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# HtrA3, a Serine Protease Possessing an IGF-binding Domain, is Selectively Expressed at the Maternal–Fetal Interface During Placentation in the Mouse

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Hemochorial placentation involves highly regulated interactions between fetal- and maternal-derived cells. HtrA3, a novel serine protease containing an insulin-like growth factor (IGF) binding domain, was previously shown to increase during early pregnancy in the mouse uterus, being dramatically upregulated post-implantation. The present study examined the regulation of *HtrA3* gene in the mouse uterus from post-implantation to late gestation. Both mRNA and protein of *HtrA3* were localized specifically in the maternal decidua. In contrast, *HtrA3* expression was below detection in trophoblasts, including the giant cells that are in direct contact with the decidua. This pattern persisted from the early stages of placentation to near term. The level of decidual *HtrA3* mRNA and its protein gradually decreased as the placenta matured. In the decidua, only the maternal decidual cells, but not blood vessels or uterine NK cells that are present in large numbers, were positive for HtrA3. The specific localization of a protease possessing an IGF-binding domain at the maternal–fetal interface suggests that HtrA3 plays a critical role in mediating maternal decidual remodelling and maintenance, likely in association with the IGF system, in placental development and function.
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**Keywords:** Serine protease; HtrA3; Uterus; Decidua; Placenta; Decidualization; Pregnancy; Placentation

## INTRODUCTION

The placenta is vital for intrauterine development of mammals, as it physically and functionally connects the fetus to the mother. Failure to establish a functional placenta results in fetal death in utero [1–3]. Even suboptimal placentation will lead to intrauterine growth restriction and a low-birth-weight fetus. For instance in mice, deletion of the insulin-like growth factor (IGF) II gene that is specifically expressed in the placenta leads to reduced growth of placentas and pups with low birth weight [4]. Moreover, defects in placentation contribute to a number of diseases of human pregnancy, including spontaneous abortion and preeclampsia [5,6]. Thus, understanding the regulation of genes critical for placentation is of relevance to fetal well-being and pregnancy outcome.

Within the mouse placenta, the fetal placental compartment, i.e., trophoblast, is in most intimate contact with the maternal decidua that lines the pregnant uterus. Placental development involves trophoblast proliferation, differentiation and invasion into the maternal tissue to anchor the placenta to the uterus. There is a developing body of evidence supporting the notion that the decidua is essential in regulating the formation and function of the placenta [7]. Particularly, several gene deletions have been reported in which impaired decidualization is associated with failure of placentation and pregnancy [8–10]. One of these is the inactivation of IL-11 Rα [11,12]; in these mice the extent of decidualization is much reduced and the mesometrial decidua is totally absent. As a result, the trophoblast giant cells expand to the area that normally contains the mesometrial decidua and the mice do not form normal placentas. Further evidence supporting the importance of maternal decidua during placentation is from a study in which IGF-binding protein (IGFBP)-1 was over-expressed in the maternal decidua with resultant impairment of placental development [13]. The proposed function of the decidua is to facilitate as well as to restrict the invasion of the trophoblast. However, the exact molecular mechanisms underlying decidual function in regulating trophoblast behaviour during placentation are not well understood.

Recently we have identified and cloned a novel gene that is dramatically upregulated in the mouse uterus in association with placentation [14]. It was predicted to encode a serine protease, which we tentatively called pregnancy-related serine protease (PRSP) [14]. Subsequently, we cloned the full mRNA

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sequence of this gene in the human [15]. Further analysis revealed that PRSP was structurally related to the previously identified mammalian HtrA (high-temperature requirement factor) proteases 1 and 2; thus we have named this new gene *HtrA3* [15].

Both in the human and mouse, two alternatively spliced *HtrA3* mRNAs (long and short forms) have been cloned [14,15]. Interestingly, the long form is predominant in the mouse uterus, whereas the expression of both forms is easily detected in the human placenta [14,15]. *HtrA3* is well conserved between the human and mouse with similarities of 87% at the mRNA and 95% at the protein level. An interesting feature of HtrA3 protein is that although it is a serine protease, it contains an IGF-binding domain at the N-terminal end immediately following the signal peptide [14,15]. HtrA3 is therefore thought to be an IGFBP-protease chimera [14,15].

In the mouse uterus, *HtrA3* mRNA expression in the endometrium is relatively low before pregnancy but it increases as pregnancy is established, especially post-implantation [14]. In situ hybridization analysis localized the *HtrA3* mRNA in the glandular and luminal epithelium of the endometrium during early pregnancy [14]. By day 8.5 of pregnancy, *HtrA3* mRNA is localized predominantly in the decidua. By day 10.5, the expression is detected predominantly in the placenta with a relatively lower level in the fetus [14]. However, the cellular origin of *HtrA3* expression in the placenta on day 10.5 and how it is regulated during subsequent development of the placenta is not known.

The present study examined the expression of *HtrA3* mRNA and protein from day 8.5 to late pregnancy in the mouse. In particular, the cellular origin of *HtrA3* expression in the different compartments of the placenta was determined from the early stages of placentation to near term, following the course of the dynamic structural changes that occur at the fetal–maternal interface during this period of time. The results provide important insights into understanding how HtrA3 protease is involved in placental development and function.

## MATERIALS AND METHODS

### Animals and tissue preparation

Swiss out-bred mice were housed and handled according to the Monash University animal ethics guidelines on the care and use of laboratory animals. All studies were approved by the Animal Ethics Committee at the Monash Medical Centre, Melbourne, Australia.

The morning of finding a vaginal plug was designated as day 0 of pregnancy. Uterine tissues were collected from non-pregnant mice and pregnant mice on days 8.5–17.5 ($n = 4$–6 per time point). For immunohistochemical analysis, intact implantation sites containing the uterine, placental and fetal tissues were fixed in 4% buffered formalin (pH 7.6) and processed as previously described [16]. For Northern and Western analysis, specific tissue compartments were dissected as described below and snap-frozen in liquid nitrogen for RNA or protein extraction. For non-pregnant mice, the entire uterus was collected. On day 8.5 of pregnancy, the entire implantation sites were sampled. From day 10.5 onwards, the uterus was first opened from the anti-mesometrial side to reveal the implantation sites. The placenta and the embryo were then separated from the uterus as illustrated in Figure 1A. This led to the exposure of the uterine site to which the placenta/embryo was attached; this site was designated as the attachment site and collected. After the dissection of the attachment sites, the reminder of the uterus was also collected. Total placenta was separated from the embryo and further dissected into two parts: the maternal decidual component (softer and paler, through which the placenta was attached to the uterus) and fetal component (firmer, through which the placenta was attached to the fetus) (Figure 1B). Although this method does not guarantee a pure population of cells in each tissue type, it does provide unique tissue compartments highly enriched with specific cells. Histologically, the uterine attachment site would be enriched mainly with the metrial triangle tissue, the decidual component with the decidual basalis and the fetal component with trophoblast cells (Figure 1B and C).

### Northern blot analysis

Total RNA was isolated and Northern analysis performed as previously published [14,17]. A cDNA fragment of 785 bp, capable of detecting both the long and short forms of mouse *HtrA3* mRNA, was used as a probe [14]. To determine lane to lane loading variation, each blot was also probed with a mouse cDNA probe for 18S ribosomal RNA. Each experiment was repeated three times using independent samples. To determine the expression of *HtrA3* mRNA in the fetus between days 10.5 and 17.5, a commercial blot containing Poly-A RNA (#m102, RNWAY Laboratories, Seoul, South Korea) was used. This blot was probed with a mouse cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (supplied together with the Poly-A RNA blot) as a loading control.

### Production of anti-HtrA3 antibody and confirmation of its specificity

An anti-HtrA3 antibody was generated in sheep against a synthetic peptide ALQVSGTPVRQC corresponding to residues 116–126 inclusive of mouse HtrA3 protein (accession no. NP_084403). This peptide is highly specific to HtrA3, common to both isoforms, and highly conserved between the mouse and human proteins [14,15]. It is also predicted to represent a highly antigenic region on HtrA3 protein using the algorithms available from the Australian Genomic Information Services (ANGIS). A cystine residue was added to the C-terminal of the peptide through which the peptide was coupled to an immunogenic carrier protein, bacterial diphtheria toxoid. The peptide was synthesized and purified by HPLC, and the identity was confirmed by ion spray mass spectrometry (Mimotopes Pty Ltd, Clayton, Vic, Australia). Sheep were