The role of miR-218-5p and miR-210-3p in human trophoblast function and placenta development

Heyam Y. Hayder

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Abstract

MicroRNAs (miRNA) are small non-coding RNAs that primarily regulate gene expression at the post-transcriptional level. Studies have shown that miRNAs play important roles in placenta development and are dysregulated in pregnancy complications such as preeclampsia (PE). One such miRNA is hsa-miR-210-3p, which is upregulated in PE. We found that miR-210-3p overexpression reduced trophoblast migration and invasion and extravillous trophoblast (EVT) outgrowth in first trimester explants. Its overexpression also downregulated endovascular trophoblast (enEVT) markers, the ability of trophoblast to form endothelial-like networks, and the mRNA levels of interleukin-1B and -8 and CXC motif ligand 1. These cytokines play a role in EVT invasion and the recruitment of immune cells to spiral artery remodelling sites. We also showed that caudal-related homeobox transcription factor 2 (CDX2) is a novel target of miR-210-3p and that CDX2 downregulation mimicked the observed effects of miR-210-3p upregulation. These findings suggest that miR-210-3p overexpression impairs spiral artery remodelling, thus contributing to PE pathogenesis.

Recently, we reported that hsa-miR-218-5p promotes trophoblast migration, invasion, differentiation down the endovascular pathway, and is downregulated in PE. Through microarray analysis, we identified neuropeptide Y (NPY) as a possible target gene of miR-218-5p. Although NPY is known to be produced by human placenta, little is known about its function during pregnancy. We found that miR-218-5p upregulates NPY pre- and mature mRNA and protein levels. It also targets NPY receptor 1 (NPY1R) mRNA at the 3' UTR, resulting in decreased mRNA levels but not the protein. Using immunohistochemistry, we detected NPY and NPY1R in first trimester placentas. Also, using immortalized EVT cell lines to examine cell proliferation, migration, and invasion, we found that NPY overexpression decreased EVT migration and invasion while silencing it increased their motility and proliferation. NPY effect on motility was attenuated by an NPY1R-specific inhibitor. Meanwhile, NPY1R overexpression decreased EVT proliferation while NPY1R antagonists increased it. Using the floating first-trimester villous model, we found that NPY knockdown or blocking NPY1R signalling increased cytotrophoblast proliferation. These findings suggest that the NPY-NPY1R signalling pathway is a negative regulator of placenta development.

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List of Abbreviations

3' UTR	3 prime untranslated regions
AGO	Argonaut
AKT	Protein kinase B
ATP	Adenosine triphosphate
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCK8	Cell counting kit 8
CDH5	Cadherin-5/Vascular endothelial cadherin/CD144
CDS	Coding DNA sequence
CDX2	Caudal type homeobox 2
CK-7	Cytokeratin 7
CREB	cAMP response element-binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
CTB	Cytotrophoblasts
CXCL1	C-X-C motif chemokine ligand 1
CXCL8	C-X-C motif chemokine ligand 8/Interleukin 8
CYC1	Cytochrome C1
DGCR8	DiGeorge syndrome critical region 8
DMSO	Dimethyl sulfoxide
dNK	Decidual natural killer cells
DPP4	Dipeptidyl peptidase-4
enEVT	Endovascular extravillous trophoblasts
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases/ classical MAP kinases
EV	Empty vector
EVT	Extravillous trophoblast
FBS	Fetal bovine serum

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GPCR	G protein-coupled receptors
GRK	G protein-coupled receptor kinase
gRNA	Guide RNA
GTP	Guanosine triphosphate
HIF1A	Hypoxia-inducible factor 1 subunit Alpha
HLA-C	Human leukocyte antigen/Major histocompatibility complex, Class I, C
HLA-G	Human leukocyte antigen/Major histocompatibility complex, Class I, G
HRE	Hypoxia response element
HUVEC	Human umbilical vein endothelial cells
iEVT	Interstitial extravillous trophoblasts
IL1B	Interleukin 1 beta
IL-6	Interleukin 6
ITGA1	Integrin subunit alpha 1
IUGR	Intra-uterine growth restriction
IVS	Intervillous space
MAPK	Mitogen-activated protein kinase
miRISC	MicroRNA-induced silencing complex
miRNA	MicroRNA
MMP	Matrix metalloproteinases
MRE	miRNA response element
mRNA	Messenger RNA
NC	Non-targeting control
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localization signal
NPY	Neuropeptide Y
NPY1R	Neuropeptide Y receptor 1

NPY2R	Neuropeptide Y receptor 2
NPY4R	Neuropeptide Y receptor 4
NPY5R	Neuropeptide Y receptor 5
npy6r	Neuropeptide Y receptor 6
PBS	Phosphate-buffered saline
PE	Preeclampsia
PECAM1	Platelet and endothelial cell adhesion molecule 1/CD31
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC-β	Phospholipase C beta
PP	Pancreatic polypeptide
Pre-miRNA	Precursor miRNA
Pre-mRNA	Premature mRNA
Pri-miRNA	Primary miRNA
PYY	Peptide YY
qRT-PCR	Quantitative real-time polymerase chain reaction
rhNPY	Recombinant human NPY
RIPA	Radioimmunoprecipitation assay
SEM	Standard error of the mean
siRNA	Small interfering RNA
SRC	Proto-oncogene tyrosine-protein kinase Src
STB	Syncytiotrophoblasts
TBST	Tris-buffered saline with Tween 20
TE	Trophectoderm
TSS	Transcription start site
U48	Small nucleolar RNA U48
U6	Small nucleolar RNA U6

Chapter One

Literature Review

Literature Review: Some parts of this chapter were published in 2018 as a review titled "**microRNAs: Crucial regulators of placental development**".

Heyam Hayder [1], Jacob A. O'Brien [1], Uzma Nadeem [1] and Chun Peng [1,2,*]

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[1]Department of Biology, York University, Toronto, ON M3J 1P3, Canada
[2]Centre for Research on Biomolecular Interactions, York University, Toronto, ON M3J 1P3, Canada
*****Correspondence: cpeng@yorku.ca (C.P.)

Authors' contributions

H.H. wrote all review parts except those listed below by J.A.O. and U.N. and I helped with the review figures generation and final reading of the review article and addressing reviewers' comments. In addition, I wrote all unpublished parts of this chapter.

J.A.O. wrote an overview of microRNAs section, review figures generation, the final reading of the review article and participated in addressing reviewers' comments.

U.N. wrote microRNAs in syncytiotrophoblasts differentiation subsection and the final reading of the review article.

C.P. was involved in conceptualization, draft preparation, figures generation, supervision and funding acquisition, the final reading of the review article, and addressing reviewers' comments.

I. Placental development and pregnancy complications

The placenta is a transient organ essential for the survival and development of mammalian embryos (Rossant and Cross, 2001). This organ plays a critical role in mediating the exchange of respiratory gases, nutrients, and waste products between the mother and the fetus (Rossant and Cross, 2001; Regnault et al., 2002; Wooding and Burton, 2008). In addition, the placenta also acts as an endocrine organ and produces many pregnancy-associated hormones and growth factors that help in sustaining the pregnancy, preventing fetus rejection by the mother's immune system, and regulating fetal growth (Rossant and Cross, 2001; Fu et al., 2013a; Ji et al., 2013). Placental development is a spatially and temporally regulated process. This allows for the increasing demands of oxygen and nutrient required by the growing fetus to be met throughout gestation (Wooding and Burton, 2008). Improper placenta formation gives rise to many pregnancy-associated conditions such as preeclampsia and intrauterine growth restriction (Genbacev et al., 1996; Rossant and Cross, 2001; Fu et al., 2013a).

I.1. Overview of placental development

Soon after fertilization, asymmetric cell division of the blastomere gives rise to different cell populations, an outer cell layer surrounding an inner cell population (Johnson and Ziomek, 1981; Viswanathan et al., 2009). The blastocyst is formed when the outer cell layer differentiates into a layer of trophoblasts termed the trophectoderm (TE), and the inner cell population differentiates into the inner cell mass (ICM). The TE will later give rise to the placenta while the ICM will develop into the embryo and the visceral endoderm (yolk sac) (Viswanathan et al., 2009; Maltepe and Fisher, 2015).

With the TE formed, the blastocyst is ready for implantation (Caniggia et al., 2000). Implantation starts with the adhesion of the TE onto the receptive decidualized endometrium through a complex network of cell-cell communication events (Red-Horse et al., 2004). Implantation leads to the invasion of the blastocyst through the extracellular matrix of the decidua by the proliferating and differentiating trophectoderm layer, embedding the blastocyst

deep into the uterine wall (Red-Horse et al., 2004; Noris et al., 2005; Wooding and Burton, 2008) **(Figure 1)**.

Once the blastocyst is embedded within the uterine wall, the process of placenta formation, termed placentation **(Figure 2)**, begins with the differentiation of cells in the TE into the different trophoblast lineages (Red-Horse et al., 2004; Maltepe and Fisher, 2015). Placentation in eutherian mammals is more complex than marsupial mammals (Moffett and Loke, 2006; Carter, 2007; Maltepe and Fisher, 2015). Moreover, among eutherian mammals, placentation varies considerably in the degree of trophoblast invasiveness from minimal invasion occurring in epitheliochorial placentation (e.g. pigs and sheep), intermediate invasion in endotheliochorial placentation (e.g. dogs and cats), and maximal invasion in hemochorial placentation (e.g. humans and rodents) (Moffett and Loke, 2006; Carter, 2007; Wooding and Burton, 2008).

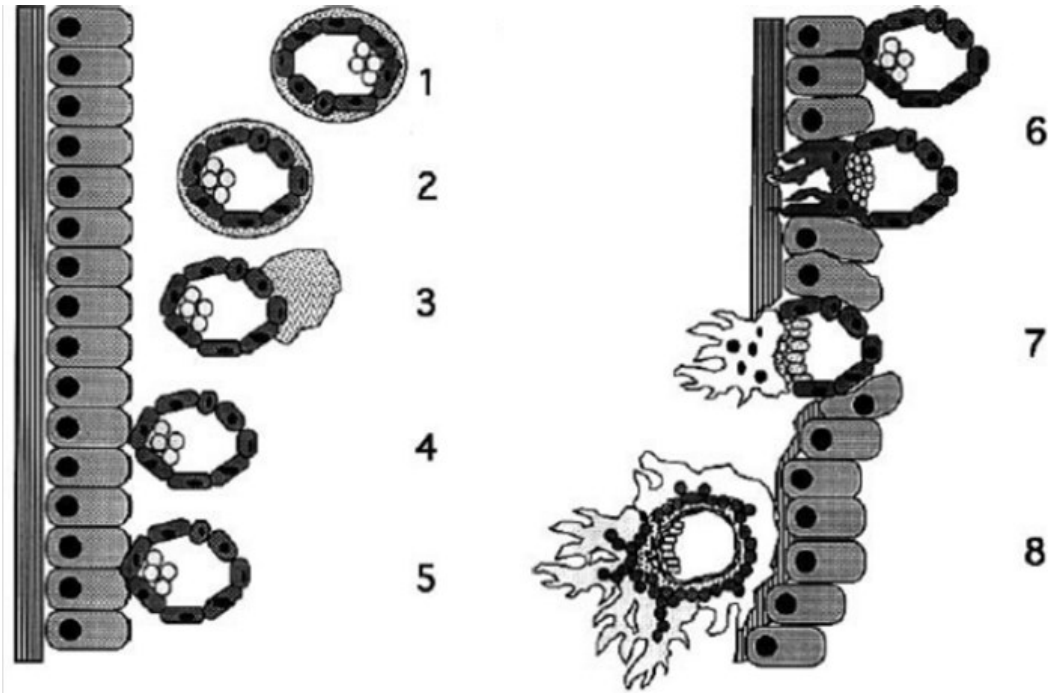

Figure 1. Embryo implantation. (1) Embryo is transported into the uterus, and **(2)** it takes the proper orientation for implantation, **(3)** then the pellucida is shed. This is followed by the three stages of implantation: **(4)** Apposition, **(5)** Adhesion and **(6)** Invasion. **(7,8)** Upon intimate contact with the endometrium, placentation can begin. Used with permission from Clancy, 2009 (Clancy, 2009).

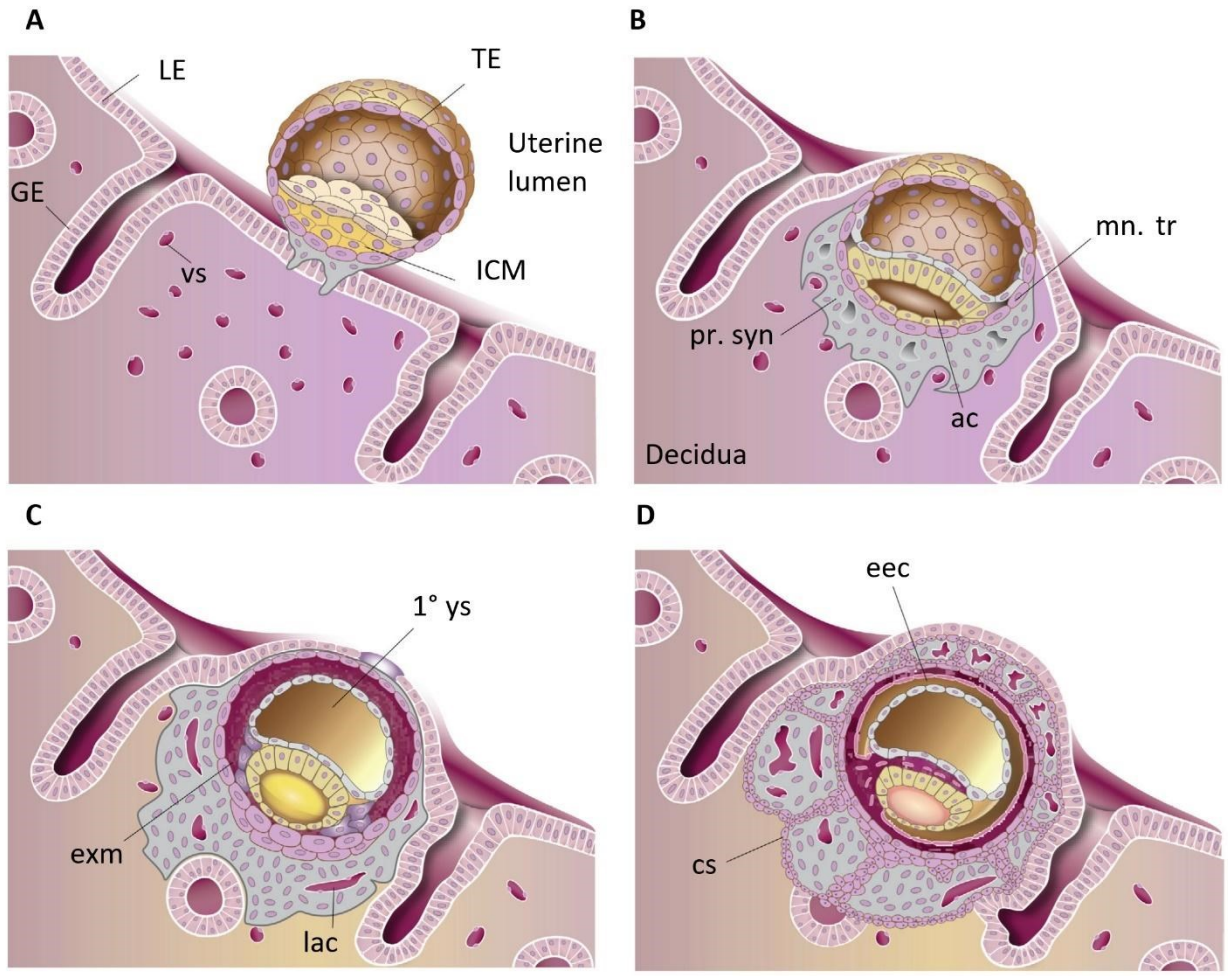

Figure 2. The early stages of placentation. (A) Blastocyst implantation occurs with the attachment of the polar trophectoderm (TE), which underlies the inner cell mass (ICM), into the surface of the uterine epithelium, the endometrium. **(B)** Next, in the first step of placentation, the pre-lacunar stage, the primary syncytium (pr. syn) invades into the transformed endometrium (decidua). **(C)** Then, in the lacunar stage, fluid-filled spaces known as lacunae (lac) start to form within the syncytium; these keep on enlarging and merging, and they later become the intervillous space. **(D)** In the primary villous stage, cytotrophoblasts proliferate and form projections that penetrate through the primary syncytium, then they merge laterally to form a continuous layer surrounding the developing embryo known as cytotrophoblast shell (cs). LE, luminal epithelium; GE, glandular epithelium; vs, blood vessels; mn. tr, mononuclear trophoblast; ac, amniotic cavity; 1° ys, primary yolk sac; exm, extra-embryonic mesoderm; eec, extra-embryonic coelom. Used with permission from Turco and Moffett, 2019 (Turco and Moffett, 2019).

In humans, placentation consists, in part, of the differentiation and proliferation of the TE to form a branching network of villi that are in direct contact with the maternal circulation while simultaneously maintaining a barrier between the fetal and maternal blood (Kaufmann et al., 2004; Wooding and Burton, 2008; Schmidt et al., 2015). The villi are the functional units of the placenta **(Figure 3)**. They facilitate and respond to the demands of the developing fetus by regulating the exchange of gases, nutrients, and wastes through the villous core, which consists of the mesenchyme and fetal blood vessels (Kaufmann et al., 2004). The tips of the branching villous network that come into direct contact with the decidua are termed the anchoring villi, while the remaining villi, which float freely in the blood-filled intervillous space, are called the floating villi (Maltepe et al., 2010).

The highly proliferative, undifferentiated cytotrophoblast (CTB) progenitor cells of the placental villi differentiate into two general pathways. CTB can either fuse to form a multinucleated monolayer of syncytiotrophoblasts (STBs) that enclose the villous stroma or differentiate into invasive extravillous trophoblasts (EVT) that infiltrate the decidualized endometrium and a portion of the myometrium (Cartwright et al., 2010). STBs function as a barrier, or more precisely, as an interface between fetal and maternal blood as well as in the production of pregnancy-associated hormones and growth factors necessary for placental and fetal development and growth (Fu et al., 2013a). The mechanisms that facilitate CTB fusion and production of the STB layer are still under investigation; however, formation of gap junctions, activation of apoptotic pathways, and the expression of endogenous retroviral proteins such as syncytin appear to be key mechanisms (Wooding and Burton, 2008).

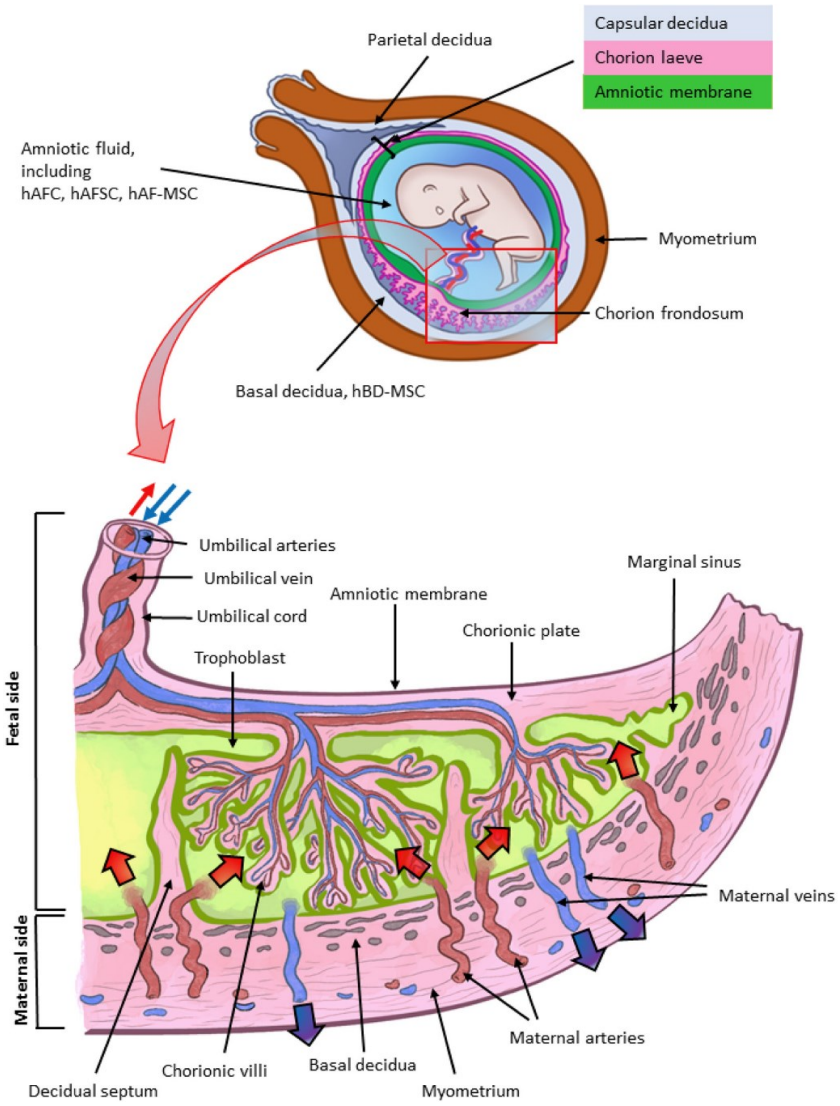

Figure 3. Structures of human placenta at term. In the pregnant uterus, the amniotic membrane adheres to the chorion laeve (so-called because it is devoid of villi), which is in touch with the capsular decidua. The amniotic cavity contains amniotic fluid with different types of detached cells: human amniotic fluid cells (hAFC), human amniotic fluid stem cells (hAFSC), and human amniotic fluid-mesenchymal stromal cells (hAF-MSC). Parietal decidua (decidua parietalis) underlines the myometrium. The basal decidua (decidua basalis), which contains human basal decidua-mesenchymal stromal cells (hBD-MSC), represents the maternal side/component of the human placenta. In contrast, the chorion frondosum, the chorionic plate and the fused amniotic membrane (placental portion) represent the fetal side/component of the human placenta. The magnified diagram shows a cross-section of a term placenta and the umbilical cord, showing the villous tree structure and the surrounding intervillous space. Arrows represent directions of blood flow. Used with permission from Silini et al. 2020 (Silini et al., 2020).

In the EVT pathway, the proliferating CTBs of the anchoring villi form a column that attaches to the uterine epithelium; subsequently, these column CTB differentiates into interstitial EVT (Anin et al., 2004; Ji et al., 2013). During early pregnancy, interstitial EVT (iEVT) invade the decidua and one-third of the myometrium, where they eventually differentiate into the multinucleated placental bed giant cells (Fu et al., 2013a). However, a subset of iEVT that reaches the walls of uterine spiral arteries are called endovascular EVT (enEVT). These cells start disrupting the vascular smooth muscle cell layer to invade into the arterial lumen and acquire endothelial-like characteristics to replace the endothelial cells in the maternal arteries in a process known as vascular mimicry (Ji et al., 2013). This results in the transformation of the spiral arteries into dilated, thin-walled vessels to ensure continuous maternal blood flow to the placenta and to maintain sufficient oxygen and nutrient supplies for the growing embryo (Anin et al., 2004; Lyall et al., 2013). Recently, endoglandular EVT (egEVT) have been identified as a potential third subtype of iEVT (Moser et al., 2010; Moser et al., 2015). Initial findings suggest that egEVT disintegrate uterine glands and open the gland lumen to the intervillous space, releasing glandular secretions to help support the embryo prior to the establishment of maternal placental blood flow (Burton et al., 2007; Moser et al., 2015), **(Figure 4)**.

I.2. Models to study human placental development

Many studies on human placental development, including the miRNA work discussed in the following sections, have been carried out using *in vitro* models, such as immortalized trophoblast and choriocarcinoma cell lines and primary cultures of trophoblasts, *ex vivo* models such as villous explants from first-trimester placentas that can be cultured with or without decidual tissue. In addition, rodents, especially mice, have also been used as *in vivo* models. It is important to recognize that each model has pros and cons. Although cell lines are easy to work with, especially with respect to transient and stable transfection of genetic material, there are significant differences in gene expression profiles between cell lines and primary trophoblasts (Bilban et al., 2010). For example, chromosome 19 miRNA cluster (C19MC) members are not expressed in HTR8/SVneo cells, while chromosome 14 miRNA cluster (C14MC) members cannot be detected in JEG-3 cells (Mouillet et al., 2011; Morales-Prieto et al., 2014).

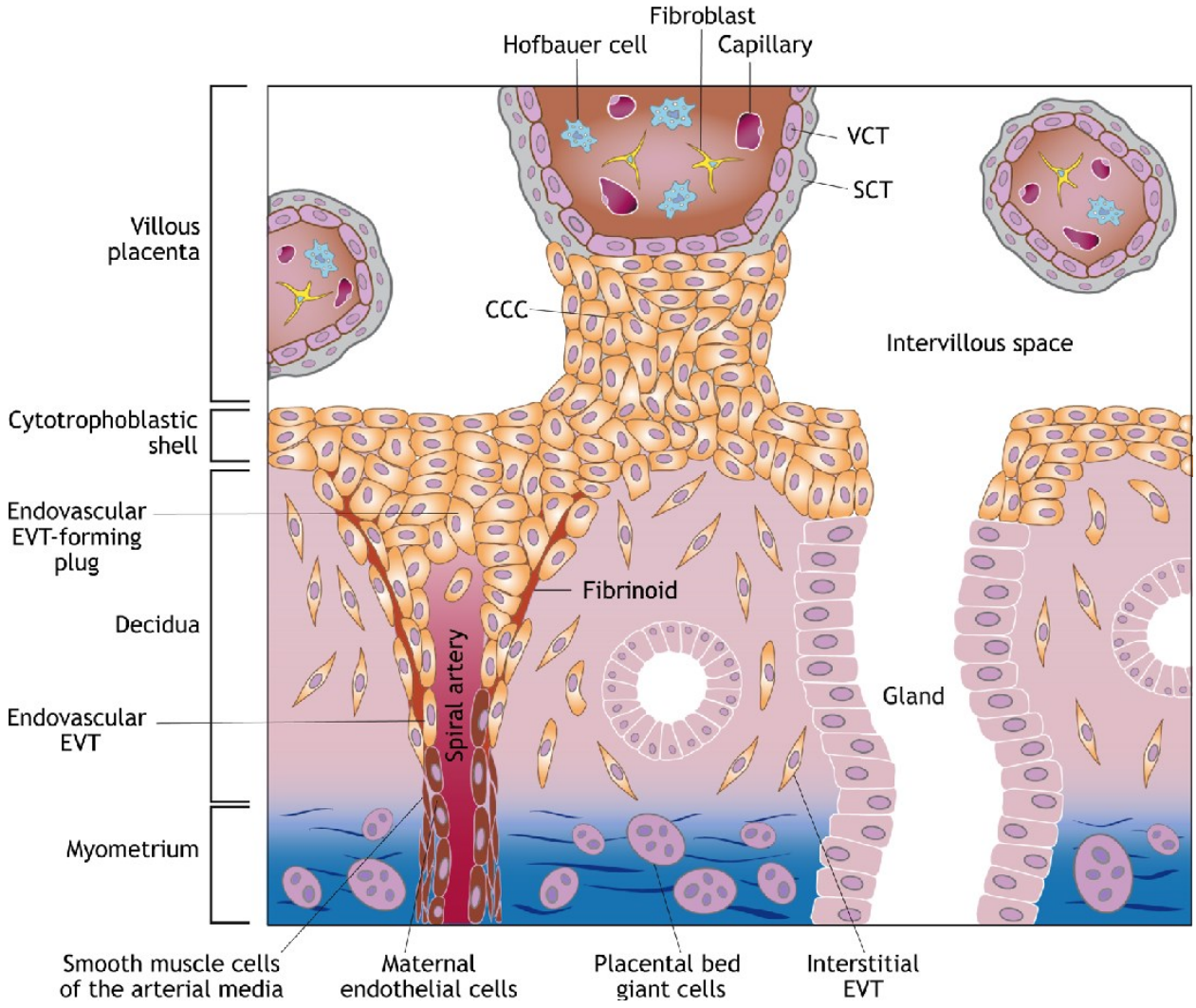

Figure 4. Trophoblast differentiation pathways. Early first trimester villi outer layer is made by syncytiotrophoblasts (SCT, grey) with villous cytotrophoblast (VCT, pink) underneath. In anchoring villi, the cytotrophoblast cell column (CCC) is in contact with the decidua where interstitial extravillous trophoblast (iEVT) populations invade the decidua and can differentiate into endovascular EVT (orange), placental bed giant cells, or endoglandular EVT. Used with permission from Turco and Moffett, 2019 (Turco and Moffett, 2019).

Also, many of these cell lines were generated decades ago; for example, HTR8/SVneo were generated in 1993 (Graham et al., 1993) while BeWo cells in 1968 (Pattillo and Gey, 1968), resulting in the accumulation of mutations over time. Moreover, culturing conditions are maintained differently in various labs around the world, which can lead to contrasting results in different studies. Therefore, there is a need for continuous validation and monitoring of cell lines and comparing their miRNA and gene expression profiles against primary cells. To overcome some of these issues, researchers have isolated primary CTBs, albeit using laborious and time-consuming protocols. They have been used mainly to study the differentiation of CTB to STB, but these cells have a limited life span and can only be used to study the short-term effects of transiently transfected miRNA, for example. There has also been continuous work in order to generate CTB stem cell lines from human embryonic stem cells, human pluripotent stem cells, or CTB from term placentas (Xu et al., 2002; Harun et al., 2006; Bai et al., 2021; Mischler et al., 2021). These stem cell lines facilitated a recent work that identified trophoblast fate-specifying miRNA (Nosi et al., 2017).

Placental villous explant and placental explant-decidua co-culture models are commonly employed as *ex vivo* models used in the study of placenta development. Importantly, villous explants maintain the cellular architecture and mimic more closely the *in vivo* environment (Miller et al., 2005). Also, the placental explants/decidua co-culture model provides insight into processes that occur at the maternal-fetal interface, such as the interaction between trophoblast and decidual cells and spiral artery remodelling (Dunk et al., 2003). However, only the short-term effect of miRNA overexpression or inhibition can be examined as these explants can be cultured only for days. It is also crucial to culture these explants under conditions that resemble *in utero* environments that match the gestational age of these explants, such as different oxygen levels, extracellular matrices, and specific culture media (Miller et al., 2005).

The mouse model provides some insights into the *in vivo* functions of miRNA, but it should be noted that there are significant differences in placentation between mouse and human that can affect the transferability of findings to humans (Wildman et al., 2006; Carter, 2007; Maltepe and Fisher, 2015; Schmidt et al., 2015; Grigsby, 2016). For example, trophoblast invasion during early mouse placentation is shallower compared to humans as it only extends into the decidua,

whereas in humans, it proceeds to the myometrium (Carter, 2007; Maltepe and Fisher, 2015; Schmidt et al., 2015). Also, mouse trophoblasts express major histocompatibility complex (MHC)-K, -D and -L, while human trophoblasts express human leukocyte antigen G (HLA-G) or HLA-C. This leads to different interaction dynamics between uterine immune cells and invading trophoblasts (Chaouat and Clark, 2015; Schmidt et al., 2015). Importantly, there are different miRNA expression profiles between human and mouse placentas. Specifically, C19MC, a chromosome 19 cluster of miRNA expressed within a single RNA transcript, is expressed only in primates with no orthologs found in rodents (Morales-Prieto et al., 2014), while miRNA of the *Sfmbt2* cluster are rodent-specific (Zheng et al., 2011; Schmidt et al., 2015; Inoue et al., 2017). Also, C14MC in humans shows a divergence in rodents where it is located on chromosome 12 and lacks multiple members found in humans (Seitz et al., 2004). Therefore, in the following discussion, we will point out which model(s) was used in each study.

I.3. Role of caudal-related homeobox transcription factor 2 in placenta development

Caudal-related homeobox transcription factor 2 (CDX2) is a transcription factor known to be vital for establishing trophectoderm (Kunath et al., 2004; Hemberger et al., 2010; Sakurai et al., 2010). While an early study reported that *CDX2* mRNA was not detected in placental cells beyond the first trimester (Hemberger et al., 2010), a recent study showed that CDX2 expression is maintained in trophoblasts throughout gestation (Weber et al., 2013; Vadakke-Madathil et al., 2019) especially in the CTB population which is considered the trophoblast progenitor cells that maintain and replenish the STB layer and the villous structure (Kolahi et al., 2017). Another study suggested that CDX2 plays a role in histone acetylation resulting in the expression of trophoblast-specific genes (Sakurai et al., 2010).

In the EVT cell line HTR8/SVneo, CDX2 was shown to be regulated by Yes-associated protein (YAP), which promoted invasion and reduced apoptosis in these cells (Sun et al., 2018). Also, overexpressing CDX2 in HTR8/SVneo promoted cell invasion through the activation of matrix metalloproteinase-9 (MMP9) and the inhibition of the MMP9 inhibitor, tissue inhibitor metalloproteinase-1 (TIMP1), and that CDX2 expression is regulated by the phosphatidylinositol-

3-kinase (PI3K) and protein kinase B (AKT) signalling pathway (Jia et al., 2014). Our lab showed that miR-210-3p can target CDX2 and that reduced CDX2 levels decreased HTR8/SVneo cells' potential to form endothelial-like networks and downregulated the expression of many enEVT markers (Hayder et al., 2021).

Using available trophoblast cell lines to investigate implantation and early events in placenta development has its limitations. Therefore, work has been done to derive trophoblast stem cells from human pluripotent stems cells or from term CTB (Harun et al., 2006; Bai et al., 2021; Mischler et al., 2021). CDX2 expression in these derived trophoblast cells is considered the hallmark of successful reprograming of pluripotent stems cells into trophoblast stem cells (Hemberger et al., 2010; Takao et al., 2011; Chang and Parast, 2017). These cells provide an important *in vitro* model to better study trophoblast differentiation and in understanding how defects in this process can lead to pathological placenta development and pregnancy-associated complications.

Interestingly, a study suggested that CDX2-positive trophoblast cells isolated from human term placenta may have a therapeutic use in humans. The authors showed that Cdx2-positive trophoblasts isolated from mouse term placentas exhibited the ability to migrate toward sites of injury in the heart in a mouse acute myocardial infarction model and differentiated into cardiomyocytes (Vadakke-Madathil et al., 2019). These cells exhibited the stem-like characteristics seen in embryonic stem cells but with the homing and immune surveillance avoidance characteristic of trophoblasts.

I.4. Disorders associated with abnormal placental development

The intricate events involved in implantation, placentation and placental vascularization are tightly regulated by complex interactions of a large group of molecules, including growth factors, hormones, cell adhesion molecules, proteases and cytokines from a variety of cell types (Rossant and Cross, 2001; Red-Horse et al., 2004). Disruption of the pathways involved in placental development, especially during the early stage of gestation, can lead to complications

later in pregnancy, such as preeclampsia (PE) and intra-uterine growth restriction (IUGR) (Norwitz, 2007).

I.4.1 Preeclampsia

Preeclampsia (PE) is one of the leading causes of maternal morbidity, mortality and premature delivery affecting 3-4 % of pregnancies (Cartwright et al., 2010; Rana et al., 2020). These numbers increase dramatically in developing countries, where 42% of all pregnancy-related deaths are attributed to this disorder (Noris et al., 2005). It is typically diagnosed after 20 weeks of gestation by the onset of hypertension (≥ 160/110 mmHg) and proteinuria (≥ 5 g in 24 h) in the mother (Leone and Einav, 2015).

The cause of PE remains elusive, yet this disorder is recognized to be placental in origin (Noris et al., 2005; Staff, 2019). Defects at any stage during placental development and spiral artery remodelling are thought to contribute to the onset of this disorder in most but not all cases (Fisher, 2015; Staff, 2019). In general, it is characteristic of PE placenta to have poor trophoblast migration and invasion into the decidua, along with inadequate placental perfusion due to insufficient spiral artery remodelling (Lala and Chakraborty, 2003; Cartwright et al., 2010) **(Figure 5).**

Studies of PE-compromised pregnancies have revealed many factors potentially responsible for abnormal placentation. Poor CTB differentiation has been observed along with an accumulation of intermediate CTB populations contributing to the overall smaller size of PE placentas (Cheng and Wang, 2009). Many differences in the EVT subpopulations and their involvement in spiral artery remodelling have been continuously observed in PE studies (Noris et al., 2005; Wang et al., 2009; Silasi et al., 2010). Interstitial EVT, which can secrete vasodilators that help prime the spiral arteries before endovascular EVT invasion, have been reported as significantly reduced both in number and density in the decidua of compromised pregnancies. Based on these and many other findings, this systemic pregnancy disorder has been summarized into two general stages: 1) abnormal placentation and 2) endothelial dysfunction and the manifestation of clinical symptoms (Cheng and Wang, 2009; Staff, 2019).

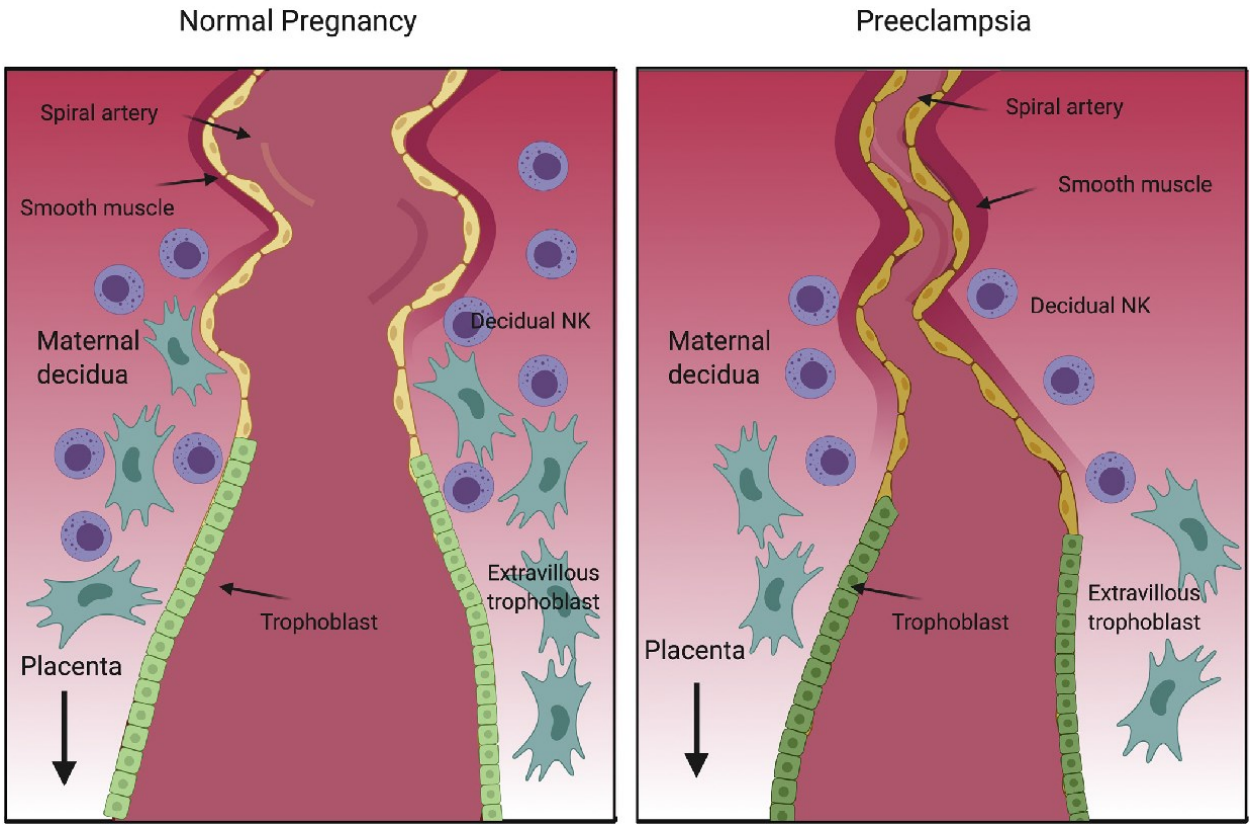

Figure 5: Placental spiral artery remodelling in normal and PE pregnancies. In normal placental development, invasive trophoblasts invade the maternal spiral arteries and differentiate from an epithelial phenotype to an endovascular phenotype. This leads to transforming these vessels from small-diameter, high-resistance vessels to large-diameter, low-resistance vessels capable of providing placental perfusion needed to meet the demands of the growing fetus (left panel). In PE, cytotrophoblasts fail to adopt an invasive endovascular phenotype. Thus, their invasion of the spiral arteries is shallow, and the spiral arteries remain as high resistance vessels with high blood velocity leading to placental malperfusion and oxidative and sheer damage of the villous structure (right panel). Used with permission from Rana et al. 2020 (Rana et al., 2020).

Studies have also shown disruption of enEVT migration and invasion, along with an increase in apoptosis (Lala and Chakraborty, 2003). Furthermore, enEVT differentiation was shown to be compromised due to a reduction in the transition from the epithelial to endovascular phenotype (Wang et al., 2009). Reported alterations in the syncytial layer are also consistent, such as an increase in shedding into the maternal circulation, a decrease in cell-cell fusion events

and a reduction in the number of nuclei (Cheng and Wang, 2009). Increased syncytial shedding may be due to a decrease in glial cells missing-1 (GCM-1), a transcription factor involved in syncytial formation and turnover regulation, as well as due to contact with higher velocity blood flow as a result of insufficient spiral artery remodelling (Baczyk et al., 2009; Cartwright et al., 2010). Furthermore, there is evidence to suggest that along with the increased syncytial shedding, there is a switch from the normal apoptotic to the necrotic pathway making the debris more pro-inflammatory compared to normal pregnancies (Paria et al., 2002). These findings coincide with the observed increase in maternal systemic inflammation in the third trimester in women with PE accompanied by clinical manifestation of an increase in blood pressure and kidney stress (Lockwood et al., 2011; Staff, 2019).

Moreover, poor placental perfusion has been shown to lead to an imbalance of angiogenic factors, which initiate widespread endothelial dysfunction and can lead to subsequent multisystem organ defects in the mother seen in severe PE (Carty et al., 2008). Soluble fms-like tyrosine kinase 1 (sFLT1), also known as soluble vascular endothelial growth factor receptor 1 (sVEGFR1), is commonly reported as being elevated in maternal serum in PE. sFLT1 upregulation correlates with the findings that vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) are reduced in PE, both of which contribute to normal CTB function (Carty et al., 2008). In addition, decreased VEGF levels contribute to the inhibition of CTB invasion and EVT-induced spiral artery remodelling (Norwitz, 2007). Studies of CTB *in vivo* have also indicated that high levels of sFLT1 decreased CTB invasiveness (Wang et al., 2009). The reason for this impaired CTB invasion and spiral artery remodelling in PE is still unclear. Currently, the only treatment option for PE is premature delivery or termination of the pregnancy (Noris et al., 2005; Rana et al., 2020).

Many studies have investigated changes in the placental transcriptome, including miRNA expression, in pregnancy complications such as PE, including severe PE, and early or late-onset PE. Studying PE pregnancies that are also compromised by other pregnancy complications such as small or large for gestational age babies, gestational diabetes, or IUGR has been used to better understand the mechanisms that give rise to these complications. More specifically, to investigate whether there are common molecular signatures between these disorders and help

identify potential biomarkers and therapeutic targets (Mayor-Lynn et al., 2011; Sõber et al., 2015; Luo et al., 2017; Zhong et al., 2019). Thus, the search for and identification of reliable and PE-specific biomarkers, including miRNA, is one of the main interests of current PE research (Lala and Chakraborty, 2003; Carty et al., 2008; Rana et al., 2020).

I.4.2 Intra-uterine growth restriction

Intra-uterine growth restriction (IUGR) is the leading cause of fetal mortality and morbidity and is clinically defined as placental insufficiency that results in a small for gestational age (SGA) fetus, where the fetus measures <10th percentile for gestational age (Cetin and Alvino, 2009; Rana et al., 2020). Although 30% of PE cases are associated with IUGR, this disorder is still seen in 8-14% of non-PE pregnancies (Cetin and Alvino, 2009).

The cause of IUGR is presumed to have fetal, maternal and placental components, where placental abnormalities are the only component clinically implicated. Studies indicate that abnormalities in spiral artery remodelling, poor placental vascularization, and insufficient villous development might all be contributing factors (Baschat and Hecher, 2004; Cartwright et al., 2010). These abnormalities alter placental metabolism and transport rate leading to fetal malnutrition and low birth weight (Cetin and Alvino, 2009). Specifically, a reduction in placental to fetal weight ratio and inefficient maternal-fetal exchange of nutrients and oxygen are the main phenotypical changes reported (Pardi et al., 2002). Malnutrition during gestation leading to low birth weights of IUGR compromised pregnancies has demonstrated an increase in poor perinatal and later-life health. Direct nutritional supplementation of the mother and/or fetus is the leading therapeutic strategy used to manage this condition. However, the timing of delivery remains the most critical aspect of IUGR compromised pregnancies (Pardi et al., 2002; Baschat and Hecher, 2004).

Our current understanding of placental development and alterations of placental tissue that lead to gestational diseases is limited. Future research must focus on the early events of gestation, such as placentation, spiral artery remodelling, and placental vascularization, to reveal the cause of PE and IUGR and contribute to the development of improved treatment options.

II. MicroRNA and their role in placental development

MicroRNAs (miRNA) have been established as major regulators of gene expression and are involved in many biological processes (Vasudevan, 2012; Jonas and Izaurralde, 2015). Since their discovery in 1993, miRNA have been of great interest to researchers, and many new advances have been made in understanding their structure, regulation, and mechanisms of action (Lee et al., 1993; Jonas and Izaurralde, 2015). Most studies have shown that miRNA suppress gene expression when bound to the 3' untranslated region (UTR) of target mRNAs by inhibiting translation and reducing mRNA stability (Behm-Ansmant et al., 2006; Chen et al., 2010; Miao et al., 2016). However, additional modes of action for miRNA, such as transcriptional regulation and activation of gene expression, have also been reported (Benhamed et al., 2012; Vasudevan, 2012; Catalanotto et al., 2016; Miao et al., 2016).

II.1. miRNA biogenesis

The vast majority of miRNA are processed through the canonical biogenesis pathway (Kim et al., 2016). Canonical miRNA biogenesis begins with the detection of the primary miRNA transcript (pri-miRNA), contained within nascent RNA, by DiGeorge Syndrome Critical Region 8 (DGCR8) and associated proteins through recognition of the RNA N6-methyladenylated GGAC motif (Alarcon et al., 2015). In complex with DGCR8 is the nuclear RNase III endonuclease Drosha which cleaves the pri-miRNA duplex proximal to the base of the characteristic hairpin structure of pri-miRNA. This produces the excised precursor (pre)-miRNA containing a 2 nucleotide 3' overhang (Han et al., 2004). Together, Drosha and DGCR8 are termed the microprocessor complex (Denli et al., 2004).

Following pri-miRNA cleavage, the pre-miRNA is exported to the cytoplasm through an exportin 5 (XPO5)/RanGTP complex and then processed by the predominantly cytoplasmic RNase III endonuclease Dicer (Denli et al., 2004; Doyle et al., 2013). This cleavage, which removes the terminal loop, produces the mature miRNA duplex from pre-miRNA (Zhang et al., 2004). The

labelling of the two strands of the miRNA duplex is based on the directionality of the strand in the pre-miRNA. The 5' end of the pre-miRNA hairpin contains the 5p strand, and the 3' end the 3p strand (previously miRNA and miRNA*). Either the 5p or 3p strand of the miRNA duplex can be loaded into the Argonaute (AGO) family of proteins (AGO1-4 in humans) in an ATP-dependent manner (Yoda et al., 2010; Ha and Kim, 2014); the strand that is loaded into AGO is termed the guide strand **(Figure 6)**.

Several non-canonical miRNA biogenesis pathways have been elucidated (Ruby et al., 2007; Babiarz et al., 2008; Yang and Lai, 2011; Abdelfattah et al., 2014; Ha and Kim, 2014) and grouped into two general categories: Drosha/DGCR8-independent and Dicer-independent. These non-canonical pathways take advantage of the cellular machinery already in place to produce canonical miRNA by producing Drosha, Dicer and Argonaute substrates from discrete RNA sources such as small hairpin RNAs (shRNA), small nucleolar RNAs and splicing products (Yang and Lai, 2011; Castellano and Stebbing, 2013; Abdelfattah et al., 2014). Drosha/DGCR8-independent pre-miRNA share a common trait in which separate processing mechanisms produce products that resemble Dicer substrates. For example, mirtrons encompass the group of pre-miRNA produced from introns during mRNA splicing. Additionally, 7-methylguanosine (m^7G)-capped pre-miRNA are transcribed such that the nascent RNA does not need Drosha cleavage and can be directly exported from the nucleus through exportin 1 (Xie et al., 2013). Moreover, the m^7G cap is thought to be the cause of a strong 3p strand bias.

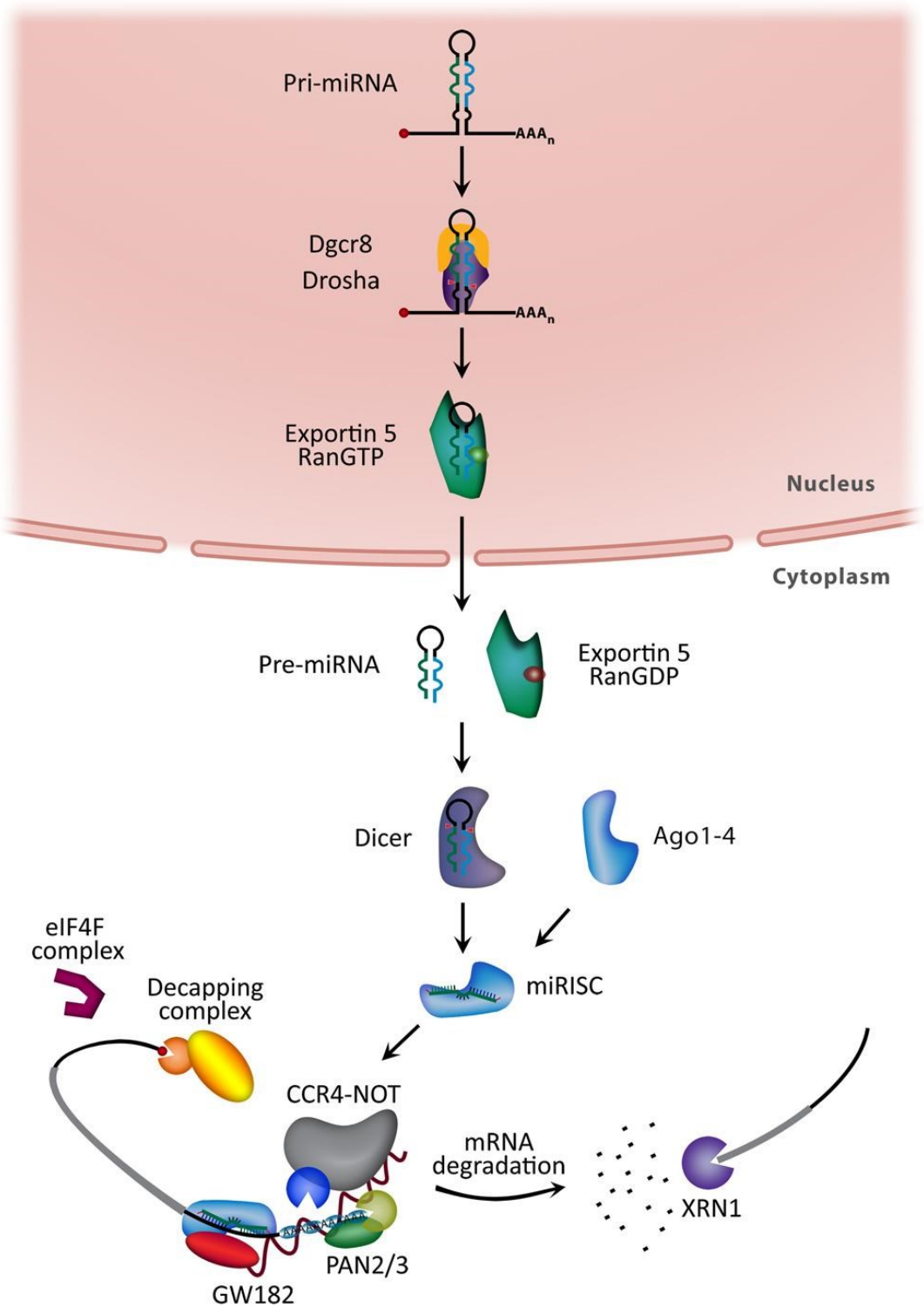

Figure 6 legend is on the next page

Figure 6: Overview of canonical microRNA biogenesis and mechanism. Canonical miRNA biogenesis is both Drosha- and Dicer-dependent. Following transcription, the primary (pri-) miRNA is identified and cleaved by the endoribonuclease, Drosha, to produce the precursor (pre-) miRNA. Nuclear export of the pre-miRNA is facilitated by the Exportin 5/RanGTP transport system. Once in the cytoplasm, the pre-miRNA is subject to terminal loop cleavage by the endoribonuclease Dicer. After cleavage, the mature miRNA duplex is loaded into the Argonaute family of proteins, and the passenger strand is degraded, forming the miRNA-induced silencing complex (miRISC). The gene regulatory power of cytoplasmic miRISC typically culminates in gene silencing by mediating induction of translation inhibition, mRNA poly(A) deadenylation and mRNA degradation via interaction at the 3′ untranslated region of target mRNA. After target association and following recruitment of GW182 and associated proteins into miRISC, translation initiation is inhibited, preventing nascent protein translation of the target mRNA molecule. It is hypothesized that miRISC-induced dissociation of the translation initiation complex, eIF4F, from the 5′ cap of mRNA and/or its functional disruption suppresses translation initiation. Interaction of GW182 with poly(A) binding proteins (PABPC) and poly(A) deadenylase complexes PAN2/3 and CCR4-NOT localizes the 3′ mRNA tail to the miRISC complex, promoting efficient target mRNA deadenylation. Complete poly(A) deadenylation leads to decapping-protein 2 (DCP2)-mediated mRNA decapping, exposing the mRNA to 5′–3′ degradation via the exoribonuclease XRN1 (Hayder et al., 2018).

Dicer-independent miRNA are processed from endogenous shRNA transcripts by Drosha and may be unique in their requirement for AGO2 to complete their processing within the cytoplasm. This group of pre-miRNA is too short to be processed by Dicer, leading to the 5′ loading of the entire pre-miRNA into AGO2 (Abdelfattah et al., 2014). Slicing of the 3p strand and 3′–5′ trimming creates a strong 5p strand bias. Although non-canonical miRNA may elicit post-transcriptional silencing capabilities and undergo regulation independent of canonical miRNA, the vast majority of miRNA are processed through the canonical biogenesis pathway, requiring both Drosha and Dicer to complete their maturation (Kim et al., 2016). However, consistent with their canonical counterparts, these non-canonical miRNA have been linked to various cellular programs such as proliferation, de/differentiation, immune response, neural development and cellular metabolism (Abdelfattah et al., 2014).

Once AGO proteins are loaded, and the miRNA duplex unwound, they form the minimal miRNA-induced silencing complex (miRISC) (Kawamata and Tomari, 2010; Fabian and Sonenberg, 2012). miRISC gains target specificity by recognition of miRNA response elements (MRE) on target RNA molecules while the degree of complementarity determines, to some extent, the mode of regulation, i.e., direct or indirect gene silencing (Ameres et al., 2007; Jonas and Izaurralde, 2015). A fully complementary miRNA:MRE promotes AGO2 endonuclease activity and cleavage of the target RNA molecule (Ameres et al., 2007). In turn, this also has the consequence of decreased miRNA stability as exact matches promote not only target cleavage but the degradation of the guide miRNA as well, although the mechanism is not well understood (Ameres and Zamore, 2013). What is known is that the guide miRNA must first undergo the 3′ addition of adenosine or uracil, which promotes 3′-5′ exonuclease activity resulting in guide miRNA degradation (Krutzfeldt et al., 2005; Ameres et al., 2010).

In humans, the frequency of exact matches on target mRNA is rare (Jonas and Izaurralde, 2015). The majority of validated MREs contain at least central mismatches to their guide miRNA, preventing AGO2 endonuclease activity. Consequently, AGO2 shifts from RNAi effector to mediator, and along with the non-endonucleolytic AGO family members, act to recruit other proteins associated with mRNA stability. This has led to the detection of the miRNA seed region (nucleotides 2-8) that are crucial for many but not all miRNA:MRE interactions (Ellwanger et al.,

2011; Xu et al., 2014b; Miao et al., 2016). In most cases, miRNA interact with the 3' UTR of target mRNAs, resulting in translation inhibition and mRNA deadenylation and decapping (Huntzinger and Izaurralde, 2011; Fabian and Sonenberg, 2012; Meijer et al., 2013; Ipsaro and Joshua-Tor, 2015).

To form a miRISC complex capable of post-transcriptional gene silencing, mRNA-bound miRISC recruits the GW182 family of proteins which acts as a scaffold to recruit other effector protein complexes (Behm-Ansmant et al., 2006). Both the PAN2-PAN3 and CCR4-NOT deadenylase complexes are recruited through the unstructured, tryptophan (W) repeats of GW182 (Christie et al., 2013; Jonas and Izaurralde, 2015). PAN2-PAN3 initially catalyzes target mRNA poly(A) deadenylation, which is promoted through the interaction of W-repeats to poly(A)-binding proteins (PABPC), bringing both the mRNA poly(A) tail and deadenylase into close proximity (Jonas and Izaurralde, 2015). The CCR4-NOT complex completes the deadenylation process and is followed by mRNA decapping facilitated by decapping protein 2 (DCP2) and associated proteins (Behm-Ansmant et al., 2006). Decapped and deadenylated mRNA are then degraded from the 5' end by the 5'-3' exoribonuclease 1 (XRN1) (Braun et al., 2012) **(Figure 6)**.

While most miRNA studies focus on how miRNA target mRNA by binding to MREs at the 3' UTR to suppress their expression, MREs have also been reported in the 5' UTR. miRISC interactions within the 5' UTR have been shown to both promote and suppress translation through mRNA-specific mechanisms, discussed in detail in (Vasudevan, 2012; Valinezhad Orang et al., 2014). Moreover, cell state-specific miRNA-mediated translational activation has been observed in human quiescent cells where nuclear AGO2 complexes with Fragile-x-mental retardation related protein 1 (FXR1) instead of GW182 (Truesdell et al., 2012). This complex was found to interact with nuclear mRNA targets which in turn led to translational activation following export to the cytoplasm (Truesdell et al., 2012).

II.2. Role of miRNA in placental development

II.2.1 miRNA in trophectoderm development and implantation

Many studies in mice suggest that miRNA play a role in trophectoderm development. Examination of mouse miRNA expression patterns during trophectoderm specification has revealed let-7, miR-21, miR-29c, miR-96, miR-125a, miR-214, miR-297, miR-376a, and miR-424 as candidates that may play a role in this process (Viswanathan et al., 2009; Nosi et al., 2017). In mouse embryonic stem cells (ESC), overexpression of miR-15b, miR-322, and miR-467 suppressed their embryonic fate and led to the induction of a trophoblast stem cell (TSC)-like phenotype. Further analysis revealed that these miRNA target transcription factors *Sall1*, *Sall4*, *Pou5f1*, and *Nanog* (Nosi et al., 2017), which are important for the maintenance of ESC self-renewal and pluripotency. In addition, the miR-302/367 cluster was found to promote TE differentiation in humans by targeting bone morphogenetic protein (BMP) inhibitors TOB2, DAZAP2, and SLAIN1 (Lipchina et al., 2011); BMP4 is a member of the transforming growth factor beta (TGFB) superfamily and is involved in promoting TE differentiation (Xu et al., 2002; Wu et al., 2008). In a human pulmonary artery cell line, miR-302 was also shown to target BMP4 receptor 2, while BMP signalling led to the transcriptional downregulation of the miRNA-302/367 gene cluster (Kang et al., 2012), which if it also occurs in trophoblasts could create an interesting signal-buffering dynamic.

Limited evidence obtained so far has suggested that miRNA play a role in regulating implantation. First, studies in mice have shown that miRNA are differentially expressed between implantation sites and inter-implantation sites in the endometrium (Chakrabarty et al., 2007; Hu et al., 2008; Geng et al., 2014). Further studies revealed that overexpression of miR-145 impaired the attachment of mouse embryos to endometrial epithelial cells by targeting insulin-like growth factor 1 receptor (*Igf1r)* (Kang et al., 2015). Finally, Dicer knockdown in mouse blastocysts altered miRNA expression and resulted in a lower implantation rate (Cheong et al., 2014). In humans, a number of miRNA in the endometrium, including miR-145, were also found to be differentially expressed between women who repeatedly fail to have successful implantation and fertile women (Revel et al., 2011). These findings suggest a possible role of miRNA in regulating

implantation; however, more studies are required to understand the functions of miRNA and their underlying mechanisms in this process.

Another important aspect of successful implantation is the interaction between the blastocyst and the maternal immune cells. Early in pregnancy, maternal uterine natural killer (uNK) cells, T cells, B cells, macrophages and dendritic cells are recruited into the endometrium at the site of implantation to help regulate placental and fetal development (Szekeres-Bartho, 2002; Bidarimath et al., 2014; Zhang et al., 2016a). Human EVT expresses a limited variety of MHC molecules, mostly HLA-G and HLA-C (Bidarimath et al., 2014; Schmidt et al., 2015; Hackmon et al., 2017). HLA-G interacts with the maternal killer immunoglobulin-like receptors expressed by uNK cells, resulting in the activation of uNK cytokine production but not its cytotoxicity response (Rajagopalan et al., 2006). This, in turn, promotes maternal immunological tolerance and placental development and vascularization (Bidarimath et al., 2014; Ratsep et al., 2015). Both miR-148a and miR-152 were found to bind the 3' UTR of *HLA-G*, amplified from the JEG-3 human trophoblast cell line, downregulating its expression and thereby reducing HLA-G mediated inhibition of natural killer cells cytotoxicity (Manaster et al., 2012). These findings suggest that miRNA play a role in regulating maternal immunological tolerance to invading iEVT. In addition, miRNA have also been shown to help regulate other maternal immune cells such as macrophages, endometrial dendritic cells and T cells in the pregnant uterus and have been extensively reviewed in (Robertson and Moldenhauer, 2014; Mori et al., 2016; Schjenken et al., 2016; Robertson et al., 2017).

Interestingly, miRNA were also shown to promote antiviral immunity in both trophoblast and non-trophoblast cells. In alignment with the role of placenta to protect the developing fetus, trophoblasts are the first line of defence against external factors that can impair fetal development. Therefore, it is not surprising that primary human trophoblasts are highly resistant to viral infection (Delorme-Axford et al., 2013). More importantly, they can confer this resistance to other types of cells when these cells uptake exosomes naturally secreted by primary trophoblasts; the exosomes were found to contain members of C19MC, miR-512-3p, miR-516b-5p and miR-517-3p (Bayer et al., 2015). C19MC miRNA initiated autophagy in recipient cells without leading to cell death which was suggested to impair viral replicability (Delorme-Axford

et al., 2013; Bayer et al., 2015). Thus, miRNA play a dynamic role in promoting decidual immune tolerance in support of the growing fetus and protecting both mother and fetus from viral infection (Mouillet et al., 2014; Ouyang et al., 2014).

II.2.2 miRNA in trophoblast differentiation, migration and invasion

Several studies have suggested that miRNA are important regulators of CTB to STB differentiation. Microarray analyses of miRNA expression profiles in primary trophoblast before and after their differentiation into STB have revealed that multiple members of C19MC such as miR-515-5p, miR-518f, miR-519c-3p and miR-519e-5p were significantly downregulated during CTB to STB differentiation (Zhang et al., 2016b). Further investigation showed that miR-515-5p targeted several genes that play critical roles in STB differentiation, including human glial cell missing-1 (GCM1) (Yu et al., 2002; Liang et al., 2010; Wakeland et al., 2017) and frizzled 5 (FZD5) (Lu et al., 2013) and significantly reduced cell fusion (Zhang et al., 2016b). Another miRNA gene cluster is the miR-17~92 family that is located on chromosome 13 and encodes six miRNA (miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a, and miR-92a) (Concepcion et al., 2012). Multiple members of the miRNA-17~92 cluster, and its paralog cluster miR-106a~363, have been found to silence GCM1 in primary cultures of human trophoblasts. These miRNA are downregulated during CTB to STB differentiation, promoting the differentiation process (Kumar et al., 2013). Studies from our lab have demonstrated that miR-378a-5p suppressed BeWo cell fusion and STB marker gene expression by targeting cyclin G2 (CCNG2), suggesting that miR-378a-5p inhibits STB differentiation (Nadeem et al., 2014).

Multiple miRNA regulate EVT differentiation, migration and invasion by targeting key pathways that regulate these processes. Early placentation occurs in a hypoxic environment, and oxygen tension has been reported to regulate many cellular processes in the placenta, including proliferation, EVT differentiation and invasion (Chang et al., 2018). However, the precise role of oxygen tension in EVT differentiation and invasion is still not well understood. As the trophoblasts invade deeper into the uterus, where oxygen levels are higher, they shift from a more proliferative phenotype to a more migratory and invasive phenotype (Genbacev et al., 1997; Kaufmann and Castellucci, 1997; Knofler, 2010). However, hypoxia was recently found to

promote EVT differentiation in a hypoxia-inducible factor (HIF)-dependent manner while inhibiting STB differentiation in primary cultures of human CTB (Wakeland et al., 2017). Thus, it is proposed that low oxygen induces the differentiation into immature EVT, but further maturation of EVT and invasion increase with rising oxygen tension (Chang et al., 2018).

Since hypoxia plays an important role in early placental development, studies have investigated its effects on miRNA expression and function (Donker et al., 2007; Mouillet et al., 2010; Fu et al., 2013a). They revealed a group of miRNA that are upregulated under hypoxia, a subset of which, hypoxamirs, are under direct regulation of hypoxia-induced transcription factors (Kulshreshtha et al., 2007). miR-210-3p is the most well-studied example of hypoxamirs, upregulated directly by HIF1A (Camps et al., 2008); additionally, it is regulated by a hypoxia-responsive transcription factor, nuclear factor kappa-B subunit p50 (NFKB1), in primary human trophoblasts (Zhang et al., 2012). It was reported that miR-210-3p inhibited migration and invasion in primary CTBs (Zhang et al., 2012), HTR8/SVneo cell line (Luo et al., 2016; Hayder et al., 2021), and primary ETVs (Anton et al., 2013) by targeting ephrin-A3 (EFNA3), homeobox-A9 (HOXA9) (Zhang et al., 2012), and thrombospondin type I domain containing 7A (THSD7A) (Luo et al., 2016) or by activating the MAPK pathway (Anton et al., 2013). Also, miR-210-3p impaired the ability of HTR8/SVneo cell line to form tube-like network and decreased many markers associated with endovascular differentiation (Hayder et al., 2021). In mice, however, knockout of mir-210-3p did not result in significant changes in fetal or placental weight, and non-severe hypoxia (12% O_2) did not increase miR-210-3p level, suggesting that miR-210-3p may be dispensable for fetal-placental development under normoxic and non-severe hypoxic conditions (Krawczynski et al., 2016). Thus, the role of miR-210-3p in hypoxia-regulated placental development requires further investigation.

MiRNA also regulate EVT differentiation and invasion by modulating growth factor signalling. An important family of growth factors in placental development is the TGFB superfamily. Many miRNA have been found to enhance EVT migration and invasion by targeting members of the TGFB family. For example, in our lab, we found that miR-376c targeted both activin receptor-like kinase 7 (ALK7) and ALK5 to impede TGFB/Nodal signalling (Fu et al., 2013b) while miR-378a-5p targeted the ligand Nodal (Luo et al., 2012) to promote migration and invasion

in HTR8/SVneo cells and EVT outgrowth in first-trimester placental villous explants. Similarly, miR-195 enhanced trophoblast invasion by targeting activin receptor type-2B, a type II receptor for Nodal and activin, in HTR8/SVneo cells (Wu et al., 2016). Moreover, miR-218-5p promoted trophoblast migration, invasion and differentiation down the endovascular pathway by targeting TGFB2 and by upregulating motility and endovascular markers such as interleukin 1 beta (IL1B), IL8 and platelet endothelial cell adhesion marker 1 (PECAM1) (Brkić et al., 2018).

Using HTR8/SVneo, JEG-3, or BeWo trophoblast cell lines, several studies have suggested that miRNA also regulate EVT motility by targeting other genes involved in cell invasion. Both miR-346 and miR-582-3p targeted endocrine gland-derived vascular endothelial growth factor (EG-VEGF) as well as matrix metalloproteinase 2 (MMP2) and MMP9, and strongly inhibited the migratory and invasive abilities of trophoblasts (Su et al., 2017). Similarly, miR-93 (Pan et al., 2017) and miR-204 (Yu et al., 2015) targeted MMP2 and MMP9, respectively, to inhibit cell invasion. Members of the C19MC, miR-519d-3p (Ding et al., 2015) and miR-520g (Jiang et al., 2017a) also targeted MMP2 and inhibited migration and invasion, while miR-520c-3p inhibited invasion by suppressing CD44, which is needed for the interaction between EVTs and decidual extracellular matrix (Takahashi et al., 2017). On the other hand, miR-21 promoted not only migration and invasion but also cell proliferation (Chaiwangyen et al., 2015). Among its targets is phosphatase and tensin homolog (PTEN), a known inhibitor of the AKT pathway. PTEN dephosphorylates phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$), leading to inactivation of AKT which is involved in trophoblast cell motility (Chaiwangyen et al., 2015). MiR-34a inhibited invasion by targeting MYC (Sun et al., 2015). MiR-20a is another such miRNA where it inhibited not only trophoblast motility but also cell proliferation by targeting forkhead box protein A1 (FOXA1) (Wang et al., 2014).

II.2.3 miRNA in trophoblast proliferation and apoptosis

Proliferation and apoptosis are essential mechanisms of proper placental development; disruption of the equilibrium between cell division and death impair placental function (Levy et al., 2000). A recent *in vivo* study in mice has demonstrated the critical role of the miR-290 cluster

in placental cell proliferation and placental growth; deletion of the miR-290 cluster resulted in the reduction of trophoblast progenitor cell proliferation and placental size (Paikari et al., 2017). In addition, many *in vitro* studies have shown that miRNA regulate trophoblast proliferation and apoptosis. For example, miR-378a-5p (Luo et al., 2012) and miR-376c (Fu et al., 2013b) enhanced HTR8/SVneo cell proliferation and survival and EVT outgrowth in villous explants by inhibiting Nodal/TGFB signalling. On the other hand, miR-195 inhibited apoptosis by targeting inducible nitric oxide synthase (iNOS) in HTR8/SVneo cells (Wang et al., 2017). Furthermore, overexpression of miR-377 and let-7a, which are upregulated in term placenta samples versus first trimester samples, decreased trophoblast proliferation by reducing ERK and/or MYC expression in first trimester placental explants (Farrokhnia et al., 2014). Together, these studies suggest a potential regulatory link between miRNA and proliferation in human trophoblasts.

Studies using multiple human trophoblast cell lines suggested a role of miRNA in the regulation of apoptosis. The miR-29 family (miR-29a/b/c) promoted apoptosis by targeting myeloid cell leukemia-1 (MCL1), an apoptosis regulator and a member of the BLC2 family (Li et al., 2013; Gu et al., 2016). Overexpression of miR-18a increased apoptosis by inducing the expression of estrogen receptor alpha (ESR1) (Zhu et al., 2015) while miR-128a induced apoptosis via the mitochondrial pathway by down-regulating BAX (Ding et al., 2016) and miR-30a-3p by inhibiting IGF1 (Niu et al., 2018). On the other hand, miR-101 targeted endoplasmic reticulum protein 44 (ERP44) to suppress ER stress-induced apoptosis (Zou et al., 2014). Again, as the majority of these studies were carried out using only cell lines, more studies are required to confirm the involvement of these miRNA in trophoblast cell proliferation and apoptosis.

II.2.4 miRNA in placental vascular development

Placenta vascularization is essential to meet the metabolic demands of the rapidly growing fetus. Delayed or reduced vascular development of the placenta can result in compromised pregnancies (Reynolds and Redmer, 2001). Placental vascular formation includes vasculogenesis, the *de novo* synthesis of vessels within the villi core and angiogenesis, the formation of new vessels from pre-existing ones (Huppertz and Peeters, 2005; Demir et al., 2007).

Recently, deletion of the miR-290 cluster in mice has been reported to cause disorganization of the vasculature in the placental labyrinth (Paikari et al., 2017), providing strong evidence that miRNA are important regulators of placenta vascular development.

Several miRNA have also been suggested to play a role in vasculogenesis and angiogenesis. It was reported that miR-126 promotes proliferation, differentiation, and migration of human endothelial progenitor cells by targeting an anti-angiogenic factor, PIK3R2 (Yan et al., 2013). Also, in pregnant rats, miR-126 was found to increase vascular sprouting, as well as placental and fetal weights (Yan et al., 2013). The importance of miR-126 in placenta vascular development is further supported by the finding that downregulation of miR-126 contributes to endothelial dysfunction (Yan et al., 2013).

VEGF is a highly regulated pro-angiogenic factor known to initiate vasculogenesis in the placenta, induce endothelial cell proliferation and migration, and inhibit apoptosis (Wang and Zhao, 2010). Several miRNA have been reported to target VEGF. For example, miR-16 targets VEGF in human umbilical endothelial cells (HUVEC), thus inhibiting proliferation, migration, and tube formation (Zhu et al., 2016b). Also, overexpressing miR-16 in mice placentas decreased placental and fetal weights and inhibited the total placental vasculature and capillary number (Zhu et al., 2016b). Similarly, miR-136 (Ji et al., 2017), miR-200c, -20a, and -20b (Hu et al., 2016), also targeted VEGF, and may exert inhibitory effects on angiogenesis. However, whether these miRNA affect placental vascular development have not been investigated. In $CD34^+$ endothelial cells isolated from human umbilical cord blood, miR-210-3p was induced by VEGF and exerted proangiogenic effects (Alaiti et al., 2012), suggesting that miR-210-3p may play a role in placental angiogenesis.

II.2.5 miRNA in trophoblast cellular metabolism

Early in pregnancy and before spiral artery plug dissolution, placental and fetal nutrients and oxygen supply are dependent on endometrial gland secretions and maternal plasma (Murray, 2012). As a consequence, first-trimester placenta has a relatively low oxygen concentration (1-3%) (Pringle et al., 2010; Murray, 2012), and placental cells rely on glycolysis for energy production rather than oxidative phosphorylation to conserve oxygen supplies

for fetal tissues (Murray, 2012; Kolahi et al., 2017; Burton et al., 2021). Moreover, HIF1A downregulates mitochondrial oxygen consumption (Papandreou et al., 2006) to reduce reactive oxygen species (ROS) production at complex 3 of the electron transport chain (ETC) in the mitochondria (Colleoni et al., 2013). The hypoxia-induced miR-210-3p has been reported to regulate cellular metabolism. Using primary human trophoblasts, it was found that overexpression of miR-210-3p reduced, while inhibition of miR-210 increased mitochondrial respiration (Muralimanoharan et al., 2012). Iron-sulfur complex assembly proteins (ISCU) and cytochrome-c oxidase assembly protein (COX10), which play important roles in the mitochondria ETC and tricarboxylic acid cycle, are targeted by miR-210-3p in human endothelial and cancer cell lines (Chan et al., 2009; Chen et al., 2010). In trophoblasts, miR-210-3p was also found to target ISCU and to reduce the expression of ISCU and COX10 (Muralimanoharan et al., 2012; Colleoni et al., 2013), suggesting that these genes are involved in miR-210-3p-regulated trophoblast mitochondrial adaptation to low oxygen.

In addition to miR-210-3p, several other miRNA are also involved in mitochondrial biogenesis and function. For example, miR-130b-3p was found to decrease signals for mitochondrial biogenesis and adaptation to oxidative stress through targeting of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1A), a major regulator of mitochondrial biogenesis and energy metabolism (Jiang et al., 2017b). Also, miR-143 overexpression in primary human trophoblasts upregulated mitochondrial complexes 1, 2, and 3 but not 4 and 5 (Muralimanoharan et al., 2016), hence improving mitochondrial function. It also targeted hexokinase-2, a rate-limiting enzyme of glycolysis, reducing the glycolysis rate in trophoblasts (Muralimanoharan et al., 2016). Together, these miRNA may help regulate trophoblast metabolic adaptation to changing oxygen levels throughout gestation.

II.3. Hsa-miR-218-5p

The human miR-218 family consists of three mature miRNA: miR-218-5p, miR-218-1-3p, and miR-218-2-3p. These miRNA are derived from two pre-miRNA, mir-218-1 and mir-218-2, located in an intron of *SLIT2* and *SLIT3* genes, respectively. Processing of the SLIT2 and SLIT3 pre-mRNA generates mir-218-1 and mir-218-2 precursors, which are further processed to produce

the three mature miRNA (Small et al., 2010). miR-218-5p sequence is the same from mir-218-1 and mir-218-2 precursors, while miR-218-1-3p and miR-218-2-3p are different.

The SLIT/ROBO pathway has been reported to play a role in neuronal axon guidance (Tong et al., 2019), angiogenesis (Fujiwara et al., 2006), endothelial cell migration (Legg et al., 2008). In placenta, the expression of SLIT/ROBO pathway in active sites of placental angiogenesis suggests a role of this pathway in regulating proper placental perfusion (Liao et al., 2010). Furthermore, *in situ* hybridization analysis for ROBO4, another receptor from this pathway, reveals that it is abundantly expressed in placental endothelial cells as well as endovascular EVT (Liao et al., 2010).

Early reports of miR-218-5p showed that it plays a role in promoting neuronal differentiation (Sempere et al., 2004; Thiebes et al., 2015). It was also shown to play critical roles in heart development (Zhou et al., 2014) and in the establishment of retinal vasculature by targeting the SLIT2/3 receptor, ROBO1, thus regulating the SLIT/ROBO pathway (Small et al., 2010). In addition, miR-218-5p was reported to promote the differentiation of osteoblasts by inducing WNT signalling (Hassan et al., 2012; Zhang et al., 2014). In cancer, miR-218-5 was shown to be a tumour suppressor that targets oncogenes involved in proliferation and invasion (Lu et al., 2015). For example, in lung cancer, miR-218-5p targets epidermal growth factor receptor (EGFR), which leads to a decrease in cell proliferation and migration (Zhu et al., 2016a). In prostate cancer, miR-218-5p targets tumour necrosis factor (TNF) receptor-associated factor (TRAF)1 and TRAF2, which led to the inhibition of nuclear factor kappa-B subunit p50 (NFKB1) signalling, thus suppressing cells migration and invasion (Peng et al., 2019).

Both our laboratory (Brkić et al., 2018) and others (Xu et al., 2014a) have reported miR-218-5p to be downregulated in PE. Our lab has also shown miR-218-5p to be important in promoting trophoblast migration, invasion and endovascular differentiation by targeting TGFB2. In addition, we showed that miR-218-5p promoted spiral artery remodelling in placenta-decidua co-culture model (Brkić et al., 2018).

II.4. Hsa-miR-210-3p

As mentioned earlier, the role of miR-210-3p in trophoblast differentiation and function has been reported in many studies (Liu et al., 2012a). It is one of the best-known hypoxamirs, a group of miRNA that is upregulated by hypoxia and is upregulated directly by binding of hypoxia-induced transcription factor 1 alpha (HIF1A) to its promoter region (Chan et al., 2012). miR-210-3p can also be regulated by another hypoxia-regulated transcription factor, NFKB1 (Zhang et al., 2012). Early placenta development during the first trimester normally occurs under low oxygen conditions (2-3% O_2); this is important for trophoblast proliferation and to help maintain the trophoblast stem population. Low oxygen conditions also reduce ROS production, thus limiting the oxidative damage to the zygotic DNA and maintaining pluripotency of embryonic cells through the stabilization of HIF family members (Burton et al., 2021). In one of our studies, we found that mi R-210-3p was highest in the first trimester (weeks 5-12) and lowest in the third trimester (week 26-40) in samples from normal pregnancies (Hayder et al., 2021). Thus, miR-210-3p is thought to play an important role in trophoblast adaptation to hypoxic environment during the early stages of placenta development (Zaccagnini et al., 2013; Krawczynski et al., 2016; Hayder et al., 2021).

We and many others have shown that miR-210-3p is upregulated in placentas from severe term PE pregnancies (Liu et al., 2012a; Muralimanoharan et al., 2012; Hayder et al., 2021). PE placentas are characterized by shallow trophoblast invasion into the decidua and poor spiral artery remodelling; this leads to placental malperfusion resulting in oxidative stress due to increased ROS, and in villous shear damage due to high velocity blood flow from the non-remodelled arteries (Burton et al., 2021). In stem cells, ROS generation is known to upregulate miR-210-3p, thus enhancing their proliferation (Kim et al., 2013), while in endometrial stromal cells, upregulation of miR-210-3p protects the cells from oxidative stress-induced cell cycle arrest (Dai et al., 2019). Therefore, it is expected to see this upregulation of miR-210-3p in PE placentas. Since miR-210-3p inhibits trophoblast migration, invasion, and differentiation down the endovascular pathway (Anton et al., 2013; Luo et al., 2016; Hayder et al., 2021), it can create a positive feed-forward loop that further impairs trophoblast function seen in PE.

On the other hand, miR-210-3p can potentially be used as a biomarker for PE. It was found to be upregulated in serum of women with PE; also, several studies have shown that miR-210-3p level in the serum of women who developed PE later in gestation was higher during the second trimester than in women who did not develop PE (Xu et al., 2014a; Munaut et al., 2016). These studies demonstrate the emerging complexities of miR-210-3p in placenta development and the need to better understand its function in pregnancy.

III. Neuropeptide Y and its receptors

III.1. Neuropeptide Y

Neuropeptide Y (NPY) is one of the most abundant and wildly distributed neuropeptide in the mammalian central and peripheral nervous system (Balasubramaniam, 1997). It belongs to a family of homologous hormones that includes peptide YY (PYY), found in the intestine, and pancreatic peptide (PP), found as the name indicates, in the pancreas. All three peptides are 36 amino acids long; NPY and PYY share 70% sequence homology, while NPY and PP share about 50% (Balasubramaniam, 1997). Moreover, NPY primary structure is highly conserved among different species (Wahlestedt and Reis, 1993; Wan and Lau, 1995). *NPY* is located on chromosome 7 and is expressed in the central nervous system and multiple tissues throughout the body. *NPY* mRNA has a single open reading frame that produces a 97 amino acid pre-pro-NPY, 28 amino acids of which act as a signal peptide that regulates its translocation into the endoplasmic reticulum (ER). Once in the ER, the signal peptide is removed leaving the pro-NPY molecule that is made of 69 amino acids. Pro-NPY is then processed into the 36 amino acids mature NPY and a 30 amino acid peptide named C-terminal flanking peptide of NPY (CPON) (Wahlestedt and Reis, 1993). CPON is, as expected, found in every cell that produces NPY, and it is normally co-stored and co-released with NPY. Although CPON was found to also be highly conserved among species, it has no known biological function. It was suggested that CPON might aid NPY to exert some of its functions; however, recent work showed CPON was not needed for NPY function in suppressing seizures within the hippocampus in rats (Soud et al., 2019).

NPY (1-36) (hereafter referred to as NPY) is susceptible to proteolytic cleavage at the amino (N)-terminal by dipeptidyl peptidase-4 (DPP4) into NPY (3-36) (Hubers et al., 2018). DPP4 is a transmembrane serine protease that selectively cleaves two amino acids from the N-terminal of peptides with proline or alanine in the second to last position. Such peptides include incretin hormones glucagon-like peptide-1 (GLP-1) and glucose dependant insulinotropic peptide (GIP) involved in glucose homeostasis (Hubers et al., 2018). DPP4 can be found on the cell membrane in different tissues as well as secreted into serum (Wagner et al., 2015). NPY can also be truncated into multiple C-terminal fragments that vary in their binding affinity to different NPY receptors (NPY1R – NPY5R) (Dumont et al., 1992; Wan and Lau, 1995; Balasubramaniam, 1997). Finally, NPY can be degraded by endoproteolytic enzymes, which leads to loss of function (Wagner et al., 2015). So far, 22 different peptidases have been identified that can process and cleave NPY; they can also be found in subcellular compartments, on the cell membrane of various tissues throughout the central nervous system and body organs, as well as secreted in different body fluids such as blood and cerebrospinal fluid (Wagner et al., 2015). This emphasizes the importance of modulating NPY function and signalling through its various receptors.

III.2. Neuropeptide Y function

NPY has been studied intensely for its role in regulating food-intake and energy metabolism, stress response and cardiovascular function (Renshaw and Hinson, 2001; Dimitrijević et al., 2005; Nguyen et al., 2011; Huang et al., 2014; Reichmann and Holzer, 2016; Hubers et al., 2018). Other studies have also looked into its role in immune function (Dimitrijević et al., 2005), ovarian cell proliferation and apoptosis (Sirotkin et al., 2015), and tumour growth and progression (Tilan et al., 2013b; Tilan and Kitlinska, 2016). For example, NPY was shown to stimulate neurogenesis in different areas of the brain, including the hippocampus (Decressac et al., 2011). In the cardiovascular system, NPY increased protein synthesis in cardiomyocytes (Goldberg et al., 1998), promoted HUVEC migration and proliferation (Movafagh et al., 2006) and increased DNA synthesis and intracellular Ca^{2+} release in vascular smooth muscle cells (Pons et al., 2003) as well as induced vasoconstriction (Crnkovic et al., 2014; Tan et al., 2018). In ovaries, NPY inhibited porcine granulosa cells proliferation and promoted apoptosis via the accumulation

of p53 (Sirotkin et al., 2015). In the adrenal gland, NPY was secreted by the noradrenaline-secreting cells of the adrenal medulla in response to stressful conditions. Also, *in vivo* work suggested that NPY stimulates aldosterone secretion from the adrenal cortex, which is involved in regulating salt and water uptake in the kidneys, thus affecting blood pressure (Renshaw and Hinson, 2001). NPY was shown to exhibit endocrine, paracrine as well as autocrine effects in different body systems, including central and peripheral nervous systems, cardiovascular, respiratory, urogenital, gastrointestinal systems and adrenal gland (Dumont et al., 1992; Renshaw and Hinson, 2001; Chronwall and Zukowska, 2004; Kuo et al., 2007; Hirsch and Zukowska, 2012).

To our knowledge, only a limited number of studies have looked into the role of NPY and its receptors in placenta development. They showed that NPY and its receptors are expressed in the decidual stromal cells, amnion epithelial cells, and chorionic cytotrophoblasts (Petraglia et al., 1993; Graf et al., 1996) and suggested a possible role of NPY in regulating placental growth and energy balance (Graf et al., 1996; Pazos et al., 2014). Also, placental and blood-derived NPY was found to bind NPY1R on the maternal side of the syncytiotrophoblasts and was linked to regulating the release of corticotropin-releasing factor and inhibin (Wharton et al., 1993; Robidoux et al., 1998; Robidoux et al., 2000). In mice, placental *NPY* mRNA level varied throughout gestation and its expression was regulated by interleukin-6 (IL-6) (Pazos et al., 2014) which is an important mediator of maternal immune response to pregnancy, endocrine placental changes and fetal brain development (Hsiao and Patterson, 2011; Shirasuna et al., 2015; Wu et al., 2017). Nevertheless, no studies have looked into the specific role of NPY or its receptors in villous development, trophoblast differentiation and trophoblast-decidual immune cell interaction.

Reports of NPY levels in pregnancy complications such as PE have been controversial. To date, two studies found NPY to be upregulated in the serum of women with PE (Khatun et al., 2000; Paiva et al., 2016), while another study found no difference in NPY serum levels between normal and PE women (Klinjampa et al., 2019). Conversely, *NPY* mRNA levels were found to be downregulated in placental tissue samples from women with PE (Dotsch et al., 1999). As for the

receptors, a study found that *NPY1R*, *NPY2R* and *NPY5R* mRNA were all downregulated in PE compared to normal samples (Klinjampa et al., 2019).

III.3. Neuropeptide Y receptors

III.3.1 NPY receptors evolution and ligand affinity

To date, there have been seven different NPY receptors (NPY1R, 2R, 4R, 5R, 6R, 7R and 8R) described in vertebrates, but only five of which can be found in mammals (NPY1R, 2R, 4R, 5R and 6R) with the exclusion of NPY3R, which has been recharacterized as a CXC chemokine receptor type 4 (CXCR4). However, the pseudogene npy6r is non-functional in many primates, including humans, and it has been lost in rats (Sundström et al., 2013; Reichmann and Holzer, 2016; Chen et al., 2020). These receptors are members of the guanine nucleotide-binding protein (G-protein)-coupled receptors (GPCR) superfamily, which contain seven transmembrane domains (Böhme et al., 2008) connected by three intra- and three extra-cellular loops. The NPY receptors belong to the Rhodopsin-like receptor family of GPCR and mediate their intracellular signalling by a class of inhibitory alpha G-protein ($G_{\alpha i/o}$) subunits (Kimple et al., 2014; Ziffert et al., 2020). In humans, there are four functional NPY receptors, NPY1R, NPY2R, NPY4R and NPY5R. They are also differentially expressed in different tissues, although they can all be found in multiple regions of the brain and the central nervous system, while NPY4R is mainly expressed in the gastrointestinal tract (Dumont et al., 1998; Pedragosa Badia et al., 2013).

NPY1R, *NPY2R* and *NPY5R* are all located on chromosome 4 and thought to have evolved through gene duplication, though this must have happened early in their evolution as these three receptors share only approximately 30% amino acid sequence identity (Ammar et al., 1996; Wraith et al., 2000) (**Table 1.1**). *NPY4R* and *npy6r* (a pseudogene, written in lower case because it is non-functional in humans due to a frameshift mutation) are encoded on chromosomes 10 and 5 respectively, and they share 44% and 51% sequence identity with NPY1R, respectively (Pedragosa Badia et al., 2013). NPY1R and NPY2R are highly conserved among vertebrates, as is NPY5R, with the exception of its third intracellular loop. NPY4R and npy6r are less conserved between species, with NPY4R appearing to be the fastest evolving of all receptor family members

(Wraith et al., 2000). Interestingly, *NPY1R* is the only member of the NPY receptor family that contains an intron within its coding region; moreover, this intron shows higher than expected sequence identity among mammals suggesting that conserving selection pressures are acting on it (Wraith et al., 2000).

***Table 1.1**: Comparison of functional NPY receptors in humans (Wraith et al., 2000; Pedragosa Badia et al., 2013)*

Receptor	NPY1R	NPY2R	NPY4R	NPY5R
Number of amino acids	384	381	375	445
Sequence identity as compared to NPY1R	100%	27%	44%	30%
Ligand	NPY, PYY >> PP	NPY, PYY >> PP	PP >> PYY, NPY	NPY, PYY > PP

NPY receptors also differ in their binding affinity to different NPY/NPY fragments (Balasubramaniam, 1997; Chronwall and Zukowska, 2004; Dimitrijević et al., 2005). NPY1R cannot tolerate NPY truncations at either the N- or C-terminals, although it can tolerate some substitutions at the C-terminal of NPY. NPY1R has the highest affinity to full-length NPY, while NPY (2-36), (3-36), (13-36) and (18-36) showed a significant loss of affinity. Also, NPY residues at position arginine^{33} and arginine^{35} were extremely important for receptor binding as substitutions in these two positions showed a reduction in receptor binding of over 5000-fold compared to wildtype NPY. Tyrosine^{36} is also important for NPY1R binding but can tolerate a substitution to phenylalanine but not to alanine (Pedragosa Badia et al., 2013). In contrast to NPY1R, NPY2R can tolerate truncations at the N-terminal of NPY, resulting in the use of NPY (3-36) and (13-36) as NPY2R agonists. Even NPY (18-36) and (22-36) were shown to be still able to bind to NPY2R. This shows the importance of the C-terminal of NPY for NPY2R binding (Pedragosa Badia et al., 2013). PP is the selective ligand for NPY4R, although NPY4R can still bind NPY and PYY at a much lower affinity. Similar to NPY1R, arginine^{33} and arginine^{35} of the ligand were also essential for NPY4R activation, and it cannot accept substitutions at these two positions (Pedragosa Badia et al.,

2013). As for NPY5R, it can tolerate truncations at the N-terminal of NPY, although not to the same extent as NPY2R. NPY arginine^{25}, tyrosine^{27}, and arginine^{35} were shown to have the highest impact on ligand binding affinity to NPY5R. NPY proline residues at positions 2, 5 and 8 also affected NPY5R binding affinity, although to a lesser extent (Pedragosa Badia et al., 2013)

III.3.2 NPY receptors intracellular trafficking and desensitization

NPY receptors belong to the GPCR superfamily, for which ligand-dependent internalization is an important aspect of their signalling regulation. Canonically, when a ligand binds to a GPCR, the receptor is activated and depending on the nature of the receptor conformational change, various signalling cascades are triggered via intracellular second messenger systems predominantly mediated by heterotrimeric α, β, γ, G proteins. Activation is then followed by receptor deactivation or desensitization and internalization to be either degraded, recycled back to the plasma membrane, or perform various intracellular signalling-related activities (Drake et al., 2006; Christofides et al., 2018). This cyclic process by which cells desensitize, internalize and recycle GPCR is necessary to prevent excessive receptor stimulation or prolonged inactivity.

In the basal state, an inactive GPCR is bound to a βγ-complex and a GDP-bound α-subunit. GPCR activation by its ligand binding induces conformational changes in the receptor. This receptor conformational change promotes GDP to GTP exchange on the Gα subunit. GTP-bound α-subunit is then dissociated from Gβγ-complex and can mediate downstream receptor signalling as can the Gβγ-complex in some cases (Wettschureck and Offermanns, 2005; Gurevich and Gurevich, 2019). Freed active receptors can bind and activate another G protein, thus providing a mean for signal amplification. GPCR desensitization starts with G_α subunit hydrolyzing GTP to GDP via its own GTPase activity; however, this reaction is both kinetically slow and thermodynamically unfavourable in the absence of GTPase activating proteins (GAP), also known as RGS proteins. The formation of G_α-GDP allows the reassembly of the $G_{\alpha\beta\gamma}$ heterotrimer, which can then bind to the receptor again. Also, receptor desensitization occurs due to modification of the receptor itself via phosphorylation of its Serine and Threonine residues at the cytosolic C-

terminal domain by one of seven G-protein regulating kinases (GRK), although other kinases were also found to be involved in receptor phosphorylation. These kinases, specifically phosphorylate active receptors. Interestingly, differential phosphorylation of a single receptor type can lead to different functionality within the same cell (Wanka et al., 2017; Christofides et al., 2018; Gurevich and Gurevich, 2019; Weinberg and Puthenveedu, 2019). However, receptor phosphorylation does not fully desensitize the receptor; for that, receptor internalization needs to occur.

Internalization of the GPCR-ligand complex is triggered by GRK phosphorylation of the receptor; this promotes the binding of β-arrestin proteins (arrestin 1-4) to the receptor (Weinberg and Puthenveedu, 2019). β-arrestin binding leads to receptor conformational changes that deactivate G-protein-coupled signalling by sterically hindering the association of G-protein to the receptor and by recruiting scaffolding proteins that can direct clathrin proteins to the receptor (Gurevich and Gurevich, 2019). Endocytosis via the formation of clathrin-coated pits (CCP) promotes the internalization of the receptor-ligand-arrestin complex. The ligand-receptor complex can then be recycled back to the plasma membrane, undergo lysosomal degradation or trafficked to different intracellular compartments (Christofides et al., 2018).

However, GPCR signalling is one of the most complex and dynamically regulated cellular processes. An example of added complexity is that arrestin proteins can serve as signal transducers for GPCR. Receptor-bound arrestins were found to promote SRC-dependent activation of mitogen-activated protein kinases ERK1/2 as well as enhance G-protein activation of ERK1/2. (Gurevich and Gurevich, 2019). Also, the formation of supercomplexes has been observed where the activated receptor is bound to both G proteins and arrestin at the same time. The structure of this supercomplex allows the internalized GPCR to promote GDP–GTP exchange, thereby activating G proteins facilitating prolonged receptor signalling (Pavlos and Friedman, 2017). Another example of GPCR signalling is that some GPCR undergo retrograde trafficking, by which a receptor can traffic through the trans-Golgi complex and endoplasmic reticulum and may even translocate into the nucleus (Christofides et al., 2018). Receptor signalling has been detected within these subcellular compartments including in the endosomes. In fact, more recently, GPCR have been found in the nucleus and that these receptors contain either a monopartite (a single stretch of amino acids) or bipartite (two stretches of amino acids separated

by a linker region) nuclear localization signals (NLS) (Lange et al., 2010; Christofides et al., 2018; Bhosle et al., 2019). There are also reports that some nuclear GPCR are independent of those on the plasma membrane and that those receptors cycle between the cytoplasm and the nucleus (Christofides et al., 2018)

Many factors affect the rate at which each of the previous steps occurs. For example, the type of ligand binding and receptor organization on the plasma membrane; organization referring to whether the GPCR is a monomer or forms an oligomer (a homo- or heterodimer) and to the way the receptor interacts with cytoskeletal and scaffolding proteins (Drake et al., 2006; Weinberg and Puthenveedu, 2019). Another factor is the strength of interaction between the GPCR and β-arrestin proteins (Christofides et al., 2018). In the case of NPY receptors, studies showed that NPY1R is desensitized and internalized rapidly in clathrin-coated pits and also rapidly recycled back to the membrane (Gicquiaux et al., 2002; Ouedraogo et al., 2008). In HEK 293 cells, NPY2R and NPY4R have comparable internalization rates to that of NPY1R, but NPY5R internalization was slower, and all three were in a clathrin-dependent manner (Böhme et al., 2008; Babilon et al., 2013). However, in the same cells, NPY2R was observed to have a prolonged desensitization period that lasts after it is recycled back into the plasma membrane, which prevents further NPY2R-mediated Gαi-signaling (Ziffert et al., 2020). These differences could be because NPY1R is internalized as a supercomplex composed of receptor, G-protein and arrestin while during the internalization of NPY2R, arrestin is released from the receptor and remains at the membrane (Wanka et al., 2018). This is due to differences between NPY1R and NPY2R C-terminals and the amino acid residues of intracellular loops 1 and 2, which results in a higher relative affinity between NPY1R and arrestin (Holliday et al., 2005; Wanka et al., 2018). That being said, both NPY1R and NPY2R were shown to also internalize in an arrestin-independent manner (Walther et al., 2010). Also, some studies showed that internalization of the NPY2R strongly depends on agonist concentration (Babilon et al., 2013).

In addition, co-expression of NPY1R and NPY5R in HEK 293 cells resulted in the formation of an NPY1R-NPY5R heterodimer (Gehlert et al., 2007). This accelerated the rate of NPY5R internalization; both receptors needed to be activated by ligand binding for heterodimer internalization to occur. Also, NPY5R activation in the heterodimer form led to greater adenylyl

cyclase inhibition than NPY5R on its own (Gehlert et al., 2007). This is interesting as *NPY1R* and *NPY5R* genes are transcribed from opposing strands of the same DNA locus, regulated by a shared promoter region (Herzog et al., 1997).

III.3.3 NPY receptors signalling and function

NPY is involved in regulating many biological functions in different body systems, all of which are exerted through one or more of its receptors. In general, on the molecular level, NPY receptors activation leads to a decrease in second-messenger cyclic adenosine monophosphate (cAMP) synthesis by inhibiting adenylyl cyclase, which leads to reduced protein kinase A (PKA) activity and the release of intracellular calcium (Ca^{2+}) (Aakerlund et al., 1990; Daniels et al., 1992; Persaud and Bewick, 2014; Tan et al., 2018). In some cell types, receptors can activate phospholipase C (PLC), either through coupling to $G_{\alpha q}$ or via the β/γ subunits of $G_{\alpha i}$. This was shown to lead to the activation of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K); both of which stimulate phosphorylation of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase 1/2 (ERK1/2) through the activation of MAPK kinase (MEK) (Goldberg et al., 1998; Persaud and Bewick, 2014). That being said, NPY signalling through its receptors has proven to be complicated because of the multi-ligand fragments/multi-receptor system that varies by cell type and ligand concentration as well as the microenvironment (Balasubramaniam, 1997; Chronwall and Zukowska, 2004; Hirsch and Zukowska, 2012; Zhang et al., 2021) **(Figure 7)**. For example, in Ewing sarcoma, NPY was shown to promote cell death through NPY1R and NPY5R signalling. However, in response to hypoxia, Ewing sarcoma cell line upregulates NPY2R and DPP4 levels (which cleaves NPY into NPY 3-36, an NPY2R/NPY5R agonist). This leads to NPY 3-36 activation of NPY2R and NPY5R, and this was shown to promote Ewing sarcoma cells migration, proliferation and angiogenic potential (Tilan et al., 2013b).

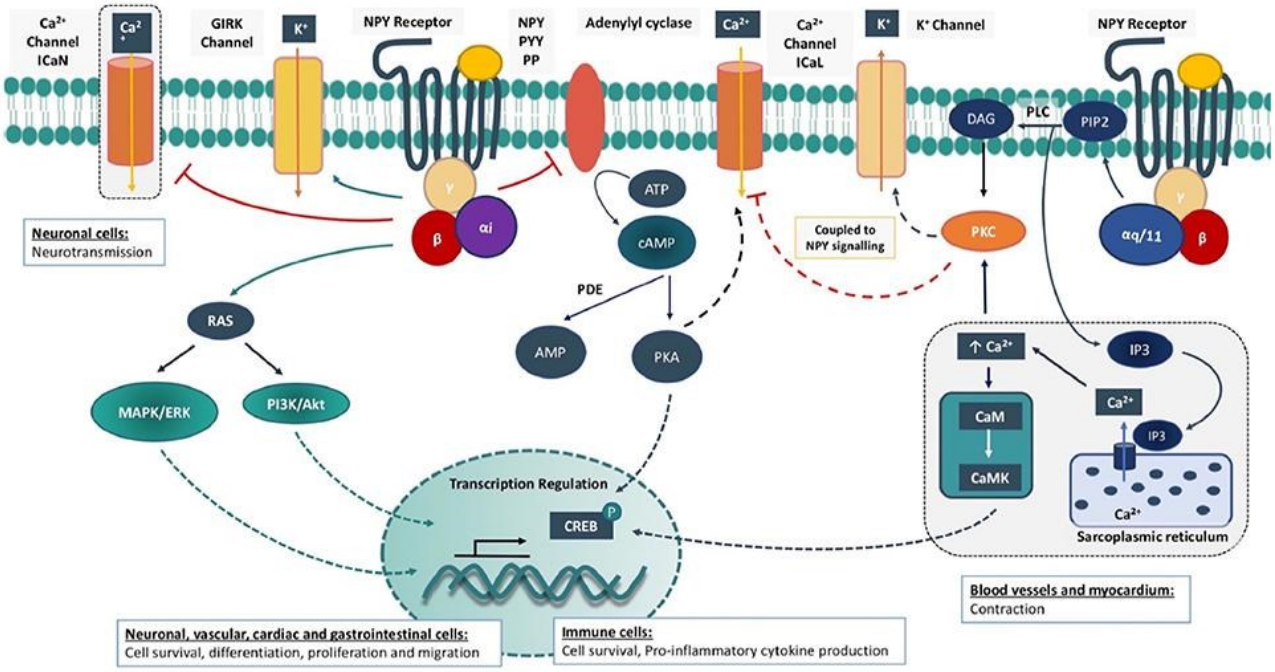

Figure 7. Neuropeptide Y receptor signalling cascades. Neuropeptide Y (NPY) receptors are G-protein coupled receptors (GPCR) and can be activated by specific peptides such as NPY, peptide YY (PYY) or pancreatic peptide (PP). The activated NPY receptors enhance the $G_{\alpha i}$ signalling resulting in the inhibition of adenylyl cyclase, thus decreasing second-messenger cyclic adenosine monophosphate (cAMP) synthesis and protein kinase A (PKA) activity. This inhibits the stimulation of L-type Ca^{2+} current (ICaL) and activation of cAMP response element binding protein (CREB). In neurons, NPY receptor activation also inhibits N-type Ca^{2+} current (ICaN). NPY receptors can also stimulate the inwardly rectifying potassium (GIRK) current, mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2), phosphatidylinositol-3-kinase (PI3K) and protein kinase B (AKT) signalling. NPY1R activation can also promote $G_{\alpha q}$ signalling to stimulate Ca^{2+} release from the sarcoplasmic reticulum via inositol triphosphate (IP3). Elevation of intracellular Ca^{2+} activates Ca^{2+}calmodulin-dependent kinase (CaMK) and protein kinase C (PKC). $G_{\alpha q}$ signalling also activates phospholipase C (PLC), leading to the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) and the formation of diacylglycerol (DAG), which further enhances PKC activation. Depending on the cell type, activation of these pathways leads to multitudes of NPY effects. Used with permission from Tan et al. 2018 (Tan et al., 2018).

However, there are many reports of well-established NPY receptors functions. For example, one of the most well-established roles of NPY1R is mediating the vasoconstrictive effect of NPY released from sympathetic nerve endings. NPY-mediated vasoconstriction via NPY1R is characterized by slow onset and long duration, especially in the small coronary arteries and cerebral bed (Zukowska et al., 2003) and is associated with increased intracellular Ca^{2+} release (Aakerlund et al., 1990). Another important role of NPY1R is the induction of food intake and the regulation of energy homeostasis in synergy with NPY5R (Babilon et al., 2013). In addition, in HEK 293 cells, NPY binding to NPY1R led to phosphorylation and activation of ERK1/2 signalling down the MAPK pathway. This activation was NPY1R internalization-independent, but it required the coactivation of insulin growth factor receptor (IGFR) in a metalloproteinase-independent pathway. (Lecat et al., 2015). Also, in mice microglia cells, which are specialized macrophages found in the central nervous system, treatment with lipopolysaccharide (LPS) resulted in a release of interleukin 1 beta (IL1B), which in turn promoted nitric oxide (NO) production through an NFKB-dependant pathway. NPY signalling through NPY1R in these cells strongly inhibited LPS-stimulated release of IL1B, NFKB activation and NO synthesis. Therefore, NPY plays a key role in modulating the inflammatory response in mice brains (Ferreira et al., 2010).

NPY2R activation by NPY stimulates capillary angiogenesis in different animal models (Abe et al., 2007; Tilan et al., 2013a), and this effect is reduced with ageing in mice due to a decreased expression of *NPY2R* and *DPP4* in endothelial cells (Kitlinska et al., 2002). Also, NPY2R activation promoted HUVEC migration and formation of tube-like networks in cooperation with NPY5R (Ghersi et al., 2001) and in NO-dependant pathways (Zukowska et al., 2003). In the central nervous system, NPY2R expression was involved in memory retention and mood disorders (Babilon et al., 2013).

NPY4R was found to inhibit gastric emptying in humans and gastrointestinal secretions. It also modulated energy homeostasis and emotional behaviour in synergy with NPY2R (Babilon et al., 2013). As for NPY5R, most reports so far showed its synergistic role with other NPY receptors, namely NPY1R and NPY2R. In conjunction with NPY1R, NPY5R promoted vascular smooth muscle cell migration and proliferation (Pons et al., 2003) while promoting vascular angiogenesis and revascularization in response to ischemic injury with NPY2R (Tan et al., 2018).

IV. Rationale, hypothesis and objectives

Placenta development is a spatially and temporally regulated process that involves a complex interaction between the fetal and maternal structures. Considering the critical roles of miRNA in embryonic development, cellular differentiation, metabolism, and pathogenesis, it is expected that they also play a role in placenta development, trophoblast differentiation and function.

A critical miRNA in trophoblast adaptation to hypoxia and oxidative stress is miR-210-3p. It is also involved in downregulating trophoblast migration and invasion by targeting multiple important pathways. Many reports have shown that miR-210-3p is upregulated in both placental tissue and maternal serum in PE, which led to it being considered a potential biomarker for PE. Therefore, we investigated potential novel targets of miR-210-3p, one of which was CDX2, a critical transcription factor in trophectoderm differentiation. CDX2 was recently shown to enhance trophoblast migration and invasion. Thus, we hypothesized that miR-210-3p might contribute to the pathogenesis of PE by targeting CDX2.

Since EVT migration, invasion and spiral artery remodelling were shown to be important mechanisms in early placenta development, and our lab has previously shown that miR-218-5p is a critical player in promoting these mechanisms through the regulation of the TGFB2 pathway, we investigated other potential pathways that may be regulated by miR-218-5p. Also, conditioned media from miR-218-5p stably overexpressing trophoblast cell line promoted trophoblast migration and invasion as compared to the control cells, suggesting that miR-218-5p downregulated secretory factors that negatively affect trophoblast motility. Our preliminary findings from microarray-screening in these stable miR-218-5p overexpressing cell line showed that the most downregulated mRNA was latexin, a tumor suppressor and a negative regulator of hematopoietic stem cells (Liu et al., 2012b). Interestingly however, the second most downregulated mRNA was that of NPY , a secreted small peptide (Klinjampa et al., 2019) . Also, in a preliminary study, knockdown of NPY using small interfering RNA (siRNA) resulted in increased trophoblast migration and invasion. In addition, *in silico* analysis showed that both *NPY* and its receptor, *NPY1R*, have potential miR-218-5p target sites. Considering the roles of

miR-218-5p in trophoblast function, we hypothesized that NPY plays a role in early human placenta development.

The objectives of this part of my study were to 1) Determine if CDX2 is indeed a target of miR-210-3p and further investigate the role of mir-210-3p in trophoblast differentiation especially towards the endovascular pathway and whether such role may be mediated by CDX2, and 2) Determine the role of the secreted peptide NPY in trophoblast differentiation and function and investigate the mechanism of miR-218-5p regulation of NPY signalling through NPY1R .

V. References

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Chapter Two

Overexpression of miR-210-3p impairs extravillous trophoblast functions associated with uterine spiral artery remodelling

Overexpression of miR-210-3p impairs extravillous trophoblast functions associated with uterine spiral artery remodelling

Heyam Hayder [1], Guodong Fu [1,†,*], Lubna Nadeem [1,†], Jacob A. O'Brien [1], Stephen J. Lye [2,3,4] and Chun Peng [1,5,*]

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[1]Department of Biology, York University, Toronto, ON M3J 1P3, Canada
[2]Research Centre for Women's and Infants' Health, Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Sinai Health System, Toronto, ON M5G 1X5, Canada
[3]Department of Obstetrics and Gynaecology, Faculty of Medicine, University of Toronto, Toronto, ON M5G, 1E2, Canada
[4]Department of Physiology, Faculty of Medicine, University of Toronto, Toronto, ON M5S, 1A8, Canada
[5]Centre for Research on Biomolecular Interactions, York University, Toronto, ON M3J 1P3, Canada
†Current address: Alex and Simona Shnaider Research Laboratory in Molecular Oncology (G.F.) and Research Centre for Women's and Infants' Health, Lunenfeld-Tanenbaum Research Institute (L.N.), Mount Sinai Hospital, Sinai Health System, Toronto, ON M5G 1X5, Canada
*Correspondence: david.fu@sinaihealth.ca (G.F.); cpeng@yorku.ca (C.P.)

Authors' contributions

H.H. was involved in conceptualization, methodology, validation, formal analysis, investigation, writing of original draft, revisions, editing, visualizations and addressing reviewers' comments.

G.F. was involved in conceptualization, methodology, validation, formal analysis, investigation, revisions, editing, and supervision.

L.N. was involved in methodology, investigation, revisions and editing.

J.A.O. was involved in the investigation, writing of original draft, revisions, editing, and addressing reviewers' comments.

S.J.L. was involved in revisions, editing, and funding acquisition.

C.P. was involved in conceptualization, methodology, writing of original draft, revisions, editing, supervision, funding acquisition and addressing reviewers' comments.

I. Summary

Hsa-miR-210-3p has been reported to be upregulated in preeclampsia (PE); however, the functions of miR-210-3p in placental development are not fully understood, and, consequently, miR-210-3p's role in the pathogenesis of PE is still under investigation. In this study, we found that overexpression of miR-210-3p reduced trophoblast migration and invasion, extravillous trophoblast (EVT) outgrowth in first trimester explants, expression of endovascular trophoblast (enEVT) markers and the ability of trophoblasts to form tube-like networks. In addition, miR-210-3p overexpression significantly downregulated the mRNA levels of interleukin-1β and C-X-C motif chemokine ligand 8 (CXCL8), as well as CXCL1. These cytokines have been suggested to play a role in EVT invasion and the recruitment of immune cells to the spiral artery remodelling sites. We also showed that caudal-related homeobox transcription factor 2 (CDX2) is targeted by miR-210-3p and that CDX2 downregulation mimicked the observed effects of miR-210-3p upregulation in trophoblasts. These findings suggest that miR-210-3p may play a role in regulating events associated with enEVT functions, and its overexpression could impair spiral artery remodelling, thereby contributing to PE.

II. Introduction

Early placental development is a highly regulated process as the trophectoderm layer differentiates into multiple trophoblast lineages to form a branching network of villi (Hayder et al., 2018). Cytotrophoblast progenitor cells differentiate into two general pathways; they can either fuse to form a multinucleated monolayer of syncytiotrophoblasts or differentiate into the invasive extravillous trophoblasts (EVT) (Ji et al., 2013). One important role of these invasive EVT is the remodelling of the maternal uterine spiral arteries. During the first trimester, EVT form aggregates (plugs) to limit the maternal blood flow into the intervillous space (IVS) (Burton et al., 2021). Toward the end of the first trimester and throughout the second trimester, EVT that are in contact with the spiral arteries differentiate into endovascular EVT (enEVT), which replace the endothelial cells of spiral arteries and transform these arteries into low resistance, large diameter vessels to establish low velocity uteroplacental perfusion (Anton et al., 2013; Burton et al., 2021). Reduced trophoblast invasion and abnormal vessel remodelling can lead to prolonged placental oxidative stress, thus contributing to trophoblast dysfunction, which is a hallmark of preeclampsia (PE) (Anton et al., 2013; Fu et al., 2013a).

MicroRNAs (miRNA) are small, non-coding, single-stranded RNA that regulate gene expression at the post-transcriptional and, to a lesser extent, transcriptional levels (O'Brien et al., 2018). They have been shown to play a vital role in many biological processes, including placenta development (O'Brien et al., 2018). Considering the importance of miRNA in placenta development, many studies have investigated the effect of oxygen on miRNA expression within the placenta (Fu et al., 2013a; O'Brien et al., 2018). A group of miRNA found to be upregulated by hypoxia, called hypoxamirs, were directly regulated by hypoxia-induced transcription factors (O'Brien et al., 2018). Hsa-miR-210-3p is one of the most well-established hypoxamirs, and it was shown to be upregulated in disparate low oxygen environments such as cancer (Dang and Myers, 2015; Ren et al., 2017), myocardial infarction (Devlin et al., 2011), ischemia (Ma et al., 2019), and PE (Chan et al., 2012; Liu et al., 2012). miR-210-3p is directly induced by the hypoxia-inducible factor 1 alpha (HIF1A) which binds to the hypoxia response element (HRE) within the miR-210-3p promoter (Chan et al., 2012). miR-210-3p can also be induced by another hypoxia-regulated transcription factor, nuclear factor kappa-B subunit p50 (NFKB1) (Zhang et al., 2012). In addition,

many studies have shown that miR-210-3p level was higher in placenta and serum from women with PE (Liu et al., 2012; Muralimanoharan et al., 2012; Zhang et al., 2012; Anton et al., 2013; Li et al., 2015). miR-210-3p plays a role in regulating cell proliferation, migration and invasion, cell cycle regulation, apoptosis, angiogenesis and mitochondrial function (Huang et al., 2010; Devlin et al., 2011; Qin et al., 2014; Dang and Myers, 2015). In trophoblasts, miR-210-3p was reported to decrease cell invasion by targeting various genes, such as iron-sulfur cluster assembly enzyme (ISCU) (Lee et al., 2011), thrombospondin type I domain containing 7A (THSD7A) (Luo et al., 2016), potassium channel modulatory factor 1 (KCMF1) (Luo et al., 2014) and fibroblast growth factor 1 (FGF1) (Li et al., 2019). miR-210-3p is also involved in the regulation of syncytialization (Wang et al., 2019) and mitochondrial respiration (Muralimanoharan et al., 2012) in trophoblasts.

Caudal-related homeobox transcription factor 2 (CDX2) is a transcription factor known to be essential for the establishment of the trophoblast lineage in the late morula stage (Kunath et al., 2004; Strumpf et al., 2005; Sakurai et al., 2010). Studies have shown that overexpression of CDX2 in the EVT cell line, HTR8/SVneo, enhances cell invasion through the activation of matrix metalloproteinase-9 (MMP9) and the inhibition of MMP9 inhibitor, tissue inhibitor metalloproteinase-1 (TIMP1) and that CDX2 expression is regulated by the PI3K/AKT pathway (Jia et al., 2014). Additionally, CDX2 is regulated by Yes-associated protein (YAP), which has been shown to promote HTR8/SVneo invasion and reduce apoptosis (Sun et al., 2018).

Uterine spiral artery remodelling is an essential part of proper placenta development. It involves a dynamic interaction among at least the decidual immune cells, EVT, endothelial and smooth muscle cells of the artery (Choudhury et al., 2017). Many autocrine and paracrine factors secreted by EVT, including cytokines, chemokines and growth factors, are known to play a role in the recruitment of immune cells to the site of remodelling (Tessier et al., 2015; Ramhorst et al., 2016). Such cytokines and chemokines include interleukin-1β (IL1B), interleukin-8/C-X-C motif chemokine ligand 8 (CXCL8) and C-X-C motif ligand 1 (CXCL1) (Bowen et al., 2002a; Bowen et al., 2002b; Hauguel-de Mouzon and Guerre-Millo, 2006; Choudhury et al., 2017). IL1B, a pro-inflammatory cytokine, has been shown to promote EVT migration (Prutsch et al., 2012) and invasion (Librach et al., 1994). CXCL8, a chemokine that is able to induce chemotaxis, has been shown to activate endothelial cell retraction and gap formation between adjacent cells

(Schraufstatter et al., 2001). CXCL1, also a chemokine, is secreted by trophoblasts and has been shown to play a role in the recruitment of regulatory T-cells needed for maternal tolerance of the fetus (Ramhorst et al., 2012), as well as having an angiogenic role (Ramhorst et al., 2016). CXCL1 expression was also shown to be induced by IL1B and to promote trophoblast invasion (Baston-Buest et al., 2017).

The role of miR-210-3p in spiral artery remodelling is largely unknown. In this study, we examined the role of miR-210-3p in EVT functions associated with spiral artery remodelling and identified CDX2 as a novel target of miR-210-3p. We report here that overexpression of miR-210-3p or silencing of CDX2 inhibited: (1) migration and invasion of HTR8/SVneo cells; (2) outgrowth of first trimester placental villous explants; and (3) the potential of EVT to form endothelial-like networks. In addition, overexpression of miR-210-3p or knockdown of CDX2 decreased the mRNA level of integrin subunit alpha 1 (ITGA1), platelet and endothelial cell adhesion molecule 1 (PECAM1), cadherin 5 (CDH5), IL1B, CXCL8 and CXCL1, which are markers of enEVT or known to promote EVT invasion and/or spiral artery remodelling. Our findings suggest that overexpression of miR-210-3p could lead to impaired maternal spiral artery remodelling, thereby contributing to the pathogenesis of PE.

III. Materials and methods

Clinical samples and tissue collection

The human placental tissue samples were collected through the BioBank program at the Research Centre for Women's and Infants' Health at Mount Sinai Hospital (MSH) (Toronto, ON, Canada) and approved by the MSH Research Ethics Board. Samples included first trimester (5–12 weeks, *n* = 8), second trimester (13–25 weeks, *n* = 10), pre-term (26–36 weeks, *n* = 13), and term (37–40 weeks, *n* = 17). First and second trimester samples were collected with informed consent from healthy patients undergoing elective termination of pregnancy at the Morgentaler Clinic (Toronto, ON, Canada). Placental samples from patients with HIV, hepatitis, or missed miscarriage were excluded. Pre-term samples were collected by caesarean section following labouring after fetal distress or spontaneous premature rupture of the membranes. All pre-term

samples were examined by a placental pathologist, and only placentas without gross abnormalities or signs of chorioamnionitis were included. Third trimester placental samples were collected from only appropriate for gestational age vaginal or caesarean section deliveries. Pre-eclamptic samples were collected from both pre-term (*n* = 14) and term (*n* = 4) pregnancies. Healthy and PE donors' clinical data are summarized in **Table 3.1**.

Table 3.1. *Clinical information for control and age-matched PE patients. Data reported represent mean ± SEM.*

	Pre-Term Control	Pre-Term PE	*p* value	Term Control	Term PE	*p* value
Gestational age (weeks)	29.83 ± 0.51	30.27 ± 0.36	0.4824	38.32 ± 0.14	37.25 ± 0.25	0.0030
Maternal age (years)	31.95 ± 1.15	30.8 ± 1.85	0.6087	33.36 ± 0.73	34.8 ± 2.21	0.4383
Systolic blood pressure (mmHg)	120.56 ± 2.9	165.13 ± 2.93	<0.0001	118.88 ± 2.11	166.8 ± 6.05	<0.0001
Diastolic blood pressure (mmHg)	73.83 ± 2.27	106.67 ± 1.9	<0.0001	77.04 ± 1.79	101.2 ± 1.46	<0.0001
Proteinuria (g/24 h)	None	3.46 ± 0.13	-	None	2.41 ± 0.25	-
% Laboring	100%	35.7%	0.0004	11.8%	75%	0.0276

Cell lines and cell culture

Experiments were conducted using an immortalized human first trimester trophoblast cell line, HTR8/SVneo, kindly provided by Dr. Charles Graham (Queen's University, Kingston, ON, Canada) and were maintained as previously described (Brkić et al., 2018; Brkić et al., 2020). Briefly, HTR8/SVneo cells were cultured in HyClone™ classical liquid media RPMI 1640 with L-glutamine (GE Healthcare Life Sciences, Ottawa, ON, Canada) supplemented with 10% fetal

bovine serum (FBS) (GIBCO® Life Technologies, Mississauga, ON, Canada) and grown in a humidified environment of 5% CO_2 and 37 °C. For oxygen tension experiments, cells were incubated in Sanyo Tri-Gas incubators set at 21%, 8%, 3% or 1% O_2 levels and grown in a humidified environment of 5% CO_2 and 37 °C.

Transient transfections and treatments

Transient transfection of miR-210-3p mimics or inhibitor or small interfering RNA oligomers (100 nM) (**Table 3.2**) was conducted using Lipofectamine RNAiMAX® reagent (Invitrogen, Life Technologies, Mississauga, ON, Canada). A modified protocol was used to optimize transfection efficiency and cell survival using a Lipofectamine volume of 2.4 µL per well in OMEM medium (Brkić et al., 2018; Brkić et al., 2020). Cells were transfected 24 h post-seeding for 5 h in 6-well plates seeded at 6.0×10^4 cells per well. Transfection mix was removed, and cells were recovered with serum-containing media for 24 h after transfection before proceeding to functional assays and for 48 h for RNA or Western blot analysis.

Table 3.2. *Oligomer sequences and reagents.*

Oligomer Name	**Sequence: 5 → 3'**
NC (non-targeting control) **GenePharma (Shanghai, China)**	Sense: UUCUCCGAACGUGUCACGUTT Anti-sense: ACGUGACACGUUCGGAGAATT
hsa-miR-210-3p **GenePharma (Shanghai, China)**	Sense: CUGUGCGUGUGACAGCGGCUGATT Anti-sense: UCAGCCGCUGUCACACGCACAGTT
siCDX2 **GenePharma (Shanghai, China)**	Sense: CCAGGACGAAAGACAAAUATT Anti-sense: UAUUUGUCUUUCGUCCUGGTT
Anti-NC **RiboBio (Guangzhou, China)**	RiboBio™ miRNA Inhibitor, Negative Control #22
Anti-hsa-miR-210-3p **RiboBio (Guangzhou, China)**	RiboBio™ anti- hsa-miR-210-3p Inhibitor

RNA extraction, reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells or tissues using RiboZol™ RNA Extraction Reagent (VWR Life Science, Mississauga, ON, Canada) following the manufacturer's protocol with some modifications. Namely, during the RNA precipitation step, instead of the recommended 10 min at room temperature in isopropanol, 30 min at −20 °C was performed. RNA concentration was measured using NanoDrop 2000 spectrophotometer (Thermo Scientific, Mississauga, ON, Canada). NCode™ miRNA first-strand cDNA synthesis kit (Invitrogen, Life Technologies, Mississauga, ON, Canada) was used to detect miR-210-3p or internal control U48 in clinical samples following the manufacturer's protocol. For measuring hsa-miR-210-3p or U6 post-transfection in cells, TaqMan® miRNA assay (Life Technologies, Mississauga, ON, Canada) was used following the manufacturer's protocol.

To measure the levels of mRNA, 1.0 µg of total RNA was reversed transcribed into cDNA using M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA, USA), following the manufacturer's protocol. qRT-PCR was performed using EvaGreen qPCR 2X Master mix (Applied Biological Materials Inc., Richmond, BC, Canada) following the manufacturer's recommendation, and samples were normalized to CYC1 internal control (Brkić et al., 2018; Brkić et al., 2020) using the $2^{-\Delta\Delta CT}$ method. Primers used were designed using NCBI primer blast and are listed in **Table 3.3**. Some of these primers have been reported previously (Brkić et al., 2018).

Table 3.3. *Primer sequences used for qRT-PCR.*

Primer Name	Sequence: 5′ → 3′
miR-210-3p	FP: GTGACAGCGGCTGAA
	RP: NCode Universal primer
U48	FP: CCCAGGTAACTCTGAGTGTGTC
	RP: NCode Universal primer
ITGA1	FP: GCTGGCTCCTCACTGTTGTT
	RP: CACCTCTCCCAACTGGACAC
PECAM1	FP: ATTGCAGTGGTTATCATCGGAGTG
	RP: CTCGTTGTTGGAGTTCAGAAGTGG
CDH5	FP: GCCAGTTCTTCCGAGTCACA
	RP: TTTCCTGTGGGGGTTCCAGT
HLA-G	FP: CTGACCCTGACCGAGACCTGG
	RP: GTCGCAGCCAATCATCCACTGGA
IL1B	FP: AATCTGTACCTGTCCTGCGTGTT
	RP: TGGGTAATTTTTGGGATCTACACTCT
CXCL8	FP: CAGAGACAGCAGAGCACACA
	RP: GGCAAAACTGCACCTTCACA
CXCL1	FP: CAGGGAATTCACCCCAAGAACA
	RP: GGATGCAGGATTGAGGCAAGC
CDX2	FP: CGCTTCTGGGCTGCTGCAAAC
	RP: CGACTGTAGTGAAACTCCTTCTCC
CYC1	FP: CAGATAGCCAAGGATGTGTG
	RP: CATCATCAACATCTTGAGCC

Transwell migration and invasion assay

Transwell migration and invasion assays were conducted using polycarbonate membrane 24-well Transwell inserts with 8 µm pore size (Costar, Corning™ Inc., Ottawa, ON, Canada). However, in the invasion assay, the Transwell inserts were coated with 100 µL of Cultrex

PathClear® growth factor reduced, phenol red-free Matrigel (Trevigen Inc., Burlington, ON, Canada) diluted with serum-free RPMI 1640 medium into a final concentration of 0.15 mg/mL. Cells were transfected with NC, siRNA, miR-210-3p, or anti-miR-210-3p 24 h prior to migration and invasion assays. Cells were then collected using Accutase (Corning™ Inc., Ottawa, ON, Canada) and seeded on the top of the Transwell insert at a density of 1.5×10^4 cells for migration, and 2.0×10^4 cells for invasion per filter. Cells were seeded onto the insert in serum-free media while 10% FBS-containing media was added outside the Transwell.

At 18 h post-seeding onto the Transwell, cells were fixed and stained with Harleco Hemacolor Staining Kit (EMD Millipore, Oakville, ON, Canada). Non-migrated/invaded cells on the top of the membranes were wiped off using a cotton swab, and membranes were cut with a blade and mounted on slides for quantification. Cells were visualized and photographed using a dissection microscope (Leica Microsystems, at 1.25×) and the numbers of cells migrated/invaded were counted using ImageJ as described previously (O'Brien et al., 2016).

First-trimester human placenta explant culture

Human placenta explant culture was performed as described previously (Caniggia et al., 1997; Nadeem et al., 2011; Fu et al., 2013b; Brkić et al., 2018). Briefly, first trimester placentas (6-9 weeks) from elective terminated pregnancies were obtained through the Research Center for Women's and Infants' Health BioBank Program and the Lunenfeld-Tanenbaum Research Institute (Toronto, ON, Canada) on the day of the procedure. Placentas were carefully dissected, and explants with potential EVT columns were placed onto 12 mm Transwell inserts (EMD Millipore, Oakville, ON, Canada) pre-coated with 200 µL of phenol red-free Matrigel (Corning™ Inc., Ottawa, ON, Canada) in a 24-well plate. Explants were then left overnight in a humidified environment of 3% O_2, 5% CO_2 and 37 °C before adding serum-free DMEM-F12 medium supplemented with 100 µg/mL Normacin™. After two days of culture, explants with successful EVT outgrowths were selected and randomly distributed for treatment with 200 nM oligomers. Explants were photographed immediately after adding the treatment and then at 24, 48 and 72 h using Leica DFC400 camera attached to a dissecting microscope. Images were analyzed for the area of EVT outgrowth using ImageJ. The total growth area was measured at each time point, then the area at each subsequent time points for each explant was divided by its initial area at

time 0 h. Before combining samples from different experiments/placentas, each experiment treatment was normalized to the average of the control group of that experiment.

Tube formation assay

Tube formation assay was conducted after 24 h recovery post-transfection (as mentioned above). Cells were stained with a 1 µM Cell Tracker™ Green CMFDA (Invitrogen, Life Technologies) in serum-free media for 45 min and then collected using Accutase (Corning™ Inc., Ottawa, ON, Canada). Cells were then seeded at the density of 2.0×10^4 cells per well into a 96-well plate pre-coated with 30 µL per well of undiluted growth factor reduced, phenol red-free Matrigel (Cultrex PathClear® 15.4 mg/mL). The plate was put into the IncuCyte® live-cell analysis system (Essen BioSience, Ann Arbor, MI, USA) for imaging at 4× for at least three days. Images were then acquired and analyzed in ImageJ using the NeuronJ plugin as previously described (Meijering et al., 2004).

Protein extraction and immunoblot analysis

After cells were transfected and allowed to recover for 48 h, cells were lysed with RIPA buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton-X, 0.5% NP-40, 0.1% SDS, pH 7.4) supplemented with Pierce protease and phosphatase inhibitors (ThermoFisher Scientific, Mississauga, ON, Canada). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Oakville, ON, Canada) overnight at 4 °C. Membranes were blocked in blocking buffer (5% skim milk in 1× Tris-buffered Saline and Tween-20) for 1 h at room temperature. Membranes were then incubated overnight at 4 °C with the primary antibodies β-Actin (Cell Signaling 3700S) 1:1000, CDX2 (Cell Signaling 3977S) 1:500. Membranes were then washed with 1× TBST buffer and probed with horseradish peroxidase-conjugated secondary antibodies (Anti-mouse (EMD Millipore 12349MI) 1:4000, Anti-Rabbit (EMD Millipore 12348MI) 1:4000) at room temperature for 1.5 h. Signals were detected using Clarity™ Western ECL substrate kit (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada).

Luciferase reporter assay

The luciferase reporter construct was generated by cloning a portion of the CDX2 3' UTR containing the predicted miR-210-3p target sequence into a pMIR-Report™ plasmid (Ambion, Life Technologies, Mississauga, ON, Canada) downstream of the luciferase gene. Briefly, the wild-type CDX2 3' UTR fragment from nucleotide 1252 to 2183 was amplified using HTR8/SVneo RNA as a template. Mutations at the seed region of the predicted miR-210-3p targeting site were introduced by PCR using primers with a substitution of four nucleotides (CACA to GTGT). Both wild type and the mutated fragments were cloned into the pMIR-Report plasmid using SpeI and PmeI restriction enzymes and finally sequenced to confirm their identities. Luciferase assay was performed using the Dual-Luciferase® reporter assay system as per the manufacturer's protocol (Promega, Madison, WI, USA). HTR8/SVneo cells were transfected with 200 ng/mL of the Luciferase construct containing wild type or mutated CDX2 3'UTR, 25 ng/mL Renilla construct (pRL-TK, Promega, Madison, WI, USA) and either 20 nM of miR-210-3p or the scrambled control. At 24 h after transfection, cells were lysed, and luciferase activity was measured.

Statistical analysis

For comparison between more than two groups, one- or two-way ANOVA, depending on the dataset, followed by Tukey's multiple comparisons analysis, was conducted using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). However, for comparison between two groups, an unpaired Student's t-test was used. A $p < 0.05$ was considered significant. To identify outliers in clinical samples, ROUT outliers test was performed (Q = 1.0%).

IV. Results

1. miR-210-3p level decreases with increasing gestation in healthy pregnancies and is upregulated in PE

To evaluate the endogenous level of miR-210-3p across gestation, we quantified its expression in placental samples from healthy pregnancies ranging from 5 to 40 weeks, collected from a Canadian cohort using qRT-PCR (**Figure 1A**). The level of miR-210-3p was significantly lower during 26-40 weeks compared to 5-12 weeks of gestation. In addition, we measured miR-

210-3p levels in placental samples from pre-term (26-36 weeks) and term (37-40 weeks) PE pregnancies and age-matched healthy controls. There was no significant difference in miR-210-3p levels between pre-term control and PE samples (**Figure 1B**). However, a significant increase in miR-210-3p levels in PE compared to the age-matched control pregnancies was observed in term samples (**Figure 1C**).

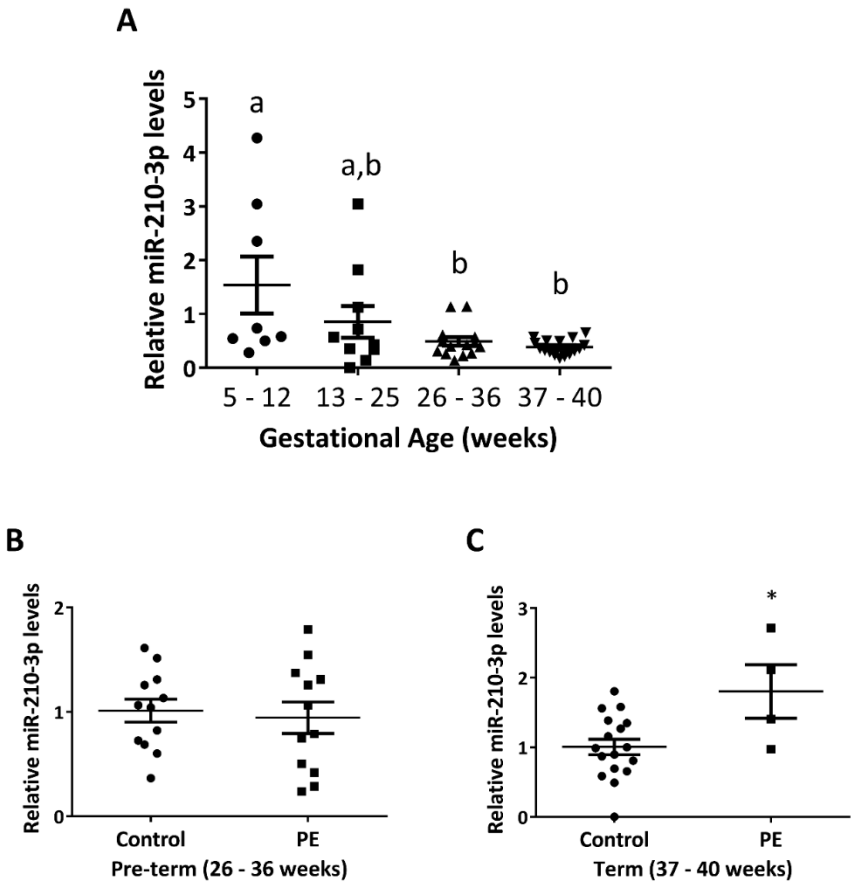

Figure 1. Expression levels of miR-210-3p in human placentas from healthy and PE pregnancies. **A.** miR-210-3p expression across gestation. miR-210-3p levels were significantly lower in the third trimester (weeks 26-40) compared to the first trimester (weeks 5-12). Different letters above bars represent statistical significance. **B.** miR-210-3p levels in pre-term (weeks 26-36) healthy controls and PE placentas. There was no significant difference in miR-210-3p between the two groups. **C.** Expression levels of miR-210-3p in healthy and PE term (weeks 37-40) placentas. miR-210-3p levels were significantly higher in PE compared to healthy age-matched controls. Data represent mean ± SEM. * $p < 0.05$.

2. miR-210-3p downregulates migration and invasion of HTR8/SVneo cells and EVT outgrowth in first trimester placental explants

To further confirm the role of miR-210-3p in trophoblast motility, Transwell migration and invasion assays were conducted. Overexpression of miR-210-3p in HTR8/SVneo cells reduced their ability to migrate and invade (**Figure 2A, 2C**) while transfection with anti-miR-210-3p promoted cell migration and invasion (**Figure 2B, 2D**). Using the first trimester placental explant model, the role of miR-210-3p in EVT outgrowth was also investigated. In explants overexpressing miR-210-3p, EVT outgrowth was significantly impeded (**Figure 2E**). On the other hand, anti-miR-210-3p significantly promoted EVT outgrowth (**Figure 2F**).

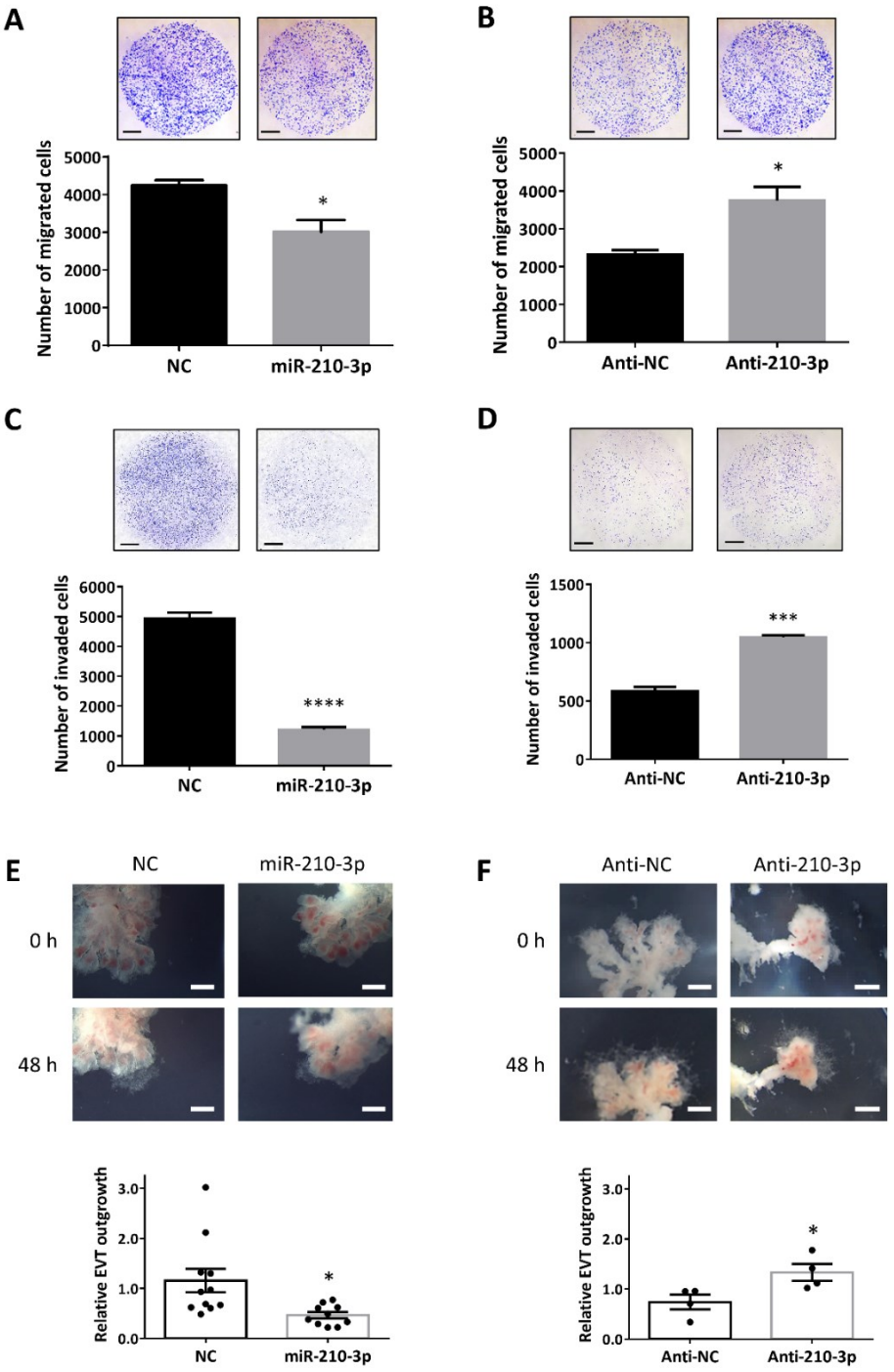

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Figure 2. miR-210-3p inhibits trophoblast migration and invasion and EVT outgrowth. A. Transwell migration assay using HTR8/SVneo cells transfected with miR-210-3p mimic (100 nM). Overexpression of miR-210-3p significantly reduced cell migration, when compared with cells transfected with a non-targeting control (NC, N = 3). **B.** Transwell migration assay with cells transfected with anti-miR-210-3p (100 nM). Inhibition of endogenous miR-210-3p significantly increased the number of migrated cells compared to the negative control (anti-NC, N = 3). **C.** Transwell invasion assay in cells overexpressing miR-210-3p. Trophoblast invasion ability was significantly decreased following miR-210-3p transfection (N = 5). **D.** Invasion assay with HTR8/SVneo cells transfected with anti-miR-210-3p. The number of invaded cells was significantly increased after anti-miR-210-3p transfection compared to anti-NC (N = 3). **E.** First-trimester (Weeks 6–9) placental explants were placed on Matrigel and treated with miR-210-3p or its control (200 nM) for 48 h. A significant decrease in EVT outgrowth in miR-210-3p-treated explants was observed (N = 11 NC, N = 10 miR-210-3p). **F.** First-trimester placental explants treated with anti-miR-210-3p (200 nM) for 48 h had a significant increase in EVT outgrowth (N = 4 in each group). Data represent mean ± SEM from representative experiments. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. Scale bars: (A-D) 100 μm; and (E,F) 1 mm.

3. miR-210-3p reduced the ability of HTR8/SVneo to form endothelial-like networks

To determine if miR-210-3p regulates the ability of trophoblasts to acquire angiogenic potentials, tube formation assays were performed. Overexpression of miR-210-3p in HTR8/SVneo cells led to a significant decrease in the total tube length and the number of branching points at both 24 and 48 h (**Figure 3A**). On the other hand, transfecting the cells with anti-miR-210-3p significantly increased the number of branching points at 48 h but had no significant effect on total tube length at both time points tested (**Figure 3B**).

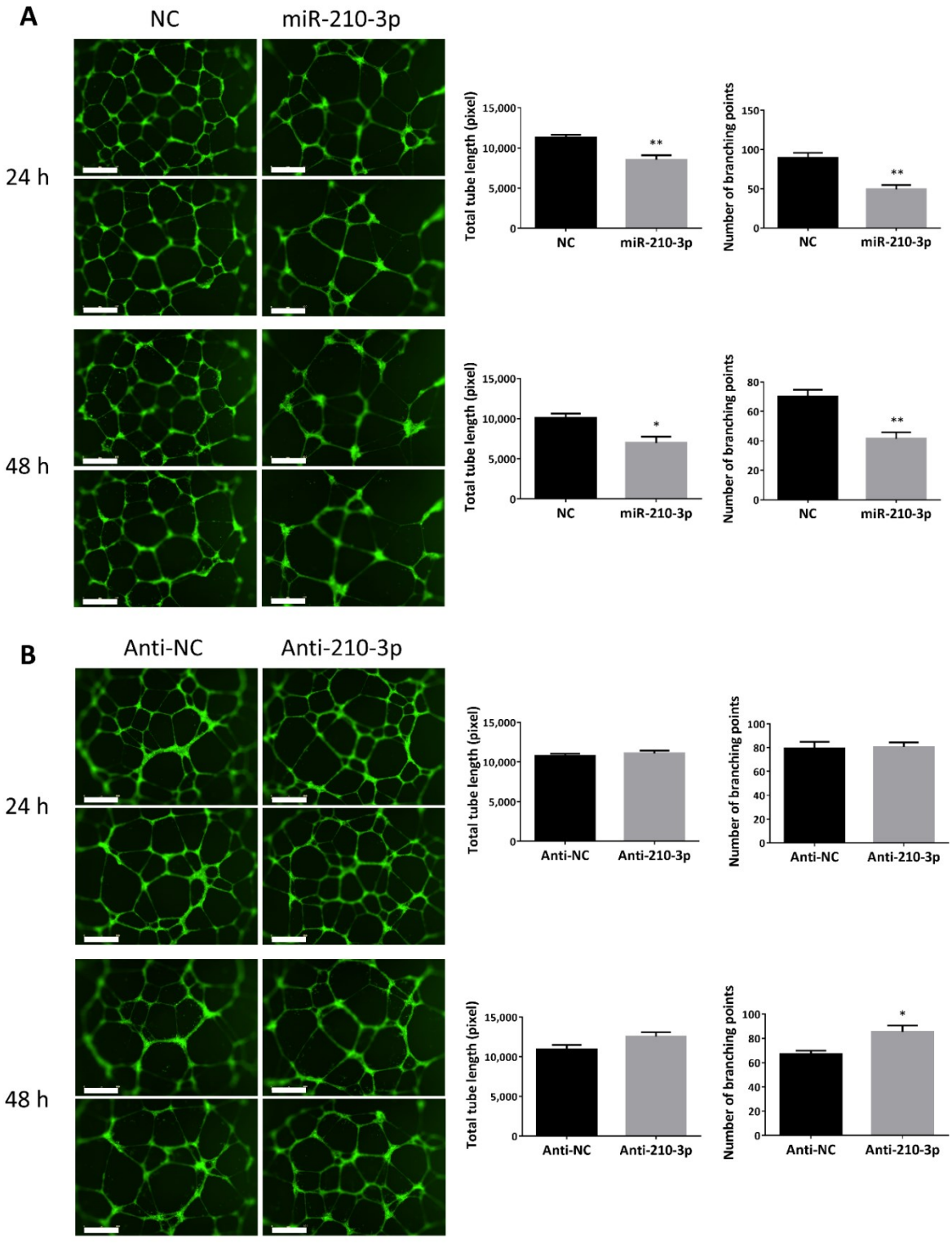

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Figure 3. Overexpression of miR-210-3p reduces the ability of trophoblasts to form endothelial-like networks. A. HTR8/SVneo cells transfected with 100 nM of miR-210-3p mimic showed a significant reduction in both the total length of the network formed and in the number of branching points at 24 and 48 h, as compared with cells transfected with non-targeting control (NC, N = 4). **B.** Cells transfected with anti-miR-210-3p (100 nM) showed no change in the total tube length and the number of branching points at 24 h but showed a significant increase in the number of branching points at 48 h (N = 5) compared to the negative control (anti-NC). Data represent mean ± SEM from representative experiments. * $p < 0.05$, ** $p < 0.01$. Scale bar is 800 µm.

4. Overexpressing miR-210-3p in HTR8/SVneo decreased the mRNA Levels of invasion and enEVT differentiation markers, especially IL1B, CXCL8 and CXCL1

To investigate the effect of miR-210-3p overexpression on known EVT and enEVT markers, we used qRT-PCR to measure the mRNA levels of these genes. HTR8/SVneo cells transfected with miR-210-3p mimics (**Figure 4A**) showed a significant decrease in key markers, ITGA1 and PECAM1, but no significant change in CDH5 and HLA-G was observed. Moreover, miR-210-3p also significantly decreased mRNA levels of the pro-inflammatory cytokine IL1B and chemokines CXCL8 and CXCL1, all of which have been shown to be important for trophoblast invasion and in the recruitment of immune cells to remodel spiral arteries (Librach et al., 1994; Jovanović et al., 2010; Baston-Buest et al., 2017; Choudhury et al., 2017). However, downregulation of endogenous miR-210-3p by anti-miR-210-3p showed no significant effects on the mRNA level of all of these markers (**Figure 4B**).

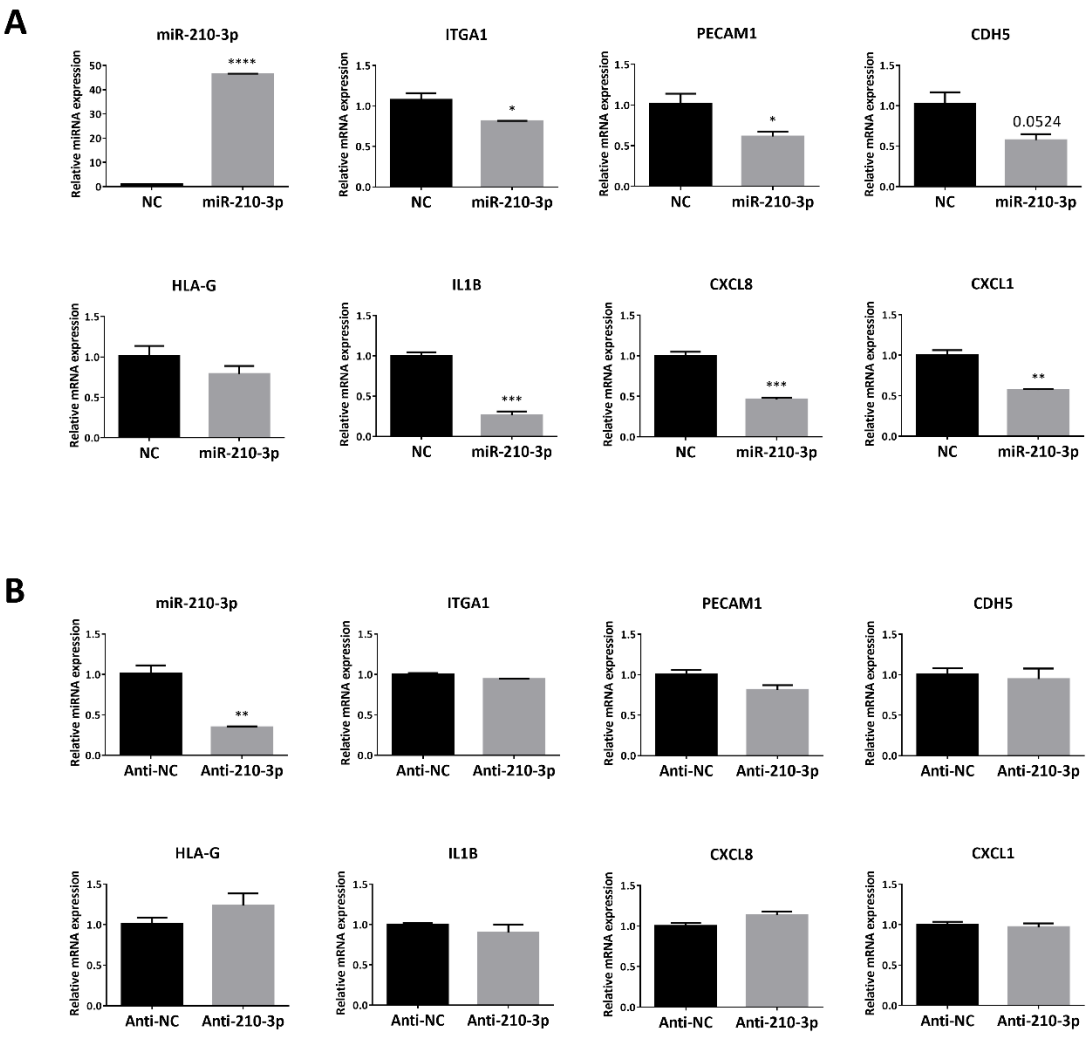

Figure 4. Overexpression of miR-210-3p downregulates mRNA levels of enEVT markers and cytokines. A. In HTR8/SVneo cells transfected with 100 nM of miR-210-3p mimic for 48 h, mRNA levels of ITGA1, PECAM1, IL1B, CXCL8 and CXCL1 were significantly decreased compared with cells transfected with its non-targeting control (NC) while a decrease in CDH5 and HLA-G mRNA levels was not significant (N = 3). **B.** Transfection of 100 nM anti-miR-210-3p for 48 h significantly decreased endogenous miR-210-3p levels compared to anti-NC miRNA inhibitor control but did not alter enEVT marker or cytokine mRNA levels (N = 3). Data represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5. Upregulation of endogenous miR-210-3p by low oxygen tension led to a decrease in IL1B, CXCL8 and CXCL1 at 1% O_2

It is well-established that miR-210-3p is upregulated in response to low-oxygen tension. We investigated if upregulating endogenous miR-210-3p by culturing HTR8/SVneo under 8%, 3% and 1% O_2 can lead to change in the mRNA levels of cytokine/chemokine IL1B, CXCL8 and CXCL1. As expected, miR-210-3p was significantly upregulated in cells cultured at 1% O_2 at both 24 (**Figure 5A**) and 48 h (**Figure 5B**). In addition, the mRNA levels of IL1B, CXCL8 and CXCL1 were all significantly downregulated at 1% O_2 (**Figure 5A, 5B**).

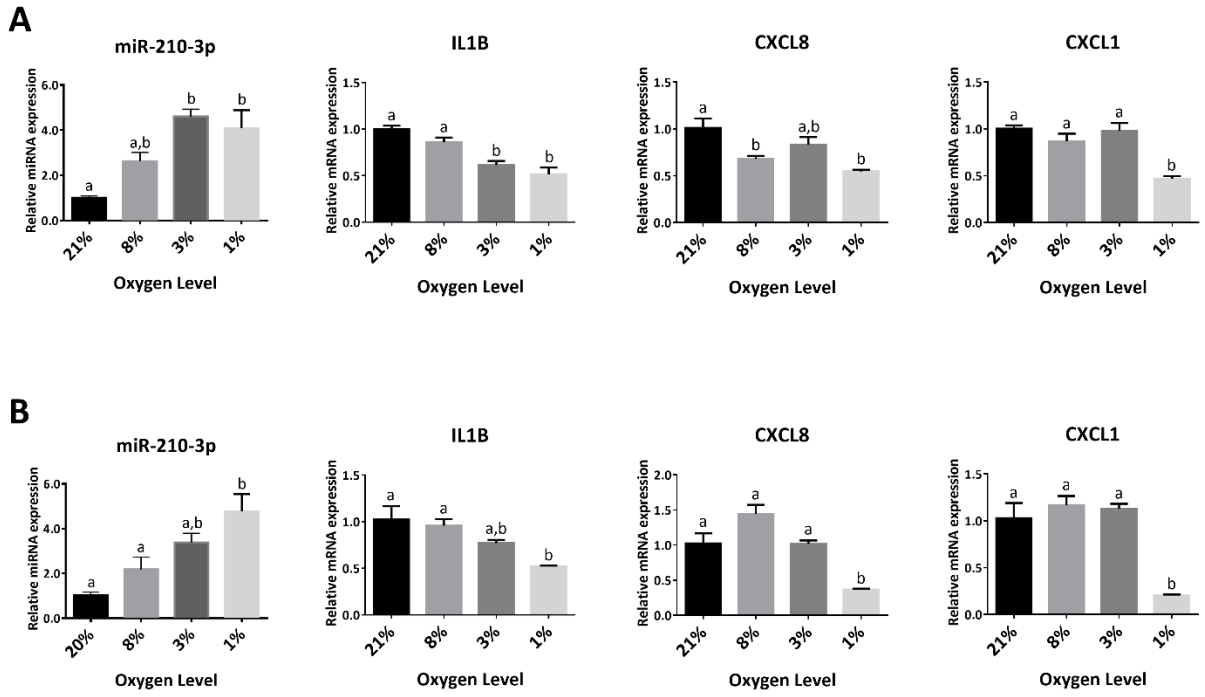

Figure 5. Low oxygen tension upregulates miR-210-3p and reduces cytokine levels. HTR8/SVneo cells cultured under different O_2 levels for 24 h **(A)** or 48 h **(B)** (N = 3). miR-210-3p levels were upregulated while IL1B, CXCL8, and CXCL1 mRNA were downregulated by low O_2 tension. Data represent mean ± SEM from representative experiments. Different letters above bars represent statistical significance. $p < 0.05$ was considered significant.

6. CDX2 is a novel target of miR-210-3p

Several genes have been reported to be targets of miR-210-3p and implicated in regulating trophoblast function (Fu et al., 2013a; Luo et al., 2014; O'Brien et al., 2018; Li et al., 2019). In search of novel miR-210-3p targets, we conducted an *in silico* search which showed that CDX2 mRNA contained a predicted miR-210-3p target site in the 3' UTR. To confirm whether CDX2 is indeed targeted by miR-210-3p, we generated a luciferase reporter with the region of the 3' UTR of CDX2 containing the predicted target site and a mutant construct with mutations in the seed region of the predicted binding site. Luciferase assays showed a decrease in activity after transfecting miR-210-3p mimic, and this decrease was abolished when the predicted miR-210-3p binding site was mutated (**Figure 6A**). In addition, miR-210-3p decreased both CDX2 mRNA (**Figure 6B**) and protein (**Figure 6C**) levels.

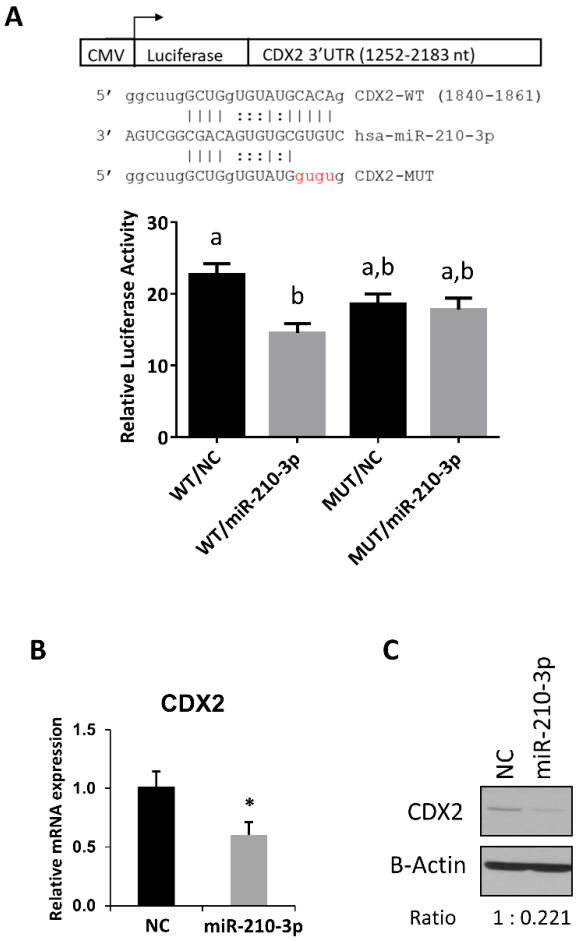

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Figure 6. CDX2 is a novel target of miR-210-3p. A. Luciferase reporter assay. The predicted miR-210-3p target site in the 3' UTR of CDX2 was cloned into a luciferase reporter vector in its wild type (WT) sequence or after introducing mutations (MUT) to disrupt the predicted miR-210-3p binding site. Cells co-transfected with WT vector and miR-210-3p mimic decreased luciferase activity compared to cells transfected with the non-targeting control (NC). No reduction in luciferase activity by miR-210-3p was observed when the putative miR-210-3p binding site was mutated. Different letters above bars denote statistical significance ($p < 0.05$) (N = 5). **B.** Overexpressing miR-210-3p led to a significant reduction in CDX2 mRNA level in HTR8/SVneo cells (N = 3). **C.** CDX2 protein levels in cells transfected with miR-210-3p mimic was decreased compared to NC. Data represent mean ± SEM from representative experiments. * $p < 0.05$.

7. CDX2 knockdown mimics the effect of miR-210-3p overexpression and led to decreased HTR8/SVneo migration and invasion and decreased EVT outgrowth in first trimester placental explants

To investigate the role of CDX2 in trophoblasts, we designed a small interfering RNA (siRNA) molecule targeting CDX2 (as shown in **Figure S1**). In Transwell migration assays, knockdown of CDX2 by siCDX2 significantly decreased cell migration (**Figure 7A**). Similarly, in Transwell invasion assay, siCDX2 decreased the number of invaded cells (**Figure 7B**). Using the first trimester explant model, we also investigated the role of CDX2 in EVT outgrowth. Treatment of the explants with siCDX2 led to a decrease in EVT outgrowth, measured at both 48 and 72 h (**Figure 7C**).

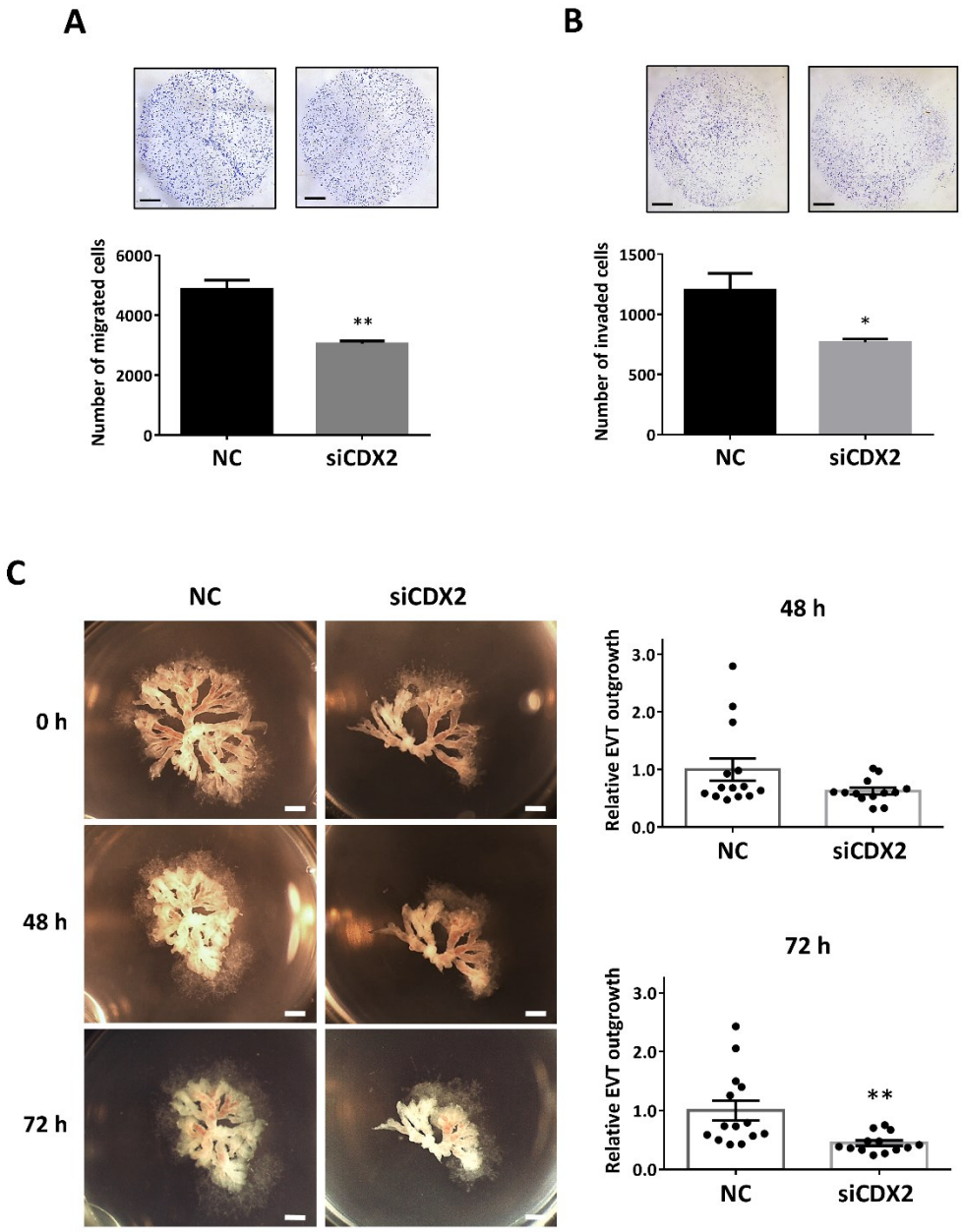

Figure 7. CDX2 promotes trophoblast migration, invasion and EVT outgrowth. A. Transwell migration assay was conducted using HTR8/SVneo cells transfected with 100 nM of siCDX2. Downregulation of CDX2 led to a significant decrease in the number of migrated cells compared to the non-targeting control (NC) (N = 3). **B.** In Transwell invasion assay, transfection with siCDX2 resulted in a significant decrease in the number of invaded cells (N = 3). **C.** First-trimester (Weeks 6–9) placental explants treated with siCDX2 (200 nM) showed a significant decrease in the area of EVT outgrowth at 72 h, when compared with NC (N = 14 NC, N = 13 siCDX2). Data represent mean ± SEM from representative experiments. * $p < 0.05$, ** $p < 0.01$. Scale bars: (A,B) 100 µm; and (C) 1 mm.

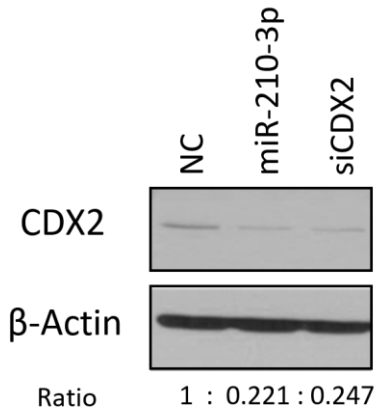

Figure S1. Validation of small interfering RNA targeting CDX2. Western blot analysis showing a decrease in CDX2 protein level 48 h post-transfection of HTR8/SVneo cells with 100 nM of non-targeting control (NC), miR-210-3p mimic (positive control), or siRNA targeting CDX2.

8. CDX2 downregulation reduced the ability of HTR8/SVneo to form endothelial-like networks

To test whether CDX2 affects the angiogenic potential of trophoblasts, HTR8/SVneo cells were transfected with siCDX2, and tube formation assays were performed. Similar to the overexpression of miR-210-3p, knockdown of CDX2 using siRNA in HTR8/SVneo resulted in a significant decrease in the total tube length and the number of branching points at both 24 and 48 h (**Figure 8A**). Interestingly, co-transfecting siCDX2 and anti-miR-210-3p led to a partial rescue of the phenotype seen with siCDX2 alone at 24 h (**Figure 8B**).

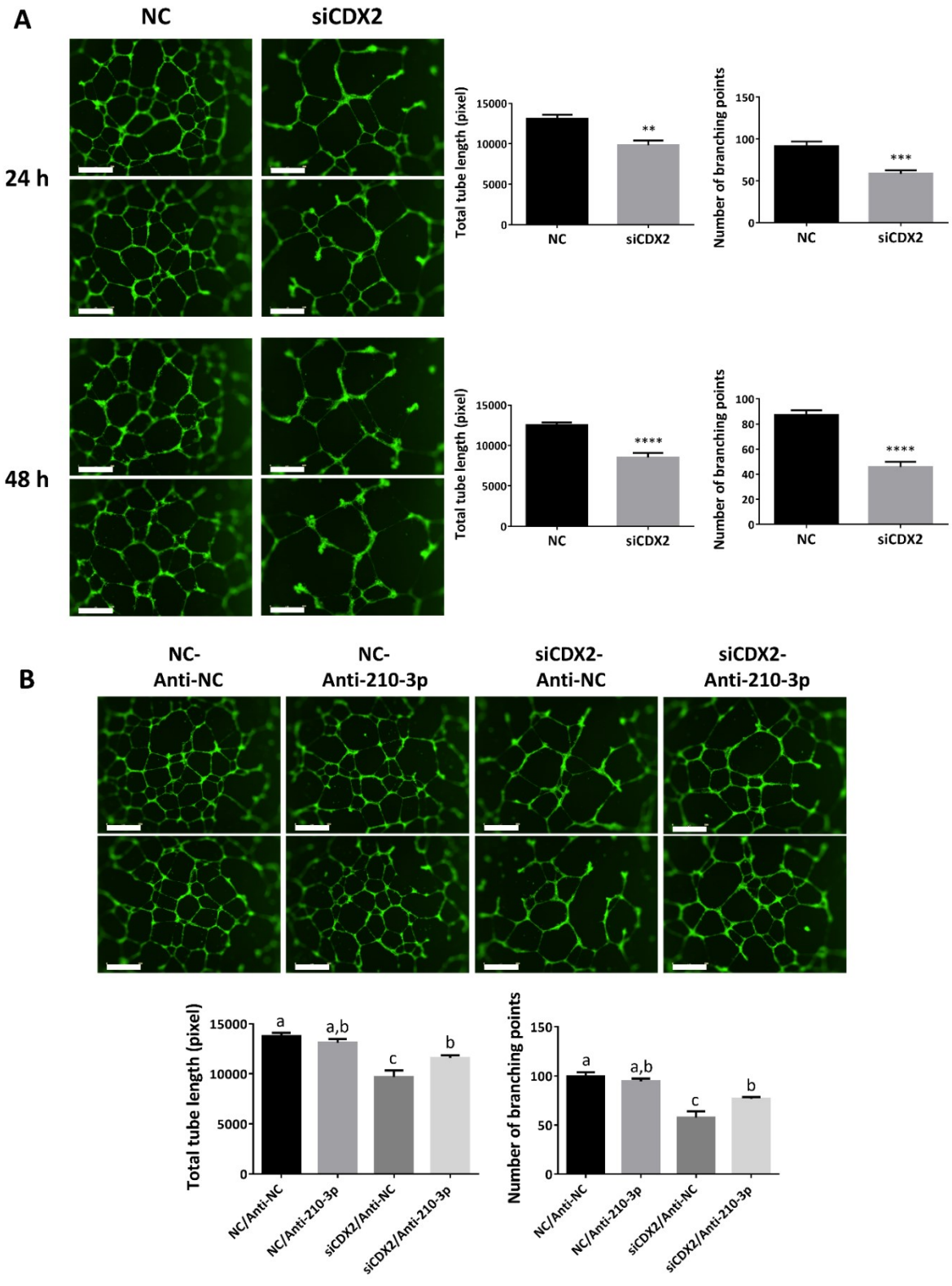

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Figure 8. CDX2 promotes trophoblast potential to form endothelial-like networks. A. Knockdown of CDX2 decreased total tube length and the number of branching points at both 24 and 48 h (N = 6). **B.** The effect of downregulating CDX2 on trophoblast ability to form endothelial-like networks was partially reversed by downregulating endogenous miR-210-3p. HTR8/SVneo cells were transfected with 100 nM of siCDX2 or its non-targeting control (NC), along with 200 nM anti-miR-210-3p or its negative control (anti-NC). siCDX2 decreased total tube length and the number of branching points, but this effect was partially reversed by anti-miR-210 (N = 6) compared to those transfected with anti-NC. Data represent mean ± SEM from representative experiments. Different letters above bars denote statistical significance ($p < 0.05$). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Scale bar is 800 µm.

9. CDX2 knockdown in HTR8/SVneo decreased the levels of EVT and enEVT differentiation markers, as well as IL1B, CXCL8 and CXCL1

To determine whether CDX2 affects multiple EVT and enEVT differentiation markers, HTR8/SVneo cells were transfected with siCDX2. Downregulation of CDX2 led to a significant decrease in the mRNA levels of ITGA1, PECAM1, CDH5, HLA-G, IL1B, CXCL8 and CXCL1 (**Figure 9**), thus mimicking the effects observed with miR-210-3p overexpression.

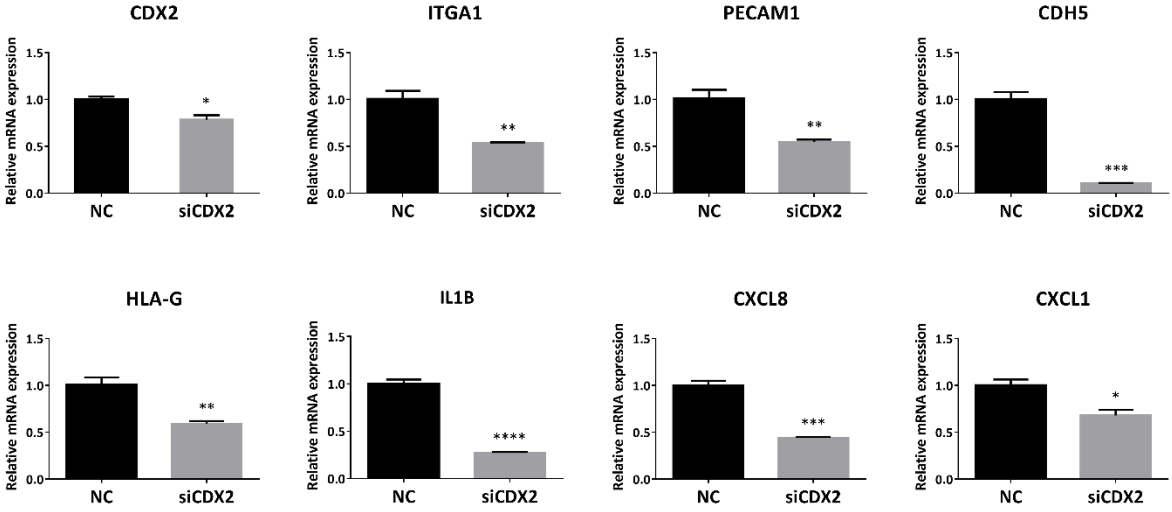

Figure 9. Silencing of CDX2 using siRNA significantly inhibited mRNA levels of enEVT markers, as well as IL1B, CXCL8 and CXCL1. HTR8/SVneo cells were transfected with 100 nM of siCDX2 or the non-targeting control (NC) and RNA was extracted 48 h post-transfection and mRNA level was measured using qRT-PCR. Data represent mean ± SEM from representative experiments (N = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

V. Discussion

MicroRNA have been shown to play an important role in every step of a successful pregnancy, including trophectoderm development, implantation and placentation (O'Brien et al., 2018). Cytotrophoblast differentiation towards both the syncytial and extravillous pathways have been shown to be regulated by multiple miRNA (Fu et al., 2013a; Ji et al., 2013; Nadeem et al., 2014; Brkić et al., 2018; O'Brien et al., 2018; Saha and Ain, 2020). Previous studies have reported that miR-210-3p inhibits trophoblast invasion (Anton et al., 2013; Luo et al., 2014; Luo et al., 2016). In this study, we showed that miR-210-3p also inhibits the outgrowth of placental explants, the expression of enEVT markers, the ability of trophoblast to form endothelial-like networks and the mRNA levels of several cytokine/chemokines known to be involved in EVT invasion and spiral artery remodelling. These novel findings suggest that miR-210-3p plays a role in regulating uterine spiral artery remodelling.

During early placental development, some cytotrophoblasts differentiate into EVT that invade the decidua. A subset of EVT further differentiates into enEVT which acquires endothelial-like properties and replaces the endothelium in uterine spiral arteries during vascular transformation. In this study, we provided several lines of evidence to suggest that miR-210-3p exerts inhibitory effects on the acquisition of an enEVT-like phenotype. First, overexpression of miR-210-3p decreased cell migration and invasion, as well as first trimester placental explant outgrowth, while inhibition of endogenous miR-210-3p promoted cell migration/invasion and explant outgrowth. Second, miR-210-3p overexpression in HTR8/SVneo cells inhibited the ability of these cells to form endothelial-like networks. Third, miR-210-3p overexpression also decreased the mRNA levels of the invasive EVT and enEVT markers ITGA1 (Ji et al., 2013; Brkić et al., 2018), CDH5 (Bulla et al., 2005), PECAM1 (Bulla et al., 2005), IL1B, CXCL8 and CXCL1 (Matsumura et al., 1997; Ji et al., 2013; Brkić et al., 2018). Although anti-miR-210-3p was able to increase cell migration, invasion and EVT outgrowth, it only significantly increased the number of branching points at 48 h and did not significantly increase enEVT marker mRNA levels. Since many factors are involved in promoting spiral artery remodelling (Albrecht and Pepe, 2020; Sato, 2020), it is likely that partial inhibition of endogenous miR-210-3p alone is insufficient to increase the angiogenic potential and enEVT marker expression.

The recruitment of uterine decidual natural killer cells (dNK) and macrophage recruitment to the spiral arteries is required to initiate the remodelling process (Ji et al., 2013; Choudhury et al., 2017). EVT have been shown to secrete different cytokines and chemokines that recruit these maternal immune cells into the spiral arteries (Bowen et al., 2002a; Hess et al., 2007; Naruse et al., 2010; Baston-Buest et al., 2017; Choudhury et al., 2017). Recently, we demonstrated that miR-218-5p promotes spiral artery remodelling by inducing enEVT differentiation and increasing cytokine/chemokine expression, including IL1B, CXCL8 and CXCL1 (Brkić et al., 2018). In the present study, we found that overexpression of miR-210-3p downregulated the mRNA level of IL1B, CXCL8 and CXCL1 in HTR8/SVneo cells. IL1B was shown to promote EVT migration (Prutsch et al., 2012), and it has been suggested that it is secreted by trophoblast to regulate their own invasion into the decidua (Hess et al., 2007). It was also shown that CXCL8 promotes trophoblasts invasion (Jovanović et al., 2010) and that it is secreted by enEVT to stimulate endothelial cells to secrete CCL14 and CXCL6, which, in turn, attract and recruit dNK cells and macrophages from the surrounding decidual tissue into the spiral arteries to accelerate the remodelling process (Choudhury et al., 2017). CXCL1 has also been suggested to play a role in trophoblast-decidual communication and to act as a chemoattractant for neutrophils and leukocytes (Red-Horse et al., 2001; Hess et al., 2007). Inhibiting the expression of these cytokine and chemokine expressions further suggests that miR-210-3p regulates events associated with spiral artery remodelling.

CDX2 is a major transcription factor important for trophectoderm lineage and maintenance of trophoblast self-renewal (Tolkunova et al., 2006; Chen et al., 2013; Malysheva et al., 2018; Xiao et al., 2020). CDX2 mRNA has been detected in multiple trophoblast cell lines such as JEG-3, TCL-1 (Hemberger et al., 2010) and HTR8/SVneo (Jia et al., 2014); however, others reported that its protein level was undetectable in placental samples beyond 15 weeks of gestation (Hemberger et al., 2010; Burton et al., 2020). Although a specific role of CDX2 in early differentiation of EVT pathways has not been reported, one study showed that CDX2 increased HTR8/SVneo motility (Jia et al., 2014). In non-human species, CDX2 was shown to regulate multiple trophoblast genes (Schiffmacher and Keefer, 2013), such as HAND1 and SOX15, both of which were shown to promote trophoblast differentiation into trophoblast giant cells in mouse (Scott et al., 2000; Yamada et al., 2006). In this study, using luciferase reporter assay, qRT-PCR

and Western blot analyses, we demonstrated that CDX2 is a target of miR-210-3p and it exerts promoting effects on enEVT functions. Specifically, we found that silencing of CDX2 mimicked the effect of miR-210-3p overexpression in inhibiting migration and invasion, first trimester EVT outgrowth, formation of endothelium-like networks, expression of invasive EVT and enEVT markers and expression of cytokine/chemokines. In addition, downregulating endogenous miR-210-3p partially attenuated the effect of siCDX2 on endothelial-like formation. These results suggest that CDX2 promotes enEVT functions associated with successful spiral artery remodelling. However, further experiments are needed to elucidate the mechanisms of how CDX2 regulates these processes.

We also investigated placental miR-210-3p levels across gestation in normal pregnancies and found that miR-210-3p levels are highest in the first trimester (weeks 5-12) and are lowest in the third trimester (weeks 26-40). During the first trimester, uterine spiral artery openings into the placental intervillous space (IVS) are obstructed by EVT plugs to protect the placenta and developing fetus from damage caused by oxidative stress. Instead, the IVS is filled with plasma and endometrial gland secretions to provide the necessary nutrients and physiological oxygen concentrations of around 2.5% prior to 10 weeks of gestation (Burton et al., 2021). The observed high level of miR-210-3p in first trimester samples suggests a role of miR-210-3p in early placentation events where trophoblasts have reduced mitochondrial function and rely heavily on glycolysis for ATP production (Kolahi et al., 2017; Burton et al., 2021). In cancer studies, miR-210-3p was shown to upregulate glycolysis (Chen et al., 2010), and, in trophoblasts, miR-210-3p targets ISCU, thus reducing mitochondrial function (Muralimanoharan et al., 2012).

Failure to remodel uterine spiral arteries into low resistance vessels leads to villous shear damage and placental malperfusion, causing oxidative stress in the placenta (Huppertz, 2018; Burton et al., 2021). This is a major mechanism underlying the development of PE, especially the severe, early-onset PE (Burton et al., 2021). Using placenta samples from healthy and PE pregnancies, we observed higher miR-210-3p levels in placentas of PE women who delivered a baby at term. However, in women who delivered at pre-term, we did not see a significant difference in miR-210-3p levels between PE patients and control subjects. Notably, the pre-term control samples were all from women who entered the labouring process, while most of the pre-

term PE women underwent caesarean section without labour. It is possible that the labouring process could affect miR-210-3p levels. It has been reported that pre-term labour altered the ratio of miR-210-3p in the amnion and chorion (Enquobahrie et al., 2016). Labour is also associated with increased NFKB1 activity in the placenta (Allport et al., 2001; Lee et al., 2003; Lindström and Bennett, 2005; Lappas and Rice, 2007; Lim et al., 2012), and NFKB1 is known to induce miR-210-3p levels (Zhang et al., 2012; Muralimanoharan et al., 2015). In addition, the majority of studies that reported an upregulation of miR-210-3p in PE only used term samples (Luo et al., 2014; Xu et al., 2014; Luo et al., 2016; Nejad et al., 2019). Interestingly, several studies have shown that miR-210-3p levels in the serum of women who developed PE later in gestation were higher during the second trimester than in women who did not develop PE (Xu et al., 2014; Munaut et al., 2016). Furthermore, it has been reported that miRNA can be secreted from trophoblasts and enter maternal circulation (Luo et al., 2009; Donker et al., 2012). This raises the intriguing possibility that increased levels of circulating miR-210-3p in the serum of women with PE may be secreted by trophoblasts damaged by oxidative-stress. The secretion of excess miR-210-3p from these trophoblasts may explain the lack of detectable intracellular upregulation of miR-210-3p in our pre-term PE samples. As gestation continues, accumulation of oxidative damage may increase intracellular levels above what can be efficiently secreted, thus leading to the observed increase of intracellular miR-210-3p in term samples. As circulating miRNA can be taken up by target cells (O'Brien et al., 2018), it is possible that enEVT within remodelling spiral arteries may be exposed to increased levels of circulating miR-210-3p, which can inhibit functions associated with successful spiral artery remodelling and contributing to a potential positive feedback of PE pathogenesis. This possibility requires further investigation.

In summary, we used multiple approaches to investigate the expression and function of miR-210-3p in human placenta. We confirmed previous findings that miR-210-3p level is upregulated in term PE. In addition, overexpression of miR-210-3p decreased trophoblast migration, invasion, placental explant outgrowth, the ability of trophoblasts to form an endothelial-like network and the expression of enEVT markers and cytokine/chemokines known to be involved in trophoblasts-immune cells communication during spiral artery remodelling. We also identified CDX2 as a novel target of miR-210-3p. Our findings suggest that miR-210-3p plays

a role in constraining enEVT functions associated with successful spiral artery remodelling, in part by downregulating CDX2. Upregulation of miR-210-3p in EVT may contribute to the impaired maternal spiral artery remodelling observed in PE. However, there are several limitations to our study. For example, we only measured mRNA levels of marker gene expression, and although we showed that miR-210-3p reduced the ability of trophoblasts to form network-like structures, more studies are required to confirm the role of miR-210-3p in enEVT differentiation and spiral artery remodelling in human pregnancy.

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Chapter Three

Regulation of neuropeptide Y by miR-218-5p and the potential role of neuropeptide Y in human placenta

Regulation of neuropeptide Y by miR-218-5p and the potential role of neuropeptide Y in human placenta (unpublished).

Heyam Hayder [1], Jacob Anderson O'Brien [1], Caroline Dunk [2,†], Jelena Brkic [1], Mohamed Salem[1], Stephen Lye [2,3,4] and Chun Peng [1,5,*]

[1]Department of Biology, York University, Toronto, ON M3J 1P3, Canada
[2]Research Centre for Women's and Infants' Health, Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Sinai Health System, Toronto, ON M5G 1X5, Canada
[3]Department of Obstetrics and Gynaecology, Faculty of Medicine, University of Toronto, Toronto, ON M5G, 1E2, Canada
[4]Department of Physiology, Faculty of Medicine, University of Toronto, Toronto, ON M5S, 1A8, Canada
[5]Centre for Research on Biomolecular Interactions, York University, Toronto, ON M3J 1P3, Canada
†Current address: Department of experimental therapeutics, Toronto General Research Institute, University Health Network, Toronto, ON M5G 2C4, Canada
*****Correspondence: cpeng@yorku.ca (C.P.)

Authors' contributions

J.A.O. generated a computer program to scan for miR-218-5p MRE on NPY DNA and NPY and NPY1R mRNA, scan *NPY* gene locus for CpG islands, designed and conducted CRISPR-cas9 NPY knockout cell line, adhesion assay quantifications, contributed to some additional figures as indicated in each figure legend.

C.D. trained me and helped perform all placental explant experiments, tissue immunohistochemistry and their data analysis.

J.B. generated the miR-218-5p overexpressing stable line and performed the microarray that showed that NPY mRNA is downregulated in these cells, thus initially contributing to the start of the NPY project.

M.S. cloned the predicted miR-218-5p target site on NPY and NPY1R into pMIR-Report vector.

C.P. supervised and contributed to all aspects of this project, including conceptualization, methodology, results, discussion, writing and funding acquisition.

I. Summary

Recent work in our lab has found that miR-218-5p promotes trophoblast differentiation along the endovascular pathway and spiral artery remodelling. To elucidate the mechanisms by which miR-218-5p exerts these functions, we used microarray analyses to compare the transcriptomes of control and miR-218-5p-overexpressing trophoblast cells. We found neuropeptide Y (NPY) mRNA to be the second most downregulated mRNA in miR-218-5p overexpressing cells. NPY is a neurotransmitter and neurohormone abundantly expressed in the brain. Its actions in humans are mediated by four G-protein coupled receptors (NPY1R, NPY2R, NPY4R and NPY5R). NPY is also produced in the placenta during pregnancy, but little is known about its role in placenta development. We found that transient overexpression of miR-218-5p upregulates NPY pre- and mature mRNA and protein levels. It also targets NPY1R mRNA at the 3' UTR and this results in decreased mRNA levels but not the protein. To investigate the role of NPY in the human placenta, we used immortalized trophoblast cell lines to examine cell proliferation, migration, and invasion after NPY overexpression or knockdown. We found that NPY overexpression decreased migration and invasion of HTR8/SVneo and Swan71 trophoblast cell lines while silencing it with siRNA increased their motility and proliferation rate. The inhibitory effect of NPY on cell migration and invasion was specific to the full-length NPY (1-36 amino acids), an NPY receptor 1 (NPY1R) agonist but not to the truncated NPY (3-36 amino acids), an NPY2R agonist. Moreover, NPY effect on motility was attenuated when cells were treated with an NPY1R-specific inhibitor. Also, NPY1R overexpression decreased HTR8/SVneo and Swan71 cells proliferation while NPY1R antagonists increased proliferation. Using immunohistochemistry, we detected NPY and NPY1R in placentas from early pregnancy, which decreased to undetectable levels toward the end of the second trimester. Finally, using the floating first-trimester villous model, we found that knockdown of NPY expression or inhibition of NPY1R signalling resulted in increased cytotrophoblast proliferation. Together, these findings suggest that the NPY-NPY1R signalling pathway negatively regulates trophoblast migration, invasion, and proliferation and may be involved in regulating early placenta development.

II. Introduction

Neuropeptide Y (NPY) is a sympathetic neurotransmitter, a neurohormone and the most abundant neuropeptide in the brain (Zukowska-Grojec et al., 1998; Zukowska et al., 2003; Tilan and Kitlinska, 2016). It is a 36 amino acid peptide, but it can be cleaved by dipeptidyl peptidase-4 (DPP4) to generate different length C-terminal fragments. NPY has been linked to obesity and tumour growth and progression. It has also been shown to play a role in regulating cardiovascular function, the immune system, stress response and smooth muscle cell proliferation (Zukowska et al., 2003; Tilan and Kitlinska, 2016).

NPY actions in humans are mediated by four G-protein coupled receptors (NPY1R, NPY2R, NPY4R and NPY5R) (Zukowska-Grojec et al., 1998; Zukowska et al., 2003; Tilan and Kitlinska, 2016) as NPY3R has been renamed CXC chemokine receptor type 4 (CXCR4). Activation of these receptors by ligand binding leads to inhibition of adenylate cyclase, which leads to a reduction in cAMP accumulation and a decrease in PKA activity (Pedragosa-Badia et al., 2013; Persaud and Bewick, 2014). Receptors activation can also lead to modulation of Ca^{2+} and K^{+} channels, the release of intracellular Ca^{2+} (Aakerlund et al., 1990; Daniels et al., 1992), an increase in phosphoinositide 3-kinase (PI3K) production by phospholipase C (PLC)-β (Pedragosa-Badia et al., 2013), activation of protein kinase C (PKC), and/or the activation of mitogen-activated protein kinase (MAPK) signalling (Goldberg et al., 1998). NPY affinity for its receptors, thus its activity, is dependent on its structure (Zukowska et al., 2003). NPY1R needs to interact with the tyrosine residues of NPY at both N- and C-terminals, but it can tolerate substituting tyrosine with phenylalanine on position 36 at the NPY C-terminal. On the other hand, the C-terminal end of the NPY activates NPY2R, and thus substitutions in the NPY C-terminal are not tolerated. NPY4R is mainly present in the gastrointestinal system and the brain and has a higher affinity to pancreatic polypeptide (PP) than NPY. Finally, NPY5R, which shares a common promoter region with NPY1R, can be activated by full-length NPY or any of its fragments (Pedragosa-Badia et al., 2013).

In pregnancy, NPY is produced by the amnion epithelial cells, the chorionic cytotrophoblasts and the decidual stromal cells (Petraglia et al., 1993). Studies on NPY

dysregulation in PE are inconsistent. *NPY* mRNA levels were reported to be downregulated in placental tissue samples from women with PE (Dotsch et al., 1999) as well as downregulated *NPY1R*, *NPY2R* and *NPY5R* mRNA levels (Klinjampa et al., 2019). Conversely, one study showed NPY serum levels did not differ between normal and PE women (Klinjampa et al., 2019). Another showed no difference in NPY plasma concentration between mild and severe preeclamptic women (Paiva et al., 2016). However, other studies reported increased plasma concentration of NPY in women with PE and that NPY accumulated mainly in the platelets (Khatun et al., 2000; Paiva et al., 2016). This is interesting as in mice, platelet NPY content is known to promote vascular smooth muscle cell proliferation, neointimal formation and monocyte/macrophage infiltration to the site of vascular injury. Also, in humans, platelet NPY levels are known to be higher in patients with peripheral vascular disease (Kuo et al., 2007), thus making NPY a potential marker for severe stress.

Currently, there are limited reports on the role of NPY or its receptors in placenta development; however, some studies suggested that NPY is the main uterine vasoconstrictor during pregnancy due to increased numbers of NPY-containing nerve fibres in uterine arteries (Khatun et al., 2000; Paiva et al., 2016). NPY has also been suggested to play a role in regulating uterine blood flow and myometrium contractility as well as a role in labour due to the progressive increase of NPY levels in the maternal circulation and amniotic fluid during labour, with the highest levels occurring at the most advanced stages (Iliodromiti et al., 2012). At the cellular level, placental and blood-derived NPY was found to bind NPY1R on the maternal side of the syncytiotrophoblasts and this regulates the synthesis and release of corticotropin-releasing factor and inhibin by trophoblasts (Wharton et al., 1993; Robidoux et al., 1998; Robidoux et al., 2000). This effect was mediated by calcium-dependent signalling via the activation of PLC-β and PI3K (Robidoux et al., 1998; Robidoux et al., 2000; Moreau et al., 2002).

In our lab, we have been investigating factors impacting trophoblast differentiation and function to better understand placenta development and possible mechanisms that might contribute to pregnancy disorders. One set of factors we have looked at are microRNAs (miRNA) (Luo et al., 2012; Fu et al., 2013; Nadeem et al., 2014; Brkić et al., 2018; Hayder et al., 2021). A recent study in our lab has found that miR-218-5p promotes trophoblast differentiation along

the endovascular pathway and spiral artery remodelling (Brkić et al., 2018). To elucidate the mechanisms by which miR-218-5p exerts these functions, we used microarray analyses to compare the transcriptomes of control and miR-218-5p-overexpressing trophoblast cells. We found NPY mRNA to be the second most downregulated mRNA in miR-218-5p overexpressing cells. In addition, *in silico* analysis showed NPY1R as a potential target gene of miR-218-5p. Therefore, we hypothesize that miR-218-5p regulates the NPY-NPY1R signalling pathway and plays a role in regulating trophoblast functions and placenta development.

III. Materials and methods

Cell lines and cell culture

Experiments were conducted using an immortalized human first-trimester trophoblast cell line, HTR8/SVneo, kindly provided by Dr. Charles Graham (Queen's University, Kingston, ON, Canada). Some experiments were repeated using another immortalized human first trimester trophoblast cell line, Swan71, generously provided by Dr. Caroline Dunk (Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, ON, Canada). Both cell lines were maintained as previously described (Brkić et al., 2018; Brkić et al., 2020). Briefly, first-trimester HTR8/SVneo cells were cultured in HyClone™ classical liquid media RPMI 1640 with L-glutamine (GE Healthcare Life Sciences, Ottawa, ON, Canada) while Swan71 in DMEM/F-12 (1:1), HEPES media (GIBCO® Life Technologies, Mississauga, ON, Canada) both of which were supplemented with 10% fetal bovine serum (FBS) (GIBCO® Life Technologies, Mississauga, ON, Canada) and grown in a humidified environment of 5% CO_2 and 37 °C. HTR8/SVneo cell line overexpressing mir-218-1 was generated (Brkić et al., 2018) and kindly provided by Dr. Jelena Brkic in our lab and maintained similarly to HTR8/SVneo parental line.

Transient transfections and treatments

Transient transfection of miR-218-5p mimic, miR-218-5p inhibitor, or small interfering RNA oligomers (150 nM) (**Table 2.1**) was conducted using Lipofectamine RNAiMAX® reagent (Invitrogen, Life Technologies, Mississauga, ON, Canada). For plasmid transfection, 500 ng per

well of plasmid were transfected using Lipofectamine 2000® reagent (Invitrogen, Life Technologies, Mississauga, ON, Canada) (**Table 2.2**). A modified protocol was used to optimize transfection efficiency and cell survival using a Lipofectamine volume of 2.4 µL per well in OMEM medium (GIBCO® Life Technologies, Mississauga, ON, Canada) (Brkić et al., 2018; Brkić et al., 2020). Cells were transfected 24 h post-seeding for 5 h in 6-well plates seeded at 6.0 × 10^4 cells/well. The transfection mix was then removed, and cells were recovered with 10% FBS containing media for 24 h after transfection before proceeding to functional assays and for 48 h for RNA, Western or dot blot analyses. Recombinant human NPY (1-36) or (3-36) were used in experiments at 1 pM concentration or, as otherwise specified, in serum-free media. Cells were pre-treated overnight prior to seeding for functional assays and also during the assay. NPY receptors inhibitor treatments were done in 10% FBS containing media at 1 nM or otherwise specified concentrations in each experiment for the assay duration.

Table 2.1 Oligomer sequences and reagents.

Oligomer Name	**Sequence: 5 → 3'**
NC (non-targeting control) GenePharma (Shanghai, China)	Sense: UUCUCCGAACGUGUCACGUTT Anti-sense: ACGUGACACGUUCGGAGAATT
hsa-miR-218-5p GenePharma (Shanghai, China)	Sense: UUGUGCUUGAUCUAACCAUGU Anti-sense: ACAUGGUUAGAUCAAGCACAA
Anti-NC	RiboBio™ miRNA Inhibitor, Negative control #22 (Guangzhou, China)
Anti-hsa-miR-218-5p	RiboBio™ anti- hsa-miR-218-5p Inhibitor RiboBio (Guangzhou, China)
siRNA-NPY GenePharma (Shanghai, China)	Sense: UGUCCCUGCUCGUGUGCCUGGGUGC Anti-sense: GCACCCAGGCACACGAGCAGGGACAGG
siRNA-NPY1R (#1) GenePharma (Shanghai, China)	Sense: GCCUUUCCUGAUCUACCAATT Anti-sense: UUGGUAGAUCAGGAAAGGCTT

siRNA-NPY1R (#2) GenePharma (Shanghai, China)	Sense: GCUCCCUCUUACCAUCUUUTT Anti-sense: AAAGAUGGUAAGAGGGAGCTT
Recombinant human NPY 1-36	Abcam Inc. ab120208 (Toronto, ON, Canada)
Recombinant human NPY 3-36	Sigma-Aldrich N9407 (Oakville, ON, Canada)
NPY1R inhibitor	BIBO 3304 trifluoroacetate TOCRIS, R&D Systems (Toronto, ON, Canada)
NPY2R inhibitor	BIIE 0246 TOCRIS, R&D Systems (Toronto, ON, Canada)
NPY5R inhibitor	CGP 71683 hydrochloride TOCRIS, R&D Systems (Toronto, ON, Canada)

Table 2.2 Plasmids

Plasmid name	**Provider**
pEGFP-N1 (EV)	Addgene #86775
NPY-pEGFP-N1	Addgene #74629
NPY1R-pEGFP-N1	GenScript GenEZ ORF Clone: OHu22213C
pMIR REPORT™ miRNA Expression Reporter	Ambion # AM5795
Renilla pRL-TK	Promega # E2241

RNA extraction, reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells or tissues using RiboZol™ RNA Extraction Reagent (VWR Life Science, Mississauga, ON, Canada) following the manufacturer's protocol with some modifications. Namely, during the RNA precipitation step, instead of the recommended 10 min

at room temperature in isopropanol, 30 min at −20 °C was performed. RNA concentration was measured using NanoDrop 2000 spectrophotometer (Thermo Scientific, Mississauga, ON, Canada). The TaqMan miRNA assay kit (Invitrogen, Life Technologies, Mississauga, ON, Canada) was used to measure miR-218-5p, miR-218-1-3p, miR-218-2-3p and U6 following the manufacturer's protocol. To measure the levels of mRNA, 1.5 µg of total RNA was reversed transcribed into cDNA by M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA, USA) using oligo-dT primers (Sigma-Aldrich, Oakville, ON, Canada) for mature mRNA and random hexamer primers (New England Biolabs, Ipswich, MA, USA) for pre-mRNA, following the manufacturer's protocol. qRT-PCR was performed using EvaGreen qPCR 2X Master mix (Applied Biological Materials Inc., Richmond, BC, Canada) following the manufacturer's recommendation, and samples were normalized to CYC1 internal control (Brkić et al., 2018; Brkić et al., 2020) using the $2^{-\Delta\Delta CT}$ method. Primers used were designed using NCBI primer blast and are listed in **Table 2.3**.

Table 2.3 Primer sequences used for qRT-PCR.

Primer Name	**Sequence: 5' → 3'**
CYC1	FP: CAGATAGCCAAGGATGTGTG
	RP: CATCATCAACATCTTGAGCC
NPY	FP: GGAAAACGATCCAGCCCAGA
	RP: CAGGGTCTTCAAGCCGAGTT
Pre-NPY (Exon 1-Intron 1)	FP: CTCGCCCGACAGCATAGT
	RP: TAGACAGACGGGTCGTAGC
NPY1R	FP: AATAAGCTGAACAGTTGACCTGC
	RP: ATCAAGGCCAGGTTTCCAGAG

Protein extraction and immunoblot analysis

After cells were transfected and allowed to recover for 48 h, cells were lysed with HEPES lysis buffer (10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.0 mM DTT, 0.1% NP-40, pH 7.9) supplemented with Pierce protease and phosphatase inhibitors (Thermo Scientific, Mississauga, ON, Canada). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to a 0.2 µm polyvinylidene difluoride membrane (EMD Millipore, Oakville, ON, Canada) overnight at 4 °C. In dot blot analysis, cell lysate was added directly into 0.2 µm nitrocellulose membrane (GE Healthcare Life Sciences, Ottawa, ON, Canada) at 1.0 × 10^4 cells/3 µL dot. Membranes were blocked in blocking buffer (5% (w/v) skim milk in 1X Tris-buffered Saline and Tween-20, TBST) for 1.5 h at room temperature. Membranes were then incubated overnight at 4 °C with the primary antibodies diluted in blocking buffer (**Table 2.4**). Membranes were then washed with 1X TBST buffer and probed with horseradish peroxidase-conjugated secondary antibodies diluted in blocking buffer at room temperature for 1.5 h. Signals were detected on X-ray film using Clarity™ Western ECL substrate kit (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada).

Table 2.4 Western and dot blot analysis antibodies

Antibody target	Manufacturer and Catalog No.	Species	Dilution
NPY	Cell Signaling (11976S)	Rabbit	1:1000
NPY1R	Abcam (ab183108)	Rabbit,	1:1000
B-Actin	Santa Cruz (SC81178), Cell Signaling (3700S)	Mouse, Mouse	1:2000, 1:1000
Anti-Rabbit secondary	Cell Signaling (7074S)	Goat	1:4000
Anti-Mouse secondary	Cell Signaling (7076S)	Horse	1:4000

Luciferase reporter assay

NPY and NPY1R mRNA were scanned for potential miRNA response element (MRE) sites using prediction software in development at our lab. The luciferase reporter construct was generated by cloning a portion of the NPY1R 3' UTR containing the predicted miR-218-5p target sequence using forward primer: TTGTGATTTCCGGTCTCGGG and reverse primer: TCTTTCGGCTCCTGAATGCT or the NPY exon 3 portion containing predicted miR-218-5p target sequence using forward primer: CCAGCCACCATGCTAGGTAA and reverse primer: CTACCAATGCATGCAGCCAC into pMIR-Report™ miRNA expression plasmid (Ambion, Life Technologies, Mississauga, ON, Canada) using HindIII and SpeI restriction enzymes (New England Biolabs, Ipswich, MA, USA) downstream of the luciferase gene. Briefly, both target fragments were amplified using HTR8/SVneo RNA as a template. The resulting PCR amplicons were cloned into the report plasmid and sequenced to confirm their identities. Luciferase assay was performed using the Dual-Luciferase® reporter assay system as per the manufacturer's protocol (Promega, Madison, WI, USA). HTR8/SVneo cells were transfected with 250 ng of the Luciferase construct containing no insert (EV) or NPY or NPY1R miR-218-5p target site inserts, and 30 ng Renilla construct (pRL-TK, Promega, Madison, WI, USA) and either 25 nM of miR-218-5p or the scrambled control. At 24 h after transfection, cells were lysed, and luciferase activity was measured.

First trimester human placenta floating villi culture and BrdU-chasing protocol

Human placental floating villi experiments were conducted as described previously (Drewlo et al., 2008). Briefly, 5-6 weeks placentas from electively terminated pregnancies were obtained through the Research Center for Women's and Infants' Health BioBank Program and the Lunenfeld-Tanenbaum Research Institute (Toronto, ON, Canada) on the day of the procedure. Informed consent was obtained from each patient, and collections were approved by the Mount Sinai Hospital's Review Committee on the Use of Human Subjects. Placentas were carefully dissected using a dissecting microscope with a gooseneck light source in a sterile plate filled with Ca^{2+}/Mg^{2+}-free 1X PBS to tease the floating villi apart. At least three villi per treatment were added to each well in a 24-well plate in phenol red-free DMEM/F-12 (1:1) with HEPES (GIBCO®

Life Technologies, Mississauga, ON, Canada) supplemented with 1% insulin/transferrin/selenium (Invitrogen, Life Technologies, Mississauga, ON, Canada) and 0.1% Normocin (Invitrogen, Life Technologies, Mississauga, ON, Canada); treatments were started at the specified concentrations listed in each figure. The plate was incubated for 24 h at 8% O_2 in a humidified environment of 5% CO_2 and 37 °C. Bromodeoxyuridine (BrdU) was then added at 100 µM to each well, and the plate was left to incubate for another 24 h at 8% O_2 in a humidified environment of 5% CO_2 and 37 °C. Tissues were then fixed in 4% paraformaldehyde in 1X PBS for 30 min, then washed with 1X PBS 2 times, 10 min each. Tissues for each treatment were embedded in HistoGel (ThermoFisher Scientific HG-4000-012, Mississauga, ON, Canada) following the manufacturer's protocol then transferred each HistoGel block into an embedding basket. Baskets were left in 10% formalin solution (neutral and buffered) in a fume hood overnight. Baskets were then put into 70% ethanol in 1X PBS at 4 °C until ready to embed. Each treatment and its control tissues were embedded into a single paraffin cube. Slides of tissues were then processed following the immunohistochemistry protocol.

Immunohistochemistry

Immunohistochemical analysis was done using the streptavidin peroxidase method. Briefly, Paraffin-embedded tissues were sectioned at 7.0 µm thickness and applied on slides. Slides with tissue sections were baked at 60 °C for at least 1 h, then cooled for 10 min. Slides were de-paraffinized in xylene (3 times, 5 min each), rehydrated in descending grades of ethanol starting at 100% ethanol (3 times, 2 min each), 95% ethanol for 2 min, quenched endogenous peroxidase activity in 3% hydrogen peroxide in methanol for 40 min, then continued rehydration in ethanol at 90%, 80%, 70% and 50% 2 min each. Slides were then washed 2 times with 1X PBS, 5 min each. Tissues were then fixed in 4% paraformaldehyde in 1X PBS for 15 min, then washed 2 times with 1X PBS, 5 min each. The antigen retrieval method for antibodies used is listed in **Table 2.5**; briefly, boiling in either 1 mM EDTA pH 9 (Dako S2367, Agilent Technologies, Mississauga, ON, Canada) or 10 mM sodium citrate pH 6 solutions. Slides were then washed 2 times with 1X PBS, 5 min each. Slides were blocked in a protein blocking buffer of 10% goat serum and 2% rabbit serum (Dako X090930-2, Agilent Technologies, Mississauga, ON, Canada) in a

humidified chamber for 1 h at room temperature. Slides were incubated with primary antibodies (**Table 2.5**) diluted in 1X PBS and incubated overnight at 4 °C in a humidified chamber. Incubation with no antibody or rabbit or mouse IgG in place of primary antibodies served as negative controls. Slides were then washed 2 times with 1X PBS, 5 min each. Slides were then incubated with anti-rabbit or anti-mouse biotinylated secondary antibodies for 1 h at room temperature in a humidified chamber. Slides were then washed2 times with 1X PBS, 5 min each, then incubated with streptavidin-HRP conjugated tertiary reagent (Invitrogen SA1007, Life Technologies, Mississauga, ON, Canada) for 1 h at room temperature in a humidified chamber, then washed 2 times with 1X PBS, 5 min each. Signals were detected using DAB$^+$ staining kit (Dako K3468, Agilent Technologies, Mississauga, ON, Canada) containing 0.02% hydrogen peroxide and 0.075% 3,3'-diaminobenzidine in 1×PBS as per the manufacturer's protocol. Slides were then counterstained using Harris hematoxylin (1:4. Sigma-Aldrich, Oakville, ON, Canada) and washed with tap water, then acidified the stain in Scott buffer (2 g sodium bicarbonate, 20 g magnesium sulphate in 1 L tap water) and washed again with tap water. Tissues were then dehydrated in ascending series of ethanol (70%, 80%, 90%, 95%, 3X 100%) 2 min each and slides cleared with xylene (2 times, 2 min each) and mounted with Cytoseal. BrdU immunohistochemistry was performed by the Mt. Sinai histology facility (University Health Network, Toronto, ON, Canada). BrdU primary antibody dilution 1:3000. Slides were scanned at the Advanced Optical Microscopy Facility (University Health Network, Toronto, ON, Canada) and viewed using the Aperio ImageScope software (Leica Microsystems, Concord, ON, Canada).

Table 2.5 Immunohistochemistry antibodies and dilutions

Antibody target	Manufacturer and Catalog No.	Species	Dilution	Antigen Retrieval
NPY	Cell Signaling (11976S)	Rabbit	1:100	EDTA
NPY1R	Abcam (ab183108)	Rabbit	1:250	EDTA
Ki67	Abcam (ab15580)	Rabbit	1:300	Sodium Citrate
CK-7	Dako (M7018)	Mouse	1:200	Sodium Citrate
HLA-G	ExBio (11-499-C100)	Mouse	1:300	Sodium Citrate

CD34	Dako (GA63261, 0.18 µg/mL)	Mouse	Ready to use	Sodium Citrate
Rabbit IgG	Abcam (ab37415)	-	1:100	EDTA
Mouse IgG	Santa Cruz (SC2025)	-	1:300	Sodium Citrate
Secondary Anti-Rabbit	Abcam (ab97083)	Donkey	1:1000	-
Secondary Anti-Mouse	Dako (E0464)	Rabbit	1:300	-

Proliferation/Cell growth assay

Cells were either transfected with siRNA or plasmid or treated 24 hours prior to assay as indicated in each figure. Cells were then collected using Accutase (Corning™ Inc., Ottawa, ON, Canada) and seeded into a 96-well plate at 3.0×10^3 cells/well (HTR8/SVneo & Swan71) in 10% FBS containing medium (with treatments if applicable). After confirming the uniform distribution of the cells in each well, the plate is then put into the IncuCyte® S4 live-cell analysis system (IncuCyte, Essen Bioscience, Ann Arbor, MI, USA) at 5% CO_2 and 37 °C and each well is scanned at 4X every 3 hours. IncuCyte software analysis of phase object density was performed until the plate reached 100% confluency.

Cell counting kit-8 (CCK-8) assay

CCK-8 assay was conducted using Bimake kit (Bimake Inc., Houston, TX, USA) following the manufacturer's protocol. Briefly, 16 h post-transfection with 50 nM siRNA targeting NPY (protocol described above), HTR8/SVneo cells were detached using Accutase (Corning™ Inc., Ottawa, ON, Canada) and seeded into five different 96-well plates, 0 h, 24 h, 48 h, 72 h and 96 h plates at a minimum of 18 wells per treatment. At each time point, 10% (v/v) of CCK-8 reagent was added directly into each well, and the reagent was left to incubate for 2 h at 5% CO_2 and 37 °C. The absorbance at 450 nm was measured for each plate using Synergy™ H4 plate reader

(BioTek, Agilent Technologies, Mississauga, ON, Canada). Values graphed were calculated as absorbance sample (450 nm) – absorbance blank (450 nm).

Transwell migration and invasion assay

Transwell migration and invasion assays were conducted using polycarbonate membrane 24-well Transwell inserts with 8 µm pore size (Costar, Corning™ Inc., Ottawa, ON, Canada). However, in the invasion assay, the Transwell inserts were coated with 100 µL of Cultrex PathClear® growth factor reduced, phenol red-free Matrigel (Trevigen Inc., Burlington, ON, Canada) diluted with serum-free medium to a final concentration of 0.15 mg/mL the day before and was left overnight in a humidified environment of 5% CO_2 and 37 °C. Cells were transfected with NC, siRNA, or plasmids 24 h prior to migration and invasion assays. Cells were then collected using Accutase (Corning™ Inc., Ottawa, ON, Canada) and seeded on top of the Transwell insert at a density of 1.5×10^4 cells/insert for migration in 200 µL and 2.0×10^4 cells/insert in 100 µL for invasion per filter. Cells were seeded onto the insert in serum-free media while 10% FBS-containing media was added outside the Transwell. At 18 h post-seeding into the Transwell, cells were fixed and stained with Harleco Hemacolor Staining Kit (EMD Millipore, Oakville, ON, Canada). Non-migrated/invaded cells on top of the membranes were wiped off using a cotton swab, and membranes were cut with a blade and mounted on slides for quantification. Cells were visualized and photographed using a dissection microscope (Leica Microsystems, at 1.25X) and the number of cells migrated/invaded were counted using ImageJ as described previously (O'Brien et al., 2016).

Wound-healing assay

Cells were transfected and recovered 24 h prior to seeding onto ImageLock 96-well plate (IncuCyte, Essen Bioscience, Ann Arbor, MI, USA) at 1.5×10^4 (HTR8/SVneo) or 1.0×10^4 (Swan71) cells/well and left to adhere overnight in a humidified environment of 5% CO_2 and 37 °C. In case of recombinant protein or inhibitors treatment, cells were seeded first onto ImageLock 96-well plate (IncuCyte, Essen Bioscience, Ann Arbor, MI, USA) at 1.5×10^4 (HTR8/SVneo) or 8.0×10^3

(Swan71) cells/well and left to adhere overnight in a humidified environment of 5% CO_2 and 37 °C. Cells were then treated with recombinant protein in a serum-free medium overnight. The next day, cells were wounded using a wound-healing device (IncuCyte, Essen Bioscience, Ann Arbor, MI, USA), then put into the incubator for 10 min, followed by washing once with serum-free media then a fresh 200 µL medium was added to each well with or without treatments as per experiment in 1% serum-containing medium. The plate was then moved into the IncuCyte® S4 live-cell analysis system (IncuCyte, Essen Bioscience, Ann Arbor, MI, USA) at 5% CO_2 and 37 °C and each well was scanned at 4X every 2 hours. IncuCyte software analysis of relative wound density was performed until wound closure occurred.

Enzyme-linked immunosorbent assay (ELISA)

HTR8/SVneo were seeded and transfected as described above, and cells were recovered for 48 h. Media was collected from multiple wells per treatment (conditioned media). Cells were then detached and counted before being lysed in NP-40 lysis buffer. NPY (1-36) was then measured using the human neuropeptide Y EIA kit (RayBiotech Life Inc., Peachtree Corners, GA, USA) following the manufacturer's protocol. Both biological and technical replicates of cell lysate and conditioned media were used without dilution. The absorbance at 450 nm was measured using Synergy™ H4 plate reader (BioTek, Agilent Technologies, Mississauga, ON, Canada). NPY (1-36) level in each sample was calculated using the generated standard curve. Normalized conditioned media values were calculated by dividing the reading of a treatment conditioned media over the number of cells per well for that treatment.

CRISPR/Cas9 plasmid generation

Forward and reverse guide RNA (gRNA) flanking the NPY transcription start site were designed using CHOPCHOP (https://chopchop.cbu.uib.no/) and ordered as ssDNA from Sigma-Aldrich (Oakville, ON, Canada). gRNA sequences were cloned into the CRISPR/Cas9 plasmid PX459 (version 2, Addgene Plasmid #62988) following the author's protocol (Ran et al., 2013).

PX459 plasmids with inserted gRNA sequences were validated by SickKid's TCAG sequencing facility (https://www.tcag.ca/).

Generation of NPY transcription start site (TSS) knockout cell lines in HTR8/SVneo

Approximately 1.0 x 10^5 HTR8/SVneo were co-transfected with 750 ng/well of forward and reverse gRNA PX459 plasmids in 6-well plates for 6 h using Lipofectamine 2000® reagent (Invitrogen, Life Technologies, Mississauga, ON, Canada). Cells were recovered in 10% FBS containing medium for 24 h and then transferred to a 10 cm plate. Cells were incubated for 2 days in a humidified environment of 5% CO_2 and 37 °C without antibiotics. Then cells were maintained in 10% FBS medium supplemented with 500 ng/mL puromycin for 5 days, replacing the antibiotic medium every 2 days. Cells were harvested with 0.05% trypsin and seeded into a 96-well plate at 1.5 cells/well. Cells were incubated for 2 hours before whole-well t_0 images were taken with the IncuCyte® S4 live-cell analysis system (IncuCyte, Essen Bioscience, Ann Arbor, MI, USA). Cells were incubated for 2 weeks to allow colonies to form, replacing the media every 3 d. Whole-well images were repeated at 2 weeks, and colonies initiating from more than one cell were disregarded. Healthy colonies with median morphology were harvested with 0.2% trypsin and transferred to 12-well plates. Clones were maintained in 6-well plates in 10% FBS medium supplemented with 500 ng/mL puromycin. Knockouts were validated by sequencing of genomic DNA at the NPY TSS. Genomic DNA was amplified with Q5 high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA), and PCR products were cleaned with the QIAquick PCR purification kit (Qiagen, Germantown, MD, USA). Cleaned PCR products were sequenced by the SickKid's TCAG sequencing facility using forward primer: TACAGGAAAAATCTGTGCGCC and reverse primer: ATTTAGCCGCCAAGAGGG.

Adhesion assay

Cells were seeded into a 48-well plate at 3.0 × 10^3 cells/well in 10% FBS containing medium and left to settle at room temperature for 5 minutes. The plate was loaded into the IncuCyte® S4 live-cell analysis system (IncuCyte, Essen Bioscience, Ann Arbor, MI, USA) at 5% CO_2

and 37 °C and phase contrast micrographs were taken every 30 minutes at 200X magnification. Cells were categorized into three phases (non-adhered, adhering, and adhered) based on their morphology during the adhering process. This was performed in NIH ImageJ by manually counting the number of cells in each category for an image to then calculate the percentage of cells in each category per image. Each image was considered n = 1.

Tube formation assay

Tube formation assay was conducted after 24 h recovery post-transfection (as mentioned above). Cells were stained with a 1 µM Cell Tracker™ Green CMFDA (Invitrogen, Life Technologies) in serum-free media for 45 min and then collected using Accutase (Corning™ Inc., Ottawa, ON, Canada). Cells were then seeded at the density of 2.0×10^4 (HTR8/SVneo) or 8.0×10^3 (Swan71) cells per well into a 96-well plate pre-coated with 30 µL per well of undiluted growth factor reduced, phenol red-free Matrigel (Cultrex PathClear® 15.4 mg/mL). The plate was put into the IncuCyte® S4 live-cell analysis system (Essen BioSience, Ann Arbor, MI, USA) for imaging at 4X for at least three days. Images were then acquired and analyzed in ImageJ using the NeuronJ plugin as previously described (Meijering et al., 2004). A more detailed protocol can be found in Appendix B.

Generation of NPY and NPY1R Swan71 overexpressing stable cells

Using the NPY, NPY1R, or the pEGFP-N1 empty vector from Table 2.2, Swan71 cells were transfected with 750 ng of each plasmid. Cells were recovered in 10% FBS containing medium for 24 h. Transfected cells were then selected for two weeks using 2.5 mg/mL of Geneticin (G418 sulphate) (GIBCO® Life Technologies, Mississauga, ON, Canada). Heterogenous populations were maintained and treated occasionally with the antibiotic. qRT-PCR and immunoblot analyses were performed to confirm overexpression.

Statistical analysis

For comparison between more than two groups, one-way ANOVA, followed by Tukey's multiple comparisons or two-way ANOVA with Sidak's multiple comparisons analysis were conducted using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). However, for comparison between two groups, unpaired Student's t-test was used with Welch's correction. For curve statistics, data points were plotted as 'mean, standard error, and sample number' within GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). X-values were first log-transformed (X = log(X + 1)) and then the data was analyzed with four parameter logistic regression (4PL). LogIC50 values, p-values for logIC50, and degrees of freedom + 1 were copied into a grouped graph. Two groups of logIC50 were compared by Student's t-test with Welch's correction and three or more groups using one-way ANOVA followed by Tukey's multiple comparisons. A $p < 0.05$ was considered significant.

IV. Results

1. NPY mRNA level is decreased while protein level is increased in mir-218-1 stably overexpressing cells

The mature miR-218-5p miRNA is processed from two genes, *MIR218-1* and *-2*, where miR-218-5p is identical between both transcripts. We used TaqMan qRT-PCR to measure all three family members in HTR-8/SVneo cells stably transfected with mir-218-1 (Brkić et al., 2018). We found that only miR-218-5p was overexpressed, and there was no significantly difference in miR-218-1/2-3p levels between the control and mir-218-1 stable cells (**Figure 1A**). Also, microarray analysis showed that NPY mRNA level was decreased by nearly 5 folds in miR-218-5p stable overexpressing cells (**Figure 1B)**. The downregulation of NPY mRNA in miR-218-5p-overexpressing cells was confirmed by qRT-PCR (**Figure 1C**). We also designed primers to measure pre-NPY mRNA. As shown in **Figure 1D**, both the pre-NPY and mature NPY mRNA were strongly downregulated in the miR-218-5p stable overexpressing cells, suggesting that miR-218-5p may inhibit NPY expression at the transcriptional level. Surprisingly, NPY protein (4 kDa) levels in the miR-218-5p-overexpressing cells were slightly upregulated (**Figure 1E**) despite a 95% decrease in mature NPY mRNA.

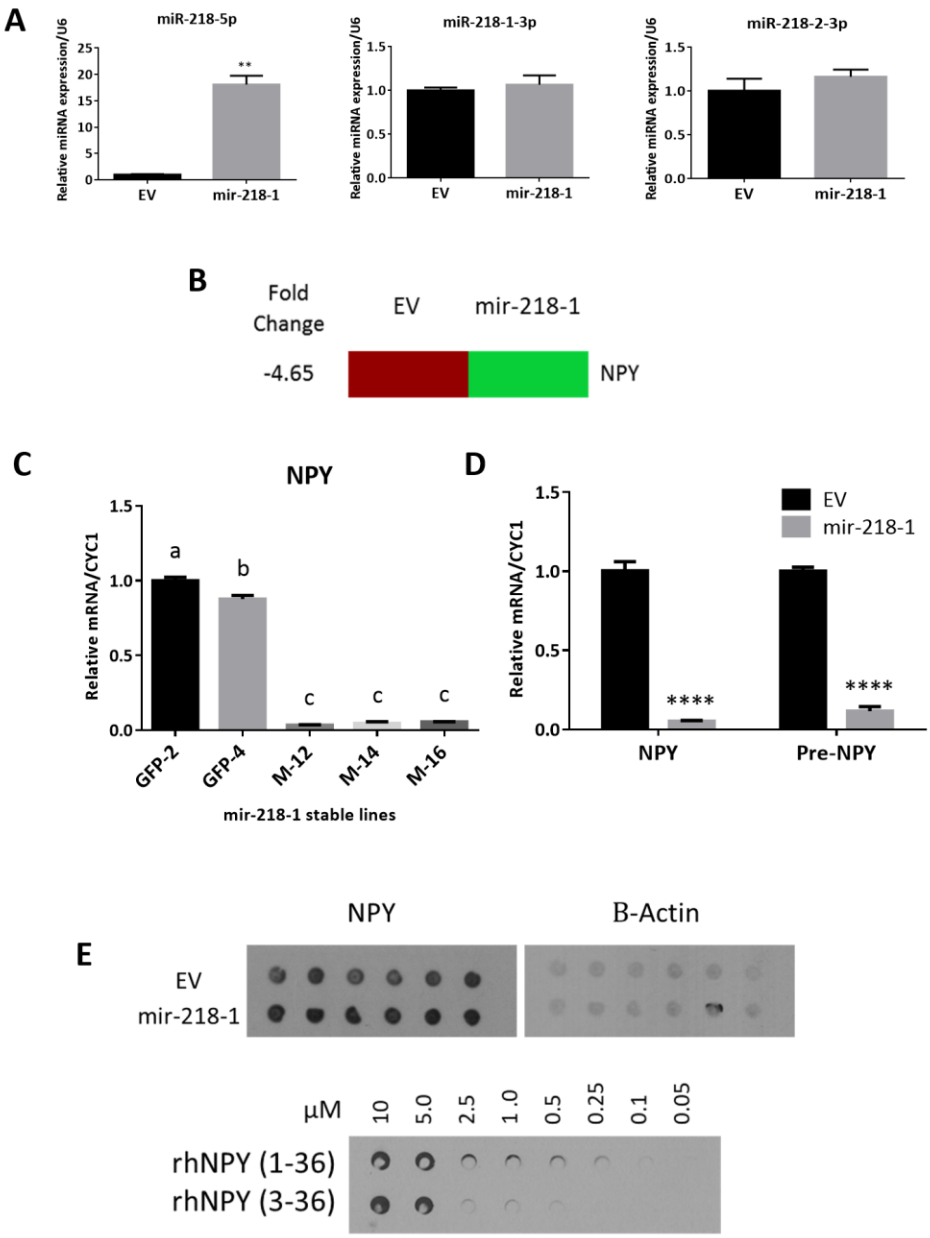


Figure 1. NPY mRNA levels decreased in mir-218-1 stably overexpressing cells. A. miR-218-5p is overexpressed in HTR8/SVneo overexpressing mir-218-1 precursor while miR-218-2-3p and miR-218-2-3p levels are unchanged. **B.** cDNA microarray showing NPY mRNA is downregulated in HTR8/SVneo overexpressing mir-218-1 precursor (conducted by Dr. Jelena Brkic). **C.** Confirmation of NPY mRNA level in different HTR8/SVneo overexpressing mir-218-1 precursor clones using qRT-PCR. **D.** Both NPY mature mRNA and pre-mRNA levels are downregulated in mir-218-1 precursor overexpressing cells. **E.** Dot blot analysis showing NPY protein levels are higher in mir-218-1 overexpressing cells. NPY antibody validation was confirmed using a serial dilution of recombinant human NPY (rhNPY) of different lengths, 1-36 and 3-36 amino acids. Jacob A. O'Brien contributed to this figure panel D. Different letters above bars represent statistical significance. Data represent mean ± SEM, N = 3. ** $p < 0.01$, **** $p < 0.0001$.

2. Transient overexpression of miR-218-5p upregulated NPY mRNA and protein despite predicted miRNA response element

Given the strong NPY mRNA downregulation observed in the mir-218-1 stable lines, we scanned the NPY gene locus for miR-218-5p miRNA response elements (MRE) and found multiple sites (**Figure 2A**). The location of these sites in relation to nearby CpG islands is shown in **Figure S1**. An MRE was predicted within exon 3 of NPY (**Figure 2B**; site 4) and was cloned into the pMIR-Report luciferase reporter vector. Transient transfection of miR-218-5p showed no significant decrease in relative luciferase activity, suggesting the predicted site is non-functional in HTR8/SVneo. Transient knockdown of endogenous miR-218-5p by transfection of anti-sense miR-218-5p (anti-miR-218-5p) led to a significant but minor decrease in relative luciferase activity (**Figure 2C**). In contrast to stable overexpression of miR-218-5p, transient transfection of miR-218-5p in HTR8/SVneo cells resulted in a significant increase in NPY pre-mRNA 24 h after transfection and in both pre- and mature mRNA at 48 h after transfection (**Figure 2D**). NPY protein was also increased at 48 h after transfection with miR-218-5p (**Figure 2E**).

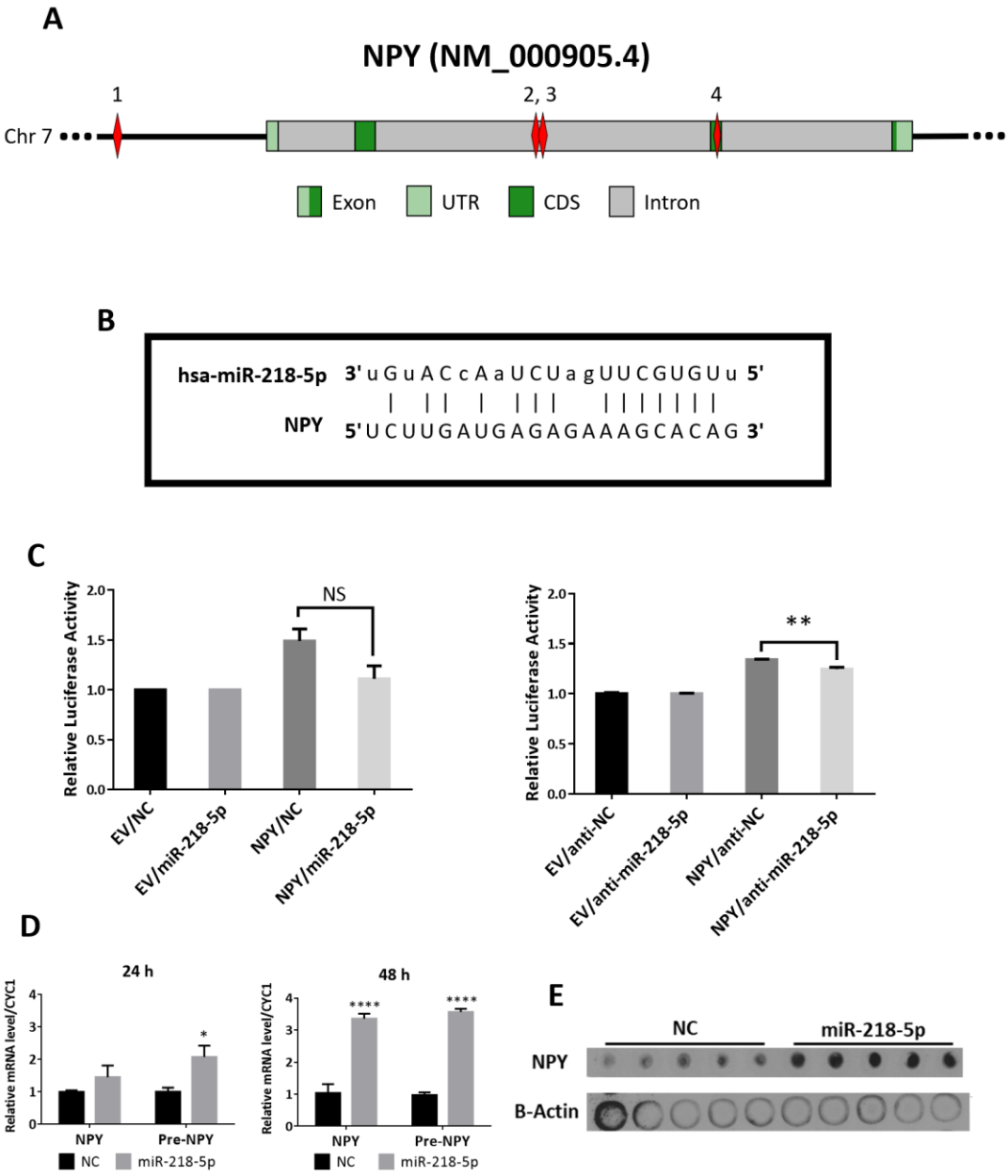


Figure 2. miR-218-5p does not target NPY at exon 3. A. Schematic diagram showing predicted miR-218-5p target sites on NPY locus indicated by the red diamond. Target site 1 is -1800 bp upstream of NPY transcription start site, sites 2 and 3 are within intron 2, and site 4 is within exon 3. **B.** miR-218-5p predicted target site on NPY exon 3 (site 4 from A) is shown. **C.** Luciferase assay using a pMIR-REPORT plasmid containing target site from B following transfection with miR-218-5p mimic or its control (NC) or anti-miR-218-5p or its control (anti-NC). **D.** NPY mature mRNA and pre-mRNA levels in cells transfected with miR-218-5p mimic or its control measured at 24 h and 48 h post-transfection (150 nM). **E.** Dot blot analysis of NPY levels (4 kDa) in cells transfected with miR-218-5p mimic or its control measured 48 h post-transfection (150 nM). Jacob A. O'Brien contributed to this figure panels D, and E. Data represent mean ± SEM, N = 3. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

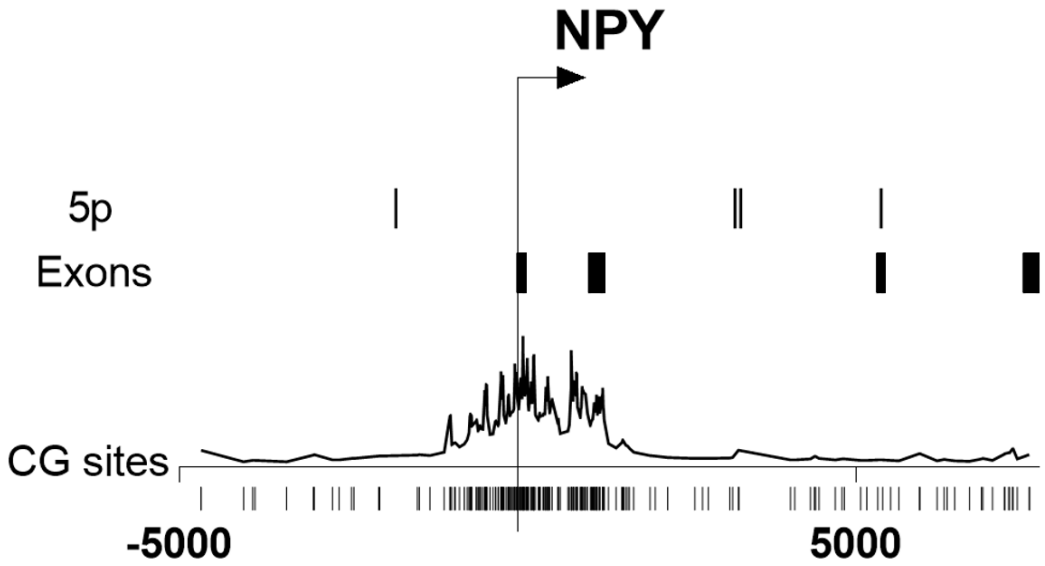


Figure S1. Schematic showing predicted miR-218-5p target sites on Neuropeptide Y (*NPY*) gene locus in relation to CpG islands. The NPY FASTA sequence, including 5 kb upstream of the transcription start site (TSS), was downloaded from NCBI and scanned for DNA-associated miRNA response elements (DMRE) for miR-218-5p. The schematic also shows 5'-CG-3' enrichment and clustering. Exon 3 contains a DMRE, and MRE following transcription. The TSS contains a CpG island. This figure was generated by Jacob A. O'Brien.

3. miR-218-5p downregulates NPY1R mRNA but increases it protein levels

MicroRNA commonly regulate multiple members of the same signalling pathway or gene network. Of the NPY receptor family, the full-length NPY (1-36) most strongly associates with NPY1R. Therefore, we investigated the potential that miR-218-5p regulates more than one aspect of NPY signalling. Scanning the NPY1R locus for potential MRE (**Figure 3A**) revealed a canonical MRE within the 3' UTR (**Figure 3B**). We then cloned a fragment of NPY1R 3'UTR that contains the potential miR-218-5p target site into a luciferase reporter construct and performed luciferase assays. We found that transient overexpression of miR-218-5p in HTR8/SVneo decreased while anti-sense knockdown of endogenous miR-218-5p increased relative luciferase activity (**Figure 3C**). Likewise, transient transfection of miR-218-5p in HTR8/SVneo led to a decrease of NPY1R mRNA (**Figure 3D**). Surprisingly, transient transfection of miR-218-5p in HTR8/SVneo increased NPY1R protein while anti-sense knockdown of endogenous miR-218-5p decreased NPY1R protein level (**Figure 3E**).

4. Gestational profile of NPY and NPY1R in human placental villi

We showed that both NPY and NPY1R are expressed in trophoblast cell lines, and we wanted to assess the spatial and temporal expression of NPY and NPY1R in the human placenta across the first and second trimesters (**Figure 4**). In week 4.1 sections, there was a weak NPY and NPY1R nuclear staining in villous cytotrophoblast and cytoplasmic staining in the syncytiotrophoblasts. In 5-8 week placenta sections, stronger NPY and NPY1R signals were observed in the nuclei of villous cytotrophoblast and the cytoplasm of syncytiotrophoblasts. There was also some staining in column cytotrophoblasts. In week 8 sections, we started to also see NPY1R staining in villous fetal macrophages (Hofbauer cells) and some NPY staining in the villous angiogenic core, the precursor structure to the villous blood vessels. Weak NPY and NPY1R signals were observed in all these cells in weeks 12–17 sections, but they disappeared almost entirely by the 18.6 week except for NPY1R staining in villous fetal macrophages (Hofbauer cells).

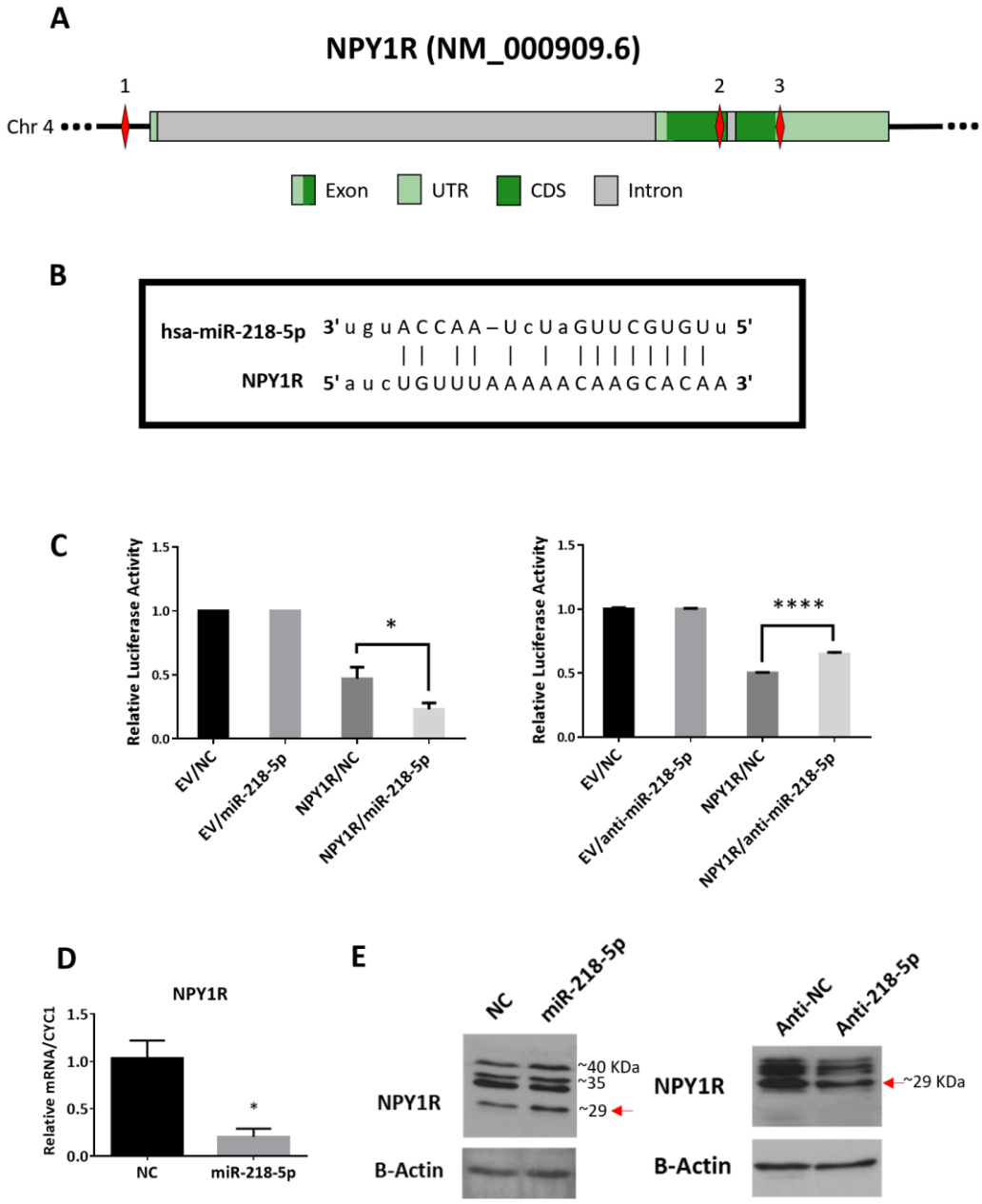
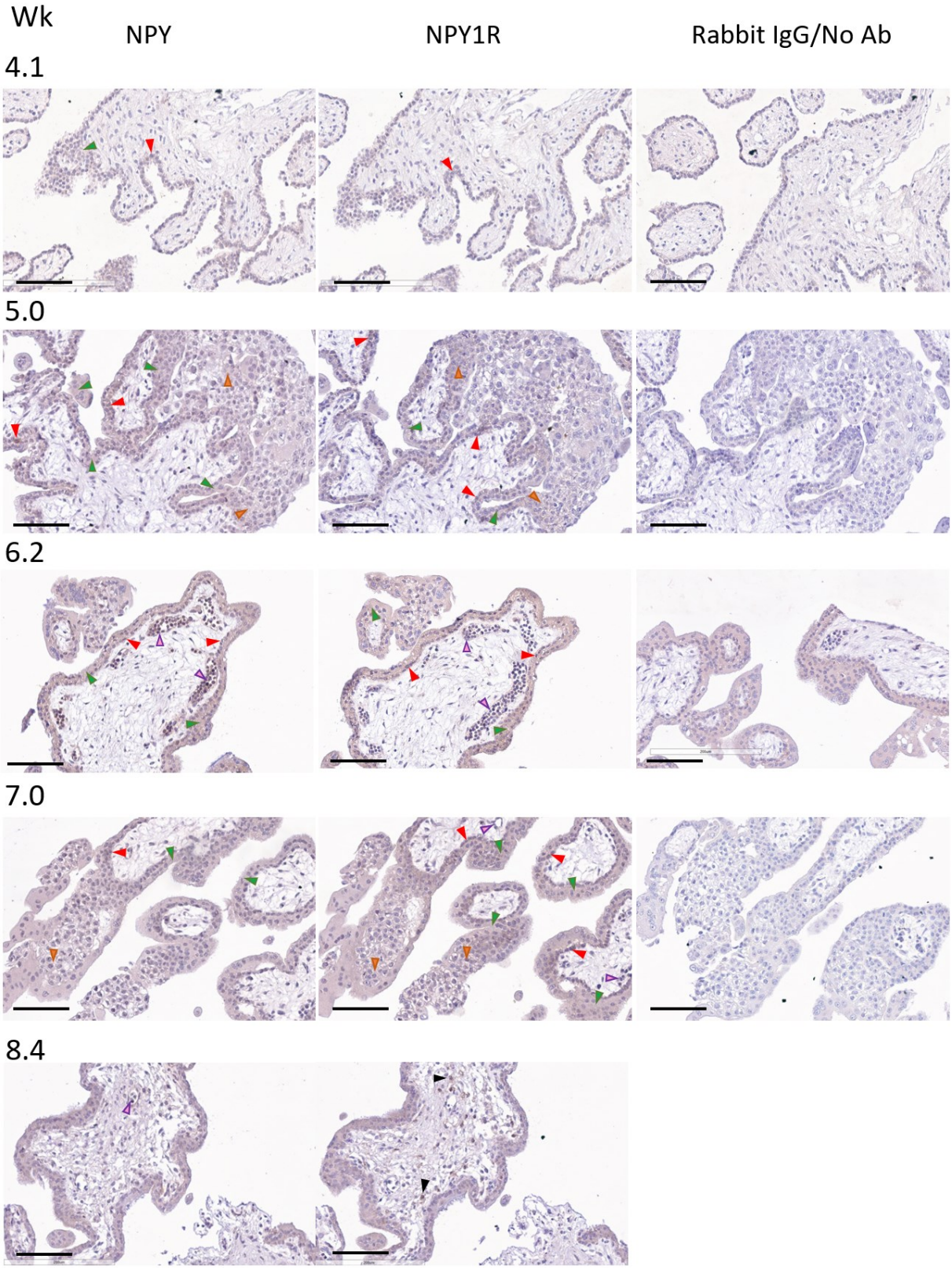


Figure 3. miR-218-5p targets NPY1R at 3' untranslated region. A. Schematic diagram showing predicted miR-218-5p target sites on NPY1R locus indicated by the red diamond. Target site 1 is 296 bp upstream of the NPY1R transcription start site and target site 2 in exon 2, 103 bp upstream of intron 2. Target site 3 is in the 3' UTR of NPY1R. **B.** miR-218-5p target site on NPY1R 3' UTR (site 3 from A) is shown. **C.** Luciferase assay using a pMIR-REPORT plasmid containing target site from B following transfection with miR-218-5p mimic or its control (NC) or anti-miR-218-5p or its control (anti-NC). **D.** NPY1R mRNA level measured in cells transfected with miR-218-5p mimic or its control measured at 48 h post-transfection (150 nM). **E.** Western blot analysis of NPY1R levels in cells transfected with miR-218-5p mimic or its control or anti-miR-218-5p or its control measured 48 h post-transfection (150 nM). Jacob A. O'Brien contributed to this figure panel E. Data represent mean ± SEM, N = 3. * $p < 0.05$, **** $p < 0.0001$.



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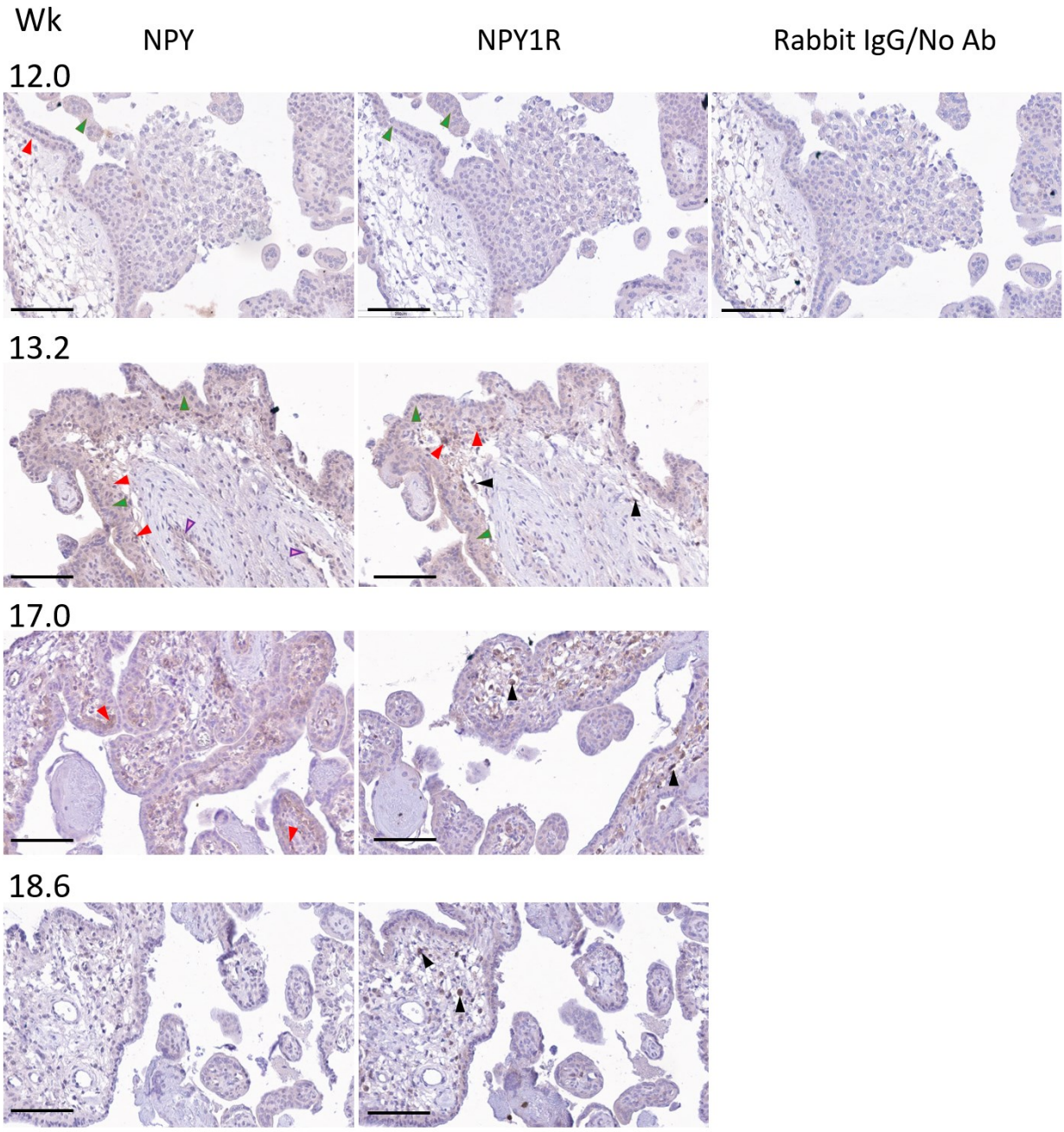


Figure 4. Gestational profile of NPY and NPY1R expression in human placenta. Immunohistochemistry was performed on placental sections across the first 20 weeks of gestation (4-19 weeks). Red arrows = cytotrophoblast nucleus, Green arrows = syncytiotrophoblasts cytoplasm, Orange arrows = column cytotrophoblast nucleus/cytoplasm, Black arrows = fetal macrophages/Hofbauer cells. Pink arrows = endothelial cells/angiogenic core. As negative controls, rabbit IgG was used on some sections, while no primary antibody was added on others. Scale bar = 100 µm.

5. NPY1R signaling inhibits trophoblast proliferation

To investigate the function of NPY in human placenta, we first used overexpression and/or knockdown approaches, as well as NPY receptors antagonists, in HTR-8/SVneo cells and in first trimester placental villi to determine if NPY regulates trophoblast proliferation. Confirmation of NPY and NPY1R overexpression and knockdown in HTR8/SVneo cells can be seen in **Figure 5** and stable overexpression of both NPY and NPY1R in Swan71 cells in **Figure S2A** and **Figure S2B** respectively. Dot blot analysis was used to detect NPY protein due to its small size (4 kDa); also, Western blot analysis of NPY1R in trophoblasts consistently showed a 29 kDa band that changes with the use of NPY1R siRNA and overexpressing plasmid whereas the expected size of NPY1R is around 44 kDa. We hypothesized that this band, and on occasions other bigger bands, are a result of NPY1R degradation in the cytoplasm. This will be discussed further in chapter 4.

In HTR-8/SVneo cells, knockdown of NPY using an siRNA (siNPY) resulted in an increase in cell proliferation, as measured by CCK8 assay (**Figure 6A**). However, both transient overexpression and knockdown of NPY increased proliferation in HTR8/SVneo as measured by plate confluency (**Figure 6B**). Overexpression of NPY1R decreased proliferation, but no significant effect was observed following NPY1R knockdown in HTR8/SVneo (**Figure 6C**). In addition, NPY1R and NPY5R selective antagonists increased proliferation, whereas NPY2R selective antagonists decreased proliferation (**Figure 6D**). We also stably transfected NPY-expressing plasmid into another trophoblast cell line, Swan71, and showed no change in proliferation rate while stable overexpression of NPY1R decreased cell proliferation (**Figure S2C**).

In the human placental villi, transfection of siNPY in 6 weeks villi led to an increase in BrdU signal and nuclear Ki67 levels in villous cytotrophoblast (**Figure 7**), as determined using immunohistochemistry. Dividing cytotrophoblast in the siNPY-treated villi also formed a more complete layer just under the syncytiotrophoblasts layer compared to a more disconnected layer in the control treatment (**Figure 7 arrows**). Interestingly, villi treated with NPY1R antagonist at the doses of 1 and 50 nM increased CTB division while NPY5R antagonists showed a slight increase in proliferation at the dose of 1 nM only. NPY2R antagonist had no effect on CTB proliferation (**Figure S3**).

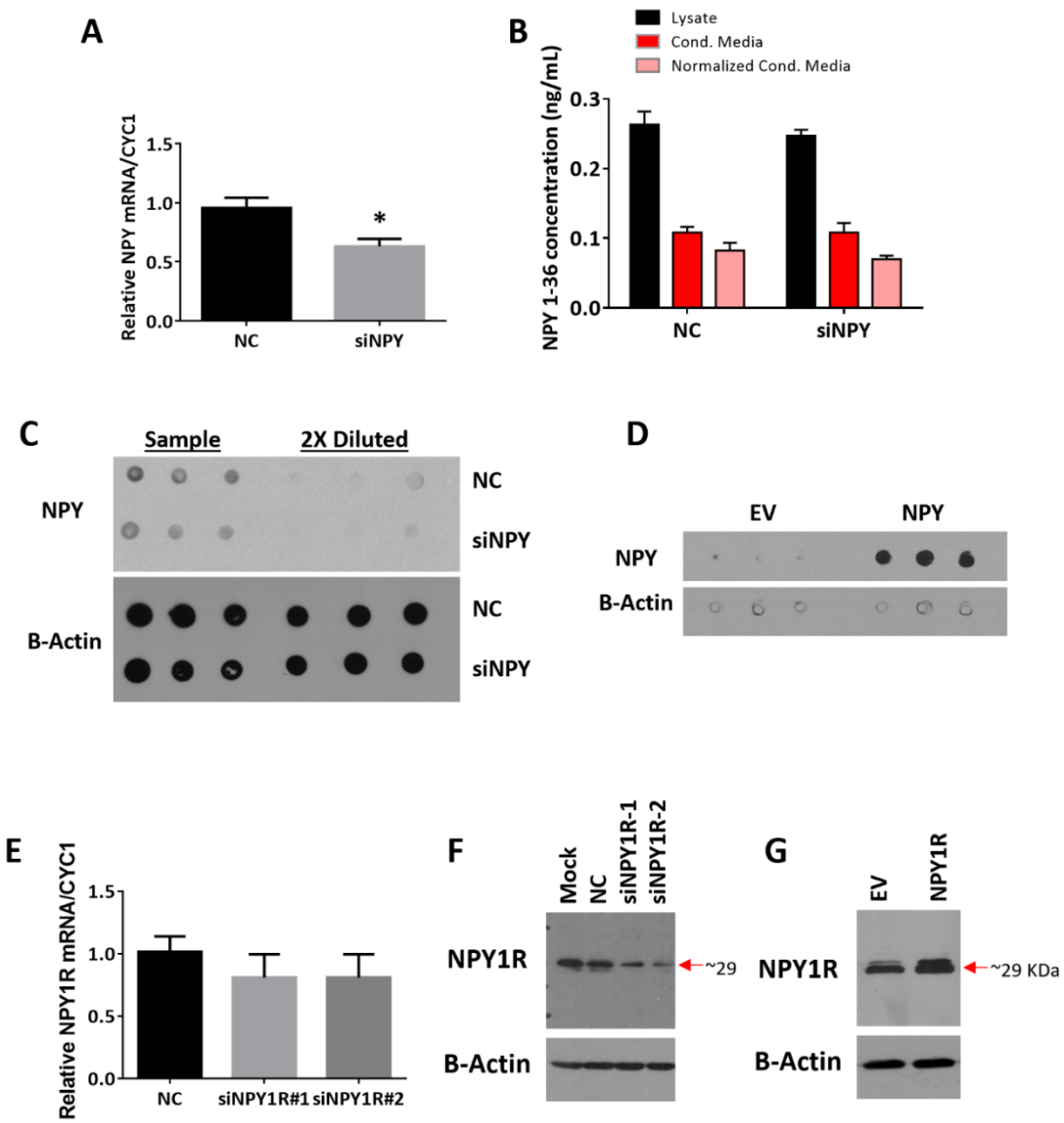
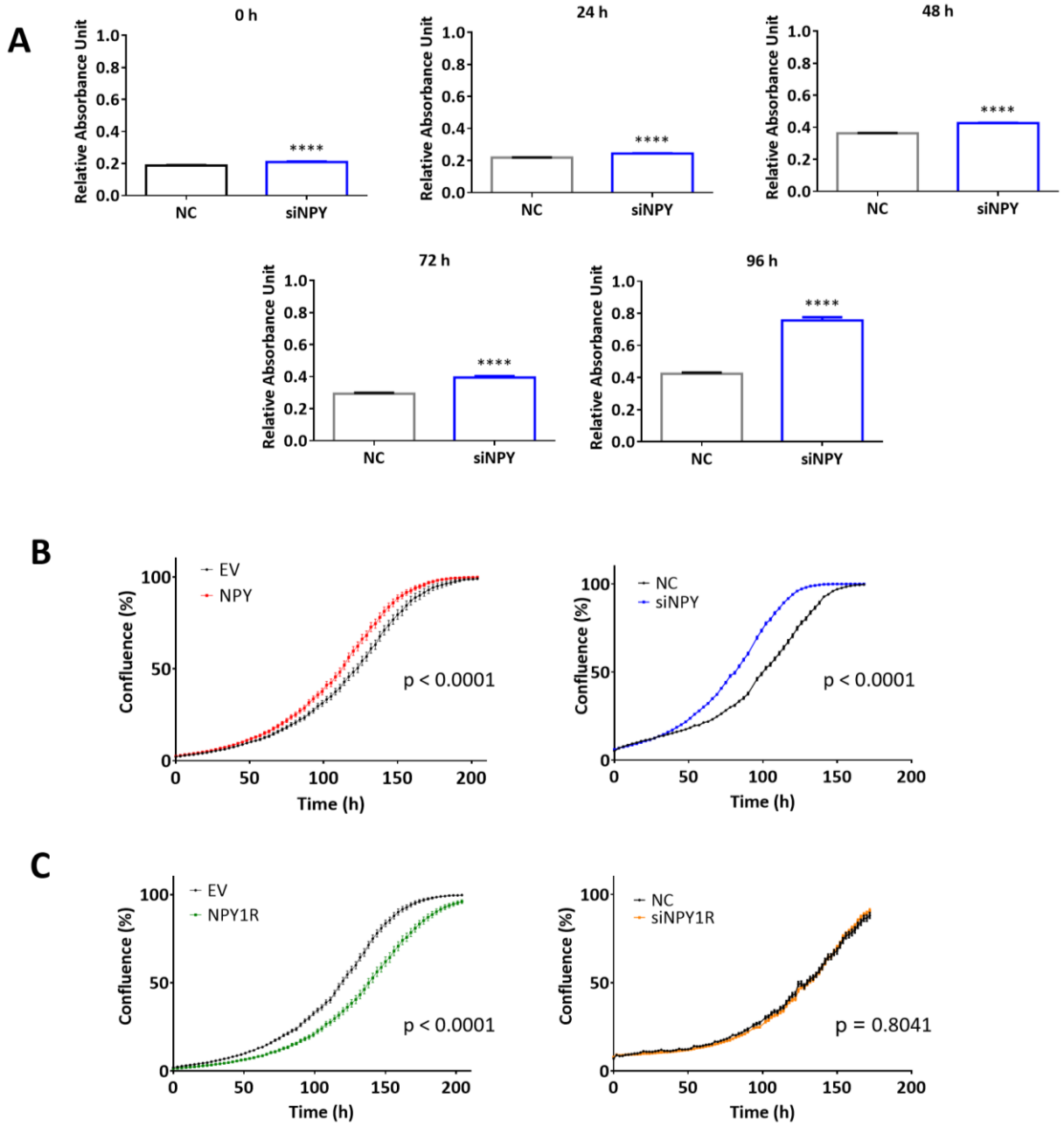


Figure 5. NPY and NPY1R knockdown and overexpression validation. A. Measured NPY mRNA level using qRT-PCR in HTR8/SVneo cells transfected with siRNA targeting NPY and recovered for 48 h (150 nM). **B.** ELISA assay using HTR8/SVneo cell lysate and conditioned medium following transfection with siNPY and measuring endogenous and secreted NPY (1-36 a.a.) levels, and secreted levels normalized to cell number. **C.** Dot blot analysis showing endogenous NPY (4 kDa) level in HTR8/SVneo cells transfected with siNPY. **D.** Dot blot analysis showing NPY level in HTR8/SVneo cells transfected with NPY overexpressing plasmid. **E.** NPY1R mRNA level measured using qRT-PCR in HTR8/SVneo cells transfected with two different siRNA targeting NPY1R and recovered for 48 h. **F.** Western blot analysis showing endogenous NPY1R levels from HTR8/SVneo cells transfected with two different siRNA targeting NPY1R. **G.** Western blot analysis showing NPY1R level in HTR8/SVneo cells transfected with NPY1R overexpressing plasmid. Data represent mean ± SEM, N = 3. * $p < 0.05$.



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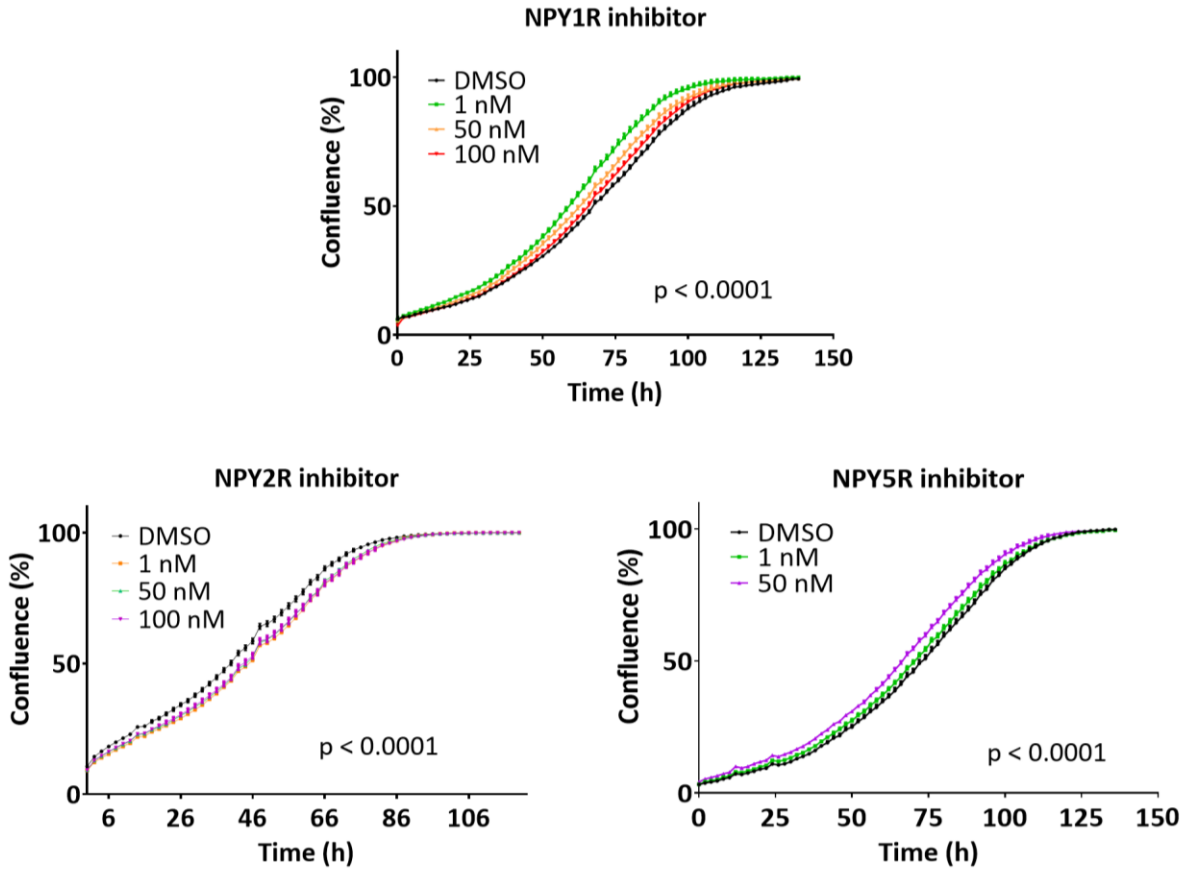

Figure 6. NPY1R downregulates trophoblast proliferation. A. CCK8 assay performed on HTR8/SVneo cells transfected with siRNA targeting NPY (siNPY) or its control (NC) and recovered for 16 h then seeded into multiple 96-well plates. Absorbance was then measured at different time points post-seeding. **B.** Proliferation assay (measuring cell confluence using IncuCyte imaging system) of HTR8/SVneo cells transfected with NPY overexpressing plasmid or its control, and in cells transfected with siNPY or its control. **C.** Proliferation assay (measuring cell confluence using IncuCyte imaging system) of HTR8/SVneo cells transfected with NPY1R overexpressing plasmid or its control, or in cells transfected with siNPY1R#2 or its control. **D.** Proliferation assay (measuring cell confluence using IncuCyte imaging system) of HTR8/SVneo cells treated with NPY1R, NPY2R, or NPY5R antagonists at different concentrations. P-value applies to DMSO vs. an inhibitor treatment. Data represent mean ± SEM, N = 3. **** $p < 0.0001$.

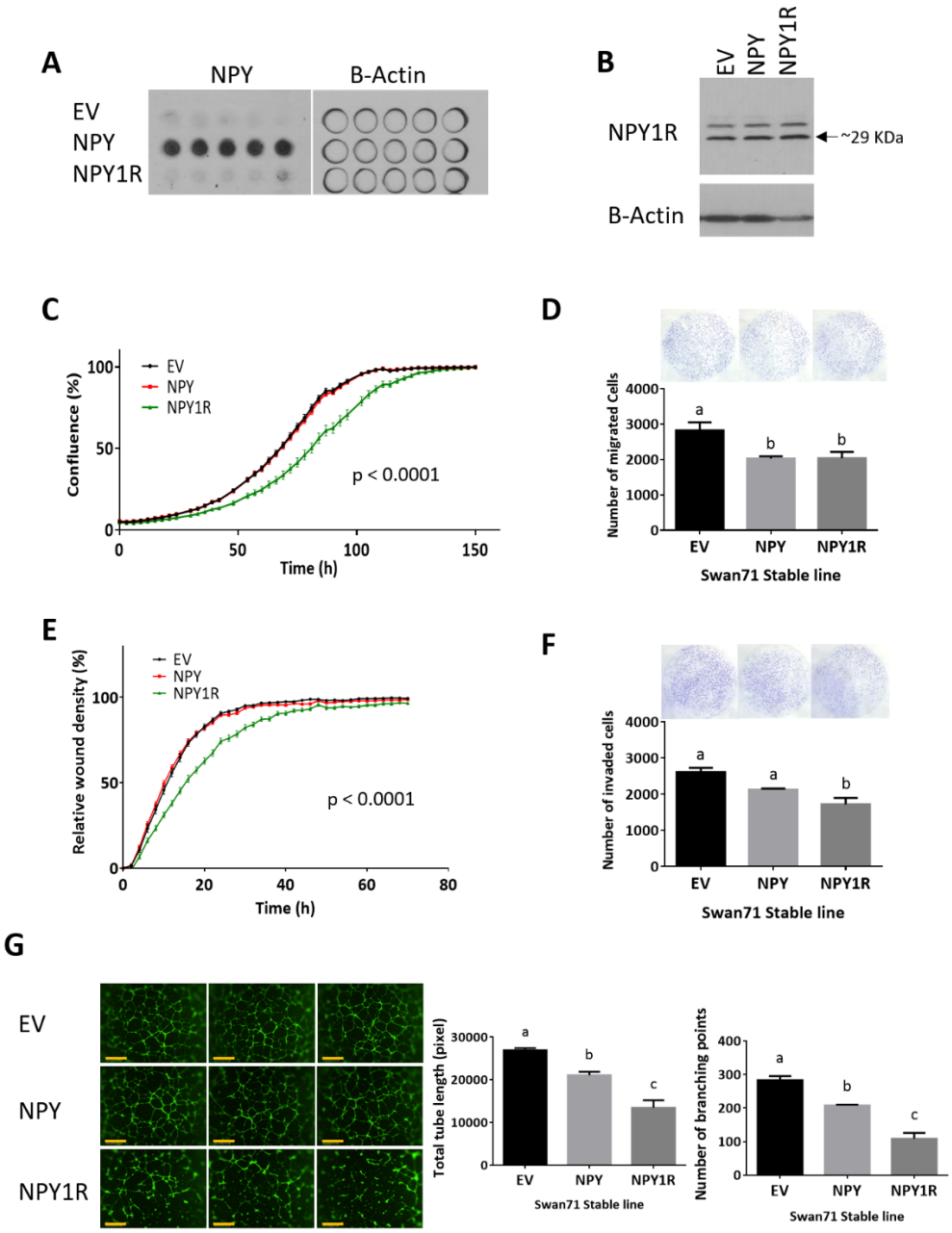

Figure S2. Functional assays using stably overexpressing NPY and NPY1R Swan71 cell lines. A. Dot blot analysis confirming the overexpression of NPY in the Swan71 stable line. **B.** Western blot analysis confirming the overexpression of NPY1R in the Swan71 stable line. **C.** Proliferation assay (measuring cell confluence using IncuCyte imaging system) of NPY and NPY1R stably overexpressing cell line. P-value applies only to EV vs. NPY1R. **D.** Transwell migration assay with Swan71 stably overexpressing cell line. **E.** Wound-healing assay using these stable lines. P-value applies only to EV vs. NPY1R. **F.** Transwell invasion assay. **G.** Tube-like network formation assay using these cells with quantification of the network total tube length (pixel) and the number of branching points. Different letters above bars represent statistical significance. Data represent mean ± SEM, N = 3. Scale bar = 800 µm.

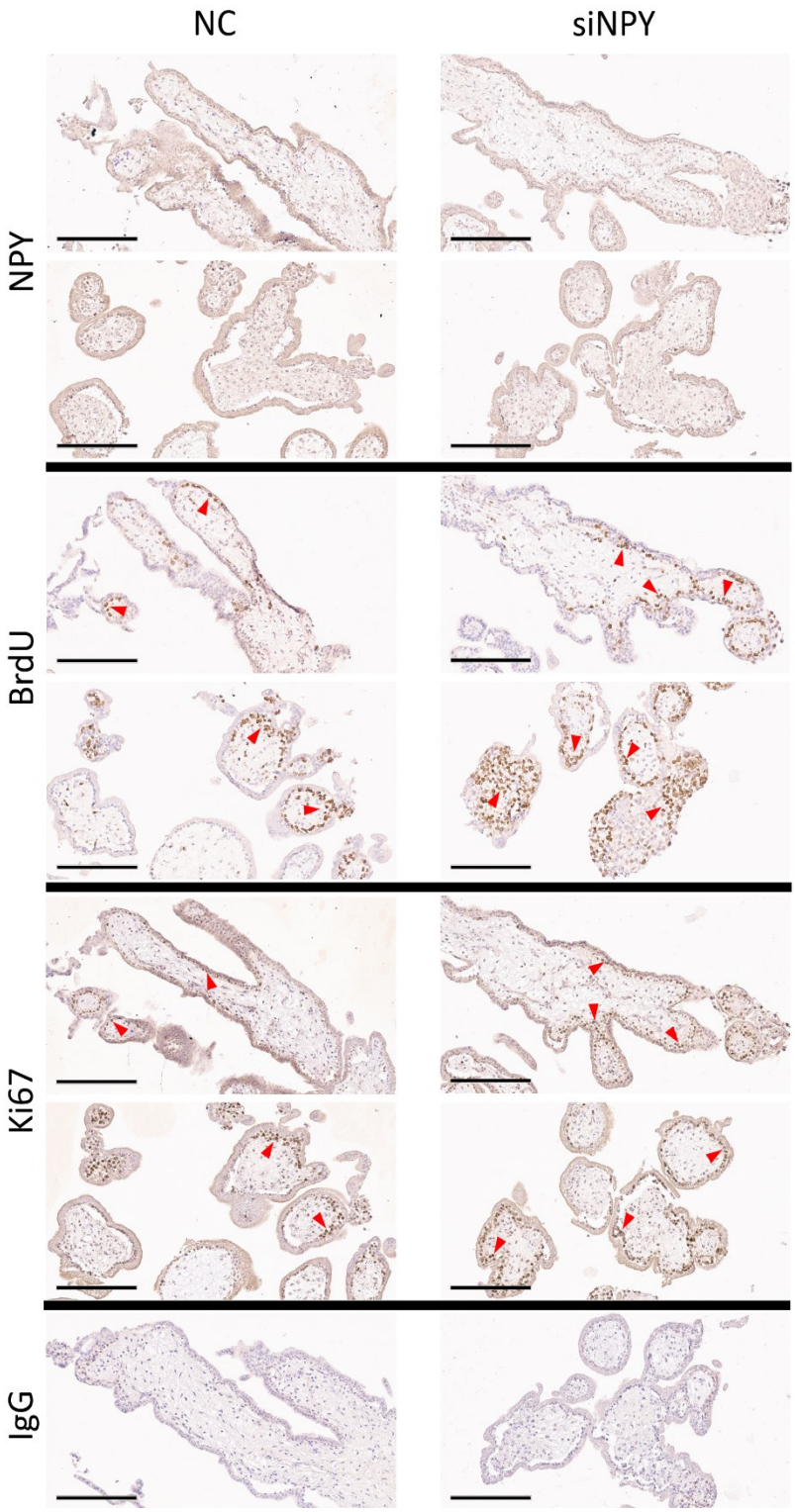

Figure 7. NPY knockdown promotes proliferation in placental floating villi model. Immunohistochemistry using first trimester (6 weeks) villi treated with siRNA targeting NPY for 48 h (BrdU was added for 24 h). Antibodies to detect BrdU and Ki67 (proliferation markers), NPY and IgG control were used. Red arrows = proliferating cytotrophoblasts, n = 3 replicates per experiment. Experiments were repeated using 3 different placentas. Scale bar = 200 µm.

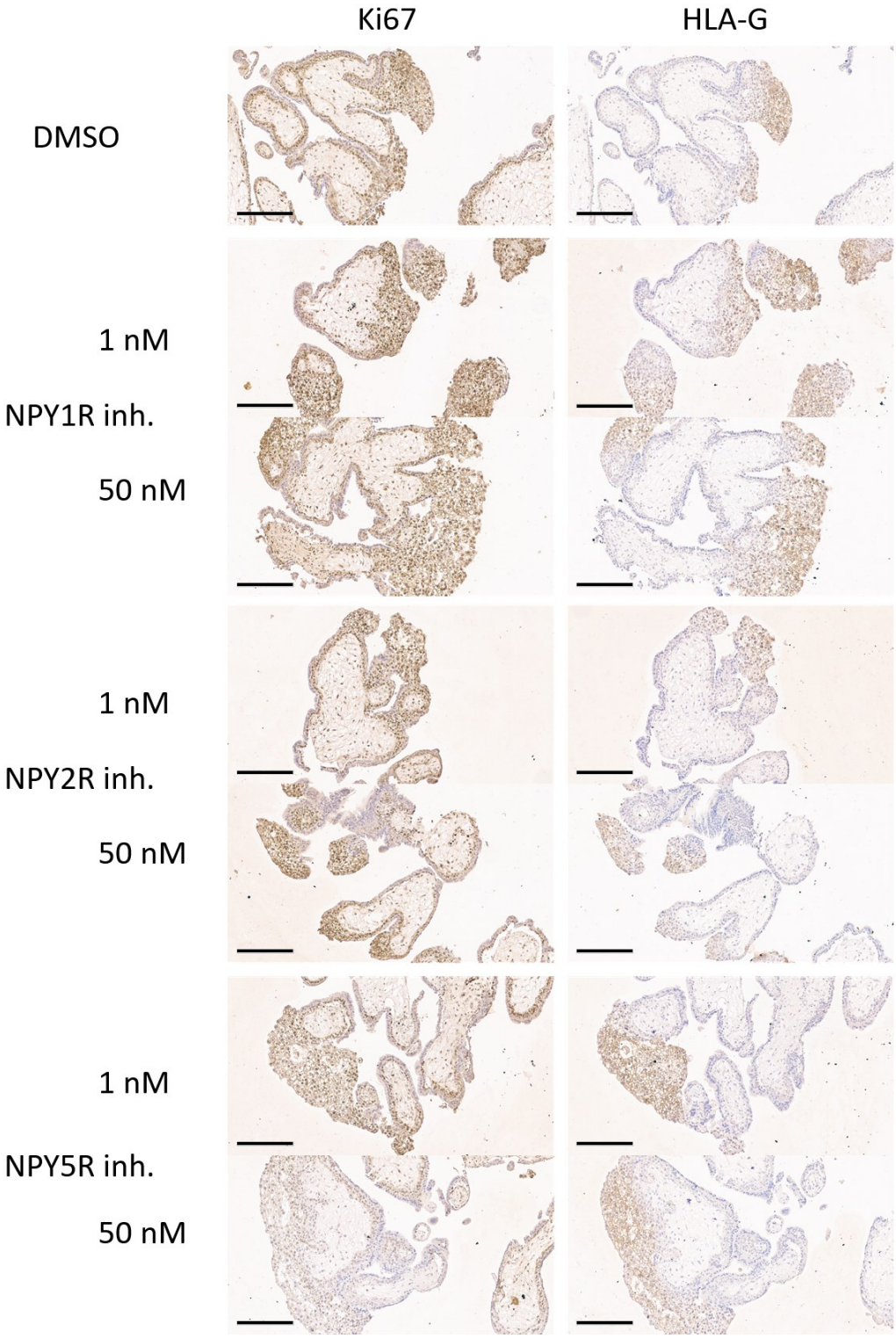

Figure S3. NPY1R inhibition increases proliferation in placental floating villi model. First trimester (5 weeks) villi treated with NPY1R, NPY2R or NPY5R antagonists at specified concentrations for 48 h. Antibodies to detect Ki67 (proliferation marker), HLA-G (EVT marker) were used. n = 3 replicates per experiment. Experiments were repeated using 2 different placentas. Scale bar = 200 µm.

6. NPY signalling via NPY1R decreases cell motility in trophoblasts

Transwell migration and wound healing assays were used to determine if NPY regulates cell migration. Treatment of HTR8/SVneo with 1 pM rhNPY (1-36) led to a decrease in cell migration in both Transwell and wound healing assays (**Figure 8A, 8B**). Conversely, the knockdown of NPY led to an increase in cell migration (**Figure 8A, 8B**). However, overexpression of NPY led to a decrease in cell migration using the Transwell assay and a slight increase of motility using the wound healing assay (**Figure 8A, 8B**). Since NPY can be processed into N-truncated fragments, which have varying affinities to different NPY receptors, we also investigated the effect of NPY (3-36), an NPY 3-36 agonist. Treatment of either rhNPY (1-36) or (3-36) at 1, 10, or 100 pM led to opposite effects, a decrease or increase of cell motility, respectively (**Figure 8C**). The most significant increase was at the 100 pM NPY 3-36 concentration, while all concentrations of the full-length rhNPY treatment decreased cell motility. The inhibitory effect of rhNPY on cell migration was attenuated by an NPY1R specific antagonist (**Figure 8D**). In addition, overexpression of NPY1R decreased while knockdown of NPY1R increased cell migration (**Figure 8E, 8F**). In Swan71 cells, stable overexpression of NPY or NPY1R decreased cell migration in Transwell assay (**Figure S2D**), while in the wound-healing assay, only NPY1R overexpression led to a decrease in wound-healing while NPY overexpression had no significant change (**Figure S2E**).

Matrigel-coated Transwell inserts were used to investigate the effect of NPY and NPY1R on cell invasion. Similar to cell migration, rhNPY treatment and NPY overexpression decreased while NPY knockdown increased cell invasion (**Figure 9A**). However, unlike cell migration, rhNPY (3-36) did not affect cell invasion (**Figure 9B**). To assay the role of NPY1R signalling in cell invasion, HTR8/SVneo were treated with an NPY1R specific antagonist with and without rhNPY. The inhibitor abrogated the effect of rhNPY on cell invasion (**Figure 9C**), and overexpression of NPY1R decreased while NPY1R knockdown increased cell invasion (**Figure 9D**). Similarly, stable overexpression of NPY1R led to a decrease in Swan71 cell invasion (**Figure S2F**). Moreover, treatment with NPY1R antagonist in 5 week placental villi, increased the number of HLA-G positive trophoblasts (**Figure S3**). These data strongly suggest that NPY1R activation decreases cell migration and invasion in trophoblasts.

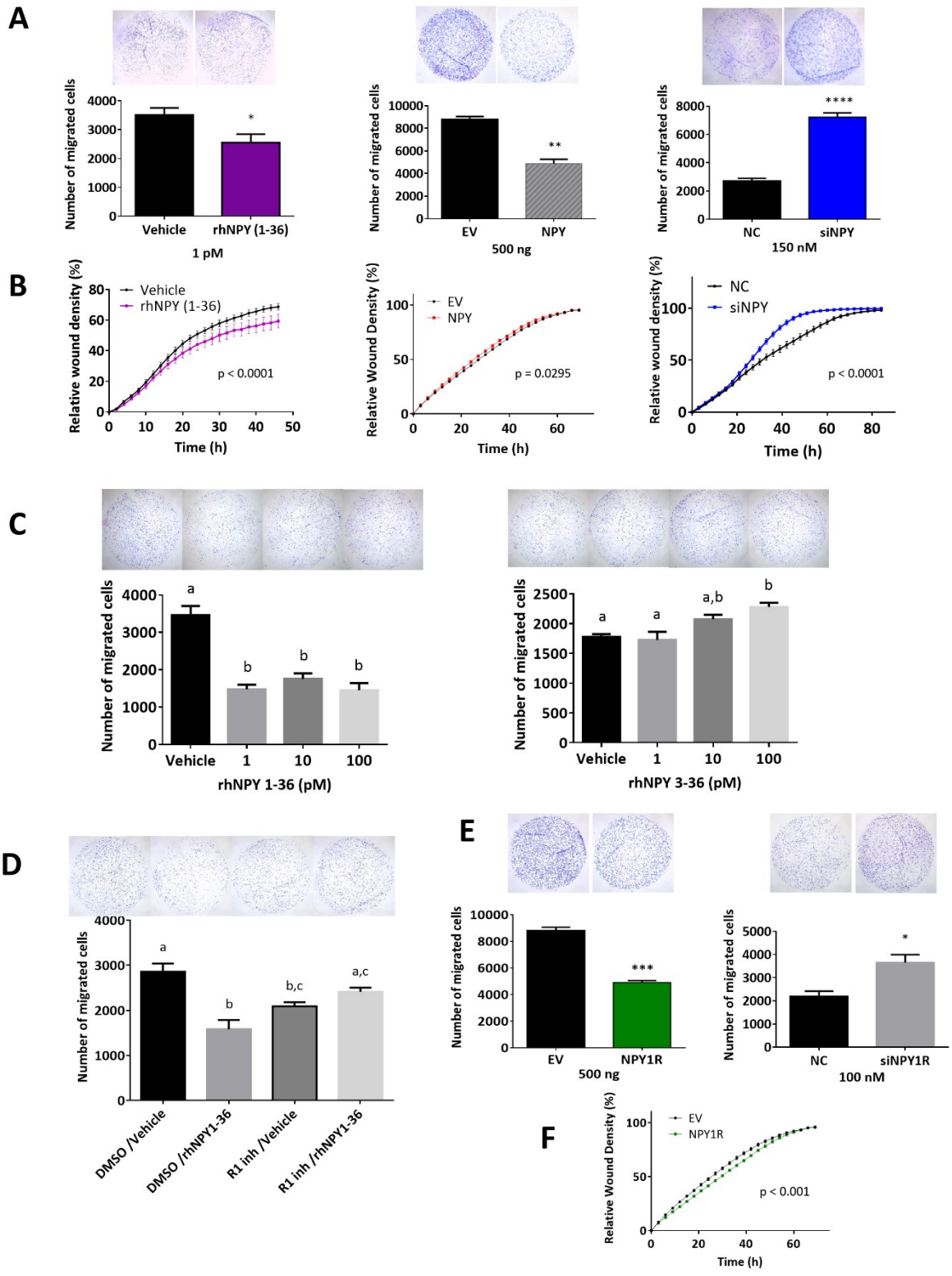

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Figure 8. NPY inhibits migration via NPY1R. A. Transwell migration assay using HTR8/SVneo cells treated with rhNPY (1-36) or transfected with NPY overexpressing plasmid or siRNA targeting NPY and their corresponding controls. **B.** Wound-healing assay using HTR8/SVneo cells treated with rhNPY (1-36) or transfected with NPY overexpressing plasmid or siRNA targeting NPY and their corresponding controls. **C.** Transwell migration assay using HTR8/SVneo cells treated with rhNPY (1-36), an NPY1R agonist, or rhNPY(3-36), an NPY2R agonist. **D.** Transwell migration assay using HTR8/SVneo cells treated with rhNPY (1-36) and NPY1R antagonist. **E.** Transwell migration assay using HTR8/SVneo cells transfected with NPY1R overexpressing plasmid or siRNA targeting NPY1R (#2) and their corresponding controls. **F.** Wound-healing assay using HTR8/SVneo cells transfected with NPY1R overexpressing plasmid. Different letters above bars represent statistical significance. Data represent mean ± SEM, N = 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

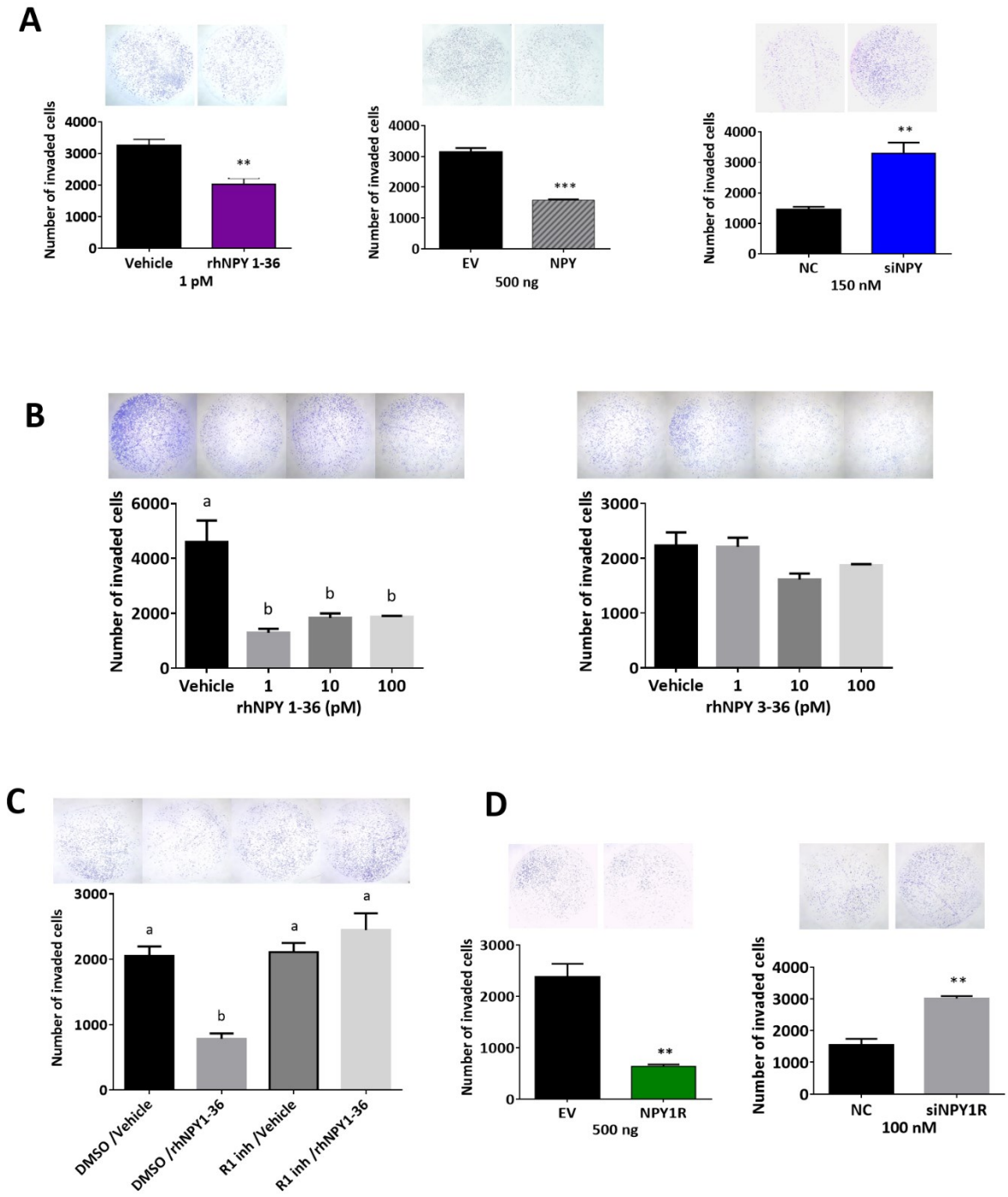


Figure 9. NPY inhibits invasion via NPY1R. A. Transwell invasion assay using HTR8/SVneo cells treated with rhNPY (1-36) or transfected with NPY overexpressing plasmid or siRNA targeting NPY and their corresponding controls. **B.** Transwell invasion assay using HTR8/SVneo cells treated with rhNPY (1-36), an NPY1R agonist, or rhNPY(3-36), an NPY2R agonist. **D.** Transwell invasion assay using HTR8/SVneo cells treated with rhNPY (1-36) and NPY1R antagonist. **D.** Transwell invasion assay using HTR8/SVneo cells transfected with NPY1R overexpressing plasmid or siRNA targeting NPY1R (#2) and their corresponding controls. Different letters above bars represent statistical significance. Data represent mean ± SEM, N = 3. ** $p < 0.01$, *** $p < 0.001$.

7. Differential expression of endogenous NPY following CRISPR/Cas9 mutagenesis and its effect on HTR8/SVneo proliferation, migration and invasion

Because we saw differences in the effect of transient versus stable NPY overexpression in proliferation assays, we wanted to investigate the effect of stable downregulation of *NPY*, using CRISPR/Cas9 and a guide RNA to target the transcription start site (TSS) of NPY. Initially, a heterogeneous population of NPY TSS knockout HTR8/SVneo was generated with an overall lower level of NPY (**Figure 10D**; δTSS NPY). This mixed population resulted from the < 100% efficiency of CRISPR/Cas9 to produce NPY TSS deletions. As was observed with siNPY, downregulation of endogenous NPY increased cell migration (**Figure 10A**), invasion (**Figure 10B**), and proliferation (**Figure 10C**).

From the δTSS NPY heterogeneous population, single-cell clones were selected to identify homozygous NPY TSS knockouts. Serendipitously, a single clone (TSS E7) had a small insertion proximal to the NPY TSS (**Figure 11A, 11B, 11C**), and this led to significant upregulation of NPY (**Figure 11D**). We first performed proliferation using the TSS E7 clone and compared it to the empty vector control clone, EV C10. We observed decreased proliferation in the TSS E7 clone (**Figure 11E**), corroborating the effect seen with exogenous NPY overexpression. However, following a visual inspection of the micrographs of TSS E7 proliferation, it was evident that E7 migrated at a higher velocity than C10. Counterintuitively, this is not what was observed when assaying the migratory potential of TSS E7 using Transwell assay, where a substantial decrease in migration was observed (**Figure 11F**). To confirm our initial visual observations of increased motility, we analyzed TSS E7 clone migration by manually calculating individual cells' migratory speed and by calculating the cells movement speed in the wound-healing assay. Both revealed that TSS E7 moved faster than EV C10 (**Figure 11F, 11G**).

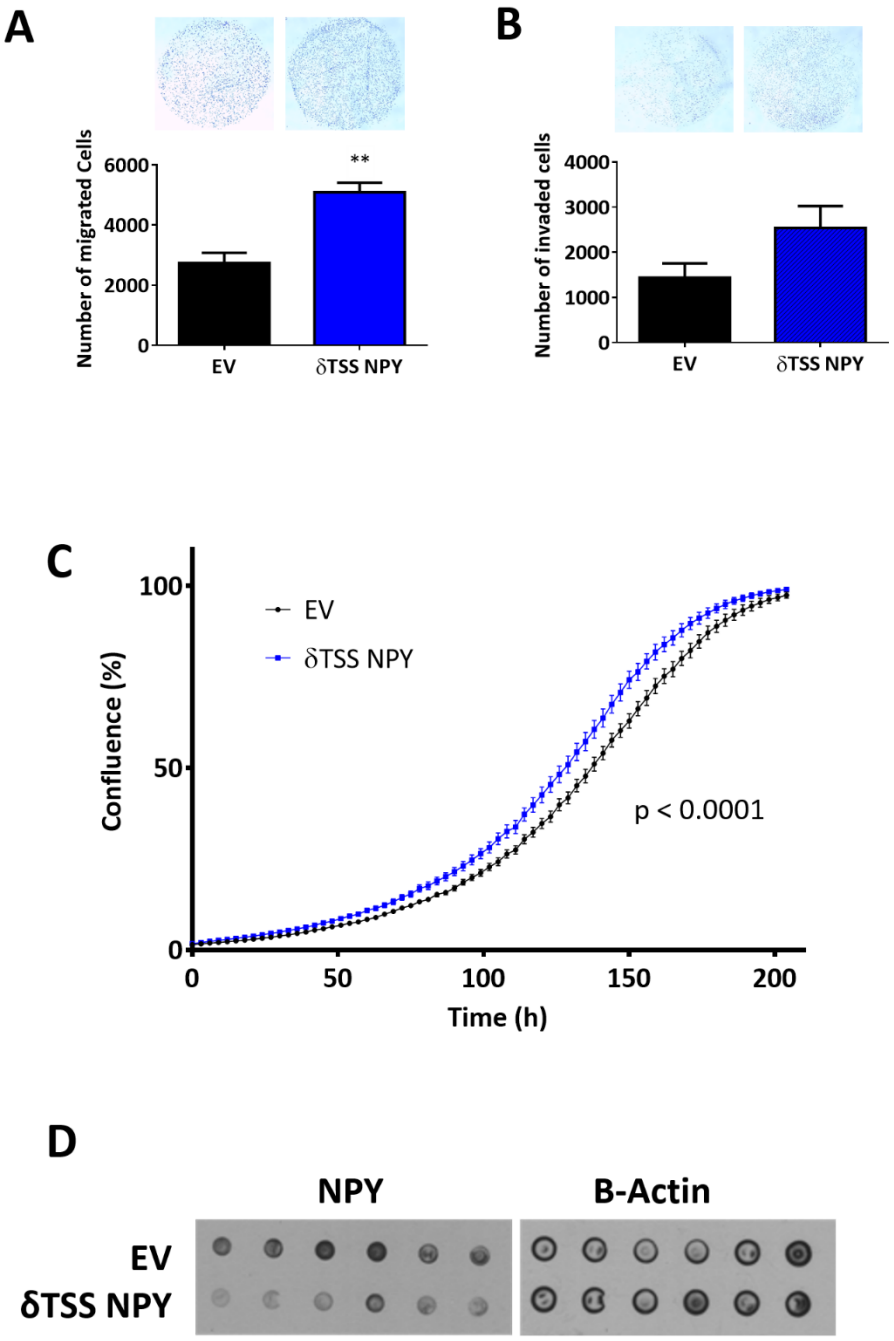
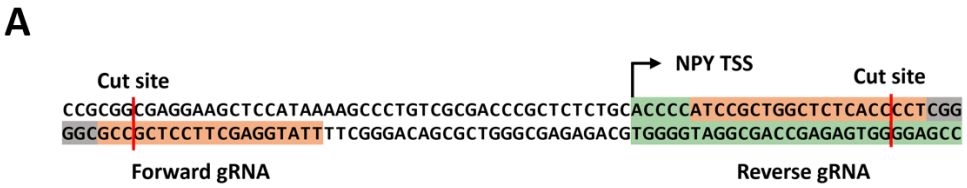
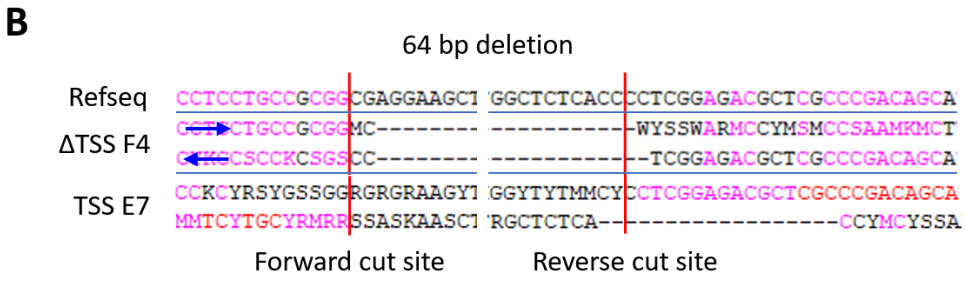
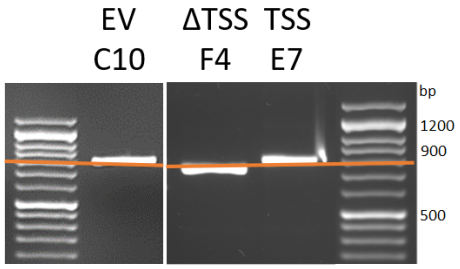
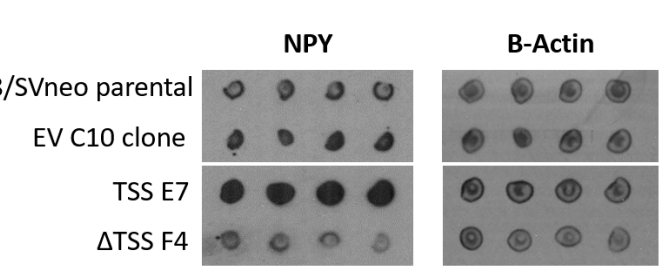


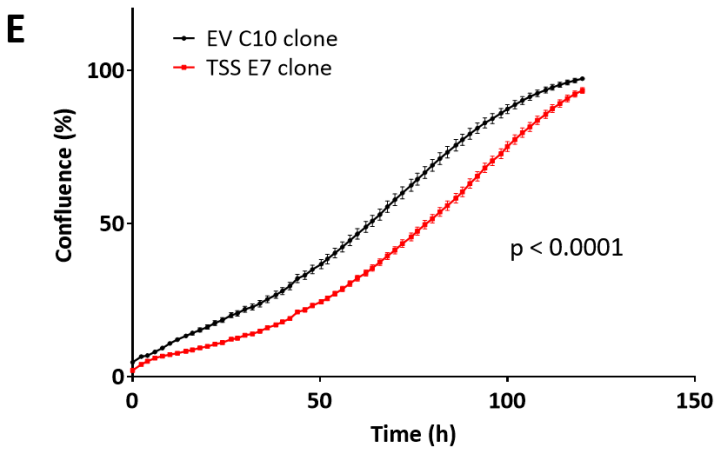
Figure 10. Functional assays using CRISPR-cas9 and gRNA targeting transcription start site (TSS) of NPY. A. Transwell migration assay using a heterogenous population of HTR8/SVneo cells transfected with PX459 CRISPR/Cas9 plasmid and guide RNA targeting NPY TSS site. **B.** Transwell invasion assay using this heterogeneous population of HTR8/SVneo. **C.** Proliferation assay (measuring cell confluence using IncuCyte imaging system) using a heterogeneous population of HTR8/SVneo cells transfected previously with PX459 CRISPR/Cas9 plasmid and guide RNA targeting NPY TSS site. **D.** Dot blot analysis showing endogenous NPY levels in this heterogeneous population. Data represent mean ± SEM, N = 3. ** $p < 0.01$.











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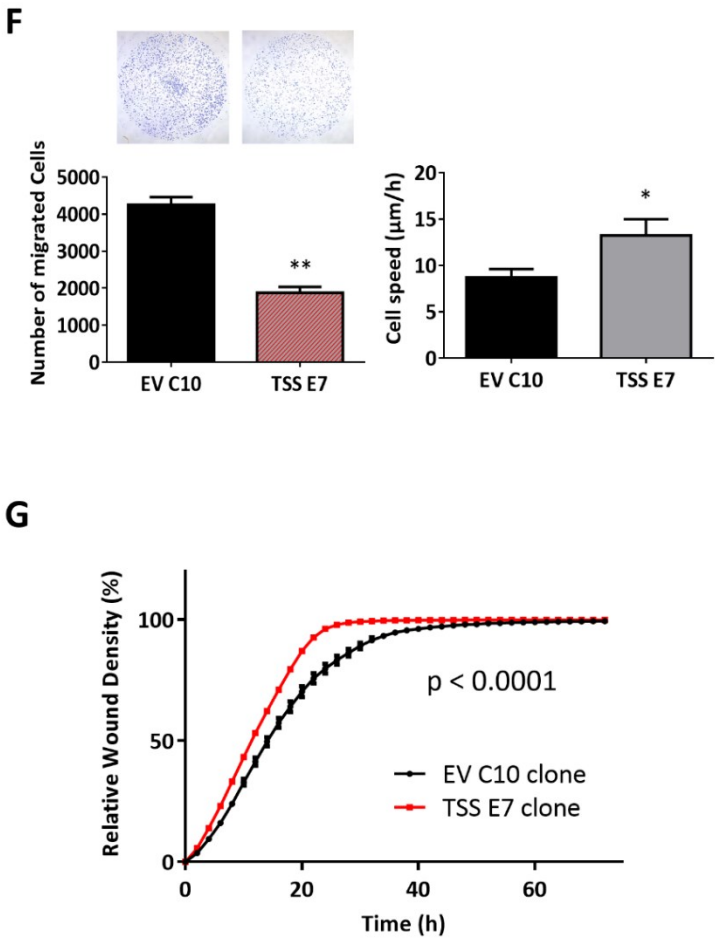

Figure 11. Functional assays using single-cell clones generated by transfecting CRISPR-cas9 and guide RNA targeting transcription start site (TSS) of NPY. A. Diagram of predicted CRISPR/Cas9 excision of the NPY TSS. Orange: guide RNA sequence, green: NPY Exon 1, grey: PAM sequence. **B.** High-fidelity PCR product of the region flanking the targeted NPY TSS sequence using genomic DNA from HTR8/SVneo single-cell clones generated with either empty vector control or with gRNA targeting NPY TSS. The PCR product was sequenced by the Sick Kids' TCAG sequencing facility using both forward and reverse primers. The arrows indicated with the forward (→) or reverse (←) primers were used for sequencing. Δ TSS F4 clone shows a deletion at the TSS site while TSS E7 clone shows a mutation at the TSS site that led to constitutive overexpression of NPY. **C.** Gel electrophoresis of PCR product using a primer flanking the NPY TSS site from single-cell clones was performed using a high-fidelity polymerase. The orange line indicates wildtype band location at ~800 bp. **D.** Dot blot analysis showing the levels of endogenous NPY in single-cell clones. **E.** Proliferation assay comparing between the wildtype clone and the E7 NPY overexpressing clone. **F.** Transwell migration assay using NPY overexpressing clone TSS E7 and a control clone EV C10. Cell movement speed was also calculated (µm / h) for both of these clones. **G.** Wound healing assay using the same single-cell clones. Clones were generated and selected by Jacob A. O'Brien. He also contributed to this figure panels A-D. Data represent mean ± SEM, N = 3. * $p < 0.05$, ** $p < 0.01$.

8. Differential expression of endogenous NPY following CRISPR/Cas9 mutagenesis and its effect on HTR8/SVneo adhesion and tube-like network formation

A critical difference between the Transwell assay and either manual determination of speed or wound healing assay is that cells are seeded into the Transwell insert in which migration happens simultaneously with adherence. In contrast, cells are given time to adhere and proliferate before quantification in the other two assays. To quantify adherence, cells were seeded into 48-well plates, and phase contrast micrographs were taken every 2 h. For each image, the cells were categorized into three groups: non-adhered, adhering, and adherent, based on their morphology (**Figure 12A**). These data revealed that the TSS E7 clone requires more time to adhere than the control. At time zero, the proportion of TSS E7 cells that are non-adherent to the plate is near 100%, whereas only 30% of control cells are non-adhered, with the remaining in the process of adhering. Over the next 6 h, we saw that it takes significantly more time for TSS E7 to fully adhere, as seen in the right panel (**Figure 12A**), where there are significantly lower percentages of adhered cells versus the control. A slower time to full adherence is also reflected in higher percentages of non-adhered and adhering in TSS E7. By 12 h, the proportion of non-adhered cells have stabilized and are not significantly different between TSS E7 and EV C10. The increased proportion of adhering and decreased proportion of adhered TSS E7 at 12 h equates to increased time required to adhere to the plate.

Given the changes in adherence, we hypothesized that there would be a difference in the tube-like structures generated in the tube formation assay, as this assay requires the ability of the cells to adhere to the matrix and each other and to differentiate to gain endothelial-like properties. We observed a decrease in total tube length and the number of branching points of the TSS E7 clone (**Figure 12B**), suggesting an overall weaker network. Similar results were obtained in the Swan71 cells stably overexpressing NPY (**Figure S2G**), where these cells also had a decrease in the total tube length and the number of branching points compared to the control cells. Moreover, this decrease was even more prominent in the NPY1R stably overexpressing cells (**Figure S2G**), suggesting that the NPY effect could be mediated by NPY1R signalling. In contrast, transient NPY overexpression showed no change in tube formation assay (**Figure S4**).

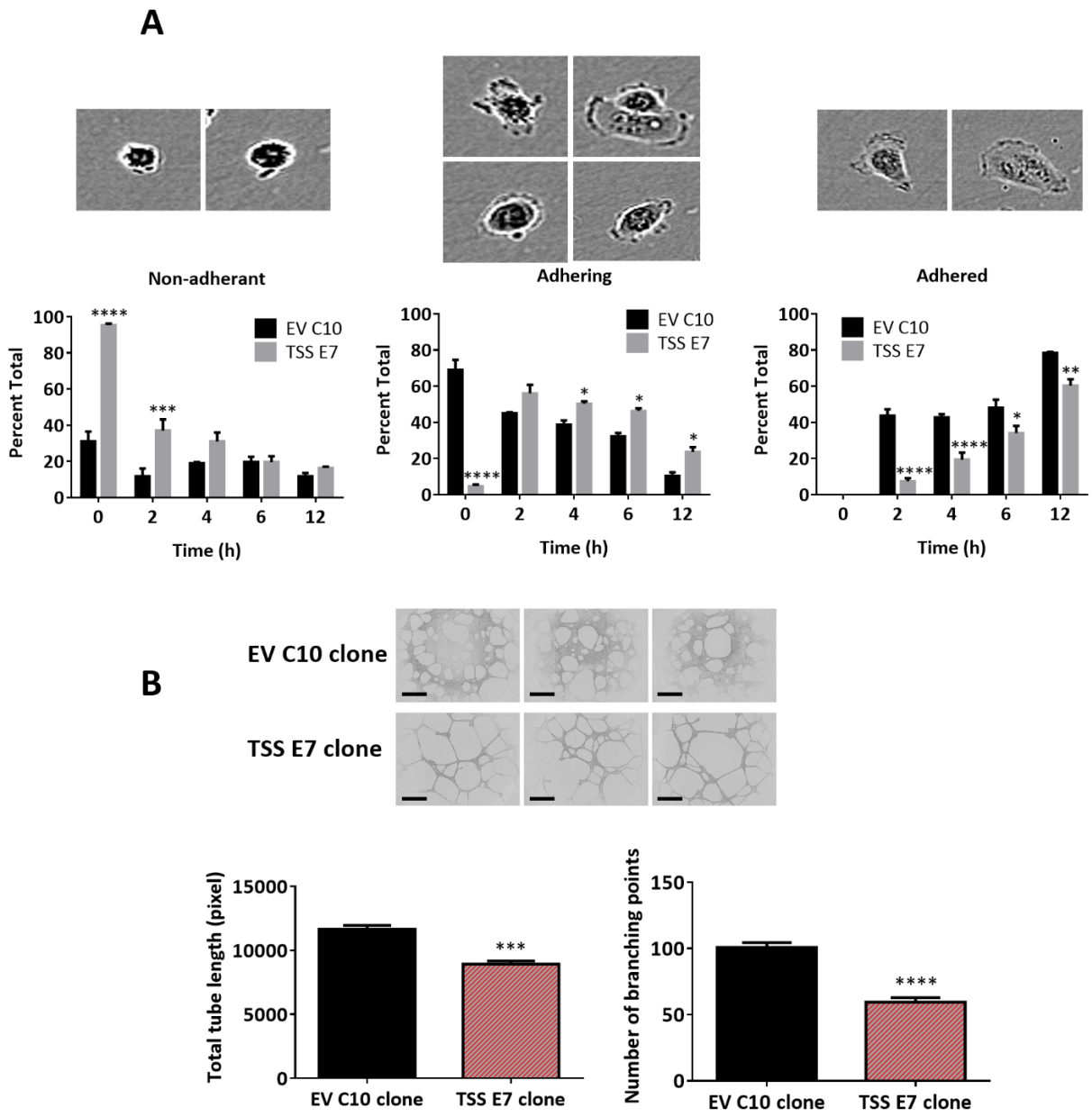

Figure 12. NPY impairs cell adhesion and reduces the ability of trophoblasts to form tube-like networks. A. Calculated percentage of cells from EV C10 and TSS E7 clones that were either non-adherent, adhering or adhered to the cell culture plate over time; N = 8. **B.** Network-like formation assay using the EV C10 clone and the NPY overexpressing TSS E7 clone. Clones were generated and selected by Jacob A. O'Brien. He also contributed to this figure panel A. Data represent mean ± SEM, N = 3. Scale bar = 800 µm. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

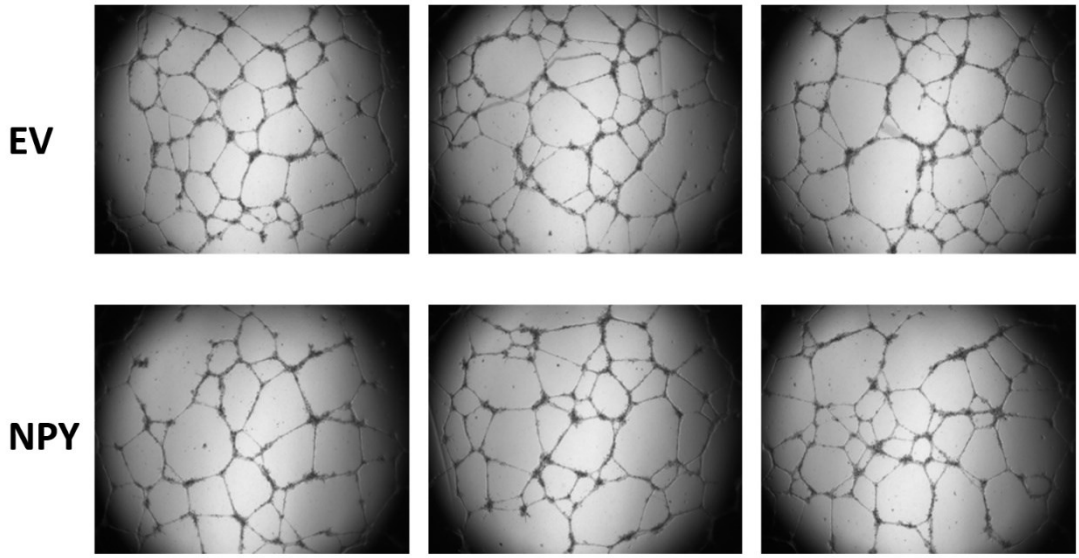

Figure S4. Network-like formation assay in transiently NPY overexpressing HTR8/SVneo. Cells were transfected with NPY overexpressing plasmid or an empty vector and recovered for 24 h at 500 ng. Cells were then seeded on top of Matrigel, and pictures were taken after 24 h. N = 3.

V. Discussion

This study was carried out to investigate whether NPY has a role in trophoblast functions associated with early placenta development and whether it is regulated by miR-218-5p. A previous study in our lab showed that miR-218-5p promoted trophoblast motility and differentiation towards the endovascular pathway (Brkić et al., 2018). In a microarray, NPY mRNA was the second most downregulated mRNA in trophoblasts overexpressing miR-218-5p. Also, we found a predicted miR-218-5p target site in NPY exon 3, but luciferase report assays revealed that this is not functional in HTR8/SVneo cells. However, we did show that NPY decreases trophoblast proliferation, migration and invasion and that a decrease in cell adhesion ability exacerbates this effect. We also showed that NPY1R mediates NPY inhibitory function and that miR-218-5p targets NPY1R at the 3' UTR.

The regulation of NPY and NPY1R by miR-218-5p is complex. While NPY pre-mRNA and mature mRNA were decreased in HTR8/SVneo stably overexpressing miR-218-5p, transient transfection with miR-218-5p mimic increased NPY pre- and mature mRNA. Meanwhile, NPY protein level was higher in mir-218-1 stable cells as well as after transient miR-218-5p transfection. The change in NPY response to stable or transient miR-218-5p overexpression suggests a difference in NPY regulation under a steady-state of miR-218-5p overexpression (~15-fold upregulation) compared to a transient overexpression. Furthermore, we suggest a de-coupling of NPY mRNA level in the mir-218-1 stable line from its protein level. This decrease in NPY transcription might be explained by the fact that mir-218-1 stable line has > 400X upregulation of Histone deacetylase 5 (mass spectrophotometry data, not shown) and the presence of a CpG island at the NPY TSS. We noticed a similar pattern of decreased mRNA level of multiple genes containing a cluster of CpG sites at their TSS in the mir-218-1 overexpressing stable line while the mRNA of genes that did not contain these CpG clusters were unchanged or upregulated. On the other hand, transient transfection of miR-218-5p increased both pre- and mature mRNA levels suggesting a possible role of miR-218-5p in regulating NPY transcription. In fact, another student in our lab, Jacob A. O'Brien, has been investigating miRNA regulation of gene transcription with miR-218-5p and NPY as his case example; he found a predicted miR-218-5p target site 1800 base pair upstream of NPY TSS that seems to be important in regulating NPY

transcription. He also found that removing this target site resulted in a decrease in NPY pre-mRNA, mature mRNA and protein levels suggesting that miR-218-5p is a positive regulator of NPY transcription.

Canonically, when a miRNA targets a gene within the 3' UTR, we expect to see a decrease in both the gene's mRNA and protein levels or protein level alone. In the case of NPY1R, we showed the miR-218-5p target site in NPY1R 3' UTR to be functional in trophoblasts using luciferase assay and that NPY1R mRNA is downregulated following transient transfection with miR-218-5p mimic. Moreover, we found that, in both HTR8/SVneo and Swan71 cell lines, some portion of NPY1R mRNA retains intron 2, leading to an in-frame premature stop codon (Figures included in Chapter 4). The premature stop codon results in an N-terminal fragment of ~29 kDa. This N-terminal fragment is not to be confused with the roughly 29 kDa fragment we see in NPY1R Western blot analyses, instead of the expected 44 kDa full-length band, as the Abcam antibody we use was generated against an antigen at the C-terminal. We also observed a predicted miR-218-5p MRE on NPY1R mRNA just before intron 2, and that preliminary data suggests that miR-218-5p promotes NPY1R intron 2 splicing. Thus, miR-218-5p may promote the generation of spliced NPY1R mRNA while simultaneously decreasing NPY1R mRNA levels. This will be discussed further in chapter 4.

Unexpectedly, the level of NPY1R protein was upregulated in miR-218-5p transfected cells and downregulated with anti-miR-218-5p transfection. The mechanism behind this upregulation in NPY1R protein level is unknown, but there are few possible explanations based on miRNA mechanisms. First, it is possible that miR-218-5p also targets a gene that promotes the degradation of NPY1R, leading to reduced NPY1R degradation. Second, miR-218-5p may increases NPY1R translation while destabilizing NPY1R mRNA. Considering the downregulation of NPY1R mRNA with miR-218-5p transfection, the observed simultaneous increase in protein level has to be a specific targeting of NPY1R translation as an increase in global translation initiation is an unlikely candidate. Enrichment of miRNA in the polysomes at the rough endoplasmic reticulum (rough ER) can lead to efficient degradation of target mRNA (O'Brien et al., 2018) and, in this case, may shift the rates of translation of NPY1R mRNA sufficiently that even with a reduced pool of mRNA, the end result is an increase in protein output. Interestingly,

in neurons, the protein level of a GluA2 subunit of AMPA-type glutamate receptor, which has a validated miR-218-5p target site in its 3' UTR, was upregulated following miR-218-5p transfection (Rocchi et al., 2019). A third potential mechanism of NPY1R protein upregulation is that the increase of spliced NPY1R mRNA may significantly impact NPY1R translation rate thus, effectively mitigating the decrease of miR-218-5p-mediated NPY1R mRNA degradation. Finally, it has been reported that miRNA can induce translational activation in the nucleus due to the recruitment of nuclear miRNA protein complexes (microRNPs) (Truesdell et al., 2012; Valinezhad Orang et al., 2014). We have observed NPY1R (this study) and miR-218-5p (O'Brien et al., unpublished) in the nucleus of trophoblasts; therefore, it is possible that miR-218-5p increases NPY1R translation in the nucleus while destabilizing its mRNA in the cytoplasm. Further studies are required to test these possibilities.

Previous reports indicated that NPY is expressed in syncytiotrophoblasts (STB) and that NPY stimulated placental corticotropin-releasing hormone synthesis and release from STB (Robidoux et al., 1998; Robidoux et al., 2000). We have also seen that both NPY and NPY1R were expressed in the cytoplasm of STB in the first 20 weeks of pregnancy. We have also detected nuclear NPY and NPY1R signals in the cytotrophoblast (CTB) layer in placental villi and, to a lesser extent, in CTB in the proliferating column and villous angiogenic cores, the precursor structures of placental vasculature. Villous CTB are stem-like progenitor cells responsible for replenishing STB cells, forming the proliferating column in the anchoring villi, and giving rise to the other trophoblast subtypes, including the extravillous trophoblast (EVT) (Ji et al., 2013). We have also detected nuclear NPY1R signal in fetal macrophages (Hofbauer cells). These cells are present throughout gestation, and like other macrophages in the body, once activated, they undergo polarization into different subtypes, each with specific functions. Hofbauer cells were shown to play a role in placental villous tree development and mesenchyme maturation, in promoting placental vasculogenesis and angiogenesis, in maintaining placental homeostasis, in placental immunology and in maternal immune tolerance to the fetus (Seval et al., 2007; Tang et al., 2011; Svensson-Arvelund et al., 2015; Loegl et al., 2016). Interestingly, in other tissues, NPY was shown to be upregulated during macrophage activation and to modulate macrophage adhesion, chemotaxis, and phagocytosis (De la Fuente et al., 1993; Schwarz et al., 1994; Zukowska et al.,

2003). In mice, NPY was shown to promote macrophage migration by upregulating matrix metalloproteinase 8 (MMP-8) via NPY1R (Wu et al., 2021). Therefore, it is likely that NPY-NPY1R signalling may play an important role in Hofbauer cell function, and will be investigated in future work.

An interesting finding from this study is the detection of NPY and NPY1R in the nucleus of trophoblasts. In placental tissues from 4 to 17 weeks of gestation, and in HTR8/SVneo and Swan71 cell lines (figures included in chapter 4), we observed strong NPY and NPY1R nuclear signals. There are increasing reports of G-protein coupled receptors (GPCR) and their ligand in the nucleus, although a complete understanding of the origin of the nuclear GPCRs is still under intense investigation (Bhosle et al., 2019). A previous study has reported NPY and NPY1R in the nuclei of human endocardial endothelial cells, and it was suggested that in these cells, NPY signals through NPY1R to increase cytosolic and nuclear Ca^{2+} concentration (Jacques et al., 2003). Interestingly, we found a predicted nuclear localization signal in the NPY1R amino acid sequence and detected NPY1R bound to chromatin in HTR8/SVneo cells (Figures included in Chapter 4). The role of nuclear NPY-NPY1R signalling in trophoblast and its contribution to our observed NPY-NPY1R functions in trophoblasts is still unclear but will be further investigated in future studies.

In this study, we have shown that NPY signalling through NPY1R decreased migration and invasion of EVT cell lines (HTR8/SVneo and Swan71). We have also seen an impaired cell adhesion in the constitutively high NPY expressing trophoblast TSS E7 clone. This was accompanied by an increase in cell movement speed. Impaired adhesion may explain why, in this clone with strong NPY overexpression, we saw a decrease in motility using the Transwell migration assay but an increase in wound-healing rate. In Transwell migration, cells seeded into the inserts have to adhere to the membrane first before migrating through the pores, while in the wound-healing assay, cells have already adhered and are in a steady-state when the wound is made. We saw a similar discrepancy in Transwell migration and wound-healing after transiently transfecting cells with NPY overexpressing plasmid albeit to a lesser extent than that in the TSS E7 clone. However, Swan71 cells stably overexpressing NPY showed a decrease in Transwell migration and no change in wound-healing closure rate from that of the control. It is possible that NPY is upregulated to a different level in each of these cell types. Additionally, or alternatively, NPY produced in these

cells could be differentially processed into different fragments, which are known to activate different NPY receptor subtypes (Balasubramaniam, 1997; Dimitrijević et al., 2005; Abe et al., 2007; Pedragosa-Badia et al., 2013). As we showed in this study, activation of NPY1R led to an inhibitory effect on cell motility while NPY2R agonist (NPY 3-36) increased trophoblast motility when used at high concentration.

Next, NPY role in the ability of trophoblasts to differentiate down the endovascular pathway and their angiogenic potential was evaluated using tube-like network formation assay. In this assay, cell migration, proliferation and adhesion are all required for good network formation (Bischoff, 1997; Ramjaun and Hodivala-Dilke, 2009). Transient overexpression of NPY had no significant difference in trophoblast ability to form a tube-like network, while stable overexpression of NPY (both TSS E7 clone and Swan71 stable line) led to a decrease in the total length of tubes formed as well as a decrease in the number of branching points. This inhibitory effect was even more prominent in cells overexpressing NPY1R. That being said, the mechanism by which NPY-NPY1R signalling led to changes in trophoblast motility, cell speed, adhesion and differentiation down the endovascular pathway is unclear and remains to be investigated in the future.

We observed that when endogenous NPY levels were reduced by siRNA or in the TSS CRISPR heterogeneous population, cell proliferation was increased. However, NPY overexpression experiments yielded inconsistent findings. In transient NPY overexpression, proliferation was increased; however, in the TSS E7 clone which has a stable NPY overexpression, cell proliferation was decreased. It is again possible that the impaired adhesion we saw in the TSS E7 clone has affected the cells by delaying their ability to initiate proliferation soon after seeding in the plate due to their delayed adherence. We did not use recombinant NPY treatment to measure cell proliferation because the assay has to be done in serum-containing media. The serum is likely to contain proteases that could lead to the cleavage of NPY into small fragments that interact with different NPY receptor subtypes (Pedragosa-Badia et al., 2013; Wagner et al., 2015). However, we used receptor antagonists to help decipher which NPY receptors elicit NPY effect on proliferation. HTR8/SVneo cells treated with NPY1R or NPY5R antagonists showed an increase in proliferation, while NPY2R antagonists showed a decrease in cell proliferation.

Moreover, transient and stable overexpression of NPY1R also led to decreased proliferation. This suggested that in trophoblasts, NPY effect on proliferation is dynamic and is dependant on which of its receptors is being activated. That is, in trophoblasts, NPY can downregulate proliferation through NPY1R and NPY5R signalling, while it can upregulate proliferation through NPY2R.

The notion that NPY may inhibit trophoblast proliferation via NPY1R and NPY5R is further supported by our findings from the floating villi cultures. Similar to the cell lines, we found that knockdown of NPY increased proliferation of CTB in 6 weeks villi. In addition, NPY1R antagonists showed an increase in CTB proliferation while NPY5R antagonist was slightly effective in upregulating proliferation at low concentration. On the other hand, NPY2R antagonist showed no observable effect on CTB proliferation. This finding suggests that NPY could regulate trophoblast proliferation, primarily via NPY1R and possibly via co-activation of NPY5R. In fact, both NPY1R and NPY5R have been shown to form homodimers and heterodimers with each other (Gehlert et al., 2007; Czarnecka et al., 2019). This dimerization led to regulation of the NPY proliferative effect in hamster ovarian cells and increased sensitivity to NPY from nanomolar range to picomolar concentration (Czarnecka et al., 2019). Trophoblast proliferation is a tightly regulated mechanism. Placental growth occurs at an incredible speed to ensure a healthy pregnancy, and restricted placental growth is associated with poor pregnancy outcomes (Genbacev et al., 1997; Fisher, 2015; Maltepe and Fisher, 2015). In rare cases, abnormally high trophoblast proliferation rate leads to gestational choriocarcinomas, a malignant disorder that is one of many gestational trophoblastic diseases (Heller, 2018; Warthan et al., 2018; Wu et al., 2018). NPY has been implicated in both tumour progression (Tilan and Kitlinska, 2016) and tumour inhibition (Ogasawara et al., 1997; DeMorrow et al., 2011), as well as to shift between both phenotypes in the same cell type in response to the microenvironment such as hypoxia due to a change in NPY receptors expression profile and the expression of DPP4 (Tilan et al., 2013). Therefore, NPY could play a vital role in modulating trophoblast proliferation and placental growth.

In summary, our study provided novel insights into the role of NPY in trophoblast function and placenta development. NPY has been a dynamic player in biology through its diverse functions in multiple body systems via differential signalling through its four receptors in humans.

Here we showed that NPY regulates trophoblast migration, invasion, and proliferation as well as impacting cell adhesion. We also showed that NPY downregulation of migration and invasion occurs via signalling through NPY1R. As for proliferation, NPY can downregulate proliferation through NPY1R and possibly through co-activation of NPY5R, while it may promote trophoblast proliferation by signalling through NPY2R. In addition, our findings also suggest a complex regulatory role of miR-218-5p in modulating NPY and NPY1R expression.

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Chapter Four

Summary and future directions

In this dissertation, I have investigated the role of miR-210-3p in extravillous trophoblast (EVT) differentiation towards the endovascular pathway via targeting caudal-type homeobox 2 (CDX2). I have also investigated the function of neuropeptide Y (NPY) in human placenta development and its regulation by miR-218-5p. Specifically, we have shown that 1) overexpression of miR-210-3p promoted the acquisition of an enEVT phenotype, and 2) CDX2 is a novel target of miR-210-3p. We have also shown that 1) a miR-218-5p predicted target site on NPY exon 3 is not functional in trophoblast but that miR-218-5p targets NPY receptor 1 (NPY1R) at the 3' UTR; 2) NPY signalling via NPY1R downregulates trophoblast migration and invasion; and 3) NPY1R signalling inhibits trophoblast proliferation.

I. Chapter Two: Summary and future directions

In chapter 2 of this dissertation, we reported for the first time that in normal pregnancy, miR-210-3p level was lower in the third trimester (26-40 weeks) compared to first trimester (5-12 weeks). During the first trimester, uterine spiral arteries are plugged by extravillous trophoblasts (EVT) to reduce maternal circulation into the intervillous space which results in a physiological oxygen level of around 2.5% in the first 10 weeks of gestation. This is important to protect the developing fetus and the early placental tissues from damage caused by oxidative stress (Burton et al., 2021). The observed high level of miR-210-3p in first trimester samples suggests a role of miR-210-3p in early placentation events where trophoblasts have reduced mitochondrial function and rely heavily on glycolysis for ATP production (Kolahi et al., 2017; Burton et al., 2021). In cancer studies, miR-210-3p was shown to upregulate glycolysis (Chen et al., 2010), and, in trophoblasts, miR-210-3p targets ISCU, thus reducing mitochondrial function (Muralimanoharan et al., 2012).

Our data also confirmed previous reports of miR-210-3p downregulation of trophoblast migration and invasion. We also reported decreased EVT outgrowth in first trimester explants model. In addition, we reported that overexpressing miR-210-3p in trophoblast significantly reduced the ability of HTR8/SVneo cells to form endothelial tube-like network. In trophoblasts,

this assay is used to assess their ability to acquire endovascular EVT (enEVT)-like phenotype. We showed that this decrease in tube-like network was associated with a significant reduction in the mRNA levels of cytokines and chemokines, specifically interleukin 1 beta (IL1B), interleukin-8 (CXCL8) and C-X-C motif ligand 1 (CXCL1). These have been involved in EVT migration, invasion and trophoblast communication with decidual natural killer cells and macrophages necessary for initiating spiral artery remodelling. This suggests that miR-210-3p regulates trophoblast functions and may play a role in the remodelling process.

To further understand the role of miR-210-3p in trophoblast differentiation down the endovascular pathway, *ex vivo* model using first trimester placenta-decidua co-culture can be used to assess trophoblast remodelling of spiral arteries. Immunohistochemistry on these co-culture models can also be used to detect different markers associated with the remodelling process such as PECAM1, HLA-G, ITGA1, CDH5 and smooth muscle actin (SMA). In addition, enzyme-linked immunosorbent assay (ELISA) can be used to measure the level of IL1B, CXCL8 and CXCL1 in the placenta-decidua co-culture conditioned medium as well as in cell lines transfected with miR-210-3p mimic.

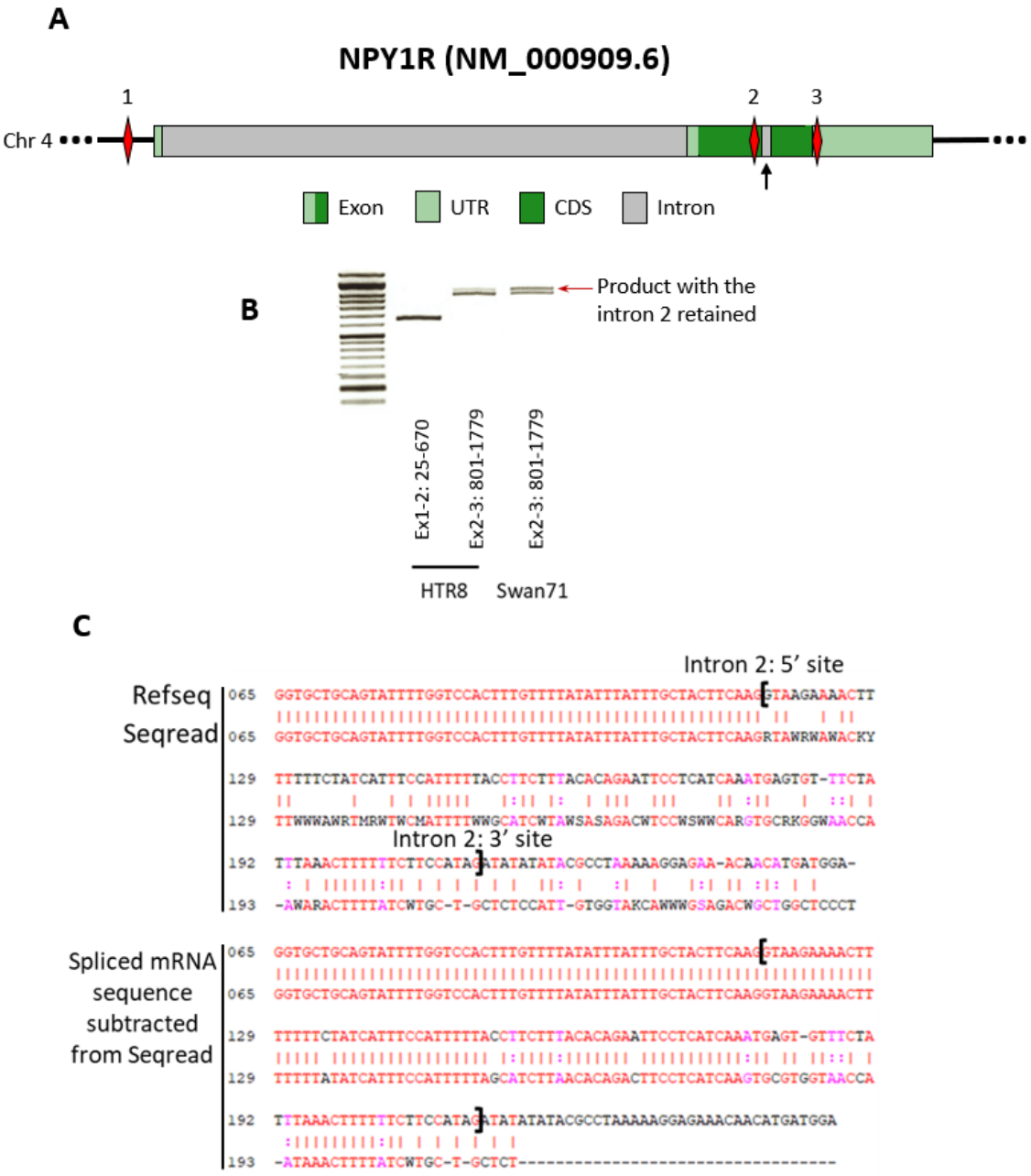
In addition, miR-210-3p is involved in regulating different aspects of trophoblast functions by targeting multiple genes; here we reported for the first time that caudal-related homeobox transcription factor 2 (CDX2) is a target of miR-210-3p. CDX2 is a transcription factor critical for the establishment of the trophoblast lineage (Sakurai et al., 2010). However, the role of CDX2 throughout gestation is not well understood. We showed that CDX2 knockdown using siRNA mimicked the effect of miR-210-3p overexpression in inhibiting migration, invasion and first trimester EVT outgrowth. We also reported that CDX2 knockdown decreased the ability of trophoblast to form endothelial tube-like networks, as well as decreased the expression of invasive EVT and enEVT markers and the expression of IL1B, CXCL8 and CXCL1. Therefore, these results suggest that CDX2 may be involved in promoting enEVT functions including processes required for a successful spiral artery remodelling.

It will also be interesting to characterize the expression levels of CDX2 across gestation using qRT-PCR and immunohistochemistry; also, these experiments will help determine which

trophoblast subtypes might express CDX2 and assess whether it is being dysregulated in PE pregnancies. Moreover, in order to further understand the mechanisms by which CDX2 regulates trophoblast migration, invasion and differentiation, we can use chromatin immunoprecipitation combined with DNA sequencing (ChIP-Seq) to detect where does CDX2 interact on the DNA in trophoblast and shed light on which genes CDX2 might be directly regulating.

II. Chapter Three: Summary and future directions

In Chapter 3 of this dissertation, we reported that miR-218-5p targets NPY1R at the 3' UTR and a decrease in NPY1R mRNA level after transfection with miR-218-5p mimic. However, this was associated with an increased NPY1R protein level. Using both HTR8/SVneo and Swan71 cell lines, we detected a retained intron in NPY1R mRNA (**Figure 1A, 1B**). The retained intron was also confirmed by sequencing (**Figure 1C, 1D**). To our knowledge, the retention of intron 2 in NPY1R mRNA is a novel finding as there are no reported transcript variants of NPY1R (NCBI and Ensembl, accessed March 25, 2022) that contain this intron making this the first report of a new NPY1R splice variant. Interestingly, cells transfected with miR-218-5p mimic showed an increase in spliced intron 2 NPY1R mRNA (**Figure 1E**). Moreover, there is a predicted miR-218-5p site approximately 100 bp upstream of intron 2 on the NPY1R mRNA (**Figure 1A, site 2**). To better understand the mechanism of miR-218-5p regulation of NPY1R, a luciferase reporter vector can be generated to validate if this miR-218-5p target site is functional in trophoblasts. The generation of silent mutations at the predicted miR-218-5p MRE, followed by RNA immunoprecipitation with and without miR-218-5p overexpression, can be used to assay argonaute 2 (AGO2) binding at the predicted MRE. PCR can then be used to assess any change in NPY1R intron 2 retention associated with the mutation of the predicted miR-218-5p MRE.



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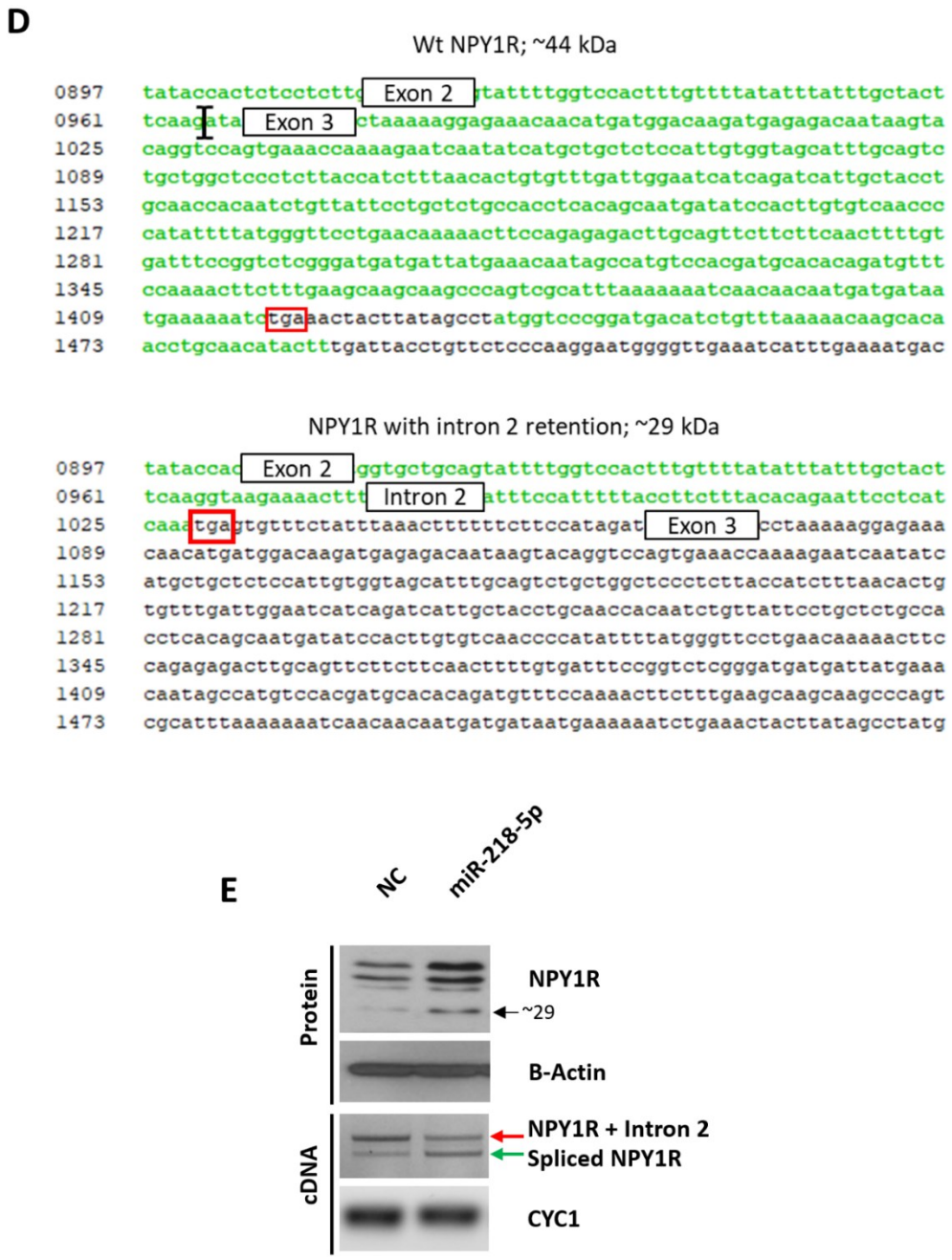

Figure 1. Detection of intron 2 retention in the mature mRNA of NPY1R in HTR8/SVneo and Swan71 trophoblast cells. A. Schematic diagram showing predicted miR-218-5p target sites on NPY1R locus indicated by the red diamonds. Target site 1 is 296 bp upstream of the NPY1R transcription start site and target site 2 in exon 2, 103 bp upstream of intron 2. Target site 3 is in the 3' UTR 0f NPY1R. Black arrow marks the retained intron. **B.** Gel electroporation showing the amplification of different regions of NPY1R mRNA variant 1 (NM_0009095.5) in HTR8/SVneo and Swan71 cDNA generated with Q5 high fidelity polymerase. Red arrow marks the Exon2-Exon3 fragments with retained intron 2. **C.** Ex2-3: 801-1779 bp PCR products from HTR8/SVneo and Swan71 were cleaned and sent for sequencing by the SickKids' sequencing facility. Upper part

compares the alignment between the reference sequence from NCBI (Refseq) to the sequence read obtained from the SickKids' facility (Seqread). Lower panel shows the Seqread sequence with intron 2 sequence removed. **D**. NPY1R open reading frame was resolved in MATLAB to detect premature stop codons within the retained intron 2 sequence. Square brackets indicate the intron sequence. The stop codon is highlighted with a red box. **E.** Western blot analysis and agarose gel electroporation of HTR8/SVneo cells transfected with miR-218-5p mimic or its control showing the effect on NPY1R mRNA intron 2 splicing. The upper band (~1100 bp, red arrow) the mRNA with the retained intron, whereas the smaller band is with the intron spliced out (~980 bp, green arrow). This figure was generated by Jacob A. O'Brien.

We have also detected nuclear NPY and NPY1R signals in cytotrophoblasts and a nuclear NPY1R signal in fetal macrophages in placental tissues (4-20 weeks) (Chapter 3). Similarly, we detected nuclear NPY and NPY1R signals in HTR8/SVneo and Swan71 cells (**Figure 2**). Using cNLS Mapper, we identified a predicted nuclear localization signal within NPY1R (**Figure 2A**). Fluorescent immunocytochemistry performed on isolated HTR8/SVneo nuclei confirmed that NPY1R is present on the nuclear envelope and is distributed throughout the nucleus (**Figure 2B**). Moreover, using chromatin fractionation as well as nuclear fractionation methods, we detected NPY1R in the nucleus and also bound to chromatin. More importantly, we detected the full-length NPY1R (~44 kDa) in those compartments, as well as, we observed less chromatin-bound NPY1R in the miR-218-5p knockout cell line than the EV cell line (**Figure 2C**) and more nuclear NPY1R in cells transfected with miR-218-5p mimic (**Figure 2D**). This suggests a possible role of miR-218-5p in NPY1R nuclear localization.

G-protein coupled receptors (GPCR) have been previously reported in the nucleus. Currently, there are four working hypotheses on the origin of nuclear GPCR, and each has some evidence to support it. The first is ligand-dependant and -independent translocation from the plasma membrane into the nucleus via importin-regulated trafficking. The second is retrograde trafficking of GPCR from the trans-Golgi network into the nucleus; both mechanisms involve the action of small GTPases**.** The third proposed mechanism is for GPCR to travel via lateral diffusion from the ER into the contiguous outer nuclear membrane, while the last proposed model is for GPCR synthesis to occur in the nucleus itself (Bhosle et al., 2019). Regardless of their origin, and

similar to GPCR in other intracellular compartments, nuclear GPCR have been shown to signal in G-protein-dependant and -independent pathways as many of their signalling molecules have been found in the nucleus (Joyal et al., 2015; Eichel and von Zastrow, 2018; Calebiro and Koszegi, 2019). In addition, the GPCR coagulation factor II receptor-like 1 (F2RL1) has been shown to regulate transcription by forming transcriptional complexes with recruited transcription factors (Joyal et al., 2014).

In addition, we also detected the presence of NPY1R ligand, NPY in the nucleus in both HTR8/SVneo and Swan71 cells (**Figure 2E. 2F**). NPY was diffused throughout the nucleus (**Figure 2E**), and miR-218-5p did not seem to affect the amount of NPY in the nucleus compared to that in the cytoplasm (**Figure 2G**). Therefore, in future work, we would like to characterize the role of nuclear NPY1R in trophoblasts and how miR-218-5p may contribute to its nuclear localization. Specifically, whether NPY is bound to nuclear NPY1R, what other proteins might be bound to NPY1R on the chromatin, and what regions of the DNA NPY1R might bind to. Also, what are the possible origins of nuclear NPY1R. As miRNA have been reported to activate the translation of some mRNA in the nucleus via the recruitment of nuclear miRNA protein complexes (microRNPs) (Truesdell et al., 2012; Valinezhad Orang et al., 2014), it will be interesting to investigate whether miR-218-5p could increase NPY1R translocation into the nucleus. Another possibility is, whether miR-218-5p can regulate NPY1R trafficking into the nucleus via importins.

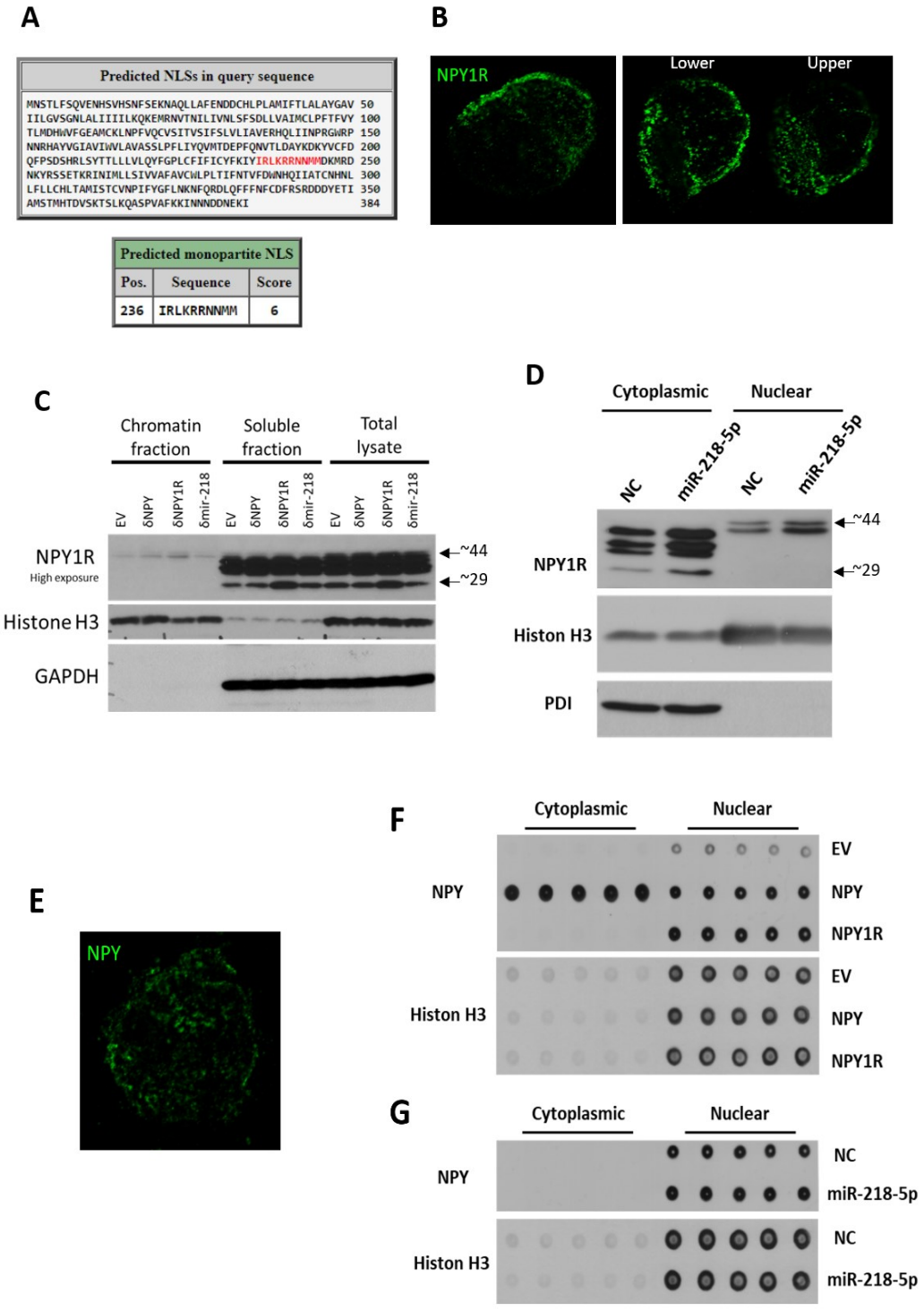

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Figure 2. NPY and NPY1R nuclear localization in HTR8/SVneo. A. cNLS Mapper was used to predict nuclear localization signal (NLS) within NPY1R. The predicted region had a score of 6 where a score of 1-2 is strictly cytoplasmic protein and a score of 9-10 is nuclear. **B.** Fluorescence immunocytochemistry on nuclei isolated from HTR8/SVneo cells showing NPY1R localization (NPY1R antibody, Abcam). **C.** Western blot analysis of heterogenous HTR8/SVneo cells transfected with CRISPR PX459 plasmid and gRNA to remove NPY, NPY1R or mir-218-1 and miR-218-2. Some cells were fractionated into chromatin-bound and soluble fractions and others kept as a whole cell lysate. **D.** Western blot analysis of HTR8/SVneo cells transfected with miR-218-5p mimic or its control and fractionated into nuclear and cytoplasmic fractions. Protein disulfide-isomerase (PDI) was used as an ER-contamination control. **E.** Fluorescence immunocytochemistry on nuclei isolated from HTR8/SVneo cells showing NPY localization (NPY antibody, Cell Signaling). **F.** Dot blot analysis of Swan71 cells stably overexpressing NPY or NPY1R or their control and fractionated into nuclear and cytoplasmic fractions. **G.** Dot blot analysis of HTR8/SVneo cells transfected with miR-218-5p mimic or its control and fractionated into nuclear and cytoplasmic fractions. Nuclei were imaged with 63X objective. Jacob A O'Brien contributed to parts B-G.

Further, our immunohistochemistry experiments detected nuclear NPY1R signals in fetal macrophages (Hofbauer cells) (**Figure 3A, and chapter 3**). We also detected nuclear NPY1R in the maternal immune cells in the decidua; including both decidua basalis (where implantation happens and the chorionic plate forms) (**Figure 3B, 3C**) and decidua parietalis (decidual tissue that lines the pregnant uterus except at the site of implantation) (**Figure 3C**) (Solders et al., 2017). To our knowledge, this is the first report of nuclear NPY1R in fetal macrophages and in uterine immune cells. Fetal macrophages play a role in placental villous tree development and mesenchyme maturation, in promoting placental vasculogenesis and angiogenesis, in maintaining placental homeostasis, in placental immune function, and in maternal immune tolerance to the fetus (Seval et al., 2007; Tang et al., 2011; Svensson-Arvelund et al., 2015; Loegl et al., 2016). Maternal immune cells such as macrophages, uterine natural killer cells and lymphocytes play a vital role during pregnancy such as in regulating trophoblast invasion into the decidua, maternal immune response to trophoblasts and the fetus, and spiral artery remodeling (Zhang et al., 2016). NPY has been reported to regulate immune functions in different tissues (Schwarz et al., 1994; Zukowska et al., 2003b; Ferreira et al., 2010; Han et al., 2012; Farzi et al.,

2015; Chen et al., 2020), including macrophage migration and cytokine release, such as interleukin-1β (IL1B) and interleukin-6 (IL-6), both of which are important for trophoblast function (Hsiao and Patterson, 2011; Brkić et al., 2018). Thus, we would like to investigate the role of NPY and particularly NPY1R signalling in fetal macrophages and decidual immune cells during pregnancy.

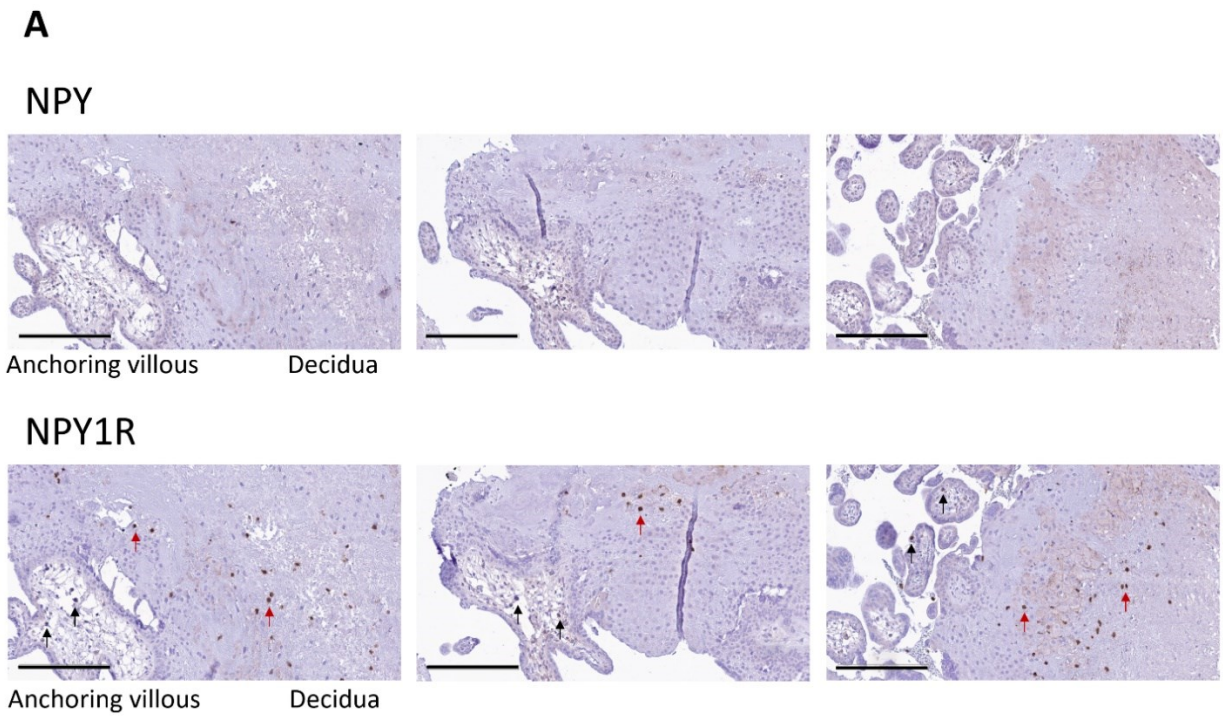

Figure 3. NPY1R is highly expressed in fetal macrophages and maternal immune cells in the decidua. A. Immunohistochemistry for NPY and NPY1R on an 18.6 week placenta and decidua basalis co-culture sections with anchoring villi observed. Black arrows indicate fetal macrophages/Hofbauer cells. Red arrows indicate maternal immune cells.

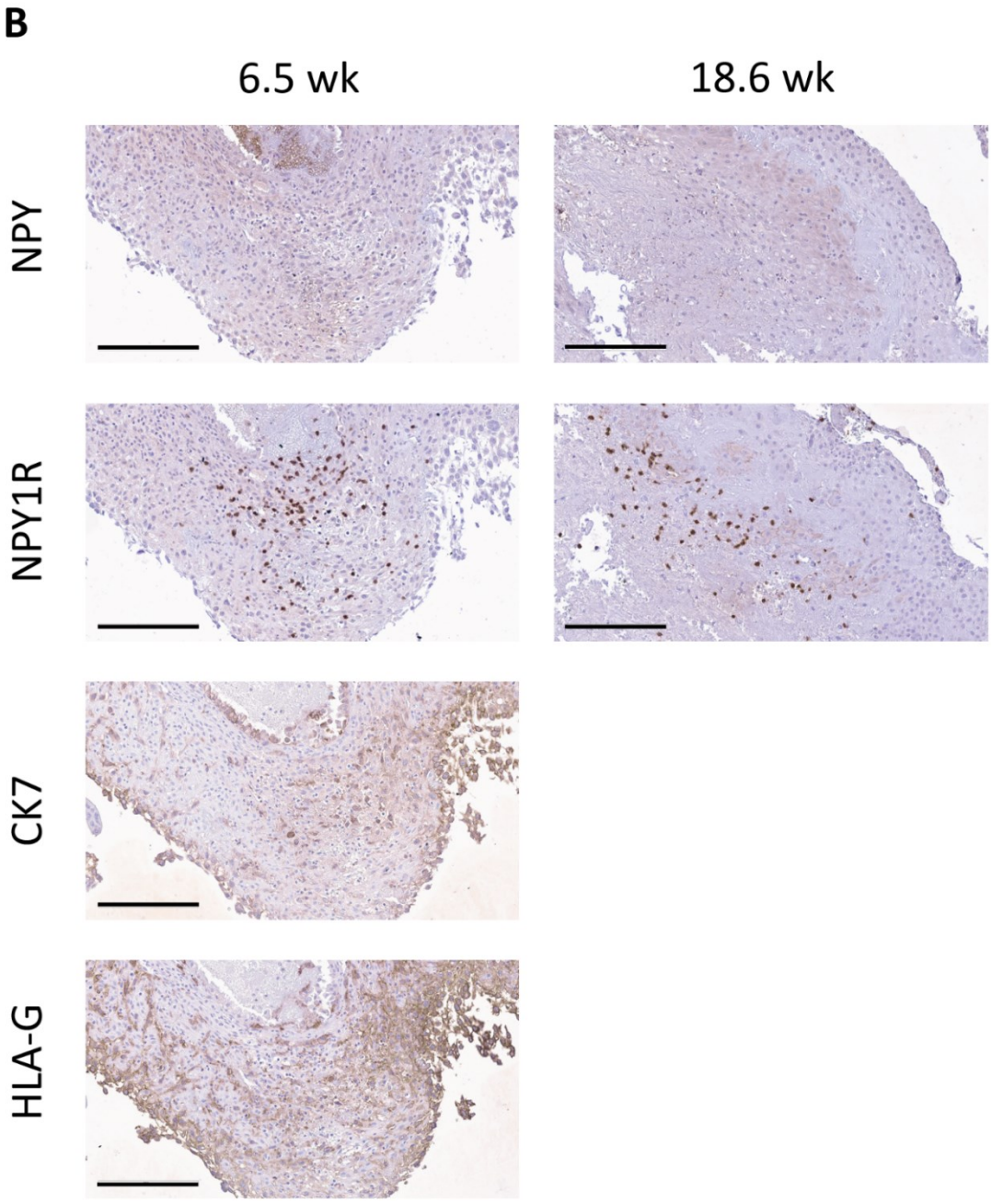

Figure 3. continued. B. Immunohistochemistry on decidua basalis sections from 6.5 and 18.6-week pregnancies detecting NPY, NPY1R, cytokeratin 7 (CK7, trophoblast marker) and major Histocompatibility Complex, Class I, G (HLA-G, EVT marker).

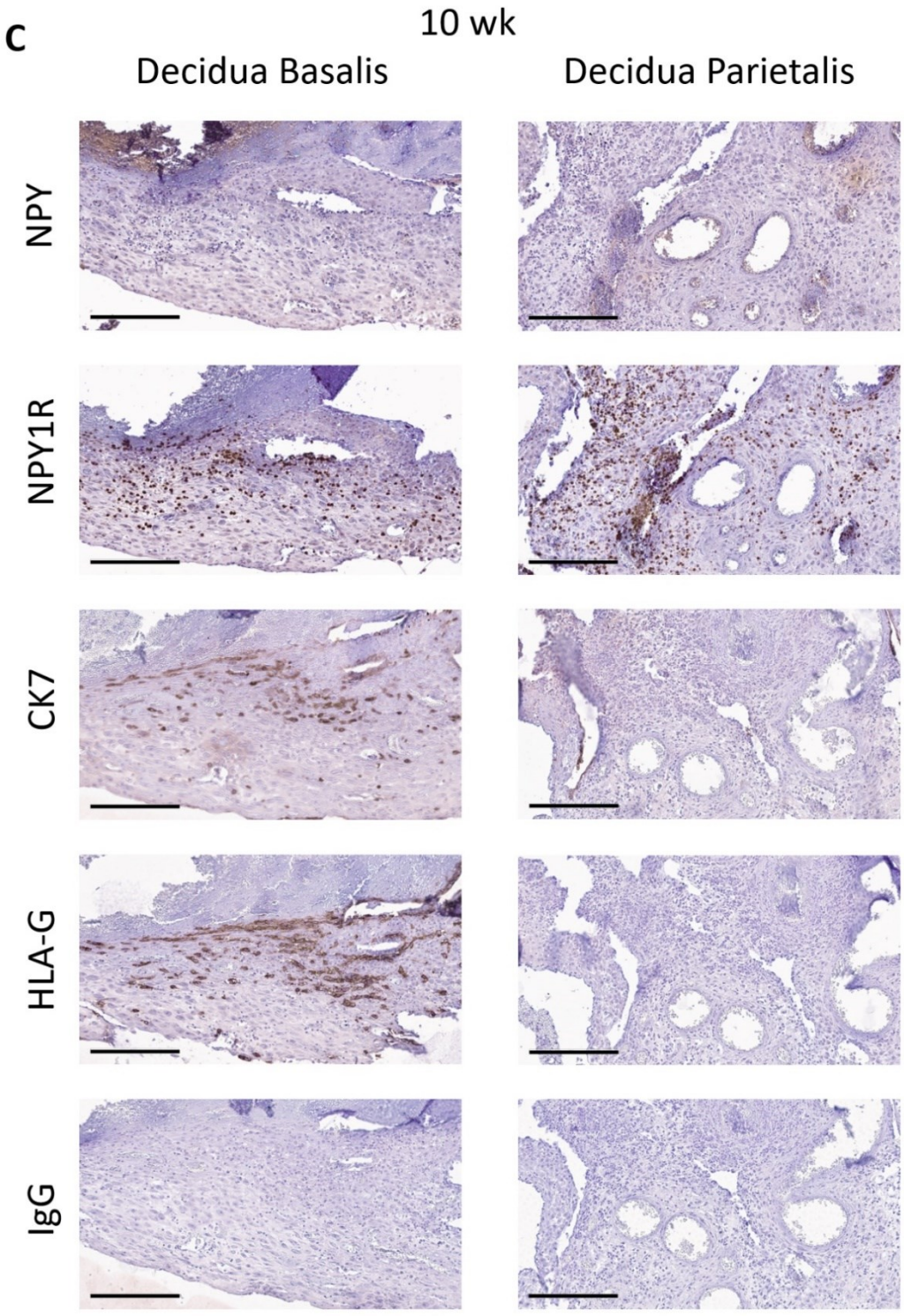

Figure 3. continued. C. Immunohistochemistry on decidua basalis and decidua parietalis sections from 10 week pregnancies detecting NPY, NPY1R, CK7, HLA-G and IgG control. Scale bar = 200 μm.

In chapter 3, western blot analyses of NPY1R consistently detected a 29 kDa fragment that changed with siRNA knockdown and plasmid overexpression of NPY1R instead of the expected full-length NPY1R band at around 44 KDa. However, following fractionation, we could detect the full-length band within the nucleus and the 29 kDa fragment in the cytoplasm (**Figure 2D**). Nevertheless, we rarely detected full-length NPY1R whenever whole cell lysate was collected from unfractionated cells. Given that the Abcam antibody epitope was at the C-terminal of NPY1R, we hypothesized that this 29 kDa band was a C-terminal NPY1R degradation product.

To verify that we were detecting a 29 kDa NPY1R C-terminal fragment, we used two additional antibodies. One targeted the middle region of NPY1R (Antibodies online; AbO), which should still detect the 29 kDa fragment, and the other detect an epitope at the N-terminal of NPY1R (R&D). The Abcam antibody detected predominantly cytoplasmic and peri-nuclear NPY1R signal (**Figure 4A**), whereas the AbO antibody was predominantly a nuclear signal (**Figure 4B**), and had a lower affinity towards the C-terminal fragment (**Figure 4C**). This subcellular distribution is consistent with Western blots of fractionated cells, where the majority of signal comes from cytoplasmic C-terminal fragments and a smaller portion of signal from full-length nuclear NPY1R (**Figure 2D**).

Lastly, we planned on performing mass spectrophotometry analysis on immunoprecipitated NPY1R; however, this has been moved to future work. Regardless, immunoprecipitating NPY1R using the R&D antibody pulled down only the full-length, whereas the Abcam antibody pulled down the full-length and C-terminal fragments (**Figure 4D**). Conversely, we can see the unbound C-terminal fragments following immunoprecipitation (IP) using the R&D antibody in columns 2 and 4 (**Figure 4D**). Taken together, these data suggest that in EVT, NPY1R is degraded within the cytoplasm but not the nucleus, resulting in the accumulation of cytoplasmic C-terminal fragments and full-length nuclear NPY1R and that this degradation is a consequence of N-terminal cleavage events of NPY1R.

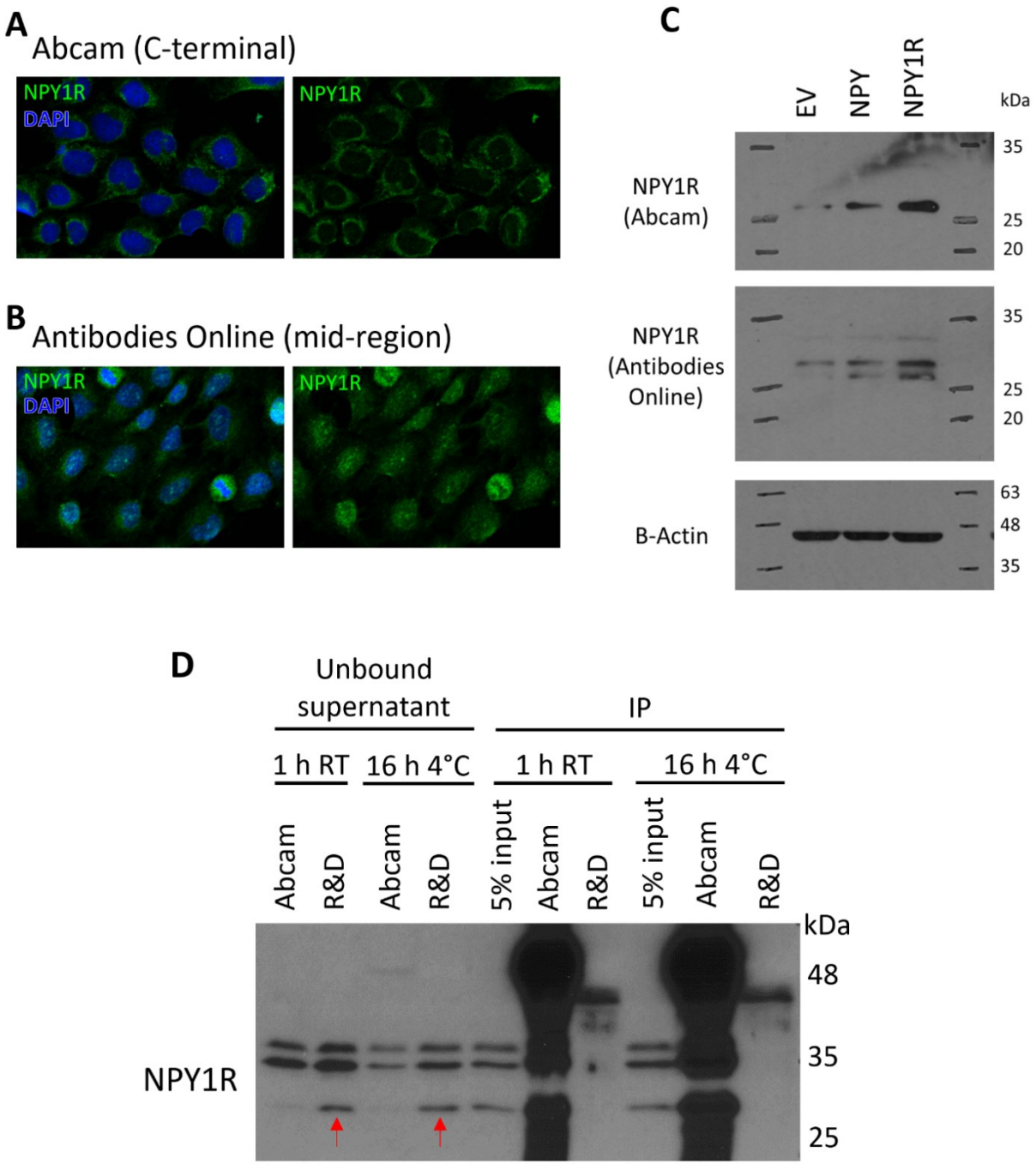

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Figure 4. Truncations of NPY1R in HTR8/SVneo. A. Fluorescence immunocytochemistry conducted on whole HTR8/SVneo cells using NPY1R antibody (Abcam- detect C-terminal). **B.** Fluorescence immunocytochemistry conducted on whole HTR8/SVneo cells using NPY1R antibody (Antibodies Online- detect middle protein region). **C.** Western blot analysis of HTR8/SVneo cells transfected with plasmid overexpressing NPY or NPY1R and their control empty vector (EV) using both the NPY1R Abcam and the Antibodies Online antibodies. **D.** Western blot analysis of immunoprecipitated NPY1R in HTR8/SVneo cell lysate. Lysate was either immunoprecipitated at room temperature (RT) for 1 h or at 4 °C for 16 h using two different antibodies against NPY1R; the Abcam epitope is at the C-terminal whereas the R&D epitope is at the N-terminal. Following immunoprecipitation (IP), the supernatant, containing unbound protein, was collected and run in columns 1-4. The red arrows indicate unbound C-terminal fragments following IP using the R&D antibody. Immunoprecipitation (IP) using the R&D antibody pulled down full length NPY1R while the Abcam antibody pulled down multiple C-terminal containing fragments. IF-ICC were imaged with 63X objective. Jacob A O'Brien contributed to part D.

In addition, we have detected NPY and NPY1R signals in angiogenic cores of 6- and 7-week placental villi (Chapter 3) and our preliminary results showed that NPY treatment in 6-week placental villi increased the number of angiogenic cores, the precursor structures to placental vasculature (Seval et al., 2007; Aplin et al., 2020), as shown by the increase in endothelial marker CD34 (Aplin et al., 2015) (**Figure 5)**. Also, treatment with NPY2R antagonist decreased the number of angiogenic cores in the placental villous structure (**Figure 5**). NPY signalling through NPY2R has been shown to promote angiogenesis in the cardiovascular system (Kitlinska et al., 2002; Zukowska et al., 2003a; Abe et al., 2007). Therefore, it will be interesting to investigate whether NPY plays a role in placental vasculature development in two ways; first by regulating fetal macrophage function through NPY1R, and second by directly promoting placental vasculogenesis and angiogenesis via NPY2R signalling.

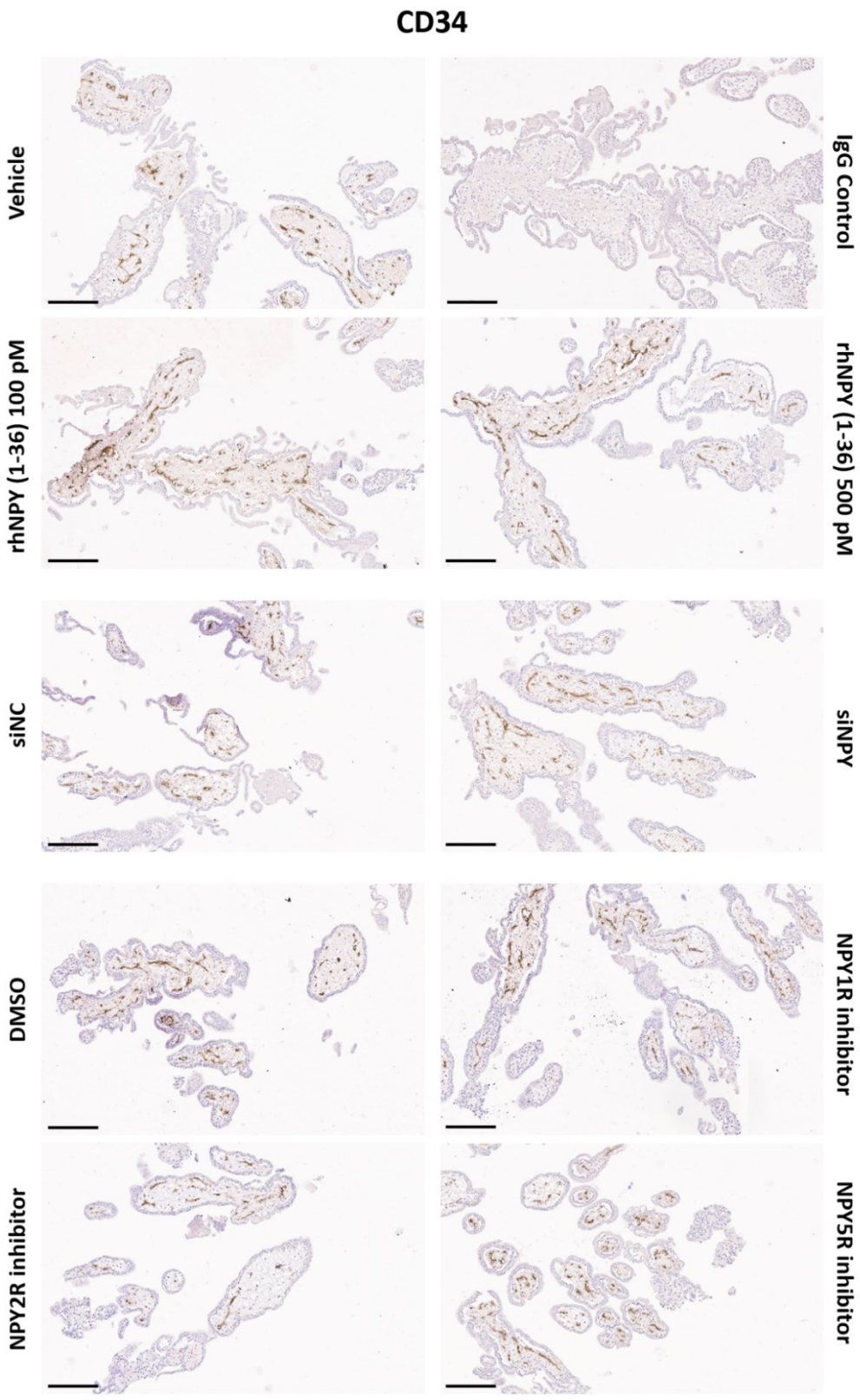

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Figure 5. NPY may promote placental angiogenic cores formation via NPY2R. Immunohistochemistry was done to detect the endothelial marker CD34 in 6.4-week placental floating villi treated for 48 h with recombinant human NPY (1-36), siRNA targeting NPY (siNPY) (200 nM) or NPY1R, NPY2R or NPY5R antagonists (1 nM). Scale bar = 200 µm. Pictures shown are representative images of two experiments, each with 3 replicates.

In chapter 3, we have also reported that NPY signalling via NPY1R decreased HTR8/SVneo and Swan71 migration and invasion and that NPY1R overexpression decreased EVT cell lines proliferation and the ability to form endothelial-like networks. To better understand the downstream mechanisms of NPY signalling in trophoblasts, we investigated different signalling pathways. We observed that NPY treatment resulted in an increase in the proto-oncogene tyrosine-protein kinase Src (SRC) activation in a time-dependant (**Figure 6A, 6B**) and dose-dependant manner (**Figure 6C**) as seen by increased phosphorylation at tyrosine 416. GPCR-β-arrestin have been reported to activate Src family kinases, including SRC, by causing conformational changes in SRC that allow SRC autophosphorylation (Miller et al., 2000; Luttrell and Luttrell, 2004; Pakharukova et al., 2020). SRC was shown to regulate cell motility and proliferation by regulating the formation of focal adhesions, cytoskeletal rearrangement and cell cycle progression via phosphorylation of cortactin, an F-actin associated protein (Huang et al., 1998; Wang et al., 2011). In addition, SRC was shown to promote trophoblast motility and differentiation (Kamei et al., 1997; He et al., 2019); it was also shown that SRC was necessary for FGF-mediated trophoblast stem cell maintenance (Lee et al., 2019). However, another study showed that the use of two different SRC inhibitors in trophoblast produced two different effects; that is, herbimycin A SRC inhibitor inhibited trophoblast morphological and hormonal differentiation, while 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d] pyrimidine (PP2) SRC inhibitor stimulated hormonal differentiation and inhibited trophoblast cell adhesion and spreading (Daoud et al., 2006). Therefore, it will be important to understand how NPY-induced SRC activation can mediate NPY function in trophoblasts.

Similarly, we detected increased phosphorylation of cAMP-response element binding protein (CREB) at serine 133 that was time- (**Figure 6A**) and dose-dependent (**Figure 6C**). CREB

phosphorylation allows the recruitment of the coactivators CREB-binding protein (CBP) and p300, which enhances CREB-dependant transcription (Johannessen et al., 2004). In EVT, CREB binding sites were shown to be present in the promotor of major histocompatibility complex, class I, C (HLA-C), and G (HLA-G); both of which play critical roles in regulating the immune response to EVT at the maternal-fetal interface (Gobin et al., 2002; Johnson et al., 2018). Also, in the placenta, calcium (Ca^{2+}) is capable of regulating transcription through binding to CREB (Baczyk et al., 2011). Interestingly, in human endocardial endothelial cells, NPY signals through NPY1R to increase cytosolic and nuclear Ca^{2+} concentration (Jacques et al., 2003). In addition, CBP and P300 were shown to help maintain hematopoietic stem cell self-renewal (Rebel et al., 2002), and in mice, histone acetylation by CBP acetyltransferases reduces trophoblast stem cell invasiveness and maintains the stemness phenotype (Kohan-Ghadr et al., 2016). In the future, we can investigate the mechanisms by which NPY-induced CREB phosphorylation can regulate trophoblast physiology.

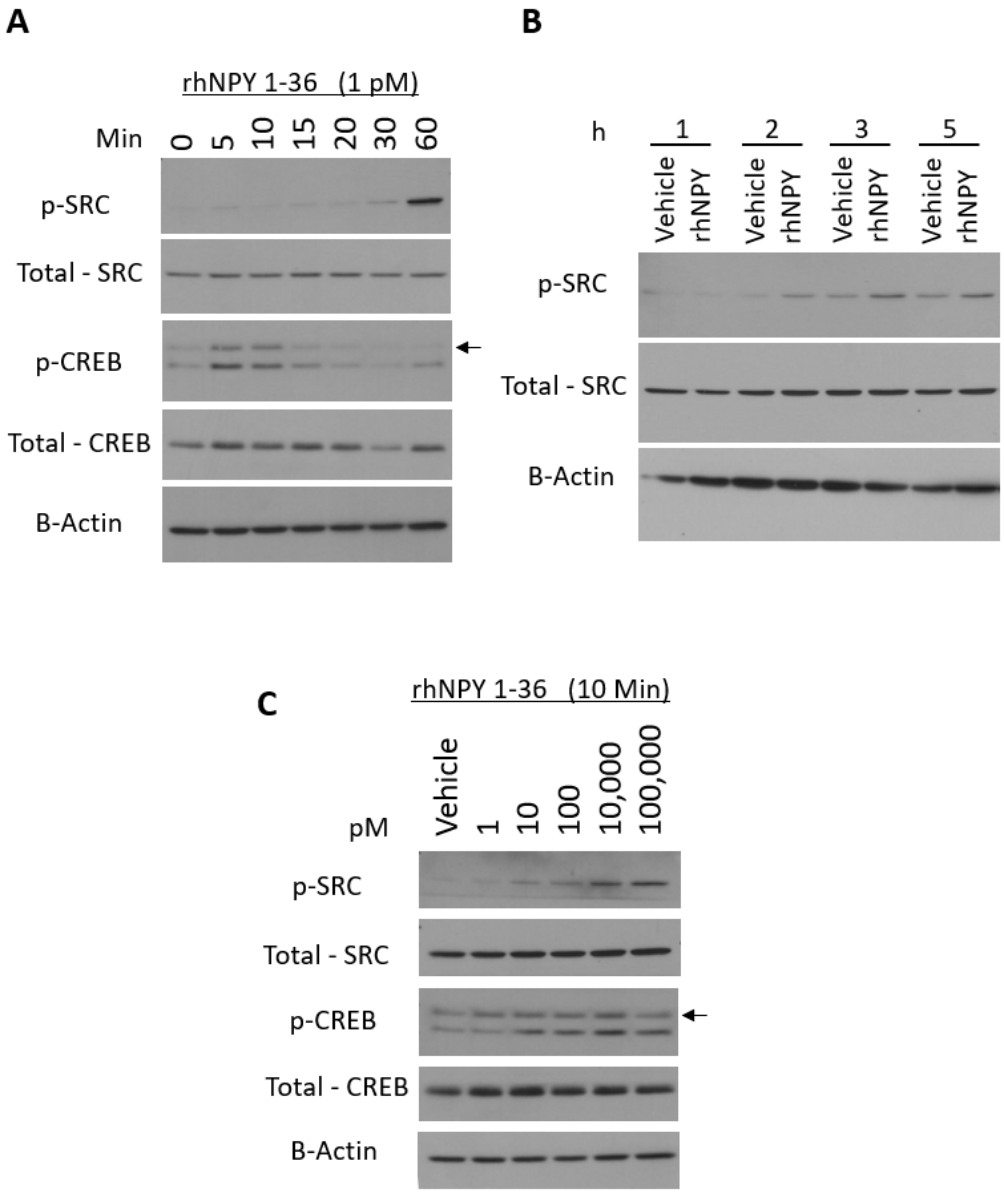

Figure 6. NPY promotes SRC and CREB phosphorylation. A. Western blot analysis of time-course treatment in minutes of HTR8/SVneo cells with recombinant human NPY (rhNPY) (1-36 a.a.) at 1 pM concentration. Phosphorylated Proto-oncogene tyrosine-protein kinase SRC (p-SRC) at tyrosine 416 (~60 kDa), indicting its activation, was detected (black arrow). Phosphorylated cAMP response element-binding protein (p-CREB) at serine 133 (transcription activating site) (~43 kDa) was detected. **B.** Western blot analysis of time-course treatment in hours of HTR8/SVneo cells with rhNPY (1-36 a.a.) at 1 pM concentration. p-SRC at tyrosine 416 was detected. **C.** Western blot analysis of HTR8/SVneo cells treated for 10 minutes with rhNPY (1-36 a.a.) at various concentrations. p-SRC at tyrosine 416 and p-CREB at serine 133 were detected (black arrow). N = 3.

III. Conclusion

MicroRNA regulation of trophoblast and placenta development continues to be a fascinating part of reproductive biology. This dissertation included work on the continually evolving miR-210-3p role in trophoblasts (Figure 7). We provided for the first time a gestational profile of miR-210-3p expression during normal pregnancy and found that miR-210-3p levels are highest in the first trimester and are lowest in the third trimester. During the first trimester, uterine spiral artery openings into the placental intervillous space are obstructed by EVT plugs to protect the placenta and developing fetus from damage caused by oxidative stress evident by physiological oxygen concentrations of around 2.5% prior to 10 weeks of gestation (Burton et al., 2021). The observed high level of miR-210-3p in first trimester samples suggests a role of miR-210-3p in early placentation events where trophoblasts have reduced mitochondrial function and rely heavily on glycolysis for ATP production (Kolahi et al., 2017; Burton et al., 2021). In cancer studies, miR-210-3p was shown to upregulate glycolysis (Chen et al., 2010), and, in trophoblasts, miR-210-3p targets ISCU, thus reducing mitochondrial function (Muralimanoharan et al., 2012).

In addition, we and others confirmed that miR-210-3p level is higher in term preeclampsia (PE). We have also reported yet another novel role of miR-210-3p in impairing EVT's acquisition of endothelial-like properties which is critical in uterine spiral artery remodelling. This remodelling is important for proper feto-placental profusion which is dysregulated in pregnancy disorders including PE. Specifically, we found that either higher miR-210-3p levels or lower CDX2 levels led to a decrease in the mRNA level of important marker genes of enEVT as well as a decrease in the mRNA level of cytokines produced by EVT. These cytokines were shown to play a role in trophoblasts and decidual cells communication to orchestrate trophoblast invasion and spiral artery remodeling. Thus, our work supports the idea of the potential use of miR-210-3p inhibitors as a treatment to reduce the severity of PE complications thus alleviating some of the damage occurring in both the mother and fetus.

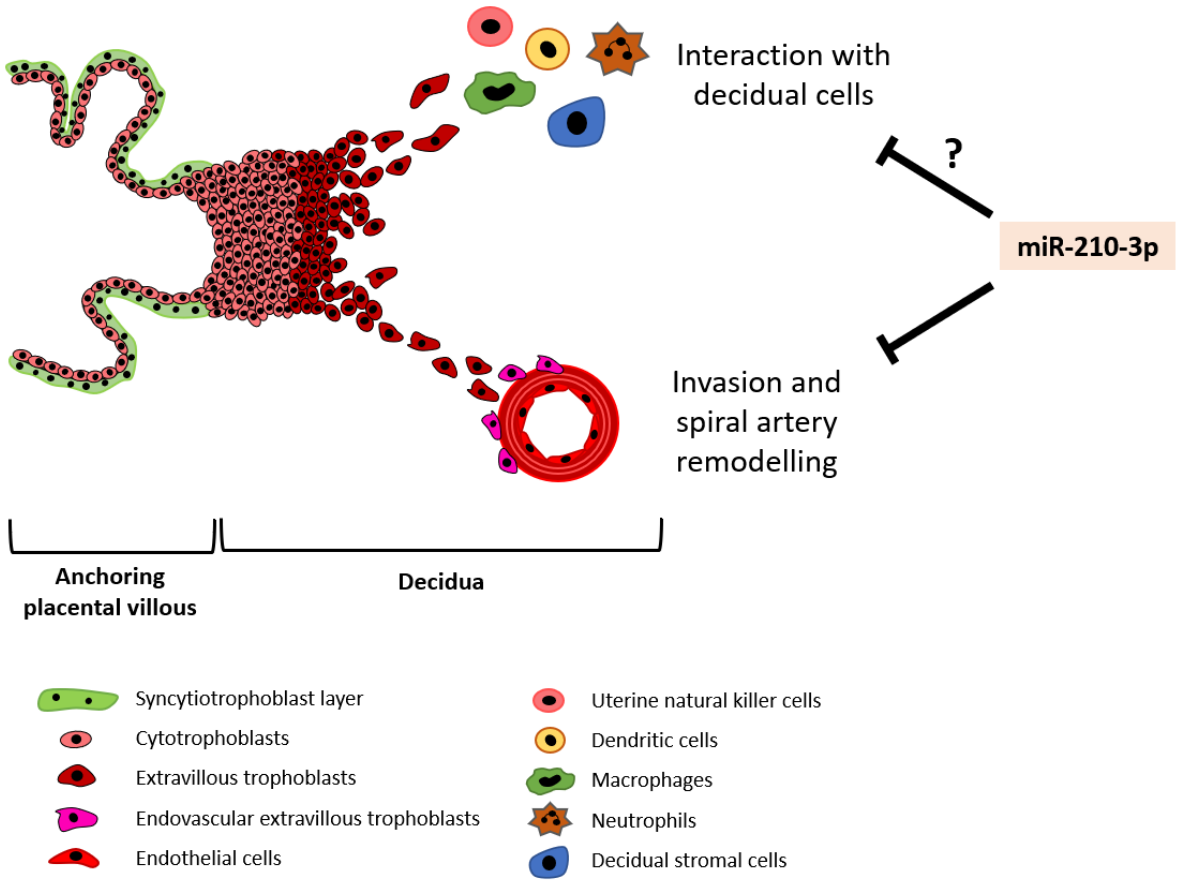

Figure 7. Proposed role of miR-210-3p in the development of preeclampsia. High miR-210-3p levels in trophoblasts early in placenta development impairs proper spiral artery remodelling in possibly two different ways. First, miR-210-3p inhibits proper extravillous trophoblasts (EVT) migration and invasion toward spiral arteries and impairs their ability to differentiate into endovascular EVT and replace endothelial cells in the arteries. Second, miR-210-3p downregulates important cytokines secreted by EVT that allow for proper communication between EVT and different types of cells in the decidua including immune cells and decidual stromal cells. This inter-cellular interaction is vital for proper EVT invasion and initiation of the spiral artery remodelling process. Thus, higher than normal levels of miR-210-3p during early placenta development could contribute to abnormal development and give rise to pregnancy diseases such as preeclampsia.

This dissertation also included more extensive work on the role of miR-218-5p in placenta development via the regulation of NPY. Our previous work showed a novel role of miR-218-5p in promoting EVT migration, invasion and differentiation down the endovascular pathway and promoting spiral artery remodelling (Brkić et al., 2018). This study identified NPY and one of its receptors, NPY1R as an important part of miR-218-5p regulatory network. It is also the first study to look in-depth into the role of NPY pathway in EVT function. We showed for the first time that NPY signalling via NPY1R downregulated EVT migration and invasion. We have also reported that NPY1R plays an inhibitory role in trophoblast proliferation. In addition, we identified a novel NPY1R splice variant that retained intron 2 containing a premature stop codon. We also reported for the first time that NPY and NPY1R are present in the nuclei of EVT and CTB and that NPY1R is strongly expressed in the nuclei of fetal macrophages and decidual immune cells.

Our work suggests that NPY may play a dynamic role in placenta development, from regulating EVT function, placental immunity and placental angiogenesis, all of which are vital for healthy pregnancy outcomes. In addition, our preliminary work suggests that miR-218-5p regulates both NPY and its receptor NPY1R at multiple levels throughout their biogenesis. In the nucleus, there is strong evidence that NPY transcription is positively regulated via a miR-218-5p binding site approximately 1800 bp upstream of the NPY transcription start site, resulting in upregulated protein levels. Conversely, NPY1R shows decreased levels of intron retention following miR-218-5p transfection. Combined with a predicted miR-218-5p miRNA response element approximately 100 nt upstream of the retained intron, this could suggest that miR-218-5p is directly regulating the alternative splicing of NPY1R mRNA. The regulation of alternative splicing by miRNA is poorly understood and to our knowledge, cases of direct regulation are rare. However, the mechanism behind this regulation likely falls into two categories, either localization of splicing machinery to NPY1R mRNA via miRISC interaction with nascent NPY1R mRNA or steric hinderance of repressor proteins that negatively regulate splicing (Maniatis, 1991).

The interaction of miRNA with nuclear RNA targets is a relatively new aspect of miRNA biology. Until recently there was little evidence of miRNA function within the nucleus. This, however, is rapidly changing (O'Brien et al., 2018). MicroRNA are known to actively degrade nuclear RNA targets. On the other hand, interaction of miRNA and mRNA targets within the

nucleus have been associated with increased levels of translation once in the cytoplasm via unknown mechanisms (O'Brien et al., 2018; Rocchi et al., 2019). In the case of NPY1R we have shown that transient overexpression of miR-218-5p both decreases NPY1R mRNA levels while increasing NPY1R protein levels, in both the nucleus and cytoplasm. The addition of miR-218-5p promoting NPY1R splicing of the retained intron raises many questions about the regulatory dynamics of NPY1R. Specifically, how does increasing NPY1R splicing while decreasing total NPY1R levels lead to an increase in NPY1R protein? We think a critical aspect of this regulation is the miRNA-guided subcellular localization of mRNA (O'Brien et al., 2018). More specifically, NPY1R mRNA with nuclear-bound miR-218-5p may be more efficiently directed to sites of translation and GPCR maturation while NPY1R mRNA within the cytoplasm at large is degraded through canonical miRNA function. The exact mechanism behind the subcellular localization is unknown but may involve splicing machinery that promotes export of nuclear mRNA (Reed and Hurt, 2002) combined with miRNA-guided shuttling to the rough ER and sites of polysomes, which is typical for many miRNA:mRNA target interactions in the cytoplasm.

Regardless of the exact mechanisms behind miR-218-5p regulation of NPY1R protein expression, the culmination of seemingly mutually exclusive results is exceptionally intriguing. Moreover, the potential discovery of novel mechanisms of gene expression in the elucidation of NPY1R regulation seems likely and at the very least would add valuable insights into the study of miRNA-directed splicing and subcellular localization of target RNA. Lastly, the regulation of NPY1R expression may one day act as a model system to investigate miRNA-mediated oppositional regulation of gene expression.

Although we provided evidence that the NPY/NPY1R signalling pathway regulates trophoblast proliferation, migration, and invasion, whether or not this pathway is involved in PE development remains to be investigated. Nevertheless, studies on NPY dysregulation in PE are inconsistent. *NPY* mRNA levels were shown to be downregulated in placental tissue samples from women with PE (Dotsch et al., 1999) as well as downregulated *NPY1R*, *NPY2R* and *NPY5R* mRNA levels (Klinjampa et al., 2019). Conversely, one study showed NPY serum levels did not differ between normal and PE women (Klinjampa et al., 2019). However, other studies reported increased plasma concentration of NPY in women with PE and that NPY accumulated mainly in

the platelets (Khatun et al., 2000; Paiva et al., 2016). Therefore, it will be important that future studies determine the mRNA and protein levels of NPY and its receptors in healthy and PE samples using bigger sample sizes; also, to consider other factors such as the severity of PE, the presence of other pregnancy complications such as IUGR, fetal weight, and gestational diabetes to help clarify if and how NPY signalling is dysregulated in PE.

IV. Supplementary materials and methods:

Immunofluorescence and immunocytochemistry assay

HTR8/SVneo cells were fractionated with 0.1% NP-40 and resuspended in 1X PBS. 500 µL of resuspension containing 1.0 x 10^4 nuclei was applied to coverslips in 12-well plates and left to adhere for 15 min at room temperature. 1X PBS was slowly aspirated and nuclei were fixed to coverslips with ice cold 1:1 methanol:acetone for 5 minutes at -20 °C. Otherwise, intact HTR8/SVneo cells were seeded on top of coverslips in 12-well plate and left to adhere and grow for 48 h. Cells were then washed with ice cold 1X PBS and fixed with ice cold 1:1 methanol:acetone for 15 minutes at -20 °C. For both nuclei and intact cells, coverslips were washed three times with ice cold 1X PBS, 5 min each then blocked with cold blocking buffer (1% BSA, 1X PBS, 0.1% Tween 20) for 1 h at room temperature with gentle rocking. Blocking buffer was then removed and primary antibodies diluted in blocking buffer were applied following dilutions in **Table 4.1** and incubated overnight at 4 °C in humidified chamber. Coverslips were washed three times with ice cold 1X PBS, 5 min each and incubated with green fluorochrome-conjugated secondary antibody diluted in blocking buffer and incubated at room temperature in the dark for 1.5 h. Coverslips were washed three times with ice cold 1X PBS, 5 min each then stained with DAPI (500 ng/mL in 1X PBS) for 10 min at room temperature in the dark. Coverslips were washed again three times with ice cold 1X PBS, 5 min each, mounted on slides and stored at 4 °C until imaged using Zeiss LSM 700 confocal microscope.

Table 4.1 Immunocytochemistry antibodies and dilutions

Antibody target	Manufacturer and Catalog No.	Species	Dilution
NPY	Cell Signaling (11976S)	Rabbit	1:400
NPY1R	Abcam (ab183108), Antibodies Online (ABIN2777014)	Rabbit, Rabbit	1:500, 1:500
Alexa fluor 488 Anti-Rabbit secondary antibody	Invitrogen (A-11008)	Goat	1:2000 (N)* 1:500 (C)*

*N = nuclei, C = intact cells.

Protein extraction and immunoblot analysis

After cell were transfected and allowed to recover for 48 h, cells were lysed with HEPES lysis buffer (10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.0 mM DTT, 0.1% NP-40, pH 7.9) supplemented with Pierce protease and phosphatase inhibitors (Thermo Scientific, Mississauga, ON, Canada). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to a 0.2 µm polyvinylidene difluoride membrane (EMD Millipore, Oakville, ON, Canada) overnight at 4 °C. In Dot blot analysis, cell lysate was added directly into o.2 µm nitrocellulose membrane (GE Healthcare Life Sciences, Ottawa, ON, Canada) at 1.0 × 10^4 cells/ 3µL dot. Membranes were blocked in blocking buffer (5% skim milk in 1X Tris-buffered Saline and Tween-20, TBST) for 1.5 h at room temperature. Membranes were then incubated overnight at 4 °C with the primary antibodies diluted in blocking buffer (**Table 4.2**). Membranes were then washed with 1X TBST buffer and probed with horseradish peroxidase-conjugated secondary antibodies diluted in blocking buffer at room temperature for 1.5 h. Signals were detected on X-ray film using Clarity™ Western ECL substrate kit (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada).

Table 4.2 Western and dot blot analysis antibodies

Antibody target	Manufacturer and Catalog No.	Species	Dilution
NPY	Cell Signaling (11976S)	Rabbit	1:1000
NPY1R	Abcam (ab183108) Antibodies Online (ABIN2777014) R&D Systems (MAB6400-SP)	Rabbit, Rabbit, Mouse	1:1000, 1:500 1:1000
p-SRC (tyrosine 416)	Cell Signaling (6943S)	Rabbit	1:1000
Total-SRC	Cell Signaling (2109S)	Rabbit	1:1000
p-CREB (serine 133)	Cell Signaling (9196S)	Mouse	1:500
Total-CREB	Cell Signaling (9104S)	Mouse	1:500
B-Actin	Santa Cruz (SC81178), Cell Signaling (3700S)	Mouse, Mouse	1:2000, 1:1000
Lamin B	Santa Cruz (SC6216)	Goat	1:1000
GAPDH	Santa Cruz (SC365062)	Mouse	1:5000
Histon H3	Abcam (ab1791)	Rabbit	1:10000
PDI	Cell Signaling (3501S)	Rabbit	1:1000
Anti-Rabbit secondary	Cell Signaling (7074S)	Goat	1:4000
Anti-Mouse secondary	Cell Signaling (7076S)	Horse	1:4000
Anti-Goat secondary	Santa Cruz (SC2020)	Donkey	1:4000

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Appendices

Appendix A: Publications

1. **Overexpression of miR-210-3p Impairs Extravillous Trophoblast Functions Associated with Uterine Spiral Artery Remodeling.**
   Hayder H, Fu G, Nadeem L, O'Brien JA, Lye SJ, Peng C. International Journal of Molecular Sciences. 2021. 22(8):3961. doi.org/10.3390/ijms22083961

2. **Multiplexed and single-cell detection of microRNA with plasmonic nanoparticle assemblies.**
   Ghotra G, Le N, **Hayder H**, Peng C, Chen J. Canadian Journal of Chemistry. 2021. 99:585-593. doi.org/10.1139/cjc-2021-0023

3. **Overview of MicroRNA biogenesis, mechanisms of actions, and circulation.**
   O'Brien J, **Hayder H**, Zayed Y, Peng C. Frontiers Endocrinology (Lausanne). 2018. 3:9:402. doi: 10.3389/fendo.2018.00402

4. **MicroRNAs: crucial regulators of placental development.**
   Hayder H, O'Brien J, Nadeem U, Peng C. Reproduction. 2018. 155(6):R259-R271. doi: 10.1530/REP-17-0603

5. **Accurate MicroRNA Analysis in Crude Cell Lysate by Capillary Electrophoresis-Based Hybridization Assay in Comparison with Quantitative Reverse Transcription-Polymerase Chain Reaction.**
   Hu L, Stasheuski AS, Wegman DW, Wu N, Yang BB, **Hayder H**, Peng C, Liu SK, Yousef GM, Krylov SN. Analytical Chemistry. 2017. 18;89(8):4743-4748. doi: 10.1021/acs.analchem.7b00707

6. **Automated Quantification and Analysis of Cell Counting Procedures Using ImageJ Plugins.**
   O'Brien J, **Hayder H**, Peng C. Journal of Visualized Experiments. 2016. 17;(117). doi: 10.3791/54719

7. **MicroRNAs in Human Placental Development and Pregnancy Complications.**
   Fu G, Brkić J, **Hayder H**, Peng C. International Journal of Molecular Sciences. 2013. 8;14(3):5519-44. doi: 10.3390/ijms14035519

Appendix B: Extended protocols

Tube Formation Assay:

Cell Culture Media:

HUVEC:

M199 supplemented with 10% non-heated FBS and streptomycin (100 µg/mL) and penicillin (100 IU/mL) and freshly added 0.05 mg/mL Endothelial Cell Growth Supplement (ECGS) (prepared with heparin).

Preparation of ECGS (Corning Catalog # 356006) stock (5 mg/mL):

1. Dissolve 100 mg ECGS (supplied by company) and 100 mg Heparin Sulfate Salt (supplied by user) in 20 mL of serum-free M199 media and mix well.
2. Filter the solution with a 0.22 µm pore size syringe filter.
3. Store in 200 µL aliquots in -80 °C. Do not refreeze thawed aliquots.
4. Use 100 µL of the 5 mg/mL solution per 10 mL of media.

HTR8/SVneo:

RPMI 1640 supplemented with 10% non-heated FBS

Using a single cell line:

Day 1

- Thaw the desired amount of growth factors-reduced Matrigel overnight on ice at 4 °C (you need 50 µL per well or 30 µL per well if using the IncuCyte); thus, the total volume of Matrigel needed depends on the number of samples and replicates for each plus an extra 100 µL.

Day 2

- Chill in the fridge a flat-bottom 96-well plate and a box of P200 tips for at least 30 min.

- Inside the cell culture hood and while keeping the Matrigel on ice, add 50 or 30 µL of Matrigel at a 45° angle while ejecting at a slow and steady rate into the top edge of the well, making sure the Matrigel covers the whole bottom before flattening the plate.
- Once all the wells are done, add 100 µL of 1X PBS into the wells surrounding the wells with the Matrigel for hydration.
- Move the plate into the 37 °C cell culture Incubator and leave for 30 min to allow the Matrigel to polymerize. Meanwhile, start preparing cells for the experiment.
- If using the IncuCyte, stain the cells with 1µM CellTracker™ dye (Invitrogen, Life Technologies) by incubating for 30-45 min. Then remove and replace with fresh medium.
- Remove culturing medium from the cell plate(s).
- Wash the cells with 1X PBS.
- Add 1 mL of Accutase® and ensure the entire surface is covered.
- Put the plate back into the incubator for 3-5 min.
- View the plate under a microscope to verify cells have detached, tap the plate gently to dislodge the cells from the surface of the plate.
- Add 3 mL of warm medium to the plate and transfer the detached cells to a sterile conical tube.
- Centrifuge the cells at 1500 rpm for 5 minutes until the cells are pelleted.
- Remove the supernatant from the tube, being careful not to dislodge the cell pellet.
- Resuspend the cells in a fresh 10% medium.
- Count the cells.
- Make a cell mixture at the desired concentration of cell/mL at the volume needed for the number of desired replicates in 10% serum-containing medium.
- Add treatment, if any, into the cell mixtures.
- Mix cells well.
- Add 100 µL of each sample into their corresponding well, releasing the cells slowly into the center of the well, making sure the pipette is in a vertical position.

- Once all samples are done, leave the cells to settle to the bottom of the well for 5 to 10 min.
- Put the plate back into the incubator for 20 h or into the IncuCyte (image at 4X every 2 h in both green fluorescence and phase contrast is recommended).

Day 3:

- If fluorescent staining is not needed, wait until the incubation time is done and image each well with the 2X lens using phase contrast.
- Discard cells/plate into the biohazard waste upon completion.

OR

- If fluorescent staining is desired, then 30 min before the imagining time, prepare 1:1000 dilution of the Calcein® stock (2 mM) in a warm serum-free medium.
- Add 100 µL of the prepared solution to each well on top of the media already in the wells for a final Calcein® dilution of 1:2000 and stain for 20 min.
- Image the plate with the 2X lens using the blue filter.
- Discard cells/plate into the biohazard waste upon completion.
- Quantify the total network length using ImageJ NeuronJ plugin.

OR

- View images taken by IncuCyte, retrieve images and quantify the total network length using ImageJ NeuronJ plugin.

Using co-culture assay (two cell lines):

Day 1

- Thaw the desired amount of growth factors-reduced Matrigel overnight on ice at 4 °C (you need 50 µL per well or 30 µL per well if using the IncuCyte); thus, the total volume of Matrigel needed depends on the number of samples and replicates for each plus an extra 100 µL.
    - Make sure to have separate plates of the cell lines to be used than stock plates. The cells will be stained with live tracker dye; thus, they cannot be used to maintain the cell line.

Day 2

- Chill in the fridge a flat-bottom 96-well plate and a box of P200 tips for at least 30 min.
- Stain the cells with 1µM CellTracker™Red CMTPX (Invitrogen, Life Technologies) or CellTracker™Green CMFDA (Invitrogen, Life Technologies),

  - Add 2 µL of the stock red dye into a warm 10 mL serum-free media and add to the "Red" cells plate. Incubate for 30-45 min. Then remove and replace with fresh media.

  - Add 1.2 µL of the stock green dye into a warm 10 mL serum-free media and add to the "Green" cells plate. Incubate for 30-45 min. Then remove and replace with fresh media.
- Inside the cell culture hood and while keeping the Matrigel on ice, add 50 or 30 µL of Matrigel at a 45° angle while ejecting at a slow and steady rate into the top edge of the well, making sure the Matrigel covers the whole bottom before flattening the plate.
- Once all the wells are done, add 100 µL of 1X PBS into the wells surrounding the wells with the Matrigel for hydration.
- Move the plate into the 37 °C cell culture Incubator and leave for 30 min to allow the Matrigel to polymerize. Meanwhile, start preparing cells for the experiment.
- Remove culturing medium from the cell plate(s).
- Wash the cells with 1X PBS.
- Add 1 mL of Accutase® and ensure the entire surface is covered.
- Put the plate back into the incubator for 3-5 min.
- View the plate under a microscope to verify cells have detached, tap the plate gently to dislodge the cells from the surface of the plate.
- Add 3 mL of warm medium to the plate and transfer the detached cells to a sterile conical tube.
- Centrifuge the cells at 1500 rpm for 5 minutes until the cells are pelleted.
- Remove the supernatant from the tube, being careful not to dislodge the cell pellet.
- Resuspend the cells in a fresh 10% medium.
- Count the cells.
- Make a cell mixture at the desired concentration of cell/mL at the volume needed for the number of desired replicates in a 10% serum-containing medium.

- Mix the cells in a one-to-one ratio for a total of 25,000 cells in 100 µL per well.
- Add treatment, if any, into the cell mixtures.
- Mix cells well.
- Add 100 µL of each sample into their corresponding well, releasing the cells slowly into the center of the well, making sure the pipette is in a vertical position.
- Once all samples are done, leave the cells to settle to the bottom of the well for 5 to 10 min.
- Put the plate back into the incubator for 20 h or into the IncuCyte (image at 4X every 2 h in green and red fluorescence and in phase contrast is recommended).

Day 3:

- Once the incubation time is done, image each well with the 2X lens using the green and blue filters without moving the plate between filter changes.
- Discard cells/plate into the biohazard waste upon completion.
- Quantify the total network length using ImageJ NeuronJ plugin.

OR

- View images taken by IncuCyte, retrieve images and quantify the total network length using ImageJ NeuronJ plugin.