Regulation of proteinases during mouse peri-implantation development: urokinase-type plasminogen activator expression and cross talk with matrix metalloproteinase 9

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Abstract

Trophoblast cells express urokinase-type plasminogen activator (PLAU) and may depend on its activity for endometrial invasion and tissue remodeling during peri-implantation development. However, the developmental regulation, tissue distribution, and function of PLAU are not completely understood. In this study, the expression of PLAU and its regulation by extracellular matrix proteins was examined by RT-PCR, immunocytochemistry, and plasminogen–casein zymography in cultured mouse embryos. There was a progressive increase in PLAU mRNA expression in blastocysts cultured on gestation days 4–8. Tissue-type plasminogen activator (55 kDa) and PLAU (a triplet of 40, 37, and 31 kDa) were present in conditioned medium and embryo lysates, and were adsorbed to the culture plate surface. The temporal expression pattern of PLAU, according to semi-quantitative gel zymography, was similar in non-adhering embryos and embryos cultured on fibronectin, laminin, or type IV collagen, although type IV collagen and laminin upregulated PLAU mRNA expression.

Immunofluorescence revealed PLAU on the surface of the mural trophectoderm and in non-spreading giant trophoblast cells. Exogenous human plasminogen was transformed to plasmin by cultured embryos and activated endogenous matrix metalloproteinase 9 (MMP9). Indeed, the developmental expression profile of MMP9 was similar to that of PLAU. Our data suggest that the intrinsic developmental program predominantly regulates PLAU expression during implantation, and that PLAU could be responsible for activation of MMP9, leading to localized matrix proteolysis as trophoblast invasion commences.

Reproduction (2011) 141 227–239

Introduction

Plasminogen activators (PAs) have been implicated in the initiation of proteolytic cascades that mediate invasive processes. PAs are serine proteases that convert plasminogen to plasmin, the active form of a broad-specificity protease that can control extracellular matrix (ECM) turnover, either directly or indirectly, by activating some members of the matrix metalloproteinase (MMP) family (Lijnen 2002, Castellino & Ploplis 2005, Rijken & Lijnen 2009).

Although Plau knockout mice are fertile, several lines of evidence indicate a role for this enzyme in the invasive process. Low implantation rates are associated with reduced PA activity (Aflalo et al. 2004, 2007). Failure of implantation is associated with reduced PA expression in embryos of the homozygous tw73 mouse mutant (Axelrod 1985). Moreover, general inhibitors of serine proteases block trophoblast invasion (Sun et al. 2007).

Trophoblast invasion in vivo is confined to the immediate pericellular environment and can be achieved by proteolytic enzymes secreted locally in discrete areas of cell–matrix contact (van Hinsbergh et al. 2006). PLAU binds with high affinity to a specific cell surface glycosylphosphatidylinositol-anchored receptor, PLAUR (Rijken & Lijnen 2009). The binding of PLAU to its receptor enhances and directs its proteolytic activity; therefore, promoting efficient plasminogen activation and plasmin formation (Stahl & Mueller 1995, Castellino & Ploplis 2005, Rijken & Lijnen 2009). PLAU expression appears to be regulated by trophoblast interactions with the ECM. Culture on fibronectin, fibronectin fragments, or laminin induces trophoblast interactions with ECM. Importantly, the question of whether exogenous plasminogen can induce the activation of pro-MMP9 secreted by mouse trophoblast cells was examined experimentally in light of MMP9 expression patterns.

Although there are several studies that have investigated PLAU secretion, function, and localization during mouse peri-implantation development, understanding of the role of cell-associated PLAU and the events that regulate its expression during blastocyst implantation is incomplete. The purpose of this study was to analyze temporal changes in mRNA levels, cellular distribution, concentration, and secretion of PLAU during mouse blastocyst development in culture and its regulation by trophoblast interactions with ECM. Importantly, the question of whether exogenous plasminogen can induce the activation of pro-MMP9 secreted by mouse trophoblast cells was examined experimentally in light of MMP9 expression patterns.

**Results**

**Plau gene expression**

RT-PCR generated single bands at the expected sizes for both Plau (194 bp) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh; 729 bp) mRNA with Plau band intensities that increased linearly between cycles 25 and 35 (Fig. 1A). The amount of Plau mRNA was estimated from densitometric scans of the amplicon bands, normalizing to the value obtained for Gapdh. Blastocysts expressed relatively low levels of Plau mRNA on gestation day (GD) 4. Thereafter, Plau mRNA expression increased until GD 8 in non-adhering embryos cultured on plates coated with BSA (Fig. 1B). Significantly higher levels of Plau mRNA were observed when the embryos were cultured on surfaces coated with type IV collagen or laminin (P ≤ 0.05, compared with the embryos cultured on BSA; Fig. 2).

![Figure 1](https://via.placeholder.com/150)

**Figure 1** Temporal expression pattern of Plau mRNA during blastocyst development. Mouse blastocysts were cultured from GD 4 to GD 8 on non-adhesive plates coated with BSA and assessed for Plau mRNA.

(A) Representative 1.2% agarose gels showing amplicons produced for Plau and Gapdh from total cellular RNA corresponding to 1.5 embryos. Cycling reactions were terminated after 25, 30, or 35 cycles, as indicated. (B) Densitometric analysis of bands produced after 30 cycles of amplification with relative pixel densities normalized to Gapdh. Means ± S.E.M. from three independent experiments are shown. *P < 0.05, relative to GD 4.
Cellular distribution of PLAU protein

Immunofluorescence and confocal microscopy revealed PLAU at the apical cell surface of the mural trophoblast after blastocysts were cultured to GD 5; however, no stain was detected in earlier stage blastocysts (Fig. 3A). The antibody labeled non-permeabilized embryos comparably to those treated with detergent prior to staining (Fig. 3B), confirming PLAU localization on the trophoblast surface. The enzyme remained at the surface of giant trophoblast cells cultured until GD 8 on BSA-coated plates (Fig. 3A and B). When the embryos were cultured on fibronectin to permit trophoblast outgrowth, PLAU appeared on the surface of large rounded cells that appeared to be adherent trophoblasts (Fig. 3C), based on their size and because PLAU is considered a trophoblast marker (Strickland et al. 1976, Behrendtsen & Werb 1997). However, it was absent from fully spread trophoblast giant cells at the periphery of the outgrowth (Fig. 3C). PLAU labeling of trophoblast cells outgrowing on fibronectin appeared more intense than that of non-adherent embryos.

Analysis of PAs during blastocyst differentiation by zymography

Zymography followed by densitometry was used for semi-quantification of enzyme activity in pooled conditioned embryo culture medium from GD 8 and GD 9, using human urinary PLAU as a standard (Fig. 4). Control zymograms developed without plasminogen produced no areas of casein lysis (data not shown). A linear response was observed between the integrated density and the concentration of enzyme standard (1.25–12.5 fg) or the amount of conditioned medium (recovered from 0.5 to 2 embryos). Up to four major bands of plasminogen-dependent caseinolytic activity were observed during peri-implantation development at the apparent molecular weights of 55, 40, 37, and 31 kDa, while the human urokinase produced a lytic zone primarily at 45 kDa (Fig. 4A). Mouse PLAU is not glycosylated and, therefore, has a lower molecular weight than glycosylated human PLAU (Marshall et al. 1990). The 55 kDa band corresponds with tissue-type plasminogen activator (PLAT), while the lower three bands are at the expected size of murine PLAU (McGuire & Seeds 1989). Using similar methodology, PLAU and PLAT produced by mouse blastocysts have been detected at 38 and 52 kDa respectively (Behrendtsen et al. 1992, Behrendtsen & Werb 1997) or at 40 and 60 kDa (Teesalu et al. 1996). Therefore, the 40 kDa band represents the high-molecular weight two-chain form of PLAU, while the 37 and 31 kDa bands are the result of its proteolytic processing. MMP3 can process human PLAU to a 31 kDa form lacking the non-catalytic chain A. There is evidence that MMP3 is present in mouse blastocysts (Brenner et al. 1989), and that MMP3 or another enzyme catalyzes PLAU activation.

Zymography revealed the temporal pattern of PLAT and PLAU production and secretion during mouse embryo peri-implantation development (Fig. 5). Both PLAU and PLAT were present in embryonic tissues and conditioned medium, and were adsorbed to the culture surface. On GD 4 and GD 5, the major type of PA produced and secreted from blastocysts was PLAT. The PLAT triplet first appeared and became the major PA form on GD 6, increasing progressively until GD 9. Levels of PLAT in conditioned medium and cell extracts decreased on GD 6, and then increased in cell extract from GD 8 to 9. Zymograms from mouse embryos cultured on a non-adhesive substrate (BSA) or on fibronectin-coated plates were compared (Fig. 5). Similar patterns of enzyme accumulation were observed except for reduced PLAT in embryo lysates from blastocysts cultured on plates coated with fibronectin. This was also observed on laminin, but not on type IV collagen (data not shown). Densitometric analysis of PLAU in zymograms prepared from embryo-conditioned medium revealed comparable levels of PLAU production during development when blastocysts were cultured in the presence or absence of ECM, including laminin and type IV collagen (Fig. 6A). Accumulation of PLAU on GD 9 in embryo lysates, conditioned medium, or the substratum was unaffected by the presence of immobilized ECM (Fig. 6B).
Plasminogen-dependent MMP9 activation

Considering that mouse blastocysts produce PLAU and pro-MMP9 in the culture, it was hypothesized that activated embryonic PLAU can use plasminogen to generate plasmin, which can proteolytically activate MMP9. Therefore, embryos were cultured for 48 h beginning on GD 7 in the absence or presence of 5, 10, or 20 \( \mu \)g/ml human plasminogen. On GD 9, MMP9 in the conditioned medium was assessed by gelatin zymography. As a control, medium without embryos was incubated with plasminogen and processed similarly. The addition of plasminogen resulted in the activation of MMP9 (Fig. 7A). Conditioned medium from embryos cultured without plasminogen produced only the zymogenic form of MMP9 (105 kDa). In the presence of 5–20 \( \mu \)g/ml plasminogen, embryos produced an additional gelatinolytic band at 88 kDa. A band with similar electrophoretic migration was observed with embryo-conditioned medium obtained on GD 9 and treated with \( p \)-aminophenyl mercuric acetate (APMA).

Production of the 88 kDa form was inhibited by \( a2 \)-antiplasmin (data not shown). Concomitantly, plasmin activity measured in the conditioned medium increased proportionally to the plasminogen concentration, and its activity required the presence of embryos (Fig. 7B). Amiloride, a specific PLAU inhibitor (Vassalli & Belin 1987), reduced the ability of embryo-conditioned medium (GD 9) to convert plasminogen to plasmin by 90% \( (P<0.05) \), and its addition with plasminogen to live embryo cultures caused a 60% \( (P<0.05) \) inhibition of plasmin production (data not shown). The milder effect of amiloride in live cultures could reflect its reduced effectiveness when PLAU is bound to cell surfaces (Dyer et al. 2002).

MMP9 expression

MMP9 expression was examined to determine if it was coordinately regulated with PLAU, since the latter enzyme may be required for MMP9 activation. RT-PCR
with primers of *Mmp9* generated a single amplicon at the expected size that increased linearly up to 40 cycles, as estimated by densitometric scanning. *Mmp9* mRNA levels, estimated after 35 cycles of amplification and normalized to *Gapdh* mRNA, increased linearly between GD 4 and GD 8 in non-adhering blastocysts (Fig. 8A and B). Gelatin zymography revealed five bands (230, 165, 105, 97, and 87 kDa) of EDTA-sensitive gelatinolytic activity in peri-implanting mouse embryos that first, and most strongly, appeared in the conditioned medium on GD 5 and increased progressively until GD 9 in both medium and embryo lysates (Fig. 8C). The number of embryo equivalents of the culture medium loaded on the zymograms was reduced at later times to prevent overloading. The 165 kDa band has not been characterized. The 230 kDa band, present in both medium and embryonic lysates, could represent the homodimeric form of MMP9 (*Goldberg et al*. 1992, *Olson et al*. 2000), while bands of 97 and 87 kDa correspond to the non-glycosylated precursors, partially active forms or fully active forms of the enzyme. As shown in Fig. 7A, APMA converts the 105 kDa pro-MMP9 (*Tanaka et al*. 1993, *Reponen et al*. 1995) to an active 87 kDa species. Embryo-conditioned medium contained a major 105 kDa band, while the 97 kDa band appeared only occasionally on GD 6 (Fig. 8C). However, embryonic lysates contained bands of 105, 97, and 87 kDa.

**Discussion**

The expression of *PLAU* and its role in MMP regulation during blastocyst differentiation was investigated, revealing the progressive accumulation of *PLAU* between GD 4 and GD 9. Expression of *Plau* mRNA increased from GD 4 to GD 8, while protein levels first appeared on the surface of mural trophoblast cells on GD 5. Zymographic detection of *PLAU* secretion into the culture medium commenced on GD 6, increasing until GD 9. Expression of *Plau* mRNA was stimulated when type IV collagen or laminin was present, but *PLAU* protein levels were apparently unaffected by an ECM substrate.

Similar patterns of *PLAU* expression during the development of cultured mouse blastocysts have been described using an enzymatic assay (*Strickland et al*. 1976, *Sellens & Sherman 1980*, *Zhang et al*. 1996), but the enzyme was assessed only in the conditioned medium or the lytic zone around the embryo. Although we used the same primers as *Harvey et al*. (1995) to estimate *Plau* mRNA, we detected it a day earlier (GD 4), probably due to differences in the mRNA extraction procedure. In both studies, however, *Plau* mRNA expression increased sharply on GD 6, suggesting transcriptional activation around the time of blastocyst hatching, as it prepares for implantation. The *Plau* promoter contains binding sites for activator proteins 1.
(TCFAP1) and 2 (TCFAP2; Cassady et al. 1991). Tcfap2c is expressed at high levels in mouse trophectoderm and giant trophoblast cells, and it is upregulated during differentiation of trophoblast stem cells (Kuckenberg et al. 2010). During implantation, Tcfap2c expression is restricted to the extra-embryonic lineages and deletion of the gene disrupts trophoblast development (Auman et al. 2002). Elimination of the transcription factor Ets2, which is also expressed specifically in the trophoblast at implantation and required for trophoblast development, leads to failure in MMP9 expression and persistence of ECM restricting trophoblast invasion (Yamamoto et al. 1998). Both Mmp9 and Plau utilize Ets-binding sites and could be coordinately regulated in the trophoblast.

The other PA, PLAT, was also detected by gel zymography in the conditioned medium and embryo extracts. PLAT was initially more prominent than PLAU but was then overtaken as PLAU expression progressively increased after GD 6. In the first 2 days, PLAT from maternal oviductal fluid was probably bound to the blastocyst surface (Carroll et al. 1993) and released into the medium. Furthermore, PLAT can be produced by parietal endodermal cells (Strickland et al. 1976), which first appear and proliferate beginning on GD 5 (Behrendtsen & Werb 1997).

**Cell surface location of PLAU**

The localization of PLAU on the external surface of mouse trophoblast cells was confirmed by confocal microscopy in fixed, non-permeabilized embryos. In mouse blastocysts, PLAU was initially detected on the surface of mural trophoblast cells (GD 5). In contrast, PLAU has been observed at all the stages of rat preimplantation development, and it is present both in the cytoplasm and on the surface of trophoblast at the early blastocyst stage (Aflalo et al. 2005, 2007). However, PLAU distribution in adherent rat blastocysts has not been studied.

Peri-implanting mouse embryos express a specific membrane receptor for PLAU (PLAUR; Harvey et al. 1995, Teesalu et al. 1996). The zymogenic and active forms of PLAU are bound to PLAUR through the

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**Figure 6** Influence of ECM on PLAU secretion during blastocyst development. Mouse blastocysts were cultured on plates coated with BSA, laminin (LN), fibronectin (FN), or type IV collagen (Col), as indicated. (A) The secretion of PLAU was analyzed daily in conditioned medium by plasminogen−casein zymography and densitometry. (B) On GD 9, medium conditioned during the final 24 h (MEDIUM), detached embryos (TISSUE), and proteins adsorbed to the culture surface (PLATE SURFACE) were recovered and analyzed for PLAU by plasminogen−casein zymography and densitometry. Data are presented as the mean and S.E.M. from three experiments. There were no statistically significant differences among the four groups in either experiment.

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**Figure 7** Plasminogen-mediated activation of MMP9. Mouse blastocysts were cultured from GD 7 to GD 9 in medium containing 0, 5, 10, or 20 µg/ml plasminogen, and the conditioned medium was processed by gelatin zymography. (A) Zymograms of human pro-MMP9 (hMMP) and blastocyst-conditioned medium. Conditioned medium (no plasminogen) was treated with APMA to activate MMP9 (APMA). The presence of plasminogen during embryo culture activated MMP9 with a dose dependency. (B) Plasmin activity was measured in medium containing plasminogen (Plg, as indicated) after 2 days of culture either without (M) or with (CM) blastocysts.
non-catalytic A chain of the active enzyme or the
NH₂-terminal portion of the single-chain proenzyme
(Quax et al. 1998). It has been observed that PLAU bound to PLAUR on the cell surface activates plasmino-
gen more efficiently than the soluble enzyme (Ellis
bound to PLAUR on the cell surface activates plasmino-
genesis and extracellular matrix degradation
(Quax et al. 1998). Our data indicate that PLAU can
produce fragments of both laminin and fibronectin
bonds to laminin z chain (Salonen et al. 1985, Moser et al. 1993,
Goldfinger et al. 1998). The binding of PLAU to ECM
has been less studied and was found to be weakly
associated with fibronectin (Salonen et al. 1985) or laminin (Goldfinger et al. 1998). Our data indicate that
ECM produced by the peri-implanting mouse blastocyst has a high capacity for PA binding. This binding may
increase the transformation of plasminogen to plasmin
(Stack et al. 1990, 1994). While type IV collagen is
resistant to plasmin cleavage, both laminin and fibro-
nectin are degraded by plasmin (Goldfinger et al. 1998,

PLAU regulation at implantation

All forms of PA secreted by trophoblast cells were
consistently bound to the culture surface, even when
exogenous adhesive substrates were not added. In
the latter case, this may be attributed to ECM or other
proteins produced by the embryos that become immobi-
lized in the substratum. The polystyrene Petri dishes used
for embryo culture avidly bind proteins, including those
exposed to the dishes before embryo culture. These
proteins and others secreted by embryonic cells during
the culture reversibly adhere to polystyrene to create a
matrix that binds secreted proteins, often through
specific protein–protein interactions. Both PLAT and
PLAU binding to the culture surfaces are dependent on
cell type (Stack et al. 1994), and both have been found
associated with ECM produced by cultured fibroblasts,
neurons, and endothelial cells (Krystosek & Seeds 1986,
Korner et al. 1993). The binding of PLAT to laminin and
fibronectin has been described previously as specific and
saturable. PLAT binds to the amino-terminus of fibro-
nectin or the carboxy-terminal globular domain of the
laminin z chain (Salonen et al. 1985, Moser et al. 1993,
Goldfinger et al. 1998). The binding of PLAU to ECM
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ECM interacts with plasminogen/plasmin through a lysine-binding site-dependent interaction (Moser et al. 1993), thereby protecting plasmin from certain inhibitors and proteases to prolong its proteolytic activity (Knudsen et al. 1986). In fluid phase, plasmin is rapidly inactivated by inhibitors. Modulation of plasminogen activation by ECM proteins may be an important regulatory mechanism during ECM proteolysis associated with cell invasion. ECM-growth promoting and thrombogenic properties are modulated by PAs and plasmin through the release of ECM-bound growth factors and activation of proenzymes like pro-MMP9 and prothrombin (Stack et al. 1990, 1994, Korner et al. 1993).

**Developmental regulation of PLAU expression and ECM interactions**

Our findings are consistent with the idea that temporal PLAU expression is regulated by an intrinsic program during blastocyst development and quantitatively modulated by interactions with ECM proteins, principally type IV collagen. Under serum-free conditions and in the absence of exogenous ECM, PLAU was expressed in a temporal pattern similar to that observed under adherent conditions, consistent with previous work (Sellens & Sherman 1980), and paralleled the developmental pattern in vivo (Teesalu et al. 1996). Zhang et al. (1996) found that ECM interactions increase PLAU expression in cultured mouse blastocyst. The present investigation only partially corroborates that data, since fibronectin did not have a stimulating effect. Although the labeling of PLAU by immunofluorescence in embryos outgrowing on fibronectin appeared stronger than that of blastocysts cultured on a non-adhesive substrate, semi-quantitative analysis of PLAU zymography suggested otherwise. There was no significant difference in PLAU proteolytic bands in embryo lysates, conditioned medium, or proteins adsorbed to the culture plate under non-adherent or adhesion-promoting conditions. If there was, indeed, elevated cell surface PLAU in embryos outgrowing on fibronectin, it did not result in greater accumulation in the medium where the greater portion of the enzyme was detected. The different results reported here and elsewhere could be due to differences in experimental conditions or contamination of ECM proteins with proteases or growth factors. Zhang et al. (1996) used a colorimetric PLAU assay under conditions that could be influenced by endogenous PA inhibitor (PAI). The zymographic procedure used in the present investigation disrupts the interaction of PLAU with its inhibitor. It is, therefore, possible that ECM regulates PLAU activity predominantly through PAI. The role of PAI in mouse trophoblast invasion merits further investigation.

Since deficiency of Plau or Mmp9 does not impair embryo implantation (Bugge et al. 1995), other proteases may be required during trophoblast invasion. Additionally, the overlapping functions of several ECM-degrading proteases may compensate for the loss of any one of them. A role for maternal PLAT acting upon the embryo or ECM cannot be excluded. The role of serine proteinases (Tang & Rancourt 2005) or other MMPs, including MMP3, MMP1/collagenase I (Brenner et al. 1989, Chen et al. 2007), stromelysin-3/ MMP11 (Lefebvre et al. 1995), or MMP14/MT1-MMP (Tanaka et al. 1998), likely contributed to trophoblast invasion either through their direct ECM-digesting activity or through activation of other proteases within a proteolytic cascade.

**Developmental regulation of MMP9 expression**

Transcription, translation, and secretion of MMP9 increased progressively during blastocyst development in parallel to PLAU. Gelatin zymography indicated that the active form of MMP9 was not secreted into the medium, although the 87 kDa form was present in embryo lysates. The 97 kDa protein, also found in embryos, may represent an intermediate in MMP9 activation. Two groups (Behrendtsen et al. 1992, Whiteside et al. 2001) have reported the presence of the active form of MMP9 in medium conditioned by developing mouse embryos cultured with either serum or a Matrigel substrate. The contribution of serum or Matrigel components (proteinases or growth factors) cannot be excluded, as Matrigel contains plasminogen (Farina et al. 1996), PAs, MMP2, and MMP9 (Mackay et al. 1993). When Matrigel is reconstituted by incubation at 37 °C, plasmin is formed, and MMP9 activation can be induced (Farina et al. 1996). Decreased PLAU activity has been observed in embryos developing in vitro compared with embryos developing ex vivo, suggesting that the activation of both proteases could be deficient in vitro due to a lack of maternal factors (Aflalo et al. 2005). Here, we have explored the potential role of the plasminogen/plasmin cascade in blastocyst MMP9 activation, which may better reflect circumstances in utero.

**Plasminogen activation and MMP9 activation**

PLAU function during embryo implantation appears to depend on the generation of plasmin, leading to the degradation of ECM components either directly or indirectly through the activation of MMP9 zymogen. The findings that the temporal pattern of MMP9 expression paralleled that of PLAU, that plasmin was generated after addition of plasminogen to the embryo culture, and that exogenous plasminogen induced the activation of MMP9 are, in fact, consistent with the existence of a proteinase cascade involving both the plasminogen/plasmin system and metalloproteinases to facilitate trophoblast invasion of the endometrium.
There is a marked increase in uterine vascular permeability on GD 5 in mice associated with the release of plasma proteins, including plasminogen, into the luminal fluid (Rockwell et al. 2002). These data suggest that maternal-embryonic interactions are required for protease activation during blastocyst implantation, with plasminogen having a key role. Furthermore, plasmin can degrade some ECM proteins, including soluble and immobilized fibronectin (Liotta et al. 1981, Hantai & Festoff 1987), and laminin (Uemura et al. 2005).

The physiological pathway for efficient MMP9 activation is unclear. Its activation requires proteolytic processing of the amino-terminal domain that can be initiated by several enzymes, including plasmin and stromelysin-1 (MMP3). The addition of plasmin to conditioned medium of GD 8 mouse blastocysts failed to activate MMP9 (Behrendtson et al. 1992). However, the activation of MMP9 by plasmin in solution is very inefficient (Goldberg et al. 1992) because of plasmin instability, and MMP3 has been considered as the physiological activator of MMP9 (Goldberg et al. 1992, Olson et al. 2000). Whether plasmin can convert pro-MMP9 to its active form is a subject of debate. Efficient activation of MMP9 by plasmin is obtained with cell-bound substrates (Mazzieri et al. 1997). Accordingly, in our experiments, PAs are bound to the cell and culture surfaces. In agreement with our findings, the addition of plasminogen induces pro-MMP9 activation in macrophages obtained from Mmp3-deficient mice (Lijnen et al. 1998). Additionally, plasminogen-dependent MMP9 activation is required for inflammatory macrophage migration (Gong et al. 2008). We cannot exclude the possibility that pro-MMP9 activation can be mediated by stromelysin-1, but we have demonstrated that maternal plasminogen can fulfill this key regulatory function during trophoblast invasion. It has been proposed that the combined action of plasminogen/plasmin and MMPs is essential for proper placental development and function (Solberg et al. 2003), particularly in the regulation of placental vascularization and decidual differentiation. The plasminogen-dependent activation of MMP9 that we report in late blastocysts is consistent with this suggestion and can partially explain how both enzyme systems interact in vivo.

In conclusion, the temporal pattern of PLAU expression in the blastocyst is dependent primarily on the intrinsic developmental program and is enhanced quantitatively through cellular interactions with laminin or type IV collagen. In light of our observation that cultured blastocysts secrete predominantly pro-MMP9 in the absence of serum, these findings suggest that developing blastocysts produce increased levels of both PLAU and latent MMP9 during the peri-implantation period, while execution of the plasminogen/ MMP proteolytic cascade requires maternal provision of plasminogen.

Materials and Methods

Production and culture of embryos

Mouse embryos were generated from 5 to 8-week-old female CF1 mice (Charles River Laboratories, Willimington, MA, USA) that were superovulated and mated with fertile B6SJL/J males (Jackson Laboratory, Bar Harbor, ME, USA), as described previously (Armant 2006). Blastocysts were collected on GD 4, where the vaginal plug was observed on the morning of GD 1. Uterine horns were removed and flushed with M2 medium (Sigma Chemical Co.), and the embryos were recovered under a Wild M3 stereo dissecting microscope (Leica, Wetzlar, Germany).

Groups of four embryos were cultured under mineral oil, in 5 μl drops of Ham’s F10 medium (Gibco BRL) containing 1 mg/ml BSA, 2.2 mM calcium lactate, 12.5 mM NaHCO3, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C using a 5% CO2/air incubator. Polystyrene Petri dishes (number 1008; Falcon, Lincoln Park, NJ, USA) were coated overnight at 4 °C with ECM proteins by applying medium containing 100 μg/ml of mouse laminin (Sigma), human type IV collagen, or human fibronectin (Gibco BRL). The next day, they were washed five times with culture medium and equilibrated in an incubator before adding embryos.

Blastocyst cultures were inspected daily between noon and 1400 h with a dissecting microscope to determine the percentage of embryos with trophoblast outgrowth, as detailed elsewhere (Armant 2006). The conditioned medium was harvested and replaced with fresh medium every 24 h. Attached or outgrowing embryos were physically detached with a flame-polished capillary tube (Sellens & Sherman 1980). Non-adherent and detached embryos were collected, rinsed five times in the culture medium, and lysed in 1× non-reducing electrophoresis sample buffer. The culture dish surface, after removing embryos, was washed five times with medium, and the adsorbed proteins were extracted with 1× non-reducing electrophoresis sample buffer. For examination of trophoblast outgrowths by immunofluorescence, blastocysts were cultured to GD 5, transferred to eight-well chamber slides (Lab-Tek, NUNC, Naperville, IL, USA), and then cultured to GD 8.

Analysis of mRNA

Total RNA was extracted from groups of 12 mouse embryos or blastocyst outgrowths using RNeasy kit (Qiagen). RNA was reverse transcribed and PCR was performed using a one-step RT-PCR kit (Qiagen). RT-PCR analysis of Gapdh mRNA, which was used as control, was performed for RNA integrity and accuracy of loading.

Specific primers used for RT-PCR of Plau, Mmp9, and Gapdh mRNA were sense 5′-GTGCCGCAACTGCTTCTT-3′ and antisense 5′-CTGTCTGGACTCTTCA-3′ (Harvey et al. 1995); sense 5′-TTAGGTCGGCGACCGCAG-3′ and antisense 5′-CCTATCCACGCGAATGACG-3′ (Harvey et al. 1995); sense 5′-GTATGTCGTTGAGCTTACT-3′ and antisense 5′-TCTCCTGAGCGCCATGTA-3′ (Ogawa et al. 2000) respectively. The RT reaction was conducted for 30 min at 50 °C. Reverse transcriptase was inactivated, and HotStar Taq DNA polymerase was activated at 95 °C for 15 min.
The amplification reaction included 25–35 cycles of heat denaturation at 94 °C for 45 s, followed by annealing of the primers for 45 s at 62 °C, and extension for 1 min at 72 °C. After the final cycle, the temperature was maintained at 72 °C for 10 min. PCR-amplified products were examined using 1.2% agarose gel electrophoresis and ethidium bromide staining. DNA bands were photographed, and the ratio of band intensity relative to Gapdh was estimated by scanning densitometry using the ImageJ (NIH, Bethesda, MD, USA) image analysis program. Because quantitative application of this method is contingent on the analysis of the PCR products during the amplification phase, before reaching the plateau, cycle relationships and dilution curves for RNA samples were determined empirically.

**Immunofluorescence microscopy**

Mouse embryos were fixed for 30 min at room temperature in PBS containing 3% paraformaldehyde and then incubated for 10 min in 0.15 M glycine, pH 7.2. Some embryos were permeabilized with 0.1% Triton X-100 for 10 min at room temperature. Embryos were rinsed through five drops of PBS containing 10 mg/ml BSA (PBS/BSA) and then incubated overnight at 4 °C with 10 μg/ml of purified goat polyclonal antibody against the carboxy-terminal domain of human PLAU (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in PBS/BSA. Primary antibody control was 10 μg/ml non-immune goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Primary antibodies were detected using 10 μg/ml fluorescein- or Texas Red-conjugated rabbit anti-goat IgG (Jackson), and nuclei were stained with 10 μg/ml non-immune goat IgG (Jackson). Immunofluorescence microscopy, using excitation wavelengths of 543, 488, and 635 nm, respectively. Images presented are representative of at least ten embryos that produce similar staining patterns.

**Zymography**

Plasminogen-dependent caseinolytic activity in the conditioned medium, cell lysates, and proteins adsorbed to the culture surfaces were studied by substrate gel electrophoresis (Legrand et al. 2001), to determine the presence of PAs and their molecular weights. Samples corresponding to 2.5–8 embryo equivalents were resolved by electrophoresis under non-denaturing conditions in 10% polyacrylamide–SDS gels containing 1 mg/ml bovine α-casein (Sigma Chemical Co.) and 10 μg/ml human plasma plasminogen (528175, Calbiochem). Prestained molecular weight markers 27–180 (SDS-7B, Sigma) were run on the same gels for molecular weight estimation. After electrophoresis, the gels were washed twice for 30 min in 2% (v/v) Triton X-100 to remove SDS and then incubated for 24 h at 37 °C in 100 mM glycine, 10 mM EDTA, pH 8.3. After staining with Coomassie blue R-250, plasminogen-dependent caseinolytic proteins were identified as clear zones of lyses against a blue background. The zymograms were imaged with the Eagle Eye II Still Video System (Stratagene, Santa Clara, CA, USA), and the zones of enzyme activity were quantified as integrated density by densitometric analysis using NIH ImageJ 1.62. Enzyme levels were expressed in arbitrary units set automatically by the software. Human urine urokinase (Calbiochem) was used as reference.

Zymography was also used for MMP9 analysis. In this case, 1 mg/ml porcine skin gelatin (G2625; Sigma Chemical Co.) was co-polymerized with the gels as enzyme substrate. Zymograms were developed for 48 h at 37 °C using 50 mM Tris–HCl, pH 7.5, containing 10 mM CaCl2, 150 mM NaCl, and 0.5% Triton X-100. As a positive control for MMP9 activation, MMPs were activated with APMA in blastocyst-conditioned medium obtained on GD 9. A freshly prepared 10 mM solution of APMA in 20% ethanol was added to conditioned medium in 50 mM Tris–HCl, pH 7.5, buffer containing 1 mM CaCl2 to give a final APMA concentration of 1 mM. The mixture was incubated at 37 °C for 2 h before electrophoresis.

**Plasmin activity**

Plasmin activity was measured using a specific chromogenic substrate, β-Val-Leu-Lys–4-nitroanilide-acetate (Sigma), which is cleaved by plasmin into a residual peptide and 4-nitroaniline (Ellis et al. 1987). The assay was carried out at 37 °C in a 0.2 ml reaction mixture containing 50 mM Tris (pH 7.4), 110 mM NaCl, and 0.3 mM of the chromogenic substrate. The absorbance of 4-nitroaniline was monitored spectrophotometrically at 405 nm. To ensure specificity, the results were confirmed by inclusion of the plasmin inhibitor, aprotinin (2 μg/ml), which inhibited nearly all observed plasmin activity.

**Statistical analysis**

Each experiment was performed at least three times. The data are presented as the mean ± S.E.M. ANOVA followed by Tukey’s post hoc test was used to compare experimental groups for statistical significance. Percentage was transformed to arcsine before statistical analysis.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

Supported, in part, by the intramural research program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, DHHS, NIH grants HD36764, HD045966, and AA12057 to D R Arman and by PAPIIT-DGAPA-UNAM, grant IN 230611 and PAPCA 2006–2007, FES-Iztacala to L A Baiza-Gutman and M G Martinez-Hernandez. Confocal microscopy was supported by center grants ES06639 and CA22453 from the NIH.
Acknowledgements

We thank Linda Mayernik for technical assistance with confocal microscopy. M G Martínez-Hernández was a predoctoral fellow of CONACYT, and this study constitutes part of her PhD dissertation for the Posgrado en Ciencias Biológicas, UNAM (México).

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Received 3 August 2010
First decision 26 August 2010
Revised manuscript received 30 October 2010
Accepted 12 November 2010