

Regulation of the uteroplacental renin–angiotensin system in human pregnancy

By

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Bachelor of Medical Science (Hons)

A thesis submitted in fulfilment of the requirements for the degree of
Doctorate of Philosophy

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on April 2013

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2	Regulation of the Renin–Angiotensin System (RAS) in BeWo and HTR–8/SVneo trophoblast cell lines.	Published	Experimental design and procedures Data analysis Manuscript preparation
3	Fetal sex affects expression of Renin–Angiotensin System components in term human decidua.	Published	Experimental design and procedures Data analysis Manuscript preparation
4	The effects of cyclic AMP, sex steroids and global hypomethylation on the expression of genes controlling the activity of the Renin–Angiotensin System in placental cell lines.	Published	Experimental design and procedures Data analysis Manuscript preparation
5	Regulation of Renin–Angiotensin System (RAS) pathways in the Human Decidua	Submitted	Experimental design and procedures Data analysis Manuscript preparation

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ACKNOWLEDGEMENTS

I would like to take this opportunity to acknowledge the help and support of my dear supervisors, Eugenie Lumbers, Kirsty Pringle and Tamas Zakar, without whom none of this would be possible. Beyond their guidance and support with all things scientific, they have also had a great and positive impact on the person that I am now. Eugenie, I feel deeply inspired each time I see you, for I see boundless energy and passion. Kirsty, your patience and knowledge, has made each and every problem and puzzle seem less daunting. Tamas, I am very grateful for all of your help and guidance.

I would like to acknowledge the head of the Mothers and Babies research centre, Professor Roger Smith and the University of Newcastle for giving me the opportunity to do a PhD in Newcastle. In addition, note that this PhD was supported by the NHMRC and HMRI.

To all my friends and colleagues at the Mothers and Babies research centre, you have made everyday much more enjoyable and frustrating moments bearable. I am especially grateful to Annie and all those on the collection roster for organising and collecting samples.

To all my friends, thank you for putting up with me, I love you all, what more can I say. To my mum and dad, I am thankful everyday that I have been blessed with such wonderful and loving parents.

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ABSTRACT

A renin–angiotensin system (RAS) exists within the decidua and the placenta and has been shown to play a significant role in the regulation of trophoblast proliferation, invasion and migration, angiogenesis and the modulation of blood flow.

As the RAS is crucial for the normal progression of pregnancy, it naturally follows then; that disruptions to the uteroplacental RAS during pregnancy may contribute to pregnancy complications such as intrauterine growth restriction (IUGR) and preeclampsia. Although many studies have linked changes in the RAS to these pathologies, our understanding of how the RAS is involved in these physiological changes is lacking, as are adequate medical interventions. This thesis attempts to address how the RAS is regulated within the uteroplacental unit.

We explored the RAS in two trophoblast (*i.e.*, placental) cell lines, HTR–8/SVneo and BeWo cells, to determine how they express the genes of the RAS and their proteins, in order to determine their value as models of the placental RAS. We found however, that HTR–8/SVneo cells expressed only the Angiotensin II (Ang II)/type 1 Ang II receptor (AT₁R) pathway, while the BeWo cells expressed only the Angiotensin 1–7 (Ang 1–7)/Mas receptor pathway. Therefore these cell lines are not good models for placental RAS, but they are useful for exploring the regulation of RAS pathways within the placenta. Our aim was then to determine if we could induce the RAS pathways not expressed, in those cell lines that lacked them.

We were also interested in the maternal decidua, as it is the main site of production of renin in the intrauterine tissues during human pregnancy and it plays a critical role in regulation of trophoblast invasion and placentation. We found that the

expression of certain RAS genes within the decidua was sex specific. Decidua is a maternal tissue, yet the sex of the fetus determines the level of genes expression of the RAS pathway. This is extremely interesting since fetal sex is a major determinant of pregnancy outcome.

We then showed that the sex specific differences in (pro)renin gene expression (*REN*) was not due to maternal sex steroid exposure. In fact, *ex vivo*, prorenin protein, along with several other RAS genes were expressed in a sex specific manner. Based on these observations, we were interested in determining how the sex of the fetus could affect RAS gene expression of a maternal tissue. In addition, we wanted to show whether this sex difference could be attenuated with cAMP stimulation.

In conclusion, this thesis shows the RAS pathways within two trophoblast cell lines, establishes a decidual explant model that expresses the RAS and demonstrates that the decidual RAS is sexually dimorphic and finally discusses how these findings may contribute to our understanding of the role fetal sex plays in determining pregnancy outcome.

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Wang Y, Pringle KG, Sykes SD, Marques FZ, Morris BJ, Zakar T, Lumbers ER, 2012

Fetal sex affects expression of Renin–Angiotensin System components in term human decidua. *Endocrinology* 153:462-468

Wang Y, Pringle KG, Chen YX, Zakar T, Lumbers ER, 2012

Regulation of the Renin–Angiotensin System (RAS) in BeWo and HTR–8/SVneo trophoblast cell lines. *Placenta* 33:634-639

Wang Y, Pringle KG, Lumbers ER, 2013

The effects of cyclic AMP, sex steroids and global hypomethylation on the expression of genes controlling the activity of the Renin–Angiotensin System in placental cell lines. *Placenta* 34:275-280

Wang Y, Pringle KG, Sykes SD, Lumbers ER

Regulation of Renin–Angiotensin System (RAS) pathways in the Human Decidua (Submitted to *Endocrinology*)

LIST OF PUBLICATIONS INCLUDED AS PART OF THIS THESIS

Sykes SD, Mitchell C, Pringle KG, Wang Y, Zakar T, Lumbers ER

Transgestational DNA methylation in the regulation of the human intrauterine Renin–Angiotensin System and prorenin processing enzymes (Submitted to *Human Reproduction*)

Lumbers ER, Pringle KG, Wang Y, Gibson KJ

The Renin–Angiotensin System from conception to old age: the good, the bad and the ugly.
(Submitted to *Clinical and Experimental Pharmacology and Physiology*)

LIST OF CONFERENCE ABSTRACTS

Wang Y, Pringle KG, Sykes SD, Zakar T, Lumbers ER, 2012

Renin regulation in the BeWo and HTR-8/SVneo Trophoblast cell lines. *Fetal and Neonatal Physiology Workshop, Sydney, Australia.*

Wang Y, Pringle KG, Lumbers ER, 2012

Effects of cAMP on sexually determined renin expression and secretion by human decidua explants. *16th Annual conference of the Perinatal Society of Australia and New Zealand (PSANZ), Sydney, Australia.*

Pringle KG, Sykes SD, Wang Y, Dekker G, Roberts CT, Lumbers ER, 2012

Fetal sex alters the expression of the uteroplacental and maternal circulating Renin-Angiotensin System; implications for maternal cardiovascular function and fetal outcome. *Gordon Research Conference on Angiotensins, Ventura, CA, USA.*

Wang Y, Pringle KG, Sykes SD, Zakar T, Lumbers ER, 2012

Regulation of Renin-Angiotensin System (RAS) pathways in BeWo and HTR-8/SVneo Trophoblast cell lines and the Human Decidua. *Gordon Research Conference on Angiotensins, Ventura CA, USA.*

Wang Y, Pringle KG, Lumbers ER, Scott RS, 2011

Interactions between Renin-Angiotensin System components in an endometrial cancer cell line; evidence for positive feedback in a tissue renin angiotensin system? *Gordon Research Symposium on Angiotensins. Ventura CA, USA.*

Wang Y, Pringle KG, Zakar T, Lumbers ER, 2011

Regulation of the Renin–Angiotensin System (RAS) pathway by cAMP in BeWo and HTR–8/SVneo trophoblast cells. *Renin-angiotensin system club, Melbourne, Australia.*

Wang Y, Pringle KG, Zakar T, Lumbers ER, 2011

Identification of Renin–Angiotensin System (RAS) pathways in BeWo and HTR–8/SVneo cells. *Society for Reproductive Biology (SRB), Cairns, Australia.*

Wang Y, Pringle KG, Sykes SD, Zakar T, Lumbers ER, 2011

Effects of fetal sex on prorenin production by the decidua. *Fetal and Neonatal Physiological Society (FNPS), Cairns, Australia.*

Wang Y, Pringle KG, Chen YX, Zakar T, Lumbers ER, 2010

Regulation of the renin angiotensin system (RAS) in a trophoblast cell line by cyclic adenosine monophosphate (cAMP) and 5'–aza–2'–deoxycytidine (AZA). *Society for Reproductive Biology (SRB), Sydney, Australia.*

LIST OF ABBREVIATIONS

μL	Microlitre
μg	Microgram
(P)RR	(pro)renin receptor
ACE	Angiotensin converting enzyme
<i>ACE1</i>	Angiotensin converting enzyme gene
<i>ACE2</i>	Angiotensin converting enzyme 2
<i>ACE2</i>	Angiotensin converting enzyme 2 gene
AGT	Angiotensinogen
<i>AGT</i>	Angiotensinogen gene
<i>AGTR1</i>	Angiotensin II type 1 receptor gene
<i>AGTR2</i>	Angiotensin II type 2 receptor gene
Ang 1–7	Angiotensin 1–7
Ang 1–9	Angiotensin 1–9
Ang I	Angiotensin I
Ang II	Angiotensin II
Ang III	Angiotensin III
Ang IV	Angiotensin IV
ANOVA	Analysis of variance
APA	Aminopeptidase A
ARBs	Angiotensin receptor blockers
AT ₁ R	Angiotensin II type 1 receptor
AT ₂ R	Angiotensin II type 2 receptor
<i>ATP6AP2</i>	(Pro)renin receptor gene
AZA	5'-aza-2'-deoxycytidine
BeWo	Choriocarcinoma derived cell line
c.p.m	Counts per minute
cAMP	Cyclic adenosine monophosphate
CRE	cAMP response element
CREB	cAMP response element-binding protein
CRH	Corticotropin releasing hormone
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
E ₂	Estradiol-17 β
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERK1	Extracellular signal-related protein kinase 1
ERK2	Extracellular signal-related protein kinase 2

EVT	Extravillous trophoblast cell
h	Hour
hCG	Human chorionic gonadotropin
HEPES	4- (2-hydroxyethyl) -1-piperazineethanesulfonic acid
HESCs	Human endometrial stromal cells
HSP	Heat shock protein
HTR-8/SVneo	Transformed first trimester extravillous trophoblast cell line
<i>IGFBP-1</i>	Insulin-like growth factor binding protein-1 gene
IGFBP-1	Insulin-like growth factor binding protein-1
JG	Juxtaglomerular
JNK	c-jun N terminal kinase
<i>KAI1</i>	Gene for the transmembrane glycoprotein of the tetraspanin family
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
<i>MAS1</i>	Protooncogene receptor gene
MMP	Metalloproteinase
MPA	Medroxyprogesterone acetate
Mas	Protooncogene receptor
mL	Millilitre
mRNA	Messenger RNA
mg	Milligram
miRNA	Micro RNA
NaHCO ₃	Sodium bicarbonate
NEP	Neutral endopeptidase
ng	Nanogram
nm	Nanometre
NSP	Nephrectomised sheep plasma
OD	Optical density
PAI	Plasminogen activator inhibitor
PEP	Prolylendopeptidase
pg	Picogram
PGHS-2	Prostaglandin H synthase-2
PI3K	Phosphatidylinositol-3 kinase
Pit-1	Positive transcription factor 1
PLZF	Promyelocytic Leukaemia zinc finger protein
PMSF	Phenylmethylsulfonyl fluoride
PRL	Prolactin
qRT PCR	Real-time quantitative reverse transcription polymerase chain reaction

RAS	Renin-Angiotensin System
<i>REN</i>	Renin gene
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
SPSS	Software package for statistical analysis
<i>SRY</i>	Sex determining region Y gene
TGF- β_1	Transforming growth factor beta 1
TIMP	Tissue inhibitors of metalloproteinase
TMB	3,3,5,5-tetramethylbenzidine
V-ATPase	Vacuolar H ⁺ -adenosine triphosphatase
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells

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Chapter One

Overview

This chapter provides a review of available literature on the uteroplacental RAS and pregnancy and briefly outlines the studies conducted for this thesis.

The overview focuses on the importance of the renin angiotensin system in normal human placentation, which is essential for nutrient, oxygen and waste exchange, especially as fetal demand increases (1-3).

1.1 HUMAN PREGNANCY AND THE RENIN-ANGIOTENSIN SYSTEM

Human pregnancy is defined as the period, usually spanning 40 weeks, from conception to birth. Pregnancy starts upon fertilisation of the ovum, progressing to implantation of the blastocyst into the decidua. Decidualisation is the process whereby the normal endometrium of the uterus is transformed to receive the implanting blastocyst; it is essential for normal pregnancy. Immediately after implantation, placental development begins with attachment of outer trophoblast layer of the blastocyst to the uterine epithelial cells. The next stage of placentation is trophoblast invasion; cytotrophoblast cells invade the decidua, myometrium and maternal spiral arterioles, changing them from muscular walled arteries to passive conduits, thus ensuring adequate nutrients and gas exchange for the developing embryo. Normal placentation is essential for fetal development by maintaining adequate and efficient oxygen and nutrient exchange between the mother and the fetus.

The developing fetus is surrounded by the amnion, which is surrounded by the chorion. The chorion is attached to the decidua of the uterine wall. The amnion develops as a cavity within the implanting embryo. Within this cavity amniotic fluid accumulates and the cavity continues to expand, eventually adhering to the chorion. Both the amnion and chorion are of fetal origin, whereas the decidua is maternal (Figure 1.1) (4, 5).

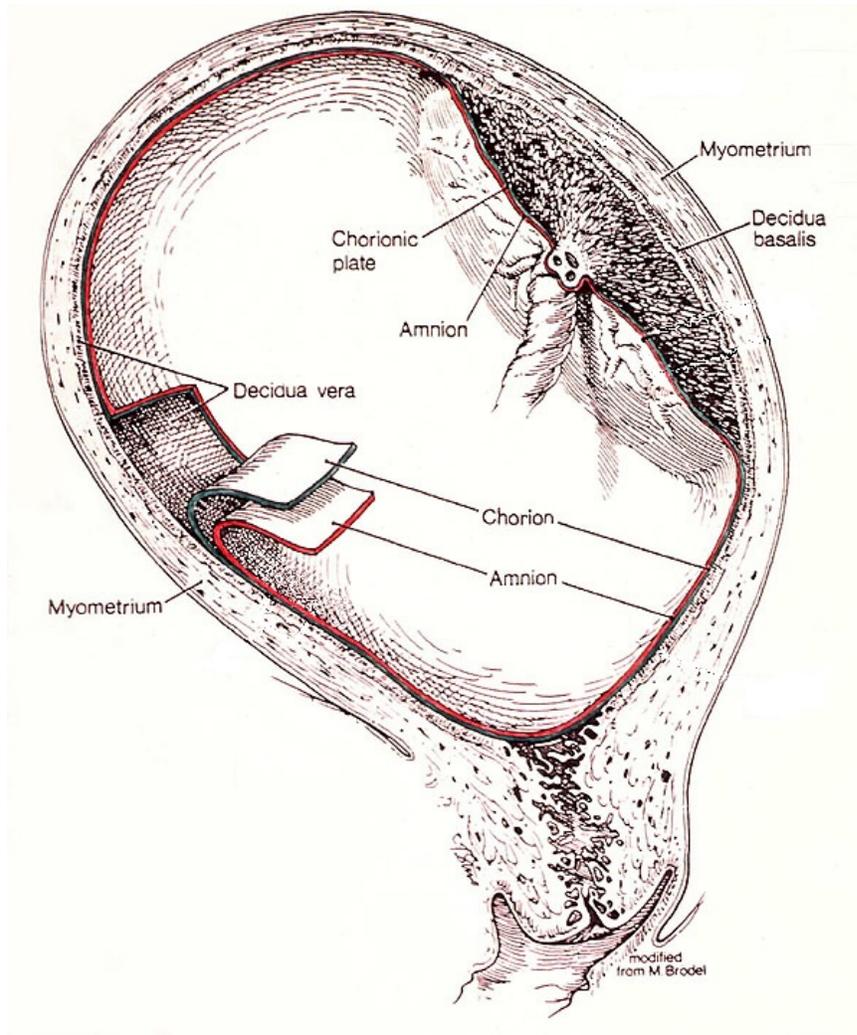


Figure 1.1 Cross section of the pregnant uterus.

The fetal chorion is shown in green and amnion in red. The Decidua basalis is the site of implantation and trophoblast invasion. (Image source: American Society for Microbiology, Larry D. Gray, Ph.D., TriHealth Laboratories, Bethesda Oak Hospital, Cincinnati, OH, USA (6))

The renin angiotensin system (RAS) has also been shown to play a major regulatory role in intrauterine tissues. The RAS consists of renin, a specific aspartyl protease that catalyses the conversion of liver/tissue derived angiotensinogen (AGT) into a decapeptide, angiotensin I (Ang I) (7, 8). Ang I is subsequently cleaved by the angiotensin converting enzyme (ACE) to form the biologically active, eight amino acid peptide, angiotensin II (Ang II). There are two main types of Ang II receptors, angiotensin II receptor type 1 (AT₁R) and angiotensin II receptor type 2 (AT₂R) (Figure 1.2).

Within the uterine tissues, Ang II acting on the AT₁R facilitates trophoblast proliferation and invasion into the maternal decidua (9), whilst Ang II acting on the AT₂R also inhibits cell growth and promotes apoptosis (10). Apoptosis is a necessary consequence of trophoblast invasion, occurring when decidual cells are eroded and when uterine spiral arterioles are transformed into dilated vessels. Within the placenta, the locally synthesised RAS is involved in the modulation of placental blood flow, angiogenesis, cell proliferation and spiral artery remodelling (4, 5, 11, 12). Research from our laboratory shows that placental renin gene (*REN*), (pro)renin receptor gene (*ATP6AP2*) and angiotensin 1 receptor gene (*AGTR1*) mRNA expression have strong correlations with the mRNA expression of an angiogenic factor, vascular endothelial growth factor (VEGF) in early gestation, implicating the RAS in placental angiogenesis (13).

It is not surprising then that disruptions to the uteroplacental RAS have been associated with pregnancy complications (14, 15). Many studies have shown that inadequate decidualisation, poor or shallow placental implantation and deficient

trophoblast invasion are associated with pregnancy complications such as preeclampsia and intrauterine growth restriction (IUGR) (16).

Several RAS changes can be observed in patients with preeclampsia compared to normal pregnancies, such as; lower levels of renin, Ang I, and Ang II, coupled with an increased sensitivity to Ang II. In addition, several studies have demonstrated the role of the AT₁R autoantibody (AT₁-AA) in the pathogenesis of preeclampsia (17). The AT₁-AA binding to the AT₁R has been shown to produce reactive oxygen species, as well as the activation of plasminogen activator inhibitor type 1 (PAI-1), which can reduce trophoblast invasion, resulting in poor placentation and placental insufficiency (18).

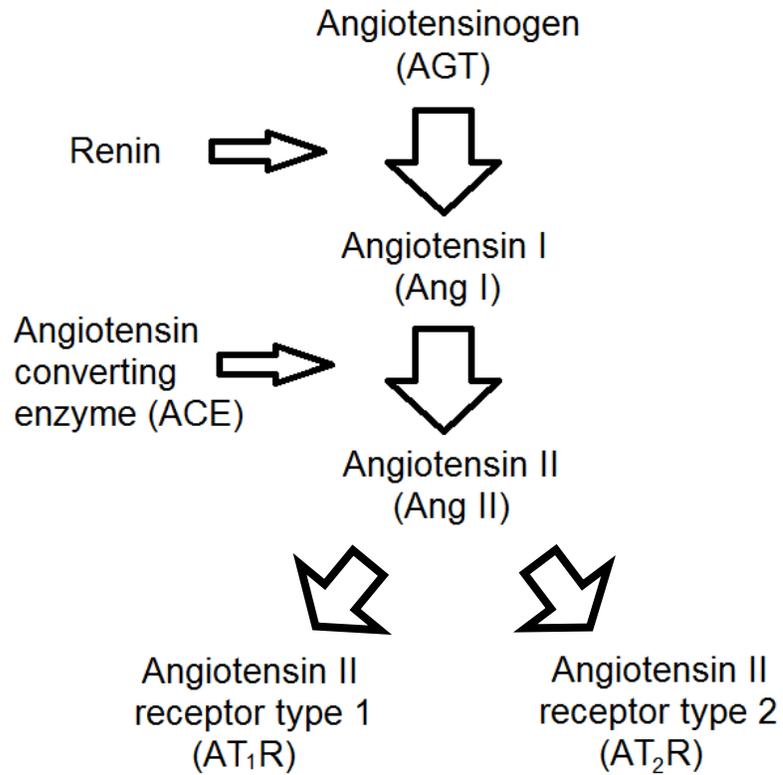


Figure 1.2 The 'classical' Renin–Angiotensin System

Angiotensinogen is cleaved by renin to form angiotensin I, which is in turn cleaved by ACE to form angiotensin II, which can bind to its angiotensin receptors, AT₁R and AT₂R.

The RAS has also been shown to be involved in fetal development; playing a critical role in normal nephrogenesis, therefore angiotensin receptor blockers (ARBs) and ACE inhibitors are contraindicated in pregnancy. A meta-analysis conducted on the outcome of neonates from pregnancies exposed to ACE inhibitors or ARBs, found that in pregnancies where ACE inhibitors had been used, only 52% of neonates were without complications, and in pregnancies where ARBs were used only 13% of newborns were without complications (19). Inhibition of the RAS during pregnancy disrupts fetal renal development. In animal models AT₁R blockade and ACE inhibition during the period of fetal renal development causes profound changes in renal morphology (20-22). These include thickening of the cortical radial arterial wall, changes to the afferent arterioles, which become smaller, thicker and fewer in number, papillary atrophy and a reduction in size and number of glomeruli (20). Renal lesions were also observed in rats treated with the ACE inhibitor enalapril during nephrogenesis. In addition, similar findings were reported when the renal RAS had been disrupted in pig animal models, which closely resembles human nephrogenesis (23). All these models demonstrate the role of the RAS in the normal renal development.

Finally, the RAS has also been implicated in the initiation of labour. Although the precise mechanism remains unclear, renin has been shown to directly stimulate prostaglandin H synthase-2 (PGHS-2) production by decidual cells (24) and previous studies from our laboratory suggests that the decidual RAS is involved in labour-associated increases in amnion *PGHS-2* abundance (25). Since PGHS-2 is an important enzyme regulating prostaglandin synthesis and prostaglandin

administration is an established method for labour induction (26), it is plausible that the decidual RAS is involved in the initiation of human labour.

The RAS has also been shown to play an important role in male reproduction, prior to fertilisation of the ovum. Ang II binding to the AT₁R causes acrosomal exocytosis in capacitated sperm in the male bovine reproductive tract (27) and has been shown to maintain sperm motility in both human and rat models (28, 29). In addition, captopril, an ACE inhibitor, decreases binding of human spermatozoa to oocytes (30). In mice, ACE knockout caused male infertility (31), which could be corrected by restoring testicular ACE (32). This shows that the RAS is involved in several critical processes within the male reproductive system that are essential in successful fertilization of the ovum.

Given that the RAS is extensively involved from conception through to birth, it is important that we understand the role and mechanism of action of the intrauterine tissue RASs in pregnancy. We should determine how renin and other components of the RAS are regulated within these tissues. To that end, my thesis describes the human RASs within the tissues of the uteroplacental unit and explores how they are regulated.

1.1.1 The decidua

During the course of a menstrual cycle and in conjunction with ovulation, there are significant changes in the endometrium to prepare it for the possible implantation of a blastocyst. At the end of the luteal phase of the menstrual cycle, if implantation has not occurred, the endometrium, which has been transformed from a proliferative growing tissue into a secretory tissue, is then shed (33). If blastocyst

implantation is successful however, the luteal type endometrial transformation becomes more substantial, with changes that involve the spiral arteries, and local immunological, endometrial, epithelial and stromal cells (34). This process is known as decidualisation, a specific transformation that only occurs in humans and other primates (35), rodents (36) and guinea pigs (37). The transformed endometrium is called the decidua and is only present during pregnancy.

Although cyclic adenosine monophosphate (cAMP) is necessary for initiation of decidualisation, it is prolonged exposure to maternal progesterone that causes the endometrial stromal cells, which are fibroblasts (elongated spindle shaped), to undergo transformation into large polygonal decidual cells (swollen cells which contain glycogen and lipids in their cytoplasm) during the proliferative phase of the menstrual cycle (33).

Decidual cells are characterised by their ability to release many cytokines, proteins and tissue factors such as prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1), thus both proteins are routinely used as markers of decidualisation (34). Exposure of the endometrium to cAMP *in vivo* has been shown to induce transient expression of the *PRL* and *IGFBP-1* genes (38), however it is progesterone stimulation that sustains the transformation of endometrial stromal cells, so it has been hypothesised that cAMP sensitises human endometrium stromal cells to progesterone. Progesterone also stimulates the influx of natural killer cells, which are a rich source of growth and angiogenic factors, and recent studies have highlighted the critical role of these immune cells in remodelling the endometrial spiral arteries prior to and during pregnancy (39).

Once blastocyst implantation is successful, trophoblast cells invade the decidua. This acts to physically anchor the developing placenta to the decidua and therefore to the uterine wall. Invasion of extravillous trophoblast into the spiral arterioles and the remodeling of these vessels creates low resistance arterial vessels that ensure adequate perfusion at the intervillous space (1, 3). Since the decidua is the site of trophoblast invasion it is not surprising that it also regulates the extent of this invasion. The decidua has a dense cellular matrix that inhibits trophoblast invasion. Furthermore, the metastasis suppressor gene *KAI1* has been shown to be present in decidual cells and its protein may be involved in intercellular communication between trophoblast cells and is also involved in regulating trophoblast invasion (34). Decidual transforming growth factor- β (TGF- β) release can inhibit trophoblast invasion through direct downregulation of trophoblast metalloproteinases (MMPs) production and through regulation of PAI-1 production, which in turn inhibits extracellular matrix (ECM) degradation (40-42)

The decidua has also been shown to be involved in determining the level of resistance of the conceptus to inflammation (43) and oxidative stress (44), as well as in dampening local maternal immune responses (34). In mice, T-cells cannot accumulate within the decidua, thus T-cell-dependent inflammatory responses to fetal alloantigens are suppressed (45). This decidua-mediated suppression of the local immune system is necessary to accommodate the invading trophoblast cells, which are fetal in origin.

1.1.2 The placenta

The placenta is the site of exchange of nutrients and oxygen; as such its functional capacity is essential for fetal development. Placental formation occurs after implantation of the embryo into the maternal endometrium. By the eighth day after fertilisation, the embryo is attached to the endometrium, at this time the embryo is surrounded by the inner mononucleated cytotrophoblast cell layer, and an outer multinucleated syncytiotrophoblast cell layer (this is the primitive syncytium). On the ninth day, lacunae are present in the syncytium, they will enlarged and fuse with each other and this establishes the beginning of the placental circulation when maternal blood seeps into these lacunae. These lacunae spaces will eventually become the intervillous space. The syncytiotrophoblast cells that covers the intervillous space is the site of exchange, as it forms a barrier which separates the maternal and fetal circulation.

At two weeks, cytotrophoblast cells break through the syncytium, these cytotrophoblast cells are known as primary villi; secondary villi are formed when the mesoderm is incorporated into the solid cytotrophoblast primary villi. The tertiary villi are formed when the mesenchyme core differentiates into blood vessels and connects the villi to the embryonic circulation. Cytotrophoblast cells proliferate to form cell columns at the tips of these villi, they act to anchoring the villi to the placenta and are known as anchoring villi. The cell columns proceed to colonise the decidual surface, fusing with other cell columns and forming a cytotrophoblast shell. Extravillous trophoblast cells are derived from the cytotrophoblast shell and differentiated into endovascular trophoblast cell and the interstitial trophoblast cell

and invade the maternal decidua to transform the maternal vasculature into low resistance, high flow vessels.

The endovascular trophoblast cells travel down the uterine vessels and form plugs that occlude the uterine arteries and so induce a hypoxic environment, which in turn stimulates angiogenesis within the placental tissue. Endovascular trophoblast cells also remodel the lumen of the maternal spiral arteries, which are now characterised by loss of endothelium, whilst the interstitial trophoblast cells travel through the decidua and remove the smooth muscle cell layers in the spiral arteries. Thus facilitating the transformation of the vessel into sinusoidal sacs, unresponsive to vasoconstriction, thereby lowering vascular resistance and increasing uteroplacental blood flow, ensuring that the increasing nutrient and oxygen demands of the fetus can be met (Figure 1.3) (1-3).

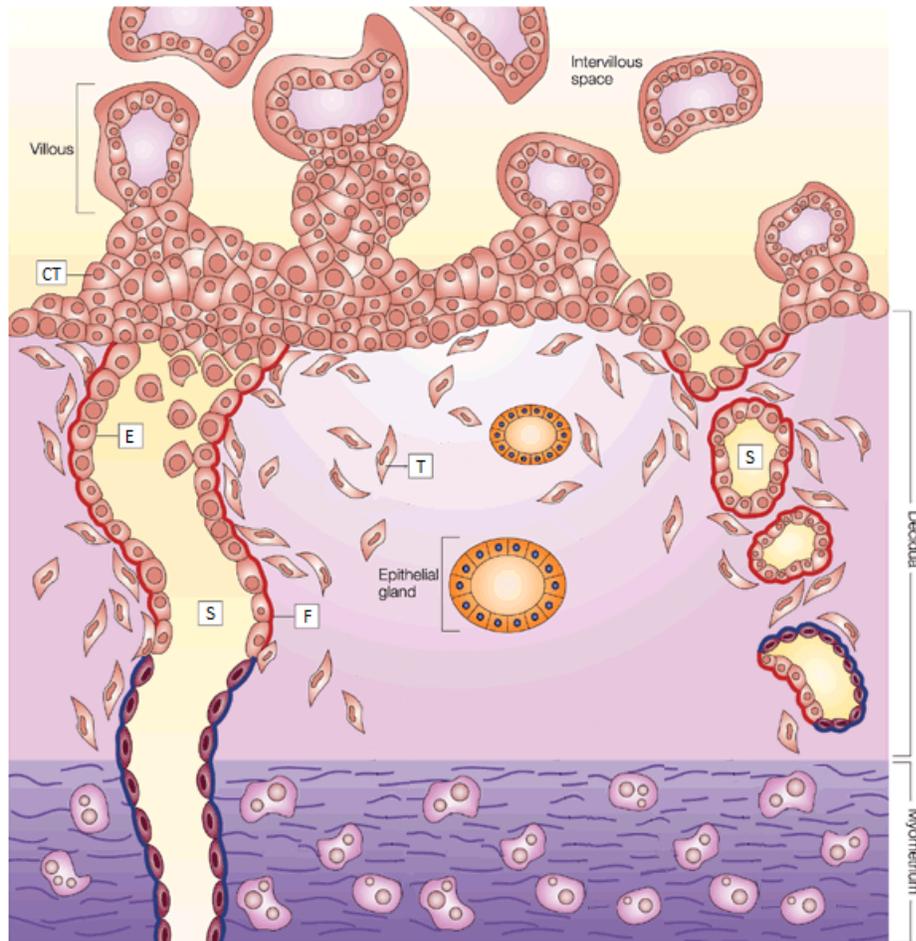


Figure 1.3 Trophoblast cell invasion and spiral artery remodelling in the first trimester of pregnancy.

Cytotrophoblasts grow out from the villi and form cell columns [CT], which anchor the placenta to the maternal decidua. The extravillous trophoblast cells [T] are derived from the cytotrophoblast shell and invade the maternal decidua to occlude the spiral arterioles [S]. The endovascular trophoblast cells [E] then transform the musculoelastic layers of the maternal spiral arteriole and replace them with a fibrinoid material [F]. (Adapted from Moffet-King, 2002 (2))

Human pregnancy is largely inefficient *i.e.*, a large number of pregnancies never progress beyond the first trimester. One reason for this may be the extensive yet incredibly complex uterine remodeling that is required to achieve sufficient placental perfusion, while at the same time not compromising the health of the mother.

On the one hand, inadequate trophoblast invasion can impair uterine vessel occlusion, which leads to reduced angiogenesis and incomplete or insufficient spiral artery remodelling, terminating in poor placental perfusion and increased shear stress in the intervillous space (12, 46). Studies have shown that shallow trophoblast invasion is often associated with placental insufficiency, IUGR and preeclampsia (47-49).

On the other hand, abnormally deep trophoblast invasion also results in obstetric complications; these are characterised by their depth. Placenta accreta is an invasion of the myometrium, placenta increta is invasion beyond the myometrium and placenta percreta is where the placenta attachment penetrates the uterine wall and possibly invades other internal organs. Deep penetration of the uterine wall is usually associated with increased likelihood of massive postpartum haemorrhage (50).

Placenta accreta is frequently associated with a reduction or an absence of the decidual layer; which is made of a dense cellular matrix that resists trophoblast invasion (see above and (51)). The depth of trophoblast invasion is the balance between invasion and resistance to this invasion. MMPs are a large family of zinc-dependent proteases produced by trophoblast cells to facilitate invasion of the decidua by degrading the ECM. Two MMPs are present in the trophoblast, MMP-2

and MMP-9, while decidual cells produce tissue inhibitors of metalloproteinases (TIMPs), which suppresses the activity of the MMPs.

1.2 THE IMPACT OF FETAL SEX ON PREGNANCY OUTCOMES

It is becoming increasingly evident that the sex of the fetus may influence neonatal morbidity and mortality (52). In several epidemiological studies focusing on the incidence of adverse pregnancy outcomes, fetal sex emerged as a major contributing factor. These studies showed that male fetuses have a higher risk for a number of adverse pregnancy outcomes; including spontaneous abortion (53), miscarriage later in pregnancy (54), stillbirth (55, 56), premature rupture of membranes and spontaneous preterm birth (57-60), gestational diabetes (61), and delivery by cesarean section (59, 62, 63). Moreover in term deliveries, a higher proportion of preeclamptic pregnancies carry a male fetus (60, 61), whereas in preterm deliveries the proportion of female babies from preeclamptic pregnancies is higher (60, 62). Female babies are also more likely to be growth restricted than male babies (59, 61).

Although the specific mechanisms responsible for these differences are unknown, there is evidence to suggest that the intrauterine tissues (and in particular the placenta) are involved in the regulation of fetal growth and survival in a sex-specific manner (52). As the uteroplacental RAS is critical in the regulation of key development periods, such as decidualisation, placentation, trophoblast invasion and angiogenesis, it is conceivable that tissue RASs within the conceptus are also regulated in a sex-specific manner.

1.3 THE RENIN–ANGIOTENSIN SYSTEM

The 'classical' circulating renin angiotensin system (RAS) is an endocrine system involved in the regulation of hemodynamics and fluid and electrolyte homeostasis. Renin, the main enzyme of the RAS, was first described by Tigerstedt and Bergman, as a compound capable of increasing arterial blood pressure (64). Blood pressure regulation is mediated through Ang II binding to the AT₁R which illicit physiological actions including; vasoconstriction, sympathetic activation and aldosterone release (65-67), whilst Ang II binding to the AT₂R is associated with vasodilation (68).

Historically, prorenin has been acknowledged as the pro–enzyme of renin, with both the active enzyme renin and prorenin released from the kidney into the circulation (8). Prorenin is processed in the juxtaglomerular (JG) cells of the renal afferent arterioles, where enzymatic cleavage of the prosegment occurs, resulting in active renin. Furthermore, it was accepted that Ang II acting through either the AT₁R or the AT₂R were the only pathways by which the RAS had biological effects.

More recent studies have identified angiotensin–like activity during pregnancy that is independent of the circulating RAS (69). Much of this activity has been attributed to locally synthesised RAS components within the uteroplacental unit, *i.e.*, local tissue RASs.

Local tissue RASs are distinct renin–angiotensin systems, existing within a specific tissue, that are involved in normal tissue functions (70-72). Tissue RASs can be found in the brain, testis, kidney and heart (69). There is evidence that suggests several local tissue RASs also exist at several sites within the uteroplacental unit including the trophoblast cells, spiral arterioles, decidua,

myometrium, amnion and chorion (12, 14). A previous study from our laboratory demonstrates the presence of RAS genes and proteins within intrauterine tissues and the placenta (Figure 1.4) (73).

The identification of local tissue RASs demonstrated that the 'classical' circulating RAS illustrated in Figure 1.2 is only a partial description of the complexity of pathways that make up the modern RAS. The modern RAS now consists of a specific prorenin receptor ((P)RR), which can non-proteolytically activate prorenin (74) to induce angiotensin-independent actions and is involved in Wnt signaling (75). It also contains the promyelocytic zinc finger (PLZF) pathway and an Ang 1-7 specific receptor, the Mas proto-oncogene (Mas) receptor pathway (74, 76, 77).

Moreover, local tissue RASs have also expanded our knowledge of RAS actions. Ang II acting through the AT₁R is pro-angiogenic and proliferative and has been shown to be involved in tumor growth and angiogenesis, by stimulating production of vascular endothelial growth factor (VEGF) (78). On the other hand, Ang II acting through its AT₂R has anti-angiogenic and anti-proliferative effects, by inhibiting VEGF and promoting apoptosis. Ang 1-7 (formed from Ang II) acting on a proto-oncogene G coupled protein receptor, the Mas receptor, has also been shown to be anti-proliferative in cultured tumour cells, through inhibition of the extracellular signal-related protein kinase 1 and 2 pathway (ERK1/ERK2) (79).

Thus the modern RAS is a complex biological system that does regulate blood pressure, fluid and electrolyte homeostasis, but is also involved in numerous other biological processes, including tissue growth, cell differentiation and angiogenesis. These tissue RASs are now preeminent areas for study, especially within intrauterine tissues.

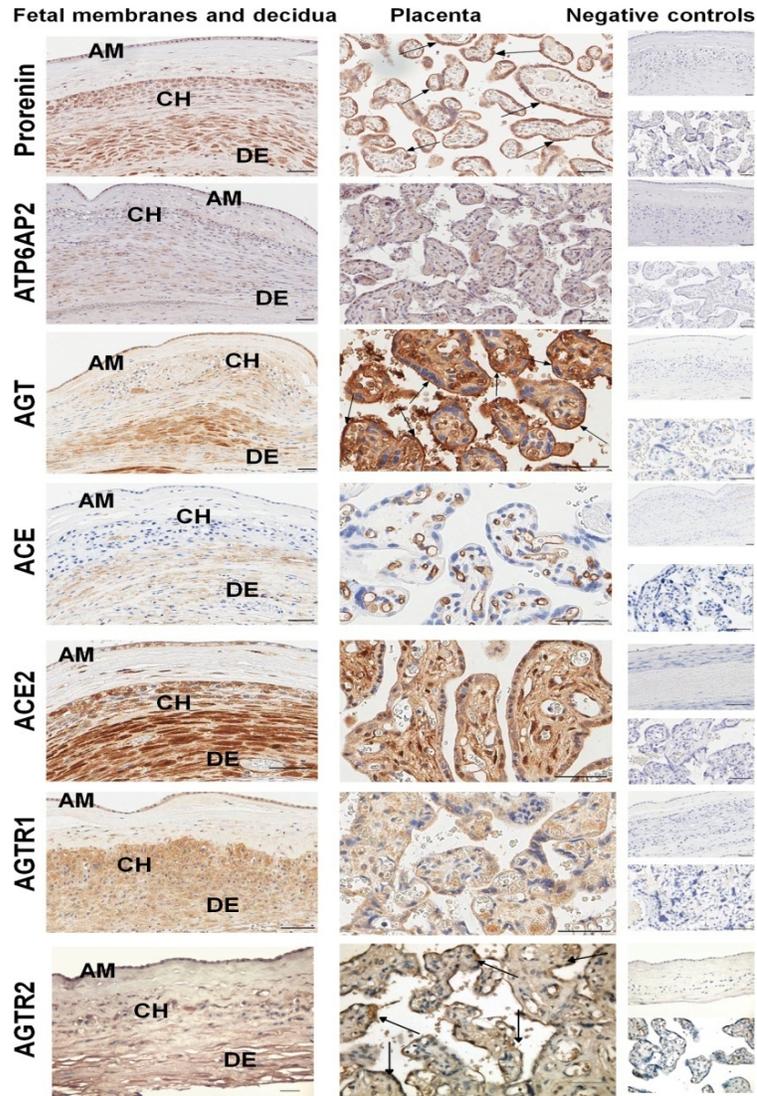


Figure 1.4 Immunohistochemical localisation of RAS proteins within the uteroplacental unit

Prorenin, AGT, ACE2 and AT₁R staining indicates their presence in amnion, chorion and decidua. Strong immunostaining for prorenin, (P)RR, AGT and AT₂R protein is present in the syncytiotrophoblast layer. AM, amnion; CH, chorion; DE, decidua. (Image source: Marques, *et al.*, 2011 (73)).

1.3.1 (Pro)renin receptor

Prorenin, under physiological conditions, does not readily cleave AGT, as its catalytic site is blocked by the prorenin prosegment, however this prosegment has been shown to unfold under low temperature or acidic pH (80). Consequently, it is believed that the activity of circulating renin is due solely to active renin secreted by the kidney, although prorenin can exist in an open conformation, its levels are negligible (70). By binding to the prorenin receptor ((P)RR) there is a conformational change in prorenin and the prosegment unfolds, revealing its catalytic site, so producing renin-like activity without proteolytic activation (Figure 1.5) (81). Bound prorenin exhibits a similar catalytic activity to that of mature active renin. Catalytic activity is the measure of how efficiently an enzyme is able to promote a reaction (74). The (P)RR has also been shown to bind mature active renin, however there is preference for the prorenin molecule (82).

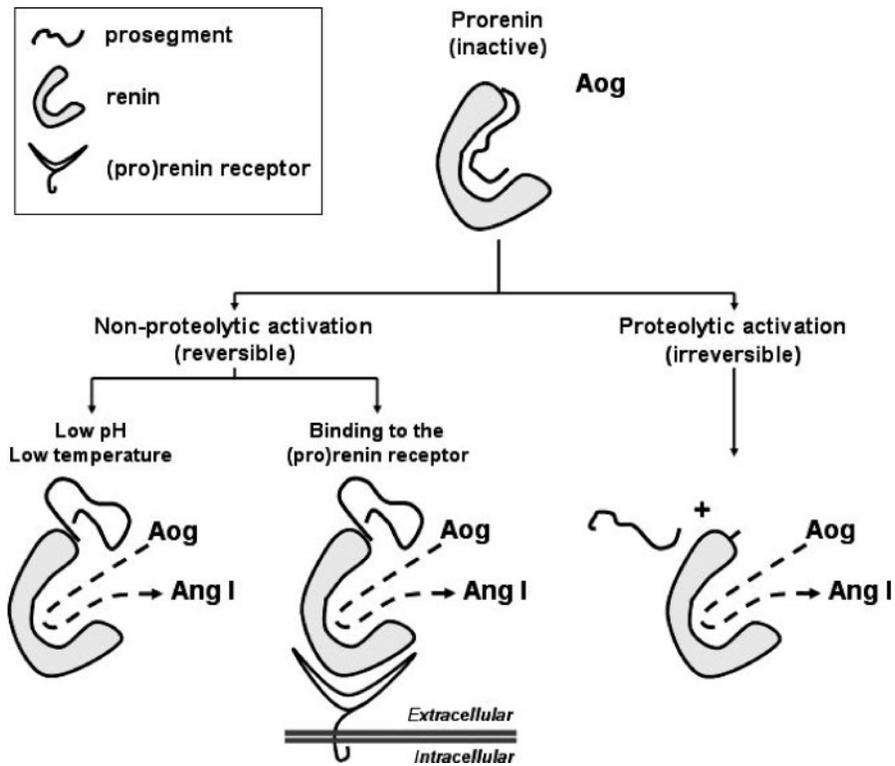


Figure 1.5 Non-proteolytic and proteolytic activation of prorenin

Non-proteolytic activation of prorenin under normal physiological conditions exists in equilibrium with the environmental pH and temperature, in addition to binding to the (pro)renin receptor. Prorenin can also be proteolytically activated by enzymatic removal of its prosegment. Adapted from Danser, *et al.*, 2007(83).

Another aspect of renin and prorenin binding to (P)RR, is activation of Ang II independent intracellular signaling cascades. The downstream effects of which include activation of TGF- β_1 , mitogen-activated protein kinases (MAPKs) p38, c-jun N terminal kinase (JNK), as well as the ERK1/2 pathway (74, 76). These second messengers subsequently have a diversity of structural and physiological effects. TGF- β_1 protein stimulates PAI-1, fibronectin, and collagen (84). It has been shown that in cardiomyocytes, p38 MAPK and heat shock protein 27 (HSP 27) are also activated by prorenin (76). HSP 27 is involved in the regulation of actin filaments, and thus in the reorganisation of cellular architecture (85).

(Pro)renin/(P)RR intracellular signaling occurs through second messenger systems, such as the induction of the p85 α subunit of the phosphatidylinositol-3 kinase (PI3K-p85 α), which is proliferative and anti-apoptotic (77). Phosphatidylinositide 3-kinase (PI3K) is involved in the TGF- β_1 stimulation of AT₁R gene expression in lung fibroblasts, in Ang II/AT₁R regulated migration and invasion of choriocarcinoma cells, and finally in the Ang II/AT₁R mediated regulation of the Na⁺ pumps in rat vascular smooth muscle cells (VSMC) (86, 87).

Furthermore, a recent study on embryonic development has shown that the (P)RR is essential for Wnt signaling. The Wnt proteins are growth factors, which are important in normal embryo development, cell differentiation, and have been shown to be involved in diseases such as cancer (75, 88). Cruciat *et al.*, hypothesised that the (P)RR acts as an adaptor between the Wnt receptor and the vacuolar H⁺-adenosine triphosphatase (V-ATPase) (75, 88).

1.3.2 Promyelocytic Leukaemia Zinc Finger (PLZF) protein

Another Ang II independent action of renin/prorenin binding to the (P)RR is through (P)RR interaction with the transcription factor PLZF, which translocates into the nucleus and suppresses (P)RR receptor gene transcription, forming a negative feedback loop (77). A secondary downstream effect of (P)RR and PLZF interaction is the activation of the PI3K–p85 α , which is associated with increased protein synthesis in cardiac hypertrophy (89).

1.3.3 Ang 1–7/ Mas receptor

Ang II can be further catalysed to form angiotensin III (Ang III) by aminopeptidase A (APA), subsequently Ang III is converted into angiotensin IV (Ang IV) (90). More significantly, Ang I can also be cleaved by angiotensin–converting enzyme type 2 (ACE2), an ACE homologue, to form angiotensin 1–9 (Ang 1–9), Ang 1–9 is further cleaved by ACE to form the heptapeptide angiotensin 1–7 (Ang 1–7). As well, ACE2 can also directly generate Ang 1–7 from Ang II at a rate faster than it is produced by the pathways described above (Figure 1.6) (91, 92).

Ang 1–7 is an endogenous ligand of the G protein–coupled receptor Mas, and has actions that counteract the effects of Ang II acting on the AT₁R, such as cell proliferation, inhibition of angiogenesis and stimulation of endothelial nitric oxide synthase (eNOS) (93). The Ang 1–7 Mas receptor axis is present in the human endothelium, endometrium, decidua and placenta (94, 95).

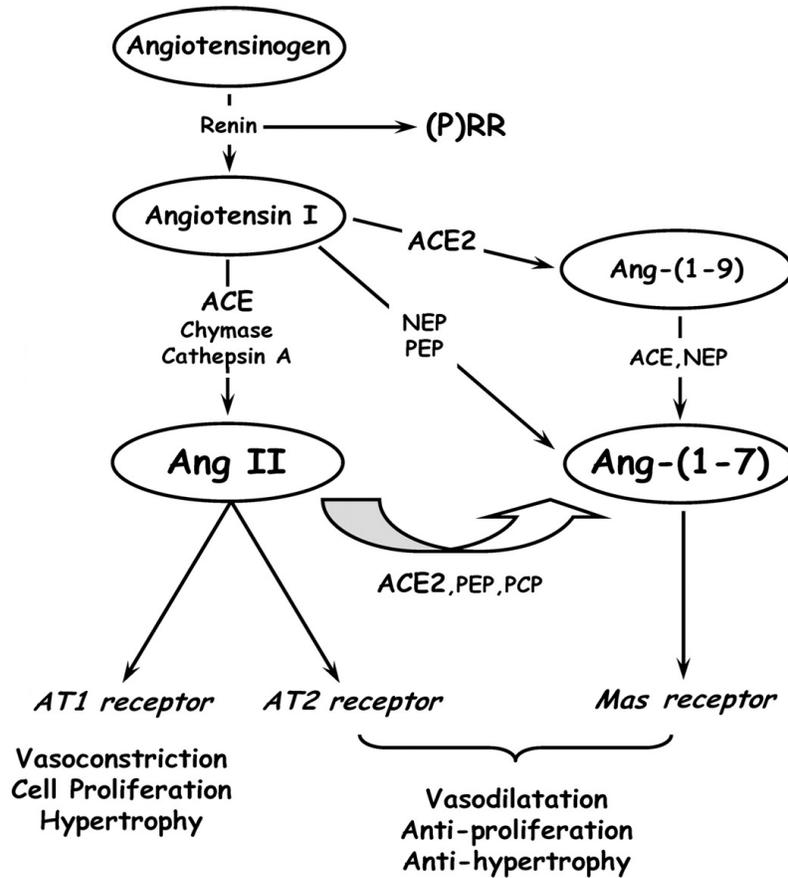


Figure 1.6 Ang 1-7 MAS receptor pathway of the Renin-Angiotensin System cascade

ACE2, prolylendopeptidase (PEP), and neutral endopeptidase (NEP) can cleave Ang I to form Ang 1-9, which is further cleaved to form Ang 1-7 by ACE and NEP. ACE2 can directly convert Ang II to Ang 1-7. Ang 1-7 bound to the MAS receptor, elicit similar actions to Ang II binding to the AT₂R (Adapted from Santos, *et al.*, 2008 (92)).

1.4 REGULATION OF THE RENIN–ANGIOTENSIN SYSTEM

Renin is a rate–limiting enzyme of the RAS cascade and, as such, is the major regulator of RAS activity. AGT however may also be rate–limiting due to its availability. Therefore during pregnancy an estrogen–dependent increase in AGT has a pronounced effect on circulating Ang II levels (96, 97).

As such, many studies have been conducted to identify factors that regulate renin secretion. During pregnancy, the decidua is a major source of renin, however the decidua does not possess dense–core renin containing secretory granules. Therefore it is widely thought that decidual prorenin secretion is constitutive and that the decidua cannot secrete active renin (5). However, several agents can modify decidual prorenin release; suggesting that although decidual prorenin synthesis and release is continuous, it is still subject to regulation. Possible regulators of renin within the uteroplacental unit are cAMP, progesterone, estradiol and hCG.

Besides these direct regulators of prorenin secretion, the methylation status at the CpG islands near promoter regions of genes can also affect their expression. Chemical agents like 5–aza–2′–deoxycytidine (AZA), can cause global hypomethylation, by blocking DNA methyltransferases (DNMTs), enzymes that facilitate the transfer of methyl groups on to DNA. Methyl groups on the CpG islands near promoter region of a gene; can prevent transcription of those genes.

Finally, Marques, *et al.*, demonstrated in a study on human hypertensive kidneys that micro RNAs (miRNAs) have post–transcriptional control on *REN* expression. The study identified two miRNAs, hsa–miR–181a and hsa–miR–663, which may be responsible for upregulating intrarenal *REN* mRNA in hypertension (98).

1.4.1 cAMP

Several studies have shown that cAMP is the main positive regulator of *REN* gene expression in the juxtaglomerular cells (99, 100) and primary decidual cells (101). cAMP increases decidual release of prorenin in a dose dependent manner; the effect is at a maximum after 72 h, with a four-fold increase over control (101). cAMP has also been shown to positively regulate renin gene expression within the uterine tissues (102, 103).

We now know that cAMP is in fact the second messenger for renin gene expression (104). Positive regulation of the prorenin gene occurs through cAMP binding to the cAMP response element (CRE), which allows the recruitment of the pituitary-specific positive transcription factor 1 (Pit-1) motif and the cAMP response element-binding (CREB) protein, resulting in the transcription of *REN* (100, 105).

1.4.2 Progesterone and Estradiol and hCG on the Renin–Angiotensin System

Downing *et al.*, demonstrated that human chorionic gonadotropin (hCG) can stimulate renin gene expression in the intrauterine tissues (106). This is of particular importance, as the developing blastocyst secretes hCG, which is responsible for maintaining the corpus luteum, which in turn maintains production of both estrogen and progesterone in early pregnancy.

It is hypothesised that estrogen and progesterone may also stimulate RAS expression in uterine tissues (107, 108). Given that at approximately 6 weeks gestation both hCG levels and maternal plasma prorenin levels are maximal, addition of hCG administration was associated with increased plasma prorenin levels(107). This hCG induced plasma prorenin increase may come from the corpus

luteum, as estrogen and progesterone can stimulate prorenin production via the ovaries. In addition, hCG and progesterone have also been shown to stimulate placental prorenin production (107, 109).

1.4.3 5-aza-2'-deoxycytidine (AZA)

Genes of the RAS pathways may also be subject to regulation through their methylation status *i.e.*, *ATP6AP2*, *ACE* and *AGTR1* genes have a high density of CpG islands in their promoter region. Despite this, previous work in our laboratory on human endometrial stromal cells (HESCs) showed that while AZA treatment increased *ACE*, *AGTR1* and *ATP6AP2* mRNA abundance, it also upregulated *REN* mRNA, which does not have CpG islands at its transcriptional start site, suggesting that AZA treatment has other effects apart from demethylation of promoter regions, perhaps it affects methylation of cytosine at other sites within the *REN* gene (unpublished observations).

1.5 STUDY OVERVIEW

Building on the available body of knowledge about the uteroplacental RAS, my aim was to identify how the sex of the fetus influenced the expression of the RAS within the human conceptus, in order to provide insight into the role fetal sex plays in the prevalence of adverse pregnancy outcomes in male and female neonates.

Our studies first describe the expression of the RAS genes within two placental cell lines. We were able to show that the HTR-8/SVneo and BeWo cell lines do not express the entire RAS cascade; in fact each cell line expressed a

different RAS pathway. The HTR-8/SVneo cells expressed the Ang II/AT₁R pathway, whereas the BeWo cells expressed the Ang 1-7/Mas receptor pathway. The purpose of studying a trophoblast cell line and a choriocarcinoma cell line was to establish their RAS profile and understand how the RAS was regulated within the trophoblast.

Since only the HTR-8/SVneo cells expressed *REN* we therefore proposed to study the effects of several agents known to stimulate *REN* expression in BeWo cells. We hoped to upregulate *REN* expression and prorenin protein in the HTR-8/SVneo cells and to induce *REN* expression in the BeWo cells. We did these experiments, in order explored the downstream effects of *REN* upregulation on the RAS cascade, in terms of gene expression, as well as the subsequent Ang II and Ang 1-7 peptide production. This is important ground work for understanding the role of the RAS in trophoblast invasion, angiogenesis and possibly cancer metastases. The work is described in Chapters 2 and 3 of this thesis, which are published papers reformatted for the purposes of the thesis (see Appendix A and B).

Aside from the RAS in trophoblast cells, we were also interested in the decidual RAS, as previous work conducted in this laboratory found a sex specific difference in decidual *REN* gene expression prior to the onset of labour. As the decidua is a maternal tissue formed only during pregnancy, in response to the implanting blastocyst, a difference in gene expression based on the sex of the fetus was very intriguing. My aim was to identify if the sex of the fetus could influence RAS gene expression in a maternal tissue, and see what effect it had on prorenin protein levels.

In order to study the decidua, we used a tissue explant model in which we sustained freshly excised decidua within a nutrient medium for 48 h. This allowed us to isolate the tissue from the maternal circulation and therefore free from the influences of any circulating fetal or maternal steroids. In addition, this tissue explant model better represents the *in vivo* environment than isolated cultured decidual cells. The decidua is a heterogeneous tissue containing many cell types, including decidual, glandular, blood vessel cells and while some cell types do replicate in culture, decidual cells do not. Therefore the explant method offered both tissue variability and representation of all cell types within the decidua.

Our study first identified the RAS gene expression profile from cultured decidual explants and compared it with similar non-incubated decidua. The sex specific difference in *REN* expression first described in non-incubated decidua was postulated to be due to differences in sex hormone levels. Therefore we postulated that incubating decidual tissue in a medium free from any sex hormones would attenuate the sex specific differences in *REN* expression. However, after 48 h incubation, the sex specific difference was maintained. In addition, we identified sex specific differences in expression of several other RAS genes. As a result, our study focused on how *REN* and prorenin protein was regulated within the decidua, especially how these sex-specific differences in decidual RAS expression could be sustained *ex vivo*. We used cAMP, other *REN* stimulators and AZA (validated through the earlier studies on HTR-8/SVneo and BeWo cells) to investigate how *REN* expression and prorenin protein levels were regulated in the decidua. This work is described in Chapters 4 and 5 of this thesis. Chapter 4 is a published article

reformatted for the purposes of this thesis (see Appendix C) and Chapter 5 is a submitted manuscript.

Chapter Two

Regulation of the Renin Angiotensin System (RAS) in BeWo and HTR–8/SVneo Trophoblast cell lines

This chapter explores expression of the renin angiotensin system (RAS), prorenin protein and angiotensin (Ang) peptide production by two established trophoblast cell lines. It provides basic knowledge about the expression and regulation of the placental RAS, which is necessary if we are to understand placentation and improve fetal and maternal outcomes.

2.1 ABSTRACT

Objectives

The Renin–Angiotensin System (RAS) is implicated in placentation. We determined which RAS pathways are present in two trophoblast cell lines (HTR–8/SVneo and BeWo cells) and the effects of cAMP, which stimulates renal renin.

Study design

The effect of cAMP on RAS gene expression and on prorenin and angiotensin peptides in HTR–8/SVneo and BeWo cells were investigated.

Results

In HTR–8/SVneo cells, prorenin mRNA (*REN*) and protein, (pro)renin receptor (*ATP6AP2*) and angiotensin II type 1 receptor (*AGTR1*) were stimulated by cAMP ($P<0.05$, $P<0.05$, $P<0.001$ and $P<0.05$, respectively). HTR–8/SVneo cells also expressed angiotensinogen (*AGT*), angiotensin converting enzyme 1 (*ACE1*), but did not express *AGTR2* or *ACE2* nor the Ang 1–7 receptor (*MAS1*).

BeWo cells did not express *REN*, and *REN* was not inducible by cAMP, but cAMP increased *ACE2* and *MAS1* (both $P<0.05$) and decreased *AGT* ($P<0.05$). BeWo cells expressed *AGT*, *ACE1*, *ACE2* and *MAS1* but not *ATP6AP2*, *AGTR1* nor *AGTR2*.

There was net destruction of Ang II in media from HTR–8/SVneo and BeWo incubations and net production of Ang 1–7 by BeWo and untreated HTR–8/SVneo cells.

Conclusion

HTR–8/SVneo cells express *REN* and produce prorenin as well as expressing other RAS genes likely to regulate Ang II/AT₁R interactions and respond to cAMP, like

renal renin–secreting cells. They are more similar to early gestation placentae and are therefore useful for studying effects of renin/ACE/Ang II/AT₁R on cell function.

BeWo cells express the ACE2/Ang 1–7/Mas pathway, which is sensitive to cAMP and therefore are useful for studying the effects of ACE2/Ang 1–7/Mas on trophoblast function.

2.2 INTRODUCTION

The placental renin angiotensin system (RAS) is important in placental development as it is involved in angiogenesis (110) and modulation of placental blood flow (11), and plays a key role in the regulation of trophoblast invasion (111, 112). Disruption of this local RAS may be associated with pregnancy complications, such as preeclampsia (14, 15).

The ‘classical’ RAS consists of renin, an enzyme secreted by the kidney that acts on angiotensinogen (AGT) to produce angiotensin I (Ang I), which is catalysed by angiotensin converting enzyme (ACE) to form angiotensin II (Ang II). The major actions of this RAS pathway are mediated by Ang II acting on the angiotensin II type 1 receptor (AT₁R) and the Ang II type 2 receptor (AT₂R). The latter has a number of actions that oppose those mediated by Ang II acting on the AT₁R (67).

Recently, additional RAS pathways have been described. These include an Ang 1–7/Mas receptor pathway, consisting of ACE2 (a homologue of ACE), which terminates the actions of Ang II by converting it to Ang 1–7. Ang 1–7 acting through the proto–oncogene receptor (Mas) has effects that oppose those of Ang II acting via the AT₁R (113). There is also a (pro)renin receptor ((P)RR) pathway, where prorenin bound to the (P)RR is non–proteolytically activated and can cleave AGT to

Ang I (74). Prorenin was previously considered to be an inactive precursor of renin, having little biological activity despite the fact that its circulating levels are 10 times higher than those of renin in non-pregnant subjects (114). Through binding to the (P)RR, prorenin acquires enzymatic activity. Additionally, it can induce intracellular signalling via angiotensin independent pathways (74, 84).

Studies have shown that the RAS may be involved in the regulation of trophoblast invasion (111) as well as spiral artery remodelling (12), and consequently, may play a role in implantation and placentation. Although we have described the expression of RAS genes and proteins in the human placenta (73), the mechanisms regulating their expression are yet unknown.

Cyclic adenosine monophosphate (cAMP) stimulates prorenin mRNA (*REN*) expression in renal juxtaglomerular cells (100). cAMP has also been shown to increase prorenin release in primary decidual cell cultures in a dose dependent manner (101). We postulated that since *REN* contains a cAMP response element (CRE) at its promoter region (115, 116), cAMP would increase expression of *REN*, as well as prorenin production. This would provide us with a tool for determining how the placental RAS regulates placental cellular function. As an initial step in determining how the placental RAS is regulated, we examined the expression of RAS genes and the secretion of prorenin and the Ang peptides, Ang II and Ang 1-7 in two trophoblast cell lines.

In this study we show that the two cell lines (HTR-8/SVneo and BeWo) express different components of the RAS pathways and report that while cAMP stimulates *REN* expression and prorenin secretion in HTR-8/SVneo cells, it does not induce *REN* expression in BeWo cells.

2.3 MATERIALS AND METHODS

Trophoblast Cell Culture

Two established trophoblast cell lines commonly used for studying placental function; HTR-8/SVneo and BeWo cells were used. HTR-8/SVneo cells are a transformed first trimester human extravillous trophoblast cell line (developed by Charles Graham, Ontario, Canada) (117), whilst BeWo cells are derived from a choriocarcinoma (118). HTR-8/SVneo and BeWo cells were cultured in phenol red-free RPMI-1640 or DMEM/F-12, respectively, supplemented with 15 mM HEPES, 1.2 g/L NaHCO₃, 1 mg/mL L-glutathione reduced, 0.1 g/L albumin fraction V, 0.65 µg/mL aprotinin, 10% fetal bovine serum and 40 µg/mL gentamicin. Cells were seeded at a density of 200,000 cells, in each well of a 6 well plate with 2 mL of incubation medium. Cells were allowed to settle for 24 h, after which the media was changed, cells were treated with either 0.3 mM 8-bromo-cAMP (Sigma-Aldrich, St. Louis, MO, USA) or vehicle. Cells were harvested and the incubation media collected at 24 and 48 h and snap frozen in liquid nitrogen for subsequent protein and mRNA analyses. Three experiments were conducted in triplicate. Cell viability was verified by measuring RNA stability and quality (data not shown).

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

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). In addition, we examine each sample's RNA integrity by running samples on a gel. RNA samples were DNase treated (Qiagen N.V., Hilden, Germany) and total RNA spiked with a known amount

of Alien RNA (Stratagene, La Jolla, CA, USA; 10^7 copies per microgram of total RNA, before the RNA is reverse transcribed using a Superscript III RT kit with random hexamers (Invitrogen). The Alien qRT PCR inhibitor alert system serves as a reference for internal standardization (119). qPCR was performed in an Applied Biosystems 7500 Real Time PCR System using SYBR Green for detection. Each reaction contained 5 μ L of SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA), RAS primers as we have described previously (13, 25, 73), cDNA reversed transcribed from 10 ng total RNA, and water to 10 μ L. Messenger RNA abundance was calculated as described previously, using the $\Delta\Delta_{CT}$ method, relative abundance is relative to Alien mRNA and a calibrator sample (a term placental sample collected at elective Caesarean section) (13, 25, 73).

Measurement of prorenin protein by ELISA

Prorenin concentration in culture media was measured using the Human Prorenin ELISA kit (Molecular Innovations Inc; Novi, MI) according to the manufacturer's instructions as described previously (120). Samples were assayed in duplicate. In our laboratory 1 ng/mL amniotic fluid prorenin measured using this technique generated 116 ng/h/mL of Ang I from AGT present in nephrectomized sheep plasma used as the source of AGT substrate. All samples were assayed on one ELISA plate. Therefore there was no inter-assay variability. Intra-assay coefficient of variation was 7.3%.

Radioimmunoassay (RIA) of Ang II and Ang 1–7

Angiotensin II was measured by radioimmunoassay (RIA) by Prosearch Pty Ltd, using the “delayed tracer addition” technique as described previously (120). Sensitivity was 3.5 pg/mL. Cross-reactivities to Ang I, Ang 1–7 and all other pertinent hormones were 0.52%, 0.0138% and < 0.1% respectively. Intra and inter-assay coefficients of variation were 6.4% and 12%, respectively.

Ang 1–7 was assayed directly by RIA by Prosearch Pty Ltd as described previously (120). Sensitivity was 14 pg/mL. Cross-reactivities to Ang I, Ang II, Ang III and Ang IV were 0.11%, 0.04%, 0.53% and 0.03%, respectively. Intra- and inter-assay coefficients of variation were 4.5% and 10%, respectively.

Data Analysis

Mann–Whitney U tests were used to determine the effects of cAMP treatment on RAS mRNA abundance at 24 and 48 h incubation and on prorenin protein, Ang II and Ang 1–7 peptide levels in the supernatant after 48 h in the BeWo and HTR–8/SVneo cells. The SPSS statistical package (SPSS for Windows, Release 17.0.0. Chicago) was used for all analyses. Significance was set at $P < 0.05$.

2.4 RESULTS

RAS mRNA abundance in HTR–8/SVneo and BeWo trophoblast cells and effects of cAMP

After 24 and 48 h incubation HTR–8/SVneo cells expressed detectable levels of most RAS mRNAs, namely *REN*, *AGT*, *ATP6AP2*, *ACE1* and *AGTR1* (Figure 2.1). *ACE2*, *AGTR2* and *MAS1* mRNA was not detected. By contrast, in

BeWo cells *REN*, *ATP6AP2*, *AGTR1* and *AGTR2* gene expression was not detected although significant amounts of *AGT*, *ACE1*, *ACE2* and *MAS1* mRNA were found after 24 and 48 h incubation (Figure 2.2).

In HTR–8/SVneo cells cAMP treatment significantly increased *REN* mRNA at both 24 and 48 h (both $P<0.001$), in addition cAMP treatment was associated with a time dependent increase in *REN* expression ($P<0.001$, Figure 2.1). At 24 h incubation only, cAMP treatment increased *ATP6AP2* and *AGTR1* mRNA abundance ($P=0.04$ and $P=0.02$, respectively). cAMP treatment did not have any effect on *AGT* and *ACE1* mRNA abundance (Figure 2.1).

REN expression in BeWo cells was not induced with cAMP treatment. After 48 h, cAMP treated BeWo cells showed a reduction in *AGT* mRNA abundance ($P=0.012$) but a significant increase in *ACE2* and *MAS1* mRNA abundance compared to vehicle treated cells after 24 and 48 h incubation ($P<0.001$, $P=0.006$; and $P<0.001$ and $P<0.001$, respectively), in addition cAMP treatment was associated with a time dependent decrease in *ACE2* expression ($P<0.001$) (Figure 2.2).

All RAS mRNA abundances are calculated relative to both Alien RNA and a placental sample, as such comparisons of relative gene expression levels can be made between the two cell lines. However, *AGT* mRNA was the only gene that showed any significant differences between the two cell lines. *AGT* is significantly lower in HTR–8/SVneo cells compared with BeWo cells after 24 and 48 h incubation (both $P<0.001$) (Figure 2.1B & 2.2A).

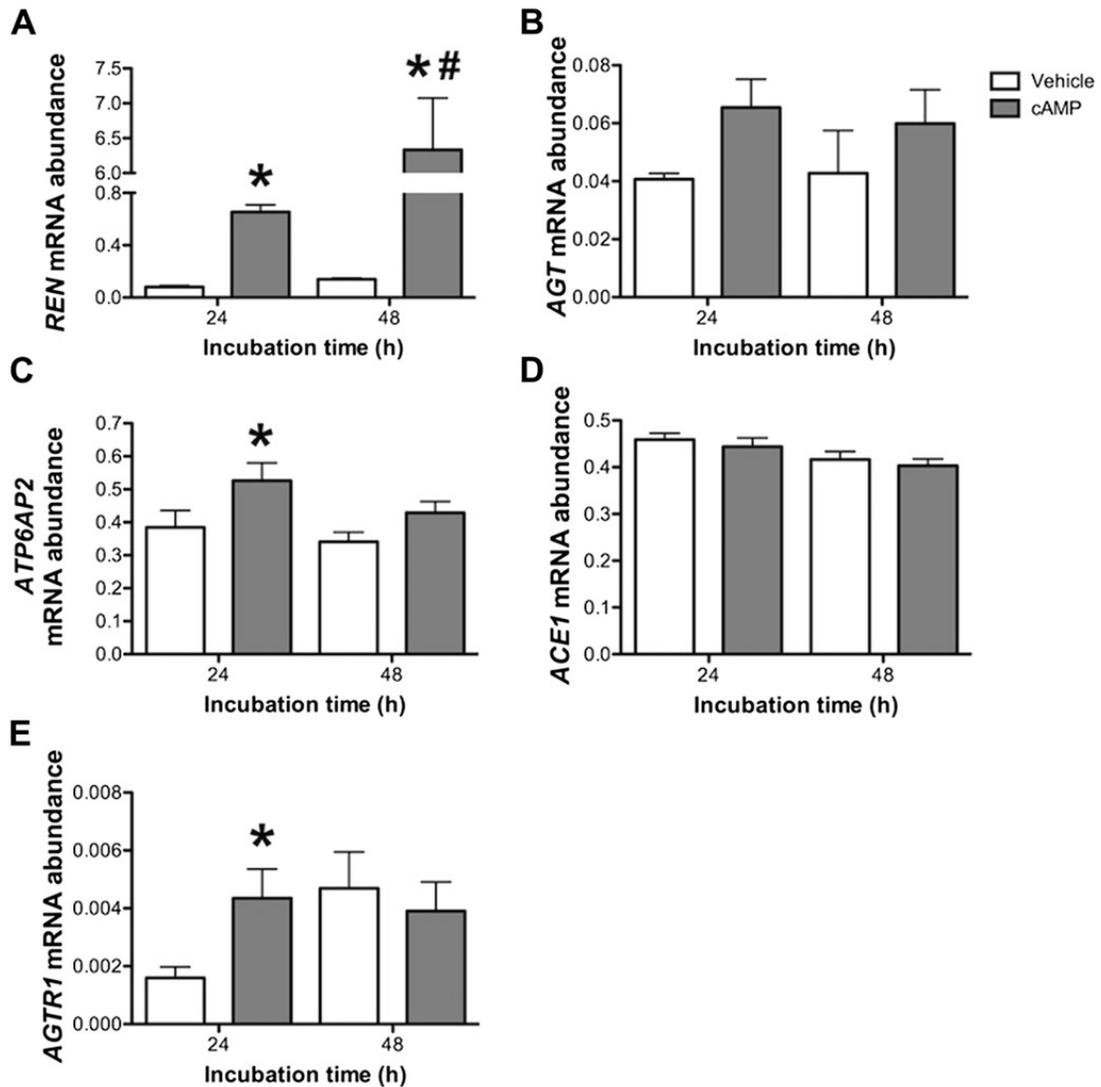


Figure 2.1 mRNA abundance of RAS genes in HTR-8/SVneo cells

(A) *REN* mRNA was expressed in HTR-8/SVneo cells and increased with cAMP treatment. In cAMP treated HTR-8/SVneo cells, *REN* expression increased with incubation time. (B) *AGT* expression did not change with cAMP treatment or incubation time. (C) *ATP6AP2* expression increased with cAMP treatment at 24 h only. (D) *ACE1* expression did not change with cAMP treatment or incubation time. (E) *AGTR1* expression increased with cAMP treatment at 24 h only (n=9; three experiments in triplicate in HTR-8/SVneo cells). * Denotes significant difference from vehicle treated controls at the same incubation time ($P < 0.05$); # Denotes significant difference in 48 h treatment from 24 h treatment of the same group ($P < 0.05$). HTR-8/SVneo cells did not express *ACE2*, *AGTR2* and *MAS1*.

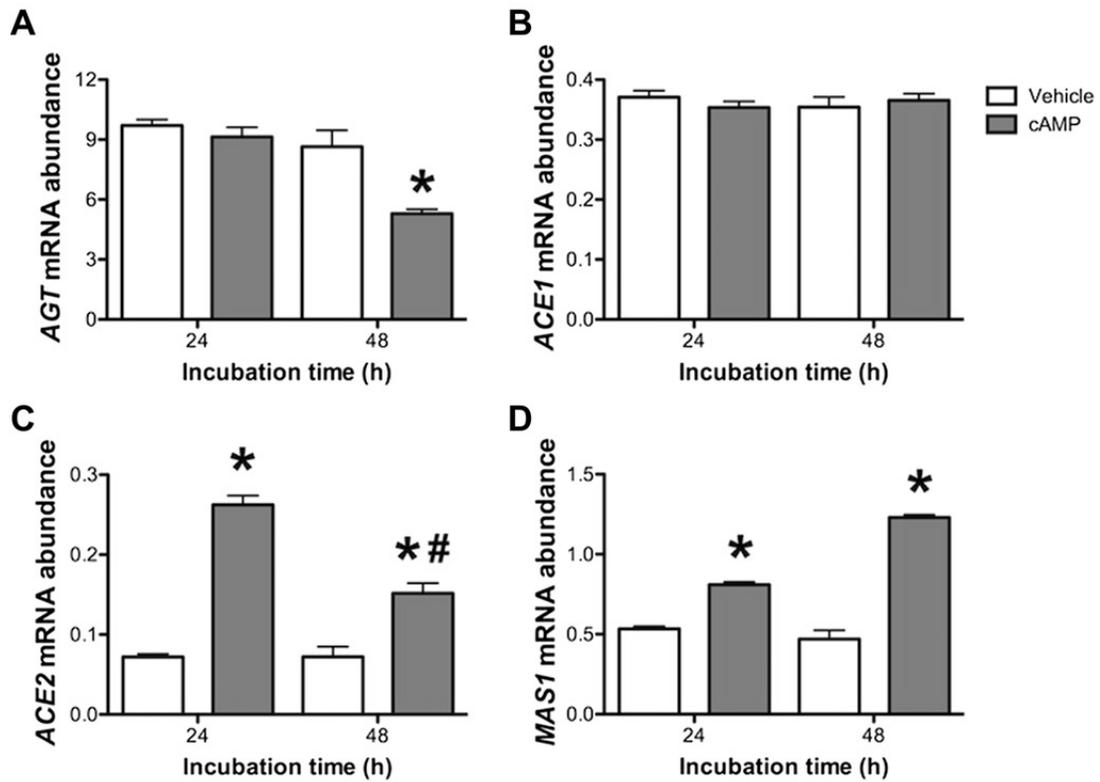


Figure 2.2 mRNA abundance of RAS genes expressed in BeWo cells

(A) cAMP treatment decreased *AGT* expression at 48 h incubation. (B) *ACE1* expression did not change with cAMP treatment or incubation time. (C) cAMP treatment increased *ACE2* expression at 24 and 48 h. *ACE2* expression in cAMP treated BeWo cells decreased with incubation time. (D) *MAS1* expression in BeWo cells increased with cAMP treatment at both 24 and 48 h (n=9; three experiments in triplicate in BeWo cells). * Denotes significant difference from vehicle treated controls at the same incubation time ($P<0.05$); # Denotes significant difference in 48 h incubation from 24 h incubation of the same group ($P<0.05$). BeWo cells did not express *REN*, *ATP6AP2*, *AGTR1* and *AGTR2*.

Prorenin, Ang II and Ang 1–7 levels in BeWo and HTR–8/SVneo cell supernatants and the effects of cAMP

Supernatants from triplicates of each of the 3 experiments were pooled and assayed for prorenin and Ang peptides. Significant amounts of prorenin were present in the supernatants of vehicle treated HTR–8/SVneo cells (Figure 2.3) and cAMP treatment was associated with increased amounts of prorenin in the supernatants collected from HTR–8/SVneo cells ($P=0.005$; Figure 2.3). Prorenin was not detected in either vehicle or cAMP treated BeWo cell supernatants.

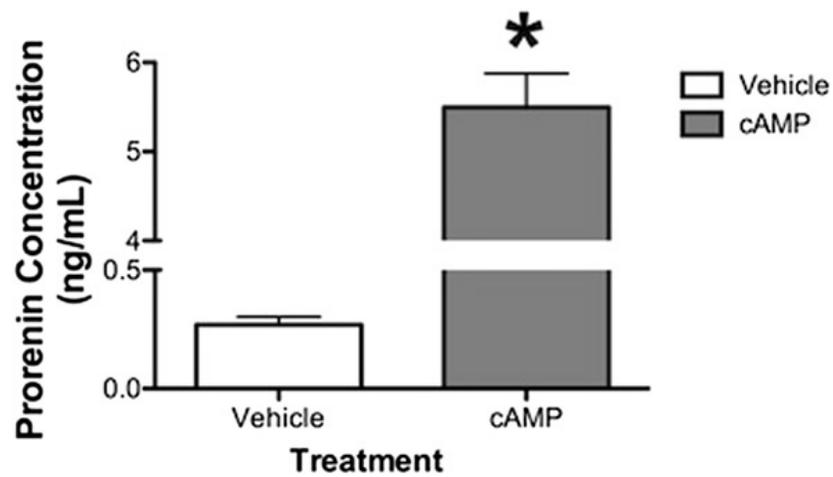


Figure 2.3 Prorenin protein (measured by ELISA)

Prorenin protein was present in the supernatant of vehicle treated HTR-8/SVneo cells after 48 h incubation. cAMP treatment significantly increased prorenin protein levels (n = 6). * Denotes significant difference to vehicle treated HTR-8/SVneo cells ($P < 0.05$).

Prior to incubation, measurable levels of both Ang 1–7 (18.4 pg/mL in DMEM–F12 and 10.81 pg/mL in RPMI–1640) and Ang II (37.33 pg/mL in DMEM–F12 and 18.56 pg/mL in RPMI–1640) were present in the culture media; therefore we have reported the amount of Ang 1–7 and Ang II found in media collected after incubation with trophoblast cell lines as net production or net destruction.

Since the levels of Ang II after incubation were less than those measured before incubation, there was a net loss of Ang II from the supernatants of both BeWo and HTR–8/SVneo cells. cAMP treatment had no effect on the net amount of Ang II present (Figure 2.4). There was net production of Ang 1–7 in media collected after incubation from both untreated HTR–8/SVneo and BeWo cultures (Figure 2.5). But there was net destruction of Ang 1–7 from HTR–8/SVneo cell supernatant during treatment with cAMP (Figure 2.5A), this was not statistically significant. In BeWo cells there was net production of Ang 1–7 in both untreated and cAMP treated cell supernatant, although like HTR–8/SVneo media, it was less if the BeWo cells had been treated with cAMP (Figure 2.5B). Due to the low number of samples, these observations were not statistically significant.

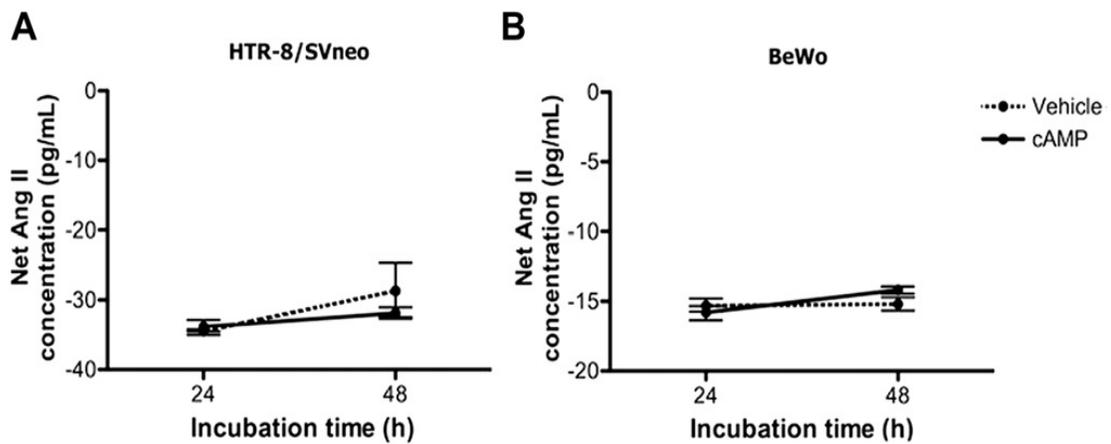


Figure 2.4 Net Ang II levels in the supernatant of HTR-8/SVneo and BeWo cells after 48 h.

(A) In the supernatant of HTR-8/SVneo cells, both vehicle and cAMP treatment resulted in a net loss of Ang II. (B) In the supernatant of BeWo cells, both vehicle and cAMP treatment resulted in a net loss of Ang II. Net loss of Ang II however, appeared to be greater in HTR-8/SVneo cells compared to BeWo cells (n = 6).

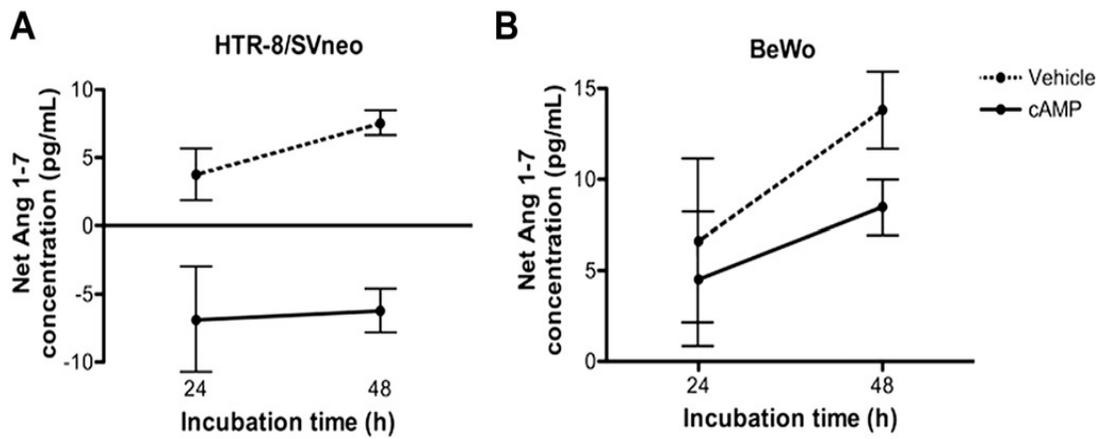


Figure 2.5 Net Ang 1–7 in the supernatant of HTR–8/SVneo and BeWo cells after 48 h.

(A) In the supernatant of HTR–8/SVneo cells vehicle treatment resulted in a net gain of Ang 1–7, whereas cAMP treatment resulted in a net loss of Ang 1–7. (B) In the supernatant of BeWo cells, both vehicle and cAMP treatment resulted in a net gain of Ang 1–7 (n = 6).

2.5 DISCUSSION

This study compared RAS gene expression within BeWo and HTR-8/SVneo cells. Although both cell lines have been used to model placental cellular functions, there are some notable differences between the two cell lines. For example, BeWo cells contain a mixture of villous and extravillous trophoblast cells, whereas the HTR-8/SVneo cells contain only extravillous trophoblast cells. In addition, BeWo cells are derived from a choriocarcinoma. In terms of the expression of RAS pathways these two cell lines were very dissimilar. Since HTR-8/SVneo cells lack both the AT₂R and the Ang 1-7/MAS receptor pathway, we would predict that any anti-angiogenic and pro-apoptotic effects of the placental RAS occurring as a result of activation of these pathways (121, 122) would not be active in this cell line. Thus any putative angiogenic and proliferative actions of the HTR-8/SVneo renin/AGT/ACE/Ang II/AT₁R pathway which is present would be unopposed by actions of Ang II via AT₂R or Ang 1-7 via the Mas receptor. This means that the role of Ang II/AT₁R in the control of placental angiogenesis could be challenged using cAMP to drive *REN*, *ATP6AP2*, *AGTR1* expression and prorenin production. The effects of this RAS pathway on placental trophoblast function can therefore be studied without interference from antagonistic effects of the RAS mediated via Ang II/AT₂R and Ang 1-7/Mas receptor interactions. Conversely, as the BeWo cell line only expressed the ACE2/Ang 1-7/MAS receptor pathway, the putative anti-angiogenic and anti-proliferative effects of this RAS pathway (113) can be studied in isolation, free from any concomitant actions of Ang II mediated by either AT₁R or AT₂R receptors. Since cAMP stimulated expression of both *ACE2* and *MAS1*, the

effects of stimulation of this pathway on angiogenesis and apoptosis can easily be investigated.

HTR-8/SVneo cells behave in a similar manner to juxtaglomerular renin secreting cells (115), where prorenin expression and production are enhanced by cAMP. Similar increases in placental *REN* expression and renin protein have been reported in villous placenta and decidual cells after treatment with cAMP (101, 102).

In HTR-8/SVneo cells, *AGTR1* mRNA is higher after cAMP treatment, similar upregulation of *AGTR1* expression has been reported in smooth muscle cells (123). In addition, *AGTR1* expression is downregulated by Ang II (123), which in cAMP treated HTR-8/SVneo cells appear to have lower Ang II levels and thus may contribute to the increase in *AGTR1* expression after cAMP treatment.

Since BeWo cells, unlike HTR-8/SVneo cells, do not express *REN*, we used 8-bromo-cAMP in an attempt to stimulate *REN* expression in this cell line, however this proved ineffective. This was perhaps surprising, given that the dose of cAMP used was highly effective in stimulating *REN* expression and prorenin production in HTR-8/SVneo cells, and that the ability of cAMP to stimulate juxtaglomerular cell renin is well recognised (115, 116). Therefore, we believe that in BeWo cells, cAMP could not access the cyclic AMP response element (CRE) of the *REN* gene. Whether this was due to heavy methylation of genes in BeWo cells, whereby the CRE in *REN* was silenced but left other genes intact (*i.e.*, *ACE2* and *MAS1*), or that BeWo cells lack the necessary transcription factors for cAMP to bind to the CRE is unknown, however as far as we are aware, this is the first study to look at the RAS pathway in this cell line. Given that both BeWo cells and the HTR-8/SVneo cells

both originated from trophoblast, it is somewhat surprising that they are so dissimilar in terms of the components of the RAS pathway that were expressed.

We have however have been able to stimulate *REN* expression in human endometrial stromal cells using an inhibitor of DNA methylation (5-Aza-2'-deoxycytidine: AZA; unpublished data) so it will be of interest to see what happens to the response of BeWo cells to cAMP when they are exposed to AZA.

Ang 1-7 and Ang II peptides were present in the culture media prior to incubation, possibly because it was supplemented with 10% fetal bovine serum. Both cell lines failed to show net production of Ang II, which may be due to the labile nature of Ang II (124), as we were unable to use protease inhibitors in the culture without threatening cell viability. Net Ang 1-7 production by BeWo cells was observed, and may have resulted from the conversion of Ang II (present in the culture media prior to incubation) to Ang 1-7 by *ACE2* in the BeWo cells, as cAMP-induced expression of both *ACE2* and *MAS1* was observed. This probably accounts for the greater production of Ang 1-7 by BeWo cells compared to HTR-8/SVneo cells (Figure 2.5). An alternative AGT processing enzyme may also have been present in the culture medium, such as chymase or cathepsin D (125-127). The latter is less likely, as it is inactive at neutral pH (125). Additionally, HTR-8/SVneo cells do not express *AGT* to the same extent as BeWo cells. If this translates into a lower rate of *AGT* synthesis, it could account for the lower rate of Ang 1-7 production.

Low *AGT* abundance and protein levels are also seen in the placenta (73). *In vivo*, placental *AGT* may not be a rate-limiting factor for Ang peptide synthesis, as *AGT* could be sequestered from the maternal circulation. However, as no external

sources of AGT exist under culture conditions, Ang II production in both HTR-8/SVneo and BeWo cells may be low.

The production of Ang 1-7 by BeWo cells in the absence of prorenin raises the interesting possibility that non-renin proteases exist, which can form Ang peptides within human intrauterine tissues. As far as we know this possibility has not been investigated, although a non-renin angiotensin system (chymase) has been described in the heart where Ang II plays a key role in cardiac hypertrophy (128).

In conclusion, we have shown that two cell lines derived from trophoblast have only some of the now well-described RAS pathways and the components of the RAS pathways that they do possess are strikingly different, as is their response to cAMP. Thus these two cell lines could be used to determine how the various placental RAS pathways regulate angiogenesis, invasion and proliferation, all of which are key features of placentation. Using HTR-8/SVneo cells we are able to study the cAMP effects on the renin/Ang II/AT₁R pathway, while further study of the RAS pathway in BeWo cells may lead to identification of other neutral proteases capable of forming Ang II, as well as providing us with the opportunity to investigate the Ang 1-7/MAS receptor pathway in isolation from effects of Ang II. Neither cell line however, truly represents the placental RAS, as all RAS genes and proteins are present in both the early and late gestation human placentae (13, 73).

Chapter Three

The effects of cyclic AMP, sex steroids and global hypomethylation on the expression of genes controlling the activity of the renin–angiotensin system in placental cell lines

In this chapter, we investigated how *REN* and prorenin protein production were regulated within the two trophoblast cell lines. As shown in Chapter Two, both trophoblast cell lines do not express all the components of the RAS. We postulated that the BeWo cells may be heavily methylated and therefore could not express *REN*. We aimed to stimulate *REN* by BeWo cells, and explore renin/prorenin actions on the RAS pathway in the HTR–8/SVneo cell line. We used several agents; cAMP, Estradiol–17 β , medroxyprogesterone acetate and AZA, which may stimulate *REN* expression.

3.1 ABSTRACT

The placental renin–angiotensin system (RAS) is involved in placentation. We have shown that prorenin mRNA (*REN*) is expressed in a first trimester trophoblast cell line (HTR–8/SVneo) but not in a choriocarcinoma cell line (BeWo). We attempted to stimulate RAS expression in these cells by cAMP, 5′–aza–2′–deoxycytidine (AZA; an inhibitor of methylation), cAMP and AZA combined, and the sex steroids medroxyprogesterone acetate (MPA) and estradiol–17 β (E_2) with and without cAMP. RAS mRNAs were measured by qPCR and prorenin concentration in supernatants measured by an ELISA. In HTR–8/SVneo cells, all treatments increased *REN* expression compared to controls and cAMP + AZA combined was more effective than either treatment alone. Prorenin levels in supernatants were similarly upregulated. In HTR–8/SVneo cells, angiotensinogen (*AGT*) mRNA expression was increased by MPA + E_2 either with or without cAMP. *AGT* expression was also significantly increased by AZA. BeWo cells did not express *REN* or prorenin and it was not inducible with any treatment. *AGT* expression was significantly increased with AZA, the combination of cAMP + AZA, and MPA + E_2 + cAMP treatments. Since cAMP, AZA, cAMP and AZA combined, or MPA and E_2 with and without cAMP in HTR–8/SVneo cells, a cell line most similar in its RAS expression to the *in vivo* placenta, these factors may affect placental RAS activity. Surprisingly, these treatments also induced *AGT* expression in BeWo cells. Whether they are involved in regulating *AGT* in choriocarcinomas *in vivo* remains to be determined.

3.2 INTRODUCTION

Studies have shown that the renin angiotensin system (RAS) is involved in the regulation of trophoblast invasion (111) and spiral artery remodelling (12) and consequently, may play a role in implantation and placentation.

Within the placenta, renin acts on angiotensinogen (AGT) to form angiotensin (Ang) I, which is catalysed by angiotensin converting enzyme (ACE) to form Ang II. Ang II can act either through the angiotensin II type 1 (AT₁R) or type 2 (AT₂R) receptor or it can be converted by ACE2 to Ang 1–7. Potentially, the placental RAS could have a number of actions, some of which may be cooperative and some antagonistic. RAS pathway genes and proteins have been detected in both the early and late gestation human placentae (13, 73, 111).

We have studied two established trophoblast cell lines that are commonly used for studying placental function: HTR–8/SVneo (a transformed first trimester extravillous trophoblast cell line developed by Charles Graham, Ontario, Canada (117)) and BeWo cells (derived from a choriocarcinoma (118)), postulating that these cell lines would show similar patterns of RAS expression to freshly obtained human placenta. This was not the case however. HTR–8/SVneo cells expressed prorenin (*REN*), angiotensinogen (*AGT*), the prorenin receptor (*ATP6AP2*), ACE (*ACE1*) and AT₁R (*AGTR1*), but not AT₂R (*AGTR2*), ACE2 (*ACE2*) nor the Ang 1–7 receptor, MasR (*MAS1*), while BeWo cells only expressed *AGT*, *ACE1*, *ACE2* and *MAS1* and not *REN* (129). Although the expression of RAS genes and proteins in the human placenta and these trophoblast cell lines has been established, the mechanisms regulating their expression are unknown.

Another difference between BeWo and HTR-8/SVneo cells is in their response to cAMP. When stimulated with cAMP, *REN* expression and prorenin protein synthesis was induced in HTR-8/SVneo cells, but not in BeWo cells (129). This was interesting, given that cAMP is a classical stimulator of *REN* expression, as seen in juxtaglomerular (99) and primary decidual cells (101). We postulated that BeWo cells lack the *REN* response to cAMP stimulation, as they are derived from a choriocarcinoma and have a high degree of polyploidy and therefore could be heavily methylated in order to be viable. Therefore, in BeWo cells, hypermethylation of DNA may prevent cAMP from binding to the cAMP response element (CRE), which is responsible for the regulation of *REN* gene transcription (115, 116). We postulated that a genome wide inhibitor of methylation such as 5'-aza-2'-deoxycytidine (AZA) might therefore initiate *REN* expression and prorenin secretion in BeWo cells, particularly in response to cAMP. AZA inhibits DNA methyltransferases, resulting in global hypomethylation as cells continue to divide. Hypomethylation could increase transcriptional protein binding at the CRE (130).

Furthermore, if as we proposed, AZA did stimulate *REN* expression through global hypomethylation of BeWo DNA, then AZA might also have a similar effect in HTR-8/SVneo cells, so that not only do cAMP (as previously shown, (129)) and AZA stimulate *REN* expression and prorenin secretion but the combination of cAMP and AZA together would be additive, having a greater effect on *REN* expression in HTR-8/SVneo cells than that seen with either agent alone.

In normal pregnancy, the developing blastocyst secretes human chorionic gonadotropin (hCG). hCG levels are maximal at about 6 weeks gestation, at which time maternal plasma prorenin levels are also at a maximum. hCG administration is

associated with increased plasma prorenin levels. hCG maintains the corpus luteum, and maintains production of both estrogen and progesterone (107, 108). The hCG induced increase in plasma prorenin in early pregnancy also comes from the corpus luteum (131). In addition, hCG stimulates placental production of prorenin, as does progesterone (107, 109). Therefore, we proposed that the combined administration of estrogen and progesterone, hormones essential for the establishment and maintenance of human pregnancy, could stimulate renin expression by both HTR-8/SVneo and BeWo cells.

In this study, therefore, we compared the effects of cAMP with the global hypomethylating agent, AZA, and their combined effects on expression of RAS genes and on prorenin secretion by the placentally derived cell lines, HTR-8/SVneo and BeWo. We also investigated the effects of cAMP, estradiol and progesterone, in combination, on *REN* expression and prorenin production and on the expression of other RAS genes.

3.3 MATERIALS AND METHODS

Trophoblast Cell Culture

HTR-8/SVneo and BeWo cells were cultured in phenol red-free RPMI-1640 or DMEM/F-12, respectively, supplemented with 15 mM HEPES, 1.2 g/L NaHCO₃, 1 mg/mL L-glutathione reduced, 0.1 g/L albumin fraction V, 0.65 µg/mL aprotinin, 10% fetal bovine serum and 40 µg/mL gentamicin. Cells were seeded at a density of 200,000 cells, in each well of a 6 well plate with 2 mL of incubation medium. Cells were allowed to settle for 24 h, after which the media was changed, cells were then treated with one of the following:

- 1) 150 μ M 8-bromo-cAMP (cAMP, Sigma-Aldrich, St. Louis, MO, USA);
- 2) 7.5 μ M 5'-aza-2'-deoxycytidine (AZA, Sigma-Aldrich);
- 3) 150 μ M cAMP with 7.5 μ M AZA;
- 4) 1 μ M medroxyprogesterone acetate (MPA, Sigma-Aldrich) with 10 nM estradiol-17 β (E₂, Sigma-Aldrich);
- 5) 1 μ M MPA with 10 nM estradiol-17 β and 150 μ M cAMP; or
- 6) Incubation medium alone (vehicle).

In a previous study we used 300 μ M 8-bromo cAMP (129) to induce prorenin release. To ensure that a significant increase in prorenin mRNA and protein levels would be seen when cAMP treatment was combined with AZA or E₂ and MPA compared with cAMP treatment alone, we dropped the cAMP dose by half, and treated the cells with 150 μ M cAMP. The concentration of AZA is within the range normally used in cell culture experiments. E₂ and MPA concentrations were selected based on previous studies examining the effects of these hormones in human endometrial stromal cells (132) and are similar to what trophoblast cells would be exposed to *in vivo* during normal human pregnancy (133). Since placental estrogen and progesterone levels increase in pregnancy in a coordinated fashion (133) we chose to only assess the effects of these hormones in combination, to better mimic the *in vivo* environment. Incubation media were collected at 24 and 48 h and cells were harvested. Cells and media were snap frozen in liquid nitrogen for subsequent protein and mRNA analyses. There were 3 independent experiments and in each experiment the treatments were in triplicate. Cell viability was assessed by an LDH cytotoxicity assay for all treatments as well as by measuring RNA stability and

quality (data not shown). None of the treatments used in this study affected cell viability at 48 h.

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

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Each sample was examined for RNA integrity, DNase treated (Qiagen N.V., Hilden, Germany), spiked with a known amount of Alien RNA (Agilent Technologies, La Jolla, CA, USA; 10^7 copies per microgram of total RNA) and reverse transcribed using Superscript III RT kit (Invitrogen). The alien qRT PCR inhibitor alert system (Agilent Technologies) served as a reference for internal standardization (119). RAS primers for qPCR have been described previously (13, 25, 73). Messenger RNA abundance was calculated using the $\Delta\Delta_{CT}$ method and relative abundance was calculated relative to Alien mRNA and a calibrator sample (term placenta).

Measurement of prorenin protein by ELISA

Prorenin levels in culture media were measured using the Human Prorenin ELISA kit (Molecular Innovations Inc; Novi, MI) according to the manufacturer's instructions. The methods and reagents used have been previously described (13, 25, 73). Samples were assayed in duplicate and on the same ELISA plate. Therefore there was no inter-assay variability. Intra-assay coefficient of variation was 10.8%.

Data Analyses

A one-way ANOVA with Bonferroni post hoc analysis was used to determine if there were any differences in RAS mRNA abundance and prorenin protein concentration in the supernatant between each treatment. GraphPad Prism (Prism version 6.0) was used for all analyses. Significance was set at $P < 0.05$.

3.4 RESULTS

cAMP stimulates prorenin in HTR-8/SVneo but not BeWo cells

In this study, we used 150 μM 8-bromo-cAMP as compared to the 300 μM used in our previous study (129). Despite this, cAMP significantly increased *REN* mRNA expression in HTR-8/SVneo cells at both 24 and 48 h incubation (both $P < 0.001$, Figure 3.1A). In supernatants from cultures of HTR-8/SVneo treated cells, there was a corresponding increase in prorenin protein concentrations at 48 h compared to vehicle ($P < 0.001$, Figure 3.1B).

In BeWo cells, cAMP was not able to induce *REN* expression or prorenin in the supernatant.

AZA alone, or in combination stimulates *REN* in HTR-8/SVneo cells

In order to determine whether *REN* expression could be stimulated in BeWo cells by inhibiting DNA methylation, the two trophoblast cell lines were treated with AZA. In HTR-8/SVneo cells, *REN* expression was significantly increased by treatment with AZA compared to vehicle at both 24 and 48 h incubation (both $P < 0.001$, Figure 3.1A) and AZA significantly increased the levels of prorenin in the supernatant ($P < 0.001$, Figure 3.1B).

To test our hypothesis that cAMP and AZA act through different pathways to stimulate *REN* expression, we compared *REN* expression in cells treated with the combination of cAMP and AZA, to either treatment alone. We found that the combination of cAMP and AZA was more effective at stimulating *REN* expression in HTR-8/SVneo cells compared to vehicle or to cAMP or AZA treatment alone at both 24 and 48 h incubation ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.05$ and $P < 0.001$ respectively, Figure 3.1A). There were corresponding increases in prorenin concentrations in the supernatants from the treated HTR-8/SVneo cells, ($P < 0.001$, Figure 3.1B). Prorenin levels however were no greater than levels measured in supernatants treated with either cAMP or AZA alone.

In contrast, AZA, alone or in combination with cAMP failed to induce *REN* expression in BeWo cells and prorenin was not detected in the supernatant.

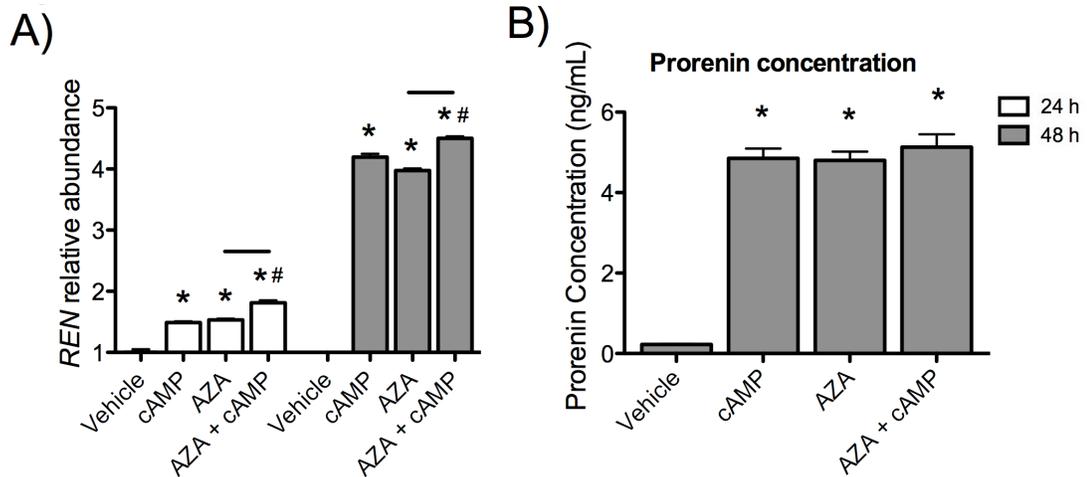


Figure 3.1 *REN* mRNA abundance in HTR-8/SVneo cells

(A) *REN* mRNA was expressed in HTR-8/SVneo cells, and increased with cAMP, AZA and the combined cAMP + AZA treatment compared to vehicle treated controls at both 24 and 48 h incubation. At 24 and 48 h incubation, the combined cAMP + AZA treated HTR-8/SVneo cells had higher *REN* expression than cAMP or AZA treated cells. (B) Prorenin protein levels measured in the supernatant at 48 h incubation were significantly higher in cAMP, AZA and cAMP + AZA treated cells compared to the controls, however there was no significant differences in prorenin protein levels between the three treatments ($n = 3$). * denotes significant difference from vehicle treated controls at the same incubation time ($P < 0.001$); # denotes significant difference from cAMP treated cells at the same incubation time ($P < 0.001$); the solid line denotes a significant difference in AZA treatments with and without the addition of cAMP ($P < 0.001$).

Effects of MPA and E₂, alone or in combination with cAMP on prorenin mRNA and protein levels

The combination of MPA & E₂ significantly increased HTR-8/SVneo cell expression of *REN* compared to vehicle at both 24 and 48 h ($P<0.001$) and was more effective at stimulating *REN* expression compared with cAMP treatment ($P<0.01$ and $P<0.001$, respectively, Figure 3.2A). Prorenin concentration in the supernatants of the MPA and E₂ treated cells was greater than vehicle ($P<0.001$, Figure 3.2B), however there was no significant difference between MPA, E₂ or cAMP treated cells.

The MPA + E₂ + cAMP mix significantly increased *REN* expression in HTR-8/SVneo cells compared to vehicle at 24 and 48 h (both $P<0.001$, Figure 3.2A) and was more effective at stimulating *REN* expression than cAMP treatment alone ($P<0.001$, Figure 3.2A) but not more effective than the combined treatment of MPA and E₂ alone. After 48 h incubation, prorenin levels in the supernatants of HTR-8/SVneo cells treated with MPA + E₂ + cAMP was significantly greater than vehicle ($P<0.001$, Figure 3.2B), which corresponds with their greater *REN* gene expression levels. There was no difference however, between prorenin levels in MPA and E₂ treated, MPA + E₂ + cAMP treated, and cAMP treated cells.

In BeWo cells, MPA + E₂, or in combination with cAMP, failed to induce prorenin gene expression or prorenin protein production.

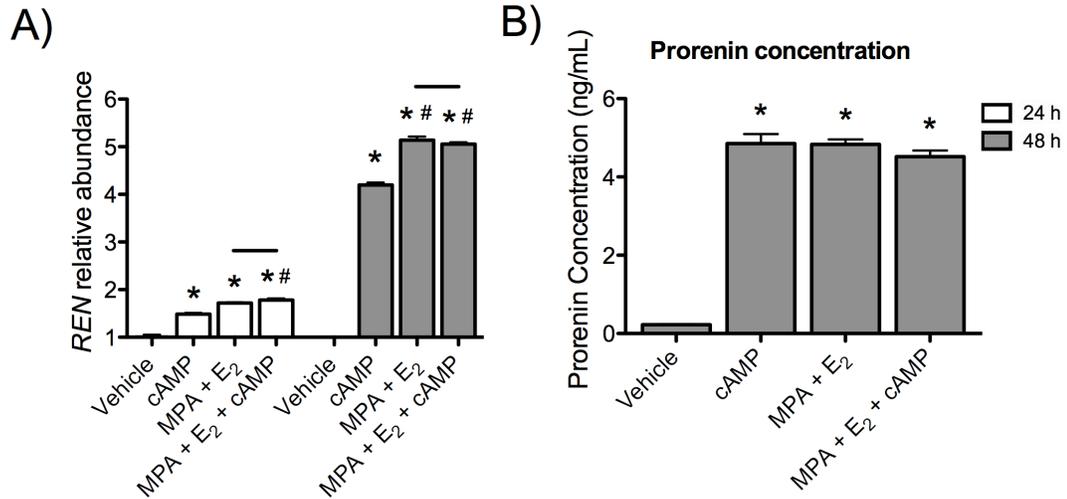


Figure 3.2 *REN* mRNA abundance in HTR-8/SVneo cells

(A) *REN* mRNA was expressed in HTR-8/SVneo cells, and increased with cAMP, MPA + E₂ and MPA + E₂ + cAMP treatment compared to vehicle treated controls at both 24 and 48 h incubation. At 24 h incubation, the combined MPA + E₂ + cAMP treated HTR-8/SVneo cells had higher *REN* expression than cAMP treated cells. Whilst at 48 h incubation, both MPA + E₂ and MPA + E₂ + cAMP treated cells had significantly higher *REN* expression than cAMP treated cells. (B) Prorenin protein levels measured in the supernatant at 48 h incubation were significantly higher in cAMP, MPA + E₂ and MPA + E₂ + cAMP treated cells compared to the controls, however there was no significant differences in prorenin protein levels between the three treatments (n = 3). * denotes significant difference from vehicle treated controls at the same incubation time ($P < 0.001$); # denotes significant difference from cAMP treated cells at the same incubation time ($P < 0.001$).

Effects of cAMP and AZA on the expression of other genes in the placental RAS pathways of HTR–8/SVneo and BeWo cells

cAMP treatment alone did not alter the expression of any of the other RAS genes studied in HTR–8/SVneo cells and had no effect on any of the RAS genes expressed by BeWo cells.

In contrast, AZA treatment of HTR–8/SVneo cells increased *AGT* mRNA abundance compared to vehicle at 24 h ($P<0.01$, Figure 3.3A). There were no significant effects however, of AZA on *ATP6AP2*, *ACE1* and *AGTR1* expression. In HTR–8/SVneo cells, combined cAMP and AZA treatment was more effective at stimulating *AGT* expression compared to vehicle ($P<0.001$) and cAMP treatment at 24 h ($P<0.05$, Figure 3.3A). There were no significant effects of the combined cAMP and AZA treatment on *ATP6AP2*, *ACE1* and *AGTR1* expression.

In BeWo cells, treatment with AZA increased *AGT* mRNA abundance at 24 h incubation compared to vehicle ($P<0.01$, Figure 3.4A). This effect was reversed by the combination of cAMP and AZA treatment ($P<0.05$, Figure 3.4A) and was no longer different from controls. Neither AZA nor cAMP had any effect on *ACE1*, *ACE2* or *MAS1* mRNA abundances in BeWo cells.

Effects of MPA and E₂, alone or in combination with cAMP the expression of other genes in the placental RAS pathways of HTR–8/SVneo cells and BeWo.

Combined MPA and E₂ treatment significantly increased expression of *AGT* at 24 h incubation ($P<0.05$) in HTR–8/SVneo cells compared to vehicle (Figure 3.3B). Like MPA and E₂ treatment alone, MPA + E₂ + cAMP combined significantly

increased HTR-8/SVneo cell expression of *AGT* compared to vehicle at 24 h incubation ($P < 0.05$, Figure 3.3B) but this was not seen at 48 h.

In BeWo cells, there was no effect of MPA and E_2 treatment alone on RAS gene expression however, the combined MPA + E_2 + cAMP treatment significantly increased *AGT* expression at 24 h incubation compared to vehicle ($P < 0.05$, Figure 3.4B). This effect was no longer observed at 48 h.

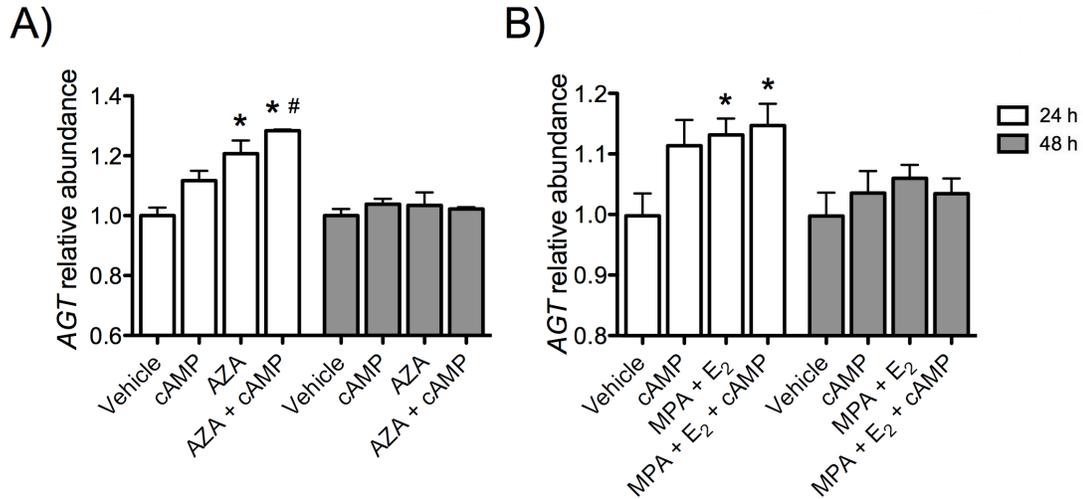


Figure 3.3 *AGT* mRNA abundance in HTR-8/SVneo cells

(A) At 24 h incubation, *AGT* mRNA was expressed in HTR-8/SVneo cells and increased with AZA and the combined cAMP + AZA treatment compared to vehicle treated controls. Also at 24 h incubation, cAMP + AZA treated HTR-8/SVneo cells had significantly greater *AGT* expression compared to HTR-8/SVneo cells treated with cAMP alone. At 48 h incubation, there was no significant difference between the expression of *AGT* in the vehicle, cAMP, AZA or the combined cAMP + AZA treated HTR-8/SVneo cells. (B) *AGT* mRNA in HTR-8/SVneo cells was increased with the combined MPA + E₂ and the MPA + E₂ + cAMP treatments at 24 h incubation compared to vehicle treated controls from the same incubation time. At 48 h incubation, there was no significant difference between the expression of *AGT* in the vehicle, cAMP, MPA + E₂ or the MPA + E₂ + cAMP treated HTR-8/SVneo cells (n = 3). * denotes significant difference from vehicle treated controls at the same incubation time ($P < 0.05$); # denotes significant difference from cAMP treated cells at the same incubation time ($P < 0.05$).

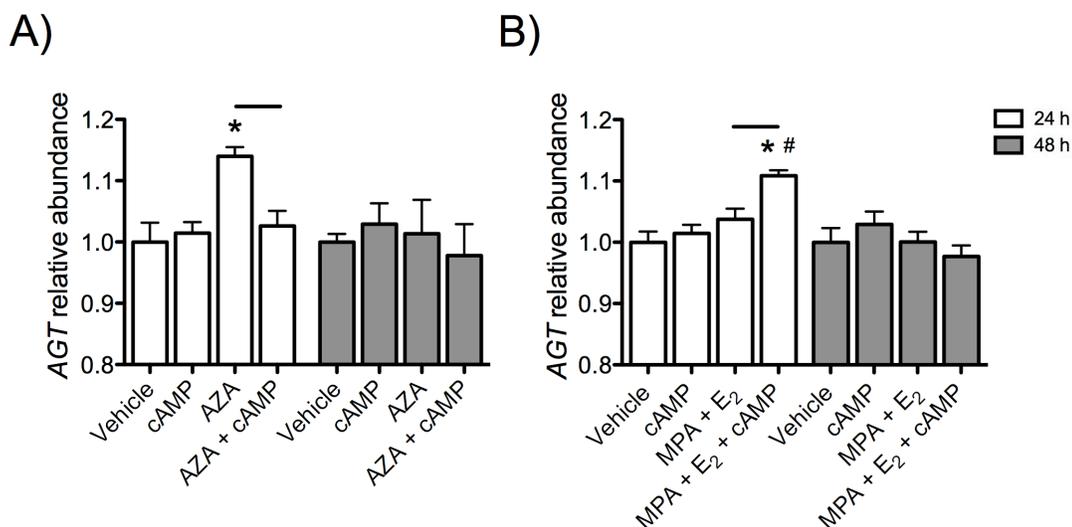


Figure 3.4 AGT mRNA abundance in BeWo cells

(A) At 24 h incubation, *AGT* expression was greater in AZA treated BeWo cells compared to vehicle treated controls, whereas *AGT* expression in cAMP or AZA + cAMP treated cells were not different from the control. Therefore AZA treated BeWo cells had significantly greater *AGT* expression compared to BeWo cells treated with the combined cAMP + AZA treatment. At 48 h incubation, there was no significant difference between the expression of *AGT* in the vehicle, cAMP, AZA or the combined cAMP + AZA treated BeWo cells. (B) *AGT* mRNA in BeWo cells was significantly increased with the combined MPA + E2 + cAMP treatment at 24 h incubation compared to vehicle treated controls and since there was no significant difference between *AGT* expression in the vehicle, cAMP or the MPA + E2 treated BeWo cells, these cells also had significantly lower *AGT* expression than the MPA + E2 + cAMP treated BeWo cells. At 48 h incubation, there was no significant difference between the expression of *AGT* in the vehicle, cAMP, MPA + E2 or the MPA + E2 + cAMP treated BeWo cells ($n = 3$). * denotes significant difference from vehicle treated controls at the same incubation time ($P < 0.05$); # denotes significant difference from cAMP treated cells at the same incubation time ($P < 0.05$); the solid line denotes significant difference in AZA or MPA + E2 treatments with and without the addition of cAMP ($P < 0.05$).

3.5 DISCUSSION

Our previous work showed that cAMP stimulated prorenin mRNA and protein levels in HTR–8/SVneo cells and supernatants but not BeWo cells, in which *REN* expression could not be detected (129). HTR–8/SVneo cells express *REN* and produce prorenin as well as expressing other RAS genes likely to regulate Ang II/AT₁R interactions and respond to cAMP, like renal renin–secreting cells. They are more similar to early gestation placentae and are therefore useful for studying effects of renin/ACE/Ang II/AT₁R on cell function (129). BeWo cells express the ACE2/Ang 1–7/Mas pathway, and therefore are useful for studying the effects of this pathway on trophoblast function.

In the present study, we wanted to find out if *REN* expression could be induced in BeWo cells and further stimulated in HTR–8/SVneo cells by reducing gene silencing through global hypomethylation using AZA, or by combined treatment with the two major hormones of pregnancy, estrogen and progesterone. We stimulated cells with cAMP alone to confirm our previous findings and to quantitate the effects of these novel treatments (relative to the cAMP induced effects) on *REN* and other RAS genes by both cell lines.

We demonstrated that cAMP, AZA, MPA and E₂ all increased *REN* expression and prorenin levels in HTR–8/SVneo cells but not in BeWo cells. On the other hand, treatment of HTR–8/SVneo cells with either AZA or MPA and E₂ had effects on renin that were independent of any effects of cAMP and in fact AZA treatment amplified the stimulatory effect of cAMP on *REN* expression. This amplified cAMP action on *REN* mRNA however, did not translate into increased levels of prorenin protein. The additive effect of the combined AZA and cAMP

treatment was minimal, suggesting that AZA is likely acting through the cAMP/CRE pathway, possibly by allowing better access for cAMP and the associated binding factors to bind to the CRE of the *REN* gene, as opposed to AZA acting independently of cAMP (130).

This is the first report that global hypomethylation of a placental cell line is associated with increased *REN* expression and prorenin production. It is also the first report that the combination of E₂ and MPA stimulates *REN* mRNA and prorenin production by trophoblast cells.

We showed, as postulated, that E₂ and MPA in combination stimulate placental *REN* expression and prorenin secretion in HTR-8/SVneo cells. While a role for estrogen and progesterone in regulating the placental RAS has been suggested, there are no conclusive studies to support this. In this study we have been able to successfully demonstrate that estrogen and progesterone are able to stimulate *REN* and *AGT* mRNA as well as prorenin protein by trophoblast cells *in vitro*. Further studies are necessary to determine whether the human placental RAS *in vivo* responds to sex steroids in a similar manner.

The addition of cAMP to the MPA and E₂ mixture had no additive effect on *REN* mRNA abundance; that is, there was no significant difference between treatments after addition of cAMP. Furthermore, MPA and E₂ together, with or without the addition of cAMP, were more effective at stimulating *REN* expression than cAMP alone. This suggests that MPA and E₂ most likely act by stimulating cAMP and, as such, additional cAMP did not further stimulate renin expression in HTR-8/SVneo cells.

All of the treatments used in this study stimulated prorenin mRNA and protein levels but only in HTR–8/SVneo cells. We propose that the reason we are unable to induce renin in BeWo cells is due to the *REN* gene in these cells not being transcribable or, that the region of the mRNA our primers are designed against is mutated or missing. In addition, we were unable to detect *AGTR1* or *AGTR2* mRNAs in BeWo cells, which may suggest that this cancer cell line has undergone significant physiological and molecular changes.

It is important to note that the lack of translation of differences in *REN* mRNA abundance to differences in prorenin protein in the media may be because all treatments resulted in maximal prorenin production. On the other hand, active renin production in these supernatants was not measured and it is possible that prorenin in these cells may have undergone activation to renin, through the release of proteases by global hypomethylation, or through stimulation by cAMP and steroid hormones, which could act on the constitutively expressed prorenin (134, 135). The ELISA we use is targeted specifically to the pro–segment of prorenin and does not detect active renin.

The treatments used in this study were selected primarily to examine their potential effects on placental *REN* and prorenin protein however we did observe significant increases in *AGT* expression in both cell lines in response to both AZA and the sex steroids. Interestingly, these increases were only transient and were no longer evident after 48 h. Whether this transient effect is due to negative feedback from the concomitant production of high levels of prorenin in HTR–8/SVneo cells, as seen in other cell types (136), is unknown. It seems more likely however, since we see similar affects in BeWo cells which do not express renin, that it is more likely

due to the rapid turnover of *AGT* mRNA to protein or that the treatments have a different mechanism of action to that against renin and require additional or prolonged stimuli to have a sustained effect.

Interestingly, both the *AGT* and *REN* gene lie on chromosome 1, however analysis has shown that there is a great genetic distance between the two genes (137). Nevertheless, AZA administration results in global hypomethylation and could cause activation of both *AGT* and *REN*. Indeed, we have demonstrated increased *AGT* and *REN* expression in HTR-8/SVneo but only increased *AGT* expression in BeWo cell lines. The combination of AZA and cAMP also had a small additive effect on *AGT* mRNA abundance in HTR-8/SVneo cells, similar to that seen in *REN*, but it blocked the effects of AZA on *AGT* in BeWo cells (Figures 3.3 & 3.4). This also supports our hypothesis that BeWo cells are physiologically abnormal.

Estrogen has also been shown to induce angiotensinogen release in rats (138). In humans, oral contraceptives, which contain synthetic estrogen, cause a dose dependent increase in plasma angiotensinogen levels (139). In addition, estrogen replacement therapy also increases angiotensinogen levels (140) due to upregulation of hepatic *AGT*. Thus it is perhaps not surprising that *AGT* expression was greater in MPA and E₂ treated cells compared to vehicle in HTR-8/SVneo cells.

In conclusion, we have shown that the two trophoblast cell lines respond very differently to a wide range of treatments, and despite the effectiveness of these agents at stimulating prorenin expression in HTR-8/SVneo cells, they were unable to induce *REN* expression in BeWo cells. As a result, we propose that AZA, progesterone and estradiol stimulate *REN* expression and prorenin protein in trophoblast cells through a cAMP-dependent pathway, although they target different

aspects of this pathway. AZA, likely acting through hypomethylation of the region around the CRE, probably allows for better binding of cAMP and other associated binding proteins, whereas progesterone and estradiol probably act by stimulating cAMP release.

Additive effects of these treatments on the placental RAS pathway could be proposed to further enhance the activity of the placental RAS through provision of more prorenin binding sites, stimulation of placental *AGT* expression and upregulation of *AGTR1*. The role(s) of epigenetic factors such as gene methylation and the sex steroids on the activity of the placental RAS *in vivo* remain to be determined, but we have demonstrated that the activity of the placental RAS, which is thought to play a role in placentation, is regulated by sex steroids and is subject to epigenetic modification.

Chapter Four

Fetal Sex Affects Expression of Renin–Angiotensin System Components in Term Human Decidua

This chapter aimed to establish RAS gene expression, prorenin protein and Ang peptides profile in isolated term decidua and in decidual explants. Previous work in our laboratory demonstrated a sex specific difference in *REN* expression in decidual tissue isolated before the onset of labour. Since decidual RAS components may also escape into the maternal circulation, they could affect maternal cardiovascular and renal function and possibly placental perfusion. We postulated that the sex specific differences in decidual *REN* expression described in this chapter were due to an effect of maternal or fetal sex hormones. If this were the case then culture of decidual explants on a medium free from sex hormones would abolish the sex-specific differences in *REN* expression. This work may provide some insight into how fetal sex could affect pregnancy outcome.

4.1 ABSTRACT

The maternal decidua expresses the genes of the renin–angiotensin system (RAS). Human decidua was collected at term either before labour (*i.e.*, caesarean delivery) or following spontaneous labour. The mRNA for prorenin (*REN*), prorenin receptor (*ATP6AP2*), angiotensinogen (*AGT*), angiotensin converting enzyme 1 and 2 (*ACE1 and ACE2*), angiotensin II type 1 receptor (*AGTR1*) and angiotensin 1–7 receptor (*MAS1*) were measured by quantitative real–time RT–PCR (RT–qPCR). Decidual explants were cultured in duplicate for 24 and 48 hours, and all RAS mRNAs, and the secretion of prorenin, angiotensin II (Ang II) and angiotensin 1–7 (Ang 1–7) was measured using RT–qPCR, ELISA and radioimmunoassay, respectively. In decidua collected before labour *REN* mRNA levels were higher if the fetus was female. In addition, *REN*, *ATP6AP2*, *AGT*, and *MAS1* mRNA abundance was greater in decidual explants collected from women carrying a female fetus, as was prorenin protein. After 24 h *ACE1* mRNA was higher in decidual explants from women with a male fetus, whereas after 48 h both *ACE1* and *ACE2* mRNA was higher in decidual explants from women with a female fetus. Ang II was present in all explants, but Ang 1–7 levels often registered below the lower limits of sensitivity for the assay. After labour decidua, when compared to non–labouring decidua, demonstrated lower *REN* expression when the fetus was female. Therefore, the maternal decidual RAS is regulated in a sex–specific manner, suggesting that it may function differently when the fetus is male than when it is female.

4.2 INTRODUCTION

It is becoming increasingly apparent that the outcome of pregnancy in relation to the health of the fetus and survival of the neonate is influenced by fetal sex. Several studies have shown that male fetuses have a higher risk of adverse pregnancy outcomes including spontaneous abortions (53), miscarriages later in pregnancy (54), stillbirths (55, 56), premature rupture of membranes (PROM) and spontaneous preterm birth (57-60), gestational diabetes (61), and delivery by caesarean section (59, 62, 63). In term deliveries, a higher proportion of preeclamptic pregnancies carry a male fetus (60, 61), whereas in preterm deliveries the proportion of females from preeclamptic pregnancies is higher (60, 62). Female babies are also more likely to be growth restricted than are males (59, 61). The causes of these differences are unknown, but it has been suggested that intrauterine tissues (and in particular the placenta) regulate fetal growth and survival in a sex-specific manner (52).

Renin is an enzyme secreted by the kidney that hydrolyzes angiotensinogen (AGT) to produce angiotensin I (Ang I), which in turn is cleaved by angiotensin converting enzyme (ACE) to form angiotensin II (Ang II). The major actions of the renin-angiotensin system (RAS) are mediated via binding of Ang II to the angiotensin II type 1 receptor (AT₁R). Ang II exerts opposing actions via the Ang II type 2 receptor (AT₂R) (67). An ACE homologue – ACE2 – can terminate the actions of Ang II by converting it to Ang 1-7, which has effects that oppose those of Ang II, and exerts these by binding to the proto-oncogene receptor (Mas) (113).

Until recently, the precursor of renin, prorenin, was considered to have little biological activity, despite the fact that its circulating levels are 10 times higher than

those of renin (114). The discovery of a (pro)renin receptor ((P)RR) that binds both renin and prorenin has changed this notion. Prorenin bound to (P)RR is active and can cleave AGT to Ang I. In addition, binding of prorenin to the (P)RR can induce intracellular signalling in its own right (74, 84).

Although the RAS is classically associated with the control of blood pressure, tissue-specific RASs have now been described in a number of organs not necessarily involved in blood pressure control (141-144). We have identified most of the RAS components in term human decidua, placenta, myometrium and fetal membranes (13, 73). Although RAS proteins are found in human fetal membranes, expression of some of the RAS genes in these membranes is very low. For example, amnion contains an abundance of renin despite low renin (*REN*) mRNA (73). Interestingly, we have shown that decidua, a maternal tissue, expresses all of the known RAS genes (13, 73). Locally-produced Ang II might affect the decidual microvasculature either by promoting angiogenesis or by altering vascular tone. Since decidual renin can escape into the maternal circulation (145), the decidual RAS may also play a role in maternal cardiovascular and renal homeostasis. Through these extrauterine and intrauterine actions, the decidual RAS could affect fetal development by altering embryonic access to nutrients.

Human recombinant renin has been shown to stimulate decidual production of prostaglandin-endoperoxide synthase 2 (PTGS2, also known as COX-2) (24), a key enzyme involved in the initiation of labour. The release of prorenin from the decidua is constitutive and the decidua is the major source of prorenin within the human uterus (13, 146). The decidual RAS could therefore play a role in the initiation of labour, since the decidua is immediately adjacent to the myometrium.

We have developed a human decidual explant model to study the decidual RAS. This explant model provides the closest possible approximation to *in vivo* because freshly isolated tissue is used. This means that all cell types are present and in the same proportions at the end of 48 hours of incubation as at the beginning. This is because the time elapsed is insufficient for any one cell type to outgrow the others. Furthermore there is no evidence of increased cell death at 48 h.

The aims of the present study were (i) to determine if the expression of decidual RAS genes was affected by fetal sex; (ii) to define the expression of RAS genes in term human decidua collected before the onset of spontaneous labour by maintaining decidua in explant culture for 24 and 48 h *ex vivo* and (iii) to determine if decidual explants could be used to study the control of expression of RAS genes and the secretion of prorenin and the angiotensin peptides Ang II and Ang 1–7. We also set out to determine if the expression of decidual RAS genes is altered with labour.

4.3 METHODS

Tissue Collection

Decidual samples were collected as described previously (13, 73). All samples were collected from uncomplicated singleton pregnancies in women aged 19 to 39. Decidual samples at term (37–40 weeks gestation) delivered by elective caesarean section in the absence of labour were collected for the measurement of decidual gene expression (n=21). Additional samples were collected for decidual explants (n=6) and decidua was collected after spontaneous labour and vaginal delivery (38–41 weeks gestation) for measurement of decidual RAS gene

expression (n=23). Women treated with non-steroidal anti-inflammatory drugs, or having a history of infection, chorioamnionitis, asthma, preeclampsia or undergoing induction of labour were excluded. Informed consent was obtained from all participants, and the study was approved by the Hunter Area Research Ethics Committee and the University of Newcastle Human Research Ethics Committee. The fetal membranes and attached decidua parietalis were isolated as a whole, apart from a 2 cm border at the edge of the placenta. Amnion was peeled from the choriondecidua, and chorion laevae were separated from the decidua by sharp dissection as described previously (147). Decidual tissue was then used in the decidual explant study or snap frozen in liquid nitrogen for subsequent RNA analyses.

Decidual Explant Culture

Entire excised deciduas were washed in tissue culture medium (phenol red-free DMEM/F-12 supplemented with 15 mM HEPES, 1.2 g/L NaHCO₃, 1 mg/mL L-glutathione reduced, 0.1 g/L albumin fraction V, 0.65 µg/mL aprotinin, 10% fetal bovine serum, 40 µg/mL gentamicin) and dissected into approximately 0.25 cm² pieces. Several pieces of decidua were selected randomly, blotted and weighed. One hundred milligrams of decidua were placed into each well of a 6 well plate with 2 mL of incubation medium. Decidual tissues and supernatants were collected after 24 and 48 h and snap frozen in liquid nitrogen for subsequent protein and mRNA analyses. Each experiment was conducted, in duplicate, using 6 separate decidual samples (3 from women carrying a male and 3 from women carrying a female baby).

Cell viability was verified by measuring lactate dehydrogenase (LDH) release after incubation, as well as by measuring RNA stability and quality (data not shown).

The media from decidual explant cultures were collected into EDTA tubes, containing a protease inhibitor cocktail (Sigma; St. Louis, MO) and Ang II and Ang 1–7 concentrations were measured by radioimmunoassay (RIA) at ProSearch International Australia (Melbourne). Prorenin in the incubation medium was measured using the Human Prorenin ELISA kit (Molecular Innovations Inc; Novi, MI).

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

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen; Carlsbad, CA). RNA samples were treated with DNase (Qiagen N.V., Hilden, Germany) before being reverse transcribed using a Superscript III RT kit with random hexamers (Invitrogen). qPCR was performed in an Applied Biosystems 7500 Real Time PCR System using SYBR Green for detection. Each reaction contained 5 μ l of SYBR Green PCR master mix (Applied Biosystems; Carlsbad, CA), RAS primers as we have described previously (13, 73), cDNA reverse transcribed from 10 ng total RNA, and water to 10 μ l. Messenger RNA abundance was calculated relative to β -actin (*ACTB*) mRNA using the ΔC_T method as described previously (13, 73).

RIA of Ang II and Ang 1–7

Ang II was measured by direct RIA in plasma using the “delayed tracer addition” technique. Each sample of medium was equilibrated for 20 h at 4°C in a total volume of 300 µL with antibody raised in rabbit against angiotensin II N-terminally conjugated to bovine thyroglobulin. Monoiodinated ¹²⁵I-angiotensin II, 10,000 c.p.m in 100 µL, was added and allowed to equilibrate for a further 16 h at 4°C, after which the bound and free phases were separated using Dextran 10-coated charcoal and centrifugation. Sensitivity was 3.5 pg/mL. Intra and inter-assay coefficients of variation were 6.4% and 12%, respectively.

Ang 1–7 was assayed directly by RIA using an antibody raised in guinea pig to Ang 1–7 N-terminally conjugated to porcine thyroglobulin, and Ang 1–7 that had been mono-iodinated with ¹²⁵I antibody. Bound ¹²⁵I-Ang 1–7 was separated from free by Dextran 10-coated charcoal, and unbound c.p.m. were compared to serially diluted standard amounts of Ang 1–7. Sensitivity was 14 pg/mL. Cross-reactivities to Ang I, Ang II, Ang III and Ang IV were 0.11%, 0.04%, 0.53% and 0.03%, respectively. Intra- and inter-assay coefficients of variation were 4.5% and 10%, respectively.

Measurement of prorenin protein by ELISA

Prorenin in culture media was measured using the Human Prorenin ELISA kit (Molecular Innovations Inc; Novi, MI) according to the manufacturer’s instructions. Prorenin in each sample was captured by an antibody immobilised onto the surface of each well of the plate. A primary antibody specific for prorenin was then applied and the unbound fraction removed by washings. For subsequent

detection by means of colour development, a secondary antibody conjugated to horseradish peroxidase. 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added. After termination of the reaction with 4 M sulphuric acid, optical density was read at 450 nm. Prorenin concentration was directly proportional to colour development and was measured using a standard curve. Samples were assayed in duplicate. In our laboratory 1 ng/mL amniotic fluid prorenin measured using this technique generated $116 \text{ ng}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$ of Ang I from angiotensinogen present in nephrectomized sheep plasma used as the source of angiotensinogen substrate. All samples were assayed on one ELISA plate. Therefore there was no inter-assay variability. Intra-assay coefficient of variation was 7.3%.

Data Analysis

Decidual RAS gene expression data were tested using the non-parametric Mann-Whitney U test. Decidual explant RAS gene expression data were tested for normality using the skewness and kurtosis test. The data set was found to be not normally distributed, and so the data were logarithmically transformed. A univariate analysis of variance (ANOVA) with the patients' ID as a covariate was then used to test for differences in RAS mRNA abundance as a function of fetal sex and incubation time in decidual explants. An independent sample *t*-test was used to determine the effects of fetal sex on prorenin, Ang II and Ang 1-7 levels in the supernatant after 48 h *ex vivo*. The SPSS statistical package (SPSS for Windows, Release 17.0.0; Chicago) was used for all analyses. Significance was set at $P < 0.05$.

4.4 RESULTS

The interaction between fetal sex and labour on decidual RAS expression

The level of *REN* mRNA in maternal decidua from non-labouring women was higher if the fetus was female ($P=0.011$). Labour only affected decidual *REN* mRNA abundance when the fetus was female; decidual *REN* mRNA level was lower after labour ($P=0.027$), such that the significant sex-associated difference in *REN* mRNA abundance seen before labour was no longer present (Figure 4.1).

There were no sex-specific effects, nor were there any interactions between fetal sex and labour on the expression of decidual *AGT*, *ACE1*, *ACE2*, *AGTR1* and *ATP6AP2* mRNAs.

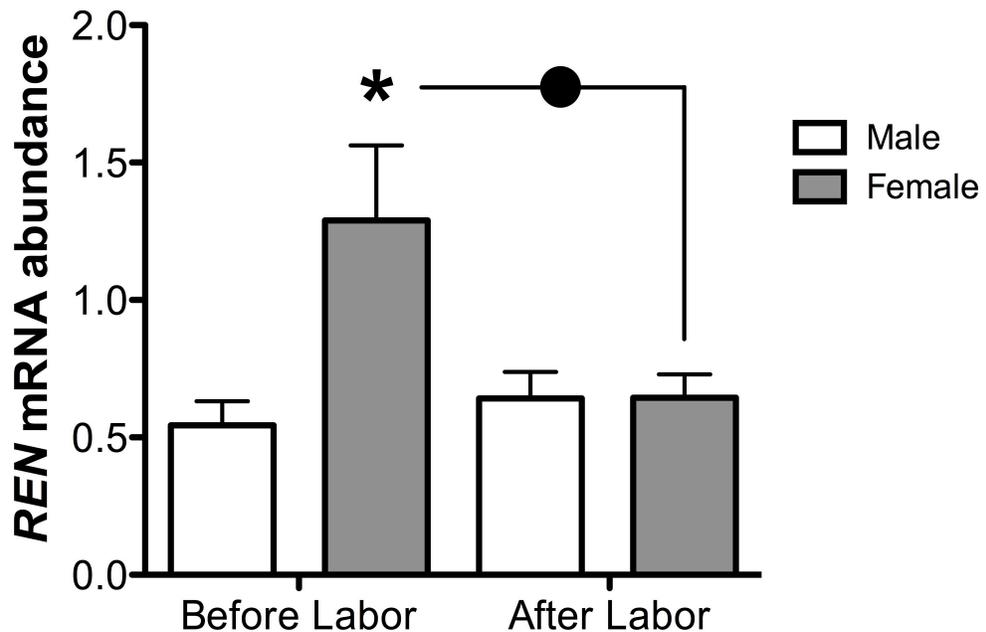


Figure 4.1 Sex differences in *REN* mRNA abundance in maternal decidua collected before or after labour.

Before labour decidual *REN* mRNA abundance was higher in women carrying a female fetus; after labour decidual *REN* levels no longer differed according to fetal sex because *REN* mRNA was lower in women with a female fetus after spontaneous labour compared with those not in labour. The n value was 21 for before labour decidual samples (n = 11 for women carrying a male fetus and n = 10 for women carrying a female fetus), and the n value was 23 for after labour decidual samples (n = 10 for women carrying a male fetus and n = 13 for women carrying a female fetus). The *closed circle* denotes the difference before and after labour ($P < 0.05$). *, Significant difference between fetal sex ($P < 0.05$).

Effects of fetal sex on *REN* mRNA and protein levels in term decidua

To determine if the sex differences in decidual *REN* mRNA persisted *in vitro*, decidual explants were cultured for 24 or 48 h. *REN* mRNA levels increased during incubation of decidual explants from women with a male fetus ($P=0.002$). *REN* mRNA abundance was, however, significantly higher at 24 and 48 h in decidual explants from women with a female fetus compared to explants from women with a male fetus ($P=0.002$ and $P=0.010$ respectively; Figure 4.2A).

Prorenin secretion into the explant supernatant, measured at 48 h *ex vivo* (over a 24 h incubation period), was significantly higher in the medium from explants of women with a female fetus ($P<0.05$). Prorenin secretion into the medium for decidual explants from women carrying a female fetus was 4.9 ± 0.95 ng/mL compared with 0.13 ± 0.06 ng/mL in decidual explants from women carrying a male fetus (Figure 4.2B).

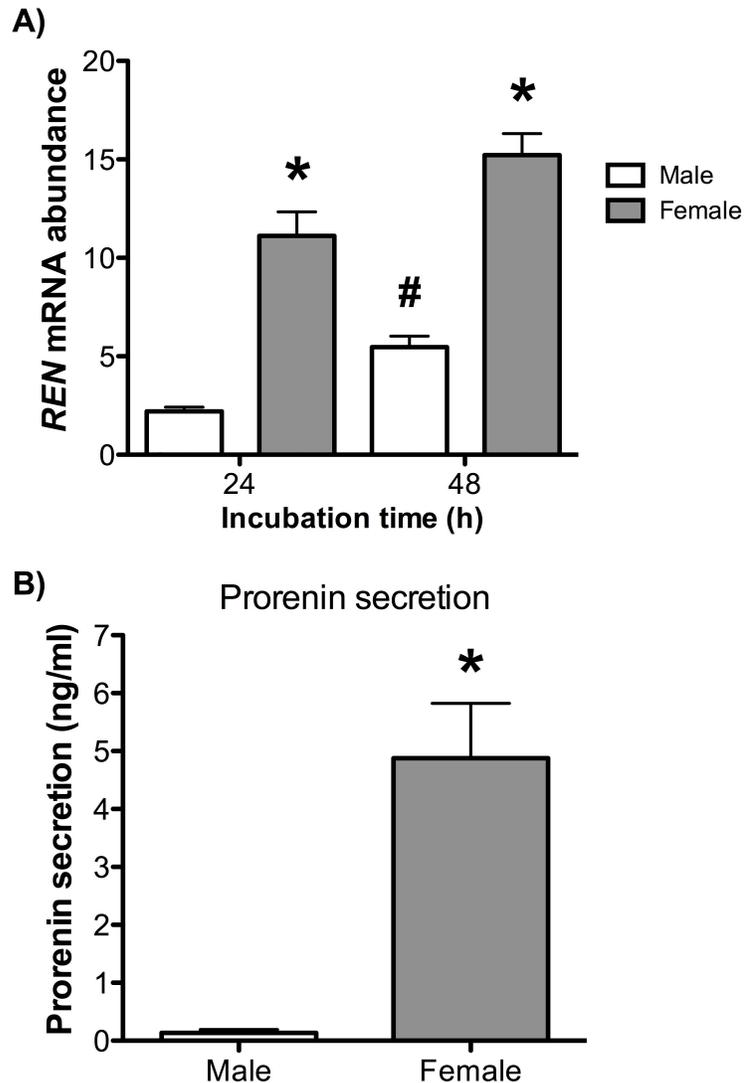


Figure 4.2 Sex differences in *REN* mRNA abundance and prorenin secretion in decidual explants.

REN mRNA levels were measured at 24 and 48 h of incubation. Prorenin was measured in medium collected from the second 24 h incubation period. A) After 24 and 48 h of incubation, decidual *REN* mRNA levels were higher in women carrying a female fetus. *REN* mRNA abundance in decidua increased with incubation time in decidua from women with a male fetus. B) The incubation medium from decidua of women with female fetuses contained significantly higher levels of prorenin than decidual explants from women with male fetuses. Data shown are for n = 4–6 decidual explants collected from three different women per group. *, Significant difference between fetal sex ($P < 0.05$); #, significant difference between incubation time within the same sex ($P < 0.05$).

Effects of fetal sex on the expression of the RAS mRNA in decidual explants

Messenger RNAs for the RAS genes *AGT*, *ACE1*, *ACE2*, *AGTR1*, *ATP6AP2* and *MAS1* were detected in decidual explants at 24 and 48 h of incubation (Figure 4.3), whereas *AGTR2* expression was not. At 24 and 48 h of incubation decidual mRNA levels of *AGT*, *ATP6AP2*, and *MAS1* were all significantly higher in decidual explants from women with a female fetus than from women carrying a male fetus. *ACE1* mRNA abundance was higher at 48 h of incubation in decidua from women with a female fetus than from women carrying a male fetus ($P=0.002$). In contrast, *ACE2* mRNA expression was lower at 24 h ($P=0.041$), but higher at 48 h ($P=0.037$), of incubation in decidua from women with a female fetus as compared to women pregnant with male fetuses.

In decidual explants isolated from women carrying a male fetus, *ACE1* mRNA levels decreased with incubation time ($P=0.010$; Figure 4.3B). In decidual explants from women who were carrying a female fetus, the levels of *ACE2* and *ATP6AP2* mRNA increased with incubation time ($P=0.041$ and $P=0.010$, respectively; Figures 4.3C and 4.3E). *AGTR1* mRNA abundance in decidual explants was not affected by either fetal sex or incubation time (Figure 4.3D).

Decidual explant medium was changed every 24 h; Ang II and Ang 1–7 levels are measured in medium collected from the second 24 h incubation. Measurable levels of Ang II were found (range of 6.9–134 pg/mL, $n=6$), whereas Ang 1–7 levels in the medium were low, often registering below the lower limits of sensitivity of the assay (*i.e.*, <14 pg/mL).

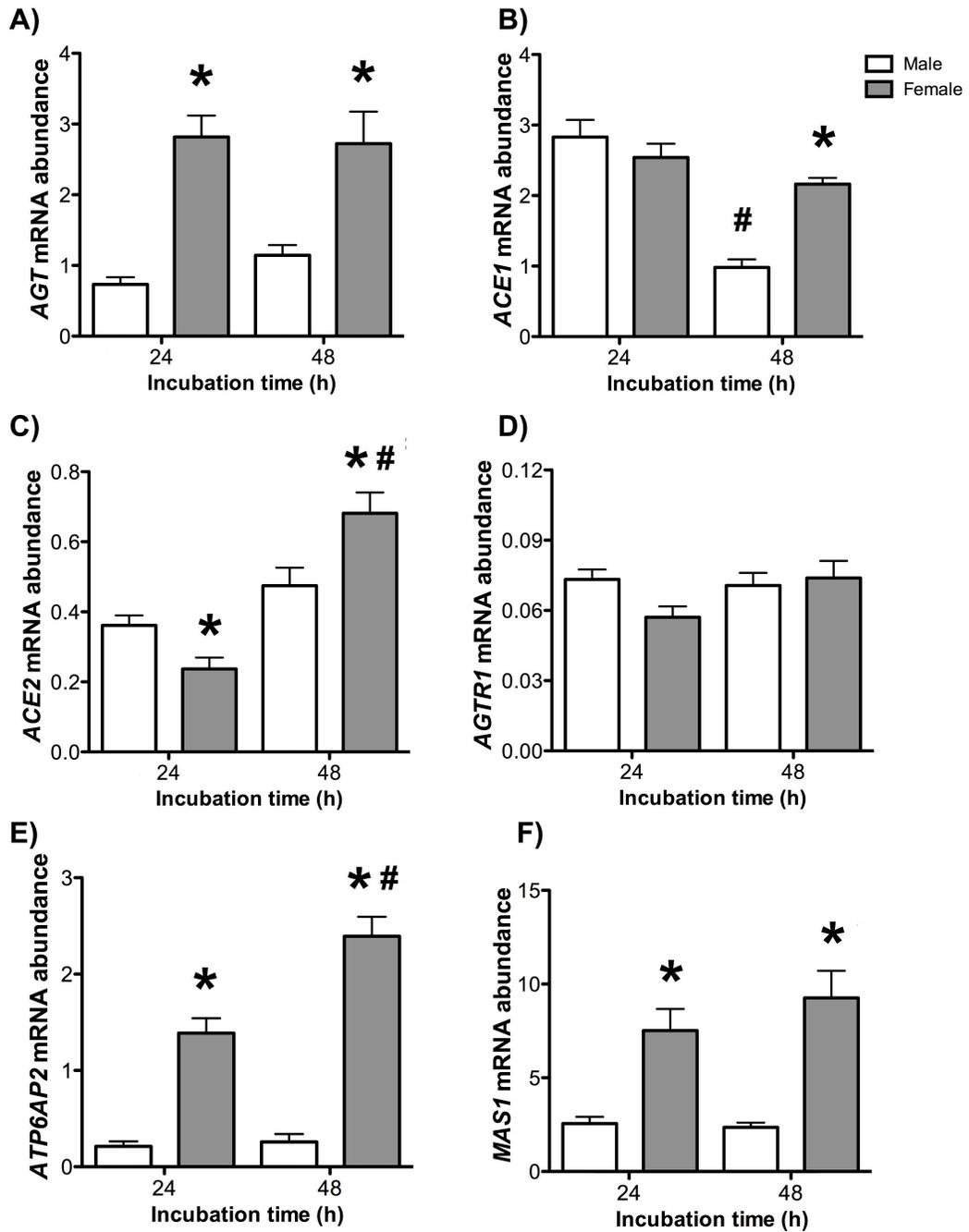


Figure 4.3 Effects of fetal sex on mRNA levels for other genes in the RAS in human decidual explants incubated for 24 and 48 h. (Figure legend on next page)

A) *AGT* mRNA levels after 24 and 48 h incubation were higher in decidual explants from women with a female fetus ($P=0.002$ and $P=0.004$). B) After 48 h incubation, *ACE1* expression was higher in women with a female fetus ($P=0.002$). *ACE1* mRNA abundance in decidual explants from women with a male fetus decreased with incubation time ($P=0.01$). C) *ACE2* mRNA levels at 24 h incubation were lower in explants from women with a female fetus but were higher at 48 h of incubation ($P=0.041$ and $P=0.037$). *ACE2* mRNA abundance increased with incubation time for decidual explants from women with a female fetus ($P=0.041$). D) *AGTR1* mRNA abundance in decidual explants was not affected by either fetal sex or incubation time. E) Decidual *ATP6AP2* mRNA levels were higher after 24 and 48 h of incubation for explants from women carrying a female fetus ($P<0.001$ and $P=0.002$). *ATP6AP2* expression was higher over the course of incubation for decidual explants from women with a female fetus ($P=0.010$). F) At 24 and 48 h of incubation, *MAS1* expression was higher in explants from women with a female fetus ($P=0.005$ and $P<0.001$). Values shown are for $n = 4-6$ decidual explants collected from three women per group. *, Significant difference between sex ($P<0.05$); #, significant difference between incubation time within the same sex ($P<0.05$).

4.5 DISCUSSION

The present study has revealed a striking effect of fetal sex on the expression of decidual *REN* mRNA. Subsequent tissue culture experiments confirmed and expanded our finding that the sex of the fetus determines the level of expression of several RAS mRNAs and the amount of prorenin secreted. As well, we showed that decidual explants secrete both Ang II and Ang 1–7 into the culture supernatant, although we have not been able to determine if the production of these peptides is influenced by fetal sex. These sex-associated differences in decidual *REN* mRNA expression and prorenin secretion provide new insights into the effects of fetal sex on adjacent tissues that are important for fetal growth and development, especially in early gestation. As mentioned in the Introduction, spontaneous abortions (53), miscarriages later in pregnancy (54), stillbirths (55, 56), premature rupture of membranes (PROM) and spontaneous preterm birth (57-60), gestational diabetes (61), and delivery by caesarean section (59, 62, 63) occur more frequently if the mother is carrying a male fetus, whereas the incidence of preeclampsia in preterm pregnancies is more common in women carrying female fetuses (60, 62). Female babies are also more likely to be growth restricted (59, 61). All of these sex differences may be related to the effects of fetal sex on the decidual RAS because we believe that the decidual RAS is involved in regulating decidual angiogenesis and perhaps placental implantation. Therefore, these sex-specific effects on decidual RAS expression and prorenin secretion may contribute to the sex differences reported in pregnancy outcomes cited above. Future studies on gene expression and protein synthesis in tissues of the uteroplacental unit should take

into account the sex of the fetus because we have shown that fetal sex is able to alter gene expression in a maternal tissue (decidua).

It is unclear how sex-specific differences of decidual RAS expression affect pregnancy outcomes because there is currently no concrete knowledge of how the RAS functions within the decidua or how the decidua contributes its RAS products to the maternal circulation. However, it is clear that the sex differences in RAS genes seen in the decidua are unique, since the fetal membranes and placenta do not show any sex specific differences in RAS gene expression (22), even though these tissues are predominantly fetal in origin. Further investigation of the RAS in the myometrium may be warranted, as the myometrium is a maternal tissue closely associated with the decidua.

In an attempt to better understand the effects of fetal sex on decidual RAS mRNA expression levels and subsequent protein secretion, we established human decidual explants. We were successful in maintaining decidua for 48 h *ex vivo*. The pattern of *REN* and *AGTR1* expression within this explant model was similar to that seen in non-labouring non-incubated decidua (a difference and no difference with fetal sex, respectively). In addition, decidual explants secreted prorenin, Ang II and Ang 1-7 into the incubation medium, suggesting that the explant model is a valid model for the study of the human decidual RAS *ex vivo*.

Not only did the sex of the fetus influence maternal decidual *REN* mRNA expression, it also affected prorenin protein secretion by the decidua. This sex difference was maintained for up to 48 h *ex vivo*. Therefore it is unlikely that fetal sex hormones were responsible for the difference. The persistence of the sex difference in expression, and the emergence of increased expression of other RAS

genes in decidua from women carrying a female fetus, suggest that decidual RAS gene expression is permanently altered by fetal sex. However, we have not as yet studied whether fetal sex affects the expression of the decidual/placental RAS in early gestation.

Amongst the various late gestation human intrauterine tissues (fetal membranes, placenta, chorion and myometrium), the decidua has the highest levels of *REN* mRNA (13, 73). High levels of prorenin (measured as enzyme activity after acid activation) have, however, been found in fetal membranes (148), and immunostaining for prorenin also shows that it is present in amnion and chorion, as well as decidua and placenta (73). Since amnion and chorion express only very low levels of *REN* mRNA, it is likely that decidual prorenin is the source of the high levels of prorenin found in amniotic fluid (13, 73, 80, 146). Since we have shown that prorenin secretion by cultured decidua is significantly higher in pregnancies with female than with male fetuses, it is reasonable to conclude that levels of prorenin in amnion and in amniotic fluid may be higher when the fetus is female than when it is male.

The secretion of prorenin into the maternal circulation from the uteroplacental unit could also be influenced by fetal sex (145). Evidence for this can be seen in mice in which a human *REN* transgene was made to be expressed only in the placenta, yet human prorenin was found in maternal plasma (149). This demonstrates the ability of placental prorenin to enter the maternal bloodstream. Since we consistently found measurable levels of Ang II in decidual explants, it is tempting to speculate that decidual Ang II might be able to escape into the maternal circulation from early in gestation. As a consequence the higher incidence of growth

restriction in female neonates (59) may be related to higher prorenin and Ang II levels causing vasoconstriction and reduced uteroplacental blood flow, possibly through downregulation of AT₂R within the uterine vasculature. We have, moreover, observed such a phenomenon when Ang II levels are raised in the pregnant ewe through exogenous infusion of Ang II for >24 h (150), in these studies, the actions of Ang II on AT₂R in the uterine vasculature of the sheep were able to offset the vasoconstrictor effects of Ang II mediated via the AT₁R (150).

In this study, several decidual end-products of the RAS, prorenin and Ang peptides were measured to determine if the changes in expression of decidual RAS genes resulted in changes in the levels of their protein end-products or peptides. The high level of expression of *REN* mRNA was coupled with a high level of prorenin secretion from cultured decidua collected from women with a female fetus. Despite these striking differences in prorenin secretion and the upregulation of expression of other RAS genes, it was not possible to demonstrate a sex difference in angiotensin peptide production, possibly because assays were performed on only 6 subjects (3 male and 3 female) and the levels of the peptides were very low. As well, there was degradation of these peptides was probably occurring concomitant with their production. Notwithstanding, it is still clear that an effect of fetal sex on the maternal decidua may be imprinted at an early stage of pregnancy, so that the differential expression of RAS components and the physiological and biochemical consequences of this may be present up to, but not after, labour.

Interestingly, it appears that the expression of decidual *REN* mRNA is downregulated following labour (Figure 4.1). If the decidual RAS plays a specific role in regulating the onset of labour, e.g., via stimulation of decidual PTGS2 production

(24), then the suppression of *REN* mRNA if the fetus is female would counterbalance this action. Alternatively, it is possible that withdrawal of the decidual RAS could affect the integrity of the fetal membranes through a reduction in production of TGF- β_1 (151), which stimulates formation of profibrotic molecules such as PAI-1, fibronectin and collagens (152), or through reduced activation of pro-inflammatory cytokines (153). These possibilities need to be tested in future studies.

In conclusion, the present study provides the first demonstration of a fetal sex-associated difference in expression of RAS genes in the maternal decidua. We have also shown that the changes in maternal decidual RAS gene expression that occur with labour depend on the sex of the fetus. Our data provide novel insights into the influences of fetal sex on decidual gene expression, where the latter is important in supporting fetal growth and development. Our findings could contribute to understanding why there are significant differences in adverse pregnancy outcomes between female and male fetuses.

Chapter Five

Regulation of the Renin Angiotensin System (RAS) pathways in the Human Decidua

This chapter focuses on how sex-specific differences in decidual RAS expression within explant cultures are sustained. In addition, we aimed to determine how the RAS is regulated within the decidua, specifically by studying the effects on decidual *REN* and prorenin protein by decidual explants using the renal *REN* stimulator, cAMP and determining if the cAMP response of the RAS in decidual explants were also fetal-sex specific.

5.1 ABSTRACT

A renin angiotensin system (RAS) exists within the decidua, the expression of which is influenced by fetal sex. Specifically, decidual renin mRNA (*REN*) is more abundant in women carrying a female compared to a male fetus. We aimed to determine whether cyclic AMP (cAMP), a renal renin regulator, also influences decidual RAS expression and whether cAMP effects are fetal sex specific. RAS gene expression was measured using qPCR and prorenin protein, renin activity, angiotensin (Ang) 1–7 and Ang II peptides were measured in the incubation medium using an ELISA or radioimmunoassay. The presence of male fetal cells within the decidua was determined by *SRY* gene expression.

cAMP treatment did not affect *REN* expression, *i.e.*, *REN* abundance was still greater in decidual explants from women carrying a female compared to a male fetus ($P<0.01$). Interestingly, cAMP was associated with a fall in supernatant prorenin levels if the fetus was female ($P<0.01$), *i.e.*, prorenin levels were no longer sexually dimorphic.

We also observed that cAMP treatment altered fetal sex specific differences in other RAS genes; it abolished sex differences in *AGT*, *ATP6AP2* and *MAS1* genes, moreover, reversed the pattern of *ACE2* and *AGTR1* expression, *i.e.*, in vehicle treated explants, *ACE2* and *AGTR1* expression were greater in decidua with a female fetus, however after cAMP treatment, *ACE2* and *AGTR1* expression were greater in decidua with a male fetus.

In conclusion, the present study demonstrated that decidual RAS response cAMP depends on fetal sex and further exploration of which may contribute to our understanding of how the decidual RAS is regulated.

5.2 INTRODUCTION

The renin angiotensin system (RAS) within intrauterine tissues has been shown to be important for the normal progression of pregnancy in both the mother and the fetus. The RAS is vital for normal placental development; as it is involved in processes such as angiogenesis (110), modulation of placental blood flow (11), and the regulation of trophoblast invasion (111, 112). Furthermore, within the decidua and myometrium, the RAS is involved in spiral artery remodelling (12). Therefore, it is not surprising that disruptions to the uteroplacental RAS have been associated with pregnancy complications, such as preeclampsia (14, 15).

The rate-limiting enzyme of the RAS is renin. It is an enzyme secreted predominately by the kidney that acts on angiotensinogen (AGT) to produce angiotensin I (Ang I). Renin is produced as a pro-enzyme, prorenin, which is enzymatically cleaved to form active renin. Historically, prorenin has been seen only as the inactive precursor of renin, having little biological activity, despite the fact that its circulating levels are 10 times higher than those of renin (114). Recently a (pro)renin receptor ((P)RR) pathway has been identified which gives prorenin the capacity to have a physiological function. Prorenin bound to the (P)RR can be non-proteolytically activated and therefore gains the ability to cleave AGT to Ang I, not unlike active renin (74).

The resulting Ang I peptide is then acted upon by angiotensin converting enzyme (ACE) to form angiotensin II (Ang II). Ang II acting on the angiotensin II type 1 receptor (AT₁R) is associated with the majority of the actions normally attributed to the classical RAS including vasoconstriction, aldosterone synthesis and secretion, angiogenesis and proliferation, whereas Ang II binding to the Ang II type 2 receptor

(AT₂R) has a number of actions that generally oppose those mediated by Ang II acting on the AT₁R (67).

Recently, the RAS has been expanded to include an Ang 1–7/Mas receptor pathway, consisting of ACE2, a homologue of ACE, which allows an alternative pathway for Ang II by converting it to Ang 1–7. Ang 1–7 acting through the proto-oncogene receptor (Mas), has also been shown to have actions that oppose those of Ang II acting through the AT₁R (113).

We have previously shown that the decidua, a maternal tissue, expresses all of the known RAS genes and that sex of the fetus can alter the expression of the RAS within the maternal decidua (13, 73). Specifically, in decidual samples collected prior to the onset of labour (*i.e.*, at delivery by caesarean section), *REN* mRNA levels were higher when the fetus was female. This fetal sex specific expression was maintained for up to 48 h *ex vivo* in decidual explant cultures (120). These findings are intriguing, particularly if we take into account the fact that pregnancy outcomes in relation to the health and survival of both neonate and the mother are influenced, in part, by the sex of the fetus (52). Therefore the examination of how RAS is regulated, but more importantly, what role fetal sex plays in regulating the RAS in our decidua explant model, may provide new insight into how fetal sex influences the prevalence of fetal and neonatal diseases and pathologies.

In this current study we postulated that cAMP would increase *REN* expression and prorenin secretion in human decidual explants, and that this stimulatory effect would abolish the sex difference seen in *REN* expression and prorenin secretion, specifically by upregulating *REN* expression in decidual explants obtained from women carrying a male fetus. It has been well documented that cAMP

stimulates *REN* gene expression in the juxtaglomerular cells of the kidney (100) and increases prorenin release from primary decidual cells in a dose dependent manner (101), through cAMP binding to the cAMP response element (CRE) (115, 116). We used a dose of cAMP that stimulates *REN* expression in a placental primary cell line (154). We also examined the expression of other RAS genes, the secretion of Ang II and Ang 1–7 peptides and prorenin concentration, as well as renin activity in these explants, in order to examine what impact the sex of the fetus has on the decidual RAS as a whole.

5.3 METHODS

Tissue collection

Decidual samples were collected as described previously (13, 73, 120). Samples were collected from uncomplicated singleton pregnancies at term (38–41 weeks gestation), delivered by elective caesarean section in the absence of labour (n=6). Women treated with non-steroidal anti-inflammatory drugs or who had a history of infection, chorioamnionitis, asthma or preeclampsia or who were undergoing induction of labour were excluded. Informed consent was obtained from all participants, and the study was approved by the Hunter Area Research Ethics Committee and the University of Newcastle Human Research Ethics Committee.

The fetal membranes and attached decidua parietalis were isolated as a whole, excluding a 2 cm border around the edge of the placenta. Amnion was peeled from the choriodecidua, and the chorion laevae was separated from the decidua by sharp dissection as described previously (13, 73, 120).

Decidual explant culture

As previously described (120), excised decidua were washed in tissue culture medium (phenol-free DMEM/F-12 supplemented with 15 mM HEPES, 1.2 g/L NaHCO₃, 1 mg/mL L-glutathione reduced, 0.1 g/L albumin fraction V, 0.65 µg/mL aprotinin, 10% fetal bovine serum and 40 µg/mL gentamicin) and dissected into approximately 0.25 cm² pieces. Several pieces of decidua were randomly selected, blotted and weighed. One hundred milligrams of decidua was placed into each well of a 6 well plate with 2 mL of incubation medium, these tissues are incubated for 24 h. After the initial 24 h equilibration period, the decidual samples were treated with either vehicle or 300 mM 8-bromo-cAMP (Sigma-Aldrich, St. Louis, MO, USA) for a further 24 h. Decidual tissues and supernatants were collected after 24h and snap frozen in liquid nitrogen for subsequent protein and mRNA analyses. Each experiment was conducted in duplicate, on 6 separate decidual samples (3 from women carrying a male and 3 from women carrying a female baby). Cell viability was verified by measuring LDH levels after incubation, as well as measuring RNA stability and quality (data not shown). The media from decidual explant cultures were collected into EDTA tubes, containing a protease inhibitor cocktail (Sigma, St. Louis, MO) for subsequent measurement of prorenin, active renin, Ang II and Ang 1-7.

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

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). In addition, we examined

each sample's RNA integrity by running samples on a gel. RNA samples were DNase treated (Qiagen N.V., Hilden, Germany) and total RNA spiked with a known amount of Alien RNA (Stratagene, La Jolla, CA, USA; 10^7 copies per microgram of total RNA) before the RNA was reverse transcribed using a Superscript III RT kit with random hexamers (Invitrogen). The Alien qRT-PCR Inhibitor Alert system (Stratagene) served as a reference for internal standardization (119). qPCR was performed in an Applied Biosystems 7500 Real Time PCR System using SYBR Green for detection. Each reaction contained 5 μ L of SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA), primers, cDNA reversed transcribed from 10 ng total RNA, and water to 10 μ L. RAS primer sequences have been described previously. To examine whether fetal cells were present in the human decidua that could regulate the RAS *ex vivo*, we determined if the cultures of decidua from pregnancies carrying male fetuses had SRY mRNA; the following primers were used, (155), forward: 5'-TGGCGATTAAGTCAAATTCGC-3', reverse: 5'-CCCCTAGTACCCTGACAATGTATT-3'. For all genes of interest mRNA abundances were calculated relative to *Alien* mRNA using the $\Delta\Delta C_T$ method as described previously (13, 73, 120).

Radioimmunoassay (RIA) of Ang II and Ang 1-7

Angiotensin II was measured by radioimmunoassay (RIA) by Prosearch Pty Ltd, using the "delayed tracer addition" technique. Each sample of medium was equilibrated for 20 h at 4°C in a total volume of 300 μ L with antibody raised in rabbit against angiotensin II N-terminally conjugated to bovine thyroglobulin. Monoiodinated 125 I-angiotensin II, 10,000 c.p.m.in 100 μ L, was added and allowed

to equilibrate for a further 16 h at 4°C, after which the bound and free phases were separated using Dextran 10-coated charcoal and centrifugation. Sensitivity was 3.5 pg/mL. Cross-reactivities to Ang I, Ang 1-7 and all other pertinent hormones were 0.52%, 0.0128% and < 0.1% respectively. Intra and inter-assay coefficients of variation were 6.4% and 12%, respectively.

Ang 1-7 was assayed directly by RIA using an antibody raised in guinea pig to Ang 1-7 N-terminally conjugated to porcine thyroglobulin, and Ang 1-7 that had been mono-iodinated with ¹²⁵I antibody. Bound ¹²⁵I-Ang 1-7 was separated from free by Dextran 10-coated charcoal, and unbound c.p.m. were compared to serially diluted standard amounts of Ang 1-7. Sensitivity was 14 pg/mL. Cross-reactivities to Ang I, Ang II, Ang III and Ang IV were 0.11%, 0.04%, 0.53% and 0.03%, respectively. Intra- and inter- assay coefficients of variation were 4.5% and 10%, respectively.

Measurement of prorenin protein by ELISA

Prorenin concentration in culture media was measured using the Human Prorenin ELISA kit (Molecular Innovations Inc; Novi, MI) according to the manufacturer's instructions. Prorenin in each sample was captured by an antibody immobilised onto the surface of each well of the plate. A primary antibody specific for prorenin was then applied and the unbound fraction removed by washings. For subsequent detection by means of colour development, a secondary antibody conjugated to horseradish peroxidase. 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added. After termination of the reaction with 4 M sulphuric acid,

optical density was read at 450 nm. Prorenin concentration was directly proportional to colour development and was measured using a standard curve. Samples were assayed in duplicate. In our laboratory 1 ng/mL amniotic fluid prorenin measured using this technique generated 116 ng/h/mL of Ang I from angiotensinogen present in nephrectomised sheep plasma used as the source of angiotensinogen substrate. All samples were assayed on one ELISA plate. Therefore there was no inter-assay variability. Intra-assay coefficient of variation was 7.3%.

Active renin Assay

Renin activity was measured using the GammaCoat plasma renin activity kit, by our pathology department according to the manufacturer's instruction. Ang I generation was performed in the laboratory, 1 mL of each medium sample was incubated at 37°C for 24 h, with 1 mL nephrectomised sheep plasma (NSP), 63 µL 100 mM EDTA, 29.4 µL 100 mM phenylmethylsulfonyl fluoride (PMSF). After which, each sample was snap frozen and sent to Hunter Area Pathology Service, where 100 µL of the sample after Ang I generation and 1 mL of tracer-buffer are added to a tube coated with rabbit raised moniodinated ¹²⁵I-angiotensin I antibodies. After 3 h at 37°C incubation, all tubes are counted in a gamma counter adjusted for iodine-¹²⁵ for one minute to measure renin activity (Ang I generation in ng/ml/h).

Data Analysis

Decidual RAS gene expression data were tested using the nonparametric Mann-Whitney U test. An independent-sample t-test was used to determine the effects of fetal sex on prorenin, Ang II and Ang 1-7 levels in the supernatant after 48

h *ex vivo*. The SPSS statistical package (SPSS for Windows, release 17.0.0; Chicago, IL) was used for all analyses. Significance was set at $P < 0.05$.

5.4 RESULTS

Effects of fetal sex and cAMP on prorenin mRNA and protein levels in decidual explants

cAMP treatment did not stimulate *REN* expression in decidual explants as we expected. The sex difference in *REN* expression described in our previous study on vehicle treated control explants was still evident in the cAMP treated explants *i.e.*, even after cAMP treatment, decidual *REN* expression remained higher in explants from women with a female fetus compared to those from women with a male fetus ($P < 0.01$, Figure 5.1A). In contrast, in vehicle treated control explants, prorenin protein levels were significantly lower after cAMP treatment of decidua from pregnancies carrying a female fetus ($P < 0.01$, Figure 5.1B), while cAMP treatment did not have an effect on explants when the fetuses were male. Thus after cAMP treatment the sex specific differences in prorenin protein levels previously reported in control explants was no longer observed.

To see if the cAMP induced reduction in prorenin levels in decidual explants (when there were female fetuses) was due to an increased conversion of prorenin to active renin we measured renin activity in the supernatants of explants. There were no sex specific differences in renin activity of untreated decidual explants. However irrespective of fetal sex, cAMP treatment was associated with a decline in renin activity which was significant if data from both types of cAMP treated explants were combined ($P < 0.05$, Figure 5.1C). There was therefore, no evidence of an increase in

the rate of conversion of prorenin to active renin in decidual explants treated with cAMP that would explain the cAMP induced reduction in decidua from female pregnancies seen in Figure 5.1B.

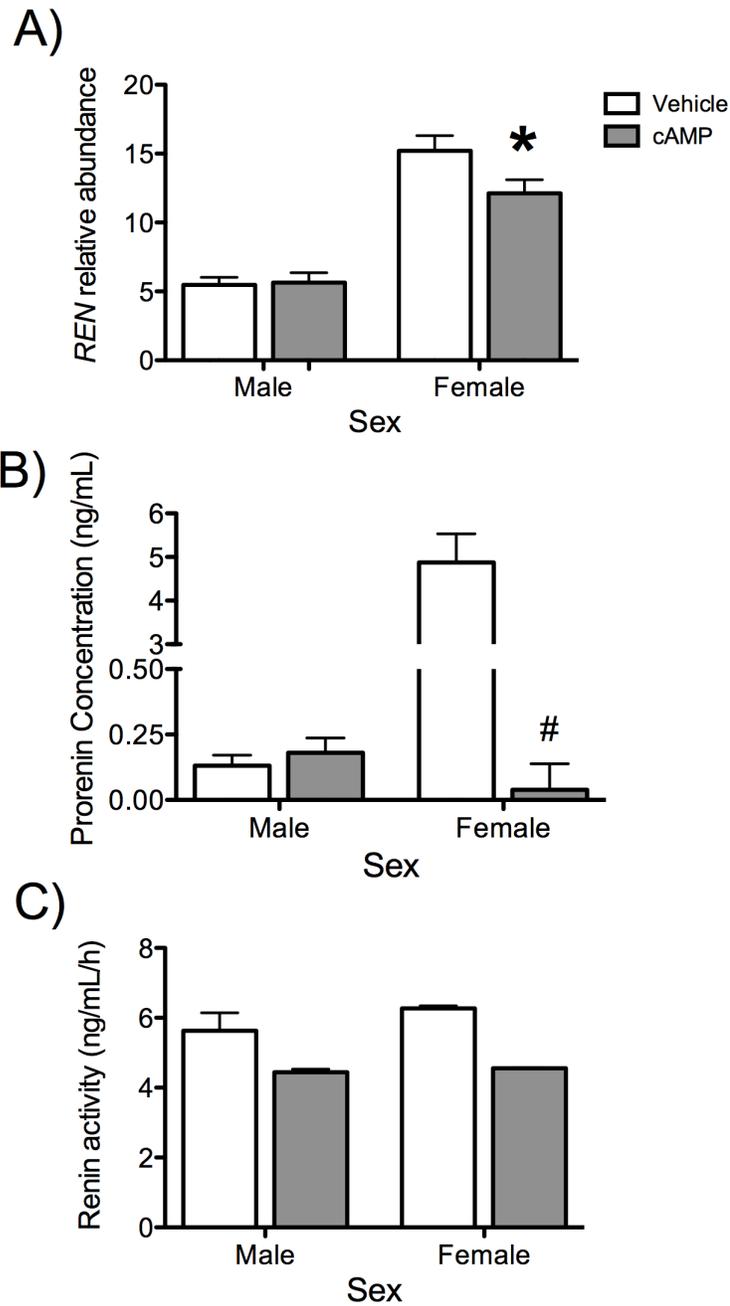


Figure 5.1 Fetal sex differences in decidual *REN* mRNA abundance as well as prorenin secretion and renin activity in the supernatant of vehicle and cAMP treated decidua explants (collected from the second 24 h incubation period). Data are represented as mean \pm SEM. (Figure legend on next page)

A) After cAMP treatment, decidual *REN* mRNA levels were higher in women carrying a female fetus compared to cAMP treated decidual explants from with a male fetus. B) Prorenin protein levels after cAMP treatment was significantly lower in decidua with a female fetus compared to vehicle treated controls, while cAMP did not affect prorenin protein levels in decidua with a male fetus. Therefore after cAMP treatment, the sex specific difference in prorenin levels was abolished. C) Renin activity in supernatant from decidual explants was not affected by fetal sex or cAMP treatment. Data shown are for n = 4–6 decidual explants collected from three different women per group. *denotes significant difference between fetal sex within the same treatment ($P < 0.05$); # denotes significant difference between treatment within the same sex ($P < 0.05$).

Decidual RAS and affects of fetal sex and cAMP

We have previously reported sex specific differences in the expression of *AGT*, *ATP6AP2*, *ACE1*, *ACE2* and *MAS1* genes in vehicle treated decidual explants; *AGTR2* was not expressed (120). Briefly, the expression of these genes was significantly higher if the fetus was female.

In this present study, we have found that as in the case of *REN* expression, cAMP treatment was associated with a reduction in the mRNA levels of *AGT*, *ATP6AP2* and *MAS1* ($P<0.01$, $P<0.01$ and $P<0.05$ respectively, Figure 5.2A–C), so that with cAMP treatment, the sex specific differences in expression seen in vehicle treated explants was abolished.

On the other hand, cAMP treatment had no effect on the levels of expression of *ACE1*, so the sex difference in *ACE1* expression observed in vehicle treated decidua was still present after cAMP treatment in women with a female fetus ($P<0.01$, Figure 5.2D). cAMP also was not able to induce *AGTR2* expression in the decidua

Furthermore we have shown that cAMP treatment can reverse the sex specific differences of some of the RAS genes. Thus in vehicle treated control explants of decidua from pregnancies carrying a female fetus there was a significantly greater level of expression of *ACE2* compared with levels in decidua collected from pregnancies carrying a male fetus. In the same study we showed that there was no significant sex specific difference in *AGTR1* expression. cAMP treatment in decidua from pregnancies with a male fetus, however, had increased expression of both *ACE2* and *AGTR1* compared with controls (both $P<0.01$, Figure 5.2E & F). On the other hand and unexpectedly, cAMP treatment was associated

with a reduction in *ACE2* mRNA abundance in decidua from pregnancies with a female fetus compared with controls ($P < 0.01$, Figure 5.2E). The outcome of these cAMP induced changes was that after cAMP treatment there was a reversal of the sex specific difference in *ACE2* expression and the appearance of a sex specific difference in *AGTR1* expression, *i.e.*, after cAMP treatment *ACE2* and *AGTR1* expression were greater in decidua with a male fetus (both $P < 0.01$, Figure 5.2E & F).

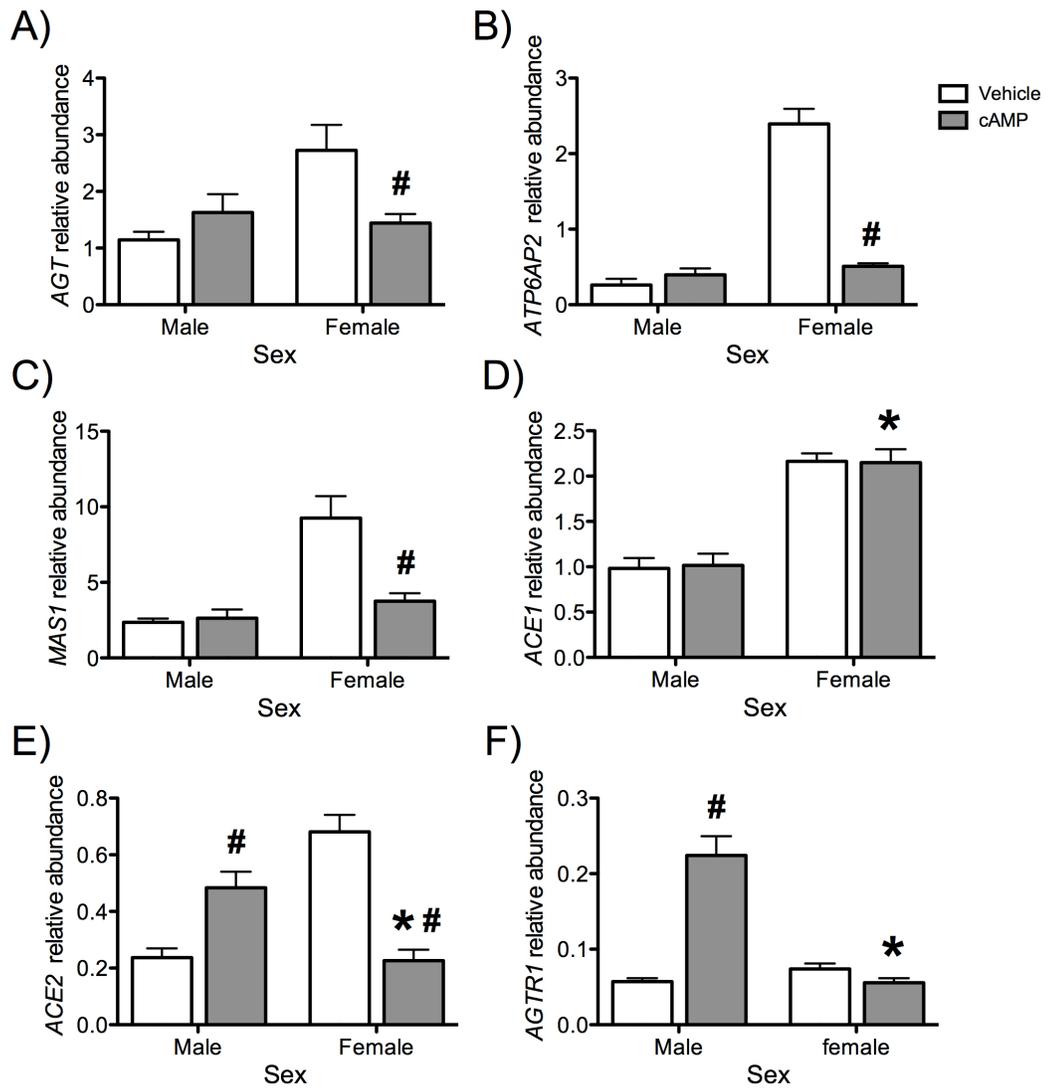


Figure 5.2 Sex specific differences in RAS gene expression in vehicle and cAMP treated decidua explants. (Figure legend on next page)

Sex specific differences in RAS gene expression in vehicle and cAMP treated decidual explants. Data are represented as mean \pm SEM. A) - C) cAMP treatment significantly lowered decidual *AGT*, *ATP6AP2* and *MAS1* mRNA levels in women carrying a female fetus compared to their vehicle treated controls. D) cAMP treatment had no effect on the levels of expression of *ACE1*, so the sex difference in *ACE1* expression observed in vehicle treated decidua was still present E) After cAMP treatment, *ACE2* expression in decidua with a male fetus was significantly greater than its vehicle treated controls. Whereas cAMP treatment significantly lowered *ACE2* mRNA levels in women carrying a female fetus compared to their vehicle treated controls. As a result, *ACE2* expression in vehicle treated decidua was greater in decidua with a female fetus, however after cAMP treatment, this sex difference was reversed, *i.e.*, *ACE2* expression was greater in decidua with a male fetus. F) cAMP treatment significantly increased *AGTR1* expression in decidua with a male fetus compared to its vehicle treated controls, whilst *AGTR1* expression in decidua with a female fetus was not affected. As a result, cAMP treatment caused a reversal of the sex difference in *AGTR1* mRNA abundance, *i.e.*, after cAMP treatment *AGTR1* expression is significantly greater in decidua with a male fetus. Data shown are for n = 4–6 decidual explants collected from three different women per group. *denotes a significant difference between fetal sex within the same treatment ($P < 0.05$); # denotes a significant difference between treatment within the same sex ($P < 0.05$).

Decidual Ang II and Ang 1–7 peptides and the effects of fetal sex and cAMP

There were no statistically significant effects of fetal sex or cAMP treatment on Ang II peptide levels in the supernatant from the decidual explants, however it looked as though Ang II levels were increased in supernatants from cAMP treated decidual explants from pregnancies with a female fetus (Figure 5.3).

There were no significant effects of fetal sex or cAMP treatment on Ang 1–7 peptide levels in the supernatant (data not shown).

***SRY* gene**

Since the sex specific differences in expression of genes of the decidual RAS, and prorenin protein levels were still evident after 48h incubation *ex vivo*, away from the fetus, we needed to determine whether if there were cells of fetal origin present in the maternal decidual explants that might still be affecting expression of the decidual RAS. We found detectable amounts of *SRY* mRNA in decidual explants obtained from women pregnant with a male fetus (data not shown), demonstrating the presence of fetal cells within the maternal decidua.

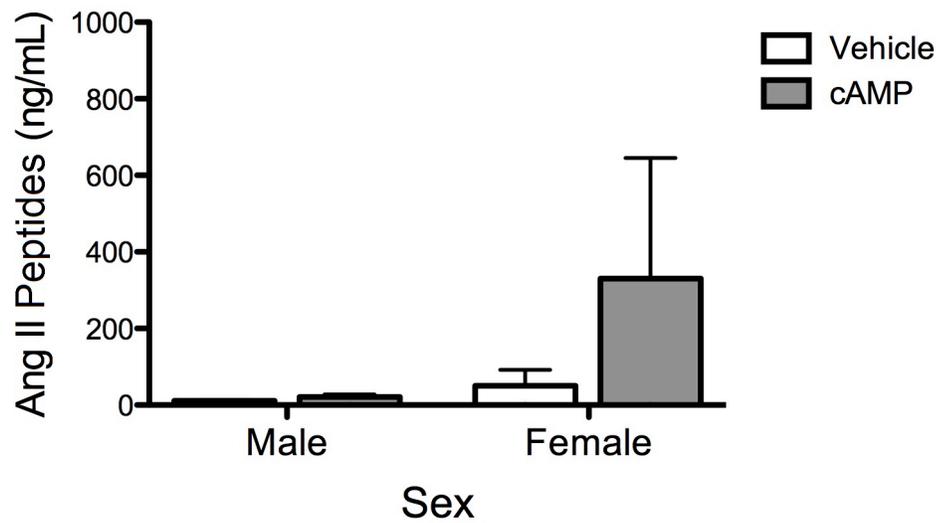


Figure 5.3 Ang II peptide levels in the supernatant of decidual explants treated with vehicle or cAMP.

Ang II levels were not affected by fetal sex or cAMP treatment. Data are represented as mean \pm SEM. Data shown are for n = 4–6 decidual explants collected from three different women per group.

5.5 DISCUSSION

We were interested in how the RAS was regulated within the decidua because, in previous independent experiments, we had found sex specific differences in decidual *REN* expression and prorenin protein levels (120). Although the decidua is a maternal tissue, the sex of the fetus might influence decidual RAS gene expression and prorenin protein secretion in both freshly isolated term tissues either through sex specific hormones or through paracrine regulation of gene expression by fetal cells invading the decidua. The sex specific differences in RAS gene expression and prorenin secretion by decidual explants after 48 h *in vitro* does suggest that the effects of fetal sex are localised to the explant tissue and not the result of endocrine secretions by the fetus (120). So perhaps it was not surprising that we identified fetal cells in maternal decidua. What is surprising is that the effects of cAMP on RAS gene expression in decidual explants were also sex specific.

It is unclear how the fetal sex specific differences in decidual expression of RAS genes and prorenin protein are maintained. One reason could be that fetal cells themselves are responsible for expression of the RAS and prorenin. The most likely source of fetal cells would be the chorion, which is adjacent to the decidua. In previous studies, however, we have found that chorion does not express *REN* and isolated choriodecidua explants contain only half the RAS mRNA abundance of decidual explants (unpublished observations). On the other hand we have found prorenin protein present in chorion, as have others (13, 148), but prorenin produced by decidua may well be taken up by fetal chorion and amnion, as decidual explants secrete prorenin (Figure 5.1). The more likely possibility is that the fetal cells may influence the decidua, or more likely a specific cell type within the decidua. As the

decidua is a heterogeneous tissue, we were not able to determine which cell type was expressing the RAS genes and proteins in this explant model. Since the fetal cells which we found to be present in decidua are foreign to the maternal immune system, their presence within the maternal decidua may elicit a specific immune response, which could account for the differences seen in the decidual RAS based on fetal sex. As both mast cells and macrophages express *REN* (22, 23) and angiotensin type 1 receptors are found on white blood cells (156). Interestingly, in species like the horse, an immunological reaction within the decidua to invading fetal cells occurs in early gestation resulting in loss of endometrial cups of fetal cells and declining levels of chorionic gonadotropin (157). In humans, chorionic gonadotropins have been shown to stimulate *REN* expression in intrauterine tissues (106).

Because cAMP is a known regulator of renal renin gene expression and regulates expression of *REN* and prorenin in placental cell lines in an endometrial cancer cell line (unpublished); we postulated that treatment of decidual explants with cAMP would stimulate *REN* expression and increase prorenin secretion in decidua from pregnancies bearing male fetuses, thereby abolishing the sex specific differences seen in isolated tissue and in vehicle treated controls. So we were in fact surprised that cAMP had no effect on decidual explant *REN* mRNA and in fact reduced secretion of prorenin by decidua from pregnancies bearing females to levels found in supernatants of control and cAMP treated decidua from women carrying male fetuses. We cannot explain why cAMP did not stimulate *REN*. We thought that the fall in prorenin secretion might be due to the fact that cAMP stimulated conversion of prorenin to active renin through stimulation of other proteases. To our surprise we found that enzymatically active levels of renin in

decidual supernatants were not affected by fetal sex (Figure 5.1C), and cAMP actually caused a small fall in active renin levels in both types of decidua. The only conclusion we can draw at this stage is that the fall in prorenin protein levels in decidua from pregnancies carrying females was not accompanied by a concomitant increase in enzymatically measured active renin levels.

Since untreated decidual explants from pregnancies carrying male fetuses had only low levels of prorenin protein but levels of active renin were similar to those found in supernatants from pregnancies with female fetuses, it is possible that there is continuous conversion of prorenin to active renin by decidua. Unpublished observations by authors of this paper, have found that in homogenised choriodecidua that was suspended in phosphate buffer for 4h at room temperature and then dialysed at pH 7.5 for 24 h, there was proteolytic activation of prorenin, as 100% of the acid-activatable renin in the homogenate and 68% of that in the supernatant was in fact active. Since choriodecidua contains only 23% of the trypsin inhibitory capacity of plasma, proteolytic activation of prorenin in isolated tissues and explants is highly likely.

Not only did cAMP have unexpected effects on *REN* and prorenin but it also had a variety of effects on other RAS genes. Thus in decidual explants from women with a male fetus it increased *AGTR1* expression, whereas in decidua with a female fetus *AGT*, *ACE2*, *MAS1*, and *ATP6AP2* mRNA levels were all lower after cAMP treatment. We are unable to identify the specific mechanisms via which cAMP caused these effects, however they do suggest that RAS pathways are responding differently to cAMP within decidua depending upon whether the fetus is male or female. One possible explanation may be that this is due to the different ratio of cell

types populating the decidua depending on the sex of the fetus as mentioned above. We have yet to demonstrate this.

Although we were able to demonstrate the presence of Ang II and Ang 1–7 peptides in the supernatant from these explants, which indicates that translation and subsequent protein synthesis has occurred, the levels detected were for the most part very low. This is because we cannot grow our explants in media which contain protease inhibitors sufficient to block breakdown of Ang peptides.

In conclusion, the present study provides new insight into how the RAS within the decidua is regulated and shows that fetal sex is an important determinant of regulation of its expression. In addition, we have provided preliminary evidence of cAMP dependent pathway/s that can influence prorenin conversion into active renin at physiological pH and temperatures. These data may have far reaching consequences in our understanding as to why there are significant sex specific differences in pregnancy outcomes (52).

Chapter Six

Conclusion

This thesis focuses on the RAS within placental trophoblast cells and the maternal decidua. We believe that both the human decidual and placental RASs are likely to be involved in regulating placental development and by extension the overall ability of the pregnancy to survive and develop, but that it also has the capacity to adapt to adverse insults during pregnancy. We are the first to study the decidual RAS and its implications with regards to fetal sex in pregnancy outcomes.

The RAS mediates several key placental events at the beginning of gestation such as Ang II's involvement in trophoblast proliferation, angiogenesis and migration (4, 5, 11). AGT has also been shown to be vital for spiral artery remodeling (12, 158). While these events occur at the beginning of pregnancy they determine the efficacy of placental exchange throughout gestation. Normal development of the placenta is vital to meet the exponentially increasing oxygen and nutrient demands of the fetus. In addition, the placenta is also an important endocrine organ in the maintenance of pregnancy through the production of estrogens and progesterone.

During pregnancy, the decidua is a major source of renin and possibly Ang II. Furthermore, Ang II has also been shown to limit trophoblast invasion via the AT₁R, through the activation of PAI-1 (111, 159). Furthermore, it is conceivable that decidual RAS components may also escape into the maternal circulation and thus may affect maternal cardiovascular and renal function and, ultimately placental perfusion.

Given the role of the RAS in placental development and the regulation of trophoblast invasion, it is not surprising that RAS has been shown in several studies to be associated with pregnancy complications; such as preeclampsia (17). Therefore, our studies have endeavored to address the regulation of the

uteroplacental RAS during gestation, as the exact mechanisms of action by which the uteroplacental RAS mediates pregnancy pathologies remains unclear. Further investigation of the uteroplacental RAS in both normal and abnormal pregnancies, may contribute to advancing management of abnormal pregnancies and improving pregnancy outcomes.

6.1 Placental RAS

We investigated the placental RAS using two trophoblast cell lines previously employed to study placental function. Our studies however indicated that the HTR-8/SVneo and BeWo cell lines were not good models for studying the placental RAS, due to each cell line only expressing part of the RAS, whereas the human placenta contains all RAS genes and proteins (13). These cell lines may, however, be well suited for investigating the effects of various RAS pathways independently, since the proliferative, pro-angiogenic renin/Ang II/AT₁R pathway is only expressed in the HTR-8/SVneo cells, whereas the anti-proliferative Ang 1-7/Mas receptor pathway is expressed only in the BeWo cells. Further investigation of these RAS pathways may help our understanding of the role(s) of the RAS pathways in normal placentation and development. These RAS pathways also have implications in abnormal proliferation and angiogenesis, which are important areas of study for metastatic spread and development of malignant cancers.

We are the first to identify a prorenin/AGT/ACE/Ang II/AT₁R pathway within the HTR-8/SVneo cells. The identification of this pro-angiogenic/proliferative pathway is consistent with previous findings that show that the HTR-8/SVneo cell line is an useful model of trophoblast invasion and migration because Ang II acting

on the AT₁R is associated with trophoblast proliferation (160) and angiogenesis (110). However, BeWo cells did not express *REN* or *ACE1*, however it did express genes of the AGT/ACE 2/Ang 1–7/Mas receptor pathway. This pathway is typically anti-proliferative (79) and anti-angiogenic (161). Initially these results may seem conflicting, given that the cell line is derived from a choriocarcinoma (a malignant trophoblastic cancer). One must consider however that the Ang 1–7/Mas receptor pathway is also responsible for vasodilation (95, 162), therefore activation of this pathway could increase blood flow and promote perfusion. One explanation for the expression of the Ang 1–7/Mas receptor pathway within the BeWo cell line may be that endometrium (choriocarcinomas that are formed after pregnancy or miscarriage often are located in the uterus (163)) unlike the decidua, does not have a dense cellular matrix (164), in addition, choriocarcinomas are extremely fast growing, in some cases, the growth is so rapid that it outgrows its own blood supply, which results in spontaneous regression (165, 166). Therefore increased blood flow in the surrounding vessels may be more beneficial for growth than extensive tissue invasion.

We explored the effects of upregulating RAS activity within the two trophoblast cell lines. We hypothesised that the RAS of the HTR–8/SVneo cell line may be suppressed, given that the HTR–8/SVneo cells are an immortalised 3rd trimester cell line, at which time the major portion of trophoblast proliferation, invasion and migration has been completed. Several agents which may promote *REN* expression were tested, along with cAMP, a known regulator of renal renin (99, 115, 116), to determine if the RAS within the HTR–8/SVneo cell line could be stimulated. We demonstrated that in HTR–8/SVneo cells, cAMP stimulation

upregulated *REN* expression and prorenin protein, as well as *AGTR1* expression, all of which could be associated with cell proliferation and angiogenesis, since the renin, Ang II, AT₁R pathway is associated with cell proliferation and angiogenesis (110, 160). Furthermore, MPA and E₂ consistently stimulated *REN* and prorenin protein in the HTR-8/SVneo cells. We propose that these agents function by stimulating secondary messenger cAMP binding to the CRE of the *REN* gene.

On the other hand, BeWo cells did not express *REN* and prorenin protein. More surprisingly, *REN* could not be induced in the BeWo cells with cAMP, sex steroids or global hypomethylation. Our study demonstrated that cAMP stimulated *REN* and prorenin protein within trophoblast cells, however whether or not these changes in gene expression and protein synthesis; translates into increased trophoblast proliferation and migration requires further investigation using angiogenesis assays.

6.2 Decidual RAS

We have established and validated an explant model to explore the regulation of the RAS within the human decidua. We demonstrated that the decidua contains all the RAS component genes, prorenin protein, Ang II and Ang 1–7 peptides, although the Mas receptor is very low in abundance or not present. We did however observe differences in RAS gene expression and prorenin protein that were determined by the sex of the fetus. These fetal sex-specific differences in *REN* were found in non-incubated decidual samples and after explant culture in the absence of steroid hormones; thereby demonstrating that it was not due to fetal or maternal sex hormones *in vivo*.

Surprisingly, in view of the effects of cAMP on HTR-8/SVneo cells, decidual *REN* and prorenin protein were not upregulated in response to cAMP stimulation. cAMP treatment did not alter the fetal sex specific differences in *REN* expression, it did however reduce *REN* mRNA abundance in decidua from women carrying female fetuses. More significantly, cAMP treatment abolished the sex-specific differences in prorenin protein secretion into the supernatant. cAMP treatment also abolished the sex-specific differences in expression of *AGT*, *ATP6AP2* and *MAS1* and upregulated *AGTR1* expression in decidua from only women carrying male fetuses.

We looked for and found fetal cells in the human decidua, however their role in determining the decidual expression of *REN* and release of prorenin is unknown. They are unlikely to be a source of prorenin because chorion has a very low level of *REN* expression and choriodecidual explants had significantly lower levels of *REN* expression than our decidual explants (unpublished observations). Therefore in future studies, we would like to isolate specific cell types from human decidua and establish individual cultures of these cells, in order to further investigate the basis for the sex-specific differences in the decidual RAS. In addition, we need to determine the effects of progesterone and estrogen on the decidual RAS, as the decidua *in vivo* is normally exposed to these sex steroids. We have demonstrated that MPA and E₂ stimulation promoted *REN* expression and prorenin protein in the HTR-8/SVneo cell line.

6.3 Conclusion

This thesis has attempted to address some of the gaps in our understanding of the uteroplacental RAS, by separately investigating both the decidual and placental RAS from normal pregnancies and exploring how the RAS is regulated.

Our studies have demonstrated that HTR-8/SVneo and BeWo cells can not represent the complete RAS within the placenta; therefore a placental explant model is required for further investigation of the placental RAS. Recently a group have investigated the RAS within the human placental cell line CRL-7548 and have shown *REN*, *AGT*, *ACE1* and *AGTR1* were expressed, in addition, Ang II peptides and AT₁R protein were detected (167) suggesting that this cell line, as well as the placental explant cultures may serve as good models of the placental RAS. Further validation is required to determine if this cell line contains all the components of the modern RAS, as outlined in Figure 1.3.

We have established the gene expression profile and prorenin protein profile within the maternal decidua and decidual explants, which serves as reference for comparing the decidual RAS from pregnancies with abnormal outcomes. It is important that further research into RAS function and regulation during pregnancy is conducted, since it is highly likely that the decidual and placental RAS do not function in isolation and may influence each other. Furthermore, each tissue RAS may even exert actions on adjacent tissues such as amnion and on maternal cardiovascular system, as well as, renal function. Therefore, it is important to know how the maternal decidua and the fetal tissues interact, in order to understand how they contribute to pregnancy outcomes, which have far reaching social and economic consequences for both mother and the fetus.

REFERENCES

1. **Liu J, Matsuo H, Laoag-Fernandez JB, Xu Q, Maruo T** 2007 The effects of progesterone on apoptosis in the human trophoblast-derived HTR-8/SV neo cells. *Mol Hum Reprod* 13:869-874
2. **Moffett-King A** 2002 Natural killer cells and pregnancy. *Nature reviews Immunology* 2:656-663
3. **Nicola C, Chirpac A, Lala PK, Chakraborty C** 2008 Roles of Rho guanosine 5'-triphosphatase A, Rho kinases, and extracellular signal regulated kinase (1/2) in prostaglandin E2-mediated migration of first-trimester human extravillous trophoblast. *Endocrinology* 149:1243-1251
4. **Jensen JA** 1964 Tissue Culture of Fetal Membranes with Special Reference to Hormonal Activity. *Obstet Gynecol* 23:935-937
5. **Poisner AM** 1998 The human placental renin-angiotensin system. *Frontiers in neuroendocrinology* 19:232-252
6. **Gray LD** Drawing of placental membranes. In. <http://www.asm.org/>: TriHealth Laboratories, Bethesda Oak Hospital, Cincinnati, OH
7. **Neves FA, Duncan KG, Baxter JD** 1996 Cathepsin B is a prorenin processing enzyme. *Hypertension* 27:514-517
8. **Reudelhuber TL, Ramla D, Chiu L, Mercure C, Seidah NG** 1994 Proteolytic processing of human prorenin in renal and non-renal tissues. *Kidney international* 46:1522-1524
9. **George AJ, Thomas WG, Hannan RD** 2010 The renin-angiotensin system and cancer: old dog, new tricks. *Nature reviews Cancer* 10:745-759
10. **Mehta JL, Khaidakov M, Hu CP, Spagnoli G, Li DY** 2009 Upregulation of Angiotensin II Type 2 Receptor (agtr2) Attenuates Atherosclerotic Lesion Formation and Enhances Apoptosis in the LDL Receptor Knockout Mice Fed High Cholesterol Diet. In: Frohlich ED, Re RN eds. *The local cardiac renin-angiotensin aldosterone system*. 2nd ed. New York: Springer; 151-163
11. **Binder ND, Laird MR, Faber JJ** 1995 Interrelationships between the renin angiotensin system and uteroplacental blood flow--a recent perspective. *Reprod Fertil Dev* 7:1437-1442
12. **Morgan T, Craven C, Ward K** 1998 Human spiral artery renin-angiotensin system. *Hypertension* 32:683-687
13. **Pringle KG, Tadros MA, Callister RJ, Lumbers ER** 2011 The expression and localization of the human placental prorenin/renin-angiotensin system throughout pregnancy: roles in trophoblast invasion and angiogenesis? *Placenta* 32:956-962
14. **Anton L, Merrill DC, Neves LA, Diz DI, Corthorn J, Valdes G, Stovall K, Gallagher PE, Moorefield C, Gruver C, Brosnihan KB** 2009 The uterine placental bed Renin-Angiotensin system in normal and preeclamptic pregnancy. *Endocrinology* 150:4316-4325
15. **Irani RA, Xia Y** 2011 Renin angiotensin signaling in normal pregnancy and preeclampsia. *Semin Nephrol* 31:47-58
16. **Kaufmann P, Black S, Huppertz B** 2003 Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia. *Biology of reproduction* 69:1-7
17. **Herse F, Staff AC, Hering L, Muller DN, Luft FC, Dechend R** 2008 AT1-receptor autoantibodies and uteroplacental RAS in pregnancy and pre-eclampsia. *Journal of molecular medicine* 86:697-703

18. **Rodriguez M, Moreno J, Hasbun J** 2012 RAS in Pregnancy and Preeclampsia and Eclampsia. *International journal of hypertension* 2012:739274
19. **Bullo M, Tschumi S, Bucher BS, Bianchetti MG, Simonetti GD** 2012 Pregnancy outcome following exposure to angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists: a systematic review. *Hypertension* 60:444-450
20. **Tufro-McReddie A, Romano LM, Harris JM, Ferder L, Gomez RA** 1995 Angiotensin II regulates nephrogenesis and renal vascular development. *The American journal of physiology* 269:F110-115
21. **Guron G, Friberg P** 2000 An intact renin-angiotensin system is a prerequisite for normal renal development. *Journal of hypertension* 18:123-137
22. **Wintour EM, Alcorn D, Albiston A, Boon WC, Butkus A, Earnest L, Moritz K, Shandley L** 1998 The renin-angiotensin system and the development of the kidney and adrenal in sheep. *Clinical and experimental pharmacology & physiology Supplement* 25:S97-100
23. **Guron G, Sundelin B, Wickman A, Friberg P** 1998 Angiotensin-converting enzyme inhibition in piglets induces persistent renal abnormalities. *Clinical and experimental pharmacology & physiology* 25:88-91
24. **Mitchell MD, Edwin SS, Pollard JK, Trautman MS** 1996 Renin stimulates decidual prostaglandin production via a novel mechanism that is independent of angiotensin II formation. *Placenta* 17:299-305
25. **Pringle KG, Zakar T, Yates D, Mitchell CM, Hirst JJ, Lumbers ER** 2011 Molecular evidence of a (pro)renin/ (pro)renin receptor system in human intrauterine tissues in pregnancy and its association with PGHS-2. *J Renin Angiotensin Aldosterone Syst* 12:304-310
26. **Mozurkewich EL, Chilimigras JL, Berman DR, Perni UC, Romero VC, King VJ, Keeton KL** 2011 Methods of induction of labour: a systematic review. *BMC pregnancy and childbirth* 11:84
27. **Gur Y, Breitbart H, Lax Y, Rubinstein S, Zamir N** 1998 Angiotensin II induces acrosomal exocytosis in bovine spermatozoa. *The American journal of physiology* 275:E87-93
28. **Vinson GP, Mehta J, Evans S, Matthews S, Puddefoot JR, Saridogan E, Holt WV, Djahanbakhch O** 1996 Angiotensin II stimulates sperm motility. *Regulatory peptides* 67:131-135
29. **Vinson GP, Puddefoot JR, Ho MM, Barker S, Mehta J, Saridogan E, Djahanbakhch O** 1995 Type 1 angiotensin II receptors in rat and human sperm. *The Journal of endocrinology* 144:369-378
30. **Kohn FM, Muller C, Drescher D, Neukamm C, el Mulla KF, Henkel R, Hagele W, Hinsch E, Habenicht UF, Schill WB** 1998 Effect of angiotensin converting enzyme (ACE) and angiotensins on human sperm functions. *Andrologia* 30:207-215
31. **Krege JH, John SW, Langenbach LL, Hodgin JB, Hagaman JR, Bachman ES, Jennette JC, O'Brien DA, Smithies O** 1995 Male-female differences in fertility and blood pressure in ACE-deficient mice. *Nature* 375:146-148
32. **Ramaraj P, Kessler SP, Colmenares C, Sen GC** 1998 Selective restoration of male fertility in mice lacking angiotensin-converting enzymes by sperm-specific expression of the testicular isozyme. *J Clin Invest* 102:371-378
33. **Fazleabas AT, Strakova Z** 2002 Endometrial function: cell specific changes in the uterine environment. *Molecular and cellular endocrinology* 186:143-147
34. **Gellersen B, Brosens IA, Brosens JJ** 2007 Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Seminars in reproductive medicine* 25:445-453
35. **Banerjee P, Fazleabas AT** 2010 Endometrial responses to embryonic signals in the primate. *The International journal of developmental biology* 54:295-302

36. **Kaloglu C, Onarlioglu B** 2010 Extracellular matrix remodelling in rat endometrium during early pregnancy: the role of fibronectin and laminin. *Tissue & cell* 42:301-306
37. **Makker A, Singh MM, Chowdhury SR, Maitra SC, Kamboj VP** 1994 Uterine estradiol and progesterone receptor concentration in relation to circulating hormone levels and histoarchitecture during high endometrial sensitivity and induced decidualization in guinea pigs. *The Journal of steroid biochemistry and molecular biology* 48:535-543
38. **Gellersen B, Brosens J** 2003 Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair. *The Journal of endocrinology* 178:357-372
39. **Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, Prus D, Cohen-Daniel L, Arnon TI, Manaster I, Gazit R, Yutkin V, Benharroch D, Porgador A, Keshet E, Yagel S, Mandelboim O** 2006 Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nature medicine* 12:1065-1074
40. **Anteby EY, Greenfield C, Natanson-Yaron S, Goldman-Wohl D, Hamani Y, Khudyak V, Ariel I, Yagel S** 2004 Vascular endothelial growth factor, epidermal growth factor and fibroblast growth factor-4 and -10 stimulate trophoblast plasminogen activator system and metalloproteinase-9. *Mol Hum Reprod* 10:229-235
41. **Staun-Ram E, Shalev E** 2005 Human trophoblast function during the implantation process. *Reproductive biology and endocrinology : RB&E* 3:56
42. **Vassalli JD, Sappino AP, Belin D** 1991 The plasminogen activator/plasmin system. *J Clin Invest* 88:1067-1072
43. **Yeh CC, Chao KC, Huang SJ** 2013 Innate immunity, decidual cells, and preeclampsia. *Reproductive sciences* 20:339-353
44. **Kajihara T, Uchino S, Suzuki M, Itakura A, Brosens JJ, Ishihara O** 2011 Human chorionic gonadotropin confers resistance to oxidative stress-induced apoptosis in decidualizing human endometrial stromal cells. *Fertility and sterility* 95:1302-1307
45. **Nancy P, Tagliani E, Tay CS, Asp P, Levy DE, Erlebacher A** 2012 Chemokine gene silencing in decidual stromal cells limits T cell access to the maternal-fetal interface. *Science* 336:1317-1321
46. **Cartwright JE, Keogh RJ, Tissot van Patot MC** 2007 Hypoxia and placental remodelling. *Advances in experimental medicine and biology* 618:113-126
47. **Kublickiene KR, Lindblom B, Kruger K, Nisell H** 2000 Preeclampsia: evidence for impaired shear stress-mediated nitric oxide release in uterine circulation. *American journal of obstetrics and gynecology* 183:160-166
48. **VanWijk MJ, Kublickiene K, Boer K, VanBavel E** 2000 Vascular function in preeclampsia. *Cardiovascular research* 47:38-48
49. **Gerretsen G, Huisjes HJ, Hardonk MJ, Elema JD** 1983 Trophoblast alterations in the placental bed in relation to physiological changes in spiral arteries. *British journal of obstetrics and gynaecology* 90:34-39
50. **Abrams ET, Rutherford JN** 2011 Framing postpartum hemorrhage as a consequence of human placental biology: an evolutionary and comparative perspective. *American anthropologist* 113:417-430
51. **Tantbirojn P, Crum CP, Parast MM** 2008 Pathophysiology of placenta creta: the role of decidua and extravillous trophoblast. *Placenta* 29:639-645
52. **Clifton VL** 2010 Review: Sex and the human placenta: mediating differential strategies of fetal growth and survival. *Placenta* 31 Suppl:S33-39
53. **Byrne J, Warburton D** 1987 Male excess among anatomically normal fetuses in spontaneous abortions. *Am J Med Genet* 26:605-611

54. **Mizuno R** 2000 The male/female ratio of fetal deaths and births in Japan. *Lancet* 356:738-739
55. **Engel PJ, Smith R, Brinsmead MW, Bowe SJ, Clifton VL** 2008 Male sex and pre-existing diabetes are independent risk factors for stillbirth. *Aust N Z J Obstet Gynaecol* 48:375-383
56. **Smith GC** 2000 Sex, birth weight, and the risk of stillbirth in Scotland, 1980-1996. *Am J Epidemiol* 151:614-619
57. **Cooperstock M, Campbell J** 1996 Excess males in preterm birth: interactions with gestational age, race, and multiple birth. *Obstet Gynecol* 88:189-193
58. **Zeitlin J, Saurel-Cubizolles MJ, De Mouzon J, Rivera L, Ancel PY, Blondel B, Kaminski M** 2002 Fetal sex and preterm birth: are males at greater risk? *Hum Reprod* 17:2762-2768
59. **Melamed N, Yogev Y, Glezerman M** 2010 Fetal gender and pregnancy outcome. *J Matern Fetal Neonatal Med* 23:338-344
60. **Vatten LJ, Skjaerven R** 2004 Offspring sex and pregnancy outcome by length of gestation. *Early Hum Dev* 76:47-54
61. **Di Renzo GC, Rosati A, Sarti RD, Cruciani L, Cutuli AM** 2007 Does fetal sex affect pregnancy outcome? *Gend Med* 4:19-30
62. **Brettell R, Yeh PS, Impey LW** 2008 Examination of the association between male gender and preterm delivery. *Eur J Obstet Gynecol Reprod Biol* 141:123-126
63. **Lieberman E, Lang JM, Cohen AP, Frigoletto FD, Jr., Acker D, Rao R** 1997 The association of fetal sex with the rate of cesarean section. *American journal of obstetrics and gynecology* 176:667-671
64. **Phillips MI, Schmidt-Ott KM** 1999 The Discovery of Renin 100 Years Ago. *News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society* 14:271-274
65. **Bourque CW** 2008 Central mechanisms of osmosensation and systemic osmoregulation. *Nature reviews Neuroscience* 9:519-531
66. **De Champlain J, Van Ameringen MR** 1972 Regulation of blood pressure by sympathetic nerve fibers and adrenal medulla in normotensive and hypertensive rats. *Circ Res* 31:617-628
67. **MacGregor GA, Markandu ND, Roulston JE, Jones JC, Morton JJ** 1981 Maintenance of blood pressure by the renin-angiotensin system in normal man. *Nature* 291:329-331
68. **Ozono R, Wang ZQ, Moore AF, Inagami T, Siragy HM, Carey RM** 1997 Expression of the subtype 2 angiotensin (AT2) receptor protein in rat kidney. *Hypertension* 30:1238-1246
69. **Bader M, Ganten D** 2008 Update on tissue renin-angiotensin systems. *Journal of molecular medicine* 86:615-621
70. **Danser AH, Deinum J** 2005 Renin, prorenin and the putative (pro)renin receptor. *Hypertension* 46:1069-1076
71. **Jan Danser AH, Saris JJ** 2002 Prorenin uptake in the heart: a prerequisite for local angiotensin generation? *Journal of molecular and cellular cardiology* 34:1463-1472
72. **Yosipiv IV, el-Dahr SS** 1996 Activation of angiotensin-generating systems in the developing rat kidney. *Hypertension* 27:281-286
73. **Marques FZ, Pringle KG, Conquest A, Hirst JJ, Markus MA, Sarris M, Zakar T, Morris BJ, Lumbers ER** 2011 Molecular characterization of renin-angiotensin system components in human intrauterine tissues and fetal membranes from vaginal delivery and cesarean section. *Placenta* 32:214-221

74. **Nguyen G, Delarue F, Burckle C, Bouzahir L, Giller T, Sraer JD** 2002 Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J Clin Invest* 109:1417-1427
75. **Nguyen G** 2011 Renin, (pro)renin and receptor: an update. *Clinical science* 120:169-178
76. **Huang Y, Wongamorntham S, Kasting J, McQuillan D, Owens RT, Yu L, Noble NA, Border W** 2006 Renin increases mesangial cell transforming growth factor-beta1 and matrix proteins through receptor-mediated, angiotensin II-independent mechanisms. *Kidney international* 69:105-113
77. **Scheffe JH, Menk M, Reinemund J, Effertz K, Hobbs RM, Pandolfi PP, Ruiz P, Unger T, Funke-Kaiser H** 2006 A novel signal transduction cascade involving direct physical interaction of the renin/prorenin receptor with the transcription factor promyelocytic zinc finger protein. *Circ Res* 99:1355-1366
78. **Herr D, Rodewald M, Fraser HM, Hack G, Konrad R, Kreienberg R, Wulff C** 2008 Regulation of endothelial proliferation by the renin-angiotensin system in human umbilical vein endothelial cells. *Reproduction* 136:125-130
79. **Gallagher PE, Tallant EA** 2004 Inhibition of human lung cancer cell growth by angiotensin-(1-7). *Carcinogenesis* 25:2045-2052
80. **Lumbers ER** 1971 Activation of renin in human amniotic fluid by low pH. *Enzymologia* 40:329-336
81. **Saris JJ, van den Eijnden MM, Lamers JM, Saxena PR, Schalekamp MA, Danser AH** 2002 Prorenin-induced myocyte proliferation: no role for intracellular angiotensin II. *Hypertension* 39:573-577
82. **Batenburg WW, Danser AJ** 2008 Prorenin and the (pro)renin receptor: binding kinetics, signalling and interaction with aliskiren. *J Renin Angiotensin Aldosterone Syst* 9:181-184
83. **Danser AH** 2007 Novel drugs targeting hypertension: renin inhibitors. *Journal of cardiovascular pharmacology* 50:105-111
84. **Saris JJ, t Hoen PA, Garrelds IM, Dekkers DH, den Dunnen JT, Lamers JM, Jan Danser AH** 2006 Prorenin induces intracellular signaling in cardiomyocytes independently of angiotensin II. *Hypertension* 48:564-571
85. **Burridge K, Wennerberg K** 2004 Rho and Rac take center stage. *Cell* 116:167-179
86. **Martin MM, Buckenberger JA, Jiang J, Malana GE, Knoell DL, Feldman DS, Elton TS** 2007 TGF-beta1 stimulates human AT1 receptor expression in lung fibroblasts by cross talk between the Smad, p38 MAPK, JNK, and PI3K signaling pathways. *American journal of physiology Lung cellular and molecular physiology* 293:L790-799
87. **Isenovic ER, Jacobs DB, Kedeas MH, Sha Q, Milivojevic N, Kawakami K, Gick G, Sowers JR** 2004 Angiotensin II regulation of the Na⁺ pump involves the phosphatidylinositol-3 kinase and p42/44 mitogen-activated protein kinase signaling pathways in vascular smooth muscle cells. *Endocrinology* 145:1151-1160
88. **Cruciat CM, Ohkawara B, Acebron SP, Karaulanov E, Reinhard C, Ingelfinger D, Boutros M, Niehrs C** 2010 Requirement of prorenin receptor and vacuolar H⁺-ATPase-mediated acidification for Wnt signaling. *Science* 327:459-463
89. **Senbonmatsu T, Saito T, Landon EJ, Watanabe O, Price E, Jr., Roberts RL, Imboden H, Fitzgerald TG, Gaffney FA, Inagami T** 2003 A novel angiotensin II type 2 receptor signaling pathway: possible role in cardiac hypertrophy. *The EMBO journal* 22:6471-6482
90. **Padia SH, Kemp BA, Howell NL, Fournie-Zaluski MC, Roques BP, Carey RM** 2008 Conversion of renal angiotensin II to angiotensin III is critical for AT2 receptor-mediated natriuresis in rats. *Hypertension* 51:460-465

91. **Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S** 2000 A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res* 87:E1-9
92. **Santos RA, Ferreira AJ, Simoes ESAC** 2008 Recent advances in the angiotensin-converting enzyme 2-angiotensin(1-7)-Mas axis. *Experimental physiology* 93:519-527
93. **Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R, Walther T** 2003 Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A* 100:8258-8263
94. **Santos RA, Brosnihan KB, Jacobsen DW, DiCorleto PE, Ferrario CM** 1992 Production of angiotensin-(1-7) by human vascular endothelium. *Hypertension* 19:II56-61
95. **Vaz-Silva J, Carneiro MM, Ferreira MC, Pinheiro SV, Silva DA, Silva-Filho AL, Witz CA, Reis AM, Santos RA, Reis FM** 2009 The vasoactive peptide angiotensin-(1-7), its receptor Mas and the angiotensin-converting enzyme type 2 are expressed in the human endometrium. *Reproductive sciences* 16:247-256
96. **Brasier AR, Li J** 1996 Mechanisms for inducible control of angiotensinogen gene transcription. *Hypertension* 27:465-475
97. **Kobori H, Navar LG** 2011 Urinary Angiotensinogen as a Novel Biomarker of Intrarenal Renin-Angiotensin System in Chronic Kidney Disease. *International review of thrombosis* 6:108-116
98. **Marques FZ, Campain AE, Tomaszewski M, Zukowska-Szczechowska E, Yang YH, Charchar FJ, Morris BJ** 2011 Gene expression profiling reveals renin mRNA overexpression in human hypertensive kidneys and a role for microRNAs. *Hypertension* 58:1093-1098
99. **Klar J, Sandner P, Muller MW, Kurtz A** 2002 Cyclic AMP stimulates renin gene transcription in juxtaglomerular cells. *Pflugers Arch* 444:335-344
100. **Germain S, Konoshita T, Fuchs S, Philippe J, Corvol P, Pinet F** 1997 Regulation of human renin gene transcription by cAMP. *Clin Exp Hypertens* 19:543-550
101. **Poisner AM, Thrailkill K, Poisner R, Handwerger S** 1991 Cyclic AMP as a second messenger for prorenin release from human decidual cells. *Placenta* 12:263-267
102. **Poisner AM, Downing GJ, Poisner R** 1994 Prorenin secretion from villous placenta: regulation by cyclic AMP and angiotensin. *Placenta* 15:487-499
103. **Downing GJ, Poisner AM** 1995 Stimulation of placental prorenin secretion by selective inhibition of cyclic nucleotide phosphodiesterases. *Biochemical pharmacology* 49:1675-1682
104. **Churchill PC** 1985 Second messengers in renin secretion. *The American journal of physiology* 249:F175-184
105. **Ying L, Morris BJ, Sigmund CD** 1997 Transactivation of the human renin promoter by the cyclic AMP/protein kinase A pathway is mediated by both cAMP-responsive element binding protein-1 (CREB)-dependent and CREB-independent mechanisms in Calu-6 cells. *J Biol Chem* 272:2412-2420
106. **Downing GJ, Maulik D, Poisner AM** 1996 Human chorionic gonadotropin stimulates placental prorenin secretion: evidence for autocrine/paracrine regulation. *J Clin Endocrinol Metab* 81:1027-1030
107. **Itskovitz J, Sealey JE, Glorioso N, Rosenwaks Z** 1987 Plasma prorenin response to human chorionic gonadotropin in ovarian-hyperstimulated women: correlation with the number of ovarian follicles and steroid hormone concentrations. *Proc Natl Acad Sci USA* 84:7285-7289

108. **Hunter MG, Baker TG** 1981 Effect of hCG, cAMP and FSH on steroidogenesis by human corpora lutea in vitro. *J Reprod Fertil* 63:285-288
109. **Sealey JE, Itskovitz-Eldor J, Rubattu S, James GD, August P, Thaler I, Levrone J, Laragh JH** 1994 Estradiol- and progesterone-related increases in the renin-aldosterone system: studies during ovarian stimulation and early pregnancy. *J Clin Endocrinol Metab* 79:258-264
110. **Buharalioglu CK, Song CY, Yaghini FA, Ghafoor HU, Motiwala M, Adris T, Estes AM, Malik KU** 2011 Angiotensin II-induced process of angiogenesis is mediated by spleen tyrosine kinase via VEGF receptor-1 phosphorylation. *Am J Physiol Heart Circ Physiol* 301:H1043-1055
111. **Williams PJ, Mistry HD, Innes BA, Bulmer JN, Pipkin FB** 2010 Expression of AT1R, AT2R and AT4R and their roles in extravillous trophoblast invasion in the human. *Placenta* 31:448-455
112. **Araki-Taguchi M, Nomura S, Ino K, Sumigama S, Yamamoto E, Kotani-Ito T, Hayakawa H, Kajiyama H, Shibata K, Itakura A, Kikkawa F** 2008 Angiotensin II mimics the hypoxic effect on regulating trophoblast proliferation and differentiation in human placental explant cultures. *Life Sci* 82:59-67
113. **Sampaio WO, Henrique de Castro C, Santos RA, Schiffrin EL, Touyz RM** 2007 Angiotensin-(1-7) counterregulates angiotensin II signaling in human endothelial cells. *Hypertension* 50:1093-1098
114. **Derckx FH, Alberda AT, de Jong FH, Zeilmaker FH, Makovitz JW, Schalekamp MA** 1987 Source of plasma prorenin in early and late pregnancy: observations in a patient with primary ovarian failure. *J Clin Endocrinol Metab* 65:349-354
115. **Pinet F, Mizrahi J, Menard J, Corvol P** 1986 Role of cyclic AMP in renin secretion by human transfected juxtaglomerular cells. *J Hypertens Suppl* 4:S421-423
116. **Pinet F, Mizrahi J, Laboulandine I, Menard J, Corvol P** 1987 Regulation of prorenin secretion in cultured human transfected juxtaglomerular cells. *J Clin Invest* 80:724-731
117. **Graham CH, Hawley TS, MacDougall RC, Kerbel RS, Khoo N, Lala PK** 1993 Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Experimental Cell Research* 206:204-211
118. **Pattillo RA, Gey GO, Delfs E, Mattingly RF** 1968 Human hormone production in vitro. *Science* 159:1467-1469
119. **Gilsbach R, Kouta M, Bonisch H, Bruss M** 2006 Comparison of in vitro and in vivo reference genes for internal standardization of real-time PCR data. *BioTechniques* 40:173-177
120. **Wang Y, Pringle KG, Sykes SD, Marques FZ, Morris BJ, Zakar T, Lumbers ER** 2012 Fetal sex affects expression of renin-angiotensin system components in term human decidua. *Endocrinology* 153:462-468
121. **Anene-Maidoh OT, Greene AS** 2009 Angiotensin 1-7 treatment induces apoptosis in human umbilical vein endothelial cells. *FASEB J Meeting abstract*:626.614
122. **Silvestre JS, Tamarat R, Senbonmatsu T, Iccchiki T, Ebrahimian T, Iglarz M, Besnard S, Duriez M, Inagami T, Levy BI** 2002 Antiangiogenic effect of angiotensin II type 2 receptor in ischemia-induced angiogenesis in mice hindlimb. *Circ Res* 90:1072-1079
123. **Chen X, Nishimura K, Hasna J, Kobayashi S, Shikasho T, Kanaide H** 1994 Protein Kinase C and protein kinase A regulate the expression of angiotensin II receptor mRNA in smooth muscle cells. *European journal of pharmacology* 15:175-183
124. **Wolf RL, Mendlowitz M, Pick J, Gitlow SE, Naftchi N** 1962 Metabolism and distribution of I-131-labeled angiotensin II. *J Lab Clin Med* 60:150-159

125. **Hackenthal E, Hackenthal R, Hilgenfeldt U** 1978 Isorenin, pseudorenin, cathepsin D and renin. A comparative enzymatic study of angiotensin-forming enzymes. *Biochimica et biophysica acta* 522:574-588
126. **Ramaha A, Patston PA** 2002 Release and degradation of angiotensin I and angiotensin II from angiotensinogen by neutrophil serine proteinases. *Arch Biochem Biophys* 397:77-83
127. **Velez JC, Bland AM, Arthur JM, Raymond JR, Janech MG** 2007 Characterization of renin-angiotensin system enzyme activities in cultured mouse podocytes. *Am J Physiol Renal Physiol* 293:398-407
128. **Crowley SD, Gurley SB, Herrera MJ, Ruiz P, Griffiths R, Kumar AP, Kim HS, Smithies O, Le TH, Coffman TM** 2006 Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. *Proc Natl Acad Sci USA* 103:17985-17990
129. **Wang Y, Pringle KG, Chen YX, Zakar T, Lumbers ER** 2012 Regulation of the renin-angiotensin system (RAS) in BeWo and HTR-8/SVneo trophoblast cell lines. *Placenta* 33:634-639
130. **Sunahori K, Juang YT, Kyttaris VC, Tsokos GC** 2011 Promoter hypomethylation results in increased expression of protein phosphatase 2A in T cells from patients with systemic lupus erythematosus. *J Immunol* 186:4508-4517
131. **Lumbers ER** 1993 The Ovary. In: Nicholls MG, Robertson JIS eds. *The Renin-Angiotensin System*. London: Gowers Medical Publishing Co.; 46.41-.12
132. **Logan CY, Nusse R** 2004 The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 20:781-810
133. **Chen JZ, Sheehan PM, Brennecke SP, Keogh RJ** 2012 Vessel remodelling, pregnancy hormones and extravillous trophoblast function. *Molecular and cellular endocrinology* 349:138-144
134. **Derx FH, Schalekamp MP, Bouma B, Kluff C, Schalekamp MA** 1982 Plasma kallikrein-mediated activation of the renin-angiotensin system does not require prior acidification of prorenin. *J Clin Endocrinol Metab* 54:343-348
135. **Derx FH, Schalekamp MP, Schalekamp MA** 1987 Two-step prorenin-renin conversion. Isolation of an intermediary form of activated prorenin. *J Biol Chem* 262:2472-2477
136. **Scheffe JH, Unger T, Funke-Kaiser H** 2008 PLZF and the (pro)renin receptor. *Journal of molecular medicine* 86:623-627
137. **Lifton RP, Jeunemaitre X** 1993 Finding genes that cause human hypertension. *Journal of hypertension* 11:231-236
138. **Gordon MS, Chin WW, Shupnik MA** 1992 Regulation of angiotensinogen gene expression by estrogen. *Journal of hypertension* 10:361-366
139. **Hollenberg NK, Williams GH, Burger B, Chenitz W, Hoosmand I, Adams DF** 1976 Renal blood flow and its response to angiotensin II. An interaction between oral contraceptive agents, sodium intake, and the renin-angiotensin system in healthy young women. *Circ Res* 38:35-40
140. **Schunkert H, Danser AH, Hense HW, Derx FH, Kurzinger S, Riegger GA** 1997 Effects of estrogen replacement therapy on the renin-angiotensin system in postmenopausal women. *Circulation* 95:39-45
141. **Cooper AC, Robinson G, Vinson GP, Cheung WT, Broughton Pipkin F** 1999 The localization and expression of the renin-angiotensin system in the human placenta throughout pregnancy. *Placenta* 20:467-474
142. **Dostal DE, Baker KM** 1999 The cardiac renin-angiotensin system: conceptual, or a regulator of cardiac function? *Circ Res* 85:643-650
143. **Engeli S, Negrel R, Sharma AM** 2000 Physiology and pathophysiology of the adipose tissue renin-angiotensin system. *Hypertension* 35:1270-1277

144. **Wagner J, Jan Danser AH, Derkx FH, de Jong TV, Paul M, Mullins JJ, Schalekamp MA, Ganten D** 1996 Demonstration of renin mRNA, angiotensinogen mRNA, and angiotensin converting enzyme mRNA expression in the human eye: evidence for an intraocular renin-angiotensin system. *Br J Ophthalmol* 80:159-163
145. **Brar HS, Do YS, Tam HB, Valenzuela GJ, Murray RD, Longo LD, Yonekura ML, Hsueh WA** 1986 Uteroplacental unit as a source of elevated circulating prorenin levels in normal pregnancy. *Am J Obstet Gynecol* 155:1223-1226
146. **Shaw KJ, Do YS, Kjos S, Anderson PW, Shinagawa T, Dubeau L, Hsueh WA** 1989 Human decidua is a major source of renin. *J Clin Invest* 83:2085-2092
147. **Johnson RF, Mitchell CM, Clifton V, Zakar T** 2004 Regulation of 15-hydroxyprostaglandin dehydrogenase (PGDH) gene activity, messenger ribonucleic acid processing, and protein abundance in the human chorion in late gestation and labor. *J Clin Endocrinol Metab* 89:5639-5648
148. **Skinner SL, Lumbers ER, Symonds EM** 1968 Renin concentration in human fetal and maternal tissues. *Am J Obstet Gynecol* 101:529-533
149. **Takimoto E, Ishida J, Sugiyama F, Horiguchi H, Murakami K, Fukamizu A** 1996 Hypertension induced in pregnant mice by placental renin and maternal angiotensinogen. *Science* 274:995-998
150. **McMullen JR, Gibson KJ, Lumbers ER, Burrell JH, Wu J** 1999 Interactions between AT1 and AT2 receptors in uterine arteries from pregnant ewes. *Eur J Pharmacol* 378:195-202
151. **Schuster N, Kriegelstein K** 2002 Mechanisms of TGF-beta-mediated apoptosis. *Cell Tissue Res* 307:1-14
152. **Jan Danser AH, Batenburg WW, van Esch JH** 2007 Prorenin and the (pro)renin receptor--an update. *Nephrol Dial Transplant* 22:1288-1292
153. **Keelan JA, Wang K, Chaiworapongsa T, Romero R, Mitchell MD, Sato TA, Brown DA, Fairlie WD, Breit SN** 2003 Macrophage inhibitory cytokine 1 in fetal membranes and amniotic fluid from pregnancies with and without preterm labour and premature rupture of membranes. *Mol Hum Reprod* 9:535-540
154. **Wang Y, Pringle KG, Lumbers ER** 2013 The effects of cyclic AMP, sex steroids and global hypomethylation on the expression of genes controlling the activity of the renin-angiotensin system in placental cell lines. *Placenta* 34:275-280
155. **Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, Wainscoat JS, Johnson PJ, Chang AM, Hjelm NM** 1998 Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *American journal of human genetics* 62:768-775
156. **Rasini E, Cosentino M, Marino F, Legnaro M, Ferrari M, Guasti L, Venco A, Lecchini S** 2006 Angiotensin II type 1 receptor expression on human leukocyte subsets: a flow cytometric and RT-PCR study. *Regulatory peptides* 134:69-74
157. **Austin CR, Short RV** 1972 Species differences. In: *Reproduction in mammals*. Cambridge, Eng.: University Press; 24
158. **Morgan T, Craven C, Nelson L, Lalouel JM, Ward K** 1997 Angiotensinogen T235 expression is elevated in decidual spiral arteries. *J Clin Invest* 100:1406-1415
159. **Ishimatsu S, Itakura A, Okada M, Kotani T, Iwase A, Kajiyama H, Ino K, Kikkawa F** 2006 Angiotensin II augmented migration and invasion of choriocarcinoma cells involves PI3K activation through the AT1 receptor. *Placenta* 27:587-591
160. **Tower CL, Lui S, Charlesworth NR, Smith SD, Aplin JD, Jones RL** 2010 Differential expression of angiotensin II type 1 and type 2 receptors at the maternal-fetal interface: potential roles in early placental development. *Reproduction* 140:931-942

161. **Soto-Pantoja DR, Menon J, Gallagher PE, Tallant EA** 2009 Angiotensin-(1-7) inhibits tumor angiogenesis in human lung cancer xenografts with a reduction in vascular endothelial growth factor. *Molecular cancer therapeutics* 8:1676-1683
162. **da Costa Goncalves AC, Leite R, Fraga-Silva RA, Pinheiro SV, Reis AB, Reis FM, Touyz RM, Webb RC, Alenina N, Bader M, Santos RA** 2007 Evidence that the vasodilator angiotensin-(1-7)-Mas axis plays an important role in erectile function. *Am J Physiol Heart Circ Physiol* 293:H2588-2596
163. **Baltazar JC** 1976 Epidemiological features of choriocarcinoma. *Bulletin of the World Health Organization* 54:523-532
164. **Kliman HJ** 2000 Uteroplacental blood flow. The story of decidualization, menstruation, and trophoblast invasion. *The American journal of pathology* 157:1759-1768
165. **Kim W, Rosen MA, Langer JE, Banner MP, Siegelman ES, Ramchandani P** 2007 US MR imaging correlation in pathologic conditions of the scrotum. *Radiographics : a review publication of the Radiological Society of North America, Inc* 27:1239-1253
166. **Ulbright TM** 2005 Germ cell tumors of the gonads: a selective review emphasizing problems in differential diagnosis, newly appreciated, and controversial issues. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 18 Suppl 2:S61-79
167. **Pan N, Frome WL, Dart RA, Tewksbury D, Luo J** 2013 Expression of the renin-angiotensin system in a human placental cell line. *Clinical medicine & research* 11:1-6

Appendix A

Regulation of the renin-angiotensin system (RAS) in
BeWo and HTR-8/SVneo trophoblast cell lines.



Regulation of the renin-angiotensin system (RAS) in BeWo and HTR-8/SVneo trophoblast cell lines

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ARTICLE INFO

Article history:
Accepted 7 May 2012

Keywords:
HTR-8/SVneo
BeWo
Renin-angiotensin system
Trophoblast
Placenta
cAMP

ABSTRACT

Objectives: The renin-angiotensin system (RAS) is implicated in placentation. We determined which RAS pathways are present in two trophoblast cell lines (HTR-8/SVneo and BeWo cells) and the effects of cAMP, which stimulates renal renin.

Study design: The effect of cAMP on RAS gene expression and on prorenin and angiotensin peptides in HTR-8/SVneo and BeWo cells were investigated.

Results: In HTR-8/SVneo cells, prorenin mRNA (*REN*) and protein, (pro)renin receptor (*ATP6AP2*) and angiotensin II type 1 receptor (*AGTR1*) were stimulated by cAMP ($P < 0.05$, $P < 0.05$, $P < 0.001$ and $P < 0.05$, respectively). HTR-8/SVneo cells also expressed angiotensinogen (*AGT*) and angiotensin converting enzyme 1 (*ACE1*), but did not express *AGTR2* or *ACE2* nor the Ang 1-7 receptor (*MAS1*).

BeWo cells did not express *REN*, and *REN* was not inducible by cAMP, but cAMP increased *ACE2* and *MAS1* (both $P < 0.05$) and decreased *AGT* ($P < 0.05$). BeWo cells expressed *AGT*, *ACE1*, *ACE2* and *MAS1* but not *ATP6AP2*, *AGTR1* nor *AGTR2*.

There was net destruction of Ang II in media from HTR-8/SVneo and BeWo incubations and net production of Ang 1-7 by BeWo and untreated HTR-8/SVneo cells.

Conclusion: HTR-8/SVneo cells express *REN* and produce prorenin as well as expressing other RAS genes likely to regulate Ang II/AT₁R interactions and respond to cAMP, like renal renin-secreting cells. They are more similar to early gestation placentae and are therefore useful for studying effects of renin/ACE/Ang II/AT₁R on cell function.

BeWo cells express the ACE2/Ang 1-7/Mas pathway, which is sensitive to cAMP and therefore are useful for studying the effects of ACE2/Ang 1-7/Mas on trophoblast function.

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1. Introduction

The placental renin-angiotensin system (RAS) is important in placental development as it is involved in angiogenesis [1] and modulation of placental blood flow [2], and plays a key role in the regulation of trophoblast invasion [3,4]. Disruption of this local RAS may be associated with pregnancy complications, such as preeclampsia [5,6].

The 'classical' RAS consists of renin, an enzyme secreted by the kidney that acts on angiotensinogen (Aogen) to produce angiotensin I (Ang I), which is catalysed by angiotensin converting enzyme (ACE) to form angiotensin II (Ang II). The major actions of this RAS pathway are mediated by Ang II acting on the angiotensin II type 1 receptor (AT₁R) and the Ang II type 2 receptor (AT₂R). The latter has a number of actions that oppose those mediated by Ang II acting on the AT₁R [7].

Recently, additional RAS pathways have been described. These include an Ang 1-7/Mas receptor pathway, consisting of ACE2 (a homologue of ACE), which terminates the actions of Ang II by converting it to Ang 1-7. Ang 1-7 acting through the protooncogene receptor (Mas) has effects that oppose those of Ang II acting via the AT₁R [8]. There is also a (pro)renin receptor ((P)RR) pathway, where prorenin bound to the (P)RR is nonproteolytically activated and can cleave Aogen to Ang I [9]. Prorenin was previously considered to be

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an inactive precursor of renin, having little biological activity despite the fact that its circulating levels are 10 times higher than those of renin in nonpregnant subjects [10]. Through binding to the (P)RR, prorenin acquires enzymatic activity. Additionally, it can induce intracellular signalling via angiotensin independent pathways [9,11].

Studies have shown that the RAS may be involved in the regulation of trophoblast invasion [3], as well as spiral artery remodelling [12], and consequently, may play a role in implantation and placentation. Although we have described the expression of RAS genes and proteins in the human placenta [13], the mechanisms regulating their expression are yet unknown.

Cyclic adenosine monophosphate (cAMP) stimulates prorenin mRNA (*REN*) expression in renal juxtaglomerular cells [14]. cAMP has also been shown to increase prorenin release in primary decidual cell cultures in a dose dependent manner [15]. We postulated that since *REN* contains a cAMP response element (CRE) at its promoter region [16,17], cAMP would increase expression of *REN*, as well as prorenin production. This would provide us with a tool for determining how the placental RAS regulates placental cellular function. As an initial step in determining how the placental RAS is regulated, we examined the expression of RAS genes and the secretion of prorenin and the Ang peptides, Ang II and Ang 1-7 in two trophoblast cell lines.

In this study we show that the two cell lines (HTR-8/SVneo and BeWo) express different components of the RAS pathways and report that while cAMP stimulates *REN* expression and prorenin secretion in HTR-8/SVneo cells, it does not induce *REN* expression in BeWo cells.

2. Materials and methods

2.1. Trophoblast cell culture

Two established trophoblast cell lines commonly used for studying placental function; HTR-8/SVneo and BeWo cells were used. HTR-8/SVneo cells are a transformed first trimester human extravillous trophoblast cell line (developed by Charles Graham, Ontario, Canada) [18], whilst BeWo cells are derived from a choriocarcinoma [19]. HTR-8/SVneo and BeWo cells were cultured in phenol red-free RPMI-1640 or DMEM/F-12, respectively, supplemented with 15 mM HEPES, 1.2 g/L NaHCO₃, 1 mg/mL L-glutathione reduced, 0.1 g/L albumin fraction V, 0.65 µg/mL aprotinin, 10% fetal bovine serum and 40 µg/mL gentamicin. Cells were seeded at a density of 200,000 cells, in each well of a 6 well plate with 2 mL of incubation medium. Cells were allowed to settle for 24 h, after which the media was changed, cells were treated with either 0.3 mM 8-bromo-cAMP (Sigma–Aldrich, St. Louis, MO, USA) or vehicle. Cells were harvested and the incubation media collected at 24 and 48 h and snap frozen in liquid nitrogen for subsequent protein and mRNA analyses. Three experiments were conducted in triplicate. Cell viability was verified by measuring RNA stability and quality (data not shown).

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

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). In addition, we examine each sample's RNA integrity by running samples on a gel. RNA samples were DNase treated (Qiagen N.V., Hilden, Germany) and total RNA spiked with a known amount of Alien RNA Stragene, La Jolla, CA, USA; 10^7 copies per microgram of total RNA, before the RNA is reverse transcribed using a Superscript III RT kit with random hexamers (Invitrogen). The Alien qRT PCR inhibitor alert system serves as a reference for internal standardization [20]. 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, Carlsbad, CA), RAS primers as we have described previously [13,21,22], cDNA reverse transcribed from 10 ng total RNA, and water to 10 µL. Messenger RNA abundance was calculated as described previously, using the $\Delta\Delta$ CT method, relative abundance is relative to Alien mRNA and a calibrator sample (a term placental sample collected at elective Caesarean section) [13,21,22].

2.3. Measurement of prorenin protein by ELISA

Prorenin concentration in culture media was measured using the Human Prorenin ELISA kit (Molecular Innovations Inc; Novi, MI) according to the

manufacturer's instructions as described previously [23]. Samples were assayed in duplicate. In our laboratory 1 ng/mL amniotic fluid prorenin measured using this technique generated 116 ng/h/mL of Ang I from Aogen present in nephrectomized sheep plasma used as the source of Aogen substrate. All samples were assayed on one ELISA plate. Therefore there was no inter-assay variability. Intra-assay coefficient of variation was 7.3%.

2.4. Radioimmunoassay (RIA) of Ang II and Ang 1-7

Angiotensin II was measured by radioimmunoassay (RIA) by Prosearch Pty Ltd, using the "delayed tracer addition" technique as described previously [23]. Sensitivity was 3.5 pg/mL. Cross-reactivities to Ang I, Ang 1-7 and all other pertinent hormones were 0.52%, 0.0138% and <0.1% respectively. Intra- and inter-assay coefficients of variation were 6.4% and 12%, respectively.

Ang 1-7 was assayed directly by RIA by Prosearch Pty Ltd as described previously [23]. Sensitivity was 14 pg/mL. Cross-reactivities to Ang I, Ang II, Ang III and Ang IV were 0.11%, 0.04%, 0.53% and 0.03%, respectively. Intra- and inter-assay coefficients of variation were 4.5% and 10%, respectively.

2.5. Data analysis

Mann–Whitney *U* tests were used to determine the effects of cAMP treatment on RAS mRNA abundance at 24 and 48 h incubation and on prorenin protein, Ang II and Ang 1-7 peptide levels in the supernatant after 48 h in the BeWo and HTR-8/SVneo cells. The SPSS statistical package (SPSS for Windows, Release 17.0.0. Chicago) was used for all analyses. Significance was set at $P < 0.05$.

3. Results

3.1. RAS mRNA abundance in HTR-8/SVneo and BeWo trophoblast cells and effects of cAMP

After 24 and 48 h incubation HTR-8/SVneo cells expressed detectable levels of most RAS mRNAs, namely *REN*, *AGT*, *ATP6AP2*, *ACE1* and *AGTR1* (Fig. 1). *ACE2*, *AGTR2* and *MAS1* mRNA was not detected. By contrast, in BeWo cells *REN*, *ATP6AP2*, *AGTR1* and *AGTR2* gene expression was not detected although significant amounts of *AGT*, *ACE1*, *ACE2* and *MAS1* mRNA were found after 24 and 48 h incubation (Fig. 2).

In HTR-8/SVneo cells cAMP treatment significantly increased *REN* mRNA at both 24 and 48 h (both $P < 0.001$), in addition cAMP treatment was associated with a time dependent increase in *REN* expression ($P < 0.001$, Fig. 1). At 24 h incubation only, cAMP treatment increased *ATP6AP2* and *AGTR1* mRNA abundance ($P = 0.04$ and $P = 0.02$, respectively). cAMP treatment did not have any effect on *AGT* and *ACE1* mRNA abundance (Fig. 1).

REN expression in BeWo cells was not induced with cAMP treatment. After 48 h, cAMP treated BeWo cells showed a reduction in *AGT* mRNA abundance ($P = 0.012$) but a significant increase in *ACE2* and *MAS1* mRNA abundance compared to vehicle treated cells after 24 and 48 h incubation ($P < 0.001$, $P = 0.006$; and $P < 0.001$ and $P < 0.001$, respectively), in addition cAMP treatment was associated with a time dependent decrease in *ACE2* expression ($P < 0.001$) (Fig. 2).

All RAS mRNA abundances were calculated relative to both Alien RNA and a placental sample, as such comparisons of relative gene expression levels could be made between the two cell lines. However, *AGT* mRNA was the only gene that showed any significant differences between the two cell lines. *AGT* was significantly lower in HTR-8/SVneo cells compared with BeWo cells after 24 and 48 h incubation (both $P < 0.001$) (Fig. 1B and Fig. 2A).

3.2. Prorenin, Ang II and Ang 1-7 levels in BeWo and HTR-8/SVneo cell supernatants and the effects of cAMP

Supernatants from triplicates of each of the 3 experiments were pooled and assayed for prorenin and Ang peptides. Significant amounts of prorenin were present in the supernatants of vehicle treated HTR-8/SVneo cells (Fig. 3) and cAMP treatment was

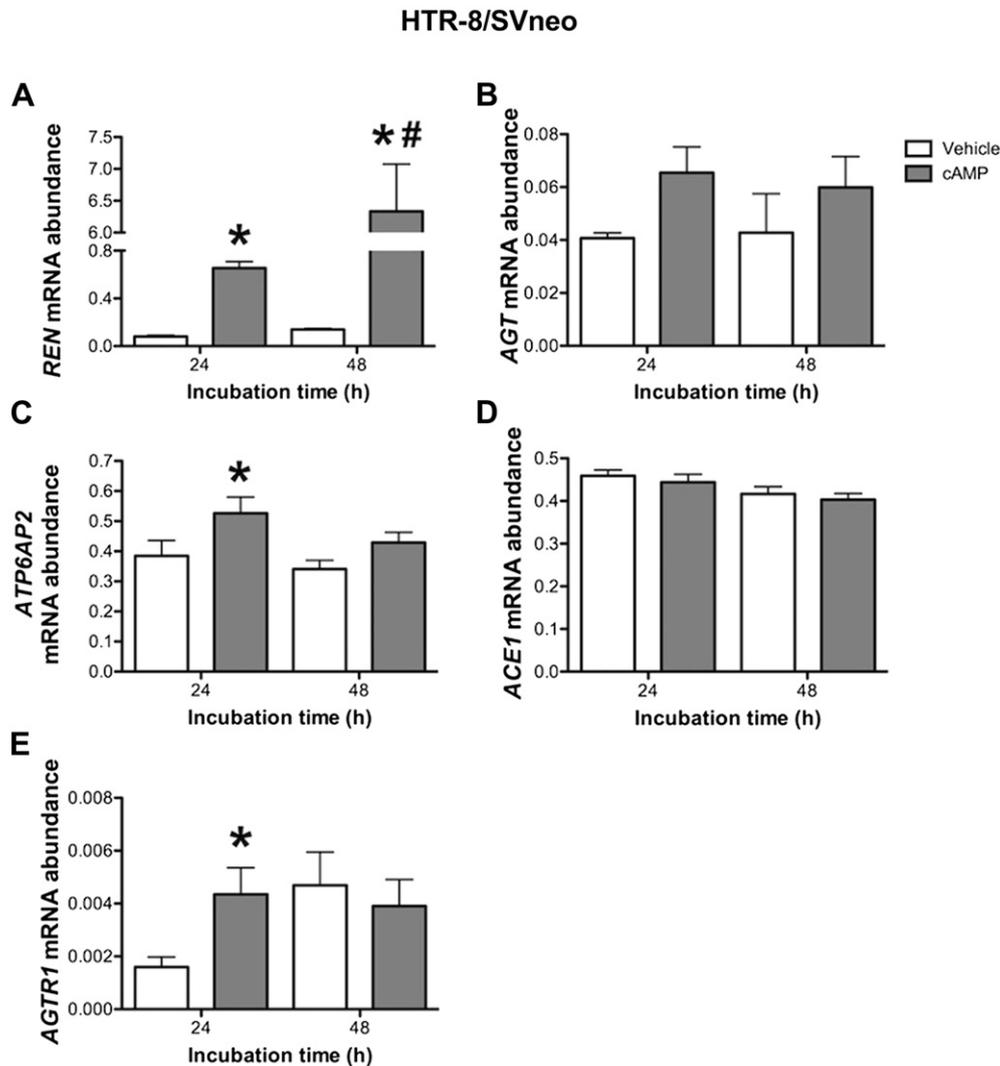


Fig. 1. mRNA abundance of RAS genes in HTR-8/SVneo cells (mean \pm SEM). (A) *REN* mRNA was expressed in HTR-8/SVneo cells and increased with cAMP treatment. In cAMP treated HTR-8/SVneo cells, *REN* expression increased with incubation time. (B) *AGT* expression did not change with cAMP treatment or incubation time. (C) *ATP6AP2* expression increased with cAMP treatment at 24 h only. (D) *ACE1* expression did not change with cAMP treatment or incubation time. (E) *AGTR1* expression increased with cAMP treatment at 24 h only ($n = 9$; three experiments in triplicate in HTR-8/SVneo cells). * Denotes significant difference from vehicle treated controls at the same incubation time ($P < 0.05$); # Denotes significant difference in 48 h treatment from 24 h treatment of the same group ($P < 0.05$). HTR-8/SVneo cells did not express *ACE2*, *AGTR2* and *MAS1*.

associated with increased amounts of prorenin in the supernatants collected from HTR-8/SVneo cells ($P = 0.005$; Fig. 3). Prorenin was not detected in either vehicle or cAMP treated BeWo cell supernatants.

Prior to incubation, measurable levels of both Ang 1-7 (18.4 pg/mL in DMEM-F12 and 10.8 pg/mL in RPMI-1640) and Ang II (37.3 pg/mL in DMEM-F12 and 18.6 pg/mL in RPMI-1640) were present in the culture media; therefore we have reported the amount of Ang 1-7 and Ang II found in media collected after incubation with trophoblast cell lines as net production or net destruction.

Since the levels of Ang II after incubation were less than those measured before incubation, there was a net loss of Ang II from the supernatants of both BeWo and HTR-8/SVneo cells. cAMP treatment had no effect on the net amount of Ang II present (Fig. 4). There was net production of Ang 1-7 in media collected after incubation from both untreated HTR-8/SVneo and BeWo cultures (Fig. 5). But there was net destruction of Ang 1-7 from HTR-8/SVneo cell supernatant during treatment with cAMP (Fig. 5A), this was not

statistically significant. In BeWo cells there was net production of Ang 1-7 in both untreated and cAMP treated cell supernatant, although like HTR-8/SVneo media, it was less if the BeWo cells had been treated with cAMP (Fig. 5B). Due to the low number of samples, these observations were not statistically significant.

4. Discussion

This study compared RAS gene expression within BeWo and HTR-8/SVneo cells. Although both cell lines have been used to model placental cellular functions, there are some notable differences between the two cell lines. For example, BeWo cells contain a mixture of villous and extravillous trophoblast cells, whereas the HTR-8/SVneo cells contain only extravillous trophoblast cells. In addition, BeWo cells are derived from a choriocarcinoma. In terms of the expression of RAS pathways these two cell lines were very dissimilar. Since HTR-8/SVneo cells lack both the AT_2R and the Ang 1-7/MAS receptor pathway, we would predict that any anti-angiogenic and pro-apoptotic effects of the placental RAS

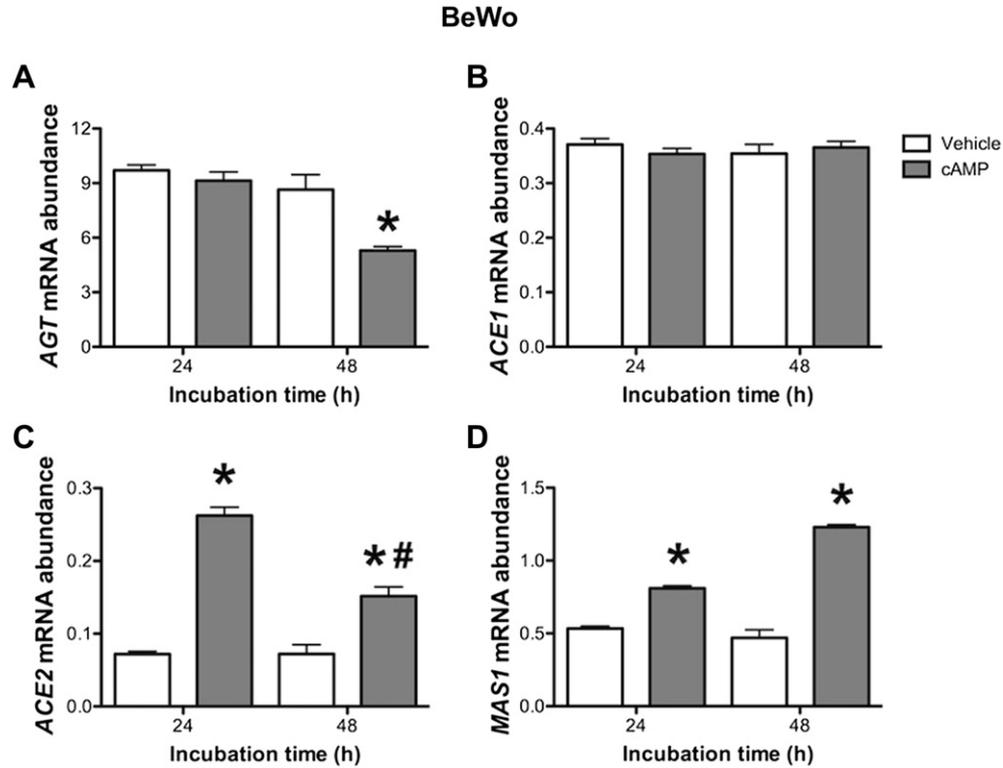


Fig. 2. mRNA abundance of RAS genes expressed in BeWo cells (mean ± SEM). (A) cAMP treatment decreased *AGT* expression at 48 h incubation. (B) *ACE1* expression did not change with cAMP treatment or incubation time. (C) cAMP treatment increased *ACE2* expression at 24 and 48 h. *ACE2* expression in cAMP treated BeWo cells decreased with incubation time. (D) *MAS1* expression in BeWo cells increased with cAMP treatment at both 24 and 48 h ($n = 9$; three experiments in triplicate in BeWo cells). * Denotes significant difference from vehicle treated controls at the same incubation time ($P < 0.05$); # Denotes significant difference in 48 h incubation from 24 h incubation of the same group ($P < 0.05$). BeWo cells did not express *REN*, *ATP6AP2*, *AGTR1* and *AGTR2*.

occurring as a result of activation of these pathways [24,25] would not be active in this cell line. Thus any putative angiogenic and proliferative actions of the HTR-8/SVneo renin/Aogen/ACE/Ang II/AT₁R pathway which is present would be unopposed by actions of Ang II via AT₂R or Ang 1-7 via the Mas receptor. This means that the role of Ang II/AT₁R in the control of placental angiogenesis could be challenged using cAMP to drive *REN*, *ATP6AP2*, *AGTR1* expression and prorenin production. The effects of this RAS pathway on placental trophoblast function can therefore be studied without interference from antagonistic effects of the RAS mediated via Ang II/AT₂R and Ang 1-7/Mas receptor interactions. Conversely, as the BeWo cell line only expressed the ACE2/Ang 1-7/MAS receptor pathway, the putative anti-angiogenic and anti-proliferative effects

of this RAS pathway [8] can be studied in isolation, free from any concomitant actions of Ang II mediated by either AT₁R or AT₂R receptors. Since cAMP stimulated expression of both *ACE2* and *MAS1*, the effects of stimulation of this pathway on angiogenesis and apoptosis can easily be investigated.

HTR-8/SVneo cells behave in a similar manner to juxtaglomerular renin-secreting cells [17], where prorenin expression and production are enhanced by cAMP. Similar increases in placental *REN* expression and renin protein have been reported in villous placenta and decidual cells after treatment with cAMP [15,26].

In HTR-8/SVneo cells, *AGTR1* mRNA is higher after cAMP treatment, similar upregulation of *AGTR1* expression has been reported in smooth muscle cells [27]. In addition, *AGTR1* expression is downregulated by Ang II [27], which in cAMP treated HTR-8/SVneo cells appear to have lower Ang II levels and thus may contribute to the increase in *AGTR1* expression after cAMP treatment.

Since BeWo cells, unlike HTR-8/SVneo cells, do not express *REN*, we used 8-bromo-cAMP in an attempt to stimulate *REN* expression in this cell line, however this proved ineffective. This was perhaps surprising, given that the dose of cAMP used was highly effective in stimulating *REN* expression and prorenin production in HTR-8/SVneo cells, and that the ability of cAMP to stimulate juxtaglomerular cell renin is well recognised [16,17]. Therefore, we believe that in BeWo cells, cAMP could not access the cyclic AMP response element (CRE) of the *REN* gene. Whether this was due to heavy methylation of genes in BeWo cells, whereby the CRE in *REN* was silenced but left other genes intact (i.e. *ACE2* and *MAS1*), or that BeWo cells lack the necessary transcription factors for cAMP to bind to the CRE is unknown, however as far as we are aware, this is the first study to look at the RAS pathway in this cell line. Given that both BeWo cells and the HTR-8/SVneo cells both originated from

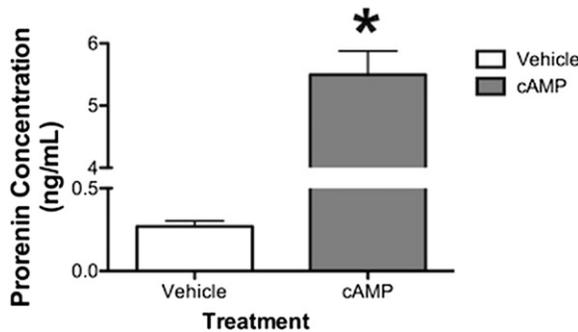


Fig. 3. Prorenin protein (measured by ELISA) was present in the supernatant of vehicle treated HTR-8/SVneo cells after 48 h incubation. cAMP treatment significantly increased prorenin protein levels ($n = 6$). * Denotes significant difference to vehicle treated HTR-8/SVneo cells ($P < 0.05$).

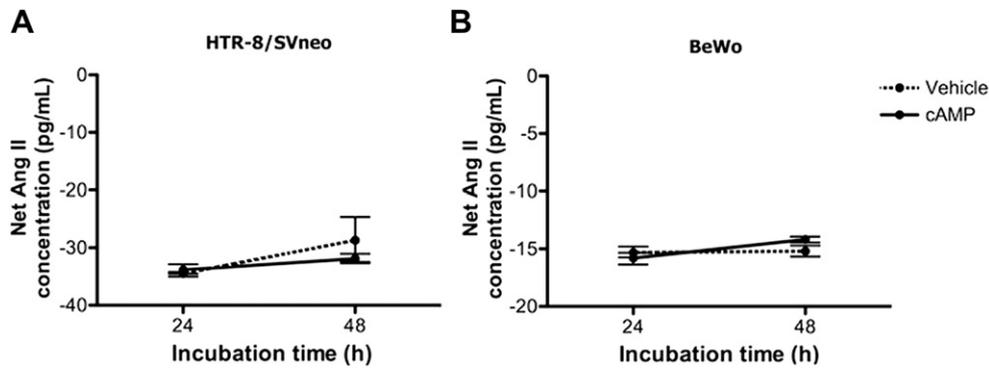


Fig. 4. Net Ang II levels in the supernatant of HTR-8/SVneo and BeWo cells after 48 h. (A) In the supernatant of HTR-8/SVneo cells, both vehicle and cAMP treatment resulted in a net loss of Ang II. (B) In the supernatant of BeWo cells, both vehicle and cAMP treatment resulted in a net loss of Ang II. Net loss of Ang II however, appeared to be greater in HTR-8/SVneo cells compared to BeWo cells ($n = 6$).

trophoblast, it is somewhat surprising that they are so dissimilar in terms of the components of the RAS pathway that were expressed.

We have however been able to stimulate *REN* expression in human endometrial stromal cells using an inhibitor of DNA methylation (5-Aza-2'-deoxycytidine: AZA; unpublished data) so it will be of interest to see what happens to the response of BeWo cells to cAMP when they are exposed to AZA.

Ang 1-7 and Ang II peptides were present in the culture media prior to incubation, possibly because it was supplemented with 10% fetal bovine serum. Both cell lines failed to show net production of Ang II, which may be due to the labile nature of Ang II [28], as we were unable to use protease inhibitors in the culture without threatening cell viability. Net Ang 1-7 production by BeWo cells was observed, and may have resulted from the conversion of Ang II (present in the culture media prior to incubation) to Ang 1-7 by *ACE2* in the BeWo cells, as cAMP-induced expression of both *ACE2* and *MAS1* was observed. This probably accounts for the greater production of Ang 1-7 by BeWo cells compared to HTR-8/SVneo cells (Fig. 5). An alternative Aogen processing enzyme may also have been present in the culture medium, such as chymase or cathepsin D [29–31]. The latter is less likely, as it is inactive at neutral pH [30]. Additionally, HTR-8/SVneo cells do not express *AGT* to the same extent as BeWo cells. If this translates into a lower rate of Aogen synthesis, it could account for the lower rate of Ang 1-7 production.

Low *AGT* abundance and protein levels are also seen in the placenta [13]. *In vivo*, placental Aogen may not be a rate-limiting

factor for Ang peptide synthesis, as Aogen could be sequestered from the maternal circulation. However, as no external sources of Aogen exist under culture conditions, Ang II production in both HTR-8/SVneo and BeWo cells may be low.

The production of Ang 1-7 by BeWo cells in the absence of prorenin raises the interesting possibility that non-renin proteases exist, which can form Ang peptides within human intrauterine tissues. As far as we know this possibility has not been investigated, although a non-renin angiotensin system (chymase) has been described in the heart where Ang II plays a key role in cardiac hypertrophy [32].

In conclusion, we have shown that two cell lines derived from trophoblast have only some of the now well-described RAS pathways and the components of the RAS pathways that they do possess are strikingly different, as is their response to cAMP. Thus these two cell lines could be used to determine how the various placental RAS pathways regulate angiogenesis, invasion and proliferation, all of which are key features of placentation. Using HTR-8/SVneo cells we are able to study the cAMP effects on the renin/Ang II/ AT_1R pathway, while further study of the RAS pathway in BeWo cells may lead to identification of other neutral proteases capable of forming Ang II, as well as providing us with the opportunity to investigate the Ang 1-7/*MAS* receptor pathway in isolation from effects of Ang II. Neither cell lines however, truly represent the placental RAS, as all RAS genes and proteins are present in both the early and late gestation human placenta [13,22].

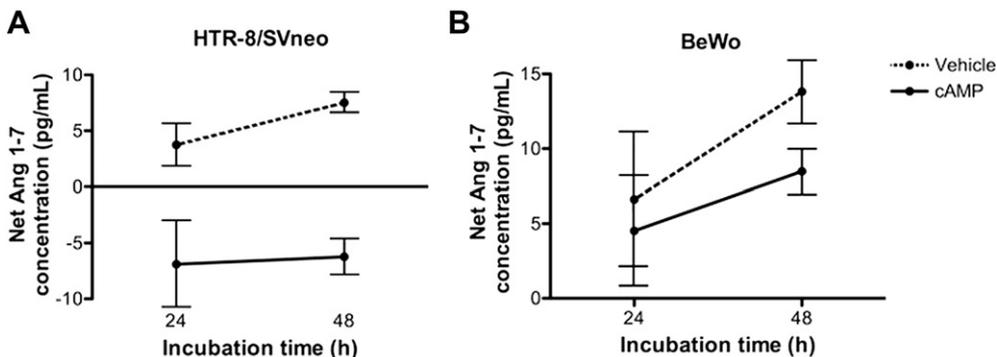


Fig. 5. Net Ang 1-7 in the supernatant of HTR-8/SVneo and BeWo cells after 48 h. (A) In the supernatant of HTR-8/SVneo cells vehicle treatment resulted in a net gain of Ang 1-7, whereas cAMP treatment resulted in a net loss of Ang 1-7. (B) In the supernatant of BeWo cells, both vehicle and cAMP treatment resulted in a net gain of Ang 1-7 ($n = 6$).

Acknowledgements

This work was supported by project grant [510746] from the National Health and Medical Research Council of Australia.

References

- [1] Buharioglu CK, Song CY, Yaghini FA, Ghafoor HU, Motiwala M, Adris T, et al. Angiotensin II-induced process of angiogenesis is mediated by spleen tyrosine kinase via VEGF receptor-1 phosphorylation. *Am J Physiol Heart Circ Physiol* 2011;301(3):H1043–55.
- [2] Binder ND, Laird MR, Faber JJ. Interrelationships between the renin-angiotensin system and uteroplacental blood flow—a recent perspective. *Reprod Fertil Dev* 1995;7(6):1437–42.
- [3] Williams PJ, Mistry HD, Innes BA, Bulmer JN, Pipkin FB. Expression of AT1R, AT2R and AT4R and their roles in extravillous trophoblast invasion in the human. *Placenta* 2010;31(5):448–55.
- [4] Araki-Taguchi M, Nomura S, Ino K, Sumigama S, Yamamoto E, Kotani-Ito T, et al. Angiotensin II mimics the hypoxic effect on regulating trophoblast proliferation and differentiation in human placental explant cultures. *Life Sci* 2008;82(1–2):59–67.
- [5] Irani RA, Xia Y. Renin angiotensin signaling in normal pregnancy and preeclampsia. *Semin Nephrol* 2011;31(1):47–58.
- [6] Anton L, Merrill DC, Neves LA, Diz DI, Corthorn J, Valdes G, et al. The uterine placental bed renin-angiotensin system in normal and preeclamptic pregnancy. *Endocrinology* 2009;150(9):4316–25.
- [7] MacGregor GA, Markandu ND, Roulston JE, Jones JC, Morton JJ. Maintenance of blood pressure by the renin-angiotensin system in normal man. *Nature* 1981;291(5813):329–31.
- [8] Sampaio WO, Henrique de Castro C, Santos RA, Schiffrin EL, Touyz RM. Angiotensin-(1-7) counterregulates angiotensin II signaling in human endothelial cells. *Hypertension* 2007;50(6):1093–8.
- [9] Nguyen G, Delarue F, Burckle C, Bouzahir L, Giller T, Sraer JD. Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J Clin Invest* 2002;109(11):1417–27.
- [10] Derkx FH, Alberda AT, de Jong FH, Zeilmaker FH, Makovitz JW, Schalekamp MA. Source of plasma prorenin in early and late pregnancy: observations in a patient with primary ovarian failure. *J Clin Endocrinol Metab* 1987;65(2):349–54.
- [11] Saris JJ, t Hoen PA, Garrelts IM, Dekkers DH, den Dunnen JT, Lamers JM, et al. Prorenin induces intracellular signaling in cardiomyocytes independently of angiotensin II. *Hypertension* 2006;48(4):564–71.
- [12] Morgan T, Craven C, Ward K. Human spiral artery renin-angiotensin system. *Hypertension* 1998;32(4):683–7.
- [13] Marques FZ, Pringle KG, Conquest A, Hirst JJ, Markus MA, Sarris M, et al. Molecular characterization of renin-angiotensin system components in human intrauterine tissues and fetal membranes from vaginal delivery and cesarean section. *Placenta* 2011;32(3):214–21.
- [14] Germain S, Konoshita T, Fuchs S, Philippe J, Corvol P, Pinet F. Regulation of human renin gene transcription by cAMP. *Clin Exp Hypertens* 1997;19(5–6):543–50.
- [15] Poisner AM, Thraillkill K, Poisner R, Handwerger S. Cyclic AMP as a second messenger for prorenin release from human decidua cells. *Placenta* 1991;12(3):263–7.
- [16] Pinet F, Mizrahi J, Laboulandine I, Menard J, Corvol P. Regulation of prorenin secretion in cultured human transfected juxtaglomerular cells. *J Clin Invest* 1987;80(3):724–31.
- [17] Pinet F, Mizrahi J, Menard J, Corvol P. Role of cyclic AMP in renin secretion by human transfected juxtaglomerular cells. *J Hypertens Suppl* 1986;4(6):S421–3.
- [18] Graham CH, Hawley TS, MacDougall RC, Kerbel RS, Khoo N, Lala PK. Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp Cell Res* 1993;206(2):204–11.
- [19] Pattillo RA, Gey GO, Delfs E, Mattingly RF. Human hormone production *in vitro*. *Science* 1968;159(3822):1467–9.
- [20] Gilsbach R, Kouta M, Bonisch H, Bruss M. Comparison of *in vitro* and *in vivo* reference genes for internal standardization of real-time PCR data. *Bio-techniques* 2006;40(2):173–7.
- [21] Pringle KG, Zakar T, Yates D, Mitchell CM, Hirst JJ, Lumbers ER. Molecular evidence of a (pro)renin/(pro)renin receptor system in human intrauterine tissues in pregnancy and its association with PGHS-2. *J Renin Angiotensin Aldosterone Syst* 2011;12(3):304–10.
- [22] Pringle KG, Tadros MA, Callister RJ, Lumbers ER. The expression and localization of the human placental prorenin/renin-angiotensin system throughout pregnancy: roles in trophoblast invasion and angiogenesis? *Placenta* 2011;32(12):956–62.
- [23] Wang Y, Pringle KG, Sykes SD, Marques FZ, Morris BJ, Zakar T, et al. Fetal sex affects expression of renin-angiotensin system components in term human decidua. *Endocrinology* 2012;153(1):462–8.
- [24] Silvestre JS, Tamarat R, Senbonmatsu T, Ichchiki T, Ebrahimian T, Isglar M, et al. Antiangiogenic effect of angiotensin II type 2 receptor in ischemia-induced angiogenesis in mice hindlimb. *Circ Res* 2002;90(10):1072–9.
- [25] Anene-Maidoh OT, Greene AS. Angiotensin 1-7 treatment induces apoptosis in human umbilical vein endothelial cells. *FASEB J*; 2009. Meeting abstract(23):626.14.
- [26] Poisner AM, Downing GJ, Poisner R. Prorenin secretion from villous placenta: regulation by cyclic AMP and angiotensin. *Placenta* 1994;15(5):487–99.
- [27] Chen X, Nishimura K, Hasna J, Kobayashi S, Shikasho T, Kanaide H. Protein kinase C and protein kinase A regulate the expression of angiotensin II receptor mRNA in smooth muscle cells. *Eur J Pharmacol* 1994;15(267(2)):175–83.
- [28] Wolf RL, Mendlowitz M, Pick J, Gitlow SE, Naftchi N. Metabolism and distribution of I-131-labeled angiotensin II. *J Lab Clin Med* 1962;60:150–9.
- [29] Velez JC, Bland AM, Arthur JM, Raymond JR, Janech MG. Characterization of renin-angiotensin system enzyme activities in cultured mouse podocytes. *Am J Physiol Ren Physiol* 2007;293(1):398–407.
- [30] Hackenthal E, Hackenthal R, Hilgenfeldt U. Isorenin, pseudorenin, cathepsin D and renin. A comparative enzymatic study of angiotensin-forming enzymes. *Biochim Biophys Acta* 1978;522(2):574–88.
- [31] Ramaha A, Patston PA. Release and degradation of angiotensin I and angiotensin II from angiotensinogen by neutrophil serine proteinases. *Arch Biochem Biophys* 2002;397(1):77–83.
- [32] Crowley SD, Gurley SB, Herrera MJ, Ruiz P, Griffiths R, Kumar AP, et al. Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. *Proc Natl Acad Sci U S A* 2006;103(47):17985–90.

APPENDIX B

The effects of cyclic AMP, sex steroids and global hypomethylation on the expression of genes controlling the activity of the renin-angiotensin system in placental cell lines.



The effects of cyclic AMP, sex steroids and global hypomethylation on the expression of genes controlling the activity of the renin–angiotensin system in placental cell lines

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ARTICLE INFO

Article history:

Accepted 22 December 2012

Keywords:

HTR-8/SVneo
BeWo
Renin–angiotensin system
Trophoblast
Placenta
cAMP
AZA
MPA
E²

ABSTRACT

The placental renin–angiotensin system (RAS) is involved in placentation. We have shown that prorenin mRNA (*REN*) is expressed in a first trimester trophoblast cell line (HTR-8/SVneo) but not in a choriocarcinoma cell line (BeWo). We attempted to stimulate RAS expression in these cells by cAMP, 5'-aza-2'-deoxycytidine (AZA; an inhibitor of methylation), cAMP and AZA combined, and the sex steroids medroxyprogesterone acetate (MPA) and estradiol-17 β (E₂) with and without cAMP. RAS mRNAs were measured by qPCR and prorenin concentration in supernatants measured by an ELISA. In HTR-8/SVneo cells, all treatments increased *REN* expression compared to controls and cAMP + AZA combined was more effective than either treatment alone. Prorenin levels in supernatants were similarly upregulated. In HTR-8/SVneo cells, angiotensinogen (*AGT*) mRNA expression was increased by MPA + E₂ either with or without cAMP. *AGT* expression was also significantly increased by AZA. BeWo cells did not express *REN* or prorenin and it was not inducible with any treatment. *AGT* expression was significantly increased with AZA, the combination of cAMP + AZA, and MPA + E₂ + cAMP treatments. Since cAMP, AZA, cAMP and AZA combined, or MPA and E₂ with and without cAMP in HTR-8/SVneo cells, a cell line most similar in its RAS expression to the *in vivo* placenta, these factors may affect placental RAS activity. Surprisingly, these treatments also induced *AGT* expression in BeWo cells. Whether they are involved in regulating *AGT* in choriocarcinomas *in vivo* remains to be determined.

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1. Introduction

Studies have shown that the renin–angiotensin system (RAS) is involved in the regulation of trophoblast invasion [1] and spiral artery remodeling [2] and consequently, may play a role in implantation and placentation.

Within the placenta, renin acts on angiotensinogen (*AGT*) to form angiotensin (Ang) I, which is catalyzed by angiotensin converting enzyme (*ACE*) to form Ang II. Ang II can act either through the angiotensin II type 1 (AT₁R) or type 2 (AT₂R) receptor or it can be converted by ACE2 to Ang 1-7. Potentially, the placental RAS could have a number of actions, some of which may be cooperative and some antagonistic. RAS pathway genes and proteins have been

detected in both the early and late gestation human placentae [1,3,4].

We have studied two established trophoblast cell lines that are commonly used for studying placental function: HTR-8/SVneo (a transformed first trimester extravillous trophoblast cell line developed by Charles Graham, Ontario, Canada [5]) and BeWo cells (derived from a choriocarcinoma [6]), postulating that these cell lines would show similar patterns of RAS expression to freshly obtained human placenta. This was not the case however. HTR-8/SVneo cells expressed prorenin (*REN*), angiotensinogen (*AGT*), the prorenin receptor (*ATP6AP2*), *ACE* (*ACE1*) and AT₁R (*AGTR1*), but not AT₂R (*AGTR2*), ACE2 (*ACE2*) nor the Ang 1-7 receptor, MasR (*MAS1*), while BeWo cells only expressed *AGT*, *ACE1*, *ACE2* and *MAS1* and not *REN* [7]. Although the expression of RAS genes and proteins in the human placenta and these trophoblast cell lines has been established, the mechanisms regulating their expression are unknown.

Another difference between BeWo and HTR-8/SVneo cells is in their response to cAMP. When stimulated with cAMP, *REN* expression and prorenin protein synthesis were induced in HTR-8/

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SVneo cells, but not in BeWo cells [7]. This was interesting, given that cAMP is a classical stimulator of *REN* expression, as seen in juxtaglomerular [8] and primary decidual cells [9]. We postulated that BeWo cells lack the *REN* response to cAMP stimulation, as they are derived from a choriocarcinoma and have a high degree of ploidy and therefore could be heavily methylated in order to be viable. Therefore, in BeWo cells, hypermethylation of DNA may prevent cAMP from binding to the cAMP response element (CRE), which is responsible for the regulation of *REN* gene transcription [10,11]. We postulated that a genome wide inhibitor of methylation such as 5'-aza-2'-deoxycytidine (AZA) might therefore initiate *REN* expression and prorenin secretion in BeWo cells, particularly in response to cAMP. AZA inhibits DNA methyltransferases, resulting in global hypomethylation as cells continue to divide. Hypomethylation could increase transcriptional protein binding at the CRE [12].

Furthermore, if as we proposed, AZA did stimulate *REN* expression through global hypomethylation of BeWo DNA, then AZA might also have a similar effect in HTR-8/SVneo cells, so that not only do cAMP (as previously shown [7]) and AZA stimulate *REN* expression and prorenin secretion but the combination of cAMP and AZA together would be additive, having a greater effect on *REN* expression in HTR-8/SVneo cells than that seen with either agent alone.

In normal pregnancy, the developing blastocyst secretes human chorionic gonadotropin (hCG). hCG levels are maximal at about 6 weeks gestation, at which time maternal plasma prorenin levels are also at a maximum. hCG administration is associated with increased plasma prorenin levels. hCG maintains the corpus luteum, and maintains production of both estrogen and progesterone [13,14]. The hCG induced increase in plasma prorenin in early pregnancy also comes from the corpus luteum [15]. In addition, hCG stimulates placental production of prorenin, as does progesterone [13,16]. Therefore, we proposed that the combined administration of estrogen and progesterone, hormones essential for the establishment and maintenance of human pregnancy, could stimulate renin expression by both HTR-8/SVneo and BeWo cells.

In this study, therefore, we compared the effects of cAMP with the global hypomethylating agent, AZA, and their combined effects on expression of RAS genes and on prorenin secretion by the placentially derived cell lines, HTR-8/SVneo and BeWo. We also investigated the effects of cAMP, estradiol and progesterone, in combination, on *REN* expression and prorenin production and on the expression of other RAS genes.

2. Materials and methods

2.1. Trophoblast cell culture

HTR-8/SVneo and BeWo cells were cultured in phenol red-free RPMI-1640 or DMEM/F-12, respectively, supplemented with 15 mM HEPES, 1.2 g/L NaHCO₃, 1 mg/mL L-glutathione reduced, 0.1 g/L albumin fraction V, 0.65 µg/mL aprotinin, 10% fetal bovine serum and 40 µg/mL gentamicin. Cells were seeded at a density of 200,000 cells, in each well of a 6 well plate with 2 mL of incubation medium. Cells were allowed to settle for 24 h, after which the media was changed, cells were then treated with one of the following:

- 1) 150 µM 8-bromo-cAMP (cAMP, Sigma–Aldrich, St. Louis, MO, USA);
- 2) 7.5 µM 5'-aza-2'-deoxycytidine (AZA, Sigma–Aldrich);
- 3) 150 µM cAMP with 7.5 µM AZA;
- 4) 1 µM medroxyprogesterone acetate (MPA, Sigma–Aldrich) with 10 nM estradiol-17β (E₂, Sigma–Aldrich);
- 5) 1 µM MPA with 10 nM estradiol-17β and 150 µM cAMP; or
- 6) incubation medium alone (vehicle).

In a previous study we used 300 µM 8-bromo-cAMP [7] to induce prorenin release. To ensure that a significant increase in prorenin mRNA and protein levels would be seen when cAMP treatment was combined with AZA or E₂ and MPA compared with cAMP treatment alone, we dropped the cAMP dose by half, and treated the cells with 150 µM cAMP. The concentration of AZA is within the range

normally used in cell culture experiments. E₂ and MPA concentrations were selected based on previous studies examining the effects of these hormones in human endometrial stromal cells [17] and are similar to what trophoblast cells would be exposed to *in vivo* during normal human pregnancy [18]. Since placental estrogen and progesterone levels increase in pregnancy in a coordinated fashion [18] we chose to only assess the effects of these hormones in combination, to better mimic the *in vivo* environment. Incubation media were collected at 24 and 48 h and cells were harvested. Cells and media were snap frozen in liquid nitrogen for subsequent protein and mRNA analyses. There were 3 independent experiments and in each experiment the treatments were in triplicate. Cell viability was assessed by an LDH cytotoxicity assay for all treatments as well as by measuring RNA stability and quality (data not shown). None of the treatments used in this study affected cell viability at 48 h.

2.2. Semi-quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Each sample was examined for RNA integrity, DNase treated (Qiagen N.V., Hilden, Germany), spiked with a known amount of Alien RNA (Agilent Technologies, La Jolla, CA, USA; 10⁷ copies per microgram of total RNA) and reverse transcribed using Superscript III RT kit (Invitrogen). The alien qRT PCR inhibitor alert system (Agilent Technologies) served as a reference for internal standardization [19]. RAS primers for qPCR have been described previously [3,4,20]. Messenger RNA abundance was calculated using the $\Delta\Delta C_T$ method and relative abundance was calculated relative to Alien mRNA and a calibrator sample (term placenta).

2.3. Measurement of prorenin protein by ELISA

Prorenin levels in culture media were measured using the Human Prorenin ELISA kit (Molecular Innovations Inc.; Novi, MI) according to the manufacturer's instructions. The methods and reagents used have been previously described [3,4,20]. Samples were assayed in duplicate and on the same ELISA plate. Therefore there was no inter-assay variability. Intra-assay coefficient of variation was 10.8%.

2.4. Data analyses

A one-way ANOVA with Bonferroni post hoc analysis was used to determine if there were any differences in RAS mRNA abundance and prorenin protein concentration in the supernatant between each treatment. GraphPad Prism (Prism version 6.0) was used for all analyses. Significance was set at $P < 0.05$.

3. Results

3.1. cAMP stimulates prorenin in HTR-8/SVneo but not BeWo cells

In this study, we used 150 µM 8-bromo-cAMP as compared to the 300 µM used in our previous study [7]. Despite this, cAMP significantly increased *REN* mRNA expression in HTR-8/SVneo cells at both 24 and 48 h incubation (both $P < 0.001$, Fig. 1A). In supernatants from cultures of HTR-8/SVneo treated cells, there was a corresponding increase in prorenin protein concentrations at 48 h compared to vehicle ($P < 0.001$, Fig. 1B).

In BeWo cells, cAMP was not able to induce *REN* expression or prorenin in the supernatant.

3.2. AZA alone, or in combination stimulates *REN* in HTR-8/SVneo cells

In order to determine whether *REN* expression could be stimulated in BeWo cells by inhibiting DNA methylation, the two trophoblast cell lines were treated with AZA. In HTR-8/SVneo cells, *REN* expression was significantly increased by treatment with AZA compared to vehicle at both 24 and 48 h incubation (both $P < 0.001$, Fig. 1A) and AZA significantly increased the levels of prorenin in the supernatant ($P < 0.001$, Fig. 1B).

To test our hypothesis that cAMP and AZA act through different pathways to stimulate *REN* expression, we compared *REN* expression in cells treated with the combination of cAMP and AZA, to either treatment alone. We found that the combination of cAMP and AZA was more effective at stimulating *REN* expression in HTR-8/SVneo cells compared to vehicle or to cAMP or AZA treatment

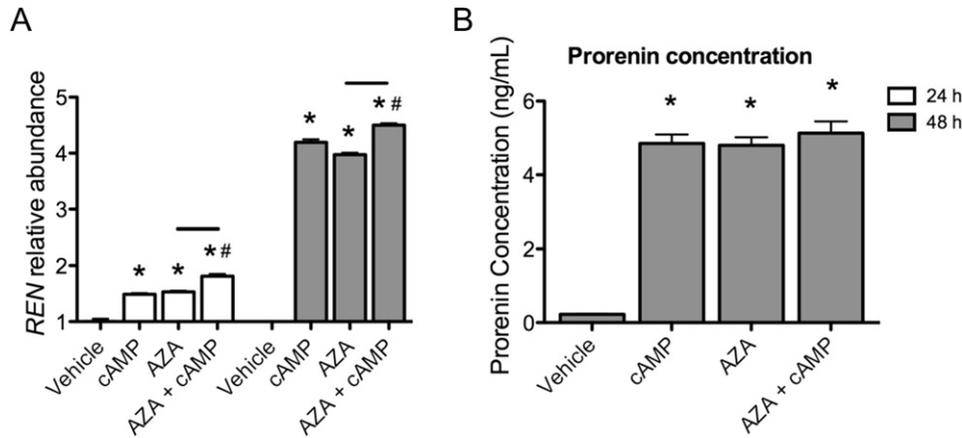


Fig. 1. *REN* mRNA abundance in HTR-8/SVneo cells (mean ± SEM). (A) *REN* mRNA was expressed in HTR-8/SVneo cells, and increased with cAMP, AZA and the combined cAMP + AZA treatment compared to vehicle treated controls at both 24 and 48 h incubation. At 24 and 48 h incubation, the combined cAMP + AZA treated HTR-8/SVneo cells had higher *REN* expression than cAMP or AZA treated cells. (B) Prorenin protein levels measured in the supernatant at 48 h incubation were significantly higher in cAMP, AZA and cAMP + AZA treated cells compared to the controls, however there was no significant differences in prorenin protein levels between the three treatments ($n = 3$). * denotes significant difference from vehicle treated controls at the same incubation time ($P < 0.001$); # denotes significant difference from cAMP treated cells at the same incubation time ($P < 0.001$); the solid line denotes a significant difference in AZA treatments with and without the addition of cAMP ($P < 0.001$).

alone at both 24 and 48 h incubation ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.05$ and $P < 0.001$ respectively, Fig. 1A). There were corresponding increases in prorenin concentrations in the supernatants from the treated HTR-8/SVneo cells, ($P < 0.001$, Fig. 1B). Prorenin levels however were no greater than levels measured in supernatants treated with either cAMP or AZA alone.

In contrast, AZA, alone or in combination with cAMP, failed to induce *REN* expression in BeWo cells and prorenin was not detected in the supernatant.

3.3. Effects of MPA and E₂, alone or in combination with cAMP on prorenin mRNA and protein levels

The combination of MPA & E₂ significantly increased HTR-8/SVneo cell expression of *REN* compared to vehicle at both 24 and 48 h ($P < 0.001$) and was more effective at stimulating *REN* expression compared with cAMP treatment ($P < 0.01$ and $P < 0.001$,

respectively, Fig. 2A). Prorenin concentration in the supernatants of the MPA and E₂ treated cells was greater than vehicle ($P < 0.001$, Fig. 2B), however there was no significant difference between MPA, E₂ or cAMP treated cells.

The MPA + E₂ + cAMP mix significantly increased *REN* expression in HTR-8/SVneo cells compared to vehicle at 24 and 48 h (both $P < 0.001$, Fig. 2A) and was more effective at stimulating *REN* expression than cAMP treatment alone ($P < 0.001$, Fig. 2A) but not more effective than the combined treatment of MPA and E₂ alone. After 48 h incubation, prorenin levels in the supernatants of HTR-8/SVneo cells treated with MPA + E₂ + cAMP was significantly greater than vehicle ($P < 0.001$, Fig. 2B), which corresponds with their greater *REN* gene expression levels. There was no difference however, between prorenin levels in MPA and E₂ treated, MPA + E₂ + cAMP treated, and cAMP treated cells.

In BeWo cells, MPA + E₂, or in combination with cAMP, failed to induce prorenin gene expression or prorenin protein production.

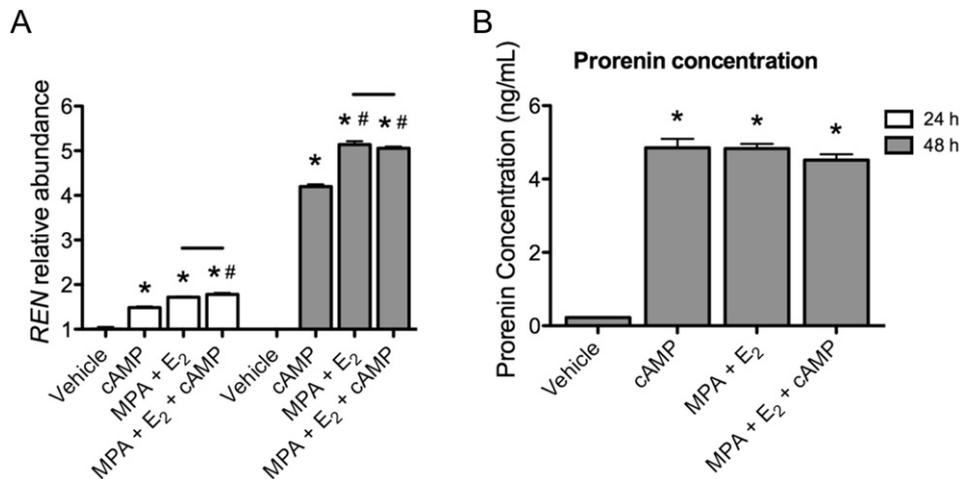


Fig. 2. *REN* mRNA abundance in HTR-8/SVneo cells (mean ± SEM). (A) *REN* mRNA was expressed in HTR-8/SVneo cells, and increased with cAMP, MPA + E₂ and MPA + E₂ + cAMP treatment compared to vehicle treated controls at both 24 and 48 h incubation. At 24 h incubation, the combined MPA + E₂ + cAMP treated HTR-8/SVneo cells had higher *REN* expression than cAMP treated cells. Whilst at 48 h incubation, both MPA + E₂ and MPA + E₂ + cAMP treated cells had significantly higher *REN* expression than cAMP treated cells. (B) Prorenin protein levels measured in the supernatant at 48 h incubation were significantly higher in cAMP, MPA + E₂ and MPA + E₂ + cAMP treated cells compared to the controls, however there was no significant differences in prorenin protein levels between the three treatments ($n = 3$). * denotes significant difference from vehicle treated controls at the same incubation time ($P < 0.001$); # denotes significant difference from cAMP treated cells at the same incubation time ($P < 0.001$).

3.4. Effects of cAMP and AZA on the expression of other genes in the placental RAS pathways of HTR-8/SVneo and BeWo cells

cAMP treatment alone did not alter the expression of any of the other RAS genes studied in HTR-8/SVneo cells and had no effect on any of the RAS genes expressed by BeWo cells.

In contrast, AZA treatment of HTR-8/SVneo cells increased *AGT* mRNA abundance compared to vehicle at 24 h ($P < 0.01$, Fig. 3A). There were no significant effects however, of AZA on *ATP6AP2*, *ACE1* and *AGTR1* expression. In HTR-8/SVneo cells, combined cAMP and AZA treatment was more effective at stimulating *AGT* expression compared to vehicle ($P < 0.001$) and cAMP treatment at 24 h ($P < 0.05$, Fig. 3A). There were no significant effects of the combined cAMP and AZA treatment on *ATP6AP2*, *ACE1* and *AGTR1* expression.

In BeWo cells, treatment with AZA increased *AGT* mRNA abundance at 24 h incubation compared to vehicle ($P < 0.01$, Fig. 4A). This effect was reversed by the combination of cAMP and AZA treatment ($P < 0.05$, Fig. 4A) and was no longer different from controls. Neither AZA nor cAMP had any effect on *ACE1*, *ACE2* or *MAS1* mRNA abundances in BeWo cells.

3.5. Effects of MPA and E₂, alone or in combination with cAMP on the expression of other genes in the placental RAS pathways of HTR-8/SVneo and BeWo cells

Combined MPA and E₂ treatment significantly increased expression of *AGT* at 24 h incubation ($P < 0.05$) in HTR-8/SVneo cells compared to vehicle (Fig. 3B). Like MPA and E₂ treatment alone, MPA + E₂ + cAMP combined significantly increased HTR-8/SVneo cell expression of *AGT* compared to vehicle at 24 h incubation ($P < 0.05$, Fig. 3B) but this was not seen at 48 h.

In BeWo cells, there was no effect of MPA and E₂ treatment alone on RAS gene expression however, the combined MPA + E₂ + cAMP treatment significantly increased *AGT* expression at 24 h incubation compared to vehicle ($P < 0.05$, Fig. 4B). This effect was no longer observed at 48 h.

4. Discussion

Our previous work showed that cAMP stimulated prorenin mRNA and protein levels in HTR-8/SVneo cells and supernatants

but not BeWo cells, in which *REN* expression could not be detected [7]. HTR-8/SVneo cells express *REN* and produce prorenin as well as expressing other RAS genes likely to regulate Ang II/AT1R interactions and respond to cAMP, like renal renin-secreting cells. They are more similar to early gestation placentae and are therefore useful for studying effects of renin/ACE/Ang II/AT1R on cell function [7]. BeWo cells express the ACE2/Ang 1-7/Mas pathway, and therefore are useful for studying the effects of this pathway on trophoblast function.

In the present study, we wanted to find out if *REN* expression could be induced in BeWo cells and further stimulated in HTR-8/SVneo cells by reducing gene silencing through global hypomethylation using AZA, or by combined treatment with the two major hormones of pregnancy, estrogen and progesterone. We stimulated cells with cAMP alone to confirm our previous findings and to quantitate the effects of these novel treatments (relative to the cAMP induced effects) on *REN* and other RAS genes by both cell lines.

We demonstrated that cAMP, AZA, MPA and E₂ all increased *REN* expression and prorenin levels in HTR-8/SVneo cells but not in BeWo cells. On the other hand, treatment of HTR-8/SVneo cells with either AZA or MPA and E₂ had effects on renin that were independent of any effects of cAMP and in fact AZA treatment amplified the stimulatory effect of cAMP on *REN* expression. This amplified cAMP action on *REN* mRNA however, did not translate into increased levels of prorenin protein. The additive effect of the combined AZA and cAMP treatment was minimal, suggesting that AZA is likely acting through the cAMP/CRE pathway, possibly by allowing better access for cAMP and the associated binding factors to bind to the CRE of the *REN* gene, as opposed to AZA acting independently of cAMP [12].

This is the first report that global hypomethylation of a placental cell line is associated with increased *REN* expression and prorenin production. It is also the first report that the combination of E₂ and MPA stimulates *REN* mRNA and prorenin production by trophoblast cells.

We showed, as postulated, that E₂ and MPA in combination stimulate placental *REN* expression and prorenin secretion in HTR-8/SVneo cells. While a role for estrogen and progesterone in regulating the placental RAS has been suggested, there are no conclusive studies to support this. In this study we have been able to

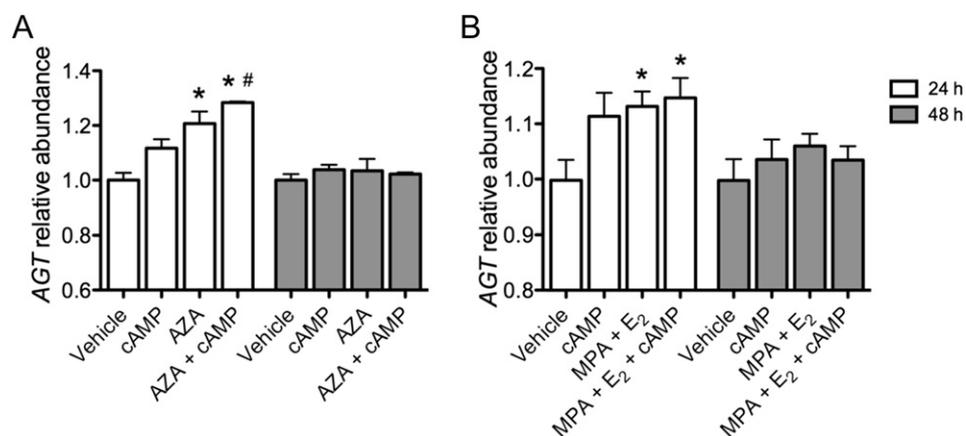


Fig. 3. *AGT* mRNA abundance in HTR-8/SVneo cells (mean \pm SEM). (A) At 24 h incubation, *AGT* mRNA was expressed in HTR-8/SVneo cells and increased with AZA and the combined cAMP + AZA treatment compared to vehicle treated controls. Also at 24 h incubation, cAMP + AZA treated HTR-8/SVneo cells had significantly greater *AGT* expression compared to HTR-8/SVneo cells treated with cAMP alone. At 48 h incubation, there was no significant difference between the expression of *AGT* in the vehicle, cAMP, AZA or the combined cAMP + AZA treated HTR-8/SVneo cells. (B) *AGT* mRNA in HTR-8/SVneo cells was increased with the combined MPA + E₂ and the MPA + E₂ + cAMP treatments at 24 h incubation compared to vehicle treated controls from the same incubation time. At 48 h incubation, there was no significant difference between the expression of *AGT* in the vehicle, cAMP, MPA + E₂ or the MPA + E₂ + cAMP treated HTR-8/SVneo cells ($n = 3$). * denotes significant difference from vehicle treated controls at the same incubation time ($P < 0.05$); # denotes significant difference from cAMP treated cells at the same incubation time ($P < 0.05$).

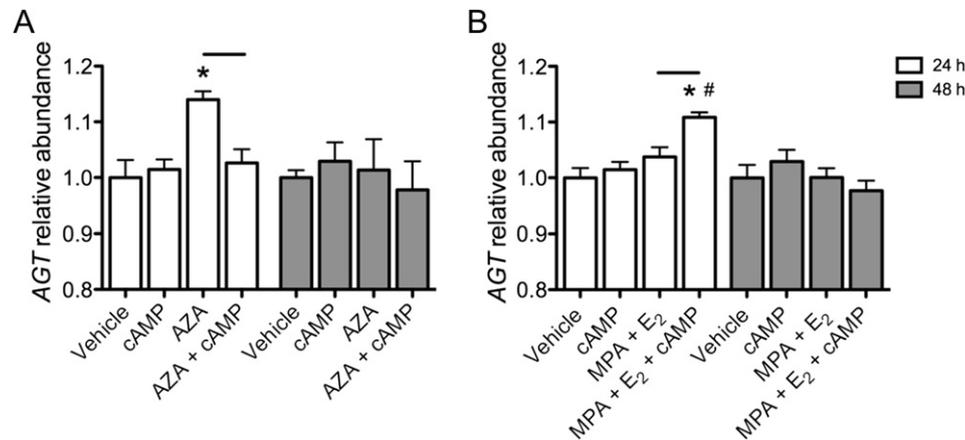


Fig. 4. *AGT* mRNA abundance in BeWo cells (mean \pm SEM). (A) At 24 h incubation, *AGT* expression was greater in AZA treated BeWo cells compared to vehicle treated controls, whereas *AGT* expression in cAMP or AZA + cAMP treated cells were not different from the control. Therefore AZA treated BeWo cells had significantly greater *AGT* expression compared to BeWo cells treated with the combined cAMP + AZA treatment. At 48 h incubation, there was no significant difference between the expression of *AGT* in the vehicle, cAMP, AZA or the combined cAMP + AZA treated BeWo cells. (B) *AGT* mRNA in BeWo cells was significantly increased with the combined MPA + E₂ + cAMP treatment at 24 h incubation compared to vehicle treated controls and since there was no significant difference between *AGT* expression in the vehicle, cAMP or the MPA + E₂ treated BeWo cells, these cells also had significantly lower *AGT* expression than the MPA + E₂ + cAMP treated BeWo cells. At 48 h incubation, there was no significant difference between the expression of *AGT* in the vehicle, cAMP, MPA + E₂ or the MPA + E₂ + cAMP treated BeWo cells ($n = 3$). * denotes significant difference from vehicle treated controls at the same incubation time ($P < 0.05$); # denotes significant difference from cAMP treated cells at the same incubation time ($P < 0.05$); the solid line denotes significant difference in AZA or MPA + E₂ treatments with and without the addition of cAMP ($P < 0.05$).

successfully demonstrate that estrogen and progesterone are able to stimulate *REN* and *AGT* mRNA as well as prorenin protein by trophoblast cells *in vitro*. Further studies are necessary to determine whether the human placental RAS *in vivo* responds to sex steroids in a similar manner.

The addition of cAMP to the MPA and E₂ mixture had no additive effect on *REN* mRNA abundance; that is, there was no significant difference between treatments after addition of cAMP. Furthermore, MPA and E₂ together, with or without the addition of cAMP, were more effective at stimulating *REN* expression than cAMP alone. This suggests that MPA and E₂ most likely act by stimulating cAMP and, as such, additional cAMP did not further stimulate renin expression in HTR-8/SVneo cells.

All of the treatments used in this study stimulated prorenin mRNA and protein levels but only in HTR-8/SVneo cells. We propose that the reason we are unable to induce renin in BeWo cells is due to the *REN* gene in these cells not being transcribable or, that the region of the mRNA our primers are designed against is mutated or missing. In addition, we were unable to detect *AGTR1* or *AGTR2* mRNAs in BeWo cells, which may suggest that this cancer cell line has undergone significant physiological and molecular changes.

It is important to note that the lack of translation of differences in *REN* mRNA abundance to differences in prorenin protein in the media may be because all treatments resulted in maximal prorenin production. On the other hand, active renin production in these supernatants was not measured and it is possible that prorenin in these cells may have undergone activation to renin, through the release of proteases by global hypomethylation, or through stimulation by cAMP and steroid hormones, which could act on the constitutively expressed prorenin [21,22]. The ELISA we use is targeted specifically to the pro-segment of prorenin and does not detect active renin.

The treatments used in this study were selected primarily to examine their potential effects on placental *REN* and prorenin protein however we did observe significant increases in *AGT* expression in both cell lines in response to both AZA and the sex steroids. Interestingly, these increases were only transient and were no longer evident after 48 h. Whether this transient effect is

due to negative feedback from the concomitant production of high levels of prorenin in HTR-8/SVneo cells, as seen in other cell types [23], is unknown. It seems more likely however, since we see similar effects in BeWo cells which do not express renin, that it is due to the rapid turnover of *AGT* mRNA to protein or that the treatments have a different mechanism of action to that against renin and require additional or prolonged stimuli to have a sustained affect.

Interestingly, both the *AGT* and *REN* genes lie on chromosome 1, however analysis has shown that there is a great genetic distance between the two genes [24]. Nevertheless, AZA administration results in global hypomethylation and could cause activation of both *AGT* and *REN*. Indeed, we have demonstrated increased *AGT* and *REN* expression in HTR-8/SVneo but only increased *AGT* expression in BeWo cell lines. The combination of AZA and cAMP also had a small additive effect on *AGT* mRNA abundance in HTR-8/SVneo cells, similar to that seen in *REN*, but cAMP blocked the effects of AZA on *AGT* in BeWo cells (Figs. 3 and 4). This also supports our hypothesis that BeWo cells are physiologically abnormal.

Estrogen has also been shown to induce angiotensinogen release in rats [25]. In humans, oral contraceptives, which contain synthetic estrogen, cause a dose dependent increase in plasma angiotensinogen levels [26]. In addition, estrogen replacement therapy also increases angiotensinogen levels [27] due to upregulation of hepatic *AGT*. Thus it is perhaps not surprising that *AGT* expression was greater in MPA and E₂ treated cells compared to vehicle in HTR-8/SVneo cells.

In conclusion, we have shown that the two trophoblast cell lines respond very differently to a wide range of treatments, and despite the effectiveness of these agents at stimulating prorenin expression in HTR-8/SVneo cells, they were unable to induce *REN* expression in BeWo cells. As a result, we propose that AZA, progesterone and estradiol stimulate *REN* expression and prorenin protein in trophoblast cells through a cAMP-dependent pathway, although they target different aspects of this pathway. AZA, likely acting through hypomethylation of the region around the CRE, probably allows for better binding of cAMP and other associated binding proteins, whereas progesterone and estradiol probably act by stimulating cAMP release.

Additive effects of these treatments on the placental RAS pathway could be proposed to further enhance the activity of the placental RAS through provision of more prorenin binding sites, stimulation of placental *AGT* expression and upregulation of *AGTR1*. The role(s) of epigenetic factors such as gene methylation, and the sex steroids on the activity of the placental RAS *in vivo* remain to be determined, but we have demonstrated that the activity of the placental RAS, which is thought to play a role in placentalation, is regulated by sex steroids and is subject to epigenetic modification.

Acknowledgments

This work was supported by a project grant [510746] to E.R. Lumbers from the National Health and Medical Research Council of Australia.

References

- [1] Williams PJ, Mistry HD, Innes BA, Bulmer JN, Pipkin FB. Expression of AT1R, AT2R and AT4R and their roles in extravillous trophoblast invasion in the human. *Placenta* 2010;31:448–55.
- [2] Morgan T, Craven C, Ward K. Human spiral artery renin-angiotensin system. *Hypertension* 1998;32:683–7.
- [3] Marques FZ, Pringle KG, Conquest A, Hirst JJ, Markus MA, Sarris M, et al. Molecular characterization of renin-angiotensin system components in human intrauterine tissues and fetal membranes from vaginal delivery and caesarean section. *Placenta* 2011;32:214–21.
- [4] Pringle KG, Tadros MA, Callister RJ, Lumbers ER. The expression and localization of the human placental prorenin/renin-angiotensin system throughout pregnancy: roles in trophoblast invasion and angiogenesis? *Placenta* 2011;32:956–62.
- [5] Graham CH, Hawley TS, MacDougall RC, Kerbel RS, Khoo N, Lala PK. Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp Cell Res* 1993;206:204–11.
- [6] Pattillo RA, Gey GO, Delfs E, Mattingly RF. Human hormone production *in vitro*. *Science* 1968;159:1467–9.
- [7] Wang Y, Pringle KG, Chen YX, Zakar T, Lumbers ER. Regulation of the renin-angiotensin system (RAS) in BeWo and HTR-8/SVneo trophoblast cell lines. *Placenta* 2012;33:634–9.
- [8] Klar J, Sandner P, Muller MW, Kurtz A. Cyclic AMP stimulates renin gene transcription in juxtaglomerular cells. *Pflügers Arch* 2002;444:335–44.
- [9] Poisner AM, Thrailkill K, Poisner R, Handwerger S. Cyclic AMP as a second messenger for prorenin release from human decidual cells. *Placenta* 1991;12:263–7.
- [10] Pinet F, Mizrahi J, Menard J, Corvol P. Role of cyclic AMP in renin secretion by human transfected juxtaglomerular cells. *J Hypertens Suppl* 1986;4:S421–3.
- [11] Pinet F, Mizrahi J, Labouladine I, Menard J, Corvol P. Regulation of prorenin secretion in cultured human transfected juxtaglomerular cells. *J Clin Invest* 1987;80:724–31.
- [12] Sunahori K, Juang YT, Kytтары VC, Tsokos GC. Promoter hypomethylation results in increased expression of protein phosphatase 2A in T cells from patients with systemic lupus erythematosus. *J Immunol* 2011;186:4508–17.
- [13] Itskovitz J, Sealey JE, Glorioso N, Rosenwaks Z. Plasma prorenin response to human chorionic gonadotropin in ovarian-hyperstimulated women: correlation with the number of ovarian follicles and steroid hormone concentrations. *Proc Natl Acad Sci U S A* 1987;84:7285–9.
- [14] Hunter MG, Baker TG. Effect of hCG, cAMP and FSH on steroidogenesis by human corpora lutea *in vitro*. *J Reprod Fertil* 1981;63:285–8.
- [15] Lumbers ER. The ovary. In: Nicholls MG, Robertson JIS, editors. *The renin-angiotensin system*. London, England: Gower Medical Publishing Co.; 1993. p. 46.1–46.12.
- [16] Sealey JE, Itskovitz-Eldor J, Rubattu S, James GD, August P, Thaler I, et al. Estradiol- and progesterone-related increases in the renin-aldosterone system: studies during ovarian stimulation and early pregnancy. *J Clin Endocrinol Metab* 1994;79:258–64.
- [17] Logan PC, Ponnampalam AP, Rahnama F, Lobie PE, Mitchell MD. The effect of DNA methylation inhibitor 5-aza-2'-deoxycytidine on human endometrial stromal cells. *Hum Reprod* 2010;25:2859–69.
- [18] Chen JZ, Sheehan PM, Brennecke SP, Keogh RJ. Vessel remodelling, pregnancy hormones and extravillous trophoblast function. *Mol Cell Endocrinol* 2012;349:138–44.
- [19] Gilsbach R, Kouta M, Bonisch H, Bruss M. Comparison of *in vitro* and *in vivo* reference genes for internal standardization of real-time PCR data. *Bio-techniques* 2006;40:173–7.
- [20] Pringle KG, Zakar T, Yates D, Mitchell CM, Hirst JJ, Lumbers ER. Molecular evidence of a (pro)renin/(pro)renin receptor system in human intrauterine tissues in pregnancy and its association with PGHS-2. *J Renin Angiotensin Aldosterone Syst* 2011;12:304–10.
- [21] Derx FH, Schalekamp MP, Schalekamp MA. Two-step prorenin-renin conversion. Isolation of an intermediary form of activated prorenin. *J Biol Chem* 1987;262:2472–7.
- [22] Derx FH, Schalekamp MP, Bouma B, Kluff C, Schalekamp MA. Plasma kallikrein-mediated activation of the renin-angiotensin system does not require prior acidification of prorenin. *J Clin Endocrinol Metab* 1982;54:343–8.
- [23] Schefe JH, Unger T, Funke-Kaiser H. PLZF and the (pro)renin receptor. *J Mol Med (Berl)* 2008;86:623–7.
- [24] Lifton RP, Jeunemaitre X. Finding genes that cause human hypertension. *J Hypertens* 1993;11:231–6.
- [25] Gordon MS, Chin WW, Shupnik MA. Regulation of angiotensinogen gene expression by estrogen. *J Hypertens* 1992;10:361–6.
- [26] Hollenberg NK, Williams GH, Burger B, Chenitz W, Hoosmand I, Adams DF. Renal blood flow and its response to angiotensin II. An interaction between oral contraceptive agents, sodium intake, and the renin-angiotensin system in healthy young women. *Circ Res* 1976;38:35–40.
- [27] Schunkert H, Danser AH, Hense HW, Derx FH, Kurzinger S, Riegger GA. Effects of estrogen replacement therapy on the renin-angiotensin system in postmenopausal women. *Circulation* 1997;95:39–45.

APPENDIX C

Fetal sex affects expression of renin-angiotensin
system components in term human decidua.

Fetal Sex Affects Expression of Renin-Angiotensin System Components in Term Human Decidua

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The maternal decidua expresses the genes of the renin-angiotensin system (RAS). Human decidua was collected at term either before labor (*i.e.* cesarean delivery) or after spontaneous labor. The mRNA for prorenin (*REN*), prorenin receptor (*ATP6AP2*), angiotensinogen (*AGT*), angiotensin-converting enzymes 1 and 2 (*ACE1* and *ACE2*), angiotensin II type 1 receptor (*AGTR1*), and angiotensin 1–7 receptor (*MAS1*) were measured by quantitative real-time RT-PCR. Decidual explants were cultured in duplicate for 24 and 48 h, and all RAS mRNA, and the secretion of prorenin, angiotensin II, and angiotensin 1–7 was measured using quantitative real-time RT-PCR, ELISA, and radioimmunoassay, respectively. In the decidua collected before labor, *REN* mRNA levels were higher if the fetus was female. In addition, *REN*, *ATP6AP2*, *AGT*, and *MAS1* mRNA abundance was greater in decidual explants collected from women carrying a female fetus, as was prorenin protein. After 24 h, *ACE1* mRNA was higher in the decidual explants from women with a male fetus, whereas after 48 h, both *ACE1* and *ACE2* mRNA was higher in decidual explants from women with a female fetus. Angiotensin II was present in all explants, but angiotensin 1–7 levels often registered below the lower limits of sensitivity for the assay. After labor, decidua, when compared with nonlaboring decidua, demonstrated lower *REN* expression when the fetus was female. Therefore, the maternal decidual RAS is regulated in a sex-specific manner, suggesting that it may function differently when the fetus is male than when it is female. (*Endocrinology* 153: 462–468, 2012)

It is becoming increasingly apparent that the outcome of pregnancy in relation to the health of the fetus and survival of the neonate is influenced by fetal sex. Several studies have shown that male fetuses have a higher risk of adverse pregnancy outcomes including spontaneous abortions (1), miscarriages later in pregnancy (2), stillbirths (3, 4), premature rupture of membranes and spontaneous preterm birth (5–8), gestational diabetes (9), and delivery by cesarean section (7, 10, 11). In term deliveries, a higher proportion of preeclamptic pregnancies carry a male fetus (5, 9), whereas in preterm deliveries the proportion of females from preeclamptic pregnancies is higher (5, 10). Female babies are also more likely to be growth restricted

than are males (7, 9). The causes of these differences are unknown, but it has been suggested that intrauterine tissues (and in particular the placenta) regulate fetal growth and survival in a sex-specific manner (12).

Renin is an enzyme secreted by the kidney that hydrolyzes angiotensinogen (*AGT*) to produce angiotensin I (Ang I), which in turn is cleaved by angiotensin-converting enzyme 1 (*ACE1*) to form angiotensin II (Ang II). The major actions of the renin-angiotensin system (RAS) are mediated via binding of Ang II to the Ang II type 1 receptor (*AT₁R*). Ang II exerts opposing actions via the Ang II type 2 receptor (*AT₂R*) (13). An angiotensin-converting enzyme homolog, angiotensin-converting enzyme 2 (*ACE2*),

can terminate the actions of Ang II by converting it to angiotensin 1–7 (Ang 1–7), which has effects that oppose those of Ang II and exerts these by binding to the pro-oncogene receptor (Mas) (14).

Until recently the precursor of renin, prorenin, was considered to have little biological activity, despite the fact that its circulating levels are 10 times higher than those of renin (15). The discovery of a (pro)renin receptor [(P)RR] that binds both renin and prorenin has changed this notion. Prorenin bound to (P)RR is active and can cleave AGT to Ang I. In addition, binding of prorenin to the (P)RR can induce intracellular signaling in its own right (16, 17).

Although the RAS is classically associated with the control of blood pressure, tissue-specific RAS have now been described in a number of organs not necessarily involved in blood pressure control (18–21). We have identified most of the RAS components in term human decidua, placenta, myometrium, and fetal membranes (22, 23). Although RAS proteins are found in human fetal membranes, expression of some of the RAS genes in these membranes is very low. For example, amnion contains an abundance of renin despite low renin (*REN*) mRNA (22). Interestingly, we have shown that decidua, a maternal tissue, expresses all of the known RAS genes (22, 23). Locally produced Ang II might affect the decidual microvasculature either by promoting angiogenesis or by altering vascular tone. Because decidual renin can escape into the maternal circulation (24), the decidual RAS may also play a role in maternal cardiovascular and renal homeostasis. Through these extrauterine and intrauterine actions, the decidual RAS could affect fetal development by altering embryonic access to nutrients.

Human recombinant renin has been shown to stimulate decidual production of prostaglandin-endoperoxide synthase 2, also known as cyclooxygenase-2 (25), a key enzyme involved in the initiation of labor. The release of prorenin from the decidua is constitutive, and the decidua is the major source of prorenin within the human uterus (23, 26). The decidual RAS could therefore play a role in the initiation of labor because the decidua is immediately adjacent to the myometrium.

We have developed a human decidual explant model to study the decidual RAS. This explant model provides the closest possible approximation to *in vivo* because freshly isolated tissue is used. This means that all cell types are present and in the same proportions at the end of 48 h of incubation as at the beginning. This is because the time elapsed is insufficient for any one cell type to outgrow the others. Furthermore, there is no evidence of increased cell death at 48 h.

The aims of the present study were: 1) to determine whether the expression of decidual RAS genes was af-

ected by fetal sex; 2) to define the expression of RAS genes in term human decidua collected before the onset of spontaneous labor by maintaining decidua in explant culture for 24 and 48 h *ex vivo*; and 3) to determine whether decidual explants could be used to study the control of expression of RAS genes and the secretion of prorenin and the angiotensin peptides Ang II and Ang 1–7. We also set out to determine whether the expression of decidual RAS genes is altered with labor.

Materials and Methods

Tissue collection

Decidual samples were collected as described previously (22, 23). All samples were collected from uncomplicated singleton pregnancies in women aged 19–39 yr. Decidual samples at term (37–40 wk gestation) delivered by elective cesarean section in the absence of labor were collected for the measurement of decidual gene expression (n = 21). Additional samples were collected for decidual explants (n = 6), and decidua was collected after spontaneous labor and vaginal delivery (38–41 wk gestation) for measurement of decidual RAS gene expression (n = 23). Women treated with nonsteroidal antiinflammatory drugs or who had a history of infection, chorioamnionitis, asthma, or preeclampsia or who were undergoing induction of labor were excluded. Informed consent was obtained from all participants, and the study was approved by the Hunter Area Research Ethics Committee and the University of Newcastle Human Research Ethics Committee. The fetal membranes and attached decidua parietalis were isolated as a whole, apart from a 2-cm border at the edge of the placenta. Amnion was peeled from the choriodecidua, and chorion laevae were separated from the decidua by sharp dissection as described previously (27). Decidual tissue was then used in the decidual explant study or snap frozen in liquid nitrogen for subsequent RNA analyses.

Decidual explant culture

Entire excised deciduas were washed in tissue culture medium (phenol red free DMEM/F-12 supplemented with 15 mM HEPES, 1.2 g/liter NaHCO₃, 1 mg/ml L-glutathione reduced, 0.1 g/liter albumin fraction V, 0.65 μg/ml aprotinin, 10% fetal bovine serum, 40 μg/ml gentamicin) and dissected into approximately 0.25-cm² pieces. Several pieces of decidua were selected randomly, blotted, and weighed. One hundred milligrams of decidua were placed into each well of a six-well plate with 2 ml of incubation medium. Decidual tissues and supernatants were collected after 24 and 48 h and snap frozen in liquid nitrogen for subsequent protein and mRNA analyses. Each experiment was conducted, in duplicate, using six separate decidual samples (three from women carrying a male and three from women carrying a female baby). Cell viability was verified by measuring lactate dehydrogenase release after incubation, as well as by measuring RNA stability and quality (data not shown).

The medium from decidual explant cultures was collected into EDTA tubes containing a protease inhibitor cocktail (Sigma, St. Louis, MO), and Ang II and Ang 1–7 concentrations were measured by RIA at ProSearch International Australia (Mel-

bourne, Australia). Prorenin in the incubation medium was measured using the human prorenin ELISA kit (Molecular Innovations Inc., Novi, MI).

Semiquantitative real-time RT-PCR

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). RNA samples were treated with deoxyribonuclease (QIAGEN N.V., Hilden, Germany) before being reverse transcribed using a Superscript III reverse transcriptase kit with random hexamers (Invitrogen). Quantitative PCR was performed in an Applied Biosystems 7500 real-time PCR system using SYBR Green for detection (Applied Biosystems, Carlsbad, CA). Each reaction contained 5 μ l of SYBR Green PCR master mix (Applied Biosystems), RAS primers as we have described previously (22, 23), cDNA reverse transcribed from 10 ng total RNA, and water to 10 μ l. mRNA abundance was calculated relative to β -actin (*ACTB*) mRNA using the comparative Ct method as described previously (22, 23).

RIA of Ang II and Ang 1–7

Ang II was measured by direct RIA in plasma using the delayed tracer addition technique. Each sample of medium was equilibrated for 20 h at 4 C in a total volume of 300 μ l with antibody raised in rabbit against angiotensin II N-terminally conjugated to bovine thyroglobulin. Monoiodinated 125 I-angiotensin II, 10,000 cpm in 100 μ l, was added and allowed to equilibrate for a further 16 h at 4 C, after which the bound and free phases were separated using Dextran 10-coated charcoal and centrifugation. Sensitivity was 3.5 pg/ml. Intra- and interassay coefficients of variation were 6.4 and 12%, respectively.

Ang 1–7 was assayed directly by RIA using an antibody raised in guinea pig to Ang 1–7 N-terminally conjugated to porcine thyroglobulin and Ang 1–7 that had been monoiodinated with 125 I antibody. Bound 125 I-Ang 1–7 was separated from free by dextran 10-coated charcoal, and unbound counts per minute were compared with serially diluted standard amounts of Ang 1–7. Sensitivity was 14 pg/ml. Cross-reactivities to Ang I, Ang II, Ang III, and Ang IV were 0.11, 0.04, 0.53, and 0.03%, respectively. Intra- and interassay coefficients of variation were 4.5 and 10%, respectively.

Measurement of prorenin protein by ELISA

Prorenin in culture medium was measured using the human prorenin ELISA kit (Molecular Innovations) according to the manufacturer's instructions. Prorenin in each sample was captured by an antibody immobilized on to the surface of each well of the plate. A primary antibody specific for prorenin was then applied and the unbound fraction was removed by washings. For subsequent detection by means of color development, a secondary antibody conjugated to horseradish peroxidase 3,3',5,5'-tetramethylbenzidine substrate was added. After termination of the reaction with 4 M sulfuric acid, OD was read at 450 nm. Prorenin concentration was directly proportional to color development and was measured using a standard curve. Samples were assayed in duplicate. In our laboratory 1 ng/ml amniotic fluid prorenin measured using this technique generated 116 ng/h⁻¹ · ml⁻¹ of Ang I from angiotensinogen present in nephrectomized sheep plasma used as the source of angiotensinogen substrate. All samples were assayed on one ELISA plate. There-

fore, there was no interassay variability. Intraassay coefficient of variation was 7.3%.

Data analysis

Decidual RAS gene expression data were tested using the non-parametric Mann-Whitney *U* test. Decidual explant RAS gene expression data were tested for normality using the skewness and kurtosis test. The data set was found to be not normally distributed, so the data were logarithmically transformed. A univariate ANOVA with the patients' identification as a covariate was then used to test for differences in RAS mRNA abundance as a function of fetal sex and incubation time in decidual explants. An independent-sample *t* test was used to determine the effects of fetal sex on prorenin, Ang II, and Ang 1–7 levels in the supernatant after 48 h *ex vivo*. The SPSS statistical package (SPSS for Windows, release 17.0.0; Chicago, IL) was used for all analyses. Significance was set at $P < 0.05$.

Results

The interaction between fetal sex and labor on decidual RAS expression

The level of *REN* mRNA in maternal decidua from nonlaboring women was higher if the fetus was female ($P = 0.011$). Labor only affected decidual *REN* mRNA abundance when the fetus was female; the decidual *REN* mRNA level was lower after labor ($P = 0.027$), such that the significant sex-associated difference in *REN* mRNA abundance seen before labor was no longer present (Fig. 1).

There were no sex-specific effects, nor were there any interactions between fetal sex and labor on the expression of decidual *AGT*, *ACE1*, *ACE2*, *AGTR1*, and *ATP6AP2* mRNA.

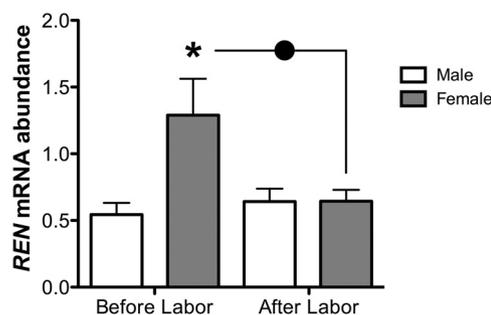


FIG. 1. Sex differences in *REN* mRNA abundance in maternal decidua collected before or after labor. Before labor decidual *REN* mRNA abundance was higher in women carrying a female fetus; after labor decidual *REN* levels no longer differed according to fetal sex because *REN* mRNA was lower in women with a female fetus after spontaneous labor compared with those not in labor. The *n* value was 21 for before labor decidual samples ($n = 11$ for women carrying a male fetus and $n = 10$ for women carrying a female fetus), and the *n* value was 23 for after labor decidual samples ($n = 10$ for women carrying a male fetus and $n = 13$ for women carrying a female fetus). The closed circle denotes the difference before and after labor ($P < 0.05$). *, Significant difference between fetal sex ($P < 0.05$).

Effects of fetal sex on *REN* mRNA and protein levels in term decidua

To determine whether the sex differences in decidual *REN* mRNA persisted *in vitro*, decidual explants were cultured for 24 or 48 h. *REN* mRNA levels increased during incubation of decidual explants from women with a male fetus ($P = 0.002$). *REN* mRNA abundance was, however, significantly higher at 24 and 48 h in decidual explants from women with a female fetus compared with explants from women with a male fetus ($P = 0.002$ and $P = 0.010$, respectively; Fig. 2A).

Prorenin secretion into the explant supernatant, measured at 48 h *ex vivo* (over a 24 h incubation period), was significantly higher in the medium from explants of women with a female fetus ($P < 0.05$). Prorenin secretion into the medium for decidual explants from women carrying a female fetus was 4.9 ± 0.95 ng/ml compared with 0.13 ± 0.06 ng/ml in decidual explants from women carrying a male fetus (Fig. 2B).

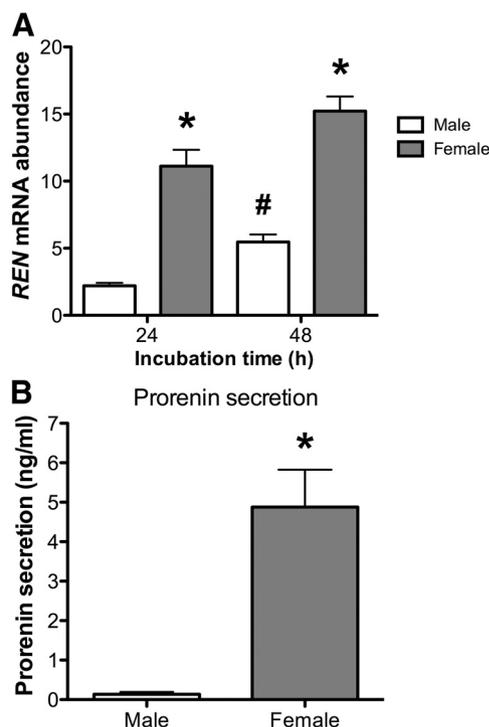


FIG. 2. Sex differences in *REN* mRNA abundance and prorenin secretion in decidual explants. *REN* mRNA levels were measured at 24 and 48 h of incubation. Prorenin was measured in medium collected from the second 24-h incubation period. A, After 24 and 48 h of incubation, decidual *REN* mRNA levels were higher in women carrying a female fetus. *REN* mRNA abundance in decidua increased with incubation time in decidua from women with a male fetus. B, The incubation medium from decidua of women with female fetuses contained significantly higher levels of prorenin than decidual explants from women with male fetuses. Data shown are for $n = 4$ –6 decidual explants collected from three different women per group. *, Significant difference between fetal sex ($P < 0.05$); #, significant difference between incubation time within the same sex ($P < 0.05$).

Effects of fetal sex on the expression of RAS mRNAs in decidual explants

mRNA for the RAS genes *AGT*, *ACE1*, *ACE2*, *AGTR1*, *ATP6AP2*, and *MAS1* were detected in decidual explants at 24 and 48 h of incubation (Fig. 3), whereas *AGTR2* mRNA was not. At 24 and 48 h of incubation, decidual mRNA levels of *AGT*, *ATP6AP2*, and *MAS1* were all significantly higher in decidual explants from women with a female fetus than from women carrying a male fetus. *ACE1* mRNA abundance was higher at 48 h of incubation in decidua from women with a female fetus than from women carrying a male fetus ($P = 0.002$). In contrast, the *ACE2* mRNA expression was lower at 24 h ($P = 0.041$), but higher at 48 h ($P = 0.037$), of incubation in decidua from women with a female fetus compared with women pregnant with male fetuses.

In decidual explants isolated from women carrying a male fetus, *ACE1* mRNA levels decreased with incubation time ($P = 0.010$; Fig. 3B). In decidual explants from women who were carrying a female fetus, the levels of *ACE2* and *ATP6AP2* mRNA increased with incubation time ($P = 0.041$ and $P = 0.010$, respectively; Fig. 3, C and E). *AGTR1* mRNA abundance in decidual explants was not affected by either fetal sex or incubation time (Fig. 3D).

Decidual explant medium was changed every 24 h; Ang II and Ang 1–7 levels were measured in medium collected from the second 24 h incubation. Measurable levels of Ang II were found (range of 6.9–134 pg/ml, $n = 6$), whereas Ang 1–7 levels in the medium were low, often registering below the lower limits of sensitivity of the assay (*i.e.* <14 pg/ml).

Discussion

The present study has revealed a striking effect of fetal sex on the expression of decidual *REN* mRNA. Subsequent tissue culture experiments confirmed and expanded our finding that the sex of the fetus determines the level of expression of several RAS mRNAs and the amount of prorenin secreted. In addition, we showed that decidual explants secrete both Ang II and Ang 1–7 into the culture supernatant, although we have not been able to determine whether the production of these peptides is influenced by fetal sex. These sex-associated differences in decidual *REN* mRNA expression and prorenin secretion provide new insights into the effects of fetal sex on adjacent tissues that are important for fetal growth and development, especially in early gestation. As mentioned in the introductory text, spontaneous abortions (1), miscarriages later in pregnancy (2), stillbirths (3, 4), premature rupture of

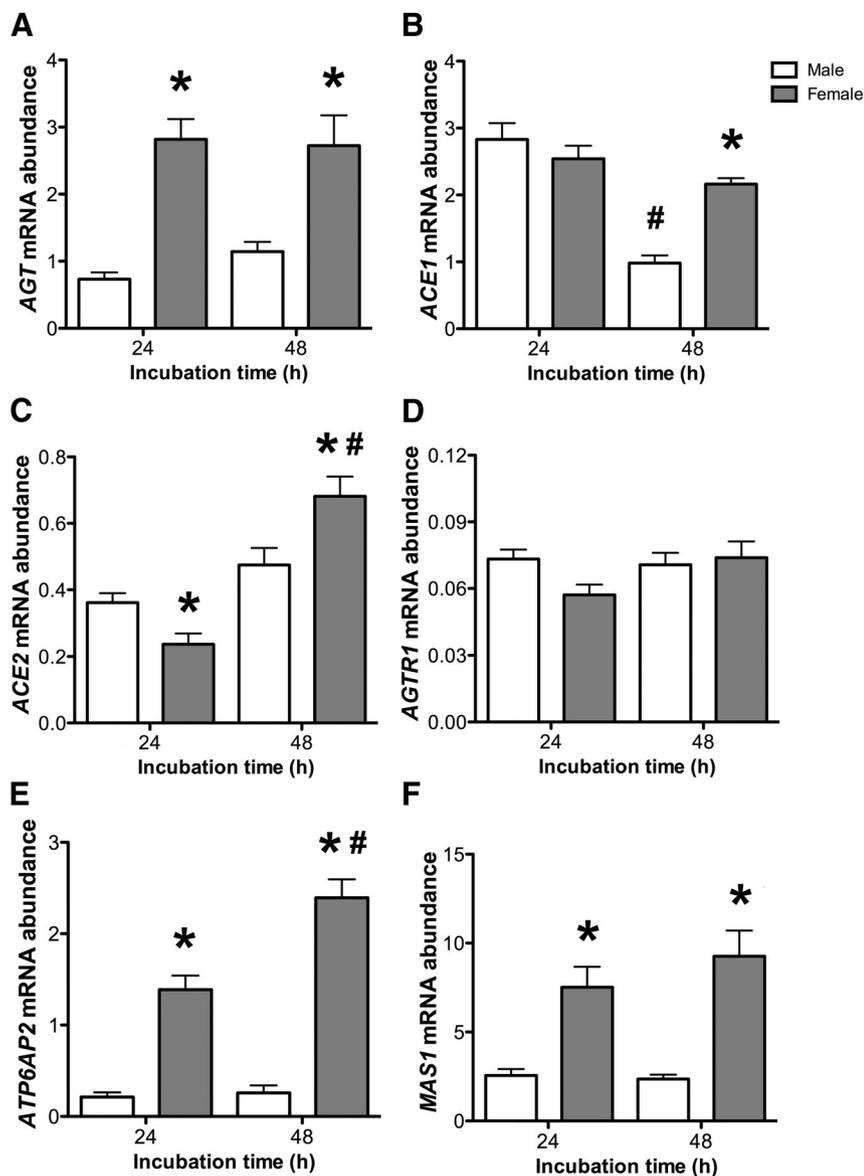


FIG. 3. Effects of fetal sex on mRNA levels for other genes in the RAS in human decidual explants incubated for 24 and 48 h. **A**, *AGT* mRNA levels after 24 and 48 h incubation were higher in decidual explants from women with a female fetus ($P = 0.002$ and $P = 0.004$). **B**, After 48 h incubation, *ACE1* expression was higher in women with a female fetus ($P = 0.002$). *ACE1* mRNA abundance in decidual explants from women with a male fetus decreased with incubation time ($P = 0.01$). **C**, *ACE2* mRNA levels at 24 h incubation were lower in explants from women with a female fetus but were higher at 48 h of incubation ($P = 0.041$ and $P = 0.037$). *ACE2* mRNA abundance increased with incubation time for decidual explants from women with a female fetus ($P = 0.041$). **D**, *AGTR1* mRNA abundance in decidual explants was not affected by either fetal sex or incubation time. **E**, Decidual *ATP6AP2* mRNA levels were higher after 24 and 48 h of incubation for explants from women carrying a female fetus ($P < 0.001$ and $P = 0.002$). *ATP6AP2* expression was higher over the course of incubation for decidual explants from women with a female fetus ($P = 0.010$). **F**, At 24 and 48 h of incubation, *MAS1* expression was higher in explants from women with a female fetus ($P = 0.005$ and $P < 0.001$). Values shown are for $n = 4$ – 6 decidual explants collected from three women per group. *, Significant difference between sex ($P < 0.05$); #, significant difference between incubation time within the same sex ($P < 0.05$).

membranes and spontaneous preterm birth (5–8), gestational diabetes (9), and delivery by cesarean section (7, 10, 11) occur more frequently if the mother is carrying a male fetus, whereas the incidence of preeclampsia in preterm

pregnancies is more common in women carrying female fetuses (5, 10). Female babies are also more likely to be growth restricted (7, 9). All of these sex differences may be related to the effects of fetal sex on the decidual RAS because we believe that the decidual RAS is involved in regulating decidual angiogenesis and perhaps placental implantation. Therefore, these sex-specific effects on the decidual RAS expression and prorenin secretion may contribute to the sex differences reported in pregnancy outcomes cited above. Future studies on gene expression and protein synthesis in tissues of the uteroplacental unit should take into account the sex of the fetus because we have shown that fetal sex is able to alter gene expression in a maternal tissue (decidua).

It is unclear how sex-specific differences of decidual RAS expression affect pregnancy outcomes because there is currently no concrete knowledge of how the RAS functions within the decidua or how the decidua contributes its RAS products to the maternal circulation. However, it is clear that the sex differences in RAS genes seen in the decidua are unique because the fetal membranes and placenta do not show any sex-specific differences in RAS gene expression (22), even though these tissues are predominantly fetal in origin. Further investigation of the RAS in the myometrium may be warranted because the myometrium is a maternal tissue closely associated with the decidua.

In an attempt to better understand the effects of fetal sex on decidual RAS mRNA expression levels and subsequent protein secretion, we established human decidual explants. We were successful in maintaining decidua for 48 h *ex vivo*. The pattern of *REN* and *AGTR1* expression within this explant model was similar to that seen in nonlaboring, nonincubated decidua (a difference and no difference

with fetal sex, respectively). In addition, decidual explants secreted prorenin, Ang II, and Ang 1–7 into the incubation medium, suggesting that the explant model is a valid model for the study of the human decidual RAS *ex vivo*.

Not only did the sex of the fetus influence maternal decidual *REN* mRNA expression, but it also affected prorenin protein secretion by the decidua. This sex difference was maintained for up to 48 h *ex vivo*. Therefore, it is unlikely that fetal sex hormones were responsible for the difference. The persistence of the sex difference in expression and the emergence of increased expression of other RAS genes in decidua from women carrying a female fetus, suggest that decidual RAS gene expression is permanently altered by fetal sex. However, we have not as yet studied whether fetal sex affects the expression of the decidual/placental RAS in early gestation.

Among the various late gestation human intrauterine tissues (fetal membranes, placenta, chorion, and myometrium), the decidua has the highest levels of *REN* mRNA (22, 23). High levels of prorenin (measured as enzyme activity after acid activation) have, however, been found in fetal membranes (28), and immunostaining for prorenin also shows that it is present in amnion and chorion as well as decidua and placenta (22). Because amnion and chorion express only very low levels of *REN* mRNA, it is likely that decidual prorenin is the source of the high levels of prorenin found in amniotic fluid (22, 23, 26, 29). Because we have shown that prorenin secretion by cultured decidua is significantly higher in pregnancies with female than with male fetuses, it is reasonable to conclude that levels of prorenin in amnion and in amniotic fluid may be higher when the fetus is female than when it is male.

The secretion of prorenin into the maternal circulation from the uteroplacental unit could also be influenced by fetal sex (24). Evidence for this can be seen in mice in which a human *REN* transgene was made to be expressed only in the placenta, yet human prorenin was found in maternal plasma (30). This demonstrates the ability of placental prorenin to enter the maternal bloodstream. Because we consistently found measurable levels of Ang II in decidual explants, it is tempting to speculate that decidual Ang II might be able to escape into the maternal circulation from early in gestation. As a consequence, the higher incidence of growth restriction in female neonates (7) may be related to higher prorenin and Ang II levels, causing vasoconstriction and reduced uteroplacental blood flow, possibly through down-regulation of AT₂R within the uterine vasculature. We have, moreover, observed such a phenomenon when Ang II levels are raised in the pregnant ewe through exogenous infusion of Ang II for more than 24 h (31); in these studies, the actions of Ang II on AT₂R in the uterine vasculature of the sheep were able to offset the vasoconstrictor effects of Ang II mediated via the AT₁R (31).

In this study, several decidual products of the RAS, prorenin and Ang peptides, were measured to determine

whether the changes in expression of decidual RAS genes resulted in changes in the levels of their protein products or peptides. The high level of expression of *REN* mRNA was coupled with a high level of prorenin secretion from cultured decidua collected from women with a female fetus. Despite the striking difference in prorenin secretion and the up-regulation of expression of other RAS genes, it was not possible to demonstrate a sex difference in angiotensin peptide production, possibly because assays were performed on only six subjects (three male and three female) and the levels of the peptides were very low. In addition, there was degradation of these peptides, probably occurring concomitant with their production. Notwithstanding, it is still clear that an effect of fetal sex on the maternal decidua may be imprinted at an early stage of pregnancy, so that the differential expression of RAS components and the physiological and biochemical consequences of this may be present up to, but not after, labor.

Interestingly, it appears that the expression of decidual *REN* mRNA is down-regulated after labor (Fig. 1). If the decidual RAS plays a specific role in regulating the onset of labor, *e.g.* via stimulation of decidual prostaglandin-endoperoxide synthase 2 production (25), then the suppression of *REN* mRNA if the fetus is female would counterbalance this action. Alternatively, it is possible that withdrawal of the decidual RAS could affect the integrity of the fetal membranes through a reduction in production of TGF- β (32), which stimulates the formation of profibrotic molecules such as plasminogen activator inhibitor type 1, fibronectin, and collagens (33), or through reduced activation of proinflammatory cytokines (34). These possibilities need to be tested in future studies.

In conclusion, the present study provides the first demonstration of a fetal sex-associated difference in expression of RAS genes in the maternal decidua. We have also shown that the changes in maternal decidual RAS gene expression that occur with labor depend on the sex of the fetus. Our data provide novel insights into the influences of fetal sex on decidual gene expression, in which the latter is important in supporting fetal growth and development. Our findings could contribute to understanding why there are significant differences in adverse pregnancy outcomes between female and male fetuses.

Acknowledgments

Dr. David Casely (Prosearch Pty. Ltd.) carried out the Ang II and Ang 1–7 RIA.

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This work was supported by Project Grant 510746 from the National Health and Medical Research Council of Australia.

Disclosure Summary: Y.W., K.G.P., S.D.S., T.Z., and E.R.L. have nothing to declare. F.Z.M. and B.J.M. have received grant support from the National Health and Medical Research Council of Australia.

References

- Byrne J, Warburton D 1987 Male excess among anatomically normal fetuses in spontaneous abortions. *Am J Med Genet* 26:605–611
- Mizuno R 2000 The male/female ratio of fetal deaths and births in Japan. *Lancet* 356:738–739
- Engel PJ, Smith R, Brinsmead MW, Bowe SJ, Clifton VL 2008 Male sex and pre-existing diabetes are independent risk factors for stillbirth. *Aust N Z J Obstet Gynaecol* 48:375–383
- Smith GC 2000 Sex, birth weight, and the risk of stillbirth in Scotland, 1980–1996. *Am J Epidemiol* 151:614–619
- Vatten LJ, Skjaerven R 2004 Offspring sex and pregnancy outcome by length of gestation. *Early Hum Dev* 76:47–54
- Cooperstock M, Campbell J 1996 Excess males in preterm birth: interactions with gestational age, race, and multiple birth. *Obstet Gynecol* 88:189–193
- Melamed N, Yogev Y, Glezerman M 2010 Fetal gender and pregnancy outcome. *J Matern Fetal Neonatal Med* 23:338–344
- Zeitlin J, Saurel-Cubizolles MJ, De Mouzon J, Rivera L, Ancel PY, Blondel B, Kaminski M 2002 Fetal sex and preterm birth: are males at greater risk? *Hum Reprod* 17:2762–2768
- Di Renzo GC, Rosati A, Sarti RD, Cruciani L, Cutuli AM 2007 Does fetal sex affect pregnancy outcome? *Gender Med* 4:19–30
- Brettell R, Yeh PS, Impy LW 2008 Examination of the association between male gender and preterm delivery. *Eur J Obstet Gynecol Reprod Biol* 141:123–126
- Lieberman E, Lang JM, Cohen AP, Frigoletto Jr FD, Acker D, Rao R 1997 The association of fetal sex with the rate of cesarean section. *Am J Obstet Gynecol* 176:667–671
- Clifton VL 2010 Review: Sex and the human placenta: mediating differential strategies of fetal growth and survival. *Placenta* 31(Suppl):S33–S39
- MacGregor GA, Markandu ND, Roulston JE, Jones JC, Morton JJ 1981 Maintenance of blood pressure by the renin-angiotensin system in normal man. *Nature* 291:329–331
- Sampaio WO, Henrique de Castro C, Santos RA, Schiffrin EL, Touyz RM 2007 Angiotensin-(1–7) counterregulates angiotensin II signaling in human endothelial cells. *Hypertension* 50:1093–1098
- Derkx FH, Alberda AT, de Jong FH, Zeilmaker FH, Makovitz JW, Schalekamp MA 1987 Source of plasma prorenin in early and late pregnancy: observations in a patient with primary ovarian failure. *J Clin Endocrinol Metab* 65:349–354
- Nguyen G, Delarue F, Burcklé C, Bouzahir L, Giller T, Sraer JD 2002 Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J Clin Invest* 109:1417–1427
- Saris JJ, 't Hoen PA, Garrelds IM, Dekkers DH, den Dunnen JT, Lamers JM, Jan Danser AH 2006 Prorenin induces intracellular signaling in cardiomyocytes independently of angiotensin II. *Hypertension* 48:564–571
- Cooper AC, Robinson G, Vinson GP, Cheung WT, Broughton Pipkin F 1999 The localization and expression of the renin-angiotensin system in the human placenta throughout pregnancy. *Placenta* 20:467–474
- Dostal DE, Baker KM 1999 The cardiac renin-angiotensin system: conceptual, or a regulator of cardiac function? *Circ Res* 85:643–650
- Engeli S, Negrel R, Sharma AM 2000 Physiology and pathophysiology of the adipose tissue renin-angiotensin system. *Hypertension* 35:1270–1277
- Wagner J, Jan Danser AH, Derkx FH, de Jong TV, Paul M, Mullins JJ, Schalekamp MA, Ganten D 1996 Demonstration of renin mRNA, angiotensinogen mRNA, and angiotensin converting enzyme mRNA expression in the human eye: evidence for an intraocular renin-angiotensin system. *Br J Ophthalmol* 80:159–163
- Marques FZ, Pringle KG, Conquest A, Hirst JJ, Markus MA, Sarris M, Zakar T, Morris BJ, Lumbers ER 2011 Molecular characterization of renin-angiotensin system components in human intrauterine tissues and fetal membranes from vaginal delivery and cesarean section. *Placenta* 32:214–221
- Pringle KG, Zakar T, Yates D, Mitchell CM, Hirst JJ, Lumbers ER 2011 Molecular evidence of a (pro)renin/(pro)renin receptor system in human intrauterine tissues in pregnancy and its association with PGH₂-J. *Renin Angiotensin Aldosterone Syst* 12:304–310
- Brar HS, Do YS, Tam HB, Valenzuela GJ, Murray RD, Longo LD, Yonekura ML, Hsueh WA 1986 Uteroplacental unit as a source of elevated circulating prorenin levels in normal pregnancy. *Am J Obstet Gynecol* 155:1223–1226
- Mitchell MD, Edwin SS, Pollard JK, Trautman MS 1996 Renin stimulates decidual prostaglandin production via a novel mechanism that is independent of angiotensin II formation. *Placenta* 17:299–305
- Shaw KJ, Do YS, Kjos S, Anderson PW, Shinagawa T, Dubeau L, Hsueh WA 1989 Human decidua is a major source of renin. *J Clin Invest* 83:2085–2092
- Johnson RF, Mitchell CM, Clifton V, Zakar T 2004 Regulation of 15-hydroxyprostaglandin dehydrogenase (PGDH) gene activity, messenger ribonucleic acid processing, and protein abundance in the human chorion in late gestation and labor. *J Clin Endocrinol Metab* 89:5639–5648
- Skinner SL, Lumbers ER, Symonds EM 1968 Renin concentration in human fetal and maternal tissues. *Am J Obstet Gynecol* 101:529–533
- Lumbers ER 1971 Activation of renin in human amniotic fluid by low pH. *Enzymologia* 40:329–336
- Takimoto E, Ishida J, Sugiyama F, Horiguchi H, Murakami K, Fukamizu A 1996 Hypertension induced in pregnant mice by placental renin and maternal angiotensinogen. *Science* 274:995–998
- McMullen JR, Gibson KJ, Lumbers ER, Burrell JH, Wu J 1999 Interactions between AT₁ and AT₂ receptors in uterine arteries from pregnant ewes. *Eur J Pharmacol* 378:195–202
- Schuster N, Krieglstein K 2002 Mechanisms of TGF- β -mediated apoptosis. *Cell Tissue Res* 307:1–14
- Jan Danser AH, Batenburg WW, van Esch JH 2007 Prorenin and the (pro)renin receptor— an update. *Nephrol Dial Transplant* 22:1288–1292
- Keelan JA, Wang K, Chaiworapongsa T, Romero R, Mitchell MD, Sato TA, Brown DA, Fairlie WD, Breit SN 2003 Macrophage inhibitory cytokine 1 in fetal membranes and amniotic fluid from pregnancies with and without preterm labour and premature rupture of membranes. *Mol Hum Reprod* 9:535–540

Appendix D

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