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Cathepsin proteases have distinct roles in trophoblast function and vascular remodelling

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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, *Cts7* and *Cts8*, are expressed in distinct but largely overlapping subsets of giant cells that are in direct contact with maternal arteries. We find that *Cts8*, but not *Cts7*, has the capacity to mediate loss of smooth muscle α-actin and to disintegrate blood vessels. Consequently, conditional ubiquitous overexpression of *Cts8* leads to midgestational embryonic lethality caused by severe vascularization 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 *Cts7* and *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 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 trophectoderm 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 decidualized 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 μm 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 polyploid 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 *Cts7* and *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

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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 into 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., 2005), and placentas from pre-eclamptic pregnancies exhibited de-regulated expression levels of cathepsins B, L and L2 (Varanou et al., 2006).

Despite their abundance in trophoblast tissues, the precise function of cysteine cathepsins in placental development remains elusive. Giant cell-expressed cathepsins B and L have been knocked out, but are dispensable during gestation even in compound mutants (Felbor et al., 2002). Although none of the eight placenta-specific cathepsins has been studied in knockout experiments yet, close evolutionary and sequence similarity has suggested overlapping functions. Such redundancy was further suggested by co-expression of: *Cts7* and *Cts8* in invading giant cells (Hemberger et al., 2000); *Ctsm*, *Ctsq*, *Ctsr* and possibly *Cts6* and *Ctsj* in sinusoidal giant cells in the labyrinth layer (Ishida et al., 2004; Nakajima et al., 2000; Simmons et al., 2007); and *Ctsm*, *Cts3* and *Ctsr* in the spongiotrophoblast of the mature mouse placenta (Bode et al., 2005; Ishida et al., 2004). Because of this likely functional overlap, an overexpression system was preferable to a knockout strategy for elucidating the function of individual cathepsins. Hence, we chose to pursue a gain-of-function analysis in trophoblast stem cells and conditional overexpression in mice to gain insights into the roles of *Cts7* and *Cts8* during development. Our data show that these two highly related cathepsins play distinct roles during embryogenesis and contribute critically to trophoblast proliferation, differentiation and remodelling of the uterine vasculature.

MATERIALS AND METHODS

Expression constructs

The open reading frames (ORF) of *Cts7* and *Cts8* were cloned into the CMV-IRES-EGFP (kindly provided by A. Nagy) and pIRES-(FLAG)hrGFP-1a (Stratagene, La Jolla, CA, USA) expression vectors. Point mutations were introduced by PCR-mediated mutagenesis targeted to the nuclear localization signal and the active site residues 1 and 3 of *Cts7*. For generation of transgenic mice, a C-terminally FLAG tagged *Cts7* ORF construct and a *Cts8*-IRES-EGFP fragment were cloned into the pCALL expression vector (Lobe et al., 1999). All constructs were sequence verified.

Cell culture

TS cell lines TS-GFP, TS-Rs26 and TS-B6 were grown under standard conditions (Tanaka et al., 1998). For transfections, Lipofectamine 2000 reagent (Invitrogen, Paisley, UK) was used. Immunofluorescence staining was performed with an anti-FLAG antibody (Sigma, Dorset, UK) diluted 1:250. Relative cell sizes were measured using the 'Cell' software (Olympus, Watford, UK).

Transgenic mouse production

$Tg^{LacZ/Cts8}$ mice were generated by pronuclear injection of the linearized plasmid into C57BL/6×CBA F1 zygotes. $Tg^{LacZ/Cts7}$ strains were obtained by electroporation 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, USA) 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 (MAB1499; R&D Systems, Minneapolis, MN, USA) 1:200; anti-Laminin (Sigma, Dorset, UK) 1:200; anti-phosphohistone H3-S10 (#06-570; Upstate, Charlottesville, VI, USA) 1:200; anti-Ki-67 (ab15580; Abcam, Cambridge, UK) 1:100; anti-FLAG M2 (F3165; Sigma, Dorset, UK) 1:300; and peroxidase-conjugated isolectin BSI-B_4 (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 Hematoxylin.

Quantitative expression analyses

Total RNA was prepared using Trizol Reagent (Invitrogen, Paisley, UK). Total RNA (20-40 μg) was electrophoresed in 1% formaldehyde gels and processed for northern blotting according to standard procedures (Sambrook et al., 2001). Signals were quantified against 18S ribosomal RNA (rRNA). For qRT-PCRs, random-primed reverse-transcribed cDNA was used and PCRs were carried out on an ABI 7700 cycler. mRNA levels were normalized against three housekeeping genes (*Gapdh*, *Dync11:2*, *Sdha*).

Western blotting

Cells were lysed in PBS/1% Triton X-100 or in 1× RIPA buffer in the presence of 20 mM dithiothreitol 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 the 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-I (*Prl3d1*) (Fig. 1A-C). *Cts7*- and *Cts8*-expressing giant cell populations

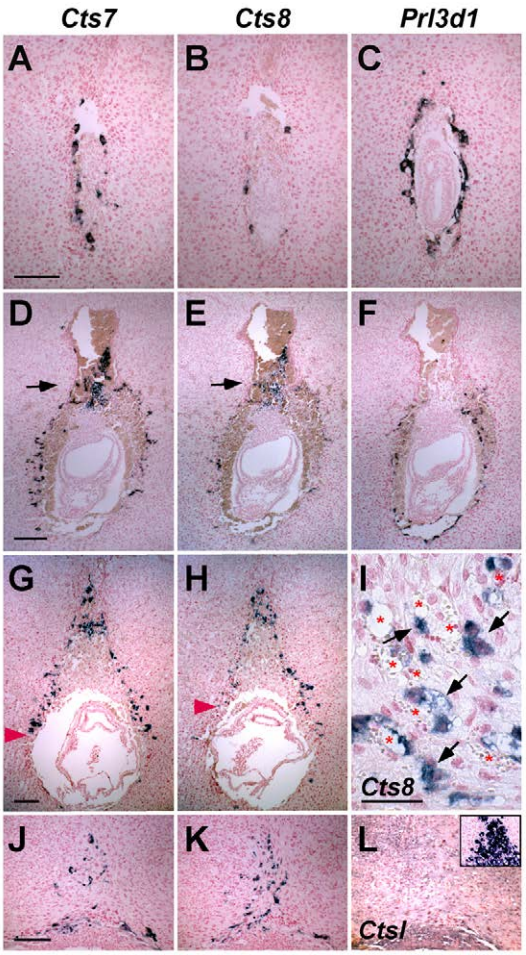

Fig. 1. Expression analysis of *Cts7* and *Cts8* on early post-implantation conceptuses. (**A-C**) In situ hybridization on E6.5 implantation sites. *Cts7* and *Cts8* expression (blue staining) is present in a subset of parietal trophoblast giant cells labelled by placental lactogen-I (*Prl3d1*). (**D-F**) Serial sections of E7.0 conceptuses. *Cts7* and *Cts8* are expressed by some parietal giant cells that are positive for *Prl3d1*, and in invasive giant cells at the margins of the ectoplacental cone (arrow). (**G**,**H**) E7.5 conceptuses. *Cts7* and *Cts8* staining is largely restricted to secondary invasive giant cells of the ectoplacental cone. The red arrowheads indicate that *Cts7* expression in parietal giant cells extends 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). *Ctsl* (L) is not expressed in this cell population. Scale bars: 200 μm in A-H,J-L; 100 μm in I.

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 further distally to parietal giant cells positioned around the midline of the implantation site (Fig. 1G,H). Again, only a subpopulation of all giant cells in the ectoplacental cone region was labelled when compared with the marker genes *Prl3d1* and proliferin (*Prl2c2*) (Fig. 1F,L inset). Remarkably, this population of *Cts7*- and *Cts8*-expressing invasive trophoblast giant cells was always associated with maternal blood vessels (Fig. 1I). It is also important to note that this expression pattern seemed to be unique to *Cts7* and *Cts8*, and was not shared by other placental cathepsins (Deussing et al., 2002), including the presumptive ancestral *Ctsl* (Fig. 1L). Expression levels declined sharply after E9.5 with only very few giant cells at the lateral edges of the placenta remaining positive for *Cts7* and *Cts8* (not shown). A second site of expression was observed in late-stage placentas from ~E14.5 onwards in labyrinth trophoblast, albeit at much reduced levels [not shown (Hemberger et al., 2000)]. Thus, expression of *Cts7* and *Cts8* was most abundant in giant cells associated with maternal arteries during the phase of trophoblast invasion.

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/CM; 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 (Hemberger et al., 2004).

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 (Gottesman, 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 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

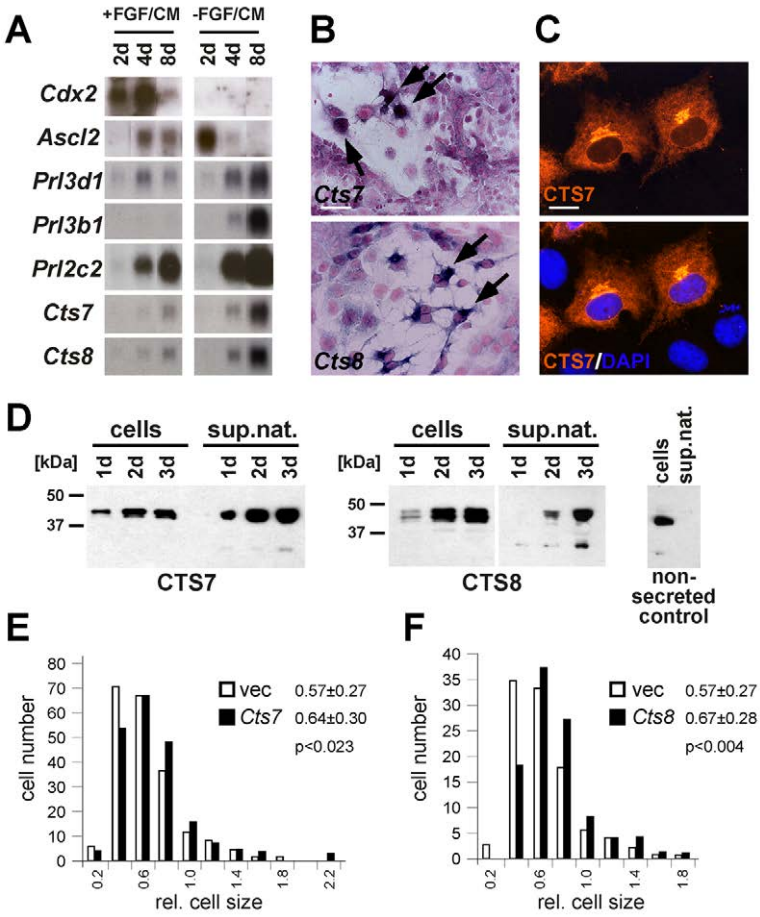

Fig. 2. Cytological characterization and effects of *Cts7*/*Cts8* expression in TS cells. (**A**) Northern blot hybridization on trophoblast stem (TS) cells grown in media promoting stem cell maintenance (+FGF/CM) or differentiation (–FGF/CM). Expression of *Cts7* and *Cts8* correlates with the profile of giant cell markers (*Cdx2*, stem cells; *Ascl2*, ectoplacental cone and spongiotrophoblast; *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 (PFPL) 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.

and CTS8. Consistent with these findings, the clonal expansion of *Cts7*/*Cts8* transfected TS cells was limited and mitotic indices were reduced by 1.5-fold to twofold (P<0.025). The reduced proliferative capacity interfered with the generation of TS cell lines that stably 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 *Cts7*/*Cts8* 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 trophoblast-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 tg^{Cts7} 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). By contrast, the efficiency of transgene activation was incomplete when the trophoblast-specific Tpbp-Cre and Cyp19-Cre lines were used, resulting in weak tg^{Cts7} expression in target cells (not shown). Importantly, live, healthy and fertile *Cts7*$^{+}$/*Cre*$^{+}$ offspring were obtained at Mendelian ratios, even from matings that yielded highest ubiquitous tg^{Cts7} expression, indicating that *Cts7* overexpression did not interfere with embryonic development to term.

CTS7 affects trophoblast proliferation and differentiation

As there was no difference in survival between the various transgenic lines, we analyzed the strongest tg^{Cts7}-overexpressing line for subtle defects in trophobast differentiation. Consistently, these placentas were characterized by a thinner spongiotrophoblast layer (Fig. 3D). This phenotype correlated with the spongiotrophoblast exhibiting strongest transgene expression, and resulted in a downregulation of spongiotrophoblast-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 tg^{Cts7} placentas (Fig. 3E).

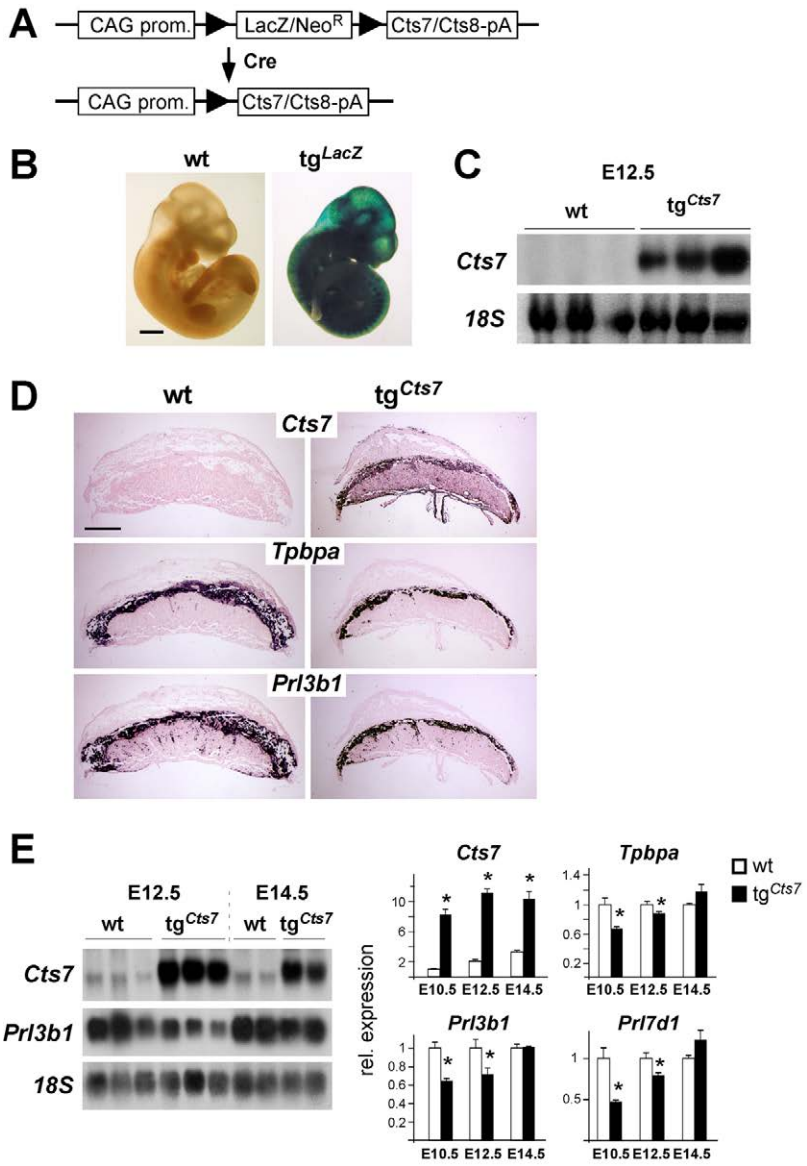

Fig. 3. Transgenic mouse models for inducible expression of *Cts7*. (**A**) General structure of the construct used. (**B**) β-Galactosidase staining proves strong, ubiquitous transgene activity before activation with Cre recombinase. (**C**) Northern blot demonstrating strong *Cts7* expression in the embryo after ubiquitous activation with Cre. (**D**) E12.5 wild-type and tg^{Cts7} (Sox2-Cre.tg^{Cts7}) placentas hybridized with *Cts7*, *Tpbpa* and *Prl3b1*. (**E**) Northern blot analysis and relative expression levels of marker genes in wild-type and transgenic (Sox2-Cre.tg^{Cts7}) placentas. **P*<0.05. Scale bars: 500 μm in B; 1 mm in D.

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 is associated with chromosome condensation and 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

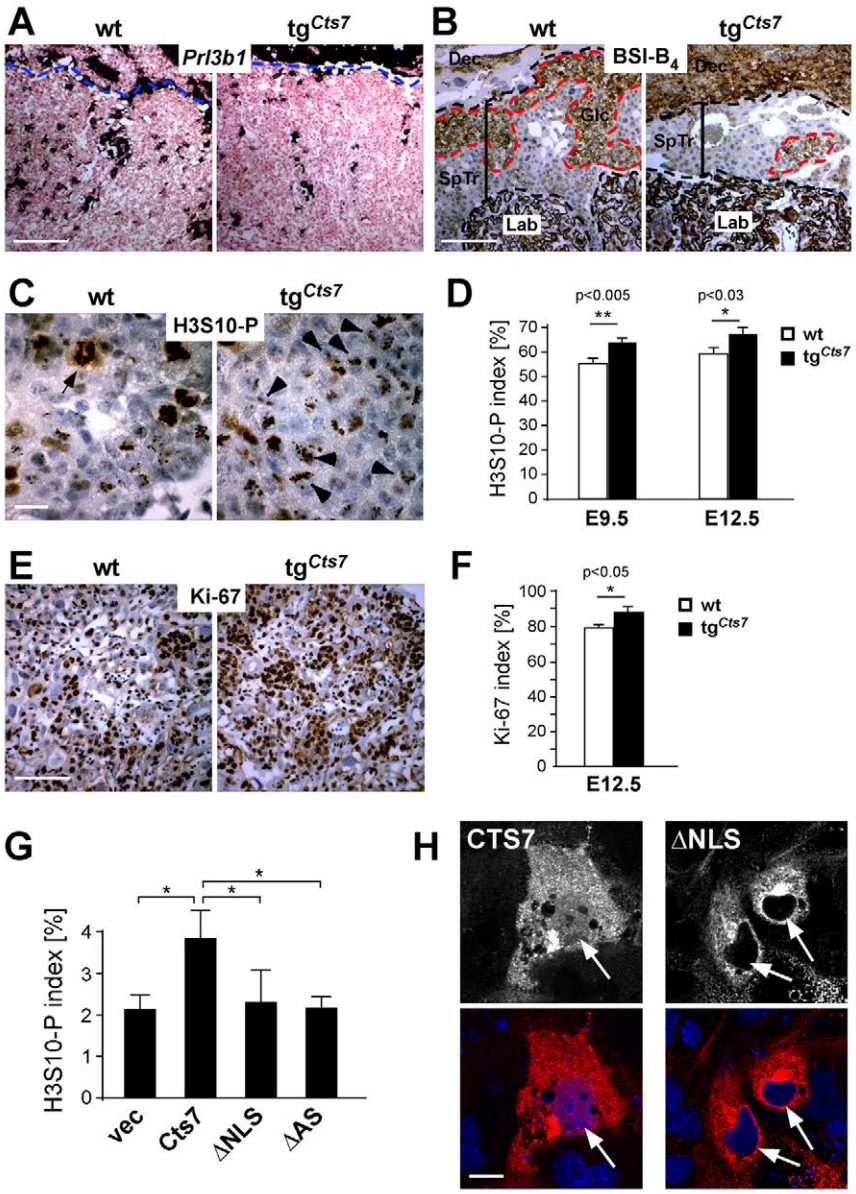

Fig. 4. Nuclear CTS7 causes trophoblast proliferation and differentiation defects. (**A**) Differentiation of sinusoidal giant cells marked by *Prl3b1* (blue) is reduced in the labyrinth layer of E12.5 Sox2-Cre.tg^{Cts7} placentas. The border to the spongiotrophoblast is indicated. (**B**) Isolectin BSI-B$_4$ staining (brown) on E12.5 placentas labelling glycogen cells (Glc, encircled in red). Bracket indicates the spongiotrophoblast (SpTr) layer. Broken black lines indicate the borders to the decidua (Dec) and labyrinth (Lab). (**C**) Anti-phosphohistone staining (H3S10-P, brown) on E9.5 trophoblast of CMV-Cre×tg^{Cts7} matings and (**D**) quantification of signals. The arrow indicates a cell in metaphase of mitosis. Arrowheads indicate some nuclei with a speckled punctate staining in the tg^{Cts7} sample. (**E**,**F**) Ki-67 staining (brown) of E12.5 placentas shows denser clusters and less differentiated (negative) trophoblast cells in transgenic (Sox2-Cre.tg^{Cts7}) placentas. Quantification in F. (**G**) Proportion of H3S10-P positive TS cells 2 days post-transfection with empty vector control (vec), wild-type *Cts7*, nuclear localization signal-mutant (ΔNLS) and active site-mutant *Cts7* (ΔAS). Accumulation of H3S10-P staining depends on nuclear localization and proteolytic activity. Transfected cells were identified by GFP expression. *P<0.05. (**H**) Confocal sectioning of TS cells expressing *Cts7* or *Cts7*-ΔNLS. CTS7 protein can be found inside the nucleus (arrows) of some cells, whereas the NLS-mutant variant is clearly excluded from this compartment. Scale bars: 200 μm in A,B; 20 μm in C; 100 μm in E; 20 μm in H.

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 tg^{Cts8} activation (Fig. 5A). When hemizygous tg^{Cts8} 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

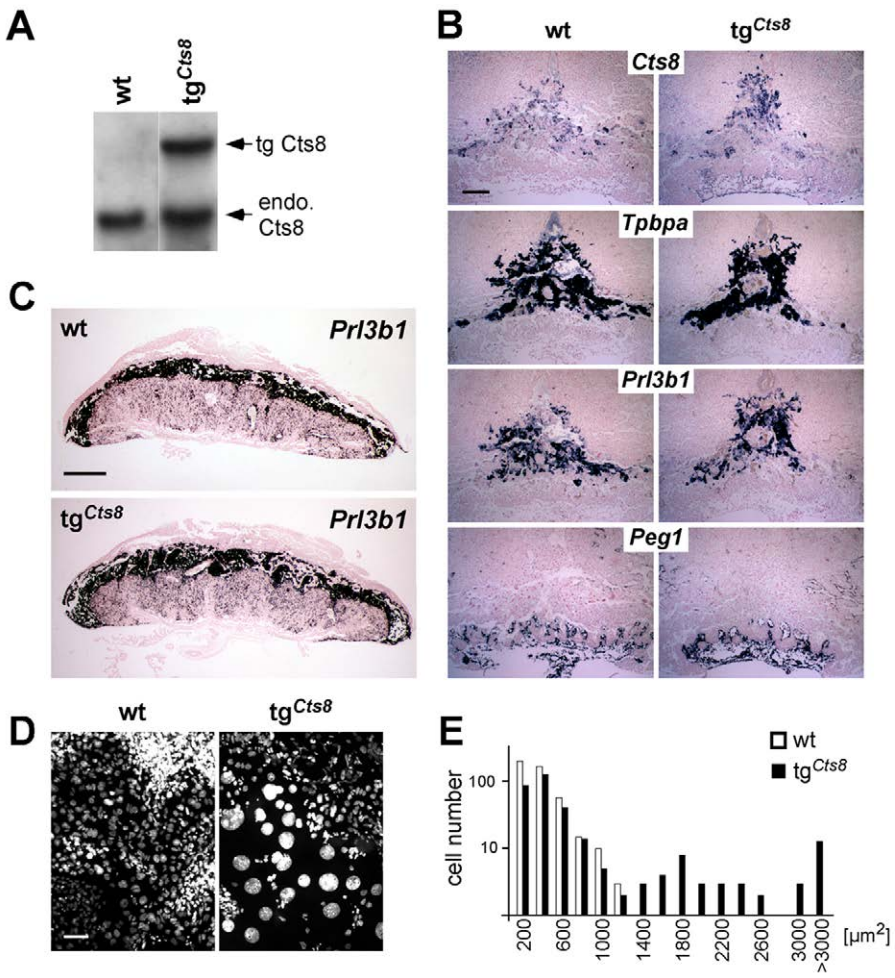

Fig. 5. *Cts8* overexpression promotes giant cell differentiation. (**A**) Northern blot hybridization with *Cts8* on wild-type and tg^{Cts8} placentas showing approximately equal amounts of endogenous (endo. Cts8) and transgenic (tg Cts8) *Cts8* mRNA. The transgenic product is a bicistronic Cts8-GFP RNA. (**B**) Marker expression analysis of E9.5 placentas after ubiquitous transgene activation (Sox2-Cre.tg^{Cts8}). 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.tg^{Cts8} 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 μm^2 was found in controls. Scale bars: 500 μm in B; 1 mm in C; 1 mm in D.

cone/spongiotrophoblast (*Tpbpa*) and extra-embryonic mesoderm (*Peg1*, *Cdh5*, *F8c*) (Fig. 5B). However, late-stage placentas of trophoblast-activated tg^{Cts8} 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×tg^{Cts8} 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 ($P<6\times10^{-15}$) 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, tg^{Cts8} 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 α-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 tg^{Cts8} 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.

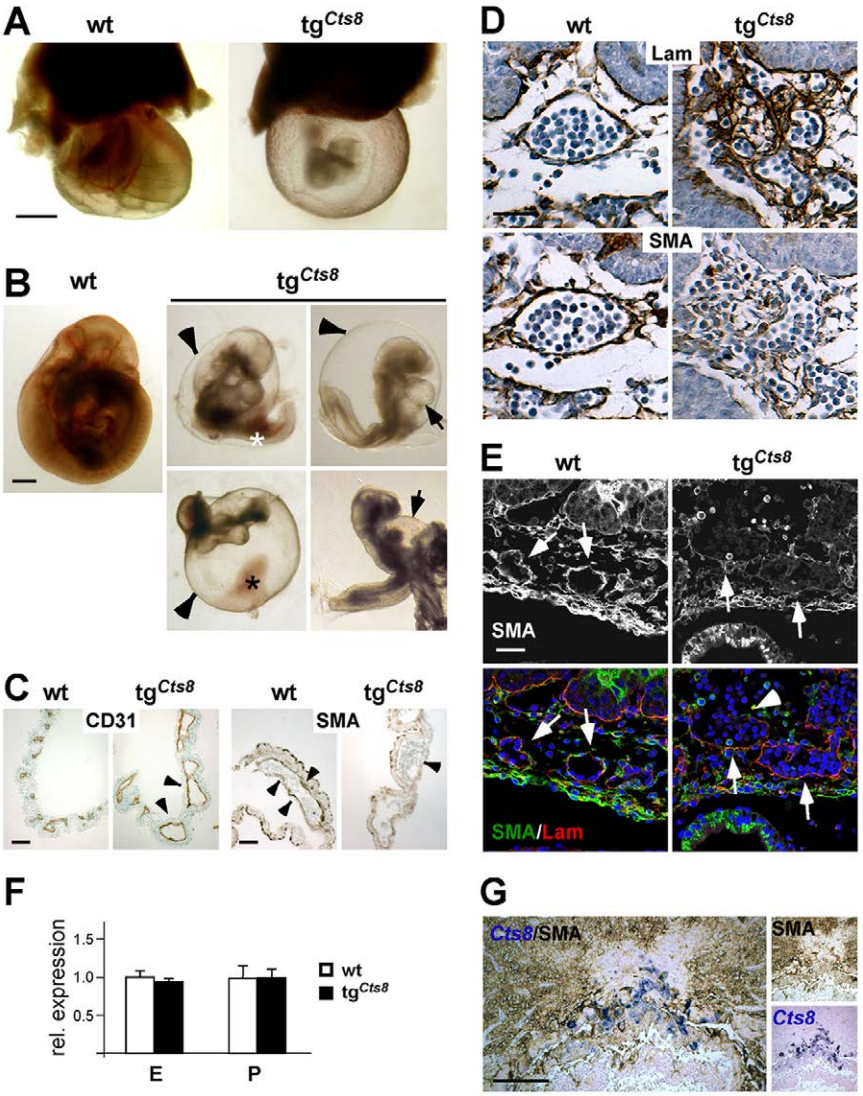

Fig. 6. *Cts8* causes vascular disintegration associated with perivascular smooth muscle degradation. (**A**) Phenotype of E10.5 tg^{Cts8} conceptuses upon ubiquitous activation of the transgene (Sox2-Cre.tg^{Cts8}). No mature vitelline vessels are observed. (**B**) Wild-type E10.5 embryo and ubiquitously activated tg^{Cts8} embryos (Sox2-Cre.tg^{Cts8} and CMV-Cre.tg^{Cts8}) at E10.5 (left-hand panels) and E9.5 (right-hand panels). Transgenic embryos are severely retarded and display an inflated amnion (arrowheads), pericardial oedema (arrows) and lack of normal heart chamber formation. Asterisks indicate the occurrence of blood pools. (**C**) Yolk sac phenotype of *Cts8*-overexpressing (Sox2-Cre.tg^{Cts8}) conceptuses. Pecam/CD31-positive endothelial cells are present in transgenic samples; however, spaces between the endodermal and mesodermal layers are dilated. Smooth muscle α-actin (SMA) staining surrounds vessels in wild-type yolk sacs, but is absent from transgenic samples (arrowheads). (**D**,**E**) Laminin (Lam) and smooth muscle α-actin (SMA) staining on consecutive sections (D) and by double immunofluorescence (E) of chorioallantoic vessels in the E9.5 placenta. Laminin staining reveals disorganized vessel structures that lack smooth muscle cells in transgenic samples (Sox2-Cre.tg^{Cts8}). Arrows indicate vessel structures that display a striking reduction of SMA staining in transgenic samples. The arrowhead indicates a site of vessel rupture where blood cells (round, DAPI positive) can be seen exiting into the surrounding tissue. (**F**) Quantitative RT-PCR analysis of SMA mRNA levels in embryos (E) and placentas (P). (**G**) Consecutive sections of E9.5 placentas stained for *Cts8* by RNA in situ hybridization (blue) and SMA by immunohistochemistry (brown) showing lack of SMA in the vicinity of *Cts8*-expressing giant cells. Scale bars: 1 mm in A; 500 μm in B; 50 μm in C; 50 μm in D; 100 μm in E; 500 μm in G.

6D,E). To determine whether the decrease in SMA amounts was regulated on the transcriptional or protein level, quantitative RT-PCRs were performed. These experiments demonstrated that mRNA levels remained unchanged in transgenic embryos and placentas (Fig. 6F). Therefore, SMA reduction occurred on the protein level presumably by direct proteolytic degradation by CTS8. An important expectation arising from these results was that, if perivascular smooth muscle degradation was a general function of CTS8 in placentation, spiral arteries should be devoid of SMA where they are in contact with trophoblast giant cells that express *Cts8* endogenously. Consistent with this expectation, the area surrounding *Cts8*-positive giant cells of the ectoplacental cone was characterized by a lack of SMA (Fig. 6G). Taken together, our approach of ectopic *Cts8* expression proved to be a powerful tool to reveal additional activities of this protease. Our findings strongly indicate that perivascular smooth muscle degradation is a physiological function of CTS8 that contributes to the remarkable artery remodelling capacity characteristic of invasive trophoblast giant cells.

DISCUSSION

Cysteine cathepsins are essential for extra-embryonic development (Afonso et al., 1997; Freeman and Lloyd, 1983), but despite their abundance in trophoblast tissues their molecular function during placentation remains elusive. Here, we have characterized two 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 prolongation 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^{Cts8} conceptuses share phenotypic similarities not only in the embryo and yolk sac, but also in the placenta. As in *Cts8*-overexpressing placentas, 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.

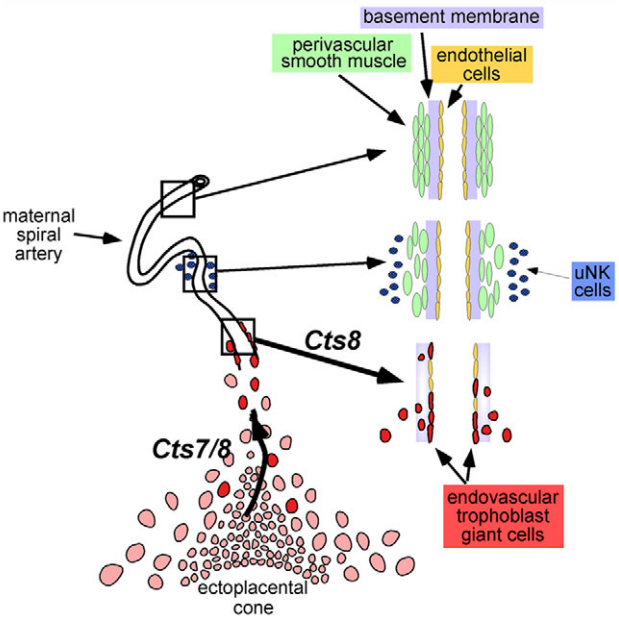

Fig. 7. Model of *Cts7* and *Cts8* function. Spiral artery remodelling is a concerted action between uterine natural killer (uNK) cells and trophoblast giant cells. *Cts8* endows trophoblast giant cells with smooth muscle-degrading functions. This activity facilitates endovascular trophoblast invasion to complete the remodelling process. In addition, *Cts7* and *Cts8* may have complementary roles in promoting differentiation of the spiral artery-associated giant cell subtype from precursors within the ectoplacental cone.

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.

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Supplementary material

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

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