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Role of proteases in implantation

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Implantation of the embryo into the endometrium is a critical step in the establishment of pregnancy and the failure of embryos to implant is a major limiting factor in the success of reproductive technologies. Furthermore, one or more of the molecules of importance at implantation could provide a suitable target for post-coital contraception. While there is considerable species variation in the extent to which the trophoblast invades the maternal endometrium and makes contact with the maternal blood supply, many of the molecular mechanisms are conserved among species. Three families of protease are involved in the matrix degradation required for implantation: the cysteine, serine and matrix metalloproteinases. Other proteases are required for the activation of regulatory molecules. Although trophoblast from all species appears to have a high invasive potential, this is limited by the presence of partner protease inhibitors, the presence of which provides restraint to this invasion. It is the balance between the proteases and their inhibitors at any focal point that determines the site and extent of trophoblast invasion. This review examines the literature regarding proteases and their inhibitors at early implantation sites across a range of species with very different forms of placentation and evaluates their common features and their dissimilarities.

Implantation of the embryo into the maternal endometrium is the first step leading to placentation and ultimately ensures that the conceptus is provided with an adequate blood supply. The extent to which the conceptus invades the endometrium varies considerably among mammals, but in all species, considerable remodelling of the endometrium occurs at the site of implantation. The barriers breached to establish connections to the blood supply include the basement membranes of the uterine epithelium and the endothelium and, to a variable extent, the endometrial interstitial matrix supporting the stroma. The aggressiveness of trophoblast is otherwise only seen with malignant cells, and trophoblast invasion shares many of the features of tumour invasion and metastasis. The cardinal difference is that trophoblast invasion is tightly controlled whereas tumour invasion progresses without restraint.

This review will examine the proteases likely to be involved in the implantation process, taking into account the interspecies differences. First, it will examine which proteases and specific inhibitors have been identified in the tissues associated with implantation, both *in vivo* and *in vitro*, and then review the functional studies that have given some indication of the relative importance of individual proteases. Finally, it will examine how these proteases might be regulated at a number of levels, including transcription, activation and inhibition.

Proteases, invasion and remodelling

Proteases with a role in extracellular matrix (ECM) degradation fall primarily into three categories: serine, cysteine and metalloproteinases (Barrett, 1994). In addition, proteases that activate key regulatory molecules, such as tumour necrosis factor α (TNF-α), will play an important but undefined role in the events of implantation. Proteases thought to be important in implantation, along with their likely substrates, are listed (Table 1). Moreover, as excessive or inappropriately timed proteolytic events need to be restrained, most known mammalian enzymes have partner protease inhibitors, which are often widely distributed in the same tissues in which the enzymes are likely to act.

Cysteine proteinases are lysosomal enzymes that act at acid pH. The best known are cathepsin B and cathepsin L, both of which cleave the N-terminal peptides of collagen that contain the covalent cross-links within and between molecules. Cathepsin B also cleaves the attachment region of proteoglycans to hyaluronan. Another member of this family, interleukin 1β-converting enzyme (ICE or caspase-1) (Tocci, 1997), releases active interleukin 1β (IL-1β) from its precursor, providing this cytokine for local actions including the regulation of transcription of matrix metalloproteinases (MMP). Cysteine proteinases are controlled by members of the cystatin superfamily which include both intracellular (cystatin A and B) and extracellular (cystatin C) forms. The invasive phenotype of many metastatic cell types is associated with the increased production and secretion of cysteine proteinases and the aberrant regulation of cystatin C, and a role could be postulated for these molecules in embryo implantation.

Serine proteinases are the largest class of mammalian protease and most act at neutral pH. Among those produced in the uterus are urokinase-type plasminogen activator (uPA), plasmin (derived from plasminogen by the action of uPA), and kallikrein, which are produced mainly by endometrial fibroblasts, while enzymes such as mast cell tryptase, neutrophil elastase and granzyme are contained in specific migratory cells within the tissue and are released on activation. Many of these enzymes exert their action on matrix degradation, in part indirectly, by proteolytic activation of MMPs. Specific inhibitors,

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Table 1. Proteases with a potential role at implantation sites

Class	Examples	pH range	Natural substrates
Cysteine	Cathepsin B	3–7	Proteoglycan, collagen 1, denatured collagen
	Cathepsin L		Proteoglycan, collagen 1, denatured collagen, fibronectin
	Interleukin-1β converting enzyme		Precursor IL-1β
Serine	uPA	6–10	Fibronectin, plasminogen
	tPA		Plasminogen
	Kallikrein		Proenzymes, including proMMP-1, proMMMP-8
	Tryptase		Collagen VI, proMMP-3
	PMN elastase		Elastin, fibronectin
Metallo-	Collagenase (MMP-1)	6–9	Collagen I, III,VII, X
	Stromelysin-1 (MMP-3)		Proteoglycans, fibronectin, laminin, collagen IV, proMMP-1, -7
	Gelatinase A (MMP-2)		Collagen IV, V, gelatin
	Gelatinase B (MMP-9)		Collagen IV, V, fibronectin, laminin
	Matrilysin (MMP-7)		ProMMP-9, proMMP-1
	Membrane-type matrix metalloproteinase-1 (MT1–MMP)		ProMMP-2, collagen 1
	TNF converting enzyme		Precursor TNFα

MMP, matrix metalloproteinase; PMN, polymorphonuclear leukocytes.

such as plasminogen activator inhibitor 1 (PAI-1), PAI-2 and protease nexin, which limit the action of PA, serve to balance the activity of many of these enzymes. Cell surface binding sites for uPA and plasminogen are present on cell membranes, often in the same cell and serve to focus the activity to the vicinity of the cell. Binding to these receptors enhances the proteolytic potential of the system markedly. Expression of uPA and PAIs is controlled mainly by transcriptional mechanisms, driven by hormones and growth factors in a cell type-specific manner. This PA–PAI–PAR–plasmin system (Fig. 1) is thought to be a key participant in the balance of matrix turnover (Vassalli *et al.*, 1991).

The third class of protease, the metalloproteinases, contains the most critical enzymes for matrix degradation and remodelling, the substrate activities of which collectively allow them to degrade virtually all the components of both interstitial matrix and basement membranes. The role of metalloproteinases in tumour invasion and metastasis is well-documented (Powell and Matrisian, 1996). The best known grouping of these enzymes are the MMPs which fall broadly into four categories: the collagenases, gelatinases, stromelysins and membrane-type MMPs. Most MMPs act extracellularly and at neutral pH and are released as latent precursors requiring activation by proteases such as plasmin, tryptase and elastase, as well as by other activated MMPs. MMPs can be regulated at a number of levels (Fig. 1). Transcription is modified by growth factors, cytokines and steroid hormones, the actions of which are tissue- and cell-type specific and vary among the enzymes. Importantly in the present context, progesterone is a negative regulator of transcription of several MMPs including MMP-1, -3 and -9 (Salamonsen *et al.*, 1997), although this can be overridden by some cytokines, including IL-1 and TNF-α (Singer *et al.*, 1997; Zhang *et al.*, 1998). Both transcriptional and post-transcriptional mechanisms regulate MMP and TIMP production; binding sites (AP-1 and PEA-3) for the transcription factors: fos and jun and Ets proteins, respectively, are present in the promoter regions of the genes (Crawford and Matrisian, 1996), while a number of Ets proteins (Kilpatrick *et al.*, 1998), as well as fos and jun, are present in endometrium. The mechanisms by which MMP gene transcription is regulated in endometrium or trophoblast are not yet known. Most MMPs are secreted in their latent forms which require activation by proteolytic cleavage of the propeptide. Cascades of enzyme activation can be set up, as some MMPs, particularly MMP-3 and MMP-7, can activate others (Salamonsen and Woolley, 1996). ProMMP-2 is unusual in that it is activated at the cell surface by the membrane type (MT)-MMPs (in the presence of tissue inhibitor of metalloproteinase 2 (TIMP-2)). Thus, the active form of this enzyme is associated closely with the cell membrane. Most studies on MMPs at implantation have localized either mRNA or immunoreactive protein in the tissue but have not produced evidence of activation. Once activated, MMPs are not necessarily available for tissue degradation, as a family of TIMPs act to restrain their action by binding their active forms with a 1:1 stoichiometry (Edwards *et al.*, 1996). Binding to α_2-macroglobulin provides additional, but less specific, inhibition. At any focal point within a tissue, MMP-catalysed proteolysis of matrix will occur only if the active form of the MMP is present in molar excess over inhibitors.

Although proteolytic degradation of the ECM and the control of that action are the logical roles for MMPs and TIMPs, respectively, other functions described for these molecules may be important. Several MMPs degrade regulatory molecules, including IL-1β (Ito *et al.*, 1996), and insulin-like growth factor

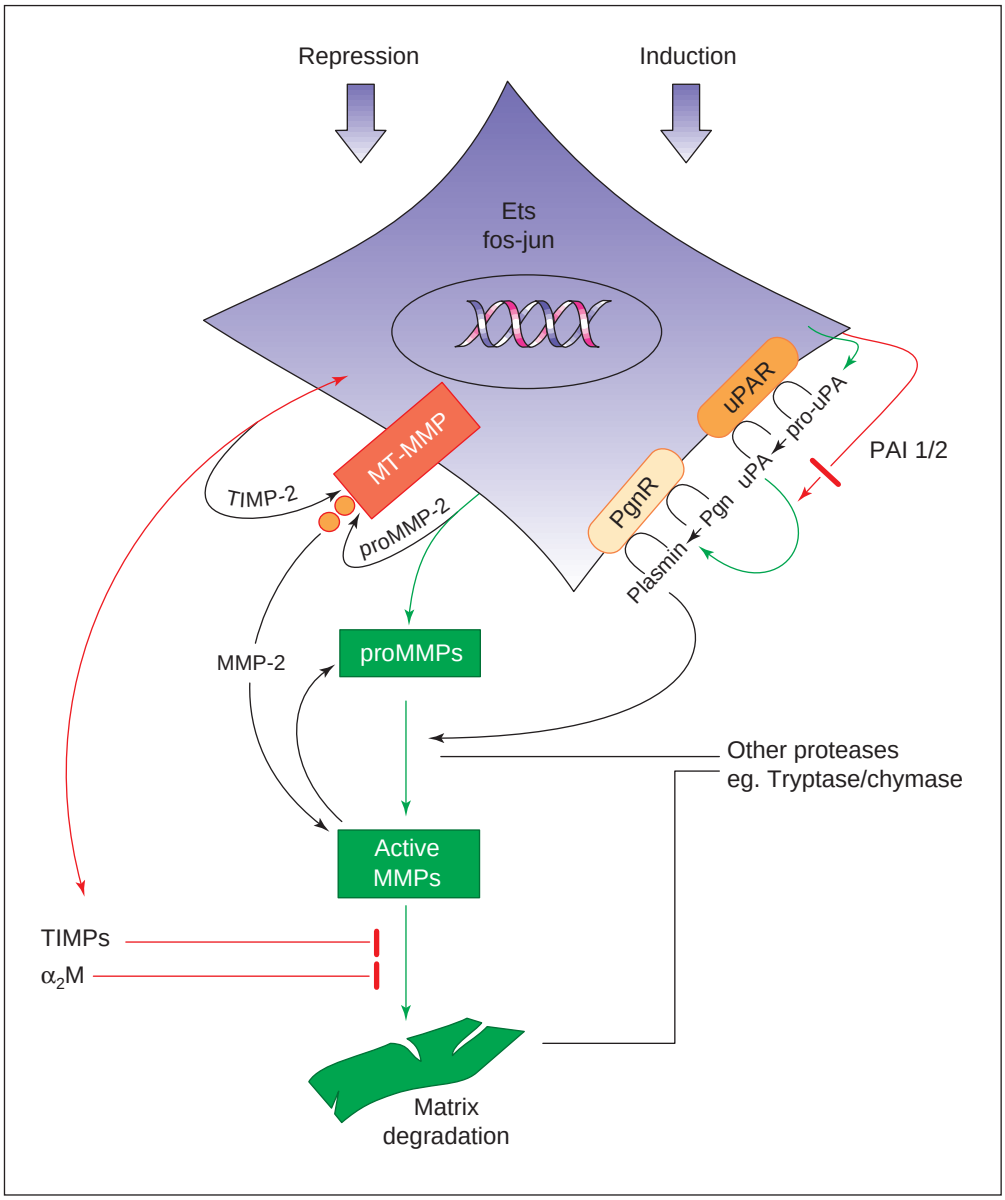

Fig. 1. Schematic representation of the potential regulation of matrix degradation by interactions between serine proteases and matrix metalloproteinases. ProMMP, latent matrix metalloproteinase; MT-MMP, membrane-type MMP; TIMP, tissue inhibitor of metalloproteinases; uPA, urokinase-type plasminogen activator; Pgn, plasminogen; R, receptor; PAI, plasminogen activator inhibitor; α_2M; α_2-macroglobulin.

binding protein 3 (Fowlkes *et al.*, 1994), while MMP-7, an epithelial cell MMP, processes molecules such as TNF-α precursor and uPA (Wilson and Matrisian, 1996). TIMP-1 and TIMP-2 have growth factor activities (Hayakawa *et al.*, 1992, 1994) and both embryotrophic (Satoh *et al.*, 1994) and steroidogenic (Boujrad *et al.*, 1995) actions have been described for TIMP-1, while TIMP-3 overexpression promotes apoptosis of vascular smooth muscle cells (Baker *et al.*, 1998). Whether any of these functions are important at implantation has not been established.

Members of other metalloproteinase groups include enzymes such as TNF-α-converting enzyme (TACE) (Black *et al.*, 1997), a member of the adamalysin protease family that releases soluble TNF-α from its membrane-bound precursor and the action of which is inhibited by the same hydroxamic acid-based inhibitors that inactivate MMPs. Other adamalysins may also have actions to shed important cell-surface proteins.

Species differences in trophoblast invasion at implantation

Both the means by which the trophoblast penetrates the uterine epithelium and makes contact with the maternal blood supply and the extent to which the trophoblast invades the maternal endometrium vary considerably among species (Fig. 2). Such

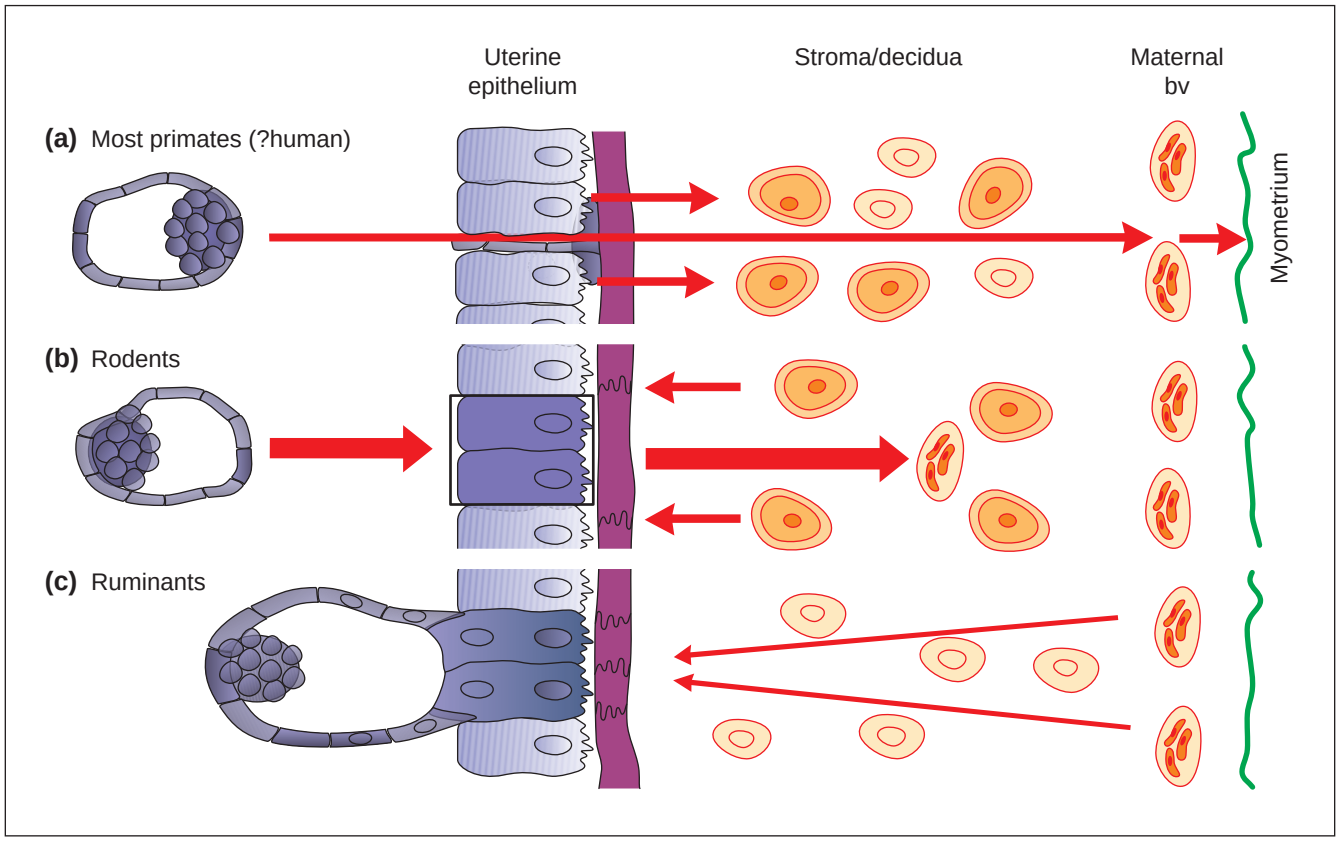

Fig. 2. Differences in the invasive steps of implantation in primates, rodents (rats and mice) and ruminants (sheep). (a) In primates, the polar trophectoderm passes between the epithelial cells, spreads across the basal lamina and then invades through the stroma to the blood vessels (bv) deep in the endometrium. (b) In rats and mice, the uterine epithelium undergoes apoptosis at implantation, the basal lamina is penetrated first by the decidual cells, and the trophoblast invasion within the endometrium is tightly constrained. (c) In sheep, the trophectoderm fuses with the luminal epithelial cells to form a syncytium. The syncytial cells penetrate the basal lamina at intervals but no further invasion occurs. Considerable development of the blood vessels brings the blood supply towards the syncytical layer.

differences must be taken into account in any discussion of trophoblast invasion. In rats and mice, which have haemochorial placentation, there is apoptosis of the uterine epithelium at the site of blastocyst apposition (Parr *et al.*, 1987) before the trophoblast invades the stroma. Originally it was assumed that the initial breaching of the epithelium and its basement membrane resulted from the invasive nature of the trophoblast but, subsequently, it was shown that, in normally implanting rats and mice, the basement membrane is first perforated by decidual cells alone (Schlafke *et al.*, 1985; Blankenship and Given, 1991). Decidual cells also penetrate the basement membranes of stromal vasculature but these are eventually replaced by trophoblast (Welsh and Enders, 1987).

In primates, including humans, the cytoplasmic protrusions of trophoblast interdigitate between the uterine epithelial cells and spread along the basement membrane underlying the luminal epithelium, before eroding this and invading into and through the stroma, eventually to tap the blood vessels (Enders, 1997). The human trophoblast is highly invasive *in vivo* and can penetrate the full thickness of the endometrium, reaching as far as the first third of the myometrium. By contrast, even in closely related primates, invasion is limited to the endometrium (Ramsey *et al.*, 1976), suggesting either particularly invasive trophoblast or less efficient inhibition of invasion in humans.

In ruminants such as sheep and cattle, in which placentation is synepitheliochorial, the trophoblast does not invade through the uterine epithelium into the uterine stroma, although there is considerable tissue remodelling and development of new blood vessels. At implantation, fetal chorionic binucleate cells fuse with those of the uterine epithelium and the syncytial cells penetrate through the basal lamina (Wooding and Flint, 1994). In other species with epitheliochorial placentation (for example, pigs), there is also little direct invasion of the trophoblast into the uterine stroma. Horses appear to be unique in that they have a subpopulation of highly invasive trophoblast cells forming the chorionic girdle that become highly invasive around day 35 of pregnancy, penetrating through the uterine epithelium into the stroma to form endometrial cups (Enders and Liu, 1991).

Trophoblast cells from most species appear to have a high degree of invasive capacity that can be demonstrated outside the uterine lumen or *in vitro*. Human trophoblast can invade at any of a number of extrauterine sites, as evidenced by the number of ectopic pregnancies in women, while mouse trophoblast penetrates basement membrane derived from the uterine

epithelium *in vitro* (Armant and Kameda, 1994) and, *in vivo*, when transplanted to an ectopic site, invades uncontrollably (Enders *et al.*, 1981). Even pig trophoblast can penetrate non-uterine epithelia and develop in ectopic sites (Samuel and Perry, 1972). However, within the microenvironment of the uterus, trophoblast invasion is strictly limited. Both the hostility of the uterine epithelium to a conceptus, apart from during the 'window of implantation', and the presence of protease inhibitors probably restrain the movement of this very invasive tissue, with the level of restraint varying with the type of placentation.

In spite of these differences, as some of the molecular events resulting in the establishment of pregnancy have come to be understood, it has become apparent that the molecules involved in trophoblast invasion are highly conserved among species (Nie *et al.*, 1997).

Expression patterns of proteases at implantation sites

It has long been suspected that proteolytic enzymes play a role in trophoblast invasion. Strickland (1976) showed that uPA was produced by mouse trophoblast, that this coincided with the invasive phase of the embryo and that it was localized to regions of the invasion. Roles for both MMPs and cathepsins have also been suggested and a number of proteases and inhibitors are produced by the conceptus and the endometrium of all species examined (Table 2). The present discussion will focus primarily on the early phases of trophoblast invasion rather than on the subsequent events of placental development.

Species with haemochorial implantation

Mouse. Cathepsin B mRNA is expressed by mouse trophoblast throughout the invasive period (days 5.5–10.5) and the proteins for cathepsins B and L are localized to the mature invasive trophoblast giant cells; the activity of these enzymes has also been demonstrated. Cathepsin L is also present within the stroma, particularly in the lateral decidualizing zone. Cystatin C, the major inhibitor of cathepsins, is a major product of the adjacent decidualizing stroma close to the embryo, suggesting a restraining role. After the completion of placental trophoblast invasion (day 11.5), the trophoblast cells express high concentrations of cystatin C, probably in association with a switch from an invasive cell type to one providing nutrients to the conceptus (Alfonso *et al.*, 1997).

There is also a very distinct and cell-specific expression pattern of the type IV collagenases, MMP-2 and MMP-9, and TIMP-1, -2 and -3, during mouse embryo implantation (Reponen *et al.*, 1995; Alexander *et al.*, 1996; Leco *et al.*, 1996; Das *et al.*, 1997). In particular, MMP-9 is expressed weakly at 4.5 days of gestation (day 0 = day of plug) with mRNA present in some rounded trophoblast cells, increasing to intense expression in the multinucleated trophoblast giant cells emerging around the embryo and penetrating the uterine surface at day 5.5. On days 6.5–8.5, MMP-9 mRNA is observed in numerous trophoblast giant cells surrounding the embryo and invading the uterine stroma. In the uterus, MMP-9 mRNA is induced in some stromal cells on day 4, at the time of blastocyst apposition but then disappears (Das *et al.*, 1997). Expression of MMP-2 differs significantly from that of MMP-9. Its mRNA is not detected in embryonic or extra-embryonic tissues but a positive reaction is seen in uterine mucosal stroma cells outside the decidualized area (Reponen *et al.*, 1995; Das *et al.*, 1997). While no TIMP-1 or -2 expression is observed in the embryo, trophoblasts or decidua, intense TIMP-3 mRNA is present in maternal cells in the area of the decidual reaction surrounding the 5.5 day early egg cylinder, and this reduces considerably by day 7.5 (Reponen *et al.*, 1995; Alexander *et al.*, 1996; Leco *et al.*, 1996). The strong expression of MMP-9 in the trophoblast at days 4.5–7.5, and of TIMP-3 in the surrounding decidua, but their reduction during the same time interval implies a crucial role for each in the implantation–invasion process (Reponen *et al.*, 1995). At present, there is little information on how TIMP-3 and MMP-9 expression is regulated directly or indirectly at implantation sites *in vivo*, although *in vitro* studies implicate leukaemia inhibitory factor (LIF) and epidermal growth factor (EGF) (Harvey *et al.*, 1995). MMP-13 (mouse collagenase) does not seem to participate in the implantation process and MMP-3 is found primarily in the myometrium with no change in expression during peri-implantation (Das *et al.*, 1997). Uterine concentrations of TIMP-1 and -2 also do not change.

Components of the urokinase system are also expressed in a finely regulated pattern during mouse implantation (Teesalu *et al.*, 1996). A high abundance of uPA mRNA is found in invasive trophoblast cells, which do not express PAI-1. Urokinase-type PA is also expressed in the endothelial cells of newly forming vessels from day 6.5. The uPA receptor and the low density lipoprotein receptor-related gene (LRP) are expressed by decidual tissue and co-localize with uPA and PAI-1, a finding of particular interest in view of the role of the two receptors in clearing uPA–PAI-1 complexes. Active uPA has been demonstrated in the ectoplacental cone region at days 7.5–8.5.

Thus, all classes of protease associated with degradation of extracellular matrix are expressed in a temporal and cell-specific pattern consistent with their likely roles in the invasive process of implantation in the mouse. Furthermore, these expression patterns are matched by those of specific inhibitors in the tissue surrounding the site of invasion.

Primates, including humans. Data localizing proteases or their inhibitors at early implantation sites in primates are not available at present, although information gleaned from *in vitro* studies (see below) suggest that there will be many similarities with the data from rats and mice. Between 6 and 40 days after attachment in macaques, MMP-1, -2 and -9, but not cathepsin B or D, are seen within intraluminal and intramural cytotrophoblast, although the cathepsins are present in uterine macrophages and stromal cells. MMP-2 has been immunolocalized in many different cell types, including all types of trophoblastic cells, decidual cells, Hofbauer's cells and villous fibroblasts within human implantation sites during the first trimester (Fernandez *et al.*, 1992).

During the secretory phase of the normal menstrual cycle, MMP protein is not detectable by immunohistochemistry in the endometrial stroma. MMP-9 is immunolocalized within the luminal and glandular epithelium during the postovulatory period and can be seen progressing towards the apical surface of the cells, being released into the uterine lumen just at the time the surface becomes receptive (Jeziorska *et al.*, 1996). The role of MMP-9 at this time is not yet understood but it may

Table 2. Proteases and inhibitors or their mRNA in the embryo–maternal axis at the time of implantation

Protease or inhibitor	Species	Location			References
		Endometrium	Trophoblast	Uterine lumen	
Cathepsin D	Rat	+			Elangovan and Moulton, 1980
	Pig	+			Roberts *et al.*, 1976
Cathepsin L	Mouse	–	+		Alfonso *et al.*, 1997
	Pig	+	–	+	Geisert *et al.*, 1997
Cathepsin B	Mouse	–	+		Alfonso *et al.*, 1997
Cystatin C	Mouse	+	–		Alfonso *et al.*, 1997
UPA	Mouse	–	+		Harvey *et al.*, 1995; Teesalu *et al.*, 1996
	Pig	+	+		Menino *et al.*, 1997
	Human	+	?		Hofmann *et al.*, 1994
PAI	Mouse	+	–		Teesalu *et al.*, 1996
	Pig	+			Mullins *et al.*, 1980
	Human	+	+		Hofmann *et al.*, 1994
MMP-2	Mouse	+	–		Reponen *et al.*, 1995; Alexander *et al.*, 1996; Das *et al.*, 1998
	Pig	–	+		Menino *et al.*, 1997
	Sheep	–	+	+	Salamonsen *et al.*, 1995
	Human/marmoset		+		Franek *et al.*, 1997
MMP-9	Mouse	+ (day 5) – / –	+		Harvey *et al.*, 1995; Reponen *et al.*, 1995; Alexander *et al.*, 1996; Teesalu *et al.*, 1996; Das *et al.*, 1998
	Pig		+		Menino *et al.*, 1997
	Sheep		+	+	Salamonsen *et al.*, 1995
	Human	+	+		Shimonovitz *et al.*, 1994; Jeziorska *et al.*, 1996
TIMP-1	Mouse	+	+ / –		Harvey *et al.*, 1995; Reponen *et al.*, 1995; Alexander *et al.*, 1996
	Pig	+	+		Menino *et al.*, 1997
	Sheep	+			Salamonsen *et al.*, 1995
	Human	+			Zhang and Salamonsen, 1997
TIMP-2	Mouse	+	+ / –		Harvey *et al.*, 1995; Reponen *et al.*, 1995
	Pig	+	+		Menino *et al.*, 1997
	Sheep	+			Hampton *et al.*, 1995
	Human	+			Zhang and Salamonsen,1997
TIMP-3	Mouse	+			Harvey *et al.*, 1995; Reponen *et al.*, 1995; Alexander *et al.*, 1996; Teesalu *et al.*, 1996; Das *et al.*, 1998
	Pig	+	+		Menino *et al.*, 1997
	Human	+			Zhang and Salamonsen.,1997
MT1-MMP	Mouse		+		Tanaka *et al.*, 1998
	Human	+			J. Zhang and L. Salamonsen, unpublished

cleave components of the glycocalyx. MT1-MMP is also detectable in endometrial epithelium from day 20 of the normal cycle (J. Zhang and L. Salamonsen, unpublished) and it is postulated that this activates MMP-2 of conceptus origin in a fertile cycle, facilitating penetration of the epithelial basement membrane. TIMP-1, -2 and -3 are present in human endometrium across the cycle in all cellular compartments (Zhang and Salamonsen, 1997) and are strongly expressed in decidual cells late in the cycle. These inhibitors would provide a substantial barrier to invasion.

Endometrial leukocytes are an additional source of both enzymes and regulatory molecules that may modify enzyme production by adjacent cells. In mid-luteal phase human endometrium, these are primarily macrophages, neutrophils, large granular lymphocytes and mast cells (see Salamonsen and Woolley, 1996). Activation of mast cells in normal human endometrium has been observed around day 20 of the cycle (Jeziorska *et al.*, 1995). Mast cell tryptase cleaves collagen VI (Kielty *et al.*, 1993) and, thus, is likely to contribute to the preparation of the stromal matrix for implantation in humans. Mast cell tryptase is also capable of establishing a cascade of MMP activation by its initial cleavage of the propeptide from proMMP-3 (Salamonsen and Woolley, 1996). The role of endometrial leukocytes during placentation in women has been reviewed (Loke and King, 1995). Both numerical and functional changes in endometrial leukocytes have been observed in

infertile women (Stewart-Akers *et al.*, 1998), supporting a role for them in the establishment of pregnancy.

Urokinase-type PA and PAI-1 and -2 have been localized in all three forms of trophoblast (cytotrophoblast, intermediate and syncytiotrophoblast) at the maternal–fetal interface in unruptured ectopic pregnancies (Hofmann *et al.*, 1994). Whether these factors are important at earlier stages of the implantation process remains to be determined.

Species with epitheliochorial placentation

Uterine flushings from pregnant ewes at the time of implantation contain MMP-2 and MMP-9, and these have been immunolocalized in trophoblast on day 17 and detected in media after trophoblast culture (Salamonsen *et al.*, 1995). Periimplantation pig embryos produce uPA and MMP-2 and -9 (Mullins *et al.*, 1980; Fazleabas *et al.*, 1983; Menino *et al.*, 1997), although the MMPs are not detectable by immunohistochemistry, suggesting low expression (Menino *et al.*, 1997). In sheep and pigs, a variety of protease inhibitors, including PAI and TIMPs, are produced both by the trophectoderm (Menino *et al.*, 1997) and the endometrium (Salamonsen *et al.*, 1995) and probably limit the invasive activity of the conceptus (Roberts *et al.*, 1993; Salamonsen *et al.*, 1995; Menino *et al.*, 1997). The endometrium of these species also has the potential to produce a range of MMPs (Salamonsen *et al.*, 1995), and their focal production is doubtless important for the extensive vascular changes associated with implantation.

Several lysosomal enzymes, including cathepsins B1, D and E and leucine aminopeptidase (see Roberts and Bazer, 1988), are also synthesized and released into the uterine lumen by the endometria of species that display non-invasive epitheliochorial types of implantation. A cathepsin L-type polypeptide in the endometrium and in uterine flushings of gilts appears to be regulated by progesterone. Its concentration increases markedly during the attachment phase and it may assist with placental attachment through the limited proteolysis of the glycocalyx on the maternal epithelium and trophoblast (Geisert *et al.*, 1997).

Production of proteases by components of the embryo–maternal axis *in vitro*

Functional studies in vitro

Mouse trophoblast produces proteolytic enzymes *in vitro*. Outgrowths from cultured mouse blastocysts release components from subjacent radiolabelled ECM after attachment (Glass *et al.*, 1983) and, when cultured with an ECM purified from mouse endometrium on day 4 of pregnancy, this trophoblast migrates through the matrix in a manner resembling trophoblast invasion *in vivo* (Armant and Kameda, 1994). This migration is associated with the production of proteolytic enzymes, including MMP-9 (Behrendtsen *et al.*, 1992). Although these data appear to be at odds with the observations that decidual cells are the first cell type to breach the basement membrane, clearly neither the restraining influences nor the normal regulatory controls of gene expression and enzyme activation are present in the *in vitro* environment. Both TIMP-1 (Behrendtsen *et al.*, 1992) and inhibitors of cysteine proteinases (Barbiarz *et al.*, 1992) prevent mouse trophoblast invasion *in vitro*, suggesting that at least two protease families are involved in the invasion.

Preimplantation human embryos, collected as oocytes after superovulation and cultured until the 32-cell stage, display collagen IV-degrading enzyme activity. The secretion of the enzyme(s) increases with time in culture, suggesting an increasing capacity for invasion. The enzyme activity produced per cell is up to 60 000 times greater than that produced by a normal fibroblast (Puistola *et al.*, 1989). Studies on the invasiveness of human trophoblast have used cytotrophoblast cells of first trimester pregnancy to study implantation, applying the rationale that these cells retain almost all the properties of the trophectodermal cells of the blastocyst. Once attached to a thick layer of ECM, these trophoblasts invade it (Fisher *et al.*, 1989; Shimonovitz *et al.*, 1994). They secrete several gelatin-degrading proteases, particularly proMMP-9 and proMMP-2 (Fisher *et al.*, 1989; Bischof *et al.*, 1991; Librach *et al.*, 1991), and these activities can be abolished by inhibitors of metalloproteinases but not by inhibitors of other types of proteases (Fisher *et al.*, 1989; Bischof *et al.*, 1991). In contrast, trophoblastic vesicles derived *in vitro* from marmoset monkey trophoblast, released predominantly MMP-2 when cultured on laminin and fibronectin, suggesting that, if MMP-9 is necessary for trophoblast invasion, then the appropriate stimulatory factors were not present under the culture conditions used (Franek *et al.*, 1997). Human endometrial fibroblasts are the major endometrial source of a number of MMPs, including MMP-3 (Salamonsen and Woolley, 1996). Co-culture of such cells with human cytotrophoblasts in a system where direct contact between the cells did not occur, inhibited the production of MMP-3 by the fibroblasts. This inhibition appeared to be tissue-specific, as similar inhibition did not occur when the fibroblasts were derived from skin. Such inhibition, if it occurs *in vivo*, may provide a mechanism by which invasion is arrested during trophoblast cell migration (Bellingard *et al.*, 1996). Neutralizing antibodies against MMP-9 inhibit ECM degradation by both mouse blastocyst outgrowths and human cytotrophoblasts *in vitro*, suggesting that MMP-9 is the common effector of trophoblast invasion (Librach *et al.*, 1991; Behrendtsen *et al.*, 1992) in these two species in which implantation is highly invasive.

Trophoblast-derived proteases have been identified *in vitro* in species with epitheliochorial placentation. MMPs are produced by ovine trophoblast in culture: latent and active forms of MMP-2 and -9 have been identified, although no proteases that are active against casein (including MMP-1 and MMP-3 along with a number of serine proteases) can be detected. Similarly, trophoblast cells from the equine chorionic girdle have matrix-degrading activities and produce both MMP-2 and MMP-9 when cultured on the artificial basement membrane, Matrigel (Vagoni *et al.*, 1995). This invasion was inhibited by 1,10,phenanthroline, a specific inhibitor of MMPs, but not by aprotinin and bestatin, which inhibit serine proteases and amino peptidase, respectively.

Thus, essentially the same proteases and inhibitors are associated with implantation in all species, regardless of the extent of invasion, the exception being that MMP-2 appears not to be a trophoblast product in mice. In other species, MMP-2 is produced by trophoblast *in vitro* and is detectable in the uterine lumen during early implantation. Whether

Table 3. Targetted deletion or overexpression of protease and related genes

Protease or related gene	Deletion (–) or overexpression (++)	Reproductive disturbance	Reference
uPA	–	No effect on litter size Some reduction in decidual size	Carmeliet *et al.*, 1994
uPA R	–	Nil	Bugge *et al.*, 1995
uPA + tPA + PAI	–	Reduced fertility, some fibrin deposits in uterine subepithelial stroma	Carmeliet *et al.*, 1995
MMP-3	–	Nil	Rudolph-Owen *et al.*, 1997
MMP-7	–	Nil	Rudolph-Owen *et al.*, 1997
MMP-9	–	Nil	Cited in Rinkenberger *et al.*, 1997
α_2-Macroglobulin	–	Nil	Umans *et al.*, 1995
TIMP-1	++	No effect on litter size. Decidual size reduced, embryo displaced mesometrially in uterus	Alexander *et al.*, 1996. Rinkenberger *et al.*, 1997
IL-1 R	–	Slight reduction in mean litter size	Abbondanzo *et al.*, 1996
IL-1R + TNF p55R	–	Nil	Cited in Abbondanzo *et al.*, 1996
TGF-β1	–	Nil	Letterio *et al.*, 1994
Ets2	–	Death of –/– embryos by day 8.5, decreased trophoblast MMP-9, failure of extraplacental zone proliferation.	Yamamoto *et al.*, 1998

IL, interleukin; MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; TIMP, tissue inhibitor of metalloproteinases; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

MMP-2 and MMP-9 are both activated at implantation sites remains to be determined.

Functional studies in vivo

Strategies to establish whether proteases are critical for implantation include treatment with specific synthetic protease inhibitors during early pregnancy, immunoneutralization of protease action and examination of reproductive function in genetically modified mice (Table 3). Such experiments have provided some *in vivo* support to the concept that proteases are important for implantation, although, in general, interference with protease production or activity has not resulted in a reduction in live births.

Studies in mice that are deficient in individual MMPs have provided a diversity of results. Mice with a mutated *MMP-7* gene (Rudolph-Owen *et al.*, 1997) have no evidence of significant reproductive dysfunction, in that they are fertile and can produce multiple litters. MMP-7 has been associated with the involution of the rat uterus (Sellers and Woessner, 1980) and is also present in human endometrial epithelium, although a role in implantation has yet to be established. MMP-3-deficient mice (Rudolph-Owen *et al.*, 1997) have no obvious defect in reproductive function as determined by the average litter size. In both these groups of mice, no deficiencies in the expression of other MMPs were observed. It is possible that there is compensation for the loss of one MMP family member by overexpression of other family members: this is an artefact of the mechanism used for targeted gene deletion. Alternatively, active MMPs may trigger a pathway that represses the expression of other MMPs with similar substrates, to control the extent of matrix degradation, and this negative feedback loop may be disrupted in the MMP-targeted mice.

Given that MMP-9 is a major product of invasive trophoblast, it might be expected that specific disruption of its gene, or inhibition of its action, would severely disrupt trophoblast invasion. Neither targeted disruption of the *MMP-9* gene (Alexander *et al.*, 1996) nor overexpression of the gene for TIMP-1 (Rinkenberger *et al.*, 1997) resulted in a decrease in the number of live births, although both affected the extent of decidualization at implantation sites and resulted in misorientation of embryos. Targeted deletion of the conserved DNA-binding domain of the Ets2 transcription factor, which results in deficient expression of MMP-9, did not actually block implantation *per se*, but did result in the retardation and death of homozygous embryos (Yamamoto *et al.*, 1998). Similarly, injection of MMP inhibitors, including MPI (Alexander *et al.*, 1996) and doxycycline (L. Salamonsen and M. Rechtman, unpublished), into pregnant mice and rats, respectively, before implantation, did not block the implantation process, although both treatments slowed the development and remodelling of the decidual zone; the width and length of this zone was reduced in treated mice. In spite of the apparent defects in decidualization, all animals were fertile and did not exhibit increased embryo resorption (Alexander *et al.*, 1996; Rinkenberger *et al.*, 1997) or numbers of implantation sites on day 7 (L. Salamonsen and M. Rechtman, unpublished). However, these data do suggest that MMPs facilitate decidual morphogenesis and development.

Injection of E64, a synthetic inhibitor of cathepsin B and L, into pregnant females at the stage of blastocyst attachment,

resulted in complete failure of implantation at high doses and in stunted embryo development and reduced decidual reaction at lower doses (Alfonso *et al.*, 1997). Like the MMPs, these cathepsins are necessary for uterine decidualization, and have an additional role in normal embryo development.

In contrast with the MMPs and the cathepsins, the plasminogen activators do not appear to be essential for mouse development or fertility. Mice with combined deficiency of tissue-type PA (tPA) and uPA survived embryonic development, with both single- and double-deficient mice appearing normal at birth (Carmeliet *et al.*, 1994, 1995). Similarly, disruption of the gene encoding uPA receptor, which focuses plasmin-mediated pericellular proteolysis and clears uPA-inhibitor complexes from the cell surface (Bugge *et al.*, 1995), did not affect development or reproduction, including implantation. Furthermore, targeted inactivation of α_2-macroglobulin does not affect fertility in homozygous animals (Umans *et al.*, 1995).

Partial functional overlap with regard to both sites of expression and substrate specificity probably explains why loss or inhibition of any one component does not necessarily compromise overall extracellular matrix remodelling or turnover. The interplay between the various protease systems could perhaps be better understood by the application of combinations of inhibitors of the various protease classes.

Regulation of protease activity at implantation

Proteases (for example, the matrix metalloproteinases: Fig. 1) can be regulated at a number of levels, including transcription, stability of the mRNA, activation and inhibition. From the data currently available, it is likely that most, if not all, of these mechanisms are used at implantation.

Regulation of transcription

Many cytokines, growth factors and steroid hormones have been implicated in the regulation of proteases, in a range of cell types. Some control endometrial or trophoblast proteases and inhibitors *in vitro*. Given the diversity of the known regulators, a high degree of overlap in their functions is likely and few appear to be essential for blastocyst implantation. Leukaemia inhibitory factor (LIF) regulates mouse trophoblast MMP-9 and uPA expression *in vitro* (Harvey *et al.*, 1995), and maternal LIF, produced by the luminal epithelium at implantation, is critical for implantation in mice, although not necessarily in other species (Stewart *et al.*, 1992; Vogiagis *et al.*, 1997). Given that MMP-9-deficient mice were able to carry litters to term, it is likely that LIF has additional, as yet undefined roles at implantation. The cytokine, IL-1β, found in both mouse and human uterus during the pre-implantation stages of development (Simon *et al.*, 1993; Kover *et al.*, 1995) is activated by a serine protease, ICE (Tocci 1997), and is a potent regulator of MMP production by endometrial cells and cytotrophoblast (Librach *et al.*, 1994; Salamonsen and Woolley, 1996). Injection of IL-1 receptor antagonist into mice blocked implantation (Simon *et al.*, 1994); however, in contradiction, deletion of the type 1 IL-1 receptor protein resulted in homozygous females that were fertile (Abbondanzo *et al.*, 1996). As with LIF, IL-1 is likely to have additional functions associated with implantation. It is also possible that another cytokine, such as TNF-α, compensates for the IL-1 deficiency. However, mice that are doubly deficient for both IL-1 receptor and the TNF- p55 receptor are also fertile (cited in Abbondanzo *et al.*, 1996).

Transforming growth factor β (TGFβ) promotes the deposition of ECM and inhibits the production of MMPs. TGFβ is also a potent inducer of TIMP-1 and TIMP-2 (Graham *et al.*, 1994) and may be a negative regulator of trophoblast invasion (Cross *et al.*, 1994). The literature concerning cell-specific and temporal patterns of TGFβ1, -2 and -3 expression in the uterine endometrium during the oestrous cycle and early pregnancy and in the developing embryo has been reviewed (Godkin and Dore, 1998). Although the TGFβ proteins are widespread, production of active forms requires protease cleavage (by mechanisms not yet understood) and this may control TGF action at focal points. Decidual cells may limit invasion by loading latent TGFβ into the ECM (Das *et al.*, 1997). As trophoblast invades, plasmin generated via cell surface uPA may trigger the activation of latent TGFβ, increasing TIMP expression and helping to terminate the invasion process.

The attachment of cells to ECM molecules via cell-surface receptors also regulates protease transcription. In particular, attachment to laminin or fibronectin is considered to be required for the traction and stabilization of migrating cells as well as for inducing MMP expression (Stetler-Stevenson *et al.*, 1993). Cell mobility may also be induced by hyaluronan (Goshen *et al.*, 1996), which is widely distributed in human endometrium in the mid-luteal phase (R. Stern and L. Salamonsen, unpublished) and in mouse uterus at implantation sites (Brown and Papaioannou, 1992). In primates, in which the trophoblast does not immediately invade the basal lamina underlying the luminal epithelium, it is likely that components of this ECM play a role in regulating MMP production.

Activation of enzymes

The invasive capacity of trophoblast is restricted to the feto–maternal interface in a temporally defined manner, suggesting that the activation of proteases is a control mechanism. There are mechanisms to account for the focal activation of trophoblast proMMP-2 and proMMP-9 (Fig. 1). In other systems, proMMP-2 activation occurs in association with the plasma membrane by the action of the MT-MMPs. The role of the MT-MMPs in trophoblast invasion has not yet been defined. However, MT1-MMP is expressed in the luminal epithelium of human endometrium mid-cycle, at a time corresponding to the window of receptivity, and may function to activate proMMP-2 on the trophoblast cell surface at the basal lamina (J. Zhang and L. Salamonsen, unpublished). MT1-MMP has also been localized in early human placenta and in decidual membrane (Hurskainen *et al.*, 1998). Studies *in vitro* have not demonstrated active MMP-2 in cytotrophoblast cultures and support its likely activation *in vivo* by endometrial MT-MMPs. MMP-9 activity may also be focused at the plasma membrane by activation through the PA system. Although pro-uPA is secreted as a soluble protein, it binds to a specific cell-surface receptor. The uPA can convert the plasminogen that is abundant in most extracellular fluids to plasmin, a protease of broad specificity that activates proMMPs, including MMP-9, although, at present, the mechanisms controlling the activators are not understood. In addition, human chorionic gonadotrophin (hCG), an

important product of both cytotrophoblast and trophectodermal cells, downregulates MMP-9 and uPA activities in first trimester cytotrophoblast and this downregulation is associated with decreased invasion *in vitro* (Yagel *et al.*, 1993). Since the mRNA for MMP-9 is not affected, it can be postulated that hCG, via its action on uPA, limits the conversion of latent MMP-9 to its active form and, thus, regulates the transient invasive activity of the trophoblast.

Role of protease inhibitors

Why do carcinoma cells invade uncontrollably while trophoblast invasion is restrained? The evidence suggests that trophoblast invasion is controlled because the cells of the uterus produce particularly large quantities of specific inhibitors, which provide a protective function. In species with interstitial implantation, the decidual cells probably play an important role. In mice, these cells produce large quantities of TIMP-3 from day 5.5 to day 7.5 (Alexander *et al*., 1996; Leco *et al*., 1996) while, in humans, decidual cells express TIMP-1, -2 and -3 during the late luteal phase of the cycle (Zhang and Salamonsen, 1997). Cystatin C (Alfonso *et al.*, 1997) and PAI (Hofmann *et al.*, 1994; Teesalu *et al.*, 1996) are also appropriately present near implantation sites in these species. In both pigs and sheep, TIMPs and PAI are present in the endometrium at implantation, even though decidualization does not occur (Mullins *et al.*, 1980; Hampton *et al.*, 1995; Menino *et al.*, 1997). Broad spectrum protease inhibitors, such as α_2-macroglobulin, probably also assist in inhibiting trophoblast invasion.

Conclusions

Matrix-degrading proteases, and enzymes that activate either the proteases or regulators of their activation pathways, have an undisputed role in the early stages of embryo implantation, particularly with respect to invasion of the trophoblast. However, no one protease, or even one class of protease appears to be solely responsible for the invasion. The contribution of various inhibitors to restraining this trophoblast invasion is also without doubt. Indeed, the difference between mammalian species in the extent of invasion is likely to be attributable to such inhibitors. The major differences between tumour invasion and invasion of the trophoblast appear to be, first, the restraint imposed by endometrial inhibitors, and second, that trophoblast does not induce MMP production in adjacent cells of the host tissue as is seen with tumour invasion.

Given the centrality of the process of implantation to the continuation of mammalian survival, the evolution of a myriad of redundant and compensatory mechanisms to maintain reproductive capacity provides important safeguards. It is not surprising that the manipulation of genes for single proteases or inhibitors has not caused the failure of implantation, given the complexity of the pathways controlling the expression, activity and inhibition of the proteases and the expression of their inhibitors, together with the overlapping substrate specificities and redundancy in regulatory molecules.

The identities and roles of other proteases at implantation sites remain to be determined. However, it is becoming apparent that proteases with additional roles not necessarily related to matrix degradation will also be of importance to the implantation process.

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References

Key references are indicated by asterisks.

Abbondanzo SJ, Cullinan EB, McIntyre K, Labow MA and Stewart CL (1996) Reproduction in mice lacking a functional type 1 Il-1 receptor *Endocrinology* **137** 3598–3601

***Alexander CM, Hansell EJ, Behrendtsen O, Flannery ML, Kishani NS, Hawkes SP and Werb Z** (1996) Expression and function of matrix metalloproteinases and their inhibitors at the maternal–embryonic boundary during mouse embryo implantation *Development* **122** 1723–1736

***Alfonso S, Romagnano L and Babiarz B** (1997) The expression and function of cystatin C and cathepsin B and cathepsin L during mouse embryo implantation and placentation *Development* **124** 3415–3425

Armant DR and Kameda S (1994) Mouse trophoblast cell invasion of extracellular matrix purified from endometrial tissue: a model for periimplantation development *Journal of Experimental Zoology* **269** 146–156

Baker AH, Zaltsman AB, George SJ and Newby AC (1998) Divergent effects of tissue inhibitor of metalloproteinase-1, -2 or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation and death *in vitro*. *Journal of Clinical Investigation* **101** 1478–1487

Barbiarz B, Romagnano L and Kurilla G (1992) The interaction of mouse ectoplacental cone trophoblast and uterine decidua *in vitro*. *In Vitro Cellular and Developmental Biology* **28A** 500– 508

Barrett AJ (1994) Classification of peptidases *Methods in Enzymology* **244** 1–15

Behrendtsen O, Alexander CM and Werb Z (1992) Metalloproteinases mediate extracellular degradation by cells from mouse blastocyst outgrowths *Development* **114** 447–456

Bellingard V, Hedon B, Capony F, Defrenne A, Rochefort H and Maudelonde T (1996) Identification of prometalloproteinase-3 as a major protein secreted by human endometrial fibroblasts and inhibited by coculture with trophoblast cells *Biology of Reproduction* **55** 604–612

Bischof P, Friedli E, Martelli M and Campana A (1991) Expression of extracellular matrix-degrading metalloproteases by cultured human cytotrophoblast cells: effect of cell adhesion and immunopurification *American Journal of Obstetrics and Gynecology* **65** 1791–1801

Black RA, Rauch CT, Kozolosky CJ *et al.* (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells *Nature* **385** 729–733

Blankenship TN and Given RL (1991) Penetration of the uterine epithelial basement membrane during blastocyst implantation in the mouse *Anatomical Record* **233** 196–204

Boujrad N, Ogwuegbu SO, Garnier M, Lees M, Martin BM and Papadopoulos V (1995) Identification of a stimulator of steroid hormone synthesis isolated from testis *Science* **268** 1609–1612

Brown JJG and Papaioannou VE (1992) Distribution of hyaluronan in the mouse endometrium during the periimplantation period of pregnancy *Differentiation* **52** 61–68

Bugge TH, Suh TT, Flick MJ, Daugherty CC, Romer J, Solberg H, Ellis V, Dano K and Degen JL (1995) The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility *Journal of Biological Chemistry* **270** 16 886–16 894

Carmeliet P, Schoonjans L, Kieckens L, Rean B, Degen J, Bronson R, DeVos R, van den Oord JJ, Collen D and Mulligan RC (1994) Physiological consequences of loss of plasminogen activator gene function in mice *Nature* **368** 419–424

***Carmeliet P, Bouche A, De Clercq C, Janssen S, Pollefeyt S, Wyns S, Mulligan RC and Collen D** (1995) Biological effects of disruption of the tissue-type plasminogen activator, urokinase-type plasminogen activator and plasminogen activator inhibitor-1 genes in mice *Annals of the New York Academy of Sciences* **748** 367–381

Crawford HC and Matrisian LM (1996) Mechanisms controlling the transcription of matrix metalloproteinase genes in normal and neoplastic cells *Enzyme and Protein* **49** 20–37

Cross JC, Werb Z and Fisher SJ (1994) Implantation and the placenta: key pieces of the development puzzle *Science* **266** 1506–1518

Das SK, Yano S, Wang J, Edwards DR, Nagase H and Dey SK (1997) Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in the mouse uterus during the peri-implantation period *Developmental Genetics* **21** 44–54

Das SK, Yano S, Wang J, Edwards Dr, Nagase H and Dey SK (1998) Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in the mouse uterus during the peri-implantation period *Developmental Genetics* **21** 44–54

Edwards DR, Beaudry PP, Liang TD, Kowal TD, Leco KJ, Leco PA and Lim MS (1996) The roles of tissue inhibitors of metalloproteinases in tissue remodelling and cell growth *International Journal of Obesity* **20** S9–S15

Elangovan S and Moulton BC (1980) Blastocyst implantation in the rat and the immunohistochemical distribution and rate of synthesis of uterine lysosomal cathepsin D *Biology of Reproduction* **23** 663–668

Enders AC (1997) Implantation, Embryology. In *Encyclopedia of Human Biology* 2nd edn pp 799–807 Ed. R Dulbecco. Academic Press, San Diego

Enders AC and Liu IKM (1991) Trophoblast–uterine interactions during equine chorionic girdle cell maturation, migration and transformation *American Journal of Anatomy* **192** 366–381

Enders AC, Chavez DJ and Schlafke S (1981) Comparison of implantation *in utero* and *in vivo*. In *Cellular and Molecular Aspects of Implantation* pp 365–382 Eds SR Glasser and DW Bullock. Plenum Press, New York

Fazleabas AT, Geisert RD, Bazer FW and Roberts RM (1983) Relationship between release of plasminogen activator and estrogen by blastocysts and secretion of plasmin inhibitor by uterine endometrium in the pregnant pig *Biology of Reproduction* **29** 225–238

Fernandez PL, Merino MJ, Nogales FF, Charonis AS, Stetler-Stevenson WG and Liotta L (1992) Immunohistochemical profile of basement membrane proteins and 72 kilodalton type IV collagenase in the implantation placental site *Laboratory Investigation* **66** 572–579

Fisher SJ, Cui T, Zhang L, Hartman L, Grahl K, Zhang G, Tarpey J and Damsky CH (1989) Adhesive and degradative properties of human placental cytotrophoblast cells *in vitro*. *Journal of Cellular Biology* **109** 891–902

Fowlkes JL, Enghild JJ, Suzuki K and Nagase H (1994) Matrix metalloproteinases degrade insulin-like growth factor binding protein-3 in dermal fibroblast cultures *Journal of Biological Chemistry* **269** 25 742–25 746

Franek AD, Salamonsen LA and Lopata A (1997) Marmoset monkey trophoblastic tissue growth and matrix metalloproteinase secretion in culture *Proceedings of the Australian Society for Reproductive Biology* **28** 61

Geisert RD, Blair RM, Pratt T and Zavy MT (1997) Characterizaton and proteolytic activity of a cathepsin L-like polypeptide in endometrium and uterine flushings of cycling, pregnant and steroid-treated ovariectomized gilts *Reproduction Fertility and Development* **9** 395–402

Glass RH, Aggeler J, Spindle A, Pederson RA and Werb Z (1983) Degradation of extracellular matrix by mouse trophoblast outgrowths: a model for implantation *Journal of Cellular Biology* **96** 1108–1116

Godkin JD and Dore JJ (1998) Transforming growth factor beta and the endometrium *Reviews of Reproduction* **3** 1–6

Goshen R, Ariel I, Shuster S, Hochberg A, Vlodavsky I, de Groot N, Ben-Rafael Z and Stern R (1996) Hyaluronan, CD44 and its variant exons in human trophoblast invasion and placental angiogenesis *Molecular Human Reproduction* **2** 685–691

Graham CH, Connelly I, MacDougall JR, Kerbel RS, Stetler-Stevenson WG and Lala PK (1994) Resistance of malignant trophoblast cells to both the anti-proliferative and anti-invasive effects of transforming growth factor-beta *Experimental Cell Research* **214** 93–99

Hampton AL, Butt AR, Riley SC and Salamonsen LA (1995) Tissue inhibitors of metalloproteinases in endometrium of ovariectomized, steroid-treated ewes and during the estrous cycle and early pregnancy *Biology of Reproduction* **53** 302–311

Harvey MB, Leco KJ, Arcellana-Panlilio MY, Zhang X, Edwards DR and Schultz GA (1995) Proteinase expression in early mouse embyros is regulated by leukaemia inhibitory factor and epidermal growth factor *Development* **121** 1005–1014

Hayakawa T, Yamashita K, Uchijima M and Iwata K (1992) Growth-promoting activity of tissue inhibitor of metalloproteinases (TIMP-1) for a wide range of cells *FEBS Letters* **298** 29–32

Hayakawa T, Yamashita K, Ohuchi E and Shinagawa A (1994) Cell growth promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2) *Journal of Cell Science* **107** 2372–2379

Hofmann GE, Glatstein I, Schatz F, Heller D and Deligdisch L (1994) Immunohistochemical localization of urokinase-type plasminogen activator and the plasminogen activator inhibitors 1 and 2 in early human implantation sites *American Journal of Obstetrics and Gynecology* **170** 671–676

Hurskainen T, Seiki M, Apte SS, Syrjakallio-Ylitalo M, Sorsa T, Oikarinen A and Autio-Harmainen H (1998) Production of membrane-type matrix metalloproteinase-1 (MT-MMP-1) in early human placenta. A possible role in placental implantation *Journal of Histochemistry and Cytochemistry* **46** 221–229

Ito A, Mukaiyama A, Itoh Y, Nagase H, Thogersen IG, Enghild JJ, Sasaguri Y and Mori Y (1996) Degradation of interleukin 1beta by matrix metalloproteinases *Journal of Biological Chemistry* **271** 14 657–14 660

Jeziorska M, Salamonsen LA and Woolley DE (1995) Mast cell and eosinophil distribution and activation in human endometrium throughout the menstrual cycle *Biology of Reproduction* **53** 312– 320

Jeziorska M, Nagase H, Salamonsen LA and Woolley DE (1996) Immunolocalization of the matrix metalloproteinases, gelatinase B and stromelysin-1 in human endometrium throughout the menstrual cycle *Journal of Reproduction and Fertility* **107** 43–51

Kielty CM, Lees M, Shuttleworth CA and Woolley DE (1993) Catabolism of intact type VI collagen microfibrils: susceptibility to degradation by serine proteinases *Biochemical and Biophysical Research Communications* **191** 1230–1236

Kilpatrick LM, Kola I and Salamonsen LA (1998) Differential expression of ETS1, ETS2 and ELF1 in human endometrium across the menstrual cycle *Proceedings of the Australian Society for Reproductive Biology* **29** Abstract 54

Kover K, Liang L, Andrews GK and Dey SK (1995) Differential expression and regulation of cytokine genes in the mouse uterus *Endocrinology* **136** 1666–1673

Leco KJ, Edwards DR and Schultz GA (1996) Tissue inhibitor of metalloproteinases-3 is the major metalloproteinase inhibitor in the decidualizing murine uterus *Molecular Reproduction and Development* **45** 458–465

Letterio JJ, Geiser AG, Kukarni AB, Roche NS, Sporn MB and Roberts AB (1994) Maternal rescue of transforming growth factor-β1 null mice *Science* **264** 1936–1938

***Librach CL, Werb Z, Fitzgerald ML, Chiu K, Corwin NM, Esteves RA, Grobelny D, Galardy R, Damsky CH and Fisher SJ** (1991) 92-kDa type IV collagenase mediates invasion of human cytotrophoblasts *Journal of Cellular Biology* **113** 437–449

Librach CL, Feigenbaum SL, Bass KE, Cui T, Verastas N, Sadovsky Y, Quigley JP, French DL and Fisher SJ (1994) Interleukin-1α regulates human cytotrophoblast metalloproteinase activity and invasion *in vitro*. *Journal of Biological Chemistry* **269** 17 125–17 131

Loke YW and King A (1995) *Human Implantation: Cell Biology and Immunology* pp 102–129 Cambridge University Press, Cambridge

Menino ARJ, Hogan A, Schultz GA, Novak S, Dixon W and Foxcroft GH (1997) Expression of proteinases and proteinase inhibitors during embryo–uterine contact in the pig *Developmental Genetics* **21** 68–74

Mullins DE, Bazer FW and Roberts RM (1980) Secretion of a progesterone-induced inhibitor of plaminogen activator by the porcine uterus *Cell* **20** 865– 872

Nie G, Butt AR, Salamonsen LA and Findlay JK (1997) Hormonal and non-hormonal agents at implantation *Reproduction Fertility and Development* **9** 65–76

Parr EL, Tung HN and Parr MB (1987) Apoptosis as the mode of uterine epithelial cell death during embryo implantation in mice and rats *Biology of Reproduction* **36** 211–225

Powell WC and Matrisian LM (1996) Complex roles of matrix metalloproteinases in tumor progression *Current Topics in Microbiology and Immunology* **213** 1–21

Puistola U, Ronnberg L, Martikainen H and Turpeenniemi-Hujanen T (1989) The human embryo produces basement membrane collagen (type IV collagen)-degrading protease activity *Human Reproduction* **4** 309–311

Ramsey EM, Houston ML and Harris JWS (1976) Interactions of the trophoblast and maternal tissues in three closely related primate species *American Journal of Obstetrics and Gynecology* **124** 647–652

Reponen P, Leivo I, Sahlberg C, Apte SS, Olsen BR, Thesleff I and Tryggvason K (1995) 92-kDa type IV collagenase and TIMP-3, but not 72-kDa type IV collagenase or TIMP-1 or TIMP-2, are highly expressed during mouse embryo implantation *Developmental Dynamics* **202** 388–396

Rinkenberger JL, Cross JC and Werb Z (1997) Molecular genetics of implantation in the mouse *Developmental Genetics* **21** 6–20

Roberts RM and Bazer FW (1988) The function of uterine secretions *Journal of Reproduction and Fertility* **82** 875–892

Roberts RM, Bazer FW, Baldwin N and Pollard WE (1976) Progesterone induction of lysozyme and peptidase activities in the porcine uterus *Archives of Biochemistry and Biophysics* **177** 499–507

Roberts RM, Xie S and Trout WE (1993) Embryo–uterine interactions in pigs during week 2 of pregnancy *Journal of Reproduction and Fertility Supplement* **48** 171–186

Rudolph-Owen LA, Hulboy DL, Wilson CL, Mudgett J and Matrisian LM (1997) Coordinate expression of matrix metalloproteinase family members in the uterus of normal, matrilysin-deficient, and stromelysin-1-deficient mice *Endocrinology* **138** 4902–4911

Salamonsen LA and Woolley DE (1996) Matrix metalloproteinases and their tissue inhibitors in endometrial remodelling and menstruation *Reproductive Medicine Reviews* **5** 185–203

***Salamonsen LA, Nagase H and Woolley DE** (1995) Matrix metalloproteinases and their tissue inhibitors at the ovine trophoblast–uterine interface *Journal of Reproduction and Fertility Supplement* **49** 29–37

Salamonsen LA, Butt AR, Hammond FR, Garcia S and Zhang J (1997) Production of endometrial matrix metalloproteinases but not their tissue inhibitors is modulated by progesterone withdrawal in an *in vitro* model for menstruation *Journal of Clinical Endocrinology and Metabolism* **82** 1409–1415

Samuel CA and Perry JS (1972) The ultrastructure of pig trophoblast transplanted to an ectopic site in the uterine wall *Journal of Anatomy* **113** 139–149

Satoh T, Kobayashi K, Yamashita S, Kikuchi M, Sendai Y and Hoshi H (1994) Tissue inhibitor of metalloproteinases (TIMP-1) produced by granulosa and oviduct cells enhances *in vitro* development of bovine embryos *Biology of Reproduction* **50** 835–844

Schlafke S, Welsh AO and Enders AC (1985) Penetration of the basal lamina of the uterine luminal epithelium during implantation in the rat *Anatomical Record* **212** 47–56

Sellers A and Woessner JFJ (1980) The extraction of a neutral metalloproteinase from the involuting rat uterus and its action on cartilage proteoglycan *Biochemical Journal* **189** 521–531

Shimonovitz S, Hurwitz A, Dushnik M, Anteby E, Geva-Eldar T and Yagel S (1994) Developmental regulation of the expression of 72 and 92 kd type IV collagenases in human trophoblasts: a possible mechanism for control of trophoblast invasion. *American Journal of Obstetrics and Gynecology* **171** 832–838

Simon C, Piquette GN, Frances A and Polan ML (1993) Localization of interleukin-1 type 1 receptor and interleukin-1β in human endometrium throughout the menstrual cycle *Journal of Clinical Endocrinology and Metabolism* **77** 549–555

Simon C, Frances A, Piquette GN, el Danasouri I, Zurawski G, Dang W and Polan ML (1994) Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist *Endocrinology* **134** 521–528

Singer CF, Marbaix E, Kokorine I, Lemoine P, Donnez J, Eeckhout Y and Courtoy PJ (1997) Paracrine stimulation of interstitial collagenase (MMP-1) in the human endometrium by interleukin-1α and its dual block by ovarian steroids *Proceedings of the National Academy of Sciences USA* **94** 10 341–10 345

Stetler-Stevenson WG, Aznavoorian S and Liotta LA (1993) Tumor cell interactions with the extracellular matrix during invasion and metastasis *Annual Review of Cell Biology* **9** 541–573

Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F and Abbondanzo SJ (1992) Blastocyst implantation depends on maternal expression of leukemia inhibitory factor *Nature* **359** 76–79

Stewart-Akers AM, Krasnow JS, Brekosky J and DeLoia JA (1998) Endometrial leukocytes are altered numerically and functionally in women with implantation defects *American Journal of Reproductive Immunology* **39** 1–11

Strickland S, Reich E and Sherman MI (1976) Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm *Cell* **9** 231–240

Tanaka SS, Togooka Y, Sato H, Seiki M, Tojo H and Tachl C (1998) Expression and localization of membrane type matrix metalloproteinase-1 (MT-MMP) in trophoblast cells of cultured mouse blastocysts and ectoplacental cones *Placenta* **19** 41–48

Teesalu T, Blasi F and Talarico D (1996) Embryo implantation in mouse: fetomaternal coordination in the pattern of expression of uPA, uPAR, PAI-1 and alpha 2MR/LRP genes *Mechanisms of Development* **56** 103–116

Tocci MJ (1997) Structure and function of interleukin-1β converting enzyme *Vitamins and Hormones* **53** 27–63

Umans L, Serneels L, Overbergh L, Lorent K, Van Leuven F and Van den Berghe H (1995) Targeted inactivation of the mouse alpha 2-macroglobulin gene *Journal of Biological Chemistry* **270** 19 778–19 785

Vagoni KE, Ginther OJ and Lunn DP (1995) Metalloproteinase activity has a role in equine chorionic girdle cell invasion *Biology of Reproduction* **53** 800–805

Vassalli J, Sappino A and Belin D (1991) The plasminogen activator/plasmin system *Journal of Clinical Investigation* **88** 1067–1072

Vogiagis D, Fry RC, Sandeman RM, Squires TJ and Salamonsen LA (1997) Effect of immunization against leukemia inhibitory factor on the establishment of pregnancy in sheep and cattle *Reproduction Nutrition and Development* **37** 459–468

Welsh AO and Enders AC (1987) Trophoblast–decidual cell interactions and establishment of maternal blood circulation in the parietal yolk sac placenta of the rat *Anatomical Record* **217** 203–219

Wilson CL and Matrisian LM (1996) Matrilysin: a epithelial matrix metalloproteinase with potentially novel functions *International Journal of Biochemistry and Cell Biology* **28** 123–136

Wooding FBP and Flint APF (1994) Placentation. In *Marshall's Physiology of Reproduction* 2nd edn pp 235–460 Ed. GE Lamming. Chapman and Hall, London

Yagel S, Geva TE, Solomon H, Shimonovitz S, Reich R, Finci-Yeheskel Z, Mayer M and Milwidsky A (1993) High levels of human chorionic gonadotropin retard first trimester trophoblast invasion *in vitro* by decreasing urokinase plasminogen activator and collagenase activities *Journal of Clinical Endocrinology and Metabolism* **77** 1506–1511

Yamamoto H, Flannery ML, Kupriyanov S, Pearce J, McKercher SR, Henkel GW, Maki RA, Werb Z and Oshima RG (1998) Defective trophoblast function in mice with a targeted mutation of Ets2 *Genes and Development* **12** 1315–1326

Zhang J and Salamonsen LA (1997) Tissue inhibitor of metalloproteinases (TIMP)-1, -2 and -3 in human endometrium during the menstrual cycle *Molecular Human Reproduction* **3** 735–741

Zhang J, Nie G, Wang J, Woolley DE and Salamonsen LA (1998) Mast cell regulation of endometrial stromal cell matrix metalloproteinases: a mechanism underlying menstruation *Biology of Reproduction* **59** 693–703