A disintegrin and metalloproteinase 12 (ADAM12) localizes to invasive trophoblast, promotes cell invasion and directs column outgrowth in early placental development

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ABSTRACT: During pregnancy, stromal- and vascular-remodeling trophoblasts serve critical roles in directing placental development acquiring pro-invasive characteristics. The A Disintegrin and Metalloproteinase (ADAM) family of multifunctional proteins direct cellular processes across multiple organ systems via their intrinsic catalytic, cell adhesive and intracellular signaling properties. ADAM12, existing as two distinct splice variants (ADAM12L and ADAM12S), is highly expressed in the human placenta and promotes cell migration and invasion in several tumor cell lines; however, its role in trophoblast biology is unknown. In this study, ADAM12 was localized to anchoring trophoblast columns in first trimester placentas and to highly invasive extracellular matrix-degrading trophoblasts in placental villous explants. The importance of ADAM12 in directing trophoblast invasion was tested using loss-of and gain-of-function strategies, where siRNA-directed knockdown of ADAM12 inhibited trophoblast cell invasion while over-expression promoted migration and invasion in two trophoblastic cell models. In placental villous explant cultures, siRNA-directed loss of ADAM12 significantly dampened trophoblast column outgrowth. Additionally, we provide functional evidence for the ADAM12S variant in promoting trophoblast invasion and column outgrowth through a mechanism requiring its catalytic activity. This is the first study to assign a function for ADAM12 in trophoblast biology, where ADAM12 may play a central role regulating the behavior of invasive trophoblast subsets in early pregnancy. This study also underlines the importance of ADAM12L and ADAM12S in directing cell motility in normal developmental processes outside of cancer, specifically highlighting a potentially important function of ADAM12S in directing early placental development.

Key words: A Disintegrin and Metalloproteinase 12 / cell invasion / placenta / protease / trophoblast

Introduction

Trophoblasts, placental cells of epithelial lineage, terminally differentiate from common cytotrophoblast progenitors into highly specialized cells that are essential for placental development. Differentiating along either one of two cellular pathways (the villous or invasive pathways), trophoblasts support placental endocrine function and nutrient transport to the developing fetus, as well as control endometrial arterial vascular remodeling (Pijnenborg et al., 2011). Cells committed to the invasive pathway acquire conserved cellular and molecular characteristics important in promoting cell motility that are described across multiple cell systems. Notably, invasive trophoblast subtypes, termed extravillous cytotrophoblasts (EVTs), utilize molecular machinery that impact homotypic cell–cell adhesion (Getsios et al., 1998), cell–extracellular matrix (ECM) interactions (Damsky et al., 1994; Zhou et al., 1997), cell survival (Armanit et al., 2006) and production of proteases important for promoting ECM remodeling (Bischof et al., 2002; Beristain et al., 2011) and growth factor bioavailability (Leach et al., 2004; Wright et al., 2010). Severe pregnancy disorders, like fetal growth restriction and pre-eclampsia, are associated with placental insufficiency and associate with altered trophoblast function and incomplete remodeling of maternal bed arterials (Chaddha et al., 2004; Steegers et al., 2010); however, the causative cellular processes leading to aberrant trophoblast function and placental development are poorly understood.

In recent years, members of the A Disintegrin and Metalloproteinase (ADAM) gene family have attracted considerable attention in directing diverse biological processes in development and disease. ADAMs are
multifunctional proteins belonging to the Zn\(^{2+}\)-dependent metzincin superfamily of metalloproteinases (Reiss and Saftig, 2009) and are characterized structurally by an N-terminal signal sequence followed by a prodomain, a metzincin domain, a cysteine-rich region, an epidermal growth factor (EGF) domain, a transmembrane domain and a cytoplasmic tail (Blobel, 2005). ADAMs have the ability to control cell motility by regulating ectodomain shedding of transmembrane proteins (metzincin domain) (Sahin et al., 2004), cell–cell (Iba et al., 2000) and cell–matrix adhesion (disintegrin, cysteine-rich and EGF domains) (Reiss and Saftig, 2009; Zigrino et al., 2011), as well as by participating in signal transduction pathways (Suzuki et al., 2000; Stautz et al., 2010; Leyme et al., 2012). Not all ADAM proteins harbor putative metalloproteinase activity, as determined by the presence of the conserved catalytic site consensus motif HEXGHXXGXXHD within the ‘Met-turn’ of the metalloproteinase domain. Only half of all human ADAM proteins exhibit catalytic function (Blobel, 2005; Mochizuki and Okada, 2007).

Due to their multifunctional properties, ADAM genes are attractive candidates for investigating control of early placentation and trophoblast biology. However, the roles that ADAMs play in placental establishment and trophoblast function have not been thoroughly studied. ADAM10 and ADAM17, two of the best-characterized ADAM subtypes, function as cell-surface sheddases and have been shown to control trophoblast cell invasion in vitro. Here, ADAM10 and ADAM17 cleave the cMet receptor preventing hepatocyte growth factor-induced cell invasion (Yang et al., 2012). ADAM17 has further been ascribed a role in impacting trophoblast cell survival through regulating the bioavailability of pro-apoptotic TNFα (Ma et al., 2011). Additionally, ADAM12, harboring both cell–ECM adhesive (Kawaguchi et al., 2003) and catalytic properties (Loechel et al., 1998), is highly expressed in the placenta and has recently been immunolocalized to progenitor villous cytotrophoblasts and multinuclear syncytiotrophoblast in placentas of early and late pregnancies (Kokozidou et al., 2011); however, despite its high level of expression, its importance in placental development and in controlling trophoblast cell biology remains to be elucidated.

In humans, ADAM12 exists as two splice variants; a long transmembrane form (ADAM12L) and a truncated secreted form (ADAM12S) lacking the transmembrane and cytoplasmic domains: both are active metalloproteinases (Gilpin et al., 1998; Hougard et al., 2000). Functional studies in mice identify ADAM12 as a critical regulator in myogenesis and osteoclast differentiation via effects on cell–cell fusion, though the mechanisms directing these processes are not well defined (Kurisaki et al., 2003; Verrier et al., 2004). Additionally, ADAM12 promotes cell motility in several mesenchymal and epithelial cell lineages (Albrechtsen et al., 2011; Leyme et al., 2012; Rao et al., 2012). For example, ADAM12 specifically regulates (heparin-binding) HB-EGF directed invadopodia formation through a Notch-dependent manner in multiple tumor cell lines (Díaz et al., 2013) and promotes breast cancer cell invasion and growth (Roy et al., 2011). In other studies, both long and truncated ADAM12 promote cell invasion in squamous cell carcinoma cells (Wright et al., 2010; Rao et al., 2012), while ADAM12L specifically regulates podosome formation in epithelial 293-VnR cells through a c-Src-dependent mechanism (Albrechtsen et al., 2011). Taken together, these studies identify ADAM12 as a critical regulator in tumor cell motility through multiple mechanisms, though the specific roles the individual splice variants have in directing cell invasion is still unclear. While evidence suggests a dominant role for ADAM12 in directing tumor cell invasion, surprisingly no studies exist linking ADAM12 function in regulating cell invasion in normal developmental processes.

In this study, we identify ADAM12 expression in columnar EVT subsets from early gestation placentas. ADAM12 is preferentially localized to invasive ECM-degrading EVTs ex vivo, and ADAM12 loss-of and gain-of-function strategies identify a role for ADAM12 in promoting trophoblast cell migration, invasion and column formation. We show that ADAM1S and ADAM12L regulate cell proliferation in the Jeg3 choriocarcinoma cell line, but not in invasive HTR8/SVneo trophoblastic cells, highlighting subtle differences with regard to ADAM12 function in distinct cell types. Finally, our studies provide evidence for a specific role for ADAM12S in promoting trophoblast invasion and EVT column outgrowth through a mechanism requiring its intrinsic catalytic activity.

**Materials and Methods**

**Tissues**

Samples of first trimester placental tissues were obtained from women undergoing elective termination of pregnancy (gestational ages ranging from 5 to 12 weeks). The use of these tissues was approved by the Research Ethics Board on the use of human subjects, University of British Columbia. All women provided informed written consent.

**Cell and tissue culture**

EVT cultures were propagated from first trimester placental explants essentially as described (Gettos et al., 1998). The purity of the EVT cultures was determined by immunostaining for human cytokeratin-7. Only cultures that exhibited >90% immunostaining for cytokeratin-7 were included in study. On-going EVT cultures were maintained in DMEM:F12 1:1 containing 25 mM glucose, L-glutamine, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and supplemented with 10% fetal bovine serum (FBS) (all from Gibco, Grand Island, USA) and Anti antimycotic (Wisent, St-Bruno, Canada). The EVT cell line HTR8/SVneo was a generous gift from Dr Charles H. Graham, Queen’s University, Canada. HTR8/SVneo cells were maintained in RPMI containing 25 mM glucose, L-glutamine, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and supplemented with 10% FBS. JEG-3 trophoblastic cells were purchased from (ATCC, Manassas, USA) and were cultured in DMEM media containing 25 mM glucose, L-glutamine, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and supplemented with 10% FBS.

**Placental villous explant culture**

Ex vivo placental villous cultures were established as described in Wright et al. (2010). Briefly, placental villi from 5- to 7-week-old gestation placentas obtained from patients undergoing elective termination of pregnancies, were dissected, washed in cold phosphate-buffered saline (PBS) and imbedded into Millicell cell culture inserts (0.4 μm pores, 12 mm diameter; EMD Millipore, Darmstadt, Germany) containing 200 μl of growth factor-reduced Phenol-red-free Matrigel (BD Biosciences, Mississauga, Canada). Explants, containing 400 μl DMEM/F12 1:1 (200 mM L-glutamine) in the outer chamber, were allowed to establish overnight in a humidified 37°C incubator at 3% oxygen. After ~16 h of culture, explants were either: (1) transfected with siRNA duplexes for 24 h, (2) cultured with 200 μl conditioned media (CM) derived from ADAM12/ADAM12S\(^{E351Q/pCMV6}\) transfected Jeg3 cells for 72 h or (3) cultured in 200 μl DMEM/F12 1:1 media for 24 h which was then replaced with 200 μl 1:1 DMEM/F12 1:1 for up to 120 h. All explant media were supplemented with penicillin/streptomycin and Anti antimycotic solution (100× dilution). Media was replenished every 48 h. Growing explants were imaged at indicated times.
using a Nikon SMZ 7454T tricocular dissecting microscope outfitted with a digital camera. EVT outgrowths were measured by ImageJ software. Quantification of EVT column outgrowth is calculated as the difference in column length at 96 and 0 h (μm) measured from the base of each column to the invasive EVT front at three distinct locations within each column using ImageJ software. Each treatment was conducted in duplicate and each experiment was carried out three or four times using different placenta.

**Immunohistochemistry and immunofluorescence**

Placental villi (8–10 weeks gestation) and Matrigel villous explants (5–7 weeks gestation) were fixed in 2% paraformaldehyde overnight at 4°C. EVTs were fixed in 4% paraformaldehyde for 20 min at 4°C. Tissues and explants were paraffin embedded and sectioned at 5 μm onto glass slides. Immunohistochemistry was performed following the horseradish peroxidase-3′-amino-9-ethylcarbazole (HRP-AEC) System protocol (R&D Systems, Minneapolis, USA) using antibodies diluted in 5% goat serum directed against human ADAM12 (1:50; ProteinTech, Chicago, USA), HLA-G (1:100; Exbio, Vestec, Czech Republic), cytokeratin-7 (1:200; Santa Cruz Biotechnology, Dallas, USA), Ki67 (1:100, clone SP6; Thermo Scientific, Waltham, USA) and caspase-cleaved cytokeratin-18 (1:10, clone M30; Roche, Indianapolis, USA). Antigen retrieval was performed by heating slides in a microwave for 5 × 2-min intervals in a citrate buffer (pH 6.0). For immunofluorescence, EVTs or Matrigel explants (following antigen retrieval) were incubated with sodium borohydride for 5 min, RT. Slides were blocked in 5% bovine serum albumin for 1 h, RT. Glass coverslips were mounted onto slides using a 4′,6-diamidino-2-phenylindole (DAPI) mounting media (Life Technologies) and slides were imaged immediately using a Leica DM4000B fluorescence microscope.

**Gene expression analysis**

Total RNA was prepared from placental villi, Matrigel villous explants or cell lines using TRIzol (Life Technologies). RNA purity was confirmed using a NanoDrop Spectrophotometer (Thermo Scientific) and by running RNA on formaldehyde agarose gels to observe the integrity of 18S and 28S ribosomal RNA species. For RNA isolation in Matrigel explants, 0.5 μg of glycerol (Roche) was used to increase RNA precipitation. One microgram of RNA was reverse-transcribed to cDNA using a first-strand cDNA synthesis kit (QuantaBiosciences Inc., Gaithersburg, USA) and a NanoDrop Spectrophotometer (Thermo Scientific) according to the manufacturer's protocol. Selection began 48 h post-transfection prior to experimentation. Cells were maintained in medium containing 100 μg/ml G418 sulfate. Stable transfections were performed to establish multiclonal HTR8/SVNeo and JEG3 cells constitutively expressing ADAM12L and ADAM12S. Two micrograms per milliliter of plasmid DNA was transfected using Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer’s protocol. Select began 48 h post-transfection using 400 μg/ml G418 sulfate in DEME or RPMI; after selection, cells were maintained in medium containing 100 μg/ml G418 sulfate. Transient transfection experiments were performed as above, except cultures were incubated 48 h post-transfection prior to experimentation.

To generate the ADAM12 catalytically inactive mutant expression construct, ADAM12S<sub>ES31Q</sub>, a point mutation in the metalloproteinase domain of ADAM12S was introduced using the QuikChange PCR site-directed mutagenesis kit (Agilent, Santa Clara, USA), where guanine (1059) was substituted to cytosine, resulting in a translated protein consisting of a glutamine (Q) at amino acid 351 instead of a glutamate (E), thus rendering the protein catalytically inactive (Kveiborg et al., 2005). The parent pADAM12 construct served as the template for this reaction. The following HPLC-purified primers were used: forward 5′-CGTGACCCTGGCACAT-3′; reverse 5′-GAAATTGTGGCCCAGCT-3′; reverse 5′-GAAATTGTGGCCCAGCT-3′. Dpn I-digested ADAM12S<sub>ES31Q</sub> expression constructs were transformed into Sbi3 competent cells (Life Technologies), where eight bacterial clones were grown, and DNA plasmid was isolated and sequenced to verify the guanine (1059) to cytosine point mutation.

**Cell lysis and immunoblot analysis**

Cells were washed in PBS and incubated in RIPA cell extraction buffer (20 mM Tris-HCl, pH 7.6, 1% Triton X-100, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 5 mM EDTA, 50 mM NaCl) supplemented with 200 mM Na2VO4, 2 mM phenylmethylsulphonyl fluoride and an appropriate dilution of Complete Mini, EDTA-free protease inhibition cocktail tablets (Roche), for 30 min. Protein concentrations were determined using a BCA kit (Thermo Scientific). For immunoblottting, 30 μg of cell protein lysate was resolved by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were probed using rabbit polyclonal antibodies directed against ADAM12 (ProteinTech; Figs 1, 2, 5, 6 or Abcam Cat#. 28225, Fig. 3; Toronto, Canada). The blots were stripped and re-probed with an HRP-conjugated monoclonal antibody directed against mouse β-actin (Santa Cruz Biotechnology).

**siRNA transfection**

Four siRNAs (Thermo Scientific; 25 nM) targeting the human ADAM12 mRNA transcript (A12i-5 5′-CCCAAUGGCUUUCUAAUGA-3′; A12i-6 5′-CCCAAGGAUUGUAAC-3′; A12i-7 5′-UCAAGGCAUAAUAGUAUU-3′; A12i-8 5′-CAAAUGCCUUCUAAUGAC-3′) were transfected into HTR8/SVNeo cells using Lipofectamine RNAi Max reagent (Life Technologies) according to manufacturer’s protocol. HTR8/SVNeo cells transfected with ON-TARGETplus non-silencing (NS) siRNA#1 (NS; Cat# D-001810-01-20) or cultured in the presence of transfection reagent alone, served as negative controls. For siRNA-directed silencing in Matrigel explant cultures, 200 nM A12i-5 or A12i-8 were transfected into explant cultures 24 h post-culture for 24 h.

**Expression vectors, site-directed mutagenesis and cell transfection**

Full-length human ADAM12L and ADAM12S cDNAs (GenBank Accession Nos NM_003474 and NM_021641.3) in the pCMV6-AC mammalian expression construct backbone were purchased from Origene (Rockville, USA). Expression constructs pCMV6-AC and pCMV6-AC-GFP (green fluorescence protein) served as controls (Origene). Stable transfections were performed to establish multiclonal HTR8/SVNeo and JEG3 cells constitutively expressing ADAM12L and ADAM12S. Two micrograms per milliliter of plasmid DNA was transfected using Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer’s protocol. Selection began 48 h post-transfection using 400 μg/ml G418 sulfate in DEME or RPMI; after selection, cells were maintained in medium containing 100 μg/ml G418 sulfate. Transient transfection experiments were performed as above, except cultures were incubated 48 h post-transfection prior to experimentation.
Invasion and migration assays

Cell motility assays were performed using uncoated (migration) or coated (growth factor-reduced Matrigel; Invasion) Transwells fitted with Millipore membranes (BD Biosciences; 6.5 mm filters, 8 μm pore size) as described (Beristain et al., 2012). Briefly, 2 × 10⁴ cells/200 μl of DMEM or RPMI supplemented with 1% BSA were plated in the upper chambers and cultured for the indicated times. Lower chambers contained 500 μl of DMEM or RPMI supplemented with 10% FBS. For experiments using TAPI-1 (Enzo Life Sciences, Farmingdale, USA), both upper and lower chambers contained RPMI medium as described above supplemented with 25 nM TAPI-1. Cells from the upper surface of the Millipore membrane were removed by gentle swabbing, and transmigrated cells attached to the membrane were fixed in 4% paraformaldehyde and stained with eosin. The filters were rinsed with water, excised from the Transwells and mounted upside down onto glass slides. Cell invasion was determined by counting the number of stained cells in 10 randomly selected, non-overlapping fields at 100× magnification using a light microscope. Cell invasion was tested in duplicate wells, on three independent occasions.

Proliferation and apoptosis assays

Cells were seeded in triplicate at 1 × 10³ cells/well into opaque 96-well microplates containing 100 μl of DMEM or RPMI with 5% FBS. Cells were cultured for 0, 24, 48, 72 or 96 h, after which cell proliferation was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, USA).
ADAM12 promotes trophoblast invasion

ADAM12 preferentially localizes to invasive trophoblasts

Because we identified ADAM12 to be highly expressed in placental EVT columns and cultures, we next set out to examine its expression in invasive ECM-degrading EVTs derived from placental villous explants. Placental villous explant cultures recapitulate EVT column formation ex vivo and allow the examination of columnar trophoblasts differentiating into invasive trophoblast populations (Newby et al., 2005). To this end, immunohistochemistry localized ADAM12 in placental explants to invasive trophoblast populations actively degrading and invading Matrigel ECM (Fig. 2A). Immunolocalization of HLA-G and cytokeratin-7 in serial sections of placental explants confirmed that ADAM12 primarily localizes to the villous syncytiotrophoblast and to invasive HLA-G-positive EVTs.
Supporting this observation, dual immunofluorescence labeling of ADAM12 and cytokeratin-7 in placental explants show that ADAM12 preferentially localizes to trophoblast at the leading invasive edge, but is expressed at much lower levels within cellular columns (Fig. 2B). Co-localization of ADAM12 and cytokeratin-7 (Fig. 2B) confirms that cells residing at the invasive front are of trophoblast lineage. Taken together, we conclude that ADAM12 is highly expressed by invasive EVTs, suggesting that ADAM12 may play a prominent role in directing trophoblast invasion.

Figure 2. ADAM12 localizes to the invasive front of invading EVT. (A) Images of serially sectioned Matrigel-imbedded chorionic villous explant cultures stained with antibodies directed against ADAM12, HLA-G and cytokeratin-7. (B) Images of Matrigel-imbedded villous explant dual-labeled with ADAM12 (red) and cytokeratin-7 (green) antibodies; blue indicates 4′,6′-diamidino-2-phenylindole (DAPI)-stained (blue) nuclei. ‘Villi’ indicates chorionic villi within section; ‘Col’ indicates villous trophoblast column; ‘EVT’ denotes area within Matrigel where invasive trophoblast have invaded. Images are representative of six placental explants derived from distinct placentae (n = 6). Hashed box indicates magnified area. Bars = 100 μm.
ADAM12 controls trophoblast invasion

To interrogate the importance of ADAM12 in directing an invasive phenotype in trophoblasts, we silenced ADAM12 expression in the HTR8/SVneo EVT-like cell line and subjected cells to proliferation, migration and invasion assays. Four siRNAs targeting ADAM12 (A12i-5, -6, -7, -8) and an NS control siRNA were transiently transfected into HTR8/SVneo cells; ADAM12 knockdown was assayed 48 h post transfection. qPCR analysis confirmed significant knockdown of both ADAM12L and ADAM12S transcripts in A12i-5 and -8 transfected cells (Fig. 3A). Only A12i-8 routinely knocked down endogenous ADAM12 levels of either variant >50%. HTR8/SVneo cells transfected with NS control siRNA showed equivalent levels of both ADAM12S and ADAM12L when compared with mock-transfected cells (Fig. 3A). Batch variability of the polyclonal ADAM12 antibody that we had previously used to detect both long and short ADAM12 variants prevented us from detecting specific ADAM12 protein products in endogenous cell lysates derived from siRNA transfected HTR8/SVneo cells. Using another commercially available ADAM12 antibody directed against the catalytic domain (please refer to Materials and Methods), we confirmed siRNA-directed reduction of ADAM12 protein, however, only the 68 kDa band was specifically recognized (Fig. 3B). This discrepancy in observed molecular weight from the expected molecular weights (110, 90 and 68 kDa) may explain the slight discrepancies between mRNA and protein levels observed between different siRNA duplexes since ADAM12S transcripts appear to be more efficiently silenced than ADAM12L transcripts. To determine the specificity of our two most efficient ADAM12 siRNA constructs (A12i-5 and -8), we analyzed the expression levels of ADAM9, ADAM10 and ADAM17, three ADAM metalloproteinases characterized by having functional catalytic properties similar to ADAM12. While both siRNA duplexes significantly reduced ADAM12S mRNA levels in HTR8/SVneo cells, neither altered the expression of the other ADAM subtypes (Fig. 3C), demonstrating specificity of the siRNA’s used in this study.

As ADAM12 has been shown to promote cell survival and tumor growth (Kveiborg et al., 2005; Leyme et al., 2012), we first examined whether siRNA-directed loss of ADAM12 could impact cell proliferation in HTR8/SVneo trophoblasts. Figure 3D shows that neither of the two most efficient ADAM12 siRNA’s (A12i-5 or A12i-8) affected cell proliferation over 96 h; confirmation of ADAM12 knockdown up to 96 h of culture was confirmed by qPCR analysis (Supplementary data, Fig. S2). To assess whether a reduction in ADAM12 could alter cell motility, we subjected A12i-5 and A12i-8 transfected cells to Transwell migration and invasion assays. siRNA-directed loss of ADAM12 led to a significant reduction in cell invasion and a non-significant, but decreasing trend, in migration (Fig. 3E and F). These results reveal that ADAM12, endogenously expressed by invasive trophoblastic cells, regulates cell invasion.

Loss of ADAM12 blocks EVT column outgrowth

To address the importance of ADAM12 in regulating trophoblast column outgrowth and EVT invasion ex vivo, we assayed the effects of ADAM12 inhibition in Matrigel-imbedded placental explant cultures. This ex vivo model recapitulates trophoblast outgrowth and interstitial-like cell invasion that occurs in early placental development (Newby et al., 2005). Using the broad-spectrum ADAM protease inhibitor TAPI-1, we show that treatment with the ADAM inhibitor inhibits explant outgrowth over 96 h (Supplementary data, Fig. S3), indicating that catalytically active ADAM metalloproteinases play underlying roles in promoting EVT column outgrowth.

To directly examine the importance of ADAM12 in directing trophoblast column formation, we performed siRNA-directed silencing of ADAM12 in placental explants using A12i-5 or -8 siRNA duplexes. ADAM12 knockdown resulted in profound impairment in EVT column outgrowth (Fig. 4A and B). This was in contrast to EVT outgrowth in villous explants transfected with control NS siRNA, which exhibited rapid EVT outgrowth over 96 h (Fig. 4A and B). ADAM12 knockdown was shown by qPCR analysis using cDNA templates generated from placental explants; silencing of both ADAM12L and ADAM12S variants were observed (Fig. 4C). To examine if the effect of siRNA ADAM12 knockdown on EVT column outgrowth was in part due to changes in cell survival (i.e. apoptosis) or proliferation, placental explants were immuno-labeled with antibodies directed against caspase-cleaved cytokeratin-18 or the proliferative nuclear antigen Ki67; co-staining with cytokeratin-7 ensured trophoblast lineage specificity (Fig. 4D). siRNA-directed ADAM12 knockdown did not result in altered cell apoptosis within columnar or invasive EVTs, nor did it affect cell proliferation within EVT columns. These findings establish a biological role for ADAM12 in directing EVT column outgrowth through a process most likely impacting cell invasion.

ADAM12S but not ADAM12L promotes trophoblast invasion

Studies have shown roles for both ADAM12L and ADAM12S in directing invadopodia/podosome-like formations in tumor cells and fibroblasts (Stautz et al., 2010; Díaz et al., 2013). Recent work has also ascribed a pro-invasive role specifically for ADAM12S, but not the long transmembrane variant (Roy et al., 2011). To this end, we tested the importance of ADAM12L and ADAM12S in promoting motility in two trophoblast cell models: highly invasive EVT-like HTR8/SVneo cells that express moderate levels of ADAM12, and the JEG3 choriocarcinoma cell line that express low/undetectable levels of ADAM12 (Fig. 1B and C).

The structural domains of ADAM12L and ADAM12S are shown schematically in Fig. 5A. Expression of ADAM12 in HTR8/SVneo and JEG3 cells stably transfected with the cdNA expression constructs pCMV6-ADAM12L (abbreviated as pADAM12L) or pCMV6-ADAM12S (pADAM12S) is shown (Fig. 5B and Supplementary data, Fig. S4A). Cells over-expressing the ADAM12L transmembrane variant produced protein products with molecular weights that are consistent with previous reports (Leyme et al., 2012); specifically a 110 kDa band corresponding to the pro-form, a 90 kDa band corresponding to the catalytically active form and a smaller processed band ≈65 kDa were observed (Fig. 5B and Supplementary data, Fig. S4A). Two major protein bands were detected in ADAM12S expressing cells, corresponding to the unprocessed 90 kDa and catalytically active 68 kDa isoforms, respectively (Fig. 5B and Supplementary data, Fig. S4A). qPCR analysis using primers specific to either the short or long transmembrane variant further validated the identity of the ADAM12 expression constructs (Fig. 5C). As expected, the ADAM12S splice variant, but not ADAM12L, was shown to be secreted into cell culture media (Fig. 5D), while flow cytometry analysis localized ADAM12L to the
Figure 3  Reduction of ADAM12 inhibits trophoblast invasion. (A) qPCR analysis of ADAM12L and ADAM12S in HTR8/SVneo cells transfected with control (NS) or ADAM12-directed siRNA (A12i-5, -6, -7, -8) or transfection reagent alone (-). (B) Immunoblot showing protein levels of ADAM12 in cell lysates from HTR8/SVneo cells transfected with control or ADAM12-directed siRNAs. Molecular weights (kDa) are shown to the right and β-actin indicates loading control. Arrow points to ADAM12-specific band. (C) mRNA expression levels of ADAM9, ADAM10, ADAM12S and ADAM17 in HTR8/SVneo cells transfected with reagent alone (-), control siRNA (NS) or ADAM12-directed siRNAs (A12i-5 and A12i-8). (D) Line graph depicts cell proliferation over 96 h of HTR8/SVneo cells transfected with control or ADAM12 siRNA (A12i-5 and A12i-8). Proliferation assays were evaluated by the CellTiter Glo ATP-based luminescence assay performed in triplicate on three separate occasions (n = 3). Statistical significance of data was assessed using the Student’s t-test comparing ADAM12 siRNA’s to control siRNA (NS) at each timepoint. Representative images and bar graphs showing the quantification of Transwell migration (E) and invasion (F) of HTR8/SVneo cells transfected with control siRNA (NS), ADAM12-directed siRNA (A12i-5 and A12i-8) or transfection reagent alone (-). Cell invasion and migration were performed for 24 h in duplicate, and repeated on three independent occasions (n = 6). The results are presented as mean ± s.e.m. in bar graphs (*P ≤ 0.05, **P ≤ 0.01, n.s., no statistical difference; compared with NS).
outer cell membrane (Supplementary data, Fig. S4B). For controls in these studies, the empty expression construct pCMV6 or the GFP-expressing pCMV6-GFP (pGFP) construct was used.

Consistent with our previous finding that siRNA-directed loss of ADAM12 did not affect cell proliferation in HTR8/SVneo cells, overexpression of ADAM12L or ADAM12S in HTR8/SVneo cells similarly

Figure 4 ADAM12 loss prevents EVT column outgrowth. (A) Representative images of Matrigel-imbedded chorionic villi explant cultures transfected with either control siRNA (NS) or ADAM12-directed siRNAs (A12i-5 and A12i-8). Cultures were imaged 0 and 96 h post-siRNA transfection, where (B) EVT outgrowth is calculated as the difference in column length at 96 and 0 h (µm) measured from the base of each column to the invasive front at three distinct locations (hashed lines). Quantification of column length was performed using ImageJ software; inverted images of explants (invert) provide better contrast, where ‘villi’ denotes placental villi, and hashed lines represent the column base (white) and invasive extremity (black). Matrigel explant assays were performed in duplicate from six independent placenta (n = 12); bars = 200 µm. (C) qPCR analysis showing ADAM12L and ADAM12S mRNA levels in chorionic villi Matrigel explant cultures described above. Reactions were normalized to endogenous GAPDH. (D) Representative images showing proliferative (Ki67 positive; green) and apoptotic [caspase-cleaved cytokeratin-18 (K18); green] trophoblasts within EVT columns, where nuclei are stained with DAPI (blue). EVT columns are indicated as ‘column’ and cells immuno-positive for cleaved cytokeratin-18 are highlighted with arrows; bars = 100 µm. The percentages of proliferative and apoptotic cells, calculated as the number of Ki67 or cleaved cytokeratin-18 cells into numbers of trophoblasts (cytokeratin-7; red) or total cells (DAPI; blue) are shown in bar graphs (right). The results are presented as mean ± s.e.m. in bar graphs (*P ≤ 0.05, **P ≤ 0.01).
had a negligible effect in altering proliferation (Fig. 5E). In contrast, both ADAM12 variants promoted cell proliferation in the JEG3 choriocarcinoma cell line, suggesting that ADAM12L and ADAM12S may induce differential effects contingent on the transformative state of a cell (Fig. 5F). Strikingly, in Transwell motility assays, only the ADAM12S splice variant promoted HTR8/SVneo and JEG3 cell migration and invasion, while over-expression of ADAM12L neither enhanced nor decreased cell motility (Fig. 5G and H, and Supplementary data, Fig. S4C). These functional experiments demonstrate that the secreted ADAM12S splice variant is crucial for regulating trophoblast motility.
ADAM12 promotes trophoblast invasion

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Figure 6 ADAM12S-directed invasion requires intrinsic proteolytic activity. (A) Representative Transwell images and bargraphs show cell invasion of untreated (-) or TAPI-1 treated HTR8/SVneo cells stably over-expressing ADAM12S. (B) Immunoblot shows protein expression levels of ADAM12 in HTR8/SVneo cells transiently transfected with pCMV6, pCMV6-GFP (pGFP), pADAM12S or pADAM12S<sup>D</sup>E351Q expression constructs. Molecular weights (kDa) are shown to the right and β-actin indicates loading control. (C) Representative images and bargraphs show cell invasion indices of HTR8/SVneo cells transiently over-expressing the expression constructs described above. Invasion assays were performed in duplicate on three independent occasions (n = 6). The results are presented as mean ± s.e.m. (*P ≤ 0.01).
endogenous levels of ADAM12S, and therefore any effect on column outgrowth would be specifically attributed to exogenous expression. CM from cells transfected with the empty pCMV6 construct served as a control in these experiments. Figure 7A schematically describes the experimental procedure, initiating with ADAM12S/ADAM12S\textsuperscript{D\textsubscript{E351Q}} transfection, followed by CM harvesting and treatment of placental
explants and trophoblast column imaging at 0 and 72 h post-treatment. Secretion of wild-type ADAM12S and mutant ADAM12S<sub>E351Q</sub> into CM was verified by immunoblot analysis from respective JEG3 CM (Fig. 7B). Explants treated with control pCMV6-derived CM reproducibly formed EVT column outgrowths over 72 h of culture (Fig. 7C). Strikingly, treatment with CM derived from ADAM12S-expressing cells profoundly increased EVT column outgrowth whereas ADAM12S<sub>E351Q</sub> CM generated EVT outgrowths comparable with pCMV6 control CM (Fig. 7C and D). To investigate if ADAM12S CM imparted a proliferative phenotype within EVT columns, placental explants treated with either ADAM12S or pCMV6 CM were dual-labeled with antibodies recognizing the proliferative antigen Ki67 and the trophoblast-specific marker cytokeratin-7 (Supplementary data, Fig. S5). Although proliferating cells were observed in EVT columns in both ADAM12S and control CM-treated explants, we did not observe differences in the number of proliferating cells between columns. Further, Matrigel-invasive EVTs were Ki67 negative, thus providing strong evidence for a role of ADAM12S in directing interstitial EVT invasion.

**Discussion**

In this work, we demonstrate that ADAM12 localizes to invasive trophoblast populations in vivo and ex vivo, and define a role for ADAM12 in directing trophoblast invasion. The secreted ADAM12S variant was specifically shown to promote EVT invasion and column outgrowth through a mechanism requiring functional metalloprotease activity. Prior to this study, ADAM12 was known to be highly expressed in placental tissue (Glipin et al., 1998); however, we now ascribe a molecular function for this ADAM family member in directing a key cellular aspect in trophoblast biology necessary for placental development. Specifically, our work describes a new role for ADAM12 in controlling cell invasion in a normal developmental process critical in human reproduction.

ADAM metalloproteases exert diverse biological functions supported by their complex multifunctional domains. Although only select ADAMs demonstrate intrinsic catalytic activity, all share structurally conserved ancillary domains important in regulating cellular processes like cell–ECM (integrin-mediated adhesion) and cell–cell attachment (through binding to selectins and syndecans), as well as intracellular signal transduction (SH3 domains within cytoplasmic tail) (Seals and Courtneidge, 2003). Therefore, it is not surprising that ADAM subtypes play essential roles in developmental processes such as angiogenesis, neurogenesis, organ development and cancer (Reiss and Saftig, 2009). In light of the possible metalloprotease-independent mechanisms whereby ADAM12 may influence cell motility, we tested the importance of ADAM12L in controlling trophoblast invasion, as this isoform is highly expressed in the placenta. The cytoplasmic tail of ADAM12L associates with the non-receptor tyrosine kinase Src (Stautz et al., 2010) and integrin linked kinase (Leyme et al., 2012), positioning ADAM12 within podosomes and focal adhesion structures important in regulating cell motility. Our finding that ADAM12L did not promote an invasive phenotype was surprising, but not unprecedented. Roy et al. (2011) previously demonstrated that ADAM12L over-expression in breast cancer cells did not affect invasion. The biological significance of ADAM12L expression in trophoblasts remains to be fully examined; however, our finding that its expression promoted JEG3 choriocarcinoma proliferation suggests a role for ADAM12L in directing placental growth or survival. Additionally, its localization to multinucleated syncytiotrophoblast structures suggests a putative role for ADAM12L in directing trophoblast cell–cell fusion, justifying further investigation into these possible ADAM12L regulated processes.

The biological function of the truncated ADAM12S variant has primarily been examined in cancer cell biology, in part to its restricted expression in cancer cells. ADAM12S promotes tumorigenesis in a transgenic mouse model of breast cancer (Kveiborg et al., 2005) and also stimulates chondrocyte proliferation/maturation leading to enhanced bone growth (Kveiborg et al., 2006). Further, ADAM12S gene expression is elevated in malignant human breast tissue, and its over-expression in MCF-7 breast cancer cells promotes cell proliferation, migration and invasion, as well as MCF-7 tumor growth and metastasis (Roy et al., 2011). Notwithstanding ADAM12S expression in myoblasts (Galliano et al., 2000), its expression in trophoblasts is unique with respect to normal non-transformed cell types. Prior to this study, only one immunohistochemical report localized the ADAM12S variant to trophoblasts in early and late placentas, though characterization was limited to chorionic villi devoid of proliferative EVT columns (Kokozidou et al., 2011). Our finding that ADAM12S is sufficient to promote EVT column outgrowth not only provides additional perspective to the broad biological implications of ADAM12 function in the placenta, but also highlights a previously unidentified role for ADAM12S in regulating cell migration and invasion in non-transformed cells.

ADAM metalloproteases elicit many of their biological effects through cell surface ectodomain shedding of proteins, and are not generally thought to function as ECM-degrading proteinases (Reiss and Saftig, 2009). Capable of regulating the bioavailability of growth factors and growth factor receptors, as well as other cell-surface proteins, ADAMs promote or restrain cell invasion, respectively. For example, in keratinocyte wound healing, ADAM10, ADAM12 and ADAM17 regulate cell migration through HB-EGF shedding and subsequent EGFR activation (Tokumaru et al., 2000; Asakura et al., 2002). Likewise, ADAM12-mediated cleavage of delta-like 1, a Notch receptor ligand, promotes cell invasion (Sahlgren et al., 2008; Sonoshita et al., 2011). Interestingly, a recent study linking Notch activity and ADAM12 gene expression with enhanced HB-EGF/EGFR signaling in invadopodia highlights the interconnectedness of these pathways in modeling cell motility (Díaz et al., 2013). With EGFR and Notch signaling shown to be important in directing trophoblast column outgrowth, invasion and survival (Hunkapiller et al., 2011), combined with our observation that ADAM12S is highly expressed by invasive trophoblasts, it is likely that ADAM12 facilitates early placental development through cross-talk with these signaling pathways.

ADAM12S is elevated in serum of pregnant women (Saharavand et al., 2011). As insulin-like growth factor binding proteins (IGFBPs) -3 and -5 are specific substrates for ADAM12S (Shi et al., 2000), it is possible that ADAM12S is responsible for increasing the pool of bioavailable IGFII during pregnancy through IGFBP proteolysis. IGF-II plays a critical role in early placental development by regulating trophoblast function (Regnaut et al., 2002), and thus mechanisms controlling IGF signaling may very well play central roles in placentaclination. Evidence linking ADAM12 to healthy placentaclation extends from studies that have correlated lower serum levels of ADAM12S in pregnant women with preeclampsia and fetal growth restriction (Laigaard et al., 2005; 2006; Cowans and Spencer, 2007). As placentas from women with preeclampsia or fetal growth restriction are often underdeveloped and poorly vascularized, a possible interaction exists between ADAM12
levels in pregnancy and optimal placental function. Although gene targeting of ADAM12 in mice does not affect embryo viability, ADAM12 deletion does impact perinatal survival (Kurisaki et al., 2003), suggesting placental dysfunction or inadequate placentation may exist in ADAM12 knockout mice. As mice express only the long ADAM12 variant and not the short secreted isoform, it is difficult to draw direct comparisons between the biological functions and importance of human ADAM12 variants with mouse genetic studies. Our finding that ADAM12L promotes JEG3 proliferation but not EVT invasion indicates that in mice ADAM12 may direct trophoblast growth and/or homeostasis but not trophoblast motility.

In summary, we have identified a novel role for ADAM12 in directing an invasive phenotype in human trophoblasts. Showing distinct biological functions for ADAM12S and ADAM12L in directing aspects of trophoblast biology, we assign a specific role for ADAM12S in promoting cell motility and trophoblast column outgrowth in early pregnancy. These findings highlight ADAM12 as a critical molecule in human placental development. Further, as the importance of ADAM12 in the progression of specific cancers is becoming evident, insights gained from future studies examining molecular mechanisms controlling ADAM12 function in these systems may well shed light on key downstream processes controlled by ADAM12 in placental development or that are dysregulated in aberrant pregnancies.

### Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/online.

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### Authors’ roles

M.A. and A.G.B. contributed to the conception and design of experiments. M.A., S.P., K.I. and A.G.B. performed experiments and collected and analysed data. M.A. and A.G.B. conceived and designed the experiments. M.A., S.P., K.I. and A.G.B. performed experiments, collected and interpreted data. M.A., S.P., K.I. and A.G.B. have approved publication of the manuscript.

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### Conflict of interest

None declared.

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