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 trophoblast 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.

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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).

ECM adhesion and proteolysis play important roles during embryo implantation. The specialized interaction between embryonic and maternal tissues is unique to mammalian development and includes invasion of the uterus by the trophoblast as the initial step in placentation (Armant 2005). Trophoblast cells must adhere to the ECM, secrete ECM-degrading proteinases, and penetrate the remodeled matrix. Several proteolytic

enzymes have been implicated in trophoblast invasion of the endometrium, including the PA/plasmin system (Strickland *et al.* 1976, Sappino *et al.* 1989, Teesalu *et al.* 1996, Zhang *et al.* 1996), MMPs (Peters *et al.* 1999, Whiteside *et al.* 2001, Chen *et al.* 2007), cathepsins (Afonso *et al.* 1997, 1999), and implantation-specific serine proteinases (Tang & Rancourt 2005, Sharma *et al.* 2006, Oozono *et al.* 2008). Mouse embryos dramatically increase secretion of functional proteinases at implantation. The major proteinases secreted are urokinase-type plasminogen activator (PLAU; Strickland *et al.* 1976, Zhang *et al.* 1996) and MMP9 (92 kDa type IV collagenase; Behrendtsen *et al.* 1992, Peters *et al.* 1999, Whiteside *et al.* 2001, Chen *et al.* 2007). PLAU and MMP9 are expressed in primary trophoblast cells both *in vitro* and *in vivo* (Sappino *et al.* 1989, Harvey *et al.* 1995, Reponen *et al.* 1995, Teesalu *et al.* 1996, Das *et al.* 1997, Peters *et al.* 1999, Whiteside *et al.* 2001).

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 PLAU secretion by blastocysts, partially as a result of increased steady-state levels of *Plau* mRNA (Zhang *et al.* 1996). The proteolysis-stimulating activity of fibronectin is inhibited by hexapeptides containing the integrin-recognition sequence Arg-Gly-Asp.

Aside from its role in ECM remodeling, the binding of catalytically active or inactive PLAU to its receptor can stimulate cell proliferation, migration, and invasion (Alfano *et al.* 2005, Caceres *et al.* 2008). It has also been suggested that the cell surface-associated plasminogen/plasmin system can activate or shed growth factors, including hepatocyte growth factor (Naldini *et al.* 1995, Mars *et al.* 2005, Matsuoka *et al.* 2006), latent transforming growth factor β (Godar *et al.* 1999, Yehualaesht *et al.* 1999, Leksa *et al.* 2005), basic fibroblast growth factor, dimeric platelet-derived growth factor-D (Ehnman *et al.* 2009), vascular endothelial growth factor (Noel *et al.* 2004, Lee *et al.* 2005), and heparin-binding epidermal growth factor-like growth factor (HBEGF; Guerrero *et al.* 2004, Roztocil *et al.* 2005, Caceres *et al.* 2008).

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 ($P \leq 0.05$, compared with the embryos cultured on BSA; Fig. 2).

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 $\pm$ S.E.M. from three independent experiments are shown. * $P < 0.05$, relative to GD 4.

Figure 2 Effect of adhesion to ECM on *Plau* mRNA expression during trophoblast outgrowth. Blastocysts were cultured on BSA, fibronectin (FN), laminin (LN), or type IV collagen (Col) from GD 5 to GD 8. On GD 8, total RNA was extracted to estimate *Plau* or *Gapdh* mRNA, as in Fig. 1. (A) Representative gels of amplicons produced for *Plau* and *Gapdh* after 30 cycles of amplification. (B) Densitometric analysis of amplicon bands, normalized to *Gapdh*. Means $\pm$ S.E.M. from three independent experiments are shown. * $P < 0.05$, relative to culture on BSA.

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 PLAU 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).

Figure 3 Immunofluorescence localization of PLAU in developing blastocysts. (A) Mouse blastocysts were labeled on GD 4 (a, b) and GD 5 (c–f) with either non-immune goat IgG (a, c, and e) or goat anti-PLAU antibody (b, d, and f) with DAPI counterstaining, as described in the Materials and Methods section. The images were obtained by fluorescence conventional (a, b, c, and d) or confocal (e and f) microscopy. (B) Non-adhering mouse blastocysts were cultured on BSA-coated surfaces until GD 6 (a–c), GD 7 (d–f), or GD 8 (g–i) and labeled with either non-immune goat IgG (a, d, and g) or goat anti-PLAU antibody (b, c, e, f, h, and i), as in (A), before imaging by confocal microscopy. Prior to labeling, embryos were either permeabilized with detergent (a, b, d, e, g, and h) or non-permeabilized to restrict labeling to the cell surface (c, f, and i). (C) Mouse blastocysts cultured to GD 8 on fibronectin (FN) were labeled with either non-immune goat IgG (a) or goat anti-PLAU antibody (b), as in (A). Nuclei appear blue, and fluorescein- or Texas Red-conjugated secondary antibody appears green (A and B) or red (C). At least ten embryos were examined in each treatment group. Bar = 50 μ m.

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 μ 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 μ 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 α 2-anti-plasmin (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

Figure 4 Plasminogen-casein zymography of conditioned medium. (A) Human PLAUI (hPLAU) and conditioned medium from embryos cultured to GD 9 (CD) were analyzed by plasminogen-casein zymography, along with molecular weight (MW) markers. (B–D) Varying amounts of blastocyst-conditioned medium (B) or hPLAU standard (C) were analyzed by the same procedure and semi-quantified by densitometric analysis (D). Data are presented as the mean and S.E.M. from three experiments. Results of a linear regression are shown in the graph.

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 PLAUI and its role in MMP regulation during blastocyst differentiation was investigated, revealing the progressive accumulation of PLAUI 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 PLAUI 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 PLAUI protein levels were apparently unaffected by an ECM substrate.

Similar patterns of PLAUI 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

Figure 5 Temporal pattern of PA proteins during development. Plasminogen-casein zymography of conditioned medium, extracts of protein adsorbed to the culture plate surface, and embryo lysates was conducted on the indicated days of gestation, using blastocysts cultured on plates coated with BSA or fibronectin (FN). Assays used two embryo equivalents for conditioned medium and three embryo equivalents for adsorbed proteins or lysates. The zymograms are representative of at least three separate experiments.

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.

(TCFAP1) and 2 (TCFAP2; Cassady *et al.* 1991). *Tcfap2c* is expressed at high levels in mouse trophoblast and giant trophoblast cells, and it is upregulated during differentiation of trophoblast stem cells (Kuckenber *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 trophectoderm 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

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.

Figure 8 MMP9 expression during blastocyst development. Blastocysts were cultured to the indicated gestation day (GD), and *Mmp9* and *Gapdh* mRNA were amplified by RT-PCR (A) and then quantified by densitometry to determine the developmental pattern of *Mmp9* mRNA (B), or the conditioned medium and embryo lysates were analyzed by gelatin zymography (C), as described in the Materials and Methods. Means and S.E.M. from three independent experiments are shown in (B). Conditioned medium equivalent to 100, 25, 8, 3, and 3 embryos was used on GD 5, 6, 7, 8, and 9 respectively for zymography shown in (C). All lysate samples contained 12 embryo equivalents. Human MMP9 (hMMP9) was used as reference. All zymograms shown are representative of three or more separate experiments.

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 plasminogen more efficiently than the soluble enzyme (Ellis *et al.* 1991). Therefore, the peri-implanting blastocyst can form plasmin beginning on GD 5, approximately the time *in vivo* when the embryo binds to the luminal epithelium. At that time, PLAU could participate in focalized proteolysis of ECM in the vicinity of trophoblast cells. Membrane-associated proteolytic systems contribute significantly to trophoblast invasion of the endometrium (Cohen *et al.* 2006, Fujiwara 2007).

Cell surface PLAU could facilitate communication between fetal and maternal cells by activating pro-forms of intercellular signaling molecules, either directly or indirectly as part of a protease cascade. Of particular interest is HBEGF, which is expressed specifically by

luminal epithelial cells adjacent to the blastocyst at the beginning of GD 5 (midnight) and appears to have a critical role during implantation (Das *et al.* 1994, Lim & Dey 2009). Like other members of the EGF family, HBEGF is initially produced as a transmembrane protein that is secreted through the cleavage and shedding of its extracellular domain by a metalloproteinase. HBEGF and its receptors are expressed in both trophoblast and uterine epithelium, and expression of the transmembrane form of HBEGF is considered the earliest molecular response of uterine epithelium to blastocyst signals (Jessmon *et al.* 2009, Lim & Dey 2009). Acceleration of blastocyst differentiation *in vitro* is dependent upon HBEGF shedding in GD 5 mouse embryos (Liu & Armant 2004), possibly through the contribution of PLAU to the proteolytic activation of the HBEGF sheddase. It has been demonstrated in cancer cells that PLAU induces HBEGF shedding and transactivation of its receptor through a process mediated by MMPs (Guerrero *et al.* 2004), possibly MMP9 (Lucchesi *et al.* 2004, Hurtado *et al.* 2007), and that PLAU can induce the expression of HBEGF (Caceres *et al.* 2008).

PA adsorption to the culture surface

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 immobilized 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 fibronectin or the carboxy-terminal globular domain of the laminin α 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 fibronectin are degraded by plasmin (Goldfinger *et al.* 1998, Bonnefoy & Legrand 2000, Uemura *et al.* 2005).

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 1 (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-degrading 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 (Behrendtsen *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, Willmington, MI, 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'-GTGCCGCACTGCTTCATT-3' and antisense 5'-CGTGCTGGTACGTATCTTCA-3' (Harvey *et al.* 1995); sense 5'-TTGAGTCCGGCAGACAATCC-3' and antisense 5'-CCTTATCCACGCGAATGACG-3' (Harvey *et al.* 1995); and sense 5'-GTATGTCGTGGAGTCTACTG-3' and antisense 5'-TACTCCTTGGAGGCCATGTA-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 4',6'-diamino-2-phenylindole HCl (DAPI; Calbiochem, La Jolla, CA, USA). Embryos were incubated with a cocktail of secondary antibody and DAPI at 37 °C for 60 min, rinsed, mounted on slides with permanent mounting medium (Chemicon, Tamecula, CA, USA), and stored in the dark at 4 °C. Antigens labeled with Texas Red (red) or fluorescein (green) and nuclei stained with DAPI (blue) were viewed in a Zeiss (Thornwood, NY, USA) 310 confocal scanning laser microscope, using excitation wavelengths of 543, 488, and 364 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, D-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 $\pm$ 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.

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