The role of microRNAs that target the renin-angiotensin system in placental development and function

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Declaration

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I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

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By signing below, I confirm that Anya Arthurs contributed to the papers and publications included in this thesis. Their contribution is outlined within individual chapters.

Signed: Kirsty G Pringle

Statements of contribution

By signing below, I confirm that Anya Arthurs contributed upward of 50% towards data collection/analysis and manuscript preparation for all the publications included in this thesis for which I am a co-author.

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Abbreviations

ACE	Angiotensin converting enzyme
Ago2	Argonaute 2
AGT	Angiotensinogen
Ang	Angiotensin
ANGPT	Angiopoietin
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AT_XR	Angiotensin II Type x Receptor
AVP	Arginine vasopressin
BMI	Body mass index
C14MC	Chromosome 14 cluster
C19MC	Chromosome 19 cluster
CTB	Cytotrophoblast
CVE	Chorionic villus explant
CYR61	Cysteine-rich protein 61
DMEM	Dulbecco's modified eagle media
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-related kinase
EV	Extracellular vesicle

EVT	Extravillous trophoblast
g-ASCF	Glycerol substituted artificial cerebrospinal fluid
hCG	Human chorionic gonadotrophin
hCS	Human chorionic somatomammotrophin
HIF	Hypoxia-inducible factor
HPL	Human placental lactogen
HRP	Horseradish peroxidase
IGF	Insulin-like growth factor
IUGR	Intrauterine Growth Restriction
LNA	Locked nucleic acid
MAPK	Mitogen activated protein kinase
miRNA	microRNA
mRNA	Messenger RNA
nc-RNA	Non-coding RNA
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
p85α-PI3K	p85α-phosphoinositol 3-kinase
PAI	Plasminogen activator inhibitor
pc	Post-coital
PCR	Polymerised chain reaction
PE	Preeclampsia
PLAP	Placental alkaline phosphotase
PPAR-γ	Peroxisome proliferator-activated receptor
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
(P)RR	(Pro)renin receptor

PTB	Preterm birth
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
RAS	Renin-angiotensin system
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
SEM	Standard error of the mean
SGA	Small for Gestational Age
STB	Syncytiotrophoblast
TBS	Tris buffered saline
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
UTR	Untranslated region
V-ATPase	Vacuolar-ATPase
VEGF	Vascular endothelial growth factor

Abstract

This thesis explores the role of microRNAs (miRNAs) that target the placental renin-angiotensin system (RAS) in placental development and function. The placental RAS contributes to trophoblast proliferation, migration and invasion, as well as vasoconstriction and angiogenesis. As such, the placental RAS is essential for adequate placental development and function. miRNAs targeting RAS mRNAs are present in the developing placenta, and their expression varies across gestation, along with RAS expression. Furthermore, changes in expression of these miRNAs are seen in pregnancy complications. This research investigated:

- the effects of low oxygen tension, such as the low oxygen environment of the first trimester placenta, on expression of miRNAs targeting the placental RAS
- the effect of miRNAs targeting the placental RAS on the functional ability of trophoblasts to proliferate, and
- the *in vitro* and *in vivo* roles of miR-155 in the placenta.

I found that in HTR-8/SVneo cells (an immortalised human extravillous trophoblast cell line) cultured in low oxygen tension (~1%), there was suppressed expression of ten miRNAs predicted to target the RAS. Furthermore, this allowed increased expression of two critical RAS components. This would suggest that a low oxygen environment encourages placentation by suppressing miRNA expression to allow RAS activity. These experiments were then repeated in first trimester chorionic villus explants (CVE) obtained from primary tissue, where the expression of only four of the tested miRNAs was suppressed by low oxygen tension. The RAS components that were increased in this low oxygen milieu were also different to those increased in the cell line experiments. These experiments taken together illustrate the differing roles of miRNAs and the RAS in the extravillous trophoblasts and the chorionic villi.

Investigation into the effect of miRNAs predicted to target the RAS on trophoblast proliferation showed that treatment with mimics of these miRNAs suppressed, or completely inhibited, trophoblast proliferation. In the case of many miRNAs tested, a dose-dependent response was observed, with higher mimic concentrations leading to lower proliferation of the cells. The effect of these miRNAs on their intended RAS targets was also assessed, with 7 of the 9 miRNAs suppressing mRNA expression of their RAS targets. These experiments demonstrated the functional effect of miRNA dysregulation of placental development through trophoblast proliferation. This has particular implications for a number of pregnancy complications that arise from poor placental development such as fetal growth restriction and preeclampsia.

Finally, the role of miR-155 in placentation was observed, as a miR-155$^{-/-}$ mouse model revealed the consequences of miR-155 deletion. Placentae from miR-155$^{-/-}$ dams had significantly larger labyrinthine zones (responsible for substrate transfer to the fetus), but no change in placental weight, indicating an increase in the labyrinth zone to placental area ratio that would suggest that the placenta has improved efficiency of substrate transfer. These dams also had larger fetuses, possibly as a consequence of the changes in placental morphology. As mIR-155 is known to target the angiotensin II type 1 receptor (AT_1R), mRNA and protein was measured and was significantly increased in miR-155$^{-/-}$ placentae. Additionally, *in vitro* investigation into the functional effects of miR-155, utilising a miR-155 mimic, showed that treatment with this miRNA decreases trophoblast proliferation in a dose-dependent manner. Altogether, this study clarified the importance of appropriate miR-155 regulation during pregnancy. This was particularly important as miR-155 upregulation is seen in preeclampsia (PE), a dangerous pregnancy complication.

Therefore, through utilising a cell line, primary tissue explants and a murine model, I have been the first to demonstrate the effects of various miRNAs targeting the placental RAS on

RAS expression, both in and out of a low oxygen milieu, trophoblast proliferation, placental morphology and fetal growth.

1. Introduction

Placentation is an integral part of healthy pregnancy. As the organ of exchange between mother and fetus, the human placenta must develop so that it provides the fetus with nutrients and oxygen, and removes the products of fetal metabolism. Thus, the placenta is responsible for an optimum environment for fetal growth and development. Babies that are born small for gestation age (SGA) or with intrauterine growth restriction (IUGR) and/or due to pregnancy complications such as preeclampsia (PE) and preterm birth (PTB), frequently experience inadequate placental function. As a result, these babies are often predisposed to developing numerous chronic diseases later in life [2].

The human placental renin-angiotensin system (RAS) contributes to trophoblast proliferation, migration, and angiogenesis in the early developing placenta [3-7]. The major morphological development of the human placenta occurs in the first trimester of gestation, when blood supply to the developing placenta is restricted and the oxygen tension is low [8]. Expression of many RAS mRNAs and proteins is enhanced by this hypoxic milieu [9], and their expression decreases with advancing gestation [3]. One mechanism that is postulated to regulate the expression of RAS mRNAs are post-transcriptional factors [10], such as microRNAs (miRNAs).

miRNAs have been shown to alter the expression of placental genes, including placental RAS mRNAs [11]. The expression of miRNAs that have RAS mRNAs as potential targets has also been shown to be lowest in the first trimester and increase across gestation [12], which might account in part for the high expression of their target RAS mRNAs in early gestation [3]. Furthermore, placental miRNA expression profiles are different in women with PE compared with women with uncomplicated pregnancies [12, 13]. Whilst the developmental and

pathophysiological role of miRNAs targeting the RAS has been investigated in other organs, such as the kidney [14, 15], the role of miRNAs targeting placental RAS mRNAs has not been studied and so it forms the basis of this thesis.

1.1 The Human Placenta

The placenta is a transient organ formed during pregnancy. It typically weighs 300-500g and is 14-17.5 cm in diameter. The placenta has embryonic and maternal components, and connects the developing fetus to the mother's uterine wall. It detaches from the uterus at birth. The placenta plays a critical role in waste and gas exchange between the fetus and the mother during pregnancy and provides nutrients to the fetal circulation as required. The placenta also secretes hormones to maintain pregnancy and functions as a partial barrier to infection. However, certain viruses, drugs and alcohol, can cross into the placenta and result in birth defects (teratogens).

1.2 Placentation

Placentation (the process of placental development) occurs early in the first trimester of pregnancy. A fertilised ovum goes through a number of cell divisions in the fallopian tube on its way to the uterus. At the point where the fertilised ovum contains ~100 cells and a cavity, it is termed the blastocyst. The developing blastocyst obtains its nutrition from uterine endometrial secretions for 1 to 3 days before implantation in the uterus. Implantation occurs when trophoblast cells over the surface of the blastocyst burrows into the uterine wall. Once this has occurred trophoblasts and other adjacent cells proliferate to form the placenta and fetal membranes. The ovarian corpus luteum, driven by human chorionic gonadotrophin (hCG), secretes progesterone to maintain the pregnancy and promote swelling of the endometrial cells to store nutrients. These nutrients are used by the embryo for development

and growth as the trophoblast cells invade the decidua, which arises from the process of decidualisation. This process involves a dramatic morphological and functional differentiation of the endometrial cells, which is essential for healthy pregnancy.

1.2.1 Placental Structure

Once formed, the placenta is composed of a fetal portion, derived from the chorionic sac, and a maternal portion, derived from the endometrium. The fetal side is referred to as the chorionic plate in the mature placenta and contains the villi. These closely packed villous structures are highly vascularised, containing branches from the umbilical blood vessels and their capillary networks. The chorionic villi project into the intervillous space where they are bathed in maternal blood. In the terminal regions of these villi, the majority of fetal-maternal exchange occurs. The maternal side encompasses the decidua basalis. Maternal blood moves from the spiral arteries in the decidua basalis into the intervillous space. Endometrial veins then drain this space [16] (Figure 1.1).

1.2.2 Placental cell types

The placenta consists mainly of trophoblasts, which arise from the outer trophectoderm layer of the blastocyst. Trophoblasts, in three subtypes: cytotrophoblasts (CTBs), extravillous trophoblasts (EVTs) and the syncytiotrophoblast (STB), go on to form the major part of the placenta [17]. The cellular activities of these placental cells, such as proliferation,

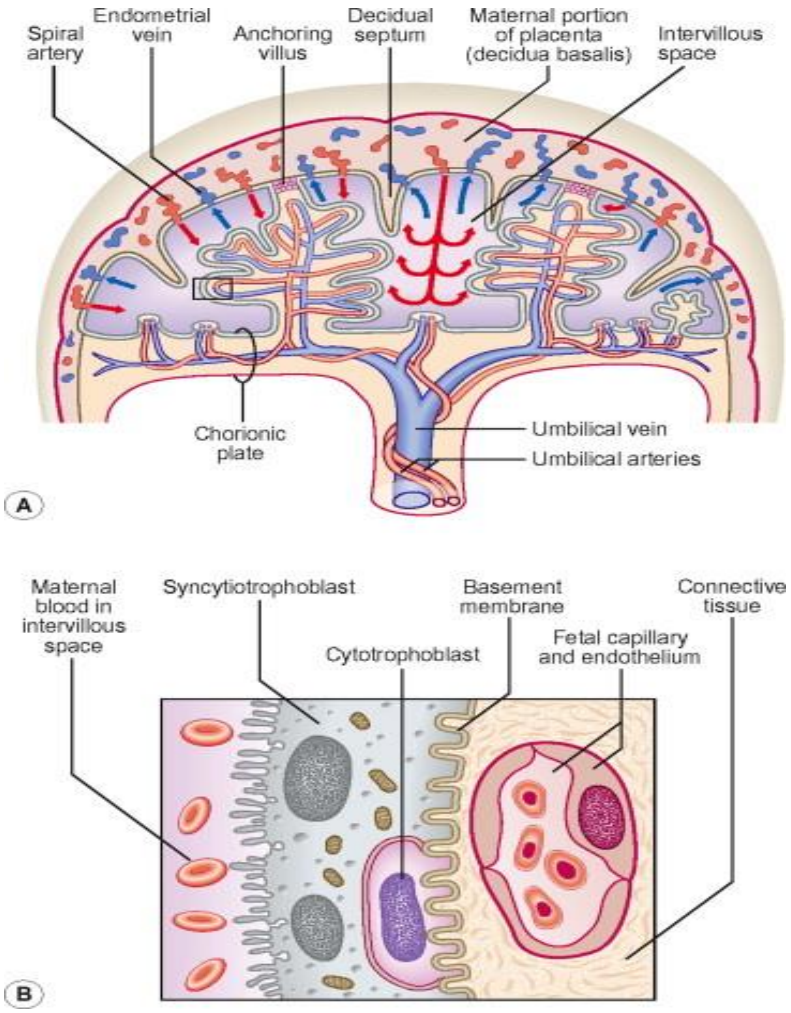

Figure 1.1: Structure and cell types of the placenta.

A The placenta is composed of a maternal portion (decidua basalis) which contains the endometrial veins and spiral arteries to distribute and receive blood flow, and the chorionic plate which contains the umbilical vein and arteries to transport blood to and from the fetus. Between these two segments is the intervillous space, where the chorionic villi are bathed in the maternal blood, facilitating nutrient, gas and waste exchange. **B** This close up diagram illustrates the exchange surface of the chorionic villi. The intervillous space is filled with maternal blood that surrounds the villi. The syncytiotrophoblast lines the perimeter of the villi and facilitates the exchange of nutrients and wastes between the maternal and fetal circulations. Cytotrophoblasts lie directly beneath the syncytiotrophoblast and readily fuse to form the syncytiotrophoblast. A basement membrane separates this from the connective tissue containing fetal capillaries and the endothelium within the villi. Sourced from [1].

differentiation, invasion, as well as promoting cell differentiation, decidualisation and angiogenesis, are pivotal for a healthy pregnancy [18]. A number of other cell types, such as fibroblasts, endothelial cells and Hofbauer cells, are also found in the placenta, but will not be explored in this review.

Villous cytotrophoblasts

CTBs are considered the 'stem cell' for the placenta as they continuously proliferate and can differentiate into the EVTs or continuously fuse to form and maintain the STB. The chorionic villi are composed of CTBs with a layer of STB around the perimeter that allow gas exchange and facilitate nutrient transfer.

The syncytiotrophoblast

The STB forms as a result of the specialised fusion of CTBs, to become a large continuous multinucleated cell layer that covers the entire surface of the chorionic villi and is in direct contact with maternal blood [19]. The STB not only provides a structural and biochemical barrier between the mother and fetus during pregnancy, but it also functions as an endocrine system that produces multiple growth factors and hormones. This includes hormones such as hCG for the maintenance of pregnancy, and others responsible for fetal growth and development [20, 21]. The STB also plays a role in immune tolerance. It undergoes continuous renewal as it is able to shed aged nuclei and fuse with fresh CTBs to replenish cellular organelles and nutrients. For this reason, the STB has minimal transcriptional activity of its own [22].

There are a number of known factors involved in syncytialisation, including cell junction proteins (Connexin-43, E-cadherin), growth factors (epidermal growth factor (EGF), vascular

endothelial growth factor (VEGF)), membrane proteins (syncytin 1 and 2), kinases (p3-mitogen activated protein kinase (MAPK)), hormones (hCG) and enzymes, such as furin. Furin is pro-protein convertase that can proteolytically activate VEGF (as well as insulin-like growth factor 1 and receptor) and which promotes trophoblast syncytialisation [23, 24].

The STB can experience oxidative stress, often contributed to by mitochondrial dysfunction, drugs, metabolic disorders, paternal genotype and maternal diet. Oxidative stress leads to degradation of the STB, which can result in early termination of pregnancy. Chronic oxidative stress can be induced by poor placental perfusion and maladaptation of the mitochondria. Initiation of antioxidant enzymes can resolve oxidative stress in some cases, allowing the pregnancy to continue [25].

Extravillous trophoblasts

CTB cells at the tips of anchoring villi proliferate and break through the overlying syncytium, to form cell columns. From these cell columns, CTBs differentiate to form EVTs, which have an important role in invading and remodelling the maternal spiral arterioles, where they replace the endothelial lining and musculoelastic tissue. EVTs invade deep into the uterine wall (as far as the first third of the myometrium) and are directly implicated in the anchoring of chorionic villi in the uterus. This process of invasion and vascular remodelling is essential in early development and is coupled with the EVTs plugging the spiral arteries. This results in a low oxygen environment within the placenta as the spiral arteries are remodelled. In this process, the loss of elasticity and increased luminal diameter of the spiral arteries decreases resistance in these vessels, allowing increased maternal blood flow in the second trimester [26]. Fetal genotype, the maternal immune system and the endometrial environment all influence extravillous trophoblast invasion of the endometrium [27]. At the time when arteries are

unplugged and maternal circulation enters the intervillous space, there is a rise in intraplacental oxygen tension [25].

Oxygen therefore plays a pivotal role in placentation, as the oxygen tension within the placenta changes during pregnancy. This allows increased placental development early in gestation to provide maximal substrate transfer to the growing fetus.

1.2.3 Oxygen tension

During the first trimester of pregnancy, the placenta develops in a low oxygen environment. Low oxygen in the developing placenta stimulates endothelial cell proliferation and increases the number of capillary loops and the terminal villi. This increases the surface area available for contact with the maternal blood, thus maximising oxygen and nutrient transfer to allow for optimal fetal development [28].

It has been proposed that an oxygen concentration gradient, established between the lumen of the uterus (~3% O_2, 23 mmHg) and the myometrium (~12% O_2, 91 mmHg), drives EVT migration from the anchoring villi of the placenta into the decidua and myometrium. The intraluminal EVT cells occlude the spiral arteries. This action limits the passage of maternal blood into the placenta [29], maintaining a low oxygen environment that is necessary for normal early placental and fetal development. This low oxygen milieu allows expression of pro-proliferative genes, including those of the RAS (Section 1.4), to promote rapid placentation [9]. Vascular smooth muscle cells migrate out of the vessels and/or undergo apoptosis. They are then replaced by EVTs and there is fibrin deposition. Approximately 100-150 spiral arteries are converted from high resistance low flow vessels to high flow low resistance vessels with a diameter of ~2mm [30].

In low oxygen/hypoxic conditions, Hypoxia Inducible Factor 1α (HIF-1α) is stabilised, leading to the production of VEGF. This results in angiogenesis and vascularisation. Towards the end of the first trimester the onset of low resistance, high capacity blood flow to the placenta is achieved by the dislodging of the intraluminal EVT plugs. Following this, the placental intervillous space is perfused with maternal blood, establishing effective materno-fetal exchange [31].

It has been proposed that inadequate invasion by EVTs into the myometrium is associated with an inability to remodel uterine spiral arteries. Aberrant spiral artery remodelling has been implicated in the subsequent development of pregnancy complications, including PE, compromised fetal growth and spontaneous PTB. Pregnancy complications such as PE and IUGR may be consequences of incomplete spiral arteriolar blockage during the first trimester. This results in a change from low resistance, high blood flow to a high resistance turbular flow, which is damaging to the villous structure. The retention of smooth muscle in the spiral arteries also increases the risk of spontaneous vasoconstriction and ischaemia–reperfusion injury, generating oxidative stress (see Section in 1.3.3 Oxidative stress) [32]. This incomplete spiral artery remodelling maintains a low oxygen environment into the second and third trimesters, which negatively affects fetal development as increases in maternal blood occur too early, before these processes are complete [33].

1.2.4 Hormones

A number of hormones are required to maintain a healthy pregnancy. hCG is synthesised by the STB and stimulates CTB proliferation and differentiation [34]. Synthesis of hCG begins before implantation, and production peaks at approximately 10-12 weeks of pregnancy before declining, however it remains elevated for the duration of pregnancy [35]. hCG is also detectable in early pregnancy tests. It is responsible for maintaining the maternal corpus

luteum, which secretes estrogen and progesterone [36], as well as preventing menstruation [35]. However, by the end of the first trimester, the placenta is able to source enough of these steroids and the corpus luteum is no longer required [37].

Estrogens are formed *de novo* in the adrenal glands of the mother and the fetus from androgenic steroid compounds. These weak androgen compounds are then transported in the blood to the placenta where the trophoblasts convert them to estradiol, estriol and estrone [35]. These estrogens then stimulate CTB proliferation and differentiation [34]. High levels of estrogens work to enlarge the mother's uterus, as well as the breasts and their ductal structure [35]. Estrogens also work with progesterone during the latter half of pregnancy to block the effects of prolactin, inhibiting lactation during pregnancy [38]. Toward the end of pregnancy, estrogens relax the sacroiliac joints and pubic symphysis to ease the movement of the baby through the birth canal [35].

Progesterone is produced independently by the placenta from cholesterol precursors, and almost all progesterone produced by the placenta enters the maternal circulation. Progesterone serves many important functions, some of which occur prior to implantation. Progesterone is implicated in the preparation and maintenance of the endometrium to allow implantation [37]. It plays a role in development of the decidual cells in the uterine endometrium [35] and suppression of maternal immunologic responses to fetal antigens, preventing the rejection of the trophoblast [37]. Progesterone is also responsible for inhibiting myometrial contractions that may lead to spontaneous labour, and for preparing the breasts for lactation along with estrogens [35].

Human placental lactogen (HPL), also known as chorionic somatomammotropin (hCS), begins being secreted by the placenta approximately five weeks into pregnancy, and secretion increases proportionally with growth of the placental mass. The exact function of hCS is unknown, although it is secreted in volumes many times greater than all the other pregnancy

hormones combined [35]. hCS is known to enhance fetal growth by increasing protein synthesis [39], as well as having weak actions similar to human growth hormone [35]. hCS also serves important metabolic functions as it decreases insulin sensitivity and glucose utilisation and increases fatty acid use for ATP production in the mother [39]. It also prepares the mammary glands for lactation after parturition [35].

Other hormones that play important roles in pregnancy include glucocorticoids and aldosterone, thyroid hormones such as thyroxin and human chorionic thyrotropin, as well as parathyroid hormone and relaxin. All of these hormones, coupled with adequate placental development are required for healthy pregnancy. Without this, pregnancy complications can arise.

1.3 Pregnancy Complications

Placental insufficiency is a consequence of inadequate placentation, and contributes to pregnancy complications including PE, IUGR, PTB and spontaneous abortion [40]. IUGR, PE and PTB are all dangerous complications of pregnancy that contribute to infant morbidity and mortality.

1.3.1 Intrauterine Growth Restriction

IUGR refers to a condition in which a fetus is unable to achieve its genetically determined potential size, and is associated with increased perinatal morbidity and mortality [41]. IUGR is most commonly caused by placental dysfunction leading to inadequate fetal nutrition and hypoxia and potentially intrauterine death. Additionally, babies who have IUGR are at risk of

developing chronic diseases such as hypertension, diabetes mellitus and cardiovascular disease in later life, and have increased risk of preterm birth [42].

1.3.2 Preterm Birth (PTB)

PTB is defined as birth prior to 37 weeks of gestation, and occurs in ~11% of live births. More than 50% of infant mortality cases across the world are attributed to this [43]. Should premature infants survive the immediate postnatal period, their risk of developing short- and long-term health problems is significantly increased [44-48], due to immaturity of organs as well as complications from inflammation and oxidative stress [49-52].

1.3.3 Preeclampsia

PE is a pregnancy disorder that affects ~5-8% of pregnancies worldwide. PE is a major cause of maternal and neonatal morbidity and mortality [53-56]. Stillbirth is more common in PE pregnancies, and one third of infants born from PE pregnancies are growth restricted [57, 58]. Preterm delivery also occurs twice as often as for normotensive pregnancies [58]. It is well established in the literature that an adverse intrauterine environment is highly correlated with chronic disease development in later life [2, 56, 59].

Clinical risk factors for PE, as stated by the Royal Australian and New Zealand College of Obstetricians and Gynecologists, include having a previous PE pregnancy, primigravidity, BMI of over 35 during pregnancy, chronic hypertension or pre-existing diabetes [60]. Symptoms of PE include a rapid onset of proteinuria, as well as an increase in blood pressure and swelling of the hands, feet and face [61]. This is due to increased vascular permeability in the glomeruli and peripheral vessels. If untreated, PE can progress to eclampsia, which is characterised by seizures. PE often presents in the third trimester of pregnancy and worsens over time [54, 55].

PE prevention and treatment is limited due to the potential for adverse drug effects on the fetus. Patients can take aspirin, magnesium sulphate and calcium supplements, as well as antihypertensives; namely, methyldopa and labetalol however these are of limited benefit [57].

Although the etiology and pathogenesis of PE are still unknown, the placenta causes the clinical symptoms of PE which usually resolve with its removal [62, 63]. The pathogenesis of PE can be divided into two stages. The first stage is not associated with any clinical symptoms; at this stage cytotrophoblastic endothelialisation is impaired and there is inadequate spiral artery invasion. Consequently, there is poor placentation and the placenta is ischemic and hypoxic [54]. Physiologically, improper invasion and plugging of the spiral arterioles are observed in PE [64], and there is a premature increase in maternal blood flow into the placenta, which results in a premature increase in oxygen tension. Additionally, insufficient plugging of the maternal arteries is believed to lead to episodes of ischemia, followed by reperfusion, generating free radicals and oxidative stress [33]. It has been shown that in women with PE, reduced cytotrophoblast differentiation into EVTs results in impaired maternal vascular invasion [65]. Insufficient spiral arteriole remodelling leads to episodes of ischemia and reperfusion. Endothelial damage, resulting in microvascular dysfunction, is a consequence of the resulting chronic hypoxia [56].

The second stage is characterised by the appearance of abnormal placental factors in the maternal circulation and altered maternal physiology. The placenta releases anti-angiogenic factors (including prostaglandins, cytokines, soluble endoglin and sFlt-1, acting in an anti-angiogenic capacity) into the maternal circulation. This results in dysregulation of pro-angiogenic factor production (including VEGF, transforming growth factor-β (TGF-β), insulin-like growth factor 1 and placental growth factor) [66]. Thus, levels of anti-angiogenic factors are shown to be increased, and pro-angiogenic factors decreased in women with PE [67-72]. These alterations lead to inflammatory responses and systemic endothelial dysfunction [73].

PE prevention is fairly limited because symptoms do not appear until late in gestation, after the initial impairment in placental development. A viable biomarker present in early gestation is necessary for early detection of PE as any therapy would need to begin before week 16 of gestation [25], i.e. early treatment is essential to prevent onset of clinical symptoms [65, 74]. Co-peptin is a suggested biomarker for PE. Co-peptin is the peptide pro-segment of the arginine vasopressin (AVP) gene, which is secreted in an equimolar ratio to AVP. The short biological half-life of AVP makes it inappropriate as a clinical biomarker, but its function and secretion are able to be measured from the quantity of co-peptin present in a sample. The improved kinetic qualities of copeptin make it a viable biomarker, and studies are now investigating its potential [75].

PE detection and early intervention is important for a number of reasons; it is particularly important for preserving placental vasculature and promoting development. Early resolution of the disease could prevent a large amount of damage to the placenta. The majority of this damage arises from oxidative stress.

Oxidative Stress

The term oxidative stress is used to describe a disruption in the balance between the production of damaging reactive oxygen species (also known as free radicals) and antioxidant defences [76]. Oxidative stress damages trophoblasts, triggering the detachment and release of cell fragments and extracellular vesicles into the maternal circulation [77]. These extracellular vesicles can be carried into the maternal circulation, resulting in maternal endothelial dysfunction [56].

Abnormal placental development, and poor uterine and placental perfusion, can lead to oxidative stress and the release of an excess of reactive oxygen species [78]. An indicator of

endothelial dysfunction in this situation is the impaired bioavailability and synthesis of nitric oxide, which acts as a vasodilator and regulates angiogenesis [56]. Oxidative stress in the placenta can also be caused by the endocrine disruptor group, phthalates. These molecules can also induce oxidative stress-responsive miRNA upregulation, and high levels of these miRNAs have been found in maternal urine samples in PTB, low birth weight and spontaneous abortions (see more in Section 1.5) [78].

Disruptions to, and abnormal development of the placenta is associated with pregnancy complications, as mentioned above. Adequate development of the placenta is essential for optimal growth of the baby. Much of the physiological and pathophysiological development of the placenta can be attributed to the actions of the RAS.

1.4 The Renin-Angiotensin System

The circulating RAS maintains blood pressure, blood volume and salt and water balance. This system is able to increase sympathetic activity, tubular Na^+/Cl^- reabsorption and K^+ excretion, aldosterone secretion, cause arteriolar vasoconstriction and anti-diuretic hormone secretion to retain salt water and to control blood pressure. However, there are also tissue RASs which have important functions in physiological as well as pathological, tissue growth, invasion and angiogenesis [40, 79]. A tissue RAS exists the placenta (Figure 1.2).

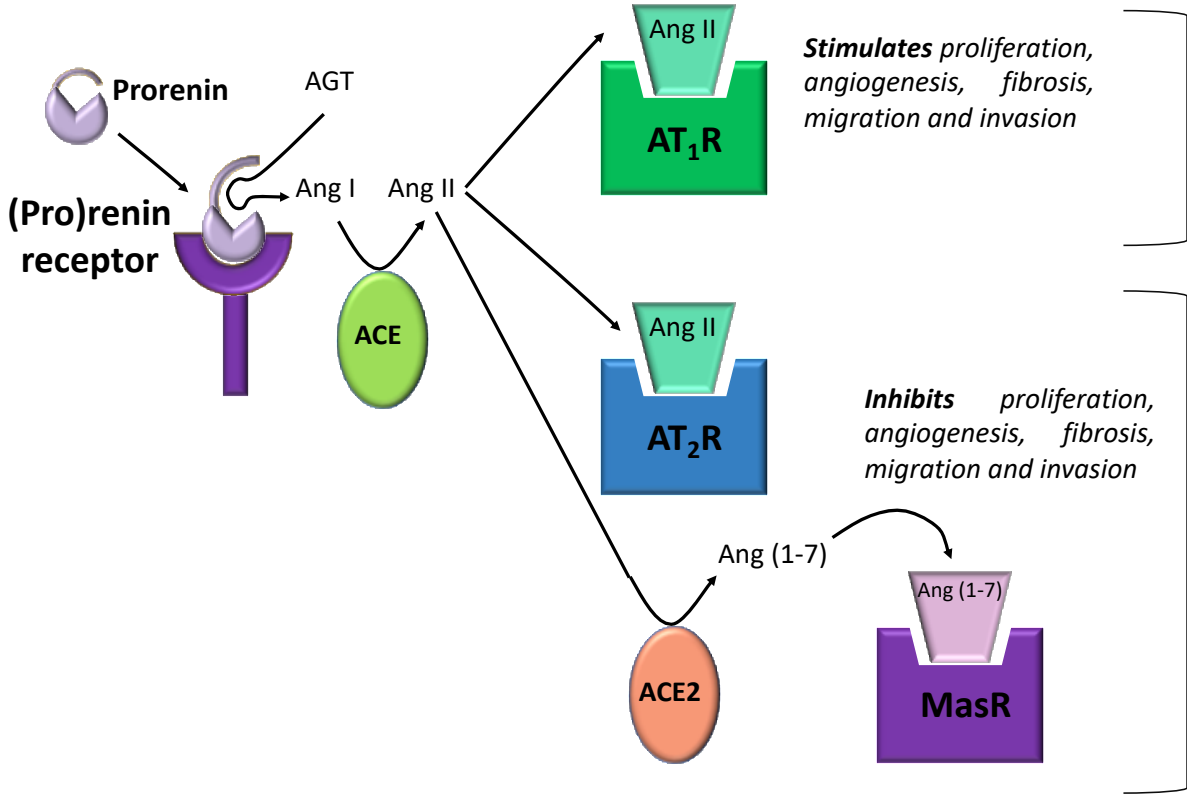

Figure 1.2: The placental RAS

The placental RAS pathway begins with the binding of prorenin to the (pro)renin receptor. This catalyses angiotensinogen (AGT) conversion to Angiotensin (Ang) I. The angiotensin converting enzyme (ACE) converts Ang I to Ang II, which can act upon the AT_1R, the angiotensin II type 2 receptor (AT_2R), and, after conversion to Ang-(1-7) by ACE2, the Mas receptor.

For prorenin to be activated, the 28 amino acid pro-segment must either unfold or be removed so that the catalytic site is exposed. Prorenin can be activated by binding to the (pro)renin receptor ((P)RR/ATP6AP2), which is an essential component of a vacuolar ATPase (V-ATPase). Prorenin binding to (P)RR induces intracellular signalling via MAPK and Wnt/β-catenin signalling [80]. Prorenin/renin can also activate the RAS cascade by converting angiotensinogen (AGT), produced by the liver, into angiotensin I (Ang I). Ang I is converted by the angiotensin converting enzyme (ACE) to angiotensin II (Ang II), where it is able to bind to one of two receptors; the angiotensin II type 1 receptor (AT_1R/AGTR1), which among other actions also stimulates cell migration, proliferation and growth, or the angiotensin II type 2 receptor (AT_2R/AGTR2), which has effects that predominantly antagonise the actions of Ang II mediated through the AT_1R. These include vasodilation and apoptosis [81-83]. The interaction of Ang II with AT_1R stimulates signalling pathways that include MAPK/extracellular signal-related kinase (ERK) and p85α-phosphoinositol 3-kinase (p85α-PI3K) pathways, that further contribute to growth and vascularisation of tissues [84, 85].

Ang II is subject to degradation by a number of proteolytic enzymes. Most significantly, removal of an amino acid from its carboxy terminal by ACE2 (a homologue of ACE), produces Ang–(1-7), which acting via the Mas receptor further antagonises Ang II/AT_1R-mediated signalling [86].

1.4.1 The Placental RAS

All components of the RAS can be found in the placenta, with the exception of all but very small amounts of the active form of renin, which is mainly produced by the kidneys and circulates in maternal blood. The placental RAS is expressed from at least 6 weeks of gestation [3, 40, 87-90]. Additionally, expression of these genes is variable depending on the sex of the fetus [3, 87, 91]. In the first trimester, the placenta has significantly higher

expression of prorenin (*REN*), (P)RR (*ATP6AP2*), *AGT* and *AGTR1* mRNA than term placentae [3]. In the placenta, the RAS contributes to placentation by promoting trophoblast proliferation, migration, invasion, angiogenesis and vasculogenesis [3-5, 92-98].

The effect of physiological oxygen regulation on the RAS

It has been well established that a low oxygen environment upregulates expression of the placental RAS, as well as altering pancreatic, renal and pulmonary RAS expression [40, 99-101]. It has already been established that HTR-8/SVneo cells, when incubated in 1% O_2, have increased expression of *AGTR1* and *VEGF* mRNA, as well as increased protein levels of ACE and VEGF, indicating that there is activation of the pro-angiogenic RAS pathway [9, 40].

Ang II/AT_1R signalling

As well as playing a critical role in fluid and electrolyte balance, Ang II acting via the AT_1R stimulates proliferation, migration [4] and angiogenesis [4, 102]. This occurs as the binding of Ang II to the AT_1R stimulates the release of a number of angiogenic factors including VEGF [103-105]. The importance of adequate AT_1R expression in the placenta was established in a study by Walther, *et al.*, where knock-out of the AT_1R yielded a much smaller and poorly vascularised placenta, severely impacting on fetal development [96].

A study by Delforce, *et al.*, showed that if Ang II/AT_1R signalling was inhibited by the specific AT_1R antagonist, losartan, the expression of an angiogenic/proliferative trophoblast phenotype (i.e. increased expression of the pro-angiogenic factors VEGFA mRNA and protein, angiopoietin 2 (ANGPT2) mRNA, and plasminogen activator inhibitor 1 (PAI-1/SERPINE1) mRNA and protein levels), angiogenic capacity and cell viability in HTR-8/SVneo cells was inhibited [7]. This phenotype is essential for the development of the early placenta.

1.4.2 Downstream targets

Putative downstream targets of the placental RAS pathway include VEGF [106], PAI-1/SERPINE1 and the angiopoietin-2/-1 proteins (ANGPT2/ANGPT1) [7].

VEGF is a disulphide-linked homodimeric glycoprotein production, which is stimulated by hypoxia [107]. It plays major roles in vasculogenesis and angiogenesis, and controls microvascular permeability [108]. VEGFA expression has been positively linked with PAI-1 expression in cytotrophoblasts [109]. As its name suggests, PAI-1 is the major inhibitor in the activation of the fibrinolytic protease, plasminogen [110]. PAI-1, which can be stimulated by the RAS [111], is responsible for inhibiting extracellular matrix degradation in implantation and placentation, and therefore inhibits trophoblast invasion [112]. Of note, PAI-1 is known to be increased in a number of pregnancy complications, including PE, where inadequate extravillous trophoblast invasion occurs [113].

Angiopoietins are vascular growth factors that are critical for embryonic and postnatal homeostasis and angiogenesis [114]. ANGPT1 is involved in new blood vessel maturation and stability as it mediates endothelial cell migration, adhesion and survival. Conversely, ANGPT2 antagonises ANGPT1-mediated receptor phosphorylation, promoting cell death and vascular regression [115]. However, with the addition of VEGF-A, ANGPT2 encourages neovascularisation. The ratio of ANGPT1:ANGPT2 expression is a major determinant of angiogenesis, with a higher ANGPT1:ANGPT2 ratio promoting angiogenesis [116]. The early gestation placenta favours ANGPT2 expression and experiments in placental explants have shown that this local balance is regulated by oxygen (Delforce, *unpublished observations*). Therefore, a reduction in the ANGPT2:ANGPT1 ratio in early gestation may impair angiogenesis [116].

1.4.3 The RAS in Pregnancy Complications

Dysregulation of placental RAS expression has been associated with placental insufficiency [98] and human pregnancy complications including PE and IUGR [97, 117-121].

It is also known that in pregnancies where placental insufficiency is evident such as in cases of PE [122], RAS expression is significantly altered, with increased expression within the placenta of renin, Ang II and the AT_1R [123]. Dysregulation of oxygen delivery to the placenta in pregnancy alters the placental RAS [124]. It has been shown that women who reside at high altitude have significantly higher levels of placental AT_1R, AT_2R, (P)RR and prorenin proteins than their normotensive controls at living at sea level, although mRNA levels were unaffected. In this population, the incidence of PE is significantly elevated compared with the prevalence of PE in women living at sea level [124]. Thus, the increase in placental Ang II in late PE may be secondary to the inadequate placental blood flow resulting from shallow placentation and lack of spiral artery remodelling.

Furthermore, in a murine model, maternal hypoxia in mid-late pregnancy is associated with reduced ACE, ACE2 and AGT levels, and increased AT_1R expression [125], as well as alterations in the placental vasculature [126]. The RAS may also play a compensatory role for insufficient placental perfusion [127, 128].

It is well established that the maternal RAS is critical in maintaining a healthy pregnancy [129]. Furthermore, adequate expression of the RAS in the placenta, both in normal and complicated pregnancy, is essential [95]. RAS expression is controlled by a number of factors; one of which could be post-transcriptional regulators, such as miRNAs [130].

1.5 miRNAs

miRNAs are single-stranded, non-protein coding RNAs approximately 22 nucleotides in length. miRNAs bind to approximately complementary mRNA sequences to either repress translation or degrade mRNA, depending on the degree of complementarity. In this way, miRNAs function as effective post-transcriptional modulators.

miRNA binding to a highly complementary mRNA sequence results in mRNA degradation. The polyA tail of the mRNA fragment is removed, then the RNA-Induced Silencing Complex (RISC) is dismantled. When there is poor complementarity in miRNA/mRNA binding, a stable complex is formed. A miRNA binds to the 3'-UTR of mRNA, leaving the mRNA unable to be translated due to steric hinderance. Each individual miRNA can potentially target ~60% of all genes [124]. Likewise, a single gene is able to be regulated by multiple miRNAs. Although miRNAs have inhibitory roles in gene regulation, the end result can be inhibitory or excitatory, dependent on the gene [63]. miRNAs have differing effects on cell development and growth dependent on their physiological context [131].

In 1993, the first miRNA, lin-4, was discovered in *Caenorhabditis elegans* [132, 133]. There are now four main detection methods of miRNAs; microarray, Northern Blot hybridisation, *in situ* hybridisation and real time PCR. Each detection method has its own advantages and disadvantages, as outlined in Table 1.1.

Apart from pregnancy, miRNAs have been implicated in the development of diabetic retinopathy [134], heart disease [135, 136], diabetes [137], and pancreatic cancer [138], along with many other physiological and pathological processes including hypoxia [137], ischemia and reperfusion injury [139], endothelial progenitor cell activity [137] and cellular senescence [137].

Table 1.1: Comparison of miRNA detection methods

Technique	Advantages	Disadvantages
1. Microarray	Profiles the expression of a large number of miRNAs at the same time	Results usually require validation and confirmation using larger sample sizes and more sensitive techniques (e.g. PCR)
2. Northern Blot hybridisation	Provides information on individual miRNA precursors in different tissues	Low sensitivity and specificity because miRNAs have very short sequences. Use of locked nucleic acid (LNA) probes improves sensitivity and specificity greatly as it substitutes LNAs for every third nucleotide of the probe.
3. *In situ* hybridisation	Shows localisation and abundance of miRNAs	Sensitivity of detection is low because binding affinity to target miRNAs is weak with normal DNA and RNA probes. LNA modified ISH probes increase the affinity, specificity and sensitivity of the assay.
4. Real time PCR	Improved sensitivity	Time-expensive and can often only run one miRNA per sample

1.5.1 miRNA formation

As with all genetic information, miRNA genes are found in the nucleus and are transcribed by RNA Pol II (Figure 1.3). This forms the primary-miRNA (pri-miRNA). 7-methylguanosine is added and polyadenylation occurs before the enzyme Drosha + DGCR8 (a required subunit to form the microprocessor complex) cleaves the pri-miRNA, giving rise to the precursor-miRNA (pre-miRNA). This structure is a ~70nt imperfect stem loop. The pre-miRNA is transported into the cytoplasm by Exportin-5. Once the pre-miRNA is in the cytoplasm, Dicer (an RNase III enzyme) cleaves the structure at the stem loop to produce the miRNA duplex. This duplex is unwound, with the 5' strand being the mature miRNA. The 3' strand is consequently degraded. Argonaute 2 (Ago2) is added to the mature miRNA, before being loaded into the RISC.

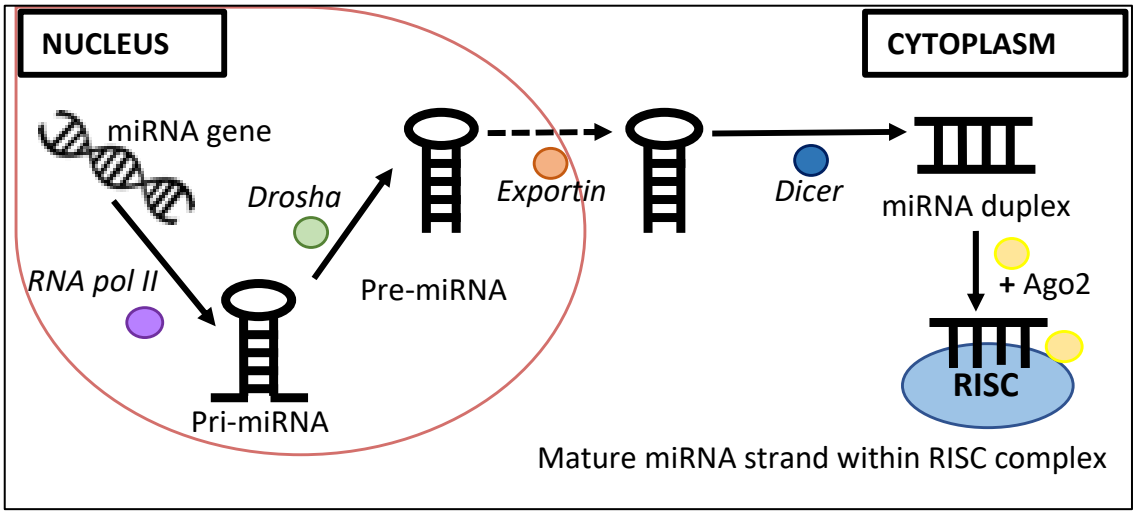

Figure 1.3: miRNA processing

Within the nucleus, miRNA genes are transcribed by RNA pol II, giving rise to pri-miRNAs. Drosha then assists in their conversion to pre-miRNAs. Exportin translocates pre-miRNAs from the nucleus to the cytoplasm, where Dicer forms the miRNA duplex. Ago2 then assists in assembling the RISC.

miRNAs are important biological phenomena, and although they exist mainly to 'fine-tune' mRNA and protein expression, the body cannot function without miRNAs. In fact, inactivation of Dicer, the enzyme required to produce functional miRNA, results in developmental defects in mice and the Dicer1 knockout is embryo-lethal [63, 130]. Moreover, depletion of Ago2, which is required for assembly of the RISC, results in fetal death [140].

1.5.2 miRNA transport

Each cell type has its own unique miRNA milieu to control gene expression [141], with certain cell types employing transport strategies for miRNAs. Extracellular miRNAs are categorised as tissue-resident (located in the tissue), or circulating, depending on their physiological movement and localisation [142]. Circulating miRNAs are predominantly transported in extracellular vesicles (EVs), which are released from cells into the extracellular space under normal and pathological conditions [130]. They are made up of a lipid-bilayer surrounding nucleic acid material. miRNAs are also able to be transported around the body attached to lipoproteins (miRNA transport summarised in Figure 1.4). Extracellular trophoblastic miRNAs can be found as either non-vesicular protein-bound miRNAs, or vesicular miRNAs, which are more common as they are shielded from degradation by circulating RNase by their lipid bilayer [143].

Various types of EVs can be produced from multiple cell types, including trophoblasts [144], and are able to act in a paracrine manner or travel to distant target cells in bodily fluids [145]. EVs can be sorted into three categories, based on their morphology [146], size, function and biogenesis [56, 144]. These are microvesicles, apoptotic bodies and exosomes. For these EVs to be successfully incorporated into cells, toll-like receptor (TLR) signalling (particularly TLR7 and TLR9) is necessary [145]. Environmental change can stimulate and suppress EV release. Hypoxia in early pregnancy [145], changes in oxygen tension [144], D-glucose

concentrations [147] and fluctuations in intracellular Ca^{2+} [56] have been shown to alter EV release and even their contents or target cells.

Microvesicles

Microvesicles are morphologically unique capsules which are actively released from the syncytiotrophoblast. They play a role in fetal-maternal cross-talk throughout pregnancy, delivering packages of information as signals or bioactive RNA. They can deliver this information locally or from distant donor cells to a target cell, and can also have immune effects. Secretion of microvesicles from the placenta can arise either by blebbing or shedding of microparticles from the plasma membrane, or by exosome release via the endosomal pathway [143]. Microvesicles are larger than exosomes, being approximately 100-1000 nm in diameter [148], and are marked with CD40 [56].

Apoptotic bodies

Apoptotic bodies are derived from cells undergoing apoptotic fragmentation and blebbing from the cell membrane containing miRNA fragments. Apoptotic bodies are involved in proliferation and differentiation of endothelial progenitor cells *in vitro* [149], or can be engulfed by phagocytes that trigger cytokine and growth factor release, including VEGF [150, 151]. Apoptotic bodies, produced by direct cell budding during apoptosis, are the largest EVs, being approximately 1000-5000 nm in diameter [152].

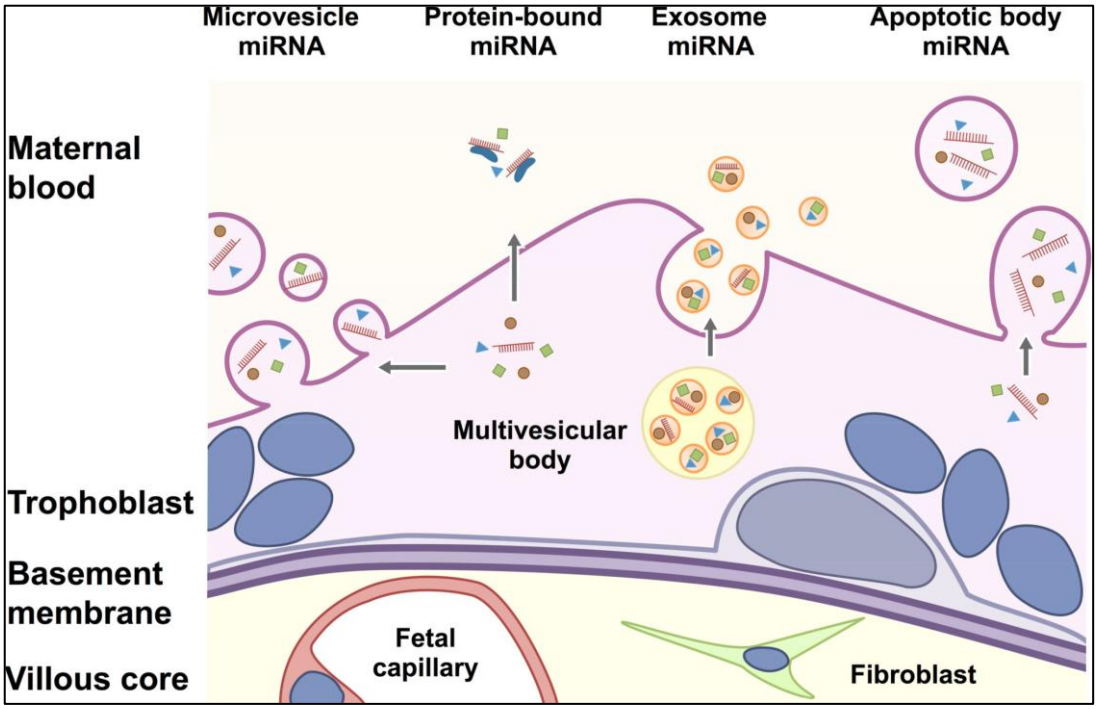

Figure 1.4: miRNA transport

miRNAs are able to be transported throughout the body through various mechanisms. They are able to be transported out of the placenta via microvesicles, as exosomes or apoptotic bodies, or bound to proteins. They are then able to exert their effects on other cells in the mother. Figure sourced from Mouillet, *et al*. [11]

Exosomes

Exosomes are the most well characterised EVs. They are encapsulated by a lipid bilayer approximately 40-120 nm in diameter (the smallest of the EVs) [56], and they are exocytosed into body fluids. They are nano-vesicles containing signalling molecules (including proteins, mRNAs, miRNAs, nc-RNAs or lipids). They can be secreted in response to received signals from nearby or distant cells, or as a reaction to the environment [28]. Endosomal membrane markers CD9, CD81, CD63 and TSG101 can be found on exosomes [56, 144].

Exosomes are able to interact with their target cells in three main ways:

- by directly activating target cell membrane receptors;
- by altering the extracellular environment of the target cell; or
- by fusing with the cell membrane of the target and releasing their contents into the cell [144].

Exosomes are endosomally derived, produced by inward budding multi-vesicular bodies. The multi-vesicular body fuses with the plasma membrane, then the exosome is exocytosed into the extracellular space [144]. They have been shown to alter cell proliferation, metabolism, apoptosis, angiogenesis and translational activity, as well as pro-inflammatory cytokine release [153].

Isolation and characterisation of exosomes is a multi-step process involving isopyknic centrifugation and classification of particle size (often by electromicroscopy) and endosomal markers (for example, immuno-affinity quantification). By fractionating the microsomal pellet on density gradient media, exosome-like nanoparticle subpopulations (diameter of 40-120 nm) can be identified. The unique exosomal subpopulation is then identified by particle density [144].

miRNAs as Biomarkers

The discovery that circulating trophoblast-derived miRNAs could reflect the physiological status of pregnancy has been ground-breaking, and as such, multiple studies have confirmed that exosomes transporting these miRNAs are potentially valuable biomarkers. miRNAs have also been used as therapeutic agents [131, 154]. As miRNAs are smaller than mRNAs, and much more stable, they are able to be extracted from blood, formalin-fixed paraffin-embedded tissue and frozen tissue, all with little to no degradation. These circulating miRNAs are resistant against enzymatic degradation, extreme pH conditions, freezing and thawing. They are also easily accessed, being present in almost all bodily fluids and excretions [155]. This makes them ideally suited for use in the diagnosis of disease.

miRNAs are currently being optimised as biomarkers for gestational diabetes mellitus [156], ovarian and breast cancer [157]. A recent study identified miRNAs as successful biomarkers for intrahepatic cholestasis of pregnancy [155]. This condition could not be diagnosed previously by measuring serum total bile acids. The discovery of circulating miRNAs that reflect the tissue injury occurring in this disease has made a urine test for these molecules possible, because they are highly stable and easily detected. Circulating miRNAs have proven to be valuable biomarkers for a number of acute and chronic liver disorders [155].

As the use of miRNAs as biomarkers is further elucidated, it calls into question the possibility of also using miRNAs as biomarkers of placental insufficiency. This is a topic of investigation for many pregnancy complications due to the unique expression patterns of miRNAs in the placenta.

1.5.3 Placental miRNAs

Placental-specific and placenta-derived miRNAs found in patient's plasma and serum can be used as biomarkers of placental dysfunction [10, 158-160], as they are released mostly by the syncytiotrophoblast as exosomes [158]. These circulating miRNAs are easily accessible, as they are present in almost all bodily fluids and excretions [155]. Placental exosomes, which are secreted from week six of gestation into the maternal circulation, are tagged with placental alkaline phosphatase (PLAP), a unique enzyme allowing exosome isolation and characterisation throughout pregnancy [144]. It is suggested that miRNAs in exosomes may play an important role in cross-talk between the embryo and the endometrium, as specific exosomal miRNAs have been shown to target pathways critical to embryo implantation [144].

Transport of miRNAs in exosomes from EVTs has been shown to promote migration and phenotypic changes in vascular smooth muscle cells *in vitro* [145]. In 1% oxygen, exosomes from EVTs have reduced expression of miRNAs known to promote spiral artery remodelling. These exosomes also decrease endothelial cell migration and are present in early gestation in the maternal circulation [28], which has the potential to disrupt placentation.

Placental miRNAs can be categorised into placenta-specific, placenta-associated and placenta-derived miRNAs. Placenta-specific miRNAs are expressed either solely or mostly in the placenta, while placenta-associated miRNAs are ubiquitously expressed in the placenta and other tissues. miRNAs that are placenta-derived are usually circulating miRNAs released from the placenta [130]. Placenta-associated miRNAs are shown to be significantly increased in serum of pregnant women compared with their non-pregnant controls, and miRNA expression levels are specific to the stage of pregnancy [161, 162]. Moreover, expression is reduced in exosomes isolated from PE pregnancies [163], although there is excess shedding [143].

In the placenta, many miRNAs are located on their respective chromosomes near to each other as a cluster. As such, one promoter can regulate an entire cluster [162, 164, 165]. The chromosome 19 cluster (C19MC), was the first to be acknowledged [166], followed by a cluster on chromosome 14 (C14MC) and the miR-371-3 cluster, which is also positioned on chromosome 19 [164]. Placental miRNAs are nearly always from C19MC and C14MC [167], with miRNAs from these clusters also observed in maternal plasma [10] and the embryo [164].

It has been found that immortalised trophoblasts cell lines (HTR-8/SVneo cells) and primary trophoblasts have similar, but slightly different, miRNA expression profiles to each other [168]. This makes HTR-8/SVneo cells a valuable tool for investigating placental miRNAs as they are easily accessible.

miRNAs have been identified in the placenta in various studies employing miRNA microarrays, with the expression of many of these miRNAs changing between early gestation and term [12]. miRNAs can also regulate a number of signalling pathways [130], including the RAS.

1.5.4 miRNAs that target the RAS

The expression of the placental and circulating RAS may be, at least in part, regulated by miRNAs. Expression of some placental miRNAs that are predicted to target the RAS are lowest in early pregnancy compared with term placenta [12]. This is at a time when the placenta is developing in a low oxygen environment and when RAS expression is highest [144]. Increased expression of RAS proteins is seen in low oxygen [9], when certain miRNAs targeting these genes are repressed [12]. Additionally, the expression of several miRNAs that target the placental RAS are reduced in the mouse placenta after maternal hypoxia [127].

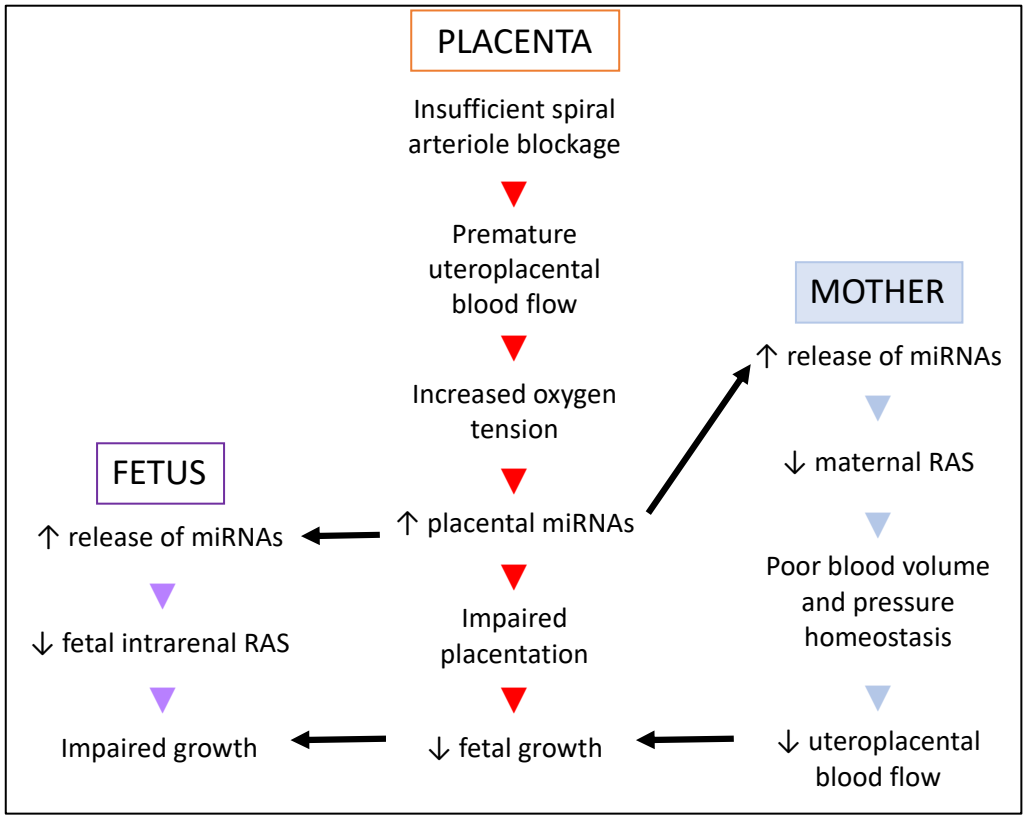

Figure 1.5: The consequences of abnormal miRNA expression on the placental RAS in early pregnancy

Irregular placental development, such as in PE, can have detrimental effects on not only placental development, but also the maternal circulation and fetal development. Premature rises in intraplacental oxygen tension can lead to early increases in placental miRNA levels, suppressing placental RAS expression and impairing placentation. These placental miRNAs can also cross into maternal circulation, suppressing the maternal RAS and ultimately decreasing uteroplacental blood flow. These factors can lead to increased miRNA levels in the fetus, impairing the fetal intrarenal RAS and fetal growth.

It has been established that placental-derived circulating miRNAs are able to enter the fetal circulation and affect fetal organ development [130]. We hypothesise, as described in Figure 1.5, that the interaction between placental miRNAs and the RAS could affect both the maternal condition and fetal development during pregnancy. Insufficient blockage of the spiral arteries, in addition to premature erosion of trophoblastic plugs, gives rise to premature onset of uteroplacental blood flow, leading to increased oxygen tension in the placenta. This allows the upregulation of placental miRNAs targeting the RAS, as well as the increased release of miRNAs in the fetus and mother. This decreases the activity of the placental RAS, impairing placentation and decreasing fetal growth. Depression of the maternal circulating RAS results in poor blood volume, pressure and water homeostasis, leading to decreased uteroplacental blood flow. This contributes to the decrease in fetal growth. Additionally, the lowered expression of the fetal intrarenal RAS contributes to diminishing fetal growth.

Placental miRNAs targeting RAS mRNAs have been associated with trophoblast invasion potential [169] and are able to affect placental development. For example, the discovery of miR-181 (known to affect the regulation of renin in the kidney) within the placenta provides evidence of post-transcriptional changes occurring within the placental RAS. These miRNAs are involved in the regulation of angiogenesis and placentation in early gestation [170].

1.5.5 miRNAs in Pregnancy Complications

It has been established from numerous studies that miRNA and DNA methylation patterns are significantly different between preterm and term infants' cord blood, affirming that transcriptional regulation plays a role in parturition [52]. In 2012, placental-specific C19MC miRNAs were found in maternal blood. Multiple C19MC circulating miRNAs have been shown to be upregulated in those patients who would go on to develop PE [65, 171]. These miRNAs

were later confirmed to have been released from trophoblasts via exosomes [172]. This study confirmed that placental miRNAs are able to transfer into the maternal and fetal circulations.

Altered placental and extracellular miRNA expression in early gestation has been demonstrated in patients with severe PE [13, 65, 171, 173-175]. In PE, circulating C19MC miRNA expression is significantly increased [65, 171, 176]. Moreover, in early spontaneous abortion [177] and in patients with recurrent spontaneous abortion [178] miRNA profiles within the placenta are dysregulated.

More importantly, miRNAs that target the placental RAS are known to be altered in pregnancy complications. A recent study by Wang & Lumbers, *et al.*, used a microarray to highlight the altered miRNA profile in the placenta across gestation, and in women with and without PE. Key miRNAs including miR-378, miR-663, miR-514 and miR-892 were significantly increased in expression in placentae from women with PE [12].

1.5.6 Complications in miRNA research

As miRNAs are able to bind to so many potential targets, many published miRNA studies draw on implied links between miRNA expression and a variable, without fully confirming the effects of the miRNA on gene expression. For example, an increase in expression of one miRNA can potentially alter the expression of multiple mRNAs, however this cannot be assumed to be the case. To address this, studies will often examine the expression of a miRNA and then the expression of their chosen mRNA target. This can elucidate a causal link between the miRNA and target mRNA. To definitively confirm that a miRNA directly interacts with a target mRNA sequence, a luciferase assay is required to be performed. This is one major difficulty in miRNA research that should be clarified to ensure reliable studies.

Another complication is that inconsistencies exist within the literature surrounding miRNA research. A study by Betoni, *et al.*, compared differentially expressed miRNAs in the placenta from women with PE from previously published data array sets with their own microarray data. They detected that ~15% of miRNAs expression were consistent across the studies [179]. A similar phenomenon was observed by others, because miRNA expression across patients also changes with gestational age and individual variation per person [130, 168, 180-182].

It has been suggested that a miRNA pool, rather than a single miRNA, should be utilised as disease biomarkers due to alterations between individuals [130]. This would correct for the individual variation in miRNA expression per person, by allowing a number of markers to be used, thereby decreasing margin of error.

To conclude, the field of miRNA research is complex but promising, with the potential to identify many key players in regulating the physiological and pathophysiological development and function of the placenta. Furthermore, the effects of these miRNAs on the placental RAS suggest that they are critical for regulation of placentation, and dysregulation of their expression is seen in pregnancy complications. For this reason, we believe further investigation into their regulation of the placental RAS is required.

1.6 Hypothesis and Aims

1.6.1 Hypothesis

In early gestation, expression of miRNAs that target the RAS are suppressed due to the low oxygen environment. This allows the placental RAS to be active and promote normal placental growth and development, in part through trophoblast proliferation. Furthermore, irregular

expression of miRNAs that target placental RAS mRNAs significantly impact on placental development and function.

1.6.2 Aims

My first aim was to determine the effects of oxygen on expression of placental miRNAs predicted to target RAS mRNAs in a placental cell line (HTR-8/SVneo cells). Following this, I studied the effects of oxygen on expression of placental miRNAs targeting RAS mRNAs in first trimester chorionic villous explants. I then aimed to find out if the miRNAs (predicted to target the RAS, and that are suppressed in low oxygen) that I had identified in aim 1 inhibited the expression of the placental RAS, and if so, did they reduce the functional capacity of trophoblasts to proliferate? Finally, I aimed to examine the role of miR-155 in placentation, as this miRNA is proposed to be involved in placental development and disease [183-190]. To fully elucidate the role of miR-155 in placental development, I examined this miRNA both *in vitro* and *in vivo*.

1.6.3 Significance

This project explores for the first time, the possibility that miRNAs that target the placental RAS, affect placentation and placental function. These disruptions would not only adversely affect pregnancy outcome but directly and indirectly compound the risk for an individual of developing early onset chronic diseases in adult life.

2. The role of oxygen in regulating miRNAs in control of the placental renin-angiotensin system

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This published manuscript examines the effects of physiological oxygen tension on expression of miRNAs and the renin-angiotensin system components in a first trimester extravillous trophoblast cell line.

Published manuscript

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2.1 Abstract

Human placental renin-angiotensin system (RAS) expression is highest in early gestation, at a time when placental oxygen tension is at its lowest (1–3%), and promotes placental development. Some miRNAs predicted to target RAS mRNAs are downregulated in early gestation. We tested the hypothesis that low oxygen suppresses expression of miRNAs that target placental RAS mRNAs, thus increasing concentrations of RAS mRNAs.

HTR-8/SVneo cells were cultured in 1%, 5% and 20% oxygen for 48 h. Differences in miRNA expression were measured on an Affymetrix miRNA microarray (n=3/group). Those predicted to target RAS mRNAs, or that were decreased in early gestation, were confirmed by qPCR (n=9/group). RAS protein levels were assessed by ELISAs or immuno-blotting.

Microarray analysis identified 4 miRNAs predicted to target RAS mRNAs that were differentially expressed between 1% and 5% oxygen. Using qPCR, 15 miRNAs that target the RAS were measured in HTR-8/SVneo cells. Five miRNAs were downregulated in 1% compared with 5% oxygen. Expression of a number of RAS mRNAs (*ATP6AP2*, *AGT*, *ACE* and *AGTR1*) were increased in either, or both, 1% and 5% oxygen compared with 20% oxygen. AGT protein levels were increased in 1% oxygen compared with 5%.

Further validation is needed to confirm that these miRNAs target RAS mRNAs directly and that placental development is partly regulated by oxygen-sensitive miRNAs that target RAS mRNAs. Since placental oxygen tension changes across gestation, changes in expression of these miRNAs may contribute to the transgestational changes in placental RAS expression and the resulting effects on placental development.

2.2 Introduction

Placentation is a complex developmental process associated with changes in expression of the placental renin-angiotensin system (RAS). The placental RAS contributes to angiogenesis and tissue growth. It could therefore have important physiological effects on the developing placenta [98]. In a model of placental insufficiency, the expression of placental RAS genes is significantly altered, i.e. expression of some mRNAs (e.g. the angiotensin II type 1 receptor (AT_1R / *AGTR1))* is increased, but expression of others (e.g. angiotensinogen (*AGT)*) is decreased [98]. Furthermore, altered expression of RAS components in human placentae is associated with pregnancy complications including intrauterine growth restriction (IUGR) and PE [117-121, 127]. Expression of most of the components of the placental RAS are highest in the first trimester of pregnancy [3], when most of the placental vasculogenesis and cell proliferation is occurring [20], and decreases with advancing gestational age. This is postulated to be due to a number of factors, including changes in the prevailing oxygen tension and possibly, changes in post-transcriptional regulation [10, 30].

The placenta develops in a very low oxygen environment in the first trimester due to the prevention of maternal blood flow by trophoblastic plugs within the uterine spiral arteries [191]. These trophoblastic plugs are dislodged from the spiral arteries, after 12–13 weeks of gestation so that blood can flow into the intervillous space [8]. In unviable pregnancies, a very different haemodynamic profile has been observed in early gestation using Doppler ultrasonography. There is an early onset and increase in the flow of blood into the intervillous space that results in premature increases in oxygen tension, that are detrimental to the growth of the embryo [192]. At the end of the first trimester, i.e., at the end of the embryonic period, continuous blood flow into the placenta is established allowing fetal access to extra oxygen and nutrients to aid further growth and development.

We have found that a low oxygen environment, such as occurs in first trimester placentae, is responsible for the upregulation of *AGTR1* mRNA and angiotensin converting enzyme (ACE) protein levels in a first trimester extravillous trophoblast cell line [9]. Other researchers have also shown that maternal hypoxia alters expression of placental RAS components [127] and such changes are associated with IUGR and PE [125, 193, 194].

MicroRNAs (miRNAs) are short, non-coding, single-stranded RNA fragments (~22 nucleotides) that repress the expression levels of specific genes. miRNAs do so by binding to target sequences in the 3'-untranslated region of mRNA transcripts, inducing mRNA degradation when there is high complementarity to the sequence or creating a stable, untranslatable miRNA:mRNA complex when binding to a sequence of poor complementarity. In this way, miRNAs can modulate expression of placental RAS genes [12]. For example, miR-155 causes reduced expression of the AT_1R gene by binding to a target sequence in *AGTR1* mRNA [171].

Interestingly, the expression of some miRNAs that are predicted to target placental RAS mRNAs increase with gestational age [12] and their expression shows variation between each person [179]. In a recent paper from our group, the expression of miR-181a-5p, miR-181a-3p, miR-181c-5p, miR-let-7, miR-34c, miR-454 and miR-625, which have RAS mRNAs as predicted targets, were examined in placental tissue from early, mid and late gestation [12]. These miRNAs were shown to be significantly downregulated in early gestation, with highest expression in late gestation. This is consistent with data showing that the mRNA expression of the AGT gene (*AGT*), *AGTR1,* (pro)renin receptor ((P)RR)/ATPase H^+ transporting accessory protein 2 gene (*ATP6AP2*) and (pro)renin gene (*REN*) is highest in early gestation – perhaps because there is downregulation of miRNAs that target the mRNAs of these genes [3].

We postulate that oxygen tension and miRNAs interact to regulate expression of the placental RAS. We propose that a low oxygen environment, such as is present in the first trimester, reduces the expression of miRNAs that target one or more placental RAS mRNAs, causing specific placental RAS genes to be more highly expressed (Figure 2.1).

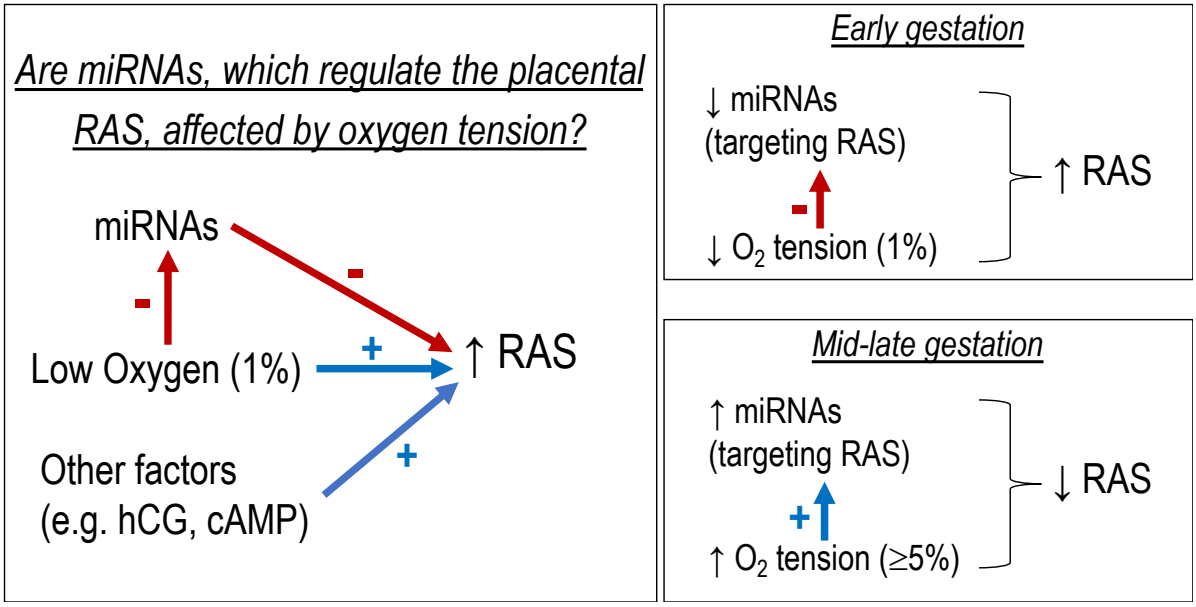


Figure 2.1: Hypothesis for the study.

The left side of the figure shows the overall hypothesis for the study. We hypothesise that low oxygen (1%) will increase expression of the renin angiotensin system (RAS), as well as suppress the expression of miRNAs which could repress RAS expression. Furthermore, we believe other factors such as hCG and cyclic AMP (cAMP) play a role in RAS expression. Red arrows depict decreased expression, blue arrows depict increased expression. The right side of the figure shows our extended hypotheses; that is, in early gestation, when the oxygen tension is low (1%), miRNAs targeting the RAS are reduced and overall RAS expression is increased. Furthermore, in mid to late gestation, when oxygen tension in the placenta is increased (≥ 5%), there is increased expression of miRNAs targeting the RAS and overall RAS expression in reduced.

2.3 Methods

Cell Culture

HTR-8/SVneo cells (an immortalised first trimester trophoblast cell line provided by Professor Charles Graham, Queens University, Ontario) were cultured at 37 °C with 5% CO_2 in air in RPMI-1640 (HyClone) supplemented with 10% v/v heat inactivated FCS (ISAFC Biosciences), 1% w/v L-glutamine and 1mg/mL Antibiotic-Antimycotic (Gibco). Three separate cultures of HTR-8/SVneo cells were made and from each of these 3 cultures, three sets of $2x10^5$ cells from passages 10-20 were plated (n=9). The culture medium was then replaced and cells were incubated in either standard culture conditions (20% O_2) for proof of concept that miRNA expression is sensitive to low oxygen, or in one of two sealed oxygen chambers (5% or 1% O_2 with 5% CO_2 in N_2) to represent the oxygen tension within the intervillous space during the 1st and 2nd trimesters, respectively [30] for a further 48 h, with chambers flushed every 24 h. Cells and culture media were collected into PBS and snap frozen in liquid nitrogen, then stored at −80 °C before RNA analysis. These procedures were repeated to collect cells used for protein analysis.

RNA Extraction and DNAse Treatment

Total RNA extraction of HTR-8/SVneo cells was performed using the miRNeasy kit, according to the manufacturer's instructions (*Qiagen*). The integrity of total RNA and miRNAs was examined by gel electrophoresis and quantified using the Nanodrop 2000 (data not shown). Samples were used for further analysis if the 260:280 and 260:230 nm ratios were greater than 1.8.

Microarray analysis

Total RNA (in a final concentration of 50–100 ng/μL) was provided to the Ramaciotti Centre at the University of New South Wales, Sydney for Affymetrix miRNA microarray analysis. RNA quality was analysed using a Bioanalyser (Agilent) before microarray analysis at the Ramaciotti Centre. Labelling and hybridisation to an Affymetrix Human miRNA microarray, which supports the detection of 2006 human miRNAs, was performed. miRNA microarray analysis was undertaken on n=3 samples cultured in separate experiments at each of the 3 different oxygen tensions (1%, 5% and 20%). All samples in the microarray occupied a single microarray slide. Data produced by microarray analysis were analysed using Partek Genomic Suite software.

miRNA Analysis

Fifteen miRNAs predicted to target RAS mRNAs were assessed by qPCR for their expression at 1%, 5% and 20% oxygen tension. RNA samples underwent reverse transcription to cDNA (TaqMan® miRNA Reverse Transcription Kit and RT-qPCR probes) and were analysed by quantitative PCR (RT-qPCR) using TaqMan® Universal PCR master mix, according to the manufacturer's instructions (Applied Biosystems). A list of primer sequences used can be found in Table 2.1 and Table 2.2.

Table 2.1: mRNA primer sequences

Official gene symbol	GenBank Accession #	Primer Sequence (5'- 3')	Concentration	Melt temperature
ACTB	NM_001101	Fw: CGCGAGAAGATGACCCAGAT	1000 nM	78°C
		Rv: GAGTCCATCACGATGCCAGT		
ACE	NM_000789	Fw: AAGCAGGACGGCTTCACAGA	200 nM	85°C
		Rv: GGGTCCCCTGAGGTTGATGTAT		
AGT	NM_000029	Fw: CAACACCTACGTCCACTTCCAA	200 nM	62°C
		Rv: TGTTGTCCACCCAGAACTCCT		
AGTR1	NM_000685	Fw: CCTCAGATAATGTAAGCTCATCCAC	200 nM	77°C
		Rv: GCTGCAGAGGAATGTTCTCTT		
ATP6AP2	NM_005765	Fw: CCTCATTAGGAAGACAAGGACTATCC	200 nM	60°C
		Rv: GGGTTCTTCGCTTGTTTTGC		

Fw: Forward; Rv: Reverse

Table 2.2: miRNA primer sequences

miRNA name	Mature miRNA sequence	TaqMan Assay ID
Hsa-miR-181a-3p	ACCAUCGACCGUUGAUUGUACC	000516
Hsa-miR-181a-5p	AACAUUCAACGCUGUCGGUGAGU	000480
Hsa-miR-181c-5p	AACAUUCAACCUGUCGGUGAGU	000482
Hsa-miR-34c-5p	AGGCAGUGUAGUUAGCUGAUUGC	000428
Hsa-miR-7-5p	UGGAAGACUAGUGAUUUUGUUGUU	005723_mat
Hsa-miR-625-5p	AGGGGGAAAGUUCUAUAGUCC	002431
Hsa-miR-454-3p	UAGUGCAAUAUUGCUUAUAGGGU	002323
Hsa-miR-143-3p	UGAGAUGAAGCACUGUAGCUC	002249
Hsa-miR-155	UUAAUGCUAAUCGUGAUAGGGGU	002287
Hsa-miR-514b	AUUGACACCUCUGUGAGUGGA	242955_mat
Hsa-miR-330	UCUCUGGGCCUGUGUCUUAGGC	002230
Hsa-miR-892a	CACUGUGUCCUUUCUGCGUAG	002195
Hsa-miR-378g	ACUGGGCUUGGAGUCAGAAG	462874_mat
Hsa-miR-483-3p	UCACUCCUCUCCUCCCGUCUU	002339
Hsa-miR-663	GGUGGCCCGGCCGUGCCUGAGG	002857
RNU44	CCTGGATGATGATAGCAAATGCTGACTGAA CATGAAGGTCTTAATTAGCTCTAACTGACT	001094

Semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (qPCR)

All RNA samples (n=9) underwent reverse transcription to cDNA (Superscript III First-Strand Synthesis for RT, using the manufacturer's instructions, *Thermo Fisher Scientific*). Total RNA was spiked with a known amount of Alien RNA (Stratagene), consisting of total RNA. qPCR was performed in an Applied Biosystems 7500 Real Time PCR System using SYBR Green for detection. Each reaction mixture contained 5 µL of SYBR Green PCR master mix (Applied Biosystems), RAS primers, cDNA reversed transcribed from 10 ng total RNA, and water to 10 µL. Messenger RNA abundance was calculated as described previously, using the $2^{-\Delta\Delta CT}$ method and expressed relative to *ACTB* mRNA and a calibrator (a term placental sample collected at elective Caesarean section) [3].

Protein Extraction

Total protein was extracted from HTR-8/SVneo cells using a radioimmunoprecipitation assay (RIPA) lysis and extraction buffer directly from the cell culture well. RIPA buffer (500 µL of 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% v/v NP-40, 0.5% w/v sodium deoxycholate, 100 nM sodium orthovanadate), Complete Mini Protease Inhibitor Cocktail tablets (Roche Diagnostics Australia) and 5 µL of 100 nM PMSF were added to each sample. Samples were incubated on ice for 10 min, vortexed and then centrifuged at 16200 x g at 4 °C for 10 min. Supernatants were collected. Protein was quantified using the Pierce BCA Protein assay kit (Life Technologies) according to the manufacturer's instructions.

Western Blotting

Western Blotting was performed as described previously [12]. Briefly, samples were loaded into Bis-Tris methane 4–12% gels in duplicate before electrophoresis. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane using the wet sandwich method

immersed in a transfer buffer. The PVDF membrane was then completely dried and re-activated before immunodetection. The membrane was rocked in a blocking solution (5% w/v BSA, 5% w/v skim milk in Tris buffered saline (TBS)) overnight at 4 °C. The primary antibody solution was then added (1:1000 dilution for both, the antibodies being Abcam #ab40790 for (P)RR and Abcam #ab124734 for AGTR1) and samples were incubated at 22 °C on a rocker ((P)RR for 2 h and AGTR1 for 4 h). The secondary anti-rabbit antibody solution was added (Millipore, Burlington, MA, USA; #12-348, 1:5000) and incubated at 22 °C on a rocker for 1 h. Membranes were rinsed before signal detection using an Amersham ECL detection kit (GE Healthcare Life Science) and Amersham Imager 600. Membranes were then stripped using 0.2 M NaOH and, using a rabbit polyclonal anti-β-actin antibody (Abcam; ab8227, 1:5000), were used to detect β-actin for normalisation. The ratio of the protein of interest to β-actin was averaged for duplicate lanes and differences between blots were corrected using an internal control (a pooled placental sample).

Enzyme-Linked Immunosorbent Assays (ELISAs)

Commercially available ELISAs were used to measure concentrations of prorenin (Molecular Innovations, MI, USA), angiotensinogen (IBL International, Hamburg, Germany) and ACE (Duoset, R&D systems, MN, USA) using methods described previously [195].

Statistics

Statistical analysis was undertaken using GraphPad Prism 7. A one-way ANOVA (non-parametric) test was conducted using Tukey's multiple comparisons test. Differences between groups were considered significant for $p \leq 0.05$.

Partek Genomic Suite software was used to analyse microarray data. A robust multi-array analysis (RMA) was performed, including log_2 transformation, background corrections, quantile normalisation and summarisation of probe features, which produced a set of expression signal intensities. To identify differences in expression of miRNAs at different oxygen tensions, unsupervised hierarchical clustering was conducted between the treatment groups. Correction for multiple testing was performed using the Benjamini -Hochberg method, with a default false discovery rate [196]. Particular attention was paid to those miRNAs predicted to target RAS mRNAs (as described on miRNA predictors mirbase.org, mirdb.org, targetscan.org, and targetexplorer.ingenuity.com) with 90% complementarity or higher.

2.4 Results

miRNA Microarrays of HTR-8/SVneo Cells Cultured in 1%, 5% and 20% O_2

Comparison of the expression of miRNAs in HTR-8/SVneo cells cultured in 1% versus 20% O_2 showed significant differences in the expression of 355 miRNAs (Listed in Appendices; GEO submission GSE121593) (PCA analysis can be found in Supplementary Figure 2.1, and unsupervised hierarchical clustering is shown in Figure 2.2). In 1% O_2, the expression of 192 miRNAs was increased and 163 miRNAs was decreased compared with cells cultured in 20% O_2 (Figure 2.3). This included miR-210-3p, known to be regulated by hypoxia (termed a hypoximiR), which was increased by 7.6-fold in 1% O_2 compared with 20% O_2 (p = 6.5E–08). Of those that were differentially expressed, 6 miRNAs were predicted to target RAS mRNAs, with 2 being increased and 4 being decreased compared with 20% O_2 (Table 2.3).

Table 2.3: Microarray levels of miRNAs, that are predicted to target renin-angiotensin system mRNAs, in 1% compared with 20% oxygen tension.

Transcript ID (Array Design)	**Target RAS mRNA**	**p-value (1% vs. 20%)**	**Fold-Change (1% vs. 20%)**
Hsa-miR-625-5p	*ATP6AP2*	3.3E–04	1.8
Hsa-miR-892b	*AGT*	4.9E–02	1.3
Hsa-miR-155-5p	*AGTR1*	4.3E–02	–1.2
Hsa-miR-34b-5p	*AGTR1*	3.5E–02	–1.3
Hsa-miR-181a-5p	*REN, ACE*	1.1E–03	–1.5
Hsa-miR-454-3p	*ATP6AP2*	3.1E–02	–1.7

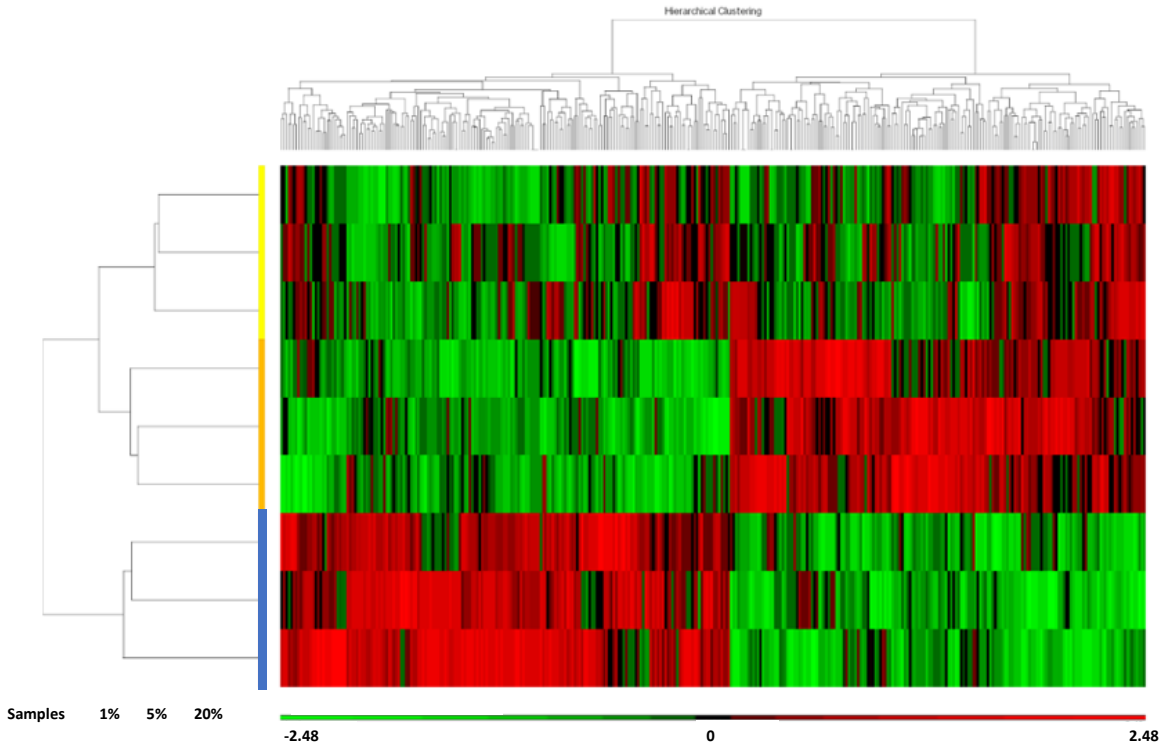


Figure 2.2: Unsupervised hierarchical clustering of miRNAs differentially expressed in 1%, 5% and 20% oxygen tensions.

This figure depicts the miRNA microarray data through unsupervised hierarchical clustering. N=3 samples per oxygen tension. The coloured bar to the left of the graph indicates the group to which each sample belongs. 1% oxygen is shown in blue, 5% oxygen is shown in orange and 20% oxygen is shown in yellow.

Comparison of the expression of miRNAs in HTR-8/SVneo cells cultured in 1% versus 5% O_2 showed significant differences in the expression of 354 miRNAs (Appendices). Compared with cells cultured in 5% O_2, cells grown in 1% O_2 showed increased expression of 184 miRNAs and decreased expression of 170 miRNAs (Figure 2.3). One of these, the hypoximiR, miR-210-3p, was increased by 2.6-fold in 1% O_2 compared with 5% O_2 (p = 6.0E–06). Of those that were differentially expressed between 1% and 5% oxygen, 4 were predicted to target RAS mRNAs (Table 2.4), of which 3 were down-regulated in 1% oxygen compared with expression in 5% O_2.

Comparison of the expression of miRNAs in HTR-8/SVneo cells cultured in 5% versus 20% O_2 showed significant differences in the expression of 261 miRNAs (Appendices). In 5% O_2, 158 miRNAs had increased expression and 103 miRNAs had decreased expression compared with cells cultured in 20% O_2 (Figure 2.3). The hypoximiR, miR-210-3p, was increased by 3-fold in 5% O_2 compared with 20% O_2 (p = 2.7 E–06). Furthermore, 8 of these miRNAs were predicted to target RAS mRNAs (Table 2.5).

Table 2.4: Microarray levels of miRNAs, that are predicted to target renin-angiotensin system mRNAs, in 1% compared with 5% oxygen tension.

Transcript ID (Array Design)	**Target RAS mRNA**	**p-value (1% vs. 5%)**	**Fold-Change (1% vs. 5%)**
hsa-miR-514a-3p	AGT, AGTR1	4.7E–02	1.16
hsa-miR-330-3p	REN	4.7E–03	–1.26
hsa-miR-625-5p	ATP6AP2	5.3E–03	–1.39
hsa-miR-181a-5p	REN, ACE	2.9E–03	–1.41

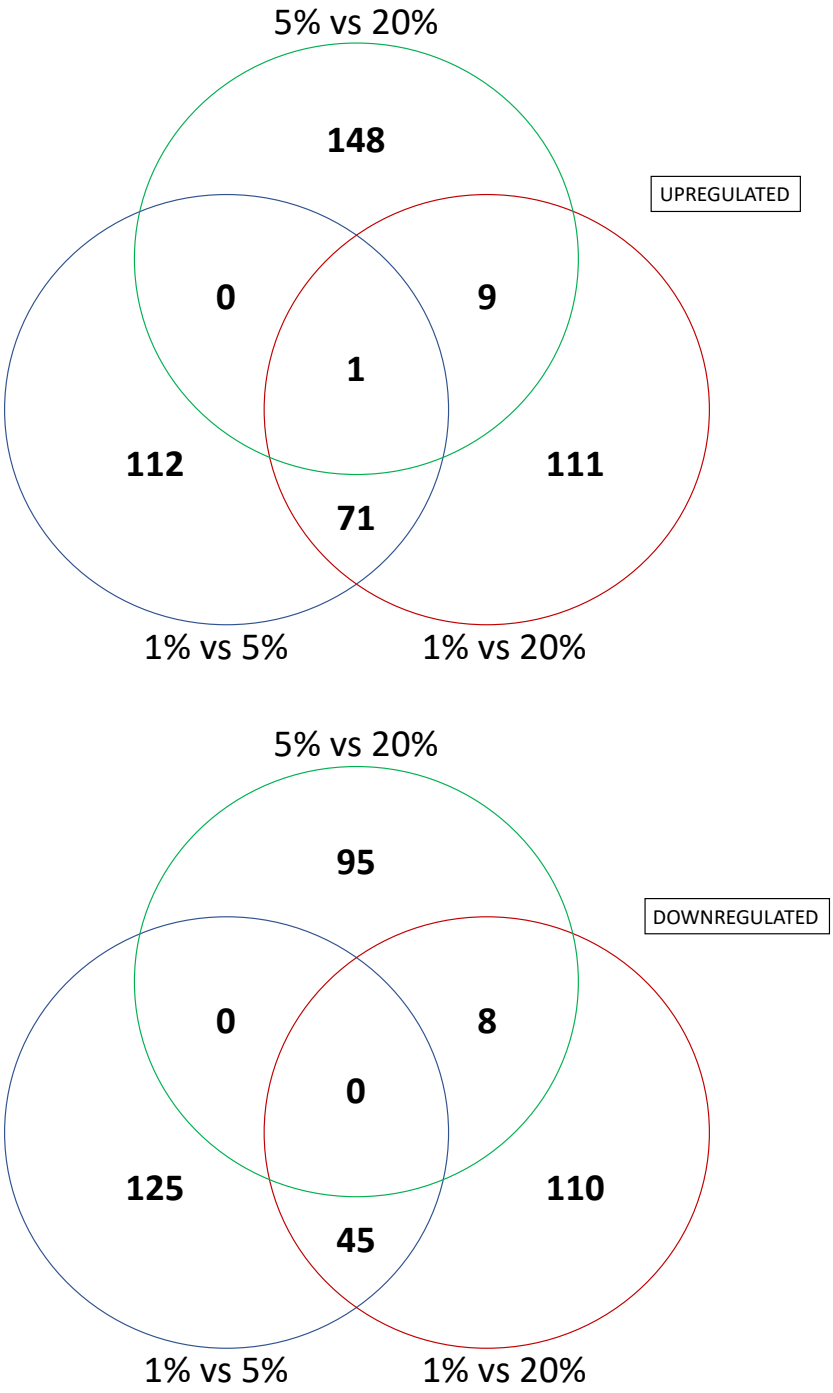


Figure 2.3*:* Venn diagram depicting miRNAs upregulated and downregulated between 5% and 20% oxygen, between 1% and 5% oxygen, and between 1% and 20% oxygen. The top diagram shows miRNAs upregulated between the oxygen tensions. The bottom diagram shows miRNAs downregulated between the oxygen tensions. The green circle (above) depicts miRNAs shown in the microarray to be altered between 5% and 20% oxygen

tensions. The blue circle (left) depicts miRNAs shown in the microarray to be altered between 1% and 5% oxygen tensions. The red circle (right) depicts miRNAs shown in the microarray to be altered between 1% and 20% oxygen tensions. The intersection of the three circles indicates miRNAs that are altered in 5% versus 20% oxygen, 1% versus 20% oxygen, and in 1% versus 5% oxygen.

Table 2.5: Microarray levels of miRNAs, that are predicted to target renin-angiotensin system mRNAs, in 5% compared with 20% oxygen tension.

Transcript ID (Array Design)	**Target RAS mRNA**	**p-value (5% vs. 20%)**	**Fold-Change (5% vs. 20%)**
Hsa-miR-330-3p	*REN*	4.2E–02	–1.27
Hsa-miR-625-5p	*ATP6AP2*	2.2E–02	–1.27
Hsa-miR-514a	*AGT, AGTR1*	1.6E–02	–1.27
Hsa-miR-378c	*REN, ACE*	1.5E–02	–1.38
Hsa-miR-892b	*AGT*	1.2E-02	–1.42
Hsa-miR-378i	*REN, ACE*	7.5E–03	–1.47
Hsa-miR-378a-3p	*REN, ACE*	1.5E–02	–1.49
Hsa-miR-454-3p	*ATP6AP2*	4.9E–03	–2.30

Effect of O_2 on Expression of miRNAs that Target the RAS in HTR-8/SVneo Cells

At all oxygen tensions, the HTR-8/SVneo cells expressed miRNAs predicted to target RAS mRNAs. These miRNAs were hsa-miR-34c-5p, hsa-miR-454-3p, hsa-miR-514b, hsa-miR-330, hsa-miR-892a, hsa-miR-378g, hsa-miR-181a-5p, hsa-miR-181a-3p, hsa-miR-483-3p, hsa-miR-663b, hsa-miR-181c-5p, hsa-miR-7-5p, hsa-miR-143-3p, hsa-miR-625-5p and hsa-miR-155.

The expression of miRNAs shown to be significantly altered by oxygen tension in the microarray analysis, and predicted to target RAS mRNAs, was confirmed using qPCR. qPCR showed that the following miRNAs were significantly reduced in 1% O_2 compared with cells cultured in 20% O_2 for 48 h: hsa-miR-34c-5p (p = 0.03; Figure 2.4A), hsa-miR-454-3p (p = 0.04; Figure 2.4B), hsa-miR-514b (p = 0.002; Figure 2.4C), hsa-miR-330 (p = 0.034; Figure 2.4D), hsa-miR-892a (p = 0.02; Figure 2.4E), hsa-miR-378g (p = 0.02; Figure 2.4F), and hsa-miR-181a-5p (p = 0.043; Figure 2.4G). Table 2.6 summarises these results.

Additionally, the expression of hsa-miR-34c-5p (p = 0.03; Figure 2.4A), hsa-miR-454-3p (p = 0.03; Figure 2.4B), hsa-miR-514b (p = 0.02; Figure 2.4C) and hsa-miR-378g (p = 0.002; Figure 2.4F) was significantly reduced in cells cultured in 1% O_2 compared with cells cultured in 5% O_2 (see also Table 2.7). Expression of hsa-miR-892a was downregulated in cells cultured in 5% O_2 compared with cells cultured at 20% O_2 (p = 0.004; Figure 2.4E).

Hsa-miR-625-5p and hsa-miR-155, which are predicted to target *ATP6AP2* and *AGTR1* mRNAs, respectively, were expressed in HTR-8/SVneo cells, but their expression was not affected by oxygen tension (data not shown).

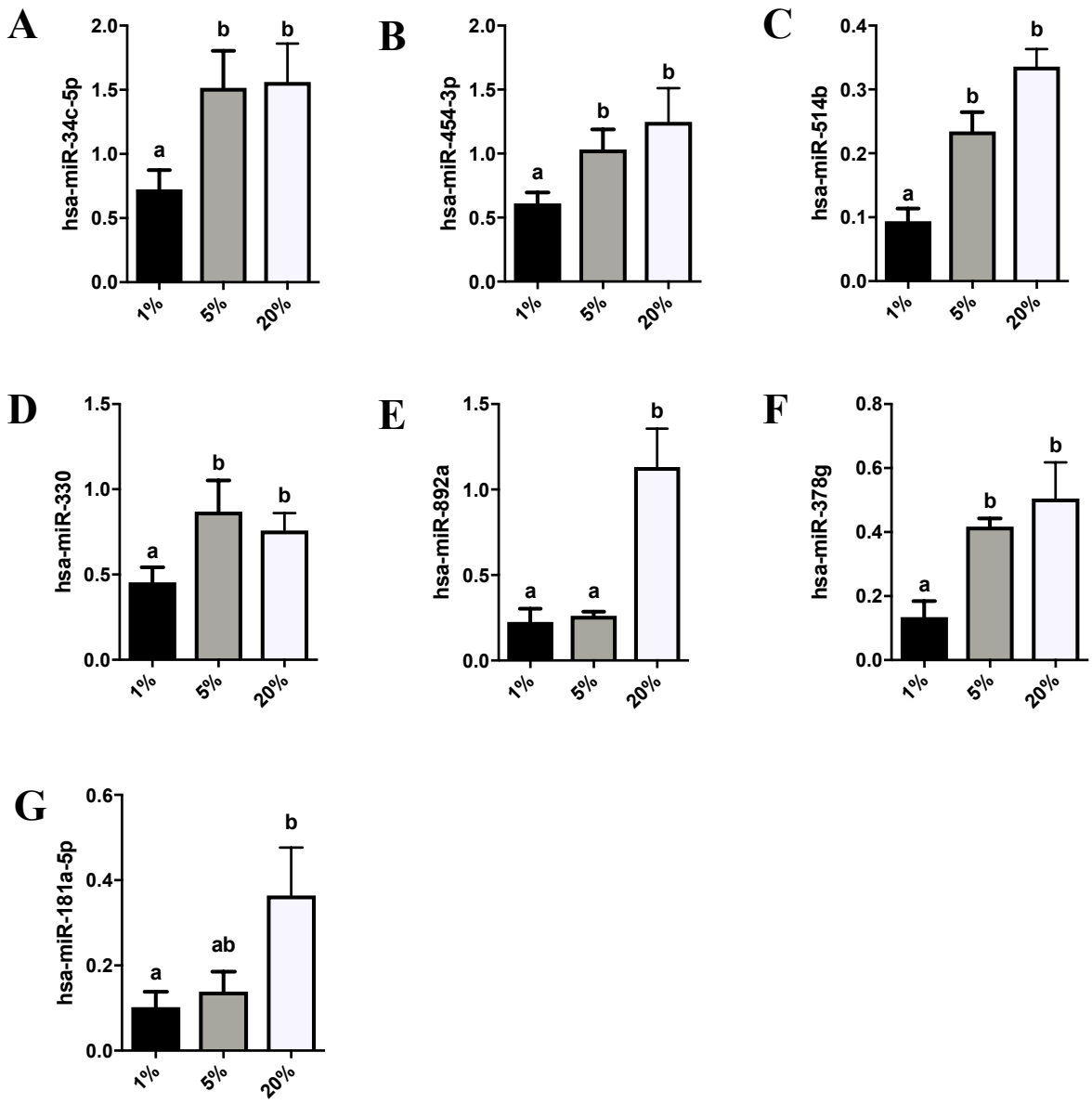


Figure 2.4: Oxygen regulates the expression of placental miRNAs (that were also altered in the microarrays) that are predicted to target RAS mRNAs.

Levels of **A** miR-34c-5p, **B** miR-454-3p, **C** miR-514b, **D** miR-330, **E** miR-892a, **F** miR-378g, and **G** miR-181a-5p are significantly downregulated in HTR-8/SVneo cells cultured in 1% O_2 compared with those cultured in 20% O_2. *Relative abundance is presented as mean ± SEM. N = 3 experiments, each in triplicate. The same letter above each bar indicates that groups are not different from each other. A different letter above the bar indicates that the groups are different from each other (all $p < 0.05$).*

The expression of a number of other miRNAs predicted to target RAS mRNAs, but that did not show oxygen dependent expression on the microarray, were previously shown to be decreased in first trimester compared with term placentae [12]. These were also examined using qPCR. This showed that hsa-miR-181a-3p (p = 0.03; Figure 2.5A), hsa-miR-483-3p (p = 0.01; Figure 2.5B) and hsa-miR-663b (p = 0.01; Figure 2.5C) were in fact significantly downregulated in cells incubated in 1% O_2 compared with 20% O_2.

Expression of hsa-miR-483-3p (p = 0.01; Figure 2.5B) was also significantly decreased in 1% O_2 when compared with cells cultured in 5% O_2, and expression of hsa-miR-663b (p = 0.02; Figure 2.5C) was decreased in 5% O_2 samples compared with 20% O_2 samples.

Hsa-miR-181c-5p, hsa-miR-7-5p, and hsa-miR-143-3p were detected in all samples but no differences in the expression of these miRNAs in cells cultured at different oxygen tensions were detected using qPCR (data not shown).

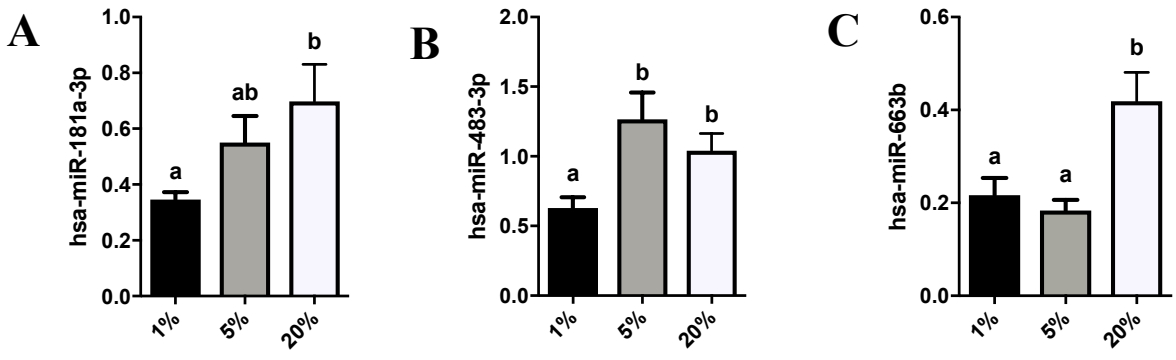


Figure 2.5: Oxygen regulates the expression of placental miRNAs (that were not shown to be differentially expression in the microarrays) that are predicted to target renin angiotensin system (RAS) mRNAs.

Levels of **A** miR-181a-3p, **B** miR-483-3p, and **J** miR-663b are significantly downregulated in HTR-8/SVneo cells cultured in 1% O_2 compared with those cultured in 20% O_2. *Relative abundance is presented as mean ± SEM. N = 3 experiments, each in triplicate. The same letter above each bar indicates that groups are not different from each other. A different letter above the bar indicates that the groups are different from each other (all $p < 0.05$).*

Effect of O_2 on RAS mRNA and Protein Expression in HTR-8/SVneo Cells

At all oxygen tensions, HTR-8/SVneo cells expressed *REN*, *ACE*, *AGT*, *AGTR1*, and *ATP6AP2* mRNAs.

Levels of *REN* and *AGT* mRNAs were not significantly different in cells cultured in 1% and 20% O_2 ($p > 0.05$; respectively Figure 2.6A, 2.6F), although levels of *AGT* mRNA were significantly upregulated in 5% O_2 compared with 20% O_2 ($p = 0.009$).

Levels of prorenin protein were significantly increased in the cell culture supernatant, and decreased in cell lysate in 1% O_2 compared with 20% O_2 ($p = 0.009$, $p = 0.017$, respectively; Figure 2.6B, 2.6C), but expression of total prorenin protein (inclusive of protein in both the cell lysate and supernatant) was increased in cells cultured in 1% O_2 compared with 20% O_2 ($p = 0.017$; Figure 2.7A). Expression of AGT protein was significantly increased in supernatant and in cell lysate ($p < 0.001$, $p = 0.001$ respectively; Figure 2.6G, 2.6H) in 1% compared to 20% O_2. Expression of AGT protein in supernatant and cell lysate was also significantly increased in 5% O_2 when compared with 20% oxygen ($p = 0.002$ and $p = 0.041$, respectively) and in 1% O_2 when compared with 5% O_2 in cell lysate ($p = 0.034$).

Expression of *ACE, AGTR1* and *ATP6AP2* mRNAs were significantly increased by culture in 1% O_2, ($p < 0.001$, $p = 0.03$, and $p = 0.001$; respectively, Figure 2.6I, 2.6L, 2.6D) and 5% O_2 ($p = 0.002$, $p = 0.001$, $p < 0.001$; respectively) compared with cells cultured in 20% O_2 for 48 h. Expression of *ACE* mRNA was also significantly increased in low oxygen (1%; $p = 0.008$; Figure 2.6I) compared with cells cultured in 5% O_2. Expression of *ATP6AP2* mRNA was also significantly increased in 5% oxygen compared with 20% oxygen ($p = 0.03$; Figure 2.6D).

ACE protein was significantly increased in both cell culture supernatant and cell lysate in 1% O_2 compared with 20% O_2 ($p < 0.001$, $p = 0.032$ respectively; Figure 2.6J, 2.6K).

Concentrations of AGTR1 and (P)RR proteins were not significantly different between cells cultured in 1% and 20% O_2 ($p > 0.05$; respectively Figure 2.6M, 2.6E).

For simplicity, a summary of the differences in protein levels between 1% and 20% and between 1% and 5% oxygen tensions are presented in Tables 2.6 and 2.7, respectively.

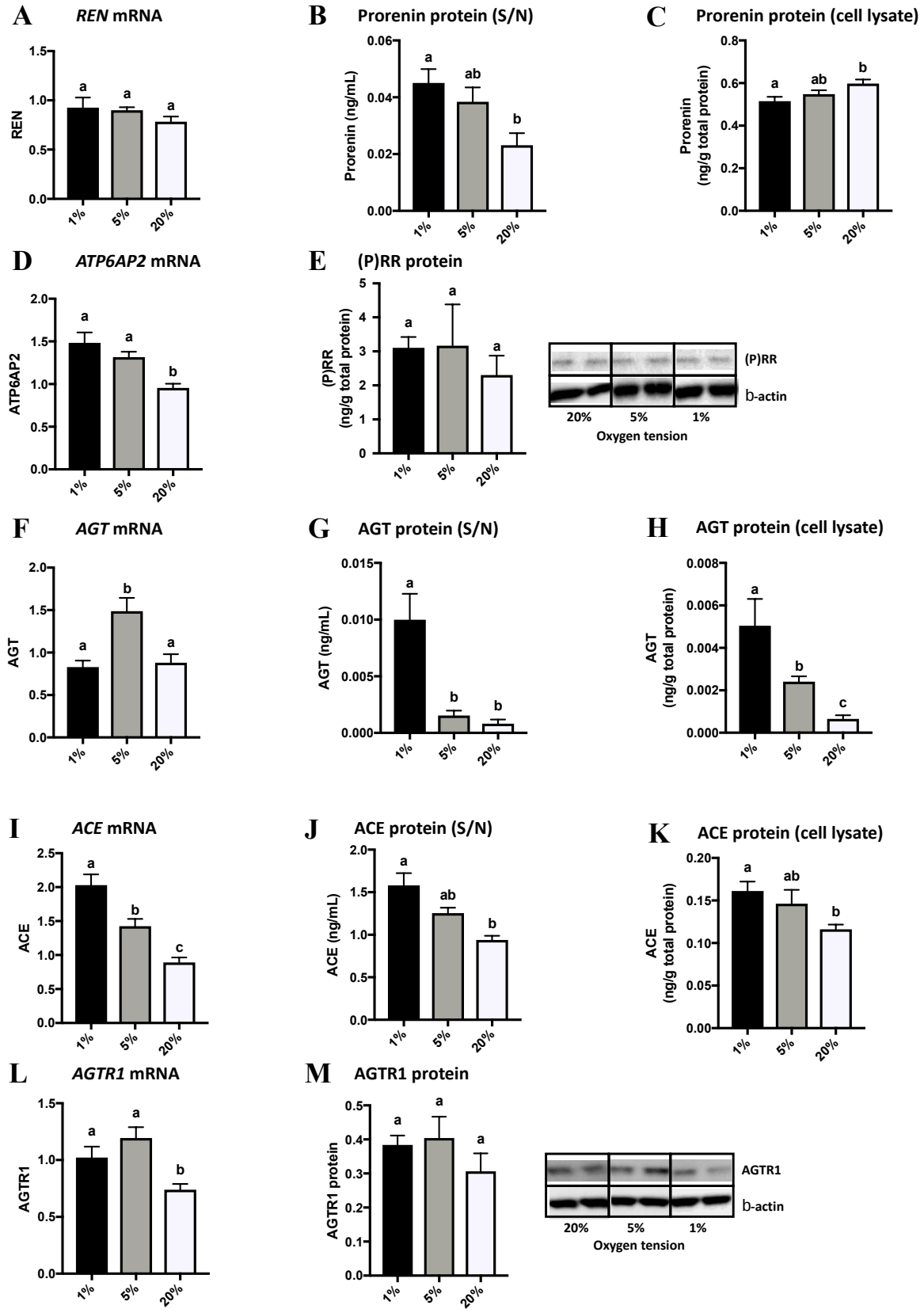


Figure 2.6: Oxygen regulates the expression of placental renin angiotensin system (RAS) RNAs and proteins.

Levels of **A** *REN* mRNA, **B** prorenin protein in supernatant (S/N), **C** prorenin protein in cell lysate, **D** *ATP6AP2* mRNA, **E** (P)RR protein, **F** *AGT* mRNA, **G** AGT protein in supernatant, **H** AGT protein in cell lysate, **I** *ACE* mRNA, **J** ACE protein in supernatant, **K** ACE protein in cell lysate, **L** *AGTR1* mRNA, **M** AGTR1 protein; in HTR-8/SVneo cells cultured in 1% O_2 and 5% O_2 compared with those cultured in 20% O_2. *Relative abundance is presented as mean ± SEM. N = 3 experiments, each in triplicate. The same letter above each bar indicates that groups are not different from each other. A different letter above the bar indicates that the groups are different from each other (all $p < 0.05$). Immunoblot images are all sourced from the same gel, and have been spliced for aesthetics only.*

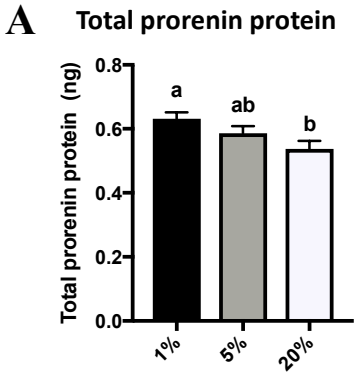


Figure 2.7: Oxygen regulates the expression of total prorenin protein.

Expression of total prorenin protein; in HTR-8/SVneo cells cultured in 1% O_2 was significantly higher than those cultured in 20% O_2. *Relative abundance is presented as mean ± SEM. N = 3 experiments, each in triplicate. The same letter above each bar indicates that groups are not different from each other. A different letter above the bar indicates that the groups are different from each other (all p < 0.05).*

Table 2.6: Summary of changes in the levels of selected miRNAs and predicted target mRNAs and proteins of the renin-angiotensin system in 1% oxygen tension compared with 20% oxygen tension.

miRNA name	Effects of low oxygen (measured by microarray) n=3	Effects of low oxygen (measured by qPCR) n=9	Target RAS genes	Effects of low oxygen on target mRNA n=9	Effects of low oxygen on target protein n=9
hsa-miR-34	↓	↓	*AGTR1*	↑	-
hsa-miR-454-3p	↓	↓	*ATP6AP2*	↑	-
hsa-miR-514	-	↓	*AGT*	-	↑
(514a-3p / 514b)			*AGTR1*	↑	-
hsa-miR-330-3p	-	↓	*REN*	-	↑
hsa-miR-892	↑	↓	*AGT*	-	↑
hsa-miR-378	-	↓	*REN*	-	↑
(378a / c / i)			*ACE*	↑	↑
hsa-miR-181a-5p	↓	↓	*REN*	-	↑
			ACE	↑	↑
hsa-miR-181a-3p	-	↓	*REN*	-	↑
hsa-miR-483-3p	-	↓	*ACE*	↑	↑
			ACE2	Not measured	Not measured
			AGT	-	↑
			AGTR1	↑	-
hsa-miR-663b	-	↓	*REN*	-	↑
hsa-miR-181c-5p	-	-	*AGT*	-	↑
hsa-miR-7-5p	-	-	*AGTR1*	↑	-
hsa-miR-143-3p	-	-	*ACE2*	Not measured	Not measured
hsa-miR-625-5p	↑	-	*ATP6AP2*	↑	-
hsa-miR-155-5p	↓	-	*AGTR1*	↑	-

↑ indicates upregulation, ↓ indicates downregulation, - indicates no change.

Table 2.7: Summary of changes in the levels of selected miRNAs and predicted target mRNAs and proteins of the renin-angiotensin system in 1% oxygen tension compared with 5% oxygen tension.

miRNA name	Effects of low oxygen (measured by microarray) n=3	Effects of low oxygen (measured by qPCR) n=9	Target RAS genes	Effects of low oxygen on target mRNA n=9	Effects of low oxygen on target protein n=9
hsa-miR-34	-	↓	*AGTR1*	-	-
hsa-miR-454-3p	-	↓	*ATP6AP2*	-	-
hsa-miR-514 (514a-3p / 514b)	↑	↓	*AGT*	↓	↑
			AGTR1	-	-
hsa-miR-330-3p	↓	↓	*REN*	-	-
hsa-miR-892	-	-	*AGT*	↓	↑
hsa-miR-378 (378a / c / i)	-	↓	*REN*	-	-
			ACE	↑	-
hsa-miR-181a-5p	↓	-	*REN*	-	-
			ACE	↑	-
hsa-miR-181a-3p	-	-	*REN*	-	-
hsa-miR-483-3p	-	↓	*ACE*	↑	-
			ACE2	Not	Not measured
			AGT	↓	↑
			AGTR1	-	-
hsa-miR-663b	-	-	*REN*	-	-
hsa-miR-181c-5p	-	-	*AGT*	↓	↑
hsa-miR-7-5p	-	-	*AGTR1*	-	-
hsa-miR-143-3p	-	-	*ACE2*	Not	Not measured
hsa-miR-625-5p	↓	-	*ATP6AP2*	-	-
hsa-miR-155-5p	-	-	*AGTR1*	-	-

↑ indicates upregulation, ↓ indicates downregulation, - indicates no change.

2.5 Discussion

We have found that the prevailing oxygen tension affects the expression of miRNAs in a human first trimester placental trophoblast cell line (HTR-8/SVneo). Results from our microarrays showed that the expression of 374 miRNAs were significantly different in cells incubated in 1% O_2 compared with cells incubated in 20% O_2. Of these, 9 miRNAs have been predicted to target the RAS and the oxygen-dependent changes in their expression was established by PCR. A further 6 miRNAs predicted to target the RAS, that were not significantly altered on the microarray, but that are decreased in first trimester placentae [12], were examined and 3 were shown to have decreased expression in 1% O_2 when samples were tested by RT-qPCR.

Interestingly, we found discrepancies in the expression of miRNAs upon attempting to validate the microarray results. This may have been due to the limited number of samples per oxygen tension (n=3) used for the microarray analysis, whilst a larger number of samples (n=9) were used for qPCR, and so non-significant results from the microarray may in fact be false negatives. It is, moreover, well known that qPCR is a more sensitive method for detecting differences in miRNA expression than microarrays [197], and we have used the microarray data as an initial indicator for miRNAs that may be of value for further study. For ease of interpretation of our results, Tables 2.6 (comparing 1% and 20% oxygen tensions) and 2.7 (comparing 1% and 5% oxygen tensions) summarise the miRNAs studied, their expressions in the microarray and qPCR data, their predicted RAS mRNA targets, and the targets' mRNA and protein expressions. We hypothesised that a lower oxygen tension would repress expression of miRNAs that target the RAS, allowing higher expression of RAS mRNA and protein (Figure 2.1); this is consistent with results shown in Table 2.7.

It should be noted that for some miRNAs (miR-34, miR-514, miR-892 and miR-378) an alternative isoform of the miRNA was used for the qPCR analysis to what was detected on the

microarray, due to the availability of primers. However, all isoforms are less than 2 nucleotide bases different from each other. As miRNAs are able to bind with various degrees of complementarity, we do not believe the isoforms chosen differ in their mRNA target binding, nor would the expression of the alternate miRNA isoform differ from the isoform measured on the microarray.

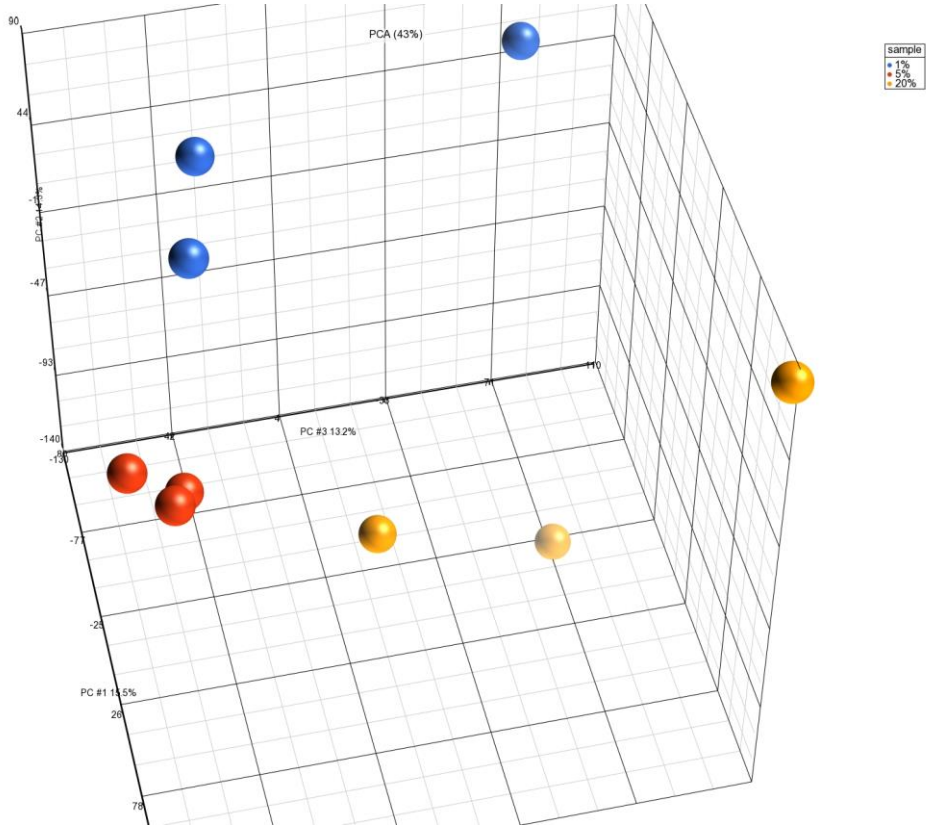
As mentioned in the Methods section, comparing between 1% and 20% oxygen tensions allowed proof of concept that oxygen was responsible for the regulation of miRNA expression. However, with regards to placental physiology, only 1% (7.6 mm Hg) and 5% (38 mm Hg) oxygen tensions are physiologically relevant, as they reflect the oxygen tensions in the chorionic villi in the first and second trimesters [30], respectively. Interestingly, as shown in Figure 2.2, miRNA expression in 5% oxygen was more similar to that seen in 20% oxygen than it was to the expression of miRNAs in 1% oxygen, suggesting that the use of 20% oxygen tension is not completely irrelevant and perhaps accentuates effects that are more subtle at 5%. However, for simplicity we have focussed our discussion around physiological differences in comparison of expression of cells incubates in 1% and 5% oxygen.

It has previously been shown that some components of the placental RAS are most highly expressed in the first trimester [3] and that culture of HTR-8/SVneo cells in low oxygen increased ACE protein levels [9]. Many factors can affect placental RAS gene expression; only one of these is placental miRNAs. Our data show that downregulation of miRNAs targeting most RAS mRNAs may boost RAS expression in 1% O_2 compared to 20% O_2 (Table 2.6), confirming our hypothesis that oxygen regulates these factors. In our physiologically relevant model of 1% compared with 5% oxygen, the level of expression of ACE and AGT and miRNAs targeting these mRNAs are significantly altered (Table 2.7). As well, in early gestation, when oxygen tension is lowest, the suppression of miRNAs that target prorenin, prorenin receptor, angiotensinogen and ACE mRNAs is greatest [12].

We propose that a low oxygen milieu suppresses the expression of miRNAs that target RAS mRNAs, allowing increased expression of RAS mRNAs and proteins (Figure 2.1). The increased AGT protein levels would result in a considerable increase in the potential of the placental RAS to modify placental cellular function and angiogenesis through increased production of angiotensin II and increased levels of AT_1R.

There are a number of reasons why miRNA and mRNA interactions are complex. MiRNAs are known to have many potential targets, because they require only partial complementarity to a target mRNA to mediate their effects. Although a specific miRNA may have high (>85%) complementarity to a certain mRNA target sequence, this mRNA sequence is still only one of many possible targets. MiRNAs are also diverse in their functions, with the potential to bind to target mRNAs and reduce protein synthesis [198, 199] by downregulating gene translation, nuclear mRNA transcript stability or modulating alternative splicing when reimported into the nucleus [200]. They also possess the ability to act as messengers in cell-to-cell communication, such as when they are exported into the extracellular space and are able to act in an autocrine, paracrine [201], exocrine or endocrine capacity. For this reason, it is not possible to draw a direct link at this stage between miRNA expression and mRNA and expression.

In conclusion, we have shown that a number of miRNAs, that are predicted to target placental RAS mRNAs, are regulated by oxygen. Some of these miRNAs are repressed in early gestation when placental oxygen tension is lowest. This repression could result in activation of the placental RAS, so facilitating its role in normal placentation. MiRNAs downregulated in late-onset preeclamptic placentae are oxygen-sensitive, and their downregulation in these relatively hypoxic placentae, may also be responsible for activation of the placental RAS at this time. This may lead to inappropriate effects on fetal blood flow to the placenta and possibly increased vasoconstriction of maternal spiral arterioles, thus compromising fetal oxygen and nutrient supply.



Supplementary Figure 2.1: Principal component analysis (PCA) examining miRNAs differentially expressed between oxygen tensions.

This diagram depicts the miRNA microarray data through PCA analysis. N=3 per oxygen tension group. 1% oxygen is shown in blue, 5% oxygen is in red and 20% oxygen is shown in yellow.

3. Oxygen-induced regulation of the renin-angiotensin system in first trimester chorionic villi; what is the role of microRNAs?

This prepared manuscript examines the effects of physiological oxygen tension on expression of miRNAs and the renin-angiotensin system components in first trimester chorionic villus explants sourced from primary tissue.

Manuscript Under Revision

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Anya L Arthurs*	Laboratory procedures Data analysis Manuscript preparation, revision and submission	
Sarah J Delforce*	Laboratory procedures Data analysis Manuscript preparation, revision and submission	
Eugenie R Lumbers	Experimental design Manuscript corrections	
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3.1 Abstract

Placentation occurs in a low oxygen environment that promotes angiogenesis and proliferation, during which expression of the placental renin-angiotensin system (RAS) is highest. In HTR-8/SVneo cells (a first trimester extravillous trophoblast (EVT) cell line), expression of RAS components are significantly upregulated in a low oxygen milieu and are associated with suppression of miRNAs predicted to target these placental RAS mRNAs. We postulated that incubation of first trimester human chorionic villous explants (CVE) in low oxygen would increase the expression of RAS components and that this would be associated with decreased expression of the miRNAs that target RAS mRNAs.

Human first trimester CVE (6-12 weeks gestational age) were cultured in 1%, 5% or 20% O_2 for 48h (n=6-8). Culture of CVE in low oxygen (1% O_2) stimulated angiotensin II type 1 receptor (AT_1R) protein and *VEGFA* mRNA levels. Angiotensin converting enzyme 2 (ACE2) mRNA and protein levels were significantly suppressed in CVE in 1% O_2. *ACE*, prorenin (*REN)* and angiotensinogen (*AGT)* mRNAs were suppressed in CVE in low oxygen. miR-663 (targeting *REN*), miR-330 (targeting *REN*), miR-892 (targeting *AGT*) and miR-454 (targeting *ATP6AP2*) were significantly decreased by culture in low oxygen, however these were not associated with upregulation of RAS genes they are known to target.

This study confirms that a low oxygen milieu regulates the expression of some RAS components in first trimester human placenta, promoting pro-angiogenic/proliferative AT_1R signalling and inhibiting ACE2 anti-angiogenic/proliferative signalling. However, not as a result of miRNAs predicted to target the RAS in early gestation placentae.

3.2 Introduction

The renin-angiotensin system (RAS) plays a significant role in normal placental development, growth and function. The placental RAS and other tissue renin-angiotensin systems are known to promote vascular development, cell growth and invasion [203].

Tissues only secrete prorenin, an enzymatically inactive precursor of renin. For prorenin to be activated, the 28 amino acid pro-segment must either unfold or be removed so that the catalytic site is exposed and cleavage of angiotensin I (Ang I) from angiotensinogen (AGT) can occur. Prorenin can be activated by binding to the (pro)renin receptor ((P)RR), which is an essential component of a vacuolar ATPase (V-ATPase). Ang I is converted to Ang II by angiotensin converting enzyme (ACE). Ang II is the major biologically active peptide of the cascade and acts mainly by binding to the angiotensin II type 1 receptor (AT_1R). As well as playing a critical role in fluid and electrolyte balance, the Ang II/AT_1R interaction promotes proliferation, angiogenesis, migration, invasion and fibrosis by stimulating the release of a number of factors including vascular endothelial growth factor (VEGF) [103-105].

The interaction of Ang II with AT_1R stimulates signalling pathways that include mitogen activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) and p85α-phosphoinositol 3-kinase (p85α-PI3K) pathways, that further contribute to growth and vascularisation in tissues [84, 85]. Ang II can also act via the angiotensin II type 2 receptor (AT_2R) and have effects that are opposite to those produced by the Ang II/AT_1R. These include vasodilation and apoptosis [81-83]. Ang II is subject to degradation by a number of proteolytic enzymes. Most significantly, removal of an amino acid from its carboxy terminal by ACE2 (a homologue of ACE), produces Ang–(1-7), which acting via the Mas receptor further antagonises Ang II/AT_1R -mediated signalling [86].

We have previously shown that most of the genes involved in the RAS cascade are expressed in the human placenta, and that the expression of prorenin (*REN)*, (P)RR (*ATP6AP2)*, *AGT*, *ACE2* and AT_1R *(AGTR1)* mRNAs are significantly higher in first trimester placentae compared with term [204]. In the first trimester, when expression of these placental RAS components is highest, the prevailing oxygen tension is lowest. Rodesch *et al.,* among others, showed that the oxygen content within first trimester intervillous space is between 1-3% O_2, whereas closer to the decidual bed it is between 5-8% O_2 [205]. At this time development of the early placenta is characterised by proliferation of cytotrophoblast cells and angiogenesis under the influence of the hypoxic milieu [206-209].

We have also previously shown that culture of HTR-8/SVneo cells in a low oxygen environment (1% O_2) is associated with increased expression of *AGTR1* and *VEGFA* mRNA as well as ACE and VEGFA protein levels [10]. These findings show that a low oxygen environment stimulates the capacity of HTR-8/SVneo cells to produce Ang II and activate AT_1R intracellular signalling. Furthermore, if Ang II/AT_1R signalling is inhibited by the specific AT_1R antagonist, losartan, the expression of an angiogenic/proliferative trophoblast phenotype (i.e. increased expression of the pro-angiogenic factors VEGFA mRNA and protein, angiopoietin 2 (ANGPT2) mRNA, and plasminogen activator inhibitor 1 (SERPINE1) mRNA and protein levels), angiogenic capacity and cell viability in HTR-8/SVneo cells is inhibited [210].

There are miRNAs that target the human placental RAS and their pattern of expression across gestation suggests that they might influence the gestational pattern of expression of the human placental RAS [211]. We have also shown that the expression of ten miRNAs found in a first trimester EVT cell line (HTR-8/SVneo), which are predicted to target placental RAS genes, are down-regulated when cells are incubated in 1% O_2. This suppression of miRNAs in cells cultured in 1% O_2 occurs in association with increased expression of their predicted RAS mRNA targets, namely, *ACE*, *AGTR1* and *ATP6AP2* mRNAs [212].

To determine whether these findings could be replicated in chorionic villous explants (CVE) and if low oxygen induced changes in the expression of the same 10 miRNAs and their putative placental RAS targets, we studied the effects of oxygen tension on the expression of these 10 miRNAs and on RAS target mRNAs and their proteins in first trimester CVE.

3.3 Methods

Ethics

This research was approved by the Hunter Area Research Ethics Committee and the University of Newcastle Human Research Ethics Committee.

Culture of Early Gestation Placental Explants

Early gestation placental tissue (6-12 weeks, Newcastle, Australia; gestational age was established using the date of the last menstrual period and ultrasound) was obtained following elective termination of pregnancy. No correlation between gestational age and miRNA expression levels was determined. All samples were obtained with written, informed consent. Samples were collected in glycerol substituted artificial cerebrospinal fluid (g-ASCF) containing 250 mM glycerol, 26 mM $NaHCO_3$, 11 mM glucose, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, and 2.5 mM $CaCl_2$ and bubbled with Carbinox (95% O_2, 5% CO_2) to achieve a pH of 7.3, made fresh on the day of collection. All samples were transported on ice and arrived in the laboratory within 1h of the procedure. Villous tips were dissected from the tissue using an Olympus SZ51 dissecting microscope.

100 mg of placenta was placed in each well of a 6-well plate and 2 mL of complete culture medium (DMEM supplemented with 10% fetal bovine serum (SAFC Biosciences, Darmstadt,

Germany), 2 % antibiotic-antimycotic (ThermoFisher Scientific, Massachusetts, United States) and 1% L-glutamine (ThermoFisher Scientific) was added to each well. Explants were equilibrated overnight in 5% CO_2 in room air at 37°C. After 24h, 2 mL of complete media, containing 1 % antibiotic-antimycotic, was added to each well and explants were placed into oxygen chambers for 48h (1%, 5% and 20% O_2). Chambers were flushed every 24 h. After 48h explants and culture media were snap frozen in liquid nitrogen and stored at −80°C for subsequent protein and mRNA analyses. Between six and eight individual placentae were obtained and explants were cultured in duplicate at each O_2 tension and levels of mRNA and miRNA measured. A further six to eight individual placentae were obtained to measure proteins (n=6-8/group in duplicate).

RNA extraction and DNase Treatment

Total RNA was extracted from these placental explants using TRIzol Reagent according to the manufacturer's instructions (Invitrogen). Explants were homogenised with 1 mL of TRIzol in the Precellys24 homogenizer (5000 rpm, 2 x 30 sec, with 20 sec rest in between). DNase I treatment was performed on all samples (Qiagen). The integrity of total RNA and miRNAs was examined by gel electrophoresis and quantified using the Nanodrop 2000 (data not shown).

Semi-quantitative real-time reverse transcriptase polymerase chain reaction (qPCR)

All RNA samples underwent reverse transcription to cDNA (Superscript III First-Strand Synthesis for RT) as per the manufacturer's instructions (Thermo Fisher Scientific). qPCR was performed in an Applied Biosystems 7500 Real Time PCR System using SYBR Green for detection. Each reaction contained 5 µL of SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA), RAS primers (Table 3.1), cDNA reversed transcribed from

Table 3.1: Primers used for PCR analysis

Official gene symbol	GenBank Accession #	Primer Sequence (5'- 3')	Concentration	Melt temperature
ACTB	NM_001101	Fw: CGCGAGAAGATGACCCAGAT Rv: GAGTCCATCACGATGCCAGT	1000 nM	78°C
ACE	NM_000789	Fw: AAGCAGGACGGCTTCACAGA Rv: GGGTCCCCTGAGGTTGATGTAT	200 nM	85°C
ACE2	NM_021804	Fw: GCAAGCAGCTGAGGCCATTATA Rv: ATCTTCAATCAACTGGCCGC	400 nM	78°C
AGT	NM_000029	Fw: CAACACCTACGTCCACTTCCAA Rv: TGTTGTCCACCCAGAACTCCT	200 nM	62°C
AGTR1	NM_000685	Fw: CCTCAGATAATGTAAGCTCATCCAC Rv: GCTGCAGAGGAATGTTCTCTT	200 nM	77°C
ATP6AP2	NM_005765	Fw: CCTCATTAGGAAGACAAGGACTATCC Rv: GGGTTCTTCGCTTGTTTTGC	200 nM	60°C
VEGFA	M32977	Fw: CTACCTCCACCATGCCAAGT Rv: GCAGTAGCTGCGCTGATAGA	400 nM	75°C

Abbreviations: Fw (forward), Rv (reverse)

10 ng total RNA, and water to 10 µL. Messenger RNA abundance was calculated as described previously, using the $2^{-\Delta\Delta CT}$ method and expressed relative to ACTB mRNA and a calibrator sample (a term placental sample collected at elective Caesarean section) [204].

Quantification of miRNA Expression by qPCR

Total RNA (5 ng) was reverse transcribed to generate cDNA using the Taqman™ miRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions as previously described [211]. Pre-designed Taqman™ probes (ThermoFisher Scientific) were used to determine miRNA levels in placental explants. cDNA was amplified using the TaqMan™ ProAmp Master Mix (Applied Biosystems). qPCR analysis was performed using TaqMan™ Universal PCR mix No AmpErase UNG and TaqMan™ miRNA Assays (Applied Biosystems). Results were quantified using a 7500 Real-Time PCR System (Applied Biosystems). The expression levels of ten miRNAs (miR-181a-5p, miR-181a-3p, miR-330, miR-892a, miR-378g, miR-514b, miR-454, miR-34c-5p, miR-483-3p and miR-663b) were determined by calculating $2^{-\Delta\Delta CT}$ using RNU44 (a highly conserved small nucleolar RNA in the growth arrest specific 5 transcript) as the house-keeping gene.

Protein extraction

Whole cell protein was isolated from placental explants using radioimmunoprecipitation assay buffer (RIPA, 50 mM Tris–HCl, 158 mM NaCl, 1% Triton X–100, 1% sodium dodecyl sulphate, SDS) supplemented with a Pierce Halt™ complete protease inhibitor cocktail kit (Thermo Fisher Scientific). Protein was quantified using the Pierce BCA Protein assay kit (Life Technologies) according to the manufacturer's instructions.

Quantification of protein levels by Immunoblotting

ACE2, AT_1R and (P)RR proteins were measured by immunoblot. The Novex® NuPAGE® SDS-PAGE Gel System (Thermo Fisher Scientific) was used for electrophoresis of the protein in 4–12% Bis-Tris 1.5 mm gels, and then transferred onto PVDF membrane (Thermo Fisher Scientific).

Membranes were blocked for 1h at room temperature in 5% BSA (Bovine Serum Albumin)/5% skim milk powder in 0.1% TBS–T before being incubated with the respective primary antibody (Table 3.2) overnight at 4°C. The membrane was then incubated with the secondary antibody (1:5000, goat anti-rabbit IgG antibody, HRP-conjugate (12-348, Merck, Burlington, Massachusetts, United States)) in 3% skim milk powder in 0.1% TBS–T for 1h at room temperature. Protein bands were detected using an ECL detection kit (Amersham, GE Healthcare, Illinois, United States) and imaged using an Amersham Imager 600 (Amersham, GE Healthcare).

Membranes were then stripped in 0.1M NaOH to allow detection of the internal control (β–actin/ACTB). After blocking (5% skim milk in 0.1% TBS–T) for 1h at room temperature, membranes were incubated with the primary antibody (Table 3.2) for a further 1h at room temperature. Membranes were then incubated with the secondary antibody and detected as described above. The density of each band (determined by the Amersham Imager 600 analysis software) was corrected for its respective loading control (ACTB) and further normalized to an internal control sample (pooled term placenta collected at Caesarean section) on each membrane. Samples were run in duplicate and averaged for the final analysis.

Table 3.2: Antibodies for Western Blotting

Protein Name	Protein Size (~kDa)	Protein Concentration (µg)	Primary Antibody	Manufacturer	Ab concentration
ACE2	97	5	Rabbit polyclonal to Angiotensin Converting Enzyme 2 (ab15348)	Abcam (Cambridge, UK)	1:1000
AGTR1	40	5	Rabbit monoclonal to Angiotensin II Type 1 Receptor (ab124734)	Abcam (Cambridge, UK)	1:1000
ATP6AP2	40	10	Rabbit polyclonal to ATP6AP2 (ab40790)	Abcam (Cambridge, UK)	1:1000
ACTB	42	N/A	Rabbit polyclonal to beta Actin (ab82270)	Abcam (Cambridge, UK)	1:5000

ELISAs

Placental ACE and AGT proteins were measured by ELISA. The human ACE Duoset ELISA kit (R&D Systems) was used to measure placental ACE protein. The human SERPINE8 Duoset ELISA kit (R&D Systems) was used to measure placental AGT protein. All proteins were assayed on single plates. The intra-assay coefficients of variation were 4.92% and 4.14% respectively for ACE and AGT.

Statistics

Data were analysed using Graphpad Prism, version 7.0. Data points that lay >2 standard deviations from the mean were considered outliers and were removed from the analysis. One-way non-parametric ANOVA was used to determine the overall effects of oxygen on the relative abundance of mRNA, miRNA and protein expression. Kruskal Wallis test with Dunn's multiple comparisons test was then used to determine differences between each oxygen tension. To determine the associations between the abundance of particular RAS mRNAs and miRNAs known to target these components, Spearman's non–parametric correlations were used. Significance was set at $p<0.05$ for all data.

3.4 Results

Effect of O_2 on RAS mRNA and protein levels in first trimester placental explants

Measurable amounts of *REN, ATP6AP2, AGT, ACE, ACE2* and *AGTR1* mRNA in human first trimester CVE were identified at all 3 oxygen tensions (Figures 3.1, 3.2 and 3.3).

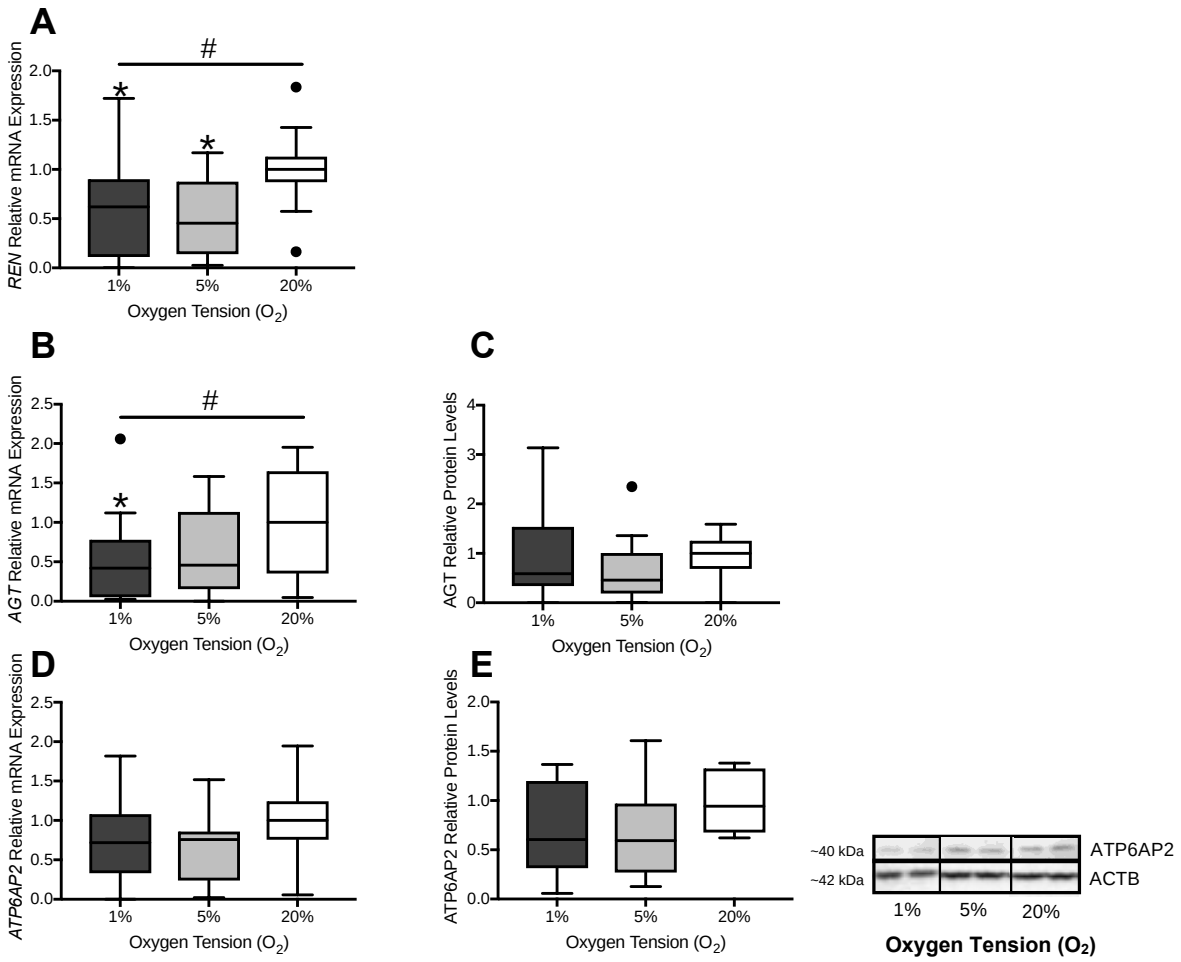


Figure 3.1: Effect of oxygen on *REN, AGT*, and *ATP6AP2* mRNA and protein in first trimester chorionic villi.

A *REN* mRNA was significantly affected by O_2 tension; levels in 1% and 5% were less than 20% O_2. **B** *AGT* mRNA was significantly affected by oxygen tension. *AGT* mRNA expression in 1% O_2 was less than in cells cultured in 20% O_2. **C** AGT protein levels were not affected by O_2. Both **D** *ATP6AP2* mRNA and **E** ATP6AP2 protein levels were not altered by O_2.

*Data are expressed as median and interquartile range (IQR). n=6. *P<0.05. Single dots represent data points that were outside the IQR. Immunoblot images are all sourced from the same gel, and were spliced for aesthetics only.*

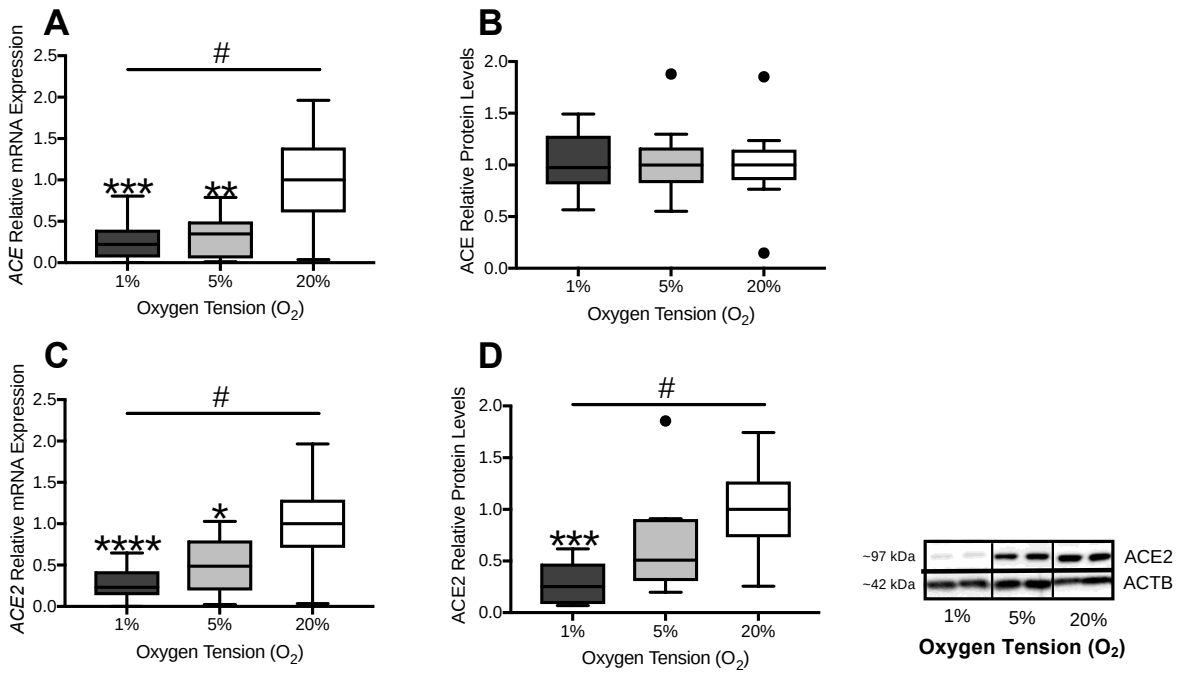


Figure 3.2: Regulation of angiotensin converting enzymes in first trimester chorionic villi by oxygen.

A *ACE* mRNA, **C** *ACE2* mRNA and **D** ACE2 protein levels were significantly affected by oxygen. **A** *ACE* mRNA expression was significantly lower in villous explants cultured in either 1 or 5% O_2 when compared with 20%. **B** ACE protein levels within villi (measured by ELISA) were not affected by O_2. Both **C** *ACE2* mRNA and **D** ACE2 protein levels (measured by immunoblot) were significantly lower in villi cultured in 1% O_2 when compared with villi cultured in 20% O_2. **C** *ACE2* mRNA expression was also reduced when villi were cultured in 5% O_2 compared with levels in villi cultured in 20% O_2. *Data area expressed as median and IQR. n=6/group. *P<0.05, **P<0.01, ***P<0.001,****P<0.0001. (IQR). n=6. *P<0.05. Single dots represent data points that were outside the IQR. Immunoblot images are all sourced from the same gel, and were spliced for aesthetics only.*

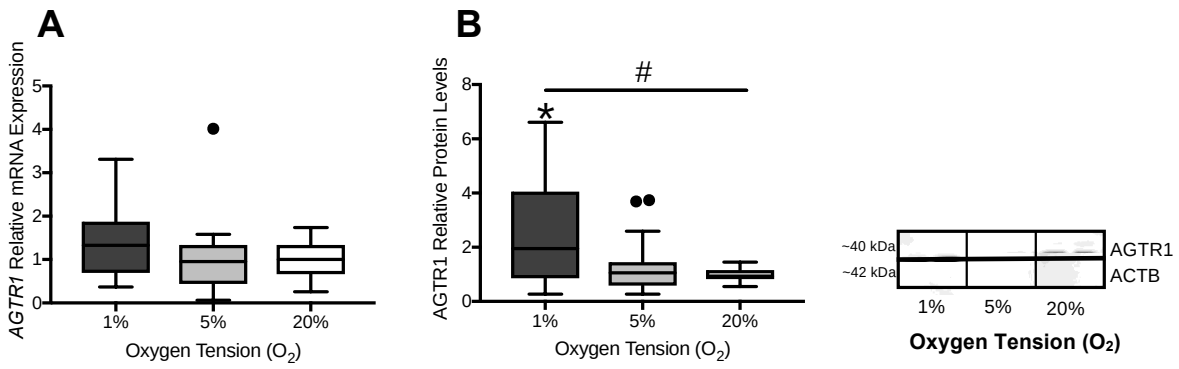


Figure 3.3: Effect of oxygen on *AGTR1* mRNA and protein levels in first trimester placental villous explants.

A *AGTR1* mRNA expression in villous explants was not affected by oxygen tension but **B** AT_1R protein levels however were affected by oxygen. Placental explants cultured in 1% O_2 had significantly greater levels of AT_1R protein compared with levels in villi cultured in 20% O_2. *Data are expressed as median and IQR. n=6/group. *P<0.05. (IQR). n=6. *P<0.05. Single dots represent data points that were outside the IQR. Immunoblot images are all sourced from the same gel, and were spliced for aesthetics only.*

REN mRNA was significantly less if CVE were cultured in 1% and 5% O_2 compared with the abundance of *REN* in CVE incubated in 20% O_2 (p=0.007 and p=0.020 respectively; Figure 3.1A). Prorenin protein was not measured in this study. *AGT* mRNA expression was also less in CVE cultured in 1% O_2 when compared with those cultured in 20% O_2 (p=0.044; Figure 3.1B). AGT protein levels however, were not affected (Figure 3.1C). Neither *ATP6AP2* mRNA nor (P)RR protein levels were significantly affected by oxygen.

Both *ACE* and *ACE2* mRNAs were significantly suppressed when CVE were cultured in 5% or 1% O_2 compared with cultures incubated in 20% O_2 (p=0.001 and p=0.0002, and p=0.021 and p=0.0001 respectively; Figure 3.2A, 3.2C). ACE protein levels were however, unaffected by O_2 (Figure 3.2B, 3.2D). ACE2 protein levels were significantly less in CVE cultured in 1% O_2 compared with those cultured in 20% O_2 (p=0.0006; Figure 3.2D), but not compared with those cultured in 5% O_2.

CVE cultured in low oxygen (1% O_2) tended to have higher levels of expression of *AGTR1* mRNA but this was not significant (p=0.195; Figure 3.3A). However, AT_1R protein levels were greater when CVE were cultured in 1% O_2 compared with CVE cultured in 20% O_2 (p=0.033; Figure 3.3B).

Effect of O_2 on a pro-angiogenic target of placental RAS activation; vascular endothelial growth factor (VEGF) mRNA expression in first trimester placental explants

VEGFA mRNA expression was significantly affected by the O_2 tension in which CVE were incubated (p=0.022; Figure 3.4). *VEGFA* mRNA expression was significantly greater in explants cultured in 1% O_2 when compared with expression in those cultured in 20% O_2 (p=0.017; Figure 3.4).

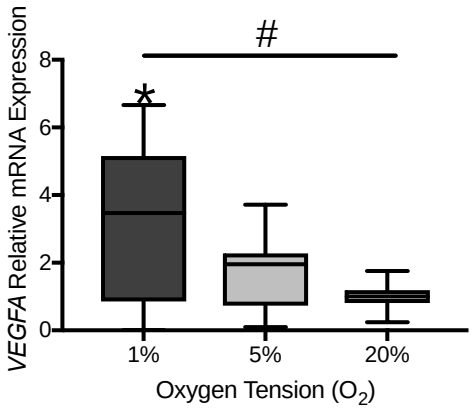


Figure 3.4: Regulation of *VEGFA* mRNA expression in first trimester placental villous explants by oxygen.

VEGFA mRNA abundance was affected by the oxygen tension at which explants were incubated. *VEGFA* mRNA expression was greater in villi incubated in 1% O_2 compared with culture in 20% O_2.

*Data are expressed as median and interquartile range. n=8/group. *P<0.05.*

Effect of O_2 tension in first trimester placental explants on expression of miRNAs predicted to target the RAS

Of the ten miRNAs tested; miR-663b (predicted target: *REN*), miR-330 (*REN*), miR-181a-3p (*REN*), miR-181a-5p (*REN, ACE*), miR-378g (*REN, ACE*), miR-892 (*AGT*), miR-483-3p (*AGT, ACE, ACE2, AGTR1*), miR-454 (*ATP6AP2*), miR-34c-5p (*AGTR1*) and miR-514b (*AGT, AGTR1*), only the abundance of miR-514b was too low to be detected in CVE at any oxygen tension (data not shown).

There was an effect of O_2 tension on the expression of 4 out of the 10 miRNAs studied (Figure 3.5). Expression of miRNAs; miR-663 (*REN*), miR-892 (*AGT*) and miR-454 (*ATP6AP2*) in 1% O_2 was less than their expression in 20% O_2 (p=0.0005, p=0.0001 and p=0.014; Figure 3.5A. 3.5F and 3.5H). In addition, expression of miR-663, miR-330 and miR-892 was lower in explants cultured in 5% O_2 compared with 20% O_2 (p=0.010, p=0.014 and p=0.035; Figure 3.5A, 3.5B and 3.5F).

The expression of miR-181a-3p (*REN*; Figure 3.5C), miR-181a-5p (*REN, ACE*; Figure 3.5D), miR-378g (*REN, ACE*; Figure 3.5E) miR-483-3p (*AGT, ACE, ACE2, AGTR1*; Figure 3.5G) and miR-34c-5p (*AGTR1*; Figure 3.5I), were not affected by the O_2 tension in which explants were incubated.

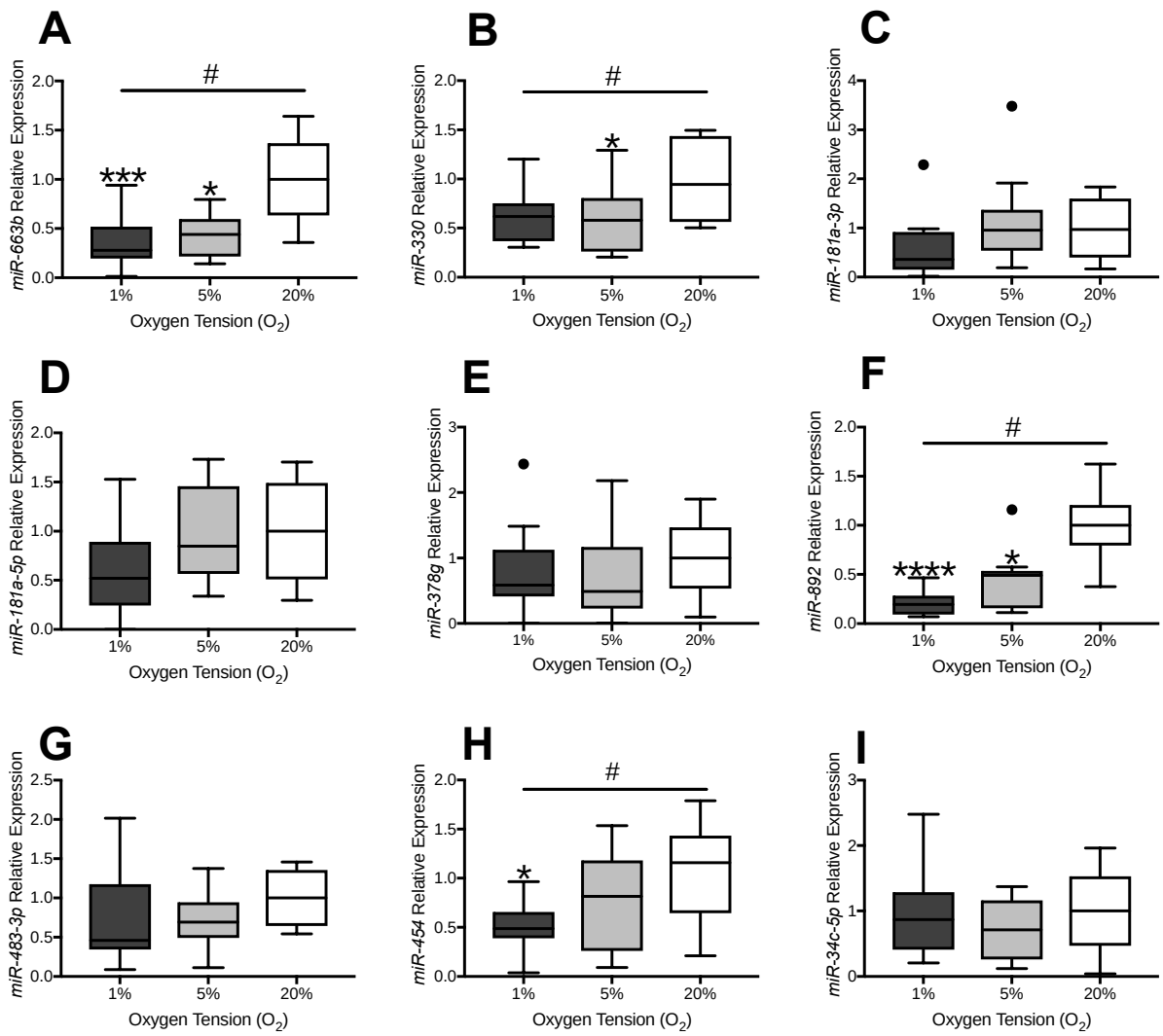


Figure 3.5: Regulation of miRNAs predicted to target RAS components by oxygen tension in first trimester chorionic villous explants (CVEs).

Culture of CVEs in 5% and 1% O_2 significantly reduced the expression of **A** miR-663 *(REN)* and **F** miR-892 *(AGT)* compared with culture in 20% O_2. **B** miR-330 *(REN)* expression was supressed by culture in 5% O_2 when compared with culture in 20% O_2. **H** miR-454 *(ATP6AP2)* expression was significantly supressed by culture in 1% O_2 when compared with culture in 20% O_2. Whilst **D** miR-181a-5p *(REN, ACE)* levels tended to be suppressed in CVEs when cultured in low oxygen, this failed to reach significance. Expression of **C** miR-181a-3p *(REN)*, **E** miR-378g *(REN, ACE)*, **G** miR-483-3p *(AGT, ACE, ACE2, AGTR1)* and **I** miR-34c-5p

(AGTR1) were unaffected by O_2 tension. *Data are expressed as median and IQR. n=6/group. *P<0.05, ***P<0.001,****P<0.0001. (IQR). n=6. *P<0.05. Single dots represent data points that were outside the IQR. Immunoblot images are all sourced from the same gel, and were spliced for aesthetics only.*

3.5 Discussion

In first trimester CVEs, culture in low O_2 was associated with decreased expression of *REN*, *AGT*, *ACE* and *ACE2* mRNAs as well as ACE2 protein but the expression of AGTR1 protein was increased. This study has also demonstrated that the expression of four miRNAs (miR-663 (*REN*), miR-330 (*REN*), miR-892 (*AGT)* and miR-454 (*ATP6AP2*)), that have RAS mRNAs as their potential targets, were lower in CVEs cultured in 1% and/or 5% O_2 compared with 20% O_2.

REN mRNA expression was downregulated in low oxygen compared to 20% O_2 in cultured first trimester CVEs (Figure 3.1A). Interestingly, miR-330 and miR-663, which are predicted to target *REN* with high complementarity, were also downregulated in low oxygen (Figures 3.5B and 3.5A, respectively). However, miR-181a-3p, which is predicted to target *REN* and was downregulated in 1% O_2 in HTR-8/SVneo cells [212] was unaffected by O_2 in CVEs. As there is evidence that miR-330 [15] and miR-663 [14] downregulate *REN* in other tissues, it may be that other factors are overshadowing the effect of the miRNA:mRNA interactions or simply that this relationship is not occurring in first trimester chorionic villi. Similar to this, *AGT* mRNA was also significantly downregulated in low oxygen (Figure 3.1B), with no change in AGT protein, while miR-892, which is predicted to target *AGT* mRNA, was also downregulated in low oxygen.

In this study, oxygen had no effect on the expression of *ATP6AP2* mRNA or its protein levels in CVEs (Figures 3.1D and 3.1E, respectively). Interestingly, HTR-8/SVneo cell expression of *ATP6AP2* mRNA tends to be increased when cells are cultured in 1% O_2 [212, 214], although there were no changes in (P)RR protein levels [212]. There are a few other reports suggesting that oxygen might regulate *ATP6AP2* mRNA and protein levels. At term, placentae from women living at high altitude have significantly higher levels of (P)RR protein compared with placentae from women living at sea level, but there are no changes in *ATP6AP2* mRNA

expression [215]. Furthermore, both hypoxia and ischemia/reperfusion have been associated with increased (P)RR protein in a H9c2 rat cardiomyocyte cell line [216] and hypoxia induces (P)RR protein levels in a pre-adipose cell line (3T3-L1) without affecting *ATP6AP2* mRNA expression [217]. This might indicate that the regulation of (P)RR is cell specific and perhaps the complex composition of CVEs masks the effects of oxygen on expression and regulation by miRNAs.

Culture of first trimester CVEs in low oxygen (1% O_2) suppressed mRNA and protein levels of *ACE* and *ACE2* mRNA (Figures 3.2B and 3.2D, respectively). While placental *ACE2* mRNA decreases with gestational age, *ACE* mRNA is significantly increased in term placentae [204]. This may be due to the localisation and roles of these two enzymes within the placenta. ACE protein is localised to the fetal endothelium of chorionic villi, and increases as fetal vascular endothelium in the chorionic villi becomes more abundant with increasing gestational age [218]. Antenatal hypoxia is associated with a reduction in placental *ACE* mRNA levels in mice [219]. ACE2 protein is localised to the syncytiotrophoblast and villous stroma [204]. *ACE2* mRNA was significantly reduced by maternal hypoxia in two studies in mice [219, 220] but different results were found in regard to ACE2 protein. As mentioned, ACE2 is responsible for the conversion of pro-proliferative/pro-angiogenic/vasoconstrictive Ang II to the antagonist Ang-(1-7). Thus, low levels of *ACE2* mRNA and protein in first trimester chorionic villi cultured in low oxygen may indicate that Ang II/ AT_1R signalling is dominant and regulated by oxygen.

Culture in low oxygen (1% O_2) was associated with increased expression of *AGTR1* mRNA and protein in CVEs (Figures 3.3A and 3.3B, respectively). This finding is similar to that seen in HTR-8/SVneo cells in response to oxygen [214]. This increase in *AGTR1* mRNA in HTR-8/SVneo cells cultured in low oxygen is associated with a downregulation of a number of miRNAs (miR-34c-5p, miR-514b and miR-483-3p) known to target *AGTR1* [212]. In first trimester CVEs however, miR-34c-5p and miR-483-3p were not significantly affected by oxygen (Figure 3.5). Furthermore, miR-514b (belonging to the placenta-specific chromosome

19 miRNA cluster) [221] was undetectable in first trimester chorionic villi at all oxygen tensions (data not shown).

Culture of CVEs in low oxygen (1% O_2) increased the expression of *VEGFA* (Figure 3.4) in first trimester chorionic villi as it did in HTR-8/SVneo cells [214]. In HTR-8/SVneo cells cultured in low oxygen, the specific AT_1R antagonist, losartan, significantly inhibited the low oxygen induced expression of angiogenic factors *VEGFA*, plasminogen activator inhibitor-1 (*SERPINE1)* and angiopoietin-2 *(ANGPT2* mRNA [210]). This indicates that similar mechanisms of Ang II/ AT_1R -mediated angiogenesis may be occurring between both HTR-8/SVneo cells and first trimester CVEs cultured in low oxygen, however it does not appear that AT_1R is directly regulated by miRNAs.

miR-892, which was significantly downregulated by low oxygen in CVEs, is not only predicted to target AGT but also peroxisome proliferator-activated receptor γ (PPAR-γ) mRNA. Through targeted inhibition of PPAR-γ mRNA, miR-892 could affect *VEGFA* expression. Ligand binding to PPAR-γ has been shown to stimulate VEGF gene expression, by interacting with the PPAR-γ responsive element in the VEGF gene promoter [222]. Decreased expression of miR-892 would allow upregulation of PPAR-γ and, in turn, the angiogenic factor VEGF, which is consistent with the pro-angiogenic environment in the early gestation placenta.

Failure to detect miR-514b in CVEs and the lack of response of any of the other miRNAs shown to be suppressed when HTR-8/SVneo cells are incubated in 1% O_2 could contribute to these differences in oxygen sensitivity of RAS genes and proteins in CVEs and HTR-8/SVneo cells that we have observed. The lack of response to O_2 of miRNAs; miR-181a-5p (*REN, ACE*), miR-181a-3p (*REN*), miR-483-3p (*AGT*, *ACE*, *ACE2, AGTR1*) and miR-378g (*REN, ACE*) may explain, in part, why *ACE* mRNA abundance was low in CVEs cultured in 1% O_2. On the other hand, there was upregulation of AT_1R protein levels despite the lack of an effect

of oxygen on expression of miR-34c (*AGTR1*). Since we do not find parallel changes in either the sensitivity to O_2 of CVEs and HTR-8/SVneo cell miRNAs that target RAS genes and proteins, microarrays are required to analyse the profile of expression of miRNAs in CVEs.

It is interesting to note that there is a clear difference in the oxygen-regulated expression of RAS genes in CVEs compared with that previously demonstrated in HTR-8/SVneo cells [214]. In CVEs, *REN* and *AGT* mRNA were lower in 1% O_2 cultures and there was no effect of O_2 on the (P)RR. In HTR-8/SVneo cells on the other hand, prorenin and AGT protein levels were increased in low O_2 cultures and *ATP6AP2* mRNA abundance was increased [212]. Compared with HTR-8/SVneo cells, however, the abundance of all RAS mRNAs in CVEs was greater than in HTR-8/SVneo cells (Delforce SJ., Morosin SK., Tamanna S., Drury HR., Quinn RK., Lim R., Tadros MA., Lumbers ER., Pringle KG., Unpublished observations). In contrast, expression of the same four miRNAs that were reduced by low oxygen in CVEs have also been shown to be reduced in HTR-8/SVneo cells incubated in 1% O_2 [212] and/or their expression is low in early gestation human placentae [211], when RAS expression is maximal [218].

These differences in sensitivity to oxygen in terms of expression of miRNAs that target the RAS and of expression of RAS genes probably reflect differences in the populations of cell types in CVEs compared with the homogeneity of HTR-8/SVneo cells. HTR-8/SVneo cells are extravillous trophoblasts (EVTs). The unique locations and functions of chorionic villous trophoblasts compared with EVTs might explain their different expression patterns and sensitivities to oxygen. Perhaps miRNAs that target RAS mRNAs in EVTs are more sensitive to O_2 than those located in chorionic villi, although there is some overlap, as shown by the oxygen sensitivity of miR-663b (*REN*), miR-330 (*REN*), miR-892 (*AGT*), and miR-454 (*ATP6AP2*).

Differences in gestational ages of the chorionic villous explants compared with HTR-8/SVneo cells could also contribute to differences in O_2 sensitivity. In a cohort of samples which contained placental tissues collected early in the first trimester and later, i.e. first versus early second trimester, the greatest expression of RAS genes was found very early in gestation in accordance with the very high levels of prorenin found in the human blastocyst [204, 223]. A second study using a smaller subset of slightly later gestational ages (10-11 weeks) did not yield such marked differences in placental RAS expression [211]. In the current study we studied chorionic villous explants aged 6-12 weeks, with a mean age of 9.2 weeks, perhaps we would find a more marked sensitivity to oxygen if we examined CVEs with a lower mean gestational age (i.e. 6-8 weeks) [204].

Overall, we have added to the evidence that oxygen regulates RAS components and miRNAs that are predicted to target RAS mRNAs within the developing placenta. Furthermore, we have demonstrated that regulation of both the RAS and miRNAs known to target them by oxygen is different between a first trimester extravillous trophoblast cell line, HTR-8/SVneo, and first trimester chorionic villous explants. Further investigation of cell specific regulation of miRNAs and the RAS is warranted to further tease out the interaction with oxygen and contribution to placental development in both uncomplicated and complicated pregnancies.

4. miRNA mimics that target the placental renin-angiotensin system inhibit trophoblast proliferation

Arthurs AL, Lumbers ER, Pringle KG. *MicroRNA mimics that target the placental renin-angiotensin system inhibit trophoblast proliferation.* Molecular Human Reproduction (2019) **25**(4): 218–227

This published manuscript examines the functional effects of treatment with mimics of miRNAs that target the renin-angiotensin system on trophoblast proliferation. These experiments were performed in a first trimester extravillous trophoblast cell line.

Accepted Manuscript

Author	**Contribution**	**Signature**
Anya L Arthurs	Laboratory procedures Data analysis Manuscript preparation, revision and submission	
Eugenie R Lumbers	Experimental design Manuscript corrections	
Kirsty G Pringle	Experimental design Manuscript corrections	

4.1 Abstract

In early gestation, the human placental RAS is upregulated and plays a role in placental development. Among other functions, signaling through the angiotensin II type 1 receptor (AT_1R) initiates proliferation. Many miRNAs targeting placental RAS mRNAs are downregulated at this time. We propose that in early gestation miRNAs that target the placental RAS are downregulated, allowing for the increased RAS expression and proliferation required for adequate placentation.

HTR-8/SVneo cells were used to assess the effect of nine miRNA mimics (at 0.08, 0.16, 0.32 and 0.64 ng/μL) on trophoblast cell proliferation and predicted RAS target mRNAs. The effect of the miRNA mimics on the rate of cell proliferation was assessed using the xCELLigence real-time cell analysis system over 48h. Levels of miRNAs and predicted RAS target mRNAs were determined by RT-PCR (qPCR, n=9/group). Statistically different levels of expression were determined.

All nine miRNA mimics significantly affected the proliferation rates of HTR-8/SVneo cells. Five of the miRNA mimics (miR-181a-5p (predicted to target: *REN, ACE*), miR-378 (*REN, ACE)*, miR-663 *(REN)*, miR-483-3p (*ACE, ACE2, AGT, AGTR1)* and miR-514 (*AGT*)) were associated with a dose-dependent reduction in cell proliferation. Seven of the mimics significantly decreased expression of at least one of their predicted target RAS mRNAs. Our study shows that miRNAs targeting placental RAS mRNAs play a role in controlling trophoblast proliferation. As placentation is largely a process of proliferation, changes in expression of these miRNAs may be partly responsible for the expression of the placental RAS, proliferation and placentation.

4.2 Introduction

During gestation, the placenta undergoes rapid trophoblast proliferation and development to cater to the needs of the growing fetus. The proliferative arm of the placental renin-angiotensin system (RAS) includes the following components: (pro)renin receptor ((P)RR), prorenin, the angiotensin converting-enzyme (ACE), angiotensinogen (AGT), angiotensin (Ang) I and II, and the Ang II type 1 receptor (AT_1R). This arm of the RAS is involved in cell and tissue growth [98] and its expression in the placenta changes over gestation [3]. Importantly, irregular expression of the RAS is associated with placental complications, insufficient placental growth and intrauterine growth restriction [118]. Expression of the majority of the placental RAS components are highest in the first trimester of pregnancy, when the placenta is developing most rapidly. As gestation continues, expression of the placental RAS decreases [3], which is suggested to be partly due to the actions of post-transcriptional regulators, including microRNAs [12].

It is well established that miRNA profiles in both the placenta and the maternal circulation are significantly changed in pathological pregnancies [12, 13, 65, 171, 175, 224-229], with a number of miRNAs predicted to target the placental RAS being among those that are altered. For example, miR-378, miR-514, miR-663 and miR-892 were found to be overexpressed in placentae from women with PE [12], a disease of placental insufficiency. Importantly, dysregulation of placental RAS mRNA expression is also seen in women with PE [95, 230, 231].

During pregnancy, many miRNAs predicted to target the placental RAS have a level of expression that is reciprocal to that of placental RAS genes; that is, in early pregnancy (<12 weeks) when RAS expression is highest, miRNAs predicted to target these RAS mRNAs are at their lowest [12]. We hypothesise that endogenous miRNAs assist in the post-transcriptional control of RAS gene expression to regulate normal placental growth. The direct effects of

RAS-targeting miRNAs on trophoblast proliferation have not yet been determined, so that the links between RAS expression, reciprocal miRNA expression and placental growth are by association only. We propose that miRNAs interact with the placental RAS to inhibit translation, and this results in decreased trophoblast proliferation. We have tested this hypothesis using miRNA mimics, compounds that mimic endogenous miRNAs, and measured the effects of increased expression of miRNAs that target the RAS on proliferation of extravillous trophoblast cells.

4.3 Methods

Cell Culture

HTR-8/SVneo cells were cultured at 37°C with 5% CO_2 in room air in RPMI-1640 medium (HyClone, GE Healthcare Australia Pty Ltd., Paramatta, Australia) supplemented with 10% heat inactivated fetal calf serum (FCS; SAFC Biosciences, Castle Hill, Australia) and 1% L-glutamine. 2-5 x 10^5 cells from passages 10-20 were plated in each well of a 6-well plate with 2 mL of incubation medium per well and allowed to equilibrate for 24 h (n=3 experiments, each in triplicate). There was no relationship between the number of cells plated and the cell index trajectory. After this 24 h equilibration period, 0.32 ng/μL of miRNA miRVana mimics (Life Technologies, Thermo Fisher Scientific, Waltham, USA) were added to each well, using Lipofectamine as a transfection vector. Only one dose was used for transfection of mimics for RNA and protein analysis purposes. Other cells were treated either by adding Lipofectamine (Thermo Fisher Scientific) alone (vehicle control) or by addition of a scrambled control (Life Technologies), where nucleotides are scrambled in a random order, in conjunction with Lipofectamine (n=3 experiments, each in triplicate). After 48 h, cells and culture media were collected and snap frozen in liquid nitrogen at −80 °C before RNA and protein analysis.

Proliferation Analysis

Fifty μL of incubation medium was added to each well of an xCELLigence E-plate 16 (ACEA Biosciences Inc., San Diego CA) and allowed to equilibrate at room temperature for 30 min. A background reading in the xCELLigence Real-Time Cell Analysis Multi Plate (RCTA MP) system was then conducted. 2-5 x 10^4 HTR-8/SVneo cells from passages 10-20 (cultured as above) were plated in each well with an additional 100 μL of incubation medium and again allowed to equilibrate for 30 min at room temperature. 24 h after cell plating, miRNA miRVana mimics, scrambled mimics or vehicle (lipofectamine) control were added to each well at 0.08, 0.16, 0.32 or 0.64 ng/μL. As the proliferation plates have gold microelectrodes on the bottom of each well, the proliferation of cells impedes electrical conductance. Cell index was generated as a measure of the electrical resistance as cells proliferated. The cell index was measured every 30 min for 48 h in the xCELLigence RCTA MP system. After 48h of incubation with treatment, data were collected and analysed. The rate of proliferation was determined by calculating the slope of the line (cell index) between each individual measurement, for each of the three technical replicates within each experiment, and combining for the average. The xCELLigence plots, showing the mean cell index (of n=3 experiments, each in triplicate) at each time point for each miRNA mimic, are presented in Supplementary Figure 4.4.

RNA Extraction and DNase Treatment

Total RNA extraction of HTR-8/SVneo cells was performed using the miRNeasy kit, according to the manufacturer's instructions (Qiagen, Chadstone, Australia). DNase I treatment was performed on all samples (Qiagen). The integrity of total RNA and miRNAs was examined by gel electrophoresis and quantified using the Nanodrop 2000 (data not shown). Samples were used for further analysis if the 260:280 ratios were greater than 1.8.

miRNA Analysis

MicroRNA expression of miRNAs were measured by qPCR. Total RNA (5 ng) samples underwent reverse transcription to cDNA (TaqMan miRNA Reverse Transcription Kit and probes; Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's instructions. Samples then underwent quantitative PCR (RT-qPCR) using TaqMan Universal PCR master mix, according to the manufacturer's instructions (Applied Biosystems, Thermo Fisher Scientific). Results were quantified using a 7500 Real-Time PCR System (Applied Biosystems). The expression levels of nine miRNAs (miR-181a-5p, miR-181a-3p, miR-663, miR-378, miR-34c, miR-892, miR-514, miR-454 and miR-483-3p) were determined by calculating $2^{-\Delta\Delta CT}$ using RNU44 (a highly conserved small nucleolar RNA in the growth arrest specific 5 transcript) as the house-keeping gene.

qPCR

All RNA samples underwent reverse transcription to cDNA (Superscript III First-Strand Synthesis for RT, Thermo Fisher Scientific) using the manufacturer's instructions. Total RNA was spiked with a known amount of Alien RNA (10^7 copies per µg of total RNA; Stratagene, Agilent; Santa Clara, USA). qPCR was performed in an Applied Biosystems 7500 Real Time PCR System using SYBR Green for detection. Each reaction contained 5 µL of SYBR Green PCR master mix (Applied Biosystems), RAS primers as described previously [12], cDNA reversed transcribed from 10 ng total RNA, and water to 10 µL. Messenger RNA abundance was calculated using the $2^{-\Delta\Delta CT}$ method and expressed relative to ACTB mRNA and a calibrator (a term placental sample collected at elective caesarean section).

Statistics

Statistical analysis was undertaken using GraphPad Prism 7. A one-way ANOVA (non-parametric) was conducted using a Kruskal Wallis test with Dunn's multiple comparisons test. Where only two groups were compared, a Mann-Whitney test was conducted. Differences between groups were considered significant where $p \leq 0.05$.

4.4 Results

The effect of the negative controls and scrambled controls on miRNA expression, RAS mRNA expression and the rate of cell proliferation was first tested to ensure that any effects of the mimics were specific (Suppl. Figures 4.1-4.3). The scrambled control mimic had no effect on miRNA expression (Suppl. Figure 4.1), RAS mRNA expression (Suppl. Figure 4.2) or the rate of cell proliferation (Suppl. Figure 4.3) compared to vehicle control treated cells. Therefore, the vehicle control was used for all subsequent experiments as the control for the miRNA mimics.

The effect of mimics for miRNAs predicted to target REN and ACE mRNAs on RAS gene expression and cell proliferation in HTR-8/SVneo cells

HTR-8/SVneo cells expressed all of the examined miRNAs that were predicted to target RAS mRNAs. HTR-8/SVneo cells treated with miR-181a-5p and miR-378 mimics had significantly elevated levels of miR-181a-5p ($p < 0.001$; Figure 4.1A) and miR-378 ($p = 0.040$; Figure 4.1E), respectively, when compared with the vehicle (lipofectamine) control. In addition, *REN* mRNA expression was decreased in cells treated with miR-181a-5p mimic ($p = 0.032$; Figure 4.1B) or miR-378 mimic ($p = 0.0009$; Figure 4.1F). The abundance of *ACE* mRNA was unchanged with the addition of the miR-181a-5p mimic (Figure 4.1C) but was decreased with the addition of the miR-378 mimic ($p = 0.002$; Figure 4.1G).

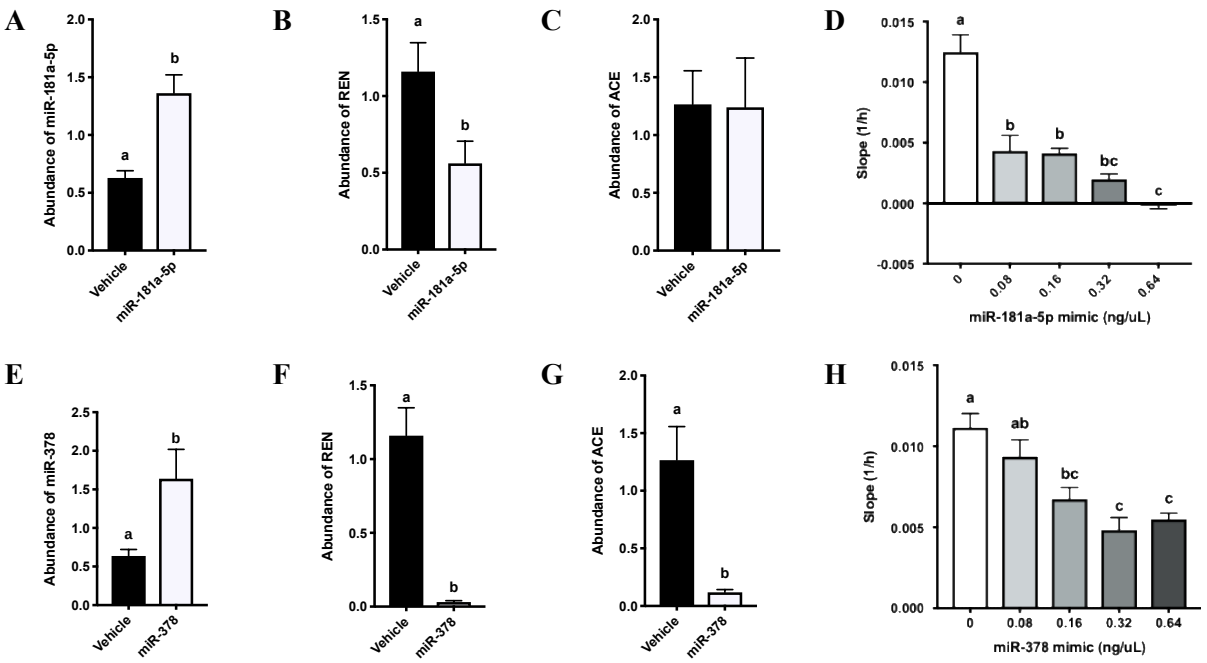


Figure 4.1: Effect of miR-181a-5p and miR-378 mimics on miR-181a-5p and miR-378 expression, respectively, as well as the effects of these mimics on *REN* and *ACE* mRNA expression and HTR-8/SVneo cell proliferation.

A In HTR-8/SVneo cells treated with 0.32 ng/μL miR-181a-5p mimic, miR-181a-5p expression was significantly increased and **B** *REN* mRNA expression was decreased compared to the vehicle control but **C** *ACE* mRNA expression was unchanged. **D** The rates of cell proliferation in cells exposed to the miR-181a-5p mimic were significantly reduced by all concentrations of miR-181a-5p mimic compared to the vehicle control (0 ng/μL mimic). **E** In HTR-8/SVneo cells treated with 0.32ng/μL miR-378 mimic, miR-378 expression was significantly increased compared with the vehicle control and **F** *REN* and **G** *ACE* expression were significantly decreased. **H** The rate of cell proliferation in cells exposed to the miR-378 mimic was significantly decreased at 0.16, 0.32 and 0.64 ng/μL compared to the vehicle control (0 ng/μL mimic*). Data are presented as mean +/- SEM. N = 3 experiments, each in triplicate. The same letter above bars indicates that groups are not different from each other. A different letter above bars indicates that groups are different (all p < 0.05).*

At miR-181a-5p mimic concentrations of 0.08, 0.16, 0.32 and 0.64 ng/μL, the rate of cell proliferation was significantly decreased compared with the vehicle (lipofectamine) control ($p < 0.0001$ for all; Figure 4.1D). The decrease in proliferation was greater in cells that were transfected with 0.64 ng/μL of mimic compared with cells transfected with 0.08 and 0.16 ng/μL ($p = 0.0004$, $p = 0.0008$; respectively). Treatment with the miR-378 mimic significantly decreased proliferation at 0.16, 0.32 and 0.64 ng/μL compared with the vehicle control ($p = 0.008$, $p < 0.0001$, $p = 0.0002$, respectively; Figure 4.1H). Treatment with 0.32 and 0.64 ng/μL mimic were also associated with decreased rates of proliferation compared to treatment with 0.08 ng/μL of mimic ($p = 0.0008$, $p = 0.005$; respectively).

The effect of mimics for miRNAs predicted to target REN mRNA on RAS gene expression and cell proliferation in HTR-8/SVneo cells

Cells exposed to a mimic for miR-181a-3p and miR-663 had significantly elevated levels of miR-181a-3p ($p = 0.049$; Figure 4.2A) and miR-663 ($p = 0.013$; Figure 4.2D), respectively, compared with the vehicle (lipofectamine) control. These cells also had decreased expression of *REN* mRNA when treated with the miR-181a-3p mimic ($p = 0.033$; Figure 4.2B) and the miR-663 mimic ($p = 0.039$; Figure 4.2E) compared to the vehicle control.

The rate of cell proliferation was significantly reduced following transfection of the miR-181a-3p mimic at a concentration of 0.64 ng/μL compared with vehicle control and cells transfected with 0.08 and 0.16 ng/μL of mimic ($p = 0.036$, $p = 0.002$, $p = 0.036$, respectively; Figure 4.2C).

Proliferation was significantly decreased in cells treated with miR-663 mimic at concentrations of 0.08, 0.16, 0.32 and 0.64 ng/μL compared with the vehicle control ($p = 0.018$, $p = 0.003$, $p < 0.0001$, $p < 0.0001$ respectively; Figure 4.2F). Proliferation was also significantly decreased at 0.64 ng/μL of miR-663 mimic compared to 0.08 ng/μL ($p = 0.047$).

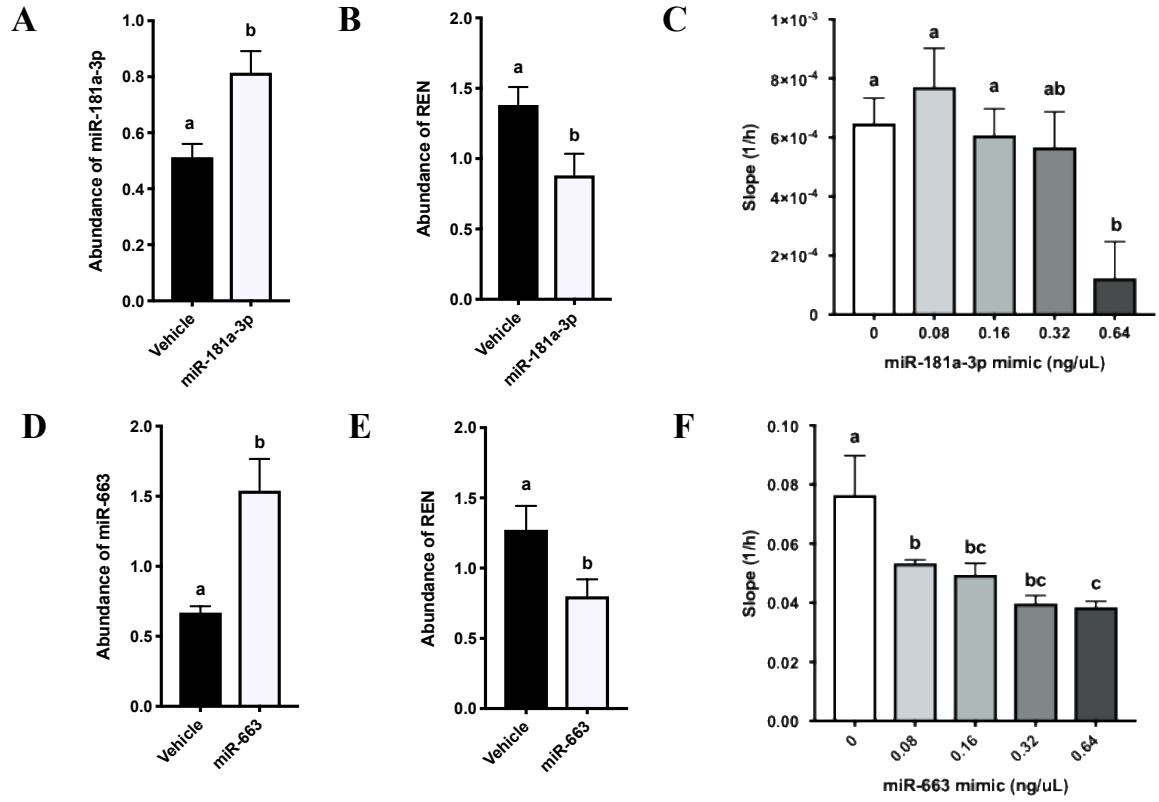


Figure 4.2: Effect of miR-181a-3p and miR-663 mimics on miR-181a-3p and miR-663 expression respectively, as well as the effects of these mimics on *REN* mRNA expression and HTR-8/SVneo cell proliferation.

A In HTR-8/SVneo cells treated with 0.32 ng/μL miR-181a-3p mimic, miR-181a-3p expression was significantly increased and **B** *REN* mRNA expression was significantly decreased compared with vehicle (lipofectamine) control. **C** The rates of cell proliferation of cells transfected with miR-181a-3p mimic was significantly decreased at 0.64 ng/μL compared to vehicle control (0 ng/μL mimic). **D** In HTR-8/SVneo cells treated with 0.32 ng/μL miR-663 mimic, miR-663 expression was significantly increased and **E** *REN* expression was significantly decreased compared with the vehicle control. **F** The rate of cell proliferation of cells when exposed to miR-663 mimic was significantly decreased at 0.08, 0.16, 0.32 and 0.64 ng/μL compared to the vehicle control (0 ng/μL mimic). *Data are presented as mean +/- SEM.*

N = 3 experiments, each in triplicate. The same letter above bars indicates that groups are not different from each other. A different letter above bars indicates that groups are different (all $p < 0.05$).

The effect of mimics for miRNAs predicted to target ATP6AP2 mRNA on RAS gene expression and cell proliferation in HTR-8/SVneo cells

Cells treated with a mimic for miR-454 had significantly increased expression of miR-454 compared with the vehicle control (p = 0.049; Figure 4.3A). *ATP6AP2* mRNA expression was significantly decreased (p = 0.036; Figure 4.3B). Cell proliferation was abolished by transfection with all concentrations of miR-454 (0.16 ng/μL compared with vehicle control; p = 0.0048, and 0.32 ng/μL compared with vehicle control (p = 0.0329; Figure 4.3C).

The effect of mimics for miRNAs predicted to target AGTR1 and AGT mRNAs on RAS gene expression and cell proliferation in HTR-8/SVneo cells

Cells treated with mimics for miR-34c and miR-892 had significantly increased expression of miR-34c (p = 0.002; Figure 4.4A) and miR-892 (p = 0.034; Figure 4.4D), respectively, compared with the vehicle control. *AGTR1* mRNA expression was not significantly changed with addition of miR-34c mimic (Figure 4.4B) nor was *AGT* mRNA expression changed with addition of the miR-892 mimic (Figure 4.4E).

Proliferation was significantly decreased with the addition of 0.64 ng/μL of the miR-34c mimic compared to 0.08 and 0.32 ng/μL (p < 0.001, p = 0.003; respectively, Figure 4.4C).

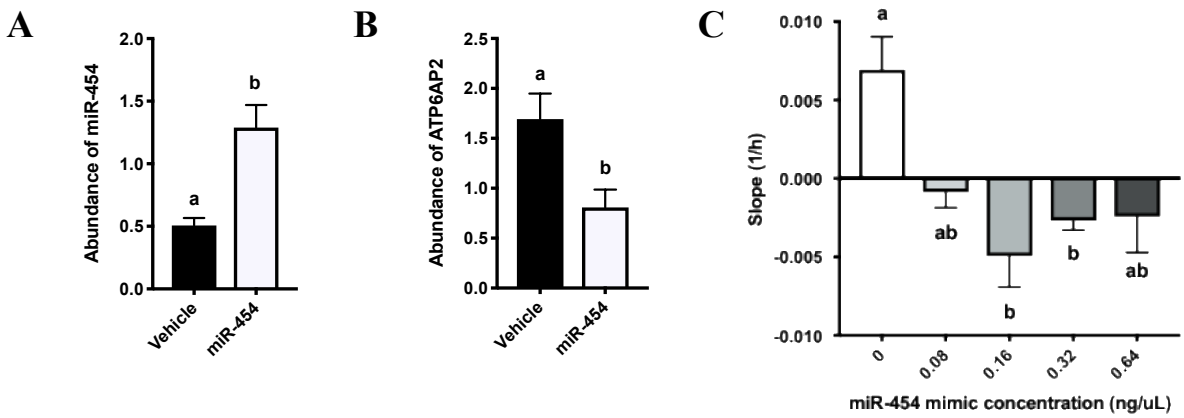


Figure 4.3: Effect of miR-454 mimic on miR-454 and *ATP6AP2* mRNA expression, and on HTR-8/SVneo cell proliferation.

A In HTR-8/SVneo cells treated with 0.32 ng/μL miR-454 mimic, miR-454 expression was significantly increased and **B** *ATP6AP2* expression was significantly decreased when compared to vehicle (lipofectamine) control. **C** Proliferation was abolished by all doses of miR-454 mimic. *Data are presented as mean +/- SEM. N = 3 experiments, each in triplicate. The same letter above bars indicates that groups are not different from each other. A different letter above bars indicates that groups are different (all $p < 0.05$).*

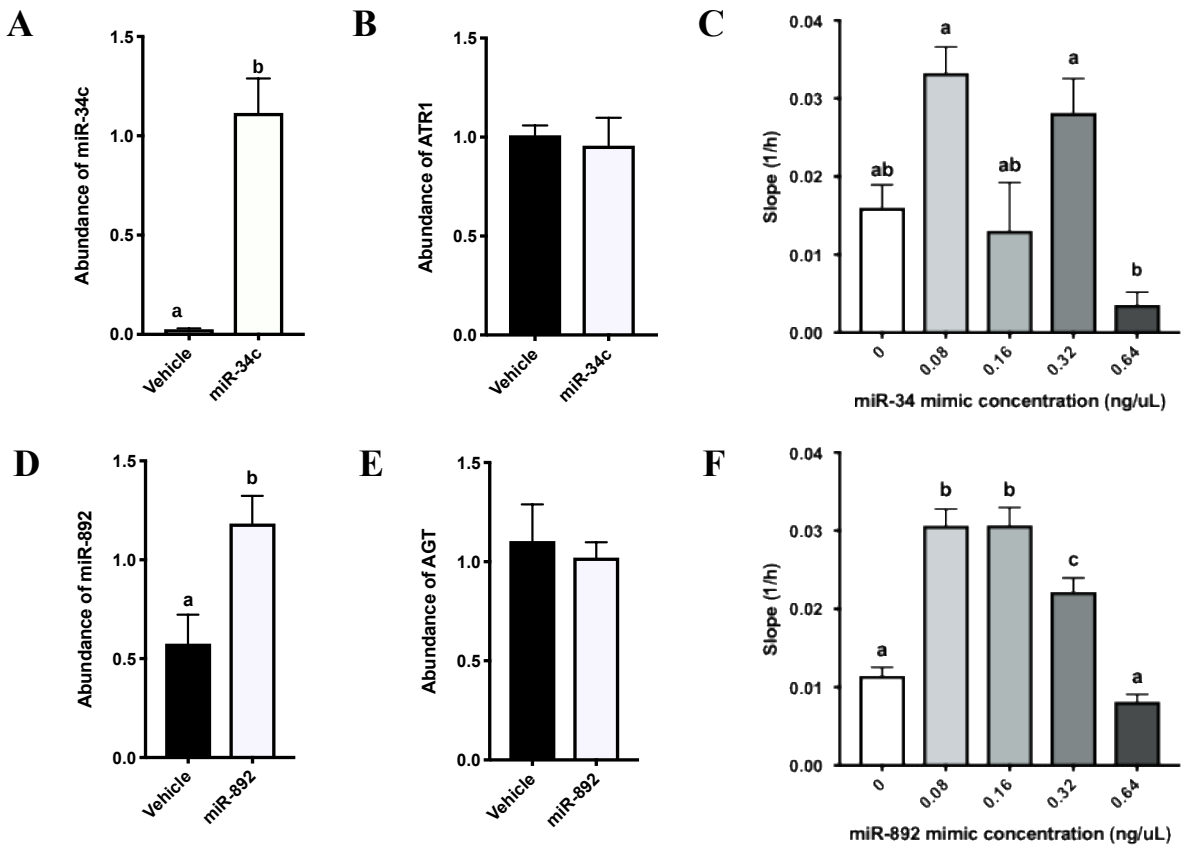


Figure 4.4: Effect of miR-34c and miR-892 mimics on miR-34c and *AGTR1* expression, and on miR-892 and *AGT* expression, respectively, as well as the effects of these mimics on HTR-8/SVneo cell proliferation.

A In HTR-8/SVneo cells treated with 0.32 ng/μL miR-34c mimic, miR-34c expression was significantly increased but **B** *AGTR1* mRNA was not altered compared to the vehicle control. **C** Rates of cell proliferation of cells when exposed to miR-34c mimic was significantly decreased at 0.64 ng/μL when compared to 0.08 and 0.32 ng/μL. **D** In HTR-8/SVneo cells treated with 0.32 ng/μL miR-892 mimic, miR-892 expression was significantly increased but **E** *AGT* expression was not significantly altered compared to the vehicle control. **F** Rates of cell proliferation of cells when exposed to miR-892 mimic were significantly decreased at 0.64 ng/μL compared with 0.08, 0.16 and 0.32 ng/μL, and significantly increased at 0.08, 0.16 and 0.32 ng/μL compared with vehicle (0 ng/μL mimic) control and 0.64 ng/μL. *Data are presented as mean +/- SEM. N = 3 experiments, each in triplicate. The same letter above bars indicates*

that groups are not different from each other. A different letter above bars indicates that groups are different (all $p < 0.05$).

Proliferation was significantly decreased at 0.64 ng/μL of miR-892 mimic compared with 0.08, 0.16 and 0.32 ng/μL (all $p < 0.001$; Figure 4.6C). Proliferation rates at 0.08 and 0.16 ng/μL were also significantly increased compared with the vehicle control (both $p < 0.001$) and 0.32 ng/μL ($p = 0.008$, $p = 0.007$; respectively). The rate of cell proliferation was significantly upregulated when transfected with 0.32 ng/μL of mimic compared to the vehicle control ($p = 0.002$).

Cells treated with a mimic for miR-514 demonstrated significantly increased abundance of miR-514 ($p = 0.039$; Figure 4.5A) and decreased *AGTR1* and *AGT* mRNA abundance ($p = 0.031$, $p = 0.043$; Figure 4.5B, 4.5C) compared with the vehicle control. Treatment with the miR-514 mimic significantly decreased proliferation at 0.08, 0.16, 0.32 and 0.64 ng/μL compared to the vehicle control ($p = 0.009$, $p = 0.004$, $p < 0.0001$, $p = 0.004$, respectively; Figure 4.5D). Proliferation rates of cells transfected with 0.16, 0.32 and 0.64 ng/μL concentrations was also less than that of cells treated with 0.08 ng/μL of mimic ($p = 0.044$, $p = 0.012$, $p = 0.046$, respectively).

The effect of mimics for miRNAs predicted to target AGTR1, AGT and ACE mRNAs on RAS gene expression and cell proliferation in HTR-8/SVneo cells

Following treatment with a miR-483-3p mimic, cells exhibited increased expression of miR-483-3p ($p = 0.009$; Figure 4.6A) and decreased *AGTR1* mRNA levels ($p = 0.049$; Figure 4.6B) compared with the vehicle control. Expression of *AGT* and *ACE* mRNAs were not changed compared with the vehicle control (Figure 4.6C and 4.6D, respectively). Proliferation rates of cells treated with 0.32 and 0.64 ng/μL miR-483-3p mimic were significantly decreased compared with the vehicle control ($p < 0.0001$ for both; Figure 4.6E) and 0.16 ng/μL ($p = 0.0055$, $p < 0.0001$, respectively). Proliferation was also decreased at a miR-483-3p mimic concentration of 0.64 ng/μL compared with 0.08 ng/μL ($p = 0.0009$).

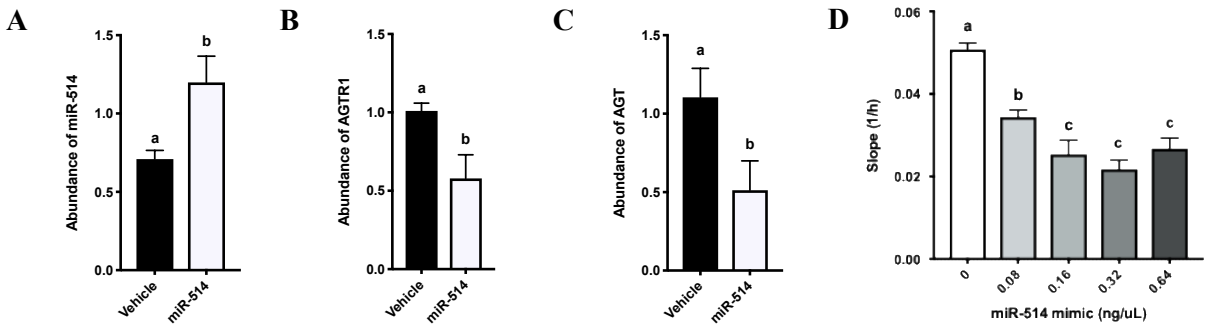


Figure 4.5: Effect of miR-514 mimic on miR-514, *AGTR1* and *AGT* expression, and on HTR-8/SVneo cell proliferation.

A In HTR-8/SVneo cells treated with 0.32 ng/μL miR-514 mimic, miR-514 expression was significantly increased and **B** *AGTR1* and **C** *AGT* mRNA were significantly decreased compared with vehicle control. **D** Rates of cell proliferation of cells when exposed to miR-514 mimic were significantly decreased at 0.08, 0.16, 0.32 and 0.64 ng/μL compared with the vehicle control (0 ng/μL mimic). *Data are presented as mean +/- SEM. N = 3 experiments, each in triplicate. The same letter above bars indicates that groups are not different from each other. A different letter above bars indicates that groups are different (all p < 0.05).*

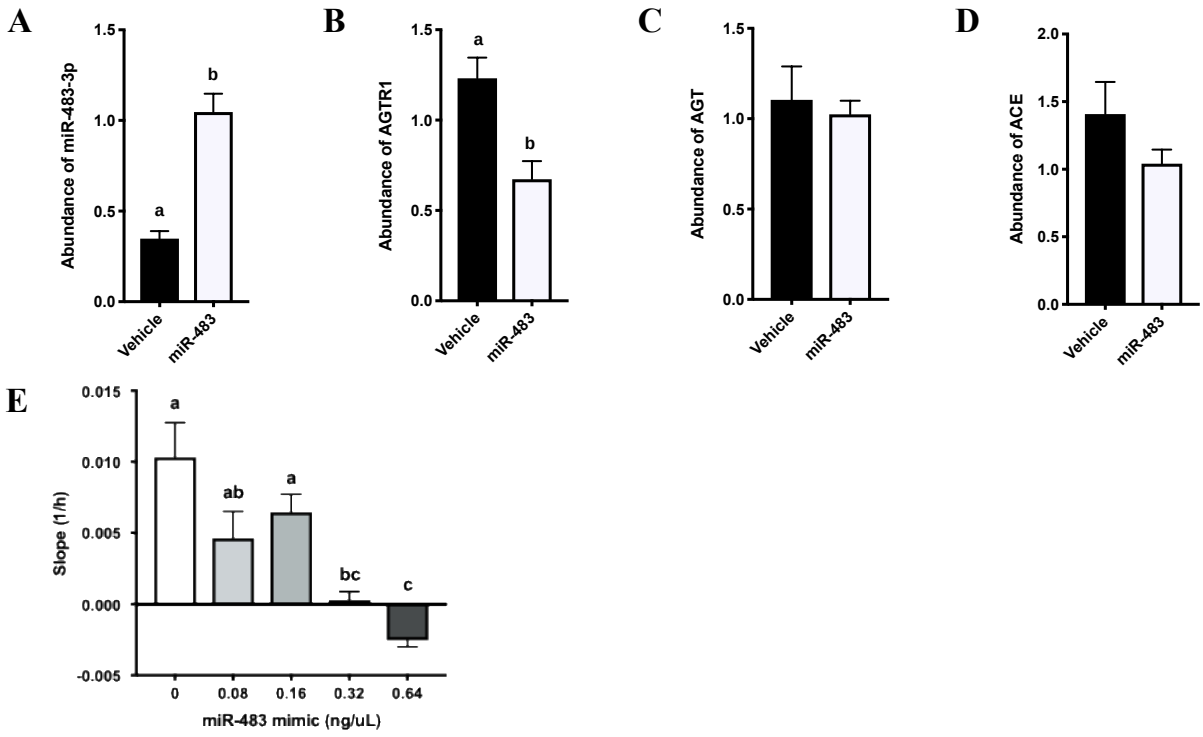


Figure 4.6*:* Effect of miR-483-3p mimic on miR-483-3p, *AGTR1*, *AGT*, and *ACE* expression, and on HTR-8/SVneo cell proliferation.

A In HTR-8/SVneo cells treated with 0.32 ng/μL miR-483-3p mimic, miR-483-3p expression was significantly increased and **B** *AGTR1* expression was significantly decreased when compared to the vehicle control. Expression of **C** *AGT* and **D** *ACE* mRNA were not significantly altered compared to the vehicle control. **E** Rates of cell proliferation of cells when exposed to miR-483-3p mimic were significantly decreased at 0.32 and 0.64 ng/μL compared to the vehicle control (0 ng/μL mimic). *Data are presented as mean +/- SEM. N = 3 experiments, each in triplicate. The same letter above bars indicates that groups are not different from each other. A different letter above bars indicates that groups are different (all p < 0.05).*

4.5 Discussion

We have previously measured the expression of miRNAs predicted to target the placental RAS throughout gestation and found a negative relationship between the miRNAs and their RAS targets [12]. RAS mRNAs were significantly increased in the first trimester of gestation, when placentation is greatest, and at this time miRNAs targeting these RAS mRNAs were downregulated. Thus, we hypothesised that miRNAs targeting placental RAS mRNAs are able to inhibit or slow placental development as measured by the rate of proliferation of extravillous trophoblasts which invade the decidua and plug maternal arterioles. In this study, we have shown that these miRNAs do target the placental RAS and reduce the rate of proliferation of HTR-8/SVneo cells. Treatment with their specific miRNA mimic was associated with increased cellular expression of all 9 miRNA mimics studied, compared with the vehicle. Of these 9 miRNA mimics, 7 successfully downregulated one or more of their predicted RAS target mRNAs; these were miR-181a-3p, miR-181a-5p, miR-378, miR-454, miR-483-3p, miR-514 and miR-663.

All nine miRNA mimics had some effect on trophoblast proliferation, although this effect varied. Five of the mimics tested (miR-181a-5p, miR-378, miR-663, miR-483-3p and miR-514) decreased trophoblast proliferation in a dose-dependent manner, with increasing mimic concentrations resulting in decreasing proliferation rates. These mimics decreased expression of *REN* (miR-181a-5p, miR-378, miR-663), *AGT* (miR-514), and *ACE* (miR-378). Another one of the mimics (miR-181a-3p; mRNA target, *REN*) tended to be associated with a dose-dependent fall in proliferation rate, but a significant decrease in cell proliferation only occurred with the highest dose. Furthermore, miR-892 (target, *AGT*) and miR-34c (target, *AGTR1*) mimics had biphasic effects on cell proliferation. Lastly, miR-454 (target*, ATP6AP2*) was cytotoxic, with cell death occurring particularly at the highest concentrations of the mimic.

Mimics targeting *REN* mRNA successfully downregulated *REN* mRNA expression and displayed a dose-dependent effect. With the knowledge that the placental RAS pathway, initiated by prorenin, is implicated in trophoblast proliferation and placental development [79], the effects of miR-181a-5p, miR-663 and miR-378 on *REN* mRNA and the associated decrease in proliferation rate of extravillous trophoblasts, explains in part why they have been associated with placental complications [130]. Mature miR-181a is associated with downregulation of the TGF-β pathway and has been shown to inhibit proliferation in mesenchymal stem cells [232]. Importantly, differential expression of miR-181a-5p has been detected in placentae from women delivering preterm [232]. MiR-378, which also downregulated *ACE* mRNA expression, has been linked with trophoblast migration and invasion by targeting Nodal, a gene encoding for a secreted ligand of the TGF-β protein superfamily. Its dysregulation contributes to PE [166]. miR-663 is also dysregulated in late-onset PE [12].

The fourth miRNA mimic that targets *REN* mRNA, miR-181a-3p, tended to cause a dose-dependent decrease in proliferation as seen in the other mimics targeting *REN*, but its effects were only significant at the highest dose. The relationship between miR-181a-3p and *REN* is well defined [14], as is the positive correlation between *REN* and proliferation. A causal link is suggested in the literature between miR-181a-3p, *REN* and cell proliferation in kidneys [14] but this has not been investigated in trophoblasts.

Interestingly, addition of the miR-454 mimic, which successfully downregulated *ATP6AP2* mRNA expression in HTR-8/SVneo cells, induced cell death at all concentrations. This is evident from the negative slope of the cell index lines (Figure 4.3, Suppl. Figure 4.4E) of cells treated with any concentration of the miR-454 mimic. As cells die, they detach from the bottom of the wells and are no longer measured by the xCELLigence machine. This differs from cell quiescence, as the cells simply do not proliferate and, as shown, there is a flat line with no

gradient. Expression of (P)RR is downregulated in the placentae from preeclamptic women and furthermore, (P)RR knock-out has been shown to be embryo-lethal (Burcklé and Bader, 2006). As miR-454 moderately downregulates (P)RR expression, this could explain the cytotoxic effect we see as the cells experienced cell death.

It should also be noted, however, that a slightly lower cell index was seen for all miR-514 mimic treatment groups compared to other mimics plated with the same cell number (miR-378, miR-181a-5p, miR-892) (Suppl. Figure 4.4). However, the small increase in the slope of the cell index line seen in the negative control cells is comparable to the slope seen in cells treated with the miR-181a-3p mimic. Therefore, we suggest that this is variation between batches of cells, as opposed to an error in experimental procedure. Furthermore, the initial low cell index, or small increase in the slope of the line, as seen in other mimic experiments did not lead to cell death. Thus we can conclude that cell death seen by cells treated with the miR-454 mimic was a consequence of treatment with the mimic.

Little has been published regarding the role of miR-892 in the growing placenta. In a microarray that we previously carried out, miR-892 was one of three RAS-targeting miRNAs significantly upregulated in placentae from early-onset preeclamptic women compared with uncomplicated [12]. miR-892 is predicted to target the RAS gene *AGT* with <95% sequence complementarity. AGT undergoes catalytic reduction to form Ang I in the RAS cascade, and then Ang I is converted to Ang II. Ang II acts predominantly on the AT_1R, stimulating proliferation [234]. Downregulation of AGT by miR-892 should, predictably, downregulate proliferation. However, it is possible that miR-892 is working by repressing another predicted target, peroxisome proliferator-activated receptor-γ (PPAR-γ). PPAR-γ represses vascular endothelial growth factor (VEGF) gene expression [222] and produces an anti-proliferative environment; thus, targeted inhibition by miR-892 could result in an increase in proliferation upon treatment with this mimic, which is consistent with our results. This should be confirmed by further studies.

MiR-514 is predicted to target both *AGT* and *AGTR1* mRNA, although there are no current studies that confirm this. We have shown that miR-514 does reduce both *AGT* and *AGTR1* mRNA expression and trophoblast proliferation in a dose-dependent manner. MiR-514 is a placenta-specific miRNA and is known to decrease cell proliferation by targeting protein tyrosine phosphatase Shp2 (Lui and Forbes, 2012). Its expression is dysregulated in PE, with unusually high levels of miR-514 found in placentae from early-onset PE [12]. miR-483-3p is also predicted to target both *AGT* and *AGTR1* mRNA, as well as *ACE* and *ACE2* mRNA, however only *AGTR1* expression was reduced. Trophoblast proliferation was also reduced in a dose-dependent manner, with no proliferation seen in cells treated with the highest doses of the mimic. We believe this could be due to the vast number of targets that miR-483-3p acts upon. miR-483-3p is not only predicted to target 4 RAS targets (mentioned above), but also a number of other important genes, thereby prohibiting cell proliferation. For example, miR-483-3p has been shown to negatively regulate trophoblast proliferation and IGF-1 expression (Forbes, 2012), which is known to promote proliferation and inhibit apoptosis in human trophoblast cells [237]. Additionally, IGF-2-derived intronic miR-483-3p is overexpressed in placentae from women delivering infants with macrosomia [238].

miR-34c is also predicted to target *AGTR1* mRNA, although we did not find any significant change in its expression. Suppression of mature miR-34 has been shown to increase trophoblast invasion [169] but we are unable confirm this observation. By combining previous knowledge of the miRNA's effects on proliferation, we can only conclude that more sequences than just *AGTR1* mRNA were targeted by miR-34c.

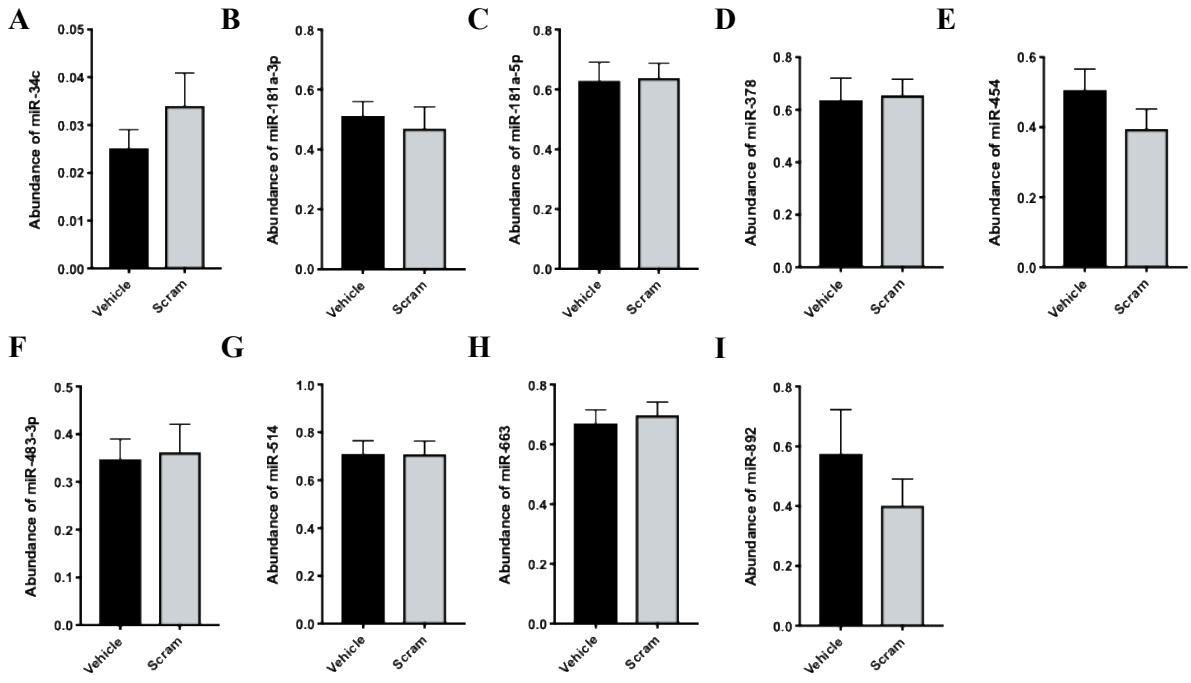
One limitation of this study that will be investigated in the future is the effect of these miRNA mimics on RAS protein expression. Although we have shown that there are significant alterations in the RAS mRNA levels when treated with these miRNA mimics, as miRNAs are

known to be post-transcriptional regulators, it would be beneficial to examine their effects on translation of their targets.

Another limitation of this study is the variation in proliferation rates of cells as measured by the xCELLigence machine. Most experiments were plated using the same protocol, there was however a large degree of variability in proliferation of the cells, despite using the same cell line. This could be due to the sensitivity of the xCELLigence machine, as even small changes in positioning, temperature and humidity can affect results. Where possible, replicate experiments were completed within a short period to minimise differences, however this could not be done for separate mimic experiments. Therefore, differences seen were between mimic experiments, but not within experiments.

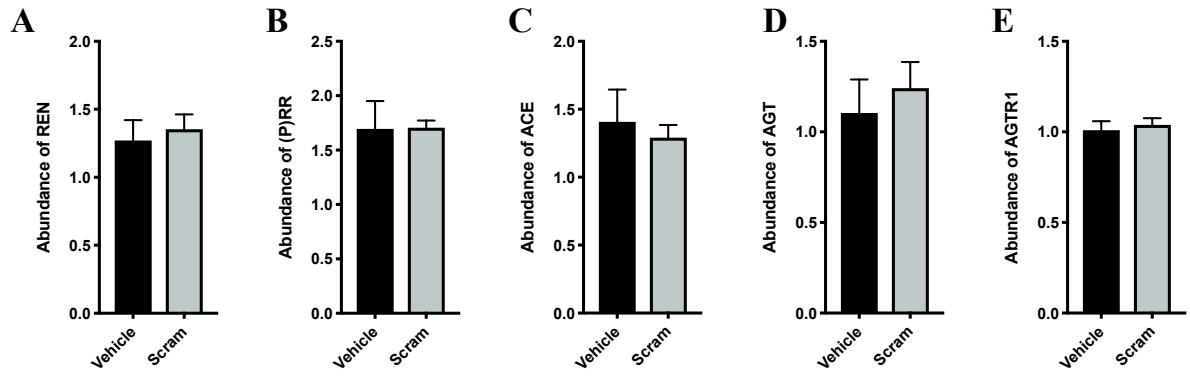
In summary, we have explored the varied effects of miRNAs targeting the placental RAS on HTR-8/SVneo cells, and confirmed that a number of miRNAs, miR-181a-3p, miR-181a-5p, miR-378, miR-663, miR-483-3p and miR-514, both target the predicted RAS mRNAs and decrease cell proliferation. We have shown therefore that miRNAs that have a changing pattern of expression in the human placentae throughout gestation and in PE, which we predicted would target the placental RAS, do in fact do so. In addition, we have shown that the expression of almost all of the placental RAS mRNAs are suppressed by at least one of the 9 miRNA mimics that we studied. Bearing in mind that there is very high expression of prorenin in early gestation [3, 239] it is impressive that four of the mimics we studied have *REN* mRNA as a target. It is also worth speculating that a combination of these miRNAs could be of greater value in treating other conditions associated with over expression of the RAS (e.g. hypertension, diabetic nephropathy and retinopathy).

Supplementary Figures



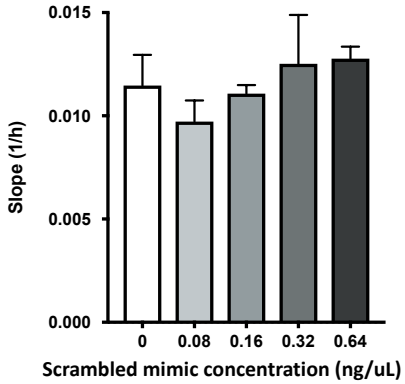
Supplementary Figure 4.1: Effect of scrambled miRNA mimic compared with vehicle on miRNA expression.

In HTR-8/SVneo cells treated with 0.32 ng/μL scrambled mimic, the expression of **A** miR-34c, **B** 181a-3p, **C** miR-181a-5p, **D** miR-378, **E** miR-454, **F** miR-483-3p, **G** miR-454, **H** miR-663 and **I** miR-892 were not significantly changed compared with the vehicle control. *Data are presented as mean +/- SEM. N = 3 experiments, each in triplicate.*



Supplementary Figure 4.2: Effect of scrambled miRNA mimic compared with vehicle on RAS mRNA expression.

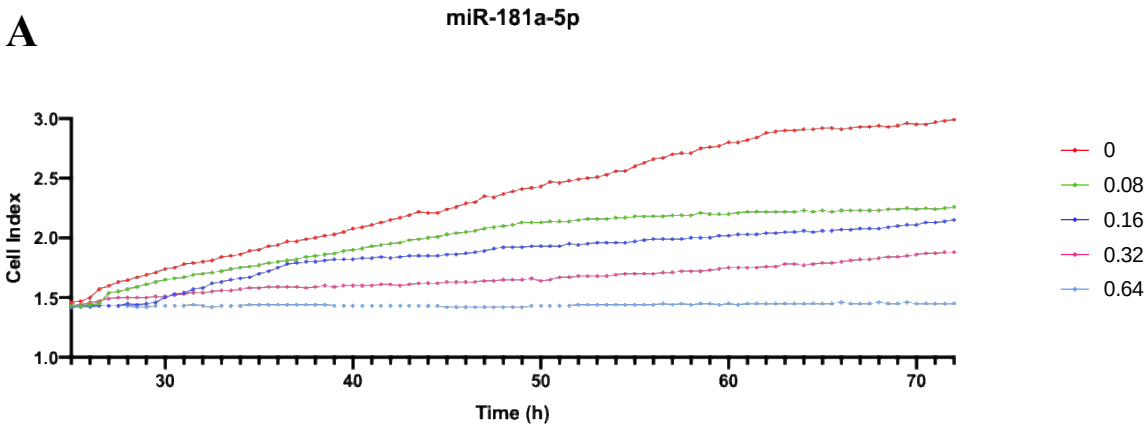
A In HTR-8/SVneo cells treated with 0.32 ng/μL scrambled mimic, the mRNA abundance of *REN*, **B** *ATP6AP2* ((P)RR), **C** *ACE*, **D** *AGT* and **E** *AGTR1* were not significantly changed compared with the vehicle control. *Data are presented as mean +/- SEM. N = 3 experiments, each in triplicate.*



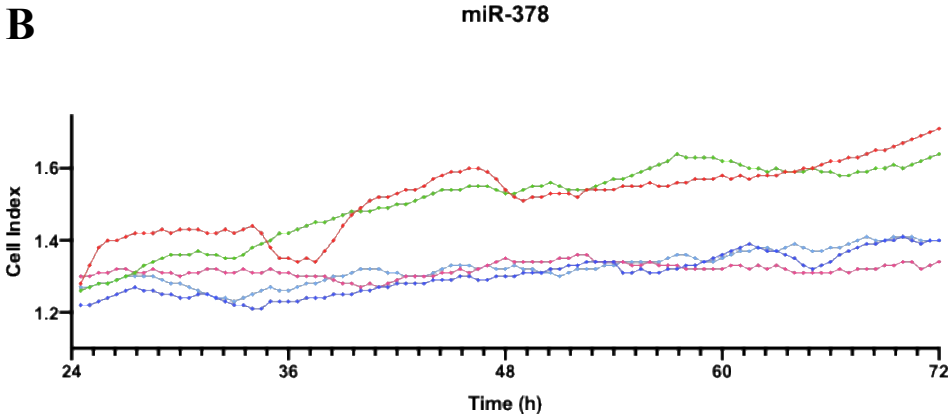
Supplementary Figure 4.3: Effect of scrambled mimic at 0.08, 0.16, 0.32 and 0.64 ng/μL on HTR-8/SVneo cell proliferation.

In HTR-8/SVneo cells treated with scrambled mimic at 0.08, 0.16, 0.32 and 0.64 ng/μL cell proliferation was not significantly altered between groups or compared with vehicle control (0 ng/μL). *Data are presented as mean +/- SEM. N = 3 experiments, each in triplicate.*

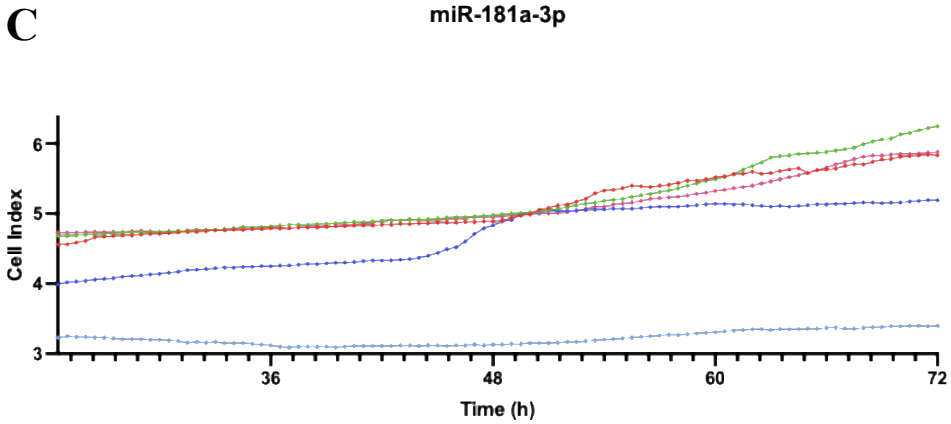
A


miR-181a-5p
Cell Index
3.0
2.5
2.0
1.5
1.0
30
40
50
60
70
Time (h)
0
0.08
0.16
0.32
0.64

B


miR-378
Cell Index
1.6
1.4
1.2
24
36
48
60
72
Time (h)

C


miR-181a-3p
Cell Index
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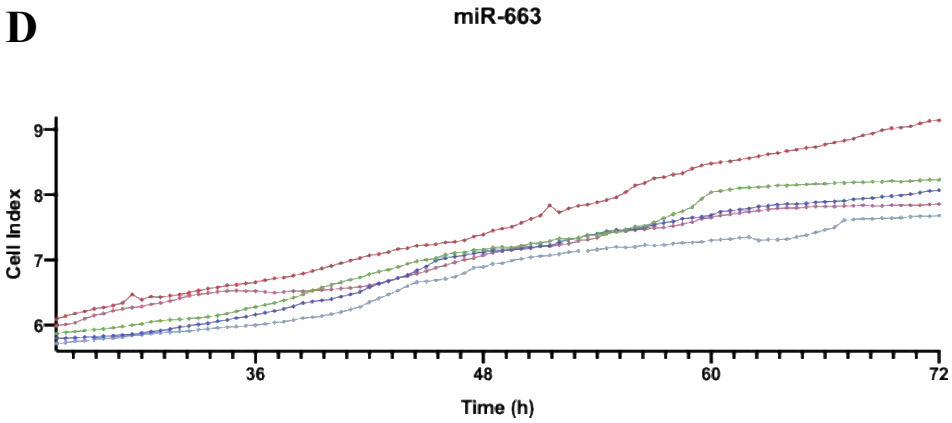
5
4
3
36
48
60
72
Time (h)

D


miR-663
Cell Index
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8
7
6
36
48
60
72
Time (h)

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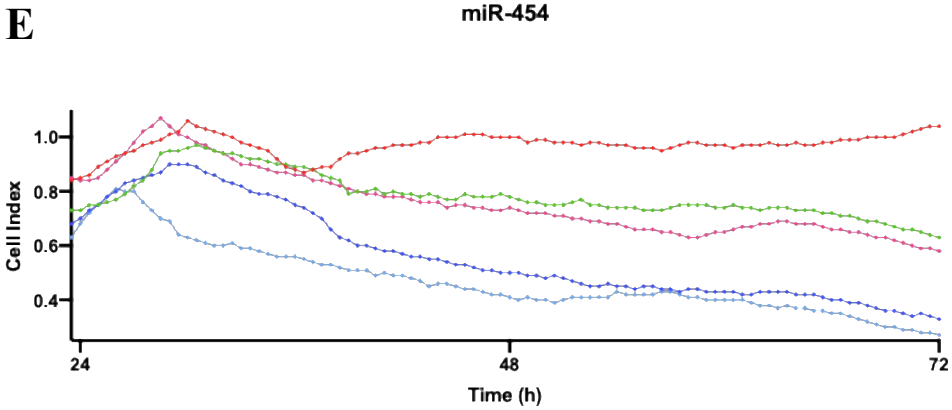
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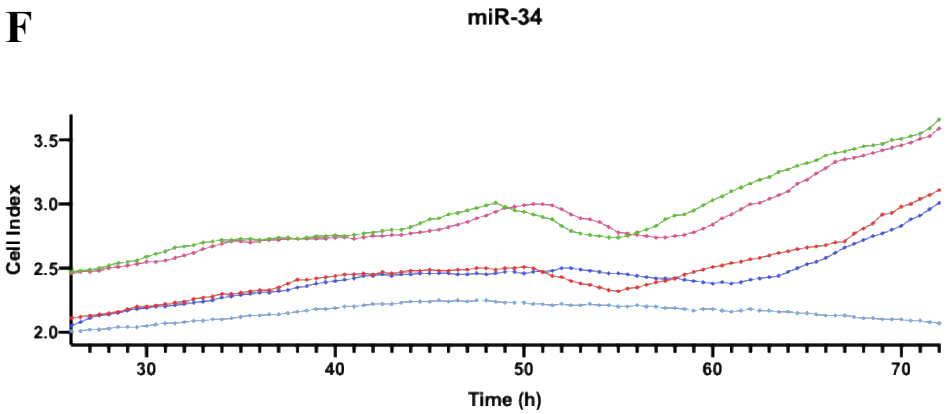
0.4
24
48
72
Time (h)

F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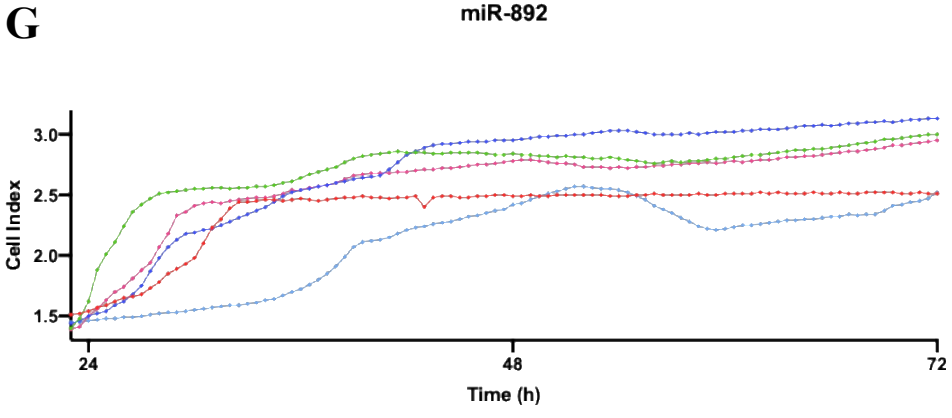
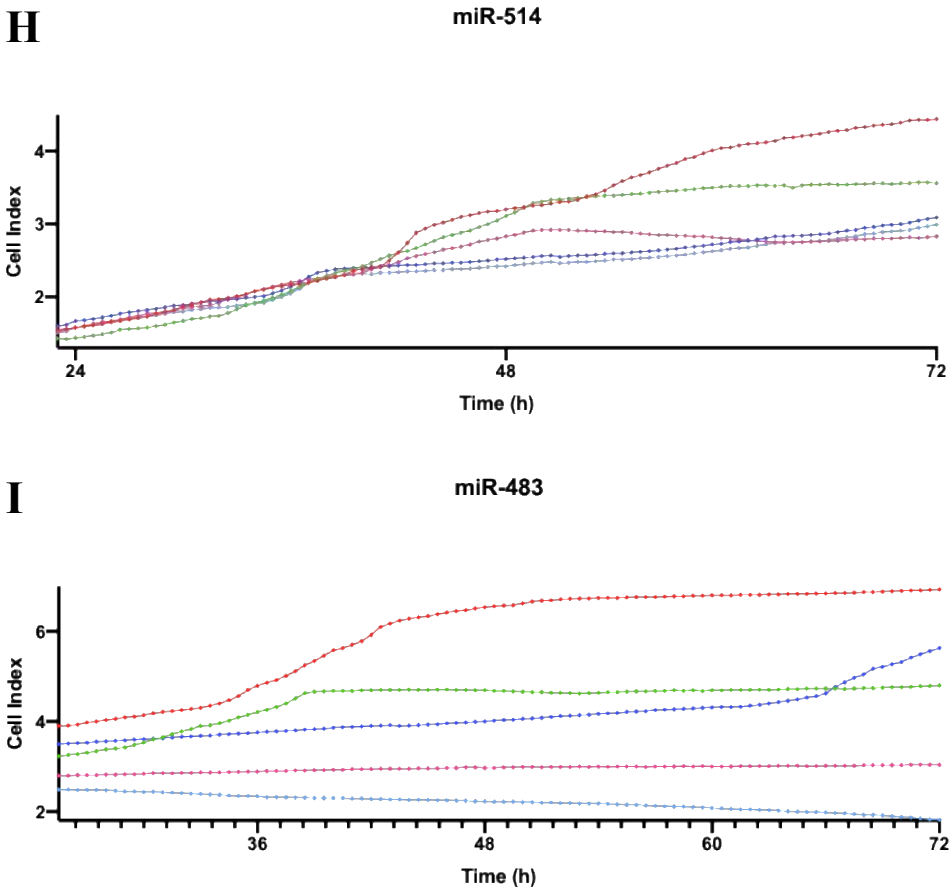
2.0
30
40
50
60
70
Time (h)

G


miR-892
Cell Index
3.0
2.5
2.0
1.5
24
48
72
Time (h)



Supplementary Figure 4.4: Merged xCELLigence line trajectories for each mimic experiment showing the effects of each mimic on HTR-8/SVneo cell proliferation.

A In HTR-8/SVneo cells treated with miR-181a-5p mimic, **B** miR-378 mimic, **C** miR-181a-3p mimic, **D** miR-663 mimic, **E** miR-454 mimic, **F** miR-34 mimic, **G** miR-892 mimic, **H** miR-514 mimic and **I** miR-483 mimic, proliferation was significantly decreased compared to the vehicle control (0 ng/μL mimic, shown in red). *Data are presented as mean +/- SEM. N = 3 experiments, each in triplicate.*

5. The role of miR-155 in placentation

This manuscript prepared for submission utilises a knockout mouse model to examine the role of miR-155 in placental development.

Prepared Manuscript for Submission

Author	**Contribution**	**Signature**
Anya L Arthurs	Laboratory procedures Data analysis Manuscript preparation, revision and submission	
Eugenie R Lumbers	Experimental design Manuscript corrections	
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Kirsty G. Pringle	Experimental design Manuscript corrections	

5.1 Abstract

Many miRNAs play vital roles in placentation, and miR-155 in particular has been implicated in the development of both healthy and abnormal placentae. miR-155 has also been shown to directly interact with angiotensin type II receptor 1 (AT_1R) (*AGTR1)* mRNA. Binding of Angiotensin II to the AT_1R is pro-proliferative and contributes to placental development, hence miR-155 by downregulating expression of AT_1R may inhibit placental growth. We therefore tested the hypothesis that miR-155 suppresses trophoblast proliferation and differentiation, and reduces placental development, because it inhibits expression of *AGTR1* mRNA.

A miR-155$^{-/-}$ mouse model was used to measure the effects of miR-155 in the placenta. Homogenous mating occurred before pregnant miR-155$^{+/+}$ (controls) and miR-155$^{-/-}$ mice were sacrificed at post-coital day 17.5 and tissues were collected. Placentae and fetuses were weighed and compared. Tissue sections were stained with haemotoxylin and eosin and mounted prior to morphometric analysis of mid-sagittal placental labyrinth and junctional zones. Differences in placental miR-155 and *AGTR1* mRNA levels were confirmed by qPCR, and differences in AT_1R protein expression were determined by immunoblotting (n = 4-9 placentae / dam, for 10 dams total).

To determine the effects of miR-155 on trophoblast proliferation, HTR-8/SVneo cells were cultured with a miR-155 mimic (at 7.5, 15, 30 and 60 pM concentrations). The effect of the mimic on the rate of cell proliferation was assessed using the xCELLigence real-time cell analysis system over 48 h. Levels of miR-155 and *AGTR1* mRNA were also determined by qPCR and differences in the levels of expression determined.

miR-155$^{-/-}$ dams produced pups that were significantly heavier, with unchanged placental weight and fetal to placental weight ratios. Moreover, placentae from miR-155$^{-/-}$ dams had significantly larger labyrinth zones and labyrinth to placental area ratios compared to controls.

miR-155 could not be detected in placentae from miR-155$^{-/-}$ dams, but *AGTR1* mRNA and protein were significantly increased. Treatment with 30pM of miR-155 mimic increased miR-155 and decreased *AGTR1* mRNA levels, and concentrations of 30 and 60 pM significantly decreased the rate of trophoblast cell proliferation.

Our study showed that placental miR-155 reduces *AGTR1* mRNA and protein, and decreases trophoblast cell proliferation. It also limits labyrinth zone growth and fetal weight. These changes show that placental miR-155 controls placental growth by suppressing AT_1R. Further studies are being completed to further analyse the effects of miR-155 on placental structure using dual-labelling so that changes in placental cell types can be determined.

miR-155 is essential for normal placental, and hence fetal, growth. Since miR-155 is secreted and dysregulated in PE, it may be able to be used as a biomarker or potential therapeutic target.

5.2 Introduction

Placentation is a finely tuned process, relying on complex integrated genetic control. Post-transcriptional regulation, for example by microRNAs (miRNAs), also plays a role in this process [11]. miRNAs are short, non-coding RNA fragments which largely act by repressing translation. One miRNA which has been suggested to regulate placental development is miR-155 [183, 184, 240-242]. Importantly, miR-155 has been shown to modulate the expression of a renin-angiotensin system (RAS) mRNA [243].

The circulating RAS is well known for its roles in water and salt homeostasis, but tissue RASs exist including a placental RAS that plays a role in placental development [3]. The placental RAS is activated by the inactive precursor to renin, prorenin, when it is non-proteolytically activated, by binding the prorenin receptor ((P)RR), revealing its active site. Ang I is cleaved from angiotensinogen by prorenin and is converted to angiotensin II (Ang II) which acts via the angiotensin II Type I Receptor (AT_1R) to stimulate proliferation, migration [4] and angiogenesis [4, 102].

Dysregulation of placental RAS expression has been associated with placental insufficiency [98] and human pregnancy complications including PE and IUGR [97, 117-121]. Levels of a number of the genes and proteins of the placental RAS are highest in early gestation [3] including the AT_1R. Interestingly, placental expression of AT_1R (*AGTR1*) mRNA is high in preeclamptic placentae [95] when maternal vascular AT_1Rs are downregulated [244].

miRNAs are able to target multiple mRNA sequences, but in particular, miR-155 has been shown to cause a reduction in expression of *AGTR1* mRNA in umbilical vein endothelial cells [245]. Indeed, polymorphisms in the 3'-UTR of the *AGTR1* mRNA inhibit miR-155 binding to this sequence [243]. Additionally, unusually high levels of expression of miR-155 have been

found in placentae from patients with PE and patients with small for gestational age babies [175].

The importance of adequate AT_1R expression in placental development was established by Walther, *et al* who showed that placental knock-out of the AT_1R was associated with much smaller and more poorly vascularised placentae, that adversely impacted on fetal development [96]. Therefore, the potential effect of miR-155 on *AGTR1* mRNA expression, and how this affects placentation is worth investigating.

In this study we examined the role of miR-155 in placental development by studying placental morphology and fetal development in a miR-155 knockout mouse model. The levels of expression of AT_1R in miR-$155^{-/-}$ placentae were also measured. To show the direct effects of miR-155 on placental growth and AT_1R expression, we also looked at its effects on trophoblast proliferation using HTR-8/SVneo cells.

5.3 Methods

Ethics

All experimental protocols were approved by the University of Adelaide Animal Ethics Committee (approval number: M-2014-023, M-2016-009), using methods in accordance with the Australian code for the care and use of animals for scientific purposes.

Animal work

Bic/*miR-155* (miR-155$^{-/-}$) mice on a C57Bl/6 background generated by Prof Klaus Rajewsky (CBR Institute for Biomedical Research, Harvard Medical School) [246] were purchased from The Jackson Laboratory (Bar Harbor, ME, USA, Stock No: 007745) and subsequently bred in-house at the University of Adelaide. C57Bl/6J (B6; miR-155$^{+/+}$) wild-type control female mice were purchased from Animal Resource Centre, Perth. All mice were co-housed in specific pathogen free conditions at the University of Adelaide Medical School Animal House on a 12-hour light–dark cycle and were administered food and water *ad libitum*. Experimental females were 8-12 weeks and males were 10 weeks to 12 months in age.

For mating experiments: *miR-155*$^{+/+}$ and *miR-155*$^{-/-}$ females were mated with the same genotype males, and 1-2 adult female mice were caged with 1 male. Mice were checked for a vaginal plug daily (checked between 8am and 10am) and females on the day of plug detection were designated post coitum (pc) day 0.5. Mated females were housed in groups of 1-4 females per cage.

Mice were sacrificed using cervical dislocation between 10 am and 12 pm on pc day 17.5. Each viable fetus was dissected from the amniotic sac and umbilical cord and the fetuses and

placentae were weighed. Placental tissues were either cryofrozen, or fixed in 10% formalin and washed in phosphate buffered saline before embedding in paraffin.

RNA Extraction and DNase Treatment

Total RNA was extracted from crushed cryofrozen placental tissue using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. Tissues (~0.15g) were homogenised with 1.5mL of TRIzol in the Precelleys24 homogeniser (5000 RPM, 2 x 30 sec, 20 sec, MANU). DNase I treatment (Qiagen) was performed on all samples. The integrity of the total RNA and miRNAs were examined by gel electrophoresis and quantified using the Nanodrop 2000 (data not shown). Samples were used for further analysis if the 260:280 and 260:230 nm ratios were greater than 1.8.

miRNA Analysis

Expression of miR-155 was measured by qPCR. Total RNA (5 ng) samples underwent reverse transcription to cDNA (TaqMan miRNA Reverse Transcription Kit and probes, Assay ID #002623 for miR-155, #001094 for RNU44; Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's instructions. Samples then underwent quantitative PCR (RT-qPCR) using TaqMan Universal PCR master mix, according to the manufacturer's instructions (Applied Biosystems, Thermo Fisher Scientific). Results were quantified using a 7500 Real-Time PCR System (Applied Biosystems). The expression of miR-155 was determined by calculating $2^{-\Delta\Delta CT}$ using RNU44 (a highly conserved small nucleolar RNA in the growth arrest specific 5 transcript) as the house-keeping gene.

Semi-quantitative reverse transcriptase polymerase chain reaction (qPCR)

All RNA samples underwent reverse transcription to cDNA according to the manufacturer's instructions (Superscript III First-Strand Synthesis for RT, *Thermo Fisher Scientific*). Total RNA was spiked with a known amount of Alien RNA (Stratagene), (10^7 copies per µg of total RNA). qPCR was performed in an Applied Biosystems 7500 Real Time PCR System using SYBR Green for detection. Each reaction mixture contained 5 µL of SYBR Green PCR master mix (Applied Biosystems), AT_1R primers (as described previously [12]), cDNA reversed transcribed from 10 ng total RNA, and water to 10 µL. Messenger RNA abundance was calculated as described previously, using the $2^{-\Delta\Delta CT}$ method and expressed relative to Alien mRNA and a calibrator (a pooled mouse kidney tissue sample) [3].

Protein Extraction

Protein was isolated from tissues using a radioimmunoprecipitation assay buffer (RIPA, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X–100, 1% sodium dodecyl sulphate, SDS) supplemented with a Pierce Halt™ complete protease inhibitor cocktail tablet (Thermo Fisher Scientific), in a ratio of 250 µL buffer to 200 mg of tissue. Samples were then homogenised using the Precelleys24 homogeniser (5000 RPM, 2 x 30 sec, 20 sec, MANU) every 10 mins for 30 mins, and cooled on ice in between. Protein levels were measured using the Pierce BCA Protein assay kit (Life Technologies) according to the manufacturer's instructions.

Immunoblotting

Immunoblotting was performed as described previously [12]. Briefly, samples were loaded into Bis-Tris methane 4–12% gels in duplicate before electrophoresis (Invitrogen, California, United States). Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific) using the wet sandwich method immersed in a transfer buffer. The PVDF membrane was then completely dried and re-activated before

immunodetection. The membrane was rocked in a blocking solution (5% BSA, 5% skim milk in Tris buffered saline (TBS)) for 2 h on a rocker at 22°C. The primary antibody solution was then added (1:1000 dilution, Abcam #ab18801) and samples were incubated at 4°C overnight. The secondary anti-rabbit antibody solution was added (Millipore, Burlington, MA, USA; #12-348, 1:5000) and incubated at 22°C on a rocker for 1 h. Membranes were rinsed before signal detection using an Amersham ECL detection kit (GE Healthcare Life Science) and Amersham Imager 600. Membranes were then dried and stripped using 0.2M NaOH and, using a rabbit polyclonal anti-β-actin antibody (Abcam; ab8227, 1:5000), were used to detect β-actin for normalisation. The ratio of the protein of interest to β-actin was averaged for duplicate lanes and differences between blots were corrected using an internal control (a pooled mouse kidney sample).

Morphometric analysis

The mid-sagittal placental labyrinth and junctional zones were morphometrically analysed, as previously described [247]. Briefly, tissue sections were stained with haemotoxylin and eosin and mounted before imaging. QuPath software was used to measure the cross-sectional areas of the labyrinth, junctional zone and total placenta. From these measurements, ratios of labyrinth to junctional zone, labyrinth to total placenta and junctional zone to total placenta were calculated.

Cell Culture

HTR-8/SVneo cells (an immortalised first trimester trophoblast cell line provided by Professor Charles Graham, Queens University, Ontario) were cultured at 37°C with 5% CO_2 in RPMI-1640 (HyClone) supplemented with 10% heat inactivated FCS (ISAFC Biosciences) and 1% L-glutamine (Gibco). Three separate cultures of HTR-8/SVneo cells were made and from each

of these 3 cultures; three sets of $2x10^5$ cells from passages 10-20 were plated (n=9). After 48 hours, 30pM of the miRVana mimic (Life Technologies) was transfected into each well, using Lipofectamine (Thermo Fisher Scientific) as the transfection vector. Other wells containing cells were treated with Lipofectamine alone (negative control) or with the addition of a scrambled control (where nucleotides are scrambled in a random order to confirm sequence order is specific). Cells and culture media were collected into PBS and snap frozen in liquid nitrogen, then stored at −80°C before RNA analysis.

Proliferation Analysis

Fifty μL of incubation medium was added to each well of an xCELLigence E-plate 16 (ACEA Biosciences Inc., San Diego CA) and allowed to equilibrate at room temperature for 30 min. A background reading in the xCELLigence Real-Time Cell Analysis Multi Plate (RCTA MP) system was then carried out. 1×10^4 HTR-8/SVneo cells from passages 10-20 (cultured as above) were plated in each well with an additional 100 μL of incubation medium and again allowed to equilibrate for 30 min at room temperature. 24 h after cell plating, miRNA miRVana mimics, scrambled mimics or vehicle (lipofectamine) control were added to each well at 0, 7.5, 15, 30 or 60 pM. As the proliferation plates have gold microelectrodes on the bottom of each well, the proliferation of cells impedes electrical conductance. Cell index was generated as a measure of the electrical resistance as cells proliferated. The cell index was measured every 30 min for 48 h in the xCELLigence RCTA MP system. After 48h of incubation with treatment, data were collected and analysed. The rate of proliferation was determined by measuring the slope of the line (cell index) over time.

Statistics

Statistical analysis was undertaken using SPSS Statistics. Where multiple placentae or pups per mother were assessed, a linear mixed model with random intercept accounting for maternal familial correlation was used. Where a single placenta from each mother was assessed, a Mann-Whitney test was used. Graphs were generated using GraphPad Prism 8. Differences between groups were considered significant for $p \leq 0.05$.

5.4 Results

Placental and fetal weights from miR-155$^{+/+}$ (control) and miR-155$^{-/-}$ mice

Pups (n = 4-9 pups / dam, for a total of 10 dams) from miR-155$^{-/-}$ dams had significantly higher fetal weights than pups from control dams ($p = 0.003$; Figure 5.1A) but placental weight (Figure 5.1B) and fetal to placental weight ratios (Figure 5.1C) were not significantly different.

Placental Morphology in control and miR-155$^{-/-}$ mice

Placentae from miR-155$^{-/-}$ mice had a significantly larger labyrinth zone ($p = 0.02$; Figure 5.2A) and labyrinth zone to placental area ratio ($p = 0.04$; Figure 5.2B) compared with placentae from control mice. However, the size of the junctional zone (Figure 5.2C), junctional zone to placental area ratio (Figure 5.2D) and labyrinth zone to junctional zone ratio (Figure 5.2E) were not different between control and miR-155$^{-/-}$ mice. This is shown in representative images of placentae from control (Figure 5.2F) and miR-155$^{-/-}$ (Figure 5.2G) mice.

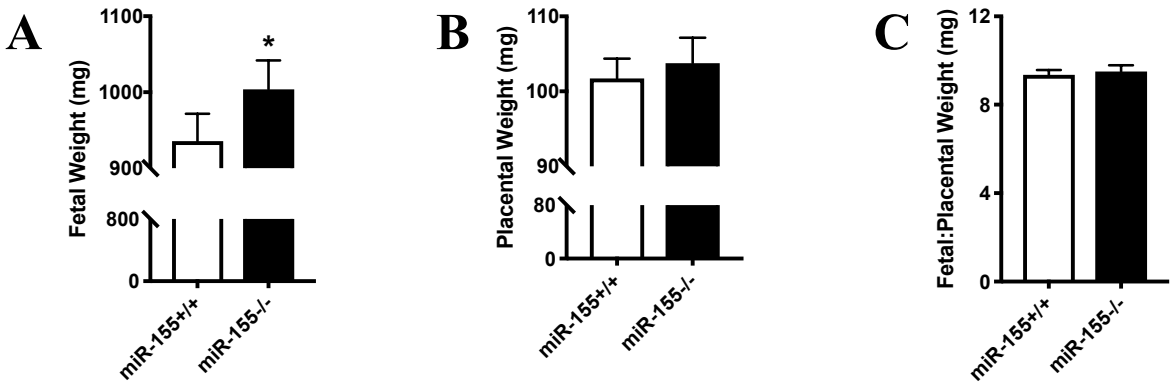


Figure 5.1: miR-155$^{-/-}$ dams produced pups that were significantly heavier but placental weight and fetal to placental weight ratios were unchanged compared to the control.

A The fetal weights for pups from miR-155$^{-/-}$ dams were greater than the weights of pups from control dams, but **B** placental weights and **C** fetal to placental weight ratios between the two groups were similar. *Data are represented as mean ± SEM. N = 4-9 pups and placentae / dam for 10 dams total. * indicates a significant difference (*$p < 0.05$*).*

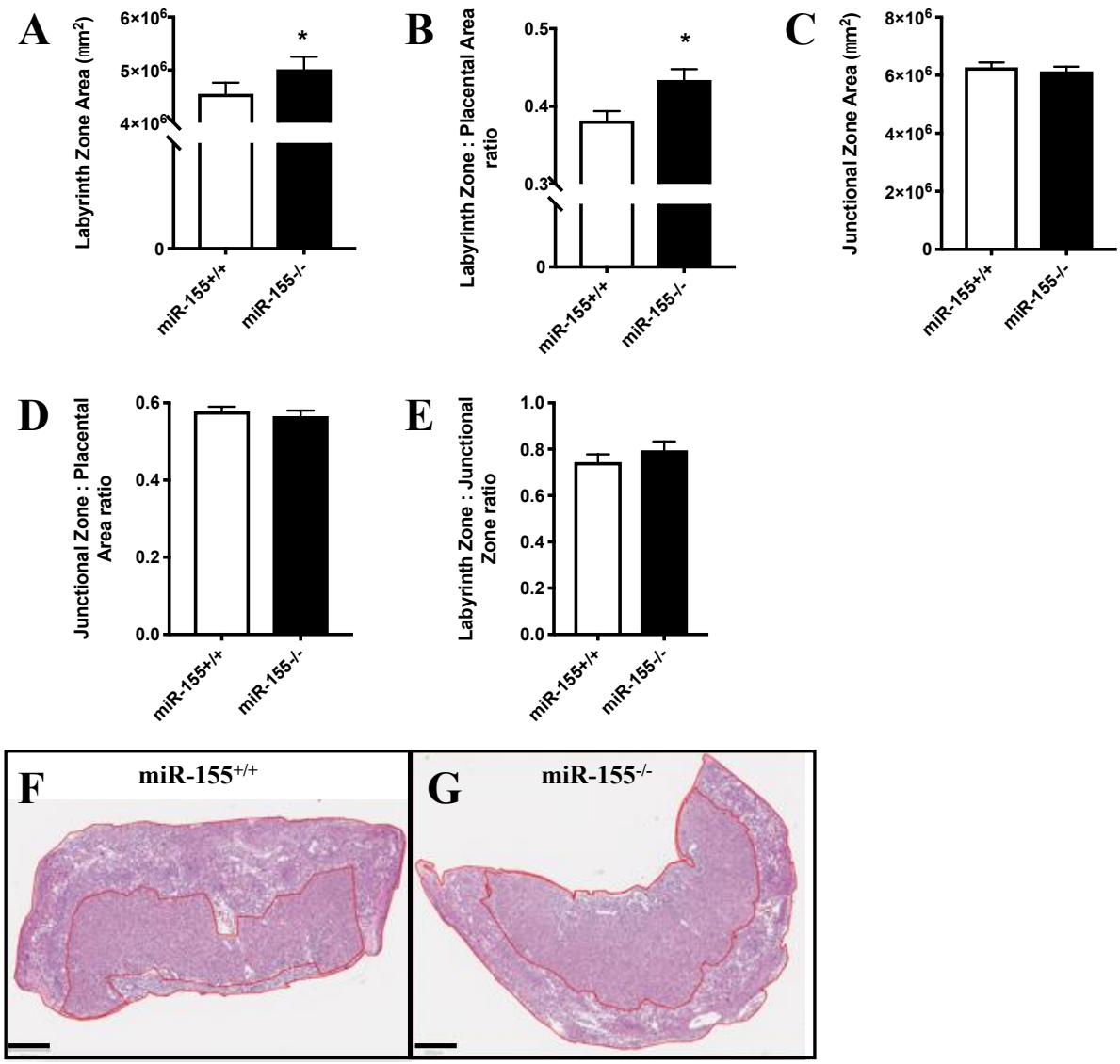


Figure 5.2: Placentae from miR-155$^{-/-}$ dams had significantly larger labyrinth zones and labyrinth to placental area ratios compared to the control.

A Labyrinth zone area and **B** labyrinth zone to placental area ratio from placentae from miR-155$^{-/-}$ dams were greater than the area from control dams, however **C** junctional zone area, **D** junctional zone to placental area ratio and **E** labyrinth zone to junctional zone area between the two groups were similar. **F** The labyrinth zone (outlined in red) from the placenta of a control dam was smaller than **G** the labyrinth zone from the placenta of a 155$^{-/-}$ dam. *Data are represented as mean ± SEM. N = 4-9 pups and placentae / dam for 10 dams total. * indicates a significant difference (*p < *0.05).*

miR-155 and AGTR1 mRNA, and AT_1R protein expression, in control and miR-155$^{-/-}$ mice

miR-155 expression was only found in placentae from control mice, however *AGTR1* mRNA and AT_1R protein were detected in all placentae. The expression of miR-155 was less in placentae from miR-155$^{-/-}$ compared with control mice (p = 0.0003; Figure 5.3A) and *AGTR1* mRNA was increased in placentae from miR-155$^{-/-}$ compared with control mice (p = 0.01; Figure 5.3B).

As well, AT_1R protein was significantly increased in placentae from miR-155$^{-/-}$ mice compared with controls (p = 0.004; Figure 5.3C).

Effect of miR-155 on trophoblast proliferation in HTR-8/SVneo cells

Cells treated with a mimic for miR-155 had significantly increased abundance of miR-155 (p = 0.003; Figure 5.4A) and significantly decreased abundance of *AGTR1* mRNA (p = 0.005; Figure 5.4B) compared with the control. Treatment with 30 and 60 pM of miR-155 mimic significantly decreased trophoblast proliferation compared with the control (p = 0.005, p < 0.001, respectively; Figure 5.4C). Proliferation was also significantly decreased at 30 and 60 pM compared with 7.5 pM (p = 0.047, p < 0.001, respectively). The cell index trajectory for 30 and 60 pM mimic concentrations (magenta and cyan, respectively) can be seen in Figure 5.5D, and it can be seen that the slopes of both are significantly less than control and 7.5 pM mimic slopes (red and blue, respectively), which shows that cell proliferation was reduced.

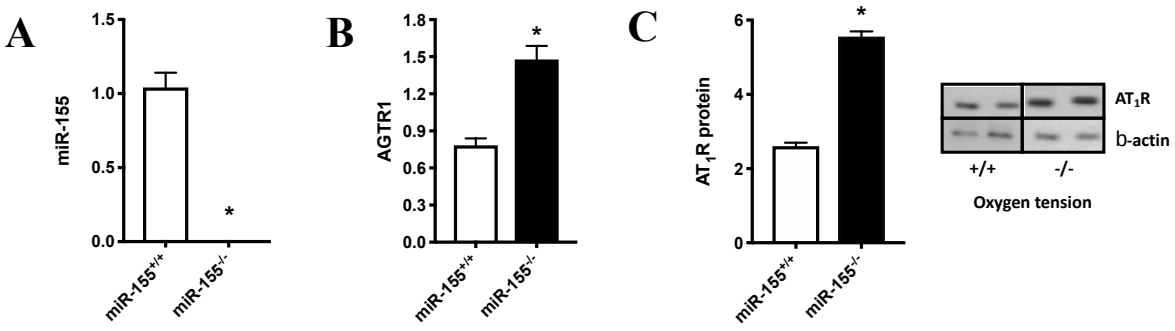


Figure 5.3: miR-155 was not detected in placentae from miR-155$^{-/-}$ dams and *AGTR1* mRNA and protein were significantly increased in placentae from miR-155$^{-/-}$ dams compared to the control.

A Expression of miR-155 was significantly higher in placentae from control mice and was not able to be detected in placentae from miR-155$^{-/-}$ mice. **B** *AGTR1* mRNA expression was significantly upregulated in placentae from miR-155$^{-/-}$ mice compared with controls. **C** AT_1R protein levels was significantly higher in placentae miR-155$^{-/-}$ compared to control mice. *Data are represented as mean ± SEM. N = 4-9 placentae / dam for 10 dams total. * indicates a significant difference (*$p < 0.05$*).*

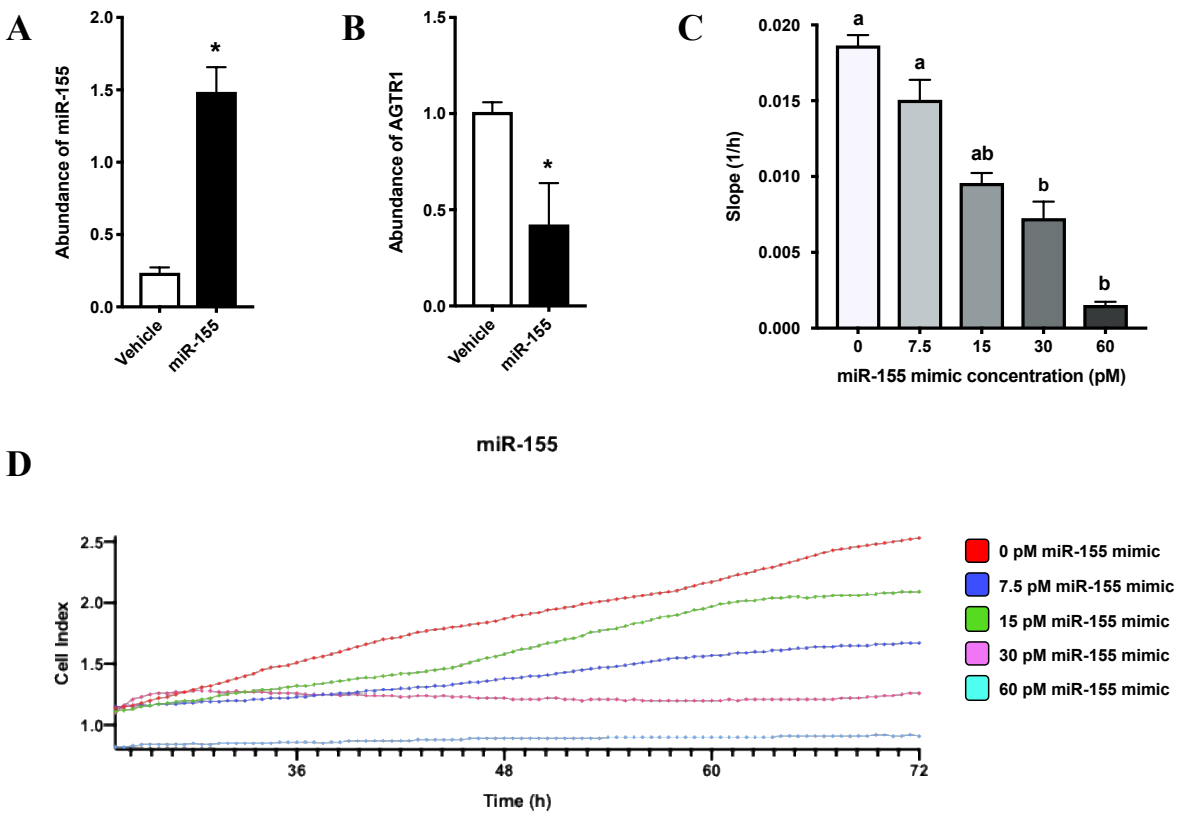


Figure 5.4: The effects of the miR-155 mimic on the abundance of miR-155 and *AGTR1* mRNA, and the rate of cell proliferation in HTR-8/SVneo cells.

A miR-155 expression was significantly increased compared with the control and **B** *AGTR1* mRNA was significantly decreased compared with the control. **C** The rate of proliferation of cells transfected with miR-155 was significantly decreased by treatment with a miR-155 mimic at 30 and 60 pM. **D** The slopes of the lines for both 30 and 60 pM mimic concentrations (magenta and cyan, respectively) are significantly less than control and 7.5 pM mimic slopes (red and blue, respectively), showing that cell proliferation was reduced. *Data are presented as mean ± SEM. N = 3 experiments, each in triplicate. * indicates a significant difference to the vehicle control (*$p < 0.05$*). The same letter above bars indicates that groups are not different from each other. A different letter above bars indicates that groups are different (all* $p < 0.05$*).*

5.5 Discussion

We have shown that miR-155 affects placental cell function and placental development, and that miR-155 knockout disrupts placental morphology by altering labyrinth and junctional zone development. We can also conclude that this occurs at least in part, due to the inhibitory action of miR-155 on *AGTR1* mRNA expression.

The impact of miR-155 deletion was easily observed when the placental morphology was analysed. The murine placenta can be subdivided into two distinctive morphological zones. The labyrinth, or labyrinthine zone, is closest to the fetus and is considered to be the functional zone of the placenta where nutrient, gas and waste exchange occurs [248]. This area is comprised of filaments of fetal tissue containing fetal capillaries among pools of maternal blood. As three layers of fetal-derived trophoblasts separate the fetal capillaries and maternal blood, the murine placenta is categorised as haemotrichorial [249]. The junctional zone is the next cellular compartment containing spongiotrophoblasts and glycogen cells [250]. This zone is hypothesised to be mainly involved in the structural, hormonal and immunological properties of the placenta, and removal of this zone is embryolethal [251]. Bordering the junctional zone is the maternal decidua.

In placentae from miR-155$^{-/-}$ mice, there was a significant increase in the labyrinth zone area, as well as the labyrinth zone to total placenta area ratio. This increase in labyrinth zone size suggests that there was increased trophoblast differentiation, resulting in increased substrate transfer capacity of the placenta and in turn, an increased ability to accelerate growth and sustain a larger fetus [252]. Indeed, at the same gestational age miR-155$^{-/-}$ mice did have larger fetuses than controls. As the placental weights were unchanged between the control and miR-155$^{-/-}$ litters, the increased proportion of the labyrinth zone in miR-155$^{-/-}$ placentae would suggest that the placenta is more efficient because of its relatively greater functional capability. Further study into specific cell type analysis may be of benefit in assessing the

degree of cell differentiation in the placentae of miR-155$^{-/-}$ dams compared with miR-155$^{+/+}$ placentae.

Adequate trophoblast proliferation is essential for placental growth. We showed that treatment with a miR-155 mimic decreased HTR-8/SVneo cell proliferation in a dose-dependent manner, which supported previous studies that also found that miR-155 decreases cell proliferation [184, 186]. This is coherent with our findings that miR-155 deficient placenta are more efficient through larger labyrinth zone area, although we did not detect a significant difference in placental weight between the two groups.

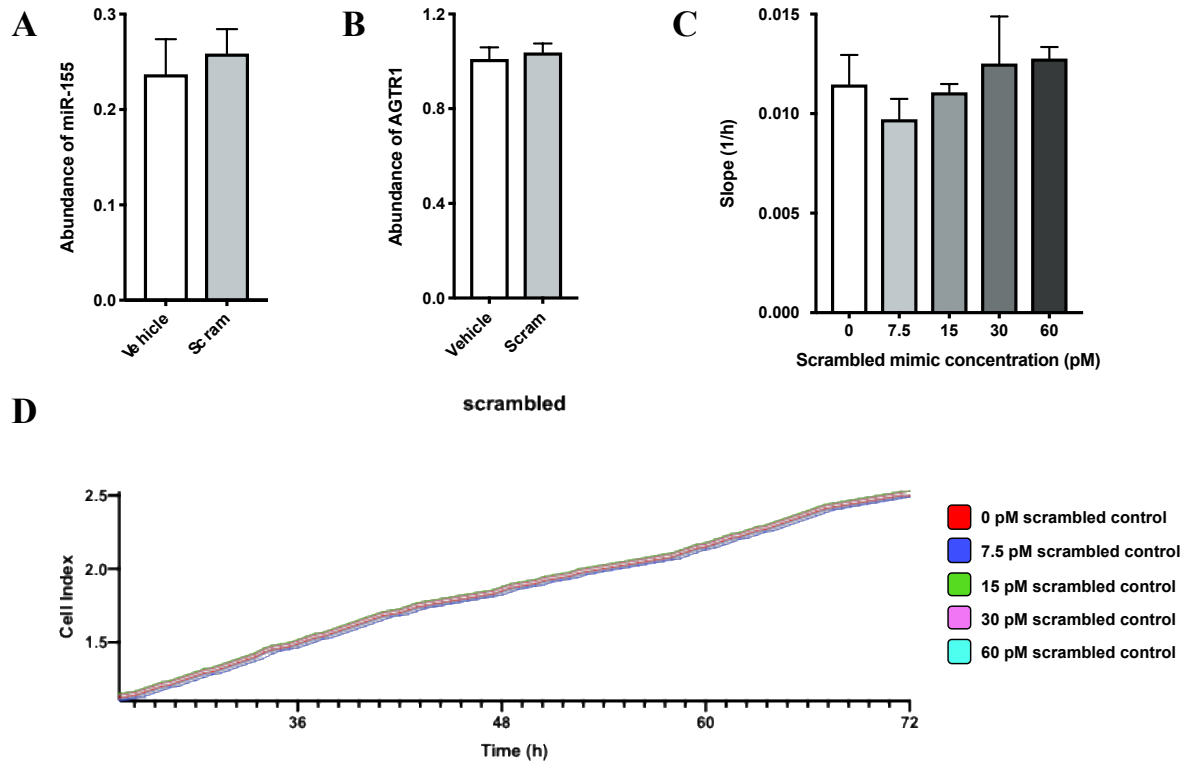
The actions of miR-155 are complex, as it is known to regulate many targets. Within the placenta, studies have associated miR-155 expression with downregulation of cysteine-rich protein 61 (CYR61), which is an important angiogenic regulating factor during pregnancy [185]. Importantly, CYR61 is essential for placental vascular integrity [253] and is shown to be decreased in PE [254]. miR-155 has also been implicated in the suppression of the PTEN 30-untranslated region, leading to reduced AP-1/NF-kB pathway activity [241]. Notably, Hromadinova *et al.,* have shown that miR-155 directly interacts with the 3'-UTR of *AGTR1* mRNA and several studies have explored the interaction of miR-155 and *AGTR1* in the placenta [189, 190].

We have shown that culturing trophoblast cells with a miR-155 mimic decreased both *AGTR1* mRNA and AT_1R protein, supporting our finding that placentae from miR-155$^{-/-}$ dams had low levels of *AGTR1* mRNA. We conclude that the effects of miR-155 on placental development and morphology are at least partly due to its regulation of AT_1R expression.

It should be noted that in PE there is a much higher Ang II sensitivity; therefore, even at a lower AT_1R expression level, Ang II effectiveness may still be enhanced. It has been claimed (largely based on animal models e.g. related to AT_1R autoantibodies, which are expressed in

PE and responsible for elevation of sFlt1, soluble Endoglin, oxidative stress and endothelin-1) that blockade of the RAS may be beneficial in PE, however RAS blocking drugs are teratogenic and not recommended in pregnancy. This is consistent with our data as in PE the RAS is dysregulated and placental growth is suboptimal, just as increased expression of miR-155 hindered trophoblast proliferation.

This study confirms the importance of adequate Ang II/AT_1R signalling in placentation and reinforces findings of a recent study by Delforce, *et al.*, that show that specific inhibition of the AT_1R by losartan inhibits expression of the angiogenic / proliferative trophoblast phenotype. This phenotype is highly correlated with expression of pro-angiogenic factors and cell viability [7]. Binding of Ang II to the AT_1R has also been implicated in cytotrophoblast secretion of many hormones essential for healthy pregnancy, including placental lactogen, human chorionic gonadotropin and pregnancy-specific glycoprotein [255], as well as estradiol [256]. Furthermore, placental AT_1R capacity and affinity are reduced in PE [257] but expression is upregulated [258]. As mentioned previously, AT_1R knockout in the placenta severely impedes placental growth and vasculogenesis [96]. Even intrauterine growth restriction is associated with reduced placental *AGTR1* mRNA and AT_1R protein [259], which is consistent with our results as miR-$155^{-/-}$ placentae expressed significantly higher levels of AT_1R mRNA and protein, and produced larger fetuses.



Supplementary Figure 5.1: Abundance of miR-155 and AGTR1 mRNA measured by qPCR, and proliferation levels measured using the xCELLigence RTCA system show that there were no differences between negative and scrambled controls.

A miR-155 expression and **B** *AGTR1* mRNA were not significantly different in scrambled and negative controls. **C** The rate of proliferation of cells transfected with miR-155 and **D** the trajectories for all scrambled mimic concentrations were not significantly different. *Data are presented as mean ± SEM. N = 3 experiments, each in triplicate.*

6. Discussion

This thesis focuses on the RAS within placental trophoblasts and chorionic villi. I propose that miRNAs that target placental RAS mRNAs, regulate RAS gene expression and influence placental development. I have shown that dysregulation of these miRNAs has a critical impact on the ability of placental cells to proliferate and on placental development. I am the first to study miRNAs that target placental RAS mRNAs and their effects on placental development.

In early gestation, the placental RAS acts predominantly via Ang II/AT_1R binding to stimulate trophoblast proliferation [102], as well as migration and vascular development [4]. Furthermore, the RAS is known to mediate spiral artery remodelling, allowing adequate blood supply to access the chorionic villi in the second trimester [260], and dysregulation of the RAS in the placenta impedes placental development and function [98]. As these events occur early in gestation, they are responsible for establishing the placenta and providing an appropriate environment for the growing fetus. For optimal fetal and maternal health, the placenta must provide nutrients and oxygen to meet the increasing requirements of the fetus throughout gestation and it must maintain the pregnancy through endocrine secretions of estrogens and progesterone.

miRNAs are present throughout gestation and change according to the stage of pregnancy. They have considerable effects on the placental RAS, as they can influence expression of all the RAS mRNAs and proteins that I have studied. While miRNAs play an important physiological role in ‘fine-tuning’ mRNA translation, when expressed inappropriately they can also play a role in the pathophysiology of disease. In addition, there is evidence that placental miRNAs are able to escape into both the fetal and maternal circulations [130]. This raises concerns for both the mother and the fetus, as the RAS is both a circulating system, that

regulates blood pressure and fluid balance, and is also expressed in multiple developing tissues [79]. Inappropriate levels of miRNAs that target the RAS could therefore affect the development of the fetal kidney [261], heart [262], eye [263] and brain [264]. As miRNAs are also secreted into the maternal circulation, they may not only disturb placental perfusion and fetal development, but also renal regulation of maternal fluid and electrolyte homeostasis. It is not surprising that there is abnormal expression of both the RAS and miRNAs targeting the RAS in the maternal circulation and the placenta in pregnancy complications associated with placental insufficiency, such as PE and IUGR [12, 65, 176, 180].

The studies outlined in my thesis have described possible mechanisms that regulate miRNAs that target the RAS and therefore RAS mRNA expression and the effects of these miRNAs on the placenta (Figure 6.1). However, the exact miRNAs that control this system in each individual person will vary, and the *in vivo* targets of these miRNAs are still unknown. Further investigation of miRNA expression and control of the placental RAS will improve our understanding of placentation and placental development, as well as potentially identifying a panel of miRNAs that could be biomarkers or therapeutic targets for pregnancy associated complications.

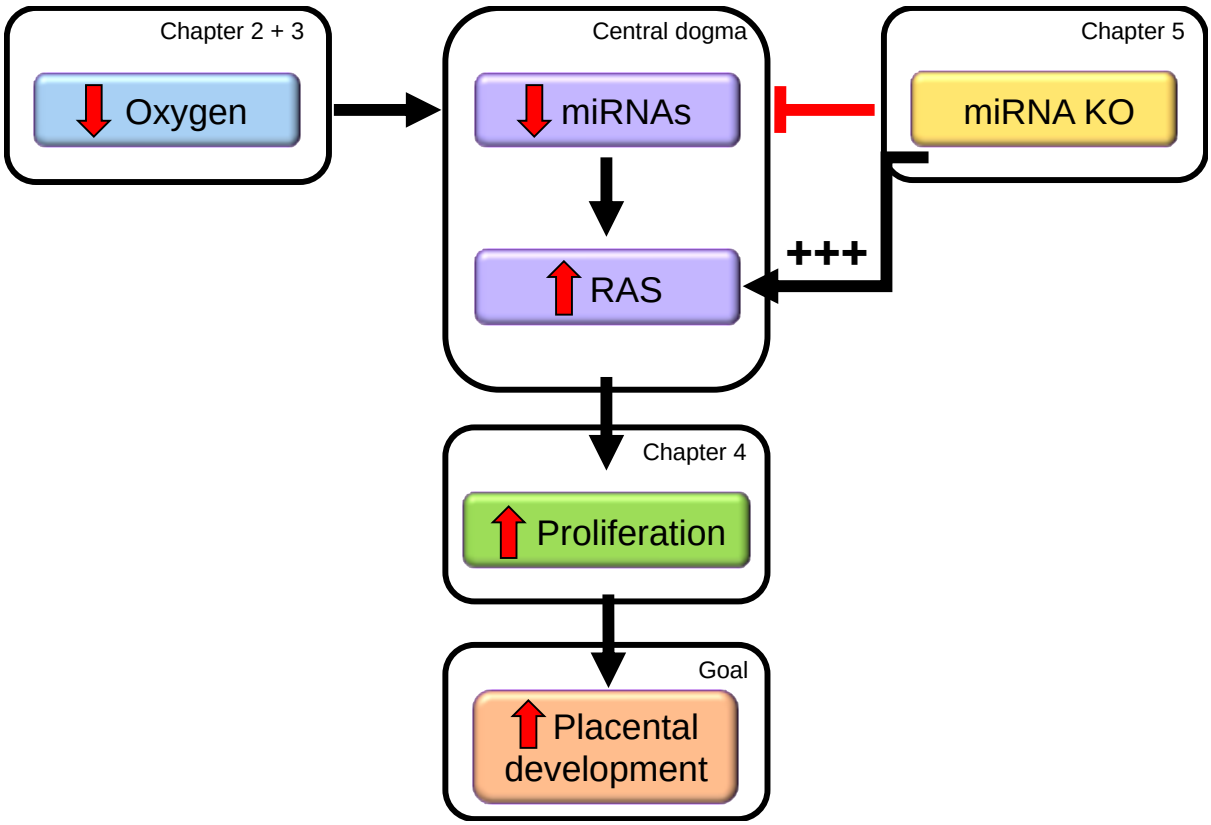


Figure 6.1: Pathways studied in this thesis

This thesis focused on the central hypothesis that downregulation of miRNAs targeting RAS mRNAs allows upregulation of RAS gene expression in early gestation, thus promoting placental development. Chapters 2 and 3 showed that a low oxygen tension, similar to that seen in the first trimester, supported this central proposition in HTR-8/SVneo cells and first trimester chorionic villous explants, respectively. Chapter 4 showed that miRNAs that are predicted to target the RAS and which are decreased in first trimester placenta, repress placental RAS gene expression, leading to decreased proliferation of trophoblasts. Chapter 5 utilised a miR-155 knock-out model to show that inhibition of this miRNA allowed increased expression of its target RAS mRNA, AGTR1, which was associated with increased placental efficiency.

Exploration of miRNAs that control the placental RAS *in vitro*

I have successfully demonstrated that culture of HTR-8/SVneo cells in a low oxygen tension similar to that experienced by the first trimester placenta (1% O_2) not only downregulates the expression of multiple miRNAs targeting RAS mRNAs, but also regulates the expression of over 200 miRNAs in trophoblasts (Chapter 2), which could have a host of different targets that could be investigated in future studies. Furthermore, we found that the expression of numerous RAS components were increased in a low oxygen environment compared with control. These results are consistent with findings from a previous study showing that culture of HTR-8/SVneo cells in low oxygen stimulates levels of RAS mRNAs and proteins [9].

I also demonstrated that some miRNAs known to target RAS mRNAs are suppressed by low oxygen tension in first trimester CVEs. However, many miRNAs and RAS mRNAs and proteins were not expressed in the same way as in HTR-8/SVneo cells. Only four of the tested miRNAs were significantly reduced in low oxygen, and many RAS mRNA and protein levels were unaffected by low oxygen. It has been shown that CVEs have different expression of the RAS compared with HTR-8/SVneo cells (Delforce *et al, unpublished observations)* so it is perhaps not surprising that they also have different miRNA profiles. One explanation for the difference is that HTR-8/SVneo cells are a pure population of immortalised first trimester extravillous trophoblasts, whereas the CVEs are comprised of multiple cell types, including CTB, STB, endothelial and stromal cells. Not only are these explants comprised of a heterogenous cell population, but because they are being sourced from primary tissue, they are subject to maternal stresses, genes and hormones. This could alter the miRNA and gene expression profile of the tissue. In addition, oxygen tension would be expected to influence EVTs and CVEs differently due to their different locations and functions within the placenta. EVTs are stimulated by oxygen to adopt an invasive or proliferative phenotype [265]. EVTs closest to the chorionic villi experience a lower oxygen tension and adopt more of a proliferative phenotype, working towards placental development. However, EVTs in the vicinity

of the maternal decidua have a slightly higher oxygen tension and thus invade into the maternal tissue to plug and remodel the spiral arteries [28]. However, we can conclude that low oxygen stimulates CVEs and EVTs to participate in their respective functions in placental development by repressing miRNAs and stimulating the RAS.

While the studies outlined in Chapters 2 and 3 examined the role of oxygen in regulating the expression of miRNAs that target the placental RAS, it should be noted that oxygen is only one of many potential factors regulating miRNA expression throughout gestation. Many factors are involved in regulating miRNA expression including DNA methylation, chromatin modifications, various transcription factors and silencing elements, hormonal alterations occurring during pregnancy, and autoregulatory feedback mechanisms [266, 267].

I then explored the functional effects of these miRNAs that target RAS mRNAs on trophoblast proliferation (Chapter 4), showing that when most of these miRNAs, when in excess, decreased proliferation in a dose-dependent manner. Importantly, many of the miRNA mimics that yielded dose-dependent decreases in trophoblast proliferation were shown to target *REN* mRNA. Prorenin itself stimulates cell proliferation and placental development through independent binding to the prorenin receptor [268] as well as through initiating the traditional Ang II/AT_1R cascade [269]. In this way, prorenin is a rate-limiting step in initiating proliferation. Our results show for the first time that all investigated mimics of miRNAs targeting *REN* mRNA significantly reduced proliferation.

We postulate therefore that in early gestation, when oxygen tension in the placenta is low, miRNAs targeting RAS mRNAs are suppressed. This allows increased expression of the RAS, stimulating placentation by encouraging trophoblast proliferation. As placentation is facilitated through not only trophoblast proliferation but also migration, invasion and angiogenesis, in future studies we would like to examine the effects of these miRNAs on these properties of trophoblasts using the same study design and technology.

In chapters 2 and 4, I describe experiments in which I used HTR-8/SVneo cells to examine the role of miRNAs on placental RAS expression and trophoblast proliferation. While my studies have shown that this cell line has a very similar miRNA expression profile to primary placental cells [168] we are aware that HTR-8/SVneo cells do not express the anti-proliferative arm of the RAS [270] and thus are only a valid model for examining miRNAs targeting the Ang II/AT_1R arm of the RAS. Therefore, I also examined the expression of the RAS pathway components and miRNAs that target those mRNAs in freshly collected first trimester placental tissue (Chapter 3).

Whilst the same number of cells were plated for each proliferation study in Chapter 4, it should be noted that cells used were not always at the same level of confluency before plating in the xCELLigence wells. This could be a source of variation in results, as cells at higher confluency levels are at different cell cycle states (e.g. entering senescence) compared to cells at a lower confluency.

One limitation of these studies is the small number of miRNAs that were explored. We selected a subpopulation of miRNAs from the HTR-8/SVneo cell oxygen tension microarray for validation, however there were many miRNAs that could target the placental RAS that were not validated. Only the identified miRNAs from Chapter 2 were examined for their effect on trophoblast proliferation (Chapter 4) and for their expression in CVEs (Chapter 3). While studying the individual effects of many miRNAs on proliferation may not be viable on a large scale, it could be advantageous to identify all miRNAs affected by oxygen tension in CVEs to construct a complete dataset. Therefore, in future studies we would like to complete a miRNA microarray on CVEs cultured in different oxygen tensions to identify differential miRNA expressions.

Examination of miRNAs that control the placental RAS *in vivo*

I utilised a miR-155 knockout model in the mouse to explore the regulation of the placental RAS by this miRNA in placentation. I showed that overexpression of miR-155 in HTR-8/SVneo cells downregulates AT_1R expression and that knocking out miR-155 in a mouse model results in increased placental AT_1R mRNA and protein levels. I propose that the miR-155 knockout, and subsequent AT_1R upregulation, is associated with increases in placental vasculogenesis, proliferation and differentiation [3, 4, 94, 96, 123], resulting in a larger placental labyrinthine zone and more efficient placenta, as evidenced by an increased in fetal weight (Chapter 5).

Initially it seemed as though knockout of miR-155 may be advantageous for placental physiology because it increased labyrinth zone area. Since the labyrinth zone facilitates nutrient and oxygen transfer to the fetus, this would explain why miR-$155^{-/-}$ dams produced larger babies. The nutrient and oxygen transfer capacity of miR-$155^{-/-}$ placenta would be much greater due to their greater placental labyrinth to placental area ratio. Although the junctional zone does not greatly contribute to fetal development, it has been shown to also be essential for placental development. Placentae without a junctional zone lead to death of the embryo [251]. Overall this study indicates that knockout of miR-155 may increase the development of the labyrinth zone and miR-155 may need to be present in small amounts in early gestation in order for the placenta to develop adequate junctional to labyrinth zone ratios.

I also demonstrated that miR-155 effectively suppresses AT_1R mRNA and protein expression and I believe that miR-155 exerts its effects on placental development through its efficient control of the AT_1R. Not only is the AT_1R extremely important for successful placentation [96], it is also dysregulated in pregnancy complications such as PE [95, 184] where placental development is impeded [271]. This upregulation of the AT_1R in PE could be due to hypoxia in the placenta; that is, shallow placentation that is characteristic of PE leads to late gestation

hypoxia, suppression of miR-155 expression and inappropriately late upregulation of AGTR1 mRNA.

In the summarising of Chapter 6, it is suggested that AT_1R are upregulated in PE due to hypoxia; however, this seems inconsistent with the increased miR-155 levels in PE, which would be expected to downregulate AT_1R expression. Whilst there is a contradiction between increased AT_1R in PE and increased miR-155, miRNAs have a vast number of potential target mRNAs, and also can regulate pathways. As miR-155 is not the only regulator of AT1R, a simple inverse relationship between their expression cannot be assumed.

The results from this study have remarkable potential to be translated into clinical practice, as I have shown that the AT_1R and miR-155 work together in the human placenta to optimise placental development and function. Furthermore, miR-155 levels in the placenta [184] and in maternal serum [242] are significantly upregulated in PE. As miR-155 is easily detected in maternal serum, a simple blood test might identify high-risk pregnancies early in gestation. This represents a valuable opportunity for early identification of women at risk of developing PE and prompt intervention to ensure clinical management of symptoms. I propose that further investigation into the gestational profile of miR-155 serum levels across pregnancy in women with PE should occur, as this would be beneficial to isolate key time points at which this miRNA could be used for monitoring purposes.

In future studies, it would be beneficial to determine whether the overexpression of serum miR-155 levels in PE is due to placental secretion of miR-155. This would elucidate whether the miR-155 dysregulation that is seen within the placenta in PE is contributing to system-wide effects of the disease, as placenta-derived miRNAs are able to travel into, and can potentially exert their effects in, the maternal system [159]. By isolating exosomes containing miR-155 from maternal serum and determining whether they are tagged with PLAP, we could establish whether this miRNA originated from the placenta. Furthermore, this protocol could

be used to examine miRNAs from the placenta that are present in umbilical cord blood and could be transferred into the fetal circulation, as it has previously been suggested that placental-derived miRNAs could enter into the fetal circulation and affect organ development [130, 272]. The developing kidney is susceptible to unfavourable intrauterine environments [273], and adequate RAS expression is essential for fetal kidney development [274]. Furthermore, changes in expression of the RAS [275] and miRNAs that can target this system [276] are seen in cases of inadequate nephrogenesis. Thus, the invasion of placental miRNAs into the fetal circulation could alter the fetal RAS and in turn, disrupt fetal kidney development.

It is evident that appropriate miR-155 expression is critical for adequate placentation. I showed that miR-155 expression is unaffected by oxygen (Chapters 2 and 3) and previous studies have shown that miR-155 does not significantly change in its expression across gestation [12]. The stable expression levels of miR-155 across pregnancy indicates that it is required across gestation in small amounts. As the complete knockout of miR-155 allowed labyrinth zone development and larger fetal growth, we can conclude that modest expression of miR-155 is beneficial for placental development and in turn, optimal substrate transfer to the fetus, enabling growth. However, overexpression of miR-155 represses trophoblast proliferation (Chapter 4) and is observed in PE [183, 184, 190]. Clearly complex control of this miRNA's expression is required to improve placentation and pregnancy outcomes.

Notably, the effects of miR-155 knockout on placental development are not solely due to the subsequent upregulation of the AT_1R. This can be clearly observed by comparing the relative roles of miR-155 and the AT_1R in placental development. My study showed that miR-155 knockout allows labyrinth growth and fetal growth; however, a previous study which utilised an AT_1R knockout showed that this was detrimental to vascularisation of the labyrinth and overall placental size. Whilst there is overlap in the actions of miR-155 and the AT_1R, this suggests that other cells and targets are affected by miR-155 expression as well. Indeed, miR-155 has been shown to regulate secretion of numerous chemokines, cytokines and

transcription factors in CD4+ T cells [277], as well as playing critical roles in function of other immune cells including B cells [278]. Studies have also implicated miR-155 in inducing apoptosis in dendritic cells [279] and in cytotrophoblast differentiation [280]. The effects of miR-155 are widespread and further studies should investigate the direct mechanisms by which miR-155 affects placental development.

6.1 Conclusion

Overall, this thesis describes the role of miRNAs as post-transcriptional regulators of RAS genes in the placenta. I have shown that oxygen represses miRNA expression and upregulating RAS gene expression, both in a placental cell line and in first trimester chorionic villous explants. Furthermore, I have demonstrated that many miRNAs targeting placental RAS mRNAs decrease expression of their target RAS mRNAs and subsequently decrease trophoblast proliferation *in vitro*. Finally, I have explored the role of miR-155 in placental development and shown that knocking out miR-155 increased placental AT_1R expression, increased placental efficiency with larger labyrinth zones, and resulted in increased fetal weights. Thus, the work described in this thesis contributes new knowledge to the field of miRNA regulation of the placental RAS and how they both contribute to proper placental development and function.

7. References

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8. Appendices

Appendix 1: Microarray data depicting miRNAs with altered expression between 1% and 5% O_2 tensions

Transcript ID (Array Design)	p-value (1% vs. 5%)	Fold-Change (1% vs. 5%)
hsa-miR-4521	1.61E-02	-3.04049
hsa-miR-4767	4.13E-04	-2.26156
hsa-let-7d-3p	2.86E-02	-2.10307
hsa-miR-4271	1.15E-02	-1.94978
hsa-miR-6846-5p	4.52E-02	-1.94377
hsa-miR-8075	3.79E-02	-1.88116
hsa-miR-1234-3p	4.65E-02	-1.77731
hsa-miR-6734-5p	1.15E-02	-1.67516
ENSG00000212378	6.58E-03	-1.59925
U78	6.58E-03	-1.59925
hsa-miR-6894-5p	4.96E-02	-1.59732
hsa-miR-4699-5p	9.58E-03	-1.56585
hsa-mir-3120	4.73E-02	-1.53698
hsa-miR-4529-3p	3.20E-02	-1.5275
hsa-miR-6880-5p	2.81E-02	-1.51695
U78	4.11E-03	-1.51517
U50	3.17E-02	-1.4718
U79	8.66E-03	-1.47083
U14B	5.47E-03	-1.46268
hsa-miR-27b-3p	1.67E-03	-1.45911
hsa-let-7b-3p	2.91E-03	-1.4459
hsa-miR-30c-5p	2.75E-02	-1.44482
hsa-miR-376a-3p	1.94E-02	-1.44302

hsa-miR-32-3p	2.79E-02	-1.44157
hsa-miR-7109-5p	3.85E-02	-1.42284
hsa-miR-4423-3p	1.84E-02	-1.40297
hsa-miR-18b-5p	3.25E-02	-1.39577
hsa-miR-7112-3p	8.23E-03	-1.39095
hsa-mir-365a	2.22E-02	-1.38881
hsa-miR-4490	3.41E-02	-1.38532
hsa-miR-5697	2.52E-03	-1.38308
hsa-miR-4434	3.05E-03	-1.37618
ENSG00000221398	1.33E-02	-1.3725
U14B	1.35E-02	-1.37069
hsa-miR-4672	2.18E-02	-1.36983
ENSG00000212604	1.67E-02	-1.36527
hsa-miR-126-5p	8.05E-03	-1.36127
hsa-miR-3945	8.89E-03	-1.35844
hsa-miR-499a-5p	4.95E-02	-1.35667
HBII-202	3.46E-03	-1.35409
hsa-miR-6780b-3p	1.45E-02	-1.35395
ENSG00000212615	5.96E-03	-1.34789
hsa-miR-6715a-3p	2.49E-02	-1.34241
ENSG00000238608	3.42E-02	-1.34203
hsa-mir-4750	4.31E-02	-1.34063
hsa-mir-4735	2.98E-03	-1.33135
hsa-miR-6735-5p	4.22E-02	-1.32735
hsa-miR-4520a-5p	1.37E-02	-1.32366
hsa-miR-4520b-5p	1.37E-02	-1.32366
hsa-miR-383-3p	1.36E-02	-1.32207
hsa-miR-7157-3p	1.83E-02	-1.31917
ENSG00000238807	2.48E-02	-1.31886
hsa-mir-6883	9.49E-03	-1.31833
hsa-mir-1236	2.69E-02	-1.31808

hsa-mir-4284	9.28E-03	-1.31496
ENSG00000238575	3.94E-02	-1.31291
hsa-miR-3658	1.41E-02	-1.3128
hsa-miR-4296	3.43E-02	-1.31004
hsa-miR-3189-3p	3.38E-02	-1.30968
U42A	2.03E-02	-1.30835
hsa-miR-6783-3p	4.30E-02	-1.30813
hsa-miR-4722-5p	4.61E-02	-1.30504
hsa-miR-30a-3p	5.11E-04	-1.30492
ENSG00000252133	3.92E-02	-1.30005
ENSG00000252724	3.92E-02	-1.30005
ENSG00000252878	3.92E-02	-1.30005
HBII-52-23	2.51E-02	-1.29766
hsa-mir-5583-1	1.02E-02	-1.29654
hsa-mir-383	1.97E-02	-1.29593
hsa-mir-4323	2.48E-02	-1.29514
hsa-let-7a-3	7.43E-03	-1.29372
hsa-miR-4457	6.14E-03	-1.29182
hsa-mir-4760	1.02E-03	-1.29126
hsa-mir-4510	3.62E-02	-1.29081
ENSG00000239046	2.51E-02	-1.28908
ENSG00000239100	8.82E-03	-1.28883
hsa-mir-4666b	3.15E-02	-1.28756
hsa-miR-632	3.08E-03	-1.28562
hsa-miR-6847-3p	4.07E-02	-1.28511
hsa-miR-5700	1.82E-02	-1.2838
ENSG00000201042	4.91E-02	-1.28196
hsa-miR-7162-5p	3.58E-02	-1.28107
hsa-miR-449b-3p	3.05E-02	-1.27924
hsa-miR-9-3p	4.51E-02	-1.27921
hsa-miR-6885-3p	1.75E-02	-1.2765

ENSG00000238940	3.82E-02	-1.2737
hsa-mir-7844	2.57E-02	-1.27264
ENSG00000238770	4.93E-02	-1.2699
hsa-miR-503-5p	7.30E-03	-1.26845
ENSG00000252284	1.81E-02	-1.26471
hsa-miR-5685	4.88E-02	-1.25787
hsa-miR-6828-5p	3.11E-02	-1.25552
hsa-miR-15b-5p	2.70E-02	-1.2523
U44	2.11E-02	-1.25227
hsa-mir-4260	2.78E-03	-1.25005
ENSG00000238403	3.47E-02	-1.24677
hsa-mir-4726	4.11E-02	-1.24536
hsa-mir-128-1	2.48E-02	-1.24216
hsa-mir-302a	5.10E-03	-1.23913
hsa-miR-96-3p	1.10E-02	-1.23748
hsa-miR-7150	1.22E-02	-1.23726
ENSG00000238805	4.09E-02	-1.23161
hsa-miR-3941	4.72E-02	-1.23024
hsa-mir-147b	2.46E-02	-1.22972
hsa-miR-1204	4.98E-02	-1.22948
hsa-mir-1471	2.76E-02	-1.22807
hsa-let-7g-3p	4.12E-02	-1.22589
hsa-miR-4501	3.40E-02	-1.22472
hsa-miR-3183	3.53E-02	-1.22401
HBII-52-23	1.87E-03	-1.22136
hsa-miR-544b	1.60E-02	-1.22059
hsa-miR-5701	5.17E-03	-1.21926
ENSG00000238718	4.46E-02	-1.21819
hsa-mir-548a-3	3.99E-02	-1.21772
hsa-mir-3657	8.93E-03	-1.21641
hsa-mir-208b	4.33E-02	-1.21114

hsa-miR-6718-5p	4.77E-02	-1.20794
hsa-mir-4426	4.05E-02	-1.20564
hsa-miR-365a-3p	6.34E-03	-1.20509
hsa-miR-365b-3p	6.34E-03	-1.20509
ENSG00000238319	4.52E-02	-1.20367
hsa-miR-4535	5.78E-03	-1.2035
hsa-mir-1255a	3.21E-02	-1.20324
hsa-miR-6513-3p	2.35E-02	-1.19924
ENSG00000212567	2.34E-02	-1.19796
hsa-miR-28-5p	2.12E-03	-1.18782
hsa-mir-5688	6.78E-03	-1.18689
ENSG00000201036	2.76E-02	-1.18541
hsa-mir-5188	3.71E-02	-1.18505
U33	4.74E-02	-1.18391
U75	7.18E-03	-1.18327
hsa-mir-4727	3.27E-02	-1.17853
hsa-miR-6853-3p	4.27E-02	-1.17804
hsa-mir-4433	2.29E-02	-1.17605
hsa-mir-6810	2.90E-02	-1.17465
ENSG00000253090	2.81E-02	-1.17461
hsa-miR-6887-3p	2.79E-02	-1.1726
hsa-miR-3198	3.57E-02	-1.17222
ENSG00000251974	2.04E-03	-1.16802
hsa-miR-580-3p	4.36E-02	-1.16528
hsa-miR-514a-3p	4.69E-02	-1.16469
hsa-mir-4762	3.52E-02	-1.16306
hsa-miR-23b-3p	9.15E-03	-1.15742
ENSG00000221083	3.42E-02	-1.15723
ENSG00000252441	3.27E-02	-1.1545
ENSG00000268145	3.27E-02	-1.1545
hsa-miR-3973	1.56E-02	-1.15372

hsa-miR-1537-3p	3.93E-02	-1.15351
hsa-mir-6758	8.77E-03	-1.15067
ENSG00000212589	4.18E-02	-1.14948
hsa-miR-506-5p	1.51E-02	-1.14866
mgh28S-2409	3.57E-02	-1.14824
ENSG00000252765	2.70E-02	-1.14783
hsa-mir-1278	2.70E-02	-1.1478
hsa-miR-598-3p	6.66E-03	-1.14748
hsa-miR-4736	2.23E-02	-1.13861
ENSG00000201710	4.31E-02	-1.13359
U58A	3.72E-02	-1.13293
hsa-mir-517c	2.50E-02	-1.13271
hsa-miR-3177-5p	4.88E-02	-1.11276
hsa-miR-409-3p	3.61E-02	-1.106
ENSG00000238974	9.28E-03	-1.10357
hsa-miR-4266	2.15E-02	-1.10286
ENSG00000202269	2.31E-02	-1.09669
hsa-mir-3978	1.22E-02	-1.09466
hsa-miR-149-5p	3.94E-03	-1.09319
hsa-mir-4700	5.30E-03	-1.08871
155	8.45E-03	-1.07968
ENSG00000253076	4.46E-02	-1.07624
ENSG00000239157	4.59E-02	-1.07116
hsa-miR-577	4.96E-02	1.05886
hsa-miR-151a-5p	4.65E-02	1.06913
hsa-miR-4678	2.64E-02	1.07164
ENSG00000239045	4.55E-02	1.07206
hsa-mir-508	2.84E-02	1.07421
ENSG00000238407	4.91E-02	1.07935
hsa-miR-107	2.71E-02	1.08328
hsa-miR-103a-3p	3.98E-03	1.0833

hsa-mir-548b	2.17E-02	1.09171
hsa-miR-24-3p	3.17E-02	1.09266
hsa-miR-203b-3p	4.27E-02	1.09341
hsa-mir-4686	4.85E-02	1.10231
ENSG00000238466	5.00E-02	1.10667
hsa-mir-4749	4.15E-02	1.10693
ENSG00000199196	1.14E-02	1.11303
ENSG00000238748	4.46E-02	1.11645
hsa-miR-1243	1.22E-02	1.12293
U98b	2.13E-02	1.12303
hsa-let-7e-5p	4.41E-02	1.12808
hsa-mir-4272	2.09E-02	1.13059
ENSG00000238458	3.09E-02	1.13297
hsa-miR-5007-5p	1.80E-02	1.13839
ENSG00000238922	1.89E-03	1.14262
hsa-mir-3149	1.41E-02	1.14347
hsa-mir-2277	3.75E-02	1.14371
hsa-miR-3115	3.57E-02	1.14474
ENSG00000201957	1.74E-02	1.14945
hsa-miR-3680-5p	3.11E-02	1.15175
hsa-mir-4487	2.71E-02	1.15918
ENSG00000201674	4.39E-02	1.16147
ENSG00000268305	4.39E-02	1.16147
ENSG00000200385	3.60E-02	1.16774
hsa-mir-4433b	1.71E-02	1.1687
ENSG00000201245	1.21E-02	1.17075
hsa-mir-140	1.23E-02	1.17213
hsa-mir-6085	3.96E-02	1.17243
hsa-mir-5787	1.82E-03	1.17409
ENSG00000239008	4.23E-02	1.1753
hsa-miR-191-5p	9.99E-04	1.18172

hsa-mir-4766	2.90E-02	1.18259
ENSG00000212590	1.07E-02	1.18278
hsa-mir-5687	4.13E-02	1.1835
hsa-mir-1247	2.59E-02	1.19076
hsa-miR-4665-3p	3.91E-02	1.19296
hsa-mir-194-1	3.98E-02	1.1937
14qII-26	3.90E-02	1.19423
14qII-26	3.97E-02	1.19446
hsa-miR-1180-3p	7.89E-03	1.19537
hsa-miR-7153-5p	4.47E-02	1.19549
ENSG00000207410	2.76E-02	1.19584
hsa-miR-488-5p	6.07E-03	1.19701
hsa-mir-6799	4.63E-02	1.19826
14qII-9	4.14E-02	1.19905
hsa-mir-1301	3.67E-02	1.20009
HBII-180B	1.52E-02	1.20075
hsa-mir-4320	1.59E-02	1.20282
hsa-miR-100-3p	4.19E-02	1.20668
hsa-mir-152	1.29E-02	1.20732
ENSG00000238297	5.20E-03	1.20759
hsa-miR-4480	2.53E-02	1.2096
hsa-mir-4441	3.57E-02	1.21076
hsa-miR-4265	3.00E-02	1.21287
hsa-mir-4309	4.02E-02	1.21463
hsa-miR-106b-3p	2.17E-02	1.22114
hsa-miR-6747-5p	8.75E-03	1.22133
hsa-miR-652-3p	4.57E-02	1.22157
hsa-mir-770	4.17E-02	1.22826
hsa-miR-4668-3p	3.55E-02	1.22873
hsa-mir-548aw	1.20E-02	1.22958
hsa-mir-6756	1.76E-02	1.22972

hsa-miR-4484	4.20E-02	1.23231
hsa-mir-3976	2.26E-03	1.23641
hsa-miR-4745-3p	2.99E-02	1.23814
hsa-mir-302e	4.09E-02	1.239
hsa-mir-10a	2.02E-02	1.2395
hsa-miR-93-3p	2.99E-02	1.23954
hsa-mir-6821	3.05E-02	1.24466
hsa-miR-6126	2.76E-02	1.24519
ENSG00000201129	3.01E-02	1.24819
hsa-miR-320d	2.73E-02	1.24831
hsa-miR-4438	4.39E-02	1.24878
hsa-mir-452	1.66E-02	1.24939
ENSG00000252932	2.19E-02	1.25471
14qI-7	8.05E-03	1.25498
hsa-miR-302c-3p	3.24E-02	1.2558
ACA48	4.47E-02	1.25727
hsa-miR-330-3p	4.65E-02	1.26052
hsa-mir-941-1	1.26E-02	1.26061
hsa-mir-941-2	1.26E-02	1.26061
hsa-mir-941-3	1.26E-02	1.26061
hsa-mir-941-4	1.26E-02	1.26061
hsa-mir-3185	4.48E-02	1.26062
hsa-mir-195	7.23E-03	1.26804
hsa-mir-6734	9.58E-04	1.27043
hsa-miR-431-3p	2.70E-02	1.273
hsa-miR-324-5p	1.36E-02	1.28111
hsa-miR-1301-3p	4.19E-02	1.28453
ENSG00000238936	1.91E-03	1.28811
hsa-mir-4677	3.22E-02	1.28981
ENSG00000200620	4.75E-02	1.29263
hsa-miR-875-5p	1.87E-02	1.29511

hsa-miR-31-5p	2.21E-03	1.29763
hsa-miR-639	2.23E-02	1.2991
hsa-miR-4435	3.41E-02	1.30841
14qII-21	1.08E-02	1.3085
hsa-mir-4491	2.23E-02	1.3125
hsa-miR-99b-5p	1.66E-02	1.32575
hsa-miR-665	1.86E-02	1.33107
hsa-miR-425-3p	3.89E-02	1.33193
ENSG00000207119	2.08E-02	1.33317
mgU6-53	2.47E-02	1.33436
hsa-miR-423-5p	2.74E-02	1.34461
hsa-miR-493-3p	1.24E-02	1.35163
ENSG00000238939	2.76E-03	1.35797
hsa-miR-4532	3.09E-02	1.35864
hsa-miR-6827-3p	4.39E-02	1.36252
hsa-miR-320c	5.61E-03	1.36284
hsa-mir-299	9.02E-03	1.36813
hsa-miR-339-5p	2.15E-03	1.37481
14qI-9	2.90E-02	1.37806
hsa-miR-4479	4.61E-02	1.38625
hsa-miR-625-5p	5.26E-03	1.39237
hsa-miR-3064-5p	4.25E-02	1.39284
U87	9.63E-03	1.39457
hsa-miR-629-5p	2.50E-02	1.3962
hsa-miR-574-3p	5.32E-04	1.39645
hsa-miR-6803-3p	3.81E-02	1.40392
hsa-miR-3200-3p	7.06E-04	1.40518
hsa-miR-181a-5p	2.89E-03	1.40566
hsa-miR-132-3p	1.76E-04	1.40837
hsa-mir-4690	2.61E-02	1.40937
hsa-miR-127-3p	2.43E-03	1.41098

ENSG00000264452	4.17E-02	1.42181
hsa-mir-4540	3.42E-02	1.42296
hsa-miR-1911-5p	1.49E-02	1.42723
hsa-miR-339-3p	1.38E-02	1.43439
hsa-miR-370-3p	4.27E-03	1.43443
hsa-miR-1292-5p	2.18E-02	1.43481
hsa-miR-320b	4.99E-03	1.43837
hsa-miR-5591-5p	2.20E-02	1.44221
ACA21	4.96E-02	1.46339
ACA20	4.18E-02	1.47233
hsa-mir-4449	2.47E-03	1.47314
hsa-miR-345-5p	1.91E-03	1.47898
ACA66	2.30E-02	1.48735
hsa-miR-320a	1.15E-03	1.50006
hsa-miR-4510	1.04E-02	1.50796
hsa-miR-1306-5p	2.76E-02	1.51944
hsa-miR-140-3p	3.68E-03	1.53451
hsa-miR-941	2.17E-02	1.53833
hsa-miR-181a-2-3p	4.68E-05	1.54683
hsa-miR-4443	3.99E-03	1.55442
hsa-miR-3661	2.06E-02	1.57271
hsa-mir-3609	4.78E-03	1.57418
hsa-miR-1271-5p	2.17E-04	1.5811
ACA20	2.40E-02	1.59057
HBII-289	3.17E-02	1.59567
hsa-mir-3687	3.03E-03	1.61145
ACA57	9.41E-03	1.61476
hsa-miR-181b-5p	2.85E-04	1.62275
hsa-miR-99b-3p	6.77E-03	1.62373
HBII-52-25	2.45E-02	1.64648
hsa-miR-760	3.71E-02	1.65839

hsa-miR-542-5p	3.30E-02	1.67015
hsa-miR-299-3p	1.05E-04	1.69443
ACA57	1.73E-02	1.69649
hsa-miR-138-5p	7.53E-03	1.71127
SNORA38B	1.56E-02	1.71518
SNORA38B	3.95E-02	1.72384
hsa-miR-641	3.12E-02	1.76236
hsa-miR-485-5p	4.11E-03	1.7836
ENSG00000201009	3.33E-02	1.89928
U46	3.33E-02	1.89928
hsa-mir-210	1.53E-03	1.93012
hsa-miR-3617-5p	4.11E-02	2.01107
hsa-miR-193b-3p	3.87E-03	2.01983
hsa-miR-3200-5p	3.60E-02	2.19059
hsa-miR-193b-5p	2.58E-03	2.20093
hsa-miR-34a-5p	3.25E-02	2.2157
hsa-miR-127-5p	2.80E-02	2.23915
hsa-miR-491-5p	2.27E-02	2.2842
hsa-miR-550a-3p	3.26E-03	2.29047
hsa-miR-1307-5p	4.59E-02	2.40018
hsa-miR-210-3p	5.96E-06	2.57491
hsa-miR-212-3p	2.86E-03	3.97696

Appendix 2: Microarray data depicting miRNAs with altered expression between 1% and 20% O_2 tensions

Transcript ID (Array Design)	p-value (1% vs. 20%)	Fold-Change (1% vs. 20%)
hsa-miR-4521	2.08E-03	-5.65225
hsa-miR-1184	5.93E-03	-2.5647
hsa-miR-8075	1.36E-02	-2.27789
hsa-miR-222-5p	2.44E-02	-2.27191
hsa-miR-376a-3p	1.05E-03	-1.98112
hsa-miR-4284	3.81E-02	-1.88948
hsa-mir-4321	8.70E-03	-1.80859
hsa-miR-3613-3p	4.64E-02	-1.76633
hsa-miR-6746-5p	2.12E-02	-1.74773
hsa-miR-6889-5p	3.71E-02	-1.71855
hsa-mir-6776	2.11E-02	-1.66209
hsa-miR-1286	4.95E-02	-1.63897
hsa-miR-29a-3p	4.87E-03	-1.62559
hsa-miR-6760-5p	3.47E-03	-1.61309
ENSG00000212378	6.25E-03	-1.60765
U78	6.25E-03	-1.60765
U50	1.58E-02	-1.58717
hsa-mir-4521	9.17E-03	-1.57499
U79	4.33E-03	-1.56563
U78	3.37E-03	-1.54178
hsa-miR-30a-3p	3.45E-05	-1.53892
hsa-miR-503-5p	4.99E-04	-1.5007
hsa-miR-1185-1-3p	3.46E-03	-1.47914
hsa-miR-4775	2.53E-02	-1.47472
hsa-miR-9-3p	7.61E-03	-1.46901
hsa-miR-6859-3p	3.17E-02	-1.46113
ENSG00000221148	4.35E-02	-1.45242
hsa-miR-136-5p	2.02E-02	-1.4483

hsa-miR-1185-2-3p	9.42E-03	-1.44222
ENSG00000238951	3.93E-02	-1.43592
hsa-miR-6734-5p	4.54E-02	-1.43586
hsa-mir-4284	3.10E-03	-1.41377
hsa-miR-4767	2.56E-02	-1.40857
hsa-miR-3675-3p	4.68E-02	-1.40741
hsa-miR-1208	2.05E-02	-1.39888
ENSG00000212604	1.26E-02	-1.39466
hsa-miR-27b-3p	3.21E-03	-1.39328
HBII-52-15	4.72E-02	-1.37527
hsa-miR-3129-3p	1.78E-02	-1.37416
ENSG00000238852	4.25E-02	-1.37029
hsa-miR-424-3p	1.94E-02	-1.36592
U27	1.85E-02	-1.36566
hsa-miR-4303	3.16E-02	-1.36127
hsa-mir-4527	2.09E-02	-1.3587
hsa-mir-320e	4.30E-02	-1.35514
hsa-mir-503	1.25E-03	-1.35494
hsa-miR-4804-5p	1.08E-02	-1.3529
U14B	1.59E-02	-1.34816
hsa-miR-431-5p	4.49E-02	-1.3461
U27	2.87E-02	-1.34516
hsa-miR-3658	9.78E-03	-1.34504
hsa-miR-18a-5p	6.56E-03	-1.33626
hsa-mir-589	2.62E-02	-1.33497
hsa-miR-1204	1.41E-02	-1.33432
hsa-mir-1202	1.53E-02	-1.33054
ENSG00000238918	3.85E-02	-1.32794
hsa-miR-7702	4.29E-02	-1.32781
hsa-mir-6722	3.32E-02	-1.32526
hsa-miR-4423-3p	3.73E-02	-1.32468

hsa-mir-5583-1	7.25E-03	-1.32329
hsa-miR-4483	4.45E-02	-1.32288
hsa-miR-518c-5p	3.76E-02	-1.31901
ENSG00000239046	1.80E-02	-1.31807
hsa-mir-6814	4.24E-02	-1.31483
hsa-mir-5690	5.14E-03	-1.31362
hsa-miR-4637	2.74E-02	-1.3133
hsa-mir-548h-2	2.87E-02	-1.31228
hsa-mir-4755	3.81E-02	-1.31188
hsa-mir-4477b	4.49E-02	-1.30807
hsa-mir-563	2.20E-02	-1.30697
hsa-mir-7150	3.77E-02	-1.3031
ENSG00000252543	1.09E-04	-1.29947
ACA17	4.97E-02	-1.29676
U76	4.47E-02	-1.29608
hsa-miR-4434	8.18E-03	-1.2955
hsa-miR-28-5p	2.55E-04	-1.29265
HBII-202	7.69E-03	-1.29126
hsa-miR-487a-3p	1.18E-02	-1.28742
ENSG00000238670	1.34E-02	-1.2858
hsa-mir-6783	4.68E-02	-1.28418
hsa-miR-6876-3p	2.73E-02	-1.28261
U17b	4.19E-02	-1.28151
ENSG00000238522	2.18E-02	-1.27708
hsa-mir-892b	4.89E-02	-1.27587
ENSG00000200538	2.10E-02	-1.27354
hsa-miR-126-5p	2.33E-02	-1.27058
ENSG00000252236	3.28E-02	-1.26896
hsa-miR-4262	8.70E-03	-1.26665
ENSG00000212615	1.68E-02	-1.26579
hsa-mir-1281	2.82E-04	-1.26454

U14B	4.35E-02	-1.26158
hsa-miR-3168	4.98E-02	-1.26023
hsa-miR-20a-5p	3.58E-02	-1.25756
hsa-mir-6075	2.88E-02	-1.25492
hsa-miR-632	5.03E-03	-1.25479
hsa-miR-29a-5p	3.49E-02	-1.25327
hsa-miR-409-3p	9.66E-04	-1.25207
hsa-miR-4687-5p	4.82E-02	-1.25165
hsa-mir-3132	2.74E-02	-1.24831
U75	1.99E-03	-1.24582
hsa-miR-620	1.43E-02	-1.24395
hsa-mir-3161	4.08E-02	-1.24019
U75	1.08E-02	-1.23806
hsa-mir-7705	2.79E-02	-1.23712
U33	2.07E-02	-1.23551
hsa-mir-8075	2.50E-02	-1.23482
ENSG00000201810	3.45E-02	-1.23378
U30	2.86E-02	-1.23044
hsa-miR-494-3p	1.53E-02	-1.22944
ENSG00000264997	4.03E-02	-1.22821
hsa-miR-6502-3p	2.89E-02	-1.22627
hsa-miR-4457	1.74E-02	-1.22326
hsa-miR-421	1.78E-02	-1.2227
hsa-miR-4658	2.99E-02	-1.21939
hsa-miR-3591-3p	5.51E-03	-1.21885
hsa-miR-6863	3.65E-02	-1.21845
hsa-mir-5739	4.20E-02	-1.21533
hsa-miR-100-5p	2.77E-03	-1.21492
ENSG00000201810	2.78E-02	-1.2136
hsa-mir-6850	4.93E-02	-1.21022
hsa-miR-422a	4.38E-02	-1.20631

ENSG00000238739	4.15E-02	-1.20421
hsa-miR-558	4.12E-02	-1.19995
hsa-mir-525	8.10E-04	-1.19862
hsa-mir-5696	3.67E-02	-1.19856
hsa-miR-106a-5p	3.88E-02	-1.19781
hsa-mir-1258	2.39E-02	-1.19719
hsa-mir-6502	1.48E-02	-1.19625
ENSG00000238747	4.58E-02	-1.19406
hsa-mir-525	1.20E-03	-1.19328
hsa-mir-1278	1.00E-02	-1.19191
hsa-miR-5697	3.61E-02	-1.1918
hsa-mir-4433	1.74E-02	-1.18963
ENSG00000212589	1.85E-02	-1.18881
hsa-miR-221-3p	1.31E-03	-1.18832
hsa-miR-511-3p	1.99E-02	-1.18794
hsa-mir-3125	4.62E-02	-1.18732
hsa-miR-548x-3p	4.94E-02	-1.18662
hsa-miR-6747-5p	1.72E-02	-1.18629
hsa-mir-6758	3.66E-03	-1.18456
hsa-miR-5191	2.13E-02	-1.18376
hsa-mir-6500	3.77E-02	-1.1768
ENSG00000238910	3.86E-02	-1.17589
hsa-mir-6805	9.95E-03	-1.17483
hsa-mir-708	3.71E-02	-1.17366
hsa-mir-3186	4.22E-02	-1.1657
hsa-mir-4653	2.83E-02	-1.16416
ENSG00000200652	2.51E-02	-1.16246
HBII-52-23	7.78E-03	-1.16034
hsa-mir-4735	4.72E-02	-1.15978
mgh28S-2411	2.76E-02	-1.15959
hsa-mir-1-2	4.43E-02	-1.1564

ENSG00000238693	2.99E-02	-1.15437
hsa-mir-3978	1.40E-03	-1.15344
ENSG00000212224	1.66E-02	-1.15053
hsa-mir-302a	3.11E-02	-1.14991
hsa-mir-5688	1.65E-02	-1.14976
hsa-miR-4654	4.94E-02	-1.1477
hsa-miR-6866-3p	1.64E-02	-1.14677
ENSG00000251974	3.96E-03	-1.14534
hsa-miR-548ag	4.85E-02	-1.1407
hsa-miR-23b-3p	1.85E-02	-1.13175
hsa-mir-604	4.31E-02	-1.12537
hsa-miR-506-5p	4.51E-02	-1.10946
hsa-miR-149-5p	1.94E-03	-1.10838
hsa-mir-8068	4.09E-02	-1.10775
hsa-mir-7850	2.74E-02	-1.10591
hsa-miR-598-3p	2.67E-02	-1.10395
hsa-miR-151a-3p	3.88E-02	-1.10139
hsa-miR-583	2.53E-02	-1.08985
ENSG00000239157	2.57E-02	-1.08406
ENSG00000239157	2.82E-02	-1.08406
hsa-mir-489	2.79E-02	-1.07183
hsa-miR-4678	2.64E-02	1.07164
hsa-mir-3688-2	4.82E-02	1.07223
ENSG00000201541	3.66E-02	1.0848
hsa-miR-6515-3p	3.32E-02	1.08906
HBII-52-8	4.96E-02	1.09053
hsa-miR-103a-3p	2.29E-03	1.0937
hsa-mir-4678	2.62E-02	1.09811
hsa-miR-203b-3p	3.17E-02	1.1019
ENSG00000238801	4.40E-02	1.10942
hsa-mir-7854	4.67E-02	1.11022

U98b	3.04E-02	1.11161
hsa-mir-423	2.57E-02	1.11271
hsa-mir-181b-2	4.94E-02	1.11597
hsa-miR-342-3p	4.24E-02	1.12108
hsa-miR-1185-5p	4.79E-02	1.12654
hsa-mir-340	3.95E-02	1.13351
hsa-mir-4433b	3.68E-02	1.13627
hsa-miR-1243	7.76E-03	1.13716
ENSG00000238409	4.82E-02	1.13928
ENSG00000212590	2.88E-02	1.14043
hsa-miR-5007-5p	1.63E-02	1.14201
hsa-miR-4774-5p	4.76E-02	1.14351
hsa-mir-6734	1.50E-02	1.14382
hsa-mir-4744	3.43E-02	1.14943
hsa-miR-4790-3p	3.18E-02	1.14965
hsa-mir-6835	1.90E-03	1.15154
hsa-miR-654-5p	3.60E-02	1.15347
ENSG00000201541	1.95E-02	1.15378
hsa-miR-155-5p	4.26E-02	1.15888
hsa-miR-655-5p	1.41E-03	1.15958
ENSG00000239153	4.93E-02	1.1646
ENSG00000253042	3.43E-04	1.16802
ENSG00000202377	4.96E-02	1.16949
hsa-mir-3146	4.22E-02	1.17177
hsa-mir-5787	1.68E-03	1.17697
hsa-miR-6865-3p	3.86E-02	1.18086
HBII-180B	2.03E-02	1.1857
ENSG00000238556	3.67E-02	1.18639
hsa-miR-191-5p	8.73E-04	1.18686
hsa-miR-1537-3p	1.98E-02	1.18713
hsa-mir-1285-2	4.19E-02	1.19107

hsa-miR-4685-3p	4.35E-02	1.19324
hsa-miR-31-5p	1.30E-02	1.19504
HBII-52-48	4.64E-02	1.20342
hsa-mir-3118-5	4.12E-02	1.20452
hsa-mir-103b-2	4.69E-02	1.20825
hsa-mir-1290	1.28E-02	1.20838
hsa-mir-4272	2.99E-03	1.20882
ENSG00000238496	2.81E-02	1.20971
hsa-let-7e-5p	6.98E-03	1.20999
hsa-miR-148a-5p	4.69E-02	1.21111
hsa-miR-425-5p	2.72E-02	1.21129
hsa-mir-5702	1.86E-02	1.21174
hsa-miR-3135a	3.42E-02	1.21195
hsa-miR-6508-5p	1.43E-02	1.2132
ENSG00000238858	1.05E-02	1.21555
hsa-mir-4749	2.39E-03	1.21835
hsa-miR-93-3p	3.96E-02	1.2198
hsa-miR-7704	3.78E-02	1.22314
hsa-mir-558	1.03E-02	1.22351
hsa-mir-4309	3.38E-02	1.22642
hsa-miR-1282	3.37E-02	1.22775
hsa-mir-3116-1	4.51E-03	1.2303
hsa-mir-3116-2	4.51E-03	1.2303
hsa-miR-3192-5p	3.42E-02	1.2304
hsa-mir-7515	2.48E-02	1.23348
ENSG00000238801	1.52E-03	1.2396
hsa-mir-6513	5.69E-03	1.23996
hsa-mir-195	1.12E-02	1.24013
hsa-miR-345-5p	2.67E-02	1.24256
hsa-miR-6879-3p	3.45E-02	1.2428
hsa-mir-194-1	1.83E-02	1.24291

ENSG00000252888	2.27E-02	1.2459
hsa-miR-4288	3.92E-02	1.24982
hsa-mir-92a-2	3.15E-02	1.25085
hsa-mir-941-1	1.45E-02	1.25133
hsa-mir-941-2	1.45E-02	1.25133
hsa-mir-941-3	1.45E-02	1.25133
hsa-mir-941-4	1.45E-02	1.25133
hsa-miR-34b-5p	3.48E-02	1.25485
14qII-21	1.99E-02	1.26151
hsa-miR-544a	4.73E-02	1.26571
hsa-mir-2277	3.37E-03	1.26684
hsa-miR-6874-3p	2.20E-03	1.27196
hsa-miR-132-3p	1.14E-03	1.27398
hsa-mir-6085	6.98E-03	1.2763
hsa-mir-299	2.18E-02	1.28914
hsa-mir-657	4.03E-02	1.29281
HBII-234	3.86E-02	1.29339
hsa-miR-1237-3p	4.00E-02	1.29566
hsa-miR-125a-5p	1.44E-02	1.29861
hsa-mir-492	3.41E-02	1.29921
hsa-miR-3907	1.78E-02	1.30702
hsa-miR-8069	4.90E-02	1.31241
hsa-miR-3200-3p	2.23E-03	1.31303
hsa-mir-345	4.86E-02	1.31446
hsa-mir-5196	2.61E-02	1.32118
ENSG00000238329	1.87E-02	1.32543
hsa-miR-3677-3p	4.27E-03	1.32751
ENSG00000206761	1.65E-02	1.33496
ENSG00000238939	3.66E-03	1.33499
ENSG00000239176	4.53E-02	1.33544
hsa-mir-4449	9.67E-03	1.33622

hsa-miR-324-3p	3.44E-02	1.33933
hsa-mir-4734	2.61E-03	1.34024
hsa-miR-99b-5p	1.38E-02	1.34308
hsa-miR-6888-3p	4.70E-02	1.34358
hsa-miR-187-3p	3.32E-02	1.34664
ENSG00000252727	5.22E-03	1.35916
hsa-mir-92a-2	2.59E-02	1.35922
hsa-miR-6829-3p	6.62E-03	1.35952
hsa-miR-4265	3.77E-03	1.3666
hsa-miR-1292-5p	3.59E-02	1.37192
hsa-miR-140-3p	1.44E-02	1.37269
hsa-mir-6772	4.75E-02	1.37604
hsa-mir-4463	2.66E-02	1.37764
hsa-miR-1271-5p	1.45E-03	1.37894
ENSG00000206761	1.30E-02	1.38127
hsa-miR-339-3p	2.07E-02	1.38652
hsa-miR-4253	3.69E-02	1.38691
hsa-mir-615	7.61E-03	1.38851
hsa-miR-4443	1.44E-02	1.39333
hsa-mir-4304	4.55E-02	1.39692
hsa-miR-3688-3p	9.76E-03	1.40767
hsa-mir-125a	1.50E-02	1.41004
ENSG00000207516	1.86E-02	1.41586
hsa-miR-127-3p	2.20E-03	1.42092
hsa-miR-320c	3.01E-03	1.42268
hsa-miR-320d	3.41E-03	1.42977
hsa-miR-639	5.64E-03	1.43337
ENSG00000252727	1.24E-02	1.44851
hsa-miR-941	3.76E-02	1.45043
hsa-miR-6836-3p	2.75E-02	1.45178
hsa-mir-6829	5.03E-03	1.45386

14qI-7	6.08E-04	1.465
hsa-miR-615-3p	8.85E-03	1.47048
hsa-miR-1911-5p	1.04E-02	1.47199
ACA57	2.27E-02	1.47436
hsa-miR-5705	4.80E-02	1.47438
hsa-miR-339-5p	7.50E-04	1.47745
hsa-miR-320b	3.44E-03	1.48137
hsa-miR-629-5p	1.25E-02	1.48531
hsa-miR-320a	1.09E-03	1.50625
hsa-miR-181a-5p	1.11E-03	1.50862
ENSG00000239063	2.69E-02	1.51971
ACA57	4.11E-02	1.52254
hsa-mir-4634	3.91E-02	1.52923
HBII-52-25	4.31E-02	1.5331
U15A	1.88E-02	1.53376
hsa-miR-299-3p	3.01E-04	1.54789
hsa-miR-4516	2.11E-02	1.55021
hsa-miR-4707-3p	3.79E-02	1.55873
hsa-miR-125a-3p	4.47E-02	1.55889
hsa-miR-532-3p	9.49E-03	1.57818
hsa-miR-7515	2.74E-02	1.58498
hsa-mir-3609	4.25E-03	1.59225
hsa-miR-769-5p	7.13E-03	1.60242
ACA63	3.79E-02	1.60499
hsa-miR-3605-5p	4.53E-02	1.61121
HBII-85-24	4.45E-02	1.61249
hsa-miR-574-3p	7.23E-05	1.61393
hsa-miR-425-3p	4.58E-03	1.61423
hsa-miR-193b-3p	2.06E-02	1.61879
hsa-miR-550a-3-5p	4.26E-02	1.6218
HBII-85-23	4.00E-02	1.63756

hsa-miR-4314	9.50E-03	1.6382
hsa-miR-760	4.01E-02	1.63995
hsa-miR-3661	1.43E-02	1.63997
hsa-miR-4479	8.86E-03	1.64312
hsa-miR-500a-5p	3.67E-02	1.6437
14qI-3	9.64E-03	1.65516
hsa-miR-181b-5p	1.58E-04	1.71357
HBII-85-29	3.18E-02	1.71379
hsa-miR-454-3p	3.07E-02	1.71703
HBII-289	1.79E-02	1.71865
hsa-miR-485-5p	4.98E-03	1.74336
hsa-miR-625-5p	3.26E-04	1.76707
hsa-miR-629-3p	3.73E-02	1.81234
hsa-miR-187-5p	4.48E-02	1.81563
hsa-miR-6723-5p	3.88E-02	1.84705
hsa-mir-3687	8.12E-04	1.85403
hsa-miR-331-5p	2.79E-02	1.89986
hsa-miR-181a-2-3p	4.37E-06	1.92735
hsa-miR-3972	1.92E-02	1.95364
hsa-miR-193b-5p	5.64E-03	1.95566
hsa-miR-6813-5p	3.47E-02	2.01607
hsa-miR-3663-3p	2.00E-02	2.06407
hsa-miR-550b-2-5p	1.53E-02	2.06743
14qII-12	3.06E-02	2.0792
hsa-miR-3615	3.90E-02	2.08159
hsa-miR-127-5p	3.81E-02	2.09777
14qII-12	1.04E-02	2.106
hsa-miR-3065-5p	3.24E-02	2.46292
hsa-miR-550a-5p	1.53E-02	2.50198
hsa-mir-210	2.47E-04	2.52267
hsa-miR-3617-5p	1.15E-02	2.63333

hsa-miR-3917	8.52E-04	2.63627
hsa-miR-7706	2.80E-02	3.00083
hsa-miR-212-3p	6.43E-03	3.20479
hsa-miR-328-3p	1.93E-02	4.0448
hsa-miR-210-3p	6.46E-08	7.6074

Appendix 3: Microarray data depicting miRNAs with altered expression between 5% and 20% O_2 tensions

Transcript ID (Array Design)	p-value (5% vs. 20%)	Fold-Change (5% vs. 20%)
hsa-miR-542-5p	6.58E-03	-2.13294
hsa-miR-3613-3p	1.79E-02	-2.08324
ENSG00000201009	3.84E-02	-1.85246
U46	3.84E-02	-1.85246
hsa-miR-6799-3p	2.05E-02	-1.74605
hsa-miR-4668-5p	2.97E-02	-1.73212
hsa-miR-1286	4.11E-02	-1.685
hsa-miR-1238-3p	6.75E-03	-1.64323
hsa-miR-758-3p	2.06E-02	-1.59138
hsa-miR-550a-3p	4.04E-02	-1.57987
hsa-mir-4321	3.10E-02	-1.54365
hsa-miR-6760-5p	6.92E-03	-1.51139
hsa-miR-1185-2-3p	5.67E-03	-1.50608
hsa-miR-323b-3p	4.21E-02	-1.50427
ENSG00000238951	2.59E-02	-1.49984
hsa-miR-378a-3p	1.45E-02	-1.49031
hsa-miR-378i	7.54E-03	-1.46892
hsa-miR-8067	2.73E-02	-1.44952
hsa-miR-6747-5p	3.97E-04	-1.44885
hsa-mir-6891	4.29E-02	-1.43275
ACA67	2.68E-02	-1.42207
hsa-mir-892b	1.21E-02	-1.42051
ENSG00000221139	3.20E-02	-1.41337
hsa-miR-1185-1-3p	6.95E-03	-1.40177
hsa-mir-1202	7.45E-03	-1.40038
hsa-miR-1208	2.31E-02	-1.38483
hsa-miR-378c	1.56E-02	-1.3841

ENSG00000252543	3.26E-05	-1.38242
ENSG00000238936	5.59E-04	-1.37771
hsa-mir-548h-2	1.51E-02	-1.3767
hsa-miR-422a	4.93E-03	-1.37591
hsa-miR-376a-3p	3.38E-02	-1.3729
hsa-miR-199a-5p	1.31E-02	-1.36538
hsa-miR-136-5p	4.28E-02	-1.35351
hsa-let-7i	3.54E-02	-1.34378
hsa-mir-6894	1.80E-02	-1.34315
hsa-miR-302c-3p	1.27E-02	-1.33434
hsa-miR-4510	4.34E-02	-1.32971
hsa-mir-3158-1	4.08E-02	-1.3192
U32B	1.15E-02	-1.31366
hsa-mir-132	4.41E-02	-1.31153
hsa-mir-3910-1	1.23E-02	-1.3111
hsa-mir-3910-2	1.23E-02	-1.3111
ENSG00000212214	2.69E-02	-1.30646
hsa-miR-487a-3p	9.60E-03	-1.30342
hsa-miR-5004-3p	7.12E-03	-1.3006
hsa-mir-6511a-1	2.25E-02	-1.29947
hsa-mir-6511b-1	2.25E-02	-1.29947
hsa-mir-6511b-2	2.25E-02	-1.29947
hsa-mir-6511a-2	2.25E-02	-1.29947
hsa-mir-6511a-3	2.25E-02	-1.29947
hsa-mir-6511a-4	2.25E-02	-1.29947
ENSG00000252000	1.51E-02	-1.29828
hsa-mir-5004	2.92E-02	-1.297
hsa-miR-4484	1.86E-02	-1.29605
hsa-miR-518c-5p	4.74E-02	-1.29564
hsa-miR-1302	1.84E-02	-1.28949
hsa-miR-4804-5p	2.23E-02	-1.28864

ENSG00000212391	3.60E-02	-1.28572
hsa-miR-488-5p	1.19E-03	-1.28442
hsa-mir-320b-2	4.12E-02	-1.28377
hsa-mir-514a-1	1.58E-02	-1.27081
hsa-mir-514a-2	1.58E-02	-1.27081
hsa-mir-514a-3	1.58E-02	-1.27081
hsa-miR-330-3p	4.22E-02	-1.26906
hsa-miR-3121-5p	2.36E-02	-1.26668
hsa-miR-3168	4.95E-02	-1.26071
U38A	3.97E-02	-1.25909
hsa-mir-4491	4.53E-02	-1.25116
hsa-mir-6884	2.41E-02	-1.25033
hsa-miR-7153-5p	1.96E-02	-1.24986
hsa-mir-6756	1.29E-02	-1.24968
hsa-mir-3125	1.80E-02	-1.24749
hsa-miR-1343-3p	4.37E-02	-1.24329
ENSG00000239137	3.84E-02	-1.24239
hsa-mir-503	7.39E-03	-1.23487
hsa-mir-3161	4.48E-02	-1.23307
hsa-miR-18a-5p	2.59E-02	-1.23303
hsa-mir-3176	1.95E-02	-1.23086
ENSG00000201245	3.76E-03	-1.22569
hsa-mir-4285	4.04E-02	-1.22334
ENSG00000252402	4.67E-02	-1.22047
ENSG00000252799	4.67E-02	-1.22047
hsa-mir-3976	3.38E-03	-1.21598
hsa-miR-6866-3p	3.37E-03	-1.21478
hsa-miR-6126	4.49E-02	-1.21124
ENSG00000251878	4.00E-02	-1.21053
hsa-miR-4665-3p	3.03E-02	-1.20854
hsa-mir-4743	2.17E-02	-1.20547

hsa-mir-525	8.45E-04	-1.19688
hsa-miR-4654	2.05E-02	-1.19164
hsa-miR-4759	4.51E-02	-1.18844
hsa-mir-3117	4.79E-02	-1.1869
hsa-miR-221-3p	1.38E-03	-1.18614
U104	2.85E-02	-1.18432
hsa-miR-503-5p	3.07E-02	-1.1831
hsa-mir-6745	2.89E-02	-1.18223
hsa-mir-3665	3.77E-02	-1.18199
hsa-miR-6781-3p	4.62E-02	-1.1803
ENSG00000238932	4.06E-02	-1.17992
hsa-miR-4262	3.66E-02	-1.17991
hsa-miR-30a-3p	5.75E-03	-1.17932
hsa-mir-525	1.87E-03	-1.17586
hsa-mir-1281	2.03E-03	-1.1753
ENSG00000238739	2.59E-02	-1.17347
hsa-miR-100-5p	7.34E-03	-1.17178
hsa-mir-1302-2	2.81E-02	-1.17051
hsa-mir-1302-3	2.81E-02	-1.17051
hsa-mir-1302-9	2.81E-02	-1.17051
hsa-mir-1302-10	2.81E-02	-1.17051
hsa-mir-1302-11	2.81E-02	-1.17051
hsa-miR-421	4.49E-02	-1.17017
hsa-mir-1247	4.06E-02	-1.16694
hsa-miR-3591-3p	1.66E-02	-1.16637
ENSG00000251778	2.31E-02	-1.16229
hsa-mir-4686	8.86E-03	-1.16213
ENSG00000212224	1.45E-02	-1.15588
hsa-miR-6777-3p	1.69E-02	-1.15507
hsa-mir-6805	1.72E-02	-1.15215
ENSG00000199196	3.81E-03	-1.14578

hsa-miR-4753-5p	3.64E-02	-1.14027
hsa-mir-365b	3.52E-02	-1.13723
hsa-miR-409-3p	1.62E-02	-1.13207
hsa-mir-3149	1.95E-02	-1.13183
hsa-miR-583	5.34E-03	-1.13166
ENSG00000238297	4.98E-02	-1.11403
ENSG00000238922	5.39E-03	-1.11361
ENSG00000238748	4.90E-02	-1.11309
hsa-mir-6734	3.88E-02	-1.1107
hsa-mir-7850	2.81E-02	-1.1052
hsa-miR-28-5p	4.47E-02	-1.08826
ENSG00000253076	4.89E-02	1.07413
hsa-miR-4527	4.58E-02	1.07555
ENSG00000202269	4.18E-02	1.08173
ENSG00000199934	4.58E-02	1.08391
hsa-mir-4700	5.89E-03	1.08663
hsa-miR-130a-5p	5.32E-03	1.08852
hsa-miR-6515-3p	3.38E-02	1.08859
hsa-miR-26a-5p	2.22E-02	1.09588
ENSG00000238974	1.24E-02	1.0966
hsa-miR-152-3p	4.45E-02	1.10173
hsa-miR-301a-5p	2.97E-02	1.11697
ENSG00000239093	3.97E-02	1.11857
hsa-mir-522	3.77E-02	1.12214
hsa-mir-4278	4.92E-02	1.12843
hsa-miR-3973	2.71E-02	1.13232
hsa-mir-517c	1.83E-02	1.14434
ENSG00000238801	1.60E-02	1.14524
hsa-mir-7854	1.74E-02	1.14595
hsa-mir-4260	2.46E-02	1.14627
hsa-miR-6887-3p	4.36E-02	1.15092

hsa-miR-380-3p	1.90E-02	1.15355
hsa-miR-574-3p	2.70E-02	1.15573
hsa-miR-655-5p	1.51E-03	1.15728
hsa-miR-6874-3p	1.88E-02	1.16216
hsa-mir-6863	4.44E-02	1.16678
14qI-7	3.79E-02	1.16735
hsa-mir-6810	3.15E-02	1.17052
ENSG00000221083	2.49E-02	1.17225
hsa-mir-3657	2.15E-02	1.17226
hsa-miR-95-5p	4.07E-02	1.17555
hsa-mir-6513	1.89E-02	1.17742
hsa-mir-7-2	3.49E-02	1.17766
hsa-mir-1199	3.40E-02	1.18083
hsa-miR-365a-3p	1.05E-02	1.18155
hsa-miR-365b-3p	1.05E-02	1.18155
hsa-mir-5702	3.19E-02	1.18168
ENSG00000251992	3.36E-02	1.18409
hsa-mir-5188	3.69E-02	1.18542
hsa-mir-340	1.15E-02	1.18733
hsa-mir-6835	6.69E-04	1.18818
ENSG00000253042	1.88E-04	1.18907
hsa-miR-3196	2.84E-02	1.18949
hsa-mir-1284	4.61E-02	1.19215
ENSG00000252727	4.98E-02	1.19218
hsa-mir-296	4.22E-02	1.19404
hsa-miR-204-5p	1.64E-02	1.19759
hsa-mir-1255a	3.33E-02	1.20107
hsa-mir-3116-1	7.96E-03	1.20124
hsa-mir-3116-2	7.96E-03	1.20124
hsa-mir-6883	4.70E-02	1.20154
hsa-miR-4774-5p	1.28E-02	1.20801

hsa-mir-1471	3.54E-02	1.2116
ENSG00000202377	2.22E-02	1.21592
ENSG00000207094	2.55E-02	1.21612
hsa-mir-6716	4.08E-03	1.21614
hsa-miR-98-3p	1.14E-02	1.21683
hsa-let-7f-2	4.74E-02	1.21898
hsa-mir-642a	2.97E-02	1.22384
hsa-miR-544b	1.51E-02	1.22388
hsa-miR-4328	2.48E-02	1.22688
hsa-miR-4715-5p	9.92E-03	1.23317
hsa-mir-4799	3.62E-02	1.23398
hsa-miR-3529-3p	2.08E-02	1.23432
hsa-miR-1185-5p	4.64E-03	1.2348
hsa-miR-125a-5p	3.21E-02	1.2375
ENSG00000253090	8.79E-03	1.23793
ENSG00000238496	1.75E-02	1.23987
hsa-miR-142-5p	3.66E-02	1.24056
hsa-miR-3941	3.96E-02	1.24371
hsa-miR-181a-2-3p	1.95E-03	1.24601
ENSG00000201042	2.83E-02	1.25108
hsa-miR-4535	2.21E-03	1.25356
ENSG00000238801	1.11E-03	1.25627
hsa-mir-6829	3.85E-02	1.2577
HBII-85-21	4.99E-02	1.2604
hsa-miR-3621	3.56E-02	1.2605
hsa-miR-5700	2.37E-02	1.26326
hsa-miR-625-5p	2.18E-02	1.26911
hsa-miR-3149	4.40E-02	1.27242
hsa-let-7a-2	4.49E-02	1.27362
hsa-mir-208b	1.78E-02	1.27489
HBII-108	3.80E-02	1.27867

hsa-miR-6838-5p	1.04E-02	1.2817
hsa-miR-6736-3p	2.13E-02	1.28202
hsa-mir-6512	3.49E-02	1.28332
hsa-miR-615-3p	4.65E-02	1.28786
hsa-mir-5685	4.30E-02	1.29171
hsa-miR-4501	1.33E-02	1.29361
hsa-miR-4488	2.19E-02	1.29765
hsa-miR-6847-3p	3.34E-02	1.30367
hsa-mir-615	1.87E-02	1.30476
hsa-miR-6780b-3p	2.43E-02	1.30547
ENSG00000238541	2.20E-02	1.30574
ENSG00000201042	3.78E-02	1.30733
hsa-mir-657	3.46E-02	1.30752
hsa-mir-936	4.47E-02	1.31201
hsa-miR-9-5p	4.82E-02	1.3137
hsa-mir-1224	2.00E-02	1.32022
hsa-miR-4722-5p	3.88E-02	1.32286
ENSG00000221398	1.97E-02	1.33383
ACA4	4.01E-02	1.34398
hsa-mir-4760	4.46E-04	1.34826
hsa-let-7d	3.92E-02	1.3492
hsa-mir-4734	2.28E-03	1.35091
hsa-miR-1295a	3.32E-02	1.35472
ENSG00000238608	3.02E-02	1.35579
hsa-let-7b-3p	7.06E-03	1.35788
hsa-miR-129-2-3p	2.89E-02	1.35804
hsa-miR-6885-3p	6.41E-03	1.36016
hsa-miR-4699-5p	4.08E-02	1.3648
hsa-miR-1537-3p	1.18E-03	1.36937
hsa-miR-499a-5p	4.40E-02	1.3714
hsa-miR-5010-5p	2.75E-02	1.37168

hsa-mir-3934	2.06E-02	1.38382
hsa-mir-6505	2.55E-02	1.38399
hsa-miR-3189-3p	1.53E-02	1.39178
hsa-miR-6807-5p	1.38E-02	1.39517
U15A	4.57E-02	1.40074
hsa-miR-6735-5p	2.10E-02	1.40732
hsa-miR-510-5p	1.13E-02	1.40908
hsa-miR-7157-3p	6.81E-03	1.41661
ENSG00000239176	2.23E-02	1.42077
14qI-3	3.98E-02	1.42278
ENSG00000212411	2.63E-02	1.42986
hsa-miR-3688-3p	6.13E-03	1.46091
ENSG00000212579	1.27E-02	1.48804
hsa-mir-1909	2.48E-02	1.52048
hsa-miR-6757-5p	2.24E-02	1.52736
hsa-miR-6781-5p	1.58E-02	1.53743
hsa-miR-4767	6.51E-03	1.60557
hsa-miR-148b-3p	2.13E-02	1.62404
HBII-85-23	3.78E-02	1.65046
hsa-miR-3917	9.09E-03	1.81826
14qII-12	2.28E-02	1.85243
hsa-miR-3972	1.77E-02	1.98134
hsa-miR-7515	3.35E-03	2.10874
hsa-miR-454-3p	4.93E-03	2.29952
hsa-miR-210-3p	2.68E-06	2.95443