The Renin-Angiotensin System in Endometrial Cancer

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Human Physiology
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August 2018
DECLARATION STATEMENT

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

Signed………………………………

7th August 2018
Date……………………………………
ACKNOWLEDGEMENTS

“Good teachers know how to bring out the best in students”

– Charles Kuralt

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Abstract

Endometrial cancer is one of the fourth most common cancer in the developed world and its incidence is increasing rapidly. Several studies have shown that there is an upregulation of the pro-angiogenic arm of the renin angiotensin system in endometrial cancer. Endometrial cancers express both prorenin and (pro)renin receptor ((P)RR) mRNA and have significantly greater levels of these proteins than normal adjacent endometrial tissue. Prorenin acting via the (P)RR can activate both RAS dependent and independent signaling pathways.

To determine the functional role of (P)RR in endometrial cancer growth, we used siRNA transfection to knock down (P)RR expression in three endometrial cancer cell lines (Ishikawa, HEC-1A and AN3CA). All three of the endometrial cancer cell lines examined (Ishikawa, HEC-1A and AN3CA) expressed (P)RR and prorenin (REN) mRNA, however levels of (P)RR and AGTR1 were much higher in Ishikawa cells. Transfection with a (P)RR siRNA resulted in knockdown of (P)RR at both gene and protein level in three cell lines. Furthermore, there was a significant reduction in endometrial cancer cell growth (proliferation and cell viability) in Ishikawa and AN3CA cells.

Several studies show that (P)RR is released in a soluble form (s(P)RR) into blood and urine. We therefore hypothesized that s(P)RR could be released from endometrial cancer cells and that levels of s(P)RR in blood and uterine fluid would be elevated in women with endometrial cancer. The levels of s(P)RR were measured with a specific s(P)RR ELISA it was found that all three cell lines secrete s(P)RR into the cell culture supernatant. Also, we found that there was significant amount of s(P)RR levels in plasma samples.

Therefore, we postulated that endometrial cancer growth can be inhibited by drugs that block Angiotensin (Ang) II/Ang II type 1 receptor interactions and prorenin/(P)RR mediated signaling pathways.
Further we looked at the individual effects and combined effects of RAS blockers with ATP6AP2 siRNA on cell viability and cell proliferation in three endometrial cancer cell lines. There was no effect of aliskiren (a renin inhibitor) on cell viability in HEC-1A and AN3CA cell lines. Perindoprilat (an ACE inhibitor) and losartan (an AT1R receptor antagonist) had no effect on the cell viability of any cell line. Another AT1R antagonist, telmisartan, which also acts as a selective agonist of peroxisome proliferator-activated receptor gamma (PPAR-γ), did however significantly reduce the viability of the three cell lines (Ishikawa 75%, HEC-1A 50% and AN3CA 60%). The combination of telmisartan + troglitazone had a similar effect to that of telmisartan on its own. Aliskiren + perindoprilat reduced the viability of HEC-1A cells, there was no effect in Ishikawa and AN3CA cells. Conversely, the combination of (P)RR siRNA + telmisartan significantly reduced cell viability and cell proliferation in Ishikawa cells. We also looked at the effect of ovarian steroids (estrogen and progesterone) on RAS expression in two other cancer cell lines (an endometrial cancer cell line (RL-952) and a breast cancer cell line (MCF-7)). Treatment with estrogen had no effect on RAS expression in RL-952 or MCF-7 cells.

This study is the first to characterize the functional role of prorenin and (P)RR in endometrial cancer, and to demonstrate that drugs that inhibit the (P)RR and the RAS pathway could reduce endometrial cancer growth. Finally, measurement of s(P)RR could be used as a biomarker for endometrial cancer detection.
**Conference Abstracts**

Effect of prorenin receptor ((P)RR) knock down and telmisartan on endometrial cancer growth, *Cancer Research, 2017; (77)-13*

Riazuddin Mohammed, Sarah J. Delforce, Yu Wang, Nicole M. Verrills, Eugenie R. Lumbers, Kirsty G. Pringle

Effect of (P)RR knockdown and Telmisartan on endometrial cancer growth, *Australian Society for Medical Research (ASMR), HMRI, June-2, Newcastle, 2017- Australia.*

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The prorenin receptor as a novel therapeutic target for the treatment of endometrial cancer, *Sydney Cancer Conference, September (22-23), Sydney, 2016- Australia.*
Riazuddin Mohammed, Sarah J. Delforce, Yu Wang, Nicole M. Verrills, Eugenie R. Lumbers, Kirsty G. Pringle

The prorenin receptor as a novel therapeutic target for the treatment of endometrial cancer,
*Australian Society for Medical Research (ASMR), Hunter Medical Research Institute, HMRI, Newcastle, April 2016 -Australia.*

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The prorenin receptor as a novel therapeutic target for the treatment of endometrial cancer,
*Gordon Research Seminar, February (20-21), Italy 2016.*

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Riazuddin Mohammed, Sarah J. Delforce, Yu Wang, Nicole M. Verrills, Eugenie R. Lumbers, Kirsty G. Pringle

The prorenin receptor as a novel therapeutic target for the treatment of endometrial cancer,
*Australian Society for Medical Research (ASMR), HMRI, April 2015, Newcastle, Australia*
Abbreviations

A-LAP - Adipocyte-derived leucine aminopeptidase
ACE - Angiotensin converting enzyme
ACE-1 - ACE mRNA
ACEI - Angiotensin converting enzyme inhibitor
ACTB - Beta-actin mRNA
ADH - Antidiuretic hormone
AGT - Angiotensinogen
AGT - AGT mRNA
AGTR1 - AT1R mRNA
AGTR2 - AT2R mRNA
Ang - Angiotensin
APA - Aminopeptidase A
ARB - Angiotensin receptor blocker
AT1R - Angiotensin receptor blocker
AT2R - Angiotensin II type 2 receptor
ATP6AP2 - (P)RR mRNA
BCA - Bicinchoninic acid assay
cDNA - Complementary deoxyribonucleic acid
CI - Confidence interval
CT - Cycle threshold
D - Deletion
DNA - Deoxyribonucleic acid
DMSO - Dimethyl sulfoxide
DMEM - Dulbecco Modified Eagle Medium
dNTP - Deoxynucleotide triphosphate
DTT - Dithiothreitol
EC - Endometrial cancer
ECC-1 - Endometrial carcinoma cell line 1
EDTA - Ethylenediaminetetraacetic acid
EGF - Epidermal growth factor
ELISA - Enzyme linked immunosorbent assay
ER - Estrogen receptor
ER-α - Estrogen receptor alpha
ERK - Extracellular signal-regulated kinases
FGF-β Fibroblast growth factor beta
FIGO - International Federation of Gynecology and Obstetrics
Fwd - Forward
G - Grade
h - Hour
HEC-1A - Human endometrial carcinoma cells
HRP - Handle region peptide
HRT - Hormone replacement therapy
hVSMCs - Human vascular smooth muscle cells
I - Insertion
IHC - Immunohistochemistry
IRAP - Insulin regulated aminopeptidase
LRP5/6 - Low density lipoprotein receptor-related protein 5/6
MAPK - Mitogen-activated protein kinase
MAS1 - Mas receptor mRNA
MEM- Minimum essential media
MgCl₂- Magnesium chloride
mM- Millimolar
MRI -Magnetic resonance imaging
mRNA- messenger ribonucleic acid
MVD - Microvascular density
NaOH- Sodium hydroxide
ng/ml-Nanogram/milliliter
nt- Nucleotide
NTC - Non template control
PBS - Phosphate buffered saline
PCR- Polymerase chain reaction
pg/ml-picogram/milliliter
PPAR-γ - Peroxisome proliferator-activated receptor gamma
PVDF- Polyvinylidene difluoride
qPCR - Quantitative polymerase chain reaction
RAS- Renin angiotensin system
REN- Prorenin mRNA
Rev- Reverse
RIPA buffer- Radioimmunoprecipitation assay buffer
RNA- Ribonucleic acid
RR - Relative risk
RT- Reverse transcription
s- Seconds
siRNA- Small interfering ribonucleic acid
TBE - Tris/Borate/EDTA

Tm - Melting temperature

UV - Ultraviolet

V-ATPase - Vacuolar-type H+ ATPase

VEGF - Vascular endothelial growth factor

VEGF - VEGF mRNA

w/v - weight/volume
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CHAPTER 1 - INTRODUCTION

1.1 Endometrial cancer

Endometrial cancer is one of the most common gynaecological malignancies. Globally, around 140,000 women are diagnosed with endometrial cancer each year, making it the fourth most prominent cancer in women [1]. The incidence of endometrial cancer is rising and is associated with increasing age and obesity. It generally occurs in the 6th and 7th decades of life [2] and is commonly diagnosed in pre and post-menopausal women [3]. The disease is uncommon in women before the age of 40, with only 20% of cases occurring before menopause [4].

The most common risk factors for endometrial cancer include: obesity, early menarche, advanced age at menopause, menstrual disorders, polycystic ovary syndrome, diabetes mellitus, Lynch syndrome and tamoxifen treatment for breast cancer. However, the etiology of endometrial cancer remains unknown [5]. Traditionally, endometrial cancers are classified as either type-1 or type-2 based on clinical and epidemiological observations [6].

Based on histopathological characteristics, endometrial cancers can be classified into three of subtypes: adenocarcinoma (90%), uterine sarcoma (2-5%) or adenosquamous sarcoma [7]. Type-1 endometrial cancer, is estrogen dependent and is the most common endometrial cancer, accounting for approximately 80% of all cases. Histologically, most type-1 endometrial cancers are endometrioid adenocarcinomas of low histological grade [8]. This type of cancer is associated with obesity and/or a rise in estrogen levels unopposed by progesterone, which leads to endometrial hyperplasia. The risk factors for type-1 endometrial cancer include obesity, nulliparity, diabetes, and hypertension [9]. These tumours are
minimally invasive, well differentiated and are generally diagnosed in the early stage. Thus type-1 endometrial cancers usually have a good prognosis.

Type-2 endometrial cancer accounts for most of the remaining 10%-20% of all endometrial carcinomas. They are non-estrogen dependent and generally occur in non-obese women. They are very aggressive, poorly differentiated, and are associated with a poor prognosis; typically they are high-grade tumours [10].

Invasion and metastatic spread are necessary for cancer progression. In the early stages of endometrial cancer, the most commonly affected sites include the pelvic viscera and adnexa, as well as the pelvic region and para-aortic lymph nodes; a low incidence of distant metastases occurs through haematogenous spread. The mechanism underlying aggressive transformation and distribution is poorly understood [11, 12].

1.2 EMT (epithelial mesenchymal transition)/tumour invasion

In order to minimize cancer mortality, it is important to understand how cancer spreads. Endometrial cancer can spread by direct extension of the tumour through the myometrium, a process which involves removal of dead cells that are shed through the fallopian tubes, lymphatic distribution and/or haematogenous dissemination [13, 14].

In endometrial cancer, the presence of myometrial invasion is considered an important prognostic factor. For this to occur, endometrial cancer cells must undergo dramatic remodeling, a process known as epithelial mesenchymal transition (EMT). This is a reversible de-differentiation process, which involves conversion of epithelial cancer cells into de-differentiated cells with mesenchymal properties [15].
The following changes occur during EMT;

- Molecular changes
- Morphological changes
- Phenotypical changes

EMT is a process in which epithelial cells lose their polarity and cell-cell contacts and undergo transformation to attain a migratory phenotype that includes an increase in motility and migration. The epithelial cells then form a sheet like structure that is maintained by specialized junction structures that include tight junctions, desmosomes, gap junctions and adherent junctions. Of these, adherent junctions (e.g. E-cadherin) have a prominent role in assembling and constructing lateral epithelial cell-cell adhesions [16].

When EMT occurs, mesenchymal cells are able to detach, penetrate through basement membranes and intrude into surrounding tissue to metastasize at secondary sites (Fig. 1.1) [17]. These changes are not essentially noticeable during EMT, but a single cell’s acquisition of the ability to migrate and invade the extracellular matrix is considered a functional hallmark of EMT [14].

In recent years, various pathways have emerged that can down regulate E-cadherin and, as a result, can stimulate EMT. The various factors involved are transforming growth factor (TGF-β), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and Wnt/β-catenin signaling pathways [11, 14]. The majority of these pathways repress E-cadherin through modulation of a set of pleiotropically acting transcription factors that include a family of snail (snail and Slug) and basic helix loop-loop-helix (E47 and Twist) families, along with two double zinc finger and homeodomain (Zeb 1 and Zeb2) factors [18].
These signaling pathways are expressed in a number of malignant tumors and experimental models of cancer formation [18, 19], and have been accepted as critical inducers of EMT. Although EMT has been described, the precise molecular pathways responsible for triggering the process are still not clear.

Figure 1.1. Schematic representation of the molecular, morphological and phenotypical changes occurring in EMT [14].

1.3 Endometrial cancer grading

Endometrial cancers are categorized based on grade and tumour stage. The staging of endometrial cancers is based on surgical and pathological criteria, produced by the International Federation of Gynaecologic Oncology (FIGO) (Table 1.1) [20].
### Table 1.1: International Federation of Gynaecology and Obstetrics (FIGO) Staging System for Endometrial Cancer

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<td>Tumour confined to the uterus, no invasion or invasion of less than one half of the myometrial thickness</td>
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<tr>
<td>Stage IB</td>
<td>Tumour confined to the uterus with invasion of more than one half of the myometrial thickness</td>
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<tr>
<td>Stage II</td>
<td>The tumour invades the cervical stroma, but does not extend beyond</td>
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<tr>
<td>Stage IIIA</td>
<td>The tumour invades the uterine serosa or adnexa</td>
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<td>Stage IIIB</td>
<td>Vaginal and/or parametrial involvement</td>
</tr>
<tr>
<td>Stage IIIC</td>
<td>The tumour has spread to pelvic or para aortic lymph nodes</td>
</tr>
<tr>
<td>Stage IIIC1</td>
<td>Pelvic lymph node involvement</td>
</tr>
<tr>
<td>Stage IIIC2</td>
<td>Para aortic lymph node involvement (with or without pelvic nodes)</td>
</tr>
<tr>
<td>Stage IVA</td>
<td>Tumour invasion of the bladder and/or bowel mucosa</td>
</tr>
<tr>
<td>Stage IVB</td>
<td>Distant metastases including abdominal metastases and/or inguinal lymph nodes</td>
</tr>
</tbody>
</table>

Faria *et al.* 2015 [2]

Magnetic resonance imaging (MRI) is used to assess the depth of myometrial invasion, the most important morphologic prognostic factor.

Beddy *et al.* classified endometrial cancers into three grades; grade 1 (G1) cancers that comprise of ≤5% of solid components and are well differentiated; G2 are moderately differentiated and account for around 6-50% of solid components; and G3 cancers, which are poorly differentiated and account for <50% of solid components. Current treatments for endometrial cancer include surgery, chemotherapy, radiotherapy, and hormonal therapy [21, 22].

As discussed above, type-1 endometrial cancer is oestrogen-dependent and often occurs in obese women. The relationship between endometrial cancer and obesity is related to elevated levels of estrogens, which are unopposed by progesterone [23]. It has been widely established
that excess oestrogen has been associated with an increased risk of cancer [2]. One system that is upregulated by estrogen and plays key roles in regulating proliferation and apoptosis in a variety of physiological and pathological conditions is the renin-angiotensin system (RAS). Very little research has been done to investigate the role of the RAS in endometrial cancer.

As such, I postulate that high estrogen production unopposed by progesterone could cause activation of the endometrial RAS and promote tumourigenesis.

1.4 Renin-angiotensin system

The renin-angiotensin system (RAS) consists of a cascade of precursors of angiotensin II (Ang II), which regulate blood pressure, water-sodium balance, and tissue homeostasis (Fig.1.2) [24]. Renin is an aspartyl protease secreted by the kidney into blood, but it is also present in many tissues as an enzymatically inactive molecule, prorenin. The substrate for (pro)renin is angiotensinogen (AGT), a large 62-KDa α2-globulin substrate secreted by the liver, from which a decapeptide, angiotensin I (Ang I), is cleaved [25]. Ang I is further cleaved by angiotensin converting enzyme (ACE) to produce the biologically active octapeptide angiotensin II (Ang II) [26]. Ang II elicits most of its actions by acting on the Ang II type I receptor (AT1R).

The blood pressure raising action of Ang II includes stimulation of aldosterone synthesis and secretion, vasoconstriction, stimulation of sympathetic outflow and inhibition of vagal tone through actions within central nervous system. Aside from these actions, Ang II also stimulates angiogenesis and cell proliferation. By contrast, Ang II also acts via the Ang II/AT2R and generally opposes the actions of Ang II acting via the AT1R, i.e. it
causes vasodilation and apoptosis [27]. Ang II is further processed to either Ang III or Ang IV, which is a ligand for insulin-regulated amino peptidase (IRAP) (Fig. 1.2B) [28].

Recently, an additional arm of the RAS has been described, that also opposes the actions of Ang II acting via AT₁R. This pathway involves the cleavage of a single amino acid from either Ang I or Ang II by angiotensin converting enzyme 2 (ACE2), which is a homologue of ACE, to form Ang(1-9) or Ang(1-7), respectively (Figure 2). Ang(1-9) can then be cleaved to form Ang(1-7) through the actions of ACE. Ang(1-7) acts on the Mas receptor and exerts actions that counterbalance the RAS system, i.e. Ang(1-7)/Mas is anti-angiogenic and pro-apoptotic (Fig. 1.2B) [29].
**Figure 1.2.** (A) The renin-angiotensin system cascade. Angiotensinogen is converted to angiotensin (Ang) I via renin, which can further be converted to the biologically active peptide Ang II by angiotensin converting enzyme (ACE). Ang II acts upon its receptors angiotensin II type 1 receptor (AT₁R) and angiotensin II type 2 receptor (AT₂R). Ang II acting via the AT₁R promotes angiogenesis and cell proliferation while acting via AT₂R antagonizes AT₁R stimulation. Both Ang I and II can be further processed by ACE2, a
homologue of ACE, to produce Ang (1-7), which acts upon the Mas receptor to antagonize AT1R activation. Further processing of Ang II by amino peptidase A (APA) forms Ang IV, which acts via angiotensin II type 4 receptor (AT4R) to promote vascularization and inflammation [30, 43]. (B) Amino acid sequence of angiotensin peptides [31].

1.5 Tissue renin-angiotensin systems

Apart from the circulating RAS; the system also exists in a variety of tissues including the kidney, placenta, eye, ovaries, heart and endometrium. Tissue renin-angiotensin systems differ from the circulating RAS, as only the kidney can secrete active renin. All other tissues produce prorenin, the inactive precursor of renin [32, 33]. As such the (pro)renin receptor ((P)RR), allows prorenin to become catalytically active in tissues and results in local production of Ang II.

The (P)RR was discovered by Nguyen et al. in 2002 [34]. This receptor binds to both active renin and prorenin [35]. When prorenin binds to the (P)RR, it, like active renin, can form Ang I from AGT (Fig. 1.3) [34, 36].

Tissue renin-angiotensin systems are known to stimulate angiogenesis, cell proliferation and migration, all of which may be involved in the growth and spread of cancer. These effects are primarily mediated via Ang II acting on the AT1R [34].
Prorenin binding to the (pro)renin receptor ((P)RR) non-proteolytically activates the classical RAS pathway, promoting the formation of Ang II and also directly stimulates (P)RR-mediated intracellular signalling pathways. I propose that these pathways are involved in stimulating the growth and spread of endometrial cancer.

Prorenin bound to the (P)RR not only activates the RAS cascade to produce Ang II (Fig. 1.3) but also stimulates proliferative and pro-angiogenic intracellular signalling pathways independent of the RAS cascade (Fig. 1.3). For example, the prorenin/(P)RR interaction can cause translocation of promyelocytic leukaemia zinc finger protein (PLZF) to the nucleus where it activates the p85α subunit of phosphatidylinositol-3 kinase (PI3K-p85α), leading to proliferation and decreased cell death [52].

Nuclear translocation of PLZF also suppresses expression of (P)RR via a negative feedback pathway (Figure 1.3) [37]. The (P)RR is also known as ATPase H(+) -transporting lysosomal accessory protein 2 (ATP6AP2) because it is associated with vacuolar H(+) -ATPase (V-ATPase). V-ATPase is a multi-subunit proton pump that plays an important role in maintaining intracellular pH [38].
(P)RR has been shown to be involved in Wnt/β-catenin signaling, as an adapter between V-ATPase and Wnt receptors [36]. The (P)RR can also activate the Wnt/β-catenin signalling pathway, as Wnt ligands bind to frizzled (FZD)/low-density lipoprotein receptor related protein complex (LRP6), which is internalized by V-ATPase. In this way, β-catenin is stabilized and accumulation of β-catenin leads to cell proliferation. β-catenin is directed towards the nucleus and stimulates fibroblast growth factor (FGF-9), an oncogene that is overexpressed in ovarian cancer (Fig. 1.3) [39]. Binding of prorenin to the (P)RR also increases DNA synthesis and cell proliferation via phosphorylation of ERK 1/2 (extracellular signal-regulated kinases) and mitogen activated protein kinases (MAPK). This in turn activates transforming growth factor (TGF-β1) leading to enhanced proliferation (Fig. 1.3) [40]. Prorenin/(P)RR mediated pathways are proliferative and angiogenic hence they could be involved in cancer progression.

1.6 Soluble (pro)renin receptor (s(P)RR)

A novel form of the (P)RR, soluble (P)RR (s(P)RR) has also been discovered [41]. There are therefore three different molecular forms of (P)RR: (1) a full-length transmembrane protein; (2) a soluble (P)RR found in plasma and urine; and (3) a truncated form composed of the transmembrane and cytoplasmic domains (Fig. 1.4). It has been proposed that s(P)RR serves as a biomarker, reflecting tissue (P)RR levels [42].

![Figure 1.4. Site at which the (P)RR can be cleaved by the protease furin. The soluble 28KDa (P)RR is a putative biomarker of (P)RR synthesis [41].](image-url)

Figure 1.4. Site at which the (P)RR can be cleaved by the protease furin. The soluble 28KDa (P)RR is a putative biomarker of (P)RR synthesis [41].
Cousin et al. showed that the (P)RR can be cleaved by a pro-protein convertase, furin, or by Site1 protease in the trans Golgi to generate s(P)RR (Fig. 1.5) [41, 44]. Soluble (P)RR is secreted into the extracellular space and can be detected in the blood stream [45] and in conditioned media cultured from glomerular epithelial cells [45, 46, 47, 48, 49] by a specific ELISA [42]. In human plasma, the normal interquartile range is 20.9-26.5 ng/ml [47]. The levels of s(P)RR are unaltered by any physiological changes like posture and circadian rhythms [42] but may be elevated in patients with chronic kidney disease [50]. Furin, which cleaves the extracellular domain of (P)RR to form s(P)RR, is overexpressed in the colon [51], head and neck [52], breast and ovarian cancers [53, 54], and is consistently elevated in endometrial cancer cell lines and in endometrial cancers [55]. Similarly, (P)RR levels are high in endometrial cancers [56]. Thus, it is likely that the levels of s(P)RR are elevated in blood and uterine fluid from women with endometrial cancer.

**Figure 1.5. Proposed interactions between (P)RR, V-ATPase and s(P)RR and the role of furin in forming the 28KDa soluble segment of (P)RR (s(P)RR).** Cousin et al. were the first to propose that the extracellular domain of (P)RR is cleaved by furin in the trans-Golgi to generate s(P)RR, which is secreted [41]. Consequently, membrane bound (P)RR can exist in 2 forms, full-length or truncated (P)RR. The V-ATPase may be complexed with either of these forms. Soluble (P)RR binds to renin and prorenin. It should be noted that the intracellular localization of (P)RR and soluble (P)RR has not been clarified.
1.7 The renin-angiotensin system in angiogenesis

During reproductive life, the endometrium undergoes cyclical morphological changes. The endometrium is shed during each menstrual cycle and is completely rebuilt in preparation for blastocyst implantation. This process is dependent on angiogenesis and cell proliferation [57]. Angiogenesis is the formation of new blood vessels, is important in embryogenesis, wound healing, endometrial remodelling during the menstrual cycle and decidualization. Angiogenesis is also involved in ischemic peripheral vascular disease, retinopathy, neoplastic growth and metastasis of malignant tumours [58].

Ang II promotes angiogenesis and upregulates the expression of various angiogenic factors like platelet derived growth factor (PDGF) and fibroblast growth factor-β (FGF-β) [59]. One of the most potent angiogenic factors is vascular endothelial growth factor (VEGF), Ang II acting via the AT1R stimulates angiogenesis [60]. Tumour growth dependent on angiogenesis is reduced in AT1R null mice, because there is decreased VEGF protein expression [61]. On the other hand, Ang II acting via the AT2R antagonizes the effects of AT1R and inhibits VEGF expression [62].

Cell proliferation is crucial for endometrial growth during the proliferative phase of the menstrual cycle. Ang II acting via the AT1R stimulates cell proliferation; it also causes transactivation of epidermal growth factor (EGF) [61]. There is increased cell proliferation in cancer cell lines in response to Ang II stimulation, which are partly mediated by PI3K and MAPK signaling [63]. In contrast, activation of AT2R not only inhibits angiogenesis but also inhibits cell proliferation, thereby promoting apoptosis by decreasing ERK pathway activation [64].
1.8 The renin angiotensin system in endometrial cancer

Several studies show that the RAS exists in the ovary, placenta and endometrium under both physiological and pathological conditions. Moreover, many components of the RAS are expressed in endometrial cancer [65]. Watanabe et al. were the first to demonstrate that the RAS components, Ang II and AT1R were expressed in endometrial cancerous tissue and cancer cell lines, as is adipocyte-derived leucine amino peptidase (A-LAP). A-LAP belongs to the M1 family of Zinc-metallopeptidases, which, when present in endometrial cancer, hydrolyse Ang II [66, 67].

Watanabe et al. and Shibata et al. also confirmed the localization of VEGF, which was earlier reported by Guidi et al. to be upregulated in endometrial cancer [59]; their immunohistochemical studies revealed localisation of Ang II, AT1R and VEGF proteins in endometrial cancers [67]. Piastowska-Ciesielska et al. showed the presence of the proliferative arm of the RAS pathway, as well as (AT2R), oestrogen receptor alpha (ER-α) and VEGF, in endometrial cancer cells [68]. They also investigated the relative expression of RAS genes in both endometrial cancer and normal endometrium, and found that the expression of AT1R, AT2R and ER-α was much higher in tumour samples compared with controls. For example, expression of AGTR1 mRNA was found to be eight times higher in endometrial cancers compared with normal endometrium, suggesting that Ang II may be involved in tumour development [68].

Apart from overexpression of RAS genes, Freitas-Silva et al. showed that a polymorphism in ACE gene was associated with an increased risk of endometrial cancer. This polymorphism is due to an insertion (I) or deletion (D) of a 287 base pair of Alu-repetitive sequence in intron 16 of the ACE gene [69].
Other studies have also shown that Ang II is actively involved in the growth of endometrial cancers. *In vivo* studies by Shibata *et al.* have demonstrated that the proliferative arm of the Ang II/AT₁R pathway is involved in tumour growth. Furthermore, Ang II and the AT₁R are expressed in a variety of human tumours [70]. The presence of AT₁Rs have been reported in various malignant tumour tissues like skin cancer [71], prostate cancer [72], pancreatic cancer [73] and laryngeal cancer [74]. Shibata *et al.* were the first to report the presence of AT₁R in human ovarian carcinomas. More importantly, the presence of AT₁R was also documented in other gynaecological malignancies, such as cervical and endometrial cancer [75]. Shibata *et al.* showed that Ang II stimulated cell proliferation and VEGF secretion by acting through the AT₁R in gynecological malignancies like endometrial cancers, cervical cancers and ovarian cancers *in vitro* [67, 76].

Thus, the AT₁R is present in a wide range of human cancers and in particular, female specific cancers [68]. In human endometrial carcinoma cells (HEC-1A), VEGF secretion is increased by Ang II in a dose-dependent manner [67]. Further investigation using animal models found that overexpression of A-LAP, which hydrolyzes Ang II, in endometrial cancer cells resulted in decreased VEGF expression, reduced vessel density and central necrosis [77].
Previous studies have shown that both prorenin and the (P)RR (Fig. 1.6) are present in greater abundance in endometrial cancer tissue compared with normal adjacent endometrium [78].

**Figure 1.6.** Immunohistochemical staining of (A) prorenin and (B) (pro)renin receptor ((P)RR) protein in both normal adjacent endometrium (left) and tumour (right) of women with endometrial cancer [78].

In addition, other components of the proliferative, pro-angiogenic Ang II/AT₁R pathway, namely AGT, ACE and AT₁R (Fig. 1.7), are also highly expressed in endometrial cancers [78].
1.9 RAS blocking drugs

Two classes of drugs commonly used in the treatment of hypertension have been proposed for the treatment of endometrial cancer; angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs). They act by blocking Ang II production or its binding to the AT1R [79].

1.9.1 Angiotensin converting enzyme inhibitors (ACEIs)

ACEIs have different lipophilic/hydrophilic properties that determine tissue bioavailability, which may result in different affinities to ACE. When compared with hydrophilic ACEIs, lipophilic ACEIs bind to ACE for a longer period of time and show low dissociation, which could be useful for blocking tissue ACE [80]. Lever et al. carried out a retrospective study of over 5000 patients who were undergoing treatment with different anti-hypertensive drugs. They found that patients taking ACEIs over a period of 15 years had a lower incidence of cancer. Patients who were on ACEIs also had a significantly reduced relative risk (RR) for cancer-associated deaths (0.72, CI 0.55-0.92). They further reported that the patients undergoing treatment with ACEIs, exhibited a prominent decrease in relative risk.
for female specific cancers (0.37, CI 0.12-0.87) [81]. Makar et al. showed that patients who were treated with ACEIs/ARBs for four years also exhibited a lower risk for colorectal cancer [82].

1.9.2. Angiotensin receptor blockers (ARBs)

There are seven different ARBs available, one of which, losartan, was first approved for clinical use and has an excellent tolerability and safety profile [83, 84]. Koyama et al. has demonstrated that losartan increased the percentage of viable cells in an endometrial cancer cell line (Ishikawa cells, Fig. 1.8). They also demonstrated that telmisartan, another ARB, decreased the percentage of viable cells in endometrial cancer cell lines (Ishikawa, HEC-59 and HHUA, Fig. 1.9).

**Figure 1.8. Effect of losartan on the viability of endometrial cancer cell lines.** Ishikawa, HEC-59, and HHUA endometrial cancer cell lines were treated with various concentrations of losartan (1-100 μM) or vehicle control for 48 hours (Modified from Koyama et al. 2014) [85].
Figure 1.9. Effect of telmisartan on the viability of endometrial cancer cell lines. Ishikawa, HEC-59, and HHUA endometrial cancer cell lines were treated with various concentrations of telmisartan (1-100 µM) or control for 48 hours. There is a significant reduction in the percentage of viable cells when treated with increasing concentrations of telmisartan. * denotes $P < 0.05$ and ** denotes $P < 0.01$ when compared to control (Modified from Koyama et al. 2014)[85].

In vivo studies by the same group demonstrated that telmisartan reduced human endometrial tumour growth (Fig 1.10A). Tumours treated with telmisartan, exhibited weaker staining for Ki67, a marker for cell proliferation (Fig. 1.10B) [85]. Similarly, telmisartan was able to inhibit cell proliferation and tumour growth both in vivo and in vitro in esophageal cancer and prostate cancer cell lines [86, 87].
Figure 1.10. Effect of telmisartan on a mouse model of endometrial cancer. (A) There is a significant decrease in tumour size with telmisartan treatment. (B) Tumours treated with telmisartan displayed a weak staining for Ki67 compared with control, as denoted by **, P<0.01 (Modified from Koyama et al.)[85].

Bensson et al, found that telmisartan, an ARB, not only blocks the AT1R but can also act as a partial agonist of peroxisome proliferator-activated receptor gamma (PPAR-γ). Telmisartan can activate 25-30% of PPAR-γ, when compared to full PPAR-γ agonists troglitazone and pioglitazone, which are Thiazolidines (a new class of anti-diabetic drugs) that also regulate differentiation of cancer cells [88].
1.10 PPARs and PPAR-γ agonists

Peroxisome proliferator activated receptors (PPARs) belong to the nuclear receptor family of ligand activated transcription factors. They are considered to be important immunomodulatory factors and fatty acid regulators [88, 89].

They interact with peroxisome proliferator response elements (PPRES) in the promoter region of the target genes, which are involved in fatty acid transport, lipid metabolism and glucose homeostasis [90]. There are three isoforms that have a conserved binding domain, but they differ in their transactivation domain according to their tissue of distribution [91]. PPAR-α is highly abundant in the liver, kidney, small intestine, and heart and is also involved in fatty acid catabolism, whereas PPAR-γ, which is abundant in adipose tissue, plays an important role in adipocyte differentiation [90].

Park et al. demonstrated that troglitazone, a PPAR-γ agonist, decreased cell viability in thyroid cancer cell lines [92]. In another study carried out by Motomura et al., treatment with troglitazone reduced cell growth in human pancreatic carcinoma cells [93].

1.11 Renin inhibitors

Another class of drugs that target the RAS are renin inhibitors. Aliskiren, a direct renin inhibitor, specifically binds to the active site (S3bp) of renin [94] and blocks the formation of Ang I from AGT [95]. Unlike ACEI, renin inhibitors do not display side effects like coughing as they do not interfere with kinin metabolism [96]. Approximately 40-80% of renin activity is inhibited by aliskiren; therefore, it causes a significant reduction in Ang II formation [97].
1.12 Drugs that target the (P)RR

1.12.1 (P)RR antagonists

Since the discovery of (P)RR, discussion has focused on (P)RR inhibitors, which could block prorenin binding to (P)RR and therefore inhibit Ang II mediated effects, and which could be useful as anti-hypertensive agents [98].

Handle region peptide (HRP) binds to the (P)RR and prevents prorenin binding. This in turn, blocks activation of (P)RR-mediated intracellular signalling and formation of Ang II [34]. Ichihara et al. demonstrated that HRP inhibited the renal conversion of Ang I to Ang II and inhibited diabetic nephropathy [99].

Our group is the first to test the effects of HRP on (P)RR expression in MCF-7 cells (a breast cancer cell line). We showed that HRP inhibited the expression of VEGF in MCF-7 cells (Fig. 1.11A). HRP also down regulated (P)RR (ATP6AP2) mRNA expression, which could inhibit the (P)RR-mediated effects on cell proliferation and apoptosis (Fig. 1.11B), (Wang et al. unpublished). Similarly, in vivo studies carried out on diabetic models and AT1R knock out mice show that HRP can prevent development of glomerulosclerosis [100].

**Figure 1.11.** Effect of HRP on (A) VEGF and (B) (P)RR/ATP6AP2 mRNA abundance in MCF-7 cells. HRP (1\(\mu\)M) inhibits VEGF and (P)RR mRNA expression in MCF-7 cells (Wang et al. unpublished).
Based on these results, it is postulated that (P)RR can exert pathological actions via Ang II independent pathways. [101]. In contrast, other studies did not show any beneficial effects of HRP. Susic et al. found in their spontaneous hypertensive rat model that HRP did not ameliorate cardiac hypertrophy [102]. Several studies also showed that there was no effect of HRP in other animal models. In double transgenic animals that over express renin and AGT, as well as in Goldblatt hypertensive animals, no reduction in renal and cardiac pathologies were observed when they were treated with HRP [103, 104]. The above studies suggest that the efficacy of HRP warrants further investigation.

1.12.2 (P)RR knockdown by ATP6AP2 siRNA

Shibayama et al. revealed that (P)RR is important for Wnt/β-catenin dependent proliferation. They were the first group to demonstrate that a (P)RR siRNA inhibited (P)RR expression and decreased cell proliferation in pancreatic ductal adenocarcinoma (PDAC) cells [105]. In vivo studies by the same group show that there is a decrease in cell number and tumour size when there is inhibition of (P)RR expression with a siRNA (Fig 1.12) [105].
**Figure 1.12** (A) Effect of (P)RR siRNA transfection on cell number, as assessed by direct cell counting of Wnt3a-treated PDAC cell lines. (P)RR siRNA treatment prevented the Wnt3a-induced increase in cell number. *P<0.05 vs. scrambled siRNA cells; § P<0.05 vs. vehicle-treated cells without Wnt3a stimulation; #, P<0.05 vs. scrambled siRNA-treated cells without Wnt3a stimulation; ¶, P<0.05 vs. (P)RR siRNA cells without Wnt3a stimulation (modified from Shibayama et al.). (B) (P)RR siRNA knock down: Xenograft formation in vivo by scrambled siRNA-(left) or (P)RR siRNA-(right) transfected PDAC cells at four weeks post injection (modified from Shibayama et al.) [105].

*In vitro* studies by Sakoda *et al.* found similar actions of (P)RR in human vascular smooth muscle cells (hVSMCs). They were able to knock down (P)RR expression with a siRNA and demonstrate that it blocked prorenin induced ERK activation. They confirmed that stimulation of (P)RR activates ERK phosphorylation in hVSMCs [82]. Huang *et al.* were the
first to show that stimulation of renin by binding to the (P)RR triggers intracellular signalling pathways independent of the formation of Ang II. They demonstrated that transfection of the (P)RR siRNA into mesangial cells to silence (P)RR expression blocked ERK 1/2 activation [83]. As well, in breast cancer cell lines the rate of proliferation was decreased when transfected with a (P)RR siRNA [106]. However, until now, no studies have examined the role of prorenin/(P)RR interactions in endometrial cancer.

In summary, very little research has been done to investigate the role of the RAS in endometrial cancer. Dysregulation of ovarian steroids may lead to activation of the endometrial renin angiotensin system to promote tumourigenesis.

Endometrial cancers are generally identified at a late stage which leads to a high mortality and morbidity. A specific biomarker could mean earlier detection. Measuring s(P)RR in the circulation or in uterine fluids collected following lavage might show that it is a non-invasive and early marker for endometrial cancer.

We know that components of the endometrial RAS are overexpressed in endometrial cancer cells and tissues, and from the above studies that drugs that target the RAS may reduce proliferation and angiogenesis in endometrial cancer. Given that these drugs have very few side effects, they could be used in conjunction with existing therapies to treat endometrial cancer; especially when many patients are already on some form of blood pressure lowering medication. We postulate that the prorenin/(P)RR interaction plays a major role in endometrial cancer growth. Therefore, blocking the prorenin/(P)RR alone, or in combination with AT1R antagonists, could reduce the progression and/or recurrence of endometrial cancer.
**Figure 1.13.** Sites of action of RAS blockers. Handle region peptide (HRP) (1a) inhibits the non-proteolytic activation of prorenin and subsequently inhibits the conversion of AGT to Ang I. (P)RR siRNA (1b) lowers (P)RR expression and, as a result, prorenin is unable to bind to the (P)RR. Renin inhibitors (2) act directly on renin and prevent the cleavage of Ang I from Angiotensinogen. Angiotensin converting enzyme inhibitors (ACEIs) (3) inhibit the conversion of Angiotensin I to Ang II resulting in reduced Ang II formation. Angiotensin receptor blockers (ARBs) (4) act directly and inhibit the activation of AT1R proliferative pathway.
1.13 Hypotheses

- Endometrial cancer growth *in vitro* is inhibited by drugs that block Ang II/AT$_1$R interactions (ARBs, ACEIs) and/or prorenin/(P)RR (renin inhibitors, (P)RR siRNA, handle region peptide) mediated signaling pathways.

- Drugs that block prorenin/(P)RR are more effective than drugs that block the Ang II/AT$_1$R interaction

- Plasma/uterine fluid levels of s(P)RR are biomarkers for endometrial cancer.

- Estrogen stimulates the expression of the endometrial RAS, but progesterone inhibits the RAS in endometrial cancer cell lines.
1.14 References


Chapter 2 - Materials and Methods

2.1 Chemicals

Renin inhibitors (VTP-27999 and aliskiren) were obtained from Medchem express and prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO). The (P)RR inhibitor, handle region peptide (HRP, H-Arg-Ile-Phe-Leu-Lys-Arg-Met-Pro-Ser-Ile-OH) was obtained from Auspep Pty Ltd. HRP was prepared as a 10 mM stock solution. The (P)RR siRNA and negative control siRNA were obtained from Invitrogen Life Technologies. (P)RR siRNA was prepared as 20 μM stock solution.

The ACEI (perindoprilat), ARBs, (losartan and telmisartan) and PPAR-γ agonist (troglitazone) were obtained from Sigma Aldrich and prepared as 10 mM stock solutions. Perindoprilat, telmisartan and troglitazone were dissolved in DMSO (Ajax Chemicals). The final concentration of DMSO used for telmisartan and troglitazone was 0.1%. Estrogen (estradiol) and progesterone were obtained from Sigma Aldrich and were prepared as 10 mM stock solutions. All the stock solutions were stored in aliquots at -20°C.

2.2 Cell culture

2.2.1 Cell lines

Five cancer cell lines were used, out of which four were endometrial epithelial cancer cell lines (Ishikawa, HEC-1A, AN3CA and RL-952). After genotyping, the remaining one cell line (ECC-1) was found to be a breast cancer cell line (MCF-7). All five cancer cell lines were obtained from American Type Culture Collection (ATCC; Manassas, USA). All the procedures for cell culture were carried under aseptic conditions in a class II biosafety cabinet. To reduce the risk of contamination the biosafety cabinet was always exposed to UV for 20 min before and after use of the cabinet.
Ishikawa and AN3CA cells were cultured and maintained in minimum essential media (MEM; Sigma Aldrich, United states) supplemented with 5% heat inactivated fetal bovine serum (Bovogen Biologicals), and 1% antibiotic-antimycotic (Gibco). For RL-952 cells the growth medium used was DMEM media (Sigma Aldrich) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS) (Bovogen Biologicals), 1% antibiotic-antimycotic (Gibco), and 1% L-glutamine. Similarly, the growth medium for HEC-1A cells was McCoy’s 5A media (Sigma Aldrich) supplemented with 10% heat inactivated fetal bovine serum (Bovogen Biologicals), 1% antibiotic-antimycotic (Gibco), and 1% L-glutamine. For MCF-7 cells, the growth medium used was DMEM (Sigma Aldrich) supplemented with 10% (HI-FBS) (Bovogen Biologicals), and 1% antibiotic-antimycotic (Gibco).

### 2.2.2 Passaging cells

All cell lines were cultured in T75 and T150 sterile tissue culture flasks (Greiner bio-one) at 37°C in a humidified condition with 5% CO₂. Once 80% of the cells had become confluent they were passaged. Culture media was removed from the flask and cells were washed thrice with 5 x 10 ml PBS. Two ml (T75) or 3 ml (T150) of 1x trypsin-EDTA (Invitrogen Life Technologies) was added and the flasks were incubated at 37°C for approximately 1-2 min until cells were detached. Media, containing FBS was added to inactivate trypsin. The cell suspension was pipetted up and down, collected and counted manually in the haemocytometer. Cells were then passaged into new flasks with fresh media containing FBS and antibiotics or plated for an experiment.

### 2.2.3 Freezing and thawing

Confluent cells were detached by trypsinization and approximately 200,000 to 300,000 cells/ml were frozen in 10% sterile DMSO, 10% FBS, and 80% media in cryotubes. These
cryotubes was kept in a freezing container at -80°C overnight. Later the cryotubes were moved to storage in liquid nitrogen.

Cells were thawed in a warm water bath and the cell suspension transferred to a new falcon tube to which 3-5 ml of warm media was added and pipetted up and down to allow mixing. The falcon tube was centrifuged at 1000g for 5 min, the supernatant was discarded and 3 ml of fresh media was added and the resuspended cells placed into a (T25) tissue culture flask. Another 4 ml of fresh media was added to the tissue culture flask and the flask was gently swirled to ensure uniform distribution of media; it was then incubated at 37°C.

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

**2.3.1 RNA Extraction**

After specific treatment, the cells were washed with PBS three times. The cells were collected with help of cell scraper and were snap frozen in a liquid nitrogen to get cell pellets. RNA was extracted from cell pellets using an RNeasy-mini Kit according to the manufacturer’s instructions (Qiagen, Netherlands). To disrupt the snap frozen cell pellets 350 μL RLT buffer/β-Mercaptoethanol (Sigma) was added. To ensure that there were no cell clumps the tubes were vortexed or pipetted for 1 min. Cells were then incubated for 2 min at room temperature.

After incubation, equal amounts (350 μL) of 70% ethanol were added and mixed and the mixture (700 μL) was transferred to an RNeasy column. The columns were subjected to centrifugation at 10,000 rpm for 15s and the flow through was discarded. To wash membrane bound RNA, 700 μL of RW1 buffer was added to the RNeasy column and centrifuged for 15s at 10,000 rpm and the supernatant discarded. To remove traces of salts from pellets, 500 μL RPE buffer was added and centrifuged at 10,000 rpm for 15s. 500 μL RPE buffer was then added to each column and centrifuged for 2 min at 10,000 rpm.
The RNeasy columns were placed in a new 2 ml collection tube and centrifuged at maximum speed at 13,000 rpm for 1 min. To elute the RNA, the RNeasy column was transferred to new sterile 1.5 ml eppendorf tube and 30 μL of RNase free water was added directly to top of the filter pad inside the column and centrifuged at 10,000 rpm for 1 min.

2.3.2 RNA Quantification and assessment of purity

A Nanodrop spectrophotometer (Thermo Fisher Scientific, MA, United States) was used to quantify the concentration of RNA in ng/μL and purity was checked by using $A_{260}: A_{280}$ and $A_{260}/A_{230}$ ratio.

2.3.3 DNase treatment

The extracted samples were then treated with a DNase I digest to remove any contaminating DNA in the sample. From each sample, 5 μg of RNA was mixed with the following: 2.5 μl DNase Buffer (Qiagen), 0.5 μl RNase inhibitor (Qiagen) and 0.5 μl DNase I (Qiagen). The total volume was then made up to 25 μl with RNA free water and incubated for 30 min at 37°C. To the above tubes, 2.5 μl of 1 mM EDTA was added and tubes incubated for 5 min at 65°C. To determine the amount of RNA in samples, samples were again quantified by Nanodrop spectrophotometer (Thermo Fisher Scientific, MA, United States). Samples that had $A_{260}:A_{280}$ ratios above 1.8 were considered for further steps.

2.3.4 Reverse transcription

Reverse transcription was carried out using a Superscript III Synthesis System (Invitrogen, CA, United states) according to the manufacturer’s instructions and an ABI 7500 RT-PCR (Applied Biosystems, CA, United States). All samples were prepared with both a positive (+RT) and a negative (-RT) reaction. For –RT tubes, 1 μl dH₂O was used in place of
Superscript III Reverse Transcriptase. -RT was used for each sample in the qPCR as a control for genomic DNA contamination.

Tubes were labelled for both (+RT) and (-RT) reactions and 0.5 or 1 ng of RNA was added to each tube. To this, 1 μl of random hexamers (50 μg/μl) and 1 μl of 10 mM dNTP mix were added; the final volume was 10 μl. Samples were incubated at 65°C for 5 min in a thermocycler, ABI 7500 RT-PCR system (Applied Biosystems, CA, United States). After incubation, samples were placed on ice and 2 μl of 10X RT buffer, 4 μl of 25 mM MgCl₂, 2 μl of 0.1M DTT, 1 μl of RNase OUT (40U/μl) and either 1 μl of Superscript III RT (200 U/μl) (Invitrogen) or 1 μl dH₂O were added to each tube to obtain a final volume of 20 μl. All the tubes were spun and then placed back into the ABI 7500 RT-PCR (Applied Biosystems, CA, United States) where they were incubated for 25°C for 10 min (Denature), 50°C for 50 min (cDNA synthesis), 85°C for 5 min (Terminate) and 4°C indefinitely. Then 1 μl of RNase H was added and tubes incubated at 37°C to remove the RNA. This step was only performed in +RT tubes. cDNA samples were then diluted to 5 ng/μl by adding 80 μl of milli-Q water to each tube and stored at -20°C for qPCR.

2.3.5 Real Time Polymerase Chain Reaction

qPCR was carried out in an Applied Biosystems 7500 Real Time PCR system and SYBR Green was used for detection. All the samples were assayed in duplicate (-RT were also run along with +RT). The volume of reaction mixture contained 5 μl of SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA) for RAS genes shown in (Table 1), 10 ng of cDNA reversed transcribed from 0.5 or 1 ng of total RNA, and water to make a final volume of 10 μl.

The plate was sealed and centrifuged for 1 minute at 1000rpm to ensure the contents are mixed thoroughly. PCR was carried out using β-Actin (ACTB) as housekeeping gene. Relative
mRNA abundance was calculated using $2^{-\Delta\Delta CT}$ and was expressed to β-Actin and a calibrator sample (term placenta).

### Table 2.1. Primers used in real time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Bank Accession No.</th>
<th>Primer sequence (5'-3')</th>
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<th>Annealing temp (°C)</th>
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### 2.4 siRNA transfection

Once the cells were confluent, they were trypsinized (Invitrogen Life Technologies) and cells were counted and seeded in 6 well plates (Greiner Bio-one) 24h prior to transfection. Cells were plated 1.5 X $10^5$ cells /well for experiments requiring 24 or 48h incubation. For 72h and
96h incubations 7 X 10^4 cells were plated with 2 ml of culture media. The media did not have antibiotics. Cells were 70-80% confluent at the time of transfection.

The next day, a transfection mix was prepared according to manufacturer’s protocol. 3 different types of ATP6AP2 siRNA (Life Technologies, CA United States) were tested in 2 cell lines (Ishikawa and HEC-1A); only siRNA-2 was used for AN3CA cells. Based on their ability to knock down expression of ATP6AP2, we selected siRNA-2 for cell proliferation and cell viability studies in all 3 cell lines.

Twelve point five microliters of 20 μM ATP6AP2 siRNA was diluted in 250 μl of Opti-MEM reduced serum and kept aside (Solution A). For the negative control siRNA, 2.5 μl of (10 nM) –VE control siRNA was diluted with 247.5 μl of Opti-MEM (Solution A). In another falcon tube, 5 μl of Lipofectamine 2000 reagent (Invitrogen) was mixed with 245 μl Opti-MEM (Gibco) and incubated for 15 min (Solution B). Solutions A and B were then combined and incubated together for a further 15 min. After incubation, media (without antibiotics) was added to the falcon tube and pipetted up and down to ensure uniform mixing. This produced a final concentration of 125 nM for the ATP6AP2 siRNA and 10nM for the negative control siRNA. To ensure that differences in the concentrations of the negative control and ATP6AP2 siRNA were not impacting on the results in any of the studies, Ishikawa cells were cultured with three different concentrations (5nM, 50nM and 125nM) of each siRNA (Appendix A1). Transfection with ATP6AP2 siRNA at one of three different concentrations (5nM, 50nM and 125nM) resulted in ~90% knockdown of ATP6AP2 mRNA expression in Ishikawa cells compared to the negative control siRNA treated cells (P<0.0001, P<0.0001 and P<0.0001; Appendix A1). There was no significant difference however in ATP6AP2 mRNA expression between the 3 different concentrations of ATP6AP2 siRNA or negative control siRNA used (P= 0.9996, P= 0.9996, P= 0.9999, and P=0.0662, P=0.5166, P=0.9907 respectively).
The non-transfected control was made by mixing 40 μl lipofectamine with 460 μl of Opti-MEM alone to make 500 μl/well. The final volume of each treatment was made up to 2 ml/well with the respective media but without antibiotics. Old media was removed from the 6 well plates and 2 ml of transfection media was added to each well. To ensure uniform distribution of the siRNAs, the plates were mixed gently by rocking back and forth and incubated in a humidified atmosphere containing 5% CO₂ at 37°C. Media was changed after 24h of transfection and fresh media added. Supernatant and cell pellets were collected at 24h, 48h, 72h & 96h after transfection. For each time point, the supernatant was collected and frozen and stored at -80°C. Wells were washed with ice cold PBS (3 times) and to each well 1 ml of ice-cold PBS was added. Cell pellets were collected with help of cell scraper and pellets were snap frozen and stored at -80°C for subsequent protein and mRNA analysis.

### 2.5 Cell viability assay

Cell viability was determined in 96-well plates by Resazurin assay. The assay was optimised based on the growth rate of each cell line. Based on the proliferation rate of each cell line, cells were seeded at a density of 1.5 X 10^2/100 μL for Ishikawa, 5 X 10^3/100 μL for HEC-1A and 2.5 X 10^2/100 μL for AN3CA cells into each well of 96-well flat-bottomed microplate (Thermo Scientific) and incubated overnight. The following day 100 μL of experimental medium containing various concentrations (0.1 μM, 1 μM, 10 μM and 100 μM) of renin inhibitors (Aliskiren or VTP-27999; Medchem Express), Handle Region Peptide (HRP; Auspep Pty Ltd), ACE inhibitor (perindoprilat; Sigma Aldrich), angiotensin receptor blockers (losartan, telmisartan; Sigma Aldrich) and PPAR-γ agonists (troglitazone; Sigma Aldrich) were added and incubated.

For the ATP6AP2 siRNA (ATP6AP2 siRNA; Life Technologies) a concentration of (125 nM) was used. Negative control siRNA (Life Technologies, at 10 nM) and non-transfected cells
were used as controls. Transfection with siRNA is described above. Furthermore, 20μL of resazurin (Biovision) was added to each well at around 43h and incubated for 5h. At 48h, fluorescence intensity was measured by Fluostar optima (BMG LABTECH, Germany) with excitation wavelength at 530nm and emission wavelength at 590nm.

#### 2.6 Cell proliferation by xCELLigence machine

Cell proliferation was carried out by using xCELLigence RTCA DP instrument (ACEA Biosciences, China) that was placed in a humidified incubator maintained at 37°C and 5% CO₂. 16 well E-plates (ACEA Biosciences) were used to study the rate of cell proliferation. Each well has gold microelectrodes at the bottom of the well for impedance-based detection of cellular attachment (Fig. 2.1). Prior to beginning an experiment, 100 μl of media was added to each well. This was to ensure that media and E-plate surface achieve equilibrium. After leaving the device at room temperature for 30 min, a background impedance reading was recorded.

![Overview of xCELLigence machine](image)

**Figure 2.1** Overview of xCELLigence machine. A side view of a single well is shown before and after cells have been added. In the absence of cells electric current flows freely through culture medium, completing the circuit between the electrodes. As cells adhere to and proliferate on the electrodes, the current flow is impeded and provides an extremely sensitive readout of cell number, cell size/morphology, and cell-substrate attachment quality [1].
For the three cell lines (Ishikawa, HEC-1A and AN3CA), the density of cells seeded was based on their proliferation rate (described in cell viability method). Cells were harvested from exponential phase cultures using the standard trypsin detachment procedure (see section 2.5).

Another 100 μL of media containing cells was added to the E-plates, the E-plates were inserted into the xCELLigence machine and left for 30 min to allow the cells to attach to the plate. After 30 min, the xCELLigence machine was started and left overnight. After 24h, the experiment was paused, and plates removed. Old media was removed from the wells of E-plates and 200μL of experimental medium containing various concentrations (0, 0.1μM, 1μM, 10μM and 100μM) of RAS inhibitors or siRNA were added to each well. Plates were returned to the xCELLigence machine and run for 48h. Cell proliferation was continuously monitored by cell index, the change in electrical impedance which is recorded by the xCELLigence system.

2.7 Preparation of cells for western blotting

2.7.1 Protein extraction by RIPA buffer

Protein from frozen cell pellets were extracted using RIPA buffer (2x stock solution + 10%NP-40 + 10% NA deoxycholate + 100nM Na3VO4 + protease inhibitor cocktail + mQH2O. The master mix (100μl RIPA buffer + 1μl of phenylmethane sulfonyl fluoride, (PMSF)) was added to each sample. Samples were vortexed and incubated on ice for 30 min. During the 30 min of incubation, samples were vortexed every 10 min. After incubation, samples were centrifuged at 13,000 rpm at 4°C for 10 min. The supernatant was collected, and protein quantification was carried out by bicinchoninic acid assay (BCA Assay).
2.7.2 BCA assay

Protein samples were thawed on ice and RIPA diluent was prepared (60μl RIPA in 540 μl of mQH₂O). Standard curve was prepared from 2000 μg/ml to 25 μg/ml. To each sample 48 μl of RIPA diluent was added, followed by 25 μl of standards and samples to plate. To each well, 200 μl of working reagent was added. For uniform mixing the plate was gently rocked and incubated at 37°C for 30min. The plate was read at 570nm on the SpectroStar Nano plate reader to determine the quantity of proteins of each well.

2.7.3 Western blot

This technique is used to separate and detect specific protein of interest from cell lysates or solutions with different proteins. After diluting the samples to the determined concentration of 10μg/μL and preparing them with NuPAGE LDS and a reducing agent (Invitrogen), they were heated at 70°C for 10min prior to loading on a NuPAGE 1.0mm gel, alongside Magic-Mark and Pre-Stain ladders. An amnion calibrator was used on each gel to check for the quality of the western blot. Gels were placed in a mini cell tank and the inner chamber filled with running buffer (50ml MOPS in 950ml MQH₂O) and 2 cm in the outer chamber.

To each inner chamber, 500 μl of antioxidant was added and it was finally topped up with running buffer. Samples were loaded and air bubbles removed with the help of a Pasteur pipette. Using an Invitrogen Power Ease 500 at 200V, 120mA and 25W, the gels were run for 60 min. When the electrophoresis was completed the gel cassettes were opened. Gels were washed with transfer buffer and the wells and thick bottom edge were cut off with help of sharp gel knife. The gels were then transferred into a new dish with transfer buffer and the gel allowed to soak for 1 minute.

Transfer buffer (50ml NuPAGE 20x transfer buffer stock + 200ml methanol+ 750ml MQH₂O + 1ml antioxidant) was prepared and PVDF membrane (7x9cm) and Whatman filter
paper (7x9cm) were specifically cut according to size of the gel. Blotting gauze was soaked in the transfer buffer for around 15min. PVDF membranes were activated by placing in methanol for 1 min, followed by soaking in transfer buffer. The gel on a bed of blotting gauze and 2 filter papers was put into a chamber containing electrodes and covered by the PVDF membrane.

More filter papers and gauzes were added to the top of the chamber. The chamber was then placed into the tank and the inner chamber filled with transfer buffer until the pads were submerged. Air bubbles, which might interfere with transfer process, were eradicated from the chamber with gentle tapping. The outer chamber was filled with cold MQH₂O. Proteins were transferred to the membrane with a Invitrogen Power Ease 500 at 30V, 160mA, and 17W for 60 min. The membrane was taken out of the chamber and was dried between 2 filter papers for 40 min and reactivated in methanol for 1 min followed by subsequent rinsing in mQH₂O for 5 min prior to immunodetections.

### 2.7.4 Immunodetection

The membrane was then rinsed with TBST (20x TBS + 950ml MQH₂O + 5ml tween-20) for 5min and was placed in a blocking solution (5% skim+ 5% BSA in TBST) on a rocker at 4°C overnight. The membrane was then incubated with the (P)RR primary antibody (Abcam, Ab40790, 1/1000 dilution) on a rocker at room temperature for 2h. To reduce the amount of background staining, the membrane was then rinsed with TBST three times for 5 min each followed by rinsing once in TBS for 5 min. Then the membrane was incubated with anti-rabbit secondary antibody (1/5000 dilution, Abcam-Ab672) for 1h at room temperature. After rinsing the membrane as described above, the signal was detected by using an ECL Detection kit (GE Healthcare) and the Amersham Imager 600.

Membranes were then stripped with 0.2M NaOH and ran as described above for β-actin (Abcam-AB8227) (1h in blocking solution at room temperature, 1h in primary antibody
The intensity of the band of interest was measured using the Amersham Imager 600 and divided by the intensity of the band of β-actin for each sample. This ratio was then corrected for variability across gels by dividing it by the intensity of the band of interest of the calibrator for each gel.

2.7.5 Enzyme linked immunosorbent assay (ELISA)

A human s(P)RR ELISA kit from IBL (Catalogue #27782) was used to determine the amount of s(P)RR in sample conditioned media, plasma and uterine lavage fluid. Prior to loading the plate, the samples were diluted 1/5 in the EIA buffer provided by the kit and wash buffer was prepared.

Standard dilutions were prepared from a stock of 16,000 pg/mL to 125 pg/mL. EIA buffer was used as a test blank. 100 μL per well of standards, calibrator, blank and samples were loaded in duplicate. The plate was then incubated overnight at 4°C. The following day, after making up all the required reagents, the wells were washed with 300 μl of wash buffer five times. Wash buffer was removed by blotting against an absorbent towel.

To each well, 100 μl of labelled antibody was added. To reagent blank wells, 100 μl of wash buffer was added. The plate was incubated for 60 min at 4°C. After incubation, the plate was washed five times as described before, followed by the addition of 100 μl of chromogen to each well. The plate was wrapped in foil to avoid direct light exposure and incubated for 30 min at room temperature. To each well, 100 μl of stop solution was added and the plate was gently rocked to ensure uniform mixing. The plate was read at 450nm on the SpectroStar Nano plate reader to determine the optical density of each well. The average value of standard duplicates was used (normalised to blank) to plot the standard curve, linear regression provided the equation of the line (Fig. 2). This equation was used to calculate the concentration of samples.
based on the average absorbance of sample duplicates, also normalised to blank. The final concentration of s(P)RR in pg/mL was found by multiplying the values by the dilution factor.

2.8 References

Chapter 3 - Role of (pro)renin receptor ((P)RR) in endometrial cancer cell growth in three endometrial cancer cell lines

3.1 Introduction

Endometrial cancers are the most common malignant tumors of the female genital tract, and their prevalence is increasing [1, 2]. The renin-angiotensin system (RAS) has been shown to stimulate angiogenesis and proliferation, both of which potentiate tumor growth. The RAS, which is known to stimulate blood pressure and fluid and electrolyte homeostasis, may also play a role in the spread of endometrial cancer. Recently, we have shown that components of the RAS, including prorenin, (pro)renin receptor ((P)RR), angiotensinogen (AGT), angiotensin II type 1 receptor (AT\textsubscript{1}R) and angiotensin converting enzyme (ACE) are expressed in endometrial cancers of all grades [3].

The (pro)renin receptor ((P)RR, ATP6AP2), a transmembrane protein, is located on the X chromosome and encodes a 350 amino acid protein with a single transmembrane domain [4]. The (P)RR is widely expressed in a variety of tissues including the kidney, heart, brain and endometrium. The (P)RR was discovered by Nguyen et al. in 2002, and is capable of binding both renin and prorenin [5]. In vitro studies show that the affinity of prorenin binding to the (P)RR is 3-4-fold higher than binding of active renin to the (P)RR, suggesting that prorenin is the preferred ligand for (P)RR [6, 7].

Binding of prorenin to the (P)RR leads to a conformational change in prorenin in which the pro-segment moves out of its catalytic cleft and is now accessible to AGT. Thus causing non-proteolytic activation of prorenin [8]. AGT is further cleaved to Angiotensin (Ang) I, which is converted by ACE to Ang II, which can act via either AT\textsubscript{1}R or AT\textsubscript{2}R or is converted by ACE2 to Ang-(1-7). Binding of prorenin to the (P)RR also activates intracellular signaling
pathways independent of the formation of Ang II [9]. For example, the prorenin/(P)RR interaction can cause translocation of promyelocytic leukaemia zinc finger protein (PLZF) to the nucleus where it activates the p85α subunit of phosphatidylinositol-3 kinase (PI3K-p85α), leading to proliferation and decreased cell death. Nuclear translocation of PLZF also suppresses expression of (P)RR via a negative feedback pathway [10].

The (P)RR is also known as ATPase H (+)-transporting lysosomal accessory protein 2 (ATP6AP2) because of its association with a vacuolar H+-ATPase (V-ATPase). V-ATPase is a multi-subunit proton pump that plays an important role in maintaining intracellular pH [11, 12]. (P)RR can also activate the Wnt/β-catenin signaling pathway, as Wnt ligands bind to frizzled/low-density lipoprotein receptor related protein complex (FZD/LRP6), which is internalized by V-ATPase [13]. This complex is essential for survival of murine cardiomyocytes and podocytes [14, 15]. Binding of prorenin to the (P)RR also increases DNA synthesis and cell proliferation via phosphorylation of extracellular signal-regulated kinases 1/2 (ERK 1/2) and mitogen activated protein kinases (MAPK) [16]. This, in turn, activates transforming growth factor (TGF-β1), leading to enhanced proliferation of mesangial and vascular smooth muscle cells [14, 17, 18]. Thus prorenin/(P)RR mediated pathways are proliferative and angiogenic so they could be involved in facilitating the growth and spread of cancer. Several studies have shown that the (P)RR is involved in the pathophysiology of diabetic nephropathy, kidney ischemia, cardiac fibrosis and some cancers [19-22].

Previous studies have shown that transfection with an siRNA directed against the (P)RR significantly reduced cell proliferation in breast carcinoma cell lines [17]. Also, (P)RR siRNAs are able to inhibit ERK 1/2 activation in human vascular smooth muscle cells and mesangial cells [23, 24]. In vivo studies show that transfection with a (P)RR siRNA reduced tumour growth in a mouse model of pancreatic ductal adenocarcinoma [22]. The role of
prorenin/(P)RR interactions in endometrial cancer however have not yet been investigated. Despite the evidence that (P)RR is upregulated in endometrial cancer tissue [9] and that (P)RR signaling can contribute to cell growth [123], there are no documented reports on the functional role of (P)RR in endometrial cancer cells.

We postulated that knocking down (P)RR expression with a specific siRNA or preventing prorenin binding to (P)RR with the decapeptide, handle region peptide (HRP), which inhibits prorenin binding to (P)RR [8], would inhibit proliferation in endometrial cancer cells.

3.2 Materials and Methods

3.2.1 Cell Culture

Three endometrial epithelial cancer cell lines, Ishikawa, AN3CA and HEC-1A, were obtained from the American Type Culture Collection (ATCC; Manassas, USA). Ishikawa and AN3CA cells were cultured and maintained in minimum essential media (MEM; Sigma Aldrich, Missouri, USA) supplemented with 5% heat inactivated fetal bovine serum (HI-FBS, Bovogen Biologicals, Victoria, Australia), and 1% antibiotic-antimycotic (Gibco, Waltham, USA). The growth medium for HEC-1A cells was McCoy’s 5A media (Sigma Aldrich) supplemented with 10% HI-FBS (Bovogen Biologicals), 1% antibiotic-antimycotic (Gibco), and 1% L-glutamine. All cell lines were incubated in tissue culture flasks at 37°C with 5% CO2 under humidified conditions. Confluent cells were detached by trypsinization (1X trypsin-EDTA, Invitrogen Life Technologies).

3.2.2 siRNA Transfection

The (P)RR gene was knocked down in the three endometrial epithelial cancer cell lines (Ishikawa, HEC-1A and AN3CA) by ATP6AP2 siRNA transfection. ATP6AP2 specific
siRNAs were introduced to the cells by transfection using Lipofectamine® 2000 Transfection Reagent (Invitrogen) and Opti-MEM. The transfection procedure was carried out according to the manufacturer’s recommendations (Life Technologies). Briefly, on the day before transfection, cells were plated at 1.5 x 10^4 cells/well in 6 well plates with 2 ml of growth medium without antibiotics. Plates were then incubated at 37°C. After 24h, the media were removed and transfection was performed by adding 125 nM ATP6AP2 Stealth RNAi™ siRNA, or 5 nM negative control siRNA (medium GC content) in Lipofectamine® 2000 Transfection Reagent and Opti-MEM. Vehicle non-transfected controls were also included and contained Lipofectamine and Opti-MEM in growth media. Media were changed after 24h of transfection and fresh media without antibiotics added. After 48h of transfection the supernatant was collected from each well. Wells were washed with ice cold PBS three times. Cell pellets were snap frozen and stored at -80°C for subsequent protein and mRNA analysis.

Three different ATP6AP2 siRNAs were tested in 2 cell lines (Ishikawa and HEC-1A). Based on the efficacy of the three siRNAs (siRNA-1, siRNA-2, siRNA-3) in reducing ATP6AP2 mRNA abundance, siRNA-2 was selected for use in cell proliferation and cell viability studies.

3.2.3 Real-time reverse transcriptase polymerase chain reaction (qPCR)

For gene expression analysis, cells were plated in 6-well plates at 7.5 x 10^3 cells per well for 48h. Cell pellets were then snap frozen and stored at -80°C. Total RNA extraction was carried out using an RNeasy mini kit as per the manufacturer’s instructions (Qiagen). The concentration and purity of extracted RNA was determined by a Nanodrop (ND-1000 spectrophotometer) and the RNA integrity was confirmed by gel electrophoresis. RNA samples were then DNase treated (Qiagen) and RNA was spiked with a known amount of Alien RNA
(about $10^7$ copies per μg of total RNA), before the RNA was reverse transcribed using the SuperScript™ III Reverse Transcriptase kit with random hexamers (Invitrogen).

qPCR was performed in Applied BioSystems 7500 Real Time PCR system using SYBR Green for detection. The reaction mixture contained 5μl of SYBR Green; 2 μl of cDNA reverse transcribed from 10 ng of total RNA, primer mix and water to a final volume of 10 μl/well. The expression of (P)RR ($ATP6AP2$) and vascular endothelial growth factor ($VEGF$) was measured. Primer sequences and their concentrations are described in Table 1.

Messenger RNA abundance was calculated relative to β-actin ($ACTB$) mRNA and compared with a calibrator sample (term human placenta) which was incorporated into each run. Relative abundance was thereby calculated as $2^{-\Delta\Delta CT}$.

Table 3.1 Primers used in real time PCR.

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3.2.4 Western blot

Total protein was extracted from cell pellets with the addition of 100μl RIPA buffer + 1μl of PMSF to each sample. Proteins were quantified by BCA assay. Samples were diluted to 10μg/μL, prepared by adding NuPAGE LDS and reducing agent and incubated at 70°C for 10min prior to loading on a NuPAGE 1.0mm gel, alongside a Magic-Mark and Pre-Stain
ladders. A calibrator was used on each gel as a loading control. Samples were loaded and allowed to run for 60 min. Membrane transfer was done on an Invitrogen Power Ease 500 at 30V, 160mA, and 17W for 60 min. The membrane was then dried for 40 min and reactivated in methanol for 1 min followed by subsequent rinsing in mQH$_2$O for 5 min.

The membrane was rinsed with TBS-T for 5 min and placed in a blocking solution (5% skim + 5% BSA in TBST) on a rocker at 4°C overnight. The membrane was then incubated with the (P)RR primary antibody (Abcam, ab40790, 1/1000 dilution) at room temperature for 2h after which the membrane was rinsed with TBS-T three times for 5 min, followed by rinsing once in TBS for 5 min. The membrane was then incubated with an anti-rabbit secondary antibody (Abcam, 1/5000 dilution) for 1h at room temperature. The signal was detected using an ECL Detection kit (GE Healthcare) and the Amersham Imager 600.

3.2.5 Cell viability

Cell viability was determined by resazurin assay in 96-well flat-bottomed microplates (Thermo Scientific). Based on the growth rate of each cell line, cells were seeded at a density of 1.5 x 10$^2$/100 μL for Ishikawa, 5 x 10$^3$/100 μL for HEC-1A and 2.5 x 10$^2$/100 μL for AN3CA cells and incubated overnight. The following day, 100 μL of experimental medium containing various concentrations (0.1, 1, 10 and 100 μM) of Handle Region peptide (HRP) (HRP, H-Arg-Ile-Phe-Leu-Lys-Arg-Met-Pro-Ser-Ile-OH, Auspep Pty Ltd, Victoria, Australia) were added or siRNA transfection experiments were carried out as described above. After 43 h of incubation with HRP or siRNA transfection, 20 μL of resazurin (Biovision) was added to each well and cells were incubated for a further 5 h. At 48 h fluorescence intensity was measured by Fluostar optima (BMG LABTECH, Germany) with an excitation wavelength of 530nm and emission wavelength of 590nm.
3.2.6 Cell proliferation

Cell proliferation was assessed using an xCELLigence RTCA DP instrument (ACEA Biosciences, China) in a humidified incubator maintained at 37°C and 5% CO₂. Sixteen well E-plates were used; each well has gold microelectrodes at the bottom of the well for impedance-based detection of cells, given as a cell index. Prior to the start of the experiment, 100 μl of media was added to each well. This is to ensure that the media and E-plate surface achieve equilibrium. After leaving the device in the incubator for 30 min, a background impedance reading was recorded.

Following the background reading, 100 μL of media containing cells were added to the E-plates and loaded into the xCELLigence machine. The density of cells seeded was based on their proliferation rate (as described in cell viability method). After 30 min of incubation, in order to allow the attachment of cells to the plate, the xCELLigence machine was started and left overnight. After 24h, the media was removed and 200 μL of experimental medium containing ATP6AP2 siRNA or negative control siRNA were added per well as were their respective vehicle controls. Cells were then incubated for a further 48h with the cell index recorded every 15 minutes. The rate of proliferation was determined by calculating the slope of the line between the beginning of the treatment and the end of the experiment (48h after treatment).

3.2.7 Statistics

All experiments were performed in triplicate and were repeated on three separate occasions. GraphPad Prism (version 6.0) was used for all statistical analyses and statistical
significance was set at P<0.05. One way and two-way ANOVA statistical analysis were used and multiple comparisons were done by Tukey’s multiple comparison test.

3.3 Results

3.3.1 ATP6AP2 mRNA expression in endometrial cancer cell lines

Three endometrial cancer cell lines, Ishikawa, HEC-1A and AN3CA were cultured and the expression of ATP6AP2 mRNA measured. ATP6AP2 mRNA abundance was significantly higher in Ishikawa cells compared with HEC-1A and AN3CA cells (P<0.0001 and P<0.0001). AN3CA cells have an intermediate amount of (P)RR (P<0.0001 and P<0.0002; Fig. 3.1).

Figure 3.1. mRNA expression of ATP6AP2 in Ishikawa, HEC-1A and AN3CA endometrial cancer cell lines. Results are expressed as mean ± SEM. All three experiments were performed independently in three different occasions. ****: P<0.0001; ***: P<0.0001; P<0.0002. “mRNA expression is expressed relative to the expression in term placenta”.

3.3.2 Effect of ATP6AP2 siRNA on ATP6AP2 mRNA abundance in endometrial cancer cell lines

The effects of 3 different ATP6AP2 siRNAs (siRNA-1, 2 & 3) on ATP6AP2 mRNA abundance were first validated in Ishikawa and HEC-1A cells. siRNA transfection with any
one of these 3 ATP6AP2 siRNAs resulted in ~90% knockdown of ATP6AP2 mRNA expression in Ishikawa and HEC-1A cells compared with negative control siRNA treated cells (P<0.0001 and P<0.0002; Fig. 3.2A & 3.2B). Based on their effect on ATP6AP2 mRNA expression in these two cell lines, we selected siRNA-2 for transfection in AN3CA and for functional studies on cell viability and proliferation. In AN3CA cells, siRNA transfection also appeared to decrease ATP6AP2 mRNA expression however due to the large variance in the controls this did not reach statistical significance (Fig. 3.2C).

Initially, (P)RR knockdown by transfection was studied using all the three siRNAs (1, 2 & 3). Based on their knock down efficiency in Ishikawa and HEC-1A cells, siRNA-2 was selected for further studies. Hence knockdown studies using siRNA-1 and -3 to investigate their effects on ATP6AP2 mRNA abundance in AN3CA cells were not carried out. In functional experiments, siRNA-3 was also explored in addition to siRNA-2.

**Figure 3.2** ATP6AP2 mRNA abundance in (A) Ishikawa, (B) HEC-1A, and (C) AN3CA cells after 48h incubation with one of three ATP6AP2 siRNAs, negative control siRNA (-VE Control) or vehicle (non-transfected). Results are expressed as mean ± SEM. All experiments were performed in triplicate on three different occasions. **: P<0.0002; ***: P<0.001; for comparison versus –VE control siRNA. “mRNA expression is expressed relative to the expression in term placenta”.
3.3.3 Effect of ATP6AP2 siRNA on ATP6AP2 protein expression in endometrial cancer cell lines

Transfection with an ATP6AP2 siRNA resulted in significant knockdown of (P)RR protein levels in all three endometrial cancer cell lines (Fig. 3.3). We used two different ATP6AP2 siRNAs (siRNA 2 and 3) in Ishikawa cells. Transfection with ATP6AP2 siRNA resulted in ~60% knockdown of (P)RR protein expression in Ishikawa and AN3CA cells compared to the negative control siRNA (P<0.0001 and P<0.0022; Fig. 3.3A & 3.3C). In HEC-1A cells, siRNA transfection resulted in ~50% knockdown of (P)RR protein compared to the negative control siRNA (P<0.015; Fig. 3.3B).
Figure 3.3 Relative (P)RR protein levels in (A) Ishikawa, (B) HEC-1A, and (C) AN3CA cells after 48h of transfection with one of two ATP6AP2 siRNAs, negative control siRNA (-VE Control) or vehicle (non-transfected). Representative western blots are presented, β-actin was used as an internal control. Results are expressed as mean ± SEM. All experiments were performed in triplicate on three different occasions; *: P<0.0149; **: P<0.0022; ****: P<0.0001; for comparison versus –VE control siRNA.
3.3.4 Effects of ATP6AP2 siRNA on Endometrial Cancer Cell Function

Effect of ATP6AP2 siRNA on cell proliferation in endometrial cancer cell lines

To investigate the effects of ATP6AP2 siRNA on cell proliferation using xCELLigence, all three siRNAs (siRNA-1, 2 & 3) were used in Ishikawa and HEC-1A cells. For AN3CA cells, only siRNAs-2 & 3 were used. Transfection with the negative control siRNA significantly reduced the rate of cell proliferation in all three cell lines compared to the non-transfected control (P<0.0062, P<0.001, P<0.001; Fig 3.4). In Ishikawa and HEC-1A cells all three ATP6AP2 siRNAs significantly reduced cell proliferation compared to the negative control siRNA (P<0.0001 and P<0.0001; Fig. 3.4A & 3.4B).

Both siRNA-2 & -3 significantly reduced cell proliferation in AN3CA cells (P<0.0001; Fig.3.4C). In HEC-1A cells, there was a significant reduction in cell proliferation when cells were transfected with siRNA-3, but siRNA-1 & 2 had no effect on cell proliferation compared to negative control siRNA (P<0.05, Fig. 3.4B).
Figure 3.4 Effect of ATP6AP2 siRNAs on the rate of cell proliferation in (A) Ishikawa, (B) HEC-1A, and (C) AN3CA cell lines. Cells were cultured for 48h after siRNA transfection and the slope of change in cell index was measured. Data are presented as mean ±SEM. *: P<0.05; **: P<0.01; ***: P<0.001.

3.3.5 Effect of ATP6AP2 siRNA on endometrial cancer cell viability (resazurin assay)

We studied the effects of ATP6AP2 siRNA-2 on cell viability in three endometrial cancer cell lines. In Ishikawa and AN3CA cell lines, siRNA transfection to knock down ATP6AP2 expression resulted in a small (30%) but significant decrease in the number of viable
cells at 48h, compared with the negative control siRNA (P<0.0001 and P<0.0001, Fig. 3.5A & 3.5C). In HEC-1A and AN3CA cells, there was a significant increase in the number of viable cells after transfection with the negative control siRNA, compared with that of the non-transfected control (P<0.0024 and P<0.0001) and there was no effect of (P)RR siRNA relative to non-transfected control (Fig. 3.5B).

Figure 3.5 Effect of ATP6AP2 siRNA on cell viability in (A) Ishikawa, (B) HEC-1A, and (C) AN3CA cell lines. Cells were cultured for 48h after siRNA-2 transfection; absorbance was measured by resazurin assay. Bar graphs represent mean ± SEM. *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.
3.3.6 Effect of ATP6AP2 siRNA on VEGF mRNA expression in endometrial cancer cell lines

We also looked at the mRNA expression of vascular endothelial growth factor (VEGF) in Ishikawa, HEC-1A and AN3CA cells in response to (P)RR siRNA. There was very low expression of VEGF in the non-transfected controls of all three cell lines (Fig. 3.6).

Transfection with a negative control siRNA appeared to stimulate VEGF expression in Ishikawa cells (Fig 3.6A) and did stimulate VEGF expression in HEC-1A cells (P<0.0019; Fig. 3.6B). In AN3CA cells, VEGF mRNA abundance was greater in (P)RR siRNA treated cells compared with both transfected and non-transfected negative controls (P<0.0001, P<0.0001) (Fig. 3.6C).
Figure 3.6 VEGF mRNA expression in (A) Ishikawa (B) HEC-1A and (C) AN3CA after 48h incubation with ATP6AP2 siRNA-2. Results are expressed as mean ± SEM. All experiments were performed independently on three different occasions **: P<0.01; ****: P<0.0001; for comparison versus -VE control cells. “mRNA expression is expressed relative to the expression in term placenta”.

3.3.7 Effect of a peptide inhibitor of ATP6AP2 on endometrial cancer cell viability

The addition of the (P)RR inhibitor, HRP, which prevents the binding of prorenin to the (P)RR [8], had no effect on cell viability in any of the three endometrial cancer cell lines (Fig. 3.7).
Figure 3.7 Effect of (P)RR inhibitor, HRP, on cell viability in three endometrial cancer cell lines. Cells were cultured for 48h after treatment with HRP and absorbance was measured by resazurin assay. Results are expressed as mean ± SEM. All experiments were performed on three independent occasions in triplicate.

3.4 Discussion

The actions of the (P)RR in endometrial cancer are unclear. Research shows that the (P)RR plays an important role in angiogenesis and proliferation. It is also an essential component of a V-ATPase and so plays a role in intracellular alkanisation and extracellular acidification, and activates the Wnt/β-catenin pathway [33]. Thus, through all these actions as well as its potential role in activating the RAS, it could play a very significant role in the spread of endometrial cancer. We were the first to identify the (P)RR in endometrial cancerous tissue [9].

The present study shows for the first time that the (P)RR is also expressed in three endometrial cancer cell lines (Fig. 3.1). In this study we focused on the effects of (P)RR on endometrial cancer cell function, i.e. its effects on cell proliferation and viability. This is the first study to show that the (P)RR is involved in endometrial cancer growth and viability. We know that binding of prorenin to the (P)RR can activate the RAS cascade to form Ang II. Concurrently, prorenin binding to the (P)RR can also initiate intracellular signaling pathways [8]. Both of these pathways can stimulate angiogenesis and proliferation. Several studies have
shown that the (P)RR plays a role in tumorigenesis and cancer formation [19, 22]. We have shown that the (P)RR is highly abundant in endometrial cancers [3], and now we have described its expression in three endometrial cancer cell lines (Fig. 3.1).

All three cell lines are derived from epithelial endometrial cancers (Ishikawa, HEC-1A and AN3CA). *ATP6AP2* expression was however variable. The Ishikawa cell line had significantly higher *ATP6AP2* mRNA abundance when compared with AN3CA cells and HEC-1A cells. Abundance was less in AN3CA and least in HEC-1A (Fig 3.1). Similarly, Figure 3.3 shows that the levels (P)RR protein in non–transfected controls was highest in Ishikawa cells, lower in AN3CA and least in HEC-1A cells. Thus there was agreement between comparative levels of mRNA and protein in the three cell lines.

As (P)RR is highly expressed in endometrial cancers [9] and in endometrial cancer cell lines (Figure 3.2) and prorenin binding to the (P)RR could activate intracellular signaling pathways [10], we proposed that knocking down the (P)RR with an *ATP6AP2* siRNA or inhibiting prorenin/(P)RR binding with HRP, would decrease cell proliferation and viability either by blocking RAS pathways involved in Ang II-induced angiogenesis and cell growth or by blocking the Ang II independent effects of (P)RR (described above) on cellular function.

In the first part of this study, we showed that (P)RR was knocked down after 48h with *ATP6AP2* specific siRNAs in three endometrial cancer cell lines. An approximately 90% knockdown of *ATP6AP2* mRNA expression was achieved in Ishikawa and HEC-1A cells, and 60% in AN3CA cells, although this did not reach statistical significance (Fig. 3.2C). These effects were consistent at 72h and 96h post-transfection (Appendix A2).

Furthermore, *ATP6AP2* siRNA treatment resulted in a remarkable reduction in the expression of (P)RR protein in all three endometrial cancer cell lines (Ishikawa 75%, HEC-1A 55% and AN3CA-65%) (Fig. 3.3). Similar results have been observed in pancreatic ductal adenocarcinoma cells [22].
Transfection with an ATP6AP2 siRNA significantly reduced the rate of cell proliferation in both Ishikawa and AN3CA cells (Fig. 3.4A & 3.4C) but the effect of knockdown on the rate of cell proliferation in HEC-1A cells was not as obvious, only being observed in siRNA-3 treated cells (Fig. 3.4B). Our results are similar to the effects of (P)RR knockdown described in breast and pancreatic cancer [17, 22]. It should be noted that levels of (P)RR mRNA and protein were lowest in HEC-1A (Fig. 3.1 & 3.3B) so it is perhaps not surprising that the effects of knockdown of its (P)RR on cell proliferation rates and viability were less. Also, ATP6AP2 siRNAs significantly reduced the viability of both Ishikawa and AN3CA cells (Fig. 3.5A & 3.5C). Surprisingly, knockdown of (P)RR had no effect on the viability of HEC-1A cells (Fig. 3.5B).

Previous studies have shown that in tissue specific knockout mice, (P)RR knockdown in cardiomyocytes or glomerular podocytes resulted in death after birth as a result of heart failure [14, 25, 26]. Also, loss of (P)RR leads to decreased cell proliferation in cancer cells, suggesting that the (P)RR affects tumour viability [27]. So, it is not surprising that (P)RR knockdown by siRNA caused a significant decrease in cell proliferation and cell viability in two endometrial cancer cells (Ishikawa, AN3CA). Similar effects of (P)RR siRNA have also been documented in other cancers [22, 28].

Tumour growth and spread is heavily dependent upon angiogenesis. VEGF induces angiogenesis and monoclonal antibodies against VEGF are used in the treatment of a number of cancers. Therefore, we looked at the effects of knocking down (P)RR in the three cell lines on their ability to express VEGF. Surprisingly there was virtually no expression of VEGF in the non-transfected control cells in any of the cell lines (Figure 3.6), and the effects of transfection of negative control and ATP6AP2 siRNAs on the abundance of VEGF mRNA in these cell lines was variable and confusing. In Ishikawa and HEC-1A cells, transfection with the negative control siRNA was associated with increased in VEGF mRNA and there was no
effect of ATP6AP2 siRNA (Fig. 3.6A & 3.6B). In AN3CA cells transfection with the negative control siRNA had no effect on VEGF mRNA while (P)RR knockdown in fact caused a significant increase in its expression (Fig. 3.6C). Therefore, it is not possible to conclude that (P)RR has any effect on VEGF expression in these cell lines. VEGF is an important angiogenic factor in early tumorigenesis and increased expression of VEGF may be important for tumour growth and spread; later in tumorigenesis it may be less important [32]. It is however interesting to note that transfection with either the specific siRNA or the scrambled siRNA caused increased expression of VEGF, suggesting that the processes or chemicals involved in transfecting cells changes the levels of expression of genes that can confound experimental objectives.

We also used a synthetic peptide, HRP, that is said to block the binding of prorenin to (P)RR [8]. It did not have any effect on cell viability in any of the three cell lines (Ishikawa, HEC-1A and AN3CA) (Fig. 3.7). HRP may not bind to the cell membrane because of its poor membrane solubility. Although HRP has been shown to block prorenin binding to (P)RR other studies have shown that HRP doesn’t affect the cardiovascular and renal actions of (P)RR [29-31]. Since there was no effect of HRP on cell viability in the three cell lines, HRP was not studied further. Ichihara et al. demonstrated that HRP inhibited the non proteolytic activation of prorenin [34].

In conclusion, we have shown for the first time, that (P)RR mRNA and protein are expressed in three epithelial endometrial cancer cell lines. Knockdown of (P)RR expression reduced the cell viability and proliferative capacity of two of these cell lines. Therefore, knocking down the (P)RR could be a novel therapeutic strategy for treatment of certain forms of endometrial cancer. Further studies are required to delineate those cancers which are most susceptible to the growth inhibiting actions of (P)RR knockdown.
3.5 References


Chapter 4 - Soluble (P)RR in human plasma, uterine fluid and supernatant from three endometrial cancer cell lines

4.1 Introduction

The (pro)renin receptor ((P)RR), binds both prorenin and renin, and converts prorenin into an active enzyme. It was discovered in 2002 and is expressed in the kidney, heart, eye, vascular smooth muscle and placenta [1, 2]. The (P)RR is also known as ATPase H (+)-transporting lysosomal accessory protein 2 (ATP6AP2) because it forms part of a vacuolar H+-ATPase (V-ATPase) complex. V-ATPase is a multi-subunit proton pump which also plays a significant role in maintaining intracellular pH [4, 5]. The (P)RR is also involved in the Wnt/β-catenin signaling pathway, acting as an adapter between V-ATPase and the Wnt receptor [3].

The (P)RR is ubiquitously expressed and has the potential to induce effects through its interactions with prorenin and/or renin. Apart from activating the classical RAS pathway, (P)RR can act independently of prorenin (e.g. via Wnt and ERK1/2 pathways). Recently, a soluble form of (P)RR, (s(P)RR), has been discovered [1].

The (P)RR has three different molecular forms: (1) the full length transmembrane protein; (2) the soluble (P)RR (s(P)RR) found in plasma and urine [6]; and (3) a truncated form composed of the transmembrane and cytoplasmic domains (Fig.1.1)

Binding of both full length (P)RR and s(P)RR with prorenin can activate prorenin, which can then generate angiotensin (Ang) I from angiotensinogen (AGT). Therefore, s(P)RR serves as a biomarker, reflecting tissue RAS status independent of its activity as a V-ATPase [7].

Several studies have shown that there is an association between s(P)RR levels and diseases such as gestational diabetes [8], preeclampsia [9-11], chronic kidney disease [12],
hypertension, heart failure [13, 14] and pancreatic ductal adenocarcinoma [15], which suggests that (s)PRR may be a useful biomarker for disease.

Cousin et al. showed that the (P)RR could be found in the Golgi apparatus where it is cleaved by the pro-protein convertase, furin or by Site 1 protease to generate s(P)RR [6]. s(P)RR is secreted into the extracellular space and can be detected by a specific ELISA [7] in the blood stream [16] and in conditioned media from cultured glomerular epithelial cells [17, 18]. In human plasma from chronic kidney disease patients, the normal interquartile range is 20.9–26.5 ng/ml [19]. s(P)RR levels are unchanged by posture and circadian rhythms [7] but may be raised in people with chronic diseases and in pregnant women [19].

The s(P)RR is overexpressed in colon [20], head, breast and ovarian cancers [21, 22] and is constantly elevated in endometrial cancer cell lines. Since the levels of (P)RR are high in endometrial cancers [23], we postulated that levels of s(P)RR would be increased in supernatant collected from cultured endometrial cancer cell lines and in blood and uterine fluid (collected as a result of uterine lavage) from women with endometrial cancer.

4.2 Materials and Methods

Soluble (P)RR was measured in the blood and uterine fluid (collected by uterine lavage) collected from healthy women and from women who had endometrial cancer as well as in supernatant collected in vitro from endometrial cancer cell cultures (see chapter 3).

4.2.1 Patient samples

This research was approved by the University of Newcastle Human Research Ethics Committee and The Hudson Research Institute, Melbourne. Most plasma and uterine lavage fluid samples were obtained from the Hudson Research Institute. Patients were aged between 37 to 50 yrs. Plasma from healthy women were also collected from participants were aged
between 37 and 50 years. We examined 35 samples of uterine fluid (7 samples for control, 11 samples from women with Grade-1 cancers, 13 samples from women with Grade-2 cancers, 2 samples from women with Grade-3 endometrial cancer and 2 samples from serous endometrial cancer). We measured s(P)RR in plasma from 40 women, 11 control, 12 Grade-1, 13 Grade-2, 2 Grade-3 endometrial cancer and 2 samples from women with serous endometrial cancer.

4.2.2 Supernatant Media

In Chapter 3, I described experiments in which we ‘knocked down’ expression of (P)RR using an siRNA specific for ATP6AP2. Three endometrial epithelial cancer cell lines (Ishikawa, HEC-1A and AN3CA cells) were either not transfected (control) or were transfected with either scrambled siRNA (negative control) or with siRNA that targets ATP6AP2. Supernatant was collected after 48h of incubation and stored at -80°C for determination of s(P)RR.

4.2.3 Enzyme linked immunosorbent assay (ELISA)

A human s(P)RR ELISA kit from IBL (Catalogue #27782) was used to measure s(P)RR levels in plasma, uterine fluid and cell culture supernatant. Prior to assay, supernatant samples were diluted 1/5, lavage samples were diluted in 1/2 and plasma in 1/10 in the EIA buffer provided with the kit and the wash buffer was prepared.

Standard curve: Standard dilutions were prepared from a stock solution and ranged from 16,000 pg/mL to 125 pg/mL. EIA buffer was used as a test blank. 100 µl per well of standard, calibrator, blank or samples were loaded in duplicate. The 96-well plate was incubated overnight at 4°C. The next day, the wells were washed with 300 µl of wash buffer (1% BSA, 0.05% Tween 20 in PBS) 5 times. Excess wash buffer was removed by blotting against an absorbent towel.
To each well, 100 μl of labelled antibody was added. To reagent blank wells, 100 μl of wash buffer was added. The plate was incubated for 60 min at 4°C. After incubation, the plate was washed 5 times, and 100 μl of chromogen added to each well. The plate was wrapped in foil to avoid direct light exposure and incubated for 30 min at room temperature. Then 100 μl of stop solution was added to each well and the plate gently rocked to ensure uniform mixing. The plate was read at 450nm on the SpectroStar Nano plate reader to measure the optical density of each well. The average value of the duplicate standard duplicates was used (normalized for blank) to plot the standard curve, linear regression was used to derive the equation of the line of best fit. This equation was used to calculate the concentration of samples based on the average absorbance of sample duplicates, also normalised to blank. The final concentration of s(P)RR in pg/mL was found by multiplying the values obtained by the dilution factor.

4.2.4 Statistics

GraphPad Prism (Version 7.0) was used for all statistical analyses and statistical significance was set at P<0.05. One-way ANOVA was used and multiple comparisons were carried out using Dunnett’s multiple comparison test.

4.3 Results

4.3.1 Soluble (pro)renin receptor (s(P)RR) concentration in supernatant samples of three endometrial cancer cell lines

s(P)RR concentrations were found to be significantly higher in the cell culture supernatant collected from Ishikawa cells compared to HEC-1A cells (Fig; 4.1).
4.3.2 Soluble (pro)renin receptor (s(P)RR) concentrations in supernatant samples from three endometrial cancer cell lines

Levels of s(P)RR in supernatant from non-transfected Ishikawa cells (17 ng/ml) was higher than in supernatant from HEC-1A (10 ng/ml) or AN3CA cells (12 ng/ml) respectively. The concentration of s(P)RR was found to be higher in non-transfected controls and in the cells transfected with negative control siRNA in all three cell lines compared with levels in supernatants collected from cells that had been transfected with ATP6AP2 siRNA-2 which ‘knocks down’ (P)RR mRNA (Fig. 4.2A, 4.2B and 4.2C). Levels of s(P)RR protein in supernatants from Ishikawa cells treated with ATP6AP2 siRNA were decreased by approximately 70% compared to the negative controls (P<0.001; Fig. 4.2A). In HEC-1A and AN3CA cells, siRNA transfection was associated with a ~60% reduction in s(P)RR protein levels compared with levels in cells treated with the negative control siRNA (P<0.0001; Fig. 4.2B and 4.2C).
Figure 4.2. Soluble (P)RR levels (ng/ml) in cell culture supernatant from (A) Ishikawa (B) HEC-1A and (C) AN3CA cells collected 48h after transfection with either ATP6AP2 siRNA-2, a negative control siRNA (-VE Control) or vehicle (non-transfected). Results are expressed as mean ± SEM. All 3 experiments were performed independently in 3 different occasions. **: $P<0.0001$; ***: $P<0.0001$; for comparison versus –VE control.
4.3.3 Soluble (pro)renin receptor (s(P)RR) levels in human plasma and uterine fluid

Soluble (P)RR levels were significantly higher in plasma samples collected from higher grades of cancer (Grade-1, Grade-2 and Grade-3) when compared to the plasma samples collected from healthy women (P<0.0030; P<0.0001 and P<0.0059 Fig. 4.3A). There was no significant difference in s(P)RR levels in uterine fluid from women with different grades of cancer and serous endometrial cancer when compared to healthy controls (Fig. 4.3B).

Figure 4.3. Soluble (pro)renin receptor (s(P)RR) levels (ng/ml) concentration in (A) plasma and (B) uterine fluid samples from women without cancer (control) or women with endometrial cancer of different grades. Results are expressed as mean ± SEM. **: P<0.0001; for comparison versus control samples.

4.4 Discussion

In the present study we found that s(P)RR was abundant in cell culture supernatant from three endometrial cancer cell lines (Ishikawa, HEC-1A and AN3CA) (Fig 4.2). Knockdown of (P)RR by ATP6AP2 siRNA significantly reduced levels of s(P)RR in supernatant from all three endometrial cancer cell lines. We also found that s(P)RR concentration in plasma significantly
increased with increasing grades of cancer (Grade-1, 2 & 3) and serous endometrial cancer compared to control plasma samples.

From Chapter 3, we know that the (P)RR mRNA expression and protein was present in three endometrial cancer cell lines and the expression was highest in Ishikawa when compared to the other two cell lines. In Ishikawa cells, higher amounts of (P)RR are expressed and may contribute to the higher rate of proliferation of this cell line compared with the other two cell lines. Since knockdown by ATP6AP2 siRNA reduced both the cell viability and rate of proliferation (see Chapter 3). Cousin et al. also found that soluble prorenin secretion from cultured glomerular epithelial cells was greater the greater, the level of expression of (P)RR [6].

The concentration of s(P)RR in media was also higher in Ishikawa cells compared with HEC-1A and AN3CA cells (Fig 4.1). This may be because (P)RR is most highly expressed in Ishikawa cells (Chapter-3). In all three cell lines, s(P)RR levels in cell culture supernatant was higher in non-transfected cells and cells treated with negative control siRNA compared with media from cells in which (P)RR was knockdown by ATP6AP2 siRNA (Fig 4.2A, 4.2B and 4.2C).

These findings suggest that cancer cell proliferation and viability, which must be considered as powerful contributors to malignancy in terms of spread of cells and metastasis, is highest in cells expressing high levels of (P)RR. Furthermore, levels of expression of (P)RR influence the level of production of s(P)RR. Since (P)RR knockdown reduces both (P)RR and s(P)RR levels (Fig. 4.1), (P)RR may be a therapeutic agent for treatment of endometrial cancer and measurement of levels of s(P)RR could be used to track the success of treatment of endometrial cancer.

To test these possibilities further, we measured s(P)RR in plasma and uterine fluid from women with endometrial cancer of varying grades and compared levels with samples from
women who did not have endometrial cancer. s(P)RR levels were much higher in plasma samples from women with both epithelial adenocarcinoma and serous endometrial cancer (P<0.0001; Fig. 4.3A) compared with samples from women who did not have cancer.

A study by Kreienbring et al. showed that there was no significant difference between the levels of s(P)RR in ovarian cancer and healthy patients [24]. This may suggest that high s(P)RR levels in plasma is specific to endometrial cancers only.

In samples of uterine fluid however we could find no significant difference between s(P)RR levels in tumours of varying grades, nor between these samples and serous endometrial cancer or control samples (Fig 4.3B). We do not know how much fluid was used in each case to collect uterine fluid and it is possible that there was variable dilution of uterine fluids by washings used to collect the samples. Data shown in (Fig 4.3B) tend to support this proposition. Levels of s(P)RR in control samples were all low and within a narrow range, suggesting that levels were consistently low. In samples from women with grade 1 and 2 endometrial cancers where there are a number of samples (as compared with grade 3 and serous cancer) levels were extremely variable. In fact, many samples had significantly more s(P)RR than control samples.

On the other hand, it is possible that the variability is due to the levels of (P)RR within the various tumours as has been described above in our cancer cell lines (see Ishikawa vs HEC1-A and AN3CA). It should be noted that in a previous study in which we measured levels of expression of RAS genes and proteins, it was demonstrated that the RAS is highly expressed in endometrial tumors and the adjacent endometrium [23]. While levels of (P)RR were higher in cancerous tissue, the expression was highly variable with some tissues expressing undetectable amounts of (P)RR. If our hypothesis is correct that cell lines in which (P)RR is most abundant are the most proliferative and viable, then we would anticipate that those tumours with the highest expression of (P)RR would be the most malignant. However, there was no significant difference in (P)RR expression with tumour grade [23]. It is possible
however that a relationship between (P)RR and potential malignancy occurs early in the ontogenesis of the cancer. Tumours that have become clinically invasive may have escaped dependency on the activity of the (P)RR. Therefore, it could be extremely worthwhile pursuing the role of (P)RR and the potential of s(P)RR as a biomarker for early detection of endometrial cancer.

In conclusion, we have shown for the first time that s(P)RR levels were high in the supernatant from three endometrial cancer cell lines (Ishikawa, HEC-1A and AN3CA) as well as in human plasma samples from different tumour grades (Grade-1, 2 & 3) and serous endometrial cancer. Therefore, measurement of plasma s(P)RR in women at risk of endometrial cancer may help in the early detection of this cancer and its severity.
4.5 References


CHAPTER 5 - Effect of renin-angiotensin system (RAS)

blockers in endometrial cancer cell lines

5.1 Introduction

Endometrial cancer is the most common gynecological malignancy affecting women in the western world [1]. Risk factors for endometrial cancer include obesity, hypertension and diabetes, all of these are linked to activation of the renin-angiotensin system (RAS) [2].

The RAS consists of a cascade of precursor proteins, that are converted by enzymes into active peptides that regulate blood pressure, water-sodium balance, and tissue homeostasis [3, 4]. It may also play an important role in the growth and spread of endometrial cancer. Previous studies have shown that various components of the RAS are expressed in several cancers including lung, pancreas, brain, stomach, prostate, skin and cervical cancers [5].

Recently, we showed that key genes controlling the synthesis and activity of RAS peptides like prorenin (REN), (pro)renin receptor ((P)RR, ATP6AP2), angiotensinogen (AGT), angiotensin II type 1 receptor (AT\(_1\)R, AGTR1) and angiotensin converting enzyme (ACE) are highly expressed in endometrial cancer tissues [8].

Watanabe et al. showed that endometrial cancer cells treated with the major effector peptide of the RAS, angiotensin II (Ang II), had increased vascular endothelial growth factor (VEGF) expression and endothelial cell migration; these were suppressed when breakdown of Ang II was enhanced by overexpression of adipocyte-derived leucine amino peptidase (A-LAP) [7]. A-LAP belongs to the M1 family of zinc-metallopeptidases, which hydrolyse Ang II [6, 7].

In tissues, the RAS depends on the (P)RR to bind and activate prorenin, thus activating the RAS cascade. Binding of prorenin to the (P)RR leads to a conformational change so that
the pro-segment of prorenin is unfolded from the catalytic cleft and its active site becomes available for cleavage of AGT to Ang I, i.e. there is non-proteolytic activation of prorenin [9]. Ang I is then cleaved by ACE to produce the biologically active octapeptide Ang II [10-12]. Ang II elicits most of its actions by acting on AT_1R, and in tissues, stimulates angiogenesis and cell proliferation. In addition, binding of prorenin to the (P)RR can activate intracellular signaling pathways independent of the formation of Ang II [9, 15].

Renin inhibitors (aliskiren and VTP-27999) block the catalytic actions of renin/prorenin, thus preventing the formation of Ang II [16]. Aliskiren, a direct renin inhibitor, specifically binds to the active site (S3^P) of renin [17] and blocks the formation of Ang I from AGT [18]. Approximately 40-80% of renin activity is inhibited by aliskiren. Therefore, it causes a significant reduction in Ang II formation [19]. The effects of renin inhibitors on cancer spread is at present unknown.

Two drugs that block the RAS, angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs), are commonly used anti-hypertensive agents. They act by blocking Ang II production or its binding to the AT_1R [20, 21]. Makar et al. showed that patients who were treated with ACEIs/ARBs for four years, exhibited lower risk for colorectal cancer [21]. Lever et al. also showed that patients undergoing treatment with ACEIs for hypertension had a reduced risk of cancer, particularly female-specific cancers [22]. Previous studies have shown that ARBs inhibited the growth of several cancers both in vivo and in vitro, including colon [26], gastric [27], breast [28], and endometrial cancers [25].

Several studies have shown that Ang II promotes cell proliferation and Ang II activation can be inhibited by using ARBs that antagonize its interaction with the AT_1R. Out of seven different ARBs available, losartan was the first to be approved for clinical use and has an excellent tolerability and safety profile [23, 24].
Telmisartan is another orally effective ARB that is widely used for lowering blood pressure. Apart from being an anti-hypertensive agent, telmisartan has also been shown to reduce proliferation of colon cancer cells [26]. Telmisartan decreased the percentage of viable cells in an endometrial cancer cell line and reduced human endometrial tumour growth \textit{in vivo} in a mouse model [25]. Telmisartan also inhibited esophageal and prostate cancer cell proliferation and tumour growth both \textit{in vivo} and \textit{in vitro} [30, 31]. Telmisartan not only blocks the \textit{AT}1\textit{R} but also acts as a partial agonist of peroxisome proliferator-activated receptor gamma (PPAR-γ) [32]. Telmisartan can activate 25-30\% of PPAR-γ when compared with full PPAR-γ agonists like troglitazone and pioglitazone, which are thiazolidines (a new class of anti-diabetic drug) that can also regulate differentiation of cancer cells [32]. Park \textit{et al.}, demonstrated that troglitazone, a PPAR-γ agonist, decreased cell viability in thyroid cancer cell lines [33]. In another study which was carried out by Motomura \textit{et al.}, treatment with troglitazone inhibited growth of human pancreatic carcinoma cells [34].

There is good evidence that the RAS is overexpressed in endometrial cancers [8] and act to promote cell proliferation and angiogenesis. Therefore, we postulated that endometrial cancer growth would be inhibited by drugs that inhibit prorenin (aliskiren, VTP-27999), Ang II formation (ACEI, perindoprilat), or the interaction between Ang II and \textit{AT}1\textit{R} (losartan and telmisartan). We tested the effects of these drugs in \textit{‘in vitro’} studies using three endometrial cancer cell lines.

\textbf{5.2 Methods}

Most methods used to carry out these experiments are the same as those described in Chapter 2 & 3.

\textbf{5.2.1 Drugs}
One hundred microliters of experimental medium containing various concentrations (0.1, 1, 10 and 100 μM) of renin inhibitors (aliskiren or VTP-27999; Medchem Express), ACE inhibitors (Perindoprilat; Sigma Aldrich), ARBs (losartan, telmisartan; Sigma Aldrich) or a PPAR-γ agonist (troglitazone; Sigma Aldrich) were added to cultured cells to measure their effects on cell viability or proliferation. Vehicle controls containing the same amount of DMSO were included in all experiments.

5.2.2 Statistics

All experiments were performed in triplicate with each dose being tested in duplicate. Each experiment was repeated on three separate occasions. GraphPad Prism (Version 7.0) was used for all statistical analyses and statistical significance was set at P<0.05. One way and two-way ANOVA statistical analyses were used and multiple comparisons were done using Tukey’s multiple comparison test. A drug was only considered to have a significant effect if a dose of the drug significantly reduced cell viability on cell proliferation compared with control (i.e. Tukey’s multiple comparison showed that the significance of any difference between the effect of the drug and the effect of the vehicle control was less than 5%).

5.3 Results

5.3.1 RAS mRNA expression in endometrial cancer cell lines

Three endometrial cancer cell lines (Ishikawa, HEC-1A and AN3CA) were cultured and RAS mRNA abundances measured. There were significant differences between these three cell lines in their expression of RAS genes. REN mRNA abundance was highest in Ishikawa cells compared with both HEC-1A and AN3CA cells (P<0.001 and P<0.001) but there was no difference between HEC-1A and AN3CA cells (Fig. 5.1A). AGTRI mRNA abundance was also highest in Ishikawa cells compared with the other two cell lines (P<0.0001, Fig. 5.1E) as
was \( ACE \) mRNA abundance (\( P<0.00001 \), Fig. 5.1C). On the other hand, \( AGT \) and \( ACE2 \) mRNA abundances were greatest in HEC-1A cells compared with Ishikawa and AN3CA cells (\( P<0.0001 \), Fig. 5.1B & D). Therefore, the Ishikawa cell line expressed \( REN, ACE1 \) and \( AGT/R \) most abundantly while HEC-1A cells expressed \( AGT \) and \( ACE2 \) mRNA most abundantly.

![Graphs A-E](image.png)

**Figure 5.1** Abundances of (A) prorenin (\( REN \)) (B) angiotensinogen (\( AGT \)) (C) angiotensin converting enzyme (\( ACE \)) (D) angiotensin converting enzyme 2 (\( ACE2 \)) (E) and Angiotensin II Type 1 Receptor (\( AGT/R \)) mRNAs in Ishikawa, HEC-1A and AN3CA cells. Results are expressed as mean ± SEM. Three experiments were performed independently in triplicate. ***: \( P<0.001 \); ****: \( P<0.0001, 0.0001, 0.0001 \) and 0.0001 respectively.

### 5.3.2 Effect of renin inhibitors (aliskiren and VTP-27999) on cell viability in endometrial cancer cell lines

Treatment with a renin inhibitor, VTP-27999, significantly reduced the viability of HEC-1A and Ishikawa cells (one-way ANOVA: Ishikawa, \( P=0.02 \); HEC-1A, \( P<0.0007 \)).
Ishikawa cells, only VTP-27999 at 100uM significantly inhibited cell viability whereas in HEC-1A cells a significant effect was seen at both 10μM and 100μM of VTP-27999 (P<0.05 and P<0.01; Fig. 5.2A). There was no significant effect of VTP-27999 on the viability of AN3CA cells (Fig. 5.2A). Treatment with aliskiren, another renin inhibitor, also reduced Ishikawa cell viability, this was significant at 100μM (P<0.0003; Fig. 5.2B). There was no effect of aliskiren on the viability of HEC-1A and AN3CA cells (Fig. 5.2B).

**Figure 5.2** Effect of renin inhibitors, (A) VTP-27999, and (B) aliskiren, on cell viability in three endometrial cancer cell lines. The concentrations are (0.1 – 100 μM) and there is a vehicle control. Cells were cultured with the drugs for 48h; absorbance was measured by resazurin assay. Results are expressed as mean ± SEM. N=3 independent experiments in triplicate. P denotes the results of the One-Way ANOVA examining the effect of treatment within each cell line. *: P<0.05; ***: P<0.001, denotes significant difference from control within the same cell line.
5.3.3 Effect of an ACE inhibitor on cell viability in endometrial cancer cell lines

Fig. 5.3 shows that there was no effect of perindoprilat on cell viability in any of the three endometrial cancer cell lines tested.

Figure 5.3 Effect of an ACE inhibitor (perindoprilat) on cell viability in the three endometrial cancer cell lines; the concentrations used were (0.1 - 100μM) or vehicle alone (control). Cells were cultured for 48h with perindoprilat and absorbance was measured by resazurin assay. Results are expressed as mean ± SEM. N=3 independent experiments in triplicate.

The ACE inhibitor, perindoprilat, did not display any effect on cell viability in the three endometrial cancer cell lines (Ishikawa, HEC-1A and AN3CA), and low concentrations of renin inhibitors had little effect on cell viability, although an effect was observed at higher concentrations. Therefore, the effects of the renin and ACE inhibitor on cell proliferation was not studied further.

5.3.4 Effects of angiotensin receptor blockers (ARBs) on cell viability and cell proliferation in endometrial cancer cell lines

Two ARBs were tested and variable responses by the three endometrial cancer cell lines were obtained. In all three endometrial cancer cell lines treatment with losartan at 100 μM was
associated with a significant increase in cell viability compared with the vehicle control (Ishikawa, P<0.01; HEC-1A, P<0.0001; and AN3CA, P<0.0001; Fig. 5.4A).

When the three cell lines were treated with telmisartan, there was, however, a dose-dependent reduction in cell viability (one-way ANOVA, Ishikawa, P<0.0001; HEC-1A, P<0.0001; and AN3CA, P<0.0001; Fig. 5.4B). In Ishikawa cells, this effect was first seen with 0.1μM, and in HEC-1A and AN3CA cells it was first seen at 1μM (P<0.0001; Fig. 5.4B).

The effects of losartan and telmisartan (ARBs) on the rate of proliferation of the three cell lines was also examined. 10μM losartan was associated with a significant increase in the rate of proliferation of Ishikawa cells. By contrast, at 100μM there was a significant reduction in the growth rate of Ishikawa cells (P<0.0001, Fig. 5.4C). There was no effect of losartan on the proliferation rates of either HEC-1A or AN3CA cells (Fig. 5.4C).

A concentration of 100μM telmisartan caused both Ishikawa and AN3CA cells to undergo cell death as indicated by the negative slope (Ishikawa, P<0.0001 and AN3CA, P<0.0001; Fig. 5.4D) but telmisartan at 0.1μM was associated with a small increase in the rate of proliferation of Ishikawa cells (P<0.05, Fig. 5.4D). Telmisartan had no effect on the rate of proliferation of HEC-1A cells (Fig. 5.4D). Negative slope values indicate that the cells are dying.
Figure 5.4 Effect of ARBs, losartan and telmisartan, on cell viability (A and B) and the rate of cell proliferation (C and D) in the three endometrial cancer cell lines. The concentrations used were 0.1 - 100 μM and the vehicle control. Cells were cultured for 48h with ARBs. Cell viability was measured by resazurin assay. Rate of cell proliferation was calculated as the rate of change in cell index (slope) by xCELLigence. Results are expressed as mean ± SEM. Three experiments were performed independently in triplicate. Tukey’s values a: P<0.05; b: P<0.01; c: P<0.001; d: P<0.0001, denotes significant difference from control within the same cell line.
5.3.5 Effect of a PPAR-γ agonist (troglitazone) on cell viability and cell proliferation in three endometrial cancer cell

To see if the effects of telmisartan, an ARB and partial PPAR-γ agonist, could be mimicked by troglitazone, a PPAR-γ agonist, we examined the effects of troglitazone on cell viability and proliferation in three endometrial cancer cell lines. Fig. 5.5A shows that troglitazone treatment reduced the viability of Ishikawa cells and AN3CA cells, both at a dose of 100μM. There was no effect of troglitazone on HEC-1A cells (Fig. 5.5A).

At a concentration of 1μM, troglitazone was associated with a significant increase in the rate of cell proliferation of HEC-1A cells (Fig. 5.5B); as the concentration of troglitazone increased, cell proliferation decreased. This effect was significant at 100μM (P<0.05 Fig. 5.5B). Treatment with troglitazone at 100μM also decreased the rate of cell proliferation in Ishikawa and AN3CA cells (P<0.05 and P<0.001, respectively; Fig. 5.5B).

![Figure 5.5](image_url)

**Figure 5.5** Effect of troglitazone, a PPAR-γ agonist, on (A) cell viability and (B) cell proliferation in three endometrial cancer cell lines; the concentrations used were 0.1 - 100μM or the vehicle as control. Cells were cultured for 48h with troglitazone. Cell viability was measured by resazurin assay. Rate of cell proliferation was calculated as the rate of change in cell index (slope) by xCELLigence. Results are expressed as mean ± SEM. Three experiments were performed independently in triplicate. a: P<0.05; C: P<0.001; d: P<0.0001.
5.4 Discussion

Endometrial cancer cell lines show variable levels of RAS mRNA expression. All three cell lines (Ishikawa, HEC-1A and AN3CA) are derived from endometrial epithelial cancer cells. We observed greater abundances of REN, ACE1 and AGTR1 mRNA in Ishikawa cells (Fig 5.1A, 5.1C & 5.1E). These RAS components are most likely to promote angiogenesis and tumourigenesis. In HEC-1A cells ACE2 and AGT were most highly expressed (Fig 5.1B & 5.1D). The lowest expression of RAS genes occurred in AN3CA cells (Fig. 5.1).

It has been documented that binding of prorenin to the (P)RR can activate the RAS cascade to form Ang II. Similarly, prorenin binding to (P)RR can also initiate intracellular signaling pathways [11]. Both of these pathways can stimulate angiogenesis and cell proliferation. The effects of prorenin and (P)RR interactions were discussed in Chapter 3. In this study, we examined the effects of RAS blockers on cell proliferation and cell viability. This study demonstrated that renin inhibitors (VTP-27999 and aliskiren) were only effective in reducing cell viability in cells which had high expression of REN mRNA. ACE inhibitors were ineffective in all three cell lines. Telmisartan, which is both an ARB and a partial PPAR-γ agonist, and troglitazone (a PPAR-γ agonist) significantly reduced the viability and proliferation rate of all three endometrial cancer cell lines.

Effect of renin and ACE inhibitors on cell viability

To the best of our knowledge this is the first study to report the effect of renin inhibitors (aliskiren and VTP-27999) in endometrial cancer cells. Renin binding to the (P)RR can cleave angiotensinogen to form Ang-1 and thereby activating the classical RAS pathway to form Ang II [35]. So, we postulated that renin inhibitors (aliskiren, VTP-27999) would prevent the formation of Ang I from AGT and prevent formation of Ang II.
Both aliskiren and VTP-27999 reduced the viability of Ishikawa and HEC-1A cells (Fig. 5.2A, Fig. 5.2B). However, there was no effect in AN3CA cells (Fig. 5.2A & 5.2B). This may be related to the low level of expression of REN in AN3CA cells compared with Ishikawa and HEC-1A cells (Fig. 5.1A). With renin inhibitors (aliskiren and VTP-27999) we have only examined cell viability. Future studies are required to examine whether the reduction in cell viability is due to increase in apoptosis or a decrease in cell proliferation.

Perindoprilat, an ACE inhibitor, which prevents the conversion of Ang I to Ang II did not have any effect on the viability of any of the three endometrial cancer cell lines (Fig. 5.3). With perindoprilat, we were able to perform only cell viability studies, more studies are required to validate the effect of perindoprilat on the rate of cell proliferation.

**Effect of ARBs on cell proliferation and cell viability**

We know that Ang II acting via the AT1R receptor can stimulate both cell proliferation and angiogenesis, which can lead to cancer progression [36]. Research shows that Ang II plays an important role in tumour growth and angiogenesis [37]. The level of expression of AT1R is high in endometrial cancer [10] and it is expressed in all three endometrial cancer cell lines (Fig. 5.1E). The level of expression of AT1R in these endometrial cancer cell lines was however variable. Ishikawa cells had the highest level of expression (Fig. 5.1E).

As AT1R is expressed in all three endometrial cancer cell lines, Ang II binding to the AT1R might regulate their rate of proliferation and their viability and we proposed that both ARBs (losartan, telmisartan) would prevent the binding of Ang II to AT1R, reduce cell viability and inhibit cell proliferation.

Surprisingly, at high concentrations, losartan significantly increased the viability of all three cancer cell lines (Fig. 5.4A). Koyama et al. also showed that losartan increased the viability of endometrial cancer cells [25]. It was surprising therefore that at 100μM losartan
significantly reduced the rate of proliferation of Ishikawa cells (Fig. 5.4C). Different signaling pathways control cell viability and cell proliferation, therefore it is possible that Ang II/AT$_1$R cell signaling has different effects on these pathways.

Telmisartan, another ARB, significantly reduced the viability of all three endometrial cancer cell lines (Fig. 5.4B) and at higher concentrations, significantly reduced the rates of proliferation in Ishikawa and AN3CA cells (P<0.0001; Fig. 5.4D). Thus telmisartan was much more effective in inhibiting cancer growth than losartan. This may be because telmisartan not only acts as an antagonist at the AT$_1$R but is a partial PPAR-γ agonist. PPAR-γ regulates glucose and insulin metabolism [38]. Koyama has described similar effects of telmisartan in endometrial cancers both in vitro and in vivo [25] and other studies show that telmisartan inhibits proliferation in gastric cancer [39], breast [40] and other cancer cells [30, 31, 41].

To see if the effects of telmisartan were mimicked by a PPAR-γ agonist, we looked at the effects of troglitazone, a full PPAR-γ agonist on cell proliferation and viability. Like telmisartan, troglitazone reduced the viability of both Ishikawa and AN3CA cells but there was no effect on HEC-1A cells (Fig. 5.5A & Fig. 5.5B). However, troglitazone significantly reduced the rate of cell proliferation in all three endometrial cancer cell lines (Fig 5.5A & 5.5B). Thus our findings are similar to those seen in other studies on the effects of troglitazone on cancer cell growth [42-44].

Ishikawa was the most proliferative of the three endometrial cancer cell lines studied. Furthermore, the levels of expression of the RAS genes that drive the interaction of Ang II with its AT1R are greatest in Ishikawa cells. This suggests that susceptibility of endometrial cancers to the anti-cancer effects of aliskiren, and telmisartan will be greatest in those endometrial cancers expressing high levels of REN, ACE or AGTR1. Thus, these genes may be biomarkers for indicating the efficacy of drugs that act on the RAS in the treatment of cancer.
In conclusion, we have demonstrated for the first time that variable levels of mRNA expression of RAS genes; *REN, ACE, ACE2, AGT* and *AGTR1* occur in endometrial epithelial cancers. This is also the first study to examine the effect of renin inhibitors in endometrial cancer cell lines. Compared with other RAS blockers (losartan and perindoprilat), aliskiren was more effective in reducing cell viability. Levels of expression of the RAS genes may be biomarkers for anti-cancer drug efficacy. Telmisartan consistently reduced cell proliferation and cell viability in all three endometrial cancer cell lines. Future studies examining the efficacy of these RAS blockers in a mouse model of endometrial cancer may shed more light on the potential of RAS blockers to inhibit endometrial cancer growth.
5.5 References


20. Goldberg, A I., Dunlay, M C., et al., Safety and tolerability of losartan potassium, an angiotensin II receptor antagonist, compared with hydrochlorothiazide, atenolol, felodipine


Chapter 6 - Combined effect of renin-angiotensin system (RAS) blockers in endometrial cancer cell lines

6.1 Introduction

In Chapter 3, I described how I successfully ‘knocked down’ (P)RR mRNA and protein expression using a specific (P)RR siRNA in three endometrial cancer cell lines. Transfection with this (P)RR siRNA reduced the cell proliferation and cell viability of Ishikawa and AN3CA cells but had no effect on HEC-1A cells.

In Chapter 5, I described experiments in which I studied the effects of drugs that blocked the different components of the renin-angiotensin system (RAS) on the proliferation and viability of three endometrial cancer cell lines. Of all the RAS inhibitors tested, only telmisartan and troglitazone (an ARB and partial PPAR-γ agonist) were effective in inhibiting cell proliferation and viability in thses cell lines. Therefore, I wanted to see if they would be more powerful if used in combination or when used in combination with a (P)RR siRNA. This chapter describes the combined effects of RAS blockers and a (P)RR siRNA on cell proliferation and cell viability in three endometrial cancer cell lines.
6.2 Methods

Most methods used to carry out these experiments are the same as those described in Chapter 2 & 3.

6.2.1 Drugs

One hundred microliters of experimental medium containing various concentrations (0.1, 1, 10 and 100 μM) with combinations of a renin inhibitor (aliskiren; Medchem Express), an ACE inhibitor (Perindoprilat; Sigma Aldrich), one of two ARBs (losartan and telmisartan; Sigma Aldrich) and a PPAR-γ agonist (troglitazone; Sigma Aldrich) were added to cultured cells in 96-well plates and to wells in an xCELLigence plate to measure effects on cell viability or proliferation, respectively. Vehicle controls containing the same amount of DMSO were included in all experiments. Cells were also transfected with either a negative control or ATP6AP2 siRNA (siRNA-2) as described in Chapter 3 and treated with either losartan, telmisartan, troglitazone or perindoprilat (see Chapter 5).

6.2.2 Statistics

All experiments were performed in triplicate and were repeated on three separate occasions. GraphPad Prism (Version 7.0) was used for all statistical analyses and statistical significance was set at P<0.05. One way and two-way ANOVA statistical analyses were used, and multiple comparisons were done using Tukey’s multiple comparison test.

6.3 Results

6.3.1 The combined effects of an ARB (telmisartan) and a PPAR-γ agonist (troglitazone)

To see if there was an additive effect on cell viability of an ARB and a PPAR-γ agonist, we treated the three cell lines with equal volumes and concentrations of telmisartan +
troglitazone (0.1 μM of telmisartan + 0.1 μM troglitazone, 1 μM of telmisartan + 1 μM troglitazone, 10 μM of telmisartan + 10 μM troglitazone or 100 μM of telmisartan + 100 μM troglitazone). Analysis by 2-way ANOVA showed that there was a significant effect of both treatment and dose in Ishikawa and HEC-1A cells (All P<0.001; Fig. 6.1A & 6.1B). In AN3CA cells, only a significant effect of dose was observed (P<0.001; Fig. 6.1C).

Telmisartan on its own significantly reduced the cell viability of the three cell lines (Fig. 6.1). In comparison, troglitazone decreased the cell viability in Ishikawa and AN3CA cells but it had no effect on HEC-1A cell viability (Fig. 6.1). These effects were similar to those described previously (Chapter-5) (Fig. 5.4B, Fig. 5.5A).

Telmisartan was significantly more effective in reducing cell viability in Ishikawa and HEC-1A cells than Troglitazone (both P<0.0001). The combination of telmisartan (ARB) and troglitazone (ARB and PPAR-γ agonist) was significantly more effective at reducing the viability of Ishikawa and HEC-1A cells compared with troglitazone alone (P<0.0001). However, the combined effect of telmisartan + troglitazone was not significantly different from the effect of telmisartan on its own (Fig. 6.1).
Figure 6.1 The effect of an ARB (telmisartan) and a PPAR-γ agonist (troglitazone) alone or in combination on the viability of (A) Ishikawa, (B) HEC-1A, or (C) AN3CA cells. Cells were cultured for 48h after treatment and absorbance was measured by resazurin assay. Results are expressed as mean ± SEM. Three experiments were performed independently in triplicate. Superscripts denote a significance difference to 0 μM control within the same treatment group (denoted by the colour); a: P<0.05; b: P<0.01; d: P<0.0001.
6.3.2 The effects of combined treatment with aliskiren (a renin inhibitor) and perindoprilat (an ACEI) on cell viability

To determine whether treatment with a renin inhibitor (aliskiren) and an ACE inhibitor (perindoprilat) in combination had a greater effect on cancer cell viability than either of these drugs alone, we treated the three cell lines with equal concentrations of aliskiren and perindoprilat in combination. Analysis by 2-way ANOVA showed that there was a significant effect of both treatment and dose in all three cell lines (Fig. 6.1).

In Ishikawa cells, treatment with 100 μM of aliskiren alone significantly reduced cell viability compared with vehicle treated control cells (P<0.0001; Fig. 6.2A). This effect of aliskiren on cell viability in Ishikawa cells is the same as that described in Chapter 5 (Fig. 5.2B). Aliskiren was significantly more effective in reducing cell viability in Ishikawa cells than perindoprilat (P=0.012) or aliskiren + perindoprilat in combination (P=0.0007). Surprisingly, the combination of aliskiren + perindoprilat had no effect on cell viability in Ishikawa cells (Fig. 6.2A) suggesting that perindoprilat abolished the toxic effects of aliskiren.

In contrast to Ishikawa cells, neither aliskiren nor perindoprilat on their own had any effect on the viability of HEC-1A cells however treatment with both aliskiren and perindoprilat, at 10 μM (10 μM of aliskiren + 10 μM perindoprilat) or 100 μM (100 μM of aliskiren + 100 μM perindoprilat) was associated with a significant reduction in the viability of HEC-1A cells compared with vehicle controls (P<0.0009 and P<0.0001; Fig. 6.2B). Therefore, combined treatment of HEC-1A cells with a renin inhibitor and an ACEI had a synergistic interaction in reducing cell viability and was more effective than aliskiren (P=0.0001) or perindoprilat alone (P=0.036).

In AN3CA cells, the combination of both aliskiren and perindoprilat at 100 μM significantly reduced the viability of the cells (P=0.0031; Fig. 6.2C). On its own, perindoprilat at either 1 μM or 10 μM, increased the viability of AN3CA cells compared with controls.
(P=0.0034 and P=0.039, respectively; Fig. 6.2C). Aliskiren alone had no effect on the viability of AN3CA cells. At 1 μM aliskiren alone significantly reduced the viability of AN3CA cells compared to the combination of aliskiren + perindoprilat or perindoprilat alone (P=0.0159 and P=0.0020; Fig. 6.2C).
Figure 6.2. The effect of a renin inhibitor and ACE inhibitor, in combination (aliskiren + perindoprilat) on cell viability in (A) Ishikawa, (B) HEC-1A, and (C) AN3CA cells. Cells were cultured for 48h after treatment and absorbance was measured by resazurin assay. Results are expressed as mean ± SEM. Three experiments were performed independently in triplicate. Superscripts denote a significance difference to the 0 µM control within the same treatment group (denoted by the colour): a: \( P<0.05 \); c: \( P<0.001 \); d: \( P<0.0001 \).
6.3.3 The effects of treatment with a renin inhibitor (aliskiren) and an ARB (losartan) in combination

When Ishikawa cells were treated with aliskiren and losartan, either alone or in combination, there was a significant effect of treatment and an interaction between treatment and dose (Fig. 6.3A), although no effect of dose was observed. In Ishikawa cells, the combination treatment of aliskiren + losartan had no effect on cell viability even though treatment with aliskiren alone at 10 μM or 100 μM significantly reduced the viability of Ishikawa cells compared with control (P<0.0319 and P<0.0001; Fig. 6.3A). Losartan treatment alone also had no effect on the viability of Ishikawa cells. Thus aliskiren was significantly more effective at inhibiting cell viability of ishikawa cells compared with losartan or the combined treatment of aliskiren and losartan (both P<0.0001). Furthermore, it would appear that combining an ARB with a renin inhibitor inhibited the cytotoxic effects of the renin inhibitor.

In HEC-1A cells, there was a significant effect of treatment on cell viability. However, when each treatment was examined alone, neither aliskerin or losartan, alone or in combination significantly altered cell viability compared to the vehicle control (Fig. 6.3B). Despite this, cells treated with aliskiren had significantly increased viability overall compared with cells treated with losartan alone (P=0.018) or aliskiren and losartan combined (P<0.0001).

In AN3CA cells, there was a significant effect of treatment, dose and an interaction between treatment and dose on cell viability (Fig. 6.3B). The combination of aliskiren + losartan did not reduce cell viability, instead there was a significant increase in cell viability at 100 μM (100 μM of aliskiren + 100 μM losartan) compared with control (P<0.0038; Fig. 6.3C). Overall, the combination of aliskiren and losartan significantly increased cell viability compared with losartan alone (P=0.025) but was not significantly different from aliskiren alone. Losartan (100 μM) on its own also significantly increased cell viability compared with
control (P<0.001; Fig. 6.3C). Since aliskiren alone did not have any effects on AN3CA cell viability, the effects of the combined treatment must be due to losartan.

**Figure 6.3.** The effect of a renin inhibitor (aliskiren) and an ARB (losartan) alone and in combination on cell viability in (A) Ishikawa, (B) HEC-1A, and (C) AN3CA cells. Cells were cultured for 48h after treatment and absorbance was measured by resazurin assay. Results are expressed as mean ± SEM. Three experiments were performed independently in triplicate. Superscripts denote significance difference to 0µM control within the same treatment group (denoted by the colour); a: P<0.05; b: P<0.01; c: P<0.001; d: P<0.0001.
6.3.4 The combined effects of (P)RR siRNA and RAS blockers on cell growth and cell viability

For this study, cells were transfected with one dose of the ATP6AP2 siRNA (125nM), and the effects of increasing doses of the RAS blocking drug or telmisartan were studied.

6.3.4.1 The combined effects of an ACEi (perindoprilat) and ATP6AP2 siRNA on cell viability

From Chapter 3 we know that ATP6AP2 siRNA on its own significantly reduced cell viability in two of the three endometrial cancer cell lines. Therefore, we combined treatment with an ATP6AP2 siRNA and perindoprilat (ACE inhibitor) to see if there were any additive effects on cell viability. For the combined treatment, cells were transfected with one dose of the ATP6AP2 siRNA (125nM) and increasing doses of perindoprilat. Treatment with an ATP6AP2 siRNA decreased cell viability in Ishikawa cells relative to the negative siRNA control (P<0.0001, Fig. 6.4A). There was no effect of the ATP6AP2 siRNA in HEC-1A and AN3CA cells (Fig. 6.4B and 6.4C). Apart from the effects of (P)RR knockdown on cell viability, there was no additional effects of perindoprilat. This suggests that there is no synergistic effect on cell viability of ATP6AP2 siRNA + perindoprilat.
Figure 6.4. Combined effect of ATP6AP2 siRNA and ACE inhibitor (ATP6AP2 siRNA + perindoprilat) on cell viability in (A) Ishikawa, (B) HEC-1A, and (C) AN3CA cells. The concentrations used are (0.1μM - 100μM) or the vehicle (control). Cells were cultured for 48h and absorbance was measured by resazurin assay. Results are expressed as mean ± SEM. All experiments were performed independently in triplicate. Superscripts denote significance difference to 0μM control within the same treatment group (denoted by the colour); b: P<0.01. * denotes significant effect of (P)RR siRNA in the absence of perindoprilat compared to the -VE control siRNA.
6.3.4.2 The combined effects of an ARB (losartan) and an ATP6AP2 siRNA on cell viability

Treatment with the ATP6AP2 siRNA decreased cell viability in both Ishikawa and AN3CA cells relative to the negative control siRNA alone (P<0.0001 and P<0.0001). There was no effect of ATP6AP2 siRNA on the viability of HEC-1A cells (Fig. 6.5B). Combined treatment with the negative control siRNA and losartan was associated with an increase in cell viability in all three endometrial cancer cell lines (P<0.05, P<0.01 and P<0.001, Fig. 6.5 A, 6.5 B & 6.5 C). Although combined treatment with ATP6AP2 siRNA and losartan (0.1 μM, 1 μM, 10 μM) had the same effect. Losartan (as shown above) was responsible for the increase in cell viability (Fig. 6.5A & 6.5C).
Figure 6.5. Combined effect of ATP6AP2 siRNA and losartan (ARB) (ATP6AP2 siRNA + losartan) on cell viability in (A) Ishikawa, (B) HEC-1A, and (C) AN3CA cells. Cells were cultured for 48h with doses of the drug and absorbance was measured by resazurin assay. Results are expressed as mean ± SEM. All experiments were performed independently in triplicate. a: P<0.05; b: P<0.01; c: P<0.001; d: P<0.0001. * denotes significant effect of (P)RR siRNA in the absence of losartan compared to the -VE control siRNA.
6.3.4.3 The combined effect of treatment with an ATP6AP2 siRNA and an ARB (telmisartan) on cell viability

In Ishikawa cells, combined treatment with an ATP6AP2 siRNA and telmisartan was associated with a significant reduction in the number of viable cells compared with the ATP6AP2 siRNA alone (P<0.0001; Fig 6.6A). Therefore, there was positive interaction between (P)RR knockdown and treatment with telmisartan in reducing cancer cell viability in Ishikawa cells.

Figure 6.6. Combined effect of 125nM ATP6AP2 siRNA and telmisartan (ATP6AP2 siRNA + telmisartan) on cell viability in (A) Ishikawa, (B) HEC-1A, and (C) AN3CA cells. Cells were cultured for 48h and absorbance was measured by resazurin assay. Superscripts denote significance difference to 0 µM control within the same treatment group (denoted by the colour); c: P<0.001, compared with -VE control + DMSO control. * denotes significant effect of (P)RR siRNA in the absence of telmisartan compared to the -VE control siRNA.
6.3.4.4 Effects of combined treatment with ATP6AP2 siRNA and telmisartan (ARB) on cell proliferation

Both ATP6AP2 siRNA and telmisartan, when used alone, significantly reduced cell proliferation in both Ishikawa and AN3CA cells compared with negative control siRNA + DMSO control (P<0.0001 and P<0.0001, and P<0.0001 and P<0.0001 respectively, Fig 6.7). However, the effect of telmisartan on its own in this experiment was significantly less than that observed previously (see Figure 5.5), suggesting that the negative control siRNA inhibited the suppressive effects of telmisartan on cell proliferation.

ATP6AP2 siRNA was also combined with telmisartan (ARB) to examine whether there was an additive effect on cell proliferation. A concentration of 125 nM for the ATP6AP2 siRNA and 50μM of telmisartan was used. In Ishikawa cells, combined treatment of ATP6AP2 siRNA with telmisartan significantly reduced both cell viability and cell proliferation compared to the negative control siRNA + DMSO control (P<0.0001; Fig. 6.6A and 6.7A). Similarly, when AN3CA cells were treated with both ATP6AP2 siRNA + telmisartan, there was a significant decline in cell growth compared to the negative control siRNA + DMSO control (P<0.0001; Fig. 6.7C), but the effect was lower than ATP6AP2 siRNA treatment alone (Fig. 6.7C). The combination of ATP6AP2 siRNA + telmisartan did not show any effect in HEC-1A cells (Fig. 6.7B).
Figure 6.7. Combined effect of ATP6AP2 siRNA + telmisartan on cell proliferation in (A) Ishikawa, (B) HEC-1A, and (C) AN3CA cells. Cells were cultured for 48h after treatment and the rate of change in cell index (slope) was measured by xCELLigence. Slope is defined as a change in cell index with respect to a particular time range [6]. All experiments were performed independently in triplicate. Results are expressed as mean ± SEM. Different superscripts (i.e. a, b, c) on graphs denote a statistically significant difference between groups.

6.3.4.5 Combined effect of an ATP6AP2 siRNA and troglitazone (PPAR-γ agonist) on cell proliferation in three endometrial cancer cell lines

The ATP6AP2 siRNA reduced cell proliferation in both Ishikawa and AN3CA cells compared with negative control siRNA + DMSO control (P=0.029 and P<0.0001, respectively, Fig 6.8). In contrast, troglitazone was only effective in reducing cell proliferation in AN3CA cells (P=0.0039). The combination of ATP6AP2 siRNA and troglitazone had no effect on cell
proliferation in Ishikawa cells but significantly reduced proliferation in AN3CA cells (P=0.002, Fig 6.8A, 6.8B).

In Figure 5.5B the effect was solely induced by troglitazone, but in Figure 6.8 the negative control siRNA inhibited cell proliferation so no effects of troglitazone were able to be measured.

**Figure 6.8.** Combined effect of ATP6AP2 siRNA + troglitazone (50μM) on cell proliferation in (A) Ishikawa, (B) HEC-1A, and (C) AN3CA cells. Cells were cultured for 48h after treatment and the rate of change in cell index (slope) was measured by xCELLigence. Slope is defined as a change in cell index with respect to a particular time range [6]. All experiments were performed independently in triplicate. Results are expressed as mean ± SEM. Different superscripts (i.e. a, b, c) on graphs denote a statistically significant difference between groups.
6.4 Discussion

Binding of prorenin to the (P)RR might activate the RAS pathway and results in the formation of Ang II which stimulates a number of intracellular signaling pathways [1]. Prorenin binding to the (P)RR can also activate intracellular signaling pathways [2]. Both of these actions of the (P)RR can result in increased angiogenesis and cell proliferation [3]. In Chapter 3 the effects of (P)RR on cell proliferation and cell viability were described and in Chapter 5 I described the effects of drugs that block the RAS or stimulate PPAR-gamma on cell proliferation and cell viability. In this chapter I investigated the effects of combination treatments with RAS blocking drugs and also their actions when combined with knockdown of the (P)RR.

In the first part of study I examined the combined effects of telmisartan and troglitazone on cell viability in three endometrial cancer cell lines. Since the combined effects of telmisartan and troglitazone were similar to the effects of telmisartan on its own (Fig. 6.1), we suggest that telmisartan might be acting as both an ARB and PPAR-γ agonist. Further studies are required to evaluate the potential of telmisartan as an ARB and PPAR-γ agonist; this could be carried out by using GW9662, a PPAR-γ antagonist.

Aliskiren and perindoprilat in combination did not have any synergistic effect in Ishikawa cells, but in HEC-1A cells the combined treatment significantly decreased the viability of cells compared with aliskiren and perindoprilat alone (Fig. 6.2B). HEC-1A cells have higher AGT and ACE2 mRNA abundance compared with Ishikawa and AN3CA cells (Chapter 5, Figure 5.1) which might result in greater Ang-(1-7) production. Ang-(1-7) acts on the Mas receptor and has actions that oppose those of the Ang II/AT1R pathway (i.e. it is anti-angiogenic and anti-apoptotic) [4, 5]. In AN3CA cells the effects of aliskiren and perindoprilat were negligible, likely because this cell line has the least abundance of RAS genes (Chapter 5,
In Ishikawa cells, which express *REN* most abundantly, the efficacy of aliskiren in reducing cell viability was greater than the combination treatment.

Combined treatment of aliskiren + losartan did not have a synergistic effect on cancer cell viability (Fig. 6.3). Losartan has increased the cell viability while aliskiren decreases the cell viability so combining each other cancels each other out. Similarly, combined treatment of *ATP6AP2* siRNA + perindoprilat did not have any synergistic effect on cancer cell viability (Fig. 6.4A, 6.4B & 6.4C). Furthermore, there was no synergistic effect of (P)RR knockdown and ARB blockade on cancer cell viability in any of the three cell lines (Fig. 6.5A, 6.5B & 6.5C).

In contrast the combination of *ATP6AP2* siRNA + telmisartan had an additive anti-cancer effect in reducing cell growth and cell viability in Ishikawa cells (Fig. 6.7A & 6.6A). However, there was no synergistic effect observed in HEC-1A and AN3CA cells.

In Chapter 5 I demonstrated that telmisartan on its own was effective in reducing the cell viability in the three cell lines (Figure 5.4), however the effect of telmisartan was reduced when combined with the negative control siRNA (Fig. 6. 6). In Appendix 3 the impact of the negative control siRNA on the effect of telmisartan was explored and shows that it does indeed reduce the effectiveness of telmisartan in inhibiting cell proliferation (Fig. A3 (A-C)).

Thus (P)RR knockdown in combination with telmisartan was more effective in reducing cell viability and proliferation in Ishikawa cells compared with either treatment alone but not in other cell lines. Additionally, we investigated the combined effects of troglitazone (a PPAR-γ agonist) with *ATP6AP2* siRNA and found that the combined treatment did not have synergistic effect on any of the three cell lines (Fig. 6.8A, 6.8B & 6.8C).

In conclusion, this is the first study to examine the combined effects of *ATP6AP2* siRNA and RAS blockers on endometrial cancer cell viability and proliferation. Out of all the RAS blockers used, telmisartan consistently reduced cell proliferation and cell viability (Chapter 5).
and $ATP6AP2$ siRNA decreased the cell proliferation and cell viability in two out of three endometrial cancer cell lines (see Chapter 3). Aliskiren (a renin inhibitor) reduced cell viability only in Ishikawa cells, which specifically express high levels of $REN$ mRNA (Chapter 5). Here I demonstrate that the combination treatment of $ATP6AP2$ siRNA with telmisartan acts in a synergistic manner to further reduce the cell growth and viability of Ishikawa cells. There was no effect of the ACE inhibitor and no synergistic effect was observed when RAS blockers were combined. Therefore, I propose that the combination of telmisartan and $ATP6AP2$ siRNA might be useful as a novel therapy to treat particular forms of endometrial cancer. Additional studies are required to comprehensively understand the potential of RAS blockers to inhibit endometrial cancer growth.
6.5 References


Chapter 7 - Effect of ovarian steroids (estrogen & progesterone) on the renin-angiotensin system (RAS)

expression in MCF-7 and RL-952 cells

7.1 Introduction

During reproductive life, the endometrium undergoes cyclical morphological changes. The endometrium is shed during each menstrual cycle and is completely revitalized in preparation for blastocyst implantation [1].

The menstrual cycle is divided into two phases, the 1st phase is the proliferative phase when the hormone estrogen is dominant and the 2nd phase is the secretory phase, which is controlled by both estrogen and progesterone [2]. Under the influence of these ovarian steroids (estrogen and progesterone), cyclical changes in the endometrium occur. These include proliferation of stroma and epithelial cells and alterations in the expression of the major renin-angiotensin system (RAS) components [4, 5]. When levels of steroids decline there are further changes in the endometrium, i.e. vasospasm, necrosis and shedding of the superficial layer of endometrium [3,4].

Angiotensin II (Ang II) and Vascular endothelial growth factor (VEGF) can mediate angiogenesis, proliferation and cell growth [6]. Piastowska-Ciesielska et al. showed that the proliferative arm of the RAS pathway, as well as Ang II type 2 receptor (AT₂R), estrogen receptor alpha (ER-α) and VEGF are present in endometrial cancer cells and are increased during the proliferative phase of the menstrual cycle [9]. We have shown that endometrial cancer cell lines express RAS genes (Chapters 3 & 5) [10].
Treatment with ovarian steroids results in increased mRNA and protein abundance of VEGF both in vitro [11, 12] and in vivo [13, 14]. Treatment with estrogens also increases the proliferation of breast and endometrial cancer cells [15-17].

To date, the effect of ovarian steroids on the expression of RAS in breast and endometrial cancer cell lines has not been investigated. We postulated that in a breast cancer cell line (MCF-7) and in an endometrial cancer cell line (RL-952) estrogens would stimulate expression of the genes of the RAS, whereas progesterone would inhibit them. In this chapter, endometrial cancer cell lines ECC-1 and RL-952 were used. These cell lines were not used for further experiments as ECC-1 was found to be a breast cancer cell line (MCF-7) after genotyping and RL-952 cells not express angiotensinogen (AGT).

7.2 Methods

7.2.1 Cell Culture and Treatments

MCF-7 and RL-952 cells were cultured in DMEM (Sigma Aldrich, Missouri, USA) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS, Bovogen Biologicals, Victoria, Australia), and 1% antibiotic-antimycotic (Gibco, Waltham, USA). Both cell lines were incubated in tissue culture flasks at 37°C in a humidified incubator with 5% CO2. Confluent cells were detached by trypsinization (1X trypsin-EDTA, Invitrogen Life Technologies).

Cells were seeded at a density of 200,000 cells per well in a 6-well plate with 2 mL of incubation medium per well. Cells were allowed to settle for 24h, after which the media was changed and replaced with fresh media containing various concentrations (0.1nM - 100nM) of either 17[alpha]-estradiol (E2) or progesterone (medroxy-progesterone acetate, MPA; Sigma Aldrich, Missouri, USA) and incubated for 48h. Wells were washed with ice cold PBS (3 times). Cell pellets were snap frozen and stored at -80°C for subsequent protein and mRNA
analysis. Both cell lines were genotyped, and it was found that what we thought were ECC-1 cells were in fact a breast cancer cell line (MCF-7), and RL-952 cells were confirmed to be an endometrial epithelial cancer cell line.

7.2.2 Real-time reverse transcriptase polymerase chain reaction (qPCR)

qPCR was carried out as discussed in Chapters 2 & 5. The expression of RAS genes: renin (REN), (pro)renin receptor (ATP6AP2), angiotensin converting enzyme (ACE1), angiotensin converting enzyme 2 (ACE2), angiotensin II type 1 receptor (AGTR1), and vascular endothelial growth factor (VEGF) was measured. Furthermore, estrogen and progesterone receptor (ESR1 and PR, respectively) expression were also measured. Primer sequences and their concentrations are described in Table 7.1.

Messenger RNA abundance was calculated relative to β-actin (ACTB) mRNA and compared with a calibrator sample (term human placenta) which was incorporated into each run. Relative abundance was thereby calculated as \(2^{-\Delta\Delta CT}\).
Table 7.1. Primers used in real time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Bank Accession number</th>
<th>Primer Sequence (5'-3')</th>
<th>Conc'n (nM)</th>
<th>Annealing temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>REN</td>
<td>NM_000537</td>
<td>F: CCGACAGACACCACCACCTT</td>
<td>200nM</td>
<td>77°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ATGTCCACACCTCGTCCCTCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP6A2</td>
<td>NM_005765</td>
<td>F: ACAATGAAGTGGACCTGCTTTTCTT</td>
<td>100nM</td>
<td>76°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTTTGGCTAAGATGCTTATGACGAGACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE</td>
<td>NM_000789</td>
<td>F: CAGGTGGTGTTGGAAACGATATGC</td>
<td>200nM</td>
<td>77°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TCTCTGTGGGTGTTGGTGTGTAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE2</td>
<td>NM_021804</td>
<td>F: AAGCAGTCACGATTGTGGGACTCT</td>
<td>200nM</td>
<td>75°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AAGACCATCCACCTCCACTCTCAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGTR1</td>
<td>NM_000685</td>
<td>F: GCCTCCTCGCAATGATTCCA</td>
<td>100nM</td>
<td>82°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CGTTGTTCGTACCTGCCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>M32977</td>
<td>F: CTACCTCCACCATGCAATTCCA</td>
<td>400nM</td>
<td>81°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GCAGTAGTGTCGCTGATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTB</td>
<td>NM_001101</td>
<td>F: CGGCATCGTCACCAACTG</td>
<td>1000nM</td>
<td>77°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AAGGTGTGTTGACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESR1</td>
<td>NM_00125.3</td>
<td>F: TGAAAGGTGGGATACGAAAGAC</td>
<td>200nM</td>
<td>78°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CATCTCTCGGCCTGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>NM_000926.4</td>
<td>F: GTGGGAGCTGTAAGGTTCTTCTTTAA</td>
<td>200nM</td>
<td>77°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AACGATGCAGTCTTTTCTTCCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

7.2.3 Statistics

All experiments were performed in triplicate and were repeated three times. Graph Pad Prism (Version7.0) was used for all statistical analyses and statistical significance was 5%, data are represented as mean ± SEM. “Student’s” t-test, One-way ANOVA statistical analysis and Tukey’s multiple comparison test were used to detect significant effects.
7.3 Results

7.3.1 Expression of RAS genes in RL-952 and ECC-1 cells

Two cell lines (MCF-7 and RL-952 cells) were cultured and examined for RAS mRNA expression. *ATP6AP2* mRNA abundance was significantly higher in RL-952 cells compared to MCF-7 cells (P<0.009; Fig. 7.1A).

MCF-7 cells expressed both *REN* and *ACE* to a greater extent than RL-952 cells (P<0.016 and P<0.0001; Fig. 7.1B, 7.1C). By contrast, *ACE2* and *VEGF* expression was significantly higher in RL-952 cells compared with MCF-7 cells (P<0.0001; Fig. 7.1D & 7.1E).
There was no significant difference in AT1R expression between MCF-7 and RL-952 cells. mRNA expression of ATP6AP2, ACE2 and VEGF was found to be very abundant in RL-952 cells.

**Figure 7.1.** mRNA expression of (A) (pro)renin receptor (ATP6AP2), (B) prorenin (REN), (C) angiotensin converting enzyme (ACE), (D) angiotensin converting enzyme 2 (ACE2), (E) vascular endothelial growth factor (VEGF) and (F) angiotensin II type 1 receptor (AGTR1) in MCF-7 cells and RL-952 cells. Results are expressed as mean ± SEM and were analysed by “student’s” t test. Three experiments were performed independently in triplicate. “mRNA expression is expressed relative to the expression in term placenta”.

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7.3.2 Response to Estrogen (E\textsubscript{2}) in MCF-7 and RL-952 cells

Treatment with estrogen 0.1-100 nM exhibited variable effect on RAS expression in RL-952 and MCF-7 cells (Table 7.2). There was a significant effect of estrogen on ACE2 in RL-952 cells. Furthermore, there was an effect of estrogen on VEGF abundances in both MCF-7 and RL-952 cells and also Tukey’s test shows a significant increase at 100 nM (P<0.0263 and P<0.0001) (Appendix-A4).

**Table 7.2.** Summary of ANOVA, residual values and P values for the effect of estrogen (E\textsubscript{2}) on RAS genes in MCF-7 and RL-952 cell lines.

<table>
<thead>
<tr>
<th>Gene</th>
<th>MCF-7 Cells</th>
<th>RL-952 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP6AP2</td>
<td>F (4, 55) = 0.2669, P=0.8</td>
<td>F (4, 46) = 0.81, P=0.5</td>
</tr>
<tr>
<td>REN</td>
<td>F (4, 46) = 1.511, P=0.2</td>
<td>F (4, 46) = 2.322, P=0.07</td>
</tr>
<tr>
<td>ACE</td>
<td>F (4, 40) = 0.9708, P=0.4</td>
<td>F (4, 45) = 0.2378, P=0.9</td>
</tr>
<tr>
<td>ACE2</td>
<td>F (4, 40) = 1.665, P=0.2</td>
<td>F (4, 45) = 3.266, P=0.019</td>
</tr>
<tr>
<td>VEGF</td>
<td>F (4, 40) = 3.087, P=0.0263</td>
<td>F (4, 86) = 7.271, P=0.0001</td>
</tr>
<tr>
<td>AGTR1</td>
<td>F (4, 37) = 0.9709, P=0.4</td>
<td>F (4, 45) = 0.4897, P=0.7</td>
</tr>
</tbody>
</table>
7.3.3 Effects of medroxyprogesterone acetate on the expression of the RAS and VEGF in MCF-7 and RL-952 cells

Treatment with progesterone (0.1-100 nM) didn’t have any effect on the expression of RAS mRNAs or VEGF in MCF-7 and RL-952 cells (Table 7.3).

Table 7.3 Summary of ANOVA, Residual values and P values for the effect of progesterone (MPA) on RAS genes in MCF-7 and RL-952 cell lines.

<table>
<thead>
<tr>
<th>Gene</th>
<th>MCF-7 Cells</th>
<th>RL-952 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF/Residual Values</td>
<td>ANOVA</td>
</tr>
<tr>
<td>ATP6AP2</td>
<td>F (4, 42) = 0.727</td>
<td>P=0.5</td>
</tr>
<tr>
<td>REN</td>
<td>F (4, 40) = 0.881</td>
<td>P=0.4</td>
</tr>
<tr>
<td>ACE</td>
<td>F (4, 29) = 0.7156</td>
<td>P=0.5</td>
</tr>
<tr>
<td>ACE2</td>
<td>F (4, 30) = 0.578</td>
<td>P=0.6</td>
</tr>
<tr>
<td>VEGF</td>
<td>F (4, 30) = 1.65</td>
<td>P=0.1</td>
</tr>
<tr>
<td>AGTR1</td>
<td>F (4, 29) = 0.9059</td>
<td>P=0.4</td>
</tr>
</tbody>
</table>

7.3.4 Estrogen (ESR1) and progesterone (PR) receptors in RL-952 cells

To make certain that RL-952 cells had both estrogen and progesterone receptors we measured the expression of estrogen (ESR1) and progesterone (PR) receptors in RL-952 cells. There were no detectable levels of PR mRNA in RL-952 cells, but ESR1 mRNA was detected (Fig 7.2).
Figure 7.2 mRNA expression of estrogen (ESR1) and progesterone (PR) receptors in RL-952 cells. Results are expressed as mean ± SEM and were analysed by “student’s” t test. All experiments were performed independently in triplicate. ****: P<0.0001. “mRNA expression is expressed relative to the expression in term placenta”.

7.4 Discussion

In the present study, we have shown that RL-952 cells express a greater abundance of ATP6AP2, ACE2 and VEGF than MCF-7 cells. In MCF-7 cells, REN and ACE are highly expressed (Fig 7.1). When compared with the other three endometrial cancer cell lines used in Chapters 3-6 (i.e. Ishikawa, HEC-1A and AN3CA) REN mRNA expression was higher in MCF-7 and RL-952 cells (see Chapter 5). mRNA expression of ATP6AP2 was more abundant in RL-952 cells than in MCF-7 cells (Fig 7.1A). The expression of ATP6AP2 was less in RL-952 than in Ishikawa and AN3CA cells but higher than in HEC-1A cells (see Chapter 3). Future studies need to be carried out to investigate the effects of estrogen and progesterone on RAS expression in Ishikawa, HEC-1A and AN3CA cells lines.

ACE2 mRNA expression was more abundant in RL-952 cells than in MCF-7 cells (Fig 7.1). The expression of ACE2 was found to be highest in RL-952 cells compared with all other endometrial cancer cell lines (Chapter 5 & Fig 7.1D).

ACE mRNA was more abundant in MCF-7 cells than in RL-952 (Fig. 1), HEC-1A and AN3CA cells, but lower than in Ishikawa cells. Also, mRNA expression of AGTR1 in MCF-7
and RL-952 cells was found to be higher than in HEC-1A and AN3CA cells, but the expression was lower compared to Ishikawa cells (Fig 7.1 & Chapter 5.1E).

The variability of RAS expression in these endometrial cancer cells (RL-952, Ishikawa, HEC-1A and AN3CA) and MCF-7 cells reflects the variability in expression of these genes in endometrial cancer tissue [10] and may also reflect the variability between cancer types (endometrial versus breast).

Treatment with 100nM of E₂ caused upregulation of mRNA expression of VEGF in both RL-952 and MCF-7 cells (Table 7.2). Similar results have been described in endometrial epithelial cancer cells [11, 12]. Estrogen also significantly increased ACE2 mRNA expression in RL-952 cells (Table 7.2). Treatment with progesterone had no effect on either MCF-7 and RL-952 cells (see Table 7.3) which is not surprising as no PR mRNA was undetectable in RL-952 cells (Fig.7.2).

We looked at variety of endometrial cancer cell lines, of which Ishikawa was the fastest growing and had the highest expression of RAS mRNAs, followed by HEC-1A, RL-952 and AN3CA cells. From the above data we can say that estrogen does not have an effect on the expression of the majority of RAS components in both MCF-7 and RL-952 cells. Treatment with estrogen suppressed ACE2 mRNA in RL-952 cells expression but increased VEGF expression in both cell lines. Therefore, we suggest that the ACE2 /MAS-R pathway may suppress angiogenesis when there is an increase in VEGF. So may be estrogens are cancer inducing but involving different pathway.

In conclusion, I have shown that several components of the RAS are variably expressed in both RL-952 and MCF-7 cancer cell lines. VEGF expression was significantly increased by estrogen treatment in both MCF-7 and RL-952 cells, there was a significant effect of estrogen on ACE2 expression in RL-952 cells. Progesterone had no effect in either cell line.
7.5 References


Chapter 8 – Conclusions

8.1 Discussion

The focus of this thesis was to determine if endometrial cancer growth could be inhibited by drugs that block Ang II/AT1R interactions and/or prorenin/(P)RR mediated signaling pathways and also to assess the use of s(P)RR as a potential non-invasive biomarker for the detection of endometrial cancer.

Prorenin binding to the (P)RR can activate the RAS to form Ang II, concurrently the (P)RR can activate intracellular signaling pathways independent of the formation of Ang II [1]. Both of these pathways stimulate angiogenesis and cell proliferation, which may in turn stimulate the growth of cancer cells. There are a number of studies that suggest that RAS blockers (ARBs and ACEis) could be used to reduce cancer growth [2-6]. However, to date, no studies have looked at the role of prorenin/(P)RR interactions in the growth of endometrial cancer.

In Chapter 3, I demonstrated for the first time that there was a higher level of expression of (P)RR mRNA and protein in Ishikawa cells compared with levels found in two other endometrial cancer cell lines (HEC-1A and AN3CA). This is the first study to identify (P)RR mRNA and protein in endometrial cancer cell lines and expands on previous work from our laboratory that showed that the (P)RR was present in endometrial cancer tissue [1]. This was also the first study to determine if the (P)RR in endometrial cancer could be ‘knocked-down’ at both the gene and protein level using a specific ATP6AP2 siRNA. I successfully knocked-down the (P)RR in all three endometrial cancer cell lines. Furthermore, knocking down both (P)RR mRNA and protein was associated with a significant reduction in endometrial cancer cell growth (proliferation and cell viability) in two of the three cell lines. HRP, which is a synthetic peptide that blocks prorenin binding to the (P)RR [7], had no effect
however on cell viability. Therefore, (P)RR knockdown could be a novel therapeutic pathway in the treatment of certain forms of endometrial cancer. Our studies have shown that there are variable levels of expression of (P)RR in endometrial cancers [1]. I predict, therefore, that the efficacy of ‘knock-down’ treatment could depend on the phenotype of the endometrial cancer in terms of its level of expression of (P)RR.

In Chapter 4, I describe studies in which I investigated the potential role of s(P)RR as a biomarker for endometrial cancer. Usually, endometrial cancer is detected when it has begun to cause symptoms (e.g. bleeding), by which time the spread of the tumour is often quite advanced. Since perimenopausal women often experience abnormal patterns of uterine bleeding, women with endometrial cancer have to be identified against this background of other causes of uterine dysfunction. As well, factors such as obesity and long-term use of tamoxifen to treat breast cancer increase the risk of developing endometrial cancer in perimenopausal women. To definitively eliminate this risk, these women, especially those who are on tamoxifen, often have to have repeated uterine curettage. This means that there is a real need to develop a simple and efficient non-invasive diagnostic test.

The (pro)renin receptor is known to be released in a soluble form (s(P)RR) into blood [1, 9] and urine [10]. We postulated that s(P)RR could be released from endometrial cancer cells and that levels in blood and uterine fluid would be higher in women with endometrial cancer than in women without this cancer. Therefore, we measured the levels of s(P)RR in supernatant from the three endometrial cancer cell lines (Ishikawa, HEC-1A and AN3CA), and in blood and uterine lavage fluid, collected from women with different tumor grades and from women without endometrial cancer.

We measured the s(P)RR levels using a specific s(P)RR ELISA and found that endometrial cancer cells secrete s(P)RR and that there is a significant increase in s(P)RR concentration in human plasma samples from women with endometrial cancer. These finding
raise the potential for s(P)RR to be used as a diagnostic test to distinguish between women with and without endometrial cancer. Therefore, I propose that measuring s(P)RR may help to diagnose early detection of cancer and its severity.

Having shown that the (P)RR is a potential therapeutic target for the treatment of endometrial cancer and that its cleavage product, s(P)RR, is a putative biomarker for endometrial cancer, in Chapter 5 and 6 I studied the effects of drugs that block the activity of the RAS on endometrial cancer cell growth and viability. I also looked at the efficacy of a combination treatment with these drugs. For comparison we found we had to study the effects of a PPAR-γ agonist, troglitazone, because one of the drugs that block the Ang II-AT₁R, is also a PPAR-γ agonist. Before we could carry out these experiments, we looked at the expression of RAS components in the three endometrial cancer cell lines used in this thesis.

I found that *REN, ACE, ACE2, AGT* and *AGTR1* were all variably expressed in these endometrial epithelial cancer cell lines. Expression of *AGTR1* was highest in Ishikawa cells. *ACE2* and *AGT* were found to be most abundant in HEC-1A cells, whereas all of these RAS genes had limited expression in AN3CA cells.

I then tested the effects of a renin inhibitor (aliskiren), an ACE inhibitor, perindoprilat, and two AT₁R blocking drugs (ARBs, losartan and telmisartan) on cell proliferation and cell viability in endometrial cancer cell lines. Aliskiren reduced cell viability to some extent. This is the first study to examine the effect of renin inhibitors in endometrial cancer cell lines. The ACE inhibitor had no effect, but telmisartan, an ARB, caused a pronounced reduction in cell proliferation and cell viability in all three cell lines.

As explained above, telmisartan has been shown to be a partial PPAR-γ agonist. It is known from the literature that troglitazone (also a PPAR-γ agonist) [11] has no known effects on Angiotensin receptors) but in my studies, also reduced endometrial cancer cell viability and proliferation. Since telmisartan was effective in reducing endometrial cancer growth in all three
cell lines, this effect may be partly due to its interaction with PPAR-\(\gamma\); this was further explored in Chapter 6.

Suffice to say, since many women are safely treated for hypertension with ARBs, including telmisartan, our findings raise the possibility that in women with hypertension and endometrial cancer, telmisartan could be the drug of choice for maintaining their high blood pressure.

Having investigated the individual effects of RAS blocking drugs on endometrial cancer cell function and finding that only two drugs, aliskiren and telmisartan, had any effect on cell viability/proliferation, we decided to find out if a combination of RAS blocking drugs or the combination of a RAS blocking drug and (P)RR knockdown with a specific siRNA would be more effective than use of a single drug in blocking cell proliferation and viability. Only a limited number of drugs showed increased efficacy when used in combination. What was surprising to us was that our prediction that combination therapy with telmisartan and troglitazone would be more effective in reducing endometrial cancer cell viability than treatment with telmisartan was not the case. Combined treatment with telmisartan and troglitazone had a similar effect to that of telmisartan on its own.

Therefore, this data suggests that telmisartan acts both by blocking the AT\(_1\)R and as a PPAR-\(\gamma\) agonist to reduce the growth of endometrial cancer. Further studies are required to validate the hypothesis that telmisartan exerts its anti-cancer effects by blocking both AT\(_1\)R and PPAR-\(\gamma\) receptors.

This finding further strengthens the proposition that this drug could be promoted not only as an effective anti-hypertensive but also as an effective adjunct treatment for endometrial cancer.

Combined treatment with aliskiren + perindoprilat had no effect on the viability of Ishikawa and AN3CA cells but it did reduce the viability of HEC-1A cells. As HEC-1A cells
express more AGT (Chapter 5, Fig. 5.1B), increased amounts of Ang I might be converted to Ang-(1-7). Ang-(1-7) acting on Mas receptor has actions that oppose those of the AngII/AT1R pathway (i.e. antiangiogenic and antiapoptotic) [12, 13], thus this combination of anti-RAS drugs is more efficacious in this cell line.

The combination of (P)RR siRNA and telmisartan caused a significant reduction in cell viability and cell growth only in Ishikawa cells. Since Ishikawa cells express more (P)RR and AT1R than HEC-1A and AN3CA cells, this might explain the greater efficacy of these drugs than when given alone.

In Chapter 7 we looked at two other cancer cell lines (an endometrial cancer cell line (RL-952) and a breast cancer cell line (MCF-7) to see if either of the major ovarian steroids (estrogen and progesterone) altered expression of components of their RAS pathway. Estrogen treatment increased the expression of VEGF and ACE2 in RL-952 cells and ACE2 expression in MCF-7 cells. Progesterone had no effect on RL-952 RAS gene expression because this cell line was later found to not express progesterone receptors. We concluded that estrogen treatment does not up regulate the expression of RAS genes that lead to activation of the Ang II-AT1R in these two cell lines. The downstream effects of oestrogens on ACE2 expression were not investigated. The ability of estrogen treatment to enhance the expression of VEGF may well contribute to its carcinogenic potential as tumour induced neovascularisation is critical for tumour growth and spread.

8.2 Limitations and future directions

In Chapter 3, I demonstrated that knockdown of (P)RR mRNA and protein level by a specific (P)RR siRNA occurred in all three cell lines studied but I did not investigate further the mechanism via which (P)RR knock down affected cell growth and viability. Further studies are therefore required to identify these downstream targets. This can be carried out by
immunoblotting in order to evaluate whether the downstream targets are affected by (P)RR knockdown.

We also need to know the stage of cell cycle affected by the (P)RR siRNA. This can be studied by using flow cytometry. Further studies using a mouse model will give us a better insight into the feasibility of using (P)RR knock down as a therapy for endometrial cancer.

As stated in Chapter 4, low sample numbers and contamination of uterine lavage samples by blood is a major limitation of this study. More samples are required to validate the potential of s(P)RR plasma and uterine fluid levels, as potential biomarkers and a large clinical cohort needs to be investigated to determine the prognostic potential of plasma and uterine (P)RR levels as biomarkers. This would require recruitment of women are at a risk of developing uterine cancer (e.g. women on long term tamoxifen therapy).

Telmisartan significantly reduced the rate of proliferation and the viability of endometrial cancer cells. To confirm that the effect of telmisartan is also through PPAR-γ, GW9662 (PPAR-γ antagonist) can be tested with telmisartan.

We haven’t measured the downstream effects of telmisartan. Further studies are also required to see if telmisartan would be an effective adjunct therapy when used in conjunction with currently used anti-cancer drugs (e.g. Doxorubicin, Carboplatin etc). Gene expression can be assessed by qPCR and western blot can be used to measure protein expression.

We showed that telmisartan + (P)RR siRNA had an additive effect in inhibiting endometrial cell proliferation and viability. However, we have not determined the effects of this combined treatment on downstream pathways to identify how these treatments are exerting their effects. Proteomics using mass spectrometry may be a useful technology to determine these effects.

Future studies focusing on uncovering the signalling pathways via which the (P)RR is working through to elicit its effects may add to the current knowledge and identify additional
therapeutic targets. Prorenin binding to the (P)RR can activate the classical RAS pathway to form Ang II. Furthermore, Ang II acting via the AT1R receptor can activate signaling pathways to activate mitogen activated protein kinase p38, extracellular signal-regulated kinase and down targets the heat shock protein and tumour growth factor-β (TGF-β). Simultaneously, when prorenin binds to the (P)RR, it can also activate other signaling pathways independent of the formation of Ang II. Prorenin/(P)RR interaction causes translocation of promyelocytic leukaemia zinc finger protein (PLZF) to the nucleus, where it activates p85α subunit of phosphatidylinositol-3 kinase (PI3K-p85α) leading to proliferation. (P)RR can also activate the Wnt/β-catenin signaling pathway, as Wnt ligands bind to frizzled (FZD)/low-density lipoprotein receptor related protein complex (LRP6), which is internalized by V-ATPase. In this way, β-catenin is stabilized, and accumulation of β-catenin leads to cell proliferation. It is highly likely that these pathways are activated by (P)RR in endometrial cancer.

Finally, we have not looked at estrogen and progesterone receptor expression in MCF-7 cells. We would like to see if there are any receptors in MCF-7 cells to determine the effects of steroid treatment. The main limitation of this study is that we have used only one endometrial and one breast cancer cell line. Studies on more endometrial cancer cell lines would give us a better understanding of the effects of steroids on RAS expression.

8.3 Conclusion

In summary, these are the first studies to describe a functional role for prorenin and (P)RR in endometrial cancer, and to demonstrate that some drugs that block the (P)RR and the RAS inhibit cancer growth. I have also shown that s(P)RR has the potential to be used as a biomarker for endometrial cancer.
8.4 References


Appendix

A1. Effect of ATP6AP2 and negative control siRNA (5nM, 50nM and 125nM) on ATP6AP2 mRNA abundance in Ishikawa cells

Transfection with ATP6AP2 siRNA at one of three different concentrations (5nM, 50nM and 125nM) resulted in ~90% knockdown of ATP6AP2 mRNA expression in Ishikawa cells compared to the negative control siRNA treated cells (P<0.0001, P<0.0001 and P<0.0001; Fig. A1). There was no significant difference in ATP6AP2 mRNA expression between the 3 different concentrations of ATP6AP2 siRNA or negative control siRNA used (P= 0.9996, P= 0.9996, P= 0.9999, P= 0.0662, P= 0.5166, P= 0.9907 respectively).

Ishikawa

![Graph showing ATP6AP2 mRNA abundance in Ishikawa cells after incubation with different concentrations of ATP6AP2 siRNAs, negative control siRNA, and vehicle.](image)

Figure A1. ATP6AP2 mRNA abundance in Ishikawa cells after incubation with one of 3 concentrations (5nM, 50nM and 125nM) of ATP6AP2 siRNAs, negative control siRNA (-VE Control) or vehicle (non-transfected). Results are expressed as mean ± SEM. All experiments were performed in triplicate on three different occasions. d: P<0.0001; d: P<0.0001; d: P<0.0001; for comparison versus –VE control siRNA.
A2. Effect of ATP6AP2 siRNA on ATP6AP2 mRNA abundance in endometrial cancer cell lines at 72h and 96h post transfection

Transfection with three ATP6AP2 siRNAs (siRNA-1, 2 & 3) resulted ~90% knockdown of ATP6AP2 mRNA abundance in both HEC-1A and AN3CA cells after 72h and 96h (P<0.0001, P<0.0001, and P<0.0004, P<0.0001, respectively, Fig. A2 (B) & (C)).

In Ishikawa cells transfection with any one of the three ATP6AP2 siRNA resulted in ~70% knock down of ATP6AP2 mRNA abundance at 72h (P<0.0001; Fig. A2 (A)). After 92h the siRNA transfection also appeared to decrease ATP6AP2 mRNA expression, however there was a large variance in the controls and this did not reach statistical significance (Fig. A2 (A)).

**Figure A2.** ATP6AP2 mRNA abundance in (A) Ishikawa, (B) HEC-1A, and (C) AN3CA cells after 72 and 96h incubation with one of three ATP6AP2 siRNAs, negative control siRNA (-VE Control) or vehicle (non-transfected). Results are expressed as mean ± SEM. All experiments were performed in triplicate on three different occasions. ****: P<0.0001; ****: P<0.0001; ****: P<0.0001; ****: P<0.0001; ****: P<0.0004; for comparison versus –VE control siRNA.
A3. Effect of telmisartan in the presence or absence of the negative control siRNA on cell viability in three endometrial cancer cell lines.

Treatment with telmisartan significantly reduced the cell viability in three endometrial cancer cell lines when compared to the vehicle control (P<0.0001, P<0.0001 and P<0.0001; Fig. A3(A-C)). In Ishikawa cells, this effect was first observed with 1μM, in HEC-1A and AN3CA cells it was observed at 100μM (P<0.0001, P<0.0001 and P<0.0001; Fig. A3 (A-C)). When telmisartan was combined with the negative control siRNA, the individual effect of telmisartan was diminished (Fig. A3 (A-C)).
Figure A3. Combined effect of negative control + telmisartan on cell viability in (A) Ishikawa, (B) HEC-1A, and (C) AN3CA cells. Cells were cultured for 48h and absorbance was measured by resazurin assay. Superscripts denote significant difference between telmisartan treatment compared to negative control+ telmisartan; a: P<0.0310, P<0.0197; b: P<0.0088, P<0.0027; c: P<0.0003; d: P<0.0001, P<0.0001, P<0.0001.
A4. Dose response curve for effect of estrogen on MCF-7 and RL-952 cells

Treatment with estrogen at 100 nM significantly increased VEGF mRNA expression in both MCF-7 and RL-952 cells (P<0.0263 and P<0.0001; Fig. A4 (A) & (B)).

**Figure A4.** mRNA expression of VEGF in (A) MCF-7 and (B) RL-952 cells in response to estrogen (0.1-100 nM). Results are expressed as mean ± SEM. All experiments were performed independently in triplicate *: P<0.0263; ****: P<0.0001; for comparison versus control.