Cathepsin proteases have distinct roles in trophoblast function and vascular remodelling

Mark Screen\(^1\), Wendy Dean\(^1\), James C. Cross\(^2\) and Myriam Hemberger\(^{1,3,*}\)

Trophoblast giant cells are instrumental in promoting blood flow towards the mouse embryo by invading the uterine endometrium and remodelling the maternal vasculature. This process involves the degradation of the perivascular smooth muscle layer and the displacement of vascular endothelial cells to form trophoblast-lined blood sinuses. How this vascular remodelling is achieved at the molecular level remains largely elusive. Here, we show that two placenta-specific cathepsins, \textit{Cts7} and \textit{Cts8}, are expressed in distinct but largely overlapping subsets of giant cells that are in direct contact with maternal arteries. We find that \textit{Cts8}, but not \textit{Cts7}, has the capacity to mediate loss of smooth muscle \(\alpha\)-actin and to disintegrate blood vessels. Consequently, conditional ubiquitous overexpression of \textit{Cts8} leads to midgestational embryonic lethality caused by severe vasculature defects. In addition, both cathepsins determine trophoblast cell fate by inhibiting the self-renewing capacity of trophoblast stem cells when overexpressed in vitro. Similarly, transgenic overexpression of \textit{Cts7} and \textit{Cts8} affects trophoblast proliferation and differentiation by prolonging mitotic cell cycle progression and promoting giant cell differentiation, respectively. We also show that the cell cycle effect is directly caused by some proportion of \textit{Cts7} localizing to the nucleus, highlighting the emerging functional diversity of these typically lysosomal proteases in distinct intracellular compartments. Our findings provide evidence for the highly specialized functions of closely related cysteine cathepsin proteases in extra-embryonic development, and reinforce their importance for a successful outcome of pregnancy.

**KEY WORDS:** Cysteine cathepsins, Placenta, Trophoblast differentiation, Vascular remodelling

**INTRODUCTION**

Formation of a functional placenta is essential for intrauterine development of the mammalian embryo. Placental development depends on the proper differentiation of various trophoblast cell types that descend from the trophoderm layer of the blastocyst. Most of our insights into key factors and pathways that regulate placental development come from studies in the mouse (Rossant and Cross, 2001). Here, after implantation of the blastocyst, a specialized trophoblast population termed giant cells initiates an invasive process during which they penetrate deeply into the surrounding decidua of the maternal uterine stroma. Invasion of trophoblast giant cells is tightly regulated and occurs only in the area surrounding the ectoplacental cone. It is specifically targeted towards maternal spiral arteries and results in a remodelling of the uterine vasculature that is characterized by a number of remarkable features: as far as several 100 \(\mu\)m outside the main placental border, trophoblast giant cells displace the endothelial cell lining of maternal spiral arteries and adopt pseudo-endothelial cell function (Adamson et al., 2002; Hemberger et al., 2003). The perivascular smooth muscle layer and the elastic lamina of spiral arteries are lost, which enables an extensive dilation of these vessels (Adamson et al., 2002; Pijnenborg et al., 2006). Spatially correlated with the site of trophoblast invasion (and possibly induced by trophoblast-produced factors), spiral arteries converge to form a few large canals that funnel maternal blood into the placenta. Thus, giant cell invasion initiates a cascade of processes that are essential for development of the foeto-maternal circulatory interface of the placenta.

Trophoblast giant cells differentiate by exiting the mitotic cell cycle and undergoing repeated rounds of endoreduplication. This process results in extremely large, highly polyplloid cells with a DNA content of up to 1000 \(N\) whose chromosome arrangement may even be, at least in part, polytene (Goncalves et al., 2003; Varmuza et al., 1988). Based on the gestational stage of their differentiation and on gene expression patterns, giant cells can be grouped into at least four distinct classes represented by (1) parietal giant cells surrounding the implantation site, (2) giant cells associated with maternal spiral arteries and (3) with maternal blood canals, and (4) giant cells within sinusoidal spaces in the placental labyrinth (Simmons et al., 2007). Within the uterine bed, invasive characteristics are displayed only by giant cells associated with maternal spiral arteries. We are particularly interested in determining the molecular framework that regulates their differentiation and invasive properties. For this purpose, a cDNA subtraction library specific for invasive trophoblast was generated and extensively analysed by array technology (Hemberger et al., 2001; Hemberger et al., 2000). Two of the genes identified from this screen were the cathepsin proteases \textit{Cts7} and \textit{Cts8}. Intriguingly, expression of these two proteases was confined to extra-embryonic tissues and exhibited a strict temporal and spatial correlation with trophoblast giant cell invasion (Hemberger et al., 2000).

Cysteine cathepsins are a main component of the proteolytic breakdown machinery in lysosomes. In addition to this general proteolytic function, cathepsins are also implicated in a variety of specific cellular processes, such as apoptosis, angiogenesis, cell proliferation and invasion (Turk et al., 2001). They play essential physiological roles in antigen presentation, bone remodelling and epidermal homeostasis, and several family members have been associated with tumour development and metastasis (Joyce and...
Hanahan, 2004). Cts7 and Cts8 belong to a placenta-specific group of papain-like cysteine cathepsins (Deussing et al., 2002; Sol-Church et al., 2002). This group consists of eight closely related genes that are located in a dense cluster on mouse chromosome 13, and that may have arisen by repeated gene duplication of cathepsin L on the same chromosome. Evolution of a placenta-specific cathepsin group and high expression levels of ubiquitously expressed family members in trophoblast tissues (Afonso et al., 1997; Varanou et al., 2006) suggests the importance of cysteine cathepsins for placental development. Direct evidence for their requirement in extra-embryonic tissues has been provided by the administration of cysteine protease inhibitors to pregnant mice and rats. This treatment causes embryonic lethality associated with a failure of extra-embryonic tissues to develop (Afonso et al., 1997; Freeman and Lloyd, 1983). Cathepsins have also come into the spotlight for their clinical significance in human pregnancy-associated disorders. Recurrent spontaneous miscarriage has been associated with increased decidual levels of cathepsins B and H (Nakanishi et al., 1983). Cathepsins have also come into the spotlight for their importance of cysteine cathepsins for placental development.

Transgenic mouse production
TgCts7−/−/Cts7 mice were generated by pronuclear injection of the linearized plasmid into C57BL/6×CBA F1 zygotes. TgCts7−/−/Cts8−/− mice were obtained by electroperoration of E14 ES cells that were subsequently used for blastocyst injections. Cre-expressing lines used were CMV-Cre and Sox2-Cre, which both confer ubiquitous transgene induction upon maternal transmission (Hayashi et al., 2003; Schwenk et al., 1995), as well as the spongiotrophoblast-specific Tpbp-Cre and pan-trophoblast Cyp19-Cre lines (Wenzel et al., 2007).

Histology and in situ hybridization
Pregnant females were dissected at the gestational age indicated, counting the morning of the vaginal plug as E0.5. For lacZ staining, embryos were fixed in 0.25% glutaraldehyde and stained for β-galactosidase according to standard protocols. For embedding, tissues were fixed overnight in 4% paraformaldehyde and processed for routine paraffin histology. In situ hybridization was performed with digoxigenin-labelled riboprobes according to a standard protocol. Signals were detected with an anti-DIG alkaline phosphatase-conjugated antibody (Roche, Basel, Switzerland), followed by colour reaction using NBT and BCIP (Promega, Madison, WI) and counterstaining with nuclear Fast Red (Sigma, Dorset, UK).

Immunohistochemistry and immunofluorescence
Immunostaining was carried out on paraffin sections treated with 100 μg/ml Proteinase K or boiling in 0.01 M sodium citrate (pH 6.5). Antibodies and dilutions were: anti-CTS7 (MABI499; R&D Systems, Minneapolis, MN, USA) 1:200; anti-Laminin (Sigma, Dorset, UK) 1:200; anti-phosphohistone H3-810 (#060-570; Upstate, Charistottesville, Va, USA) 1:200; anti-Ki-67 (ab15580; Abcam, Cambridge, UK) 1:100; anti-FLAG M2 (#F3165; Sigma, Dorset, UK) 1:300; and peroxidase-conjugated isoelectric BSI-B4 (L5391; Sigma, Dorset, UK) 1:60. Smooth muscle α-actin was detected with the IMMH-2 kit (Sigma, Dorset, UK). Sections were counterstained with DAPI or Hematoxilyn.

Western blotting
Cells were lysed in PBS/1% Triton X-100 or in 1× RIPA buffer in the presence of 20 mM diithiothreitol and protease inhibitors (Sigma, Dorset, UK). Routine SDS-PAGE electrophoresis and western blotting were carried out using 10% Bis-Tris polyacrylamide gels (Invitrogen, Paisley, UK) (Sambrook et al., 2001). Anti-FLAG antibody (Sigma, Dorset, UK) was used at 1:5000. The processing pattern of endogenous and transfected CTS7 was confirmed with an anti-CTS7 antibody used at 1:1000. Signals were detected using the ECL Plus Detection system (GE Healthcare, Chalfont, UK).

RESULTS
Cts7 and Cts8 define a unique subset of trophoblast giant cells
When first identified, Cts7 (‘Epcs24’) and Cts8 (‘Epcs68’) were described in parietal giant cells of the yolk sac and the ectoplacental cone of E7.5 conceptuses (Hemberger et al., 2000). To determine the onset and sites of expression more precisely, serial gestational stages were analysed from early post-implantation development until the second half of pregnancy (E5.5-E14.5). Both cathepsins were found as early as E5.5 in parietal trophoblast giant cells surrounding the implantation site, where they demarcated a distinct subset when compared with the giant cell marker placental lactogen-1 (Prl3d1) (Fig. 1A–C). Cts7- and Cts8-expressing giant cell populations
largely overlapped, although Cts8 expression was somewhat weaker and confined to fewer giant cells. Coinciding with the onset of trophoblast invasion, a striking shift in expression was observed towards invasive giant cells around the ectoplacental cone (Fig. 1D-L). This effect was particularly pronounced for Cts8, whereas Cts7 expression extended slightly more distally compared with that of Cts8. I) Every Cts8-positive giant cell (arrows) is in contact with a maternal blood vessel (asterisks). J-L) Ectoplacental cone area of E8.5 conceptuses. Cts7 (J) and Cts8 (K) expression is restricted to a subset of trophoblast giant cells compared to the pan-giant cell marker proliferin (Prl2c2) (inset in L). Cts8 (L) is not expressed in this cell population. Scale bars: 200 μm in A-H,J-L; 100 μm in I.

To gain first insights into the roles of Cts7 and Cts8, we pursued a gain-of-function strategy by transient transfections of TS cells. As the transfection efficiency of TS cells is notoriously low, we used bicistronic expression constructs that conferred concomitant cathepsin and GFP expression to identify transfected cells. Assessing only GFP-positive and, hence, transfected cells, we found that both cathepsins caused an increase in cell and nuclear sizes as early as 24 hours post transfection, indicative of the initiation of giant cell differentiation (Fig. 2E,F). Co-cultured cells and cathepsin-conditioned media showed no effect (not shown), demonstrating that cell size enlargement was a cell-autonomous, intracellular function of CTS7.

**CTS7 and CTS8 are mainly localized to endo-/lysosomes and can be secreted**

Taking advantage of tagged expression constructs, the subcellular localization of CTS7 and CTS8 was investigated in TS cells and in heterologous COS-7 cells. Following the typical distribution that has been described for other cathepsins (Wang et al., 1998), the majority of CTS7 and CTS8 was localized to the cytoplasm with a punctate staining pattern indicative of a predominant lyso- and endosomal localization (Fig. 2C). As other cathepsins can be secreted (Götterson, 1978), we assessed whether CTS7 and CTS8 also shared this characteristic. Indeed, both cathepsins were readily detected in the supernatant of transfected TS and COS-7 cells, whereas non-secreted control proteins were not found in the medium (Fig. 2D). No difference in the post-translational processing pattern was observed between the tagged expression constructs and the endogenously expressed proteases, indicating that the constructs were normally processed into active cathepsin enzymes. These data suggested a potential dual role of CTS7 and CTS8 in the intra- and extracellular environments.

**Cts7 and Cts8 overexpression interferes with TS cell maintenance**

To determine whether this highly specific expression pattern was recapitulated in vitro, we characterized Cts7 and Cts8 expression in trophoblast stem (TS) cells. TS cells can be maintained in a multipotent state when cultured in the presence of FGF4 and embryonic feeder cell-conditioned medium (CM). Upon withdrawal of these components, TS cells differentiate into all trophoblast cell types but predominantly into giant cells (Tanaka et al., 1998). Both cathepsins were upregulated with the onset of giant cell differentiation (–FGF/C; Fig. 2A), indicating that the normal regulation of expression is recapitulated in cultured TS cells. In situ hybridization on TS cells confirmed giant cell-specific expression of Cts7 and Cts8 (Fig. 2B); however, similar to the situation in implantation sites, only a subset of giant cells was labelled. Feasibly, it is this cathepsin-positive subset that endows TS cells with the invasive capacity that is characteristic of trophoblast in vivo and that is recapitulated by TS cells in vitro (Hembeler et al., 2004).
Cts7 and Cts8. Consistent with these findings, the clonal expansion of Cts7/Cts8 overexpressed these proteases. Thus, Cts7 and Cts8 reduced the proliferation rates of TS cells and primed them towards differentiation.

Mouse models to characterize the functions of Cts7 and Cts8 in vivo

Because of the likely redundancy among the eight closely related placental cathepsins that would have interfered with a significant outcome of a knockout strategy and because of the observed effects of overexpressing TS cells, we chose to study the function of Cts7 and Cts8 in conditional transgenic mouse models where Cre recombinase (Lobe et al., 1999) expression was under control of Cre recombinase (Lobe et al., 1999) (Fig. 3A,B). This system allowed us to investigate the roles of Cts7/Cts8 by gain-of-function analysis in trophoblast cells in their physiological environment, including potential paracrine effects on neighbouring decidual or extra-embryonic mesodermal cells. Importantly, it also enabled the examination of potential effects at ectopic sites where functions of these proteases may be revealed that are masked in trophoblast cells by the pre-existing endogenous expression of both cathepsins.

Cts7 overexpression is compatible with embryonic development to term

Conditional overexpression of Cts7 was evaluated in three independent transgenic lines after ubiquitous and trophectoderm-specific transgene activation using the CMV-Cre, maternally transmitted Sox2-Cre, Tpbp-Cre and Cyp19-Cre lines (Hayashi et al., 2003; Schwenk et al., 1995; Simmons et al., 2007; Wenzel et al., 2007). Matings to CMV-Cre and Sox2-Cre females conferred ectopic, ubiquitous tgCts7 expression to the embryos proper (Fig. 3C) and strong (up to eightfold) Cts7 overexpression to placentas where particularly high Cts7 levels were observed in spongiotrophoblast, the chorionic plate and in sinusoidal giant cells (Fig. 3D,E). As there was no difference in survival between the various transgenic offspring were obtained at Mendelian ratios, even from matings that yielded highest ubiquitous tgCts7 expression in target cells (not shown). Importantly, live, healthy and fertile Cts7+/Cre offspring correlated with the profile of giant cell markers (Cdx2, stem cells; Ascl2, ectoplacental cone and spongiosotrophoblast; Prl3d1, primary, parietal giant cells; Prl3b1, secondary giant cells; Prl2c2, all giant cells). (B) In situ hybridization on TS cell grown for 4 days in differentiation medium. Some giant cells (blue, arrows) are positive. (C) Immunostaining of Cts7 showing localization to the perinuclear area, the Golgi, and to the cytoplasm in a granular pattern indicative of endo- and lysosomal localization. (D) Western blots of transfected TS cells and their supernatants. Cts7 and Cts8 are secreted into the medium; intracellular control proteins (PPPL) were not detected in the supernatant. (E,F) Relative cell size measurements of TS cells 2 days after transfection with empty GFP-expression vector and Cts7-GFP (E) or Cts8-GFP (F). Cathepsin expression causes a significant shift towards larger cell sizes. Scale bars: 40 μm in B, 20 μm in C.

CTS7 affects trophoblast proliferation and differentiation

As there was no difference in survival between the various transgenic lines, we analyzed the strongest tgCts7-expressing line for subtle defects in trophoblast differentiation. Consistently, these placentas were characterized by a thinner spongiotrophoblast layer (Fig. 3D,E). This phenotype correlated with the spongiosotrophoblast exhibiting strongest transgene expression, and resulted in a downregulation of spongiosotrophoblast-expressed genes, such as Tpbpa, Prl2c2, proliferin-related protein (Prl7d1) and placental lactogen-II (Prl3b1) relative to controls at E10.5 and E12.5 (Fig. 3D,E). By E14.5, however, the proportional size of placental layers was similar to that of controls and, accordingly, relative expression of these genes reached comparable levels in wild-type and tgCts7 placentas (Fig. 3E).
Because of the timing of this placental growth defect, we investigated the differentiation of cell types that form after E10.5 in more detail. Thus, the appearance of Prl3b1-positive sinusoidal giant cells as well as glycogen cells was analyzed (Coan et al., 2006), both representing cell types in which the transgene was highly expressed. Consistent with reduced Prl3b1 expression levels, fewer sinusoidal giant cells had formed by E12.5 (Fig. 4A). Similarly, initial differentiation of glycogen cells was sparse in E12.5 tg Cts7 placentas. Compared with wild-type controls and placentas carrying the inactive (tg LacZ/Cts7+/Cre–) transgene, glycogen cells contributed only 32.82±2.32% to the spongiotrophoblast layer compared with 47.22±2.43% in controls (P<0.00015; Fig. 4B). Thus, although all trophoblast cell types could be formed in principle, Cts7 overexpression caused a delay in their differentiation.

A nuclear role of CTS7 in mitotic slowdown

In the absence of overt differences in apoptotic rates, we analyzed trophoblast proliferation by using Ki-67 and histone H3 phosphorylation (H3S10-P) as markers. Ki-67 is characteristic of all proliferating cells but is lost upon terminal differentiation (Gerdes et al., 1991), whereas H3S10-P accumulates in late G2 and M phase of the cell cycle (Hendzel et al., 1997). Unexpectedly, a subtle increase was observed for both markers in tg Cts7 placentas (~9% for Ki-67; ~8% for H3S10-P). However, closer examination revealed that this increase was chiefly due to cells exhibiting a punctate H3S10-P staining pattern, whereas fewer cells with a meta- or telophase arrangement of chromosomes were seen (Fig. 4C,D). This phenotype was particularly obvious at E9.5 but was still present at E12.5. Accordingly, these placentas exhibited larger patches of Ki-67-positive cells in the spongiotrophoblast and labyrinth. The Ki-67 staining pattern also highlighted the absence of differentiated trophoblast cells in these areas (Fig. 4E,F). These findings indicated that Cts7 overexpression was associated with a slower progression through the cell cycle, in particular through mitosis, and provided a possible explanation for the observed delay in trophoblast differentiation.

Next, we aimed to determine whether the proliferation defect depended on the proteolytic activity and involved a direct nuclear function of CTS7. Such a nuclear role was feasible because the CTS7 propeptide sequence contains a predicted bipartite nuclear localization signal (defined by a motif consisting of two consecutive basic residues, 10 intervening amino acids and a
minimum of three out of five additional basic residues; Prosite motif PDOC00015). Interestingly, we found that the increase in H3S10-P staining caused by Cts7 was abolished when point mutations were introduced into the catalytic site or the nuclear localization signal (Fig. 4G), thus indicating that proteolytic activity and nuclear localization were essential for the role of CTS7 in affecting cell proliferation. S-phase progression as assessed by BrdU incorporation rates was comparable for all transfected protein variants (not shown), reinforcing that the main effect of CTS7 was restricted to late G2 and/or M phase of the cell cycle.

As these findings suggested that some amount of CTS7 was located to the nucleus, we re-examined the cellular localization by detailed confocal microscopy of transfected TS and COS-7 cells. Although CTS7 was mainly confined to the cytoplasm, optical sectioning revealed unambiguously nuclear CTS7 signals that were absent from cells transfected with the NLS mutant variant (Fig. 4H). Thus, a small fraction of CTS7 was present in the nucleus where it most probably exerts a direct role in chromosome condensation and mitotic progression.

**Cts8 overexpression enhances giant cell differentiation**

For Cts8, one transgenic line was obtained that exhibited ubiquitous lacZ expression and in which the floxed lacZ/Neo reporter cassette could be efficiently removed by CMV-Cre and Sox2-Cre, while transgene activation was again incomplete when the trophoblast-specific Tpbp-Cre and Cyp19-Cre expressors were used. Analysis of placentas showed approximately equal amounts of endogenous Cts8 and transgenic Cts8-IRES-GFP mRNA upon strongest tgCts8 activation (Fig. 5A). When hemizygous tgCts8 mice were mated to ubiquitous or inner cell mass-specific (paternal transmission of Sox2-Cre) Cre expressors, no live transgenic offspring were obtained. By contrast, foetuses survived to term when Cts8 expression was induced trophoblast specifically. Hence, lethality was due to Cts8 expression in the embryo and/or extra-embryonic endo- and mesoderm. Consistent with this observation, trophoblast differentiation occurred largely normally in conceptuses derived from matings to all four Cre lines at mid-gestation, as determined by expression and distribution of markers of giant cell differentiation (Prl3d1, Prl3b1, Prl2c2, Cts7), ectoplacental...
cone/spongiotrophoblast (Tpbpa) and extra-embryonic mesoderm (Peg1, Cdh5, F8c) (Fig. 5B). However, late-stage placentas of trophoblast-activated tgCts8 exhibited an enlarged giant cell/spongiotrophoblast layer compared with control placentas (Fig. 5C). The effect of Cts8 on giant cell differentiation was particularly obvious when the trophoblast layer of E9.5 CMV-Cre\textsuperscript{tgCts8} placentas was dissected and grown in culture for 2 days, a system where transgene activation could be easily detected by the concomitantly induced GFP expression. In this combination with in vitro culture, strikingly more and larger giant cells (P<6/\textsuperscript{10}\textsuperscript{-15}) were observed in the transgenic samples (Fig. 5D,E). These findings showed that, similar to our previous results in overexpressing TS cells, Cts8 primed diploid trophoblast cells towards differentiation into giant cells, but that (mild) overexpression in trophoblast is not detrimental for normal development to term.

**CTS8 has the capacity to disintegrate blood vessels**

We next sought to determine whether the phenotype resulting from ectopic activation of Cts8 could reveal additional functions of this protease that may be masked in trophoblast by its endogenous Cts8 expression. Analysis of E9.5-E16.5 conceptuses determined that the embryonic lethality caused by ubiquitous Cts8 expression occurred around mid-gestation. At this stage, the vast majority of transgenic embryos were severely growth retarded and were characterized by a complete lack of blood vessels (Fig. 6A,B). Instead of the mature vitelline vasculature, transgenic yolk sacs contained only the honeycomb-like pattern of blood islands. Blood pools were frequently observed in the yolk sac, pericardium and the embryonic trunk suggesting that haematopoiesis was initiated, but erythrocytes were not obviously restricted to vessels. The two layers of the yolk sac were only loosely attached to each other and CD31-positive endothelial cells enclosed much wider and often empty spaces (Fig. 6C). Thus, although vasculogenesis seemed to proceed normally, Cts8 expression caused a severe defect in embryonic and yolk sac angiogenesis. Furthermore, tgCts8 embryos exhibited a hugely inflated amniotic cavity and severe heart defects, including pericardial oedema and absence of atrial and ventricular chamber formation (Fig. 6B). Additional common malformations included a kinking of the neural tube and neural tube closure defects, as well as a massive increase in cells undergoing apoptosis (see Fig. S1 in the supplementary material).

**CTS8 causes vascular breakdown by smooth muscle degradation**

To explain the vascularization defect in Cts8 transgenic conceptuses, the perivascular support lining was examined by staining for smooth muscle \(\alpha\)-actin (SMA=Acta2). Whereas the vitelline vessels of wild-type yolk sacs were surrounded by a thin, continuous layer of smooth muscle cells, the enlarged blood spaces of tgCts8 yolk sacs were devoid of SMA staining (Fig. 6C). The same finding was observed in the chorioallantoic vasculature of the placenta. Vessel outlines were present in transgenic placentas, as detected by laminin staining; however, they appeared disorganized. Ruptured blood vessels and free foetal blood cells were frequently observed. Importantly, these areas were characterized by a striking reduction of SMA staining (Fig. 6D). This finding together with the observed haematopoiesis strongly suggested that Cts8 expression compromised the integrity of the perivascular support layer, leading to vessel rupture and subsequent haemorrhage. Consistently, we observed frequent extravasations of blood islands and free foetal blood cells into the surrounding stroma and surrounding tissues, with a pronounced increase in blood cell numbers and haematopoiesis (Fig. 6E,F). These findings indicate that, similar to our previous studies in overexpressing TS cells (21), Cts8 may act as an efficient ‘disintegrin’ of mesenchymal support layers in both the embryo and in the placenta.

---

**Fig. 5. Cts8 overexpression promotes giant cell differentiation.** (A) Northern blot hybridization with Cts8 on wild-type and tgCts8 placentas showing approximately equal amounts of endogenous (endo. Cts8) and transgenic (tg Cts8) Cts8 mRNA. The transgenic product is a bicistronic Cts8-GFP RNA. (B) Marker expression analysis of E9.5 placentas after ubiquitous transgene activation (Sox2-Cre.tgCts8). Note the particularly strong transgene expression in extra-embryonic mesoderm and parietal endoderm. No striking phenotype is observed at this stage. (C) Prl3b1 expression on E16.5 placentas after trophoblast-specific Cts8-activation by mating to Tpbp-Cre mice. The giant cell/spongiotrophoblast layer is enlarged. (D) Trophoblast tissue dissected from E9.5 control and ubiquitously induced Sox2-Cre.tgCts8 placentas after 2 days culture stained with DAPI. Fewer diploid but more and bigger giant cells are observed. (E) Quantification of nuclear sizes. No giant cell larger than 1200 \(\mu\text{m}^2\) was found in controls. Scale bars: 500 \(\mu\text{m}\) in B; 1 mm in C; 1 mm in D.
abundance in trophoblast tissues their molecular function during the mitotic slowdown. This effect may also account for the inability to delay in differentiation of trophoblast cell types, such as glycogen and sinusoidal giant cells, may be a direct consequence of this functional diversity of this morphologically similar cell type. Cts8 expression proved to be overexpressed (Sox2-Cre.tgCts8) defined by a combination of their localization and gene expression. According to this classification, Cts8 and Cts7 are expressed in newly emerging placenta-specific cathepsins, Cts7 and Cts8, and provide evidence for their role in trophoblast proliferation, differentiation and vascular remodelling at the site of trophoblast invasion.

Cts7 and Cts8 define a unique subset of trophoblast giant cells that is generally characterized by an invasive behaviour and by interaction with maternal spiral arteries. The previously underappreciated diversity of trophoblast giant cells has recently been highlighted by the description of four subtypes that can be defined by a combination of their localization and gene expression (Simmons et al., 2007). According to this classification, Cts7 and Cts8 mark an exclusive set of parietal and spiral artery-associated giant cells. This pattern suggests a common function between these cells and establishes another giant cell subgroup, emphasizing the functional diversity of this morphologically similar cell type.

Our data indicate that overexpression of CTS7 causes a postponement of the cell cycle in G2 and/or M phase, which may be due to a subtle defect in chromosome condensation. The observed delay in differentiation of trophoblast cell types, such as glycogen and sinusoidal giant cells, may be a direct consequence of this mitotic slowdown. This effect may also account for the inability to maintain the self-renewing, proliferative capacity of Cts7-overexpressing TS cells. As Cts7 is expressed in newly emerging...
giant cells during gestation, it is feasible that the delay in chromosome condensation and cell cycle progression contributes to the switch towards endoreduplication. In this context, it is interesting to note that MENT (myeloid and erythroid nuclear termination stage-specific protein), a nuclear protein that can act as an inhibitor of cathepsins L and L2, was identified as a heterochromatin-associated protein mediating chromatin condensation (Grigoryev et al., 1999; Irving et al., 2002). Hence, it is possible that a similar protease-inhibitor relationship is disturbed in trophoblast cells upon CTS7 (over)expression and interferes with the progression of mitotic chromatin condensation.

Our evidence for a direct nuclear role of CTS7 in this process is intriguing, but not entirely unexpected as a nuclear function of some cathepsins has recently been reported. Most strikingly, it has been shown that nuclear CTSL mediates the proteolytic degradation of a transcription factor (Goulet et al., 2004). However, in contrast to CTSL, where alternate translation start sites give rise to isoforms that lack a signal peptide, nuclear CTS7 localization is achieved mainly by an internal nuclear localization signal that, when abrogated, rescues the chromosome condensation defect and leads to significantly weaker nuclear immunostaining signals.

As to CTS8, our data indicate that this cathepsin may have complementary functions to CTS7 in promoting giant cell differentiation (Fig. 7), an effect that was particularly obvious when signals from surrounding cells such as decidual stroma, maternal blood and/or endothelial cells were removed in an in vitro context. Hence, within the uterine bed, the differentiation-promoting effect of Cts8 is rather mild and an increase in fully differentiated giant cells becomes only obvious in late-stage placentas upon prolonged overexpression. This is consistent with the biological function of Cts8-expressing giant cells that need to remain comparatively small to exhibit their highly invasive properties (Hemberger, 2008).

Most notably, however, our transgenic approach revealed a dramatic functional difference between the closely related cathepsins CTS7 and CTS8, in that (ubiquitous) Cts8 overexpression causes midgestational embryonic lethality whereas Cts7 is tolerated even at high levels. The most significant effect of CTS8 is that it interferes with normal embryonic and vitelline angiogenesis, and leads to a reduction of the perivascular support structure, most probably by direct degradation of smooth muscle α-actin. As SMA ablation allows development into adulthood (Schildmeyer et al., 2000), CTS8 may well have additional targets whose proteolysis leads to the observed embryonic lethality. The Cts8 gain-of-function phenotype resembles that of knockout models of many factors required for normal vascular development, haematopoiesis, cell adhesion and communication, and of the TGFβ signalling network. Thus, these factors represent good candidates for potential substrates of CTS8. Particularly noteworthy is the TGFβ receptor Alk1 (activin receptor-like kinase 1) as Alk1-deficient and tg^\text{Cts8} conceptuses share phenotypic similarities not only in the embryo and yolk sac, but also in the placenta. As in Cts8-overexpressing placenta, allantoic mesoderm invasion into the chorionic trophoblast is largely undisturbed, but chorioallantoic vessels are severely dilated and fused (Hong et al., 2007; Oh et al., 2000). Moreover, Alk1 deficiency leads to enhanced expression of a number of proteases (Oh et al., 2000), and TGFβ signalling is known to regulate cathepsins negatively (Gerber et al., 2000). Hence, Cts8 activation in TGFβ/Alk1 mutants could provide an explanation for the almost identical phenotypes. As mutations of the TGFβ signalling cascade also lead to disruptions in vascular smooth muscle support, this defect may be a direct consequence of aberrant Cts8 expression.

Importantly, our approach of activating Cts8 expression at ectopic sites revealed a major effect of this protease on the integrity of blood vessels. As it is only Cts8-positive trophoblast giant cells that are in contact with mature vessels in the early conceptus, this activity of CTS8 would not have been recognized by trophoblast-restricted overexpression only. In the later placenta where foetal blood vessels are present in the labyrinth, poor transgene inducibility in the lining syncytiotrophoblast layer III and differences in the ultrastructure of labyrinthine vessels compared with spiral arteries may explain the lack of an overt placental phenotype. Our findings suggest that CTS8 produced by giant cells in direct contact with maternal spiral arteries mediates their localized remodelling by degradation of perivascular smooth muscle cells. Such a paracrine effect is feasible because secreted cathepsins can function in the extracellular milieu as well as inside exposed cells (Nielsen et al., 2007). Weakening of the vessels could then facilitate endovascular giant cell invasion and the formation of trophoblast-lined blood sinuses; these are essential processes for the normal progression of pregnancy.

Partial loss of arterial smooth muscle cells starts in the metrial triangle of the decidua, some distance away from trophoblast giant cells, and is mediated by uterine natural killer (uNK) cells (Adamson et al., 2002). In addition, remodelling of the smooth muscle layer is incomplete in uNK cell-deficient females (Croy et al., 2000). Thus, a model emerges where in the environment of the uterine bed, a concerted action of uNK cells and trophoblast giant cells is necessary to mediate normal spiral artery remodelling (Fig. 7). This dual regulation from both the foetal and maternal side represents an intriguing control mechanism to prevent excessive arterial wall degradation and uterine bleeding. Having established this blood vessel-disintegrating function of CTS8 that is not shared by the co-
expressed CTS7, it will now be interesting to investigate whether CTS8 is essential for trophoblast-mediated vascular remodelling in knockout approaches.

Cysteine cathepsins are implicated in a variety of cellular processes and are involved in numerous pathological conditions that include extracellular matrix remodelling and invasion. Here, we provide molecular insights into the role of the two placenta-specific cathepsins, CTS7 and CTS8, in trophoblast differentiation and function. This knowledge will further our understanding of the physiological and pathological significance of cathepsins. Our data demonstrate that, despite their close ontological and genetic relationship, highly related placental cathepsin proteases have distinct non-redundant functions during development and contribute crucially to a successful outcome of pregnancy.

We are grateful to Dr Simon Walker for expert help with confocal microscopy and image analysis, and to Ms Anaïs Delourme for contributing to the histological analyses. This work was supported by a Career Development Award of the Medical Research Council (UK) to M.H.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/19/3311/DC1

REFERENCES


