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The Role of NUMB in Human Extravillous Trophoblast Cell Migration and Survival

By

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ABSTRACT

Placental growth and development depend on highly regulated extravillous trophoblast (EVT) function, such as proliferation, migration and invasion. NUMB has been reported to play a role in cell differentiation and proliferation. An established cell line (HTR8/SVneo) was used as a model to study the role of NUMB in invasive extravillous trophoblast cell migration and survival in the human placenta. Immunofluorescence showed the presence of NUMB at the plasma membrane and the cytoplasmic region in HTR8/Svneo cells. NUMB isoform 1 significantly increased EVT migration while NUMB knockdown decreased the rate of migration as compared to the negative control. Overexpression of Numb 1,3,4 and 8 did not influence cellular viability whereas Numb 2 overexpression decreased trophoblast viability significantly. We have demonstrated for the first time that Numb is expressed in human invasive extravillous trophoblasts and that its role is isoform-dependent. Dysregulation of NUMB expression/function may play a role in placental pathologies such as preeclampsia and IUGR.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
bp	Base pairs
BrdU	Bromodeoxyuridine (5-bromo-2-deoxyuridine)
BSA	Bovine serum albumin
cDNA	Complementary deoxynucleic acid
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethylsulphoxide
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ERK 1/2	Extracellular signal-regulated kinase1/2
EVT	Extravillous trophoblast
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3' phosphate dehydrogenase
GFP	Green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IGFII	Insulin-like growth factor II
IgG	Immunoglobulin G
IHC	Immunohistochemistry
kDa	kilodalton
KO	Knock-out
LB	Lysogeny Broth
MAPK	Mitogen-activated protein kinase
M-MLV RT	Murine Leukemia Virus reverse transcriptase
MMP	Matrix metalloproteinase

mRNA	Messenger ribonucleic acid
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PTB	Phosphotyrosine binding
PBS	Phosphate buffered saline
PI3K	Phosphotidylinositol 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
PRR	Proline rich region
RCF	Relative centrifugal force
RNA	Ribonucleic acid
RPMI 1640	Roswell ark Memorial Institute 1640
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of mean
shRNA	Short hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
S.O.C.	Super Optimal Catabolite repression medium
TAE	Tris-acetate-EDTA
TBS-T	Tris buffered saline Tween-20
UV	Ultraviolet
WB	Western blot

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CHAPTER 1: INTRODUCTION

1. Placental Development

i. Developmental stages:

Prelacunar Stage

Placental development begins at the stage of implantation known as apposition. At this initial stage, which takes place at day 6 to 7 after fertilization, there exists a close communication between the extra-embyronic tissue of the embryo and uterine mucosa (endometrium) of the mother. That is, the blastocyst becomes implanted into the nutrient filled, highly vascular decidua basalis and forms the maternal face of the placenta. The blastocyst is made up of two layers: an external layer composed of trophoblast, which acts as the "stem cell" setting the foundation for both fetal and placental development, and an innermost layer, the embryoblast, which forms the umbilical cord, embryo and amnion (Benirschke and Kaufmann, 2000).

During the second week after fertilization, implantation continues and is mediated by proliferating and invading trophoblasts. Trophoblasts of the embryonic pole of the blastocyst fuse with neighbouring cells to form the syncytiotrophoblast layer. The underlying cytotrophoblast still remain unfused and do not come in contact with maternal tissue. The progressive invasion allows for additional parts of the blastocyst to come in contact with maternal tissue and the increased proliferation and fusion of cytotrophoblast results in a thickened, solid layer of syncytiotrophoblast which is characteristic of the prelacunar stage (Benirschke and Kaufmann, 2000; Edmonds and Dewhurst, 2007; Larsen, 1997).

Lacunar Stage

The lacunar stage (day 8-13) is marked by the initiation of formation of lacunae (small intrasyncytial vacuoles within the syncytiotrophoblastic mass) which are separated from one another by trabeculae (syncytiotrophoblasts septa) at the implantation pole. As the syncytiotrophoblastic mass expands, it covers the entire surface of the blastocyst, and forms a system of lacunae, which further subdivides the outermost trophoblastic portion of the blastocyst (Benirschke and Kaufmann, 2000). This subdivision consists of three layers (Benirschke and Kaufmann, 2000):

(1) The *primary chorionic plate*, which consists of a stratum of cytotrophoblastic layers. Two weeks after fertilization, the inner surface of the cytotrophoblastic layer becomes surrounded by mesenchymal cells which transform into the extraembryonic mesenchyme.

(2) The *lacunar system, along with the trabeculae,* become the intervillous space and the anchoring villi

(3) By day 15 after fertilization, cytotrophoblasts surround the trabeculae and form the outer most layer of the trophoblast, the *trophoblastic shell*, which faces the endometrium.

Day 12 after fertilization also marks the completion of implantation. The embryo is completely embedded within the endometrium with a surrounding surface layer of syncytiotrophoblast. At this point the chorion is also produced as mesenchymal cells surround the inner trophoblastic surface (Edmonds and Dewhurst, 2007).

The uteroplacental circulatory system becomes evident late in the second week. The newly formed trophoblastic lacunae and the preexisting lacunae anastomose with

maternal capillaries, establishing the maternal perfusion of the entire lacunar system (Larsen, 1997).

Early Villous Stages

The villous stage is marked by the presence of maternal erythrocytes within the lacunae and the presence of cytotrophoblastic projections called primary villi during the second week of gestation. Proliferation and branching initiate the development of villous trees which are housed in the maternal blood filled intervillous space. The next two days are marked by the presence of mesenchymal cells which invade the expanding villi, converting them into secondary villi. Towards the third week of pregnancy, the secondary villi become transformed into tertiary villi, which consist of an outer syncytiotrophoblastic layer and an inner cytotrophoblastic layer. This transition is marked by the appearance of blood vessels within the mesenchymal core of the villi and considerable branching. Villi usually cover the entire chorion but, by the end of the third month, they become localized to the embryonic pole, known as the chorion frondosum. The placenta is then clearly composed of two components: (1) the maternal portion consisting of the basal plate, which is derived from the decidua basalis, and (2) the fetal portion consisting of the chorionic plate, which is formed by the chorion frondosum (Benirschke and Kaufmann, 2000; Carlson, 1999; Edmonds and Dewhurst, 2007; Mitchell and Sharma, 2005). An illustration of the human placenta and the trophoblast distribution is represented by Figure 1.

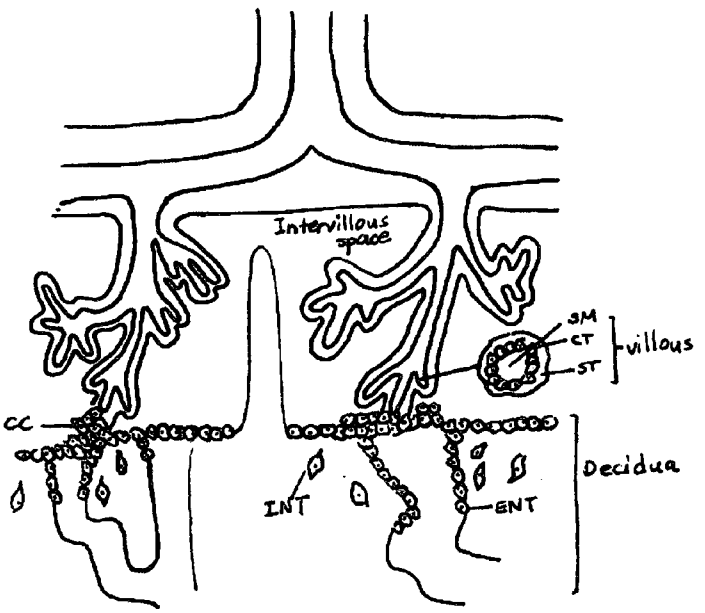


Figure 1. Cross Section of the Human Placenta. The human placenta is composed of villi situated in an intervillous space. Each villous is composed of a stromal cell (SM) core surrounded by an internal cytotrophoblast (CT) and an outer syncytiotrophoblast (ST) layer. Anchoring villi form trophoblast cell columns (CC) with the maternal decidua. The cell population (extravillous trophoblast) from these cell columns invade the decidua and differentiate into interstitial (INT) and endovascular (ENT) trophoblasts to remodel the spiral arteries.

The syncytiotrophoblast layer produces the outer most region of the villi and are covered by a larger number of microvilli. The microvilli increase the total surface area of the placenta and aid in transport, providing the developing fetus with an efficient nutrient supply (Nishimura *et al.*, 2004; Rama and Rao, 2003). Under hypoxic conditions, cytotrophoblastic cell columns penetrate the surrounding syncytiotrophoblastic layer and form cytotrophoblastic shells around maternal decidual cells. These cell columns are characteristic of anchoring villi and serve as attachment points to the trophoblastic shell, and therefore position the placenta to the uterine wall (Nishimura *et al.*, 2004). Importantly, all surfaces of the villi, chorionic plate as well as the cytotrophoblastic shell are bathed in continually exchanging maternal blood and, as a result, the human placenta is considered hemochorial. The proliferating cell columns at distal ends of the anchoring villi give rise to extravillous trophoblast cells (EVT) (Pollheimer and Knofler, 2005), which invade the decidua continuously throughout the first trimester of pregnancy (Chaddha *et al.*, 2004). Extravillous trophoblasts are an invasive cell type which penetrates the decidual bed to differentiate into interstitial trophoblasts or endovascular trophoblasts. Interstitial trophoblasts invade the decidual stroma to replace the arterial wall with matrix-type fibrinoid and allow adequate blood flow into the intervillous space (Cartwright *et al.*, 2002; Chaddha *et al.*, 2004). The endovascular trophoblasts are persistent throughout the first trimester of pregnancy and proliferate to form clumps, which in turn occlude the spiral arterioles to prevent maternal blood flow from entry into the intervillous space (Figure 1). By the end of the first trimester the disintegration of the endovascular trophoblast and the first inflow of maternal blood into the intervillous space is observed (Chaddha *et al.*, 2004; Huppertz *et al.*, 2006). As a result, blood vessels of

low resistance and high capacitance are formed to facilitate increased blood flow to the developing fetus (Bauer *et al.*, 2004; Cartwright *et al.*, 2002). Furthermore, as soon as intravillous fetal blood circulation is established, both placental (i.e. maternal) and fetal blood come into close proximity with one another. With advancing gestation, the placenta grows and gives rise to mature villi and larger capillaries which lay close to a thinner syncytiotrpohoblast layer. The cytotrophoblastic cell layer also disappears, and the intervillous space functions as an area in which exchange of respiratory gases (oxygen, carbon dioxide, and carbon monoxide) occurs by simple diffusion. In addition, transfer of nutrients and electrolytes (amino acids, fatty free acids, carbohydrates and vitamins), diffusion of maternal antibodies (maternal immunoglobulin) and production of hormones (progesterone, estrogenic hormones and human chorionic gonadotropin) occur in this specialized space (Carlson, 1999; Moore and Persaud, 2008; Sadler, 2004).

At the end of the fourth month, the placenta is composed of 10-38 cotyledons, villi groups separated by placental septa. The placenta starts to grow in diameter rather than in thickness, covering approximately 15-30% of the internal surface of the expanding uterus. At term, a healthy placenta is discoid shaped (approximately 20 cm in diameter), 3 cm thick and weighs 500g (1/6 of the fetal weight) (Benirschke and Kaufmann, 2000; Moore and Persaud, 2008; Pansky, 1982).

ii. Placental Circulation

The placental circulation requires the contribution of both the maternal and fetal circulations (Carlson, 1999; Moore and Persaud, 2008). The fetal circulatory system houses both umbilical and placental vessels. The two umbilical arteries within the placenta provide a means of transportation for fetal blood to reach the placenta. Small extensions from these arteries displace blood into the chorionic villi and then break into capillary networks in terminal chorionic villi where fetal-maternal exchange occurs (Carlson, 1999). Venous branches from the villous capillary beds are directed to the umbilical vein and to the fetus. The maternal circulation is not bound within vessels, but 80-100 spiral arteries drain into the villous space under reduced pressure, situating the villi in 50 ml of oxygenated nutrient-enriched maternal arterial blood. The endometrial veins drain waste products into the maternal circulation and the oxygenated fetal blood from the placenta is carried through the umbilical vein. Deoxygenated fetal blood is directed from the fetus to the placenta through the umbilical arteries (Benirschke and Kaufmann, 2000; Edmonds and Dewhurst, 2007; Moore and Persaud, 2008).

iii. Placental Apoptosis

Apoptosis, also known as programmed cell death, is a naturally occurring phenomenon during normal placental development. It is observed in villous trophoblasts throughout gestation and increased apoptosis is seen in the syncytium at term (Gruslin *et al.*, 2001; Jurisicova *et al.*, 2005). This type of cell death is crucial for the regulation of syncytial turnover. Indeed, syncytiotrophoblasts of the villi are under continuous

renewal. However, the proliferative properties within terminal villi are retained by the cytotrophoblasts. As fusion of particular cytotrophoblasts occurs to eventually join the syncytial layer, these cells initiate the expression of apoptosis-related proteins required for syncytial fusion and transfer them, in addition to the nuclei and cytoplasmic components of the cytotrophoblast, into the syncytiotrophoblast. The apoptotic cascade remains at rest immediately after fusion due to the up-regulation of anti-apoptotic proteins. Upon reactivation of the apoptotic cascade, syncytiotrophoblast undergo nuclei aging, shrinkage and are eventually packed into tight clumps known as syncytial knots. Syncytial knots are then released from the syncytiotrophoblast, into the maternal circulation to eventually be engulfed by pulmonary macrophages (Huppertz *et al.*, 2006).

Normal placental growth occurs in an elaborate array of events which include the maintenance of cell populations by proliferation and cell death. Pregnancies complicated by intrauterine growth restriction (IUGR) as well as preeclampsia (PET) have also shown an increase in cytotrophoblast apoptosis (Erel *et al.*, 2001). PET, in particular, has been shown to be associated with increased end-stage apoptosis in the syncytiotrophoblast, rendering the function of the villous layer inefficient in mediating nutrient transport and, in turn, causing fetal growth arrest and distress (Scifres and Nelson, 2009). Furthermore, in early onset PET and IUGR, apoptosis affects the invasive extravillous trophoblast by reducing the number of endovascular trophoblasts in the maternal arterial walls (Heazell and Crocker, 2008; Huppertz *et al.*, 2006). As a result, apoptosis could represent a mechanism by which reduced maternal blood flow to the placenta contributes to altered placental phenotype associated with both preeclampsia and intrauterine growth restriction (DiFederico *et al.*, 1999).

2. Molecular Dynamics Governing Cellular Migration/Invasion During Human Placental Development

Healthy uteroplacental homeostasis depends on highly regulated extravillous trophoblast functions, such as proliferation, migration and invasion. Several studies have shown that these functions are regulated by growth factors, growth factor binding proteins, adherence junction molecules (epithelial-cadherin) and cell surface integrins (Kabir-Salmani *et al.*, 2003; Rahnama *et al.*, 2006; Widmer *et al.*, 2007).

Integrins recognize a variety of extracellular matrix (ECM) components and mediate cell adhesion, migration, invasion and activate signal transduction pathways, including the mitogen-activated protein (MAP) kinase and phosphatidylinositol 3 (PI3) kinase pathways (McKinnon *et al.*, 2001). During normal trophoblast migration/invasion, integrin expression is altered in EVT in response to signals induced by adhesion to the ECM. This "switch" in integrin expression results in a change from an epithelial phenotype of the cytotrophoblasts to an endothelial one. This requires downregulation of integrins $\alpha6\beta4$ and upregulation of integrins $\alpha5\beta1$ and $\alpha1\beta1$ (Fukushima *et al.*, 2003; Lim *et al.*, 1997; Nishimura *et al.*, 2004; Zhou *et al.*, 1993), which further promotes the trophoblast to migrate, invade and remodel the maternal arterioles

Invasion by trophoblast cells is a multistep process which also involves attachment to a basement membrane or extracellular matrix (ECM) components followed by degradation and subsequent migration through the degraded components. Matrix metalloproteinase-9 (MMP-9; Gelatinase B) is part of the MMP family of neutral proteinases that regulate and catalyze the degradation of the ECM. MMP-9 is important

in placental development and plays a major role in the degradation and reconstitution of the ECM in uterine endometrium (Behrendtsen *et al.*, 1992). Epithelial growth factor (EGF) has been shown to up-regulate MMP-9 activity in a variety of cell types, and activates the P13K/Akt signaling (involved in regulating cell proliferation and survival (Ferretti *et al.*, 2007) and MAPK/extracellular-signal regulated kinase (ERK) signaling (promotes cell motility) in HTR8/SVneo (EVT) cells (Forbes and Westwood, 2008; Qiu *et al.*, 2004).

The insulin-like growth factor (IGF) is a family of peptide growth factors, which include IGF-I, IGF-II, six IGF binding proteins (IGFBP) and four cell surface receptors [type 1 or 2 IGF receptors (IGFR-I and IGFR-II), insulin receptor (IR) and IGFR-I/IR receptor] (Forbes and Westwood, 2008; McKinnon *et al.*, 2001). IGF-II is produced by the trophoblast and stimulates invasion by promoting migration without affecting proliferation (Irving and Lala, 1995). This cellular process is governed by the ability of IGF-II to signal through the IGFR- I or II (Forbes and Westwood, 2008; Fowden, 2003; Shields *et al.*, 2007) and in turn, activating the MAPK pathway and promoting EVT cell migration (Hamilton *et al.*, 1998; McKinnon *et al.*, 2001).

Although trophoblast migration has been shown to be upregulated through IGF signaling, hypoxia has also been shown to influence trophoblast fate, such that studies have revealed that low oxygen tension promotes trophoblast proliferation while the increase in oxygen promotes trophoblast differentiation and invasion (Jurisicova *et al.*, 2005). Furthermore, it has been shown that in response to hypoxic conditions, epidermal growth factors derived from maternal sources and the syncytiotrophoblast, mediate

syncytial fusion of the post-proliferative cytotrophoblast to regenerate the damaged syncytiotrophoblast (Benirschke and Kaufmann, 2000).

3. Intrauterine Growth Restriction (IUGR)

Intrauterine growth restriction (IUGR) is a condition characterized by failure of the fetus to reach its growth potential. As such, birth weight is below the 5th centile for the given gestational age (Cetin and Antonazzo, 2009; Roberts and Post, 2008). IUGR is one of the leading causes of perinatal morbidity and mortality (Peleg *et al.*, 1998) and its incidence is approximately 5 percent in the general population. Fetal causes of IUGR include aneuploidy (fetal chromosomal anomalies such as sex chromosome abnormalities, trisomy 13 and 18) and multifetal gestation. Maternal causes include drug and cigarette exposure, medical complications such as hypertension, anemia, diabetes mellitus, chronic pulmonary disease, and congenital infections such as malaria and rubella. Uteroplacental insufficiency makes up 25-30% of the causes of IUGR. This may be related to Mullerian anomalies (e.g. septate uterus), chronic placental abruption, placental infarcts (i.e. necrotic villous tissue), placenta previa, and placental infections among others. In all cases however, placental function and growth are compromised. As a result, there is deficient oxygen and nutrient supply to the fetus, which ultimately results in impaired growth. Pregnancies complicated by IUGR show a reduced placental size and function (Norwitz and Schorge, 2001; Reece and Hagay, 2007; Roberts and Post, 2008). Inadequate trophoblast migration and invasion are linked to restricted placental development and pose as a leading cause of IUGR.

The inactivation of adhesion molecules and integrins has been shown to be linked to insufficient endothelial cell invasion into the spiral arteries. For example, impaired trophoblast migration is associated with inefficient up-regulation of MMP-9 and unsuccessfully digests the extracellular matrix of the feto-placental bed. Therefore, the role of EVT migration and invasion of the arterioles is essential for the completion of the remodeling process, including the replacement of the endothelium by the endovascular trophoblast. Defects in trophoblast adhesion molecule expression may provide an explanation for reduced spiral arteries remodeling, poor placentation and inadequate placental perfusion (Lala and Chakraborty, 2003).

Although cellular and molecular abnormalities have provided explanations for impaired placental function, the clinical detection of IUGR is based on Doppler ultrasound, placental morphology, and clinical evaluation of the mother. Ultrasound allows for measurements of the fetal head, abdominal circumference and femur length which are computed in an estimated weight. This can be further compared to average fetal weight at a given gestational age (Peleg *et al.*, 1998). Placental morphology can vary from a thick globular placenta to one displaying several infarcts. Doppler flow allows for the monitoring of blood as it flows through blood vessels in the fetal brain and the umbilical cord. In situations of uteroplacental insufficiency, high resistance flow is depicted in the umbilical arteries and increased velocity is apparent in the fetal middle cerebral artery as a result of redistribution of blood to preserve brain function (APA, 2009; Beckman *et al.*, 2006).

Furthermore, placental insufficiencies, deprivation of maternal nutrients, as well as increased maternal stress, influence the fetal environment and lead to physiological

readjustments throughout development (Jurisicova *et al.*, 2005). It is now believed that early exposure to such adverse intrauterine environments can reprogram the fetal hypothalamic-pituitary-adrenal (HPA) axis. As a result, HPA axis dysregulation can pose long term health risks in adult life with development of the metabolic syndrome as well as immune or inflammatory diseases (Lesage *et al.*, 2006). Hence, newborns affected by IGUR have an increased risk of development of adult onset diseases such as cardiovascular disease, type 2 diabetes, hypertension, arteriosclerosis, hyperlipidemia, and cognitive diseases later in life (Ross and Beall, 2008; Sitras *et al.*, 2009).

4. Preeclampsia (PET)

Preeclampsia is a multisystem syndrome strictly associated with pregnancy (usually within the third trimester) and characterized by hypertension and proteinuria. It is the most common cause of maternal mortality globally (Carlson *et al.*, 2004). If left untreated, preeclampsia can develop into eclampsia, leading to convulsions and coma. Women at risk of developing preeclampsia are often those with a personal or family history or suffering from medical problems including hypertension, renal disorders, autoimmune diseases and diabetes. Women over 40 and teenagers as well as women carrying multiple fetuses also have elevated risks of developing preeclampsia (Taeusch *et al.*, 2005).

Preeclampsia is characterized by endothelial dysfunction, which is thought to result from factors released by the placenta secondary to its exposure to oxidative stress. Major pathological changes associated with PET include decidual arteriolopathy, infarcts,

abruption placentae (detachment of the placentae from the decidual bed) and Tenny-Parker changes, which are characterized as excessive budding of syncytial cytoplasm and agglomeration of the nuclei, producing "knots" at the villous surface. These Tenney Parker changes suggest the presence of uteroplacental ischemia in preeclampsia (Lyall and Belfort, 2007).

In addition, PET placentae are characterized by reduced villi number, which eventually undergo atrophy and degeneration. PET infarcts are often located in the central region of the placentae and are common at the placental edge, where they appear "plastic", commonly white in appearance and firm. These placental infarcts are usually not invaded by fibrinoid tissue, as seen in many other organs, and lack the spongy texture most common to healthy placentae (Benirschke and Kaufmann, 2000).

Perinatal outcome of preeclamptic pregnancies are impacted by gestational age among several other factors including maternal well being. These infants are of low birth weight, premature, malnourished and may face cognitive disabilities, cerebral palsy and epilepsy (http://yourtotalhealth.ivillage.com). As a result of the reprogramming of structural and metabolic systems in response to limited nutrient supply, these changes may be the origin of a number of diseases in adulthood, including coronary heart disease and diabetes (Jurisicova *et al.*, 2005).

Although preeclampsia is usually monitored by blood testing, urinalyses as well as routine Doppler ultrasonography, which allows for the monitoring of the vasculature of the uterine arteries, as in IUGR, the only available treatment is delivery (Taeusch *et al.*, 2005).

5. Placental Insufficiency in IUGR and PET

Both IUGR and PET are contributing factors to maternal and fetal mortality and morbidity. Although an expectant mother's medical history contributes tremendously to a healthy pregnancy, it is however not the only culprit. A vast majority of studies have linked insufficient maternal arterial remodeling and poor EVT cell invasion as key factors contributing to preeclampsia and IUGR (Caniggia *et al.*, 2000; Chaddha *et al.*, 2004; Cross, 2003; Kaufmann *et al.*, 2003; Rahnama *et al.*, 2006; Widmer *et al.*, 2007). Human placental bed biopsies have revealed that incomplete trophoblast differentiation along the invasive pathway, as a result of cell-cycle arrest, results in shallow trophoblast invasion and inadequate remodeling of the uterine arteries. As a result, fetal development is affected by poor nutrient uptake and inadequate oxygen tension (Caniggia *et al.*, 2000; Chaddha *et al.*, 2004; Cross, 2003). Furthermore, these placentae show an increase in endovascular apoptosis (Kaufmann *et al.*, 2003) and enter a proliferative pathway (rather than an invasive pathway), culminating in a reduction in trophoblast differentiation (Goldman-Wohl and Yagel, 2002). In PET, shallow trophoblast invasion is associated with the inability of the trophoblast to modulate the alpha1 integrin switch at the mRNA and protein level (Lim *et al.*, 1997) and in turn, inhibit trophoblast differentiation (Fukushima *et al.*, 2003). The continuous expression of adhesion molecules that characterize the stem cell population of villous cytotrophoblasts inhibits invasion of the extravillous trophoblast. During normal pregnancy, invasive trophoblasts undergo an epithelial to endothelial cell transformation and express endothelial cell specific adhesion molecules $\alpha 5\beta 1$ and $\alpha 1\beta 1$ (Goldman-Wohl and Yagel, 2002). In preeclampsia, the

trophoblasts do not acquire the endothelial cell phenotype, and their migration towards the spiral arteries into the decidual stoma is reduced significantly.

In addition, pregnancies affected by IUGR and PET show an increase fluctuation in placental oxygen concentration. In turn, antioxidant defenses at the maternal-fetal interface can become permeated, resulting in oxidative stress. Consequently, oxidative stress causes cellular damage, and therefore impairs normal extravillous trophoblast function and may lead to cell death (Jauniaux *et al.*, 2000). Studies examining trophoblast apoptosis in pregnancies complicated with IUGR have shown increased expression of the transcription factor p53 in human placental villi (Levy *et al.*, 2002). In addition, *in vitro* studies have revealed the exposure of villous trophoblasts to hypoxic conditions causes an increased p53 expression (Heazell and Crocker, 2008; Jurisicova *et al.*, 2005). As a result, it is postulated that the increased apoptosis observed in villous trophoblast may be a result of exposure to a noxious stimulus apparent in IUGR and PET (Heazell and Crocker, 2008).

PET is distinguished from IUGR by the specific failure of villous trophoblast differentiation and IUGR from PET by the specific failure of extravillous trophoblast differentiation (Huppertz *et al.*, 2006). Table 1 depicts the differences in cellular behavior in response to IUGR and PET and the effect of both conditions on the vasculature of the placental system resulting from inadequate cytotrophoblast and extravillous trophoblast function. The upstream factors contributing to the extravillous trophoblasts invasive properties, such as trophoblast cell turnover and the integrin switch promoting differentiation (Zhou *et al.*, 1993) are associated with differentiation during

earlier development. Together, they provide an understanding to the reasons behind restricted placental blood flow to the developing fetus and insufficient nutrition uptake.

Table 1. Features of early onset intrauterine growth restriction and preeclampsia

	IUGR	Preeclampsia
Trophoblast specific insult	Extravillous (2^{nd} trimester)	Villous (1^{st} trimester)
Trophoblast invasion	Shallow	Shallow
Maternal spiral arteries	Poor transformation	Inadequate/incomplete invasion
Inadequate Blood flow	Uterine arteries Umbilical	Placental bed Uterine arteries Umbilical arteries
Fetal growth restriction[a]	Yes	Severity level and in association with IUGR

(Huppertz, 2006)

[a]fetal growth restriction (less than 3^{rd}, 5^{th} or 10^{th} percentile for given gestational age)

6. NUMB

i. NUMB protein structure

NUMB was first described in *Drosophila* as a mutation during neural sensory organ development (Uemura *et al.*, 1989). It is now well established that NUMB plays a role in neurogenesis. NUMB is characterized by an N-terminal phosphotyrosine binding (PTB) domain and a proline rich region (PRR) domain in the c-terminus of the protein (Li *et al.*, 1997; Li *et al.*, 1998). The PTB domain is 20% homologous to the adapter Src protein at the amino acid level (Yaich *et al.*, 1998) and critical for NUMB function (Bork and Margolis, 1995). The PRR domain of NUMB contains several putative Src homology 3 (SH3) domain binding sites as well as Esp15 homology (EH) domain binding motifs, DPF and NPF (Dho *et al.*, 1999). The EH domain is a component of the endocytic machinery (Santolini *et al.*, 2000).

As a result of alternate splicing, four known NUMB isoforms exist, with sizes 65, 66, 71 and 72 kDa (Figure 2), differing from one another on the basis of an 11 amino acid sequence insert within the phosphotyrosine binding (PTB) domain region of the N terminus and a 49 amino acid sequence insert at the C terminus region, the Proline Rich Region (PRR) (Bani-Yaghoub *et al.*, 2007; Dho *et al.*, 1999). These isoforms are cell-specific and are expressed only in mammalian counterparts, as only one NUMB isoform exists in *Drosophila* development.

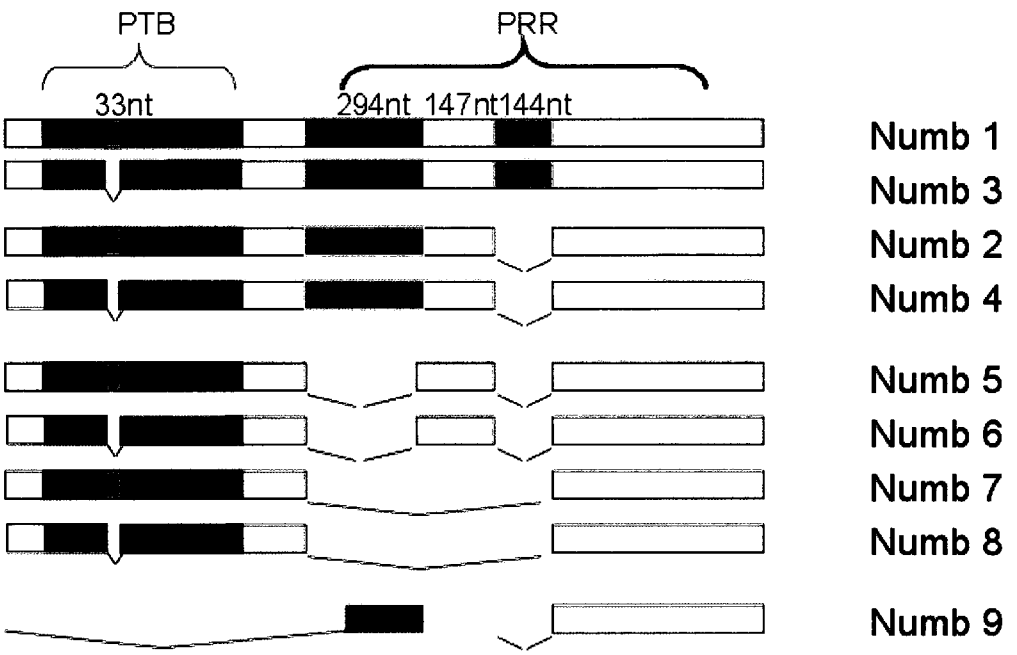


Figure 2. Schematic representation of NUMB isoforms 1, 2, 3 and 4, and novel NUMB isoforms 5, 6, 7, 8 and 9.

ii. NUMB function

It is well established that by means of asymmetric cell division, NUMB is a determinant of cell-fate during *Drosophila* and mouse neurogenesis which promotes distinct fates in the resultant daughter cells (Zhong *et al.*, 2000). NUMB expression is localized to adult tissues and mouse embryos, suggesting its function being non-specific. Interestingly, only two of the four NUMB isoforms which contain the PTB insert are localized to the plasma membrane: NUMB 1 and 3 (Dho *et al.*, 1999). These two isoforms are further translocated into the cytosol via Protein Kinase C (PKC), a serine/threonine protein kinase activated by Ca^{2+} (Alberts *et al.*, 2002).

Studies using chromaffin PC12 cells demonstrated that NUMB isoforms with short PTB enhance differentiation upon growth factor stimulation to a similar degree as NUMB isoforms with long PTB (Alberts *et al.*, 2002; Pedersen *et al.*, 2002; Tyson *et al.*, 2003), suggesting that differentiation is independent of the length of the PRR. Recent studies have also shown that NUMB expression can be switched on and off during development of specific neuronal functions on the basis of the PTB and PRR. NUMB 1 and 3 are only switched on in undifferentiated cortical progenitors while NUMB 2 and 4 are expressed throughout differentiation during mouse neurogenesis (Bani-Yaghoub *et al.*, 2007). *Drosophila* outer optic anlages of the larval brain studies have shown that expression of individual NUMB isoforms during neurogenesis is specific with respect to the developmental stage of specific cell types. Neural epithelial stem cells in the outer optic anlage of the *Drosophila* larval brain were used as a model for studying NUMB distribution during cellular proliferation and differentiation. Isoforms with the long PRR promoted proliferation without affecting differentiation during early neurogenesis and

isoforms with the short PRR inhibited stem cell proliferation and promoted neural differentiation in late neurogenesis (Toriya *et al.*, 2006). Null mouse studies have further shown severe defects during neural development in addition to embryonic death at day 11.5 (Zhong *et al.*, 2000).

iii. NUMB function and Notch signaling during asymmetric cell division

The study of NUMB and its role in Notch signaling have been studied extensively. The Notch receptor is a single transmembrane receptor involved in controlling cell fate decisions during development. Upon activation by the delta ligand, the Notch receptor undergoes a series of cleavage events leading to the translocation of the intracellular domain to the nucleus and up-regulation of transcription factors (Chapman *et al.*, 2006). As a cell fate determinant, NUMB could antagonize Notch signaling by promoting its endocytosis during asymmetric cell division (Gold *et al.*, 2009).

Asymmetric cell division refers to the unequal distribution of specific molecules to two daughter cells (Alberts *et al.*, 2002). As a result of the vertical place of spindle formation, NUMB is unequally distributed to one of the two daughter cells (Cayouette and Raff, 2002). The daughter cell which inherits NUMB differentiates into a new cell, while the other daughter cell remains identical to the original cell, thus maintaining neural progenitor cells (Cayouette and Raff, 2002; Shen *et al.*, 2002). NUMB influences cell fate by interaction of its PTB and PRR domains. The PTB binds to the cytosolic tail of Notch. The PRR domain consists of two motifs: DPF and NPF. The DPF motif binds

α adaptin, a subunit of the AP2 complex which is a major component of clathrin coated vesicles (Santolini *et al.*, 2000; Smith *et al.*, 2004; Tang *et al.*, 2005). On the other hand, the NPF motif associates with proteins containing Esp15 homology, a component of the endocytic machinery (Salcini *et al.*, 1997), to stimulate Notch receptor endocytosis (Cayouette and Raff, 2002; Smith *et al.*, 2004). Eps15 must be released from the AP2 complex for NUMB to bind to it, therefore the Esp15 complex is disassembled upon clathrin-coat assembly (Santolini *et al.*, 2000).

iv. The role of NUMB and p53

Double minute 2 protein, MDM2, an oncogene, has been show to be up-regulated in several human tumor cells. It interacts with p53 at its N-terminal transactivation domain (Oliner *et al.*, 1993), thus preventing p53 transactivation of target genes (Momand *et al.*, 1992) and promoting its degradation via the ubiquitin-proteosome pathway (Haupt *et al.*, 1997). The p53-MDM2 complex has been shown to bind to the N-terminal (PTB domain) of NUMB, by MDM2 (Juven-Gershon *et al.*, 1998), which in turn, functions as the ubiquitin ligase towards NUMB (Yogosawa *et al.*, 2003), tand prevents p53 degredation. (Colaluca *et al.*, 2008). Furthermore, NUMB has been shown to act as an oncosupressor in primary breast tumor cells. The loss of NUMB causes a decrease in tumor suppressor p53 and an up-regulation of the Notch receptor (Colaluca *et al.*, 2008; Pece *et al.*, 2004).

To date, the role of NUMB/Notch signaling and p53 in cellular apoptosis has not been thoroughly investigated. Only one group investigated the regulation of apoptosis by

NUMB/Notch signaling during the development of the serotonin lineage in *Drosophila*. Cells experienced over-proliferation in the absence of Notch signaling but acquired an apoptotic status upon Notch activation and NUMB inhibition. Furthermore, p53 counteracted Notch-mediated apoptosis and rescued cells from undergoing apoptosis (Lundell *et al.*, 2003).

v. NUMB in cell migration

To date the role of NUMB in cell migration remains unclear. One study examined Notch expression in migrating peripheral glial cells and showed increased Notch activity and reduced levels of NUMB expression during migration (Edenfeld *et al.*, 2007). , Nishimura *et al.* (2007) carried out an extensive study in HeLa cells with respect to NUMB localization in migration. It was concluded that NUMB was an important component of the machinery for directional trafficking in migrating cells. NUMB also bound to integrin beta 1 and 3 via the PTB domain at the leading edge and colocalized with focal adhesions in migrating HeLa cells. NUMB knockout inhibits intergrin endocytosis and HeLa cell migration (Nishimura and Kaibuchi, 2007). Although these studies examined NUMB localization during cellular migration, the influence of NUMB isoforms 1-4 on cellular migration and the mechanism by which NUMB functions, if any, in promoting migration is still unclear.

vi. NUMB and the Placenta

To date no studies have systematically examined the expression and the role of NUMB in the human placenta. , Zilian *et al.* (2001) developed NUMB knockout mice and investigated the role of NUMB in mouse neurogenesis. It was observed that null NUMB (-/-) mice died at embryonic day 11.5 as a result of insufficient differentiation and proliferation of various cell types involved in maintaining neurogenesis, spinal tube-closure defects, abnormal cardiovascular development and signs of pericardial edema. In the same study, examination of NUMB $^{-/-}$ placentas showed a significant decrease in spongiotrophoblast number as well as placental thickness, suggesting that placental dysfunction may contribute to the fetal demise observed (Zilian *et al.*, 2001). Although the placentae of NUMB -/- mice were examined, the expression or distribution of NUMB in null mice placentae was not compared to that of the control. Another study which focused on Wnt signaling, revealed Numb mRNA expression in different tissue types including placental tissue (Katoh, 2006), but failed to recognize the extent of NUMB expression and distribution across placental cell types.

As a result, our lab has focused its attention to the role of NUMB in the human placenta. We have shown for the first time, NUMB distribution at the maternal-fetal interface and, in particular, within decidual and extravillous trophoblast cells (Figure 3). The mRNA transcripts of NUMB isoforms 1-4 are present in both human placental tissue as well as the invasive human extravillous trophoblast cell line, HTR8/SVneo. Furthermore, we have for the first time, uncovered the presence of NUMB isoforms 5, 6, 7, 8 and 9 in both human placental tissue and HTR8/SVneo, and have successfully sequenced each individual novel isoform and produced their corresponding expression

vectors in addition to NUMB isoforms 2 and 4. The phosphorylation status of these novel isoforms was also investigated. It appears that NUMB isoforms 5, 6, 7 and 8, but not NUMB 9, are all subjected to phosphorylation. NUMB expression in HTR8/SVneo and human placental tissue of first, second and third trimester was also compared. NUMB isoforms 1-4 were predominantly expressed in HTR8/SVneo and NUMB isoform 8 was the predominant isoform expressed throughout the first, second and third trimesters of gestation (Figure 3).

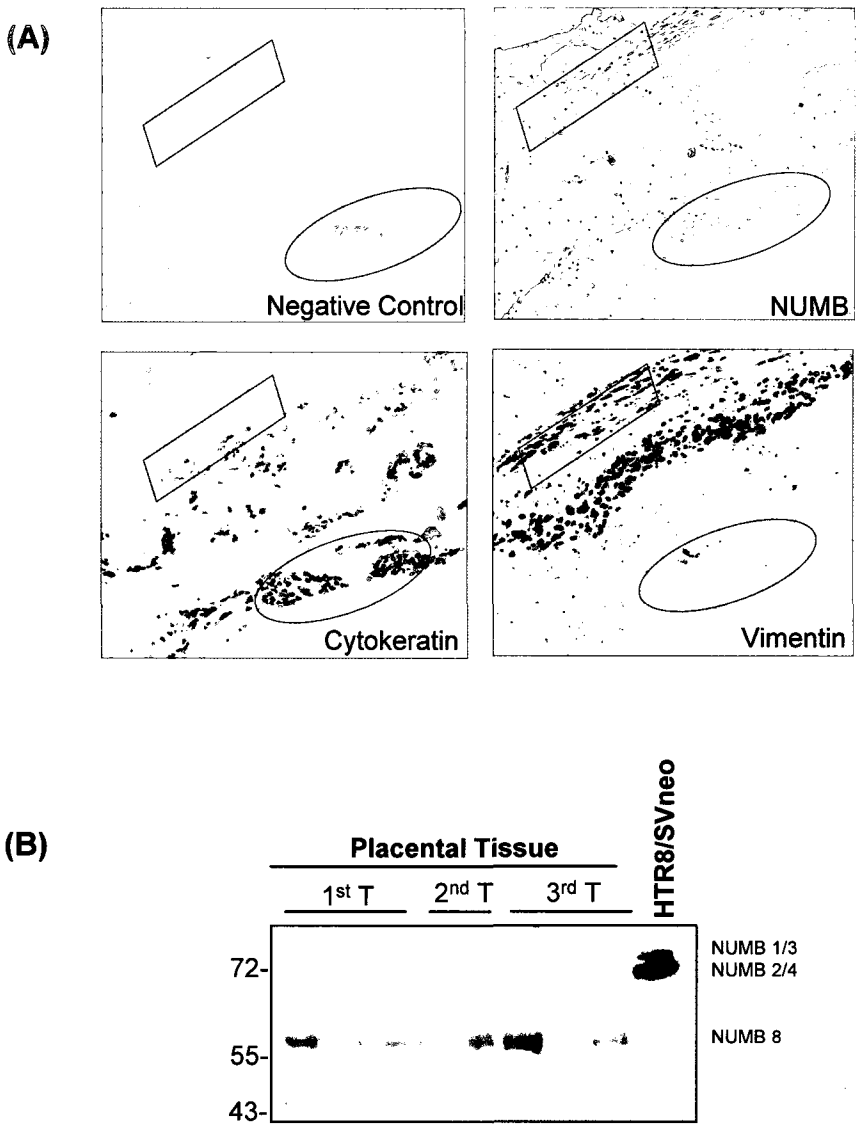


Figure 3. Characterization of NUMB in the Human Placenta and Human Extravillpus Trophoblast (EVT; HTR8/Svneo) cells. (A) Immunohistochemistry of NUMB and cell specific markers (cytokeratin and vimentin, as extravillous cytrophoblast and decidual stromal cell markers respectively) at the maternal-fetal interface of adjacent first trimester human placental tissue sections. Goat IgG was used as negative control. (B) Protein was extracted from human placental tissue at 1st, 2nd and 3rd trimester immediately after termination or birth and HTR8/SVneo. NUMB protein was then examined by Western blot analysis.

7. Rationale

PET and IUGR are the major causes of fetal and maternal morbidity and mortality. Due to the lack of available treatment, delivery remains the only option to reduce morbidity and mortality and in the case of PET, cure the patient. Evidence suggests that abnormalities involving trophoblasts are involved in the etiology of PET and IUGR. For instance, impaired trophoblast invasion and increased apoptosis of the same cells are associated with PET and IUGR. However, the exact mechanisms by which these processes are regulated are not fully elucidated. Hence, it is important to investigate trophoblast function to better understand the molecular pathology of these conditions, such that potential therapeutic agents may arise.

Zillian *et al.* 2001 demonstrated that NUMB $^{-/-}$ mice died at E11.5. In investigating the potential reason to explain the demise observed, investigators noted the presence of important placental ultrastructural anomalies in these null mice. Preliminary results in our lab have shown NUMB localization at the maternal-fetal interface of first trimester human placental tissue in both decidual and trophoblast cells. Since NUMB has been shown in other systems (nervous system in particular) to be involved in the regulation of cell migration and apoptosis, we postulated that this could also be true in the trophoblast. This therefore prompted us to examine the possible role of NUMB in the human placenta. .

8. Hypothesis

NUMB is expressed in the human extravillous trophoblast and individual NUMB isoforms play a role in EVT cell migration and/or apoptosis.

9. Overall Objective

To examine the expression and role of NUMB in human extravillous trophoblast (HTR8/SVneo) cell migration.

Specific Objectives

1. Successfully up-regulate NUMB isoforms 1 and 3 and down regulate NUMB protein content in HTR8/SVneo cells

2. To examine the role of NUMB in EVT cell migration

3. To examine the effect of NUMB on EVT cell survival

CHAPTER 2: MATERIALS AND METHODS

1. MATERIALS

Culture media (RPMI-1640), fetal bovine serum, penicillin, streptomycin, trypsin, Ethylenediaminetetraacetic acid (EDTA), Tetramethylethylenediamine (TEMED), (Moloney Murine Leukemia Virus reverse transcriptase (M-MLV) reverse transcriptase, pEF6 mammalian cell expression vector, Lipofectamine Plus reagent, Lipofectamine 2000, primer sets used for PCR, SlowFade, TOPO® cloning kit, Super Optimal Catabolite (SOC) repression medium, Alexafluor488 goat anti-mouse secondary antibody were purchased from Invitrogen Corporation (Burlington, ON, Canada). Both negative shRNA-GFP and NUMB shRNA-GFP were were purchased from SAbiosciences Corporation (Frederick, MD, USA). Calf Intestinal Alkaline Phosphatase and corresponding buffer, EcoR1 and EcoR1 buffer, PageRuler™ Prestained Protein Ladder (SM#0671), and MassRuler™ DNA were purchased from Fermentas Life Sciences (Burlington, ON, Canada). DC Protein Assay kit, secondary IgG HRP conjugated antibodies (goat anti-rabbit and goat anti-mouse), acrylamide (electrophoresis grade), N, N'-methylene bis-acrylamide, Tris, sodium dodecyl sulphate (SDS), nitrocellulose membranes, SDS polyacrylamide gel electrophoresis (SDS-PAGE), and molecular weight standards were purchased from BioRad (Hercules, CA, USA). Enhanced chemiluminescence (ECLTM) kit and Hyperfilm ECLTM high performance chemiluminescence film was purchased from Amersham Life Sciences (Oakville, ON, Canada). Both anti-NUMB goat and rabbit polyclonal antibodies (ab4147, ab41410) as well as mouse monoclonal anti-GAPDH were purchased from Abcam (Cambridge, MA, USA) and Cytokeratin 18 and rabbit and mouse IgG were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA, USA). The Gel Extraction Kit, QIA prep Spin Miniprep Kit, Plasmid Miniprep kit and HotStartTaq DNA polymerase were purchased from QIAGEN (Mississauga, ON, Canada).

The Boyden chamber insert was purchased from VWR International (Mississauga, ON, Canada). Enhanced chemiluminescence (ECL^{TM}) kit and Hyperfilm ECL^{TM} high performance chemiluminescence film were purchased from Amersham Life Sciences (Oakville, ON, Canada). Tween-20 and 10% formalin were purchased from Fisher Scientific (Kalamazoo, MI, USA) and Toluidine Blue O was purchased from JT Baker Chemical Co. (Phillipsburg, NJ, USA). Hoechst 33258, Dimethylsulphoxide (DMSO) , (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ethidium bromide, Brd-U and mouse anti-BrdU monoclonal antibody (B8434) were purchased from Sigma-Aldrich Canada Ltd.(Oakville, ON, Canada). Vectorshield with DAPI was purchased from Vector Laboratories Inc.(Burlingame, CA, USA). All Falcon culture ware was purchased from BD sciences (Mississauga, ON, Canada). The human invasive extravillous trophoblast cell line, HTR8/SVneo, was a gift from Dr. C. Graham (Queen's University, Kingston, ON, Canada).

2. METHODS

i. Immunocytochemistry

HTR8/SVneo cells, a human invasive extravillous trophoblast cell line, were plated on 8 well chamber slides (BD Falcon; BD Biosciences, Bedford, USA), incubated overnight and fixed in 4% paraformaldehyde (EM Sciences, Hatfield, PA, USA) in 1X PBS for 1 h at 4°C. Cells were then washed 3 times with 1X PBS for 5 min, and incubated in blocking buffer (2% normal goat serum, 1% BSA and 0.4% saponin10mM in PBS, 10mM sodium azide) for 30 min at RT (room temperature) and subsequently incubated overnight at 4°C in either rabbit polyclonal anti-human NUMB (1:250; ab41410; 0.5 mg/ml in 1X PBS; Abcam Inc., Cambridge, MA, USA) or mouse-monoclonal anti-cytokeratin 18 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were blocked once again (20 min, RT), and incubated (1h, RT) with corresponding fluorescent secondary antibodies [Alexafluor488 (1:500) and Texas Red (1:300)] in blocking buffer. Cells were washed (3 x 5 min) with 1X PBS and incubated in equilibrium buffer (30% glycerol, 0.01% sodium azide, 1X PBS; 10 min) and sealed with SlowFade (Invitrogen Corporation, Burlington, ON, Canada). To determine the extent of non-specific immunostaining of NUMB, primary antibodies were substituted with rabbit and mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as negative controls. Images were captured by confocal microscopy using the (Olympus IX70 microscope equipped with a Bio-Rad MRC1024 laser-scanning confocal unit) at 400X magnification. The program used to analyze images was LaserSharp image acquisition software.

ii. NUMB Expression Vector construction:

a. Polymerase chain reaction (PCR)

Primer sequences spanning the NUMB coding region with Spe 1 and Not 1 enzymatic sites at each end were used to amplify NUMB isoforms 1 and 3 within the NUMB 1 and NUMB 3 vector (constructs provided by Dr. Mahmud Bani-Yaghoub, National Research Council, Ottawa, Canada). The PCR reaction was performed by mixing 1 µl cDNA, 0.4 µl of forward 5'TAATACGAGTCACTATAGGGA3' and reverse 5'TAGAAGGCACAGTCGAGG3' primers (Invitrogen Corporation, Burlington, ON, Canada), 0.4 µl dNTP (10 mM each of dATP, dCTP, dGTP, and dUTP), 0.2 µl HotStart Taq DNA polymerase (QIAGEN, Mississauga, ON, Canada), 2 µl 10X PCR buffer (Fermentas Life Sciences, Burlington, ON, Canada) for a final volume of 20 µl. HotStart Taq DNA Polymerase was activated at 95°C(15min), followed by an amplification cycle at 94°C (30sec), 56°C (30sec), and 72°C (45sec), which was repeated for 35 cycles. A final cycle at 72°C (10 min) was carried out for each reaction.

b. Gel Extraction

PCR products were resolved by 1% agarose gel electrophoresis (1 g agarose in 100ml 1X TAE) and bands of various sizes were cut out and isolated, using the Qiagen Gel Extraction Kit (QIAGEN Inc., Mississauga, ON) and according to the manufacturer's instructions. In brief, DNA fragments were dissolved in buffer solution and incubated at 50 °C for 10 min. Following this, 1 gel volume of isopropanol was added to the dissolved gel to obtain a maximum yield of DNA. The solution obtained was then placed

in a QIAquick spin column, washed and eluted to obtain a final concentration of approximately 50 µl of DNA for each band.

Ligation was carried out using a 1:5 vector to insert ratio master mix consisting of 2 µl dH_2O, 1 µl 10X T4 Buffer, 1 µl T4 DNA Ligase, 1 µl pEF6 mammalian cell expression vector into which NUMB isoforms 1 and 3 insert was added and incubated at RT for 2 h.

c. Transformation

After the cloning reaction (kit provided by Invitrogen Corporation, Burlington, ON, Canada), 1 µl of the cloning reaction product was mixed with TOPO10 OneShot chemically competent cells *E. coli* (20µl) for the transformation reaction. After a 30 min incubation to open competent cells and heat shock to seal cells once DNA containing transformant is integrated into the cell, 250 µl of Super Optimal Catabolite (S.O.C.) repression medium (Invitrogen Corporation, Burlington, ON, Canada) was added to the tubes, which were transferred to ice for 2 min. Each transformation was spread onto ampicillin resistant plates (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl, pH 7.0, 15 g/L agar) and incubated at 37°C overnight. Individual white colonies, which contained the ampicilin resistant transformed plasmid containing target DNAwere then picked to be cultured overnight in lysogeny broth (LB) (1% Tryptone, 0.5% Yeast Extract, 1% NaCl, pH 7.0) containing 50 µg/ml of ampicillin. The plasmid DNA was isolated using the QIA prep Spin Miniprep Kit from QIAGEN Inc. (Mississauga, ON, Canada). In brief, samples were centrifuged at 4000 rpm in a swinging bucket centrifuge and bacterial cell pellets were resuspended and transferred into a microcentrifuge tube. Cells were then

lysed and precipitated to isolate DNA and eliminate cell debris. Once the QIAprep spin column was washed with buffer, it was placed in a clean 1.5 ml microcentrifuge tube, and DNA was eluted to give a final solution of approximately 50µl. Restriction Digest was carried out on samples to ensure proper insert size using digestive enzyme EcoR1 (1µl), EcoR 1 buffer (2µl) (Fermentas Life Sciences, Burlington, ON, Canada) and select plasmid DNA. Water was added to a final volume of 20 µl and samples were incubated at 37 °C for 1 h and subjected to gel electrophoresis on 1% agarose and ethidium bromide (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Bands were examined under ultra violet light.

iii. Sequencing and NUMB isoforms 1 and 3 confirmation

The pEF6 mammalian cell expression vector with insert, M13 forward and reverse primers (supplied with pEF6 mammalian cell expression vector), was sent to the National Research Council, Neurogenesis & Brain Repair Group, (Ottawa, ON, Canada) for sequencing.

Overexpression of NUMB isoforms 1 and 3 was confirmed by a 24 h transfection and Western blot analysis (in addition to NUMB isoforms 2, 4, 5-9), and was compared to the endogenous NUMB in the HTR8/SVneo cell line.

iv. Cell Culture

The HTR8/SVneo cell line was derived from human invasive extravillous trophoblast cells. They are immortalized non-tumor cells, highly proliferative and undifferentiating in culture. They were cultured under 5 % CO_2, 95 % O_2 at 37°C in 10 % fetal bovine serum (FBS) (Invitrogen Corporation, Burlington, ON, Canada), fungizone (0.625 μg/ml), streptomycin (100 μg/ml) and penicillin (100 units/ml) in RPMI 1640 medium (Life Technologies Inc., Brooklyn, NY, USA).

v. NUMB Overexpression

HTR8/SVneo cells were plated (2 X 10^5) in 1 ml of complete media (RPMI 1640 and Fetal Bovine Serum) for a 12 wells plate overnight to a confluence of 70%. NUMB vectors containing isoforms 2, 4, 5, 6, 7, 8 and 9 were then transfected into HTR8/SVneo using Lipofectamine and Plus Reagent (Invitrogen Corporation, Burlington, ON, Canada). A mixture containing 1μg of NUMB isoform cDNA, 50 μl serum free RPMI 1640 media and 5 μl Plus reagent was incubated at room temperature for 15 minutes. In parallel, a mixture of 2μl Lipofectamine reagent and 0.4 ml serum free RPMI 1640 media was prepared. These two mixtures were combined, mixed by vortex, and incubated (15 mins) to allow for the formation of DNA complexes. Once the reaction was complete, it was added to the wells containing 0.5 ml fresh serum-free medium drop wise. After 3-6 h, serum-free media was replaced with 1ml complete medium for 24 h.

vi. NUMB Down-regulation (siRNA)

Cells were plated in 1 ml of complete media (RPMI 1640 and 10% FBS) on a 12-well plate overnight to 50-80% confluence (depending on post-transfection incubation period). The cells were incubated (RT, 5 min) in a mixture (final volume of 125 µl) consisting of serum free media and RiboJuice^{TM} (NOVAGEN, A Brand of EMD Chemicals, Inc., Gibbstown, NJ, USA) or Lipofectamine 2000 (Invitrogen Corporation, Burlington ON, Canada) and with siRNA for an additional 20 min accordingly to stock concentration. The mixture was then added drop-wise to cells containing 525 µl complete media for 6 hrs and changed to 1ml complete media and incubated for 24-72 h at 37°C. Thereafter, cells were harvested for NUMB protein content determination by Western Blotting (WB).

vii. NUMB Down-regulation (shRNA)

Cells were plated into each well in 1 ml of complete media (RPMI 1640 and 10% FBS) on a 12-well plate overnight to a confluence of 40%. Two mixtures containing (1) 250 µl serum free media and Negative shRNA; 5' GGAATCTCATTCG ATGCATAC 3' or NUMB shRNA; 5' TCCTGCTCTTAGCCAGAAG AT 3' (1µg/ml; SAbiosciences corporation, Frederick, MD, USA) and (2) serum free media and 4.5 µl Lipofectamine 2000 (Invitrogen Corporation, Burlington, ON, Canada) were incubated separately for 15 min and then combined and incubated for additional 15 min. The final reaction mixture was added drop-wise to the wells containing complete media and, after 6 h, media was

replaced with complete media for 48 h and once again for an additional 24h at 37 °C. After 72h, cells were harvested for further analyses.

vii. Scratch (Wound Healing) Assay

HTR8/SVneo cells (transfected with individual NUMB isoforms or NUMB down-regulated) in 12 well plates were subjected to scratches using the ends of 200 µl pipette tips. Media was changed to complete media after the scratch and plates were incubated in 37°C for 6 h. Images were taken at 100X magnification (Zeiss SV 11 Apo stereo light microscope) at the initial time of scratch and after 6 hours incubation. Plates were marked initially at specific locations where images were captured (Zeiss AxioCam MRc the digital camera) and analyzed (AxioVision software v3.1; Zeiss, Jena, Germany) in order that images could be taken again at the same location after incubation period.

viii. Boyden Chamber Migration Assay

HTR8/SVneo cells (following overexpression or down-regulation) were lifted and dispersed with 2 mM EDTA/1XPBS for 10 mins at 37°C in 12 wells plates. Cells were then washed with RPMI1640 containing 0.5 % FBS and spun down at 900 *x g* for 5 minutes. The cell pellet was then resuspended in RPMI1640 containing 0.5% FBS and a 200 µl cell suspension aliquots containing $5X10^5$ cells were added into the Boyden Chamber insert (8µm; VWR International, Mississauga, ON, Canada) in a 24 well plate. An additional 600 µl 0.5% FBS in medim was added to the lower portion of the insert

and incubated (24h, 37 °C). Thereafter, cells on the surface of the insert were removed by scraping with a cotton swab. Cells which migrated to the lower portion of the insert were then stained with 1% toluidine blue O (JT Baker Chemical Co., Phillipsburg, NJ, USA) in 10% buffered formalin (Fisher Scientific, Kalamazoo, MI, USA) for 1h at RT. After several washes with dH_2O, the membrane was removed from the insert with a small scalpel blade, mounted on a microscope slide and sealed using Cytoseal 60 (Kalamazoo, MI, USA) and a coverslip. Cells that migrated through the chamber were counted by bright field light microscopy.

x. Proliferation Assay

The cells were transfected for 24 h in 12 well plates and incubated with 10 μM Brd-U (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) for 6h in complete media. They were then washed with 1X PBS, fixed in acid ethanol (90% EtOH, 5% Acetic Acid, 5% H_2O) for 30 min at 4°C. Cells were then washed with 1X PBS (3 times), incubated in 2M HCl (20 min at RT), and then in 0.1M NaB_4O_7 (10 min at RT). They were washed with 1X PBS (3 times) and incubated in blocking solution (1X PBS, 0.5% Tween20, 1% BSA; 20 min at RT) and then incubated (1 h at RT) with anti-Brd-U (Sigma-Aldrich Canada Ltd., Oakville, ON; 1:500 in PBS/BSA). Thereafter, cells were washed (3 times), and incubated with Alexafluor488 goat anti-mouse secondary antibody (1:250; 45 min at RT; Invitrogen Corporation, Burlington, ON, Canada), washed again with 1X PBS (3 times) and mounted with Vectorshield mounting medium with DAPI (Vector

Laboratories Inc., Burlingame, CA, USA). Proliferating cells were viewed directly by fluorescent microscopy.

xi. MTT (3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl-tetrazolium bromide) Cell Viability Assay

Following a 24 h transfection of individual NUMB isoforms 1, 2, 3, 4 and 8 and control empty vector in a 12 well plate, HTR8/SVneo cells were incubated in 25 µl of MTT (0.5 mg/ml) (Sigma-Aldrich Canada Ltd., Oakville, ON) for 1 h. Following media removal, 500 ml of DMSO was added to each well and aliquots of 100 µl of each sample were transferred to a flat bottom 96 well plate and absorbance was measured at 550 nm.

xii. Apoptosis Assessment (Hoechst)

At the end of the culture period, cells were trypsinized (250 µl Trypsin), collected (in complete media) and resuspended in 10 % formalin (Fisher Scientific, Kalamazoo, MI, USA) containing Hoechst stain (12.5 ng/ml; Sigma-Aldrich Canada Ltd., Oakville, ON) and incubated in the dark for 24-48 h at 4 °C. Cells were then spotted onto slides and assessed for apoptotic morphology (blebbing, chromatin condensation, nuclear fragmentation) by fluorescent microscopy and images were taken by QCapture Pro 6. Percentage of apoptotic cells was determined from a total cell number of approximately 500 cells from randomly selected fields.

xiii. Protein Extraction

Cells were harvested and resuspended in lysis buffer (for 100 ml, 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EGTA 10 mM sodium pyrophosphate, 1.5 mM $MgCl_2$, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 μg/ml Aprotinin, 1 mM Na_3VO_4). Proteins were left on ice (1 h) and centrifuged (4°C, 30 min) at 13, 000 x g to enable the collection of the supernatant. Cell lysate was stored at -20°C for further future analysis.

Protein bioassay was carried out on these samples using the Bradford method (BioRad DC protein bioassay kit; Hercules, CA, USA). BSA (BioRad, Hercules, CA, USA) ranging from 0.059.-3.75 μg/μl was used as standards to calculate protein concentration via spectrophotometry (software). Samples containing 4X loading buffer (200mM Tris-HCl, 400 mM dithiothreitol (DTT), 8 % (w/v) SDS, 40 % (v/v) glycerol and 0.4 % (w/v) bromophenol blue; pH 6.8) were boiled (5 min) to denature proteins before SDS-PAGE gel.

xiv. Western Blot

Proteins (25μg) were separated on 10 % SDS-Polyacylamide gel electrophoresis and electro-transferred onto nitrocellulose membranes. The membranes were blocked (1 h at RT) in blotto (5 % skim milk in Tris-buffered saline pH 7.6 with 0.05 % Tween 20 (TBS-T) and incubated (4°C overnight) in corresponding primary antibodies. Primary antibody bound membranes were washed (3 times, 10 min) in 1X TBS-T [400 ml 10X TBS (12.11 g Tris, 87.65g NaCl, 800 ml dH2O, pH 7.2) added to 3600 ml H_2O and 2 ml

Tween-20 (Fisher Scientific, Canada)] and incubated (1 h at RT) with secondary antibody of Horseradish peroxidase conjugated human immunoglobin (IgG) in blotto, according to the corresponding primary antibodies. Membranes were washed (3 times, 10 min) and immunosignals were visualized, in accordance to manufacturer's instructions and using the Enhanced Chemiluminescence Detection Kit (Amersham Life sciences, Oakville, ON, Canada) Film was developed on HyperFilm MP (Amersham life sciences, Oakville, ON, Canada) and results were analyzed densitometrically, using Alpha Image Pro and PRISM 3.0 (GraphPad Software Inc.).

xv. Statistical Analysis

Experimental results of at least three independent experiments were expressed as the mean $\pm$ SEM. Data were analyzed by PRISM 3.0 (Graphpad Software Inc.) and analysis was based on results obtained from performing t-test and by analysis of variance (ANOVA). The Tukey post-hoc test was used to assess differences between groups. Statistical significance was inferred at $p<0.05$.

Table 2. Antibodies used for Western blotting (WB) and Immunocytochemisrty (ICC)

Antigen	Species raised against	Species	Monoclonal or Polyclonal	Working dilution	Manufacturer	Application
NUMB	Human	Goat	Polyclonal	1:5000	Abcam	WB
NUMB	Human	Rabbit	Polyclonal	1:250	Abcam	ICC
pErk1/2	Human	Rabbit	Polyclonal	1:1000	Cell Signaling	WB
pAKT	Human	Rabbit	Polyclonal	1:1000	Cell Signaling	WB
P53	Human	Mouse	Monoclonal	1:1000	Santa Cruz	WB
GADH	Human	Mouse	Polyclonal	1:20000	Abcam	WB

CHAPTER 3: RESULTS

LOCALIZATION AND OPTIMIZATION STUDIES

1. NUMB localization in HTR8/SVneo cells

NUMB was localized to the subcellular region of HTR8/SVneo by immunocytochemistry and confocal microscopy. HTR8/SVneo and NUMB positive NT2/D1 anembryonic carcinoma cell line, used to investigate Numb expression by Verdi et. Al, 1999, were plated into 8 chamber slides overnight and immunostained for NUMB (1:250) and cell type specific marker cytokeratin 18 (1:100) the following day. NUMB was visualized by immunofluorescence using secondary antibodies tagged with Alexafluor488 (NUMB; 1:500) and Texas Red (Cytokeratin 18; 1:300). HTR8/SVneo cells stained positive for NUMB (Figure 4a) and cytokeratin. NUMB was found to be localized to the cytoplasmic region of EVT cells (Figure 4b-c). NUMB reactivity was confirmed in NT2/D1 cells, in which NUMB was localized to the cytoplasm (Figure 4e).

2. NUMB isoforms 1 and 3 overexpression constructs

Previously, NUMB isoforms 2, 4, 5-9 had been successfully overexpressed in EVT cells. As a result, the overexpression vector constructs containing NUMB 1 and 3 were required in order to investigate their role and function in EVT cells. Our collaborator at the National Research Council (Dr. M. Bani-Yaghoub) had generously provided vectors containing the NUMB 1 and 3 sequences. In order to overexpress NUMB protein in EVT cells, these sequences were first subcloned into the pEF6 mammalian cell expression vector. The PCR products containing the full length of

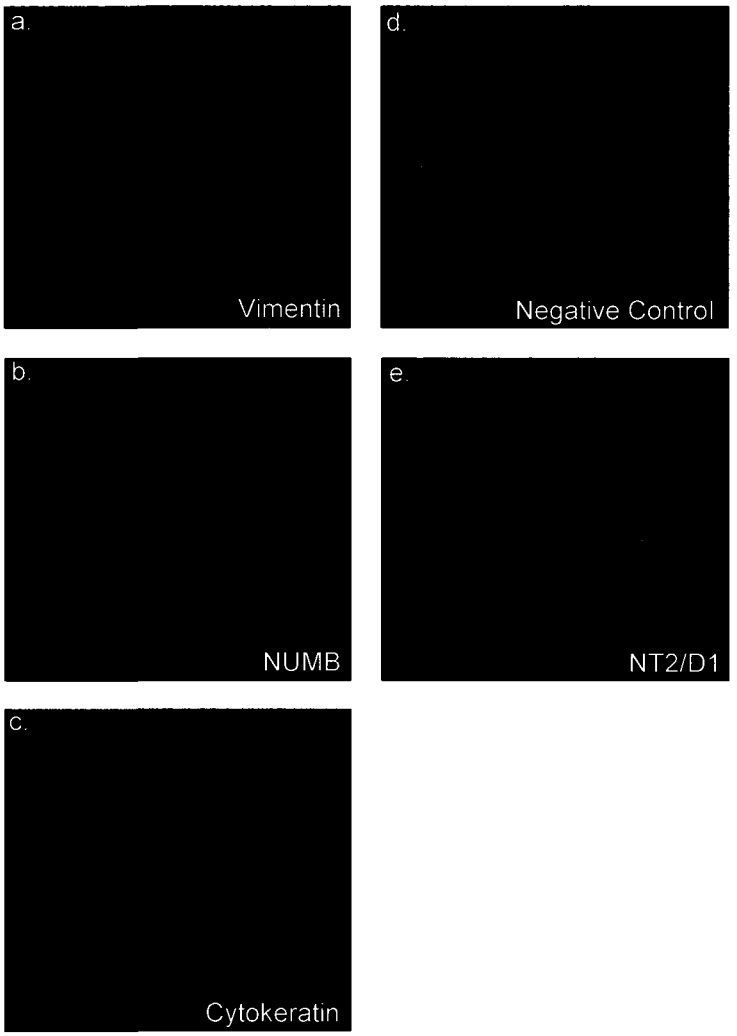


Figure 4. Subcellular localization of NUMB in human extravillous trophoblast (EVT) cells. Confocal microscopy analysis of human EVT cells (HTR8/Svneo) demonstrates the cytoplasmic localization of NUMB as determined by co-localization to the cytoplasmic marker cytokeratin (a-c). Normal IgG was used as a negative control (d) and NUMB immunoreactivity was confirmed in NT2/D1 cells as a positive control (e).

NUMB isoforms 1 and 3 with Spe1 and Not 1 enzymatic digestive sites were verified on 1% agarose gel (Figure 5), with expected band sizes of 2062 bp (NUMB 1) and 2029 bp (NUMB 3). When compared to the DNA ladder (1kb), the banding patterns were consistent and within the range of the known band sizes of the DNA ladder.

Once the vector containing the insert was transformed into *E. coli*, grown overnight and colonies were purified for NUMB isoform 1 or 3 plasmids. The pEF6 mammalian cell expression vector with insert, M13 Forward and Reverse primers were then sent to the National Research Council, Neurogenesis & Brain Repair Group, (Ottawa, ON, Canada) for sequencing. Sequence analysis indicated that NUMB 1 and 3 cDNA was incorporated into the expression vectors successfully.

To verify NUMB 1 and NUMB 3 overexpression, the protocol for the overexpression of NUMB isoforms 2, 4, 5-9 was applied to EVT cells, using these NUMB 1 and 3 overexpression vectors (24h). Western blot analysis verified that NUMB 1 and 3 are overexpressed in EVT cells, as are their counterparts NUMB 2, 4, 5-9 (Figure 6).

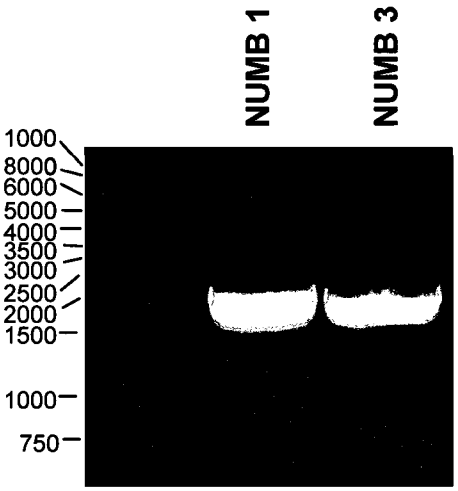


Figure 5. Subcloning of NUMB 1 and 3. (A) PCR products of NUMB 1 and NUMB 3 with digestive site Not1 and Spe 1 were cut out of the plasmid, isolated and ligated into the pEF6 mammalian cell expression vector.

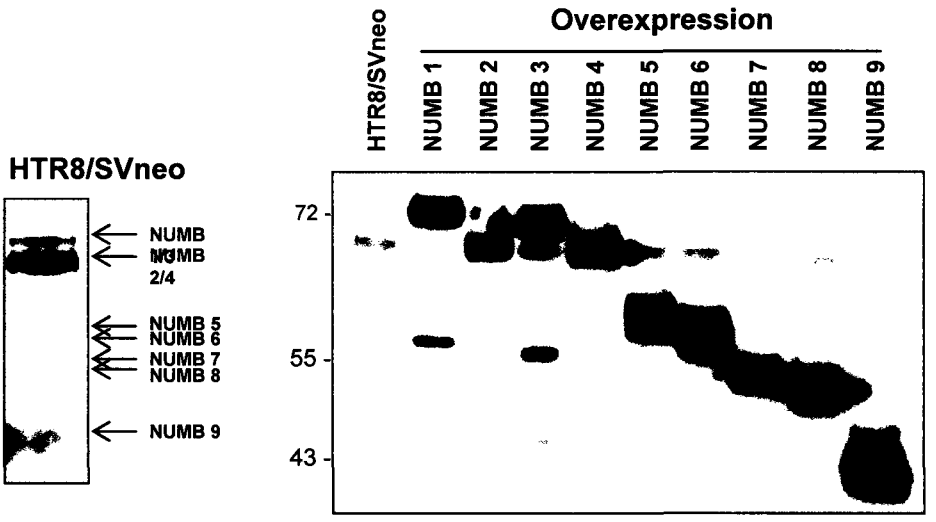


Figure 6. NUMB Expression in EVT cells. Left: Expression of endogenous NUMB isoforms 1-9 in HTR8/SVneo cells. Right: Overexpression of NUMB isoforms 1-9. Cells were seeded in 12 well-plate and transfected with individual NUMB isoforms 1-9 (1 μg; 24 h). Over-expression was verified by Western blot.

3. Optimization of NUMB down-regulation by siRNA

In order to study the role of NUMB in extravillous trophoblast function, we used NUMB siRNA which targeted an mRNA sequence specific to NUMB to inhibit NUMB protein formation. Three different siRNA sequences were used: siRNA 1 (sense: 5'CCAGACGAUAGAGAAAGUUtt3', antisense: 5'AACUUUCUCUAUCGUCUGGtc 3'), siRNA 2 (sense: 5'CCGGAAAAUGUAGCUUCCCtt3', antisense: 5'GGGAAGC UACAUUUUCCGGtg3') and siRNA3 (sense: 5'GGACCUCAUAGUUGACCAGtt3', antisense: CUGG UCAACUAUGAGGUCCtt3'). All three NUMB siRNA targeted the PTB region of NUMB, which is common to NUMB isoforms 1-7 but partially missing for NUMB 8 and absent for NUMB 9. A scrambled sequence against human mRNA (sense: 5'AUGAACGUGAAUUGCUCAAtt3', antisense: 5'UUGAGCAAUCACGUUCAtt3') from Thermo Scientific (Waltham, MA, USA) was used as a control in the knockdown system. Several attempts with respect to transfection reagents (Ribojuice^{TM} and Lipofectamine 2000), incubation periods (from 24-72h), as well as combinations of siRNA were used to down-regulate NUMB protein. Table 3 lists all the different combinations of optimization experiments carried out to down-regulate NUMB protein content. Experiments which used "siRNA 3" to down-regulate NUMB resulted in a greater decrease in NUMB protein content but this pattern of decrease failed to produce consistent results, regardless of reagent (Figure 7). Furthermore, one experiment using a combination of NUMB siRNA (1+2) and (2+3) resulted in decreased NUMB protein content (Figure 8), but this response was inconsistent. Furthermore, regardless of volume of reagent or media (both serum free and complete media) down-regulation of NUMB protein content by siRNA in the presence of Ribojuice^{TM} and

Lipofectamine did not differ greatly but incubation of NUMB siRNA in Ribojuice caused an increased number of EVT to shed off the plates and into the surrounding media. Close physical examination showed signs of unhealthy cell morphology.

Table 3. Optimization conditions of NUMB down regulation using siRNA

siRNA (1, 2, 3)	siRNA (nM)	Reagent (3 µl)	Incubation (h)
1, 2, 3	0, 50, 100, 200	Ribojuice	24 and 48
(1+2+3)	0, 50, 100, 200	Ribojuice	24 and 48
1, 2, 3	0, 50,100, 200	Lipofectamine 2000	24 and 48
1, 2, 3	0, 15, 30, 60	Ribojuice	48
1, 2, 3	0, 7.5, 15, 30	Ribojuice	48
(1+2), (2+3)	0, 7.5, 15, 30	Ribojuice	48
(1+2)	0, 7.5, 15, 30	Ribojuice	24 and 36
1, 2, 3	0, 7.5, 15, 30	Ribojuice	72
(1+2), (2+3)	0, 7.5, 15, 30	Ribojuice	72
1, 2, 3	0, 7.5, 15, 30	Lipofectamine 2000	72
(1+2), (2+3)	0, 7.5, 15, 30	Lipofectamine 2000	72

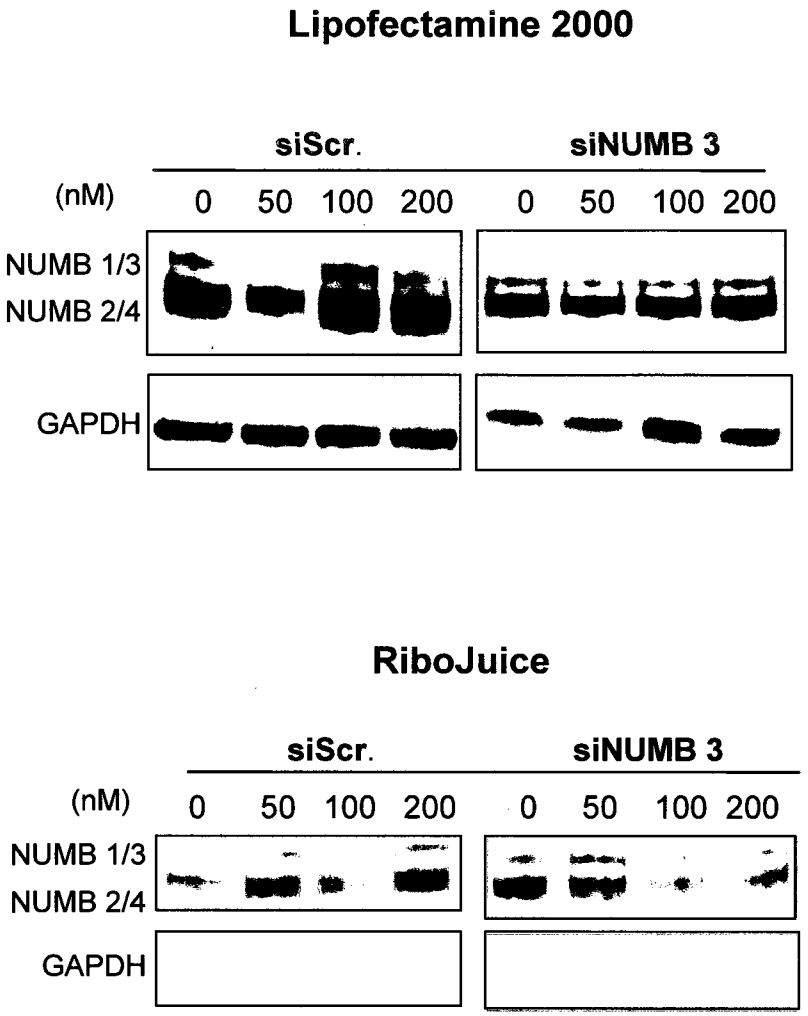


Figure 7. NUMB down-regulation using siRNA in the presence of Lipofectamine 2000 and RiboJuice. Cells were plated overnight in 12 well plates and subjected to si-NUMB 3* for 24 h in the presence of 3 µl Lipofectamine or Ribojuice in complete media for 24 h at 37C. Cells were harvested and NUMB down-regulation was verified by Western blot.

*See text page 49

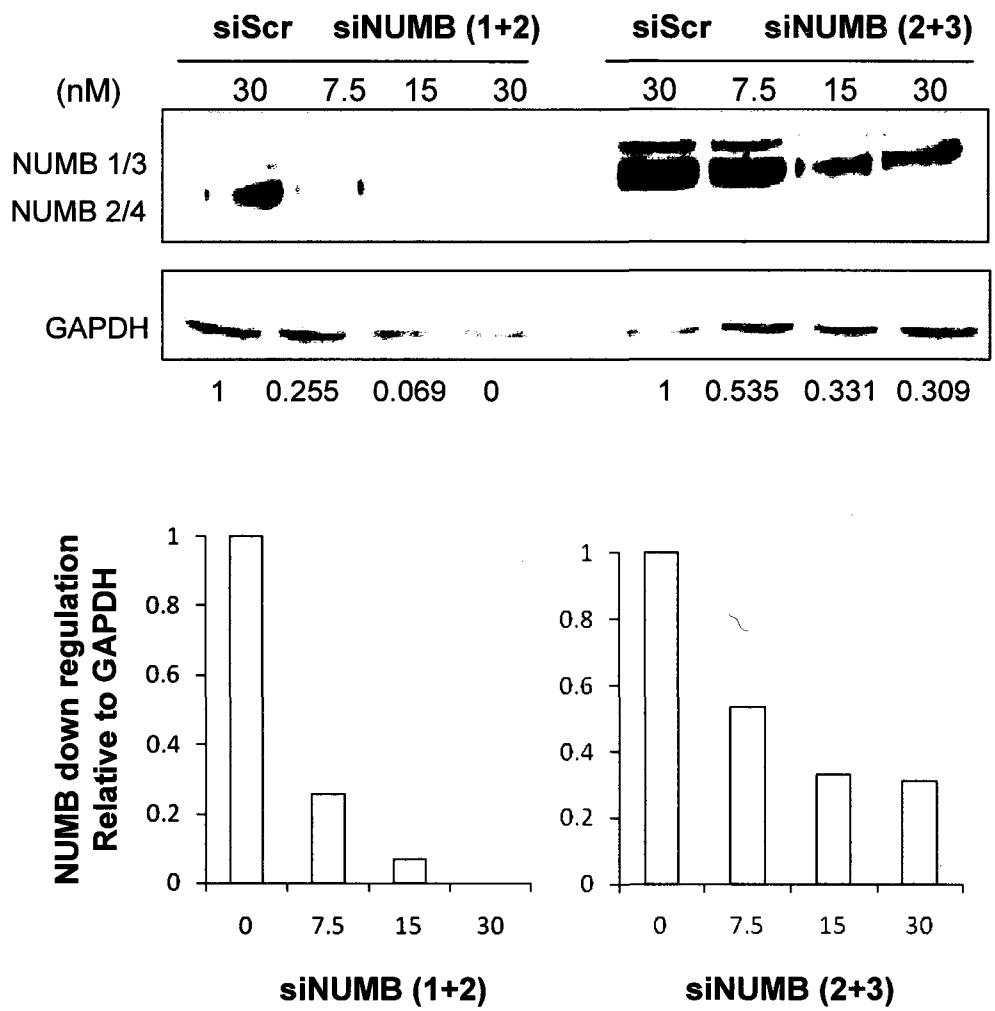


Figure 8. Optimization of si-NUMB (1+2) and (2+3). Cells were plated in 12 well plates and subjected to different concentrations of combination si-NUMB (1+2)* or (2+3)* and control si-Scramble for 48h in complete media at 37°C. NUMB knockdown was confirmed by Western blot.
*See text page 49

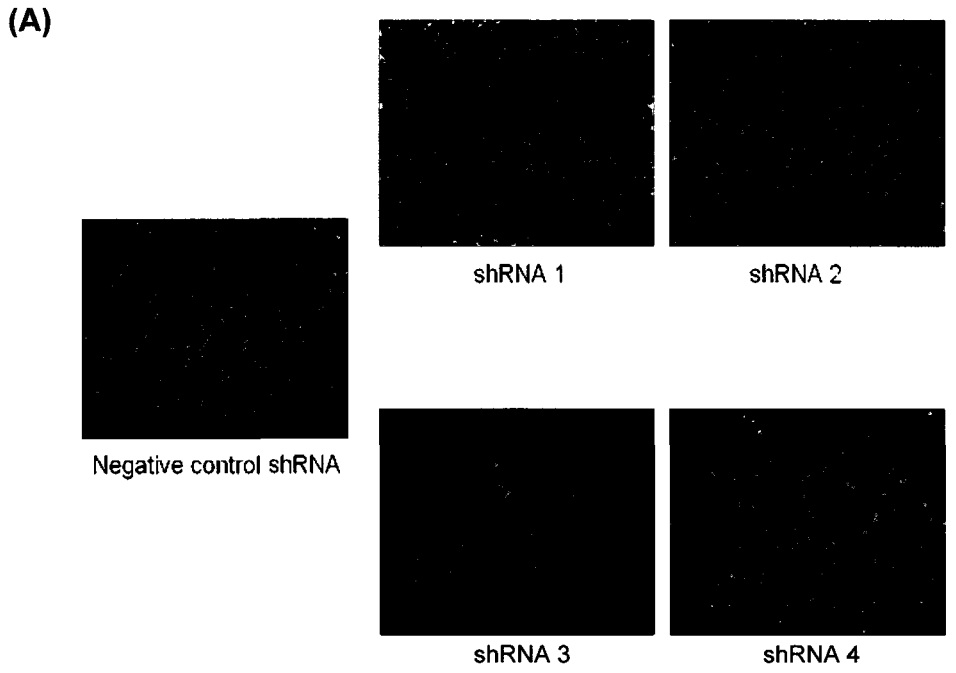
4. Optimization of NUMB down-regulation protocol using shRNA

The inconsistent results obtained from siRNA experiments lead us to use short hairpin RNA (shRNA) as an alternative to NUMB down-regulation. Four shRNA tagged with a Green fluorescent Protein (GFP) namely, shRNA 1 (insert sequence: CCAGATGGTGGCCAACGTATT) shRNA 2 (insert sequence: TCTCCTACCTTCCA AGCTAAT) shRNA 3 (insert sequence: TGATGCTAGTCGGACCACTTT) and shRNA 4 (insert sequence: TCCTGCTCTTAGCCAGAAGAT), as well as a negative control vector (insert sequence: GGAATCTCATTCGAATGCATAC) were amplified before experiments pertaining to NUMB down-regulation were conducted. Sequences corresponding to shRNA 1 and 4 both spanned the PRR which is common to all NUMB isoforms 1-9, therefore efficient for complete NUMB knockdown. Sequences corresponding to shRNA 2 and 3 spanned PTB regions uncommon to all NUMB isoforms, therefore should result in less efficient NUMB protein down-regulation. Following amplification, DNA content determined spectrophotometrically was as follows: Negative control (0.17μg/ml), shRNA 1 (0.26μg/ml), shRNA 2 (0.1μg/ml), shRNA 3 (0.09μg/ml) and shRNA 4 (0.09μg/ml). Several different conditions using Lipofectamine2000 as the transfection reagent were investigated to down-regulate NUMB as depicted in Table 4. Visualization of the GFP-tag at 48 h post-transfection by fluorescent microscopy showed a larger number of cells to express GFP when treated with shRNA 1 and shRNA 2 (Figure 9a). The number of cells expressing shRNA 4 was comparable to those of the control group, and the number of cells expressing shRNA 3 was much lower than the control group. Western blot analyses in the first replicate showed that NUMB protein was down-regulated at 48 h by shRNAs 2, 3 and 4, but not 1

(Figure 9b). Upon replication, results were inconsistent. For example, in the second replicate, NUMB protein content for all NUMB isoforms 1-9 was not down-regulated but in the third replicate, NUMB protein was down-regulated by shRNA 2 and 4. Due to the inconsistency of these results, the shRNA protocols still remained questionable. After several changes to the conditions under which shRNA was being delivered to EVT cells, it was seen that shRNA 1 and/or 4 caused a consistent and significant down-regulation (approx 80%; $p<0.01$) of NUMB isoforms 1-4 after a 72h incubation and were therefore used in further experiments. Figure 10 depicts NUMB down-regulation of the dominant NUMB isoform 1-4 using shRNA 1, as shRNA 2 and 3 still produced inconsistent NUMB down-regulation and were therefore abandoned.

Table 4. Optimization conditions of NUMB down-regulation using shRNA

shRNA (μg)	Lipofectamine2000 (μl)	Serum-free media (μl)	Incubation (h)
1	5	250	24
1	5	250	48
1	2	125	24 and 48
1	3.5	250	24 and 48
1	4.5	250	72



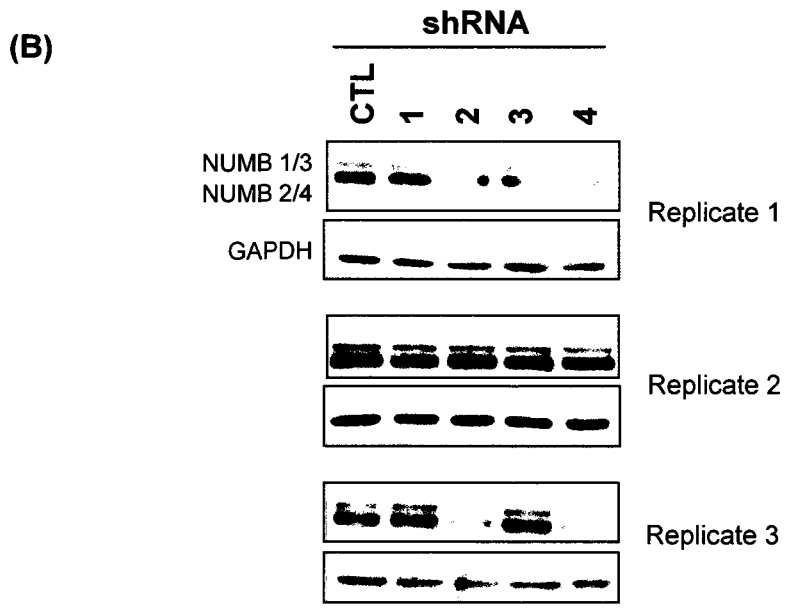


Figure 9. Inconsistent NUMB down-regulation using four NUMB shRNAs. Cells were seeded in 12 wells plates overnight and treated with shRNA (1μg/ml) for 48 hr at 37°C. Cells were then (A) visualized for GFP incorporation at 48h and (B) harvested for NUMB protein content.

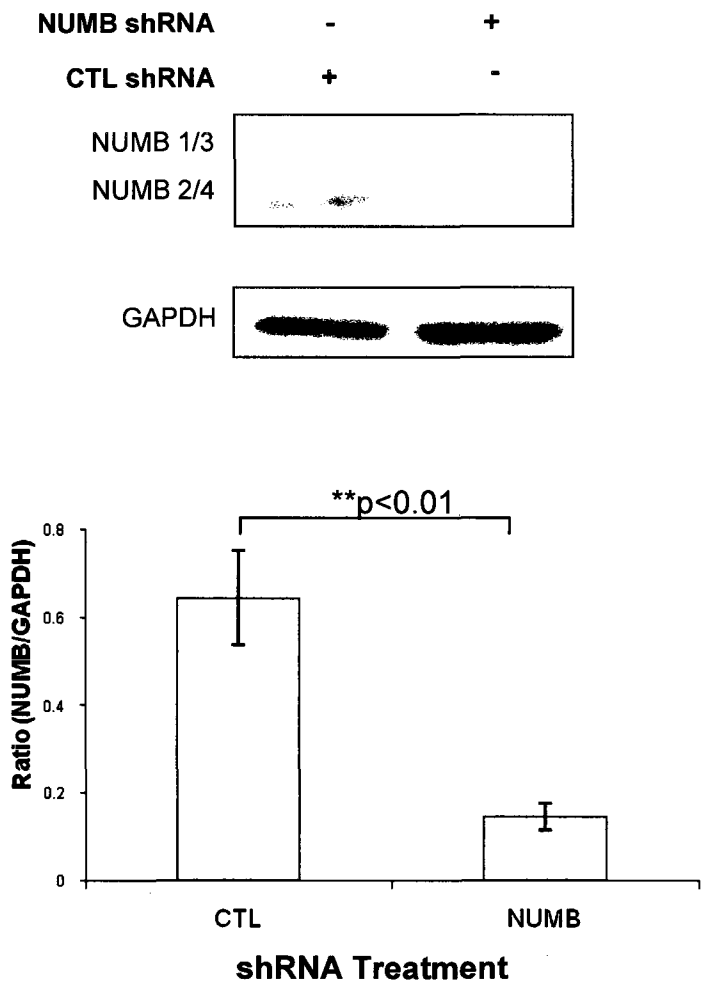


Figure 10. Optimization of NUMB down-regulation by shRNA 1. Cells were seeded in a 12- well plate and incubated in shRNA 1 (1μg/ml) for 48 h in complete media. At the 48 h time point, media was replaced with 1 ml of fresh complete media and re-incubated for an additional 24h in 37°C. Cells were collected at 72 hr for further analyses. Complete NUMB knockdown was verified by Western blot. **p<0.01 (relative to control shRNA; n=3).

FUNCTIONAL STUDIES

ROLE OF NUMB IN EVT CELL MIGRATION

5. The effect of NUMB silencing on EVT cell migration

The role of NUMB was primarily investigated by gene silencing. Two different approaches were taken to study the role of NUMB in EVT cell migration: the classical Boyden chamber assay, which was used as a quantitative approach, and the wound healing assay which was a qualitative methodol allowing the visualization of cell migration towards the "wound". NUMB protein content was down-regulated in 72 h by NUMB shRNA 4 (1μg/ml) when compared to control shRNA (1μg/ml) in HTR8/SVneo cells. NUMB down-regulation was confirmed by Western blotting, which showed 80-90% NUMB down-regulated in 3 independent experiments (Figure 11a). Statistical analysis (t-test) revealed that NUMB silencing resulted in significant reduction in the number of cells that migrated across the Boyden chamber when compared to the control group ($p<0.01$) (Figure 11b). The scratch assay showed that after 6 h of the initial scratch made to the plate NUMB knockout cells did not migrate towards the wound as quickly as the control cells and by 6h the gap started to seal off as a result of EVT migration towards the scratch by approximately 2 folds (Figure 11c).

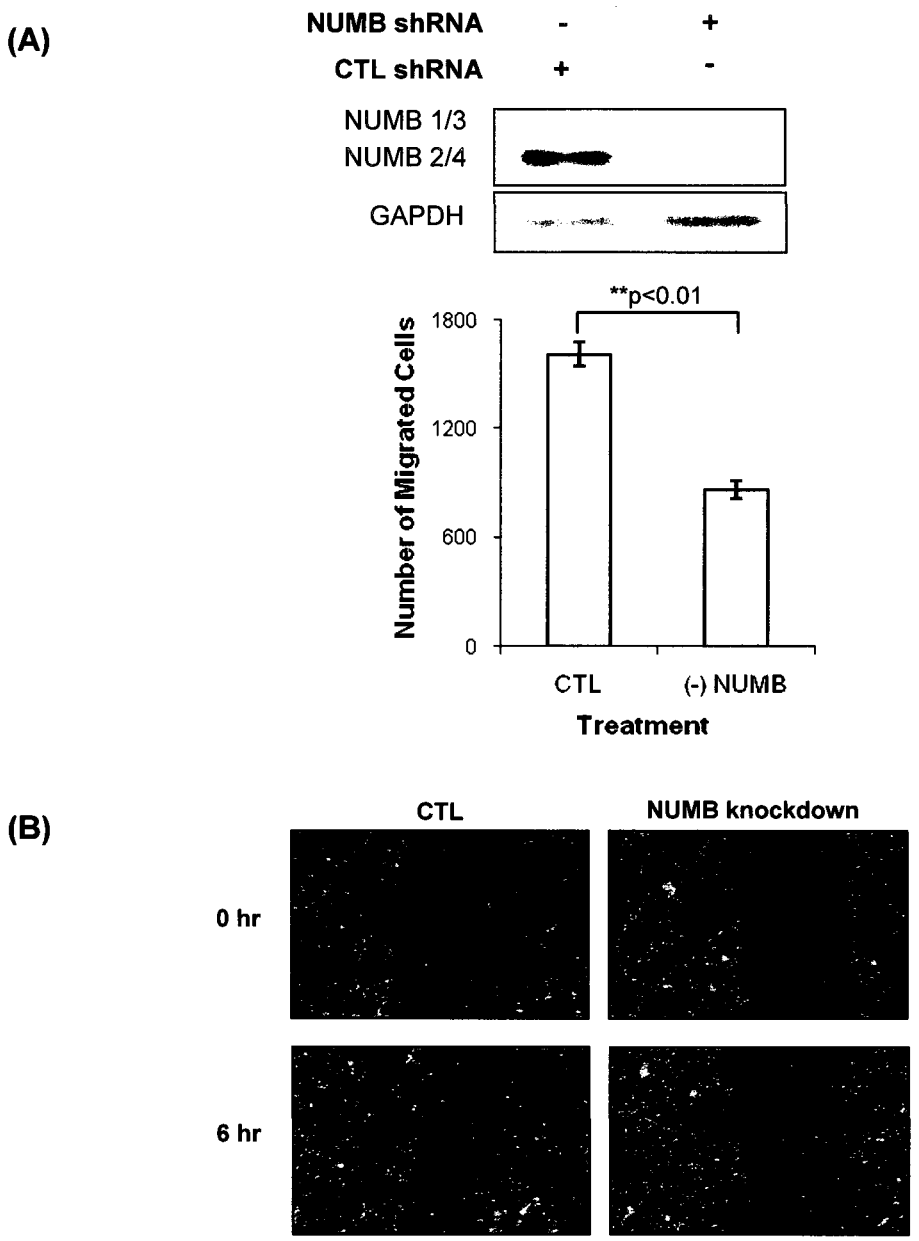


Figure 11. The effect of NUMB down-regulation on extravillous trophoblast (EVT) cell migration. HTR8/SVneo cells were seeded in 12 wells plates overnight and transfected with shRNA 4 (1μg/ml; 72h) (A) Down-regulation of NUMB protein verified by Western blot and cell migration was determined by the (B) Boyden chamber migration assay (**p<0.01 (relative to Control shRNA) and (C) wound healing assay separately (n=3).

6. The effect of NUMB overexpression on EVT cell migration

Since NUMB 1, 2, 3 and 4 were predominantly expressed in HTR8/SVneo cells and NUMB 8 consistently in human placental tissues throughout gestation, the focus of this study was to determine whether or not individual NUMB isoforms 1-4 and 8 play a role in EVT cell migration. NUMB isoforms 1-4 and 8 (1μg cDNA) as well as control vector (1μg cDNA) were transfected into HTR8/SVneo cells for 24 h before being transferred to the Boyden Chamber (24 h incubation) for migratory studies or subjected to a scratch assay on the surface of the 12 well plates in which they were being treated. Cells overexpressing NUMB isoform 1 exhibited a significant increase in cell migration across the Boyden chamber when compared to the control group ($p<0.01$) (Figure 12b). In contrast, NUMB isoform 4 significantly reduced cell migration when compared to the control group ($p<0.05$), whereas overexpression of NUMB isoforms 2, 3 and 8 did not have a significant impact on cell migration.

After the 6h incubation period from the initial scratch, microscopy showed that cells over-expressing NUMB isoform 1 migrated towards the gap in greater numbers when compared to control cells (Figure 12c). Cells which over-expressed exogenous NUMB isoforms 2, 3, 4 and 8 did not move towards the gap in greater numbers when compared to control cells.

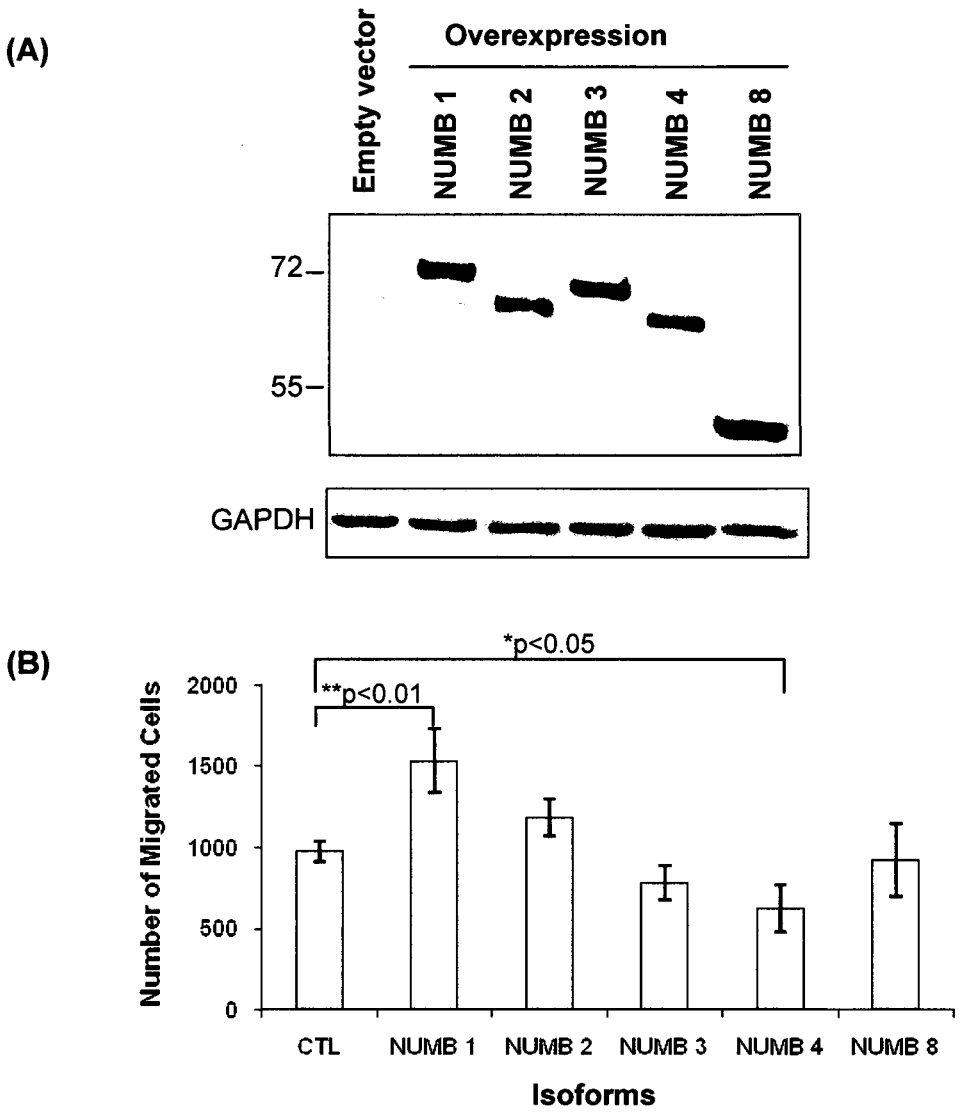


Figure 12. Isoform specific effects of NUMB on extravillous trophoblast (EVT) cell migration. NUMB isoforms 1-4 and 8 were overexpressed in HTR8/Svneo cells (1ug/ml; 24h) and verified by (A) western blot. Cell migration was determined by (B) the Boyden chamber migration assay. *p<0.05 and **p<0.01 [relative to Control (empty vector); n=4] and (C) the wound healing assay separately(n=3).

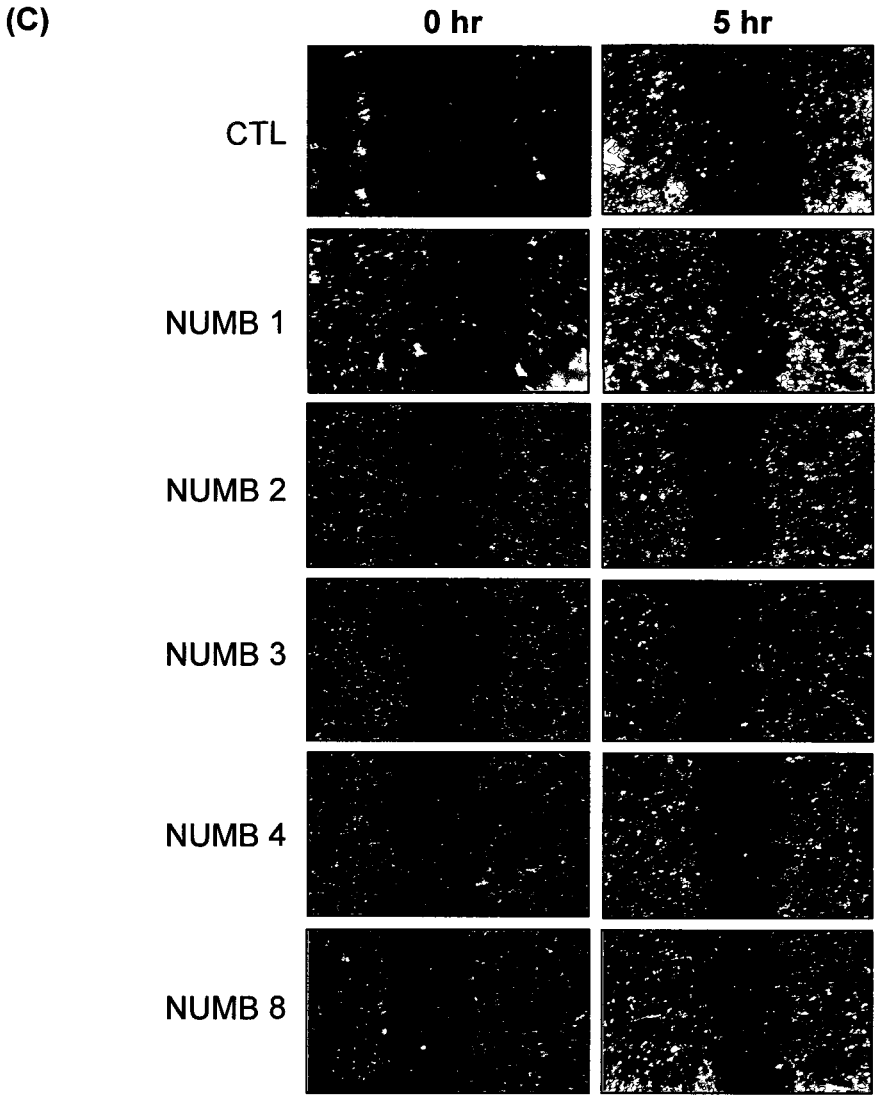


Figure 12. Isoform specific effects of NUMB on extravillous trophoblast (EVT) cell migration. NUMB isoforms 1-4 and 8 were overexpressed in HTR8/Svneo cells (1ug/ml; 24h) and verified by (A) western blot. Cell migration was determined by (B) the Boyden chamber migration assay. *$p<0.05$ and **$p<0.01$ [relative to Control (empty vector); n=4] and (C) the wound healing assay separately (n=3).

7. The effect of NUMB 1 on EVT cell proliferation

Although a significant increase in cell migration upon up-regulation of NUMB isoform 1 was observed in the previous study, whether this effect was partly due to an increase in proliferation remained a possibility. To exclude this, we performed a proliferation assay. Cells were transfected with NUMB isoform 1 and control vector (1μg/ml cDNA) for 24 h and subjected to Brd-U (10μM) incorporation for 6 h. Brd-U incorporated cells were visualized under fluorescent microscopy (Figure 13a). Statistical analysis indicated that NUMB 1 overexpression did not increase cell proliferation when compared to the control cell population (Figure 13b).

ROLE OF NUMB IN EVT CELL SURVIVAL AND APOPTOSIS

8. The effect of NUMB overexpression on EVT cell viability

EVT cell survival was considered when cells were being induced to express high levels of NUMB isoforms 1-4 and 8, and control vector (1μg/ml cDNA). As a result, a MTT assay was performed on HTR8/SVneo cells transfected with individual NUMB isoforms 1-4 and 8 in serum free media. Overexpression of NUMB 2 significantly reduced cell survival as compared to the control ($p<0.01$) (Figure 14). However, EVT cell survival upon transfection of NUMB isoforms 1, 3, 4 and 8 was not significantly affected.

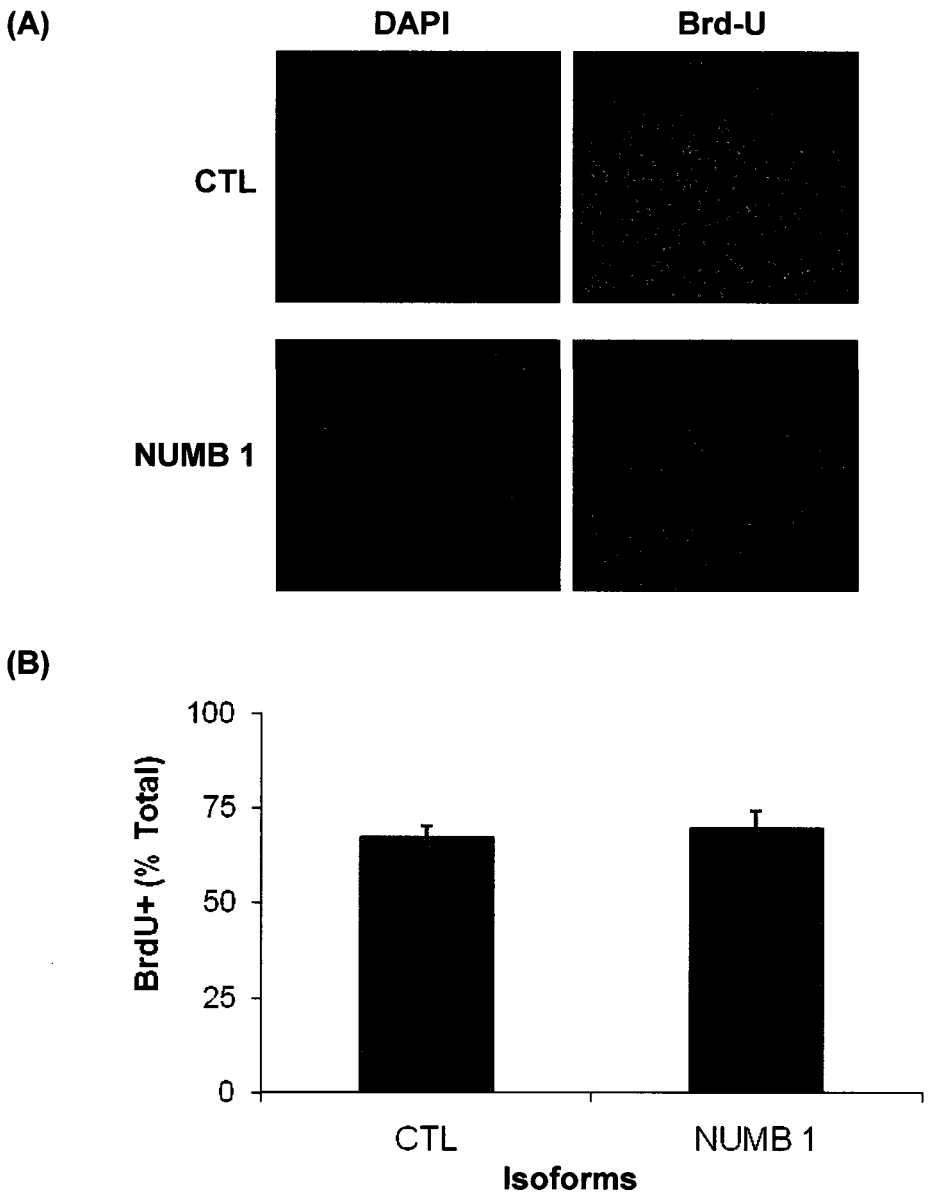


Figure 13. The effect of NUMB 1 overexpression on extravillous trophoblast (EVT) cell proliferation. (A) Overexpression of NUMB 1 in HTR8/Svneo cells (1ug/ml; 24h) had no effect on cell proliferation as determined by Brd-U incorporation assay (10μM; 6h). Total cell numbers were obtained from nuclear counts (DAPI) and cell proliferation was quantified (B) (n=3).

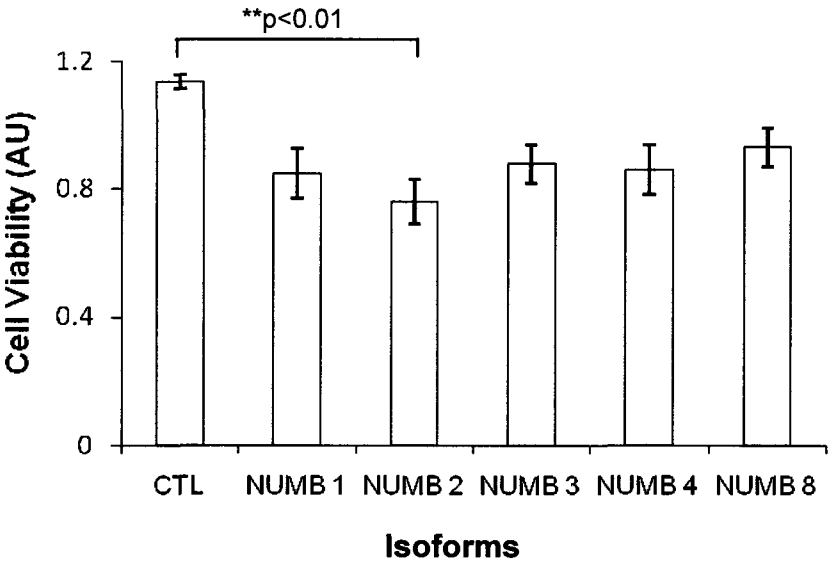


Figure 14. The effect of NUMB overexpression on extravillous trophoblast (EVT) cell viability. NUMB isoforms 1-4 and 8 were over-expressed in HTR8/SVneo cells (1μg/ml; 24h) in serum free media. After 24hrs, 25ul of MTT (0.5mg/ml) was added to each well containing serum free media and incubated in 37C for 30 minutes followed by absorbance measurement at 550nm [**p<0.01 [relative to Control (empty vector); n=3].

9. The effect of NUMB silencing on EVT apoptosis

Previously, we have shown that NUMB isoform 2 overexpression significantly decreased cell survival when compared to the control ($p<0.01$). To explain this observation, we examined whether or not apoptosis is altered. Apoptotic cells were characterized by nuclear fragmentation and condensation using Hoechst stain. Down-regulation of NUMB in EVT cells had no significant influence on EVT cell apoptosis as compared to the control group (Figure 15). NUMB down-regulation was confirmed by Western blot analysis.

10. The effect of NUMB overexpression on EVT apoptosis

In order to determine whether or not specific NUMB isoforms play a role in the regulation of apoptotic cell death (and in particular isoform 2), HTR8/SVneo cells were transfected for 24hrs with individual isoforms 1-4 and 8, and control vector (1μg/ml cDNA). Apoptotic cells were characterized by nuclear condensation and fragmentation (Figure 16a). NUMB 1-4 and 8 overexpression in EVT cells was confirmed by Western analysis. Overexpression of NUMB 2 ($p<0.05$) and 4 ($p<0.01$) significantly increased EVT apoptosis by approximately 2 fold (Figure 16b). NUMB 1, 3 and 8 failed to significantly alter apoptotic cell death compared to the control.

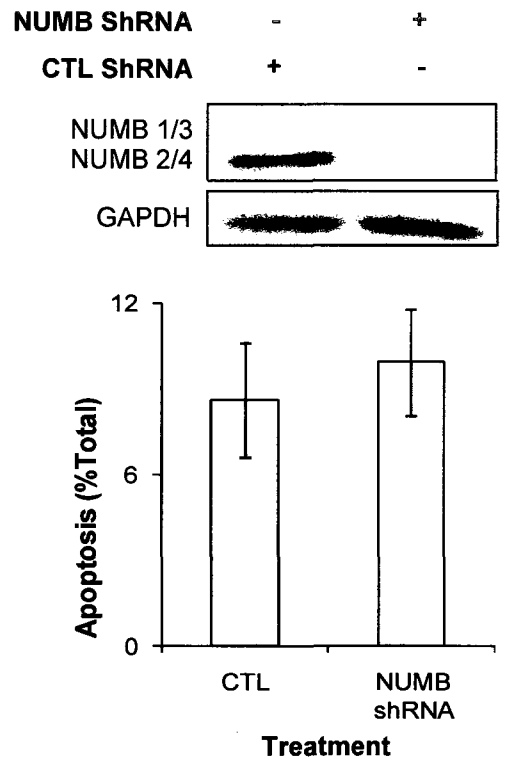


Figure 15. The effect of NUMB down-regulation on human extravillous trophoblast (EVT) cells apoptosis. Down-regulation of NUMB in HTR8/Svneo cells with shRNA (1ug/ml; 72 h) and apoptosis was assessed by Western blot and Hoechst nuclear staining, respectively. A total of 500 cells within several fields of view was analyzed to determine significance [*$p<0.05$ (relative to Control shRNA); n=3].

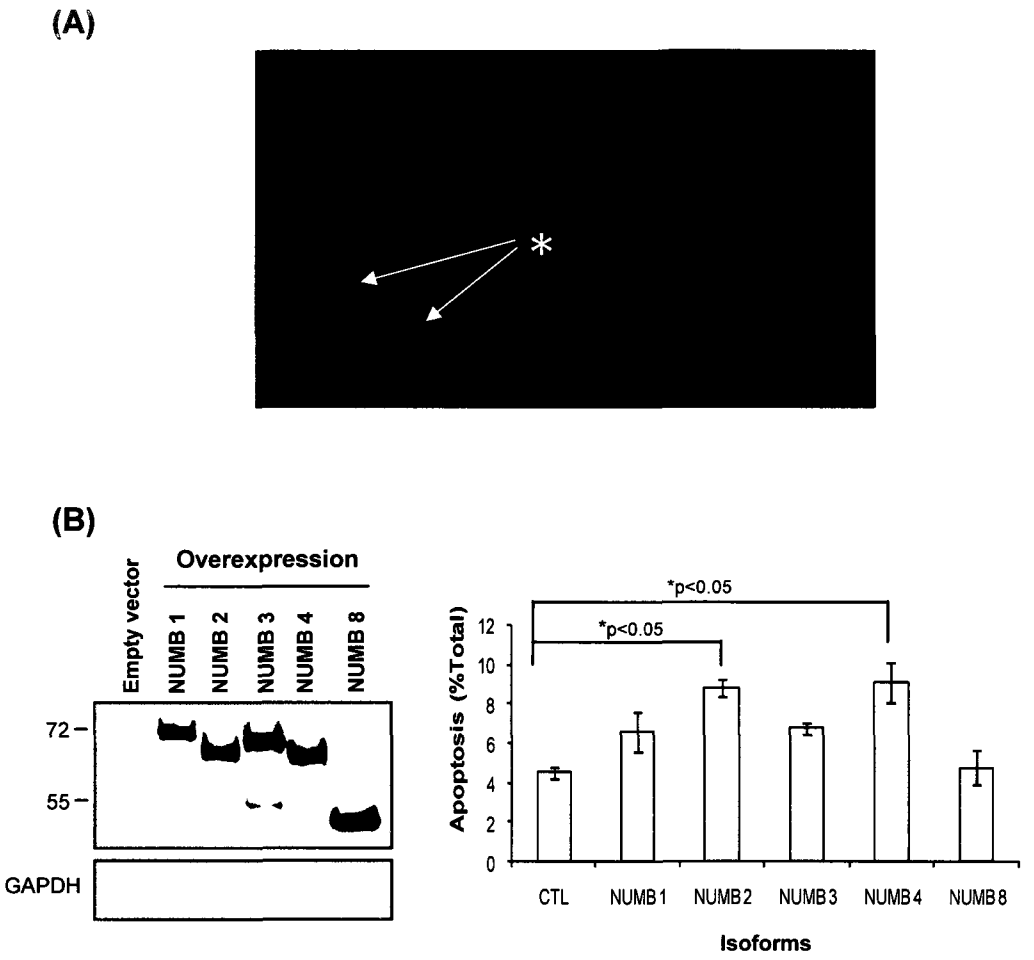


Figure 16. Isoform specific effects of NUMB on human extravillous trophoblast (EVT) cell apoptosis. A) Apoptotic HTR8/SVneo cells were identified morphologically (nuclear staining with Hoechst 33258; 12.5 ng/ml) based on phenotypes of nuclear condensation and fragmentation (*). B) NUMB isoforms were transfected in HTR8/Svneo cells (μg; 24h), confirmed by Western blot and apoptosis was assessed by Hoechst. A total of 500 cells within several fields of view was assessed to determine significance [*p<0.05 (relative to Control (empty vector); n=3]

ROLE OF NUMB IN EVT CELLS: SIGNALLING PATHWAYS

11. Does NUMB affect p53 content in EVT?

Studies examining the role of NUMB in breast carcinomas have shown that NUMB associates with p53, a transcription factor known to be involved in the induction of apoptosis. Therefore, we next examined the role of NUMB in the regulation of p53 content in HTR8/SVneo cells. The influence of overexpression of individual NUMB isoforms on p53 levels was determined after 24 h of transfection and compared to endogenous levels of NUMB and p53 in EVT cells. Western Blot analysis indicated that p53 levels did not significantly change in HTR8/SVneo cells expressing high levels of NUMB 1, 2, 3, and 4 (Figure 17). Interestingly, p53 levels were reduced in cells expressing high levels of NUMB 8 although this failed to reach statistical significance.

12. Does NUMB signaling occur via the MAPK and PI3K pathways?

To date, there is little information on the signaling pathways mediating the action of NUMB. IGF-II-induced EVT cell migration is known to involve both the MAPK (ERK1/2) and PI3-K pathways. Therefore, we tested whether or not NUMB signaling occurs through these pathways in the presence and absence of IGF-II. Individual NUMB isoforms 1-4 and 8 were over-expressed in HTR8-Svneo cells which were then treated with IGF-II for 15 minutes. Western blot analyses showed that the overexpression of NUMB did not up-regulate phospho-ERK1/2 or AKT content when compared to the control. In contrast, a significant increase in phosphorylation of both ERK1/2 and AKT was evident after the addition of IGF-II (Figure 18).

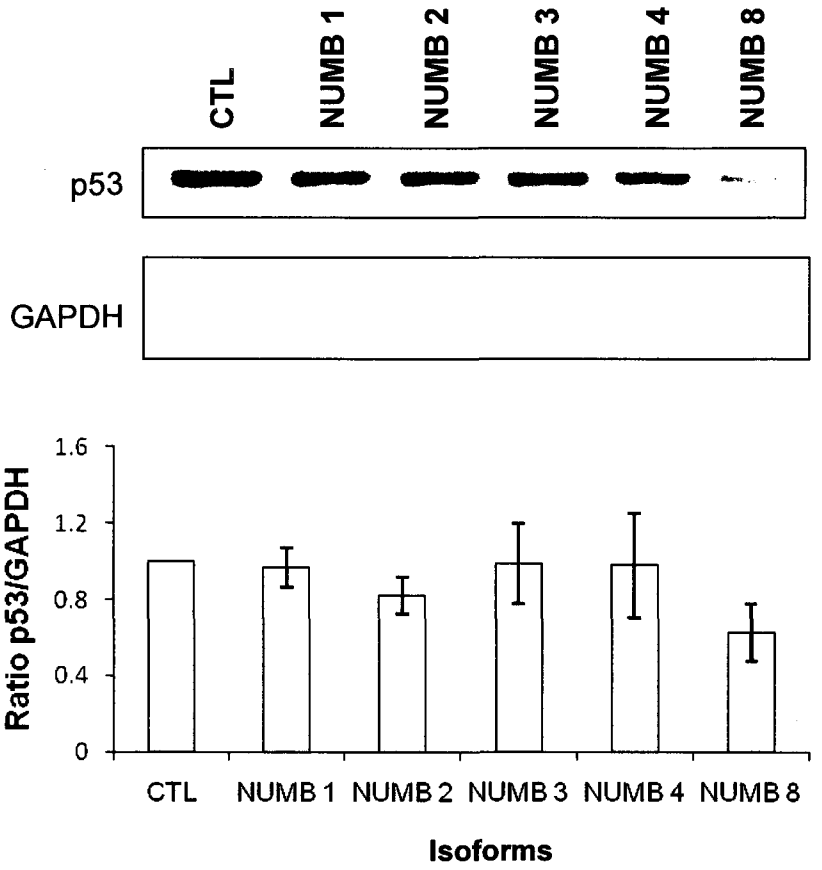


Figure 17. The effect of NUMB up-regulation on p53. HTR8/SVneo cells were seeded in a 12 well plate and transfected with individual NUMB isoforms 1-4 and 8 (1 μg/ml; 24). Cells were then harvested and analyzed for p53 protein content by Western blot (n=3).

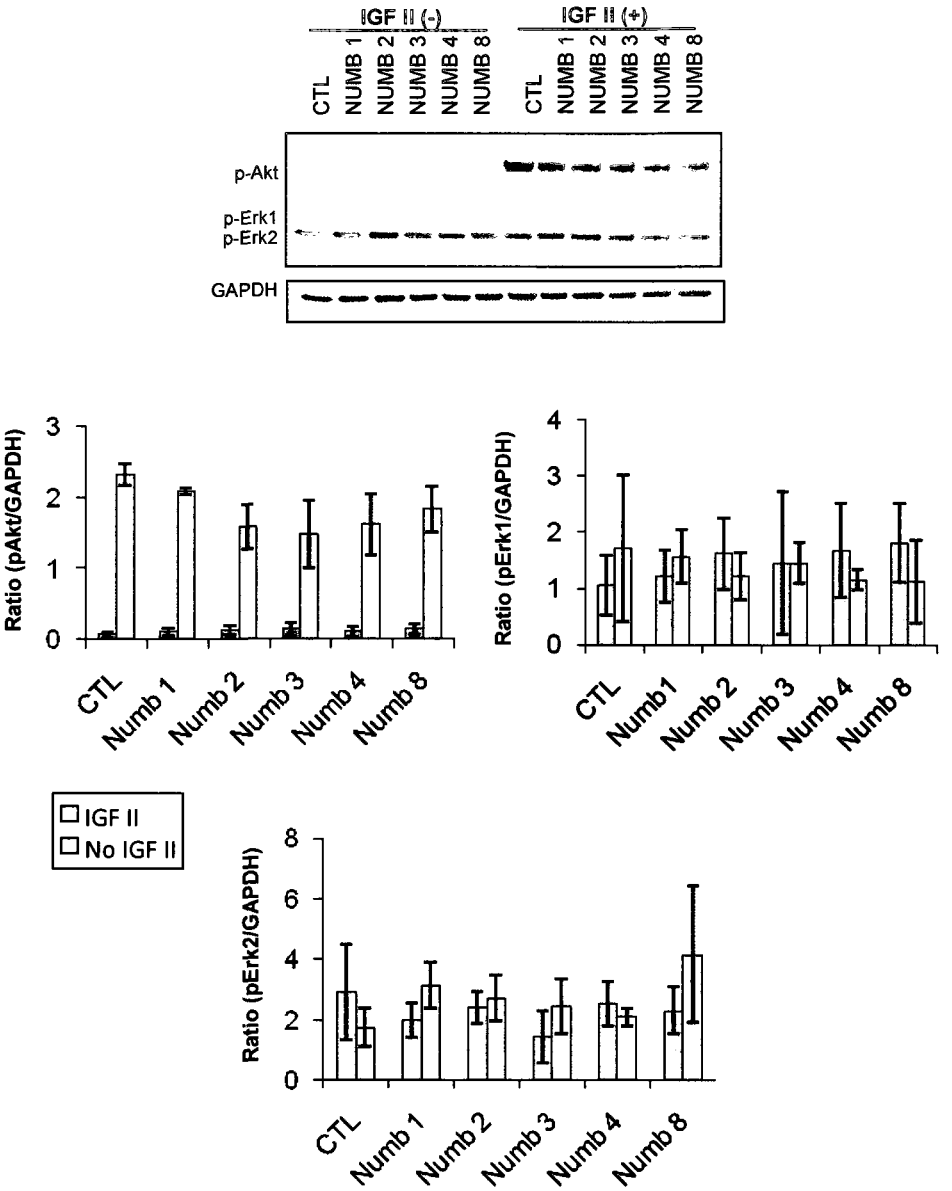


Figure 18. The effect of NUMB on MAPK and PI3K signaling pathways in EVT migration. The cells were seeded in 12 well plates and transfected with individual NUMB isoforms 1-4 and 8 (1ug; 24h). Cells were cultured in the absence or presence of IGF-II (10ng/ml; 15 min), harvested and then analyzed by (top)Western blot for (left) p-Akt, (right) p-Erk1 and (bottom) p-Erk2 relative to GAPDH (n=3).

CHAPTER 4: DISCUSSION

1. Localization of NUMB in HTR8/SVneo cells

Our earlier findings revealed NUMB localization at the maternal-fetal interface of first trimester human placental tissue. A closer investigation into the maternal-fetal interface revealed NUMB to be expressed predominantly by the human invasive extravillous trophoblast cells. As a result, NUMB localization in the EVT cell line, HTR8/SVneo was examined.

We have, for the first time, localized NUMB to the cytoplasmic region of the human invasive extravillous trophoblast cell line HTR8/SVneo. Our findings are consistent with the observation that NUMB is present in the cytoplasm of P19 Madin-Darby canine kidney cells and human embryonal carcinoma cells NT2/D1 (which as a result, were used as the positive control in our HTR8/SVneo NUMB-localization studies) (Bani-Yaghoub *et al.*, 2007; Dho *et al.*, 1999). In addition to cytoplasmic NUMB localization, P19 cells also exhibit the presence of NUMB at the plasma membrane. To determine if NUMB is also found at the plasma membrane in human EVT cells, co-localization studies using plasma membrane markers (e.g. integrins) specific to HTR8/SVneo cells are required. Integrins contain α and β subunits which bind to extracellular matrix proteins and trigger cell motility. Invasive EVT up-regulate the expression of α1β5 and α1β1. The co-localization of NUMB and integrin β1 would not only provide strong support for the localization of NUMB at the plasma membrane, but would further validate the localization of NUMB and β1 as observed in HeLa cells and Chinese Human Ovary (CHO) cells (Calderwood *et al.*, 2002; Nishimura and Kaibuchi, 2007). This co-localization could allow for a possible role for NUMB in integrin

signaling and recycling in EVT. While several studies have shown the importance of integrin regulation during extravillous trophoblast migration (Burrows *et al.*, 1996; Juliano *et al.*, 2004) it is important to note that NUMB acquires a major role in integrin endocytosis during directional migration (Nishimura and Kaibuchi, 2007). Hence, these localization studies may provide evidence for the role of NUMB in integrin-regulated EVT cellular migration, specifically, $\alpha 1\beta 1$, as extravillous trophoblast migration into the maternal decidua is essential for trophoblast invasion and arteriole remodeling.

Unfortunately, we were unable to distinguish individual NUMB isoforms in our confocal studies as the antibody used recognized the C-terminus PRR domain present in all NUMB isoforms (Figure 2). Due to the combination of insertion and deletion sequences within the PTB and PRR domains that frame the structural component of individual NUMB isoforms, the isolation of individual NUMB isoforms was difficult. The localization of NUMB 1 and 3 at the plasma membrane, and NUMB 2 and 4 in the cytosol was evident in P19 Madin-Darby canine kidney cells following transient transfection with NUMB pEGFP constructs (Dho *et al.*, 1999). Thus, exogenous pEGFP-vector based analyses would allow for the visualization of individual NUMB isoforms within EVT cells, but also NUMB distribution, as well as pathways and molecules associated with NUMB.

Although the physiological role of NUMB in the placenta has never been examined, neural studies indicate, NUMB is required for cell-fate decisions. Although the placental histology of null NUMB mice placenta do not highlight the effect of knockout studies on the placental giant cells which function similarly to the invasive extravillous trophoblast, it can be noted that the maternal decidua of null NUMB placenta

appear thicker than the control group, perhaps due to the inability of the giant cells to invade the uterine wall. Furthermore, as depicted by earlier immunohistological studies of healthy human placental tissue NUMB is expressed in human extravillous trophoblasts. Pregnancies affected by IUGR and PET have shown insufficient trophoblast function to be associated with hypoxic conditions, poor invasion and inadequate arterial remodeling. Although mouse studies have shown null NUMB knockout lethality at embryonic day 11.5, it is possible the same trend with respect to placental dysfunction and neurogenesis may be evident in the human model to a lesser degree. Hence, altered NUMB expression during human placental development could spare fetal life at the expense of fetal growth, but consequently pose cognitive abnormalities in later life. To investigate the effect of NUMB on IUGR placenta, a comparison between NUMB distribution in healthy and IUGR placentae, in particular the EVT would be required.

2. The effect of NUMB EVT on cell migration

The role of NUMB in extravillous trophoblast migration was investigated following NUMB knockdown in HTR8/SVneo cells with the use of the Boyden chamber migration assay and the wound healing assay. NUMB down-regulation by siRNA transfection failed to consistently decrease NUMB protein content in HTR8/SVneo cells, although it was efficient in other cell systems (Colaluca *et al.*, 2008). These differences in efficacy may be attributed to a greater importance of NUMB function or a problem

with the stability and delivery of the siRNA, as noted in many cell systems (Whitehead *et al.*, 2009).

Our second approach to down-regulate NUMB involved the use of shRNA. NUMB down-regulation by shRNA caused a significant decrease in NUMB protein content, which could be due to increased shRNA transfection stability compared to the siRNA or target specificity (Whitehead *et al.*, 2009). Expression of NUMB-shRNA resulted in consistent NUMB knockdown and a significant decrease in cellular migration. These results were as expected since NUMB down-regulation has been shown to reduce directional cell migration in HeLa cells (Nishimura and Kaibuchi, 2007). This decrease in trophoblast cell migration resulting from NUMB down-regulation could be explained by ineffective integrin endocytosis (Nishimura and Kaibuchi, 2007). From a molecular aspect, NUMB down-regulation may affect pathways associated with EVT cellular migration such as the phosphoinositide 3-kinase (PI3K; via Akt activation) and mitogen-activated protein kinase (MAPK; via Erk activation) (Caswell and Norman, 2008). Both PI3K and MAPK pathways are activated via the IGF1R following endocytosis, thus enabling activation of proliferation, differentiation, migration or cell survival. Upon autophosphorylation of the intracellular domain of IGF1R, high affinity binding sites become available for proteins with SH2 domains or PTB domains. Once bound, signaling proteins become activated and trigger intracellular responses (Alberts *et al.*, 2002). NUMB down-regulation could possibly play an important role independent of direct integrin endocytosis in continued activation of the MAPK and PI3K pathways by enabling continued binding of the IGF1R to its associated molecules. NUMB's PTB domain may directly bind to the IGF1R for endocytic receptor recycling and as a result,

allow for continued activation of IGF1R at the endosomal membrane (Caswell and Norman, 2008).

Overexpression of NUMB isoform 1 in EVT cells significantly increased cell migration in the Boyden chamber and wound healing assay. This increase could be linked to a number of mechanisms, but it is important to rule out the influence of proliferation on migration. Both NUMB 1 and NUMB 3 have been shown to promote cell proliferation, and therefore maintain cell numbers during neurogenesis (Bani-Yaghoub *et al.*, 2007). As a result, it was important to investigate the effect of proliferation on NUMB 1 induced migration. Using Brd-U incorporation as an assessment of cell proliferation, no significant change in proliferation was noted following NUMB 1 up-regulation. Also, the effect of growth factors in media was minimal. Although media containing 10% FBS was used for the wound-healing assay, the qualitative results were unremarkable, and similar to those of the Boyden chamber assay, which required 0.05% FBS.

IGF-I- and IGF-II-induced EVT migration is known to be mediated through the IGF1R which, in turn, activates the MAPK and PI3K pathways (Forbes and Westwood, 2008). As a result, we monitored the MAPK pathway after up-regulating NUMB isoforms 1-4 and 8 in the presence and absence of IGF-II. Up-regulation of NUMB isoforms 1-4 and 8 did not influence phospho-Erk1 and -Erk2, or phospho-Akt. Thus, NUMB may not directly activate the MAPK pathway even upon IGF-II stimulation.

The role of the Rho GTPases in trophoblast migration has been extensively studied (Nicola *et al.*, 2008; Pollheimer and Knofler, 2005; Shields *et al.*, 2007; Shiokawa *et al.*, 2002). The Rho GTPases are a family of intra-signaling molecules

belonging to the Ras superfamily, which includes Rho, Rac and Cdc42. EVT express RhoA, Ras and CdC42 Rho GTPases during trophoblast cell migration (Shiokawa *et al.*, 2002). These Rho GTPases are activated when bound to GTP and are regulated by 3 proteins: guanine nucleotide exchange factor (GEFs), GTPase activity proteins (GAPs) and guanine nucleotide disassociation inhibitors (GDIs) (Etienne-Manneville and Hall, 2002; Heasman and Ridley, 2008). Many cellular processes, such as cell polarity, migration, morphogenesis, vesicle transport regulation, adhesion and cell division, are activated upon activation of Rho GTPase by GTP. During coordinated epithelial cell movement, Cdc42 controls cell polarity by communicating with the partitioning defective (PAR) complex (PAR6-PAR3-atypical protein kinase) as well as promoting filipodia formation and allowing the cell to sense its environment. Epithelial cell migration is partly controlled by Rac activation to the leading edge and lamellapodia formation (Heasman and Ridley, 2008). During dendritic spine development of cultured hippocampal neurons, NUMB binds to the Cdc42-specific GEF Intersectin, and enhances its GEF activity for filopodia formation (cytoskeletal actin-projections involved in cell motility). During mammalian asymmetric cell division, NUMB has been shown to be localized to the apical surface of neuronal progenitors and overlap with the PAR complex, providing epithelial polarity (Casanova, 2007; Kim and Walsh, 2007). Therefore, NUMB may play an important role in Rho GTPase signaling as up-regulation of NUMB isoform 1 increases binding of GEFs to their Rho GTPase counterparts, thus allowing for increased cellular migration by promoting cell polarity or formation of cytoskeletal projections such as lamellapodia and filopodia, which are involved in leading edge cell motility, in the absence of integrin stimulation.

One signaling molecule upstream of Rho GTPase is the focal adhesion kinase (FAK). During EVT migration, active focal adhesion kinase-Src signaling complexes regulate binding and phosphorylation of GAPS and Rho-GEFs (Pollheimer and Knofler, 2005). It is therefore possible that NUMB Src homology within the PRR binds FAK and recruits GEFs to appropriate Rho GTPases during EVT cell migration. This may further activate the serine/threonine effector, p21-associated kinase (PAK), which plays a role in cell migration and adhesion (Fournes *et al.*, 2003).

Our study of individual NUMB isoforms 1-4 and 8 on cell migration revealed that HTR8/SVneo cell migration is increased by NUMB isoform 1 and decreased by NUMB isoform 4. NUMB 1 and NUMB 4 are structurally different at the PTB and PRR domains. NUMB 4 lacks the 11 amino acid insert within the PTB and a 49 amino acid insert within the central region of the PRR (Dho *et al.*, 1999). The requirement of both inserts may be an important aspect of NUMB function in EVT cell migration.

3. The effect of NUMB on EVT apoptosis

Apoptosis, or programmed cell death, is a naturally occurring cellular process during placental development. Apoptosis is required for placental homeostasis associated with trophoblast turnover in the villi, for example. Pregnancies affected by IUGR and PET are associated with increased in endovascular EVT apoptosis. We investigated the role of NUMB in EVT cell apoptosis and observed that complete NUMB down-regulation had no significant effect on EVT apoptosis. In contrast, the overexpression of NUMB isoforms 2 and 4 significantly increased cell apoptosis. The lack of an effect of

NUMB down-regulation on apoptosis could be explained by the presence of a compensatory mechanism which recruits other molecules with similar function to sustain cell survival. The effect of NUMB 2 and 4 on EVT death could be explained by two phenomena. First, the up-regulation of NUMB 4 resulted in reduced migration. The high apoptotic counts could explain the reduced number of cells during migration. Second, the up-regulation of NUMB 2 and 4 had a negative effect on HTR8/SVneo cell survival. The HTR8/SVneo cell line is an immortalized non-differentiating cell line obtained from first trimester pregnancies (Graham *et al.*, 1993). NUMB 2 and 4 have been previously shown to induce cell differentiation during neurogenesis (Bani-Yaghoub *et al.*, 2007). Therefore, the inability of the HTR8/SVneo extravillous trophoblast cell line to differentiate into the interstitial and endovascular extravillous trophoblast may justify the increased levels of apoptosis with the overexpression of NUMB 2 and NUMB 4.

4. Apoptosis and p53

To date, only one group has examined NUMB and p53 and have used primary breast tumor cells. NUMB has been shown to form a tricomplex with MDM2 and the tumour suppressor p53 and prevents its degradation. In chemoresistant cells, reduced NUMB levels corresponded to reduced levels of p53. In addition, high levels of NOTCH signaling, lead to reduced NUMB protein levels (Chapman *et al.*, 2006; Colaluca *et al.*, 2008). p53, a transcription factor, induces the expression of genes involved in cell cycle arrest and apoptosis in response to cellular stress (Carter and Vousden, 2008). p53 is expressed in placental tissue of healthy pregnancies and those complicated by IUGR and

PET (Heazell and Crocker, 2008). The regulatory role of p53 in extravillous trophoblast has not been established due to the lack of tissue specimens of placental bed biopsies (Huppertz *et al.*, 2006). Reports on the level of p53 in cases affected by IUGR and PET are contradictory as some have indicated no change while others an increase or decrease in p53 protein levels (Heazell and Crocker, 2008). Although no consensus exists with regards to the up-regulation of p53 levels in extravillous trophoblast of IUGR and PET placentae, the increased levels of p53 in pregnancies complicated by IUGR or PET could be a result of inadequate invasion of the spiral arterioles in the first trimester. In turn, poor arteriole remodeling results in a hypoxic environment that ultimately causes apoptosis of EVT. We therefore examined the levels of p53 in HRT8/SVneo cells following up-regulation of NUMB protein isoforms 1-4 and 8. No significant change in total p53 protein content was observed, even in samples over-expressing NUMB isoforms 2 and 4, which showed an increase in cellular apoptosis. It is possible that NUMB up-regulation could have activated alternative intrinsic pathways associated with extravillous trophoblast cell death. Although no direct link between NUMB isoform 1-4 up-regulation was observed, samples over-expressing NUMB isoform 8 showed a slight but non-significant decrease in p53 expression.

5. Cell viability

Cell viability was significantly reduced with exogenous NUMB isoform 2. NUMB 4, which is missing both the 11 amino acid and the 49 amino acid sequences within the PTB and PRR, does not affect cell viability as significantly as NUMB 2. It is

postulated that the absence of the 11 amino acid sequence within the PTB domain alone is detrimental to cell survival, resulting in reduced cell viability.

6. Insight to human sample studies

Unfortunately, human placental samples obtained from healthy and IUGR/PET placental bed biopsies were unavailable for the current study. As a result we were unable to investigate the role of individual NUMB isoforms 1-4 and 8 in EVT *in vivo*. Our previous results showed NUMB isoform 8 to be predominantly expressed throughout gestation. Although the current study focused on the role of NUMB isoforms 1-4 and 8 in EVT cell migration and survival, it is evident that NUMB 8 does not influence EVT cell migration, which may explain low levels of NUMB 8 in HTR8/SVneo. It is possible that explant culture and primary culture systems established from human placental tissue may allow for a further investigation of the possible role of NUMB 8.

CHAPTER 5: CONCLUSION

Studies have revealed strong similarities between the cellular structures and molecular mechanisms governing mouse and human placental development. The null NUMB knockout mouse study revealed that placental morphology of knockout mice was severely altered in addition to fetal growth (Zilian *et al.*, 2001). However, this study did not examine NUMB across the null mouse placenta. As a result, we have, for the first time, demonstrated the presence of NUMB in the human placenta and in a well characterized human invasive extravillous trophoblast cell line, HTR8/SVneo. With the use of the HTR8/SVneo human invasive extravillous trophoblast derived cell line, we have for the first time, revealed the role of NUMB in trophoblast cell migration and cell survival. We have further shown that NUMB is localized to the cytoplasm in HTR8/SVneo cells and its role is isoform-specific. We have successfully demonstrated the effect of NUMB down-regulation and overexpression on EVT cell migration, which is isoform-specific. NUMB 1 promotes cell migration without the influence of proliferation, while NUMB 4 reduces cell migration. Cell viability is reduced by NUMB 2 overexpression which could potentially be a result of increased apoptosis. NUMB overexpression has no significant effect on p53 level. It is postulated that apoptotic regulation may occur through a pathway which remains to be investigated. Our studies on the regulation of EVT cell migration and survival indicate that the PI3K and MAPK pathways are not involved in the action of NUMB.

The results of the present study suggest that NUMB plays an important role in cellular processes involved in human extravillous trophoblast function. Although the role of NUMB in pathologies afflicted with inadequate trophoblast invasion at the maternal

fetal interface (PET and IUGR) remains unclear, it is important to characterize NUMB expression at the maternal fetal interface of these placentae, as NUMB distribution may potentially impact trophoblast invasion during development and play a major role in regulating fetoplacental growth.

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