Androgen Receptor Mediated Activity In The Ovary: Implications For Polycystic Ovary Syndrome.

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THESIS ABSTRACT

Context
The expression of androgen receptors (AR) in follicular granulosa cells (GC) of mammals suggests a role for direct AR-mediated androgen activity in the regulation of folliculogenesis, however this role and the mechanistic pathways involved have not been fully characterised. In women, excess androgen is a characteristic feature of polycystic ovary syndrome (PCOS), but AR-mediated activity has not been widely investigated in relation to the pathophysiology of this disorder.

Hypotheses
The current thesis tested two general hypotheses related to AR activity in PCOS: 1) The polymorphic (CAG)n repeat region in the AR gene, which has functional implications for receptor activity, influences the manifestation of PCOS and 2) AR signalling is disrupted in GC from women with PCOS.

Results
In a cross-sectional population analysis, this thesis reports an association between PCOS and long CAG repeat tracts in the AR gene, which functionally represent reduced androgen sensitivity. The association was further enhanced by compensating for the influence of X chromosome inactivation (XCI) on expression of specific AR alleles. Preferential expression of long CAG repeat tracts positively correlated with serum testosterone levels in PCOS patients. In an analysis of sister pairs with the same CAG repeat genotype at the AR locus, different patterns of XCI were evident when sisters had a different clinical manifestation of PCOS. Collectively, these results provide evidence that supports the hypothesis that the (CAG)n polymorphism in the AR influences the manifestation of PCOS, the effects of which are modulated by variable allele expression via a mechanism involving XCI. These findings accord with the concept that both genetic and environmental factors are determinants of this disorder.
At the level of the ovary, AR-mediated signalling in follicular GC was influenced by proximity to the oocyte in both pigs and humans. In particular, the ability of androgen to directly induce porcine GC proliferation in vitro was dependent upon presence of the oocyte or the oocyte mitogen, growth differentiation 9 (GDF9). This finding provides a potential mechanism to explain how androgens may enhance early follicle growth. Granulosa cells from women with PCOS had normal mRNA expression for AR signalling molecules, but GC surrounding the oocyte in vivo had reduced AR protein content and diminished responses to androgen in culture as compared to those from normal ovaries. GC from women with PCOS also expressed mRNA for an androgen-regulated serine protease (hKLK3), which did not occur in normal GC. Therefore, follicular GC from women with PCOS have evidence of perturbed AR-mediated signalling which is likely to contribute to the pathophysiology of this disorder. As AR-mediated signalling is influenced by the oocyte, the differences in AR-mediated signalling in GC from women with PCOS may be indicative of dysregulated signals emanating from the oocyte.

**Conclusion**

The results of this thesis indicate that abnormal AR action occurs in PCOS, but further investigation is required to determine whether this phenomenon represents a primary disruption or a secondary consequence of another primary disruption in the sequence of events that leads to aberrant folliculogenesis in this disorder.
DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made.

I give consent for this thesis, when deposited in the University of Adelaide library, to be available for photocopying and loan.

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PUBLICATIONS ARISING FROM THIS THESIS

2005


**Hickey** TE, Legro RS, and Norman RJ. Epigenetic modification of the X chromosome influences susceptibility to polycystic ovary syndrome. *Journal of Clinical Endocrinology and Metabolism* (submitted 12 January 2006)

2004


2002


ABSTRACTS ARISING FROM THIS THESIS

2005

Hickey TE, Alvino HJ, Gilchrist RB, and Norman RJ. Granulosa cells from polycystic ovaries have increased expression of androgen receptor regulated kallikrein 3, but normal expression of androgen receptor and two androgen receptor co-activators. Proc 87th Annual Meeting of the Endocrine Society, San Diego, CA, USA. Abstract # P2-242, pg 415. (Winner, Australian Women in Endocrinology Award)

Hickey TE, Milner CR, Sheetz B, Legro RS, and Norman RJ. Differential patterns of X-inactivation among sisters in family groups with polycystic ovary syndrome. Annual Meeting of the Androgen Excess Society, San Diego, CA, USA. (Winner, New Investigator Award)

Hickey TE, Marrocco DL, Amato F, Ritter LJ, Norman RJ, Gilchrist RB, and Armstrong DT. Androgens augment the mitogenic effects of oocyte-secreted factors and growth differentiation factor 9 on porcine granulosa cells. Annual Meeting of the Society for Reproductive Biology, Perth, Australia. (Winner, New Investigator Award)

Hickey TE, Milner CR, Sheetz B, Legro RS, and Norman RJ. Differential patterns of X-inactivation among sisters in family groups with polycystic ovary syndrome. 48th Annual Meeting of the Endocrine Society of Australia, Perth, Australia. (Finalist, New Investigator Award)

Hickey TE, Alvino HJ, Gilchrist RB, and Norman RJ. Androgen receptor mediated activity in ovarian granulosa cells: Implications for polycystic ovary syndrome. Annual Meeting of the Australian Society for Medical Research, SA division, Adelaide.
Hickey T, Alvino H, Gilchrist R, and Norman, R. Androgen receptor associated signalling in ovarian granulosa cells from normal and polycystic ovaries. National Meeting of the Australian Society for Medical Research, Couran Cove, Queensland.


2004


Hickey TE, and Norman RJ. Expression of prostate specific antigen in the ovary. Annual Meeting of the Australian Society for Medical Research, SA division, Adelaide.


2003

Hickey TE, and Norman, RJ. Expression of androgen receptor and enhancement of FSH signaling by dihydrotestosterone in a granulosa tumour cell line. Proc. 46th Annual Meeting of the Endocrine Society of Australia, Melbourne. Abstract # 169, pg 120.


2002


2001

Hickey TE, Chandy A, Norman RJ. Androgen receptor CAG repeat polymorphism and X-chromosome inactivation in Australian Caucasian women with polycystic ovaries. Proc 83rd Annual Meeting of the Endocrine Society, Denver CO, USA. Abstract # 0R19-3, pg 102.

1999

Hickey TE, and Norman RJ. Variation of polymorphic trinucleotide repeat in the androgen receptor in Caucasian women with polycystic ovary syndrome. 42nd Annual Scientific Meeting of the Endocrine Society of Australia, Melbourne. Abstract #63, pg 91.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A4</td>
<td>Androstenedione</td>
</tr>
<tr>
<td>AMH</td>
<td>Anti-Mullerian Hormone</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Analysis of Covariance</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>ARA</td>
<td>Androgen Receptor Associated protein</td>
</tr>
<tr>
<td>ARE</td>
<td>Androgen Response Element</td>
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<tr>
<td>ASRM</td>
<td>American Society for Reproductive Medicine</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchinonic Acid</td>
</tr>
<tr>
<td>B-TCM</td>
<td>Bicarbonate-buffered Tissue Culture Medium</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>C</td>
<td>Carbon</td>
</tr>
<tr>
<td>CAG</td>
<td>Cytosine Adenosine Guanine (nucleotide sequence)</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB Binding Protein</td>
</tr>
<tr>
<td>CC</td>
<td>Cumulus Cells</td>
</tr>
<tr>
<td>CCG</td>
<td>Cytosine Cytosine Guanine (nucleotide sequence)</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CL</td>
<td>Corpus Luteum</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>COC</td>
<td>Cumulus Oocyte Complex</td>
</tr>
<tr>
<td>COOH</td>
<td>Carboxyl group</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>CPM</td>
<td>Counts Per Minute</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP Response Element Binding Protein</td>
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<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
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<tr>
<td>DBD</td>
<td>DNA Binding Domain</td>
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<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
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<td>DO</td>
<td>Denuded Oocytes</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E1</td>
<td>Estrone</td>
</tr>
<tr>
<td>E2</td>
<td>Estradiol</td>
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<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
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<td>EDTA</td>
<td>Ethylenediamine Tetraacetic Acid</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinase</td>
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<tr>
<td>ESHRE</td>
<td>European Society for Human Reproduction and Embryology</td>
</tr>
<tr>
<td>FAI</td>
<td>Free Androgen Index</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
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<tr>
<td>FHL</td>
<td>Four and a half LIM domains</td>
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<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
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<tr>
<td>FSHR</td>
<td>FSH Receptor</td>
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<tr>
<td>GATA</td>
<td>Family of transcription factors</td>
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<td>GC</td>
<td>Granulosa Cells</td>
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<tr>
<td>GDF</td>
<td>Growth Differentiation Factor</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotrophin Releasing Hormone</td>
</tr>
<tr>
<td>GEE</td>
<td>General Estimating Equation</td>
</tr>
<tr>
<td>GLC</td>
<td>Granulosa Lutein Cells</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>HA</td>
<td>Hyperandrogenic</td>
</tr>
<tr>
<td>hCG</td>
<td>Human Chorionic Gonadotrophin</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-cyclohexyl-2-aminoethanesulfonic acid</td>
</tr>
<tr>
<td>HIC</td>
<td>Hydrophobic Interaction Chromatography</td>
</tr>
<tr>
<td>hKLK</td>
<td>Human Kallikrein</td>
</tr>
<tr>
<td>HPO</td>
<td>Hypothalamic Pituitary Ovarian Axis</td>
</tr>
<tr>
<td>HSD</td>
<td>Hydroxysteroid Dehydrogenase</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat Shock Protein</td>
</tr>
<tr>
<td>H-TCM</td>
<td>HEPES-buffered Tissue Culture Medium</td>
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<td>ICSI</td>
<td>Intracytoplasmic Sperm Injection</td>
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<td>IGF</td>
<td>Insulin-like Growth Factor</td>
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<td>IGF Binding Protein</td>
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<td>IGFR</td>
<td>IGF Receptor</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>INS</td>
<td>Insulin gene</td>
</tr>
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<td>IRS</td>
<td>Insulin Receptor Substrate</td>
</tr>
<tr>
<td>IU</td>
<td>International Units</td>
</tr>
<tr>
<td>IVF</td>
<td>In Vitro Fertilization</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo-Daltons</td>
</tr>
<tr>
<td>KITL</td>
<td>Kit Ligand</td>
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<td>LBD</td>
<td>Ligand Binding Domain</td>
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<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
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<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
</tr>
<tr>
<td>LHR</td>
<td>LH Receptor</td>
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<td>LIM</td>
<td>A conserved cysteine-rich protein domain</td>
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<tr>
<td>MAP</td>
<td>Mitogen Activated Protein</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MAPK</td>
<td>MAP Kinase</td>
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<td>MGC</td>
<td>Mural Granulosa Cells</td>
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<td>mg</td>
<td>Milligrams</td>
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<td>ml</td>
<td>Millilitres</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetres</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut-Off</td>
</tr>
<tr>
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<td>Sodium Chloride</td>
</tr>
<tr>
<td>ND</td>
<td>Not Determined</td>
</tr>
<tr>
<td>NH$_2$</td>
<td>Amino group</td>
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<tr>
<td>NICHD</td>
<td>National Institute of Child Health and Development</td>
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<td>NIH</td>
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<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>OHF</td>
<td>OH-Flutamide (hydroxyflutamide)</td>
</tr>
<tr>
<td>OSF</td>
<td>Oocyte Secreted Factor(s)</td>
</tr>
<tr>
<td>P450arom</td>
<td>P450 Aromatase</td>
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<tr>
<td>P450scc</td>
<td>P450 Side chain cleavage</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PBR</td>
<td>Peripheral-type Benzodiazepine Receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PCO</td>
<td>Polycystic Ovaries</td>
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<tr>
<td>PCOS</td>
<td>Polycystic Ovary Syndrome</td>
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<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
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<tr>
<td>PI-3</td>
<td>Phospho-inosital-3</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>-------------</td>
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<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
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<td>pmol</td>
<td>Picomoles</td>
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<tr>
<td>PVA</td>
<td>Polyvinyl Alcohol</td>
</tr>
<tr>
<td>rhFSH</td>
<td>recombinant human FSH</td>
</tr>
<tr>
<td>rhIGF1</td>
<td>recombinant human IGF1</td>
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<tr>
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<td>Radioimmunoassay</td>
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<td>Radioimmunoprecipitation Assay buffer</td>
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<td>Ribonucleic Acid</td>
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<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>SAS</td>
<td>Statistical Analysis Software</td>
</tr>
<tr>
<td>SBMA</td>
<td>Spinobulbar Muscular Atrophy</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecylsulphate</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>SHBG</td>
<td>Steroid Hormone Binding Globulin</td>
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<tr>
<td>SMAD</td>
<td>Mothers Against Decapentaplegic protein</td>
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<td>SPSS</td>
<td>Scientific Products SYSTAT Software</td>
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<tr>
<td>SRC</td>
<td>Steroid Receptor Coactivator</td>
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<tr>
<td>StAR</td>
<td>Steroidogenic Acute Regulatory protein</td>
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<tr>
<td>T</td>
<td>Testosterone</td>
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<tr>
<td>TCM</td>
<td>Tissue Culture Medium</td>
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<tr>
<td>TET</td>
<td>Tetrachlorofluorescein</td>
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<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
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<tr>
<td>TRAM</td>
<td>Thyroid Receptor Activator Molecule</td>
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<tr>
<td>TSX</td>
<td>Transsexual</td>
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<tr>
<td>µCi</td>
<td>Microcurie</td>
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<tr>
<td>Abbreviation</td>
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<td>ug</td>
<td>Micrograms</td>
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<td>U</td>
<td>Units</td>
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<tr>
<td>URR</td>
<td>Upstream Regulatory Region</td>
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<tr>
<td>UTR</td>
<td>Untranslated Region</td>
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<tr>
<td>v/v</td>
<td>volume / volume</td>
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<tr>
<td>VNTR</td>
<td>Variable Nucleotide Triplicate Repeat</td>
</tr>
<tr>
<td>XCI</td>
<td>X Chromosome Inactivation</td>
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Chapter 1  Literature Review

1.1  Introduction

Androgens are steroid hormones predominantly produced by the gonads and adrenal glands of both sexes. While males have larger quantities of circulating androgens compared to females, this category of sex hormone still plays an important role in normal female physiology, including healthy reproductive functioning. Excessive androgen production in women is associated with ovarian dysfunction, and is the cardinal feature of polycystic ovary syndrome (PCOS), a leading cause of female infertility. Androgens act directly within cells by binding the androgen receptor (AR) and, in the classic manner of steroid hormone action, influence the expression of androgen-responsive target genes. This steroid hormone receptor is expressed in ovarian cells, which exist in an environment that is relatively high in androgens compared to other female body compartments. These characteristics clearly implicate AR-mediated activity as being important for normal ovarian function, but to date specific roles and mechanistic pathways for such activity remain obscure. Presumably, the presence of excess androgens in the ovaries of women with PCOS would disrupt AR-mediated signalling, but this concept has not been widely investigated in relation to the pathophysiology of this disorder. As a prelude to the work presented in this thesis, the following literature review will summarize current knowledge concerning normal ovarian function, abnormal ovarian function in PCOS, characteristic features of the AR and its presence in ovarian cells, and finally, AR dynamics in relation to PCOS.

1.2  General Ovarian Anatomy and Physiology

1.2.1  Ovarian anatomy

Two ovaries are located in the pelvic cavity of women, each suspended between the peritoneum and the uterus by ligaments. The body of an ovary (Figure 1.1) is largely
Figure 1.1. Anatomy of the ovary as described in the thesis text. Source of original drawing is unknown.
comprised of stromal tissue, stratified into an inner medulla and outer cortex, which is encapsulated by the ovarian surface epithelium and an outermost covering of connective tissue (tunica albuginea). The cortical region of the ovary contains immature germ cells (oocytes), each surrounded by a single layer of squamous granulosa cells; collectively, this forms a structure called a primordial follicle. Throughout a female’s lifespan, primordial follicles are stimulated to grow and appear at varying stages of development depending upon the hormonal milieu characteristic of a particular age and/or stage of the menstrual cycle.

During active reproductive years, fluid-filled antral follicles of various sizes appear in the ovary. These have the following distinct characteristics (Figure 1.2): a basement membrane separating the inner follicle chamber from a vascularized layer of surrounding theca cells that have differentiated from the stroma; a cavity filled with follicular fluid called the antrum; an inner wall of mural granulosa cells (MGCs); and an oocyte encased in layers of cumulus granulosa cells (CCs), which are intimately attached to the oocyte via gap junctions. At this stage of development, the oocyte and accompanying CCs are called a cumulus-oocyte complex (COC). Antral follicle growth peaks in the form of large, pre-ovulatory (Graafian) follicles (Figure 1.1). Apart from size, these follicles have the characteristic feature of an expanded COC. Expansion involves changes to the extracellular matrix surrounding cumulus cells and is necessary for ovulation to occur [1, 2]. Following ovulation of the COC into the reproductive tract, the follicle is remodelled into the corpus luteum (CL) which secretes the hormones necessary to establish and maintain pregnancy should it occur; in the absence of pregnancy, CL are reabsorbed. Atretic follicles are also a feature of the ovary, present throughout the lifespan. Atresia occurs when follicle development has been arrested, with subsequent death and resorption of follicular cells [3].

For medical purposes, transabdominal or transvaginal 2- or 3-dimensional ultrasonography are currently the most practical and reliable methods to observe and
**Figure 1.2:** Structure of an antral follicle. The basement membrane separates the epithelial granulosa cells from the mesenchymal thecal and stromal cells. Granulosa cells that remain attached to the oocyte following antrum formation are called cumulus cells (CC), and together these form a cumulus-oocyte complex (COC). Granulosa cells that remain associated with the basement membrane following antrum formation are called mural granulosa cells (MGC). These two granulosa cell subtypes are phenotypically distinct. The antrum becomes filled with follicular fluid, which is a filtrate of blood serum that also contains the secretions of the follicular cells.
characterise features of the ovary, with the transvaginal approach providing greater resolution and less disruption from fatty tissue [4].

1.2.2 Ovarian physiology

The two basic functions of the ovary are: 1) to nurture and extrude mature oocytes capable of being fertilized by sperm in the reproductive tract and 2) to produce steroid hormones for both endocrine and intracrine purposes. Endocrine regulation of both of these functions occurs through the hypothalamic-pituitary-ovarian (HPO) axis. Gonadotrophin releasing hormone (GnRH) from the hypothalamus stimulates the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from pituitary gonadotrophs. Both gonadotrophins act in an endocrine manner on follicular cells via specific receptors that are differentially expressed at various stages of follicle development [5]. In part, this stimulates production of steroid hormones and inhibins [6], which in turn act in an endocrine manner on the hypothalamus and pituitary to regulate gonadotrophin release.

As gonadotrophins arrive in the ovary via the circulation, their direct effects are largely confined to follicles that have acquired vascularization in the course of development, which mainly occurs during reproductive years. The ovary also produces many internal factors that control the growth of non-vascularized follicles and modulate the action of gonadotrophins within vascularized follicles. Of these internal factors, this literature review will focus on insulin-like growth factors (IGFs) and oocyte-secreted factors (OSFs), as they are involved in the work presented in this thesis. First, an overview of folliculogenesis and steroidogenesis will be presented in this thesis. First, an overview of folliculogenesis and steroidogenesis will be given, highlighting gonadotrophic regulation, followed by a description of IGFs and OSFs and their respective influences on these two ovarian processes.
1.2.2.1 Folliculogenesis

Mammalian primordial follicles are thought to form in the ovary only during embryonic development; a concept recently challenged by Johnson et al [7]. These follicles remain relatively quiescent until stimulated to undergo a sequential program of growth and differentiation called folliculogenesis (Figure 1.3). Folliculogenesis involves dynamic changes to follicle structure during which somatic cells proliferate and attain steroidogenic capacity while the oocyte grows and undergoes the final stages of meiosis. Ultimately, the purpose of folliculogenesis is to extrude a mature, cumulus cell-enclosed oocyte that is competent to undergo fertilization by sperm in the oviduct, and to create a hormone secreting structure, the CL, that primes and maintains the uterine endometrium for potential pregnancy.

Not all follicles achieve ovulatory status after being recruited into growth; cohorts of follicles begin development and are minimized at transitional stages of folliculogenesis by the process of atresia [3]. McGee and Hsueh [8] describe two recruitment phases within the course of folliculogenesis: 1) initial recruitment, in which primordial follicles begin a slow process of growth to the early antral stage and 2) cyclic recruitment, in which early antral follicles are rescued from atresia and undergo a more rapid growth process that is linked to the oestrus/menstrual cycle (Figure 1.4). Following cyclic recruitment, a selection process occurs in which one/some of the rescued follicles achieve dominance and proceed to ovulation; the number of ovulated follicles varies among species and is a genetically determined trait.

1.2.2.1.1 Initial follicle recruitment

The factors controlling initial follicle recruitment are still unclear, although nerve stimulation and/or neurotrophins may be involved (review: [9]), and the intraovarian factors stem cell factor/kit ligand (SCF/KL) [10] and basic fibroblast growth factor (bFGF) [11], are important to the process. The slow growth phase following this recruitment is considered gonadotrophin independent and involves a number of hormones, growth factors and cytokines acting in
Figure 1.3: Folliculogenesis. Follicles in the ovary undergo a regulated program of growth called folliculogenesis. This involves dynamic changes to follicle structure as depicted above and described in the thesis text. Image obtained from the internet: original source could not be identified.
Figure 1.4: Life history of ovarian follicles. Primordial follicles in the ovary are initially recruited to grow in a gonadotrophin-independent manner, a process that continues throughout the reproductive years until this follicle pool becomes depleted. Growing follicles proceed through the preantral and antral stages of development, and in the absence of sufficient stimulation by gonadotrophins they are resorbed into the ovary through the process of atresia. After puberty, cyclic recruitment commences, whereby some of the antral follicles are rescued from atresia by gonadotrophins and continue to grow. Of these, one or more will become dominant and are ovulated from the ovary. Adapted from [8].
autocrine/paracrine fashion among all components of the follicular unit (review: [12]). While most of these agents promote growth, controlled inhibition of primordial follicle activation and attenuation of preantral follicle growth is also clearly evident and important to overall synchronicity of folliculogenesis, both within one follicle and among the cohort of follicles within an ovary. The only inhibitor of primordial follicle activation identified to date is anti-Müllerian hormone (AMH) (review: [13]). The gonadotrophin-independent, preantral growth phase occurs acyclically throughout the lifespan, progressively reducing the primordial follicle pool, and there are significant species differences with respect to both the rate of growth and the controlling mechanisms that regulate this phase of folliculogenesis (Figure 1.5) [8, 12].

1.2.2.1.2 Cyclic follicle recruitment

Cyclic recruitment is considered gonadotrophin dependent and occurs only after puberty when there is sufficient FSH in circulation. This gonadotrophin rescues small antral follicles from atresia by promoting many aspects of follicle growth, including release from the inhibitory influences of AMH [13]. The FSH-dependent phase is comparatively rapid, occurring over days/weeks as compared to the many months of growth following primordial follicle activation (Figure 1.5) [8]. At the beginning of the cyclic phase, the follicular antrum enlarges, a differentiated thecal layer becomes established, granulosa cells proliferate, and the oocyte begins germinal vesicle breakdown (Figure 1.3). Granulosa cells from this cohort also begin to secrete oestrogen and inhibin B [6], which progressively exert negative feedback to the pituitary gland and hypothalamus. As a result, circulating FSH diminishes, and only some (poly-ovulators) or one (mono-ovulators) of the follicles survive, presumably those most sensitive to FSH: these are considered dominant follicles. The factors that absolutely determine dominant follicle selection are unknown, although dominant follicles have higher follicular fluid FSH, oestradiol, and potentially increased expression of gonadotrophin receptors and/or steroidogenic enzymes compared to non-selected follicles [14].
Figure 1.5: Duration of transitional phases of follicle development in human and rat ovaries. Adapted from [8].
1.2.2.1.3 Ovulation

Cyclic recruitment culminates in ovulation. As serum LH becomes elevated during pre-ovulatory growth, the combined influence of both gonadotrophins cause dominant follicles to terminally differentiate (luteinize), which includes resumption of germ cell meiosis, cumulus expansion, cessation of mural granulosa cell proliferation, and increased overall steroidogenic output. At mid-cycle, the pituitary releases a bolus surge of LH that stimulates the follicular changes that cause rupture of the follicle [5]. This process constitutes a unique inflammatory event that involves classic mediators of inflammation such as tissue macrophages and the cytokines they secrete [15, 16], as well as expression of enzymes that allow anti-inflammatory cortisol to accumulate in the local environment, presumably to ensure rapid healing of the ruptured ovary [17]. Once the follicle ruptures, granulosa and thecal cells enlarge and differentiate into luteal cells that form the corpus luteum [18].

1.2.2.2 Steroidogenesis

Steroidogenesis in the ovary represents a highly co-ordinated cascade of enzymatic events that metabolise cholesterol, a C<sub>27</sub> steroid, into three types of carbon reduced steroid hormone: progestins (C<sub>21</sub>), androgens (C<sub>19</sub>), and oestrogens (C<sub>18</sub>) (Figure 1.6). Cholesterol substrate for steroidogenesis is mainly derived from serum lipoproteins, with species differences as to which form is preferentially utilized [19]. Serum lipoproteins have specific receptors in the outer membrane of cells, selectively controlling the intake of C<sub>27</sub> steroids, which are stored as cholesterol esters in cytoplasmic lipid droplets [20, 21]. To initiate steroidogenesis, cholesterol is transported to mitochondria via the cytoskeleton and sterol carrier proteins, where it is internalized through the action of steroid acute regulatory protein (StAR) [22] and peripheral-type benzodiazepine receptor (PBR) [23]. Entry of cholesterol into the mitochondria represents a rate-limiting step in steroidogenesis that is regulated by many intracellular signaling pathways [22, 24]. Subsequently, cholesterol is metabolized by a progressive loss of carbon atoms in a series of hydroxylation reactions that are catalyzed by
Figure 1.6. Steroidogenesis. Cholesterol is transported across the cell membrane via specific lipid receptors and entry into the mitochondria requires the activity of steroidogenic acute regulatory protein (StAR). Here, cholesterol is converted to pregnenolone via the activity of cytochrome P450 side chain cleavage enzyme (CYP11a). The other enzymatic conversions take place in the endoplasmic reticulum. The enzymes involved are abbreviated as follows: cytochrome P450cyp17 (CYP17); hydroxysteroid dehydrogenase (HSD), cytochrome P450arommatase (arom) and 5α-reductase (5α-RED).
three members of the cytochrome P450 superfamily of genes [19]. Different forms of the
three steroid hormone subtypes are also produced, through the activity of various
hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$ isomerase (HSD) enzymes.

Stroma, theca and granulosa cells are all capable of steroidogenesis, but their specific
secretory patterns differ throughout follicle development and atresia [25], reflecting patterns
of steroidogenic enzyme expression [19]. Genes encoding lipoprotein receptors, StAR, and
most steroidogenic enzymes are under the control of gonadotrophins. In general,
steroidogenesis is geared toward the production of oestradiol (E$_2$) by the dominant follicle(s)
in the follicular phase (FP) and toward progesterone production (as well as E$_2$ production in
primates) in the luteal phase (LP) of the oestrus/menstrual cycle.

1.2.2.2.1 Progestin biosynthesis

The first step in steroidogenesis involves the conversion of cholesterol to pregnenolone,
catalysed by cholesterol side chain cleavage cytochrome (P450scc; CYP11a), and commonly
considered the rate-limiting step in steroid biosynthesis before the discovery of StAR.
Pregnenolone is converted to progesterone by 3$\beta$-HSD in the endoplasmic reticulum, and both
progestins can be converted to C$_{19}$ androgens via the duel activity of 17$\alpha$-hydroxylase/C17-20
lyase cytochrome (P450cyp17). P450scc and 3$\beta$-HSD are first expressed in theca cells during
the FP, increase with follicle growth, and are intensely expressed in luteal cells [19].
However, prior to the LH surge, progesterone secretion is limited and progestins largely serve
as precursor molecules for subsequent metabolism to androgens. Progesterone secretion by
follicular cells is mainly invoked at the pre-ovulatory stage of follicle development and peaks
post-ovulation, being the primary product of the CL.

1.2.2.2.2 Androgen biosynthesis

Androstenedione (A4) and testosterone are the predominant androgens in follicular fluid,
remaining at fairly constant levels throughout the various stages of antral follicle growth [25].
Intrafollicular androgens are elevated approximately 1000-fold compared to levels in general circulation; a similar situation exits in the testes. As mentioned above, A4 is a product of progesterone metabolism and can be converted to testosterone (T) by 17β-HSD; both of these androgens can be aromatised to oestrogen. In addition, follicular cells exhibit 5-α-reductase activity, which converts aromatisable androgens into non-aromatizable 5-α-reduced androgens, notably 5-α-dihydrotestosterone (DHT) [26, 27]. Theca cells are the primary source of aromatisable androgens in the follicular phase, and these are preferentially converted to 5-α-reduced androgens by follicular cells prior to dominant follicle selection [28]. The latter is consistent with observations of greater 5-α-reductase expression and activity in non-selected small antral follicles compared to larger selectable follicles [29]. Androgens also continue to be produced by cells of atretic follicles [25].

1.2.2.2.3 Oestrogen biosynthesis

Oestradiol (E2) is the primary steroid hormone product of dominant antral follicles. The aromatase cytochrome P450 complex (P450arom) is responsible for the conversion of C₁₉ steroids to C₁₈ steroids, namely A4 to estrone (E1) and testosterone (T) to E2. The same enzyme that can convert A4 to T, 17β-HSD, is also responsible for conversion of E1 to E2. The “two-cell, two-gonadotrophin” paradigm describes endocrine regulation and paracrine modulation of oestrogen synthesis within the follicle (Figure 1.7) [30, 31].

In this paradigm, LH stimulates thecal cell androgen synthesis, the products of which diffuse into granulosa cells and are converted to oestrogen via the aromatase activity induced by FSH. In early stages of antral follicle growth, theca cells exclusively express the LH receptor (LHR), and granulosa cells exclusively express the FSH receptor (FSHR), the molecular basis for the two-cell paradigm [30, 31]. However, at later pre-ovulatory stages, LHR are induced in granulosa cells and the dual stimulation by both gonadotrophins markedly enhances oestrogen output.
Figure 1.7: The two-cell, two-gonadotrophin model of oestrogen synthesis within the ovarian follicle, described in the thesis text.
Adapted from Erickson, G.F.: www.endotext.com
1.2.2.3 **Steroid action**

Ovarian steroids are required for both endocrine and intracrine purposes. Most tissues in the body respond to steroids in some manner, and relative proportions of the three classes of steroid in circulation determine sex differences in body development and function. Within the ovary, steroids act as autocrine/paracrine factors that influence both folliculogenesis and steroidogenesis through their specific receptors [32, 33]. This literature review will focus on the action of androgens, which will be covered in greater detail in subsequent sections.

1.2.2.4 **Mechanisms of gonadotrophin action**

Endocrine and intracrine factors, alone or working together, induce changes in gene expression profiles that correlate with the various stages of folliculogenesis [5, 34, 35]. The molecular effects of FSH and LH are both mediated by activation of adenylate cyclase with subsequent accumulation of intracellular cyclic adenosine monophosphate (cAMP). This mononucleotide activates protein kinase A (PKA), leading to phosphorylation of cAMP response element binding protein (CREB), thereby stimulating cAMP sensitive genes. This pathway is partly regulated through intracellular compartmentalization and/or degradation of cAMP by cell type specific phosphodiesterases (PDEs) [36]. Genes sensitive to cAMP can be considered “low tone” or “high tone”, the former generally induced by FSH alone and the latter induced by combined gonadotrophin stimulation [5, 34, 35]. Ovulatory doses of LH dramatically heighten PKA signalling and activate protein kinase C (PKC), causing an acute up-regulation of specific genes necessary for luteinization and ovulation [37, 38]. The multitude of diverse cellular responses that can be stimulated by gonadotrophins is indicative of a greater underlying complexity that involves other signalling pathways in addition to the classic PKA-CREB route. For example, protein kinase B (PKB) and members of the Forkhead transcription factor family have recently been implicated in FSH-induced intracellular signalling [39-42]. These latter studies indicate that the mitogenic and
steroidogenic effects of FSH may be mediated via pathways that are divergent from those that initiate the expression of genes involved in early granulosa cell differentiation.

1.2.2.5 Intra-ovarian regulators of ovarian function

A host of different factors are produced by the various cell types of the ovary and serve to regulate ovarian function. The following two sections represent those factors that are most pertinent to this thesis.

1.2.2.5.1 Insulin-like growth factors

Insulin like growth factors (IGFs) were first identified as factors in serum that could stimulate insulin-like effects and were subsequently designated IGF1 and IGF2 [43]. There are two receptors that specifically recognize the IGFs: the type 1 IGF receptor, which is structurally homologous to the insulin receptor, and the type 2 IGF receptor, which is identical to the mannose-6-phosphate receptor. The type 1 receptor mediates most of the actions of both IGF1 and IGF2. Signal transduction from the IGF1 receptor (IGFR) involves post-ligand receptor phosphorylation followed by phosphorylation of intracellular insulin receptor substrate (IRS) molecules. Phosphorylated IRS can subsequently activate two kinase signalling cascades: 1) phosphatidylinositol-3 kinase (PI-3) and/or 2) mitogen activated protein (MAP) kinase. In general, IGF activity influences cell replication and differentiation, as well as having anabolic effects on protein and carbohydrate metabolism. The IGFs are present in most body fluids, differentially bound to any of 6 high affinity IGF binding proteins (IGFBPs) that coordinate and regulate the biological activities of IGFs [43].

In the ovary, the follicular expression profiles of IGFs, IGF receptors and IGFBPs have been documented for many animal species, with apparent species differences [44-46]. The IGFs are able to exert both autocrine and paracrine effects in promoting the growth and differentiation of both granulosa and thecal cells, in part by augmenting the action of gonadotrophins (review; [44]). In mice, pre-antral follicle growth does not require IGF1, but
acquisition of adequate FSH responsiveness to promote antral follicle growth does [47]. Evidence of a positive feedback loop exists in most mammals whereby IGFs induce gonadotrophin responsiveness, which in turn enhances IGFR expression [45]; this synergistic action appears to drive antral follicle growth and promote thecal androgen synthesis. Binding protein expression and/or expression of proteases that cleave them, may be important factors that determine the selection of dominant follicles that emerge after cyclic recruitment [14, 48]. Conversely, initiation of atresia at any stage of folliculogenesis is generally associated with attenuation of IGF activity through increased expression of particular binding proteins and/or reduced expression of IGF and/or IGFR [46, 49, 50].

1.2.2.5.2 Oocyte secreted factors (OSFs)

Oocyte control of steroidogenesis and folliculogenesis is a more novel, but now well-established concept in reproductive biology. Historically, the oocyte was considered a passive participant in its developmental history, but in fact a dynamic interplay exists between the oocyte and surrounding somatic cells that mutually influences growth and differentiation of both cell lineages and is essential for normal fertility (reviews: [51-53]). This is apparent even at the formative stage of the follicle, which does not develop in the absence of an oocyte [54]. Once recruitment of primordial follicles is initiated, the rate of subsequent follicular development also appears to be based on a developmental program that is intrinsic to the oocyte [55]. Thus, the oocyte coordinates the follicle as both a structural and functional unit.

Oocytes and granulosa cells form a functional syncytium via heterogenous gap junctions that exist between granulosa cells and between some granulosa cells and the oocyte; the latter depends upon developmental stage of the follicle [56]. Maintenance of the gap junction network between the oocyte and its immediate surrounding granulosa cells is essential for most somatic cell effects on oocyte metabolism, growth, meiotic arrest, and maturation [57]. Factors secreted by the oocyte may also use these conduits to influence granulosa cells [58], but soluble OSFs can act in a paracrine manner independent of gap
junction communication (review: [53]). This influence is not limited to granulosa cells; soluble OSFs affect thecal cell recruitment and differentiation, particularly at early stages of folliculogenesis [59-61].

Erickson and Shimasaki [62] describe a morphogenic gradient of OSFs that exists across the follicle and is responsible for the heterogenous stratification of granulosa cell differentiation, which is particularly evident once the follicular antrum has formed. In antral follicles, two distinct granulosa cell subtypes emerge: the mural and cumulus granulosa cells, as depicted in Figure 1.2. In general, OSFs inhibit cumulus cell luteinization in the early stages of folliculogenesis by promoting proliferation and inhibiting steroidogenesis; in the absence of such factors the cumulus cells adopt a more mural cell phenotype [53]. At later stages of folliculogenesis, OSFs induce a specific differentiation of cumulus cells that enables cumulus expansion prior to ovulation [63]. This cumulus cell activity cannot be induced in mural granulosa cells.

In the past decade, growth factors specific to the oocyte have been identified and characterised, with focus on members of the transforming growth factor β (TGFβ) superfamily, particularly growth differentiation factor 9 (GDF9), bone-morphogenetic protein 15 (BMP15; also known as GDF9b), and bone-morphogenetic protein 6 (BMP6) [64, 65]. Normal folliculogenesis and fertility is dependent upon GDF9 [66], but not BMP15 [67] or BMP6 [68] in mice. Interestingly, both GDF9 and BMP15 are essential for sheep fertility [69], the latter associated with infertility and super-fertility in a dosage-sensitive manner. Expression profiles for GDF9 and BMP15 have been characterised in other animal species, including humans, with some species differences at the initial stages of folliculogenesis [70]. While first touted as being oocyte-specific, GDF9 is expressed at low levels in other ovarian and non-ovarian cells [71], but to date BMP15 expression appears to be confined to the ovary [72]. Both molecules are involved in many aspects of folliculogenesis, and can modulate FSH and/or IGF1 action in vitro [53, 73]. The degree to which they account for the effects of
whole oocytes or oocyte-conditioned media on follicular cell function is still being actively investigated [53, 64, 74]. Less is known about the role of BMP6, which is expressed by the oocyte as well as granulosa cells. In the rat, this molecule inhibits FSH action and is dramatically down-regulated by the secondary rise in FSH that occurs prior to dominant follicle selection, perhaps representing an obligatory event in pre-ovulatory follicle growth [75].

1.3 Polycystic Ovary Syndrome (PCOS)

Polycystic ovary syndrome (PCOS) is a complex endocrine disorder that afflicts approximately 3-10% of women in their reproductive years and accounts for the majority of WHO type II infertility cases, which include women considered to have normal FSH and oestrogen levels [76]. In addition to reproductive dysfunction, PCOS is associated with metabolic disturbances that carry a definitive increased lifetime risk of type 2 diabetes [77, 78], and a still putative increased risk of cardiovascular disease and endometrial cancer [79-81]. PCOS is classified as a syndrome, not a disease, for lack of a constant set of symptoms and physical parameters. As such, PCOS has been described in an evolutionary context as occupying one end of a spectrum of normal human female fertility in which genetic influences for energy conservation may negatively impact reproductive potential under particular environmental circumstances [82]. This disorder has no natural correlate among other animal species. Barnett and Abbot hypothesize that PCOS is a uniquely human phenomenon that represents manifestation of an inherent vulnerability of reproductive adaptations in primates that accommodated increasingly large-brained fetuses [83].

1.3.1 Diagnosis and general clinical characteristics

The first consensus criteria for PCOS were established at a National Institutes of Health (NIH) conference in 1990, which designated unexplained hyperandrogenaemia and chronic anovulation as the two essential diagnostic features of this disorder. Since then, another
consensus meeting on PCOS was convened at a joint American Society for Reproductive Medicine/ European Society of Human Reproduction and Embryology (ASRM/ESHRE) meeting in Rotterdam, the Netherlands, in 2003 [84]. This meeting revised the 1990 NIH diagnostic criteria to include the detection of polycystic ovaries. Under these new criteria, a women is diagnosed with PCOS if there is evidence of 2 of the 3 following characteristics: 1) oligo- or anovulation, 2) clinical and/or biochemical signs of hyperandrogenism, and 3) polycystic ovaries. Of these, hyperandrogenism is the essential feature, so other aetiologies that cause excess androgen production and mimic PCOS must be excluded: Cushing’s syndrome, non-classical congenital adrenal hyperplasia, and androgen-secreting tumours (reviewed by Kaltsas et al [85]). Epileptic drug use can also cause PCOS-like symptoms [86]. Wide variation occurs within and among all the major criteria of this disorder, and all are variably associated with other commonly observed characteristics, including hirsutism, acne, obesity, insulin dysfunction, and abnormal LH secretion [84]. Symptoms of PCOS may manifest in the peri-pubertal period [87, 88], progressively worsen through adolescence and early reproductive years [89-91], and then ameliorate in later years [92].

1.3.1.1 Hyperandrogenism

Androgen excess is considered the key element in PCOS [93], and women with this disorder represented 82% of those involved in a large study of women with clinically evident androgen excess [94]. Hyperandrogenism (HA) can be visually manifest as hirsutism, acne, seborrhea, and alopecia. Women with polycystic ovaries have a higher incidence of all of these cutaneous symptoms compared to women with normal ovaries [95], reflecting stimulation of the pilosebaceous unit by androgens. Biochemically, HA is assessed by measurement of serum androgens, including androstendione (A4), testosterone (T), dehydroepiandrosteronesulphate (DHEAS) and/or the free androgen index (FAI), which takes into account levels of circulating sex-hormone binding protein (SHBG). Both techniques for assessing HA have limitations, as documented in the Rotterdam consensus report [84] and
discussed by Azziz et al [94]. In general, women with PCOS will have significantly higher serum androgens than women without the disorder, but there is an area of overlap into the normal range for the PCOS group, and this normal range should be empirically determined by a representative population/ethnic mix.

1.3.1.2 Polycystic ovaries

Polycystic ovaries (PCO) are present in approximately 20% of the normal female population [96-100] and are associated with abnormal androgen and insulin-related parameters irrespective of otherwise normal reproductive parameters [101-104]. Histologically, PCO have a sclerotic outer capsule, a thicker ovarian epithelium, stromal hypertrophy, and an excess of developing follicles (≥ 12 follicles 2-9 mm in diameter [105]), making them larger and visually distinct from normal ovaries (Figure 1.8A). Although historically called “cysts” and thought to be atretogenic, the follicles in PCO are in fact healthy and actively steroidogenic [106], albeit in anovulatory cycles a developmental arrest occurs in the larger follicles. This arrest can be overcome in most instances by therapeutic treatment.

Transvaginal ultrasonography is the preferred method to detect PCO (Figure 1.8B) and a recent review by Balen et al [4] provides a detailed description of the newly adopted international consensus definitions for this ovarian abnormality. In general, these are: a finding of 12 or more follicles measuring 2-9 mm in diameter in one plane, or an ovarian volume > 10 cm³; if there is evidence of a dominant follicle > 10 mm in diameter or a CL, the scan is not valid. In addition, the follicles of a PCO appear in a string-like formation beneath the ovarian cortex on ultrasound whereas the follicles of a normal or multi-follicular ovary will be scattered throughout the stroma.

1.3.1.3 Menstrual dysfunction and oligo/anovulation

Chronic amenorrhoea (lack of menses for > 3 months), oligomenorrhoea (repetitive menstrual cycles > 35 days), and prolonged erratic menstrual bleeding are all characteristic of women
Figure 1.8: Appearance of polycystic ovaries

A) Surgically exposed. Arrows indicate enlarged sclerotic ovaries.

B) Transvaginal ultrasound scan. Arrows indicate multiple peripheral follicles, 2-9mm in diameter.
with PCOS. Cycles with an interval greater than 35 days are likely to be anovulatory, and are reported in 50-90% of women with PCO [95, 96, 107]. In addition, up to 40% of women with PCO and apparently normal menstrual cycles may be anovulatory if signs of hyperandrogenism are present, as assessed by low serum progesterone in the luteal phase of the cycle [94, 108]. Menstrual disturbance and anovulation are associated with increased incidence of infertility, obesity, hyperinsulinaemia/insulin resistance, and elevated serum LH concentration [109, 110].

1.3.2 Aetiology of PCOS

The aetiology of PCOS remains largely uncertain, although it is clear that both genetic and environmental factors play key roles in its pathogenesis [111]. As symptomatic characteristics of PCOS vary widely and exhibit ethnic differences [112-114], adoption of universal criteria for diagnosis is considered essential before a specific cause of PCOS can be unravelled [84, 115, 116]. It is possible that the heterogeneity observed in PCOS may be indicative of more than one distinct disorder, with areas of symptomatic overlap between them.

1.3.2.1 Fetal origins of PCOS

During fetal life, high maternal levels of serum androgen and/or insulin may influence genetic and/or metabolic programming in the fetus in a manner that predisposes the developing individual to PCOS and associated metabolic diseases [117-119]. Once initiated, the syndrome becomes a self-perpetuating pathological process whereby the abnormal activities of androgen and insulin exacerbate each other, making it difficult to delineate primary causes from secondary effects. Whether one specific hormonal disturbance precedes the other remains the topic of widespread debate, with convincing evidence on both sides [120]. However, it is plausible that one condition does not necessarily cause the other in all cases of PCOS. Instead, androgen and insulin dysfunction may represent two independent modalities that are common enough to coexist in a syndrome that afflicts a relatively large portion of the
female population [93]. The issue of causality is compounded by the fact that both of these hormonal excesses are conditioned by genetic and environmental influences.

Compiling data from pre-natally androgenized animal models and supporting clinical evidence, Abbot et al [121] put forward a unifying hypothesis for a singular origin of PCOS that encompasses the heterogeneity inherent in this disorder. These authors propose that in utero androgen excess is the primary insult, reprogramming many fetal organ systems in a way that predisposes the adult female to acquire all of the characteristic features of PCOS. According to this theory, genetic and/or environmental factors moderate the physiological consequences of the initial insult, thus determining the ultimate phenotypic expression of the disorder in adulthood and giving rise to a wide spectrum of symptoms.

The timing of prenatal androgen exposure is important in determining the degree of subsequent reproductive dysfunction [122], with early gestational exposure more damaging than later ones. Perturbation of the endocrine hypothalamus may occur with early gestational androgen excess, especially in terms of entrainment of gonadotrophin releasing hormone (GnRH) [122], a notion supported by studies at the level of GnRH neurons in sheep [123-126] and mice [127]. Various types of insulin dysfunction also vary with timing of the androgen insult: early gestational exposure impairs pancreatic β-cell secretion, while late gestational exposure impairs insulin sensitivity [128]. The former may be due to the effect of androgen on pancreatic organogenesis [122], while the latter may be due to the effect of androgen in promoting abdominal adiposity, known to be associated with insulin insensitivity [129]. Both altered pancreatic β-cell function [130, 131] and increased incidence of abdominal adiposity (reviewed by [132]) are evident in women with PCOS. Therefore, timing effects may also be a source of symptom heterogeneity in adult individuals with this disorder, supported by the finding that PCO/PCOS in adulthood segregated into two independent groups based on body weight at birth and length of gestation [117].
The remaining conundrum in the fetal origin hypothesis is how maternal androgen excess gets translated to the developing fetus, since under normal circumstances high levels of SHBG in pregnancy and aromatase activity in the placenta should buffer the effect. Abbot et al [121] suggest four possibilities: 1) inherited ovarian and/or adrenal hyperandrogenism in the female fetus; 2) altered placental enzyme function; 3) hyperandrogenism in the fetal compartment due to intrauterine growth restriction; 4) environmental chemicals (and/or drugs) crossing the placenta to stimulate fetal androgen biosynthesis directly or indirectly. So, while the origin of PCOS may be a singular type of event, the cause of this event may still be many.

**1.3.2.2 Inheritance**

The familial clustering of PCOS is described by many studies and clearly indicates that susceptibility to this disorder has a genetic foundation (Table 1.1). At least 50%, and as high as 87%, of first-degree relatives of women with PCOS will have some symptom manifestation [133, 134]. Hyperandrogenism is a primary heritable trait in families with PCOS [88, 133, 135, 136], but to date no convincing inheritance pattern has emerged. Twin studies do not support the concept of a single gene defect leading to PCOS [137] and it has now become widely accepted that this disorder is oligogenic, possibly based on a few key genes that are influenced by environmental factors such as nutritional status [138]. Discrepancies between studies for affected status criteria, lack of a male phenotype, ascertainment bias, and study sizes, have severely compromised the findings of most genetic analyses. In addition, the well-documented effects of environmental factors in determining phenotype expression increase the difficulty of achieving consistent results [139]. Moreover, with many genes involved, each may have a different mode of inheritance, each may convey different degrees of susceptibility, and each may be variably represented within or between family groups. Due to this complexity, studies involving very large, clinically well-defined cohorts will be needed to definitively identify the key genes in PCOS [140].
### Table 1.1 Family studies in polycystic ovary syndrome. Updated from [141]

<table>
<thead>
<tr>
<th>Reference</th>
<th>Criteria for affected status</th>
<th>Proposed mode of inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cooper et al. [142]</td>
<td>PCO (surgery or cauldoscopy)</td>
<td>Autosomal dominant with reduced penetrance</td>
</tr>
<tr>
<td>Givens et al. [143] and Wilroy et al. [144]</td>
<td>PCO (surgery or exam), hirsutism</td>
<td>X-linked, (dominant?)</td>
</tr>
<tr>
<td>Ferriman and Purdie [145]</td>
<td>Hirsutism ± enlarged ovaries</td>
<td>Modified dominant</td>
</tr>
<tr>
<td>Hague et al. [146]</td>
<td>PCO (U/S), clinical symptoms</td>
<td>Ratios exceeded autosomal dominant</td>
</tr>
<tr>
<td>Lunde et al. [147]</td>
<td>“Multicystic” ovaries (surgery)</td>
<td>? Autosomal dominant</td>
</tr>
<tr>
<td>Carey et al. [148]</td>
<td>PCO (U/S), premature balding in male</td>
<td>Oligogenic</td>
</tr>
<tr>
<td>Jahanfar et al. [137]</td>
<td>PCO (U/S), clinical symptoms</td>
<td>X-linked or autosomal recessive with environmental influences</td>
</tr>
<tr>
<td>Norman et al. [149]</td>
<td>PCO (U/S), hyperandrogenaemia hyperinsulinaemia</td>
<td>Not stated</td>
</tr>
<tr>
<td>Legro et al. [133]</td>
<td>Oligomenorrhoea and hyperandrogenaemia</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>Govind et al. [150]</td>
<td>PCO (U/S), clinical symptoms, premature balding in male</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>Azziz et al. [151]</td>
<td>Hyperandrogenism and menstrual irregularity</td>
<td>Autosomal dominant and X-linked not excluded, probably multigenic</td>
</tr>
<tr>
<td>Kahsar-Miller et al. [135]</td>
<td>Oligomenorrhoea and hyperandrogenaemia (hirsutism)</td>
<td>Not determined</td>
</tr>
<tr>
<td>Mao et al. [152]</td>
<td>Irregular menstrual cycles, premature balding in male</td>
<td>Co-dominant with complete penetrance</td>
</tr>
<tr>
<td>Yildiz et al. [153]</td>
<td>Hyperandrogenaemia, oligomenorrhoea and insulin resistance</td>
<td>Not stated</td>
</tr>
<tr>
<td>Legro et al. [136, 154]</td>
<td>Hyperandrogenaemia, Menstrual Irregularity and Insulin resistance (sisters and brothers)</td>
<td>Autosomal dominant</td>
</tr>
</tbody>
</table>

PCO = polycystic ovarian morphology  
U/S = ultrasound

### 1.3.2.3 Candidate genes

The search for PCOS genes has mainly involved association and linkage analyses for selected candidates [155]. These candidates reflect what is known of the pathophysiology of PCOS.
and include genes involved in: 1) steroid hormone synthesis and action, 2) gonadotrophin action and regulation, 3) energy/insulin metabolism and 4) immune function. Candidate genes in PCOS have recently been extensively reviewed [156]. The most thorough and highly acclaimed linkage analysis to date initially identified follistatin as the most likely gene to be linked to PCOS [157]. Subsequent mutation analyses failed to detect abnormalities in the follistatin gene that would incur physiological consequences [158, 159], although levels of follistatin are elevated in women with the syndrome [160] and in pre-natally androgenized sheep [161]. This suggests abnormal regulation, but the regulatory region of the follistatin gene remains uncharacterised. Further association analyses took focus away from follistatin and placed it upon a region near the insulin receptor gene on chromosome 19 [155, 162], a finding supported by Villuendas et al [163]. No mutations have been found in the insulin receptor gene in women with PCOS [164, 165], so the significance of these findings remains unclear.

Use of new techniques, such as microarray analysis, to examine global patterns of gene expression is beginning to identify new candidate genes for PCOS without the selection bias of targeted signalling pathways. Indeed, the first of these analyses to be published compared mRNA expression in theca cells from normal and PCOS ovaries and identified alterations in three molecular pathways that had previously not been explored in association with PCOS: Wnt signalling, retinoid biosynthesis and GATA6 transcriptional factor excess, with documentation that the latter two anomalies can lead to increased androgen synthesis in theca cells [166]. More recently, Jansen et al [167] took a whole-ovary approach, comparing gene expression profiles between normal, PCOS and female-to-male transsexual (TSX) ovaries, confirming disruption of Wnt signalling and identifying expression abnormalities of extracellular matrix components and immunological factors.
1.3.2.3.1 Polymorphisms

Polymorphic sequences within or near a gene of interest are often used to establish linkage to a particular disease or disorder; these are genetic sequences that vary in the population but do not represent mutations. However, many of these polymorphisms, especially regions of repeated nucleotide sequences known as microsatellites, comprise gene regulatory elements that are subject to differential epigenetic modifications (such as methylation) and/or demonstrate differential binding to transcriptional regulatory proteins. As such, microsatellite polymorphisms hold good potential for being the significant genetic factors involved in PCOS, as their characteristics could account for the dynamic phenotypic and physiological heterogeneity in this disorder as well as inconsistent results in classical genetic analyses, which do not account for epigenetic phenomenon.

The variable nucleotide triplet repeat (VNTR) region upstream of the insulin gene (INS) influences transcription of this gene as well as its down-stream neighbor, insulin-like growth factor II (IGF II) [168]. Type I alleles of INS-VNTR have been associated with Type I diabetes [169] and Type III alleles have been associated with Type II diabetes and birthweight [170]. Waterworth et al [171] originally reported an association between the Type III INS-VNTR allele and PCOS, making a provocative genetic link between this disorder and its metabolic sequelae, but this association has been contradicted by other studies [157, 172, 173].

A polymorphic variant in the 5’ regulatory region of CYP11a, the gene that encodes a rate-limiting enzyme in steroid hormone production, has also been a persistent candidate for association with PCOS [174]. Other than documented polymorphisms, there appear to be no other differences in this region between affected and unaffected family members, and the polymorphic variants were strongly associated with total serum testosterone levels [175]. The influence of this polymorphism on regulation of the CYP11a gene is currently unknown,
although abnormal regulation, expression, and mRNA stability of this gene has been reported in cultured theca cells from women with PCOS [176-178].

Polymorphisms in many other genes have also been investigated for association with PCOS, with variable reports of positive associations that have yet to be validated [174]. These include factors that influence sensitivity, availability, and/or clearance of androgens, thus exacerbating the problem of excess androgen or creating a state of hyperandrogenism in the absence of abnormal androgen secretion. The latter situation is particularly evident in women who have a mutation in the SHBG gene, leading to low or absent levels of serum SHBG and manifestation of symptoms of PCOS [179]. The contribution of mutations or variant polymorphic alleles of the SHBG gene within this disorder are still speculative [180]. Genetic variants of the LH receptor have also been associated with PCOS, but they appear to only affect a particular subgroup of women, and the association varies with ethnicity [181, 182]. Finally, the CAG repeat polymorphism within the androgen receptor gene potentially mediates androgen sensitivity and will be discussed later in this review.

1.3.3 Pathophysiology of PCOS

In terms of folliculogenesis, two primary processes appear to be disrupted in PCOS: early follicular growth (which is excessive) and dominant follicle selection (which is inhibited) [183]. Excess androgen, and perhaps in some cases increased sensitivity to androgen, is the keystone to both abnormalities, causing polycystic ovarian morphology and setting the appropriate pathological scenario for altered insulin and/or LH action to instigate follicular arrest and anovulation [93]. Ultimate pathological expression of these hormonal disturbances in a given person may be dependent upon achieving particular thresholds of all three variables, giving rise not only to the great heterogeneity of symptoms among women with PCOS, but also variable degrees of symptom expression within a person’s reproductive lifespan. The following sections will first summarize current knowledge on the
pathophysiology behind excess androgen, insulin and LH, then describe how these excesses
influence altered patterns of folliculogenesis in PCOS.

1.3.3.1 **Ovarian hyperandrogenism**

1.3.3.1.1 **Theca cell dysfunction**

The ovary is considered the main source of excess androgen production in women with
PCOS, although excess adrenal androgen production also occurs in a proportion of women
with this disorder [184, 185]. Ovarian steroidogenic responses to clinically administered
GnRH agonists or hCG are elevated in women with PCOS [186], a response that persists in
cultured theca cells from polycystic ovaries [176, 187]. Perturbed steroidogenic enzyme
expression and/or activity form a platform for excessive androgen and 17-
hydroxyprogesterone secretion in this disorder [176, 177, 188], but the primary cause is still
unknown. Increased retinoic acid biosynthesis [166], diminished repression of the CYP17
promoter [189], and/or repressed mitogen activated protein kinase (MAPK) signalling [190]
are mechanisms that have been implicated. As these characteristics persist over many theca
cell passages, they probably represent either an underlying genetic predisposition or an
incontrovertible metabolic imprint that was established in vivo [191], both consistent with
current theories on the aetiology of PCOS. As phosphorylation of signaling molecules
represents a means of activation/inactivation of most molecular pathways, an inherent
disruption of serine kinase activity could explain the multiple disruptions observed in PCOS
theca cells, as well as other cell types, and has been proposed as a potential pathological
mechanism that could underscore all abnormal manifestations in this disorder [192].

In addition to intrinsically altered steroidogenic capacity, hypertrophy of the theca and
stromal compartments is characteristic of the PCO, and may contribute to ovarian
hyperandrogenism, although in the case of stromal tissue, this may only apply to cortical
stroma as medullary stroma appears to have little steroidogenic activity in both pre- and post-menopausal ovaries [193, 194].

1.3.3.1.2 Granulosa cell dysfunction

Although theca cell dysfunction appears to be the primary cause of hyperandrogenaemia in PCOS, evidence exists for granulosa cell [195-200] abnormalities in this disorder that potentially disrupt paracrine signalling to theca cells in a way that promotes androgen synthesis. In particular, signalling molecules belonging to members of the TGFβ superfamily (including activins, inhibins, and follistatin), epidermal growth factor (EGF), and binding proteins for IGF1 have been investigated with respect to potential dysregulation leading to the pathology of PCOS [201]. These factors generally enhance or inhibit the activities of serum gonadotrophins, insulin, and/or IGF1, exerting indirect effects that may have pathological consequences if their expression or activity were abnormal. It is out of the scope of this review to discuss the evidence related to these factors, but all have been implicated in some way with the pathology of PCOS and show patterns of dysregulation that could be either genetic, environmental, or a combination of both. To date, most of these factors are considered secondary consequences that exacerbate hyperandrogenaemia rather than primary causes.

1.3.3.1.3 Oocyte dysfunction

Highlighting the importance of the follicle as an integrated unit and shedding further speculation on where the primary pathological insult in PCOS lies, oocyte dysfunction [202, 203] has been recently proposed as a potential mediator of this disorder, coincident with the current interest in the concept of oocyte-directed folliculogenesis. As discussed previously, GDF9 and GDF9b are the most well described oocyte-derived molecules thought to mediate many of the oocyte’s effects on folliculogenesis, and GDF9, but not GDF9b may be expressed at a lower level in PCOS oocytes [204]. Whether this actually translates to abnormal theca
cell steroidogenesis in this disorder remains to be determined. Human theca cells respond to GDF9, which inhibits cAMP-stimulated StAR mRNA and promoter activity while concurrently decreasing steroidogenic output [205]. Thus, GDF9 may negatively regulate thecal cell steroidogenesis and reduced levels of this oocyte paracrine factor could feasibly lead to increased androgen biosynthesis. Two studies have reported an increase in StAR abundance in the theca of polycystic ovaries [200, 206], and although StAR promoter activity is not altered in cultured PCOS theca cells [177], such cells are devoid of exposure to GDF9 once isolated. Of note, human granulosa cells express GDF9 [205], which has not yet been shown to be dysregulated in PCOS. To date, only one study has investigated mutations in GDF9/9b with respect to this disorder, finding no evidence of abnormalities in the coding regions of these genes [207], which does not rule out mutations and/or abnormal regulation of their promoter regions, which have yet to be characterised. More recently, oocyte-derived BMP6 has been shown to negatively regulate thecal androgen biosynthesis and has been targeted as a new candidate to consider in the pathogenesis of hyperandrogenism [208].

1.3.3.2 Hyperinsulinaemia / Insulin resistance

Insulin resistance and chronic hyperinsulinaemia, either induced by excessive dietary intake of high-glycemic-index foods, and/or induced in a compensatory manner by insulin resistance, is associated with a cluster of maladies referred to as the metabolic syndrome or Syndrome X, which is associated with a number of illnesses including PCOS and most non-reproductive abnormalities that are coincident with PCOS [119, 209]. A unique disorder of insulin action is evident in at least 50% of women with PCOS, characterised by tissue specific alterations in insulin signalling that involve post-receptor mediated phosphorylation events [192]. The selective insulin resistance in PCOS disrupts metabolic but not mitogenic sequelae of both insulin and IGF1 action [210]. This particular type of insulin resistance is independent of obesity, but obesity has an additive deleterious effect on insulin sensitivity [211, 212]. Insulin and IGF1 can augment LH and FSH activity, and increase thecal androgen synthesis
independent of LH augmentation [213]. This activity not only remains intact in cells from PCOS ovaries, it is also excessively stimulated in an environment of hyperinsulinaemia [119, 214]. It is probable that there are genetic components to the insulin dysfunction associated with PCOS that can be dependant and/or independent of obesity [149, 154, 215, 216].

High serum insulin and/or obesity also inhibit the synthesis of liver-derived SHBG, and decrease the levels of serum IGFBP1, thus increasing the bioavailability of serum androgen and IGF1 [119]. In turn, androgens enhance the deposition of fat over the central abdomen, a fat store that is metabolically different from peripheral fat, predominant in women with PCOS, and is associated with hyperinsulinaemia and insulin insensitivity [119, 217]. In this way androgens augment their own bioavailability by encouraging conditions that lower serum SHBG levels.

1.3.3.3 Aberrant LH secretion

The hypothalamic GnRH pulse generator appears to have decreased sensitivity to the negative feedback effects of estradiol and progesterone in many women with PCOS, leading to excess release of GnRH from the hypothalamus and a selective disruption to LH secretion from pituitary gonadotrophs [218, 219]. This disrupted neuroendocrine signalling in turn abnormally stimulates ovarian theca cells, resulting not only in excess androgen and 17-hydroxyprogesterone production, but also potentially altered patterns of FSH-stimulated granulosa cell oestrogen secretion [219]. The altered endocrine milieu emanating from the ovary provides abnormal negative feedback to the GnRH pulse generator, thus perpetuating a pathological HPO cycle. Although neuroendocrine abnormalities were once considered a potential cause of PCOS, and may be the primary perturbation in a subgroup of women [218], strong evidence supports excess ovarian (and/or adrenal) androgen as the primary impetus for the hypothalamic/pituitary dysfunction [220, 221]. A direct role for obesity, insulin resistance and hyperinsulinaemia in altering neuroendocrine function independent of androgen excess in PCOS remains controversial [222].
1.3.3.4 **Altered folliculogenesis**

1.3.3.4.1 *Early follicular growth*

Polycystic ovaries have approximately 2-6 times the normal number of primary and secondary follicles, but similar numbers of primordial follicles, indicating an overall increase in cohort size at early stages of folliculogenesis and accumulation of follicles at the pre-selection stage [105, 203, 223, 224]. Initiation of follicle growth from the primordial resting state to the primary follicle stage is unlikely to be disrupted in PCO since these ovaries have normal numbers of primordial follicles and the primordial follicle pool does not become prematurely depleted [225]. Paradoxically, there is some evidence that menopause may be delayed in women with PCOS [225]. Also, PCO secrete excess AMH [226, 227], a factor known to inhibit the primordial to primary follicle transition in mice [13]. However, most studies that include a primordial follicle count for human ovaries use biopsy material, which is most readily available, with subsequent computational analysis to achieve an estimated follicle number. This method is not ideal, and may incur computational error. Therefore, the issue of whether primordial follicle numbers are altered in PCOS should remain an open question.

In light of current data, it seems most probable that the post-initiation, early follicular growth phase is altered in PCO, possibly due to augmented growth and/or inhibition of atresia in follicular cells. Follicle excess is particularly high in the 2-5mm diameter range [105], a stage at which the developing cohort of human follicles is normally reduced by atresia [8]. Enhanced follicle survival in an environment of excess androgen can potentially be explained by a trophic/anti-apoptotic action of this steroid hormone on follicular cells of preantral/early antral follicles, as supported by studies in mice and non-human primates (reviewed by [183]). Clinical observations of women exposed to pharmacological or pathological serum androgen excess have evidence of PCO or PCO-like ovaries [85, 228-230], and antral follicle numbers in PCO are positively correlated with serum androgens [105], supporting data from animal
studies and leaving little doubt that hyperandrogenaemia, either from intra- and/or extra-ovarian sources, causes the follicle excess of PCO morphology. However, the underlying mechanisms for the follicle accumulation remain unknown, and may differ among species. Androgens directly inhibited apoptosis in cultured human cortical biopsies [231], lending strength to an anti-apoptotic role for androgens in the human ovary. However, studies conflict as to whether the incidence of atresia is abnormal in follicles of PCO, possibly because this attribute may be influenced by ovulatory status [232]. It is unknown how androgens promote follicle survival in any species, although evidence exits that excess serum androgens in primates increases expression of follicular FSH receptor (FSHR) [233] and IGF1 and its receptor [234, 235] in follicular cells.

Recently, an alternative hypothesis was proposed by Maciel et al [203] in which follicle excess is not considered the result of augmented growth or retarded atresia, but rather a stock-piling effect that results from abnormally slow growth, possibly due to dysregulation of oocyte-derived GDF9. As stated earlier, levels of GDF9 may be decreased in PCOS [204], but whether this is somehow the result of excess androgen, or its cause, requires further investigation. This slow-growth hypothesis is feasible from other perspectives as well. Elevated AMH is evident in PCOS, which does not appear to influence primordial activation as it does in mice, but could feasibly inhibit FSH-stimulated proliferation in humans. Despite abundant bioactive FSH in PCO follicles, granulosa cell numbers are deficient compared to follicles of comparable size from normal cycling ovaries, an indication that these cells do proliferate, but at a different rate [195].

1.3.3.4.2 Follicular arrest and anovulation

Despite the enlarged pool of selectable follicles in PCO, emergence of one dominant follicle does not always occur and follicles can become arrested at the pre-selection stage, which in humans occurs when a follicle is approximately 8-10mm in diameter. This arrest explains an anovulatory cycle in women with PCOS. Lack of ovulation is an inconsistent phenomenon in
this disorder, and even women with severe PCOS will have occasional ovulations [102, 110], so this disturbance is clearly less entrenched than the disturbance that increases follicle numbers. Indeed, ovulatory women with PCO may represent an occult form of PCOS, whereby mild hyperandrogenism and insulin dysfunction are below threshold levels required to initiate the molecular mechanisms that cause follicular arrest [93]. In this vein, there is general speculation that the incidence of PCOS will rise in coming years coincident with the epidemic of obesity in Western societies and those that adopt a Western lifestyle, as obesity aggravates both androgen and insulin abnormalities [236-238]. To date, no singular feature explains all cases of anovulation in women with PCOS, perhaps indicative of a common endpoint for multiple independent and/or inter-dependent molecular pathways that may be more or less prevalent in any given situation.

It is clear that hyperinsulinaemia /insulin resistance has a major influence on the incidence of follicular arrest and resistance to pharmacological ovulation induction [192, 239, 240]. However, insulin dysfunction in the absence of hyperandrogenaemia does not itself incur anovulation, again highlighting the essentiality of excess androgen in all aspects of PCOS. Improving insulin sensitivity via lifestyle modifications [241] or pharmacological intervention with insulin sensitizing drugs such as Metformin [242], can induce ovulation in women with PCOS, further substantiating the role of insulin dysfunction as a mediator of anovulation. This is supported by the pre-natally androgenized monkey model of PCOS, for manifestation of the anovulatory phenotype in these animals requires excess androgen and hyperinsulinaemia due to adiposity-related insulin resistance, not occurring in the event of either parameter alone [243]. Where and when insulin dysfunction inflicts the damage that results in follicular arrest remains uncertain. Apart from the effects of hyperinsulinaemia in exacerbating abnormal theca cell function as mentioned above, excess insulin can also adversely stimulate granulosa cells by promoting premature differentiation [110]. Willis et al [244] demonstrate that GC from anovulatory hyperinsulinaemic women with PCO respond to
LH when follicles reach 4mm in diameter as compared to GC from ovulatory PCO and normal ovaries which only acquire similar responses at the 9-10mm stage. Intrafollicular LH attenuates GC growth, promotes terminal differentiation, and can cause follicular growth arrest if prematurely elevated within the follicle [232, 245]. By inducing GC to become abnormally sensitive to LH at an early stage, the consequences of excess insulin may therefore mimic an “excess LH” effect in the absence of elevated gonadotrophin. Therefore, it is highly probable that excess insulin and LH interact in ways that produce a sliding scale of negative effects on ovulation, with one being the predominant factor at each end of the scale. For instance, Silfen et al [246] demonstrate that lean anovulatory adolescent females had more pronounced hypothalamic/pituitary disturbances and little or no insulin dysfunction whereas their obese anovulatory counterparts had the opposite profile.

Selective insulin resistance in granulosa cells may also contribute to follicular arrest by restricting energy supply, thus attenuating the rapid growth characteristic of a Graffian follicle. Granulosa cells appear to rely on utilization of glucose as a primary source of ATP, possibly due to relatively hypoxic conditions within the follicle [247]. Rice et al [248] demonstrate impaired glucose metabolism in granulosa cells of women with anovulatory PCOS but not in ovulatory women with PCO, suggesting this phenomenon influences the incidence of anovulation. Very rapid granulosa cell growth is characteristic of the Graffian follicle, and an optimal number of GC may be a prerequisite for ovulation [195, 249]. Interestingly, animal models deficient in cell cycling proteins have anovulatory phenotypes, with follicles characterized by reduced granulosa cell numbers coincident with normal or high expression of differentiation markers [249], a situation that is evident in PCOS. In the early stages of antral follicle growth, FSH stimulates granulosa cell proliferation as well as expression of differentiation genes such as aromatase, and insulin/IGF1 enhances both processes. Therefore, it appears as if the FSH-stimulated mitogenic pathway is retarded while the differentiation pathway proceeds unhindered (or is hyperstimulated) in PCOS, possibly
reflecting the selective insulin resistance in granulosa cells and the need for insulin synergy to achieve adequate mitosis.

As stated previously, the degree of small follicle excess appears to be determined by the degree of hyperandrogenism. In turn, the absolute numbers of small developing follicles may impact on the incidence of anovulation as they are higher in anovulatory compared to ovulatory PCO [105, 224, 232], and the decrease in follicle numbers associated with advancing age may be the reason that menstrual cyclicity resumes in many older women with PCOS [92, 105, 183]. In addition, resistance to ovulation induction has been associated with a larger cohort of antral follicles [183, 250]. Jonard et al [183] propose that an auto-blocking effect arises from the small follicle pool that gives rise to follicular arrest, perhaps via the inhibitory action of AMH on FSH activity. Although AMH is elevated in PCOS at both the serum and follicular level, and is partly regulated by androgens in the male, regulatory control of this molecule in the ovary is unknown [251].

1.4 The Androgen Receptor

Androgens are steroid hormones that interact with a specific androgen receptor (AR), considered the only receptor for all androgens [252], binding testosterone and DHT with highest affinity [253]. Although some non-genomic effects of steroid hormones, including androstenedione, have been documented [254, 255], steroid-bound receptors classically affect physiological changes within a cell via DNA-binding, with subsequent modulation of gene transcription [252]. Variations in receptor number, phosphorylation status, interaction with transcriptional co-factors, and polymorphic variations are all variables that can confer tissue-specific responses to androgen [256, 257], and can impart varying degrees of sensitivity to the presence of hormone regardless of serum concentration. Herein lies ample potential for association of AR dynamics with heterogeneity of androgenic characteristics in PCOS.
1.4.1 Molecular characteristics of the AR

The AR is a member of the steroid hormone superfamily of nuclear receptors whose members are structurally characterised by four general functional domains: the amino-terminal (NH$_2$-) regulatory domain, a DNA-binding domain (DBD), a hinge region, and the carboxy-terminal (COOH-) ligand-binding domain (LBD) (Figure 1.9B) [256, 257]. Of these, the DNA-binding domain is the most highly conserved and the NH$_2$- transactivation domain the least conserved among family members. The AR gene is located on the X-chromosome at Xq11-12 and contains 8 exons (Figure 1.9A).

Transcription can be initiated from two promoters within the 5’ upstream regulatory region (URR), each controlled by different transcriptional control factors [258, 259]. In addition, two mRNA transcripts (11kb and 7.5kb) appear for AR in Northern blots of male reproductive tissues, arising from differential splicing in the 3’ untranslated region (UTR), not from differential promoter use, and do not affect the open reading frame of the gene [260]. Differential promoter use infers tissue specific regulation of the transcription of AR and differential 3’UTR splicing suggests a pre-translational control mechanism that can affect mRNA stability and/or alter interaction with translational control factors. On Western blots, full length AR migrates at approximately 110kD, depending on size of polymorphic microsatellites and state of receptor phosphorylation.

1.4.2 AR activation

Without ligand, AR resides in the cytoplasm bound to heat-shock proteins (HSP) 90, HSP70, and HSP56, which stabilize its tertiary structure and prevent constitutive receptor activity [261]. Ligand binding causes a conformational change in receptor structure that results in dislodgment of HSPs and subsequent translocation of the receptor-ligand complex into the nucleus. Nuclear AR binds to androgen response elements (AREs) on DNA, usually in tandem as an antiparallel arrangement of AR monomers formed by an interaction of the NH$_2$-
Figure 1.9: Molecular characteristics of the androgen receptor (AR). A. Gene structure. Coloured rectangles represent exons that code for the same coloured domains in the receptor protein (B). AR has two promoters in the 5’ upstream regulatory region (URR) where transcription can be initiated and two splice sites in the 3’ untranslated region (UTR) that give rise to two different mRNA species. B. General steroid receptor domains and their functions. C. Polymorphic regions within the N-terminal domain of the AR. The 3’CAG repeat motif is highly polymorphic and moderates the ability of AR to transactivate gene transcription but is not necessary for that function. The 5’ CCG repeat motif is less polymorphic and is essential for the AR transactivation function.
terminal region of one AR to the COOH-terminal region of another (termed the N-C interaction)[262]. These features are depicted in Figure 1.10.

Receptor phosphorylation is an important aspect of all steroid receptor activation, although when and where this takes place varies among family members. AR is rapidly phosphorylated after being translated, a step that is essential to the acquisition of hormone binding capacity [263]. A second phosphorylation event occurs after hormone binding and is part of the transformational process that enables AR to bind DNA [263].

In addition to DNA binding, AR also requires the interaction of a variety of specific and/or general co-regulatory molecules that can either activate or repress AR-mediated gene transcription (Figure 1.10). Specific androgen receptor associated factors (ARAs) include ARA24, ARA54, ARA55, ARA70, ARA160, and FHL2 [264, 265]. Other more general steroid receptor co-regulators include cAMP response element binding protein (CBP), steroid receptor coactivator-1 (SRC-1) and thyroid receptor activator molecule-1 (TRAM-1) [266]. These can be variably involved in the formation of a complex that enhances the recruitment of RNA polymerase II to promoter sequences in the 5’ upstream regulatory region (URR) of a gene for initiation of the transcriptional process.

1.4.3 Polymorphic regions

The AR has a relatively large NH₂-terminal domain that comprises nearly half of the molecule and harbours two polymorphic trinucleotide repeat motifs (Figure 1.9C) [267]. The 3’ (CCG)ₙ microsatellite encodes a poly-glycine tract in the translated protein that does not display a broad spectrum of length variation [268-271] and is essential for activation of target gene transcription [272]. In contrast, the 5’ (CAG)ₙ microsatellite encodes a poly-glutamine tract that is highly polymorphic in the population [268, 273-276] and is not essential for transcriptional activation by AR, but modulates its capacity to do so in vitro [277-279]. The normal range of CAG repeat number is considered to be 11 to 32 tandem repeats, with a non-Gaussian distribution of alleles that display ethnic differences as to the clustering of most
Figure 1.10: General mechanism of androgen receptor action in the cell. Inactive receptors are associated with heat shock proteins (hsp) that are dislodged upon hormone binding, which in turn activates the receptor. Activated receptors are then translocated into the nucleus where they form dimers and bind to androgen response elements (ARE) in the regulatory region of target genes. Numerous transcriptional cofactors are recruited to the site in an unknown sequence of events. Specific androgen receptor activators (ARA) most likely bind to the receptor complex and help to recruit the co-factors common to all steroid hormones: steroid receptor cofactor-1 (SRC-1) and cAMP response element binding protein (CBP). These in turn are thought to remodel chromatin and recruit the TFII family of proteins to the promoter site, which facilitates the binding of RNA polymerase II. Transcription of the target gene is then initiated.
common alleles [268, 273-276]. Recently, Buchanan et al [279] show that in all ethnic populations, >90% of alleles are situated in the 16-29 repeat range, which corresponds to the optimal microsatellite length for stable receptor N-C interaction. Only higher order primates exhibit variation at this gene locus and humans have the greatest range of variant alleles [280].

1.4.3.1 Clinical ramifications of AR polymorphisms

Mutations resulting in abnormal expansion of the AR (CAG)$_n$ repeat motif cause the neurodegenerative disease spinal and bulbar muscular atrophy (SBMA) [281] and may be associated with impaired spermatogenesis [282]. Within the normal polymorphic range of CAG repeats, specific variants have been associated with hormone sensitive pathologies in men and women such as prostate and breast cancer (Figure 1.11). Association of androgenic phenotype with AR genotype is more complicated in women since one AR allele is inactivated in every cell by the early developmental process of X-chromosome inactivation (XCI) [283]. While XCI is generally considered a random process, skewed XCI can occur whereby one allele is preferentially expressed, resulting in a range of hemizygous phenotypic effects [284]. No specific pathology has been associated with variants of the (CCG)$_n$ repeat alone, however a weak association with prostate cancer has been reported, and the juxtaposition of the two homopolymeric stretches may be important [268-271].

1.4.3.2 Effect of CAG polymorphism on AR activity

In vitro studies of AR expression constructs with varying numbers of CAG repeats, translated as a poly-glutamine (polyQ) stretch of amino acids, indicate that deletion of the microsatellite incurs greater transcriptional activity of target genes than naturally occurring receptor variants [272], and within the normal polymorphic range there is an inverse relationship between transactivation and polyQ length, whereby lower repeat number effects greater receptor enhancement of target gene product [277, 279, 285-289]. All normal CAG allele variants
Androgen Receptor Gene

Figure 1.11. The (CAG)n repeat polymorphism in the N-terminal region of the androgen receptor gene. The normal polymorphic range is 11 to 32 tandem repeats. This sequence encodes a polyglutamine tract of variable length in the receptor protein that has functional consequences for receptor activity: short tracts confer relatively high transactivation ability and long tracts confer relatively low transactivation ability. This is thought to impart variable degrees of androgen action (androgenicity) among individuals and influence the manifestation of hormone-dependent diseases.
appear to have equivalent hormone binding kinetics to both T and DHT, although the differential effect of repeat number on transcriptional activation may be enhanced in the presence of excess ligand, and may be a cell-specific type of event that is dependent upon differentially expressed co-regulatory molecules that bind to this region [286-291]. Increased receptor phosphorylation has also been observed with increasing length of the polyQ tract in the mature AR protein and may affect transcriptional ability of constructs with varying repeats [280].

1.4.4 AR in the ovary

Early in vitro studies of rat ovarian granulosa cells suggested that apart from serving as precursors for the synthesis of oestrogen, androgens affected ovarian function directly through AR-mediated activity [292-295]. Subsequent to this, immunoreactive AR has been documented in the ovaries of all mammalian species studied to date, as well as some non-mammalian species including chickens [296], frogs [297] and fish [298, 299], suggesting an important role in ovarian function.

1.4.4.1 AR localization in the mammalian ovary

All ovarian cell types possess AR immunoreactivity to varying degrees among mammalian species, including ovarian surface epithelial cells, stroma, theca, granulosa, and oocytes; localization is predominantly nuclear, although cytoplasmic immunoreactivity has been observed in rat oocytes at a particular developmental stage [300]. In most species, granulosa cells exhibit the strongest signals for AR mRNA and/or protein as compared to the other cell types. Some studies suggest that stromal AR expression is differentially distributed in the ovary, being most prominent around healthy follicles and absent from the area around atretic follicles and in stroma that is distant to follicles [301, 302] but this was not observed by Suzuki et al [303]. Oocyte expression of AR has been reported in mice [304], rat [300], pig [305] and the rhesus [301] but not marmoset primate [306] species. Apart from potential
species differences in AR expression within the various cell types, differences can also arise from the use of different antibodies and stringency of immunohistochemical (IHC) techniques.

1.4.4.2 AR regulation in the ovary

In the non-human primate [306-308], pig [305], and rat ovary [309], granulosa cell AR expression has an inverse relationship with stage of follicle development and granulosa cell maturity. In these models, granulosa cell AR expression is most abundant in pre-antral and early antral follicles, declining as the latter approach the pre-ovulatory stage and acquire aromatase expression. Hillier and Tesuka [310] propose that this decline in receptor expression may be necessary for the final stages of follicle maturation to occur. In rats, AR expression begins to decline in the mural granulosa cells of selected antral follicles at the time of oestrus and disappears in a centripetal fashion toward the antrum, leaving only the innermost layer positive for AR at the time of pro-oestrus; interestingly, cumulus granulosa cells escape this down-regulation [311]. Conversely, AR expression in primordial and preantral follicles of all the above-mentioned species remains impervious to oestrus/menstrual cycle transitions. This developmental pattern of AR expression may be reversed in the human [302, 303, 312, 313] and cow [314], whereby AR expression is low or absent in the granulosa cells of primordial, preantral, and early antral follicles, incrementally increasing as antral follicles enlarge and approach the pre-ovulatory stage. Interestingly, AR expression in human follicles appears in theca cells before granulosa cells, and as the developing granulosa layer gains receptor expression, theca cells lose it [303]. After ovulation, AR progressively diminishes in luteal cells over the luteal phase in all species, and is usually absent in the CL at time of regression. It is also diminished or absent in the granulosa cells of atretic follicles, although maintained in the theca of these follicles [302, 303].

Regulation of AR expression appears to involve gonadotrophins and androgens, with apparent species differences as to timing effects and up- or down-regulation. Gonadotrophins
administered in vitro decrease AR mRNA in rat granulosa cells [309], but similar FSH treatment had no effect in porcine granulosa cells [305, 315]. In the rhesus monkey, three different in vivo treatment regimes demonstrate 1) FSH increases AR mRNA only in the granulosa cells of primary follicles [233], 2) hCG increases AR expression only in the granulosa cells of preovulatory follicles, independent of steroids [316], and 3) testosterone increases receptor expression in granulosa cells but decreases it in theca and stroma cells of most antral follicles [308]. In vitro, neither FSH nor cAMP administered alone had any effect on immature rat granulosa cell AR expression, but FSH could reverse a reduction of AR mRNA elicited by DHT [317]. Apparently, this is the only published study that examines regulation of AR expression in ovarian cells in vitro.

Although the veracity and direct/indirect nature of the effects of in vivo treatments on AR expression remain undetermined, regulation of AR by gonadotrophins and androgens in ovarian cells is consistent with cAMP response elements (CRE) [318, 319] and androgen receptor response elements (ARE) [320, 321] found in the AR gene. Also, similar regulatory profiles have been shown for AR expression in testicular Sertoli cells, which are the male equivalent of granulosa cells [322-325]. Sertoli cell AR expression is also temporally regulated depending on the maturity of the germ cells that they surround [325-327], and differentially regulated as compared to androgen-secreting Leydig cells [328]. Clearly, the control of AR expression in the ovary is complex and may differ between species, as might be expected from the observation that certain developmental patterns of AR expression may also be species specific.

1.4.4.3 Functional role of AR in the ovary

Total ablation of AR results in subfertile mice that have an elongated oestrus cycle and a luteal phase defect that persists after superovulation and partly involves a post-hCG increase in granulosa cell apoptosis [329]. Prior to hCG administration, the granulosa cell compartment is deficient in AR−/− mice, supporting the notion that direct androgen activity is
necessary for optimal granulosa cell proliferation. The fact that follicle numbers appear to be
normal in these mice suggests that follicle recruitment does not require AR-mediated activity,
at least in this species.

The use of DHT as a non-aromatizable androgen with highest affinity for the AR, and
in some cases opposition of androgen ligand binding by the non-steroidal antiandrogen
flutamide or its more potent metabolite hydroxyflutamide, are methods used to explore AR-
mediated actions both in vivo and in vitro. Early studies claimed androgens were mediators of
atresia, but later data pointed unequivocally to a trophic role in early growth and development
of ovarian follicles (reviewed by [310]). The AR is predominantly expressed in healthy
follicles of all species and in monkeys has been positively associated in vivo with markers of
cell proliferation [308] and abundance of mRNA for FSHR [233], as well as negatively
associated with markers of apoptosis [308]. Androgens also directly stimulate in vitro follicle
growth in mice, independent of FSH [330, 331], and are thought to boost human primary
follicle growth by stimulating surrounding stromal tissue to secrete survival factors [231].
However, in vitro studies using isolated granulosa cells reveal variable effects of androgens
on proliferation, at times stimulatory and at others inhibitory [332-334]. These differential
effects are likely to be due to specific experimental conditions as well as lack of modulating
factors that arise from other follicular cells.

The effects of AR-mediated activity within theca cells and oocytes have not been
widely explored despite documentation of receptor expression in these cells. In vitro, DHT
has been shown to have variable effects on steroidogenic enzyme expression and activity in
theca cells [335-337], both promoting and inhibiting androgen synthesis. Interestingly, oocyte
maturation is dependent upon a non-transcriptional AR-mediated mechanism in *Xenopus
laevis* [297], a mechanism that may be conserved in higher vertebrates [304]. Although AR
ligands have been found to reversibly inhibit in vitro oocyte maturation and prolong meiotic
arrest in mice [338-340] and pigs [341], Hammes [342] suggests this may have been due to
the difficulty of preventing spontaneous maturation upon follicle aspiration in these studies. Bovine cumulus-oocyte complexes cultured with testosterone or DHT have an increased cleavage rate [343], but these androgens had no effect on cleavage and blastocyst development in in-vitro matured pig oocytes [344]. Oocyte quality and developmental capacity has yet to be assessed in AR⁻/⁻ mice, although these mice do produce viable embryos and pups [329].

1.4.4.4 Molecular mechanisms

Numerous studies have shown that androgens augment most of the differentiative activities of FSH, and this occurs via post-receptor mediated mechanisms (reviewed by [310]). This review proposes that androgens modulate cAMP “tone” in follicular cells, by amplifying FSH-induced cAMP formation and action, perhaps through regulation of expression and/or activity of phosphodiesterases that degrade cAMP, though this has yet to be experimentally tested. In vivo studies suggest that androgens also upregulate the FSH receptor (FSHR) in early stages of follicle development, thus priming immature granulosa cells for subsequent exposure to circulating FSH [233, 345]. Regulation of the FSHR in granulosa cells is influenced by many factors, including FSH itself, but the initial trigger for its induction is still unknown [346]. Ablation of IGF1 in the ovaries of mice severely reduces FSHR expression [47], and since amplification of IGF1 signalling occurs in all follicle compartments of androgen treated monkeys [234, 235], this may be the means by which similar treatment amplifies FSHR expression. Indeed, boosting IGF1 activity could potentially be the primary mechanism by which androgens exert trophic effects on follicle growth, both in the FSH-independent phase, and the FSH-dependent phase. In support of this theory, androgens have been shown to regulate IGF1 and/or various IGF binding proteins (IGFBP) in prostate, bone, liver and uterine tissues [347-350].

In keeping with the traditional function of liganded AR as a transcriptional factor that mediates gene expression, an important key to understanding direct androgen action in the
Ovary would be the identification of AR-regulated ovarian genes. Although the expression of FSH-regulated genes (including aromatase, inhibin, and LHR) is further augmented by the presence of androgen, this is probably not an effect of AR binding to the promoters of these genes. It more probably occurs through genes regulated by AR that are involved in intracellular signalling cascades induced by FSH and/or other signalling cascades that interact with FSH signalling. To date, no such AR-regulated genes have been identified. However, mRNA for lysyl oxidase, an enzyme important to extracellular matrix deposition, is markedly induced by DHT in the absence of gonadotrophin in rat granulosa cells [351, 352]. This gene is also more highly expressed in an androgen-induced rat model of PCO [353]. Other enzymes involved in extracellular matrix modelling are differentially expressed in PCOS and TSX ovaries as compared to normal ovaries, suggesting regulation by androgens [167]. The significance of these latter findings in terms of impact on normal ovarian function and potential role in ovarian cyst formation are unknown, but may represent an important role for androgens that has not received much investigative attention.

1.4.5 AR in PCOS

Two approaches can be taken in considering the AR with respect to PCOS: 1) a causal role for altered receptor functionality and 2) a mediator role in determination of disorder phenotype. These two roles are not mutually exclusive: in fact, fulfilment of both roles is consistent with the self-perpetuating nature of PCOS.

1.4.5.1 Abnormalities of AR expression in PCOS

Few studies have addressed qualitative and/or quantitative aspects of AR expression with regard to PCOS. Using IHC, Chadha et al [354] report a persistent up-regulation of stromal AR by systemic androgen excess upon comparison of normal (albeit post-menopausal) ovaries to PCOS and TSX ovaries. However, quantitative methods were not used to draw these conclusions and such a result would be expected due to the stromal hyperplasia
characteristic of PCO. In contrast, Takayama et al [106] report normal AR immunoreactivity in PCOS ovaries, but also use non-computational visual assessment. To this author’s knowledge, no studies have looked for mutations in the AR or quantitatively addressed AR mRNA expression in PCOS.

1.4.5.2 AR (CAG)n polymorphism in PCOS

Most studies examining the role of AR in PCOS have focused on the polymorphic CAG repeat microsatellite in exon 1 of the AR gene. The first two published articles on this topic found no correlation between this genetic marker and PCOS but they are both methodologically inadequate to draw such conclusions [355, 356]. One study had a cohort of only 10 women, less than half the number of possible polymorphic alleles to be found in a population [356]. The other study had a larger cohort, but did not properly analyse the frequency of allele distribution between controls and patients [355]. With permission from the first author, a reanalysis of the data shows that Mexican-American women with PCOS had a significant skew toward alleles with lower CAG repeat numbers as compared to the control group (unpublished data). This finding was partly corroborated by Mifsud et al [357] who report a trend toward lower CAG repeat number in Asian women with anovulatory infertility, but this association was only significant when a subgroup of anovulatory women who had normal serum testosterone levels were considered alone. The latter is a good example whereby AR variants may cause increased tissue sensitivity to hormone, resulting in a hyperandrogenic ovarian phenotype in the absence of elevated androgens. It is also possible that exaggerated AR activity somehow affects androgen biosynthesis and/or secretion. Lower CAG repeat number has been associated with increased risk of post-menarchal ovarian hyperandrogenism preceded by precocious pubarche [358]. Two studies have also reported an association between lower CAG repeat number and higher serum androgens in pre- and postmenopausal women [359, 360]. The converse situation may apply to men [361].
The polymorphic CAG repeat region in AR has also been examined with respect to other androgen sensitive conditions related to PCOS including hirsutism [355, 362, 363], acne [355, 362-364], abdominal weight distribution [355, 365], irregular menstrual bleeding [366] and male pattern baldness (a putative male phenotype for PCOS) [367]. However, there is no consensus on the effect of this polymorphism in determining sensitivity to these conditions in women, partly due to the paucity of studies, small study cohorts, the variation in clinical criteria between studies, and problems associated with the effects of non-random X-inactivation of AR alleles in females.

### 1.4.5.3 Antiandrogen treatment in PCOS

Various antiandrogens have been used to treat women with PCOS [368, 369], but the one most specific for the AR is flutamide, a non-steroidal molecule that binds the receptor but does not induce transactivation of AR target genes [263, 370]. In general, most antiandrogens, including flutamide, are effective in reducing cutaneous symptoms of hyperandrogenism, especially hirsutism, theoretically through AR blockade but also through a reduction in serum androgens that occurs in most cases [369, 371-376]. The reduction in serum androgens by flutamide provides evidence that androgens are directly involved in up-regulating androgen synthesis in human adrenal glands and ovary [377]. In the case of the adrenal glands, this occurs only at the level of steroidogenic enzymes as flutamide treatment has no effect on the pituitary-adrenal axis [371, 378-381]. Conversely, an AR-mediated mechanism does appear to affect hypothalamic and/or pituitary secretion of LH as flutamide treatment can decrease serum levels of this gonadotrophin [377, 382], possibly via restoration of GnRH pulse generator sensitivity to inhibitory steroids [383]. The latter is consistent with the hypothalamic-pituitary perturbations that occur in pre-natally androgenized animals [384]. Importantly, the above effects of flutamide treatment appear to be independent of hyperinsulinaemia and insulin resistance, which is largely unaffected by antiandrogen therapy.
in PCOS [381, 385, 386] or by long term oestrogen/antiandrogen or testosterone therapies for individuals undergoing cross-sex transformation [387].

Monotherapy with flutamide has given inconclusive results on whether such therapy induces ovulation in anovulatory patients and/or improves menstrual irregularities. In two studies, groups of adolescents with PCOS were treated for a minimum of 6 months with a comparable dose of flutamide, and while both studies report reductions in serum androgens, one study observed complete reversal of all reproductive abnormalities [382], and the other observed no changes [388]. One of the main differences between these two studies was the documentation of hyperinsulinaemia in the latter study, which was not assessed in the former, leaving the possibility that these two studies represented two distinct metabolic subgroups of PCOS. Clinical investigation of older women treated with flutamide report highly variable effects on serum hormones and little or no effect on ovulatory status and/or menstrual regularity [376, 379, 389, 390].

More recently, a combined therapy of flutamide with the insulin-sensitizing drug metformin has been trialled in young women with PCOS, providing evidence of improved outcomes on all reproductive and metabolic parameters over that achieved by either agent alone [391, 392], and these beneficial effects were maintained when an oral contraceptive was added [393]. Further studies by this same group initially identified flutamide as the key ingredient within the latter combination therapy that attenuated hypoadiponectinemia, ovarian vascular hyperresistance, lean mass deficit, and central adiposity in hyperinsulinaemic, hyperandrogenic adolescents [394]. However, removing Metformin from the combination therapy produced similar results [395], indicating that these effects cannot be exclusively attributed to direct androgen action, with the possible exception of alterations to serum levels of low density lipoprotein (LDL). The effects of flutamide on body composition, lipid profiles, and vascular resistance are likely to be AR-mediated since expression of this receptor
is found in fat, especially abdominal fat [396], vascular endothelial cells, and muscle tissues [397].

In general, the effect of clinical antiandrogen treatment on ovarian cells has not been determined, making it difficult to assess whether cessation of AR-mediated activity at this level was involved in the observed improvements to ovarian function in those individuals who responded well to therapy.

1.5 Thesis Hypotheses and Aims

The general focus of this thesis was to further explore AR-mediated activity in the ovary, with intent to ascertain whether such activity is altered in PCOS. This work proceeded along two separate hypotheses and sets of aims:

1.5.1 Hypothesis #1

The (CAG)n repeat polymorphism in the AR gene influences the manifestation of PCOS.

1.5.1.1 Specific aims for hypothesis #1

Since ethnic variations are evident in allele distribution patterns for the AR (CAG)n repeat polymorphism, as well as for clinical manifestations of PCOS, associations between this genetic element and the disorder may differ between diverse racial populations, necessitating numerous studies to establish relevance. In addition, no one has attempted to factor into the analysis the variable allele expression patterns that could arise from non-random inactivation of the X chromosome in female cells. Besides potentially affecting allele distribution patterns, the latter could also influence linkage studies for PCOS at this gene locus. Urbanek et al [157] included the AR (CAG)n locus in a large linkage analysis for PCOS genes, and it was among the first selected subgroup of potential candidates for significant linkage, but was ruled out after stringent statistical adjustments for multiple testing. Differential X-chromosome inactivation (XCI) patterns could feasibly undermine positive linkage at the AR (CAG)n
locus, and account for symptomatic discrepancies between affected and unaffected/partially affected sisters with the same genotype in families with PCOS. Therefore, the following specific aims were devised:

1. Compare the distribution of AR alleles containing the (CAG)n polymorphism in populations of Australian Caucasian women with and without PCOS (Figure 1.12A).
2. Determine whether there is an association between CAG repeat number and various clinical parameters of PCOS.
3. Determine whether patterns of XCI in individuals heterozygous for the AR (CAG)n polymorphism influence the potential significance of this genetic factor in PCOS (Figure 1.12B).

3a. In population comparisons, use XCI patterns to estimate degree of allele expression and adjust for this expression in distribution and association analyses.

3b. In family groups with PCOS, compare XCI patterns between sister pairs that are genetically the same for the AR (CAG)n polymorphism but have a different clinical diagnosis of PCOS.

1.5.2 Hypothesis #2

AR-mediated activity in ovarian granulosa cells is disrupted in women with PCOS.

1.5.2.1 Specific aims for hypothesis #2

Although in vivo evidence proposes a mitogenic role for androgens, most in vitro studies suggest an anti-proliferative/pro-differentiative role. In light of the current reproductive view that the oocyte controls follicle growth, experiments in this thesis were designed to address the following specific aims (Figure 1.13):

1. Establish whether oocytes affect AR-mediated signalling in granulosa cells in vitro, using pre-pubertal pig ovaries as source material, since such experiments cannot be
Figure 1.12. First hypothesis: The (CAG)n repeat polymorphism in the N-terminal region of the androgen receptor (AR) influences manifestation of polycystic ovary syndrome (PCOS). The normal polymorphic range is 11 to 32 tandem repeats. This sequence encodes a polyglutamine tract of variable length in the receptor protein that has functional consequences for receptor activity: short tracts are associated with relatively high androgen action and long tracts are associated with relatively low androgen action. Specific aims: A. To document the distribution pattern of CAG repeat alleles in a population of women with PCOS to see if this pattern deviates from normal distribution patterns, perhaps in a manner indicative of increased androgen action and B. To determine whether the process of X-inactivation plays role in mediating the influence of specific AR alleles in PCOS.
Androgen Receptor Gene

A. Allele distribution in PCOS indicative of increased androgenicity?

B. Influence of X-inactivation:
   Favours expression of more androgenic alleles in PCOS?

Inherited alleles

CAG\text{(short)}  CAG\text{(long)}

X-inactivation

Cellular Expression

Androgenicity
AR-mediated granulosa cell functions:

1. Influenced by oocyte secreted factors (OSFs)?
2. Perturbed in women with PCOS?

Figure 1.13. Examination of AR-mediated activity in granulosa cells from antral follicles. Two granulosa cell subtypes exist in an antral follicle, partly due to variable exposure to oocyte-secreted factors (OSFs). Is androgen receptor (AR)-mediated activity in granulosa cells influenced by these OSFs? In women with polycystic ovary syndrome (PCOS), a condition associated with excess thecal cell androgen secretion, is there evidence of perturbed AR-mediated granulosa cell function?
done with human tissue for lack of access to oocytes and cells from small antral follicles.

2. Compare AR signalling in human granulosa cell subtypes that had different associations with the oocyte in vivo (i.e. cumulus versus mural granulosa cells) prior to oocyte retrieval procedures in women undergoing IVF. Do this comparison on cells obtained from the ovaries of normal cycling women and women with PCOS to address whether altered AR signalling is evident in this disorder.

1.6 Thesis Format

Although this thesis is not formally submitted as a “thesis by publication”, due to the fact that not all of its chapters have been formally published, all data-presenting chapters have been written in manuscript format, conforming to the specifications of the specific journal to which it was or will be submitted. The identities of these journals, and the contributions of the authors involved, are presented in the indicated Appendices. Chapters representing published articles have not had their content altered in the preparation of this thesis, but the subtitle formatting and figure coloration was changed to align with the general thesis outline. New insights on this data and recent studies that have relevant implications are included in the final discussion chapter.
Chapter 2 The Androgen Receptor CAG Repeat Polymorphism and X-Chromosome Inactivation in Australian Caucasian Women with Infertility Related to Polycystic Ovary Syndrome.¹

2.1 Abstract

The human androgen receptor (AR) gene contains a polymorphic trinucleotide (CAG) repeat sequence in exon 1. The number of CAG repeats may confer differential receptor activity and specific ranges of variants have been correlated with androgen sensitive disease processes. Polycystic ovary syndrome (PCOS) is a female condition characterised by androgen excess and infertility, many features of which are probably mediated through the AR. We compared frequency distributions of CAG repeat alleles, and their pattern of expression via X-inactivation analysis among 83 fertile women and 122 infertile women with PCOS, all of Australian Caucasian ethnicity. A population comparison with 831 predominantly fertile Australian women was also employed. PCR based assays were used to genotype each woman and assess allele inactivation patterns following digestion of DNA with methylation sensitive Hpa II. Infertile women with PCOS exhibited a greater frequency of CAG alleles or biallelic means >22 repeats as compared to both the fertile control group (p < 0.05) and the general population (p < 0.01). Preferential expression of longer CAG repeat alleles was also observed in PCOS and correlated with increased serum testosterone. We conclude that the AR (CAG)n gene locus and/or its differential methylation patterns influence the disease process leading to PCOS.

¹See Appendix 1
2.2 Introduction

The androgen receptor (AR) contains a polyglutamine tract of variable size in the N-terminal transactivation domain, which can modulate the receptor’s ability to enhance transcriptional events \textit{in vitro} [277]. This tract is encoded by a highly polymorphic CAG repeat microsatellite in exon 1 of the AR gene, located on the X chromosome at Xq12-13. Theoretically, an inverse relationship exists between repeat number and AR activity whereby tracts of shorter size confer greater activity than tracts of larger size. Many studies suggest that variations in androgen sensitive disease processes in both men and women are correlated to increased frequencies of either shorter or longer CAG repeat alleles [278, 362, 364, 398]. However, some clinical studies showing positive correlation are refuted by others showing no correlation [276, 363, 399]. Allele distribution patterns can differ between populations [273, 274] allowing ethnicity, as well as cohort numbers, to exert a strong influence on the variation that appears between studies. When women are the subject of investigation, the phenomenon of X-inactivation whereby one X chromosome becomes inactive in every female cell [283], further complicates interpretation. Few studies involving women take this factor into account.

Polycystic ovary syndrome (PCOS) is an endocrine disorder characterised by abnormal androgen production and/or activity that leads to changes in the control of follicle development and maturation [400]. Evidence in non-human primates [401] and transsexual women [354] treated with high doses of androgen indicate that the characteristic ovarian morphology, comprised of an enlarged ovary with numerous small follicular cysts, may be the result of direct, receptor-mediated androgen activity. In women with PCOS, this disruption often leads to chronic anovulation and subsequent infertility. Recently Mifsud \textit{et al} [357] have reported that normoandrogenic Chinese women with anovulation and polycystic ovaries had a significantly greater frequency of short AR CAG repeat alleles than hyperandrogenic Chinese women with the same condition. They suggest that the higher AR activity potentially conferred by shorter CAG repeat alleles may amplify the androgenic response in ovarian
tissues, resulting in a PCOS phenotype. Acceptance of this proposal assumes preferential expression of the allele of shorter size by skewed inactivation of the allele of larger size but patterns of X-inactivation were not assessed. This study showed no differences in CAG allele distribution between infertile Chinese women with PCOS and fertile Chinese women.

We investigated a cohort of Australian Caucasian women with PCOS to compare their patterns of CAG repeat distribution and X-chromosome inactivation against those of normal women. Vottero et al [362] have reported that preferential expression of the shorter CAG repeat allele was the sole difference between women with and without hirsutism, an androgen sensitive condition. Considering the results of these previous studies, we expected to find an association between PCOS and increased frequency or preferential expression of AR CAG repeat alleles in the lower region of the normal polymorphic spectrum, but surprisingly found the opposite to occur in our population of women.

2.3 Subjects and Methods

2.3.1 Study population

Women were recruited from infertility and antenatal clinics at The Queen Elizabeth Hospital in Adelaide, South Australia, following approval by the ethics committee of North Western Adelaide Health Services. Due to ethnic differences in allele frequency for the hAR (CAG)n polymorphism, women from distinct Asian, Indian, Middle Eastern or Aboriginal heritage were excluded from the study. The resultant study group represents women of various European cultural backgrounds and can generally be classified as Caucasian. A total of 171 subjects in an infertile relationship were recruited. All of these women were subject to the same infertility assessment protocol, which included interviews about reproductive history, pelvic ultrasound for ovarian morphology, blood tests for hormonal profiles, tracking of menstrual cycles, and height and weight measurements. Women with abnormal androgen levels were further assessed for disorders that form exclusion criteria for the diagnosis of
PCOS [402]. PCOS was based on the criteria of hyperandrogenism and anovulation with exclusion of congenital adrenal hyperplasia and Cushing’s syndrome. We also recorded the presence of polycystic ovaries (PCO) on ultrasound due to documented AR-mediated causal effects and to accord with criteria used in a previous study of CAG repeats in infertile women [357]. PCO were defined as the presence of ≥ 8 peripheral cysts <10 mm in diameter with increased ovarian stroma on ultrasound, occurring bilaterally [403]. Hyperandrogenism was diagnosed as the presence of at least two of the following: high serum androstendione (> 7.0 nmol/L), high serum testosterone (> 2.0 nmol/L), low SHBG (<20 nmol/L). Menstrual cycles were considered normal if they occurred every 23-35 days and anovulation was inferred either by repetitively abnormal (> 35 days) menstrual cycle history or serial serum progesterone levels less than 5 nmol/L in the luteal phase. A total of 122 (71%) of women fulfilled our criteria for PCOS. We recruited 83 women from an antenatal clinic to serve as controls, all of whom were Caucasian, had had a history of normal menstrual cycles, had no history or evidence of cutaneous hyperandrogenaemia, and had never sought treatment for infertility.

2.3.2 Analysis of the hAR (CAG)n gene locus

2.3.2.1 DNA extraction

Peripheral blood lymphocytes were purified from whole blood using Lymphoprep (Nycomed Pharma, Oslo, Norway), and kept frozen in saline at –20C until DNA extraction. DNA was extracted from this tissue using a QIAmp Blood Kit (Qiagen, Chatsworth, CA) protocol, quantified by spectrophotometry, and stored at –20C.

2.3.2.2 CAG allele characterisation.

Alleles were characterised by the number of CAG repeat units in the hAR microsatellite. Our technique is similar to those used in other published studies [363, 404]. Genomic DNA (100 ng) was amplified by PCR using primers that flank the hAR (CAG)n polymorphism and
include two Hpa II sites that are methylated on the inactive X-chromosome: 165-hAR-CAG-S, 5’-gtgcgcgaagtgatccagaa-3’ and 392-hAR-CAG-AS, 5’-tagcctgtggggcctctacg-3’. The sense primer was 5’-labelled with the fluorescent dye TET (tetrachloro fluorescein). PCR was performed with the DNA polymerase HotStarTaq (Qiagen, Chatsworth, CA) using reagents and protocol provided with the enzyme, and a primer annealing temperature of 65°C. PCR products were run on Genescan (ABI, Foster City, CA) under standard conditions and analysed by Genescan 3.1 software. Sequenced standards were run on each gel to correlate PCR fragment size with CAG repeat number.

### 2.3.3 X-inactivation analysis

Subjects homozygous at the AR(CAG)n gene locus were excluded from X-inactivation analysis due to an inability to distinguish between the two alleles: 13% (n = 16) PCOS, and 17% (n = 14) fertile controls. The remaining heterozygous samples including 87% (n = 106) PCOS, and 83% (n = 69) fertile controls were analysed for X-inactivation ratio by assessment of methylation status using the methylation-sensitive restriction enzyme, Hpa II (Roche Diagnostics, Mannheim, Germany). Non-methylated (active X) DNA segments digest with enzyme and are thereby unavailable for PCR amplification. Methylated (inactive X) Hpa II sites do not digest with enzyme, and remain intact for amplification. Post-digestion PCR products therefore represent methylated (inactive X) DNA sequences only.

Equivalent 2µg DNA aliquots were either digested with Hpa II (30 units) or mock-digested in digestion buffer with no enzyme. Samples were digested overnight at 37°C in a 20µl reaction volume, with a final enzyme denaturation step of 95°C for 5 minutes. Aliquots of 5µl were amplified by PCR and analysed on Genescan as described above. Total fluorescent peak areas for both alleles, and the ratio to which each allele contributed to the total, were calculated for digested and undigested samples using Genescan 3.1 software (ABI, Foster City, CA). Differences in the allele ratio between undigested and digested samples represents the degree to which one allele is more or less methylated than the other in a DNA sample. We
used calculations similar to those reported by Naumova et al [405], expressing final ratios in terms of degree of inactivation of the longer allele (which directly correlates to the degree of expression of the shorter allele).

All samples were analysed in duplicate in non-digested and digested conditions. In calculating the degree of X-inactivation for individuals, we used the average value of the duplicate samples for both digested and undigested DNA samples in the calculations. While total florescent peak areas for replicates varied, the ratio that each amplified allele contributed to total florescence remained remarkably constant, with a mean difference of 1.4%, and standard deviation of 1.5% between replicates.

**2.3.4 Allele distribution profiles**

Alleles were analysed following the conventional method of representing data of this type in women. First, total alleles were plotted whereby each woman contributed two independent values that represented both CAG repeat alleles. Three further modes of allele representation were employed, using 1) the mean value of the two alleles (biallelic mean); 2) the shorter allele alone and; 3) the longer allele alone. In the latter two instances, the use of “shorter” and “longer” is relative per individual and does not represent an absolute CAG repeat number or range of numbers. We also formulated a new method of calculating biallelic means whereby the results of X-inactivation analysis were used to create a mean value that represents differences in the expression of constituent alleles. This was achieved by multiplying each allele in a genotypic pair by its percent of total expression (100 % inactivity) and summing the two adjusted repeat values to achieve a new mean value that we call the X_weighted_biallelic_mean. Individuals homozygous at the AR (CAG)n locus were included in the distribution comparison of X_weighted_biallelic_means since variation of allele expression would not alter the mean value of alleles of equivalent repeat number.
2.3.5 Statistical analysis

All statistics were generated using SigmaStat Software (Version 2, SPSS Inc., Chicago, IL). Parameters for statistical analysis among groups lacked normal distribution profiles, requiring non-parametric statistical tests. Allele distributions between groups were analysed by dividing control profiles into two sections of approximately equal size then using the median CAG repeat value as a cut-point for division of PCOS alleles. Differences in distribution were then determined by Fisher’s Exact Test. Simple linear regressions and Spearman rank order correlations were used to compare serum testosterone and BMI values with CAG repeat number in the PCOS group. If a correlation was found, t-tests comparing alleles divided by specific CAG repeat number cut-points were used to further characterise the correlation. Frequency of skewed (≥ 80%) inactivation of one allele, as defined by Naumova et al [405], was analysed using Fisher’s Exact test. For all tests, significance was set at 5%.

2.4 Results

2.4.1 CAG allele distribution analysis

Androgen receptor CAG repeats ranged from 14-30 in control women and 8-32 in PCOS women, which reflects the normal polymorphic range reported at this genetic locus for a large cohort (n = 831) of predominantly fertile (74%) Australian Caucasian women [406]. Control allele distribution profiles corresponding to total alleles, biallelic means, and $X_{weighted\_biallelic\_means}$ were divided into two approximately equal sections at a cutpoint of $\leq 22$ CAG repeats. Short allele distributions had a cutpoint of $\leq 21$ and long allele distributions had a cut-point of $\leq 23$ to ensure equal partition. Women with PCOS had a significantly greater frequency of alleles in the upper half of the polymorphic spectrum when comparing distributions of biallelic means and long alleles with control women (Table 2.1). Interestingly, when biallelic means were adjusted for preferential allele expression via X-inactivation analysis, this difference between groups became highly significant ($p = 0.04$ for
Table 2.1: Androgen receptor Exon 1 (CAG)n repeat allele distribution profiles in case and literature subjects

Values with the same superscript in the same row are significantly different from each other. NA = Not available.

<table>
<thead>
<tr>
<th>Mode of CAG allele distribution and cut-point value</th>
<th>PCOS (n = 122)</th>
<th>Fertile Controls (n = 83)</th>
<th>Female Australian Caucasian Population (n = 831)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total alleles ≤ 22</td>
<td>46%</td>
<td>57%</td>
<td>57%</td>
</tr>
<tr>
<td>Biallelic Mean ≤ 22</td>
<td>36%&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>51%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>X_Weighted_Biallelic_Mean ≤ 22</td>
<td>38%&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60%&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>Short allele ≤ 21</td>
<td>52%</td>
<td>58%</td>
<td>63%</td>
</tr>
<tr>
<td>Long allele ≤ 23</td>
<td>34%&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>51%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56%&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> = p ≤ 0.05  
<sup>b</sup> = p ≤ 0.01  
<sup>c</sup> = p ≤ 0.001
biallelic means vs. $p = 0.009$ for $X_{\text{weighted\_biallelic\_means}}$) (Figure 2.1). We also compared the Australian Caucasian data with our group data and found a very significant difference ($p < 0.001$) between this large cohort and the PCOS group for the distribution of biallelic means and long alleles (Table 2.1). In contrast, the allele distribution patterns of fertile controls were concordant with those of the larger population profile.

### 2.4.2 X chromosome inactivation analysis

The PCOS group appeared to have a greater incidence of non-random X-inactivation (alleles $\geq 60\%$ inactive) than Controls, but this was not significant (63\% vs. 49\%, $p = 0.08$) (Figure 2.2). We found no evidence of abnormal incidence of skewed X-inactivation (alleles $\geq 80\%$ inactive) in either group (14\% Controls, 15\% PCOS), which is comparable to previously published studies [404, 405].

To determine whether alleles of specific repeat number were preferentially inactivated, we characterised each genotype with non-random inactivation patterns by the specific repeat number of the constituent alleles, and compared distribution of active versus inactive alleles over the normal polymorphic range (Controls $n = 43$, PCOS $n = 65$). Alleles $>22$ repeats were preferentially inactivated compared to alleles $<22$ repeats in both groups, with no differences between groups (51\% vs 37\% Controls; 57\% vs. 26\% PCOS). However, when analysing the distribution of partner (preferentially active) alleles over the polymorphic range, Controls had 40\% and PCOS had 57\% in the $>22$ repeat range ($p = 0.03$) (Fig. 2.3). This difference explains the significant shift in frequency of $X_{\text{weighted\_biallelic\_means}}$ toward the upper region of the polymorphic range in the PCOS group as compared to Controls (Fig. 2.1).

### 2.4.3 Clinical characteristics

Fertile control and PCOS women were well matched for age (median $\pm$ SD; 28.5 $\pm$ 7.3 vs. 29.4 $\pm$ 4.8 years). All women in this study are $<50$ years old and fall within a two decade
Figure 2.1: Allele distribution comparisons corresponding to Biallelic Means (the average of two alleles) and X_Weighted_Biallelic Means (the average of two alleles after adjustment for preferential allele expression by X-inactivation analysis). A cut-point of ≤ 22 divides both Control distribution profiles into two approximately equal groups. Comparing group distributions at this cut-point shows a significant difference between Controls and PCOS for both Biallelic Means (51% vs 36%, p = 0.04) and X_Weighted_Biallelic Means (60% vs. 38%, p = 0.009).
Figure 2.2: Comparison of X-inactivation patterns in Controls (n = 69) and PCOS (n = 103). Inactivation is gauged by the relative methylation, normalised to 100%, of an allele in a genomic pair. Values on the graph represent the percent inactivation of the allele of longer CAG repeat size. Skewed inactivation is considered as ≥ 80% inactivation of one allele. There are no significant differences between groups.
**Fig. 2.3:** Distribution of AR CAG alleles with ≥ 60% activity compared to their genomic partner allele as assessed by methylation analysis. Control (n = 43) and PCOS (n = 65). The normal polymorphic spectrum is bisected by 22 repeats, the control group mode. PCOS has a greater frequency (57%) of active alleles in the >22 repeat range as compared to Controls (40%) *p = 0.03.
range, therefore age should have minimal effect on the results of X-inactivation analysis [407, 408].

A common clinical feature of PCOS is obesity and its effects may influence ovulation [241, 409]. We did not find any correlation between BMI and CAG repeat number in this study, which concurs with previously published results [355].

A positive association was found between serum testosterone and the longer CAG allele (r = 0.21, p = 0.03) in PCOS women. However, testosterone levels in these women were significantly different only when sorted by X_weighted_biallelic_means, not by longer alleles. PCOS women with X_weighted_biallelic_means ≥ 23 had significantly higher levels of testosterone (5.42 nmol/L ± 2.7) than PCOS women with means < 23 (4.1 nmol/L ± 2.6) (p = 0.01).

2.5 Discussion

Our findings indicate that infertile Australian Caucasian women with PCOS have a significantly greater frequency of longer AR CAG alleles and biallelic means (> 22 repeats) than healthy fertile women and a general population group of the same ethnicity. Although the latter group was not characterised for hyperandrogenism or menstrual abnormality, its large size provided an accurate representation of allele distribution for this ethnic group, a necessary tool when considering polymorphisms of such high variability. Few differences exist in the sub-ethnic groups that comprise the Caucasian populations of large Australian cities [410], from which this larger data set derives. Fertile control subjects in this study did not significantly differ from the general population, which increases confidence in our findings.

Preferential expression of longer CAG alleles augments the disparity found between healthy fertile women and infertile women with PCOS. It also correlates with higher levels of serum testosterone within the PCOS group. These findings endorse the use of X-inactivation analysis when comparing groups of women at the AR (CAG)n gene locus.
convention, we used DNA from peripheral blood samples to assess X-inactivation patterns, which may not reflect patterns found in other tissues. There is no data that compares peripheral blood X-inactivation patterns to those in ovarian tissues, however Van Deerlin et al [411] have reported that 8 of 9 normal cycling women have one or more ovarian follicles that express only one AR allele. Theoretically, these follicles could behave quite differently in terms of AR-mediated activity to follicles that solely express the other allele, or to follicles that express different degrees of both alleles. This is an intriguing concept that has yet to be explored in PCOS and could potentially explain variations in ovarian morphology and follicle development within the disorder.

In contrast to the current study, Mifsud et al [357] found no differences between infertile and fertile women for AR CAG repeat distribution patterns in a cohort of predominantly Chinese women resident in Singapore. Genetically, both studies reflected previously reported ethnic differences at the AR (CAG)n gene locus [273]. Clinically, anovulation and polycystic ovaries were common inclusion criteria for infertile women, but our study also included hyperandrogenaemia, a primary diagnostic criteria for PCOS. Both studies suffered from lack of a control group that had ovarian ultrasound scan and serum hormone measurements. Such control groups are difficult to attain because of the intrusive nature and cost of these procedures. In our experience, women seeking infertility treatment who definitely lacked a PCOS phenotype did not comprise an adequate control group due to diverse infertility diagnoses. A long-term prospective study involving large numbers of women is needed to confirm the preliminary findings presented by these two studies.

Despite differences, an interesting commonality emerges between the Mifsud et al [357] and current studies: higher serum testosterone was associated with frequency or greater expression of longer CAG alleles. In both studies, the relative term “long” refers to the same sector of the polymorphic spectrum and thereby assumes the same potential physiological significance. Collectively these data may indicate that hyperandrogenism as a characteristic of
PCOS correlates to long CAG repeat alleles. This would be consistent with reports that hyperandrogenism *per se* is the strongest genetically inherited characteristic in familial cases of PCOS [133, 150].

Two previous studies have investigated X-inactivation patterns of AR CAG repeat alleles in women with hirsutism, an androgen sensitive disorder [362, 363]. Increased methylation of longer CAG alleles in hirsute women was found in the former but not the latter study. We also found increased methylation of longer CAG alleles, but this was not exclusive to women with PCOS, and the longer alleles were considerably larger in terms of absolute repeat numbers than the alleles in these former studies. To our knowledge, we are the first to report preferential methylation of CAG alleles > 22 repeats within the normal polymorphic range.

It is difficult to explain how an increased frequency and/or expression of long CAG repeat alleles relates to the condition of PCOS. Our data partially contradicts data from other studies as well as our initial hypothesis, which emphasises the need for future comparative studies. It is possible that the association we found has no physiological effect and therefore is of no significance. However, incremental differences in CAG repeat number do appear to have clinical relevance, presumably due to cumulative pathological effects [412]. Also, the differential effect of CAG repeat number on AR transactivation capability *in vitro* is enhanced with elevated levels of androgen [288], so these effects may be amplified in a hyperandrogenic environment.

If we adhere to the theory of an inverse relationship between CAG repeat number and receptor activity, our results indicate that infertile women with PCOS tend to have and/or preferentially express receptors of relatively low androgenic activity compared to fertile controls. Also, those women with the lowest potential androgenic activity had higher levels of serum testosterone. This latter data has no correlate in the literature as it depends on the evaluation of X-inactivation patterns and has sufficient study numbers to span the entire
normal polymorphic spectrum. Considering the theory that PCOS may arise from an early developmental event that evolves into a self-perpetuating disease process, we are compelled to ask whether low androgenic activity could trigger a mechanism that ultimately results in increased androgen secretion. This could involve hypothalamic AR activity [361] as opposed or in addition to ovarian AR activity. In summary, we conclude that the AR CAG repeat polymorphism and its differential methylation has some influence in the manifestation of PCOS and warrants continued investigation. Although evidence suggests that the AR (CAG)n gene locus does not have the strongest linkage to PCOS in a study of 37 candidate gene loci [157], it ranked among the top ten in associative strength. Furthermore, this genetic locus has recently been linked to male pattern baldness [367], a putative male PCOS phenotype[148], providing further support to this view.
Chapter 3 Epigenetic Modification of the X Chromosome Influences Susceptibility to Polycystic Ovary Syndrome¹.

3.1 Abstract

The cause of polycystic ovary syndrome (PCOS) is unknown, although genetic and environmental influences, including intrauterine conditions and lifestyle factors, are clearly implicated. Extensive investigation into the identification of causal genes has been unsuccessful, due in part to heterogeneity of the condition and the confounding nature of environmental determinants. Some genetic studies have suggested the involvement of X-linked genes in PCOS. Since the X chromosome is subject to epigenetic modification through inactivation of one X chromosome in every female cell, we examined patterns of X chromosome inactivation (XCI) between sister pairs in families with this disorder. A different pattern of XCI was evident in the majority of cases where sister pairs had the same genotype at a selected locus on the X chromosome but exhibited a different phenotype or manifestation of PCOS. Functionally, this means that sisters may inherit the same X chromosomes, but where they do not have the same clinical presentation, the genes on these X chromosomes are likely to be differentially expressed. This finding warrants a closer inspection of X-linked genes in PCOS, one in which both genotype and epigenotype are considered. Environmental determinants of PCOS may alter clinical presentation via epigenetic modifications, which currently remain undetected in traditional genetic analyses.

¹See Appendix 1
3.2 Introduction

Polycystic ovary syndrome (PCOS) is a complex endocrine disorder characterised by unexplained hyperandrogenism, chronic anovulation, and the occurrence of polycystic ovaries [84]. Familial aggregation in this disorder implies underlying genetic influences [156], but extensive investigation into the identity of the gene(s) involved and their mode of transmission has yet to yield any valid candidates or clear inheritance patterns, due in part to the difficulties endemic to such an endeavour [140]. Among the numerous genetic studies of PCOS, a few have suggested the involvement of genes located on the X chromosome. These include a study of women with mosaicism for Turner’s Syndrome [413], two studies of family groups with PCOS [143, 144], and the only twin study of PCOS published to date [137]. The latter study suggested that the symptomatic discordance observed in both mono- and dizygotic twins with PCOS could potentially result from unequal expression of X-linked genes. Women usually do not manifest X-linked diseases unless non-random inactivation of the normal X chromosome occurs, thereby increasing expression of the abnormal allele on the opposing chromosome [284]. Significant differences in X chromosome inactivation (XCI) patterns between normal female monozygotic twins has been reported [414], and X-linked diseases can be differentially manifest in female twins due to variable patterns of XCI [415, 416]. This suggests differential XCI could occur in twin sisters discordant for PCOS, and would confound traditional linkage studies involving genes on the X chromosome. To date, the only X-linked candidate gene for PCOS is the androgen receptor (AR), which Urbanek et al [157] reported as having no significant linkage. Analysing methylation status of sequences upstream of the highly polymorphic (CAG)n repeat region in the AR gene is the most common method used to determine patterns of XCI in females[417]. In the current study, we used this method to compare sisters in family groups with PCOS to determine whether XCI lays a role in the presentation of this disorder.
3.3 Methods

Informed consent to participate in this study was sought from members of nuclear families previously recruited for genetic studies by Dr. Richard Legro in Hershey, Pennsylvania, USA, in which at least one sister had PCOS as defined by the 1990 NICHD criteria of hyperandrogenaemia (HA) and chronic anovulation [133]. A total of 40 families provided sufficient consent and genetic material to analyse at least one parent (to determine parental origin of alleles) and at least one sister pair. As described by Urbanek et al [157], sisters were considered affected if given a diagnosis of PCOS or HA, and unaffected if they had normal circulating androgen levels, were not taking any confounding medications (e.g., oral contraceptives), and had regular menstrual cycles (menses every 27-35 days). The (CAG)n variable nucleotide repeat region in Exon 1 of the human androgen receptor gene (AR) was used to genotype and determine patterns of XCI between sisters, as previously described [418]. This method allows determination of relative levels of inactivation of the two inherited X chromosomes, and by reciprocal association, the relative transcriptional activity expected from each one. Patterns of XCI were considered different between heterozygous sisters with the same genotype if: 1) opposing alleles were preferentially inactivated or 2) one sister had a random pattern of XCI and the other had a non-random pattern (defined as ≥ 60% inactivation of one allele). A logistic Generalised Estimating Equation (GEE) was used to estimate the odds ratio, comparing the odds of different clinical presentation in sister pairs with different XCI patterns or the same XCI pattern, while adjusting for the fact that some sister pairs came from the same family (SAS 9.1, Cary, NC, USA). Significance was set at P < 0.05.

3.4 Results

In total, 88 sister pairs were formed from the 40 family groups, in which 7 families provided multiple sister pairs and 33 families provided a single sister pair. Group distribution and
percent of sister pairs with the same genotype within the group were as follows: unaffected:unaffected 9 pairs (3/9;33%); unaffected:affected 47 pairs (21/47; 45%); affected:affected 18 pairs (18/32;56%). Analysis of XCI patterns (Table 3.1) shows that in the majority (85%) of cases where sister pairs had the same genotype but a different clinical presentation, a different pattern of XCI was evident, which implies a non-equivalent dose of gene products from a particular X chromosome. In contrast, this occurred in the minority (15%) of cases where sister pairs had the same genotype and the same clinical presentation. Statistically, the odds on a different clinical presentation was approximately 29 times higher in sister pairs with different XCI patterns compared to sister pairs with the same XCI pattern (OR = 28.9; 95% CI = 4.0 to 206; p=0.0008). This suggests that the odds of having a different clinical presentation are dependent upon XCI. Overall, XCI analysis was determined in a total of 28 unaffected sisters and 54 affected sisters. There was no statistical difference (p = 0.07) in distribution of XCI patterns between these two groups as a whole, indicating that affected sisters do not have a higher prevalence of non-random XCI per se. Therefore the impact of XCI had to be considered on a sister-pair basis to implicate a particular X chromosome in the disease process. An exemplary family group with multiple participating siblings is illustrated in Figure 3.1.

3.5 Discussion

PCOS is clearly a disorder conditioned by both genetic and environmental influences [111], but to date, there have been no studies that examine how environmental influences could have impact at the genetic level in this disorder. Epigenetic modification of DNA could be a means by which an environmental influence directly affects the behaviour of genes without altering the genetic code itself, and new evidence comparing epigenetic differences in monozygotic twins indicates that such differences are likely to be involved in the aetiology of complex diseases [419]. We provide the first evidence that in families affected by PCOS, differential XCI occurs in the majority of cases where phenotype does not segregate with genotype and
Table 3.1: X-inactivation analysis of sisters with the same genotype

<table>
<thead>
<tr>
<th>Group</th>
<th>n (pairs)</th>
<th>X-inactivation pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Same</td>
</tr>
<tr>
<td>Same clinical presentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unaffected:Unaffected</td>
<td>2</td>
<td>2 (100%)</td>
</tr>
<tr>
<td>PCOS:PCOS or HA:HA</td>
<td>11</td>
<td>9 (82%)</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>11/13 (85%)</td>
</tr>
<tr>
<td>Different clinical presentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unaffected:PCOS/HA</td>
<td>18</td>
<td>3 (17%)</td>
</tr>
<tr>
<td>PCOS:HA</td>
<td>7</td>
<td>1 (14%)</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>4/25 (16%)*</td>
</tr>
</tbody>
</table>

* OR= 28.9; P = 0.0008 as compared to totals for groups where sister pairs had the same clinical presentation
Figure 3.1: Example of X chromosome segregation and pattern of X-inactivation among 5 sisters in a family group with PCOS. Diagnosis for the mother could not be determined (ND). The three affected sisters all inherited the same maternal X chromosome while their unaffected sisters inherited the alternate maternal X chromosome. The graph depicts the degree to which each X chromosome is active, determined by measuring inactivation of the opposing X chromosome. Among affected sisters there is a clear difference in X-inactivation patterns between the sister with PCOS, where the paternal X is non-randomly inactivated, and the two sisters with HA who display random X-inactivation patterns. Theoretically, this provides the PCOS sister with a greater dose of gene products transcribed from the maternal X inherited by all affected sisters. The two unaffected sisters also substantially inactivate the paternal X chromosome, thereby accentuating the effect of inheriting different maternal X chromosomes.
where heterogeneous symptoms occur among affected sisters with the same genotype. This suggests that, in addition to genetic linkage, inactivation of the X chromosome is an important contributor to manifestation of the PCOS phenotype.

Mounting evidence suggests that the origins of PCOS occur in utero or early life; a concept powerfully supported by pre-natally androgenized animal models [420]. Timing of gestational androgen excess is important, as different PCOS-associated characteristics arise when the hormonal insult is administered at various stages of fetal organogenesis [420]. Clonal selection of cells that are dependent upon X-linked genes for growth and differentiation during development is the most likely cause of non-random XCI patterns in adult tissue [284], although mutations of genes involved in the X inactivation process can cause complete inactivation of a particular X chromosome [421, 422]. Naumova et al [423] report a strong sister-sister correlation in degree of XCI, consistent with our observation that sister pairs with the same genotype and the same clinical presentation had similar XCI patterns. Divergence from this norm in sister pairs with a different clinical presentation for PCOS suggests a developmental clonal selection process, perhaps one influenced by a hyperandrogenic environment. Our findings accord with the concept of an early origin for PCOS and offer a possible mechanism through which environmental conditions during gestation or early life influence gene expression patterns later in life. Variable familial XCI of an X-linked gene could also explain why the migration of PCOS in family groups tends to behave in a manner suggestive of a dominant gene effect with incomplete penetrance [424].

While our study further substantiates candidature of the AR, our results implicate any gene on the X chromosome subject to inactivation. Our analysis was performed on DNA from peripheral blood leukocytes. Examination of other cell types corresponding to specific target tissues likely to be involved with PCOS is desirable, although ovarian tissue is extremely difficult to obtain, and represents a challenge for future investigations.
We believe our findings warrant a closer inspection of X-linked genes in PCOS, one in which both genotype and epigenotype are considered. Examination of epigenetic modifications to the genetic code may be necessary to unravel some of the confounding complexities that have so far hindered the identification of causal genes for this disorder.

3.6 End of Work Involving the AR (CAG)n Polymorphism

The work presented in Chapters 2 and 3 support the hypothesis that the AR (CAG)n polymorphism influences manifestation of PCOS. This work is discussed further in the final discussion (Chapter 7). The next 3 chapters of this thesis deal with the second hypothesis outlined in Chapter 1, examining AR-mediated activity in follicular granulosa cells.
Chapter 4 Interactions Between Androgen and Growth Factors in Granulosa Cell Subtypes of Porcine Antral Follicles

4.1 Abstract

Androgens acting via the androgen receptor (AR) have been implicated in regulation of folliculogenesis in many animal species. These effects are possibly mediated via enhancement of FSH and/or IGF1 activity in granulosa cells, which contain high levels of AR protein. We examined the in vitro effect of dihydrotestosterone (DHT) on DNA synthesis and progesterone secretion by follicular cells in response to FSH and IGF1, alone or in combination. Cells from separate pools of 1-3 mm and 3-5 mm antral follicles were aspirated from gilt ovaries and fractioned into mural granulosa cells (MGCs) and cumulus-oocyte complexes (COCs) for subsequent cell culture. Androgen alone or with any combination of mitogen, had no effect on the proliferative or steroidogenic responses of MGCs from 3-5mm antral follicles. Conversely, in MGCs from 1-3mm follicles, DHT significantly enhanced proliferative responses to IGF1 and had variable influence on progesterone secretion. The effects of DHT on proliferative responses of COC were also dependent on follicle size: DHT significantly augmented either IGF1 stimulated proliferation (1-3mm follicles) or FSH-stimulated proliferation (3-5mm follicles). However, the steroidogenic responses of all COC were identical, whereby DHT significantly suppressed the steroidogenic response of granulosa cells in the presence of FSH. Addition of an AR antagonist, hydroxyflutamide, generally reversed the proliferative responses invoked by DHT but not the steroidogenic responses. We conclude that AR-mediated activity in granulosa cells of antral follicles is dependent on follicle size, is influenced by proximity of cells to the oocyte, and possibly involves both classic and non-classic steroid mechanisms.

1See Appendix 1
4.2 Introduction

Androgens are the predominant steroids produced in early follicular development and are present at high concentrations in follicular fluid at all stages of follicular growth, although the ratio of androgens to oestrogens changes as follicle growth advances and dominant follicles engage in aromatase activity [28]. Treatment effects observed with the non-aromatisable androgen 5-α-dihydrotestosterone (DHT), both in vivo [425, 426] and in vitro [293, 427-429], demonstrate that androgens have a direct influence on ovarian function. This influence is potentially mediated by the androgen receptor (AR), which has been detected in ovarian cells from all vertebrate species studied to date [296, 297, 301, 302, 430-435], suggesting a conserved receptor-mediated role for androgens in folliculogenesis. Within the ovary, granulosa cells generally display the strongest AR immunoreactivity and are exposed to the most potent AR agonists, testosterone and DHT. The latter are absorbed from the secretions of thecal cells or are internally produced through enzymatic conversion of androgen precursors [25]. Therefore granulosa cells are considered to be the main location of AR-mediated activity in the follicle and are the focus of most studies in this area of investigation.

The AR-mediated role for androgens in folliculogenesis and its mechanism of action is still being characterised. Non-human primates [401] and women [95, 354] exposed to high serum androgens develop large ovaries with increased numbers of antral follicles. Both testosterone and DHT promote in vitro follicle growth in mice [330, 331] and DHT treatment enhances ovulation rate in pigs [345]. Many of the differentiative actions of FSH on granulosa cells are augmented by AR agonists, and include cholesterol metabolism, progesterone secretion, expression of steroidogenic enzymes, and induction of aromatase activity (reviewed by [310]). The mechanism involves modulation of cAMP within granulosa cells at both pre- and post cAMP sites [310] and presumably involves the regulation of androgen-responsive genes. Although definitive proof of direct AR transcriptional regulation is still lacking, the FSHR may be one such ovarian target gene [233, 294, 345]. However, androgens may also act
via other systems that impact on FSH activity. In addition to FSHR, androgen treatment also enhances IGF1 and IGFR mRNA expression in granulosa cells [235] and oocytes [234] of rhesus monkeys. Enhancement of FSH-mediated activity by IGF1 is well documented, and also occurs at both pre- and post-cAMP sites [436, 437]. However, the effect of androgens on IGF1-mediated activity, alone or in combination with FSH, has not been explored in cultured granulosa cells.

In the current study, we have used a pre-pubertal pig model to study the effects of androgen on granulosa cell responses to FSH and IGF1. As the interaction of these two mitogens changes in the presence of oocyte-secreted factors [438], we included in our investigation the two sub-populations of granulosa cells that emerge upon antrum formation in the follicle: mural granulosa cells (MGCs) that comprise the follicle wall, and cumulus cells which are intimately connected to the oocyte via gap junctions forming a cumulus-oocyte complex (COC). These two granulosa cell subtypes are anatomically and functionally distinct (reviewed by Eppig et al. [439]), due to varying proximities to the oocyte and the vascularized thecal layer. Both subtypes display strong immunological reactivity for AR in pigs [440]. We anticipated differential androgen responses between these two granulosa cell sub-types, and to our knowledge we report for the first time that proximity of the oocyte corresponds to increased androgenic activity that manifests as altered granulosa cell responses to mitogenic stimulation.

4.3 Materials and Methods

4.3.1 Collection of follicular cells

Unless otherwise noted, all chemical reagents were purchased from Sigma (S. Louis, MO, USA). Pre-pubertal pig ovaries were collected from a local abattoir and transported to the laboratory in warm saline supplemented with penicillin G (100U/ml) and streptomycin sulphate (100mg/ml). Time from ovary excision to follicle aspiration was approximately 1-2
hours duration. Follicular cells were aspirated from antral follicles using a Precision Glide needle (0.9mm X 25mm) inserted in a 10 ml Vacutainer tube (both from Becton Dickinson, France) under vacuum; ovaries with corpora lutea and follicles that were opaque or hemorrhagic were excluded. Follicular aspirates were kept at 37°C and allowed to sediment for 5 minutes. The sediment was transferred to non-treated petri dishes containing warm 25mM HEPES-buffered tissue culture medium-199 (H-TCM; ICN, Costa Mesa, CA) supplemented with 2mM sodium pyruvate, 100 U/ml penicillin G, streptomycin sulphate (100mg/ml), and polyvinyl alcohol (PVA; 0.3mg/ml). With the aid of a dissecting microscope, all naked oocytes and cumulus-oocyte complexes (COC) were removed from the aspirates and placed into fresh H-TCM (as described above). From these, COC with good structural integrity were further selected and manually washed twice in H-TCM and twice in bicarbonate-buffered TCM-199 (B-TCM; ICN, Costa Mesa, CA, supplemented as per H-TCM) prior to culturing. Debris was removed from the remaining aspirate, which was then collected into a centrifuge tube and allowed to sediment by unit gravity for 1 min. This sediment contained large aggregates of mural granulosa cells (MGC) and was discarded. The remaining aspirate was allowed to sediment a further 30 min, creating a pellet of aggregated MGCs of a more uniform size range. The supernatant, containing mostly single cells, was discarded and the pellet was washed twice in H-TCM and twice in B-TCM, with centrifugation between washes. An aliquot of resuspended cells was manually dissociated using a 1 ml syringe with a 23 G needle and counted with a haemocytometer to determine cell density.

4.3.2 Follicle cell culture

Mural granulosa cells were cultured as aggregates in 96-well flat-bottom plates (Falcon, Franklin Lakes, NJ) at a density of 10⁶ cells/ml in a culture volume of 250µl. Cumulus-oocyte complexes (10/well) were cultured in 96-well round-bottom plates (Falcon) in a culture volume of 125µl. Culture conditions were serum-free B-TCM, supplemented as above, with
combinations of the following hormone treatments: 50mIU/ml recombinant human FSH (rhFSH; Puregon, N.V. Organon, Oss, Netherlands), 50ng/ml recombinant human IGF1 (rhIGF1; Gropep, Adelaide, Australia), 0.5µM DHT (Sigma), and 5µM hydroxyflutamide (OHF; Sigma). Cells were cultured with treatments in an atmosphere of 38.5°C, 96% humidity, 5% CO₂ in air for 18 hour followed by a 6-hour pulse of 0.8µCi tritiated thymidine ([H³]-thymidine, ICN) in the same conditions. At 24 hours a fraction of the culture media was removed and frozen (-20°C) for steroid analysis and plates were kept at 4°C until cell harvest.

4.3.3 Effects of androgens on proliferative and steroidogenic responses to FSH and IGF1 in MGCs and COCs

To test the responses of MGCs and COCs to an AR agonist, we used the non-aromatisable DHT at 0.5 µM, a concentration that approximates the level of this steroid in follicular fluid. In separate experiments, cells were obtained from either 3-5mm or 1-3mm antral follicles. Cell numbers were not restrictive for MGCs and all treatments were done on one plate in triplicates or quadruplicates, repeated in 5 experiments. As COCs were limited in number, smaller treatment groups were employed with 2-4 duplicates per treatment. Each experimental condition was repeated in a minimum of 4 experiments.

4.3.4 Effects of androgen receptor antagonist on proliferative and steroidogenic responses of MGCs and COCs to FSH, IGF1 and androgens

Since most steroids, including DHT, have documented non-genomic effects that are independent of their cognate receptor [441], the androgen receptor antagonist, hydroxyflutamide (OHF), was used to test the specificity of the observed androgen responses. MGCs or COCs were cultured under conditions selected on the basis of significant androgen effects that were observed in the above experiments. Hydroxyflutamide was employed at 5 µM, a 10-fold excess above that of DHT. Each experiment was repeated a minimum of 4
times with triplicate or quadruplicate treatments in MGCs and 2-3 duplicate treatments in COCs.

**4.3.5 Time-course of proliferative responses to FSH, IGF1 and androgens**

MGCs from 1-3mm antral follicles were cultured as described above, but for each experiment four separate culture plates were established, corresponding to 6-hourly termination points. Tritiated thymidine was added to cultures 6 hours prior to termination of culture for each time point. The experiment was repeated 3 times with 3-6 duplicated treatments per plate.

**4.3.6 Measurement of DNA synthesis and progesterone secretion**

Following culture, a fraction of media was removed and assayed for progesterone using an RIA kit (Diagnostic System Laboratories, Webster, TX) in accordance with the manufacturer’s instructions. The kit has a sensitivity of 0.25 pmol/ml, an intraassay coefficient of 8.4%, and an interassay coefficient of 12%. Incorporation of \[^{3}H\]-thymidine (ICN) was measured in cells as an indication of the degree of cellular DNA synthesis and potential proliferation [442]. Cells were harvested onto a filter-mat using a Tomtec Harvester 96, which removes unincorporated isotope in the process. Filter-mats were then saturated with scintillation fluid, and emission of beta particles by \[^{3}H\]-thymidine was detected with a Wallac microbeta counter (Fisions, Leies, UK).

**4.3.7 Data analysis**

Statistical analyses were performed with SAS software (SAS Institute, Cary, NC, USA) using 3-way ANOVAs on the log response of raw data, with blocking on experiment. Where significant effects were seen, post-hoc t-tests were used for comparison of adjusted means for those significant effects. For clarity of presentation, raw data were converted to relative data for each replicate experiment, in which the mean value for the control treatment with no hormone was designated as 1. Graphs therefore represent the mean values (± SEM) of the
summation of relative data for a group of replicate experiments. Statistical significance was set at $p < 0.05$, except where otherwise noted.

### 4.4 Results

Significant interaction between FSH and IGF1 occurred within all experiments where both mitogens were present, confirming previous observations [443, 444]. Since the focus of our study was the interaction between DHT and these factors, we report herein the statistical results relevant to these interactions for sake of brevity and simplicity of presentation.

#### 4.4.1 Effects of androgen on proliferative and steroidogenic responses of MGCs and COCs to FSH and IGF1

##### 4.4.1.1 3-5 mm antral follicles

Figure 4.1 summarizes the effects of FSH, IGF1 and DHT alone and in combinations on DNA synthesis and progesterone secretion in MGCs and COCs from 3-5mm antral follicles. Both FSH and IGF1 significantly increased DNA synthesis in MGCs, and the responses to these agents in combination were approximately additive (Fig 4.1A). Similarly, both FSH and IGF1 stimulated progesterone secretion in MGCs, with the effect of FSH considerably greater than that of IGF1. The effect of combined treatment with FSH plus IGF1 on progesterone secretion was a greater than additive response that tended toward synergism (Fig 4.1C).

Proliferative effects of FSH and IGF1 were also seen in COCs (Fig 4.1B), although the magnitudes of stimulation by both agents were considerably greater than observed in MGCs, and the response to IGF1 was substantially greater (20-fold stimulation) than the response to FSH (8-fold stimulation). A more striking difference between the two-granulosa cell sub-types was seen in response to combined treatment with FSH and IGF1, in which FSH completely obliterated the proliferative response to IGF1. The pattern of steroidogenic responses of COCs to FSH and IGF1 alone and in combination were similar to those observed in the MGCs, although the magnitude of progesterone secretion was considerably less in the COCs.
MGC from 3 – 5 mm Antral Follicles

A. DNA synthesis

![Graph showing DNA synthesis for Control, FSH, IGF1, and FSH/IGF1 conditions with fold differences indicated.]

B. Progesterone secretion

![Graph showing Progesterone secretion for Control, FSH, IGF1, and FSH/IGF1 conditions with fold differences indicated.]

(note: facing page for Figure 4.1)
COC from
3 – 5 mm Antral Follicles

B. DNA synthesis

Figure 4.1: Effect of DHT on DNA synthesis and progesterone secretion in gilt MGC (A,C) and COC (B,D) from 3-5 mm antral follicles. Cells were cultured for 24 hours in serum free media with combinations of rhFSH (50mIU/ml), rhIGF1 (50ng/ml), and DHT (0.5µM). Graphs represent data expressed as relative means (± SEM) to the control mean with no hormone, which was set at a value of 1. Raw data for control values ± SEM were as follows: (A) 536 ± 41 cpm; (B) 927 cpm ± 131; (C) 3.7 ± 0.3 nmol/L and (D) 0.9 ± 0.0 nmol/L. DHT significantly enhanced DNA synthesis in both cell types (3-way ANOVA main effects: (A) p = 0.04 and (B) p < 0.0001). Progesterone secretion was unaffected by DHT in MGCs (C) and suppressed by DHT in COCs (D) (3-way ANOVA main effect: p < 0.0001). * p < 0.05 and ** p < 0.0001 as compared to equivalent treatment with no DHT.
The non-aromatisable androgen, DHT, had a significant main effect on DNA synthesis in MGCs (p = 0.04) but there were no significant interactions between androgen and FSH or IGF1. Progesterone secretion in MGCs was globally unaffected by DHT (Fig 4.1C). To exclude the possibility of an inappropriate dose of androgen, the same treatments were repeated in combination with a range of DHT concentrations from 1nM to 1µM, with no significant effect on FSH and/or IGF1 stimulated DNA synthesis or progesterone secretion (data not shown). In COC, the 3-way interaction of FSH, IGF1 and DHT was close to significance (p = 0.06), and post-hoc tests assuming significance at this level show augmentation of FSH-stimulated proliferation by DHT (p < 0.001) (Fig 4.1B). Interestingly, although DHT did not alter proliferative responses of COC to IGF1 alone, in the presence of both mitogens, DHT reverses to a small but significant degree (p= 0.02) the inhibitory effect of FSH on IGF1 stimulated proliferation. The steroidogenic response of COC to FSH was inhibited 2-fold by DHT (p < 0.001) (Fig 4.1D), independent of the presence of IGF1. Progesterone secretion from COC under control conditions and DHT alone was undetectable and therefore arbitrarily given the value of the lowest assay standard in the graph.

4.4.1.2 1-3 mm antral follicles

Figure 4.2 summarizes the effects of FSH, IGF1 and DHT alone and in combinations on DNA synthesis and progesterone secretion in MGCs and COCs from 1-3 mm follicles. The pattern and degree of mitogen-stimulated DNA synthesis in MGCs from these follicles (Fig 4.2A) differed from that seen in MGCs from follicles 3-5 mm in diameter (Fig 4.1A). The relative degree of FSH stimulation above control values was ≈ 10-fold in small follicles compared to ≈ 2-fold in the larger ones, resulting in a 6-fold higher uptake of [³H]-thymidine in cells from smaller follicles. While in relative terms IGF1 alone was a less potent mitogen than FSH in cells from small follicles, in terms of total uptake of [³H]-thymidine, IGF1 induced similar effects in cells from both follicle sizes. Another consistent feature of responses of MGCs from both follicle sizes was the enhanced effect of combined mitogen over either mitogen
MGC from 1 – 3 mm Antral Follicles

A. DNA synthesis

C. Progesterone secretion

(Note: facing page for Figure 4.2)
Figure 4.2: Effect of DHT on DNA synthesis and progesterone secretion in gilt MGC (A, C) and COC (B, D) from 1-3 mm antral follicles. Cells were cultured for 24 hours in serum free media with combinations of rhFSH (50mIU/ml), rhIGF1 (50ng/ml), and DHT (0.5µM). Graphs represent data expressed as relative means (± SEM) to the control mean with no hormone, which was set at a value of 1. Raw data for control values ± SEM were as follows: (A) 749 ± 168 cpm; (B) 473 ± 51 cpm; (C) 13.8 ± 0.5 nmol/L; and (D) 0.09 ± 0.0 nmol/L. DHT enhanced proliferation and suppressed progesterone secretion in both cell types (3-way ANOVA main effects: (A) p < 0.001) and (B,C,D) p < 0.0001). * p < 0.005 and ** p < 0.0001 as compared to equivalent treatment with no DHT.
alone, although this effect was \( \approx 20 \)-fold in small follicles as compared to \( \approx 4 \)-fold in larger follicles, due to the greater mitogenicity of FSH in the former. Progesterone secretion from MGCs of 1-3mm follicles (Fig 4.2C) also followed similar patterns of response to FSH and IGF1 as observed in MGCs from 3-5 mm follicles, although cells from smaller follicles secreted a greater amount than cells from larger follicles under all conditions.

The effects of DHT were more ubiquitous in MGCs from 1-3mm follicles as compared to MGCs from 3-5 mm follicles; there was a highly significant main effect of DHT on both DNA synthesis \( (p < 0.001) \) and progesterone secretion \( (p < 0.0001) \) in cells from the smaller follicles. In terms of DNA synthesis, there was also a trend toward a 3-way interaction between DHT, FSH and IGF1 \( (p = 0.08) \) in MGCs from smaller follicles that was not apparent in MGCs from larger follicles. Post-hoc tests assuming this interaction was significant show an enhancement of IGF1 stimulated DNA synthesis by DHT \( (p = 0.0003) \) (Fig 4.2A). The effects of DHT on steroidogenic responses of MGCs from 1-3mm follicles were inconsistent: in some assays DHT inhibited FSH-stimulated progesterone secretion and in others it did not, leading to comparisons that were not statistically significant (Fig 4.2C). However, the main effect of DHT on progesterone secretion in cells from smaller follicles was highly significant \( (p < 0.0001) \).

Mitogen-stimulated DNA synthesis (Fig 4.2B) and progesterone secretion (Fig 4.2D) characteristic of COCs from 1-3mm follicles followed similar patterns of stimulation as observed in COCs from larger follicles (Fig 4.1B, 4.1D). In terms of DNA synthesis, baseline values for control and FSH-stimulated COCs were approximately 2-fold greater in COC from 3-5 mm follicles, but the amount of progesterone secretion was nearly identical between COCs of both follicle sizes. Stimulation by IGF1 and combined mitogen was also remarkably similar in relative and absolute terms between COC from both follicle sizes in assessment of both DNA synthesis and progesterone secretion.
In COC from 1-3mm follicles, there was a significant 3-way interaction between DHT, FSH and IGF1 (p = 0.05) in stimulating DNA synthesis, whereby DHT augmented IGF1 stimulated DNA synthesis to ≈ 50-fold over controls (p < 0.0001)(Fig 4.2B). This feature was absent in COCs from 3-5mm follicles, where the androgen effect was only evident in the presence of FSH. Dihydrotestosterone also significantly enhanced FSH-stimulated DNA synthesis in COCs from 1-3mm follicles (p < 0.01) and tended to increase DNA synthesis on its own (p = 0.07) (Fig 4.2B). In addition, as observed in COC from 3-5 mm follicles, DHT inhibited progesterone secretion by approximately 2-fold in the presence of FSH, independent of the presence of IGF1 in COCs from 1-3mm follicles (p < 0.0001) (Fig 4.2D).

4.4.2 Effect of an androgen receptor antagonist, hydroxyflutamide, on granulosa cell responses to DHT

The AR antagonist OHF was able to abolish the stimulatory effects of DHT on both FSH-stimulated DNA synthesis in COC from 3-5 mm follicles (Fig 4.3A) and IGF1-stimulated DNA synthesis in COC from 1-3 mm follicles (Fig 4.4B). However, DHT and OHF had significant 2-way interactions with IGF1 (p = 0.05 and p = 0.001, respectively), independently stimulating DNA synthesis in MGC from 1-3mm follicles, and having a greater effect than either alone when combined (Fig 4.4A). Likewise, OHF had agonistic effects similar to DHT in the inhibition of FSH-stimulated progesterone secretion in COC from 3-5 mm follicles (Fig 4.3B)(two significant 2-way interactions: FSH x DHT, p = 0.02; FSH x OHF, p = 0.001) and was more potent than DHT in suppressing progesterone secretion in the absence of FSH (significant 2-way interaction between DHT x OHF, p < 0.001). As COC from small antral follicles do not secrete measurable quantities of progesterone under control or IGF1 conditions, the effects of OHF were not examined.
COC from 3–5 mm Antral Follcles

A. DNA synthesis

B. Progesterone secretion

Figure 4.3: Effect of an AR antagonist, hydroxyflutamide (OHF), on DNA synthesis (A) and progesterone secretion (B) in gilt COC from 3-5 mm antral follicles. Cells were cultured for 24 hours in serum free media with combinations of rhFSH (50mIU/ml), DHT (0.5µM), and OHF (5µM). Graphs represent data expressed as relative means (± SEM) to the control mean with no hormone, which was set at a value of 1. Refer to Results for statistical comparisons.
1 – 3 mm Antral Follicles

A. DNA synthesis: MGC

![Bar chart showing DNA synthesis in MGC from 1-3 mm antral follicles.

B. DNA synthesis: COC

![Bar chart showing DNA synthesis in COC from 1-3 mm antral follicles.

Figure 4.4: Effect of an AR antagonist, hydroxyflutamide (OHF), on DNA synthesis in gilt MGC (A) and COC (B) from 1-3 mm antral follicles. Cells were cultured for 24 hours in serum free media with combinations of rhIGF1 (50ng/ml), DHT (0.5µM), and OHF (5µM). Graphs represent data expressed as relative means (± SEM) to the control mean with no hormone, which was set at a value of 1. Refer to Results for statistical comparisons.
4.4.3 Time-course of proliferative responses of MGC to FSH, IGF1 and androgens

Six-hourly time points were used to observe the progressive effects of mitogens and androgens on DNA synthesis in MGC from small antral follicles over a 24-hour period (Table 4.1). Under control conditions, MGC dramatically lost their ability to incorporate $[^3]$H-thymidine after 6 hours in culture, exhibiting a 50-fold decrease in uptake by 24 hours. As expected, addition of FSH and IGF1, alone or in combination, maintained a higher level of $[^3]$H-thymidine incorporation in these cells as compared to control conditions at each time point. Interestingly, at both 6 and 12 hours, MGCs exhibited a response to mitogen that is similar to that exhibited by COCs, whereby IGF1 was a more potent stimulus than FSH, and FSH tended to inhibit IGF1 stimulation when both mitogens were present ($p < 0.001$ in all instances). During this first half of the culture period, DHT had no effect on $[^3]$H-thymidine incorporation under any condition. The second 12 hour culture period was distinct from the first 12 hour period in three ways: 1) the mitogenic effect of FSH exceeded the effect of IGF1 at both 18 ($p < 0.001$) and 24 hours ($p < 0.0001$); 2) the two mitogens combined had an additive effect over each mitogen alone, and 3) responses to DHT emerged (significant 3-way interaction between DHT, FSH, and IGF1, $p <0.05$ at both 18 and 24 hours). The effect of DHT was most notable in combination with IGF1. At 18 hours DHT was able to enhance the mitogenic stimulation of IGF1, and at 24 hours there was a 2-fold increase in $[^3]$-thymidine incorporation over IGF1 alone ($p < 0.0001$ in both instances). In this series of experiments, DHT also tended to enhance FSH-stimulated DNA synthesis ($p = 0.06$).

4.5 Discussion

Androgens influence ovarian function in two ways: 1) as metabolic precursors for oestrogen synthesis and 2) as ligands for androgen receptors. The latter represents direct androgen activity and its role in folliculogenesis remains obscure. We have explored the effect of the
**Table 4.1.** Effect of hormone treatment over time on \(^{3}H\)-thymidine uptake in gilt mural granulosa cells from 1–3mm follicles.

<table>
<thead>
<tr>
<th>Duration of culture</th>
<th>DHT</th>
<th>Control</th>
<th>FSH</th>
<th>IGF1</th>
<th>FSH + IGF1</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hr</td>
<td>–</td>
<td>38,453 ± 10,590</td>
<td>35,642 ± 12,117</td>
<td>54,033 ± 15,534</td>
<td>40,813 ± 9,171</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>41,282 ± 9,226</td>
<td>30,262 ± 8,373</td>
<td>53,164 ± 12,765</td>
<td>40,419 ± 7,998</td>
</tr>
<tr>
<td>12 hr</td>
<td>–</td>
<td>9,062 ± 2,180</td>
<td>13,713 ± 3,695</td>
<td>24,254 ± 3,561</td>
<td>20,495 ± 4,879</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>9,853 ± 2,237</td>
<td>11,843 ± 3,355</td>
<td>26,774 ± 4,647</td>
<td>20,082 ± 3,878</td>
</tr>
<tr>
<td>18 hr</td>
<td>–</td>
<td>4,262 ± 1,330</td>
<td>11,573 ± 4,293</td>
<td>7,305 ± 1,763</td>
<td>16,913 ± 3,731</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4,795 ± 1,631</td>
<td>15,312 ± 6,542</td>
<td>11,750 ± 2,219*</td>
<td>18,596 ± 5,424</td>
</tr>
<tr>
<td>24 hr</td>
<td>–</td>
<td>798 ± 242</td>
<td>8,648 ± 3,389</td>
<td>1,079 ± 267</td>
<td>13,605 ± 4,702</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1051 ± 398</td>
<td>10,563 ± 3,581</td>
<td>2,547 ± 587*</td>
<td>13,595 ± 5,412</td>
</tr>
</tbody>
</table>

Data are represented as mean cpm / 2.5x \(10^5\) cells ± SEM. *p < 0.0001 as compared to equivalent treatment with no DHT.
most potent AR agonist, DHT, alone or in combination with the follicular growth factors FSH and IGF1, on granulosa cell proliferation and differentiation in 24-hour cultures of porcine granulosa cells from pre-pubertal gilts. Furthermore, we examined these cells in varying spatial and morphological proximity to oocytes, represented in terms of follicle size and structural attachment. In general, we observed that DHT stimulated proliferation and inhibited progesterone secretion, dependent upon follicle size, presence of another growth factor, and proximity to the oocyte. Furthermore, we demonstrate that the androgenic responses exhibited by ovarian cells are complex and indicative of pathways involving both classic and non-classic steroid action.

 Alone, DHT had negligible effect, solely represented by a minor increase in the proliferation of granulosa cells in COCs but not MGCs from 1-3 mm antral follicles. Although we did not examine pre-antral granulosa cells in this study, these cells have intense expression of AR protein in most species, including the pig [315], and it is feasible that androgen effects on proliferation could be stronger in these cells. Cultured pre-antral mouse follicles respond directly to androgens by increasing follicle diameter and DNA synthesis [331]. However, previous to our study, a direct in vitro effect of androgen alone on proliferation of granulosa cells had not been demonstrated for non-murine species, although the presence of AR has been positively associated with the cell cycle related nuclear antigen Ki-67 [308].

 Stimulatory effects of androgen on the proliferation of granulosa cells were most pronounced in the presence of a singular mitogen, and the specificity of that mitogen was determined by follicle size. Thus, in granulosa cells from small antral follicles, DHT had the most significant and consistent effect in the presence of IGF1 whereas in granulosa cells from medium-sized antral follicles, this effect was lacking, and androgen either had no effect (MGCs) or enhanced FSH-stimulated mitogenesis in the presence of the oocyte (COCs). Androgen also induced a small increase in FSH-stimulated proliferation in MGC from small
antral follicles, an effect preceded in a time course trial by enhancement of IGF1 stimulated mitogenesis. Studies by Vendola et al using androgen-treated monkeys as a model show marked increases in mRNA for IGF1 and IGFR [234, 235], and FSHR [401] in similar sized follicles. Our data support the hypothesis introduced by these authors that androgens may be acting in part through mediation of IGF1 activity, at least in small antral follicles. Whether this occurs through induction of FSHR, and is responsible for the effect of androgen on FSH-mediated mitogenesis remains to be determined. However, enhancement of IGF1 activity could potentially explain the effect of DHT alone on proliferation of granulosa cells in our study and of androgens on in vitro mouse follicle growth [330, 331].

Numerous studies have demonstrated the enhancement of differentiative actions of FSH by androgens, but few have examined their effect on the mitogenic capacity of this gonadotrophin. Bley et al [332] demonstrate enhanced FSH-stimulated DNA synthesis by DHT under serum-free conditions in cultured rat granulosa cells, but only after 48 hours. At 24 hours the steroid had no effect, consistent with our data for MGC from 3-5mm antral follicles. However, we observed this mitogenic response in COC from the same follicles, coincident with a suppression of FSH-stimulated progesterone secretion that was not observed in the MGCs. Only MGCs from small antral follicles, that may not have fully differentiated from the more primitive pre-antral granulosa cells, exhibited responses to DHT that were similar to those observed in COCs. An inverse relationship between proliferation and progesterone secretion has been widely demonstrated in granulosa cells, whereby factors that stimulate one activity also suppress the other [32, 38]. This pertains to androgens, which have been shown to suppress granulosa cell DNA synthesis [333, 334] and enhance granulosa cell steroidogenesis [428, 445], the opposite to our current findings. In the earlier studies, granulosa cells were cultured under conditions that promote a more luteinized granulosa cell phenotype. Collectively, these observations suggest that the differentiative differences incurred upon granulosa cells by oocyte-secreted factor(s) influence the ability of androgens
to alter both the mitogenic and steroidogenic actions of FSH and IGF-I in antral follicles. Current research in our laboratory, in which denuded oocytes are co-cultured with MGCs, is aimed at further characterising this phenomenon [74].

One means by which oocytes may affect androgenic responses is via regulation of AR expression. It has been demonstrated in rats [309], pigs [315], and monkeys [306, 308] that MGC from small follicles possess more AR protein and mRNA than larger follicles. Hillier et al [310] suggest that this reduction in AR is necessary for the transition to pre-ovulatory growth, a suggestion further supported by recent data from Cheng et al [446] in which high expression of AR in the granulosa cells of ERβ deficient mice was observed together with a block in progressive antral follicle growth. Our data provide evidence that one physiological consequence of a reduction in AR expression in MGCs is loss of the ability of androgen to stimulate DNA synthesis and suppress progesterone secretion. Furthermore, our results concord well with those of Garrett et al [315] that these events occur when antral follicles reach 3mm in diameter in the pig. Although quantitative comparisons of the expression of AR between cumulus and mural granulosa cells are lacking, two studies anecdotally observe that AR expression diminishes in a centripetal fashion toward the oocyte in rats [309] and pigs [440]. We demonstrate that androgens continue to enhance FSH-stimulated mitogenesis and suppress FSH-stimulated progesterone secretion in COC from 3-5mm follicles, suggesting that modulation of androgen activity by the oocyte may be a means of maintaining a distinct phenotype.

When studying androgen activity, it is common to use DHT as a ligand whose activity is implicitly linked with the AR, and effects induced by this androgen are generally presumed to follow classic genomic steroid receptor pathways involving modulation of the transcriptional activity of target genes. Using the AR antagonist hydroxyflutamide (OHF), we found that enhancement of mitogen-stimulated proliferation by DHT generally followed a pattern of classic steroid activity but suppression of FSH-stimulated progesterone secretion by
DHT and OHF was indicative of non-classic steroid activity. Antiandrogens have been shown to inhibit steroidogenic enzyme activity in rat testicular tissue [447], and to have differential effect on FSH and cAMP-induced steroidogenesis in rat granulosa cells [445]. The ability of OHF to antagonise certain androgenic effects but agonise others may be due to its unique interaction with the AR: it can bind the AR and induce a conformational change that can have androgenic effects on cytoplasmic signal transduction pathways [448], but is unable to induce the conformational change necessary for DNA binding and gene regulation [263]. The finding that OHF can be agonistic in the presence of DHT and IGF1 in stimulating proliferation of MGCs from small follicles, but antagonistic to proliferation under the same conditions in cumulus granulosa cells from the same follicles is difficult to explain, but again highlights the influence of the oocyte. Zhu et al have reported differential effects of OHF on proliferation of human breast cancer cells, and provide evidence that both genomic and non-genomic mechanisms are involved [448]. Our data suggests that AR-mediated influences on steroidogenesis and mitogenesis in granulosa cells are more complex than the regulation of gene transcription, although the specific mechanisms at play remain unresolved.

In summary, we demonstrate that androgens enhance proliferation and suppress differentiation in immature granulosa cells of the pig and that this activity is promoted by proximity to the oocyte. As the antrum enlarges and two granulosa cell subtypes become established, differential androgen activity emerges whereby those cells more distant from the oocyte become less responsive to androgen. This change in androgen activity appears to correlate with a reduction in AR expression and subsequent luteinization of the granulosa cells. Androgenic activity in immature granulosa cells is complex and possibly mediated by classic and non-classic steroid mechanisms; both appear to be influenced by the oocyte. Our data add to the growing compendium of evidence that the oocyte orchestrates developmental events in folliculogenesis. We suggest that control of androgenic activity can be added to its
repertoire and may be one means by which the oocyte abrogates differentiation of granulosa cells toward a luteinized phenotype.
Chapter 5  Androgens Augment the Mitogenic Effects of Oocyte Secreted Factors and Growth Differentiation Factor 9 on Porcine Granulosa Cells.

5.1 Abstract

In this study, we test the hypothesis that the growth-promoting action of androgens on granulosa cells requires paracrine signalling from the oocyte. Mural granulosa cells (MGCs) from small antral (1-3mm) pre-pubertal pig follicles were cultured in the presence or absence of denuded oocytes (DO) from the same follicles to determine whether mitogenic and/or steroidogenic responses to combinations of FSH, insulin like growth factor 1 (IGF1) and dihydrotestosterone (DHT) were influenced by oocyte-secreted factors (OSFs). To further explore the identity of such factors we performed the same experiments, substituting growth differentiation factor 9 (GDF9), a known OSF, for the DO. OSFs and GDF9 both potently enhanced IGF1-stimulated proliferation, and inhibited FSH-stimulated progesterone secretion. Alone, DHT had little effect on DNA synthesis, but significantly enhanced the mitogenic effects of OSFs or GDF9 in the presence of IGF1. DO, GDF9, and DHT independently inhibited FSH-stimulated progesterone secretion, and androgen together with DO or GDF9, caused the most potent steroidogenic inhibition. Focussing on mitogenic effects, we demonstrate that both natural androgen receptor (AR) agonists, testosterone and DHT, dose-dependently augmented the mitogenic activity of DO or GDF9. Antiandrogen (hydroxyflutamide) treatment, used to block AR activity, opposed the interaction between androgen and GDF9. In conclusion, androgens stimulate porcine MGC proliferation in vitro by potentiating the growth-promoting effects of oocytes or GDF9, via a mechanism that involves the AR. These signalling pathways are likely to be important regulators of folliculogenesis in vivo, and may contribute to the excess follicle growth that is observed in androgen-treated female animals.

1See Appendix 1
5.2 Introduction

Mammalian follicles form in the ovary during embryonic development (perhaps, also, post-natally [7]) and are cyclically stimulated to undergo a sequential program of growth called folliculogenesis by a wide variety of intra- and extra-ovarian growth factors and hormones, primarily in the reproductive years of an animal’s lifespan. Once initiated, the purpose of folliculogenesis is to nurture and ultimately extrude a mature, cumulus cell-enclosed oocyte that is competent to undergo fertilization by sperm, and subsequent embryo development, in the oviduct, and to create a hormone secreting structure that endocrinologically primes and maintains the uterine endometrium for potential pregnancy. Folliculogenesis involves dynamic changes to follicle structure during which somatic cells proliferate and attain steroidogenic capacity while the oocyte grows and acquires developmental competence. Historically, the oocyte was thought to be a passive participant in this complex process, but it is now well established that there is a dynamic interplay between the oocyte and surrounding somatic cells that mutually influences growth and differentiation of both cell lineages and is essential for normal fertility (reviews: [51-53]). Somatic cell influences on oocyte growth and maturation have been intensely studied for many decades, but the identification and characterisation of oocyte-derived molecules and their influence on somatic cell function have only been avidly pursued in recent years, with focus on members of the transforming growth factor β (TGFβ) superfamily, particularly growth differentiation factor 9 (GDF9) and growth differentiation factor 9b (GDF9b), the latter also known as bone-morphogenetic protein 15 (BMP15) [52].

Oocyte-secreted factors (OSFs) play a role in determining phenotypic differences between the two subpopulations of granulosa cells that emerge when a follicle enters the antral stage of folliculogenesis. These two subpopulations are morphologically distinct and are partly defined by their proximity to the oocyte or to the basement membrane: cumulus cells (CCs) surround and maintain direct contact via gap junctions with the oocyte, forming
the cumulus-oocyte complex (COC), while mural granulosa cells (MGCs) form the follicle wall, separated from surrounding theca cells by the basement membrane. Within this model there exist at least two chemical gradients that granulosa cell subtypes are differentially exposed to: one arising from the oocyte and one arising from the theca cells and surrounding vasculature. Soluble factors from the oocyte have been shown to impart temporal differences in granulosa cell responses to environmental factors such as FSH and insulin-like growth factor 1 (IGF1) [438, 444, 449], which are both essential for normal folliculogenesis. These studies show that CCs mainly proliferate in response to the presence of these factors whereas MGCs have a more steroidogenic phenotype. Moreover, CCs will become more steroidogenic in the absence of an oocyte [438, 450] and MGCs will become more proliferative when co-cultured with denuded oocytes [451, 452]. It appears that GDF9 and its homologue GDF9b can account for some but not all of these particular OSF-induced effects [453, 454], and an interaction between the two may be necessary for normal follicular development in vivo through as yet unresolved mechanisms [455] that are likely to differ between animal species with different ovulation rate phenotypes [52].

Locally produced ovarian steroid hormones also regulate folliculogenesis through modulation of gonadotropin and growth factor activities. During antral follicle growth, theca cells predominantly secrete aromatizable androgens, which accumulate in micromolar quantities within follicular fluid. In the early growth phase, granulosa cells preferentially metabolise these androgens to 5α-dihydrotestosterone (DHT) [26, 28, 456], a non-aromatizable androgen that has the highest affinity for the androgen receptor (AR). Granulosa cells of all mammalian species examined to date express the AR and direct, AR-mediated activity has been implicated in the control of folliculogenesis through mechanisms that involve both FSH [310] and IGF1 [234, 235]. This steroid receptor has also been identified in the oocytes of some mammalian species [300, 302, 305] and may be involved in non-classical steroid receptor signalling pathways that influence oocyte maturation [342]. In a previous
study, we observed differential effects of DHT on mitogenic and steroidogenic responses of pig COCs and MGCs to FSH and IGF1 that were dependent on follicle size and variably associated with classic steroid receptor mediated mechanisms [457]. The latter study led to our current hypothesis that there is an interaction between OSF- and androgen-stimulated signalling in granulosa cells. To address this hypothesis, we have employed an established bioassay for OSFs in which denuded oocytes are co-cultured with MGCs to ascertain whether factors derived from the oocyte can induce responses in MGCs that are characteristic of the cumulus cell phenotype. This technique has mainly been employed using mouse models [451, 452], and to a lesser extent domestic animal models [438, 449], both under various culture conditions that may or may not involve FSH and/or IGF1. We also examined whether recombinant GDF9, a known OSF, had the same effect on porcine granulosa cells as that elicited by denuded oocytes.

5.3 Materials and Methods

All chemicals were purchased from Sigma Chemical Co, (St Louis MO, USA) unless otherwise stated.

5.3.1 Collection of follicular cells

Pre-pubertal pig ovaries were collected from a local abattoir and follicular cells comprised of separate COC and aggregated MGC cell fractions were aspirated from small antral follicles (1-3mm diameter) and isolated as previously described [457]. For the current study, COCs in warm 25mM HEPES-buffered tissue culture medium-199 (H-TCM; ICN, Costa Mesa, CA) supplemented with sodium pyruvate (2 mM), penicillin G (100 U/ml), streptomycin sulphate (100mg/ml), and polyvinyl alcohol (PVA; 0.3mg/ml), were further processed to remove the surrounding cumulus cells in order to obtain denuded oocytes (DO). This was achieved by collecting pooled COC in 1ml H-TCM with supplements and vortexing them for 4 min in a 15ml centrifuge tube (Falcon, Franklin Lakes, NJ). The oocytes were allowed to pellet by unit
gravity for 1 min before being transferred to a new Petri dish containing warm H-TCM. Oocytes that were free of cumulus cells and had an intact zona pellucida were further isolated and washed twice in warm bicarbonate-buffered TCM-199 (B-TCM: ICN, Costa Mesa, CA; supplemented as per H-TCM). At the end of their isolation procedure, a small aliquot of the MGC suspension was manually dispersed by repeated pipetting to disassociate aggregated granulosa cells to enable accurate determination of cell numbers by counting with a haemocytometer.

5.3.2 Production and partial purification of GDF9

Since GDF9 is not commercially available, bioactive recombinant mouse GDF9 was produced in house from a transfected human embryonic kidney-293H cell line, generously donated by Olli Ritvos (University of Helsinki) [453, 458]. In preliminary experiments, material(s) secreted by the parent cell line were found to be inhibitory to pig, but not to mouse [453], MGC proliferation. Therefore conditioned media were subjected to purification by hydrophobic interaction chromatography (HIC).

Control conditioned media from untransfected 293H cells, and conditioned media from 293H cells expressing recombinant mouse GDF9 (max. 200 ml for 1 ml column), were concentrated approximately 20-fold by ultrafiltration, using a YM10 (10,000 MWCO) membrane (Millipore Corporation, Bedford, MA, USA). After addition of ammonium sulphate to a final concentration of 1M, the medium was loaded onto a 1 ml Phenyl Sepharose (low sub) column (Amersham Biosciences, Sydney, Australia), pre-equilibrated with 50 mM sodium phosphate, pH 7.0, 1 M ammonium sulphate. The column was washed with 13 ml of the same buffer and then eluted with a linear gradient of 50 mM sodium phosphate, pH 7.0. The procedure was carried out at room temperature, at a flow rate of 1 ml/min. One ml fractions containing GDF9 were detected by immunoblotting using mAb-GDF9-53, and by using a mouse MGC proliferation bioassay, both as previously described [453]. Fractions that were both immunoreactive and bioactive were pooled, concentrated, and dialysed with PBS.
using a Centriprep concentrator (10,000 MWCO; Millipore). After filter sterilisation, the samples were stored at −80°C. Concentration of GDF9 was determined by immunoanalysis, using a rat GDF9 preparation as standard, as previously described [453].

The relative bioactivities of conditioned media from 293H cells expressing GDF9, and media from untransformed 293H cells, before and after HIC purification, were determined by addition at increasing concentrations to cultures of MGC, alone or in the presence of 50 ng/ml recombinant human IGF1 (rhIGF1; Gropep, Adelaide, Australia). Cells were cultured and [\(^3\)H]-thymidine incorporation was determined as described below.

### 5.3.3 Follicle cell culture

MGCs were cultured as aggregates in 125μl of media (serum-free B-TCM, supplemented as above) in 96-well flat-bottom plates (Falcon) at a density of 0.5 x 10⁶ cells/ml. Variable combinations of the following treatments were added, depending upon the particular experiment: 50 mIU/ml recombinant human FSH (rhFSH; Puregon, N.V. Organon, Oss, Netherlands), 50 ng/ml recombinant human IGF1 (rhIGF1), 5-500 nM DHT (Sigma), 5-1000 nM testosterone (Sigma), denuded oocytes (DO; 5-40/well), and recombinant mouse GDF9 (GDF9; 15-120 ng/ml) . To block action of the AR, some experiments included the addition of a non-steroidal antiandrogen, hydroxyflutamide (OHF, Sigma). In these latter experiments, cells were pre-incubated for 1 hr with 100 or 1000 nM OHF, plus other non-androgen treatments, prior to the addition of DHT or testosterone. Cells were cultured with treatments in an atmosphere of 38.5ºC, 96% humidity, 5% CO₂ in air for 18 hour followed by a 6-hour pulse of 0.8 μCi tritiated thymidine ([\(^3\)H]-thymidine; ICN Biomedicals, NSW, Australia) in the same conditions. At 24 hours, a fraction of the culture medium was removed and frozen (-20ºC) for steroid analysis and plates were kept at 4ºC until cell harvest. Experiments were performed 3-6 times, in which each treatment was represented in triplicate, except in a few instances in the OHF experiments where insufficient numbers of DO necessitated some treatments in duplicate.
5.3.4 Measurement of DNA synthesis and progesterone secretion

Incorporation of \([^{3}H]\)-thymidine was measured in cells as an indication of the degree of cellular DNA synthesis and potential proliferation, using a previously described method and validation procedure [457]. Conditioned medium samples were assayed for progesterone content using a RIA kit (Diagnostic System Laboratories, Webster, TX) in accordance with the manufacturer’s instructions. The kit has a sensitivity of 0.25 pmol/ml, an intra-assay coefficient of 8.4%, and an inter-assay coefficient of 12%.

5.3.5 Data analysis

Statistical analyses for data represented in Fig. 5.2, were performed with SAS software (SAS Institute, Cary, NC, USA) using 4-way ANOVAs on mean data, derived from 6 replicate experiments in which each individual mean represented the average of triplicate data points for each treatment group, with blocking on experiment. Where significant treatment effects were detected, post-hoc t-tests were used for comparison of adjusted means for those significant effects. All other experimental data were analysed with SigmaStat software (Version 2, SPSS Inc., Chicago, IL) using 1- or 2-way ANOVAs, as appropriate, on log-transformed raw data, derived from 3 replicate experiments. Differences between groups were subsequently assessed by all pairwise multiple comparison procedures (Tukey Test). Statistical significance in all instances was set at p < 0.05.

5.4 Results

5.4.1 Partial purification of recombinant mouse GDF9

When GDF9-expressing 293H conditioned media were subjected to HIC, GDF9 bound to the Phenyl Sepharose (low sub) column. Both the precursor protein (57 kDa as monomer) and the more abundant mature form (17.7 kDa as monomer) were eluted with between 640 mM and 180 mM ammonium sulphate (Fig. 5.1A). All fractions were examined for
**Figure 5.1:** Partial purification of conditioned medium containing recombinant mouse GDF9 (GDF9) from the human embryonic kidney-293H (293H) host cell line. (A) Hydrophobic interaction chromatography (HIC) elution profile for GDF9-conditioned media and (B) Western blot of HIC elution fractions #30-41, demonstrating immunoreactive GDF9 in both the precursor (57 kDa) and mature (17.5 kDa) forms in fractions #35-40 (C) Comparison of mitogenic activity ([³H]-thymidine uptake) of conditioned GDF9-containing media and conditioned 293H control media fractions, acquired either pre- or post-HIC. Gilt MGCs were cultured for 24 hr in serum-free conditions with increasing concentrations of conditioned media ± IGF1 (50ng/ml). Data is represented as means ± SEM of treatments done in triplicate.
immunoreactive and bioactive GDF9, and only those fractions that contained immunoreactive
GDF9 (fractions 35-40; fractions 1-29 not shown) (Fig. 5.1B) also contained bioactive GDF9,
as demonstrated by potent stimulation of $[^3]$H-thymidine incorporation in cultured mouse
MGCs (data not shown). After dialysis of the pooled immunoreactive fractions, GDF9 was
shown to be enriched approximately 5 fold (from 2-6 µg GDF9/mg total protein to 10-28 µg
GDF9/mg total protein). HIC successfully removed the inhibitory factor(s) expressed by the
parent 293H cell line present in the conditioned media. Whilst control (untransfected) 293H-
conditioned medium pre-HIC dose dependently suppressed $[^3]$H-thymidine incorporation in
pig MGCs cultured with IGF1, control medium post-HIC had no such inhibitory effect (Fig.
5.1C). Furthermore, GDF9 post-HIC medium was twice as active as pre-HIC medium in
enhancing IGF1-stimulated DNA synthesis. To determine whether specific inhibitory
substances remained in the post-HIC preparation that affected FSH- but not IGF1-regulated
MGC functions, we performed experiments with post-HIC 293H conditioned media, at
equivalent concentration to the highest dose of GDF9 used in the main experiments, and
found no inhibition of FSH-stimulated DNA synthesis or progesterone secretion (data not
shown). Hence, partial purification of GDF9 by HIC was effective and necessary to generate
GDF9 that is bioactive on pig MGC; this HIC purification was also necessary for GDF9 to
promote cumulus expansion in the mouse [459].

5.4.2 Effects of DO or GDF9 ± DHT on MGC responses

Experimental results are summarised in graphs (Fig. 5.2) where all data are expressed as the
average of raw means from six replicate experiments ± SEM. Due to the complexity of the
data set, statistical significance is only indicated for responses related to the effects of DO or
GDF9 alone and in combination with DHT, as these represent the more novel responses that
are the main focus of this study. In most instances, the actions of FSH, IGF1 and DHT, alone
and in combination were as previously described [457]. In summary, FSH and IGF1 both
stimulated DNA synthesis in MGCs and had an additive effect when present together; DHT
Denuded Oocytes (DO)

A. DNA synthesis

![Graph showing DNA synthesis with control, DHT, DO, and DO + DHT treatments.]

B. Progesterone secretion

![Graph showing progesterone secretion with control, DHT, DO, and DO + DHT treatments.]

(Note: facing page for Figure 5.2)
Growth differentiation factor 9 (GDF9)

**B. DNA synthesis**

![Graph showing DNA synthesis](image)

**D. Progesterone secretion**

![Graph showing progesterone secretion](image)

**Figure 5.2:** Effect of dihydrotestosterone (DHT) and denuded oocytes (DO) or GDF9 on $^3$H-thymidine incorporation (A, B) and progesterone secretion (C, D) in gilt MGCs from 1-3 mm antral follicles. Cells were cultured for 24 h under serum-free conditions with combinations of FSH (50 mIU/ml), IGF1 (50 ng/ml), DHT (0.5 µM), and DO (0.16/µl) or GDF9 (60ng/ml). Graphs represent the means ± SEM of raw data from 6 replicate experiments in which each treatment was performed in triplicate. Statistical findings of interest are: A. Mitogenic effects of DO are greatest in the presence of IGF1 (p< 0.0001) or DHT (p = 0.01); B. Mitogenic effects of GDF9 are greatest in the presence of IGF1 (p < 0.0001) and DHT enhances this specific effect (p = 0.02); C. DO (p < 0.0001) and DHT (p = 0.04) inhibit FSH-stimulated progesterone secretion; D. GDF9 (p = 0.004) and DHT (p = 0.05) inhibit FSH-stimulated progesterone secretion.
had little additional effect, with the exception of a small, < 2-fold increase in IGF1-stimulated DNA synthesis. The effects of FSH, IGF1, and DHT, alone and in combinations, on progesterone secretion from MGCs were also consistent with previous observations [457], whereby FSH more potently stimulated progesterone secretion as compared to IGF1, and combined hormone had a synergistic effect. However, in the current study, DHT significantly inhibited (p < 0.05) FSH-stimulated steroid secretion, an effect that was not consistently observed in the previous study.

Prior to the current study, we observed that 500 nM DHT potently augmented IGF1-stimulated DNA synthesis and suppressed FSH-stimulated progesterone secretion in porcine COC from 1-3 mm antral follicles, but the same treatment of MGCs from the same follicles had little, or no effect, respectively [457]. As a continuation of this line of investigation, we herein repeated those same experimental conditions, substituting a co-culture of MGC with DO for the COC to see whether similar responses could be elicited. When HIC-purified GDF9 became available, we then repeated the same experiments with this known oocyte-secreted factor to ascertain whether it behaved similarly to the DO in the previous experiment. The doses of DO (20/well) and GDF9 (60 ng/ml) used in these exploratory experiments were chosen on the basis of similar experiments performed in mice that did not include the DHT treatment [53].

5.4.2.1 Effects of denuded oocytes (DO)

5.4.2.1.1[^H]-thymidine uptake (Figs. 5.2A and 5.3)

In the absence of any other growth factor(s), DO induced a moderate (2-fold) stimulation of DNA synthesis in MGC, however they potently stimulated[^H]-thymidine incorporation in the presence of IGF1 (p< 0.0001) (Fig. 5.2A), independent of the presence of FSH; this occurred in a dose-dependent manner (Fig. 5.3). The overall stimulatory effect of DO was significantly enhanced by DHT (p = 0.01), irrespective of the presence of IGF1 or FSH (Fig.
5.2A). Interestingly, DO tended to further increase DNA synthesis in the presence of combined FSH and IGF1, as compared to either mitogen alone, in marked contrast to previous observations with intact COC, in which suppression of DNA synthesis is a hallmark response to this combined treatment [444, 457].

**5.4.2.1.2 Progesterone secretion (Fig. 5.2C)**

Denuded oocytes also suppressed progesterone secretion from MGCs, an effect that was most significant in the presence of FSH (p < 0.001), independent of the presence of IGF1. In addition, DO significantly suppressed IGF1-stimulated progesterone secretion (p = 0.04) in a manner independent of FSH. The inhibitory effects of DHT and DO on progesterone secretion appear to be independent since there was not a significant interaction between these two factors, although, in combination, they induced the most potent suppressive cocktail in the presence of FSH and/or IGF1, as compared to either factor alone.

In summary, these DO-induced effects on hormone stimulated proliferation and progesterone secretion are indicative of a partial reversal of the differentiated MGC phenotype toward a less differentiated, more proliferative phenotype. Furthermore, this reversal was enhanced in all instances by the additional presence of DHT, particularly and most significantly in terms of proliferation.

**5.4.2.2 Effects of GDF9**

**5.4.2.2.1 [³H]-thymidine uptake (Figs. 5.2B and 5.3)**

Like DO, GDF9 alone moderately stimulated DNA synthesis in MGCs, and significantly interacted with IGF1 (p < 0.0001) (Fig. 5.2B), enhancing [³H]-thymidine incorporation in a dose-dependent manner (Fig. 5.3). However, unlike DO, the recombinant agent did not affect FSH-stimulated mitogenesis, and did not further enhance the mitogenic effects of combined IGF1 and FSH (Fig. 5.2B). Furthermore, GDF9 did not have statistically significant interactions with DHT when assessed across all treatment options, although the combination
Figure 5.3: Mitogenic effect of increasing doses of denuded oocytes (DO; 5-40/well) and GDF9 (15-120 ng/ml) on gilt MGC cultured for 24 hr under serum-free conditions ± IGF1 (50ng/ml). Graph represents the means ± SEM of raw data from 3 replicate experiments in which each treatment was performed in triplicate.
of GDF9 and DHT more potently enhanced IGF1-stimulated DNA synthesis compared to either agent alone (p = 0.02), and there was a tendency for this combination to inhibit IGF1 + FSH-stimulated DNA synthesis when either agent alone had no effect.

5.4.2.2.2 Progesterone secretion (Fig. 5.2D)

In a manner similar to DO, GDF9 significantly suppressed FSH-stimulated progesterone secretion (p < 0.0001) independent of IGF1. Unlike DO, GDF9 increased basal progesterone secretion 2-fold, and did not have any significant independent interactions with IGF1 in the modulation of progesterone secretion. Furthermore, significant interactions were not observed between GDF9 and DHT, however, as with DO, there was an obvious pattern of enhanced suppression of FSH-stimulated progesterone secretion when both of these factors were present as compared to either factor alone, and this pattern was more pronounced in the FSH + IGF1 treatment group.

In summary, recombinant GDF9 mimicked many of the actions induced by DO in porcine MGC, particularly in terms of enhancing IGF1-stimulated DNA synthesis and suppressing FSH-stimulated progesterone secretion. Interactions between the recombinant protein and DHT also showed marked similarities to those observed with DO, but confined to instances where GDF9 behaved in a DO-like manner.

5.4.3 Dose effects of DHT and testosterone on DO or GDF9-stimulated MGC

To ascertain whether both of the primary AR agonists, DHT and testosterone, show evidence of an interaction with mitogenic signalling initiated by DO and/or GDF9, a range of doses for each androgen was employed under the following constant conditions that were replicated from the initial experiments: IGF1 (50ng/ml) ± DO (20/well) or GDF9 (60 ng/ml), as depicted in Fig.5.4. In the absence of DO or GDF9, only the highest dose (500nM) of DHT (Fig 5.4A) and the two higher doses of testosterone (100nM and 1000nM) (Fig 5.4B), increased IGF1-
Figure 5.4: Dose-dependent mitogenic effects of DHT (A) and Testosterone (B). Gilt MGC were cultured for 24 hr under serum-free conditions in the presence of combinations of IGF1 (50 ng/ml), denuded oocytes (DO; 20/well), GDF9 (60ng/ml), DHT (5-500nM) and testosterone (10-1000nM). Graphs represent the means ± SEM of raw data from one of 3 replicate experiments in which each treatment was performed in triplicate. *p < 0.05 and **p < 0.001, as compared to similar treatment minus androgen.
stimulated DNA synthesis, (less than 2-fold in all instances). However, in the presence of DO+IGF1 or GDF9+IGF1, low doses of both androgens that were ineffective with IGF1 alone, led to substantial increases in DNA synthesis. The dose-dependent nature of the 3-way interactions between androgen x IGF1 x GDF9/DO were similar with DHT or testosterone.

5.4.4 Opposition of androgen-induced effects with the antiandrogen OHF

To determine whether the observed androgen effects on mitogenic signalling in GC were mediated through the AR, cells were pre-treated for one hour with the antiandrogen OHF (at double the androgen dose) prior to the addition of particular doses of DHT or testosterone. These experiments were performed in the presence of IGF1 and DO (20/well) or GDF9 (60 ng/ml), but only the results of the GDF9 experiments are presented, because OHF alone at either dose had highly variable inhibitory effects on the activity of DO. In the presence of IGF1 + GDF9, the stimulatory action of 50 nM DHT was completely inhibited by 100 nM OHF, but 500 nM DHT was only partially inhibited by 1000 nM OHF, possibly due to a small agonistic effect of the higher dose of OHF alone (Fig. 5.5A). The same doses of testosterone had potent stimulatory effects that were substantially, but not fully, inhibited by the antiandrogen (Fig. 5.5B).

5.5 Discussion

Androgen receptor (AR)-mediated activity is clearly evident in follicular cells, but its role in folliculogenesis and the mechanistic pathways involved remain uncertain. As with many growth factors and hormones, androgens have the ability to stimulate opposing actions in target cells depending upon a particular developmental program and the environmental milieu characteristic of the various stages of that program. In terms of the ovary and the process of folliculogenesis, a direct, receptor-mediated role for androgens in promoting FSH-stimulated granulosa cell (GC) differentiation has been well documented (reviewed by [310]), coinciding with evidence that androgens can inhibit GC proliferation and enhance exit from the cell cycle.
Figure 5.5: Antiandrogen treatment opposes the mitogenic effects of androgen in GDF9-stimulated cells. Gilt MGCs were cultured for 24 hr under serum-free conditions in the presence of combinations of IGF1 (50ng/ml), GDF9 (60ng/ml), DHT (50-500nM), testosterone (50-500nM), and the antiandrogen hydroxyflutamide (OHF; 100-1000nM). Graphs represent the means ± SEM of raw data from one of 3 replicate experiments in which each treatment was performed in triplicate. *p < 0.05 and **p < 0.001, as compared to similar treatment minus OHF.
[333, 334]. In the present study we show the opposite effect, whereby androgens promote GC proliferation and suppress differentiation, contingent upon the presence of oocyte-secreted factors (OSFs). These results concord with our earlier observations of differential effects of DHT on porcine COC and MGC [457], as well as those by Bley et al [332], whereby DHT enhanced rat GC proliferation under strict non-luteinizing culture conditions. With the advent of knowledge concerning the essential nature of oocyte-derived growth factors in directing the course of folliculogenesis and determining the functional stratification of follicular GC, it is plausible that exposure to such factors could be environmental determinants of differential androgen activity in GC. Collectively, these data suggest that the direct role of androgens is likely to evolve during the process of folliculogenesis, and this evolution is potentially characterised by variable degrees of interaction with signalling pathways stimulated by OSFs.

Oocyte control of folliculogenesis via OSFs now represents a firmly established concept in ovarian biology, but the mechanics of this control still require characterisation, especially in non-murine species. As previously documented in mice and cows (reviews: [51, 53]), as well as pigs [460, 461], co-culture of MGCs with DO in the present study induced changes in GC responses that correlate with a less luteinized phenotype, as characterised by increased proliferation and diminished progesterone secretion. The mitogenicity of pig DO was largely dependent on the simultaneous presence of IGF1, having little or no stimulatory effect in the absence of any other growth factors or in the presence of FSH alone. These observations concur with those reported by Li et al under similar culture conditions in the cow [438] and Brankin et al [461] under long-term culture in the pig, but differ from those observed in the mouse, in which oocytes alone exert a potent mitogenic effect that is similar to, but does not augment IGF1-stimulated MGC proliferation [452]. In contrast to the species-specific differences in mitogenicity of oocyte secretions, the inhibitory effect of oocytes on progesterone secretion from MGCs in vitro occurs mainly in the presence of FSH in all species studied (reviewed by [53]).
Overall, the non-aromatizable androgen, DHT, universally potentiated the interactions of an unknown cocktail of OSFs secreted by DO with IGF1 and/or FSH: in essence, mitogenesis was greatest and progesterone secretion was most attenuated in the combined presence of DHT and DO. While it is clear that DHT and oocytes can independently influence the activities of FSH and IGF1, our data suggest that there is an interaction between OSFs and androgen that is independent of their individual effects on signalling by these growth factors: one that nonetheless appears to bootstrap those independent effects. Therefore it is feasible to propose that OSFs either potentiate androgen signalling, or androgens potentiate oocyte signalling, or both. Since most identified OSFs are members of the TGFβ superfamily, there is precedence for such interaction from studies in the prostate whereby androgen regulates expression of TGFβ1 and its cognate receptors [462-464] and the AR interacts with intracellular SMAD signalling molecules [465, 466]. Additionally, Killian et al [467] report that an androgen-regulated protease cleaves latent TGFβ1 in osteoblast cells and proteolytically modulates cell surface receptors. In the ovary, DHT administered in vivo to hamsters induces TGFβ receptor type II mRNA in follicular cells [468, 469]. Although TGFβ1 is expressed by the oocyte, it is unlikely to be the factor responsible for the effects of oocytes and the interaction of its secreted factors with androgen in the current study, as predicated by previous studies in the cow [470] and mouse [453], and supported by our unpublished observations using the current pig model.

In the mouse model, many of the effects of OSFs on GCs can be mimicked by, but not necessarily attributed to, GDF9 [453, 459, 471]. GDF9 is a member of the TGFβ superfamily, whose expression is oocyte-specific in many species, but possibly not in primates [205, 472] or pigs [473], where expression has also been detected to a lesser degree in granulosa cells. Due to lack of commercial availability, the specific effects of GDF9 have not been widely explored outside of the mouse model. Using recombinant mouse GDF9 generated in house, we demonstrate that GDF9 generally mimicked the non-luteinizing effects of oocytes in our
culture system, especially in terms of dose-dependent increases in IGF1-stimulated proliferation. This specific mitogenic effect of GDF9 or DO was significantly augmented by DHT and testosterone, the two most potent AR agonists. Moreover, the effects of both androgens on GDF9-stimulated cells could be inhibited by the antiandrogen OHF, implicating an AR-mediated mechanism. The variable influence of OHF in the DO co-culture model (data not shown) probably indicates a direct effect of OHF and/or androgens on naked oocytes, as this variability was not observed when COC were similarly treated [457]. Although AR expression has been documented in pig oocytes [305], the dynamics of this expression has not been fully explored as in the rat, where expression is differentially localized at various developmental stages [300]. A transcription-independent, AR-mediated mechanism has been implicated in mouse oocyte maturation, under conditions in which oocytes were stripped of surrounding GC [304], giving support to the possibility that androgens in our co-culture system caused direct effects within the oocyte. It will be interesting to see whether such effects influence the expression and/or secretion of GDF9 and/or other OSFs.

One of the striking differences between the actions of DO and GDF9 in the current study was their opposing effect on proliferation in the presence of FSH and IGF1. Inhibition of IGF1-stimulated DNA synthesis by FSH is a characteristic feature of COC that distinguishes them from MGCs [444, 474] which can be replicated by co-culturing oocyctomised cumulus complexes with denuded oocytes in the cow [438]. Curiously, in the current study, GDF9 tended to induce this cumulus cell-like effect in MGC, but DO did not; in fact DO further enhanced DNA synthesis under these conditions. In addition, DHT potentiated both of these opposing effects. There are a few possible explanations for these differences: 1) the additional complexity of other factors secreted by the oocyte and/or granulosa cells in response to those factors, 2) the exact amount of GDF9 released by the DO during culture could not be determined, and was potentially in constant renewal over the culture period, in contrast to a fixed dose of recombinant GDF9, and 3) mouse oocytes seem
to predominantly secrete GDF9 in the unprocessed form while the recombinant molecule represents mainly the processed form [453]. It is provocative to speculate that the specific role of GDF9, and/or its interaction with DHT, changes as antral follicles become more vascularized and FSH intervenes on activities largely driven by IGF1 alone in earlier stages of folliculogenesis. These complex interactions, and the additional effects of other OSFs such as GDF9b, are to be explored in future studies.

Consequences of interruption to the interaction between androgens and OSFs may be inferred from studies of mice with perturbations of androgen signalling. Ablation of 5-α-reductase [475] or inactivating mutations of the AR [476] induce a sub-fertility phenotype in mice that has not yet been explored at the ovarian level. However, a similar sub-fertility is also evident in AR knockout mice, characterised by a reduction of GC numbers in large antral follicles that may result in luteal deficiency [329], suggesting an important albeit non-obligatory role for androgens in GC proliferation. Dominant follicles are characterised by exponential increases in GC numbers, and a critical mass of GC may be necessary for ovulation to occur [249]. Perhaps androgens provide the extra boost necessary to achieve this optimal growth. Indeed, pigs treated with DHT have an increased rate of ovulation [345]. Non-human primates treated with excess androgens have increased follicular growth [401] and elevated androgen in women is associated with an abnormal accumulation of antral follicle numbers (polycystic ovaries) (reviewed by [183]), supporting a growth-promoting role for androgens in an in vivo context where cells are exposed to oocyte secreted factors. However, in polycystic ovary syndrome (PCOS), ovulation is impaired due to a follicular arrest by unknown mechanisms. Such follicles are characterised by a deficient GC layer [195], and perhaps reduced expression of GDF9 in oocytes [204], giving rise to speculation that elevated androgens disturb normal GDF9 signalling [203]. Herein we provide evidence of a novel interaction between androgen and GDF9 that substantiates the feasibility of this
postulate, although the mechanistic profile of this newly discovered interaction, and its species-specific peculiarities have yet to be determined.

In conclusion, we have demonstrated that androgens directly interact with GDF9 and possibly other unknown factors secreted by the oocyte to amplify GC proliferation at early antral stages of folliculogenesis in the pig. Oocyte signalling may therefore be necessary for androgens to stimulate a proliferative rather than a differentiative pathway in follicular granulosa cells.
Chapter 6 Androgen Receptor Signalling in Human Granulosa Cell Subtypes from Normal and Polycystic Ovaries.

6.1 Abstract

Polycystic ovary syndrome (PCOS) is characterised by hyperandrogenaemia, which may disrupt androgen receptor (AR) signalling within the ovary. However, AR signalling pathways remain poorly defined in ovarian cells, and deviant AR-mediated activity has not been widely examined in relation to the pathology of PCOS. In this study, expression of AR, two AR transcriptional cofactors (ARA55 and ARA70) and two AR-regulated genes (human kallikrein 2 and 3 (hKLK2; hKLK3)) were analysed in two human GC subtypes obtained from women undergoing IVF with intracytoplasmic sperm injection (ICSI). Granulosa lutein cells (GLC) were purified from follicular aspirates, and cumulus cells (CC) were stripped from the oocyte prior to ICSI. Cumulus cells had 3-7 fold higher AR (p<0.0001) and 3-11 fold higher ARA55 (p<0.0001) mRNA expression compared to GLC. In contrast, mRNA expression of ARA70 was similarly expressed in both GC subtypes. No differences in AR, ARA55, or ARA70 mRNA expression were observed between controls and women with PCOS for either GC subtype. However, CC from PCOS ovaries had 2-fold lower (p<0.01) AR protein content compared to normal CC. Human KLK2 mRNA was universally undetectable, and hKLK3, although undetectable in GC from normal ovaries, was expressed in 82% of CC and 36% of GLC from PCOS ovaries. In cell culture experiments, dihydrotestosterone (DHT) induced expression of hKLK3 mRNA and increased the mitogenic and steroidogenic effects of FSH and/or IGF1 in CC but not GLC from normal ovaries. The latter effects were diminished or different in CC from PCOS ovaries. Collectively, these results demonstrate that AR-mediated signalling is more potent in GC adjacent to the oocyte, suggesting possible regulation by oocyte-secreted factors. Anomalies in AR-mediated

\[1^\text{See Appendix 1}\]
signalling are evident in CC from PCOS ovaries, which potentially contribute to disrupted folliculogenesis in this disorder.

**6.2 Introduction**

Polycystic ovary syndrome (PCOS) is a complex endocrine disorder of unknown aetiology that occurs in approximately 3-10% of women in their reproductive years and is characterised by unexplained hyperandrogenaemia, chronic anovulation, and polycystic ovaries (PCO) [84]. The phenomenon of PCO is more common than the disorder, being apparent in approximately 20% of women [96]. Ovulatory women with PCO may represent an occult form of PCOS, as serum androgen levels in these women tend to be of intermediate levels between women with PCOS and normal cycling women without PCO [93]. The association between hyperandrogenaemia and PCO is further substantiated by the fact that other disorders or pharmacological treatments that cause a state of hyperandrogenaemia induce PCO, and the severity of this ovarian morphology is positively associated with the degree of androgen excess (review: [183]). One of the defining features of a PCO is an abnormally large number of antral follicles 2-9 mm in diameter appearing in the cortical region of the ovary, which may be the result of enhanced early follicle growth/survival. In women with PCOS, the ovaries chronically fail to ovulate, and follicular growth becomes developmentally arrested at a diameter of approximately 10mm, suggesting inhibition of dominant follicle selection. How a state of hyperandrogenaemia causes this abnormality is currently unknown.

Pharmacological doses of testosterone or dihydrotestosterone (DHT) induce a PCO-like morphology in rhesus monkeys [401], suggesting that the ovarian perturbations mechanistically involve the androgen receptor (AR). A state of hyperandrogenaemia may disrupt normal AR-mediated signalling within the ovarian follicle, but AR signalling pathways remain poorly defined in the human ovary, and have not been widely examined in relation to the pathology of PCOS. Within mammalian ovaries, follicular granulosa cells (GC) generally have the most intense immunoreactivity for the AR, and androgens have been
shown to augment the differentiative action of FSH on this cell type in vitro (review: [310]). However, GC diverge into two distinct subtypes upon antral follicle development: mural granulosa cells (MGC) that form the follicle wall (and represent the GC used in most in vitro studies), and cumulus cells (CC) that remain attached to the oocyte. Using a prepubertal pig model, we have recently shown that AR-mediated activity is different in these two GC subtypes [457], due to the influence of oocyte-secreted factors [477]. The aim of the current study was to examine AR signalling in human GC subtypes and determine whether these pathways are disturbed in women with PCOS.

6.3 Materials and Methods

All chemicals were purchased from Sigma Chemical Co, (St Louis MO, USA) unless otherwise stated.

6.3.1 Study participants

Tissues were collected from consenting women attending Repromed infertility clinic (Adelaide, Australia) and having intracytoplasmic sperm injection (ICSI) as a means of fertilization. Women received a GnRH agonist (Synarel; Searle, Division of Monsanto South Africa Pty Ltd), recombinant FSH (Gonal F; Serono), and urinary hCG (Pregnyl; Organon) to stimulate follicle growth prior to oocyte retrieval. The study was approved by the human ethics committees of The University of Adelaide, Women’s and Children’s Hospital, and The Queen Elizabeth Hospital. Women were diagnosed with PCOS based on the 2003 Rotterdam consensus criteria of having at least two of the following criteria: 1) clinical or biochemical hyperandrogenism, 2) chronic anovulation, and 3) polycystic ovaries (PCO) on ultrasound, with the exclusion of other aetiologies [84]. In this study, all women in the PCOS group had PCO on ultrasound. Controls were normal cycling women with no evidence of the characteristic symptoms of PCOS. For gene expression experiments involving follicular cells
from a single dominant follicle, cDNA was obtained from the authors of another study involving PCOS [478].

6.3.2 Granulosa cell purification

To distinguish the fact that mural granulosa cells (MGC) used in this study were exposed to an ovulatory dose of hCG as part of the hyperstimulation protocol prior to oocyte retrieval for IVF, these cells are herein designated granulosa lutein cells (GLC). To obtain GLC, follicular aspirates were centrifuged at 3,000 rpm for 10 min at 5°C to separate follicular fluid from blood and follicular cells. The lower fraction was mixed with 15 ml cold PBS containing 2% FCS (Trace Scientific, Australia), penicillin G (100 U/ml) and streptomycin sulphate (100 U/ml) (hereafter referred to as supplemented PBS) and layered onto 7.5 ml of a 50:50 v/v mixture of Percoll (Amersham Biosciences, Australia) and supplemented PBS. A density gradient was produced by centrifugation at 3,000 rpm for 30 min at 5°C, and GLC were subsequently removed from the middle fraction with a glass pipette. These cells were gently dispersed by repeated pipetting in fresh supplemented PBS, and then subjected to a second density gradient to remove any remaining blood cell contamination. GLC were then either washed twice in cold, non-supplemented PBS prior to RNA extraction of the cell pellet, or washed once in warm supplemented PBS and 3 times in warm bicarbonate-buffered TCM-199 (B-TCM: ICN, Costa Mesa, CA) supplemented with sodium pyruvate (2 mM), penicillin G (100 U/ml), streptomycin sulphate (100mg/ml), and 10% FCS, prior to culture in the same medium.

Cumulus cells (CC) were obtained from aspirated cumulus-oocyte-complexes (COC) following a brief (< 30 sec) digestion in 75 IU/ml Hyalase (Aventis) to allow CC to be manually stripped from the oocyte. After removal of oocytes, pooled CCs were washed twice in cold PBS prior to RNA extraction or washed 3 times in warm supplemented B-TCM prior to cell culture in the same media.
6.3.3 Cell culture

For cell culture experiments examining the induction of hKLK3 mRNA expression in GC from control ovaries, cells from one individual were initially cultured at a density of $5 \times 10^4$ cells/500µl BTCM with 10% FCS in 4-well culture plates (Nunc) for 48 hours. The medium was then removed and cells were washed 3 times in serum-free BTCM before a further 24-hour culture in the same medium, with or without the addition of 500nM DHT. Due to limited numbers of CC, single treatments were set up for each individual, and the experiment was repeated on cells from 7 individuals (n=7).

For experiments examining mitogenic and steroidogenic responses of GC to hormone stimulation, cells of each GC subtype relating to individual patients were cultured for 7-14 days in 25 mm$^2$ tissue culture flasks (Falcon, Franklin Lakes, NJ, USA) in an atmosphere of 37°C, 96% humidity, and 5% CO$_2$, with a half volume media change every 3 days. GLC were plentiful and had a homogenous morphology, but CC were limited in amount and appeared in different morphological phenotypes (see next section). Following the initial culture period, cells were trypsinized and washed 3 times in serum-free supplemented BTCM. To achieve adequate CC numbers for experiments, cells from individual patients were pooled according to CC morphology (see next section) and treatment group (Control or PCOS). GLC were also pooled in accordance to profile of their corresponding CC. Cells were counted using a haemocytometer, aliquoted into 96-well flat-bottom culture plates (Falcon) at a density of 25,000 cells in 125 µl of serum-free BTCM, and treated in duplicate or triplicate with combinations of the following: 50mIU/ml recombinant human FSH (rhFSH; Puregon, N.V. Organon, Oss, Netherlands), 50ng/ml recombinant human IGF1 (rhIGF1; Gropep, Adelaide, Australia), and 50nM DHT (Sigma). Cells were cultured with treatments for 18 hour followed by a 6-hour pulse of 0.8µCi tritiated thymidine ($[^3]$H-thymidine; ICN Biomedicals, NSW, Australia) under the same conditions. At 24 hours a fraction of the culture medium was removed and frozen (-20°C) for steroid analysis and plates were kept at 4C until cell harvest.
Each experiment was repeated a minimum of 3-5 times. In some instances where CC numbers were high, cellular responses were determined for individual participants.

6.3.4 GC morphology in culture

GLC were morphologically distinct from CC in culture (Figure 6.1A). GLC were invariably large, flattened cells, which showed no visible signs of proliferation over the culture period. In contrast, CC had a range of morphological appearances, consistent with those previously described and categorized by Gitay-Goren et al [479]. Three of the categories described by these authors were predominant among the CC collected for the current study: 1) type A (Figure 6.1B), which were very rounded, established clear intercellular connections and visibly proliferated over the culture period, 2) type B (Figure 6.1C), which were more flattened, had fewer intercellular connections and were generally less proliferative, and 3) type C (Figure 6.1D), which were similar in appearance and proliferative capacity to GLC. Because initial CC cultures represented a pool of cells from all collected COCs for a particular individual, a mixture of CC subtypes was usually observed. To attain adequate numbers of cells for in vitro experiments, cultured CC were pooled into two groups according to their predominant morphology: group I (mostly type A with some type B) and group II (mostly type C with some type B). There were no apparent differences in morphology between GC collected from normal and PCOS ovaries.

6.3.5 Measurement of DNA synthesis and progesterone secretion

Incorporation of [³H]-thymidine was measured in harvested cells as an indication of the degree of cellular DNA synthesis and potential proliferation using a previously described method and validation procedure [457]. Conditioned media were assayed for progesterone content using an RIA kit (Diagnostic System Laboratories, Webster, TX) in accordance with the manufacturer’s instructions. The kit has a sensitivity of 0.25 pmol/ml, an intra-assay coefficient of 8.4%, and an inter-assay coefficient of 12%.
Figure 6.1: Morphology of granulosa lutein cells (A) and three types of cumulus cells (B-D) in culture (x20 magnification). Designation of cumulus cell morphological types was adopted from [469].
6.3.6 Gene expression

Total RNA was extracted from GC using an RNeasy Microkit (Qiagen, Clifton Hill, Victoria, Australia) with on-column DNAase treatment; yield was assessed using a Ribogreen RNA Quantification Kit (Molecular Probes, Eugene, OR, USA). To produce cDNA, 0.5 µg of RNA was reversed transcribed with an Omniscript RT Kit (Qiagen) using oligo dT primers (Promega). For real-time quantitative PCR, 3µl of a 1:10 cDNA dilution was amplified in triplicate on a GeneAmp 5700 Sequence Detection System (Applied Biosystems Pty Ltd, Victoria, Australia) using Taqman Universal PCR Master Mix (Applied Biosystems) with specific Assays-On-Demand Gene Expression Products (Applied Biosystems) for the following genes: androgen receptor (AR), androgen receptor associated proteins 55 and 70 (ARA 55 and ARA70), human kallikreins 2 and 3 (hKLK2 and hKLK3), and ribosomal protein L19 (internal control). Amplification data from the genes of interest were normalised with L19 expression data and fold-differences between the granulosa cell subtypes was calculated using the $2^{-\Delta\Delta Ct}$ method. A random CC sample from the Control group was designated as having 100% expression.

6.3.7 Western blots

For protein analysis, GC from 7-10 day cultures were lysed in 100 µl cold RIPA buffer (10mM Tris (pH7.4), 150 mM NaCl, 1mM EDTA, 1% Triton X-100) supplemented with a protease inhibitor cocktail (Complete Mini; Roche Diagnostics, Mannheim, Germany) according to manufacturer instructions. Total protein concentration was determined using the Micro BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA). For analysis, 15 ug of each sample was precipitated in ice-cold acetone, reconstituted in Laemmli buffer containing 100 mM DTT, heated for 4 min at 100C, subjected to SDS-PAGE (on a 7.5% acrylamide gel), and transferred to Hybond ECL (Amersham Biosciences). The membrane was blocked at RT for 1 hour in 2% ECL Advance blocking reagent (Amersham Biosciences)
prepared in Tris-buffered saline with 0.1% Tween 20, probed overnight at 4C with an AR antibody (N20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:5000 or an unphosphorylated MAPK antibody (Sigma) diluted 1:10,000, followed by incubation at RT for 1 hour with a horseradish peroxidase-conjugated anti-rabbit antibody (Chemicon Australia Pty Ltd, Boronia, VIC, Australia) diluted 1:100,000 (for AR) or 1:400,000 (for MAPK). Antibodies were diluted in the blocking buffer. Immunoreactive proteins were detected using the ECL Advance kit (Amersham Biosciences) according to instructions. The MAPK antibody detects unphosphorylated ERK 1 and 2 proteins, and was used as a loading control after determining that protein levels do not differ between GC cell types or between cells from normal and PCOS ovaries. Protein quantification was assessed by analysing the intensity of bands for AR and both ERK proteins using ImageJ software (Version 1.3; National Institute of Health, USA).

6.3.8 Statistics

Statistical analyses were performed with SAS software (SAS Institute, Cary, NC, USA). For gene expression studies, mixed model repeated measures ANOVA or ANCOVA were performed on relative expression data to test for cell type and treatment effects. Statistical differences in AR protein were determined by t-test. For cell culture experiments, 3-way ANOVAs were performed on raw data, with blocking on experiment. Where significant effects were seen, post-hoc t-tests were used for comparison of adjusted means for those significant effects. Statistical significance was set at p<0.05 in all instances.

6.4 Results

6.4.1 GC expression of the AR and AR co-regulatory molecules

In GC subtypes from pooled follicular samples, CC had 3-7 fold higher AR (Figure 6.2A) and 3-11 fold higher ARA55 (Figure 6.2B) mRNA expression compared to GLC, and there was a positive linear association (p<0.001) between follicular AR and ARA55 ratios. In contrast,
Figure 6.2: Gene expression analysis of (A) AR, (B) ARA55, and (C) ARA70 in granulosa lutein cells (GLC) and cumulus cells (CC) from Controls (n=11) and PCOS (n=11). Each point represents a pool of cells from all of the aspirated follicles of one individual. Horizontal bars represent the mean value for each group.
expression of ARA70 was similar between GC subtypes (Figure 6.2C). No differences in AR, ARA55 or ARA70 mRNA expression were seen between Controls and PCOS in either GC subtype. The differential mRNA expression of AR in GC subtypes and lack of difference in AR levels between Control and PCOS patients in GC subtypes was confirmed in cells derived from individual, size-matched dominant follicles using leftover cDNA from a study by Phy et al [478](Figure 6.3). There was not enough cDNA to do a similar analysis for ARA55 and ARA70.

Western blot analysis revealed differential AR expression at the protein level in GC subtypes from both groups (Figure 6.4A). CC from normal ovaries had abundant AR protein, but this was significantly reduced (p<0.01) in CC from PCOS ovaries (Figure 6.4B). Minimal or no immunoreactive receptor protein was evident in GLC from either group, disallowing further quantification. In contrast, AR protein was abundant in CC from normal ovaries, compared to CC from PCOS ovaries (Figure 6.4B).

6.4.2 GC expression of hKLK2 and hKLK3

Kallikreins are serine proteases regulated by androgens in most tissues, particularly hKLK2 and hKLK3 (the latter also known as prostate specific antigen or PSA) (review: [480]). Women with PCOS have elevated serum and urine levels of these kallikreins [481], but their expression in human ovarian GC has not previously been determined. No evidence of a real-time linear amplification profile for hKLK2 or hKLK3 was evident in CC (n=11) or GLC (n=11) from normal ovaries. GC from PCOS ovaries also had undetectable levels of hKLK2, but hKLK3 was detectable (defined as a real-time linear amplification profile that crossed the Ct threshold at ≤ 35 cycles) in 9/11 (82%) of CC and 4/11 (36%) of GLC derived from PCOS ovaries. Treatment with DHT induced hKLK3 expression in CC but not GLC from normal ovaries (Figure 6.5).
Figure 6.3: Gene expression analysis of AR in granulosa lutein cells (GLC) and cumulus cells (CC) from Controls (n=24) and PCOS (n=8). Each data point represents cells from a single size-matched pre-ovulatory follicle for one individual. Horizontal bars represent the mean value for each group.
Figure 6.4: (A) Western blot analysis for immunoreactive AR in cumulus cells (CC) and granulosa lutein cells (GLC) derived from pooled cells of aspirated pre-ovulatory follicles. A prostate cancer cell line (LNCaP) was used as a positive control for AR and detection of unphosphorylated MAPK protein used as a loading control. (B) Quantification of AR protein levels in CC from Control (n=6) and PCOS (n=6) groups. *p=0.01
**Figure 6.5:** Induction of human kallikrein 3 (hKLK3) mRNA expression by androgen in cumulus cells (CC) but not granulosa lutein cells (GLC) from normal ovaries. CC were cultured for 24 hours in serum-free media with (+) or without (--) 500nM dihydrotestosterone (DHT) prior to RNA extraction. Amplification of hKLK3 in cDNA from a prostate cancer cell line (LNCaP) was used as a positive control.
6.4.3 Mitogenic responses to DHT

The following series of results are supported by graphs that depict a representative experiment among a group of 3-5 replicate experiments, due to the variability in baseline hormone responses, particularly in experiments involving CC. Only those statistical results related to androgen action are indicated in the graphs, for clarity and simplicity.

6.4.3.1 Normal GC

As expected, GLC had a low level of $[^3]$H-thymidine uptake, which was unaffected by FSH but increased approximately 4-fold (p<0.01) by the presence of IGF1 (Figure 6.6A). These cells did not respond to DHT under any hormone treatment combinations. Group I CC were potently stimulated (p<0.001) by IGF1 (Figure 6.6B), but were unaffected by FSH alone. Interestingly, FSH inhibited (p<0.05) IGF1-stimulated uptake in CC with this morphology, a feature characteristic of similarly treated COC in other animal species [438, 444, 457]. Androgen treatment induced a 2-fold increase in FSH-stimulated thymidine uptake in Group I CC. This pattern of hormonal response was consistent over 3 replicate experiments. In contrast, basal levels of $[^3]$H-thymidine uptake were comparatively low in Group II CC, and although IGF1 significantly stimulated uptake (p<0.001) (Figure 6.6C), the magnitude of stimulation was considerably less than that observed in Group I CC. In addition, the inhibitory effect of FSH on IGF1-stimulated mitogenesis in Group I CC was reversed in Group II CC, whereby FSH consistently enhanced the effects of IGF1 (p<0.05). This effect is characteristic of similarly treated MGCs or oocytectomized cumulus complexes from other animal species [438, 444, 457], and probably reflects the more advanced state of luteinization expected in Group II CC [479]. Androgen treatment significantly increased the mitogenic effect of IGF1 in Group II CC (Figure 6.6C). This effect was observed in 5 replicate experiments, although the magnitude of these androgen effects varied from 1.5 to 5-fold.

In summary, the mitogenic responses to FSH and IGF1 in GLC and CC are consistent with those observed for other animal species and support the expectation that Group I CC
Figure 6.6: Mitogenic responses of (A) granulosa lutein cells (GLC) and cumulus cells (CC) categorized by morphology into (B) Group I (less luteinized) and (C) Group II (more luteinized). Graphs depict raw data from a representative experiment in which bars indicate the mean (±SEM) of 2-3 replicate treatments. Experiments were repeated a minimum of 3 times. *p<0.05 as compared to similar treatment without androgen.
Figure 6.7: As in Figure 6.6, but using cells derived from PCOS ovaries. *p<0.05 as compared to similar treatment without androgen.
have a less luteinized phenotype than Group II CC. Only CC were responsive to DHT, and these responses were dependent upon their specific morphological phenotype.

6.4.3.2 PCOS CC

Androgen signalling in GLC from PCOS ovaries was not examined because this tissue was always extensively infiltrated with blood vessels that were difficult to fully remove, and GLC in culture invariably developed endothelial cell contamination. Therefore, only CC were examined in this group. The pattern of mitogenic response to combinations of FSH and IGF1 by cells from PCOS ovaries (Figure 6.7) were similar to those observed for CC from normal ovaries (Figure 6.6). However, responses to DHT were more variable. Androgen always increased FSH-stimulated $[^3H]$-thymidine uptake by a small amount in Group I CC (Figure 6.7A), but this never reached the 2-fold level that was observed in CC of this morphology from normal ovaries (Figure 6.6B). In Group II CC, two types of androgen responses were observed: 1) DHT had no effect (Figure 6.7B) or 2) DHT increased the mitogenic action of FSH- and/or IGF1 (Figure 6.7C; graph depicts an experiment in which both were stimulated). Also, in Group II CC from PCOS ovaries, there was a tendency for DHT to inhibit the mitogenic stimulation of FSH+IGF1 (Figure 6.7C). This effect was not observed in CC from normal ovaries and in one replicate experiment from a single PCOS patient, DHT inhibited this stimulation by 5-fold.

In summary, CC from PCOS ovaries had similar mitogenic responses to FSH and IGF1 compared to CC from normal ovaries, but androgen responsiveness appears to be diminished or altered in these cells.

6.4.4 Steroidogenic responses to DHT

6.4.4.1 Normal GC

Secretion of progesterone from cells represented in Figure 6.6 is depicted in Figure 6.8. Basal secretion was high in GLC from normal ovaries (Figure 6.8A), and was further stimulated by
Figure 6.8: Steroidogenic responses of (A) granulosa lutein cells (GLC) and cumulus cells (CC) categorized by morphology into (B) Group I (less luteinized) and (C) Group II (more luteinized). Graphs depict raw data from a representative experiment in which bars indicate the mean (±SEM) of 2-3 replicate treatments. Experiments were repeated a minimum of 3 times. *p<0.001 as compared to similar treatment without androgen.
Figure 6.9: As in Figure 6.8, but using cells derived from PCOS ovaries. *p<0.001 as compared to similar treatment without androgen.
FSH (p<0.01) but not by IGF1 or DHT under any treatment combination. This pattern of hormone response was the same for 3 replicate GLC experiments. In contrast, baseline progesterone secretion by CC was more variable, possibly due to variable responses to the hyperstimulation protocol [482]. In Group I CC, basal secretion tended to be minimal, but was always potently stimulated by FSH (0>0.0001) and more moderately stimulated by IGF1 (p>0.001) (Figure 6.8B). Androgen consistently potentiated IGF1-stimulated progesterone secretion in Group I CC (p<0.001). In contrast, progesterone secretion from Group II CC (Figure 6.8C) was similar to that observed for (Figure 6.8A), whereby cells had a high basal level of secretion, which was not augmented by any hormone treatment.

6.4.4.2 PCOS GC

Baseline steroid secretion was also highly variable in CC from PCOS ovaries. Progesterone secretion from cells represented in Figure 6.7 is depicted in Figure 6.9. In Group I CC, patterns of response to FSH alone were similar to those observed in normal ovaries, but in cells from PCOS ovaries, IGF1 increased the steroidogenic effect of FSH (p<0.01)(Figure 6.9A,B), a feature characterisitic of mural granulosa cells in other animal species [438, 444, 457], but one not observed in any of the experiments with human CC from normal ovaries. DHT consistently increased FSH-stimulated progesterone secretion in Group I CC, but inhibited FSH action in Group II CC. Notably, DHT reduced FSH-stimulated steroid secretion close to basal levels in one individual with PCOS. In 2 of 5 replicate experiments, Group II CC from PCOS ovaries had low baseline progesterone secretion, which was not further stimulated by hormone treatment (Figure 6.9C).

In summary, FSH stimulated progesterone secretion in both GC subtypes as expected, but IGF1 did not further increase this action in cells from normal ovaries as observed in similarly treated cells from other animal species. This occurred only in CC from PCOS ovaries. Androgen only influenced progesterone secretion in the CC subtype, but had different effects in cells from normal and PCOS ovaries.
6.5 Discussion

The results of this study indicate that human GC subtypes from pre-ovulatory follicles of women hyperstimulated with gonadotrophins have differential androgen signalling whereby CC that had been attached to an oocyte in vivo have higher levels of AR and ARA55 expression and respond to androgen to in culture as compared to GLC from the follicle wall which have lower expression of androgen signalling molecules and do not respond to androgen in culture. These findings indicate a positive role for the oocyte in promoting and/or maintaining an elevated level of AR signalling in GC closely associated with it and perhaps a continuing role for direct, AR-mediated activity within the cumulus-oocyte complex after such activity is down-regulated in the outer regions of the follicle. Importantly, some aspects of AR signalling were abnormal in CC from PCOS ovaries, which may play a role in the pathophysiology of this disorder.

Immunohistochemical studies of AR expression in human ovaries show the developmental pattern of expression is markedly different to that in most other mammalian species [302, 303, 313, 354]. In these studies, the greatest nuclear immunoreactivity for receptor protein occurs in the GC of large antral follicles, when the strong signal that appears in preantral and small antral follicles of other mammalian species is greatly diminished [305, 306, 308, 309]. Our current results and those of Chang et al [483] show that AR expression in GC that form the outer perimeter of pre-ovulatory follicles is low or completely absent after exposure to pharmacological doses of gonadotrophins. Collectively, these observations imply that developmental regulation of follicular AR expression in human follicles may be different to that which occurs in other species, although a common feature is the marked reduction of AR content by endocrine factors at a particular stage of follicular development. To our knowledge, the current study is the first to document AR expression in CC that surround the oocyte; no mention or evidence of spatial AR expression within follicular GC appears in the studies of human ovaries referenced above. Comparatively high AR expression in human CC
suggests a protective effect of proximity to the oocyte on gonadotrophin-stimulated downregulation of AR. This finding accords with those of other studies that report a gradient of AR expression from the basement membrane toward the oocyte within antral follicles of the rat [309] and pig [434] ovary. Whether oocyte paracrine factors positively up-regulate AR expression in GC has yet to be determined.

Androgens appear to have a duel role in the life history of GC that may be dependent upon relative expression levels of the AR and interaction with oocyte-derived paracrine factors. Recently, we have shown that the mitogenic and steroidogenic effects of androgen on follicular GC are determined by such interactions [457, 477], explaining, at least in part, some of the discrepancies of in vitro and in vivo androgen action on follicular cells. The modulating influence of the oocyte on steroid activity in GC also pertains to oestrogen [484] The emerging scenario in non-human animal species is one in which androgens promote GC proliferation and early differentiation when AR expression is high and cells are in close proximity to the oocyte, while low AR expression and distance from the oocyte is associated with a more luteinized GC phenotype. The results of the current study confirm that a similar association may pertain to human GC since the highly differentiated GLC had very low AR expression and in vitro DHT treatment did not influence $[^{3}\text{H}]$-thymidine uptake or progesterone secretion. Although the action of DHT was only examined over a 24-hour period, Berheisal et al [485] also report no modulation of steroidogenic output by DHT in human GLC cultured over several days. In contrast, we observed that human CC formerly attached to the oocyte in vivo maintained a degree of proliferative capacity and expressed high levels of AR mRNA and protein. Furthermore, DHT treatment enhanced growth factor-stimulated mitogenic and steroidogenic activity in these cells. As these attributes were most evident in CC with a non-luteinized morphology, it is feasible to suggest that androgens act in a similar manner on non-stimulated human GC prior to the LH surge, which down-regulates AR expression and drives luteinization. Therefore, high AR expression may correlate with a
role for androgen in stimulating proliferation and steroidogenesis in GC of all animal species, but regulation of AR expression and the specific stage in which androgens exert their influence on follicular development may differ between humans and other mammalian species.

The nuclear transcriptional regulatory factor ARA55 increases AR-mediated transcription [486] and was expressed in a positive linear relationship with AR in human GC, suggesting it may be important to AR signalling in the follicle, and that the oocyte may influence expression of multiple components involved in androgen signalling pathways. Interestingly, ARA55 [487] was first identified as a transforming growth factor β (TGFβ)-induced molecule, raising the possibility that oocyte-specific members of the TGFβ superfamily regulate its expression in follicular cells. In contrast, ARA70 did not show a GC subtype-specific pattern, indicating a different means of regulation. Expression of mRNA for ARA55 and ARA70 has been reported in human GLC from women undergoing IVF [483], and localization of ARA70 transcripts has been determined in the human ovary [488], but detection of protein in ovarian tissue has not been documented for either of these molecules.

Having characterised AR signalling in GC from normal ovaries, we wanted to determine whether such signalling is altered in GC from PCOS ovaries. Although we did not see a difference at the mRNA level, CC from PCOS ovaries contained approximately one-half the protein content measured in CC from normal ovaries. This implies that regulation of AR at the transcription level is normal, but mRNA and/or protein stability may be perturbed. Immunohistochemical studies of PCOS ovaries do not support the notion of abnormal AR expression in GC [106, 354], although neither of these studies used reliable quantitative measurements. Androgens are known to regulate AR protein levels by influencing the post-transcriptional stability of AR mRNA, but this influence can be positive or negative in a cell type dependent manner [489]. Such regulation has not been explored in ovarian cells, but a state of hyperandrogenaemia could feasibly destabilize AR mRNA in cells from PCOS
ovaries. As low AR expression is characteristic of luteinized GC, reduced AR protein in CC from PCOS ovaries may also manifest as part of the process that induces premature follicle luteinization in this disorder [195, 490]. Like AR, normal mRNA expression for AR co-regulatory molecules was observed in GC from PCOS ovaries. However, it will be interesting to determine whether ARA55 protein levels are diminished in tandem with that of AR in cells from women with this disorder.

Since the oocyte appears to promote AR expression in follicular GC, another possible explanation for reduced AR expression in cells from PCOS ovaries is lack of sufficient stimulation/protective effect from oocyte paracrine factors. Although the specific factors that are involved have yet to be identified, decreased expression of GDF9 expression has been reported in PCOS oocytes [204], and this finding gave rise to the novel hypothesis that as a consequence of insufficient oocyte signalling, follicle growth is abnormally slow, resulting in a stockpile of follicles characteristic of PCO [203]. We have previously demonstrated that androgens interact with GDF9 to potentiate porcine GC proliferation [477], and if such an interaction occurs in human GC, insufficient AR expression could feasibly hinder GC proliferation, consistent with the new “slow-growth” hypothesis for follicle excess in PCOS. This theory is supported by evidence that female mice lacking a functional AR have a deficient GC layer and reduced capacity to ovulate [329]. While AR-mediated activity is not obligatory for GC proliferation, it may be required to achieve the greatly accelerated rate of GC mitosis characteristic of a dominant follicle. Insufficient GC numbers inhibit the ability of a follicle to ovulate [249], and this characteristic has been observed in follicles of anovulatory women with PCOS [195]. Therefore, reduced AR-mediated activity could be involved in the mechanism of anovulation in PCOS. Whether this represents a primary lesion or a secondary effect needs to be addressed.

Kallikreins have been implicated in the process of folliculogenesis and ovulation in rodent species [491-493], but have not been widely explored in other animal species. Most
kallikreins are regulated by androgens, including hKLK2 and hKLK3, which are unique to humans [494]. We only observed mRNA expression of hKLK3 and this mainly occurred in CC from PCOS ovaries, despite evidence of reduced AR protein in these cells. As expression could be induced in CC from normal ovaries with a high dose of DHT, excess environmental androgen may be the major contributing factor that drives hKLK3 expression in human CC, although AR content per se did not predict the level of hKLK3 transcription among breast cancer cell lines [495], suggesting the influence of variable AR co-factor expression profiles. We attempted to measure immunoreactive hKLK3 in follicular fluid and culture media using an ultra-sensitive assay that can detect this protein in female serum [481], but this was largely unsuccessful. These findings may indicate that elevated androgen can induce transcription of this gene, but transcripts do not get translated, consistent with the fact that suppression of ovarian activity does not alter immunoreactive serum hKLK3 levels in hyperandrogenic women [496, 497]. However, transcript levels of kallikreins are also relatively low in rat GC and proteins produced are generally below the level of detection by conventional means [492]. Therefore it is premature to declare that elevated hKLK3 expression in GC from PCOS ovaries has no physiological significance.

In summary, AR signalling in human GC is enhanced by proximity to the oocyte in vivo and down-regulation of this signalling may be part of the luteinization process that occurs after the LH surge in human pre-ovulatory follicles. Cumulus GC from PCOS ovaries have reduced AR protein, and are less responsive to androgen in culture, which could be indicative of pre-mature luteinization, insufficient stimulation by oocyte-derived paracrine factors, and/or an effect of excess environmental androgen on AR mRNA stability. This abnormality may inhibit pre-ovulatory follicle growth and contribute to the incidence of anovulation in PCOS. Elevated transcripts of hKLK3 are evident in GC from women with this disorder, but whether this has pathological significance requires further investigation.
Chapter 7  FINAL DISCUSSION

The overreaching goal of this thesis was to further scientific understanding of androgen receptor (AR)-mediated androgen action in the ovary and use this understanding to investigate whether perturbation of such action is or could be involved in the pathophysiology of PCOS.

The work involved two major approaches: 1) examination of a polymorphism in the AR gene that has implications for androgen sensitivity and 2) examination of AR-mediated activity in cells from the ovary. As the chapters in this thesis were written in manuscript format, the following paragraphs will discuss the thesis work in a broad, integrative manner, adding relevant information from recent literature, and proposals for future work.

7.1  CAG repeat polymorphism in the AR gene

At the commencement of my thesis, only one study [355] had explored the association between the CAG repeat polymorphism in the AR gene and PCOS. In the interval between this publication and the one arising from my thesis [418], two other studies were published on this topic [356, 357]. At this point my research differed from the other scientific reports in that we found an association between PCOS and CAG repeats in the upper end of the normal polymorphic range (long tracts) while the other studies found no association [355, 356] or an association with repeats in the lower end of the normal range (short tracts) [357]. Since then, two more studies have been published, including a report of short CAG tracts increasing the risk of precocious puberty with subsequent ovarian hyperandrogenism [358] and another report finding no association between PCOS and this polymorphism [498]. Variability among studies is to be expected, partly because each study represents a distinct ethnic group, diagnostic criteria for PCOS differ, and the large number of polymorphic variants in the normal range necessitates large study numbers to adequately reflect normal distribution patterns for a particular ethnic population, which few studies achieve. In part, confidence in the validity of our findings comes from the fact that we had access to CAG repeat distribution
data from a very large female Australian Caucasian reference population, with which we could compare our patient and control groups. Repeat distribution in our cohort of PCOS women significantly differed from that observed in the Australia-wide control group as well as our recruited control group, suggesting that the finding has a firm foundation. A drawback of our study was the lack of comprehensive data on clinical parameters that are relevant to PCOS (other than serum testosterone levels and body mass index), which did not allow statistically reliable correlations to be made with CAG repeat number. Ideally, a much larger cohort of well described Australian Caucasian women with PCOS should be studied in order to validate the association with long CAG repeat tracts and determine the influence of this AR modulator on clinical parameters of this disorder, perhaps incorporating substratification of PCOS into distinct phenotypic groups if numbers allowed.

An association between PCOS and long CAG repeat tracts has yet to be repeated by another published study. A long polyglutamine tract in the AR protein theoretically corresponds to relatively low AR function, which at first seemed counterintuitive and difficult to explain in the context of PCOS where a state that represents increased androgen action was expected. Indeed, short polyglutamine tracts may confer increased sensitivity to androgens in normoandrogenic Asian woman, thus inducing signs of hyperandrogenaemia consistent with PCOS [357]. However, all the PCOS women in our study were hyperandrogenic, leading us to speculate that low AR-mediated activity could somehow stimulate excess androgen secretion, perhaps as a compensatory mechanism. Differential AR-mediated activity in ovarian cells due to variations in length of the polyglutamine tract was not explored in this thesis. Theca cells acquire AR immunoreactivity at the preantral follicle stage in humans [303], but little is known about AR-mediated effects on thecal cell proliferation and steroidogenesis in any animal species. Human thecal tissue is difficult to acquire and usually limited in amount, but the McAllister group have developed a long-term culture system for thecal cells [188], which may be a possible avenue to explore AR-mediated effects, including
the influence of polyglutamine tract length, on steroidogenesis. The ovarian thecal-like tumour (HOTT) cell model could also be potentially employed [499]. However, the steroidogenic modulating signals influenced by AR-mediated activity could be coming from outside the thecal compartment. Within the ovary, altered AR-mediated signalling in stromal cells, granulosa cells, or oocytes, all of which potentially secrete paracrine factors that influence thecal steroidogenesis, could be culpable. In addition, modulating endocrine factors may be involved. Although our numbers were not sufficient to make reliable statistical statements, long CAG repeat tracts tended to positively correlate with serum LH levels, suggesting an effect in the brain on gonadotrophin secretion or on endocrine molecules that control that secretion. As ever, PCOS presents a tangled web of cause and effect that is difficult to unravel.

7.1.1 Influence of X-chromosome inactivation

Another difference between our examination of the AR CAG repeat polymorphism in PCOS and that of other studies was the inclusion of X-chromosome inactivation (XCI) analysis, an epigenetic phenomenon that influences expression of the two alleles of the AR gene in females. In the study involving a general population, we found that compensating for variable allele expression in the analysis of CAG repeat distribution strengthened the association between PCOS and long CAG repeat tracts, and repeat means adjusted for specific allele expression were positively correlated with serum testosterone levels [418]. Using our method, Zitzmann et al [500] also report that XCI patterns favouring expression of alleles containing long CAG repeat tracts associate with a more androgen insensitive clinical phenotype and influence the pharmacokinetics of testosterone therapy in Kleinfelter patients, who have an XXY karyotype.

Further support for an important modulating influence of XCI on AR allele expression in PCOS was provided by our study of sister pairs with this disorder. Without taking patterns of XCI into account, no linkage to the AR gene is apparent for PCOS [157]. However, our
work demonstrates that differential XCI can account for the majority of discordance between AR genotype and PCOS phenotype in family groups, thus resurrecting the AR as a viable PCOS candidate gene and potential modulator of androgen action. Importantly, this finding highlights the need to look at both genetic and epigenetic factors when considering the cause of complex diseases, especially ones clearly conditioned by environmental factors, as is the case with PCOS. The family groups involved in this study were a subset of a much larger group of families that were involved in the PCOS candidate gene study by Urbanek et al [157]. Unfortunately, the appropriate human ethics committee denied our request to use DNA from the entire available cohort, and demanded that the family members be re-consented into the AR study. Thus, the resultant study group was relatively small, although this weakness was partly offset by the highly significant final results.

### 7.1.1.1 Target tissues

Both studies involving the CAG repeat polymorphism in this thesis were conducted using DNA from peripheral blood leucocytes, leaving the question of whether patterns of XCI in these cells has any relevance to the function of tissues known to be affected by PCOS. Examination of other cell types would be desirable, but extremely difficult to obtain from sister pairs. However, the activity of leucocytes may have some functional relevance to the pathology of PCOS. Obesity and insulin resistance are evident in women with this disorder, and the new perspective of these metabolic abnormalities as being associated with an inflammatory condition [501, 502] means that variable expression of X-linked genes in immune cells could have some significance in the pathology of PCOS. Androgen receptor expression in human leukocytes is regulated by steroids [503], although quantitative differences in leukocyte AR expression has not been studied with respect to PCOS. Leukocytes and the cytokines they secrete directly affect ovarian function [16, 504], so it is feasible that a hyperandrogenic environment could directly alter their activity in the ovary.
Examination of XCI patterns in a wider variety of tissues would also help determine when the tendency toward non-random XCI occurred in individuals. If many different tissues were similarly affected, an early developmental event that involved common precursor cells for those tissues would be likely. However, if the non-random pattern occurred only in a specific cell type, the event probably happened at a later developmental stage. Models of prenatally androgenized females show that gestational timing of the hormonal insult can incur differential PCOS symptomatic effects by altering the developmental pathway of particular cell types [420]. It is plausible therefore, that such hormonal insults could cause clonal selection of particular subsets of cells within a developing tissue that are dependent upon X-linked genes for their growth and differentiation. Currently, we are examining XCI patterns in a blood sample and cumulus cells from four separate follicles for patients with and without PCOS to determine whether patterns in blood cells are similar to those in cells from the ovary. Our preliminary results indicate that a common clonal selection process may occur more frequently in blood and cumulus cells derived from PCOS ovaries. This suggests an early developmental pressure toward inactivation of a particular X chromosome in women with this disorder.

7.2 AR-Mediated Activity in the Ovary

7.2.1 Pre-pubertal pig model

From the cell culture experiments performed in the pig model, we provided evidence for the novel concept that AR-mediated activity in follicular granulosa cells (GC) is influenced, and perhaps determined by, proximity to the oocyte. The interaction between androgen and oocyte-derived factors, including GDF9, increased the mitogenic capacity of porcine granulosa cells and thus potentially promotes early follicle growth in vivo. The mitogenic action of androgens and oocytes on follicular GC only occurred in the presence of FSH or IGF1, depending on follicle size. This requirement for FSH or IGF1 appears particular to non-
rodent species, as oocytes and GDF9 are independently mitogenic in rodent species [53]. In this respect, rodent species would not be good models to study these interactions with intent to speculate about the human situation. The interactions we observed in the porcine model could not be directly investigated in the human model, partly due to inaccessibility of oocytes and GC from non-stimulated, non-preovulatory antral follicles. However, we demonstrate that AR signalling is greater in human cumulus cells as compared to granulosa lutein cells, and androgens could only stimulate proliferation in cumulus cells in the presence of FSH or IGF1, suggesting that the interactions we observed in the pig model could occur in humans at earlier stages of folliculogenesis. We attempted to treat human cells with our original preparation of recombinant mouse GDF9, but it was cytotoxic. Future experiments with HIC-purified GDF9 are planned.

Our findings in the pig model provide a potential mechanism by which androgen treatment increases pre-ovulatory follicle numbers and ovulatory events in the pig [345, 505]. Theoretically, the excess androgen increases GDF9-stimulated GC proliferation and thereby promotes follicle growth beyond normal levels, perhaps by rescuing follicles that normally would undergo atresia for lack of sufficient growth factor stimulation. Importantly, this action could occur at the level of the granulosa cell or the oocyte due to AR expression in both cell types in pigs [305]. The addition of an AR antagonist, hydroxyflutamide, to cocultures of porcine denuded oocytes and mural GC sometimes potently inhibited the mitogenic activity of the oocytes (Appendix 2; Supplementary Figure S1), suggesting that direct androgen activity may influence the formation, secretion, and/or activation of oocyte derived mitogens. While these findings support the theory that increased follicle numbers in PCOS is caused by androgens excessively stimulating follicle growth, there is growing evidence that disputes this notion. The growth-promoting effect of androgens has yet to be validated in vitro in a human model, although an increase in developing follicle numbers occurs in human ovarian cortical tissue explanted into immunodeficient male mice [506].
Still, the issue of whether there is sufficient AR in small antral human follicles to mediate such effects remains uncertain. Indeed, stromal tissue surrounding the follicles may be the direct target of androgen action, with subsequent paracrine effects on follicular cells [231]. Also, advent of the new theory that follicle growth is retarded in PCOS due to reduced expression of GDF9 [203], throws the notion of increased follicle growth in this disorder into question. Moreover, pigs treated with excess androgen have a higher rate of ovulation [345] whereas women with PCOS tend toward anovulation. Therefore, the role of AR-mediated action in the ovary may be species specific, perhaps especially so in poly- versus mono-ovular species.

Although cross-study comparisons are not quantitative, AR immunoreactivity in human ovaries is strikingly lower than that observed in poly-ovular species such as the mouse, rat, pig and marmoset (which tend to ovulate 3 oocytes). We speculate that low AR expression in the granulosa cells of small antral human follicles (and perhaps in other mono-ovulatory species) may be a natural means of limiting the growing follicle pool. In retrospect, a mono-ovulatory species such as the cow or sheep may have been preferable to the pig model to explore AR signalling in granulosa cells with intent to draw inferences to the human situation. At commencement of this thesis, AR expression in the ovaries of these animals had not been done, and our laboratory had ongoing, funded interests in porcine reproduction that influenced the choice of animal model. Since then, in situ hybridization of AR mRNA in bovine ovaries has been reported [314], suggesting a pattern similar to that observed in the human ovary. We have done some preliminary work with sheep ovaries, and have also observed patterns of AR expression similar to human ovaries. As pre-natally androgenized sheep acquire a PCOS-like condition [420], this animal may be the preferential model for future studies. Further support for this comes from new evidence that testosterone treatment increases primary follicle numbers and reduces atresia in strips of lamb ovarian cortex explanted into fertilized chicken eggs [507]. The finding of reduced atresia is particularly
interesting as GC growth and resistance to atresia may be determined by two separate
signalling systems stimulated by different oocyte-derived molecules [508]. Therefore,
androgens could feasibly promote human follicle survival without promoting follicle growth
per se, a scenario that may be more consistent with the emerging view of slow-growth follicle
excess in PCOS. This is supported by the study of Otala et al [231], which shows that an AR-
mediated mechanism increases the in vitro survival of human ovarian cortex explants.

7.2.2 Human model

7.2.2.1 AR expression

Experiments in this thesis using human cells all represented material from the final, pre-
ovulatory stage of follicle growth, artificially stimulated with pharmacological doses of
gonadotrophins. Although immunohistochemical studies show that mural GC from large
antral human follicles have greater AR expression than small follicles, our results and those of
Chang et al [483] demonstrate that this expression is markedly reduced by an IVF
hyperstimulation protocol. Our study suggests that AR mRNA expression is normal in both
GC compartments in cells derived from PCOS ovaries, but receptor protein is reduced in
cumulus cells from these patients. As the hyperstimulation protocol usually overcomes
follicular arrest in PCOS, such treatment may mask underlying abnormalities of AR
expression in follicular cells prior to gonadotrophin exposure. Cumulus cells are further away
from the vasculature, and may be less prone to the down-regulatory effects of gonadotrophins
on AR expression, therefore reduced receptor protein in these cells in PCOS patients possibly
reflect pre-stimulation conditions. Examination of non-stimulated tissue would be required to
ascertain whether reduced AR content is confined to cumulus cells or is evident in mural
granulosa cells as well. These studies would be difficult to perform with human tissue, but
may be possible in animal models that mimic PCOS via prenatal androgenization [420].
Presumably, elevated AR in large human follicles implies that AR-mediated activity is
important at this stage of folliculogenesis, and a reduced expression may play a role in the events leading to follicular arrest in PCOS. When comparing AR mRNA expression in single dominant follicles with concentrations of various hormones in the follicular fluid of those follicles, we observed a positive correlation with oestradiol levels. As dominant follicle selection is contingent upon robust oestradiol production, this preliminary finding supports the notion that AR is up-regulated in pre-ovulatory human follicles prior to the LH surge. However, this association has yet to be experimentally confirmed.

Identifying the factors that regulate AR expression in human GC will be a future theme of investigation. We have now established that gonadotrophins directly or indirectly down-regulate this steroid receptor and that the oocyte secretes factors that either actively promote AR expression or protect it from down-regulation. Regulation of the AR gene via cAMP has already been established in other cell types [318, 319] and is likely to be the mechanism through which gonadotrophins exert their effect. Positive influence on AR expression from oocyte paracrine factors is a more novel approach. Being largely derived from the oocyte, GDF9 and GDF9b (BMP15) determine many of the differences in gene expression between cumulus and mural granulosa cells [53], and will be primary candidates for further investigation. However, AR expression in Sertoli cells, the male counterpart to granulosa cells, is activated by nuclear factor kappa beta (NF-κB) [509], perhaps via secretion of tumour necrosis factor alpha (TNFα) from round spermatids. Human oocytes secrete this cytokine [510, 511], so a common germ cell mechanism that regulates somatic cell AR expression is an intriguing possibility.

7.2.2.2 Expression of AR co-regulators

Dysregulation of co-regulatory molecules that modulate the activity of the AR can be involved in manifestation of androgen-related male diseases [512], but this has not been widely explored in female disorders associated with androgen activity. Although not formally reported in this thesis, we observed mRNA expression of a panel of AR co-regulators
including ARA24, ARA54, ARA55, ARA70, and ARA160 in human GLC, a finding formally reported by Chang et al [483]. Due to restrictions of sample material, we could only select two of these for further exploration and chose ARA70 because it has been shown to be dysregulated in ovarian cancer [488, 513] and ARA55 because it is purported to be the co-regulatory molecule most specific to the AR in prostate cancer cells (personal communication with Prof. Wayne Tilley). So far, it appears that expression of these two factors is not altered in PCOS, although this has not been confirmed at the protein level. Potential involvement of the other AR co-regulatory molecules in this disorder will be done in future experiments.

### 7.2.2.3 Expression of AR-regulated kallikreins

Examination of hKLK2 and hKLK3 expression in human GC was a novel endeavour, partly based on the fact that these were well-documented AR-regulated genes and that immunoreactive serum levels were previously shown to be elevated in hyperandrogenic women. Apart from the potential physiological role they could play in ovarian function and in the pathology of PCOS if shown to be dysregulated, we hoped they could serve as an experimental endpoint to measure relative AR-mediated activity in follicular GC. Although hKLK3 was expressed by cells from PCOS ovaries, and could be induced by a high dose of androgen in normal cells, the expression was very low, indicating that it is not a robust marker of AR-mediated transcriptional activity in our system. We did make preliminary attempts to identify other AR-regulated genes by microarray analysis using cumulus cells from individual patients, comparing cells treated with DHT to untreated cells. Unfortunately, this did not prove successful, with inconsistent results between replicates, possibly due to the variability of cumulus cell phenotypes that are present in pooled follicular cells and the variability in patient responses to the hyperstimulation protocol. Future attempts to identify AR-regulated genes in human granulosa cells will still utilize cumulus cells, as we have established that these cells have active AR signalling, but it will be important to obtain cells from a single follicle of a particular size and determine the morphology of the cumulus cells prior to the
experimental procedures to minimize the types of variability that are problematic. In addition, instead of microarray analysis, which does not definitively identify the genes directly targeted by a particular transcription factor, the newer technology of chromatin immuno-precipitation (ChIP) is preferable, whereby the AR can be cross-linked to DNA where it is bound and immuno-precipitated with specific AR antibodies. Subsequent sequencing of the precipitated DNA fragments allows identification of the genes they correspond to.

Low expression of hKLK3 in human GC does not rule out a functional role for this molecule, although our attempts to detect this serine protease in sections of ovarian tissue and in follicular fluid samples indicate that the protein level may be beyond the lower limit of detection provided by these methods. The next step in this line of investigation will be to attempt measurement of protease activity in follicular fluid samples, culture media and cell extracts. In addition, other androgen-regulated kallikreins will be investigated.

7.2.2.4 Androgen effects on progesterone secretion

The results relating to androgen effects on progesterone secretion from human cumulus cells were not focussed upon in the Chapter 6 discussion in part because these results were highly variable. Such variability is likely to be due to the degree of response to the hyperstimulation protocol [514], and highlights the problem of using this tissue as experimental material. The general observation was that DHT could enhance IGF1-stimulated progesterone secretion in normal human cumulus cells of a morphology that suggested the cells had little exposure and/or response to hyperstimulation. Alternatively, cumulus cells of a morphology that suggested at least partial luteinization had little or no steroidogenic responses to androgen, a similar response to that observed in fully luteinized mural granulosa cells. In apparently non-luteinized cumulus cells from PCOS ovaries, DHT enhanced FSH-mediated progesterone secretion, a finding that should be viewed with caution since cells from PCOS ovaries are known to have exaggerated responses to FSH in vitro while such activity is inhibited in vivo [195]. Indeed, in vivo progesterone secretion may be inhibited in PCOS, and low
progesterone in relation to oestradiol may contribute to anovulation in this disorder [515]. In our work with the pig model, testosterone and DHT could inhibit FSH-stimulated cumulus cell progesterone secretion, but only testosterone had this effect in mural granulosa cells (Appendix 2; Supplementary Figure S2). Importantly, these androgen effects could not be reversed by the AR antagonist hydroxyflutamide (Appendix 2; Supplementary Figure S3), indicating that these are non-classical actions of androgen. Such non-classical actions are likely to still involve the AR, but are independent of transcription, and probably involve MAPK signalling [342, 516]. FSH-stimulated progesterone secretion was potently inhibited by DHT in cumulus cells from some but not all patients with PCOS. Due to lack of sufficient tissue, the effects of testosterone and opposition by hydroxyflutamide could not be determined. However, others have demonstrated that aromatizable androgens inhibit progesterone secretion in non-luteinized human cumulus cells [517]. Inhibition of progesterone secretion via non-classical effects of excess aromatizable androgens may be a novel mechanistic pathway that leads to anovulation. Flutamide treatment variably influences ovulatory status in women with PCOS [379, 380, 389, 390, 518], indicating the classic action of the AR may not be involved in this process. If a non-classic mechanism were involved, it would open up the possibility of treatment with selective AR modulators (SARMs) that only interfere with non-classic signalling pathways.

7.3 Summary

In a review on PCOS by Jonard et al [183] the idea is put forward that two separate processes are perturbed in PCOS: 1) early antral follicle growth/survival and 2) dominant follicle selection. It appears that excess androgen is mechanistically linked to both processes, although the underlying mechanisms remain obscure. In light of the findings contained within this thesis, both processes could directly involve the AR, which may have a different role to play at these two distinct stages of folliculogenesis. At early stages, excess androgens potentially interact with oocyte-derived mitogens to promote abnormal follicle growth and/or
survival. This interaction could also feasibly enhance the effects of androgens on granulosa cell differentiation, and contribute to the premature luteinization characteristic of granulosa cells from PCOS ovaries. As a consequence of the advanced luteinization process, AR may be down-regulated at a time when it is normally up-regulated in the human follicle. This down-regulation potentially disrupts dominant follicle selection and contributes to follicular arrest. The concept of diminished AR-mediated activity being involved in the pathophysiology of PCOS is consistent with our finding of an association between PCOS and long CAG repeat tracts in the AR gene, which is another potential indication of reduced AR-mediated activity, and may occur in a subset of women with this disorder.

Collectively, the work contained within this thesis supports both of the initial hypotheses proposed by this thesis: 1) that the AR (CAG)n polymorphism is involved in the manifestation of PCOS and 2) that AR-mediated signalling is altered in granulosa cells from PCOS ovaries. Interestingly, the results indicate that such signalling may influence PCOS in a way not previously anticipated, and is deserving of further investigative attention.

7.4 Addendum

Just prior to submission of this thesis for print, a new study involving another AR knockout mouse has appeared [519], which differs from the previous model [329], and has significant implications for the findings reported in this thesis and subsequent future directions of this work. The difference between the two models is that the first model only ablated the classic function of AR by deleting a portion of the gene that is necessary to bind DNA, whereas the second model completely ablated transcription of the AR gene by removing the methionine transcriptional start site. Presumably, the first model still produces AR (not determined by the study), albeit one that is crippled, but the second model definitively contains no AR protein whatsoever. The major finding of the new model is premature ovarian failure in homozygous knockout animals, with progressive loss of developing follicle numbers coincident with increasing rates of follicular atresia until follicle development ceases at 40 weeks of age. The
authors further their investigation by doing microarray analysis of gene expression in 3- and 8-week-old ovaries, demonstrating that a primary event in the sequence of events that lead to ovarian dysfunction is the down-regulation of kit ligand (KITL) expression. This event precedes down-regulation of GDF9 and BMP15 in the ovaries of 8-week-old animals, and was proven to be an AR-mediated event, whereas this receptor was not directly involved in the down-regulation of GDF9 and BMP15. A classic paracrine relationship exists between follicular granulosa cells and the oocyte that involves secretion of KITL from granulosa cells and expression of its receptor on the oocyte (review: [520]), but to date regulation of GDF9 and/or BMP15 by this paracrine interaction has not been explored. Regulation of KITL by androgens via an AR-mediated mechanism could feasibly provide a mechanistic explanation for the interactions we observed in co-cultures of porcine granulosa cells and denuded oocytes. Furthermore, up-regulation of KITL in a hyperandrogenic environment could have impact on fetal and adult ovarian function in a manner conducive to the development of PCOS, as this growth factor is involved in oocyte survival during ovarian organogenesis and is necessary for follicles to enter the primary growth phase throughout the reproductive lifespan [520]. Most studies involving KITL expression and activity have been done in mice and sheep, leaving broad scope for future work involving human tissues, and laying the framework for further studies in sheep, using prenatal androgen exposure as a model for PCOS.

7.5 Future Studies

Future work mentioned throughout this chapter, is briefly summarised in the following sections.

7.5.1 The AR (CAG)n polymorphism and the influence of XCI in PCOS

A collaboration with Dr Grant Townsend’s group at University of Adelaide School of Dentistry has been initiated, in which a large cohort of twins used for a previous study of X-
linked genes in tooth development will be contacted after obtaining appropriate ethics committee approval, with intent to find monozygotic sister pairs with evidence of PCOS. Study of such pairs will be important to verify whether differential XCI is evident in twins discordant for the disorder. In addition, a potential collaboration with investigators in England is being considered in which we propose to do a replicate study of XCI in family groups with PCOS. Finally, experiments are being performed comparing XCI patterns in blood and ovarian cells from women with and without PCOS.

7.5.2 AR-mediated activity in the ovary and its implications for PCOS

7.5.2.1 Androgen regulation of KITL

In light of the recent finding that KITL is directly regulated by androgens, and that up-regulation of this growth factor may be involved in the pathophysiology of PCOS, a series of experiments looking at expression of this gene in granulosa cells from normal and PCOS ovaries is planned. It would also be highly desirable, if possible, to establish collaboration with researchers who are working with models of PCOS that involve prenatal androgen exposure, to explore via immunohistochemistry the expression patterns of KITL in the ovaries of normal and androgen-exposed animals.

7.5.2.2 Regulation of AR expression in the ovary

Experiments examining the regulation of AR expression in human granulosa cells are ongoing. Potent down-regulation of AR mRNA expression in human cumulus cells by cAMP has already been confirmed in our laboratory, an effect not influenced by the additional presence of oestradiol. The next series of experiments will attempt to reverse this down-regulation by addition of denuded oocytes from other animal species or addition of known oocyte-secreted factors to the culture media, including GDF9, BMP15, and TNFα.
7.5.2.3 Non-genomic actions of androgens on granulosa cell steroidogenesis

Preliminary work examining potential non-genomic actions of androgens on human granulosa cell steroidogenesis has been done using the human granulosa tumour cell line, KGN, confirming that it is a suitable model to pursue this avenue of investigation. Future experiments will explore the effect of a wide range of androgens and selective AR modulators (SARMs), alone and in combination, on progesterone synthesis by KGN. Involvement of the intracellular AR, acting in transcription-dependent and/or transcription-independent ways, will be addressed as well as whether a membrane-bound androgen binding receptor can be detected and implicated in the action of androgens on these cells.

7.5.2.4 Altered AR-mediated signalling in PCOS

The first experiments in the continuation of this work will involve quantification of ARA55 protein in GC from normal and PCOS ovaries to determine whether protein content reflects our findings at the mRNA level. In addition, provided more tissue can be obtained, examination of a panel of AR co-activators in human GC will be done to ascertain whether they are differentially expressed between GC subtypes and between women with and without PCOS. A broader perspective on AR-regulated ovarian kallikreins will also be explored, along with their potential implications for this disorder.
Chapter 8 REFERENCES


41. Gonzalez-Robayna IJ, Falender AE, Ochsner S, Firestone GL, Richards JS. Follicle-Stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-Induced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells. Mol Endocrinol 2000; 14: 1283-1300.


74. Armstrong DT, Marrocco DL, Hickey TE, Gilchrist RB. Oocyte-secreted factor(s) augment mitogenic responses of porcine granulosa cells to insulin-
like growth factor-1 and 5-alpha-dihydrotestosterone. In: Endocrine Society; 2004; New Orleans, USA.


183. Jonard S, Dewailly D. The follicular excess in polycystic ovaries, due to intra-ovarian hyperandrogenism, may be the main culprit for the follicular arrest. Hum Reprod Update 2004; 10: 107-117.


208. Glister C, Richards SL, Knight PG. Bone morphogenetic proteins (BMP) -4, -6, and -7 potently suppress basal and luteinizing hormone-induced androgen production by bovine theca interna cells in primary culture: could ovarian hyperandrogenic dysfunction be caused by a defect in thecal BMP signaling? Endocrinology 2005; 146: 1883-1892.


244. Willis DS, Mason HD, Watson H, Franks S. Developmentally regulated responses of human granulosa cells to insulin-like growth factors (IGFs): IGF-


276. Spurdle AB, Dite GS, Chen X, Mayne CJ, Southey MC, Batten LE, Chy H, Trute L, McCredie MR, Giles GG, Armes J, Venter DJ, Hopper JL, Chenevix-


305. Cardenas H, Pope WF. Androgen receptor and follicle-stimulating hormone receptor in the pig ovary during the follicular phase of the estrous cycle. Mol Reprod Dev 2002; 62: 92-98.


358. Ibanez L, Ong KK, Mongan N, Jaaskelainen J, Marcos MV, Hughes IA, De Zegher F, Dunger DB. Androgen receptor gene CAG repeat polymorphism in
...the development of ovarian hyperandrogenism. J Clin Endocrinol Metab 2003; 88: 3333-3338.


381. Vrbikova J, Hill M, Dvorakova K, Stanicka S, Vondra K, Starka L. Flutamide suppresses adrenal steroidogenesis but has no effect on insulin resistance and secretion and lipid levels in overweight women with polycystic ovary syndrome. Gynecol Obstet Invest 2004; 58: 36-41.


393. Ibanez L, de Zegher F. Low-dose combination of flutamide, metformin and an oral contraceptive for non-obese, young women with polycystic ovary syndrome. Hum Reprod 2003; 18: 57-60.


395. Ibanez L, de Zegher F. Flutamide-metformin plus ethinylestradiol-drospirenone for lipolysis and antiatherogenesis in young women with ovarian hyperandrogenism: the key role of metformin at the start and after more than one year of therapy. J Clin Endocrinol Metab 2005; 90: 39-43.


469. Roy SK. Regulation of transforming growth factor-beta-receptor type I and type II messenger ribonucleic acid expression in the hamster ovary by gonadotropins and steroid hormones. Biol Reprod 2000; 62: 1858-1865.
483. Chang SY, Kang HY, Lan KC, Chang CY, Huang FJ, Tsai MY, Huang KE. Expression of steroid receptors, their cofactors, and aromatase in human


Chapter 9  APPENDIX 1

9.1 Details of Publications

9.1.1 Chapter 2: The androgen receptor CAG repeat polymorphism and X-chromosome inactivation in Australian Caucasian women with infertility related to polycystic ovary syndrome

9.1.1.1 Journal, authors and author affiliations

Journal of Clinical Endocrinology and Metabolism

T. Hickey, A. Chandy and R. Norman

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9.1.1.2 Author contributions

1. T. Hickey: Study theory and design, experimental procedures, patient recruitment, data representation and analyses, manuscript preparation.

2. A. Chandy: Patient recruitment

3. R. Norman: Supervisor, manuscript review

9.1.2 Chapter 3: Epigenetic modification of the X chromosome influences susceptibility to polycystic ovary syndrome.

9.1.2.1 Journal, authors and author affiliations

Journal of Clinical Endocrinology and Metabolism

Hickey TE\textsuperscript{1}, Legro RS\textsuperscript{2}, and Norman RJ\textsuperscript{1}.

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9.1.2.2 Author contributions

1. TE Hickey designed the study, analysed and interpreted data, and prepared the manuscript
2. RS Legro consented and diagnosed patients, and reviewed the manuscript
3. RJ Norman reviewed the manuscript.

9.1.2.3 Acknowledgements

The authors would like to acknowledge the role of Barbara Scheetz in the recruitment, consent, and delivery of DNA samples, Clyde Milner for technical assistance, and Lisa Yelland, Department of Public Health, University of Adelaide, for performing the statistical analysis.

9.1.3 Chapter 4: Interactions between androgen and growth factors in granulosa cell subtypes of porcine antral follicles.

9.1.3.1 Journal, authors and author affiliations

Biology of Reproduction
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9.1.3.2 Author contributions

1. TE Hickey: Study theory and design, experimental procedures, data representation and analysis, manuscript preparation
2. DL Marrocco: Technical assistance
3. RB Gilchrist: Study design, manuscript review
4. RJ Norman: Supervisor
5. DT Armstrong: Supervisor, manuscript review
9.1.3.3 Acknowledgements

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9.1.4 Chapter 5: Androgens augment the mitogenic effects of oocyte secreted factors and growth differentiation factor 9 on porcine granulosa cells

9.1.4.1 Journal, authors and author affiliations

Biology of Reproduction

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9.1.4.2 Author contributions

1. TE Hickey: Study theory and design, experimental procedures, data representation and analysis, manuscript preparation
2. DL Marrocco: Technical assistance
3. F Amato: Assistance in purification of recombinant GDF9
4. LJ Ritter: Technical assistance
5. RB Gilchrist: Supervision, manuscript review
6. DT Armstrong: Supervision, manuscript review

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9.1.5 Chapter 6: Androgen receptor signalling in granulosa cell subtypes from normal and polycystic ovaries

9.1.5.1 Publication comments

This chapter has yet to be honed for publication, and a particular journal and the final list of authors has yet to be defined. The author of this thesis designed and carried out the study in full. Lisa Yelland, Dept. of Public Health, University of Adelaide, performed some of the statistical analyses.
Chapter 10  APPENDIX 2

10.1 Supplementary Figures
Figure S1: Effect of the androgen receptor antagonist hydroxyflutamide (O HF) and denuded oocytes (DO) on DNA synthesis in gilt mural granulosa cells from 1-3 mm follicles. Cells were isolated and cultured as described in Chapter 5. Bars represent the mean (± SD) of raw data from one experiment in which each treatment was performed in triplicate. As expected, DO potently stimulated DNA synthesis. Addition of OHF significantly inhibited this stimulation. *p<0.0001 as compared to treatment with no OHF.
Figure S2: Effects of testosterone and dihydrotestosterone (DHT) on (A) DNA synthesis and (B) progesterone secretion in gilt mural granulosa cells isolated from 3-5mm follicles. Cells were isolated and cultured as described in Chapter 4. Bars represent the mean (± SEM) of relative data from 3 replicate experiments in which each treatment was performed in triplicate. Neither androgen affected DNA synthesis, but testosterone significantly inhibited progesterone secretion. *p<0.01 as compared to treatment with no androgen.
Figure S3: Effects of testosterone and the androgen receptor antagonist hydroxyflutamide (OHF) on progesterone secretion from gilt mural granulosa cells isolated from 3-5mm follicles. Cells were isolated and cultured as described in Chapter 4. Bars represent the mean (± SEM) of raw data from 3 replicate experiments in which each treatment was performed in triplicate. As expected, testosterone inhibited basal and FSH-stimulated secretion but OHF did not reverse this effect. *p<0.05 as compared to treatment with no androgen.