeed, histological analysis of mammary glands 7.5 and 12.5 dpc revealed delayed development of the lactational phenotype with delayed development of the secretory epithelium as well as an abnormal adipocyte and connective tissue stroma in galardin-treated mice of both genotypes, and to a lesser extent also in mock-treated *Plg*-deficient mice (data not shown).

The uteri were examined 24 hours after the mice had given birth. In mice of both genotypes treated with galardin, the uteri contained a number of apparently normally developed pups that had not been delivered (Table 1B,C). In addition, the uteri in seven out of eight *Plg*-deficient mice but only one out of six wild-type mice treated with galardin, contained a number of resorption sites. The uteri of mock-treated mice of either genotype did not show any resorption sites (Table 1). Persistence of the resorption sites in the uteri 24 hours after term indicates fetal deaths that have occurred at a late stage of pregnancy. Similar results were obtained in a separate experiment that included only two groups of mice: *Plg*-deficient mice and wild-type mice both treated with galardin as described above. Of the pregnant wild-type mice treated with galardin

in this experiment seven out of eight mice delivered pups, whereas none of the nine pregnant *Plg*-deficient mice treated with galardin delivered pups. Thus, the combination of *Plg*-deficiency and MMP inhibition had a synergistic effect and resulted in a high rate of embryonic lethality.

Expression of uPAR and MT-1-MMP during implantation and placentation

The localization of protease activities is determined in part by the location of their receptors. Two molecules that could localize plasmin and MMP activity are the receptor for urokinase plasminogen activator and the membrane-type MMP MT1-MMP or MMP14. We therefore next examined the expression of uPAR by immunohistochemistry, using highly specific polyclonal antibodies against mouse uPAR (Solberg et al., 2001). We also examined MT1-MMP expression by in situ hybridization. Localization studies were done on sections of implantation sites at 7.5, 8.5, 10.5 and 12.5 dpc.

In 7.5 dpc implantation sites, uPAR staining was seen in the maternal vessels, as well as in the decidua cells, with the most intense uPAR immunoreactivity in the less differentiated of these cells towards the peripheral zone of the decidua (Fig. 1C). Weak to moderate uPAR staining was also seen in the trophoblast cells, identified by staining for cytokeratin (data not shown). No staining was seen with either pre-immune rabbit IgG (Fig. 1D) or with the polyclonal immune IgG on equivalent tissue obtained from uPAR-deficient mice (data not shown). The same uPAR staining pattern was seen at 8.5 dpc implantation sites (Fig. 1G,O). At 12.5 dpc implantation sites the remaining mesometrial decidua cells (Fig. 1K), as well as the giant trophoblast cells, identified by PL1 expression were positive for uPAR immunoreactivity (Fig. 1K, and data not shown). uPAR staining was also seen in the maternal vessels and in the spongiotrophoblast cells, identified by detection of mRNA for the spongiotrophoblast marker 4311 (Fig. 1K, and data not shown). uPAR staining was also detected in the maternal vessels, as well as in the embryonic vessels present in the labyrinth layer. The embryonic vessels were easily recognized by their content of nucleated red blood cells (data not shown).

In 7.5 and 8.5 dpc implantation sites MT1-MMP (MMP-14) mRNA was detected in the peripheral zone of the undifferentiated decidual cells, as well as in cells bordering the remains of the uterus lumen. MT1-MMP was also expressed in the maternal vessels in the decidua (Fig. 1A,E,M). In addition, at 8.5 dpc a few cells scattered at the mesometrial side close to the embryo contained mRNA for MT1-MMP. These cells were not a subgroup of the PL-1 positive giant trophoblast cells, but probably a subgroup of highly differentiated decidual cells (data not shown). At later stages of the development of the placenta (10.5 and 12.5 dpc), MT1-MMP was still expressed by the undifferentiated decidual cells (data not shown), as well as in the maternal vessels (Fig. 1I). In addition, MT1-MMP mRNA was detected in cells lying in close proximity to spongiotrophoblast cells (Fig. 1I).

Delayed decidualization and angiogenesis at 7.5 dpc in *Plg*-deficient mice treated with galardin

We next examined the impact of galardin treatment on embryo implantation sites in *Plg*-deficient and littermate wild-type mice. Plugged female mice were either treated daily with 150 mg/kg of galardin or mock treated from 3.5 dpc until they were sacrificed at 7.5 dpc. In histological sections, all implantation sites from mock-treated wild-type mice had an elongated egg shape, whereas 33% of the implantation sites from galardin-treated wild-type mice and *Plg*-deficient mice and 60% of the implantation sites from galardin-treated *Plg*-deficient mice were rounded instead of egg shaped (Table 2 and Fig. 2A, parts a-d). Morphometric analysis revealed that the decidual length was significantly shorter in the mock-treated *Plg*-deficient mice than in the mock-treated wild-type mice ($P<0.001$), whereas galardin-treatment in both genotypes led to only moderately and non significantly shorter decidual length ($P>0.05$ for

both genotypes). The most pronounced difference was however seen between the galardin-treated *Plg*-deficient mice and the mock-treated wild-type mice ($P<0.0001$) (Fig. 2B).

Examination of implantation sites also revealed that secondary trophoblast giant cells showed an altered spatial distribution. These giant cells, which are marked by expression of MMP-9 mRNA, were closer to the ectoplacental cone in implantation sites from galardin-treated *Plg*-deficient mice compared with implantation sites from the other three groups of mice where the trophoblast giant cells are located at the border to the decidua (Fig. 3A, parts a–d). These results imply that galardin treatment in a plasminogen-deficient background may alter giant cell migration, or inhibit the differentiation of stem cells in the ectoplacental cone to secondary trophoblast giant cells.

Since fibrin is a major substrate for plasmin and some MMPs can degrade fibrin (Bini et al., 1996; Rømer et al., 1996; Hiraoka et al., 1998; Lund et al., 1999; Lelongt et al., 2001), we hypothesized that fibrin accumulation might be increased in implantation sites from galardin treated animals. However, there were no differences in the amount of immunoreactive fibrin seen in implantation sites from mock-treated wild-type versus galardin-treated *Plg*-deficient mice (Fig. 3A, parts e–h).

MMP-9 and plasminogen are both involved in angiogenesis (Pepper, 2001). We observed that 60% of the 7.5 dpc implantation sites from galardin-treated *Plg*-deficient mice had a decreased decidual vascularization compared with mock-treated *Plg*-deficient mice. The endothelial cells were identified by their expression of PECAM mRNA by situ hybridization (Fig. 3A, parts i–n, Table 2).

Retarded embryonic growth at 8.5 dpc in *Plg*-deficient mice treated with galardin

To study the effect of galardin and *Plg*-deficiency at later stages of implantation, we treated mice of both genotypes daily with galardin or vehicle alone from 3.5 dpc to 8.5 dpc, and then analyzed the implantation sites. Only slight differences in the size and shape of the decidua were observed in implantation sites from the four groups of mice (Fig. 2, parts e–h). At 8.5 dpc, 50% of the embryos from *Plg*-deficient mice treated with galardin were runted and resembled the mock-treated wild-type embryos at the 7.5 dpc developmental stage (Table 2, Fig. 2, parts e–h). Although MMP-9 and PL-1 mRNA were detected in the trophoblast giant cells of implantation sites from all four groups of mice (Fig. 3B, parts a–d), the expression pattern of MMP-9 in galardin-treated *Plg*-deficient mice 8.5 dpc was characteristic of the pattern of mock-treated wild-type mice 7.5 dpc (compare Fig. 3B, part d and Fig. 3A, part a). The vascularization of the decidua in 8.5 dpc galardin-treated *Plg*-deficient mice, detected by PECAM expression (Fig. 3B,n), was also like that seen in 7.5 dpc mock-treated wild-type implantation sites, indicating that the interaction of the embryos with the maternal vasculature was delayed in the galardin-treated *Plg*-deficient mice and probably contributed to the phenotype observed at 8.5 dpc.

Underdeveloped labyrinth layer and abnormal pattern of giant trophoblast cells in 12.5 dpc placentas from galardin-treated *Plg*-deficient mice

Sections of 12.5 dpc placentas from *Plg*-deficient and wild-type mice treated daily with galardin from 3.5 dpc to 12.5 dpc revealed poorly associated loose tissue in all layers of the placenta. Additionally, the decidual and spongiotrophoblast layers failed to remain associated and separated at collection. By contrast, mock-treated *Plg*-deficient and wild-type mice did not exhibit separation of the placental tissue layers (Fig. 2, parts i,j and Table 3). These findings suggest that MMPs have an important role in promoting proper adhesion between the cells of these tissue layers. Although both galardin treated wild-type and *Plg*-deficient mice exhibited placental separation, most of the wild-type embryos were viable, whereas 38% of the

implantation sites from the galardin-treated *Plg*-deficient mice either did not contain an embryo or contained a growth retarded embryo. Therefore, it is likely that the placental separation observed after galardin treatment had occurred during embryo collection. However, the placental fragility and poor association of the tissues may have contributed to the growth retardation and reduced placental development observed in the galardin-treated *Plg*-deficient mice (Fig. 2, parts i–l and Table 3).

We then analyzed whether trophoblast differentiation and/or migration was altered in the 12.5 dpc placentas of galardin-treated *Plg*-deficient mice. The expression pattern of mRNA for 4311, a marker for spongiotrophoblast cells, was virtually unaltered with galardin treatment, *Plg*-deficiency and their combination (Fig. 4, parts a–d and Table 3), suggesting that the embryo was viable. However, half of the placentas from galardin-treated *Plg*-deficient mice showed altered expression and spatial distribution of mRNA for the trophoblast giant cell marker PL-1, compared with mock-treated wild type mice and mock-treated *Plg*-deficient mice. In the affected placentas, PL-1 positive secondary trophoblast giant cells were found to accumulate both in the middle of the placenta as well as in an unusual broad band of trophoblast giant cells migrating towards the periphery. *Plg*-deficiency and MMP inhibition thus act synergistically to cause a migration or differentiation defect (Fig. 4H,L). The remaining galardin-treated *Plg*-deficient embryos and all galardin-treated wild-type embryos had a similar PL-1 expression to the mock-treated wild-type embryos (Fig. 4E–L and Table 3).

Placentas from galardin-treated *Plg*-deficient mice had a considerably and statistically highly significantly less developed and thinner labyrinth layer than those from mock-treated wild-type mice ($P<0.0001$), whereas there was no significant difference with respect to this parameter between mock-treated *Plg*-deficient mice and mock-treated wild-type mice or between galardin-treated wild-type mice and mock-treated wild-type mice ($P>0.05$ in both cases) (Fig. 2A, parts i–l). This shows that also with respect to this parameter there is synergy between *Plg* deficiency and galardin treatment.

In the galardin- and mock-treated wild-type placentas, as well as in the mock-treated *Plg*-deficient placentas, the fetal vessels were uniformly distributed throughout the labyrinth as shown by immunohistochemical staining of CD34 (Fig. 4N; not shown) and by in situ hybridization for PECAM mRNA (not shown). However, in the galardin-treated *Plg*-deficient placentas, fetal blood vessels were abnormal in a third of all placentas and not detected in the labyrinth layer by either CD34 staining (Fig. 4Q) or PECAM mRNA in situ hybridizations (not shown), indicating that *Plg*-deficiency and galardin treatment synergistically impair the fetal vessel formation. The defective vascularization of the labyrinth placenta may reduce feto-maternal exchange of gases and nutrients, causing growth retardation and, in the severely affected placentas, embryonic death. In the remaining placentas an apparent normal vessel system was observed as detected by gross histological analysis at day 12.5 dpc.

In the 12.5 dpc placentas from galardin-treated mice of both genotypes and from mock-treated *Plg*-deficient mice a large amount of fibrin was detected in the middle of the placenta surrounding the central artery. However, no increase in the amount of fibrin(ogen) was observed in the rest of the placental tissue (Fig. 4O,R). It is unlikely that impaired fibrin degradation is a primary cause of the phenotype of the placentas from galardin-treated *Plg*-deficient mice.

DISCUSSION

Our results demonstrate that the combined action of PLG and MMPs is essential for proper placental development and function. In many studies, either MMPs or the plasminogen cascade have been shown to regulate angiogenesis (Pepper, 2001). Our data point to a synergistic

relationship between the two enzyme classes in regulation of placental vascularization. Although MMP inhibition alone was sufficient to alter the differentiation of the decidua as assessed by size and shape, loss of both MMPs and PLG was necessary to alter vascularization.

These data raise the question of how the two enzyme systems interact in vivo. In wild-type mice, uPA is expressed in maternal vessels in the decidua close to the embryo and in trophoblast cells of the ectoplacental cone (Teesalu et al., 1996). As shown in the present study uPAR is expressed in the decidua cells, in the maternal vessels and in trophoblast cells, whereas MMP-9 and mColA are also expressed in the giant trophoblast cells (Alexander et al., 1996; Balbin et al., 2001). In contrast to an earlier report based on immunohistochemical analysis of cultured blastocysts, we found that MT1-MMP was expressed in maternal vessels in the decidua, in cells surrounding the remnant of the uterine lumen and in the undifferentiated decidua, but not in giant cells as described (Tanaka et al., 1998). MMP2, MMP3 and MMP11 are all expressed in the undifferentiated decidua (Alexander et al., 1996; Teesalu et al., 1999). A delay in the development of the decidua in response to protease inhibition by galardin has been demonstrated previously (Alexander et al., 1996), and could arise as a result of inhibition of several enzymes. Our results demonstrate that although inhibition of either MMPs or loss of plasminogen leads to an initial effect on decidualization at 7.5 dpc, none of the embryos in these groups of mice show signs of growth retardation. Therefore, deficiency of neither plasminogen nor MMPs alone impairs normal embryonic and placental development, probably owing to a functional overlap between the two classes of proteases (Lund et al., 1999). This redundancy may be extensive as mice deficient for both uPA and MMP11 have normal embryonic and placental development and are fertile (Teesalu et al., 1999). Likewise, mice deficient for MMP9 and PLG are viable and fertile (L.R.L., unpublished). However, this is not surprising given the role of galardin as a broad-spectrum inhibitor (Grobelny et al., 1992; Levy et al., 1998). Furthermore, each protease might have multiple and distinct functions during various stages of implantation and placental development, as well as during embryogenesis. Future genetic experiments and/or access to inhibitors specific for individual MMPs will be required to identify the MMPs involved and elucidate their functional roles and interactions with plasminogen (Johnsen et al., 1998).

The placentas from galardin-treated *Plg*-deficient mice exhibited improper vascularization and development of the labyrinth layer. The width of the labyrinth layer of placentas in galardin-treated *Plg*-deficient mice is significantly less than in the other three groups of mice. The development of the labyrinth layer is essential for embryo survival because after gestation day 10.5, all exchange of gases and nutrients takes place there. Failure to establish a proper feto-maternal circulatory system eventually will result in death and resorption of the embryo (Cross et al., 1994). Histological analysis revealed that the entire labyrinth structure in galardin-treated *Plg*-deficient mice was abnormal, both trophoblast differentiation and villous morphogenesis appeared to be defective. As fetal vessels grow into the branched villous as a secondary event, the observed lack of vessels in the labyrinth may be a secondary effect (Rossant and Cross, 2001). This phenotype is seen only in galardin-treated *Plg*-deficient mice; thus, the result strongly suggests that both plasmin and MMP activities directly or indirectly are involved in vascularization of the labyrinth, and that there is a functional overlap between the two types of activities, so that each of them alone is adequate for proper vascularization. Expression studies, showing co-localization of mRNA for uPA and MMP9 10.5 dpc in giant trophoblast cells at the fetomaternal interface (Teesalu et al., 1998; Teesalu et al., 1999) support this hypothesis. However, the functional implication of co-expression of uPA and MMP-9 can be definitively clarified only by the generation of mice deficient for both uPA and MMP9. The giant trophoblast cells were also shown to express uPAR by immunohistochemical staining, thus enabling a focalized uPA-catalyzed proteolysis at the fetomaternal interface. The functional importance of this is shown by in situ zymography data reported by Teesalu et al. (Teesalu et al., 1998), demonstrating in situ uPA-dependent proteolytic activity.

The combined loss of plasminogen and MMP inhibition also affected the normal development of the trophoblast giant cell. At 12.5 dpc the placentas from galardin-treated *Plg*-deficient mice displayed abnormal spatial distribution of trophoblast giant cells, which remained accumulated in the middle of the placenta in a condensed cluster of PL1-positive cells; in addition an abnormal multicellular layer of trophoblast giant cells was observed towards the periphery of the placenta. This finding indicates a potential defect in the migration or differentiation of these cells as a result of MMP-inhibition and lack of plasminogen. However, to distinguish clearly between a differentiation and/or migration defect future studies with additional and more specific markers (Adamson et al., 2002) will be required. A defect in giant cell migration is in accordance with previous in vitro studies of cultured blastocysts, where galardin treatment inhibited the migration of the trophoblast cells (Librach et al., 1991; Behrendtsen et al., 1992).

The placentas from galardin-treated mice of both genotypes separated during collection between the decidual layer and the spongiotrophoblast layer. This could be due to dead or dying tissue at the interface or to accumulation of maternal blood at the interface which is unable to enter into the labyrinth layer. However, it is tempting to speculate that cell-cell or cell-matrix adhesion of these layers was abnormal. Inhibition of MMPs or lack of plasminogen leads to abnormal adhesion and migration of cells. Cleavage of laminin-5 α-chain by plasmin has been shown to be important for the formation of hemidesmosomes of cells in vitro (Goldfinger et al., 1998). Furthermore, cleavage of laminin-5 γ-chain by MMP2 (Giannelli et al., 1997) and MT1-MMP (Koshikawa et al., 2000) induces migration of cells cultured on laminin 5. The mechanism for cell-cell and cell-matrix adhesion in placenta has not been elucidated in the present study, but abnormal adhesion of the cell layers due to malformation of hemidesmosomes may prevent adequate development of the maternal-fetal interface, resulting in growth retardation or fetal death.

Even though the galardin treatment was stopped after 13.5 dpc the placental insufficiency persisted and pups that reached parturition had high perinatal mortality. Malformation of placentas is known to result in intrauterine growth retardation and birth of runted embryos with poor survival prospects. Moreover, MMPs and PLG are necessary for the normal development of the mammary gland (Witty et al., 1995; Thomasset et al., 1998; Lund et al., 2000). Thus, the observed lack of milk in pup stomachs indicates that the poor postnatal survival rate of pups delivered by galardin-treated mice of both genotypes might also be due to impaired lactation resulting from impaired mammary development.

Mutation of almost every gene that regulates angiogenesis or vascular development results in placental defects of varying severity (Rossant and Cross, 2001; Adamson et al., 2002). Both MMPs and plasminogen have a role in angiogenesis in a number of developmental and pathological processes (Pepper, 2001). Our data show that MMPs and the plasminogen system synergize during the development of vascular connections between the mother and fetus. Placental development is a highly sensitive indicator of the genes involved in vascular development. The challenge remains to elucidate the molecular targets of these proteases that direct each stage of trophoblast and placental development.

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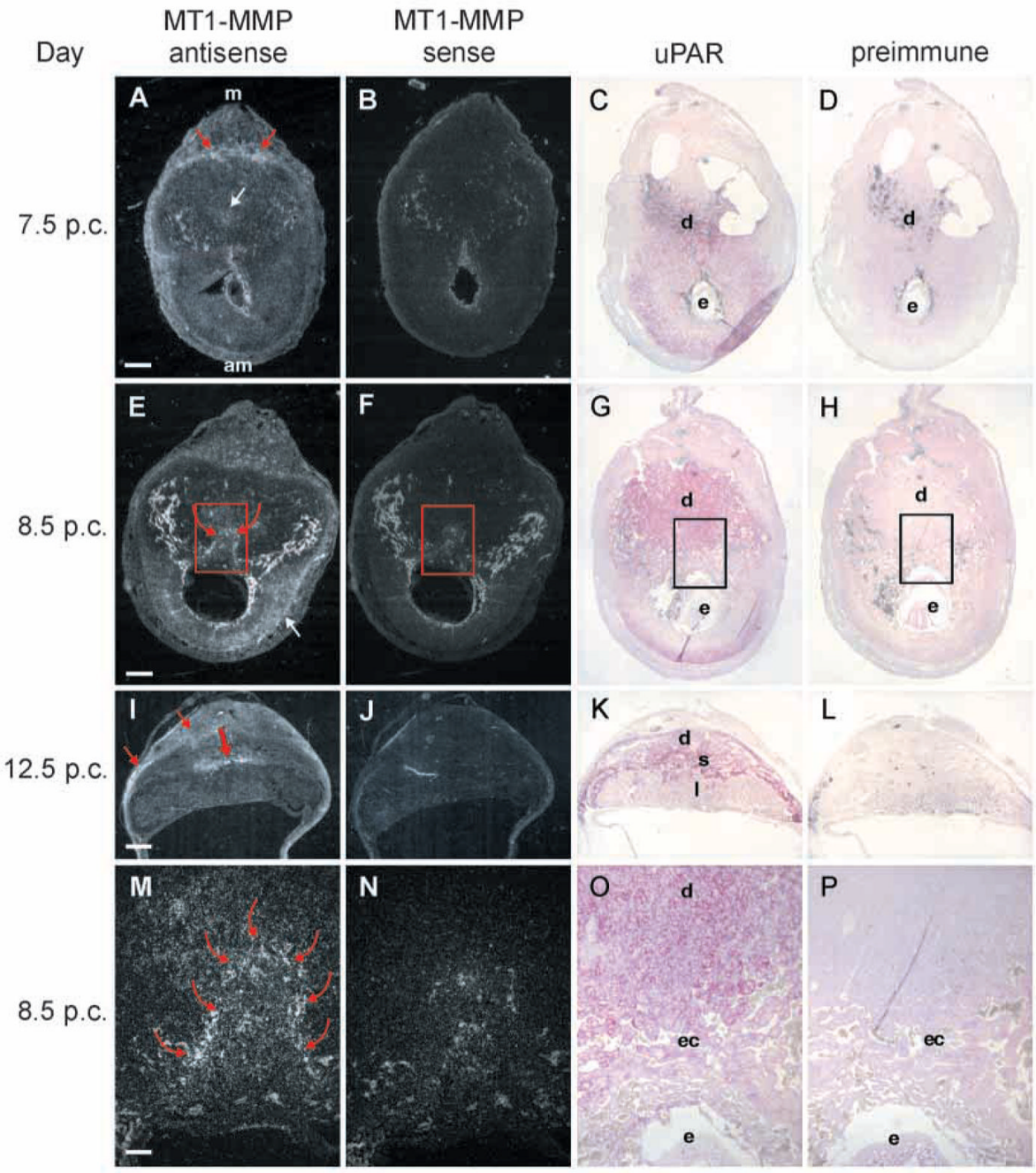


Fig. 1.

Expression of MT1-MMP and uPAR during mouse embryo implantation. Implantation sites (7.5 dpc and 8.5 dpc) and 12.5 dpc placentas from wild-type mice were analyzed by in situ hybridization for MT1-MMP mRNA (A,E,I,M) and immunohistochemistry for uPAR (C,G,K,O). As controls, a sense probe for MT1-MMP (B,F,J,N) and preimmune rabbit IgG (D,H,L,P), were included. At 7.5 (A) and 8.5 (E) dpc MT1- MMP signal was seen in cells bordering the remnants of the uterus lumen (white arrows), as well as in the undifferentiated decidua cells and the maternal vessels (thin red arrows). In addition, at 8.5 dpc a few cells present at the mesometrial side close to the embryo contained mRNA for MT1-MMP (curved red arrows). At later stages of placental development (12.5 dpc), MT1-MMP was still expressed

by the undifferentiated decidual cells, as well as in the maternal vessels (thin red arrows). In addition, MT1-MMP mRNA was detected in cells lying in close proximity to spongiotrophoblast cells (bold red arrow) (I). At 7.5 dpc (C) and 8.5 dpc (G) uPAR immunoreactivity was detected in the decidual cells, as well as in the maternal vessels. Weak staining could also be seen in the trophoblast cells. At 12.5 dpc, placentas the remaining mesometrial decidua cells, as well as the giant trophoblast cells, were positive for uPAR immunoreactivity. uPAR staining was also seen in the maternal and embryonic vessels as well as in the spongiotrophoblast cells (K). The areas boxed in E-H are shown at higher magnification in M-P, respectively. am, antimesometrial; d, decidua; e, embryo; ec, ectoplacental cone; l, labyrinth layer; m, mesometrial; s, spongiotrophoblast layer. Scale bars: 500 μm in A-L; 100 μm in M-P.

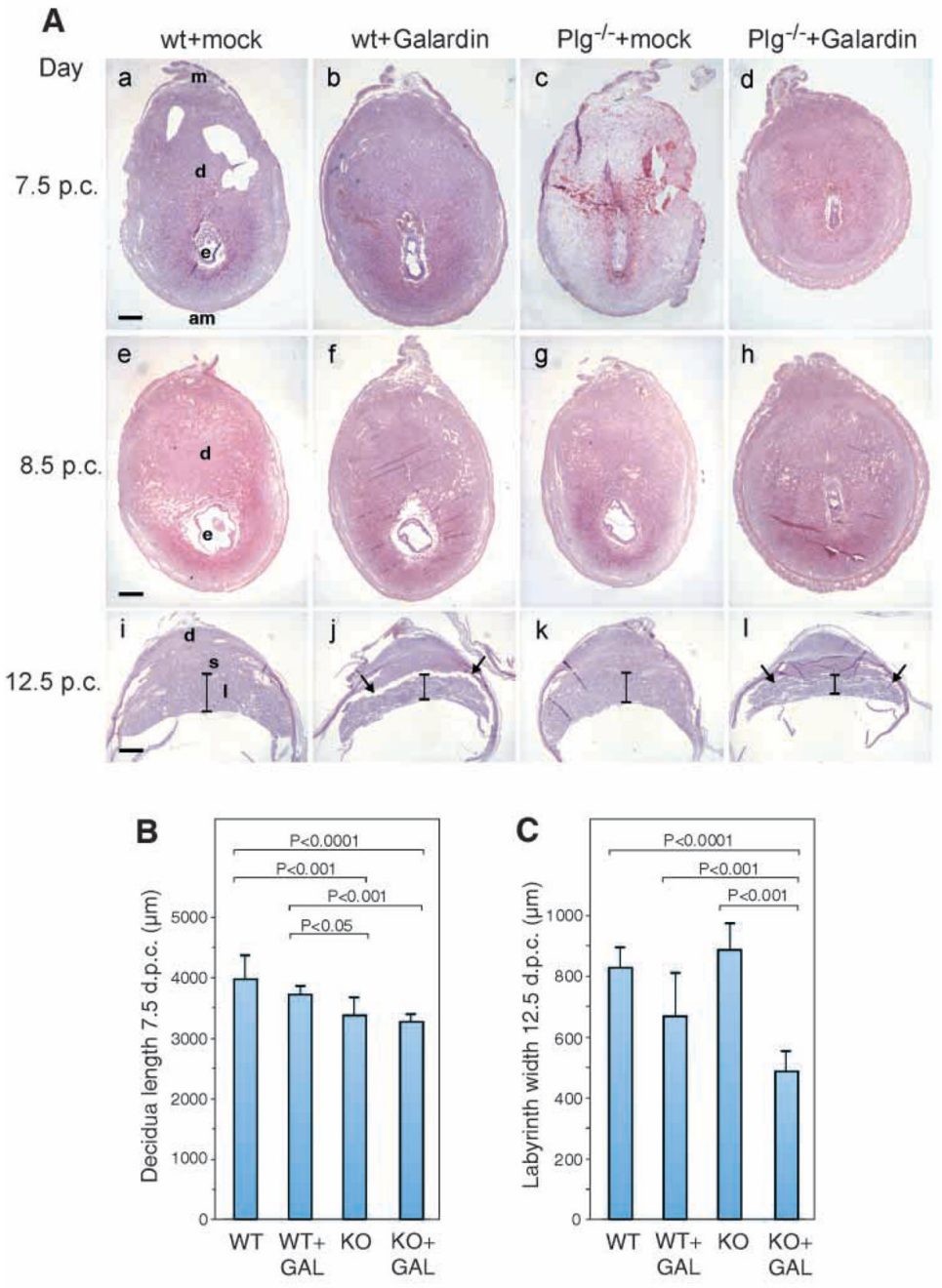
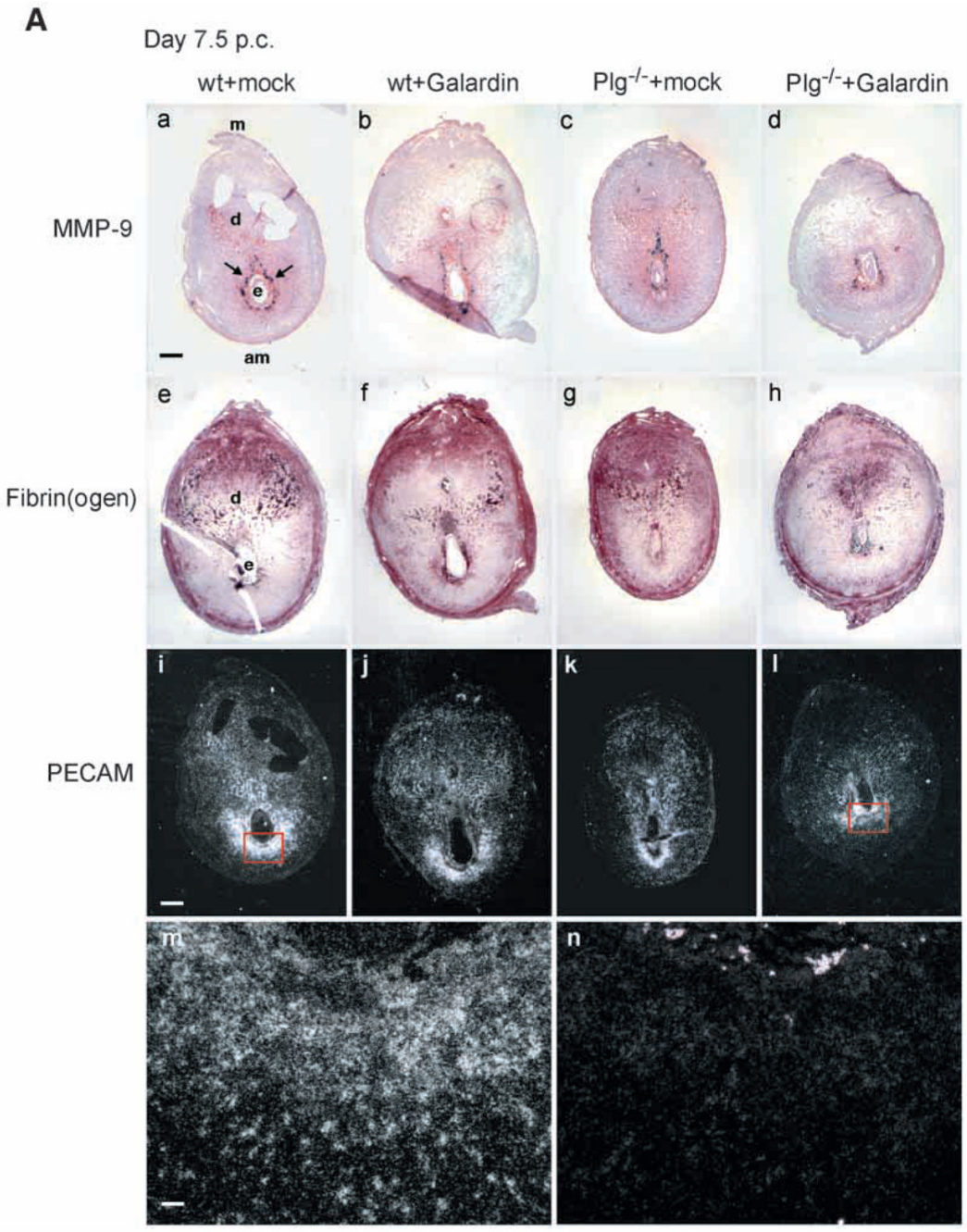


Fig. 2.

Effect of galardin treatment on 7.5 and 8.5 dpc implantation sites and 12.5 dpc placenta from wild-type and *Plg*-deficient mice. (A) Hematoxylin and Eosin stained sections of 7.5 and 8.5 dpc implantation sites and 12.5 dpc placentas from mock-treated wild-type mice (parts a,e,i), galardin-treated wild-type mice (parts b,f,j), mock-treated *Plg*-deficient mice (parts c,g,k) and galardin-treated *Plg*-deficient mice (parts d,h,l). Typical implantation sites from mock-treated wild-type mice and most of the galardin-treated wild-type and *Plg*-deficient mice had an elongated egg shape (parts a–c), whereas 60% of the implantation sites from the galardin-treated *Plg*-deficient mice were round (part d) (as they were in 33% of the mock-treated *Plg*-deficient mice and the galardin-treated wild-type mice). At 8.5 dpc, 50% of the embryos from

galardin-treated *Plg*-deficient mice were runted and at a developmental stage resembling 7.5 dpc embryo from mock-treated wild-type mice (part h). Sections of 12.5 dpc placentas from *Plg*-deficient and wild-type mice treated with galardin revealed that the tissue was loosely associated and the decidua and spongiotrophoblast layers separated upon collection (arrows in parts j,l). Placentas from galardin-treated *Plg*-deficient mice had a less developed and thinner labyrinth layer than in mock- and galardin-treated wild-type and in *Plg*-deficient mice. Length of bars (in parts i–l) indicates the size of the labyrinth layer. (B) Histograms of the decidua length at 7.5 dpc±s.d. for each genotype.
(C). Histograms of the labyrinth width at 12.5 dpc± s.d. for each genotype. am, antimesometrial; d, decidua; e, embryo; ec, ectoplacental cone; l, labyrinth layer; m, mesometrial; s, spongiotrophoblast layer. Scale bar: 500 μm.



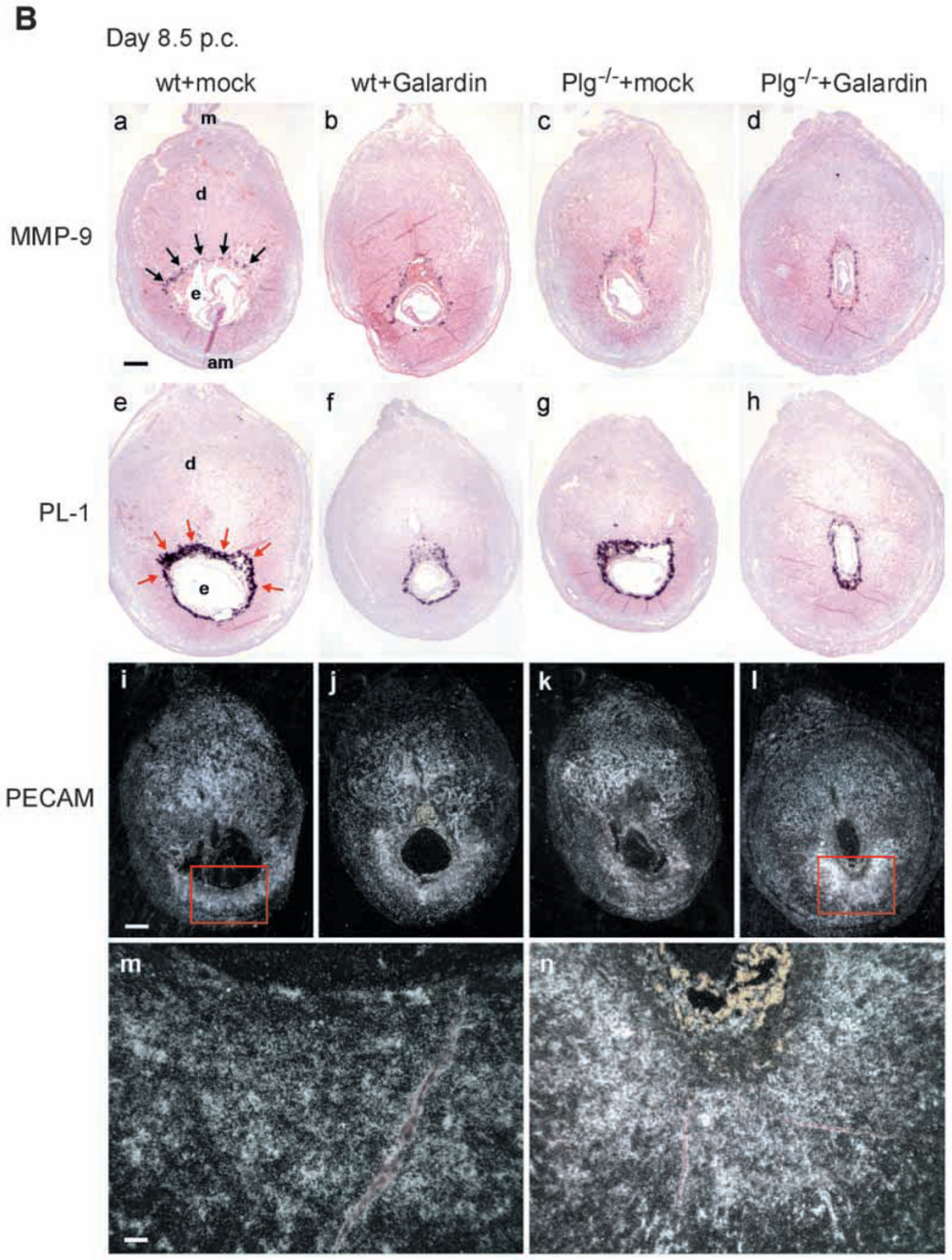


Fig. 3.

MMP9 expression, fibrin accumulation, and vascularization in implantation sites from galardin-treated wild-type and *Plg*-deficient mice. (A) At 7.5 dpc in situ hybridization for MMP9 mRNA showed expression in the giant trophoblast cells (black arrows). Few giant trophoblasts were observed in the ectoplacental cone in 7.5 dpc implantation sites from galardin-treated *Plg*-deficient mice (part d) compared with implantation sites from mock- and galardin-treated wild-type mice and from mock-treated *Plg*-deficient mice (parts a–c). Fibrin (ogen) was detected by immunohistochemical staining using rabbit anti-fibrin(ogen) (parts e–h). No difference in the fibrin(ogen) staining pattern was observed among implantation sites from the four groups of mice. Endothelial cells were detected by in situ hybridization for

PECAM mRNA (parts i–l). A decrease in the vascularization was seen in implantation sites from some galardin-treated *Plg*-deficient mice (part l), when compared with implantation sites from mock- and galardin-treated wild-type mice and from mock-treated *Plg*-deficient mice (parts i–k) (see Table 2). The areas boxed in parts i and l are shown at higher magnification in parts m and n, respectively. am, antimesometrial; d, decidua; e, embryo; m, mesometrial. Scale bar: 500 μm in parts a–l; 50 μm in parts m,n. (B) Expression of MMP-9 at 8.5 dpc was detected by in situ hybridization (parts a–d) and was present in the giant trophoblast cells (black arrows) that are identified by their expression of PL-1 mRNA (red arrows) (parts e–h). The spatial distribution of the giant trophoblast cells in 8.5 dpc implantation sites from galardin-treated *Plg*-deficient mice (part d), was similar to that of mock-treated wild-type mice 7.5 dpc implantation sites (data not shown). Endothelial cells were detected by in situ hybridization for PECAM mRNA (parts i–l). The vascularization of the decidua in 8.5 dpc galardin-treated *Plg*-deficient mice (parts l,n) was like that found at 7.5 dpc mock-treated wild-type mice (Fig. 3A, parts i,m). The areas boxed in parts i and l are shown at higher magnification in parts m and n, respectively. am, antimesometrial; d, decidua; e, embryo; m, mesometrial. Scale bar: 500 μm in parts a–l; 100 μm in parts m,n.

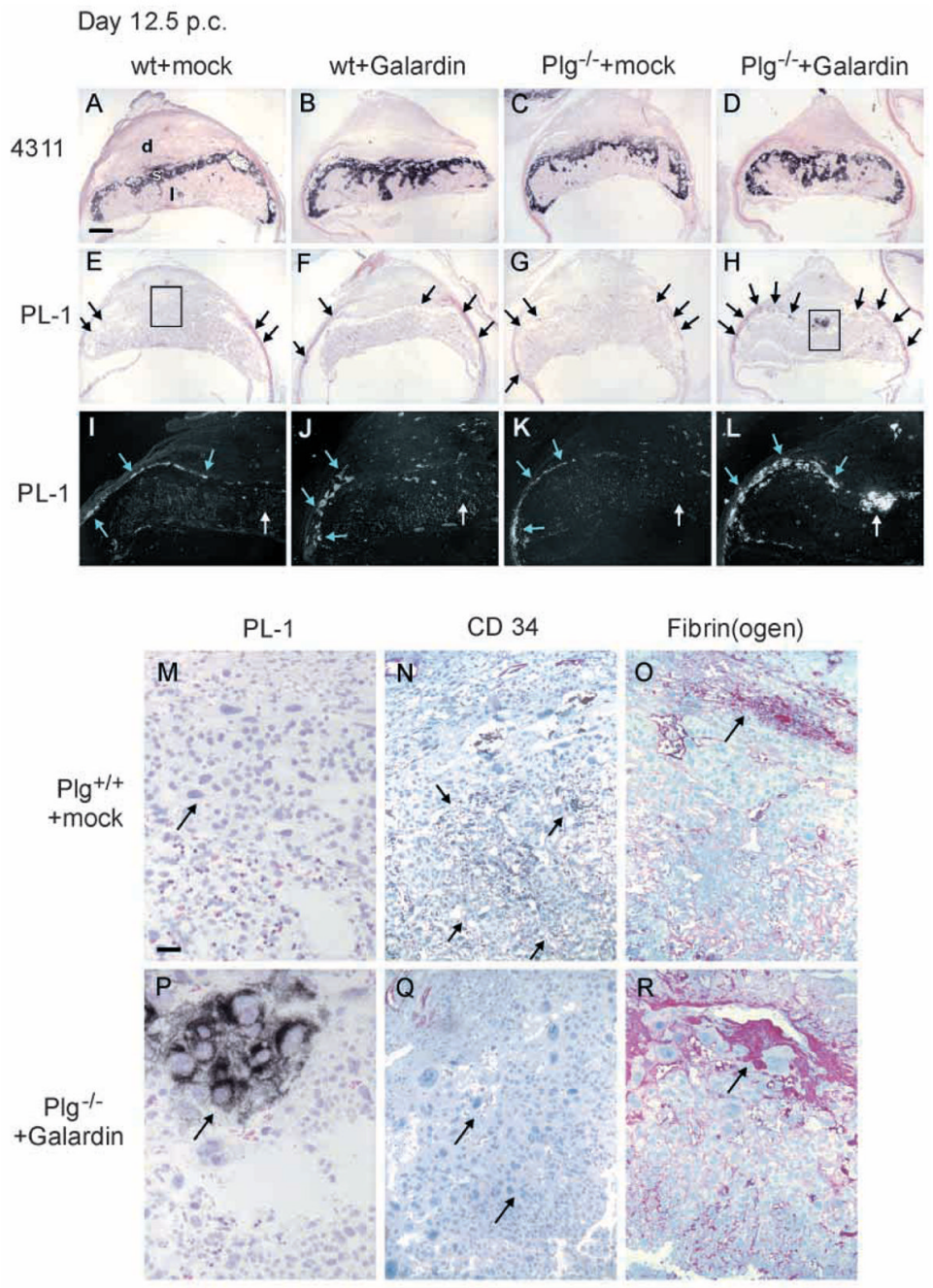


Fig. 4.

Effect of galardin treatment on 12.5 dpc placentas from wild-type and galardin-treated *Plg*-deficient mice. No difference in the distribution of the spongiotrophoblast cells identified by 4311 expression is observed between placentas from galardin-treated *Plg*-deficient mice (D) and placentas from mock- or galardin-treated wild-type mice or mock-treated *Plg*-deficient mice (A–C). Trophoblast giant cells were identified by in situ hybridization for PL-1 mRNA (brightfield, E–H,M,P; darkfield, I–L). The spatial distributions of PL1-positive cells in the central part of the placenta are shown in higher magnification of the boxed area of placentas from galardin-treated *Plg*-deficient mice (H,P), or from mock-treated wild-type mice (E,M). In mock-treated wild-type mice (E,I,M), galardin-treated wild-type mice (F,J) and mock-

treated *Plg*-deficient mice (G,K) PL-1 positive trophoblast giant cells are primarily found towards the periphery of the placenta, whereas in galardin-treated *Plg*-deficient mice (H,L,Q) PL1-positive trophoblast giant cells are also found in the central part of the placenta (black and blue arrows). In I–L, white arrows point towards the central part of the placenta. Immunohistochemical staining for the endothelial cell marker CD34 (black arrows) (N,Q) revealed that the labyrinth layer in 33% of the placentas from galardin-treated *Plg*-deficient mice was non-vascularized (Q). Immunohistochemical staining for fibrin(ogen) in placentas from mock-treated wild-type mice (O) or from galardin-treated *Plg*-deficient mice (R) showed large amounts of fibrin deposition surrounding the central arteries in the middle of the placentas (black arrows). No other differences in the amount of fibrin deposition were observed in placentas from galardin-treated *Plg*-deficient mice, when compared with placentas from the other three groups of mice (O; data not shown).The areas boxed in E and H are shown at higher magnification in M–O and P–R respectively to demonstrate the distribution of the three different markers. D, decidua; L, labyrinth layer; S, spongiotrophoblast layer. Scale bar: 500 μm in A–H; 100 μm in I–N.

Table 1

Effect of galardin on embryonic and perinatal lethality in wild-type and plasminogen-deficient mice

	Number of pregnant mice	Mice that delivered pups	Total number of pups*	Average number of pups/pregnant mouse	Number of resorptions	Twenty-four hour survival of pups	Average number of pups/pregnant mouse surviving 24 hours
$Plg^{+/+}$ + mock	7	100% (7)	43	6.2	0	100%	6.2
$Plg^{-/-}$ + mock	7	100% (7)	49	7.0	0	100%	7.0
$Plg^{+/+}$ + galardin	6	83% (5)	44†	7.3	9	42%	3.1
$Plg^{-/-}$ + galardin	8	25% (2)	16‡	2.0	41	38%	0.8

*Includes fully developed pups born plus pups remaining in the uterus.

†Eight of these pups remain in the uterus of two mice 24 hours after birth of the first pups.

‡Eight of these pups remain in the uterus of four mice 24 hours after birth of the first pups.

Table 2

Analysis of 7.5 and 8.5 dpc implantation sites

	Implantation sites (7.5 dpc)				Implantation sites (8.5 dpc)		
	Number of mice	Number of sites examined*	Less vascularization	Round decidua	Number of mice	Number of sites examined*	Runted embryos†
$Plg^{+/+}$ + mock	4	7	0% (0)	0% (0)	3	9	0% (0)
$Plg^{-/-}$ + mock	4	6	16% (1)	33% (2)	4	8	0% (0)
$Plg^{+/+}$ + galardin	4	9	11% (1)	33% (3)	4	11	0% (0)
$Plg^{-/-}$ + galardin	3	10	60% (6)	60% (6)	4	8	50% (4)

*Subset of total number of sites.

†Number in brackets show the actual number of embryos examined having this phenotype.

Table 3

Analysis of 12.5 dpc implantation sites

	Number of miceexamined	Number of sites examined*	Separation of decidual/spongiotrophoblast†	Number of resorptions	Abnormal spongiotrophoblast†,‡	Abnormal giant trophoblast†,§	Non-vascular labyrinth†
$Plg^{+/+}$ + mock	3	9	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)
$Plg^{-/-}$ + mock	3	9	0% (0)	0% (0)	11% (1)	0% (0)	0% (0)
$Plg^{+/+}$ + galardin	3	11	100% (11)	18% (2)¶	9% (1)	0% (0)	0% (0)
$Plg^{-/-}$ + galardin	5	24	100% (24)	38% (9)**	4% (1)	46% (11)	33% (8)

*Subset of total number of sites.

†Numbers in brackets show the actual number of embryos examined with this phenotype.

‡Detected by expression of mRNA for the marker 4311.

§Detected by expression of mRNA for the marker PL1.

¶These sites were almost totally resorbed.

**These sites were either much smaller than 12.5 dpc (n=5) or deficient embryo (n=4).

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