

**THE ROLE OF ACTIVIN IN REGULATING HUMAN TROPHOBLAST INVASION
AND VASCULAR MIMICRY**

by

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Abstract

During implantation, extravillous cytotrophoblast (EVT) differentiation follows invasive interstitial and endovascular routes. Aberrant placentation due to restricted trophoblast invasion and spiral artery remodeling is responsible for the pathogenesis of common complications during pregnancy, including miscarriage, preeclampsia and IUGR. Activin A, which is abundantly produced at the maternal-fetal interface, exerts its stimulatory effect in trophoblast invasion. Whether two additional activin isoforms, activin B and AB exert similar effects as activin A in trophoblast invasion remains unknown. We hypothesized that mesenchymal adhesion molecule neural cadherin (N-cadherin) and matrix metalloproteinase 2 (MMP2) play essential roles in activin-induced trophoblast invasion. In addition, the effect of activin A on trophoblast endovascular differentiation has never been studied. Vascular endothelial growth factor-A (VEGF-A) is well recognized as a key regulator in trophoblast endovascular differentiation and activin A has been shown to stimulate VEGF-A expression in human hepatocellular carcinoma cells. Whether and how activin A might regulate VEGF-A production in human trophoblasts and its relationship to endovascular differentiation has yet to be determined. An established immortalized EVT cell line HTR8/SVneo and primary cultures of human EVT cells were used as study models. Trophoblast invasiveness and endovascular differentiation were assessed by matrigel-coated transwell assays and endothelial-like tube formation assays following exposure to recombinant human activin. Small interfering RNA (siRNA)-mediated knockdown approaches were used to investigate the molecular determinants of activin-mediated functions. In summary, our results demonstrate that activin A, B and AB produce comparable increases in human trophoblast cell invasion by up-regulating N-cadherin expression. Activin A induces human trophoblast cell invasion by up-regulating SNAIL-mediated MMP2 expression through ALK4 in

a SMAD2/3-SMAD4-dependent manner. In addition, activin A induces human trophoblast cells endothelial-like tube formation by up-regulating VEGF-A expression in a SMAD2/3-SMAD4-dependent manner. Our findings provide important insights into the molecular mechanisms underlying the dual effects of activin on human EVT_s, and enhance our understanding of the establishment of pregnancy under normal or pathological conditions.

Preface

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I designed and conducted all the testing as well as statistical analysis. I wrote the manuscript, which was further refined by Dr. Christian Klausen and my supervisor Dr. Peter C.K. Leung. Dr. Hua Zhu assisted me in culturing primary human EVT cells.

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I was responsible for experiments design and the performance of all the experiments in this chapter. I analyzed the research data and wrote the manuscript, which was revised by Dr. Hua Zhu, Dr. Christian Klausen and my supervisor Dr. Peter C.K. Leung.

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List of abbreviations

ACVR	Activin receptor
ALK	Activin receptor-like kinase
ANOVA	Analysis of variance
BSA	Bovine serum albumin
°C	Degrees Celsius
cDNA	Complementary DNA
DMEM	Dulbeco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal-regulated kinase
EVT	Extravillous cytotrophoblast
g	Gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h	Hour
IUGR	Intrauterine growth restriction
kDa	Kilodalton

min	Minutes
ml	Milliliter
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
μL	Microliter
nM	Nanomolar
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
R-SMAD	Receptor-regulated SMAD
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
siRNA	Small interfering RNA
SMAD	Sma- and Mad-related protein
SV40	Simian vacuolating virus-40
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinases
TBS	Tris-buffered saline
Tris-HCl	Tris (hydroxyl methyl)-aminomethane-hydrochloric acid
VEGF	Vascular endothelial growth factor

ZEB

Zinc finger-E-box-binding homeobox

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Chapter 1: Introduction

1.1 Placenta

Pregnancy loss occurs in approximately 30-50% of conceptions and the incidence goes down with an increase of gestational age (1). Up to 15% of clinical pregnancies never come to term and approximately 10% of couples have severe fertility problems (2, 3). Furthermore, changes in human lifestyle, such as late childbearing and unhealthy diets, will increase the risk of placental-related disorders worldwide (4). Notwithstanding the rapid development and application of assisted reproductive technologies over the last decades, the live-birth rate following *in vitro* fertilization still rarely exceeds 30-35% in Canada (5). All these placental-related problems call for our better understanding of the biology of embryonic implantation and placentation.

As a disposable organ formed from tissues of two genetically distinct organisms, the placenta exhibits multiple functions that are essential for the establishment and maintenance of successful pregnancy. The development of the placenta, which is also referred as placentation, begins upon the implantation of the blastocyst into the maternal endometrium, followed by anchoring the embryo to uterus and remodeling of the spiral arteries to ensure sufficient blood supply to fetus (6). The appearance of the human placenta is discoid, since the distribution of chorionic villi over the uterine surface is arranged in a circular plate. The human placenta is a highly invasive organ that invades into maternal tissues and vasculature (7). According to the degree to which maternal uterine tissue has been modified, human placenta is classified as a haemochorial type, characterized by maternal blood in direct contact with fetal trophoblast (8). The proper placentation provides foundation for the fully display of a series of placental functions including endocrine functions (9), immunity protection (10), waste excretion, nutrition

supply (11) and gas exchange. Inadequate formation and maturation of the placenta leads to a series of pregnancy unfavorable outcomes, including miscarriage, preeclampsia, intrauterine growth restriction (IUGR), preterm birth and stillbirth (12-14).

1.2 Implantation

Implantation is the initial stage of placentation that begins around day 6-7 after fertilization. It is a critical period of reproduction since 50% of pregnancy failure occurs at this time. In vitro fertilization (IVF) and assisted reproductive technologies (ART) show less than a 25% success rate, and this likely involves a problem with implantation (15). Implantation is a highly coordinated process where specialized cells located in the outer layer of a blastocyst, the trophoblasts, establish contact with a specialized tissue of the mother, endometrium (16). Successful implantation requires the synchronized adaptation of both endometrium and embryo to ensure a continuous blood supply to fetus. The endometrium undergoes proliferation and differentiation to form a “receptive window” for the embryo (17), while trophoblasts lining the outer layer of the blastocyst differentiate along different pathways (18).

1.2.1 Endometrial proliferation and differentiation

The endometrium undergoes hormonally dependent changes following fertilization (19). This decidualization process leads to a “uterine receptivity window”, which occurs in the late secretory phase of the menstrual cycle and is critical for implantation of the blastocyst (17). Decidualization is induced *in vivo* by progesterone (20), with the morphological change into rounded cells, the influx of specific uterine natural killer cells that are characterized by abundant expression of CD56 (21), the remodeling of spiral arteries and the release of prolactin and

insulin-like growth factor binding protein -1 (IGFBP-1) (22), as well as an increase in the mesenchymal cell marker vimentin (23). Thus, the endometrium becomes permissive to the blastocyst implantation process.

1.2.2 Early development of the fertilized zygote

By day 5, fluid is absorbed by the zygote, resulting in a large inner cavity to form a blastocyst. The inner cell mass of the blastocyst gives rise to the embryo. The outer cell mass of the blastocyst forms a single layer linking the blastocyst cavity and is now termed trophoblast (24). Implantation starts from the blastocyst's attachment to the uterine luminal epithelium. The carbohydrate binding protein, L-selectin, which is located on the trophoblast surface, mediates the attachment by binding to oligosaccharide molecules expressed at the surface of the uterine epithelial cells (25).

1.2.3 Trophoblast differentiation

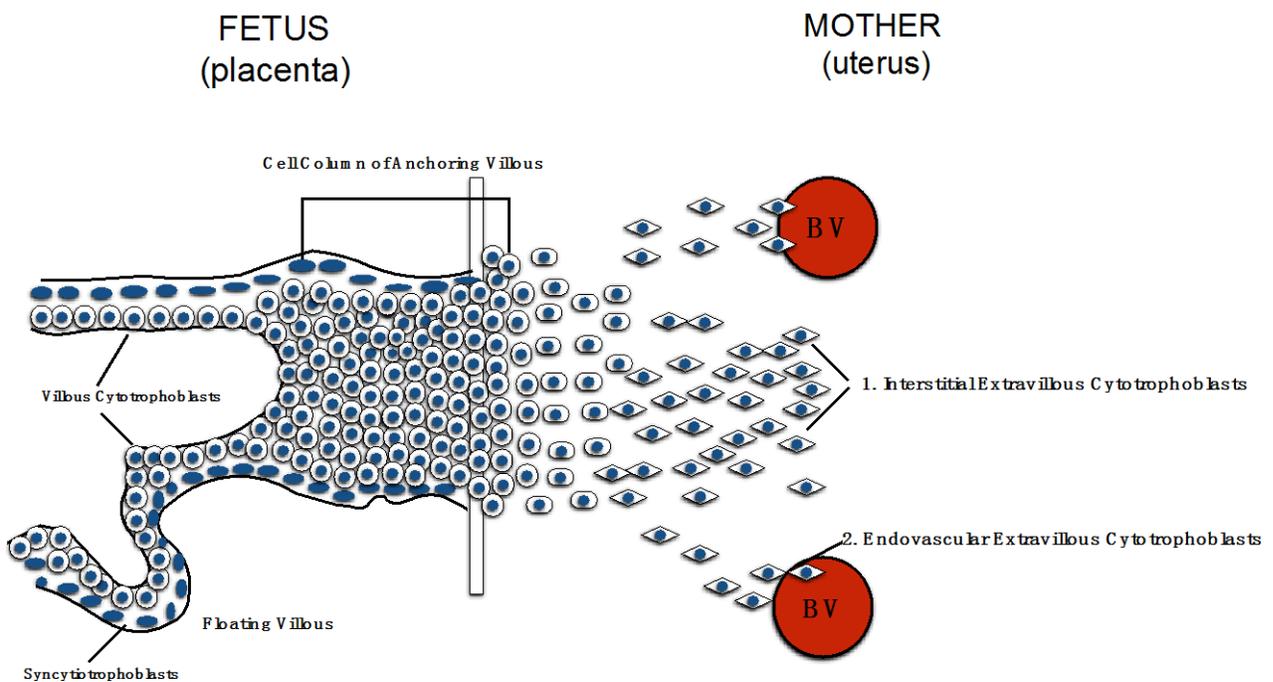
After the establishment of interaction, the trophoblast cells proliferate, differentiate and invade the stroma to form chorionic villi that are composed of two cell layers. The outer layer of villi is lined with multinucleated syncytiotrophoblasts whereas the inner cell layer is comprised of mitotically active cytotrophoblasts (26). Syncytiotrophoblast cells are located at the outer layer of blastocyst. The syncytiotrophoblast secretes progesterone, leptin, human chorionic gonadotropin (hCG) and human placental lactogen (HPL). Syncytiotrophoblasts lack proliferative ability and instead are maintained by the fusion of underlying cytotrophoblasts. The fusion process involves endogenous retroviral envelope protein (27, 28). Cytotrophoblasts are interior to the syncytiotrophoblast layer. Villous cytotrophoblasts proliferate continuously to

support the rapid growth of placenta (29). Proliferation marker Ki67 and cell cycle regulator cyclin A have been detected in villous cytotrophoblasts (30). From days 9 to 12, columns of cytotrophoblast cells outgrow towards the outer layer of the conceptus. Then cytotrophoblast cells located in outer layer of anchoring chorionic villi become invasive, these extravillous trophoblasts (EVTs) gradually detach from the cell columns followed by maternal uterine remodeling (31).

1.2.4 EVT interstitial invasion and endovascular differentiation

At around 8 week during implantation, subpopulations of highly invasive extravillous cytotrophoblasts (EVT) derived from cell columns extend through the outer syncytial cytotrophoblast layer and invade into the maternal uterine tissues, followed by the remodeling of the uterine environment for a successful human pregnancy (32, 33). These detached EVT cells invade into maternal decidua and vasculature during the first trimester of pregnancy by two separate routes: interstitial and endovascular invasion (Figure 1.1). Interstitial EVT cells invade into the decidua to anchor the placenta to the maternal uterus, whereas endovascular EVT cells migrate into lumen of maternal spiral arterials, acquire an endothelial-like phenotype and modify the vessels into low-resistance dilated conduits to ensure a constant delivery of maternal nutrients to the placenta (34). Inadequate trophoblast invasion and failures in vascular transformation are related to many unfavorable outcomes of pregnancy, such as first-trimester miscarriage, preeclampsia, intrauterine growth restriction (IUGR), choriocarcinoma and hydatiform moles (12, 35-37). These unfavorable pregnancy outcomes are common and directly threaten the well being of mothers and their fetuses. Thus, a better understanding of molecular

mechanisms of human trophoblast invasion and vascular formation regulation is urgently needed and would benefit the diagnosis and therapy of these pregnancy-related diseases.



Adapted from Zhou et al., 1997. *J.Clin. Invest.* 99:2139-2151

Figure 1.1 Different development of extravillous trophoblast derived from an anchoring chorionic villous at approximately 10 weeks

Cell column cytotrophoblasts give rise to interstitial extravillous cytotrophoblasts (iEVT) invading the uterine decidua and endovascular extravillous cytotrophoblasts (eEVT) migrating into the maternal spiral arteries. BV stands for blood vessel.

1.2.5 EVT dysfunction and pregnancy complications

Trophoblast invasion and endovascular differentiation is tightly regulated by numerous hormones, growth factors and cytokines (33). Aberrant trophoblast invasion and restricted EVT endovascular differentiation have severe consequences for the health of both the mother and fetus. In particular, first-trimester miscarriage is the most common complication of human reproduction and mostly presents with reduced cytotrophoblast invasion (35). Also, preeclampsia and intrauterine growth restriction (IUGR) are two clinically dangerous pregnancy disorders that characteristically present with insufficient trophoblast invasion and inadequate remodeling of the maternal and fetal vasculature (12, 36, 38-40). Preeclampsia occurs in approximately 3-14% of pregnant women and results in >100,000 maternal deaths per year all over the world (41). By contrast, uncontrolled trophoblast invasion results in choriocarcinoma and hydatiform moles (37). The incidence of choriocarcinoma varies from 0.05 to 0.17 per 1000 live births in different geographical regions. If patients have been diagnosed with hydatidiform mole before, the risk of choriocarcinoma occurrence is approximately 10% in the future (42). These unfavorable outcomes can have severe consequences for the health of both the mother and fetus. A better understanding of the regulation of human trophoblast invasion, as well as the underlying molecular mechanisms, will improve the diagnosis and treatment of infertility and disorders of pregnancy.

1.3 Models in placenta research

1.3.1 Animal models

Due to the ethical limitation of *in vivo* studies on human placentation, animal models have been utilized as substitute approaches to learn the molecular and cellular mechanisms during human pregnancy. Although exhibiting different gross structures from the human, animal models provide essential insights into understanding certain aspects of the human placentation process, including blastocyst attachment, uterine spiral artery remodeling, interstitial trophoblast invasion and endovascular trophoblast differentiation. Since human placenta is classified to discoid haemochorial type, animal placentas, which belong to the haemochorial type, are more adequate for investigating trophoblast differentiation and maternal blood vasculature modification.

1.3.1.1 Rodent animals

Nearly all rodent placentas belong to the haemochorial type that are comparable to human placenta (43). Mouse is close to primates in an evolutionary context, the availability of embryonic stem cells provides useful information in gene targeting and transgenic lines development (44). Moreover, its small size and short generation time make the manipulation of mouse models more feasible. However, it should be noted that the high rate of mutation and gross appearance difference make the mouse an inappropriate model for chromosome painting (45) and morphological evidence. The shorter gestation and poorly developed young are largely different from the human. Besides, trophoblast invasion is limited in the mouse and uterine arteries remodeling depends on maternal factors (46). Guinea pig is a good rodent model known to develop pregnancy toxemia (47). The sheep is well established as a model for studying fetal

physiology (48). The ovine placenta is epitheliochorial with no trophoblast invasion that is quite different (49).

1.3.1.2 Non-human primates

Primates are close to humans in terms of lineage and likely to resemble humans in biology. The baboon has been proven to be a model for endometriosis (50). Studies in comparative genomics are primarily focused on primates (51). In all of the great apes, the interstitial implantation and the villous part of placenta look identical to the human one. Their placenta are villous and haemochorial. For the monkeys, the villous structure, the remodeling of spiral arteries following trophoblast invasion (52) and the pattern of circulation in the intervillous space show similarities to the human placenta (53). Especially, the initial stages of implantation are remarkably similar in monkeys and human (54). Syncytiotrophoblast initially invade through the uterine epithelium to attach blastocyst into the endometrium (55). Further implantation of the monkey lacks interstitial trophoblast invasion, but shows a strong resemblance to the way spiral arteries are invaded and modified (52). Thus, primates close to humans are important models for investigating the pathogenesis of pre-eclampsia and IUGR (intrauterine growth restriction). In particular, symptoms that resemble preeclampsia have been observed in monkeys (56) and great apes (57).

1.3.2 Primary cultures of human trophoblastic cells

The trophoblastic subpopulations from term placenta can be obtained from enzyme digestion and subsequent gradients centrifuge or immunoselection (58, 59). These isolated mononucleate cytotrophoblasts undergo fusion *in vitro* which mimics syncytiotrophoblasts formation *in vivo*.

Besides morphologic changes, there is also an increase in the secretion of β -hCG, which correlates with trophoblast differentiation and fusion (60). Cytokeratin-7 is used to differentiate trophoblasts from other cell types. However, this method cannot separate EVT cells and cytotrophoblasts (61).

As previously reported, human first trimester EVT cells can be mechanically isolated from first trimester chorionic villous explants (6-12 weeks gestation) (62). Briefly, distal regions of first trimester placental villi are isolated and minced into small pieces, and then cultured in culture flasks. After 3-4 days, non-attached pieces are washed away. Villous tissue fragments are cultured for a further 5-7 days to allow for EVT outgrowth, and EVT cells are subsequently separated from attached fragments by trypsinization. These cells express EVT specific markers (e.g. HLA-G) and are stained positive for cytokeratin-7 (epithelial marker) and negative for vimentin (mesenchymal marker). Since villous fibroblasts also outgrow from villous fragments, fibroblast contamination is the major challenge during the culture of first trimester primary EVTs.

1.3.3 Immortalized trophoblastic cell lines

1.3.3.1 HTR8/SVneo, first trimester immortalized EVT cell line

The HTR8/SVneo cell line was established by transfecting simian virus 40 large T antigen into first trimester human trophoblast. HTR8/SVneo cells maintain the morphology and the majority of gene expression profile of parental cells while acquiring an extended lifespan. Parental (HTR8) cells can only be cultured for 12-14 passages, whereas HTR8/SVneo cells can be cultured at least for 32 passages. Both HTR8/SVneo cells and parental cells exhibit similar *in*

in vitro invasive capacities, and are positive for cytokeratin staining, identifying their epithelial (trophoblastic) phenotype. Both cell lines secrete mostly MMP2 collagenases as determined by zymography. The extended lifespan and sustained phenotypic properties provide an important tool for studying placental biology (63). The effects of a variety of cytokines and growth factors on trophoblast migration, invasion, proliferation and apoptosis as well as the underlying molecular mechanism have been revealed by studies using HTR8/SVneo cells (64-67). Recently, studies have demonstrated the intrinsic capacity of the immortalized HTR8/SVneo cells for endothelial-like tube formation when cultured on matrigel, which is the mimicry of endothelial cells in the vascular system. Investigators utilize the ability of HTR8/SVneo cells to mimic endothelial cells via angiogenesis to form endothelial-like tubes *in vitro* upon culture on matrigel (68, 69) to investigate the regulation and underlying molecular mechanisms of EVT endovascular differentiation by different factors (68-71).

1.3.3.2 Choriocarcinoma cell lines

BeWo, JAR and JEG-3 are three commonly used choriocarcinoma cell lines for studying placental functions. These cell lines were established from human choriocarcinoma cells (hCC) derived from first trimester trophoblast. The preservation of certain characteristics of early placental trophoblasts supports the use of these cells as suitable models to investigate different aspects of trophoblast physiology and pathology. Although they share similar characteristics of early placental trophoblast like the production of chorionic gonadotropin and steroids, they differ significantly in other aspects, such as their degree of differentiation and proliferative activity. For example, BeWo and JAR cells exhibit less differentiation than JEG-3 cells, but show higher

proliferation rates (72-74). Thus, it is important to choose appropriate cell lines for different placental functional studies.

BeWo cells produce an easily detected hormone marker, β -hCG (75), which is secreted by syncytiotrophoblasts under physiological condition. Thus, this human hormone-synthesizing trophoblastic cell system has been widely used as a functional model for trophoblast syncytialization. JEG-3 cells exhibit low invasiveness and have been used to study trophoblast differentiation along the invasive pathway (76). JAR cells express several molecular transporters for serotonin, taurine and glucose and have been used to study placenta transport mechanisms and delineate certain aspects of the regulation of these transporters (77-79). Moreover, due to their malignant derivative, BeWo, JAR and JEG-3 cells provide convenient approaches for investigating the pathological mechanisms of choriocarcinoma and their responses to chemotherapy *in vitro* (80, 81).

1.4 TGF- β superfamily in placentation

Since in normal pregnancy, interstitial/endovascular invasion does not extend beyond the decidua and the first third of the underlying myometrium, this process is thought to be precisely regulated by a plethora of regulating factors such as growth factors, cytokines, interleukins, chemokines, hormones, angiogenic growth factors, adhesion molecules, proteases, matrix-derived components and oxygen tension (33, 82-84). Trophoblast invasion is regulated by trophoblastic factors in an autocrine manner and by uterine factors in a paracrine manner (85). Regulators such as hormones, cytokines, growth factors and extracellular matrix glycoproteins have been reported to induce invasion of trophoblast and choriocarcinoma cells (86). Among these, the transforming growth factor-beta (TGF- β) superfamily members including TGF- β 1,

TGF- β 2, TGF- β 3, activin, inhibin, bone morphogenetic protein (BMP), Müllerian inhibiting substance (AMH), and several other proteins are abundantly and dynamically expressed in the endometrium and placenta (87). The TGF- β superfamily members are believed closely related to tissue remodeling events and reproductive processes during the menstrual cycle and establishment of pregnancy. During the implantation process, TGF- β superfamily members display divergent functions in the regulation of trophoblast invasion. For example, activin A can promote trophoblast invasion by inducing MMP expression, whereas TGF- β inhibits trophoblast invasion, suggesting the relative balance of TGF- β members participates in modulating trophoblast invasion (87).

1.4.1 Activin and inhibin

Activin and inhibin proteins are disulphide-linked heterodimeric or homodimeric proteins that have almost directly opposite effects but share similar or identical β subunits (88). Activins are homo or heterodimers of inhibin β subunits (89). Inhibins are heterodimers of a common α subunit and a β subunit. Depending on the subunits combination, there are three primary isoforms of activins [activin A (β A β A), activin AB (β A β B) and activin B (β B β B)] and two primary isoforms of inhibins [inhibin A (α β A) and inhibin B (α β B)] (90, 91). Two other β subunits (β C and β E) have been found in human (92). However, little is known about their biological significance.

1.4.2 Activin isoforms in placenta

As growth and differentiation factors, activins have been shown to regulate multiple biological activities in a variety of tissues, including hormone secretion, embryonic development, spermatogenesis, inflammation and carcinogenesis (90, 93-96). However, most studies regarding the functions of activins have focused on activin A, without actually testing the effects of other activin isoforms. Although controversial, a number of studies have demonstrated different roles for activin isoforms via differential expression (97), receptor (98) and antagonist binding affinity (99), as well as signaling pathways (100-103). The human placenta and endometrium express both subunits and are capable of producing activins A ($\beta A\beta A$), AB ($\beta A\beta B$) and B ($\beta B\beta B$) (104-106). Though activin A has been shown to enhance human trophoblast cell invasion (107, 108), whether the two additional activin isoforms, activin B and AB, which also exist at the maternal-fetal interface, could exert similar pro-invasive effects remains unknown. Moreover, whether activin could induce human trophoblast acquisition of the endothelial phenotype needs to be elucidated.

1.4.3 TGF- β receptors and signaling pathways

The canonical TGF- β signaling pathway involves the activation of a complex of type I and type II transmembrane serine/threonine kinase receptors and later participation of intracellular effectors, SMADs (109). TGF- β superfamily members first bind to extracellular domain of type II receptor, then a type I receptor is recruited. After that, receptor SMADs (R-SMADs) 1/5/8 or 2/3 are phosphorylated by the type I receptor. A complex of R-SMADs with a common SMAD4 is formed and then translocates into the nucleus to modulate gene expression. There are five type II receptors (TGF β R2, ACVR2A, ACVR2B, BMPR2 and AMHR2) and seven type I receptors,

ACVRL1, ACVR1, BMPR1A, ACVR1B, TGF β RI, BMPR1B and ACVR1C, which are also called activin receptor-like kinase (ALK) 1-7, recognized in mammals (110). In addition to the SMAD-dependent canonical pathway, TGF- β family can activate SMAD-independent non-canonical pathways, such as MAPK, Rho GTPase and PI3K/Akt (111).

1.4.4 Activin receptors and signaling pathways

Activins, similar to other members of the TGF-beta superfamily, exert biological effects by activation of heteromeric complexes of type I and type II transmembrane serine-threonine kinase receptors (112). Specifically, after activin A binds to the extracellular domain of constitutively active activin type II (ActR2A) or activin type IIB (ActR2B) receptor, activin type I receptors are recruited and transphosphorylated. Among seven activin receptor-like kinase (ALK) receptors, activin A binds to ActRIB (ALK4) and ActRI (ALK2) with high affinity (113). Phosphorylation of activin type I receptors leads to the recruitment and phosphorylation of receptor-regulated SMAD2/3. Phosphorylated SMAD2/3 then bind with common SMAD4 to form heterotrimeric complexes that translocate into the nucleus where they function as transcriptional activators or repressors to modulate gene expression (114).

1.4.5 Activin in placentation and trophoblast invasion

In non-pregnant endometrium, β A and β B subunits are primarily produced by endometrial glands. The expression levels of these two subunits are highest in the secretory phase of menstrual cycle (104, 115). Although inhibin α subunit can be expressed in glandular epithelium, the α subunit level is much lower than β A and β B subunits (116). This expression pattern indicates that activins might play important roles in either the preparation for

decidualization of the uterus, or the differentiation of the embryo for the following implantation step (87). During pregnancy, β A and β B subunits are abundant in decidualizing human stromal cells and in the decidua of the early stage of pregnancy (104, 117). However, activins expression by invasive cytotrophoblast cells is low *in vivo* (118), suggesting that maternally derived activins are the key sources of activins at the maternal-fetal interface. A recent study has shown activin A levels in serum and placenta increase with gestational age. The mean placental activin A concentration is 9 ng/ml in normal pregnancies and 54 ng/ml in severe preeclampsia (66). Receptors for activins (ActRs) are detected in human pre-implantation embryos and are up-regulated at the blastocyst stage (119). Immunohistochemical staining for β A and β B subunits was clearly observed in syncytiotrophoblasts and cytotrophoblasts of hydatidiform mole and invasive mole, indicating activins may play a role in trophoblastic proliferation and invasion (120). Activin A has been shown to stimulate the outgrowth of cytotrophoblast cells from first trimester chorionic villous explants (107), as well as the invasion of purified first trimester cytotrophoblasts (108). In addition, EVT outgrowth was antagonized by low-dose follistatin, the activin binding protein, indicating an important pro-invasive effect of endogenous activin (107).

1.5 Cadherins

Cadherins are transmembrane glycoproteins that mediate calcium-dependent cell-cell adhesion (121). Based upon molecular characteristics, the cadherin family can be subdivided into classical cadherins (type I and type II cadherins), desmosomal cadherins, and seven-pass transmembrane or flamingo cadherins (122). All classical cadherin members are composed of an extracellular domain that includes one or more cadherin repeats, and transmembrane and cytoplasmic domains. Both type I and type II cadherins have five extracellular repeats. Type I

differs from type II in the presence of a histidine, alanine, valine (HAV) tripeptide within the most N-terminal extracellular repeat (EC1), which is essential for homotypic interaction of the cadherins. Type I and type II cadherins are transmembrane components of specialized cell junction regions called adherens junctions (123). Their cytoplasmic domains interact with several adaptor proteins such as catenins and vinculin, which link the cadherin to the actin cytoskeleton and facilitate the clustering into the junctional structure (123). E-cadherin (epithelial-cadherin, CDH1), N-cadherin (neural cadherin, CDH2), P-cadherin (placental cadherin, CDH3), R-cadherin (retinal cadherin, CDH4) and M-cadherin (mytubule cadherin, CDH15) belong to type I classical cadherins, while VE-cadherin (vascular endothelial cadherin, CDH5) and OB-cadherin (osteoblast cadherin, CDH11) belong to type II classical cadherins. Classical cadherins are critical for normal development, and alterations in cadherin function and expression have been implicated in tumorigenesis (124, 125).

1.5.1 N-cadherin in cancer invasion

N-cadherin is a mesenchymal classical type I cadherin with well-described effects on cell invasiveness in a variety of cancers (126). N-cadherin, which belongs to classical/type I cadherins, is composed of five extracellular cadherin repeats, a transmembrane and two cytoplasmic subdomains. Since Hazen et al. (127) reported the expression of mesenchymal adhesion molecule N-cadherin was correlated with breast carcinoma cell invasion, an increasing number of studies have identified the important role of N-cadherin in mediating cancer invasion (128-130). It has been proposed that N-cadherin, as an invasion promoter, could be a novel target for antitumor therapy (126).

1.5.2 Expression of N-cadherin in placental tissues

The process that trophoblasts differentiate along the invasive pathway is thought to be similar with those observed during tumor progression (131). Similarly, the molecular mechanisms underlying trophoblast and cancer cell invasion both involve the regulated expression of cell-cell adhesion molecules (switching from E-cadherin to N-cadherin) (132, 133). Our lab has demonstrated N-cadherin expression by immunohistochemistry (IHC) in EVT's in placental tissue showing higher expression levels of N-cadherin in EVT's compared to other cell components of placental tissue (134). In poorly invasive BeWo and JEG-3 human choriocarcinoma cells, N-cadherin expression levels were significantly lower compared to that in highly invasive HTR8/SVneo cells, suggesting that N-cadherin is important in mediating cell invasion. We have recently demonstrated N-cadherin promotes invasive behavior in HTR8/SVneo cells (135). However, whether or not N-cadherin mediates activin-regulated trophoblast invasion remains unknown.

1.6 Matrix Metalloproteinases (MMPs)

Trophoblast invasion involves not only the regulation of cellular adhesion but also the remodeling and degradation of extracellular matrix (ECM) components. This process requires the coordinated regulation of MMPs as well as their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (136, 137). The MMP family in humans is composed of at least 17 zinc-dependent endopeptidases collectively capable of degrading essentially all components of the extracellular matrix (ECM) (137). MMPs become active after the pro-peptide domain of initially synthesized MMP zymogens is removed. It is well characterized that the overexpression of MMPs contributes to tumor invasion (138, 139). MMP2 (gelatinase A) and MMP9 (gelatinase

B) are the two most well-studied MMPs that can degrade the collagen component in the ECM, thus promoting cancer cell invasion (140). A decreased invasive capacity of tumor cells has been observed when the endogenous inhibitors of MMPs known as tissue inhibitors of metalloproteinases (TIMPs) are expressed (141, 142).

1.6.1 Expression and function of MMPs in placenta

Matrix metalloproteinases (MMPs) are considered crucial for remodeling the uterine environment by the specific degradation/activation of extracellular matrix (ECM) components during implantation (143). MMP2 (gelatinase A) and MMP9 (gelatinase B), as well as some of their well-known substrates (collagen I, IV, V and fibronectin) have been detected in human decidua (144). In first trimester placental tissues, MMP2 is mainly expressed in EVT cells whereas MMP9 is expressed in both villous cytotrophoblasts and EVT cells (145). In addition, MMP2 is abundantly expressed in EVT of ectopic pregnancies (146). This pattern of expression suggests MMP2 and MMP9 are associated with trophoblast invasiveness, and is consistent with their peak expression in the first trimester when trophoblast invasion is highest (147). Indeed, the invasiveness of purified cytotrophoblast cells have been shown to be limited by treatment with an MMP2 neutralization antibody (145). Interestingly, MMP9 expression was found to be weak or absent in preeclamptic placentas, which could be related to impaired trophoblast invasiveness in preeclampsia (148). The activity of these two gelatinases is tightly regulated by tissue inhibitors of metalloproteinase 1 and 2 (TIMP1 and 2), which have been detected not only in human decidua cells (149), but also EVT cells (150). It has been shown that TGF- β inhibits trophoblast invasion through reducing MMP9 levels in placental explants (151). Activin A induces MMP2 production in cytotrophoblast and promotes cytotrophoblast invasion and

outgrowth from placental villous tips (107). Our lab has demonstrated the involvement of MMP2 and MMP9 in human trophoblast invasion and vascular formation (Unpublished data). However, the roles of MMP2 and MMP9 in activin-induced trophoblast invasion and vascular formation as well as the underlying molecular mechanisms are not fully understood.

1.6.2 Transcriptional factor SNAIL and SLUG in cell invasion and MMPs expression

SNAIL (SNAI1) and SLUG (SNAI2) are zinc-finger transcription factors (152) that were first identified as transcriptional repressors of E-cadherin (153). SNAIL and SLUG are both involved in epithelial-mesenchymal transition during embryonic development and the acquisition of invasive properties during cancer progression (154). Indeed, SLUG expression is positively correlated with that of MMP2 in ovarian carcinoma (155), and SNAIL has been shown to enhance MMP2 expression and migration of human bone mesenchymal stem cells and hepatocellular cancer cells (156, 157). In skin cancer cells, promoter-luciferase analysis demonstrated that SNAIL could increase MMP2 expression by binding to its promoter region (158).

1.7 The regulation of EVT endovascular differentiation

1.7.1 Factors regulate EVT endovascular differentiation

Due to the intrinsic capacity for endothelial-like tube formation while cultured on matrigel, the immortalized first trimester HTR8/SVneo cells have been used to investigate the underlying molecular mechanisms of EVT endovascular differentiation (68-70). Aldo et al. observed HTR8/SVneo cells synchronized with an endothelial cell capillary network while co-cultured on

matrigel and eventually replaced endothelial cells (69). Von Dadelszen et al. used HTR8/SVneo cells to investigate the effect of decidual natural killer (dNK)-derived conditioned medium (CM) on EVT endovascular differentiation (68). Lala et al. identified VEGF-A as a promoter of EVT endovascular differentiation in HTR8/SVneo cells (70).

1.7.2 Vascular endothelial growth factor-A (VEGF-A) in angiogenesis

VEGF-A is a well-characterized mediator of physiological and pathological angiogenesis and regulates cell survival, migration and proliferation of endothelial cells through activating multiple tyrosine kinase signaling pathways (159). Currently, many VEGF-A inhibitors are utilized clinically as a strategy for preventing angiogenesis in several malignancies (160).

1.7.3 Expression and function of VEGF-A in placenta

Clinically, a decreased free-form of VEGF-A accompanied with increased anti-angiogenic factor soluble fms-like tyrosine 1 (sFlt-1) levels has been implicated in the pathogenesis of preeclampsia by preventing extensive spiral artery remodeling (161, 162). The VEGF gene family is composed of VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PLGF). As the most potent angiogenic factor in the VEGF family, VEGF-A was first isolated from ascetic fluid and identified as a vascular permeability factor (163). The expression level of VEGF-A is closely related to physiological and pathological angiogenesis (164). In the placenta, the expression of VEGF-A has been described in normal and preeclamptic pregnancy (165). Specifically, endovascular EVT cells express markers of endothelial cells, including VEGF-A and its receptors (18), migrate toward the maternal decidua followed by replacing the endothelial cells of spiral arteries (69, 166). The crucial role of VEGF-A during embryonic development has

been proven by single VEGF-A allele missing mouse embryos, which die in utero between day 11 and 12 due to defective vascularization (167). In addition, neutralizing VEGF-A monoclonal antibody infusion into pregnant Sprague-Dawley rats caused hypertension and proteinuria, the typical features of preeclampsia (168). Lala et al. demonstrated that VEGF-A promoted EVT endovascular differentiation, as measured by increased endothelial-like tube formation after recombinant VEGF-A treatment in HTR8/SVneo cells (70).

1.7.4 Growth factors regulate VEGF-A expression

The up-regulation of VEGF-A can be induced by hypoxia and a variety of growth factors as well as cytokines. It has been reported that hypoxia-inducible factor (HIF) mediates hypoxia-induced VEGF-A expression in endothelial cells (169). Activin A, epidermal growth factor (EGF), TGF- α , TGF- β , FGF, IL-1 α and IL-6 have been shown to increase VEGF-A gene expression in different cell types (170, 171). Likewise, in HTR8/SVneo cells, VEGF-A production is up-regulated in response to TNF- α and TGF- β (172). The possible effect of activin A on VEGF-A expression in placenta has yet to be determined.

Based on the background discussed above, I hypothesize that activin, which is produced by EVT cells and maternal decidual cells, promotes human extravillous cytotrophoblasts invasive and endovascular differentiation by regulating the expression of several key invasive (Cadherins & MMPs) and angiogenic (VEGF-A) related genes in an autocrine or paracrine manner.

Chapter 2: Rationale and objectives

2.1 Rationale

Successful placentation requires that extravillous cytotrophoblasts (EVTs) terminally differentiate into invasive stromal and endovascular remodeling EVT cells (18). Invasive stromal EVT cells invade into the decidua and anchor the placenta to the maternal uterus, whereas endovascular EVT cells penetrate the lumen of distal maternal spiral arterials and subsequently acquire an endothelial-like phenotype and replace the pre-existing endothelial cells lining the uterine vessels. An adequate remodeling of the uterine vasculature system ensures a constant exchange of key molecules between maternal and fetal circulations (34).

Activin A, which belongs to the transforming growth factor- β (TGF- β) superfamily, is a homodimer of the inhibin β A subunit. The expression of activin A is elevated in endometrial stromal and decidual cells (104) and modestly elevated in EVT cells (173). Receptors for activin A have also been detected in human trophoblast cells (119). Although activin A has been shown to enhance human trophoblast cell invasion (107, 108), whether the two additional activin isoforms, activin B and AB, which also exist at the maternal-fetal interface, exert similar effects remains unknown. We have recently demonstrated that mesenchymal adhesion molecule neural cadherin (N-cadherin) levels are much higher in highly invasive HTR8/SVneo human EVT cells than poorly invasive BeWo and JEG-3 choriocarcinoma cells, and that N-cadherin promotes invasive behavior in HTR8/SVneo cells (135). However, whether or not N-cadherin mediates activin-regulated trophoblast invasion remains to be elucidated.

Besides the regulation of cell-cell adhesion molecules, the trophoblast invasion process also involves the remodeling and degradation of extracellular matrix (ECM) by MMPs as well as

their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs). MMP2 (gelatinase A) and MMP9 (gelatinase B) are the two mostly studied MMPs that can degrade the collagen component in ECM. It has been reported that activin A promotes cytotrophoblast invasion by inducing MMP2 production (107). Indeed, the invasiveness of purified cytotrophoblast cells is limited by a MMP2 neutralization antibody in a dose-dependent manner (145). The abundantly expressed MMP2 is detected in EVT cells in ectopic pregnancies (146); however, whether the expression of MMP2 is essential for the pro-invasive effect of activin A has yet to be determined. Among seven activin receptor-like kinase (ALK) receptors, activin A binds to ActRIB (ALK4) and ActRI (ALK2) with high affinity (113). SNAIL and SLUG are both involved in epithelial-mesenchymal transition (EMT) during embryonic development and the acquisition of invasive properties during cancer progression (154). The involvement of TGF- β type I receptors (ALKs) and potential transcriptional factors such as SNAIL and SLUG that are often associated with invasive phenotypes in activin A-induced trophoblast invasion is unknown.

During maternal spiral arterial remodeling, EVT cells acquire an endothelial phenotype and replace the endothelial cells lining the lumen of vessels (174). The mimicry of endothelial cells in the vascular system by EVT cells is often referred to as EVT endovascular differentiation (174), and this primarily represents the ability of EVT cells to mimic endothelial cells in angiogenesis to form endothelial-like tubes *in vitro* upon culture on Matrigel (68, 69). The endovascular differentiation is regarded as a complex process requiring coordinated regulation by essential autocrine and paracrine growth factors. Vascular endothelial growth factor-A (VEGF-A) is well recognized as a key regulator in trophoblast endovascular differentiation (70). The possible roles of activin A associated with VEGF-A in regulating trophoblast endovascular differentiation and the underlying molecular mechanisms have yet to be determined.

Taken together, these results lead us to propose that activin produced by EVT cells and maternal decidual cells could regulate trophoblast invasion and endovascular differentiation in an autocrine or paracrine manner.

2.2 Overall hypothesis

The overall hypothesis of this study is that activin, which is abundantly expressed at the maternal-fetal interface, promotes human extravillous cytotrophoblasts' invasive and endovascular differentiation by regulating the expression of several key invasive and angiogenic related genes.

2.3 Aim of the study

The general aim of my study is to investigate the role of activin in regulating human trophoblast invasion and vascular mimicry as well as the underlying molecular mechanisms during early pregnancy.

2.4 Study models

The HTR8/SVneo immortalized human extravillous cytotrophoblast (EVT) cell line and primary cultures of human EVT cells were used as study models. Basically, HTR8/SVneo cells are more stable and have relatively longer life span (20-30 passages) compared to primary EVT cells (2-5 passages). Although having limited number of cells, primary EVT cells are more representative of the *in vivo* situation compared to HTR8/SVneo cells. The combination of these two study models compensates the limitations of each other and provides more conclusive results. The HTR8/SVneo immortalized human EVT cell line was generously provided by Dr. P.K. Lala

at the Western University (63). The use of primary human EVT cells for this study was approved by the Children's and Women's Hospital Research Ethics Board of the University of British Columbia and all patients provided informed written consents. Human EVT cells were mechanically isolated from first trimester chorionic villous explants (6-10 weeks gestation) (62).

2.5 The specific objectives of this study

Objective 1: To investigate the involvement of N-cadherin in activin isoforms-regulated human trophoblast invasion. (Presented in Chapter 3)

- (1) To compare the effects of three different activin isoforms (activin A, B and AB) on trophoblast invasion.
- (2) To determine the effects of activin isoforms on N-cadherin expression
- (3) To examine the role of N-cadherin in activin isoforms-regulated trophoblast invasion.
- (4) To investigate the effects of activin isoforms on SMAD signaling pathway.
- (5) To investigate the SMAD2/3-SMAD4 signaling mediated up-regulation of N-cadherin.
- (6) To confirm observed results in primary cultures of human first trimester EVT cells.

Objective 2: To investigate the contribution of MMPs and TIMPs in activin A-regulated trophoblast invasion. (Presented in Chapter 4)

- (1) To examine the effect of activin A on MMPs and TIMPs expression.
- (2) To investigate the effect of activin A on SNAIL and SLUG expression.
- (3) To determine the effect of activin A on SMAD signaling pathway.
- (4) To determine the specific TGF- β type I receptor(s) (ALK4/5/7) involved in activin A-induced SMAD2/3 phosphorylation as well as up-regulation of SNAIL, SLUG and MMP2.

- (5) To investigate the SMAD2/3-SMAD4 signaling-mediated up-regulation of SNAIL, SLUG and MMP2.
- (6) To identify the involvement of SNAIL and SLUG in activin A-up-regulated MMP2 expression.
- (7) To confirm the observed results in primary cultures of human first trimester EVT cells.

Objective 3: To investigate the effect of activin A on trophoblast endothelial-like tube formation and underlying molecular mechanisms. (Presented in Chapter 5 and Appendix B)

- (1) To characterize the cell phenotype and gene expression of invasive EVTs and endovascular EVTs.
- (2) To examine the effect of activin A on trophoblast endothelial-like tube formation.
- (3) To study the effect of activin A on the expression of VEGF-A.
- (4) To investigate the role of VEGF-A in activin A-induced human trophoblast endothelial-like tube formation.
- (5) To determine the effect of activin A on SMAD signaling pathway.
- (6) To investigate the SMAD2/3-SMAD4 signaling mediated up-regulation of VEGF-A.

Chapter 3: Activin A, B and AB increase human trophoblast cell invasion by up-regulating N-cadherin

3.1 Introduction

During implantation, highly invasive extravillous cytotrophoblasts (EVTs) extend from cell columns through the outer syncytial trophoblast layer to invade the maternal decidua where they aid in remodeling the uterine environment to support human pregnancy (32, 33). Trophoblast invasion must be tightly controlled because insufficient invasion is related to first-trimester miscarriage, preeclampsia and intrauterine growth restriction (IUGR), whereas uncontrolled invasion is associated with choriocarcinoma and hydatiform moles (12, 35-37). These unfavorable outcomes can have severe consequences for the health of both the mother and fetus. A better understanding of the regulation of human trophoblast invasion, as well as the underlying molecular mechanisms, will improve the diagnosis and treatment of infertility and disorders of pregnancy.

Human trophoblast invasion into the maternal endometrium is tightly regulated by numerous hormones, growth factors and cytokines (33, 82, 83). Activins are members of the transforming growth factor- β (TGF- β) superfamily that are homo or heterodimers of inhibin β A and β B subunits (89). The human placenta and endometrium express both subunits and are capable of producing activins A (β A β A), AB (β A β B) and B (β B β B) (104-106). As growth and differentiation factors, activins have been shown to regulate multiple biological activities in a variety of tissues, including hormone secretion, embryonic development, spermatogenesis, inflammation and carcinogenesis (90, 93-96). However, most studies regarding the functions of activins have focused on activin A, without actually testing the effects of other activin isoforms.

Although controversial, a number of studies have demonstrated different roles for activin isoforms via differential expression (97), receptor (98) and antagonist binding affinity (99), as well as signalling pathways (100-103).

Activin A has been shown to stimulate the outgrowth of cytotrophoblast cells from first trimester chorionic villous explants (107), as well as the invasion of purified first trimester cytotrophoblasts (108). In addition, EVT outgrowth was antagonized by the activin binding protein follistatin, indicating important pro-invasive effects of endogenous activin (107). Similar to other members of the TGF- β superfamily, activins exert their biological effects via activation of heterotetrameric complexes of type I and type II transmembrane serine-threonine kinase receptors (112). Phosphorylation of activin type I receptors leads to the recruitment and phosphorylation of receptor-regulated SMAD2/3. Phosphorylated SMAD2/3 bind with common SMAD4 to form heterotrimeric complexes that translocate into the nucleus where they function as transcriptional activators or repressors to modulate gene expression (114).

Cadherin molecules mediate calcium-dependent cell-cell adhesion in specialized cell junction regions called adherens junctions (123). N-cadherin is a mesenchymal classical type I cadherin with well-described effects on cell invasiveness in a variety of cancers (126-130). Trophoblast differentiation along the invasive pathway is thought to share many features associated with the acquisition of invasiveness during tumor progression (131). Indeed, the molecular mechanisms underlying both trophoblast and cancer cell invasion include the regulated expression of cell-cell adhesion molecules, in particular a switch from E-cadherin to N-cadherin (132, 133). We have recently demonstrated that N-cadherin levels are much higher in highly invasive HTR8/SVneo human EVT cells than poorly invasive JAR, BeWo and JEG-3 choriocarcinoma cells (Figure 3.1), and that N-cadherin promotes invasive behavior in HTR8/SVneo cells (135). However,

whether or not N-cadherin mediates activin-regulated trophoblast invasion remains to be elucidated.

The purpose of our study was to compare the effects of all three activin isoforms on human trophoblast cell invasion and to define the molecular mechanisms involved, in particular the roles of N-cadherin and SMAD2/3 signaling. Our results demonstrate that treatment with activin A, B or AB produces comparable increases in human trophoblast cell invasion and N-cadherin expression. Moreover, the effects of activins on trophoblast invasion are mediated by the up-regulation of N-cadherin via activation of SMAD2/3-SMAD4-dependent signaling.

3.2 Materials and methods

Culture of HTR8/SVneo immortalized human EVT cells

The HTR8/SVneo simian virus 40 large T antigen-immortalized human EVT cell line was generously provided by Dr. P.K. Lala at the Western University (63). The choriocarcinoma cell lines, BeWo, JAR, JEG-3, are obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle Medium/nutrient mixture F-12 Ham (DMEM/F12; Life Technologies, Grand Island, NY) supplemented with 100 U/ml penicillin (Life Technologies), 100 µg/ml streptomycin (Life Technologies), and 10% (vol/vol) fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). Cultures were incubated at 37°C in a humidified 5% CO₂/air atmosphere and the medium was changed every other day.

Primary human EVT isolation and culture

Human EVT cells were mechanically isolated from first trimester chorionic villous explants (6-12 weeks gestation) as previously reported (62). Briefly, extravillous tissues were washed in

Dulbecco's Modified PBS (HyClone Laboratories) and minced into small pieces that were cultured in DMEM (Sigma-Aldrich, Oakville, ON) containing 10% (vol/vol) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cultures were incubated in flasks at 37°C in a humidified 5% CO₂/air atmosphere for 3-4 days, after which non-attached pieces were washed away. Villous tissue fragments were cultured for a further 5-7 days to allow for EVT outgrowth, and EVT cells were subsequently separated from attached fragments by trypsinization. The purity of EVT cultures was assessed immunocytochemically and showed 100% positive staining for cytokeratin-7 (epithelial marker) together with no staining for vimentin (mesenchymal marker). The use of the cells for this study was approved by the Children's and Women's Hospital Research Ethics Board of the University of British Columbia.

Antibodies and reagents

Phospho-SMAD2 (Ser^{465/467}) rabbit monoclonal antibody, SMAD2 (L16D3) mouse monoclonal antibody, Phospho-SMAD3 (Ser^{423/425}) (C25A9) rabbit monoclonal antibody, SMAD3 (C67H9) rabbit monoclonal antibody and SMAD4 polyclonal antibody were purchased from Cell Signaling Technology (Beverly, MA). Purified mouse monoclonal anti-N-cadherin and purified mouse Anti-E-cadherin antibodies were obtained from BD Biosciences (Mississauga, ON). Mouse monoclonal anti- α -tubulin (B-5-1-2) (sc-23948) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The TGF- β type I receptor inhibitor SB431542 (#S4317) was purchased from Sigma Aldrich. Cleaved caspase 3 antibody was obtained from Cell Signaling Technology. Cycloheximide was obtained from BioVision (California, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased from

Bio-Rad Laboratories (Hercules, CA). Recombinant human activin A, activin B and activin AB were obtained from R&D Systems (Minneapolis, MN).

Matrigel-coated transwell invasion assay

Cell invasiveness was measured using 24-well transwell inserts (pore size 8 μm ; BD Biosciences) coated with 40 μl of growth factor-reduced Matrigel (BD Biosciences) at a concentration of 1 mg/ml. Inserts were seeded with 1×10^5 cells in 250 μl DMEM/F12 medium supplemented with 0.1% (vol/vol) FBS, and medium with 10% (vol/vol) FBS (750 μl) was added to the lower chamber. Cells were incubated at 37°C for 48 h after which non-invading cells were wiped from the upper side of the membrane and cells on the lower side were fixed with cold methanol (-20°C) and air dried. Cell nuclei were stained with Hoechst 33258 (Sigma-Aldrich) and counted using a Zeiss Axiophot epifluorescent microscope and Northern Eclipse 6.0 software (Empix Imaging, Mississauga, Ontario, Canada). Triplicate inserts were used for each individual experiment and five microscopic fields were counted per insert.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Cells were washed with cold PBS and total RNA was extracted with TRIzol Reagent (Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed using 2 μg of total RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI) in a final volume of 20 μl . RT-qPCR was performed on the Applied Biosystems 7300 real-time PCR system equipped with 96-well optical reaction plates. Each 20- μL RT-qPCR reaction contained 1 \times SYBR Green PCR Master Mix (Applied Biosystems), 12 ng cDNA and 150 nM of each specific primer. The primers used were: N-cadherin, 5'-GGA CAG TTC CTG AGG GAT

CA-3' (forward) and 5'-GGA TTG CCT TCC ATG TCT GT-3' (reverse); VE-cadherin, 5' - CAG CCC AAA GTG TGT GAG AA-3' (forward) and 5' - CGG TCA AAC TGC CCA TAC TT-3' (reverse); E-cadherin, 5'-ACA GCC CCG CCT TAT GAT T-3' (forward) and 5'-TCG GAA CCG CTT CCT TCA-3' (reverse); human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GAG TCA ACG GAT TTG GTC GT-3' (forward) and 5'- GAC AAG CTT CCC GTT CTC AG -3' (reverse); SMAD2, 5'-GCC TTT ACA GCT TCT CTG AAC AA-3' (forward) and 5'-ATG TGG CAA TCC TTT TCG AT-3' (reverse); SMAD3, 5'-CCC CAG CAC ATA ATA ACT TGG-3' (forward) and 5'-AGG AGA TGG AGC ACC AGA AG-3' (reverse); SMAD4, 5'-TGG CCC AGG ATC AGT AGG T-3' (forward) and 5'-CAT CAA CAC CAA TTC CAG CA-3' (reverse); SNAIL, 5'-CCC CAA TCG GAA GCC TAA CT-3' (forward) and 5'-GCT GGA AGG TAA ACT CTG GAT TAG A-3' (reverse); SLUG, 5'-TTC GGA CCC ACA CAT TAC CT-3' (forward) and 5'- GCA GTG AGG GCA AGA AAA AG -3' (reverse); TWIST, 5'-GGA GTC CGC AGT CTT ACG AG-3' (forward) and 5'-TCT GGA GGA CCT GGT AGA GG-3' (reverse); RUNX2, 5'-GGA GTG GAC GAG GCA AGA GTT T-3' (forward) and 5'-AGC TTC TGT CTG TGC CTT CT GG-3' (reverse); ZEB1, 5'-GCA CCT GAA GAG GAC CAG AG-3' (forward) and 5'TGC ATC TGG TGT TCC ATT TT-3' (reverse); ZEB2, 5'-GTA CCT TCA GCG CAG TGA CA-3' (forward) and 5'-CAG GTG GCA GGT CAT TTT CT-3' (reverse).

The specificity of each assay was confirmed by dissociation curve analysis and agarose gel electrophoresis of PCR products. Assay performance was validated by assessing amplification efficiencies by means of calibration curves, and ensuring that the plot of log input amount versus ΔCq has a slope with an absolute value of <0.1 . At least three separate experiments were performed and each sample was assayed in triplicate. A mean value was used for the

determination of mRNA levels by the comparative Cq method with GAPDH as the reference gene and using the formula $2^{-\Delta\Delta Cq}$.

Western blot

Cells were washed with cold phosphate buffered saline (PBS) twice and lysed in ice cold lysis buffer (Cell Signaling Technology) with added protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined using the DC protein assay kit (Bio-Rad Laboratories) with bovine serum albumin (BSA) as the standard. Equal amounts of protein (30 μ g) were separated by SDS-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene fluoride membrane. Membranes were blocked with TBS containing 5% (wt/vol) non-fat dry milk for 1 h and incubated overnight at 4°C with primary antibodies diluted in Tris-buffered saline (TBS) with 5% (wt/vol) non-fat dried milk and 0.1% (vol/vol) Tween-20. Bound antibodies were visualized using HRP-conjugated secondary antibody, ECL or SuperSignal West Femto chemiluminescent substrates (Thermo Fisher, Waltham, MA), and CL-XPosure film (Thermo Fisher). Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mM β -mercaptoethanol and 1% wt/vol SDS) at 50°C for 30 min and reprobbed with anti- α -tubulin antibody as a loading control. Densitometric quantification was performed using Scion Image software with α -tubulin as the internal control for normalization.

Small interfering RNA (siRNA) transfection

Fifty percent confluent cells were transfected for 48h with 20 nM specific gene targeting ON-TARGET*plus* SMART*pool* siRNA or 20 nM siCONTROL NON-TARGETING*pool* siRNA (Thermo Scientific, Lafayette, CO) using Lipofectamine RNAiMAX and Opti-MEM I according

to the manufacturer's instructions (Life Technologies). Transfection efficiency was assessed by RT-qPCR or Western blot analysis.

MTT assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) assay was used to assess cell viability. HTR8/SVneo cells were seeded in 24-well plates (2×10^4 cells/well in 1 mL of medium) and treated every 24 h with vehicle or activin isoforms in medium containing 1% (vol/vol) FBS for a total of 72 h. Cells were incubated with 0.5 mg/mL MTT for 4 h, the medium was replaced with 1 mL DMSO and absorbances were measured at 490 nm using a microplate reader.

Study protocol

Study groups comprised cells treated with activin A, SB431542 and/or siRNA targeting N-cadherin, SMAD2, SMAD3 and/or SMAD4, whereas the corresponding control groups were comprised of cells treated without activin A or with equivalent amounts of DMSO or non-targeting control siRNA. For studies involving HTR8/SVneo cells at least three independent experiments were performed, each on a separate passage. For studies conducted in primary human EVT cells at least three independent experiments were performed on separate primary cultures of human EVT cells.

Statistical analysis

Results are presented as the mean \pm SEM of at least three independent experiments, and were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons of means

using PRISM software (GraphPad Software, Inc., San Diego, CA). Means were considered significantly different if $P < 0.05$ and are indicated by different letters.

3.3 Results

Activins A, B and AB increase HTR8/SVneo cell invasion

Activin A has been shown to increase human trophoblast cell invasion; however, whether activin B and AB exert similar effects is unknown. To examine the effects of activin isoforms on trophoblast cell invasion, Matrigel-coated transwell invasion assays were performed following treatment of HTR8/SVneo human immortalized EVT cells with 50 or 100 ng/ml recombinant human activin A, B or AB. Consistent with previous studies, treatment with activin A significantly enhanced HTR8/SVneo cell invasion in a concentration-dependent manner. Likewise, treatment with activin B or AB induced HTR8/SVneo cell invasion to a similar extent as that of activin A (Figure 3.2A). SB431542 is a specific inhibitor for TGF- β type I receptors (175). Application of the TGF- β type I receptor inhibitor SB431542 (10 μ M) for 1 h prior to treatment with activin isoforms completely abolished their effects on HTR8/SVneo cell invasion (Figure 3.2B). It has been reported that high concentrations of activin A (25-100 ng/ml) can induce apoptosis in HTR8/SVneo cells as well as primary cultured cytotrophoblast cells (66). We used MTT assays and measured cleaved caspase 3 levels to determine whether the effects of activin isoforms on trophoblast cell invasion could be influenced by changes in cell viability or apoptosis. Treatment with 50 ng/ml activin A, B or AB for as much as 72 h did not influence HTR8/SVneo cell viability (Figure 3.2C). Moreover, Western blot analysis showed that treatment for 24 h with activin isoforms (50 ng/ml or 100 ng/ml) did not influence cleaved caspase 3 protein levels (Figure 3.2D).

Activins A, B and AB increase N-cadherin expression

Knockdown of N-cadherin has been shown to reduce human trophoblast cell invasion (135). To investigate whether activin isoforms up-regulate of N-cadherin, HTR8/SVneo cells were treated with 50 ng/ml activin A, B or AB for 3, 6, 12, 24 or 48 h. RT-qPCR analysis showed that treatment with activins up-regulated N-cadherin mRNA levels in a time-dependent manner (Figure 3.3A). Similarly, Western blot analysis showed that treatment with activin isoforms increased N-cadherin protein levels (Figure 3.3B). As with cell invasiveness, these effects on N-cadherin mRNA and protein levels were abolished by co-treatment with SB431542 (Figure 3.3C and D). Importantly, the stimulatory effects of activins on N-cadherin mRNA and protein levels were confirmed in primary human EVT cells propagated from first trimester chorionic villous explants (Figure 3.3E and F).

Basal and activin-induced cell invasion are attenuated by siRNA-mediated depletion of N-cadherin

Treatment with siRNA was used test whether N-cadherin is involved in activin-induced HTR8/SVneo cell invasion. Western blot analysis showed that transfection with siRNA targeting N-cadherin for 48 h suppressed both endogenous and activin-induced N-cadherin protein levels (Figure 3.4A). Matrigel transwell invasion assay results revealed that treatment with N-cadherin siRNA attenuated both basal and activin isoform-induced HTR8/SVneo cell invasion (Figure 3.4B). Furthermore, the crucial role of N-cadherin in mediating basal and activin-induced trophoblast cell invasion was confirmed in primary human EVT cells propagated from first trimester chorionic villous explants. Treatment with N-cadherin siRNA decreased both

endogenous and activin A-induced N-cadherin mRNA and protein levels (Figure 3.4C) as well as Matrigel transwell invasion (Figure 3.4D).

SMAD2/3 signaling is required for activin-induced up-regulation of N-cadherin

To investigate whether activin isoforms exert their pro-invasive effects through canonical SMAD-dependent signaling, SMAD2/3 phosphorylation was measured by Western blot following treatment of HTR8/SVneo cells with 50 ng/ml activin A, B or AB for 30 or 60 min. All three activin isoforms significantly, and equally, increased the phosphorylation of both SMAD2 and SMAD3 (Figure 3.5A). Alternatively, HTR8/SVneo cells were treated for 60 min with activin A, B or AB in the presence or absence of 10 μ M SB431542. As with cell invasiveness and N-cadherin expression, activin-induced phosphorylation of SMAD2 and SMAD3 was abolished by treatment with SB431542 (Figure 3.5B). Though SMAD2 and SMAD3 are highly homologous, they can mediate different cellular functions in a context-dependent manner (176). To investigate the individual and/or combined roles of SMAD2 and SMAD3 in activin-induced N-cadherin up-regulation, HTR8/SVneo cells were treated with activin isoforms following siRNA-mediated depletion of SMAD2 or SMAD3 (knockdown confirmed by RT-qPCR). Depletion of either SMAD2 or SMAD3 alone attenuated, but did not completely abolish, the up-regulation of N-cadherin mRNA by activins (Figure 3.6A and B). However, combined knockdown of SMAD2/SMAD3 completely abolished the stimulatory effects of activin isoforms on N-cadherin mRNA levels (Figure 3.6C). To confirm the role of SMAD signaling in activin-regulated N-cadherin expression, HTR8/SVneo cells were treated with activin isoforms following siRNA-mediated depletion of common SMAD4. Down-

regulation of SMAD4 completely abolished the stimulatory effects of activins on N-cadherin mRNA and protein levels (Figure 3.6D and E).

3.4 Discussion

The expression of inhibin β subunits and production of activins has been described in normal and pathological human endometrium and placenta (173). Inhibin β A and β B subunits are highly expressed in the endometrium during the secretory phase of the menstrual cycle (115). Additional studies have demonstrated elevated expression in endometrial stromal and decidual cells (104, 105) as well as modest expression in invasive EVT_s (106). Receptors for activins have been detected in human pre-implantation embryos and are up-regulated at the blastocyst stage (119). In preeclampsia, a common complication of pregnancy characterized by shallow trophoblast invasion (36), up-regulation of activin A levels in serum and placenta (66) as well as placental ActRIIA levels (177) suggests important functions for activin during placentation. However, activin A has been shown to promote the invasion of cytotrophoblasts at 6-8 and 8-10 weeks, but not 10-12 weeks gestation (108), indicating a window for the pro-invasive effects of activin early in gestation. While these findings may, at first, appear contradictory, it should be noted that the earliest comparison measurements of serum activin A levels were at 15-18 weeks gestation and no significant differences were observed until 25-30 weeks, when patients may already present with preeclampsia (66). Thus, it is uncertain to what degree the elevation of activin A or ActRIIA later in pregnancy contributes to the development of preeclampsia, as opposed to reflecting disease manifestation or a homeostatic response. Evidence to date suggests the effects of activin on trophoblasts may vary depending on gestational age. Pro-invasive effects exerted early in pregnancy are likely to be important for spiral artery remodeling which starts

from 10 weeks gestation in humans (7), whereas pro-apoptotic effects could manifest at later gestational stages and in association with preeclampsia.

Inhibin β A and β B subunits share 63% amino acid sequence identity, however differences in their patterns of synthesis suggest independent functional roles (178, 179). Indeed, mice lacking either of these two subunits exhibited different phenotypic defects and no functional overlap was found (180). Thus, while we did not identify any differences in the effects of exogenous treatment with activin isoforms, it does not rule out *in vivo* differences based on cell type-specific subunit expression. Nevertheless, some studies have identified differential effects of activin isoforms that are likely attributable to variations in receptor expression or binding affinity. For example, ActRIIA levels are significantly increased in preeclampsia (177), and ActRIIA has been shown to bind activin A with higher affinity than activin B (98). Activin A and AB affect epidermal growth factor-inhibited DNA synthesis in the same manner, whereas activin B has no effect (101). Likewise, activin A, B and AB inhibited hepatocyte growth factor synthesis in human lung fibroblasts with different potencies (100). In addition, inhibin B ($\alpha\beta$ B) has higher affinity for ActRIIB than inhibin A ($\alpha\beta$ A) and only inhibin A binding to ActRIIB was significantly enhanced in the presence of betaglycan. However, the binding of both inhibin A and B to ActRIIA was potentiated in the presence of betaglycan even though their affinities to betaglycan were thought to be equivalent (181). Lastly, the biological activity of activin isoforms may be influenced by differential binding and neutralization by endogenous antagonist proteins. In particular, it has been reported that the neutralization of activin A and B by follistatin or follistatin-like 3 are not identical, so that in tissues where both activins are produced, their relative activities could be quite different (99). In this context, putative isoform- or gestational age-specific effects of activins on human placental cell types are likely to involve multiple

mechanisms, and future studies aimed at addressing this question will be of interest.

TGF- β superfamily members display divergent functions in regulating trophoblast invasion during implantation. TGF- β 1, β 2 and β 3 have been shown to suppress the invasiveness of EVT cells derived from placental explants by down-regulating the production and activity of matrix metalloproteinase 9 (MMP9) (151), whereas activin A enhanced their invasive capacity by up-regulating the production and activity of MMP2 (107). Recently, inhibition of trophoblast invasion by TGF- β 1 was also shown to involve the suppression of VE-cadherin expression by SMAD2/3-induced SNAIL production. TGF- β 1-induced SNAIL production was primarily mediated by SMAD2, whereas up-regulation of SLUG was largely SMAD3-dependent (67). Interestingly, treatment of trophoblasts with activin isoforms also up-regulates SNAIL and SLUG in an SB431542- and SMAD4-sensitive manner (Figure 3.7, Figure 4.2 and Figure 4.5), however siRNA-mediated depletion of SNAIL or SLUG does not affect activin-induced N-cadherin expression (Figure 3.8). Activin isoforms also exert suppressive effects on VE-cadherin only at the 24 h time interval and not at the earlier time intervals (Figure 3.9) that, like TGF- β 1, are likely to be mediated by SNAIL, though this has not been confirmed. In contrast, the up-regulation of N-cadherin mRNA as early as 3 hours after treatment with activin suggests direct binding of SMAD2/3-SMAD4 to the N-cadherin promoter. Indeed, chromatin immunoprecipitation and promoter luciferase analyses have demonstrated that SMAD4 cooperates with Notch to regulate N-cadherin promoter activity in endothelial cells (182). Overall, the divergent functions of TGF- β and activin on trophoblast invasion cannot be fully explained by cadherin dynamics and suggests important roles for additional molecular mechanisms, be they SMAD-dependent or SMAD-independent.

In summary, the present study demonstrates for the first time that N-cadherin is involved in activin-induced trophoblast invasion. Moreover, activin A, B and AB produce equivalent increases in human trophoblast cell invasion by up-regulating N-cadherin expression in a SMAD2/3-SMAD4-dependent manner. Specifically, activin A (homodimer of inhibin β A) binds to a heterotetrameric receptor complex comprised of type I (ALK4) and type II (ACVR2A/B) receptors. Ligand-induced activation of the receptor complex results in the activation of receptor-regulated SMAD2/3. Phosphorylated SMAD2/3 forms a heterotrimeric complex with common SMAD4 that translocates into the nucleus where it binds DNA and increases the production of N-cadherin, which is an essential mediator of activin A-induced human trophoblast cell invasion (Figure 3.10). Our findings provide important insights into the molecular mechanisms underlying the pro-invasive effects of activins on human EVT_s, and enhance our understanding of the establishment of pregnancy under normal or pathological conditions.

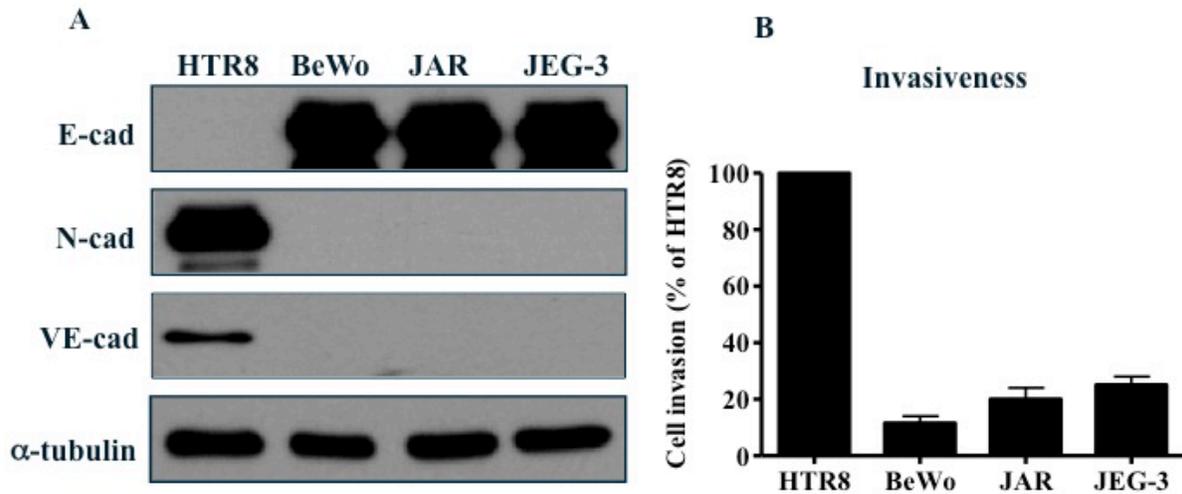
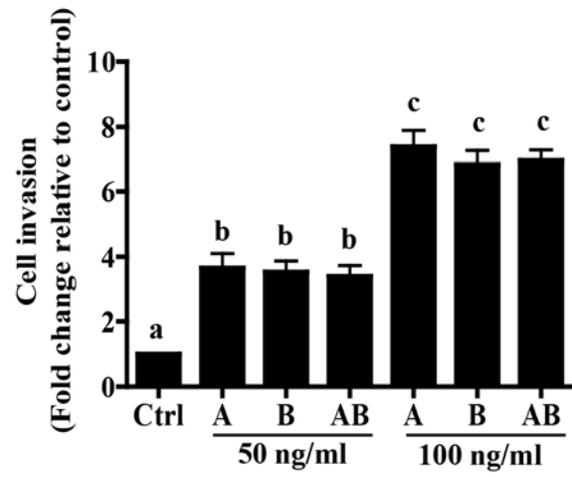
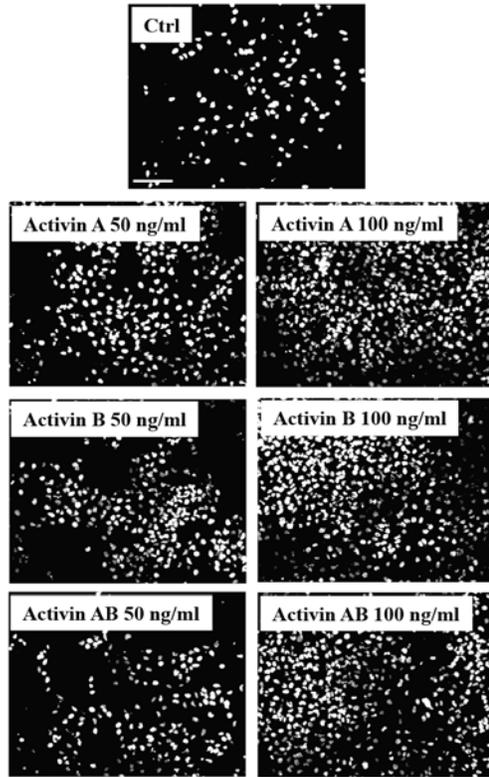


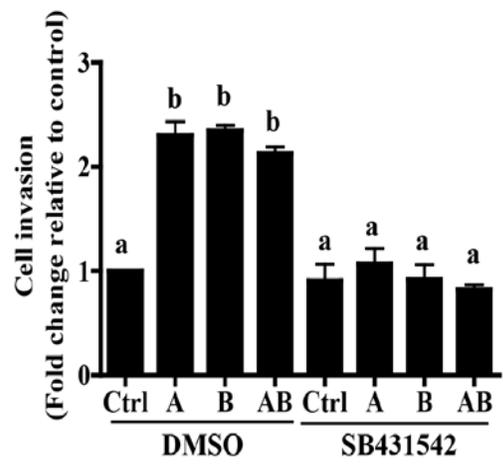
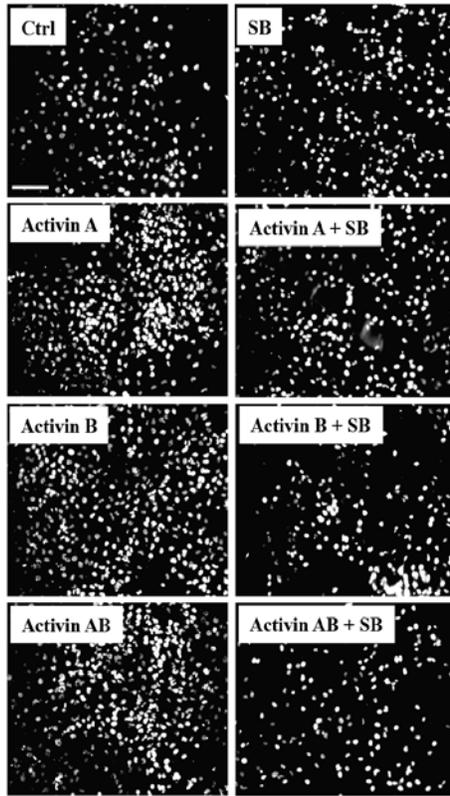
Figure 3.1 Protein levels of E-cadherin, N-cadherin and VE-cadherin as well as basal invasive capacity in HTR8/SVneo, BeWo, JAR and JEG-3 cells

A, Cells were lysed with lysis buffer and the cell lysate was analyzed by Western blot with anti-E-cadherin, anti-N-cadherin, anti-VE-cadherin antibodies and with anti- α -tubulin antibody as the loading control. Representative results from Western blot are presented. **B**, Relative basal invasiveness of the HTR8/SVneo and choriocarcinoma cell lines, BeWo, JAR and JEG-3. The invasion assay results are expressed as the mean \pm SEM of at least three independent experiments. Groups without a common letter are statistically different from each other ($p < 0.05$).

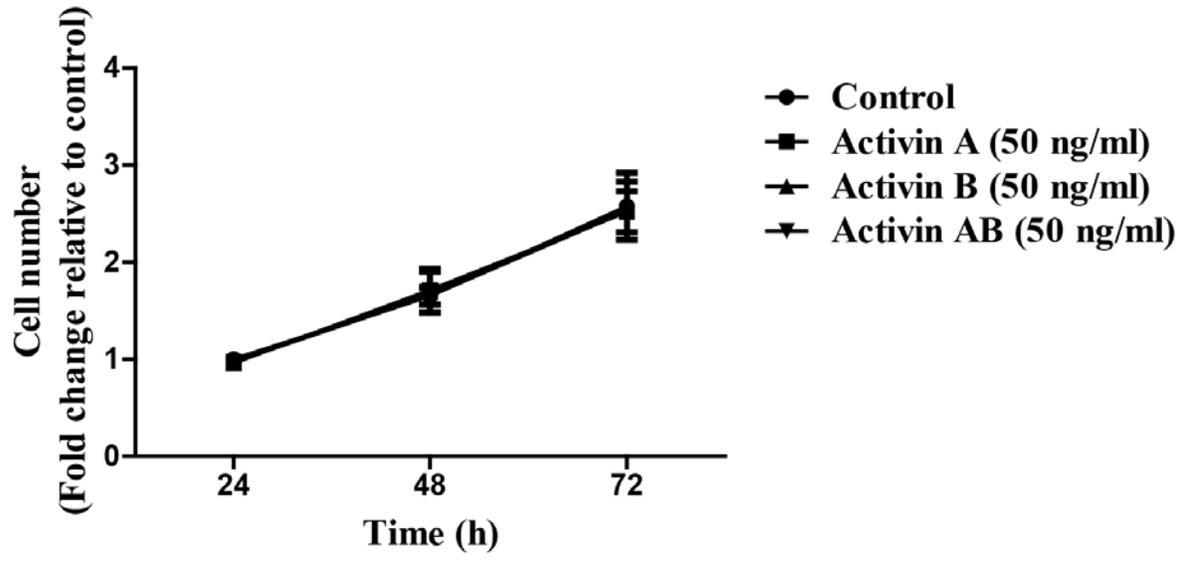
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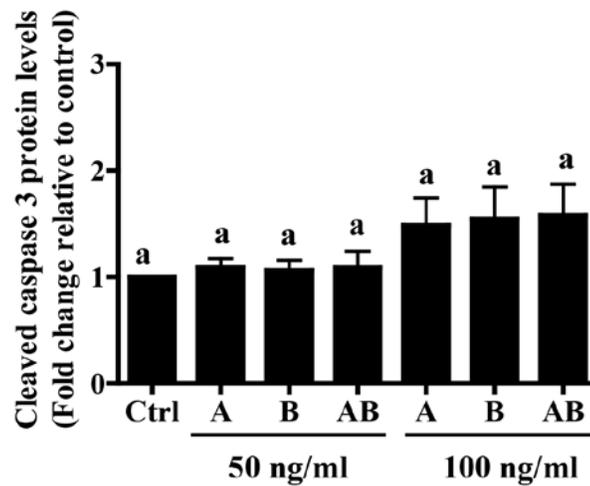
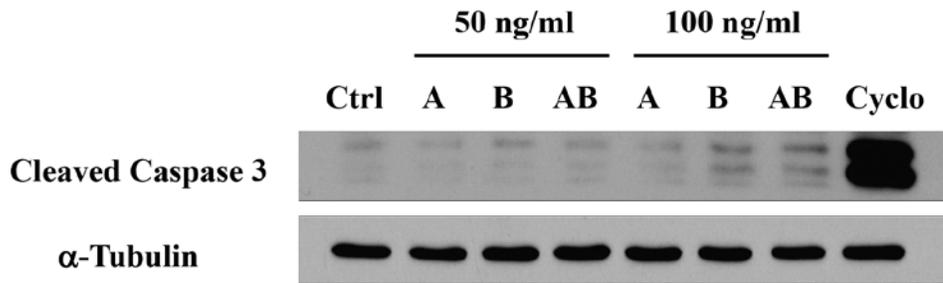
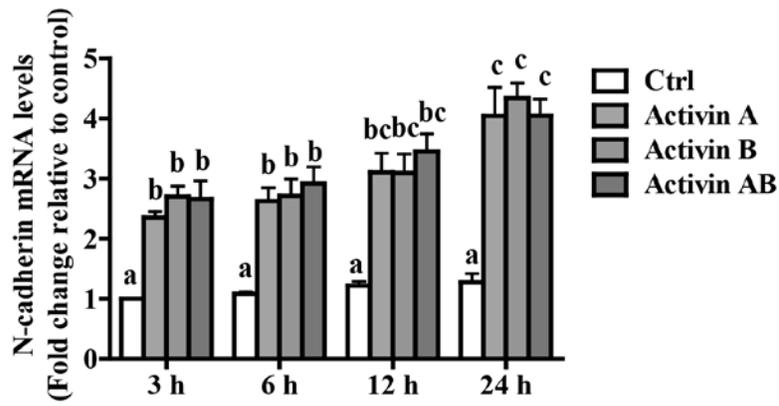


Figure 3.2 Activin A, B and AB produce comparable increases in HTR8/SVneo cell invasion that can be blocked by the TGF- β type I receptor inhibitor SB431542

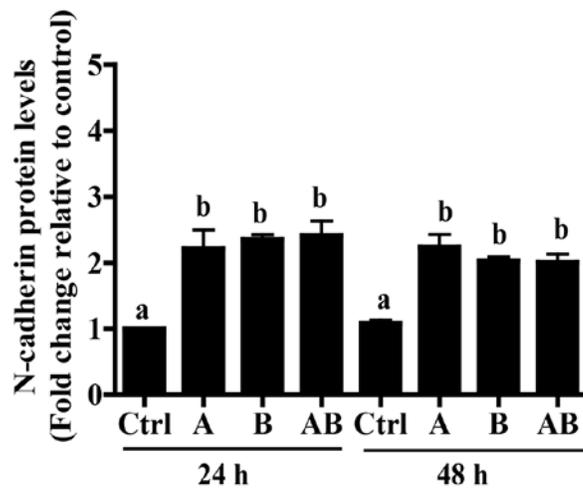
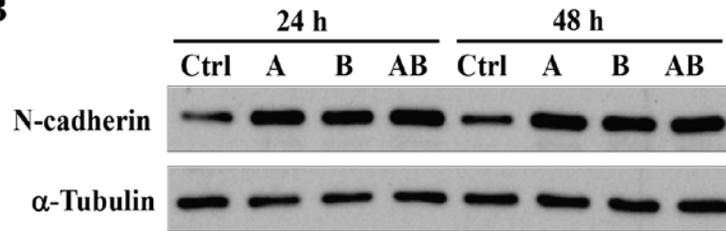
A, HTR8/SVneo cells were treated without (Ctrl) or with 50 or 100 ng/ml activin A (A), B (B) or AB (AB) and cell invasiveness was examined by Matrigel-coated transwell invasion assay. **B**, HTR8/SVneo cell invasiveness was assessed following pretreatment for 1 h with vehicle control (DMSO) or 10 μ M SB431542 prior to treatment without (Ctrl) or with 50 ng/ml activin A, B or AB. Left panels show representative images of the invasion assays (scale bar: 200 μ m) and right panels show summarized quantitative results. **C**, HTR8/SVneo cells were seeded in 24-well

plates (2×10^4 cells/well in 1 mL of medium) and the next day were treated every 24 h without (Ctrl) or with 50 ng/ml activin A, B or AB in medium containing 0.1% FBS. Cell viability at 24, 48 and 72 h after treatment was examined by MTT assay. **D**, HTR8/SVneo cells were treated for 24 h without (Ctrl) or with 50 or 100 ng/ml activin A, B, AB or positive control (200 μ M cycloheximide (Cyclo)). Cleaved caspase-3 protein levels were examined by Western blot and data were normalized to α -tubulin. The upper panel shows a representative Western blot and the lower panel shows summarized quantitative results. The quantitative results of invasion assay and Western blot analysis are expressed as the mean \pm SEM of at least three independent experiments. Groups without a common letter are statistically different from each other ($p < 0.05$).

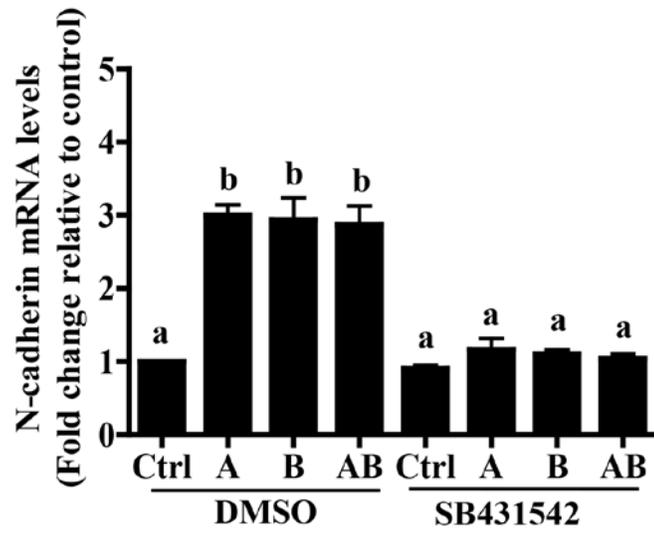
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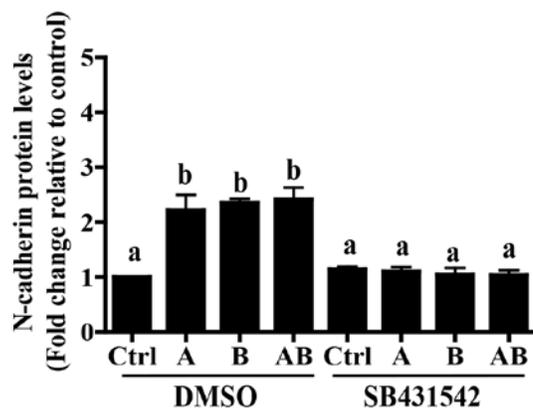
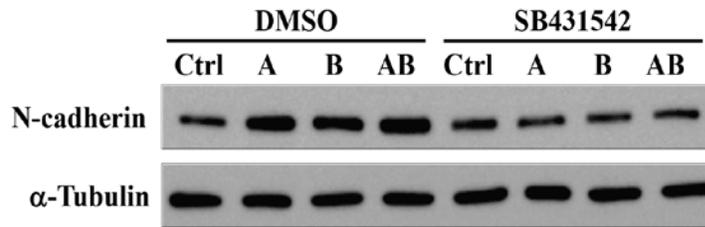
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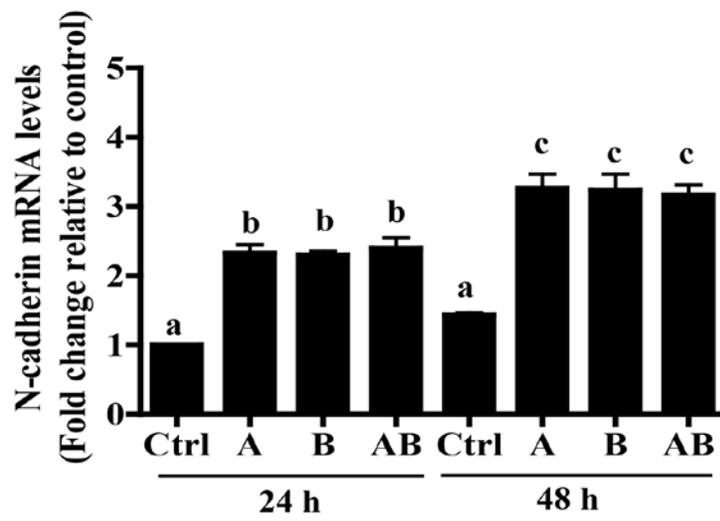
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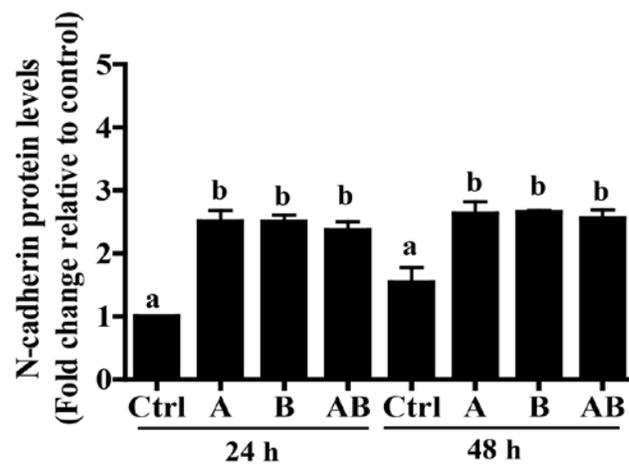
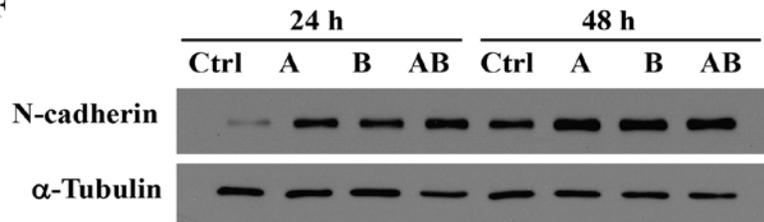
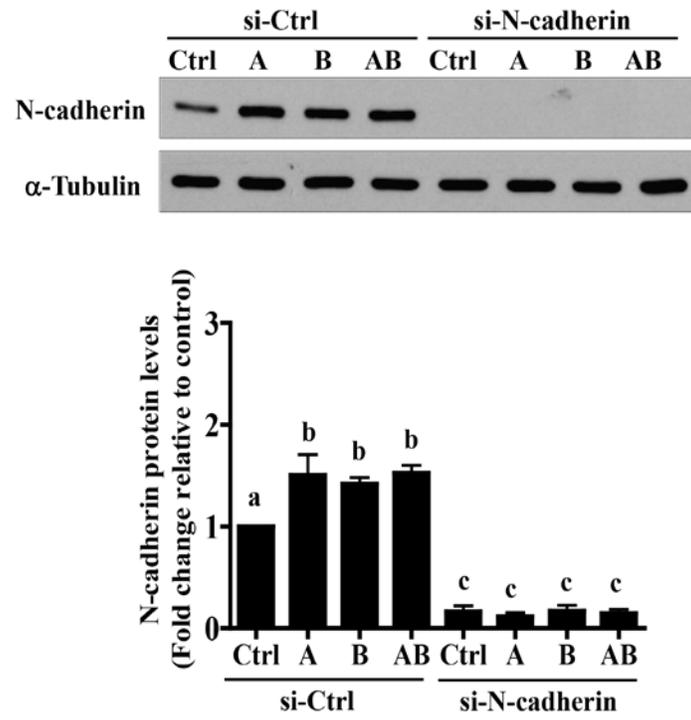


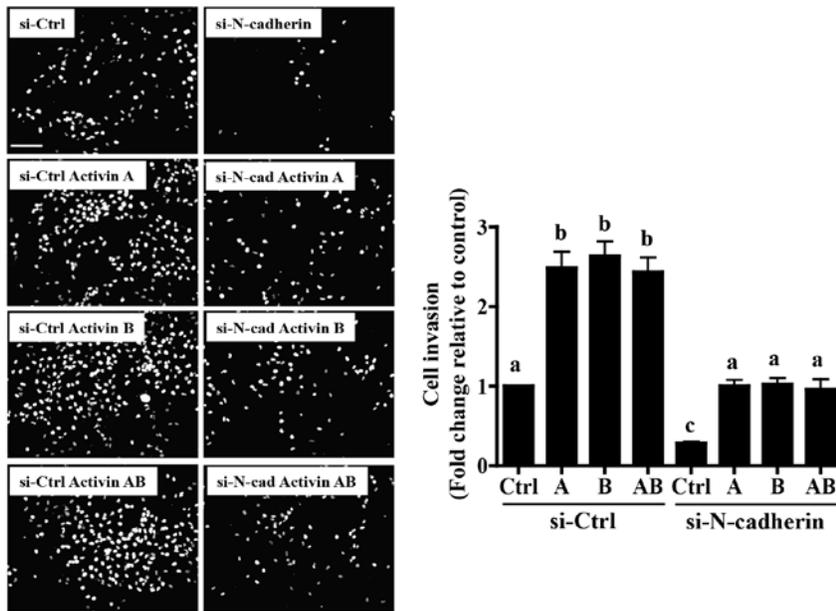
Figure 3.3 Activin A, B and AB produce comparable increases in N-cadherin in HTR8/SVneo and primary human EVT cells

A, HTR8/SVneo cells were treated without (Ctrl) or with 50 ng/ml activin A, B or AB for different lengths of time (3, 6, 12 or 24 h) and N-cadherin mRNA levels were examined by RT-qPCR with GAPDH as the reference gene. **B**, HTR8/SVneo cells were treated for 24 or 48 h without (Ctrl) or with 50 ng/ml activin A, B or AB, and N-cadherin protein levels were examined by Western blot and normalized to α -tubulin. The upper panel shows a representative Western blot and the lower panel shows summarized quantitative results. **C** and **D**, HTR8/SVneo cells were pretreated for 1 h with vehicle control (DMSO) or 10 μ M SB431542 prior to treatment without (Ctrl) or with 50 ng/ml activin A, B or AB for 24 h. **C**, N-cadherin mRNA levels were examined by RT-qPCR with GAPDH as the reference gene. **D**, N-cadherin protein levels were examined by Western blot and normalized to α -tubulin. Quantitative results are expressed as the mean \pm SEM of at least three independent experiments. **E** and **F**, Primary cultures of human EVT cells were treated for 24 or 48 h without (Ctrl) or with 50 ng/ml activin A, B or AB. **E**, N-cadherin mRNA levels were examined by RT-qPCR with GAPDH as the reference gene. **F**, N-cadherin protein levels were examined by Western blot and normalized to α -tubulin. The upper panel shows a representative Western blot and the lower panel shows summarized quantitative results expressed as the mean \pm SEM of at least three independent experiments from different primary cultures of human EVT cells. Groups without a common letter are statistically different from each other ($p < 0.05$).

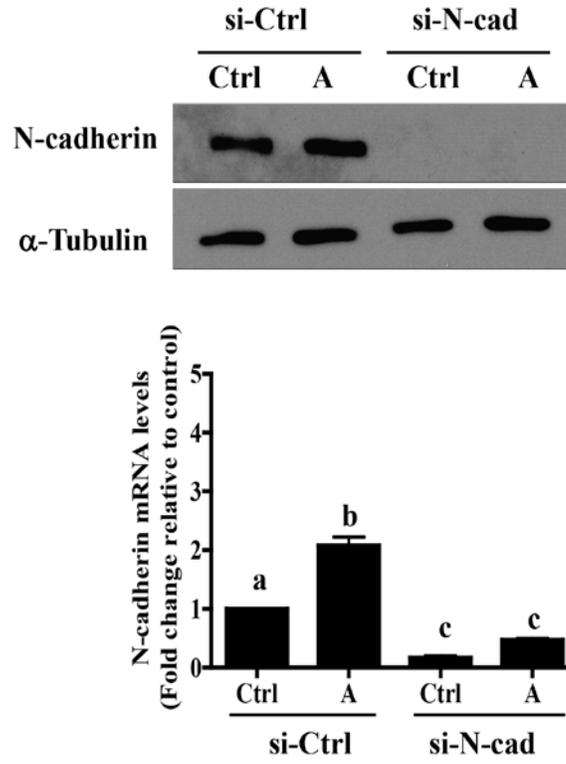
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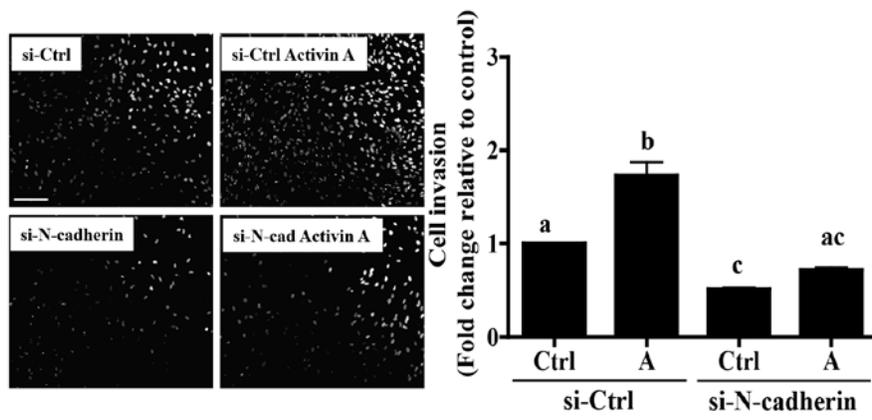
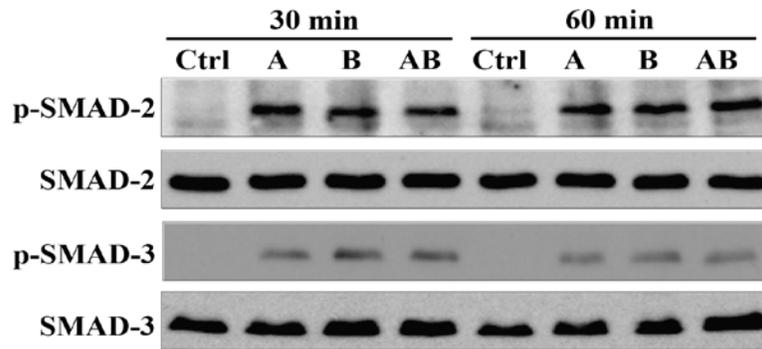


Figure 3.4 Basal and activin-induced cell invasion are attenuated by siRNA-mediated down-regulation of N-cadherin

A and **B**, HTR8/SVneo cells were transfected for 48 h with non-targeting control siRNA (si-Ctrl) or siRNA targeting N-cadherin (si-N-cadherin) prior to treatment without or with 50 ng/ml activin A, B or AB. **A**, 24 h after treatment N-cadherin protein levels were examined by Western blot and normalized to α -tubulin. **B**, Cell invasiveness was examined by Matrigel-coated transwell assay. The left panel shows representative images of the invasion assay and the right panel shows summarized quantitative results. **C** and **D**, Primary cultures of human EVT cells were transfected for 48 h with non-targeting control siRNA (si-Ctrl) or siRNA targeting N-cadherin (si-N-cad) prior to treatment without or with 50 ng/ml activin A. **C**, The upper panel shows a representative Western blot of N-cadherin protein levels and the lower panel shows summarized quantitative results of N-cadherin mRNA levels. **D**, Cell invasiveness was examined by Matrigel-coated transwell assay. The left panel shows representative images of the invasion assay and the right panel shows summarized quantitative results expressed as the mean \pm SEM of at least three independent experiments from different primary cultures of human EVT cells. Groups without a common letter are statistically different from each other ($p < 0.05$).

A



B

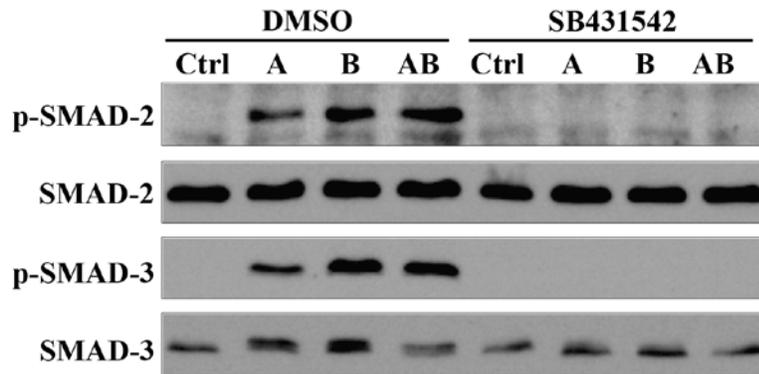
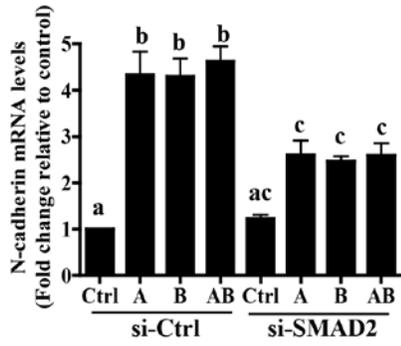
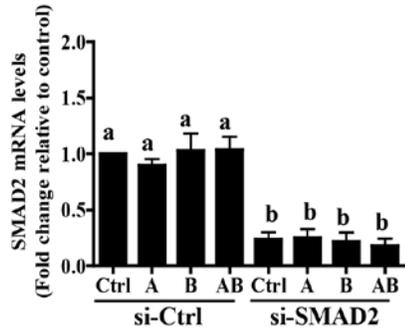
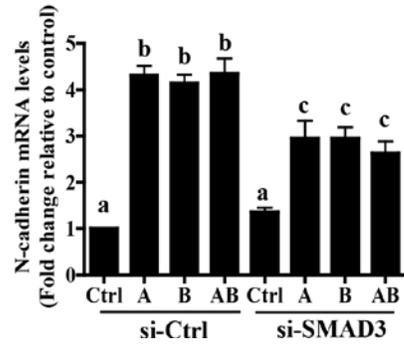
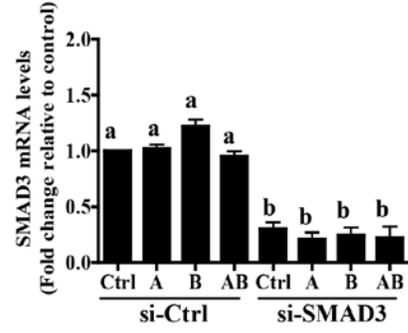
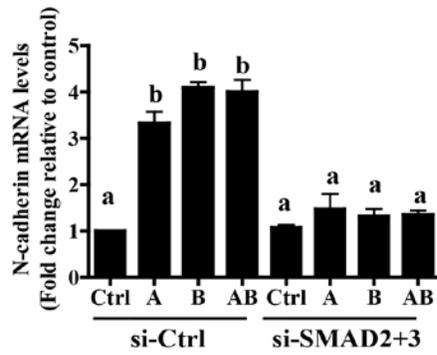
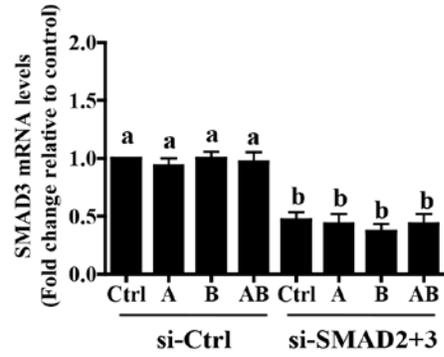
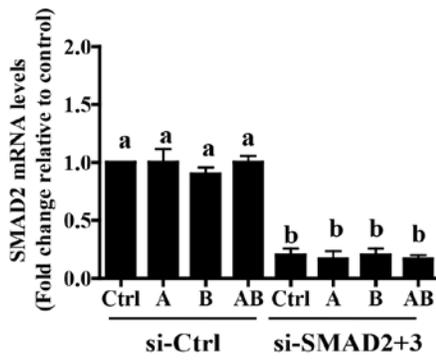


Figure 3.5 Activin A, B and AB induce comparable increases in the phosphorylation of SMAD2 and SMAD3 that are abolished by SB431542

A, HTR8/SVneo cells were treated for 30 or 60 min without (Ctrl) or with 50 ng/ml activin A, B or AB. Levels of phosphorylated SMAD2 (p-SMAD2) and SMAD3 (p-SMAD3) were examined by western blot with phospho-specific antibodies. Membranes were stripped and reprobed with antibodies for total SMAD2 and SMAD3. **B**, HTR8/SVneo cells were pretreated for 1 h with vehicle control (DMSO) or 10 μ M SB431542 prior to treatment for 60 min without (Ctrl) or with 50 ng/ml activin A, B or AB. Levels of phosphorylated and total SMAD2 and SMAD3 were measured by Western blot as described for **A**.

A**B****C**

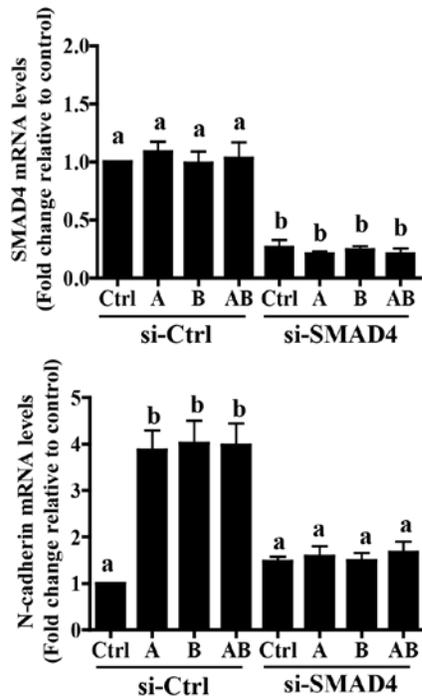
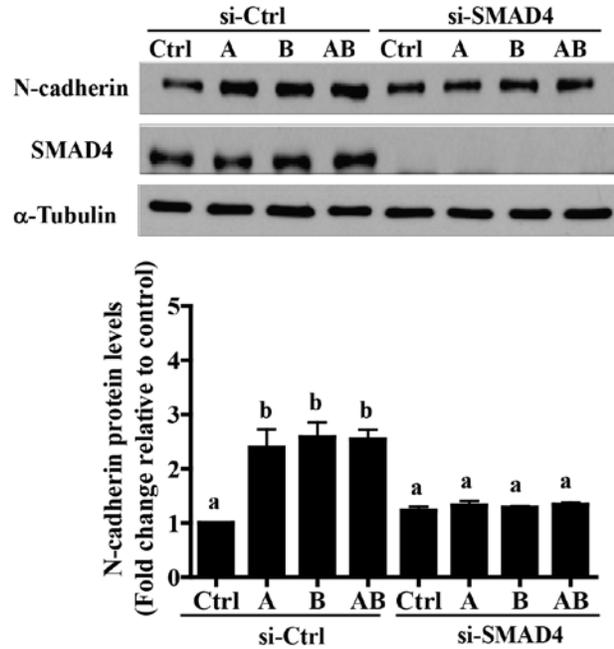
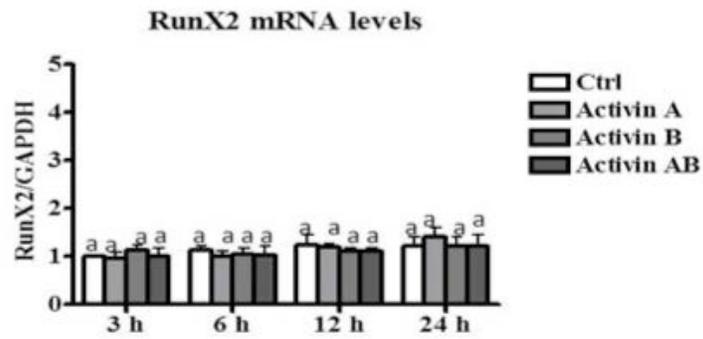
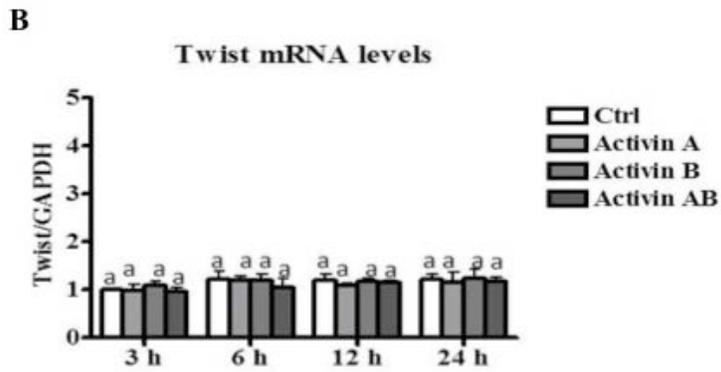
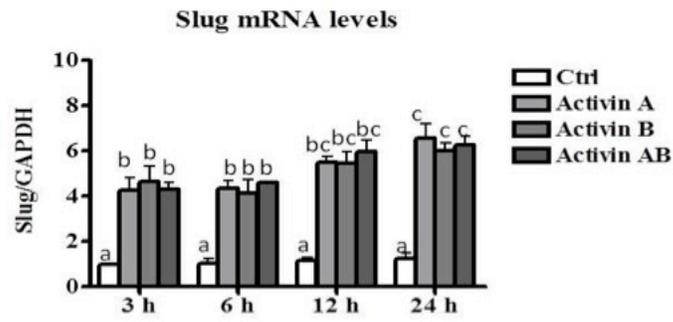
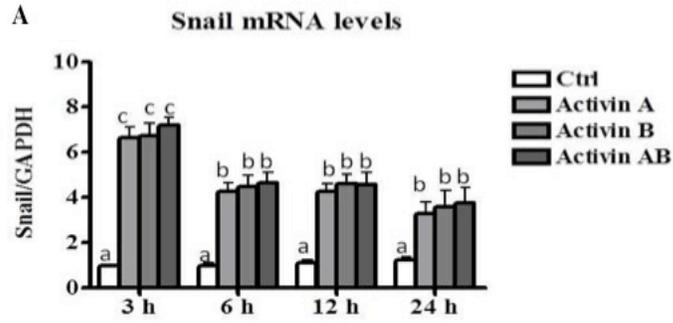
D**E**

Figure 3.6 Activin-induced up-regulation of N-cadherin in HTR8/SVneo cells is abolished by combined knockdown of SMAD2/SMAD3 or depletion of SMAD4

A-D, Cells were transfected for 48 h with 20 nM non-targeting control siRNA (si-Ctrl) or 20 nM siRNA targeting SMAD2 (si-SMAD2), SMAD3 (si-SMAD3), SMAD2 and SMAD3 (si-SMAD2+3) or SMAD4 (si-SMAD4) prior to treatment for 24 h without (Ctrl) or with 50 ng/ml activin A, B or AB. RT-qPCR was used to examine the knockdown efficiencies of each siRNA treatment (upper panels) as well as effects on N-cadherin mRNA levels (lower panels). **E**, Cells were transfected with siRNA targeting SMAD4 and treated with activins as described for **A-D**. SMAD4 and N-cadherin protein levels were examined by Western blot and normalized to α -tubulin. The upper panel shows a representative western blot and the lower panel shows

summarized quantitative results expressed as the mean \pm SEM of at least three independent experiments. Groups without a common letter are statistically different from each other ($p < 0.05$).



C

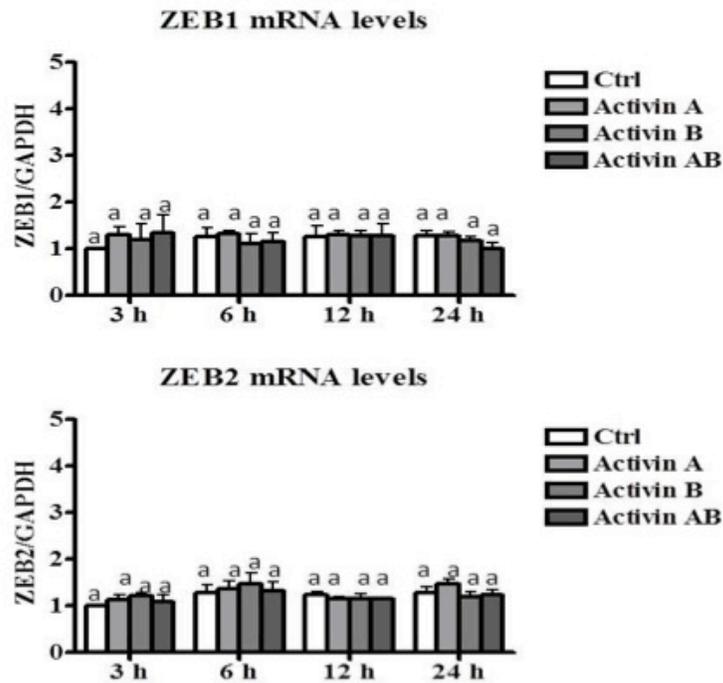


Figure 3.7 Activin isoforms up-regulate Snail and Slug, but not Twist, RunX2, ZEB1 and ZEB2, mRNA levels in HTR8/SVneo cells in time-dependent manner

A-C, HTR8/SVneo cells were treated without (Ctrl) or with 50 ng/ml activin A, activin B or activin AB for different lengths of time (3, 6, 12 or 24 h). Snail, Slug, Twist, RunX2, ZEB1 and ZEB2 mRNA levels were examined by RT-qPCR. Data were normalized to GAPDH. The results are expressed as the mean \pm SEM of at least three independent experiments. Groups without a common letter are statistically different from each other ($p < 0.05$).

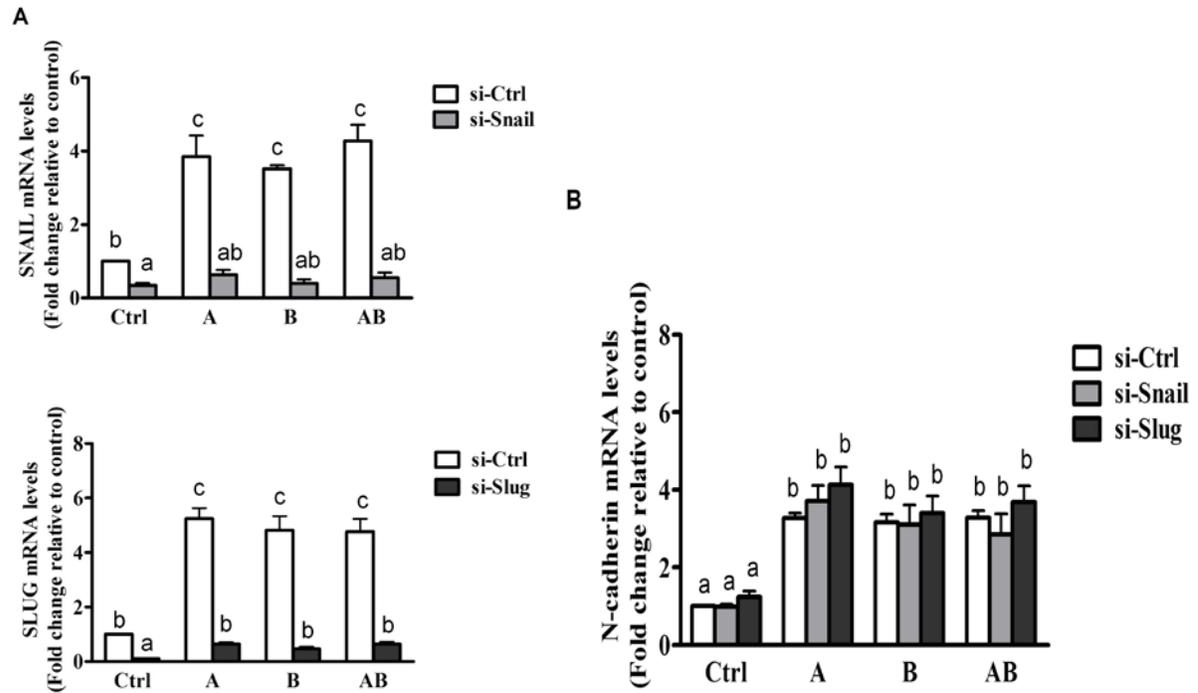


Figure 3.8 Depletion of SNAIL or SLUG does not attenuate activin isoforms-induced N-cadherin upregulation

A and B, Cells were transfected for 48 h with 20 nM non-targeting control siRNA (si-Ctrl) or 20 nM siRNA targeting SNAIL (si-SNAIL) or SLUG (si-SLUG) prior to treatment for 24 h without (Ctrl) or with 50 ng/ml activin A (A), activin B (B) or activin AB (AB). **A**, SNAIL, SLUG and N-cadherin mRNA levels were examined by SYBR RT-qPCR with GAPDH as the reference gene. The results are expressed as the mean \pm SEM of at least three independent experiments. Groups without a common letter are statistically different from each other ($p < 0.05$).

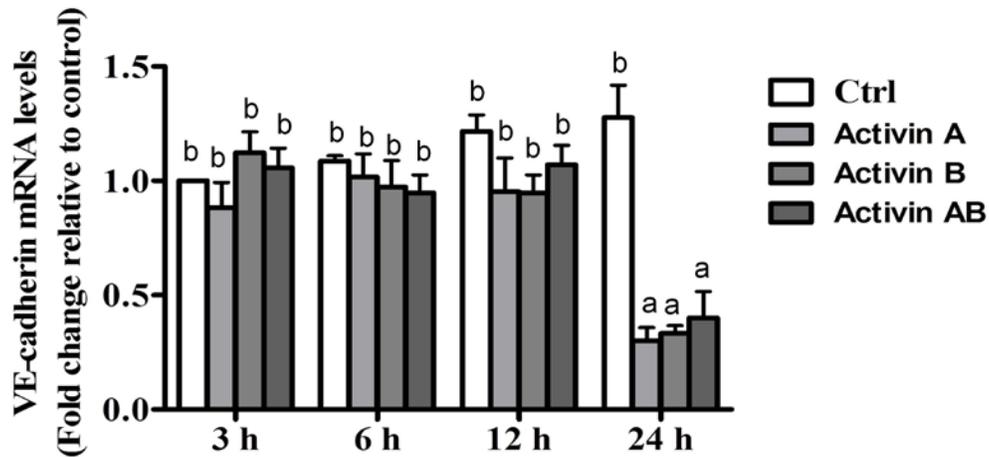


Figure 3.9 Activin isoforms decrease VE-cadherin mRNA levels in HTR8/SVneo cells

A, HTR8/SVneo cells were treated without (Ctrl) or with 50 ng/ml activin A, B or AB for different lengths of time (3, 6, 12 or 24 h) and VE-cadherin mRNA levels were examined by RT-qPCR with GAPDH as the reference gene. The results are expressed as the mean \pm SEM of at least three independent experiments. Groups without a common letter are statistically different from each other ($p < 0.05$).

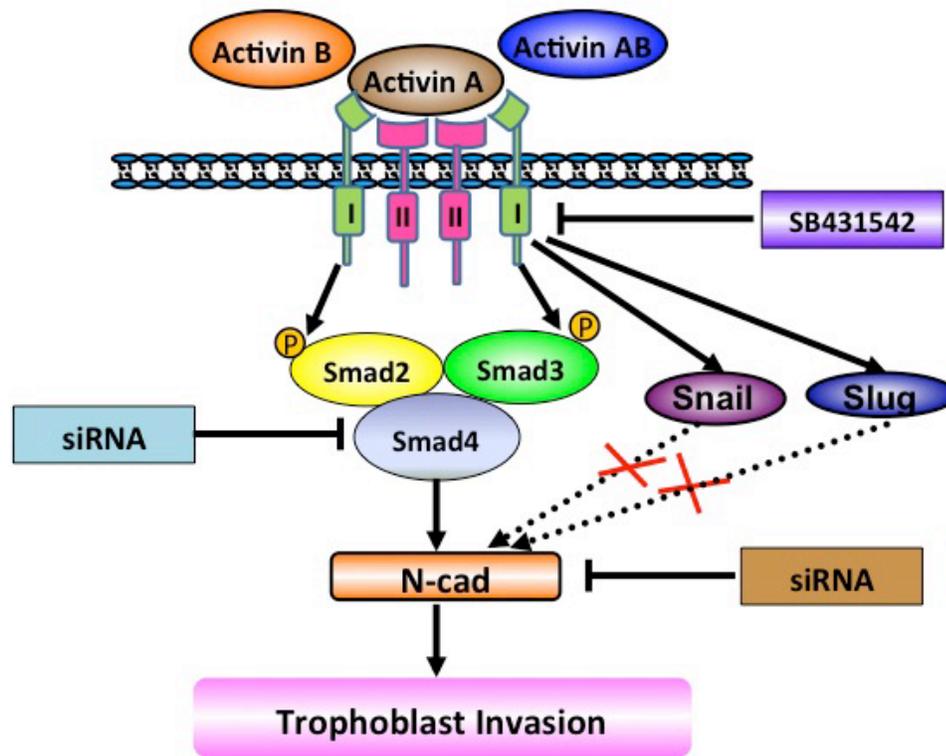


Figure 3.10 A schematic illustration of Chapter 3 data

Treatment of HTR-8/SVneo cells with Activin A, B or AB produced comparable increases in cell invasion (Figure 3.2), N-cadherin expression (Figure 3.3) and SMAD2/3 phosphorylation (Figure 3.5). In addition, treatment with activin isoforms up-regulated the mRNA levels of SNAIL and SLUG, but not TWIST, RUNX2, ZEB1 or ZEB2 (Figure 3.7). Co-treatment with the TGF- β type I receptor inhibitor SB431542 abolished activin-induced cell invasion, up-regulation of N-cadherin, phosphorylation of SMAD2/3 and up-regulation of SNAIL and SLUG (Figure 3.2, 3.3, 3.5 and 4.2). Knockdown of SMAD2, 3, 2+3 or 4 (Figure 3.6), but not SNAIL or SLUG (Figure 3.8), reduced the effects of activin isoforms on N-cadherin. Importantly, activin isoform-induced cell invasion was attenuated by siRNA-mediated down-regulation of N-cadherin (Figure

3.4). Activin A, B and AB did not influence the proliferation or apoptosis of HTR8/SVneo cells (Figure 3.2). In primary cultures of human EVT cells, activin A increased cell invasion and N-cadherin expression. Activin A-induced primary EVT invasion was attenuated by siRNA-mediated down-regulation of N-cadherin (Figure 3.4).

Chapter 4: Activin A increases human trophoblast invasion by inducing SNAIL-mediated MMP2 up-regulation through ALK4

4.1 Introduction

Highly invasive extravillous cytotrophoblasts (EVTs) derived from chorionic villi invade into the endometrium and proximal third of the myometrium and subsequently remodel the uterine environment to ensure the supply of essential nutrients and oxygen to the fetus (7). This tightly regulated trophoblast invasion process is temporally and spatially influenced by various hormones, growth factors as well as cytokines, and plays a vital role in supporting normal human pregnancy (183). Aberrant trophoblast invasion is related to many unfavorable pregnancy-related outcomes including first trimester miscarriage, preeclampsia, intrauterine growth restriction and choriocarcinoma (184). A better understanding of the physiological and molecular mechanisms related to trophoblast invasion will inform diagnosis and the development of therapeutic strategies for pregnancy complications.

Activin A belongs to the transforming growth factor- β (TGF- β) superfamily and is a homodimer of inhibin β A subunits. As with other TGF- β superfamily members, activin A is implicated in multiple physiological and pathological processes in a variety of tissues (185). Both activin A and its receptors have been detected in human endometrium and placenta (119, 173). Moreover, studies have shown that activin A stimulates the outgrowth of cytotrophoblast cells from first trimester chorionic villous explants (107), as well as the invasion of purified first trimester cytotrophoblasts (108). We have previously demonstrated that the mesenchymal adhesion molecule neural cadherin (N-cadherin) is a key mediator of activin A-induced human trophoblast cell invasion (186). However, activin A has also been shown to enhance MMP2

expression within villous cytotrophoblast cells (107), though it is not known if this up-regulation is essential for the pro-invasive effect of activin A, or whether transcription factors other than SMADs are involved.

The biological effects of activin A are mediated by the activation of heterotetrameric complexes of two type I and two type II transmembrane serine-threonine kinase receptors (187). Specifically, after activin A binds to the activin type II (ActRII) or activin type IIB (ActRIIB) receptors, activin type I receptors are recruited and transphosphorylated. Among the seven known activin receptor-like kinase (ALK) receptors, activin A binds to ActRIB (ALK4) and ActRI (ALK2) with high affinity (113). Phosphorylated activin type I receptors then recruit and phosphorylate receptor-regulated SMAD2/3, which then bind with common SMAD4 to form heterotrimeric complexes that translocate to the nucleus and act as transcription factors to modulate downstream gene expression.

Matrix metalloproteinases (MMPs) are critical for the extensive remodeling of the uterine extracellular matrix during implantation (143). MMP2 (gelatinase A) and MMP9 (gelatinase B), as well as some of their well-known substrates (collagen I, IV, V and fibronectin) have been detected in human decidua (144). In first trimester placental tissues, MMP2 is mainly expressed in EVT cells whereas MMP9 is expressed in both villous cytotrophoblasts and EVT cells (145). In addition, MMP2 is abundantly expressed in EVT of ectopic pregnancies (146). This pattern of expression suggests MMP2 and MMP9 are associated with trophoblast invasiveness, and is consistent with their peak expression in the first trimester when trophoblast invasion is highest (147). Indeed, the invasiveness of purified cytotrophoblast cells was shown to be limited by treatment with an MMP2 neutralization antibody (145). Interestingly, MMP9 expression was found to be weak or absent in preeclamptic placentas, which could be related to impaired

trophoblast invasiveness in preeclampsia (148). The activity of these two gelatinases is tightly regulated by tissue inhibitors of metalloproteinase 1 and 2 (TIMP1 and TIMP2), which have been detected not only in human decidua cells (149), but also EVT cells (150).

SNAIL (SNAI1) and SLUG (SNAI2) are zinc-finger transcription factors (152) that were first identified as transcriptional repressors of E-cadherin (153). SNAIL and SLUG are both involved in epithelial-mesenchymal transition during embryonic development and the acquisition of invasive properties during cancer progression (154). Indeed, SLUG expression is positively correlated with that of MMP2 in ovarian carcinoma (155), and SNAIL has been shown to enhance MMP2 expression and migration of human bone mesenchymal stem cells and hepatocellular cancer cells (156, 157). In skin cancer cells, promoter-luciferase analysis demonstrated that SNAIL could increase MMP2 expression by binding to its promoter region (158).

The objective of this study was to investigate the contribution of MMP2/9 and TIMP1/2 to activin A-induced trophoblast invasion, and to define the type I receptors and transcriptional factors involved, in particular the roles of ALK4, SNAIL, SLUG and SMAD2/3-SMAD4. Our results demonstrate that activin A increases human trophoblast cell invasion by inducing SNAIL-mediated MMP2 up-regulation via ALK4- and SMAD2/3-SMAD4-dependent signaling.

4.2 Materials and methods

Culture of HTR8/SVneo immortalized human EVT cells

The HTR8/SVneo immortalized human EVT cell line (63) was generously provided by Dr. P.K. Lala (Western University). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Life Technologies) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Hyclone,

GE Healthcare Life Sciences), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies). Cultures were incubated at 37°C in a humidified 5% CO₂/95% air atmosphere and the medium was changed every other day. Prior to growth factor treatment, cells were maintained in medium supplemented with 0.1% (vol/vol) FBS for 24 h.

Primary human EVT isolation and culture

This study was approved by the Research Ethics Board of the University of British Columbia and all patients were provided with informed written consent. First trimester human placentas (6-10 weeks gestation) were obtained from women undergoing elective termination of pregnancy, and human EVT cells were isolated from chorionic villous explants as previously described (62). Briefly, chorionic villi were washed in Dulbecco's Modified PBS (HyClone, GE Healthcare Life Sciences) and minced into small pieces which were cultured in DMEM with 10% FBS for 3-4 days. Non-attached pieces were washed away and the villous fragments were cultured for a further 5-7 days to allow for EVT outgrowth. EVT cells were subsequently separated from attached fragments by trypsinization, and their purity was confirmed by positive immunocytochemical staining for cytokeratin-7 (epithelial marker) together with negative staining for vimentin (mesenchymal marker).

Antibodies and reagents

Phospho-SMAD2 (Ser^{465/467}) rabbit monoclonal antibody, SMAD2 (L16D3) mouse monoclonal antibody, Phospho-SMAD3 (Ser^{423/425}) (C25A9) rabbit monoclonal antibody, SMAD3 (C67H9) rabbit monoclonal antibody, SMAD4 polyclonal antibody, SNAIL mouse monoclonal antibody (#3895) and SLUG rabbit monoclonal antibody (#9585) were purchased from Cell Signaling

Technology. Mouse monoclonal anti-MMP2 antibody was obtained from NeoMarkers. Mouse monoclonal anti- α -Tubulin (B-5-1-2; sc-23948) antibody was obtained from Santa Cruz Biotechnology. The TGF- β type I receptor inhibitor SB431542 (#S4317) was purchased from Sigma-Aldrich. Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Bio-Rad Laboratories. Recombinant human activin A was obtained from R&D Systems.

Matrigel-coated transwell invasion assay

Cell invasiveness was measured using 24-well transwell inserts (pore size 8 μ m; BD Biosciences) coated with 40 μ l of growth factor-reduced Matrigel (1 mg/ml; BD Biosciences). Inserts were seeded with 1×10^5 cells in 250 μ l DMEM medium supplemented with 0.1% FBS, and 750 μ l of medium with 10% FBS were added to the lower chamber. Cells were incubated at 37°C for 48 h after which non-invading cells were wiped from the upper side of the membrane and cells on the lower side were fixed with cold methanol (-20°C) and air dried. Cell nuclei were stained with Hoechst 33258 (Sigma-Aldrich) and counted using a Zeiss Axiophot epifluorescent microscope and Northern Eclipse 6.0 software (Empix Imaging, Mississauga, Ontario, Canada). Five microscopic fields were counted per insert, triplicate inserts were used for each individual experiment, and each experiment was repeated at least three times.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Cells were washed with cold PBS and total RNA was extracted with TRIzol Reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed using 2 μ g of total RNA, random primers and M-MLV reverse transcriptase

(Promega) in a final volume of 20 μ l. SYBR Green or TaqMan RT-qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates..TaqMan gene expression assays for ACVR1B (ALK4; Hs00244715_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs02758991_g1) were purchased from Applied Biosystems (Life Technologies). Each 20 μ l TaqMan RT-qPCR reaction contained 1 \times Taqman Gene Expression Master Mix (Applied Biosystems), 50 ng cDNA, and 1 \times specific Taqman gene expression assay containing primers and probe. Alternatively, each 20 μ l SYBR Green RT-qPCR reaction contained 1 \times SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA, and 250 nM of each specific primer. The primers used were: MMP2, 5'-TAC ACC AAG AAC TTC CGT CTG T-3' (forward) and 5'-AAT GTC AGG AGA GGC CCC ATA-3' (reverse); MMP9, 5'-GGA CGA TGC CTG CAA CGT-3' (forward) and 5'-CAA ATA CAG CTG GTT CCC AAT CT -3' (reverse); Tissue inhibitor of metalloproteinase 1 (TIMP1), 5'-ACC ATG GCC CCC TTT GA-3' (forward) and 5'-CAG CCA CAG CAA CAA CAG GAT-3' (reverse); TIMP2, 5'-AGC ATT TGA CCC AGA GTG GAA-3' (forward) and 5'-CCA AAG GAA AGA CCT GAA GGA-3' (reverse); SNAIL, 5'-CCC CAA TCG GAA GCC TAA CT-3' (forward) and 5'-GCT GGA AGG TAA ACT CTG GAT TAG A-3' (reverse); SLUG, 5'-TTC GGA CCC ACA CAT TAC CT-3' (forward) and 5'-GCA GTG AGG GCA AGA AAA AG-3' (reverse); GAPDH, 5'-GAG TCA ACG GAT TTG GTC GT-3' (forward) and 5'- GAC AAG CTT CCC GTT CTC AG -3' (reverse); SMAD2, 5'-GCC TTT ACA GCT TCT CTG AAC AA-3' (forward) and 5'-ATG TGG CAA TCC TTT TCG AT-3' (reverse); SMAD3, 5'-CCC CAG CAC ATA ATA ACT TGG-3' (forward) and 5'-AGG AGA TGG AGC ACC AGA AG-3' (reverse); SMAD4, 5'-TGG CCC AGG ATC AGT AGG T-3' (forward) and 5'-CAT CAA CAC CAA TTC CAG CA-3' (reverse). The specificity of each assay was validated by dissociation curve analysis

and agarose gel electrophoresis of PCR products. Assay performance was validated by assessing amplification efficiencies by means of calibration curves, and ensuring that the plot of log input amount versus ΔCq has a slope with an absolute value of < 0.1 . At least three separate experiments were performed and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative Cq method with GAPDH as the reference gene and using the formula $2^{-\Delta\Delta Cq}$.

Western blot analysis

Cells were washed twice with cold PBS and lysed in ice cold lysis buffer (Cell Signaling Technology) with added protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at $20,000 \times g$ for 10 min at $4^\circ C$ to remove cellular debris, and protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories) with BSA as the standard. Equal amounts of protein (30 μg) were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes (GE Healthcare Life Sciences). Membranes were blocked with Tris-buffered saline (TBS) containing 0.1% (vol/vol) Tween-20 and 5% (wt/vol) non-fat dry milk for 1 h and then incubated with primary antibodies against phospho-SMAD2 (1:500), phospho-SMAD3 (1:500), SMAD4 (1:500), MMP2 (0.4 $\mu g/ml$), SNAIL (1:2000) or SLUG (1:500) overnight at $4^\circ C$. Bound antibodies were visualized using peroxidase-conjugated secondary antibody, ECL or SuperSignal West Femto chemiluminescent substrates (Pierce, Thermo Scientific), and CL-XPosure film (Thermo Scientific). Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mM β -mercaptoethanol and 1% (wt/vol) SDS) at $50^\circ C$ for 30 min and reprobbed as described above with antibodies against α -tubulin (1:50000), SMAD2

(1:2000) or SMAD3 (1:500) as loading controls. Densitometric quantification was performed using Quantity One 1-D Analysis software (version 4.6.6) using α -tubulin, SMAD2 or SMAD3 for normalization.

Small interfering RNA (siRNA) transfection

Fifty percent confluent cells were transfected the day after seeding with 20 nM ON-TARGET*plus* non-targeting control pool siRNA or ON-TARGET*plus* SMART*pool* siRNAs targeting human MMP2, ALK4, SNAIL, SLUG, SMAD2, SMAD3 or SMAD4 (Dharmacon, GE Healthcare Life Sciences) using Lipofectamine RNAiMAX and Opti-MEM I according to the manufacturer's instructions (Invitrogen, Life Technologies). Knockdown efficiency was assessed by RT-qPCR and/or Western blot analysis.

Study protocol

Study groups were comprised of cells treated with activin A, SB431542 and/or siRNA targeting ALK4, MMP2, SNAIL, SLUG, SMAD2, SMAD3 and/or SMAD4, whereas the corresponding control groups were comprised of cells treated without activin A or with equivalent amounts of DMSO or non-targeting control siRNA. For studies involving HTR8/SVneo cells at least three independent experiments were performed, each on a separate passage. For studies conducted with primary human EVT cells at least three independent experiments were performed on separate primary cultures of human EVT cells.

Statistical analysis

Results are presented as the mean \pm SEM of at least three independent experiments. For

experiments involving only two groups, the data were analyzed by t-test using PRISM software (GraphPad Software Inc.). Multiple group comparisons were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons of means using PRISM software. Means were considered significantly different if $P < 0.05$ and are indicated by different letters.

4.3 Results

Activin A increases HTR8/SVneo cell invasion by up-regulating MMP2 expression through ALK4

Our previous studies demonstrated that depletion of N-cadherin reduced, but did not completely abolish, activin A induced HTR8/SVneo cell invasion, suggesting the involvement of additional molecular mediators (186). To investigate the involvement of MMP2, MMP9, TIMP1 and/or TIMP2 in activin A-induced human trophoblast cell invasion, we first examined the time-dependent effects of activin A on their mRNA levels in HTR8/SVneo cells. RT-qPCR results show that treatment with activin A (50 ng/ml) increased MMP2 mRNA levels in a time-dependent manner, without affecting the mRNA levels of MMP9, TIMP1 and TIMP2 (Figure 4.1A and B). Consistent with previous studies (107), Western blot analysis showed that treatment with activin A for 24 or 48 h up-regulated pro-MMP2 protein levels (Figure 4.1C). Next, we investigated the effects of pre-treatment with the TGF- β type I receptor (ALK4/5/7) inhibitor SB431542 (175), which was previously shown to completely inhibit activin A-induced HTR8/SVneo cell invasion (186). Application of 10 μ M SB431542 for 1 h prior to treatment with activin A completely abolished its effects on MMP2 mRNA and protein levels (Figure 4.1D). To verify that MMP2 up-regulation is necessary for activin A-induced HTR8/SVneo cell invasion, cells were pre-treated with siRNA targeting MMP2 prior to treatment with activin A.

RT-qPCR and Western blot showed that transfection with siRNA targeting MMP2 for 48 h before treatment with 50 ng/ml activin A suppressed both basal and activin A-induced MMP2 expression (Figure 4.1E). Importantly, Matrigel transwell invasion assay results revealed that pre-treatment of HTR8/SVneo cells with MMP2 siRNA significantly attenuated, but did not completely inhibit, activin A-induced invasion (Figure 4.1F). To test whether ALK4 mediates the stimulatory effects of activin A on MMP2 up-regulation and HTR8/SVneo cell invasion, cells were pre-treated with siRNA targeting ALK4 prior to treatment with activin A. RT-qPCR and Western blot showed that transfection with siRNA targeting ALK4 for 48 h prior to treatment with 50 ng/ml activin A abolished the stimulatory effects of activin A on MMP2 mRNA and protein levels (Figure 4.1G). Consistent with the effects of SB431542, pre-treatment with ALK4 siRNA completely abolished activin A-induced HTR8/SVneo cell invasion (Figure 4.1H).

Activin A increases SNAIL and SLUG expression in HTR8/SVneo cells

Trophoblast invasion resembles epithelial-mesenchymal transition that occurs during tumor progression (132). Next, we studied the effect of activin A on epithelial-mesenchymal transition-related transcription factors associated with cell invasion (SNAIL, SLUG, TWIST, RUNX2, ZEB1 and ZEB2 (188)). Transcription factor mRNA levels were measured in HTR8/SVneo cells following treatment with 50 ng/ml activin A for 3, 6, 12 or 24 h. RT-qPCR results show that activin A up-regulated the mRNA levels of SNAIL and SLUG (Figure 4.2A), but not TWIST, RUNX2, ZEB1 or ZEB2 (Figure 3.7). Western blot results show that activin A also increased SNAIL and SLUG protein levels (Figure 4.2B). As for MMP2, the effects of activin A on SNAIL and SLUG mRNA and protein levels were abolished by pre-treatment with SB431542

(Figure 4.2C and D). Similarly, RT-qPCR and Western blot analysis showed that pre-treatment with ALK4 siRNA for 48 h abolished the up-regulation of SNAIL and SLUG mRNA and protein levels by activin A (Figure 4.2E and F).

Activin A-induced MMP2 up-regulation is attenuated by depletion of SNAIL, but not SLUG, in HTR8/SVneo cells

Treatment with siRNA targeting SNAIL or SLUG was used to investigate their involvement in activin A-induced MMP2 up-regulation in HTR8/SVneo cells. RT-qPCR and Western blot analysis showed that pre-treatment with SNAIL or SLUG siRNA for 48 h specifically suppressed both endogenous and activin A-induced SNAIL or SLUG mRNA (Figure 4.3A) and protein (Figure 4.3B and C) up-regulation. Interestingly, depletion of SNAIL attenuated the up-regulation of MMP2 mRNA and protein levels by activin A, whereas knockdown of SLUG had no effect (Figure 4.3).

Activin A induces SMAD2/3 phosphorylation via ALK4 in HTR8/SVneo cells

Next we sought to determine whether canonical SMAD-dependent signaling is involved in the pro-invasive effect of activin A on human trophoblast cells. SMAD2/3 phosphorylation was measured by Western blot following treatment of HTR8/SVneo cells with 50 ng/ml activin A for 10, 30 or 60 min. As shown in Figure 4.4A, treatment with activin A significantly increased the phosphorylation of both SMAD2 and SMAD3 at all time-points examined. Next, we examined the effects of pre-treatment with 10 μ M SB431542 (1 h) or ALK4 siRNA (48 h) on activin A-induced SMAD2/3 phosphorylation (60 min). Consistent with our findings for cell invasiveness and MMP2, SNAIL and SLUG expression, activin A-induced phosphorylation of SMAD2 and

SMAD3 was inhibited by pre-treatment with SB431542 (Figure 4.4B) or ALK4 siRNA (Figure 4.4C).

SMAD2/3 signaling is required for activin A-induced up-regulation of SNAIL and MMP2 in HTR8/SVneo cells

The individual and combined roles of SMAD2 and SMAD3 in activin A-induced SNAIL and MMP2 up-regulation were investigated by treating HTR8/SVneo cells with activin A (50 ng/ml, 24 h) following siRNA-mediated depletion of SMAD2 or SMAD3 (48 h). The efficiency and specificity of knockdown for each siRNA treatment was confirmed by RT-qPCR (Figure 4.5A-C). Depletion of either SMAD2 or SMAD3 alone attenuated, but did not completely abolish, the up-regulation of SNAIL and MMP2 mRNA by activin A (Figure 4.5A and B). However, combined knockdown of SMAD2/SMAD3 completely abolished the stimulatory effect of activin A on SNAIL and MMP2 mRNA levels (Figure 4.5C). To further confirm the role of SMAD signaling in activin A-induced SNAIL and MMP2 expression, HTR8/SVneo cells were treated with activin A following siRNA-mediated depletion of common SMAD4. Knockdown of SMAD4 completely abolished the stimulatory effects of activin A on SNAIL and MMP2 mRNA and protein levels (Figure 4.5D and E).

Activin A induces SNAIL-mediated MMP2 expression via ALK4 in primary human EVT cells

The crucial roles of ALK4 and SNAIL in mediating the up-regulation of MMP2 by activin A were further confirmed in primary human EVT cells propagated from first trimester chorionic villous explants. Primary human EVT cells were treated with activin A (50 ng/ml, 24 h)

following siRNA-mediated depletion of ALK4, SMAD4 or SNAIL (48 h). As shown in Figure 6A and B, depletion of ALK4 or SMAD4 abolished the up-regulation of SNAIL and MMP2 mRNA and protein levels by activin A. Furthermore, knockdown of SNAIL attenuated the stimulatory effects of activin A on MMP2 expression (Figure 4.6A and B).

4.4 Discussion

The present study provides important insights into molecular mechanisms underlying activin A-induced human trophoblast cell invasion. Our results demonstrate for the first time that MMP2 is involved in activin A-induced trophoblast invasion. In addition, the up-regulation of MMP2 by activin A is mediated by SNAIL and is dependent on ALK4 and SMAD2/3-SMAD4 signaling.

Both MMP2 and MMP9 are considered to be important mediators of trophoblast invasion. Although MMP2 and MMP9 have similar substrate specificities, differential temporal and spatial expression patterns of MMP2 and MMP9 in the placenta suggests unique biological roles or responses to local factors. In first trimester placental tissues, MMP2 is mainly expressed in EVT cells whereas MMP9 is expressed in both villous cytotrophoblasts and EVT cells (145). In 6-8 week trophoblast cells, MMP2 is the main gelatinase regulating invasion whereas both MMP2 and MMP9 participate in 9-12 week trophoblast cell invasion (189). Interestingly, epidermal growth factor (190) and interleukin-1 α (191) have been shown to induced trophoblast invasion by up-regulating the expression of MMP9 without affecting MMP2, whereas activin A induces MMP2, but not MMP9, production in human trophoblast cells (107, 118). Leptin has been shown to increase trophoblast invasion by up-regulating MMP2 without affecting MMP9 expression (192). However, tumor necrosis factor- α was found to suppress the invasion of EVT cells despite inducing MMP9 expression (193). Together, these studies suggest that trophoblast MMP2 and

MMP9 expression can be differentially regulated by multiple hormonal factors utilizing different signaling pathways. Future studies will be required to determine how these signals are integrated at the promoter level for both MMP2 and MMP9.

The human genome encodes seven type I receptors (ALK1-7), of which only ALK2 and ALK4 are suggested to bind activin A with high affinity (113). We now demonstrate that activin A-induced human trophoblast cell invasion, SMAD2/3 phosphorylation, and SNAIL, SLUG and MMP2 expression are mediated by ALK4. These findings are in agreement with previous studies implicating ALK4, but not ALK7, in activin A-induced human trophoblast cell apoptosis and SMAD2 phosphorylation (66). Although ALK2 mRNA levels are 10-times higher than those of ALK4 in HTR8/SVneo cells (TaqMan RT-qPCR, Figure 4.7), ALK2 is not likely to mediate the effects of activin A in our studies because SB431542 does not inhibit ALK2 (175). Interestingly, reductions in HTR8/SVneo cell invasion approaching statistical significance were observed following depletion of ALK4 alone. This is suggestive of autocrine activin A-induced invasiveness and is consistent with modest activin A production by invasive EVT cells (106) compared to endometrial stromal and decidual cells (104, 105), which are considered to be the major source of activin A at the maternal-fetal interface.

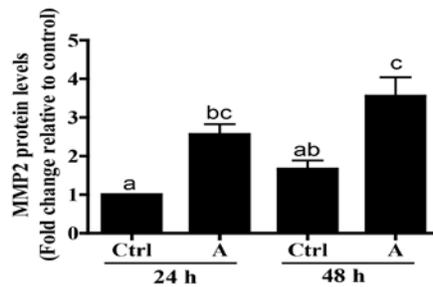
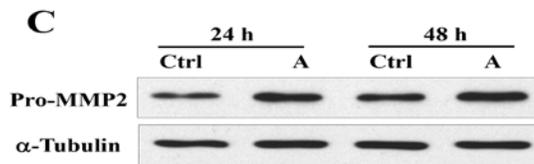
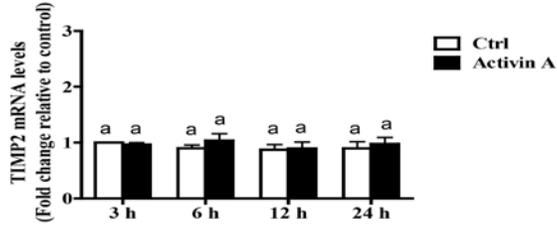
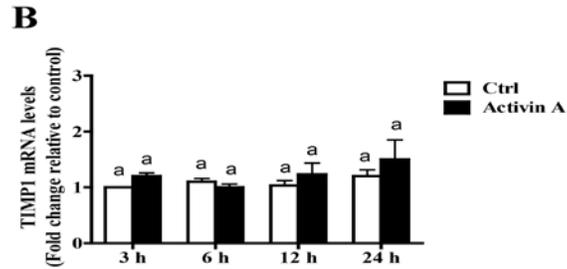
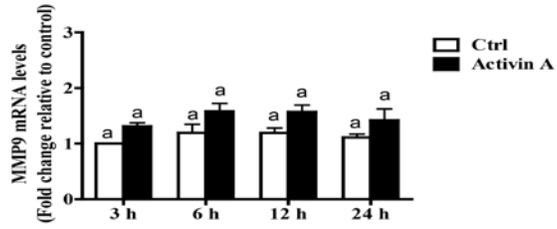
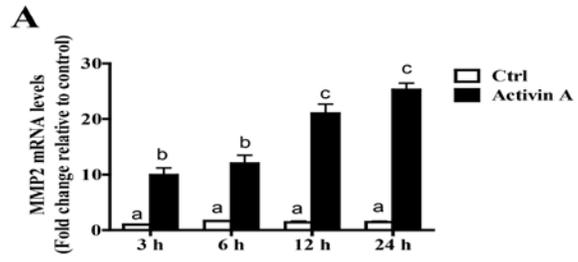
SNAIL and SLUG have been shown to play distinct roles in breast cancer cell invasion (194). Overexpression of SNAIL has been reported to increase MMP2 expression by binding to its promoter region in skin cancer cells (158). Previous studies have demonstrated the expression of SNAIL in human EVT cells (195), and levels of SNAIL have been shown to be reduced in preeclamptic compared to normal placentas (196). Interestingly, we have previously shown that depletion of SNAIL alone does not affect the basal invasiveness of human trophoblast cells (67). Rather, the present findings suggest that SNAIL, but not SLUG, mediates the up-regulation of

MMP2 by activin A in human trophoblast cells. However, activin A-induced MMP2 expression was only partially reduced by depletion of SNAIL, suggesting the involvement of additional transcriptional regulators. Knockdown of SMAD4 or SMAD2+3 abolished the up-regulation of MMP2 by activin A, suggesting either direct regulation by SMADs themselves or the involvement of additional factors. Interestingly, siRNA-mediated depletion of SNAIL or SLUG does not affect activin A-induced N-cadherin expression (Figure 3.8), which has also been shown to mediate activin A-induced trophoblast invasion (186). However, SNAIL was shown to be an important mediator of the suppressive effects of TGF- β 1 on vascular endothelial-cadherin expression and cell invasion in human trophoblast cells (67). Together, these studies suggest a complex role for SNAIL in mediating both pro- and anti-invasive effects depending on the ligand involved.

TGF- β 1 inhibits trophoblast invasion by down-regulating the production and activity of MMP9 in placental explants (151), whereas activin A induces trophoblast invasion by up-regulating MMP2 production. Although the anti-invasive effects of TGF- β 1 and pro-invasive effects of activin A are mediated by ALK5 (67) and ALK4, respectively, they both involve the activation of SMAD2/3-SMAD4 signaling and the induction of SNAIL. The mechanisms underlying such differential regulation by apparently similar signaling pathways are not well understood. However, TGF- β 1-ALK5 have been shown to induce a number of SMAD-independent signaling pathways, including MAPK (ERK, p38 and JNK), phosphoinositide 3-kinase and Rho family small GTPase signaling (197, 198). In addition to regulating a variety of transcription factors, some of these pathways (e.g. ERK, p38 and JNK) can modulate the functions of SMAD proteins by phosphorylating an internal linker region which is unique from the C-terminal region that is phosphorylated by ALK5 (197). Thus, the differential effects of

TGF- β 1 and activin A could result from differences in the activation of SMAD-independent signaling pathways, though this type of signaling is much less well defined for activin A-ALK4. Nevertheless, our studies provide important insights into the molecular mechanisms underlying the divergent functions of TGF- β 1 and activin A on trophoblast invasion.

In summary, the present study demonstrates that activin A up-regulates SNAIL expression via ALK4-induced SMAD2/3-SMAD4 signaling in human trophoblast cells. Elevated SNAIL contributes to the up-regulation of pro-MMP2 expression which plays a key role in promoting trophoblast cell invasion. Specifically, activin A (homodimer of inhibin β A) binds to a heterotetrameric receptor complex comprised of type I (ALK4) and type II (ACVR2A/B) receptors. Ligand-induced activation of the receptor complex results in the phosphorylation and activation of ALK4, leading to the activation of receptor-regulated SMAD2/3. Phosphorylated SMAD2/3 forms a heterotrimeric complex with common SMAD4 that translocates into the nucleus where it binds DNA and increases the transcription of *SNAIL* and *SLUG*. The up-regulation of SNAIL, but not SLUG, subsequently contributes to the production of MMP2, which is an essential mediator of activin A-induced human trophoblast cell invasion (Figure 4.8). By providing important insights into the molecular mechanisms mediating the pro-invasive effects of activin A on human trophoblast cells, our findings add to our understanding of physiological and pathological trophoblast invasion, providing candidate biomarkers for early diagnosis and targets for therapy in trophoblast invasion dysfunction-related pregnancies.



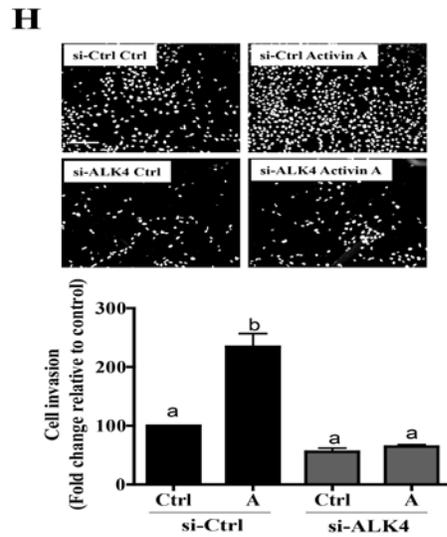
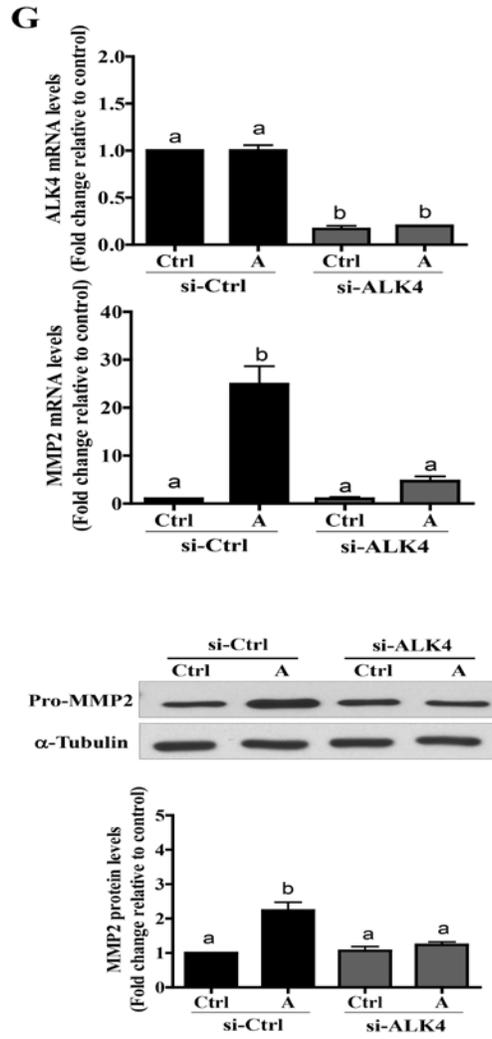
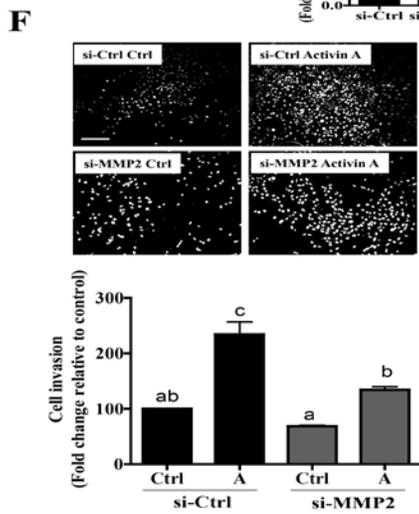
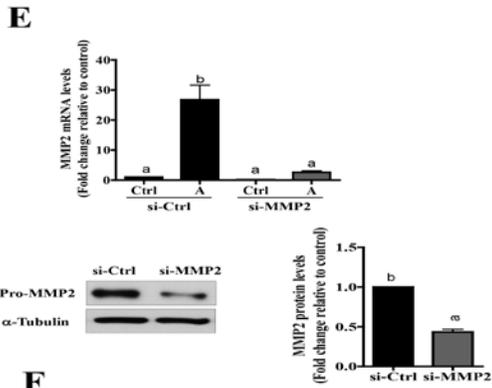
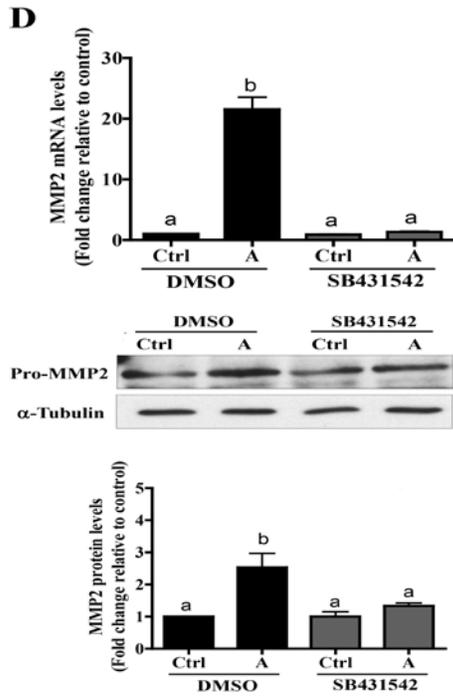
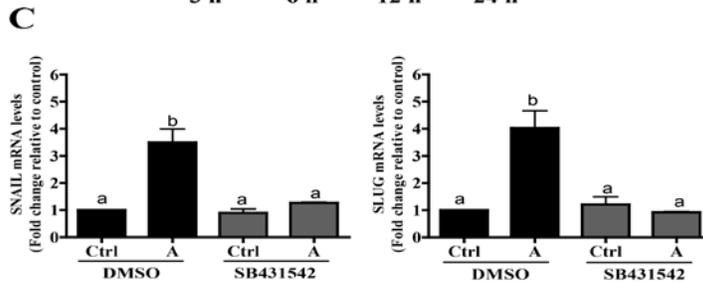
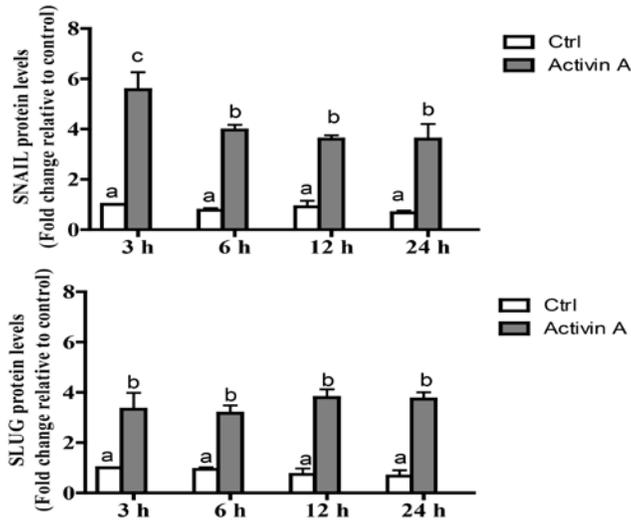
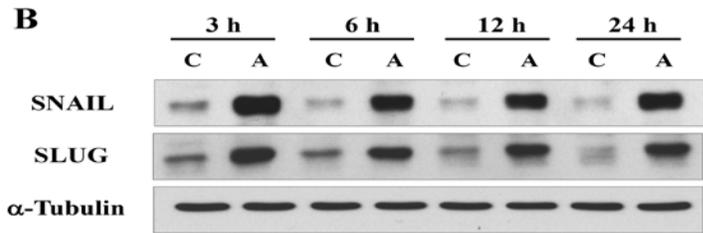
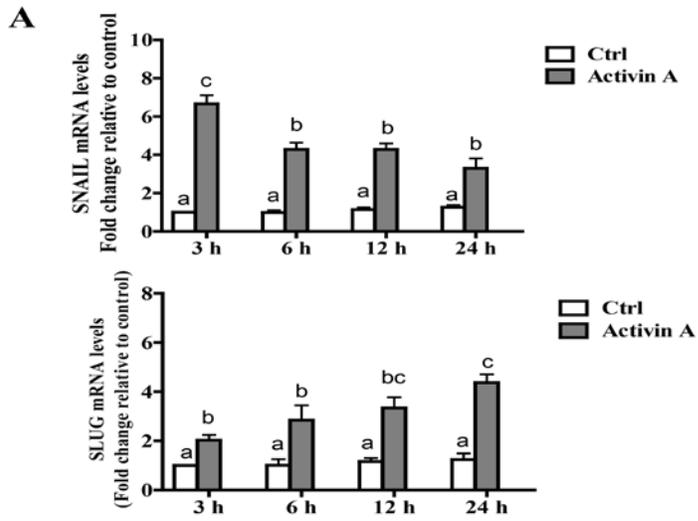


Figure 4.1 Activin A increases HTR8/SVneo cell invasion by up-regulating MMP2 expression through ALK4

A and **B**, HTR8/SVneo cells were treated without (Ctrl) or with 50 ng/ml activin A for different lengths of time (3, 6, 12 or 24 h) and MMP2, MMP9, TIMP1 and TIMP2 mRNA levels were examined by RT-qPCR with GAPDH as the reference gene. **C**, HTR8/SVneo cells were treated for 24 or 48 h without or with 50 ng/ml activin A (A) and MMP2 protein levels were examined by Western blot and normalized to α -tubulin. The upper panel shows a representative Western blot and the lower panel shows summarized quantitative results. **D**, HTR8/SVneo cells were pretreated for 1 h with vehicle control (DMSO) or 10 μ M SB431542 prior to treatment without or with 50 ng/ml activin A for 24 h. MMP2 mRNA and protein levels were examined by RT-qPCR and Western blot, respectively. **E** and **F**, Cells were transfected for 48 h with 20 nM non-targeting control siRNA (si-Ctrl) or 20 nM siRNA targeting MMP2 (si-MMP2) prior to treatment for 24 h without or with 50 ng/ml activin A. **E**, RT-qPCR and/or Western blot were used to examine the knockdown efficiency of MMP2 siRNA treatment as well as its effects on activin A-induced MMP2 mRNA levels. **F**, The effects of MMP2 siRNA treatment on activin A-induced HTR8/SVneo cell invasion were examined by Matrigel-coated transwell assay. The upper panel shows representative images of the invasion assay and the lower panel shows summarized quantitative results. **G** and **H**, HTR8/SVneo cells were transfected for 48 h with 20 nM non-targeting control siRNA (si-Ctrl) or 20 nM siRNA targeting ALK4 (si-ALK4) prior to treatment for 24 h without or with 50 ng/ml activin A. **G**, RT-qPCR and/or Western blot were used to examine the knockdown efficiency of ALK4 siRNA treatment as well as its effects on activin A-induced MMP2 mRNA and protein levels. **H**, The effects of ALK4 siRNA treatment on activin A-induced cell invasiveness were examined by Matrigel-coated transwell assay.

Quantitative results are expressed as the mean \pm SEM of at least three independent experiments.

Different letters indicate statistically significant differences ($P < 0.05$).



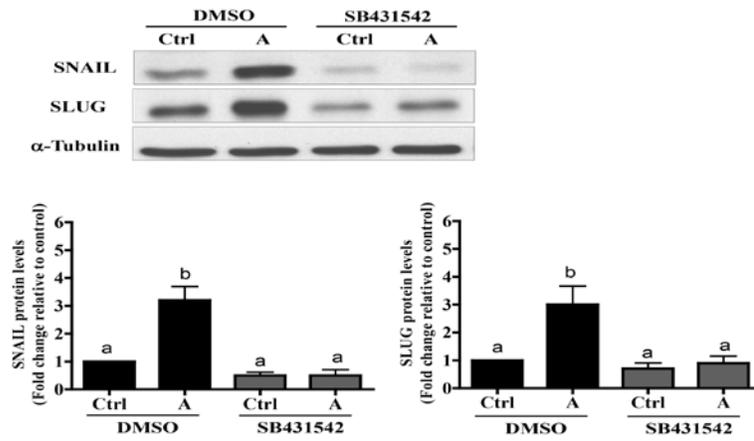
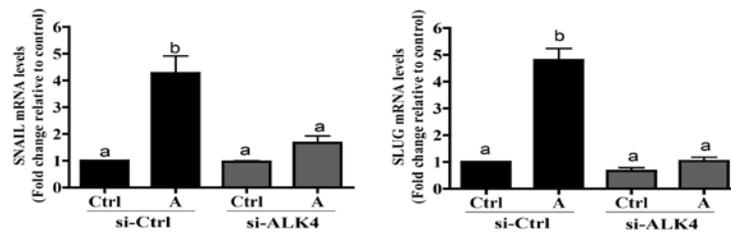
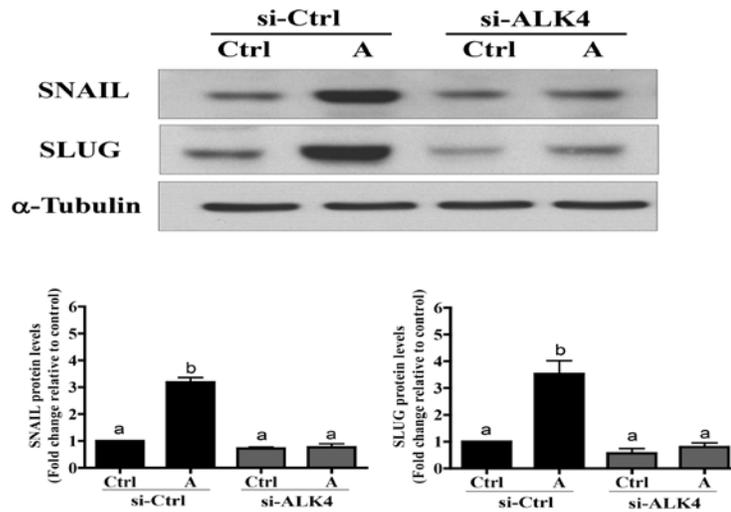
D**E****F**

Figure 4.2 Activin A increases SNAIL and SLUG mRNA and protein levels via ALK4 in HTR8/SVneo cells

A and **B**, HTR8/SVneo cells were treated without (Ctrl) or with 50 ng/ml activin A (A) for different lengths of time (3, 6, 12 or 24 h). **C** and **D**, HTR8/SVneo cells were pretreated for 1 h with vehicle control (DMSO) or 10 μ M SB431542 prior to treatment without or with 50 ng/ml activin A for 24 h. **E** and **F**, Cells were transfected for 48 h with 20 nM non-targeting control siRNA (si-Ctrl) or 20 nM siRNA targeting ALK4 (si-ALK4) prior to treatment for 24 h without or with 50 ng/ml activin A. **A**, **C** and **E**, SNAIL and SLUG mRNA levels were examined by RT-qPCR with GAPDH as the reference gene. **B**, **D** and **F**, SNAIL and SLUG protein levels were examined by Western blot and normalized to α -tubulin. The upper panel shows a representative Western blot and the lower panel shows summarized quantitative results expressed as the mean \pm SEM of at least three independent experiments. Different letters indicate statistically significant differences ($P < 0.05$).

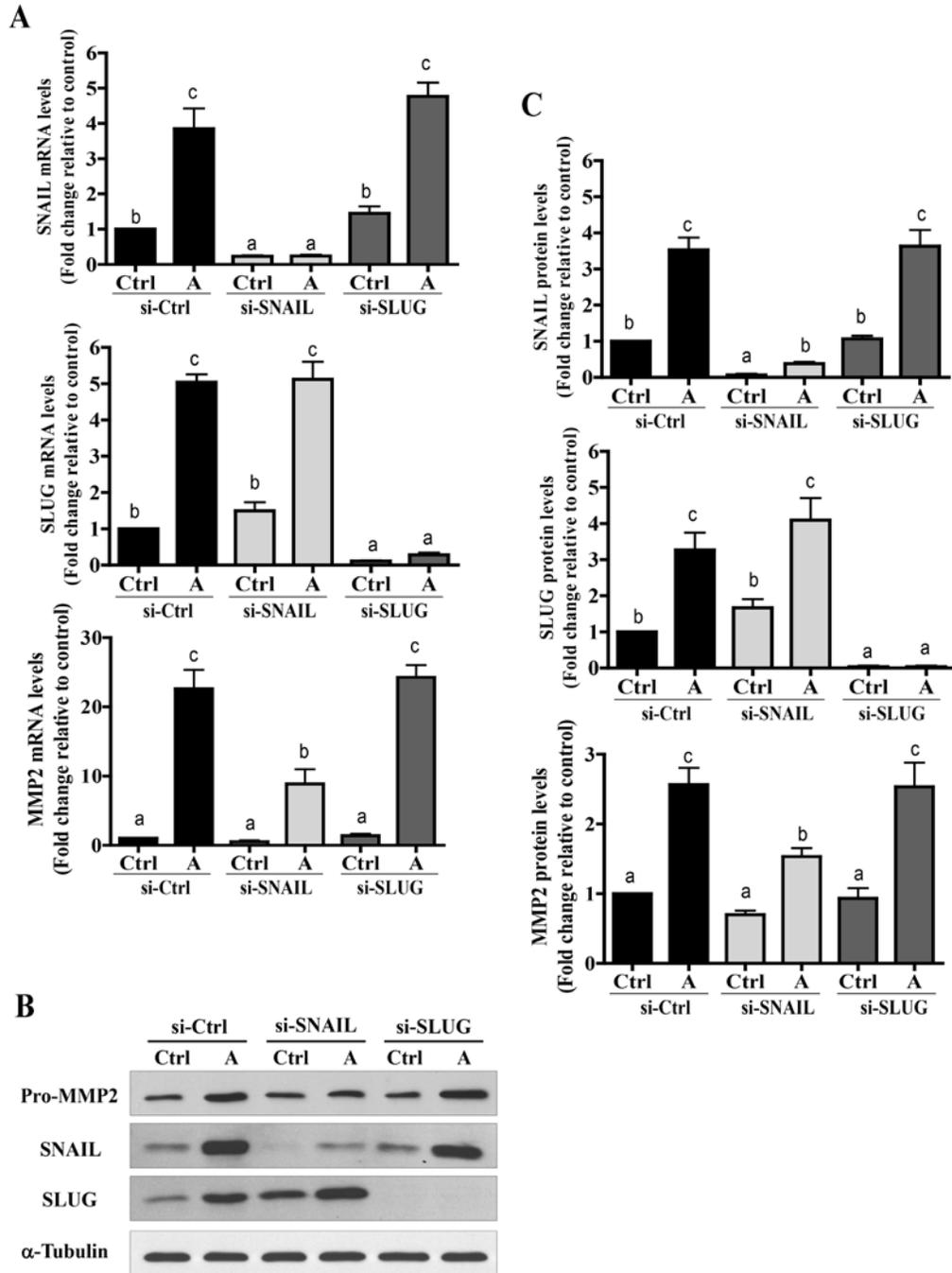


Figure 4.3 Depletion of SNAIL, but not SLUG, attenuates activin A-induced MMP2 up-regulation in HTR8/SVneo cells

A-C, Cells were transfected for 48 h with 20 nM non-targeting control siRNA (si-Ctrl) or 20 nM

siRNA targeting SNAIL (si-SNAIL) or SLUG (si-SLUG) prior to treatment for 24 h without (Ctrl) or with 50 ng/ml activin A (A). **A**, SNAIL, SLUG and MMP2 mRNA levels were examined by RT-qPCR with GAPDH as the reference gene. **B** and **C**, SNAIL, SLUG and MMP2 protein levels were examined by Western blot and normalized to α -tubulin. **B**, A representative Western blot. **C**, Summarized quantitative results expressed as the mean \pm SEM of at least three independent experiments. Different letters indicate statistically significant differences ($P < 0.05$).

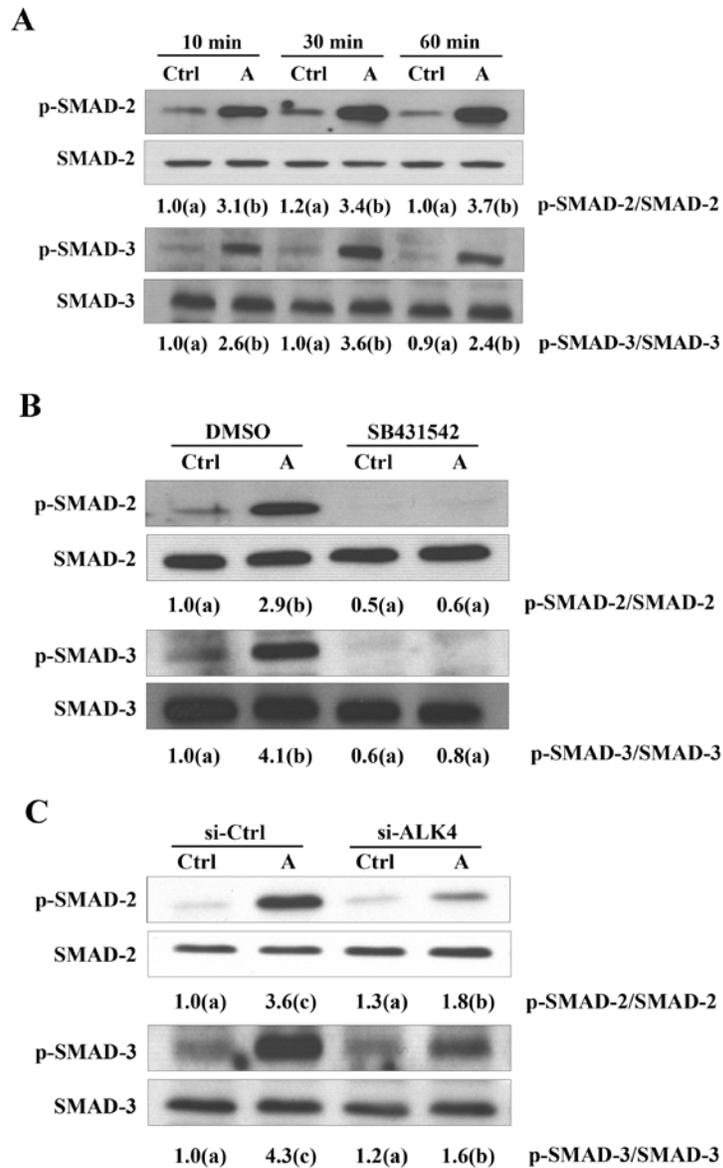
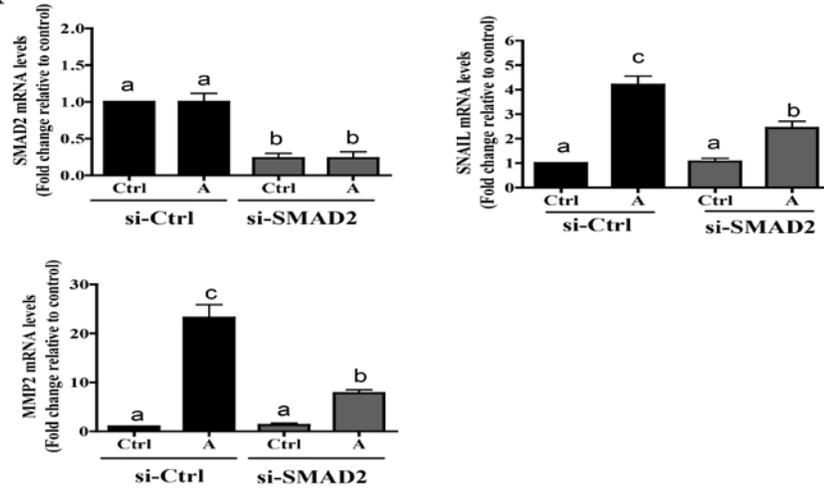
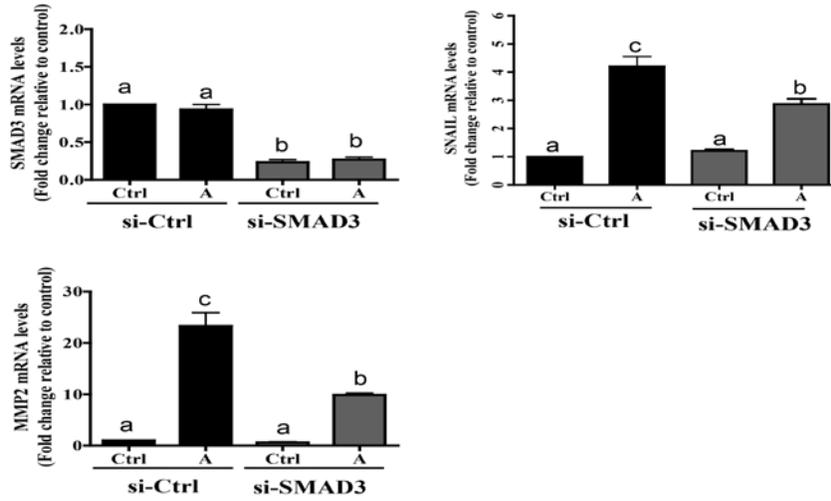
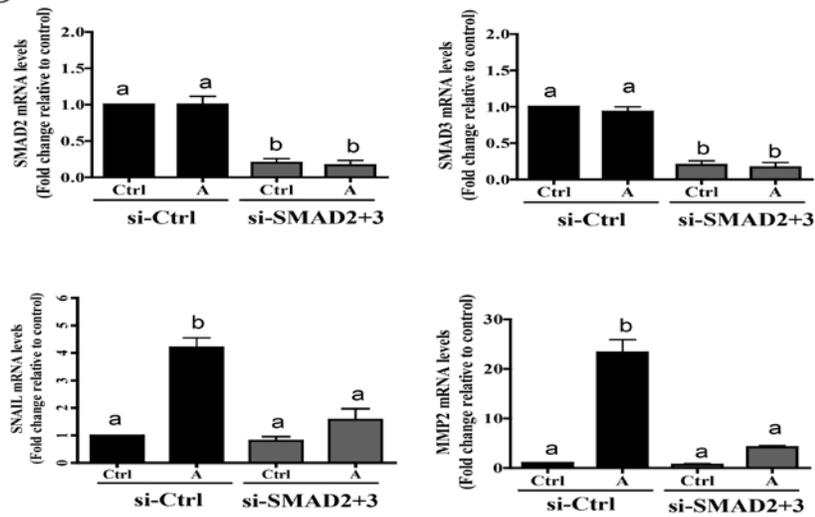


Figure 4.4 Activin A induces SMAD2/3 phosphorylation via ALK4 in HTR8/SVneo cells

A, Cells were treated for 10, 30 or 60 min without (Ctrl) or with 50 ng/ml activin A (A). **B**, Cells were pretreated for 1 h with vehicle control (DMSO) or 10 μ M SB431542 prior to treatment for 60 min without or with 50 ng/ml activin A. **C**, Cells were transfected for 48 h with 20 nM non-targeting control siRNA (si-Ctrl) or 20 nM siRNA targeting ALK4 (si-ALK4) prior to treatment

for 60 min without or with 50 ng/ml activin A. **A-C**, Levels of phosphorylated SMAD2 (p-SMAD2) and SMAD3 (p-SMAD3) were examined by Western blot with phospho-specific antibodies. Membranes were stripped and reprobbed with antibodies for total SMAD2 and SMAD3. Summarized quantitative results were normalized to total SMAD2 or SMAD3 and are displayed numerically under the representative Western blots as the mean of at least three independent experiments. Different letters in brackets indicate statistically significant differences ($P < 0.05$).

A**B****C**

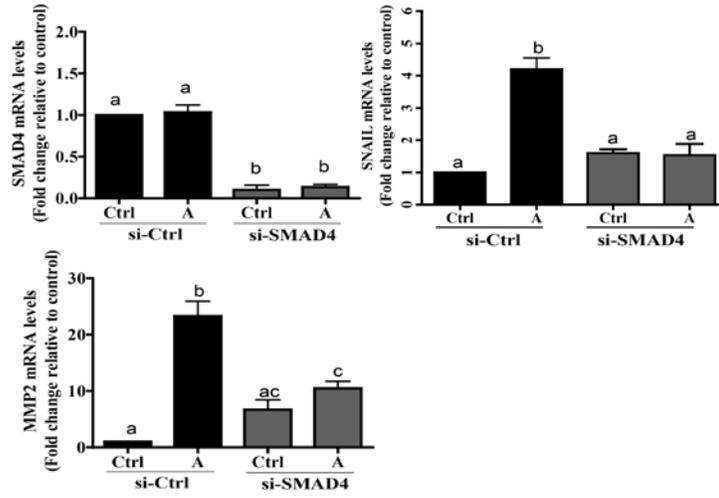
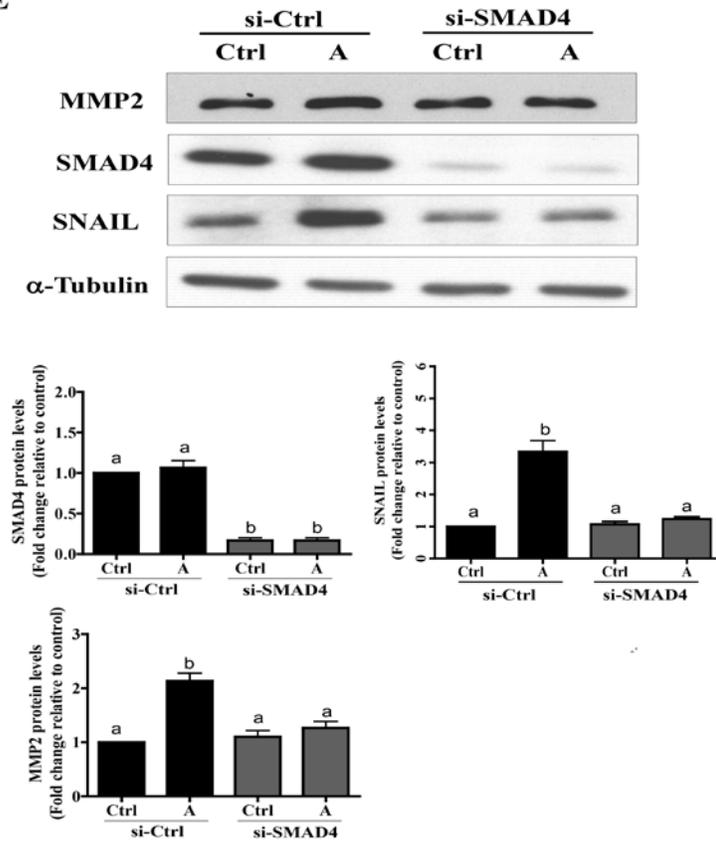
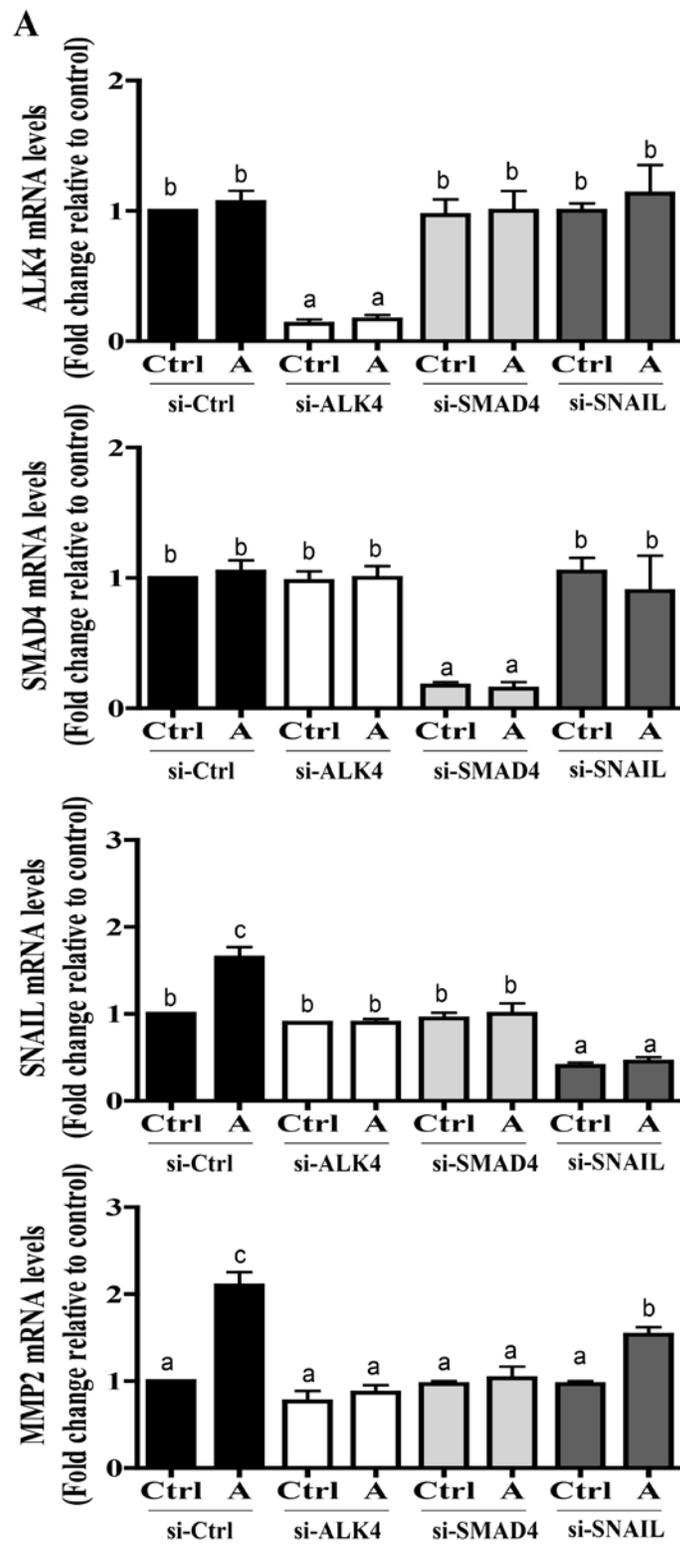
D**E**

Figure 4.5 Activin A-induced up-regulation of SNAIL and MMP2 is abolished by combined knockdown of SMAD2/SMAD3 or depletion of SMAD4

A-D, HTR8/SVneo cells were transfected for 48 h with 20 nM non-targeting control siRNA (si-Ctrl) or 20 nM siRNA targeting SMAD2 (si-SMAD2), SMAD3 (si-SMAD3), SMAD2 and SMAD3 (si-SMAD2+3) or SMAD4 (si-SMAD4) prior to treatment for 24 h without (Ctrl) or with 50 ng/ml activin A (A). RT-qPCR (with GAPDH as the reference gene) was used to examine the knockdown efficiencies of each siRNA treatment as well as their effects on activin A-induced SNAIL and MMP2 mRNA levels. **E**, Cells were transfected with 20 nM non-targeting control siRNA or 20 nM siRNA targeting SMAD4 and treated without or with activin A as described for **A-D**. SMAD4, MMP2, SNAIL and SLUG protein levels were examined by Western blot and normalized to α -tubulin. The upper panel shows a representative Western blot and the lower panel shows summarized quantitative results expressed as the mean \pm SEM of at least three independent experiments. Different letters indicate statistically significant differences ($P < 0.05$).



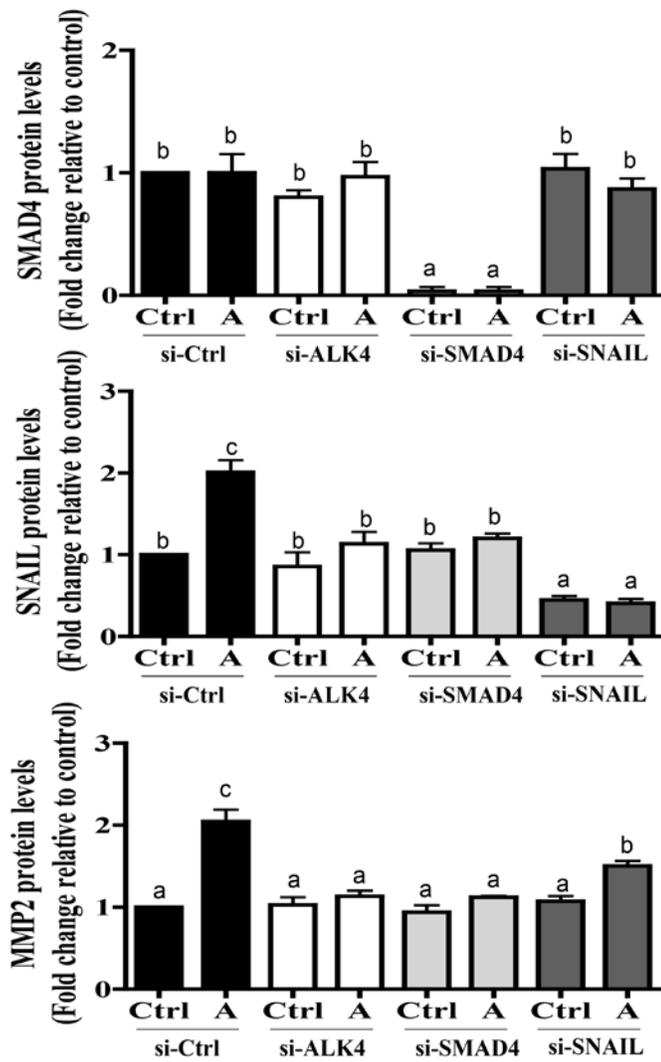
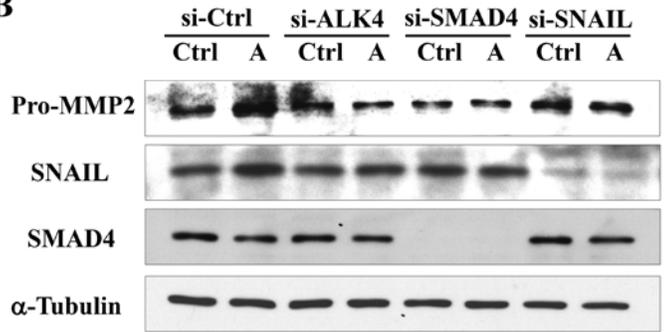
B

Figure 4.6 Activin A induces SNAIL-mediated MMP2 expression via ALK4 and SMAD4 in primary human EVT cells

A and B, Primary cultures of human EVT cells were transfected for 48 h with 20 nM non-targeting control siRNA (si-Ctrl) or 20 nM siRNA targeting ALK4 (si-ALK4), SMAD4 (si-SMAD4) or SNAIL (si-SNAIL) prior to treatment for 24 h without (Ctrl) or with 50 ng/ml activin A (A). **A,** RT-qPCR (with GAPDH as the reference gene) was used to examine the knockdown efficiencies of each siRNA treatment as well as their effects on SNAIL and MMP2 mRNA levels. **B,** MMP2, SNAIL and SMAD4 protein levels were examined by Western blot and normalized to α -tubulin. The upper panel shows a representative Western blot and the lower panel shows summarized quantitative results expressed as the mean \pm SEM of at least three independent experiments. Different letters indicate statistically significant differences ($P < 0.05$).

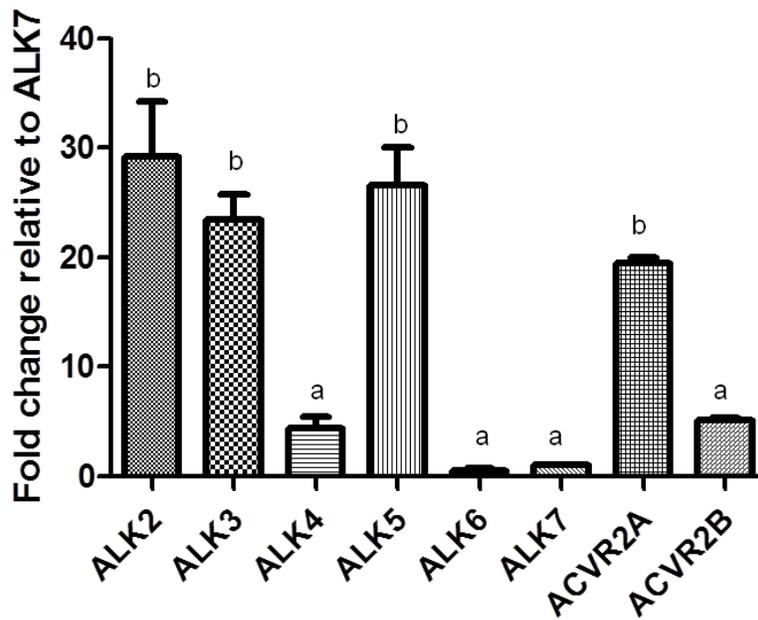


Figure 4.7 Expression of TGF- β type I and type II receptors in HTR8/SVneo cells

Cells were washed with cold PBS and total RNA was extracted with TRIzol Reagent (Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed using 2 μ g of total RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI) in a final volume of 20 μ l. RT-qPCR was performed on the Applied Biosystems 7300 real-time PCR system equipped with 96-well optical reaction plates. ALK2-7, ACVR2A and ACVR2B TaqMan primers were purchased from Life Technologies. The mRNA levels of ALK2-7, ACVR2A and ACVR2B were examined by TaqMan gene expression assays (Applied Biosystems), each 20- μ L RT-qPCR reaction contained 1 \times Taqman Gene Expression Master Mix (Applied Biosystems), 50 ng cDNA and 1 \times Taqman primer probe. At least three separate experiments were performed and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative Cq method with GAPDH as the reference gene and using the formula $2^{-\Delta\Delta Cq}$. The quantitative results of RT-qPCR analysis are

expressed as the mean \pm SEM of at least three independent experiments. Different letters indicate statistically significant differences ($P < 0.05$).

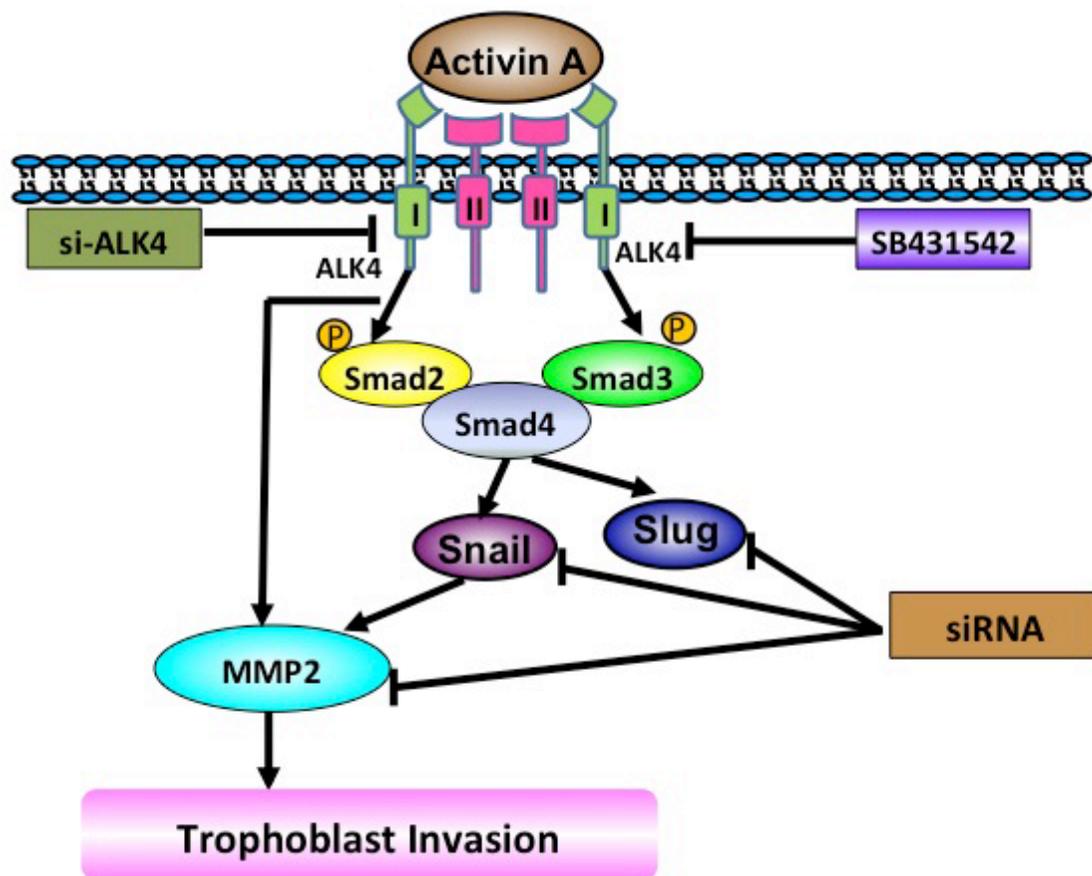


Figure 4.8 A schematic illustration of Chapter 4 data

Treatment of HTR8/SVneo cells with activin A increased the production of SNAIL, SLUG and MMP2, without altering that of MMP9, TIMP1, TIMP2, TWIST, RUNX2, ZEB1 or ZEB2 (Figure 4.1, 4.2 and 3.7). Similarly, activin A up-regulated the mRNA and protein levels of SNAIL and MMP2 in primary EVT cells (Figure 4.1, 4.2 and 4.6). Knockdown of SNAIL attenuated activin A-induced MMP2 up-regulation in HTR8/SVneo and primary EVT cells (Figure 4.3 and 4.6). In HTR8/SVneo cells, activin A-induced production of SNAIL and MMP2 was abolished by pre-treatment with the TGF- β type I receptor (ALK4/5/7) inhibitor SB431542 or siRNA targeting ALK4, SMAD2/3 or common SMAD4 (Figure 4.1, 4.2 and 4.5). Likewise,

knockdown of ALK4 or SMAD4 abolished the stimulatory effects of activin A on SNAIL and MMP2 expression in primary EVT cells (Figure 4.6). Importantly, activin A-induced HTR8/SVneo cell invasion was attenuated by siRNA-mediated depletion of ALK4 or MMP2 (Figure 4.1).

Chapter 5: Vascular endothelial growth factor-A (VEGF-A) mediates activin A-induced human trophoblast endothelial-like tube formation

5.1 Introduction

Successful placentation requires that extravillous cytotrophoblasts (EVTs) terminally differentiate into invasive stromal and endovascular remodeling EVT cells (18). Invasive stromal EVT cells invade into the decidua and anchor the placenta to the maternal uterus, whereas endovascular EVT cells penetrate the lumen of distal maternal spiral arterials and subsequently acquire an endothelial-like phenotype and replace the pre-existing endothelial cells lining the uterine vessels. An adequate remodeling of the uterine vasculature system ensures a constant exchange of key molecules between maternal and fetal circulations (34). The mimicry of endothelial cells in the vascular system by EVT cells is often referred to as EVT endovascular differentiation (174), and this primarily represents the ability of EVT cells to mimic endothelial cells in angiogenesis to form endothelial-like tubes *in vitro* upon culture on Matrigel (68, 69). Impaired spiral artery remodeling caused by restricted EVT endovascular differentiation is responsible for the development of preeclampsia and intrauterine growth restriction (IUGR), two common dangerous complications during pregnancy (38-40).

The most studied decidua-derived growth factor to date, VEGF-A, has been shown to regulate EVT endovascular differentiation (70, 199, 200). Clinically, decreased free-form VEGF-A accompanied by increased anti-angiogenic factor soluble fms-like tyrosine 1 (sFlt-1) has been implicated in the onset of preeclampsia by preventing extensive spiral artery remodeling (161, 162). VEGF-A belongs to the VEGF gene family that consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PLGF). As the most potent angiogenic factor in the VEGF

family, VEGF-A was first isolated from ascetic fluid and identified as a vascular permeability factor (163). Specifically, endovascular EVT cells that express markers of endothelial cells, including VEGF-A and its receptors (18), migrate toward the maternal decidua and subsequently replace the endothelial cells of spiral arteries (69, 166). The crucial role of VEGF-A during embryonic development has been proven by single VEGF-A allele missing mouse embryos, which die between days 11 and 12 of pregnancy due to defective vascularization (167). Moreover, neutralizing VEGF-A monoclonal antibody infusion in rats caused hypertension and proteinuria, the typical features of preeclampsia (168).

Activin A, which belongs to the transforming growth factor- β (TGF- β) superfamily, is a homodimer of the inhibin β A subunit. The expression of activin A is elevated in endometrial stromal and decidual cells (104) and modest in EVT cells (173). Receptors for activin A have also been detected in human trophoblast cells (119). Activin A exerts its wide biological effects via binding to heterotetrameric complexes of type I and type II transmembrane serine-threonine kinase receptors (112). The pro-invasive effect of activin A on first trimester EVT has been demonstrated both *in vivo* and *in vitro* (107, 108); in addition, activin A was found to stimulate VEGF-A gene expression in human hepatocellular carcinoma cells (171). However, the role of activin A associated with VEGF-A on EVT endovascular differentiation remains to be determined.

Our study aims to investigate the regulatory effect of activin A on EVT endovascular differentiation by analyzing endothelial-like tube formation in HTR8/SVneo cells and to further identify the involvement of the key angiogenic factor VEGF-A as well as the underlying molecular mechanisms.

5.2 Materials and methods

Culture of HTR8/SVneo immortalized human EVT cells

The HTR8/SVneo simian virus 40 large T antigen-immortalized human EVT cell line was generously provided by Dr. P.K. Lala at the Western University (63). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 100 U/ml penicillin (Life Technologies), 100 µg/ml streptomycin (Life Technologies), and 10% (vol/vol) fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). Cultures were incubated at 37 °C in a humidified 5% CO₂/air atmosphere, and the medium was changed every other day. Twenty-four hours prior to activin A or SB431542 treatment, regular 10% (vol/vol) FBS DMEM culture medium was replaced with 0.1% (vol/vol) FBS DMEM to minimize the possible inferences resulting from factors in FBS.

Antibodies and reagents

Phospho-SMAD2 (Ser^{465/467}) (138D4) rabbit monoclonal antibody, SMAD2 (L16D3) mouse monoclonal antibody, Phospho-SMAD3 (Ser^{423/425}) (C25A9) rabbit monoclonal antibody, SMAD3 (C67H9) rabbit monoclonal antibody and SMAD4 polyclonal antibody were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti- α -tubulin (B-5-1-2) (sc-23948) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The TGF- β type I receptor inhibitor SB431542 (#S4317) and recombinant VEGF-A were purchased from Sigma-Aldrich. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Bio-Rad Laboratories (Hercules, CA). Recombinant human activin A and the human VEGF-A Quantikine ELISA Kit were obtained from R&D Systems (Minneapolis, MN).

Endothelial-like tube formation assay

Twenty-five microliters of growth factor-reduced Matrigel (BD Biosciences) at a concentration of 10 mg/ml diluted 1:1 (vol/vol) with 0.1% (vol/vol) FBS DMEM medium was added to 96-well plates and incubated for 2 h at 37 °C to solidify. HTR8/SVneo cells in 0.1% (vol/vol) FBS DMEM with or without activin A treatment (50 ng/ml) were subjected to the tube-formation assay by seeding on the GFR Matrigel-coated plates (4×10^4 cells/well) and incubating at 37 °C and 5% CO₂/air for 24 h. Digital images (40× magnification) were taken using a Nikon Eclipse TE300 microscope with Northern Eclipse 6.0 software (Empix Imaging, Mississauga, Ontario, Canada). The number of branching points and total tube length were quantified using the National Institutes of Health (NIH) ImageJ software. Branching points were considered as a point from which two or more tubes branched.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Cells were washed with cold PBS, and total RNA was extracted with TRIzol Reagent (Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed using 2 µg of total RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI) in a final volume of 20 µl. RT-qPCR was performed on the Applied Biosystems 7300 real-time PCR system equipped with 96-well optical reaction plates. Each 20-µL RT-qPCR reaction contained 1×SYBR Green PCR Master Mix (Applied Biosystems), 12 ng cDNA and 150 nM of each specific primer. The primers used were VEGF-A, 5'-CGAGGGCCTGGAGTGTGT-3' (forward) and 5'-CGCATAATCTGCATGGTGATG-3' (reverse); human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GAG TCA ACG GAT TTG GTC GT-3' (forward) and

5'-GAC AAG CTT CCC GTT CTC AG-3' (reverse); SMAD2, 5'-GCC TTT ACA GCT TCT CTG AAC AA-3' (forward) and 5'-ATG TGG CAA TCC TTT TCG AT-3' (reverse); SMAD3, 5'-CCC CAG CAC ATA ATA ACT TGG-3' (forward) and 5'-AGG AGA TGG AGC ACC AGA AG-3' (reverse); and SMAD4, 5'-TGG CCC AGG ATC AGT AGG T-3' (forward) and 5'-CAT CAA CAC CAA TTC CAG CA-3' (reverse). The specificity of each assay was confirmed by dissociation curve analysis and agarose gel electrophoresis of PCR products. The assay performance was validated by assessing amplification efficiencies by means of calibration curves and ensuring that the plot of log input amount versus ΔCq had a slope with an absolute value of <0.1 . At least three separate experiments were performed, and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative Cq method with GAPDH as the reference gene and using the formula $2^{-\Delta\Delta Cq}$.

Western blot

Cells were washed with cold phosphate-buffered saline (PBS) twice and lysed in ice cold lysis buffer (Cell Signaling Technology) with added protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined using the DC protein assay kit (Bio-Rad Laboratories) with bovine serum albumin (BSA) as the standard. Equal amounts of protein (30 μ g) were separated by SDS-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene fluoride membrane. Membranes were blocked with TBS containing 5% (wt/vol) non-fat dry milk for 1 h and incubated overnight at 4 °C with primary antibodies diluted in tris-buffered saline (TBS) with 5% (wt/vol) non-fat dried milk and 0.1% (vol/vol) tween-20. Bound antibodies were visualized using HRP-conjugated secondary antibody, ECL or SuperSignal West Femto chemiluminescent substrates (Thermo Fisher, Waltham, MA), and CL-XPosure film (Thermo

Fisher). Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mM β -mercaptoethanol and 1% wt/vol SDS) at 50 °C for 30 min and re-probed with anti- α -tubulin antibody as a loading control. Densitometric quantification was performed using Scion Image software with α -tubulin as the internal control for normalization.

Small interfering RNA (siRNA) transfection

Cells that were 50% confluent were transfected for 48 h with 20 nM specific gene targeting ON-TARGET*plus* SMART*pool* siRNA or 20 nM siCONTROL NON-TARGETING*pool* siRNA (Thermo Scientific, Lafayette, CO) using Lipofectamine RNAiMAX and Opti-MEM I according to the manufacturer's instructions (Life Technologies). The transfection efficiency was assessed by RT-qPCR or Western blot analysis.

MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) assay was used to assess cell viability. HTR8/SVneo cells were transfected with 20 nM siRNA targeting VEGF-A or CONTROL and seeded in 24-well plates (2×10^4 cells/well in 1 mL of 10% FBS DMEM medium). The culture media were replaced every 24 h with DMEM containing 0.1% (vol/vol) FBS. Cell viability was tested at 24, 48 and 72 h after transfection together with specific time point controls. At each time point, cells were incubated with 0.5 mg/mL MTT for 4 h, the medium was replaced with 1 mL DMSO, and absorbances were measured at 490 nm using a microplate reader (Wallac VICTOR³).

Human VEGF-A enzyme-linked immunosorbent assay (ELISA)

A total of 1×10^5 cells were seeded in a 6-well dish with 2 mL 10% (vol/vol) FBS DMEM before being starved with 0.1% (vol/vol) FBS DMEM overnight. HTR-8/SVneo cells were then treated with or without activin A (50 ng/ml) for 1, 3, 6, 12 or 24 h. After treatments, the cell culture supernatant and corresponding protein lysates were collected. The concentrations of free-form VEGF-A in conditioned medium were assayed by the human VEGF-A ELISA kit (R&D Systems) according to the manufacturer's instructions. The detection limit of VEGF-A was 1000 pg/mL. Intra- and inter-assay coefficients of variation were less than 10%. Protein concentrations were determined with the DC protein assay kit (Bio-Rad Laboratories) with BSA serving as the standard. VEGF-A values were normalized to total cellular protein content.

Study protocol

Study groups were composed of cells treated with activin A, SB431542 and/or siRNA targeting VEGF-A, SMAD2, SMAD3 and/or SMAD4, whereas the corresponding control groups were composed of cells treated without activin A or with equivalent amounts of DMSO or non-targeting control siRNA. For each study, at least three independent experiments were performed, each with a separate passage of cells.

Statistical analysis

Results are presented as the mean \pm SEM of at least three independent experiments and were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons of means using PRISM software (GraphPad Software, Inc., San Diego, CA). Means were considered significantly different if $P < 0.05$ and groups without a common letter are statistically different

from each other.

5.3 Results

Activin A induces HTR8/SVneo cell endothelial-like tube formation

HTR8/SVneo cells treated with or without activin A were subjected to an endothelial-like tube formation assay, and 50 ng/ml VEGF-A treated HTR8/SVneo cells served as a positive control. The acquisition of the endothelial phenotype of HTR8/SVneo cells cultured on 1:1 diluted Matrigel-coated plates was significantly enhanced after activin A treatment, with increased total branch points and total tube length (Figure 5.1A). Application of the TGF- β type I receptor inhibitor SB431542 (10 μ M) for 1 h prior to treatment with activin A completely abolished its stimulatory effect on HTR8/SVneo cell tube formation (Figure 5.1B). The possible influence of activin A on HTR8/SVneo cell viability or apoptosis was previously excluded by the results from an MTT assay and consistent cleaved caspase 3 protein levels measured by Western blot (Figure 3.2C and D).

Activin A increases VEGF-A expression

Treatment with activin A up-regulated VEGF-A mRNA levels, with the highest level at 1 h after treatment. ELISA showed that treatment with activin A increased free-form VEGF-A levels in culture medium, with the highest levels at 3 h after treatment (Figure 5.2A). Similarly, as observed in the endothelial-like tube formation assay, these effects on VEGF-A mRNA and free protein levels in the medium were abolished by pre-treatment with SB431542 (Figure 5.2B). To check the possible effect of Matrigel on basal VEGF-A expression, HTR8/SVneo cells were cultured on Matrigel versus plastic for 24 h and both VEGF-A mRNA and free-form protein

levels in conditioned medium were then examined. The basal levels of VEGF-A of HTR8/SVneo cells under these two culture conditions showed no significance difference (Figure 5.7A and B).

Basal and activin A-induced endothelial-like tube formation are attenuated by siRNA-mediated depletion of VEGF-A

Treatment with siRNA targeting VEGF-A was used to investigate whether VEGF-A is involved in activin A-induced trophoblast tube formation. RT-qPCR analysis and ELISA showed that transfection with 20 nM siRNA targeting VEGF-A for 48 h suppressed both endogenous and activin A-induced VEGF-A mRNA and free-form protein levels in culture medium, as measured by RT-qPCR and ELISA (Figure 5.3A). MTT assays were used to check the possible influence of siRNA targeting VEGF-A on cell viability. MTT results showed that knockdown of VEGF-A for as little as 24 h and as much as 72 h did not influence HTR8/SVneo cell viability (Figure 5.3B). Endothelial-like tube-formation assay results revealed that treatment with VEGF-A siRNA attenuated both basal and activin A-induced HTR8/SVneo cells tube formation on Matrigel (Figure 5.3C). Thus, VEGF-A promoted HTR8/SVneo cell endothelial-like tube formation without altering cell viability.

SMAD2/3 signaling is required for activin A-induced up-regulation of VEGF-A

Activin A treatment significantly increased the phosphorylation of both SMAD2 and SMAD3 (Figure 5.4A). Moreover, activin A-induced phosphorylation of SMAD2 and SMAD3 was abolished by treatment with SB431542 (Figure 5.4B). To analyze the individual roles of SMAD2 and SMAD3 in activin A-induced VEGF-A up-regulation, HTR8/SVneo cells were treated with activin A following siRNA-mediated depletion of SMAD2 or SMAD3. The knockdown

efficiencies were confirmed by RT-qPCR and Western blot (Figure 5.5A and B). Depletion of either SMAD2 or SMAD3 completely abolished the up-regulatory effects of activin A on VEGF-A on both mRNA and protein levels (free-form) (Figure 5.5C). To confirm the role of SMAD signaling in activin A-regulated VEGF-A expression, HTR8/SVneo cells were treated with activin A following siRNA-mediated depletion of common SMAD4. The knockdown efficiencies of SMAD4 were confirmed by RT-qPCR analysis and Western blot (Figure 5.6A and B). Down-regulation of SMAD4 completely abolished the stimulatory effects of activin A on VEGF-A mRNA and free-form protein levels (Figure 5.6C).

5.4 Discussion

For the first time, we demonstrate that activin A, which is critical for trophoblast invasion, also promotes trophoblast endovascular differentiation in an immortalized EVT cell line, HTR8/SVneo, as measured by increased endothelial-like tube formation. Moreover, activin A promotes trophoblast endothelial-like tube formation through up-regulating VEGF-A expression in a SMAD2/3-SMAD4-dependent manner.

Due to the intrinsic capacity for endothelial-like tube formation when cultured on Matrigel, the immortalized HTR8/SVneo cells have been widely used to investigate the underlying molecular mechanisms of EVT endovascular differentiation (68-70). Aldo et al. observed that HTR8/SVneo cells synchronized with the endothelial cell capillary network when co-cultured on Matrigel and eventually replaced the endothelial cells (69). Von Daldelszen et al. used HTR8/SVneo cells to investigate the effect of decidual natural killer (dNK)-derived conditioned medium on EVT endovascular differentiation (68). Lala et al. identified VEGF-A as a promoter of EVT endovascular differentiation in HTR8/SVneo cells (70). Interestingly, we not only found

that activin A stimulated endothelial-like tube formation in HTR8/SVneo cells but also revealed that this trophoblast endovascular differentiation was promoted by activin A-induced VEGF-A expression. Because the use of Matrigel in culture has been reported to affect gene expression in HTR8/SVneo cells (201), we compared the expression of VEGF-A mRNA and protein levels between HTR8/SVneo cells cultured on plastic and diluted (1:1) Matrigel to exclude the possible interference of Matrigel on VEGF-A expression. Our results showed that both mRNA and free-form protein levels of VEGF-A were not altered after cells were cultured on Matrigel versus plastic for 24 h (Figure 5.7A and B).

VEGF-A is a well-characterized mediator of physiological and pathological angiogenesis and regulates cell survival, migration and proliferation of endothelial cells through activating multiple tyrosine kinase signaling pathways (159). Currently, many VEGF-A inhibitors are utilized clinically as a strategy for preventing angiogenesis in several malignancies (160). During pregnancy, decreased free-form VEGF-A is responsible for the onset of preeclampsia, a dangerous complication caused by the failure of trophoblasts to mimic a vascular adhesion phenotype (39, 161, 162, 202). VEGF-A exerts its functional role by interaction with its receptors, among which, Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2) are the most well-studied tyrosine kinase receptors that mediate the effect of VEGF-A on angiogenesis (203). A soluble form of VEGFR-1 (sFlt-1) can bind with VEGFR-2 to form a heterodimeric complex, preventing the binding of VEGF-A to VEGFR-2 and subsequent signaling (204), whereas most physiological and pathological functions of VEGF-A are mediated by the activation of VEGFR-2 (205). VEGFR-2 knockout in mice is lethal due to impaired development of endothelial cells (206). Dr. Lala's group reported the expression of VEGFR-2 in HTR8/SVneo cells and identified VEGFR-2 as the principle mediator in VEGF-A-stimulated human trophoblast endovascular

differentiation (70). In the present study, the essential role of VEGF-A in activin A-induced trophoblast tube formation was confirmed by using siRNA-mediated VEGF-A knockdown. MTT results showed that siRNA-mediated VEGF-A knockdown attenuated tube formation without changing cell viability. At present, the most studied downstream target of VEGF-A and its receptor is vascular endothelial cadherin (VE-cadherin) (207, 208). Although up-regulated VE-cadherin expression has been reported as a molecular marker for increased trophoblast endothelial-like tube formation (70), VE-cadherin was found to be critical to the endothelial survival function of VEGF-A without promoting initial vessel assembly (207). Others have reported that VE-cadherin inhibits VEGF-A-induced angiogenesis in endothelial cells by blocking VEGFR-2 phosphorylation (209). Further underlying molecular mechanisms regarding VEGF-A-induced trophoblast tube formation and EVT endovascular differentiation need to be explored.

The up-regulation of VEGF-A can be induced by hypoxia and a variety of growth factors as well as by cytokines. It has been reported that hypoxia-inducible factor (HIF) mediates hypoxia-induced VEGF-A expression in endothelial cells (169). Epidermal growth factor (EGF), TGF- α , TGF- β , FGF, IL-1 α and IL-6 have been shown to increase VEGF-A gene expression in different cell types (170). Likewise, in HTR8/SVneo cells, VEGF-A production is up-regulated in response to TNF- α and TGF- β (172). In our study, activin A increased VEGF-A expression in human trophoblast cells by activation of the TGF- β type I receptor. The results confirmed that after blockage of the activation of type I receptor by a specific inhibitor, SB431542, the phosphorylation of SMAD2/3 completely vanished. Furthermore, by siRNA-mediated knockdown of SMAD2/3/4, activin A-induced VEGF-A expression was attenuated. Indeed, chromatin immunoprecipitation and promoter luciferase have demonstrated interactions of

SMAD2 and SMAD3 with TCF4 and β -catenin at the VEGF-A promoter after TGF- β 1 treatment in smooth muscle cells (210). Additionally, activin A has been shown to stimulate VEGF-A gene expression in human hepatocellular carcinoma cells through the formation of a trimeric complex consisting of SMAD2, SMAD3 and SMAD4 at the VEGF-A promoter region (30). Our results indicated that the activation of SMAD2/3/4 is required for VEGF-A responsiveness to activin A treatment in human trophoblast cells. In our study, the up-regulation of VEGF-A mRNA as early as 1 h after treatment with activin A suggests the direct binding of SMAD2/3-SMAD4 to the VEGF-A promoter.

In summary, our study describes the stimulatory effects of activin A on trophoblast endothelial-like tube formation. VEGF-A, a candidate early diagnosis biomarker and therapeutic target for preeclampsia and intrauterine growth restriction, mediates activin A-induced endothelial-like tube formation. Moreover, SMAD2/3-SMAD4 signaling is crucial for activin A-induced VEGF-A production and endothelial-like tube formation. Specifically, activin A (homodimer of inhibin β A) binds to a heterotetrameric receptor complex comprised of type I (ALK4) and type II (ACVR2A/B) receptors. Ligand-induced activation of the receptor complex leads to the activation of receptor-regulated SMAD2/3. Phosphorylated SMAD2/3 forms a heterotrimeric complex with common SMAD4 that translocates into the nucleus where it binds DNA and increases the transcription of VEGF-A. The up-regulation of VEGF-A, subsequently contributes to activin A-induced human trophoblast cell endothelial-like tube formation (Figure 5.8). Our results provide important insights into the cellular and molecular mechanisms underlying spiral artery remodeling during early pregnancy

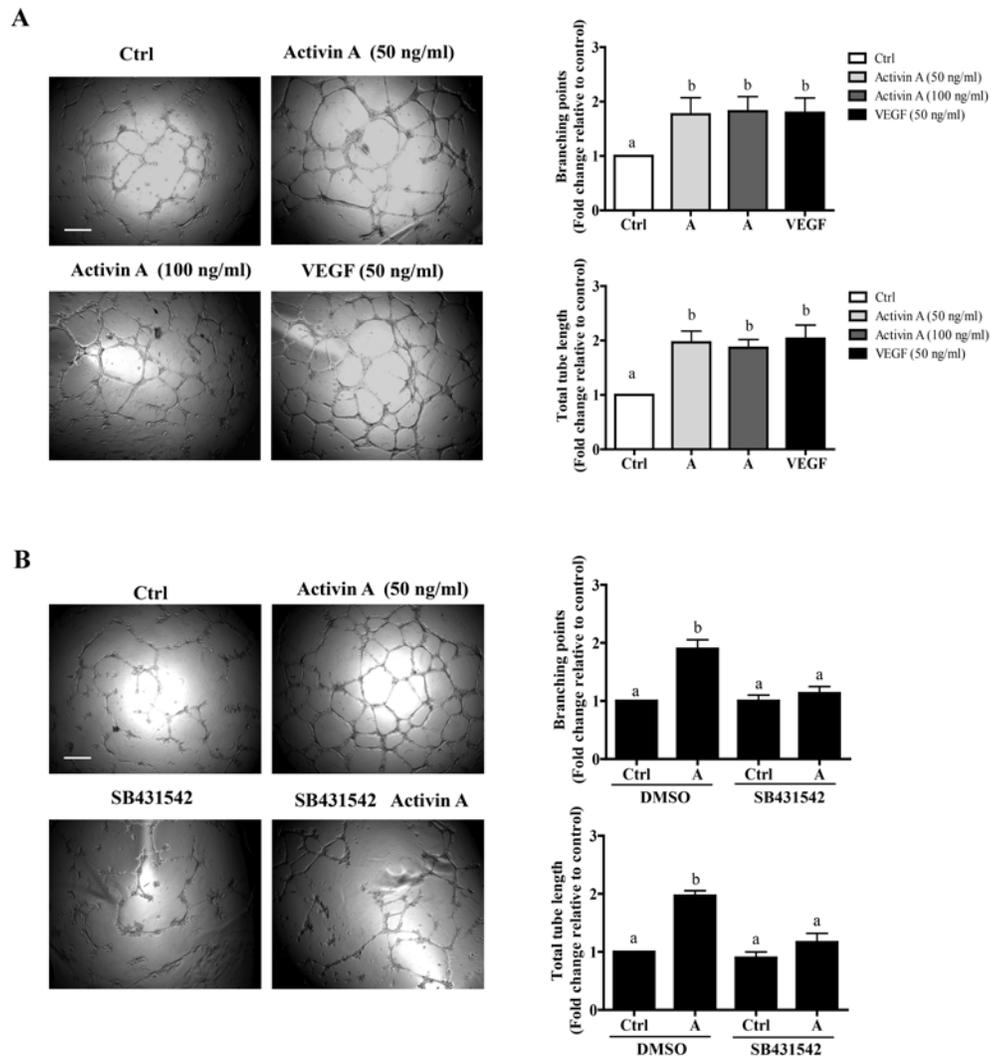


Figure 5.1 Activin A induces HTR8/SVneo cell endothelial-like tube formation, which can be abolished by the TGF- β type I receptor inhibitor SB431542

A, HTR8/SVneo cells were treated without (Ctrl) or with 50 or 100 ng/ml activin A (A) or 50 ng/ml VEGF-A (VEGF), and the acquisition of the endothelial phenotype was examined by an endothelial-like tube-formation assay. **B**, HTR8/SVneo cell endothelial-like tube formation was assessed following pretreatment for 1 h with vehicle control (DMSO) or 10 μ M SB431542 prior to treatment without (Ctrl) or with 50 ng/ml activin A. **A** and **B**, Left panels show representative images of the endothelial-like tube formation assays (scale bar: 400 μ m), and right panels show

summarized quantitative total branching points and total tube length results. Analysis of total tube length produced identical results as analysis of branching points. The quantitative results of these two parameters are expressed as the mean \pm SEM of at least three independent experiments. Groups without a common letter are statistically different from each other ($p < 0.05$).

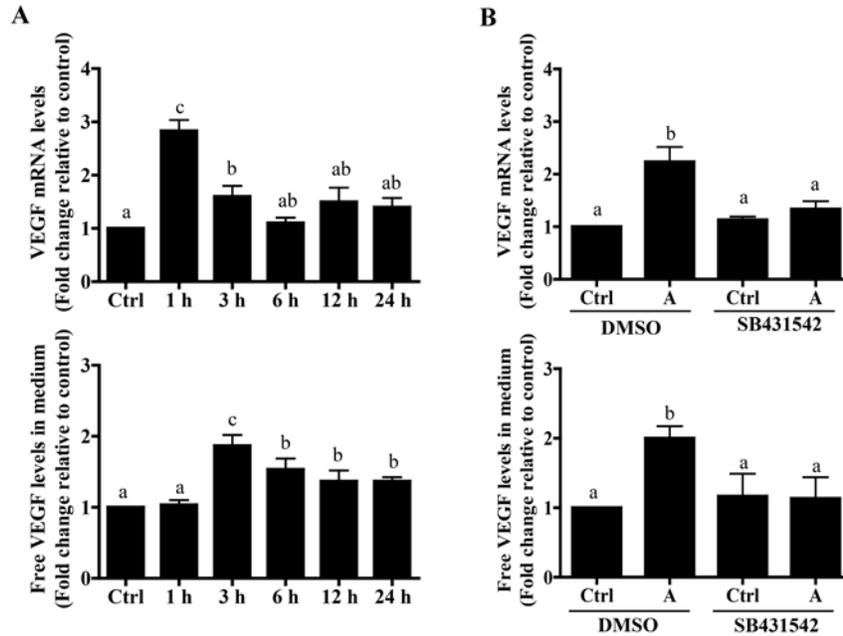


Figure 5.2 Activin A up-regulates the expression of VEGF-A mRNA and free-form protein levels in culture medium in HTR8/SVneo cells

A, HTR8/SVneo cells were treated without (Ctrl) or with 50 ng/ml activin A (A) for different lengths of time (1, 3, 6, 12 or 24 h), and *VEGF-A* (VEGF) mRNA levels were examined by RT-qPCR with GAPDH as the reference gene (Upper panel). Free-form VEGF-A protein levels in cultured medium were examined by ELISA and normalized to the total cellular protein content (Lower panel). **B**, HTR8/SVneo cells were pretreated for 1 h with vehicle control (DMSO) or 10 μ M SB431542 prior to treatment without (Ctrl) or with 50 ng/ml activin A for 1 and 3 h. VEGF-A mRNA levels were examined after 1 h treatment by RT-qPCR with GAPDH as the reference gene (Upper panel). Free-form VEGF-A protein levels in culture medium were examined by ELISA after a 3 h treatment and normalized to the total cellular protein content (Lower panel).

Quantitative results are expressed as the mean \pm SEM of at least three independent experiments.

Groups without a common letter are statistically different from each other ($p < 0.05$).

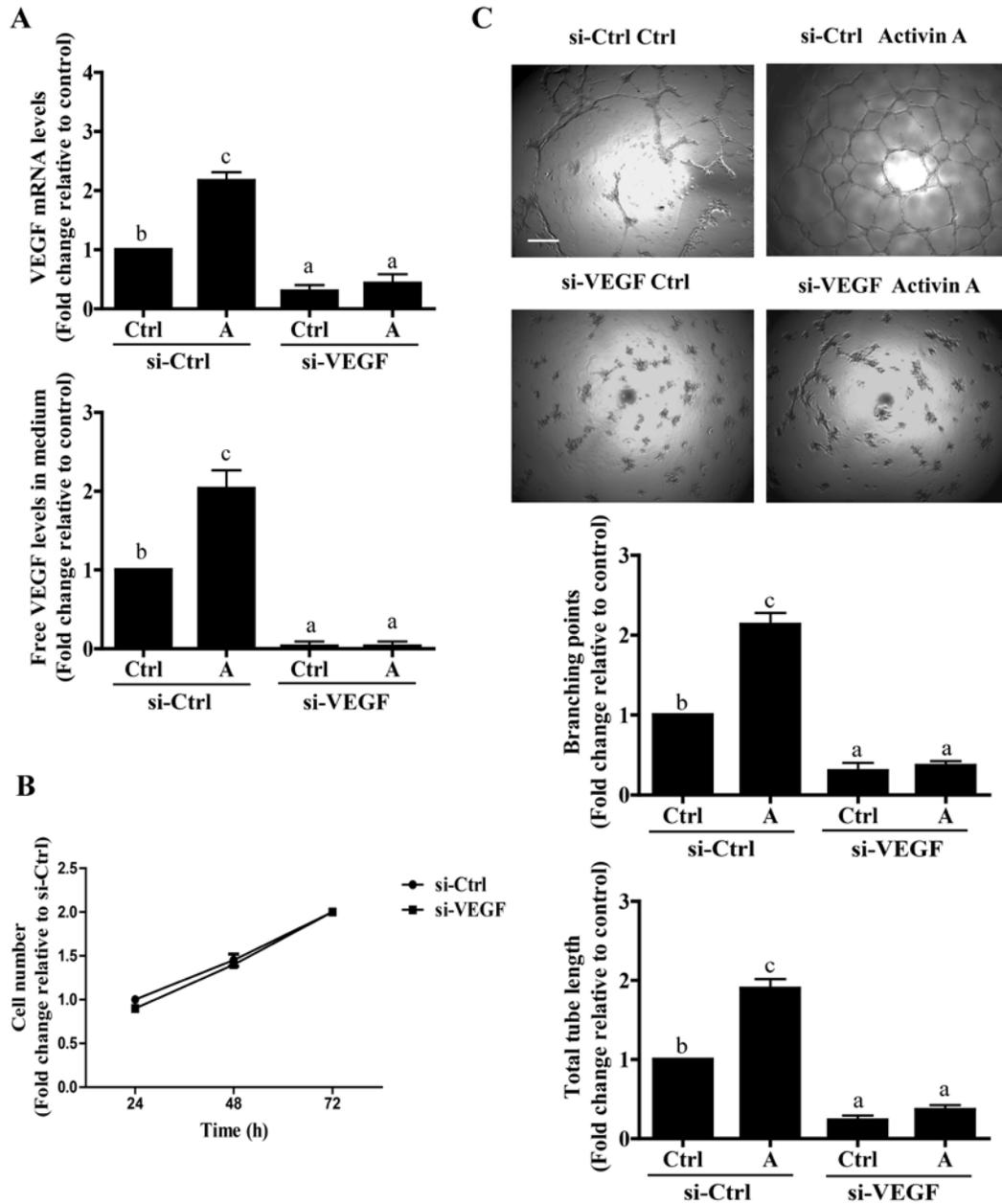


Figure 5.3 Basal and activin A-induced cell acquisition of endothelial phenotype are attenuated by siRNA-mediated down-regulation of VEGF-A

A, HTR8/SVneo cells were transfected for 48 h with 20 nM non-targeting control siRNA (si-Ctrl) or siRNA targeting VEGF-A (si-VEGF-A) prior to treatment without or with 50 ng/ml

activin A for 1 and 3 h. VEGF-A (VEGF) mRNA levels were examined after 1 h treatment by RT-qPCR with GAPDH as the reference gene (Upper panel). Free-form VEGF-A protein levels in culture medium after 3 h treatment were examined by ELISA and normalized to the total cellular protein content (Lower panel). **B**, HTR8/SVneo cells were transfected with si-RNA targeting VEGF-A or Ctrl and seeded in 24-well plates (2×10^4 cells/well in 1 mL of medium). The medium was replaced every 24 h with DMEM containing 0.1% FBS. Cell viability at 24, 48 and 72 h after treatment was examined by MTT assay. **C**, HTR8/SVneo cells were transfected for 48 h with 20 nM non-targeting control siRNA (si-Ctrl) or siRNA targeting VEGF-A (si-VEGF-A) prior to the endothelial-like tube-formation assay. The upper panel shows representative images of the endothelial-like tube-formation assay (scale bar: 400 μ m), and the lower panels show summarized quantitative total branching points and total tube lengths. The summarized quantitative results are expressed as the mean \pm SEM of at least three independent experiments. Groups without a common letter are statistically different from each other ($p < 0.05$).

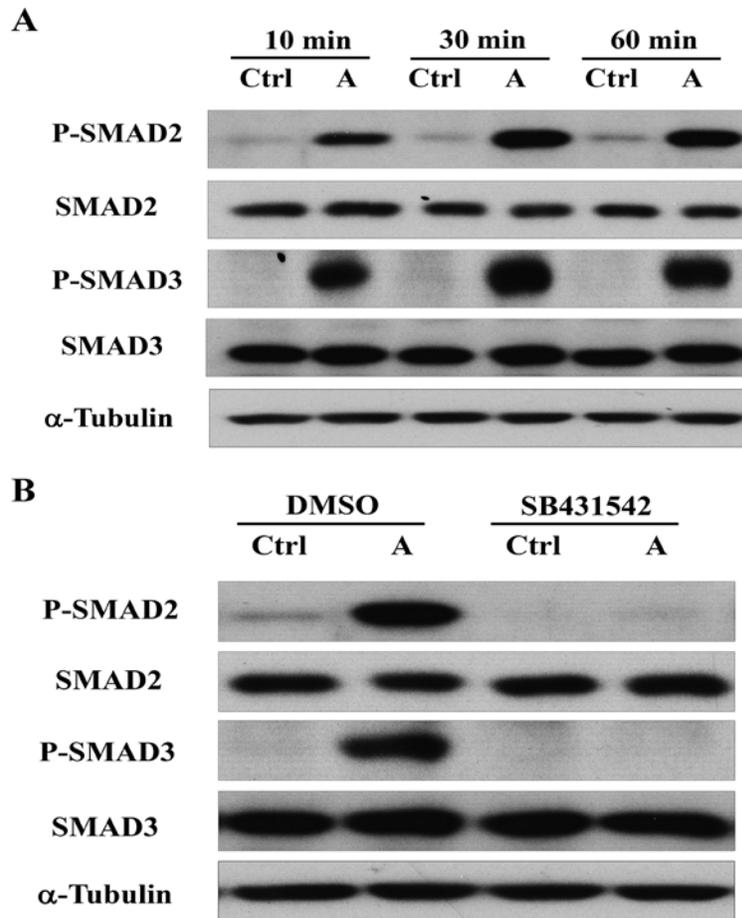


Figure 5.4 Activin A phosphorylates SMAD2 and SMAD3, which is abolished by SB431542

A, HTR8/SVneo cells were treated for 10, 30 or 60 min without (Ctrl) or with 50 ng/ml activin A (A). Levels of phosphorylated SMAD2 (p-SMAD2) and SMAD3 (p-SMAD3) were examined by Western blot with phospho-specific antibodies. Membranes were stripped and re-probed with antibodies for total SMAD2, SMAD3 and α -tubulin. **B**, HTR8/SVneo cells were pretreated for 1 h with vehicle control (DMSO) or 10 μ M SB431542 prior to treatment for 60 min without (Ctrl) or with 50 ng/ml activin A (A). Levels of phosphorylated and total SMAD2, SMAD3 and α -tubulin were measured by Western blot as described in **A**.

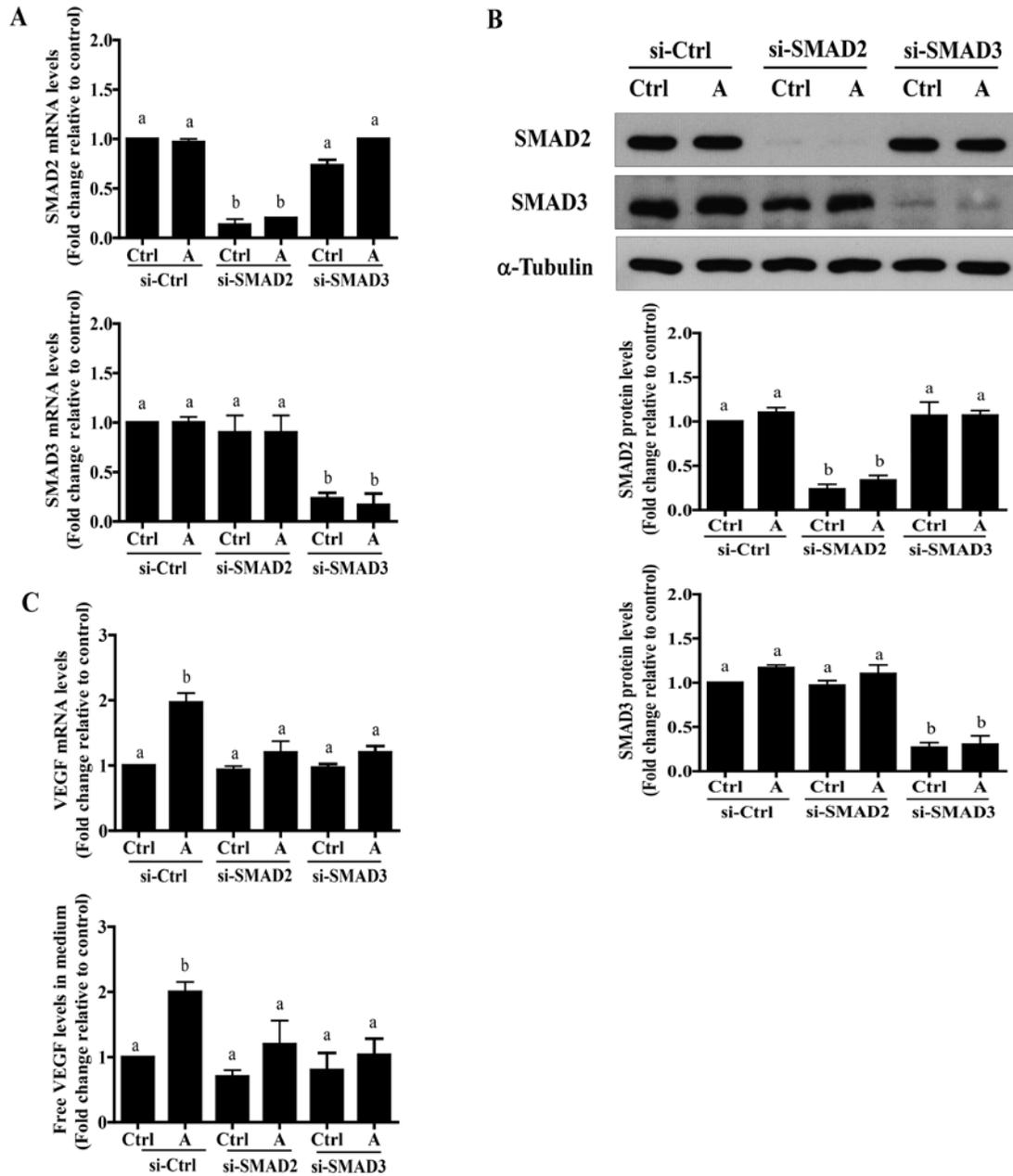


Figure 5.5 Activin A-induced up-regulation of VEGF-A in HTR8/SVneo cells is abolished by knockdown of SMAD2 or SMAD3

A-C, Cells were transfected for 48 h with 20 nM non-targeting control siRNA (si-Ctrl) or 20 nM siRNA targeting SMAD2 (si-SMAD2) or SMAD3 (si-SMAD3) prior to treatment for 1 and 3 h without (Ctrl) or with 50 ng/ml activin A (A). **A**, The knockdown efficiencies of si-SMAD2 and

si-SMAD3 were examined by RT-qPCR after 1 h activin A treatment. **B**, The knockdown efficiencies of siSMAD-2 and siSMAD-3 after 3 h activin A treatment were further examined by Western blot and normalized to α -tubulin. The upper panel shows a representative Western blot, and lower panels show the summarized quantitative results. **C**, After 1 h activin A treatment, VEGF-A (VEGF) mRNA levels were examined by RT-qPCR (upper panel). After 3 h activin A treatment, the concentrations of free-form VEGF-A in culture medium were examined by ELISA and normalized to the total cellular protein content (lower panel). The summarized quantitative results are expressed as the mean \pm SEM of at least three independent experiments. Groups without a common letter are statistically different from each other ($p < 0.05$).

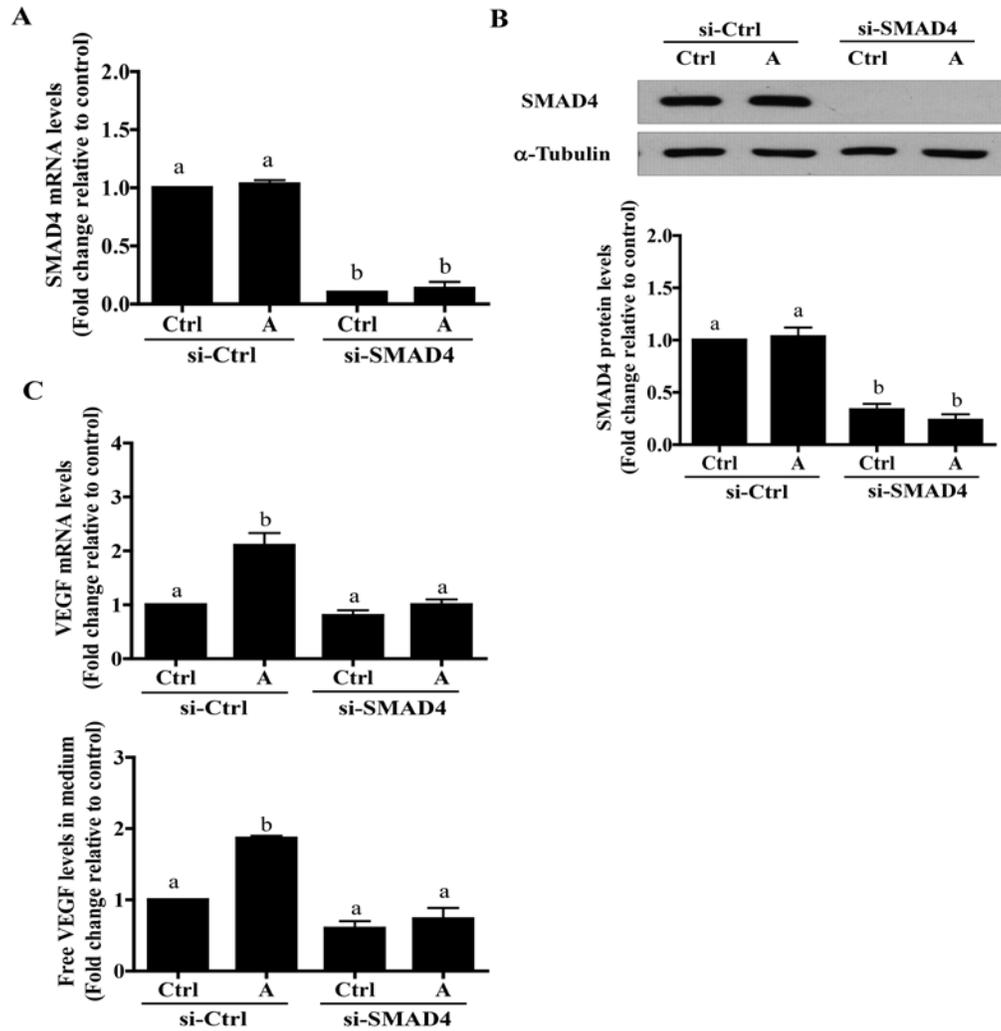


Figure 5.6 siRNA-mediated depletion of SMAD4 abrogates activin A-up-regulated VEGF-A expression

Cells were transfected for 48 h with 20 nM non-targeting control siRNA (si-Ctrl) or 20 nM siRNA targeting SMAD4 prior to treatment for 1 and 3 h without (Ctrl) or with 50 ng/ml activin A (A). **A**, The knockdown efficiency of si-SMAD4 treatment was examined by RT-qPCR after 1 h treatment. **B**, The knockdown efficiency of si-SMAD4 was further examined by Western blot and normalized to α -tubulin after 3 h treatment. The upper panel shows a representative Western blot, and the lower panel shows the summarized quantitative results. **C**, After 1 h activin A

treatment, VEGF-A (VEGF) mRNA levels were examined by RT-qPCR (upper panel). After 3 h activin A treatment, the concentrations of free-form VEGF-A in culture medium were examined by ELISA and normalized to the total cellular protein content (lower panel). The summarized quantitative results are expressed as the mean \pm SEM of at least three independent experiments. Groups without a common letter are statistically different from each other ($p < 0.05$).

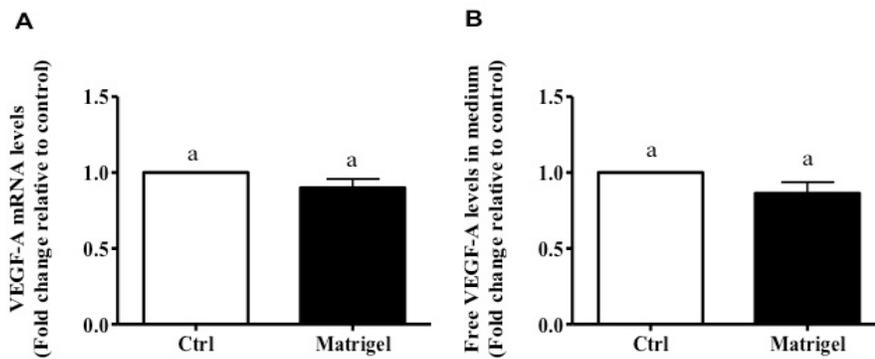


Figure 5.7 VEGF-A mRNA and free-form protein levels in HTR8/SVneo cells are not altered after cells were cultured on Matrigel versus plastic for 24 h

A and **B**, A total of 1.2×10^6 HTR8/SVneo cells in 1.2 mL 0.1% (vol/vol) FBS DMEM were then seeded in the GFR matrigel (5mg/ml)-coated or regular 6-well dish and incubated at 37°C and 5% CO₂/air for 24 h. **A**, HTR8/SVneo cells cultured on plastic dish or 1:1 diluted matrigel were collected and assayed by RT-qPCR with corresponding SYBR primers. Relative gene expression levels are presented as the mean \pm SEM relative to GAPDH and normalized to cells grown on plastic, given a value. **B**, the conditioned medium were collected and subjected to free-form VEGF-A ELISA assay. The concentrations of free-form VEGF-A were normalized to the total cellular protein content. The summarized quantitative results are expressed as the mean \pm SEM of at least three independent experiments. Groups without a common letter are statistically different from each other ($p < 0.05$).

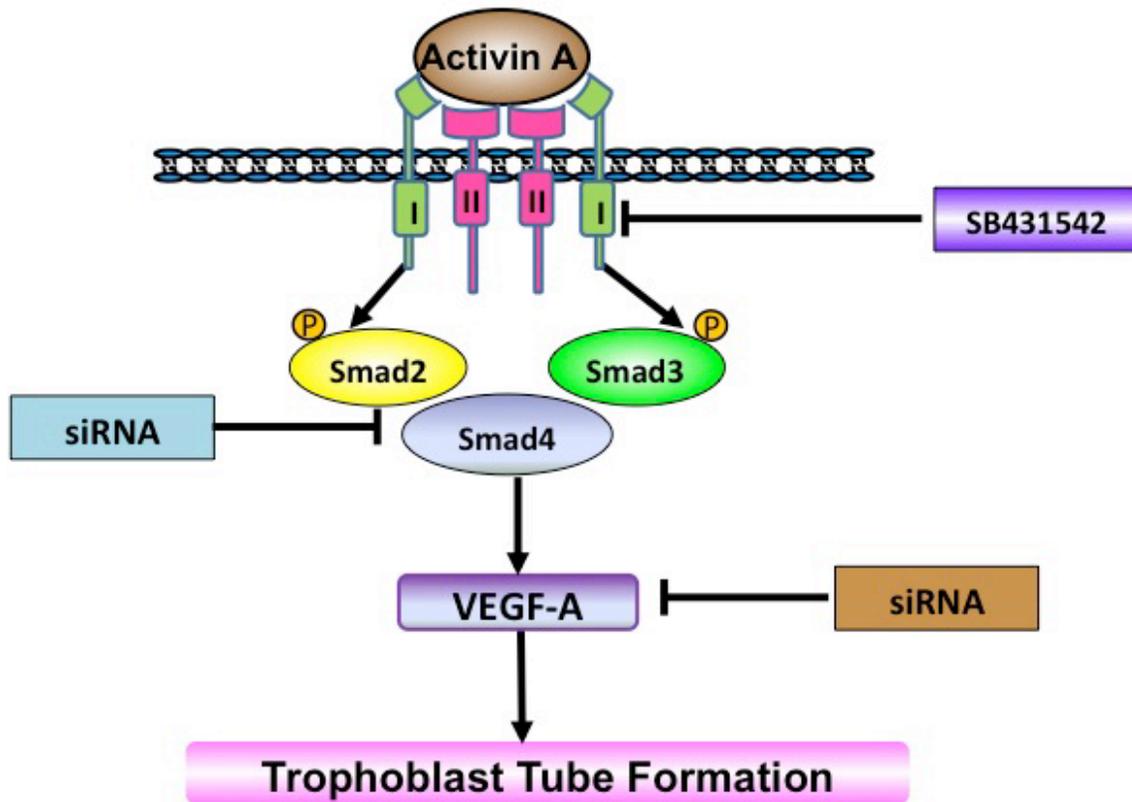


Figure 5.8 A schematic illustration of Chapter 5 data

Activin A increased the formation of endothelial-like tubes of HTR-8/SVneo cells cultured on Matrigel. In addition, treatment with activin A induced SMAD2/3 phosphorylation and up-regulated the mRNA and free-form protein levels of VEGF-A. Co-treatment with the TGF- β type I receptor inhibitor SB431542 abolished activin A-induced HTR8/SVneo cell endothelial-like tube formation, phosphorylation of SMAD2/3 and up-regulation of VEGF-A. Importantly, activin A-induced HTR8/SVneo cell endothelial-like tube formation was attenuated by siRNA-mediated down-regulation of VEGF-A. Knockdown of SMAD2, 3, 2+3 or 4 abolished the effect of activin A on VEGF-A up-regulation. si-RNA mediated knockdown of VEGF-A did not influence the viability of HTR8/SVneo cells.

Chapter 6: Conclusion

6.1 Conclusion

The objective of the current thesis was to investigate the potential roles of decidua- and placenta-derived activin in regulating human trophoblast invasion and endovascular differentiation as well as the underlying molecular mechanisms. Our results provide insight into the cellular and molecular events regulated by activin in trophoblast cells during human implantation.

In **Chapter 3**, the effects of all three activin isoforms on human trophoblast cell invasion and the underlying molecular mechanisms, in particular the roles of N-cadherin and SMAD2/3 signaling were studied. All three activin isoforms produced comparable increases in HTR8/SVneo cell invasion as well as N-cadherin expression. In addition, the up-regulatory effect of activin isoforms on N-cadherin was confirmed in primary cultures of human trophoblast cells. Interestingly, siRNA-mediated down-regulation of N-cadherin attenuated basal and activin-induced invasion of both HTR8/SVneo and primary trophoblast cells. All three activin isoforms induced equivalent phosphorylation of SMAD2 and SMAD3. Importantly, activin-stimulated cell invasion, up-regulation of N-cadherin as well as activation of SMAD2/SMAD3 were abolished by the TGF- β type I receptor inhibitor SB431542 in HTR8/SVneo cells. Furthermore, knockdown of SMAD2/3 or common SMAD4 abolished the stimulatory effects of all three activin isoforms on N-cadherin expression. These findings provide important insights into the molecular mechanisms underlying the pro-invasive effects of activins on human EVT, and enhance our understanding of the establishment of pregnancy under normal or pathological conditions.

As described in **Chapter 4**, Since I did not observe any different effects among three activin isoforms, in this part, I used activin A as a representative to identify the contribution of MMP2/9 and TIMP1/2 in activin A-regulated trophoblast invasion and the involvement of the type I receptors, transcriptional factors as well as signaling pathways. In both HTR8/SVneo and primary human EVT cells, the mRNA and protein levels of SNAIL and MMP2 were increased after treatment with activin A, and siRNA-mediated down-regulation of SNAIL attenuated activin A-induced MMP2 up-regulation. Importantly, siRNA-mediated depletion of MMP2 abolished activin A-induced HTR8/SVneo cell invasion. Up-regulation of SNAIL and MMP2 expression by activin A was abolished by the TGF- β type I receptor inhibitor SB431542 or siRNA-mediated depletion of ALK4. Furthermore, activin A induced the phosphorylation of SMAD2 and SMAD3, and knockdown of SMAD2/3 or common SMAD4 abolished the stimulatory effects of activin A on SNAIL and MMP2 expression. These findings benefit the understanding of molecular mechanisms under physiological and pathological trophoblast invasion circumstances and promote the development of diagnostic and therapeutic methods for those trophoblast-dysfunction related diseases.

As presented in **Chapter 5**, the effect of activin A on human trophoblast endothelial-like tube formation and the possible involvement of VEGF-A in this process were identified. Activin A induced HTR8/SVneo endothelial-like tube formation and VEGF-A expression. Importantly, basal and activin A-induced cell tube formation were attenuated by siRNA-mediated down-regulation of VEGF-A. Activin A activated the SMAD2/3 signaling pathway by phosphorylation of SMAD2 and SMAD3. Pre-treatment with the TGF- β type I receptor inhibitor SB431542 abolished activin A-induced trophoblast tube formation, up-regulation of VEGF-A and phosphorylation of SMAD2/3. Moreover, siRNA-mediated down-regulation of SMAD2,

SMAD3 or common SMAD4 abolished the stimulatory effect of activin A on VEGF-A expression. These results provide important insights into the cellular and molecular mechanisms underlying spiral artery remodeling during early pregnancy.

In **Appendix**, the cell phenotype and gene expression in HTR8/SVneo cells cultured on matrigel versus plastic were characterized. Matrigel induced HTR8/SVneo cells to form endothelial-like tubes, and decreased VE-cadherin as well as MMP2 mRNA levels whereas increased SLUG as well as MMP9 mRNA levels. Characterization of the cell phenotype and gene expression benefits the understanding of EVT endovascular differentiation and provides insights into further investigation on underlying molecular mechanisms.

In summary, these results describe two essential EVT differentiation routes regulated by activin during human implantation and the underlying novel molecular mechanisms. Decidua- and placenta-derived activin plays vital roles in promoting the acquisition of invasive properties of trophoblast cells by up-regulating N-cadherin and SNAIL-mediated MMP2, and inducing the acquisition of the endothelial phenotype of trophoblasts by increasing VEGF-A expression. Collectively, the illustration of these mechanisms suggests the dual roles of activin in regulating trophoblast differentiation during early pregnancy in an autocrine or paracrine manner (Figure 6.1).

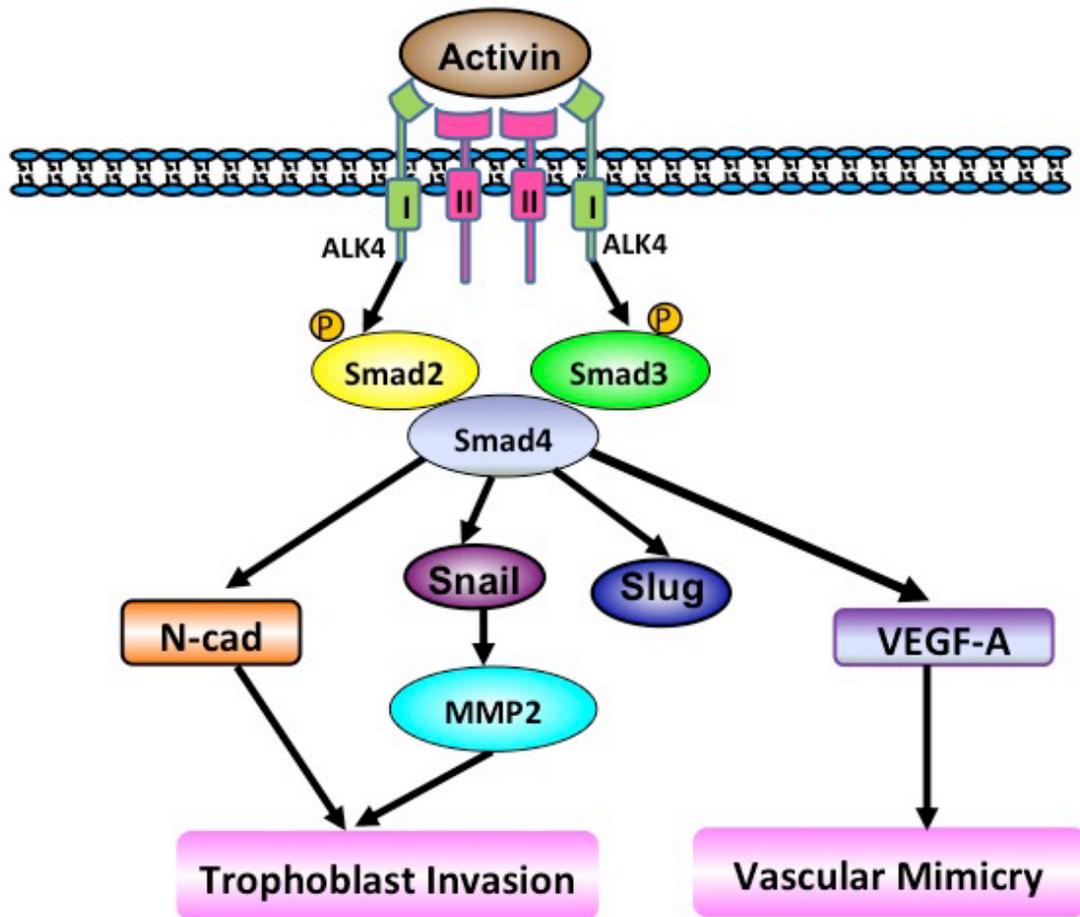


Figure 6.1 A schematic diagram of the proposed mechanisms of activin regulating trophoblast invasion and vascular remodeling

During implantation, activin A induces in trophoblast cells the acquisition of invasive properties and the endothelial phenotype through ALK4. Activin A, B, and AB do not show any difference in regulating cell invasion and N-cadherin expression. The activated SMAD2/3-SMAD4 signaling pathway mediates the increase of N-cadherin by transcriptional machineries. SMAD2/3-SMAD4-mediated up-regulation of the MMP2 transcriptional activator SNAIL

contributes to the up-regulation of MMP2 and, subsequently, to cell invasion. Besides promoting in trophoblast cells the acquisition of invasive properties, activin A induces in trophoblast cells the acquisition of the endothelial phenotype, which is essential in trophoblast endovascular differentiation. Activin A promotes trophoblast cells endothelial-like tube formation by up-regulating a key angiogenic factor VEGF-A, and the activated SMAD2/3-SMAD4 signaling pathway mediates the increase of VEGF-A by transcriptional machineries. Thus, activin A plays an essential role in regulating human trophoblast invasion and vascular mimicry during early pregnancy.

6.2 Overall discussion of this study

6.2.1 How to interpret the opposing effects of activins and TGF- β s in regulating trophoblast invasion?

The expression of TGF- β s (TGF- β 1, β 2 and β 3) and activins (activin A, B and AB) in the endometrium and placenta has been reported (87). Although both TGF- β s and activins belong to the TGF- β superfamily due to their structure similarities and shared SMAD2/3-SMAD4 downstream signaling pathway (89, 211), they display divergent functions in regulating trophoblast invasion during implantation.

Changes in cadherin expression profiles are essential for trophoblast invasive behavior. Our Chapter 3 data demonstrate activins promote trophoblast invasion by up-regulating N-cadherin expression in a SMAD2/3-SMAD4 dependent manner, whereas inhibition of trophoblast invasion by TGF- β 1 was shown to involve the suppression VE-cadherin expression by SMAD2/3-induced SNAIL production. TGF- β 1-induced SNAIL production was primarily mediated by SMAD2, whereas up-regulation of SLUG was largely SMAD3-dependent (67). Interestingly, our Chapter 3 data demonstrate treatment of trophoblasts with activin isoforms also up-regulates SNAIL and SLUG in an SB431542- and SMAD4-sensitive manner, however siRNA-mediated depletion of SNAIL or SLUG does not affect activin-induced N-cadherin expression (Figure 3.7). Activin isoforms also exert suppressive effects on VE-cadherin (Figure 3.8) that, like TGF- β 1, are likely to be mediated by SNAIL, though this has not been confirmed. In contrast, the up-regulation of N-cadherin mRNA as early as 3 hours after treatment with activin suggests direct binding of SMAD2/3-SMAD4 to the N-cadherin promoter. Indeed, chromatin immunoprecipitation and promoter luciferase analyses have demonstrated that

SMAD4 cooperates with Notch to regulate N-cadherin promoter activity in endothelial cells (182). Overall, the divergent functions of TGF- β s and activins on trophoblast invasion cannot be fully explained by cadherin dynamics and suggests important roles for additional molecular mechanisms.

The regulated expression and activation of MMPs in trophoblasts also plays important role in regulating trophoblast invasion. TGF- β 1, β 2 and β 3 have been shown to suppress the invasiveness of EVT cells derived from placental explants by down-regulating the production and activity of matrix metalloproteinase 9 (MMP9) (151), whereas activin A enhanced their invasive capacity by up-regulating the production and activity of MMP2 and our Chapter 4 data demonstrate that SNAIL mediates activin A-induced MMP2 up-regulation (107). It has been reported that TGF- β 1 exerts its anti-invasive effect through ALK5 (67), whereas both SB431542 and ALK4 knockdown data in Chapter 4 indicated the essential role of ALK4 in mediating activin A-induced trophoblast invasion, MMP2 and SNAIL up-regulation, as well as SMAD2/3 phosphorylation. Thus, although TGF- β 1 and activin A belong to the same superfamily, sharing same SMAD2/3-dependent signaling pathway and SMAD2/3-induced SNAIL up-regulation, their effects on human trophoblast invasion are totally opposite, the target genes they regulate and the type I receptors they use are distinct. Our present studies provide important insights into the molecular mechanisms underlying the divergent functions of TGF- β s and activins on trophoblast invasion.

6.2.2 What is the potential pathological importance of activin signaling in pre-eclampsia?

In preeclampsia, a common complication of pregnancy characterized by shallow trophoblast

invasion (36), up-regulation of activin A levels in serum and placenta (66) as well as placental ActRIIA levels (177) suggests important functions for activin during placentation. However, activin A has been shown to promote the invasion of cytotrophoblasts at 6-8 and 8-10 weeks, but not 10-12 weeks gestation (108), indicating a window for the pro-invasive effects of activin early in gestation. While these findings may, at first, appear contradictory, it should be noted that the earliest comparison measurements of serum activin A levels were at 15-18 weeks gestation and no significant differences were observed until 25-30 weeks, when patients may already present with preeclampsia (66). Thus, it is uncertain to what degree the elevation of activin A or ActRIIA later in pregnancy contributes to the development of preeclampsia, as opposed to reflecting disease manifestation or a homeostatic response. Evidence to date suggests the effects of activin on trophoblasts may vary depending on gestational age. Pro-invasive effects exerted early in pregnancy are likely to be important for spiral artery remodeling which starts from 10 weeks gestation in humans (7), whereas pro-apoptotic effects could manifest at later gestational stages and in association with preeclampsia.

6.2.3 Is there any combined effect of activin on N-cadherin and MMPs expression?

Structurally, N-cadherin regulates cell polarization by connecting to the actin cytoskeleton via α -catenin and β -catenin. Functionally, it has been reported that N-cadherin regulates cell-cell adhesion by activating the non-receptor tyrosine kinase Fer and RhoGTPase Rac1 (212). Besides, interaction between N-cadherin and FGFR (fibroblast growth factor receptor) leads to the activation of FGF downstream signaling and subsequently increased cell invasiveness (213, 214). N-cadherin also functions with PDGFR (platelet-derived growth factor receptor) to sustain MAPK signaling pathway activation and increase cell motility and MMPs secretion (215). In my

study, I observed that activin treatment increased trophoblast invasion by up-regulating N-cadherin and MMP2 expression. However, I did not check the expression level of MMP2 after siRNA-mediated knockdown of N-cadherin or the expression level of N-cadherin after MMP2 knockdown. Further studies are needed to check the possibility of mutual influences between N-cadherin and MMP2 up-regulation after activin treatment.

6.2.4 What are the molecular mechanisms shared by activin and GnRH in regulating trophoblast invasion and vascular mimicry?

GnRH is a decapeptide that mainly secreted by hypothalamic neurons. Both activin and GnRH are expressed in first trimester invasive EVT cells and have been shown to promote trophoblast invasion (104-106, 108, 216, 217). My study demonstrates that decidua- and placenta-derived activin plays vital roles in promoting the acquisition of invasive properties of trophoblast cells by up-regulating N-cadherin and SNAIL-mediated MMP2, and inducing the acquisition of the endothelial phenotype of trophoblasts by increasing VEGF-A expression in a SMAD2/3-SMAD4 dependent manner. Dr. Bo Peng from our lab has studied the molecular mechanisms underlying the role of GnRH in regulating trophoblast invasion and vascular mimicry. His findings reveal GnRH promotes trophoblast cell invasion by up-regulating the expression of TWIST-mediated N-cadherin (134), c-FOS- and c-JUN-mediated cadherin-11 (218) as well as RUNX2-mediated MMP2/9, and inducing the acquisition of the endothelial phenotype of trophoblasts by increasing N-cadherin, cadherin-11 as well as MMP2 and MMP9 expression (Unpublished data). Collectively, although activin type I and type II receptors are serine-threonine receptors whereas GnRHRs are classical G-protein coupled receptor (GPCR), common molecular mechanisms are involved in activin and GnRH-regulated trophoblast invasion and vascular remodeling.

Comparing and contrasting the molecular mechanisms involved in activin and GnRH-regulated trophoblast invasion and vascular mimicry sheds light on the regulating of implantation and placentation during early pregnancy and provides candidate targets for other growth factors in regulating trophoblast behaviors.

6.2.5 Does the increase of HTR8/SVneo cell invasiveness represent the differentiation of villous cytotrophoblast to invasive EVT?

The epithelial-mesenchymal transition (EMT) describes the conversion of a cell from an immotile epithelial phenotype to a motile mesenchymal phenotype and has been implicated in development and tumor progression (124). Loss of E-cadherin accompanied without or with the gain of N-cadherin is the hallmark of EMT (219). During implantation, the villous cytotrophoblasts bind together tightly at the first, then gradually lose their tight assembly, become loosely attached and invasive. The process of villous cytotrophoblasts differentiate into EVT cells resembles EMT during cancer progression. Similarly, the loss of E-cadherin and gain of N-cadherin is a remarkable change of cytotrophoblast invasive differentiation. In poorly invasive BeWo, JAR and JEG-3 chorionic carcinoma cells, E-cadherin is positive whereas N-cadherin is negative. In highly invasive HTR8/SVneo cells, N-cadherin is positive whereas E-cadherin is absent (Figure 3.1). Although my study demonstrates treatment of activins significantly increased the invasiveness of HTR8/SVneo cells, HTR8/SVneo cells exhibit high invasiveness without any growth factor treatment. Thus, I am aware that the change from relatively low invasive to relatively high invasive phenotype in HTR8/SVneo cells may not fully represent the full progression of EVT invasive differentiation. No effective method has been found to maintain the villous trophoblast in an “intermediate” differentiated status. Establishment of trophoblastic

cell population that expresses both endogenous E-cadherin and N-cadherin would provide essential study model for investigating the differentiation of villous cytotrophoblasts to invasive EVTs.

6.3 Limitations of this study

Due to the ethical limitation of *in vivo* human placentation model and inaccessibility of non-human primates models, we take advantage of the available HTR8/SVneo immortalized human EVT cell line, which has been proven as an important tool for delineating placental function and molecular mechanisms, to check our overall hypothesis. The HTR8/SVneo cell line was established by transfecting simian virus 40 large T antigen into first trimester human trophoblast. HTR8/SVneo cells maintain the morphology and the majority of gene expression profiles of parental cells while acquiring extended lifespan, thus providing us the stable and feasible model for the entire study. We have to admit that cell properties might change as the extended culture *in vitro* and the existence of differences between immortalized cells and primary cells. Fortunately, although limited in cell numbers, primary human EVT cells are available for my study as approved by the Children's and Women's Hospital Research Ethics Board of the University of British Columbia. The confirmatory studies performed in primary human EVT cells further verified the results we obtained in HTR8/SVneo cells, the approach of using an immortalized EVT cell line and cultured first trimester EVT cells together allows me to investigate the detailed molecular mechanisms and provides more confirmative results, which could better reflect the *in vivo* situation.

I am aware that all the experiments presented in this thesis were performed in an *in vitro* system, which can not exactly represent the *in vivo* condition. Especially in my study,

trophoblast cells were cultured in conditioned medium alone, whereas *in vivo* situation, these cells interact with decidual cells and maternal immune cells, particularly decidual natural killer cells. The culture of a specific type of cells allows us to examine the effect of a specific growth factor on this unique cell type by excluding interference; however, the complexity of *in vivo* situation and possible mutual effects of these two cell types can not be reflected by our study approach. Human first trimester placental villi and decidual explants co-culture system that mimic the *in vivo* environment will provide more important insights into implantation biology (220).

6.4 Future directions

These results increase our understanding of the cellular and molecular mechanisms of the highly expressed activin at the maternal-fetal interface in regulating human trophoblast invasion and vascular remodeling. However, more detailed and comprehensive studies using appropriate study models regarding the functional roles of activin during implantation are needed to advance our knowledge about human pregnancy. Future directions should include the following aspects:

- 1) Trophoblast cells interact with decidualized endometrial stromal cells *in vivo*, studies on first trimester placental villi and decidual explants co-culture system *in vitro* could provide more information about *in vivo* situation.
- 2) The main source of activin A at the maternal-fetal interface is maternal decidualized endometrial stromal cells compared to trophoblast cells. Although activin A has been reported to promote the decidualization process during implantation, the underlying molecular mechanisms remain to be investigated.

- 3) The molecular mechanisms underlying trophoblast cell invasion involve the regulated expression of cell-cell adhesion molecules by cadherins, the specific degradation/activation of extracellular matrix (ECM) components by MMPs, but also the regulated expression of cell-adhesion molecules by integrins. Thus, the possible roles of integrins, such as integrin $\alpha v \beta 3$, in activin-induced trophoblast invasion and vascular differentiation are unknown.
- 4) Our results reported the effect of activin A on VEGF-A expression during trophoblast endovascular differentiation, whether placental growth factor (PlGF), s-flt and other factors involved in placental angiogenesis could be regulated by activin A is still unknown.
- 5) A subpopulation of cytotrophoblast cells fuse together to form syncytiotrophoblast cells, which are essential for hormone production and pregnancy maintenance. In our study, we focused on studying the differentiation of EVT cells, the assessment of the effects of activin A on BeWo cells fusion and syncytin expression needs to be further investigated.
- 6) Decidual cells and maternal immune cells secrete a variety of growth factors to regulate the interaction between trophoblast and decidual cells, to study the effects of TGF- β s, BMPs, VEGF-A, EGF etc. located at the maternal-fetal interface on trophoblast differentiation could provide us a more comprehensive picture about the coordinated regulation of trophoblast differentiation by divergent growth factors.

6.5 Significance and translational potential

The present study provides the first comprehensive research of the functional roles and mechanisms of decidua and placenta derived activin in the regulation of human trophoblast differentiation. Moreover, this study increases our understanding of the roles of N-cadherin, MMP2 in activin-induced trophoblast invasion, and emphasizes the impact of VEGF-A on

activin-induced trophoblast endovascular differentiation. These results provide important insights into our understanding of the molecular mechanisms underlying activin-regulated human implantation, and promote the development of diagnostic and therapeutic methods for those trophoblast-dysfunction related diseases. In summary, activin functions as an implantation promoter during implantation in early pregnancy.

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Appendix

Appendix A Characterization of cell phenotype and gene expression in HTR8/SVneo cells cultured on matrigel

A.1 Introduction

Due to the intrinsic capacity for endothelial-like tube formation while cultured on relative high concentration matrigel (5-10 mg/ml), the immortalized first trimester HTR8/SVneo cells have been used to investigate the underlying molecular mechanisms of EVT endovascular differentiation (68-70); however, while cultured on plastic for migration assay or relative low concentration matrigel (1 mg/ml) for matrigel invasion assay, HTR8/SVneo cells exhibit invasive ability without forming endothelial-like tubes (67, 186). During implantation, a subpopulation of EVT cells follows endovascular differentiation pathway by acquiring endothelial phenotype and subsequently replacing endothelial cells lining in maternal spiral arteries, whereas the other subpopulation of EVT cells follows interstitial invasive differentiation pathway by invading into maternal interstitial decidua and anchoring placenta to uterus (18, 34). Thus, the dual phenotypes of HTR8/SVneo cells, which stand for cytotrophoblasts involved in endothelial-like tube formation and invasive cytotrophoblasts respectively, are induced by different concentrations of matrigel. To date, the cellular and molecular mechanisms underlying these processes are not well understood.

This study aims to quantify the effect of matrigel (5 mg/ml) used in previous endothelial-like tube-formation assay (Chapter 5) on expression of genes commonly referred to as markers of EVT invasive potential including N-cadherin, VE-cadherin, MMP-2, MMP9, ITGB1, ITGAV,

ITGB3, SNAIL and SLUG, as well as endothelial phenotype including VE-cadherin and VEGF-A to shed lights on cellular mechanisms regulating EVT invasive and endovascular differentiation.

A.2 Materials and Methods

Culture of HTR8/SVneo immortalized human EVT cells

The HTR8/SVneo simian virus 40 large T antigen-immortalized human EVT cell line was generously provided by Dr. P.K. Lala at the Western University (63). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 100 U/ml penicillin (Life Technologies), 100 µg/ml streptomycin (Life Technologies), and 10% (vol/vol) fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). Cultures were incubated at 37°C in a humidified 5% CO₂/air atmosphere and the medium was changed every other day.

Culture of HTR8/SVneo cells on plastic or diluted matrigel

600 µl of Growth factor-reduced matrigel (BD Biosciences) at a concentration of 10 mg/ml diluted 1:1 (vol/vol) with 0.1% (vol/vol) FBS DMEM medium was added to 6-well dish and incubated for 2 h at 37°C to solidify. A total of 1.2×10^6 HTR8/SVneo cells in 1.2 mL 0.1% (vol/vol) FBS DMEM were then seeded in the GFR matrigel-coated or regular 6-well dish and incubated at 37°C and 5% CO₂/air for 24 h. Digital images (40× magnification) were taken using Nikon Eclipse TE300 microscopy with Northern Eclipse 6.0 software (Empix Imaging, Mississauga, Ontario, Canada).

Reverse transcription quantitative real-time PCR (RT-qPCR)

After 24 h culture, cells cultured on diluted matrigel have been mixed with polymerized matrigel. To collect these cells, upper medium were sucked out, and matrigel-coated plate was wash with cold PBS twice. Then 1mL cold PBS was added and the plate was put on ice for 10 min. After the depolymerization of matrigel, the total liquid was sucked from plate followed by spinning down cells. Trizol was then added into the tube with cell pallet and total RNA was extracted with TRIZol Reagent (Life Technologies) according to the manufacturer's instructions. Cells cultured on plastic were washed with cold PBS and total RNA was extracted with the same method. Reverse transcription was performed using 2 µg of total RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI) in a final volume of 20 µl. RT-qPCR was performed on the Applied Biosystems 7300 real-time PCR system equipped with 96-well optical reaction plates. Each 20-µL RT-qPCR reaction contained 1×SYBR Green PCR Master Mix (Applied Biosystems), 12 ng cDNA and 150 nM of each specific primer. The primers used were: N-cadherin, 5'-GGA CAG TTC CTG AGG GAT CA-3' (forward) and reverse 5'-GGA TTG CCT TCC ATG TCT GT-3'; VE-cadherin, 5'- CAG CCC AAA GTG TGT GAG AA-3' (forward) and 5'- CGG TCA AAC TGC CCA TAC TT-3' (reverse); MMP2, 5'-TAC ACC AAG AAC TTC CGT CTG T-3' (forward) and 5'-AAT GTC AGG AGA GGC CCC ATA-3' (reverse); MMP9, 5'-GGA CGA TGC CTG CAA CGT-3' (forward) and 5'-CAA ATA CAG CTG GTT CCC AAT CT-3' (reverse); ITGB1, 5'-TGG ACA ATG TCA CCT GGA AA -3' (forward) and 5'-AGC TCC TTG TAA ACA GGC TGA A-3' (reverse); ITGAV, 5'-GTT GCT ACT GGC TGT TTT GG-3' (forward) and 5'-CTG CTC CCT TTC TTG TTC TTC-3' (reverse); ITGB3, 5'-ACA CTG GCA AGG ATG CAG TGA ATT GTA C-3' (forward) and 5'-CGT GAT ATT GGT GAA GGT AGA CGT GGC-3' (reverse); VEGF-A, 5'-CGA GGG CCT GGA GTG

TGT-3' (forward) and 5'-CGC ATA ATC TGC ATG GTG ATG-3' (reverse); SNAIL, 5'-CCC CAA TCG GAA GCC TAA CT-3' (forward) and 5'-GCT GGA AGG TAA ACT CTG GAT TAG A-3' (reverse); SLUG, 5'-TTC GGA CCC ACA CAT TAC CT-3' (forward) and 5'-GCA GTG AGG GCA AGA AAA AG-3' (reverse); GAPDH, 5'-GAG TCA ACG GAT TTG GTC GT-3' (forward) and 5'-GAC AAG CTT CCC GTT CTC AG-3' (reverse). The specificity of each assay was confirmed by dissociation curve analysis and agarose gel electrophoresis of PCR products. Assay performance was validated by assessing amplification efficiencies by means of calibration curves, and ensuring that the plot of log input amount versus ΔCq has a slope with an absolute value of <0.1 . At least three separate experiments were performed and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative Cq method with GAPDH as the reference gene and using the formula $2^{-\Delta\Delta Cq}$.

Study protocol

Study groups comprised cells cultured on 1:1 diluted matrigel-coated culture dish, whereas the corresponding control groups were comprised of cells cultured on plastic culture dish. For each study, at least three independent experiments were performed, each on a separate passage.

Statistical analysis

Results are presented as the mean \pm SEM of at least three independent experiments. Paired data comparison was evaluated by t-test method. Means were considered significantly different if $P < 0.05$ and are indicated by asterisk (*).

A.3 Results

Diluted matrigel induced HTR8/SVneo cells to form endothelial-like tubes

HTR8/SVneo cells formed endothelial-like tubes on diluted matrigel. Cells rearranged to form branching points, which were linked by endothelial-like tubes. This phenomenon is similar to tubes formed by endothelial cells *in vitro* upon culture on matrigel, whereas the arrangement of cells cultured on plastic was not changed and tube formation was not observed.

Diluted matrigel decreased VE-cadherin and MMP2 mRNA levels whereas increased SLUG and MMP9 mRNA levels

Compared with cultures on plastic, VE-cadherin (CDH5) and MMP2 mRNA levels were significantly decreased whereas SLUG and MMP9 mRNA levels were statistically increased on diluted matrigel. The mRNA levels of N-cadherin (CDH2), ITGB1, ITGAV, ITGB3, VEGF-A, SNAIL and SLUG showed no difference in cells cultured for 24 h on plastic or diluted matrigel.

A.4 Discussion

Human immortalized EVT cell lines, particularly HTR8/SVneo and TCL1, have been shown to mimic endothelial cells by forming endothelial-like tubes *in vitro* upon culture on GFR-matrigel (68-70, 199, 221, 222). The formation of endothelial-like tubes by HTR8/SVneo cells cultured on matrigel in our study is consistent with previous studies.

GFR-matrigel is a biological active matrix composed of over 1200 proteins (223). Its relevance to EVT cells differentiation requires further investigation. Down-regulated MMP2 expression is a feature of endothelial cell angiogenesis (224), it also suggests decreased invasive capacity. Although MMP9 is also a parameter of cell invasiveness (143), the relatively low

expression of MMP9 compared to MMP2 in EVT cells suggest the effect of up-regulated MMP9 in increasing invasive capacity might be offset by decreased MMP2 (145). SLUG has been shown to promote cell invasiveness and vascular development (154, 225). The up-regulated SLUG suggests increased endothelial phenotype. Notably, vascular endothelial cadherin (VE-cadherin) is involved in angiogenesis process in endothelial cells (208). Up-regulated VE-cadherin expression has been reported as a molecular marker for increased trophoblast endothelial-like tube formation (70). However, in our study, the mRNA levels of VE-cadherin in HTR8/SVneo cells cultured on diluted (1:1) matrigel for 24 h showed 80% decrease compared to that cultured on plastic (Figure A.2). In addition, activin A treatment for 24 h significantly reduced VE-cadherin mRNA levels in HTR8/SVneo cells cultured on diluted (1:1) matrigel as cultured on plastic (Figure 3.9). While these findings may, at first, appear contradictory, our results suggest that decreased VE-cadherin does not necessarily inhibit trophoblast tube formation. Especially, it should be noted that the expression of VE-cadherin on endothelial cells inhibits VEGF-A-induced angiogenesis by blocking VEGFR-2 phosphorylation (209). Thus, VE-cadherin is not the determinative molecule of trophoblast tube formation. Further investigation regarding specific molecular markers of trophoblast tube formation is needed.

Due to the interference of matrigel in extracted cells collected from cultures on matrigel, the measurement for the protein levels of target genes is restricted. Characterization of the phenotype and underlying molecules differences between HTR8/SVneo cells cultured on plastic and matrigel provide insights into understanding EVT cells invasive and endovascular differentiation during early pregnancy.

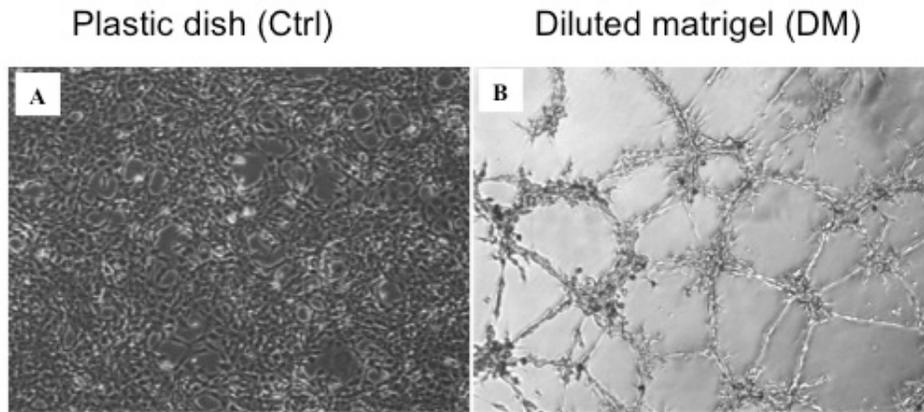


Figure A.1 Cell phenotypes of HTR8/SVneo cells cultured on plastic dish (Ctrl) or diluted matrigel (DM) after 24 h

A, A total of 1.2×10^6 HTR8/SVneo cells in 1.2 mL 0.1% (vol/vol) FBS DMEM were then seeded in a 6-well plastic dish (Ctrl) and incubated at 37°C and 5% CO₂/air for 24 h. **B**, 600 ul of growth factor-reduced Matrigel (BD Biosciences) at a concentration of 10 mg/ml diluted 1:1 (vol/vol) with 0.1% (vol/vol) FBS DMEM medium was added to 6-well plates and incubated for 2 h at 37°C to solidify. A total of 1.2×10^6 HTR8/SVneo cells in 1.2 mL 0.1% (vol/vol) FBS DMEM were then seeded in the GFR matrigel-coated 6-well dish (DM) and incubated at 37°C and 5% CO₂/air for 24 h. Compared with cultures on plastic, HTR8/SVneo cells formed endothelial-like tubes on diluted matrigel.

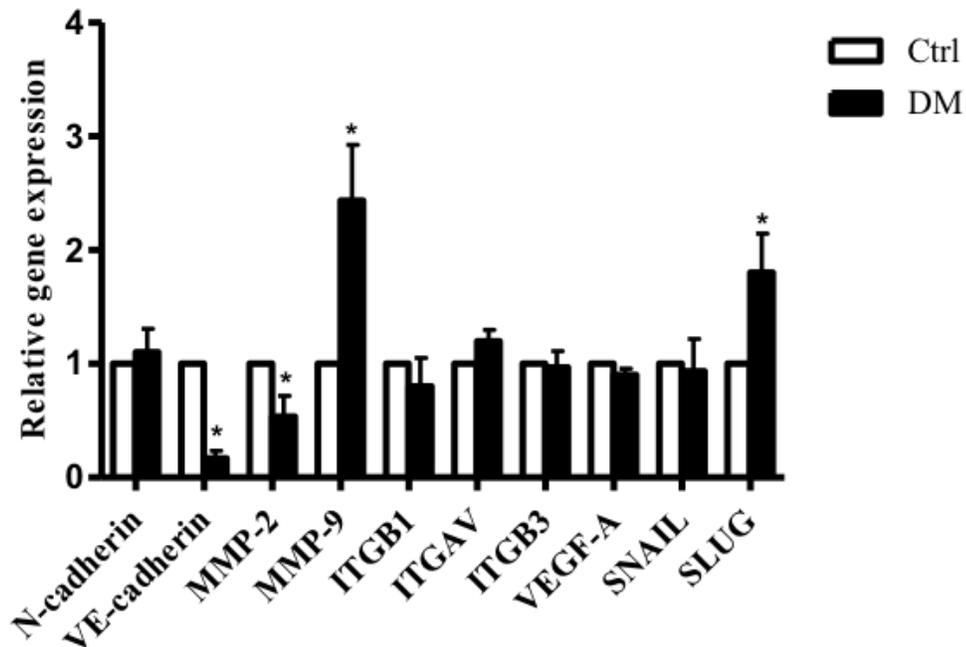


Figure A.2 Characterization of relative gene expression of HTR8/SVneo cells cultured on plastic dish (Ctrl) or diluted matrigel (DM)

HTR8/SVneo cells cultured on plastic dish or diluted matrigel were collected and assayed by RT-qPCR with corresponding SYBR primers. Relative gene expression levels are presented as the mean \pm SEM relative to GAPDH and normalized to cells grown on plastic, given a value of 1. At least three independent experiments were repeated. Asterisk (*) means statistically difference evaluated by t-test method.