A DISINTEGRIN AND METALLOPROTEINASE 12 (ADAM12) LOCALIZES TO INVASIVE TROPHOBLAST, PROMOTES CELL INVASION AND DIRECTS COLUMN OUTGROWTH IN EARLY PLACENTAL DEVELOPMENT

by

Mahroo Aghababaei

B.Sc., The University of Waterloo, 2011

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies

(Reproductive and Developmental Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

July 2015

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ABSTRACT

Placental development is a highly regulated process requiring signals from both fetal and maternal uterine compartments. Within this complex system, trophoblasts, placental cells of epithelial lineage, form the maternal-fetal interface controlling nutrient, gas and waste exchange. The commitment of progenitor villous cytotrophoblasts to differentiate into diverse trophoblast subsets is a fundamental process in placental development. Differentiation of trophoblasts into invasive stromal- and vascular-remodeling subtypes is essential for uterine arterial remodeling and placental function. Inadequate placentation, characterized by defects in trophoblast differentiation, may underlie the earliest cellular events driving pregnancy disorders such as preeclampsia and fetal growth restriction. Molecularly, invasive trophoblasts acquire characteristics defined by profound alterations in cell-cell and cell-matrix adhesion, cytoskeletal reorganization and production of proteolytic factors.

A Disintegrin and Metalloproteinase 12 (ADAM12) is a multifunctional protein belonging to the ADAM family of metalloproteinases. ADAM12 exists as two alternatively spliced isoforms: ADAM12L (a transmembrane form) and ADAM12S (a truncated secreted form). ADAM12 is highly expressed in the human placenta and promotes matrix remodelling, cell proliferation and invasion in tumorigenesis. Importantly, low ADAM12 serum levels have been associated with pregnancies linked to poor pregnancy outcome. In spite of this knowledge, the importance of ADAM12 in directing trophoblast biology in early placentation is poorly understood. In this thesis, I have determined that both ADAM12 isoforms are expressed in distal ends of trophoblast columns, highly-invasive matrix-degrading trophoblasts and the invasive HTR8/SVneo

trophoblastic cell line. Utilizing loss-of and gain-of-function strategies, I have demonstrated that ADAM12S plays a central role in promoting trophoblast invasion, as well as extravillous cytotrophoblast (EVT) column outgrowth through a mechanism requiring its intrinsic proteolytic activity. Moreover, I have identified cAMP signalling as the upstream regulator of ADAM12 expression and function in trophoblasts. Collectively, these findings describe a novel role for ADAM12 in directing an invasive phenotype in trophoblasts, and highlights ADAM12 as a key protease in human placental development.

PREFACE

Dr. Alexander G. Beristain generated the hypothesis for this work. Dr. Alexander G. Beristain and I designed and performed the experiments in Chapter 3 except for the FACS purification of placental cells, which was performed by Sofie Perdu. I conducted all the experiments in Chapter 4. Dr. Alexander G. Beristain, Sofie Perdu and I collected and analyzed data. This thesis was read and approved by Drs. Alexander G. Beristain, Geoffrey L. Hammond, Chinten James Lim, Dan Rurak and Wendy Robinson.

A version of chapter 1 has been published. Aghababaei, M. & Beristain, A. G. The Elsevier Trophoblast Research Award Lecture: Importance of metzincin proteases in trophoblast biology and placental development: A focus on ADAM12. *Placenta* 1–9 (2015). doi:10.1016/j.placenta.2014.12.016.

A version of chapter 3 has been published. Aghababaei, M., Perdu, S., Irvine, K. & Beristain, A G. A disintegrin and metalloproteinase 12 (ADAM12) localizes to invasive trophoblast, promotes cell invasion and directs column outgrowth in early placental development. *Mol. Hum. Reprod.* **20,** 235–49 (2014).

This thesis was conducted under ethical approval from the University of British Columbia/ Children's and Women's Health Centre of British Columbia Research Ethics Board (Certificate H1300640).

TABLE OF CONTENTS

ABSTRACT ..... **ii**

PREFACE ..... **iv**

TABLE OF CONTENTS ..... **v**

LIST OF TABLES ..... **ix**

LIST OF FIGURES ..... **x**

LIST OF ABBREVIATIONS ..... **xii**

ACKNOWLEDGEMENTS ..... **xvii**

DEDICATION ..... **xix**

CHAPTER 1. INTRODUCTION **1**

1.1 Overview 1

1.2 Implantation and placentation 3

1.3 Models to study human placentation 8

1.3.1 Animal models 8

1.3.2 *Ex vivo* and *in vitro* models 11

1.3.2.1 Villous explant cultures and primary trophoblast cells 11

1.3.2.2 Immortalized trophoblast cell lines 13

1.3.2.3 Choriocarcinoma cell lines 14

1.4 Regulation of trophoblast invasion by metzincin metalloproteinases 15

1.5 ADAMs and their role in development 17

1.5.1 Phenotypes of ADAM deficient mice 18

1.5.2 ADAMs at different stages of pregnancy 20
1.5.3 ADAMs during placental development 21
1.6 ADAM12 22
1.6.1 ADAM12-mediated regulation of cell differentiation, proliferation and motility 23
1.6.2 ADAM12 and cell fusion 24
1.6.3 ADAM12 function during neovascularization and placental development 25
1.7 cAMP 26
1.7.1 cAMP effectors 27
1.7.1.1 cAMP/PKA signalling 28
1.7.1.2 Cyclic nucleotide-gated (CNG) ion channels 29
1.7.1.3 Exchange protein directly activated by cAMP (Epac) 30
1.7.2 cAMP levels in human pregnancy 31
1.7.3 Effects of cAMP on endocrine functions of trophoblasts 31
1.7.4 Effects of cAMP on trophoblast syncytialization 32
1.7.5 Regulation of trophoblast invasion by cAMP 32
1.8 Hypothesis and rationale 33
CHAPTER 2. MATERIALS AND METHODS 39
2.1 Placental tissue collection 39
2.2 Cell and tissue culture 39
2.3 Placental villous explant culture 40
2.4 Reagents and antibodies 41
2.5 RNA preparation and real-time PCR 42

2.6 Immunoblotting 43
2.7 ADAM12 siRNA transfection 44
2.8 Expression constructs and plasmid DNA transfection 45
2.9 Cell viability analysis 46
2.10 Immunohistochemistry and immunofluorescence 47
2.11 FACS purification of placental cells 48
2.12 Invasion and migration assays 49
2.13 Dual luciferase reporter assay 50
2.14 Statistical analysis 50

CHAPTER 3. CHARACTERIZING THE FUNCTIONAL IMPORTANCE OF ADAM12 IN DIRECTING TROPHOBLAST INVASION AND EVT COLUMN FORMATION 54

3.1 Introduction 54
3.2 Results 55
3.2.1 ADAM12 localizes to the multinucleated syncytiotrophoblast and EVT anchoring columns in first trimester human placenta 55
3.2.2 ADAM12 preferentially localizes to invasive ECM-degrading EVTs 57
3.2.3 ADAM12 is preferentially expressed by invasive trophoblasts 57
3.2.4 Loss of ADAM12 inhibits trophoblast invasion 58
3.2.5 ADAM12 promotes EVT column outgrowth 59
3.2.6 ADAM12S, but not ADAM12L, promotes trophoblast invasion 60
3.2.7 ADAM12S's intrinsic metalloproteinase activity is necessary in directing trophoblast invasion 61

3.2.8 Secreted ADAM12S variant is sufficient to direct EVT column outgrowth ... 62

3.3 Discussion ... 82

CHAPTER 4. IDENTIFYING THE MOLECULAR MECHANISM REGULATING ADAM12 EXPRESSION AND FUNCTION ... 87

4.1 Introduction ... 87

4.2 Results ... 88

4.2.1 cAMP treatment induces *ADAM12* expression by regulating its promoter activity ... 88

4.2.2 cAMP regulates ADAM12 expression through a PKA-independent mechanism ... 89

4.2.3 ADAM12 mRNA but not protein expression is regulated by Epac signalling ... 91

4.2.4 cAMP treatment promotes HTR8/SVneo cells migration ... 91

4.2.5 cAMP-induced cell migration is partially blocked by ADAM12 knockdown 92

4.3 Discussion ... 102

CHAPTER 5. GENERAL DISCUSSION, SUMMARY OF RESULTS AND CONCLUSIONS ... 105

5.1 General discussion ... 105

5.2 Summary of results and future directions ... 108

5.3 Conclusions ... 110

REFERENCES ... 113

LIST OF TABLES

Table 2.1 List of all the antibodies used in this thesis..... 42

Table 2.2 List of the accession number and the target sequence for each ADAM12-directed siRNA..... 45

LIST OF FIGURES

Figure 1.1 Schematic diagram of trophoblast differentiation 37

Figure 1.2 Structure of human ADAM12L and ADAM12S isoforms 38

Figure 2.1 Primary EVTs cultured on a collagen-coated plate 51

Figure 2.2 Primary EVTs are positive for Cytokeratin-7..... 51

Figure 2.3 Light microscope image of a first trimester chorionic villous explant imbedded on Matrigel 52

Figure 2.4 Schematic diagram of the 1237-bp promoter fragment of the ADAM12 gene 53

Figure 3.1 ADAM12 localizes to the multinucleated syncytiotrophoblast layer and EVT anchoring columns in first trimester placental villi 64

Figure 3.2 ADAM12 is preferentially expressed by invasive ECM-degrading EVTs 66

Figure 3.3 ADAM12 is preferentially expressed by invasive trophoblasts 67

Figure 3.4 Loss of ADAM12 inhibits trophoblast invasion 69

Figure 3.5 ADAM12 inhibition or loss prevents EVT column outgrowth 73

Figure 3.6 ADAM12S, but not ADAM12L, promotes trophoblast invasion 76

Figure 3.7 ADAM12S' intrinsic metalloproteinase activity is necessary in directing trophoblast invasion 78

Figure 3.8 Secreted ADAM12S variant is sufficient in directing EVT column outgrowth 81

Figure 4.1 *ADAM12* expression and promoter activity are regulated by cAMP 94

Figure 4.2 cAMP regulates ADAM12 *expression* through a PKA-independent mechanism 96

Figure 4.3 cAMP promotes *ADAM12* expression through an Epac-independent mechanism 98

Figure 4.4 An increase in the intracellular cAMP level promotes trophoblast migration 99

Figure 4.5 ADAM12 knockdown abolishes cAMP-induced trophoblast migration 101

Figure 5.1 Schematic diagram demonstrating ADAM12 localization in human placental villi 112

LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
ADAM	A Disintegrin and Metalloproteinase
ADAMTS	A Disintegrin and Metalloproteinase with ThromboSpondin motif
AKAP	A kinase Anchoring Proteins
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
C	Catalytic
CaM	Calmodulin
cAMP	Cyclic adenosine 3′-5′-monophosphate
Cdx2	Caudal type homeobox 2
cEVT	Columnar extravillous cytotrophoblasts
cGMP	Cyclic guanosine monophosphate
CNG	Cyclic nucleotide-gated
CNS	Central nervous system
CRE	cAMP response element
Ct	Cytoplasmic tail
Cys	Cysteine-rich region
DAPI	4’, 6-Diamidino-2-Phenylindole
DIS	Disintegrin domain
DLL1	Delta like-1
EC	Endothelial cells

Ecad	E-cadherin
ECM	Extracellular matrix
eEVT	Endovascular extravillous cytotrophoblasts
EG	Endometrial gland
EGF	EGF- like domain
Eomes	Eomesodermin
Epac	Exchange protein directly activated by cyclic AMP
ER	Endoplasmic reticulum
ErbB	Erb-b2 receptor tyrosine kinase
EVT	Extravillous cytotrophoblast
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
Flk-1	Fetal liver kinase 1
FSK	Forskolin
G protein	Guanine nucleotide binding protein
GCM1	Glial cell missing homolog 1
GEF	Guanine nucleotide exchange factor
GLuc	Gaussia luciferase
GnRH	Gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
Hand1	Heart and neural crest derivatives expressed 1

HB-EGF	Heparin-binding EGF-like growth factor
HBSS	Hanks Balanced Salt Solution
hCG	Human chorionic gonadotropin
HGF	Hepatocyte growth factor
HLA-G	Major histocompatibility complex, class I, G
HNSCC	Head and neck squamous cell carcinoma
hPL	Human placental lactogen
hPMSC	Human placental multipotent mesenchymal stromal cells
HRP	Horseradish peroxidase
hTERT	Human telomerase reverse transcriptase
IBMX	3- isobutyl-1-methylxanthine
iEVT	Interstitial extravillous cytotrophoblasts
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
ILK	Integrin-linked kinase
IUGR	Intrauterine growth restriction
JNK	c-Jun N-terminal kinase
K7	Cytokeratin-7
LIF	Leukemia inhibitory factor
MAP1A	Microtubule-associated protein 1A
MAPK	Mitogen-activated protein kinase
MAS	Membrane- anchored substrate

MC	Mesenchymal core
MCAM	Melanoma cell adhesion molecule
MEK	Mitogen-activated protein kinase kinase
MMP	Matrix metalloproteinase
MP	Metalloproteinase Domain
mSA	Maternal spiral artery
MSK1	Mitogen- and stress-activated protein kinase-1
MUC	Mucin
MΦ	Macrophages
NES	Nuclear export signal
NF-κB	Nuclear factor-kappa B
P-LAP	Placental leucine aminopeptidase
PACSIN3	Protein Kinase C and Casein Kinase Substrate In Neurons
PAPP-A	Pregnancy-associated plasma protein-A
PBS	Phosphate-buffered saline
PDE	Phosphodieserase
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKI	Protein kinase inhibitor
R	Regulatory
Rap	Ras-associated protein
ROCK2	Rho-associated protein kinase 2

SEAP	Secreted alkaline phosphatase
siRNA	Small interfering RNAs
SS	Secreted substrate
Stra13	Stimulated by retinoic acid 13
SV40	Simian Virus 40
SVMP	Snake Venom Metalloproteinase
synCT	Syncytiotrophoblast
TACE	Tumor necrosis factor-α-converting enzyme
TGC	Trophoblast giant cell
TGF- β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinase
TM	Transmembrane domain
TNF-α	Tumor necrosis factor-α
uNK	Uterine Natural Killer cell
uPA	Urokinase plasminogen activators
VCAM-1	Vascular cell adhesion molecule 1
vCT	Villous cytotrophoblast
VE-cadherin	Vascular endothelial cadherin

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, Dr. Alexander G. Beristain, for his continuous support, encouragement and guidance throughout my MSc studies. To my supervisory committee members, Drs. Sai Ma, Geoffrey L. Hammond and Chinten James Lim, I am extremely grateful for all your guidance and insightful suggestions over the past few years. To Mrs. Roshni Nair, I cannot thank you enough for always being so supportive, caring and kind.

To all the current and former members of Beristain Lab: Kimberley Irvine, sofie Perdu, Yoona Kim, Lauren De Leuca and Drs. Hoa Le and Barbara Castellana, thank you so much for all your help along the way. I will always remember the times we spent together. To all my amazing friends and colleagues at the Child and Family Research Institute (CFRI), you have made my stay at CFRI/UBC unforgettable, thank you!

I would like to extend my deepest gratitude to my family and friends for their unconditional love and endless support. To my Mom and Dad, thank you for believing in me and for allowing me to peruse my goals. You have always told me to aim high and work hard. Without you, I would not be the person I am today. I am incredibly fortunate to have you as my parents.

To my beloved sister, Mahvand and my brother-in-law, Amir, thank you for supporting me since the first day I decided to move to Canada. Words cannot express how much your love has meant to me. To my nephews, Reza and Ryan, you are the sunshine of my life. Just the thought of you is enough to make me smile and happy. To my dearest friends Negin, Oranous, Sima and Simin, your love, support and friendship means the world to me, thank you!

And finally, I wish to express a sincere thank you to all the women who participated in our study, this project could not have been completed without you.

Dedicated to my beloved parents, Effat and Ahmad ,

who have always loved me unconditionally!

تقدیم به پدر و مادر عزیزم،

به آنان که وجودم جز هدیه وجودشان نیست...

CHAPTER 1. INTRODUCTION

1.1 Overview

Human reproduction is an inefficient process where the average fertility rate is about 20% per reproductive cycle with a further 30% chance of embryonic loss once conception is achieved [1–4]. Although chromosomal abnormalities and aberrant embryo implantation are considered to be the major causes of early pregnancy failures, improper placentation can also affect both the mother and fetus, resulting in poor pregnancy outcomes. Therefore, successful establishment of the placenta is one of the pivotal events in early pregnancy, bridging maternal and fetal circulatory systems. Cellular interactions between fetal trophoblasts (placental cells of epithelial lineage), maternal stromal (tissue-supporting) and immune cells coordinate placental development [5]. In addition to facilitating fetal-maternal attachment to the uterine wall, the placenta functions as the key endocrine organ during pregnancy and regulates physiological exchange of gases and nutrients between the fetus and mother [5]. Critically, the placenta also acts as an immunological barrier as well as an immunomodulator, instructing maternal tolerance towards fetal tissue [6,7].

The human placenta is hemochorial, meaning the epithelial cells of the placenta (called trophoblasts) directly interact with maternal blood [8]. In addition to humans, hemochorial placentation is also observed in other species, including most rodents, rabbits, bats, hedgehogs, monkeys and apes [9]. Although sharing structural similarities with other types of hemochorial placentae, the human placenta represents the most invasive example of placentation, characterized by extensive infiltration of trophoblasts

into uterine arteries and stroma; these invasive characteristics are thought to be essential for optimal/healthy placental function [8]. Despite strong indications that pregnancy disorders are linked with abnormal placental development, little is known about the underlying molecular events affecting trophoblast differentiation into invasive cell subsets in these conditions.

Numerous studies have shown that cancer cell invasion and trophoblast invasion share similar biochemical pathways, although the latter is stringently controlled. Among the major molecular candidates regulating trophoblast and cancer cell invasion are the gene members of Metzincin family of metalloproteinases including matrix metalloproteinases (MMPs) and A Disintegrin and Metalloproteinases (ADAMs). Indeed, extracellular matrix (ECM) degrading MMPs are the most widely studied Metzincins in trophoblast biology where they have been shown to control trophoblast differentiation into invasive cell subsets. However, very little knowledge exists regarding the importance of ADAM family of proteases in trophoblast biology. The overall objective of this thesis is to elucidate the functional importance of the ADAM protease, ADAM12, in promoting trophoblast differentiation along the invasive extravillous cytotrophoblast (EVT) pathway. Moreover, work presented in this thesis identifies an upstream signalling pathway controlling ADAM12 expression and function.

The following introduction will present the current knowledge regarding human implantation and placentation, as well as the *in vivo, ex vivo and in vitro* models available to study placental development and trophoblast differentiation. Moreover, I will describe the significance of Metzincin metalloproteinases in regulating trophoblast differentiation along the invasive pathway. The importance of ADAMs, and in particular ADAM12, in

directing diverse cellular processes important in placental development will then be addressed. Lastly, the involvement of cAMP signalling in regulating trophoblast biology will be reviewed, with particular emphasis on the role of cAMP in mediating the expression of genes associated with trophoblast differentiation.

1.2 Implantation and placentation

Following fertilization, the one-cell zygote undergoes successive mitotic divisions during passage along the oviduct and within the uterine cavity; at this stage of development the embryonic mass is referred to as the blastocyst [10]. The blastocyst consists of two populations of cells: an outer epithelial layer (known as the trophectoderm) which gives rise to the cellular units of the placenta, and the inner cell mass, which differentiates into multiple cell lineages that together make up the embryo [11]. Successful implantation depends on synchronized cross-talk between a receptive endometrial epithelium and a competent blastocyst. The period during which the endometrium is conductive to embryo implantation is called the "window of implantation" [12]. The cellular changes that occur before and during this time include increased uterine vascular permeability, angiogenesis and enlargement and differentiation of stromal fibroblasts into highly secretory decidual cells [12]. Decidualized stromal cells express numerous growth factors and cytokines necessary for early blastocyst development and pregnancy maintenance. It has been shown that both progesterone and estrogen regulate decidualization and uterine neovascularization; however, the exact mechanism of action of these steroid hormones remains elusive [13,14]. Concurrent with the decidual reaction, a massive leukocyte infiltration takes place, which optimizes uterine

angiogenesis and maintains maternal-fetal tolerance by promoting the secretion of angiogenic and immunosuppressive factors [15–17].

Another characteristic indicative of a receptive endometrium is the appearance of cellular protrusions known as pinopodes on the apical surface of the endometrial epithelium [10]. Although the exact function of pinopodes during human implantation remains to be fully elucidated, it has been suggested that the human blastocyst adheres preferentially to pinopode-presenting endometrial cells *in vitro* [18].

The process of embryo implantation is classified into three stages: apposition, adhesion and invasion. During the apposition stage, the outer trophectodermal layer of the blastocyst becomes closely apposed to the epithelial lining of the endometrium, resulting in an unstable adhesion of the blastocyst to the endometrial epithelium. This is followed by a much stronger adhesion of the blastocyst to the luminal epithelial cells. In the subsequent stage, trophoblast cells continue to proliferate and form a primitive multinucleated syncytium, which invades the luminal epithelium and the underlying stroma. The invasion process continues until the entire blastocyst is embedded within the endometrium [10].

The three phases of embryo implantation are regulated by a variety of cell adhesion molecules, growth factors, cytokines and hormones produced by both maternal and fetal cells. Among these factors, selectins and integrin families of adhesion receptors and their ligands are considered to mediate the initial blastocyst-uterine interaction [10,19]. Specifically, the binding of L-selectin receptors expressed on trophoblasts to their oligosaccharide ligands expressed on uterine luminal epithelial cells plays a crucial role during apposition [20]. Following the initial contact mediated by L-selectin, subsets of

integrin cell adhesion molecules, such as α1β1, α4β1 and αvβ3 initiate a stronger attachment between the blastocyst and endometrial lining [21,22]. Among several cytokines and growth factors studied in humans, leukemia inhibitory factor (LIF), Interleukin-11 (IL-11) and the heparin-binding EGF-like growth factor/ Erb-b2 receptor tyrosine kinase 4 (HB-EGF/ErbB4) complex play key roles during pre-implantation and implantation, where aberrant expression of these factors is linked to unexplained recurrent miscarriage and infertility [23–25].

Shortly after blastocyst implantation, establishment of the placenta begins. During placental development, progenitor cytotrophoblasts residing within the trophectodermal layer differentiate along one of two cellular pathways: (i) the villous pathway or (ii) the invasive pathway [26] (Figure 1.1A and B). These two distinct differentiation events give rise to all the diverse subsets of trophoblasts that make up the functioning placenta. It is widely accepted that first trimester cytotrophoblasts are bipotential progenitor cells capable of differentiating along either one of the two above-mentioned pathways [27]. However, there are several lines of evidence suggesting the existence of two distinct cytotrophoblast progenitor populations by 8 weeks of gestation. This implies that trophoblast differentiation into specialized subsets is controlled via a more complex cellular hierarchy than what was previously thought [28,29].

Mitotically active villous cytotrophoblasts (vCTs) differentiating along the villous pathway undergo a process of cell-cell fusion with neighboring cells to form a multinucleated syncytial structure called the syncytiotrophoblast (synCT) (Figure 1.1B). The synCT is mitotically inactive, and therefore this external cellular layer is maintained by continuous fusion of underlying vCTs with overlying synCT [29].

The synCT is essential for pregnancy as it facilitates nutrient and gas exchange between maternal and fetal circulations and is also the primary source of most growth factors, cytokines and hormones required for pregnancy maintenance [26]. Recent studies have identified the transcription factor glial cell missing homolog 1 (GCM1) and two of its transcriptional targets, the retroviral envelope proteins Syncytin-1 and -2, to be the principal molecular mediators controlling trophoblast fusion [30–32]. The importance of these molecular factors in placental function is highlighted by the observation that their expression levels are significantly reduced in placentas from preeclamptic pregnancies, suggesting that inadequate placental function, in part characterized by aberrant syncytial function, is linked to severe complications of pregnancy [33,34].

Differentiation along the invasive pathway initiates at specific locations of placental attachment to the uterine wall. At these areas of contact, proliferating vCTs breach the syncytial layer forming multicellular columns of trophoblasts that are now termed columnar extravillous cytotrophoblasts (EVTs); these cells physically anchor the placenta to maternal endometrial tissue [26] (Figure 1.1A and B). EVTs residing within distal portions of anchoring columns give rise to specialized trophoblasts that invade into and remodel maternal uterine stroma and spiral arteries. It is noteworthy that besides fetal-factors, factors derived from maternal leukocytes (i.e. macrophages and uterine natural killer (uNK) cells) and endometrial gland secretions strictly regulate EVT differentiation and function [17,35,36]. EVTs that migrate into spiral arterials are called endovascular EVTs while EVTs that invade into endometrial stroma are collectively referred to as interstitial EVTs [26] (Figure 1.1B and C). Invasion continues as far as the inner third of the myometrium where interstitial EVTs either differentiate into trophoblast

giant cells (TGCs) or undergo apoptosis, where either of these cellular events limits excessive trophoblast invasion [37]. The exact function of TGCs, which appear as large multinucleated aggregates, remains unknown. However, evidence suggests that they are responsible for local production of pregnancy-specific hormones, such as human placental lactogen (hPL) and human chorionic gonadotropin (α-hCG), and may therefore play roles in pregnancy maintenance [37].

Trophoblast-directed remodeling of endometrial arteries, characterized by disorganization and erosion of vascular smooth muscle cells, infiltration of arterial walls by endovascular EVTs, replacement of endothelial cells by trophoblasts and replacement of elastic fibers by fibrinoid material, ultimately leads to loss of vascular contractility [8]. This is a key event in early pregnancy that results in increased and continuous blood flow towards the intervillous space (the site of oxygen and nutrient exchange) of the placenta [8]. Inadequate or incomplete arterial remodeling is tightly associated with placental insufficiencies linked to pregnancy disorders such as preeclampsia and intrauterine growth restriction (IUGR), and may be the earliest cellular dysfunctions leading to their development [26].

The remodeling of maternal tissues by invasive EVTs involves molecular factors important in controlling cell-matrix (integrins) and cell-cell (cadherins) interactions, as well as proteases important in regulating cell-cell or cell-matrix adhesion turnover and ECM degradation [38]. Spatial and temporal control of protease expression patterns is crucial for successful placentation.

1.3 Models to study human placentation

The process of human implantation and early placental development remains the so-called "black box" of reproduction since access to human samples is extremely limited for obvious ethical reasons. However, several *in vivo* animal models and *in vitro* tissue/cell culture systems have been developed to uncover the cellular and molecular events that take place during the earliest stages of pregnancy.

1.3.1 Animal models

Even though no animal model faithfully recapitulates human pregnancy, animal models can provide useful information regarding key maternal-fetal interactions. Rodents such as mice and rats have long been used as experimental models due to their small size, high fecundity, short gestation period and low housing costs [39]. Indeed, studies using transgenic and tissue-specific conditional knockout mice have significantly contributed to our understandings of key genes and signalling pathways involved in trophoblast differentiation and trophoblast stem cell maintenance. For instance, rodent models have shown that the fibroblast growth factor (FGF) signalling pathway is required for maintaining the trophoblast stem cell niche [5]. Specifically, pre-implantation embryonic lethality, caused by defects in trophectoderm differentiation and trophoblast stem cell formation, have been observed in mice carrying mutations in FGF4/Fgfr2 proteins or the key downstream targets of FGF signalling, namely transcription factors caudal type homeobox 2 (Cdx2) and eomesodermin (Eomes) [40–44]. Conversely, trophoblast differentiation in mice is associated with suppression of FGF signalling and elevation in the activity of the transcription factor GCM1 and several basic helix-loop-helix

transcription factors such as Hand1 and Stra13 [45]. One of the main conclusions obtained from these studies is that different molecular pathways are involved in the maintenance of trophoblast stem cells and the formation of differentiated trophoblast subtypes. Whether these molecular pathways are evolutionary conserved and can be applied to human placental development require further investigation. So far, gene expression studies have identified human homologues for several above-mentioned mouse genes including Hand1 and Stra13 [46,47], however, it is still unknown if aberrant expression of these proteins is associated with human pregnancy complications. There is only one study suggesting a correlation between reduced GCM1 expression and preeclampsia [34].

Even though rodents (mice and rats) and humans share structurally related hemochorial placentae, fundamental differences exist limiting the efficacy of rodents as experimental models for human implantation and placentation. In both mice and rats, the process of decidualization is blastocyst-dependent and occurs post-implantation, whereas in humans this process occurs spontaneously during the late secretory phase of the menstrual cycle [48]. Moreover, in mice and rats, the area of physiological exchange is called the labyrinth, which is analogous to the human placental villous tree. However, three trophoblast layers separate maternal and fetal circulations within the labyrinth of rodents placentas, where only the synCT layer forms the human placental barrier; trophoblast organization into labyrinthine structures in rodents is quite distinct to that of the human villous tree [5].

Mice have proven to be invaluable model organisms for studying the biology and regulation of decidual lymphocytes shown to regulate trophoblast function, particularly uNK cells [49]. However, maternal immunological response to pregnancy seems to be

slightly different between these species and humans [50,51]. For instance, while human uNK cells appear during the late secretory phase of every menstrual cycle, mouse uNK cells only appear after blastocyst implantation [52,53]. There also seems to be much higher levels of B cells in the mouse decidua than in human decidua [54,55]. Studies have also shown that trophoblast invasion is fairly shallow in mice and is limited to the decidua [56]. Interestingly, it has been suggested that rats might by the most appropriate rodent model for the studies of trophoblast invasion and spiral artery remodelling since the depth of trophoblast invasion in this species is similar to that observed in humans [57]. One of the main differences between rats and humans, which limits their usefulness as models to study human placentation, is the number of spiral arteries delivering maternal blood to the placenta. While there are only three spiral arteries in rats, maternal blood is delivered to the human placenta through approximately 80-100 spiral arteries [8,58]. Considering all these variations, it is quite challenging to extrapolate and generalize findings obtained from rodent models to human pregnancy.

Experimental studies on domestic ruminants such as sheep have provided valuable information regarding fetal physiology and placental gas exchange [59]. However, unlike humans, the sheep has the least invasive, epitheliochorial type of placentation, in which maternal tissue is completely intact and does not directly interact with fetal trophoblasts [60]. Therefore, the sheep is not considered as a useful model of human placentation.

From a phylogenetic standpoint, non-human primates are the most appropriate models to study human pregnancy. In particular, all of the great apes possess hemochorial placentas characterized by deep trophoblast invasion and extensive vascular remodeling

[9,61]. Trophoblast invasion and spiral artery transformation have also been observed in several Old World monkeys such as baboons and macaques [62,63]. Given the extensive similarities between the placenta of human and non-human primates, studies on these species significantly advance our understanding of human pregnancy. However, the ethical concerns regarding the use of primates are much greater than any other animal models mainly because of their genetic closeness to humans and endangered status. Moreover, primates have a much slower rate of breeding compared to most other animal models and studies involving these species are extremely costly.

1.3.2 *Ex vivo* and *in vitro* models

Besides animal models, a wide range of *in vitro* and *ex vivo* tissue/cell culture systems has been developed to examine the process of human pregnancy. Human placental tissues and trophoblastic cell lines have been widely used to investigate the mechanisms involved in trophoblast differentiation.

1.3.2.1 Villous explant cultures and primary trophoblast cells

Several protocols have been developed for the isolation of mononuclear cytotrophoblast cells from human placentae by enzymatic digestion, density gradient centrifugation and immuno-selection [64,65]. Isolated term trophoblast cells have been shown to spontaneously differentiate and form multinucleated syncytiotrophoblast in culture [64]. This feature has allowed researchers to discover several signalling pathways involved in trophoblast differentiation and syncytium formation such as cAMP and Wnt

signalling pathways [66,67]. A major drawback in using these cultures is that they can only be used for short-term experiments since they lose their proliferative capacity in culture.

To investigate the underlying mechanisms regulating the early stages of trophoblast differentiation, studies have been performed using first trimester placentae obtained from elective terminations of pregnancy. Villous explant cultures established from these samples mimic the early stages of human placentation including EVT column formation and outgrowth, making them suitable models to study the signalling pathways regulating EVT biology and function [68]. Upon culturing villous tissue on a matrix-coated culture plates, densely packed trophoblasts expand from EVT columns and differentiate along the invasive pathway and acquire an ECM-degrading phenotype while expressing EVT-specific markers such as HLA-G and integrin $\alpha 1\beta 1$ [69]. These cultures have been extensively used to examine the effects of oxygen tension on human trophoblast survival, function and differentiation. Unlike primary cytotrophoblasts, which can be stored frozen for later use, explant cultures cannot be stored and explants should be established immediately after tissue harvesting. This is probably one of the main disadvantages of using these cultures. Moreover, explant cultures are more difficult to genetically manipulate.

Isolated first trimester trophoblasts are appropriate primary cell models when studying trophoblast migration and invasion. Upon culturing these cells on a matrix-coated plate, they differentiate into invasive trophoblasts expressing EVT-specific markers (i.e. HLA-G) as well as gelatin-degrading proteases MMP2 and MMP9 [70]. These cultures have been used for blocking the expression or function of different genes by means of different strategies, such as the use of small interfering RNAs (siRNAs) and

small molecule inhibitors. Interestingly, co-culture of primary EVTs with spiral arteries have proven to be a useful model for studying endovascular trophoblast invasion [71]. However, maintaining primary cultures of trophoblasts is quite challenging due to their limited lifespan, progression to senescence, and unwanted culture contamination by other placental cells, particularly fibroblasts [72].

1.3.2.2 Immortalized trophoblast cell lines

Several immortalized trophoblast cell lines have been established by transfecting first trimester placentae with Simian Virus 40 (SV40) or human telomerase reverse transcriptase (hTERT). Among them, HTR8/SVneo, SGHPL-4, SGHPL-5 and Swan 71 cell lines are widely used to investigate the key molecular mechanisms regulating EVT migration and invasion [73–76]. HTR8/SVneo cells exhibit an unlimited lifespan and express some trophoblast specific markers such as cytokeratin-7 and hCG, however, these cells do not express the EVT-specific marker HLA-G under unstimulated conditions, however, HTR8/SVneo cells can be induced to express HLA-G when grown on Matrigel [76,77]. Despite the differences in gene expression profiles of HTR8/SVneo cells and primary EVTs, HTR8/SVneo cells possess several characteristics associated with primary EVTs, including their ability to invade the Matrigel by up-regulating the expression of integrin α1 as well as several proteases such as MMP2 [76,77].

SGHPL-4 and -5 cells are EVT-derived cell lines, which are commonly used until passage 25 while maintaining the expression of HLA-G, hPL and integrin subunits α1, α3 and β1 [38,73,78]. The recently established Swan 71 cells are also positive for cytokeratin-

7 and HLA-G, however, unlike SGHPL group of cells, they can be maintained in culture for over 100 passages without undergoing cellular senescence [75].

Loss-of-function and gain-of-function experiments performed on these cell lines have provided valuable insights into the function of various genes during trophoblast proliferation and differentiation. Although the above-mentioned cell lines have been widely used as surrogates for invasive EVTs, it should be kept in mind that extending cellular lifespan may lead to altered patterns of gene expression not observed in mitotically inactive EVTs *in vivo* or in primary trophoblast cultures. Indeed, DNA microarray analyses have suggested that there is a significant difference between the gene expression profiles of these cell lines and primary EVTs, suggesting that experiments performed on these cells should be verified in primary trophoblast cultures [79].

1.3.2.3 Choriocarcinoma cell lines

Choriocarcinoma-derived cell lines such as BeWo, JEG3 and JAR have long been considered as useful in vitro model systems for studying trophoblast biology.
The BeWo cell line is the first established choriocarcinoma cell line, possessing several characteristics of the mononuclear vCTs, including cytokeratin-7 and integrin α6 expression [78]. Moreover, these cells are capable of producing several steroids and pregnancy specific hormones such as hCG and progesterone [80]. BeWo cells have been widely selected as an in vitro cell culture model to study the process of trophoblast syncytialization since they can be induced to differentiate into multinucleated syncytial–like structures via cAMP treatment [81].

The highly proliferative JEG3 cell line retains several features associated with

syncytiotrophoblasts such as hCG, hPL and progesterone production [82,83]. However, these cells also express HLA-G and they have been considered as a model of columnar EVTs [84]. Although JEG3 cells are not spontaneously invasive, they have been used to examine the functions of placental hormones and their effects on trophoblast invasion.

JAR cells possess many characteristics of vCTs, such as cytokeratin-7 and integrin α6 expression, while lacking the expression of EVT-specific marker, HLA-G [78]. Similar to BeWo choriocarcinoma cell line, JAR cells can differentiate into multinuclear cells and secrete a variety of steroids and peptide hormones [85]. Compared to the above-mentioned choriocarcinoma cell lines, JAR cells are more aggressive and they constitutively synthesize a broad range of ECM degrading enzymes [86,87]. Moreover, the ability to differentiate into multicellular spheroids makes JAR cells a suitable model for studying blastocyst implantation *in vitro* [88].

Clearly trophoblast cell lines are important tools for placental research, and indeed they have provided invaluable insights regarding trophoblast biology. However, it should be kept in mind that similar to immortalized trophoblasts, choriocarcinoma cells do not truly recapitulate primary trophoblast populations, and results obtained from these studies should be interpreted with caution.

1.4 Regulation of trophoblast invasion by metzincin metalloproteinases

Similar to the mechanism by which cancer cells spread from their site of origin and invade to surrounding tissues, invasive trophoblasts penetrate into decidual stroma aided in part through mechanisms controlling ECM proteolysis. Among the best-characterized family of proteases regulating trophoblast invasion are members of the

metzincin metalloproteinase gene family. Metzincins, belonging to the largest class of proteases, are aptly named due to a conserved zinc-binding motif (HEXXHXXGXXH) within their catalytic active site [89]. Three histidine residues residing within the metzincin catalytic domain serve as zinc ligands while a glutamic acid residue functions as a catalytic base during hydrolysis [89]. All members of the metzincin family have a conserved methionine residue within their 1,4-β-turn located downstream of the zinc-binding active site [89], which is fundamental for the catalytic function and structural integrity of metzincins [90–92].

The metzincin superfamily can be further classified into astacins, serralysins, matrixins, pappalysins, adamalysins, snapalysins and leishmanolysins [89]. To date, the matrixins, which comprise the matrix metalloproteinases (MMPs), are known to be instrumental in trophoblast invasion. Almost all members of human MMP gene family and their endogenous inhibitors, tissue inhibitor of metalloproteinases (TIMPs), are expressed by invasive trophoblasts [93]. In particular, MMP2 and MMP9, commonly referred to as gelatinases, and TIMP-1, -2, -3, and -4 direct cell invasion by controlling remodeling of multiple ECM proteins [94–96]. The importance of MMPs in placentation was recently demonstrated by Plaks *et al*. who showed that MMP9 deficient mice exhibit abnormal placental development and trophoblast differentiation, and also harbor clinical features resembling preeclampsia [97].

Pregnancy-associated plasma protein-A (PAPP-A), a secreted protease belonging to the pappalysin subfamily of metzincins, is highly expressed in the human placenta and localizes to diverse trophoblast subtypes [98]. Its expression is easily detectable in maternal serum, and interestingly PAPP-A serum levels tightly correlate with poor pregnancy

outcomes, where low levels associate with preeclampsia [98,99]. PAPP-A promotes trophoblast invasion through cleavage of its substrates insulin-like growth factor binding proteins (IGFBP)-4 and -5, leading to increased IGF bioavailability and pro-survival/pro-growth IGF signalling [100,101].

While the significance of MMPs in EVT biology has been an intense focus over the past decade, the importance of Adamalysin proteases is slowly being recognized. For example, A Disintegrin and Metalloproteinase with ThromboSpondin motifs-12 (ADAMTS-12), was shown to regulate trophoblast differentiation along the invasive pathway via a proteolytic independent mechanism that necessitated integrin-ECM mediated interaction [102]. Additionally, the importance of the ADAM family of proteases in regulating trophoblast biology has recently been investigated. Their ability to affect multiple cellular processes central to trophoblast differentiation along the invasive pathway positions ADAMs as attractive molecular candidates in regulating placental development [103].

1.5 ADAMs and their role in development

ADAMs are multifunctional proteins, which, together with ADAMTSs and Snake Venom Metalloproteinases (SVMPs), constitute the Adamalysin family of zinc-dependent proteinases [103]. ADAMs are proteins of about 750 amino acids long and are structurally characterized by an N-terminal signal sequence followed by a prodomain, a metalloproteinase domain, a disintegrin domain with a cysteine-rich region, an EGF domain, a transmembrane domain and a cytoplasmic tail [103]. Forty ADAM family members have been identified in the mammalian genome, where of these, only 21

ADAMs have been described in humans [103]. Although most ADAM family members are expressed as membrane-bound proteins, select members are expressed as multiple splice variants consisting of both membrane bound and secreted isoforms [103]. Only 12 human ADAMs (ADAM8, 9, 10, 12, 15, 17, 19, 20, 21, 28, 30, and 33) contain the canonical HEXXHXXGXXH zinc-binding consensus sequence within their metalloproteinase domain and are thus predicted to possess metalloproteinase activity [103]. The importance of select ADAMs in development has been interrogated in mice, identifying biological roles in fertilization, central nervous system (CNS) development and heart organogenesis. This will be explained in more detail in the following section.

1.5.1 Phenotypes of ADAM deficient mice

To interrogate the biological significance of individual ADAM proteases during normal development, several research groups have examined the phenotypes of ADAM deficient mice. Studies have shown that mice lacking ADAM1a, ADAM2 or ADAM3 are viable, however, male mice are infertile due to defects in sperm migration into the oviduct and subsequent deficiencies in sperm-egg binding and fusion [104,105]. Despite the high expression levels of ADAM8 within trophoblasts of the developing placenta, ADAM8$^{-/-}$ mice do not display any placental defects and are viable and fertile [106]. It was previously reported that ADAM9 deficient mice are viable and do not show any histopathological abnormalities, however, a recent study demonstrated that these mice develop retinal degeneration [107,108]. ADAM10 null mice die at day 9.5 of embryogenesis possibly due to severe retardation in heart and CNS development. Interestingly, the phenotypes of these homozygous mutant embryos are similar to mouse models with

supressed Notch signalling [109].

Similar to ADAM10 knockout mice, ADAM17 deficient mice are not viable, presumably because of pulmonary hypoplasia and abnormal cardiac development [110,111]. By embryonic day 18.5, ADAM17$^{-/-}$ embryos have a significantly higher heart-to-body weight ratio, which could be caused by cardiac hypertrophy or increased cardiomyocyte proliferation [111]. Interestingly, histological analysis of the ADAM17$^{-/-}$ placentas revealed impaired maturation of the spongiotrophoblast layer, which may contribute to the high embryonic lethality seen in these mice [112]. Although ADAM11 and ADAM15 genes are not required for mouse development and survival, mice lacking ADAM11 exhibit defects in spatial learning and motor coordination and ADAM15$^{-/-}$ mice show significant reduction in pathological neovascularization [113,114]. About 30% of ADAM12 deficient mice die during the early neonatal period and 30% of viable homozygotes display a reduction in their interscapular brown adipose tissue [115]. ADAM12 null mice were reported to be more resistant to obesity induced by high-fat diet and exhibit improved insulin sensitivity compared to wild-type mice [116]. Interestingly, the same phenotypic alterations have recently been observed in mice lacking Notch1 or its downstream effector Rbpj in their adipose tissues, linking ADAM12 with Notch signalling to metabolic maintenance [117]. While 80% of ADAM19 knockout mice die postnatally from congenital heart defects, all ADAM22 null mice die before weaning due to severe ataxia and hypomyelination of peripheral nerves [118,119]. On the other hand, ADAM33 deficient mice are viable, fertile and do not show any pathological abnormalities [120].

Notwithstanding studies that have directly examined the phenotypes of mice lacking individual members of ADAM family, other studies have investigated the roles of

individual metalloproteinases in regulating tumorigenesis. In these experimental settings, ADAMs have been shown to regulate cellular processes that are not only the hallmarks of cancer but also important during normal development, including placentation, such as cell proliferation, survival and invasion [121].

It is worth mentioning that although several of these ADAMs including ADAM10, 12, 15, 19 and 33 are highly expressed in the murine placenta [122], the placental phenotypes of mice lacking these ADAMs remain to be determined.

1.5.2 ADAMs at different stages of pregnancy

The functional importance of ADAMs during human implantation, decidualization and parturition remains to be further elucidated, however, there is a growing body of evidence suggesting the involvement of these proteases during the early stages of pregnancy in mice. Changing expression levels of ADAM8, 9, 10, 12, 15 and 17 in mouse uterus during the oestrous cycle and peri-implantation period suggests these proteases may play a role during endometrial remodelling [123,124]. Particularly, ADAM12 is expressed in decidualized stromal cells at the implantation site where its expression is controlled by progesterone [125]. The biological significance of ADAM12 during mouse decidualization is further supported by the observation that loss of ADAM12 significantly blocks progestin-mediated stromal cell differentiation in culture [125].

Although there is no clear evidence for the involvement of ADAM12 during human implantation and decidualizion, reduced levels of ADAM12 in serum of women with ectopic pregnancies indicate its possible involvement in blastocyst implantation [126,127]. It has also been suggested that ADAM17, which is highly expressed in the

receptive-phase human endometrium, might facilitate blastocyst implantation by mediating MUC1 ectodomain release [128].

To our knowledge, nothing is known about the role of ADAMs during normal parturition in mice and humans, however, the significantly increased levels of ADAM17 and 28 in the late-pregnant mouse uterus suggest their involvement in the remodelling of the uterine ECM during labor [122].

1.5.3 ADAMs during placental development

The importance of ADAMs in placental development is poorly defined, however, few studies have suggested the importance of ADAMs 10, 12 and 17 in pregnancy complications inferring that they may play an important role in placental function [129–132]. ADAM12 has been shown to be highly expressed in the human placenta throughout the course of pregnancy [133], and low serum levels of ADAM12 associate with spontaneous pre-term delivery and IUGR [131,134]. However, the use of ADAM12 as a reliable biomarker for preeclamptic pregnancies is controversial, where reduced ADAM12 serum levels have been positively and inversely correlated with preeclampsia [135,136]. These discrepancies are in contrast with the consistent association of reduced ADAM12 in trisomy 13, 18 and 21 pregnancies, demonstrating its usefulness as a biomarker for detecting chromosomal anomalies [137–139]. Besides ADAM12, evidence suggests that ADAM17 (also known as tumor necrosis factor-α [TNF-α]-converting enzyme or TACE) may contribute to the pathogenesis of preeclampsia by cleaving and activating the pro-form of TNF-α, impacting apoptosis and cell survival within the maternal-fetal interface [132]. ADAM10-mediated cleavage of protocadherins has also been suggested to negatively

impact pregnancy outcomes [129].

Apart from associative studies, only a few studies have examined the importance of ADAMs experimentally. For example, ADAM19, which is expressed in trophoblasts in early pregnancy [140,141], negatively regulates cell invasion in the JEG3 trophoblastic cell line [140]. Taken together, further studies are required to elucidate the biological significance of ADAMs in placental development and trophoblast differentiation.

1.6 ADAM12

While screening for fertilin α and β (ADAM1 and 2) related fusion genes, Yagami-Hiromasa and colleagues discovered a novel ADAM gene family member called meltrin α [142]. Subsequent studies identified two alternatively splice variants of meltrin α: ADAM12L (a long transmembrane form) and ADAM12S (a short secreted form) [143]. The domain structure of ADAM12L is similar to the canonical ADAM transmembrane protein, while the secreted ADAM12S variant lacks the transmembrane and cytoplasmic domains (Figure 1.2) [143].

ADAM12, containing the conserved HEXXHXXGXXH zinc-binding consensus sequence is predicted to be an active protease, and consistent with this, multiple substrates have been identified including IGFBP-3 and -5, heparin-binding EGF-like growth factor (HB-EGF) and certain ECM components such as fibronectin and type IV collagen [144–146]. Thought to function predominantly as a cell surface sheddase, ADAM12 is predicted to regulate the bioavailability of many growth factors, cell adhesion molecules and other membrane-bound proteins.

The underlying mechanisms regulating ADAM12 expression and function are not well characterized, however, Protein Kinase C and Casein Kinase Substrate In Neurons (PACSIN3) as well as hypoxia-activated Notch signalling have been shown to regulate ADAM12 cleavage of proHB-EGF [145,147]. Since early placentation occurs in a hypoxic environment, Notch-mediated regulation of ADAM12 may function as a key mechanism in trophoblast differentiation.

Both ADAM12 splice variants have been reported to play key roles in the progression of human cancers and diseases by controlling cell proliferation and invasion [148]. Through its intrinsic proteolytic, cell adhesive and intracellular signalling properties, ADAM12 can also direct many cellular processes important in trophoblast biology.

1.6.1 ADAM12-mediated regulation of cell differentiation, proliferation and motility

ADAM12L-mediated shedding of HB-EGF, an EGFR ligand, or the Notch ligand delta like-1 (DLL1) activates signalling pathways which ultimately control cellular proliferation, differentiation and migration [145,149,150]. Cleavage of DLL1 by ADAM12 can either stimulate Notch signalling in a cell-autonomous manner or suppress Notch signalling in neighbouring cells [149,150]. Specifically, ADAM12-induced down-regulation of Notch signalling results in accelerated myogenic differentiation [150].

As the biological roles for Notch and EGFR signalling in controlling trophoblast invasion are well established, an underlying role for ADAM12 in regulating these pathways seems highly possible [151,152]. Notably, there appears to be a reciprocal interaction between ADAM12 and Notch signalling, where Notch signalling can also regulate ADAM12 expression and function. Indeed, a recent study reported that

inhibition of canonical Notch signalling increases ADAM12L expression and trophoblast invasion [153].

Importantly, ADAM12's non- proteolytic domains also impact cell migration. For instance, SH3-mediated interaction of ADAM12L's cytoplasmic tail with the c-Src and integrin-linked kinase (ILK) regulates the formation of invadopodia and focal adhesion structures important in cell migration [154,155]. Further, the disintegrin and cysteine-rich domains of ADAM12 play roles in cell adhesion and stress fiber formation through syndecan- and integrin-mediated associations that facilitate cell-ECM attachment [156,157]. ADAM12-mediated recruitment and activation of MMP14 at the cell surface induces gelatin degradation and invadopodia formation and this interaction is proposed to be important in driving cell invasion in cancer [158,159]. It is interesting to note that ADAM12 isoforms can exert distinct effects on their target cells. For instance, overexpression of either ADAM12 isoform increases proliferation of breast cancer cells, however, only the secreted variant (ADAM12S) is capable of promoting breast cancer invasion and metastasis [160]. Likewise in transgenic mice, ADAM12S promotes longitudinal bone growth due to enhanced chondrocyte proliferation and maturation [161].

1.6.2 ADAM12 and cell fusion

ADAM12 was originally cloned through cDNA library screening of putative myoblast fusion genes [142], and not surprisingly, ADAM12 is expressed in several fusogenic cell types including osteoclasts (bone), myoblasts (muscle) and trophoblasts (placenta) [162–164]. The presence of a cell-fusion peptide sequence within its cysteine rich region, and the ability to interact with integrins and cytoskeletal scaffolds (i.e. via

disintegrin and cytoplasmic domains) positions ADAM12 as an attractive molecule in directing cell-cell fusion [143].

Mechanistically, little is known about how ADAM12 directs cell fusion in any of these cellular systems, however during myoblast fusion, the interaction between the cytoplasmic tail of ADAM12L and α-actinin-2 has been shown to be important [163]. Although α-actinin-2 is a muscle specific actin-binding protein, the presence of non-muscle α-actinin along with ADAM12 within the synCT layer suggests that these proteins might synergistically regulate trophoblast cell fusion.

1.6.3 ADAM12 function during neovascularization and placental development

ADAM12 may control other events important in placental development. For example, ADAM12S is known to increase local IGF bioavailability required for proper placentation and optimal fetal development by cleaving IGFBP-3 and -5 [144]. A recent study by Fröhlich, C. *et al*. demonstrated that ADAM12 mediates ectodomain shedding of several membrane-anchored endothelial proteins including vascular endothelial cadherin (VE-cadherin), receptor tyrosine kinase Tie-2, fetal liver kinase 1 (Flk-1), and vascular cell adhesion molecule 1 (VCAM-1) which together affect vascular permeability [165]. Cleavage of these endothelial proteins by ADAM12 might potentially regulate neovascularization and vascular permeability in the placenta. Serving as a sheddase, ADAM12 can influence the activity of other proteinases such as placental leucine aminopeptidase (P-LAP) whose catalytic function is important in maintaining homeostasis during pregnancy [166].

Taken together, ADAM12 has unique characteristics that enable it to affect processes common in many developmental pathways, and therefore may play a fundamental role in directing trophoblast biology.

1.7 cAMP

Cyclic adenosine 3′-5′-monophosphate (cAMP) is the first second messenger identified to relay extracellular signals to intracellular effector proteins [167]. cAMP has been shown to regulate a wide variety of cellular processes including cell proliferation, differentiation, invasion and migration. cAMP signalling initiates upon the binding of G-protein coupled receptors (GPCRs) to the extracellular ligands such as neurotransmitters and hormones [168]. All GPCRs contain seven transmembrane α-helices and are associated with heterotrimeric guanine nucleotide binding proteins (G proteins).

Heterotrimeric G proteins are composed of three distinct subunits, namely Gα, Gβ, and Gγ [168]. To date, about 20 Gα, 5 Gβ and 12 Gγ subtypes have been identified which assemble in various combinations and form more than 1000 potential G protein heterotrimers [169,170]. Based on the sequence similarity and function of their α subunits, the heterotrimeric G proteins are grouped into four main families (G_s, $G_{i/o}$, G_q/G_{11} and $G_{12/13}$) with each having distinct downstream effectors. While members of the G_s and $G_{i/o}$ family mediate stimulation or inhibition of various isoforms of adenylyl cyclases, G_q/G_{11} and $G_{12/13}$ regulate the activity of other proteins such as phospholipase C isozymes, phospholipase A2 and the small GTPase RhoA [171–175].

Despite the tremendous diversity of GPCRs, their ligands and the G proteins associated with them, these receptors share a conserved mechanism of action. Ligand

binding to GPCRs results in G proteins cycling between active and inactive states; the inactive state occurs when Gα binds to GDP and retains high affinity for the Gβγ dimer, while ligand-activated GPCRs undergo a conformational change enabling them to function as guanine nucleotide exchange factors (GEF) promoting the exchange of GDP for GTP on the Gα subunit which results in Gα's dissociation from the Gβγ complex. The GTP-bound α subunit and the freed Gβγ complex function independently and regulate various effector molecules [176].

It is worth mentioning that an alternate "clamshell" model has been proposed suggesting that Gα and Gβγ subunits exert their biological functions without complete dissociation from each other [177,178]. Termination of GPCR signalling occurs following the hydrolysis of GTP to GDP by the intrinsic GTPase activity of the G proteins [179].

As it was mentioned before, the downstream effector of the active G_s protein is the enzyme adenylyl cyclase (AC), which upon activation converts cytoplasmic adenosine triphosphate (ATP) to cAMP [180]. Moreover, the intracellular cAMP concentration is regulated by a family of cyclic nucleotide-degrading enzymes known as phosphodiesterases (PDEs), which hydrolyze the phosphodiester bond to convert cAMP to AMP [181]. Discretely positioned ACs and PDEs generate compartmentalized pools of cAMP, leading to selective activation of its local effectors [182].

1.7.1 cAMP effectors

The main intracellular targets of cAMP are protein kinase A (PKA), cyclic nucleotide-gated (CNG) ion channels and exchange protein directly activated by cAMP

(Epac) [183]. These cAMP mediators may act independently, oppose each other or have synergistic effects.

1.7.1.1 cAMP/PKA signalling

For many years, the heterotetrameric PKA holoenzyme was the only known downstream effector of cAMP. Inactive PKA is composed of two catalytic (C) and two regulatory (R) subunits, where each of the two R subunits contains two cAMP-binding domains/pockets. To date, four types of R subunits (RIα, RIβ, RIIα and RIIβ) and four different C subunits (Cα, Cβ, Cγ and Prkx) have been identified [184]. While Cα, Cβ and Cγ isoforms can form holoenzymes with both RI and RII subunits, Prkx exclusively associates with type I R subunits [185]. In general, PKA isozymes are classified into PKA type I and II based on the associated R subunit, and they are all activated in the same manner [186].

Binding of cAMP to the R subunits triggers conformational changes that lead to the dissociation of the holoenzymes' catalytic subunits from its regulatory subunits. Free C subunits are able to phosphorylate serine/threonine residues of cytoplasmic proteins or they can translocate to the nucleus and mediate the phosphorylation of several other nuclear proteins including transcription factors [183]. Therefore, the cAMP/PKA signalling pathway can regulate a variety of biological processes.

PKA activation and subcellular localization are partly controlled by cAMP gradients and via interaction of its R subunits with A kinase Anchoring Proteins (AKAPs). AKAPs are a heterogeneous group of scaffold-like proteins that target PKA to discrete cellular locations (i.e. ER, Golgi, polar aspects of cell) [187,188].

1.7.1.2 Cyclic nucleotide-gated (CNG) ion channels

CNG ion channels belonging to the voltage-gated cation channel superfamily are considered to be important mediators of cAMP signalling [189]. Although transcripts encoding CNG channels have been found in a variety of tissues, these channels are highly localized in retinal photoreceptors and olfactory neurons, thus playing pivotal roles in sensory transduction [190–192].

CNG channels are tetramers composed of A and B subunits. To date, four A subunits and two B subunits have been identified, which co-assemble in different combinations to give rise to functional channels [193]. CNG channels differ from each other in terms of their ligand selectivity. While some have higher affinity for cGMP, others do not discriminate between cAMP or cGMP [194,195].

The opening of voltage-independent channels requires the binding of cAMP or cGMP to the cyclic nucleotide-binding domain located at the C terminus. Activation and opening of the channels are accompanied by an influx of extracellular cations such as Na^+, K^+ and Ca^{2+}, all of which have been shown to trigger various cellular processes. Downstream events resulting from cation influx activate multiple signalling pathways. For example, as intracellular Ca^{2+} concentrations increase, free Ca^{2+} binds to calmodulin (CaM; a calcium-binding protein), and the Ca^{2+}-CaM complex interacts with CNG channels reducing their affinities for cyclic nucleotide ligands. This leads to inactivation of the ion channel and membrane repolarization [196]. Ca^{2+}-CaM complex also modulate CNG channels by stimulating the activity of PDEs and promoting cAMP/cGMP hydrolysis [197].

1.7.1.3 Exchange protein directly activated by cAMP (Epac)

The characterization of cAMP-mediated effects acting independently of PKA or CNG led to the identification of Epac proteins [198]. Two isoforms of Epac have been identified, Epac1 and Epac2, both of which have been shown to play important roles in cAMP siganling [199,200]. Unlike PKA, Epac protein is expressed as a single polypeptide chain containing an N-terminal regulatory region followed by a C-terminal catalytic region. While both Epac variants share a conserved catalytic domain structure, the regulatory regions of Epacs differ slightly; Epac1's regulatory region contains a single cAMP-binding domain while Epac2 possesses two cAMP-binding domains. The presence of the additional cAMP binding site in Epac2 suggests the enzyme may be more responsive to changes in cAMP gradients, however studies have shown that the second binding site has low affinity for cAMP and does not potentiate Epac activation. Indeed, the exact function of this additional cAMP binding site has yet to be determined [200].
In the absence of intracellular cAMP binding, the regulatory region of Epac protein sterically hinders the interaction between the catalytic region and effector target molecules. The auto-inhibitory effect of the regulatory region is relieved following cAMP binding, making the catalytic domain fully active [201,202].

Both Epac proteins act as cAMP-dependent GEFs and activate small GTPases, Ras-associated protein 1 and 2 (Rap1 and 2), which have been reported to regulate diverse biological functions such as cell adhesion, migration and differentiation [202,203]. Besides Rap GTPases, Epac proteins directly interact and activate other effector molecules such as Ras GTPases, c-Jun N-terminal kinases (JNKs), secretory granule-associated protein Rim2 and microtubule-associated protein 1A (MAP1A), thereby

regulating diverse cellular processes such as exocytosis and microtubule assembly [204–207]. Despite these findings, the detailed mechanisms by which Epac proteins regulate the activity of their downstream effectors remain to be elucidated.

1.7.2 cAMP levels in human pregnancy

During normal pregnancy, plasma cAMP levels increase progressively and promote myometrial relaxation necessary for pregnancy maintenance. cAMP exerts this effect most likely through regulation of intracellular calcium concentration and phosphorylation of myosin-light chain kinase [208,209]. Not surprisingly, cAMP concentration drops precipitously before the onset of labor to drive myometrial contraction [210].

Molecular components central to cAMP synthesis and breakdown (i.e. GPCRs, ACs, G proteins and PDEs) are expressed in the human placenta, indicating that cAMP signalling plays an important role in human placentation [211–215]. Indeed, the activity of ACs and induction of intracellular cAMP are regulated by several molecules produced by decidual cells and/or trophoblasts, such as prostaglandins, catecholamines, and chorionic gonadotrophin [216–218]. Once synthesized, cAMP acting through its downstream effector proteins, PKA and Epac, plays a central role in different aspects of trophoblast biology and placental development [219,220].

1.7.3 Effects of cAMP on endocrine functions of trophoblasts

cAMP has been shown to modulate the endocrine function of trophoblasts by stimulating the production and secretion of several proteins such as hCG, inhibin and

gonadotropin-releasing hormone (GnRH), as well as progesterone, all of which are essential for successful maintenance of pregnancy [219,221–223]. cAMP also regulates aromatase activity in cultured trophoblasts, suggesting its involvement in directing trophoblast-mediated estrogen production during pregnancy [224].

1.7.4 Effects of cAMP on trophoblast syncytialization

Trophoblast cells isolated from term placentae undergo spontaneous differentiation and form syncytial structures in culture. Fusion of these cells is associated with an increase in intracellular cAMP levels, supporting the notion that cAMP is involved in cytotrophoblast differentiation [225]. Interestingly, treatment of cultured human cytotrophoblasts with exogenous cAMP analogs further enhances the differentiation and syncytialization of these cells [220]. Syncytium formation can also be stimulated in BeWo choriocarcinoma cells by cAMP [81], where cAMP-mediated differentiation is accompanied by a concomitant increase in expression of several fusogenic proteins including GCM1, Syncytin-1 and -2 as well as gap junction protein Connexin 43 [226]. Other studies have determined that both Epac and PKA signalling pathways associate with cAMP-directed trophoblast fusion [219,220]. Collectively, cAMP signalling mediates synCT formation by regulating the expression of genes involved in the cell fusion.

1.7.5 Regulation of trophoblast invasion by cAMP

Although a few studies have suggested an inhibitory effect on trophoblast invasion by cAMP, recent reports have shown that increases in intracellular cAMP levels promote trophoblasts invasion and migration [227–231]. In one study, cAMP signalling

induced trophoblast invasion by driving the expression and secretion of MMP2 and 9 [229,232]. The stimulatory effect of cAMP on trophoblast invasion is further supported by a recent report showing that cAMP/PKA signalling promotes trophoblast adhesion and migration via a Rap1-dependent mechanism [233]. Conversely, elevated cAMP levels inhibit IGF-II-mediated trophoblast invasion [227]. Therefore, the effect of cAMP on trophoblast migration and invasion remains controversial, and possibly highlights the complexity in PKA regulation through possible mechanisms affecting intracellular localization, transient activity and substrate availability. Further studies are required to investigate the underlying mechanisms by which cAMP regulates trophoblast invasion.

1.8 Hypothesis and rationale

Acquisition of an invasive phenotype by EVTs is a fundamental process in early placentation, where shallow trophoblast invasion and inadequate maternal vascular remodelling associate with several pregnancy complications. In addition to paracrine factors produced by decidual cells, several trophoblast factors have been shown to regulate trophoblast migration and invasion including EVT-specific proteases such as MMPs and urokinase plasminogen activator (uPA). These proteases have been shown to mediate a wide variety of cellular processes including ECM remodelling, growth factor and cytokine availability, cell survival and apoptosis [234]. Members of the ADAM family of metalloproteinases, sharing structural and biological similarities to MMPs, are also among the major candidates regulating trophoblast invasion; however, the roles of ADAM proteases in trophoblast biology have not been tested. Differential global gene expression analysis comparing gene signatures from vCTs to invasive EVTs identified

several ADAM members, including ADAM12, 19 and 28, to be highly and preferentially expressed by EVTs [235]. In particular, ADAM12 has previously been shown to be highly expressed in human placental tissues [143]. Along these lines, maternal ADAM12 serum levels significantly increase during pregnancy and are reciprocally detected at lower concentrations in high-risk pregnancies such as IUGR [131]. Despite the high expression levels of ADAM12 in the placenta, its regulation and biological importance in placental development is unknown.

The second messenger cAMP regulates trophoblast differentiation along both the villous and invasive pathways [236,237]. Specifically, cAMP signalling induces trophoblast invasion by promoting the expression of several key proteases, such as MMP2, MMP9 and uPA [229,238]. Importantly, a recent study by our group has identified a role for cAMP/PKA signalling in driving ADAM12 expression during trophoblast fusion [239]. Therefore, it is likely that cAMP signalling also regulates ADAM12 expression in the invasive trophoblast subtype.

Given that ADAM12 is highly expressed in the human placenta, directs tumor cell migration and invasion, and is linked to major pregnancy complications, **I set out to test the hypothesis that ADAM12 plays a fundamental role in regulating trophoblast differentiation along the invasive pathway, and that ADAM12 expression and function are regulated through a cAMP-dependent mechanism.**

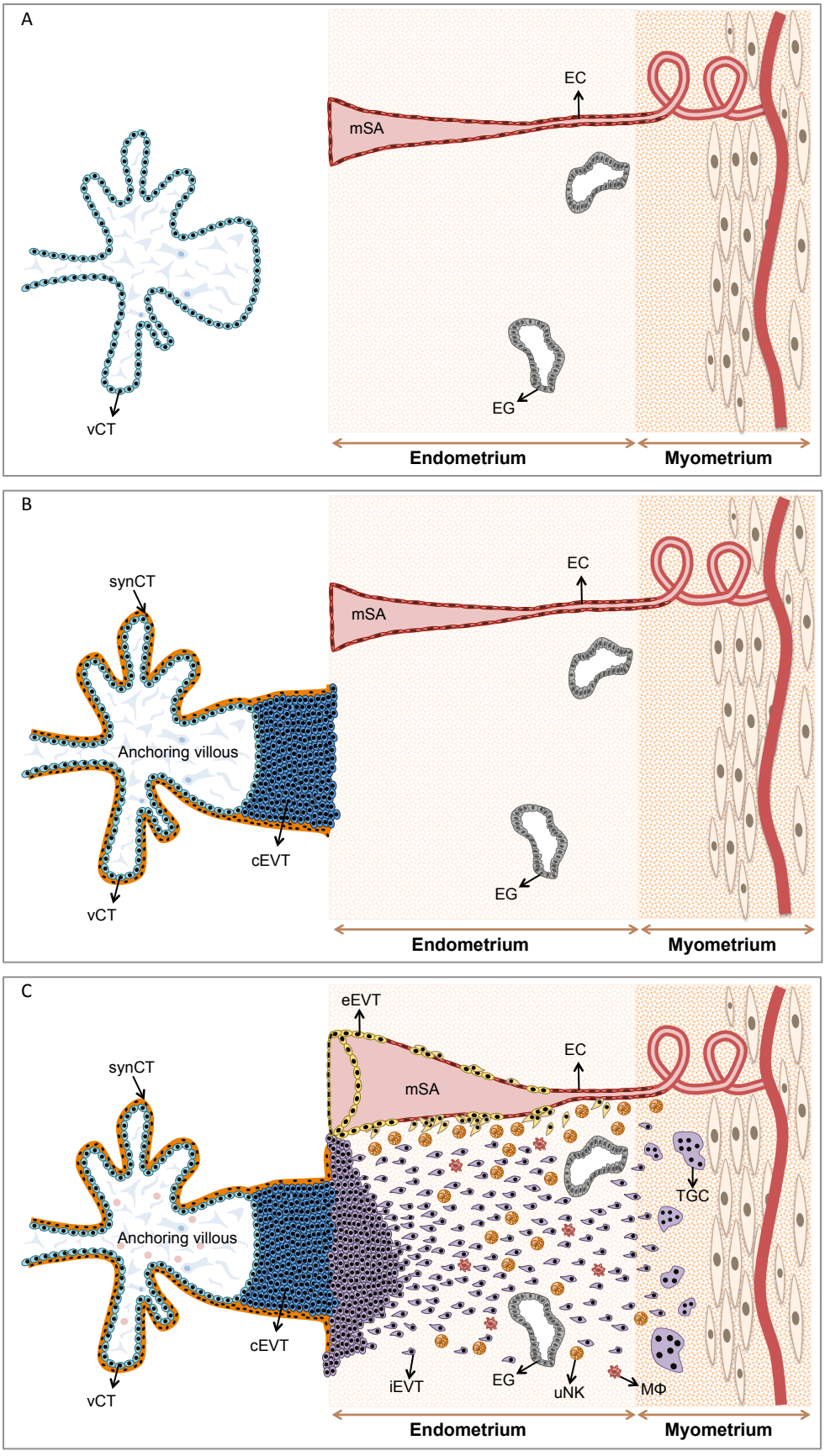
The specific aims of my research are as follows:

Aim 1. To examine ADAM12 localization in first trimester placenta and to determine the importance of ADAM12 in promoting invasion in primary EVTs and *ex vivo* placenta culture systems.

Aim 2. To elucidate whether ADAM12S's intrinsic metalloproteinase activity is essential in directing trophoblast invasion.

Aim 3. To determine the effects of cAMP signalling pathway on ADAM12 expression and function.

These aims directly interrogate the significance of ADAM12 in directing trophoblast invasion and migration, a critical part of the placentation that is often dysregulated in pregnancy complications harboring severe placental pathologies.


A
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eEVT
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synCT
mSA
TGC
Anchoring villous
cEVT
vCT
iEVT
EG
uNK
MΦ
Endometrium
Myometrium

Figure 1.1 Schematic diagram of trophoblast differentiation. (A-C) Mononuclear villous cytotrophoblasts differentiate into multinucleated syncytiotrophoblast or invasive EVTs. As trophoblasts differentiate along the invasive pathway, they migrate into and remodel decidual stroma and arterioles. This trophoblast-mediated remodelling of maternal stroma and vasculature ultimately increases the blood flow towards the placenta and fetus. vCT, villous cytotrophoblast; synCT, syncytiotrophoblast; cEVT, columnar extravillous cytotrophoblast; eEVT, endovascular extravillous cytotrophoblast; iEVT, interstitial extravillous cytotrophoblast; mSA, maternal spiral artery; EC, endothelial cell; EG, endometrial gland; uNK, uterine Natural Killer cell; MΦ, macrophages; TGC, trophoblast giant cell.

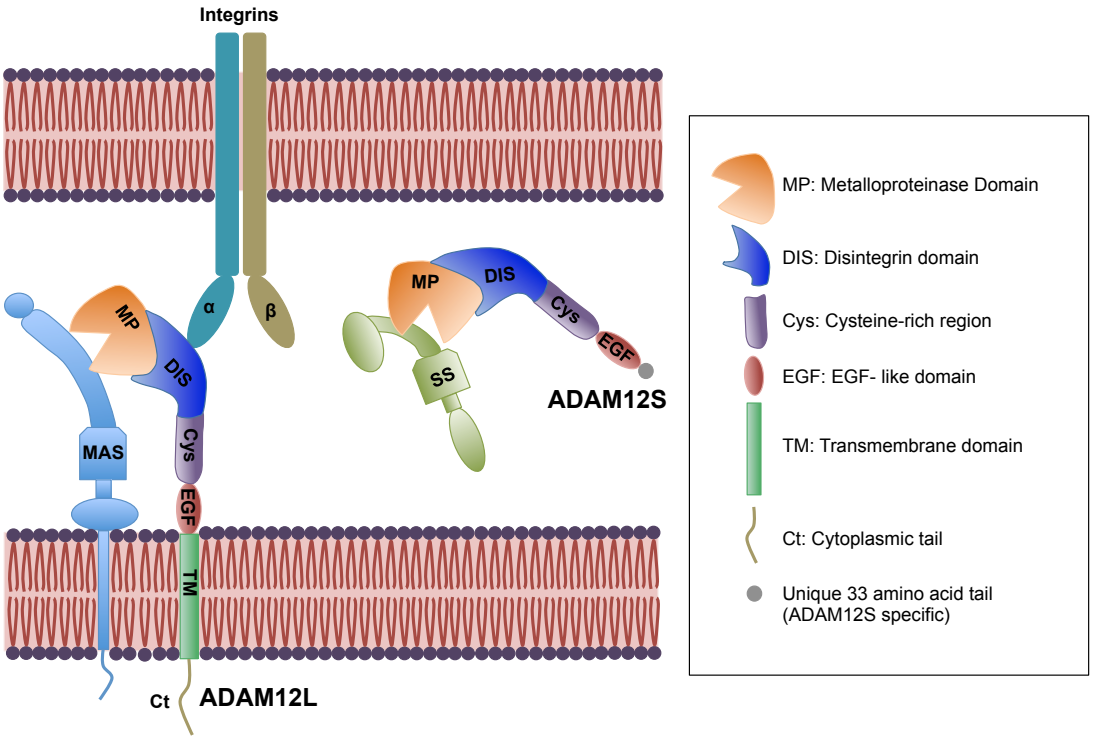


Figure 1.2 Structure of human ADAM12L and ADAM12S isoforms. Depicted are generic membrane-bound and soluble ADAM12 substrates. ADAM12 can interact with integrin subunits through its disintegrin domain. MP, Metalloproteinase domain; DIS, Disintegrin domain; Cys, Cysteine-rich region; EGF, EGF- like domain; TM, Transmembrane domain; Ct, Cytoplasmic tail; SS, secreted substrate; MAS, membrane-anchored substrate.

CHAPTER 2. MATERIALS AND METHODS

2.1 Placental tissue collection

Placental specimens (5-12 weeks of gestation) were obtained from women undergoing elective surgical terminations of pregnancy at BC Women's Hospital's Comprehensive Abortion and Reproductive Education (CARE) Program. All participants provided written informed consent. Ethics approval for this study was obtained from the University of British Columbia Research Ethics Board.

2.2 Cell and tissue culture

The human HTR8/SVneo cell line was kindly provided by Dr. Charles Graham, Queen's University, ON, Canada. HTR8/SVneo cells were cultured in RPMI 1640 medium containing 25 mM glucose, L-glutamine, and supplemented with 10% fetal bovine serum (FBS) and 0.1% antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin).

The human choriocarcinoma JEG3 cells were purchased from the American Type Culture Collection (ATCC, Manassas, USA) and cultured in DMEM medium supplemented with 25 mM glucose, L-glutamine, 10% FBS and 0.1% antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin).

To obtain primary EVT cultures, first trimester placental villi were minced and plated on collagen-coated plates (Figure 2.1). After 1-2 days of culture, non-adherent fragments were washed and EVTs propagating from the placental explants were maintained in phenol-red free DMEM:F12 (1:1) medium supplemented with 25 mM

glucose, L-glutamine, 15 mM HEPES, 10% FBS, and 0.1 % antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin and Antibiotic-Antimycotic) (all purchased from Gibco by Life Technologies, Grand Island, USA). Only cultures that were >90% positive for cytokeratin-7 were used for the studies (Figure 2.2).

2.3 Placental villous explant culture

To establish first trimester villous explant cultures, placentas obtained from women undergoing elective surgical termination of pregnancy were washed in cold phosphate-buffered saline (PBS; Gibco by Life Technologies; Cat# 14190-144). Small pieces of placental villi (from 5-7 weeks of gestation) were dissected under the microscope and imbedded into 12 mm Millicell cell culture inserts with 0.4 μm pore filters (EMD Millipore, Darmstadt, Germany) coated with 200 μL growth-factor reduced Phenol-red free Matrigel (BD Biosciences, Mississauga, Canada) (Figure 2.3).

All the explant cultures were maintained in phenol-red free DMEM:F12 (1:1) medium containing 25 mM glucose, L-glutamine, 15 mM HEPES, and supplemented with 0.1 % antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin and Antibiotic-Antimycotic) unless otherwise stated. Explant cultures were imaged at different time points using a Nikon SMZ 7454T triocular dissecting microscope equipped with a digital camera. The area of EVT column outgrowth was measured from the base of each EVT column to the invasive front of invading EVTs at three different locations within each EVT column using ImageJ software (Figure 2.3). At least three different placentas were used for each experiment and all treatments were performed in duplicate.

2.4 Reagents and antibodies

The two polyclonal antibodies directed against human ADAM12 were purchased from ProteinTech (Cat# 14139-1-AP; Chicago, USA) and Abcam (Cat# 282225; Toronto, Canada). The antibodies against HLA-G and Ki67 were obtained from Exbio (clone 4H84; Cat# 11-499-C100; Vestec, Czech Republic) and Thermo Scientific (clone Sp6; Cat# RM-9106-S0; Waltham, USA), respectively. The two Cytokeratin-7 antibodies used in the study were purchased from Santa Cruz Biotechnology (Cat# sc-8421; Dallas, USA) and Ventana Medical Systems (Cat# 790-4462; Tucson, USA). The antibody detecting Caspase-cleaved cytokeratin-18 was obtained from Roche (clone M30; Cat# 12140322001; Indianapolis, USA). β-actin antibody was purchased from Santa Cruz Biotechnology (Cat# sc-47778; Dallas, USA). E-cadherin was obtained from BD Biosciences (clone 36, Cat#; San Diego, USA). The ADAM inhibitor (TAPI-1) was purchased from Enzo Life Sciences. Forskolin, IBMX, 8-Br-cAMP and the Epac-selective cAMP analog (8-CPT-2Me-cAMP) were all purchased from Sigma-Aldrich. cAMP-dependent protein kinase peptide inhibitor (PKI) and H89 were purchased from Promega and Sigma-Aldrich, respectively. The concentrations of all the antibodies used in the study are shown in Table 2.1.

Table 2.1 List of all the antibodies used in this thesis

Antibody	Clonality/Source	Applications (concentrations)
ADAM12 (ProteinTech)	Polyclonal/Rabbit	Immunoblotting (1:1000) Immunofluorescence (1:50) Immunohistochemistry (1:50)
ADAM12 (Abcam)	Polyclonal/Rabbit	Immunoblotting (1:1000)
Caspase-cleaved cytokeratin-18 (Roche)	Monoclonal/Mouse	Immunofluorescence (1:10)
Cytokeratin-7 (Santa Cruz)	Monoclonal/Mouse	Immunofluorescence (1:200) Immunohistochemistry (1:100)
Cytokeratin-7 (Ventana Medical Systems)	Monoclonal/Rabbit	Immunofluorescence (1:75)
E-cadherin (BD Biosciences)	Monoclonal/Mouse	Immunofluorescence (1:400)
HLA-G (Exbio)	Monoclonal/Mouse	Immunofluorescence (1:100) Immunohistochemistry (1:100)
Ki67 (Thermo Scientific)	Monoclonal/Rabbit	Immunofluorescence (1:100)
β-actin (Santa Cruz)	Monoclonal/ Mouse	Immunoblotting (1:8000)

2.5 RNA preparation and real-time PCR

Total RNA was extracted from placental villi, villous explant cultures, HTR8/SVneo cells or JEG3 cells using TRIzol reagent (Life Technologies). For extracting RNA from villous explant cultures, 0.5 μg of glycogen was used to improve RNA purification. One μg of RNA was reverse-transcribed using qScript™ cDNA

synthesis kit from QuantaBiosciences. Reverse-transcription PCR was performed using an ABI thermal cycler, and the synthesized cDNA was subjected to quantitative PCR (qPCR) ΔΔCT analysis using ABI Viia 7 Sequence Detection System or ABI 7500. qPCR was done using either SYBR Green FastMix (QuantaBiosciences) or TaqMan Universal PCR Master Mix with UNG (Life Technologies).

Following is the list of primers used in this study: ADAM12S (forward 5'-GT GACAAGTTTGGCTTTGGAG-3', reverse 5'- GTGAGGCAGTAGACGCAT G-3'), ADAM12L (forward 5' -GACAATGGGAGACTGGGC-3', reverse 5'-GTGG ATCTGGGCACTTGG-3') and GAPDH (forward 5'-AGGGCTGCTTTTAACTCTGGT-3', reverse 5' -CCCCACTTGATTTTGGAGGGA -3'). The following TaqMan primer/probe sets used in the study: ADAM12S (Hs00222216_m1), ADAM12L (Hs01106111_m1) or β-actin (Hs99999903_m1). The TaqMan qPCR assay was only used to analyze ADAM12 gene expression in Figure 3.3. The cycle threshold (CT) values were used for quantitative analyses of relative gene expression. All the values were normalized to β-actin or GAPDH transcripts.

2.6 Immunoblotting

Placental villi, HTR8/SVneo cells or JEG3 cells were lysed in RIPA 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, 200 mM Na_3VO_4, 2 mM phenylmethylsulphonyl fluoride), and an appropriate dilution of Complete Mini protease inhibitor cocktail tablet (Roche). Protein concentration was assessed using Bio-Rad DCTM protein assay kit and 20-30 μg of total protein was subjected to SDS-PAGE and

transferred to nitrocellulose membranes (Pall Life Sciences). The membranes were probed at 4ºC with specific primary antibodies. After overnight incubation with the primary antibodies, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit or anti-mouse antibodies; Cell Signalling). The signals were detected using enhanced chemiluminescence substrate (ECL; PerkinElmer). All the membranes were stripped and re-probed with a horseradish peroxidase (HRP)-conjugated β-actin antibody to normalize the amounts of protein loaded. To examine the secreted ADAM12 variant, serum-free conditioned medium was first concentrated using Amicon Ultra Centrifugal Filter Units (EMD Millipore) and then subjected to SDS-PAGE. The densitometric analysis was performed using ImageJ software.

2.7 ADAM12 siRNA transfection

The siRNAs targeting ADAM12 as well as non-silencing (NS) siRNA were purchased from Dharmacon (ON-TARGET plus set of 4 siRNAs, Cat# LQ-005118-00-0002; ON-TARGET plus Non-targeting siRNA #1, Cat# D001810-01-20). The target sequences and the accession number for all the four siRNAs are shown in table 2.2. HTR8/SVneo cells were grown to 30%-40% confluency and transfected with 25 nM of siRNA oligos using Lipofectimen RNAiMAX reagent (Life Technologies). Cells transfected with NS siRNA or cultured in the presence of RNAiMAX transfection reagent alone, served as negative controls. For siRNA-mediated ADAM12 silencing in villous explant cultures, explants were first allowed to adhere to the Matrigel. After 24 h

of culture, siRNA transfection was carried out using 200 nM of siRNA oligos (A12i-5 or A12i-8).

Table 2.2 List of the accession number and the target sequence for each ADAM12-directed siRNA

ADAM12 siRNA	Accession number	Target Sequence
A12i-5	J-005118-05	5'-CCAAUGCCGUUUCCAUAGA-3'
A12i-6	J-005118-06	5'-CCAGAGGAAUGUAUGAAUC-3'
A12i-7	J-005118-07	5'-UCAAGGCAACUAAGUAUGU-3'
A12i-8	J-005118-08	5'-CAAAUGCUCUGUAAGUCAG-3'

2.8 Expression constructs and plasmid DNA transfection

HTR8/ SVneo and JEG3 cells were stably/ transiently transfected with pCMV6-AC mammalian expression constructs encoding full length human ADAM12L and ADAM12S (OriGene, Rockville, USA). The pCMV6-AC and pCMV6-AC-GFP (green fluorescent protein) expression constructs were served as controls (OriGene). Transfections were carried out using 4 μg/mL of plasmid DNA and Lipofectamine 2000 transfection reagent (Life Technologies). For stable transfection experiments, 48 h post transfection, 400 μg/mL Geneticin (G418) was added to the media as a selective agent. After selection, cells were maintained in the media containing 100 μg/mL G418.

To generate the ADAM12S mutant construct (ADAM12S$^{\Delta E351Q}$), a point mutation in the proteolytic site (E351Q) of ADAM12 was introduced using QuikChange PCR site-

directed mutagenesis kit (Agilent, Santa Clara, USA) while the parent pADAM12S construct was used as the template. The following HPLC-purified primers were used: forward 5'- CGTGACCCTGGCACATCAGCTGGGCCACAATTTC-3', reverse 5'-GAAATTGTGGCCCAGCTGATGTGCCAGGGTCACG-3'. Following the reaction, the products were digested with DpnI, and were transformed into Stbl3 competent cells (Life Technologies). DNA plasmid was isolated from several bacterial colonies and sent for sequencing to confirm the introduction of a single point mutation. This point mutation renders the protein proteolytically inactive since it causes a single-base substitution of guanine for cytosine resulting in a protein which contains glutamine (Q) at amino acid position 351 instead of a glutamate (E) [240].

2.9 Cell viability analysis

Cell viability was measured with the ATP-based CellTiter-Glo Luminescent Cell Viability Assay purchased from Promega. This assay determines the number of metabolically active cells based on quantitation of the ATP present in the culture. Briefly, cells were seeded at a density of 1 x 10^3 cells/well into opaque-walled 96-well microplates containing 100 µL of media supplemented with 5% FBS. The cells were then incubated at 37ºC for 12, 24, 48, 72 or 96 h. The viability assays were performed according to the manufacturer's instructions and luminescence was measured using a Victor^{3}V Multilabel Counter.

To determine cell proliferation or apoptosis in villous explant cultures, immunofluorescent staining was performed using the following two antibodies: ki67 (a proliferation marker) or caspase-cleaved cytokeratin-18 (an apoptosis marker). Villous

explants were dual-labeled with cytokeratin-7 and Ki67 and the percentage of proliferating cells within each EVT column was calculated as the ratio of ki67 positive cells to cytokeratin-7 positive cells. Dual labeling for cytokeratin-7 and caspase-cleaved cytokeratin-18 could not be performed because of antibody incompatibility. Therefore, the percentage of apoptotic cells was calculated by dividing the number of caspase-cleaved cytokeratin-18 positive cells into the total number of cells present in each EVT column (DAPI positive).

2.10 Immunohistochemistry and immunofluorescence

Villous explants and placental villi were fixed in 2% paraformaldehyde overnight at 4ºC, paraffin embedded, serially sectioned at 5 μm thickness and mounted on glass microscope slides. EVT cultures were fixed using 4% paraformaldehyde for 20 min at 4ºC. Immunohistochemistry was performed using the horseradish peroxidase 3-amino-9-ethylcarbazole (HRP-AEC) system protocol obtained from R&D systems (Minneapolis, USA). Antigen retrieval was performed by heating slides in a microwave for 2 mins (5x) in a citrate buffer (pH 6.0). Antibodies directed against ADAM12, cytokeratin-7 and HLA-G were diluted in 5% goat serum and used in the assay.

For immunofluorescence, following antigen retrieval, sections were incubated with sodium borohydride for 5 min at room temperature (RT) and permeabilized with 0.2% Triton X-100. Subsequently, sections were blocked in 5% normal goat serum for 1h (RT) and incubated overnight with antibodies directed against human ADAM12, cytokeratin-7, HLA-G, E-cadherin, Ki67 and caspase-cleaved cytokeratin-18. The concentration of all the antibodies used in the study is shown in Table 2.1. Following

overnight incubation with the primary antibodies, slides were washed with PBS and incubated with species-specific Alexa Fluor 488- or 568- conjugated secondary antibodies (Life Technologies) for 1 h at RT. After washing with PBS, coverslips were mounted onto glass slides with mounting media containing 4', 6-Diamidino-2-Phenylindole (DAPI). Images were captured using a Leica DM4000B fluorescent microscope.

2.11 FACS purification of placental cells

Placental villi single cell suspensions were generated from fresh first trimester placental specimens (N=4) by enzymatic digestion and analyzed by flow cytometry following protocols adapted from Joshi *et al*. and Beristain *et al*. [241,242]. Briefly, placental villi were digested for 1.5 h at 37°C in Hanks Balanced Salt Solution (HBSS), 750 U/mL collagenase and 250 U/mL hyaluronidase. Organoids obtained after vortexing were subjected to red blood cell lysis in 0.8% NH_4Cl, further dissociation in 0.25% trypsin for 2 min, 5 mg/mL dispase with 0.1 mg/mL DNase I for 2 min, and filtered through a 40 μm mesh to obtain single cells. Contaminating immune and endothelial cells were removed from the cell admixture by EasySep immuno-magnetic bead purification (all reagents obtained from StemCell Technologies Inc, Vancouver, Canada). Following magnetic bead exclusion, 2.5 x 10^6 cells were blocked with Fc receptor antibody (eBioscience, San Diego, CA, USA), and incubated with the following antibodies on ice for 30 min: anti-CD45 (clone 2D1, eBioscience), anti-CD49f-PE-Cy7 (clone GoH3, eBioscience) and anti-HLA-G-PE (clone 87G, eBioscience). Dead cells were excluded from analysis by staining with 7AAD (eBioscience). The cell surface markers CD49f and HLA-G were

used to identify placental trophoblast cell populations, while CD45 was used to identify and exclude contaminating immune cells that were not completely removed by EasySep magnet purification. FACS analysis was performed using FACSDiva (BD, San Diego, CA, USA) and FlowJo software (Tree Star, Inc. Ashland, USA). Cell sorting was performed on a FACSAria (BD) into tubes containing ice-cold HBSS/2% FBS.

2.12 Invasion and migration assays

Trophoblast invasion and migration were performed using uncoated (migration) or Matrigel-coated (invasion) Transwell inserts containing 6.5 mm filters with 8 μm pore size (BD Biosciences). Assays were performed according to the manufacturer's protocol. Briefly, cells were suspended in media containing 0.1% BSA and seeded in the upper chambers of Transwell inserts at 2.5 x 10^4 cells/chamber. The media in the bottom chamber were supplemented with 10% FBS and served as chemoattractant. For experiments looking at the effect of TAPI-1 or cAMP on cell invasion, 25 nM TAPI-1 or 25 μM Forskolin was added to the media in both upper and lower chambers.

After 24-72 h of incubation, cells were fixed with 100% methanol for 20 min at 4ºC and stained with hematoxylin and eosin (Sigma-Aldrich). The inserts were washed with dH_2O and cells residing at the upper surface of the membrane were removed using cotton swabs. The filters were then cut off from the inserts and mounted on glass slides. Cell invasion and migration were determined by counting the number of stained cells in five random fields at 10x magnification using an inverted microscope (Leica DM4000B).

2.13 Dual luciferase reporter assay

The 1237-bp ADAM12 promoter sequence inserted upstream of the Gaussia luciferase (GLuc) and secreted alkaline phosphatase (SEAP) reporter genes in the pEZX-PG04 vector was purchased from GeneCopoeia (pEZX-ADAM12; GeneCopoeia Cat# HPRM13238-PG04). The ADAM12 promoter fragment comprises the sequence from position -1000 to +237 relative to the transcription start site (Figure 2.4). HTR8/SVneo cells were transiently transfected with the ADAM12 promoter luciferase construct (pEZX-ADAM12) or the promoterless vector containing the luciferase reporter genes (pEZX-control; GeneCopoeia Cat# NEG-PG04) using Lipofectamine 2000 transfection reagent. After 24 h of culture, cells were treated with or without cAMP analogs (8-Br-cAMP or 8CPT-2Me-cAMP). Secreted GLuc and SEAP were assayed from the conditioned medium 48 h post transfection, using a Secrete-Pair™ Dual Luminescence assay Kit (GeneCopoeia; Cat# SPDA-D010). Luminescence was measured using Victor^{3}V plate reader and reported as the ratios of Gaussia luciferase normalized to secreted alkaline phosphatase in the same sample.

2.14 Statistical analysis

Statistical analyses were performed using GraphPad Prism software. All the comparisons were made using ANOVA and two-tailed Student's t-test. Cellular invasion indices were analyzed by one-way ANOVA followed by the Tukey multiple comparison test. Analysis of the ADAM12 promoter activity was performed using one-tailed Student's t-test. Data are presented as mean ± s.e.m. P-values less than 0.05 were considered statistically significant.

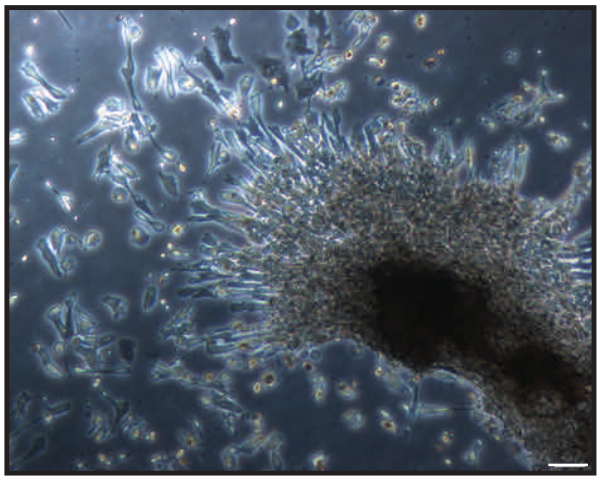


Figure 2.1 Primary EVTs cultured on a collagen-coated plate. Light microscopy observation of EVT propagation on a collagen-coated plate. Scale bars= 100 μm.

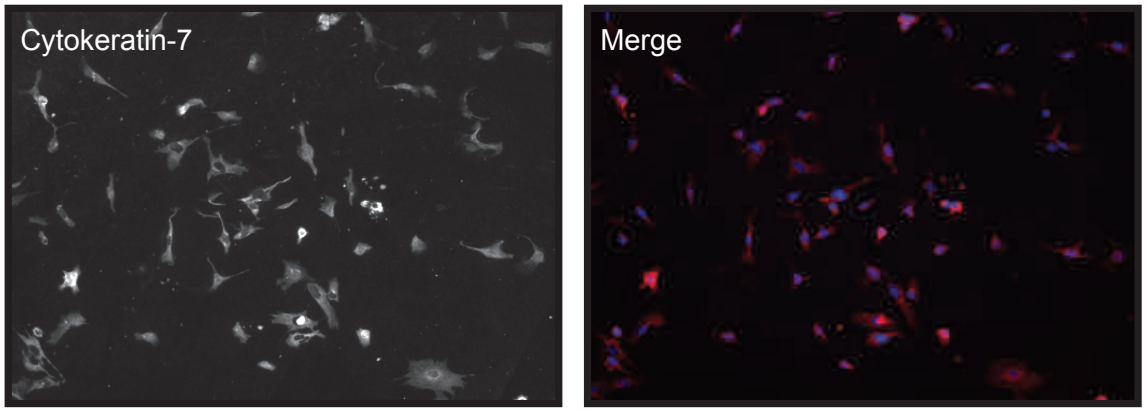


Figure 2.2 Primary EVTs are positive for Cytokeratin-7. Immunofluorescence labeling of primary EVT cultures (7 weeks gestation) with antibody directed against cytokeratin-7 (red). Blue indicates DAPI-stained nuclei.

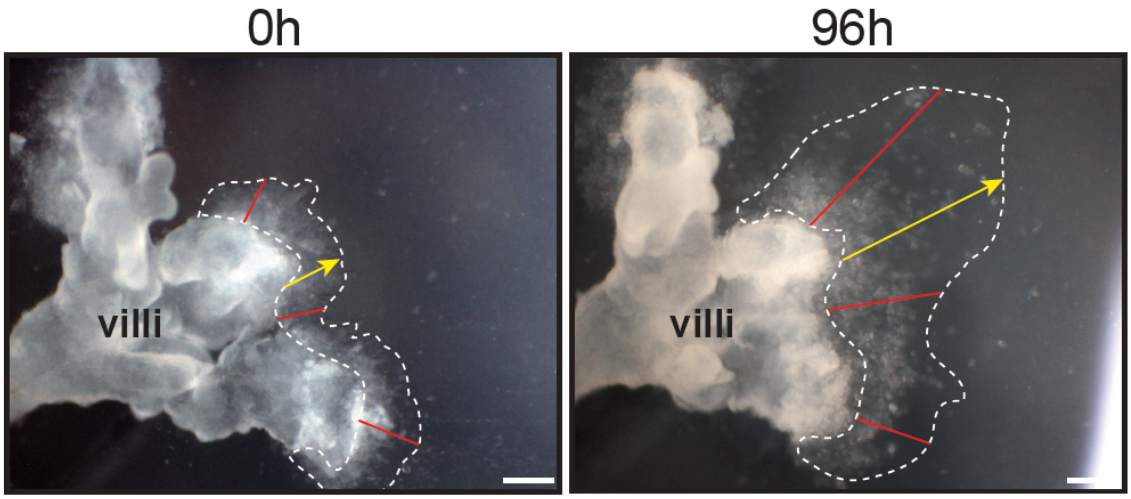


Figure 2.3 Light microscope image of a first trimester chorionic villous explant imbedded on Matrigel. EVT column outgrowth is calculated as the difference in column length at 96 and 0 h (μm), where column length is measured from the base of each column to the invasive front at three distinct locations (red lines). Yellow arrows indicate the direction of trophoblast invasion. 'Villi' denotes placental villi. Scale bars= 100 μm.

Figure 2.4 Schematic diagram of the 1237-bp promoter fragment of the ADAM12 gene. Several transcription factor binding sites were predicted using PROMO software. TSS indicates the transcription start site.

CHAPTER 3. CHARACTERIZING THE FUNCTIONAL IMPORTANCE OF ADAM12 IN DIRECTING TROPHOBLAST INVASION AND EVT COLUMN FORMATION

3.1 Introduction

Members of the ADAM family are multidomain proteins that play important roles in a variety of physiological and pathological conditions by directing diverse cellular processes such as cell proliferation, differentiation and invasion. A considerable amount of evidence supports the idea that ADAM12 is involved in a variety of life-threatening diseases including cardiac hypertrophy, musculoskeletal disorders and chronic asthma [243–246]. Both ADAM12 isoforms are highly expressed in several cancers including breast cancer, small cell lung cancer, colon cancer, bladder cancer and liver cancer, where their elevated expression is correlated with cancer progression, tumor cell invasion and metastasis [160,247–252]. Through its proteolytic and/or ancillary domains, ADAM12 can exert diverse biological functions important not only in pathological conditions but also in normal development.

As mentioned earlier, ADAM12 is also highly expressed in the human placenta and its serum levels tend to increase throughout pregnancy [131,143]. This is in contrast with pregnancies complicated by chromosomal abnormalities, IUGR and spontaneous preterm delivery, where ADAM12 serum levels are significantly reduced [133,134]. Moreover, gene expression profiling of first trimester placenta identified ADAM12 to be highly expressed in the invasive trophoblast population [235]. Despite these intriguing findings, no further studies have looked at the significance of ADAM12 in placental development.

Serving as a key regulator of cancer cell invasion, it is possible that this protease might also play a role during trophoblast invasion. Therefore, in this chapter I sought to determine the significance of ADAM12 in regulating trophoblast invasion by addressing the following two aims.

Aim 1. To examine ADAM12 localization in first trimester placenta and to determine the importance of ADAM12 in promoting invasion in primary EVTs and *ex vivo* placenta culture systems.

Aim 2. To elucidate whether ADAM12S's intrinsic metalloproteinase activity is essential in directing trophoblast invasion.

3.2 Results

3.2.1 ADAM12 localizes to the multinucleated syncytiotrophoblast and EVT anchoring columns in first trimester human placenta

As a first step in understanding the importance of ADAM12 in placental development, ADAM12's localization within first trimester human placental villi was examined. Serially sectioned placental villi were immunostained with antibodies directed against ADAM12, E-cadherin (epithelial marker), cytokeratin-7 (pan-trophoblast marker) and HLA-G (EVT-specific marker) (Figure 3.1A). Immunofluorescence staining confirmed previous findings that ADAM12 is highly expressed within the synCT layer [164]. Interestingly, ADAM12 positivity was observed within anchoring EVT columns, where cells residing in the middle and distal end of the EVT column were strongly

positive for the ADAM12 signal (Figure 3.1A). Dual labeling with cytokeratin-7 and HLA-G confirmed the EVT identity of ADAM12-positive cells.

Since the antibody used in our immunofluorescence analysis does not discriminate between ADAM12L and ADAM12S splice variants, we examined the expression of individual ADAM12 isoforms in cells isolated from placental villi using a fluorescence activated cell sorting (FACS) and quantitative PCR strategy. Using CD49f and HLA-G, which are vCT and EVT markers respectively, three distinct populations of cells were identified and isolated from placental villi; vCTs ($CD49f^{+}HLA\text{-}G^{-}$), column EVTs ($CD49f^{lo}HLA\text{-}G^{+}$) and mesenchymal core cells ($CD49f^{-}HLA\text{-}G^{-}$) (Figure 3.1B). The cell-type purity of these cells was verified by qPCR gene expression analysis of cytokeratin-7 (pan-trophoblast marker), HLA-G (EVT-specific marker) and vimentin (mesenchymal marker), where whole placental villi served as a positive control (Figure 3.1C). Consistent with ADAM12 immunofluorescence data, gene expression analysis confirmed that ADAM12S and L isoforms are abundantly expressed in column EVTs compared to vCTs (Figure 3.1D). Moreover, although ADAM12L expression did not significantly differ between column EVTs and mesenchymal core cells, the expression of ADAM12S isoform was found to be significantly higher in column EVTs (Figure 3.1D). Taken together, these findings suggest that ADAM12 isoforms are differentially expressed in distinct placental cell types. While ADAM12L expression is less defined to specific cell types, the ADAM12S variant, which encodes for the secreted form, is preferentially expressed by column EVTs.

3.2.2 ADAM12 preferentially localizes to invasive ECM-degrading EVTs

ADAM12 localization within columnar EVTs prompted us to further examine its expression in highly invasive ECM-degrading EVTs using placental villous explant cultures. These cultures, which mimic *in vivo* EVT column formation and trophoblast invasion, allow for the examination of trophoblast differentiation along the invasive EVT pathway *ex vivo*. ADAM12 immunohistochemistry in placental explants showed that ADAM12 is highly expressed by invasive matrix-degrading EVTs, highlighting a possible role for ADAM12 in promoting trophoblast invasion (Figure 3.2A). Staining serially sectioned placental explants with antibodies directed against cytokeratin-7 and HLA-G confirmed our previous finding that ADAM12 localizes to the synCT layer and the EVT column (Figure 3.2A). Dual labeling of placental explants with antibodies directed against ADAM12 and cytokeratin-7 further confirms that ADAM12 preferentially localizes to EVTs at the invasive front (Figure 3.2B).

3.2.3 ADAM12 is preferentially expressed by invasive trophoblasts

Following examination of ADAM12 localization within first trimester placenta, we aimed to investigate the expression of both ADAM12 isoforms in primary EVTs propagated from placental villi, and invasive HTR8/SVneo and poorly-invasive JEG3 trophoblastic cell lines. Since the invasive potential and cellular composition of trophoblasts is influenced by gestational age [253,254], we further grouped our EVT cultures as early (<10 weeks gestation) or late (≥10 weeks gestation). Total RNA and protein from placental villi functioned as a positive control. Both ADAM12L and ADAM12S mRNA transcripts were shown to be highly expressed in early and late EVTs, and significant

transcript levels were detected in invasive HTR8/SVneo cells while poorly-invasive JEG3 cells had undetectable levels (Figure 3.3A).

ADAM12 immunoblotting in these cells and tissues further supported our gene expression findings. Probing with an antibody that recognizes both ADAM12L and ADAM12S variants, we detected three major proteins bands corresponding to ADAM12 isoforms (68, 90 and 110 kDa), where ADAM12 protein products were found to be highly abundant in placental villi and primary EVTs, moderately abundant in HTR8 cells and undetectable in JEG3 cells (Figure 3.3B). Taken together, ADAM12 expression in primary EVT cultures and the invasive HTR8/SVneo cells suggests that ADAM12 may regulate trophoblast invasion.

3.2.4 Loss of ADAM12 inhibits trophoblast invasion

To test the functional importance of ADAM12 in regulating trophoblast invasion, HTR8/SVneo cells were transiently transfected with four siRNAs targeting both ADAM12 isoforms (A12i-5, -6, -7, -8) or a non-specific (NS) siRNA control. The ADAM12 knockdown efficiency was confirmed at the mRNA and protein level by qPCR analysis or immunoblotting, respectively (Figure 3.4A and B). Two ADAM12 siRNAs (A12i-5 and A12i-8) generated the most efficient knockdown compared to control NS siRNA or mock (transfection reagent alone)-transfected cells (Figure 3.4A). Batch-to-batch variability of the previously used polyclonal ADAM12 antibody prevented us from specifically detecting ADAM12 in endogenous cell lysates, as the new batch of antibody only gave us nonspecific bands. Therefore, in order to confirm siRNA-mediated

reduction of ADAM12 protein, another commercially available polyclonal antibody was used, which only detected the 68kDa ADAM12 protein band (Figure 3.4B).

To test whether loss of ADAM12 impairs trophoblast invasion and migration, HTR8/SVneo cells transfected with ADAM12 siRNAs were subjected to Matrigel-coated/uncoated Transwell invasion/migration assays. ADAM12 knockdown led to a significant reduction in trophoblast invasion and a non-statistically significant, but decreasing trend, in trophoblast migration (Figure 3.4C and D). To rule out the possibility that manipulating ADAM12 expression affected cell proliferation and not cell invasion/migration, siRNA transfected cells were subjected to proliferation assay. Notably, ADAM12 knockdown had no effect on cell proliferation (Figure 3.4E) over the 96 h culture time course. Together, these results suggest that endogenous ADAM12 expression is important for directing trophoblast invasion.

3.2.5 ADAM12 promotes EVT column outgrowth

To examine the functional importance of ADAM12 in regulating EVT column outgrowth, we tested the effects of ADAM12 protease inhibition on EVT column formation using a broad-spectrum ADAM protease inhibitor, TAPI-1. Treatment of villous explant cultures with 25 nM TAPI-1 significantly blocked EVT column outgrowth over 96 h of culture (Figure 3.5A and B).

To more specifically examine the importance of ADAM12 in regulating trophoblast column outgrowth, placental explant cultures were transfected with ADAM12 (A12i-5 or -8) or non-silencing control (NS) siRNAs and cultured over 96 h. ADAM12 knockdown significantly inhibited EVT column outgrowth (Figure 3.5C and D).

Endogenous knockdown of ADAM12 was confirmed by qPCR analysis (Figure 3.5E). To rule out the possibility that ADAM12 suppression may impact *ex vivo* cell survival or cell proliferation, serial sections of explant cultures were probed with immunofluorescence antibodies targeting caspase-cleaved cytokeratin-18 (M30 clone; apoptosis marker) or Ki67 (proliferation marker). Microscopic quantification of positive/negative cells within cell columns demonstrated that ADAM12 knockdown does not affect apoptosis or cell proliferation (Figure 3.5F). Therefore, these findings suggest that ADAM12 promotes EVT column formation most likely by promoting trophoblast invasion.

3.2.6 ADAM12S, but not ADAM12L, promotes trophoblast invasion

To elucidate the individual roles of ADAM12 isoforms in directing trophoblast invasion, HTR8/SVneo and JEG3 cells were stably transfected with pCMV6, pCMV6-GFP (pGFP), pCMV6-ADAM12L (pADAM12L) or pCMV6-ADAM12S (pADAM12S) expression constructs. Immunoblotting of whole cell lysates confirmed the overexpression of both isoforms in the two trophoblastic cell lines (Figure 3.6A and B). For these studies, the empty pCMV6 backbone construct or pGFP expression construct functioned as controls. In agreement with our ADAM12 knockdown studies, overexpression of ADAM12L or ADAM12S did not affect HTR8/SVneo cells proliferation (Figure 3.6C). In contrast, overexpression of either isoforms significantly increased the proliferation of JEG3 choriocarcinoma cells (Figure 3.6D). This suggests that ADAM12 isoforms may exert diverse effects on different subtypes of trophoblasts.

Strikingly, in Transwell invasion assays, ectopic expression of only ADAM12S promoted trophoblast invasion, while ADAM12L over-expression had no effect (Figure 3.6E and F).

3.2.7 ADAM12S's intrinsic metalloproteinase activity is necessary in directing trophoblast invasion

To elucidate whether the metalloproteinase domain of ADAM12S is required in promoting trophoblast invasion, HTR8/SVneo cells overexpressing ADAM12S were subjected to Transwell invasion assays in the presence or absence of the hydroxamate-based ADAM protease inhibitor, TAPI-1. In this experiment, HTR8/SVneo cells transfected with pCMV6 construct served as a control. TAPI-1 treatment significantly reduced ADAM12S-mediated trophoblast invasion compared to control, indicating that its proteolytic activity is important in promoting trophoblast invasion (Figure 3.7A).

To specifically interrogate the importance of ADAM12S's metalloproteinase domain in promoting trophoblast invasion, HTR8/SVneo cells were transiently transfected with the parent wild-type ADAM12S (pADAM12S) construct or a mutant ADAM12S construct (pADAM12S$^{\Delta E351Q}$) harbouring a single point mutation within its proteolytic domain [240]. Ectopic expression of wild-type and mutant forms of ADAM12S was confirmed by immunoblotting; protein lysates from cells transfected with empty pCMV6 or pGFP served as controls (Figure 3.7B). Over-expression of wild-type ADAM12S significantly promoted trophoblast invasion as shown in Figure 3.7C. However, proteolytic-dead ADAM12S failed to promote an invasive phenotype,

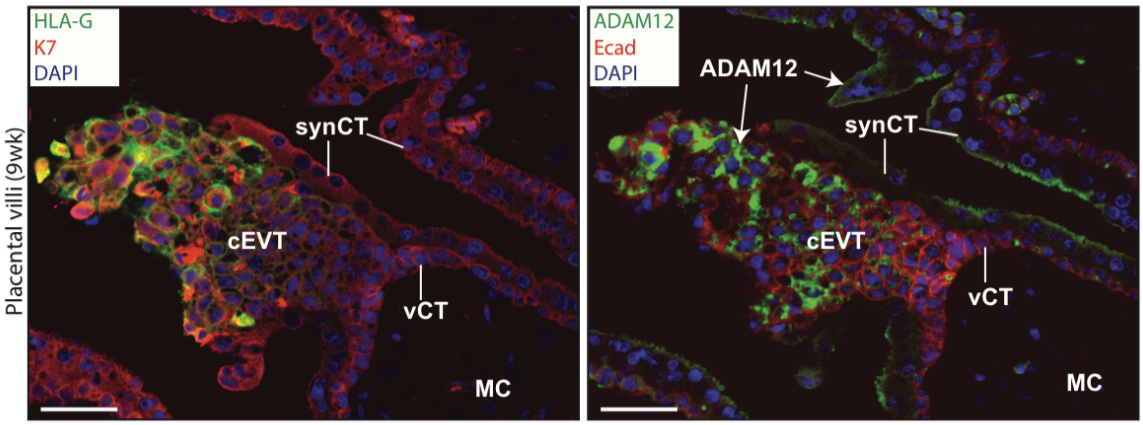
confirming the importance of ADAM12S's intrinsic proteolytic activity in directing trophoblast invasion.

3.2.8 Secreted ADAM12S variant is sufficient to direct EVT column outgrowth

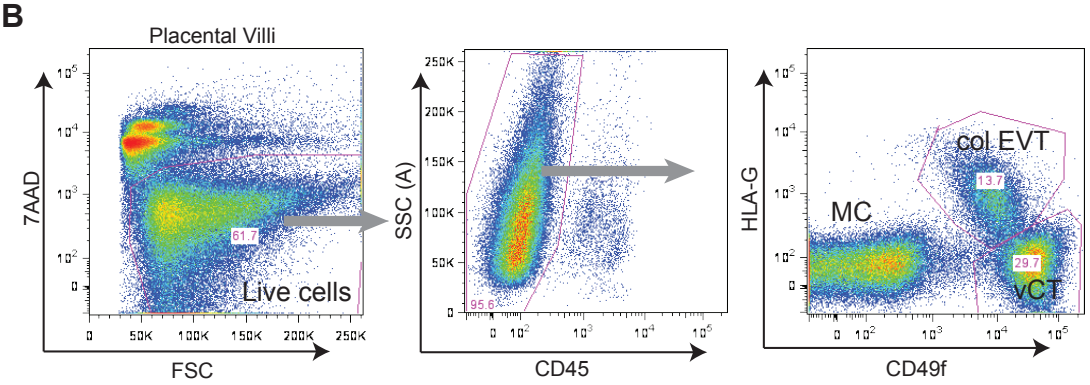
To investigate the importance of secreted ADAM12S in directing *ex vivo* EVT column outgrowth, villous explants were cultured in the presence or absence of conditioned media (CM) containing ectopically expressed ADAM12S. CM derived from pCMV6, pGFP, or pADAM12S$^{\Delta E351Q}$ expressing JEG3 cells served as controls in this experiment (Figure 3.8A and B). The JEG3 cell line was selected to over-express ADAM12S since these cells express low/undetectable levels of ADAM12 and any observed effect would be specific to the exogenous ADAM12S protein. The secretion of both mutant and wild-type ADAM12S into CM was confirmed by immunoblotting (Figure 3.8B). Importantly, CM derived from only wild-type ADAM12S cells significantly promoted EVT column outgrowth (Figure 3.8C and D).

Finally, to exclude the possibility that ectopic soluble ADAM12S might alter column EVT proliferation, dual labelling of serially sectioned ADAM12S and control CM-treated explant cultures for Ki67 and cytokeratin-7 was performed. Quantification of these images via ImageJ analysis showed that soluble ADAM12S does not affect column EVT proliferation (Figure 3.8E and F). These results further confirm our previous finding that ADAM12 specifically promotes EVT column outgrowth by inducing trophoblast invasion, without affecting cellular proliferation.

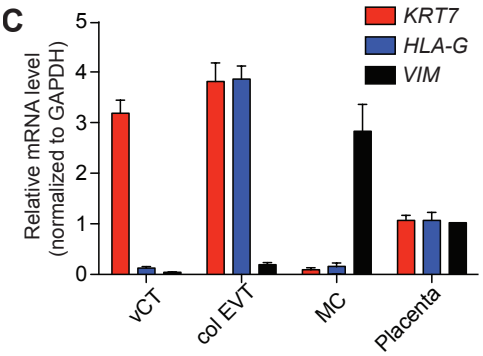
A


HLA-G
K7
DAPI
synCT
cEVT
vCT
MC
Placental villi (9wk)
ADAM12
Ecad
DAPI
ADAM12
synCT
cEVT
vCT
MC

B


Placental Villi
7AAD
FSC
61.7
Live cells
SSC (A)
CD45
95.6
HLA-G
CD49f
col EVT
13.7
MC
29.7
vCT

C


KRT7
HLA-G
VIM
Relative mRNA level (normalized to GAPDH)
vCT
col EVT
MC
Placenta

D

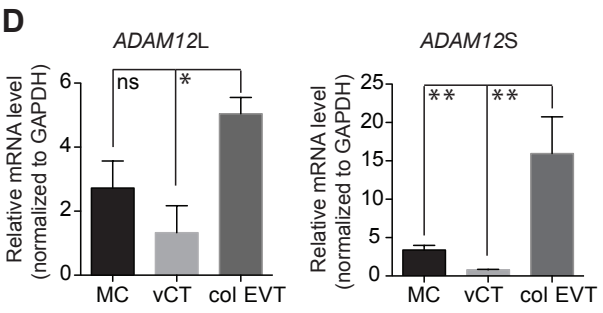
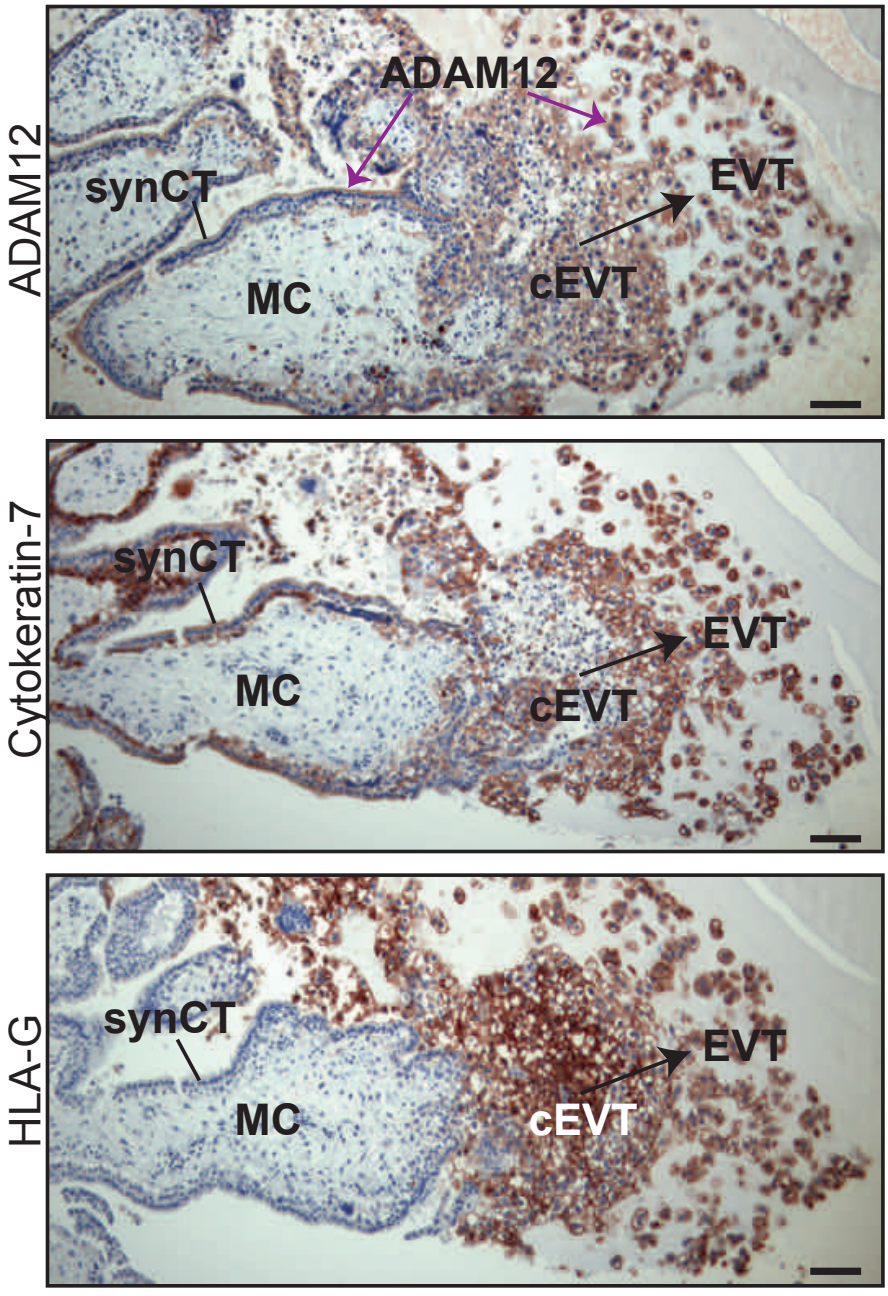

ADAM12L
ADAM12S
Relative mRNA level (normalized to GAPDH)
ns
*
**
**
MC
vCT
col EVT

Figure 3.1 ADAM12 localizes to the multinucleated syncytiotrophoblast layer and EVT anchoring columns in first trimester placental villi. (A) Representative serial sections of 9 weeks gestation human placental villi dual-labeled with HLA-G (green; left) and cytokeratin-7 (red; left) or ADAM12 (green; right) and E-cadherin (red; right). White arrows indicate ADAM12 expression. Nuclei are stained with 4', 6-Diamidino-2-Phenylindole (DAPI; blue). K7, cytokeratin-7; Ecad, E-cadherin; cEVT, columnar extravillous cytotrophoblast; synCT, syncytiotrophoblast; vCT, villous cytotrophoblast; MC, mesenchymal core. Scale bars= 100 μm. (B) Representative fluorescence activated cell-sorting (FACS) plots demonstrating the trophoblast isolation strategy used to purify mesencymal core (MC) cells, columnar EVTs (col EVT) and villous cytotrophoblasts (vCT). Live cells, depleted of $CD31^+$ (endothelial) and $CD45^+$ (immune) cells using immune-magnetic beads, were positively gated by 7AAD exclusion. Cells were further segregated by excluding $CD45^+$ immune cells and by cell surface labeling of HLA-G and CD49f. Cell subtype proportions are indicated within each gated population (percent of cells within FACS plot). (C) Trophoblast subtype purity was assessed by qPCR analysis targeting the pan-trophoblast marker cytokeratin-7 (K7), the EVT-marker HLA-G, and the mesenchymal lineage marker vimentin (VIM); placental villi (Placenta) served as positive control and relative gene expression reference. (D) qPCR analysis of ADAM12L and ADAM12S mRNAs in FACS-purified mesenchymal core cells, vCTs and column EVTs. GAPDH mRNA was used for normalization. Results are presented as mean ± s.e.m. in bar graphs (*P≤ 0.05, **P≤ 0.01) from 4 distinct placental villi specimens (n=4).

A


ADAM12
ADAM12
synCT
MC
cEVT
EVT
Cytokeratin-7
synCT
MC
cEVT
EVT
HLA-G
synCT
MC
cEVT
EVT

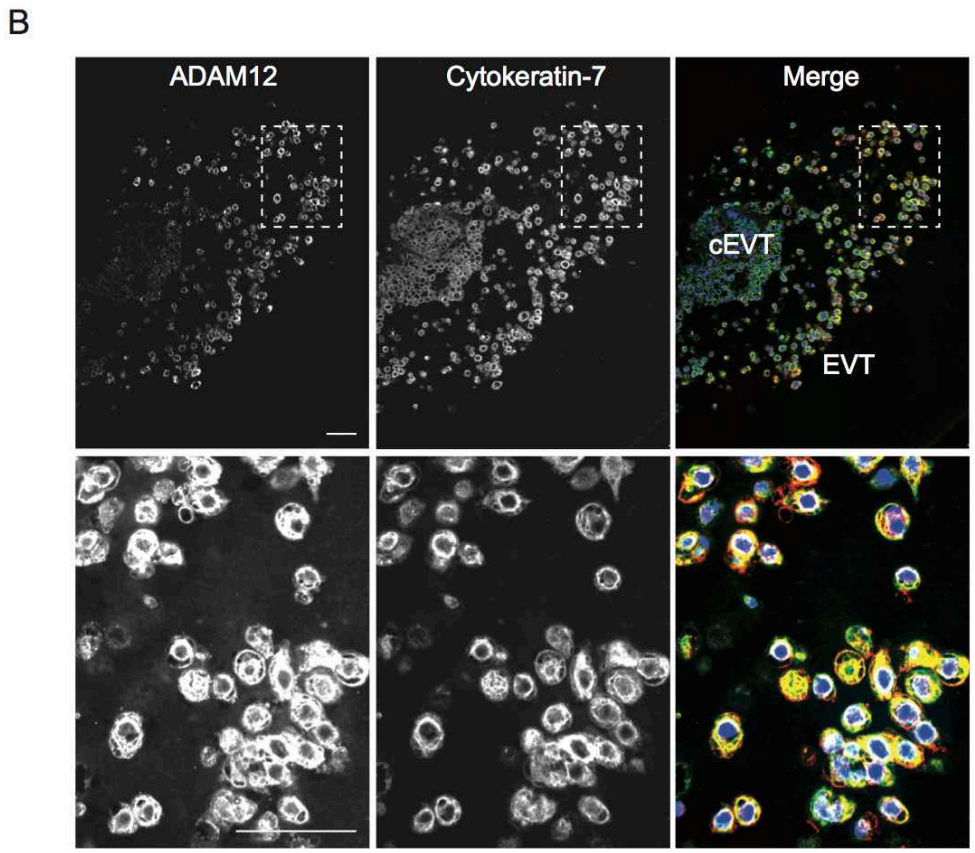


Figure 3.2 ADAM12 is preferentially expressed by invasive ECM-degrading EVTs. (A) Representative images of Matrigel-imbedded villous explants immunostained with antibodies directed against ADAM12, cytokeratin-7 and HLA-G. Black arrows indicate the direction of trophoblast invasion. Purple arrows indicate ADAM12 expression. (B) Dual immunofluorescence labeling of ADAM12 (red) and cytokeratin-7 (green) in Matrigel-imbedded villous explants. Nuclei are stained with 4', 6-Diamidino-2-Phenylindole (DAPI; blue). White dashed box indicates magnified area. Immunostaining was performed on six villous explant cultures obtained from six distinct placentae. MC, mesenchymal core; cEVT, columnar extravillous cytotrophoblast; synCT, syncytiotrophoblast; EVT, area of extravillous cytotrophoblast invasion through matrigel. Scale bars= 100 μm.

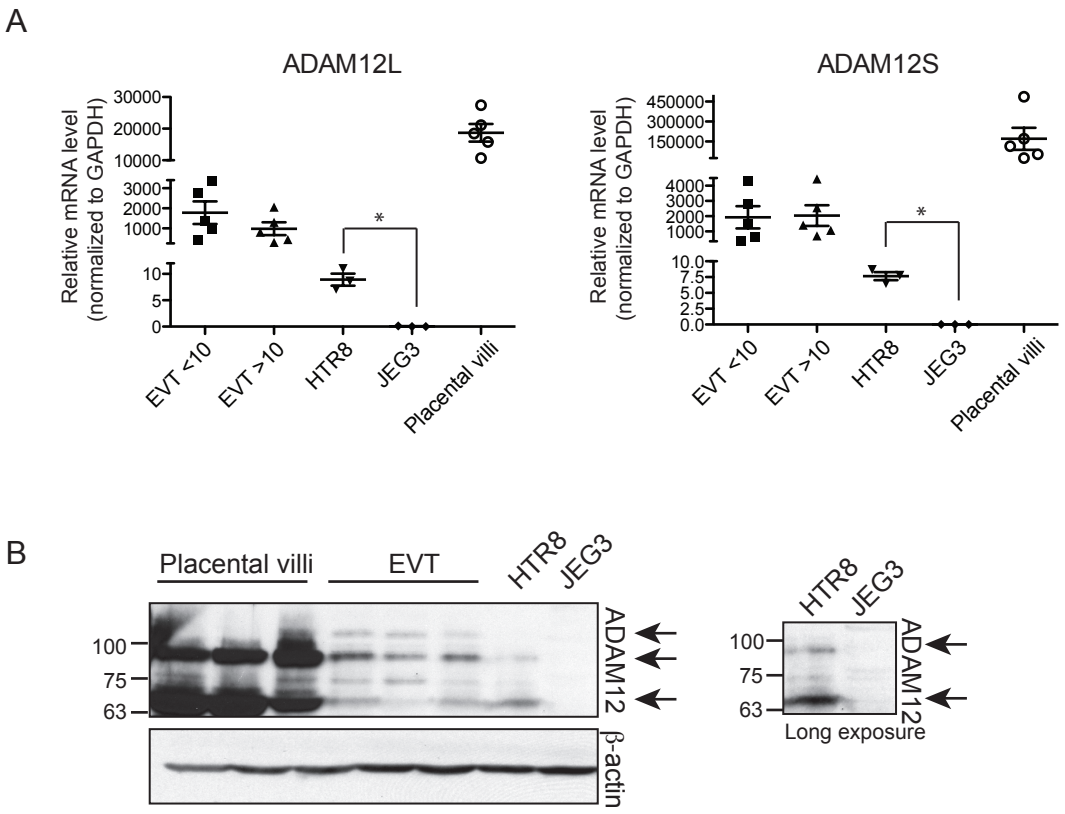


Figure 3.3 ADAM12 is preferentially expressed by invasive trophoblasts. (A) qPCR analysis of ADAM12L and ADAM12S mRNA levels in first trimester placental villi (n=5), EVTs propagated from placental villi (< 10 or ≥10 weeks gestation, n=10), and the two trophoblastic cell lines HTR8/SVneo (n=3) and JEG3 (n=3), which model invasive and proliferative columnar EVTs, respectively. (B) Immunoblot analysis of ADAM12 in first trimester placental villi, primary EVTs obtained from these tissues, and HTR8/SVneo and JEG3 trophoblastic cell lines (left). A longer exposure of the same membrane is also shown on the right to better visualize ADAM12 in HTR8/SVneo and JEG3 cells. Molecular weights (kDa) are shown to the left and arrows indicate ADAM12-specific protein bands. β-actin was used as the loading control. HTR8=HTR8/SVneo. Data are presented as mean ± s.e.m (*$P \leq 0.05$).

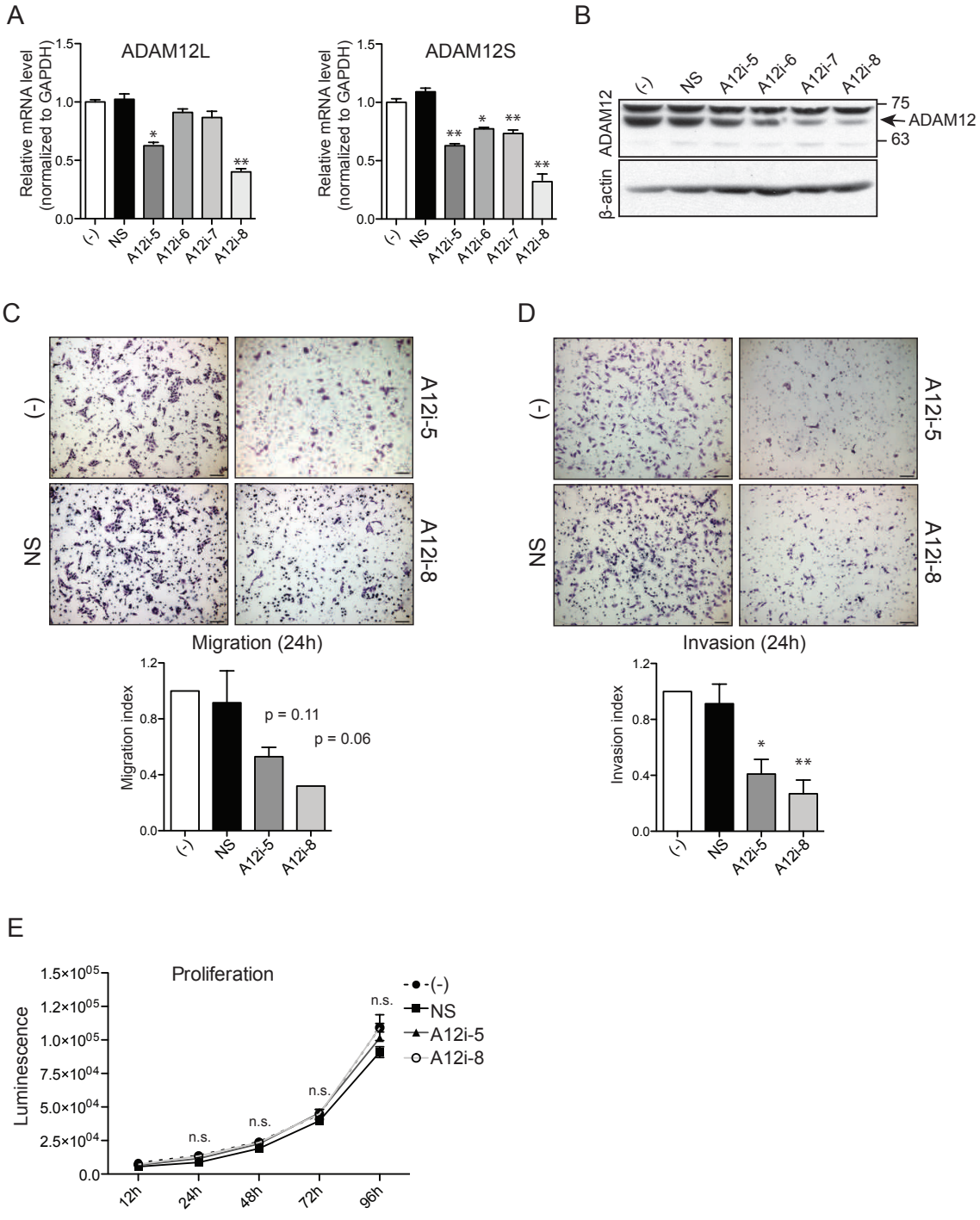
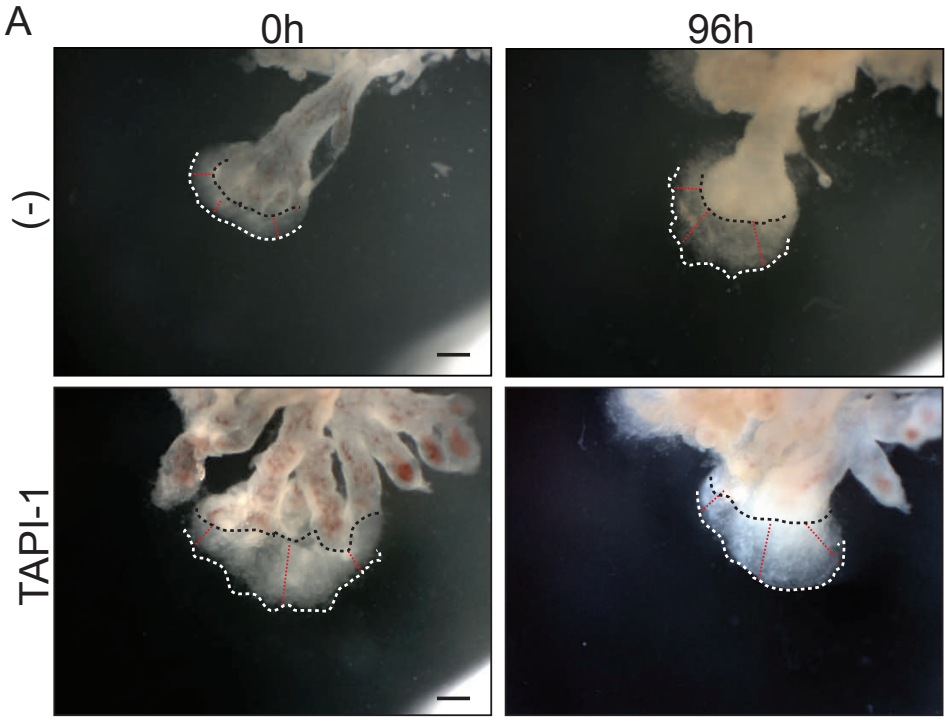
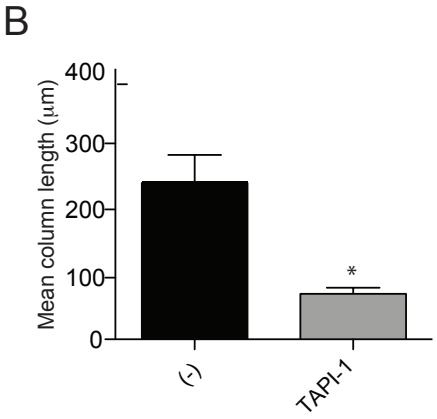
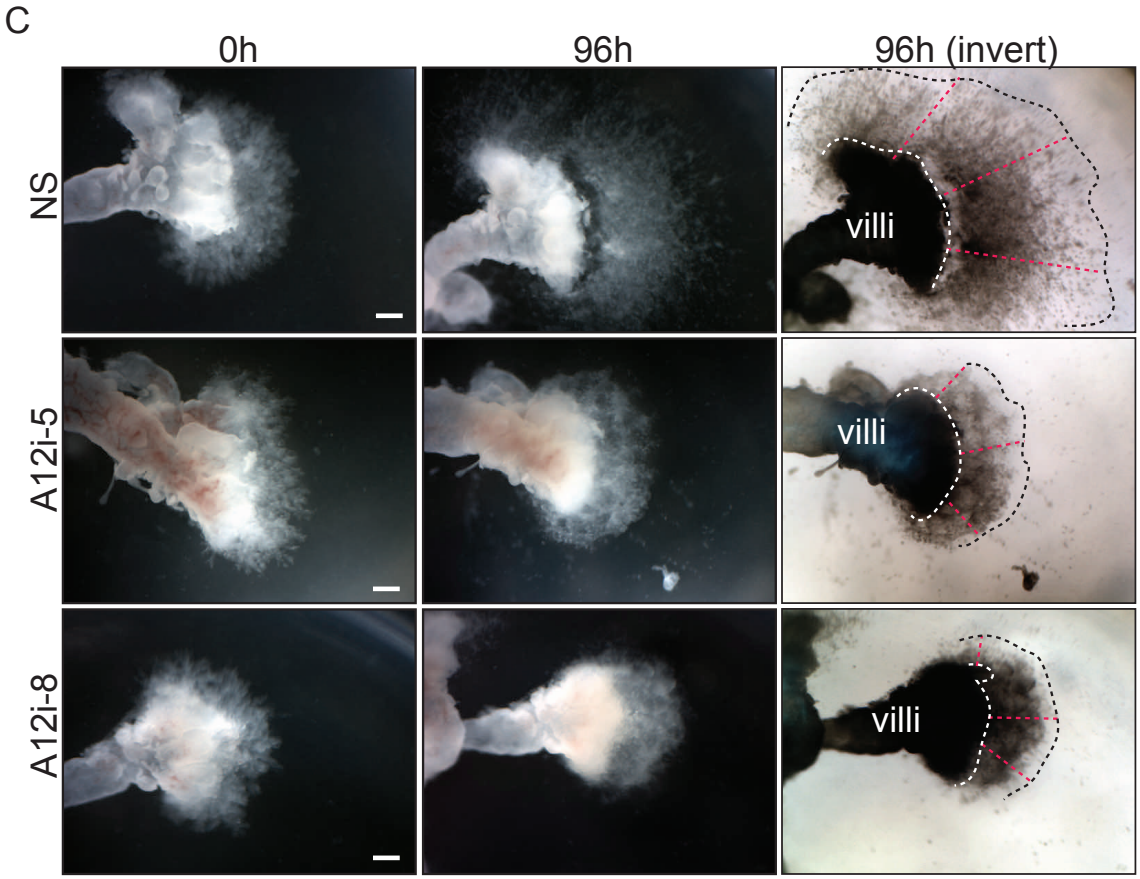
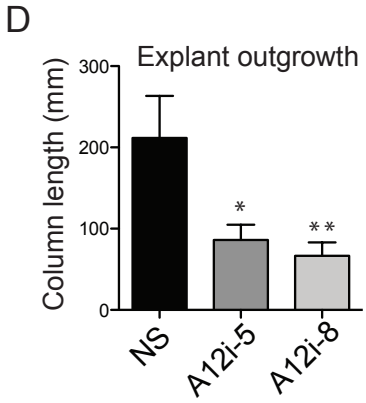

A
ADAM12L
ADAM12S
Relative mRNA level (normalized to GAPDH)
(-)
NS
A12i-5
A12i-6
A12i-7
A12i-8
B
ADAM12
β-actin
75
63
C
D
Migration (24h)
Invasion (24h)
Migration index
Invasion index
p = 0.11
p = 0.06
E
Proliferation
Luminescence
12h
24h
48h
72h
96h
n.s.

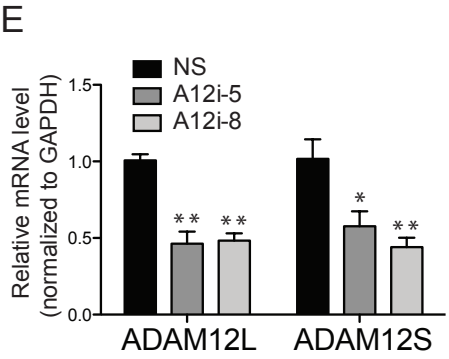
Figure 3.4 Loss of ADAM12 inhibits trophoblast invasion. (A) qPCR analysis of ADAM12 mRNA and (B) immunoblot analysis of ADAM12 levels in HTR8/SVneo cells transfected with non-silencing control (NS), ADAM12-directed siRNA (A12i-5, -6, -7, -8) or transfection reagent alone (-). Molecular weights (kDa) are shown to the right and the arrow indicates ADAM12-specific band. β-actin serves as the loading control. (C and D) Representative images showing Transwell migration and invasion of HTR8/SVneo cells transfected with NS, A12i-5, A12i-8 or transfection reagent alone (-). Invasion and migration assays were performed for 24 h in duplicate and repeated on three independent occasions (n=6). Bar graphs show the quantification of cell invasion and migration. Migration and invasion indices were calculated as the ratio of the number of ADAM12-siRNA transfected cells migrating/invading to the number of NS transfected cells. (E) Quantitative analysis of cell proliferation over 96 h of HTR8/SVneo cells transfected with control or ADAM12-directed siRNAs. Proliferation assay was performed in triplicate and repeated three times. Data are presented as mean ± s.e.m (*$P \leq 0.05$, **$P \leq 0.01$; n.s., not statistically significant when compared to NS transfected cells).


A
0h
96h
(-)
TAPI-1


B
Mean column length (μm)
400
300
200
100
0
*
(-)
TAPI-1


C
0h
96h
96h (invert)
NS
A12i-5
A12i-8
villi
villi
villi


D
Explant outgrowth
Column length (mm)
300
200
100
0
*
**
NS
A12i-5
A12i-8


E
NS
A12i-5
A12i-8
Relative mRNA level
(normalized to GAPDH)
1.5
1.0
0.5
0.0
**
**
*
**
ADAM12L
ADAM12S

F

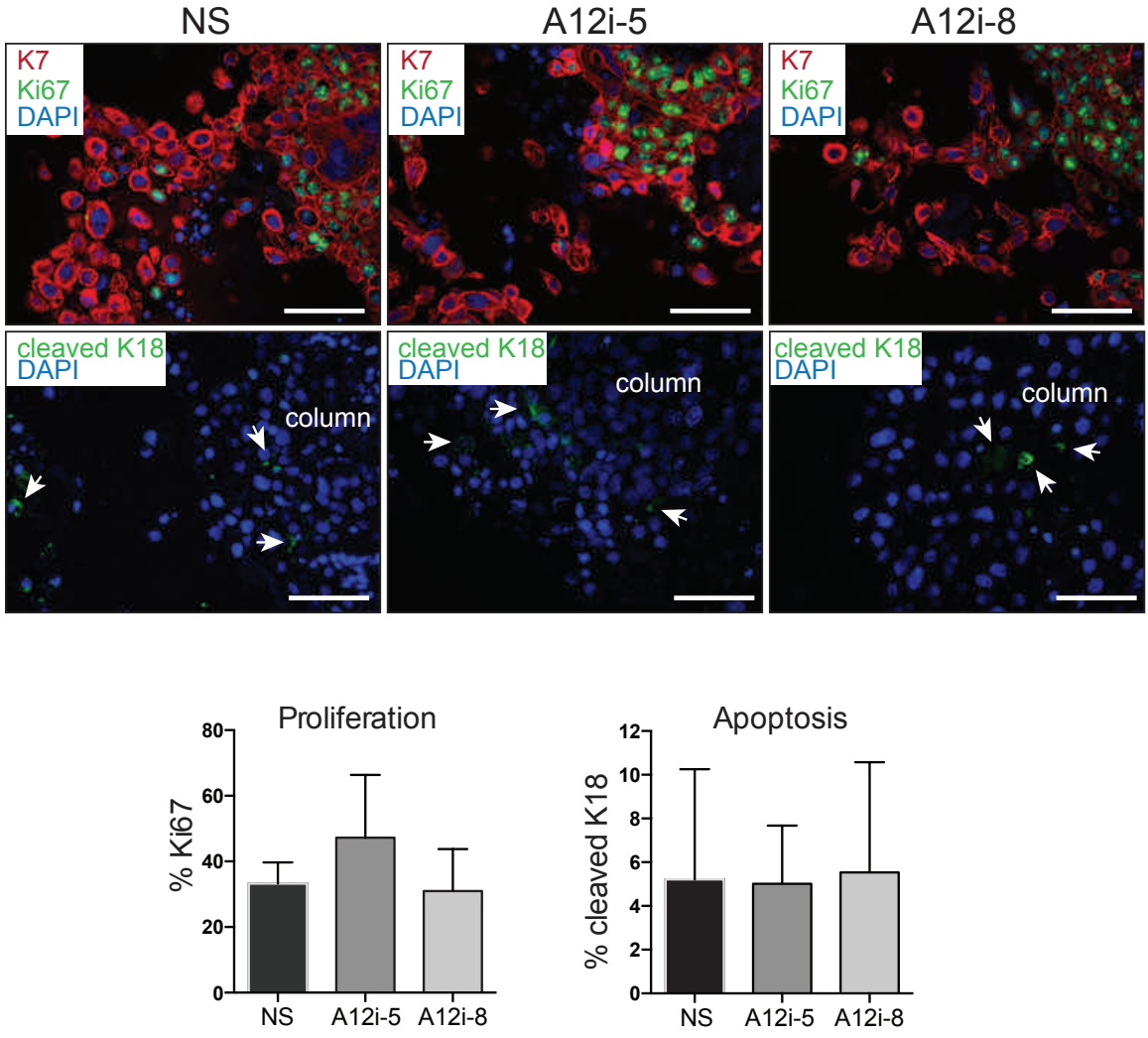

NS
A12i-5
A12i-8
K7
Ki67
DAPI
cleaved K18
DAPI
column
Proliferation
% Ki67
80
60
40
20
0
NS
A12i-5
A12i-8
Apoptosis
% cleaved K18
12
10
8
6
4
2
0
NS
A12i-5
A12i-8

Figure 3.5 ADAM12 inhibition or loss prevents EVT column outgrowth. (A) Representative images of chorionic villous explant cultures imbedded in Matrigel and treated with 20 nM TAPI-1 (broad-spectrum ADAM inhibitor). Images were taken 0 and 96 h post treatment. EVT column outgrowth is calculated as the difference in column length at 96 and 0 h (μm), where column length is measured from the base of each column (black hashed lines) to the invasive front (white hashed lines) at three distinct locations (red hashed lines). Quantification of column length was performed using ImageJ software. Explant cultures were established in duplicate using three distinct placentae (n=6); bars= 200 μm (B) Bar graph represents the quantification of EVT column outgrowth. (C) Representative images of Matrigel-imbedded chorionic villous explant cultures transfected with non-silencing control (NS) or ADAM12-directed siRNAs (A12i-5 or -8). Images were taken 0 and 96 h post transfection. Images at 96 h were inverted to provide a better visualization. Column length is measured from the base of each column (white hashed lines) to the invasive front (black hashed lines) at three distinct locations (red hashed lines). Explant cultures were established in duplicate using six distinct placentae (n=12); bars= 200 μm (D) Bar graph represents the quantification of EVT column outgrowth. (E) qPCR analysis of ADAM12L and ADAM12S mRNA levels in chorionic villous explant cultures transfected with control (NS) or ADAM12-directed siRNAs. (F) Representative images of EVT columns stained with Ki67 (proliferation marker; green), caspase-cleaved cytokeratin-18 (apoptosis marker; green) or cytokeratin-7 (pan-trophoblast marker; red). Nuclei are stained with DAPI (blue). Caspase-cleaved cytokeratin-18 positive cells are indicated with white arrows. Bar graphs represent the percentage of proliferative and apoptotic cells. The percentage of cellular proliferation

and apoptosis was calculated as the number of ki67-positive or caspase-cleaved cytokeratin-18-positive cells into the number of trophoblasts (cytokeratin-7-stained) or total cells (DAPI stained), respectively. K7, cytokeratin-7; cleaved K18, caspase-cleaved cytokeratin-18; column, villous trophoblast column; villi, placental villi. Scale bars= 100 μm. Data are presented as mean ± s.e.m in bar graphs (*$P \leq 0.05$, **$P \leq 0.01$).

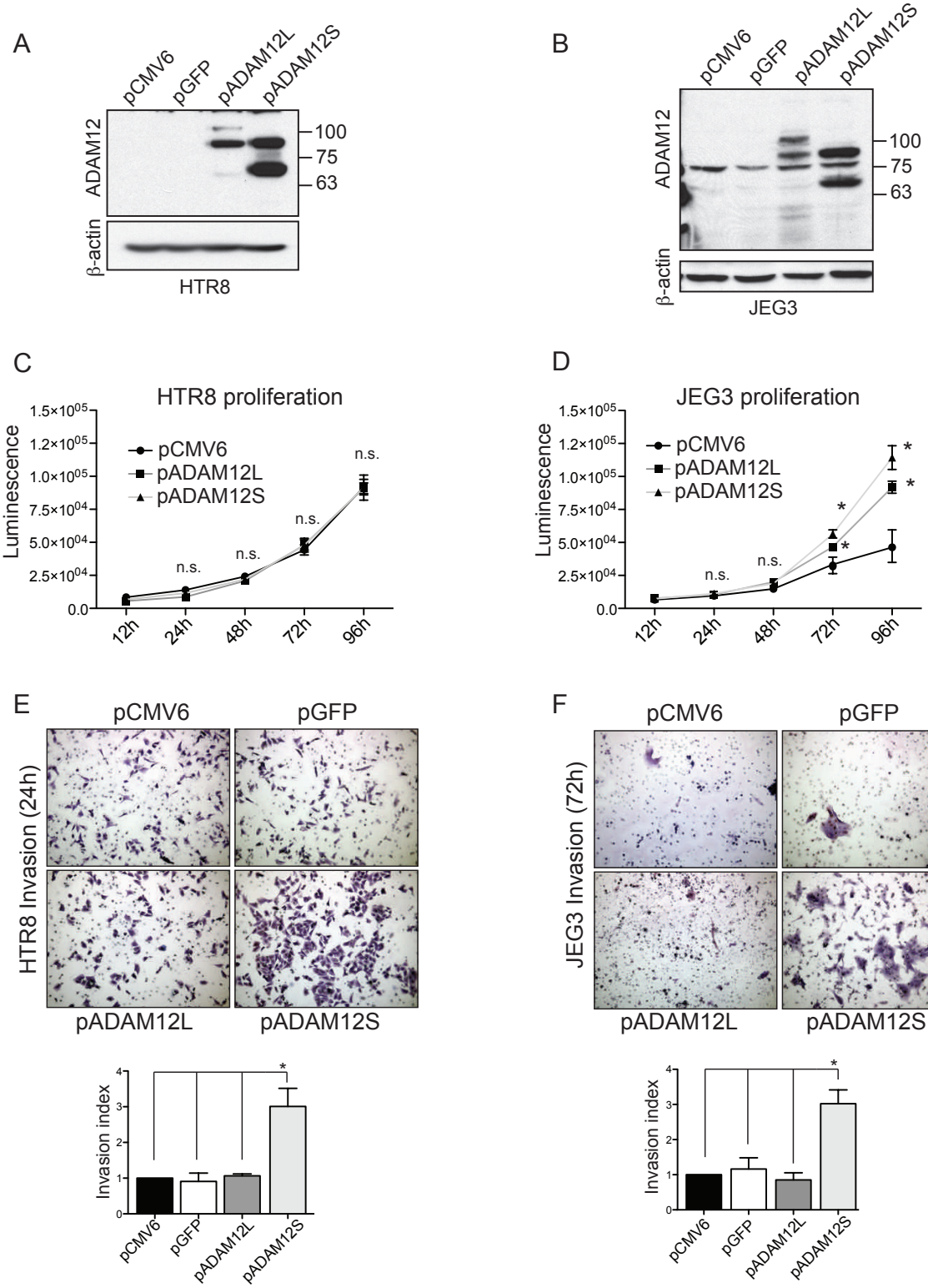
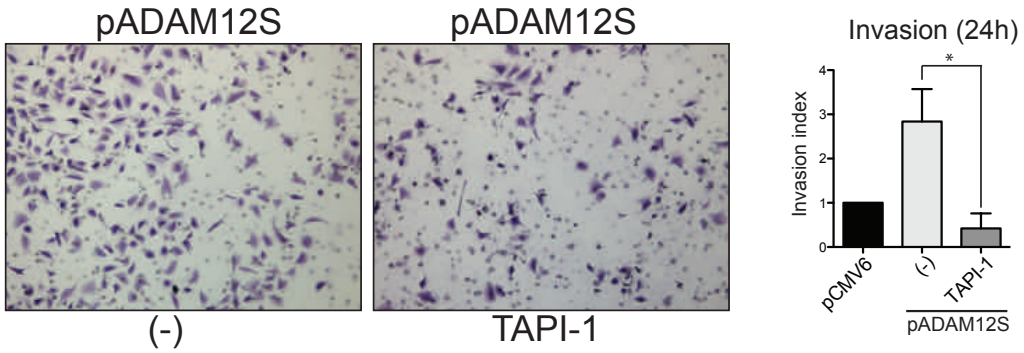
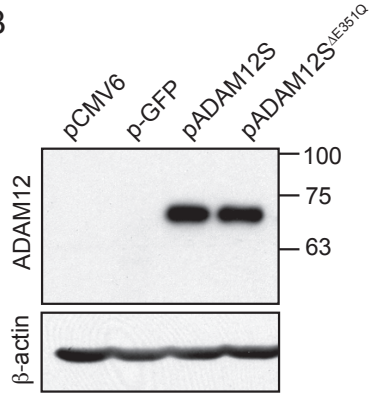

A
pCMV6
pGFP
pADAM12L
pADAM12S
ADAM12
β-actin
100
75
63
HTR8
B
JEG3
C
HTR8 proliferation
Luminescence
pCMV6
pADAM12L
pADAM12S
n.s.
12h
24h
48h
72h
96h
D
JEG3 proliferation
*
E
HTR8 Invasion (24h)
Invasion index
F
JEG3 Invasion (72h)

Figure 3.6 ADAM12S, but not ADAM12L, promotes trophoblast invasion. (A and B) Immunoblot analysis of ADAM12L and ADAM12S in HTR8/SVneo and JEG3 cells stably transfected with pCMV6, pCMV6-GFP (pGFP), pCMV6-ADAM12L (pADAM12L) or pCMV6-ADAM12S (pADAM12S) expression constructs. Molecular weights (kDa) are shown to the right and β-actin serves as the loading control. (C and D) HTR8/SVneo and JEG3 cells stably transfected with pCMV6, pADAM12L and pADAM12S expression constructs were subjected to proliferation assays over 96 h. Proliferation assays were performed in triplicate and were repeated on three independent occasions. Data are presented as mean ± s.e.m (*$P \leq 0.01$; n.s., not statistically significant when compared to pCMV6 transfected cells). (E and F) HTR8/SVneo and JEG3 cells stably transfected with the above-mentioned constructs and subjected to Matrigel-coated Transwell invasion assays over 24 or 72 h. Bar graph shows the quantification of cell invasion. Invasion assays were performed in duplicate on three independent occasions (n=6). Invasion index was calculated as the ratio of the number of ADAM12 overexpressing cells invading through the Matrigel to the number of pCMV6 transfected cells. Data are presented as mean ± s.e.m. (*$P \leq 0.01$).

A


pADAM12S
pADAM12S
(-)
TAPI-1
Invasion (24h)
Invasion index
*
pCMV6
(-)
TAPI-1
pADAM12S

B


pCMV6
p-GFP
pADAM12S
pADAM12S$^{\Delta E351Q}$
ADAM12
β-actin
100
75
63

C

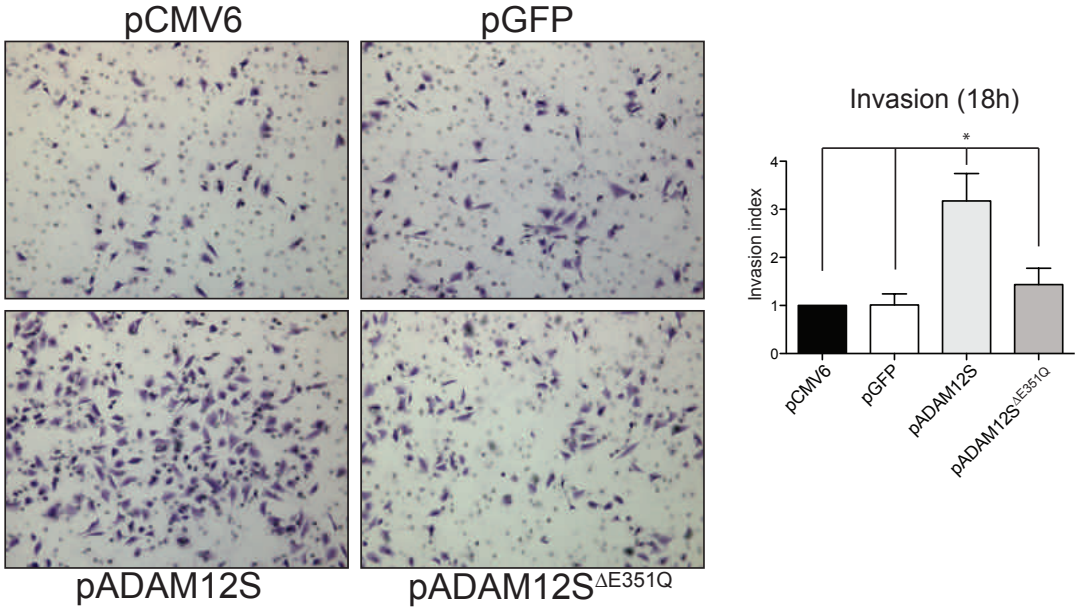
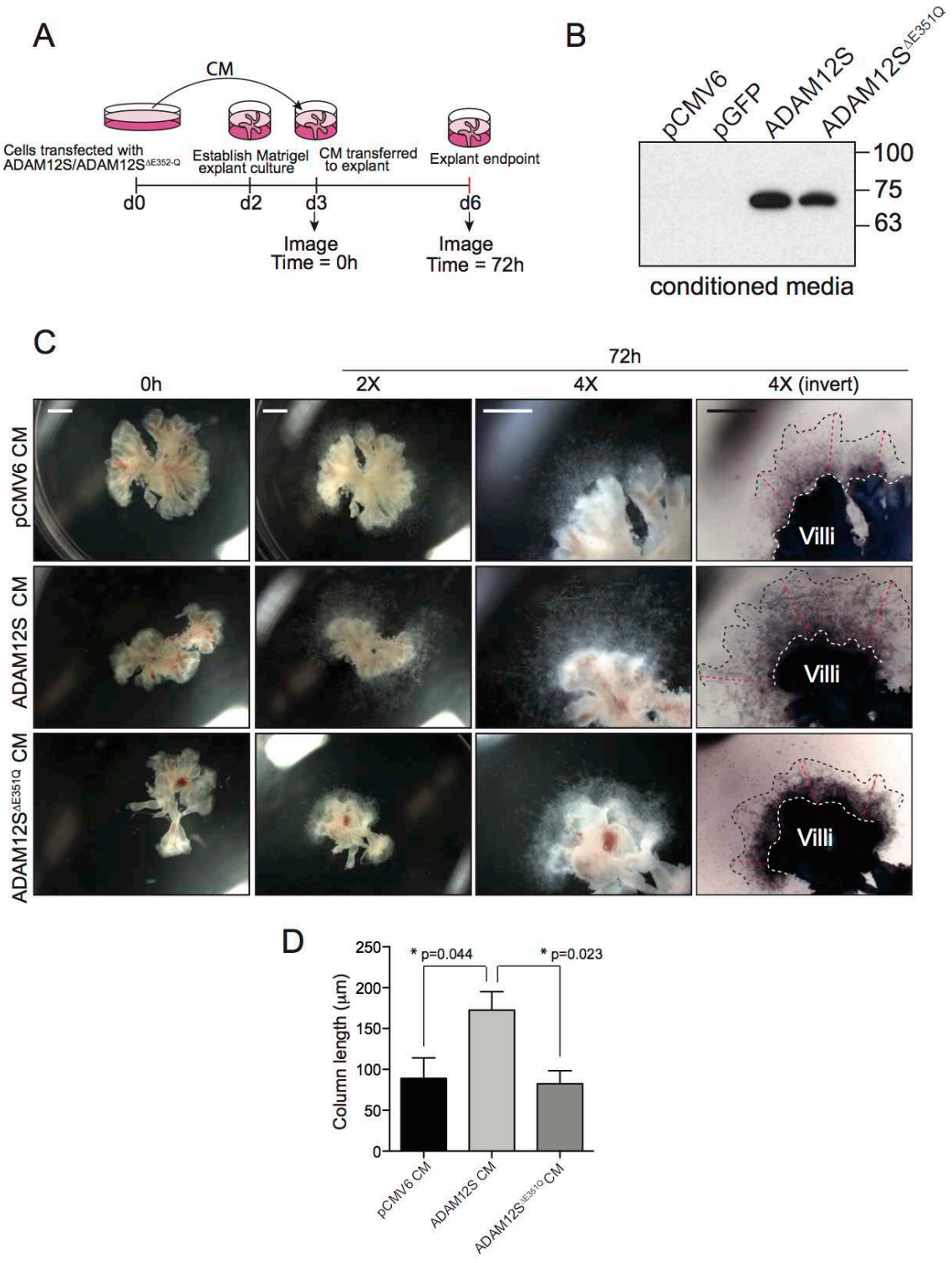
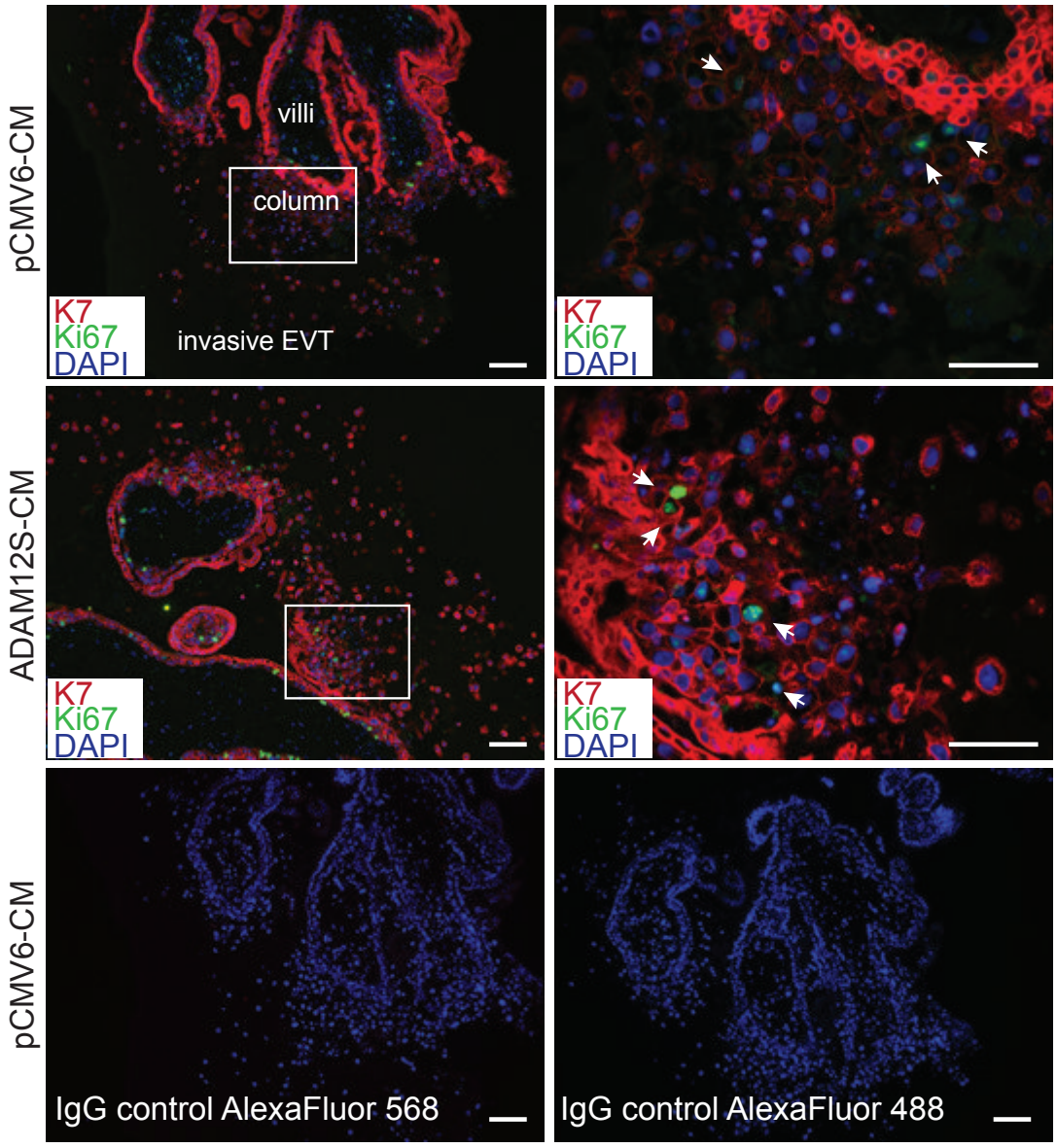

pCMV6
pGFP
pADAM12S
pADAM12S$^{\Delta E351Q}$
Invasion (18h)
Invasion index
*
pCMV6
pGFP
pADAM12S
pADAM12S$^{\Delta E351Q}$

Figure 3.7 ADAM12S' intrinsic metalloproteinase activity is necessary in directing trophoblast invasion. (A) HTR8/SVneo cells stably overexpressing ADAM12S were subjected to Transwell invasion assay in the presence or absence of 20 nM TAPI-1. Bar graph shows the quantification of cell invasion. Invasion index was calculated as the ratio of the number of TAPI-1-treaded cells invading through the Matrigel to the number of control cells (B) Immunoblot analysis of ADAM12 in HTR8/SVneo cells transiently transfected with pCMV6, pCMV6-GFP (pGFP), pCMV6-ADAM12S (pADAM12S) or pADAM12S$^{\Delta E351Q}$ (mutant ADAM12S harboring a single point mutation within its proteolytic domain) expression constructs. Molecular weights (kDa) are shown to the right and β-actin serves as the loading control. (C) Representative images of Matrigel-coated Transwell invasion assay of HTR8/SVneo cells transiently transfected with the above-mentioned expression constructs. Invasion assay was performed in duplicate on three independent occasions (n=6). Bar graph shows the quantification of cell invasion. Invasion index was calculated as the ratio of the number of ADAM12S overexpressing cells invading through the Matrigel to the number of pCMV6 transfected cells. Data are presented as mean ± s.e.m. (*$P \leq 0.01$).


A
CM
Cells transfected with ADAM12S/ADAM12S$^{\Delta E352-Q}$
Establish Matrigel explant culture
CM transferred to explant
Explant endpoint
d0
d2
d3
d6
Image Time = 0h
Image Time = 72h
B
pCMV6
pGFP
ADAM12S
ADAM12S$^{\Delta E351Q}$
100
75
63
conditioned media
C
0h
72h
2X
4X
4X (invert)
pCMV6 CM
ADAM12S CM
ADAM12S$^{\Delta E351Q}$ CM
Villi
Villi
Villi
D
* p=0.044
* p=0.023
Column length (µm)
250
200
150
100
50
0
pCMV6 CM
ADAM12S CM
ADAM12S$^{\Delta E351Q}$ CM

E


pCMV6-CM
villi
column
K7
Ki67
DAPI
invasive EVT
K7
Ki67
DAPI
ADAM12S-CM
K7
Ki67
DAPI
K7
Ki67
DAPI
pCMV6-CM
IgG control AlexaFluor 568
IgG control AlexaFluor 488

F

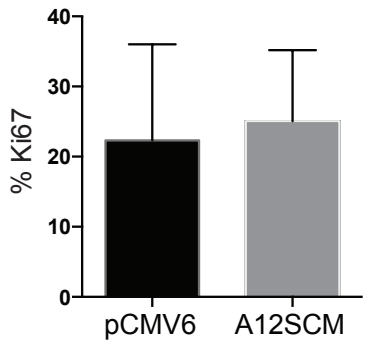

% Ki67
40
30
20
10
0
pCMV6
A12SCM

Figure 3.8 Secreted ADAM12S variant is sufficient in directing EVT column outgrowth. (A) Schematic diagram shows the experimental procedure. CM, conditioned media; d, day. (B) Immunoblot analysis of CM harvested from JEG3 cells transiently transfected with pCMV6, pGFP, pADAM12S or pADAM12S$^{\Delta E351Q}$ expression constructs. Membrane was probed with an antibody directed against ADAM12. Molecular weights (kDa) are shown to the right. (C) Representative images of chorionic villous explant cultures treated with CM harvested from JEG3 cells transfected with the above-mentioned constructs. Images were captured at 0 and 72 h of culture at 2X and 4X magnification. EVT column outgrowth is calculated as the difference in column length at 72 and 0 h (μm), and measured from the base of each column (white hashed lines) to the invasive front (black hashed lines) at three distinct locations (red hashed lines). Explant cultures were established in duplicate using three distinct placentae (n=6). (D) Bar graph represents the quantification of EVT column outgrowth. Scale bars= 400 μm. (E) Representative images of explant cultures dual-labeled with Ki67 (green) and cytokeratin-7 (k7; red) antibodies. Nuclei are stained with DAPI (blue). White box indicates magnified area and white arrows show ki67-positive cells within EVT column. The specificity of the secondary antibodies was confirmed by staining the explant cultures with secondary antibodies alone (goat anti-mouse Alexa Fluor 586; goat anti-rabbit Alexa Fluor 488). Villi, placental villi; column, EVT column. Scale bars= 100 μm. (F) Bar graph represents the percentage of proliferative cells calculated as the number of ki67-positive cells into the number of trophoblasts (cytokeratin-7-positive). Immunofluorescence analysis was performed in duplicate on two independent occasions (n=4). Data are presented as mean ± s.e.m. (*$P \leq 0.05$).

3.3 Discussion

In this study, we demonstrate that ADAM12 localizes to invasive trophoblast populations and promotes trophoblast invasion. Specifically, ADAM12S isoform was shown to regulate trophoblast invasion through a mechanism requiring its intrinsic proteolytic activity. Previous studies have shown that ADAM12 is highly expressed in human placenta, and its reduced levels are associated with several pregnancy complications, however, this is the first study to describe a role for this protease in directing trophoblast invasion and EVT column formation, both of which are fundamental processes during placental development [143,164].

ADAMs are multidomain proteins capable of regulating diverse biological processes, including cellular adhesion, intracellular signal transduction and proteolysis. To date, over-expression of several ADAMs, including ADAM10, 12 and 17, have been observed in a variety of human carcinomas, where they have been shown to promote tumor growth and metastasis by regulating the bioavailability of several growth factors, growth factor receptors, as well as other membrane-bound proteins [145,255,256]. Specifically, ADAM12-mediated cleavage of HB-EGF has been shown to promote tumor cell invasion in an EGFR-dependent or -independent manner [145,257,258]. Since previous studies have suggested a stimulatory effect for HB-EGF/EGFR signalling on trophoblast invasion, it seems possible that ADAM12 promotes trophoblast invasion and EVT column outgrowth by induction of HB-EGF processing and subsequent activation of EGFR signalling [259]. Besides HB-EGF, IGFBP-3 and -5 are the other known substrates for ADAM12 where their proteolysis increases the bioavailability of IGF-I and IGF-II, both of which are considered to be important mediators of trophoblast invasion, placental development and

fetal growth [144,260–262]. Since ADAM12 expression and IGFBP degradation increase during pregnancy, we can speculate that ADAM12-mediated cleavage of IGFBP-3 and -5 might be a potential mechanism for increasing IGF activity during pregnancy.

Although we were able to assign a role only for the ADAM12S isoform in promoting an invasive phenotype, a recent publication by Biadasiewicz *et al*. has suggested the involvement of both isoforms in regulating trophoblast invasion [263]. Localization of ADAM12L to the cell surface is required for its biological functions, however, not much is known about the mechanism in which ADAM12L translocates to the cytoplasmic membrane. Studies have shown that both transmembrane and cytoplasmic domains of ADAM12L are rate-limiting factors for its export from endoplasmic reticulum (ER) and Golgi apparatus, and its subsequent localization to the cell membrane [264,265]. Indeed, removal of the cytoplasmic domain of ADAM12L has been shown to prevent its intracellular retention and facilitate its plasma membrane localization [265]. Therefore, it is possible that due to using a truncated form of ADAM12L, which lacks the cytoplasmic tail, Biadasiewicz *et al*. observed a more pronounced effect of this variant in promoting trophoblast invasion [263]. Even though we could not assign a role for ADAM12L in regulating trophoblast invasion, it does not rule out the possibility of the involvement of this variant in regulating other cellular pathways important in placental development, including trophoblast proliferation and survival. Therefore, further studies are required to examine the biological significance of ADAM12L during trophoblast differentiation. Since ADAM12L is the only ADAM12 isoform expressed in mice, studies on mouse models might reveal a role for this variant in trophoblast biology and placental development.

Our finding that only ADAM12S promotes trophoblast invasion was not really surprising since others have also reported functional differences between ADAM12 isoforms in different biological systems. For instance, while both ADAM12 variants have been reported to promote cell invasion of head and neck squamous cell carcinoma (HNSCC), only the secreted ADAM12S isoform is associated with *in vitro* invasion and *in vivo* metastasis of breast cancer and small cell lung cancer cells [160,248,266].

ADAM12 isoforms have been previously reported to exert growth-promoting or -inhibiting effects on certain types of transformed and non-transformed cells, perhaps by regulating the bioavailability of growth factors and cytokines. For example, while ADAM12 has been shown to induce chondrocyte and adipocyte proliferation, its expression seems to suppress myoblast and osteoblast proliferation [116,161,267,268]. Other studies have also shown that ADAM12 expression promotes cellular proliferation and tumour growth in several types of cancers including breast cancer, osteosarcoma and prostate cancer [160,269,270]. Sharing several similarities to cancer cells including their proliferative and invasive capacities, it was expected that ADAM12 isoforms also regulate trophoblast proliferation. However, our results showed that ADAM12 does not alter trophoblast proliferation, even though both ADAM12 isoforms significantly increased JEG3 choriocarcinoma proliferation. This contradictory result further suggests that ADAM12 isoforms may impart different biological effects across diverse cellular systems likely involving differences in cell signalling networks (i.e. state of transformation) and the cellular niche.

Although we showed that the intrinsic proteolytic activity of ADAM12 is necessary for its role in promoting trophoblast invasion, the possible involvement of its

ancillary domains in other aspects of trophoblast differentiation and function should not be underestimated. Studies have shown that the disintegrin and cysteine rich domains of ADAM12 interact with syndecans and several types of integrins including α7β1, α5β1 and αvβ3 [156,159,271,272]. This interaction has been suggested to regulate diverse cellular processes such as cell adhesion, spreading and migration [157,273]. As trophoblasts acquire an invasive phenotype, ADAM12 and integrin β1 expression significantly increases [274]. Therefore, one can hypothesize that ADAM12 interaction with integrin β1 may affect lamellipodia formation and promote trophoblast adhesion. Moreover, ADAM12 and α9β1 interaction has been previously reported to mediate myoblast adhesion and fusion, suggesting possible involvement of ADAM12 and integrins in trophoblast fusion and syncytialization [275].

The prodomain and the cytoplasmic domain of ADAM12 are also considered to be important regulators of its activity. For example, ADAM12 has been shown to mediate MMP14 activation and subsequent tumor progression as a function of its prodomain [276]. Likewise, the cytoplasmic domain of ADAM12 has been reported to physically interact with several proteins including c-Src and ILK, where this interaction is suggested to promote the formation of invadopodia and focal adhesion structures important in cell migration [154,155]. Taken together, it should be kept in mind that ADAM12 can regulate trophoblast biology not only through its intrinsic proteolytic activity but also via mechanisms involving its ancillary domains.

In summary, our study describes a novel role for ADAM12 in regulating trophoblast invasion and EVT column formation, and highlights ADAM12 as a key regulator of early placental development. Our findings suggest that placental trophoblasts

are responsible for the elevated levels of ADAM12 detected in the serum of pregnant women and that defects in trophoblast differentiation and ADAM12 expression may be the underlying causes of several pregnancy complications such as IUGR and preeclampsia. Further studies are required to elucidate the underlying mechanisms regulating ADAM12 expression and function within human placental trophoblasts.

CHAPTER 4. IDENTIFYING THE MOLECULAR MECHANISM REGULATING ADAM12 EXPRESSION AND FUNCTION

4.1 Introduction

Despite the central roles that ADAM12 plays in normal and pathological conditions, little is known about regulatory mechanisms controlling *ADAM12* gene expression. Transforming growth factor-beta (TGF-β) regulates *ADAM12* expression in mammary epithelial cells and activated hepatic stellate cells [249,277]. Within these cell types, TGF-β drives *ADAM12* transcription via activation of phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase kinase (MEK), nuclear factor-kappa B (NF-κB) and Smad 2/3 signalling pathways [249,277,278]. In addition to TGF-β, EGFR and HER2 signalling pathways have been reported to induce ADAM12 expression through PI3K and c-Jun N-terminal kinase (JNK) pathways [266]. A recent study has also suggested a role for IL-1β in mediating ADAM12 expression in mouse myoblasts [279]. Although regulatory circuits central in mediating ADAM12 expression are beginning to become elucidated, no information exists with respect to the effects of these growth factors and cytokines on ADAM12 expression in the placenta.

GPCRs agonists such as angiotensin II have been previously suggested to induce ADAM12 expression through post-translational activation of MMP7, ultimately leading to the development of hypertension and cardiac hypertrophy in mouse models [280]. One of the intracellular signalling molecules produced upon GPCR activation is the second messenger cAMP, which has been shown to regulate a wide variety of biological processes including cell proliferation, invasion and migration. Importantly, cAMP has

been shown to regulate trophoblast invasion and migration[220,229,231]; however, the exact mechanisms by which cAMP promotes trophoblast invasion are not well understood. Studies have suggested that cAMP signalling promotes trophoblast invasion by enhancing the expression and secretion of key proteases involved in this process including MMP2, MMP9 and uPA [229,232,238]. Interestingly, as depicted in Figure 2.4, the 1200 bp promoter region of *ADAM12* contains at least one cAMP response element (CRE) indicating that cAMP may play a key role in activating *ADAM12* transcriptional activity. Indeed, we have recently shown that cAMP/PKA signalling induces syncytialization of primary vCTs and BeWo choriocarcinoma cells by up-regulating *ADAM12* expression [239]. This finding suggests that cAMP signalling might also regulate *ADAM12* expression in other trophoblast subtypes. Therefore, in this chapter I sought to determine the effects of the cAMP signalling on *ADAM12* expression and function in the invasive trophoblast populations.

4.2 Results

4.2.1 cAMP treatment induces *ADAM12* expression by regulating its promoter activity

To examine the role of cAMP signalling in regulating ADAM12 expression, HTR8/SVneo cells were cultured over 72 h in the presence of the cell permeable cAMP analog (8-Br-cAMP) or with the adenylyl cyclase activator, forskolin (FSK). cAMP induction resulted in an increase in ADAM12L and ADAM12S mRNA levels, as determined by qPCR analysis. Specifically, the increase in ADAM12 mRNA levels was

notable by 24 h of culture and then consistently subsides over 48 and 72 h (Figure 4.1A and B). Immunoblotting protein lysates of HTR8 cells treated with cAMP identified a 68 kDa product that peaked by 72 h of culture; this band size corresponds to the active cleaved form of ADAM12 (Figure 4.1C).

To examine if cAMP is capable of activating *ADAM12*'s promoter activity, HTR8/SVneo cells were transiently transfected with a luciferase promoter reporter construct containing a 1237-bp *ADAM12* promoter fragment (pEZX-A12); the empty pEZX-con construct served as a control for these studies. Following transfection, cells were cultured with or without cAMP, and *ADAM12* promoter activity was analyzed after 24 h. As shown in Figure 4.1D, cAMP treatment significantly increased the luciferase activity driven by the *ADAM12* promoter. Together, these findings demonstrate that cAMP induces *ADAM12* expression by directly inducing the activity of its regulatory promoter sequence.

4.2.2 cAMP regulates ADAM12 expression through a PKA-independent mechanism

To directly interrogate the effect of PKA signalling on ADAM12 expression, HTR8/SVneo cells were treated with 8-Br-cAMP in the presence or absence of the PKA inhibitors, PKI or H89. PKI contains a pseudo-substrate sequence, which binds to PKA's catalytic subunits and prevents it from phosphorylating its downstream substrates, thereby blocking PKA enzymatic activity [281]. PKI can also inhibit PKA function through its nuclear export signal (NES) sequence, which upon binding to the PKA catalytic subunit induces its export from the nucleus [282,283]. On the other hand, H89 inhibits PKA activity by competing with ATP for binding to the catalytic subunits of the kinase [284].

While PKI has been shown to be a PKA-specific inhibitor, H89 has been suggested to inhibit the function of eight other kinases including Rho-associated protein kinase 2 (ROCK2) and mitogen- and stress-activated protein kinase-1 (MSK1) [285].

To our surprise, co-treatment of HTR8/SVneo cells with either inhibitor in combination with cAMP actually potentiated ADAM12 mRNA and protein expression (Figure 4.2A and B). Importantly, treatment with either PKI or H89 alone did not affect baseline ADAM12 levels, indicating that steady-state PKA activity does not regulate ADAM12 expression (Figure 4.2A and B).

To further investigate this unexpected finding, the effect of cAMP-directed PKA activation on ADAM12 expression was tested in primary EVT cultures. Consistent with our previous findings that cAMP induced ADAM12 expression in HTR8 cells, FSK and IBMX (an inhibitor of PDEs) treatment increased presence of the ADAM12S isoform, but not the ADAM12L variant (Figure 4.2C). Moreover, treatment with the PKA antagonist, PKI, in combination with FSK/IBMX resulted in a modest, but significant, increase in ADAM12S mRNA levels while ADAM12L mRNA levels were not affected (Figure 4.2C). Consistent with our findings in HTR8 cell line, PKI treatment in the absence of FSK/IBMX did not affect ADAM12 expression in EVTs. Taken together, these surprising findings suggest that PKA does not directly regulate ADAM12 expression. However, the observed ADAM12 induction upon cAMP treatment in combination with PKA antagonists suggests that the PKA holoenzyme competes for intracellular cAMP with other downstream effectors of cAMP.

4.2.3 ADAM12 mRNA but not protein expression is regulated by Epac signalling

To examine the possibility of PKA-independent events responsible for cAMP induction of *ADAM12*, we investigated the involvement of Epac proteins, which are cAMP-dependent GEFs for small GTPases Rap1 and Rap2 [286]. The ability of the Epac-selective cAMP analog (8-CPT-2Me-cAMP [287]) to stimulate *ADAM12* promoter activity was first examined using our previously described 1200bp *ADAM12* promoter fragment luciferase construct (Figure 2.4). 8-CPT-2Me-cAMP treatment enhanced *ADAM12* promoter activity, as shown in Figure 4.3A. To further elucidate the effect of Epac regulation on *ADAM12* expression, we next examined the effect of the Epac-specific cAMP analog on ADAM12 mRNA and protein levels using qPCR and immunoblotting approaches. Treatment with the cAMP analog increased ADAM12 mRNA levels, however, this effect was not mirrored at the protein level, as 8-CPT-2Me-cAMP did not induce the ADAM12 68 kDa product in HTR8 cells (Figure 4.3B and C). Based on these findings, Epac activation appears to not be sufficient for mediating the effect of cAMP-induced *ADAM12* expression in trophoblastic cells.

4.2.4 cAMP treatment promotes HTR8/SVneo cells migration

Despite the well-recognized effects of cAMP in regulating trophoblast endocrine functions and synCT formation, its role in mediating trophoblast differentiation along the invasive pathway is controversial. Thus, we investigated the effect of cAMP on trophoblast migration using the HTR8/SVneo cell line. HTR8/SVneo cells were subjected to Transwell migration assays in the presence or absence of FSK/IBMX; in these studies DMSO served as the negative control. Our results show that cAMP

induction leads to a pronounced increase in trophoblast migration (Figure 4.4).

4.2.5 cAMP-induced cell migration is partially blocked by ADAM12 knockdown

To investigate the involvement of ADAM12 in cAMP-induced trophoblast migration, we tested the effect of FSK/IBMX in ADAM12 silenced HTR8 cells. Specifically, HTR8/SVneo cells were transfected with one of two ADAM12-directed siRNAs (A12i-5 or A12i-8), and following 24 h of culture, were subjected to Transwell migration assays in the presence of FSK/IBMX. The extent of siRNA-directed ADAM12 knockdown was assessed via qPCR and immunoblot analyses (Figure 4.5A and B). Notably, loss of ADAM12 significantly dampened the pro-migratory effect of FSK/IBMX (Figure 4.5C). However in this experiment, only A12i-8 siRNA inhibited baseline and cAMP-directed trophoblast migration (Figure 4.5C). These findings indicate ADAM12 is a downstream effector in cAMP-induced trophoblast migration.

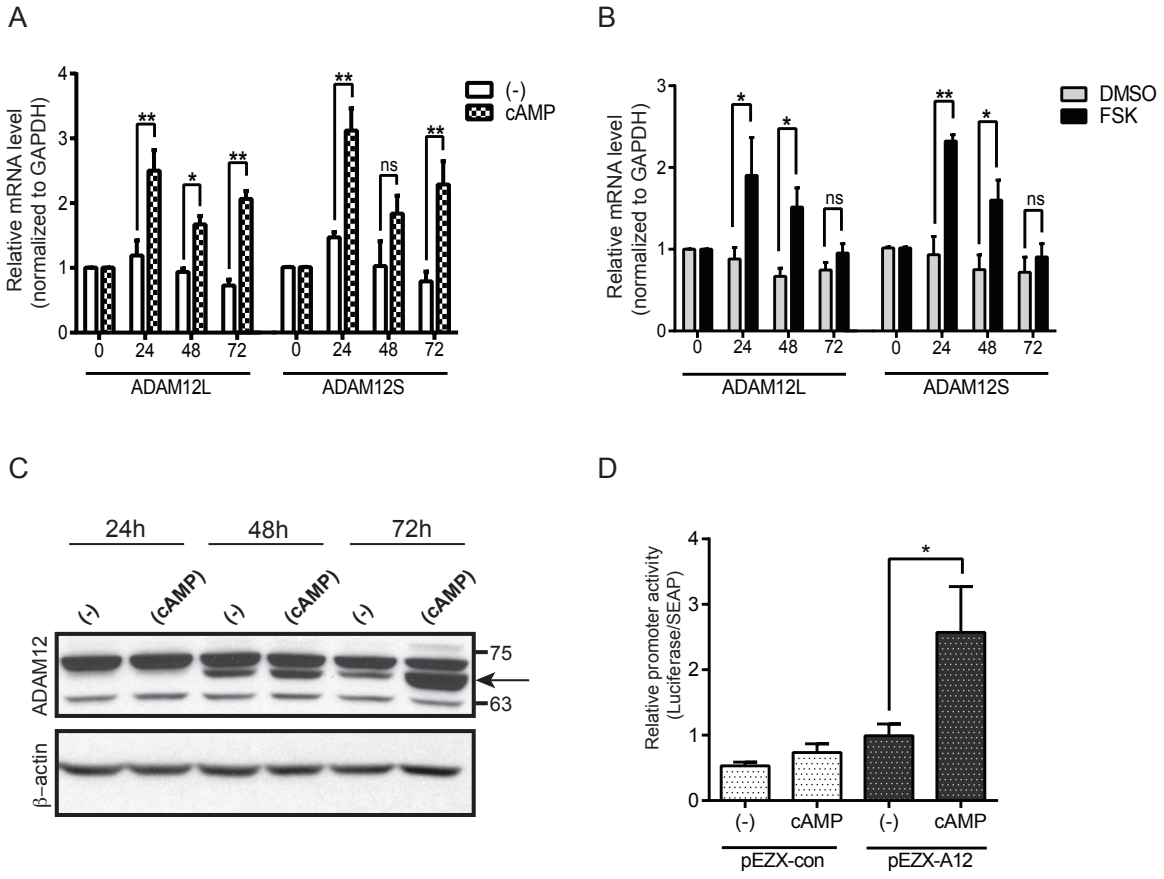

A
Relative mRNA level (normalized to GAPDH)
(-)
cAMP
0 24 48 72 0 24 48 72
ADAM12L
ADAM12S
B
Relative mRNA level (normalized to GAPDH)
DMSO
FSK
0 24 48 72 0 24 48 72
ADAM12L
ADAM12S
C
24h
48h
72h
(-)
(cAMP)
ADAM12
β-actin
75
63
D
Relative promoter activity (Luciferase/SEAP)
(-) cAMP (-) cAMP
pEZX-con
pEZX-A12

Figure 4.1 *ADAM12* expression and promoter activity are regulated by cAMP. (A and B) qPCR analysis of ADAM12L and ADAM12S mRNA levels in HTR8/SVneo cells treated with 1 mM 8-Br-cAMP or 25 μM Forskolin over 72 h. (C) Immunoblot representing ADAM12 in HTR8/SVneo cells treated with 1 mM 8-Br-cAMP over 72 h. Molecular weights (kDa) are shown to the right and the arrow indicates ADAM12-specific protein band. β-actin serves as the loading control. (D) Analysis of the effect of 8-Br-cAMP treatment on *ADAM12* promoter activity in HTR8 cells transiently transfected with either the *ADAM12* promoter reporter construct (pEZX-A12) or the control vector (pEZX-con). Promoter activity was analyzed 24 h after 8-Br-cAMP treatment. Gene expression and promoter activity experiments were performed in triplicate and repeated three times. Data are presented as mean ± s.e.m. (*$P \leq 0.05$, **$P \leq 0.01$; n.s., not statistically significant when compared to control). cAMP, 8-Br-cAMP; FSK, forskolin.

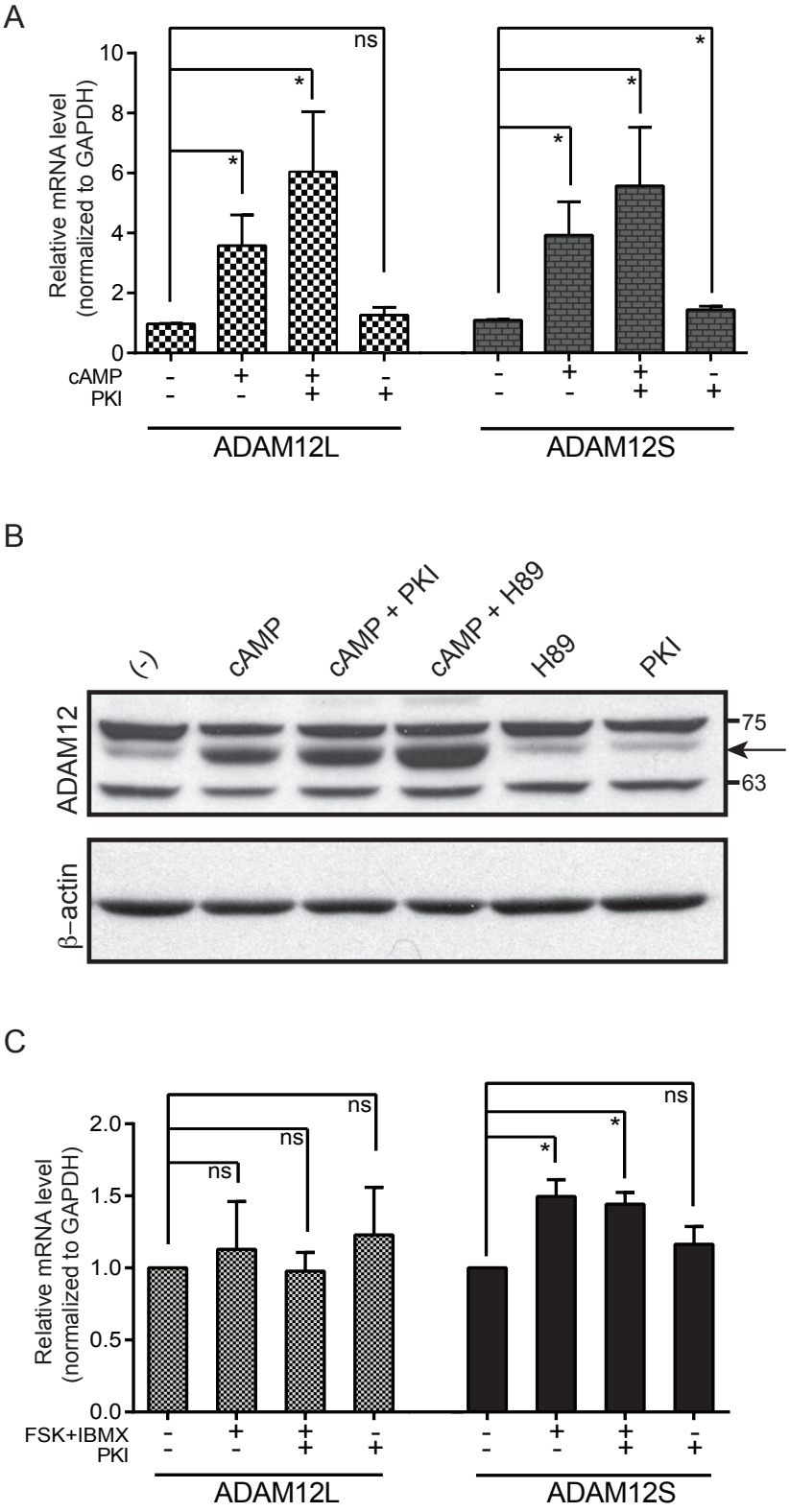
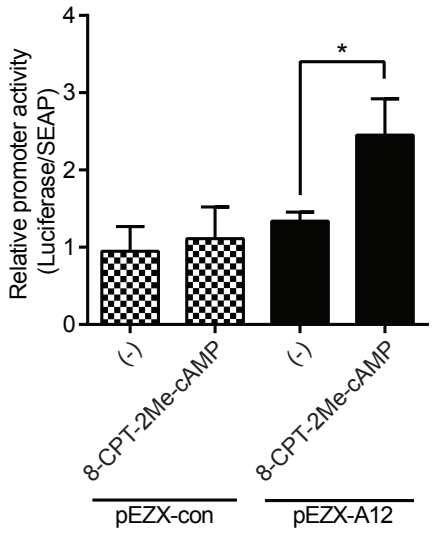
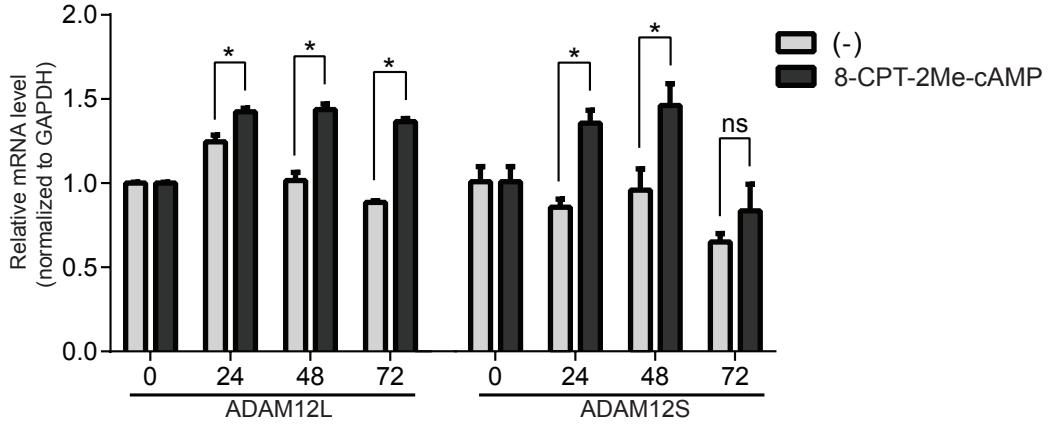

A
Relative mRNA level
(normalized to GAPDH)
10
8
6
4
2
0
ns
*
*
*
*
*
cAMP
PKI
ADAM12L
ADAM12S
B
(-)
cAMP
cAMP + PKI
cAMP + H89
H89
PKI
ADAM12
β–actin
75
63
C
Relative mRNA level
(normalized to GAPDH)
2.0
1.5
1.0
0.5
0.0
ns
ns
ns
ns
*
*
FSK+IBMX
PKI
ADAM12L
ADAM12S

Figure 4.2 cAMP regulates ADAM12 *expression* through a PKA-independent mechanism. (A) qPCR analysis of ADAM12L and ADAM12S mRNA levels in HTR8/SVneo cells treated with 1 mM 8-Br-cAMP in the presence or absence of PKI for 24 h. (B) Immunoblot representing ADAM12 in HTR8 cells treated with 8-Br-cAMP in the presence or absence of PKA inhibitors (PKI or H89) (72 h). Molecular weights (kDa) are shown to the right and the arrow indicates the ADAM12-specific band. β-actin serves as the loading control. (C) qPCR analysis of ADAM12L and ADAM12S mRNA levels in primary EVTs obtained from first trimester placentae (n=3) and treated with 25 μM FSK and 100 μM IBMX in the presence or absence of PKI for 24 h. Gene expression experiments were performed in triplicate and repeated three times. Data are presented as mean ± s.e.m. (*$P \leq 0.05$; n.s., not statistically significant when compared to control). cAMP, 8-Br-cAMP; FSK, forskolin.

A


Relative promoter activity
(Luciferase/SEAP)
4
3
2
1
0
*
(-)
8-CPT-2Me-cAMP
(-)
8-CPT-2Me-cAMP
pEZX-con
pEZX-A12

B


Relative mRNA level
(normalized to GAPDH)
2.0
1.5
1.0
0.5
0.0
*
*
*
*
*
ns
(-)
8-CPT-2Me-cAMP
0
24
48
72
0
24
48
72
ADAM12L
ADAM12S

C

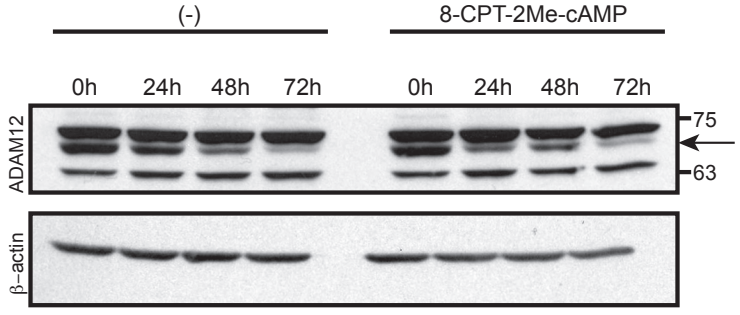

(-)
8-CPT-2Me-cAMP
0h
24h
48h
72h
0h
24h
48h
72h
ADAM12
75
63
β-actin

Figure 4.3 cAMP promotes *ADAM12* expression through an Epac-independent mechanism. (A) Analysis of the effect of 8CPT-2Me-cAMP treatment on *ADAM12* promoter activity in HTR8 cells transiently transfected with either the *ADAM12* promoter reporter construct (pEZX-A12) or the control vector (pEZX-con). Promoter activity was analyzed 24 h after 8CPT-2Me-cAMP treatment. (B) qPCR analysis of ADAM12L and ADAM12S mRNA levels in HTR8/SVneo cells treated with 100 μM 8CPT-2Me-cAMP over 72 h. Gene expression and promoter activity experiments were performed in triplicate and repeated three times. (C) Immunoblot shows the levels of ADAM12 in HTR8/SVneo cells treated with 100 μM 8CPT-2Me-cAMP over 72 h. Molecular weights (kDa) are shown to the right and the arrow indicates the ADAM12-specific band. β-actin serves as the loading control. Data are presented as mean ± s.e.m. (*$P \leq 0.05$; n.s., not statistically significant when compared to control).

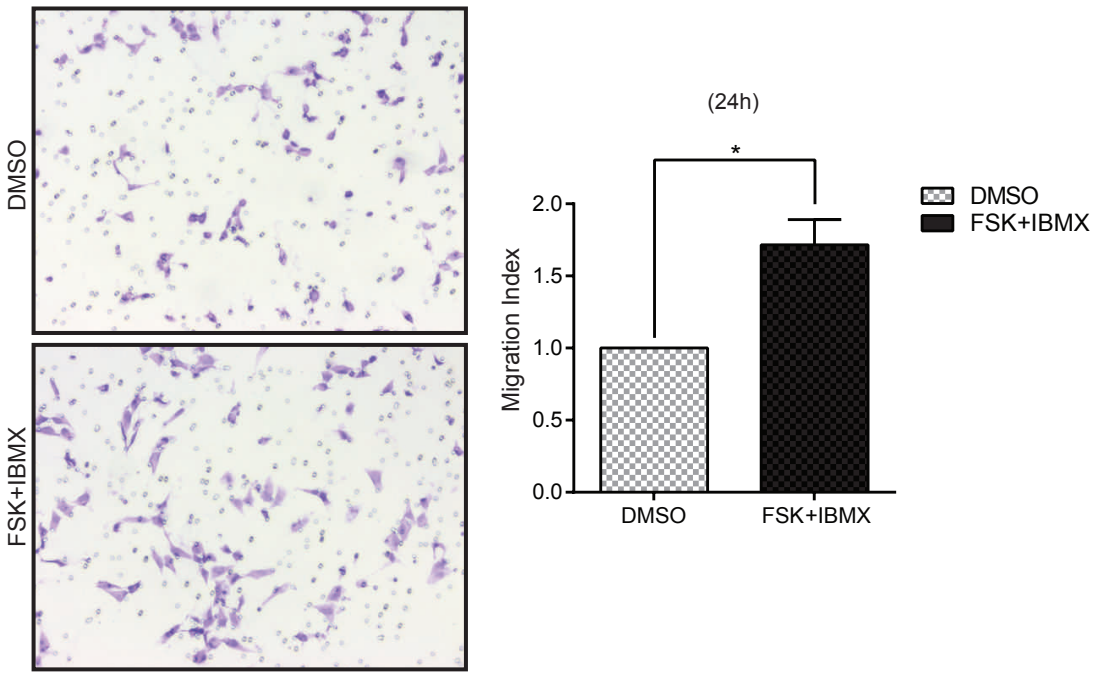


Figure 4.4 An increase in the intracellular cAMP level promotes trophoblast migration. Representative images of HTR8/SVneo cells subjected to collagen-coated Transwell migration assay in the presence or absence of 25 μM FSK and 100 μM IBMX. Migration assay was performed in duplicate and repeated on three independent occasions (n=6). Bar graph shows the quantification of cell migration. Migration index was calculated as the ratio of the number of FSK/IBMX-treaded cells migrating to the number of DMSO-treated cells. Data are presented as mean ± s.e.m. (*$P \leq 0.05$). FSK, forskolin.

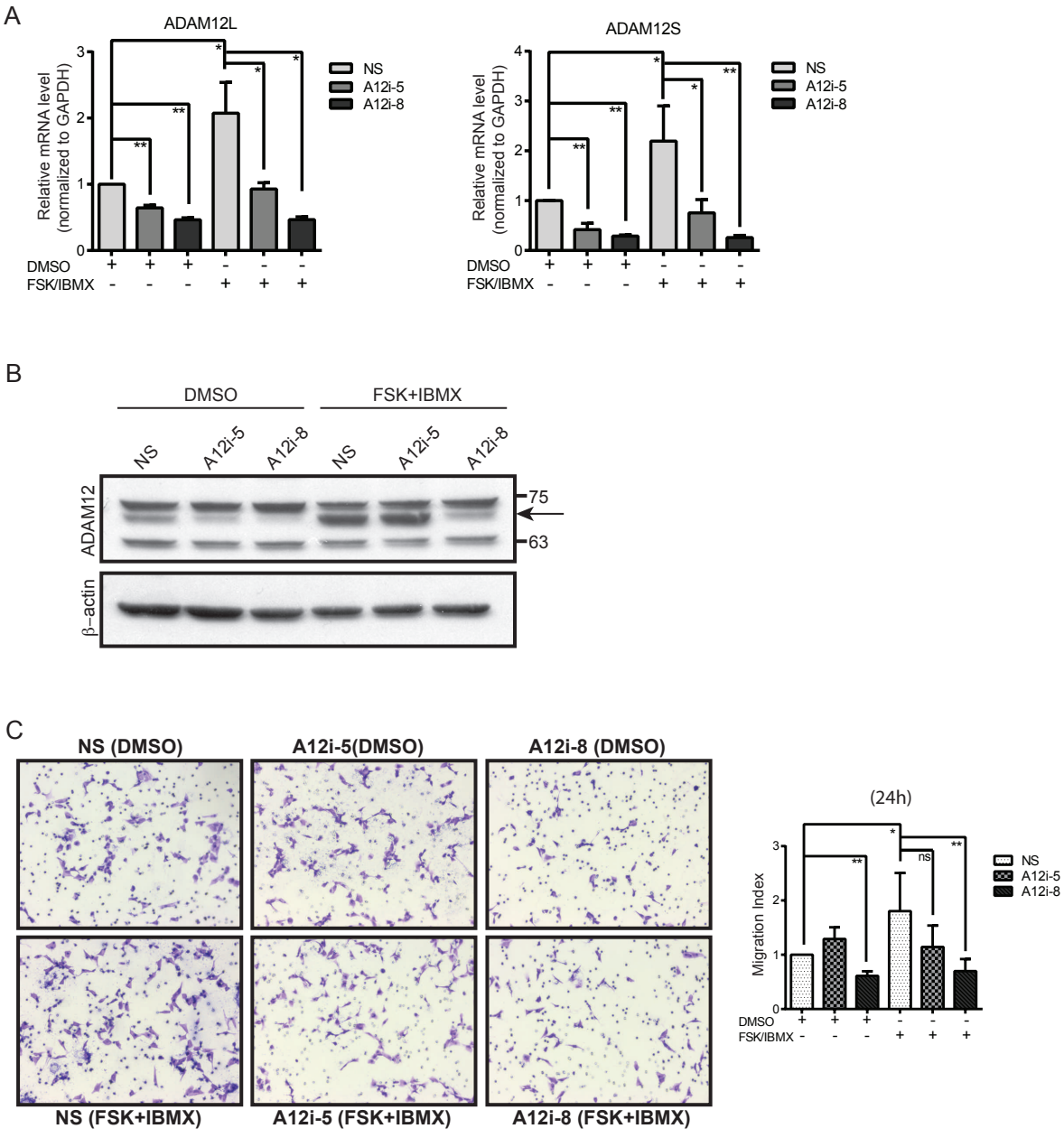

A
ADAM12L
Relative mRNA level (normalized to GAPDH)
NS
A12i-5
A12i-8
DMSO
FSK/IBMX
ADAM12S
B
DMSO
FSK+IBMX
NS
A12i-5
A12i-8
ADAM12
β-actin
75
63
C
NS (DMSO)
A12i-5(DMSO)
A12i-8 (DMSO)
NS (FSK+IBMX)
A12i-5 (FSK+IBMX)
A12i-8 (FSK+IBMX)
(24h)
Migration Index
ns

Figure 4.5 ADAM12 knockdown abolishes cAMP-induced trophoblast migration. (A and B) qPCR and Immunoblot analyses of ADAM12 expression in HTR8/SVneo cells transfected with two ADAM12-directed siRNAs (A12i-5 and A12i-8) and treated with DMSO or 25 μM Forskolin and 100 μM IBMX. Molecular weights (kDa) are shown to the right and the arrow indicates the ADAM12-specific band. β-actin serves as the loading control. (C) Representative images of HTR8/SVneo cells transfected with ADAM12-directed siRNAs and subjected to Transwell migration assay in the presence of DMSO or FSK/IBMX. Migration assay was performed in duplicate and repeated on three independent occasions (n=6). Bar graph shows the quantification of cell migration. Migration index was calculated as the ratio of the number of ADAM12-siRNA transfected cells migrating (in the presence or absence of FSK/IBMX) to the number of NS transfected cells (treated with DMSO). Data are presented as mean ± s.e.m. (*$P \leq 0.05$, **$P \leq 0.01$; n.s., not statistically significant when compared to control, NS). FSK, forskolin.

4.3 Discussion

In this study, we demonstrate that an increase in intracellular cAMP increases the expression of ADAM12S isoform and directly induces *ADAM12* promoter activity. Moreover, we provide evidence that ADAM12 is a key downstream effector in cAMP-directed trophoblast migration. Prior to this work, several studies have shown that trophoblast differentiation is associated with marked increases in intracellular cAMP levels [225]. Importantly, our previous study showed that cAMP signalling induces *ADAM12* expression during trophoblast fusion [239]. We now describe a role for cAMP signalling in mediating *ADAM12* expression within the invasive trophoblast populations.

Although our previous study suggested a role for PKA signalling in mediating the effect of cAMP on ADAM12 expression during trophoblast fusion [239], we were not able to assign a specific role for either PKA or Epac in regulating cAMP-directed ADAM12 levels in invasive trophoblasts. This suggests that a non-canonical pathway mediates the cAMP-induced ADAM12 expression in EVTs. Indeed, a few studies have also suggested the existence of alternative intracellular transducers responsible for mediating the action of cAMP, however, the identity of these effector molecules remains to be determined. For example, cAMP-induced expression of krox-20, a master regulator of Schwann cells differentiation, was shown to be independent of either PKA or Epac [288]. Consistent with this finding, another study suggested that a non-PKA/Epac signalling pathway mediates the effect of cAMP on IL-6 expression in mouse cardiac fibroblasts [289]. As previously mentioned, some of the effects of cAMP are mediated through CNG ion channels, however, whether these channels play a role in cAMP-induced ADAM12 expression requires further investigation.

Trophoblasts possess all the cellular machinery required for cAMP synthesis including multiple GPCRs, ACs and G proteins [218]. Once synthesized, cAMP plays a central role in controlling trophoblast differentiation and endocrine function mostly through regulation of genes involved in these cellular processes. However, discrepancies exist in the literature regarding the role of cAMP in the regulation of trophoblast migration and invasion, where both stimulatory and inhibitory effects have been reported. For instance, increased levels of cAMP was found to inhibit IGF-II-induced HTR8/SVneo cells migration [227]. This is in contrast to a recent study showing that hypoxia-mediated induction of adenosine A2B receptor expression induces cAMP synthesis, leading to an increase in HTR8/SVneo trophoblast cell invasion and their integration into endothelial cell monolayers [231]. Hepatocyte growth factor (HGF) produced by human placental multipotent mesenchymal stromal cells (hPMSCs) has also been found to promote trophoblast cAMP production leading to an increase in their adhesive and migratory potential. In this later study the effects of cAMP were found to be mediated through a PKA/Rap1 dependent mechanism[233]. Similarly, cAMP signalling was suggested to mediate trophoblast migration and invasion by inducing the expression and secretion of various proteases including uPA, MMP2 and MMP9 [229,232,238,290]. cAMP/PKA signalling has also been reported to induce the expression of the melanoma cell adhesion molecule (MCAM), shown to be selectively expressed by invasive EVTs and contributes to their invasive phenotype [236,291,292]. These findings are in line with the results of our study showing that cAMP signalling induces the migratory potential of HTR8/SVneo cells through an ADAM12-dependent mechanism.

The controversial effects of cAMP in regulating cell invasion and migration have

also been observed in other cellular systems. For instance in one report, cAMP was suggested to inhibit pancreatic cancer cell migration and invasion through inhibitory regulation of RhoGTPase, whereas another study showed that cAMP induces pancreatic cancer cell migration *in vitro* and distant metastasis *in vivo* through an Epac-dependent mechanism [293–295]. The dichotomous effects of cAMP on cell migration have also been reported in gastric cancer, where cAMP-mediated inhibition of RhoA activation and actin reorganization was suggested to inhibit cell migration [296]. However, cAMP-induced MMP2 and MMP9 production and activation was found to have a stimulatory effect on gastric cancer cells migration [297].

Several possibilities may account for these disparate findings, including the use of different in vitro cell model systems and different treatment regimens that have been applied to induce the intracellular cAMP level. For instance, some studies have relied on the use of cell permeable cAMP-analogs or adenylyl cyclase activators, which by-pass GPCRs, and directly elevate cellular cAMP levels. However, others have used agents that specifically mediate the activation of GPCRs involved in the induction of endogenous cAMP synthesis. Increasing the intracellular levels of cAMP via the above-mentioned pathways results in differential spatiotemporal distribution of cAMP, which in turn may lead to the activation of different effector molecules and cellular pathways.

In summary, the results presented here indicate that cAMP signalling regulates *ADAM12* expression as well as function in invasive trophoblasts. We have also shown that cAMP treatment induces the migratory phenotype of trophoblasts partly through an ADAM12-dependent mechanism. However, further studies are required to identify the cAMP responsive effector molecules regulating *ADAM12* expression.

CHAPTER 5. GENERAL DISCUSSION, SUMMARY OF RESULTS AND CONCLUSIONS

5.1 General discussion

Terminal differentiation of progenitor vCTs into invasive stromal- and vascular-remodelling EVTs is a key process in placental development. Indeed, inadequate trophoblast invasion, leading to defects in placentation and placental perfusion, is associated with the onset of diverse pregnancy complications such as IUGR and preeclampsia [298,299]. Trophoblast invasion is spatiotemporally regulated by a plethora of paracrine factors expressed by different cell types including decidual stromal cell, uNKs, macrophages and uterine glands. Moreover, acquisition of an invasive phenotype is mediated by a complex network of autocrine factors expressed by trophoblasts such as growth factors, cytokines, adhesion molecules and multiple families of proteases [38].

ADAM12 is a member of the ADAM family of metalloproteinases, which due to their multidomain structures have the ability to interact with a diverse array of proteins, thereby mediating distinct cellular functions such as signal transduction, cell-cell and cell-matrix adhesion and proteolysis. Although *ADAM12* expression is relatively low in most of the adult tissues, its expression significantly increases during normal developmental events (neonatal skeletal muscle/bone and placenta), cellular turnover and growth (regenerating muscle) and in instances of tissue pathology, especially cancer [142,148,163,300,301]. The highly restricted expression of *ADAM12* is probably due to the presence of a negative regulatory element at its 5'-UTR, which acts as a transcriptional repressor by acquiring a Z-DNA confirmation [302]. Binding of Z-DNA binding proteins to

this repressor element further stabilizes the Z-DNA confirmation and transcriptional repression. Interestingly, several Z-DNA binding proteins have been identified in low-*ADAM12* expressing cells while these proteins are absent in high-*ADAM12* expressing placental cells [302]. Since reduced ADAM12 levels have been observed in pregnancies complicated with chromosomal anomalies, IUGR and spontaneous pre-term delivery [134,138,303], it is tempting to speculate that the presence of Z-DNA binding proteins might be responsible for the low levels of ADAM12 observed in these pregnancies, as well as in several subtypes of trophoblasts, including vCTs.

Like other ADAMs, ADAM12 is first synthesized as a zymogen, which gets activated upon the cleavage of its prodomain by furin or other endopeptidases [304,305]. Several studies have suggested that the prodomain remains non-covalently associated with the rest of the protease [144,306]. Although crystal structure of ADAM12 is not yet available, electron microscopy has suggested that the mature form of ADAM12 is a four-leafed clover, where one of the leaves represents the prodomain [307]. This finding suggests a role for the prodomain in mediating ADAM12 functions in the extracellular space, where it might regulate the accessibility of the metalloproteinase domain to its substrates. Solving the crystal structure of ADAM12 can indeed enhance our knowledge about its exact mechanism of action.

Since ADAM12's discovery, distinct functions have been assigned to its individual domains. For instance, the prodomain was suggested to regulate proper folding of the protein as well as keeping the proteolytic domain in a latent form. Once the prodomain is cleaved, the metalloproteinase domain is responsible for mediating ECM degradation and ectodomain shedding of membrane-bound proteins and cell surface

receptors. ADAM12-mediated cleavage of various substrates, such as fibronectin, type IV collagen, IGFBP-3 and -5, HB-EGF and EGF, has been suggested to impact diverse cellular functions such as cell migration, invasion and growth [144,146,245,308,309]. Besides serving as a sheddase, ADAM12 has been shown to regulate cellular adhesion through a mechanism that requires the interaction of its cysteine-rich and disintegrin domains with cell adhesion molecules such as syndecans and integrins [156,157]. While the exact function of the EGF-like domain is unknown, the cytoplasmic domain of ADAM12L was suggested to mediate signal transduction by interacting with proteins harbouring Src homology domains, including the non-receptor tyrosine kinases c-Src and Yes and the adaptor protein, Grb2 [310].

Given its ability to regulate diverse cellular processes, it is not surprising that ADAM12 has been suggested to play key roles in the development of a variety of human malignancies such as melanoma, bladder cancer, liver cancer and breast cancer [249,252,311,312]. ADAM12 has been shown to regulate tumorigenesis by inhibiting tumor cell apoptosis and inducing cancer cell proliferation, migration and invasion [311,313]. In addition to cancer development, ADAM12 is linked to other pathologies including cardiac hypertrophy and osteoarthritis, where its expression is aberrantly elevated in these two conditions [245,314]. These findings place ADAM12 as a potential biomarker and drug target for various diseases. Indeed, research efforts have focussed on assessing the sensitivity and accuracy of ADAM12 as a biomarker for breast, prostate and bladder cancer [146,252,269].

Despite the negative effects of *ADAM12* overexpression in the above-mentioned pathologies, it has been shown that the serum level of ADAM12S increases by more than 60 fold by the third trimester in healthy pregnancies, indicating an underlying importance

of ADAM12 in the normal development and maintenance of pregnancy[133]. Supporting this notion, studies have shown that serum concentrations of ADAM12 are reduced in pregnancies complicated with chromosomal anomalies, IUGR and spontaneous pre-term delivery [134,138,303].

Despite a growing body of evidence linking *ADAM12* expression with various pathological and physiological conditions, the molecular mechanisms controlling its expression and functions are largely unknown. So far, only a few well-known signalling pathways such as Notch, TGF-β and EGF have been suggested to mediate the expression of *ADAM12* [249,266,315,316]. Therefore, further studies are required to identify the potential upstream regulators of *ADAM12* expression.

In spite of the high levels of *ADAM12* expression in the human placenta, and its association with diverse pregnancy complications, the importance of ADAM12 in trophoblast biology is still poorly understood. Recent work in our laboratory has suggested a role for ADAM12S in mediating the differentiation of vCTs into multinucleated syncytial structures. Here, ADAM12S was suggested to induce trophoblast fusion by mediating the ectodomain shedding of E-cadherin and disrupting the intercellular boundaries [239]. Moreover, microarray analysis of placental trophoblasts has shown high expression levels of *ADAM12* in invasive EVTs, suggesting a role for this protease in mediating EVT biology, and in particular EVT invasion [235].

5.2 Summary of results and future directions

Utilizing first trimester placental tissues, trophoblastic cell lines and *ex vivo* explant cultures; ADAM12 expression was identified in distal columnar EVTs, highly-

invasive matrix-degrading EVTs and the invasive HTR8/SVneo trophoblastic cells (Figure 5.1). Notably, a specific role for the secreted isoform, ADAM12S, in promoting trophoblast invasion and EVT column formation was demonstrated. Moreover, *ADAM12* gene expression and promoter activity were shown to be in part regulated by cAMP signalling. While this effect was independent of PKA activity, a role for Epac signalling in inducing *ADAM12* promoter activity and ADAM12 mRNA levels was suggested. We also provided evidence that cAMP signalling promotes trophoblast migration partly through regulation of *ADAM12* expression. Together, work presented in this thesis underline the importance of ADAM12 in regulating EVT column formation and trophoblast cell invasion, and highlight a novel upstream factor controlling *ADAM12* expression and function in the placenta.

Further investigation is required to examine the biological significance of ADAM12L during placental development and trophoblast differentiation, as this isoform is highly expressed in the human placenta [143]. Moreover, studies remain to be done to elucidate the molecular mechanisms by which cAMP induces *ADAM12* expression in trophoblasts and also to examine whether cAMP signalling regulates *ADAM12* expression *in vivo*. Although we were not able to assign a role for Epac in cAMP-mediated induction of ADAM12 levels, further studies using Epac inhibitors and Epac-directed siRNAs are required to confirm our findings. Likewise, whether Epac signalling regulates *ADAM12* expression in primary EVT cultures requires further investigation. It would also be of interest to determine if ADAM12 induces trophoblast invasion by regulating the expression and activity of other proteases, as a role for this protease in mediating the activation of MMP14 has been recently recognized [276]. Since ADAM12

functions as a cell surface sheddase and modulates the bioavailability of different growth factors and cell surface receptors, the findings presented in this thesis can be extended by identifying novel ADAM12 substrates and interacting proteins, which might play roles in ADAM12-mediated trophoblast invasion. We have recently shown that ADAM12-mediated cleavage of E-cadherin potentiates trophoblast fusion and syncytialization [239]. Interestingly, it is widely accepted that loss of E-cadherin expression or function induces tumor cell invasion and metastasis [317]. As down-regulation of E-cadherin is also required for trophoblast differentiation into an invasive phenotype [318,319], it is possible that ADAM12 promotes trophoblast invasion by inducing E-cadherin cleavage.

5.3 Conclusions

Metzincins are evolutionary conserved proteases that instruct diverse biological functions across multiple organ systems. By sharing several conserved domains, metzincin proteases have partially redundant/overlapping roles in controlling cellular processes such as motility, survival, growth and fusion. Thus, assigning specific roles for individual metalloproteinases in placental development is often a difficult task. Individual MMPs have been shown experimentally to be crucial in directing aspects of trophoblast biology, while the function of other metzincin protease members remains elusive. Recent findings examining the function of ADAM family members in disease and development highlight the biological potential of these unique proteases in controlling placentation. Specifically, findings presented in this thesis have suggested a critical role for ADAM12 in regulating trophoblast invasion and EVT column outgrowth. Its value as a possible biomarker for aberrant pregnancies, along with still unknown cellular functions in

trophoblast survival (possibly through establishing growth factor gradients) highlight ADAM12, as well as related ADAM family members, as attractive candidates in controlling placental development.

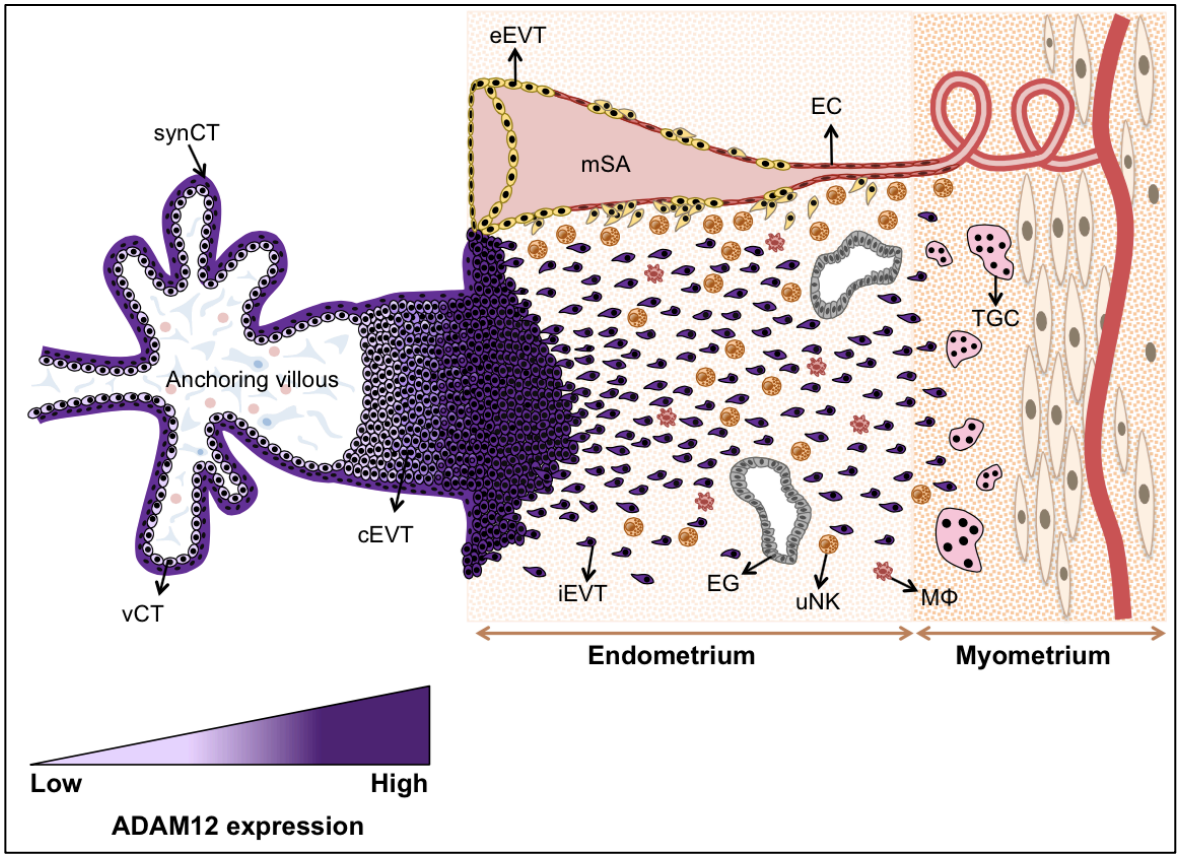


Figure 5.1 Schematic diagram demonstrating ADAM12 localization in human placental villi. ADAM12 is primarily localized to the multinucleated synCT layer, distal columnar EVTs and highly-invasive interstitial EVTs. vCT, villous cytotrophoblast; synCT, syncytiotrophoblast; cEVT, columnar extravillous cytotrophoblast; eEVT, endovascular extravillous cytotrophoblast; iEVT, interstitial extravillous cytotrophoblast; mSA, maternal spiral artery; EC, endothelial cell; EG, endometrial gland; uNK, uterine Natural Killer cell; MΦ, macrophages; TGC, trophoblast giant cell.

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