Androgen synthesis, metabolism and action in the developing ovarian follicle

by

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Abstract

The ovarian follicle is the major site of sex steroid production in females, hormones that are crucially required for normal ovarian function and female reproduction. The biosynthesis and metabolism of androgens by individual follicles throughout development has been difficult to study because androgens and androgen precursors are structurally similar molecules present in low concentrations. Androgens have dual dose-dependent effects on folliculogenesis, with reduced or exaggerated levels of androgens being deleterious for follicle development. Few studies have shown the whole spectrum of androgen action using a single experimental model.

Here we employed tandem mass spectrometry to measure sex steroid production by murine follicles, cultured in an alginate encapsulated, 3-dimensional model. We showed developmental stage-dependant FSH-stimulated androgen and oestrogen secretion. When follicles were cultured in the presence of non-aromatisable 5α-dihydrotestosterone, endogenous androgen production decreased. Following exposure to the universal sex steroid precursor dehydroepiandrosterone, high androgen generation was achieved by immature follicles. We described androgen receptor-mediated growth-promoting effects of androgen supplementation in developing follicles. However, when androgen exposure was gradually increased, we first observed suppression of oocyte development, followed by stagnation of follicle growth.

These data provide the rationale for androgen treatment in women with low ovarian reserve, but call for caution as over-replacement might cause harm to oocyte quality. Furthermore, this study describes mechanisms which might contribute to the follicular developmental arrest that is observed in androgen excess conditions such as polycystic ovary syndrome.

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I would like to dedicate this thesis to my husband Charles-Emmanuel and to our sons Augustin, Baudouin, Grégoire, Ambroise and Adrien. Without Charles-Emmanuel's love and support, his enthusiasm and confidence in this work, none of this would have been possible!

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Abbreviations

17β-HSD	17β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
2D	2-dimensional
3D	3-dimensional
ACTH	adrenocorticotrophic hormone
A'dione	androstenedione
AKR1C3	aldoketoreductase type 3
AMH	anti-Müllerian hormone
AMH-R2	anti-Müllerian hormone receptor 2
APS	autoimmune polyendocrine syndrome
AR	androgen receptor
ART	assisted reproductive techniques
ARE	androgen response elements
ARKO	androgen receptor knock-out
ATP	adenosine triphosphate
BMP15	bone morphogenetic factor 15
cDNA	complementary deoxyribonucleic acid
CO2	carbondioxide
CRP	c-reactive protein
CYP11A1	cytochrome P450 11A1
CYP17A1	cytochrome P450 17A1
CYP19A1	cytochrome P450 17A1 or placental aromatase
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DHT	5α-dihydrotestosterone
DNA	deoxyribonucleic acid
E1	oestrone
E2	oestradiol
EGF	epidermal growth factor
eIVFG	encapsulated *in vitro* follicle growth
FGF	fibroblast growth factor
FOXO3	forkhead box O3
FSH	follicle-stimulating hormone
FSH-R	follicle-stimulating hormone receptor
GC-MS	gas chromatography mass spectrometry
GDF9	growth differentiation factor 9
GnRH	gonadotrophin-releasing hormone
HCG	human chorionic gonadotrophin
HDL	high-density lipoprotein
HPLC	high performance liquid chromatography
HSD	hydroxyl steroid dehydrogenases
IBMX	phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine
ICSI	intracytoplasmatic sperm injection
IGF-1	insulin-like growth factor-1
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
LC-MS/MS	liquid chromatography tandem mass spectrometry
LDL	low-density lipoprotein
LH	luteinising hormone
LH-R	luteinising hormone receptor
LOR	low ovarian reserve
mRNA	messenger ribonucleic acid
MTBE	tert-butyl-methyl-ether
NADPH	nicotinamide adenine dinucleotide phosphate
OC	oral contraceptive
OHSS	ovarian hyperstimulation syndrome
P4	progesterone

P450scc	cholesterol side-chain cleavage enzyme
PAI	primary adrenal insufficiency
PCO	polycystic ovaries
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
PMSG	pregnant mare serum gonadotrophin
POMC	proopiomelanocortin
POF	premature ovarian failure
Prog	progesterone
PTEN	phosphatase and tension homolog
rFSH	recombinant follicle-stimulating hormone
RIA	radioimmunoassay
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
SD	standard deviation
SF1	steroidogenic factor 1
SHBG	sex hormone binding globulin
StAR	steroidogenesis acute regulatory protein
STS	steroid sulfatase deficiency
SULT	sulfotransferase
T	testosterone
TGF	tumor growth factor
VEGF	vascular endothelial growth factor
WSD	Western-style diet
Δct	delta cycle threshold

1 General Introduction

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M.Lebbe, T.K.Woodruff.

Molecular Human Reproduction, 2013, 19(12): 828-837.

1.1 Androgen synthesis and metabolism in females

1.1.1 Androgen biosynthesis

In women, circulating androgens are mainly derived from the adrenal glands and the ovary. The universal sex steroid precursor dehydroepiandrosterone (DHEA) and its sulphated ester DHEAS originate mostly from the adrenal gland (Arlt, 2006). The conjugated DHEAS, the inactive storage form, is the most abundant steroid hormone in the human circulation (Labrie, 2010). In the adrenal, ovary and peripheral target cells, DHEA is downstream metabolized to androstenedione and testosterone, and both can be aromatized into oestrogens (Longcope, 1996). Testosterone can further be converted to the much more potent 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase (Arlt, 2006). The increased androgenic potency of DHT is attributed to its stronger interaction with the androgen receptor (AR), compared with testosterone (Grino et al., 1990). The peripheral target cells of sex steroid action, such as skin, liver, fat, muscle, breast and brain cells, readily take up non-conjugated, lipophilic DHEA (Labrie, 2010). The tissue-specific downstream metabolism of DHEA depends on the intracellular availability and activity of steroidogenic enzymes (Arlt et al., 1998). This means that circulating levels of androgens and their precursors do not necessarily reflect target cell concentrations (Lebbe M et al, 2012).

1.1.1.1 Adrenal androgen synthesis

The adrenals are small yellowish glands located at the upper pole of the kidneys. The adrenal gland consists of an outer cortex and an inner medulla and is responsible for secretion of the adrenocortical steroids and catecholamines respectively (Nussey and Whitehead, 2001). The adrenal cortex is divided in three separate zones, from outside to inside: the zona

glomerulosa, producing mineralocorticoids (principally aldosterone), the zona fasciculata, producing glucocorticoids (dominantly cortisol in humans, corticosterone in rodents) and the zona reticularis, the site of adrenal androgen synthesis in humans (mainly DHEA) (Figure 1-1).

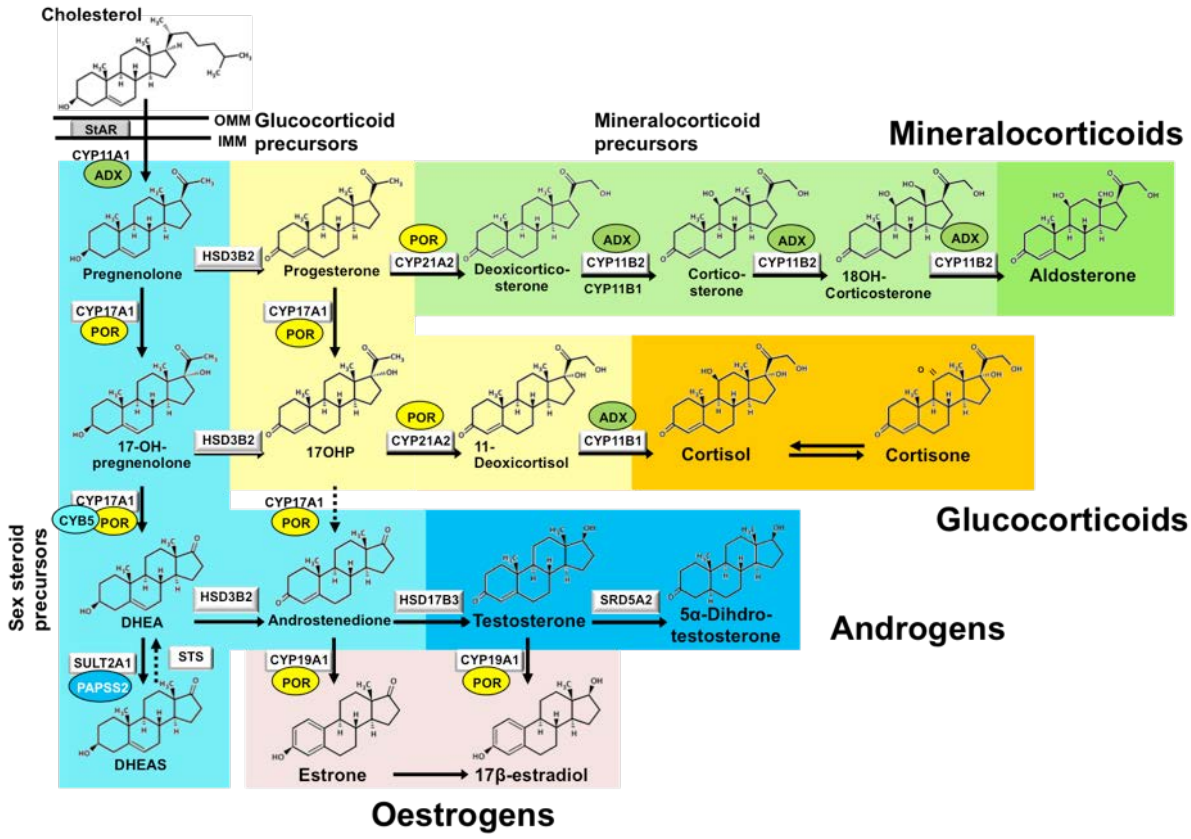


Figure 1-1 Steroidogenesis in the adrenal glands, divided in the zona glomerulosa (green), fasciculata (yellow), and reticularis (blue), with synthesis of mineralocorticoids, glucocorticoids and adrenal androgens respectively. DHEA is taken up by peripheral tissues, and metabolised to active androgens (dark blue) and oestrogens (pink). Reprinted with kind permission of Prof. Wiebke Arlt, University of Birmingham

All biologically active steroids are derived from cholesterol, that is delivered to the inner compartment of the mitochondria by steroidogenic acute regulatory protein (StAR), enabling CYP11A1 (Cytochrome P450 enzyme 11A1) or P450scc (cholesterol side-chain cleavage

enzyme)-mediated conversion to pregnenolone in a first rate-limiting step (Miller and Auchus, 2011) (Figure 1-1). The P450 enzymes, members of the heme-containing protein superfamily, and contributing to steroid hormone production are membrane-bound to the mitochondria (CYP11A) or the endoplasmatic reticulum (CYP17A1 and CYP19A1) (Payne and Hales, 2004). The CYP enzymes catalyze selective irreversible reactions in steroidogenic cells, by hydroxylation and cleavage of the steroid substrate, utilizing nicotinamide adenine dinucleotide phosphate (NADPH) as electron donor. The weak precursor hormones are stepwise converted to potent bioactive hormones via CYP enzymes, which catalyze unidirectional reactions, or hydroxysteroid dehydrogenases (HSD), which can act in a bidirectional way. HSD and 5α-reductase are found in peripheral target tissues of steroid action (Miller and Auchus, 2011).

In humans, the adrenal zona reticularis has a pronounced CYP17A1 activity, and abundant DHEA is produced, most of it being sulfated to DHEAS by SULT2A1 (sulfotransferase 2A1), and small amounts converted to androstenedione by 3β-HSD2 (Idkowiak et al., 2011) (Figure 1-1) In contrast, the mouse adrenal gland does not express CYP17A1 (Perkins and Payne, 1988), and does not produce DHEA; corticosterone is the main glucocorticoid in rodents (Brock and Waterman, 1999).

1.1.1.2 Ovarian steroidogenesis

The ovaries are bilateral, white, oval-shaped glands, localized in the posterior wall of the broad ligament in the supra-pelvic peritoneal cavity (Nussey and Whitehead, 2001). The ovarian follicle, the basic structure of the ovary, contains an oocyte surrounded by specialized endocrine cells (inner granulosa and outer theca cells, separated by a basal membrane) and is the other main site of androgen production in women. Androgens are the mandatory precursors of oestrogen synthesis, the main sex steroid in females. Ovarian steroid hormone secretion

occurs in a cyclical pattern, with estradiol secretion during the follicular phase and progesterone production during the luteal phase of the menstrual cycle. Testosterone levels modestly increase at midcycle in women (Braunstein et al., 2011)

From the 1960s onward, the idea arose that follicular oestrogen secretion results from a collaboration between granulosa and theca cells, named as 'two cell, two gonadotropin' theory (Bjersing and Carstensen, 1967). In this concept of follicular steroidogenesis, the theca cells produce androgens, under stimulation of Luteinizing Hormone (LH), which induces the crucial androgen-synthetizing enzyme CYP17A1, that is solely expressed in theca cells (Fortune and Armstrong, 1977, Liu and Hsueh, 1986) (Figure 1-2). The main androgen produced in women is androstenedione, from which small amounts are converted to testosterone in the theca cells via 17β-HSD1 (Figure 1-2). Most of the androstenedione diffuse across the basal lamina to the granulosa cells, which express Follicle Stimulating Hormone (FSH) receptors on the cell membrane, inducing aromatase enzyme (CYP19A1) expression (Figure 1-2). Aromatase converts both androstenedione to estrone, and testosterone to estadiol (Dorrington et al., 1975, Makris and Ryan, 1977). Estone is converted to estradiol, the main oestrogen in the circulation, via 17β-HSD1 (Figure 1-2). During follicle maturation, theca and granulosa cells become progressively more active in the production of steroid hormones, under the influence of FSH and LH. Circulating androgens and oestrogens, in turn, provide feedback regulation to the hypothalamic-pituitary axis, thereby adjusting hypothalamic gonadotrophin-releasing hormone (GnRH) production and pituitary LH and FSH release, allowing for continuous monitoring of sex steroid hormone concentrations (Figure 1-3).

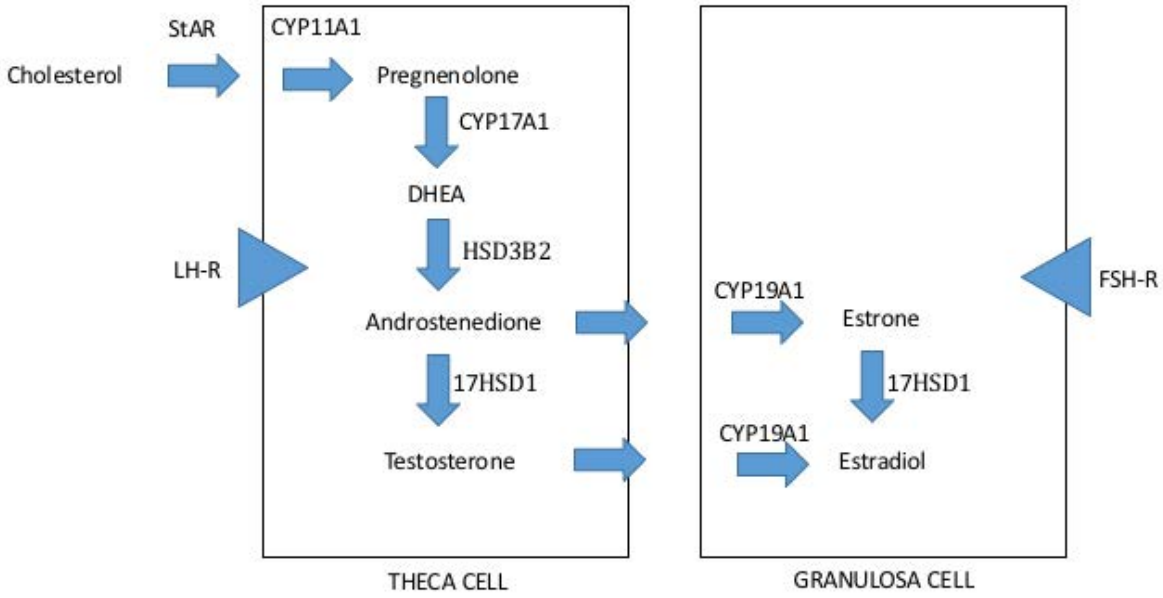


Figure 1-2 Two cell, two gonadotropin concept of oestrogen synthesis in the follicle focusing on steroidogenic enzyme expression in granulosa and theca cells: steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage cytochrome P450 (CYP11A1), 17-hydroxylase (CYP17A1), 3β-hydroxysteroid dehydrogenase (HSD3B2) and 17β-hydroxysteroid dehydrogenase (17HSD1). CYP17A1 expression is induced solely in theca cells under the influence of LH and CYP19A1 (aromatase) expression is found uniquely in granulosa cells, stimulated by FSH.

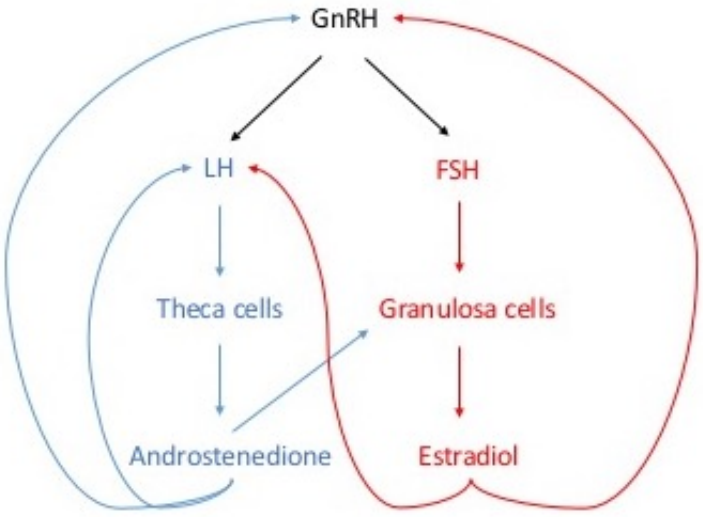


Figure 1-3 Two cell, two gonadotrophin hypothesis and neuro-endocrine axis. Pituitary LH stimulates theca cells to produce androstenedione, that diffuses to the granulosa cells for conversion to estradiol under FSH-stimulation. The sex steroids feedback to the hypothalamo-pituitary unit to regulate gonadotrophin releasing hormone (GnRH) and LH secretion.

1.1.1.3 Androgen levels in the female lifespan

Circulating androgen levels fluctuate with age. Immediately after birth, levels of DHEA and DHEAS show a transient peak, reflecting fetal adrenal androgen production, that rapidly disappears (Arlt, 2004). Around the age of 8 years, DHEA is increasingly produced in the adrenal gland, and converted to active androgens in androgen-responsive hair follicles, progressively manifesting with appearance of pubic hair, a phenomenon called adrenarche (Idkowiak et al., 2011). Serum levels of DHEAS, androstenedione and testosterone increase and peak in the early reproductive years, followed by a progressive decline with advancing age, as illustrated by a cross-sectional study of 1423 healthy women (Davison et al., 2005). The high inter-individual variations in circulating DHEAS levels and their decrease over time reflect a genetic component in androgen synthesis (Orentreich et al., 1984). (Figure 1-4). A large study of serum testosterone and DHT, measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) in 985 women in the third to ninth decade, showed overall lower values for testosterone compared with previous measurements by immunoassays, especially for the lower concentrations (Shiraishi et al., 2008) Androstenedione levels also decrease with advancing reproductive age, as shown in a recent study measuring these androgen levels by LC-MS/MS in a cohort of 995 women aged 20-80 years (Haring et al., 2012) Menopause refers to the cessation of oestrogen production by the ovary, but the postmenopausal ovary continues to secrete androgens, as a result of the increased LH drive (Adashi, 1994). This means that

menopause does not significantly affect circulating androgen levels (Burger et al., 2000). At the end of life, circulating DHEAS levels have dropped to 10-20% of young adult levels, a phenomenon sometimes called ‘adrenopause’, which only refers to the decline in adrenal androgen secretion, as glucocorticoid and mineralocorticoid secretion are maintained with advancing age (Lebbe M et al, 2012).

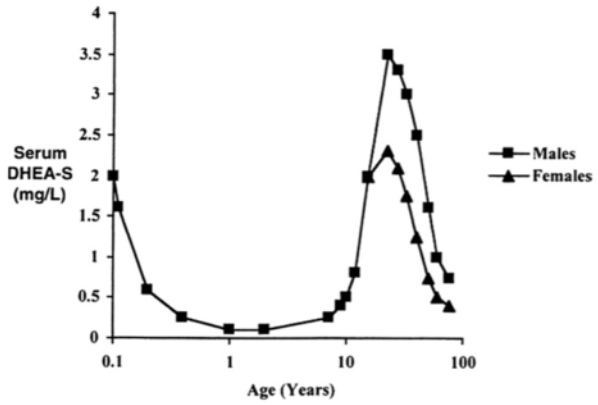


Figure 1-4 Concentrations of DHEAS as function of age. Derived from data in Orentreich et al, 1984. Reprinted from Miller and Auchus with kind permission of Endocrine Society, Copyright 2011.

1.1.2 Androgen metabolism in peripheral target tissues

Androgen metabolism refers to the tissue-specific generation of androgens, derived from circulatory DHEA, and depending on the expression and the activity of peripheral steroidogenic enzymes, including isoforms of 3β-HSD, 17β-HSD and 5α-reductase, in the specific target tissue (Labrie, 2010) (Figure 1-5).

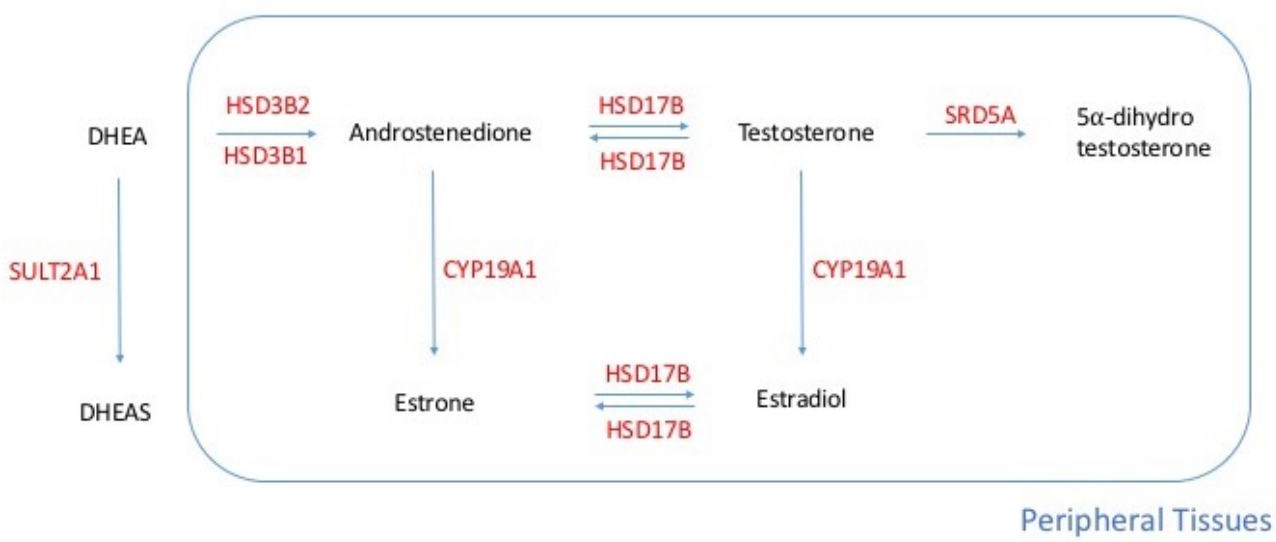


Figure 1-5 Peripheral sex steroid metabolism. Circulatory DHEA is taken up by peripheral tissues and converted to androstenedione, which can be either converted to testosterone or aromatized to estrone (E1). Testosterone can be metabolized to the more potent dihydrotestoserone (DHT) or aromatized to estradiol (E2). The enzymes responsible for these reactions are mentioned in red.

The HSD enzymes include the 3β-HSDs and 17β-HSDs and belong to the dehydrogenase reductase superfamily. They regulate steroid potency by catalyzing the conversion of keto- and hydroxysteroid pairs using the pyridine nucleotide cofactors NAD(P)H/NAD(P)$^+$ (Miller and Auchus, 2011) (Figure 1-6). Their physiological role is the biosynthesis versus inactivation of androgens and oestrogens in peripheral tissues.

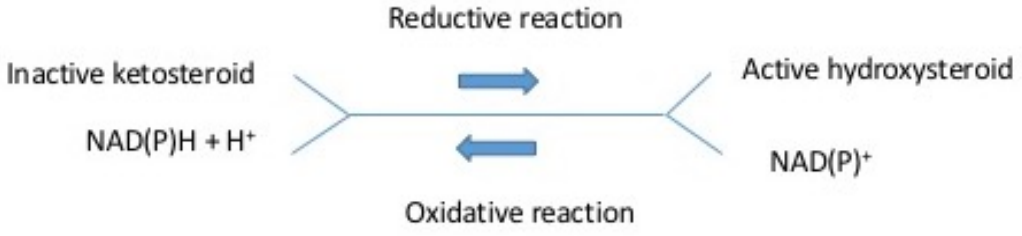


Figure 1-6 HSDs interconvert inactive and active steroids. The direction of the reaction in vivo depends mainly on the availability of the pyridine nucleodide NAD(P)H/NAD(P)+ cofactor.

3β-HSD/isomerases are bound to mitochondrial or microsomal membranes, depending on the cell type in which they are expressed (Payne and Hales, 2004). Multiple isoforms of 3β-HSD have been isolated in human (Simard et al., 1996) and mouse (Payne et al., 1997). The different isoforms are chronologically numbered, which means that the same number in a different species is not orthologous (Payne and Hales, 2004, Rasmussen et al., 2013). Six isoforms of 3β-HSD are discovered in mouse and 5 in humans. Human 3β-HSD I and II and mouse 3β-HSD I and VI are involved in the biosynthesis of active sex steroid hormones, and some of their characteristics have been outlined in Table 1-1 (based on (Payne and Hales, 2004)).

Table 1-1 Characteristics of human and mouse 3β-HSD involved in active biosynthesis of steroid hormones

Human Gene	Mouse Gene	Human Protein	Mouse Protein	Reaction catalyzed	Tissue distribution
HSD3B1	Hsd3b6	3β-HSD I	3β-HSD VI	Preg -> Prog DHEA ->A'dione	Placenta, Uterus Skin
HSD3B2	Hsd3b1	3β-HSD II	3β-HSD I	Preg -> Prog DHEA ->A'dione	Ovary,Testis, Adrenal

Abbreviations: Preg (pregnenolone), Prog (progesterone), DHEA (dehydroepiandrosterone), A'dione (androstenedione).

17β-HSD are membrane bound or soluble enzymes, catalyzing the final step in sex steroid biosynthesis by interconverting androstenedione and testosterone, and estrone and estradiol. Multiple 17β-HSD isoforms are identified, and the nomenclature for these enzymes has assigned orthologs for the same number among species (Adamski and Jakob, 2001). 17β-HSD isoforms 1, 3 and 7 are predominant in gonadal tissue. Human 17β-HSD1 has substrate specificity for estrone, while mouse 17β-HSD1 also efficiently converts androstenedione to testosterone (Puranen et al., 1997). 17β-HSD5 account for most of the extra-gonadal peripheral reduction of androstenedione, and is also called AKR1C3 (aldoketoreductase 1C3) (Miller and Auchus, 2011).

5α-reductases perform irreversible transformations, and activate testosterone to DHT, which is the most active androgen in skin, vulva and clitoris (Arlt et al., 1998). Type 1 5α-reductase is found in skin, liver, adrenal and ovaries while the type 2 isoform is confined to genital skin and prostate (Jenkins et al., 1992).

1.1.3 Measuring androgen levels

The access to a sensitive, specific and reproducible assay for the measurement of androgen levels is challenging, particularly in the case of low sample concentrations and in the presence of other steroids with closely related structures which may cross-react the assay (Rosner et al., 2010). For the past five decades, immunoassay has been the routine assessment for androgen concentrations, and for steroid hormones in general. A great deal of our knowledge about the role of sex steroid hormones in normal cycling women through the reproductive lifespan (puberty, menstrual cycle, pregnancy, menopause) and endocrine-related diseases is based on studies using these techniques (Lebbe M et al, 2012). Mass spectrometry-

based assay methods have been used since 1960 for the measurement of steroid hormones, and have recently started to replace immunoassays for steroid quantification in larger clinical and research laboratories (Rosner et al., 2007). This section aims to introduce both assays, and discuss the main applications, advantages and disadvantages.

1.1.3.1 Immunoassays for steroid hormone measurements

Antibodies were first developed for large proteins, such as insulin, growth hormone, LH and FSH, which provoke immunogenic reactions when administered to experimental animals, f.ex. rabbits or mice (Yalow and Berson, 1959). Steroids are smaller molecules, and require coupling to a protein, such as albumin or thyroglobulin, to evoke antibody generation (Abraham, 1969). Steroid hormone measurement using immunoassays are subject to different issues, which might have negative effects on the sensitivity and specificity of the assay. Steroids have low affinity for the employed antibodies, and are generally present in low concentration in biological samples. Moreover, the presence of steroid molecules with similar structure induces risks of cross-reactivity (Taylor et al., 2015). The immunoassays utilizing pre-assessment extraction with organic solvent (liquid-liquid extraction) and chromatographic fractioning have shown to perform with good sensitivity and specificity (Janse et al., 2011). The more recent 'direct' assays have suppressed the sample purification steps, and run on automated platforms, using chemiluminescence, fluorescence or enzymatic color reactions as detection methods (Taylor et al., 2015). Although the development of monoclonal antibodies has improved the analytical performance, a lower sensitivity and specificity is often achieved. (Stanczyk, 2006). The advantages of the automated assays are their wide availability, reduced cost and ease of use. Standardization of specific assays is difficult, because of differences in antibodies and kit formulations purchased from different suppliers (Taylor et al., 2015).

1.1.3.2 Mass spectrometry-based steroid hormone measurement

The mass spectrometer is a device that measures the mass-charge ratio of charged particles (m/Q). It is composed of a sample inlet device, an ionization source, an analyzer and an ion detector. The initial steroid mixture is first separated using chromatography, either gas chromatography (GC) or liquid chromatography (LC).

GC-MS is employed to analyze the metabolites of steroid hormones and precursors. GC vaporizes liquid analytes, and separates them in a heated column based on different speed resulting from different interactions with the liquid medium in the column. Since steroid metabolites present as glucuronide and sulfate conjugates, these charged are removed by chemical derivatization, making them less polar and more volatile and stable in order to improve chromatographic resolution (McDonald et al., 2011). GC-MS is the method of reference for urinary steroid profiling, and remains at the forefront for identifying and studying steroid metabolic disorders (Krone et al., 2010)

LC-MS requires less sample preparation, and does not rely on chemical derivatization of steroid analytes. LC and High performance LC (HPLC), retain steroids in the column based on polarity of the mobile phase versus the stationary phase. The introduction of tandem mass spectrometry (MS/MS), utilizing quadrupole mass analyzers, which fragment the sample inside the instrument and analyze the generated products, have modernized the MS-field considerably (Shackleton, 2010). LC-MS/MS is the method of choice in routine clinical laboratories, because of its superior sensitivity and specificity with regard to immunoassays, the possibility to measure several different steroids simultaneously and the high throughput capacity (Grebe and Singh, 2011). The LC-MS/MS methods require thorough, standardized validation and appropriate technical training to optimize their analytical performance (Taylor et al., 2015). Importantly, the employed assay requires calibration against approved standards (Wierman et

al., 2014a). Kushnir *et al*. published a standardized sample preparation technique using for the quantification of steroid hormones, and made available reference ranges for testosterone in pre- and post-menopausal women (Kushnir et al., 2010).

1.1.3.3 Which method to choose?

Choosing the correct assay for research or clinical purposes might be a difficult, and will depend on the specific scientific or diagnostic question, the expected steroid concentration in the biological sample, the availability of the technique, costs and technical and analytical training (Taylor et al., 2015) A Sex Steroid Assay Reporting Task Force, issued by the Endocrine Society, has recently highlighted the requirements for analytical validity and quality of the steroid hormone assay, with regard to accuracy, sensitivity, specificity, and reproducibility (Wierman et al., 2014b). This statement arises following a vigorous debate, provoked by an editorial promoting MS assays for reporting steroid hormone measurement (Handelsman and Wartofsky, 2013) and indicates a place for both immunoassay and MS in the current state of steroid hormone measurements, although MS will eventually take the lead in the future.

For testosterone assays, the sensitivity of immunoassays is sufficient to detect circulating levels in male subjects, but inaccurate to test samples from women or children, in which case MS is recommended (Rosner et al., 2007). Another problem for androgen measurement by immunoassay is the high level of cross-reactivity between structurally similar molecules, the most abundant circulating steroid DHEAS, present in μM concentrations, will compete and interfere with low testosterone levels (<10 nM) for antibody binding (Taylor et al., 2015).

The Endocrine Society position on estradiol assays states that good quality immunoassays and validated MS methods are both convenient for measuring levels present in normal or high concentrations (such as in *in vitro* fertilization or IVF settings), but inaccurate for determining very low levels, as present in non-reproductive tissues or in male patients (Rosner et al., 2013). With both immunoassays and MS-based methods, current analytical performance for estradiol measuring needs improvement (Vesper et al., 2014), and in particular sensitivity in the low pM range. Both techniques have reported ultrasensitive methods, but the availability of these techniques is extremely limited (Klein et al., 1994, Owen et al., 2014).

1.1.3.4 Free testosterone levels

Sex hormone binding globulin (SHBG) and albumin are the main binding proteins for testosterone in the circulation. Free fractions of testosterone are calculated using total testosterone, concentrations of binding proteins and dissociation coefficients between testosterone and SHBG and testosterone and albumin (Sodergard et al., 1982). A commonly used calculation of free androgen index is total testosterone X 100/SHBG blood level (Vermeulen et al., 1999). The most accurate way of measuring free testosterone levels are done by equilibrium dialysis, but this technique is not routinely used in clinical practice (Vermeulen et al., 1999).

1.1.4 The role of androgens in women

1.1.4.1 Adrenarche and gonadarche

The human adrenal zona reticularis starts to mature and becomes steroidogenically active around the age of 6-8 years in both boys and girls. Its regulation is still poorly understood.

This developmental process is termed adrenarche, and manifests with increasing circulatory levels of DHEA, DHEAS and androstenedione (Idkowiak et al., 2011). DHEA is converted to testosterone and DHT in target cells of androgen actions, including androgen-responsive skin and bone cells, resulting in the appearance of pubic and axillary hair, oily skin, a transient growth spurt and bone maturation. In parallel, increased estrone and estradiol levels can be observed in pre-pubertal girls, and might be derived from androstenedione through peripheral tissue-specific aromatization (such as in adipose tissue) (Biro et al., 2014).

The onset of steroidogenesis in the gonads, ovary and testicles, is called gonadarche, and is triggered by increasing gonadotropin releasing hormone (GnRH) pulse frequency and amplitude with subsequent LH release from the pituitary gland. It manifests with breast development and menarche in girls, and testicular and scrotal enlargement in boys. Adrenarche is usually observed before gonadarche, but it remains unclear if and how both events are related (Idkowiak et al., 2011).

1.1.4.2 Androgens and female sexuality

Androgens are important drivers of female sexual desire, increasing interest in sexual activity and response to sexual stimulation (Bancroft, 2002) Female Androgen Deficiency Syndrome is defined by self-reported symptoms of decreased well-being and libido occurring in women with testosterone levels in the lower quartile of normal and adequate estradiol levels (Bachmann et al., 2002). This definition, provided by the Princeton statement, has been widely criticized, because of the imprecise clinical symptoms, the heterogeneity in androgen assays, and the lack of normative age-related testosterone levels in females (Wierman et al., 2006). The diagnosis has been revised, and the American Psychiatric Association states that Hypoactive Sexual Desire Disorder is defined by deficiency or absence in sexual desire causing marked

personal distress or relational difficulties, and associated with decreased or absent physical arousal (*Diagnostic and Statistical Manual of Mental Disease*) (Association, 2013). In the latest guidelines of the Endocrine Society for androgen therapy in women, a 3-6 months trial of testosterone for postmenopausal women with diagnosed hypoactive sexual desire disorder is suggested, after exclusion of contraindications, and with the aim of restoring midnormal premenstrual testosterone levels (Wierman et al., 2014a). This suggestion is based on evidence suggesting an increased sexual satisfaction in postmenopausal women receiving 300 μg/d transdermal testosterone, alone (Davis et al., 2008) or with associated estradiol therapy (Shifren et al., 2000, Simon et al., 2005, Buster et al., 2005, Shifren et al., 2006)

1.1.4.3 Androgens and bone metabolism

Bone mass in women is critically dependent on bioavailable oestrogen. In premenopausal women, bone mineral density is not correlated with sex steroid levels, but in postmenopausal women >60 years, an association between bioavailable oestrogen and testosterone with cortical and trabecular bone mineral density was observed (Khosla et al., 2005) . Randomized controlled studies on combined testosterone and oestrogen replacement in surgically menopausal women show increased bone mineral density in the combined treatment group versus estradiol alone (Barrett-Connor et al., 1999, Watts et al., 1995) In naturally menopausal women, the effect of addition of testosterone to oestrogen replacement is controversial, either positive (Garnett et al., 1992) or insignificant (Miller et al., 2000).

1.1.4.4 Androgens and body composition

The relation between endogenous testosterone levels and lean body mass in women depends on menopausal status. Casson et al reported that increased circulating androgen levels in naturally postmenopausal women is associated with reduced body fat, increased aerobic capacity and increased insulin sensitivity (Casson et al., 2010). The same group found that in healthy premenopausal women without hyperandrogenism, circulating testosterone is a predictor of increased fat mass, but not visceral adiposity, and no association is found with resting energy or substrate metabolism (Keller et al., 2011)

1.1.4.5 Androgens and cardiovascular health

A prospective population-based study of 639 postmenopausal women found age-adjusted increased risk for coronary heart disease in women with total and free testosterone levels in the highest and lowest quintiles (Laughlin et al., 2010). These data suggest a U-shaped association of bioavailable testosterone and incidence of cardiovascular disease (Laughlin et al., 2010). In younger women with polycystic ovary syndrome (PCOS), a condition characterized by increased circulating androgen levels, no increase of coronary heart disease was observed during long-term follow-up, despite the presence of a higher incidence of cardiovascular risk factors, such as diabetes mellitus, hypertension, hyperlipidaemia and obesity (Wild et al., 2000a, Wild et al., 2000b). Low levels of SHBG, associated with obesity and resulting in increased free testosterone but without change in total testosterone concentrations, were associated with impaired cardiovascular outcome (Guthrie et al., 2004).

The described effects of postmenopausal testosterone replacement on lipid metabolism in women is conflicting. Some randomized controlled studies showed beneficial effects of combined estradiol and testosterone administration, leading to decreased triglycerides levels (Chiuve et al., 2004, Watts et al., 1995), increased HDL-cholesterol (Barrett-Connor et al.,

1999), decreased LDL (Davis et al., 1995) and total cholesterol (Davis et al., 2000) compared with estradiol only. Other studies failed to prove any effect of testosterone on lipid parameters (El-Hage et al., 2007, Nathorst-Boos et al., 2006), or reported detrimentally decreased HDL-cholesterol levels (Penotti et al., 2001, Shafighi et al., 2012, Raisz et al., 1996, Watts et al., 1995). The long-term effects of these altered lipoprotein concentrations induced by oral testosterone have not been investigated in these studies.

1.2 Androgen signaling

1.2.1 The androgen receptor

Androgens exert their action mainly through the androgen receptor (AR). The AR is a member of the steroid and nuclear receptor superfamily. The AR gene is located on the X chromosome (Xp11-12) and contains 8 exons (Gelmann, 2002). AR protein contains 919 amino acids and contains 3 major functional domains: a N-terminal modulatory domain with activation function 1, a DNA-binding domain containing a nuclear localization signal and a C-terminal ligand-binding domain with activation function 2. There is also a small hinge region, localized between the DNA and ligand-binding domain (Gao et al., 2005).

The unbound AR is associated with a heat-shock protein complex and usually resides in the cytoplasm. Upon ligand-binding, the heat-shock protein dissociates from the AR protein, that will further undergo dimerization and phosphorylation before translocating to the nucleus. The DNA-binding domain is directed to androgen-response elements in the promotor or enhancer region of androgen-responsive genes. The AR-DNA complex further necessitate the presence of co-activators or co-repressors to ensure AR-related gene expression (Gao et al., 2005).

The human and rodent AR structure and function are quite similar (Lubahn et al., 1988).

In females, AR is expressed in the ovary, breast, liver, bone, muscle, adipose tissue, brain and skin (De Gendt and Verhoeven, 2012). In the ovarian follicle, ARs are identified in the oocyte, granulosa, and theca cells (Sen and Hammes, 2010). In cattle, non-human primates and women, AR is expressed in ovarian cortical stroma cells (Weil et al., 1998, Horie et al., 1992, Yang and Fortune, 2006). In rodents, bovine, primates and human, AR are detected in granulosa cells from the primary stage onward, and their concentration peaks in the antral follicle stage (Sen and Hammes, 2010, Yang and Fortune, 2006, Weil et al., 1998, Rice et al., 2007). The expression of AR decreases as the follicle reaches the preovulatory stage (Prizant et al., 2014).

1.2.2 Non-genomic androgen signaling

As for other steroids, it is now commonly accepted that many effects of androgens depend on other complex signaling pathways, including rapid non-genomic signaling. The reported non-genomic effects related to physiological concentrations of testosterone appear to involve the classical AR, with downstream activation of the MAPK-ERK pathways (Kousteni et al., 2001). Further evidence points toward increased AR transcriptional activation following rapid non-genomic signaling, with creation of an autocrine stimulatory loop between AR and its ligand (Heinlein and Chang, 2002b, Heinlein and Chang, 2002a).

Evidence for non-genomic androgen actions in ovarian tissue is elegantly described in the frog *Xenopus laevis* oocyte, where progesterone and testosterone are capable of promoting oocyte maturation *in vitro* (Lutz et al., 2001). Inhibitory signals maintain high intracellular cAMP levels within the frog oocyte, keeping it in meiotic arrest. Testosterone has the ability to overcome and release these inhibitory signals, via transcription-independent activation of

MAPK-pathways, and permit meiosis to progress (Lutz et al., 2003). The same group has further demonstrated that testosterone-induced maturation equally occurs in mouse oocytes (Gill et al., 2004). In the frog as well as the mouse oocytes, androgen-triggered maturation was found attenuated in the presence of the AR antagonist flutamide, indicating that classical AR in the oocytes are involved in the non-genomic signaling cascade (Lutz et al., 2001, Gill et al., 2004). Transcription-independent upregulation of FSH-R protein following androgen-treatment was also described in mouse ovaries (Sen et al., 2014). Increasing attention focuses on extra-nuclear non-genomic androgen signaling pathways, including the activation of the scaffold protein paxillin, which, in turn, increases AR-mediated transcription, by retaining AR in the nuclear compartment (Sen et al., 2014). Another example of non-genomic action of testosterone in the ovary is the rapid activation of PI3-K/Akt pathway documented in neonatal mouse ovaries, an effect blocked by the AR-antagonist flutamide (Yang et al., 2010).

1.2.3 Antiandrogens

AR antagonists or androgens directly bind or block the AR, and prevent the biological effects of androgens to occur. Steroidal antiandrogens, such as cyproterone acetate or medroxyprogesterone acetate, share an analogue chemical structure with androgens and bind the AR (Helsen et al., 2014). Non-steroidal antiandrogens bind the AR, and impair the AR binding to DNA. First generation non-steroidal antiandrogens include flutamide and bicalutamide (Helsen et al., 2014). Enzalutamide, also known as MDV3100, was introduced in 2009 as a second generation non-steroidal antiandrogens, with stronger AR affinity and has a dual action of AR blockade: it prevents AR translocation to the nucleus and inhibits AR binding to ARE on DNA (Tran et al., 2009). The working mechanism of Enzalutamide is depictured in Figure 1-7.

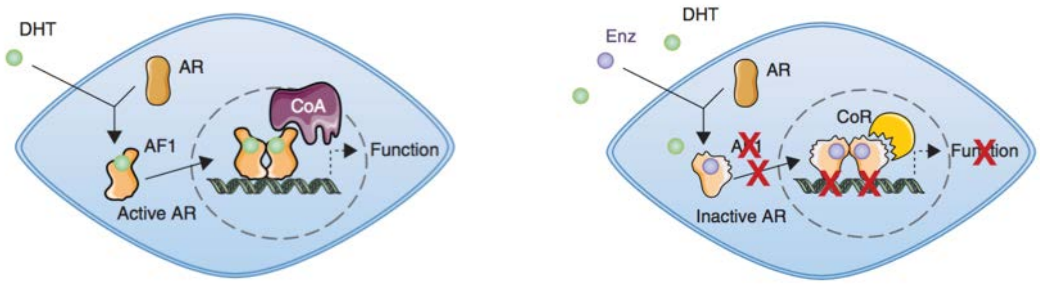


Figure 1-7 Mechanism of action of Enzalutamide (MDV). DHT binds the AR and induces the formation of a co-activator binding platform (AF1), nuclear translocation and transcription of AR-responsive genes. Enzalutamide (Enz) inhibits the formation of AF1 and prevents nuclear translocation, making it a potent antiandrogen. Adapted from Helsen et al, with kind permission of BioScientifica Limited. Copyright 2014.

1.3 Physiology of the ovary

1.3.1 Function and structure of the ovary

The ovary is the central female reproductive organ, a gonad and endocrine gland. It houses a fixed reserve of gametes and ensures the cyclic release of (a) mature fertilizable oocyte(s) (Figure 1-8). The single follicle is the fundamental unit of the ovary and is composed of an oocyte surrounded by specialized endocrine cells (granulosa and theca cells). It produces peptide hormones and sex steroids that modulate the maturation of the oocyte and regulate follicle cell growth and differentiation through local auto- and paracrine signaling pathways. Secreted into the blood stream, androgens and oestrogens exert endocrine effects and prepare the reproductive organs for fertilization. Following ovulation, the remaining follicle differentiates into a corpus luteum, responsible for progesterone production, required to allow for implantation and early embryo development (Lebbe and Woodruff, 2013).

The outer cortex of the ovary is a rigid, avascular environment, containing the immature primordial follicle reserve, and surrounded by the ovarian surface epithelium. It consists of tightly packed spindle-shaped fibroblasts, smooth muscle, endothelial and inflammatory cells, as well as precursor theca cells. Its role is to sustain the ovarian architecture by providing structural support to the growing follicles. The stroma secretes a variety of cytokines, chemokines and growth factors that steadily co-regulate—in an autocrine and paracrine manner—the early development of its enclosed follicles (Lebbe and Woodruff, 2013). The inner medulla has a more elastic composition and consists of loose connective tissue and harbors the ovarian vasculature. It is home to the developing pool of follicles, as well as the corpus luteum and albicans, remaining products of the follicle following ovulation (Figure 1-8).

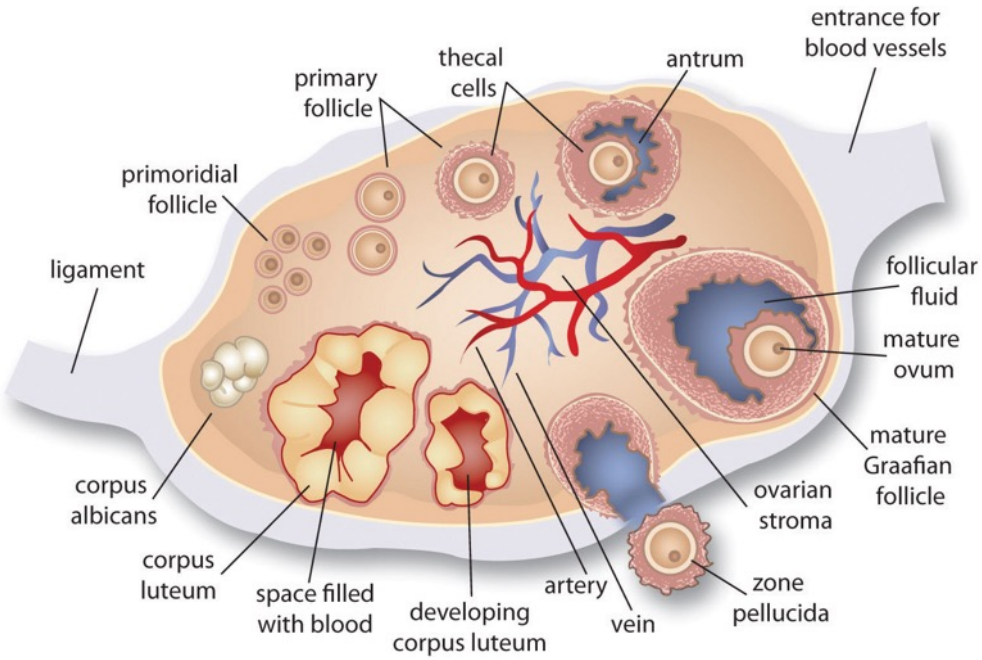


Figure 1-8 Schematic overview of the ovary, showing the main cell types and follicle stages. The follicle consists of an oocyte or ovum surrounded by granulosa cells and theca cells. Preovulatory follicles contain a central fluid-fuild space called an antrum. After ovulation, the somatic cells differentate in a corpus luteum. The central area of the ovary is the medulla, the

outer layer the cortex, surrounded by the ovarian surface epithelium. Reprinted from Sexual bodies: anatomy and physiology, by Marcus Tye with kind permission of Flat World Knowledge.

1.3.2 Folliculogenesis and oogenesis *in vivo*

Follicles form during embryonic development, and establish the dormant primordial pool. Once the primordial follicle activated, it is called a primary follicle, that develops into a secondary pre-antral follicle in the gonadotrophin-independent stage. This developmental process takes approximately 6 months in women (Gougeon, 1996). While the majority of growing follicles are lost in atresia, a small cohort of antral follicles is recruited for further growth, dominance, and ovulation under the cyclic stimulation of gonadotrophins during the follicular phase of the menstrual cycle. The terminal follicular differentiation is called luteinization and occurs during the luteal phase.

1.3.2.1 Bipotential gonad and formation of germ cell syncytia

During the first stages of fetal life, the mammalian gonad, localized in the urogenital ridge, is 'bipotential', meaning that it develops similarly in both male and female embryos. Sexual differentiation of the gonad starts when primordial germ cells, that migrate from the yolk sac, colonize the gonad (Richards and Pangas, 2010). Research in the last decades has identified factors, mainly in the mouse, that regulate the active process of differentiation of the bipotential gonad to an ovary, with simultaneous suppression of testis-determining pathways. Following colonization, the primordial germ cells multiply through mitosis and differentiate to oogonia that form syncytia or germ cell nests (Pepling and Spradling, 1998). Oogonia will undergo the first stages of meiosis and get arrested in prophase I (Pangas and Rajkovic, 2006).

After meiotic recombination, each oocyte holds a unique genetic constitution (Gosden and Lee, 2010).

1.3.2.2 Primordial follicle formation

As illustrated in Figure 1-9, syncytia will gradually break down, which happens by invasion of single layers of flattened epithelial cells, pre-granulosa cells, into the germ cell nests. Pre-granulosa cells envelop the individual oocytes to form the primordial follicle and surrounding stroma cells (Tingen et al., 2009). This happens prenatally in humans, and a few days after birth in mice (Peters et al., 1975). An important number of oocytes will get lost in this process, as their count drops from approximately 6 million to 1-2 million at birth in humans, and 10,000 in mice (Gosden, 1987, Pepling and Spradling, 2001). The primordial follicle pool represents the reserve of follicles available. Even though this follicle reserve seems to subsist in a resting state, it is continuously and tightly regulated by follicular fate decisions: most follicles will undergo programmed cell death, a large number stay quiescent, and only a few will be selected into the growing pool (Tingen et al., 2009, Kim, 2012, Sanchez and Smitz, 2012). A central dogma in reproductive biology claims that the quantity and maintenance of the primordial follicle pool determines the individual woman's reproductive lifespan. By puberty, approximately 400,000 primordial follicles remain, and menopause sets in when the follicle count is approximately 1,000 (te Velde et al., 1998). The concept of a fixed non-renewable ovarian reserve has recently been challenged, by the hypothesis of neo-oogenesis derived from human germ cell stem cells (Johnson et al., 2004, Johnson et al., 2005, White et al., 2012). The current opinion regarding the existence of neo-oocyte in women is divided in the field, and their physiologic relevance remains unproven (Notarianni, 2011).

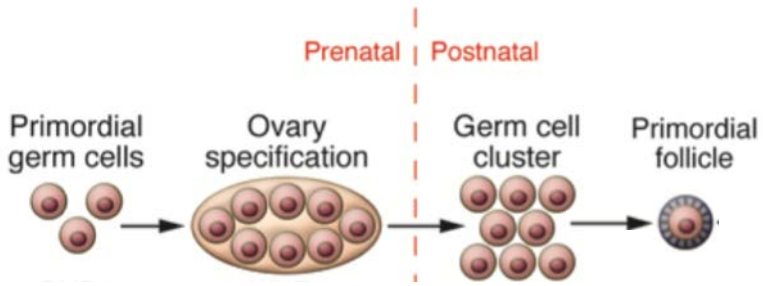


Figure 1-9 Prenatal and early postnatal ovarian follicle development in the mouse. Primordial germ cells increase in number and migrate to the bipotential gonad, where they differentiate to oocytes. Closely after birth, clusters or syncytia of germ cells break down to form primordial follicles. Adapted and reprinted from Richards et al, with kind permission of American Society for Clinical Investigation, Copyright 2010.

1.3.2.3 Primordial follicle activation

Primordial follicles recruited to enter the growing pool exhibit a change in the epithelial cell shape, from squamous to cuboidal, with subsequent proliferation of granulosa cells. On molecular level, the follicle activation process remains largely a mystery, although progress has been made in the discovery of genetic regulation of follicle activation, mainly through work in genetically modified mice. It is believed that follicle fate regulation in the primordial follicle pool is maintained by various forms of inhibitory signals (Adhikari and Liu, 2009). The phosphoinoside 3-kinase (PI3K) pathway has been identified as a key regulator for the status of primordial follicles (Reddy et al., 2008, Castrillon et al., 2003) (Figure 1-10). A basal degree of intra-oocyte PI3K activation is required for maintaining survival of the oocyte throughout the long dormancy of these follicles (Reddy et al., 2010). An elevated intra-oocyte PI3K balance results in oocyte growth and follicle activation (Zheng et al., 2012). Similarly, PTEN (phosphatase and tensin homolog) reverses PI3K action and ensures sustained dormancy of the primordial follicles. Condition Gdf-Cre PTEN knock-out mice exhibit premature ovarian

failure due to rapid follicle depletion as a result of global primordial follicle activation (Reddy et al., 2008). Another negative regulator of the PI3K pathway is FOXO3 (forkhead box O3) (Castrillon et al., 2003, Liu et al., 2007, John et al., 2008). Inactivation of these PI3K repressors by KIT ligand and other growth factors plays a crucial role in the recruitment of the primordial follicle, at least in mice (Sanchez and Smitz, 2012).

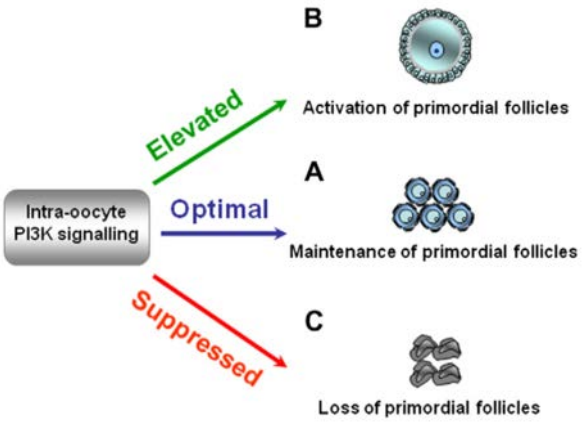


Figure 1-10 Presumed roles of intra-oocyte phosphoinoside 3-kinase (PI3K) signaling in controlling the fate of the primordial follicle pool. An optimal PI3K balance maintains follicle survival during dormancy, elevated PI3K signaling leads to follicle activation, while suppressed PI3K signaling leads to follicle loss. Adapted and reprinted from Zheng et al, with kind permission of Elsevier, Copyright 2012.

1.3.2.4 Primary and secondary follicles

Primary follicles initiate growth of both their oocyte and granulosa cells, which requires a considerable generation of metabolic energy. The oocyte will secrete glycoproteins, that will assemble in a circumferential way to form a tick porous shell or coat, the zona pellucida (Wassarman et al., 1996).

Homogenous follicle growth and differentiation will strongly depend on oocyte-somatic cell bidirectional communication. Metabolic cooperation between both compartments is essential for the survival of the oocyte. Oocytes are not able to carry out some essential metabolic processes, such as glycolysis, uptake of amino acids and synthesis of cholesterol. The oocyte manages these metabolic deficiencies by cooperating with the surrounding granulosa cells (Sugiura and Eppig, 2005)). It has been shown that, rather than being solely a passive recipient of nutritional support, the oocyte has a commanding role on the metabolic gene expression in these somatic cells, by the secretion of paracrine factors such as growth differentiation factor 9 (GDF9), and the closely related bone morphogenetic factor 15 (BMP15) and fibroblast growth factors (FGFs) (Sugiura et al., 2007, Su et al., 2008). On the other hand, GDF9 and BMP15 collaborate in enhancing somatic cell growth and differentiation (Gosden and Lee, 2010). Intercellular communication between oocyte and somatic cells happens through heterologous gap junctions (Kidder and Mhawi, 2002), diffusion, secreted factors and endocytosis. This intercellular communication of great importance for the follicle, which must stay a coordinated unit until ovulation.

Anti-Müllerian hormone (AMH), a member of the tumor growth factor (TGF)-β superfamily, was first discovered as a testicular glycoprotein causing regression of the Müllerian ducts during male embryonic sexual differentiation (Müllerian inhibiting substance) (Cate et al., 1986). In females, AMH is produced exclusively by granulosa cells, and production starts in the primary follicle stage. Its role is less obvious than in male sexual development, but it has become clear that AMH exerts inhibitory actions on the activation of primordial follicles, in rodents (Durlinger et al., 2002) and humans (Carlsson et al., 2006).

By acquiring a second layer of granulosa cells, the follicle enters the secondary stage, and its growth will remain under control of intra-ovarian regulators until the pre-antral stage (Richards and Pangas, 2010).

1.3.2.5 Pre-antral to antral follicle transition

As the granulosa cell layers increase around the growing oocyte, pre-theca cells are recruited from the surrounding stoma and differentiate to theca cells by acquisition of steroidogenic function (Young and McNeilly, 2010). Theca cell layers form along the basal membrane that surrounds the granulosa cell layers. The follicle builds its own capillary bed within the theca cell layer, enabling it to interact with systemic endocrine factors, essential for the switch to the gonadotrophin-dependent phase, and for delivering the required nutrient and growth factors for the granulosa cells and the oocyte.

Theca cell maturation and androgen generation are initially solely under auto/paracrine control, by factors secreted by the oocyte and the granulosa cells, before becoming responsive to LH and insulin during later development (Lebbe and Woodruff, 2013). GDF-9, secreted by the oocyte, is essential for theca cell function, as GDF-9 knockout mice fail to develop a theca cell layer (Dong et al., 1996). More specifically, GDF-9 has shown to induce expression of the key enzyme of androgen biosynthesis, CYP17A1, in pre-antral follicles (Orisaka et al., 2009, Vitt et al., 2000b), while suppressing CYP19A1 activity (Vitt et al., 2000a). BMP-4, BMP-6 and BMP-7, also members of the TGF-β superfamily, have opposite effects, and act via insulin-like peptide 3 to decrease CYP17A1 expression; they are potent inhibitors of thecal androgen secretion (Glister et al., 2005, Glister et al., 2013). Gremlin, chordin, and follistatin, which are local binding factors, have the ability to reverse BMP protein's function and exhibit stimulatory effects on theca cell function (Glister et al., 2005). During follicle maturation granulosa cells

produce activin that locally suppresses androgen production (Knight et al., 2012). Activins act by facilitating FSH secretion and action, thereby enhancing estradiol secretion. Inhibins are secreted by the antral follicles, their structure is closely related to activins, sharing common β-subunits, but they are functional antagonists of activins and decrease FSH action (Figure 1-11). Insulin, working in synergy with LH, via receptors expressed on theca cells, induces CYP17 expression and increases thecal androgen production (Franks et al., 1999). KIT ligand promotes thecal differentiation via Erk1/2 –mediated up-regulation of steroidogenic factor 1 (SF-1) (Jin et al., 2005).

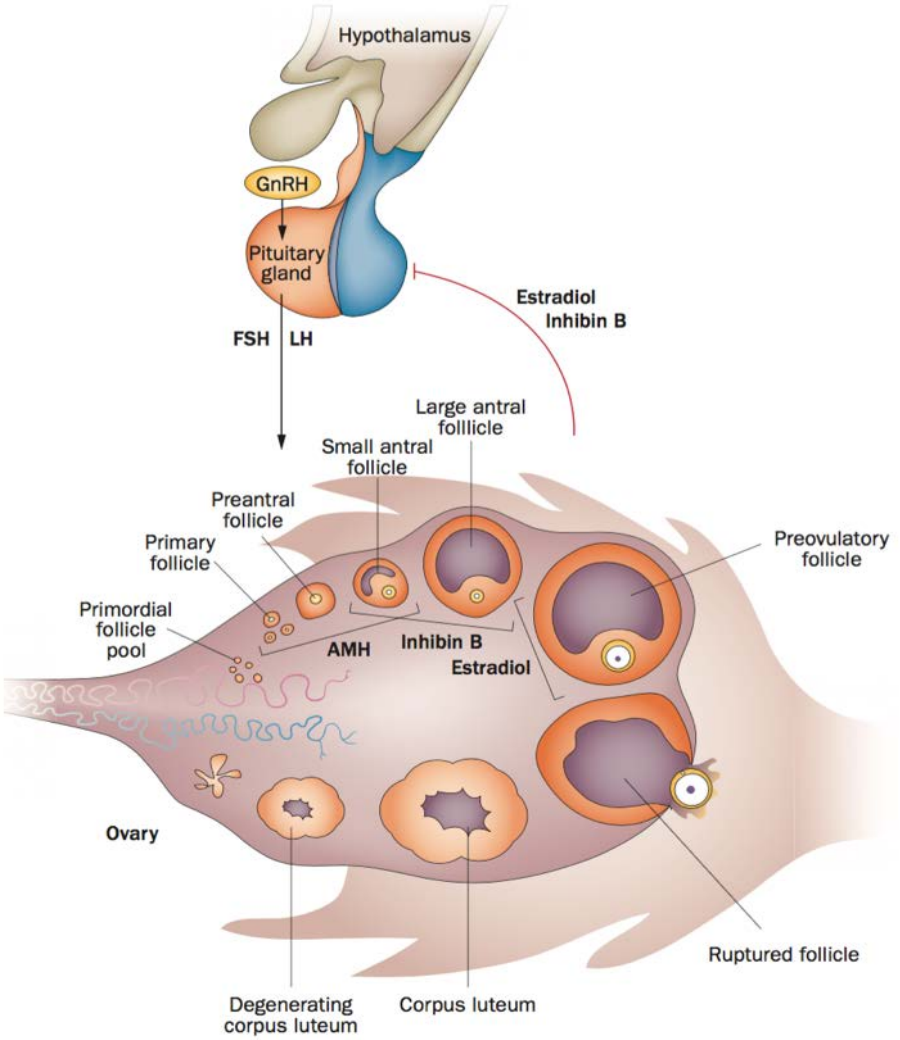


Figure 1-11 Schematic overview of the different stages of folliculogenesis. AMH, Inhibin B and Estradiol secretion and interaction with hypothalamo-pituitary axis. Reprinted from Visser et al, with kind permission from Macmillan Publishers Ltd: Nature Reviews. Copyright 2012.

AMH levels peak in follicles reaching the stage of FSH selection (preantral stage in mice, antral stage in women, Figure 1-11), and decline in mural granulosa cells (but not in cumulus cells) as the follicle enters the gonadotrophin-dependent phase (Durlinger et al., 2002, La Marca et al., 2009, Weenen et al., 2004, Andersen et al., 2010, Dumesic and Richards, 2013). When AMH levels are high, FSH sensitivity of the follicle remains low. In fact, AMH functions as a break for FSH-recruitment of antral follicles (Durlinger et al., 2001). Its level has to decrease to allow the follicle to be recruited by FSH. *In vitro*, AMH diminishes the FSH-induced expression of aromatase (CYP19A1) (Grossman et al., 2008), thereby increasing the ration of androgen/oestrogen in the follicle. AMH levels reflect antral follicle count, and serum AMH level has become an surrogate marker for follicular reserve in clinical care (Pigny et al., 2006, Visser et al., 2012, Kristensen et al., 2012).

The development of a cavitation or fluid-filled central cavity, called antrum, is a conspicuous finding in most mammals, including rodents and humans. The graafian follicle is characterized by antrum formation that accompanies the exponential granulosa cell proliferation and aromatase (CYP19A1) expression, and happens in response to an intrinsic paracrine factor (Gore-Langton and Daniel, 1990). Antral follicular fluid is composed by transudates of plasma, and follicular secretions (Edwards, 1974). Physiologically, the antrum acts as a barrier, limiting passive diffusion of blood-derived gaseous, nutritive and hormonal substances across the follicular wall of the rapidly expanding avascular follicle harboring the maturing oocyte (Gore-Langton and Daniel, 1990). Structurally, the antrum divides the granulosa cell population into central cumulus oophorus cells, and mural granulosa cells

proliferating between the antral cavity lining and the basal membrane. Both granulosa cell subpopulations are morphologically and functionally distinct, in their steroidogenic capacities (Hillensjo et al., 1981), AMH production (Ueno et al., 1989), and LH-receptor distribution (Camp et al., 1991). The oocyte plays an important paracrine role in the trans-differentiation of granulosa cells to cumulus oophorus cells (Eppig et al., 1997). In mural granulosa cells, FSH has proliferative effects, and induces LH-receptor expression, while in in cumulus cells FSH does not induce LH-receptor but stimulates production and secretion of hyaluronic acid, resulting in mucification and cumulus cell expansion, a prerogative for ovulation (Eppig, 1979).

1.3.2.6 Selection of the dominant follicle(s)

FSH restricts the growing follicle cohort in order to allow a dominant follicle to emerge in mono-ovulating species, or a larger amount of follicles in poly-ovulators. In women, the rise in FSH during the luteal-follicular transition triggers the selection of the follicle that will ovulate next, which happens when sufficient FSH is accumulated in the follicular fluid for a threshold level to be reached (Welt et al., 1997). The oestrogen and inhibin production by the dominant follicle will provide feedback to the hypothalamic-pituitary axis and down-regulate FSH secretion, leading to atresia of the remaining cohort of growing follicles (Welt et al., 1997)

The oocyte of the dominant follicle will continue to develop and accumulate all key regulatory factors required for fertilization (Gosden and Lee, 2010). It is important to keep in mind that, while the spermatozoon essentially makes a genetic contribution for embryogenesis, the oocyte, far more voluminous, is the main cytoplasmic donor, having accumulated all RNA, proteins and key organelles required for post-fertilization embryo development (Figure 1-12), (Gosden and Lee, 2010).

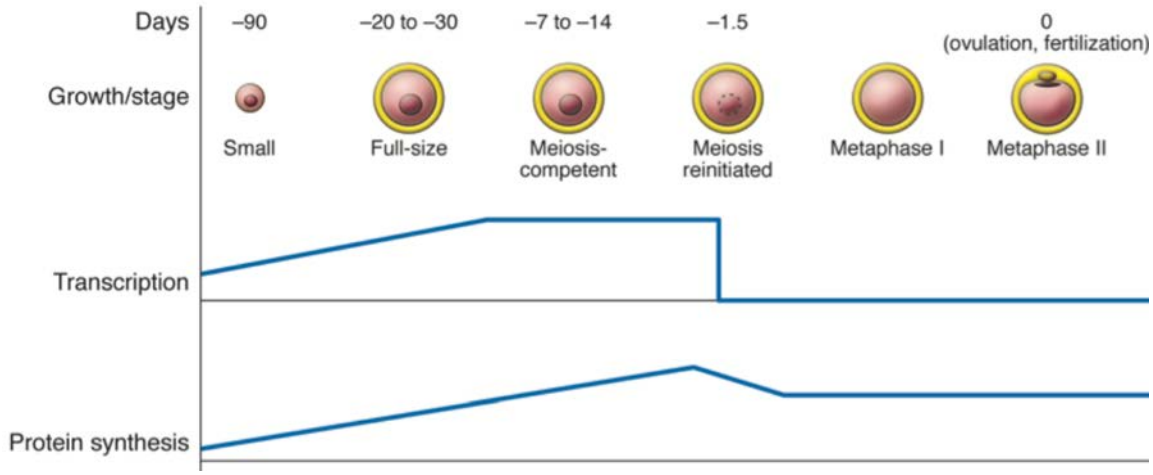


Figure 1-12 Schematic overview of molecular and cellular development of human oocytes. Oocyte development is initiated by oocyte growth in small follicles. Competence to resume meiosis is reached shortly before ovulation. Transcription becomes silenced after resumption of meiosis. Protein synthesis increases to a plateau when the oocyte is fully grown. Adapted and reprinted from Gosden and Lee with kind permission from the American Society for Clinical Investigation. Copyright 2010.

The somatic follicular compartments and the antral cavity rapidly expand in the pre-ovulatory follicle, resulting in large estradiol secretion, essential for establishing endometrial proliferation and generating the gonadotrophin surge prior to ovulation.

1.3.2.7 Ovulation

Ovulation is the unique phenomenon by which the mature pre-ovulatory follicle, triggered by the pituitary mid-cycle LH-surge, ruptures and releases a fertilizable oocyte, and transdifferentiates the somatic cells to form the corpus luteum (Richards et al., 1998) (Figure 1-11).

Ovulation is considered to be a triad of events: cumulus expansion, follicular rupture and luteinization. In response to the LH surge occurring prior to ovulation, cumulus cells start

to secrete hyaluronic acid, leading to mucidification and cumulus cell expansion (Eppig et al., 1997). The oocyte will complete its first meiotic division as pictured in Figure 1-13. The oocyte, until that point arrested in the prophase of meiosis I, first starts dissolving the envelope of its nucleus, a process called germinal vesicle breakdown. A spindle, which is a microtubular structure allowing chromosomal movements during cell division, will assemble and align the diploid set of chromosomes in the center of the oocyte (called the metaphase). The spindle relocates to the pole of the cell, while homologous pairs of chromosomes exchange genetic information by forming cross-overs between them, before they segregate in 2 haploid sets (anaphase). Finally, an asymmetric cell division takes place with the formation and extrusion of the first polar body. The DNA content of the oocyte has been divided, to become an egg, still maintaining the majority of the cytoplasm, containing critical stores of maternal components essential for fertilization and pre-implantation embryo development. The egg is arrested in metaphase II, which it will complete upon fertilization (Clift and Schuh, 2013).

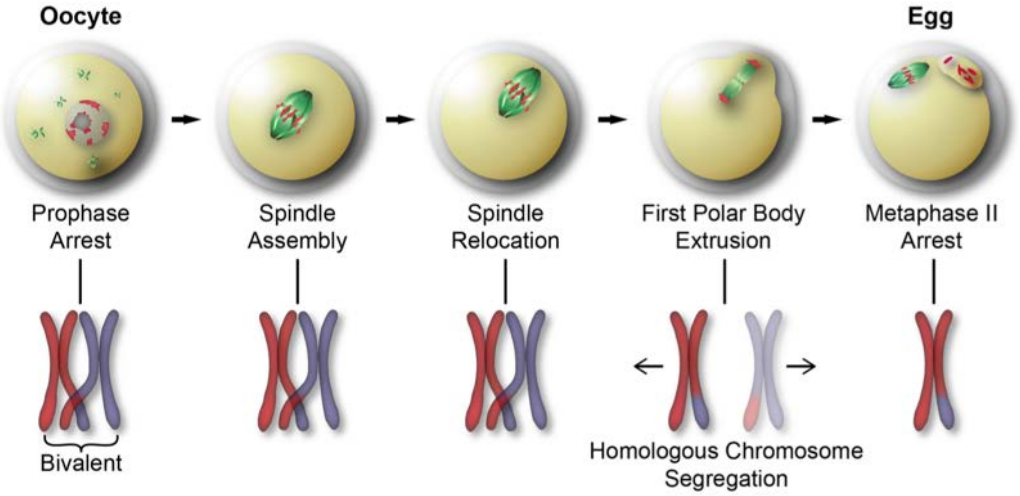


Figure 1-13 Schematic image of oocyte maturation and chromosome configuration. Following germinal vesicle envelope breakdown, the spindle assembles and moves to the surface of the oocyte. Half of the homologous chromosomes segregates and is extruded in the polar body.

Around the remaining chromosomes a spindle assembles. The egg arrests in metaphase II until fertilization. Adapted from Clift and Schuh, with kind permission from Nature Publishing Group. Copyright 2013.

Progesterone-receptor, a down-stream target of LH, is known to be an essential mediator of the follicular rupture (Lydon et al., 1996). Progesterone-receptor activation triggers a precisely localized inflammatory cascade, with vascular changes and appearance of matrix metalloproteinases breaking down the extracellular matrix and ovarian surface epithelium to permit extrusion of the oocyte into the oviduct or fallopian tube (Robker et al., 2000).

Luteinization refers to the terminal differentiation of the granulosa and theca cells, with a steroidogenic shift toward progesterone secretion, development of vascularization and cessation of proliferation. The corpus luteum is essential for the uterine implantation and early survival of the embryo (Murphy, 2000). If the egg is not fertilized, the corpus luteum will involute to a corpus albicans or fibrous scar.

1.3.3 Reproductive cycle

1.3.3.1 Menstrual cycle

The duration of the menstrual cycle is typically 28 ± 3 days and is divided into two phases: the follicular or proliferative phase, and the luteal or secretory phase (Figure 1-14).

As shown in Figure 1-3 and Figure 1-11, pituitary gonadotrophin secretion is basically under negative feedback control by estradiol, progesterone and inhibin (Welt et al., 1997). Rising estradiol levels provide negative feedback at the level of the hypothalamus and inhibit GnRH and consequently FSH secretion. Rising estradiol levels also provide positive feedback with stimulation of LH secretion at the level of the pituitary gland. Decreasing estradiol levels

will suppress LH secretion (Welt et al., 1997). Inhibin is secreted in 2 forms, A and B, by the follicle and inhibit FSH secretion. Inhibin B is produced by the dominant follicle and peaks in the follicular phase. Inhibin A is produced by the corpus luteum and peaks in the luteal phase (Messinis, 2006).

The follicular phase starts on the first days of the menstrual bleeding, and lasts until ovulation, occurring mid-cycle (typically day 14). Pituitary FSH, that begins to rise in the last days of the previous menstrual cycle, as a results of falling levels of ovarian steroids, will cyclically recruit a cohort of 2-5mm antral follicles for further growth and maturation (Baerwald et al., 2012). One follicle will emerge as dominant, and is thought to have the highest FSH-responsiveness and most abundant estradiol and inhibin-B secretion. As a result, FSH levels will decrease and the remaining cohort of antral follicles will die by apoptosis. The rising estradiol levels stimulate endometrial and myometrial growth in the uterus, and the cervix produces increased amounts of mucus, reducing the vaginal acidity, making it more accessible for the entry of sperm. Immediately before ovulation, estradiol levels will reach a peak and the enhanced LH secretion will regulate the peri-ovulatory events. The pre-ovulatory LH surge is accompanied by a smaller peak in FSH levels, which might be physiologically significant (Kol and Humaidan, 2010).

At the start of the luteal phase, following ovulation, progesterone secretion by the corpus luteum increases and provides negative feedback to the hypothalamic-pituitary unit, and plasma levels of LH and FSH decrease. Progesterone is known to raise the basal body temperature by 0.3-0.5^{0}C, and this observation is used to track ovulation. Progesterone increases endometrial glandular secretions, and is required to render the endometrium receptive for blastocyst implantation. A rise in oestrogen, produced by the corpus luteum, causes further inhibition of gonadotrophin secretion, causing corpus luteum suppression with decreased oestrogen and

progesterone secretion, and FSH levels begin to rise to initiate the next cyclical recruitment of antral follicles. At the level of the endometrium, intense vasospasm occurs in the arterioles, leading to ischemia, desquamation and menstrual bleeding.

Alternatively, the wane of the corpus luteum is prevented by blastocyst implantation. The production of human chorionic gonadotrophin (HCG) by the embryo, which has a similar action as LH, maintains corpus luteum survival and progesterone production for 8-12 weeks, until the placenta takes over this function.

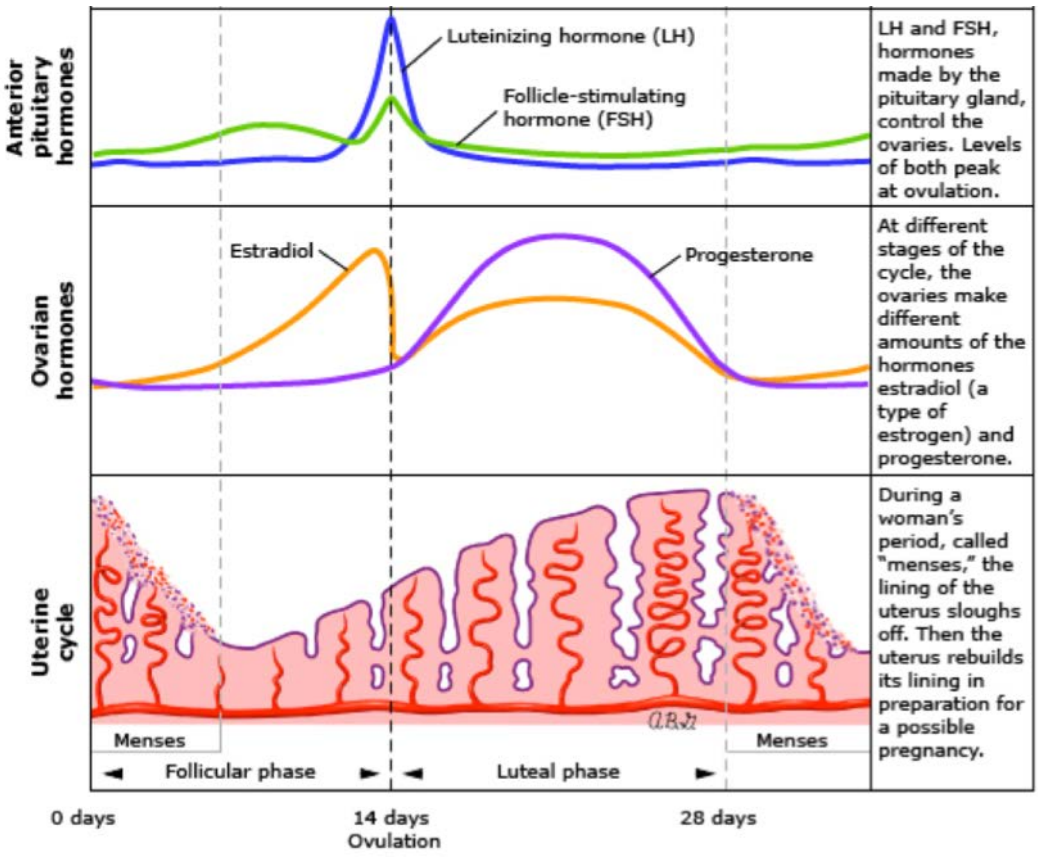


Figure 1-14 The menstrual cycle, with circulating levels of gonadotrophins (LH and FSH), ovarian steroid hormones (estradiol, progesterone) and cyclical endometrial changes, during the follicular and luteal phase. Adapted and reprinted with kind permission from UpToDate.

1.3.3.2 Estrous cycle

In rodents, the reproductive cycle lasts 4-5 days, and is called the estrous cycle. To determine the different cycle phases (proestrous, estrous, metestrous and diestrus), investigators typically use vaginal smear cytology and assess the proportion of epithelial cells, cornified cells and leucocytes (Evans and Long, 1922) (Table 1-2).

Table 1-2 Characteristics of the mouse estrous cycle, with regard to vaginal smear cytology and levels of pituitary and ovarian hormones (based on (Caligioni, 2009))

Estrous cycle phase	Vaginal smear cytology	LH and FSH levels	Ovarian hormones
Proestrous	Nucleated epithelial cells	Surge during the night	E2 level peak, followed by P4
Estrous	Anucleated cornified epithelial cells	low	E2 remain elevated in the morning, then fall back to basal levels
Metestrous	Mix of leucocytes, epithelial and cornified cells	low	Low
Diestrous	Leucocytes	low	E2 starts to rise

Abbreviations: E2: estradiol, P4: progesterone

1.3.4 Folliculogenesis and oogenesis *in vitro*

Alongside the development of assisted reproduction with its clinical implications, scientists have been working for more than 40 years on techniques to mature follicles and oocytes *in vitro,* with the aim to improve and widen the applications of reproductive biology. In 1977, John Eppig showed for the first time, that mouse oocytes were able to grow and mature *in vitro,* if cultured in the presence of granulosa cells, thereby proving the crucial interactions between both cells (Eppig, 1977). Since then, *in vitro* culture of ovarian follicles and oocytes has provided novel knowledge on follicle biology and the mechanisms involved in oocyte maturation. An important clinical application of *in vitro* follicle growth and maturation is under development for women desiring to spare their fertility before undergoing gonadotoxic oncology treatments. Oncofertility has emerged as a new discipline, linking reproductive medicine and oncology, and researches treatment alternative treatment options for ovarian tissue transplantation in female cancer patients of reproductive age (Jeruss and Woodruff, 2009, Woodruff, 2010).

Follicle culture has been achieved *in vitro*, employing 2-dimensional (2D) or adhesive systems, or more recently, in spherical 3-dimensional (3D) systems.

1.3.4.1 Pioneering methods of follicle culture

In 2D culture systems, different types of substrates are used, such as culture dishes (Eppig, 1977, Cortvrindt et al., 1996) or collagen trans-wells (Eppig and Schroeder, 1989), to which the granulosa cells adhere, allowing the follicle to expand on a flat surface.

The follicle structure is remodeled, and although gap-junctions and basal membrane continuity might be disrupted (Desai et al., 2010), this method have been proven successful in producing live offspring in rodents following *in vitro* follicle culture, oocyte maturation and IVF (Eppig

and Schroeder, 1989). Its application in larger mammals, including human (Abir et al., 1997), is hampered by the abnormal follicle expansion and aberrant paracrine signaling (Ksiazkiewicz, 2006).

1.3.4.2 Encapsulated in vitro follicle growth

To overcome the challenges faced while culturing follicles in 2D, novel non-adherent techniques, allowing for 3D follicle proliferation, have recently been developed, including 'floating' and 'encapsulated' models (Brito et al., 2014) (Figure 1-15). For the purpose of this thesis, the focus is on the alginate encapsulated 3D follicle culture technique, developed by Dr Teresa Woodruff, in collaboration with Dr Lonnie Shea at Northwestern University, a decade ago.

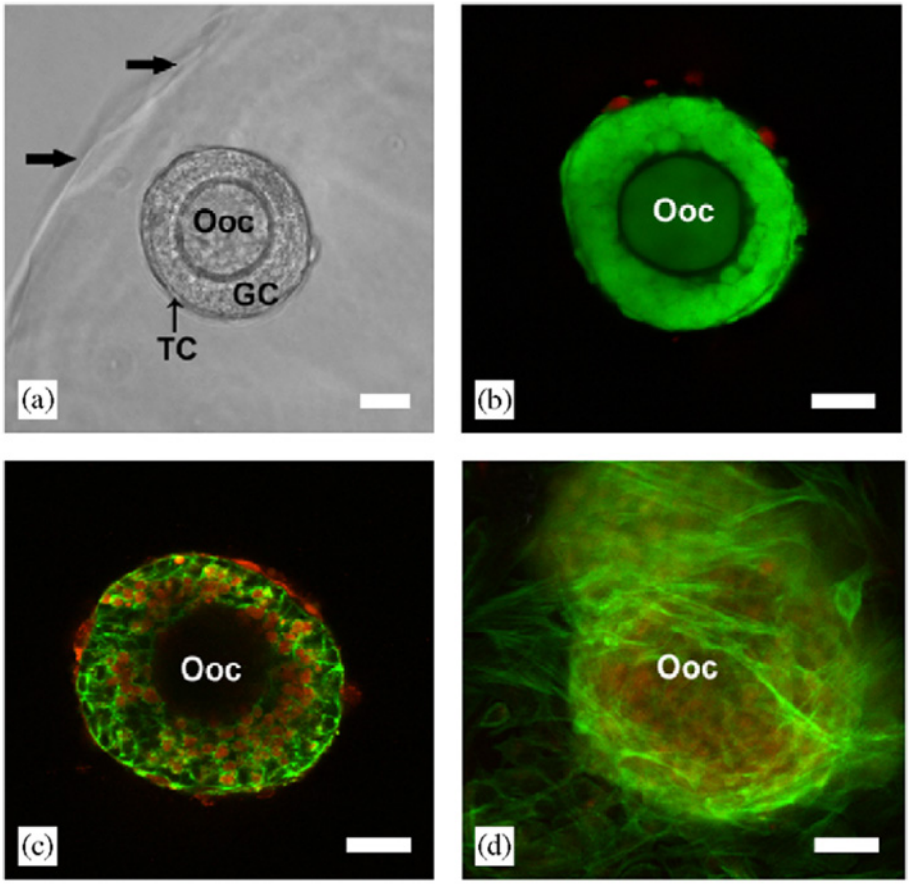


Figure 1-15 (a) Secondary mouse follicle encapsulated in an alginate bead (edge of bead indicated by arrows). (b) Follicles stained for viability 1 day after encapsulation are healthy. (c,d) Follicles cultured in alginate beads (c) maintain their morphology at day 4 of culture, while follicles cultured on 2D substrates (d) have a disrupted follicular architecture. Scale bar = 30 μm. Reprinted from Kreeger et al, with kind permission of Pergamon, Copyright 2006.

Alginate is a polysaccharide of repeating β-D-mannuronic and α-L-guluronic units, isolated from cell walls of brown algae. It is liquid in its natural form, but cross-links in the presence of calcium, and forms a solid-like hydrogel used to surround the follicle (Figure 1-16).

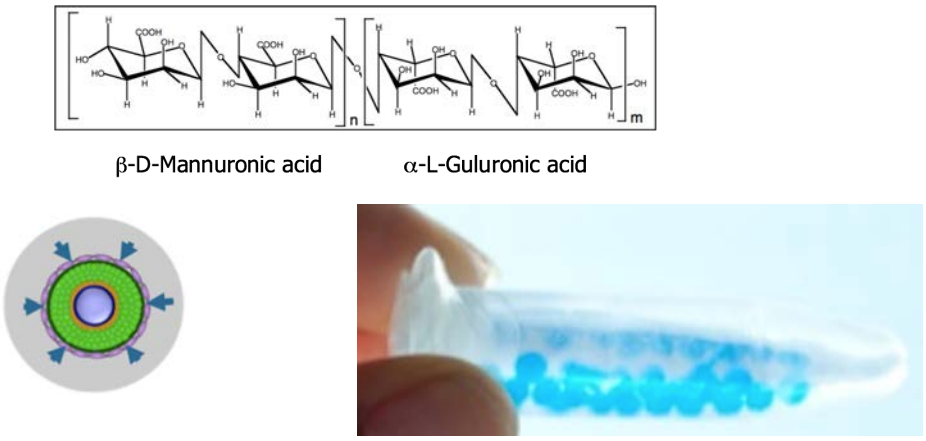


Figure 1-16 Alginate is a polysaccharide, derived from seaweed, that crosslinkes in the presence of calcium to form a solid gel, used to encapsulate ovarian follicles. Courtesy to the Woodruff Lab.

Having achieved success with live birth of healthy offspring in mice (Xu et al., 2006a) (Figure 1-17), these 3D model equally support spherical expansion of follicles isolated from larger mammals, including murine, bovine, goat, canine, non-human primate, and human-derived follicles (Xu et al., 2006a, Araujo et al., 2014, Silva et al., 2015, Songsasen et al., 2011, Min Xu, 2009, Xu et al., 2009). Studies across species have enabled comparative assessments

of biology unique to each species, for example, the physical rigidity of the biomaterial, while providing important new insights into the conserved mechanisms governing the follicle development (Woodruff and Shea, 2011).

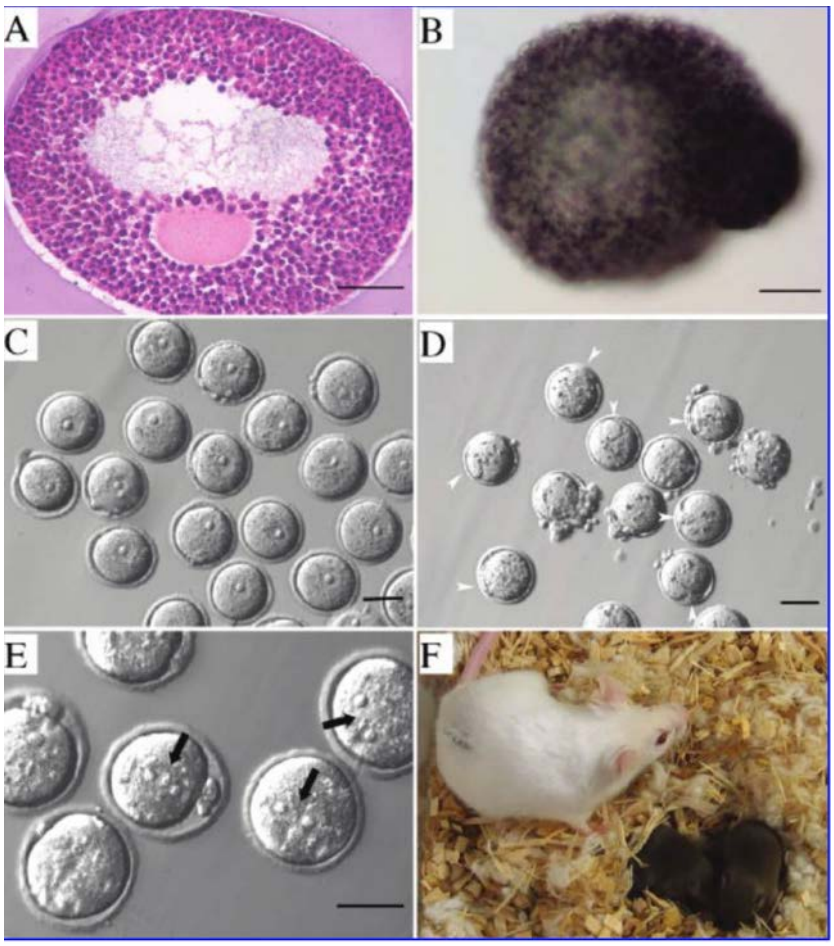


Figure 1-17 (A) After 8 days of culture, immature follicle reached the pre-ovulatory stage (B) an outer theca cell layer indicated by 3b-hydroxysteroid dehydrogenase staining. (C) Meiotically arrested cultured oocytes (D) Resumption of meiosis after exogenous HCG stimulation. (E) Metaphase II oocytes fertilized in vitro, and (F) resulting in live offspring after transfer into the oviduct of pseudopregnant mouse. Bar 1⁄4 100 mm (A, B), 50 mm (C–E). Reprinted from Xu et al, with kind permission of Mary Ann Liebert INC Publishers, Copyright 2006.

Alginate is non-degradable and non-adhesive, and adaptable, making it a unique tissue-engineered system to support in vitro follicle growth (Kreeger et al., 2005). Through chemical changes, extra-cellular matrix proteins, such as collagen, fibronectin or laminin, can be incorporated in the alginate scaffold, which has shown to improve the rate of meiotically competent oocytes (Kreeger et al., 2006). Additionally, alginate concentrations can be modified, which leads to changes in the rigidity of the alginate bead and consequently variation in the forces exerted on the enclosed follicle. Decreased alginate concentrations have been found to improve follicle growth and oocyte meiotic competence (Xu et al., 2006b, West et al., 2007).

1.3.4.3 Follicle culture medium and additives

In vitro follicle culture is dependent on various additives within the culture medium, such as nutrients, gonadotrophins, energy substrates, antioxidants and vitamins, involved in *in vivo* endocrine and paracrine regulation of follicle growth and maturation (Brito et al., 2014). Follicle culture protocols may include serum, containing many different substrates and growth factors, or include purified proteins such as albumin and fetuin (Demeestere et al., 2005). The addition of FSH and insulin, transferrin and selenium in follicle culture medium has shown to improve follicle growth and morphology, oocyte maturation and steroidogenesis (Silva et al., 2004, Demeestere et al., 2005). The optimized culture protocol for murine alginate-based follicle culture consists of α-minimal essential medium (MEM), substituted with insulin-selenium-transferrin, FSH, bovine serum albumin (BSA) and fetuin (Xu et al., 2006a).

1.4 Effect of androgens on the on the different stages of follicular development

1.4.1 Pharmacological studies

1.4.1.1 Primordial to primary follicle transition

Testosterone enhances in a non-genomic way the intra-oocyte PI3K balance in mouse primordial follicles, thereby increasing by more than two-fold the ratio of primary to primordial follicles (Yang et al., 2010). A cortical autograft experimental model in female sheep treated with DHEA for 10 weeks resulted similarly in an increased activation of primordial follicles (Narkwichean et al., 2014). Rapid non-genomic activation of the PI3K/Akt pathway by androgens has been described in other target cells of androgen action (Baron et al., 2004, Kang et al., 2004, Cinar et al., 2007). In lamb ovaries, the increase of primary follicle numbers following a 5-day incubation with testosterone, was mainly attributed to a decreased rate of atresia (Qureshi et al., 2008). In rhesus monkeys, testosterone treatment equally appears to promote primordial follicle activation, through elevated intra-oocyte IGF1 (insulin growth factor 1) signaling (Vendola et al., 1999a, Vendola et al., 1999b). This androgen action is probably non-genomic, as AR is not detected, or is below limit of detection, in primordial follicles (Weil et al., 1998). Nevertheless, it is interesting that IGF1 receptor-mediated protection from apoptosis is similarly achieved through activation of PI3 kinase (Shelton et al., 2004).

1.4.1.2 Primary to secondary follicle transition

Intercellular communication is of paramount importance for follicle development and survival. Gap junctions are intracellular channels, allowing direct exchange of small molecules between cells. Connexins are building stones of gap junctions, and connexin 43 is the main

connexin found in gonads. In fetal bovine ovaries, exposure to the anti-androgen flutamide at mid-gestation decreases the expression of connexin 43 and the amount of granulosa cell-oocyte gap junctions (Knapczyk-Stwora et al., 2013). On the contrary, in the mouse ovary (Yang et al., 2015b), and in a luteinized granulosa cell line (Wu et al., 2010a), a down-regulation of connexin 43 expression is observed in androgen-excess conditions. A non-identical granulosa cell differentiation status between both studies could possibly explain the observed dual effect.

Testosterone, but not estradiol, stimulates, in a dose-dependent way, the acquisition of a second layer of granulosa cells in fetal bovine follicles, and this effect is blocked by the AR antagonist flutamide (Yang and Fortune, 2006). The proliferative properties of testosterone could be partly mediated through increasing the availability of metabolic energy to the cell, such as enhanced glucose-metabolism via Glut4 pathways (Sato et al., 2008). In rhesus monkeys, AR expression positively correlates with granulosa cell growth and androgen treatment up-regulates the expression of AR in the granulosa cells of healthy pre-antral and antral follicles (Weil et al., 1998). Similar findings have been observed in mouse ovarian stroma cells (Yang et al., 2015b) and ovaries from testosterone-treated transsexual women (Chadha et al., 1994).

1.4.1.3 Pre-antral to antral follicle transition

Immunohistochemistry and steroid measurements performed in *in vitro*-cultured murine follicles, elegantly showed that the AR protein translocate from the cytoplasm to the nucleus in granulosa and theca cells at the moment the follicle starts to produce androgens (Lenie and Smitz, 2009). Indeed, the timeframe surrounding antrum formation is a critical stage of androgen action, where AR expression is at its highest (Weil et al., 1998, Lenie and Smitz, 2009, Yang et al., 2015b). Murine follicles cultured in the presence of anti-androgen serum or

with the AR-antagonist Casodex, grew smaller and showed impaired antrum formation, and these features were rescued when adding androstenedione or DHT (Murray et al., 1998). Treatment of pre-antral follicles with androgen precursors such as DHEA, DHEAS and androstenedione, employed at doses ranging from 10^{-11} to 10^{-7} M, have equally shown to provoke a dose-dependent increase in murine follicle size during culture (Wang et al., 2001). In a steroid-depleted environment, obtained by trilostane treatment, a steroid synthesis inhibitor, pre-antral macaque follicles in 3D alginate-encapsulated culture exhibited decreased survival, grew smaller and developed less frequently an antrum compared with control follicles (Rodrigues et al., 2015). High dose (50 ng/ml) testosterone and DHT treatment (Rodrigues et al., 2015), as well as estradiol treatment (100 pg/ml and 1ng/ml) (Ting et al., 2015) were able to rescue follicle survival and antrum formation to control levels. In this primate model, both androgens and oestrogens are survival and growth factors in pre-antral and antral follicles (Ting et al., 2015).

Moreover, a clear synergistic effect between androgens and FSH on pre-antral and antral follicle growth has been demonstrated. Vendola and Weil treated female adult rhesus monkeys with short term implants of testosterone or DHT, and demonstrated a robust stimulation of antral follicle growth, with upregulation of granulosa cell AR and FSH-R expression (Weil et al., 1999, Vendola et al., 1998). In individual follicles, the AR and FSH-R expression was positively correlated (Weil et al., 1999). Similarly, in small human antral follicles of naturally cycling women, a positive correlation between androgen levels in follicular fluid and FSH-R expression in granulosa cells has been observed (Nielsen et al., 2011). It is important to note that, in humans, the AR expression precedes the FSH-R expression during follicle development (Rice et al., 2007). The concept is that locally produced androgens act via granulosa cell AR to promote FSH-induced granulosa cell differentiation. On a molecular level, ligand-activated AR

enhances FSH action by stimulating cAMP-mediated post-receptor signaling (Hillier and Tetsuka, 1997). A recent study started to unravel the underlying mechanisms of androgen action in the antral follicle, and showed that androgen increase FSH-R protein expression, but not mRNA, and enhance the mRNA expression of miR125, which protects the follicle from atresia (Sen et al., 2014).

1.4.1.4 Pre-ovulatory follicle, ovulation and oocyte maturation

Administration of 1 mg testosterone (but not the higher doses) during the follicular phase in gilts, increased FSH-R expression in pre-ovulatory follicles, augmented estradiol levels and resulted in a higher number of corpus lutea (Cardenas and Pope, 1994, Cardenas et al., 2002). Immature mice, superovulated with pregnant mare serum gonadotrophin (PMSG), had an improved response when treated with low dose DHT (50mg/kg), but not with higher doses, and cyproterone acetate, an AR antagonist, abolished the androgen effect (Ware, 1982). A subsequent study in rats, stimulated with PMSG, showed that at lower DHT doses (4 injections of 1mg/kg DHT), ovulation rates were decreased as a result of decreased follicle development and decreased estradiol secretion (Conway et al., 1990). A recent experiment confirmed again increased ovulatory rates when gonadotrophin-stimulated mice are exposed to low dose androgen treatment (0.25 mg DHT), while in high dose (25 mg DHT) androgen conditions this effect was lost (Sen et al., 2014). From these different studies it is clear that optimal intra-follicular androgen levels are required to allow the ovary to achieve full-potential oocyte yield.

Androgen levels are also of importance for oocyte meiotic maturation. Gill et al. showed that testosterone (250 nM) was capable of triggering meiotic resumption in mouse oocytes kept arrested *in vitro* in prophase I by phosphodiesterase inhibitor 3-isobutyl-1-methylxan- thine

(IBMX) (Gill et al., 2004). A study published more than 20 years ago, showed that short-term exposure of murine oocytes to supraphysiological doses of testosterone (μM range) clearly inhibited their capacity to resume meiosis, as well as post-fertilization embryo development (Anderiesz and Trounson, 1995). Romero et al cultured mouse follicle with FSH and aromatisable androgens and observed that meiotic maturation was compromised in follicles exposed to androgen concentrations above 200 nM (Romero and Smitz, 2010).

1.4.2 Genetic studies

Another approach to delineate AR actions in the ovary is provided by studying the phenotype of the female global androgen receptor knockout (ARKO) mouse. With the help of recent conditional gene-targeting approach three different mice have been generated, containing a targeted deletion of an exon of the AR. Their specific features were the subject of a detailed review by Walters, KA (Walters, 2015). ARKO^{Ex1} (Shiina et al., 2006), ARKO^{Ex2} (Hu et al., 2004) and ARKO^{Ex3} (Walters et al., 2007, Cheng et al., 2013) mice carry a heterozygous deletion of AR exon 1, 2 and 3 respectively. All global ARKO mice were subfertile (had fewer pups per litter), showed compromised oocyte and follicle development, altered gonadotrophin regulation and decreased ovulation rates (Walters, 2015).

Further, 2 granulosa cell specific ARKO mice were generated: GC-ARKO^{Ex2} (Sen and Hammes, 2010) and GC-ARKO^{Ex3} (Walters et al., 2012b), resulting in subfertility, longer oestrus cycles, increased atretic follicles, reduced ovulation rates and premature follicle depletion for the GC-ARKO ^{Ex2} mice. The oocyte specific ARKO mouse generated by Sen and Hammes (Sen and Hammes, 2010) exhibited maintained fertility. This means that nearly all reproductive defects observed in the female global ARKO mouse could be explained by the lack of AR in granulosa cells (Sen and Hammes, 2010).

1.5 Androgen excess and polycystic ovary syndrome (PCOS)

1.5.1 Introduction to PCOS

PCOS is the most frequent endocrine disorder in women and the major cause of oligoanovulation. PCOS is a heterogeneous condition, with a range of reproductive and long-term metabolic complications, that are further aggravated if obesity is present. It affects 5%-20% of women of reproductive age, depending on the diagnostic criteria employed, and manifests clinically predominantly with menstrual dysfunction and hirsutism (March et al., 2010, Sirmans and Pate, 2013).

1.5.2 Etiology and pathophysiology of PCOS

The etiology of PCOS is still unclear, but likely results from a genetic-environmental interaction.

Published case-control genetic studies have yielded multiple candidate genes, but due to the lack of large cohort studies, their individual importance remains elusive (Kosova and Urbanek, 2013). Genome-wide association studies have mapped genetic susceptibly loci in Chinese (Chen et al., 2011, Shi et al., 2012, Lee et al., 2015) and European ancestry women (Hayes et al., 2015) with PCOS phenotypes; several identified genes are involved in the regulation of gonadotropin secretion and action, underlying the crucial role of FSH and LH in the pathogenesis of PCOS.

The presence of obesity, sedentary lifestyle and unhealthy diet are the main environmental factors, aggravating the PCOS phenotype (Diamanti-Kandarakis et al., 2006a).

Hyperandrogenism and insulin resistance are cardinal features of PCOS (Figure 1-18).

1.5.2.1 Hyperandrogenism

Hyperandrogenemia is the most consistent feature in PCOS, and originates from the ovaries, adrenal gland, as well as peripheral tissue generation from DHEA and increased peripheral conversion of testosterone to DHT (Ehrmann et al., 1995, Stewart et al., 1990) .

Ovarian excess androgen production is related to an intrinsic abnormality of theca cell function, possibly resulting from enhanced steroidogenic enzyme activity (Nelson et al., 2001, Franks and Hardy, 2010). The gonadotrophin secretion is altered in PCOS, with a plasma ratio of LH to FSH levels that is characteristically increased. It is believed that oestrogens, derived from peripheral aromatization of androgens, exert positive feedback with pituitary hypersecretion of LH and negative feedback with hyposecretion of FSH (Rebar et al., 1976). The increased LH further drives thecal androgen production. The inappropriately low FSH levels are responsible for deficient dominant follicle selection, follicular arrest and anovulation (Hillier, 1994, Franks, 1995).

An adrenal contribution to androgen excess might exist in some patients, manifesting by increased circulating DHEA and DHEAS levels (Kumar et al., 2005). The adrenal androgen excess may result from qualitative changes in the androgen secreting enzyme CYP17A1, with increased 17,20 lyase activity, promoting androgen production, although this hypothesis could not be proofed *in vitro* (Azziz et al., 1998, Martens et al., 2000).

In simple obesity, almost one quarter of circulating testosterone originates from peripheral conversion of androstenedione, a reaction mediated by 17β-hydroxysteroid dehydrogenase (also called aldoketoreductase type 3, AKR1C3), mainly occurring in adipose tissue (Quinkler et al., 2004). New evidence points towards increased AKR1C3 activity in subcutaneous adipose tissue in women with PCOS, driving adipose androgen generation (O'Reilly et al., 2015). In women with PCOS, significantly increased 5α-reductase activity in

peripheral target tissues, such as in the liver, is a recognized contributor to the excess androgen production (Fassnacht et al., 2003). Increased 5α-reductase activity was found in both obese and nonobese PCOS women, and correlated with BMI, insulin levels and homeostasis model assessment (Stewart et al., 1990, Vassiliadi et al., 2009). Increased 5α-reductase activity was also observed in daughters of women with PCOS (Torchen et al., 2016), thereby amplifying androgen action in target tissues.

1.5.2.2 Insulin resistance

Insulin resistance is the diminished ability of insulin to mediate its metabolic actions for the production and uptake of glucose and the breakdown of lipids, resulting in a compensatory increased insulin secretion, called hyperinsulinemia (Kahn, 1985). Insulin resistance is present in up to 70% of the PCOS patients, in obese as well as lean patients (Brennan et al., 2009). In PCOS, the peripheral metabolic insulin resistance has a selective pattern, affecting muscle and adipose tissue but not the ovary (Diamanti-Kandarakis and Dunaif, 2012). Even in the presence of insulin resistance, insulin interacts with insulin-like growth factor to increase CYP17A1 activity and androgen production in the theca cells (Bergh et al., 1993)

Obesity is a known causative factor for insulin resistance, but seems to have an exacerbated effect in obese PCOS women compared to BMI-matched control women (Manneras-Holm et al., 2011). Abdominal, or central, obesity has an enhanced metabolic activity and is more detrimental for the development of insulin resistance, compared with subcutaneous obesity (Barber and Franks, 2013).

Androgen excess and insulin resistance are closely associated in PCOS. A mechanistic or causative link between both conditions has not been established yet. Abnormal kinase hyperphosphorylation of both the insulin receptor and CYP17A1, causing insulin resistance

and androgen excess respectively, has been investigated in fibroblasts of PCOS women, but this study failed to proof this hypothesis (Martens et al., 2000).

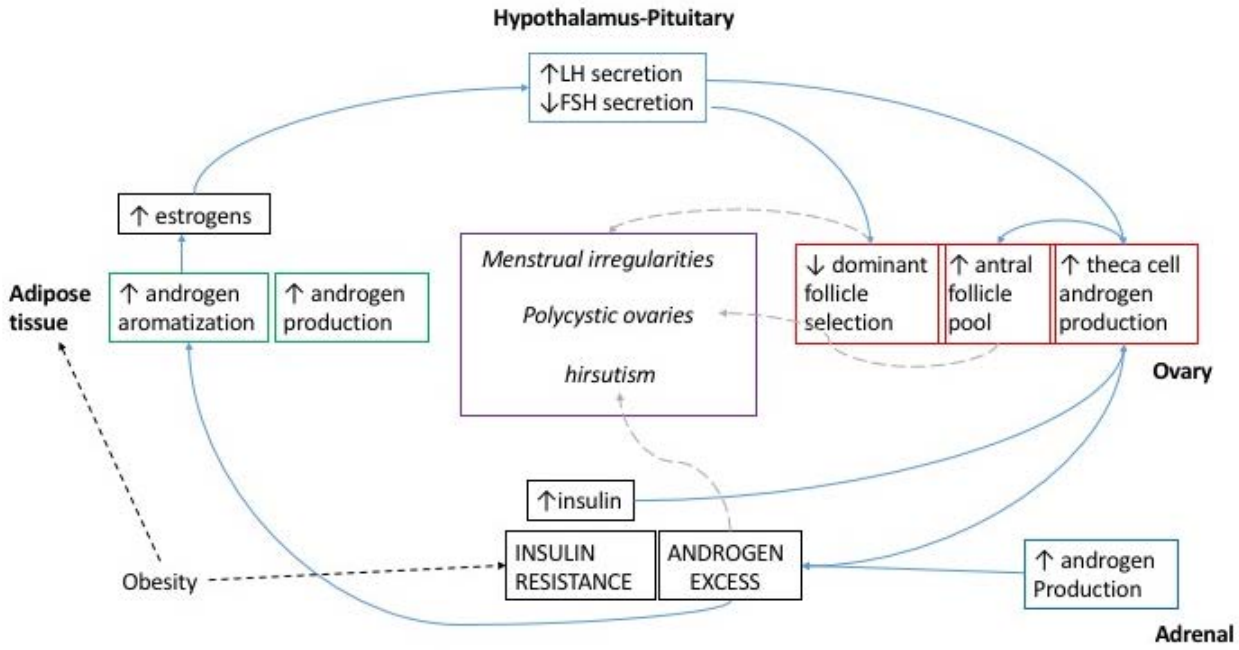


Figure 1-18 Current understanding of PCOS pathophysiology. Androgen excess and insulin resistance perpetuate the vicious cycle of increased ovarian and extra-ovarian androgen production and adipose tissue oestrogen generation. The latter results in deficient gonadotropin secretion, contributing to the ovarian hyperandrogenenism and the deficient dominant follicle selection, leading to chronic anovulation. Obesity reinforces the vicious cycle.

1.5.3 Diagnostic criteria

The Rotterdam criteria for the diagnosis of PCOS (Rotterdam, Eshre Asrm-Sponsored PCOS consensus workshop group), established in 2003 and revised in 2004 (Rotterdam, 2004), resulted from a general agreement on 3 characteristic features of PCOS. Because the condition is heterogeneous, not all patients are equally affected, and 2 out of the 3 following criteria are

required for diagnosis: clinical and/or biochemical signs of hyperandrogenism, menstrual disturbancies (oligo-or anovulation) and the presence of polycystic ovaries on ultrasound.

The Rotterdam criteria have extended the previously published National Institute of Health (NIH) criteria, focusing on hyperandrogenemia and menstrual irregularities, by adding a subgroup of patients with polycystic ovaries (PCO) on ultrasound, coupled with either signs of androgen excess or chronic anovulation. Recent guidelines for PCO diagnosis recommend the presence of ≥25 follicles on ultrasound, and an ovarian volume > 10ml (Dewailly et al., 2014b). In 2006, the Androgen Excess Society has reinforced the notion that hyperandrogenism is the cardinal feature of PCOS, and should be present at diagnosis, together with menstrual disorders and/or PCO (Azziz et al., 2006).

The diagnostic criteria for PCOS, summarized in Table 1-3, all have to be preceded by the exclusion of other causes of androgen-excess or related disorders, such as congenital adrenal hyperplasia (CAH) and its non-classical form, Cushing's disease, androgen-producing adrenal or ovarian tumours, idiopathic or racial hirsutism, hyperprolactinaemia and thyroid disorders (Sirmans and Pate, 2013, Pasquali et al., 2016a).

Table 1-3 Diagnostic criteria for PCOS, to be employed after exclusion of other causes of androgen excess or related disorders

Diagnostic criteria	NIH criteria 1992	Rotterdam criteria 2004	Androgen Excess Society criteria 2006
Clinical and/or biochemical hyperandrogenism	obligatory	2 out of 3 criteria required	obligatory
Oligo/amenorrhea	obligatory	2 out of 3 criteria required	1 out of the 2 remaining criteria required
Polycystic ovaries on ultrasound		2 out of 3 criteria required	1 out of the 2 remaining criteria required

Using the diagnostic criteria, four different combinations and PCOS phenotypes are determined: i) hyperandrogenism and oligoamenorrhea, ii) hyperandrogenism, polycystic ovarian morphology without oligoamenorrhea, iii) oligoamenorrhea and polycystic ovarian morphology without hyperandrogenism and iv) hyperandrogenism, oligoamenorrhea and polycystic ovarian morphology.

1.5.4 Genetics of PCOS

PCOS is a common and heritable disease, as observed in families and twin studies (Legro et al., 1998). Hyperandrogenemia is found in daughters of women with PCOS during puberty, which may result from genetic factors or epigenetic modifications of androgens in utero during specific developmental time-frames (Sir-Petermann et al., 2009) A large genome-wide association study in women with National Institute of Health PCOS diagnosis (hyperandrogenism and anovulation), of European ancestry has determined 3 loci of genetic susceptibility, associated with the highest risk for metabolic morbidities (insulin resistance and dysglycemia), and reproductive hormone levels (Hayes et al., 2015). The different loci discovered in this study are discussed below, and add to another list of PCOS susceptibility loci in Chinese patients diagnosed according to the Rotterdam criteria (Shi et al., 2012). It is considered that these variants only account for the minority of the PCOS-heritability (Hayes et al., 2015)

1.5.4.1 Chromosome 11p14. FSHB/ARL14EP locus

FSHB encodes the FSH β polypeptide, and was strongly associated with LH levels as well as PCOS diagnosis in European (Hayes et al., 2015) and Asian (Tian et al., 2016) women

. These findings strongly suggest that gonadotrophin dysregulation is involved in the etiology of PCOS. FSH levels are relatively deficient in PCOS and LH-secretion is increased, contributing to anovulation and androgen excess.

1.5.4.2 Chromosome 8p32.1 GATA4/NEIL2 locus

The GATA4 proteins are zinc-finger transcription factors that have promotors in many genes, and are involved in gonadal development (Gustin et al., 2016) and steroidogenesis (George et al., 2015). *NEIL 2* encodes a protein involved in DNA repair.

1.5.4.3 Chromosome 9q22.32 c9orf/FANCC locus

This locus has also been found to be associated with PCOS (Rotterdam criteria) in Han-Chinese (Shi et al., 2012) and encodes for proteins involved in proteolysis and activation of peptides (aminopeptidase O), and DNA-repair (FANCC) (Hayes et al., 2015)

1.5.5 Clinical signs and symptoms of PCOS

The Stein-Leventhal syndrome was first described in 1935 in 7 women with amenorrhea, hirsutism and bilateral polycystic ovaries (Stein, 1955). The long-standing debate and difficulties surrounding a consensus regarding diagnostic criteria for PCOS result from the heterogeneous clinical manifestations of this syndrome. In most cases, PCOS comes to clinical attention because of menstrual irregularities, leading to subfertility, or skin manifestations of hyperandrogenism, such as hirsutism or acne. In later life, the metabolic challenges come at the forefront of medical attention in PCOS women: insulin resistance and increased risk of type 2 diabetes, hepatic steatosis (Kelley et al., 2014) and an enhanced cardiovascular risk profile,

especially in the presence of obesity. Lifestyle measurements, such as weight reduction and exercise, are considered to be the most effective means to normalize the menstrual cycle and prevent metabolic complications (Jayasena and Franks, 2014).

1.5.5.1 Biochemical hyperandrogenism

Total serum testosterone concentration is the first-line laboratory test for diagnosing PCOS. Immunoassays, in particular direct platform assays, are largely inaccurate for measuring testosterone levels in the female range (Rosner et al., 2007), and mass-spectrometry based assays are now considered to be the most appropriate technique, but require age- and ethnicity-based reference ranges (Conway et al., 2014) Two recent studies have demonstrated the superior sensitivity and specificity of LC-MS/MS-measured androstenedione in diagnosing hyperandrogenism in women with PCOS, compared with testosterone (O'Reilly et al., 2014, Pasquali et al., 2016b). Free androgen index (FAI), calculated by the ratio between total testosterone and SHBG (Vermeulen et al., 1999) is the most sensitive measure of hyperandrogenism in PCOS (Conway et al., 2014). Circulating DHEA and DHEAS levels are heterogeneously affected among PCOS women, and are not conferred diagnostic value, but are related to varying degrees of adrenal contribution to androgen excess.

1.5.5.2 Clinical hyperandrogenism

Clinical signs of androgen excess are hirsutism, excess hair growth, in a male-type pattern, acne and androgenic alopecia (Legro et al., 2013). Hirsutism is a common feature, and present in up to three quarter of women with PCOS (DeUgarte et al., 2006). The severity of hirsutism is recorded clinically using the modified Ferriman-Gallwey index (Yildiz et al., 2010)

whereby terminal hair growth and distribution is examined in 9 areas of the body and a score of > 8/36 is considered pathological (Figure 1-19). This score has been criticized, because of the large variability of the scoring system with poor agreement between patient and medical staff observations (Wild et al., 2005). Younger age, larger waist circumference and higher serum testosterone levels were independent predictors of hirsutism in an observational study of 1297 PCOS patients (Panidis et al., 2013).

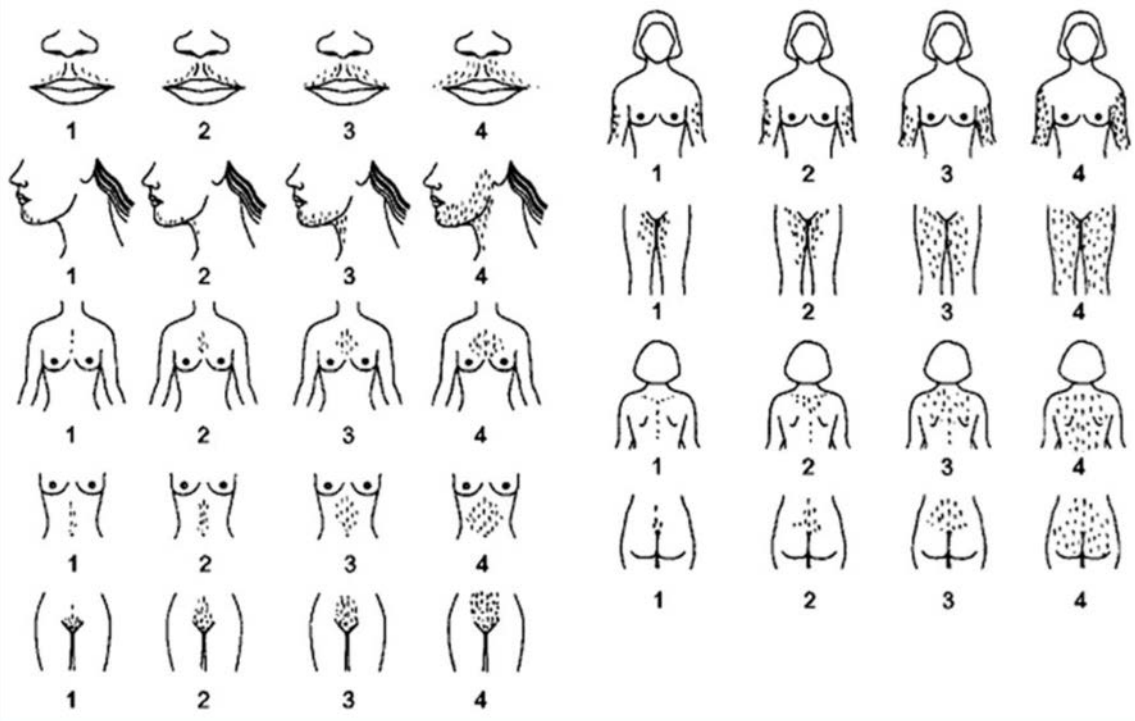


Figure 1-19 Modified Ferriman-Gallwey score. Terminal hair growth is scored from 1 (minimally present) to 4 (equivalent to hairy male), in 9 body areas: upper lip, chin, chest, arm, upper and lower abdomen, lower back and tights. If no terminal hair is present in a specific area, the score is 0. A sum of scores > 8, is considered as clinically relevant hirsutism. Reprinted from Yildiz, with kind permission of Oxford University Press, Copyright 2010.

A recent study observed that the modified Ferriman-Gallwey score did not correlate with blood total testosterone levels, which indicate that local androgen metabolism at the

pilosebaceous unit, dependent on local steroidogenic enzyme expression, circulating androgen levels and local androgen receptor expression, determine the severity of hirsutism (Pasquali et al., 2016b).

Mild hirsutism requires local cosmetic treatment (waxing or shaving), and topical facial Eflornithine cream inhibiting hair growth, which may reduce the frequency of hair removal. An alternative consists of permanent photo-epilation therapy by laser (Legro et al., 2013).

The addition of systemic treatments, aiming to reduce androgen production or decrease the sensitivity of the hair follicle to androgens, are recommended for severe hirsutism (Jayasena and Franks, 2014). Both the ESHRE/ASRM (Fauser et al., 2012) and the Endocrine Society (Legro et al., 2013) guidelines suggest oral contraceptive pills as first line systemic treatment to improve hirsutism, acne and menstrual irregularities for those PCOS patients not seeking fertility. The first generations of progestin were related to testosterone and had varying degrees of androgenic effects, while the recent progestins are designed to have progesterone-receptor activity and have no or even anti-androgenic effects (such as drosperidone or cyproterone acetate) (Yildiz, 2015).

1.5.5.3 Oligo/Amenorrhea

Women with PCOS experience uterine bleedings that are unpredictable, with regard to the onset and the duration of the periods (Burgers et al., 2010). This reflects the disturbed menstrual pattern, with dysfunctional follicle maturation and failure of the emergence of a dominant follicle, resulting in chronic anovulation (Franks, 1995). Circulating oestrogen levels in PCOS women are normal or even increased, resulting in normal endometrial proliferation, and the vaginal bleedings are frequently oestrogen-breakthrough bleedings. Secondary

amenorrhea refers to the absence of menstruation for ≥ 6 months in a women with previous periodic bleedings, while oligomenorrhea is used to design < 9 menstruations per year.

Lifestyle measurements and weight loss (5-10% of total body weight) are the primary treatment in overweight and obese PCOS patients, and have the ability to restore menstrual regularity and ovulation (Moran et al., 2009). If weight reduction is unsuccessful, and fertility is not warranted, low dose combined oral contraceptive pills have the benefit of providing regular withdrawal bleedings (Fauser et al., 2012, Legro et al., 2013). Oral contraceptives (OC) contain a combination of low dose of oestrogens, generally ethinyl estradiol in a dose of 20-35 μg and a progestin (0.1-0.3 mg). Oestrogens and progestins in OC exert negative feedback on the hypothalamus, with suppression of FSH and LH secretion, and subsequently absence of dominant follicle secretion, ovulation, endometrial priming and fertile cervical mucus secretion (Christin-Maitre, 2013). The hormonal preparations might have adverse cardiovascular and metabolic outcomes in some PCOS patients, such as the occurrence of venous thrombosis or the increased risk of diabetes (Yildiz, 2015). To prevent these complications, a thorough patient and family history and clinic examination is required, before prescribing OC. The World Health Organisation (WHO) has published Medical eligibility criteria for contraceptive use (Yildiz, 2015).

Metformin, an insulin sensitizer, in combination with weight loss, can ameliorate ovulation rates in women with PCOS (Moghetti et al., 2000).

1.5.5.4 Anovulation and infertility

Oligomenorrheic women with PCOS have a reduced fecundability, meaning that they may take a longer time to conceive, but their final family size is comparable to that of the population as a whole (Fauser et al., 2012). Chronical anovulation and amenorrhea however,

leads to infertility in approximately 40% of women with PCOS (Sirmans and Pate, 2013). In most instances, ovulation can be induced pharmacologically, if lifestyle measures have proven to be ineffective. Clomiphene citrate is the first line treatment (Legro et al., 2013). It is an orally administrated oestrogen receptor antagonist, that is given in the first days of the menstrual cycle to decrease the negative feedback of oestrogen on gonadotropin secretion. At the adequate dose, it has the ability to raise plasma FSH levels to adequate follicular phase levels, and stimulate ovarian follicle maturation (Thessaloniki, 2008). Metformin is sometimes used in combination with Clomiphene citrate, but its benefit in ovulation induction is not proven (Jayasena and Franks, 2014). In a double blind randomized controlled trial, grouping 288 PCOS patients treated in multiple centers, ovulation rates following clomiphene treatment were 72%, and following clomiphene plus metformine 64% (Moll et al., 2006). Alternative ovulation induction techniques are low dose gonadotrophin stimulation, laparoscopic ovarian drilling (Hendriks et al., 2014), or IVF.

1.5.5.5 Metabolic risks

Obesity is a common, but not necessary, feature of PCOS, and when present, it aggravates the adverse metabolic profile of the patient, because of its independent association with type 2 diabetes mellitus and cardiovascular morbidity (Galani and Schneider, 2007). Insulin resistance and subsequent hyperinsulinemia drive androgen excess and lay the basis for the increased risk for developing glucose intolerance and type 2 diabetes later in life (Moran et al., 2010). However, overt deterioration of glucose metabolism is almost uniquely observed in obese women with PCOS, with BMI levels > 30 kg/m^2, while lean PCOS women display intrinsic insulin resistance, but to a less extent compared with their obese peers (Pasquali and Gambineri, 2013)

Women with combined hyperandrogenemia, anovulation and polycystic ovarian morphology, the most severe form of PCOS, are clearly at increased risk developing cardiovascular events (Wild et al., 2010). These patients generally have increased body weight, but still have a higher degree of central adiposity, dyslipidaemia and insulin resistance compared with age- and BMI-aged controls (Fauser et al., 2012). However, PCOS is a highly heterogeneous condition, and affected women can move between phenotypes depending on alterations in body weight, diet and exercise, with subsequent modifications of their metabolic risk profile (Welt and Carmina, 2013).

It has become recently clear that androgen excess is an independent driver of metabolic risk, and that increasing severity of androgen excess is associated with higher prevalence of insulin resistance and adverse metabolic risk profile (O'Reilly et al., 2014, Pasquali et al., 2016b).

1.5.6 Ovarian manifestations of PCOS

1.5.6.1 Intrinsic deregulation of early follicular development

The characteristic histological feature of PCOS ovaries is an increased number of growing follicles, with the presence of atretic cystic follicles, in an enlarged collagenous stroma (Hughesdon, 1982). A similar ovarian phenotype is found in patients with adrenal androgen excess, such as congenital adrenal hyperplasia (White and Bachega, 2012, London, 1987), indicating that androgens are essential contributors to the exaggerated follicle development. Morphological studies indicate that the dysfunctional follicular development in PCOS starts at the early gonadotrophin independent stages. Ovarian cortical biopsies from anovulatory PCOS women showed a 6 times increase in density of pre-antral follicle numbers compared to normal

ovaries, including primordial and primary follicles (Webber et al., 2003). The balance between resting and growing follicles in PCOS is clearly disturbed, but the underlying mechanisms remain poorly understood (Franks and Hardy, 2010). AMH, known to play a role in suppressing primordial follicle recruitment, has been implicated in this process. Immunostaining for AMH in cross-sections of normal and PCOS ovaries have shown that primary and transitional follicles from ovaries of anovulatory PCOS women express less AMH protein (Stubbs et al., 2005). The relative AMH-deficiency in the early stage PCOS follicles could possibly contribute to the increased follicle activation. On the other hand, studies have shown, that, when sustained in *in vitro* culture, PCOS follicles exhibit decreased atresia rates, and survived longer than normal follicles (Webber et al., 2007). Decreased atresia and prolonged longevity would be another explanation for the increased pool of growing follicles observed in PCOS. However, *in vivo*, atresia rates in PCOS ovaries were comparable with normal follicles (Maciel et al., 2004).

The endocrine cells of PCOS follicles show signs of intrinsic deregulations. Human granulosa and theca cells from normal cycling women and PCOS patients exhibit different behavior in culture, especially with regard to their gonadotrophin sensitivity and the response to insulin. Granulosa cells from anovatory PCOS women were steroidogenically more active and produced 6-10 times more estradiol in response to FSH than normal granulosa cells (Mason et al., 1994). This is probably explained by the increased expression of FSHR on PCOS granulosa cells, induced by the local androgen excess (Mason et al., 1994). PCOS theca cells showed a more than 10-fold increase in basal and LH-induced androstenedione production (the main ovarian androgen secreted *in vivo*) during primary culture, compared with normal theca cells (Gilling-Smith et al., 1994). Production of progesterone, 17-OH progesterone, DHEA and testosterone were also increased, in basal condition, and this was more pronounced following forskolin-stimulation, mimicking LH-induction (Nelson et al., 1999). Moreover, mRNA

expression studies in PCOS-derived theca cells demonstrated increased expression of steroidogenic enzymes CYP11A1, CYP17A1 and 17β-HSD in human theca cells, and the activity of CYP17A1, 3β-HSD and 17β-HSD was increased compared with theca cells obtained from normally cycling women (Nelson et al., 1999). Normal small pre-antral follicles were found to become responsive to LH-stimulation when they reached 9.5-10 mm diameter, while the PCOS follicles showed premature LH-responsiveness, that became obvious at diameters of only 4 mm (Willis et al., 1998). The androgen excess and the inadequate response to LH override the FSH-induced aromatase expression and prematurely switched the granulosa cell steroid outcome from oestrogen to progesterone (Willis et al., 1998).

Furthermore, 5α-reductase activity significantly increased in PCOS follicles compared with control follicles (Jakimiuk et al., 1999), and the accumulation of 5α-reduced androgen further suppressed aromatase activity (Agarwal et al., 1996).

Insulin, at physiological doses, interacts with insulin-like growth factor to increase CYP17A1 activity and androgen production in ovarian theca cells (Bergh et al., 1993). Insulin resistance, frequently associated with PCOS, results in compensatory hyperinsulinaemia, which in turn fuels the androgen hypersecretion (Diamanti-Kandarakis and Dunaif, 2012). As mentioned earlier, insulin resistance in PCOS appears to be selective. Studies in human granulosa-lutein cells, obtained at egg retrieval following gonadotrophin-stimulation in the setting of IVF, have shown that ovarian steroidogenesis in PCOS remained responsive to the stimulatory effects of insulin (Rice et al., 2005). However, the metabolic effects of insulin, i.e. glucose uptake and utilization, were decreased in granulosa-lutein cells obtained from anovulatory PCOS women (Rice et al., 2005) or even absent in PCOS women with diagnosed insulin resistance (Fedorcsak et al., 2000).

1.5.6.2 Stromal hyperplasia, rigidity, hypervascularity, and inflammation

Histological examination of polycystic ovaries revealed a highly increased thickness of the cortical, but mostly the subcortical medullar stroma, associated with hypervascularity (Hughesdon, 1982). The enlarged ovarian stromal volume can be measured by ultrasound and correlates with the degree of hyperandrogenism (Fulghesu et al., 2007, Kyei-Mensah et al., 1998). Microarray data from PCOS ovarian tissue showed differential expression of genes known to be involved in extracellular matrix organization (Jansen et al., 2004). On the protein level, several molecules involved in fibrogenesis were upregulated in PCOS stroma (Ma et al., 2007). Increased levels of basic fibroblast growth factor (bFGF) were measured in serum and follicular fluid of PCOS women (Artini et al., 2006). The enhanced fibroblast proliferation and extracellular matrix deposition results in increased rigidity of the ovarian cortex (Lebbe and Woodruff, 2013). An important research question is whether the stromal hyperrigidity contributes to the intrinsically altered steroidogenic behavior of the antral follicles in PCOS (Woodruff and Shea, 2011). Although 'rigid' or non-permissive culture conditions in murine *in vitro* follicular culture were associated with decreased steroid production in multi-layered follicle (Xu et al., 2006b), the effect of rigidity on smaller follicles has not been studied yet.

The blood flow velocity, examined by color Doppler ultrasound, was greatly increased in polycystic ovaries (Zaidi et al., 1995), mainly in the cortex (Delgado-Rosas et al., 2009). Circulating vascular endothelial growth factor (VEGF) levels were typically increased in PCOS women, and were correlated with the Doppler measurements of ovarian stromal blood flow (Agrawal et al., 1998). Laparoscopic ovarian drilling, a surgical technique aiming to reduce the ovarian volume, reduced Doppler measurements of ovarian stromal blood flow (Parsanezhad et al., 2003), with concomitant decrease of plasma levels of VEGF (El Behery et al., 2011) and androgens (Kaaijk et al., 2000). The increased cortical blood flow in PCOS maintains the

exaggerated connective tissue proliferation and, importantly, supplies the enclosed early-staged follicles with inadequate amounts of oxygen, metabolic and endocrine factors (Lebbe and Woodruff, 2013). The profoundly altered follicular microenvironment in PCOS is an important disruptor of the normal follicle dynamics (Figure 1-20).

PCOS is also characterized by a chronic low-grade inflammatory process, which is more prominent in the presence of obesity, and manifests with elevated plasma levels of C-reactive protein (CRP), inflammatory cytokines (IL-6 and others), and leucocytes (Diamanti-Kandarakis et al., 2006b). The contribution of these inflammatory parameters in the pathogenesis of PCOS remains unclear. It has been postulated that, as follicle become activated to grow, it chemically attracts stromal macrophages, which stay associated with this follicle throughout its maturation (Tingen et al., 2011). In benign prostate hypertrophy, stromal hyperplasia is partly mediated through the infiltration of macrophages, and their recruitment is described as an androgen-dependent mechanism (Izumi et al., 2013). Similar epithelial-stromal interactions might possibly play a role in the pronounced stromal hyperplasia occurring in PCOS (Lebbe and Woodruff, 2013).

The communication between the ovarian follicular and stromal compartment is a crucial notion that contributes to the complex pathogenesis of PCOS (Lebbe and Woodruff, 2013), (Figure 1-20).

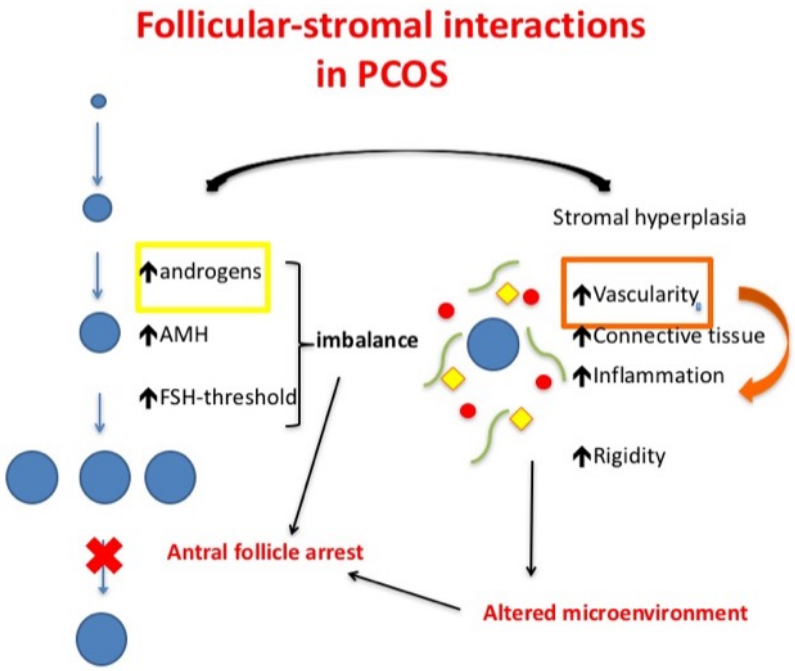


Figure 1-20 Working model for follicular-stromal interactions in the pathogenesis of PCOS. Follicular arrest results from the imbalance between androgens, FSH and AMH. Circulating FSH-levels are inappropriately low and dominant follicle selection fails. The PCOS cortex shows signs of remodeling, partly mediated by increased local androgen concentrations, and maintained by increased cortical blood flow. Reprinted and adapted from Lebbe et al, with kind permission of Oxford University Press. Copyright 2013.

1.5.6.3 Oocyte quality in PCOS

The oocyte quality in PCOS has mainly been studied in the context of assisted reproduction, in infertile PCOS women not responding to ovulation-induction by clomiphene. As mentioned earlier, PCOS is characterized by an increased FSH sensitivity. During controlled ovarian stimulation, the requirement for exogenous gonadotrophins is reduced (Sahu et al., 2008, Mulders et al., 2003). To diminish the risk of ovarian hyperstimulation syndrome (OHSS) low-dose gonadotropin protocols have been developed for PCOS, facilitating the development of an appropriate amount of follicles (Kumar et al., 2011). Nevertheless, at the time of egg

retrieval, the yield is typically increased in PCOS, compared with non-PCOS controls (Heijnen et al., 2006, Weghofer et al., 2007). The fertilization potential of PCOS oocytes is decreased and indicative of compromised oocyte quality (Urman et al., 2004). The chromosomal organization and euploidy rates appear to be normal in PCOS (Sengoku et al., 1997, Weghofer et al., 2007). But the microenvironment of the oocyte, the follicular fluid, contains hallmarks of metabolic dysregulation contributing to the impaired oocyte quality, and more pronounced in obese PCOS patients, with elevated free androgen index and free fatty acids (Niu et al., 2014). The cytoplasmic oocyte development is clearly altered in PCOS, and reflected by a distinctly abnormal gene expression profile (Wood et al., 2007).

The increased oocyte yield counter-balances the reduced oocyte quality and the final result is similar clinical pregnancy and live birth rates between PCOS patients and controls following assisted reproduction (Bailey et al., 2014, Weghofer et al., 2007, Heijnen et al., 2006, Urman et al., 2004). A recent retrospective study reported decreased pregnancy and live birth rates in obese PCOS patients compared with lean PCOS patients (Bailey et al., 2014).

In vitro maturation (IVM) has recently become an alternative for standard assisted reproductive techniques, IVF and intracytoplasmatic sperm injection (ICSI) (Lindenberg, 2013). The main aim of this technique is to avoid the risk of ovarian hyperstimulation syndrome in PCOS, as the oocytes are retrieved from unstimulated cycles, but the success rates in terms of live birth are inferior to IVF (Das et al., 2014, Walls et al., 2015). Another concern regarding *in vitro* maturation, is the presence of high rates of meiotic abnormality in the immature oocytes, but no difference is observed in chromosomal and spindle configuration between PCOS and control oocytes (Zhu et al., 2015).

1.5.7 Animal models of female androgen excess

1.5.7.1 Rodent models

Patient material for research is scarce in PCOS, and *in vivo* research faces ethical limitations. Hence, the use of animal models reproducing some of the reproductive and metabolic features of PCOS is of invaluable aid in the study of the pathogenesis and management of PCOS. Currently, rodent, sheep and non-human primate models of PCOS have been extensively studied (Caldwell et al., 2014). Rodent models have the advantage to be affordable, require easy logistical care, have a short reproductive cycle, brief gestational periods, and can be genetically manipulated (Caldwell et al., 2014).

Numerous rodent models have been generated over the last 50 years, to mimic one or more of the PCOS traits in women, employing a variety of treatments and manipulations, and are reviewed in (Walters et al., 2012a). Hyperandrogenism is the most consistent PCOS feature, and Table 1-4 summarizes the phenotypes of the recent rodent models employing prenatally or postnatally administered testosterone or DHT.

Table 1-4 Ovarian and extra-ovarian phenotype in rodent PCOS models, generated by administration of pre-natal or postnatal testosterone (T) or dihydrotestosterone (DHT). Abreviations: estradiol (E2), progesterone (P4), luteinizing hormone (LH).

Reference	Type of rodent and treatment	Intra-ovarian effects	Extra-ovarian effects
(Sullivan and Moenter, 2004)	Mouse, prenatal DHT	Irregular oestrous cycles	↑T and ↑LH
(Beloosesky et al., 2004)	Rat, postnatal T	Cysts, ↑↑pre-antral follicles	Delayed puberty, ↓P4,↑oocyte

			degeneration, insulin resistance
(Wu et al., 2010b)	Rat, prenatal T and DHT	Irregular oestrous cycles, ↑pre-antral and antral, ↓pre-ovulatory follicles, ↓corpus luteum	↑T, ↑P4, ↑E2, ↑LH
(Roland et al., 2010)	Mouse, prenatal DHT	Irregular oestrous cycles	↑T ↑LH ↑fasting glucose, impaired glucose tolerance, ↑size of visceral adipocytes
(van Houten et al., 2012)	Mouse, postnatal DHT	Anoestrous, ↑atretic follicles, cysts	↑body weight, ↑size of adipocytes, ↑leptin, ↓adiponectin, glucose intolerance
(Moore et al., 2013)	Mouse, prenatal DHT	Irregular oestrous cycles, ↑antral follicles,↓ovulation	↑T, ↑LH
(Caldwell et al., 2014)	Mouse, prenatal DHT	Irregular cycles,↓ovulation,	Adipose hypertrophy

	Mouse, postnatal long-term DHT	↓preantral follicle health Plus ↓antral follicle health and acyclicity	Plus ↑body fat, dyslipidemia

It is important to keep in mind that no single model entirely replicates the full-blown spectrum of PCOS in women. The phenotype heterogeneity of the rodent models is dependent on the time, duration and dose of androgen exposure and the use of aromatisable testosterone likely to induce oestrogen-dependent events. The use of long-term (>3 weeks) DHT in the postnatal pre-pubertal mouse induces most of the PCOS traits found in women (van Houten et al., 2012, Caldwell et al., 2014).

1.5.7.2 Non-human primate models

In utero exposure of non-human primates to androgens has generated model animals that exhibit striking similarities to PCOS women, with regard to anovulation, polycystic ovarian morphology, hyperandrogenism, LH hypersecretion and insulin resistance. These primate studies are highly informative, but come with an elevated cost, and require long developmental periods, and are, in contrast to rodents, not amendable to genetic engineering (Caldwell et al., 2014).

Table 1-5 highlights the ovarian and systemic characteristics of the reported primate PCOS models, generated by gestational or postnatal androgen exposure.

Table 1-5 Ovarian and systemic characteristics on primate models of PCOS, generated by prenatal or postnatal administration of testosterone (T) or dihydrotestosterone (DHT). Abbreviations: progesterone (P4), luteinizing hormone (LH) and Western Style Diet (WSD).

Reference	Treatment	Intra-ovarian features	Extra-ovarian features
(Dumesic et al., 1997)	Prenatal T	none	↑LH and ↑LH/FSH
(Vendola et al., 1998)	Postnatal T and DHT	↑number of antral follicles, enlarged ovaries	N/A
(Abbott and Bacha, 2013)	Prenatal T in early gestation	Oligomenorrhea, enlarged polyfollicular ovaries	↑LH, insulin resistance, abdominal obesity, hyperlipidaemia
(McGee et al., 2012)	Postnatal pre-pubertal T	No effect on ovarian morphology, menstrual cyclicity or ovulation rates	↑ LH pulse frequency, no clear metabolic features
(McGee et al., 2014)	Postnatal pre-pubertal T, and Western Style Diet (WSD) to induce obesity	WSD ± T result in ↑ number of small antral follicles and ↓ maximum follicle size. T + WSD: ↑number of small antral follicles in the luteal phase and ↓P4	WSD increased body fat from <2 to 15-19% LH pulse frequency ↑ in control to similar levels as T-treated animals, LH pulse amplitude ↓ WSD ± T ↓insulin sensitivity after 1 year
(Bishop et al., 2015)	Western-Style Diet (WSD),	WSD ± T result in ↑numbers of small antral follicles, ↑number	differential gene expression for steroid,

	with and without pre-pubertal T treatment	of atretic follicles, ↑CYP17A1 staining, differential gene expression for ovarian pathways	carbohydrate, lipid metabolism pathways

Adult primates, exposed during gestation with testosterone, exhibit a variety of ovarian, hormonal and neuro-endocrine PCOS traits (Dumesic et al., 1997, Abbott et al., 2013). These studies raise the question of a fetal origin in PCOS, and to whether in utero exposure to androgen excess, coupled with hyperinsulinaemia and/or hyperglycemia, might predispose or aggravate PCOS in the offspring (Abbott and Bacha, 2013). This interesting hypothesis is challenged by a large prospective human cohort study, failing to observe a relationship between maternal or umbilical cord androgen concentrations and incidence of adolescence PCOS in the child (Hickey et al., 2009). However, the animal studies teach us that the time of androgen exposure during gestation is critical for the outcome of the offspring. Early-mid gestational androgen exposure resulted in LH hypersecretion and adverse metabolic outcomes (such as increased abdominal adiposity and insulin resistance), while androgen excess during late gestation provoked no LH-hypersecretion and induced a milder metabolic risk profile (Walters, 2015).

Feeding Rhesus monkeys with Western-style diet, rich in lipids and carbohydrates, generated obesity, which in turn, independent of androgen levels, provoked some PCOS features, such as LH hypersecretion and exaggerated small antral follicle development. However, obesity combined with androgen excess, clearly exacerbated the PCOS phenotype, inducing increased ovarian androgen secretion, luteal insufficiency, insulin-resistance, and altering the metabolic gene expression (Xu et al., 2015, Bishop et al., 2015, McGee et al., 2014).

In vitro alginate-encapsulated culture revealed that pre-antral follicles from Western-diet fed androgen-excess monkeys had impaired survival, decreased oestrogen and AMH production and produced meiotically-incompetent oocytes (Xu et al., 2015).

1.6 Reproductive dysfunction in androgen deficiency syndromes

1.6.1 Adrenal androgen deficiency

1.6.1.1 Manifestations and diagnosis of adrenal deficiency

There are two forms of adrenal insufficiency: primary and secondary failure. Primary adrenal failure (PAI) or Addison's disease is a rare disorder, affecting predominantly women between 30 and 40 years of age, and resulting from auto-immune destruction of the steroidogenic cells in the adrenal cortex (Lebbe and Arlt, 2012). Secondary adrenal insufficiency results from deficient adrenocorticotropic hormone (ACTH) production, due to hypopituitarism related to a hypothalamic-pituitary disease or to negative feedback suppression of ACTH by pharmacological glucocorticoid treatment (for asthma or rheumatic disease for example) (Arlt and Allolio, 2003).

Adrenal insufficiency manifests with signs and symptoms related to a decreased levels of glucocorticoid, mineralocorticoid (in PAI only) and adrenal androgen hormones, and their intensity depends on the onset and the extent of adrenal hypofunction. Classical symptoms include asthenia and excessive fatigability, gastro-intestinal complaints, weight loss, arterial hypotension and hyperpigmentation (in PAI only) (Table 1-6). Occasionally PAI presents with a life-threatening adrenal crisis, a fulminating shock with severe hypotension, acute abdominal pain, vomiting and increased body temperature (Arlt and Allolio, 2003).

Table 1-6 Clinical presentation of primary and secondary adrenal insufficiency, with symptoms related to deficient production of glucocorticoid, mineralocorticoid and adrenal androgen synthesis.

Signs or Symptoms in Adrenal Insufficiency
a. Related to Glucocorticoid Deficiency • Weakness • Asthenia, fatigue • Weight loss • Hypoglycemia
b. Related to Mineralocorticoid Deficiency (occurs in PAI only) • Anorexia, nausea, vomiting, abdominal pain • Orthostatic hypotension, syncope • Salt craving • Hyponatremia, low bicarbonate levels • Hyperkalemia
c. Related to Adrenal Androgen Deficiency • Low energy levels, decreased well-being • Reduced libido • Decrease in axillary and public hair
d. Cutaneous and mucosal hyperpigmentation (in PAI only), related to an increase in proopiomelanocortin (POMC)

The diagnosis of adrenal insufficiency relies on the demonstration of a decreased adrenal glucocorticoid reserve following exogenous ACTH stimulation in the presence of a suggestive

clinical context. In routine clinical practice, the short synacten test (SST) is considered a safe and reliable tool with established predictive diagnostic value. The cut-off for adrenal insufficiency is usually defined as plasma cortisol levels of < 500 nmol/L (18 μg/dL) 30 or 60 minutes after 250 μg ACTH administration (Bornstein et al., 2016). Plasma ACTH levels further distinguish between primary (high ACTH) and secondary (low ACTH) forms of adrenal insufficiency.

1.6.1.2 Management of adrenal insufficiency

All patients with adrenal insufficiency require long-life hormonal treatment and surveillance, to ensure replacement of the physiological daily cortisol and aldosterone production (Arlt, 2009). In case of adrenal crisis, emergency hydrocortisone injection and fluid administration are life-saving (Arlt, 2009). A trial of adrenal androgen replacement, that can be achieved by 25-50 mg oral DHEA daily, can be tried in women with PAI suffering from low libido, decreased energy levels, or depressive symptoms, despite optimal glucocorticoid and mineralocorticoid hormone replacement (Bornstein et al., 2016), although the Endocrine Society guideline do not recommend this option, because of the paucity of data supporting efficacy and long-term safety (Wierman et al., 2014a). Patients diagnosed with adrenal insufficiency should remain under specialized care and benefit from adequate education regarding their disease and treatment needs.

1.6.1.3 Fertility issues in primary adrenal insufficiency

Subfertility is commonly referred to as the inability for a couple to achieve pregnancy after at least 12 months of regular unprotected sexual intercourse (Zegers-Hochschild et al.,

2009). Subfertility is caused by a variety of male and female factors. The diagnosis of PAI in a women of reproductive age has repercussion on female fertility (Lebbe and Arlt, 2012). In a national Norwegian survey reporting on 269 female patients with PAI, a significant decline in fertility was observed; the standardized incidence ratio (SIR) of live birth was 0.97 before the diagnosis of PAI, and dropped to 0.69 after establishment of the diagnosis. Eight % of females experienced premature ovarian insufficiency following PAI, but even when excluding this subgroup, fertility in PAI women remained reduced at 0.72 (Erichsen et al., 2010). An Italian cohort study of 258 female PAI patients, from which 52 (20.2%) developed premature ovarian insufficiency, confirmed the strong association between both conditions; and observed increased prevalence in patients harboring an autoimmune polyendocrine syndrome (APS) (Reato et al., 2011).

Premature ovarian failure is defined as hypergonadotrophic amenorrhea, with hypogonadism and infertility, occurring in a woman before the age of 40 (Goswami and Conway, 2007). Autoimmune premature ovarian failure is characterized by the presence of autoimmune antibodies directed against steroidogenic enzymes, such as 21-hydroxylase, 17-hydroxylase or side-chain cleaving enzyme; leading to progressive exhausting of the follicular pool (Falorni et al., 2012). This type of premature ovarian failure is found almost exclusively in association with auto-immune PAI (Hoek et al., 1997), but might not be the sole factor responsible for decreased fertility rates found in these patients. It is firmly established that Addison's patients suffer from impaired subjective health, and increased mortality (Bancos et al., 2015) which are likely to impact on reproductive function.

The relative contribution of decreased adrenal DHEA production on ovarian function and the implications for subfertility in PAI remain unknown. Controlled clinical trials studying the effects of DHEA replacement on reproductive outcomes in women with PAI are currently

lacking. A recently published clinical case relates a spontaneous pregnancy following DHEA replacement in a previously infertile Addison's patient with severe hypoandrogenism (Gleicher et al., 2016).

1.6.2 Physiological androgen deficiency

1.6.2.1 Age-related decline in circulating androgen levels and in ovarian reserve

As illustrated in Figure 1-4, circulating DHEAS levels peak in the second decade, followed by a progressive decline with age. This age-dependent decline in DHEAS level is parallel, but more pronounced, compared with the decrease in serum testosterone (Orentreich et al., 1984), and also shows important inter-individual variability (Spencer et al., 2007), suggestive of a genetic influence.

The ovarian reserve consists of the resting pool of primordial follicles, which is the most abundant pool of follicles, that diminishes over time by atresia and entry into the growing follicle pool. In routine clinical practice, the growing pool of follicles is assessed by measuring early follicular phase FSH levels, circulating AMH levels or by antral follicle count using ultrasound measurement (Dewailly et al., 2014a). It is important to realize that these surrogate markers only target the pool of growing follicles, as the primordial follicle pool cannot be clinically assessed. A woman's ovarian reserve is compared to age-related reference values and classified as normal for age, or low (low ovarian reserve, LOR).

The DHEAS curve over time mimics closely the natural decline in fecundability observed over a woman's reproductive lifespan; with a significant decrease beginning around the age of 32 years and a steep decline observed after age 37 (Faddy et al., 1992). Indeed, age-related reduction in androgen levels parallels decrease in antral follicle count and circulating levels of

AMH (Nardo et al., 2009). The decrease in AMH and Inhibin B concentrations with advancing age is associated with a progressive increase in circulating levels of FSH (American College of et al., 2014). Advancing maternal age is characterized by decreased oocyte quality, as manifested by increasing aneuploidy and miscarriage rates (Balasch and Gratacos, 2012).

1.6.2.2 Low ovarian reserve and poor ovarian response

The diminished fertility potential with advancing age as most important determinant is termed low ovarian reserve (LOR), sometimes also referred to as premature ovarian ageing. If the abnormal ovarian reserve is accompanied by a raise of FSH into the menopausal range (> 40 IU/l) in a women younger than 40 years of age, the diagnosis of premature ovarian failure (POF) or primary ovarian insufficiency is made (Goswami and Conway, 2007).

Patients with low ovarian reserve do almost invariably have low success chances to achieve pregnancy spontaneously, and their subfertility is mostly addressed through *in vitro* manipulation of gametes and embryos, in a variety of treatments commonly called assisted reproductive techniques (ART); IVF is one of them (International Committee for Monitoring Assisted Reproductive et al., 2009). Although the overall success rates in achieving live birth with IVF are around 20% to 50% (Zegers-Hochschild et al., 2009), women with low ovarian reserve respond poorly to IVF; their outcomes are characterized by poor follicular development following gonadotropin stimulation, low oocyte yield, decreased oocyte quality and low pregnancy and live-birth rates (Reproductive et al., 2011). Poor ovarian response is the term used to indicate a reduced follicular response to ovarian stimulation, yielding a decreased numbers of oocytes for assisted reproduction (American College of et al., 2014). Poor ovarian response is defined by the presence of 2 out of 3 of the following criteria: advanced maternal

age, a previous episode of poor ovarian response, or an abnormal ovarian reserve test (AMH or antral follicle count) (Ferraretti et al., 2011).

1.6.2.3 Androgen treatment in low ovarian reserve

In the last decade androgen treatment has been used in reproductive medicine to 'boost' early follicle growth and development in the context of low ovarian reserve, and subsequently improve the success rates for live birth following ART. The aim of androgen treatment is to benefit from the described evidence of AR-mediated stimulatory effect on preantral follicle growth and survival, and the priming role on FSH-action, leading to increased follicle development and improved oocyte yield following ovarian stimulation (Prizant et al., 2014), although specific underlying mechanisms remain poorly understood.

DHEA, which has been studied since more than 20 years for its anti-ageing properties (Morales et al., 1994, Arlt, 2004), has first shown to improve ovarian response to gonadotrophin stimulation in a small series published in 2000 (Casson et al., 2000). A few years later, DHEA as pre-treatment in IVF procedures in women with low ovarian reserve, was reported to improve oocyte yield and fertilization rates (Barad and Gleicher, 2006). Since then, DHEA has been widely used as adjuvant therapy for women with low ovarian reserve or ovarian ageing undergoing ART, and case reports indicate improved pregnancy rates (Barad et al., 2007, Gleicher and Barad, 2011), and reduced incidence of miscarriage (Gleicher et al., 2009). Testosterone has also, but to a lesser extent, been used as adjuvant therapy in IVF cycles for low responders, and beneficial effects on pregnancy rates have been reported (Kim et al., 2011). However, the clinical benefits of androgen supplementation in low ovarian reserve patients as an adjunct to ovarian stimulation remain highly debated (Sunkara et al., 2011) and even controversial (Urman and Yakin, 2012). A recent meta-analysis of a very limited number of

controlled randomized trials (Wiser et al., 2010) and case-control studies (Barad and Gleicher, 2005, Gleicher et al., 2010) regarding the use of adjuvant DHEA demonstrated no effect on pregnancy and miscarriage rates in the treatment group (Narkwichean et al., 2013). The interpretation of the results of the studies is hampered by the different androgens used (DHEA and testosterone), different doses and durations of treatment, and by the fact that most reports fail to indicate baseline measurements of androgens in the patients (Fanchin et al., 2011). A recent Cochrane Database Review on 8 randomized controlled trials (n=878 participants) employing DHEA as adjunct therapy in poor responders, compared with placebo or no treatment, showed an association with increased live birth rates (OR 1.88, 95% CI 1.30 to 2.71) (Nagels et al., 2015). These numbers suggest that women who have 12% of chance of ongoing pregnancy or live birth if they do not receive treatment, increase their chances to 15-26% if treated with DHEA (Nagels et al., 2015). Although this increased success rate might seem convincing, the authors conclude that the quality of evidence was moderate, because of major limitations in the methodology of the included studies (Nagels et al., 2015).

However, in the subgroup of women diagnosed with low ovarian reserve and concomitant hypoandrogenism, the rationale for androgen replacement is more obvious, and effectively improved IVF outcomes have been reported (Gleicher et al., 2013a, Gleicher et al., 2013b).

Oral DHEA or transdermal testosterone are currently the most utilized androgen treatment options (Arlt, 2006). DHEA has the advantage of being a precursor hormone, that is converted to downstream sex steroids in target tissues that possess the appropriate steroidogenic enzyme machinery (Labrie, 2010). Therefore, DHEA supplementation rarely leads to supraphysiological circulating androgen level and systemic adverse virilizing effects

Despite the weak clinical evidence, DHEA and testosterone supplementation are widely used in fertility clinics, illustrating the need for appropriately powered prospective randomized

trials to optimally guide evidence-based clinical practice (Sunkara et al., 2012). Women with low ovarian reserve clearly benefit from adequate measurements of adrenal and ovarian androgens, although the physician faces the sensitivity limitations of the current assays in precise measurements of female androgens in the lower quartile range (Wierman et al., 2006). Of note, the latest guidelines released by the Endocrine Society recommend against the generalized use of DHEA or testosterone for infertility because of insufficient evidence, and absence of clear indications (Wierman et al., 2014a).

1.7 Research hypothesis and aims of the study

This thesis aims to investigate the synthesis and metabolism of androgens in the different stages of folliculogenesis, and the effects of exogenous androgen exposure on follicle development and steroidogenesis, using a murine *in vitro* encapsulated three-dimensional follicle culture system and steroid metabolic profiling by liquid chromatography tandem mass spectrometry (LC-MS/MS).

My hypothesis is that individual follicles progressively acquire the capacity to produce androgens *in vitro*, and that androgens are converted to oestrogens in the final stages of follicle development. I further hypothesize that addition of exogenous androgens or androgen precursors promotes follicle growth and steroid production in the early stages but impedes on antral follicle development.

1.7.1 The endogenous sex steroid production by follicles in physiological conditions

The steroidogenic function of the developing ovarian follicle has not been studied in detail yet, because of the difficulty to mimic *in vivo* conditions in the laboratory, and because

of the low concentrations of endogenous steroids synthetized, which render adequate measurements difficult. Previously published steroid hormone data were performed employing immunoassays, which are known to be less accurate compared to mass-spectrometry because they achieve lower sensitivity and specificity, and are prone to cross-reactivity (Stanczyk, 2006).

- I will measure the endogenous sex steroid production in murine follicles as they acquire steroidogenic capacity and develop to pre-ovulatory follicles, in basal conditions, with minimal FSH-stimulation, employing LC-MS/MS.
- I will study the gene expression profiles of steroidogenic enzymes during follicle maturation and examine the association between sex steroid concentrations and steroidogenic gene expression results.

1.7.2 The effect of exogenous androgens on endogenous follicular steroid production

Androgen treatment is widely used in reproductive medicine, with the aim to increase fertility is women with low ovarian reserve, but indications and benefits of such treatment remain largely debated (Sunkara et al., 2012, Urman and Yakin, 2012, Gleicher et al., 2011, Narkwichean et al., 2013). I will examine if and to which extent the endogenous steroid production of the growing follicle is influenced by exposure to exogenous androgens.

- I will examine the LC-MS/MS steroid metabolome in follicles exposed to the potent androgen DHT
- I will study the metabolism of the androgen-precursor DHEA, employed as a steroid substrate, by developing ovarian follicles.

1.7.3 The androgen receptor-mediated effects of androgens on follicle development

A few studies have looked at the direct effects of androgens at the single follicle level, in mice (Murray et al., 1998, Wang et al., 2001, Romero and Smitz, 2010, Tarumi et al., 2012, Tarumi et al., 2014, Sen et al., 2014) and non-human primates (Rodrigues et al., 2015). Although further knowledge on follicular androgen action has been gained through these studies, interpretation of androgen-mediated actions remained hampered by the use of aromatisable androgens or the lack of AR-blockade.

- I will expose developing follicles to the non-aromatisable androgen DHT and examine the effects on follicle morphology and survival, steroid production, mRNA expression of target genes of follicle development and oocyte quality.
- I will antagonize the androgen effects on the follicle, employing the highly selective anti-androgen enzalutamide (MDV), and explore the differential biological actions in comparison to DHT.

1.7.4 The effects of increasing androgen provision to the growing follicle

Most studies examining androgen-excess effects on ovarian physiology are using a whole animal or whole organ approach. The direct consequences of supra-physiological doses of androgens on individual maturing follicles and oocytes need further attention.

- I will expose the maturing follicles to increasing doses of the androgen precursor DHEA, and examine the effects on follicle morphology, target gene expression, steroid and peptide hormone production and oocyte quality.
- To delineate the androgen and oestrogen-dependent effects, I will compare these findings with those observed in follicles treated with an increased dose of DHT or estradiol (E2).

- I will use a whole organ approach, employing *in vitro* culture of neonatal CD1 mice ovaries, to test the effects of increasing concentrations of DHT on ovarian follicle dynamics, evaluated by follicle counting.

2 Methods

2.1 Murine Encapsulated In Vitro Follicle Culture

2.1.1 Animals

CD1 mice were housed and bred in [redacted] in a temperature and light-controlled (12 h light, 12 h dark cycle) environment, and were provided with unrestricted access to water and chow (Mouse Diet 20 Mouse Diet 20 (PicoLab 5058)). Non-weaned pups (day 15-17) were culled at [redacted], in a laminar flow hood, by cervical dislocation and the female reproductive tract was excised. The procedure was carried out in accordance with the UK Animal Act 1986.

2.1.2 Follicle isolation procedure

Following excision, the female reproductive tissue was transported in L15 Glutamax medium (Thermo Fisher Scientific, Loughborough, UK) supplemented with 1% Fetal Bovine Serum (FBS, Sigma-Aldrich, Gillingham, Dorset, UK) and 0.5 % Penicillin-Streptomycin (Thermo Fisher Scientific) in a carrier-incubator at 37°C. Upon arrival in the laboratory, ovaries were removed from their bursa (Figure 2-1) with the aid of forceps and using a dissection scope, on a 37°C heated, temperature controlled stage, in a laminar flow hood.

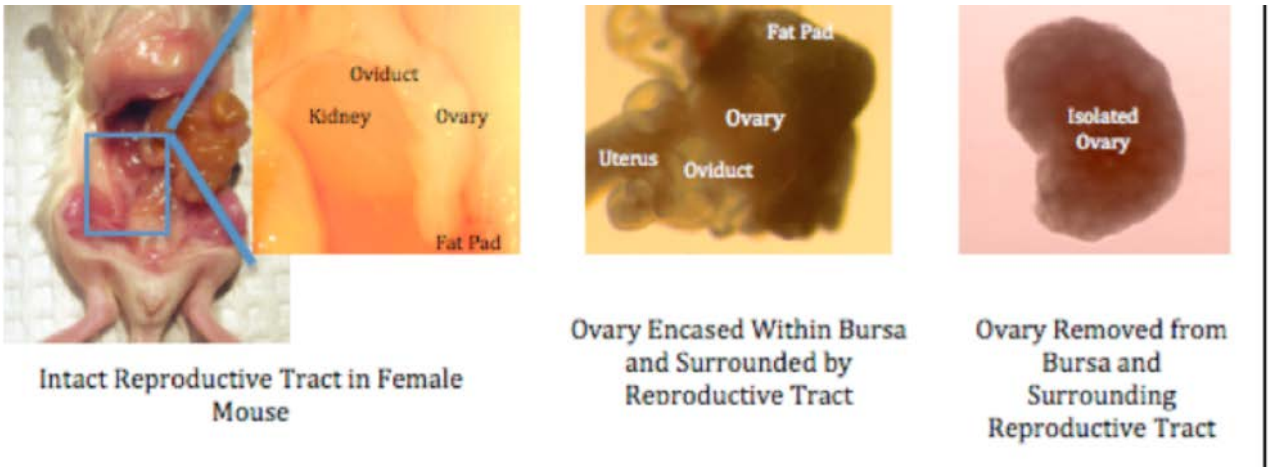


Figure 2-1Mouse ovarian dissection; excision of the female reproductive tract, followed by removal of the ovary from its bursa under the dissection scope. Courtesy of the laboratory of Dr.T.K. Woodruff, Northwestern University Chicago (Follicle handbook_2014).

The released ovaries were transferred to a dish containing L15 medium supplemented with 0.1% DNase I (Lorne Laboratories Limited, Reading, UK) and 0.1% Liberase TM (Roche, Life Science, West Sussex, UK), and placed on a shaker in a 37°C 5% carbon dioxide (CO2) incubator for 35-40 minutes. After addition of 10% FBS, to terminate the enzymatic reaction, secondary multilayered follicles (diameter 150-180 μm) were mechanically isolated employing insulin-gauge needles under a dissection scope. Follicles were placed in maintenance medium containing minimal essential medium (α-MEM Glutamax, Life Technologies Ltd, Paisley, UK) with 1% FBS and 0.5% Penicillin-Streptomycin for 2-3 hours in a 37°C 5% CO2 incubator.

2.1.3 Follicle encapsulation

2.1.3.1 Preparation

Alginate (NovaMatrix, Sandvika, Norway) was lyophilized in the laboratory of Dr. T.K. Woodruff, at Northwestern University Chicago, and shipped to the University of

Birmingham as part of the collaboration in this project. The day prior to follicle culture, lyophilized alginate was reconstituted with sterile solution PBS without Ca^{2+} **(**Dulbecco's Phosphate Buffered Saline without Calcium Chloride & Magnesium Chloride, 1X, Gibco, Life Technologies) to a concentration of 0.5%. After brief vortexing, the reconstituted alginate vial was left on orbital shaker overnight, or until completely dissolved. Before use, the tube was vortexed and centrifuged briefly to remove any air bubbles.

Calcium solution was prepared by mixing 50 mM $CaCl_2$ and 140 mM NaCl in 500 mL MilliQ water until dissolvation, and followed by sterile filtering with a 0.22 μm Millipore Stericup filter (Millipore, Watford, UK), in the laminar flow hood.

To construct a mouth pipette, used to collect the follicles, YGON tubing (Thermo Fisher Scientific) was employed to insert a mouth piece into one end (Sigma-Aldrich), and a 0.22 μm MILLEX GP Filter (Millipore) at the other end. The filter was connected to an aspirator tube (Sigma-Aldrich), which has on the other side a 0.2 μm Syringe Filter (Nalgene, Thermo Fisher Scientific) placed. The tip part of a Pasteur pipette (VWR, Lutterworth, UK) was broken, and the sharp edges were melted under a flame, to obtain the glass pipette, which was placed into the cut end of a rubber bulb (Thermo Fisher Scientific). The glass pipette was closed using the rubber stopper from the aspirator tube (Sigma-Aldrich). A hole was performed in the stopper to allow the stripper tip (200 μm, Origio Ltd, Fareham, UK) to be placed (Figure 2-2).

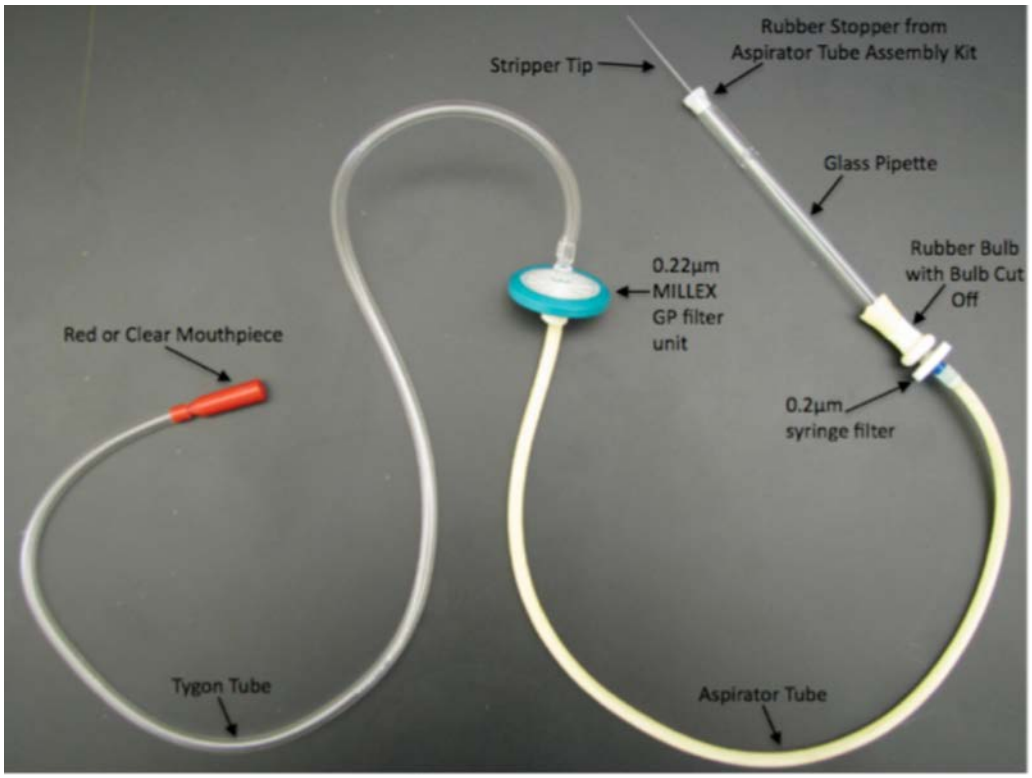


Figure 2-2Schematic of a mouthpipette. Courtesy of the laboratory of Dr. T.K.Woodruff (follicle handbook_2014).

2.1.3.2 Technique

Following isolation, follicles were transferred, with the use of a mouthpipette, to maintenance medium containing minimal essential medium (α-MEM Glutamax, Life Technologies) with 1% FBS and 0.5% Penicillin-Streptomycin, and left to recover for 2-3 hours in a 37°C 5% CO2 incubator.

Five mL of calcium solution was prepared in the outer ring of an IVF dish, and 0.5 mL of alginate was placed in the center ring. Under the dissection scoop in the laminar flow hood, follicles were transferred to dissection media for encapsulation. The mouth pipette was used to pick up 3 follicles, which were washed in alginate, and individually sucked up by a 10 µL pipette, set to 5 µL, while gently filling up the tip with alginate. The alginate/follicle content

was slowly expelled from the tip, perpendicular over the calcium solution, with the droplet gently touching the calcium solution and allowing the bead to crosslink for 2 minutes, with slight modifications to the previously described protocol (Xu et al., 2006a). The beads are transferred with a forceps to a new dish of maintenance medium in in a 37°C 5% CO2 incubator for a recovery period of 2-3 hours.

2.1.4 Follicle culture and imaging

2.1.4.1 Follicle culture medium

Alginate beads were transferred to a 96-well plate, with each well containing 100 µl culture medium, consisting of α-MEM Glutamax supplemented with 3 mg/ml bovine serum albumine (BSA, MP Biomedicals, Leicester, UK), 10 mIU/ml recombinant follicle-stimulating hormone (rFSH, Gonal-f, Merck Sereno, Feltham, UK), 1 mg/ml bovine fetuin (see below) and 5 µg/ml insulin, 5 µg/ml transferrin and 5 µg/ml selenium (ITS, Sigma-Aldrich). Fetuin, a glycoprotein added to follicle culture medium to prevent hardening of the zona pellucida (Schroeder et al., 1990), was dialyzed and lyophilized at the laboratory of Dr. T.K. Woodruff, at Northwestern University Chicago, and provided to the University of Birmingham as part of the collaboration in this project. Follicles were cultured for 6 days in 37°C 5% CO2 incubator. Media change (50 µl) was performed on alternate days; i.e. day 2 and 4 of culture.

2.1.4.2 Follicle treatments

For the treatment conditions, culture medium was supplemented with 25 or 50 nM DHT (Sigma-Aldrich); 100, 200 or 500 nM DHEA (Sigma-Aldrich); and 10 or 25 nM E2 (Sigma-Aldrich). The steroid concentrations employed were based on published dose-response

experiments (Wang et al., 2001, Sen et al., 2014). For androgen receptor-blockade, Enzalutamide (MDV) (Axon Medchem, Groningen, T) was used at the dose of 1 μM, based on its IC50 value (Tran et al., 2009).

2.1.4.3 Follicle imaging

Following plating, encapsulated follicles were imaged using Nikon Eclipse Te300 light microscope (Leica, Nikon) with 10 X objective, on a 37°C, temperature-controlled heating plate. Those follicles with intact alginate beads, and with preserved integrity of the oocyte and somatic cell compartment were selected for culture. Imaging was repeated on day 2, 4 and 6 of culture. Images were analyzed using Image J Software (National Institutes of Health, Bethesda, MD, USA) (Figure 2-3). Follicles sizes were obtained by averaging two perpendicular measurements of follicle diameters. The movement of the oocyte to an eccentric position with appearance of a fluid-filled space determined the presence of an antrum. Follicles were classified as dead if the oocyte or somatic compartment appeared shrunken or dark, if their interphase was compromised or the alginate bead disrupted. Only surviving follicles were included in the data analysis.

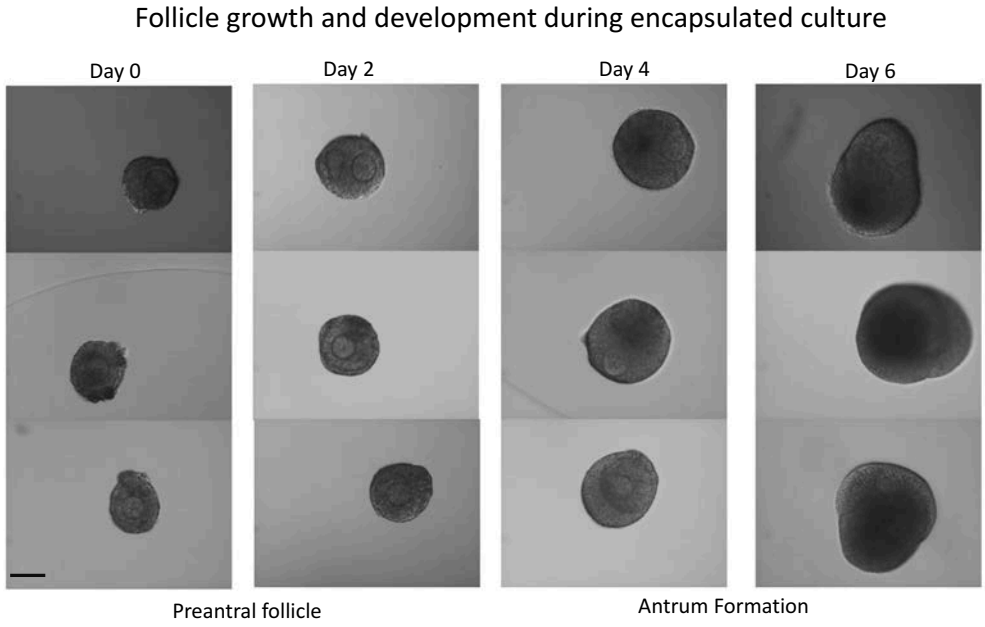


Figure 2-3 Follicle growth and development during the 6-days of alginate-encapsulated culture. Antrum formation start around day 4 in basal conditions. Scale-bar 100 μm

2.2 In vitro follicle maturation

2.2.1 Removal of follicles from alginate beads

Alginate lyase (Sigma-Aldrich) was added to maintenance medium (10 IU lyase/mL of media) and equilibrated in the 37°C 5% CO2 incubator for 30 minutes. All culture medium was removed from the wells containing alginate beads and 100 μl alginate lyase-maintenance medium mix was added to each well. The 96-well plate was transferred to the incubator for 25-30 minutes. A mouthpipette with a bend 600 μm stripper tip was used to remove the follicles from the beads.

2.2.2 Follicle maturation medium

Follicles isolated from their alginate bead were transferred in a dish containing

maturation medium composed of α-MEM Glutamax, 10 % FBS, 1.5 IU/mL human chorionic gonadotropin (HCG, Sigma-Aldrich), and 5 ng/mL epidermal growth factor (EGF) (BD Biosciences, Oxford, UK) for a washing step. Next, the follicles were placed in the center of an IVF dish containing maturation media and incubated for 16 hours at 37°C, 5 % CO2.

2.2.3 Oocyte quality and maturation status

After 16 hours in maturation medium, follicles released their oocyte-cumulus complexes. Oocytes were denuded from the surrounding cumulus cells by treatment with 0.3 % hyaluronidase (Sigma-Aldrich) for 2-3 minutes in the 37°C, 5 % CO2 incubator, followed by and gentle aspiration, using a 100 μl pipette tip. The oocytes were transferred to L15 medium and covered by ultra-pure oil, followed by imaging. The oocyte quality was assessed and follicles were classified as healthy, or degenerated based on the cytoplasmic appearance (Figure 2-4). Healthy oocytes were further divided as mature, or meiosis II, if a polar body was visible in the perivitelline space. Healthy oocytes that hadn't resumed meiosis were classified as immature, and contained meiosis I and germinal vesical staged oocytes (Figure 2-4).

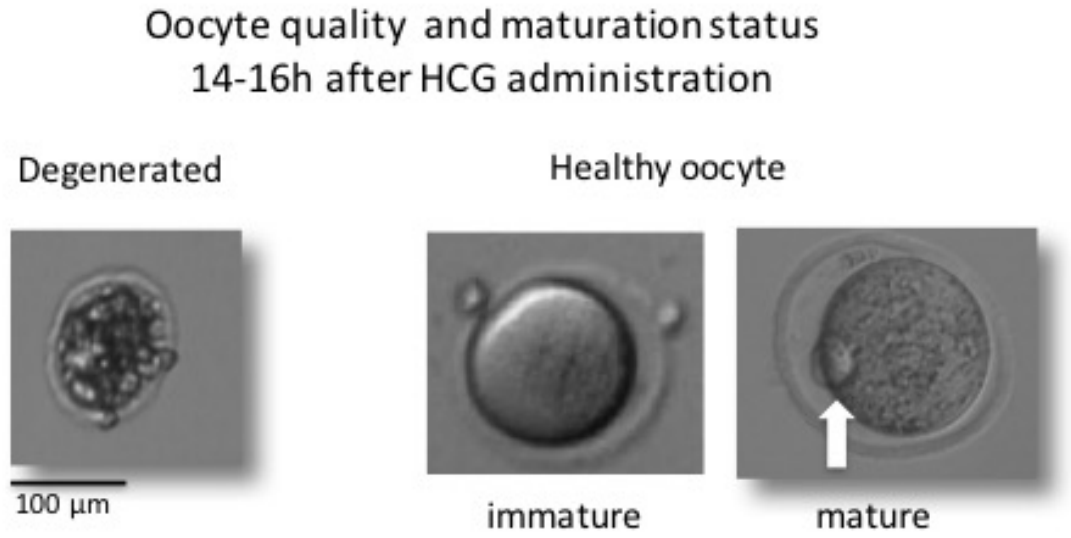


Figure 2-4 Oocyte quality and nuclear maturation following in vitro maturation at the end of culture. Completion of the first meiotic division is visualised by the presence of the first polar body in the perivitilline space (white arrow).Scale bar 100 µm.

2.3 Neonatal mouse ovarian culture

CD1 mice were housed and bred in [redacted]. A temperature, humidity, and light (12hL/12hD) controlled barrier facility was used to house and breed the mice. Mice were provided with food and water ad libitum. The mice were fed Teklad Global (Madison, WI) irradiated chow (2919 or 2916) that does not contain soybean or alfalfa meal but does contain minimal phytoestrogens. Five-day old CD1 mice were culled by cervical dislocation and female reproductive tracts were excised. Ovaries were removed from the ovarian bursa and washed twice with culture medium, consisting of α-minimal essential medium (αMEM Glutamax) supplemented with recombinant FSH (10 mIU/mL; A. F. Parlow, National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases), bovine serum albumin (3 mg/mL), bovine fetuin (1

mg/mL; Sigma-Aldrich, St. Louis, MO), insulin (5 ng/mL), transferrin (5 ng/mL), and selenium (5 ng/mL), as previously described (Jin et al., 2010). Ovaries were transferred into 6-well plates with tissue culture well inserts (nontissue culture treated; Millicell- CM, 0.4-um pore size; Millipore Corp., Billerica, MA). Into the compartment below the membrane insert, 1500 uL of culture medium was added, such that ovaries on the membrane were covered with a thin film of medium. Up to six ovaries were placed in each well. Ovaries were cultured in basal conditions or in medium supplemented with DHT (Sigma-Aldrich) at concentrations of $1x10^{-7}$ M and $1x10^{-8}$ M with stock solutions prepared in ethanol, and diluted in growth medium. Control medium contained the equivalent amount of ethanol as treatment medium. The ovaries were incubated at 37C, 5% CO2, for 4 days (96 hours).

2.4 Ovarian histology and follicle counting

At the end of the culture period, ovaries were fixed overnight in modified Davidson solution at 4°C, transferred to 70% ethanol, and then dehydrated in an ethanol series and embedded in paraffin wax. Sections (5 μn) were stained with hematoxylin and eosin (H&E).

20 X images were taken from each 5^{th} section, using a color camera and microscope. The number of follicles at each developmental stage was counted and averaged in three serial sections from the largest cross-sections through the center of the ovary. Only follicles that contained an oocyte nucleus were counted, and follicles were classified according to morphological criteria, as described by Bristol-Gould et al (Bristol-Gould et al., 2006). Primordial follicles have a small oocyte that is partially or completely surrounded by squamous or cuboidal granulosa cells, and are generally found in clumps near the borders of the ovary. Primary follicles contain larger oocytes that are completely surrounded by cuboidal granulosa cells, and are found either in isolation or in small groups throughout the ovary. Those follicles,

with a larger oocyte then the primary follicles, and surrounded by more than one layer of granulosa cells, were counted as secondary follicles, only on the section where the nucleolus of the oocyte was present.

2.5 Steroid analysis by Liquid Chromatography Mass Spectrometry

As previously described (O'Reilly et al., 2014), follicle culture supernatant (approximately 1 ml, pooled from 30-100 follicle incubations) was placed in sylinised glass TLC tubes, and 20 µl of internal standard was added. Linear calibration series were prepared prior to each steroid extraction (Table 2-1).

Table 2-1Sample calibration series for follicle culture media steroid measurement

Calibration number	**Added volume of steroid stock preparation (µl)**	**Added volume of cell media (µl)**	**Concentration (ng/ml)**
C0	0	1000	0
C1	0.5	999.5	0.5
C2	1	999	1
C3	2.5	997.5	2.5
C4	5	995	5
C5	10	990	10
C6	25	975	25
C7	50	950	50
C8	100	900	100
C9	250	750	250

Steroids were extracted via liquid-liquid extraction, by adding 3 ml of tert-butyl-methyl-ether (MTBE) to each sample, followed by vortexing and freezing for 1 hour. The upper liquid phase of the samples was then gradually transferred (approximately 800 µl per cycle) to a 96 well plate employing glass Pasteur pipettes, with alternating cycles of evaporation at 55°C under nitrogen. Samples were reconstituted with 125 µl of methanol: water mixture (50:50) and frozen at -20°C until ready for analysis. Steroids were quantified by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Waters Xevo Mass Spectrometer with Acquity uPLC systems with following settings: electrospray ionization source with capillary voltage at 4.0 kV, temperature source at 150°C, desolvation temperature of 500°C. Steroid identification was based on an identical retention time and 2 identical mass transitions when compared to an authentic reference compound. Quantification was performed relative to a calibration series (0.5-250 ng/ml of each steroid) with an appropriate internal standard. All measurements were performed in triplicate, except treatment condition DHT+MDV and DHEA100nM were assessed in duplicate because of shortage of biological material.

2.6 Gene expression studies

2.6.1 RNA extraction

At the end of cultures, we pooled 18-30 follicles for each experimental condition, which were immediately flash frozen in liquid nitrogen, and stored at -80°C until RNA extraction.

Total RNA was extracted from the follicles using the Qiagen RNeasy Micro Kit (Qiagen, Manchester, UK). All steps were performed on ice to prevent RNA degradation. Typically, 350 µl of buffer RTL Plus was added to the follicles, followed by disrupting and homogenizing the tissue using a Tissue Ruptor (Bio-Vortexer Laboratory Tube Mixer,

Research Products International, IL, USA). The lysate was centrifuged for 3 minutes at full speed. The supernatant, containing the RNA, was transferred to a genomic DNA Eliminator Spin column. Following centrifuge for 30 seconds at >8000 x g, the flow-through was discarded. In a next step, RNA extraction was performed using 350 µl of 70% ethanol to be added to the flow-through, and mixed by pipetting. The sample was transferred to an RNeasy MinElute spin column placed in a 2 ml collection tube, and centrifuged for 15 seconds at full speed, and the flow-through was discarded. In the following cleaning step, 700 µl of Buffer RW1 and 500 µl Buffer RPE were respectively added to the RNeasy MinElute spin column, followed by 15 seconds of maximum speed centrifugation and discard of the flow-through. The next washing step consisted of addition of 500 µl of 80% ethanol to the RNeasy MinElute spin column, with a 2 minutes' centrifugation step. The RNeasy MinElute spin column was placed in a new 2 ml collection tube and centrifuged, with open lid, at full speed for 5 minutes to dry the membrane. The RNeasy MinElute spin column was finally placed in a new 1.5 ml collection tube and 14 µl RNase-free water used to suspend the RNA, followed by centrifuging for 1 minute at full speed.

2.6.2 RNA quality examination

The concentration and purity of the extracted follicular RNA was determined using NanoDrop technology (ND1000, Thermo Fisher Scientific).

The quality of total RNA was examined employing Agilent R6K Screen Tape System (Agilent Technologies, Cheshire, UK), which performs RNA quality control, following sample loading, analysis and imaging. Samples were prepared using the R6K Screen Tape assay kit following the manufacturer's instructions, and performed at room temperature in RNA-free conditions. Briefly, 1 µl of RNA sample or high sensitivity RNA ladder were mixed with 4 µl

of R6K Sample Buffer. The samples and ladders were denaturated, by heating to 72°C for 3 minutes, and then placed on ice for 2 minutes, and briefly centrifuged to remove material attached to the lid or cover. The samples and the ladder were loaded into the 2200 TapeStation with appropriate loading tips, the software was updated with sample name and the remaining of the analysis was automated.

2.6.3 Complementary DNA (cDNA) generation and reverse transcription

RNA obtained from the follicle cultures was diluted to a final concentration of 50-100 ng/μl and converted to cDNA using a AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies), according to the instructions of the manufacturer. In this protocol, RNA was reverse transcribed into cDNA, in a microcentrifuge tube, by adding the following reagents, in order:

- RNAse free water to a total volume of 16.5 μl
- 2 μl AccuScript Reverse Transcription Buffer (10x)
- 3 μl Random Primers
- 0.8 μl dNTP mix (25nM each dNTP)
- the amount of RNA required to obtain 100 ng

The generated mixture was heated to 65°C for 5 minutes to denature RNA, followed by cooling at room temperature, which allowed primers to anneal to RNA. After this step, the following components were added to the reaction, in order, to a final reaction volume of 20 μl:

- 2 μl of 100 mM DTT
- 1 μl of AccuScript RT
- 0.5 μl of RNase to inhibit ribonuclease

The reaction mix was incubated at room temperature for 10 minutes, to allow for extension of the primers. Then, the microcentrifuge tube was placed in a temperature-controlled block, at 42°C for 60 minutes, to permit cDNA synthesis. The reaction was stopped by incubating the reaction mix at 70°C for 15 minutes. Samples were kept on ice for PCR amplification, or stored at -20°C until further use.

2.6.4 Real-time Polymerase Chain Reaction (RT-PCR)

2.6.4.1 Principle

PCR is a highly sensitive and specific method to determine the presence of a target gene in a sample of cDNA, reverse transcribed from RNA. The double stranded DNA is exposed to high temperature which allows for denaturation to single strands. The temperature is then lowered to permit specific primers to anneal to their complementary single DNA strand. TaqMan probes are designed to bind the primers that are bound to their specific complementary DNA. The probes are conceived with a fluorescent reporter and a quencher. The latter suppresses the fluorescent signal of the reporter if the probe is unbound to the primer. At a specific extension temperature, DNA polymerase amplifies targeted DNA by elongating the primer. By binding the primer on the cDNA, the quencher of the TaqMan probe is removed by the DNA polymerase, which results in emission of a fluorescent signal. This process is amplified, by repeating the described cycle for a dedicated amount of times. The real-time PCR machine measures the increasing fluorescent signal each cycle. The higher the amount of target gene presence, the lower the cycle number at which the fluorescent signal is first detected, a number referred to as CT value.

2.6.4.2 Method

The target gene expression levels were assessed using an ABI sequence detection system (Perkin-Elmer Applied Biosystems, Warrington, UK). All analyses were assessed in 10 µl final volume, containing 2 X TaqMan Universal PCR Master mix (5.0µl, Thermo Fisher Scientific), TaqMan Gene Expression Assay (20 X) of interest (0.5 µl) (Table2-2), nuclease free water (4.0 µl) and 0.5 µl cDNA template. All reactions were normalized against the housekeeping genes 18S rRNA and Ribosomal Protein L-18 (Rpl-18) rRNA. Data were expressed as ΔCT values, referring to CT of target gene minus CT of housekeeping gene.

Table 2-2TaqMan Gene Expression Assays used (purchased from Life Technologies, Thermo Fisher Scientific)

Gene	Protein encoded	Assay ID
star	steroidogenic acute regulatory protein	Mm00441558_m1
cyp11a1	cholesterol side-chain cleavage cytochrome P450	Mm00490735_m1
cyp17a1	17-hydroxylase	Mm00484040_m1
cyp19a1	aromatase	Mm00484049_m1
hsd3b1	3β-hydroxysteroid dehydrogenase	Mm01261921_mH
hsd17b1	17β-hydroxysteroid dehydrogenase	Mm00501692_g1
AR	androgen receptor	Mm00442688_m1
FSH-R	follicle stimulating hormone receptor	Mm00442819_m1
LH-R	luteinizing hormone receptor	Mm00442931_m1
AMH-RII	anti-Müllerian hormone receptor 2	Mm00513847_m1
GDF9	growth and differentiation factor 9	Mm00433565_m1
BMP15	bone morphogenic protein 15	Mm00437797_m1

AMH	anti-Müllerian hormone	Mm00431795_g1
RPL18	ribosomal protein 18	Mm02745785_g1

2.7 Statistical Analysis

Both graphical expression of experimental data and statistical analysis were performed with Prism 6 (Graph Pad) Software. P-values, reflecting the probability of an observed results arising by chance, was used to express statistical significance. A P-value <0.05 was set, as per convention, to reject the null hypothesis. P-values were calculated using the following statistical tests:

- Independent t-tests were employed to compare continuous variables (steroid hormone and dCT values) between 2 treatment conditions. Matched or repeated measurements were analyzed using paired t-tests, while unpaired t-tests were used for independent measurements.
- One-Way ANOVA with post-hoc Tukey test was employed to analyze means of continuous variables (follicle and oocyte size, inhibin-B measurements) between 3 or more treatment groups.
- Contingency analysis with Fisher's exact test was used to compare categorical variables (survival, presence of an antrum, oocyte maturity).

All studies were performed at least in three independent experiments, unless otherwise specified.

3 *In vitro* folliculogenesis, oogenesis and steroidogenesis under physiological conditions

3.1 Introduction

The final stages of follicular development and the process of ovulation result from a tightly regulated communication between the hypothalamic-pituitary axis and the ovary. The follicle becomes able to produce steroids in the secondary stage, just prior to antrum formation, when the theca cells are recruited to form a layer around the basal membrane surrounding the granulosa cell-oocyte unit (Young and McNeilly, 2010) (Figure 3-1). Theca cells differentiate, acquire steroidogenic function, and start producing small amounts of androgens, under the influence of granulosa cell-produced factors, such as activin and BMPs, and oocyte-derived signals such as GDF-9, and growth-factors such as insulin and IGF-1 (Logan et al., 2002). In the gonadotrophin-dependent antral stage, thecal androgen production is largely under pituitary control, by LH, transported by the bloodstream, and androgens acts as substrate for aromatase, with FSH regulating estradiol secretion in granulosa cells (Edson et al., 2009). Estradiol production from the dominant follicle triggers the pre-ovulatory LH surge, which in turn, prompts the follicle to release its mature oocyte, that is now able to get fertilized and give rise to the next generation.

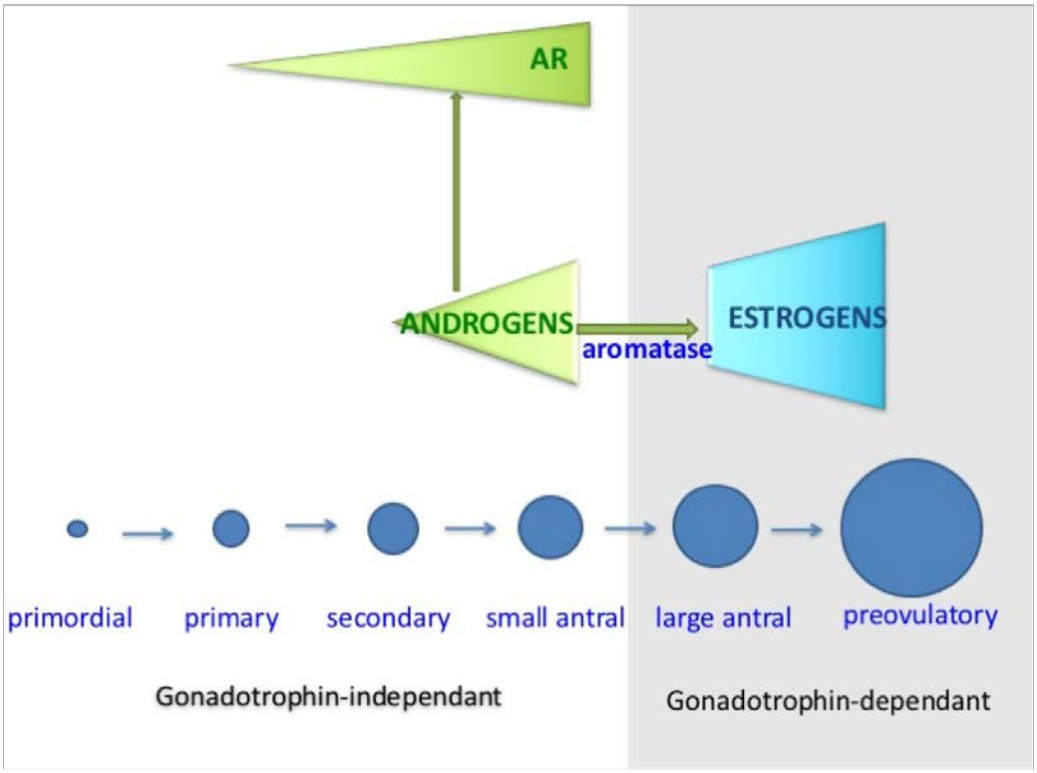


Figure 3-1 Androgen and oestrogen synthesis throughout the different stages of follicle development. Androgens production occurs in the theca cells, from the secondary stage onward, and conversion to oestrogens occurs in the granulosa cells via aromatase (CYP19A1) activity in the antral follicle. Reprinted and adapted from Lebbe and Woodruff, with kind permission of Oxford University Press, copyright 2013.

The alginate encapsulated *in vitro* follicular culture technique has proven to recapitulate all key events of folliculogenesis and oogenesis, known to occur during *in vivo* development (Xu et al., 2006a). Coordinated follicle growth, in 3- dimensions, with preserved follicle architecture, and, importantly, maintained paracrine signaling between the oocyte and the somatic compartment, is a major advantage of this technique (Kreeger et al., 2006). The follicle encapsulation provides a superior value to the follicle culture technique, compared with historical, 2-dimentional culture models, which have inherent adhesive properties, leading to a

partial dissociation in communication between the oocyte and its surrounding cells (Brito et al., 2014). Follicle function, including steroid and peptide hormone production, has been documented in mouse follicles grown in alginate beads, using immunoassays (Skory et al., 2015, Tingen et al., 2011, Jin et al., 2010, West-Farrell et al., 2009), steroid measurements include androstenedione, estradiol and progesterone (Figure 3-2). The important knowledge that has been gained through these results is the fact that the follicle is capable of sex steroid production *in vitro* (West et al., 2007*),* in a culture system employing a constant dose of rFSH, without addition of LH or FSH, in serum-free medium.

Here I repeat the study of folliculogenesis and oogenesis in basal conditions, employing the murine encapsulated in vitro follicle growth (eIVFG) system. I have trained in this technique at the laboratory of Dr. Teresa Woodruff at Northwestern University Chicago, and transferred the method to the University of Birmingham, and intended to validate my performance with this technique against published data. Following validation, I add new knowledge to this culture system, by examining endogenous steroid production by mass spectrometry-based steroid metabolome profiling, which has not been done before.

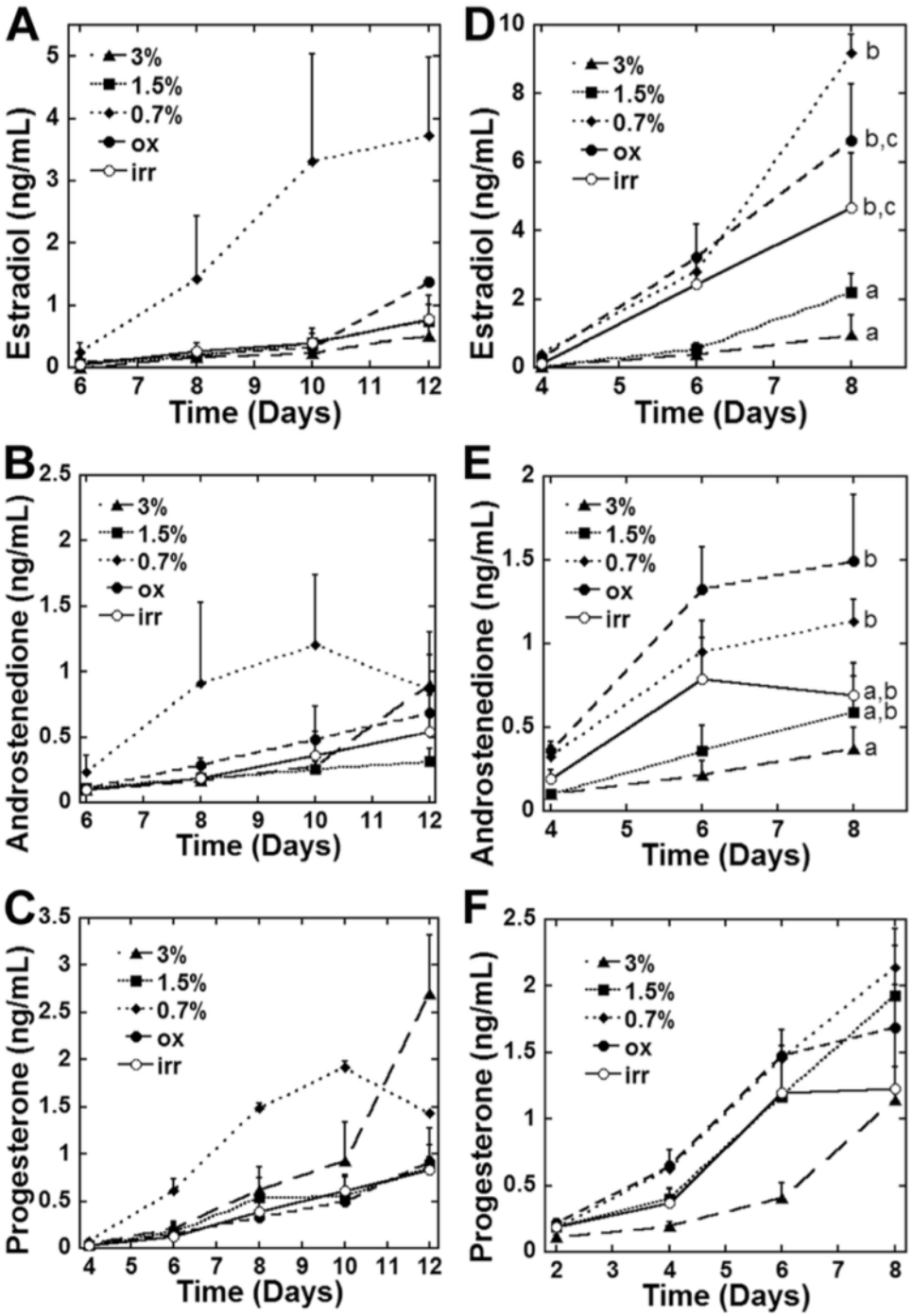


Figure 3-2 Estradiol, androstenedione and progesterone production by two-layered (A-C) and multilayered (D-F) secondary follicles during in vitro alginate encapsulated culture, for alginate concentrations at 3%, 1.5%, 0.7% and for oxidized (ox) and irradiated (irr) alginate. Steroids are measured by immunoassays in culture medium. Reprinted from (West et al., 2007) with kind permission from Pergamon, copyright 2007.

3.2 Methods

3.2.1 Research strategy

In this chapter, I will first examine the morphological characteristics of murine follicles, cultured in the alginate encapsulated system, in basal physiological conditions, with regard to follicle survival, growth, and oocyte quality. These characteristics will be compared with those previously published with the same method, in order to validate the quality of the method transfer. I will then explore the ability of LC-MS/MS methods to detect and quantify endogenous sex steroid production in developing murine follicles during *in vitro* culture. This is challenging, as follicular steroids are likely to be barely identifiable in view of the small amounts secreted by individual follicles. To overcome this challenge, conditioned media from follicles cultured in similar conditions will be pooled prior to steroid extraction.

3.2.2 Murine encapsulated *in vitro* follicle growth (eIVFG)

Immature multilayered secondary follicles, with a diameter of 150-180 μm, were mechanically isolated from pre-pubertal 15-17 day old CD1 female mice pups, after enzymatic digestion employing L15 medium supplemented with 0.1% DNase I and 0.1% Liberase TM, for 35-40 minutes on a shaker in a 37°C 5% CO2 incubator. Following a recovery period during which the follicles stayed in maintenance medium (α-MEM Glutamax medium with 1% FBS and 0.5% Penicillin-Streptomycin) in the CO2 incubator, individual follicles were examined under the dissection microscope and encapsulated in 0.5 % alginate, with slight modifications to the protocol described by Xu et al. Alginate-encapsulated follicles were placed in separate wells of a 96-well plate, each containing 100 μl culture medium, consisting of α-MEM

Glutamax supplemented with 3 mg/ml BSA, 10 mIU/ml rFSH, 1 mg/ml fetuin and 5 µg/ml ITS. Following plating, encapsulated follicles were imaged using Nikon Eclipse Te300 light microscope and healthy follicles with intact alginate beads were selected for culture. Follicles were maintained for 6 days in 37°C 5% CO2 incubator and media change (50 µl) and imaging was performed on alternate days. Images were analyzed using Image J Software.

3.2.3 In vitro follicle maturation

As previously described (Xu et al., 2006a), fully-grown follicles were, at the end of day 6 of culture, retrieved from the alginate bead and transferred to maturation medium composed of α-MEM Glutamax, containing 10 % FBS, 1.5 IU/mL HCG, and 5 ng/mL EGF for 16 h at 37°C, 5 % CO2. Oocytes were denuded from the surrounding cumulus cells by treatment with 0.3 % hyaluronidase and gentle aspiration. The oocytes were classified as mature, or metaphase II, if a polar body was visible in the perivitelline space. Healthy oocytes that hadn't resumed meiosis were classified as immature. Unhealthy oocytes were considered as degenerated.

3.2.4 RNA extraction, reverse transcription and real-time PCR

At day 6 of culture, 18-30 follicles grown in similar conditions were pooled and flash frozen in liquid nitrogen. RNA was purified from the follicles using the Qiagen RNeasy Micro Kit according to the instructions of the manufacturer. RNA quantity and quality were assessed employing NanoDrop technology and Agilent High Sensitivity R6K Screen Tape System. RNA was diluted to a concentration of 50-100 ng/µl using RNA free water, and reverse transcribed to complementary DNA (cDNA) using a AccuScript High Fidelity 1st Strand cDNA Synthesis Kit. mRNA expression levels were assessed using real-time PCR technology, employing

reactions containing Taqman Universal PCR Master mix, probe-primer mix for the target gene, and 4.5 µl cDNA (100 ng), as published previously (Gathercole et al., 2007). All reactions were normalized against the housekeeping genes 18S and Rpl-18. Data were expressed as ΔCT values. We examined the expression of key steroidogenic enzymes, i.e. star, cyp11a1, cyp17a1, cyp19, hsd3b1 and hsd17b1, after confirmation of their presence and isotope-specificity in mouse ovarian tissue, based on published data (Payne and Hales, 2004).

3.2.5 Follicle culture steroid extraction and LC-MS/MS analysis

Because endogenous steroid production from individual follicles was expected to occur in very low concentrations that were below the detection limit of LC-MS/MS, conditioned media of 72-105 follicles, cultured in similar conditions, were pooled at day 2, 4 and 6 of culture. Media was stored at minus 80°C until analysis. Pooled follicle culture supernatant (approximately 1 ml) was placed in sylinised glass TLC tubes, and 20 µl of internal standard was added. Linear calibration series were prepared prior to each steroid extraction (Table 2-1). Three ml of tert-butyl-methyl-ether (MTBE) was added to each sample, followed by vortexing and freezing for 1 hour. The upper liquid phase of the samples was then gradually transferred to a 96 well plate employing glass Pasteur pipettes, with alternating cycles of evaporation at 55°C under nitrogen. Samples were reconstituted with 125 µl of methanol: water mixture (50:50) and frozen at -20°C until ready for analysis. The LC-MS/MS measurements for control conditions were performed in triplicate. Data were expressed as mean ± SD, and were normalised to 100 follicles.

3.2.6 Statistical analysis

Statistical analysis was performed with Prism 6 (Graph Pad) Software, using unpaired t-test to analyze steroid concentrations and paired t-test to examine ΔCT values from matched follicles at the beginning (Day 0) and end (Day 6) of culture. All experiments were independently performed at least in triplicate, unless otherwise specified.

3.3 Results

3.3.1 Physiological follicle development in the alginate encapsulated *in vitro* follicle growth system (eIVFG)

3.3.1.1 Follicle growth, antrum formation and survival during culture

The baseline characteristics, with biological and technical replicates, of follicles grown in basal standard conditions, i.e. in serum-free media, under a constant dose of 10 mIU/ml rFSH, are outlined in Table 3-1. Figure 3-3 is a representative image of a secondary multilayered follicle at the moment of encapsulation in the alginate bead, at day 0, the start of culture.

Table 3-1 Baseline characteristics of follicles cultured in control conditions

Condition	Starting size (mean ± SD)	Biological replicates	Technical replicates
Control follicles	163.6 ± 9.9 μm	156	10

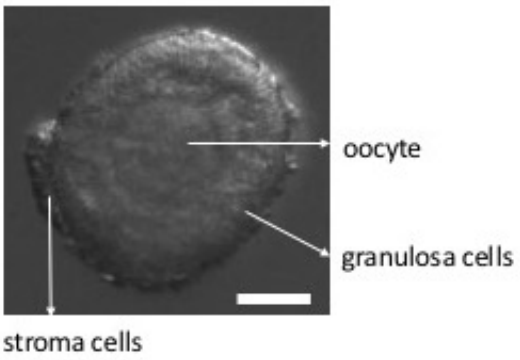


Figure 3-3 Light-microscopic representative image of a multi-layered secondary follicle at the start of culture, containing an oocyte, several layers of granulosa cells, and some attached ovarian stroma cells; scale-bar:50 μm.

Over the 6 days of culture, control follicles grew from 163.6±9.9 μm to a final follicle size of 296±45 μm. The increasing follicle size in basal conditions is outlined in Table 3-2 and Figure 3-4.

Table 3-2 Growth characteristics of follicles cultured in control conditions

Day of culture	Follicle size (mean ± SD)	Follicle number
0	163.6±9.9 μm	156
2	185.9±23.5 μm	135
4	232.6±34.5 μm	100
6	296.0±45.0 μm	88

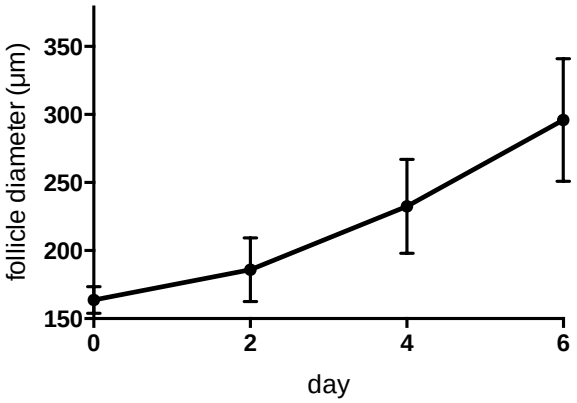


Figure 3-4 Follicle growth of follicles during eIVFG in basal conditions. Data are expressed as mean ± SD. Follicle numbers are outlined in Table 3-2.

An important developmental step during *in vivo* follicle development is the acquisition of an antrum, a fluid-filled cavity formed in the centre of the follicle. The alginate eIVFG system allows for 3D expansion of follicle growth, maintaining the *in vivo* architecture, and therefore permits antrum generation *in vitro (Brito et al., 2014)*. The development of the antrum is light-microscopically assessed by the outward movement of the oocyte, from a central to an eccentric position in the follicle. Figure 3-5 shows a light-macroscopic image of a small and large antral follicle during development. In basal conditions, a mean (SD) of 45±22 % of total follicles achieved antrum formation on day 4 of culture, and this percentage increased at 83±16 % at the day 6 of culture.

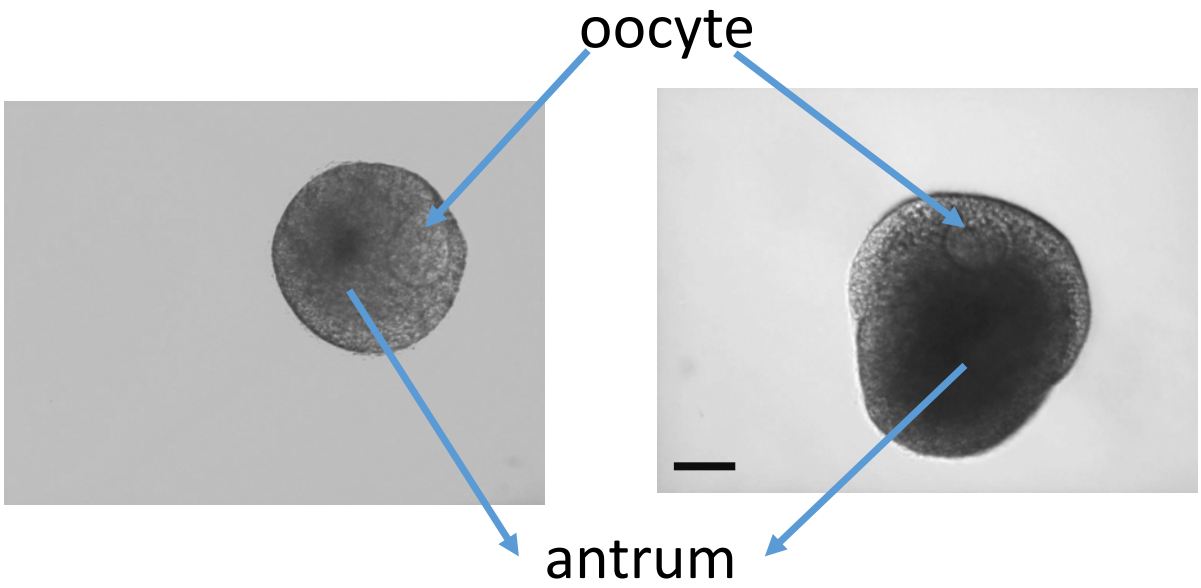


Figure 3-5 Representative light-microscopic images of small (left) and large (right) antral follicles, grown in alginate beads allowing for 3-dimensional growth expansion and maintenance of in vivo follicle architecture. The antrum is the dark area that first displaces the ovary, and later fills the entire follicle centre, hiding the oocyte for the sight. Scale-bar: 50 µm

Follicles were considered dead during culture if the oocyte or somatic cells looked unhealthy, if their integrity was disrupted, or in case of rupture of the alginate bead. Alginate gel-embedded follicles had a declining survival rate over time, as illustrated in Figure 3-6. Only survival follicles were included in data analysis.

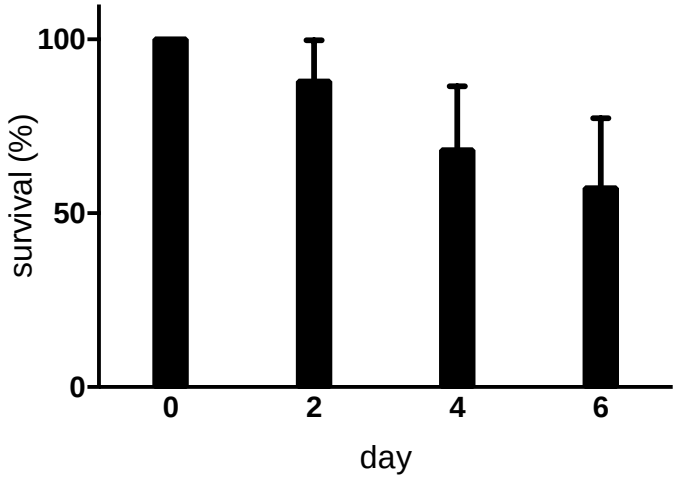


Figure 3-6 Follicle survival at day 0, 2, 4 and 6 of culture, in basal condition. Data are expressed as mean (SD).

3.3.1.2 Oocyte development in physiological conditions

At the end of culture, follicles were removed from their alginate beads and exposed to HCG and EGF to induce ovulation. Oocytes were recovered, denuded from the attached cumulus cells, imaged, and assessed for size and nuclear maturation status.

In basal conditions, a mean (SD) of 69±11 % of the retrieved oocytes (n=26) completed the first meiotic division, and had a mean (SD) size of 68.1±4 μm; while 27±9 % stayed arrested in meiosis I, and measured 68.1±2 μm. The oocyte maturation status in control conditions is illustrated in Figure 3-7.

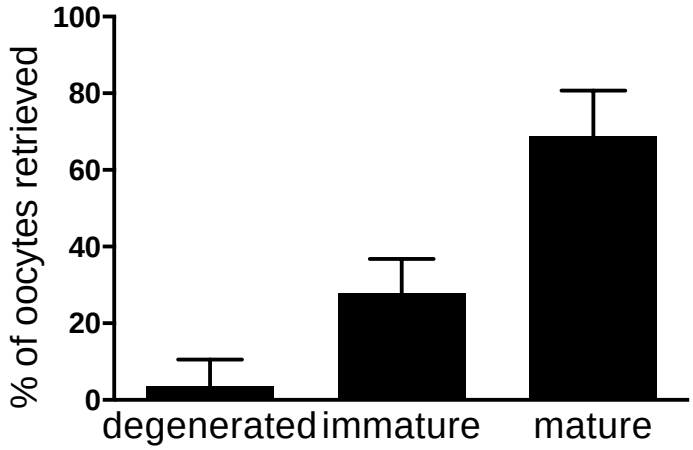


Figure 3-7 Oocyte meiotic maturation in standard conditions (n=26). Mature oocytes have extruded a polar body, while immature oocytes are healthy gametes arrested in Meiosis I. Dead or shrunken oocytes are considered degenerated. Data are expressed as mean ± SD.

3.3.1.3 mRNA analysis of target genes of follicle development

At the start and the end of culture, 30 follicles were pooled and flash frozen in liquid nitrogen, and RNA was extracted to allow for qRT-PCR analysis of target genes of folliculogenesis.

With ongoing follicle maturation, we noticed a significant upregulation of LH-receptor expression (Table 3-3). The gene expression of AR was stable during follicle development. Oocyte-derived factor GDF9 and granulosa cell-derived BMP15, molecules involved in paracrine signalling, were significantly downregulated as the follicle entered the later stages of follicle development (Table 3-3). AMH, also involved in early follicle development, was significantly downregulated at the end of culture, however mRNA expression of its receptor, AMH-RII remained unchanged during follicle development (Table 3-3).

Table 3-3 Relative mRNA expression of target genes of follicle development, expressed as ΔCT values (normalized against 18S ribosomal RNA) ± SD (for each triplicate experiment from 30 follicles from each group pooled prior to RNA extraction), in follicles at day 0 and 6 of follicle culture in basal conditions.

Gene	Protein encoded	Day 0	Day 6	p
AR	androgen receptor	13.8±1.5	14.7±0.09	0.3
FSH-R	follicle stimulating hormone receptor	16.2±1.2	12.8±1.0	0.2
LH-R	luteinizing hormone receptor	16.1±0.6	11.7±0.2	0.005
AMH-RII	anti-Müllerian hormone receptor 2	14.8±1.1	14.8±1.1	0.9
GDF9	growth and differentiation factor 9	12.6±0.5	18.3±0.4	<0.001
BMP15	bone morphogenic protein 15	14.2±0.9	17.9±0.5	0.02
AMH	anti-Müllerian hormone	8.9±0.8	20.1±0.1	0.001

3.3.2 Endogenous steroid synthesis under physiological culture conditions

3.3.2.1 LC-MS/MS steroid synthesis profiling in the developing follicle

Steroid concentrations were measured by LC-MS/MS in media collected on days 2, 4 and 6 of folliculogenesis, pooling media from 72-105 follicle incubations from each timepoint; data were normalized to 100 follicles. Raw data of steroid measurements are mentioned in Table 3-4.

Table 3-4 Steroid hormone production by the cultured follicles, measured by LC-MS/MS, in pooled follicle culture medium (72-105 follicles) at day 2, 4 and 6 of culture, and normalised to 100 follicles. Data are expressed as mean ± SD (n=3 measurements).

Steroid	Concentration at day 2 (nmol/l)	Concentration at day 4 (nmol/l)	Concentration at day 6 (nmol/l)
Progesterone (Prog)	0.6±0.5	0.8±0.7	11.0±1.2
DHEA	1.4±1.4	1.9±1.4	9.6±3.4
Androstenedione (Adione)	0.1±0.1	2.3±0.2	47.5±1.3
Testosterone	0	5.1±2.4	148.2±16.7
DHT	0	0.7±1.2	2.6±3.7
Estrone (E1)	0.5±1.0	0.9±0.9	14.4±1.4
Estradiol (E2)	0	0.7±1.3	138.1±8.3

At day 2 of culture, steroid concentrations were close to the lower limit of detection (0.5-2.0 nmol/L) (Figure 3-8). At day 4, the androgen precursors DHEA and androstenedione and biologically active testosterone increased to quantifiable levels and oestrogens became detectable (Figure 3-8). By day 6 we observed a significant increase in androgen precursors and active androgens, with the most potent androgen, DHT, emerging in quantifiable levels for the first time. At that time point, progesterone was present in quantifiable amounts, and significant oestrogen generation was observed, in the majority biologically active E2. At day 6 of culture, testosterone was the main androgen secreted in culture (with a mean (SD) of 71±4% of total androgens), followed by androstenedione (22±0.5%), DHEA (5±1%)

and low but quantifiable amounts of DHT. E2 was the principal oestrogen secreted (90±0.3% of total oestrogens) (Table 3-4 and Figure 3-8).

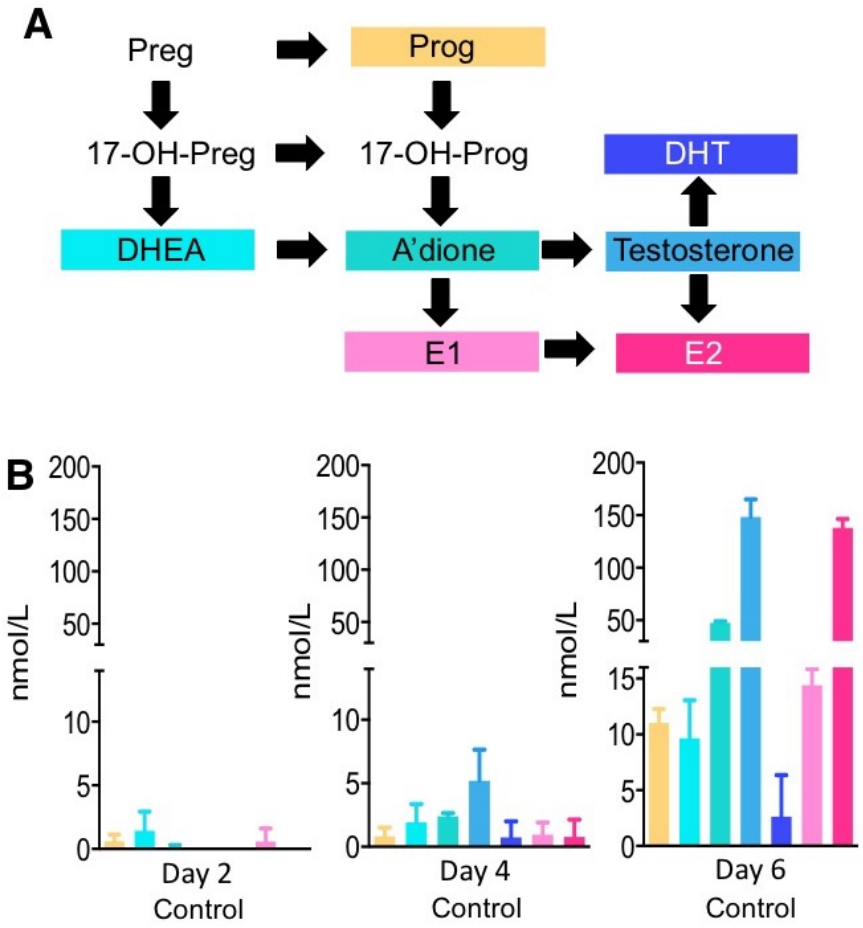


Figure 3-8 Steroid hormone production by the cultured follicles in physiological conditions, measured by LC-MS/MS, in pooled follicle culture medium (72-105 follicles) at day 2, 4 and 6 of culture, and normalised to 100 follicles. Data are expressed as mean ± SD (A) Schematic showing the steroidogenic pathway, with colour coding of the different steroids assessed in culture medium: Progesterone (Prog), Dehydroepiandrosterone (DHEA), Androstenedione (A'dione), Testosterone, Dehydrotestosterone (DHT), Estrone (E1) and Estradiol (E2). (B) Endogenous steroid production in basal conditions.

3.3.2.2 Steroidogenic enzyme expression in the developing follicle

Corresponding to the increasing generation of active sex steroids across follicle development, steroid enzyme mRNA was also accumulating with time. A significant increase in mRNA expression of 17β-hydroxysteroid dehydrogenase (hsd17b1; $p<0.001$, Table 3-5) was measured by day 6. This enzyme catalyzes the conversions of androstenedione to testosterone and estrone to estradiol and is FSH responsive (Peltoketo et al., 1999). Concurrently, mRNA levels of the FSH regulated aromatase (cyp19a1) gene, responsible for activation of androgens to oestrogens, significantly increased with follicle maturation ($p<0.01$, Table 3-5). In line with the increasing levels of progesterone, side-chain cleavage enzyme, cyp11a1 also increased ($p<0.01$, Table 3-5). These experiments showed that the murine follicle is equipped with the necessary steroidogenic machinery to produce sex steroids in a developmental stage -dependent fashion (Figure 3-9).

Table 3-5 Relative mRNA expression of target genes of steroidogenesis, expressed as ΔCT values (normalized against 18S ribosomal RNA) ± SD (for each triplicate experiment from 30 follicles from each pooled prior to RNA extraction), in follicles at day 0 and 6 of follicle culture in basal conditions.

Gene	Enzyme encoded	Day 0	Day 6	P value
star	steroidogenic acute regulatory protein	15.4±0.4	15.3±0.7	0.8
cyp11a1	cholesterol side-chain cleavage cytochrome P450	13.9±0.4	12.5±0.1	0.006
cyp17a1	17-hydroxylase	13.3±0.05	12.8±0.07	0.4

cyp19	Aromatase	15.5±0.09	12.3±1.3	0.04
hsd3b1	3β-hydroxysteroid dehydrogenase	10.9±1.4	9.6±0.3	0.2
hsd17b1	17β-hydroxysteroid dehydrogenase	15.2±0.1	11.1±0.2	0.002

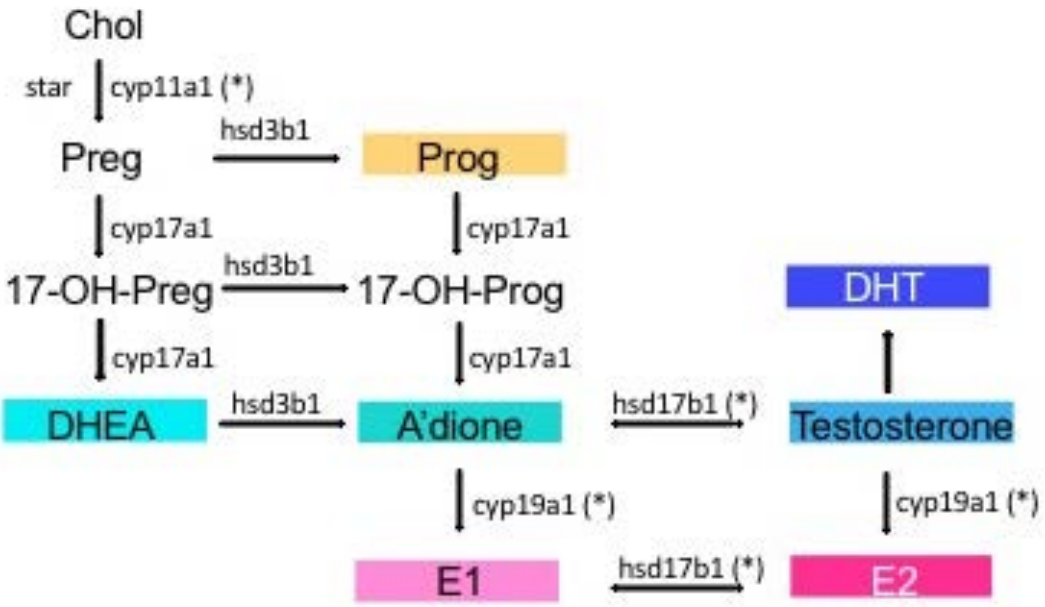


Figure 3-9 Schematic representation of steroidogenic pathway in the follicle with indication of enzymes which gene-expression was upregulated during in vitro follicle development (indicated by an asterix), ensuring increasing active sex steroid and progesterone synthesis with ongoing follicle maturation.

3.4 Discussion

Folliculogenesis and oogenesis in murine eIVFG systems have been described in detail previously (Xu et al., 2006a). I have repeated these experiments, in order to validate the quality of the eIVFG culture methods, that I have transferred to the University of Birmingham following training in the laboratory of Dr Teresa Woodruff, who has developed the technique

in collaboration with Dr Lonnie Shea. Slight modifications in the culture protocol have been introduced, such as the use of 0.5% alginate instead of 1.5%, a different encapsulation technique, and a culture period that has been shortened from 8 to 6 days. Of note, a different recombinant FSH preparation was used in my study. Follicle growth characteristics (mean follicular size at day 6 in my study 296 ± 45 versus 300 μm) , as well as oocyte size (68 ± 4 versus 68 ± 2 μm) and percentage mature oocytes (69 ± 11% versus 70 ± 9%) were similar in my hands compared with those published in the reference study. The capacity of the oocyte grown *in vitro* to develop to a stage where it is able to transition to meiosis II, is considered to be the ultimate arbiter of follicle culture quality (Hartshorne, 1997). This means that my produced outcomes can be considered valid. However, the survival rates at the end of culture were significantly lower compared to those obtained in the pilot study (57 ± 20% versus 93±2%). This could be related to inferior follicle quality at the start of culture, fewer experience with the technique, or to animal- or laboratory-related factors. Only surviving follicles have been included in the data analysis, which means that the generated results were not adversely impacted by the lower survival rates.

My study extends the application of the eIVFG technique by coupling it to modern LC-MS/MS steroid profiling. I report, for the first time, a quantitative multi-steroid metabolome of the developing follicle, indicative of FSH-stimulated endogenous production of androgens, oestrogens and progestins *in vitro,* in a folliculogenesis-stage dependant manner. The steroid hormone pattern reported was in accordance with the current knowledge of follicular steroid production, and consistent with *in vivo* hormone dynamics (Hillier, 1994). Androgen secretion was quantifiable around day 4, which corresponds to the developmental stage of antrum formation and gonadotrophin responsiveness. Oestrogen biosynthesis increased sharply between day 4 and 6 of culture, corresponding to aromatase expression as the follicle reached

ovulatory maturity. At day 6, progesterone secretion started to increase slightly, prior to luteinisation. Gene expression profiles showed upregulated gonadotrophin expression with follicle maturation. Parallel to increasing FSH-R expression, FSH-dependant steroidogenic enzymes, Hsd17b1 and Cyp19, were significantly increased, as well as LH-R. Upregulation of LH-R prepares the follicle for ovulation, i.e. cumulus cell expansion, oocyte meiotic maturation and luteinisation. According to the 2-cell, 2-gonadotrophin hypothesis, LH stimulates androstenedione production by theca cells, which provides substrate for oestrogen biosynthesis by granulosa cells (McNatty et al., 1979). In the employed culture system however, maturing follicles produce significant amounts of estradiol in a LH-free and serum-free medium. This means that follicular cells are able to constitutively produce androgens in the absence of provision of cholesterol and LH. Paracrine theca cell LH-independent androgen production is known to occur under the influence of insulin (Barbieri, 1994, Erickson et al., 1990), present in physiological amounts in the employed culture medium. Expression of Star, a theca cell marker (Kiriakidou et al., 1996), remained stable during follicle culture, which suggests that *de novo* theca cell formation was limited, and granulosa-theca cell trans-differentiation possibly accounts for the observed androgen production.

During the development from immature to pre-ovulatory follicle, testosterone was the predominant steroid, closely followed by estradiol in the terminal follicle, in a 1/1 ratio. Interestingly, the steroid distribution of the day 4 murine follicle resembled the pattern observed in follicular fluid of the androgen-dominant follicle in regularly menstruating women (Kushnir et al., 2009). In human however, androstenedione was the main androgen secreted, and progesterone production was more prominent compared with mice.

In my study, LC-MS/MS measured similar (West-Farrell et al., 2009) or lower (Xu et al., 2006a, Jin et al., 2010) concentrations for androstenedione and estradiol, compared to those

obtained in similar culture conditions but measured with immunoassays. These differences could be explained by fact that immunoassays are prone to cross-reactivity, which may lead to falsely increased concentrations (Stanczyk, 2006). Interestingly, androstenedione and estradiol immunoassays showed significantly increased concentrations in follicles grown in adherent 2D-culture systems, compared with non-adherent alginate systems, despite similar follicle growth and oocyte quality parameters, a finding attributed to differences in theca cell development (Sanchez et al., 2012). A major advantage of mass-spectrometry is the ability to simultaneously measure multiple steroid concentrations, while immunoassays are limited to one molecule. The method used in this study has been validated (O'Reilly et al., 2014), and we believe that the concentrations of steroids presented here are a true representation of the dynamic endogenous steroid production in murine follicles. The ultimate goal would be to apply this method to study human sex steroid production *in vitro*.

4 Effects of exogenous androgen exposure and antagonism on the developing follicle

4.1 Introduction

Encapsulated *in vitro* ovarian follicle culture (eIVFC) is a powerful tool that has enabled the study of autonomous follicle growth, oocyte maturation, ovulatory mechanics and signaling pathways (Kreeger et al., 2006, West-Farrell et al., 2009, Hornick et al., 2013, Skory et al., 2015). This 3D culture system takes advantage of biomaterials that maintain germ and somatic cell coupling and has been applied to a wide variety of species including murine, bovine, goat, canine, non-human primate, and human-derived follicles (Xu et al., 2006a, Araujo et al., 2014, Silva et al., 2015, Songsasen et al., 2011, Min Xu, 2009, Xu et al., 2009). Studies across species have enabled comparative assessments of biology unique to each species, for example, the physical rigidity of the biomaterial, while providing important new insights into the conserved mechanisms governing the follicle development

A particularly challenging question is the direct role of androgens on ovarian follicle development and oocyte maturation; eIVFC is ideally suited to evaluate the direct effect of these steroid hormones in an isolated, individual follicle. Although androgens are vital for female fertility, recent studies only begin to unravel the underlying mechanisms of action. Androgens exert their effect through the androgen receptor, a member of the nuclear receptor superfamily (Gelmann, 2002). In rodents, primates and human, androgen receptor is detected in granulosa cells from the primary stage onward, and its concentration peaks in the antral follicle stage (Sen and Hammes, 2010, Weil et al., 1998, Rice et al., 2007). Using the eIVFC conditions, testosterone was shown to directly increase macaque secondary follicle survival and growth supporting the notion that androgens regulate follicle dynamics (Rodrigues et al., 2015). Further support of this notion is derived from the murine androgen receptor global knockout models, in which females are subfertile and have reduced follicle development, poor oocyte

quality, altered gonadotrophin regulation and decreased ovulation rates (Hu et al., 2004, Shiina et al., 2006, Walters et al., 2007, Cheng et al., 2013). Elegant studies utilizing a granulosa cell-specific murine androgen receptor deletion model have produced a mirror image of these findings, thereby proving that androgen receptor-mediated androgen action is crucial for the development and function of the ovarian follicle (Sen and Hammes, 2010, Walters et al., 2012b). Increasing attention focuses on non-genomic androgen signaling pathways, including the activation of the protein paxillin, which, in turn, increased androgen receptor-mediated transcription, crucial mechanisms shown to be involved in the androgen-mediated promotion of follicle growth and survival (Sen et al., 2014). Several 2D murine follicle culture studies employed exogenous administration of androstenedione (Romero and Smitz, 2010, Murray et al., 1998, Wang et al., 2001, Tarumi et al., 2012) or testosterone (Wang et al., 2001, Romero and Smitz, 2010), and have shown intrafollicular convertion to estradiol, as measured by immunoassay. These powerful studies provide the impetus to assess the role of androgens more closely in the eIVFC setting and to couple this analysis with the steroid metabolome at each stage of follicle development.

Here we examined the impact of exogenous administration of DHT and the selective androgen receptor antagonist enzalutamide (MDV) on follicular steroid production and development.

4.2 Methods

4.2.1 Research strategy

In this chapter, I will study the direct effects of AR-mediated androgen action on follicular development. My outcomes will include follicle survival, follicle growth, target gene

analysis, steroid and peptide hormone production, oocyte growth and nuclear maturation status. To allow me to delineate if the observed androgen effects are mediated through the androgen receptor, I will use the potent AR agonist DHT, and the selective AR-antagonist enzalutamide (MDV).

4.2.2 Follicle treatments

Exogenous androgen exposure to the follicles was examined by the addition of 25 nM of DHT to the basal growth medium. The concentration employed was based on published dose-response experiments (Sen et al., 2014). All steroid treatments were diluted as appropriate in serum-free α-MEM media. Androgen-receptor antagonism was obtained by treatment with enzalutamide (MDV), at the dose of 1 μM, based on its IC50 value (Tran et al., 2009). Fresh treatments, at the initial dose, were provided during the media change at day 2 and 4.

4.2.3 *In vitro* follicle culture and oocyte maturation

Immature multilayered secondary follicles, with a diameter of 150-180 μm, were mechanically isolated from pre-pubertal 15-17 day old CD1 female mice pups, after enzymatic digestion using L15 medium supplemented with 0.1% DNase I and 0.1% Liberase TM, for 35-40 minutes on a shaker in a 37°C 5% CO2 incubator. Following a recovery period during which the follicles stayed in maintenance medium (α-MEM Glutamax medium with 1% FBS and 0.5% Penicillin-Streptomycin) in the CO2 incubator, individual follicles were examined under the dissection microscope and encapsulated in 0.5 % alginate, with slight modifications to the protocol described by Xu et al. (Xu et al., 2006a). Alginate-encapsulated follicles were placed in separate wells of a 96-well plate, each containing 100 μl culture medium, consisting of α-

MEM Glutamax supplemented with 3 mg/ml BSA, 10 mIU/ml rFSH, 1 mg/ml fetuin and 5 µg/ml ITS. Following plating, encapsulated follicles were imaged using Nikon Eclipse Te300 light microscope and healthy follicles with intact alginate beads were selected for culture. Follicles were maintained for 6 days in 37°C 5% CO2 incubator and media change (50 µl) and imaging was performed on alternate days. Images were analyzed using Image J Software. As previously described (Xu et al., 2006a), fully-grown follicles were, at the end of day 6 of culture, retrieved from the alginate bead and transferred to maturation medium composed of α-MEM Glutamax, containing 10 % FBS, 1.5 IU/mL HCG, and 5 ng/mL EGF for 16 h at 37°C, 5 % CO2. Oocytes were denuded from the surrounding cumulus cells by treatment with 0.3 % hyaluronidase and gentle aspiration. The oocytes were classified as mature, or metaphase II, if a polar body was visible in the perivitelline space. Healthy oocytes that hadn't resumed meiosis were classified as immature. Unhealthy oocytes were considered as degenerated.

4.2.4 Real-time qPCR analysis of target genes

Target genes of steroidogenesis and follicle development were analysed by real-time qPCR as described in Section 2.4 and according to a previously published protocol (Gathercole et al., 2007). To resume, at the end of culture, 21-28 pooled follicles cultured in similar treatment conditions (control, DHT or MDV) were flash frozen in liquid nitrogen, followed by RNA extraction with quality control, cDNA generation and qRT-PCR analysis using specific primers and probes and normalised cDNA concentrations. All reactions were normalized against the housekeeping genes 18S and Rpl-18. Data were expressed as dCT values.

4.2.5 LC-MS/MS analysis of steroid hormones

Conditioned media of 32-97 follicles, cultured in similar conditions, were pooled at day 2, 4 and 6 of culture. Media was stored at minus 80°C until analysis. Pooled follicle culture supernatant (approximately 1 ml) was placed in sylinised glass TLC tubes, and 20 µl of internal standard was added. Linear calibration series were prepared prior to each steroid extraction (Table 2-1). Three ml of tert-butyl-methyl-ether (MTBE) was added to each sample, followed by vortexing and freezing for 1 hour. The upper liquid phase of the samples was then gradually transferred to a 96 well plate employing glass Pasteur pipettes, with alternating cycles of evaporation at 55°C under nitrogen. Samples were reconstituted with 125 µl of methanol: water mixture (50:50) and frozen at -20°C until ready for analysis. The LC-MS/MS measurements for DHT conditions were performed in triplicate, for DHT+MDV conditions in duplicate. Data were expressed as mean ± SD, and were normalised to 100 follicles.

4.2.6 Peptide hormone measurement

Inhibin B was measured by ELISA at Anhs Lab (Kit AL-107, Webster, Texas) as part of the collaboration established with Dr Teresa Woodruff. The assay was performed on 1/5 diluted conditioned media. The dynamic range of the assay is 6, 12.7-1300 pg/ml, the limit of detection is 1.6 pg/ml.

4.2.7 Statistical analysis

Statistical analysis was performed with Prism 6 (Graph Pad) Software, using one-way ANOVA with post-hoc Tukey test to analyze follicle growth at each time point, inhibin-B measurements at each time point, and oocyte size at day 6 between multiple treatment

conditions. Fisher's exact test was used for survival, antrum formation and nuclear maturation status. Unpaired t-test was employed to analyze differences in steroid concentrations and dCT values between treatment versus control conditions, for independent experiments. All experiments were independently performed at least in triplicate, unless otherwise specified.

4.3 Results

4.3.1 Follicle growth, survival and oocyte maturation

We examined the morphological characteristics of the developing follicle by imaging the individual follicles at days 0, 2, 4 and 6 of culture, under control conditions (n=156) and after incubation with DHT (n=150) and MDV (n=89), respectively. We studied follicle growth, by measuring follicle diameters, the appearance of the antrum, defined by the movement of the oocyte to an eccentric position with appearance of a fluid-filled space, as well as follicle survival.

DHT-treated follicles exhibited increased growth at all examined stages of follicular development compared with control follicles (Figure 4-1), reaching a final average diameter of 336±34 μm, compared to 296±45 μm in controls ($p<0.0001$ at day 2, 4 and 6) (Figure 4-2). Conversely, MDV-treated follicles showed significantly restricted growth compared to control follicles (Figure 4-1), reaching a final diameter of 255±51 μm ($P<0.0001$ at day 2, 4 and 6) (Figure 4-2).

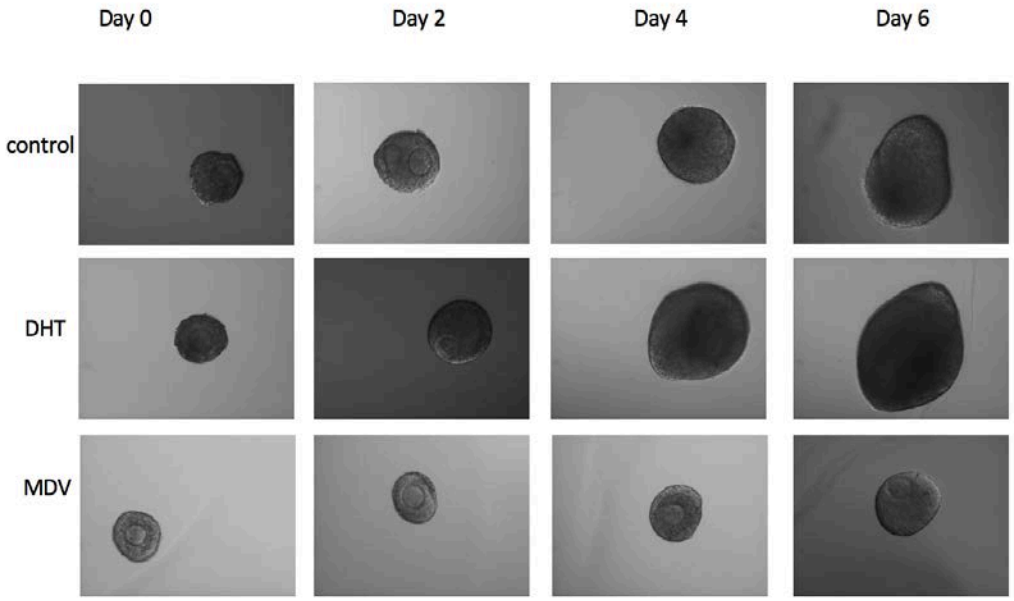


Figure 4-1 Representative light-microscopic images of control follicles, DHT- and MDV-treated follicles at day 0, 2, 4 and 6 of culture. DHT-treated follicles exhibited increased growth, and MDV-treated follicles decreased growth compared with control follicles.

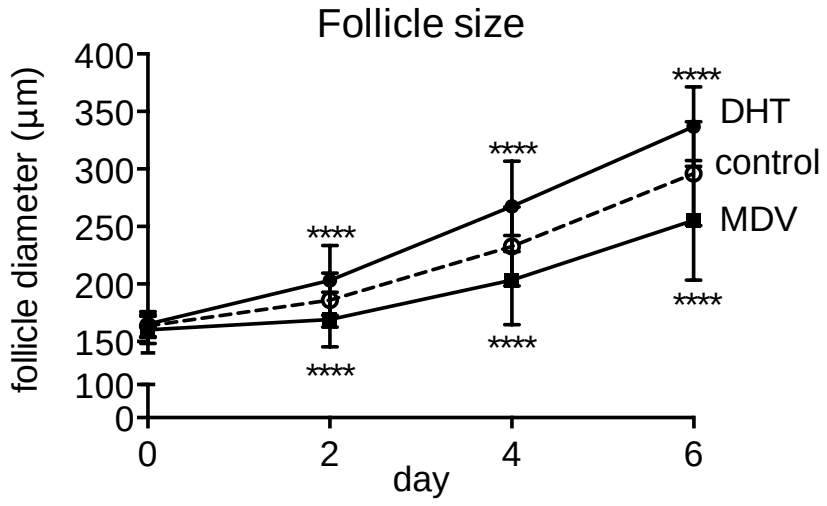


Figure 4-2 Follicle diameters at the start, and at day 2, 4 and 6 of culture for control follicles (dotted line), follicles treated with 25 nM DHT, and follicles exposed to enzalutamide (MDV). DHT-exposed follicles had an overall increased follicle growth, while MDV-treated follicles

*exhibited overall growth restriction, to a similar extent. Data are expressed as mean ± SD for 89-156 follicles. ****p< 0.0001 for the comparison of control to DHT and MDV respectively.*

Inhibin B production, a marker of granulosa cell mass (Lenie and Smitz, 2009), was significantly decreased in MDV-challenged follicles at day 6 of culture (737±358 versus 3563±992 in DHT-exposed follicles, p=0.01) (Figure 4-3).

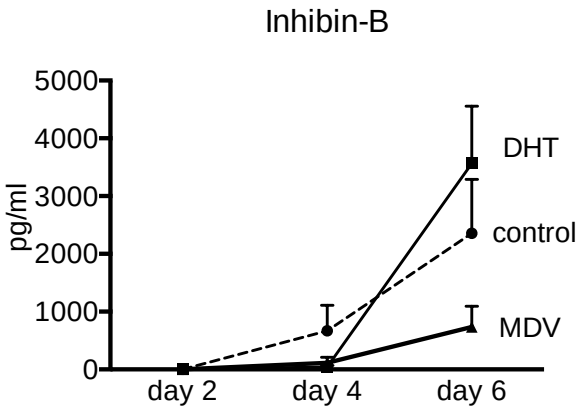


Figure 4-3 Inhibin-B concentrations, measured by ELISA, in DHT-, MDV- and untreated follicles, on day 2, 4, and 6 of culture. Data represent mean ± SD for 6 follicles for each treatment condition. Inhibin-B was significantly decreased in MDV conditions at day 6, compared with DHT conditions.

DHT supplementation resulted in significant acceleration of the pre-antral to antral follicle transition, with also a higher total number of follicles reaching the antral stage at day 6. At day 4 of culture, an antrum was present in 77±17% of the DHT-treated follicles, compared to 45±22% of control follicles (p<0.0001), and at day 6 this percentage increased at 99±2 in the DHT-group compared with 83±16% in the control group (p<0.001) (Figure 4-4). By contrast, MDV-treated follicles showed evidence of delayed antrum formation, with 35±17% rates at day 4 (p<0.05) and 69±42% at day 6 (p=0.1) (Figure 4-4).

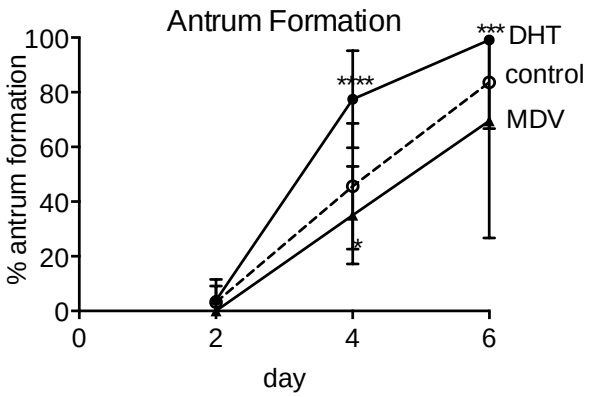


*Figure 4-4 Antrum formation in control follicles (dotted line), DHT 25 nM and MDV-follicles. DHT follicles achieve earlier and more complete antrum formation, compared with controls, and MDV-treated follicles have delayed antrum formation. Data are expressed as mean ± SD for 7-10 experiments. * <0.05, ***<0.001, ****< 0.0001 for the comparison of controls to DHT and MDV, respectively.*

We also observed a significantly increased survival rate of DHT-treated follicles, compared with control follicles (69±22 % versus 57±20 %, P=0.01), indicating that they were better protected from follicle atresia; survival of MDV-treated follicles did not differ from controls (54±28 versus 57±20 %, ns) (Figure 4-5).

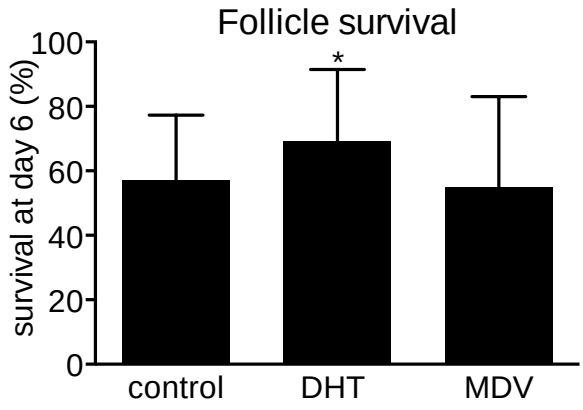


Figure 4-5 Follicle survival at the end of culture, in control follicles, follicles treated with 25 nM DHT, and follicles exposed to the AR-antagonist enzalutamide (MDV). DHT-exposed follicles have increased follicle survival ($P<0.05$). Data are expressed as mean ± SD for 7-10 independent experiments. ≤0.01*

Taken together, these results document a crucial role of androgens in the in follicle growth and antrum formation and protection from atresia.

At day 6 of follicle culture, we performed *in vitro* maturation, employing HCG and EGF to induce ovulation. Oocytes were denuded from cumulus cells and examined for size and nuclear maturation status, expressed as percentage meiosis II. Neither DHT exposure nor MDV treatment significantly impacted on oocyte size or nuclear maturation, as compared to control follicles (Figure 4-5).

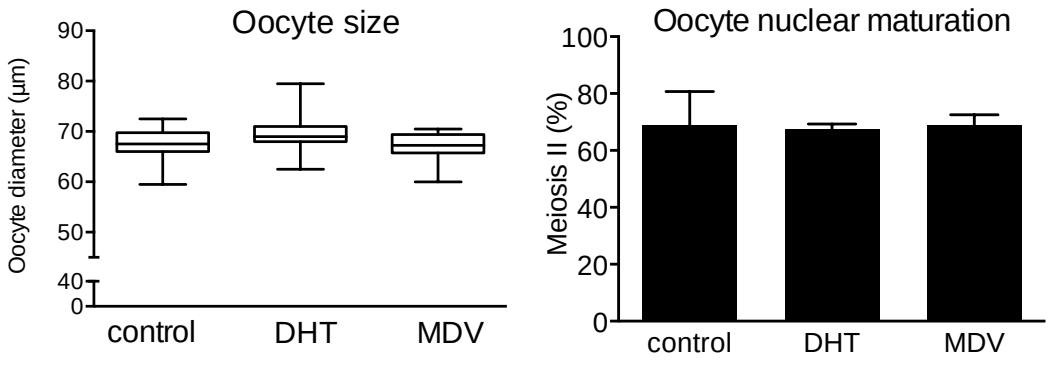


Figure 4-6 Oocyte status in control follicles, follicles treated with 25 nM DHT and follicles treated with MDV, with regard to size (left) and nuclear maturation (right). No difference were observed in either conditions. Data are expressed as mean ± SD for nuclear maturation, oocyte size is presented in box and whisker plots with boxes representative of the interquartile range and whiskers of the 5th and 95th centile, respectively, for at least 3 experiments, 14-30 oocytes per group.

At the end of culture, 21-28 follicles cultured in the presence of DHT or MDV, were pooled and flash frozen in liquid nitrogen, and RNA was extracted to allow for qRT-PCR analysis of target genes of folliculogenesis (Table 4-1). The differential gene expression for GDF-9, BMP-15 and AMH, with upregulated levels in DHT-treated follicles and downregulated levels in MDV-exposed follicles (Table 4-1) were likely to reflect changes in growth and maturation status in the treated follicles.

Table 4-1 Relative mRNA expression of target genes of steroidogenesis and follicle development, expressed as ΔCT values (normalized against 18S ribosomal RNA) ± SD (for each triplicate experiment from 21-28 follicles from each treatment group pooled prior to RNA extraction), in follicles treated with 25 nM DHT or MDV at day 6 of culture. P values reflect differential expression between DHT and MDV conditions.

Gene	Protein encoded	DHT	MDV	p
AR	androgen receptor	17.0±0.0	15.5±1.1	0.07
FSH-R	follicle stimulating hormone receptor	14.9±0.9	14.9±0.8	1
LH-R	luteinizing hormone receptor	13.1±1.3	13.7±1.0	0.5
AMH-RII	anti-Müllerian hormone receptor 2	16.0±0.9	16.7±1.1	0.2
GDF9	growth and differentiation factor 9	19.7±0.8	17.6±0.9	0.03
BMP15	bone morphogenic protein 15	20.5±0.8	16.7±1.6	0.02
AMH	anti-Müllerian hormone	20.2±0.9	18.2±0.8	0.04

4.3.2 Exogenous androgen exposure and antagonism reveals an androgen receptor-mediated autocrine feedback loop in the follicle

To determine the impact of androgens on follicular function, we explored the effects of exogenous androgen administration on follicular steroidogenesis. For this we used exposure to DHT, the most potent androgen, that, in contrast to testosterone, cannot be converted to oestrogens by aromatase activity. We compared the effects of DHT on follicular steroidogenesis to the effects of additional administration of the highly selective androgen receptor antagonist enzalutamide (MDV).

No significant changes in steroid production between the different treatment conditions were observed at day 2 and day 4 (Figure 4-7; Table 4-2 and 4-3). However, endogenous synthesis of DHEA, androstenedione and testosterone was clearly altered in DHT and DHT+MDV conditions at day 6 (Figure 4-7; Table 4-2 and 4-3). In response to exposure to 25 nM of DHT, day 6 follicles showed a significantly lower endogenous androgen production (androstenedione + testosterone, 80±15 nmol/L vs. 208±10nmol/L in controls, $p<0.01$); this decrease was most prominent for testosterone ($p<0.01$) and androstenedione ($p<0.01$). In the presence of androgen receptor antagonism, DHT+MDV, this effect was reversed and follicles showed increased endogenous androgen production (490±256 nmol/L, $P<0.05$ vs. DHT-treated follicles).

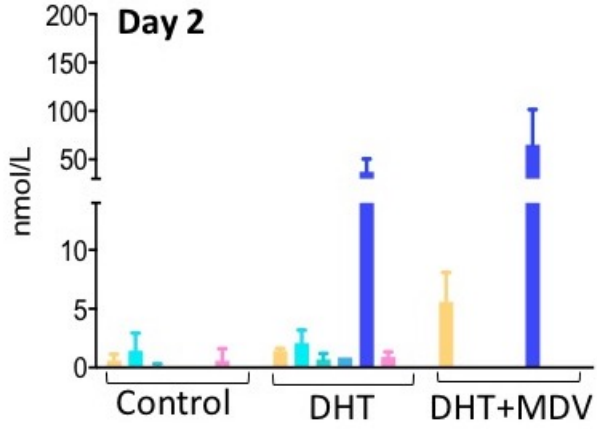
Table 4-2 Steroid hormone production by the cultured follicles, exposed to 25 nM DHT throughout culture. Steroids are measured by LC-MS/MS, in pooled follicle culture medium from 63-97 follicle incubations at day 2, 4 and 6 of culture, and normalised to 100 follicles. Data are expressed as mean ± SD (n=3 measurements).

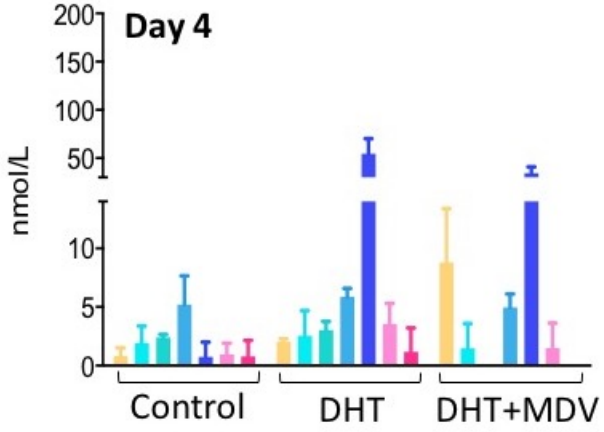
Steroid	Concentration at day 2 (nmol/l)	Concentration at day 4 (nmol/l)	Concentration at day 6 (nmol/l)
Progesterone (Prog)	1.4±0.2	2.0±0.2	10.9±0.5
DHEA	2.0±1.1	2.5±2.1	3.3±1.4
Androstenedione (Adione)	0.7±0.4	3.3±0.7	18.3±5.2
Testosterone	0.8±0.0	5.8±0.7	59.1±10.9
DHT	37.2±13.1	54.6±15.4	66.2±28.3
Estrone (E1)	0.89±0.4	3.5±1.7	5.8±5.1
Estradiol (E2)	0	1.1±2.0	77.2±10.8

Table 4-3 Steroid hormone production by the cultured follicles, exposed to 25 nM DHT + MDV throughout culture. Steroids are measured by LC-MS/MS, in pooled follicle culture medium from 32-50 follicle incubations at day 2, 4 and 6 of culture, and normalised to 100 follicles. Data are expressed as mean ± SD (n=2 measurements).

Steroid	Concentration at day 2 (nmol/l)	Concentration at day 4 (nmol/l)	Concentration at day 6 (nmol/l)
Progesterone (Prog)	5.5±2.4	8.7±4.6	45.2±2.3
DHEA	N/A	1.4±2.0	41.9±42.4
Androstenedione (Adione)	0	0	76.0±52.5
Testosterone	0	4.9±1.1	371.8±246.0
DHT	65.1±36.3	34.4±6.4	131.0±103.9

Estrone (E1)	0	1.49±2.1	4.3±6.1
Estradiol (E2)	0	0	169.6±75.9





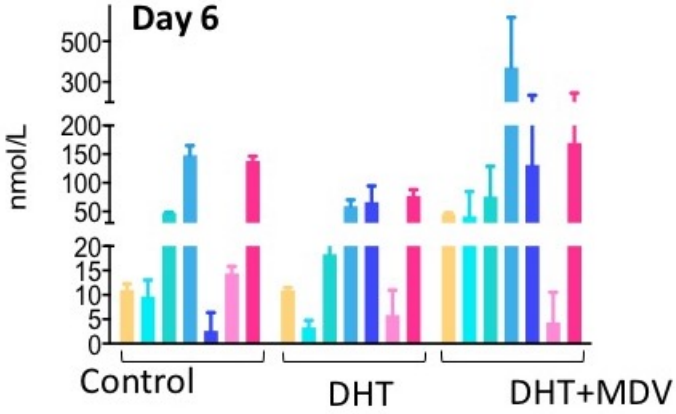


Figure 4-7 Steroid profile in control follicles, and follicles treated DHT 25 nM ± androgen-receptor antagonist MDV (enzalutamide) at day 2, day 4 and day 6 of culture, measured by LC-MS/MS in pooled conditioned medium from 32-97 follicles. Data are normalized to 100 follicles, and expressed as mean ± SD (n=3 measurements for control and DHT, 2 experiments for DHT+MDV condition). The color coding refers to Figure 3-9: Progesterone (yellow), DHEA (light green-blue), Androstenedione (dark green-blue), Testosterone (light blue), Dehydrotestosterone (dark blue), Estrone (light pink) and Estradiol (dark pink).

Corresponding mRNA expression analysis showed no changes in steroidogenic enzymes between DHT- and MDV-treated follicles, except for 17-hydroxylase (cyp17), which expression was decreased in MDV-treated follicles (Table 4-4). Moreover, a significant downregulation of androgen receptor mRNA in DHT-treated follicles (ΔCT 17.0±0.9 vs. 14.7±0.0 in untreated follicles, $p<0.05$) and a trend toward upregulation of androgen receptor expression in MDV-treated follicles (ΔCT 15.5±1.1, p=ns vs untreated follicles) was noticed.

Table 4-4 Relative mRNA expression of target genes of steroidogenesis and follicle development, expressed as ΔCT values (normalized against 18S ribosomal RNA) ± SD (for each triplicate experiment from 21-28 follicles from each treatment group pooled prior to RNA extraction), in follicles treated with 25 nM DHT or MDV at day 6 of culture. P values reflect differential expression between DHT and MDV conditions.

Gene	Protein encoded	DHT	MDV	p
star	steroidogenic acute regulatory protein	16.8±0.5	17.8±0.6	0.09
cyp11a1	cholesterol side-chain cleavage cytochrome P450	13.1±0.3	13.8±0.6	0.1

cyp17a1	17-hydroxylase	13.1±0.1	14.0±0.2	0.002
cyp19	Aromatase	15.1±0.9	15.6±0.8	0.5
hsd3b1	3β-hydroxysteroid dehydrogenase	10.1±0.7	10.0±0.8	0.8
hsd17b1	17β-hydroxysteroid dehydrogenase	11.9±0.7	11.6±0.8	0.6
AR	androgen receptor	17.0±0.0	15.5±1.1	0.07
FSH-R	follicle stimulating hormone receptor	14.9±0.9	14.9±0.8	1
LH-R	luteinizing hormone receptor	13.1±1.3	13.7±1.0	0.5

These findings point towards an androgen receptor-mediated feedback circuit, at the level of the follicle, involved in androgen exposure in the developing follicle.

4.4 Discussion

In this study, a detailed analysis of the AR-mediated androgen effects on the development of follicle and oocyte was performed. The current results support a crucial role of androgen action in follicle growth and survival. These observations were in line with the previously reported follicular growth-promoting effects of androgens (Murray et al., 1998, Wang et al., 2001, Sen et al., 2014, Vendola et al., 1998), their role in protecting from atresia (Sen et al., 2014) and enhancing follicle survival (Rodrigues et al., 2015). More specifically, we showed that the process of antrum formation occurred earlier and to a higher extent in DHT-treated follicles, and was impaired in AR-blocked follicles. Follicles grown in anti-androgen serum (Murray et al., 1998), or in the absence of FSH (Sen et al., 2014) or steroid-depleted follicles (Rodrigues et al., 2015), all had decreased antrum formation. These observations are

suggestive of AR-mediated androgen action in facilitating and optimizing antrum formation, a key developmental step in FSH-dependent follicle maturation.

It has been shown that during preantral follicle development, theca-cell derived androgens act via the AR and via non-genomic pathways to enhance FSH-induced proliferation and differentiation of granulosa cells (Sen et al., 2014). In basal conditions, although AR is expressed, immature follicles require approximately 4 days in culture to produce sufficient androgens to bind cytoplasmic AR and translocate it to the nucleus where AR becomes transcriptionally active (Lenie and Smitz, 2009). In case of exogenous androgen supplementation, this process occurs earlier and AR-signalling is enhanced (Lenie and Smitz, 2009), which might explain the promotion of growth and antrum formation observed in DHT-treated follicles. In the present study, differential gene-expression between DHT- and MDV-challenged follicles was not able to determine AR-responsive genes. This seems understandable, as only a very limited number of AR-induced genes in the ovary are currently known: Kit-ligand, mi-RNA125b, cyclooxygenase-2, cyclin-dependant kinase inhibitor-1/p21 and liver receptor-homolog 1 (Prizant et al., 2014). A more thorough analysis of gene expression by RNA sequencing of DHT-and MDV-treated follicles could possible provide new AR-targets.

Oocyte growth and maturation was not affected by AR agonist (DHT) or antagonist (MDV) treatment. Tarumi et al treated mouse ovarian follicles in culture with a concentration rate of 10^{-10} to 10^{-6} M DHT and found no effect on the capacity of the oocyte to resume meiosis following an ovulatory stimulus (Tarumi et al., 2014). Lenie et al found no effect on oocyte quality when treating mouse follicles during *in vitro* culture with the AR-antagonist hydroxyflutamide or bicalutamide (in a concentration range of 5nM - 5μM) (Lenie and Smitz, 2009). Only the highest dose (50μM) of AR-blockade resulted in decreased oocyte meiotic

maturation (Lenie and Smitz, 2009). Possibly, AR-modulating treatments have no adverse effect on oocytes because the AR remains inactive in the oocyte during preantral follicule development. In fact, AR expression was mainly found in the oocyte cytoplasm and was almost absent in the oocyte nucleus of preantral follicles in culture (Gill et al., 2004, Lenie and Smitz, 2009).

When follicles were exposed to exogenous DHT, a potent androgen that cannot be further converted, a compensatory mechanism was observed, resulting in diminished endogenous androgen secretion. This adaptation of steroid production was AR-mediated, as it was abolished in the presence of enzalutamide, a potent AR-antagonist, which prompted increased endogenous androgen synthesis. Moreover, in the presence of saturating concentrations of ligand, AR mRNA was downregulated. Reversely, when AR was structurally antagonised, follicular AR mRNA expression tended to increase. Taken together, these findings are indicative of a feedback circuit, at the level of the follicle, aiming to maintain sufficient AR stimulation in the developing follicle. The observed major up-and downregulation of androgen synthesis occurred independent of pituitary stimulation, and illustrates the capacity of the follicle to adapt, in an autonomous paracrine or autocrine way, to an endocrine challenge. The effects of androgens and antiandrogens on follicular steroidogenesis have not been studied in detail yet. Simultaneous addition of testosterone and flutamide to porcine follicles in culture increased progesterone synthesis, while testosterone or flutamide alone resulted in decreased follicular progesterone production (Duda et al., 2012). Administration of testosterone-enanthate, *in vivo* and *in vitro,* to adult male rats, decreased basal and HCG-induced testosterone production in testicular Leydig cells, while AR-blockade by cyproterone acetate increased testicular androgen levels (Bjelic et al., 2014). In the latter study, testosterone-enanthate administration induced significant decrease in mRNA expression levels of the steroidogenic

enzymes Star, Cyp11a1, Hsd3b1, Cyp17 and Hsd17b4, while enzyme levels in cyproterone acetate treated animals were similar to control conditions (Bjelic et al., 2014). Flutamide and casodex treatment in prepubertal male rates also resulted in elevated circulating and testicular androgen levels; the antiandrogens stimulated testicular steroidogenesis via increased gonadotrophin secretion (Rulli et al., 1995). In my study, DHT and MDV modulated follicular steroidogenesis, but how this was achieved needs further investigation. Steroid protein changes were not accompanied by alterations of gene expression levels of steroidogenic enzyme or gonadotrophin receptor, suggesting that the steroid enzyme bioactivity was directly or indirectly altered in treatment conditions.

In summary, I found that AR-mediated androgen action plays a crucial role in follicle growth, antrum development and protection from atresia. Exogenous androgen exposure resulted in decreased AR mRNA expression and reduced endogenous androgen production, while androgen antagonism reverted these effects. These findings are suggestive of an autocrine feedback loop in the follicle, involved in maintaining androgen homeostasis in the developing follicle.

The murine eIVFG system represents a multiparametric assay, suitable to study AR-mediated actions in follicle and oocyte development. This system could be used to gain more inside into the reproductive repercussions of endocrine disruptors with known androgenic or antiandrogenic effects (Diamanti-Kandarakis et al., 2009).

5 Effects of increasing concentrations of exogenous androgens and oestrogens on follicular development

5.1 Introduction

In clinical conditions of androgen excess, as observed in women with PCOS, preantral and antral follicle numbers are increased and development is arrested in the mid-antral stage, leading to chronic anovulation and subfertility (Jonard and Dewailly, 2004, Jayasena and Franks, 2014, Franks et al., 2006). PCOS follicles exhibit theca cell hypertrophy and metabolic dysfunction leading to hyperandrogenism. The follicle arrest is attributed to a hormonal imbalance between androgens and follicle-stimulating hormone (FSH), disturbing the selection of the dominant follicle that will ultimately ovulate (Franks, 1995). However, the direct intra-follicular effects of androgen excess remain poorly understood. Miscarriage risk is increased, and oocyte quality is reduced in PCOS, and assisted reproductive interventions might be necessary although still ineffective in some women (Fauser et al., 2012, Legro et al., 2013, Urman et al., 2004). PCOS is associated with metabolic dysfunctions, such as obesity, metabolic syndrome, hyperinsulinism and insulin resistance, and patients are at increased risk of type 2 diabetes and cardiovascular events (Goodarzi et al., 2011). On the other hand, in women with low ovarian follicle reserve, androgen supplementation through the androgen precursor DHEA or testosterone, is widely used to improve follicular development and fertility in assisted reproduction, although precise indications, dose and treatment duration are currently unknown (Sunkara et al., 2012, Narkwichean et al., 2013, Nagels et al., 2015).

In non-human primates, *in vivo* exposure to exogenous androgens in early gestation resulted in PCOS-like ovarian dysfunction in the adult offspring, with follicle excess, oligomenorrhea and hyperandrogenemia (Abbott et al., 1998, Eisner et al., 2002). During puberty in female primates, hyperandrogenism associated with obesity also triggered ovarian disturbances evocative of PCOS (McGee et al., 2014).

In mice, prenatal administration of DHT, specifically at day 16-18 of gestation, caused irregular estrous cysles, reduced preantral follicle development, decreased oculation and changes in adipose biology in the mature offspring (Caldwell et al., 2014). Several models of postnatal administration of exogenous testosterone or DHT in rodents triggered disrupted ovarian cyclicity, anovulation, atretic follicles, ovarian cysts, as well as hyperandrogenism, LH hypersecretion and insulin resistance (McDonald and Doughty, 1972, Ota et al., 1983, Arai et al., 1981, Manneras et al., 2007, van Houten et al., 2012, Caldwell et al., 2014). DHEA-induced rodent models of hyperandrogenism showed similar ovarian phenotypes but generally there were no associated increased neuro-endocrine drive (Roy et al., 1962, Familiari et al., 1985, Anderson et al., 1992, Lai et al., 2014). High fat diet induced metabolic alterations in DHEA-treated mice, such as impaired glucose tolerance and insulin resistance (Lai et al., 2014). Of note, long-term DHEA administration in mature mice did not provoke reproductive alterations evocative of PCOS (Caldwell et al., 2014).

Several 2D murine follicle culture studies employed exogenous administration of androstenedione (Romero and Smitz, 2010, Murray et al., 1998, Wang et al., 2001, Tarumi et al., 2012) or testosterone (Wang et al., 2001, Romero and Smitz, 2010), and have shown intrafollicular convertion to estradiol. The administration of high doses of androgens to growing follicles in culture was accompanied by decreased oocyte meiotic maturation (androstenedione and testosterone at ≥200 nM) (Romero and Smitz, 2010) and aberrant oocyte morphology (10^{-5} M androstenedione (Tarumi et al., 2012). Because aromatisable androgens were employed in this studies, it was unclear whether the observed effects were related to excess androgens or oestrogens.

These powerful studies provide the impetus to assess the role of androgen precursors more closely in the eIVFC setting and to couple this analysis with the steroid metabolome at

each stage of follicle development. We employed increasing doses of the clinically used androgen precursor DHEA, to examine the effects on follicle development, steroid metabolism and oocyte quality. To delineate androgen and oestrogen-mediated effects, follicles were treated with increased concentrations of DHT and E2. We next examined the effect of increasing DHT concentrations on the overall ovarian follicle dynamics, by performing follicle counting on cultured neonatal mouse ovaries.

5.2 Methods

5.2.1 Research strategy

In this chapter, I will first study the direct effects of the sex steroid precursor DHEA on follicular development. My outcomes will include follicle survival, follicle growth, target gene analysis, steroid hormone production, oocyte growth and nuclear maturation status. To allow me to delineate the observed effects with regard to androgen or oestrogen-mediated origin, I will compare the outcomes with those observed in follicles treated with increased doses of the non-aromatisable androgen DHT and the biologically active oestrogen E2. In a next set of experiments, DHT administration to neonatal whole ovaries in culture is studied to delineate the effects of exogenous androgens on follicle progression.

5.2.2 Follicle treatments

Growing follicles were exposed to DHEA at doses of 100, 200 and 500 nM, based on described statistically significant effects of DHEA at 10^{-9} M in preantral follicles grown in FSH-supplemented *in vitro* culture (Wang et al., 2001). The doses of DHT were adopted from published dose-response experiments (Sen et al., 2014, Wang et al., 2001), and the doses of E2

used were based on follicle culture experiments in primates (Ting et al., 2015). All steroids were diluted as appropriate in serum-free α-MEM medium. Fresh treatments, at the initial dose, were provided during media change at day 2 and 4 of culture.

5.2.3 *In vitro* follicle culture and oocyte maturation

Immature multilayered secondary follicles, with a diameter of 150-180 μm, were mechanically isolated from pre-pubertal 15-17 day old CD1 female mice pups, after enzymatic digestion using L15 medium supplemented with 0.1% DNase I and 0.1% Liberase TM, for 35-40 minutes on a shaker in a 37°C 5% CO2 incubator. Following a recovery period during which the follicles stayed in maintenance medium (α-MEM Glutamax medium with 1% FBS and 0.5% Penicillin-Streptomycin) in the CO2 incubator, individual follicles were examined under the dissection microscope and encapsulated in 0.5 % alginate. Alginate-encapsulated follicles were placed in separate wells of a 96-well plate, each containing 100 μl culture medium, consisting of α-MEM Glutamax supplemented with 3 mg/ml BSA, 10 mIU/ml rFSH, 1 mg/ml fetuin and 5 μg/ml ITS. Following plating, encapsulated follicles were imaged using Nikon Eclipse Te300 light microscope and healthy follicles with intact alginate beads were selected for culture. Follicles were maintained for 6 days in 37°C 5% CO2 incubator and media change (50 μl) and imaging was performed on alternate days. Images were analyzed using Image J Software. As previously described (Xu et al., 2006a), fully-grown follicles were, at the end of day 6 of culture, retrieved from the alginate bead and transferred to maturation medium composed of α-MEM Glutamax, containing 10 % FBS, 1.5 IU/mL HCG, and 5 ng/mL EGF for 16 h at 37°C, 5 % CO2. Oocytes were denuded from the surrounding cumulus cells by treatment with 0.3 % hyaluronidase and gentle aspiration, and their size and quality were assessed following imaging.

5.2.4 Neonatal mouse ovarian culture

Ovaries from day 5 old pups were cultures in αMEM Glutamax medium, supplemented with 10mIU/ml recombinant FSH, 3 mg/ml bovine serum albumin, 1 mg/ml bovine fetuib and 5 ng/ml insulin, transferrin and selenium, in 6-well plates with tissue culture well inserts, for 4 days in a 37°C 5%CO2 incubator, as previously described (Jin et al., 2010). Ovaries were fixed, dehydrated in ethanol, embedded in paraffin and stained with hematoxylin and eosin (H&E). Imaging and follicle counting were performed as per previously reported protocol (Bristol-Gould et al., 2006).

5.2.5 Realtime qPCR analysis

Target genes of steroidogenesis and follicle development were analysed by real-time qPCR as described in Section 2.4 and according to a previously published protocol (Gathercole et al., 2007). To resume, at the end of culture, 18-30 pooled follicles cultured in similar treatment conditions (control, DHT or MDV) were flash frozen in liquid nitrogen, followed by RNA extraction with quality control, cDNA generation and qRT-PCR analysis using specific primers and probes and normalised cDNA concentrations. All reactions were normalized against the housekeeping genes 18S and Rpl-18. Data were expressed as dCT values.

5.2.6 LC-MS/MS steroid hormone analysis

Conditioned media of 32-83 follicles, cultured in similar conditions, were pooled at day 2, 4 and 6 of culture. Media was stored at minus 80°C until analysis. Pooled follicle culture supernatant (approximately 1 ml) was placed in sylinised glass TLC tubes, and 20 µl of internal standard was added. Linear calibration series were prepared prior to each steroid extraction

(Table 2-1). Three ml of tert-butyl-methyl-ether (MTBE) was added to each sample, followed by vortexing and freezing for 1 hour. The upper liquid phase of the samples was then gradually transferred to a 96 well plate employing glass Pasteur pipettes, with alternating cycles of evaporation at 55°C under nitrogen. Samples were reconstituted with 125 µl of methanol: water mixture (50:50) and frozen at -20°C until ready for analysis. The LC-MS/MS measurements for DHEA 200 nM conditions were performed in triplicate, for DHEA 100 and 500 nM conditions in duplicate. Data were expressed as mean ± SD, and were normalised to 100 follicles.

5.2.7 Inhibin B measurement

Inhibin B was measured by ELISA at Anhs Lab (Kit AL-107, Webster, Texas) as part of the collaboration established with Dr Teresa Woodruff. The assay was performed on 1/5 diluted conditioned media. The dynamic range of the assay is 6, 12.7-1300 pg/ml, the limit of detection is 1.6 pg/ml.

5.2.8 Statistical analysis

Statistical analysis was performed with Prism 6 (Graph Pad) Software, using one-way ANOVA with post-hoc Tukey test to analyze follicle growth at each time point, Inhibin-B measurements, and oocyte size at day 6 between multiple treatment groups. Fisher's exact test was used for survival, antrum formation and nuclear maturation status. Unpaired t-test was employed to analyze steroid concentrations and dCT values, between treatment and control conditions, for independent experiments. All experiments were independently performed at least in triplicate, unless otherwise specified.

5.3 Results

5.3.1 The androgen precursor DHEA is converted to active sex steroids in the developing follicle

In this set of experiments, I tested whether the developing follicle is a target tissue for metabolism of the androgen precursor DHEA to downstream active sex steroids, and to which extent exogenous DHEA administration interfered with endogenous steroid synthesis in the follicle.

Steroid profiling by LC-MS/MS revealed that DHEA was actively metabolized by the follicle at all time points, including the immature stage (day 2) when endogenous steroidogenesis was not quantifiable yet (Figure 5-1 and Table 5-1). Supplementation with 100 nM DHEA revealed high capacity for androgen generation (androstenedione, testosterone and DHT) and high levels of aromatase activity at day 4, which appeared further enhanced by day 6 (Figure 5-1 and Table 5-1). This steroidogenic profile was accompanied by significant downregulation of mRNA levels of steroidogenic enzymes, AR and gonadotrophin receptors (Table 5-2).

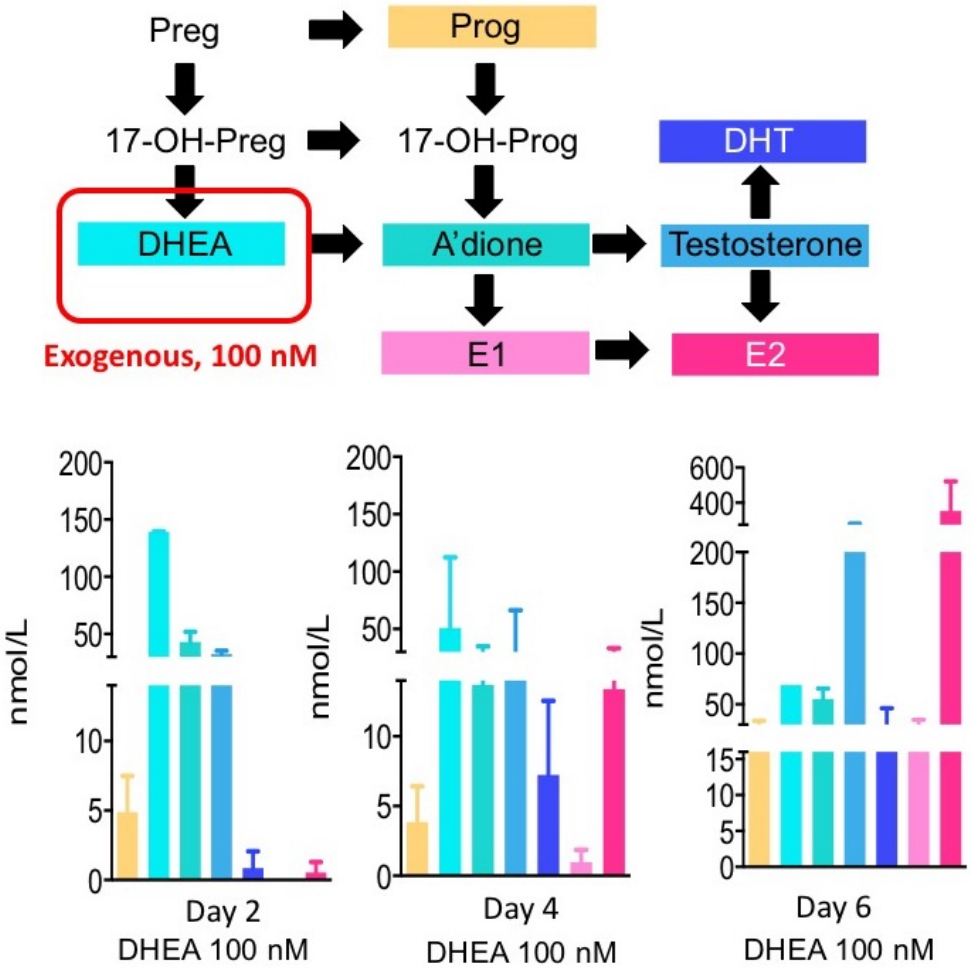


Figure 5-1 Steroid hormone profile of follicle cultured with supplementation of 100 nM DHEA, measured by LC-MS/MS, in pooled follicle culture medium (32-41 follicles) at day 2, 4 and 6 of culture, and normalised to 100 follicles. Data are expressed as mean ± SD (2 measurements). Upper panel: Schematic showing the steroidogenic pathway, with colour coding of the different steroids assessed in culture medium: Progesterone (Prog), Dehydroepiandrosterone (DHEA) (exogenous addition), Androstenedione (A'dione), Testosterone, Dehydrotestosterone (DHT), Estrone (E1) and Estradiol (E2).

When increasing DHEA concentrations to 200nM, we observed a further increase in androgen synthesis while oestrogen generation was decreased at day 4 ($p<0.01$, vs. DHEA 100

nM). A further increase of exogenous DHEA administration to 500 nM yielded a significant decrease in oestrogen generation at day 6 ($p<0.05$; vs. control, Figure 5-2 and Table 5-1).

Table 5-1 Steroid hormone production by the follicles,cultured in the presence of 100, 200 or 500 nM DHEA measured by LC-MS/MS, in pooled follicle culture medium (32-83 follicles) at day 2, 4 and 6 of culture, and normalised to 100 follicles. Data are expressed as mean ± SD (n=3 measurements for DHEA 200 nM , duplicates for DHEA 100 and 500 nM conditions).

Steroid	Prog	DHEA	A'dione	Testo-sterone	DHT	E1	E2
Day 2 DHEA 100nM	4.8± 2.6	139.3± 0.3	43.0± 8.8	32.4± 3.0	0.85± 1.2	0	0.5± 0.7
Day 2 DHEA 200nM	1.0± 0.2	120± 6.6	40.8± 2.7	31.5± 0.6	4.2± 7.4	0	0.7± 1.2
Day 3 DHEA 500nM	2.5± 0	332.1± 93	121.5± 27.5	90.7± 20.5	0	0	0
Day 4 DHEA 100nM	6.7± 2.7	119.9± 30.0	38.2± 0.8	72.4± 5.2	12.6± 7.0	0.3± 0.5	36.3± 1.8
Day 4 DHEA 200nM	1.9± 0.2	109± 47.9	34.6± 3.2	80.3± 10.0	4.9± 4.6	3.0± 0.8	4.4± 6.3
Day 4 DHEA 500nM	4.0± 0.4	287.9± 44	137.1± 33.9	236.2± 22.1	0	0	7.9± 11.2
Day 6 DHEA 100nM	27.9± 5.7	69.0± 0.0	55.2± 10.4	226.1± 56.0	23.± 22.2	20.7± 14.0	352.8± 239.0
Day 6	7.1±	107.2±	55.5±	206.3±	0	4.05±	150.1±

DHEA 200nM	0.2	2.1	2.0	15.8		6.5	9.9
Day 6 DHEA 500nM	8.3± 0	202.5± 57.2	89.0± 41.2	288.8± 50.6	0	8.2± 4.1	87.8± 17.5

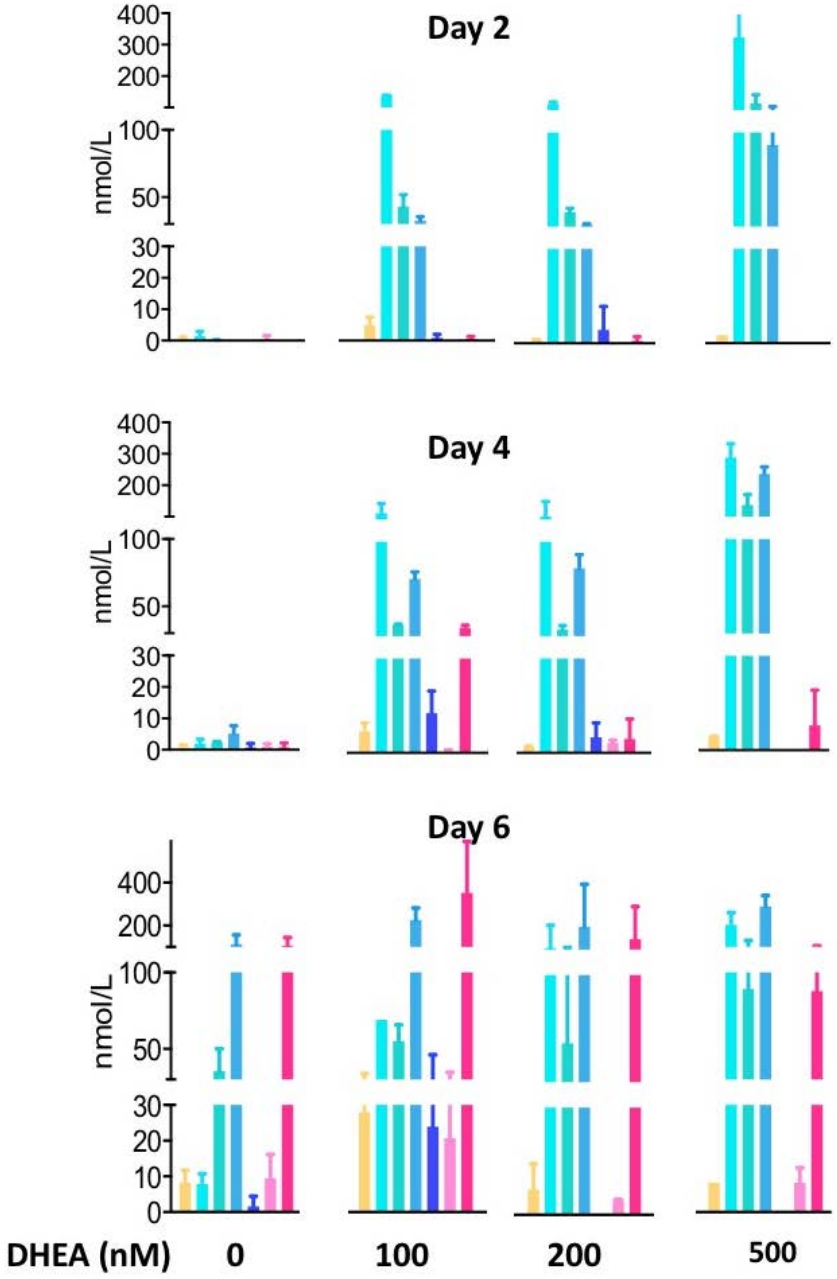


Figure 5-2 Effect of increasing DHEA supplementation (100, 200 and 500nM) on follicular steroid hormone production, compared with control conditions. Steroids were measured LC-MS/MS, in pooled follicle culture medium (32-83 follicles), and normalised to 100 follicles.

Data are expressed as mean ± SD (3 measurements for control and DHEA 200 nM and 2 measurements for DHEA 100 and 500 nM conditions. The color coding refers to Figure 5-1.

When calculating the androgen/oestrogen ratio ([androstenedione + testosterone] / [estrone + estradiol]), to assess aromatase (cyp19a1) activity (Lenie and Smitz, 2009, Kushnir et al., 2009), we found that DHEA 100 nM maintained the balance observed in control follicles (DHEA 100 nM vs. controls on day 6: 0.9±0.3 versus 1.3±0.01, p=0.3) whereas higher DHEA concentrations significantly increased the androgen/oestrogen ratio (DHEA 200 nM 1.7±0.09, $p<0.01$ vs. controls; DHEA 500 nM 4.3±1, $p<0.05$; day 6) (Figure 5-3).

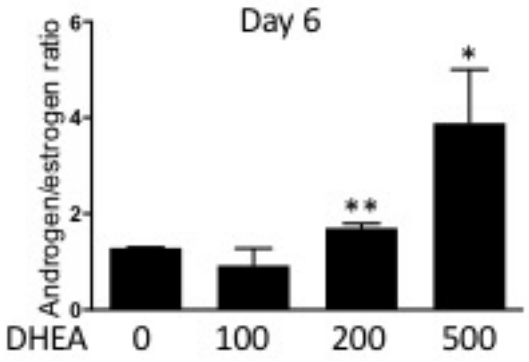


*Figure 5-3 Androgen/oestrogen ratio on day 6, for follicles treated with 100, 200 and 500 nM DHEA compared with untreated follicle; data are expressed as mean ± SD, * $p<0.05$, ** $p<0.01$ for the comparison of untreated to treated follicles.*

Increased androgen/oestrogen ratios are indicative of decreased aromatase activity. At mRNA level we found a downregulation of cyp19a1 expression with DHEA as compared to control follicles (Table 5-2).

*Table 5-2 Relative mRNA expression of steroidogenic enzymes, androgen receptor and gonadotrophin receptors, expressed as ΔCT values (normalized against 18S ribosomal RNA) ± SD (for each triplicate experiment from 18-30 follicles from each treatment group pooled prior to RNA extraction). *p<0.05, ** p<0.01 for comparison of untreated versus DHEA 100, DHEA 200 and DHEA 500nM treated conditions respectively.*

Gene	Protein encoded	DHEA 0nM	DHEA 100nM	DHEA 200nM	DHEA 500nM
star	steroidogenic acute regulatory protein	15.3±0.7	18.1±1.1 (*)	17.0±0.3 (*)	15.7±2.0
cyp11a1	cholesterol side-chain cleavage cytochrome P450	12.5±0.1	16.5±0.2 (**)	12.1±0.4	13.9±0.4 (**)
cyp17a1	17-hydroxylase	12.8±0.0	16.0±0.5 (**)	12.8±0.4	14.0±0.1 (**)
cyp19	Aromatase	12.3±0.3	15.9±0.8 (*)	14.1±0.4	14.9±0.2 (*)
hsd3b1	3β-hydroxysteroid dehydrogenase	9.6±0.3	11.4±0.5 (**)	9.9±0.3	10.5±0.1 (*)
hsd17b1	17β-hydroxysteroid dehydrogenase	11.1±0.2	12.5±0.3 (*)	11.3±0.3	12.2±0.3 (*)
AR	androgen receptor	14.7±0.0	18.5±1.1 (**)	15.6±0.8	17.2±1.7
FSH-R	follicle stimulating hormone receptor	12.8±1.0	16.6±0.9 (**)	13.7±0.2	15.6±0.6 (*)

LH-R	luteinizing hormone receptor	11.7±0.2	14.8±0.5 (**)	12.6±1.5	13.8±0.3 (**)

Taken together, the experiments utilizing exogenous DHEA administration as a probe, showed that earlier stages of the follicle development are also capable of active sex steroid generation. Moreover, increased exposure to DHEA results in inhibition of aromatase activity and consequently decreased oestrogen production.

5.3.2 Effects of increasing concentrations of exogenous androgens and oestrogens on follicular growth and survival

Next we looked at the impact of the androgen precursor DHEA on follicular development and oocyte maturation. DHEA can be converted to androgens and subsequently oestrogens; therefore, we compared this to the impact of increasing doses of non-aromatisable DHT, the most potent androgen, and the biologically active oestrogen, E2, to dissect effects that were potentially conveyed by androgens and oestrogens in a distinct fashion.

Follicle size, reflective of follicular growth, was enhanced by DHEA 100 nM ($P<0.0001$ at day 2, 4 and 6) and DHT 25 nM ($P<0.0001$ at all timepoints) (Figure 5-4); increasing androgen exposure to DHEA 200 nM neutralized this effect and a further increase to DHEA 500 nM and exposure to DHT 50 nM showed the opposite effect, with a significant reduction in follicle size ($P<0.01$ at day 6 for DHEA 500nM versus control, and $P<0.05$ at day 6 for DHT 50nM versus control) for (Figure 5-4). The higher dose of E2 (25 nM) increased follicle size significantly ($P<0.01$ at day 2, 4 and 6 compared with control) while 10 nM E2 had no effect (Figure 5-4).

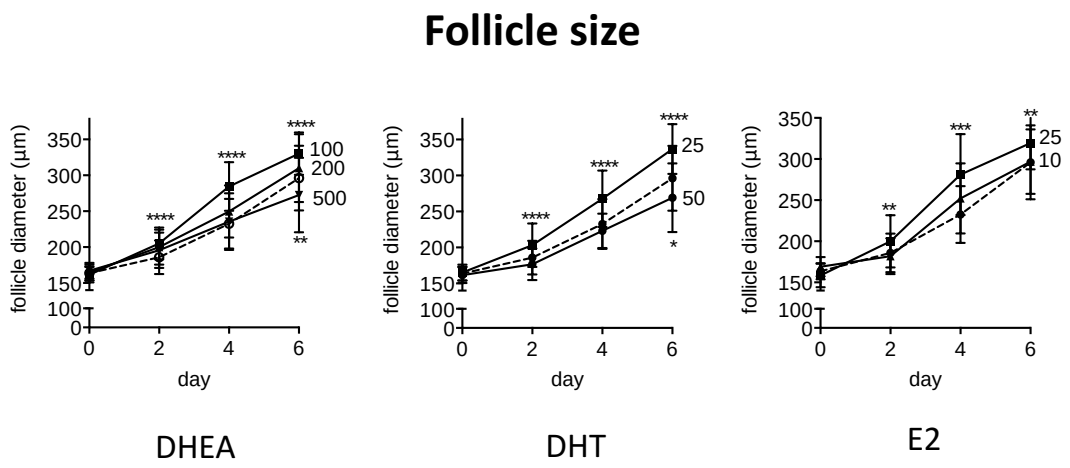


*Figure 5-4 Effect of increasing DHEA (100, 200 and 500 nM), DHT (25 and 50 nM), and E2 (10 and 25 nM) supplementation versus control (dotted lines) on follicle size at day 0, 2, 4 and 6 of culture. Data (n=31-156 follicles/condition) are expressed as mean ± SD for follicle diameter; Stars indicate significant changes between treatment and control conditions: ** p<0.01,*** p<0.001, **** p< 0.0001.*

The differences in follicle size are mirrored by changes in inhibin-B levels, a marker of granulosa cell proliferation, although the differences between treatment conditions did not quite reach statistical significance (Figure 5-5). Of note, Inhibin-B levels tended to be increased at day 4 in DHEA 100nM and E2 25n treated follicles (ns), compared with control and DHT-exposed follicles (Figure 5-5).

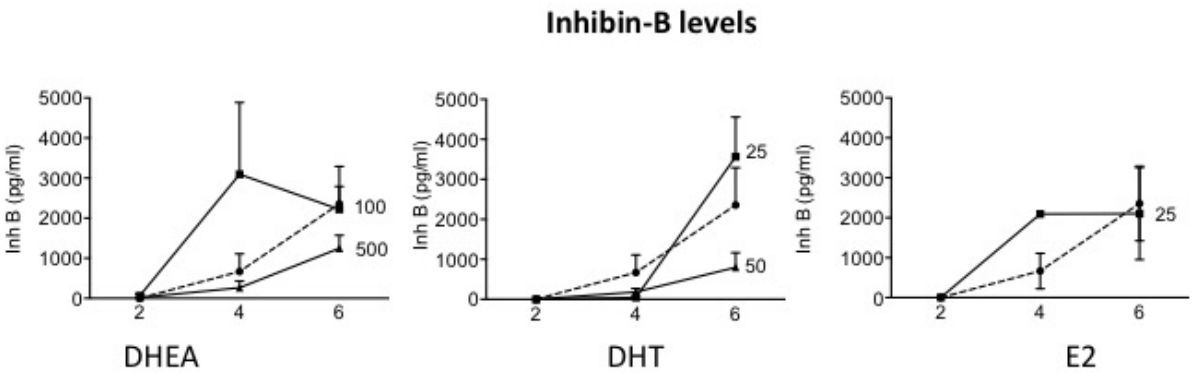


Figure 5-5 Inhibin-B levels measured by ELISA in follicles treated with DHEA (100 and 500nM), DHT (25 and 50 nM) and E2 (25nM) at day 2, 4 and 6. Data are represented by mean ± SD for n=6 follicles per condition. No statistical significant changes were noted.

Antrum formation was significantly enhanced by DHT 25 nM while increasing DHT to 50 nM reverted this effect (Figure 5-6). DHEA 100 nM appeared to have a beneficial effect on antrum formation while higher concentrations had an adverse effect, though the differences failed to reach significance (Figure 5-6). E2 had no effect whatsoever on antrum formation (Figure 5-6).

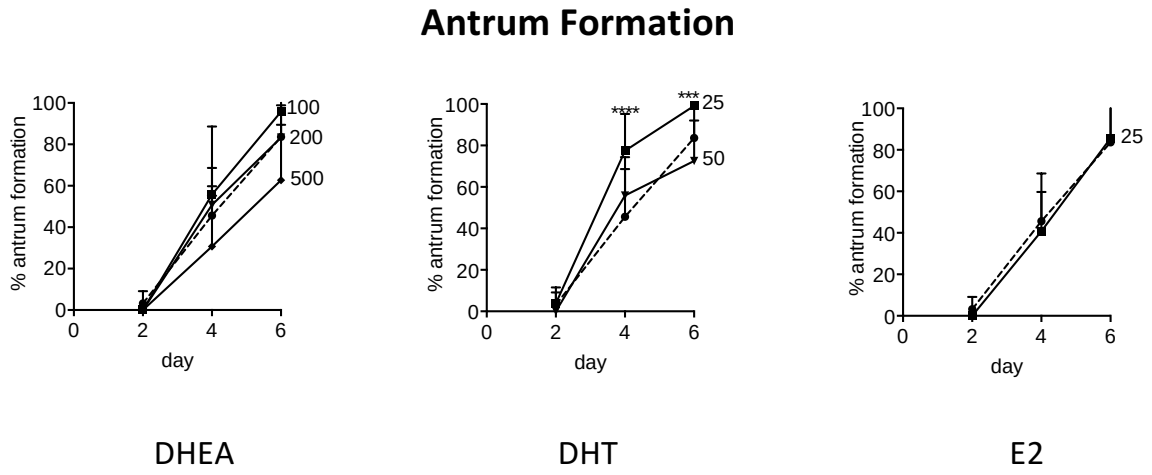


*Figure 5-6 Effect of increasing DHEA (100, 200 and 500 nM), DHT (25 and 50 nM), and E2 (10 and 25 nM) supplementation versus control (dotted lines) on antrum formation rates at day 0, 2, 4 and 6 of culture. Data (n=31-156 follicles/condition) are expressed as mean ± SD; ***p< 0.001, **** p< 0.0001.*

Follicle survival rates increased significantly with exposure to DHEA 100 and 200 nM and DHT 25 nM (Figure 5-7). This effect was lost when DHEA was increased to 500 nM and

DHT to 50 nM (Figure 5-7). By contrast, E2 at 10 nM yielded no discernible effect on follicle survival, while a significant increase was observed after increasing E2 to 25 nM (Figure 5-7).

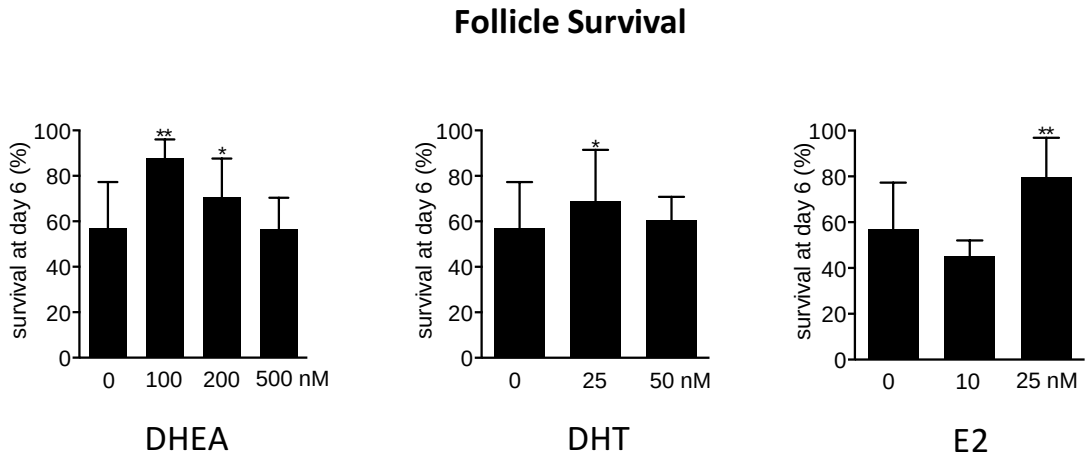


*Figure 5-7 Effect of increasing DHEA (100, 200 and 500 nM), DHT (25 and 50 nM), and E2 (10 and 25 nM) supplementation versus control (dotted lines) on follicle survival rates at day 0, 2, 4 and 6 of culture. Data (n=31-156 follicles/condition) are expressed as mean ± SD, * p<0.05, ** p<0.01.*

5.3.3 Effects of increasing concentrations of exogenous androgens and oestrogens on oocyte development and quality

Although DHEA 200 nM-treated follicles grew light-microscopically similar to control follicles (Figure 5-4), oocyte development was significantly impaired, with regard to cytoplasmic and nuclear development, as compared with control and DHEA 100 nM-exposed oocytes. Oocyte size was decreased to 62±4 μm in DHT 200 nM conditions, versus 67±3 μm in controls ($p<0.0001$) and 68.0±3 μm in DHEA 100 nM oocytes ($p<0.0001$) (Figure 5-8). Meiotic resumption for DHEA 200 nM-exposed oocytes occurred in 19±16%, versus 68±11% in control conditions ($p<0.001$) and 65±7% in DHEA 100 nM oocytes ($p<0.001$) (Figure 5-8).

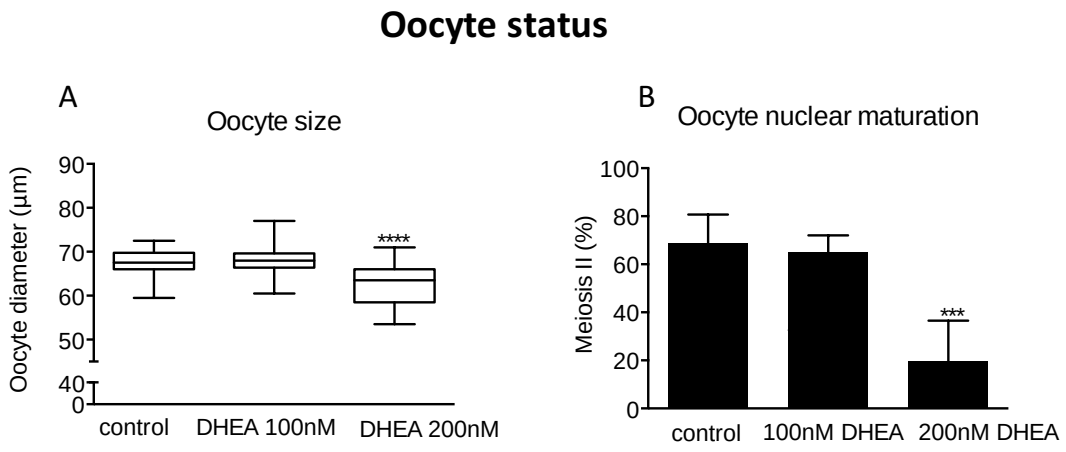


*Figure 5-8 Oocyte status in control follicles, follicles treated with 100 nM or 200 nM DHEA, with regard to (A) oocyte size and (B) oocyte nuclear maturation, for 24-48 oocytes per condition. Data are expressed as mean ± SD for nuclear maturation, oocyte size is presented in box and whisker plots with boxes representative of the interquartile range and whiskers of the 5th and 95th centile, respectively, for at least 3 experiments, 24-48 oocytes per group. *** p <0.001, **** p<0.0001*

Oocyte size was also significantly reduced by the highest concentrations of DHEA (500 nM) and the higher DHT concentration (50 nM) (Figure 5-9). By contrast, E2 exposure had no effect on oocyte size (Figure 5-9). These findings were completely mirrored when assessing oocyte nuclear maturation, which was significantly decreased by higher androgen concentrations but not affected by oestrogen administration (Figure 5-10).

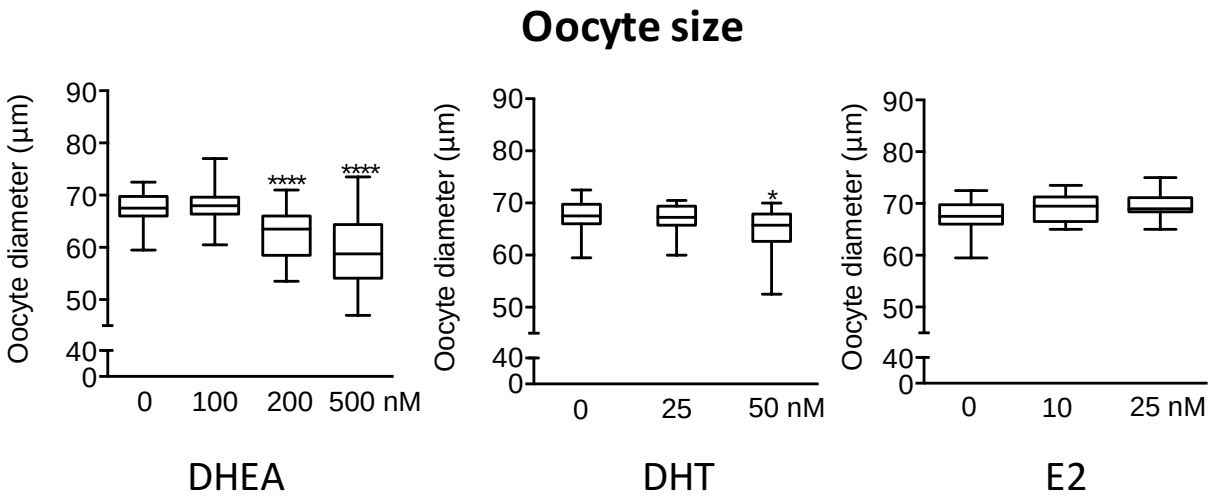


*Figure 5-9 Effect of increasing DHEA (100, 200 and 500 nM), DHT (25 and 50 nM), and E2 (10 and 25 nM) supplementation versus untreated follicles on oocyte size following in vitro maturation at day 6 of culture. Data are expressed as mean ± SD for nuclear maturation, oocyte size is presented in box and whisker plots with boxes representative of the interquartile range and whiskers of the 5th and 95th centile, respectively, for at least 3 experiment, 24-48 oocytes/condition, * p<0.05, **** p<0.0001.*

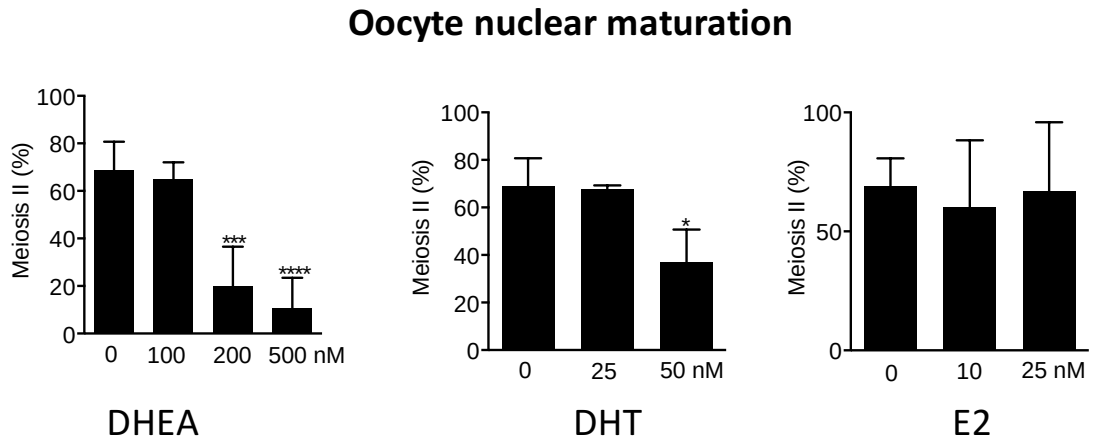


*Figure 5-10 Effect of increasing DHEA (100, 200 and 500 nM), DHT (25 and 50 nM), and E2 (10 and 25 nM) supplementation versus untreated follicles on oocyte nuclear maturation following in vitro maturation at day 6 of culture. Data (n=24-48 oocytes/condition) are expressed as mean ± SD for meiosis II rate , * p<0.05, *** p<0.001, **** p<0.0001.*

5.3.4 The impact of increasing androgen administration on ovarian follicular dynamics *in vitro*

After demonstrating that androgens promote the transition from the pre-antral to the antral stage, we tested whether androgen supplementation, at increasing concentrations, influenced follicle progression in the whole ovary, i.e. the transition from primordial to primary, and primary to secondary follicle stage. We employed neonatal CD1 mouse ovarian culture, in basal conditions and treatment with 10^{-8} and 10^{-7} M DHT for 4 days. At the end of the culture period, ovaries were fixed and stained with hematoxylin and eosin (H&E) (Figure 5-11). The number of follicles at each developmental stage was counted and were classified as primordial (a small oocyte partially surrounded by granulosa cells), primary (small oocytes completely surrounded by granulosa cells), and secondary (larger oocyte with more than one layer of granulosa cells) (Table 5-3).

DHT supplementation did not change the percentage of follicles in the primordial pool, indicating that androgen exposure did not interfere with the rate of primordial follicle activation in our system (Figure 5-12 and Table 5-3). However, observations of the follicle dynamics within the growing pool revealed ovaries exposed to the lowest DHT concentration (10^{-8} M DHT) have decreased follicle atresia compared with untreated follicles (Figure 5-13). This effect was lost in ovaries treated with a higher DHT concentration (10^{-7} M DHT), which exhibited a decreased amount of secondary follicles, indicative of a decrease in follicle progression from primary to secondary stage when compared with control ovaries and ovaries treated with a lower DHT concentration (Table 5-2 and Figure 5-13).

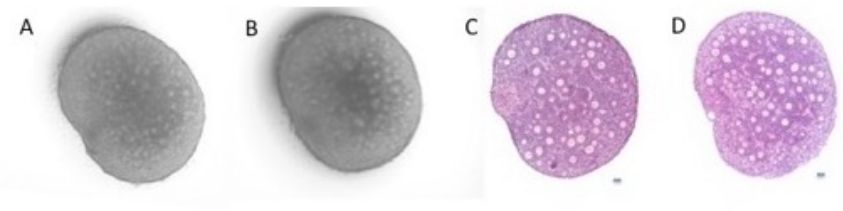


Figure 5-11 Morphology of untreated and DHT-treated ovaries at the end of culture. (A) Brightfield microscope image of a control ovary at magnification 40 ,and (B) of a 10^{-7}M DHT-treated ovary. (C) Haematoxylin-eosin (HE) stained section of a control ovary and (D) of a 10^{-7}M DHT-treated ovary at magnification 40. Scale bar = 100 μm. The number of primordial follicles and total number of growing follicles was similar between both groups.

*Table 5-3 Follicle classes in untreated ovaries, and ovaries cultured in presence of 10^{-8} or 10^{-7} M DHT. The number of different follicle classes was counted per ovary, and expressed as % of total follicles. Data represent mean ± SD (n=3 ovaries per treatment group).** p<0.01 compared with control.*

Follicle class	Untreated ovaries	10^{-8} M DHT	10^{-7} M DHT
Primordial	91.1 ± 0.8 %	87.5 ± 2.7 %	89.1 ± 4.1 %
Primary	3.1 ± 0.6 %	6.2 ± 2.7 %	6.9 ± 4.4 %
Secondary	4.2 ± 0.6 %	6.5 ± 0.3 % **	2.0 ± 0.3 % **
Atretic	1.2 ± 0.4 %	0.8 ± 0.1 %	1.5 ± 0.3 %

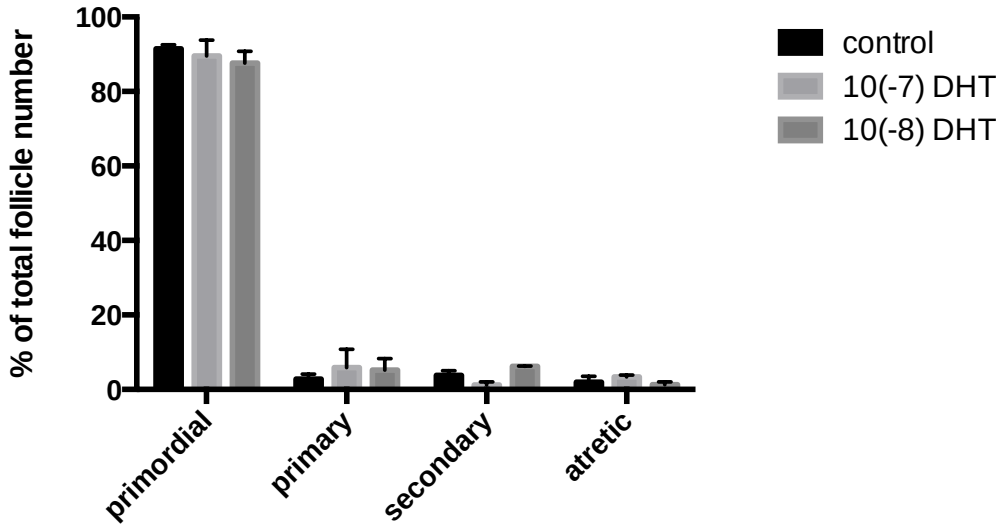


Figure 5-12 Follicle counts in ovaries in untreated ovaries, and ovaries cultured in presence of 10^{-7} or 10^{-8}M DHT. The number of different follicle classes was counted per ovary, and expressed as % of total follicles. Data represent mean ± SD (n=3 ovaries per treatment group). No significant differences were observed between groups for the different follicle classes, except for secondary follicles, which were decreased in 10^{-7} M DHT ovaries and increased in 10^{-8}M ovaries.

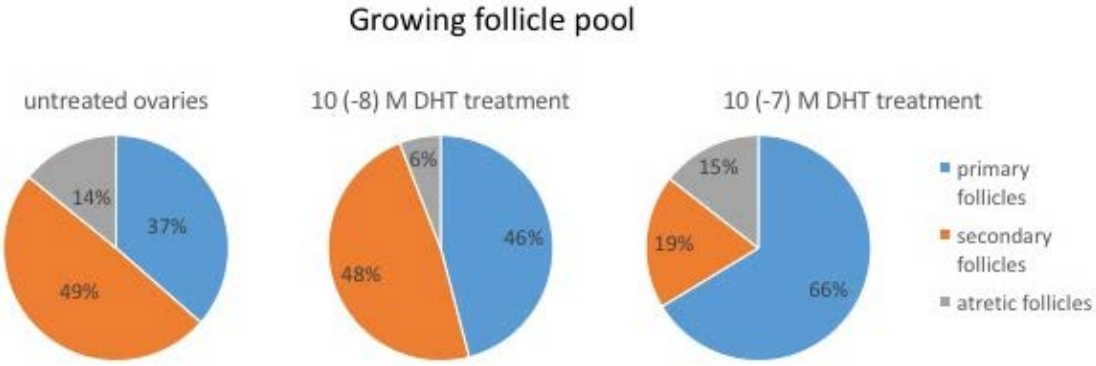


Figure 5-13 Follicle dynamics in the growing follicle pool. Primary, secondary and atretic follicles, expressed as percentage of total growing follicles, for untreated ovaries and ovaries cultured in the presence of 10^{-8} and 10^{-7}M DHT. Ovaries exposed to the lower concentrations of DHT, have a decreased amount of atretic follicles compared with controls, while exposure

to higher concentrations of DHT decreased the number of secondary follicles compared with controls and lower doses of DHT.

Taken together, the ovarian experiments pointed toward a beneficial effect of lower dose androgen supplementation, with regard to follicle survival, and a detrimental effect of higher doses of androgens on follicle progression from the primary to the secondary stage.

5.4 Discussion

Results from the current study indicate that the ovarian follicles is a target tissue for DHEA, from the early preantral stage onward. Low dose DHEA supplementation supported follicle growth and survival, without impacting on oocyte quality. Increasing DHEA exposure reversed these beneficial effects and were detrimental for oocyte development, estradiol production and follicle growth.

Employing the sex steroid precursor DHEA as steroid substrate for developing follicles, steroid metabolites could be detected for up to 3 enzymatic steps downstream of DHEA, and confirmed effective conversion to downstream androgens and oestrogens. Sex steroid production occurred earlier in DHEA-treated follicles compared with non-stimulated follicles, indicating that in the presence of steroid substrate, immature follicles are steroidogenically active and capable of major androgen synthesis. In women with PCOS adrenal DHEA and androstenedione production is clearly elevated (Mahesh and Greenblatt, 1962, Puurunen et al., 2009, Bardin and Lipsett, 1967), Circulating androgen precursors are likely to be metabolized by the small preantral PCOS follicles, thereby contributing to the ovarian hyperandrogenism. On the other hand, circulating DHEAS levels decrease from the age of 30 years on (Orentreich

et al., 1984), and lead to reduced substrate availability, which could possibly play a role in the decreased fecundability observed during the third decade.

Exposure to 100 nM DHEA had positive effects on follicle growth and survival, to a similar extent as those observed in the presence of 25 nM DHT, and these effects were mediated through the AR. Importantly, androgen treatment, at the lowest dose employed, did not impair oocyte development. These results contribute to the scientific foundation for DHEA pre-treatment in poor responder women undergoing IVF, in order to improve the developmental quality of the maturing follicles. However, the dose and duration of androgen treatment to achieve the folliculotrophic effects remain to be examined in detail, and depend on baseline circulating androgen levels in the patients.

When treating follicles with increasing concentrations of DHEA (200 and 500 nM), dysfunctional follicle development occurred, primarily characterised by a robust suppression of oocyte growth and maturation (in 200 nM DHEA conditions), and at higher doses (500 nM DHEA) accompanied by decreased aromatase enzyme activity, reduced oestrogen production, and follicular growth stagnation. The androgen-mediated downregulation of aromatase is in line with reported observations in granulosa (Yang et al., 2015a) and Leydig cells (Liu et al., 2015, Maris et al., 2015). In rats, administration of DHT was accompanied by decreased granulosa cell proliferation (Pradeep et al., 2002), suppressed aromatase activity and reduced estradiol production (Conway et al., 1990), In primates, DHT administration resulted in reduced FSH-stimulated oestrogen synthesis (Zeleznik et al., 2004) Previous work has shown that follicular steroidogenesis is modulated by the oocyte throughout development (Vanderhyden and Macdonald, 1998) Impaired oocyte development, manifested by meiotic incompetence, was associated with dysfunctional granulosa cell steroidogenesis (Goldschmit et al., 1989). In this study increasing DHEA exposure altered the ability of the granulosa cell to accumulate

estradiol in the presence of abundant substrate and at the same time suppressed oocyte development. This observation might suggest that oocyte-derived factors or oocyte-dependant signalling pathways were responsible for the decreased oestrogen output and the reduced follicle growth. It is clear that additional factors are involved, such as kinetics related to the aromatase enzyme. In fact, the enzyme might be saturated at higher concentrations of DHEA (200 and 500 nM) (Sohl and Guengerich, 2010).

Previous studies reported that *in vitro* supplementation of mouse follicles cultures with androstenedione, at doses > 200 nM (Romero and Smitz, 2010) or 10^{-5} M (Tarumi et al., 2012) was associated with decreased meiotic maturation and impaired spindle formation. The toxic effect on the oocyte was attributed to oestrogen-excess in one study (Tarumi et al., 2014), and inconclusive with regard to its androgen-mediated mechanism in the other study (Romero and Smitz, 2010). In my study, the detrimental oocyte phenotype in DHEA 200 and 500 nM treated follicles was clearly attributable to increased androgen provision to the follicle.

Although it is difficult to translate the present findings, obtained with experimental androgen concentrations in murine follicles, to human pathological follicle development in hypo-or hyperandrogenic conditions, some general implications might hold true. As others have highlighted before (Ware, 1982, Prizant et al., 2014), the maturing follicle is subject to a delicate androgen homeostasis, with a clear threshold level, beyond which the beneficial effects become deleterious. This means that over- replacement of DHEA in assisted reproductive settings, might actually harm oocyte quality and become detrimental for follicle growth. This study clearly underlies the need for adequately powered randomised controlled trials on DHEA supplementation in women with low ovarian reserve undergoing fertility work-up, which take into account baseline levels of circulating androgens, and aim toward restoring physiological DHEA concentrations.

On the other hand, results from the present study might provide novel insight into the pathophysiological mechanisms underlying antral follicle arrest observed in PCOS. I describe a gradual, oocyte-centred process of follicle developmental arrest. From this, I extrapolate that local androgen excess negatively impacts on the oocyte quality in PCOS, which in turn co-orchestrates antral follicle arrest. This statement is rendered plausible by the fact that PCOS oocytes have a decreased fertilization potential, indicative of compromised oocyte quality (Urman et al., 2004). Moreover, the cytoplasmic oocyte development is clearly altered in PCOS, and reflected by a distinctly abnormal gene expression profile (Wood et al., 2007). Also, the follicular fluid in PCOS, contains hallmarks of metabolic dysregulation, likely to contributing to the impaired oocyte quality, with elevated free androgen index (Niu et al., 2014).

To conclude, DHEA treatment in the murine eIVFG system revealed dose-dependent androgen-related effects on follicle growth and survival, steroid and peptide hormone secretion, and on the capacity of the oocyte to resume meiosis following an ovulatory stimulus. These experiments illustrate the U-shaped curve of androgen actions in the follicle and highlight the delicate androgen homeostasis required for optimal follicle development and function.

6 Final conclusions and future directions

6.1 Final conclusions

Androgen homeostasis in the developing follicle is crucial to ensure optimal follicle growth, steroidogenesis and oocyte maturation. Androgen deficiency and androgen excess are both detrimental for female reproductive health. In this study we have investigated androgen synthesis, metabolism and action in the developing murine ovarian follicle. This work has resulted from a collaboration between Dr. Teresa Woodruff, follicle biologist at Northwestern University Chicago, and Prof.Wiebke Arlt, expert in steroid metabolism at the University of Birmingham. This collaboration has allowed me to spend 9 months of training in the Woodruff Lab in 2013, as well as to be enrolled in the Frontiers in Reproduction course at the Marine Biology Lab in Woods Hole, Massachusetts, in 2014. These training periods have permitted me to set up and validate the murine encapsulated *in vitro* follicle culture system at the University of Birmingham, as a platform to study the effects of androgens in the follicle.

6.1.1 Endogenous steroid production and androgen metabolism in the developing follicle in culture

In this study, we showed for the first time FSH-stimulated, endogenous dynamic production of androgens, oestrogens and progesterone by murine follicles in 3D-culture. These data were derived from the combination of two powerful techniques and the expertise of two leading research centres. LC-MS/MS is now considered the gold standard for all steroid hormone measurements, which avoids the methodological limitations of platform-based immunoassays, known for intrinsic problems of lower sensitivity, specificity, and cross-reactivity in case of structurally linked steroid molecules. The 3D encapsulated follicle culture technique closely mimics the *in vivo* ovarian environment, and preserves the oocyte-somatic

architecture, communication and paracrine signalling. Both methods have been extensively validated by the research groups involved in this collaboration (Xu et al., 2006a, Kreeger et al., 2006, West-Farrell et al., 2009, Hornick et al., 2013, Skory et al., 2015, O'Reilly et al., 2014, Idkowiak et al., 2016). Therefore, we believe that the hormonal secretion pattern presented in this study phenocopies the *in vivo* steroid production by murine follicles.

The endogenous steroid production observed in basal conditions was modified when follicles were cultured in AR-modified conditions. The addition of exogenous DHT, a potent AR-agonist, resulted in reduced endogenous androgen secretion and reduced AR gene expression. Addition of enzalutamide (MDV), a selective AR-antagonist, abolished this effect and led to increased endogenous androgen production. These findings indicate that the ovarian follicle is equipped with a sensing mechanism, coupled to an autocrine feedback loop, aiming to maintain adequate AR-stimulation during follicular development. This regulatory mechanism occurred independent of pituitary stimulation, and was not accompanied by alterations in gene expression for steroidogenic enzymes or gonadotrophin receptors.

After addition of DHEA, the universal sex steroid precursor, to the follicle culture medium, as substrate for androgen and oestrogen generation, we observed that small preantral follicles, steroidogenically inactive in basal conditions, were capable of achieving high androgen production. Increased supplementation of DHEA substrate led to decreased oestradiol production by the preovulatory follicles. This suggests that the androgen-generating capacity of the preantral follicle may contribute to the ovarian hyperandrogenism observed in PCOS women, who often exhibit increased adrenal DHEA production.

6.1.2 Effect of increasing androgen supplementation on follicle development and oocyte maturation

It has been increasingly recognized that androgens are crucially required for optimal female fertility, but have dual effects on follicle development and ovulation (Walters, 2015, Prizant et al., 2014, Lebbe and Woodruff, 2013, Gleicher et al., 2011). In our system, we demonstrated a U-shaped curve of androgen action in normal folliculogenesis.

In Enzalutamide (MDV)-treated follicles, AR signalling was antagonized, which resulted in reduced follicle growth and significantly less follicles reaching the antral stage. Lower dose DHT (25 nM) supplementation resulted in earlier preantral to antral follicle transition, with significantly more follicles reaching the antral stage. DHT 25nM treated follicles were growth-advanced, and a similar effect was observed when supplementing follicles with a lower dose of DHEA (100nM). Low dose androgen treated follicles were better protected from atresia during culture and ovulated meiotically competent oocytes, at the same rate as untreated follicles.

Increased androgen provision (50 nM DHT and >200 nM DHEA) to the growing follicles caused a robust suppression of oocyte development, manifested by a decreased size and a reduced capacity to resume meiosis. At the highest dose of androgen exposure employed in this study (50 nM DHT and 500 nM DHEA), follicle growth was impaired, and 500nM DHEA also decreased estradiol production.

These findings are in agreement with, and complement previously published reports on the effect of androgens on follicle development (Murray et al., 1998, Romero and Smitz, 2010, Wang et al., 2001, Sen et al., 2014, Rodrigues et al., 2015). This body of work provides support for the clinical use of androgen treatment in women with androgen insufficiency seeking fertility, or in low ovarian reserve patients undergoing assisted reproduction. The observations

presented in the current study highlight the need for adequate measurements of baseline and follow-up circulating androgen levels during androgen treatment. In fact, androgen over-replacement is likely to provoke detrimental effects on follicle and oocyte.

On the other hand, this study demonstrates the oocyte-toxic effect of increased androgen provision to the follicle. In PCOS, ovarian hyperandrogenism might directly impact on oocyte development, and oocyte-derived factors or oocyte-dependent signaling pathways may contribute to antral follicular arrest in an autocrine fashion, independent of gonadotrophin stimulation. Although an increase in FSH stimulation is capable of overriding the antral follicle arrest in PCOS, the fertilization potential of individual PCOS oocytes is compromised (Heijnen et al., 2006, Urman et al., 2004). Additional factors, such as the imbalance between androgens and FSH, are known contributors to follicular arrest in PCOS women (Franks, 1995).

6.2 Future directions

6.2.1 Steroid analysis in murine target tissues of sex steroid hormones

The promising steroid hormone data obtained in this study with LC-MS/MS now allows to finely dissect androgen synthesis and metabolism at murine target tissue level. Dr Woodruff's laboratory has developed FemKUBE, an integrated *ex vivo* female reproductive tract, consisting of 3D culture of murine ovarian follicles, fallopian tube, uterus and cervix. In this bioengineered system, the ovarian follicles, cultured in a microfluidic system, are triggered, by addition of gonadotrophins, to produce steroid hormones in a pattern that mimics the human menstrual cycle. That way, the reproductive organs are exposed to cyclic levels of sex steroids, which will allow study of normal female reproductive biology in a first time, and become a tool

to explore pathophysiology and substance screening. LC-MS/MS measurements of steroid hormone levels across the different organs would represent a major addition to this technique.

6.2.2 Steroid profiling in human follicles during *in vitro* alginate encapsulated growth

The transfer of the present results to the human follicular physiological and pathophysiological development, is hindered by the use of a murine model. However, the eIVFG system was able to sustain human follicle growth, steroidogenesis and oocyte development (Xu et al., 2009). This methodology is a highly clinical relevant area of research, and developed as a fertility preserving tool for female cancer patients facing gonadotoxic treatments (Jeruss and Woodruff, 2009). Importantly, human ovarian follicles grown *in vitro* were capable of sustained steroid hormone production, in a pattern phenocopying the entire female menstrual cycle (Skory et al., 2015). Until present, all hormone measurements have been performed using immunoassays, and although important knowledge has been gained through this valuable studies, a modern mass spectrometry-based approach is warranted for this promising technique to excel.

Also, in future experiments, ovarian tissue from healthy women, women with PCOS and patients with low ovarian reserve could be employed to unravel the role of androgens in human ovarian health and disease.

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