NUMB and Syncytiotrophoblast Development and Function:
Investigation Using BeWo Choriocarcinoma Cells

By

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ABSTRACT

The role of NUMB, a protein important for cellular differentiation and endocytosis in non-placental cells, was investigated in syncytiotrophoblast development and function in the human placenta. The BeWo choriocarcinoma cell line was used as a model for villous cytotrophoblast cells and syncytiotrophoblast to investigate NUMB's involvement in differentiation and epidermal growth factor receptor (EGFR) endocytosis. NUMB isoforms 1 and 3 were found to be the predominant isoforms and were upregulated following forskolin-induced differentiation. Overexpression of NUMB isoforms 1 and 3 did not mediate differentiation or EGFR signaling. Immunofluorescence analysis revealed that NUMB colocalized with EGFR at perinuclear late endosomes and lysosomes following EGF stimulation. We have demonstrated for the first time that NUMB isoforms 1 and 3 are expressed in BeWo cells, are upregulated in forskolin-differentiated BeWo cells and are involved in ligand-dependent EGFR endocytosis in BeWo cells.

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aa	Amino acid
AEC	3-amino-9 ethylcarbazole
aPKC	Atypical protein kinase C
APP	Amyloid precursor protein
ANOVA	Analysis of variance
ARF6	ADP-ribosylation factor 6
BSA	Bovine serum albumin
CaMKI	Ca^{2+}/calmodulin-dependent protein kinase I
cAMP	Cyclic adenosine monophosphate
CT	Cytotrophoblast
DMSO	Dimethyl sulfoxide
dNUMB	*Drosophila* NUMB
E-cadherin	Epithelial cadherin
ECM	Extracellular cell matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
EH	Eps15 homology
EPS15	EGFR pathway substrate clone 15
EVT	Extravillous trophoblast
FBS	Fetal bovine serum
FV	Floating villus
GAPDH	Glyceraldehyde 3’ phosphate dehydrogenase
GCM	Glial cells missing
GFP	Green fluorescent protein
hCG	Human chorionic gonadotropin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HERV-W	Human endogenous retrovirus W
hPL	Human placental lactogen
HRP	Horseradish peroxidise
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IUGR	Intrauterine growth restriction
LAMP-2	Lysosomal-associated membrane protein 2
LSM	Laser scanning microscope
MRP	Multidrug resistance proteins
NCBI	National center for biotechnology information
OE	Overexpression

PBS	Phosphate buffered saline
PKA	Protein kinase A
PMSF	Phenylmethylsulfonyl fluoride
PRR	Proline rich region
PTB	Phosphotyrosine binding
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH3	SRC homology 3
snRNP	Small nuclear ribonucleoproteins
SOP	Sensory organ precursor
ST	Syncytiotrophoblast
STBM	Syncytiotrophoblast microvilli (or microparticles)
TBST	Tris-buffered saline Tween-20
TfR	Transferrin receptor
TGF	Transforming growth factor
WB	Western blot

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CHAPTER 1: INTRODUCTION

1.1 Formation and development of the human placenta

Placental development is a tightly regulated process that begins during the very early stages of pregnancy. Within a few days after fertilization, the human zygote undergoes holoblastic cleavage and travels through the fallopian tube. It develops into an 8-celled morula and then into a 16-celled blastocyst. Characteristic features of the blastocyst are a hollow core called a blastocoel, and two outer layers: the embryoblast, also called the inner cell mass, and the trophoblast. The embryoblast is destined to form the embryo, umbilical cord and amnion, whereas the trophoblast develops into the placenta and membranes involved in protecting and nourishing the fetus. Embryoblast-derived mesenchyme and blood vessels also comprise part of the placenta.

Differentiation of the trophoblast results in the formation of two distinct layers: the syncytiotrophoblast and the cytotrophoblast. The syncytiotrophoblast is a multinucleated layer that lines the maternal blood pool in the intervillous space. This layer forms the outer encapsulation of projections called chorionic villi and lines parts of the chorionic and basal plates. The cytotrophoblast is a reservoir of mononuclear, proliferative cells found within the chorionic villi that initially function to differentiate and fuse into the multinucleated, weakly proliferative syncytiotrophoblast and continuously replenish that layer with cytoplasmic compounds as it grows. The syncytiotrophoblast simultaneously sheds apoptotic material, referred to as syncytial knots, into the maternal circulation.

Chorionic villi consist of fetal vascular cells surrounded by mesenchyme-derived cells, cytotrophoblast cells and synyctiotrophoblast (refer to Figure 1 for cross-section representations). The two types of chorionic villi are: (i) floating and (ii) anchoring. Floating (also called terminal) villi are important for nutrient and gaseous exchange processes occurring between the fetus and the mother. Anchoring villi are responsible for maintaining mechanical stability and are formed when cytotrophoblast cells differentiate into extravillous trophoblast (EVT) cells and invade through the syncytiotrophoblast layer into the maternal tissue. During invasion, EVT cells form cell columns at the tips of the anchoring villi, lose contact with the basal lamina, migrate towards the maternal interface and invade the endometrium as well as one third of the myometrium.

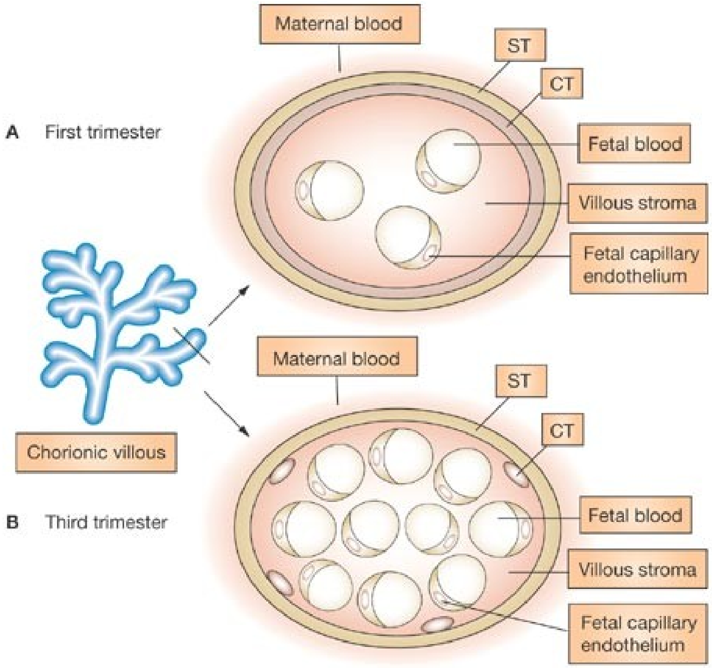


Figure 1. Schematics of first and third trimester chorionic villi cross-sections. This image is from the article, "The role of the placenta in thyroid hormone delivery to the fetus" (Chan *et al.*, 2009). Abbreviations: CT, cytotrophoblast; ST, syncytiotrophoblast.

1.2 Functions of the human placenta

The human placenta consists of a heterogeneous population of cells and tissues which have specialized functions. These different types include syncytiotrophoblast, undifferentiated cytotrophoblast cells, EVT cells, fetal vessels, connective tissues and macrophages.

The syncytiotrophoblast, which is situated at the interface between maternal and fetal circulations, is responsible for the main functions of the placenta. It facilitates the supply of essential nutrients, such as glucose, iron, lipids, amino acids and minerals, to the developing embryo and fetus from the maternal circulation (Fuchs and Ellinger, 2004) and is a site for glycogen synthesis and degradation (Fisher and Laine, 1983). The syncytiotrophoblast is responsible for the selective uptake and transport of hormones, growth factors and cytokines. This transport can occur through a variety of routes including simple diffusion, facilitated diffusion, active transport, ion channels and endocytic and exocytic processes (Desforges and Sibley, 2010). The syncytiotrophoblast also synthesizes and secretes many hormones and growth factors which are critical for the maintenance of pregnancy such as human chorionic gonadotropin (hCG), progesterone, estrogen, human placental lactogen (hPL), epidermal growth factor (EGF), and transforming growth factor (TGF-α and TGF-β).

The syncytiotrophoblast provides various types of fetal protection. The barrier it provides prevents the blood between the fetal and maternal circulations from intermingling. It helps protect against the maternal immune system and xenobiotics. The syncytiotrophoblast and the underlying villous cytotrophoblast lack class I and class II MHC antigens and protect the feto-placental unit, which is regarded as a semi-allograft, from destruction through maternal T-cell activation (Sunderland *et al*., 1981). Protein pumps of the ATP-binding cassette (ABC) transporter family in the syncytiotrophoblast actively transport out xenobiotics to limit what enters into the fetal circulation. P-glycoprotein is expressed on the maternal-facing membrane throughout gestation, with its highest expression in the first trimester (Sun *et al*., 2005), and can decrease fetal uptake of vinblastine, vincristine and digoxin (Ushigome *et al*., 2000). Multidrug resistance proteins (MRPs) are expressed in the fetal-facing membrane and in fetal endothelial cells (St-Pierre *et al*., 2000) and are hypothesized to prevent entry of organic anions and eliminate lipophilic compounds conjugated to glutathione and glucuronide. The syncytiotrophoblast also contains enzymes that can metabolize certain drugs and toxic chemicals. One of these enzymes, uridine diphosphate glucuronosyltransferase, is expressed in first-trimester and term placenta syncytium and can detoxify drugs through conjugation with glucoronic acid (Collier *et al*., 2002a and 2002b).

The other cell types of the placenta are mostly responsible for facilitating processes to initiate and maintain the functions of the syncytiotrophoblast. Undifferentiated cytotrophoblast cells are highly proliferative and can differentiate into two subtypes: (i) villous cytotrophoblast, and (ii) EVT. The main function of villous cytotrophoblast cells, also called Langhan's cells, is to be stationed as stem cells ready to differentiate and fuse into the overlying syncytiotrophoblast. However, these cells may also have endocrine activities and have been found to produce hormones such as somatostatin, gonadotropin-releasing hormone and corticotrophin-releasing factor (Nishihira and Yagihashi, 1978; Khodr and Siler-Khodr, 1978; Saijonmaa *et al*., 1988). Undifferentiated cytotrophoblast cells can also differentiate into EVT cells which are responsible for invading and remodeling spiral arteries in the endometrium to help facilitate systems of exchange between mother and fetus. Endovascular cells in the uterine spiral arteries are replaced by EVT cells that interdigitate between endothelial cells, thus increasing the flow and decreasing the resistance of maternal blood circulating to the chorionic villi. EVT cells produce and secrete various enzymes, such as matrix metalloproteinases and urokinase-type plasminogen activator, that degrade the extracellular cell matrix (ECM) to facilitate invasion progression (Bischof *et al.*, 1991; Hofmann *et al.*, 1994; Kaufmann and Castellucci, 1997).

Fetal blood vessels within the chorionic villi consist of capillaries, sinusoids, arteries and veins and are important for the delivery of oxygenated blood to the fetus. The stroma, which is separated from the syncytiotrophoblast and cytotrophoblast cells by a basement membrane, surrounds the fetal blood vessels and consists of ground substance and connective tissue cells and fibres. The stroma mostly consists of mesenchymal cells (undifferentiated stromal cells), fibroblasts or myofibroblasts depending on the maturity of the villi. Myofibroblasts are thought to help regulate intervillous blood volume (Farley *et al.*, 2004). Macrophages of mesenchymal origin, called Hofbauer cells, maintain host defense and also express cytokines and angiogenic growth factors.

Refer to Table 1 for a summary of the different components of the human placenta and their functions.

Table 1. Human placental cells and tissues

TYPE	DESCRIPTION	LOCATION	FUNCTION
Syncytiotrophoblast	multinucleated layer at maternal-fetal interface	outer layer of chorionic villi	(i) mediates exchange of nutrients and gases between maternal and fetal circulations (ii) facilitates removal of fetal waste products (iii) synthesizes, secretes and transports hormones and growth factors (iv) fetal protection
Villous cytotrophoblast cells	uninuclear	inner layer of chorionic villi, directly beneath syncytiotrophoblast	(i) act as stem cells for syncytiotrophoblast (ii) synthesize and secrete some hormones
Extravillous trophoblast cells	migratory, proliferative and invasive cells	anchoring cell columns, decidualized endometrium and myometrium, maternal blood vessels	(i) invade and remodel spiral arteries to increase flow (ii) secrete ECM-degrading enzymes
Fetal blood vessels	capillaries, sinusoids, arteries and veins	stroma within chorionic villi	(i) deliver oxygenated blood to fetus
Hofbauer cells	macrophages of mesenchymal origin	stroma within chorionic villi	(i) maintain host defense (ii) express cytokines and angiogenic growth factors
Myofibroblasts	mature form of mesenchymal cells and fibroblasts	stroma within chorionic villi	(i) regulate intervillous blood volume

1.3 Pregnancy complications associated with placental aberrations

For many years, it has been postulated that several of the complications observed during pregnancy are directly related to disturbances in early placental development and function. This association between placental defects and the progression of both maternal and fetal pathologies during gestation has been suggested as early as 1892, when it was first proposed that a thorough analysis of a diseased fetus should include a consideration of the placenta (Ballantyne, 1892). Placenta-related complications may lead to miscarriage, fetal death and maternal death. Some of these conditions include abruptio placentae (partial or complete placental detachment from the uterine wall prior to delivery), placenta accreta (excessively deep attachment of the placenta) and placenta previa (placental attachment covers the cervix). Shallow invasion of the placenta into the uterine wall is thought to be a contributing factor to the onset of two of the most prevalent placenta-mediated diseases: pre-eclampsia and intrauterine growth restriction (IUGR) (Meekins *et al*., 1994). Pre-eclampsia is defined as the onset of maternal hypertension coinciding with end organ manifestations such as proteinuria (excess secretion of serum protein into the urine) occurring *de novo* during pregnancy. It may lead to complications such as eclampsia (pregnancy-associated seizures), abnormalities in hemostasis (stoppage of bleeding) and liver or kidney failure. Pre-eclampsia occurs in about 3 to 5% of pregnancies worldwide and is a leading cause of maternal and fetal morbidity and mortality. IUGR occurs when the fetus undergoes restricted growth during its development and is physically smaller (i.e., less than the 5th percentile) than expected for its gestational age. IUGR is frequently observed in pre-eclamptic patients, although the two conditions can develop independent of each other. IUGR is the second leading cause of perinatal death in North America and is associated with significant morbidity (Bernstein and Gabbe, 1996).

The impact of pre-eclampsia and IUGR on our society persists after the baby has been delivered. Pre-eclamptic mothers are at a risk for recurrence of the condition during their next pregnancy (Skjærven *et al*., 2005) and are also at risk of development of heart disease later in life, whereas IUGR infants may suffer from health complications onwards through adulthood. A well-known theory of the association between IUGR and complications later in life is the Barker hypothesis (Barker, 1997). This hypothesis states that infants that are smaller at birth are more at risk for suffering from chronic diseases in adulthood such as coronary heart disease, diabetes mellitus, hypertension, hypercholesterolemia, hyperuricemia and stroke. It has been substantiated in many publications.

There is no cure for pre-eclampsia or IUGR during pregnancy, however the direct symptoms of pre-eclampsia end after the placenta has been properly removed post-delivery. The key triggers of these two conditions have not been completely characterized although the aberration between placental and maternal interactions appears to be a primary cause. Several contributing factors have been identified such as inflammatory defects, angiogenic abnormalities, abnormal placentation and oxidative stress (Kanasaki and Kalluri, 2009). A significant finding has been that shallow invasion and improper remodelling of the maternal spiral arteries are predisposing factors for the development of pre-eclampsia and IUGR (Meekins *et al*., 1994). An illustration of this pathogenic feature is represented in Figure 2. This leads to hypoxia (lowered oxygen levels) and ischemia (restricted blood flow). Ischemia-reperfusion injury at the maternal-fetal interface and in the placenta, which results in damage caused by excessive reactive oxygen species (ROS) due to irregular blood flow, is postulated to play a role in the etiology of pre-eclampsia (Hung *et al*., 2001).

Improper syncytiotrophoblast development and function has frequently been linked to pre-eclampsia and IUGR. In a healthy pregnancy, syncytiotrophoblast microvilli (STBM) are regularly fragmented and released into the maternal circulation as membrane-bound vesicles (Knight *et al*., 1998). This occurs as a result of apoptosis during regular syncytiotrophoblast turnover (Huppertz *et al*., 1998). Increased STBM shedding is associated with pre-eclampsia (Knight *et al*., 1998), particularly in early-onset cases (Goswami *et al*., 2006). Normal levels of STBM are thought to inhibit maternal endothelial cell growth, proliferation and function (Smárason *et al*., 1993; Cockell *et al*., 1997). Thus, elevated levels of STBM may promote pre-eclampsia through the concurrent increased hindrance of maternal endothelial activity. Specifically, this is thought to occur via increased pro-inflammatory cytokine production (Southcombe *et al*., 2011) and tissue-factor-dependent coagulation activation (Gardiner *et al*., 2011). The decreased expression of syncytins has also been associated with pre-eclampsia (Lee *et al*., 2001; Knerr *et al*., 2002; Langbein *et al*., 2008; Vargas *et al*., 2011) and IUGR (Ruebner *et al*., 2010). Syncytins 1 and 2 are fusiogenic glycoproteins encoded by the envelope genes of the human endogenous retrovirus W (HERV-W) family that help mediate the fusion of cytotrophoblast into syncytiotrophoblast. Decreased levels of syncytins would result in decreased syncytiotrophoblast formation and a deficiency in placental villous function.

The identification of molecular determinants that are involved in placental development would help contribute to more accurate predictions, diagnosis and treatments for pathological pregnancy-related conditions. Syncytiotrophoblast irregularities have frequently been linked to pre-eclampsia and IUGR. Additional studies of the development and function of the syncytiotrophoblast, which is the site of the main functions of the placenta, may contribute to a comprehensive understanding of the origins of these two conditions.

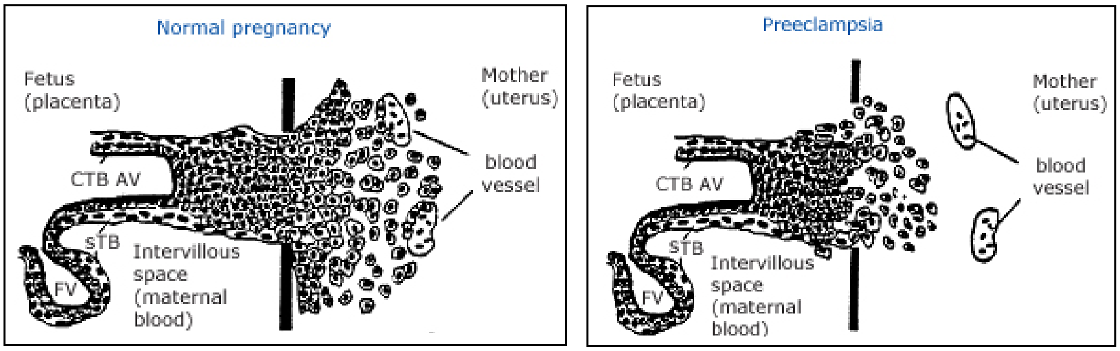


Figure 2. Comparison of trophoblast invasion during normal and pre-eclamptic pregnancies. Shallow invasion into the maternal uterine tissue is observed during pre-eclampsia as compared in normal pregnancy. Abbreviations: AV, anchoring villus; CTB, cytotrophoblast cells; FV, floating villus; STB, syncytiotrophoblast. Image modified from, "Pathogenesis of preeclampsia" (Karumanchi *et al*., 2010).

1.4 NUMB

i. NUMB structure and function

A protein that may be essential for proper human placental development is NUMB. The gene *NUMB* was first discovered in 1989 in *Drosophila melanogaster* (Uemura *et al.*, 1989). *Drosophila* NUMB (dNUMB) was described as a protein thought to be associated with cell fate determination in the sensory organ precursor (SOP) lineage. Additional studies revealed that dNUMB is a membrane-associated protein that is asymmetrically distributed in the development of the peripheral nervous system and acts as an antagonist to the NOTCH signaling pathway (Rhyu *et al.*, 1994; Spana and Doe., 1996; Guo *et al.*, 1996).

Mammalian homologues of dNUMB were discovered in rats (Verdi *et al.*, 1996), mice (Zhong *et al.*, 1996) and humans (Juven-Gershon *et al.*, 1998). Four isoforms of mammalian NUMB were first observed in rats (Verdi *et al*., 1996) and were later found in mice (Dho *et al.*, 1999), humans (Verdi *et al.*, 1999) and zebrafish (Reugels *et al*., 2006). These isoforms differ in the alternative splicing of the primary NUMB transcript and vary in their sizes of two specific regions: the phosphotyrosine-binding (PTB) domain and the proline-rich region (PRR). In humans, NUMB isoform 1 is the full-length protein, whereas NUMB isoforms 2 and 4 lack a 48-amino acid insertion in the PRR and NUMB isoforms 3 and 4 lack an 11-amino acid insertion in the PTB domain. It had been discovered that isoforms with the PRR insert (1 and 3) were involved in proliferation while those lacking it (2 and 4) were involved in differentiation. This was shown in P19 mouse embryonic carcinoma cells (Verdi *et al*., 1999), rat retinal tissue (Dooley *et al*., 2003) and mouse cortical tissue (Bani-Yaghoub *et al.* 2007). NUMB isoforms 5 and 6 were identified in human amniotic fluid cells, glioblastoma and metastatic tumor cells and were found to have a function in migratory processes through the formation of lamellipodia and filopodia (Karaczyn *et al.*, 2010).

The amino acid (aa) sequence of mammalian NUMB isoform 1 (NCBI, NP_001005743), which represents the coding region in its entirety, contains several domains and motifs that allow for its interactions with many different proteins. The PTB domain in the N-terminus, which has an alternatively spliced insert at positions 68-78aa, can bind to the NPXY motif (Chien *et al.*, 1998; Dho *et al.*, 1998; Calderwood *et al.*, 2003) and the FTNAAFD motif (Schlüter *et al.*, 2009). The PTB domain also contains eight lysine residues which are thought to interact with acidic membrane phospholipids (Dho *et al.*, 1999). The PTB insert contains three of these lysine residues and may be responsible for regulating subcellular localization in Madin-Darby canine kidney cells since NUMB isoforms 1 and 2 (contain PTB insert) associated with the cortical membrane whereas NUMB isoforms 3 and 4 (do not contain PTB insert) were located in the cytosol. The C-terminal PRR, which has an insert located at positions 366-413aa, contains several PXXP regions. The total NUMB sequence contains eight PXXP regions with the majority residing in the PRR. PXXP is the minimal consensus motif for binding to SRC homology 3 (SH3) domains found in some tyrosine kinases (Verdi *et al.*, 1996). In the C-terminus, there are two occurrences of DPF (aspartate-proline-phenylalanine) at positions 343-345aa and 614-616aa and one occurrence of NPF (asparagine-proline-phenylalanine) at positions 637-639aa. Both of these regions can bind to components of clathrin-dependent endocytic machinery. DPF associates with the α-adaptin subunit of the AP2 adaptor complex (Santolini *et al.*, 2000) and NPF associates with EGFR pathway substrate clone 15 (Eps15) homology (EH) domains (Salcini *et al*., 1997). Specific serines at positions 7aa, 276aa and 295aa are phosphorylated respectively by atypical protein kinase C (aPKC), Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI) and both aPKC and CaMKI (Tokumitsu *et al*., 2006). The phosphorylation of both of the latter two serines allows for the recruitment of 14-3-3 proteins.

The functional properties of the mammalian NUMB protein have been studied in association with several developmental processes and pathologies. Mammalian NUMB has primarily been studied in nervous system development (Yan, 2010) and has been specifically characterized in rat retinal development (Dooley *et al.*, 2003; Cayouette *et al.*, 2001), mouse pancreatic development (Yoshida *et al.*, 2003), mouse hematopoiesis (Cheng *et al.*, 2008), mouse spermatogenesis (Grisanti *et al.*, 2009), Alzheimer's disease (Chan *et al.*, 2002; Roncarati *et al.*, 2002; Kyriazis *et al.*, 2008) and intestinal epithelium development (Yang *et al*., 2011). In breast cancer, NUMB has been described as a tumour suppressor that forms a tricomplex with p53 and MDM2, a ubiquitin ligase that acts upon p53 (Pece *et al*., 2004; Colaluca *et al*., 2008). NUMB prevents the ubiquitination and subsequent degradation of the p53 tumour suppressor. Since it also inhibits the activation of the NOTCH oncogene pathway, loss of its expression would result in an aggressive tumour phenotype (Colaluca *et al*., 2008).

NUMB has also been described as an adaptor protein involved in various clathrin-dependent endocytic pathways, including the internalization of: (i) transferrin receptor (TfR) and epidermal growth factor receptor (EGFR) in A172 glioblastoma cells (Santolini *et al*., 2000), (ii) integrin-β1 in migrating HeLa cells (Nishimura and Kaibuchi, 2007), (iii) amyloid precursor protein (APP) in PC12 neuronal cells (Kyriazis *et al*., 2008), (iv) AAEC185/pSH2 type 1-piliated bacterial pathogen in 5637 bladder epithelial cells (Eto *et al.*, 2008), (v) P-selectin cell adhesion molecule in HEK293 embryonic kidney cells (Schlüter *et al.*, 2009) and (vi) NOTCH1 in C2C12 myoblast and HEK293T embryonic kidney cells (McGill *et al.*, 2009). During clathrin-dependent endocytosis, NUMB binds to and associates with other adaptor proteins including EH domain proteins and the α-adaptin subunit of the AP2 adaptor complex, through interactions with the NPF and DPF motifs in NUMB's COOH terminus (Salcini *et al.*, 1997; Santolini *et al.*, 2000). NUMB has also been shown to be involved in the clathrin-independent internalization of IL-2α receptor in CHO cells through an association with ADP-ribosylation factor 6 (ARF6) (Smith *et al.*, 2004).

ii. NUMB and placental development

The first study that considered the NUMB protein in the context of placental development established that NUMB-null (NUMB-/-) embryos exhibited defects in angiogenic remodeling, neural development and placental formation (Zilian *et al.*, 2001). These mice died by embryonic day 11.5 and it was thought that placental malformation contributed to their demise. In a second study, an *in silico* expression analysis revealed that NUMB mRNA is expressed in the human placenta (Katoh and Katoh, 2006).

Our laboratory reported that nine isoforms of NUMB were expressed in HTR-8/SVneo, a first trimester human EVT cell line (Haider *et al.*, 2011). These isoforms consisted of the six previously characterized isoforms (NUMB1-6) and three novel isoforms (NUMB7-9). A schematic representation of the alternative splicing that produces these different isoforms is shown in Figure 3. The presence of NUMB isoforms 1 to 9 in freshly isolated placental tissues was also confirmed. NUMB isoforms 1 to 4 were more abundantly expressed in HTR-8/SVneo cells, whereas NUMB isoform 8 was the predominant isoform in healthy, heterogeneous placental tissues from first, second and third trimesters. Overexpression studies in HTR-8/SVneo cells revealed that NUMB isoform 1 likely has a role in cell migration whereas NUMB isoforms 2 and 4 are potentially involved in apoptosis.

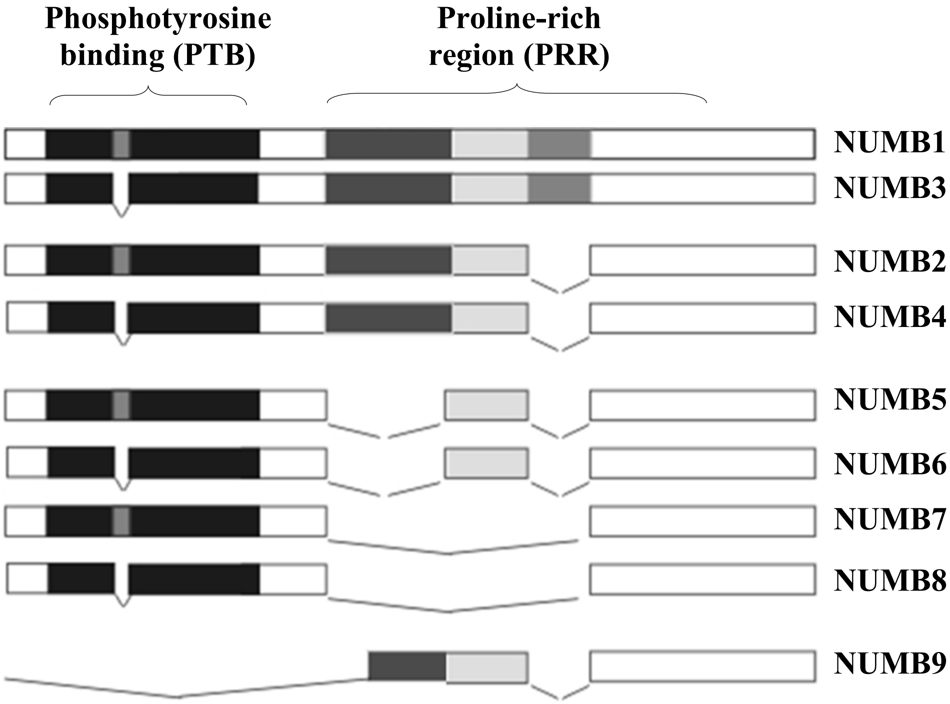


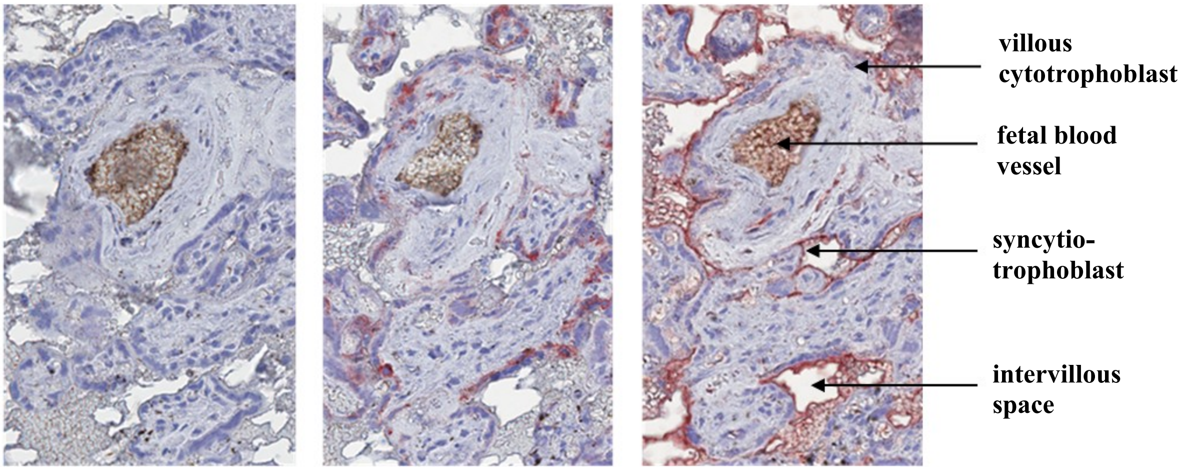
Figure 3. Schematic representation of NUMB isoforms 1 to 9. Insertions and deletions in the PTB domain and PRR vary in the different isoforms. Image was derived from Figure 1 in the article, "Characterization and role of NUMB in the human extravillous trophopblast" (Haider *et al*., 2011).

1.5 Rationale for investigation

The roles of NUMB isoforms in EVT cells have been investigated but their functions in other placental cells have not yet been identified. Preliminary data obtained by our lab revealed that NUMB is expressed in the syncytiotrophoblast layer of term chorionic villi, as shown in Figure 4. The involvement of NUMB in differentiation and endocytosis in non-placental tissues suggests that it may also be important for syncytiotrophoblast development and transport processes.

i. NUMB and villous cytotrophoblast differentiation

In non-placental cells, NUMB isoforms with the PRR insert (i.e., NUMB1 and NUMB3) have been found to be involved in proliferation while those lacking it (i.e., NUMB isoforms 2 and 4) are involved in differentiation (as described in section 1.4). These roles of NUMB isoforms in non-placental tissues suggest that they may be involved in the proliferation of villous cytotrophoblast cells and their differentiation into syncytiotrophoblast. One potential mechanism may be NUMB's regulation of the internalization of epithelial-cadherin (E-cadherin), a membrane-associated cell adhesion protein whose surface levels decrease upon the differentiation and fusion of villous cytotrophoblast into syncytiotrophoblast (Coutifaris *et al*., 1991). In MCF7 human breast adenocarcinoma cells, NUMB was found to promote E-cadherin endocytosis through association with p120 catenin (Sato *et al*., 2011), a protein that is also expressed in syncytiotrophoblast (Getsios *et al*., 2001).



(a) Control **(b) Cytokeratin 18** **(c) NUMB**

Figure 4. NUMB protein expression in human placental villi at term. Immunohistochemistry analysis of NUMB expression in term placental villi cross-section. Primary antibody - (a) none, (b) anti-cytokeratin 18 (cytotrophoblast marker, Abcam, Cambridge, MA), (c) anti-NUMB (Upstate Biotechnology, Lake Placid, NY). Secondary antibodies were HRP-conjugated and were visualized with AEC chromagen. Counterstained with Harris' hematoxylin. (Acknowledgement: Daniel Tessier, PhD candidate, Department of Biology, University of Ottawa)

ii. NUMB and EGFR endocytosis in syncytiotrophoblast

Endocytic processes in the syncytiotrophoblast are important for the transplacental transport of nutrients such as iron, lipoproteins and IgG as well as growth factors, hormones and cytokines (Fuchs and Ellinger, 2004). Receptor-mediated endocytosis is the most common mode for transport and involves the binding of a ligand to a receptor situated in the plasma membrane prior to its internalization. One of the most comprehensively studied examples of receptor-mediated endocytosis is the process by which epidermal growth factor (EGF) binds to and induces the internalization of its receptor (EGFR). EGFR can either be recycled back to the plasma membrane or degraded in lysosomes (Sorkin *et al*., 1991). The association between EGFR and lysosomes was observed in perinuclear punctuate structures in KB epithelial cells following 30 minute stimulation with 100 nM EGF (Bequinot *et al*., 1984). Lysosomal-associated membrane protein 2 (LAMP-2) is a marker for late endosomes and lysosomes (Akasaki *et al*., 1996). EGFR colocalized with LAMP-2 in serum-starved A549 lung carcinoma cells following 30 minute stimulation with 100 ng/ml EGF (Gao *et al*., 2010). In cultured syncytiotrophoblast isolated from term placentae, internalized EGF associated with endosomes and multivesicular bodies, although no degradation of the ligand or association with lysosomes was observed and no information about the receptor was available (Lai *et al*., 1986).

EGFR signaling is involved in many placental functions including cytotrophoblast differentiation (Morrish *et al*., 1987), early cytotrophoblast proliferation (Maruo *et al*., 1997), apoptosis inhibition (Johnstone *et al*., 2005; Johnstone *et al*., 2007) and hCG and hPL secretion (Morrish *et al*., 1987; Maruo *et al*., 1992). Altered EGFR activity and expression in the placenta has been found to be associated with pre-eclampsia and IUGR (Fondacci *et al.*, 1994; Faxén *et al.* 1998). EGFR is expressed in placental villi with the most intense immunoreactivity in the syncytiotrophoblast (Watanabe *et al.*, 1997). EGF is secreted by the maternal decidua and has also been found to be present in first-trimester syncytiotrophoblast, suggesting that it may act in an autocrine manner in the early placenta (Maruo *et al.*, 1997). In the second- and third-trimester placenta, EGF is expressed in cytotrophoblast cells (Ladines-Llave *et al.*, 1991).

NUMB is an adaptor protein in clathrin-dependent EGFR endocytosis in non-placental tissues. Upon internalization of EGFR in A172 human glioblastoma cells after 1 hour of incubation with 100 ng/mL EGF at 4°C, NUMB colocalized with EGFR at coated pits, coated vesicles and endosomes (Santolini *et al.*, 2000). EGFR contains a target sequence for PTB so it is possible that it binds directly to NUMB's PTB domain, although there is some data suggesting that this interaction does not occur (Verdi *et al.*, 1996; Dho *et al.*, 1998). NUMB is not directly involved in EGFR ubiquitination leading to its degradation, as was previously discovered about its interaction with NOTCH during its internalization (McGill and McGlade, 2003). However, NUMB overexpression in mouse sensory neurons has been found to induce the formation of late endosomes and lysosomes (Huang *et al.*, 2005). EGFR overexpression in the subventricular zone of mice is associated with NUMB upregulation by about 45% and NOTCH downregulation by about 60% (Aguirre *et al*., 2010). Based on these data, NUMB may be involved in sorting EGFR to endocytic compartments and promoting its degradation through the development of late endosomes and lysosomes. It is likely that NUMB would have similar roles in EGFR endocytosis in the placenta.

iii. BeWo choriocarcinoma cells as an *in vitro* model for human villous trophoblast cells

Choriocarcinoma is an aggressive, malignant neoplasm that is entirely composed of cytotrophoblast and syncytiotrophoblast in solid sheets (Benirschke, 2006). Choriocarcinoma metastases usually occur in the lung and the brain. This type of cancer is quite rare and can occur in pregnancies with or without the presence of a growing fetus.

BeWo is a human choriocarcinoma cell line derived from a cerebral metastasis (following a normal male term birth) that had been transplanted and maintained in a hamster cheek pouch for several serial transfers before it was cultured *in vitro* (Patillo and Gey, 1968). BeWo cells are frequently used as a model for villous cytotrophoblast cells since they share similar morphological and biological characteristics. They express a majority of the intracellular proteins similarly expressed by freshly isolated cytotrophoblast cells (Meyer zu Schwabedissen *et al.*, 2006). In addition, BeWo cells are capable of being artificially induced to undergo syncytialization analogous to the cyclic AMP (cAMP)-dependent differentiation and cellular fusion events observed in villous cytotrophoblast cells (Wice *et al.*, 1990, Kao *et al.*, 1992). One of the most commonly used reagents for inducing BeWo differentiation and syncytialization is forskolin, a plant-derived diterpene (Wice *et al.*, 1990). Forskolin activates adenylate cyclase which leads to the upregulation of specific proteins in BeWo cells associated with differentiation, fusion and syncytiotrophoblast function such as placental hormones (Hussa *et al.*, 1974), syncytins (Mi *et al.*, 2000) and a variety of transcription factors, adhesive molecules and amino acid transporters (Kudo *et al.*, 2004).

BeWo differentiation and syncytialization can be verified at the molecular level through analysis of the relative expression of proteins that are upregulated or downregulated. The secretion of hCG, a glycoprotein hormone, by BeWo cells is increased during differentiation to syncytiotrophoblast (Hussa *et al.*, 1974). hCG consists of two noncovalently bonded subunits (α and β) in a dimer. The α- and β-hCG subunits synthesized and secreted by BeWo cells have different molecular weights than first trimester human trophoblast hCG subunits (Takeuchi *et al.*, 1990). In cell lysates, the α- and β-hCG subunits are, respectively: (i) 19 kDa and (ii) 24 kDa in BeWo cells and (i) 21 kDa and (ii) 19 and 23 kDa in first-trimester trophoblast cells**.** In the surrounding medium, the secreted α- and β-hCG subunits are 23 and 33 kDa in BeWo cells and 21 and 31 kDa in first-trimester trophoblast cells. It is thought that hCG in BeWo cells is still functional since the molecular weight differences are attributed to slight differences in apoprotein structures. Another marker is E-cadherin, a 120 kDa protein which is decreased upon BeWo syncytialization following cAMP stimulation (Coutifaris *et al.*, 1991).

Undifferentiated BeWo cells can be used to study EGFR endocytosis as it would occur in the human syncytiotrophoblast. Both types of cells express machinery involved in clathrin-dependent endocytosis (Ockleford and Whyte, 1977; Huang *et al.*, 2003) which is the primary mechanism of EGFR internalization (Sorkina *et al.*, 1999). EGFR is expressed in BeWo choriocarcinoma cells, at levels 10-fold higher than in benign villous cytotrophoblast cells (Filla and Kaul, 1997). Similarly, EGFR is highly expressed in syncytiotrophoblast (Watanabe *et al.*, 1997), with peak cellular expression in the first trimester after 6 weeks gestation (Maruo *et al.*, 1997). LAMP-2, a late endosome and lysosome marker, is expressed in both BeWo cells (Leitner *et al.*, 2006) and syncytiotrophoblast (Leitner *et al.*, 2002). Also, similar results occur upon the addition of

EGF to the medium of BeWo cells and placental villi. A significant increase in hCG secretion can be observed following the addition of 100 ng/ml EGF to BeWo cells (Futamura *et al*., 1989) and first-trimester placental villi (Maruo *et al*., 1997). EGF also stimulates the ability of BeWo cells to invade through Matrigel^{TM} due to the upregulation of α2 integrin (Nakatsuji *et al*., 2003).

iv. Summary

This research project will investigate the specific roles of NUMB isoforms that are expressed in syncytiotrophoblast. Primary focus will be placed on differentiation and EGFR endocytosis in the syncytiotrophoblast, two processes in non-placental tissues in which NUMB has been confirmed to have important roles. The involvement of NUMB in EGFR endocytosis will be investigated since it is a characteristic model for clathrin-dependent endocytosis and EGFR signaling is essential for placental development. It has been established that a properly functioning syncytiotrophoblast is important for the progression of a healthy pregnancy. Elucidating the role of NUMB in the syncytiotrophoblast will contribute to a more complete understanding of NUMB's contribution to overall placental development and help ascertain if this protein should be investigated in the context of pre-eclampsia and IUGR. The use of BeWo cells as an *in vitro* model is sufficient to facilitate the analysis of NUMB in syncytiotrophoblast.

1.6 Hypothesis, Overall Objective and Specific Objectives

Hypothesis: NUMB is upregulated during human villous cytotrophoblast differentiation and is important for EGFR endocytosis in syncytiotrophoblast.

Overall Objective: To determine if NUMB isoforms are involved in cellular differentiation and EGFR endocytosis in BeWo cells.

Objective 1: To identify changes in the endogenous expression of NUMB isoforms in differentiated BeWo cells.

Objective 2: To examine the role of NUMB isoforms in BeWo cell differentiation.

Objective 3: To examine the role of NUMB isoforms in EGFR internalization in BeWo cells.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell culture

BeWo and HTR-8/SVneo cells were cultured in complete RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 25,000 units of penicillin and 25 mg of streptomycin (1% pen/strep). Cells were routinely maintained at 37°C with 5% CO_2.

2.2 Immunoblotting (Western blot)

After cell treatments, the medium was removed and cells were washed with 4°C phospho-buffered saline (PBS) three times quickly. Cells were resuspended in lysis buffer (containing 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 phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin and 1 mM sodium orthovanadate) and incubated on ice for 30 minutes to 1 hour. Afterwards, lysates were spun down at 10,000 rcf for 10 minutes at 4°C to pellet down and discard the cellular debris. The *DC*™ (detergent compatible) colorimetric protein assay (Bio-Rad, Mississauga, ON) was used to determine the protein concentrations of the supernatants.

Protein aliquots were resolved using tricine gels (10% separating, 5% stacking) via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membranes. The membranes were blocked for 1 hour at room temperature (RT) in blotto (5% dehydrated non-fat milk in Tris-buffered saline pH 7.6 and 0.05% Tween 20 (TBST) with 0.01% sodium azide). Membranes were washed with sterile H_2O and incubated with primary antibodies, as indicated, overnight at 4°C with shaking. Please refer to Table 2 for a complete list of the antibodies used. The next day, membranes were washed with TBST (3 times, 10 minutes each), incubated with secondary antibody conjugated with horseradish peroxidase for 1 hour at RT with shaking, and then washed again with TBST (5 times, 15 minutes each). Immunosignals were visualized by enhanced chemiluminescence (ECL) using an Amersham ECL^{TM} Advance detection kit (GE Healthcare Bio-Sciences Inc., Baie d'Urfe, QC). For quantification, protein content was determined densitometrically and normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) on the same membrane.

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

	Antigen (Product #)	Species Raised Against	Species	Monoclonal or Polyclonal	Working Dilution	Manufacturer	Application
(a)	β1 Integrin (553715)	Human	Mouse	Polyclonal	1:1000	BD Pharmingen Inc. San Diego, CA	WB
(b)	E-cadherin (4065)	Human	Rabbit	Polyclonal	1:1000	Cell Signaling Technology, Danvers, MA	WB
(c)	EGFR (sc-101)	Human	Mouse	Monoclonal	1:50	Santa Cruz Biotech., Santa Cruz, CA	ICC
(d)	EGFR (4267)	Human	Rabbit	Monoclonal	1:50	Cell Signaling Technology, Danvers, MA	ICC
(e)	EGFR (06-129)	Human	Sheep	Polyclonal	1:2000	Upstate Biotech. Inc., Lake Placid, NY	WB
(f)	GAPDH (ab8245)	Human	Mouse	Polyclonal	1:20000	Abcam Inc., Cambridge, MA	WB
(g)	hCG (ab54410)	Human	Rabbit	Polyclonal	1:1000	Abcam Inc., Cambridge, MA	WB
(h)	IgG (+Alexa Fluor 488) (A-21441)	Rabbit	Chicken	Polyclonal	1:50	Molecular Probes Inc. Eugene, OR	ICC
(i)	IgG (+HRP) sc-2768	Goat	Rabbit	Polyclonal	1:5000	Santa Cruz Biotech., Santa Cruz, CA	WB
(j)	IgG (+HRP) (170-6516)	Mouse	Goat	Polyclonal	1:5000	Bio-Rad Mississauga, ON	WB
(k)	IgG (+HRP) (170-6515)	Rabbit	Goat	Polyclonal	1:5000	Bio-Rad Mississauga, ON	WB
(l)	IgG (+Rho-damine) (sc-2092)	Mouse	Goat	Polyclonal	1:25	Santa Cruz Biotech., Santa Cruz, CA	ICC
(m)	LAMP-2 (sc-18822)	Human	Mouse	Monoclonal	1:100	Santa Cruz Biotech., Santa Cruz, CA	ICC

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

	Antigen (Product #)	Species raised against	Species	Monoclonal or Polyclonal	Working Dilution	Manufacturer	Application
(n)	NUMB(1) (ab4147)	Human	Goat	Polyclonal	1:5000	Abcam Inc., Cambridge, MA	WB
(o)	NUMB(2) (07-147)	Human	Rabbit	Polyclonal	1:50	Upstate Biotech. Inc., Lake Placid, NY	ICC
(p)	pAkt (9271)	Human	Rabbit	Polyclonal	1:1000	Cell Signaling Technology, Danvers, MA	WB
(q)	pERK 1/2 (9101)	Human	Rabbit	Polyclonal	1:1000	Cell Signaling Technology, Danvers, MA	WB
(r)	Pk (V5) (SM1691PS)	Human	Mouse	Monoclonal	1:100	Acris Antibodies Inc., San Diego, CA	ICC

(1) Antibody corresponds to a C-terminal region (positions 638-651aa in NUMB1) found in all nine NUMB isoforms.
(2) Antibody corresponds to a C-terminal region (positions 549-568aa in NUMB1) found in all nine NUMB isoforms.

2.3 Forskolin-induced differentiation BeWo cells and assessment of NUMB isoform expression

BeWo cells were seeded in 12-well plates overnight in RPMI 1640 medium containing 10% FBS and 1% pen/strep and grown to 50% confluency. The next day, the medium was removed and replaced with 1 mL of antibiotic-free RPMI 1640 medium containing 1% FBS and forskolin at various concentrations. These mixtures were prepared by adding 1 μL forskolin in DMSO (final concentrations: 5 μM, 10 μM and 20 μM) to 50 μL of media, followed by the addition of 950 μL of media (with mixing using a vortex mixer before and after). A negative control was prepared by adding 1 μL DMSO to the media instead of forskolin. HTR-8/SVneo cell lysate was used as a positive control to identify NUMB isoforms since they have been thoroughly characterized in this cell line (Haider *et al*., 2011). The cells were incubated for 48 hours or 72 hours and then harvested for immunoblotting. Blots were probed with antibodies against NUMB, E-cadherin, hCG and GAPDH (loading control). Antibody details are presented in Table 2.

Immunoblotting with an increased resolution was also completed to help distinguish between the different isoforms of NUMB. This was achieved using an increased percentage of SDS-PAGE gel (12% separating, 5% stacking) and a doubled running time (n=2). The blots were probed with NUMB antibody. Antibody details are presented in Table 2.

2.4 BeWo transfection efficiency

BeWo cells were seeded into 6-well plates containing autoclaved glass coverslips in 10% FBS, 1% pen/strep RPMI 1640 medium and grown to 80% confluency. The cells were serum-starved overnight in antibiotic-free, serum-free RPMI 1640 medium. Cells were transfected using Lipofectamine and Plus Reagent (Invitrogen Corp., Burlington, ON) with a ratio of 3:2 respectively per 1 μg of DNA. Three different approaches were used to assess transfection efficiency in BeWo cells: (i) GFP fluorescence, (ii) V5 immunodetection, and (iii) *lacZ* staining.

(i) GFP fluorescence

Green fluorescent protein (GFP) is a protein that naturally exhibits fluorescence when exposed to ultraviolet light. The transfection efficiency of BeWo cells can be visually determined after transfection with plasmids containing the GFP gene.

BeWo cells were transfected with an AP2 retroviral eGFP vector (Karaczyn *et al.*, 2010). After 24 hours, the medium was removed and the cells were rinsed briefly with PBS. The cells were fixed in 4% (w/v) paraformaldehyde in PBS for 15 minutes at RT. Following two quick ice-cold PBS washes, cells were permeabilized with 0.5% (v/v) Triton X-100 in PBS for 10 minutes at RT. Cells were washed with PBS (3 times, 5 minutes each) and then treated with Hoechst 33258 dye (B2883, Sigma-Aldrich Corporation, St. Louis, MO) at a concentration of 1 μg/mL in PBS for 10 minutes to counterstain cell nuclei. After a final wash with PBS, (3 times, 5 minutes each), coverslips were mounted onto glass microscope slides using 50% glycerol in PBS, sealed using clear acrylic nail polish, cleaned with distilled water and stored in the dark at RT. Images were taken using the 20x objective lens of a Zeiss Axioplan 2 fluorescence microscope and AxioVision software (Carl Zeiss, Toronto, ON). Transfection efficiency (%) was calculated as 100 x (number of GFP-expressing cells/total number of cells). 12 random fields and a total of 728 cells were counted (n=2).

(ii) V5 immunodetection

V5 is an epitope tag derived from a small epitope (Pk) from paramyxovirus of simian virus 5 (SV5). Cells transfected with a V5 expression vector can be incubated with an anti-V5 antibody conjugated to horseradish peroxidase (HRP) and treated with 3-amino-9 ethylcarbazole (AEC). HRP cleaves AEC and produces a red, water-insoluble precipitate which allows for assessment of transfection efficiency.

BeWo cells were transfected with pEF6/V5 vectors containing cDNA for NUMB1 and NUMB3, which were prepared using methods previously used by our laboratory (Haider *et al*., 2011). The pEF6/V5-His/*lacZ* vector (Invitrogen Corp., Burlington, ON) was used as a control plasmid. After 24 hours, cells were quickly washed twice with ice-cold PBS and were fixed with 4% (w/v) paraformaldehyde in PBS for 15 minutes at RT. Cells were washed in PBS (3 times, 5 minutes each) and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 10 minutes at RT and then washed in PBS again (3 times, 5 minutes each). The cells were then incubated with serum-free Protein Block (X0909, Dako Canada Inc., Burlington, ON) for 30 minutes at RT to block background staining. Coverslips were then placed in a humidified chamber and incubated with anti-Pk(V5) antibody (see Table 2 for antibody details) on parafilm overnight at 4°C. Two negative controls were used to assess specificity of the antibody: (a) cells that were not transfected, but incubated with the Pk(V5) antibody (b) cells transfected with the control plasmid without Pk(V5) incubation.

Following incubation with primary antibody, cells were washed with PBS (3 times, 5 minutes each) and coverslips were incubated in a humidified chamber with anti-mouse IgG-HRP polymer secondary antibody (Dako Canada Inc., Burlington, ON) on parafilm for 90 minutes at RT, in the dark. Cells were washed with PBS (3 times, 5 minutes each) and treated with AEC chromagen (K0696, Dako USA Corporation, Carpinteria, CA). After a colour change was observed, cells were washed with PBS (3 times, 5 minutes each) and counterstained with Harris' hematoxylin (R03312, BDH, VWR International, Mississauga, ON). Coverslips were mounted onto glass microscope slides using 50% glycerol in PBS, sealed using clear acrylic nail polish, cleaned with distilled water and stored in the dark at RT. Images were obtained with bright field microscopy using the 40x objective lens of an Olympus BX50 compound light microscope (Olympus Canada Inc., Richmond Hill, ON), connected to a Nikon Coolpix 990 camera (Nikon Canada Inc., Mississauga, ON). Transfection efficiency (%) was calculated as 100 x (number of V5-expressing cells/total number of cells). 10 to 16 fields and 400 to 500 cells were counted per treatment group (n=2).

(iii) lacZ staining

X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) is cleaved by β-galactosidase, the product of the *lacZ* gene, via hydrolysis. This reaction yields two products and one of them is an intensely blue precipitate (5,5'-dibromo-4,4'-dichloro-indigo). Visual detection of this precipitate will allow for assessment of transfection efficiency if cells are transfected with an expression vector containing the *lacZ* gene and subsequently stained with an X-gal solution.

BeWo cells were transfected with the pEF6/V5-His/*lacZ* vector (Invitrogen Corp., Burlington, ON). After 24 hours, cells were quickly washed twice with ice-cold PBS, fixed in 0.5% glutaraldehyde solution in PBS for 10 minutes on ice and then washed again with PBS (3 times, 5 minutes each). X-gal staining solution (2 mM $MgCl_2$, 5 mM potassium ferrocyanide trihydrate, 5 mM potassium ferricyanide and 1 mg/mL X-gal) was added and the cells were incubated overnight at 37°C. The next day, the cells were washed with PBS (3 times, 5 minutes each). Coverslips were mounted onto glass microscope slides using 50% glycerol in PBS, sealed using clear acrylic nail polish, cleaned with distilled water and stored in the dark at RT. Phase contrast images were obtained using the 20x objective of an Eclipse TE2000-U microscope (Nikon Canada Inc., Mississauga, ON). Transfection efficiency was assessed by visual inspection and not quantified due to poor separation between cells. 16 random fields were analyzed (n=1)

2.5 Overexpression of NUMB isoforms

BeWo cells were seeded in 12-well plates and grown to 80% confluency. Transfection was completed using 1 μg/mL pEF6 mammalian expression vectors containing cDNA for NUMB isoforms 1 and 3, as previously prepared by our lab (Haider *et al*., 2011). The pEF6/V5-His/*lacZ* vector was used as a control plasmid. Cells were transfected using Lipofectamine and Plus Reagent (Invitrogen Corporation, Burlington, ON) with a ratio of 3:2 respectively per 1 μg of DNA. The cells were incubated with serum-free, antibiotic-free RPMI 1640 medium containing the transfection reagents for four hours. Afterwards, the reagent-containing medium was removed and replaced with fresh antibiotic-free RPMI 1640 medium containing 1% FBS. HTR-8/SVneo cell lysate was used as a control to identify NUMB isoforms. Cell lysates were analyzed using immunoblotting methods and blots were probed with antibodies against NUMB and GAPDH (loading control) to verify overexpression. Antibody details are presented in Table 2.

2.6 Cell morphology

BeWo cells were seeded in 12-well plates and transfected with control, NUMB1 and NUMB3 overexpression vectors for 24 hours (as described in Section 2.5). Phase contrast images were obtained using the 10x and 40x objective of an Eclipse TE2000-U microscope (Nikon Canada Inc., Mississauga, ON). After images were taken, the cells were collected for analysis using immunoblotting to verify NUMB overexpression. The blots were probed with antibodies against NUMB and GAPDH (loading control). Antibody details are presented in Table 2. Morphological assessment of at least 10 random fields per group involved qualitative descriptions of (i) cell appearance and (ii) ability to form cell clusters.

2.7 Cell viability

The Vi-CELL cell viability analyzer (Beckman Coulter, Mississauga, ON) was used to assess the viability of BeWo cells. This instrument utilized an automated trypan blue dye exclusion process to determine cell concentration and viability. The increased permeability of cellular membranes upon death allowed for the entry of trypan blue dye, thus indicating which cells are non-viable, with a counting accuracy of ± 6%

BeWo cells were seeded in 6-well plates and grown to 80% confluency. Transfection of the cells with control, NUMB1 and NUMB3 vectors was completed (as described in Section 2.5). After 24-hour transfection, the medium was added to 15 mL Eppendorf tubes. Cells were washed with 1 mL PBS which was then removed and added to the respective tubes. To lift and collect the adherent cells, 0.5 mL trypsin was added and the cells were incubated at 37°C for about 3 minutes. The trypsin was neutralized with 0.5 mL complete media and the combined trypsin-medium mixture was used to gently collect cells. This mixture was added to the respective tubes as well as an additional 1 mL PBS that was used to collect any remaining cells. The tubes were capped and mixed by gentle inversion. A 0.5 mL aliquot from each tube was added to sample cups for testing with the Vi-CELL cell viability analyzer. Cell viability was reported as viable cells/mL (x 10^6). Means were calculated for each treatment group (n=3).

The remaining cell mixtures in each of the sample tubes were spun down at 900 rcf for 10 minutes at RT. The supernatant was removed by suction and the cell pellet was lysed for analysis using immunoblotting to verify NUMB overexpression. The blots were probed with antibodies against NUMB and GAPDH (loading control). Antibody details are presented in Table 2.

2.8 Overexpression of NUMB to assess effects on BeWo differentiation

BeWo cells were seeded in 12-well plates and grown to 60% confluency. Transfection of the cells with control, NUMB1 and NUMB3 vectors was completed (as described in Section 2.5). The reagent-containing medium was removed 4 to 6 hours post-transfection and replaced with antibiotic free, 1% FBS RPMI 1640 medium. The next day, the medium was replaced with 1 mL of antibiotic-free RPMI 1640 medium containing 1% FBS and either (i) DMSO or (ii) 20 µM forskolin. These mixtures were prepared as described in Section 2.3. The cells were incubated for 48 hours and then harvested for immunoblotting. HTR-8/SVneo cell lysate was used as a control to identify NUMB isoforms. Non-transfected BeWo cells treated with DMSO and 20 µM forskolin for 48 hours were negative and positive controls, respectively, for BeWo differentiation. Blots were probed with antibodies against NUMB, E-cadherin, hCG and GAPDH (loading control). Antibody details are presented in Table 2.

2.9 Overexpression of NUMB to assess effects on EGFR signaling

BeWo cells were seeded in 12-well plates and grown to 80% confluency. Transfection of the cells with control, NUMB1 and NUMB3 vectors was completed (as described in Section 2.5). The reagent-containing medium was removed 4 to 6 hours post-transfection and the cells were starved overnight in antibiotic-free, serum-free RPMI 1640 medium. Cell lysates were collected and analyzed using immunoblotting methods. The blots were probed with antibodies against NUMB, phospho extracellular signal-regulated kinases 1 and 2 (pERK 1/2), phospho Akt (pAkt) and GAPDH (loading control). Antibody details are presented in Table 2. pERK 1/2 and pAkt are downstream targets of EGFR signaling.

2.10 Streptavidin pull-down assay

The ability of EGF to induce internalization of EGFR was assessed in BeWo cells by analyzing membrane EGFR levels following EGF stimulation. EGFR in the cell membrane can be labeled with a sulfo-NHS-ester of biotin, which can bind to primary amines in membrane proteins without penetrating through the cell membrane. Since streptavidin binds to biotin with high affinity, membrane EGFR can then be isolated using agarose beads conjugated to streptavidin.

BeWo cells were seeded into 6-well plates overnight in RPMI 1640 medium containing 10% FBS and 1% pen/strep and grown to 60% confluency. Cells were starved overnight in antibiotic-free, serum-free RPMI 1640 medium. The next day, the cells were treated with antibiotic-free, serum-free RPMI 1640 medium containing 40 ng/mL EGF (Sigma-Aldrich Corporation, St. Louis, MO) at various time points (i.e., 0, 5, 10, 30, 60, 120, 240, 480 minutes). Cells were washed immediately in ice-cold PBS (3 times, 5 minutes each) and each well was incubated with 200 μL of 0.5 μg/μL EZ-Link Sulfo-NHS-biotin (Pierce Biotechnology Inc., Rockford, IL) in PBS for 1 hour at 4°C with gentle shaking. Cells were washed with PBS (3 times, 5 minutes each) and then incubated with 250 μL lysis buffer for 1 hour on ice. 30 μL of streptavidin agarose beads (Pierce Biotechnology Inc., Rockford, IL) were then added to the lysates and they were incubated overnight at 4°C with gentle inversion. The negative control for the pull-down assay was streptavidin agarose beads incubated with lysates from untreated BeWo cells (i.e., no EGF or biotin added). The next day, the supernatant was removed and the beads were washed with ice-cold TBST, two times quickly and then for 30 minutes at 4°C with gentle inversion. The TBST was removed and 50 μL of 1X Laemmli buffer was added to the beads. The mixtures were boiled for 10 minutes and then analyzed using immunoblotting methods. The blots were probed with antibodies against EGFR and β1-integrin (loading control). Antibody details are presented in Table 2. Total levels of EGFR were represented by the input lysates and membrane EGFR levels were represented by the group subjected to streptavidin pull-down.

2.11 Immunofluorescence (immunocytochemistry)

BeWo cells were seeded into 6-well plates containing autoclaved glass coverslips in 10% FBS, 1% pen/strep RPMI 1640 medium. The cells were serum-starved overnight in antibiotic-free, serum-free RPMI 1640 medium. Cells were incubated with EGF at 40ng/mL (Sigma-Aldrich Corporation, St. Louis, MO) for 30 minutes. Following EGF treatment, the medium was removed and cells were quickly washed twice with ice-cold PBS. Cells were fixed in 4% (w/v) paraformaldehyde in PBS for 15 minutes at RT, washed with PBS (3 times, 5 minutes each) and permeabilized using 0.5% (v/v) Triton X-

100 in PBS. Cells were washed in PBS (3 times, 5 minutes each), incubated with serum-free Protein Block (Dako Canada Inc., Burlington, ON) for 30 minutes at RT to block background staining and then washed again in PBS (3 times, 5 minutes each). Coverslips were incubated overnight with primary antibodies in a humidified chamber cell-side down on parafilm at 4°C. The antibodies used were for detecting NUMB, EGFR and LAMP-2 (see Table 2). The next day, the coverslips were washed in PBS (3 times, 5 minutes each) and incubated with secondary antibodies conjugated to the fluorescent dyes Alexa Fluor 488 and rhodamine (see Table 2) in a humidified chamber, cell-side down on parafilm for two hours at RT in the dark. Following a PBS wash (3 times, 5 minutes each), the coverslips were incubated with Hoechst 33258 dye (Sigma-Aldrich Corporation, St. Louis, MO) in PBS, at a concentration of 1 μg/mL, for 10 minutes to counterstain cell nuclei. Coverslips were mounted onto glass microscope slides using 50% glycerol in PBS, sealed using clear acrylic nail polish, cleaned with distilled water and stored in the dark at RT. Confocal fluorescence images were obtained using a Zeiss LSM 510 Meta microscope (Carl Zeiss, Toronto, ON). Immunofluorescence was detected at 488 nm and 543 nm using 40x and 63x oil immersion objectives. Image analysis and merging was completed using Zeiss Zen confocal software (Carl Zeiss, Toronto, ON). Three replicates were performed and approximately ten to fifteen fields were analyzed for each treatment. Colocalization was assessed by examining the extent of overlap between the red and green channels in the merge images. The most representative images were chosen to be presented.

2.12 Statistical analysis

GraphPad Prism (version 5.00, GraphPad Software Incorporated, San Diego, CA) was used to calculate means and to perform one-way and two-way analysis of variance (ANOVA) with Tukey or Bonferroni post-hoc tests, respectively, to determine if means were significantly different from one another. Error bars were presented as +/- standard error of the mean (SEM). Differences were inferred to be statistically significant at $p<0.05$.

CHAPTER 3: RESULTS

3.1 NUMB protein expression in undifferentiated and forskolin-treated BeWo cells

Western blot analysis of BeWo cell lysates revealed a prominent band corresponding to NUMB isoforms 1 and 3 (Figure 5). A second weaker band corresponding to NUMB isoforms 2 and 4 was also present. Single bands were observed for the pairs of isoforms since they only differ by 1 kDa each. The immunoblotting conditions (see Materials and Methods, section 2.2) did not allow for visible resolution between them. The other NUMB isoforms (i.e., isoforms 5 to 9) were not found to be significantly expressed.

Treatment of BeWo cells with forskolin (5, 10 and 20 μM) for 48 hours resulted in the upregulation of α- and β-hCG (which are both indications of BeWo differentiation) and the upregulation of NUMB1 and/or NUMB3 (Figure 6a). E-cadherin downregulation (which is an indication of BeWo fusion) was minimal and therefore quantification analysis was not performed. Extending the above forskolin treatment for 72 hours resulted in the upregulation of α- and β-hCG and NUMB1 and/or NUMB3 as well as the downregulation of E-cadherin. Quantification analysis revealed that the NUMB1 and/or NUMB3 were significantly upregulated ($p<0.05$) by approximately 50% for all forskolin treatments and E-cadherin was significantly downregulated ($p<0.05$) by approximately 50%, 60% and 75% for 5, 10 and 20 μM forskolin treatments respectively.

Immunoblotting conditions were modified to increase resolution between the different NUMB isoforms in BeWo cells treated with 5, 10 and 20 μM forskolin for 72 hours (Figure 7). Two bands which corresponded to NUMB1 and NUMB3 in the HTR-8/SVneo lysates were observed, which suggested that both isoforms were upregulated in forskolin-differentiated BeWo cells. However, a prominent band overlapping NUMB4 was observed for all treatments, although this was not observed when similar lysates were run under regular conditions (see Figure 6a,b). The identity of this band was unknown although it was thought to be an artifact.

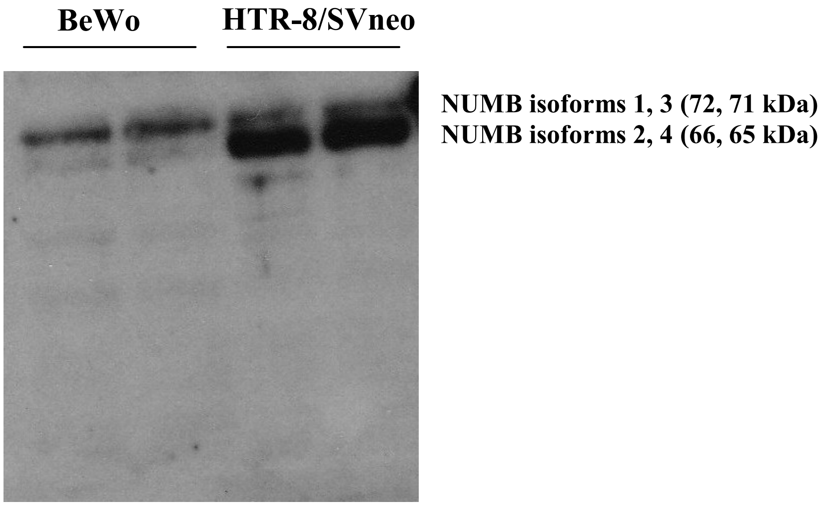


Figure 5. **NUMB protein expression in BeWo and HTR-8/SVneo cells.** Western blot analysis of cell lysates from BeWo cells (human choriocarcinoma cell line) and HTR-8/SVneo cells (human EVT cell line). HTR-8/SVneo.

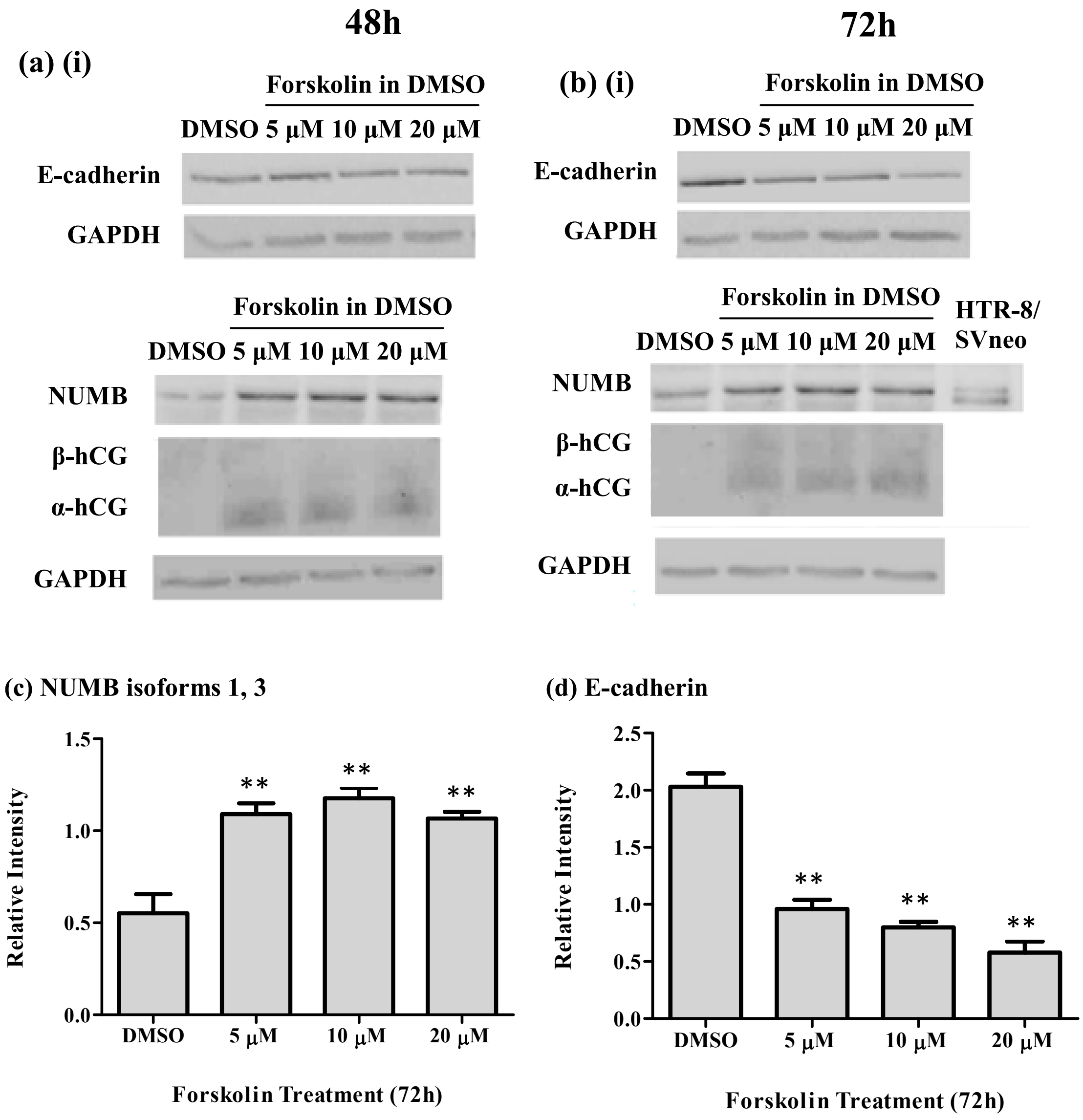


Figure 6. NUMB protein expression in forskolin-treated BeWo cells.
(a), (b) - BeWo cells at 50% confluency were treated with various concentrations of forskolin in antibiotic-free RPMI 1640 medium containing 1% FBS. Cells were incubated at 37°C and 5% CO_2 for (a) 48 hours or (b) 72 hours. Cell lysates were analyzed using Western blot methods. Loss of E-cadherin expression and an increase in hCG (α and β subunit) expression are markers for BeWo differentiation. HTR-8/SVneo cell lysate was used as a positive control to help identify NUMB isoforms. (c), (d) - Immunoblot quantification performed using ImageJ software (National Institutes of Health, Bethesda, MD) for (c) NUMB isoforms 1/3 and (d) E-cadherin. Relative intensity defined as: (a) NUMB/GAPDH, (b) E-cadherin/GAPDH. (n=3, One-way ANOVA, Tukey post-test, ** $p<0.01$).

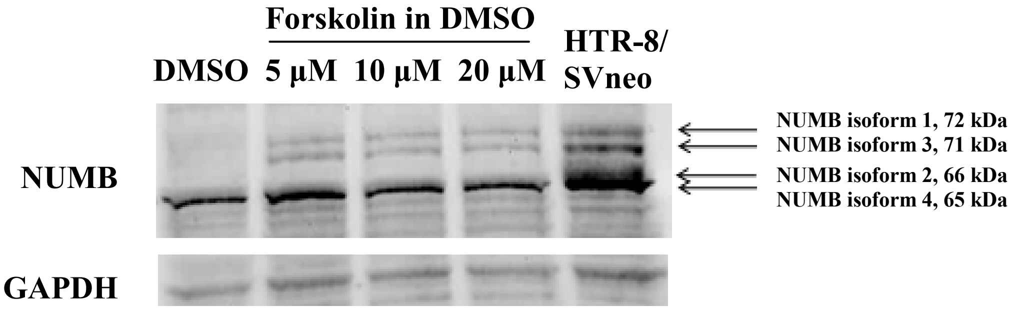
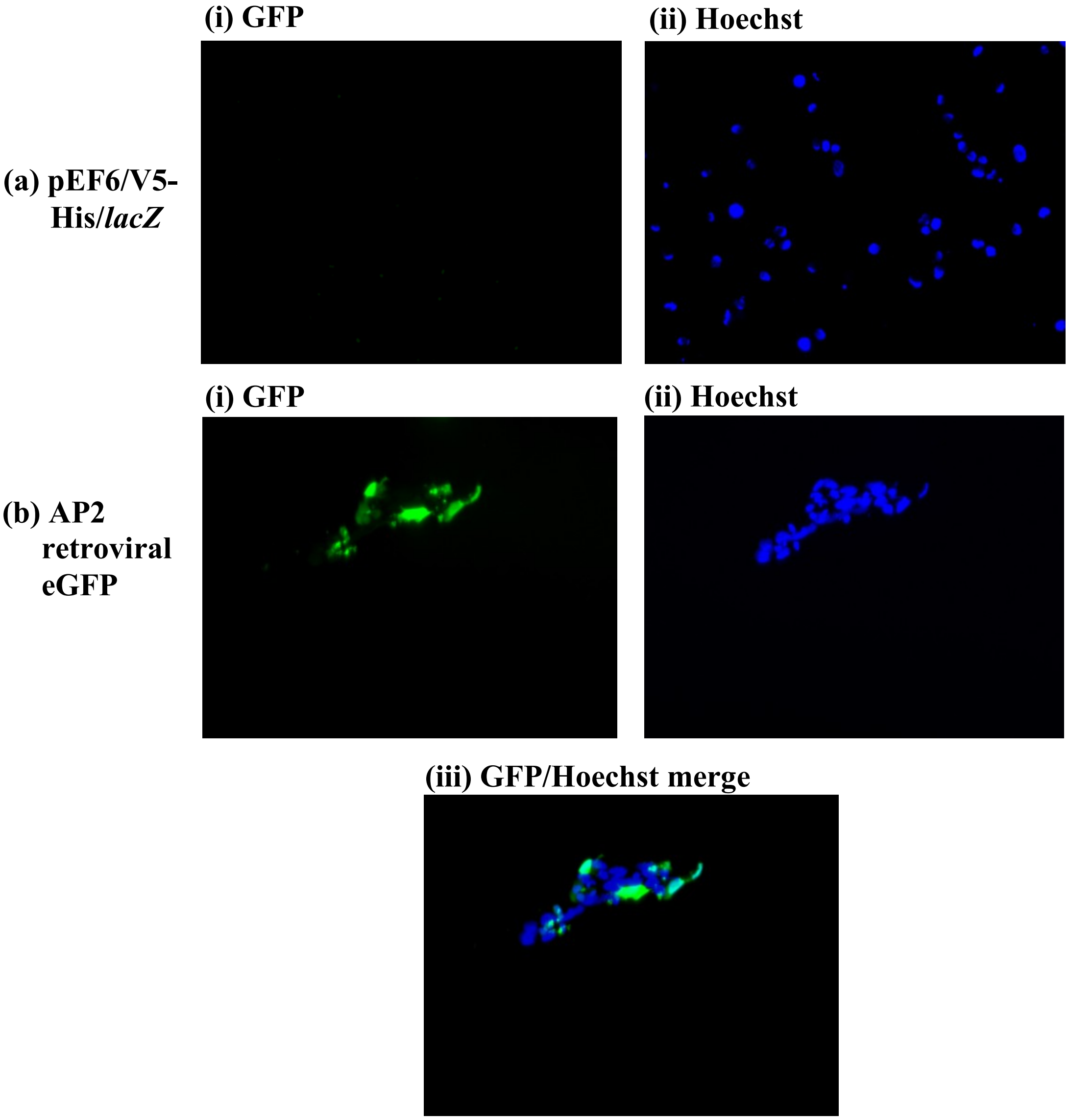


Figure 7. Resolution of NUMB isoforms in forskolin-treated BeWo cells. BeWo cells at 50% confluency were treated with various concentrations of forskolin in antibiotic-free RPMI 1640 medium containing 1% FBS. Cells were incubated at 37°C and 5% CO_2 for 72 hours. Cell lysates were analyzed using Western blot methods. A different percentage of tricine gel (12% separating, 5% stacking) and a doubled running time were used to facilitate resolution between the different NUMB isoforms. HTR-8/SVneo cell lysate was used as a positive control to help identify NUMB isoforms. The identity of the dark band overlapping the bands representing NUMB isoform 4 is unknown. (n=2)

3.2 BeWo cell transfection efficiency assessment

To determine if our laboratory's previously established cell transfection methods would be appropriate for transfecting BeWo cells, three methods for assessing transfection efficiency were used. In the first approach, BeWo cells were transfected with AP2 retroviral eGFP vector and transfection efficiency was determined by counting the number of cells exhibiting GFP fluorescence (Figure 8). Overexpression of BeWo cells with the eGFP vector was achieved at 47.5% transfection efficiency (12 random fields, 728 total cells, n=2). The second approach involved transfecting BeWo cells with pEF6/V5 expression vectors and visualizing transfected cells by means of an HRP-conjugated V5 antibody detected with AEC chromagen (Figure 9). The vectors contained NUMB cDNA and were the same as the vectors used in experiments described later on in this study (with the V5 epitope tag excluded). Overexpression was completed with the following transfection efficiencies: Control, 33.9%; NUMB1, 32.1%; and NUMB3, 34.6% (10 to 16 fields, 400 to 500 cells/group, n=2). The third approach utilized a chromagenic visualization method in which X-gal would be cleaved by β-galactosidase (*lacZ)* in transfected cells (Figure 10). Overexpression of BeWo cells with pEF6/V5-His/*lacZ* vector was achieved with a moderately high transfection efficiency of approximately 40-50% (16 random fields). The numbers of transfected cells could not be quantified due to poor resolution of cells.



(a) pEF6/V5-His/*lacZ*

(i) GFP

(ii) Hoechst

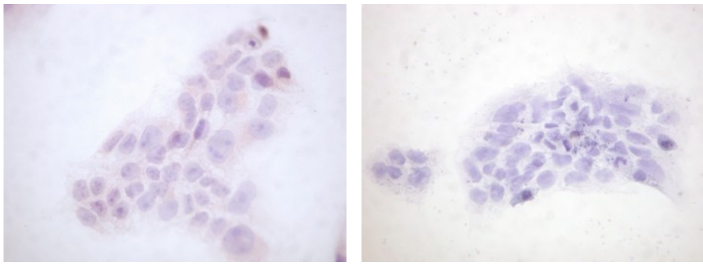
(b) AP2 retroviral eGFP

(i) GFP

(ii) Hoechst

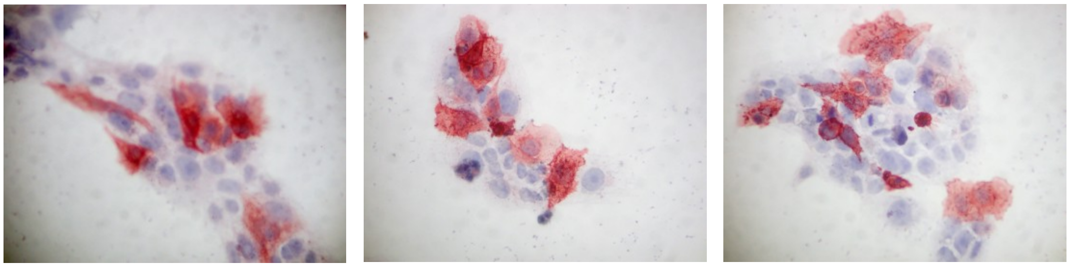
(iii) GFP/Hoechst merge

Figure 8. BeWo transfection efficiency assessment using GFP fluorescence. BeWo cells were seeded on coverslips in 6-well plates and grown to 80% confluency. Cells were transfected with (a) pEF6/V5-His/*lacZ* vector (negative control) and (b) AP2 retroviral eGFP vector for 24 hours. After fixation and permeabilization, cells were incubated with 1 μg/mL Hoechst dye for 10 minutes. Immunofluorescence images were obtained using the 20x objective lens of a Zeiss Axioplan 2 fluorescence microscope and AxioVision software (Carl Zeiss, Toronto, ON). (n=2)



(a) Cells
+ V5 antibody

(b) Control OE
- V5 antibody



(c) Control OE
+ V5 antibody

(d) NUMB1 OE
+ V5 antibody

(e) NUMB3 OE
+ V5 antibody

Figure 9. BeWo transfection efficiency assessment using V5 immunodetection. BeWo cells were seeded on coverslips in 6-well plates and grown to 80% confluency. Cells were transfected for 24 hours with (b, c) pEF6/V5-His/*lacZ* vectors (control plasmid) and (d, e) pEF6-V5 vectors containing cDNA for NUMB1 and NUMB3. After fixation and permeabilization, cells were incubated with V5 antibody overnight (a, c-e). The next day, coverslips were incubated with anti-mouse IgG-HRP polymer, followed by detection with AEC chromagen and counterstaining with Harris' hematoxylin. Images were obtained using bright field light microscopy with 40x objective lens of an Olympus BX50 compound light microscope (Olympus Canada Inc., Richmond Hill, ON), connected to a Nikon Coolpix 990 camera (Nikon Canada Inc., Mississauga, ON). (n=2)

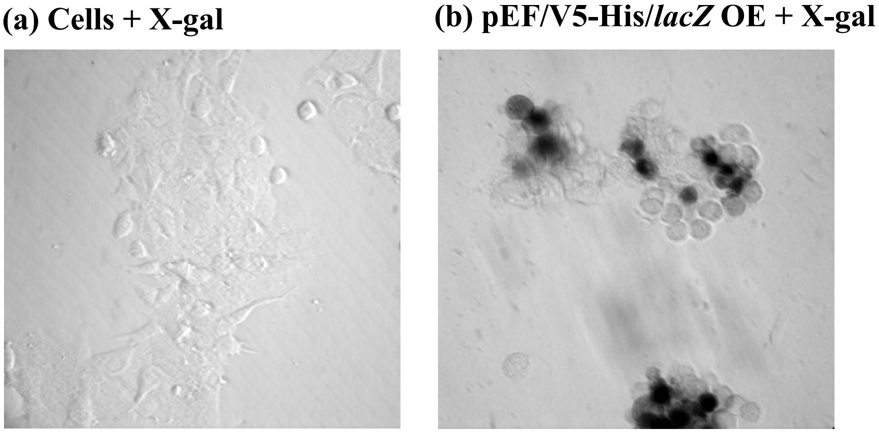


Figure 10. BeWo transfection efficiency assessment using *lacZ* staining. BeWo cells were seeded on coverslips in 6-well plates and grown to 80% confluency. Cells were transfected for 24 hours with (a) no vector (negative control) and (b) pEF6/V5-His/*lacZ* vector. After fixation, coverslips were incubated with 1mg/mL X-gal in staining solution overnight. Images were obtained using the 20x objective of an Eclipse TE2000-U microscope (Nikon Canada Inc., Mississauga, ON). (n=1)

3.3. Effects of NUMB1 and NUMB3 overexpression on BeWo cell morphology and viability

To demonstrate that the overexpression of NUMB isoforms was not detrimental to BeWo cells and to help provide some insight about NUMB function, cell morphology and cell viability were assessed following transfection with NUMB expression vectors. BeWo cells were transfected with pEF6 expression vectors containing cDNA for NUMB isoforms 1 and 3. The other NUMB isoforms were henceforth not studied since they were not found to be significantly upregulated in differentiated BeWo cells. BeWo cell morphology was qualitatively assessed following overexpression with NUMB isoforms 1 and 3 (Figure 11) in a minimum of 10 random fields per group. Images taken with the 10x objective lens (Figure 11a) revealed there were no visible differences between aggregations of cells. Images taken with a higher magnification (Figure 11b, c) allowed for more detailed analysis. The BeWo cells in all treatment groups appeared to be irregularly shaped and of similar sizes. Also, the ability of BeWo cells to cluster (without fusion) was retained following NUMB overexpression. BeWo cell viability following NUMB overexpression was assessed using the trypan blue exclusion method (Figure 12). There were no significant changes observed in viable cell concentrations when the control and non-transfected cells were compared with cells overexpressing NUMB1 and NUMB3.

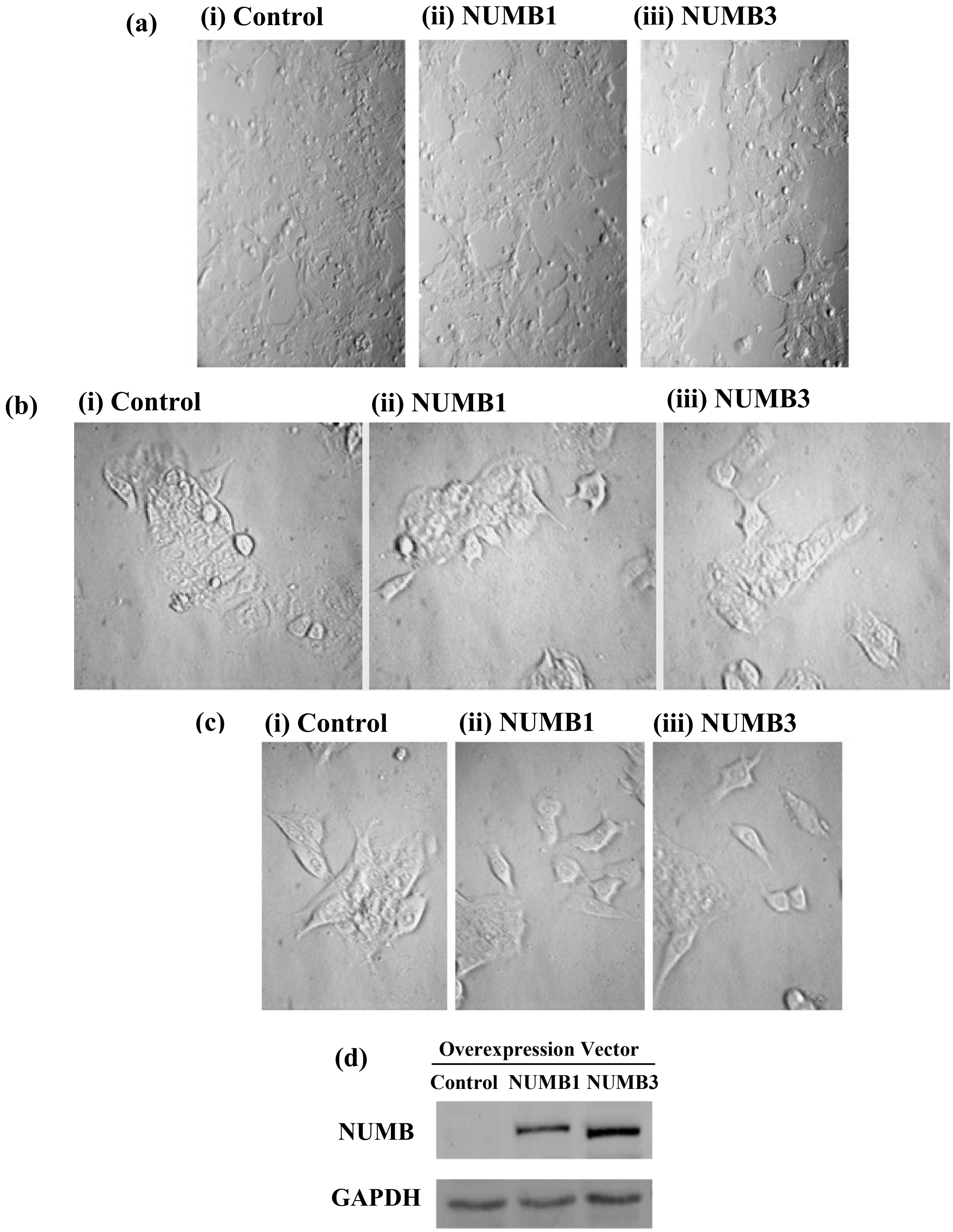


Figure 11. Assessment of BeWo cell morphology following NUMB1 and NUMB3 overexpression. BeWo cell morphology was analyzed using phase contrast microscopy following 24-hour transfection with (i) pEF6/V5-His/*lacZ* vector (control plasmid) and (ii, iii) pEF6 vectors containing cDNA for NUMB 1 and 3. Images were obtained using the (a) 10x objective lens and (b, c) 40x objective lens of an Eclipse TE2000-U microscope (Nikon Canada Inc., Mississauga, ON) with focus on (b) cell clusters and (c) individual cells. (d) Western blot analysis of NUMB overexpression. (n=2)

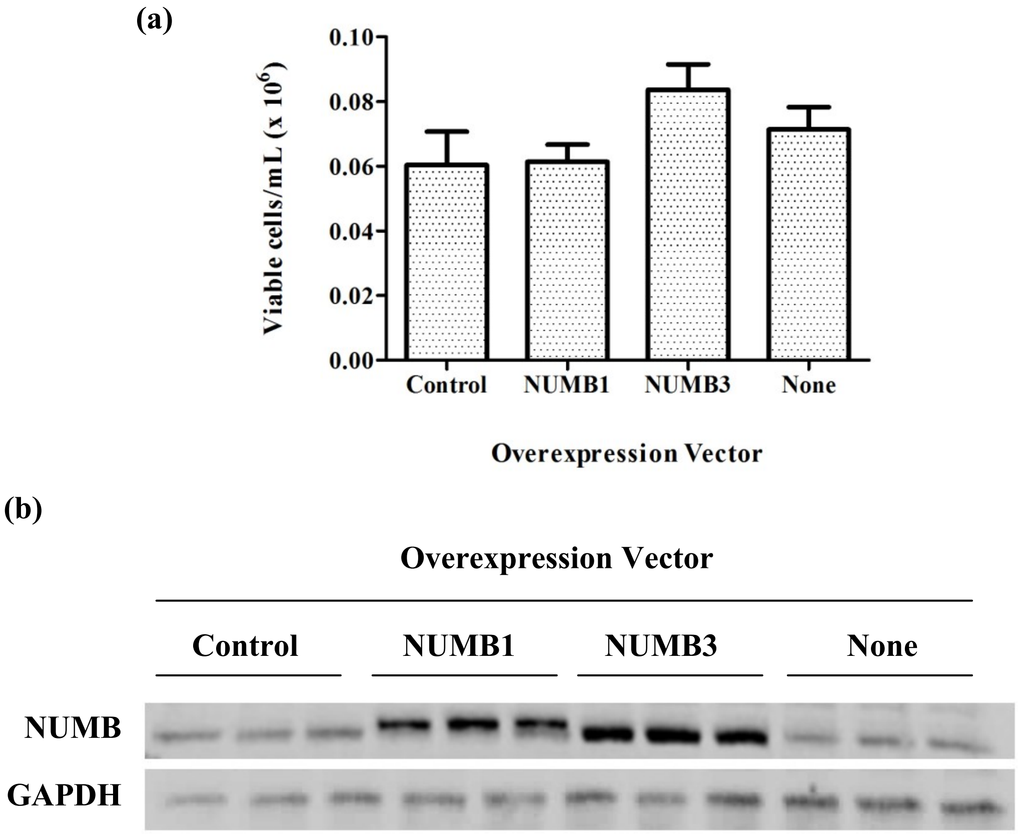


Figure 12. Assessment of BeWo cell viability following NUMB1 and NUMB3 overexpression. (a) Cell viability was assessed using the trypan blue exclusion method with a Vi-CELL cell viability analyzer (Beckman Coulter, Mississauga, ON) following 24-hour transfection with pEF6/V5-His/*lacZ* vector (control plasmid) and pEF6 vectors containing cDNA for NUMB1 and NUMB3. ($p > 0.05$, One-way ANOVA, Tukey post-test, n=3). (b) Western blot analysis of NUMB overexpression.

3.4 Effects of NUMB1 and NUMB3 overexpression on BeWo differentiation

Since NUMB isoforms 1 and 3 were upregulated in differentiated BeWo cells, they potentially have a role in the process of BeWo differentiation or fusion. This was studied by determining if NUMB1 and NUMB3 overexpression directly influenced BeWo differentiation, or facilitated the process indirectly when differentiation had already initiated. Differentiation was confirmed by observing hCG upregulation and E-cadherin downregulation. BeWo cells were transfected with control, NUMB1 and NUMB3 overexpression vectors for 24 hours and then incubated for 48 hours in the presence of either DMSO or 20 μM forskolin (Figure 13a,b). The 48 hour time point was selected since E-cadherin downregulation will have only started to occur, allowing for the assessment of any increased sensitization to the forskolin. The BeWo differentiation controls were cells that were not transfected with overexpression vectors but were treated with either DMSO (negative control) or 20 μM forskolin (positive control) for 48 hours. The differentiation controls confirmed that E-cadherin downrgulation, α and β-hCG upregulation and NUMB 1/3 upregulation (although NUMB upregulation was not very clear in Figure 13a due to image quality). No changes in the expression of E-cadherin and hCG were observed between the control and NUMB1 or NUMB3 overexpression groups. To verify this, E-cadherin expression was quantified for all treatments (Figure 13c) and no significant difference was observed ($p > 0.05$, Two-way ANOVA).

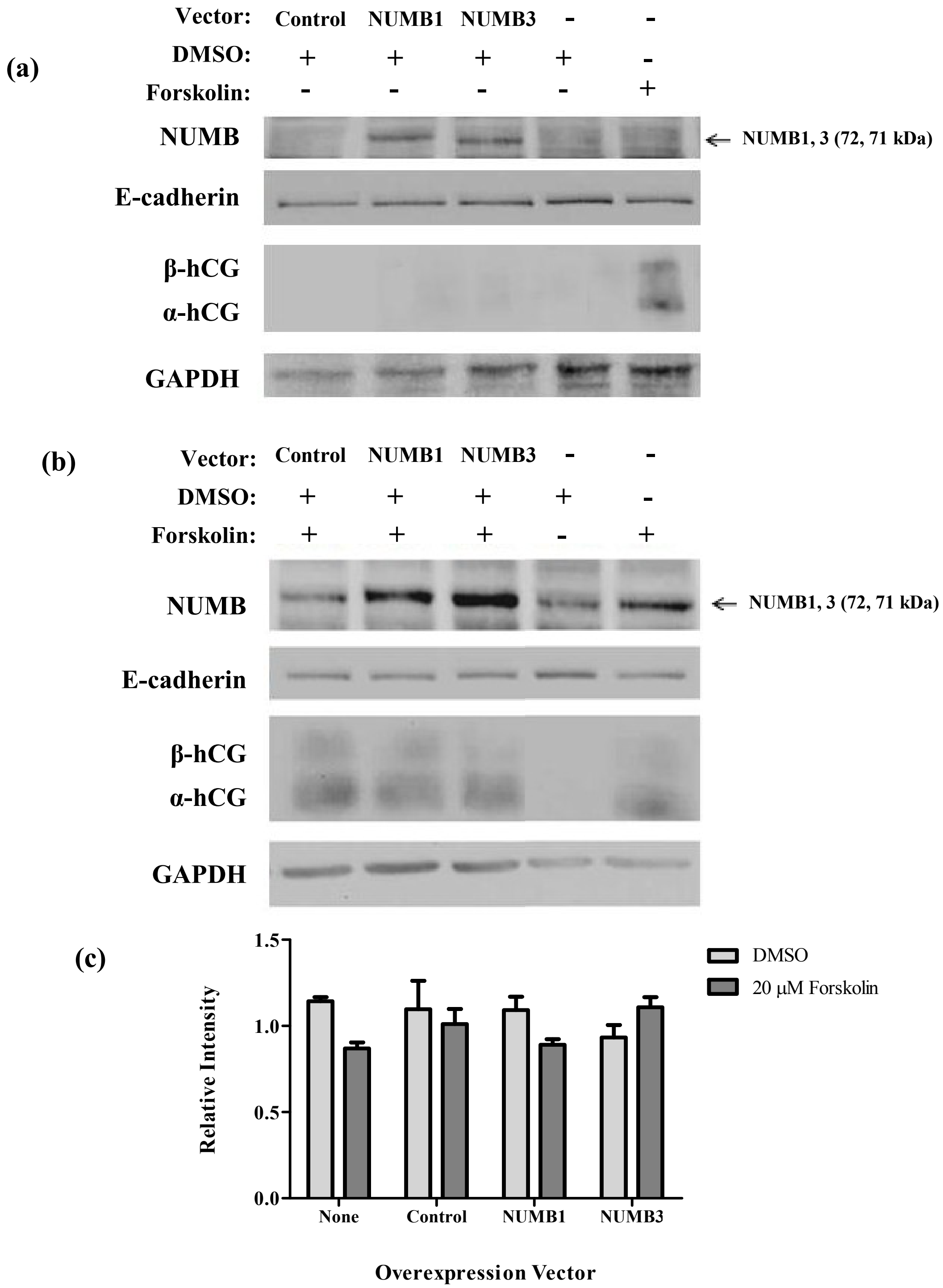


Figure 13. Influence of NUMB1 and NUMB3 overexpression on BeWo differentiation. (a,b) BeWo cells were transfected for 24 hours with pEF6/V5-His/*lacZ* vector (control plasmid) and pEF6 vectors containing cDNA for NUMB1 and NUMB3. They were then treated with (a) DMSO or (b) 20 μM forskolin for 48 hours. Cell lysates were analysed using Western blot methods. (c) Quantification of E-cadherin was performed using ImageJ software (National Institutes of Health, Bethesda, MD). Relative intensity: E-cadherin/GAPDH ($p > 0.05$, Two- way ANOVA, Bonferroni post-test, n=3; n=2 for 'None' group only).

3.5 Effects of NUMB1 and NUMB3 overexpression on EGFR signaling in BeWo cells

Since NUMB isoforms 1 and 3 were not found to be involved in BeWo differentiation, their potential roles in cell signaling were investigated. EGFR signaling is involved in a variety of different functions in trophoblast cells. NUMB1 and NUMB3 were overexpressed in BeWo cells to assess if they were involved in EGFR signaling, as confirmed by upregulation of the downstream targets pERK 1 and 2 and pAkt (Figure 14). The cells were transfected for 24 hours then were either untreated (to see if NUMB1 or NUMB3 initiate EGFR signaling) or treated with 40 ng/mL EGF for 10 minutes (to see if NUMB1 or NUMB3 potentiate EGFR signaling). In both cases, no changes were observed in pERK 1 and 2 or pAkt expression as compared to the control. The length and dosage of EGF treatment was approximated from the literature. Previous studies involving the incubation of BeWo cells with EGF have focused on analyzing its effects on hCG secretion, which requires a prolonged incubation (i.e., 12 to 48 hours). Hence, the necessary EGF concentration for inducing signaling was inferred from previous experiments completed by our lab (Qiu *et al*., 2004) and the duration time of 10 minutes was selected to include potential signaling from endosomes (Hopkins *et al*., 1990; Wang *et al*., 2002).

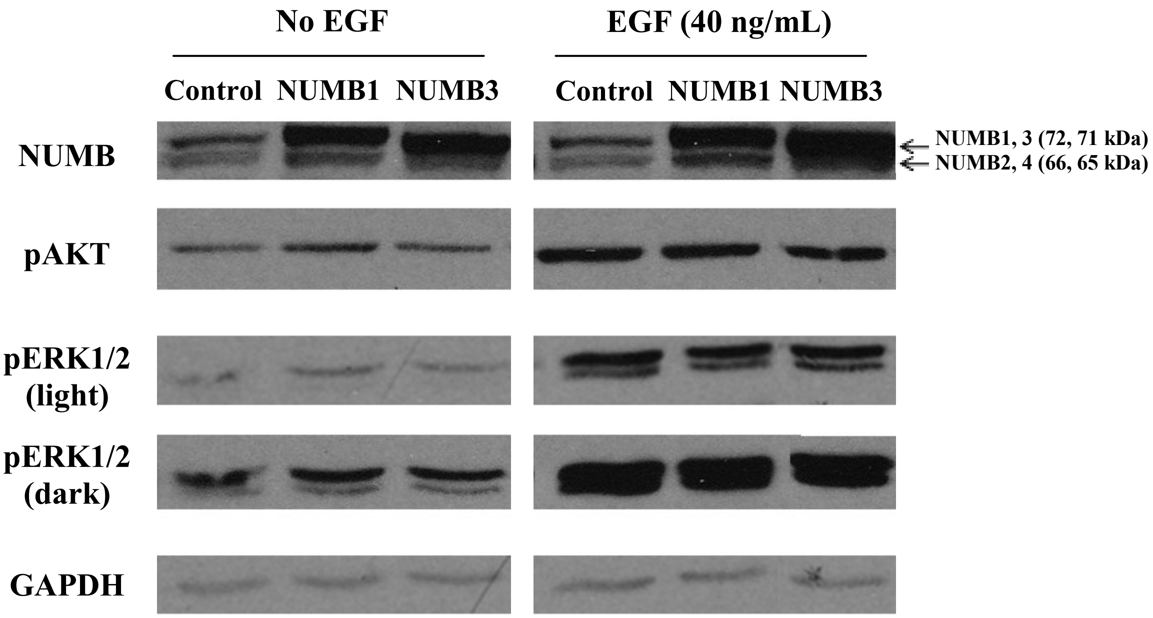


Figure 14. Influence of NUMB1 and NUMB3 overexpression on EGFR signaling in BeWo cells. BeWo cells were transfected for 24 hours with pEF6/V5-His/*lacZ* vector (control plasmid) and pEF6 vectors containing cDNA for NUMB 1 and 3. They were then treated with or without 40 ng/mL epidermal growth factor for 10 minutes. Cell lysates were analysed using Western blot methods. Antibodies against pERK 1/2 and pAkt were used since they are downstream targets of EGFR signaling. The terms "light" and "dark" refer to different exposure times for pERK 1/2. (n=2)

3.6 Membrane EGFR levels in EGF-treated BeWo cells

Although NUMB isoforms 1 and 3 did not influence EGFR signaling in BeWo cells, it was possible that they could be involved in steps downstream of signaling events during EGFR internalization (discussed in Section 4.2). An experiment was completed to confirm that EGF-induced EGFR internalization would occur in BeWo cells. Following biotinylation, BeWo cells were treated with 40 ng/mL EGF for different time points within the range of 15 and 480 minutes (Figure 15). Ligand-induced EGFR internalization was previously observed to occur rapidly in different kinds of cells.. Internalized EGFR was detected in early and late endosomes following EGF incubation for 1 to 5 minutes and 10 to 20 minutes, respectively (reviewed in Sorkin and Waters, 1993). In this study, three bands were detected using the EGFR antibody. The middle band (approximately 170 kDa) was thought to represent non-phosphorylated wild-type EGFR, whereas the top (approximately 175 kDa) and bottom (approximately 165 kDa) bands were considered to represent EGFR mutants. EGFR mutants are frequently observed in carcinoma cells (reviewed in Kuan *et al*., 2001). There was a decrease of wild-type EGFR by approximately 75% after 15 minute incubation with EGF, with further decreases observed as time progressed. This rapid internalization of EGFR suggested that prolonged incubations with EGF (i.e., over 60 minutes) would likely not be necessary for studying EGFR endocytosis in BeWo cells.

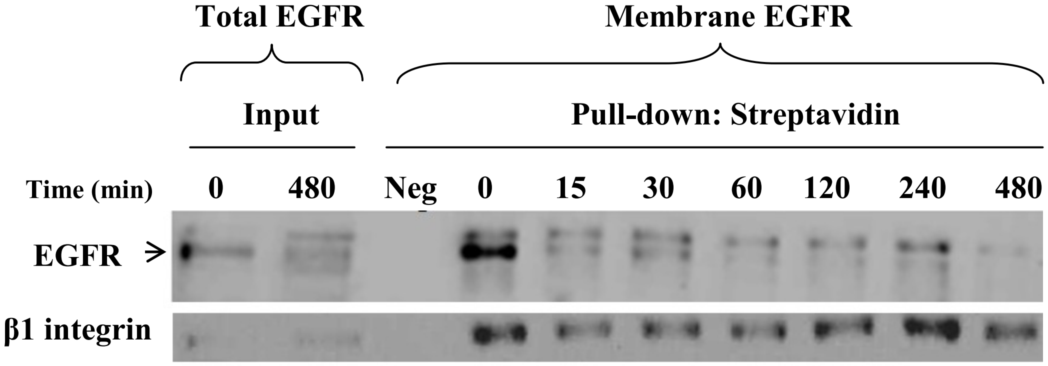


Figure 15. Membrane EGFR levels in EGF-treated BeWo cells. BeWo cells were treated with 40 ng/mL EGF for various times and then incubated with 0.5 µg/µL biotin. Cell lysates were collected following EGF treatment. Streptavidin agarose beads were used to pull down biotinylated proteins. The beads were isolated and proteins were analyzed using Western blot methods. Membranes were probed with antibodies against EGFR and β1 integrin (loading control). Wild-type EGFR was represented by the middle band of the three bands observed, as indicated by the small arrow. (n=2)

3.7 Effects of EGF on the subcellular localization of NUMB, EGFR and LAMP-2 in BeWo cells

To investigate if NUMB was involved in EGFR endocytosis, immunofluorescence was used to study the colocalization of NUMB with EGFR upon EGF stimulation. BeWo cells were treated with 40 ng/mL EGF for 30 minutes. This time point was selected based on data suggesting that 30 minutes is the approximate time it takes for EGFR to reach late endosomes and lysosomes (reviewed in Waterman and Yarden, 2001). Following EGF treatment, the cells were incubated with antibodies against NUMB, EGFR and LAMP-2 (which is a late endosomal/lysosomal marker) and visualized with secondary antibodies conjugated to fluorescent tags (Figures 16 to 18). The NUMB antibody used recognized a region in the C-terminal region present in all isoforms. Before EGF treatment, NUMB was expressed throughout the cytosol with some clustering at perinuclear regions. After EGF treatment, increased localization of NUMB to perinuclear regions was observed. EGFR expression was localized to cell membranes before EGF treatment and increased localization to perinuclear regions was observed following EGF stimulation. LAMP-2 was localized to perinuclear regions in cells before and after EGF treatment. Increased colocalization was observed between NUMB and EGFR (Figure 16) and between EGFR and LAMP-2 (Figure 18) in EGF-treated BeWo cells. Similar extents of colocalization of NUMB with LAMP-2 were observed in BeWo cells with and without EGF treatment (Figure 17).

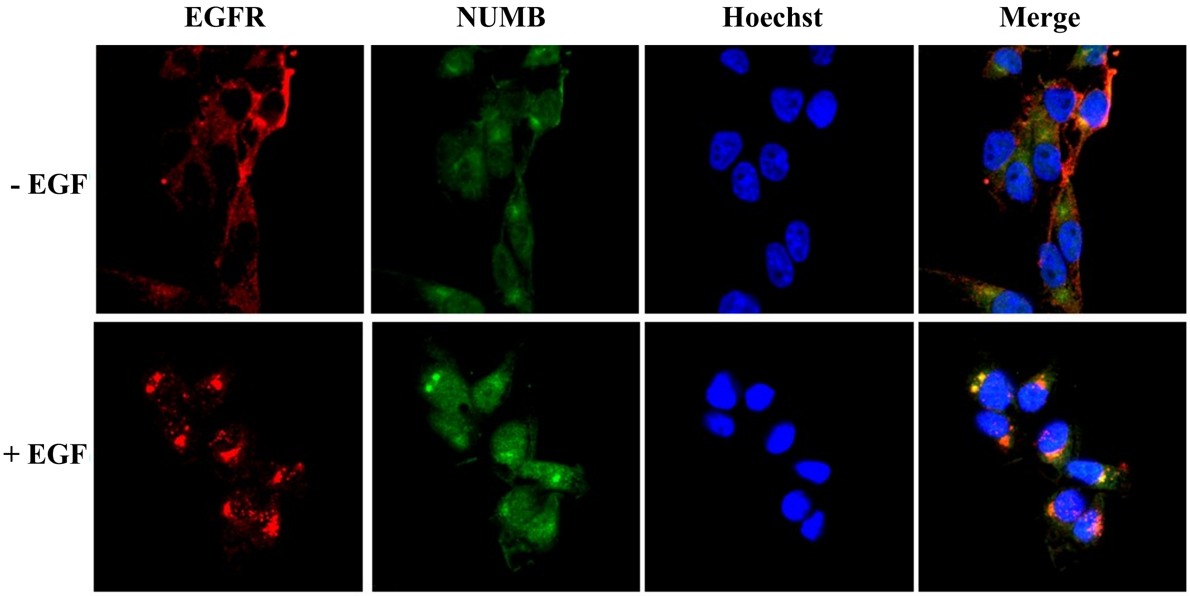


Figure 16. Colocalization of EGFR and NUMB in EGF-treated BeWo cells. BeWo cells were treated with 40 ng/mL EGF for 30 minutes. Cells were incubated with antibodies against EGFR (in mouse) and NUMB (in rabbit) overnight followed by incubation with secondary antibodies conjugated to fluorescent tags (anti-rabbit-FITC, anti-mouse-rhodamine). Confocal fluorescence images were obtained using the 63x oil immersion objective of a Zeiss LSM 510 Meta microscope (Carl Zeiss, Toronto, ON). Image analysis and merging was completed using Zeiss Zen confocal software (Carl Zeiss, Toronto, ON). (n=3)

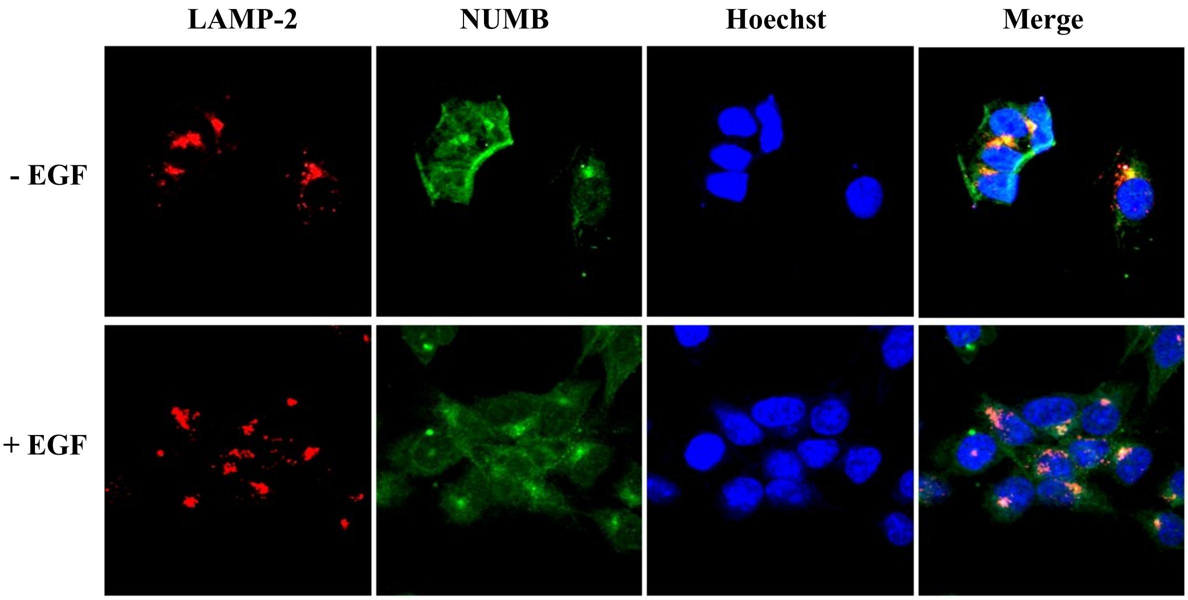


Figure 17. Colocalization of LAMP-2 and NUMB in EGF-treated BeWo cells. BeWo cells were treated with 40 ng/mL EGF for 30 minutes. Cells were incubated with antibodies against LAMP-2 (in mouse) and NUMB (in rabbit) overnight followed by incubation with secondary antibodies conjugated to fluorescent tags (anti-rabbit-Alexa Fluor 488, anti-mouse-rhodamine). Confocal fluorescence images were obtained using the 63x oil immersion objective of a Zeiss LSM 510 Meta microscope (Carl Zeiss, Toronto, ON). Image analysis and merging was completed using Zeiss Zen confocal software (Carl Zeiss, Toronto, ON). (n=3)

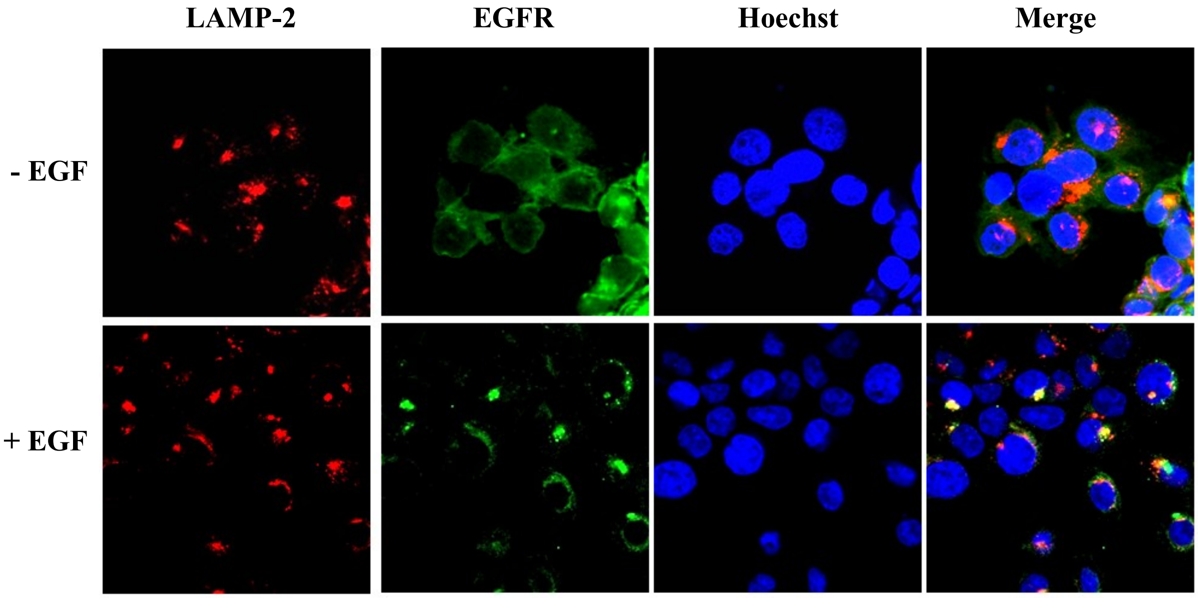


Figure 18. Colocalization of LAMP-2 and EGFR in EGF-treated BeWo cells. BeWo cells were treated with 40 ng/mL EGF for 30 minutes. Cells were incubated with antibodies against LAMP-2 (in mouse) and EGFR (in rabbit) overnight followed by incubation with secondary antibodies conjugated to fluorescent tags (anti-rabbit-Alexa Fluor 488, anti-mouse-rhodamine). Confocal fluorescence images were obtained using the 63x oil immersion objective of a Zeiss LSM 510 Meta microscope (Carl Zeiss, Toronto, ON). Image analysis and merging was completed using Zeiss Zen confocal software (Carl Zeiss, Toronto, ON). (n=3)

CHAPTER 4: DISCUSSION & CONCLUSIONS

The adaptor protein NUMB mediated cell migration and apoptosis in HTR8/SVneo first-trimester EVT cells (Haider *et al*., 2011). In this study, the roles of NUMB in the placenta were further investigated in undifferentiated and forskolin-differentiated BeWo choriocarcinoma cells, which are widely accepted models of villous cytotrophoblast cells and syncytiotrophoblast respectively. NUMB was postulated to be involved in differentiation and EGFR endocytosis.

4.1 NUMB isoform expression in the placenta

We have demonstrated NUMB1 and NUMB3 to be the predominant isoforms expressed in undifferentiated and differentiated BeWo cells (Figures 5 to 7). These isoforms were identified based on their sizes and by using HTR-8/SVneo first-trimester EVT cells as positive controls. The relative levels of the alternatively spliced NUMB transcripts in BeWo cells still need to be quantified. This can be accomplished through RNA extraction, reverse transcription and quantitative PCR using specific primers designed to identify the individual isoforms.

The data from this study supplemented previous data collected by our laboratory on NUMB expression in the placenta. In HTR-8/SVneo cells, the predominant isoforms were NUMB2 and NUMB4, followed by NUMB1 and NUMB3 (Haider *et al*., 2011). In heterogeneous placental tissue from all trimesters, all NUMB isoforms were present (including 1 and 3) but NUMB8 was identified as the isoform with the highest expression (Haider *et al*, 2011). The specific subcellular localizations of the individual NUMB isoforms could not be specified since the NUMB antibody used for ICC/IHC in the previous and the current study corresponded to C-terminal regions at positions 600-651aa and 549-568aa in NUMB1, respectively, which is found in all nine of the NUMB isoforms. However, general NUMB staining was observed in EVT cells at the maternal-fetal interface in first and second trimester placental tissue (Haider *et al*., 2011). In a recent IHC analysis of term placental villi, preliminary results indicated that NUMB staining was restricted to the syncytiotrophoblast (Figure 4). In summary, the collective data (see Table 3) suggested that NUMB isoforms have cell type-specific expression patterns in the placenta. Whether or not these NUMB isoforms retain their functions in different types of placental cells remains to be determined.

Table 3. NUMB expression in the placenta: Summary of current data

	BeWo	**HTR-8/SVneo**	**Placental tissue**			
Gestational age	unknown	first trimester	first trimester	second trimester	third trimester	term
Predominant NUMB isoforms	NUMB1 NUMB3	NUMB2* NUMB4*	NUMB8*	NUMB8*	NUMB8*	NUMB8*
Localization of NUMB (not isoform-specific)	villous CT and ST	EVT*	EVT* decidua*	EVT* decidua*	n/a	ST

EVT – extravillous trophoblast cell
CT - cytotrophoblast
ST – syncytiotrophoblast
* Haider *et al*., 2011

4.2 Regulation of NUMB1 and NUMB3 in BeWo cells

The increased expression of NUMB isoforms 1 and 3 following forskolin treatment in BeWo cells (Figures 6 and 7) may indicate a novel pathway for NUMB protein regulation that has previously not been described. Several other mediators of NUMB expression in non-placental tissues have been reported in the literature. Translational repression of NUMB by Musashi-1, an RNA-binding protein, was described in NIH 3T3 mouse embryonic fibroblast cells (Imai *et al*., 2001). NUMB inhibition was also found to be regulated by miR-146a microRNA (Kuang *et al*., 2009) in C2C12 mouse myoblast cells, and by high levels of NOTCH (Chapman *et al*., 2006) in C2C12 cells co-cultured with 3T3-J1 mouse embryonic fibroblast cells. Proteolytic degradation of NUMB was found to be mediated by the E3 ubiquitin-protein ligases SIAH-1 (Susini *et al*., 2001) and LNX (Nie *et al*., 2002) in 293T human embryonic kidney cells and by MDM2 (Yogosawa *et al*., 2003) in U2OS human osteosarcoma cells. Upregulation of NUMB was observed following EGFR overexpression in the mouse subventricular zone (Aguirre *et al*., 2010). Thus, the current study represents the first time an induced upregulation of the NUMB protein has been described in a human cell line.

The specific regulator of NUMB isoforms 1 and 3 expression within the forskolin pathway still needs to be identified but several possibilities can be derived from the literature. Forskolin-induced differentiation and fusion of BeWo cells occurs via the sequential activation of adenylyl cyclase, cAMP and protein kinase A (PKA). PKA activates glial cells missing homolog 1 (GCM1, also known as GCMa) which is a transcription factor that enhances the expression of fusogenic proteins called syncytins (Knerr *et al*., 2005). The methods used in studying the upregulation of RhoE, a Rho

GTPase, in BeWo cells differentiated with dibutyryl cAMP (Collett *et al*., 2012) can be used to ascertain the level of the forskolin pathway at which NUMB is being regulated. In this study, a PKA inhibitor was added prior to dibutyryl cAMP treatment to determine if RhoE upregulation was PKA-dependent. Also, the extent of RhoE upregulation was assessed following dibutryl cAMP treatment in hypoxic, fusion-inhibiting conditions to determine if its regulation was fusion-dependent. Another approach would be to activate specific stages in the forskolin pathway in BeWo cells and then assess the extent of NUMB1 and NUMB3 upregulation. Repeating this experiment using different cell lines would also help determine if NUMB1 and NUMB3 regulation is fusion-dependent (e.g., if using HTR-8/SVneo cells) or restricted to placental cells (e.g., if using HeLa cells).

The upregulation of NUMB by forskolin in BeWo cells has introduced the possibility that NUMB expression can be controlled by the same pathway in other types of NUMB-expressing cells. In the placenta, EVT cells and decidual cells have also been reported to express NUMB (Haider *et al*., 2011). The addition of forskolin inhibited cell proliferation and cell migration in HTR-8/SVneo cells (Biondi *et al*., 2006; Biondi *et al*., 2008) and induced the decidualization of endometrial stromal cells (Tamura *et al*., 2007). If forskolin is found to also upregulate NUMB in these cells, it is possible that NUMB is associated with these processes.

During neurogenesis, NUMB isoforms 1 and 3 are upregulated in proliferative cells and downregulated in differentiated cells (as described in section 1.4). Thus, our finding of the upregulation of these isoforms in forskolin-treated BeWo cells represents a novel association between these isoforms and differentiated cells. It remains to be determined if this correlation is restricted to syncytiotrophoblast. An important consideration is that NUMB1 and NUMB3 mRNA were reported to be overexpressed in cervical squamous cell carcinoma (CSCC) cells (Chen *et al*., 2009) and that NUMB1 and NUMB3 expression may be related to the proliferative ability of choriocarcinoma cells. However, the further upregulation of these isoforms in BeWo cells following forskolin-induced differentiation increases the likelihood that their expression is not merely attributed to the behaviour of carcinoma cells.

4.3 Investigating the roles of NUMB1 and NUMB3 in BeWo cells

NUMB isoforms 1 and 3 both contain an 11 amino acid insert in the proline-rich region (PRR) that is not found in any of the other isoforms. The increased expression of these isoforms in differentiated BeWo cells suggests that this sequence may be important for one of the functions of the syncytiotrophoblast. It was previously reported that NUMB1 overexpression in HTR-8/SVneo cells increased cell migration (Haider *et al*., 2010). Since cell migration is not an important function of the syncytiotrophoblast, NUMB1 must have a different role in this cell type.

The roles of NUMB1 and NUMB3 in BeWo cells were investigated using overexpression methods. The vectors used for experimental analysis did not contain biomarkers for assessing transfection efficiency. Therefore, their transfection efficiencies were estimated by testing three similar vectors using Lipofectamine and Plus Reagent (Invitrogen Corp., Burlington, ON) (Figures 8 to 10). Transfection efficiencies ranged between 32 to 48%, which was not surprising considering that some studies have previously reported that BeWo cells are difficult to transfect via lipofection (Forbes *et al*., 2009). Although transfection was achieved with only moderate levels of efficiency, the ability to deliver transfection reagents quickly and accurately using lipofection methods deemed this approach to be appropriate for streamlining assessments of NUMB function in BeWo cells. Following transfection of BeWo cells with overexpression vectors containing cDNA for NUMB1 and NUMB3, cell viability and morphology were found to be comparable between non-transfected and transfected cells (Figures 11 and 12). This indicated that NUMB1 and NUMB3 overexpression did not initiate or promote necrosis or apoptosis. The overexpression of NUMB1 and NUMB3 also did not mediate BeWo differentiation and as evidenced by unaltered expression of E-cadherin and hCG (Figure 13). NUMB knockdown experiments (as discussed in section 4.6) still need to be completed to confirm that these isoforms are not required for differentiation to occur.

Since NUMB1 and NUMB3 were not found to be directly involved in villous cytotrophoblast differentiation, it was postulated that they might be involved in a cellular process occurring in the syncytiotrophoblast, such as EGFR endocytosis. EGFR signaling is important for several placental functions (see section 1.5, ii) and coincides with the clathrin-dependent endocytosis of its receptor. Overexpression of NUMB1 and NUMB3 did not mediate EGFR signaling as indicated by unaltered expression of downstream proteins under the specified conditions (Figure 14). However, this data was not conclusive. It may be possible that NUMB may inhibit or promote signaling in the presence of EGF under different conditions (i.e., lower EGF concentration or shorter duration of treatment). NUMB knockdown experiments (as discussed in section 4.6) should be completed to determine if NUMB expression is required for EGFR signaling to occur.

Although NUMB1 and NUMB3 overexpression was not found to downregulate EGFR signaling (Figure 14), it was still possible for NUMB to be involved in EGFR internalization and degradation in BeWo cells. Although receptor internalization usually leads to signaling attenuation, EGFR signaling can also continue to occur from endosomes and is sufficient to stimulate signal transduction (Wang *et al*., 2002). Ligand-induced EGFR internalization was confirmed in BeWo cells with a net reduction of EGFR with prolonged EGF treatment (Figure 15), which suggested that EGFR was either being degraded or stored within the cells. However, this did not exclude that EGFR could also be recycled back to the membrane.

To further investigate the fate of EGFR, ICC fluorescence experiments were completed to analyze the localization of NUMB, EGFR and LAMP-2 (late endosomal/lysosomal marker) in EGF-treated BeWo cells (Figures 16-18). Previous studies revealed that NUMB and internalized EGFR colocalize in A172 glioblastoma cells (Santolini *et al*., 2000) and that EGF-stimulated EGFR can accumulate in compartments downstream of early endosomes (Oksvold *et al*., 2001, Sanchez *et al*., 1998, Bequinot *et al*., 1984). However, the association between NUMB, EGFR and later stages of endocytosis had not yet been studied together. The NUMB antibody did not allow for distinguishing between different isoforms so the specific subcellular localization of NUMB isoforms 1 and 3 and their individual levels of colocalization with EGFR and LAMP-2 could not be assessed. Pairwise analyses of NUMB, EGFR and LAMP-2 following EGF treatment revealed that they were all colocalizing at the same perinuclear regions which still need to be specifically identified (Figures 16 to 18). The colocalization of NUMB and EGFR was EGF-dependent since they were separately localized before ligand stimulation (Figure 16). Although EGFR was primarily localized to the membrane without EGF treatment, some NUMB was still found to colocalize with

LAMP-2. This data suggested that NUMB associates with EGFR upon EGF-induced internalization. The relevance of this interaction in the placenta and the necessity of NUMB for EGFR endocytosis are yet to be determined.

4.4 Postulated role of NUMB in EGFR endocytosis

The mechanism of ligand-induced clathrin-mediated endocytosis (CME) of EGFR has been studied extensively in the literature (Madshus and Stang, 2009). Several adaptor proteins involved in the internalization and post-endocytic trafficking of EGFR have been identified, such as GRB2, c-Cbl, AP-2 and EPS15 (Sorkina *et al.*, 1991; Burke *et al.*, 2001). Please refer to Figure 19 for a diagram summarizing the current model for EGFR endocytosis, which mentions some of the adaptor proteins that have been identified (Madshus and Stang, 2009). Other adaptor proteins, such as NUMB, may be involved in facilitating post-endocytic trafficking leading to receptor degradation.

NUMB does not directly bind to autophosphorylated EGFR (Verdi *et al.*, 1996) but it may interact with adapter proteins in a complex bound to internalized EGFR. Two such proteins are EGFR substrate clone 15 (Eps15) and adaptor protein 2 (AP-2), which are both important for the CME of EGFR (Carbone *et al.*, 1997; Sorkina *et al.*, 1999; Torrisi *et al.*, 1999; Goh *et al.*, 2010). In various EGF-treated cells, NUMB co-immunoprecipitated with Eps15 (Salcini *et al.*, 1997) and colocalized with Eps15 in endosomes (Santolini *et al.*, 2000) and plasma membrane punctae (Dho *et al.*, 2006). NUMB had also been found to interact with AP-2 (Tokumitsu *et al.*, 2006). Thus, Eps15 and AP-2 may be the proteins that NUMB interacts with in BeWo cells and in syncytiotrophoblast during EGFR endocytosis. Immunoprecipitation experiments using placental cells should be completed to confirm if NUMB associates with EGFR in a complex and to identify NUMB-associated proteins.

The colocalization of NUMB with EGFR in perinuclear regions similar to the distribution of late endosomes and lysosomes suggested that NUMB is involved in trafficking EGFR to the degradative pathway. It is known that NUMB is not involved in the ubiquitination of EGFR (McGill and McClade, 2003). Repeating the experiments indicated in Figures 16 through 18 with a shorter duration of EGF treatment (i.e., 10 minutes instead of 30 minutes) and using an early endosome marker (e.g., Rab5) would help ascertain if NUMB localizes to EGFR in earlier stages of its internalization.

NUMB associated with late endosomes and lysosomes before and after EGF stimulation in BeWo cells (Figure 17). This suggested that NUMB was constitutively localized to this region and interacts with endocytic machinery. It is possible that NUMB is also involved in the attenuation of other internalized receptors such as the transferrin receptor, which is essential for iron transport across the syncytiotrophoblast. The colocalization of NUMB with the transferrin receptor was reported in A172 human glioblastoma cells (Santolini *et al.,* 2000). Also, NUMB may be involved in recycling internalized proteins back to the cell surface. NUMB had previously been found to recycle integrin-β1 to the leading edge of HeLa cells to promote directional migration (Nishimura and Kaibuchi, 2007).

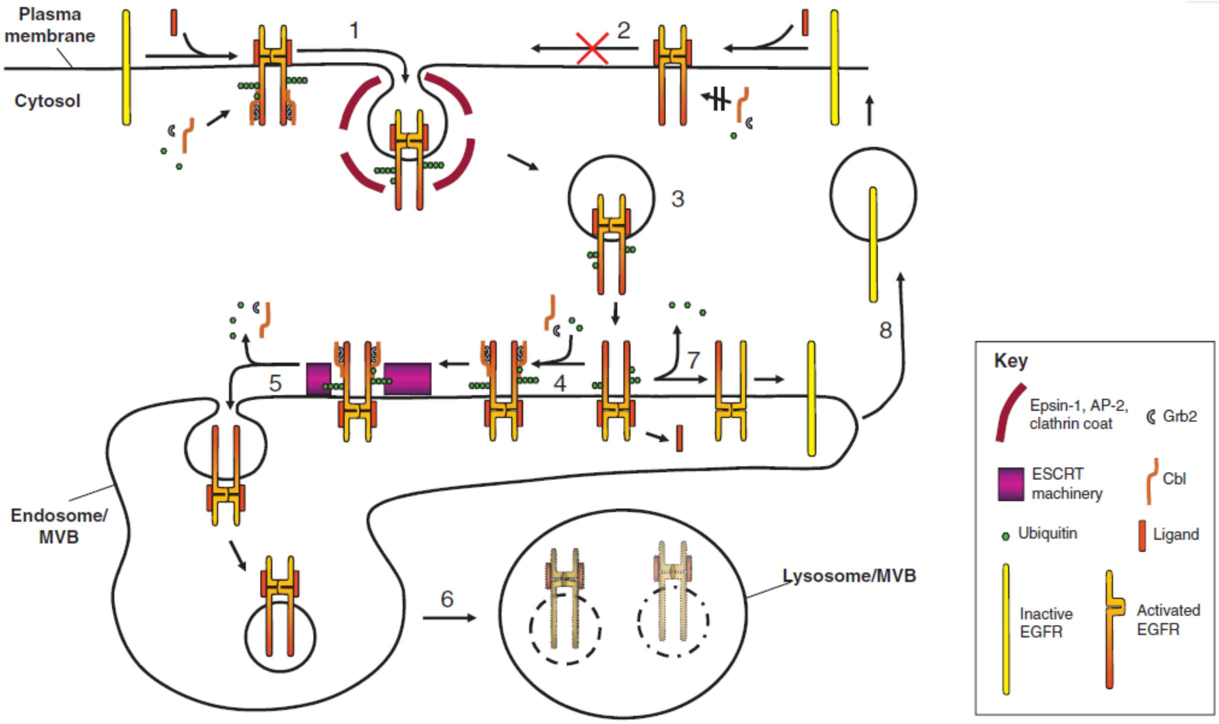


Figure 19. Current model for EGFR endosomal trafficking. This diagram is Figure 2 from a review article about EGFR internalization and trafficking (Madshus and Stang, 2009). The following text is taken directly from its caption. On ligand binding, EGFR is activated, ubiquitylated and recruited into clathrin-coated pits (1). However, when ubiquitylation of activated EGFR is blocked, its recruitment into coated pits is inhibited (2). We propose that EGFR is partially deubiquitylated following internalization (3), but that ligand-bound EGFR is reubiquitylated (Longva *et al*., 2002) (4). Through interaction with the ESCRT machinery on early endosomes, EGFR is sorted to inner vesicles of endosomes (5) and finally to MVBs and lysosomes for degradation (6). If, however, the EGFR ligand dissociates at low pH, EGFR that localizes to endosomes is deactivated and deubiquitylated (7) and recycled (8) to the plasma membrane.

4.5 Study limitations

BeWo, a well-established choriocarcinoma cell line, was used as an *in vitro* model to make inferences about NUMB expression and function in human villous cytotrophoblast cells and syncytiotrophoblast. An advantage to using cell lines such as BeWo to study cellular processes is that they can usually be relied upon to have homogenous characteristics and predictable behaviours. This consistency can be difficult to achieve when using primary cell cultures since incomplete isolation procedures can result in contamination by surrounding cells from the tissues of origin. A disadvantage to using BeWo cells is that they are choriocarcinoma cells and have different gene expression profiles from normal trophoblast cells isolated from term placentae (Garcia and Castrillo, 2004). Thus, the extrapolation of this experimental data to *in vivo* conditions must keep this in consideration. An analysis of NUMB isoform expression and function in isolated villous cytotrophoblast cells and syncytiotrophoblast during early placental development is required to confirm the results from this study in a more physiological context. Determining the localization of individual NUMB isoforms in placental villi still needs to be completed and this could be achieved by using isoform-specific antibodies.

4.6 Future directions

Loss-of-function experiments should be completed to further investigate NUMB’s role in BeWo cells. NUMB isoform 1 and 3 overexpression had revealed that these isoforms were not required for BeWo differentiation or EGFR signaling. A complementary phenotype (i.e., no changes in molecular endpoints) observed following NUMB knockdown would corroborate the conclusions that NUMB is not required for these specific cellular events in BeWo cells. Our laboratory had successfully downregulated NUMB expression with shRNA constructs in HTR-8/SVneo cells using lipofection methods (Haider *et al*., 2011). However, since BeWo cells are somewhat difficult to transfect, a lentiviral vector should be used to introduce the NUMB shRNA vectors into these cells. GCM1 knockdown was completed successfully in BeWo cells using this approach (Chang *et al*., 2011). An alternative method for downregulating NUMB expression would be to use anti-sense morpholino oligomers. This method was used to downregulate NUMB in cortical cells and involved the delivery of the oligomers to the cells via endosomes using the Endo-Porter peptide (Bani-Yaghoub *et al*., 2007). A disadvantage to using shRNA and morpholinos is that the available NUMB constructs target all isoforms, thus preventing the examination of the functions of the individual isoforms. A method for circumventing this issue would be to perform isoform-specific rescue following NUMB knockdown, an approach successfully used for analyzing NUMB isoform function following morpholino treatment in cortical cells (Bani-Yaghoub *et al*., 2007) and shRNA treatment in adenocarcinoma tissues (Misquitta-Ali *et al*., 2011). Also, the development of splice-blocking morpholinos would be a novel approach for studying NUMB isoform-specific functions in cells. However, additional information would first have to be retrieved about the mechanism of alternative splicing of the NUMB transcript before specific morpholino targets (e.g., snRNP binding sites) could be identified.

4.7 Conclusions

The roles of NUMB in villous cytotrophoblast cells and syncytiotrophoblast were investigated using undifferentiated and forskolin-differentiated BeWo choriocarcinoma cells. Our studies have revealed that NUMB1 and NUMB3 were the predominant endogenous isoforms and were both upregulated after forskolin treatment. Although NUMB1 and NUMB3 did not mediate BeWo differentiation or EGFR signaling, we demonstrated EGF-induced EGFR translocation from the cell membrane to NUMB-positive perinuclear regions resembling late endosomal/lysosomal distribution patterns. This data demonstrated that NUMB is likely an endocytic protein in BeWo cells and may be involved in EGFR internalization leading to degradation. Now that a potential role for NUMB in BeWo cells has been established, the next logical steps would be to further investigate it by completing NUMB knockdown in BeWo cells and designing experiments using primary trophoblast cell cultures. Overall, our findings supported the importance of NUMB in placental development and function and warrant further investigation.

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