A novel murine tryptase involved in blastocyst hatching and outgrowth

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Before implantation the blastocyst is maintained within a proteinaceous coat, the zona pellucida, which prevents polyspermy and ectopic pregnancy. An extracellular trypsin-like activity, which is necessary for hatching from the zona pellucida *in vitro*, is localized to the abembryonic pole of the blastocyst. Upon hatching, the extracellular matrix-degrading proteinases urokinase plasminogen activator (uPA) and matrix metalloproteinase 9 (MMP-9) are thought to promote blastocyst invasion. However, gene disruption experiments have demonstrated that uPA and MMP-9 are dispensable and, thus, that other key enzymes are involved in implantation. In this study, a novel implantation serine proteinase (ISP1) gene, which is distantly related to haematopoietic tryptases and represents

a novel branch of the S1 proteinase family, was cloned. ISP1 is expressed throughout morulae and blastocysts during hatching and outgrowth. Abrogation of ISP1 mRNA accumulation using antisense oligodeoxynucleotides disrupts blastocyst hatching and outgrowth *in vitro*. The results of this study indicate that the ISP1 gene probably encodes the long sought after 'hatching enzyme' that is localized to the abembryonic pole during hatching *in vitro*. ISP1 is the earliest embryo-specific proteinase to be expressed in implantation and may play a critical role in connecting embryo hatching to the establishment of implantation competence at the abembryonic pole of the blastocyst.

Introduction

Implantation involves an intricate co-ordination of endometrial and blastocyst factors for the correct attachment of the embryo and its subsequent controlled invasion into maternal deciduum (for review see Rinkenberger *et al.*, 1997; Carson *et al.*, 2000). Within the uterus, the blastocyst must shed the zona pellucida before fostering an intimate relationship with the maternal deciduum. Thinning of this proteinaceous structure precedes hatching and is thought to result from both internal pressures presented by the growth of the blastocyst and the presence of uterine and embryo-derived 'lysins' (Montag *et al.*, 2000). In particular, an embryo-derived, extracellular 'trypsin-like' activity is crucial for the completion of hatching *in vitro* and has been localized histochemically to the abembryonic pole where hatching is initiated (Perona and Wassarman, 1986; Sawada *et al.*, 1990; Hwang *et al.*, 2000). This apical surface is also the first to become adhesive *in utero* and orients the blastocyst within the implantation chamber (Kirby *et al.*, 1967).

On release from the zona pellucida, several extracellular matrix proteins promote blastocyst attachment and outgrowth *in vitro* (Carson *et al.*, 1993). For example, heparin

sulphate proteoglycan occurs on the surface of abembryonic trophoblasts. Attachment and outgrowth of blastocysts *in vitro* is inhibited by heparinase or soluble heparin (Farach *et al.*, 1987). Localized heparin sulphate may also facilitate the embryo–uterine dialogue and blastocyst implantation competence through the localized secretion of maternal heparin binding-epidermal growth factor (HB-EGF). Secreted HB-EGF promotes blastocyst hatching and outgrowth *in vitro* (Das *et al.*, 1994). In turn, a transmembrane form of HB-EGF, which is expressed on the surface of uterine epithelia, may mediate blastocyst adherence through this localized heparin sulphate proteoglycan and the apically expressed EGF receptor, ErbB4 (Raab *et al.*, 1996; Paria *et al.*, 1999; Wang *et al.*, 2000).

The embryo–uterine interaction and the eventual integration of the embryo into the maternal crypt are also mediated by extracellular matrix-degrading proteinases that are secreted by the invading trophoblasts. However, the nature of a potential implantation proteinase cascade is poorly understood. On day 5 of embryogenesis, blastocyst urokinase plasminogen activator (uPA) occupies receptors on the trophoblast cell surface, where it is thought to activate ubiquitous plasminogen locally and to initiate decidual extracellular matrix degradation (Teesalu *et al.*, 1996). Plasmin is also thought to activate trophoblastic matrix metalloproteinase 9 (MMP-9), a matrix metalloproteinase that cleaves several extracellular matrix components and which is thought to give the embryo its invasive character (Harvey *et al.*, 1995; Alexander *et al.*, 1996).

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Inhibitor studies indicate that both uPA and MMP-9 are likely to play principal roles in blastocyst outgrowth during implantation (Behrendtsen *et al.*, 1992; Werb *et al.*, 1992). In sharp contrast, targeted mutagenesis studies have indicated that either proteinase is dispensable in implantation (Carmeliet *et al.*, 1994; Vu *et al.*, 1998). These latter observations question the potential interaction of the uPA/plasmin and MMP-9 proteinases within the presumed implantation proteinase cascade and indicate that other proteinases may be important in implantation. Some of these proteinases have started to be identified (Lefebvre *et al.*, 1995; Afonso *et al.*, 1997; Vu *et al.*, 1997); however, we sought to identify additional serine proteinases that may be involved in implantation. Here, the cloning of a novel 'trypsin-like' implantation serine proteinase (ISP1) is described and it is proposed that the ISP1 gene encodes the long sought after 'trypsin-like' hatching enzyme. In addition, it is demonstrated that abrogation of ISP1 gene expression prevents both hatching and blastocyst outgrowth *in vitro*, thereby indicating that this hatching enzyme may also play an important early role in the initiation of implantation.

Materials and Methods

Animals and treatments

CD1 mice were obtained at the age of 6–7 weeks from Charles River Canada (St Constant, PQ) and were maintained in a standard laboratory animal facility with controlled temperature (20°C) and lighting (lights on between 07:00 h and 19:00 h). The maintenance and treatment of the animals were in full compliance with standard laboratory animal care protocols approved by the University of Calgary's Animal Care Committee.

Female mice were paired with adult males to obtain natural pregnancies and were checked once a day for the presence of a vaginal copulatory plug as an indication of mating. For embryo collection, day 0.5 corresponded to mid-day of the day on which a vaginal plug was detected. Pregnant dams were killed on a specific day of embryo development by cervical dislocation after which uteri and oviducts were removed surgically before isolation of embryos, either by dissection or flushing (Hogan *et al.*, 1994).

Embryo culture

Morulae were collected from oviducts of superovulated dams on day 2.5 of pregnancy into M2 medium (Hogan *et al.*, 1994). For hatching, morulae were cultured in microwells for approximately 24 h at 37°C, 5% CO₂ in air in KSOMaa medium (Erbach *et al.*, 1994). In embryo outgrowth, hatched blastocysts were cultured for an additional 48 h at 37°C, 5% CO₂ in air in Dulbecco's modified Eagle's medium (DMEM) plus 5% (v/v) fetal bovine serum on microwells coated with extracellular matrix derived from

10% (v/v) Triton-X100-treated mouse embryo fibroblasts (Behrendtsen *et al.*, 1995).

Preparation of embryo RNA

Total RNA was collected from embryos plus deciduum (implantation sites on day 6.5 of embryo development), embryos (days 8.5, 11.5 and 13.5 of embryo development) and placentae (days 11.5 and 13.5 of embryo development) using Trizol (Life Technologies, Rockville, MD). Hatching blastocysts (100 at 50% hatch (time at which 50% of embryos have hatched from their zona pellucida)) were collected by centrifugation at 3000 *g* for 5 min for Trizol RNA preparation. RNA from outgrowing blastocysts was collected by Trizol lysis directly in microwells. Poly (A)⁺ RNA was enriched from day 6.5 embryo plus deciduum total RNA using oligo (dT) cellulose chromatography (Sambrook *et al.*, 1989).

Serine proteinase active site RT-PCR cloning

Total RNA (1 µg) from day 6.5 embryo plus deciduum was reverse transcribed using Superscript II (Life Technologies) and was used as a template for active site PCR using degenerate His (5'-CGGAATTCTI(ACT)TI(AT)(GC)IGC(AG-CT)G(AGCT)CA(CT)TG-3') and Ser (5'-GCCGATCCA(AG)-IGGICCC(ACGT)(CG)(TA)(AG)TC(AGCT)CC-3') active site primers (Prendergast *et al.*, 1991). Each 12.5 µl PCR reaction used 0.5 µl cDNA in 10 mmol Tris-HCl l⁻¹, pH 9.0, 50 mmol KCl l⁻¹, 1.5 mmol MgCl₂ l⁻¹, 130 µmol dNTPs l⁻¹, 1 µmol l⁻¹ each primer and 1 U Taq polymerase (Amersham Pharmacia, Piscataway, NJ). Forty rounds of thermal cycling consisted of 1 min at 94°C, 2 min at 55°C and 2 min at 72°C. The amplification products were precipitated with ethanol, cleaved at flanking 5' *Eco*RI and 3' *Bam*HI sites designed in the primer ends, eluted from a 1% (w/v) agarose gel and cloned into *Eco*RI/*Bam*HI cut pBluescript KS⁺ (Stratagene, La Jolla, CA). The inserts of individual clones were screened by restriction analysis (Sambrook *et al.*, 1989), dye-terminator sequenced (Applied Biosystems, Foster City, CA) and compared with the Genbank sequence database using the BLAST program provided by the NCBI network server (Altschul *et al.*, 1997).

Library construction, cDNA cloning and sequence analysis

Poly(A)⁺ RNA from day 6.5 embryo plus deciduum (10 µg) was converted to random- and oligo(dT)-primed double stranded cDNA using the SuperScript cDNA cloning kit (Life Technologies). *Not*I-*Eco*RI adapters were ligated to the cDNA; the adapted cDNA was size selected by gel exclusion chromatography using a Sephacryl S-500 HR column (Life Technologies) and excess linkers were removed using Gene Clean (Qbiogene, Carlsbad, CA). Adapted cDNA (1 µg) was ligated with 5 µg dephosphorylated *Eco*RI-cut λGT10 (Amersham Pharmacia) and packaged into phage using Gigapack Gold II (Stratagene,

La Jolla, CA). Recombinant phages (2×10^6) were amplified on plates and the pooled lysates were frozen (-80°C) in 7% (v/v) dimethylsulphoxide (DMSO) (Sambrook *et al.*, 1989).

A 478 bp ISP1 PCR sub-fragment was used to screen 5×10^5 plaques from this library and resulted in the identification of two cDNA clones bearing a 1.3 kb insertion. An internal *Bam*HI site within the ISP1 cDNA clone permitted the directional cloning of 0.5 and 0.8 kb *Eco*RI–*Bam*HI fragments into pBluescript KS⁺ (Stratagene) for cycle sequencing (Applied Biosystems). The nucleotide sequence was translated into protein sequence using the Swiss Protein ExPASy tool (<http://expasy.cbr.nrc.ca/tools/dna.html>). Twelve serine proteinase peptides identified from a BLAST identity search were aligned with ISP1 using Clustal W (Higgins, 1994; <http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html>).

Expression analysis

Poly (A)⁺ RNA from day 6.5 embryo plus deciduum (5 µg) was subjected to electrophoresis through a 1.2% (w/v) formaldehyde–agarose gel alongside an RNA high molecular mass ladder (Life Technologies). After transfer to Hybond N⁺ (Amersham Pharmacia), the membrane was probed with the 1.2 kb ³²P-labelled ISP1 cDNA fragment.

The presence of ISP1 transcripts in embryos and placenta was monitored using RT–PCR. Total RNA (1 µg) was reverse transcribed and amplified using primers specific for ISP1 (ISP1_{for}: 5'-GGAGCAGGAAGCTTCTGAACA-3'; ISP1_{rev}: 5'-GTCAAAGATGGCCACAGC-3') and forty rounds of thermal cycling (1 min at 94°C, 2 min at 60°C and 2 min at 72°C). The RT–PCR amplification of GAPDH (as a control for mRNA loading) is described elsewhere (Arcellana-Panlilio and Schultz, 1993). The predicted 175 and 380 bp amplification products were separated on a 2% (w/v) agarose gel.

Whole embryo RNA *in situ* hybridization using digoxigenin-labelled RNA probes was performed essentially as described by Rancourt and Rancourt (1997). The ISP1 probe comprised the 478 bp RT–PCR subclone in pBSKS⁺. The antisense probe was synthesized using T3 polymerase after plasmid linearization with *Eco*RI. The sense probe was synthesized using T7 polymerase after plasmid linearization with *Bam*HI. All experiments were performed with the sense RNA probe in parallel to detect non-specific hybridization.

Histochemical staining of stryptsin activity was performed essentially as outlined by Perona and Wassarman (1986). Embryos were collected as early blastocysts in M2 medium and were fixed lightly in 1.25% (w/v) glutaraldehyde in 0.25 mol sucrose I⁻¹, 50 mmol sodium phosphate I⁻¹ (pH 7.5) for 5 min at 4°C. After fixation, the blastocysts were placed in 50 mmol sodium phosphate I⁻¹ (pH 7.5) containing the substrate *N*-α-benzoyl-DL-arginine β-naphthylamide (0.56 mmol I⁻¹; Sigma, St Louis, MO) and Fast Garnet GBC salt (1.86 mmol I⁻¹; Sigma), were incubated for 5 min at room temperature and washed in 50 mmol sodium phosphate I⁻¹ (pH 7.5).

Antisense oligodeoxynucleotide studies

In antisense oligodeoxynucleotide studies, harvested blastocysts were placed in 0.001% (v/v) L-αlysophosphatidylcholine for 60 s (Jones *et al.*, 1997) and were transferred to microdroplets equilibrated with 2.5 µmol I⁻¹ or 5.0 µmol I⁻¹ oligodeoxynucleotide or an equal volume of H₂O (Behrendtsen *et al.*, 1995). Two antisense oligodeoxynucleotides were designed against regions surrounding the initiation codon of ISP1: AS1 (5'-TCTAACTACCGCT-TAACAACG-3') situated upstream and AS2 (5'-GAACTCT-TCTAACTACCGTCT-3') lying downstream. A control oligodeoxynucleotide, SS1, (5'-ACGGTAGTTAGAAGAGT-TCT-3') represented the scrambled sense sequence surrounding the initiation codon. The oligodeoxynucleotides were designed using OligoTM software and were synthesized and purified by R. Pon (University of Calgary DNA Services, Calgary). Blastocysts were scored at 20, 30, 40 and 60 h for progress in hatching. In these studies, both AS1 and AS2 interfered specifically with blastocyst hatching. However, AS1 was found to be more effective than AS2 and was used in all subsequent experiments. After 8 h of treatment, some blastocysts were assayed for the presence of ISP1 transcripts using RT–PCR. After 24 h of treatment, some blastocysts were assayed for stryptsin activity using histochemical staining. In outgrowth studies, blastocysts were allowed to hatch and were then transferred to microdroplets equilibrated with oligodeoxynucleotide or water. Progress in outgrowth was monitored over a period of 5 days.

Results

Encoding of a novel serine proteinase in implantation by the ISP1 gene

Previous investigations of implantation, both *in vivo* and *in vitro* via blastocyst invasion assays, have indicated that a cascade of proteinases mediates the embryo–uterine interaction and the integration of the embryo into uterine deciduum during implantation. After evidence of genetic redundancy among implantation proteinases in the cascade was found, we initiated experiments to identify additional serine proteinases that may play a vital role in implantation. An active site RT–PCR strategy was followed (Prendergast *et al.*, 1991) using RNA from day 6.5 implantation sites to reveal novel serine proteinase sub-cDNAs. At this stage in implantation the embryo is fully engaged in invading the deciduum. Amplification with degenerate primers surrounding the active site His and Ser regions gave rise to a number of fragments ranging in size from 0.4 to 0.5 kb, consistent with the size of known serine proteinase His-Ser sub-cDNAs (Fig. 1a). Upon cloning and sequence characterization of these sub-cDNAs, a number of serine proteinases were identified including uPA, tissue plasminogen activator (tPA), granzyme D, granzyme F and two previously unidentified entities that we called ISPs (ISP1: present study; ISP2: O'Sullivan *et al.*, in press).

Fig. 1. Identification of the murine implantation serine proteinase 1 (ISP1) gene in mouse implantation site RNA. (a) Active site RT-PCR of day 6.5 embryo plus deciduum total RNA using S1 proteinase degenerate His and Ser oligonucleotide primers. Arrows indicate three PCR fragments obtained in the vicinity of 0.4–0.5 kb. (b) Northern blot analysis of day 6.5 embryo plus deciduum poly (A)⁺ RNA (5 µg) revealed a mRNA species of 1.3 kb (arrow) when hybridized with a random prime-labelled 1.2 kb ISP1 cDNA clone.

ISP1 represents a novel branch of the tryptase subfamily of S1 proteinases

Northern blot analysis of day 6.5 embryo plus deciduum poly (A)⁺ RNA using the 478 bp ISP1 sub-cDNA fragment indicated that the ISP1 transcript was 1.3 kb (Fig. 1b). A 1.2 kb full-length cDNA clone for ISP1 was isolated by generating and screening a day 6.5 mouse embryo plus deciduum cDNA library in λGT10. Sequence analysis of ISP1 revealed a predicted protein of 273 amino acids (Fig. 2). In BLAST identify searches (Altschul *et al.*, 1997), it was found that ISP1 had a high degree of sequence similarity with the haematopoietic serine proteinases; the most similar was mouse mast cell protease 6 (45% amino acid identity; Lutzelschwab *et al.*, 1997). Other mast cell proteases showed similar degrees of sequence identity. The next closest subfamilies contain chymotrypsins and elastases with approximately 38% shared amino acid identity. The relationship of ISP1 to the S1 peptidase family is clear as it shares the conserved His and Ser active site moieties (IHPQW and GDGGGPL), in addition to the common N-terminal sequence (IVVG) of mature tryptases (Fig. 2; Smyth *et al.*, 1996).

ISP1 gene expression in preimplantation embryos and in vitro hatching and outgrowth

Given its sequence properties, we hypothesized that the ISP1 gene encodes the previously described trypsin-like protease, strypsin, involved in blastocyst hatching (Perona and Wassarman, 1986). Consistent with this hypothesis, RT-PCR confirmed that ISP1 is expressed during hatching and embryo outgrowth (Fig. 3a), and is detectable throughout all stages of preimplantation development from the zygote stage (Fig. 3b). Beyond implantation, ISP1 expression was detected faintly in day 11.5 and 13.5 placentae, but not in day 8.5, 11.5 or 13.5 embryos (S. L. Rancourt, unpublished). In agreement with our previous RT-PCR expression data, stronger *in situ* hybridization staining was observed in morulae compared with blastocysts (Fig. 4). In the blastocyst, ISP1 RNA expression was observed throughout the embryo. Here, equivalent staining of blastomeres was noted, although staining appeared more intense within the multilayer inner cell mass than in the monolayer trophoblast (Fig. 4d).

Antisense abrogation of ISP1 gene expression and strypsin activity in blastocysts

A possible role for ISP1 in hatching was examined by determining whether ISP1 mRNA could be reduced in blastocysts by treatment with antisense oligodeoxynucleotides (Behrendtsen *et al.*, 1995; Jones *et al.*, 1997) and result in an alteration of the hatching process (Fig. 5). Two antisense oligodeoxynucleotides were designed surrounding the initiation codon of ISP1 covering the regions immediately upstream (AS1) and downstream (AS2). A control oligodeoxynucleotide (SS1) represented the scrambled sense sequence surrounding the initiation codon. Blastocysts were scored at 20, 30, 40 and 60 h for progress in hatching. In these studies, both AS1 and AS2 interfered specifically with blastocyst hatching (Table 1; Fig. 6). However, AS1 was found to be more effective than AS2 and was used in all subsequent experiments.

When blastocysts were treated with the AS1 oligodeoxynucleotide, it was determined that the accumulation of ISP1 transcripts was reduced at least 100-fold after 8 h of culture (Fig. 3c). A significant reduction was not observed in the corresponding SS1 control oligodeoxynucleotide-treated blastocysts, which had similar ISP1 transcript content to untreated controls. AS1 oligodeoxynucleotide-treated blastocysts also displayed reduced strypsin activity compared with untreated or control oligodeoxynucleotide-treated blastocysts. In control oligonucleotide-treated blastocysts (Fig. 5d), localized strypsin activity was observed histochemically at the abembryonic pole of blastocysts. In contrast, strypsin activity was absent in antisense oligodeoxynucleotide-treated blastocysts (Fig. 5e). Consistent with our hypothesis, this observation indicates that the ISP1 gene probably encodes the strypsin activity that is responsible for hatching.

Fig. 2. Predicted amino acid sequence for murine implantation serine proteinase 1 (ISP1) and alignment with related serine proteinases. Identical amino acids are marked by black boxes and conservative substitutions are noted by grey boxes. ISP1 is most similar to mouse mast cell proteinase (MMCP-6) with which it shares 45% identity. Chymotrypsin and murine elastase (MusELAST2) both share 38% identity with ISP1. Arrows indicate predicted pre- and pro-cleavage sites. The S1 proteinase active site consensus sequences His ([LIVM]-[ST]-A-[STAG]-H-C) and Ser [DNSTAGC]-[GSTAPIM VQH]-x(2)-G-[DE]-S-G-[GS]-[SAPHV]-[LIVMFYWH]-[LIVMF YSTANQH]) are underlined. HumTRYP1: human tryptase 1; RMCP-7: rat mast cell proteinase 7.

Antisense disruption of hatching *in vitro*

Blastocysts treated with the antisense oligodeoxynucleotides displayed a considerable impairment in hatching over time (Table 1; Fig. 6). Compared with oligodeoxynucleotide-treated and untreated controls, a significant percentage of antisense oligodeoxynucleotide-treated blastocysts did not hatch. Other treated blastocysts displayed a delay in hatching, indicating that antisense treatment could transiently inhibit hatching until the concentration of oligodeoxynucleotide in the media decreased.

Control oligodeoxynucleotide-treated blastocysts developed and hatched normally (Fig. 5a), mirroring untreated blastocysts that were cultured in parallel (not shown). Starting at about 20 h, the zona pellucida became thin and the blastocysts emanated through ruptures at the abembryonic pole. In contrast, most of the antisense oligodeoxynucleotide-treated blastocysts grew until they compressed

and thinned the zona pellucida wall (Fig. 5c), but were unable to cause it to rupture. After 60 h trapped inside the zona pellucida, antisense-treated blastocysts began to die and shrink away from the wall (Fig. 5b).

Antisense disruption of blastocyst invasion

As ISP1 gene expression had been observed previously during blastocyst outgrowth *in vitro*, an attempt was made to prevent blastocyst outgrowth after hatching had occurred (Fig. 7). Control oligodeoxynucleotide-treated blastocysts adhered to and invaded extracellular matrix after 2 days in culture, not unlike untreated control blastocysts (Fig. 7a,b). ISP1 antisense oligodeoxynucleotide-treated blastocysts took 5 days to adhere to the matrix, but growth was so delayed that the blastocysts never reached the size or extent of the outgrowth observed with control embryos (Fig. 7c,d). This observation indicates that ISP1 expression is probably also vital for the initiation of blastocyst attachment to

Fig. 3. Implantation serine proteinase 1 (ISP1) gene expression in preimplantation mouse embryos. ISP1 and GAPDH gene expression in early embryos (approximately $n = 100$) were detected by RT-PCR. (a) ISP1 gene expression in blastocysts undergoing hatching (lane 1) or outgrowth (lane 2) *in vitro*. (b) ISP1 transcripts in preimplantation embryos collected as zygotes (lane 3), morulae (lane 4) and blastocysts (lane 5), in addition to implantation sites (lane 6). (c) ISP1 gene expression in blastocysts at day 3.0 before exposure to antisense oligodeoxynucleotides (lane 7). ISP1 transcripts were not detected in blastocysts treated for 8 h in medium containing 2.5 μmol antisense oligodeoxynucleotide I⁻¹ (lane 8), but were detected in blastocysts cultured in the presence of 2.5 μmol control oligodeoxynucleotide I⁻¹ (lane 9).

extracellular matrix and subsequent outgrowth during implantation.

Discussion

In this study, a novel serine proteinase gene, ISP1, has been cloned by virtue of its expression at the day 6.5 implantation site. Previously, serine proteinase active site RT-PCR of RNA derived from preimplantation embryos identified hepsin, a membrane-associated serine proteinase also expressed in kidney and liver. Hepsin was proposed initially to represent the mammalian hatching enzyme (Vu *et al.*, 1997), but this possibility was ruled out in subsequent gene disruption studies (Wu *et al.*, 1998). Interestingly, ISP1 was not identified in this previous search for preimplantation serine proteinases. Similarly, hepsin was not identified in the screen in the present study, although it is not known whether this serine proteinase is expressed during implantation. Nonetheless, based on the distinction of the degenerate primers used in both studies, the possibility exists that additional serine proteinases involved in implantation may be identified (for example, O'Sullivan *et al.*, in press).

ISP1 is expressed during blastocyst hatching and outgrowth. On the basis of this expression and the similarity of tryptases to trypsin, it was hypothesized that ISP1 encodes the trypsin-like activity, strypsin, that was first identified in blastocyst hatching by Perona and Wassarman (1986). In agreement with this hypothesis, two antisense oligodeoxynucleotides targeted against ISP1 are able to specifically interfere with blastocyst hatching *in vitro* in a concentration- and time-dependent manner. Over time, it was observed that some blastocysts are able to escape hatching arrest, presumably due to degradation of oligodeoxynucleotide within culture media. Indeed, if oligodeoxynucleotides were administered in fresh medium over time, a prolonged interference on hatching was observed (C. M. O'Sullivan, unpublished). Similarly, fresh oligodeoxy-

Fig. 4. Expression of the murine implantation serine proteinase 1 (ISP1) gene in morulae and blastocysts. (a,b) Morulae and (c,d) blastocysts were stained using whole mount *in situ* hybridization with (a,c) sense and (b,d) antisense ISP1 probes. (a,c) Staining is not observed using the sense probe as a control. (b) Intense ISP1 expression is observed in all of the blastomeres of the morula. (d) In the blastocyst, ISP1 gene expression occurs within the trophoblast and inner cell mass. Equivalent staining is observed, although monolayer trophoblast staining appears weaker than that of the multilayer inner cell mass.

nucleotide medium was required to affect blastocyst outgrowth over 5 days. If oligodeoxynucleotides are allowed to dissipate in medium, blastocysts are able to escape the block on outgrowth (C. M. O'Sullivan, unpublished). The observation that blastocysts can escape hatching and outgrowth arrest indicates that the antisense oligodeoxynucleotides are not toxic. Indeed, blastocysts do not die as a consequence of antisense oligodeoxynucleotide treatment (C. M. O'Sullivan, unpublished). However, embryonic death does occur when blastocysts fail to hatch or outgrow after a period of time.

Antisense oligodeoxynucleotides that are targeted against the initiation codon of mRNAs interfere with translation and result in the degradation of target transcripts (Schlingensiepen and Brysch, 1992). Antisense oligodeoxynucleotide treatment of ISP1 specifically blocks the accumulation of ISP1 mRNA in blastocysts 8 h after treatment. As with our observations on hatching, this blockage is transient, as ISP1 mRNA content returns almost to normal after 24 h of treatment (data not shown). After 24 h of antisense oligodeoxynucleotide treatment, strypsin activity was absent at the abembryonic pole.

As the ISP1 gene is expressed throughout the blastocyst and strypsin activity is localized extracellularly to the distal pole of the blastocyst, these results indicate that ISP1

Fig. 5. Murine implantation serine proteinase 1 (ISP1) antisense oligodeoxynucleotides inhibit murine blastocyst hatching and stryptsin histochemical staining *in vitro*. (a) Control oligodeoxynucleotide-treated blastocysts showing successfully hatched blastocysts. As the zona pellucida becomes thinner, the blastocyst emerges through a small rupture that forms on the abembryonic pole at hatching. The black arrow represents a blastocyst in the middle of hatching. The white arrow shows an empty cask after hatching. (b) Antisense oligodeoxynucleotide-treated blastocysts showing degenerated embryos one day after failing to hatch. Note that in these embryos the zona pellucida is thickened. (c) One day earlier, the same embryos as in (b) developed normally until they were pressed against the zona pellucida. The black arrows show the very thin zonae pellucidae that occur when blastocysts are fully expanded, but fail to hatch in the presence of ISP1 antisense oligonucleotides. (d) Control oligodeoxynucleotide-treated blastocysts showing normal stryptsin activity staining. The histochemical stain is concentrated at the abembryonic pole (white arrow). (e) Antisense oligodeoxynucleotide-treated blastocysts display little stryptsin activity at the abembryonic pole (white arrow).

Table 1. Percentage of unhatched mouse blastocysts after oligodeoxynucleotide administration

Oligodeoxynucleotide	Percentage hatched after time (of 100 blastocysts)			
	20 h	30 h	40 h	60 h
Blank	10	30	65	80
SS1 (2.5 $\mu\text{mol l}^{-1}$)	10	16	58	90
AS1 (2.5 $\mu\text{mol l}^{-1}$)	0	0	16	37
AS2 (2.5 $\mu\text{mol l}^{-1}$)	0	20	35	40
SS1 (5.0 $\mu\text{mol l}^{-1}$)	10	39	61	77
AS1 (5.0 $\mu\text{mol l}^{-1}$)	10	10	20	20
AS2 (5.0 $\mu\text{mol l}^{-1}$)	0	5	35	45

SS1 is a scrambled sense oligodeoxynucleotide of DNA sequence surrounding the initiation codon of implantation serine proteinase 1 (ISP1). AS1 and AS2 are antisense oligodeoxynucleotides surrounding the initiation codon of ISP1 covering the region immediately upstream (AS1) and downstream (AS2).

Fig. 6. Mouse implantation serine proteinase 1 (ISP1) antisense oligonucleotides inhibit blastocyst hatching in a time dependent manner. One hundred blastocysts (day 3.5 of embryo development) were cultured in the presence of water (□), 2.5 $\mu\text{mol l}^{-1}$ scrambled sense SS1 oligodeoxynucleotide (▨) or antisense AS1 oligodeoxynucleotide (■). The percentage hatching was determined 20, 30, 40 and 60 h after addition of oligodeoxynucleotide. Values are mean $\pm$ SE ($n = 3$).

Fig. 7. Murine implantation serine proteinase 1 (ISP1) antisense oligodeoxynucleotides inhibit mouse blastocyst outgrowth onto extracellular matrix *in vitro*. (a) Prehatched blastocyst treated with a control oligodeoxynucleotide invades normally into extracellular matrix. (b) A control oligonucleotide-treated blastocyst, showing invading trophoblasts (black arrow). (c) Prehatched blastocysts treated with antisense oligodeoxynucleotide fail to invade into extracellular matrix and do not develop beyond the blastocyst stage. (d) A blastocyst failing to invade; invading trophoblasts are not apparent. The refraction of light that is noticed (arrow) is from the extracellular matrix previously laid down on these dishes.

protein is either recruited to the abembryonic pole for activity or is translated preferentially in apical trophoblasts. A significant reduction of localized strypsin activity in blastocysts was observed consistently when ISP1 mRNA accumulation was abrogated. The predicted molecular mass of ISP1 (approximately 27 000 Da) is considerably smaller than the native molecular mass of strypsin (74 000 Da), a finding which indicates that ISP1 must probably multimerize for activity. Indeed, tryptases including mouse mast cell proteinases multimerize for activity and are assembled with the assistance of heparin sulphate proteoglycans (Lindstedt *et al.*, 1998; Huang *et al.*, 2000). Consistent with this idea of ISP1/strypsin assembly, the abembryonic pole of the blastocyst is rich in heparin sulphate proteoglycan (Farach *et al.*, 1987). As heparinase digestion has demonstrated a requirement for this heparin sulphate bed in blastocyst attachment and outgrowth (Farach *et al.*, 1987), it is likely that this bed is also required for hatching. Similarly, the actions of maternal heparin sulphate binding-EGF in stimulating blastocyst hatching and outgrowth may be

explained by the pH dependence of tryptase activation (Lindstedt *et al.*, 1998; Huang *et al.*, 2000) and the changes in ion flux that occur downstream of HB-EGF binding to the ErbB4 receptor (Wang *et al.*, 2000).

Owing to the difficulty in cloning and characterizing strypsin, the importance of strypsin in facilitating the hatching process has recently been considered less important than blastocyst growth and equally ill-defined uterine 'lysins' in promoting zona pellucida thinning (Montag *et al.*, 2000). Recent studies suggest that the 'focal' hatching that occurs *in vitro* is distinct from hatching *in utero*, in which the zona pellucida appears to 'dissolve' after thinning (Gonzales and Bavinster, 1995; Montag *et al.*, 2000; Gonzales *et al.*, 2001). As hatching occurs approximately 1 day earlier *in utero* than *in vitro*, Gonzales and Bavinster (1995) have described hatching *in vitro* as an artefact characterized by the absence of a uterine 'lysin proteinase'. Indeed, there is evidence to suggest that a hormonally regulated proteinase associated with uterine secretions may also contribute to hatching (Orsini and McLaren, 1967;

Joshi and Murray, 1974; Rosenfeld and Joshi, 1981) and that this proteinase is regulated by progesterone (Denker, 1977). O'Sullivan *et al.* (in press) describe the cloning and characterization of the ISP2 gene, which encodes a trypsin homologue that is expressed in the endometrial glands during early pregnancy. As this gland provides the major source of uterine secretions during implantation and the ISP2 gene is expressed in a progesterone-dependent manner, it is speculated that ISP2 may encode a uterine lysin, which extrinsically facilitates blastocyst hatching.

The existence of uterine proteinases associated with zona lysis *in vivo* does not minimize the importance of trypsin in blastocyst hatching, as it has been demonstrated that trypsin is necessary and sufficient to effect hatching *in vitro*. However, trypsin may play a secondary role in hatching *in utero* and serve as a contingency factor for failed zona pellucida lysis. Hence, similar to the observations on implantation, genetic redundancy may also be operating in blastocyst hatching to ensure that a successful pregnancy is generated.

The ISP1 mRNA knockdown experiments have helped to clarify the mechanism of hatching. During the antisense experiments, zona pellucida thinning was observed in conjunction with blastocyst growth. However, when blastocysts failed to hatch and died within the zona pellucida, regression of the embryo resulted in a reversal of zona pellucida thinning. This observation confirms the hypothesis that zona pellucida thinning is dependent on blastocyst expansion but indicates that proteinases may not be involved in thinning. Nonetheless, blastocyst growth and zona pellucida thinning may play an important regulatory role in ensuring that hatching occurs at an appropriate time, by exposing lysin- or trypsin-specific proteolytic sites within this proteinaceous sheath.

After Mintz (1972) and Pinsker *et al.* (1974) first suggested that the enzyme responsible for hatching might also be an implantation initiation factor, Gonzales and Bavinster (1995) predicted that the enzyme responsible for focal hatching *in vitro* might really be the enzyme responsible for facilitating blastocyst attachment and invasion. Through our discovery of ISP1, we have confirmed this additional role for the hatching enzyme in facilitating implantation competence. Indeed, the abembryonic pole of the blastocyst becomes competent to attach and invade into extracellular matrix *in vitro*, and this competence occurs as a function of localized heparin sulphate proteoglycan and the action of heparin binding EGF (Farach *et al.*, 1987; Das *et al.*, 1994). ISP1/trypsin may participate in a continuum that connects blastocyst hatching to extracellular matrix attachment and outgrowth. Historically, hatching and outgrowth have been viewed as unrelated molecular events. Although serine proteinase inhibitors affect both hatching (Perona and Wassarman, 1986) and outgrowth (Kubo *et al.*, 1981; Behrendtsen *et al.*, 1992), these studies have focused on the respective roles of trypsin in hatching and uPA in outgrowth. We note that most, if not all, of these inhibitors, including bis[5-amido-2-benzimidazole], are effective

against trypsinases (Compton *et al.*, 1998; Sanderson, 1999) and suggest that their action in affecting outgrowth may have been directed to the extracellular matrix degrading potential of ISP1/trypsin.

With hindsight, it seems reasonable that a localized proteinase involved in degrading the zona pellucida might also be involved in initiating the degradation of extracellular matrix that occurs in blastocyst outgrowth. ISP1/trypsin may also participate indirectly in extracellular matrix degradation through the activation of other proteinases such as MMP-9, which is activated by trypsinases *in vivo* (Keski-Oja *et al.*, 1992; Lohi *et al.*, 1992). Although removal of the zona pellucida barrier has long been viewed as the critical first step in implantation, the results of the present study demonstrate that the role of the hatching proteinase in implantation may be more active than passive. On the basis of its early expression, ISP1/trypsin may be a lynch pin in the cascade of proteinase activity during implantation. Hence, further investigation into the function of ISP1/trypsin *in vivo* is warranted.

The role of the hatching proteinase in facilitating embryo attachment and outgrowth may also serve to explain why assisted hatching procedures performed in fertility clinics have failed to promote the successful implantation of human embryos (for review see De Vos and Van Steirteghem, 2000). Indeed, embryos from women of advanced age frequently fail to hatch *in vitro* and may be devoid of hatching enzyme activity (Bider *et al.*, 1997). Having identified the mouse ISP1 gene, an important diagnostic tool in human fertility may result, while physical preparations of ISP1/trypsin may potentially be used to improve the success of assisted reproduction.

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