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UNIVERSITY OF TECHNOLOGY SYDNEY

THE EFFECTS AND MECHANISMS OF PAEONIFLORIN ON MURINE OVARIAN CELLS FOR THE TREATMENT OF POLYCYSTIC OVARIAN SYNDROME

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Certificate of Original Authorship

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I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This research is supported by an Australian Government Research Training Program Scholarship.

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Abstract

Polycystic Ovarian Syndrome (PCOS) is a complex disorder associated with various reproductive, metabolic and cardiovascular abnormalities and is present in approximately 15% of women of reproductive age. The hallmarks include androgen excess, ovulatory dysfunction and insulin resistance which is believed to play a role in the pathogenesis of the disorder. Although the exact mechanisms of PCOS are unknown, intrinsic dysfunction of ovarian theca and granulosa cells are also thought to contribute to altered steroid production and follicle development which may explain the clinical features of the syndrome.

Metformin, an insulin sensitising agent may improve both metabolic and reproductive aspects of the disorder, however, the development of new therapeutic agents for PCOS is still required. Women with PCOS are inclined to seek complementary and alternative treatment options such as Chinese Herbal Medicine, warranting further investigation into the efficacy of the herbs commonly used. Paeoniflorin, the major compound of the herb, Radix Paeoniae Albus has demonstrated the ability to ameliorate insulin resistance in animal models, however the effects and mechanisms of paeoniflorin for the treatment of PCOS has yet to be elucidated.

This study therefore used a dexamethasone-induced *in vitro* model of PCOS in murine theca and granulosa cells to determine the effects of paeoniflorin on secretion of key hormones testosterone, progesterone and oestradiol, cell

proliferation as well as the molecular mechanisms in which paeoniflorin may regulate steroid production. Dexamethasone (10 μM) increased theca cell androgen production and adversely affected oestradiol: progesterone ratios in granulosa cells. Meanwhile, paeoniflorin (100 μg/mL) decreased androgen production in dexamethasone-induced theca cells and maintained normal oestradiol: progesterone ratios in granulosa cells. In theca cells, this was shown to be through downregulation of cholesterol side-chain cleavage enzyme and 17,20-lyase protein expression. Paeoniflorin also increased mRNA gene expression of *CYP11A1* which may indicate influence over transcription factors or post-translation modifiers, particularly in relation to cell differentiation.

Together, these results suggest that firstly, dexamethasone can be considered a useful *in vitro* model of PCOS in murine ovarian cells. Secondly, paeoniflorin may be a novel agent for the treatment of PCOS by ameliorating hyperandrogenism and improving ovarian function. Further research into the effect of paeoniflorin in differentiation of theca cells as well as the molecular mechanisms in which paeoniflorin attenuates hormones in granulosa cells is needed. Finally, this research can potentially support future animal or clinical studies to further improve the treatment options and quality of life for women with PCOS.

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Publications

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Abbreviations

AMH	Anti-mullerian hormone
AR	Androgen receptor
BPA	Bisphenol A
BSA	Bovine serum albumin
CC	Clomiphene citrate
CHM	Chinese herbal medicine
CM	Complete medium
COC	Combined oral contraceptive pill
CRP	C-reactive protein
CVD	Cardiovascular disease
CYP11	Cytochrome p450scc
CYP17	Cytochrome P45017
DEX	Dexamethasone
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
E2	Oestradiol
ECL	Enhanced chemiluminescent
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
G	Gravity
GDF-9	Growth differential factor 9
GLUT4	Glucose transporter 4
GnRH	Gonadotropin-releasing hormone
HPLC	High performance liquid chromatography
IL-6	Interleukin 6
IR	Insulin resistance
IRS-1	Insulin receptor substrate 1

IVF	In vitro fertilisation
LH	Luteinising hormone
LHR	Luteinising hormone receptor
MAPK	Mitogen-activated protein kinases
NAFLD	Non-alcoholic fatty liver disease
OCP	Oral contraceptive pill
P	Progesterone
PCOM	Polycystic ovarian morphology
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PFE	Paeoniflorin extract
PI3K	Phosphoinositide 3 kinase
PR	Progeterone receptor
qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RT	Reverse transcriptase
SHBG	Sex hormone binding globulin
T	Testosterone
T2DM	Type 2 diabetes mellitus
TNFα	Tumour necrosis factor alpha

Contents

CHAPTER 1

Introduction

CHAPTER 1: INTRODUCTION

1.1 Polycystic Ovarian Syndrome

1.1.1 Definition, Diagnosis and Clinical Features

Polycystic Ovarian Syndrome (PCOS) is a complex disorder associated with various reproductive, metabolic and cardiovascular abnormalities (Balen 2017) and is present in up to 15% of women of reproductive age (Papalou et al. 2017). According to the Rotterdam criteria, the overarching diagnostic features include ovulatory dysfunction, hyperandrogenism and polycystic ovarian morphology (PCOM) with any two of these three features satisfying a diagnosis of PCOS (Figure 1.1). PCOS is therefore, largely heterogeneous with multiple phenotypes that differ in signs, symptoms and their severity. Importantly, PCOS is a systemic disorder that will affect a woman throughout her life, even beyond the reproductive years (Delitala et al. 2017).

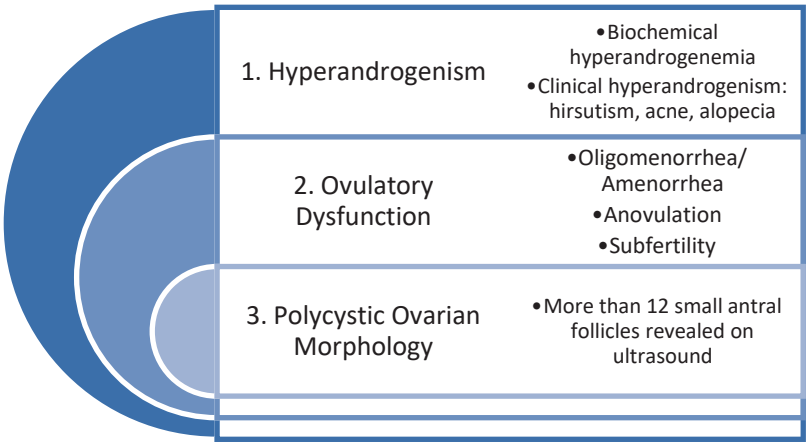

Figure 1.1. Diagnostic Criteria of PCOS according to the Rotterdam Criteria. Two of the three of the criteria including hyperandrogenism, ovulatory dysfunction and polycystic ovarian morphology is diagnostic of PCOS.

Hyperandrogenism

Hyperandrogenism is frequently considered to be the key diagnostic hallmark of PCOS and was pertinent to early descriptions of the disorder in 1935 by Stein and Leventhal. It is

characterised by either increased serum androgens (hyperandrogenemia) or relevant clinical manifestations relating to androgen function and occurs in approximately 60-80% of the PCOS population (Franks 2006). Independently, hyperandrogenism has demonstrated a link to irregular menstrual cycles, obesity and infertility in early studies of the syndrome (Smith et al. 1979, Wild et al. 1983). More recently a positive correlation with hyperandrogenism and insulin resistance (IR) has been identified (Barber et al. 2015).

In the context of PCOS, the term, *androgen* refers to the family of hormones; dihydrotestosterone (DHT), testosterone (T), androstenedione and dehydroepiandrosterone (DHEA). T is the most frequently used biomarker of hyperandrogenemia in PCOS (Münzker et al. 2015) and can be identified in various forms including elevated free testosterone (unbound to protein), bioavailable testosterone or total testosterone. Ovarian derived androgens, androstenedione and testosterone account for approximately half of total androgen production in women, with androgens also derived from adrenal glands (DHEA) and to a lesser extent adipose tissue (Wang et al. 2012, Münzker et al. 2015). Metabolites of T, such as DHT, may also be elevated in PCOS. Recent research is also showing that the different types of hyperandrogenemia may influence the phenotype of PCOS especially when comparing adrenal and ovarian hyperandrogenism (Lerchbaum et al. 2012), however further research in this area is required.

Sex Hormone Binding Globulin (SHBG), a glycoprotein synthesised in the liver can also be used in the assessment of hyperandrogenemia as it regulates bioavailability of androgens by binding to them. SHBG is frequently low in PCOS women and therefore can magnify hyperandrogenemia (Fan et al. 2013). Hyperinsulinemia is also known to suppress SHBG production (Jayagopal et al. 2003) linking PCOS to IR. SHBG is therefore becoming more

prevalent as a diagnostic marker for hyperandrogenemia in PCOS as well a surrogate marker of IR in these women.

Medication use such as the oral contraceptive pill (OCP) may also influence the way hyperandrogenemia manifests and therefore a whole panel of serum androgens and the different forms of testosterone is often encouraged when screening for hyperandrogenemia in PCOS (Karakas 2017).The preferred method of quantification of these androgens is by liquid chromatography tandem mass spectrometry (LCMS/MS) as they are now considered more sensitive than immunoassays (Keevil 2014).

Clinical features related to hyperandrogenism primarily include hirsutism which is the excessive growth of androgen dependent hair and occurs in 70% of women with PCOS (Zhao et al. 2013). However, evidence of hyperandrogenism can also be seen in acne and male pattern baldness (alopecia).These manifestations may be related to increased activity of the enzyme 5-α reductase in sebaceous glands and hair follicles which converts T to DHT (Metwally 2012). These clinical findings in women with PCOS may relate, at least in part, to the increased prevalence of depressive and anxiety disorders due to lowered self-esteem (Cooney et al. 2017).

Ovulatory Dysfunction

Ovulatory dysfunction is the most common diagnostic feature seen in PCOS women and is present in over 95% of these women (Barthelmess et al. 2014). This is defined as oligomenorrhea (irregular menstrual cycles less than 9 menses per year), amenorrhea (absence of menstrual cycle for at least three months) or anovulation. A consequence of ovulatory dysfunction in PCOS, infertility, is considered to be the most common cause of

anovulatory infertility with 40% of women with PCOS affected (Legro et al. 2007, Sirmans et al. 2014).

Polycystic Ovarian Morphology

PCOM is identified by ultrasound where there is a presence of more than 12 small, antral follicles or an increase in ovarian volume more than 10 mL (Goodman et al. 2015). However, in more recent and therefore, more sensitive ultrasound technology, the presence of more than 25 cysts is considered to be diagnostic of PCOS (Goodman et al. 2015). Regardless of the technology, the morphology of multiple small antral follicles is suggestive of follicular arrest in PCOS and may coincide with ovulatory dysfunction (Franks et al. 2000). Despite the name of the disorder, PCOM is not an essential diagnostic criterion of PCOS as it has since been argued that multiple cysts on ultrasound is not exclusive to the syndrome (Murphy et al. 2006).

Elevated serum anti-Mullerian hormone (AMH) has also been suggested as an emerging surrogate marker of PCOS with studies showing AMH more than 5 ng/mL may be a predictor of PCOS (Karakas 2017). More specifically, it may be used as a reflection of follicle number in the ovary, with AMH correlating with antral follicle count identified by ultrasound (Dewailly et al. 2014). AMH is also shown to correlate with having all three other diagnostic features of PCOS and therefore a more severe phenotype (Koninger et al. 2014).

Other Clinical Features

Changes to hypothalamic and pituitary hormones such as gonadotropin-releasing hormone (GnRH), luteinising hormone (LH) and follicle stimulating hormone (FSH) that help regulate

the menstrual cycle are frequently observed in women with PCOS. In particular, increased LH or LH: FSH ratios may be correlated with hyperandrogenism and follicular arrest in PCOS (Moore et al. 2017) as LH stimulates androgen production in the ovaries and helps to regulate oestrogen and progesterone secretion.

Common clinical features also extend to metabolic disturbances such as IR, dyslipidaemia, impaired glucose tolerance, non-alcoholic fatty liver disease (NAFLD), central adiposity or obesity (Brzozowska et al. 2009, Flannery et al. 2013, El Hayek et al. 2016). IR, defined as impaired response to insulin in tissues (Copps et al. 2012) is observed in approximately 60-80% of women with PCOS (Barthelmess et al. 2014). This not only links PCOS to metabolic syndrome but also implicates IR in the pathogenesis of the disorder. Interestingly, IR is highly correlated with hyperandrogenic phenotypes of PCOS (Barber et al. 2015). Insulin resistance in PCOS also increases the risk of other cardiometabolic disorders such as type 2 diabetes mellitus (T2DM) and hypertension in later life (Ranasinha et al. 2015).

Various other disorders that affect the reproductive hormones and ovarian volume may also need to be excluded from the diagnosis of PCOS. Such disorders include Cushing's, disease, hypothyroidism and congenital adrenal hyperplasia (Brzana et al. 2014, Singla et al. 2015, Williams et al. 2016).

1.1.2 Epidemiology and Aetiology

PCOS is the most common endocrinopathy among women of childbearing age, affecting approximately 5-15% of these women (Parker 2015). However, estimates are convoluted by differences in diagnostic criteria and subsequent heterogeneity in the presentation of the syndrome (Hsu et al. 2007). When PCOS was first described in 1935 by Stein and Leventhal,

the development of multiple cysts was thought to be the primary defect in the disorder. Subsequently, as ultrasound technology improved, the presence of ovarian cysts became the most important feature for diagnosis. However, since then revisions made by the National Institute of Health (NIH) in 1990, ultrasound was no longer mandatory for diagnosis with evidence of hyperandrogenism and ovulatory dysfunction suffice. This was later replaced by the Rotterdam Criteria in 2003 in which four distinct phenotypes were identified: 1) hyperandrogenism and ovulatory dysfunction; 2) ovulatory dysfunction and PCOM; 3) hyperandrogenism and PCOM; and 4) all three of the criteria. Hyperandrogenism is, however, compulsory for the diagnosis of PCOS according to the Androgen-Excess Society (AES). In one study, the prevalence of PCOS was 6.1%, 19.9% and 15.3% according to the NIH, Rotterdam and AES criteria respectively (Yildiz et al. 2012) demonstrating the disparity among the standards used.

Additionally, ethnicity may influence the prevalence and phenotype of PCOS. Community based studies have also reported a prevalence as high as 15% in the Aboriginal population in Australia when diagnosed according to the NIH criteria (March et al. 2010). IR and other metabolic derangements are also more prominent in non-Caucasian groups, in particular South Asians (Wijeyaratne et al. 2013). Meanwhile, hirsutism is a less common presentation in Japanese and Chinese women compared to a Caucasian population, however they may have a higher prevalence of PCOM (Huang et al. 2016). Meanwhile, African-American and Hispanic women may be more prone to obesity (Williamson et al. 2001).

Genetic origins of PCOS have therefore been explored with the syndrome found to be more prevalent when a first-degree relative is also affected by hyperandrogenism, PCOS or metabolic syndrome (Deligeoroglou et al. 2009). In one study, 35% of mothers and 40% of

sisters of women with PCOS also shared the diagnosis (Kahsar-Miller et al. 2001). Paternal history of diabetes mellitus and hypertension has also been observed to be higher in women with PCOS compared to women without PCOS (Cheng et al. 2015). In another study, monozygotic twins were twice as likely to have both twins affected compared to dizygotic twins (Vink et al. 2006).

Currently, no specific gene has been successfully identified for PCOS in genomic wide variation studies (GWAS), which is probably reflective of the heterogeneity and complexity of the syndrome. However, approximately 11 susceptibility loci have been identified with many of these candidates linked to obesity, metabolic syndrome and the steroidogenic pathway including insulin receptor (*INSR*), fat mass–and obesity-associated gene (*FTO*), follicle stimulating hormone receptor (*FSHR*) and luteinising hormone receptor gene (*LHR*) (Chen et al. 2011, Jones et al. 2016).

Although genetics may play a part, epigenetic and the *in utero* environment may be more relevant in the aetiology of PCOS as identified in numerous animal models. As such, many of these studies have examined the impact of exposure to excessive androgens on the developing foetus which may program PCOS in adult life (Steckler et al. 2005, Franks 2012). For example, pre-natal androgen exposure in a primate model was shown to imitate hyperandrogenism and ovulatory dysfunction in later life (Abbott et al. 2008). *In utero* androgen exposure may also result in reduced or no corpus luteum formation, PCOM and thecal cell hyperplasia in sheep and rodents (Veiga-Lopez et al. 2008, Caldwell et al. 2014). However, these models failed to evaluate relevant parameters or presented contradictory evidence as to whether pre-natal androgen exposure can emulate the metabolic hallmarks of PCOS.

Post-natal environmental factors may also epigenetically influence PCOS and the various phenotypes (Diamanti-Kandarakis et al. 2012). One proposed factor is the influence of diet with an excess of simple-sugars or carbohydrates. For example, chronic and excessive sugar intake has been implicated in the development of metabolic syndrome (Yki-Jarvinen 2014, Lustig 2016). However, studies relating to PCOS specifically are limited and have failed to pinpoint the exact macronutrients that may contribute to the disorder (Merkin et al. 2016). Despite this, various diets including a high-protein/low carbohydrate diet may improve reproductive and metabolic outcomes in PCOS (Moran et al. 2013) as obesity is shown to increase the severity of hyperandrogenemia, hirsutism and IR (Lim et al. 2013), contributing to the PCOS phenotype.

Many studies have identified similar profiles of inflammatory markers in PCOS, metabolic syndrome and T2DM which are also known to influence insulin resistance (González 2012). Chronic low-grade inflammation and endothelial dysfunction may therefore contribute to the development of PCOS. In *in vitro* studies, pro-inflammatory cytokines such as tumour necrosis-factor alpha (TNFα) and interleukin-6 (IL-6) are shown to adversely affect ovarian cell proliferation and increase androgen production (Roby et al. 1990, Path et al. 1997). In clinical studies, women with PCOS had significantly higher levels of plasma inflammatory markers such as C-reactive protein (CRP) and TNFα compared to women without PCOS (Nehir Aytan et al. 2016). Additionally, anti-inflammatory cytokines, interleukin-27, interleukin-35 and interleukin-37 were all decreased in these women with PCOS.

A pro-inflammatory state can also be explained by increased abdominal adiposity or adverse adipose tissue function (Spritzer et al. 2015) with the adipokine, leptin, being consistently found to higher in women with PCOS compared to healthy controls (Lecke et al. 2011,

Radwan et al. 2017). These findings may suggest leptin resistance and therefore disordered macronutrient metabolism in PCOS. Meanwhile, gherlin levels were found to be lower in obese, hirsute women with PCOS compared to weight matched controls without PCOS (Glintborg et al. 2006).

While genetics are understood to play a role in inflammation, it has also been suggested that diet may contribute to inflammation in PCOS with one study demonstrating that the ingestion of glucose activates oxidative stress and the release of TNF-α, IL-6 and CRP (González et al. 2014, González et al. 2014). Chronic, low-grade inflammation is now known to have deleterious effects systemically and is implicated in endothelial dysfunction in cardiovascular disease. Therefore, inflammation could also explain why PCOS women are at higher risk of dyslipidaemia and cardiovascular disease. Homocysteine, a now recognised risk factor for cardiovascular disease, is frequently elevated in women with PCOS compared to controls (Maleedhu et al. 2014).

The composition and diversity of microorganisms in the gut microbiome has now been established as crucial for multiple aspects of health (Clemente et al. 2012). Dysbiosis in the gut-microbiome has also been linked to both metabolism and inflammation and is currently an emerging area of research for PCOS. Notably, the gut microbiome has demonstrated the capacity to influence oestrogen levels (Baker et al. 2017) and has also been linked to the development of metabolic syndrome (Mazidi et al. 2016). One particular bacterial species identified in the human microbiome, *Clostridium scindens*, demonstrated the ability to increase the testosterone production (Ridlon et al. 2013). In letrozole-treated mice, the reproductive and metabolic defects of PCOS were observed when the abundance and variability of species of the microbiome were decreased (Kelley et al. 2016). Therefore,

altered make-up of the gut microbiome may help explain why IR and inflammation frequently occur in women with PCOS.

Endocrine disruptors that modulate hormone production and metabolism may shape the phenotype of PCOS. Bisphenol A (BPA) used in plastic is known to have hormone-like properties in the body and therefore is one of the most widely studied endocrine disruptors. In one study, BPA was found to be correlated with hyperandrogenism and significantly higher in serum of women with PCOS compared to a control population (Kandaraki et al. 2011) suggesting a pathophysiological involvement in the syndrome. BPA exposure *in vitro* was shown to increase androgen production in rat theca-interstitial cells (Zhou et al. 2008). This relationship is thought to be bi-directional as it is also proposed that androgens influence hepatic clearance of BPA (Takeuchi et al. 2006).

Neuroendorcinological factors have also been suggested in the aetiology of PCOS. Women with PCOS are frequently observed to have increased levels of LH or LH/FSH ratios (Roland et al. 2014). This suggests disruption to the hypothalamus-pituitary axis LH increases ovarian steroidogenesis. Logically, the effects of insulin on cells from these tissues have been explored *in vitro* demonstrating that it enhances the release of LH and FSH (Adashi et al. 1981) to promote ovarian androgen production.

1.1.3 Pathogenesis

While the precise pathogenesis of PCOS is still unclear, it is agreed that the disorder arises from a culmination of genetic and environmental factors that adversely affect multiple body systems, across the hypothalamus-pituitary-ovarian axis. Varying degrees of impaired ovarian hormone production (steroidogenesis), disrupted insulin signalling and chronic low-

grade inflammation (González 2012) may combine and contribute to the development of PCOS.

Early understandings of PCOS were centred upon the idea that the microenvironment of ovaries in women with PCOS were functionally different (Rosenfield et al. 2016) leading to hyperandrogenism. While this is still a relevant theory, insulin resistance has more recently added a layer of meaning to this understanding. However, at this stage it is unclear whether insulin is a causative or aggravating factor.

Considering ovulatory dysfunction and hyperandrogenism are major clinical features of the disease, understanding the pathogenesis of PCOS has been mainly centred on understanding folliculogenesis (the process of follicle maturation) and steroidogenesis (steroid hormone production). These processes are highly regulated by the cells that comprise ovarian follicles, the theca and granulosa cells.

Folliculogenesis

At the time of puberty, women have approximately 300,000-400,000 immature, primordial follicles (Coccia et al. 2008). In the first half of every menstrual cycle, known as the follicular stage, several of these follicles mature with the help of granulosa cells which encase the egg (ovum) and secrete reproductive hormones (mainly oestrogen) in response to follicle stimulating hormone (FSH). This facilitates follicle maturation from primordial follicles to primary and secondary follicles respectively (Figure 1.2).

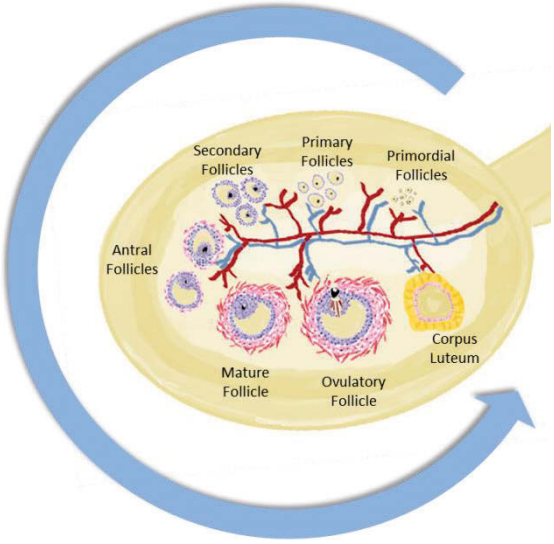

Figure 1.2: Folliculogenesis, ovulation and luteinisation of the ovarian follicle. Multiple primordial follicles begin to mature to primary follicles from puberty. With the help of oestrogen secretion from granulosa cells, the follicle further develops. Secondary follicles then recruit theca cells to further facilitate folliculogenesis. One dominant antral follicle is then selected as the mature follicle. The ovum then leaves the follicle in the process known as ovulation in response to a surge of luteinising hormone (LH). The remaining granulosa and theca cells of the mature follicle luteinise to form progesterone secreting cells.

At the stage of secondary follicle formation, theca cells begin to form around the granulosa cells from pre-cursor cells (Young et al. 2010). Theca cells of the antral follicles become steroidogenic, secreting androgens in response to LH, aiding oestrogen secretion from granulosa cells (Edson et al. 2009) to further mature antral follicles. Eventually, one follicle is selected as the dominant follicle for ovulation which is facilitated by and a sudden surge of LH at mid-cycle. Ovulation is characterised by the ovum successfully bursting from the dominant follicle which allows for fertilisation.

Antral follicles that do not reach ovulation undergo atresia (Teramoto et al. 2016). However, these cells along with the remaining cells of the follicle remain steroidogenic and differentiate or 'luteinise' to form the corpus luteum (Teramoto et al. 2016) and secrete progesterone to facilitate embryo plantation for pregnancy or to prepare the body for the next menstrual cycle.

In PCOS, inherent morphological and functional differences of the follicle and the cells that comprise them may exist (Chang et al. 2013). For example, in women with PCOS and PCOM, antral follicles may fail to mature, resulting in accumulation of small antral follicles (identifiable on ultrasound). This process is referred to as follicular arrest and subsequently inhibits dominant follicle selection, ovulation and corpus luteum formation, manifesting as ovulatory dysfunction in PCOS.

As previously mentioned, women with PCOS may have increased levels of LH. Persistently high LH may contribute to anovulation in PCOS (Franks et al. 2008). However there are many other novel factors that contribute to folliculogenesis including insulin growth-like factor 1 (IGF1), growth differentiation factor-9 (GDF-9) and bone morphogenetic protein (BMP-15) (Erickson et al. 2001) that may be altered in PCOS.

Steroidogenesis

As theca and granulosa cells may be inherently different in PCOS, their role in coordinating steroidogenesis is also implicated in the disorder contributing to hyperandrogenemia. The steroidogenic pathway (Figure 1.3) is initiated in theca cells mainly by the effects of luteinising hormone (LH) from the anterior pituitary which facilitates the conversion of cellular cholesterol into pregnenolone. This is regulated by the mitochondrial cytochrome, p450 side chain cleavage enzyme (P450scc) from the Cytochrome superfamily encoded by *CYP11A1*. This is the rate-limiting step in the steroidogenic pathway (Shan et al. 2016). Pregnenolone can then be converted into either progesterone or de-hydroepiandrosterone (DHEA) via the Δ4 and Δ5 pathways respectively. Cytochrome P450 17A1 (*CYP17A1*) and 3β-Hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (*3β-HSD*) together coordinate this pathway

which result in the synthesis of androstenedione, a pro-hormone of testosterone and estrogen. While testosterone is further synthesised in theca cells, androgens (both androstenedione and testosterone) can be converted into oestrogens, oestrone and oestradiol in adjacent granulosa cells through cytochrome P450 19A1 (aromatase).

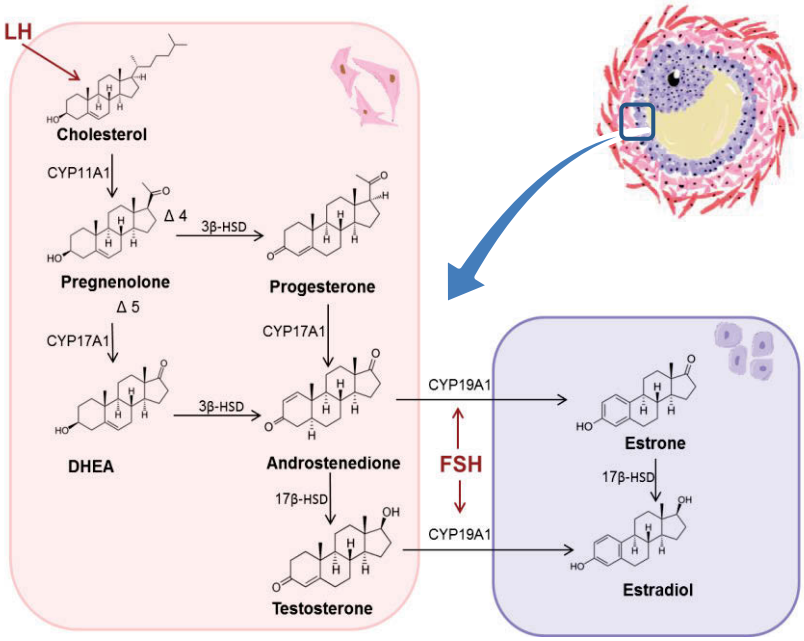

Figure 1.3 Ovarian steroidogenesis in theca and granulosa cells. The steroidogenic pathway is initiated by the effects of luteinising hormone (LH) on theca cells which stimulates the synthesis of pregnenolone from cholesterol. This is regulated by the enzyme, p450 side chain cleavage enzyme (CYP11A1) and is the rate-limiting step of the pathway. Pregnenolone is then converted to progesterone or de-hydroepiandrosterone which is further synthesised to androgen, androstenedione. Cytochrome P450 17A1 (CYP17A1) and 3β-Hydroxysteroid dehydrogenase/Δ5-4 isomerase (3β-HSD regulate these conversions. Androstenedione is either used for testosterone production or synthesised to oestrogens by adjacent granulosa cells by aromatase (CYP19A1).

Ovulation and luteinisation

Ovulation is a dynamic and coordinated process that is initiated by a surge in LH that results in the eruption of the oocyte from the follicle. While the molecular processes that characterise ovulation are extremely complex, it is known that this LH triggers various intracellular and transcription signalling in theca and granulosa cells that promote ovulation of the follicle and therefore the differentiation of these cells (Russell et al. 2007).

Post-ovulation, theca and granulosa cells undergo structural remodelling and functional changes and secrete high amounts of progesterone. This de novo steroidogenesis is achieved by the differentiation or luteinisation of theca and granulosa cells which involves the regaining of cholesterol bubbles and expression of CYP11A1 to synthesise pregnenolone and progesterone respectively (Okada et al. 2016). This process, like folliculogenesis and ovulation is highly regulated and relies on several factors working together synergistically. Many reports have identified altered gene expression between pre-ovulatory follicles and post-ovulatory follicles (Rodgers et al. 1986, Leo et al. 2001). It has also been reported that epigenetic changes via DNA methylation and histone modification play a part in this process by chromatin remodelling, for example *CYP11A1* and steroidogenic acute regulatory protein (*StAR*) mRNA hypomethylated during luteinisation in rats *in vivo* (Lee et al. 2013, Okada et al. 2016).

Other factors that regulate luteinisation include various growth factors, transcription factors, cytokines and hormones secreted by theca and granulosa cells themselves are implicated. For example, luteinising hormone receptor (*LHR*) and progesterone receptor (*PR*) are critical to both ovulation and luteinisation and increase in expression while undergoing these processes. Deletion of PR in mice inhibits ovulation despite exogenous hormone stimulation (Richards et al. 2002).

Angiogenesis is also involved in cell differentiation as it has been shown that vascularisation progressively increases towards and beyond ovulation. For these reasons, factors such as vascular endothelial growth factor (VEGF) are implicated in determining the fate of the cells (Young et al. 2010). Physiological endoplasmic reticulum stress may also play a role in cell luteinisation by mediating atresia (Yang et al. 2017) via the unfolded protein response (UPR)

and various factors such as glucose-regulated protein (GRP78), activating transcription factor 6 (ATF6) and protein kinase-like ER kinase (PERK) (Papalou et al. 2017).

Theca cells

Theca cells are highly differentiated ovarian cells that are the origin of the ovarian steroidogenic pathway and provide structural support for the developing oocyte. As the primary site of androgen production (Tajima et al. 2007) they are therefore implicated in PCOS. Notably, androstenedione production was shown to be 20-fold higher in cultured PCOS theca cells compared to theca cells derived from healthy females (Gilling-Smith et al. 1994). This effect persists in long-term culture over multiple passages suggesting a basic dysfunction of these cells in PCOS (Nelson et al. 1999).

Theca cells can be further divided into theca interna, theca interstitial and theca externa cells. Theca interna and interstitial cells first appear in secondary follicles and mainly function to produce steroid hormones in response to LH. On the other hand, theca externa cells are rich in collagen and fibroblastic in morphology and therefore primarily provide structural support for the developing follicle (Figure 1.4).

Theca cells are abundant in mitochondria, smooth ER and lipid vesicles which reflects their primary function of hormone secretion. CYP11A1 is localised to the mitochondria of theca cells whereas the remaining steroidogenic enzymes are found in the endoplasmic reticulum (Magoffin 2005).

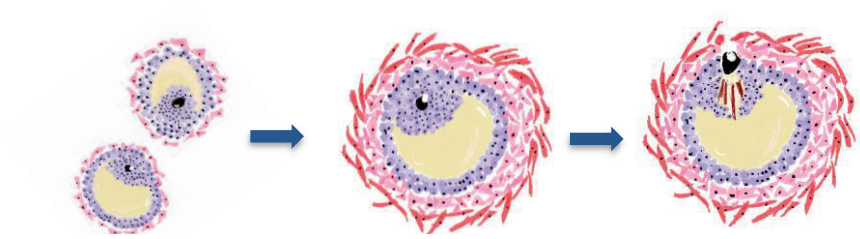

Figure 1.4 Antral follicle development towards ovulation. Antral follicles are characterised by thecal cell recruitment (indicated in pink) that surround granulosa cell (in purple) which together secrete necessary hormones for follicle maturation and ovulation. Theca external cells (in red) provide structural support throughout this process.

Expression of *CYP17* and steroidogenic acute regulatory protein (*StAR*) gene expression in PCOS and non-PCOS theca cells are significantly varied (Wickenheisser et al. 2000, Cadagan et al. 2016). Unique molecular signatures of PCOS theca cells have also been observed in microarray analysis in other studies, displaying an increased expression of other steroidogenic enzymes such as CYP11A and 3β-HSD as well as novel markers such as GATA6 and DENND1A (Wood et al. 2003, Strauss et al. 2015).

PCOS theca cells may consequently be intrinsically hypersensitive to hormones such as LH, insulin and insulin-like growth factor (IGF-1) with over expression of their respective receptors per theca cell (Magoffin 2005). Recently, theca from PCOS women compared to controls have demonstrated a significant increase in the protein expression of LHR (Comim et al. 2013).

Granulosa Cells

Granulosa cells are oestrogen secreting cells primarily responsible for the growth of the follicles and begin to proliferate as early as the primary follicle stage. This is mainly in response to follicle stimulating hormone (FSH), however, studies have also shown that insulin and IGF1 also play a part in granulosa cell development (Adashi 1998).

Granulosa cells communicate with theca cells to synthesise oestrogens from androgens. This paracrine relationship is essential to steroidogenesis. Aromatase (*CYP19A1*) is primarily responsible for this synthesis of oestrogen. Genetic modification to aromatase may therefore be implicated in PCOS and alter the rate in which androgens are synthesised.

Polycystic ovarian morphology may also provide insights into functional differences of granulosa cells in PCOS. For example, anti-müllerian hormone (AMH) has been observed to be increased in PCOS granulosa cells compared to normal controls (Pellatt et al. 2007). AMH has been identified as being a regulator of primordial follicle recruitment and the response of granulosa cells to FSH (Visser et al. 2006). Increased AMH expression in granulosa cells may therefore contribute to abnormal folliculogenesis in PCOS (Homburg et al. 2014).

Despite unique intrinsic molecular signatures of ovarian theca and granulosa cells in women with PCOS, the metabolic changes also exhibited by these women remain unexplained. For these reasons, the role of insulin resistance (IR) in the pathogenesis of PCOS has been explored more recently in this area of research.

Insulin resistance in the pathophysiology of PCOS

Metabolic syndrome and obesity frequently coincide with PCOS and subsequently IR has been identified as the link with studies showing that up to as many as 90% of women with PCOS are insulin resistant (Barber et al. 2015, Zhu et al. 2016). Meanwhile, the lean contingency of PCOS are also found to have altered insulin metabolism (Chang et al. 1983). Evidence of IR appears early in the clinical picture of PCOS, as adolescent girls with PCOS have been shown to have profound reduction in peripheral insulin sensitivity, hepatic insulin resistance and elevated levels of insulin (Lewy et al. 2001) suggesting IR central to the

pathophysiology of PCOS. The ways in which IR and hyperinsulinemia contribute to PCOS are summarised in Figure 1.5.

Furthermore, IR is positively correlated with hyperandrogenism in PCOS as well as the most severe phenotypic presentation of the disorder (Palomba et al. 2010, Barber et al. 2015). Consequently, studies have investigated the role of insulin-sensitisers in the treatment of PCOS with promising results both clinically (Young-Mo et al. 2015) and experimentally (Qu et al. 2009).

Interestingly, insulin acts as a co-gonadotrophin with synergistic effects with LH on ovarian tissues by initiating steroidogenesis (Cadagan et al. 2016). Therefore, it has been hypothesised that IR in peripheral tissues and subsequent hyperinsulinemia adversely affect insulin-sensitive ovaries to increase androgen production and disrupt the menstrual cycle. This has been coined as the 'selective insulin resistance' theory (Rojas et al. 2014). Insulin receptors are found in both theca and granulosa cells and therefore abundant insulin at the site of the ovary may augment normal insulin dependent pathways, such as the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinases (MAPK) pathways, thereby increasing steroidogenesis (Lan et al. 2015, Li et al. 2016). However further characterisation of these pathways in granulosa and theca cells are warranted.

Insulin has shown to play a role in stimulating physiologic androgen production as it appears to have a synergistic effect with LH to initiate the steroidogenic pathway (Zhang et al. 2000, Spritzer 2014). Moreover, studies have also highlighted that insulin alone significantly increases androgen production in ovarian thecal cell cultures (Nestler et al. 1996). Insulin may also independently stimulate androgen production may be through the increased rate

of proliferation of theca cells. This has been observed in vitro in a rat-derived theca cells in which the addition of insulin (1 µg/mL) resulted in a 50% increase in proliferation of cells (Will, Palaniappan et al. 2012), although androgen secretion was not quantified.

Furthermore, hyperinsulinemia may augment the function of the pituitary gland to alter the amplitude and frequency of LH release or dysregulate adrenal signalling to increase hyperandrogenism in PCOS (Rojas et al. 2014) with further research in this area needed.

IR has been linked to decreased levels of sex-hormone binding globulin (SHBG) which is also a common clinical finding in women with PCOS (Abu-Hijleh et al. 2016). Hepatically derived SHBG is responsible for the binding and metabolism of sex hormones, with a particular affinity for testosterone. Therefore decreased SHBG increases free androgens in serum and may link PCOS to other metabolic disorders such as NAFLD (Vassilatou 2014).

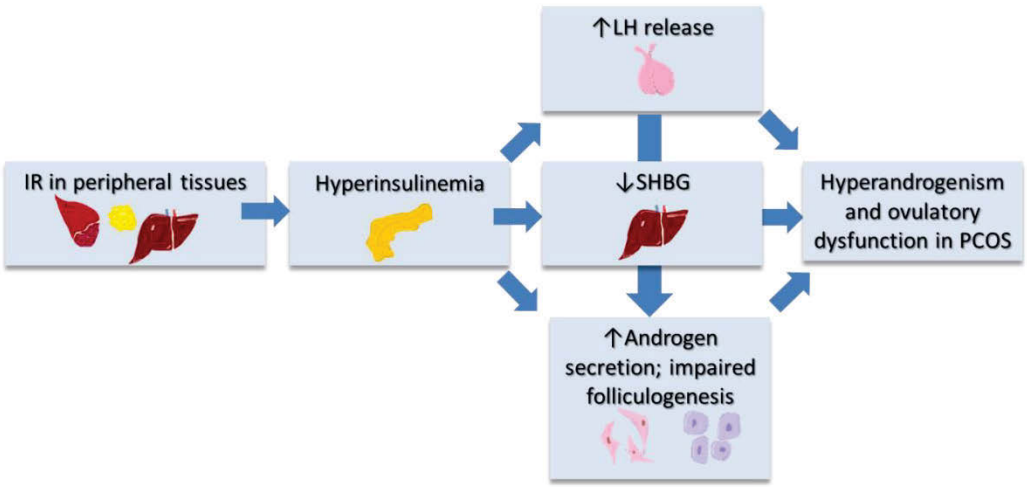

Figure 1.5 The role of insulin resistance and hyperinsulinemia in the pathogenesis of PCOS. Insulin resistant (IR) tissues such as muscle, adipose and the liver result in compensatory hyperinsulinemia. This in turn may modulate the function of the pituitary gland, liver and ovary and their respective hormone secretion of luteinising hormone (LH), sex hormone binding globulin (SHBG) and androgens to manifest as the clinical signs and symptoms of PCOS.

1.1.4 Complications of PCOS

Although the presentation of PCOS may not be life-threatening, the syndrome is associated with an increased risk of morbidity compared to the normal population (Hart et al. 2015). Clinical signs such as acne and hirsutism may not be as pronounced with aging. However, hormonal abnormalities such as hyperandrogenism are shown to persist after menopause (Markopoulos et al. 2011, Shah et al. 2014) which is associated with an increased risk of coronary artery disease and cardiovascular events (Shaw et al. 2008). Weight issues are also shown to persist in women with PCOS. One study identified that 91% of 401 women with PCOS surveyed were overweight, obese or extremely obese (Glueck et al. 2005) with 78% of women aged 31-42 were in the obese-extremely obese category. Metabolic abnormalities may also progress after menopause with insulin resistance and dyslipidaemia more common in post-menopausal women with PCOS (Shah et al. 2014). Large retrospective and prospective cohort studies have frequently demonstrated an increased prevalence of Type 2 diabetes mellitus (Wang et al. 2011, Mani et al. 2013). It is estimated that this risk of developing diabetes is quadruple –fold and has an earlier onset compared to the normal population (Moran et al. 2015) therefore regular screening for cardiovascular and metabolic parameters is encouraged.

Enduring hormonal abnormalities in women with PCOS may also increase the risk endometrial cancer which is almost three times more likely to develop in women with PCOS compared to those without (Chittenden et al. 2009). Infertility is another major complaint in 40% of women with PCOS (Legro et al. 2007, Sirmans et al. 2014), however after conception, this group of women are more likely to experience obstetric and neonatal problems when compared to healthy controls (Boomsma et al. 2006) including a significantly increased risk of gestational diabetes, pre-eclampsia, pregnancy induced hypertension, miscarriage and

pre-term birth (Palomba et al. 2015). Due to these compounding reproductive and metabolic risks, PCOS is a considerable economic burden on the health-care system and in Australia alone is estimated to cost $800 million per year (Shorakae et al. 2014).

1.1.5 Conventional Treatment of PCOS

Treatment strategies for PCOS are often heterogeneous as they should be individualised according to the phenotype of the woman and also aim to reduce hyperandrogenism, restore menstrual cyclicity, promote ovarian function and ameliorate any metabolic abnormalities. In insulin resistant and obese PCOS women, the latter is especially important, given that signs and symptoms are generally more severe (Al-Azemi et al. 2003, Hirschberg 2009). Therefore lifestyle changes such as regular exercise and diet to target weight loss are important and as little as 5-10% weight reduction can show significant improvement (Norman et al. 2004). However, lifestyle modifications may be difficult to adhere to long-term as often found in clinical trials (Ayyad et al. 2000).

There are currently no approved medications for treating PCOS and 'off-label' therapeutic options are limited. The oral contraceptive pill (OCP), is commonly prescribed to ameliorate hyperandrogenism and regulate the menstrual cycle. Combined oral contraceptive (COCs) containing both ethinylestradiol and progestins may dampen the hypothalamus-pituitary-ovarian axis to reduce steroidogenesis (Rocca et al. 2015) or increase SHBG (Wiegratz et al. 2003) thereby ameliorating hyperandrogenism and related clinical signs and symptoms such as acne and hirsutism.

However, the OCP is not appropriate for women wanting to conceive and its use may be associated with long-term side effects such as deep vein thrombosis or aggravation of

obesity, insulin resistance, hyperlipidaemia and hypertension which will in turn exacerbate PCOS (Diamanti-Kandarakis et al. 2003, Kulshreshtha et al. 2016). In non-obese and obese women with PCOS, administration of the OCP has resulted in decreased insulin sensitivity within as little as 3 months (Korytkowski et al. 1995, Nader et al. 1997). Additionally, the improvements to hormone levels may only be temporary as demonstrated in a study, which showed that levels of total testosterone and DHEAS were back to baseline within 4 weeks after discontinuation of an OCP comprised of drospirenone 3 mg and ethinyl-estradiol 30 µg (Sánchez et al. 2007). Obesity may also hinder the ability of the OCP to improve serum androgens as well as clinical signs and symptoms of hyperandrogenism such as hirsutism (Cibula et al. 2001). Furthermore, a consensus of dose of ethinylestradiol and type of progestin is unclear for both efficacy and risk of side effects (Baillargeon et al. 2005).

Anti-androgens may also be used to address severe clinical and biochemical hyperandrogenism if the OCP alone is not enough. However, anti-androgens such as finasteride and flutamide must be combined with the OCP to prevent known teratogenic effects to the foetus induced by these agents (Danesh et al. 2015). Additionally, antiandrogen use has been linked to fatal liver toxicity (Andrade et al. 1999, Nakano et al. 2015) and therefore only recommended for short term use. Gonadotropin releasing hormone agonists (GnRHa) may also be used as a therapy for hyperandrogenism by blocking LH and FSH release. However, this may result in oestrogen suppression associated with vaginal dryness, hot flashes, mood swings and loss of bone density (Azziz et al. 1995, Archer et al. 2004).

Insulin sensitizers are an emerging therapy for PCOS as IR has shown to be central to the pathogenesis of PCOS. Metformin is the most commonly used insulin-sensitising agent used for the treatment of PCOS and works by suppressing hepatic gluconeogenesis and

improving insulin sensitivity in peripheral tissues. Side effects of metformin may include nausea, diarrhoea and abdominal bloating and frequently monitoring liver enzymes may be necessary (Archer et al. 2004). While the use of metformin for metabolic abnormalities in PCOS is understandable, the insulin sensitiser has also been clinically shown to reduce ovarian volume, improve hyperandrogenemia and increase regularity of menstruation (Farimani Sanoee et al. 2011, Suvarna et al. 2016). These effects may be exhibited in non-obese women with PCOS and normal insulin sensitivity suggesting an independent action on steroidogenesis (Baillargeon et al. 2004). This has been confirmed in *in vitro* studies, in which metformin was shown to regulate thecal proliferation and reduce androgen production (Attia et al., 2001; Will et al., 2012; Di Pietro et al., 2015). However, the effects of metformin on hyperandrogenism are controversial as other results conflict these findings (Lashen 2010) or only reported in small clinical studies. Ironically, the combination of metformin with the OCP may reduce metformin's effectiveness in improving insulin sensitivity (Iwata et al. 2015). Therefore, more studies are needed to identify the efficacy of metformin, understand its interactions with the OCP and establish the safety profile for long term-use of the use (Tang et al. 2003).

Infertility in PCOS is generally treated with medications that induce ovulation such as Clomiphene Citrate (CC) or Letrozole. Ovarian drilling and *In Vitro* Fertilisation (IVF) are more extreme, expensive and invasive methods and are therefore typically reserved as a last resort.

1.1.6 Chinese Herbal Medicine

As demonstrated by the literature, PCOS is complex and multi-faceted in its presentation and it is unlikely that one drug can successfully manage all clinical signs and symptoms. Therefore, the combination of therapies and the use of complementary and alternative

medicines such as Chinese Herbal Medicine (CHM) may be important adjunctive therapies. CHM and their extracts have been successfully used in the clinical treatment of PCOS. Additionally, multiple herbs are usually combined in a formula allowing for a multi-targeted approach which can be individualised to a phenotype and are generally associated with fewer side effects (Yeh et al. 2003).

Chinese Herbal Medicines (CHM) have been traditionally used to address disorders related to menstruation and fertility (Huang et al. 2008) among a wide variety of other health issues. More recently, research into the pharmacological characterisation and mechanisms of action of Chinese herbs and their active compounds has become increasingly popular and is a potential source of new drug developments (Pan et al. 2014). This has paralleled a growing interest in complementary and alternative medicines by consumers. For example, in a survey of 493 women with PCOS in Australia, it is known that over 70% have used complementary medicines to treat PCOS, while 76.6% of women had consulted with a complementary medicine healthcare practitioner (Arentz et al. 2014). Multi-disciplinary approaches are therefore being increasingly recommended for the treatment of PCOS.

Subsequently, clinical and experimental studies of Chinese herbs for the treatment of PCOS have been investigated which are detailed in a review article published earlier this year (Ong et al. 2017). Clinically, some of these agents can successfully improve the regularity of the menstrual cycle, improve metabolic parameters and ameliorate hyperandrogenism (Qu and Ong 2015) (Appendix II). *In vitro* studies, have shown that compounds from Chinese herbs such as berberine from Rhizoma Coptidis and cryptotanshinone from Salvia Miltiorrhiza can reverse testosterone levels in media secretion (Zhao et al. 2011, Xiong et al. 2012) by improving insulin signalling and inhibiting the steroidogenic pathway.

In animal models of PCOS, ginsenosides from Panax Ginseng reduced antral follicle number and increased the number of corpora lutea to improve PCOM and ovulatory dysfunction (Jung et al. 2011). Hyperandrogenism, ovulatory dysfunction, ovarian morphology and metabolic parameters of PCOS were all significantly improved by cryptotanshinone in a DHEA-induced rat model (Yu et al. 2014) by modulating expression of CYP17A1 and androgen receptor (AR).

Clinical trials have shown that berberine can increase menstrual frequency and regularity whilst improving androstenedione and total testosterone in women with PCOS (Orio et al. 2013) and has comparable effects to metformin in improving metabolic profiles (Wei et al. 2012).

Paeoniflorin

Paeoniflorin is the major constituent of Radix Albus Paeoniae Lactiflorae *(Bai Shao)*, and a commonly used herb in Chinese Herbal Medicine for the treatment of gynaecological disorders. Paeoniflorin has exhibited anti-androgenic properties by inhibiting the production of testosterone and encouraging the aromatising of testosterone into oestrogen in ovaries *in vivo* (Takeuchi et al. 1991, Grant et al. 2012). In the context of insulin resistance, paeoniflorin has also demonstrated positive effects, for example, in 3T3-L1 cells treated with TNF-α to induce IR, paeoniflorin decreased protein expression of IRS-1 to improve insulin sensitivity (Kong et al. 2013). In an NAFLD model, insulin resistant rats demonstrated a significant improvement to homeostatic model assessment of insulin resistance (HOMA-IR) and blood glucose levels when treated with paeoniflorin. Meanwhile, in the same study, expression of key genes related to IR and glucose metabolism, peroxisome proliferator–activated receptor γ (*PPARγ*), phosphoenolpyruvate carboxykinase (*PEPCK*) and glucose 6-phosphatase

(*G6Pase*) were also improved by the herbal compound (Zhang et al. 2015). Hypoglycaemic effects and the improvement of insulin sensitivity have also been confirmed in another animal studies (Hsu et al. 1997, Zheng et al. 2013). Further, paeoniflorin has also exhibited mild anti-coagulant effects that contribute to the improvement of blood circulation and protect against cardiovascular disease (Koo et al. 2010). Currently, the effects of paeoniflorin on ovarian cells for the treatment of PCOS have not yet been examined.

1.1.7 *In Vitro* models of PCOS in theca and granulosa cells

Due to the invasiveness of obtaining human theca and granulosa cells, *in vitro* induction of PCOS in more readily accessible, animal derived theca and granulosa cells has therefore been explored. Emulation of PCOS type characteristics may include increased cell proliferation, increased androgen production in theca cells or contribution to follicular arrest in granulosa cells. These have been achieved by exposure to various reagents including LH, BPA, insulin, and dexamethasone. However, at this stage such studies are also limited with further work warranted.

Dexamethasone, a glucocorticoid, which can modulate insulin signalling, has been included as a model for inducing PCOS *in vitro*. In porcine theca cells, dexamethasone decreased insulin receptor substrate 1 (*IRS-1*) and glucose transporter 4 (*GLUT-4*), and increased *PPARγ* mRNA expression which was shown to directly contribute to increased T secretion as well as *CYP17A1* mRNA expression (Qu et al. 2009, Zhao et al. 2011).

Although not specifically trying to induce PCOS conditions, dexamethasone has shown in earlier studies to be protective against apoptosis in human granulosa cells (Sasson et al. 2002) which may correlate to impaired cell maturation. Dexamethasone potentiated the

effects of IGF-1 to stimulate mRNA expression of *CYP11A1* in porcine granulosa cells demonstrating ability to upregulate steroidogenesis (Urban et al. 1994). In pre-ovulatory rat follicles, dexamethasone also inhibited progesterone secretion (Huang et al. 2001). These studies together suggest that dexamethasone adversely affects both theca and granulosa cell *in vitro*.

Other models have shown that insulin alone increases rat theca cell proliferation at a concentration that exceeds the insulin receptor saturation threshold to and disrupt insulin-dependent signalling pathways (Will et al. 2012). This effect was reversed by treatment with metformin. The synergism of insulin, IGF-1 and LH has also demonstrated the ability to increase androstenedione production in rat theca cells (Cara et al. 1988). The combination of insulin and IGF-1 in bovine theca cells also increased cell proliferation, androstenedione production and expression of LHR (Stewart et al. 1995).

Bisphenol A exposure to rat ovarian theca cells increased production of testosterone and mRNA expression of CYP17A1 (Zhou et al. 2008). In the same study, progesterone synthesis was also increased in granulosa cells. In murine granulosa cells, BPA also contributed to cell cycle arrest and apoptosis (Xu et al. 2002). TNF-α has also demonstrated the ability to increase rat theca cell proliferation which may link to an increase in steroidogenically active cells (Spaczynski et al. 1999).

Low levels of LH have shown to increase androstenedione production in a dose-dependent manner in ovine theca cells (Campbell et al. 1998). However, after 48 hours the higher doses of LH switched to favouring progesterone secretion and an inhibition of androstendione production. Morphological changes were also observed in these cells reflecting both

morphological and functional luteinisation. The luteinisation of granulosa cells is also widely reported in the literature (Portela et al. 2010) and is therefore a consideration for inducing PCOS conditions *in vitro*.

1.2 Aims of Thesis

PCOS is a common and life-long condition that adversely affects the reproductive, metabolic and cardiovascular aspects of a woman's health and therefore research into developing therapeutics is vital. The wider aim of this study is to therefore discover new natural therapeutics for PCOS that can ameliorate both reproductive and metabolic components of the disorder. This is informed both by an overview of the literature as well as clinical experience as a qualified Chinese medicine practitioner.

Theca and granulosa cells are a primary target for understanding PCOS as they are the major site of hormone production and are involved in folliculogenesis which are undoubtedly disturbed in PCOS. However, these cells, in particular, theca cells are difficult to obtain from humans without oophorectomy. Additionally, due to an unclear pathogenesis, cell culture and animal models of PCOS are diverse with currently no established cell line or 'gold-standard' animal model for studying PCOS (Indran et al. 2016).

Certain Chinese herbs may ameliorate clinical signs and symptoms associated with PCOS, however the molecular mechanisms in which they work are unclear, and therefore the clinical knowledge needs to be brought back to the bench top. Increased used of herbal medicines by women with PCOS alone encourages an investigation into their potential mechanisms (Arentz et al. 2017). Paeoniflorin is a suitable candidate as it has been traditionally used to treat reproductive disorders and more recently identified as having insulin-sensitizing

properties. However, the effects of Paeoniflorin on theca and granulosa cells specifically have not yet been elucidated. This project therefore aimed to:

1. Establish an *in vitro* model of PCOS in murine theca and granulosa cells
2. Examine the effects and define molecular mechanisms in which Paeoniflorin ameliorates PCOS, particularly in relation to hyperandrogenism which is a major hallmark of the disorder

This is especially important due to the increasing prevalence of PCOS, particularly in Australia, as well as a lack of patient satisfaction with existing treatment options. Pharmacotherapies now need to look beyond short-term cosmetic fixes and aim to prevent long term complications such as diabetes, NAFLD and cardiovascular disease whilst still preserving ovarian function.

It is hypothesised that because insulin resistance is a key component to the pathogenesis of PCOS, reagents that disturb insulin signaling may be useful in inducing PCOS-type conditions *in vitro*. Additionally, Paeoniflorin, which has previously shown to improve insulin sensitivity, may also have beneficial effects on theca and granulosa cells under PCOS-like conditions. These effects may be related to modulation of hormone levels and expression of key enzymes and genes involved in the steroidogenic pathway.

CHAPTER 2

The Effects of Paeoniflorin Extract on Proliferation and Secretion of Reproductive Hormones in Ovarian Cells

CHAPTER 2: THE EFFECTS OF PAEONIFLORIN EXTRACT ON PROLIFERATION AND SECRETION OF REPRODUCTIVE HORMONES IN OVARIAN CELLS

2.1 Introduction

The functional and molecular characterisation of theca and granulosa cells from women with PCOS is crucial to further the understanding and development of therapeutics for the disorder. Functionally, theca cells from women with PCOS have demonstrated excessive androgen production (Chang et al. 2013), while granulosa cells have been shown to have altered rates of atresia (Webber et al. 2003) which correlate with the syndrome's diagnostic characteristics, hyperandrogenism and follicular arrest respectively.

A comprehensive understanding of theca and granulosa cell dysfunction is not fully elucidated, which, in part is due to the difficulty and invasiveness of obtaining primary human cells. This problem also arises in investigating therapeutics for PCOS warranting the development of experimental animal and cell models. Ironically, these types of studies are relatively limited and there is no established method of inducing PCOS in these models (Indran et al. 2016), reflecting the complexity and uncertainty of the disorder's pathogenesis.

However, many of these models are now centred on exploring the effects of insulin resistance (IR) as this is believed to play a large role in the pathogenesis of PCOS as outlined in Chapter 1. Therefore, recent *in vitro* studies of PCOS have included culturing ovarian cells with insulin and dexamethasone (DEX) which have shown to significantly increase thecal androgen production (Zhao et al. 2011, Cadagan et al. 2016, Faubert et al. 2016). While the use of insulin is easily understood, the use of DEX, a corticosteroid, is comparatively more novel and theorised to disrupt post-receptor insulin signalling and glucose metabolism

(Zhao et al. 2011). In addition, luteinising hormone (LH) may also be combined with these to also induce hyperandrogenic theca cells by stimulating steroidogenesis.

In the context of IR, herbal extracts that have shown to have positive effects in other disorders of metabolic syndrome have become of interest in the treatment of PCOS. Paeoniflorin, from the herb Radix Paeoniae Alba, has demonstrated hypoglycaemic actions *in vitro* (Juan et al. 2010) as well as capability to reverse insulin resistance in rats with NAFLD (Zhang et al. 2015). Clinically, this herb is frequently used by Chinese medicine practitioners in the treatment of female reproductive disorders and it is therefore hypothesised that paeoniflorin may improve ovarian function in PCOS.

This study therefore set out to establish an *in vitro* model of PCOS in murine ovarian cells as well as investigate the effect of paeoniflorin as a new therapeutic agent for the treatment of PCOS. This was carried out by measurement of key hormones, testosterone (T), progesterone (P) and oestradiol (E_2).

2.2 Materials and Methods

2.2.1 Materials

Ovarian theca and granulosa cells were isolated from 4-week-old, non-pregnant mice provided by the Human Reproduction Unit, Sydney Centre for Developmental and Regenerative Medicine, Kolling Institute for Medical Research. McCoy's 5A media was provided by Sigma Aldrich (Missouri, USA) while Fetal Bovine Serum (FBS), Penicillin-Streptomycin (100x) and Trypsin- Ethylenediaminetetraacetic acid (EDTA) (0.25%, phenol red) were purchased from GIBCO (Auckland, NZ). 0.4% Trypan Blue Solution was provided by

Thermo Fisher Scientific (Massachusetts, United States) and collagenase type IV, bovine serum albumin (BSA) and media 199 from Sigma Aldrich.

DEX and LH were supplied from Sigma Aldrich (Missouri, USA) and insulin (Humulin) from Eli Lilly, (Indianapolis, USA). Paeoniflorin extract (PFE) was provided from Tasly Pharmaceuticals (Tianjin, CN) standardised to 98% paeoniflorin, determined by high-performance liquid chromatography (HPLC). All other herbal compounds were also provided by Tasly Pharmaceuticals.

SYBR green I was provided by Thermo Fisher and dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich (Missouri, USA). All other chemicals including 10 mM Tris, 2.5 mm EDTA, 0.1% Triton X-100 were provided by Sigma Aldrich (Missouri, USA).

2.2.2 Cell Culture

Briefly, ovaries were aseptically removed from 25 sacrificed female mice (50 ovaries) and transferred to the lab where they were placed in small falcon dishes with ice-cold McCoy's 5A media for isolation adapted from previously described methods (Tian et al. 2015). Surrounding tissue on the ovaries was removed with sterile forceps and granulosa cells were then released by puncturing the follicles with a 25-gauge needle under a stereomicroscope. The media in the falcon dish containing granulosa cells were then transferred to 15 mL falcon tubes and centrifuged at 150 g for 5 min at 4°C. Granulosa cells were then re-suspended in complete culture medium (CM) which was comprised of McCoy's 5A medium supplemented with 10% FBS and 1% penicillin-streptomycin (pH 7.2). Cells were subsequently counted by staining 50 µL of cells suspension with an equal volume of 0.4% trypan blue and a haemocytometer. Cells were then seeded at a density of $1x10^6$ in T25 flasks and maintained in an incubator at 37°C, 5% CO_2. CM was replaced every 48 hours until

confluent. Cells were cultured until at least passage 3 after which, cells were used for further experiments.

Theca cells were then isolated from the remaining follicle tissue by incubating for 1 h at 37°C in 0.4 mL of collagenase IV solution (4 mg/ml of collagenase, 10 mg/ml of BSA in M199 medium) in a 15 mL falcon tube to facilitate tissue digestion. Ovarian tissue was agitated by briefly pipetting suspension up and down at intervals of 15 min during the incubation period. Cells were then centrifuged at 1000 g for 5 min and washed with CM three times. After the third wash, cells were counted, seeded and maintained as previously described for the granulosa cells.

2.2.3 *In vitro* PCOS Induction Test

$1x10^5$ of a co-culture of granulosa and theca cells were seeded in contact in a 4:1 ratio (granulosa cells 8 x 10^4 cells and theca cells 2 x 10^4 cells) (Gregoraszczuk et al. 2008) in 24-well plates and incubated for 48 h at 37°C to allow for anchorage. Seeding density was determined by previous experiments. Cell media was subsequently replaced with CM or CM with combinations of insulin (100 nM), luteinising hormone (5 µg/mL) and DEX (1 and 10 µM). Cells were then incubated for a further 24 h at which point, cell media was collected and stored at -20°C for later hormone determination.

2.2.4 Herbal Screening Test

The co-culture of granulosa and theca cells were seeded as previously described in 24 well plates and treated with either DEX (10 µM) or DEX in combination with the following compounds: paeoniflorin, ginsenoside, berberine or silibinin (10 µg/mL) to determine effect on testosterone secretion.

2.2.5 Paeoniflorin Treatment

Paeoniflorin extract (PFE) was dissolved in Milli-Q® water as various 1000-fold stock solutions (1 mg/mL, 10 mg/mL and 100 mg/mL) and then filtered through a 0.2 µm filter. Stock solutions were prepared fresh for each experiment and discarded after use to ensure similarity between experiments. 1 µL/mL of each stock solution was added to DEX media to make final dilutions of 1, 10 and 100 µg/mL. Theca cells were seeded into a 24-well plate at a density of $1x10^5$ cells. After 48 h, CM was replaced with either CM, CM with DEX (10 µM), or CM, DEX (10 µM) and PFE (1, 10 and 100 µg/mL) for a further 24 h. Cell media was then collected and stored as previously described. This was also carried out in granulosa cells and formed the treatment protocol for all other experiments, however for granulosa cells, media was also collected prior to cell treatment (48 h after seeding) to compare E_2: P ratios.

2.2.6 Cell Proliferation Assay

$2.5x10^4$ cells were seeded into 96-well plates and incubated in 200 µL CM for 48 h. Media was removed and replaced with CM and PFE (1, 10, 100 and 1000 µg/mL). After 24 h, plates were removed from the incubator, media 'flicked-off' and frozen at -80°C as previously described (McGowan et al. 2011) . This was also carried out at 48 h. Frozen plates were then thawed and 200 µL of SYBR Green I (1:8000) in a lysis buffer (10 mM Tris, 2.5 mm EDTA, 0.1% Triton X-100) was added to each well. Plates were then left for a further 72 h at 4°C in the dark. Fluorescence intensity was then measured at 485 nm excitation and 530nm emission filters using a microplate reader and Magellan Software (Tecan, Zürich, Switzerland).

2.2.7 Testosterone, Oestradiol and Progesterone Measurement

Media was collected and analysed the using the Cobas 8000 modular analyzer (Hoffman-La Roche Ltd, Basel, Switzerland) for testosterone and the ARCHITECT *i*2000SR immunoassay

analyzer (Abbott, Illinois, USA) for E_2 and P measurement. For P measurement, cell culture media was diluted 1:5 times with sterile Milli-Q® water before measurement. Untreated culture medium was used as a background and results were expressed as a percentage of control after background subtraction.

2.2.8 Statistical Analysis

Data from all experiments were expressed as the mean ±SEM and analysed using one-way ANOVA followed by post-hoc analysis with Dunnett's multiple comparison test to either the control or DEX group with GraphPad Prism software version 6 (California, USA). Data from hormone assays are represented as a percentage (%) of control group. E_2: P ratios are expressed as E_2 (ng/mL)/P (ng/mL). Statistical significance was set at $p<0.05$.

2.3 Results

2.3.1 The effects of dexamethasone on testosterone, oestradiol and progesterone secretion

Figure 2.1 shows the reproductive hormone levels secreted by in culture media from the PCOS induction test. The high dose DEX (10 μM) group significantly increased T secretion ($p<0.05$) (Figure 2.1A) in the theca and granulosa co-culture. In this co-culture, significant E_2 production ($p<0.05$) at both concentrations of DEX (Figure 2.1B) were also observed. There were no other significant changes in other treatment groups when compared to control for T or E. Additionally, insulin with DEX (1 μM) and DEX alone (1 μM and 10 μM) inhibited P production ($p<0.05$, $p<0.01$, $p<0.001$ respectively) (Figure 2.1C). E_2:P ratio was also increased in DEX treated cells when compared to untreated cells ($p<0.01$) (Figure 2.1D).

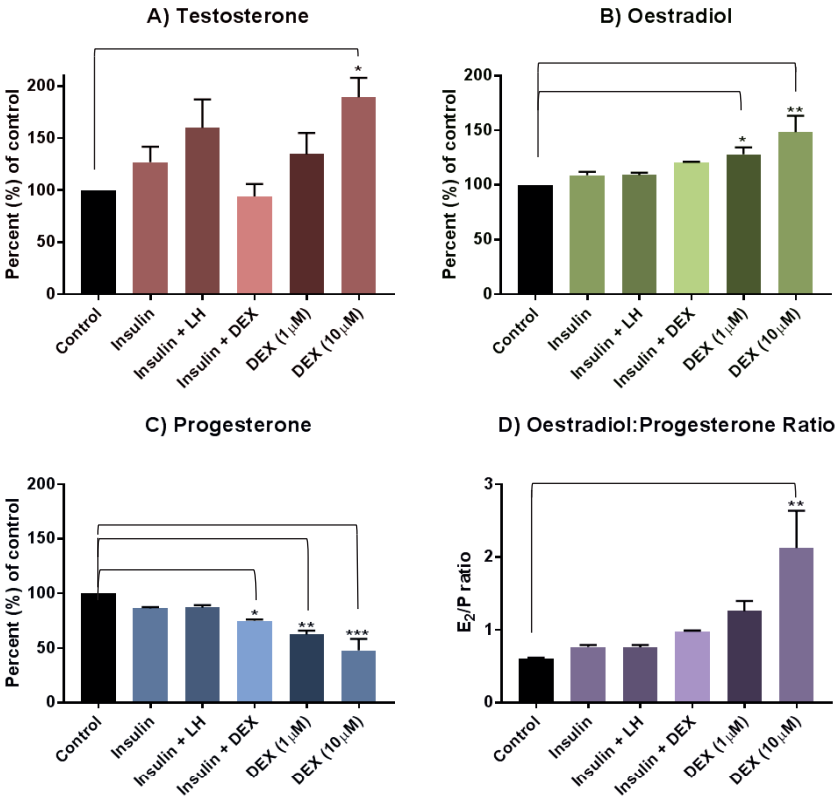

Figure 2.1 PCOS induction test in theca-granulosa co-culture. Cells were treated with control media (control group), insulin, luteinising hormone (LH), dexamethasone (DEX) or combinations of these for 24 h. Media was then collected and testosterone (A), oestradiol (B) and progesterone (C) measured. Results are represented as a percent (%) of control and expressed as the mean± SEM from three replicates (n=3). *p<0.05, **p<0.01, ***p<0.05 compared to control group.

2.3.2 The effects of paeoniflorin extract on ovarian cell proliferation

From the SYBR green proliferation assay, ovarian cells treated with 1, 10, 100 and 1000 µg/mL PFE did not exhibit any significant changes to fluorescence intensity. This was observed at both 24 and 48 h when compared to the control group as shown in Figure 2.2 (p=0.7423 and 0.8342 respectively).

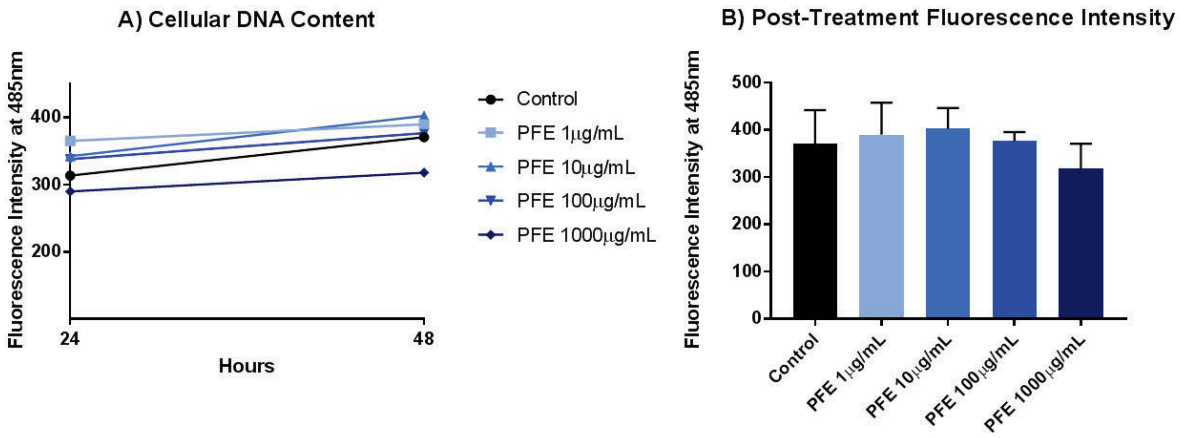

Figure 2.2 SYBR green proliferation assay of PFE-treated theca-granulosa co-culture. SYBR green assay was carried out on PFE treated ovarian cells. Following treatment, plates were treated with SYBR solution and incubated for 72 h in the dark at 4°C. Fluorescence intensity was then determined at 485 nm to show effects of PFE on cell proliferation at 24 and 48 h. Data was collected from 3 separate experiments (N=3).

2.3.3 The effects of paeoniflorin on dexamethasone-treated theca and granulosa cells

In the initial herbal screening test, paeoniflorin appeared to reverse the effects of DEX on T levels compared to other herbal compounds (N=2) as shown in Figure 2.3.

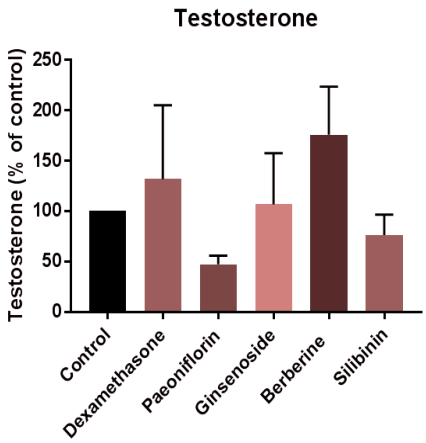

Figure 2.3 Herbal screening test of herbal compounds on testosterone levels. Granulosa and theca cells were treated with control media (control group), dexamethasone (10 µM) alone or combined with herbal compounds, paeoniflorin, ginenoside, berberine or silibinin (10 µg/mL) for 24 h. T levels in culture media were then measured. Results are displayed as a percent of untreated control cells. Data was collected from 2 separate experiments (N=2).

In further experiments using theca cells only, DEX treated cells exhibited significant increases to T ($p<0.05$). This, however was reversed by the high dose PFE (100 µg/mL) treatment group ($p<0.05$). Furthermore, P levels were suppressed by DEX, which was again protected by the effects of PFE (100 µg/mL) as shown in Figure 2.4.

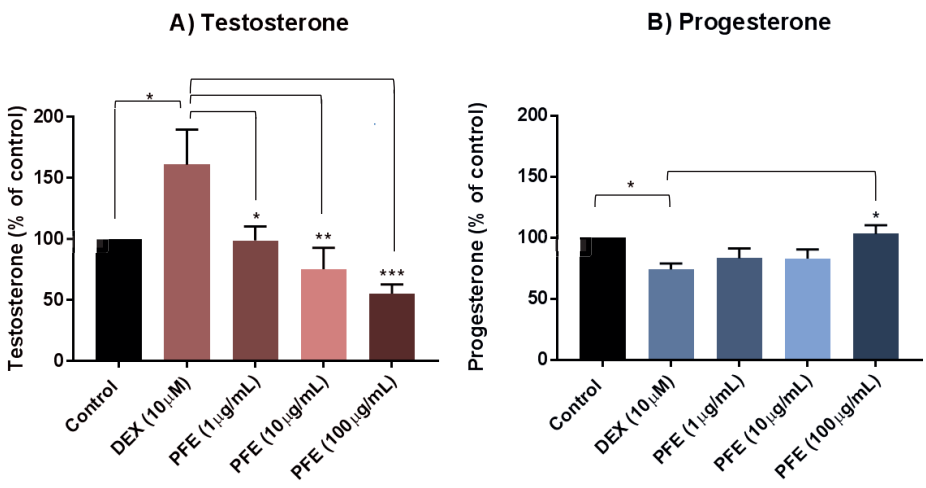

Figure 2.4 Testosterone and progesterone analysis of paeoniflorin (PFE) treated theca cells. Cells were treated control media (control group), with dexamethasone (DEX) or DEX in the presence of PFE (1, 10 and 100 µg/mL). Media was collected 24 h post treatment and collected for assay of testosterone (A) and progesterone (B). Results are represented as a percentage (%) of control group as the mean ±SEM from four replicates (n=4). *$p<0.05$, **$p<0.01$, ***$p<0.05$ compared to DEX treatment group

Similar effects were observed in DEX treated granulosa cells as DEX inhibited P secretion ($p<0.05$) which was reversed by PFE (100 µg/mL). DEX also increased E_2 secretion compared to control cells ($p<0.05$) (Figure 2.5A). While PFE appeared to reverse this trend, this effect was not considered significant.

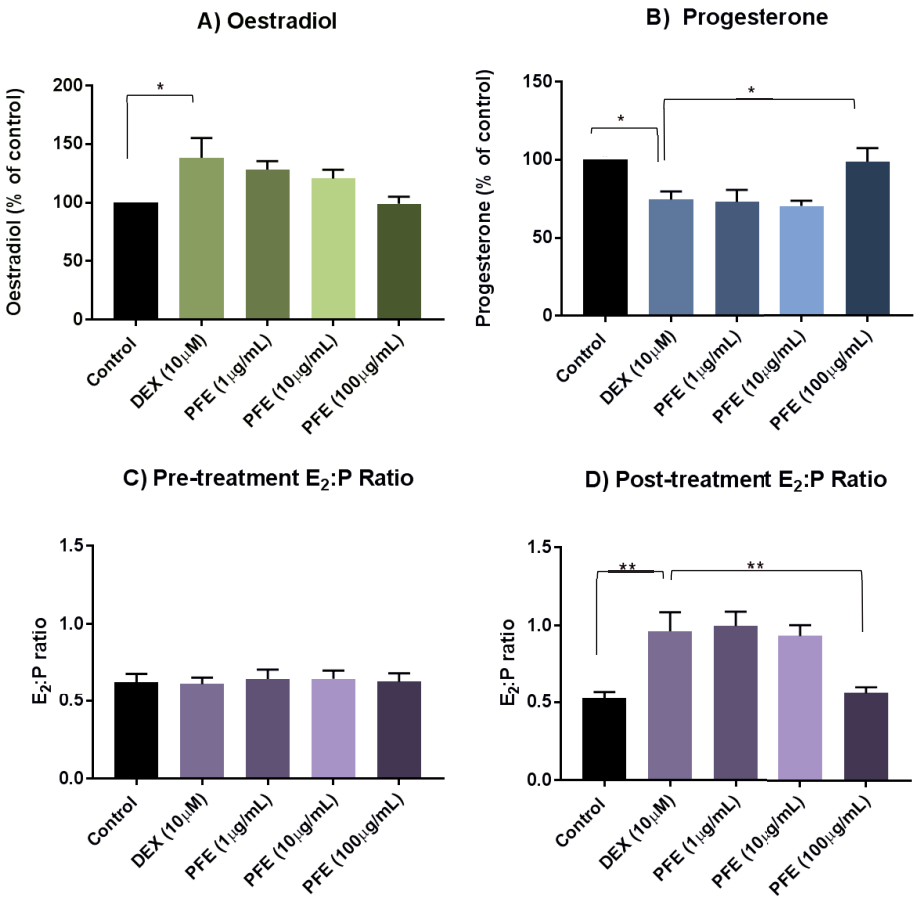

Figure 2.5 Oestradiol, progesterone and their ratios in paeoniflorin (PFE) treated granulosa cells. Granulosa cells were treated with dexamethasone (DEX) or DEX in the presence of PFE (1-100 μg/mL). Media was collected before treatment and 24 h post treatment for assay. Oestradiol and progesterone results are represented as a percentage (%) of control as the SEM± and ratios (E_2: P) from three independent replicates (n=3) *p<0.05, **p<0.01 compared to DEX treatment group.

Prior to cell culture treatment, oestradiol/progesterone (E_2/P) ratios were similar among all groups (Figure 2.5C). However, after 24-hour treatment, E_2/P ratios in control cells slightly decreased, while DEX significantly increased E_2/P ratio. PFE (100 μg/mL) significantly decreased E_2/P ratios compared to the DEX model group ($p<0.01$) as shown in Figure 2.5D.

2.4 Discussion

The results from this study indicate that DEX (10 μM) is an appropriate model for PCOS in murine theca and granulosa cells. More specifically, high-dose DEX significantly increased T secretion in the ovarian co-culture in the model test and with similar results in theca cells alone. While DEX has been used in previous studies to increase androgen production in theca cells, this has only been observed in porcine models at a concentration of 1 μM (Qu et al.) In this study, DEX therefore emulated biochemical hyperandrogenism in murine theca cells, the key clinical feature in PCOS. DEX did not, however significantly increase T in the herbal screening test which is likely due to the small sample size and large standard error. Time limitations restricted repeats of this particular experiment, however this work could be further explored in relation to other herbal compounds in future studies.

Additionally, LH and insulin in the model test failed to significantly increase androgen secretion which is contrary to previously reported studies (Stewart et al. 1995, Zhang et al. 2000, Cadagan et al. 2016) that used comparable concentrations in human, bovine and porcine theca cells. This could be due to the measurement of T as opposed to androstenedione which precedes T in the steroidogenic pathway and is up to 10 times more sensitive. Alternatively, the variation in species may account for this difference, however in the future, other concentrations of LH and insulin could be explored. To the best knowledge, this is the first time murine theca cells have been induced to increase testosterone production *in vitro* without prior *in vivo* interventions. In rat cells, bisphenol-A, an environmental endocrine disruptor has shown to increase testosterone secretion, however was not included as a model in this study.

Previously, it has been shown that PFE (100 μg/mL) combined with another herbal compound, glycyrrhizin, can suppress testosterone: androstenedione ratio in rat ovaries

(Takeuchi et al. 1991). The current study therefore highlighted a similar finding in which PFE was able to reduce T in DEX treated murine theca. The correlation between hyperandrogenism and IR has now been clearly established, as has the effects of paeoniflorin on modulating insulin and glucose metabolism highlighted in Chapter 1. These findings, among others, may therefore account for, at least in part, the ability of paeoniflorin to improve hyperandrogenism. Further investigations into the molecular mechanisms in which paeoniflorin modulates steroidogenesis and insulin signalling in ovarian cells is required.

This study also revealed that DEX has an inhibitory effect on P secretion. This was observed in both low (1 μM) and high (10 μM) concentrations of DEX in the co-culture model test as well as at the higher concentration in theca and granulosa cells alone. This effect appeared to be inversely related to increased E_2 in DEX model group. A previous study has also demonstrated that DEX in pre-ovulatory rat follicles, inhibited progesterone secretion by modulating cholesterol transport mediated by steroidogenic acute regulatory protein (StAR) (Huang et al. 2001) which may explain this effect in the present study. However, other reports have demonstrated that progesterone synthesis is enhanced by DEX in immature granulosa cells (Adashi et al. 1981), contrary to these findings. Immature granulosa cells may therefore behave differently in response to DEX compared to cells that are closer to luteinisation.

Luteinisation is a physiological process for ovarian cells which promotes ovulation and prevents excessive follicle accumulation (Amsterdam et al. 2003). Many existing studies report this phenomenon of 'functional luteinisation' of theca and granulosa cells *in vitro* (Campbell et al. 1998, Portela et al. 2010, Shimizu et al. 2016) which is characterised by cells changing from favouring oestrogen production to progesterone secretion which usually

occurs after 72 h in normal culture conditions. E_2 and insulin growth-like factor (IGF1) have been observed as pro-survival factors that may contribute to follicle immaturity and arrest (Carou et al. 2017). Furthermore, in women with PCOS, immature follicles were observed to secrete more E_2 than the non-arrested follicles of the same ovaries (Willis et al. 1998).

Conversely, increased P levels are considered to be pro-apoptotic (Braw et al. 1981, Amsterdam et al. 2003). Indeed, it has been demonstrated that follicles from women with PCOS have prolonged survival compared to non-PCOS controls that normally undergo hypertrophy and atresia (Webber et al. 2007) These studies collectively highlight that hormonal differences in granulosa cells are linked to anovulation and PCOM in PCOS. DEX therefore impairs functional luteinisation in murine ovarian cells evidenced by the significant increase in E_2/P ratio.

Importantly, paeoniflorin (100 µg/mL) significantly reversed this effect of DEX in granulosa cells and could potentially improve conditions for ovulation especially when P is insufficient due to anovulation and corpus luteum deficiency. P is essential for conception and implantation of the embryo and therefore further paeoniflorin may potentially be a candidate for adjunct therapy for hormonally related subfertility.

Paeoniflorin was also observed to be safe for theca and granulosa cells. DNA-labelling proliferation assays using dyes such as SYBR green I measure fluorescence intensity which can accurately reflect cell proliferation and cell cycle arrest (McGowan et al. 2011). PFE treated groups did not significantly affect cell proliferation when compared to control and did not exhibit apoptotic effects, even at higher concentrations (1000 µg/mL). However, cellular DNA content increased modestly when comparing the 24 and 48 h time points. Therefore, cells are still somewhat proliferative but not in a log-phase growth stage.

Interestingly, a decline in proliferative capacity has been shown to be indicative of functional luteinising of theca and granulosa cells (Plant et al. 2014). Both control and PFE treated cells in this SYBR assay were not highly proliferative. Besides changes to proliferation and hormone secretion, other markers of luteinisation include architectural changes in cell morphology such as hypertrophy of theca and granulosa cells which can be identified by microscopy. Therefore, observing morphological changes and labelling apoptotic pathways in cells treated with DEX and PFE using live cell imaging could be investigated for a deeper understanding of cell luteinisation in future experiments.

The inherent luteinisation of these cells however, meant that cell numbers were often difficult to obtain for plating experiments. Hormone levels also seemed to somewhat decline throughout cell passages. Cells for all experiments were used from passage 3-5 and a percent of control was therefore used to adjust for the difference in passage number.

In summary DEX treatment altered both theca and granulosa hormone profiles to resemble those characteristic of PCOS and therefore may be considered a useful *in vitro* model in murine ovarian cells. These changes were reversed by high-dose PFE (100 μg/mL) in both cell types which may be therapeutically important for all phenotypes of PCOS. Determining the effects of paeoniflorin in primary human PCOS cells and exploring the molecular mechanisms related to the steroidogenic pathway are warranted. In doing so, paeoniflorin may be considered a therapeutic agent in the treatment of PCOS through inhibiting testosterone secretion and promoting the luteinisation.

CHAPTER 3

The Molecular Mechanisms of Paeoniflorin Extract In Ovarian Theca Cells

CHAPTER 3: THE MOLECULAR MECHANISMS OF PAEONIFLORIN EXTRACT IN OVARIAN THECA CELLS

3.1 Introduction

The molecular signature of PCOS is primarily related to the dysfunction of genes and proteins that regulate steroidogenesis, folliculogenesis and luteinisation coordinated by theca and granulosa cells. Due to the discovery of insulin receptors in ovarian tissue and an evident correlation between PCOS and metabolic syndrome, defective insulin signalling has also been implicated when considering the molecular basis of the syndrome. However, the exact molecular mechanisms of PCOS currently remain enigmatic due to the complex endocrine, paracrine and autocrine regulation of theca and granulosa cells which are poorly understood.

Excessive androgen production is an important clinical feature of PCOS and the molecular understanding of hyperandrogenism in PCOS surrounds the idea of dysregulated steroidogenesis in theca cells. Studies have frequently shown that members of the CYP superfamily that regulate the ovarian steroidogenic pathway are upregulated (McAllister et al. 2015) in PCOS. More specifically, CYP11A1 as the rate-limiting step of the steroidogenic pathway is frequently observed to be overexpressed in PCOS tissue and plays an important role in determining the rate of ovarian hormone production (Mary et al. 2015). Similarly, CYP17A1 is upregulated in PCOS and regulates the synthesis of androgens in theca cells (Wickenheisser et al. 2005).

In the previous chapter, it has been demonstrated that paeoniflorin extract reverses dexamethasone (DEX) induced hyperandrogenism and maintains progesterone levels in

ovarian theca cells. As theca cells are the origin of ovarian hyperandrogenism, the molecular mechanisms in which DEX and paeoniflorin affect protein and gene expression are warranted. More specifically, the expression of P450 side chain cleavage encoded by *CYP11A1* and 17, 20 lyase encoded by *CYP17A1* were determined by western blotting, immunofluorescence staining and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

3.2 Materials and Methods

3.2.1 Materials

Cell culture reagents were the same as those used in the previous chapter. Mouse monoclonal anti-CYP17A1 and rabbit polyclonal anti-CYP11A1 rabbit antibodies were provided by Abcam (Cambridge, UK) and secondary antibodies (goat anti-mouse and goat anti-rabbit horseradish peroxidase conjugated) from Santa Cruz Biotechnology (Texas, USA). Other western blotting reagents including radioimmunoprecipitation assay buffer (RIPA) and 2X Laemmli loading buffer were provided by Sigma Aldrich (Missouri, USA) and enhanced chemiluminescent (ECL) substrate from Thermo Fisher Scientific (Massachusetts, USA). Gel reagents including glycerol were provided by Research Products International (Illinois, USA), Acrylamide from Bio-Rad (California, USA), ammonium persulfate and tetramethylethylenediamine (TEMED) from Sigma.

Bovine Serum Albumin (BSA) was provided by Sigma Aldrich and Hoesct® 33342 trihydrochoride trihydrate from Thermo Fisher while goat anti-rabbit Alexa Fluor 488 and goat anti-mouse IgG Alexa Fluor 568 secondary antibodies from Santa Cruz Biotechnology. Goat serum was from Thermo Fisher and Vectashield Antifade Mounting Medium for

Fluorescence H-1000 from Vector Laboratories, Inc. (California, USA) which were also used for immunofluorescence staining.

10X PCR buffer, MgCl2 (4 mM), Random N10 Hexamers (2.5 µM), RNAse inhibitor (20µ/µL) and Reverse Transcriptase were all provided by Applied Biosystems (California, USA) and 0.5 dNTPs from Sigma Aldrich. RNA extraction kit was provided by QIAGEN (Hilden, Germany) and RNA purification reagents from Promega (Wisconsin, USA). CYP11A1, CYP17A1 and β-actin oligonucleotide primers were supplied by Sigma Aldrich and Powerup SYBR green master mix from Thermo Fisher.

EasyLadder I molecular weight marker was supplied by Bioline (London, UK), Gelred nucleic acid gel stain from Biotium (California, USA) and gel loading dye, purple (6X) from New England Bio Labs Inc. (Massachusetts, United States).

3.2.2 Western Blotting

Theca cells were treated for 24 h with control media, DEX media (10 µM) or DEX with paeoniflorin media (1, 10 and 100 µg/mL) as described in Chapter 2. After 24 h, cells were washed and lifted with 0.25% trypsin-EDTA and cell suspension was then counted using 0.4% Trypan Blue solution (Thermo Fisher). Subsequently, cells were transferred into Eppendorf tubes, washed and centrifuged at 10, 000 g three times. RIPA buffer was added to the pellets and then frozen in liquid nitrogen for approximately 30 s. This was followed by quick thawing in room temperature water, vortexing and centrifuging. This process was repeated another two times to facilitate cell lysis.

Protein concentration was determined by Bradford assay. Total protein (8 µg) in 1X loading buffer was then separated on 10% SDS-page gels by electrophoresis for 90 min at 110 V.

Following this, gels were transferred onto polyvinylidene fluoride (PVDF) membranes and blocked in 5% skim milk in phosphate buffered saline (PBS) with Tween-20. Membranes were then immunoblotted with primary antibodies CYP11, CYP17 and β-actin (all 1:1000 dilution) overnight before washing and probing with secondary antibodies (1: 10,000) for 1 h. Protein bands were detected using Pierce Chemiluminescent HRP Substrate, exposing membranes using the Amersham Imager 600 (Illinois, USA). Protein bands were subsequently analyzed using Image J software. The data were normalised to housekeeping protein, β-actin and expressed as a relative density to the control group's band density.

3.2.3 Immunofluorescence staining

Theca cells were grown on sterilised glass coverslips and after 24 h treatment (as previously described), were washed twice with PBS and fixed in plate with 4% paraformaldehyde for 30 min. Following this, cells were permeabilised in PBS with Tween-20 and Triton X-100 (0.06% Tween-20 and 0.04% Triton X-100) for 30 min at room temperature. This was then aspirated and cells were washed twice with PBS. Cells were subsequently blocked with 5% BSA solution with 10% goat serum for 1 h.

Cells were then stained with primary antibodies (1: 500 CYP11 and 1: 500 CYP17) or PBS (for negative control) at room temperature for 1 h. Coverslips were then washed twice for 5 min each with PBS followed by staining with secondary antibodies (1: 400 goat anti-rabbit Alexa Fluor 488 and 1:400 goat anti-mouse IgG Alexa Fluor 568) or PBS (for secondary negative control) for 30 min. Cells were then washed twice with PBS before counterstaining the cell nuclei with Hoescht® (1: 1000) for 10 min. This was followed by a final wash with PBS.

Coverslips were then removed from the 24 well plates using clean forceps and mounted onto glass slides with mounting medium and sealed. All slides were then visualised under an epifluorescent microscope (Olympus BX51, Tokyo, Japan) and captured with digital camera (DP70, Tokyo, Japan), using a 40X objective. Each coverslip was imaged on the same day in 6 random positions. Randomisation was achieved by using the DAPI nucleus channel only. Fluorescence intensity was subsequently analysed using Fiji Image J software by masking individual cells in a field of view.

3.2.4 Quantitative reverse transcriptase polymerase chain reaction

After cell treatment, cells were lifted as previously described and transferred to Eppendorf tubes. Cells were then centrifuged at 10,000 g and washed three times with ice cold PBS. After washing, cells were transferred into a lysis buffer consisting of 1X PCR buffer in nucleotide free water and 1 U/µL RNAse inhibitor. Cells were then snap frozen by submerging in liquid nitrogen for 30 sec. Cells were lysed by a total of three rounds of snap-freezing, thawing and vortexing.

RNA was then extracted with an RNA extraction kit used according to the manufacturer's (QIAGEN) instructions. Briefly, samples were centrifuged at 4000 g for 3 min with 'RLT' buffer then added and mixed using pipette tip. The cell lysates were then centrifuged for 3 min at 20,000 G at room temperature. The supernatant was collected and pipetted into a new microcentrifuge. 70% ethanol was then added to the clear lysate, mixed and transferred to an RNeasy spin column with collection tube. Columns were then centrifuged for 15 s at 10,000 g. The flow through was discarded and 'RW1' buffer added to spin column. This was centrifuged for a further 15 s. Again, the flow-through was discarded and 'RPE' buffer was then added to the spin column. Columns were centrifuged for 2 min at 10,000 g. The

collection tube was then discarded and replaced with a microcentrifuge for storage of RNA. RNase-free water was then directly added to the spin column and centrifuged for 1 min to elute the RNA.

RNA content was then quantified using the NanoDrop One spectrophotometer (Thermo Fisher Scientific) and purified with DNAse treatment. Samples were adjusted to make 50 ng/10 µL or RNA based on the concentrations determined by NanoDrop. 0.1 U/µL DNase and 10x DNase were added to samples and incubated at 37°C for 30 min. Stop buffer was then added to each reaction and incubated at 99°C for 2 min. Samples were then stored at -80°C for further use.

Purified samples were then combined with 1X PCR buffer, MgCl2 (4 mM), 0.5 dNTPs, Random N10 Hexamers (2.5 µM), reverse transcriptase, RNAse inhibitor and nucleotide free water. For control samples, no RT and/or no RNA sample was added to Eppendorf tube. Samples were then run at 25°C for 10 min for primer annealing followed by 42°C for 30 min for cDNA synthesis. cDNA was then diluted (1: 1.5) in nucleotide free water and Powerup SYBR green master mix was added to samples in a 96-well plate with sequence-specific primers (see table 3.1)

Table 3.1 Primer sequences of *CYP11, CYP17* and *β-actin*

Genes	Primers (5'-3')	PCR product (bp)
CYP11	Forward: 5'-AAAGGGAGCTGGTACCTCTACT-3' Reverse: 5'-AAACTGACTCCAAAGTGCCCA-3'	122
CYP17	Forward: 5'-TGGAGGCCACTATCCGAGAA-3' Reverse: 5'-CACATGTGTGTCCTTCGGGA-3'	112
β-actin	Forward: 5'-CTTTGCAGCTCCTTCGTTGC-3' Reverse: 5'-CCATCACACCCTGGTGCCTA-3'	113

The well plate was then sealed, centrifuged and amplified using the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). Thermal cycling was programmed for 10 min at 95°C as initial denaturation, followed by 40 cycles of denaturation at 15 sec for 95°C, annealing for 1 min at 60°C, extension for 15 sec for 95°C for final extension for 1 min at 60°C.

After qRT-PCR, cycle threshold (Ct) values used for calculating relative expression of the averages of CYP11 or CYP17 from three replicates and were normalised to the reference gene (*β-actin*). Melt curves were used to assess the specificity of the PCR products. Relative expression levels were then calculated using the $2^{-\Delta\Delta Ct}$ value and data was then expressed as relative mRNA gene level after adjusting for initial DNAse treatment volumes.

PCR products were then mixed with loading buffer and run on 2% agarose gels with Gelred™ in 1x Tris/Borate/EDTA (TBE) buffer at 100V for 1 h and 20 min to ensure single bands. Bands and nucleic acid molecular weight marker were visualised using the UV transilluminator and captured using the Carestream MI Standard Edition™ Software.

3.2.5 Statistical Analysis

All statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test in GraphPad Prism software. Statistical significance was determined at $p<0.05$ and all data are expressed as the mean ±SEM.

3.3 Results

3.3.1 The effects of dexamethasone on CYP17 and CYP11 protein expression

According to the western blot results, DEX significantly increased protein expression of CYP17 ($p<0.05$) compared to the control group as shown in Figure 3.1. DEX however, did not significantly increase protein expression of CYP11.

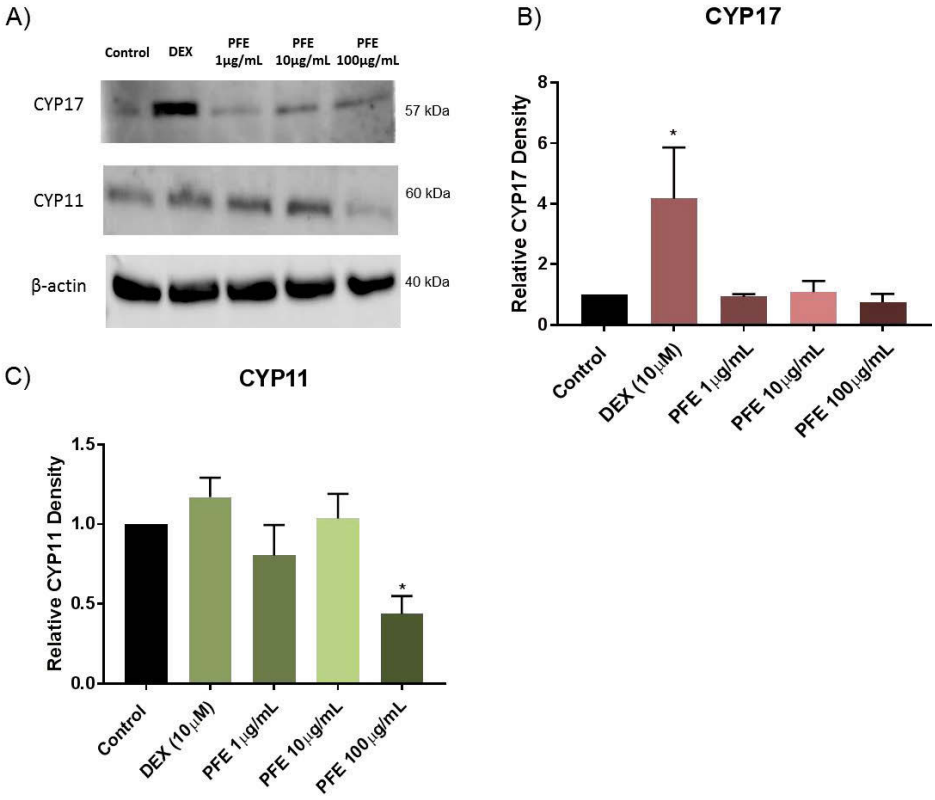

Figure 3.1 Western blot analysis of CYP17 and CYP11 protein expression in theca cells. Cells were treated with control media (control group), dexamethasone (DEX) media (10 μM) or DEX with paeoniflorin (PFE) (1, 10 or 100 μg/mL). After treatment, protein expression of CYP17 (3.1B) and CYP11 (3.1C) were revealed by western blotting and quantitated using Image J software. Data are expressed as relative density of the control group after normalising to housekeeping protein B-actin. *$p<0.05$ compared to the control group (N=3-4)

3.3.2 The effects of paeoniflorin on CYP11 and CYP17 protein expression

The PFE treatment groups at all concentrations (1, 10 and 100 μg/mL) significantly decreased protein expression of CYP17 ($p<0.05$) compared to the DEX group ($p<0.05$). Meanwhile, the high-dose PFE group (100 μg/mL) significantly decreased CYP11 protein expression ($p<0.05$) when compared to all groups (Figure 3.1).

This trend was reflected in the immunofluorescence staining, with decreased fluorescence intensity in PFE treated cells (100µg/mL). However, statistical significance was not determined (n=2) (Figure 3.2)

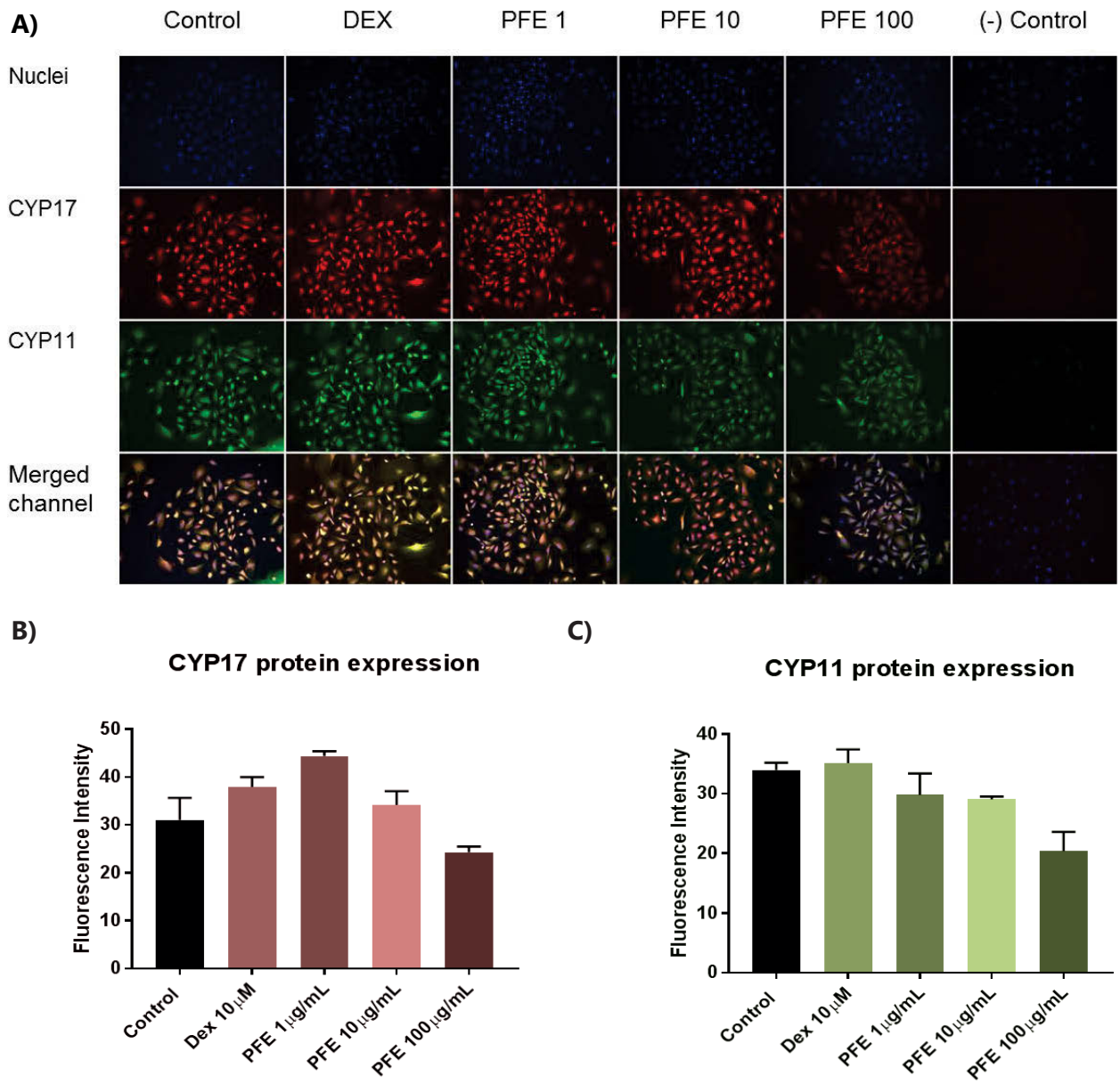

Figure 3.2 Immunofluorescence staining of CYP17 and CYP17. Cells were treated with control media (control), dexamethasone (DEX) media (10 µM) or DEX with paeoniflorin (PFE) (1, 10 or 100 µg/mL) and then stained with CYP11, CYP17 antibodies or no antibodies for negative control ((-) control). Cells were visualised under an Olympus BX51 fluorescent microscope (Tokyo, Japan) and captured with DP70 digital camera (Tokyo, Japan), using a 40X objective (3.2A). Each coverslip was imaged on the same day in 6 random positions using the nucleus channel only. Preliminary data of fluorescence intensity was subsequently analysed using Fiji Image J software (3.2B and 3.2C) (N=2-3).

3.3.3 The effect of paeoniflorin on *CYP11* and *CYP17* gene expression

According to the qRT-PCR data, *CYP11* gene expression was significantly increased in the high-dose PFE group (100μg/mL) compared to the control and DEX group ($p<0.05$). Paeoniflorin treatment at all concentrations did not significantly alter *CYP17* gene expression. Generally, DEX appeared to increase *CYP17* expression, however statistical significance was not reached.

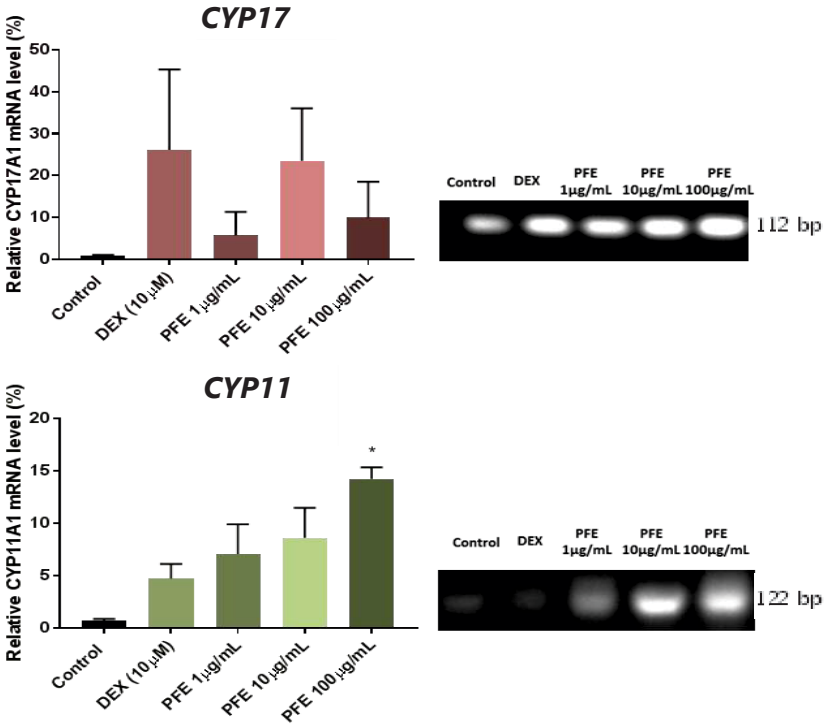

Figure 3.3 Quantitative reverse transcriptase polymerase chain reaction analysis of *CYP17* and *CYP11* mRNA expression. Theca cells were treated with control media (control), dexamethasone (DEX) media (10 μM) or DEX with paeoniflorin (PFE) (1, 10 or 100 μg/mL). Following treatment, cells were harvested and RNA extracted and purified. cDNA was then synthesised and amplified for sequence specific primer of *CYP11A1* and *CYP17A1* . Data is expressed as relative mRNA gene level normalised to housekeeping gene from three separate experiments. *$p<0.05$ compared to the control group (N=3).

3.4 Discussion

Ovarian steroidogenesis is initiated by cholesterol transport and synthesis of cholesterol to pregnenolone by enzymatic activity of steroidogenic acute regulatory protein (StAR) and

CYP11 respectively in theca cells. Meanwhile, the synthesis of androgens is primarily dependent on CYP17 which converts progestins to androgens. However, unlike StAR, only CYP11 and CYP17 are consistently shown to be differently expressed in PCOS tissue (Nelson et al. 2001, Wickenheisser et al. 2005). From the western blot analysis, DEX treatment was shown to significantly increase protein expression of CYP17 in murine theca cells. This finding may therefore explain the increase in testosterone secretion by these cells (as described in Chapter 2) through upregulation of androgen synthesis. DEX also modestly increased CYP11 protein expression; however, this was not statistically significant which suggests the main way in this study in which DEX increased testosterone secretion was through this upregulation of CYP17.

While DEX also tended to increase mRNA expression of *CYP17*, this was not statistically significant. Contrary to this, a similar study in porcine theca demonstrated that DEX did significantly increase *CYP17* mRNA expression (Zhao et al. 2011). The failure to reach statistical significance may be due to technical error suspected by a relatively large standard error. Alternatively, post-translational modifications could be involved in the discrepancy between protein and mRNA results. For example, serine phosphorylation of *CYP17* has been shown to increase CYP17 enzyme activity (Zhang et al. 1995). This may therefore shed light on the molecular links between insulin resistance and hyperandrogenism in PCOS as serine phosphorylation of insulin receptor proteins can diminish their capacity to activate PI 3-kinase and therefore contribute to insulin resistance when compared to tyrosine phosphorylation (Le Marchand-Brustel et al. 2003, Copps et al. 2012).

Meanwhile, it is widely observed that use of glucocorticoids such as DEX can induce whole-body insulin resistance (Geer et al. 2014). One of the involved mechanisms may also be the

rapid increase of serine phosphorylation which glucocorticoids have demonstrated (Solito et al. 2003, Ferris et al. 2012). Altered insulin signalling induced by DEX in theca cells has previously been established evidenced by reduced glucose uptake, decreased insulin receptor substrate -1 (*IRS-1*) and glucose transporter 4 (*GLUT-4*) mRNA expression and increased peroxisome proliferator–activated receptor γ (*PPAR-γ*) mRNA expression (Zhao et al. 2011) which was also linked to increased *CYP17* mRNA expression and hyperandrogenism. Future investigation into the effects of DEX on phosphorylation of CYP17 and CYP11 and other post translational protein modifications in theca cells may therefore provide further insights.

Paeoniflorin at all concentrations (1, 10 and 100 µg/mL) was shown to be protective of the effects of DEX on CYP17 protein expression as all PFE groups were decreased to similar levels of the control group. However, the high concentration of Paeoniflorin also decreased protein expression of CYP11 compared to all groups including the control and DEX model group. Therefore, Paeoniflorin (100 µg/mL) downregulated both CYP11 and CYP17 contributing to a decrease in T levels. This dual mechanism may explain the dose-dependent manner in which PFE reduces T. To the best knowledge, this is the first time paeoniflorin has been described to have an effect on CYP11 and CYP17 protein expression.

The ability of paeoniflorin to modulate CYP11 and CYP17 protein expression was also reflected in the immunofluorescence stain visually and from preliminary data from two to three experiments. However, due to the passage of cell used, cell proliferation was decreased which impacted the ability to repeat the experiments due to sub-optimal cell numbers for seeding. Contributing to this oversight was number of cells used for optimising cell culture conditions as there was limited information in the literature about these primary cells and

this was the first time theca cells were cultured in this particular lab. For the same reason, time was also another constraint which prevented a further of the immunofluorescence stain. Despite this, the immunofluorescence data allowed for a visual confirmation of the cell type as in ovarian cells, CYP17 is expressed only in theca cells (Havelock et al. 2004, Magoffin 2005). Contrastingly, CYP11 can be found in theca, granulosa and luteinised versions of these cells.

Interestingly, paeoniflorin at the highest concentration (100 µg/mL) significantly increased *CYP11* mRNA expression which was contrary to the trend in the protein results. This may be due to the luteinisation of paeoniflorin treated cells. This corresponds to the increase in progesterone levels found in the high dose paeoniflorin treated cells (100 µg/mL) compared to the DEX model. Progesterone and *CYP11* expression in immature follicles, follicles undergoing early atresia and progressed atresia have previously been compared with the study finding an increased progesterone level associated with progressed atresia was associated with increased *CYP11* mRNA expression (Pan et al. 2012) with similar findings in a more recent study in maturing porcine theca (LaVoie 2017). Furthermore, *CYP11* expression was shown to rapidly increase during granulosa cell luteinisation in rats via histone modification and chromatin structure remodelling (Okada et al. 2016) indicating that luteinisation is heavily orchestrated by epigenetic influences.

This, however, does not fully explain why the protein expression of CYP11 was decreased by paeoniflorin. Therefore transcriptional, translational and post translational factors could potentially be involved as the CYP superfamily are highly regulated by these various processes (Aguiar et al. 2005). Transcription of the gene *CYP11* could potentially be increased while translation or post-translation modifications could decrease the protein

expression. Transcription factors from the nuclear hormone receptor family including steroidogenic factor 1 (SF1) and androgen receptor (AR) are well characterised as having a regulatory role of CYP11 gene expression (Mizutani et al. 2015). GATA-binding factor 6 (GATA-6) has been shown to be a transcriptional activator of *CYP11* and is frequently observed to be differently expressed in theca cells from women with PCOS ((Wood et al. 2003) Ho, Wood et al. 2005, McAllister, Legro et al. 2015). Paeoniflorin has previously been shown to induce heat shock proteins (Yan et al. 2004) , transcription factors that have also been implicated in PCOS (Park et al. 2012). Therefore, further investigation into paeoniflorin's ability to modulate transcription factors in steroidogenesis is warranted with attention to differences in pre-luteinised and luteinised cells.

Post-translation factors such as phosphorylation could also play a role as there is evidence to suggest that phosphorylation of CYP11 leads to increased enzyme activity (Vilgrain et al. 1984, Aguiar et al. 2005). Therefore, one mechanism in which paeoniflorin may work could be through decreased phosphorylation. Indeed, the insulin sensitiser, metformin was shown to decrease steroidogenesis and CYP11 protein expression due to decreased phosphorylation in granulosa cells (Tosca et al. 2007). Paeoniflorin has known insulin sensitising properties (Ma et al. 2017) and therefore may act similarly to metformin in post-translation of CYP11 in ovarian cells at 100 µg/mL. Other mechanisms involving *CYP11* translation or degradation time or protein could also be altered with further experiments needed.

Overall, the DEX model was shown to increase protein expression of CYP17 which may contribute to the increase in steroidogenesis observed in theca cells. Paeoniflorin protected against this effect at all doses and additionally reduced CYP11 protein expression at the higher concentration (100 µg/mL) which might correlate with the dose-dependent way in

which testosterone secretion was decreased in Chapter 2. *CYP11* mRNA expression was significantly increased by paeoniflorin with further investigation into the transcriptional and post-transcriptional factors required.

CHAPTER 4

Final Discussion, Future Directions and Conclusions

CHAPTER 4: FINAL DISCUSSION, FUTURE DIRECTIONS AND CONCLUSIONS

4.1 Discussion and Future Directions

PCOS is an increasingly common disorder that warrants further research. Not only does the syndrome impact reproduction and metabolism but is recognised as a stepping stone to more severe diseases such as T2DM and cardiovascular disease. In addition to this, there are currently limited therapeutic options for managing PCOS and further work is needed to ensure the development of better therapeutic options to prevent the associated sequelae.

To meet these needs, further research into the pathogenesis of PCOS is required. Basic scientific studies in particular are worthwhile as they can provide a deeper understanding of the physiological microenvironment of the ovary and its dysfunction in relation to steroidogenesis, folliculogenesis and ovulation. Given the complexity of the feedback mechanisms of the reproductive hormones that regulate these processes alone, it is no wonder that other factors such as insulin signalling and regulation of theca, granulosa and luteal cells are not well understood. One of the aims of this study was to develop a cell culture model of PCOS in murine theca and granulosa cells as this had not yet been established. As reported in Chapter 2, DEX, a glucocorticoid that can disrupt insulin signalling was successfully able to emulate features of PCOS *in vitro* in these cells. More specifically, significantly increased testosterone secretion in theca cells and increased oestradiol: progesterone ratios in granulosa cells which could correspond to hyperandrogenism and ovulatory dysfunction in PCOS respectively. In theca cells, the main finding for this increase in testosterone was through upregulation of CYP17 protein expression.

Chinese herbal medicine has long been used to treat disorders related to gynaecological and reproductive dysfunction. Herbs used clinically in the treatment of disorders such as PCOS have shown to help ameliorate menstrual irregularities and signs of hyperandrogenism such as acne as well as helping to improve fertility outcomes (Qu et al. 2015). One advantage of herbal medicine is the use of multiple herbs in a formula, and therefore, when combined can target multiple signs, symptoms and mechanisms of diseases. PCOS is a heterogeneous disorder with various phenotypes and may involve systemic processes such as inflammation and oxidative stress. Additionally, it predisposes the risk to other metabolic disorders such as NALFD and T2DM further complicating therapeutic options. Therefore, Chinese herbal formulae may be a valuable therapeutic adjunct to conventional therapy in its ability to individualise for the phenotype, ameliorate multiple targets simultaneously and prevent the onset of comorbidities.

Chinese herbal medicines may also be a novel therapy for PCOS as they are generally associated with fewer side effects compared to conventional drugs (Yeh et al. 2003). This is particularly important for conditions such as PCOS that are life-long and may require ongoing treatment. Furthermore, many women with PCOS are already exploring natural treatments for their disorder which alone should warrant exploration into the mechanisms of herbal medicine.

Paeoniflorin is a unique candidate for PCOS as it has shown to also positively impact other relevant facets of the disorder including insulin resistance and adipose tissue dysfunction (Jiang et al. 2012, Zhang et al. 2015). In Chapter 2 of this study, paeoniflorin (100μg/mL) reduced DEX induced androgen production in theca cells in a dose-dependent manner which may implicate a beneficial role for paeoniflorin in ameliorating hyperandrogenism.

Therefore, one of the mechanisms in which paeoniflorin may help decrease thecal steroidogenesis is through improving insulin signalling in these cells. Previous studies have demonstrated that indeed, Chinese herbal compounds, such as berberine can benefit expression of GLUT-4, IRS-1 and PPAR-γ in theca cells (Zhao et al. 2011). While this was not carried out in this study, this could be a future avenue for research. Despite this, at least some of the mechanisms in which Paeoniflorin decreases androgen production was elucidated in this research, and that was through the downregulation of CYP11 and CYP17. A novel finding in relation to the mechanisms of paeoniflorin was the increase in mRNA expression of *CYP11*. This highlights the potential for future research into the transcriptional, translational and post-translational factors in which paeoniflorin interacts with theca cells. In general, there is more that can be learned about these factors that influence PCOS, especially those shown to both upregulate CYP11 and CYP17 enzymatic activity as well as regulation of cellular proliferation, differentiation and apoptosis. For example, the GATA transcription factor family may be useful candidates. GATA-6 has been shown to be associated with hyperandrogenism by promoting CYP11 and CYP17 enzyme activities in theca cells (Wood et al. 2004). Other studies have shown that increased expression of GATA-6 and decreased GATA-4 is linked to apoptosis of the ovarian follicle (Gillio-Meina, Hui et al. 2003). Furthermore, GATA-4 expression has been shown to be correlated with increased cell proliferation and decreased apoptosis in both mouse and human granulosa cells (Heikinheimo, Ermolaeva et al. 1997) and therefore may play a role in granulosa differentiation. As mentioned, DEX in the current project was shown to increase steroidogenesis in theca cells and decrease functional luteinisation in granulosa cells. GATA-4 and GATA-6 may therefore be altered by DEX and paeoniflorin.

Another finding in relation to paeoniflorin was that it protected against adverse oestradiol: progesterone ratios. As previously discussed, this may suggest the functional luteinisation of the cells in culture. Due to time limitations, the molecular mechanisms of the way in which paeoniflorin modulates hormones in granulosa cells were not covered. Anti-mullerian and CYP19 which controls the synthesis of androgens However, this study has outlined some key areas to investigate in the future, particularly in relation to functional luteinisation. Differences in paeoniflorin-treated cell shape and size through live cell imaging may provide further insights into when and how luteinisation occurs. Additionally, further markers of functional luteinisation could be assessed including those involved with cell differentiation and physiological apoptosis such as endoplasmic reticulum stress.

One shortcoming of this study was that there were no morphological studies conducted due to time constraints. However, the immunofluorescence images presented in Chapter 3 allowed for a visual confirmation of molecular characteristics of the theca cell type as CYP17A1 is expressed only in theca cells (Magoffin 2005). CYP11A1 expression is also specific to murine theca cells when compared to granulosa cells (Tian et al., 2015). Cells stained with both primary antibody and secondary antibody showed high fluorescence compared to cells stained with secondary antibody only therefore indicative of thecal cell cultures. Despite this, the purity of this should be identified in the future, particularly when using co-cultures of theca-granulosa cells. Another priority would be to investigate molecular and/or morphological characteristics in granulosa cells.

Androgen synthesis is not exclusive to the ovaries and in PCOS adrenally-derived androgens may also be increased. It would therefore be useful to explore the effects of Paeoniflorin on

other cell types in adrenal tissue and could potentially help other disorders that are characterised by androgen excess.

4.2 Conclusions

Dexamethasone was a useful model of PCOS in primary murine theca and granulosa cells as it emulated hyperandrogenism which was predominately through increased CYP17 protein expression and may additionally impair luteinisation. Paeoniflorin, a naturally derived compound reversed the effects of dexamethasone by decreasing testosterone production in theca cells. This was due to downregulation of CYP11 and CYP17 protein which regulate the ovarian steroidogenic pathway. mRNA expression of CYP11 and CYP17 in theca cells and their transcription factors need to be further clarified. In granulosa cells, paeoniflorin protected against adverse oestradiol: progesterone ratios which suggests that the compound may positively influence cell development and luteinisation, however the molecular mechanisms of this have yet to be defined. In conclusion, paeoniflorin has demonstrated effects *in vitro* that may support clinical research and future use of paeoniflorin in the treatment of PCOS with further experiments warranted.

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