

The Implantation Serine Proteinases: Potential Therapeutic Footholds in Female Fertility

Review Article

Derrick E. Rancourt
Depts. of Oncology
and
Biochemistry &
Molecular Biology
University of Calgary,
Calgary, Alberta,
Canada, T2N 4N1;

Tel 1-403-220-2888;
FAX 1-403-283-8727;
rancourt@acs.ucalgary.ca

Abstract

Hatching of the blastocyst from the zona pellucida represents an important first step in implantation and the establishment of a successful pregnancy. Investigation in the mouse model system has revealed two serine proteinase systems associated with hatching: strypsin, a localized blastocytic enzyme responsible for the focal hatching of the embryo in vitro; and lysin, a uterine luminal serine proteinase that lyses the zona pellucida at the time of hatching. Due to limitations in applying biochemistry to the study of hatching, much confusion has arisen over the respective roles of these two proteolytic systems. Recently, we have used RT-PCR to reveal two novel genes (denoted implantation serine proteinases, ISP 1&2) encoding tryptases that are expressed during the initiation of implantation in mouse. Based on functional studies, we suggest that ISP1 and ISP2 encode strypsin and lysin respectively. We have noted that ISP1/strypsin plays two critical roles in implantation. Although it is essential for hatching in vitro, more importantly, ISP1 is required for the initiation of blastocyst invasion into ECM. We suggest that the localized expression of strypsin/ISP1 is necessary for the orientation specific invasion of the blastocyst. In the absence of lysin/ISP2 (i.e. during assisted reproduction), strypsin plays a critical role in hatching. Hatching disorders are common in women of older reproductive age. In demonstrating a potential link between hatching and implantation, our work helps to explain why artificial hatching of these embryos often fails to yield successful pregnancies. Having identified the mammalian hatching enzymes, potential therapeutics and indicators of pregnancy success may be developed. Moreover, these proteinases represent ideal targets for the development of non-steroid based contraceptives.

Implantation in the Human and Murine Experimental Model

Implantation is a critical stage of reproduction where the embryo attaches to the uterine wall in order to garner nutrition from the mother's blood supply. Failure results in spontaneous abortion of the fetus. Based on similarities with humans, implantation has been modeled in the murine experimental system. Modern molecular genetics techniques, such as targeted gene disruption, have begun to provide insights into the potential genetic causes for implantation failure (for recent reviews see Carson, 2000; Rikenberger *et al*, 1997).

Implantation is a complex process composed of several developmental phases: blastocyst hatching, attachment and invasion (Fig. 1). Prior to implantation, the mammalian blastocyst is maintained within a proteinaceous coat, the *zona pellucida*, which prevents polyspermy and ectopic implantation. Hatching from the *zona pellucida* is considered to be the first step in implantation. It is generally accepted that hatching is mediated by two proteolytic systems (Gonzales and Bavinster, 1995; Perona and Wassarman, 1986). First a serine proteinase(s), lysin, is secreted into the uterine lumen in response to progesterone, which lyses the *zona pellucida* externally on day 4 of pregnancy (Orsini and McLaren, 1967; Joshi and Murray, 1974; Denker, 1977). A second serine proteinase, strypsin, is secreted by the embryo one day after lysin and acts more as a factor in implantation. However, in situations where embryos are removed from the uterus (i.e. during assisted reproduction), embryonic strypsin plays a primary hatching role in the absence of lysin. *In vitro*, hatching occurs focally due to the localized activity of strypsin at the abembryonic pole. As it is this pole that first attaches to uterine tissue, it has been suggested that strypsin's primary role may be in initiating implantation (Gozales and Bavinster, 1995).

The hormones estrogen and progesterone are necessary to synchronize the embryo and uterine receptivity in implantation. Prior to hatching on day

four, estrogen and progesterone are important in preparing uterine proliferation and differentiation. After hatching, an estrogen spike creates a period of receptivity between the embryo and the uterus, allowing the embryonic trophoblast and uterine epithelium to interact (Paria *et al*, 1993). This embryo-uterine dialogue is orchestrated by the local traffic of cytokines between the embryo, luminal, epithelium and endometrial glands. In mice, EGF and LIF have been found to play principle roles in blastocyst attachment and invasion, lying downstream of this estrogen spike (Das *et al*, 1994). Interestingly, the invading (abembryonic) pole of the blastocyst is rich in the heparin sulfate proteoglycan (perlecan), which is necessary for blastocyst invasion *in vitro* (Carson, 1993). Attachment of the invading pole of the blastocyst is thought to be mediated by the expression of heparin binding EGF, which is tethered to the surface of the uterine epithelium (Raab *et al*, 1996). EGF, in turn, has been found to promote blastocyst invasion, which is thought to occur by signaling through the EGF receptor, ErbB4, which appears on the apical surface of the invading trophoblasts (Paria *et al*, 1999, Wang *et al*, 2000). LIF is a regulatory cytokine that is secreted from endometrial glands at the onset of implantation and is necessary to sustain adhesion and invasion (Stewart *et al*, 1992). In the absence of LIF, luminal EGF family members are not expressed and blastocysts fail to attach (Song *et al*, 2000). As LIF expression is estrogen-dependent, it has been suggested that the window of implantation may be LIF-mediated (Song *et al*, 2000).

Proteinases and Embryonic Invasion During Implantation

Under cytokine instruction, proteinases and their corresponding inhibitors are thought to mediate the fine balance between trophoblast invasion and decidual anti-invasion. Classically, proteinases secreted by trophoblasts, uPA and MMP9, have been thought to be the main orchestrators of invasion (Rinkenberger *et al*, 1997). Their expression is upregulated by cytokines such as LIF and EGF and their enzymatic activity is regulated by the inhibitors PAI 1 & 2 and TIMP3, which are expressed on the border of trophoblast invasion (Harvey *et al*, 1995; Teesalu *et al*, 1996, Leco *et al*, 1996). Inhibitor studies have suggested that both uPA and MMP9 play principle roles in embryo outgrowth during implantation (Behrendtsen *et al*, 1992; Werb

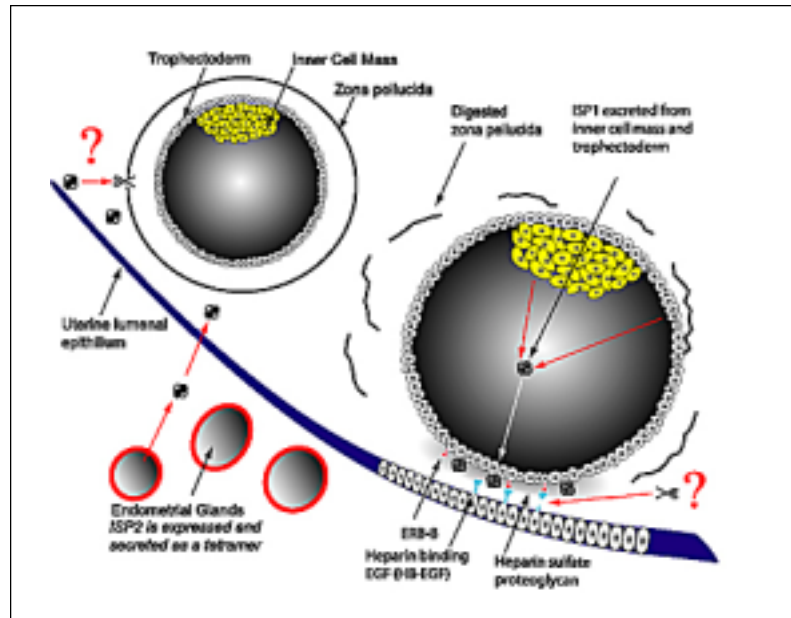


Figure 1. Prior to implantation, the blastocyst is maintained within a proteinaceous coat, the *zona pellucida*, which prevents polyspermy and ectopic pregnancy. Implantation is initiated by hatching, whereupon the blastocyst is free to interact with the uterine luminal epithelium. Hatching is mediated by a progesterone regulated serine proteinase that is secreted into the uterine fluid. Based on the expression pattern of ISP2, we hypothesize that ISP2 tetramer may represent the hatching enzyme. Following hatching, blastocyst invasion is initiated by the interaction of the heparin sulfate proteoglycan-rich, abembryonic pole with the uterine luminal epithelium. Heparin binding-EGF, which is tethered to this epithelial surface, is important in invasion. Here, ISP1 tetramer may be recruited to the heparin sulfate bed in order to participate directly in invasion or via the proteolytic liberation of luminal HB-EGF.

et al, 1992). In sharp contrast, however, targeted mutagenesis studies have indicated that either proteinase is dispensable in implantation (Carmeliet *et al*, 1994; Vu *et al*, 1998). These latter observations have questioned the importance of uPA and MMP9 within the presumed implantation proteinase cascade and have suggested that other proteinases may be important in implantation. Indeed, other proteinase and antiproteinases have begun to be localized to the embryo-decidual border (Afonso *et al*, 1997; Lefebvre *et al*, 1992; Hurskainen *et al*, 1998).

The ISPs: Novel Trypsins Expressed During Implantation

Using serine proteinase active site RT-PCR, we have identified two murine genes encoding novel trypsinases that are expressed at the time of blastocyst hatching and implantation (O'Sullivan *et*

al, 2000a; O'Sullivan *et al*, 2000b). We have coined these implantation serine proteinase genes as ISP1 and 2. Sequencing of a full-length ISP cDNA clones demonstrated that the ISP genes encode a novel serine proteinase related to tryptases. In BLAST identity searches, we found that the ISPs shared a moderate amount of sequence similarity with haematopoietic tryptases and shared conserved His and Ser active site moieties, in addition to the common N-terminal sequence (IVVG) of mature tryptases. Maximum parsimony analysis suggests that the ISPs represent distinct branches of the S1 proteinase superfamily that diverged from the elastase/chymotrypsin and mast cell proteinase clusters at approximately the same time (Fig. 2). The evolution of these tryptases is consistent with our suggestion that the ISPs play overlapping roles in hatching and implantation.

ISP 1: A Novel Tryptase Involved in Embryo Hatching and Invasion

We have demonstrated that the ISP1 gene is expressed in pre-implantation embryos and is necessary for successful blastocyst hatching *in vitro* (O'Sullivan *et al*, 2001). Accordingly, we have

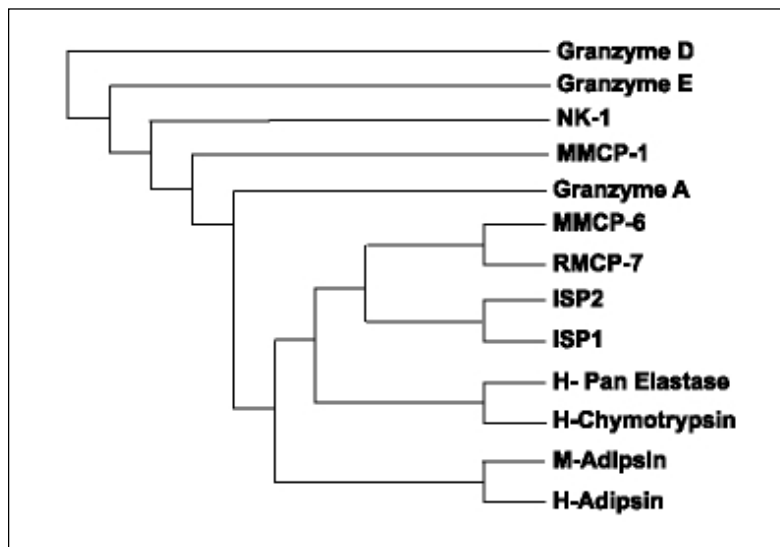


Figure 2. Dendrogram showing the relationship of amino acid sequences between representative serine proteinases. Serine proteinase sequences identified from a BLAST identity search were aligned using Clustal W and an unrooted tree was constructed using maximum parsimony analysis. The ISPs represent a distinct branch of the S1 proteinase superfamily that first diverged from the elastase/chymotrypsin and mast cell proteinase clusters at approximately the same time.

hypothesized that ISP1 encodes the elusive mammalian hatching enzyme, strypsin. Prior to implantation, strypsin is localized to the abembryonic pole of the blastocyst (Perona and Wassarman, 1986). Based on inhibitor studies, strypsin activity is necessary for hatching *in vitro*. In antisense studies, we have demonstrated that specific abrogation of ISP1 gene expression can prevent hatching *in vitro*. This affect on hatching is specific, dose-dependent and is completely reversible. In the absence of ISP1 expression, strypsin activity no longer localizes to the abembryonic pole. Interestingly, it is this pole that first becomes adhesive *in utero* and orients the embryo for invasion (Kirby *et al*, 1967). As tryptases are tetramerized on beds of heparin sulfate proteoglycan (Lindstedt *et al*, 1998), we hypothesize that ISP1 monomers that are expressed throughout the embryo must tetramerize at the abembryonic pole to form strypsin (Fig 3).

Through our discovery of ISP1, we have confirmed the prediction that the enzyme responsible for focal hatching *in vitro* might really be the enzyme responsible for facilitating blastocyst attachment and invasion (Gozales and Bavinster, 1995). In the absence of ISP1 expression, we observe that blastocysts also fail to outgrow *in vitro*. We hypothesize that ISP1 participates in a continuum that connects blastocyst hatching to ECM attachment and outgrowth. Historically, hatching and outgrowth have been viewed as unrelated molecular phenomena. While serine proteinase inhibitors have been demonstrated to affect both hatching and ECM invasion, these studies have focused on the respective roles of strypsin in hatching and uPA in invasion (Perona and Wassarman, 1986; Kubo *et al*, 1981). We have noted that most, if not all of these inhibitors, are effective against tryptases and have suggested that their action in affecting outgrowth may be directed against the ECM-degrading potential of strypsin.

Interestingly, heparinase and heparin sulfate has been found to abrogate embryo attachment and outgrowth *in vitro* (Carson *et al*, 1993) and may act, in part, by interfering with strypsin activity. In hindsight, it seems reasonable that a localized proteinase involved in degrading the *zona pellucida* might also be involved in initiating the degradation of ECM that occurs in blastocyst outgrowth. ISP1/strypsin may also participate indirectly in ECM degradation through the activation of other proteinases, such as uPA and MMP9. As EGF has previously been found to activate blastocyst out-

growth *in vitro* (Das *et al.*, 1994), localized stryptsin could also participate by freeing bound HB-EGF for signaling to the ErbB4 receptor. Indeed, other serine proteinases have been found to facilitate HB-EGF signaling (Kalmes *et al.*, 2000), and support this idea.

ISP2: A Novel Progesterone Regulated Trypsinase in Uterine Glands

Recently the action of stryptsin during hatching has been characterized an artifact, since focal hatching does not occur *in utero* (Gozales and Bavinster, 1995). Instead, it is generally accepted that release of the embryo from the *zona pellucida* occurs via the action of lysin proteinase (Gonzales *et al.*, 2001). Although not well characterized, lysin is secreted into the uterine lumen on day 4. Pregnancy manipulation studies in mouse and hamster have demonstrated that lysin activity in uterine fluid is embryo independent, yet is dependent upon progesterone signaling (Gonzales *et al.*, 2001; Orsini and McLaren, 1967; Joshi and Murray, 1974; Rosenfeld and Joshi, 1981). Based on functional studies, we hypothesize that ISP2 encodes the elusive lysin proteinase, and that like ISP1, ISP2 may multimerize to form a lysin (O'Sullivan *et al.*, 2001b). ISP2 expression is limited to the uterine endometrial gland, which is the major source of uterine secretions in early pregnancy. Based on *in situ* hybridization staining, ISP2 expression is first detected in day 4 pregnancy. In artificial pregnancy, we have demonstrated that ISP2 is expressed in oil-induced deciduomas, and in control uteri treated with estrogen and progesterone alone. These observations suggest that, like lysin, ISP2 expression is embryo-independent and hormonally regulated. Indeed, when the day 4 estrogen pulse is prevented by ovariectomy, ISP2 expression is only observed when progesterone is administered. Similarly, administration of antiprogesterin prevents ISP2 expression, suggesting that ISP2, like lysin, is progesterone regulated.

Based on all of the above functional data, including the putative relationship of ISP2 to ISP1 and stryptsin, we hypothesize that the ISP2 gene encodes lysin. Our observation that two related tryptases are derived separately from the embryo and uterus to effect hatching reiterates the hypothesis that genetic redundancy has evolved to ensure successful implantation. Although hatching and implantation have been viewed as unrelated molecular phenomena, our data suggests that these

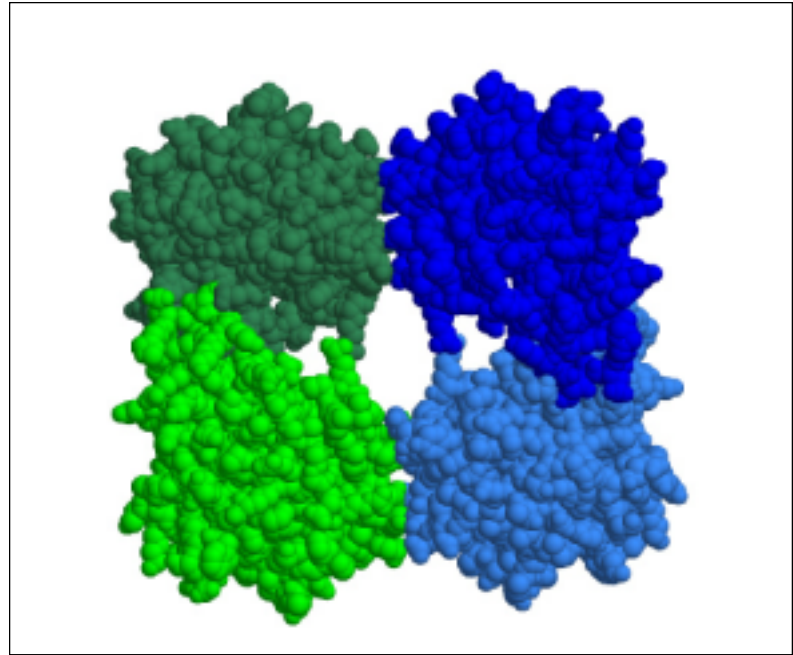


Figure 3. An ISP1 tetramer was modeled using SwissPDB Viewer and RasMol. The mouse ISP1 protein sequence was blasted against the ExPdb database and high sequence similarity to human beta tryptase was discovered (BLAST score: 2×10^{-60}). Human beta- tryptase was then used as a modeling template. The mouse ISP1 sequence was superimposed over the different chains of the human beta-tryptase tetramer using SwissPDB structural alignment algorithms.

two events may be intimately connected. While ISP1/stryptsin may facilitate implantation, the possibility exists that lysin could also participate in the early stages of implantation. Indeed, Mintz and colleagues first suggested that the uterine enzyme responsible for zona lysis is also an implantation initiation factor (Mintz *et al.*, 1972; Pinsker *et al.*, 1974). Here, we envision that after lysis, the abembryonic bed of heparin sulfate could also recruit lysin to the site of apposition and invasion.

Footholds in Female Fertility

One of the most pressing issues of the new millennium is overpopulation. Although the birth control pill has been available for many years, it is only used by 18% of women of childbearing age in the U.S., and an even smaller percentage in the developing world, due primarily to concerns over side effects. Having identified two proteinases responsible for hatching of the mammalian embryo and having demonstrated that that hatching is essential for embryonic development, we have discovered important targets for the generation of nonsteroidal

contraceptives. Since a number of specific tryptase inhibitors have recently been developed (Sanderson, 1999), it should be possible to identify pharmaceuticals, which inhibit hatching.

While contraception has given women the freedom to postpone childbearing until later in life, this has created its own set of problems. In women of advanced childbearing years, more than half of normal pregnancies fail. Chronic pregnancy failure, especially in older women, has led to the field of assisted reproduction, whereby embryos are fertilized, cultured *in vitro* and subsequently transferred back into the mother. However, less than 15% of embryo transfers result in successful pregnancy. Here it has been recognized that embryos derived from women of older childbearing years frequently fail to hatch (Bider *et al*, 1994). While physical methods for hatching embryos have been developed, these have not been found to significantly improve pregnancy outcome (Mandelbaum, 1996). Our observation that stryptsin is important both for *in vitro* hatching and the initiation of implantation, may help to explain why, in assisted reproduction clinics, human embryos that fail to hatch *in vitro*, also fail to implant, despite the successful implementation of artificial hatching. Accordingly, we anticipate that the ISPs may be important prognostic indicators for successful implantation. In addition, administration of the hatching proteinase may improve pregnancy outcome, both by emulating the normal hatching process *in vitro* and potentially enhancing implantation.

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