6-28-2012 12:00 AM

The Role of Nodal in the Regulation of Bi-Potential Trophoblast Progenitor Cells

Alia Cloutier-Bosworth
The University of Western Ontario

Supervisor
Lynne-Marie Postovit
The University of Western Ontario Joint Supervisor
Peeyush Lala
The University of Western Ontario

Graduate Program in Anatomy and Cell Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

The Role of Nodal in the Regulation of Bi-Potential Trophoblast Progenitor Cells

(Spine title: Nodal Regulates Bi-Potential Trophoblast Progenitors)

(Thesis format: Monograph)

by

Alia Cloutier-Bosworth

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

THE UNIVERSITY OF WESTERN ONTARIO
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

CERTIFICATE OF EXAMINATION

Supervisors

Dr. Lynne-Marie Postovit

Dr. Peeyush Lala

Supervisory Committee

Dr. Andy Babwah

Examiners

Dr. Alison Allan

Dr. Peter Merrifield

Dr. Dean Betts

The thesis by

Alia Cloutier-Bosworth

entitled:

The Role of Nodal in the Regulation of Bi-Potential Trophoblast Progenitor Cells

is accepted in partial fulfillment of the requirements for the degree of Master of Science

Date_____

_____

Chair of the Thesis Examination Board

ABSTRACT

The human placenta develops from highly proliferative and phenotypically plastic cells called trophoblasts. Bi-potential trophoblast stem cells differentiate into the villous pathway to form the syncytiotrophoblast layer and the extravillous trophoblast (EVT). The HTR-8/SVneo cell line is widely used to study trophoblast biology. These cells variably express villous-specific or EVT-specific genes depending on conditions. Such phenotypic plasticity is indicative of a bi-potential cytotrophoblast progenitor. Preliminary work has shown that similar to progenitors *in situ*, a subpopulation of HTR-8/SVneo cells expresses $\alpha6\beta4$ integrin. This $\alpha6\beta4^{high}$ subset exhibits enhanced clonogenicity and differentiation capacity. This cell line also expresses Nodal, a stem cell-associated factor that sustains the pluripotency of embryonic stem cells and is re-expressed in certain cancers. I hypothesized that the $\alpha6\beta4^{high}$ subset within HTR-8/SVneo is enriched with bi-potential cytotrophoblast progenitor-like cells that are maintained by Nodal signaling. Our results revealed that the $\alpha6\beta4^{high}$ subset expresses greater amounts of Nodal protein relative to the $\alpha6\beta4^{low}$ subset. To investigate the role of Nodal in regulating trophoblast progenitors, stable Nodal knock-down and over-expressing cells were analyzed. It was found that Nodal is required for clonogenicity, maintenance of enhanced $\alpha6\beta4$ expression and endovascular differentiation along the EVT pathway. These results suggest that the $\alpha6\beta4^{high}$ population may represent a villous cytotrophoblast progenitor cell in which Nodal regulates clonogenicity and capacity for differentiation along the EVT pathway.

Key Words: Placenta, Trophoblast, HTR-8/SVneo, BeWo, Nodal, Trophoblast differentiation, Endothelial Tube-Formation, hCG.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr. Lynne-Marie Postovit and Dr. Peeyush K. Lala, for accepting me into their lab family and helping me develop into a successful young scientist.

I would also like to thank all the members of the Postovit and Lala laboratories, for their friendship and guidance during my past two years here: Padmalaya Das, Daniela Quail, Guihua Zhang, Michael Jewer, Scott Findlay, Krista Vincent, Leanna Dunn, Girish Gannareddy, Mousumi Majumder, Mauricio Rodriguez Torres, and Xiping Xin.

I also thank my supervisory committee members: Dr. Alison Allan and Dr. Andy Babwah for their support and input.

Lastly, I'd like to thank my friends and family, especially Alex Coe for cheering me up when the going got tough, and my Mom, Dad, and sisters who are constant sources of joy and inspiration. Thank you for your support and encouragement. I could not have done this without you.

TABLE OF CONTENTS

CERTIFICATE OF EXAMINATION ii
ABSTRACT iii
ACKNOWLEDGEMENTS iv
TABLE OF CONTENTS v
LIST OF FIGURES vi
LIST OF TABLES viii
LIST OF ABBREVIATIONS ix
Chapter One: Introduction 1
1.1 The Placenta 2
1.1.1 Blastocyst Implantation 2
1.1.2 Development of Villi 4
1.1.3 Trophoblast Invasion 4
1.1.4 Spiral Arterial Remodeling 6
1.1.5 Regulation of Trophoblast Differentiation 7
1.1.5.1 Cytotrophoblast – Proliferation 7
1.1.5.2 Syncytiotrophoblast – Fusion 10
1.1.5.3 EVT – Migration & Invasion 11
1.1.6 Models of Placental Development 13
1.2 Nodal 15
1.2.1 The Nodal Signaling Pathway 16
1.2.2 Regulation of Nodal Signaling 18
1.2.3 Nodal and Development 20
1.2.4 Nodal and Cancer 22
1.2.5 Nodal and Placentation 26
1.3 Rationale 30
1.4 Hypothesis 32
Chapter Two: Materials & Methods 33
Chapter Three: Results 41
Co-Authorship Statement 42
Chapter Four: Discussion 64
4.1 HTR-8/SVneo & BeWo: Models for Trophoblast Self-Renewal & Differentiation 66
4.2 Nodal Signaling & Cytotrophoblast Progenitors 68
4.3 Nodal & Trophoblast Self-Renewal/Proliferation 71
4.4 Nodal & Trophoblast Differentiation 74
4.5 Experimental Limitations 77
4.6 Future Directions 78
References: 79
Appendix: Supplemental Figures 94
Curriculum Vitae 101

LIST OF FIGURES

Figure 1. Human Blastocyst Implantation 3

Figure 2. The Chorionic Villi 5

Figure 3. Regulation of Trophoblast Differentiation 8

Figure 4. The Nodal Signaling Pathway 17

Figure 5. Integrin $\alpha6\beta4^{high}$ cells express higher levels of Nodal protein compared to $\alpha6\beta4^{low}$ cells. 44

Figure 6. Integrin $\alpha6\beta4^{high}$ cells do not exhibit altered growth rate or proliferation relative to $\alpha6\beta4^{low}$ and unsorted controls. 45

Figure 7. Stable Nodal knock-down in HTR-8/SVneo trophoblast cells. 47

Figure 8. Nodal knock-down decreases expression of integrin $\alpha6\beta4$. 48

Figure 9. Stable Nodal over-expression in BeWo choriocarcinoma cells. 49

Figure 10. Nodal over-expression increases expression of integrin $\alpha6\beta4$. 50

Figure 11. Nodal knock-down reduces HTR-8/SVneo clonogenicity. 52

Figure 12. Nodal over-expression increases BeWo clonogenicity. 53

Figure 13. Nodal knock-down decreases HTR-8/SVneo growth rate but increases proliferation. 54

Figure 14. Nodal over-expression does not affect BeWo growth or proliferation. 55

Figure 15. Nodal knock-down decreases the ratio of metabolically active to apoptotic cells in HTR-8/SVneo. 57

Figure 16. Nodal over-expression decreases the ratio of metabolically active cells to apoptotic cells in BeWo. 58

Figure 17. Nodal knock-down increases hCG mRNA but not secreted protein in HTR-8/SVneo cells. 59

Figure 18. Nodal over-expression does not affect hCG expression in BeWo cells. 60

Figure 19. Nodal is required for HTR-8/SVneo tube-formation and VE-Cadherin expression. 62

Figure 20. Nodal over-expression increases BeWo tube-formation. 63

Figure A1. Analysis of $\alpha6\beta4$ expression in HTR-8/SVneo cells. 95

Figure A2. Isolation of $\alpha6\beta4^{high}$ and $\alpha6\beta4^{low}$ subsets from HTR-8/SVneo. 96

Figure A3. Characteriziation of $\alpha6\beta4^{high}$ and $\alpha6\beta4^{low}$ subsets from HTR-8/SVneo. 97
Figure A4. Loss of HLA-G expression in HTR-8/SVneo. 98

LIST OF TABLES

Table A1. Top 20 increased miRNA and corresponding decreased target genes in $\alpha6\beta4^{high}$ relative to $\alpha6\beta4^{low}$ subset..... 100

LIST OF ABBREVIATIONS

ActRIIB	Activin Receptor IIB
ALK	Activin Like Kinase
CSF-1	Colony Stimulating Factor-1
CT	Cytotrophoblast
BMP	Bone Morphogenic Protein
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	EGF Receptor
EGF-CFC	Epidermal Growth Factor-Cripto-1/FRL-1/Cryptic
EVT	Extravillous Trophoblast
FGF4	Fibroblast Growth Factor-4
hCG	Human Chorionic Gonadotropin
hESC	Human Embryonic Stem Cell
HLA	Human Leukocyte Antigen
HTR-8/SVneo	Human Trophoblast-8 SV/neo
IGF	Insulin-like Growth Factor
IGFBP-1	IGF Binding Protein-1
IGFR	IGF Receptor
MMP	Matrix Metalloproteinase
PAI	Plasminogen Activator Inhibitor
PGE2	Prostaglandin E2
PI3K	Phosphoinositide-3 Kinase

PlGF	Placenta Growth Factor
shRNA	Short Hairpin Ribonucleic Acid
SMAD	Mothers Against DPP Homolog
SPC	Subtilisin-like Proprotein Convertase
ST	Syncytiotrophoblast
TGF-β	Transforming Growth Factor-beta
TIMP	Tissue Inhibitors of Metalloproteinase
TSC	Trophoblast Stem Cell
uPA	Urokinase Plasminogen Activator
VEGF	Vascular Endothelial Growth Factor
VEGFR	VEGF Receptor

Chapter One: Introduction

1.1 The Placenta

The placenta is an organ required by Eutherian mammals for the successful development of the fetus during pregnancy (reviewed by (Herr et al., 2010)). It supports the development of a small number of fetuses within the protective maternal organism through the establishment of a close fetal-maternal exchange network. The placenta also accomplishes a variety of metabolic, hormonal and immunological functions necessary for successful pregnancy. The three main types of placentae, which are categorized according to their degree of uterus invasion, are the noninvasive "epitheliochorial" placenta, the moderately invasive "endotheliochorial" placenta, and the highly invasive "haemochorial" placenta (Chakraborty et al., 2002). The human placenta is of the third category and behaves as a highly invasive tumour-like organ, which invades the maternal uterine endometrium and vasculature, establishing vital fetal-maternal exchange (Lala & Hamilton, 1996).

1.1.1 Blastocyst Implantation

The human placenta develops from highly proliferative and phenotypically plastic cells called trophoblasts, which are derived from the trophectoderm of the pre-implantation blastocyst (Lala & Hamilton, 1996). These trophoblast cells are mononucleated and form a single layer that surrounds the inner cell mass and blastoceole of the blastocyst (Huppertz, 2008). The trophoblasts adjacent to the inner cell mass, known as polar trophoblasts, induce implantation by attaching to the uterine epithelium and penetrating the basement membrane and underlying connective tissue (Lala & Graham, 1990) (**Figure 1**).

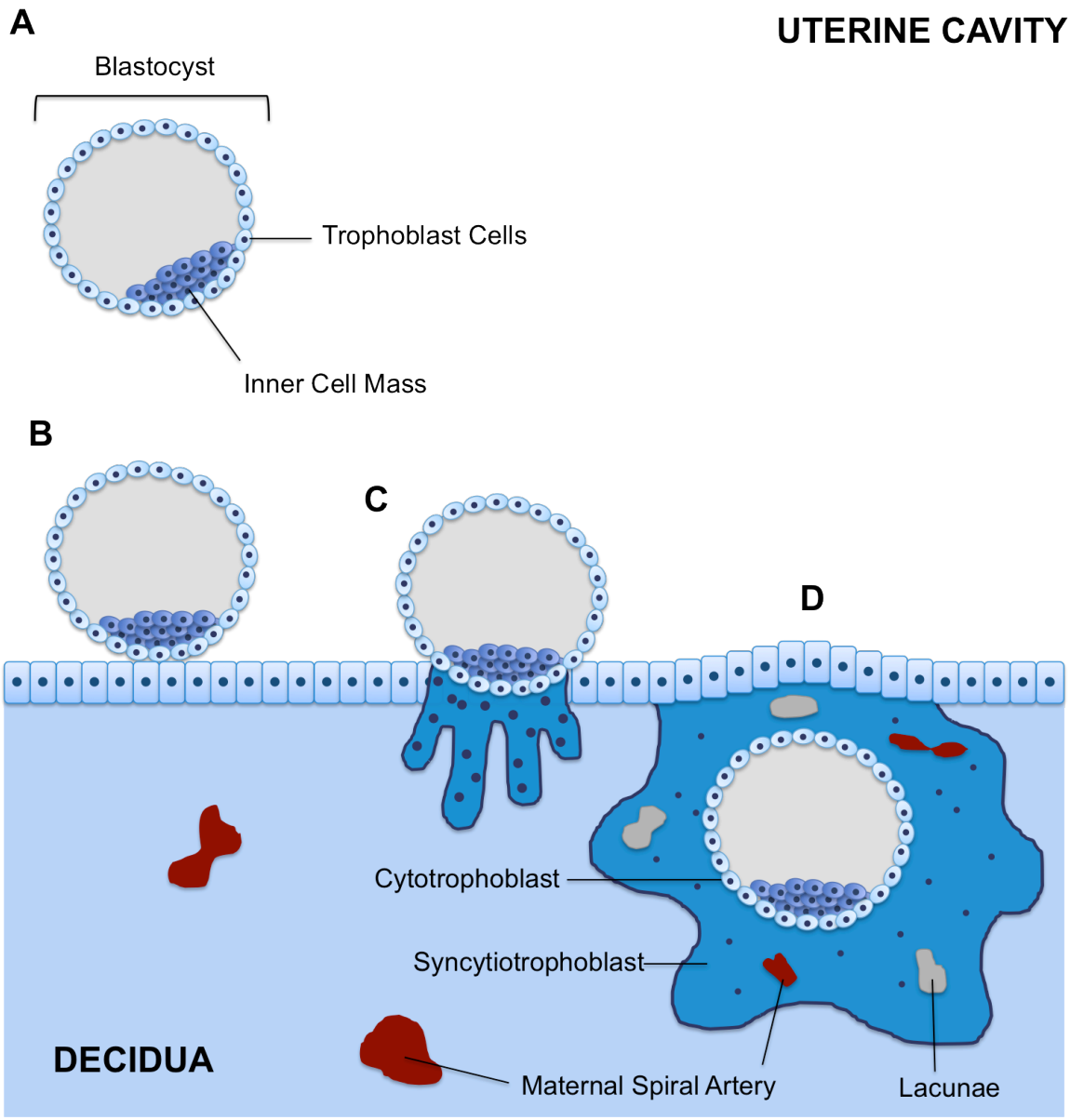

Figure 1. Human Blastocyst Implantation

Blastocyst implantation involves a number of steps: Transport – the blastocyst enters the uterine cavity (A). Adhesion – The blastocyst adheres to the endometrial epithelium (B). Invasion & Syncytialization – The polar trophoblasts penetrate the uterine epithelium and basement membrane. Ten days post-fertilization, the invading trophoblasts fuse to form the multinucleated syncytiotrophoblast, which invades the endometrium (C). Villous formation – Fluid-filled spaces form within the syncytiotrophoblast and coalesce to form lacunae which eventually develop into the intervillous space. The chorionic villi develop from the syncytiotrophoblast between the large lacunae. The former trophectodermal cells become the cytotrophoblast stem cells which maintain syncytiotrophoblast turnover (D). Adapted from Moore and Persaud, 1998.

1.1.2 Development of Villi

Approximately ten days after fertilization the invading polar trophoblasts fuse to form multinucleated syncytiotrophoblasts (ST) (Anin et al., 2004; Jones & Fox, 1991). Fluid-filled spaces develop within the syncytiotrophoblast, forming lacunae. These lacunae coalesce to form the intervillous spaces, while the syncytiotrophoblast develop into the chorionic villi. Around weeks 10-12 of pregnancy, the intervillous spaces become filled with maternal blood from ruptured endometrial capillaries. The maternal blood bathes two types of chorionic villi: floating villi, which aid in the exchange of maternal and fetal circulations, and anchoring villi, which contain migratory and invasive cytotrophoblasts and anchor the placenta to the uterus (Bischof & Irminger-Finger, 2005) (**Figure 2**).

1.1.3 Trophoblast Invasion

Each villus is comprised of two layers of trophoblasts. The outer non-proliferative, multinucleated syncytiotrophoblast layer is in direct contact with maternal blood and mediates the exchange of gas, nutrients, and waste between the maternal and fetal circulations (Lunghi et al., 2007; Yamamoto-Tabata et al., 2004). The syncytiotrophoblast is also the site of hormone production, including progesterone and human chorionic gonadotropin (hCG), which maintains the hormonal activity of the corpus luteum during pregnancy. The inner layer of proliferative, mononucleated trophoblasts called, cytotrophoblasts, acts as a source of stem cells for the dynamic syncytiotrophoblast within the floating villi, and the invasive and migratory cytotrophoblast within the anchoring villi (Huppertz, 2008). Cytotrophoblast cells within the floating villi proliferate and daughter cells differentiate and fuse with the overlying syncytiotrophoblast layer to maintain the multinucleated mass.

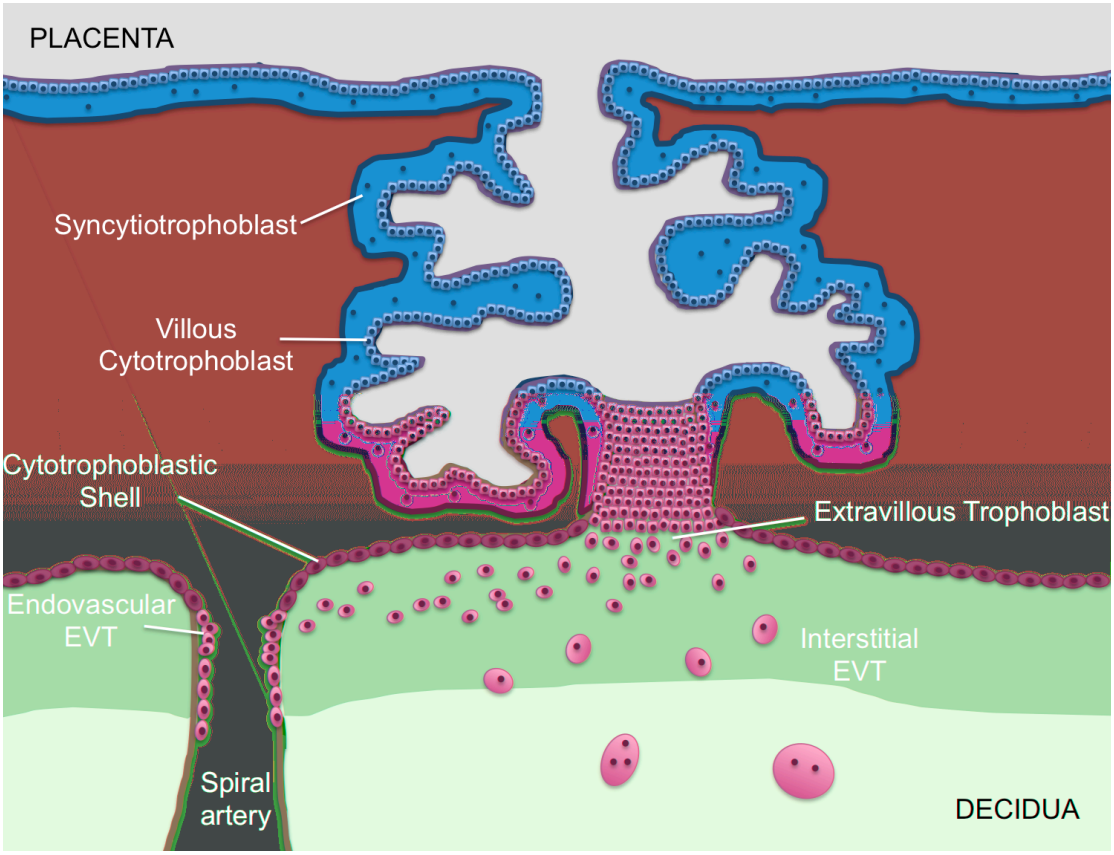

Figure 2. The Chorionic Villi

By weeks 10-12 of pregnancy, the intervillous space has developed and is filled with blood from maternal spiral arteries. The blood bathes the chorionic villi, both floating and anchoring. Each villi is lined by two layers of trophoblast, the outer non-proliferative, multinucleated syncytiotrophoblast (ST) and the inner layer of polarized, mononucleated, proliferative cytotrophoblasts (CT). A subpopulation of cytotrophoblast rapidly proliferate, forming a cell column at the tips of anchoring villi and invade the decidualized endometrium as extravillous trophoblast (EVT). The EVT cells differentiate into three populations: (1) Interstitial EVTs which invade as far as the inner third of the myometrium and eventually differentiate into giant multinuclear cells, (2) Endovascular EVTs which surround and remodel the maternal spiral arteries to allow for ample blood flow during the second and third trimesters of pregnancy, and (3) Cytotrophoblastic shell, a cell layer separating the decidua from the sinusoids. Adapted from Graham & Lala, 1992.

A subpopulation of cytotrophoblast in anchoring villi rapidly proliferate, form a cell column, and subsequently differentiate and migrate into the decidualized endometrium as extravillous trophoblast (EVT) cells. The proliferative capacity of cells within the column is gradually reduced as they migrate and invade the decidua. Three populations of EVT cells form: (a) interstitial EVTs which invade up to the inner third of the myometrium and fuse into giant multinuclear cells; (b) endovascular EVTs which target and invade maternal spiral arteries, replacing the endothelial cells lining the vessels; and (c) cytotrophoblastic shell, a cell layer that separates the decidua from the sinusoids (Aldo et al., 2007; Espinoza et al., 2006; Pijnenborg et al., 2006; Huppertz, 2008).

1.1.4 Spiral Arterial Remodeling

Extensive blood flow into the intervillous space is required to support the increasing needs of the growing fetus during the second and third trimesters of pregnancy (Charnock-Jones et al., 2004; Torry et al., 2007). This is accomplished by the remodeling of the maternal spiral arteries which leads to the disappearance of the smooth muscle layer from the vessel wall and the replacement of endothelial cells by endovascular EVTs. These trophoblast subsequently deposit a fibrinoid matrix (comprised of fibronectin, collagen type IV and laminin) along the vessel wall, substituting the normal elastic and collagenous extracellular matrix (Frank et al., 1994; Whitley & Cartwright, 2010), thereby transforming these high-resistance, low-flow muscular arteries into large flaccid vessels lacking vasomotor control (Burton et al., 2009). The resulting vessels experience a 10-fold increase in diameter, which increases the total blood volume delivered to the intervillous space by 3 to 4-fold while reducing pressure (Kliman, 2000; Thaler et al., 1990; Whitley & Cartwright, 2009).

1.1.5 Regulation of Trophoblast Differentiation

Distinct trophoblast cell types develop during placental development, namely the syncytiotrophoblast and EVT, which contribute to fetal-maternal exchange and invasion of the uterine endometrium and its vasculature, respectively (Lunghi et al., 2007). Each lineage is derived from villous cytotrophoblasts, the stem cell population in the placenta. Cytotrophoblasts are polarized, mononucleated and rapidly proliferating epithelial cells that line the basement membrane of the chorionic villi. Their proliferation and differentiation are strictly regulated by a variety of endogenous and exogenous molecules such as transcription factors, adhesion molecules, hormones, growth factors, cytokines and oxygen levels (**Figure 3**).

1.1.5.1 Cytotrophoblast – Proliferation

The cytotrophoblast stem cells maintain syncytiotrophoblast and EVT turnover, however, several transcription factors function to inhibit differentiation in favor of cytotrophoblast proliferation. The factors Hash-2 and Id-2 maintain cytotrophoblast proliferation, and are downregulated upon differentiation (Janatpour et al., 1999; Janatpour et al. 2000). A study of cytotrophoblasts transduced to constitutively express Id-2 found that these cells remained undifferentiated, as cyclin B expression was maintained and $\alpha 1$ integrin expression was suppressed (Janatpour et al., 2000). More recently, Hemberger and colleagues (2010) identified ELF5 as an important transcription factor for the maintenance of human cytotrophoblast stem cells. ELF5 is highly expressed in first trimester villous cytotrophoblast, but not in the more differentiated syncytiotrophoblast or EVT cells, and is strongly down-regulated towards the end of gestation. They established that ELF5, along with CDX2 and EOMES, forms a mutually

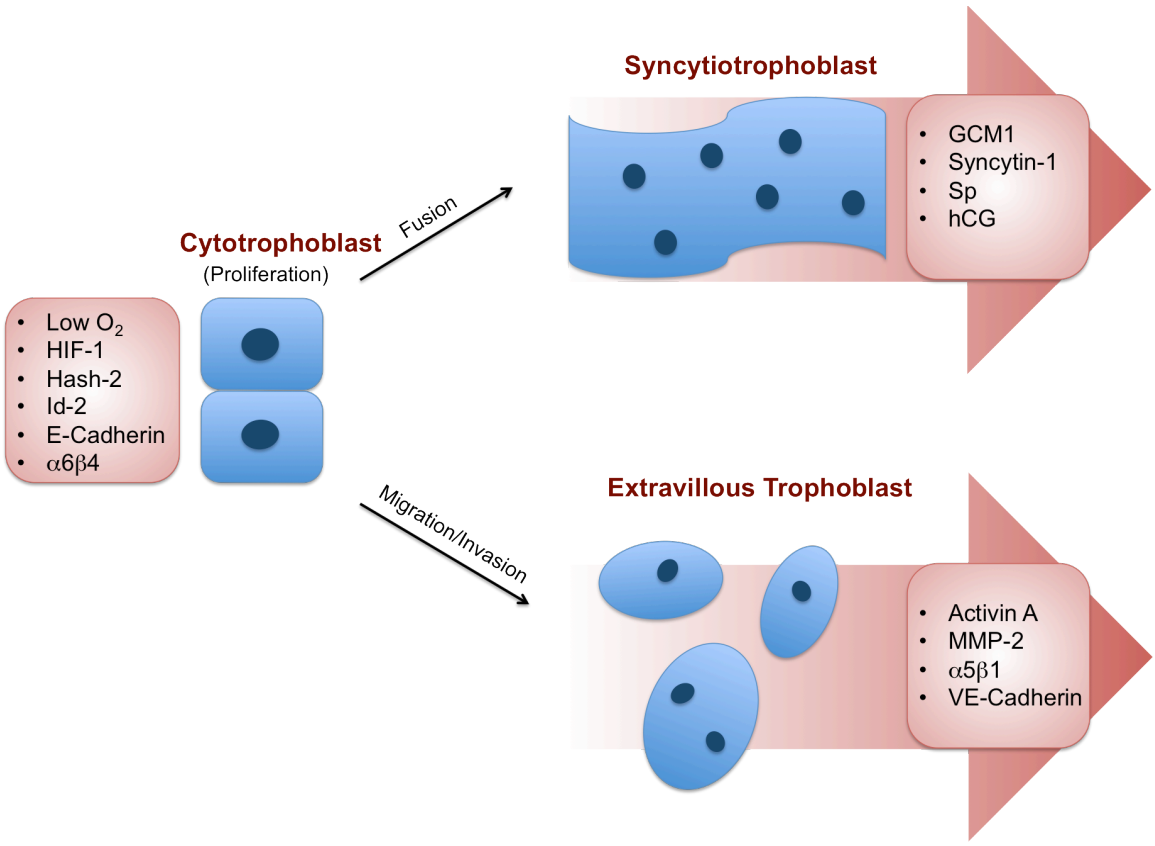

Figure 3. Regulation of Trophoblast Differentiation

Schematic diagram of cytotrophoblast (CT) differentiation to syncytiotrophoblast (ST) or extravillous trophoblast (EVT), together with key factors involved in the regulation of these events. The box on the left lists factors stimulating CT proliferation, thus inhibiting differentiation. Boxes on the right list factors stimulating ST or EVT differentiation. Adapted from Lunghi et al., 2007.

interacting trophoblast transcriptional network, and that $ELF5^+/CDX2^+$ cytotrophoblasts (which persist until 13 weeks of gestation) may represent a highly proliferating, self-renewing trophoblast stem cell population. Additionally, they have shown that in contrast to placental trophoblasts, ELF5 is hypermethylated and chiefly repressed in hESC as well as hESC derived trophoblast cell lines, outlining the importance of epigenetic regulation in trophoblast fate determination.

Several studies have established the critical role of oxygen tension in the control of trophoblast differentiation. Blastocyst implantation, placentation, and early embryonic development occur in a relatively low oxygen environment (Lash et al., 2002). Low O_2 levels stimulate cytotrophoblast proliferation and inhibit differentiation, while high O_2 levels inhibit proliferation and stimulate differentiation (Genbacev et al. 1997; Caniggia et al. 2000). Hypoxia stimulates hypoxia inducible factor-1 (HIF-1) and Hash-2, which maintains cytotrophoblast proliferation and inhibits differentiation, respectively (Janatpour et al., 1999; Janatpour et al., 2000, Baczyk et al., 2004). Cytotrophoblasts also express TGF-β3, an inhibitor of trophoblast differentiation that impedes the acquisition of the invasive phenotype (Genbacev et al., 1996; Caniggia et al., 2000). In accordance with the above findings, it has been shown that invading cytotrophoblasts downregulate the expression of both HIF-1 and TGF-β3, with HIF-1 acting upstream of TGF-β3 (Caniggia et al., 1999; Caniggia et al., 2000).

A variety of growth factors produced at the fetal-maternal interface were shown to promote cytotrophoblast proliferation in both autocrine (trophoblast derived) and paracrine (decidua-derived) manners. They include EGF-R ligands such as EGF, TGF-α and amphiregulin, VEGF, PlGF, CSF-1 (Chakraborty et al., 2002; Lala & Chakraborty,

2003). On the other hand, the negative regulation of proliferation is primarily paracrine, mediated by decidua-derived factors such as TGF-β and a TGF-β-binding leucine-rich proteoglycan, decorin (Graham et al., 1992; Lysiak et al., 1995; Xu et al., 2002).

1.1.5.2 Syncytiotrophoblast – Fusion

Trophoblast differentiation into the peri-implantation invasive syncytiotrophoblast is initiated upon attachment to the endometrial epithelium, where decidua-derived factors stimulate fusion and the acquisition of an invasive phenotype. Syncytial fusion occurs through the retroviral envelope protein, Syncytin. The transcription factor, glial cell missing 1 (GCM1) along with AP-2 and Sp transcription factor families are upregulated in pre-fusing cytotrophoblasts and induce expression of Syncytin (Yu et al., 2002; Frendo et al., 2003; Baczyk et al., 2004). It is well established that syncytialization can be initiated by certain cytokines, such as epidermal growth factor (EGF) which is expressed by both decidual and trophoblastic cells (Hofmann et al., 1991; Dakour, et al., 1999) and has been shown to stimulate the production of hormones, including human placental lactogen (hPL) and hCG (Maruo et al., 1995). hCG, itself regulates several processes during pregnancy, including promotion of syncytiotrophoblast generation through activation of the cAMP pathway (Licht et al., 2001; Yang, Lei, & Rao, 2003). Forskolin directly activates adenlyate cyclase, thereby increasing cAMP, and is commonly used to induce trophoblast fusion (Wice et al., 1990; Drewlo et al., 2008). Conversely, TGF-β1 has been shown to inhibit the formation of syncytiotrophoblast (Morrish, Bhardwaj, & Paras, 1991). TGF-β was found to be elevated in plasma of preeclamptic mothers, suggesting it may play a role in the impaired implantation associated with this disease (Caniggia et al., 2000). Lastly, physiological factors are also important regulators of

trophoblast differentiation: For example rising oxygen levels promote syncytial fusion (Jiang et al., 2005).

1.1.5.3 EVT – Migration & Invasion

Uterine invasion by trophoblast cells is critical for successful pregnancy, and is thus stringently regulated by a network of factors during gestation (Lala & Hamilton, 1996; Caniggia et al., 2000a; Chakraborty et al., 2002; Anin et al., 2004; Knofler, 2009). While a multiplicity of factors, both autocrine and paracrine, promote trophoblast migration and invasion, only a few molecules produced by the decidua or the syncytiotrophoblast negatively regulate these functions (Lala & Chakraborty, 2003). For example, IGFII, a trophoblast derived factor stimulates trophoblast migration by binding to IGFRII (McKinnon et al., 2001). Similarly, trophoblast-derived uPA promotes trophoblast migration as well as invasiveness. The migration-promoting action is due to binding of the non-catalytic amino-terminal domain to high affinity uPA receptors expressed by the EVT at the invasive front in a polarized manner (Liu et al., 2003), whereas its catalytic domain promotes matrix degradation by plasmin-induced activation of the MMPs (Lala & Chakraborty, 2003). Endothelin-1 produced by trophoblast and the decidua promotes EVT migration by elevation of intracellular Ca^{2+} (Chakraborty et al., 2003). A variety of decidua-derived factors also stimulate EVT motility. For example, IGFBP-1 which mediates pro-migratory action by binding to the $\alpha5\beta1$ integrin via its RGD domain (Gleeson et al., 2001). Activin A is a pro-migratory factor abundantly secreted by decidual cells (Jones et al., 2002). Activin A has been shown to upregulate MMP-2 expression in cytotrophoblast cells, enhancing their outgrowth from villous cell columns (Caniggia et al., 1997). Decidua-derived PGE2 promotes trophoblast migration

via signaling through the prostaglandin receptor, EP1 and elevation of intracellular calcium (Nicola et al., 2005) and EP2/EP4 by regulating Rho kinases and MAPK (Nicola et al., 2007; Nicola et al., 2008).

Factors negatively regulating EVT migration and invasiveness are primarily paracrine in origin. For example, TGF-β, produced by the syncytiotrophoblast and the decidua, is a potent inhibitor of EVT migration and invasion (Graham & Lala, 1992). The anti-invasive function is mediated by an upregulation of the endogenous tissue inhibitor of MMPs (TIMP)-1 (Graham & Lala, 1991; Graham et al., 1992) and downregulation of uPA (Graham, 1997). The anti-migratory function is due to an upregulation if integrins making the cells more adhesive (Irving & Lala, 1995). It is important to note that the inhibitory effects of TGF-β are lost in choriocarcinoma cells (eg. JAR and JEG-3 cell lines); this may be partially attributed to the downregulation of Smad-3, a downstream TGF-β signaling molecule (Xu et al., 2003). TNF-α produced by decidual macrophages negatively regulates trophoblast invasiveness via an induction of nitric oxide (Cartwright et al., 1999). Decorin, a decidua-derived proteoglycan, negatively regulates trophoblast migratory function by binding to multiple tyrosine kinase receptors, in particular VEGFR2 (Iacob et al., 2008). The VEGFR2 binding domain of decorin has recently been mapped (Khan et al., 2011).

Integrins also play a role during EVT differentiation (Bischof et al., 1990). Integrins are both receptors and adhesion molecules that bind specific extracellular matrix (ECM) proteins and regulate adhesion, migration, invasion, cytoskeleton reorganization and cellular signaling (Albelda & Buck, 1990). Cytotrophoblasts initially express integrin α6β4, a laminin-5 receptor that maintains their adherence to the villous

basement membrane (Korhonen et al. 1991; Damsky et al. 1994; Zhou et al., 1997). As these cytotrophoblast migrate off the basement membrane and differentiate into EVTs along the invasive pathway, they down regulate integrin α6β4 and up-regulate α1β1 and α5β1 integrins, a laminin-2 and fibronectin receptor, respectively to promote invasiveness by binding to the specific laminins and fibronectins abundantly expressed in the decidua (Irving et al., 1995; Irving & Lala, 1995; Church et al., 1996). α5β1 also functions as a migration promoting receptor for decidua-derived IGFBP-1 (Gleeson et al., 2001).

Additionally, studies have revealed an epithelial-to-endothelial transition essential for placental development and the remodeling of maternal uterine vasculature. For example, cytotrophoblasts in the chorionic villi initially express the adherins junction protein, E-Cadherin, a marker of polarized epithelium. Upon migration and differentiation, however, these cells down regulate E-Cadherin and up-regulate VE-Cadherin, an adhesion marker characteristic of endothelial cells (Zhou et al., 1997).

1.1.6 Models of Placental Development

Trophoblast cell culture models have provided significant insight into placental development, particularly so for human studies, where *in vivo* experimentation is not feasible. Until recently, the lack of human trophoblast stem and progenitor cell lines has significantly deterred our understanding of the mechanisms regulating trophoblast differentiation and how these mechanisms become unraveled in placental disorders. Previous studies have employed BMP4 treated hESC (Xu et al., 2002a; Das et al., 2007) primary CTs (Kliman et al., 1986; Fisher et al., 1989), chorionic villous explants (Genbacev et al., 1993), virally transformed trophoblast cell lines (Chou et al., 1978), and

choriocarcinoma cell lines (Pattillo et al., 1968). However, there are many inherent issues with the models. For example, hESC derived trophoblast models may not accurately represent true trophoblast cells, as they may not fully acquire the lineage-specific epigenetic signature (Hemberger et al., 2010); use of short-term primary cultures prohibit studies involving genetic manipulation that require long-term culture; primary cultures are often heterogeneous, complicating the identification of cells responsible for specific effects; and choriocarcinoma cells often behave differently from normal trophoblast due to their malignant phenotype.

Fisher and colleagues recently isolated a trophoblast progenitor cell line from the human chorion, offering a promising model for the study of trophoblast differentiation (Genbacev et al., 2011). Immunolocalization of pluripotency factors (Oct4) and trophoblast markers (cytokeratin 7, GCM1, Eomes, and Syncytin) in early gestation placenta identified the chorion as a putative source for trophoblast progenitors. These cells were isolated by enzymatic digestion and maintained in the presence of FGF2 (required for mouse stem cell self-renewal) and the ALK4/5/7 small molecule inhibitor, SB431542 (thought to inhibit pluripotency and trigger emergence of trophoblast progenitors) at 20% oxygen. The resulting colonies were dissociated and grown as monolayers and were found to express GATA3/4, CK7, Eomes, geminin, GCM1, neonatal FCγ receptor, and integrin α6. They were able to successfully induce differentiation with FGF4 and EGF on Matrigel, which resulted in a mixed population of mononuclear and multinuclear cells that expressed CK7, geminin, GCM1, Eomes, HLA-G, hPL, hCG, and Syncytin. These cells also exhibited increased invasion and the associated integrin switching from α6 to α1 as observed in invasive cytotrophoblast cells

in situ. Two human trophoblast progenitor cell lines have been generated thus far and continuously cultured for up to 25 passages without differentiation, promising invaluable insight into the mechanisms involved in trophoblast differentiation and placental development.

The immortalized human trophoblast cell line, HTR-8/SVneo, produced by introducing the gene encoding the simian virus (SV) 40 large T antigen into a human first trimester primary EVT cell line, HTR-8 (Graham et al., 1993), is widely used to study trophoblast biology. This cell line has been shown to be morphologically similar to its parental cell line and to express many of the markers of highly migratory EVT cells *in situ*, including cytokeratin 7, 8 and 18, placental type alkaline phosphatase, high affinity urokinase-type plasminogen activator (uPA) receptor, and integrins $\alpha1$, $\alpha3$, $\alpha5$, αv, $\beta1$ and the vitronectin receptor $\alpha v\beta3/\beta5$ (Irving et al., 1995; Irving & Lala, 1995; McKinnon et al., 2001; Gleeson et al., 2001; Nicola et al., 2005). These cells have also been observed to variably express villous-specific genes such as β-hCG and EVT-specific genes such as HLA-G depending on conditions (Zdravkovic et al., 1999). Such phenotypic plasticity is suggestive of a bi-potential cytotrophoblast progenitor population. Interestingly, this cell line also expresses the stem cell-associated factor, Nodal (Munir et al., 2004).

1.2 Nodal

Nodal is an embryonic morphogen that plays crucial roles during embryogenesis (Schier, 2003; Hendrix et al., 2007; Strizzi et al., 2008). Nodal is also a stem cell associated factor that maintains the pluripotency of embryonic stem cells, and is down-regulated with differentiation (Schier, 2003; Hendrix et al., 2007). Nodal is a member of

the TGF-β superfamily. This family encompasses 40 structurally related cell growth and differentiation factors, many of which are abundantly and dynamically expressed in the endometrium and placenta throughout gestation, including TGF-β and Activin. Recently, Nodal and its extracellular inhibitor, Lefty, have also been implicated in the regulation of placentation (Lowe et al., 2001; Ma et al., 2001; Guzman-Ayala et al., 2004; Munir et al., 2004; Tang et al., 2005; Nadeem et al., 2011). However, the precise role of Nodal signaling in the regulation of trophoblast differentiation remains unclear and is the focus of this study.

1.2.1 The Nodal Signaling Pathway

Nodal signals extracellularly by binding the heterodimeric complex between type I (ALK4/7) and type II (ActRIIB) activin-like kinase receptors (**Figure 4**). ActRIIB activates ALK4/7 via phosphorylation, which then phosphorylates and activates cytoplasmic Smad2/3. Phosphorylated Smad2/3 associates with Smad4 and translocates to the nucleus where it associates with various transcription factors, including forkhead box HI (FoxH1) and Mixer, and binds to the promoters of numerous target genes to regulate gene expression. The epidermal growth factor-cripto FRL1 cryptic (EGF-CFC) family co-receptor, Cripto, associates with Nodal and the ALK4 receptor to aid binding and increase Nodal signaling (Yeo & Whitman, 2001; Reissmann et al., 2001; Bianco et al., 2002). Cripto has also been demonstrated to interact with ALK7 (Reissman et al., 2001) and therefore assists Nodal signaling through both ALK4 and ALK7 receptors. However, while Nodal requires Cripto to bind ALK4, Nodal can bind directly to ALK7 without the assistance of Cripto (Reissman et al., 2001). It was recently discovered that Cripto interacts with the Nodal pro-domain along with the convertases, Furin and Pace4,

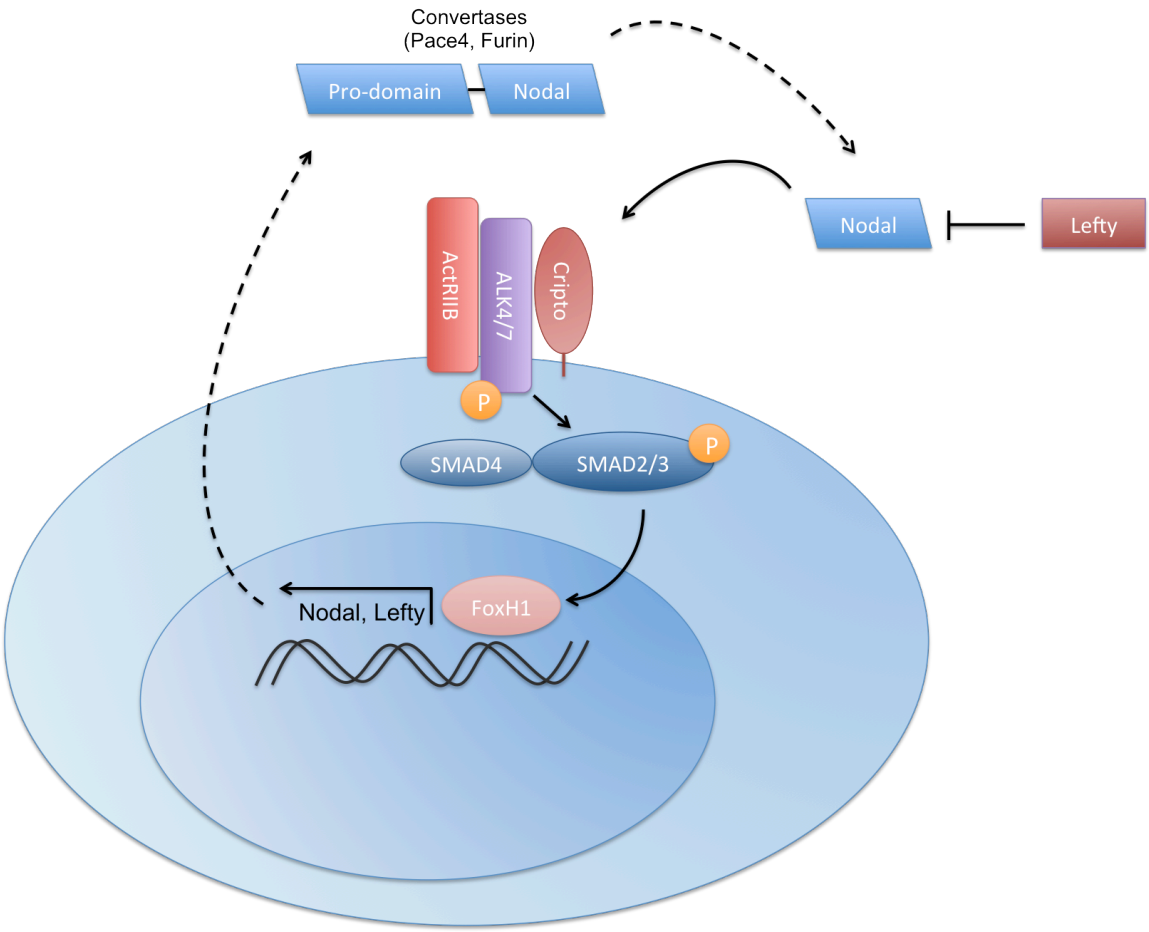

Figure 4. The Nodal Signaling Pathway

Nodal is initially secreted as a proprotein and must be activated via cleavage by the convertases Pace4 and Furin. Nodal signaling is propagated through the type I and type II activin-like kinase receptors, ALK4/7 and ActRIIB, as well as the coreceptor, Cripto. Nodal signaling is transmitted intracellularly via the phosphorylation of Smad2/3 and its association with Smad4 and transcription factors. The extracellular molecule, Lefty inhibits Nodal signaling. Adapted from Hendrix et al. 2007.

suggesting that it forms a Nodal-processing complex at the plasma membrane, providing yet another mechanisms by which Cripto enhances Nodal signaling (Blanchet et al., 2008).

The type I ALK receptors are not only critical for signal propagation but also signal specificity. For example, while Nodal has been shown to signal through both ALK4 and ALK7, the downstream signaling from these receptors produce dramatically different biological responses. Activin signaling through ALK4 promotes cytotrophoblast outgrowth and MMP-2 secretion (Caniggia et al., 1997). Conversely, Nodal signaling through ALK7 inhibits EVT invasion (Nadeem et al., 2011). Moreover, multiple ligands can signal through the same ALK receptor, for example both Nodal and Activin signal through ALK4 (Massague et al., 1998). In this case, presence of the co-receptor Cripto determines which ligand signals through the receptor (Yeo & Whitman, 2001): The presence of Cripto enhances Nodal signaling, while blocking Activin signaling (Yeo & Whitman, 2011; Yan et al., 2002).

1.2.2 Regulation of Nodal Signaling

Nodal signaling is regulated at multiple steps that include post-translational modifications, proteolytic cleavage, extracellular inhibitors, and epigenetic mechanisms. Nodal is initially secreted as a pro-protein and contains two post-translational modification sites for N-linked glycosylation within the pro-domain region. To become activated, Nodal must be cleaved by the subtilisin-like proprotein convertase (Spc) enzymes, Spc1 (Furin) and Spc4 (PACE4) (Beck et al., 2002). Studies in zebrafish revealed that the N-linked glycosylations stabilize the pro-domain and inhibit cleavage (Le Good, et al., 2005). Once cleaved, protein stability is compromised, resulting in a

reduced signaling range (Blanchet et al., 2008; Constam, et al., 2009). Therefore, cleavage promotes autocrine signaling, while N-linked glycosylation promotes paracrine signaling.

The Nodal signaling pathway is also self-regulated, including the up-regulation of its own expression in a positive feedback loop as well as the up-regulation of its endogenous inhibitors, Lefty. Lefty is another member the TGF-β superfamily and exists as two isoforms, Lefty1 and Lefty2. Lefty1/2 inhibits Nodal signaling by direct interaction with Nodal or Cripto in the cellular microenvironment or at the cell surface (Schier, 2003). Cerberus is another antagonist of Nodal signaling. It is a member of the DAN family of proteins that contain cysteine knots found in members of the TGF-β superfamily. Cerberus blocks extracellular Nodal signaling through direct ligand binding. Lastly, Tomoregulin-1 is a transmembrane protein that inhibits Nodal signaling through its association with Cripto (Cheng et al., 2003).

Nodal transcription is also thought to be regulated by epigenetic mechanisms. The Nodal gene contains a CpG island near its transcription start site, and studies in melanoma cells have correlated levels of methylation of this region with Nodal expression levels (Postovit et al., 2007). MicroRNA may also play a role in Nodal regulation. For example, miR-430 has been shown to block translation of the zebrafish Nodal equivalent, *squint* (Choi et al., 2007). It was recently observed that miR-218 expression is decreased in preeclamptic placenta compared to their gestational controls (Zhu et al., 2009) and two potential target sites were identified in the 3'UTR of Nodal (Brkic et al., 2012). These findings are supported by the report that Nodal inhibits trophoblast migration and invasion and is overexpressed in preeclamptic placenta,

suggesting that decreased miR-218 may lead to enhanced Nodal expression and preeclampsia, and may thereby prove to be a promising target for treatment (Nadeem et al., 2011).

1.2.3 Nodal and Development

Nodal was discovered through a retroviral induced mutation in the mouse called 413.d. Embryos homozygous for the mutation exhibited dramatic tissue malformations by 7.5 days of development and were embryonic lethal (Conlon et al., 1991). Eventually, the mutation was mapped to a novel TGF-β superfamily growth factor called Nodal (Zhou et al., 1993). Nodal has since been shown to be a critical regulator of embryonic development; primarily in the establishment of anterior-posterior axis patterning and left-right asymmetry (Schier, 2003).

Nodal is initially expressed in the mouse epiblast shortly after implantation, and is maintained through a positive feed-back loop (Ang & Constam, 2004). Nodal is activated by convertases expressed in the adjacent extraembryonic ectoderm, which leads to the induction of Lefty1 in the distal visceral endoderm, which eventually develops into the anterior visceral endoderm (Schier, 2003). The restriction of Nodal signaling to the proximal posterior region of the epiblast, by Lefty1 in the anterior visceral endoderm, is crucial for anterior visceral endoderm displacement and positioning of the anterior-posterior axis (Zhou et al., 1993; Smith et al., 1995; Schier, 2003; Shiratori & Hamada, 2006; Schier, 2009). Following gastrulation, Nodal becomes restricted to the node in the anterior primitive streak, hence the designation “Nodal” (Schier, 2003). The node is where left-right axis formation occurs as Lefty1 restricts Nodal signaling to the left side

of the embryo, where it facilitates mesoderm formation until it is downregulated and lost at approximately 8 dpc (Collignon et al., 1996).

It has recently been shown that Nodal also regulates epigenetic phenomenon to control stem cell differentiation (Dahle et al., 2010; Xi et al., 2011). Embryonic development is regulated by both intercellular signaling and epigenetic mechanisms to establish lineage specific patterns of gene expression. Histone acetylation and methylation alter chromatin structure and therefore the accessibility of DNA (reviewed in Dahle et al., 2010). Smad complexes can bind DNA and recruit histone acetyltransferases to stimulate transcription, or histone deacetyltransferases to inhibit transcription (Massague et al., 2005). Alternatively, these histone marks can also serve as binding sites for transcriptional regulators, for example, trimethylation of Lys^{27} of Histone H3 (H3K27me3) acts as a binding site for Polycomb repressive complex 1, which inhibits gene expression (Schuettengruber & Cavalli, 2009). Dahle and colleagues (2010) demonstrated that the Nodal signaling complex, Smad2/3, binds the histone demethylase, Jmjd3, and recruits it to the histone mark to counter inhibition by Polycomb and enable transcription of downstream target genes. Epigenetic regulation enables the maintenance of genomic programs that may be stably maintained across cell generations, but are also able to respond to environmental cues (Mohn & Schubeler, 2009). For example, while homeostasis genes generally have an active chromatin state, it has recently been observed that master regulators of differentiation are transcriptionally silenced, but are poised for activation by specific developmental signals (Young, 2011). Xi et al. (2011) have shown that Nodal utilizes the poised histone mark H3K9me3 to trigger differentiation of embryonic stem cells. Nodal signaling yields a TRIM33-Smad2/3 complex that binds

H3K9me3 on the promoters of Gsc and Mixl1 (mesendoderm regulators), displacing chromatin-compacting factor HP1γ, which exposes Nodal response elements (also known as Activin Response Elements, ARE) and enables Smad4-Smad2/3 binding for Pol II recruitment and differentiation.

Nodal signaling has also been implicated in the maintenance of human embryonic stem cell (hESC) pluripotency (Vallier et al., 2004; James et al., 2005). It has been shown that Nodal induces activation of Nanog transcription through Smad2/3; while Nanog in turn interacts with Smad2/3 to inhibit the transcriptional activity of the Nodal signaling pathway, and thereby prevent endoderm differentiation (Vallier et al., 2009). Furthermore, Nodal/Activin inhibition in hESC through the receptor inhibitor, SB431542, was found to induce neuroectoderm formation (Smith et al., 2008; Vallier et al., 2009; Patani et al., 2009). Taken together, these studies indicate a role for Nodal in maintaining hESC pluripotency by inhibiting neuroectoderm and mesendoderm differentiation. Additionally, it has recently been suggested that Nodal also maintains the pluripotent phenotype of cancer cells.

1.2.4 Nodal and Cancer

Nodal signaling has been implicated in several cancer types, including testicular, prostate, liver, endometrial, ovarian, melanoma, breast, glioma, and pancreatic (Lawrence, 2010; Adkins et al., 2003; Cavallari et al., 2012; Papageorgiou et al., 2009; Xu et al., 2004; Topczewska et al., 2006; Strizzi et al., 2008; Lee et al., 2010; Lonardo et al., 2011). It is thought that, similar to its effect on hESC, Nodal maintains an undifferentiated, pluripotent phenotype in cancer cells, increasing aggressiveness and tumourigenicity (Topczewska et al., 2006; Postovit et al., 2006; Kelusa et al., 2006;

Postovit et al., 2006a; Strizzi et al., 2008). While Nodal is undetectable in normal melanocytes, it has been shown to become minimally expressed in poorly invasive, radial growth phase melanoma and highly expressed in invasive vertical growth phase melanoma and metastatic melanoma (Topczewska et al., 2006). Furthermore, inhibition of Nodal signaling via the small molecule inhibitory drug, SB431542, or morpholino oligonucleotides led to a significant reduction in metastasis and tumour formation, as well as a loss of plasticity, as indicated by upregulation of Tyrosinase and downregulation of Vascular Endothelial (VE)-Cadherin and Keratin 18, resulting in a more differentiated melanocyte-like cell (Topczewska et al., 2006).

Nodal expression has also been implicated in breast cancer. Immunohistochemical analysis of human breast tissue microarrays revealed that Nodal has a significant positive correlation with breast cancer progression, being completely absent in normal beast tissue (Postovit et al., 2008). Nodal knock-down via morpholino oligonucleotides, shRNA, or the small molecule inhibitor SB431542 in the highly aggressive MDA-MB-231 breast cancer cell line, decreased invasion and clonogenicity *in vitro* and tumorigenicity in nude mice (Quail et al., 2010). Correspondingly, when MDA-MB-231 are treated with conditioned medium from hESC containing Lefty, these functional capabilities decreased, concomitant with reduced Nodal mRNA and protein expression (Postovit et al., 2008). Conversely, treatment of the poorly aggressive human breast cancer cell line, T47D with human recombinant Nodal resulted in an increase in invasion, self-renewal and anchorage independent growth (Quail et al., 2010). Furthermore, the phenotypic plasticity of the commonly designated $CD44^{+}/CD24^{-}$ breast cancer stem cell (Al-Hajj et al., 2003) has been shown to be mediated by the Nodal/Activin signaling pathway, as

treatment of this population with the small molecule inhibitor, SB431542 significantly inhibited its ability to undergo phenotypic switching (Meyer et al., 2009).

Following the above results suggesting that the embryonic stem cell derived Lefty can inhibit the tumorigenicity of cancer cells, Cavallari and colleagues (2012) wanted to determine if adult stem cells also possess this anti-tumoral property. They demonstrate that conditioned medium from human liver stem cells (HLSC-CM) inhibits the *in vitro* growth and promotes apoptosis in the hepatoma cell line, HepG2 and this effect was attributed to the presence of Lefty A in the HLSC-CM, as silencing Lefty A abrogated the anti-proliferative and pro-apoptotic effects. Furthermore, intra-tumor administration of HLSC-CM inhibited the *in vivo* growth of HepG2 cells in SCID mice. Conversely, CM from bone marrow-derived mesenchymal stem cells did not produce this anti-tumor effect, suggesting only certain adult stem cells are able to inhibit tumor growth through inhibition of the Nodal signaling pathway, similar to embryonic stem cells (Cavallari et al., 2012).

Nodal signaling has also been linked to endometrial cancer (Papageorgiou et al., 2009). This study showed that while Nodal and its co-receptor, Cripto, are expressed in the stroma and epithelium of the endometrium during the normal proliferative phase of the menstrual cycle, they are significantly upregulated in patient biopsies of endometrial carcinoma and show a positive correlation with cancer grade (Papageorgiou et al., 2009). Additionally, Nodal's endogenous inhibitor, Lefty, was present in normal epithelium and surrounding stroma during the late secretory and menstrual phases, however was not present in endometrial cancer tissues (Papageorgiou et al., 2009).

Nodal has similarly been found to correlate with highly invasive glioma cells (Lee et al., 2010). These authors found a positive correlation between Nodal expression and grade in human glioma tissue (Lee et al., 2010). Additionally, when they overexpressed Nodal in glioma cells exhibiting low endogenous levels of Nodal, they found a significant increase in invasion, MMP-2 secretion/activity, proliferation *in vitro*, and tumour growth *in vivo* (Lee et al., 2010). Furthermore, when Nodal was knocked down in glioma cells expressing high endogenous levels of Nodal, there was a significant decrease in invasion, MMP-2 section/activity, proliferation *in vitro*, and tumour growth *in vivo* (Lee et al., 2010).

Recent work in pancreatic cancer has also implicated an important role for Nodal and Activin signaling (Lonardo et al., 2011). This group found elevated levels of both Nodal and Activin in pancreatic "cancer stem cells" (CSC) that formed non-adherent spheroids and expressed pluripotency markers, compared to more differentiated adherent pancreatic cancer cells (Lonardo et al, 2011). The addition of recombinant Nodal to these CSCs increased spheroid formation, size, and invasion *in vitro*, while inhibition of Nodal/Activin signaling with SB431542 eliminated the CSC subpopulation and increased susceptibility to gemcitabine chemotherapy *in vitro,* and *in vivo* in an orthotopic mouse model (Lonardo et al., 2011).

The pro-metastatic role of Nodal is not so clear-cut. One study of ovarian cancer found that Nodal overexpression resulted in a decrease in the number of metabolically active and proliferating cells (Xu et al., 2004). This was attributed to the upregulation of cyclin G2 (CCNG2) (Xu et al., 2008) via the Forkhead box class O transcription factor, FoxO3a (Fu & Peng, 2011). They found that when ovarian surface epithelium cells,

IOSE-394, and ovarian cancer cells, OV2008, were treated with recombinant Nodal or transfected with Nodal plasmid DNA, there was a significant increase in FoxO3a proteins levels, while silencing of ALK7 or Smad4 using small interfering RNAs (siRNAs) decreased protein levels. Using IP assays they then showed that Smad2/3 forms a complex with FoxO3a and that this complex binds the promoter of CCNG2 to enhance transcription (Fu & Peng, 2011). While these findings appear contradictory to the widely reported pro-metastatic role of Nodal in cancer, it has been noted that overexpression of Nodal in cancer cells already expressing Nodal tends to promote apoptosis, whereas overexpression of Nodal in cancer cells that do not express Nodal tends to favour metastases (Quail et al., 2010a). These seemingly contradictory results shed light on the concentration-dependent multi-functionality of Nodal signaling. This is consistent with other members of the TGF-β superfamily, which have various concentration-dependent effects during embryonic development and cancer progression (Hoffman, 1992; Soderberg et al., 2009; Tian & Schiemann, 2009; Bachman & Park, 2005).

1.2.5 Nodal and Placentation

As mentioned above, Nodal, its co-receptor Cripto, and its inhibitor, Lefty, are all found within normal endometrial stromal and epithelial cells during various stages of the menstrual cycle (Papageourgiou et al., 2009). Nodal mRNA was found to be highest during the proliferative and early secretive phases and almost completely absent by the mid-secretory phase, while Lefty mRNA appears beginning in the late-secretory phase and continues throughout menstruation, after which it disappears with the reappearance of Nodal in the early-proliferative phase. Cripto on the other hand was expressed throughout the cycle, increasing in expression from the early-proliferative phase until the

late secretory phase, after which it rapidly decreases during menses (Papageorgiou et al., 2009). Additionally, Nodal expression is also observed in the mouse and human placentae (Ma et al., 2001; Nadeem et al., 2011). In the mouse, Nodal mRNA is restricted to the spongiotrophoblast compartment (Ma et al., 2001), however in the human, Nodal protein has been localized within all trophoblast lineages, with expression greatest in the cytotrophoblasts (Nadeem et al., 2011). Nodal expression was found to decrease during the progression of pregnancy, being absent in the syncytiotrophoblast and extravillous trophoblast and only minimally expressed in the cytotrophoblast by the third trimester (Nadeem et al., 2011).

In addition to its presence in the placenta, a role for Nodal during placentation has been indicated by the aberrant placental development observed in the Nodal null homozygous mouse. These placentae displayed enlargement of the polyploid giant cell layer at the expense of the diploid spongiotrophoblast and labyrinth layers (Ma et al., 2001). Additionally, expression of Nodal was found to inhibit trophoblast giant cell differentiation in the rat choriocarcinoma cell line, Rcho-1 (Ma et al., 2001). Following these findings, the authors postulated that Nodal functioned to direct trophoblast fate towards the labyrinth lineage, while maintaining the thin layers of giant cells and spongiotrophoblast (Ma et al., 2001). More recent work, however, demonstrated that Nodal, acting through FGF4, maintains a microenvironment that inhibits mouse trophoblast stem cell differentiation (Guzman-Ayala et al., 2004). This work has since translated to human studies, with establishment of the first human trophoblast progenitor cell line using the Nodal/Activin inhibitor, SB431542 (Genbacev et al., 2011). The rational being that inhibiting Nodal signaling in the trophoblast stem cell population

would decrease expression of pluripotency factors as well as enable the emergence of more differentiated progenitor cells. It may be the case that a Nodal gradient is present in which the more pluripotent trophoblast stem cells express high Nodal levels, bi-potential cytotrophoblast progenitors moderate levels, and differentiated trophoblasts little to no Nodal.

Nodal and its receptor, ALK7, have been detected in the placenta throughout gestation, suggesting it regulates differentiated trophoblasts, in addition to TSCs (Roberts et al., 2003). Roberts and colleagues were the first to demonstrate that ALK7 and Nodal transcripts are expressed in the placenta throughout gestation (weeks 6-40), with Nodal expression being highest between weeks 12-29 (Roberts et al., 2003). Additionally, they discovered that the ALK7 receptor transcript is alternatively spliced, yielding four isoforms (ALK7, tALK7, sALK7a, sALK7b) all of which are translated to protein (Roberts et al., 2003). The full-length ALK7 and truncated ALK7 were also expressed throughout gestation, with expression of truncated ALK7 significantly increasing in the third trimester of pregnancy, while the soluble forms of ALK7 showed variable expression. As the truncated isoform may possess reduced affinity for the ligand, it was postulated to act as an antagonist of full-length ALK7, reducing Nodal signaling during the later stages of pregnancy (Roberts et al., 2003).

Work in human trophoblast and choriocarcinoma cell lines has implicated a role for Nodal in the regulation of trophoblast proliferation and apoptosis. Nodal over-expression was found to decrease the number of actively proliferating cells while increasing the number of apoptotic cells. Furthermore, constitutively active ALK7 was found to recapitulate these effects, while a kinase-deficient ALK7 or dominant-negative

Smad2/3 was found to block the effects (Munir et al., 2004). Trophoblast migration and invasion were also affected by Nodal signaling. In this case, Nodal over-expression or constitutively active ALK7 decreased cell migration and invasion, while Nodal or ALK7 knock-down had the reverse effect (Nadeem et al., 2011). In placental explant culture, treatment with recombinant human Nodal was shown to inhibit trophoblast outgrowth, while Nodal small-interfering RNA increased EVT migration. The inhibitory effects of Nodal were found to be mediated by increased secretion of tissue inhibitor of metalloproteinase (TIMP)-1 and inhibited matrix metalloproteinase (MMP)-2 and MMP-9 activity (Nadeem et al., 2011). Taken together, these results indicate an important role for Nodal/ALK7 signaling in human placentation.

Given the importance of Nodal signaling in placental development, it is not surprising that Nodal and members of the Nodal signaling pathway have been implicated in placental disorders. For example, the Nodal antagonist Lefty has been associated with impaired implantation and infertility. Tabibzadeh and colleagues (2000) demonstrated that in contrast to normal fertile women who exhibit low levels of Lefty protein in the endometrium during the receptive phase, infertile women expressed significantly higher levels of Lefty during this phase. Furthermore, successful pregnancy occurred when treatment of these infertile women involved the downregulation of Lefty (Tabibzadeh et al., 2000). This group went on to study implantation in mice and found that when they transfected Lefty, either by retroviral vector or liposome-mediated DNA transfer, into the uterine horns of pregnant mice, implantation failure occurred (Tang et al., 2005). This implantation failure was associated with a lack of decidualized stroma suggesting Lefty plays an important role in this process, however an affect of Lefty on extraembryonic

tissues remains possible (Tang et al., 2005). Seemingly contradictory to this, work in the Peng lab has shown that Nodal and ALK7 are strongly upregulated in the placentae of women with preeclampsia, a multisymptomatic disease thought to arise from reduced trophoblast invasion (Nadeem et al., 2011). This is in accordance with their findings that Nodal has an inhibitory effect on trophoblast proliferation, migration and invasion (Munir et al., 2004; Nadeem et al., 2011). While it is apparent that Nodal signaling plays an important role in implantation and placentation, controversy remains over exactly how Nodal regulates trophoblast differentiation and how this affects placentation.

1.3 Rationale

As reviewed earlier, loss of α6β4 integrin is one of the hallmarks of the transition of trophoblast progenitors to EVT cells *in situ*. This phenomenon has been faithfully recapitulated *in vitro*, showing that EVT cells migrating out of chorionic villus explants in culture (Irving et al., 1995) as well as short-lived primary EVT cell lines including HTR-8 cells (Irving & Lala, 1995) had a paucity of α6β4 expressing cells. In contrast, a detailed analysis of the integrin profile of several SV40 Tag immortalized derivatives of HTR-8 cells, including HTR-8/SVneo, RSVT-2 and RSVT-2C cell lines revealed a significant incidence of α6β4 expressing cells. This indicated SV40 Tag integration in the small α6β4 bearing pool in HTR-8 cells leading to immortalization (Lala et al., 1995 abstract). Of the above three immortalized cell lines, two (RSVT-2 and RSVT-2C) were shown to have acquired a premalignant phenotype, whereas HTR-8/SVneo cell line remained normal except for immortalization (Khoo et al., 1998a; Khoo et al., 1998b; Lala et al., 1999).

Preliminary work by others in the lab has validated these findings in HTR-8/SVneo cells, showing that similar to the progenitors *in situ*, a subpopulation of these cells express α6β4 integrin (Figure A1-A,B). Furthermore, an $\alpha6\beta4^{high}$ subpopulation can be isolated using Fluorescence Activated Cell Sorting (FACS), a method for sorting a heterogeneous cell line into two or more subpopulations based upon the specific light scattering and immunofluorescent characteristics of each cell (Figure A2-A,B). This $\alpha6\beta4^{high}$ subpopulation has been shown to have a significantly higher colony formation capacity than the heterogeneous HTR-8/SVneo population, while the $\alpha6\beta4^{low}$ subpopulation has a lower clonogenic capacity relative to the $\alpha6\beta4^{high}$ and heterogeneous cells (Figure A3-A). Additionally, compared to the unsorted and $\alpha6\beta4^{low}$, the $\alpha6\beta4^{high}$ cells are better able to differentiate along the villous pathway, as exhibited by hCG expression (Figure A3-B) and extravillous pathway, exemplified by enhanced invasion (Figure A3-C) and endovascular differentiation including VE-Cadherin expression (Figure A3-D) and endothelial tube-like formation (Figure A3-E) (Zhong et al., 2008).

This cell line also expresses Nodal, an embryonic morphogen and stem cell-associated factor that sustains the pluripotency of embryonic stem cells. While Nodal is expressed in both the endometrium (during the peri-implantation period) and the placenta (throughout the first trimester of pregnancy), and has been shown to play roles in regulating trophoblast proliferation/apoptosis and migration/invasion, its precise role in regulating cytotrophoblast differentiation remains unclear.

Thus, in this study I examined the effects of Nodal on the regulation of $\alpha6\beta4^{high}$ cytotrophoblast progenitor cells, as well as their ability to undergo differentiation along the villous and extravillous trophoblast lineages.

1.4 Hypothesis

The $\alpha 6\beta 4^{high}$ subset within HTR-8/SVneo is enriched with bi-potential cytotrophoblast progenitor-like cells that are maintained by Nodal signaling.

The study consists of three aims:

1. To isolate and characterize proliferation and Nodal expression in $\alpha 6\beta 4^{high}$ HTR-8/SVneo cytotrophoblasts.

2. To examine effects of loss of function of Nodal on hallmarks of cytotrophoblast progenitors: (1) expression of integrin $\alpha 6\beta 4$, (2) self renewal & proliferation, and (3) differentiation capacity along both villous and extravillous pathways.

3. To examine effects of gain of function of Nodal on cytotrophoblast progenitor hallmarks.

Chapter Two: Materials & Methods

Materials: RPMI 1640, DMEM, and F-12K medium, fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (DPBS), and 0.05% Trypsin-EDTA, and puromycin used in cell culture were purchased from Invitrogen. BD Falcon cell culture flasks (150cm^2 and 75cm^2), 6-well plates, 12-well plates, and growth factor reduced (GFR) matrigel were from BD Biosciences. Gentamicin was from HyClone Laboratories. Geneticin was from Gibco. M-PER® Mammalian Protein Extraction Reagent, HALT™ Protease Inhibitor Cocktail and Restore™ Plus Western blot stripping buffer were from Pierce. Anti-Nodal antibody (clone WS65) was from Santa Cruz Biotechnology. Anti-β-Actin primary antibody, and goat anti-rabbit IgG and goat anti-mouse IgG secondary antibodies was from LI-COR Biosciences. Bovine serum albumin (BSA) and SB431542 (Nodal/Activin Inhibitor) were purchased from Sigma. Recombinant human Nodal was from R&D Systems. Human ELISA kit for hCG (BQ047F) was from BioQuant. RNeasy Mini Kit from Qiagen, High Capacity cDNA Reverse Transcription Kit and Master Mix from Applied Biosystems.

Cell Culture: HTR-8/SVneo cells were grown in RPMI medium 1640 supplemented with 10% FBS, 100 μg/ml gentamicin, unless otherwise specified, at 37°C and 5% CO_2. Stable Nodal knock-down HTR-8/SVneo cells and scrambled control cells were generated by transfection via lipofectamine of a pGIPZ lentiviral vector (Open Biosystems) containing either an $shRNA_{Nodal}$ (GI311709, clone 6) or $shRNA_{scramble}$ (TR30013). Clones were selected with 500 ng/ml puromycin. Cells were grown in RPMI 1640 supplemented with 10% FBS, 100 μg/ml gentamicin, and 200ng/ml puromycin at 37°C and 5% CO_2 unless otherwise specified. Successful transfection was verified by

GFP immunofluorescence. Nodal knock-down was verified by Western blot in comparison to scramble control-transfected cells. The choriocarcinoma cell line, BeWo (ATCC) were grown in DMEM supplemented with 10% FBS and 100 μg/ml gentamicin unless otherwise specified, at 37°C and 5% CO_2. Stable Nodal over-expressing BeWo clones were generated by transfection with Arrest-In of a pcDNA3.3-TOPO vector (Life Technologies) containing the Nodal open reading frame cloned from H9 hESCs. Clones were selected with 500 μg/ml Geneticin. Cells were grown in DMEM supplemented with 10% FBS, 100 μg/ml gentamicin and 100 μg/ml Geneticin unless otherwise specified, at 37°C and 5% CO_2. Successful Nodal over-expression was verified by Western Blot in comparison to non-transfected parental cells. All transfections completed by Guihua Zhang.

Fluorescence Activated Cell Sorting (FACS): HTR-8/SVneo cells grown in complete medium (RPMI 1640, 10% FBS, and gentamicin) were washed, trypsinized and centrifuged for 5 min at 2,500 RPM. The cells were then re-suspended in FACS buffer (DPBS + 3% FBS) to a concentration of approximately 10 million cells/ml and 100μl aliquots were incubated in monoclonal FITC-conjugated rat Anti-Human CD49f (α6) (BD Pharmingen) and PE-conjugated rat Anti-Human CD104 (β4) (BD Pharmingen) (including the appropriate isotype controls) on ice for 1 hour in the dark. Cells were then washed twice with FACS buffer and resuspended/pooled in 4 ml for a final concentration of 5 million cells/ml. The cell suspension was brought to the London Regional Flow Cytometry Facility for FACS analysis. The resulting $\alpha6\beta4^{high}$ and $\alpha6\beta4^{low}$ populations were then plated and incubated at 37°C, 5% CO_2 for 24hrs until analysis. For flow

cytometry, cells were prepared as above, except incubated in monoclonal PE-Cy5-conjugated rat Anti-Human CD49f (α6) (BD Pharmingen) and PE-conjugated rat Anti-Human CD104 (β4) (BD Pharmingen) (including the appropriate isotype controls) and resuspended in 0.5ml for a final concentration of 2 million cells/ml. Flow cytometry was conducted at the London Regional Flow Cytometry Facility. Quadrant gates set according to isotype controls were used to determine positive populations; gates set according to the top 20% of the singlet population in the appropriate control cells were used to determine the percentage of $\alpha6\beta4^{high}$ cells in the Nodal knock-down and over-expressing cells.

Cell Growth Assay: Cells were plated into 6-well plates at a density of 1 X 10^4 cells per well in triplicate and incubated in complete medium at 37°C. Cell numbers were determined on days 1, 3, 5, 7 and 9 by hemocytometry. Doubling time was calculated at mid-exponential phase according to the formula: $\mathrm{DT} = \frac{T_f - T_i}{3.32(logN_f - logN_i)}$ where T_i is initial time point, T_f is final time point, N_i is initial cell number, and N_f is final cell number (McAtier & Daview, 1998).

BrdU Cell Proliferation Assay: Cells were plated into 96-well plates at a density of 2 X 10^3 cells per well and incubated for 24h at 37°C. The 5-bromo-20-deoxy-uridine (BrdU) proliferation assay was performed as described by the manufacturer (Roche). Briefly, 20µl/well of BrdU labeling solution was added to cells and incubated for 24hr at 37°C. Cell were then fixed with 100µl fix solution for 1hr at room temperature followed by incubation with anti-BrdU antibody conjugated with peroxidase for 90 min. A substrate

solution was then added to each well, and absorbance was measured at 370nm with a reference wavelength at 492nm using an ELISA plate reader.

Viability/Cytotoxicity Assay: Cells were plated into 96-well plates at a density of 3.25 X 10^2 cells per well and incubated for 1, 3, 5, 7 and 9 days at 37°C to mimic the cell densities of the manual counting growth assay. The LIVE/DEAD assay was performed as described by the manufacturer (Life Technologies). Briefly, dead control wells were treated with DMSO and incubated for 15 min. Live control wells were washed 3X with DPBS. Wells were then labeled with 100 µl/well of appropriate dye solution and incubated for 45 min at room temperature in the dark. Fluorescence was then measured using a microplate reader (FLUOstar Omega, BMG Labtech).

Limiting Dilution Clonogenicity Assay: Cell suspensions were serially diluted in complete medium to a final concentration of 10 cells/ml. The equivalent of one cell, or 100 µl, was added per well of a 96 well plate and incubated at 37°C for 7-14 days. Cells treated with rhNodal or SB431542 were treated on days 0, 3, 6, and 9 if applicable. Clonogenicity was assessed by counting the number of colony positive wells; a well is considered positive when it contains a colony with 32 or more cells.

RT-qPCR: Total RNA was extracted after specific treatments using RNeasy Mini Kit. Respective cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit, and real time quantitative PCR analysis was performed using 2ug of cDNA and TaqMan® gene expression human primer/probe sets. Primer/probes included Nodal

(Nodal; Hs00250630_s1), hCG (CGB; Hs00361224_gH), and VE-Cadherin (CDH5; Hs00174344_m1). HPRT1 primer/probe was from Applied Biosystems (4333768-1005027). To determine the relative quantitation of gene expression, the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$) was used (Pfaffl, 2001). The final mRNA levels were normalized according to their Ct values from the standard curves and expressed in relation to respective HPRT1 level.

Western Blot Analysis: Cells were washed with ice-cold DPBS and lysed in M-PER® lysis buffer supplemented with HALT™ protease inhibitor cocktail. Cell lysates were sonicated, centrifuged and supernatant protein was quantified using the BCA protein assay kit as described by the manufacturer. Equal amounts of protein (5-10ug) were separated on 10% SDS-PAGE gels and transferred to a PVDF membrane. Membranes were blocked in 0.5% non-fat milk in TBS-T (20mM tris-base, 0.14M NaCl, pH 7.8, 0.1% Tween-20) at 4°C, overnight. Membranes were probed for Nodal (Santa Cruz, clone WS65; 100 ng/ml) for 1 hr at room temperature, then washed with TBS-T and incubated in fluorescent-conjugated rabbit or mouse secondary antibodies (LiCor, polyclonal; 50 ng/ml) for one hour at room temperature in the dark. Fluorescence was detected using the Odyssey Infrared Imager (Mandel). All experiments were performed at least 3 times.

Syncytialization: Cells were grown in F-12K medium containing 10% FBS with or without 50uM Forskolin. Syncytialization was quantified according to hCG expression as measured by qRT-PCR and ELISA.

Endothelial-like Tube Formation Assay: Growth factor reduced (GFR) matrigel, diluted 1:1 with basal RPMI 1640 media, was added to 12-well plates and incubated for 1hr at 37°C to solidify. Cells in serum free RPMI were seeded on the matrigel-coated plates (100,000 cells per plate) and incubated at 37°C, 5% CO_2 for 24 hours. Pictures were taken after 18 hours of three random fields of view using a Leica EC3 camera. The total number of branch points was quantified using the NIH ImageJ software.

Gene/miRNA Array: Single stranded complimentary DNA (sscDNA) was prepared from 200 ng of total RNA as per the Ambion WT Expression Kit for Affymetrix GeneChip Whole Transcript WT Expression Arrays and the Affymetrix GeneChip WT Terminal Labeling kit and Hybridization User Manual. Total RNA was converted to cDNA, followed by *in vitro* transcription to make cRNA. 5.5 ug of single stranded cDNA was synthesized, end labeled and hybridized, for 16 hours at 45°C, to Human Exon 1.0 ST arrays. One microgram of total RNA was labeled using the Flash Tag Biotin HSR kit from Genisphere. Samples were then hybridized to Affymetrix miRNA 2.0 arrays for 16 hours at 45°C. All washing steps were performed by a GeneChip Fluidics Station 450 and GeneChips were scanned with the GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA) using Command Console v1.1. Probe level (.CEL file) data was generated using Affymetrix Command Console v1.1. Probes were summarized to miRNA or gene level in Partek Genomics Suite v6.6 (Partek, St. Louis, MO) using the RMA algorithm (Irizarry et al., 2003). Partek was used to determine ANOVA p-values and fold changes for genes and fold changes for miRNAs. Species annotations were added and used to filter only those miRNAs found in *Homo sapiens*. The gene targets for

increased *Homo sapiens* miRNAs were determined using TargetScan v5.2 and correlated with 1.5 fold, p-value 0.05 decreased genes.

Statistical Analysis: Data are presented as mean ± standard error (SEM) for parametric data, and median ± interquartile range (IQR) for nonparametric data. Parametric data was analyzed by a one-way ANOVA followed by a post-hoc TUKEY's test using Graph Pad Prism 5 software, while nonparametric data was analyzed by a one-way ANOVA on ranks followed by a post-hoc Dunn's test using SigmaStat 3.5. Differences between two treatment groups were considered significant at $P < 0.05$.

Chapter Three: Results

Co-Authorship Statement

Tranfections were done by Guihua Zhang.

Experiments in Figures A1 and A3 were done by Charles Zhong and Lynne Postovit.

The α6β4^{high} subset of HTR-8/SVneo expresses higher levels of Nodal protein compared to the α6β4^{low} *subset*

Following the preliminary results suggesting the α6β4^{high} subset of HTR-8/SVneo contains a more phenotypically plastic population compared to the α6β4^{low} and unsorted control cells, we sought to determine whether Nodal expression correlates with this more progenitor-like phenotype. FACS analysis was used to isolate α6β4^{high} and α6β4^{low} subpopulations from HTR-8/SVneo cells (Figure 5-A,B) and Nodal expression was measured at the mRNA and protein level using qRT-PCR and Western blot analysis, respectively (Figure 5-C,D). The α6β4^{high} subpopulation expressed higher levels of Nodal protein compared to the α6β4^{low} subpopulation, but similar levels compared to the unsorted control cells. These results were passage dependent, as later passages exhibited no change in Nodal protein. Interestingly, Nodal protein expression did not correlate with mRNA levels, as there was no significant difference in Nodal mRNA expression between the three populations examined (Figure 5-C).

The α6β4^{high} and α6β4^{low} *subsets of HTR-8/SVneo display equal rates of growth and proliferation*

We next sought to determine whether this progenitor-like phenotype and Nodal protein expression correlated with proliferation. Growth rate was measured via manual counting and proliferation via the BrdU assay. The doubling times for the α6β4^{high} and α6β4^{low} subsets were 23.4 and 26.1 hours, respectively, which were not significantly different ($P = 0.274$, $n = 3$, Figure 6-A). Accordingly, BrdU analysis also revealed no significant difference in proliferation between the α6β4^{high} and α6β4^{low} subsets (Figure 6-B).

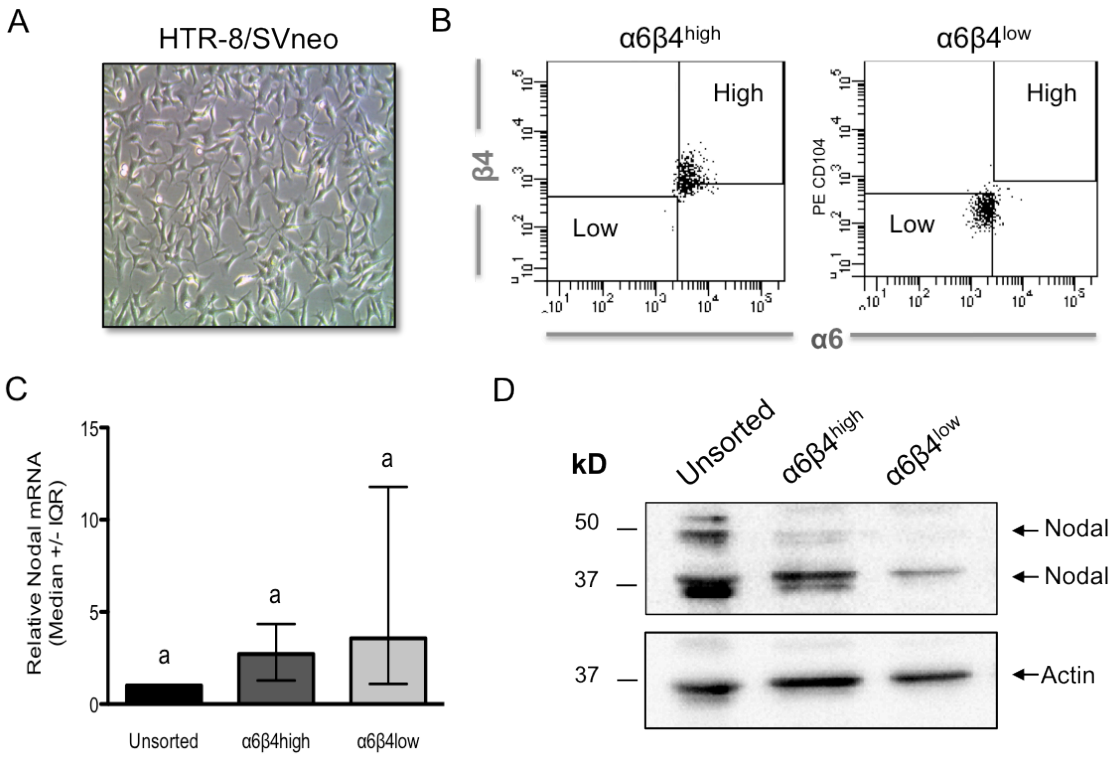

Figure 5. Integrin α6β4^{high} cells express higher levels of Nodal protein compared to α6β4^{low} cells.

HTR-8/SVneo cells (A) were harvested and incubated with IgG isotype controls, FITC conjugated rat anti-human CD49f IgG (specific for α6 integrin chain) and/or PE conjugated rat anti-human CD104 (specific for β4 integrin chain). FACS analysis was used to isolate α6β4^{high} (top 20%) and α6β4^{low} (bottom 20%) of unsorted singlet population (B). RNA was isolated immediately following FACS. cDNA was synthesized and qRT-PCR was used to quantify Nodal mRNA expression relative to the HPRT1 housekeeping gene (C). Nodal mRNA was equivalent within the three cell populations examined. Cell lysates were prepared immediately following FACS and analysed for Nodal protein by Western blot (D). Nodal protein was higher in α6β4^{high} cells compared to α6β4^{low} cells, while lower in α6β4^{low} cells compared to unsorted control cells. qRT-PCR data are represented as a median ± interquartile range (IQR) for three independent experiments. Different characters indicate significant differences ($p<0.05$).

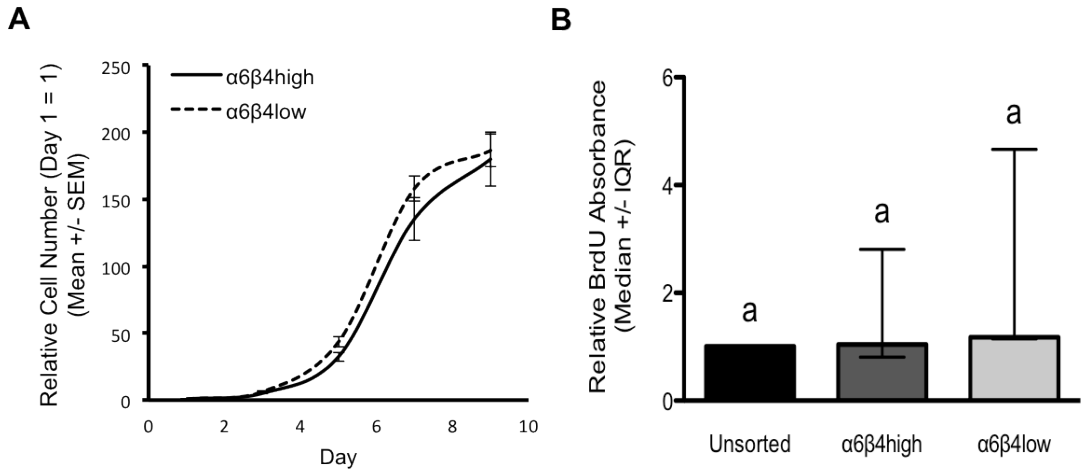

Figure 6. Integrin $\alpha6\beta4^{high}$ cells do not exhibit altered growth rate or proliferation relative to $\alpha6\beta4^{low}$ and unsorted controls.

Integrin $\alpha6\beta4^{high}$ and $\alpha6\beta4^{low}$ subpopulations were isolated by FACS from HTR-8/SVneo cells and plated at 10^4 cells/well in 6-well plates in triplicate and incubated at at 37°C, 5% CO_2. Cells were harvested and manually counted using a haemocytometer on alternate days from day 1 to day 9 (A). The $\alpha6\beta4^{high}$ subpopulation did not exhibit an altered rate of growth relative to the $\alpha6\beta4^{low}$ subpopulation, as the doubling times were 23.3 and 26.1 hours, respectively (P=0.274). Cells were also plated at 2 x 10^3 cells/well of 96-well plates in complete RPMI. Cells were incubated at 37°C, 5% CO_2 for 24 hours. BrdU labeling solution was added to the culture medium and cells were further incubated for 24 hours. Cells were fixed with 100 ul/well fix solution for 30 min at room temperature and incubated with anti-BrdU antibody conjugate with peroxidase for 90 min. A substrate solution was then added into each well, and absorbance was measured (B). Similarly, proliferation in the $\alpha6\beta4^{high}$ cells was not significantly different from that of either $\alpha6\beta4^{low}$ or unsorted control cells. Proliferation data are represented as a median ± interquartile range (IQR) for five independent experiments with each condition measured 8 times. Different characters indicate significant differences ($p<0.05$).

Nodal sustains a higher level of α6β4^{high} expressing cells

As the α6β4^{high} expressing cells were found to express higher levels of Nodal protein relative to the α6β4^{low} expressing cells, we were interested in determining whether Nodal expression correlates with expression of integrin α6β4. HTR-8/SVneo cells stably transfected with Nodal shRNA were generated (Figure 7-A) and flow cytometry analysis was completed for the α6 and β4 integrin chains. Knock-down of Nodal was verified by Western blot analysis (Figure 7-B). Nodal knock-down resulted in a decreased shift in intensity of both α6 and β4 integrin chains (Figure 8-A). Examining the α6 and β4 subunits separately, the median intensity of the α6 subunit decreased by approximately 1.6-fold, while the median intensity of the β4 subunit decreased by 2-fold and was significantly different from controls (Figure 8-B,C,D). Following our observations with Nodal knock-down on the expression of α6 and β4 integrin chains, we were interested in determining the effect of Nodal over-expression. To do so, BeWo cells were transfected with a plasmid containing the Nodal open reading frame (Figure 9-A) and flow cytometry analysis for the α6 and β4 integrin chains was completed as above. BeWo were chosen as previous studies have shown they express low levels of Nodal relative to HTR-8/SVneo. Over-expression of Nodal was verified by Western blot analysis (Figure 9-B). As expected, Nodal over-expression caused an upward shift in the intensity of both α6 and β4 integrin chains (Figure 10-A). The median intensity of the α6 subunit increased 1.7-fold, while that of the β4 subunit increased by 1.6-fold (Figure 10-B,C,D).

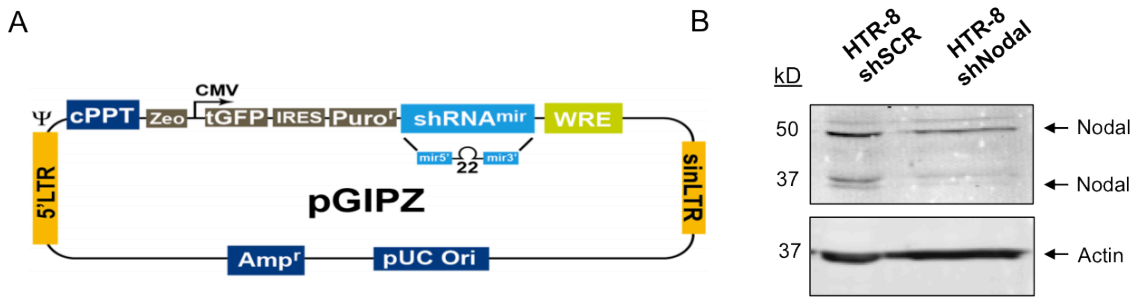

Figure 7. Stable Nodal knock-down in HTR-8/SVneo trophoblast cells.

HTR-8/SVneo cells were harvested and transfected via lipofectamine with a pGIPZ lentiviral vector containing either an $shRNA_{Nodal}$ or $shRNA_{scramble}$ control (A). Cell lysates were prepared and analyzed for Nodal protein by Western blot (B). Nodal protein (50 and 37kDa) decreased in the Nodal knock-down cells (shNodal) compared to scrambled control cells (shSCR).

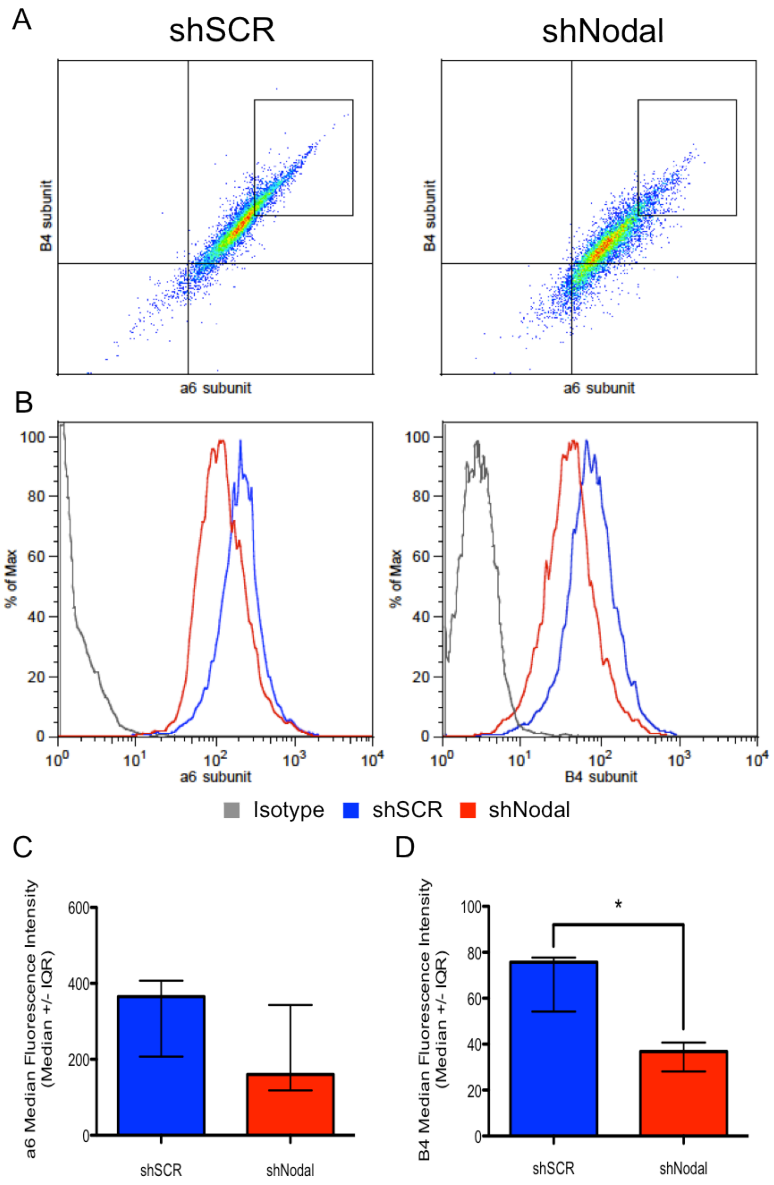

Figure 8. Nodal knock-down decreases expression of integrin α6β4.

HTR-8/SVneo scrambled control (shSCR) and shRNA$_{Nodal}$ knock-down (shNodal) cells were harvested and incubated with IgG isotype controls, PE-Cy5 conjugated rat anti-human CD49f IgG (specific for α6 integrin chain) and/or PE conjugated rat anti-human CD104 (specific for β4 integrin chain). Flow cytometry was used to examine expression of the α6 and β4 integrin subunits. Scatter plots of the intensity of α6 and β4 subunits contain square gates to designate top 20% of control cells and represent α6β4^{high} cells (A). Nodal knock-down caused a decrease in the intensity α6β4 expression. Histograms display intensity of α6 and β4 subunits separately (B). Expression of the α6 and β4 subunits decreased by 1.6 and 2-fold, respectively (C,D). Flow cytometry data are represented as a median ± interquartile range (IQR) for three independent experiments. (*) indicates significant differences ($p<0.05$).

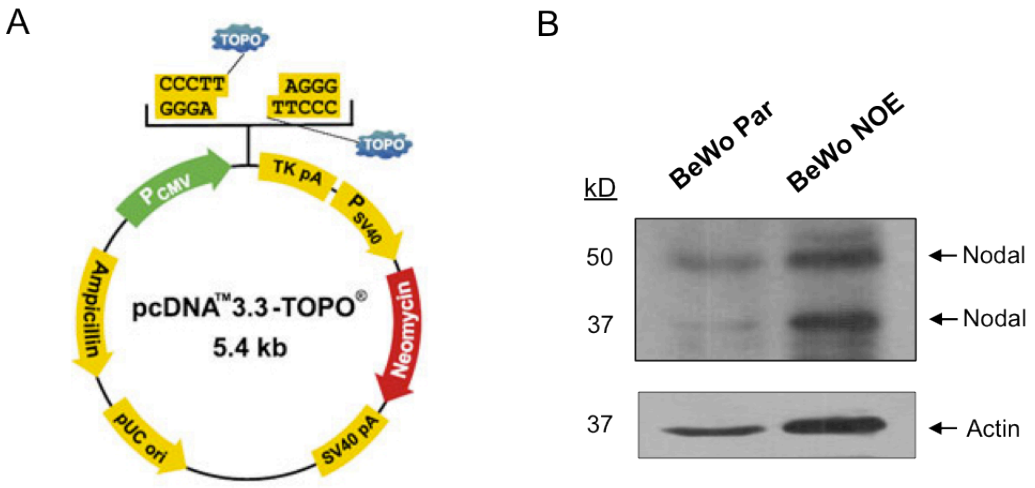

Figure 9. Stable Nodal over-expression in BeWo choriocarcinoma cells.

BeWo cells were harvested and transfected with Arrest-In with a pcDNA3.3-TOPO vector containing the Nodal open reading frame cloned from H9 hESCs (A). Cell lysates were prepared and analyzed for Nodal protein by Western blot (B). Nodal protein (37kDa) increased in the Nodal over-expressing cells (NOE) compared to BeWo parental (BeWo) cells.

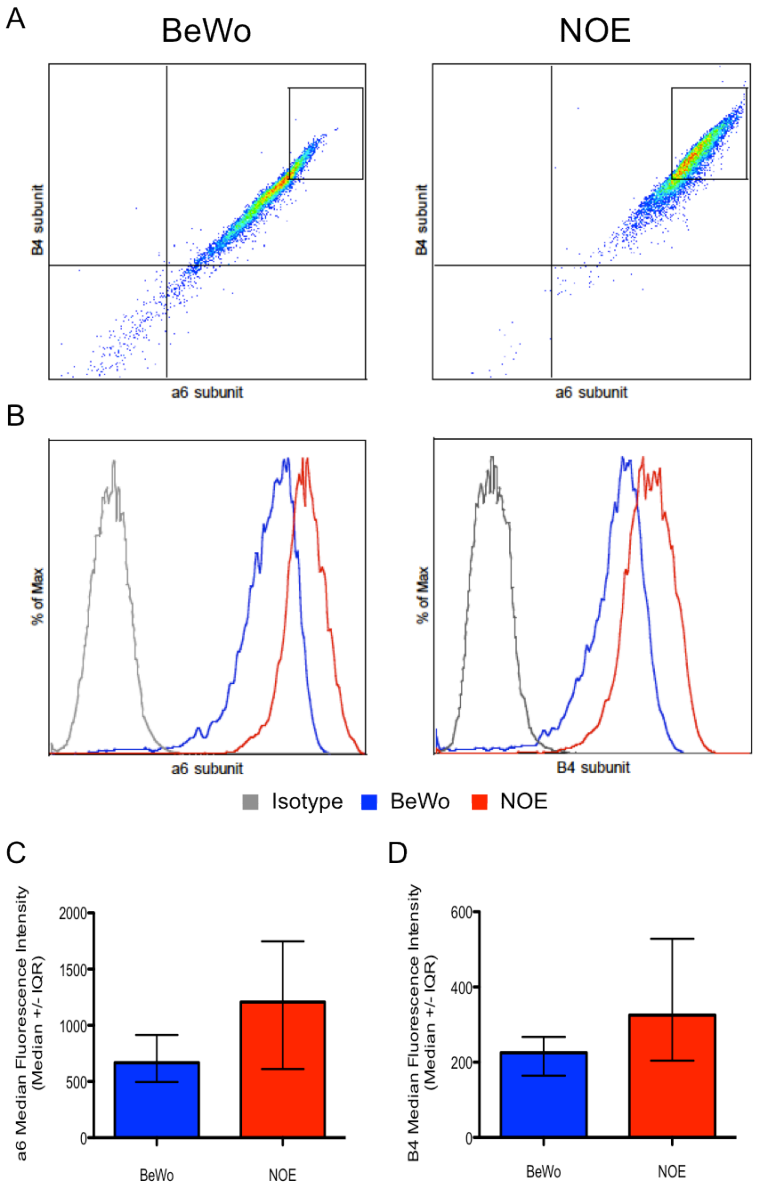

Figure 10. Nodal over-expression increases expression of integrin α6β4.

BeWo parental (BeWo) and Nodal over-expressing (NOE) cells were harvested and incubated with IgG isotype controls, PE-Cy5 conjugated rat anti-human CD49f IgG (specific for α6 integrin chain) and/or PE conjugated rat anti-human CD104 (specific for β4 integrin chain). Flow cytometry was used to examine expression of the α6 and β4 integrin subunits. Scatter plots of the intensity of α6 and β4 subunits contain square gates to designate top 20% of control cells and represent $\alpha6\beta4^{high}$ cells (A). Nodal over-expression caused an increase in the intensity α6β4 expression. Histograms display intensity of α6 and β4 subunits separately (B). Expression of the α6 and β4 subunits increased by 1.7 and 1.6-fold, respectively (C,D). Flow cytometry data are represented as a median ± interquartile range (IQR) for three independent experiments. (*) indicates significant differences ($p<0.05$).

Nodal expression maintains colony forming capacity in HTR-8/SVneo & BeWo

In order to determine if Nodal plays a role in HTR-8/SVneo colony formation, a limiting dilution clonogenic assay was performed. Nodal knock-down significantly decreased HTR-8/SVneo colony forming capacity and this effect of Nodal knock-down was partially rescued via treatment with 100ng/mL rhNodal (Figure 11). Conversely, Nodal over-expression in BeWo significantly increased colony forming capacity and this effect of Nodal over-expression was partially reversed by treatment with 10 μM of the Nodal/Activin inhibitor, SB431542 (Figure 12).

Nodal expression differentially regulates HTR-8/SVneo and BeWo proliferation

As Nodal appears to increase colony formation capacity, we sought to determine if this was associated with an affect on proliferation. Growth and proliferation were assayed by manual counting and the BrdU assay, respectively. Nodal knock-down in HTR-8/SVneo significantly decreased the rate of growth as the doubling time increased from 32.2 to 54.0 hours ($P=0.015$, $n = 3$, Figure 13-A). Conversely, in the BrdU proliferation assay, Nodal knock-down significantly increased proliferation by 12%, while treatment with recombinant human Nodal partially reversed this phenotype, although this was not significant (Figure 13-B). In contrast to HTR-8/SVneo, Nodal over-expression had no significant effect on BeWo doubling time, with BeWo parental cells doubling every 24.5 hours while the Nodal over-expressing cells doubled every 20.3 hours ($P=0.101$, $n = 3$, Figure 14-A). Similarly, BeWo proliferation measured by BrdU assay was also not significantly different (Figure 14-B).

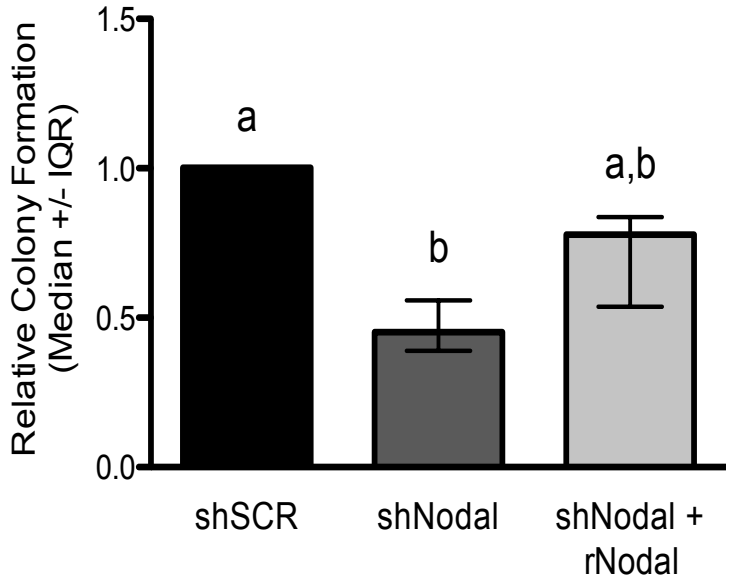

Figure 11. Nodal knock-down reduces HTR-8/SVneo clonogenicity.

HTR-8/SVneo scrambled control (shSCR) and $shRNA_{Nodal}$ knock-down (shNodal) cells were serially diluted and plated at one cell per well of 96 well plate in complete RPMI. shNodal cells were also plated in the presence of 100ng/ml recombinant human Nodal (rNodal). Nodal knock-down significantly decreased colony formation, while the presence of rNodal partially rescued the phenotype. Clonogenicity data are represented as a median ± interquartile range (IQR) for three independent experiments. Different characters indicate significant differences ($p<0.05$).

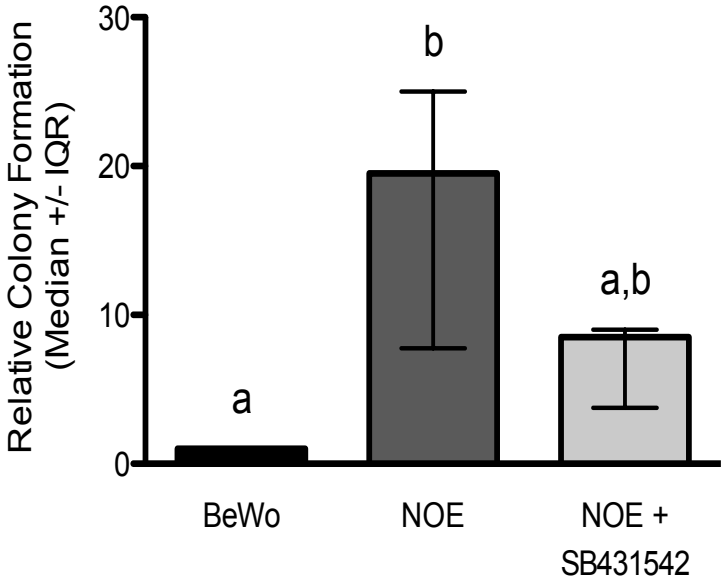

Figure 12. Nodal over-expression increases BeWo clonogenicity.

BeWo parental (BeWo) and Nodal over-expressing (NOE) cells were serially diluted and plated at one cell per well of 96 well plate. NOE cells were also plated in the presence of 10 μM Nodal/Activin inhibitor, SB431542. Nodal over-expression significantly increased colony formation, while the presence of Nodal/Activin inhibitor partially inhibited colony formation. Clonogenicity data are represented as a median ± interquartile range (IQR) for three independent experiments. Different characters indicate significant differences ($p<0.05$).

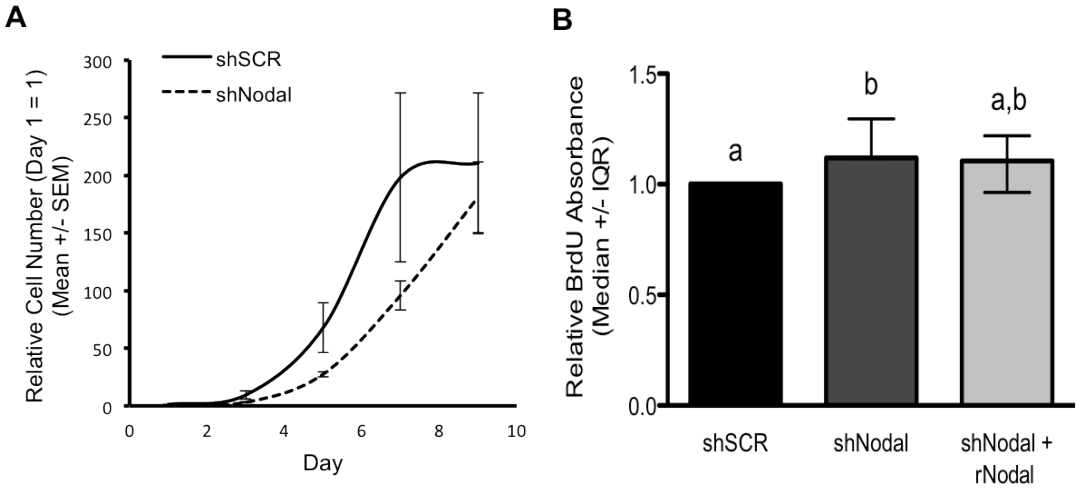

Figure 13. Nodal knock-down decreases HTR-8/SVneo growth rate but increases proliferation.

HTR-8/SVneo scrambled control (shSCR) and $shRNA_{Nodal}$ knock-down (shNodal) cells were plated at 10^4 cells/well in 6-well plates in triplicate and incubated at at 37°C, 5% CO_2. Cells were harvested and manually counted using a haemocytometer on alternate days from day 1 to day 9 (A). Growth rate was determined relative to day 1 cells counts. Nodal knock-down significantly decreased the rate of growth with doubling time increasing from 32.2 to 54.0 hours (P=0.015). Cells were also plated at 2 x 10^3 cells/well of 96-well plates in complete RPMI. Cells were incubated at 37°C, 5% CO_2 for 24 hours. BrdU labeling solution was added to the culture medium and cells were further incubated for 24 hours. Cells were fixed with 100 ul/well fix solution for 30 min at room temperature and incubated with anti-BrdU antibody conjugate with peroxidase for 90 min. A substrate soltion was then added into each well, and absorbance was measured (B). Nodal knock-down significantly increased proliferation, while the Nodal/Activin inhibitor, SB431542 partially rescured this phenotype. BrdU proliferation data are represented as a median ± interquartile range (IQR) for five independent experiments with each condition measured 8 times. Different characters indicate significant differences ($p<0.05$).

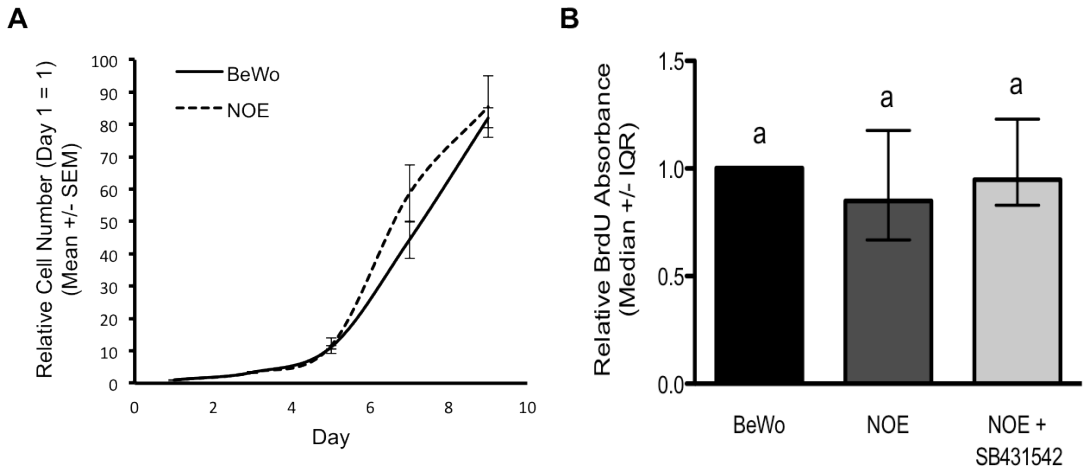

Figure 14. Nodal over-expression does not affect BeWo growth or proliferation.

BeWo parental (BeWo) and Nodal over-expressing (NOE) cells were plated at 10^4 cells/well in 6-well plates in triplicate and incubated at at 37°C, 5% CO_2. Cells were harvested and manually counted using a haemocytometer on alternate days from day 1 to day 9 (A). Growth rate was determined relative to day 1 cell count. Nodal over-expression did not signficantly affect rate of growth. The doubling times for BeWo and Nodal over-expressing cells were 24.5 and 20.3 hours, respectively (P=0.101). Cells were also plated at 2 x 10^3 cells/well of 96-well plates in complete DMEM. Cells were incubated at 37°C, 5% CO_2 for 24 hours. BrdU labeling solution was added to the culture medium and cells were further incubated for 24 hours. Cells were fixed with 100 ul/well fix solution for 30 min at room temperature and incubated with anti-BrdU antibody conjugate with peroxidase for 90 min. A substrate soltion was then added into each well, and absorbance was measured (B). Neither Nodal over-expression or treatment with 10uM Nodal/Activin inhibitor, SB431542, had a significant affect on BeWo proliferation. BrdU proliferation data are represented as a median ± interquartile range (IQR) for five independent experiments with each condition measured 8 times. Different characters indicate significant differences ($p<0.05$).

Nodal differentially regulates proliferation & apoptosis in HTR-8/SVneo and BeWo cells.

Given the results observed between the growth curves and proliferation of HTR-8/SVneo cells, we decided to do a viability/cytotoxicity assay to determine the ratio of metabolically active cells to apoptotic cells. Using the LIVE/DEAD assay, Nodal knock-down in HTR-8/SVneo cells was found to significantly decrease the number of metabolically active cells starting at day 3 (Figure 15-A), while increasing the number of apoptotic cells between days 3 to 5 (Figure 15-B). Of note, there was no difference at day 1 when BrdU assays were conducted. When the cells were at high confluence (days 7 and 9) apoptosis was approximately equal between the control and Nodal knock-down cells. Contrary to the above results, Nodal over-expression in BeWo cells also led to a significant decrease in the number of metabolically active cells starting at day 7 (Figure 16-A), while increasing the number of apoptotic cells on the same day (Figure 16-B). However, at day 1 there was no effect on proliferation, which confirms our BrdU results.

Nodal expression does not affect secreted hCG protein

To determine the effect of Nodal on the ability of HTR-8/SVneo cells to differentiate along the villous pathway, cells were treated with Forskolin (an inducer of syncytialization and hCG production) and RNA and conditioned medium were collected. qRT-PCR and ELISA were conducted for human chorionic gonadotropin (hCG). Regarding hCG mRNA expression, basal and induced expression was greatest after Nodal knock-down in HTR-8/SVneo cells (Figure 17-A). However, secreted hCG was not detected in either shSCR nor shNodal even after treatment with Forskolin (Figure 17-B). In BeWo cells, Nodal over-expression did not alter basal or induced secretion of hCG (Figure 18-A,B).

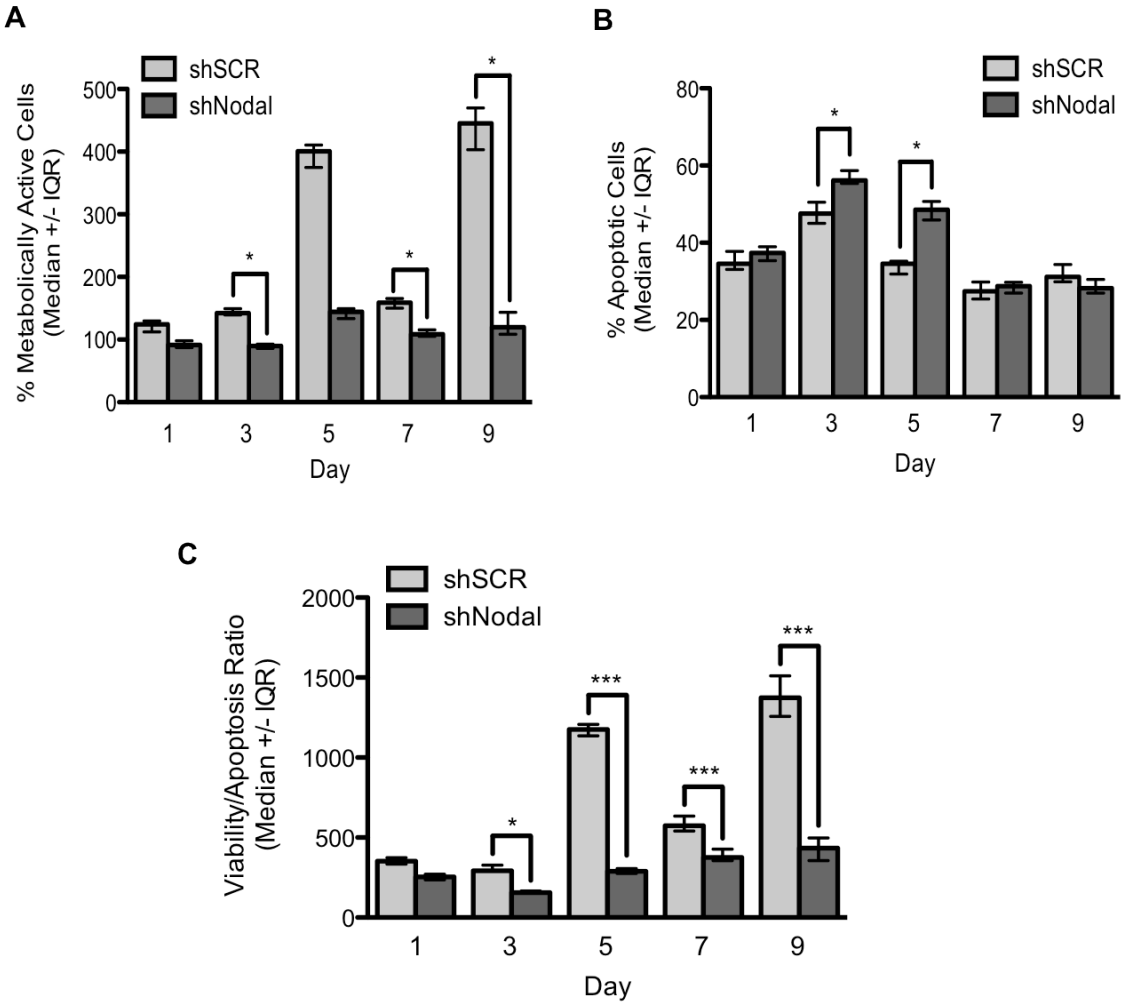

Figure 15. Nodal knock-down decreases the ratio of metabolically active to apoptotic cells in HTR-8/SVneo.

HTR-8/SVneo scrambled control (shSCR) and shRNA$_{Nodal}$ knock-down (shNodal) cells were plated at 3.25 x 10^2 cells/well in 96-well plates (n=8). Cells were incubated at 37°C, 5% and analyzed using the LIVE/DEAD assay on alternate days from day 1 to day 9. Briefly, dead control wells were treated with DMSO and incubated for 15 min. Live control wells were washed 3X with DPBS. Wells were then labeled with 100 µl/well of appropriate dye solution and incubated for 45 min at room temperature in the dark. Fluorescence was then measured using a microplate reader. Nodal knock-down moderately decreased the percentage of metabolically active cells (A) and slightly increased the percentage of apoptotic cells (B). LIVE/DEAD data are represented as median ± interquartile range (IQR) with n=8. Experiment was completed three independent times with similar results. (*) indicates significant differences ($p<0.05$).

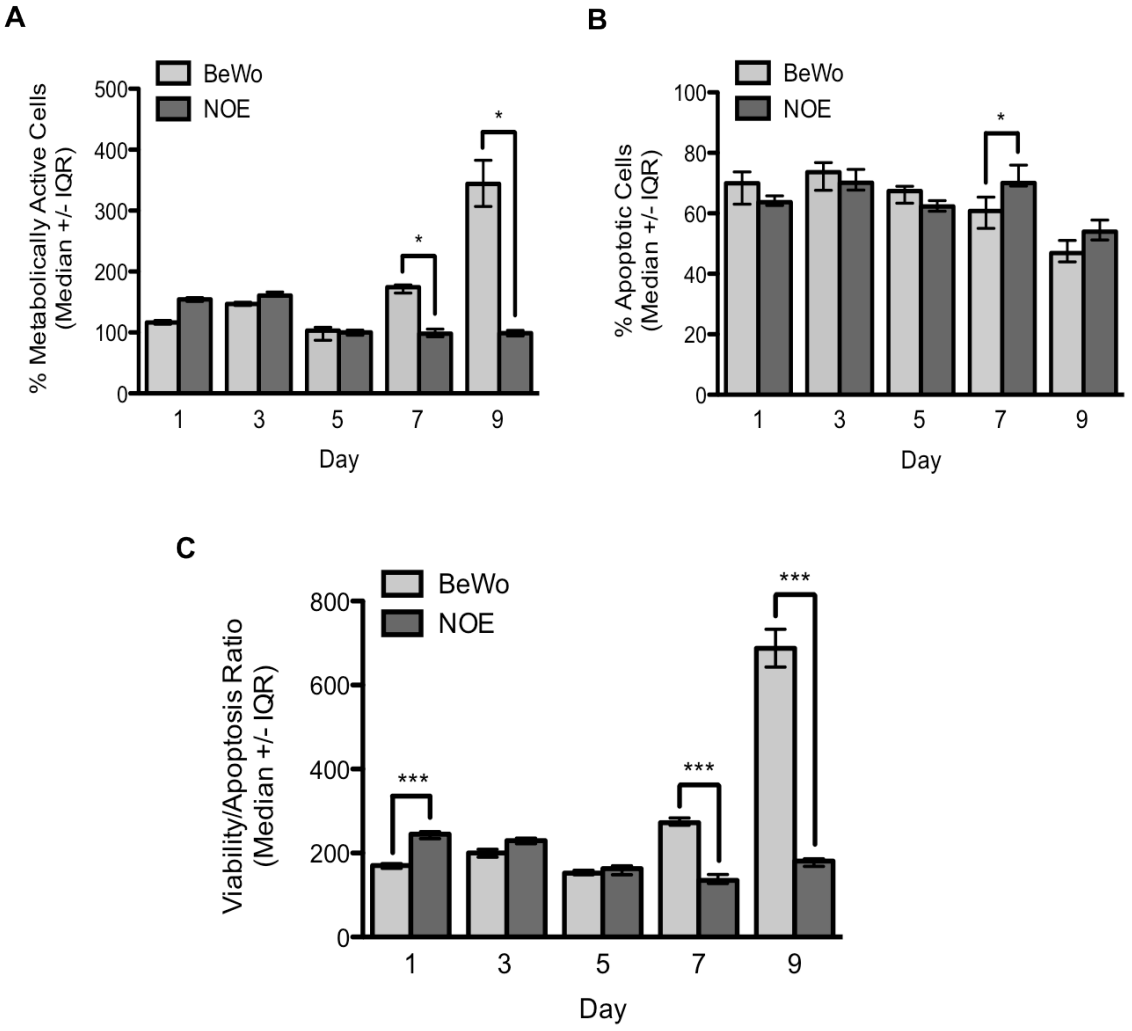

Figure 16. Nodal over-expression decreases the ratio of metabolically active cells to apoptotic cells in BeWo.

BeWo parental (BeWo) and Nodal over-expressing (NOE) cells were plated at 3.25 x 10^2 cells/well in 96-well plates (n=8). Cells were incubated at 37°C, 5% and analyzed using the LIVE/DEAD assay on alternate days from day 1 to day 9. Briefly, dead control wells were treated with DMSO and incubated for 15 min. Live control wells were washed 3X with DPBS. Wells were then labeled with 100 µl/well of appropriate dye solution and incubated for 45 min at room temperature in the dark. Fluorescence was then measured using a microplate reader. Nodal over-expression decreased the percentage of metabolically active cells (A) however had no significant effect on the percentage of apoptotic cells (B). LIVE/DEAD data are represented as median ± interquartile range (IQR) with n=8. Experiment was completed three independent times with similar results. (*) indicates significant differences ($p<0.05$).

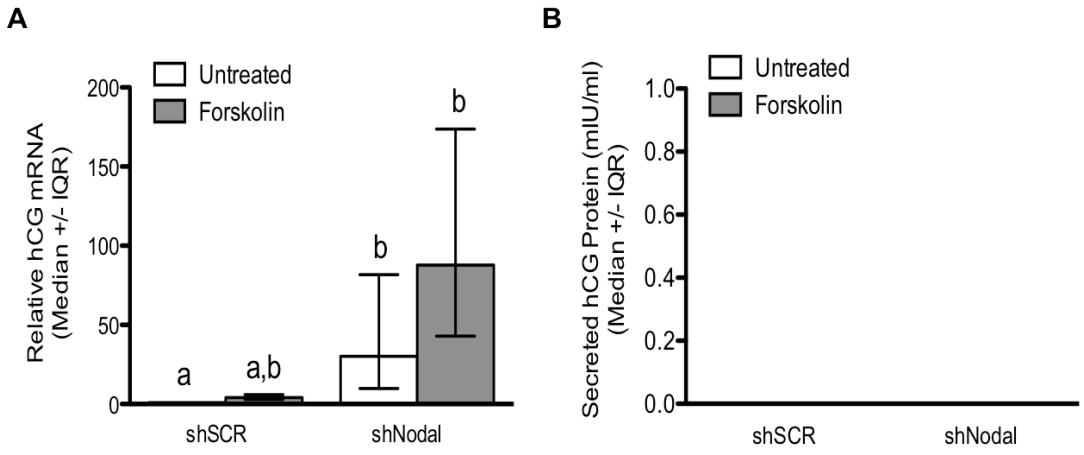

Figure 17. Nodal knock-down increases hCG mRNA but not secreted protein in HTR-8/SVneo cells.

HTR-8/SVneo scrambled control (shSCR) and $shRNA_{Nodal}$ knock-down (shNodal) cells were plated in 6-well plates in complete F-12K medium with or without 50 uM Forskolin and incubated at 37°C, 5% CO_2 for 48 hours. Conditioned medium was collected and RNA was extracted. qRT-PCR was used to quantify human chorionic gonadotropin (hCG) mRNA (A). hCG mRNA expression increased with Nodal knock-down. ELISA was used to quantify secreted hCG protein (B). Secreted hCG was not detected in either shSCR or shNodal cells. Data are represented as a median ± interquartile range (IQR) for three independent experiments with each condition being tested in triplicate. Different characters indicate significant differences ($p<0.05$).

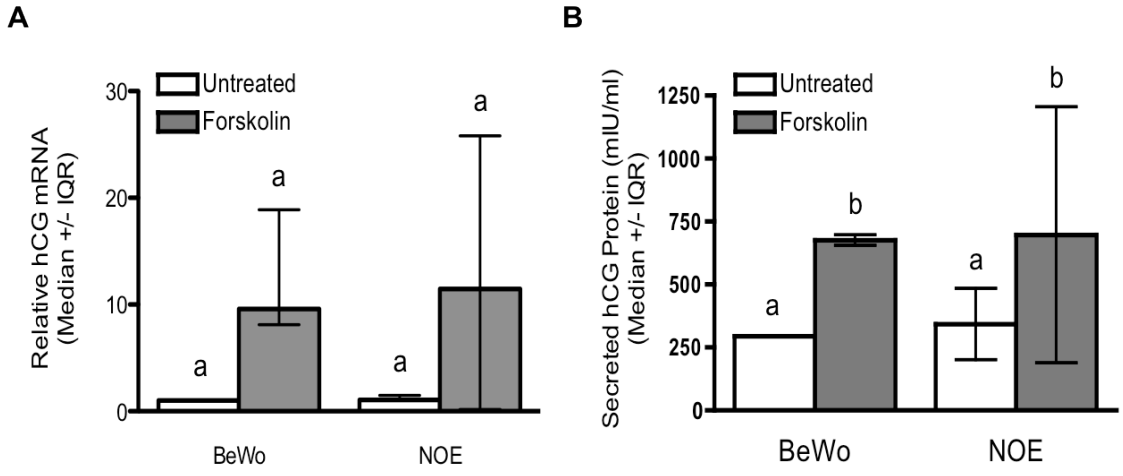

Figure 18. Nodal over-expression does not affect hCG expression in BeWo cells.

BeWo parental (BeWo) and Nodal over-expressing (NOE) cells were plated in 6-well plates in complete F-12K medium with or without 50 uM Forskolin and incubated at 37°C, 5% CO_2 for 48 hours. Conditioned medium was collected and RNA was extracted. qRT-PCR was used to quantify human chorionic gonadotropin (hCG) mRNA (A). Foskolin treatment did not significantly increase hCG mRNA in either cell type. ELISA was used to quantify secreted hCG protein. Forskolin treatment did increase hCG secretion in both BeWo and NOE, however basal and induced amounts were equivalent between the two cell lines. Data are represented as a median ± interquartile range (IQR) for three independent experiments with each condition being tested in triplicate. Different characters indicate significant differences ($p<0.05$).

Forskolin treatment increased hCG mRNA expression as well as secreted protein comparably between parental and Nodal over-expressing cells (Figure 18-A,B).

Nodal expression is required for endothelial-like tube formation capacity.

To determine the effect of Nodal on the ability of HTR-8/SVneo cells to differentiate along the extravillous (endovascular) pathway, a vascular-like tube-formation assay was performed along with qRT-PCR for VE-Cadherin mRNA. Nodal knock-down significantly decreased the ability of HTR-8/SVneo cells to form tubes on Matrigel (Figure 19-A,B). Nodal knock-down also decreased the expression of VE-Cadherin mRNA relative to HTR-8/SVneo scrambled controls (Figure 19-C). In agreement with what was observed in the Nodal knock-down HTR-8/SVneo cells, Nodal over-expression in BeWo led to increased tube formation capacity relative to parental control cells (Figure 20-A,B). VE-Cadherin expression, however, did not increase in BeWo Nodal over-expressing cells compared to BeWo parental cells (Figure 20-C).

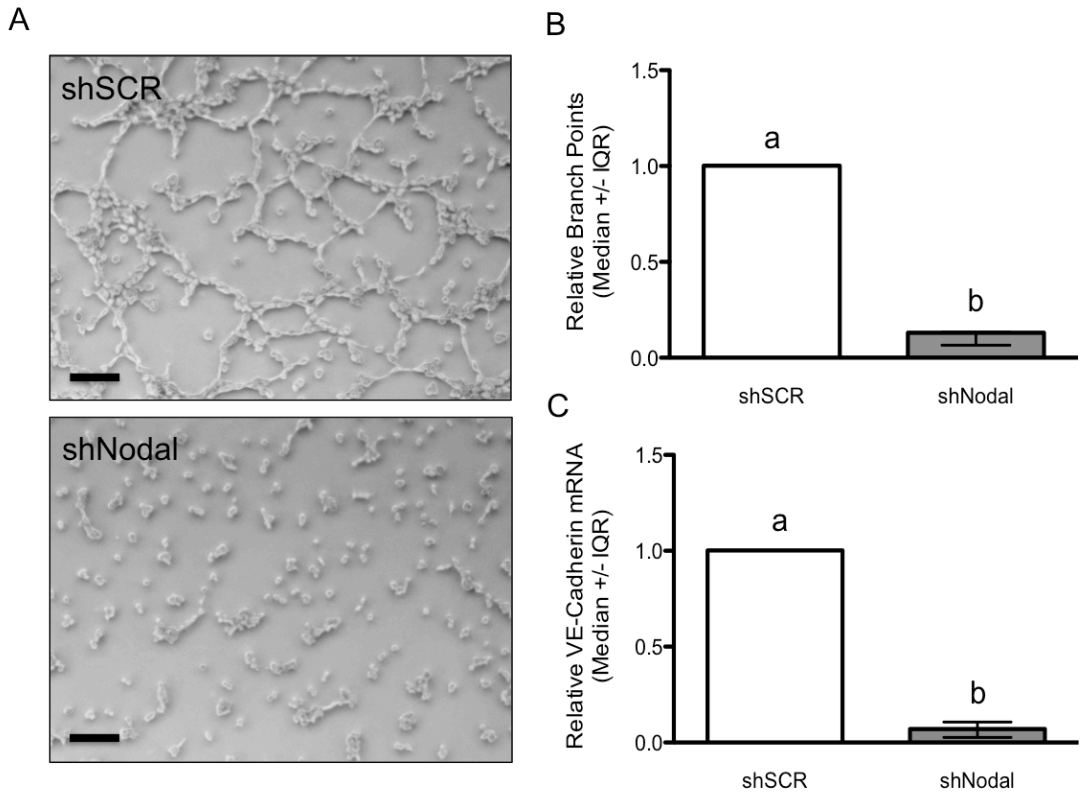

Figure 19. Nodal is required for HTR-8/SVneo tube-formation and VE-Cadherin expression.

HTR-8/SVneo scrambled control (shSCR) and $shRNA_{Nodal}$ knock-down (shNodal) cells were seeded on 12-well plates coated with a thin layer of diluted growth factor reduced (GFR) Matrigel^{TM} in serum-free medium. Cells were incubated at 37°C, 5% CO_2. Pictures were taken after 18 hours of incubation (A). Scale bar represents 100 µm. Tube formation decreased with Nodal knock-down. The extent of tube formation was measured by quantifying the number of branch points using ImageJ software (B). RNA was extracted from HTR-8/SVneo shSCR and shNodal cells. cDNA was synthesized and qRT-PCR was used to quantify VE-Cadherin mRNA (C). VE-Cadherin expression decreased with Nodal knock-down. Data are represented as a median ± interquartile range (IQR) for three independent experiments with each condition being tested in triplicate. Different characters indicate significant differences ($p<0.05$).

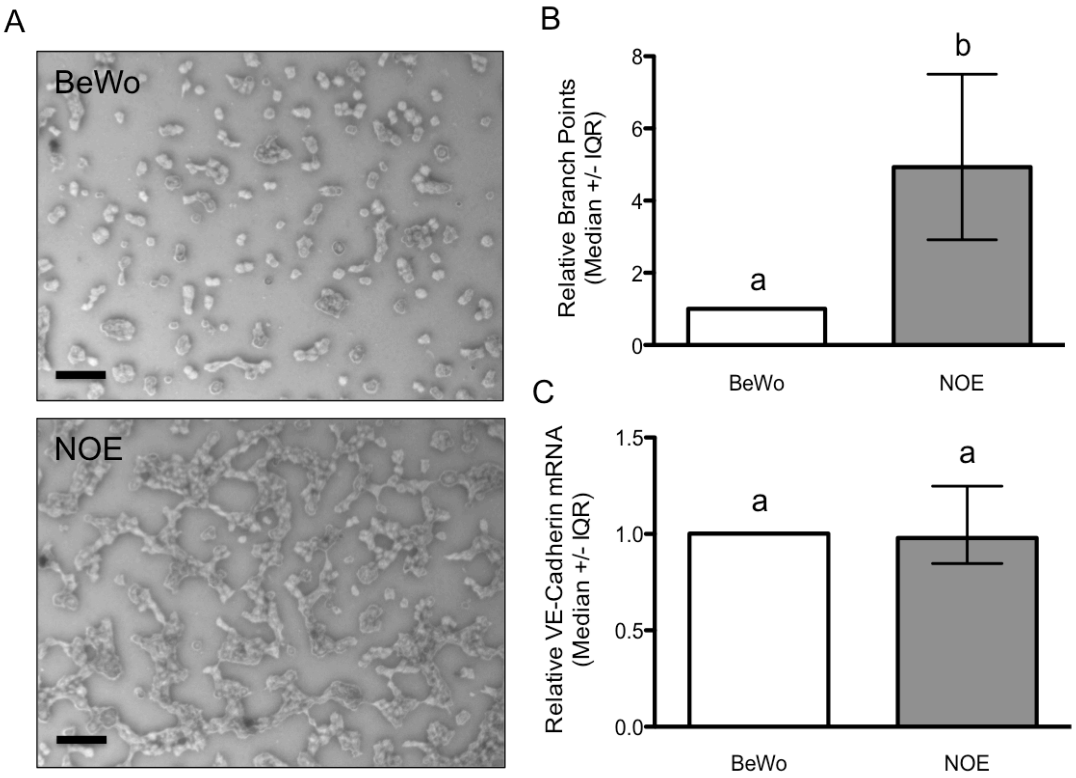

Figure 20. Nodal over-expression increases BeWo tube-formation.

BeWo parental (BeWo) and Nodal over-expressing (NOE) cells were seeded on 12-well plates coated with a thin layer of diluted growth factor reduced (GFR) Matrigel^{TM} in serum-free medium. Cells were incubated at 37°C, 5% CO_2. Pictures were taken after 18 hours of incubation (A). Scale bar represents 100 μm. Tube formation increased with Nodal over-expression. The extent of tube formation was measured by quantifying the number of branch points using ImageJ software (B). RNA was extracted from BeWo and NOE cells. cDNA was synthesized and qRT-PCR was used to quantify VE-Cadherin mRNA (C). VE-Cadherin expression did not increase with Nodal over-expression. Data are represented as a median ± interquartile range (IQR) for three independent experiments with each condition being tested in triplicate. Different characters indicate significant differences ($p<0.05$).

Chapter Four: Discussion

The placenta is an organ required by Eutherian mammals for the successful development of the fetus during pregnancy (reviewed by (Herr et al., 2010)). The human placenta develops from highly proliferative and phenotypically plastic cells called trophoblasts, which are derived from the trophectoderm of the pre-implantation blastocyst (Lala & Hamilton, 1996). Cytotrophoblast stem cells, which adhere to the basement membrane of chorionic villi via the integrin α6β4, self-renew and differentiate along the villous and extravillous pathways to form the specific tissues of the placenta (Huppertz, 2008). When the mechanisms regulating this self-renewal and differentiation are disrupted, various placental disorders can occur, including impaired implantation and preeclampsia. Due to a lack of suitable cytotrophoblast models, the factors responsible for regulating these processes have not been fully elucidated.

Recent work in our lab has shown that a subpopulation of HTR-8/SVneo cells expresses α6β4 integrin and that an $\alpha6\beta4^{high}$ subset can be isolated using FACS (Zhong et al., 2008). This subset exhibits significantly higher colony forming capacity, a measure of self-renewal, as well as enhanced ability to differentiate along the villous pathway, as indicated by increased hCG expression, as well as the extravillous pathway, illustrated by enhanced invasion, VE-Cadherin expression, and endothelial-like tube formation (Zhong et al., 2008).

The present study aimed to further characterize this $\alpha6\beta4^{high}$ cytotrophoblast progenitor subpopulation in the HTR-8/SVneo cell line, as well as to determine the role of Nodal on trophoblast self-renewal, proliferation, and differentiation capacity. Utilizing FACS and Western blot analysis, I discovered that the $\alpha6\beta4^{high}$ subset expresses higher

levels of Nodal protein, relative to the $\alpha6\beta4^{low}$ subset. Subsequently, I examined loss and gain of functions of Nodal and determined that Nodal maintains high levels of $\alpha6\beta4$ and is required for trophoblast self-renewal and differentiation along the extravillous pathway, however does not appear to affect differentiation along the villous pathway. While the current findings lend support for my hypothesis, further work will be required to determine if these cells are indeed bi-potential with the capacity to differentiate along the villous pathway in addition to the extravillous pathway.

4.1 HTR-8/SVneo & BeWo: Models for Trophoblast Self-Renewal & Differentiation

Studies examining the biology of trophoblasts have traditionally utilized primary trophoblast cultures produced from the enzymatic dispersion of placental tissue (Kliman et al., 1986) or short-lived primary cell lines generated from explants of first trimester chorionic villi (Graham et al., 1992; Irving et al., 1995). The use of such cultures, however, is often not suitable for certain studies due to their limited lifespan and inherent heterogeneity in enzyme-dispersed cells (Graham et al., 1993). Primary cell lines produced by the explant method live for around 3-4 passages, 12-15 passages in the case of the HTR-8 line (Irving et al., 1995). As a result, trophoblast cell lines generated from normal, immortalized cells or malignant trophoblast (choriocarcinoma) cells have been used to study trophoblast biology and placental development (King et al., 2000).

This study employed the immortalized human trophoblast cell line, HTR-8/SVneo, which is widely used to study trophoblast biology. These cells were immortalized by introducing the simian virus 40 (SV40) large T antigen into the short-lived first trimester EVT cell line, HTR-8 (Graham et al., 1993). The parental HTR-8 was generated by propagation of EVT cells migrating out of first trimester chorionic villus

explants. The immortalized HTR-8/SVneo cells are morphologically similar to parental (HTR-8) cells, exhibiting an epithelial phenotype, as well as cytokeratin 7 and hCG expression. They also retained the ability to be regulated by TGF-β, a function that is often lost in malignant choriocarcinoma cells (Graham et al., 1993). Expression of class 1 MHC subclass HLA-G is a well-known marker for the EVT lineage. The HLA-G gene had previously been cloned from HTR-8 cells (Zdravkovic et al., 1999). HTR-8 and HTR-8/SVneo cells at earlier passages were previously shown to express HLA-G (Zdravkovic et al., 1999; Kalkunte et al., 2008; Khanet et al., 2010). The HTR-8/SVneo cells used in the current study appear to have lost HLA-G expression (Appendix, Figure A4). These results are similar to the findings of the loss of HLA-G expression by tumour cells in culture in the case of melanoma (Rouas-Freiss et al., 2005), ovarian carcinoma (Malmberg et al., 2002), renal cell carcinoma (Bukur et al., 2003) and gastric cancer (Ye et al., 2009) cells. The down-regulation observed in gastric cancer has been attributed to hypermethylation of the HLA-G gene promoter (Ye et al., 2009). Thus, a similar mechanism may have mediated the silencing of HLA-G in the presently used HTR-8/SVneo cells.

The choriocarcinoma cell line, BeWo, was selected for the Nodal over-expression studies as these cells express low basal levels of Nodal protein and exhibit a more differentiated phenotype, making them a suitable model for examining whether enhanced Nodal expression can revert cells toward a less differentiated, progenitor cell type. This cell line was established from a trophoblastic tumour of the placenta and produces progesterone, hCG, and placental lactogen in culture. While choriocarcinoma cell lines

have been used extensively to study trophoblast biology, in certain circumstances they may behave differently from normal trophoblasts due to their malignant phenotype.

4.2 Nodal Signaling & Cytotrophoblast Progenitors

The strict regulation of cytotrophoblast progenitor self-renewal and differentiation is a critical component in the establishment of successful pregnancy. Our results suggest that Nodal may play a key role in this process, as Nodal protein level was found to be elevated in our cytotrophoblast progenitor model, the $\alpha6\beta4^{high}$ subpopulation in HTR-8/SVneo, relative to the $\alpha6\beta4^{low}$ subpopulation. Accordingly, Nodal and members of the Nodal signaling pathway have been associated with various stem and progenitor cell types, including human embryonic and mouse epiblast stem cells, mammary gland progenitors, cancer stem-cell phenotypes (e.g. breast and prostate), as well as trophoblast stem cells (Vallier et al., 2009; Strizzi et al., 2008; Meyer et al., 2009; Lonardo et al., 2011; Guzman-Ayala et al., 2004). The Fisher group (2011) recently implicated the importance of Nodal signaling in the maintenance of trophoblast stem cells. They found that the Nodal/Activin inhibitor, SB431542 was required to derive trophoblast progenitors able to differentiate along the villous and extravillous pathways, suggesting that Nodal may function to maintain a more undifferentiated trophoblast stem cell (Genbacev et al., 2011). It is possible that our $\alpha6\beta4^{high}$ subpopulation encompasses this more undifferentiated trophoblast stem cell population; however further studies will be required to demonstrate this. These studies may include examining the expression of trophoblast stem cell associated transcription factors, including ELF5, Cdx2, and Eomes to determine how stem-like this population is. Additionally, the epigenetic signature of this subpopulation may be examined to determine whether or not the specific epigenetic

marks characteristic of trophoblast stem cells are retained (Dahle et al., 2010). In the current study, we attempted to determine if the miRNA expression profile of this subpopulation differed from that of the $\alpha6\beta4^{low}$ subpopulation using a combined miRNA/exon array, and whether these changes were characteristic of more stem-like trophoblasts (Appendix, Table A2). While differences were observed between the two populations, none of our increased miRNA were cited in the literature as being associated with trophoblast stem cells. These results will hopefully help direct future studies into the specific miRNA expression pattern of trophoblast stem/progenitor cells.

In addition to enhanced Nodal expression in our $\alpha6\beta4^{high}$ cytotrophoblast subpopulation, we also found that in both HTR-8/SVneo and BeWo cells, Nodal increases expression of the integrin α6 and β4 subunit chains resulting in an apparent increase in the percentage of $\alpha6\beta4^{high}$ cells. While this is an indirect measurement utilizing integrin subunits, heterodimer surface expression can be inferred from such studies as (1) individual α or β subunits are not transported to the cell surface (Springer et al., 1987; Heino et al., 1989), (2) α6 and β4 co-localization has been confirmed using immunocytochemistry in HTR-8/SVneo, and (3) while α6 may form heterodimers with β1 and β4, immunoprecipitation studies show that α6 preferentially associates with β4 rather than β1 (Hemler et al., 1989).

While α6β4 may function as an adhesion molecule on the basal surface of the cytotrophoblast progenitor population for laminin-5 molecules on the basement membrane *in situ,* this integrin may also maintain the self-renewal capacity of the progenitors. Increased clonogenicity of $\alpha6\beta4^{high}$ trophoblast cells even in the absence of

laminin coating of the wells in our study supports the second possibility. This is consistent with recent reports in other tissues. For example, a stem cell associated function has been ascribed to α6β4 for a small subset of progenitor cells in the adult lung alveolar epithelium, capable of self-renewal and differentiation into mature cell types *in vitro* and *in vivo* (Chapman et al., 2011). Furthermore, α6 has been reported to be associated with stem cell functions in mesenchymal (Lee et al., 2009; Yu et al., 2012), mammary epithelial (Stingl et al., 2006), and hematopoietic stem cells (Qian et al., 2006). In the case of mesenchymal stem cells, this has been shown to be via SOX2 and OCT4 (Yu et al., 2012). These studies combined with our results suggest that Nodal may mediate the maintenance of cytotrophoblast stem cells, at least in part through the expression of integrin α6β4.

Proper regulation of integrin α6β4 expression is important for implantation and normal placental development. During implantation, trophoblasts modulate their integrin expression profile to bind specific laminins and fibronectins expressed in the decidua and thereby derive the traction required for migration. Zhou et al. (1993) have shown that regulation of integrin expression is deregulated in preeclamptic placentae, which exhibit limited trophoblast invasion. They found that invasive extravillous trophoblast cells fail to down-regulate integrin α6β4, which characterizes non-migratory, epithelial cells, including villous cytotrophoblast and is not associated with an invasive phenotype (Zhou et al., 1993). Additionally, these cells fail to up-regulate integrin α1β1, a fibronectin receptor normally associated with invasive EVT cells. Thus, this altered integrin expression pattern in preeclamptic trophoblast may underlie the impaired trophoblast invasion associated with this syndrome. By extension, deregulated Nodal signaling may

be contributing to this altered integrin expression profile. Further studies into the regulation of trophoblast integrin expression by Nodal may thus prove important for discovering therapeutic treatments for this disease.

4.3 Nodal & Trophoblast Self-Renewal/Proliferation

An important hallmark of cytotrophoblast progenitors is the ability to self-renew and proliferate. We found that Nodal appears to regulator self-renewal in both HTR-8/SVneo and BeWo cells. In the mouse, Nodal signaling in the epiblast has been shown to sustain Oct4 and Fgf4 transcription, thereby maintaining trophoblast stem cell self-renewal in the adjacent extraembryonic ectoderm (Guzman-Ayala et al., 2004). In contrast, Nodal appears to differentially regulate growth and proliferation in HTR-8/SVneo and BeWo cell lines. In the former, Nodal knock-down decreased the rate of growth while minimally increasing proliferation. This discrepancy was explained by an increase in apoptosis in the Nodal knock-down cells. Thus, while Nodal decreased proliferation, it simultaneously decreased apoptosis resulting in an overall elevated metabolically active to apoptotic cell ratio in HTR-8/SVneo. These results support our hypothesis that Nodal maintains a proliferative cytotrophoblast progenitor population. These findings are also in accordance with previous reports that TGF-β/Activin signaling maintains proliferation of mouse trophoblast stem cells (Erlebacher et al., 2004). This study, using the mouse trophoblast cell line, TSInk4a, demonstrated that TGF-β or Activin in the presence of FGF4 is necessary and sufficient to maintain trophoblast stem cell proliferation as measured by manual counting over 9 days. In an apparent paradox, TGF-β normally inhibits cell-cycle-progression in nontransformed epithelial cells, by regulating the transcription of cell-cycle regulators, including downregulation of c-Myc, a

ubiquitous transcription factor critical for cell-cycle progression, and Id family members, which promote cell cycle progression and inhibit cell differentiation via basic helix-loop-helix transcription factors (Chen et al., 2002; Kang et al., 2003). As such, Erlebacher and colleagues (2004) went on to show that FGF4 signaling prevents TGF-β induced down-regulation of c-Myc, thereby elucidating a mechanism by which TGF-β can act as a growth promoter rather than inhibitor in trophoblast stem cells. It is thus possible that Nodal is acting in concert with other factors to maintain trophoblast proliferation in HTR-8/SVneo.

Conversely, we discovered that Nodal did not significantly alter growth rate or proliferation in BeWo cells, however at high confluence and stress, Nodal over-expression decreased the number of metabolically active cells while increasing the number of apoptotic cells. One potential explanation for the discrepancy in Nodal function between HTR-8/SVneo and BeWo is the malignant nature of the BeWo cell line. It has previously been demonstrated that TGF-β signaling can become deregulated in choriocarcinoma cells. Graham et al. (1994) showed that while TGF-β inhibited proliferation and invasion in the normal HTR-8/SVneo trophoblast cells, the choriocarcinoma cell lines, JAR and JEG-3 were unresponsive to TGF-β (Graham et al., 1994). It has been suggested that a potential mechanism by which tumorigenic cells escape TGF-β induced growth arrest is via mutation of Smads, or the acquisition of oncogenic proteins capable of inhibiting Smad function (Massague et al., 2000; Derynck et al., 2001). For example, Smad3 expression was found to be lost in JAR and JEG-3 choriocarcinoma cells and generation of a Smad3-restituted JAR cell line partially restored its response to TGF-β (Xu et al., 2003). BeWo cells, however, have been shown

to express both Smad2 and Smad3 mRNA (Munir et al., 2004). Thus, while it is possible that tumorigenic transformation has rendered BeWo cells resistant to Nodal-induced proliferation, the mechanism is unlikely to be due entirely to defects at the level of the receptor or Smad signaling pathway, as Nodal is functional in regulating clonogenicity and tube-formation in these cells. Further studies will be required to elucidate the mechanisms underlying the resistance of BeWo cells to Nodal-induced growth.

A study by Munir et al. (2004) stated that Nodal inhibits proliferation and stimulates apoptosis in trophoblast cells. While these results contradict our findings in HTR-8/SVneo, the majority of their work was conducted on the choriocarcinoma cell line, JEG-3. Accordingly, their results more closely reflect those observed here in BeWo cells. Additionally, while they show that Nodal or constitutively active ALK7 (caALK7) decreases the number of metabolically active HTR-8/SVneo cells in an MTT assay, they failed to examine simultaneous rates of apoptosis. They instead examined expression of p27, Cdk2, cyclin E and cyclin D1 by Western blot. Their data show that in both JEG-3 and HTR-3/SVneo cells, both Nodal and caALK7 increased expression of p27 and decreased expression of cyclin D1 and Cdk2, (Munir et al, 2004). While these results suggest impaired cell cycle progression, they do not measure overall cell growth, which may explain the discrepancy as our results show that while Nodal decreases proliferation in HTR-8/SVneo cells, it simultaneously decreases apoptosis, resulting in an overall stimulatory effect on growth.

Alternatively, these differences may be a result of concentration dependent effects of Nodal. The study by Munir over-expressed Nodal in cells that express high endogenous levels of Nodal, while here we knock-down Nodal in cells that express high

endogenous Nodal and over-expressed Nodal in cells that express low endogenous Nodal. Therefore, these studies may be highlighting a potential concentration-dependent signaling mechanism. Work by other members of our lab showed that treatment of T47D and MCF-7 breast cancer cells or HTR-8/SVneo trophoblast cells with 0, 50, 100 or 500 ng/ml of rhNodal resulted in bi-phasic migration response, with low levels of rhNodal stimulating migration while higher levels reduced migration to basal levels (Quail et al., 2011; Law et al, 2011). Such concentration dependent effects of Nodal signaling are also seen in zebrafish and mouse development, where different levels of Nodal signaling induce different genes and thus cell fates; for example, in the mouse, high levels of Nodal signaling are thought to be required for node and anterior mesendoderm formation, while lower levels induce formation of posterior mesoderm (Lowe et al., 2001). Further work is needed to clarify exactly how Nodal signaling affects trophoblast self-renewal and proliferation, and whether concentration-dependent affects are involved.

4.4 Nodal & Trophoblast Differentiation

Currently, the full complement of factors responsible for maintaining the multipotent state as well as directing the differentiation of human cytotrophoblast progenitor cells remains unknown. Our results suggest that Nodal may be an important factor that allows trophoblasts to respond to microenvironmental cues directing differentiation. We show that Nodal is critical for enabling both HTR-8/SVneo and BeWo cells to adopt an endothelial-like tube morphology in response to the matrix components provided by Matrigel. This is reminiscent of the epithelial-to-endothelial transition that occurs as cytotrophoblast cells invade and remodel spiral arteries along the extravillous pathway. Accordingly, it is well established that Nodal maintains

pluripotency in human embryonic stem cells and mouse epiblast stem cells via upregulation of the key pluripotency factor, Nanog (Vallier et al., 2009). Thus, Nodal may similarly upregulate key multipotency factors in trophoblast stem cells. Additionally, work in the mouse suggests that Nodal also directs trophoblast differentiation (Ma et al., 2001). Work with Nodal null mutants revealed complete loss of spongiotrophoblast and labyrinth layers concomitant with expansion of the giant cell layer. Conversely, decreased Nodal signaling in a hypomorphic mutant led to expansion of the giant cell and spongiotrophoblast layers (Ma et al., 2001). Furthermore, expression of Nodal in the rat choriocarcinoma cell line, Rcho-1 was found to inhibit trophoblast giant cell differentiation (Ma et al., 2001). Taken together, these findings suggest that in the mouse, Nodal directs trophoblast fate towards the labyrinth lineage (homologous to the human syncytiotrophoblast), while maintaining the thin layers of giant cells and spongiotrophoblast (the human cytotrophoblast homologue) (Ma et al., 2001).

It has recently been suggested that dose-dependent Nodal signaling strictly balances mouse trophoblast self-renewal and differentiation in conjunction with the alleged Smad4 inhibitor, ectodermin (Ecto or TRIM33) (Morsut et al., 2010). Utilizing $Ecto^{-/-}$ mutants, Morsut and colleagues (2010) demonstrate that "unrestrained" Nodal/Smad4 signaling leads to trophoblast differentiation (indicated by upregulation of 4311 and GCM1) and depletion of the trophoblast stem cell compartment (indicated by loss of Eomes, Cdk2, and Bmp4). These authors concluded that Nodal signaling functions to stimulate trophoblast differentiation, while Ecto inhibits this to promote self-renewal, thus governing the expansion and differentiation of trophoblast progenitors (Morsut et al., 2010). While this study provides a nice mechanism for the dose regulation

of Nodal signaling, the purported role of Ecto as a Nodal/Smad4 inhibitor remains controversial. A recent paper by Xi et al. (2011) demonstrates that Ecto (here TRIM33) actually mediates Nodal-induced activation of master regulators of differentiation in hESCs. They show that in response to Nodal signaling, TRIM33 acts as a binding partner for Smad2/3 and that this complex binds the poised histone mark, H3K9me3 in the promoters of mesendoderm regulators, Gsc and Mixl1, freeing Nodal responsive elements to enable Nodal-induced differentiation (Xi et al., 2011). It is feasible therefore that TRIM33 (or Ecto) may instead function as an enhancer of Nodal signaling for specific downstream targets, rather than an inhibitor of Nodal signaling and differentiation in trophoblast stem cells.

The above studies appear to contradict the work of Guzman-Ayala et al (2004) and the current study suggesting that Nodal signaling maintains trophoblast stem cell self-renewal. A potential explanation for this discrepancy can be derived from the dual role of Nodal signaling observed in hESCs, where Nodal signaling maintains pluripotency through expression of the transcription factor Nanog, however in conjunction with BMP4 induces differentiation into mesendoderm and subsequently endoderm (Vallier et al., 2009). It is thus possible that Nodal may maintain trophoblast stem cell self-renewal via upregulation of FGF4, however in conjunction with Ecto/TRIM33, Nodal may convert into an inductive signal for differentiation. Future studies will be required to untangle the full complement of Nodal downstream targets as well as potential co-activators and co–repressors mediating self-renewal and differentiation in trophoblast stem cells.

While our results suggest Nodal is required to enable trophoblasts to differentiate along the extravillous pathway, we did not see an effect in differentiation along the villous pathway. This may be due to our use of the single syncytiotrophoblast marker, hCG. That hCG may not be an adequate marker of syncytiotrophoblast differentiation is indicated by a study demonstrating a dissociation between morphological and biochemical differentiation in JEG-3 cells (Al-Nasiry et al., 2006). These authors found that treatment with Forskolin led to increased secretion of hCG, however did not induce syncytial fusion as indicated by E-cadherin staining. Conversely, Nodal may be acting to direct trophoblast fate towards the extravillous trophoblast lineage. Future studies should employ more markers of syncytiotrophoblast, including the fusogenic protein, Syncytin.

4.5 Experimental Limitations

The proposed conclusions in this study rely on results obtained from an *in vitro* model using the immortalized extravillous trophoblast cell line, HTR-8/SVneo and the malignant choriocarcinoma cell line, BeWo. Despite the widespread use of these cell lines for the examination of trophoblast functions in *in vitro* assays, such as cell migration and syncytial fusion, they do not describe the full situation *in situ*. This study provides an initial overview of the possible mechanism by which Nodal affects trophoblast self-renewal, proliferation, and differentiation along the villous and extravillous pathways.

In this study we used individual antibodies to the α6 and β4 integrin subunits as a marker for cytotrophoblasts. This is an indirect measurement of the integrin α6β4 and results should be taken at face value. In the future, a greater number of cytotrophoblast makers should be utilized, such as Eomes, Cdk2, and ELF5. Additionally, as mentioned

above, a greater number of markers for syncytiotrophoblast differentiation should be used, such as Syncytin, as well as a functional assay, such as syncytial fusion. While the limiting dilution clonogenic assay is a good initial test for self-renewal, the true test must include dissociation of the resulting colony and re-plating of individual cells to prove that the clonal cells retained the ability to self-renew over multiple passages. Finally, the majority of experiments presented here were completed with an n = 3. To strengthen the results, a greater number of replicates should be completed.

4.6 Future Directions

Notwithstanding these potential limitations, the work done in the current study provides novel insights into the mechanisms by which Nodal regulates trophoblast self-renewal and differentiation. To gain greater confidence in our results, experiments should be repeated in the primary trophoblast progenitor cell line recently produced by the Fisher lab (Genbacev et al., 2011). Due to the complex nature of the Nodal signaling cascade, future studies should aim to utilize a wide range of Nodal concentrations to further elucidate the putative concentration-dependent dose response. Additionally, future studies should aim to unravel the full complement of co-activators and co-repressors, and how these mediate the effects of Nodal signaling in trophoblast stem cell self-renewal and differentiation. Finally, given the growing number of studies supporting a role for epigenetic regulation of Nodal signaling, further work should be completed to determine how this affects downstream targets in the various trophoblast cell types.

References:

Al-Nasiry, A., Spitz, B., Hanssens, M., Luyten, C., and Pijnenborg, R. (2006). Differential effects of inducers of Syncytialization and apoptosis on BeWo and JEG-3 choriocarcinoma cells. *Hum Reprod* 21(1): 193-201.

Albelda, S.M., and Buck, C.A. (1990). Integrins and other cell adhesion molecules. *FASEB J* 4: 2868-2880.

Adkins, H.B., Bianco, C., Schiffer, S.G., Rayhorn, P., Zafari, M., Cheung, A.E., et al. (2003). Antibody blockade of the Cripto CFC domain suppresses tumor cell growth in vivo. *J Clin Invest* 112(4): 575-587.

Aldo, P., Krikun, G., Visintin, I., Lockwook, C., Romero, R. and Mor, G. (2007). A novel three-dimensional in vitro system to study trophoblast-endothelium cell interactions. *Am J Repro Immunol* 58: 98-110.

Ang, S.L., and Constam, D.B. (2004). A gene network establishing polarity in the early mouse embryo. *Semin Cell Dev Biol* 15(5): 555-561.

Anin, S., Vince, G. and Quenby, S. (2004). Trophoblast invasion. *Human Fertility* 7, 169-174.

Bachman, K.E., and Park, B.H. (2005). Duel nature of TGF-beta signaling: tumor suppressor vs. tumor promoter. *Curr Opin Oncol* 17(1): 49-54.

Baczyk, D., Satkunaratnam, A., Nait-Oumesmar, B., Huppertz, B., Cross, J.C., Kingdom, J.C. (2004). Complex patterns of GCM1 mRNA and protein in villous and extravillous trophoblast cells of the human placenta. *Placenta* 25:553-559.

Baczyk, D., Dunk, C., Huppertz, B., Maxwell, C., Reister, F., Giannoulias, D., and Kingdom, J.C.P. (2006). Bi-potential behaviour of cytotrophoblasts in first trimester chorionic villi. *Placenta 27,* 367-374.

Beck, S., Le Good, J.A., Guzman, M., Ben-Haim, N., Roy, K., Beermann, F., and Constam, D.B. (2002). Extraembryonic proteases regulate Nodal signaling during gastrulation. *Nat Cell Biol* 4(12): 981-985.

Bianco, C., Adkins, H.B., Wechselberger, C., Seno, M., Normanno, N., De Luca, A., Sun, Y., Khan, N., Kenney, N., Ebert, A., Williams, K.P., Sanicola, M., and Salomon, D.S. (2002). Cripto-1 activates Nodal- and ALK4-dependent and -independent signaling pathways in mammary epithelial cells. *Cell Mol Biol* 22(8): 2586-2597.

Bischof, P., Martelli, M. and Campana, A. (1994) The regulation of endometrial and trophoblastic metalloproteinases during blastocyst implantation. *Contracept Fertil Sex* 22 48–52.

Bischof, P. and Irminger-Finger, I. (2005). The human cytotrophoblastic cell, a mononuclear chameleon. *Int. J. Biochem* 37, 1-16.

Blanchet, M.H., Le Good, J.A., Mesnard, D., Oorschot, V., Baflast, S., Minchiotti, G., Klumperman, J., and Constam, D.B. (2008). Cripto recruits Furin and PACE4 and controls Nodal trafficking during proteolytic maturation. *EMBO J* 27(19): 2580-2591.

Borges, M., Bose, P., Frank, H.-G., Kaufmann, P., and Potgens, A.J.G. (2003). The two-colour fluorescence assay for the measurement of syncytial fusion between trophoblast-derived cell lines. *Placenta* 24, 959-964.

Brkic, J., Fu, G., Ye, G., Nadeem, L., Lye, S., Wang, Y.L., and Peng, C. Potential role of miR-218 in the pathogenesis of preeclampsia. In *Southern Ontario Reproductive Biology conference proceedings, 40th Annual Meeting.* 2012. Guelph, ON.

Bukur, J., Malenica, B., Huber, C., Seliger, B. (2003). Altered expression of non-classical HLA class Ib antigens in human renal cell carcinoma and its association with impaired immune response. *Hum Immunol* 64: 1081-1092.

Burrows, T.D., King, A. & Loke, Y.W. (1993) Expression of integrins by human trophoblast and differential adhesion to laminin and fibronectin. *Hum Reprod* 8: 475-484.

Burton, G., Woods, A., Jauniaux, E. and Kingdom, J. (2009). Rheological and physiological consequences of conversion of the maternal spiral arteries for utero-placental blood flow during human pregnancy. *Placenta* 30: 473-482.

Caniggia, I., Lye, S.J., Cross, J.C. (1997). Activin is a local regulator of human cytotrophoblast cell differentiation. *Endocrinology 138*(9): 3976-3986.

Caniggia, I., Grisaru-Gravnosky, S., Kuliszewsky, M., Post, M., Lye, S.J. (1999) Inhibition of TGF-beta 3 restores the invasive capability of extravillous trophoblasts in preeclamptic pregnancies. *J Clin Invest* 103: 1641-1650.

Caniggia, I., Winter, J., Lye, S.J., Post, M. (2000). Oxygen and placental development during the first trimester: implications for the pathophysiology of pre-eclampsia. *Placenta* 21(Suppl A): 25-30.

Caniggia, I., Mostachfi, H., Winter, J., Gassmann, M., Lye, S.J., Kuliszewski, M., Post, M. (2000a). Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGFβ(3). *J Clin* Invest 105: 577–587

Cartwright, J.E., Holden, D.P., and Whitley, G.S. (1999). Hepatocyte growth factor regulates human trophoblast motility and invasion: a role for nitric oxide. *Br J Pharmacol* 128**:** 181-189.

Cavallari, C., Fonsato, V., Herrera, M.B., Bruno, S., Tetta, C., and Camussi, G. (2012). Role of Lefty in the anti tumor activity of human adult liver stem cells. *Oncogene:* 1-8.

Chakraborty, C., Gleeson, L., McKinnon, T. and Lala, P. (2002). Regulation of human trophoblast migration and invasiveness. *Can J Physiol Pharmacol* 80: 116-124.

Chapman, H.A., Li, X., Alexander, J.P., Brumwell, A., Lorizio, W., Tan, K., Sonnenberg, A., Wei, Y., and Vu, T.H. (2011). Integrin α6β4 identifies an adult distal lung epithelial population with regenerative potential in mice. *J Clin Invest* 121(7): 2855-2862.

Charnock-Jones, D., Kaufmann, P. and Mayhew, T. (2004). Aspects of human fetoplacental vasculogenesis and angiogenesis: I. Molecular regulation. *Placenta* 25: 103-113.

Chen, C-R., Kang, Y., Siegel, P.M., and Massague, J. (2002). E2F4/5 and p107 as Smad cofactors linking the TGFβ receptor to c-myc repression. *Cell* 110: 19-32.

Choi, W.Y., Giraldez, A.J., and Schier, A.F. (2007). Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. *Science 318*, 271–274.

Chou, J.Y. (1978). Establishment of clonal human placental cells synthesizing human choriogonadotropin. *Proc Natl Acad Sci USA* 75: 1854-1858.

Church, H., Vicovac, L., Williams, J., Hey, N. and Aplin, J. (1996). Laminins 2 and 4 are expressed by human decidual cells. *Lab Invest* 74, 21-32.

Collignon, J., Varlet, I., and Robertson, E.J. (1996). Relationship between asymmetric nodal expression and the direction of embryonic turning. *Nature* 381(6578): 155-158.

Conlon, F.L., Barth, K.S., and Robertson, E.J. (1991). A novel retrovirally induced embryonic lethal mutation in the mouse: assessment of the developmental fate of embryonic stem cells homozygous for the 413.d proviral integration. *Dev* 111(4): 969-981.

Constam, D.B. (2009). Riding shotgun: a dual role for the epidermal growth factor-Cripto/FRL-1/Cryptic protein Cripto in Nodal trafficking. *Traffic* 10(7): 783-791.

Dakour, J., Li, H., Chen, H., and Morrish, D. W. (1999). EGF promotes development of a differentiated trophoblast phenotype having c-myc and junB proto-oncogene activation. *Placenta* 20:119–126.

Damsky, C., Librach, C., Lim, K., Fitzgerald, M., McMaster, M., Janatpour, M., Zhou, Y., Logan, S. and Fisher, S. (1994). Integrin switching regulates normal trophoblast invasion. *Development* 120, 3657-3666.

Das, P., Ezashi, T., Schulz, L.C., Westfall, S.D., Livingston, K.A., and Roberts, R.M. (2007). Effects of FGF2 and oxygen in the BMP4-driven differentiation of trophoblast from human embryonic stem cells. *Stem Cell Res* 1: 61-74.

Derynck, R., Akhurst, R.J., and Balmain, A. (2001). TGF-b signalling in tumor suppression and cancer progression. *Nat Genet* 29: 117-129.

Drewlo, S., Baczyk, D., Dunk, C., and Kingdom, J. (2008). Fusion assays and models for the trophoblast. *Methods in Molecular Biology,* vol. 475: Cell Fusion: Overviews and Methods, 363-382.

Drewlo, S., Leyting, S., Kokozidou, M., Mallet, F., and Potgens, A.J.G. (2006). C-terminal truncations of syncytin-1 (ERVWE1 envelope) that increase its fusogenicity. *Biol Chem 387*, 1113-1120.

Espinoza, J., Romero, R., Mee Kim, Y., Kusanovic, J., Hassan, S., Erez, O., Gotsch, F., Gabor Than, N., Papp, Z. and Jai Kim, C. (2006). Normal and abnormal transformation of the spiral arteries during pregnancy. *J Perinat Med* 34: 2131-2138.

Fisher, S.J., Cui, T.Y., Zhang, L., et al. (1989). Adhesive and degradative properties of human placental cytotrophoblast cells in vitro. *J Cell Biol* 109: 891-902.

Frank, H., Malekzadeh, F., Kertschanska, S., Crescimanno, C., Castellucci, M., Lang, I., Desoye, G. and Kaufmann, P. (1994). Immunohistochemistry of two different types of placental fibrinoid. *Acta Anat (Basel)* 150: 55-68.

Frendo, J-L., Olivier, D., Cheynet, V., Blond, J-L., Boston, O., Vidaud, M., Rabreau, M., Evain-Brion, D., and Mallet, F. (2003). Direct involvement of HERV- W env glycoprotein in human trophoblast cell fusion and differentiation. *Mol Cell Biol* 23:3566-3574.

Hemberger, M., Udayashankar, R., Tesar, P., Moore, H., and Burton, G.J. (2010). ELF-5-enforced transcriptional networks define an epigenetically regulated trophoblast stem cell compartment in the human placenta. *Hum Mol Gen* 19(12): 2456-2467.

Hendrix, M.J., Seftor, E.A., Seftor, R.E.B., Kasemeier-Kulesa, J., Kulesa, P.M., and Postovit, L-M. (2007). Reprogramming metastatic tumour cells with embryonic microenvironments. *Nat Rev* 7: 246-255.

Genbacev, O., Jensen, K.D., Powlin, S.S., et al. (1993). In vitro differentiation and ultrastructure of human extravillous trophoblast (EVT) cells. *Placenta* 14: 463-475.

Genbacev, O., Joslin, R., Damsky, C.H., Polliotti, B.M., Fisher, S.J. (1996). Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia. *J Clin Invest* 97:540-550.

Genbacev, O., Zhou, Y., Ludlow, J.W., and Fisher, S.J. (1997). Regulation of human placental development by oxygen tension. *Science* 277: 1669–1672.

Genbacev, O., Donne, M., Kapidzic, M., Gormley, M., Lamb, J., Gilmore, J., Larocque, N., Goldfien, G., Zdravkovic, T., McMaster, M.T., and Fisher, S.J. (2011). Establishment of human trophoblast progenitor cell lines from the chorion. *Stem Cells* 29: 1427-1436.

Gleeson LM, Chakraborty, C., McKinnon T and Lala PK. (2001). Insulin like growth factor-binding protein 1 stimulates human trophoblast migration by signalling through integrin alpha5beta1 via MAPK pathway. *J Clin Endocrinol Metabolism* 86, 2484-2493.

Graham, C.H. and Lala, P.K. (1992). Mechanisms of placental invasion of the uterus and their control. *Biochem Cell Biol* 70: 867-874.

Graham, C.H., Lysiak, J., McCrae, K. and Lala, P.K. (1992). Localization of transforming growth factor-beta at the human fetal-maternal interface: role in trophoblast growth and differentiation. *Biol Reprod* 46: 561-572.

Graham, C.H., Hawley, T.S., Hawley, R.G., MacDougall, J.R., Kerbel, R.S., Khoo, N. and Lala, P.K. (1993). Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp. Cell Res.* 206, 204-211.

Graham, C.H., Connelly, I., MacDougall, J.R., Kerbel, R.S., Stetler-Stevenson, W.G., and Lala, P.K. (1994). Resistance of malignant trophoblast cells to both the anti-proliferative and anti-invasive effects of transforming growth factor-beta. *Exp Cell Res* 214(1): 93-99.

Graham, C.H. (1997) Effect of transforming growth factor-b on the plasminogen activator system in cultured first trimester human cytotrophoblasts. *Placenta* 18: 137–143.

Guzman-Ayala, M., Ben-Haim, N., Beck, S., and Constam, D.B. (2004). Nodal protein processing and fibroblast growth factor 4 synergize to maintain a trophoblast stem cell microenvironment. *Proc Natl Acad Sci USA* 101(44): 15656-15660.

Heino, J., Ignotz, R.A., Helmer, M.E., Crouse, C., and Massague, J. (1989). Regulation of cell adhesion receptors by transforming growth factor-b. Concomitant regulation of integrins that share a common b1 subunit. *J Biol Chem* 264: 380-388.

Hemler, M., Crouse, C., and Sonnenberg, A. (1989). Association of the VLA a6 subunit with a novel protein. *J Biol Chem* 264: 6529-6535.

Hendrix, M.J., Seftor, E.A., Seftor, R.E., Kasemeier-Kulesa, J., Kulesa, P.M., and Postovit, L.M. (2007). Reprogramming metastatic tumor cells with embryonic microenvironments. *Nat Rev Cancer* 7(4), 246–255.

Herr, F., Baal, N., Widmer-Teske, R., McKinnon, T. and Zygmunt, M. (2010). How to study placental vascular development? *Theriogenology* 73, 817-827.

Hofmann, G.E., Scott, R.T. Jr., Bergh, P.A., and Deligdisch, L. (1991). Immunohistochemical localization of epidermal growth factor in human endometrium, decidua, and placenta. *J Clin Endocrinol Metab* 73(4): 882-887.

Hoffmann, F.M. (1992). TGF-beta family factors in Drosophila morphogenesis. *Mol Reprod Dev* 32(2): 173-178.

Huppertz, B. (2008). The anatomy of the normal placenta. *Journal of Clinical Pathology* 12, 1296-1302.

Iacob, D., Cai, J., Tsonis, M., Babwah, A., Chakraborty, C., Bhattacharjee, R.N., and Lala, P.K. (2008). Decorin-mediated inhibition of proliferation and migration of the human trophoblast via different tyrosine kinase receptors. *Endocrinology* 149(12): 6187-6197.

Irizarry, R.A., Bolstad, B.M., Collin, F., Cope, L.M., Hobbs, B., Terence, P., &Speed, T.P. (2003). Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Research* 31(4): e15.

Irving, J.A., Lysiak, J.J., Graham, C.H., Hearn, S., Han, V.K.M., and Lala, P.K. (1995). Characteristic's of trophoblast cells migrating from first trimester chorionic villus explants and propagated in culture. *Placenta* 16(5): 413-433.

Irving, J. and Lala, P.K. (1995). Functional role of cell surface integrins on human trophoblast cell migration: regulation by TGF-beta, IGF-II, and IGFBP-1. *Exp. Cell Res.* 2: 419-427.

James, D., Levine, A.J., Besser, D., and Hemmati-Brivanlou, A. (2005). TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development* 132(6): 1273-1282.

Janatpour, M.J., Utset, M.F., Cross, J.C., Rossant, J., Dong, J., Israel, M.A., Fisher, S.J. (1999). A repertoire of differentially expressed transcription factors that offers insight into mechanisms of human cytotrophoblast differentiation. *Dev Genet* 25:146-157.

Janatpour, M.J., McMaster, M.T., Genbacev, O., Zhou, Y., Dong, J., Cross, J.C., Israel, M.A., Fisher, S.J. (2000). Id-2 regulates critical aspects of human cytotrophoblast differentiation, invasion and migration. *Development* 127: 549-558.

Jiang, B., and Mendelson, C.R. (2005). O2 enhancement of human trophoblast differentiation and hCYP19 (aromatase) gene expression are mediated by proteasomal degradation of USF1 and USF2. *Mol Cell Biol* 25: 8824-8833.

Jones, C. and Fox, H. (1991). Ultrastructure of the normal human placenta. *Electron Microscope Reviews* 4: 129-178.

Jones, R.L., Salamonsen, L.A. and Findlay, J.K. (2002) Activin A promotes human endometrial stromal cell decidualization in vitro. *J Clin Endocrinol Metab* 87: 4001–4004.

Kang, Y., Chen, C-R., and Massague, J. (2003). A self-enabling TGF-b response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Mol Cell* 11: 915-926.

Khan, G.A., Gannareddy, G., Lala, N., DiGugliemo, J., and Lala, P.K. (2011). Decorin is a novel VEGFR-2 binding antagonist for human extravillous trophoblast. *Mol Endorinol* 25(8): 1431-1443.

Khoo, N.K.S., Bechberger, J.F., Shepherd, T., Bond, S.L., McCrae, K., Hamilton, G.S., and Lala, P.K. (1998a). SV40 Tag transformation of the normal invasive trophoblast results in a premalignant phenotype: Mechanisms responsible for hyperinvasiveness and resistance to antiinvasive action of TGFb. *Intl J Cancer* 77: 429-439.

Khoo, N.K.S., Zhang, Y., Bechberger, J.F., Bond, S.L., Hum, K., and Lala, P.K. (1998b). SV40 Tag transformation of the normal invasive trophoblast results in a premalignant phenotype: II. Changes in gap junctional intercellular communication. *Intl J Cancer* 77: 440-448.

King, A., Thomas, L. and Bischof, P. (2000). Cell culture models of trophoblast II: Trophoblast cell lines- A workshop report. *Placenta* 14, S113-S119.

Kliman, H. (2000). Uteroplacental blood flow. The story of decidualization, menstruation, and trophoblast invasion. *Am J Pathol* 157: 1759-1768.

Kliman, H.J., Nestler, J.E., Sermasi, E., et al. (1986). Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. *Endocrynology* 118: 1567-1582.

Knofler, M. (2009). Critical growth factors and signalling pathways controlling human trophoblast invasion. *Int J Dev Biol* 54: 269-280.

Korhonen, M., Ylanne, J., Laitinen, L., Cooper, H.M., Quaranta, V. and Virtanen, I. (1991). Distribution of the α_1- α_6 integrin subunits in human developing and term placenta. *Lab Invest* 65: 347-356.

Kulesa, P.M., Kasemeier-Kulesa, J.C., Teddy, J.M., Margaryan, N.V., Seftor, E.A., Seftor, R.E., et al. (2006). Reprogramming metastatic melanoma cells to assume a neural crest cell-like phenotype in an embryonic microenvironment. *Proc Natl Acad Sci USA* 103(10): 3752-3757.

La Good, J.A., Joubin, K., Giraldez, A.J., Ben-Haim, N., Beck, S., Chen, Y., Schier, A.F., and Constam, D.B. (2005). Nodal stability determines signalling range. *Curr Biol* 15(1): 31-36.

Lala, P.K. and Graham, C.H. (1990). Mechanisms of trophoblast invasiveness and their control: the role of proteases and protease inhibitors. *Cancer and Metastasis Reviews* 9, 369-379.

Lala, P.K., Khoo, N.K.S., and Singh, R.K. (1995). Integrin expression by the normal invasive trophoblast, SV40 Tag transformed premalignant trophoblast and choriocarcinoma cells. *Placenta* 16: Abstract 40.

Lala, P.K. and Hamilton, G. (1996). Growth factors, proteases and protease inhibitors in the maternal-fetal dialogue. *Placenta* 17, 545-555.

Lala, P.K., Khoo, N.K.S., Guimond, M.J., and Chakraborty, C. (1999). Control mechanisms in human trophoblast proliferation and invasiveness and their derangement during trophoblastic tumor progression. *Trophoblast Res* 13: 119-136.

Lala, P.K., and Chakraborty, C. (2003). Factors regulating trophoblast migration and invasiveness: Possible derangements contributing to pre-eclampsia and fetal injury. *Placenta* 24(6): 575-587.

Lash, G.E., Postovit, L-M., Matthews, N.E., Chung, E.Y., Canning, M.T., Pross, H., Adams, M.A., Graham, C.H. (2002). Oxygen as a regulator of cellular phenotypes in pregnancy and cancer. *Can J Physiol Pharmacol* 80:103-109.

Lee, R.H., Seo, M.J., Pulin, A.A., Gregory, C.A., Yiostalo, J., and Prockop, D.J. (2009). The CD34-like protein PODXL and α6-integrin (CD49f) identify early progenitor MSCs with increased clonogenicity and migration to infarcted heart in mice. *Blood* 113(4): 816-826.

Lee, C.C., Jan, H.J., Lai, J.H., Ma, H.I., Hueng, D.Y., Gladys-Lee, Y.C., Cheng, Y.Y., Liu, L.W., Wei, H.W., and Lee, H.M. (2010). Nodal promotes growth and invasion in human gliomas. *Oncogene* 29: 3110-3123.

Licht, P., Russu, V. and Wildt, L. (2001) On the role of human chorionic gonadotropin (hCG) in the embryo-endometrial microenvironment: implications for differentiation and implantation. *Semin Reprod Med* 19:37-47.

Lonardo, E., Hermann, P.C., Mueller, M.T., Huber, S., Balic, A., Miranda-Lorenzo, I., Zagorac, S., Alcala, S., Rodriguez-Arabaolaza, I., Ramirez, J.C., Torres-Ruiz, R., Garcia, E., Hidalgo, M., Cebrian, D.A., Heuchel, R., Lohr, M., Berger, F., Bartenstein, P., Aicher, A., and Heeschen, C. (2011). Nodal/Activin signaling drives self-renewal and tumorigenicity of pancreatic cancer stem cells and provides a target for combined drug therapy. *Cell Stem Cell* 9: 433-446.

Lowe, L.A., Yamada, S., and Kuehn, M.R. (2001). Genetic dissection of nodal function

in patterning the mouse embryo. *Development* 128(10): 1831-1843.

Lunghi, L., Ferretti, M., Medici, S., Biondi, C. and Vesce, F. (2007). Control of human trophoblast function. *Reproductive Biology and Endocrinology* 5, 6-20.

Luo, S.S., Ishibashi, O., Ishikawa, G., Ishikawa, T., Katayama, A., Mishima, T., Takizawa, T., Shigihara, T., Goto, T., Izumi, A., Ohkuchi, A., Matsubara, S., Takeshita, T., and Takizawa, T. (2009). Human villous trophoblasts express and secrete placenta-specific microRNAs into maternal circulation via exosomes. *Biol Reprod 81*, 717-729.

Lysiak, J.J., Hunt, J., Pringle, G.A., and Lala, P.K. (1995). Localization of transforming growth factor beta and its natural inhibitor decorin in the human placenta and decidual throughout gestation. *Placenta* 16(3): 221-231.

Ma, T.G., Soloveva, V., Tzeng, S-J., Lowe, L.A., Pfendler, K.C., Iannaccone, P.M., Kuehn, M.R., and Linzer, D.I.H. (2001). Nodal Regulates Trophoblast Differentiation and Placental Development. *Dev Biol 236,* 124-135.

Mack, G.S. (2007). MicroRNA gets down to business. *Nat Biotechnol 25*, 631-638.

Malmberg, K.J., Levitsky, V., Norell, H., de Matos, C.T., Carlsten, M., Schedvins, K., Rabbani, H., Moretta, A., Soderstrom, K., Levitskaya, J., Kiessling, R. (2002). IFN-gamma protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism. *J Clin Invest* 110: 1515-1523.

Maruo, T., Matsuo, H., Otani, T., and Mochizuki, M. (1995). Role of epidermal growth factor (EGF) and its receptor in the development of the human placenta. *Reprod Fertil Dev* 7(6):1465-1470.

Massague, J. (1998). TGFβ signal transduction. *Annu Rev Biochem* 67: 753-791.

Massague, J. and Chen, Y-G. (2000). Controlling TGF-b signaling. *Genes Dev* 14: 627-644.

Massague, J., Seoane, J., and Wotton, D. (2005). Smad transcription factors. *Genes Dev* 19: 2783-2810.

McAtier, J.A., and Daview, J. Basic cell culture: A practical approach. Davies, J.M. (Ed) p. 125 (Oxford University Press Inc, New York, 1998).

McKinnon T, Chakraborty, C., Gleeson LM, Chidiac, P. and Lala, P.K. (2001). Stimulation of human extravillous trophoblast migration by IGF-II is mediated by IGF type 2 receptor involving inhibitory G protein(s) and phosphorylation of MAPK. *J Clin Endocrinol Metabolism* 86: 3665-3674.

Meyer, M.J., Fleming, J.M., Ali, M.A., Pesesky, M.W., Ginsburg, E., and Vonderhaar, B.K. (2009). Dynamic regulation of CD24 and the invasive, CD44posCD24neg phenotype in breast cancer cell lines. *Breast Cancer Res* 11: R82.

Minchiotti, G., Parisi, S., Liguori, G., Signore, M., Lania, G., Adamson, E.D., et al. (2000). Membrane-anchorage of Cripto protein by glycosylphosphatidylinositol and its distribution during early mouse development. *Mech Dev* 90(2): 133-42.

Mohn, F. and Schubeler, D. (2009). Genetic and epigenetics: Stability and plasticity during cellular differentiation. *Trends Genet* 25: 129-136.

Moore, K.L., and Persaud, P.V.N. (1998). The Developing Human: Clinically Oriented Embryology (7th Ed.).

Morrish, D.W., Bhardwaj, D., and Paras, M.T. (1991). Transforming growth factor beta 1 inhibits placental differentiation and human chorionic gonadotropin and human placental lactogen secretion. *Endocrinology* 129: 22–26.

Munir, S., Xu, G., Wu, Y., Yang, B., Lala, P.K., and Peng, C. (2004). Nodal and ALK7 inhibit proliferation and induce apoptosis in human trophoblast cells. *J Biol Chem 279*(23), 31277-31286.

Nadeem, L., Munir, S., Fu, G., Dunk, C., Baczyk, D., Caniggia, I., Lye, S., and Peng, C. (2011). Nodal signals through activin receptor-like kinase 7 to inhibit trophoblast migration and invasion: implication in the pathogenesis of preeclampsia. *Am J. Pathol. 178*(3): 1177-1189.

Nicola, C., Timoshenko, A. V., Dixon, S. J., Lala, P. K. and Chakraborty, C. (2005). EP1 receptor-mediated migration of the first trimester human extravillous trophoblast: the role of intracellular calcium and calpain. *J. Clin. Endocrinol. Metab* 90, 4736-4746.

Nicola, C., Chirpac, A., Lala, P.K., and Chakraborty, C. (2008). Roles of Rho guanosine 5'-triphosphatase A, Rho kinases, and extracellular signal regulated kinase (1/2) in prostaglandin E2-mediated migration of first-trimester human extravillous trophoblast. *Endocrinology* 149(3): 1243-1251.

Papageorgiou, I., Nicholls, P.K., Wang, F., Lackmann, M., Makanji, Y., Salamonsen, L.A., et al. (2009). Expression of nodal signalling components in cycling human endometrium and in endometrial cancer. *Reprod Biol Endocrinol* 7:122.

Patani, R., Compston, A., Puddifoot, C.A., Wyllie, D.J., Hardingham, G.E., Allen, N.D., et al. (2009). Activin/Nodal inhibition alone accelerates highly efficient neural conversion from human embryonic stem cells and imposes a caudal positional identity. *PLoS One* 4(10): e7327.

Pattillo, R.A., Gey, G.O., Delfs, E. et al. (1968). Human hormone production in vitro. *Science* 159: 1467-1469.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nuc Acid Res* 29(9): 2001-2007.

Pijnenborg, R., Vercruysse, L., and Hanssens, M. (2006). The uterine spiral arteries in human pregnancy: facts and controversies. *Placenta* 27: 939-958.

Postovit, L-M., Seftor, E.A., Seftor, R.E., and Hendrix, M.J. (2006). Influence of the microenvironment on melanoma cell fate determination and phenotype. *Cancer Res* 66(16): 7833-7836.

Postovit, L-M., Seftor, E.A., Seftor, R.E., and Hendrix, M.J. (2006a). A three-dimensional model to study the epigenetic effects induced by the microenvironment of human embryonic stem cells. *Stem Cells* 24(3): 501-505.

Postovit, L-M., Seftor, E.A., Seftor, R.E., and Hendrix, M.J. (2007). Targeting Nodal in malignant melanoma cells. *Expert Opin Ther Targets* 11(4): 497-505.

Postovit, L-M., Margaryan, N.V., Seftor, E.A., Kirschmann, D.A., Lipavsky, A., Wheaton, W.W., Abbott, D.E., Seftor, R.E., and Hendrix, M.J. (2008). Human embryonic stem cell microenvironment suppresses the tumorigenic phenotype of aggressive cancer cells. *Proc Natl Acad Sci USA* 105(11): 4329-4334.

Postovit, L.M., Margaryan, N.V., Seftor, E.A., Strizzi, L., Seftor, R.E.B., and Hendrix, M.J.C. (2009). Plasticity underlying multipotent tumor stem cells. *Cancer Drug Discovery and Development: Stem Cells and Cancer,* 99-112.

Quail, D.F., Zhang, G., Walsh, L., Broughton, H., Taylor, M.J., Hess, D., Lewis, J., Putman, D., and Postovit, L-M. The embryonic protein Nodal is a dual regulator of breast cancer initiation and angiogenesis. In *American Association for Cancer Research* conference proceedings, 101st Annual Meeting. 2010. Washington, D.C.

Quail, D.F., Taylor, M.J., and Postovit, L-M. (2010a). Microenvironmental Regulation of Cancer Stem Cell Phenotypes. *Current Stem Cell Research & Therapy* 7(3): 197-216.

Qian, H., Tryggvason, K., Jacobsen, S.E., and Ekblom, M. (2006). Contribution of α6 integrins to hematopoietic stem and progenitor cell homing to bone marrow and collaboration with α4 integrins. *Blood* 107: 3503-3510.

Reissmann, E., Jornvall, H., Blokzijl, A., Andersson, O., Chang, C., Minchiotti, G., Persico, M.G., Ibanez, C.F., and Brivanou, A.H. (2001). The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. *Genes Dev* 15(15): 2010-2022.

Roberts, H.J., Hu, S., Qui, Q., Leung, P.C.K., Caniggia, I., Gruslin, A., Tsang, B., and Peng, C. (2003). Identification of novel isoforms of activin receptor-like kinase 7 (ALK7) generated by alternative splicing and expression of ALK7 and its ligang, Nodal, in human placenta. *Biol Reprod 68*, 1719-1726.

Rote, N.S. (2005). Letter to the editor: Intercellular fusion of BeWo. *Placenta 26*, 686.

Rouas-Freiss, N., Bruel, S., Menier, C., Marcou, C., Moreau, P., and Carosella, E.D. (2005). Switch of HLA-G alternative splicing in a melanoma cell line causes loss of HLA-G1 expression and sensitivity to NK lysis. *Int J Cancer* 117: 114-122.

Schier, A.F. (2003). Nodal signaling in vertebrate development. *Annu Rev Cell Dev Biol* 19: 589-621.

Schier, A.F. (2009). Nodal morphogens. *Cold Spring Harb Perspect Biol* 1(5): a003459.

Schilling, B. and Yeh, J. (2000) Transforming growth factor-beta(1), -beta(2), - beta(3) and their type I and II receptors in human term placenta. *Gynecol Obstet Invest* 50: 19–23.

Schuettengruber, B., and Cavalli, G. (2009). Recruitment of Polycomb group complexes and their role in the dynamc regulation of cell fate choice. *Development* 136: 3531-3542.

Shiratori, H., and Hamada, H. (2006). The left-right axis in the mouse: from origin to morphology. *Development* 133(11): 2095-2104.

Smith, W.C., McKendry, R., Ribisi Jr., S., Harland, R.M. (1995). A nodal-related gene defines a physical and functional domain within the Spemann organizer. *Cell* 82(1): 37-46.

Smith, J.R., Vallier, L., Lupo, G., Alexander, M., Harris, W.A., and Pedersen, R.A. (2008). Inhibition of Activin/Nodal signaling promotes specification of human embryonic stem cells into neuroectoderm. *Dev Biol* 313(1): 107-117.

Soderberg, S.S., Karlsson, G., and Karlsson, S. (2009). Complex and context dependent regulation of hematopoiesis by TGF-beta superfamily signaling. *Ann N Y Acad Sci* 1176:55-69.

Springer, T.A., Spurr-Michaud, S., Tisdale, A., Elwell, J., and Gipson, I.K. (1987). The lymphocyte function-associated LFA-1, CD2 and LFA-3 molecules: cell adhesion receptors of the immune system. *Annu Rev Immuno* 5: 223-252.

Stingl, J., Eirew, P., Ricketson, I., Shackleton, M., Vaillant, F., Choi, D., Li, H.L., and Eaves, C.J. (2006). Purification and unique properties of mammary epithelial stem cells. *Nature* 439(7079): 993-997.

Strizzi, L., Postovit, L-M., Margaryan, N.V., Seftor, E.A., Abbott, D.E., Seftor, R.E., et al. (2008). Emerging roles of nodal and Cripto-1: from embryogenesis to breast cancer progression. *Breast Dis* 29: 91-103.

Strizzi, L., Hardy, K.M., Seftor, E.A., Costa, F.F., Kirschmann, D.A., Seftor, R.E.B., Postovit, L.M., and Hendrix, M.J.C. (2009). Development and cancer: At the crossroads of Nodal and Notch signaling. *Cancer Res 69*(18), 7131-7134.

Tabibzadeh, S., Mason, J.M., Shea, W., Cai, Y., Murray, M.J., and Lessey, B. (2000). Dysregulated expression of ebaf, a novel molecular defect in the endometrium of patients with infertility. *J Clin Endocrinol Metab* 85(7): 2526-2536.

Tamura, R.N., Rozzo, C., Starr, L., Chambers, J., Rheicharat, L.F., Cooper, H.M. and Quaranta, V. (1990). Epithelial integrin a6b4: complete primary structure of a6 and variant form of b4. *J Cell Biol* 111: 1593-1604.

Tang, M., Taylor, H.S., and Tabibzadeh, S. (2005). In vivo gene transfer of lefty leads to implantation failure in mice. *Hum Repro 20*(7): 1772-1778.

Thaler, I., Manor, D., Itskovitz, J., Rottern, S., Levit, N., Timor-Tritsch, I. and Brandes, J. (1990). Changes in uterine blood flow during human pregnancy. *Am J Obstet Gynecol* 162: 121-125.

Tian, M., and Schiemann, W.P. (2009). The TGF-beta paradox in human cancer: an update. *Future Oncol* (2): 259-271.

Topczewska, J.M., Postovit, L-M., Margaryan, N.V., Sam, A., Hess, A.R., Wheaton, W.W., et al. (2006). Embryonic and tumorigenic pathways converge via Nodal signaling: role in melanoma aggressiveness. *Nat Med* 12(8): 925-32.

Torry, D., Leavenworth, J., Chang, M., Maheshwari, V., Groesch, K., Ball, E. and Torry, R. (2007). Angiogenesis in implantation. *J Assist Reprod Genet* 24: 303-315.

Vallier, L., Reynolds, D., and Pedersen, R.A. (2004). Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. *Dev Biol* 275(2): 403-421.

Vallier, L., Mendjan, S., Brown, S., Chang, Z., Teo, A., Smithers, L.E., et al. (2009). Activin/Nodal signalling maintains pluripotency by controlling Nanog expression. *Development* 136(8): 1339-1349.

Whitley, G. and Cartwright, J. (2009). A trophoblast-mediated spiral artery remodelling: a role for apoptosis. *J Anat* 215: 21-26.

Whitley, G. and Cartwright, J. (2010). Cellular and molecular regulation of spiral artery remodelling: lessons from the cardiovascular field. *Placenta* 31: 465-474.

Wice, B., Menton, D., Geuze, H., and Schwartz, A.L. (1990). Modulators of cyclic AMP metabolism induce syncytiotrophoblast formation in vitro. *Exp Cel Res* 186: 306-316.

Xi, Q., Wang, Z., Zaromytidou, A-I., Zhang, X.H-F., Chow-Tsang, L-F., Lui, J.X., Kim, H., Barlas, A., Manova-Todorova, K., Kaartinen, V., Studor, L., Mark, W., Patel, D.J., and Massague, J. (2011). A poised chromatin platform for TGF-b access to master regulators. *Cell* 147: 1511-1524.

Xu, G., Guimond, M.J., Chakraborty, C., and Lala, P.K. (2002). Control of proliferation, migration, and invasiveness of human extravillous trophoblast by decorin, a decidual product. *Biol Reprod* 67(2): 681-689.

Xu, G., Chakraborty, C. and Lala, P.K. (2003) Reconstitution of Smad3 restores TGF-beta response of tissue inhibitor of metalloprotease-1 upregulation in human choriocarcinoma cells. *Biochem Biophys Res Commun* 300: 383–390.

Xu, G., Zhong, Y., Munir, S., Yang, B.B., Tsang, B.K., and Peng, C. (2004). Nodal induces apoptosis and inhibits proliferation in human epithelial ovarian cancer cells via activin receptor-like kinase 7. *J Clin Endocrinol Metab* 89(11): 5523-5534.

Xu, G., Bernuado, S., Fu, G., Lee, D.Y., Yang, B.B., and Peng, C. (2008). Cyclin G2 is degraded through the ubiquitin-proteasome pathway and mediates the antiproliferative effect of activin receptor-like kinase 7. *Mol Biol Cell* 19(11): 4968-4979.

Xu, R.N., Chen, X., Li, D.S. (2002a). BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* 20: 1261-1264.

Yamamoto-Tabata, T., McDonagh, S., Chang, H., Fisher, S. and Pereira, L. (2004). Human cytomegalovirus interleukin-10 downregulates metalloproteinase activity and impairs endothelial cell migration and placental cytotrophoblast invasiveness in vitro. *Journal of Virology* 78, 2831-2840.

Yan, Y.T., Liu, J.J., Luo, Y., E, C., Waltiwanger, R.S., Abate-Shen, C., and Shen, M.M. (2002). Dual roles of Cripto as a ligand and coreceptor in the nodal signaling pathway. *Mol Cell Biol* 22(13): 4439-4449.

Yang, M., Lei, Z.M., and Rao, C.V. (2003). The central role of human chorionic gonadotropin in the formation of human placental syncytium. *Endocrinology* 144: 1108–1120.

Yeo, C., and Whitman, M. (2001). Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms. *Mol Cell* 7(5): 949-957.

Young, R.A. (2011). Control of the embryonic stem cell state. *Cell* 144: 940-954.

Yu, C., Shen, K., Lin, M., Chen, P., Lin, C., Chang, G.D., and Chen, H. (2003) GCMa regulates the syncytin-mediated trophoblast fusion. *J Biol Chem* 277:50062-50068.

Yu, K-R., Yang, S-R., Jung, J-W., Kim, H., Ko, K., Han, D.W., Park, S-B., Choi, S.W., Kang, S-K., Scholer, H., and Kang, K-S. (2012). CD49f enhances multipotency and maintains stemness through the direct regulation of OCT4 and SOX2. *Stem Cells* 30: 876-887.

Zdravkovic M, Boagye-Mathiessen G, Guimond M-J, Hager H, Ebbesen P, and Lala P.K. (1999) Susceptibility of MHC class I expressing extravillous trophoblast cell lines to killing by natural killer cells. *Placenta*. 20: 431-440.

Zhong, C., Lala, P.K., and Postovit, L-M. (2008). Isolation of cytotrophoblast stem cells from the HTR-8/SVneo cell line. International Federation of Placental Associations Annual Meeting. Graz, Austria. Abstract.

Zhou, X., Sasaki, H., Lowe, L., Hogan, B.L. and Kuehn, M.R. (1993). Nodal is a novel TGF-beta-like gene expressed in the mouse node during gastrulation. *Nature* 361(6412): 543-547.

Zhou, Y., Damsky, C.H., Chiu, K., Robarts, J.M., and Fisher, S.J. (1993). Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblast. *J Clin Invest* 91: 950-960.

Zhou, Y., Fisher, S., Janatpour, M., Genbacev, O., Dejana, E., Wheelock, M. and Damsky, C. (1997). Human cytotrophoblasts adopt a vascular phenotype as they differentiate: A strategy for successful endovascular invasion? *J. Clin. Invest.* 99: 2139-2151.

Zhou, Y., Damsky, C.H., and Fisher, S.J. (1997a). Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective defective endovascular invasion in this syndrome? *J Clin Invest* 99: 2152-2164.

Appendix: Supplemental Figures

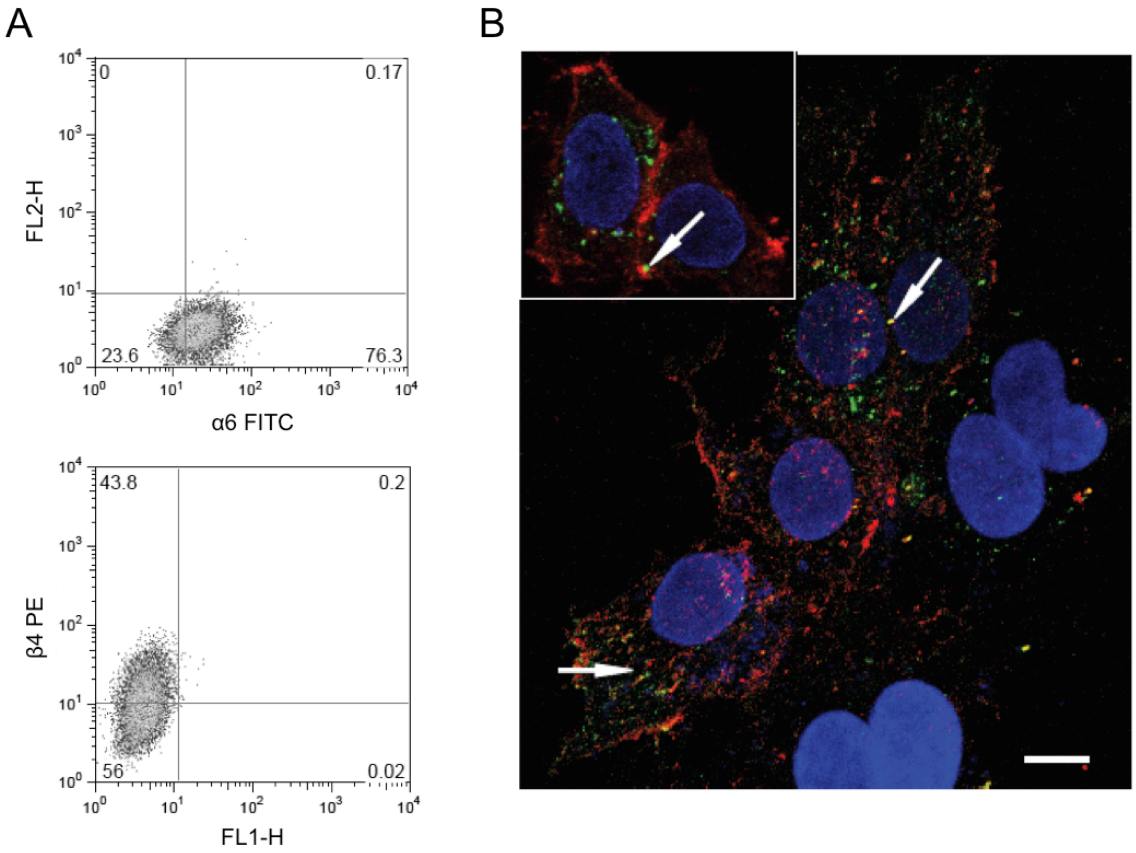

Figure A1. Analysis of α6β4 expression in HTR-8/SVneo cells.

Flow cytometry analysis of α6 (top) and β4 (bottom) integrin subunits in HTR-8/SVneo cells (A). Confocal microscopy indicating colocalization of α6 (red) and β4 (green) integrin subunits (B). White arrows indicate areas of colocalization. Scale bar represents 10 μm. Completed by Lynne Postovit.

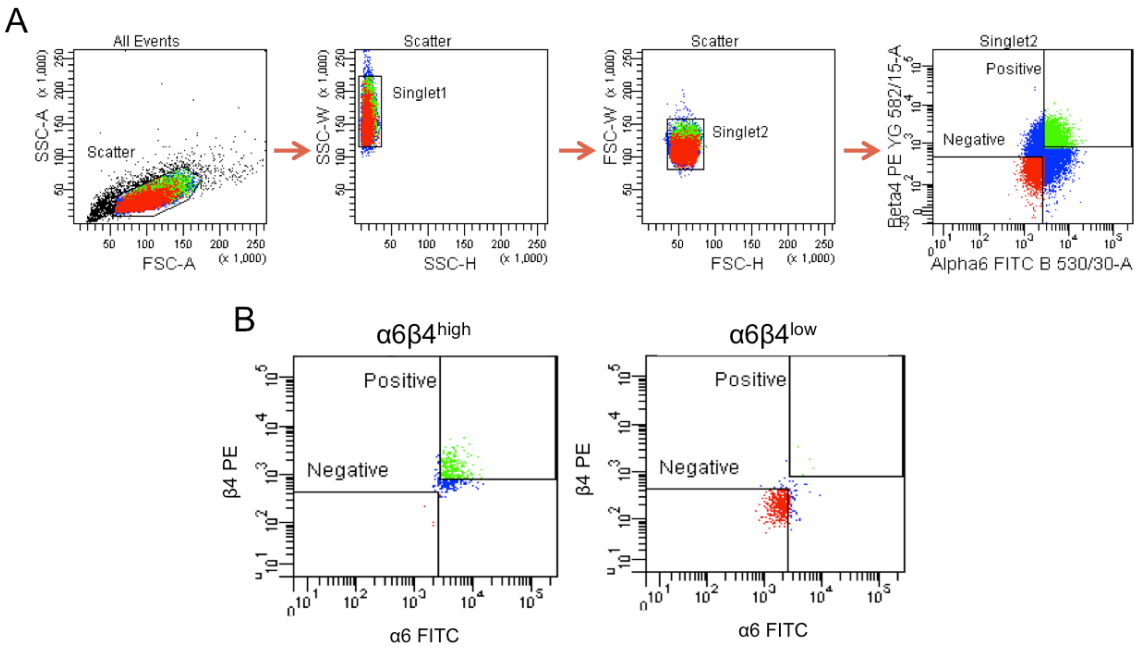

Figure A2. Isolation of $\alpha 6\beta 4^{high}$ and $\alpha 6\beta 4^{low}$ subsets from HTR-8/SVneo.

HTR-8/SVneo cells were harvested and incubated with IgG isotype controls, FITC conjugated rat anti-human CD49f IgG (specific for α6 integrin chain) and/or PE conjugated rat anti-human CD104 (specific for β4 integrin chain). Signals obtained with isotype controls were gated as negative prior to analysis of integrin expression. Singlets were gated according to FSC and SSC height and width signals. The top 20% of HTR-8/SVneo cells were sorted as $\alpha 6\beta 4^{high}$ while the bottom 20% were sorted as $\alpha 6\beta 4^{low}$. Post sorting, the purity for the $\alpha 6\beta 4^{high}$ subset was very high, with 1.2% of singlets detected as negative. The $\alpha 6\beta 4^{low}$ had 0.9% of singlets in the positive range.

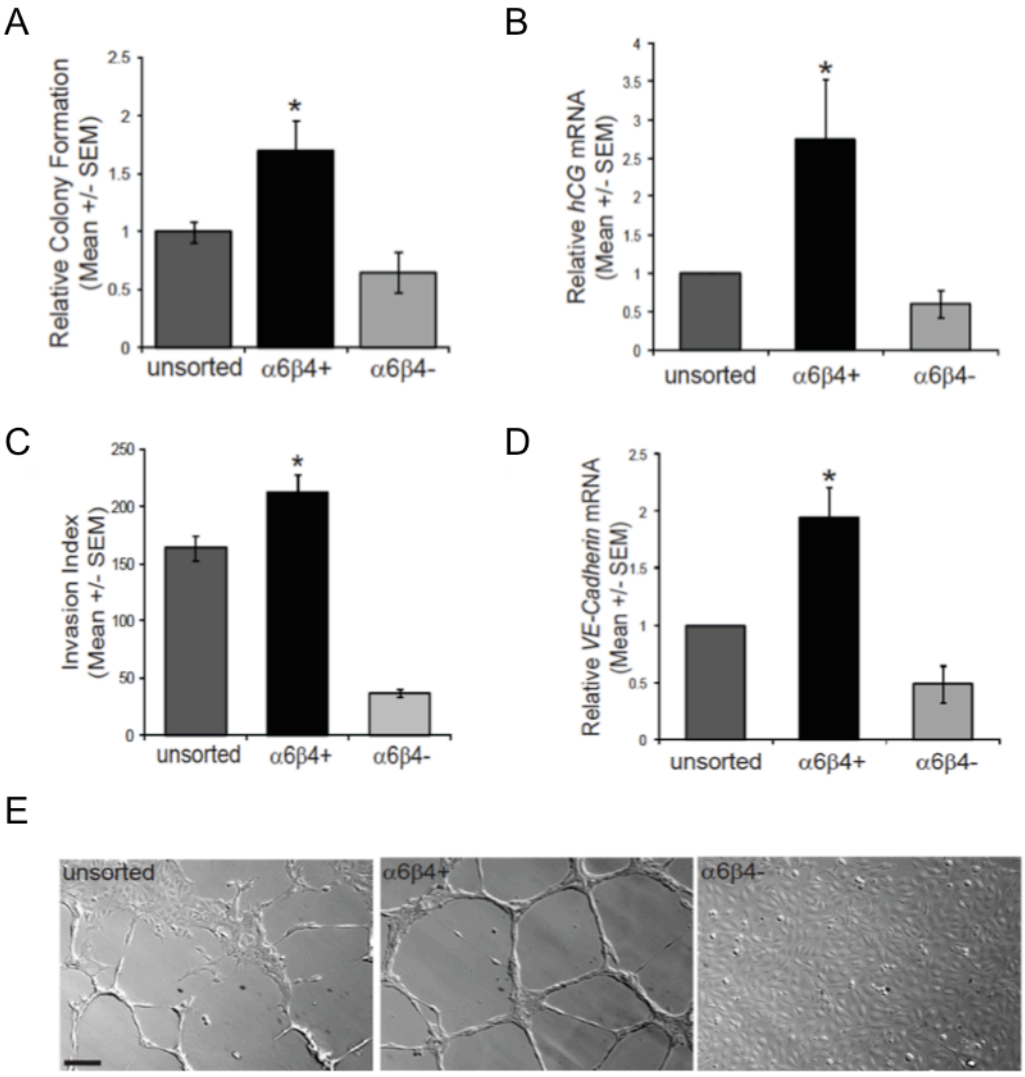

Figure A3. Characteriziation of $\alpha 6\beta 4^{high}$ and $\alpha 6\beta 4^{low}$ subsets from HTR-8/SVneo.

The integrin $\alpha 6\beta 4^{high}$ subset exhibited greater clonogenicity (C) and differentiation along the villous, exhibited by greater hCG expression (D), and extravillous pathway, exhibited by enhanced invasion (E), VE-Cadherin expression (F) and tube formation capacity (G). Scale bar represents 100 μM. (*) indicates significant differences ($p<0.05$) between treatment and control. Completed by Charles Zhong and Lynne Postovit.

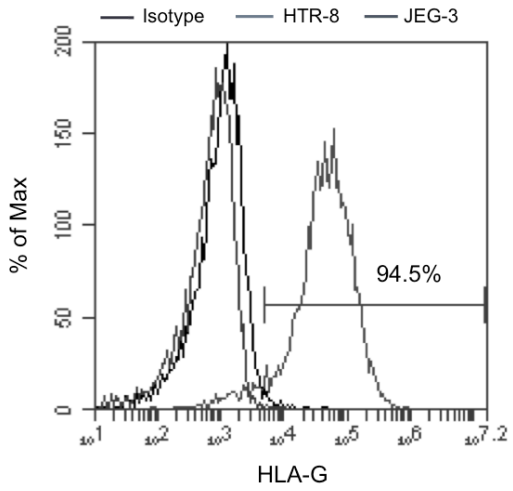

Figure A4. Loss of HLA-G expression in HTR-8/SVneo.

Flow cytometry analysis of surface HLA-G expression in the cell line, HTR-8/SVneo showing that these cells no longer express HLA-G. Conversely, 94.5% of the positive control, JEG-3 cells express HLA-G.

Table A1. Top 20 increased miRNA and corresponding decreased target genes in $\alpha 6\beta 4^{high}$ relative to $\alpha 6\beta 4^{low}$ subset.

RNA was extracted from $\alpha 6\beta 4^{high}$ and $\alpha 6\beta 4^{low}$ subsets. Single stranded cDNA was prepared, and 5.5 ug was end labeled and hybridized, for 16 hours at 45°C, to Human Exon 1.0 ST arrays. One microgram of total RNA was labeled and hybridized to Affymetrix miRNA 2.0 arrays for 16 hours at 45°C. Probe level (.CEL file) data was generated and Probes were summarized to miRNA or gene level in Partek Genomics Suite using the RMA algorithm. Partek was used to determine ANOVA p-values and fold changes for genes and fold changes for miRNAs. Species annotations were added and used to filter only those miRNAs found in *Homo sapiens*. The gene targets for increased *Homo sapiens* miRNAs were determined using TargetScan v5.2 and correlated with a minimum 1.5-fold, decreased genes.

miRNA	Fold-change	Target Gene	Fold-change	GO Biological Process
miR-1825	4.8014	SERPINE1	-2.0512	Response to reactive oxygen species
miR-1244	3.2412	NEXN	-1.592	Regulation of cell migration
miR-940	3.0888	PA2G4	-2.397	rRNA processing
miR-188-5p	3.0048	EFNB2	-1.5292	Lymph vessel development
miR-483-3p	2.6697	ADAMTS6	-1.6584	Proteolysis
miR-1224	2.4332	CSNK1G1	-1.5628	Protein phosphorylation
miR-204	2.3285	SSR3	-1.6564	Cotranslational protein targeting to membrane
miR-153	2.2739	EFNB2	-1.5292	Lymph vessel development
miR-647	2.0546	PRMT6	-1.9422	Base-excision repair
miR-381	1.9757	HMGN2	-1.9907	Chromatin organization
miR-766	1.9718	PDSS2	-1.6405	Ubiquinone biosynthetic process
miR-1286	1.9145	HMGN2	-1.9907	Chromatin organization
miR-370	1.8766	IRS1	-1.7703	Positive regulation of mesenchymal cell proliferation
miR-1228	1.8272	HOXA11	-1.6167	Skeletal system development
miR-1299	1.8187	ADAMTS6	-1.6584	Proteolysis
miR-935	1.7923	SUMO2	-3.9763	Protein sumoylation
miR-664	1.7863	SSR3	-1.6564	Cotranslational protein targeting to membrane
miR-1276	1.7823	HIST1H2AA	-1.9953	Nucleosome assembly
miR-599	1.78	CSNK1G1	-1.5628	Protein phosphorylation
miR-550	1.7715	ADAMTS6	-1.6584	Proteolysis

Curriculum Vitae

Alia Cloutier-Bosworth

EDUCATION

Doctor of Medicine (MD)
Start Sept. 2012 — *Dalhousie University* — Halifax, NS

Master's Degree (MSc)
Sept. 2010-June 2012 — *The University of Western Ontario* — London, ON

- Dept. of Anatomy & Cell Biology
- Courses: ACB9520 Research Seminars in Cell & Neurobiology (90%) and ACB9555 Advanced Topics in Cell Biology (91%)

Bachelor of Science Honours (BScH)
Sept. 2006- Apr. 2010 — *Queen's University* — Kingston, ON

- Major, Minor: Biology and Psychology.
- Cumulative Average: 85% (Dean's Scholar)

Ontario Secondary School Diploma
Sept. 2002- June 2006 — *School of Life Experience (S.O.L.E.)* — Toronto, ON

ACADEMIC AWARDS/FUNDING

- Schulich Graduate Scholarship – University of Western Ontario $7,000 (2011-12)
- Ontario Graduate Scholarship (OGS) – $15,000 (2011-12)
- Schulich Graduate Scholarship – University of Western Ontario $7,000 (2010-11)
- NSERC Alexander Graham Bell Canada Graduate Scholarship (CGS) – $17,500 (2010-11)
- Research in Biology Thesis Poster Competition – Queen's University $100 (2010)
- NSERC Undergraduate Student Research Award (USRA) – $4,500 (2009)
- Chancellor's Award – Queen's University $36,000 (2006)
- President's Award – University of Western Ontario $16,000 (2006) – Declined
- The Governor General's Academic Medal (2006)
- S.O.L.E. Graduating Class Valedictorian (2006)

PUBLICATIONS

Neena Lala[1], Gannareddy V. Girish[1], Alia Cloutier-Bosworth, and Peeyush K. Lala. (2012). Mechanisms in Decorin Regulation of Vascular Endothelial Growth Factor-induced Human Trophoblast Migration and Acquisition of an Endothelial Phenotype. *Biology of Reproduction*, published online June 13. [1]Equal contribution.

PRESENTATIONS

Poster Presentation: Alia Cloutier-Bosworth, Lynne-Marie Postovit, and Peeyush K. Lala. *Investigating the Role of Nodal in the Regulation of Integrin α6β4^{high} Trophoblast Progenitor Cells*, AACR Annual Meeting 2012, Chicago, IL

Poster Presentation: Alia Cloutier-Bosworth, Lynne-Marie Postovit, and Peeyush K. Lala. *Investigating the Role of Nodal in the Regulation of Integrin α6β4^{high} Trophoblast Progenitor Cells*, Anatomy and Cell Biology Research Day 2011, London, ON

Poster Presentation: Alia Cloutier-Bosworth, Lynne-Marie Postovit, and Peeyush K. Lala. *Isolation and Characterization of Trophoblast Stem Cell-like Progenitors*, Oncology Research and Education Day 2011, London, ON

Poster Presentation: Alia Cloutier-Bosworth, Lynne-Marie Postovit, and Peeyush K. Lala. *Isolation and Characterization of Trophoblast Stem Cell-like Progenitors*, Southern Ontario Reproductive Biology 2011, London, ON

Poster Presentation: Alia Cloutier-Bosworth, Lynne-Marie Postovit, and Peeyush K. Lala. *Isolation and Characterization of Trophoblast Stem Cell-like Progenitors*, Developmental Biology Research Day 2011, London, ON

Poster Presentation: Alia Cloutier-Bosworth and William Plaxton. *The Role of Phosphate Nutrition in the Proteolytic Turnover of Secreted Purple Acid Phosphatases of Arabidopsis thaliana*, Biology Research Day 2010, Kingston, ON

SIGNIFICANT RESEARCH EXPERIENCE

MSc Research Project
Sept. 2010-Present *Dept. of Anatomy & Cell Biology, UWO* London, ON

- Isolating and characterizing α6β4^{hi} cytotrophoblast progenitors within the HTR-8/SVneo trophoblast cell line in order to better understand mechanisms regulating trophoblast proliferation and differentiation. Also examining role of Nodal on these mechanisms.

Research in Biology Thesis Project
Sept. 2009-Apr. 2010 *Biology Dept. Queen's University* Kingston, ON

- Examined the role of phosphate nutrition in the proteolytic turnover of secreted purple acid phosphatases, AtPAP12 and AtPAP26, in *Arabidopsis thaliana* seedlings.

NSERC USRA Research (Paid)
May 2009-Aug. 2009 *Biology Dept. Queen's University* Kingston, ON

- Analyzed ability of *Arabidopsis thaliana* to utilize nucleic acids as their sole source of phosphate. Also maintained suspension cell cultures of *A. thaliana.*

Research Assistant
Sept. 2008-Apr. 2009 *Biology Dept. Queen's University* Kingston, ON

- Assisted Queen's Masters student with research involving the evolution of self-incompatibility in the plant, *Camessonia tanacetifolia.*
- Responsibilities included DNA extraction, quantification, and PCR.

Work Study Research Assistant (Paid)
Jan. 2008-Apr. 2008 *Biology Dept. Queen's University* Kingston, ON

- Assisted Queen's PhD student with data analysis and entry regarding macro-invertebrate, *Bythotrephes,* invasion into the Great Lakes.

EXTRACURRICULAR ACTIVITIES

First Aid Clinic Volunteer
Mar. 2011-Present *The Salvation Army* London, ON

- Provide first aid care to homeless and impoverished individuals around the downtown London area. Responsibilities include checking vital signs, caring for wounds, dispensing multivitamins, and educating about the community health service options available.

In-Clinic Volunteer
Jan. 2011-Present *Canadian Blood Services* London, ON

- Responsible for creating a positive, welcoming and comfortable donor experience that promotes donor satisfaction and retention at all Canadian Blood Services donor clinics.

Science Mentor
Sept. 2010-Present *Let's Talk Science* London, ON

- Lead science demonstrations and fun, exciting hands-on activities at local elementary schools to increase problem-solving, communication and teamwork skills, while increasing science literacy and making science fun.

Reading Companion
Sept. 2010-Present *Participation House* London, ON

- Read short stories and articles to persons with significant physical and/or developmental disabilities, and stimulate discussion to provide support and social interaction.

Faculty of Graduate Studies Representative for Medicine & Dentistry
Sept. 2010-Present *Graduate Student Council*, UWO London, ON

- Act as liaison between the Faculty of Graduate Studies and the Faculty of Medicine & Dentistry.

President & Co-founder
Jan. 2008-Jan. 2009 *AIR (Africa is Real) Queen's Chapter* Kingston, ON

- Certified Queen's Club. Helped raise funds and awareness for the poverty and AIDS in Africa through fundraisers and social events.

Psychiatric Recreation Coordinator/Cardiac Rehab Volunteer
Sept. 2006-Dec., 2008 *Hotel Dieu Hospital* Kingston, ON

- Provided companionship and support to patients. Helped reduce anxiety and boredom by implementing, organizing, and introducing recreational/leisure activities to patients and their visitors.
- Motivated and aided patients during their prescribed exercise regimes. Helped lead the warm-up and cool-down stretches.