

Investigating mechanisms of Premature Ovarian Insufficiency using a mouse model



Sairah Sheikh
Linacre College
University of Oxford

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I dedicate this thesis to my parents

Without their unconditional love and support none of my success
would be possible

كُنْ فَيَكُونُ

*When He decrees a matter,
He only says to it,
'Be'
And it is.
(Qur'an 2:117)*

Abstract

Premature ovarian insufficiency (POI) occurs in 1% of reproductive-age women. The ovarian manifestation ranges from the presence of a variable population of follicles (follicular) to the absence of follicles (afollicular), and in the majority of cases the cause is unknown.

The Double Mutant (DM) mouse model of follicular POI, arises from the oocyte-specific deletion of *Mgat1* and *C1galt1* required for the generation of O- and N-glycans respectively. DM females are subfertile at 6-weeks, infertile by 9-weeks and undergo POI by 3-months of age and exhibit increasing numbers of primary follicles and a depletion of follicles at the later stages. This is accompanied by elevated gonadotropins and decreased sex steroids, which is characteristic of POI in women.

In this thesis, characterisation of follicle development reveals multiple defects at various stages of development including the presence of abnormally large primary follicles resulting a block in follicle development, and luteinisation of unruptured preovulatory follicles resulting in anovulation and infertility. Analysis of *in vitro* follicle development demonstrated that follicles retain the potential to develop indicating that intra-ovarian factors may be playing a role in the follicle dysfunction. Finally, reaggregated ovaries demonstrate that although DM germ cells retain the potential to develop follicles, DM somatic cells are imprinted with the POI phenotype.

These findings highlight new avenues for investigation to further elucidate the mechanisms underpinning POI as well as targets for treatment.

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Abbreviations

3 β HSD	3 beta-hydroxysteroid dehydrogenase
AMH	Anti-müllerian hormone
ART	Assisted reproductive technology
BL	Basal lamina
BMP-15	Bone morphogenetic protein 15
cAMP	Cyclic adenosine monophosphate
CL	Corpus luteum
COC	Cumulus-oocyte-complex
DAB	3,3'-diaminobenzidine
DM	Double mutant
ECM	Extracellular matrix
EtOH	Ethanol
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
GC	Granulosa cell
GDF-9	Growth differentiation factor 9
GlcNAcT-1	<i>N</i> -acetylglucosaminyltransferase I
GnRH	Gonadotropin-releasing hormone
H&E	Haematoxylin and eosin
HA	Hyaluronic acid
HABP	Hyaluronic acid binding protein
HPG	Hypothalamic-pituitary-gonadal
IHC	Immunohistochemistry
LH	Luteinising hormone
LUF	Luteinised unruptured follicle
PCOS	Polycystic ovarian syndrome
POF	Premature ovarian failure
POI	Premature ovarian insufficiency
RO	Reaggregated ovary
ROS	Resistant ovary syndrome
T-synthase	β 1,3-galactosyltransferase
TGF- β	Transforming growth factor beta
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
ZP	Zona pellucida

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List of published papers

1. Kaune H, Sheikh S, Williams SA. Analysis of in vitro follicle development during the onset of premature ovarian insufficiency in a mouse model. *Reprod Fertil Dev.* 2016.
2. Grasa P, Sheikh S, Krzys N, Millar K, Janjua S, Nawaggi P, et al. Dysregulation of follicle development in a mouse model of premature ovarian insufficiency. *Reproduction.* 2016;152(5):591-601.

Introduction

1.1 Ovarian function

1.1.1 Overview of follicle development

The ovary is a heterogeneous organ with two key functions; generation and release of a mature egg and the production of steroid hormones essential for puberty, follicle development, and supporting the ensuing pregnancy (5, 6). Follicles are the functional unit of the ovary of which the most simple and abundant is the primordial follicle. Establishment of the primordial follicle pool occurs *in utero* in humans, around 16-weeks' gestation (6-8), and a few days after birth in mice (5, 9, 10). Throughout a woman's reproductive life, primordial follicles are actively recruited from this quiescent pool, and when the number of primordial follicles is reduced to a critical threshold of ~1000, reproduction ceases and they enter menopause (11-13).

Once recruited, the primordial follicle develops through the primary, secondary, preantral, antral, and preovulatory stages culminating with the ovulation of a mature egg in a process called follicle development (6). However, not all follicles complete this development process with the majority of follicles undergoing atresia via apoptosis (6, 7, 14, 15). In humans, follicle development takes approximately 6 months, whilst in mice this process is 19-21 days long (16).

Complex bidirectional interactions between the oocyte and somatic cells (granulosa and theca cells) orchestrate follicle development. The somatic cells provide a nurturing environment to the oocyte via signalling growth factors and nutrients. The oocyte reciprocates by secreting factors that regulate proliferation and differentiation of granulosa cells (GCs) and ultimately follicular growth (17-23).

1.1.1.1 Classification of follicles

A method of classifying follicles was established in 1968 (24) and is used in this thesis. Follicles are classified by the number of GCs surrounding the oocyte on the largest cross-section of the follicle, whilst taking its morphological appearance into account (Figure 1.1).

Primordial follicles, classified as type 1 and 2 follicles, have a small oocyte and a few surrounding flat pregranulosa cells. Primary follicles are split into two types; type 3a, which has a complete layer of up to 20 GCs, and type 3b, which have 21 to 60 GCs. Secondary follicles are classified as type 4 follicles with 61 to 100 cells. Preantral follicles are classified as type 5a (101 to 200 GCs) or type 5b (201 to 400 GCs). Antral follicles are classified as type 6 (401 to 600 GCs) and type 7 follicles (>600 GCs) and finally the preovulatory follicle is classified as a type 8 follicle.

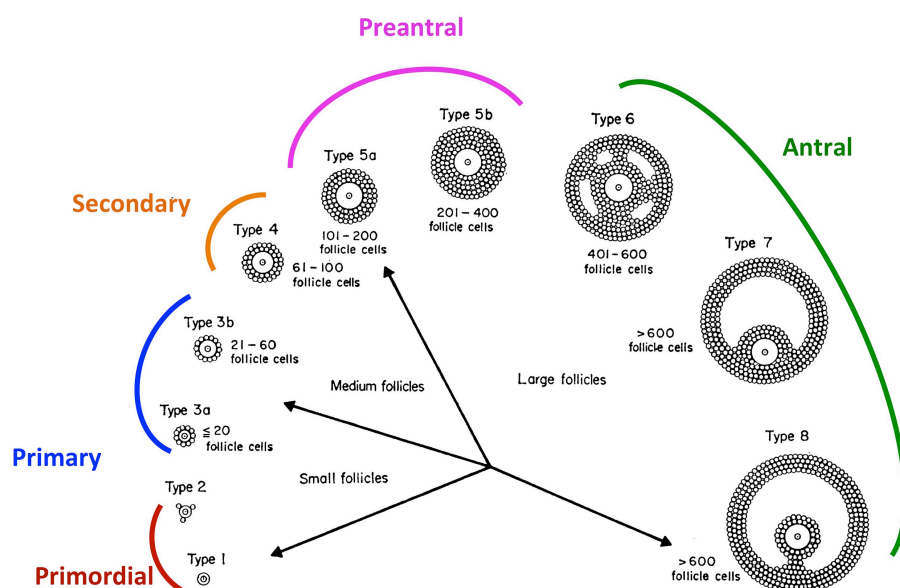


Figure 1.1 Classification of follicles according to Pedersen and Peters criteria established in 1968 (24).

1.1.2 Follicle development

Primordial follicles are composed of a meiotically quiescent oocyte surrounded by a single layer of squamous pregranulosa cells encapsulated by a basal lamina (BL) (Figure 1.2) (5, 10, 24). The BL maintains the integrity of the follicle and influences follicle growth by regulating the molecules that enter the follicle (25, 26) and is discussed in detail in Section 1.1.2.1. The activation of a quiescent primordial follicle is associated with a number of changes, including growth of the oocyte (15, 28, 29) and the differentiation (27) and proliferation of the surrounding pregranulosa cells to become cuboidal GCs (Figure 1.2) (6, 27, 30, 31). The differentiation of the squamous pregranulosa cells to cuboidal GCs is the hallmark of follicle activation, and the resulting single GC layered follicle is now referred to as a primary follicle (Figure 1.2).

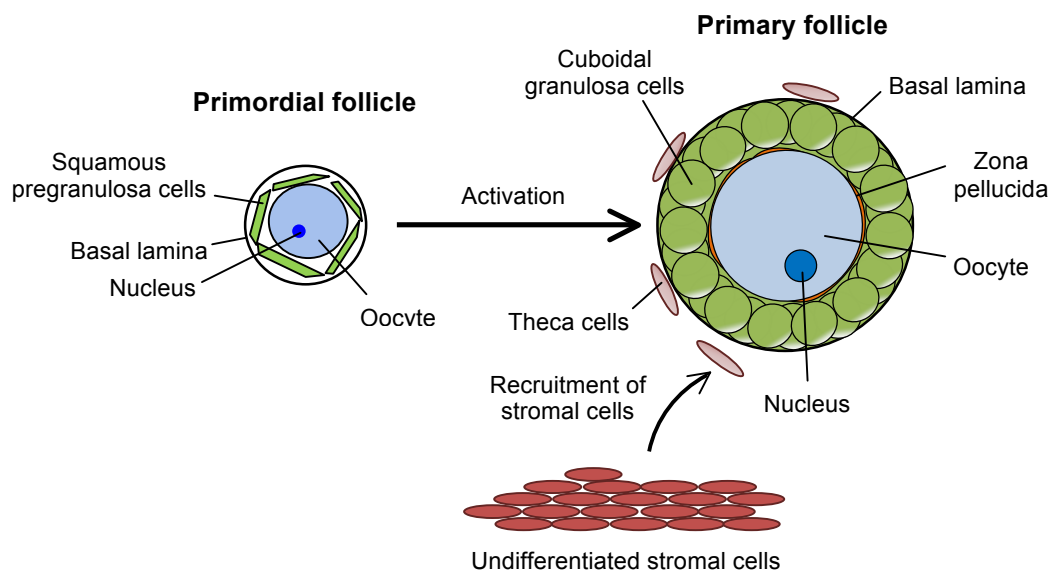


Figure 1.2 Primordial follicle activation. The activation and transition of a primordial follicle to a primary follicle involves a number of changes including oocyte growth and GC differentiation. At the primary follicle stage the oocyte starts secreting zona pellucida proteins and the theca cell layer begins to develop.

At the primary stage of follicle development, stromal cells are recruited outside the BL to start forming the theca cell layer (32), and the oocyte begins secreting zona pellucida (ZP) glycoproteins (Figure 1.2) (33). As the primary follicle develops (Figure 1.3), there is continuous oocyte growth and proliferation of GCs, resulting in the formation of additional GC layers, and proliferation and vascularisation of the theca cell layer (15, 32).

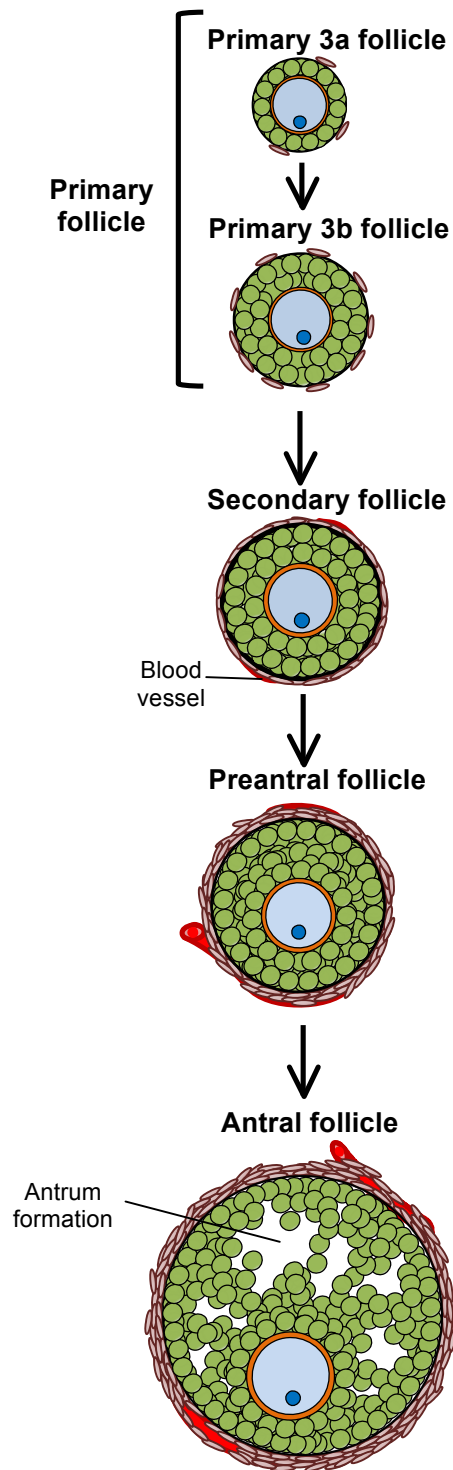


Figure 1.3 Development of the follicle from the primary stage to the antral stage. Follicle growth is associated with growth of the oocyte and proliferation of the accompanying granulosa and theca cells. Follicles will develop from the primary stage (split into the 3a and 3b stage), to form multilayered secondary and preantral follicles, before acquiring an antral cavity to form antral follicles.

Proliferation of both the GCs and theca cells results in the formation of the multi-layered preantral follicle (Figure 1.3). During these early stages of follicle development, although follicles are responsive to gonadotrophins, follicular growth is gonadotrophin independent (6, 34-36). However gonadotrophins are crucial for progression of follicle development to the antral stage (34, 37) since in female mice lacking follicle-stimulating hormone β subunit (FSH β) (34) or follicle-stimulating hormone receptor (FSHR) (36), follicle development is arrested at the preantral stage.

Follicle-stimulating hormone (FSH) secreted from the pituitary gland, augments proliferation of GCs of the preantral follicle and the development of several small fluid-filled antral cavities to form an antral follicle. Antrum formation is another hallmark of follicle development, and in antral follicles, oocyte growth is complete. Follicular fluid contains low molecular weight serum proteins and growth factors crucial to follicle, GC and oocyte development, and is thought to derive from components synthesised from GCs and blood flowing through the thecal capillaries (38-41).

During the transition of an antral follicle to a preovulatory follicle, the fluid-filled antral cavities coalesce to form a large antrum and GC proliferation decreases. This antral cavity facilitates the location dependant differentiation of GCs in the preovulatory follicle (42). GCs in close proximity to the BL become the mural GCs, whilst those surrounding the oocyte differentiate into cumulus cells (Figure 1.4) (5, 24).

The ovulatory luteinising hormone (LH) surge induces cumulus expansion and ovulation of the cumulus oocyte complex (COC) from the follicle (Figure 1.4). Cumulus cells play a vital role in maintaining the oocyte in meiotic arrest (43) and facilitate the transport of the ovulated egg into the oviduct (43, 44). The LH surge will induce a number of structural changes to the follicle following ovulation. The BL breaks down and theca and mural GCs invade the follicular cavity and proliferate to form a highly vascularized endocrine organ, the corpus luteum (CL) in a process known as luteinisation (Figure 1.4) (45).

The primary function of the CL is to produce progesterone to support the ensuing pregnancy (6). If, however, pregnancy does not occur, the CL undergoes morphological and functional regression, characterised by the cessation of progesterone production and breakdown of cellular components resulting in CL regression (46-48).

The vast majority of follicles will undergo atresia at various stages of development (7, 15) via programmed cell death (apoptosis) characterised by DNA fragmentation, cell shrinkage, and membrane blebbing (49, 50). Apoptosis in follicles is thought to occur primarily due to the depletion of anti-apoptotic factors such as oestrogen and gonadotrophins which inhibit apoptosis, or due to the increase of pro-apoptotic factors such as androgens (49) death ligand-receptor systems, and members of the B-cell lymphoma 2 protein family (51-53).

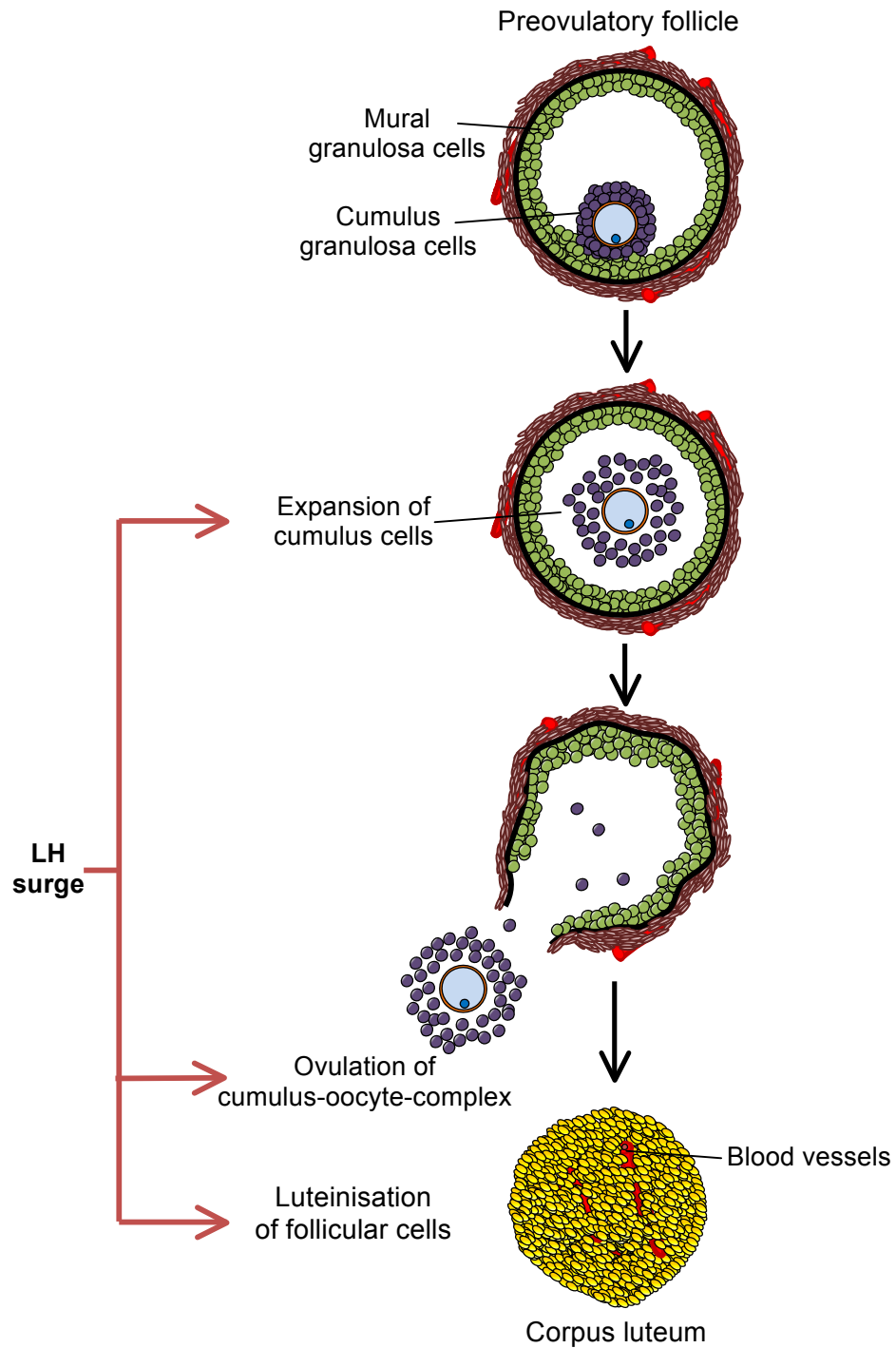


Figure 1.4 Cumulus expansion, ovulation and corpora lutea formation. Following the LH surge, cumulus cells undergo expansion and the cumulus-oocyte complex is ovulated. The remaining follicular cells transition to a highly vascularised corpus luteum.

1.1.2.1 Function of the basal lamina

The major components of the BL are proteoglycans and glycoproteins, including type IV collagen, laminin, fibronectin, and heparin sulphate (54-58). The BL encapsulates the GCs and oocyte, separating them from theca cells to create a localised avascular microenvironment within the follicle (26, 54). During the course of follicle development, the BL expands to accommodate the growing follicle before degrading during ovulation to allow the release of the COC (26, 54, 59, 60). The BL is thought to regulate follicular growth, by controlling entry of molecules into the follicle by size and charge (57, 61-63), which affects GC proliferation and differentiation (54, 58, 64). Therefore any changes to the BL are likely to modify follicle growth and function (57).

1.1.2.2 Theca cells

Theca cells are thought to originate from fibroblast-like precursors from within the stroma in response to factors secreted from the activated oocyte (65, 66) and play a number of important roles during follicle development including steroidogenesis (discussed in Section 1.1.3.1). The theca cell layer is vascularised providing communication with the hypothalamic-pituitary-gonadal (HPG) axis and essential nutrients to the follicle (32). After ovulation, thecal cells undergo luteinisation and contribute to the CL.

1.1.2.3 Zona pellucida synthesis

At the primary follicle stage, the oocyte secretes ZP proteins as filaments (33), which creates the ZP shell as the oocyte matures eventually developing to a thickness of 7 μm (67, 68). The ZP in mice is composed of three sulphated

glycoproteins ZP1, ZP2 and ZP3, which are located on chromosomes 19, 7 and 5 respectively (69, 70). ZP2 and ZP3 are present in equal amounts and constitute ~80% of the total ZP protein whilst ZP1 accounts for 20% (71, 72).

Studies using transgenic mice have revealed several vital functions of the ZP, which including protection of the oocyte during growth and transport once ovulated, preventing polyspermy during fertilisation and protecting the embryo in the reproductive tract (71, 73-77). Mice lacking ZP1 (*Zp1^{-/-}*) have a thinner ZP around their eggs, due to reduced cross linking and as a result were subfertile (78). On the contrary, mice lacking ZP2 (*Zp2^{-/-}*) or ZP3 (*Zp3^{-/-}*) were infertile due to defects in oocyte maturation and fertilisation (79, 80). Furthermore, whilst eggs from *Zp2^{-/-}* mice had a thin ZP, eggs from *Zp3^{-/-}* mice completely lacked a ZP. These findings illustrate the importance of a functional ZP.

1.1.3 Regulation of follicular development

Follicle development is regulated by the coordinated action of the HPG axis (6, 37, 81), autocrine and paracrine intra-ovarian factors (82, 83).

1.1.3.1 The two cell-two gonadotrophin theory

Gonadotrophin-releasing hormone (GnRH), a vital regulator of the HPG axis, is released from the hypothalamic neurons in the arcuate nucleus to the hypophyseal portal circulation, in a pulsatile manner (84, 85). In the anterior pituitary, GnRH binds to and stimulates the GnRH receptor resulting in the secretion of gonadotrophins, LH and FSH, into the circulation (86-90).

The levels of FSH and LH are regulated by both positive and negative feedback mechanisms by other hormones such as oestrogen, progesterone and anti-müllerian hormone (AMH) (6, 91), which are discussed further in Sections 1.1.3.2 & 1.1.3.3.

Both FSH and LH are glycoproteins composed of a common α subunit but unique β subunit which confers receptor specificity (92). The receptors for both FSH and LH (FSHR and LHR) are G-protein coupled receptors and are found on GCs and theca cells respectively (93). The paracrine interaction between GCs and theca cells and between FSH and LH are required for oestrogen synthesis giving rise to the two cell-two gonadotrophin theory (Figure 1.5) (94-100).

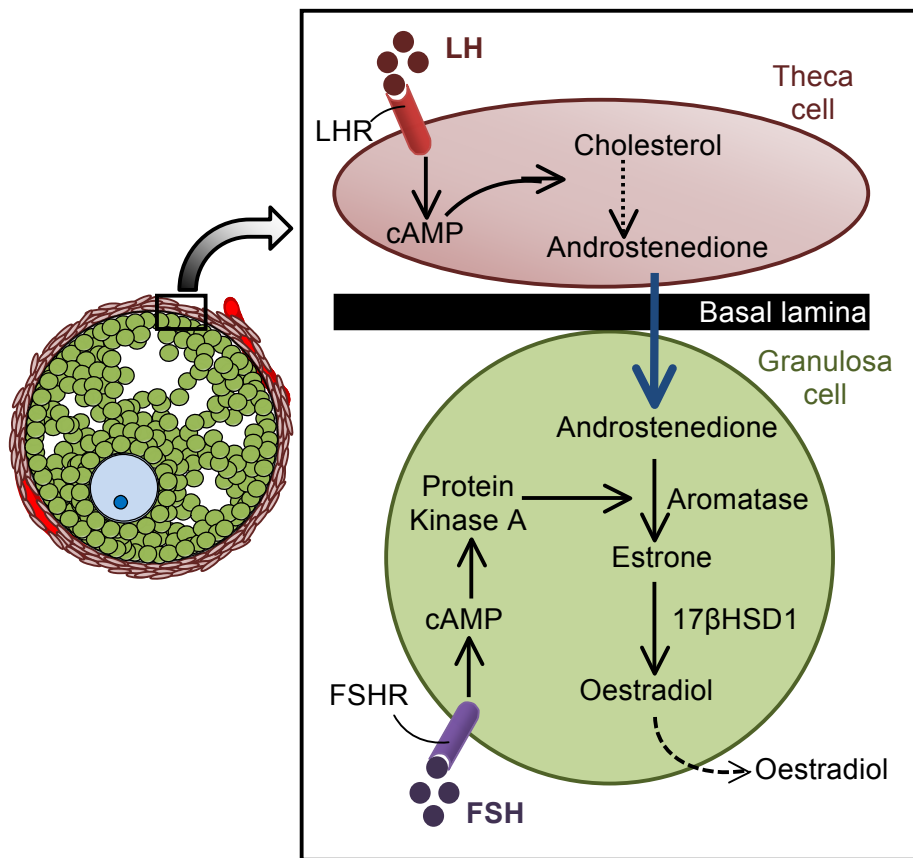


Figure 1.5 The two cell-two gonadotrophin theory for oestradiol production. Luteinising hormone (LH) binds to its receptor (LHR) on theca cells, results in stimulation of the adenylyl cyclase pathway (cAMP), resulting in the conversion of cholesterol to androstenedione by a number of enzymes in a cascade of steps (dotted line). The androstenedione synthesised in theca cells freely diffuses through the basal lamina into granulosa cells. In granulosa cells, follicle-stimulating hormone (FSH) binds to its receptor (FSHR) activating the adenylyl cyclase pathway, stimulating the granulosa cell to produce aromatase. The androstenedione is first converted to estrone by aromatase and then to estradiol by 17β-Hydroxysteroid dehydrogenase 1 (17βHSD1).

1.1.3.2 Ovarian steroids

The ovarian steroids, oestrogens, progestogens and androgens are all produced in the ovary from cholesterol, and play important roles in follicle development (101).

1.1.3.2.1 Oestradiol

The production of oestrogen has been discussed in Section 1.1.3.1. Oestrogen acts through a positive feedback mechanism to enhance the follicle's responsiveness to FSH and LH, resulting in further oestrogen production (102, 103). It is well-known that oestrogens mediate mitogenic effects on GCs by inhibiting apoptosis (104), and induce GC proliferation by maintaining FSHR levels on GCs (105, 106). Oestrogen also plays a role in ovulation since it mediates the upregulation of the LHR on GCs (102, 103, 105, 107).

Oestrogens signal via the oestrogen receptors (ER) of which there are two forms: ER α and ER β (108, 109). The role of these ERs in ovarian function has been elucidated from ER α (ER α KO) and ER β (ER β KO) knockout mice. Both ER α KO and ER β KO possess normal ovaries at birth, however ER α KO are infertile due to aberrant follicle growth whilst ER β KO are subfertile due to defects in ovulation (110-112).

Insight into the role of oestrogen in ovarian function comes from the aromatase knockout mouse (ArKO), which lacks aromatase and therefore is unable to produce oestrogen (113, 114). ArKO females are infertile due to a block in follicle development at the antral stage resulting in anovulation; administration

of oestrogen to these mice facilitates ovulation (115). Eventually ArKO females undergo premature ovarian insufficiency due to the depletion of the primordial follicle pool (113-115), indicating that oestrogen also plays a role in maintaining quiescence of the primordial follicle pool.

1.1.3.2.2 Progesterone

Following ovulation, the follicle undergoes extensive remodelling to form a CL, which secretes progesterone. Progesterone plays key roles during ovulation, by regulating GC function and follicle rupture, as well as during implantation and the maintenance of pregnancy (116, 117).

Progesterone mediates its effects through the progesterone receptor (PR), which similar to the ER, exists in two forms PR-A and PR-B (118). The differential action of these receptor is illustrated through PR-A (PR-AKO) (119) and PR-B (PR-BKO) (120) knockout mice. PR-AKO are infertile due to a failure to ovulate resulting in the presence of unruptured preovulatory follicles in the ovary (119) whilst fertility is not affected in PR-BKO mice (120). This illustrates the differences in function of the two receptors and demonstrates that the effects of progesterone on ovulation are largely mediated through PR-A.

1.1.3.2.3 Androgens

Androgens act via the androgen receptor (AR) and play a major role in the regulation of follicle development. As previously discussed, androgens are required as a substrate for the production of oestradiol and thus indirectly stimulate follicle growth. However, androgens also modulate GC apoptosis (104)

and inhibit FSH-stimulated LHR expression by GCs (121) which is vital for successful ovulation. The administration of dihydrotestosterone, a non-aromatisable androgen, to mice resulted in subfertility due to a reduction of large follicles (122). Furthermore, female mice lacking AR were also subfertile due to defective follicular development and reduced ovulation, evident by a marked reduction in the number of CL (123).

1.1.3.3 The TGF- β superfamily

The transforming growth factor beta (TGF- β) superfamily include AMH, bone morphogenetic proteins (BMPs) and growth differentiation factors (GDF) amongst others (124) and have diverse functions that regulate follicle development.

1.1.3.3.1 AMH

The glycoprotein AMH, in addition to inhibiting Müllerian duct development (125), has a central role in primordial follicle activation. However, unlike other members of the TGF- β superfamily, AMH also has an inhibitory role in follicle development. AMH is expressed by primary follicle GCs and is continuously produced until the antral follicle stage (126). The role of AMH has been elucidated from AMH knockout mice which exhibit a depletion of the primordial follicle pool accompanied by increased numbers of growing follicles as a result of unregulated recruitment of primordial follicles (127).

1.1.3.3.2 Oocyte-secreted factors GDF9 and BMP 15

Growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) are glycoproteins synthesised in oocytes from the primary follicle stage onwards and are essential for fertility (128, 129)(31-33). GDF-9 signalling is mediated through the BMPRII and ALK5 receptors resulting in the activation of the intracellular SMAD2/3 pathway (130, 131). BMP-15 signalling is mediated through the BMPRII and ALK6 receptors activating the SMAD 1/5/8 pathway (132). Therefore the secretion of these two proteins results in a synergistic effect (133).

Insight into the role of BMP-15 and GDF-9 in follicle development come from the BMP-15 (*Bmp-15*^{-/-}) and GDF-9 deficient (*Gdf-9*^{-/-}) mice. Whilst *Bmp-15*^{-/-} females are subfertile (134), *Gdf-9*^{-/-} females are infertile due to a block in follicle development at the primary 3b stage (135). Analysis of *Gdf-9*^{-/-} primary follicles revealed the presence of abnormally large oocytes (136) indicating that GDF-9 is required for the primary to secondary follicle transition. However, a subsequent study has demonstrated that these effects are a result of GC upregulation of Inhibin A (137), since mice lacking both GDF-9 and Inhibin A have follicles at the preantral stage (138). These studies revealed that although GDF-9 is not essential as a direct promoter for early follicular growth, it mediates its effects through suppression of Inhibin A, which in turn regulates development of the follicle. Mutations in GDF-9 and BMP-15 have also been linked with pathological conditions such as premature ovarian insufficiency (POI) (139, 140).

1.2 Premature Ovarian Insufficiency

Throughout a woman's reproductive life span, follicles are actively recruited from the quiescent primordial pool, and when the number of primordial follicles is reduced to a critical threshold of ~1000, reproduction ceases and they enter menopause (11-13). Thus, the finite nature of the primordial follicle pool serves as a ticking clock to the timely onset of menopause and any imbalances in activation rate can culminate in conditions such as premature ovarian insufficiency (POI).

POI occurs in women less than 40 years of age and is a clinical condition characterized by amenorrhea (primary or secondary) for 4 months, oestrogen deficiency and hypergonadism (FSH levels greater than 40 mIU/ml). POI affects approximately 1-3% of women under 40 years of age (141-143), however, the prevalence in the UK is thought to be around ~7% (144). The diagnosis of POI carries enormous impact for a woman and their partners from both a physical and psychological perspective.

1.2.1 Causes of POI

POI is highly heterogeneous in nature and despite several causal factors being identified; the majority of cases (~70%) are idiopathic (141, 143, 145-148).

1.2.1.1 Genetic causes

X-chromosome abnormalities are responsible for the majority of POI cases with ovarian dysgenesis (143, 149, 150). Two genetic causes of POI which are readily tested using karyotype analysis are Turner syndrome and Fragile X (FMR1) syndrome.

Turner syndrome is characterised by the absence of the second X chromosome (45 X) and affects 1 in 3000 live female births (151). The absence of one X chromosome leads to developmental delays, short stature, congenital lymphedema and gonadal dysgenesis (152, 153). *In utero*, patients have few primordial follicles in the ovaries, which undergo follicle depletion due to apoptosis resulting in streak ovaries and hence POI by adult life (149, 154). The mechanisms behind this are unknown.

Fragile X syndrome is caused by the expansion of a CGG trinucleotide repeat in the 5' untranslated region of the *FMR1* gene resulting in mental retardation and autism in affected males and POI in women (155). The normal repeat length of *FMR1* is less than 40 repeats, the premutation is defined as 55 to 200 repeats and over 200 repeats is the full mutation (156). The *FMR1* gene codes for a RNA binding protein called fragile X mental retardation protein (FMRP). In the full mutation, no FMRP is produced, whilst in the premutation, there is a normal amount of FMRP but excess messenger RNA production (157). The *FMR1* premutation is responsible for approximately 4-5% of cases of POI overall (158, 159). Accumulation of the excess messenger RNA is thought to be the cause of ovarian damage, since *FMR1* is found to be expressed in the germ cell population in mice (160) and humans (161, 162), however, the exact mechanism is unknown.

Autosomal mutations have also been associated with POI. Galactosaemia is caused by a lack of galactose-1-phosphate uridylyltransferase (GALT).

Galctosemia associated POI occurs due to the toxic effects of galactose that results in impaired folliculogenesis and accelerated follicular atresia (163-165).

1.2.1.2 Iatrogenic causes

Advances in chemotherapy treatments have resulted in improved survival rates among cancer patients. One of the side effects of many chemotherapy regimes in female survivors is the onset of POI. The risk of developing POI following chemotherapy is dependent on type of drug since alkylating agents are highly ovotoxic in comparison to platinum agents or plant alkaloids (166). Other risk factors include age, dose and interval of treatment regime (167). The mechanism of chemotherapy induced POI is the destruction of growing follicles and hence removal of inhibitory signals resulting in the overactivation of primordial follicles as described by the burn-out theory (168), which is discussed in Section 1.2.5.1.

1.2.1.3 Autoimmune causes

Approximately 30% of cases of idiopathic POI are thought to have an autoimmune cause (169); however in the majority of cases, diagnosis is difficult due to the lack of high sensitivity and specificity tests (169, 170). POI has been associated with hypothyroidism (in 25-60% of patients) (171, 172), Addison disease (in 10-20% of patients) (173) and autoimmune polyglandular syndrome type I (in ~40 of patients) (173).

1.2.2 Nosological classification of POI

The nomenclature of the condition has been a matter of debate for several years (143, 174, 175). Elevated urinary gonadotrophins in non-cycling women, described as premature menopausal cases, were first reported in the 1930s (176) and later termed primary ovarian insufficiency by Fuller Albright in 1942 (177). Since then, the condition has been described using different names including premature ovarian failure (POF), primary ovarian insufficiency, resistant ovary syndrome (ROS) hypergonadotropic hypogonadism and premature menopause reflecting its heterogeneous aetiology (175).

In 2013 a conference on POI was held by European Society of Human Reproduction and Embryology (ESHRE) during which the nomenclature of the condition was discussed and the outcomes later published (178). Prior to this meeting, there was a mutual consensus (174, 175, 179, 180) to use the term ovarian insufficiency as opposed to ovarian failure, since ovarian insufficiency can be used to describe a wide range of impaired ovarian function, as demonstrated by 5-10% of women, who despite being diagnosed with POI, are able to conceive (181). Furthermore, the term failure has a negative impact as patients can view themselves as the failure. Primary ovarian insufficiency is the preferred term by the National Institutes of Health (174, 175) and the consensus in Spain (182) as they argue the term 'primary' emphasises that the primary defect in ovarian dysfunction is within the ovary, rather than a secondary cause. Whilst this reasoning is not disputed, the directive of the ESHRE conference attendees was to use the term 'premature' ovarian insufficiency since the terms 'primary' and 'secondary' are used to classify amenorrhea by gynaecologists, but the terms are

used in reference to the ovary by endocrinologists, thus creating confusion in a clinical setting (183). In this situation we find the ESHRE guidelines provide an apt alternative; the term 'premature' should be used as this refers to the onset of ovarian insufficiency, which occurs prematurely regardless of aetiology.

As it stands, the current nomenclature has limitations in clinical practice since the term POI alone does not adequately reflect the spectrum of ovarian function. Usually, disease classification or nosology is based on knowledge of aetiology, pathogenesis, and clinical diagnosis. However, the clinical presentation of women diagnosed with POI is heterogeneous. During the earlier stages of POI, women present with the presence of follicles at all stages up to the preovulatory stage (184-189), whilst others present with a total depletion of ovarian follicles. The mechanisms behind these manifestations are discussed in detail later on.

1.2.2.1 Sub-classification

Despite the advances made by ESHRE, further classification of POI into subtypes based on histological analyses is required. Recognizing these subtypes would allow clinicians to distinguish between patients with and without follicles; since the presence of follicles indicates that recovery of ovarian function, either spontaneous or induced, might be possible.

In the past, POI has been subdivided into four clinical states (180), mainly based on FSH levels, fertility and amenorrhea detailed in Table 1.1. The four clinical states are occult POI, biochemical POI, overt POI and POF. The occult, biochemical and overt stages of POI are reversible and patients can move from

one state to another, however these subdivisions do not provide clinical utility in identifying patients that could benefit from fertility treatment.

Table 1.1 The four clinical states of POI assessed by FSH levels, fecundity and amenorrhea

Clinical states	FSH levels	Fecundity	Amenorrhea	Reversible
Occult	Normal	Normal	No	Yes
Biochemical	Normal	Reduced	No	Yes
Overt	Elevated	Reduced	No	Yes
POF	Elevated	Reduced	Yes	No

To this end, we propose classification into 2 different subgroups as established by Fox (190); follicular or afollicular POI, depending on the presence or absence of follicles respectively. However, longitudinal examination of women with POI have reported cases of follicular POI that have progressed to an afollicular POI demonstrating the progressive nature of the condition (158, 181, 183, 187, 191-195). This indicates that the presentation of follicular POI is not permanent, and represents a continuum of declining ovarian function, which will ultimately lead to afollicular POI.

1.2.3 Resistant ovary syndrome

ROS was first described in 1969 as Savage syndrome and is now a well-known cause of hypergonadotrophic amenorrhoea. Since manifestations of ROS are identical to POI (196), it is thought 10% of total POI cases are ROS (197). The characteristic histological finding is abundant primary follicles and an arrest at the antral stage of development (198). It has been suggested that ROS is an early stage of ovarian failure before the follicles are lost; this theory is supported in the

literature by several reports demonstrating that POI cases will transition via a resistant state (158, 199, 200). It seems reasonable to postulate that ROS represents a state before the progression to irreversible ovarian failure (afollicular POI), and we propose ROS to be classified as a follicular form of POI.

1.2.4 Presentations and relationships between different stages of POI

Overall, there is a continuum of declining ovarian function that ultimately leads to afollicular POI and the cessation of ovarian function. However, before reaching this irreversible endpoint, there are a number of presentations with varying degrees of ovarian function in women diagnosed with POI summarised in Figure 1.6. Most women diagnosed with POI will have a normal menstrual history and may have been fertile (Figure 1.6A). Others have dysfunctional ovaries and despite the diagnosis of POI, spontaneously conceive (192), indicating the unpredictable ovarian function present. In addition, preovulatory follicles may undergo premature luteinisation, which has been shown to be a major mechanism in follicle dysfunction observed (Figure 1.6B) (184). As discussed in section 1.2.3, a number of women will transition via a resistant ovary state, unable to respond to gonadotrophin stimulation (Figure 1.6C) whilst others will present with only primordial follicles (Figure 1.6D) before complete ovarian failure (Figure 1.6E) where ovaries lack follicles. However not all patients will go through each of these stages, with some reaching the afollicular stage shortly after birth.

Separation of POI patients depending on the presence or absence of follicles could help identify independent aetiologies. In the past, the distinction

between two different ovarian phenotypes (follicular and afollicular) has been crucial in finding a relationship between POI and FSHR mutations (201, 202). Furthermore, by distinguishing between the follicular and afollicular forms of POI and determining the stage at which follicle growth is arrested could orientate research and also subsequent screening for candidate genes. This will improve treatment outcomes, provide regulatory guidance for intervention and provide an opportunity to identify underlying biological mechanisms.

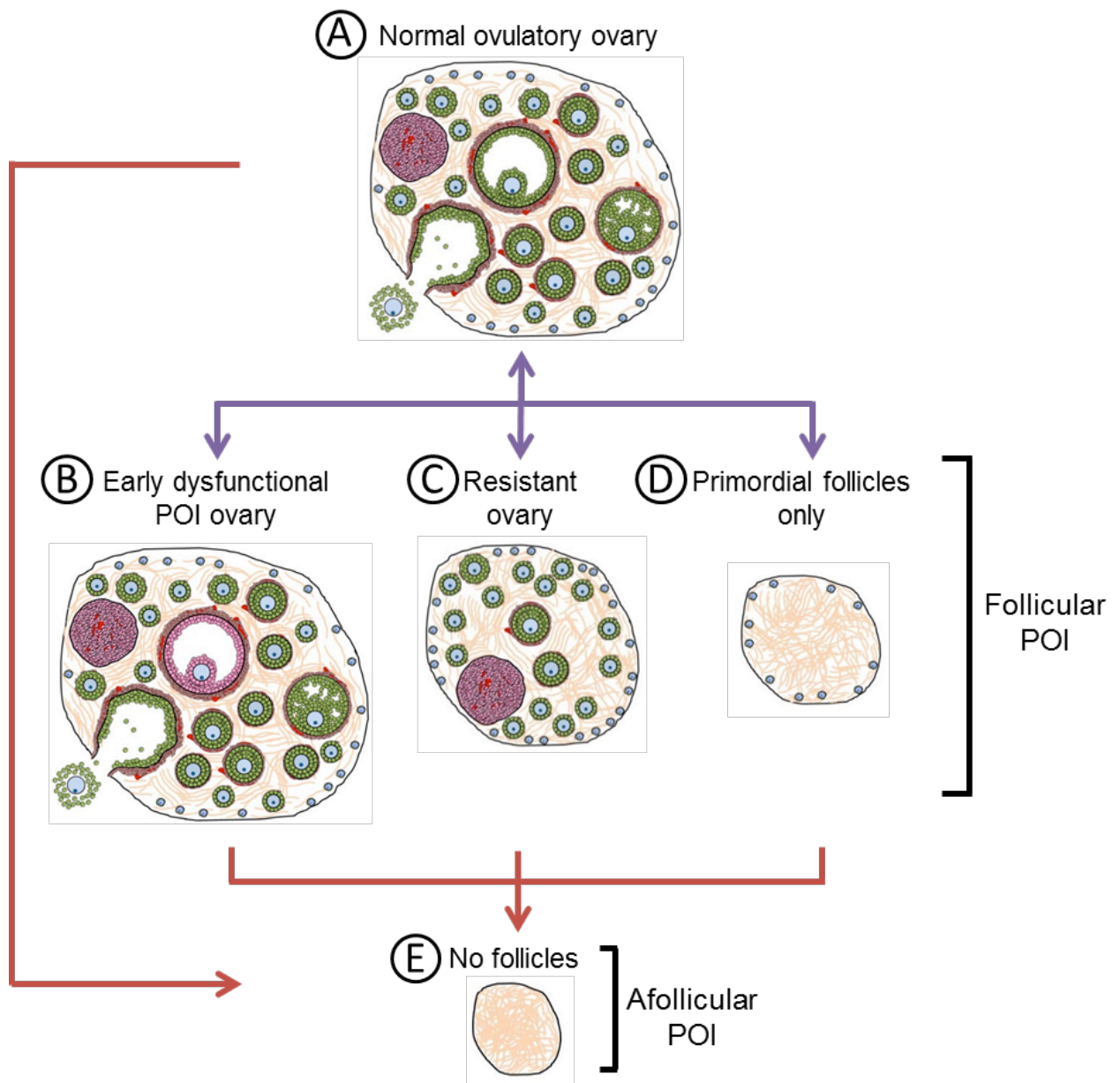


Figure 1.6 Stages of ovarian function in POI diagnosed with POI. (A) The majority of women diagnosed with POI undergo a normal puberty and are fertile, whilst others have (B) dysfunctional ovaries and although some follicles spontaneously ovulate undergo premature luteinisation (pink follicle). (C) Some women will transition through a resistant ovary state where follicles present are unresponsive to gonadotrophin stimulation, whilst others present with (D) only primordial follicles that will eventually become depleted leading to the (E) afollicular stage. Purple arrows indicate reversible states, whereas red arrows are irreversible.

1.2.5 Mechanisms of POI: insights from mouse models

As discussed, POI has a heterogeneous spectrum of ovarian manifestations resulting in the absence or presence of follicles. Since the size and activation rate of the primordial follicle pool determines the reproductive lifespan and fertility (Figure 1.7A) (5, 6), any process affecting folliculogenesis and/or follicle development will affect the reproductive lifespan. There are two known mechanisms for the development of POI, follicle depletion and follicle dysfunction. Insights into how these normal processes are affected in POI have come from mouse models of POI and patient case studies.

To identify mouse models of POI, a PubMed based literature search was conducted using a strict diagnostic criterion of significantly elevated FSH levels, since this is the criterion to diagnose women with POI; the results are summarised in Table 1.2.

Table 1.2 Mouse models of POI that exhibit increased FSH levels

	Mouse model	Reproductive Phenotype	References
Depletion of germ cells	Germ cell deficient	Reduced germ cell numbers	(203, 204)
	White spotting variant (c-kit gene)	Reduced germ cell numbers	(205)
Overactivation of primordial follicles	Pten KO	Overactivation of primordial follicles	(206)
	Foxo3a KO	Overactivation of primordial follicles	(207)
	Omcg1 KO	Elimination of early growing oocytes	(208)
	Inhibin KO	GC tumor development	(209)
	Follistatin KO	Reduced numbers of ovarian follicles, ovulation and fertilization defects	(210)
	Gpr3 KO	Fragmented oocytes after superovulation	(211)
	Mdm2 KO	Early follicular loss in mice	(212)
	Murine Uromodulin-like 1 overexpression	Few discernible follicles in the cortical region	(213)
	Kiss1r haplo-insufficient	Progressive loss of antral follicles	(214)
	Liver Kinase b1 KO	Overactivation of primordial follicle pool	(215)
	KitL KO	Arrested oocyte maturation.	(216)
	Tsc1 KO	Follicular depletion in early adulthood	(217)
	3-phosphoinositide-dependent protein kinase-1 KO	Majority of primordial follicles are depleted	(218)
	Taf4b KO	Follicles blocked at pre-antral stage	(219, 220)
Follicle dysfunction	GDF-9 KO	Block at primary follicle stage	(135)
	FSHR KO	Block before antral stage	(36)
	ArKO	Disrupted folliculogenesis and ovulation failure	(114)
	DM	Transition from subfertile to infertile; and undergo POI by 3-months of age; Primordial follicles present	(1)

1.2.5.1 Follicle depletion

Follicle depletion results in ovaries devoid of follicles and can be attributed to either a developmental reduction in the initial follicle pool (due to reduced germ cell formation or postnatal destruction of germ cells) (Figure 1.7B) or to the overactivation of primordial follicles resulting in an accelerated expenditure (Figure 1.7C).

Two mouse models of POI, the germ-cell deficient mouse (203, 204) and the White spotting variant mouse (205), are infertile due to a significant developmental reduction of germ cells resulting in a small primordial follicle pool.

One potential mechanism of primordial follicle depletion is the ‘burnout’ theory (168). In the absence of growing follicles, (which may be destroyed for example by chemotherapy), there are reduced inhibitory paracrine factors, in particular AMH. This lack of inhibitory factors results in increased recruitment of primordial follicles, thus, “burning out” the ovarian reserve, resulting in a follicular POI. The absence of inhibitory signals such as Pten (206) and Foxo3a (207) using knockout mice, that under normal circumstances prevent primordial follicle activation, also result in the overactivation and depletion of primordial follicles resulting in a follicular POI.

1.2.5.2 Follicle dysfunction

Follicle dysfunction resulting in follicular POI arises from a pathological process that prevents follicle maturation (Figure 1.7D). Manifestations vary from the presence of primordial follicles only, which are unable to activate, to the

presence of follicle at various stages of development including the presence of preovulatory follicles that are unable to ovulate and undergo luteinisation (142, 174, 184, 185, 188). The FSHR mutation is a known cause of POI in a small proportion of women (201) and also in a mouse model (36); both exhibit the presence of follicles, usually before the antral stage, that are unable to respond to FSH stimulation. Other mouse models of follicular POI include the GDF-9 knockout mouse (135), which exhibits a block in follicle development at the primary stage, and the aromatase knockout mouse (114), which exhibits disrupted folliculogenesis and a failure to ovulate. All the mouse models of follicular dysfunction discussed above are never fertile and through our search, only one model was found of follicular POI that is fertile before the onset of POI, this is the Double Mutant (DM) mouse model (1).

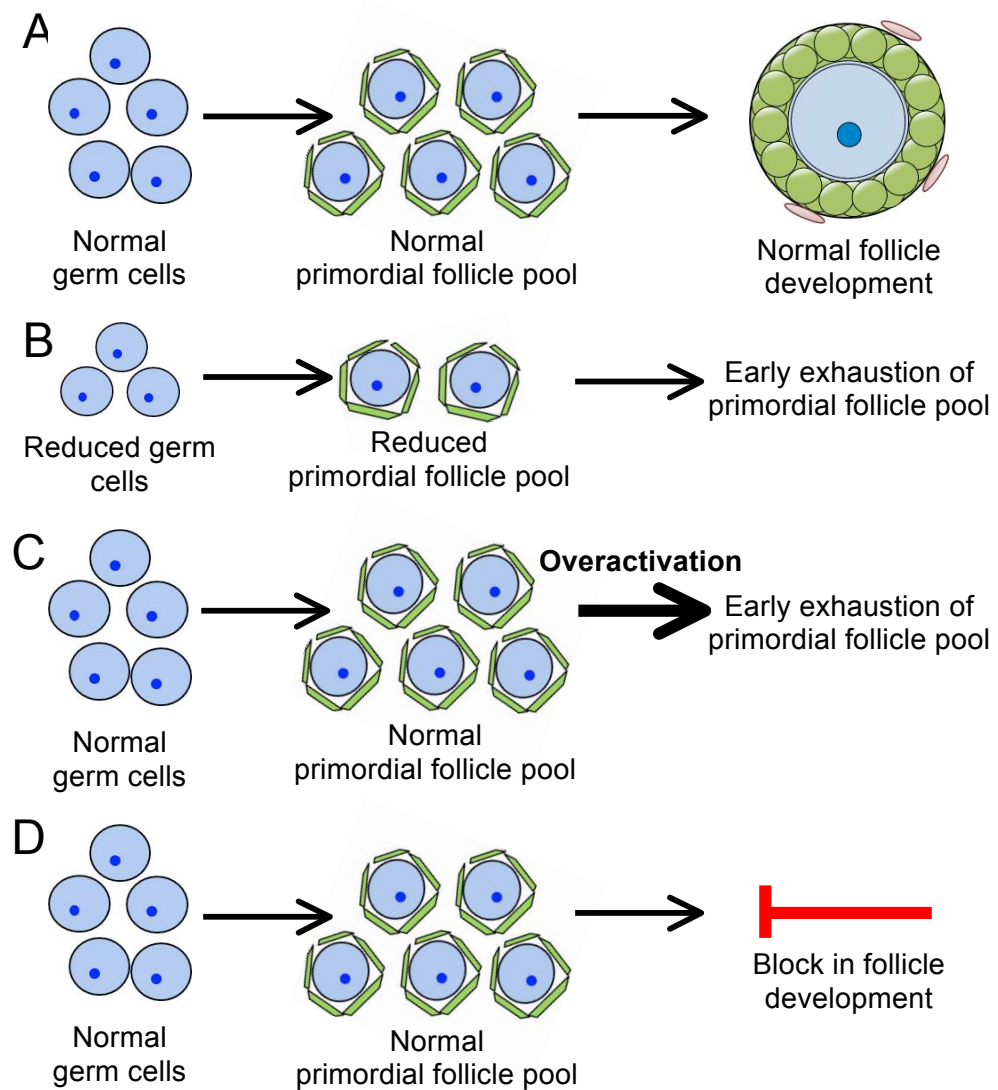


Figure 1.7 Mechanisms of POI. (A) Representation of normal follicle development. Afollicular POI can occur due to (B) a reduced germ cell pool resulting in the reduction of the primordial follicle pool or (C) due to the overactivation of primordial follicle. (D) Follicular POI results in the presence of follicles however various pathologies prevent follicle maturation.

1.3 The Double Mutant mouse model

1.3.1 Genetics of the DM mouse

The genetic origin of the DM mouse is oocyte-specific deletion of two glycosyltransferase genes, *C1galt1* (also known as *T-syn*) and *Mgat1*, that respectively encode β 1,3-galactosyltransferase (T-synthase) and *N*-acetylglucosaminyltransferase I (GlcNAcT-1). T-synthase is required for the generation of core 1-derived O-glycans (221, 222) whilst GlcNAcT-1 is required for the synthesis of complex and hybrid *N*-glycans (223, 224).

The oocyte-specific deletion is achieved using *Cre-LoxP* technology; the gene to be deleted is flanked by *loxP* sites and upon expression of the *Cre* recombinase enzyme (a type 1 topoisomerase), deletion is achieved (225). Expression of the *Cre* recombinase can be controlled by a promoter, which in the DM is the oocyte-specific *Zp3*; *Zp3* is expressed only in the developing oocyte from the primary stage of follicle development (33) therefore deletion will occur from this stage onwards. DM females (*Mgat1*^{F/F}*C1galt1*^{F/F}:*Zp3Cre*) carry floxed alleles of *Mgat1* and *C1galt1* and a *Zp3Cre* recombinase transgene, whilst Control females lack the *ZP3Cre* transgene (*Mgat1*^{F/F}*C1galt1*^{F/F}) (1, 226). The *Zp3Cre* transgene does not affect fertility (226, 227).

1.3.2 Glycosylation

Glycosylation is the most common form of post-translational modification and glycoproteins play essential roles as secreted, receptor and binding proteins (228). Changes in the glycosylation may have major implications in the functioning

of proteins and have been associated with a number of diseases such as rheumatoid arthritis (229) and diabetes (230).

Glycoproteins also play a critical role in female reproduction and include hormones such as FSH, LH (231) and AMH (232), as well as oocyte-secreted GDF-9 and BMP-15 (233). Recently, a number of essential roles of oocyte generated O- and N-glycans have been implicated in various aspects of follicle development including maintaining BL integrity (57, 234), GC function (235) and follicle selection (234, 236).

1.3.2.1 N-glycans

N-glycosylation, a major post-translational modification of proteins, occurs in the endoplasmic reticulum (ER) and Golgi apparatus (Figure 1.8). First in the ER, monosaccharides are added onto a dolichol pyrophosphate to form a lipid-linked oligosaccharide (237). The oligosaccharides are transferred by the oligosaccharyltransferase (OST) complex onto the asparagine (Asn) residues of the Asn-X-Serine/Threonine (Ser/Thr) sequence of the nascent polypeptide (237).

Following the removal of glucose residues by glucosidases, and the removal of a mannose residue by α -mannosidase I, the high mannose N-glycoprotein is transported to the Golgi. Further mannose residues are removed by Golgi α -mannosidases, followed by the elongation of the chain by various glycosyltransferases to form hybrid or complex N-glycans (238). The elongation process is initiated by the addition of N-acetylglucosamine (GlcNAc) residues to the N-glycan core structure by six different GlcNAc-glycosyltransferases of which

GlcNAcT-1 is the first. GlcNAcT-1 initiates the synthesis of a large variety of structurally different complex type *N*-glycans. In the absence of GlcNAcT-1, glycoprotein-bound *N*-glycans are therefore entirely of the high mannose *N*-glycoprotein type (238).

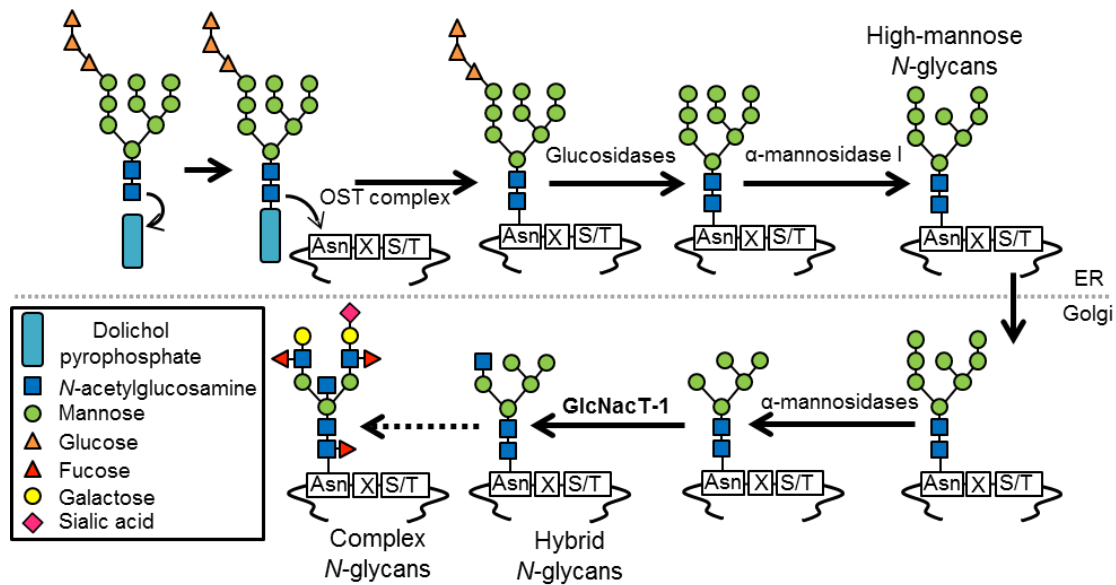


Figure 1.8 A schematic diagram of high mannose, hybrid and complex *N*-glycan synthesis pathway.

The mouse model carrying an oocyte-specific deletion of *Mgat1* was generated (235). Ablation of hybrid and complex *N*-glycans, resulted in a sustained decrease in fertility and fewer pups per litter. Analysis of ovarian morphology revealed the presence of a thinner ZP and aberrant development of preovulatory follicles (235) demonstrating the role of complex and hybrid *N*-glycans for the generation of a developmentally competent oocyte. However fertilization, blastogenesis, and implantation proceeded normally in their absence (227).

1.3.2.2 O-glycans

The production of mucin type O-glycans (Figure 1.9) occurs in the Golgi apparatus, and is initiated by the attachment of *N*-acetylgalactosamine (GalNAc) to Ser and Thr residues to produce the Tn-antigen (*N*-acetylgalactosamine-serine/threonine). This step is catalysed by up to 20 GalNAc- transferases (GalNAc-Ts) (239). The Tn-antigen is then elongated by core 1 β 1, 3-galactosyltransferase (C1galt1). The function of C1galt1 is dependent on the presence of the chaperone core 1 β 3GalT specific molecular chaperone (Cosmc) (240). C1galt1 catalyses the addition of a galactose molecule to the Tn-antigen to form core 1 O-glycans also known as the T-antigen, which are precursors to more complex O-glycans. O-glycans play important roles in cell-cell interactions and receptor signalling (238).

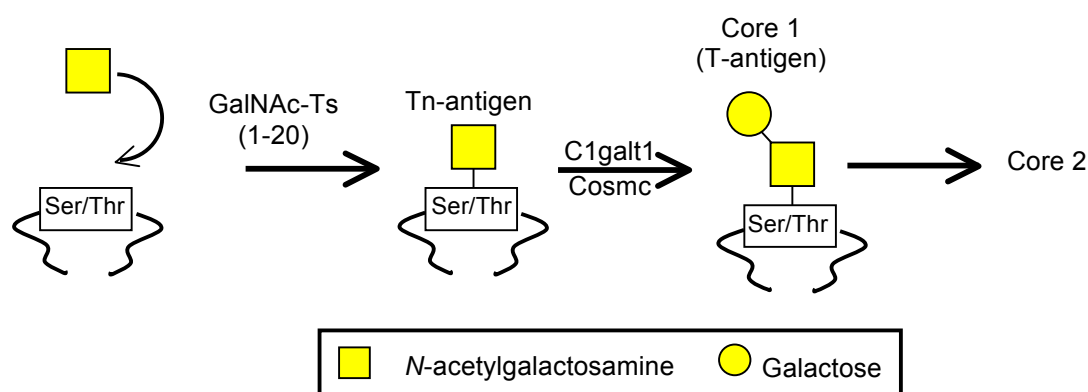


Figure 1.9 A schematic diagram of the Core O-glycan synthesis pathway. GalNAc-transferases (GalNAc-Ts) transfer *N*-acetylgalactosamine (GalNAc) to Serine (Ser) and Threonine (Thr) residues to produce the Tn-antigen. T-synthase enzyme encoded by *C1galt1* gene required for the synthesis of core 1 O-glycans by transferring galactose to *N*-acetylgalactosamine on the Tn-antigen.

The oocyte-specific deletion of *C1galt1* (234) results in a sustained increased fertility in mice which is attributed to increased ovulation rate due to increased numbers of mature follicles due to decreased apoptosis but increased ratio of GDF-9:BMP-15 resulting in the generation and survival of more follicles (2). Ovarian histology revealed the presence of some abnormalities of ovarian morphology, such as an altered ZP structure and the presence of multiple-oocyte follicles (2, 57). Furthermore, it was determined that complex O-glycans were not essential for blastogenesis and implantation, but were required for development around midgestation (234).

1.3.3 DM phenotype

As discussed, oocyte-specific deletion of *Mgat1* (235) results in a sustained decrease in fertility whilst oocyte-specific deletion of *C1galt1* (234) results in a sustained increase in fertility. It is interesting to discover that oocyte-specific deletion of both *C1galt1* and *Mgat1* results in an age-dependant decrease in ovarian function and fertility.

Prepubertal DM females at 3-weeks of age have grossly normal ovaries and contain follicles at all stages of development. At 6-weeks of age, females are subfertile producing a smaller litter (~50%) than Controls, which is attributed to delayed embryo development, since ovulation rate is normal (236). Furthermore, in 6-week DM ovaries, there is delayed CL regression resulting in a larger and more heterogeneous CL population (236) due to attenuated rates of luteal cell apoptosis (4). By 9-weeks of age, DM females are infertile, and undergo POI by 3-months of age (1, 236) accompanied by an altered endocrine profile, characterized by elevated gonadotrophins and decreased sex steroids, confirming the DM mouse as a model of POI (Figure 1.10) (1).

The drop in fertility of DM females is also accompanied by a dysregulation of follicle development, which progressively worsens as female age increases. Ovaries contain decreasing numbers of developing follicles at the later stages of development, accompanied by increased number of follicles at the primary 3a stage. Since this accumulation of primary 3a follicles is accompanied by an equivalent number of primordial follicles at 3-months of age to Controls, this is indicative of a block in follicle development.

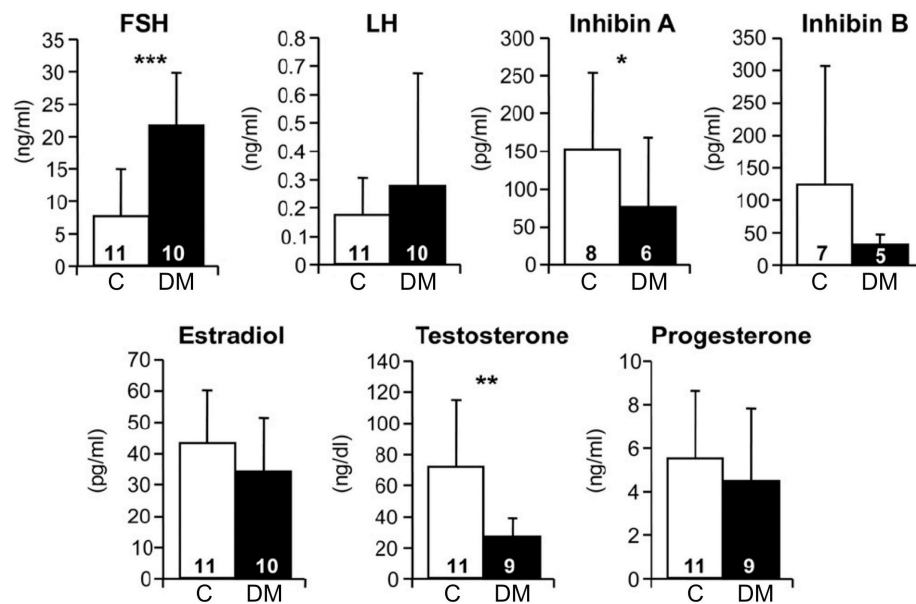


Figure 1.10 Endocrine profile of Control and DM females at 3-months. At 3 months of age, DM females exhibit increased serum gonadotropin and decreased sex steroids compared to Controls. The number in the bar represents number of animals. * $P < 0.05$; ** $P < 0.005$; *** $P = 0.001$. Adapted from Williams et al. (217).

The DM mouse model phenotype is concordant with clinical features of POI and there is a well-defined timeline for the onset of POI, the transition from a fertile state to an infertile state, which makes it an attractive research model for POI. This provides a remarkable opportunity to explore possible mechanisms underlying the onset of POI and investigate how the structure and function of follicles are modified during this period with the aim of developing interventions to restore normal ovarian function. Since DM females undergo this age-dependant demise of ovarian function before undergoing POI, the overarching hypothesis of this thesis is that follicle development cannot be rescued in the DM mouse model and to address this hypothesis we will address the following aims:

- To characterise morphological differences in primary follicles between Control and DM and assess how this is affected by age.
- To characterise follicle development in DM females when they transition from a subfertile state at 6-weeks to an infertile state at 9-weeks.
- To characterise follicle development in DM females when they transition from a subfertile state at 6-weeks to an infertile state at 9-weeks.
- To evaluate if DM follicle development can be normalised under controlled *in vitro* conditions.
- To assess DM germ and somatic cell function using the reaggregated ovary technique.
- To determine if replacing the oocyte environment is able to rescue DM follicle development.
- To investigate if transplantation of a newborn ovary with a DM ovary could rescue follicle development.

Material and Methods

2.1 Generation of the Double Mutant mouse

DM mice homozygous for floxed *Mgat1* and *C1galt1* alleles and carrying a *Zp3Cre* recombinase transgene were generated as described in Williams et al (226). The floxed alleles are deleted when exposed to *Cre* recombinase, the expression of which is controlled by the promoter of oocyte-specific zona pellucida 3 protein (*Zp3*). *Zp3* is expressed only in the developing oocyte from the primary stage of follicle development (33). DM females carry floxed alleles of *Mgat1* and *C1galt1* and a *ZP3Cre* recombinase transgene ($Mgat1^{F/F}C1galt1^{F/F}:ZP3Cre$), whilst Control females lack the *Zp3Cre* transgene ($Mgat1^{F/F}C1galt1^{F/F}$) (1, 226). The *Zp3Cre* transgene does not affect fertility (226, 227).

Mice were maintained in individually ventilated cages in 12:12 h light-dark cycles unless specified otherwise. Food and water was provided *ad libitum*.

2.2 Mouse colonies

2.2.1 Mouse colony management system

There were two systems to manage mouse colonies, the first was Dr. Williams excel sheets and the second was the official Sanger system used by the Home Office which was first introduced in 2015. Dr. Williams excel sheets were used keep track of breeding cage productivity, genotyping of newborns, mice allocations and use of mice and were updated on a weekly basis. The Sanger system was used to send requests for ear biopsies, setting up breeding cages and culling requests.

2.2.2 Mouse breeding regime

Breeding cages were set-up as pairs/trios consisting of one male (*Mgat1^{F/F} C1galt1^{F/F}:Zp3Cre*) and one/two females (*Mgat1^{F/F} C1galt1^{F/F}*). Breeding cages were set-up when mice reached 6-weeks of age and gestation time is 19-21 days. Mice are able to have litters every 3 weeks due to the post-partum oestrous, a phenomenon in which females enter oestrus and are receptive to mating after littering down.

2.2.3 Timed-mating

If a large number of females were required for experiments, temporary plugging cages were set-up using the breeding cage males (*Mgat1^{F/F} C1galt1^{F/F}:Zp3Cre*) and 9-10 females (*Mgat1^{F/F} C1galt1^{F/F}*). Two or three females were placed with each male and the next morning at ~9am, plugs were detected by using a probe. If a plug was detected then females were put into a stock cage, and the male was given a replacement female. Before littering down, the females were split into 2 females per cage with the male to utilise the post-partum oestrous.

2.2.4 Ear clipping and weaning

When the newborn pups were 12 days of age, they were ear clipped for identification and genotyping purposes. To ear clip, the mouse was held by scruffing and an ear biopsy taken using the biopsy tool (Vet-tech, Congleton, UK) and placed into a sterile microcentrifuge tube. Between each mouse, the tools were cleaned with 70% ethanol (EtOH, Sigma-Aldrich, Dorset, UK).

When mice were 3 weeks old, they were weaned into stock cages. Males and females are weaned into separate cages; females may be joined together at a later date, however since males are territorial, unless weaned within a short period (2-3 days maximum) they cannot be housed together. This also applied to males who were initially together but had been separated for any purpose.

2.3 Ethical approval

All experiments using mice were carried out with the approval of the Local Ethical Review Panel at the University of Oxford under licence in accordance with the UK Animals (Scientific Procedures) Act 1986 in a project license held by Dr. Williams (Project license number 30/3352).

2.4 Genotyping

2.4.1 DNA extraction

To obtain DNA for analysis, a single ear biopsy from each female was digested in 51 µl of a 1.5% mixture of Proteinase K (Roche diagnostics, Mannheim, Germany) in buffer (100mM Tris-HCl, 5mM EDTA, 0.2M NaCl and 0.2% SDS) overnight at 55°C. A shorter protocol for same day genotyping was also established; for this, the ear biopsy was cut into quarters using a sterile blade and digested in the Proteinase K buffer solution for 2 h.

Following digestion, the samples were centrifuged at 13,200 rpm for 8 min and 40 µl of supernatant was boiled at 99°C for 10 min to deactivate the Proteinase K. The sample was then diluted with 100 µl of distilled water (Gibco, Life technologies, UK) and stored at 4°C.

2.4.2 Polymerase chain reaction (PCR)

Each PCR reaction mix (25 µl) contained distilled water, PCR buffer (Bioline Reagents, London, UK), magnesium chloride (MgCl₂) solution (Bioline), deoxyribonucleotide triphosphates (dNTPs) (Roche, Mannheim, Germany), primers (Eurogentec, Liege Science Park, Belgium), DNA and Taq polymerase (Bioline). Quantities of each reagent are detailed in Table 2.1.

Table 2.1. Volumes of PCR reaction mixes

Reagents	PCR reaction mix (μl)		
	<i>Zp3Cre</i>	<i>C1galt1</i>	<i>Mgat1</i>
Distilled water	18.75	19.05	19.3
10x PCR buffer	2.5	2.5	2.5
50 mM MgCl ₂	0.75	0.75	0.5
10 mM dNTPs	0.5	0.5	0.5
10 mM Primer 1	0.5	0.5	0.5
10 mM Primer2	0.5	0.5	0.5
Taq polymerase	0.5	0.2	0.2
DNA	1	1	1

The primers used to detect the *Cre* transgene were PS502 and PS607 (227), to detect the *C1galt1* floxed allele were FB33 and FB34 (241), and the *Mgat1* floxed allele were PS585 and PS586; primer sequences are stated in Table 2.2.

Table 2.2. Sequences of primers used for the detection of *Mgat1*, *C1galt1* and *Cre* transgene.

	Primer	Sequence
<i>Mgat</i>	PS585	5'-TGCAAGCCAACACTTGTCTC-3'
	PS586	5'-GAGACCTGCTTACTGCAGCC-3'
<i>C1galt1</i>	FB33	5'-GTCCACAGAGGGCCATTAGA-3'
	FB34	5' TGCTGATCCACAGAGGAAGA-3'
<i>Cre</i>	PS502	5'-GGACATGTTTCAGGGATCGCCAGGCG-3'
	PS607	5'-CCATGAGTGAACGAACCTGG-3'

PCR was performed in three steps after an initialisation step. The initialisation step was for 2 min at 94°C followed by 35 cycles of denaturation (30 sec at 94°C), annealing (for *C1galt1*: 30 sec at 66°C, for *Mgat1* and *Cre*: 30 sec at 56°C) and extension (1 min at 72°C).

2.4.3 Analysis of PCR products by gel electrophoresis

For electrophoresis, a 1.5% agarose gel with 0.01% ethidium bromide (Fisher Scientific, Loughborough, UK) was prepared by heating a mixture containing 3 g of agarose (VWR, Leicestershire, UK) in 200ml TAE buffer. TAE buffer is composed of Trizma base (T1503, Sigma-Aldrich, Dorset, UK), Na₂EDTA (Fisher Scientific, Loughborough, UK) and acetic acid glacial (Fisher Scientific). A 19-well gel comb was placed in the agarose mixture to create DNA loading wells and the mixture left to set for 1 h. PCR products were mixed with 2 µl of gel loading dye (New England Biolabs, Hitchin, UK) and loaded into the agarose gel. For *Cre* genotyping 8 µl of product was loaded and for *Mgat1* and *C1galt1* genotyping 15 µl of product. The first and last well of each lane was loaded with a 100bp DNA ladder (New England Biolabs). Samples were run at 100 millivolts for 55 min and the gel visualised using the LI-COR Odyssey CLx Imaging System.

2.5 Histological processing

2.5.1 Tissue collection and Fixation

Mice were culled by cervical dislocation and death was confirmed by exsanguination. Using sterile microdissection scissors (Fine Science Tools, InterFocus Ltd, Linton, UK) and Dumont #5 forceps (Fine Science Tools), each ovary was dissected into a microcentrifuge tube containing sterile phosphate buffered saline (PBS). When transferred to the lab, the ovaries were cleaned of the bursa and fat under the stereomicroscope (Leica DM2500). The clean ovaries were transferred into labelled microcentrifuge tubes containing 10% buffered

formalin (Sigma-Aldrich) and were fixed for 8 h before being transferred to 70% EtOH and placed in the fridge at 4°C until embedding.

2.5.2 Embedding process

Before starting the embedding process, cassettes labelled with mouse/ovary/experiment details, and plastic moulds and bijou tubes containing paraffin wax, were placed in the oven at 62°C. If the tissue was too small to visualise, it was stained in a 1:10 eosin (Sigma-Aldrich) to 50% EtOH solution for 30 min. The embedding process involves two steps; the dehydration step and the embedding step.

2.5.2.1 Dehydration

The tissue is transferred to PBS before being dehydrated using increasing concentrations of EtOH followed by xylenes (Fisher Scientific) (Table 2.3). For EtOH, if a 96-well plate was used, then 200 µl of reagent was used per well; or if a 48-well plate was used then 500 µl/well was used. When transferring the tissue to xylene, glass embryo dishes containing 1ml of xylene were used, since xylene melts the plastic plates.

A blunt forcep or a wide bore 200 µl pipette tip was used to transfer the tissue from well to well to avoid damage to the tissue.

Table 2.3. Reagents and timings of the dehydration step of embedding

Reagent	Time
50% EtOH	15 min
70% EtOH	15 min
80% EtOH	15 min
95% EtOH	15 min
100% EtOH I	15 min
100% EtOH II	15 min
100% EtOH II	15 min
Xylene I	20 min
Xylene II	20 min
Xylene III	20 min

2.5.2.2 Embedding

Following dehydration, the tissue was carefully transferred into the centre of the plastic moulds containing the melted paraffin wax (provided by Richard Stillion, Sir William Dunn School of Pathology) and incubated at 62 °C for 2 h. After 2 h the labelled cassette was placed on top of the mould, if required the mould was topped up with wax, and the position of the ovary was checked to ensure it was in the centre. The oven was turned off and the wax was left to set overnight. The next morning the moulds were placed in the fridge for 1 h to help loosen the wax block from the mould.

2.6 Sectioning

Paraffin blocks were serially sectioned at 5 µm using a microtome (Leica, Nussloch, Germany) and sections were mounted onto microscope slides (SuperFrost Plus, VWR). Slides were heated in the oven at 62°C for 1 h and stored in slide boxes (Fisher Scientific) until required.

2.7 Haematoxylin & eosin staining

A regressive haematoxylin staining protocol was used for staining. There are 4 main steps for the haematoxylin (Shandon Gill 2 haematoxylin; Fisher Scientific) and eosin (H&E) protocol. These are dewaxing, rehydration, staining, and dehydration, which are detailed in Table 2.4.

Table 2.4. Details of the four steps of H&E staining protocol including reagent and duration.

Step	Reagent	Duration
Dewax	Xylene I	8 min
	Xylene II	4 min
	Xylene III	4 min
Rehydration	100% EtOH I	3 min
	100% EtOH II	3 min
	90% EtOH	2 min
	70% EtOH	2 min
	50% EtOH	3 min
	Tap water	3 min
Staining	Harris haematoxylin	2 min
	Wash in running water	5 min
	Destain in acid alcohol (1% HCL in 70% EtOH)	5 sec
	Wash in running water	5 min
	80% EtOH	1 min
	Eosin alcoholic	1 min
Dehydration	95% EtOH	30 sec
	100% EtOH I	45 sec
	100% EtOH II	1 min
	100% EtOH III	2 min
	Xylene I	8 min
	Xylene II	8 min

2.7.1.1 Mounting

Slides were mounted with coverslips using Depex (Fisher Scientific) and left on a flat surface to dry overnight.

2.7.1.2 Imaging

Sections were visualised using a DM2500 Leica microscope (Microscope services Ltd, Woodstock, UK) and imaged using a MicroPublisher 5.0 RTV camera (Qimaging, Microscope services Ltd).

2.8 *LacZ* staining

2.8.1 Genotyping

Tail tips of P0/P1 pups or ear biopsies from older *LacZ* mice and a positive and negative control were incubated in 100 µl of X-gal solution at 37°C for 1 hour. Tissue from mice carrying the *LacZ* gene stained blue at the end of the process. Components of the solutions are detailed in Table 2.5.

2.8.2 *LacZ* staining of tissue

The tissue was fixed in 200 µl of gluteraldehyde fixative for 1 h at room temperature and permeabilised by washing three times (15 min each) in 200 µl of detergent rinse solution. Finally, the tissue was incubated overnight in 100 µl of X-gal staining solution at 37°C followed by three 15 min detergent rinses and kept in PBS at 4°C until wax embedding. Details of the components of the solutions are detailed in Table 2.5.

Table 2.5. Composition of X-gal staining solutions required for fixation, detergent rinse and X-gal staining.

Solution	Reagents	Volume for 1ml solution (µl)
Fixation	1M MgCl ₂	2
	25% glutaraldehyde	8
	0.1M PBS (pH 7.3)	990
Detergent rinse	1M MgCl ₂	2
	1% sodium deoxycholate	10
	1% NP-40	20
	0.1M PBS (pH 7.3)	968
X-gal staining	1M MgCl ₂	2
	1% sodium deoxycholate	10
	250 mM potassium ferrocyanide	20
	250 mM potassium ferricyanide	20
	50mg/ml X-gal	20
	1% NP-40	20
	1M Tris (pH 7.3)	20
	0.1M PBS (pH 7.3)	888

Morphometric analysis of primary follicle development

3.1 Introduction

Approximately 50% of women diagnosed with POI have follicular POI (184-186, 242), and 5-10% of women diagnosed with POI conceive spontaneously (243). Although several factors have been associated with POI, the mechanisms that cause ovarian dysfunction are poorly understood and ~70% of cases are idiopathic. Therefore further research is warranted to decipher the underlying mechanisms and develop treatments for affected patients; models of POI provide a useful tool to this end.

The DM mouse is a model of follicular POI. DM females exhibit a clearly defined timeline for the onset of the condition since females transition from being subfertile at 6-weeks to infertile at 9-weeks and undergo POI by 3-months of age (1). The drop in fertility of DM females was also accompanied by a dysregulation of follicle development.

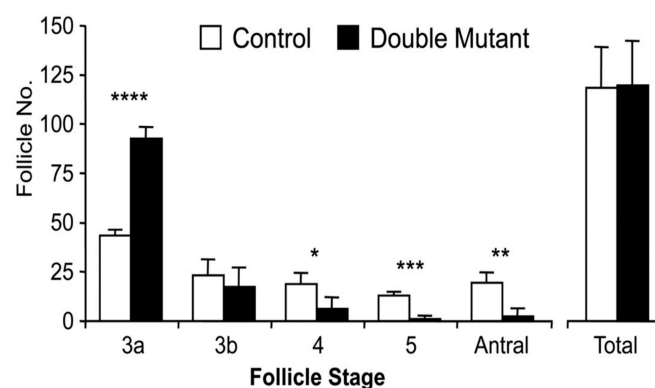


Figure 3.1 Follicle counts for 3-month old Control and DM mice. DM ovaries (n=3 mice) have an increased number of primary follicles accompanied by decreased number of developing follicles compared to Controls (n=3 mice); however total follicle numbers did not differ. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Adapted from Williams et al. (1)

Analysis of follicle numbers in DM ovaries at 3-months of age revealed increased numbers of primary follicles compared to Controls (Figure 3.1) (1, 236, 244), which suggested a block at the primary stage of follicle development existed. Therefore, we hypothesised that there are abnormal follicles in the DM ovary that are unable to progress past the primary stage of follicle development.

3.2 Aim

- To characterise morphological differences in primary follicles between Control and DM and assess how this is affected by age.

To address this, primary follicles were analysed in postnatal (8-days), pre-pubertal (3-week), post-pubertal sub-fertile (6-weeks) and infertile POI (3-months) DM ovaries. Using morphometric analysis we analysed the relationship between GC number and follicle and oocyte size, the packing density of GCs, GC proliferation and changes in the theca cell compartment.

3.3 Materials and Methods:

3.3.1 Mice

Female mice with floxed *C1galt1* and *Mgat1* alleles (*C1galt1*^{F/F} *Mgat1*^{F/F}) were Controls and mice also carrying a Zp3Cre recombinase transgene were experimental DM (*C1galt1*^{F/F} *Mgat1*^{F/F}:Zp3Cre) (1, 226).

3.3.2 Ovarian histology and analysis

Ovaries were collected at 8-days, 3-weeks, 6-weeks and 3-months of age and fixed, paraffin embedded and sectioned (Section 2.5). Every 10th serial section was stained with H&E (Section 2.7). Only morphologically healthy primary follicles with a central oocyte and a visible nucleus were assessed. Primary follicles were classified based on the number of GC in a cross section (245) as either primary 3a (9-20 GCs) or primary 3b (21-60 GCs) follicles. Follicle area (excluding theca cells) and oocyte area (excluding zona pellucida) were measured using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The follicle basement membrane was used as the follicle boundary. GC area was obtained by subtracting oocyte area from follicle area.

3.3.3 Immunohistochemistry and histochemistry

Immunohistochemistry (IHC) was used to detect the presence of Ki67. Histochemistry using hyaluronic-binding protein was used to detect the presence of hyaluronic acid (HA). Sections were dewaxed with xylene and rehydrated using decreasing concentrations of ethanol and ddH₂O. Slides were washed in Tris Buffered Saline (TBS: 0.1M Tris pH 7.5 and 0.3M NaCl) with 0.05% Tween-20

(TBST). Endogenous peroxidase was quenched by washing with 3% H₂O₂ (Fisher Scientific) in Phosphate Buffered Saline (PBS) for 5 min. For HA detection, sections were blocked with 2% FCS (Sigma-Aldrich) in PBS for 1h, & for Ki67 detection, with 1.5% normal goat serum (Vectastain ABC Kit, Vector Laboratories, Peterborough, UK) in TBS for 1h. The sections were then incubated with either 0.25mg/ml biotinylated HA binding protein (HABP, Seikagaku, Tokyo, Japan) at 1:50, or rabbit anti-Ki-67 antibody (ab66155; Abcam, Cambridge, UK) at 1:100 and incubated overnight at 4°C. After 3 washes with TBST, all sections were incubated with biotinylated anti-rabbit IgG secondary antibody (Vectastain ABC Elite Kit) for 1 h, followed by ABC solution (Vectastain ABC Elite Kit) for 30 min. A 3,3'-Diaminobenzidine (DAB) peroxidase substrate kit (Vectastain ABC Elite Kit) was used to develop the stain. The slides were counterstained with haematoxylin, dehydrated, mounted and imaged as described in Section 2.7.

For analysis of Ki67 staining, granulosa cells (GCs) were classified as either Ki67 negative or positive using an assessment scale (Section 3.4.7). For HA, theca cells were counted and then classified as positive if at least 50% of the cell was in contact with detectable HA.

3.3.4 Statistical Analysis

Statistical analyses were performed using Prism GraphPad software (GraphPad Software, Inc., version 4.0b, 2004). D'Agostino & Pearson normality test was applied to test Gaussian distribution of samples size $n > 10$. Where sample size $n < 10$, the Shapiro-Wilk test of normality was used. The unpaired *t*-test was used to analyse differences between samples with normal distribution. The Mann–

Whitney U test was performed to detect differences between groups with non-parametrical distribution and equal variances. A Welch's correction was applied to groups with non-parametrical distribution and unequal variances. To assess correlation between GC number and area, if normally distributed, the Pearson correlation coefficient was tested; for non-parametric distribution, the non-parametric Spearman correlation was tested. Differences between GC number and area at the different ages were analysed using linear regression. To compare mean area between ages, a one-way ANOVA with Tukeys' multiple comparison was used. A few groups were not normally distributed, for these the Kruskal Wallis with Dunn's multiple comparisons test was used. Unpaired *t* test was used to assess differences in theca cell area whilst a Kruskal Wallis with Dunn's multiple comparison test was used to compare theca cell numbers. A *P* value ≤ 0.05 was considered significant.

3.3.5 Disclaimer

For this chapter, the IHC experiments detecting Ki67 using 6-week old mice were conducted by Dr. Jassimran Bhansal (FHS student in Dr. Williams's lab) and the results were analysed by me. For the HA histochemistry experiments detecting HABP and subsequent analysis were conducted by Grace Brown (FHS student) under my supervision.

3.4 Results

3.4.1 Primary 3a follicles: Morphology of follicle, oocyte or GC area

As this study was focusing on primary follicles, primary 3a (9-20 GCs) and 3b (21-60 GCs) follicles with a visible nucleus were analysed at 8-days, 3-weeks, 6-weeks and 3-months in Control and DM ovaries. Images revealed (Figure 3.2) that primary follicles from 8-day ovaries looked larger than primary follicles from older ovaries despite having the same number of GCs (as categorized by GC number) indicating differences in GC density.

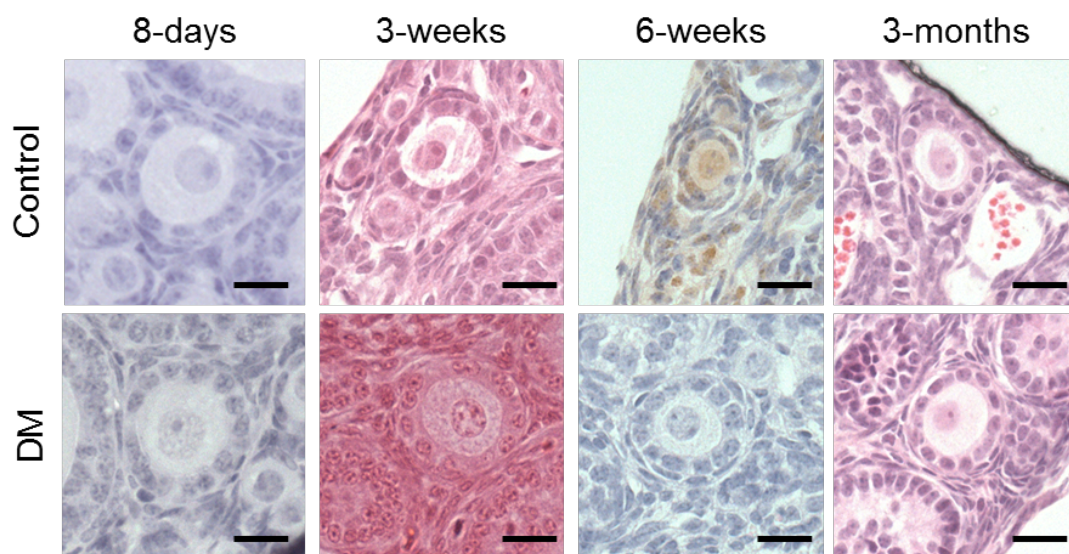


Figure 3.2. Images of primary 3a follicles in Control and DM ovary sections at 8-days, 3-weeks, 6-weeks, and 3-months. Scale bar= 20 μ m.

3.4.2 Primary 3a follicles: Analysis of linear relationship between follicle, oocyte or GC area with GC number

To investigate these differences, first, the relationship of how follicle, oocyte and GC area related to GC number in primary 3a follicles was assessed. In both Control (Figure 3.3A) and DM (Figure 3.3B) ovaries at all ages, follicle, oocyte and GC area increased proportionally with GC number. Although area increased as expected with GC number, follicles from 8-day ovaries appeared to be larger as first observed (Figure 3.2), and followed a different growth trajectory to follicles from the other ages.

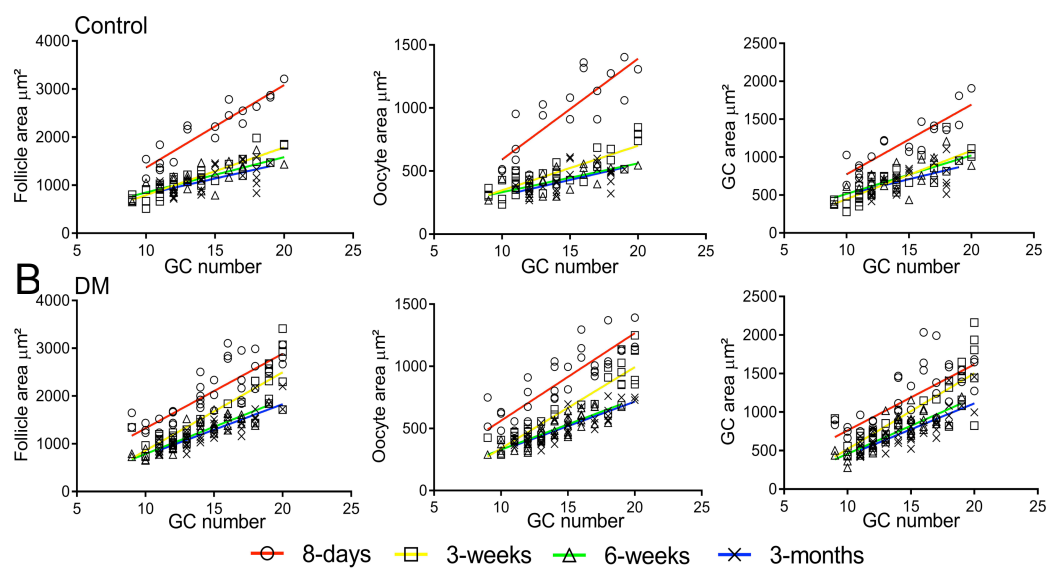


Figure 3.3 Analysis of primary 3a follicles. The relationship between follicle, oocyte and GC area with GC number in primary 3a follicles in (A) Control and (B) DM was analysed at 8-days (open circles; Control n=3 mice, n=19 follicles, DM n=3 mice, n=38 follicles), 3-weeks (open squares; Control n=3 mice, n=37 follicles, DM n=3 mice, n=36 follicles), 6-weeks (open triangles; Control n=3 mice, n=23 follicles, DM n=3 mice, n=38 follicles) and 3-months (crosses; Control n=3 mice, n=22 follicles, DM n=3 mice, n=46 follicles).

A linear regression revealed that there were significant differences in slopes between all areas (follicle, oocyte and GC) at 8-days and all other ages for Controls, whilst no differences in slopes existed between the other ages (Table 3.1). For DM, a significant difference in the slopes existed when comparing follicle and oocyte areas at 8-days to 6-weeks or 3-months however when comparing GC area and all areas (follicle, oocyte and GC) at 8-days to 3-weeks although the slopes did not differ, the intercepts did. Further differences in areas existed between 3-weeks to 6-weeks and 3-months (Table 3.1).

Table 3.1 Analysis of linear relationship between follicle, oocyte and GC area with GC number in primary 3a follicles in Control and DM ovaries at 8-days, 3-weeks, 6-weeks and 3-months. Linear regression data for 3a follicles showing *P* values. If slopes are not significantly different, then differences between intercepts were calculated.

	Ages	Control		DM	
		Slopes (<i>P</i> values)	Intercepts (<i>P</i> values)	Slopes (<i>P</i> values)	Intercepts (<i>P</i> values)
3a follicles	8-days vs 3-weeks	<0.0001	N/A	0.56	<0.0001
	8-days vs 6-weeks	<0.0001	N/A	0.001	N/A
	8-days vs 3-months	0.0002	N/A	0.0184	N/A
	3-weeks vs 6-weeks	0.09	0.3	0.0154	N/A
	3-weeks vs 3-months	0.0619	0.0075	0.0037	N/A
	6-weeks vs 3-months	0.71	0.2567	0.6400	0.1412
3a oocytes	8-days vs 3-weeks	<0.0001	N/A	0.56	<0.0001
	8-days vs 6-weeks	0.0002	N/A	0.001	N/A
	8-days vs 3-months	0.0013	N/A	0.0003	N/A
	3-weeks vs 6-weeks	0.08	0.005	0.0008	N/A
	3-weeks vs 3-months	0.21	0.0003	0.0002	N/A
	6-weeks vs 3-months	0.85	0.48	0.8021	0.364
3a GCs	8-days vs 3-weeks	0.0225	N/A	0.473	0.0038
	8-days vs 6-weeks	0.0124	N/A	0.4525	<0.0001
	8-days vs 3-months	0.006	N/A	0.2539	<0.0001
	3-weeks vs 6-weeks	0.28	0.55	0.09	0.0007
	3-weeks vs 3-months	0.09	0.2032	0.0304	N/A
	6-weeks vs 3-months	0.5363	0.2282	0.6400	0.1574

3.4.3 Primary 3a follicles: Analysis of average follicle, oocyte or GC area within Control and DM ovaries

To assess if there was a difference in 3a follicle area between 8-day, 3-week, 6-week and 3-month ovaries, we determined average follicle, oocyte and GC area at each age. Control 8-day ovaries (Figure 3.4A) had larger follicles, and increased oocyte and GC area than those from 3-week, 6-week and 3-month ovaries. No further differences were observed when comparing the older ovaries (3-week, 6-week and 3-month).

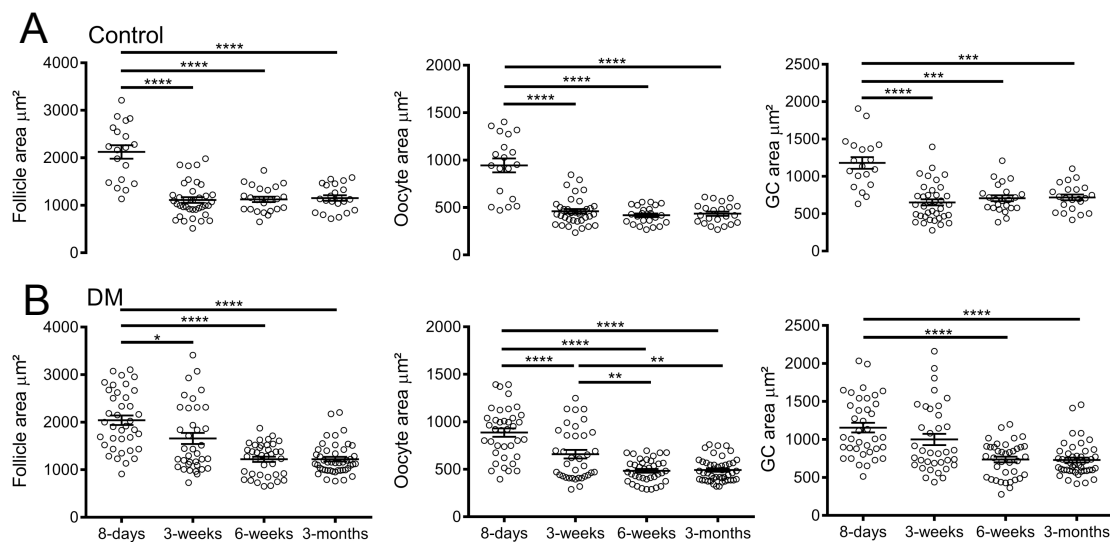


Figure 3.4 Analysis of primary 3a follicles. Comparison of follicle, oocyte and GC area of primary 3a follicles within (A) Control and within (B) DM ovaries at the different ages. Results are expressed as mean±SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

A similar pattern was observed in 3a follicles from DM 8-day ovaries (Figure 3.4B) compared to 3a follicles from 3-week, 6-weeks and 3-months ovaries; however GC area did not decline as uniformly as in Controls and thus did not differ at 3-weeks. The oocyte size remained higher and more variable in DM at

3-weeks resulting in a difference when comparing oocyte area at 3-weeks to 6-weeks and 3-months.

3.4.4 Primary 3a follicles: Comparison of areas between Control and DM ovaries

To determine if the 3a follicle dysregulation in DM ovaries was due to the presence of abnormal sized follicles, we compared follicle, oocyte and GC areas between Control and DM at the 4 different ages (Figure 3.5).

No differences in follicle, oocyte and GC area between Control and DM were observed at the earliest (8-days) (Figure 3.5A) or the latest (3-months) age analysed (Figure 3.5D). However, at 3-weeks of age, DM follicles were larger than Control follicles due to increased follicle, oocyte and GC area (Figure 3.5B). Furthermore, at 6-weeks of age oocyte area was larger in DM ovaries compared to Controls, whilst follicle and GC area did not differ (Figure 3.5C).

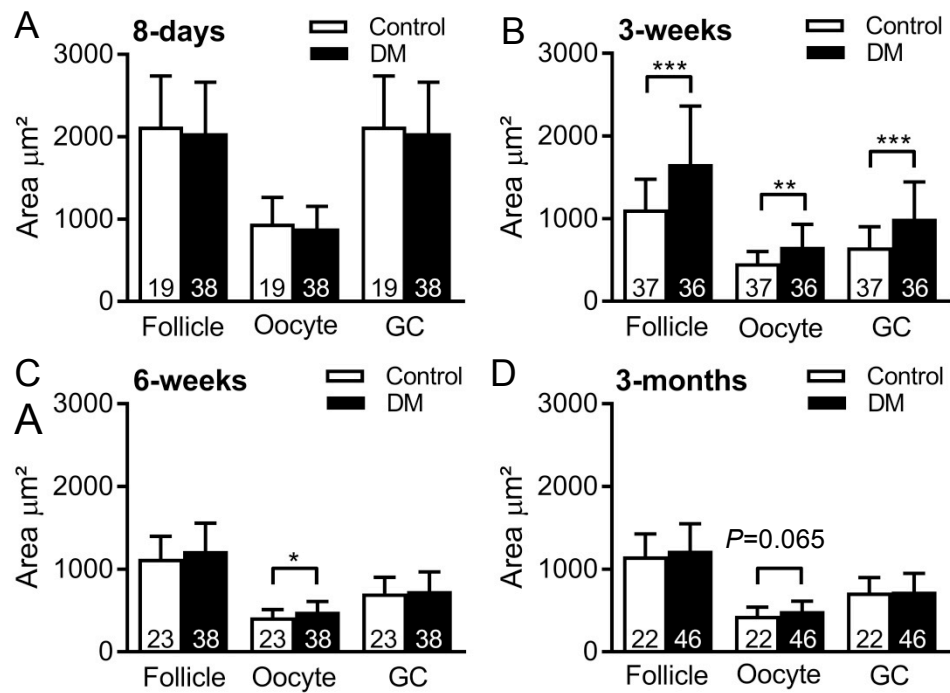


Figure 3.5 Comparison of follicle, oocyte and GC area of primary 3a follicles between Control and DM at (A) 8-days, (B) 3-weeks, (C) 6-weeks and (D) 3-months. Numbers in bars represent follicle numbers. Results are expressed as mean $\pm$ SD. ** $P\leq 0.01$, *** $P\leq 0.001$.

3.4.5 Analysis of primary 3b follicles

Having examined primary 3a follicles, we then analysed primary 3b follicles (21-60 GCs) to ascertain if the changes observed in 3a follicles persisted. Once again images (Figure 3.6) revealed that primary follicles from 8-day ovaries looked larger than primary follicles from older ovaries, indicating differences in GC density.

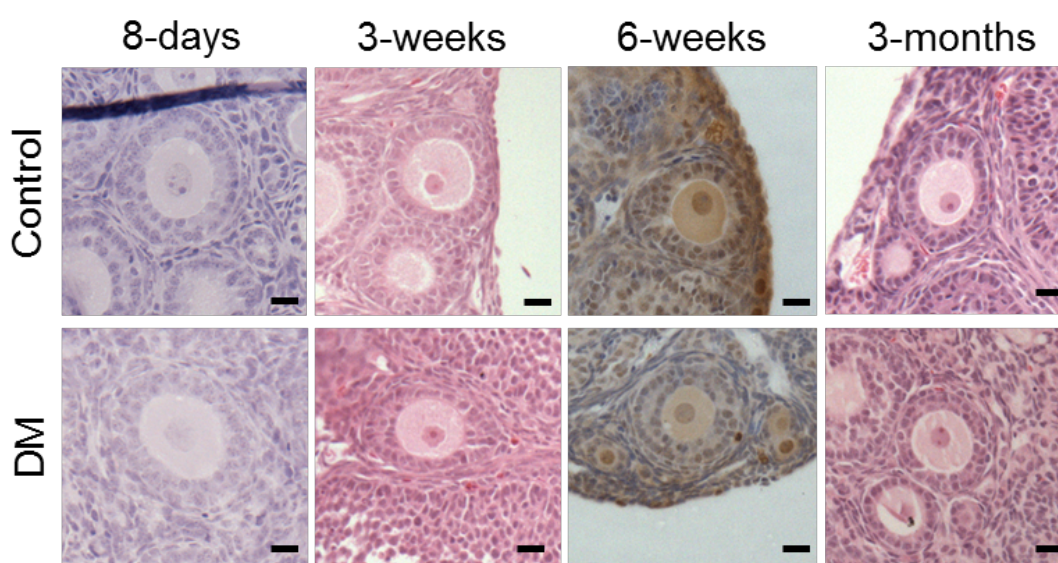


Figure 3.6 Images of primary 3b follicles in Control and DM ovary sections at 8-days, 3-weeks, 6-weeks, and 3-months. Scale bar= 20 μ m.

As expected, follicle, oocyte and GC area of 3b follicles increased proportionally with GC number in Control (Figure 3.7A) and DM (Figure 3.7B) at all ages.

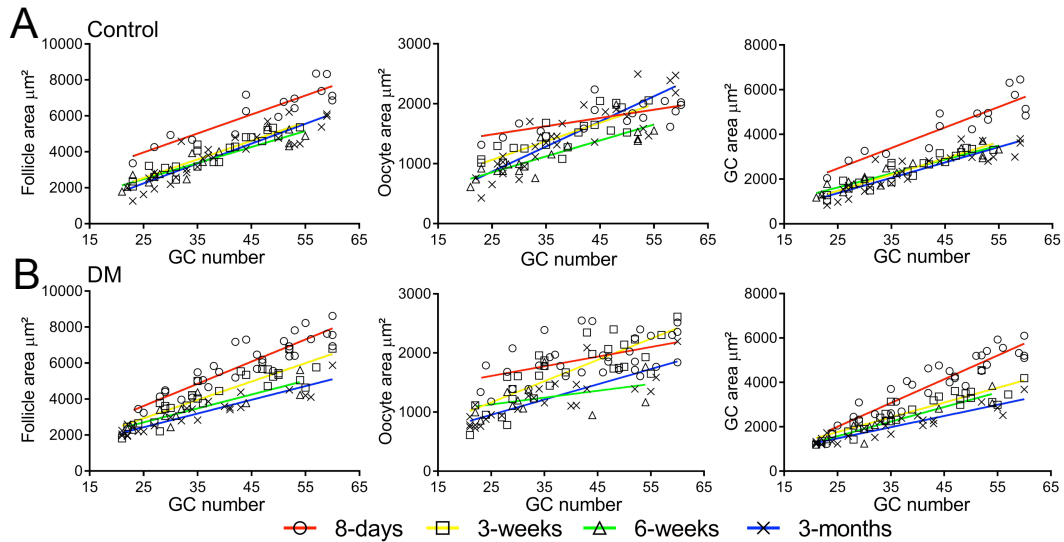


Figure 3.7 Analysis of primary 3b follicles. The relationship between follicle, oocyte and GC area with GC number in primary 3b follicles in (A) Control and (B) DM was analysed at 8-days (open circles; Control n=3 mice, n=16 follicles, DM n=3 mice, n=34 follicles), 3-weeks (open squares; Control n=3 mice, n=21 follicles, DM n=3 mice, n=29 follicles), 6-weeks (open triangles; Control n=3 mice, n=17 follicles, DM n=3 mice, n=11 follicles) and 3-months (crosses; Control n=3 mice, n=28 follicles, DM n=3 mice, n=25 follicles).

The average size of 3b follicle and oocyte area at each age was determined (Figure 3.8). Similar to Control 3a follicles, Control 3b follicles (Figure 3.8A) from 8-day ovaries were larger with an increased follicle and GC area than the older ages, however oocyte area mirrored the plateau observed in Figure 3.7A, and was only larger compared to 6-weeks (Figure 3.8B).

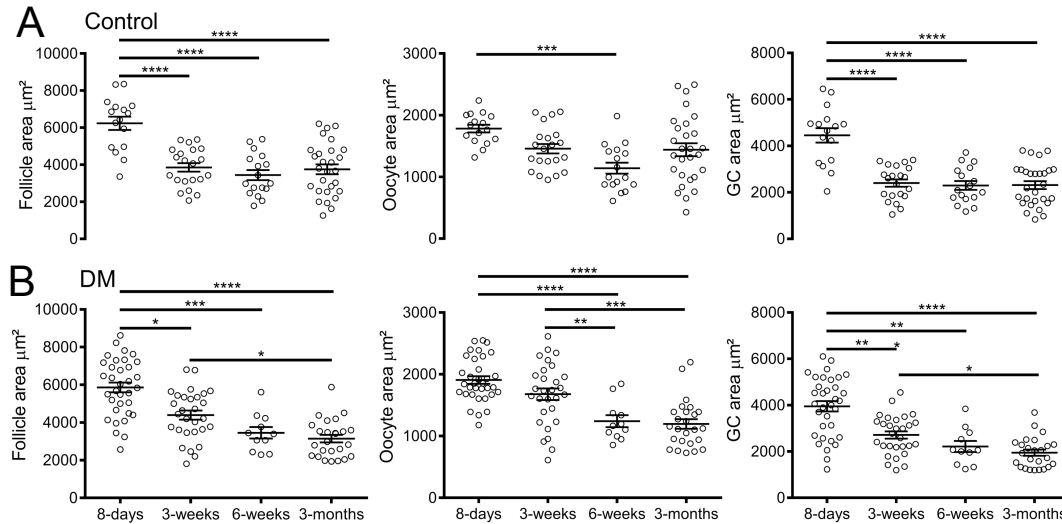


Figure 3.8 Analysis of primary 3b follicles. Comparison of follicle, oocyte and GC area of primary 3b follicles within (A) Control and within (B) DM ovaries at the different ages. Results are expressed as mean $\pm$ SEM. * $P\leq 0.05$, ** $P\leq 0.01$, *** $P\leq 0.001$, **** $P\leq 0.0001$.

A different pattern was observed amongst DM 3b follicles (Figure 3.8B), 8-day ovaries compared to 6-week and 3-month ovaries, contained larger follicles and oocytes accompanied with increased GC area; however follicle and GC area were increased but oocyte area did not decline as uniformly as in Controls and thus when comparing follicles from 8-days to 3-weeks oocyte size did not differ at 3-weeks. The oocyte size remained higher and more variable in DM at 3-weeks relating in a difference when comparing oocyte area at 3-weeks to 6-weeks; whilst follicle, oocyte and GC area was larger when comparing 3-weeks to 3-months.

Finally, comparison of 3b follicle, oocyte and GC area between Control and DM ovaries revealed no differences (Figure 3.9).

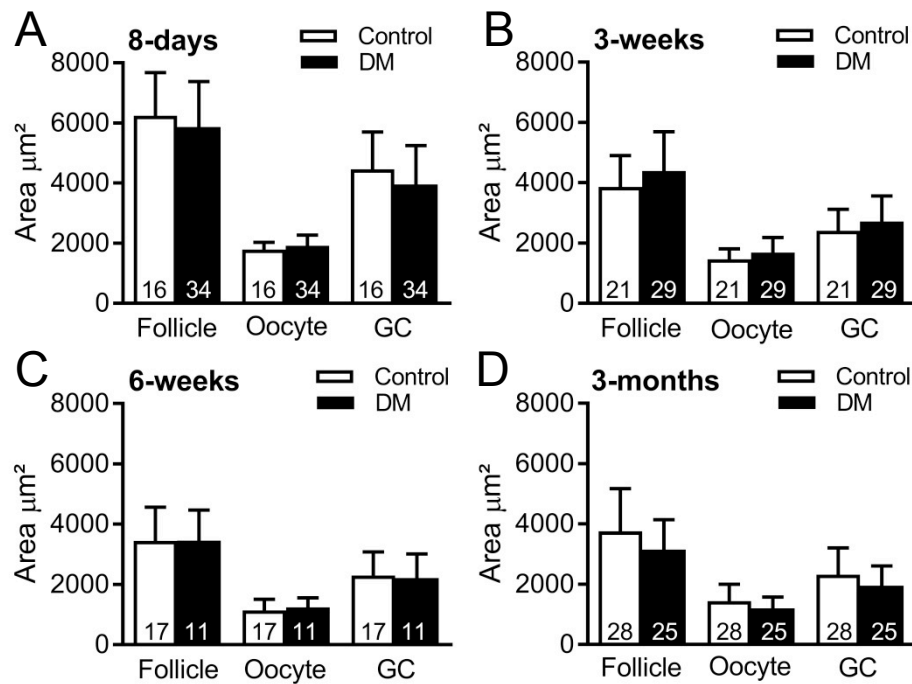


Figure 3.9 Comparison of follicle, oocyte and GC area of primary 3b follicles between Control and DM at (A) 8-days, (B) 3-weeks, (C) 6-weeks and (D) 3-months. Numbers in bars represent follicle numbers. Results are expressed as mean±SD.

3.4.6 Analysis of number of GC layers

When analysing follicle images, it became apparent that despite having the same range of GCs, some follicles had a partial second layer of GCs. To determine if follicles at the different ages were developing faster (i.e. were they developing a second layer sooner), follicles were classified as either follicles with 1 layer of GCs (1 layer), follicles that had started developing the second layer (1+ layers) or follicles that had developed 2 complete layers (2 layers) (Figure 3.10A). The proportion of 3a follicles (Figure 3.10B) and 3b follicles (Figure 3.10C) in these categories was analysed.

We first observed that a higher proportion of primary 3a and 3b follicles had 1+ and 2 layers from 8-day ovaries, which was in agreement with our observation that 8-day ovaries contain larger follicles. At 3-weeks of age, a higher proportion of DM 3a (Figure 3.10B) and 3b follicles (Figure 3.10C) had 1+ layer compared to Controls, suggesting DM follicles were developing differently, which is consistent with the larger follicles observed in DM 3-week ovaries (Figure 3.5B).

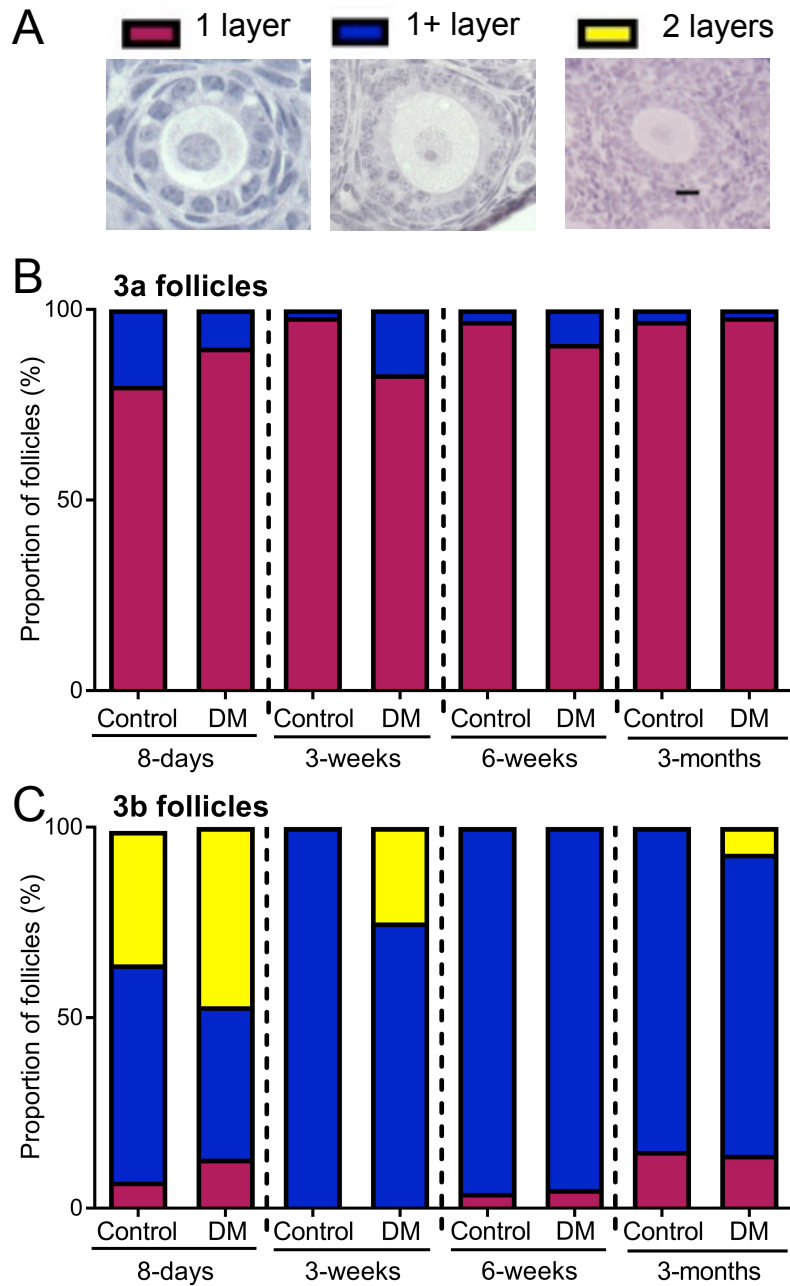


Figure 3.10 Analysis of GC layer formation. (A) All primary follicles were classified as follicles with 1 layer of GC (1 layer), follicles that had started developing the second layer (1+ layer) or follicles that had developed 2 complete layers (2 layers). The proportion of (B) primary 3a and (C) primary 3b follicles classified with 1 layer (magenta), 1+ layer (blue) and 2 layers (yellow) were analysed.

3.4.7 Ki67 labelling of follicles at 3- and 6-weeks of age

Since differences in area between Control and DM follicles existed at 3- and 6-weeks, and follicles from DM 3-week ovaries develop a second layer of GC earlier, we examined whether these changes were accompanied by changes in GC proliferation (Figure 3.11A). The proportion of Ki67 positive GCs per follicle were analysed in all primary follicles and for 3a and 3b follicles separately using the GC assessment scale (Figure 3.11B).

At 3-weeks of age (Figure 3.11C) when DM ovaries contain larger follicles than Control, the proportion of Ki67 positive GCs was higher in all primary follicles from DM ovaries than Controls however when separated into 3a and 3b follicles, significance was not quite achieved when comparing Control to DM. Since in 6-week DM ovaries we do not see an increase in follicle or GC area accompanying the presence of large oocytes (Figure 3.5C), we examined changes in GC proliferation (Figure 3.11D). Although no differences were observed in the percentage of Ki67 positive GCs in all primary follicles, when split into 3a and 3b, DM 3a follicles contained a lower proportion of proliferating GCs compared to Controls; no differences existed at the 3b stage. Therefore, although the oocyte continues to grow this is not accompanied by an increased proportion of proliferating GC surrounding DM oocytes in 3a follicles, and thus as observed, overall follicle size does not vary between Control and DM (Figure 3.5C).

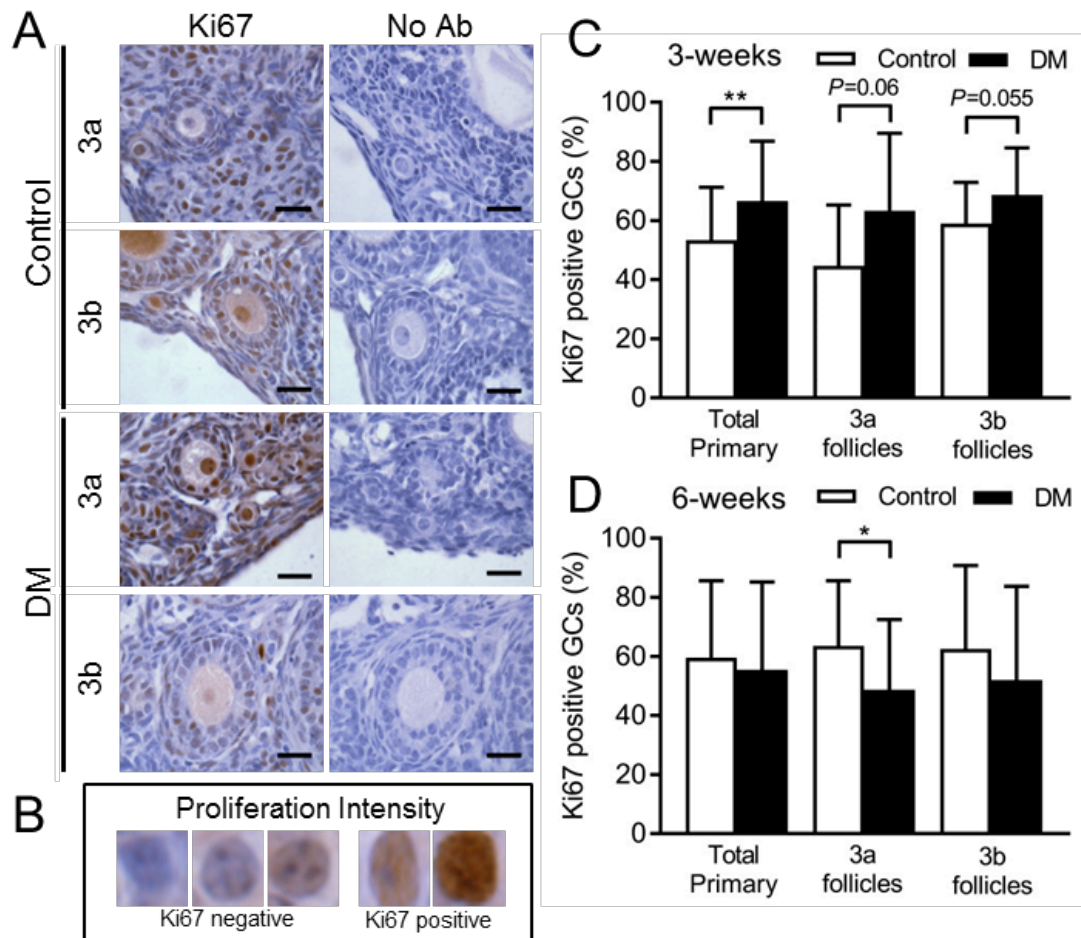


Figure 3.11 Analysis of GC Ki-67 labelling. (A) Images of Ki67 labelling of GCs of primary 3a and 3b follicles from Control and DM. (B) GC proliferation intensity was used to assess percentage of Ki67 positive GC cells in primary follicles from (C) 3-week and (D) 6-week Control (3-week total follicles n=28, 3a follicles n=11, 3b follicles n=17; 6-week total follicles n=33, 3a follicles n=16, 3b follicles n=14) and DM ovaries (3-week total follicles n=34, 3a follicles n=13, 3b follicles n=21; 6-week total follicles n=50, 3a follicles n=28, 3b follicles n=14). Scale bar= 25 μ m. * $P \leq 0.05$, ** $P \leq 0.01$.

3.4.8 Analysis of theca cell compartment

Since GC proliferation at 6-weeks was reduced in DM 3a follicles, this prompted us to assess theca cell development, since factors secreted by GCs are thought to recruit and promote theca cell proliferation. Histochemistry was used to detect HA, which localizes to the theca cell layer. In both Control and DM ovaries, HA was detected in the extracellular matrix (ECM) of the theca and GCs of primary follicles (Figure 3.12A).

Upon analysis of HA distribution, it was noted that for some theca cells HA was not detected in the surrounding ECM. To investigate these differences, first, all theca cells were analysed and classified as HA positive (HA+) theca or HA negative theca (Figure 3.12B). HA+ theca cells were classified as a theca cell with at least 50% of the cell in contact with detectable HA. Next, theca cell area and number were quantified in 3a and 3b follicles (Figure 3.12C). There were no differences in theca number or area in Control and DM 3a or 3b follicles. However, DM 3a follicles had a greater proportion of HA+ theca cells, which did not persist at the 3b stage. Comparison of follicle, oocyte and GC area of follicles with 100% HA+ theca did not differ between Control and DM for this cohort of follicles (data not shown).

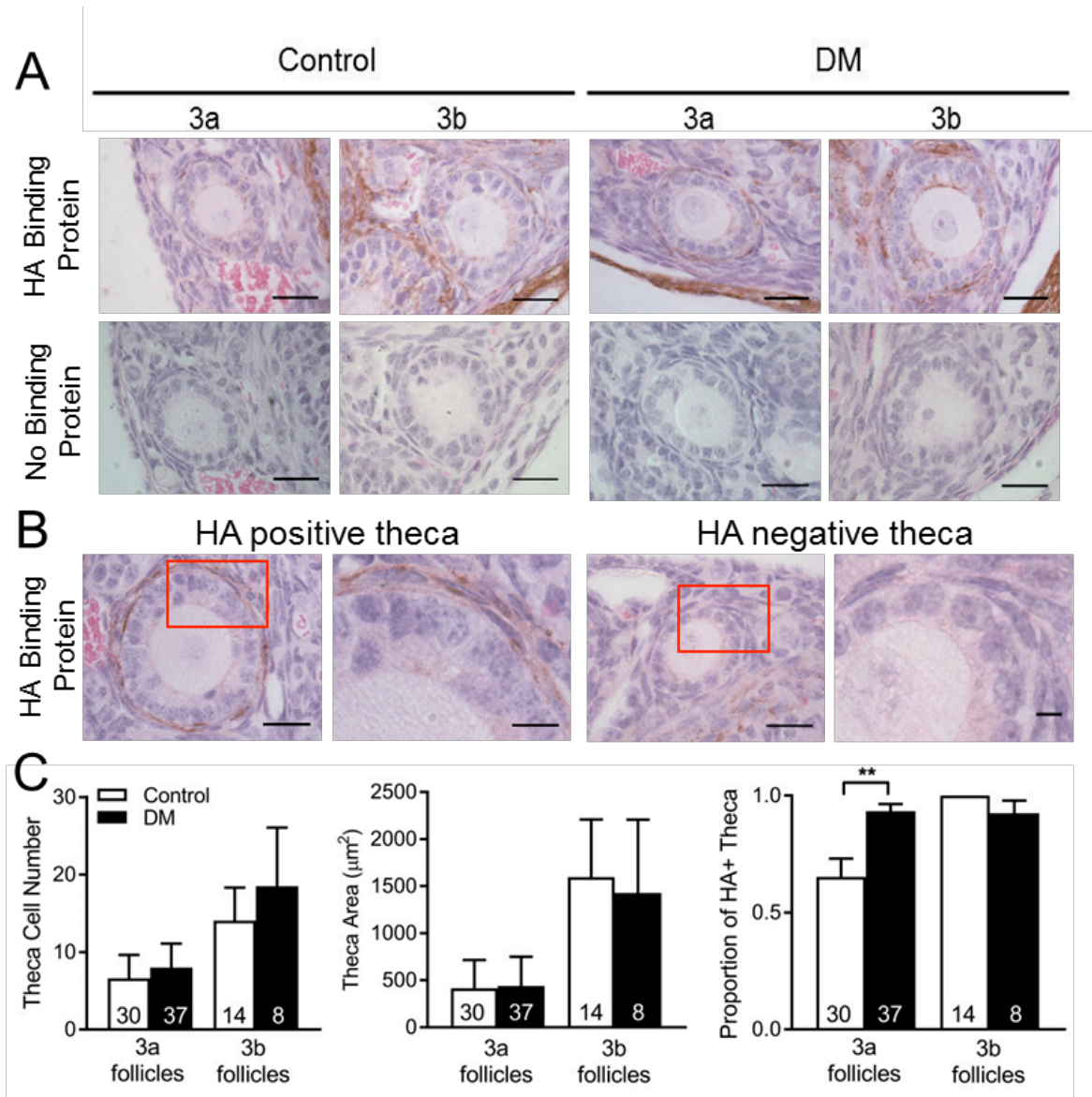


Figure 3.12 Analysis of theca cells and HA production. (A) HA was localised to the theca cell compartment of primary 3a and 3b follicles in Controls and DM. Scale bar= 50 μ m. (B) Theca cells were classified as HA positive (HA+) theca or HA negative theca. The area in the red box is enlarged to the right showing HA+ and HA negative theca. Scale bar= 10 μ m. (C) No differences existed in theca area and total theca number in 3a or 3b follicles between Control and DM however DM 3a follicles had a greater proportion of HA+ theca than Controls. Numbers in bars represent follicle numbers. Results are expressed as mean $\pm$ SD. ** $P\leq 0.01$.

3.5 Discussion

Whilst follicle dysregulation is one of the known causes of POI (246), the mechanisms and aetiology underlying the development of POI are unknown. In the DM mouse model of follicular POI, follicle development is modified with an increase in primary follicle numbers accompanied by fewer developing follicles indicative of a block in follicle development (1, 236, 244). DM females are sub-fertile at 6-weeks of age, producing one small litter, infertile by 9-weeks of age and undergo POI by 3-months of age. We hypothesised that there is an accumulation of primary 3a follicles due to the presence of abnormal follicles that are unable to progress past the primary stage of follicle development. Here, we investigated morphology of primary follicles in postnatal (8-days), pre-pubertal (3-week), post-pubertal sub-fertile (6-weeks) and infertile POI (3-months) DM ovaries.

First, in functionally wildtype Control ovaries (*C1galt1^{F/F} Mgat1^{F/F}*), this study revealed interesting aspects of early follicle development. Primary follicles from 8-day ovaries are larger in size, with larger oocytes than primary follicles in 3-week, 6-week and 3-month Control ovaries. Follicles in the 8-day ovaries are the first cohort of follicles activated shortly after birth, responsible for establishing the HPG axis, and perhaps may be functionally different to follicles that will follow, which we have shown is reflected in differences in size. The difference in developmental dynamics of follicles from 8-day ovaries may also be attributed to differences in location and rate of follicle growth, with follicles developing in the medulla in 8-day ovaries growing faster (5, 247-251) than follicles from older ovaries, which are located in the cortex. These differences in location of growth may be affecting the developmental dynamics of follicles we observed.

Furthermore, in Controls, the GCs of primary follicles from 8-day ovaries are less compact than primary follicles from the other older ages, which may be accredited to differences in the composition of ovarian stroma. Neonatal ovarian stroma is composed of fibroblast-like cells and undifferentiated stroma before the interstitium develops at P11 (252) and perhaps is more permissive of follicle growth resulting in the less compact GCs observed in 8-day ovaries.

A vital difference between 8-day ovaries and the older ages is the lack of larger developing follicles. Since these larger follicles are present in 3-week, 6-week and 3-month ovaries, there is an upregulation of extracellular matrix components, such as laminin and collagen around these larger follicles (64) which could physically restrict growth of the primary follicles as it is known that dominant growing follicles inhibit neighbouring follicles (253). Furthermore, these large follicles secrete hormones such as AMH, which is known to inhibit the growth of smaller follicles and FSH-induced follicle growth (127, 254, 255) and could ultimately result restrict follicle growth in these ovaries.

Another difference between the younger (8-day) and older mice which may be impacting GCs, is the neonatal gonadotrophin surge which occurs in mice during the first 2 weeks post-birth before declining to adult levels (248, 256-261). GCs from neonatal ovaries are reported to have large intercellular spaces and well defined round nuclei (261), as observed in 8-day ovaries. GCs of mice deprived of endogenous gonadotrophins, by administration of rabbit anti-rat gonadotrophins, have reduced intercellular spaces and irregular shaped nuclei (261) similar to our

observations of older ovaries (3-weeks, 6-weeks and 3-month). The authors speculated (261) that gonadotropin deficiency resulted in increased intracellular pressure, since GC membranes were in constant contact with neighbouring GCs and the basal lamina, resulting in more compact cells. Additionally, a decline in GC proliferation as GCs reach a maximal packing density has been observed, suggesting that a degree of contact inhibition occurs in GCs on the basal lamina (250). Since administration of FSH to gonadotropin deprived mice was able to partially reverse effects of gonadotropin deficiency (261), this indicates that FSH plays a role in the integrity of the GC membrane. Therefore, we speculate the gonadotropin surge in neonatal mice stimulates the advanced follicle growth observed in 8-day ovaries.

Moving on to compare DM to Controls, in addition to the presence of larger follicles in 8-day DM ovaries, further differences were observed when comparing follicles from 3- and 6-week Control and DM ovaries. When compared to Controls, DM ovaries at 3-weeks contained abnormally large primary 3a follicles and oocytes, accompanied by accelerated development of a second GC layer and increased GC proliferation. It has recently been shown that the absence of O-glycans in oocytes altered follicle development with follicles showing higher sensitivity to FSH and reduced apoptosis (2, 234). Therefore, it is plausible the DM follicles are more sensitive to FSH mediated GC proliferation, which results in the precocious follicle growth observed.

Contrary to 3-weeks, 6-week DM primary 3a follicles contain larger oocytes, accompanied by decreased GC proliferation when compared to Controls. Since

the main difference between 3- and 6-week DM ovaries is the presence of CL, we believe these may be playing a role. We have previously reported an accumulation of CL in 6-week DM ovaries due to a delayed CL regression (236, 244). The presence of increased CL in DM ovaries at 6-weeks of age may be preventing GC proliferation by physically restricting expansion since CL exert anti-folliculogenic effects on neighbouring follicles (262). Furthermore, secretion of progesterone by CL inhibits early follicle growth by suppressing the FSH-mediated proliferation of GCs (263-266), which may be preventing DM GCs from proliferating whilst the oocyte continues to grow, resulting in the discrepancy observed.

As GCs play a vital role in oocyte viability by maintaining prophase I arrest (267), therefore the changes in GC proliferation observed in DM ovaries at 3- and 6-weeks, may affect meiotic progression of oocytes and hence prevent these follicles from further development in DM ovaries. Although various studies have reported that GC proliferation is not directly associated with oocyte growth (5, 250, 268, 269), the reciprocal signalling between oocyte and GCs is required for normal follicle development to occur (23) and therefore any disruption will affect follicle development potentially leading to the block in follicle development as observed in DM ovaries. Furthermore, at both age's abnormalities in DM follicles exist only at the 3a stage, suggesting that follicles that get through this block are normal.

Contrary to previous reports that theca cells first appear at the secondary follicle stage (reviewed by Young and McNeilly (32)), we confirm the presence of theca cells in primary follicles, by morphological analysis. Furthermore, we confirm the localization of HA in the ECM of theca cells surrounding some primary follicles

(39, 270, 271). During follicular growth, since angiogenesis is restricted to the theca cell layer and the presence of HA is known to have anti-angiogenic activity (272, 273), we postulate the increased HA in the DM theca ECM may restrict the nutrients available to the follicle as well as being a source of rigidity that constrains follicle growth and theca cell proliferation, resulting in a reduced theca cell layer in developing follicles as previously reported (244). Furthermore, in PCOS ovaries, where there is an upregulation of ECM components, a phenomenon termed 'stockpiling' ensues which results in a 6-fold increase in primary follicles (274, 275).

Our results demonstrate that presence of morphologically abnormal follicles in DM ovaries are causing the observed block in follicle development; however, there were a number of limitations. To avoid any bias, during the experiments and analysis, the investigator was blind to the genotype of the mice, which were called group 1 and group 2. In addition, the number of mice used per age group were low (n=3-4), however the mice used in both groups (Control and DM) were littermates from 3-4 different litters, which ensures we have a representative sample. Furthermore, ovaries from 6-week and 3-month females were collected post-mating and therefore there are no variations due to different timings in oestrous cycle.

For IHC experiments, since all experiments were not conducted at the same time, there may be variation in the intensity of staining between experiments. This was controlled for within each experiment, since DAB exposure time was kept consistent and each experiment was repeated a minimum of three times and

contained an equal number of slides from all mice. Since Ki67 staining intensity was visually quantified, this has limitations due to lack of uniformity and consistency in quantification. However, it has been demonstrated that this manual method is the gold standard in assessing the Ki67 proliferative index of a number of tumours including gastroenteropancreatic neuroendocrine tumours (276). To limit variation in future studies, the use of software such as Image J could be adopted to quantify the intensity of DAB staining per cell as a percentage, which will be less subjective.

In conclusion, the results of this chapter demonstrate that (i) the first cohort of activated follicles, in both Control and DM ovaries, are larger in size, perhaps reflecting the functional difference of this cohort of follicles and that (ii) there are differences in follicle dynamics in the prepubertal and postpubertal DM ovaries compared to Control. Precocious follicle growth is observed in 3-week ovaries with follicles developing a second GC layer earlier. At 6-weeks, DM ovaries contain abnormally large primary 3a follicles accompanied with decreased GC proliferation but increased proportion of HA positive theca. These findings support our hypothesis that the presence of morphologically abnormal follicles may be responsible for the accumulation of primary follicles, resulting in a block of follicle development and ultimately POI. It remains to be determined whether follicles that develop pass the follicle development block in DM ovary are morphologically and functionally normal since only primary follicles were assessed in this chapter.

**Characterisation of follicle development
during the onset of Premature Ovarian
Insufficiency**

4.1 Introduction

There is a clearly defined onset of POI in the DM mouse model; females are subfertile at 6-weeks, infertile at 9-weeks and undergo POI by 3-months of age (1, 236). This drop in fertility of DM females is accompanied by a dysregulation of follicle development and an altered endocrine profile at 3-months (1). Although DM females have decreased fertility at 6-weeks, they have a normal ovulation rate; however, DM ovaries contain a larger and more heterogeneous population of CL (236). By 3-months of age, DM ovaries lack developing follicles and abnormal luteinised structures are present (1). In the previous chapter, morphometric analysis of primary follicles revealed the presence of abnormal follicles, which may be causing the accumulation of primary follicles.

As DM females age, there is a demise in ovarian function, indicating the absence of oocyte-specific *N*- and *O*-glycans is affecting follicle development in an age-dependant manner. Indeed, the absence of oocyte-specific Core 1 *O*-glycans results in changes in the follicle structure such as the theca cell layer (1). Therefore we hypothesise that the absence of oocyte-specific *N*- and *O*-glycans is inducing morphological and functional changes in DM follicles.

4.2 Aim

- To characterise follicle development in DM females when they transition from a subfertile state at 6-weeks to an infertile state at 9-weeks.

To address this, we used ovary sections from 6- and 9-week old mice and analysed follicle numbers, apoptosis using the TUNEL assay, and levels of laminin and 3 β -hydroxysteroid dehydrogenase using immunohistochemistry.

4.3 Materials and Methods

4.3.1 Mice

Female mice with floxed *C1galt1* and *Mgat1* alleles (*C1galt1*^{F/F} *Mgat1*^{F/F}) were Controls and mice also carrying a Zp3Cre recombinase transgene were experimental DM (*C1galt1*^{F/F} *Mgat1*^{F/F}:Zp3Cre) (1, 226).

4.3.2 Ovarian histology and follicle counts

To ensure ovaries were collected from mice at the same stage of the oestrous cycle, females were joined with males at 6- or 9-weeks of age and ovaries collected on the day after mating as determined by a vaginal plug. Ovaries were collected from females that mated within 7 days of joining to ensure the age group was contained to 6-7 and 9-10 weeks. Ovaries were weighed, fixed in 10% buffered formalin (Sigma-Aldrich) for 8 h and fixed, paraffin embedded and sectioned (Section 2.5).

To determine follicle numbers, every 10th serial section was stained with H&E for analysis (Section 2.7). Only morphologically healthy follicles with a central oocyte and a visible nucleus were assessed. Follicles were classified as follows (Section 1.1.1.1): primary (3a and 3b) secondary (type 4), preantral (5a and 5b) and antral (including 5a+A, 5b+A (which develop an early antrum (A)), 6 and 7). The number of luteinising unruptured follicles (LUFs) were also recorded. LUFs are large antral follicles that contain an oocyte and show signs of GC luteinisation.

4.3.3 Immunohistochemistry

Immunohistochemistry (IHC) was performed on 5 μm paraffin embedded sections to detect laminin and 3β -hydroxysteroid dehydrogenase (3β HSD). Sections were dewaxed with xylene and rehydrated using decreasing concentrations of ethanol in ddH₂O. Slides were washed in Tris Buffered Saline (TBS: 0.1M Tris pH 7.5 and 0.3M NaCl) with 0.05% Tween-20 (TBST). Endogenous peroxidase was quenched using 3% H₂O₂ (Fisher Scientific) in PBS for 5 min. Non-specific primary antibody binding was blocked using goat serum in TBS (NGS: Vectastain ABC Elite kit, Vector Laboratories). Primary antibodies were diluted in the blocking solution and incubated overnight at 4°C. The rabbit anti-laminin (L9393; Sigma-Aldrich) was used at 1:500 and anti- 3β HSD (generously donated by Professor Ian Mason from the University of Edinburgh) was used at 1:2000 dilution; blocking solution was used as a negative control. After 3 washes with TBST, sections were incubated with biotinylated anti-rabbit IgG secondary antibody (Vectastain ABC Elite Kit) for 1 h, followed by ABC solution (Vectastain ABC Elite Kit) for 30 min. A 3,3'-Diaminobenzidine peroxidase substrate kit (Vector Labs) was used to visualise localisation. The slides were counterstained with haematoxylin, dehydrated, mounted with DEPEX (VWR, Leicestershire, UK) and imaged. Follicles were classified as positive or negative for the presence of 3β HSD in GCs, whilst laminin detection was classified as low or high. The number of theca cell layers was counted and the depth of theca layer measured using Image J (National Institutes of Health, Bethesda, Maryland, USA).

4.3.4 TUNEL assay

Apoptosis of follicles was detected on formalin fixed ovarian sections from 6- and 9-week Control and DM females using the TUNEL assay (Apoptag kit; Merck Millipore Watford, Hertfordshire). Sections were dewaxed using xylenes and rehydrated in increasing concentrations of ethanols. Sections were then incubated with 20 µg/ml of Proteinase K (Roche Diagnostics, Welwyn Garden City, Hertfordshire, United Kingdom) for 15 min at room temperature followed by 3% H₂O₂ (Thermo Fisher Scientific) in PBS for 5 min to block endogenous peroxidase. Following this, sections were incubated with equilibration buffer for 10 min at room temperature before incubation with 30% terminal deoxynucleotidyl transferase enzyme in reaction buffer for 1 h at 37°C. Control sections were incubated with reaction buffer only. Sections were then incubated with stop buffer for 10 min at room temperature, followed by 30 min incubation at room temperature with anti-digoxigenin conjugate. Using the 3,3'-Diaminobenzidine peroxidase substrate kit, antigen-specific localization was revealed. The slides were dehydrated, mounted with Depex, and imaged.

Only follicles sectioned through the oocyte were analysed and follicles were assessed as being either healthy, containing low or high levels of apoptosis, or dead. Healthy follicles had no apoptotic cells and a healthy oocyte. Follicles classified with low levels of apoptosis had some apoptotic cells and a healthy oocyte. Follicles classified with high levels of apoptosis had numerous apoptotic cells but an intact follicle structure. Follicles classified as dead or dying follicles had lost their follicular structure and/or oocyte structure and had large amounts of apoptosis in GCs and oocyte blebbing.

4.3.5 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism software (GraphPad Software, Inc., version 4.0b, 2004). D'Agostino & Pearson normality test or Shapiro-Wilk normality test was applied to test Gaussian (normal) distribution of the samples. The Mann–Whitney U test was performed to detect differences between groups with non-parametrical distribution and equal variances. Unpaired T test was used to analyse samples with normal distribution. A one-way ANOVA followed by multiple comparisons test was used to analyse the mean number of follicles per ovary. Results are presented as mean±SD/SEM. $P \leq 0.05$ was considered significant.

4.3.6 Disclaimer

For this chapter, the experiments were conducted by MSc and FHS students (detailed below) whilst I reanalysed the results and generated the data. The following students in Dr. Williams's lab conducted experiments; Dr. Nicole Krzys (FHS student) assessed follicle development and conducted 3 β HSD IHC experiment. Dr. Sabah Janjua (FHS student) did basal lamina and theca cell analysis, and laminin IHC. Dr. Pamela Nawaggi (MSc student) carried out the TUNEL assay.

The results of this chapter have been published in collaboration with Dr. Patricia Grasa, Dr. Nicole Krzys, Dr. Sabah Janjua and Dr. Pamela Nawaggi (4).

4.4 Results

4.4.1 Follicular development in DM post-pubertal females is altered

As reported previously, DM females undergo POI by 3-months of age and this is accompanied by a decrease in the number of developing follicles from the secondary stage onwards (1). Therefore we first wanted to investigate the onset of ovarian failure defined by a decline in follicle numbers. Follicle development was evaluated (in ovaries collected post-mating to ensure cycle synchronization) at two time points, when females were subfertile at 6-weeks (Control 6.5 ± 0.1 weeks; $n=3$, DM 6.4 ± 0.1 weeks; $n=3$) and infertile at 9-weeks (Control 9.4 ± 0.0 weeks; $n=3$, DM 9.4 ± 0.1 weeks; $n=3$) (hereafter referred to as 6-weeks and 9-weeks) (Figure 4.1).

First, morphological analysis of Control ovaries at both 6- and 9-weeks demonstrated the presence of many follicles including antral follicles, however DM ovaries, at both ages, predominantly exhibited early stage follicles and numerous corpus luteum (CL) (Figure 4.1A). Classification of follicles into the stages revealed significantly more primary follicles in the DM ovaries compared to Controls at both ages (Figure 4.1B). Further detailed analysis of follicles, by classifying into the sub-groups revealed DM ovaries contained more 3a follicles than Controls, with an increase of 116% at 6-weeks and 140% at 9-weeks (Fig. 1C), consistent with the previously reported findings at 3 months (1).

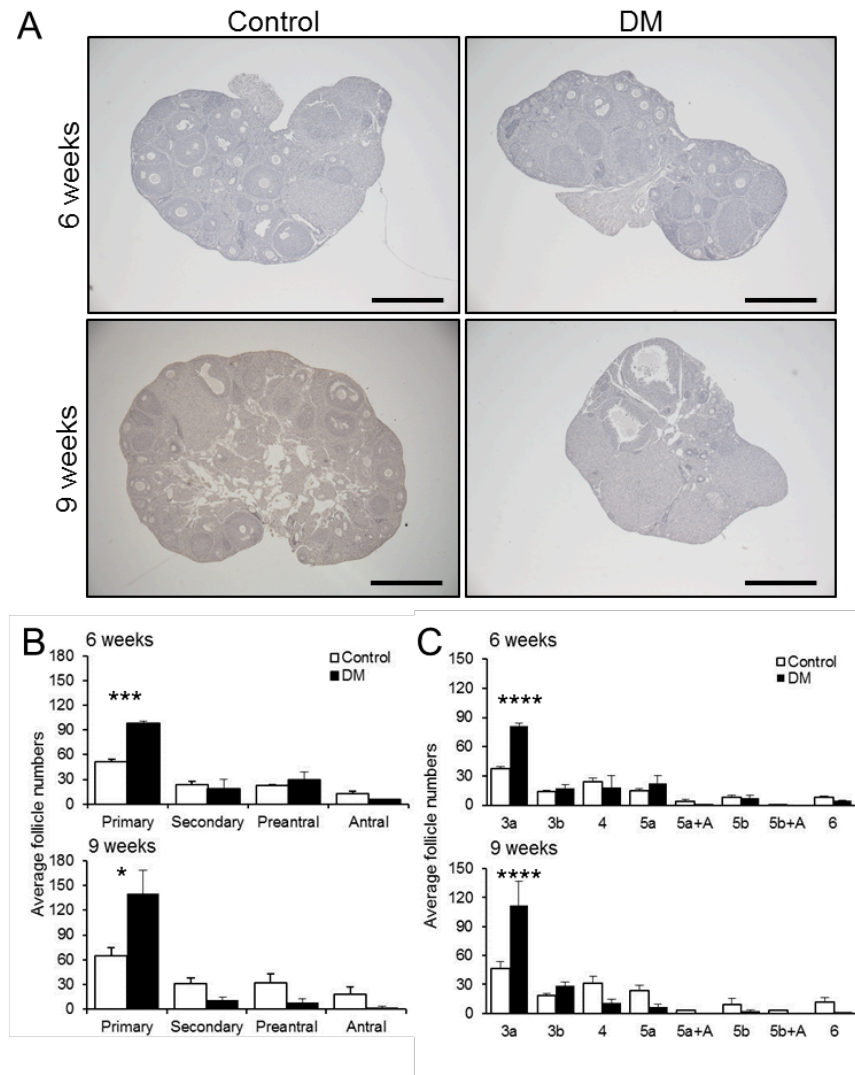


Figure 4.1 Assessment of follicle development in 6- and 9-week old mouse ovaries. (A) Ovarian morphology at 6- and 9-weeks revealed the presence of all types of follicles and CL in Control ovaries whereas DM ovaries mainly display the presence of early follicles and CL with very few developing follicles. Scale bar 500µm. (B) Average number of follicles at the primary, secondary, preantral and antral stages of development from 6- and 9-week old Control (n=3 mice) and DM (n=3 mice) ovaries collected the day after ovulation. (C) Average number of follicles at each discrete stage of development (3a, 3b, 4, 5a, 5a+A, 5b, 5b+A and 6 as per Grasa et al. (2)) from 6- and 9-week old Control (n=3 mice) and DM (n=3 mice) ovaries collected the day after ovulation. (B-C) Results are expressed as mean±SEM. * $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Figures from publication (4).

4.4.2 DM ovaries at 9-weeks contain a higher proportion of healthier follicles

Apoptosis was assessed in follicles from 6- and 9-week ovaries by TUNEL analysis and follicles were classified as either being healthy, having low or high levels of apoptosis, or dying/dead (Figure 4.2A). Although no differences in the proportion of Control versus DM follicles in each of these categories was observed at 6-weeks, at 9-weeks of age, of the few follicles that were present in DM ovaries, a higher proportion of these follicles were healthier compared to Controls (Figure 4.2B).

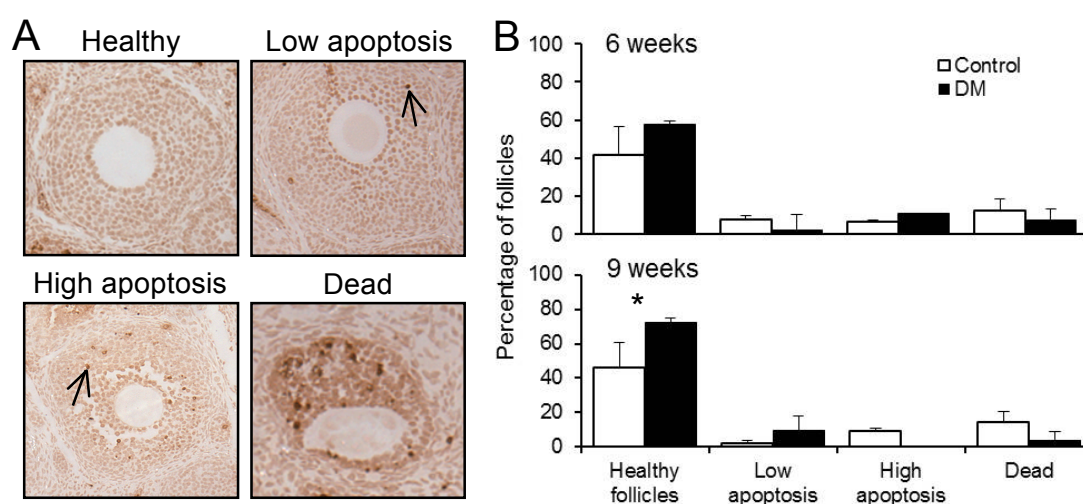


Figure 4.2 Assessment of apoptosis follicles from 6- and 9-week old mouse ovaries.

(A) Representative images of TUNEL staining scale used to assess levels of apoptosis in follicles. Arrow shows apoptotic cells. (B) Levels of apoptosis detected in follicles at 6-weeks of age and (Controls $n=96$ follicles; $n=3$ mice, DM $n=90$ follicles; $n=3$ mice) 9-weeks of age (Controls $n=116$ follicles; $n=3$ mice, DM $n=34$ follicles; $n=3$ mice). Results are expressed as mean $\pm$ SD. * $P\leq 0.05$. Figures from publication (4).

4.4.3 DM follicles have a modified basal lamina

Next, to determine if the follicles present in DM ovaries at 9-weeks of age were morphologically healthy, first we assessed the integrity of the basal lamina (BL) (Figure 4.3). Follicles were classified as having a BL that was less than 50% ($\leq 50\%$) defined, where the BL was too thin to identify or was indistinguishable from the surrounding stroma, or more than 50% defined ($>50\%$) defined, where the BL was clearly seen surrounding the majority of the follicle (Figure 4.3A). Compared to Control follicles, a higher proportion of DM follicles had $\leq 50\%$ defined BL at follicle stage 4 and 5a (Control $n=11$ follicles; $n=3$ mice, DM $n=9$ follicles; $n=3$ mice) (Figure 4.3B).

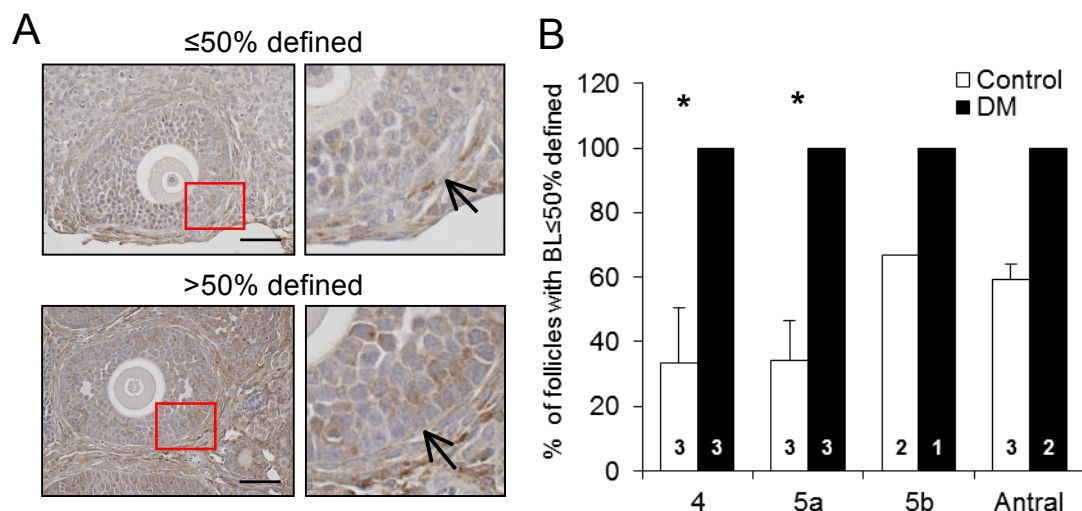


Figure 4.3 Analysis of basal lamina. (A) Representative images BL was classified as either $\leq 50\%$ or $>50\%$ BL defined. Follicles classified as $\leq 50\%$ defined have large regions of the follicle where the BL is undistinguishable from surrounding GC and theca cells whereas follicles in classified as $>50\%$ defined BL surrounding the majority of the follicle. The area in the red box is enlarged to the right showing $\leq 50\%$ defined or $>50\%$ defined BL. (B) Proportion of Control and DM follicles at 9-weeks of age (Control $n=11$ follicles; $n=3$ mice, DM $n=9$ follicles; $n=3$ mice) classified as having less than or equal to 50% or more than 50% BL defined. Results are expressed as mean $\pm$ SEM. Numbers in columns represent number of follicles. * $P \leq 0.05$. Figures from publication (4).

4.4.4 DM follicles have a decreased number and depth of theca cell layers

Next, morphology of the theca cell layer was assessed. Analyses revealed that DM follicles at the secondary stage of development have a decreased theca depth compared to Control follicles (Figure 4.4A), which is attributed to a reduced number of theca cell layers compared to Control follicles (Control n=106 follicles; n=3 mice, DM n=28 follicles; n=3 mice) (Figure 4.4B).

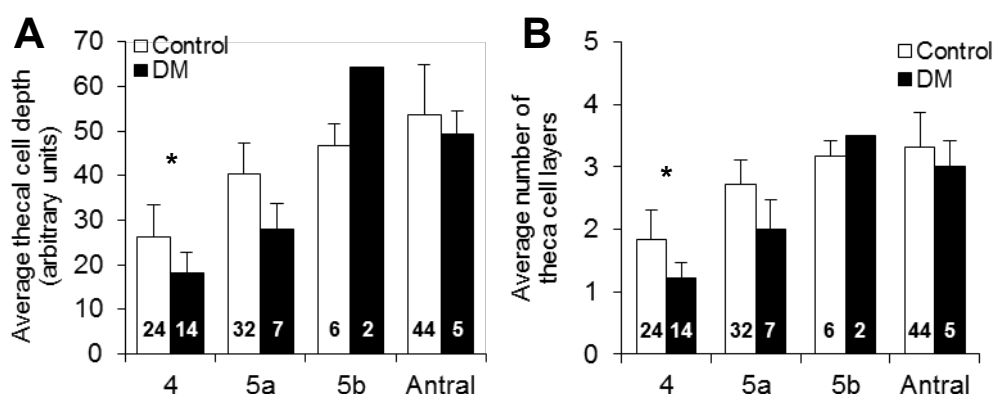


Figure 4.4 Analysis theca cell layer. (A-B) The theca cell depth and the number of theca cell layers are both reduced in DM follicles compared to Controls at 9-weeks of age (Control n=106 follicles; n=3 mice, DM n=28 follicles; n=3 mice). Results are expressed as mean±SEM. Numbers in columns represent number of follicles. * $P \leq 0.05$. Figures adapted from publication (4).

4.4.5 DM follicular theca cell layer has increased laminin content

Finally, extracellular (ECM) laminin content within the thecal compartment was analysed by immunohistochemistry and categorised as 'high', where intense staining of laminin was detected around the entire BL, or 'low' content, where there was faint or no visible staining. The percentage of DM follicles with high laminin content was higher at the secondary stage when compared to Controls

and levels did not drop until the antral stage whereas Control follicles exhibited a gradual increase in laminin content during development (Figure 4.5A).

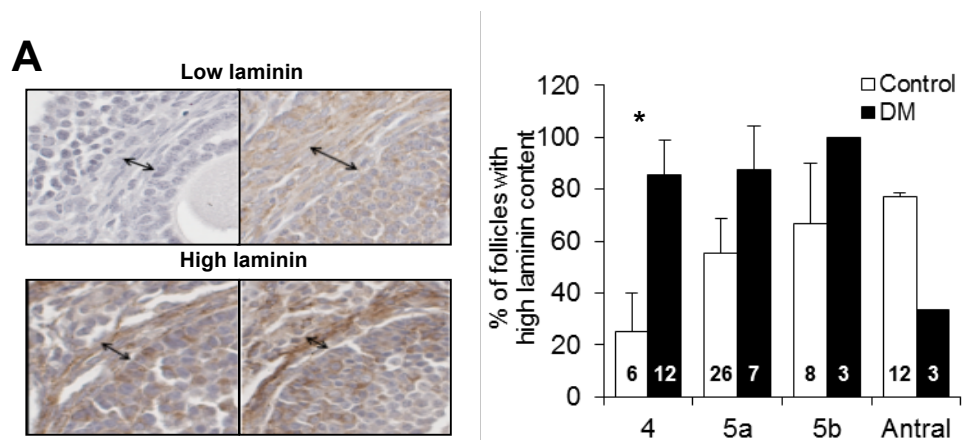


Figure 4.5 Analysis of theca cell laminin by immunohistochemistry. (A) Laminin detection in ovary sections using immunohistochemistry revealed DM follicles at the secondary stage (stage 4) have a higher laminin content in the theca compartment than Controls at 9-weeks of age. Results are expressed as mean $\pm$ SEM. Numbers in columns represent number of follicles. * $P\leq 0.05$. Figures adapted from publication (4).

4.4.6 DM follicles have equivalent 3 β HSD expression to Control follicles

To assess steroidogenic function, changes in the levels of 3 β HSD, an enzyme involved in the synthesis of progesterone, were assessed using IHC. Follicles were classified as either positive or negative for detection of 3 β HSD in the GCs (Figure 4.6A). Positive 3 β HSD follicles were present in Control and DM ovaries at both 6- and 9-weeks of age, however no differences in the proportion of positive 3 β HSD follicles between DM and Control ovaries were found (6-weeks: Control n=23 follicles; n=3, DM n=24 follicles; n=3 and 9-weeks: Control n=70 follicles; n=3, DM n=15 follicles; n=3) (Figure 4.6B).

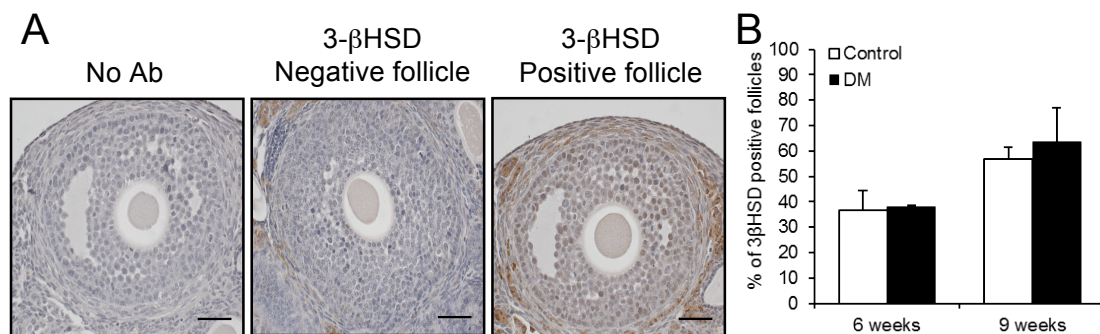


Figure 4.6 3 β HSD detection in Control and DM ovarian follicles using IHC. (A) Follicles not incubated with antibody (No Ab), follicles incubated with 3 β HSD antibody and were either negative for 3 β HSD (3 β HSD negative follicle) or 3 β HSD was detected (3 β HSD positive follicle) at 6- and 9-weeks of age (scale bar=50 μ m). (B) Proportion of follicles in Control and DM ovaries either positive or negative for 3 β HSD detection. (Control n=3, DM n=3). Results are expressed as mean $\pm$ SEM. Figures adapted from publication (4).

4.4.7 The decrease in ovulation rate in DM ovaries at 9-weeks post-mating is due to premature luteinisation of preovulatory follicles

During 3β HSD analysis, the presence of large unruptured preovulatory follicles was detected in both Control and DM ovaries. Since ovaries were collected post-mating, we did not expect any preovulatory follicles since they should have ovulated. These large unruptured preovulatory follicles had GCs staining strongly positive for 3β HSD and thus were classified as luteinised unruptured follicles (LUFs) (Figure 4.7A). Interestingly, the oocytes of some DM LUFs lacked cumulus cells (Figure 4.7A1 and 2), though LUFs with cumulus cells were also present (Figure 4.7A3).

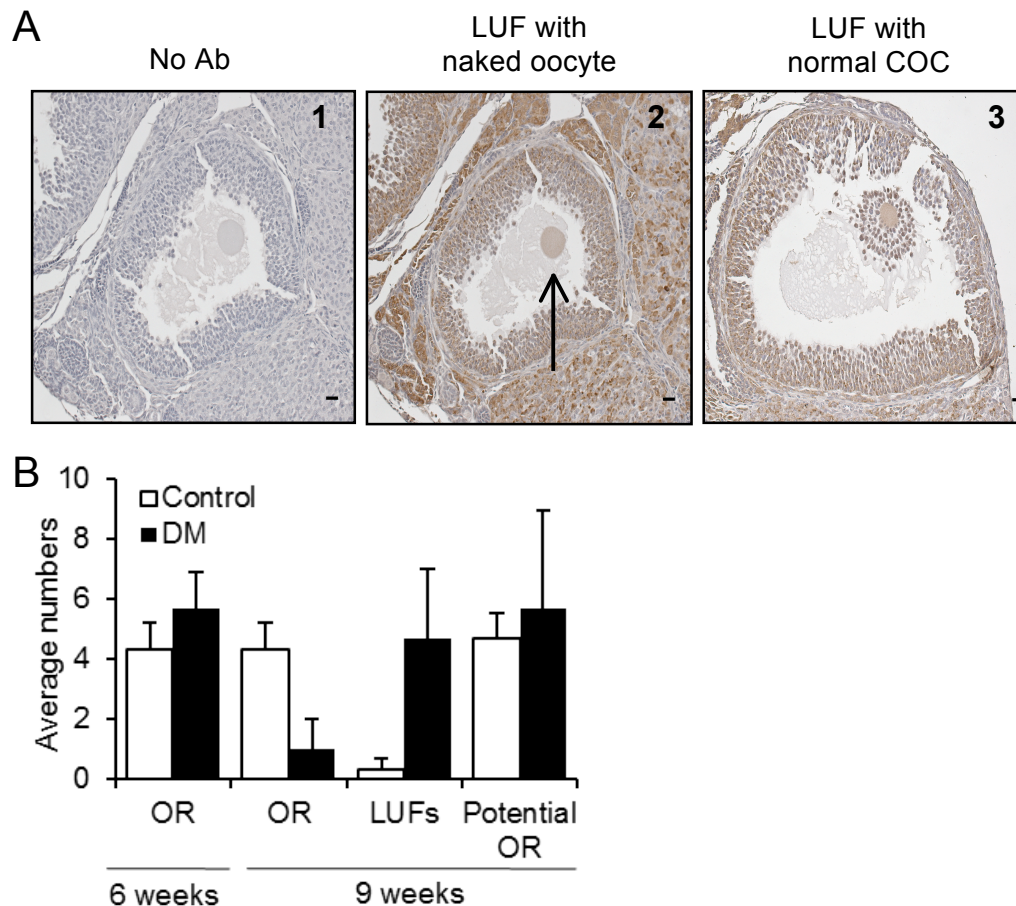


Figure 4.7 Detection of LUFs through 3β HSD detection using IHC in Control and DM ovaries. (A1-3) Luteinised unruptured follicles (LUFs) in Control and DM ovaries at 9-weeks of age. Scale bars correspond to 100 μ m. (A1) Follicles not incubated with antibody. (A2) Arrow shows an oocyte free of cumulus cells in a LUF. (A3) A cumulus oocyte complex (COC) within a LUF. (B) To assess if LUFs accounted for the previously reported drop in ovulation rate (OR) in DM between 6- and 9-weeks of age, the right ovary OR for Control and DM ovaries at 6- and 9-weeks of age was assessed by number of eggs collected from the right ovary. The OR at 9-weeks was added to the number of LUFs present in the right ovary at 9-weeks to calculate the 'Potential OR' (Control n=3 mice, DM n=3 mice). Results are expressed as mean $\pm$ SEM. Figures from publication (4).

Next, we wanted to determine if premature luteinisation of preovulatory follicles was the cause for previously observed decreased ovulation in DM females at 9-weeks. To do so, first we examined the ovulation rate of the right ovary (determined by the number of eggs collected from the right oviduct) and the numbers of LUFs present in the right ovary (counted by histological analysis). The number of LUFs in Control ovaries was very low, with less than one found on average, however, in DM ovaries, >4 on average were present (Figure 4.7B). To compare ovulation rate, we also determined ovulation rate at 6-weeks of age. The ovulation rate at 6-weeks did not differ between DM (5.67 ± 2.08 eggs ovulated; $n=3$ mice) and Controls (4.33 ± 1.53 eggs ovulated; $n=3$ mice) (Figure 4.7B); overall average ovulation rate per mouse did not differ between Controls and DM (both ovaries: DM 8.00 ± 3.00 ; $n=3$ Control 8.33 ± 0.58 ; $n=3$), consistent with previous findings (236). However, at 9-weeks of age, although the eggs ovulated from the right ovary for Controls (4.33 ± 1.53 eggs ovulated) was consistent with right ovary ovulation rate for Controls at 6-weeks of age (4.33 ± 1.53 eggs ovulated), the DM right ovary ovulation rate had declined dramatically (1.00 ± 1.73 eggs ovulated) (both ovaries: DM 1.0 ± 1.73 eggs ovulated; $n=3$ mice Control 9.00 ± 2.65 eggs ovulated; $n=3$ mice).

Next, a 'potential ovulation rate' was calculated, which was determined by adding the number of eggs collected from the right oviduct and the number of LUFs present in the right ovary (assessed by histologically analysis) (Figure 4.7B). The 'potential ovulation rate' calculated for DM females at 9-weeks of age matched that of DM females at 6-weeks of age, suggesting the decrease in

ovulation rate at 9-weeks of age may be due to premature luteinisation of preovulatory follicles.

4.5 Discussion

Although several factors have been associated with POI, the mechanisms that cause ovarian dysfunction are poorly understood. Since DM females exhibit a rapid deterioration of ovarian function from a subfertile state at 6-weeks to being infertile at 9-weeks and exhibiting POI at 3 months of age (1, 236), this provides us with the opportunity to investigate how the structure and function of follicles is modified during the onset of POI with the aim of elucidating the mechanism(s) responsible for the reproductive dysfunction.

Follicle counts at both 6- and 9-weeks of age revealed increased numbers of primary follicles, which can be attributed to either an over activation of primordial follicles or a block in follicle development. As the increase in primary follicle number does not correspond with an increase in the number of developing follicles, this suggests that the disruption of glycans impacts either growth of follicles or their progression to advanced FSH-dependent stages.

We hypothesised that the absence of oocyte-specific *N*- and *O*-glycans is inducing morphological and functional changes in DM follicles resulting in the age-dependant demise of ovarian function. Our findings support this hypothesis since ovaries from prepubertal DM mice at 3 weeks of age are grossly normal and contain follicles at all stages of development, whilst postpubertal ovaries have a dysregulation of follicle development. Recently, it has been identified that two waves of follicle activation exist within the mouse ovary, the first (prepubertal) and second (postpubertal) waves (251, 277, 278). A recent study (251) has shown this first wave of follicles are responsible for the induction of puberty, the establishment

of the hypothalamic-gonadal axis and are ovulated, thus contributing to early fertility. These are progressively replaced by follicles from the second wave, which last through the remaining reproductive lifespan. The decline in DM ovarian function is temporally similar to the demise of the first wave of follicle development. In the DM mouse, some follicles grow and ovulate in early adulthood, when the first wave of follicle development contribute to fertility, however at 3-months of age, when only the second wave of follicle development exists in the ovary, DM females undergo POI. Hence we postulate that in DM females there are defects in the post-pubertal wave of follicle development, which is driving the POI phenotype. Unfortunately, we have been unable to determine a way to test this hypothesis at the present time since the reporter mouse used to reveal these two waves utilises floxed genes and a *Cre* recombinase and since our DM mice contain floxed genes and a *Cre* recombinase, the reporter system would no longer function as designed if the two transgenics were combined.

Ovaries from 9-week DM females contained a higher proportion of healthier follicles compared to Controls. This potentially indicates that follicles that pass the developmental 'block' are more likely to survive and may be linked to the altered endocrine profile of DM in particular the increased FSH levels (1). FSH has been reported to promote survival of developing follicles (279), therefore the increased FSH levels in DM females, due to decreased ovarian negative feedback, may play a role in promoting follicle survival. Oocyte-specific factors are also known to play a role in apoptosis and we have recently shown that female mice generating oocytes devoid of O-glycans have a higher expression ratio of GDF-9:BMP-15 accompanied with reduced apoptosis (2, 234). Furthermore, mutations in GDF-9

and BMP-15 have been reported in some women with POI and are believed to play a role in ovary dysfunction (139, 140) and hence we propose the high FSH levels and possible alterations in GDF-9 and BMP-15 could be promoting DM follicle survival.

Next, we assessed the structure of the surviving follicles to determine if these were morphologically normal. Secondary DM follicles had a thinner theca cell layer and an indistinct BL. At the secondary follicle stage, theca cells are recruited from the stroma and produce testosterone which is crucial for the growth and development of the follicles (32). In chapter 1, we demonstrate that there is no difference in theca cell number at the primary stage between Control and DM, indicating their recruitment to the follicle is not affected. Therefore, a reduced theca cell layer at the secondary stage indicates a problem with proliferation of theca cells, which could in turn affect adequate follicle development and in combination with the decreased number of developing follicles found in DM ovaries could explain the low serum levels of testosterone found at 3 months of age (1).

Previously, we have shown that the lack of Core 1 O-glycans on oocyte glycoproteins regulate the expression of ECM proteins and also the form and function of the BL (57). As discussed in section 1.1.2.1, the BL regulates follicle growth by controlling entry and exit of molecules into the follicles, therefore the changes in the structure of the BL we observe in DM follicles may alter the traffic of regulatory molecules that are required for follicle remodelling and for the release of the oocyte at ovulation. The ECM protein laminin has been reported to

play a role in luteinisation however the reports are conflicting. Rat GCs cultured with laminin were stimulated to produce progesterone and exhibited luteinising effects (280). However, when human GCs were cultured with laminin, progesterone synthesis was suppressed (281). Thus, alterations in laminin levels and structure of the BL may prevent ovulation of the oocyte from preovulatory follicles, resulting in their premature luteinisation at 9-weeks of age.

Successful ovulation implies meiotic resumption and release of the oocyte, and the structural remodelling and luteinisation of the follicle. These processes are regulated by complex signalling between the somatic cells and the oocyte in response to a properly timed LH surge (83, 282, 283). Alterations in signalling, as a consequence of modified structure/function of oocyte-glycoproteins and their paracrine interactions, would result in premature luteinisation and ovulation would likely be compromised as we observe in DM ovaries and has been reported in karyotypically normal women with POI (184). Previously we have reported that 9-week DM females are infertile with scarce ovulations (236). In this study, DM ovaries collected after ovulation contain LUFs, suggesting the absence of both O- and N-glycans results in defects in preovulatory follicles resulting in a failure to ovulate. The exact mechanism by which preovulatory follicles fail to ovulate is unclear, however, evidence that the high circulating FSH levels in DM females may be inducing luteinisation comes from an *in vivo* study by van de Lagemaat *et al.* (284), who demonstrated oral administration of short-acting FSHR agonist induced luteinisation of preovulatory follicles in various female animals. It has been suggested the FSHR agonist mimics the rise of cAMP in GCs that is seen following the LH surge (285) and thus induce a suboptimal ovulation that results in

the generation of a LUF. Indeed, it has been established that the actions of FSH are mediated through secondary messengers including cAMP (286, 287) and therefore we speculate that the high circulating FSH levels in DM are responsible for the ovulation failure, resulting the production of LUFs.

The results from this chapter present data that may improve our understanding of the biology of POI. However, the study has a number of possible limitations to consider. Foremost, the number of mice used per age group was low (n=3); to control for this, each group (Control and DM) contained a littermate from 3 different litters, which ensures we have a representative sample. Furthermore, to avoid any bias during the experiments and analysis the investigator was blind to the genotype of the mice. Also, to limit variations due to differences in timings in oestrous cycle, ovaries from 6-week and 3-month females were collected post-mating which ensures all mice are at the same stage of the cycle. Once again for IHC experiments, the staining intensity was visually quantified; therefore this approach has the limitation of being semi-quantitative. Since analysis was performed at the same time, variations are equally represented in both groups however, to validate our findings future experiments could adopt the use of software such as Image J, which will accurately and consistently quantify staining.

In summary, the results of this chapter support our hypothesis that the lack of complex *N*- and *O*-glycans results in multiple defects in follicle development. These defects are leading to the premature luteinisation of preovulatory follicles and as a result sub-optimal ovulation and infertility occur in DM females at 9-weeks of age. Although rodent ovarian function and luteal control is different to

women, many of our reported findings have already been observed in women suffering from POI. Thus, identifying these follicular modifications have provided insight into the mechanisms driving the development of POI and highlight the opportunity to develop new fertility treatments that would restore ovarian function.

**Analysis of *in vitro* follicle development
during the onset of Premature Ovarian
Insufficiency**

5.1 Introduction

It is believed that 50% of women with POI have the follicular form (184, 185), this indicates that significant numbers of women could benefit from therapies focusing on reactivation of follicle development.

Ovarian function is normal in DM females since they undergo puberty and are initially fertile at 6-weeks (1); however females become infertile by 9-weeks and undergo POI by 3-months of age (1, 236). Since the genetic modification is oocyte-specific, it is conceivable that follicle development is compromised by extra-follicular factors. DM ovaries contain a larger and more heterogeneous population of CL, which may be affecting follicle development since CL exert anti-folliculogenic effects on neighbouring follicles. Therefore, if intra-follicular mechanisms are still intact in DM follicles after the onset of POI, it is likely that follicle growth could resume if the extra-follicular blocking signalling is removed. Furthermore, the DM mouse presents a POI phenotype comparable with follicular POI displayed by women and therefore provides a useful model to study POI follicle development *in vitro*.

DM ovaries contain more 3a follicles as characterised in Chapter 3; however they also contain developing follicles characterised in Chapter 4. In the previous chapters we observe structural differences in the ECM surrounding DM follicles compared to Controls. Therefore, we hypothesise that the increased ECM surrounding DM follicles is restricting follicle growth and development to the advanced stages.

5.2 Aims

- To evaluate if DM follicle development can be normalised under controlled *in vitro* conditions

To address this, we dissected preantral follicles and cultured these *in vitro* for 6-days. Follicle culture has resulted in live births in mice (288-290), and is a powerful tool that allows us to study the dynamics of follicle growth in real time. Utilising this technique will allow us to study the rate of DM follicle development compared to Controls, since follicles are at the same stage at the start of culture. Furthermore this will allow us to observe how DM follicles grow in a hormonally controlled environment compared to Controls, free of any external stimuli.

5.3 Material and methods

5.3.1 Mice

Age-matched Control and DM female littermates at 3-, 6- and 9-weeks of age were designated for follicle culture.

5.3.2 Follicle culture

Before collecting ovaries (~2 hours), culture media was made and equilibrated in the incubator at 37°C, 5%CO₂ in air.

Culture media was composed of 30 µl of minimum essential medium (MEM Alpha Modification, HyClone-Thermo, Fisher Scientific) supplemented with 140 mM ascorbic acid (Sigma-Aldrich), 5% fetal bovine serum (FBS, Biosera, Ringmer, UK; same lot number used in all experiments) and 2.5 IU/ml recombinant human FSH (r-hFSH, Gonal-F, Merck Serono, Feltham, UK). The culture medium, was filtered using a 22 µm pore syringe filter, placed in individual wells of a 96-well plate and overlaid with 75 µl of silicone oil (Dow Corning, BDH, VWR) to prevent evaporation of the media. The culture media was equilibrated in the incubator at 37°C, 5%CO₂ in air.

Mice were culled by cervical dislocation and death confirmed by exsanguination and ovaries were dissected and placed in warm Leibovitz's L-15 culture medium (HyClone-Thermo, Fisher Scientific) supplemented with 3mg/ml of bovine serum albumin (BSA fraction V, Fisher Scientific). To ensure there was limited variation in temperature when transferring ovaries from the animal house to

the laboratory, ovaries were transported in a polystyrene box containing a heat pad.

In the lab, ovaries were placed in glass embryo dishes containing the BSA supplemented Leibovitz's L-15 culture medium. The bursae and fat surrounding the ovary was removed and the ovary was cut into quarters. Each quarter was placed into a separate embryo dish containing fresh media and placed on the heated microscope stage of a stereomicroscope (Leica M125; Microscope Services Ltd., Woodstock, United Kingdom). Using 30G insulin needles, preantral follicles ~140 to 200 μm in diameter, with an undamaged basal lamina and some theca tissue surrounding the follicle were microdissected. Any excess extracellular matrix (ECM) or attached tissue/ neighbouring follicles were carefully removed using acupuncture needles. Using a mouth pipette, follicles were placed into individual wells of the 96-well plate and cultured for up to 6 days as previously described (291, 292).

Follicles were evaluated daily and transferred to a new well with freshly prepared culture medium. Images were also taken daily with a Leica M125 microscope (Leica Microsystems) and MicroPublisher 5.0 RTV camera (Qimaging). Not all follicles survived for the 6 days of culture; therefore only viable follicles that grew beyond day 3 were included for the follicular growth curve analysis. A preliminary dose response experiment was conducted by Dr. Heidy Kaune using 2.5, 5 and 7.5 IU of rFSH. Dr. Kaune found that follicle development was equal under all conditions and therefore, a concentration of 2.5 IU of rFSH was selected (data not shown).

5.3.3 Morphology assessment

Certain morphological features, such as an intact basal lamina, a visible oocyte and antrum formation were assessed daily throughout the culture period. Multiple measurements of the diameter of the follicle from the edge of the basal lamina, using an ocular reticle at 5x magnification were taken daily. Measurements were confirmed using the images taken ImageJ software (version 1.46r, National Institute of Health). To avoid any bias, during analysis the investigator was blind to the genotype of the mice.

5.3.4 Statistical analysis

Statistical analysis was carried out using Prism software version 4.0b (GraphPad Software, Inc., version 4.0b, 2004). D'Agostino & Pearson normality test was applied to test Gaussian (normal) distribution of the samples. The Mann–Whitney U test was performed to detect differences between groups with non-parametrical distribution and equal variances. Unpaired *t*-test was used to analyse samples with normal distribution.

5.3.5 Disclaimer

Experiments using 6-week mice were conducted by Dr. Heidy Kaune (a previous PhD student in Dr. Williams group) and analysis and generation of data for this chapter was shared equally.

The results have been published in collaboration with Dr. Heidy Kaune (3).

5.4 Results

5.4.1 Follicle culture using 9-week old DM was not possible due to isolation of inadequate numbers of preantral follicles

For each follicle culture experiment, ~12 follicles per mouse were dissected within 1 hour. In order to isolate the follicles, we gently teased the Control ovaries apart between follicles, which allowed isolation of numerous intact follicles. However, we were unable to tease the 9-week old DM ovaries apart as this resulted in the shredding of follicles in the process. We then attempted to cut out follicles from the 9-week old DM ovaries using microdissection scissors; however we were also unsuccessful in doing so. From 9 DM females, 30% of DM females yielded zero follicles, 55% of DM female's yielded one follicle, and one female yielded two follicles. Since, we were unable to obtain sufficient numbers of intact follicles from the DM ovaries, follicle culture was not conducted at this age.

Whilst dissecting DM ovaries to isolate follicles, we noticed that DM ovarian tissue was more rigid and fibrous than Controls, especially at 9-weeks of age, which gave the ovaries a distinctive opaque appearance compared to Controls (Figure 5.1).

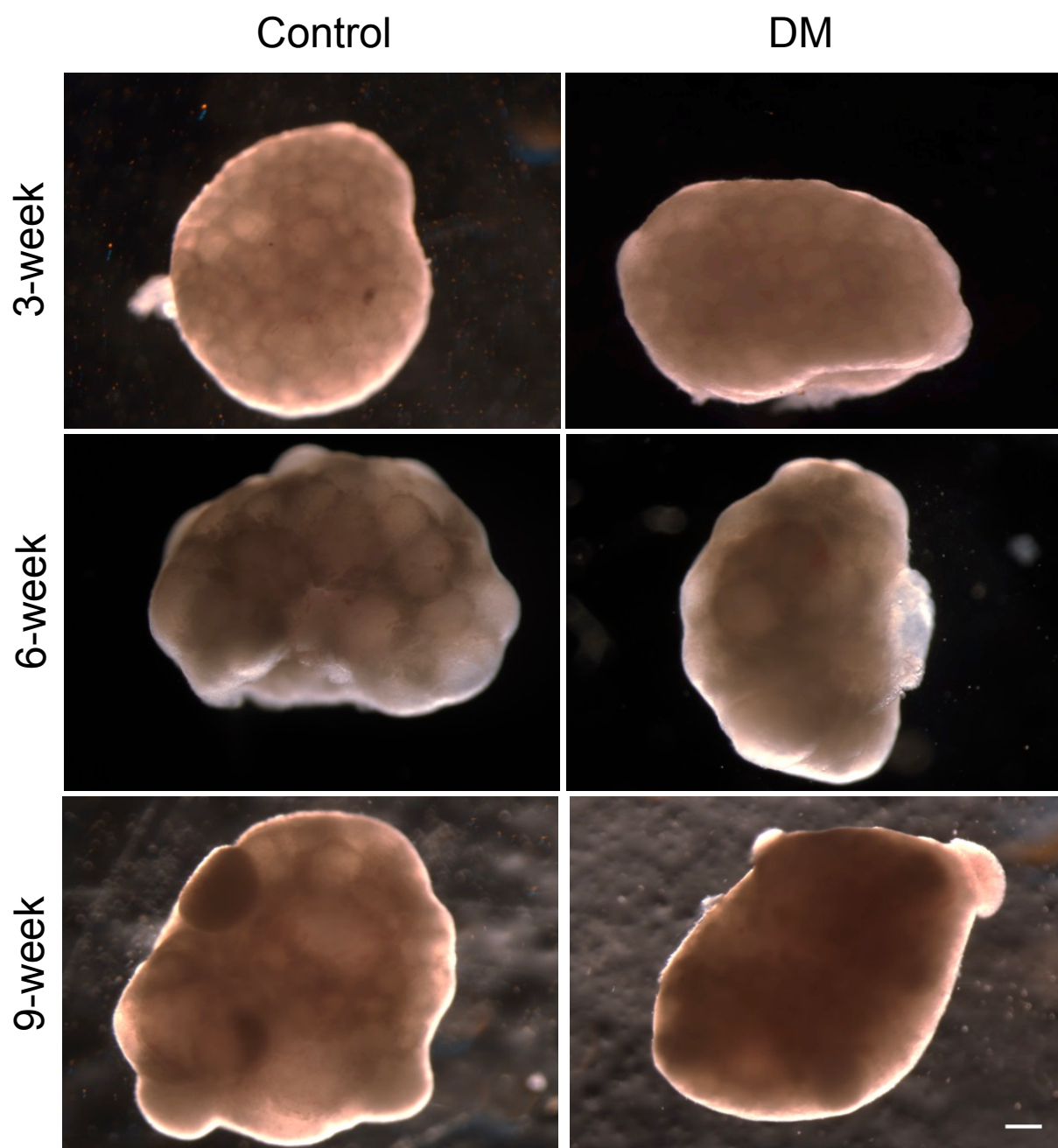


Figure 5.1 Images of Control and DM ovaries. Representative images of Control and DM ovaries from 3-, 6- and 9-week old females. Scale bar 200 μ m. Figures from publication (3).

5.4.2 A decreased survival rate of follicles isolated from 3- and 6-week old DM females is observed *in vitro*

Twelve ovarian follicles were dissected from each 3- and 6-week DM (3-weeks n=8 mice and 6-weeks n=4 mice) and Control female (3-weeks n=7 mice and 6-weeks n=4 mice) and cultured for 6 days (Figure 5.2). Follicles that stopped growing on day 0 or stopped growing by day 3 of culture were classified as unviable and were excluded from the growth analysis.

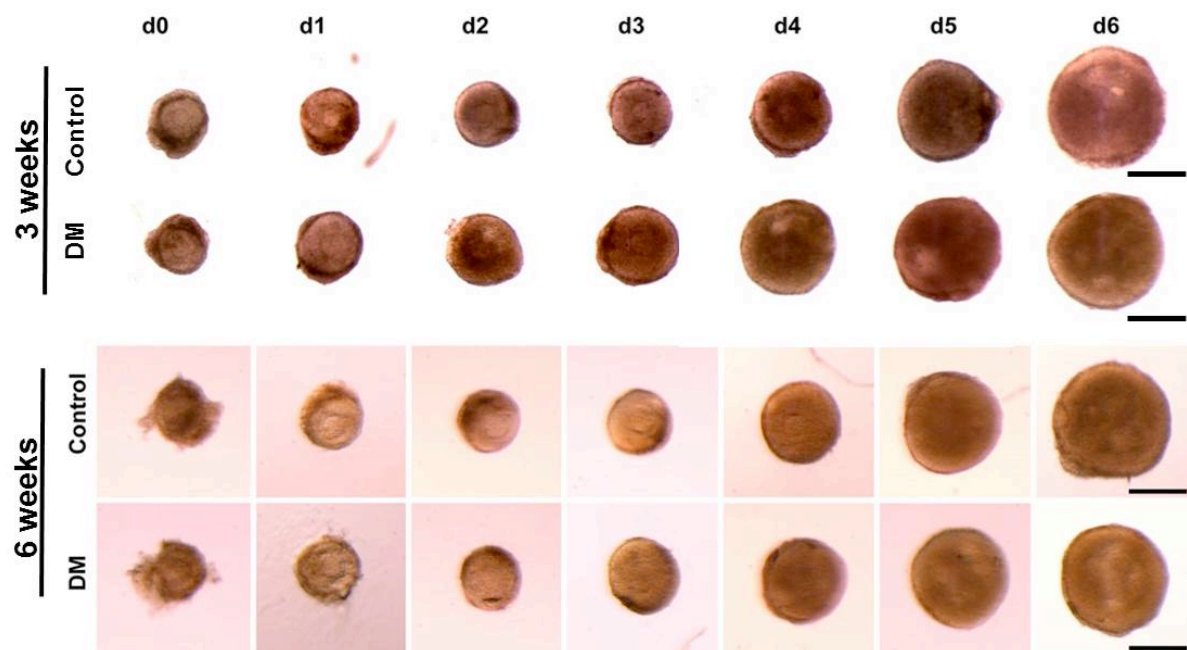


Figure 5.2 Ovarian follicle development *in vitro*. Representative images of follicle grown *in vitro* from 3-week old (Control n=69 follicles n=7 mice, DM n=56 follicles n=8 mice) and 6-week old (Control n=42 follicles n=4 mice, DM n=23 follicles n=4 mice) Control and DM females. Ovarian follicles were dissected (d0) and cultured with 2.5 IU of r-hFSH for up to 6 days. Scale bar 200 μ m. Figures from publication (3).

The survival rate of follicles was significantly decreased in DM compared with Controls at both, 3-weeks (Control $98.81 \pm 1.2\%$ versus DM $87.18 \pm 4.2\%$) and 6-weeks (Controls $97.92 \pm 2.1\%$ versus DM $50.57 \pm 8.5\%$). Furthermore, an age-dependant decrease in survival rate of DM follicles was observed from 3- to 6-weeks (3-weeks $87.18 \pm 4.2\%$ versus 6-weeks $50.57 \pm 8.5\%$) (Figure 5.3A).

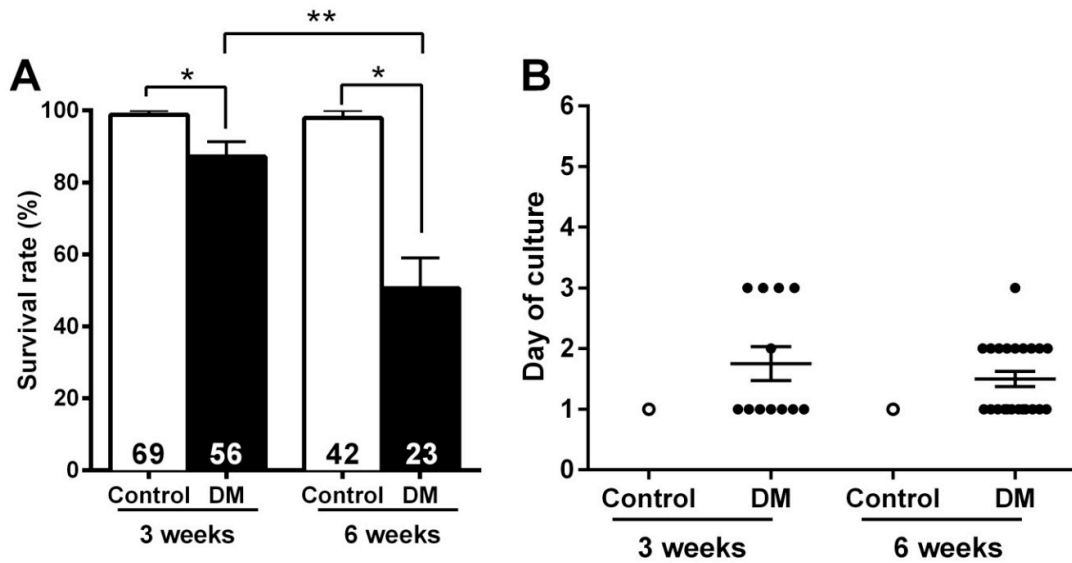


Figure 5.3 Survival rate and growth arrest of Control and DM follicles during *in vitro* culture. (A) Survival rate of Control and DM ovarian follicles dissected from 3- (Control n=69 follicles n=7 mice, DM n=56 follicles n=8 mice) and 6-week (Control n=42 follicles n=4 mice, DM n=23 n=4 mice) old females. Growth arrest of follicles dissected from (B) 3- and 6-week old females and assessed as the day when follicle growth stopped during culture. Results are expressed as mean $\pm$ SEM of follicles. * $P \leq 0.05$, ** $P \leq 0.01$. Figures from publication (3).

Next, we analysed the day on which the follicles stopped growing, however only one Control follicle stopped growing at both the 3- and 6-week cultures thus we were unable to compare this aspect between Control and DM. Analysis of DM follicle growth termination between 3- and 6-weeks revealed no difference in the day the follicles stopped growing (Figure 5.3B).

The diameter at collection (d0) of the unviable follicles was compared with the diameter of the follicles that grew normally in order to assess whether the survival rate was related with differences in the type of follicle collected in both groups or was intrinsic to the mice genotype. However, no differences were detected in the diameter between viable and unviable Control and DM follicles collected at 3- and 6-weeks (3 weeks: Control viable $183.7 \pm 1.5 \mu\text{m}$ n=69 follicles, DM viable $182.5 \pm 1.9 \mu\text{m}$ n=56 follicles, Control unviable $182.4 \pm 4.6 \mu\text{m}$ n=8 follicles, DM unviable $175.8 \pm 2.1 \mu\text{m}$ n=25 follicles; 6 weeks: Control viable $185.3 \pm 2.0 \mu\text{m}$ n=42 follicles, DM viable $188.0 \pm 4.8 \mu\text{m}$ n=23 follicles, Control unviable $195.4 \pm 8.3 \mu\text{m}$ n=4 follicles, DM unviable $177.7 \pm 4.0 \mu\text{m}$ n=24 follicles).

5.4.3 Follicles from 3- and 6-week old DM females have accelerated growth *in vitro*

Surprisingly, the final diameter reached by DM follicles, was similar to the Control group in both 3- and 6-week follicles demonstrating that DM follicles are able to grow and develop *in vitro* (Figure 5.4). However, the growth curve of DM follicles differed to Controls. Follicles (n=56) collected from 3-week DM females grew significantly larger for the duration of the culture but had reduced growth during the final day of culture and thus were the same size as Controls (n=69) at the end of the culture period (Figure 5.4A). A similar situation was observed in follicles (n=23) isolated from 6-week DM females, however follicles were significantly larger than Controls (n=42) for the first 3 days of culture only (Figure 5.4B).

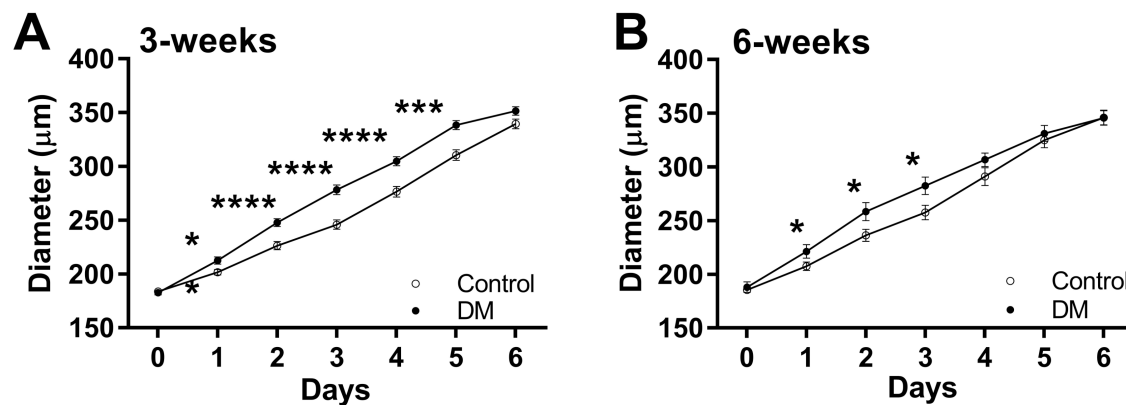


Figure 5.4 Assessment of Control and DM follicle development. (Growth curve of Control and DM follicles dissected from (A) 3- (Control n=69 follicles n=7 mice, DM n=56 follicles n=8 mice) and (B) 6-week old (Control n=42 follicles n=4 mice, DM n=23 follicles n=4 mice) females; results are expressed as mean±SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Figures from publication (3).

5.4.4 *In vitro* antrum development of DM follicles differs to

Controls at 3- and 6-weeks

The day and the size of the follicle when the antrum was first detected was assessed. There was no difference in the day that a follicular antrum developed between Control and DM in both 3- and 6-weeks follicles (Figure 5.5C and D), however the diameter of DM follicles when the antrum was first visible, was increased compared with Controls at both ages (DM $263.0 \pm 2.2 \mu\text{m}$ and Controls $246.1 \pm 2.4 \mu\text{m}$ for 3-week follicles and DM $288.6 \pm 6.4 \mu\text{m}$ and Controls $266.7 \pm 5.9 \mu\text{m}$, respectively for 6-week follicles) (Figure 5.5E and F).

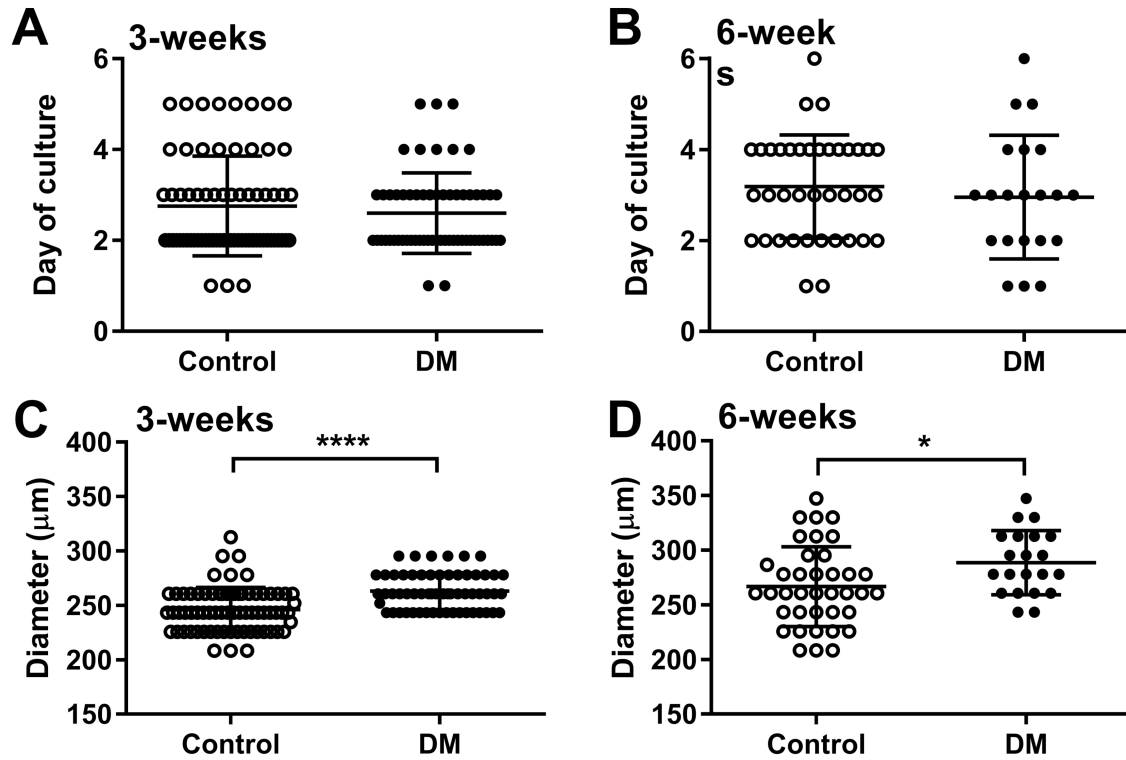


Figure 5.5 Assessment of Control and DM antrum formation *in vitro*. Day of culture when Control and DM follicles isolated from (C) 3- and (D) 6-week mice developed antrum. (E-F) Size of Control and DM follicles isolated from (E) 3- and (F) 6-week mice when an antrum was first detected. Results are expressed as mean±SD. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Figures from publication (3).

5.5 Discussion

Understanding follicle development during the onset of POI and thus developing therapies focusing on reactivation of follicle development for women with follicular POI is essential (184, 185). Glycans have important functions, including providing structural components to the ECM, modification of protein properties, mediating protein trafficking and cell signalling (293, 294). Therefore we hypothesised the absence of hybrid and complex *N*- and core 1-derived O-glycans, is likely to be disturbing intra-ovarian signalling in DM ovaries and inducing changes in the ECM which is ultimately affecting follicle progression.

In order to eliminate potential suppressing intra-ovarian factors, we isolated preantral follicles from 3- and 6-week old DM ovaries to evaluate growth under controlled *in vitro* environment. The survival rate of DM follicles exacerbates with age with less follicles surviving at 6-weeks, showing an age-dependent decrease in survival. Furthermore, survival rate of DM follicles was less than Controls indicating that intrinsic follicular factors are present in preantral follicles before and after puberty in DM females preventing follicle development. Interestingly, DM follicles that did survive initially grew faster than Control follicles but reached an equivalent final size to Controls supporting our hypothesis. Since the growth of DM follicles slows towards the end of culture, it is possible that if cultured for longer periods of time, they may stop growing altogether. However this is also the case for Controls as follicle size is finite and shortly after day 6 of culture using this system, they either ovulate an egg and structurally collapse, or die. Therefore, it is unknown if this decrease in DM follicle growth rate was due to follicle deterioration or due to physiological constraints and the fact that a larger size was attained

prematurely. However, although Control or DM follicle growth or egg competence was not assessed beyond 6-days of culture, culture of wildtype preantral follicles for 6-days is sufficient for maturation of oocytes *in vitro* (295) and therefore it is not unreasonable to assume that eggs could be retrieved from DM follicles at day 6.

The finding that DM ovaries have observable changes in rigidity and density, compared with Controls that is exacerbated with age and coincides with the decreased survival rate of DM follicles, indicates that components of the ECM are affected and therefore it is likely that intra-ovarian factors and communication are abnormal in the DM mouse as hypothesised. The rigidity observed in DM ovaries is unlike ovaries of aged mice, which are fibrous due to the loss of growing follicles which changes the relative proportions of cortex:medulla, and is therefore considered abnormal. This also indicates that these modifications accumulate with age and are coincident with the change in the DM phenotype from fertile to infertile and may provide a cause for the previously reported block in primary follicle development (1) and also for the ovary dysfunction as observed in PCOS. Changes in ECM components such as laminin and collagen IV have been reported in mice with an oocyte-specific deletion of *C1galt1* (57) and in the previous chapter we have reported changes in the ECM composition of the DM ovary, which leads to a disruption of follicle development and anovulation. This is also evident in women with PCOS who have a denser ovarian cortex due to higher levels of collagen than the cortex of normal ovaries (296).

Recently, the role of the environmental rigidity in follicle development has been investigated through the use of alginate in follicle culture systems. Follicles

cultured in a less rigid matrix exhibit an increased follicular growth and a higher rate of antrum and theca formation than follicles cultured in a more rigid environment (297, 298). The increased density observed in DM ovaries could potentially act as a growth-restriction agent to limit the expansion and development of the DM follicles. Our results demonstrate that the removal of DM follicles from their ovarian environment to a less-rigid environment in culture appears to have allowed DM follicles to have better development as they initially grew faster. We speculate that the more rigid ECM surrounding the isolated DM follicles provides support for the follicle in culture mimicking the normal ovarian environment, whereas removing Control follicles from the ovary to a culture environment results in the disruption of their follicular architecture and removal of structural support, limiting their ability to grow as well in culture initially. This hypothesis is supported by the fact that DM follicles grew faster during the first days of culture and were larger than Control follicles when they developed an antrum. The antrum cavity is filled with follicular fluid composed of serum transudate as well as secretory products of GCs such as mucopolysaccharides, steroids and hormones (299-301). The composition of the follicular fluid is related to the physiological status of the follicle and has been suggested to reflect the fertility potential of the oocyte inside in both, humans (302-304) and cattle (305).

The study has a number of possible limitations to consider. Females were not assessed for timing of oestrous cycle; however since we used littermates, any variations due to different timings in oestrous cycle are equally represented in both groups. To avoid any bias during the experiments and analysis the investigator was blind to the genotype of the mice. FBS is rich in growth factors, vitamins,

hormones and other nutrients that are essential for cell proliferations (306); however, since FBS is batch produced there is variation in composition of FBS with each batch, as well as with FSH activity. To limit this variation, we have used the same batch of both FBS and FSH for all experiments.

In summary, the findings of this chapter support our hypothesis since DM follicles are able to grow *in vitro* reaching the same size as Control follicles at 3- and 6-weeks of age. However, the survival rate of DM follicles is compromised with age and also when compared to Control follicle survival at both ages. Furthermore, the observable changes in the ECM of the DM ovary could be affecting follicle development *in vivo* since the growth curve of DM follicles differs to Controls demonstrated by an initial acceleration in follicle growth and a different pattern in antrum development. Despite these uncertainties, our results demonstrate that a population of DM follicles retain the potential to develop better *in vitro* and therefore follicle culture offers a reliable method to generate antral follicles from preantral after the onset of POI in these females.

**Analysis of DM germ and somatic cell
function using the reaggregated ovary
technique**

6.1 Introduction

Approximately 50% women diagnosed with POI have follicles present in their ovaries (184, 185) indicating that a significant number of women could benefit from therapies focusing on reactivation of follicle development.

Currently, *in vitro* activation is the only method for women with POI to have their own biological child (307). However this has not been successful for all women with only 2/37 women giving birth and in this instance there is a need to develop further treatments.

DM females are fertile at 6-weeks, infertile at 9-weeks and progress to POI by 3-months of age exhibiting the classical features of POI pathology including lack of ovulation, altered hormone levels and abnormal follicle development (1). DM ovaries have an increased number of primary follicles at the 3a stage accompanied by a decrease in developing follicles indicating a block of follicle development at the primary stage (1, 4, 24). In Chapter 3, we found the presence of abnormal follicles containing large oocytes, demonstrating a discrepancy between oocyte and GC communication. Furthermore, similar to the DM, women diagnosed with resistant ovary syndrome, have a characteristic histological finding of abundant primary follicles and an arrest at the antral stage of development (198). Therefore, the DM mouse is a valuable model to explore potential interventions to rescue egg development and restore fertility after the onset of POI.

Previously, to determine if a normal endocrine environment could facilitate the resumption of DM ovarian follicle development, ovarian tissue from 9-week DM or Control mice was transplanted under the kidney capsule of immunocompatible sibling Control females for 4 weeks. Whilst both Control whole ovaries (Figure 6.1A) and graft ovaries (Figure 6.1C) from mice at 9 weeks of age contain follicles at all stages of development as well as multiple CL, DM whole ovaries (Figure 6.1B) and graft ovaries (Figure 6.1D) contained only small follicles.

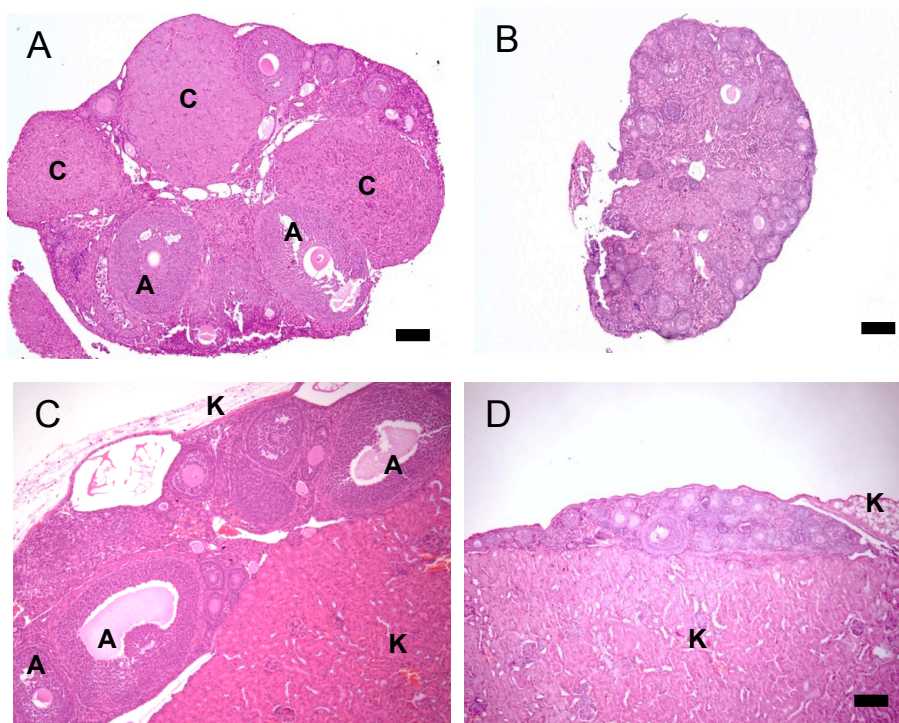


Figure 6.1 Control and DM ovarian morphology and follicle density. (A) Control 9-week ovaries contain numerous follicles at different stages of development and corpora lutea whereas (B) DM 9-week ovaries contain only small follicles. After 4 weeks of ovarian tissue transplantation to a wildtype immunocompatible host (C) Control graft tissue had normal ovarian morphology containing all stages of follicle development whilst (D) DM graft tissue exhibited morphology comparable to a 9-week DM ovary. AF: antral follicle, Cl: corpus luteum, KC: kidney capsule, K: kidney tissue. Scale bar= 100 μ m.

Comparison of follicle development between Control and DM grafts (Figure 6.2A) revealed significantly more primary follicles in DM grafts, accompanied by a decrease in follicle from the secondary stage onwards (Figure 6.2A). Analysis of 3a and 3b primary follicles in graft ovaries revealed over a 100% increase in 3a follicles in DM ovaries compared to Control, whilst 3b follicle numbers did not differ (Figure 6.2B). Therefore, follicle development in the DM grafted ovaries was not normalised by exposure to a normal hormonal environment as the block at the 3a stage of follicle development still existed in the grafted ovaries.

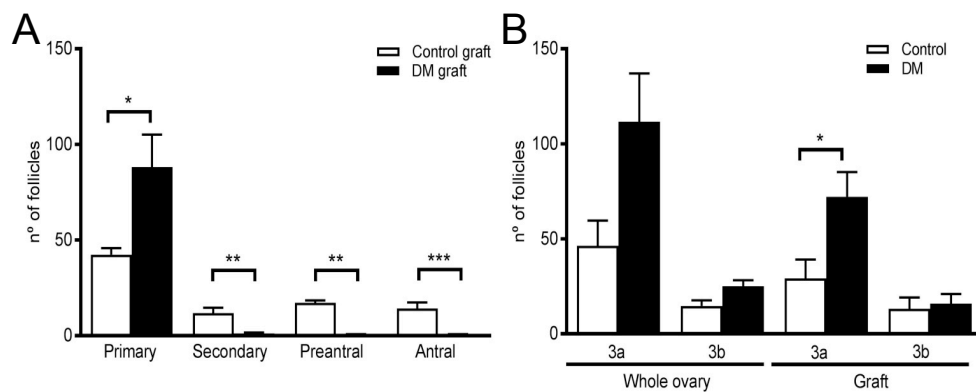


Figure 6.2 Comparison of Control and DM follicle development in whole ovaries to ovarian tissue graft. (A) Comparison of the number of follicles at each stage of development in the Control and DM ovarian tissue graft. (B) Number of primary follicles (3a and 3b) in Control and DM whole ovary and ovarian tissue graft. Results are expressed as mean $\pm$ SEM. *P<0.05, **P<0.01, ***P<0.001.

Successful follicle development is dependent on appropriate endocrine support and requires intricate two-way communication between the oocyte and the somatic cells (22). The oocyte produces factors that modulate somatic cell development and differentiation inducing follicle development (20, 133, 308). In addition, oocyte development is regulated by factors secreted by somatic cells that

also provide optimum conditions for the oocyte to develop competence for ovulation and fertility (309, 310).

Since normalising the endocrine environment did not rescue DM follicle development, we investigated the role of the ovarian somatic cells on the developmental potential of DM oocytes using the reaggregated ovary (RO) technique. The RO technique is based on the differential separation of ovarian germ and somatic cells followed by the reaggregation of both cellular types allowing the generation of a reaggregated pellet with two different cell sources. Generation and analysis of ROs enables us to determine the separate influence of the somatic and germ cells on follicle development and was established using newborn somatic and germ cells from mice up to 12 days of age (22, 311, 312).

Although DM mice have an oocyte-specific deletion, DM females quickly transition from a fertile state at 6-weeks to undergoing POI by 3-months of age, affecting the entire ovarian morphology. In the previous chapter we demonstrate that removal of DM follicles from the current architecture to an *in vitro* environment is able to support follicle growth. Therefore, we hypothesise that modifying the oocyte environment using the RO technique could rescue the ability of DM germ cells to coordinate follicle development.

6.2 Aims

- To assess DM germ and somatic cell function using the RO technique.
- To determine to replace oocyte environment is able to rescue DM follicle development.

To address this, we assessed germ cell function by generating ROs using DM germ cells and newborn somatic cells, whilst to assess somatic cell function, ROs were generated using DM somatic cells and newborn germ cells. ROs were transplanted into an immunocompromised mouse and were retrieved after 21 days and follicle development was assessed by histological analysis.

6.3 Material and methods

6.3.1 Mice

To generate ROs assessing DM germ cell function, DM or Control females, aged between 9-10-weeks were used as the source of germ cells and neonatal (P0-P1) B6SJLF1/J mice (WT) or mice ubiquitously expressing the *LacZ* gene (313) were used as the source of somatic cells.

To generate ROs assessing DM somatic cell function, the reciprocal cells were used; DM or Control females, aged between 9-10-weeks were used as the source of somatic cells and neonatal (P0-P1) B6SJLF1/J mice (WT) or mice ubiquitously expressing the *LacZ* gene (313) were used as the source of germ cells.

6.3.1.1 Breeding

To produce large numbers of mice from the DM colony (Per RO: n=7-10 mice were required), mice were bred by timed-mating (section 2.2.3).

To produce B6SJLF1/J pups, the first generation (F1) produced by mating C57BL/6 female mice with SJL/J males by plugged mating. To produce *LacZ* expressing pups, C57BL/6 female mice were mated with *LacZ* expressing males by timed-mating; newborns were genotyped as described in section 2.6.1.

In order to avoid host rejection of the transplanted ROs, immunocompromised mice (C.B-17 SCID or B6Rag1) were used as recipients since these mice lack functional immune systems.

LacZ expressing mice were generously provided by Professor Elizabeth Robertson, Sir William Dunn School of Pathology, University of Oxford. SJL/J, C57BL/6 and C.B-17 SCID mice were obtained from Harlan. B6Rag1 mice were generously provided by Professor Fiona Powrie, Nuffield Department of Medicine, University of Oxford.

Mice were kept under controlled conditions in 12:12 hour light-dark cycles. Food and water was provided *ad libitum*.

6.3.2 Generation of reaggregated ovaries

The generation of ROs involved the dissociation of ovaries into germ and somatic cells followed by the exchange and combination of both cell populations to form a reaggregated pellet (Figure 6.3) as previously described (312).

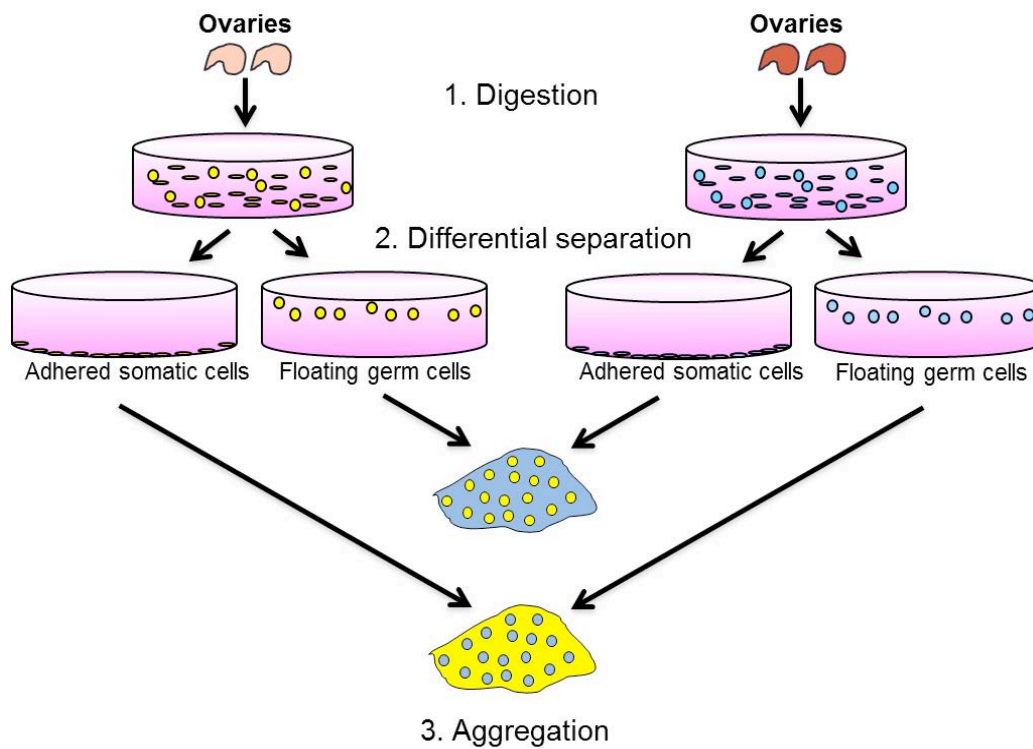


Figure 6.3 Generation of a reaggregated ovary. (1) Ovaries are digested with trypsin into a single cell suspension. (2) Somatic cells and germ cell populations are differentially separated since somatic cells are able to adhere to treated tissue culture dishes. (3) Somatic and germ cells from different sources are aggregated to form a pellet which, after overnight culture, is transplanted into an immuno-compromised mouse for 21 days.

6.3.2.1 Ovary collection and digestion

For the production of one RO, 7-10 adult mice and 4-6 neonate mice were used. Mice were culled by cervical dislocation and death was confirmed by exsanguination.

Ovaries were collected into warm sterile phosphate buffered saline (PBS; Sigma-Aldrich) supplemented with 1mg/ml BSA (PBS/BSA) (BSA fraction V, Thermo Fisher Scientific). Under the stereomicroscope, ovaries were released from the bursa and fat was removed. For adult ovaries, these were minced by cutting using 30G insulin needles until the tissue was small enough to fit through a P1000 tip. Ovarian tissue was then washed twice with PBS/BSA and transferred to a 35x10 mm dish containing 0.05% trypsin and 0.53mM EDTA (GIBCO), supplemented with 0.02% DNase-I (Sigma-Aldrich).

Ovaries were enzymatically dissociated into a single cell suspension by digesting for 15 min at 37°C and 5% CO₂ in air. After 15 min, the cells suspension was mixed by pipetting up and down repeatedly and returned to the incubator for 5 mins. This step was repeated 3 times, total digestion was 30 mins.

The single cell suspension was transferred into a 15 ml centrifuge tube with equal volume of warm Medium 199 (M199, GIBCO) supplemented with 31.3mM sodium lactate (Sigma-Aldrich), 10mg/mL Penicillin-Streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS; Biosera; same lot number used in all experiments), centrifuged at 663× *g* for 5 min. Next, the supernatant was carefully

discarded ensuring the pellet was not disturbed, followed by resuspension of the pellet in fresh M199 supplemented media.

6.3.2.2 Differential separation

The cell suspension was transferred to a 35x10 mm cell culture treated dish for overnight culture at 37°C and 5% CO₂ in air (Figure 6.4A). The following morning the non-adhered cell population was separated from the adhered cells. Non-adhered cells, which included germ cells, non-viable somatic cells and red blood cells, were collected and filtered using a 30 µm pre-separation filter (Miltenyi Biotec GmbH, Germany). This filtering step excluded any remnant undigested tissue and more developed oocytes and thus collect only germ cells from follicles up to the early secondary stage of follicle development; this was transferred to a new tissue culture dish with fresh M199 supplemented media (Figure 6.4B).

The remaining somatic cell monolayer was washed three times with PBS/BSA to remove any residual unattached cells (Figure 6.4C and D). The adhered somatic cells were then treated with trypsin for 5 min at 37°C, neutralised with equal amounts of M199 supplemented media and transferred into a 15 ml centrifuge tube and centrifuged at 663× *g* for 5 min. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in fresh M199 supplemented media and transferred into a new 35x10 mm cell culture-treated dish and cultured for a further 6 h. The second round of differential adhesion and separation was carried out to ensure complete separation of cell types.

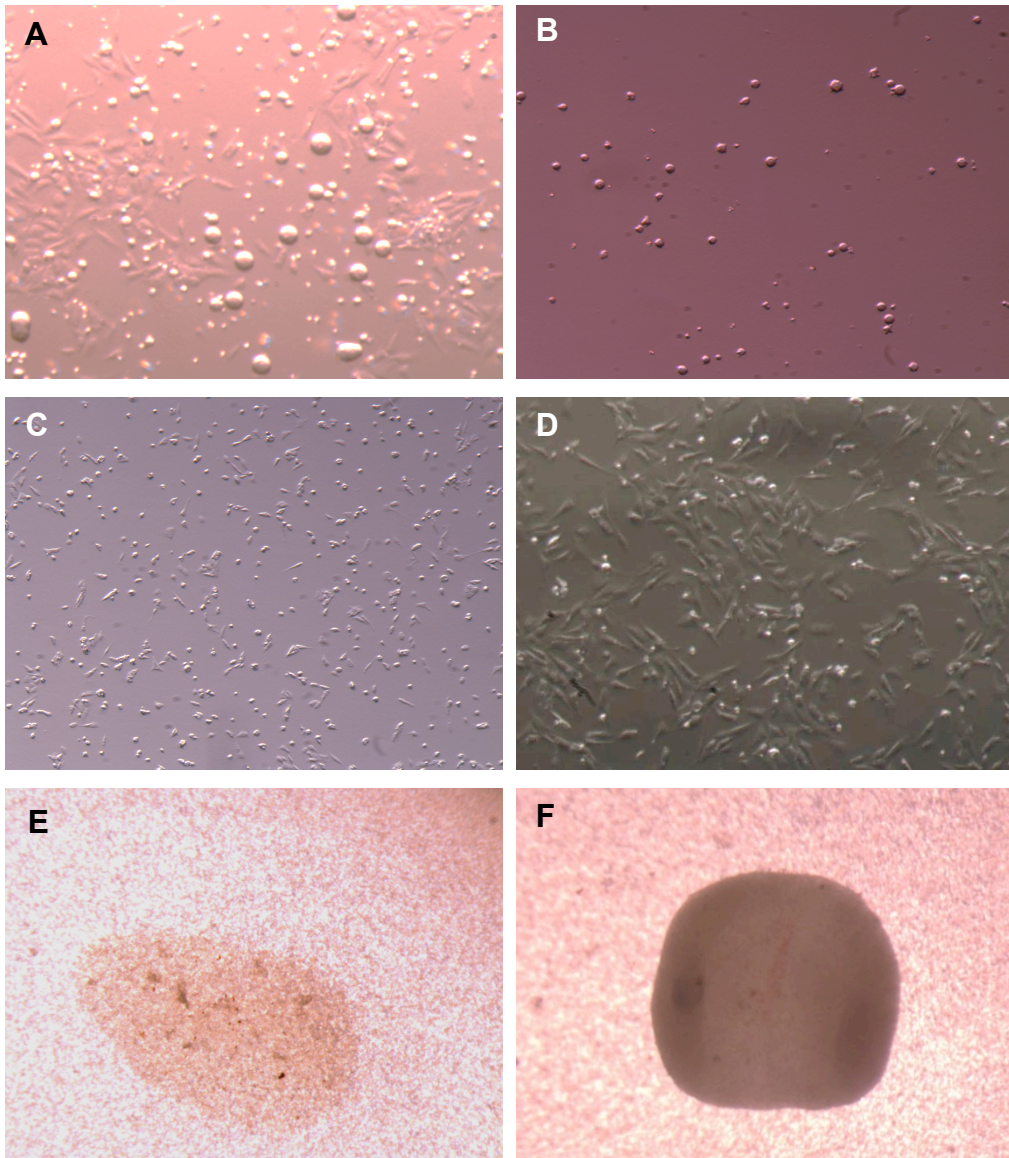


Figure 6.4 Representative images of cells and reaggregated ovaries. (A) Image of single cell suspension containing both germ and somatic cells. Following a second differential separation a clean pool of (B) germ and (C & D) somatic cells are isolated. The two cell types are combined in the desired ratio and centrifuged to form a (E) reaggregated pellet, which is left to culture overnight to form a (F) rounded pellet.

6.3.2.3 Assessing cell numbers and live/dead cells

After 6 h, germ and somatic cells were individually collected as previously, centrifuged and re-suspended in 100 µl of M199 supplemented media. To determine total cell numbers and live-dead cell numbers, 10 µl of the cell sample was mixed with 10 µl Trypan Blue (Sigma). From this mixture, 10 µl were loaded into the Neubauer Improved haemocytometer haemocytometer and cells were counted in each corner quadrant (each quadrant contains 16 squares; 4 quadrants per haemocytometer grid) and averaged. To determine the remaining total number of cells (190 µl cell suspension since 10 µl were used for cell counting) the following formula was used:

$$\begin{aligned} \frac{\text{Cells}}{\text{ml}} &= \text{Average number of cells in the quadrants} \\ &\quad \times 10^4 (\text{Volume factor for the haemocytometer}) \\ &\quad \times 2 (\text{due to 1:1 dilution with Trypan Blue}) \\ \frac{\text{Cells}}{190 \mu\text{l}} &= \frac{\text{Cells}}{\text{ml}} \times 0.2 (\text{cells were resuspended in 200 } \mu\text{l}) \\ &\quad \times 0.95 (10 \mu\text{l was taken for haemocytometer assessment}) \end{aligned}$$

6.3.2.4 Generation and overnight culture of cell pellet

For ROs made using adult germ cells and newborn somatic cells, 100% of cells isolated were combined; however for ROs made using adult somatic cells and newborn germ cells, only 30% of the newborn germ cell population was used (routine ratio used with newborn ovaries (312)).

Germ and somatic cells were combined in the desired ratio with 0.25 µg of phytohemagglutinin (PHA; Sigma-Aldrich) and centrifuged at 10,000 × g for 30 s.

To form a compact pellet, the tube was then rotated 180° and centrifuged for a further 30 s. The cell pellet is fragile at this point and is carefully transferred with a Pasteur pipette to a 24 mm diameter Millicell-PC membrane with a pore size of 4 µm (Millipore) with Waymouth medium MB 752/1 (Sigma-Aldrich) supplemented with 10% FBS for overnight culture (Figure 6.4E). Following overnight culture the pellet rounded up and was sturdy for transplantation (Figure 6.4F).

6.3.3 Surgical transplantation of reaggregated pellets

6.3.3.1 Transplantation of pellet under the kidney capsule

Recipients were anaesthetised with isoflurane and the surgical area prepared by removing the fur with clippers and cleaning with chlorhexidine solution. The anaesthetised animal was placed on a heated pad at 37°C and injected subcutaneously with 0.05-0.1 mg/kg of Buprenorphine (Vetergesic, Alstoe Limited, York, UK) to provide analgesia after surgery. Under magnification, the left kidney was exteriorised through a dorso-lateral incision and a small opening on the kidney capsule made using a pair of fine tip forceps. The pellet was placed beneath the kidney capsule with a sterile fine tip plastic pipette and pushed away from the capsule opening, and the kidney was returned to the body cavity.

6.3.3.2 Bilateral ovariectomy

Next, a bilateral ovariectomy was carried out on the recipient females. Using fine tip forceps the bursa was opened and the ovary completely exposed. The ovarian vasculature was ligated using silk suture and the ovary removed using small spring scissors. The body wall closed with interrupted suture using absorbable suture material (vicryl, Ethicon, US). Using the same skin incision, the

opposite side of the body wall was opened and the contralateral ovariectomy performed, followed by the suturing of the body wall and skin with interrupted suture (vicryl, Ethicon, US).

6.3.3.3 Mouse recovery

The animal was placed in a warm recovery chamber and evaluated constantly until fully recovered from anaesthesia. Two hours after surgery animals were returned to the animal house maintenance room and they were evaluated daily for signs of distress and infection for the first week after the surgery. During the first 48 h after surgery, mice had easy access to food pellets, mashed pellets, water and medicated jelly with meloxicam (Metacam, Boehringer Ingelheim, Bracknell, UK) to provide analgesic.

6.3.4 Collection of ROs

Pellets were grafted in the recipient mice for 21 since follicle development in mice takes 21 days. At 21 days after transplantation, mice were killed by cervical dislocation and the kidney exposed and explored under the microscope to identify the RO (Figure 6.5A). Since the RO becomes attached to the kidney capsule, the kidney capsule is carefully peeled using watchmakers' forceps and cut using small spring scissors and collected into cold PBS on ice (Figure 6.5B).

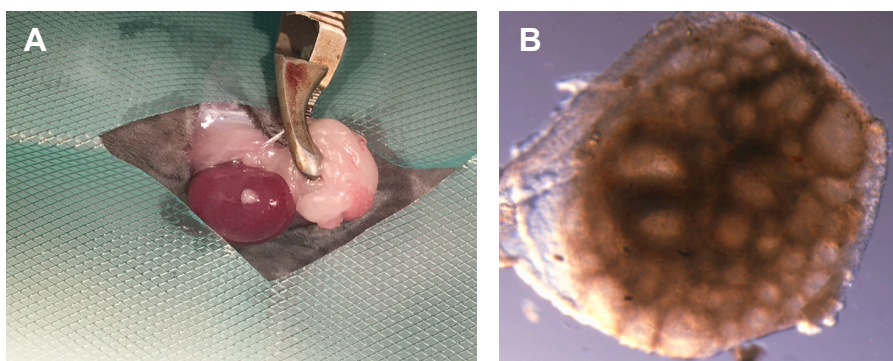


Figure 6.5 Images of collection of ROs. ROs are collected from (A) the kidney after 21 days. (B) ROs demonstrate the presence of follicles at various development stages.

6.3.5 Processing of ROs

ROs were fixed for 8 h in 10% buffered formalin (Sigma-Aldrich) before being transferred to 70% ethanol for storage at 4°C. If ROs were generated using LacZ mice, then ROs were subjected to LacZ staining as described in section 2.6.2. ROs were embedded and histologically processed (section 2.5) and all sections were used for follicle counts using the classification system described in section 1.1.1.1.

6.3.6 Statistics

Analysis was carried out using Prism software version 4.0b (GraphPad Software, Inc., version 4.0b, 2004). D'Agostino & Pearson normality test or Shapiro-Wilk normality test was applied to test Gaussian (normal) distribution of the samples. The Mann–Whitney U test was performed to detect differences between groups with non-parametrical distribution and equal variances. Unpaired *t*-test was used to analyse samples with normal distribution and equal variances

whilst Welch's correction was used to analyse samples with normal distribution and unequal variances. A One-way ANOVA followed by Dunn's multiple comparisons test was used to analyse proportion of Control and DM follicles in whole ovary versus ROs and to analyse the mean number of follicles per RO. Results are presented as mean $\pm$ SEM. $P\leq 0.05$ was considered significant.

6.4 Results

6.4.1 Formation of ROs using adult germ cells.

Previously, the generation of ROs has only been achieved using mice up to 12 days of age (22, 311). Therefore, to make ROs using adult germ cells, the protocol was tested, adjusted and evaluated in preliminary experiments. ROs were generated using neonatal (P0-1) B6SJLF1/J somatic cells and germ cells from C57/BL6 ex-breeder mice (6-month old). Twenty-one days after the transplant, gross morphological analysis, indicated the presence of multiple follicles evidenced by the presence of rounded semi-transparent structures (Figure 6.6A). Histological analysis demonstrated the presence of follicles at the preovulatory stage of development (Figure 6.6B).

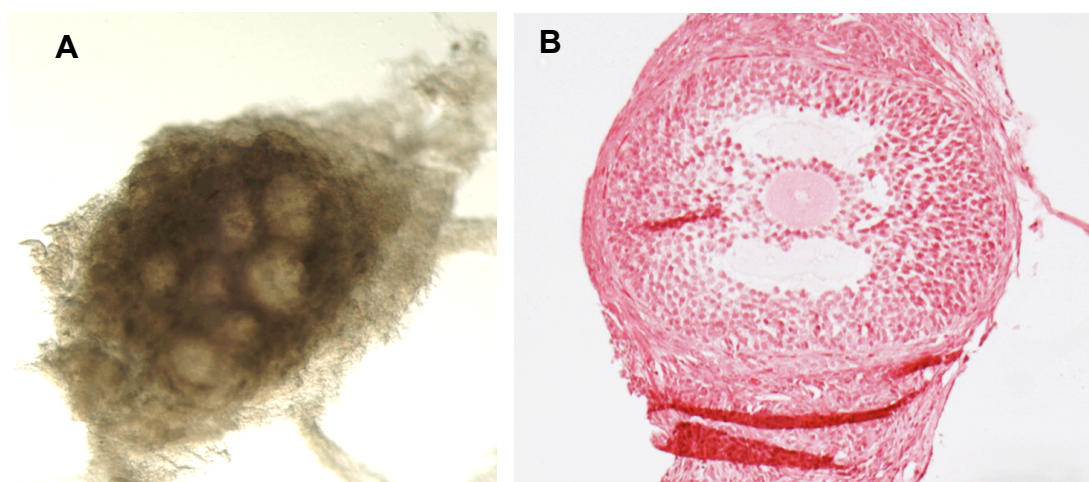


Figure 6.6 Generation of ROs using ovaries from adult mice. (A) ROs were generated using germ cells from ex-breeder adult mice and somatic cells from neonatal mice show the presence of follicles. (B) Histological analysis of ROs revealed the presence of preovulatory follicles.

6.4.2 Validation of cell isolation

To confirm efficient separation of adult germ cells and newborn somatic cells, ROs were generated using ovaries from mice ubiquitously expressing *LacZ* gene and wildtype (WT) mice (Figure 6.7A). ROs were stained for *LacZ* by incubation with X-gal (Section 2.8), revealing blue staining of the oocytes originating from the *LacZ* mice (Figure 6.7B). Histological analysis revealed no blue somatic cells (counterstained with eosin) and thus all somatic cells originated from WT ovaries, demonstrating clean somatic cell and germ cell isolation (Figure 6.7C and D).

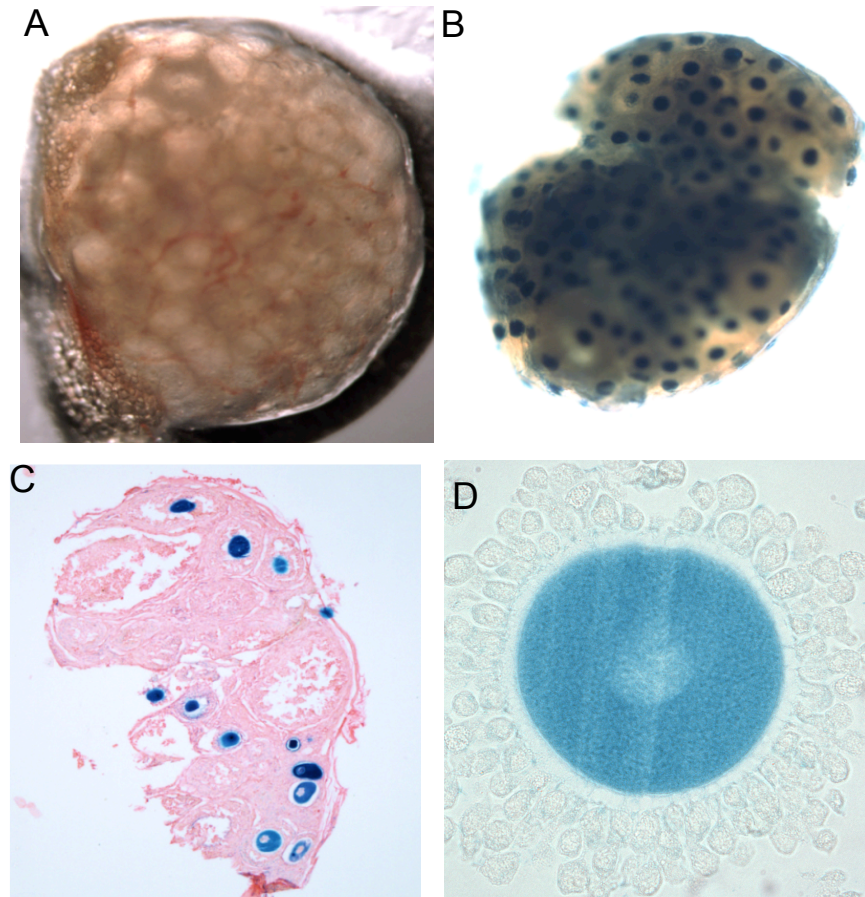


Figure 6.7 Generation of ROs to assess successful cells isolation. ROs were generated using germ cells from mice ubiquitously expressing LacZ, to demonstrate clean separation of somatic and germ cells. Image of RO 21 days post-transplantation (A) before and (B) after incubation with X-gal revealing the presence of oocytes expressing the LacZ gene stained blue and unstained somatic cells. (C) Histological analysis of RO sections confirmed 100% of oocytes were from the LacZ mice (counterstained with eosin; n=5) and (D) High magnification image of a cumulus-oocyte-complex showing blue oocyte and unstained cumulus cells.

6.4.3 DM germ and somatic cells are able to generate follicles in a reaggregated ovary

Control or DM ovaries were digested into a single population and separated into germ and somatic cells. The number of live and dead cells in each cell suspension was assessed using Trypan Blue. No differences were found in the number of Trypan blue positive dead germ cells from Control and DM ovaries and no dead somatic cells were observed in either group. Total cells numbers also did not differ between Control and DM.

ROs were generated using germ cells from 9-week old DM or Control germ cells and newborn WT somatic cells (referred to as either DM germ or Control germ ROs, respectively).

6.4.4 DM germ cells are able to overcome primary follicle arrest

First, analysis of total follicle counts revealed the presence of follicles in both Control and DM germ ROs. Surprisingly, DM germ cells were able to generate follicles to the same extent as Controls (Control: 4 ± 1.73 and DM: 2.83 ± 1.47 ; Control n=5 ROs, n=20 follicles, DM n=6 ROs, n=27 follicles).

Next, the stage at which follicles existed was categorised and follicle were counted and compared to 9-week whole ovary (Figure 6.8A and B).

Control germ ROs contained many developing follicles and these were distributed between the primary and antral stage, in numbers comparable to Control whole ovary (Figure 6.8A). Remarkably, DM germ ROs generated using

germ cells from infertile 9-week DM ovaries contained follicles at later stages of development as opposed to DM ovaries that contained predominantly primary follicles (Figure 6.8B).

Comparison of follicle numbers between Control germ and DM germ ROs revealed no differences (Figure 6.8C), however the number of follicles that developed in both DM and Control ROs were low compared to whole ovaries. Despite this, since DM ROs contained follicles at all stages of follicle development, this indicated that the block at primary development was overcome.

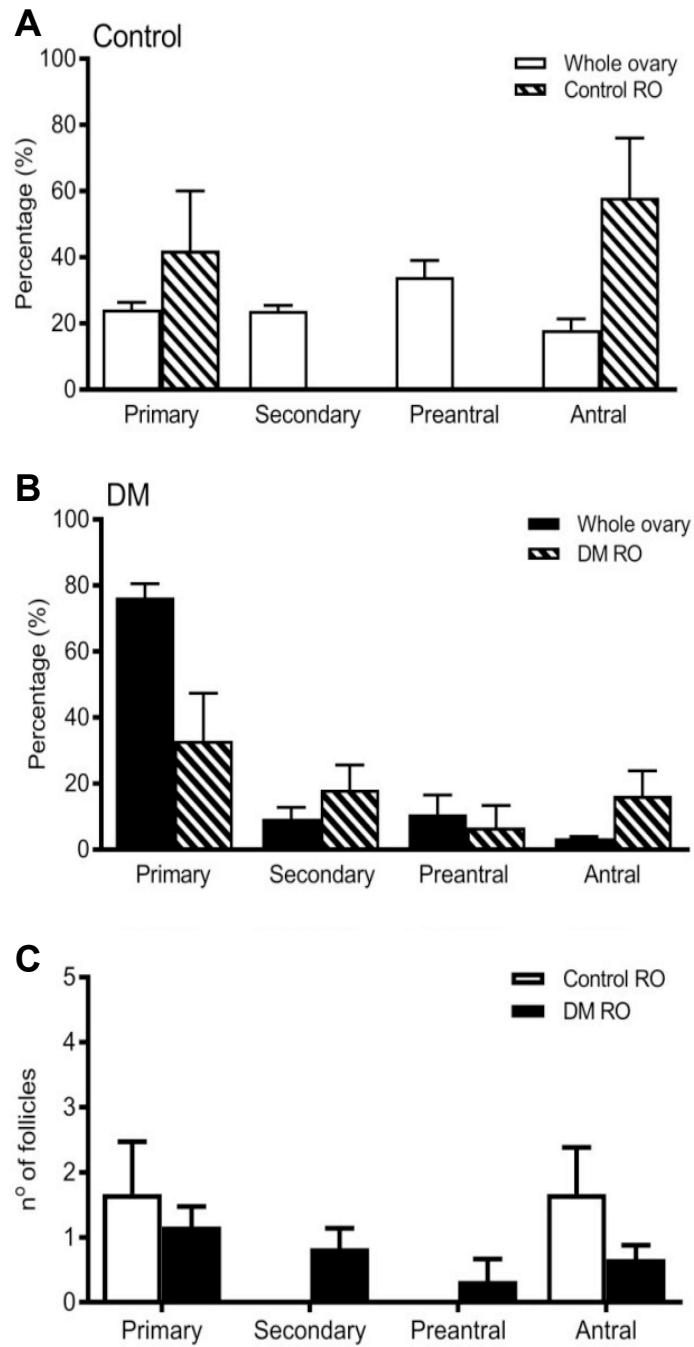


Figure 6.8 Comparison of the proportion of follicles at each stage of development in (A) Control whole ovaries (n=3) and Control ROs (n=5) and (B) DM whole ovaries (DM n=3) and DM ROs (DM n=6). (C) Comparison of follicle numbers between Control and DM ROs. Results are expressed as mean $\pm$ SEM.

6.4.5 Suboptimal follicle development observed in ROs using DM somatic cells

ROs were generated from 9-week old DM or Control somatic cells and newborn WT germ cells (referred to as either DM somatic or Control somatic ROs, respectively). Analysis of total follicle counts revealed the presence of follicles in both Control and DM somatic ROs.

The stage at which follicles existed was categorised; Control somatic ROs contained follicles at all stage of development, whereas the DM somatic ROs contained follicles predominantly at the primary stage (Figure 6.9).

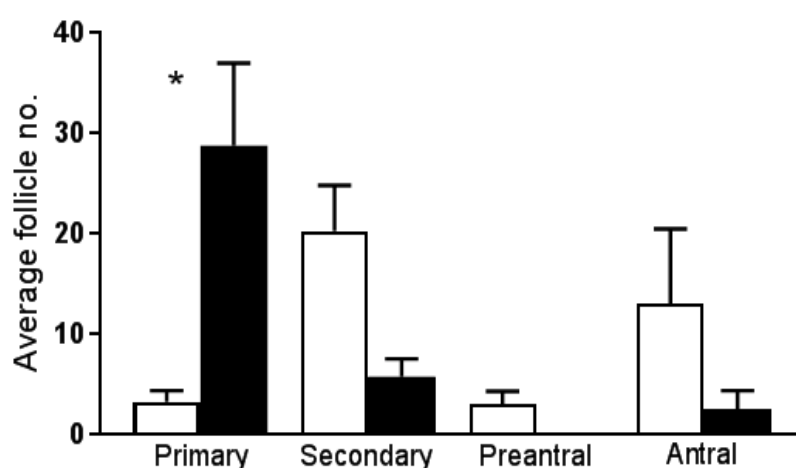


Figure 6.9 Comparison of the number of follicles at each stage of development Control and DM somatic ROs. Control somatic ROs contained follicles at all stages of development, however DM somatic ROs contained follicles predominantly at the primary stage of follicle development. Results are expressed as mean $\pm$ SD.

6.5 Discussion

DM females transition from an infertile state at 9-weeks to developing POI by 3 months of age accompanied by aberrant follicle development. Morphometric analysis of the primary follicles in Chapter 3, revealed that presence of abnormal follicles which may be contributing to the infertility and causing the POI phenotype in DM females.

Follicles are present in approximately 50% of women diagnosed with POI after diagnosis. The presence of follicles in DM ovaries after the onset of POI provides a unique opportunity to develop novel therapies to resume follicle development. The aim of this chapter was to assess the function and role of DM germ and somatic cells in the follicle dysfunction, using the RO technique.

The RO technique allows us to study the different influences of the somatic cells and germ cells individually on follicle development and the interactions that occur between the two cell types. Previously, this technique was established using newborn somatic and germ cells from mice up to 12 days of age (22, 311, 312). Here, we have implemented this technique for the first time using adult germ and somatic cells. ROs produced using 9-week Control or DM germ cells were able to generate follicles at all stages of development, from primary to antral. This was an unexpected finding since at 9-weeks of age DM females are infertile with ovulation rate just above zero (Chapter 4) and ovarian morphology revealed a lack of follicle development. These findings confirm our hypothesis, that modifying the oocyte environment using the RO technique rescued the ability of DM germ cells to coordinate follicle development.

The fact that DM 9-week oocytes retain the ability to sustain follicle development, when associated with WT somatic cells indicates that the genetically altered oocyte is inducing changes in the physiology of the somatic cell compartment resulting in aberrant follicle development and oocyte growth. Therefore, we assessed the ability of DM somatic cells to support follicle development. To our surprise, DM somatic cells exhibited a follicle development profile similar to DM whole ovaries with a block in primary follicle development and a lack of follicles developing to the later stages.

ROs generated using newborn germ cells and either Control or DM somatic cells contained over ten times the number of follicles than ROs generated using newborn somatic cells and either Control or DM germ cells. One explanation for this is the higher number of germ cells obtained from newborn ovaries compared to adult ovaries, though no differences existed between Control and DM (data not shown). Primordial follicles in newborn ovaries are dormant and have not yet undergone activation since this occurs a few days after birth in mice (5, 9, 10). However, in adult ovaries there are fewer primordial follicles since there have been numerous rounds of follicle activation.

In addition to this, there are a number of limitations of these experiments. Firstly, females were not assessed for timing of oestrous cycle when ovaries were collected; however, as with previous experiments, we used littermates to limit any variation due to different timings in oestrous cycle and thus these are equally represented in both groups. Furthermore, to avoid any bias, during the

experiments and analysis the investigator was blind to the genotype of the mice. As with the previous chapter, the same batch of both FBS and FSH were used for all experiments. Finally, the number of ROs generated using Control and DM somatic cells was low (n=3 per group). However, despite the low number of ROs generated, as previously discussed, these ROs contain a 10-fold more follicles than the ROs generated using Control and DM germ cells (n=5 per group). This suggests that the low number is unlikely to have an effect on the follicle numbers we observe.

Successful follicle development requires interactions between the follicle and the surrounding ECM environment, which remodels to adapt to the growing follicle. Our results demonstrate that the interactions between the oocyte and somatic cells are altered in the DM ovary. This indicates that the DM oocyte is affecting somatic cell physiology, imprinting the POI phenotype in this cell compartment and therefore their ability to sustain follicle development. These results provide an explanation for the failure of the DM transplantation to restore follicle development, since the existing DM somatic cells are unable to function.

The generation of ROs using adult ovaries is a newly established and requires further optimisation to establish exact germ and somatic cell number: follicle number ratios. as well as optimisation with human tissue. The results presented in this chapter indicate the RO technique as a promising therapeutic tool to re-establish follicle development in the DM and POI in humans.

**Grafting a DM ovary fragment with a
newborn ovary fragment is unable to
rescue follicle development**

7.1 Introduction

There is a growing need to identify novel interventions and treatment strategies for women diagnosed with POI who wish to conceive, since there are limited treatment options.

A treatment for women with POI who wish to conceive using their own eggs was recently developed, using *in vitro* activation (307, 314). This involved the disruption of the Hippo pathway by ovarian fragmentation followed by Akt stimulation resulting in the resumption of follicle growth. However, this regime did not work for all patients and resulted in only 2 live births out of 37 patients treated (307, 314). Furthermore, we have previously shown that fragmentation of the DM ovaries and transplantation to a normal endocrine environment was not successful in resuming follicle development (unpublished data).

Both of the above discussed treatments are based on the assumption that the existing ovarian architecture is functioning correctly and able to support follicle development. However, in the previous chapter, we demonstrate DM somatic cells were unable to support follicle development indicating that the DM oocyte-specific gene deletion is affecting somatic cell physiology. Oocyte-secreted factors are essential for GC function therefore the oocyte-specific deletion of *Mgat1* and *C1galt1* and therefore the absence of hybrid and complex N-and core 1-derived O-glycans, is likely to be disturbing intra-ovarian signalling in DM ovaries and affecting follicle progression.

It is well known that follicles secrete factors that can affect the development of other follicles (291) since co-culture of follicles *in vitro* resulted in growth inhibition (292). Previously, to test the function of chemotherapy treated ovary environment novel techniques such as the intraovarian transplantation of primordial follicles into chemotherapy induced POI mouse ovaries failed to rescue ovarian function (315) suggesting that the source of the rescue substance is the ovarian stroma or growing follicles. Furthermore, the transplantation of human adipose-derived mesenchymal stem cells into a chemotherapy-damaged ovary resulted in improvement of ovarian function (316); however there was no direct differentiation of these stem cells into oocytes or GCs. The authors speculated these stem cells may survive in the interstitium, providing essential support to oocytes.

Therefore, we hypothesised transplantation of a newborn ovary attached to a DM ovary could rescue follicle development by restoring signalling between oocytes and GCs, either by secreting factors essential for development which may be absent in the DM ovary, or by cells migrating from the newborn ovary to the DM stroma and supporting follicle development.

7.2 Aims

- To investigate if transplantation of a newborn ovary with a DM ovary could rescue follicle development

To address this, we transplanted a graft ovary, consisting of a newborn ovary joined to a DM ovary, into an immunocompromised mouse for 21 days to allow follicles to develop. Following retrieval, histological analysis was performed to assess follicle development.

7.3 Materials and Methods

7.3.1 Mice

Ovaries from littermate Control and DM female pairs aged between 9-10 weeks were combined with *LacZ* positive (*LacZ*⁺) P0-P1 ovaries. *LacZ* expressing pups were generated and genotyped as described in Chapter 6.2.1.1. In order to avoid rejection of the grafted ovaries, immunocompromised mice (B6Rag1) were used as recipients since these mice lack functional immune systems.

7.3.2 Splice experiment

Ovaries were harvested from DM or Control mice into a dish containing sterile phosphate buffered saline (PBS) and the right ovary was divided into two equal fragments by cutting with microdissection scissors under the stereomicroscope. Ovaries were harvested from a newborn *LacZ*⁺ donor mouse and the right ovary halved. In a sterile dish 0.25 µg phytohemagglutinin (PHA) was placed as a 20 µ drop, the DM or Control ovary were placed into this drop using watchmakers forceps, and the newborn ovary was placed on top ensuring the internal edges of each tissue were in contact. Both ovaries were kept in the drop of PHA until transplantation under the kidney capsule of an ovariectomised B6Rag1 mouse, as (described in section 6.3.3). Mice were sacrificed after 21 days and ovaries were collected, fixed and subjected to X-gal staining (described in section 2.8) expect ovaries were fixed for 2 h instead of 1 h. Ovaries were histologically processed (described in section 2.5) and every 10th section was used to analyse follicle development.

7.4 Results

7.4.1 Spliced ovaries are able to develop follicles *in vivo*

Since an experiment splicing ovaries was not reported in the literature, prior to conducting this experiment, we first evaluated if the two ovaries would remain adhered together and develop.

From here on we will refer to the donor (*LacZ*⁺ ovary) and the experimental (Control or DM) ovary combined together as a Control or DM graft, respectively. In total, eight grafts were transplanted (Control n=4 grafts and DM n=4 grafts) however only seven grafts were harvested from host females after 21 days (Control n=4 grafts, DM n=3 grafts) (Figure 7.1).

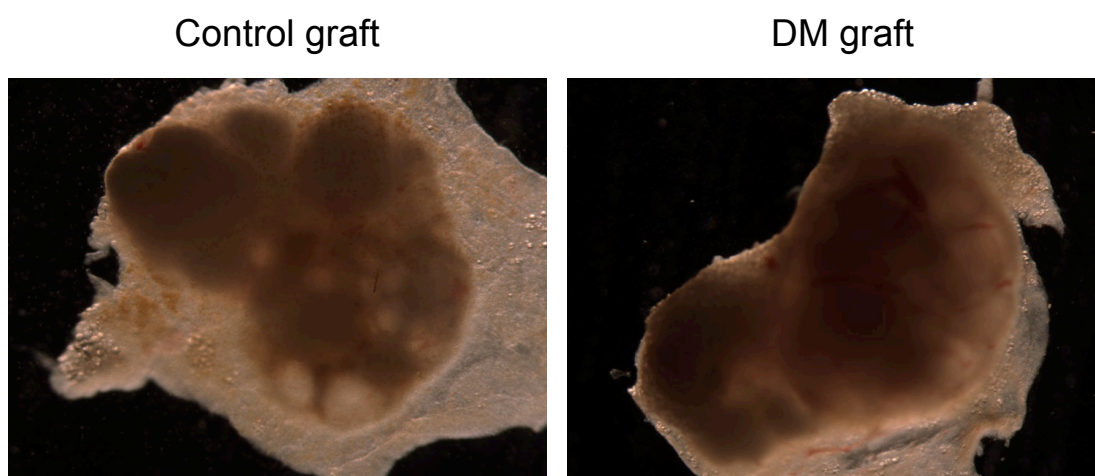


Figure 7.1 Representative images of Control and DM grafts collected 21 days after transplantation. Control or DM grafts consisting of a *LacZ*⁺ and experimental (Control or DM), harvested 21 days after surgery, contained follicles at various stages of development.

Next, we determined whether the grafts contained both experimental and *LacZ*⁺ donor ovaries. Following fixation and X-gal staining, ovaries were embedded, sectioned and mounted to determine the presence of the donor *LacZ*⁺

ovary (staining blue) and experimental Control/DM ovary (unstained). Out of the seven grafts obtained only four were technically successful (Control n=2 grafts, DM n=2 grafts); contained two ovarian pieces, one β -galactosidase staining and the other largely unstained (Figure 7.2). Two unsuccessful grafts (Control n=1 graft, DM n=1 graft) contained only the unstained experimental ovary, whilst one Control graft contained only *LacZ*⁺ ovarian tissue. For all unsuccessful grafts, we speculate that following transfer although we placed the graft away from site of insertion, it may have been dislodged and displaced from the kidney capsule whilst the mouse was moving around.

It was also noted that within each graft, size varied between the two ovaries. It was apparent in the Control grafts that the Control ovary tissue was larger than the *LacZ*⁺ ovary tissue, whereas in the DM graft, the *LacZ*⁺ ovary tissue was larger than the DM ovary tissue.

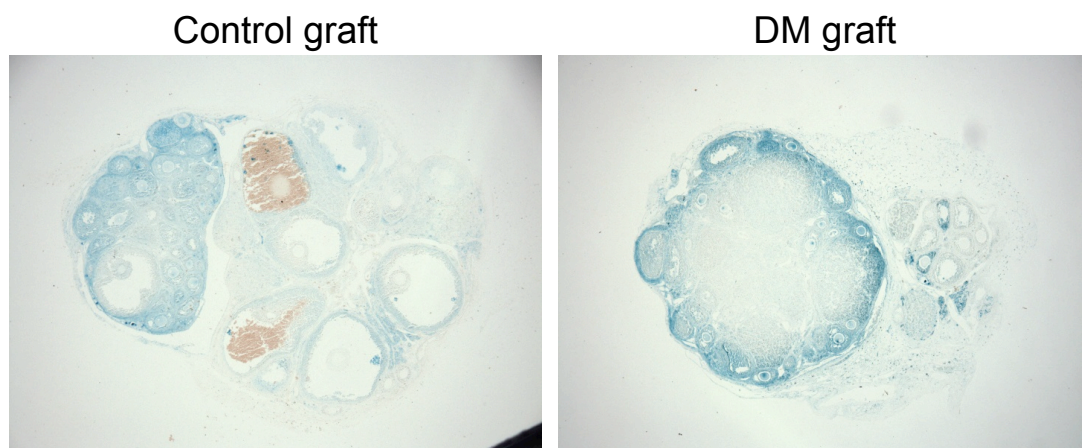


Figure 7.2 Representative images showing successful grafts containing both a *LacZ*⁺ ovary (blue) and Control or DM ovaries. Within Control grafts, the Control ovaries (unstained) are larger in size than the *LacZ*⁺ ovary (blue), whilst in DM grafts, the *LacZ*⁺ ovaries (blue) were larger in size than the DM ovary. (Control n=2 grafts, DM n=2 grafts).

7.4.2 Control grafts have more advanced follicle development compared to DM grafts

Sections were subjected to eosin staining to enhance follicles. Within each graft, follicles were present in both ovaries. It was evident that within Control grafts the Control ovary tissue had more progressed follicle development than the DM ovary tissue in the DM graft (Figure 7.3). From the successful Control grafts (n=2), a total of 6 preovulatory follicles were present in Control ovary tissue, whereas in successful DM grafts (n=2), the most developed follicles present were only at the preantral stage in one whilst in the second, only secondary follicles were visible.

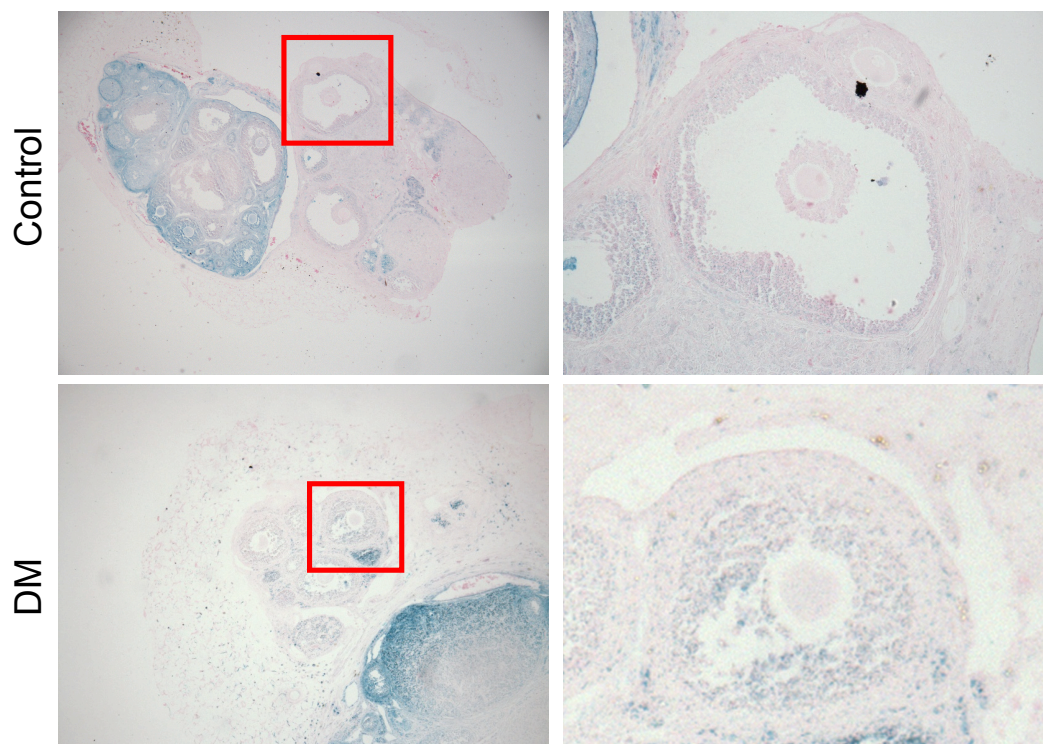


Figure 7.3 Follicle development in Control and DM spliced with *LacZ*⁺ grafts. Control ovary tissue had preovulatory follicles present, whereas DM ovary tissue had only a few preantral follicles present.

Although within Control grafts, the Control ovary was larger than the *LacZ*⁺ ovary, follicle development was equivalent in both the Control and *LacZ*⁺ ovaries since both contained preovulatory follicles. Interestingly in DM grafts, although DM ovaries did not have any preovulatory follicles, the *LacZ*⁺ ovaries did.

7.4.3 Intermingling of cells was detected by X-gal staining

Next, we wanted to assess if any cell migration occurred between the two ovaries within the graft. The contribution of cells from the *LacZ*⁺ ovarian fragments were identified by ubiquitous β -galactosidase staining. There was a *LacZ* penetration issue in the large newborn ovary; however the area of interest (adjacent to the experimental ovary) was still easily distinguishable since only the centre of the newborn ovary tissue had not stained. *LacZ* staining and hence cell mixing between the two ovaries was evident, however no blue oocytes were evident in any healthy follicles in Control or DM ovary tissue within their respective grafts. *LacZ*⁺ staining was detected in somatic cells around follicles in the stroma in both Control and DM ovary tissue (Figure 7.4). We also observed intense *LacZ* staining around dead/dying follicles including the oocyte, and inside CL (Figure 7.4).

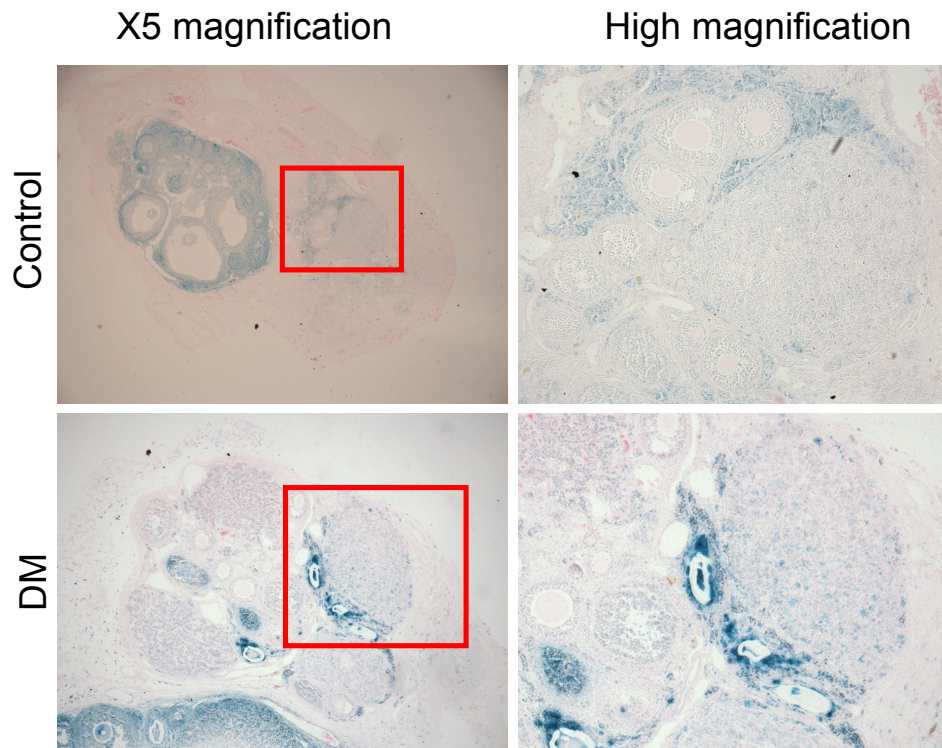


Figure 7.4 Analysis of cell migration between experimental and *LacZ*⁺ ovary. *LacZ*⁺ staining was detected in the stroma and in dying follicles containing dead oocytes and in CL.

7.5 Discussion

There is a growing need to identify novel treatments for women with POI. The experiment in this chapter was designed to test whether a graft of a newborn ovary fragment could rescue follicle development in the DM ovary by re-establishing signalling between the oocytes and GCs or preferentially replace dysfunctional somatic cells.

If the *LacZ*⁺ ovaries were able to rescue DM ovaries, we would expect follicle development to be equal in both Control and DM ovaries, however this was not the case. Although there were only two grafts per group, it is evident that follicle development has not been rescued in DM ovaries since only a few follicles progressed to the preantral stage in one whilst only secondary follicles were present in the second, whilst in Control ovaries follicles developed to the preovulatory stage, with some containing oocyte-cumulus complexes. Therefore no further analysis was carried out.

Injection of human adipose-derived mesenchymal stem cells into a chemotherapy-damaged ovary resulted in stem cells survival in the interstitium and subsequent improvement of ovarian function (316). Similar to this, although no healthy blue oocytes or GCs were detected in Control and DM ovary tissue, *LacZ* staining was located in the stroma of both ovaries. This demonstrates that there was crosstalk within the grafts however this was unable to restore follicle development in DM ovaries. Therefore since results did not indicate recovery of DM ovarian function, we decided not to pursue this experiment any further.

Conclusions and future work

Despite advancements in research and clinical studies, our understanding of the aetiology and pathophysiology of POI is severely limited. The diagnostic evaluation for POI is unavoidably complex due to the highly variable presentation of affected individuals. Since approximately 50% of women with POI have follicles, developing novel therapeutics and diagnostic tools based upon an understanding of how follicle development is affected is critical.

In the DM mouse model of follicular POI, ovarian function progressively declines with age. Though females undergo a normal puberty, they are sub-fertile at 6-weeks of age, producing one small litter, before progressing to an infertile state by 9-weeks of age and undergoing POI by 3-months of age. Since DM females undergo this age-dependant demise of ovarian function before undergoing POI, we hypothesised that follicle development could not be rescued in the DM mouse model. Therefore, the focus of this thesis was to explore potential causes of the deteriorating ovarian function prior to the onset of POI and develop interventions to rescue follicle development.

The findings of this thesis highlight new avenues for investigation to further elucidate the mechanisms underpinning POI as well as targets for treatment. Future work can be split into two parts (i) firstly, taking the existing work forward based on the results and conclusions generated in this thesis and (ii) secondly, developing alternative strategies to address the questions about the nature of the DM phenotype.

Follicle development is modified in DM females with an increase in primary follicle numbers accompanied by a depletion of developing follicles indicating a block in follicle development (1, 236, 244). Using morphometric analysis of primary follicles (Chapter 3), data from Control mice revealed novel insights into early follicle development. We report differences in follicle developmental dynamics with age, with follicles from neonatal mice having accelerated growth compared to post-pubertal mice which may be attributed to differences in location of follicles (medulla vs cortex), composition of the stroma (252), and/or the hormonal profile (248, 256-261).

In DM ovaries, we find abnormal primary 3a follicles containing large oocytes, which we speculate could be contributing to a malfunction and thus the accumulation of primary follicles (1). Deletion of the two glycosyltransferases occurs only in DM oocytes from the primary stage of follicle development onwards, which is when the block in follicle development exists. Here, we see the first evidence of malfunction between oocyte and somatic cell communication in DM, since oocyte growth and GC proliferation are not in synchrony. It is clear that the absence of oocyte produced *N*- and *O*-glycans play a role in regulating follicle growth; glycoproteins play important roles in bidirectional signalling with the surrounding GC (1, 2, 20, 23), and hence in their absence; DM primary follicles undergo precocious growth.

Next, assessment of those developing follicles that have progressed past the primary stage revealed multiple defects in follicle development prior to the presence of the POI. Assessment of follicle dynamics over time before the onset

of POI could facilitate stage-specific diagnosis of early follicle dysregulation in POI. We elucidate that the decline in ovulation rate at 9-weeks (236), resulting in an absence of a second litter in DM females (1), is due to the premature luteinisation of unruptured preovulatory follicles (LUFs). LUFs have been reported in women with POI (184) and as well as being a major pathophysiological mechanism impairing follicle function, the occurrence of LUF has been linked to many conditions such as unexplained infertility and endometriosis (317-319). This finding demonstrates the relevance of the DM as a mouse model of POI, and highlights the unique opportunity it presents to develop therapeutic interventions treatments for POI and prevention of LUFs. Future work would look to use human ovarian tissue from POI patients to understand whether a similar dysfunction in follicle development is observed in women at the clinical onset of POI.

Despite the technique of follicle culture existing for decades (320), follicle culture is still considered a research technique and not considered an assisted reproductive technology (ART). However, our results demonstrate the potential usefulness follicle culture offers as a method to generate antral follicles from women with follicular POI. Since DM females were initially fertile at 6-weeks of age, it is conceivable that follicle development is compromised by extra-follicular factors such as ECM or gonadotrophin balance. Therefore, follicle culture was conducted to assess if removal of extra-follicular blocking signal would permit follicle development to resume. Once again, we see indications that the somatic environment may be impeding follicle development since DM follicles were able to grow *in vitro*, reaching the same size as Control follicles; however survival rate of DM follicles was compromised.

There is a burgeoning body of evidence for the role of the ECM in regulating follicle development. The initial evidences for this came from studies of PCOS, where there is an upregulation of ECM components and aberrant follicle development (296). Alginate culture systems have also supported this theory, since follicles cultured in rigid matrix exhibited aberrant follicle growth and poor quality oocytes (297, 298). Recently, the role of the Hippo pathway in coordinating follicle development and ovarian function has been demonstrated within the mammalian ovary (321, 322). Pioneering work (307) demonstrated that the Hippo pathway is downregulated by fragmentation resulting in resumption of follicle development in POI ovaries; this also explains the success of techniques such as ovarian wedge resection (323), used for treating patients with PCOS to induce follicle growth. However, since the Hippo pathway is disrupted by fragmentation (307), our results from the DM transplantation experiment (unpublished data) discussed in the introduction of Chapter 6, demonstrate that fragmentation alone is not sufficient to resume follicle development, though downregulation of the Hippo pathway has not been measured directly.

Our results demonstrate that that the physiological conditions in which follicles grow play a vital role in regulating their development since ECM changes in the DM are apparent at 3-weeks of age, much earlier than the onset of POI. The increased rigidity and density of DM ovaries is largely conjecture drawn from IHC and follicle dissection experiments. Thus further experiments to determine this need to be undertaken. One method to measure rigidity in the DM ovary is using a rheometer, which has been used to measure mechanical properties using various tissues (324). If changes are detected, these could be an early marker of follicle

dysregulation and thus it may be possible to develop non-invasive diagnostic test to diagnose POI.

Currently, *in vitro* activation (185) is the only ART method for POI patients to have their own biological child; however, this has yielded a low success rate with only 62% of patients responding (307). Furthermore, this treatment is based on the assumption that the ovaries current architecture and somatic cells are able to support follicle development. However, this technique is rendered ineffective since our results in Chapter 6 reveal that DM somatic cells are unable to support successful follicle development. Since DM females have an oocyte-specific deletion and are initially fertile prior to POI, it is clear that the oocyte is inducing changes to the somatic cell compartment. These changes accumulate over time, affecting somatic cell function and inadvertently affecting oocyte function. This also explains why previously, fragmentation and transplantation of DM ovaries was unsuccessful in restoring follicle development. In this instance, the RO technique can be developed as a clinical therapeutic tool to recover follicle development in women with POI and enable them to have their own biological children since we show that follicle dysregulation can be rescued using the RO technique. The data presented in this thesis, although at the proof of concept level, may have an impact on the future therapeutic approaches to re-establishing follicle development in POI. An important next step is simply demonstrating successful follicle development in ROs generated from human ovaries.

From a biological aspect, the RO technique provides a unique opportunity to interrogate the process of follicle development. Recently the Williams lab have

successfully cultured ROs *in vitro*, surpassing the need to transplant ROs into an immunocompromised mouse. This system enables us to watch follicles develop over time and could be used to determine the effects of molecules such as hormones and growth factors on this process. Immediately after generation, a RO is a heterogeneous mixture of germ and somatic cells; how this mixture of cells coordinate follicle development is unknown. Are somatic cells surrounding the oocyte, transdifferentiated into GCs or are GCs actively recruited to the oocyte? To investigate this, ROs using specific cell lineage tracing can be generated, for example using fluorescence labelled GCs, using a GC-specific marker such as Foxl2; this would allow us to trace the fate of the cells.

Abnormalities in mitochondrial structure of GCs from small growing follicles in a fragile X mouse model of POI have been reported (325). Whether mitochondrial abnormalities or death (mitophagy) is playing a role in the demise of ovarian function in DM mice, could be elucidated by assessing mitochondria of somatic cells. In provisional experiments (in collaboration with Eszter Dombi, a PhD student in Prof. Joanna Poulton's lab (Nuffield Department Obstetrics and Gynaecology)) we have visualised mitochondrial (TOM20) co-localisation with LC3-II positive autophagosomes, which is indicative of mitophagy, using newborn somatic cells (Figure 8.1) using the protocol described in Diot et al, (326). The next step would be to compare mitochondrial content and mitophagy in DM somatic cells at the different ages. Furthermore, using technologies such as ChIP-Seq or RNA-Seq will allow deep interrogation of the DM somatic cell transcriptome and reveal the role of specific molecular and cellular mechanisms of epigenetic regulation in ovarian function.

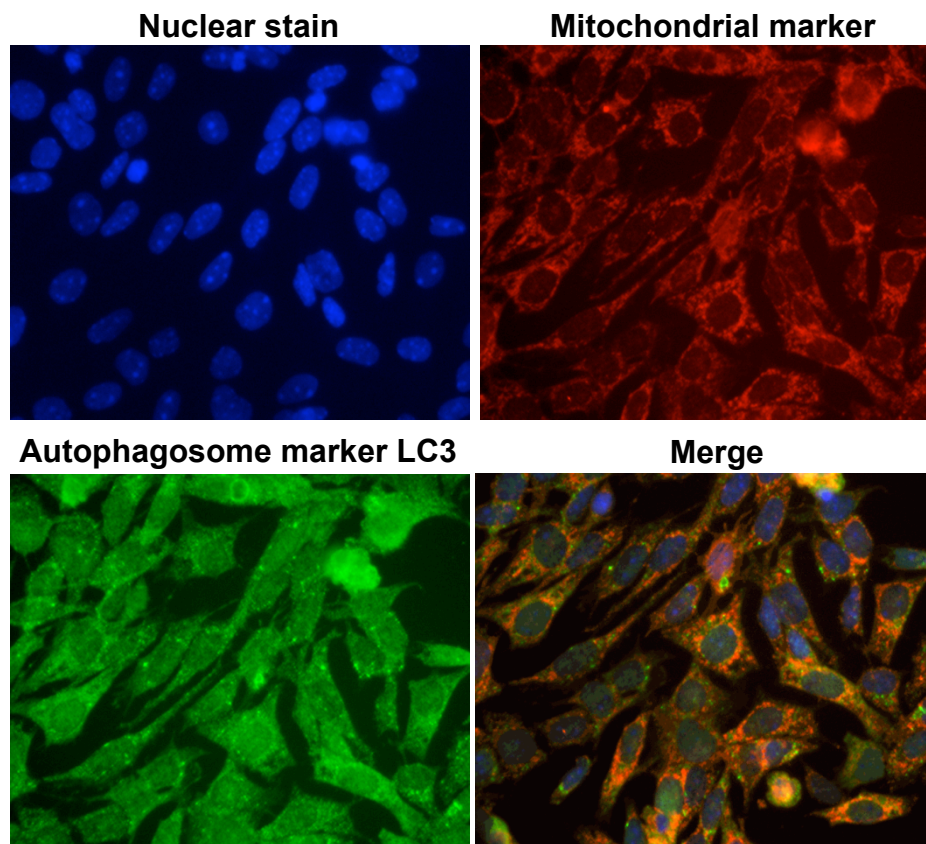


Figure 8.1 Visualisation of autophagosomes in newborn somatic cells. Newborn somatic cells were seeded in a Nunc F 96-well plate (Thermo Scientific) overnight. Cells were fixed with 4% paraformaldehyde for 15 min and immuno-stained, at room temperature in the dark, using a monoclonal anti-TOM20 (dilution 1 in 200) and a polyclonal anti-LC3 (dilution 1 in 500) antibodies for 2 hours. Alexa Fluor 488 Goat anti-rabbit (dilution 1 in 500) and Alexa Fluor 546 Goat anti-mouse (dilution 1 in 200) secondary antibodies were then incubated for 1 hour with the respective primary antibody preparations. After a DAPI staining, dilution 1 in 1000 for 5 min, the cells were imaged using the IN Cell 1000 analyser (GE Healthcare Life sciences).

Finally, in the introduction of this thesis (Section 1.2.4), we discussed the stages of ovarian failure in women and the relationships between these stages. The findings in this thesis strengthen the DM as a mouse model of POI since females exhibit all stages of ovarian dysfunction and failure (Figure 8.2). At 6-weeks of age, DM females have normal ovulating ovaries and are able to conceive (1, 236). Females quickly transition to the early dysfunctional stage at 9-weeks with the presence of LUFs. Hereafter, the follicles in the ovary do not respond to gonadotrophin stimulation (1) demonstrating the resistant ovary-like phase at 3-months. Finally, analysis of 1-year old ovaries revealed that although DM have primordial follicles there are significantly fewer compared to Controls (unpublished data). Since gene deletion occurs after the formation of the primordial follicle pool, this suggests there is an overactivation of primordial follicles, possibly due to the absence of negative feedback from more developed follicles and indicates that DM females are moving towards an afollicular state.

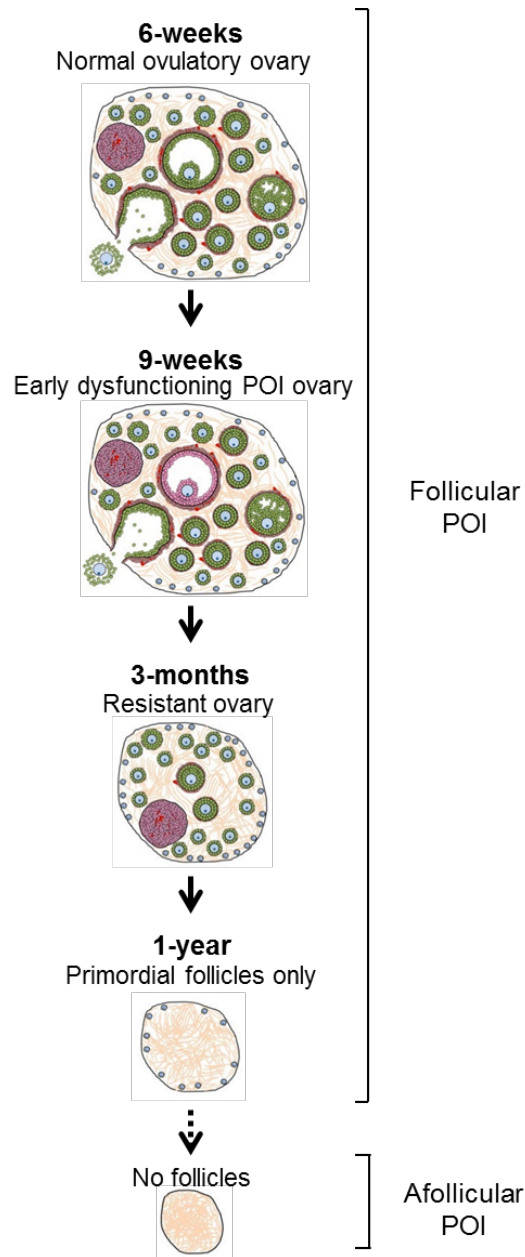


Figure 8.2 Stages of ovarian dysfunction and failure exhibited by the DM mouse model. At 6-weeks of age DM females are fertile but quickly transition to the early dysfunctional stage by 9-weeks, characterised by the presence of luteinised unruptured follicles (LUF, pink follicle). At 3-months of age, females undergo POI and are unresponsive to gonadotrophin stimulation (resistant ovary state) and by 1-year of age, DM ovaries have significantly fewer primordial follicles compared to Control ovaries. The primordial follicle pool is eventually exhausted and the ovary becomes afollicular.

With the majority of POI cases remaining idiopathic, it is extremely challenging to diagnose patients in the early stages of POI. Animal models remain an invaluable tool in understanding the pathophysiology of POI, as an inability to study women before the clinical onset of POI severely limits our ability to conduct this work into humans. The DM mouse model therefore provides a unique opportunity to develop interventions and treatments at each stage of ovarian failure to try and recover ovarian function.

References

1. Williams SA, Stanley P. Premature ovarian failure in mice with oocytes lacking core 1-derived O-glycans and complex N-glycans. *Endocrinology*. 2011;152(3):1057-66.
2. Grasa P, Ploutarchou P, Williams SA. Oocytes lacking O-glycans alter follicle development and increase fertility by increasing follicle FSH sensitivity, decreasing apoptosis, and modifying GDF9:BMP15 expression. *FASEB J*. 2015;29(2):525-39.
3. Kaune H, Sheikh S, Williams SA. Analysis of in vitro follicle development during the onset of premature ovarian insufficiency in a mouse model. *Reprod Fertil Dev*. 2016.
4. Grasa P, Sheikh S, Krzys N, Millar K, Janjua S, Nawaggi P, et al. Dysregulation of follicle development in a mouse model of premature ovarian insufficiency. *Reproduction*. 2016;152(5):591-601.
5. Hirshfield AN. Development of follicles in the mammalian ovary. *Int Rev Cytol*. 1991;124:43-101.
6. McGee EA, Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev*. 2000;21(2):200-14.
7. Baker TG. A Quantitative and Cytological Study of Germ Cells in Human Ovaries. *Proceedings of the Royal Society of London Series B, Biological sciences*. 1963;158:417-33.
8. Gondos B, Westergaard L, Byskov AG. Initiation of oogenesis in the human fetal ovary: ultrastructural and squash preparation study. *Am J Obstet Gynecol*. 1986;155(1):189-95.
9. McNatty KP, Fidler AE, Juengel JL, Quirke LD, Smith PR, Heath DA, et al. Growth and paracrine factors regulating follicular formation and cellular function. *Mol Cell Endocrinol*. 2000;163(1-2):11-20.
10. Pepling ME, Spradling AC. Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Dev Biol*. 2001;234(2):339-51.
11. Richardson SJ, Senikas V, Nelson JF. Follicular depletion during the menopausal transition: evidence for accelerated loss and ultimate exhaustion. *J Clin Endocrinol Metab*. 1987;65(6):1231-7.
12. Faddy MJ, Gosden RG, Gougeon A, Richardson SJ, Nelson JF. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. *Hum Reprod*. 1992;7(10):1342-6.
13. Faddy MJ, Gosden RG. A model conforming the decline in follicle numbers to the age of menopause in women. *Hum Reprod*. 1996;11(7):1484-6.
14. Hansen KR, Knowlton NS, Thyer AC, Charleston JS, Soules MR, Klein NA. A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause. *Hum Reprod*. 2008;23(3):699-708.
15. Peters H. The development of the mouse ovary from birth to maturity. *Acta Endocrinol (Copenh)*. 1969;62(1):98-116.
16. Pedersen T. Follicle kinetics in the ovary of the cyclic mouse. *Acta Endocrinol (Copenh)*. 1970;64(2):304-23.

17. Buccione R, Vanderhyden BC, Caron PJ, Eppig JJ. FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. *Dev Biol.* 1990;138(1):16-25.
18. Salustri A, Yanagishita M, Hascall VC. Mouse oocytes regulate hyaluronic acid synthesis and mucification by FSH-stimulated cumulus cells. *Dev Biol.* 1990;138(1):26-32.
19. Vanderhyden BC, Caron PJ, Buccione R, Eppig JJ. Developmental pattern of the secretion of cumulus expansion-enabling factor by mouse oocytes and the role of oocytes in promoting granulosa cell differentiation. *Dev Biol.* 1990;140(2):307-17.
20. Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. *Reproduction.* 2001;122(6):829-38.
21. Albertini DF, Barrett SL. Oocyte-somatic cell communication. *Reprod Suppl.* 2003;61:49-54.
22. Eppig JJ, Wigglesworth K, Pendola FL. The mammalian oocyte orchestrates the rate of ovarian follicular development. *Proc Natl Acad Sci U S A.* 2002;99(5):2890-4.
23. Matzuk MM, Burns KH, Viveiros MM, Eppig JJ. Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science.* 2002;296(5576):2178-80.
24. Pedersen T, Peters H. Proposal for a Classification of Oocytes and Follicles in Mouse Ovary. *Journal of Reproduction and Fertility.* 1968;17(3):555-7.
25. Rodgers RJ, van Wezel IL, Irving-Rodgers HF, Lavranos TC, Irvine CM, Krupa M. Roles of extracellular matrix in follicular development. *J Reprod Fertil Suppl.* 1999;54:343-52.
26. Irving-Rodgers HF, Hummitzsch K, Murdiyarso LS, Bonner WM, Sado Y, Ninomiya Y, et al. Dynamics of extracellular matrix in ovarian follicles and corpora lutea of mice. *Cell Tissue Res.* 2010;339(3):613-24.
27. Lintern-Moore S, Moore GP. The initiation of follicle and oocyte growth in the mouse ovary. *Biol Reprod.* 1979;20(4):773-8.
28. Qvist R, Blackwell LF, Bourne H, Brown JB. Development of mouse ovarian follicles from primary to preovulatory stages in vitro. *J Reprod Fertil.* 1990;89(1):169-80.
29. Elvin JA, Matzuk MM. Mouse models of ovarian failure. *Rev Reprod.* 1998;3(3):183-95.
30. Picton HM. Activation of follicle development: the primordial follicle. *Theriogenology.* 2001;55(6):1193-210.
31. Hutt KJ, Albertini DF. An oocentric view of folliculogenesis and embryogenesis. *Reprod Biomed Online.* 2007;14(6):758-64.
32. Young JM, McNeilly AS. Theca: the forgotten cell of the ovarian follicle. *Reproduction.* 2010;140(4):489-504.
33. Philpott CC, Ringuette MJ, Dean J. Oocyte-specific expression and developmental regulation of ZP3, the sperm receptor of the mouse zona pellucida. *Dev Biol.* 1987;121(2):568-75.
34. Kumar TR, Wang Y, Lu N, Matzuk MM. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet.* 1997;15(2):201-4.
35. Abel MH, Wootton AN, Wilkins V, Huhtaniemi I, Knight PG, Charlton HM. The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. *Endocrinology.* 2000;141(5):1795-803.

36. Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, et al. Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc Natl Acad Sci U S A*. 1998;95(23):13612-7.
37. Smith SS, Ojeda SR. Neonatal release of gonadotropins is essential for development of ovarian follicle-stimulating hormone receptors. *Biol Reprod*. 1986;34(1):219-27.
38. Anderson E, Albertini DF. Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. *J Cell Biol*. 1976;71(2):680-6.
39. Rodgers RJ, Irving-Rodgers HF. Formation of the ovarian follicular antrum and follicular fluid. *Biol Reprod*. 2010;82(6):1021-9.
40. Shalgi R, Kraicer PF, Soferman N. Gases and electrolytes of human follicular fluid. *J Reprod Fertil*. 1972;28(3):335-40.
41. Gosden RG, Hunter RH, Telfer E, Torrance C, Brown N. Physiological factors underlying the formation of ovarian follicular fluid. *J Reprod Fertil*. 1988;82(2):813-25.
42. Telfer E, Ansell JD, Taylor H, Gosden RG. The number of clonal precursors of the follicular epithelium in the mouse ovary. *J Reprod Fertil*. 1988;84(1):105-10.
43. Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol Reprod Dev*. 2002;61(3):414-24.
44. Chen L, Russell PT, Larsen WJ. Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. *Mol Reprod Dev*. 1993;34(1):87-93.
45. Damber JE, Cajander S, Gafvels M, Selstam G. Blood flow changes and vascular appearance in preovulatory follicles and corpora lutea in immature, pregnant mare's serum gonadotropin-treated rats. *Biol Reprod*. 1987;37(3):651-8.
46. Carambula SF, Matikainen T, Lynch MP, Flavell RA, Goncalves PB, Tilly JL, et al. Caspase-3 is a pivotal mediator of apoptosis during regression of the ovarian corpus luteum. *Endocrinology*. 2002;143(4):1495-501.
47. Davis JS, Rueda BR. The corpus luteum: an ovarian structure with maternal instincts and suicidal tendencies. *Front Biosci*. 2002;7:d1949-78.
48. Nelson JF, Felicio LS, Randall PK, Sims C, Finch CE. A longitudinal study of estrous cyclicity in aging C57BL/6J mice: I. Cycle frequency, length and vaginal cytology. *Biol Reprod*. 1982;27(2):327-39.
49. Hsueh AJ, Billig H, Tsafiriri A. Ovarian follicle atresia: a hormonally controlled apoptotic process. *Endocr Rev*. 1994;15(6):707-24.
50. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British journal of cancer*. 1972;26(4):239-57.
51. Tsujimoto H, Takeshita S, Nakatani K, Kawamura Y, Tokutomi T, Sekine I. Delayed apoptosis of circulating neutrophils in Kawasaki disease. *Clinical and Experimental Immunology*. 2001;126(2):355-64.
52. van Delft MF, Huang DC. How the Bcl-2 family of proteins interact to regulate apoptosis. *Cell research*. 2006;16(2):203-13.
53. Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol*. 2008;9(3):231-41.
54. Irving-Rodgers HF, Rodgers RJ. Extracellular matrix of the developing ovarian follicle. *Semin Reprod Med*. 2006;24(4):195-203.

55. Luck MR, Zhao Y, Silvester LM. Identification and localization of collagen types I and IV in the ruminant follicle and corpus luteum. *J Reprod Fertil Suppl.* 1995;49:517-21.
56. Luck MR, Zhao Y. Identification and measurement of collagen in the bovine corpus luteum and its relationship with ascorbic acid and tissue development. *J Reprod Fertil.* 1993;99(2):647-52.
57. Christensen AP, Patel SH, Grasa P, Christian HC, Williams SA. Oocyte glycoproteins regulate the form and function of the follicle basal lamina and theca cells. *Dev Biol.* 2015;401(2):287-98.
58. Berkholtz CB, Lai BE, Woodruff TK, Shea LD. Distribution of extracellular matrix proteins type I collagen, type IV collagen, fibronectin, and laminin in mouse folliculogenesis. *Histochemistry and cell biology.* 2006;126(5):583-92.
59. van Wezel IL, Rodgers RJ. Morphological characterization of bovine primordial follicles and their environment in vivo. *Biol Reprod.* 1996;55(5):1003-11.
60. Juengel JL, Sawyer HR, Smith PR, Quirke LD, Heath DA, Lun S, et al. Origins of follicular cells and ontogeny of steroidogenesis in ovine fetal ovaries. *Mol Cell Endocrinol.* 2002;191(1):1-10.
61. Perloff WH, Schultz J, Farris EJ, Balin H. Some aspects of the chemical nature of human ovarian follicular fluid. *Fertil Steril.* 1954;6(1):11-7.
62. Shalgi R, Kraicer P, Rimon A, Pinto M, Soferman N. Proteins of human follicular fluid: the blood-follicle barrier. *Fertil Steril.* 1973;24(6):429-34.
63. Hess KA, Chen L, Larsen WJ. The ovarian blood follicle barrier is both charge- and size-selective in mice. *Biol Reprod.* 1998;58(3):705-11.
64. Berkholtz CB, Shea LD, Woodruff TK. Extracellular matrix functions in follicle maturation. *Semin Reprod Med.* 2006;24(4):262-9.
65. Erickson GF, Magoffin DA, Dyer CA, Hofeditz C. The ovarian androgen producing cells: a review of structure/function relationships. *Endocr Rev.* 1985;6(3):371-99.
66. Honda A, Hirose M, Hara K, Matoba S, Inoue K, Miki H, et al. Isolation, characterization, and in vitro and in vivo differentiation of putative thecal stem cells. *Proc Natl Acad Sci U S A.* 2007;104(30):12389-94.
67. Rankin T, Dean J. The molecular genetics of the zona pellucida: mouse mutations and infertility. *Mol Hum Reprod.* 1996;2(11):889-94.
68. Phillips DM, Shalgi R. Surface architecture of the mouse and hamster zona pellucida and oocyte. *J Ultrastruct Res.* 1980;72(1):1-12.
69. Lunsford RD, Jenkins NA, Kozak CA, Liang LF, Silan CM, Copeland NG, et al. Genomic mapping of murine Zp-2 and Zp-3, two oocyte-specific loci encoding zona pellucida proteins. *Genomics.* 1990;6(1):184-7.
70. Epifano O, Liang LF, Dean J. Mouse Zp1 encodes a zona pellucida protein homologous to egg envelope proteins in mammals and fish. *J Biol Chem.* 1995;270(45):27254-8.
71. Bleil JD, Wassarman PM. Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Dev Biol.* 1980;76(1):185-202.
72. Shimizu S, Tsuji M, Dean J. In vitro biosynthesis of three sulfated glycoproteins of murine zonae pellucidae by oocytes grown in follicle culture. *J Biol Chem.* 1983;258(9):5858-63.
73. Snell WJ, White JM. The molecules of mammalian fertilization. *Cell.* 1996;85(5):629-37.

74. Modlinski JA. The role of the zona pellucida in the development of mouse eggs in vivo. *J Embryol Exp Morphol.* 1970;23(3):539-47.
75. Wassarman PM, Liu C, Litscher ES. Constructing the mammalian egg zona pellucida: some new pieces of an old puzzle. *J Cell Sci.* 1996;109 (Pt 8):2001-4.
76. Wassarman PM, Litscher ES. Influence of the zona pellucida of the mouse egg on folliculogenesis and fertility. *Int J Dev Biol.* 2012;56(10-12):833-9.
77. Shur BD, Hall NG. A role for mouse sperm surface galactosyltransferase in sperm binding to the egg zona pellucida. *J Cell Biol.* 1982;95(2 Pt 1):574-9.
78. Rankin T, Talbot P, Lee E, Dean J. Abnormal zonae pellucidae in mice lacking ZP1 result in early embryonic loss. *Development.* 1999;126(17):3847-55.
79. Rankin TL, O'Brien M, Lee E, Wigglesworth K, Eppig J, Dean J. Defective zonae pellucidae in Zp2-null mice disrupt folliculogenesis, fertility and development. *Development.* 2001;128(7):1119-26.
80. Rankin T, Familari M, Lee E, Ginsberg A, Dwyer N, Blanchette-Mackie J, et al. Mice homozygous for an insertional mutation in the Zp3 gene lack a zona pellucida and are infertile. *Development.* 1996;122(9):2903-10.
81. Richards JS, Fitzpatrick SL, Clemens JW, Morris JK, Alliston T, Sirois J. Ovarian cell differentiation: a cascade of multiple hormones, cellular signals, and regulated genes. *Recent Prog Horm Res.* 1995;50:223-54.
82. Elvin JA, Clark AT, Wang P, Wolfman NM, Matzuk MM. Paracrine actions of growth differentiation factor-9 in the mammalian ovary. *Mol Endocrinol.* 1999;13(6):1035-48.
83. Shimasaki S, Moore RK, Otsuka F, Erickson GF. The bone morphogenetic protein system in mammalian reproduction. *Endocr Rev.* 2004;25(1):72-101.
84. Levine JE, Ramirez VD. Luteinizing hormone-releasing hormone release during the rat estrous cycle and after ovariectomy, as estimated with push-pull cannulae. *Endocrinology.* 1982;111(5):1439-48.
85. Antunes JL, Carmel PW, Housepian EM, Ferin M. Luteinizing hormone-releasing hormone in human pituitary blood. *Journal of neurosurgery.* 1978;49(3):382-6.
86. Baba Y, Matsuo H, Schally AV. Structure of the porcine LH- and FSH-releasing hormone. II. Confirmation of the proposed structure by conventional sequential analyses. *Biochem Biophys Res Commun.* 1971;44(2):459-63.
87. Schally AV, Arimura A, Kastin AJ, Matsuo H, Baba Y, Redding TW, et al. Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle-stimulating hormones. *Science.* 1971;173(4001):1036-8.
88. Collins TJ, Parkening TA, Smith ER. Plasma and pituitary concentrations of LH, FSH and prolactin after injection of GnRH in aged female C57BL/6 mice. *Neurobiology of aging.* 1981;2(2):125-31.
89. Clarke IJ, Cummins JT, Findlay JK, Burman KJ, Doughton BW. Effects on plasma luteinizing hormone and follicle-stimulating hormone of varying the frequency and amplitude of gonadotropin-releasing hormone pulses in ovariectomized ewes with hypothalamo-pituitary disconnection. *Neuroendocrinology.* 1984;39(3):214-21.
90. Belchetz PE, Plant TM, Nakai Y, Keogh EJ, Knobil E. Hypophysial responses to continuous and intermittent delivery of hypophthalamic gonadotropin-releasing hormone. *Science.* 1978;202(4368):631-3.
91. Burns KH, Matzuk MM. Minireview: genetic models for the study of gonadotropin actions. *Endocrinology.* 2002;143(8):2823-35.

92. Baenziger JU, Green ED. Pituitary glycoprotein hormone oligosaccharides: structure, synthesis and function of the asparagine-linked oligosaccharides on lutropin, follitropin and thyrotropin. *Biochim Biophys Acta*. 1988;947(2):287-306.
93. Camp TA, Rahal JO, Mayo KE. Cellular localization and hormonal regulation of follicle-stimulating hormone and luteinizing hormone receptor messenger RNAs in the rat ovary. *Mol Endocrinol*. 1991;5(10):1405-17.
94. Moon YS, Tsang BK, Simpson C, Armstrong DT. 17 beta-Estradiol biosynthesis in cultured granulosa and thecal cells of human ovarian follicles: stimulation by follicle-stimulating hormone. *J Clin Endocrinol Metab*. 1978;47(2):263-7.
95. Brown JB. Pituitary control of ovarian function--concepts derived from gonadotrophin therapy. *The Australian & New Zealand journal of obstetrics & gynaecology*. 1978;18(1):46-54.
96. Leung PC, Armstrong DT. Interactions of steroids and gonadotropins in the control of steroidogenesis in the ovarian follicle. *Annu Rev Physiol*. 1980;42:71-82.
97. Hillier SG. Current concepts of the roles of follicle stimulating hormone and luteinizing hormone in folliculogenesis. *Hum Reprod*. 1994;9(2):188-91.
98. Dorrington JH, Moon YS, Armstrong DT. Estradiol-17beta biosynthesis in cultured granulosa cells from hypophysectomized immature rats; stimulation by follicle-stimulating hormone. *Endocrinology*. 1975;97(5):1328-31.
99. Fortune JE, Armstrong DT. Androgen production by theca and granulosa isolated from proestrous rat follicles. *Endocrinology*. 1977;100(5):1341-7.
100. Hillier SG, De Zwart FA. Evidence that granulosa cell aromatase induction/activation by follicle-stimulating hormone is an androgen receptor-regulated process in-vitro. *Endocrinology*. 1981;109(4):1303-5.
101. Drummond AE. The role of steroids in follicular growth. *Reprod Biol Endocrinol*. 2006;4:16.
102. Zhuang LZ, Adashi EY, Hsueh AJ. Direct enhancement of gonadotropin-stimulated ovarian estrogen biosynthesis by estrogen and clomiphene citrate. *Endocrinology*. 1982;110(6):2219-21.
103. Wang XN, Greenwald GS. Synergistic effects of steroids with FSH on folliculogenesis, steroidogenesis and FSH- and hCG-receptors in hypophysectomized mice. *J Reprod Fertil*. 1993;99(2):403-13.
104. Billig H, Furuta I, Hsueh AJ. Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. *Endocrinology*. 1993;133(5):2204-12.
105. Goldenberg RL, Vaitukaitis JL, Ross GT. Estrogen and follicle stimulation hormone interactions on follicle growth in rats. *Endocrinology*. 1972;90(6):1492-8.
106. Reilly CM, Cannady WE, Mahesh VB, Stopper VS, De Sevilla LM, Mills TM. Duration of estrogen exposure prior to follicle-stimulating hormone stimulation is critical to granulosa cell growth and differentiation in rats. *Biol Reprod*. 1996;54(6):1336-42.
107. Farookhi R, Desjardins J. Luteinizing hormone receptor induction in dispersed granulosa cells requires estrogen. *Mol Cell Endocrinol*. 1986;47(1-2):13-24.
108. Petersen DN, Tkalcevic GT, Koza-Taylor PH, Turi TG, Brown TA. Identification of estrogen receptor beta2, a functional variant of estrogen receptor beta expressed in normal rat tissues. *Endocrinology*. 1998;139(3):1082-92.
109. Couse JF, Lindzey J, Grandien K, Gustafsson JA, Korach KS. Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and

- estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. *Endocrinology*. 1997;138(11):4613-21.
110. Kregge JH, Hodgins JB, Couse JF, Enmark E, Warner M, Mahler JF, et al. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc Natl Acad Sci U S A*. 1998;95(26):15677-82.
 111. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci U S A*. 1993;90(23):11162-6.
 112. Schomberg DW, Couse JF, Mukherjee A, Lubahn DB, Sar M, Mayo KE, et al. Targeted disruption of the estrogen receptor-alpha gene in female mice: characterization of ovarian responses and phenotype in the adult. *Endocrinology*. 1999;140(6):2733-44.
 113. Fisher CR, Graves KH, Parlow AF, Simpson ER. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proc Natl Acad Sci U S A*. 1998;95(12):6965-70.
 114. Britt KL, Drummond AE, Cox VA, Dyson M, Wreford NG, Jones ME, et al. An age-related ovarian phenotype in mice with targeted disruption of the Cyp 19 (aromatase) gene. *Endocrinology*. 2000;141(7):2614-23.
 115. Britt KL, Saunders PK, McPherson SJ, Misso ML, Simpson ER, Findlay JK. Estrogen actions on follicle formation and early follicle development. *Biol Reprod*. 2004;71(5):1712-23.
 116. Graham JD, Clarke CL. Physiological action of progesterone in target tissues. *Endocr Rev*. 1997;18(4):502-19.
 117. Niswender GD. Molecular control of luteal secretion of progesterone. *Reproduction*. 2002;123(3):333-9.
 118. Teilmann SC, Clement CA, Thorup J, Byskov AG, Christensen ST. Expression and localization of the progesterone receptor in mouse and human reproductive organs. *J Endocrinol*. 2006;191(3):525-35.
 119. Mulac-Jericevic B, Mullinax RA, DeMayo FJ, Lydon JP, Conneely OM. Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. *Science*. 2000;289(5485):1751-4.
 120. Mulac-Jericevic B, Lydon JP, DeMayo FJ, Conneely OM. Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. *Proc Natl Acad Sci U S A*. 2003;100(17):9744-9.
 121. Jia XC, Kessel B, Welsh TH, Jr., Hsueh AJ. Androgen inhibition of follicle-stimulating hormone-stimulated luteinizing hormone receptor formation in cultured rat granulosa cells. *Endocrinology*. 1985;117(1):13-22.
 122. Nandedkar TD, Munshi SR. Effect of dihydrotestosterone on follicular development, ovulation and reproductive capacity of mice. *J Reprod Fertil*. 1981;62(1):21-4.
 123. Hu YC, Wang PH, Yeh S, Wang RS, Xie C, Xu Q, et al. Subfertility and defective folliculogenesis in female mice lacking androgen receptor. *Proc Natl Acad Sci U S A*. 2004;101(31):11209-14.
 124. Knight PG, Glistner C. TGF-beta superfamily members and ovarian follicle development. *Reproduction*. 2006;132(2):191-206.
 125. Munsterberg A, Lovell-Badge R. Expression of the mouse anti-mullerian hormone gene suggests a role in both male and female sexual differentiation. *Development*. 1991;113(2):613-24.

126. Hirobe S, He WW, Lee MM, Donahoe PK. Mullerian inhibiting substance messenger ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic activity. *Endocrinology*. 1992;131(2):854-62.
127. Durlinger AL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA, et al. Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. *Endocrinology*. 1999;140(12):5789-96.
128. McPherron AC, Lee SJ. GDF-3 and GDF-9: two new members of the transforming growth factor-beta superfamily containing a novel pattern of cysteines. *J Biol Chem*. 1993;268(5):3444-9.
129. Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ, Matzuk MM. The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. *Mol Endocrinol*. 1998;12(12):1809-17.
130. Roh JS, Bondestam J, Mazerbourg S, Kaivo-Oja N, Groome N, Ritvos O, et al. Growth differentiation factor-9 stimulates inhibin production and activates Smad2 in cultured rat granulosa cells. *Endocrinology*. 2003;144(1):172-8.
131. Kaivo-Oja N, Bondestam J, Kamarainen M, Koskimies J, Vitt U, Cranfield M, et al. Growth differentiation factor-9 induces Smad2 activation and inhibin B production in cultured human granulosa-luteal cells. *J Clin Endocrinol Metab*. 2003;88(2):755-62.
132. Moore RK, Otsuka F, Shimasaki S. Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. *J Biol Chem*. 2003;278(1):304-10.
133. Peng J, Li Q, Wigglesworth K, Rangarajan A, Kattamuri C, Peterson RT, et al. Growth differentiation factor 9:bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions. *Proc Natl Acad Sci U S A*. 2013;110(8):E776-85.
134. Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, et al. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Mol Endocrinol*. 2001;15(6):854-66.
135. Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM. Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature*. 1996;383(6600):531-5.
136. Carabatsos MJ, Elvin J, Matzuk MM, Albertini DF. Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. *Dev Biol*. 1998;204(2):373-84.
137. Elvin JA, Yan C, Wang P, Nishimori K, Matzuk MM. Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. *Mol Endocrinol*. 1999;13(6):1018-34.
138. Wu X, Chen L, Brown CA, Yan C, Matzuk MM. Interrelationship of growth differentiation factor 9 and inhibin in early folliculogenesis and ovarian tumorigenesis in mice. *Mol Endocrinol*. 2004;18(6):1509-19.
139. Dixit H, Rao LK, Padmalatha VV, Kanakavalli M, Deenadayal M, Gupta N, et al. Missense mutations in the BMP15 gene are associated with ovarian failure. *Human genetics*. 2006;119(4):408-15.
140. Laissue P, Christin-Maitre S, Touraine P, Kuttann F, Ritvos O, Aittomaki K, et al. Mutations and sequence variants in GDF9 and BMP15 in patients with premature ovarian failure. *European journal of endocrinology / European Federation of Endocrine Societies*. 2006;154(5):739-44.
141. Coulam CB, Adamson SC, Annegers JF. Incidence of premature ovarian failure. *Obstet Gynecol*. 1986;67(4):604-6.

142. Meskhi A, Seif MW. Premature ovarian failure. Current opinion in obstetrics & gynecology. 2006;18(4):418-26.
143. Shelling AN. Premature ovarian failure. Reproduction. 2010;140(5):633-41.
144. Islam R, Cartwright R. The impact of premature ovarian failure on quality of life: results from the UK 1958 Birth Cohort. OXFORD UNIV PRESS; 2011. p. 108-.
145. Rebar RW. Premature ovarian failure. Obstet Gynecol. 2009;113(6):1355-63.
146. Rebar RW, Erickson GF, Yen SS. Idiopathic premature ovarian failure: clinical and endocrine characteristics. Fertil Steril. 1982;37(1):35-41.
147. Vujovic S. Aetiology of premature ovarian failure. Menopause international. 2009;15(2):72-5.
148. Laissue P, Vinci G, Veitia RA, Fellous M. Recent advances in the study of genes involved in non-syndromic premature ovarian failure. Mol Cell Endocrinol. 2008;282(1-2):101-11.
149. Hewitt JK, Jayasinghe Y, Amor DJ, Gillam LH, Warne GL, Grover S, et al. Fertility in Turner syndrome. Clin Endocrinol (Oxf). 2013;79(5):606-14.
150. Goswami D, Conway GS. Premature ovarian failure. Hormone research. 2007;68(4):196-202.
151. Sybert VP, McCauley E. Turner's syndrome. N Engl J Med. 2004;351(12):1227-38.
152. HH T. A syndrome of infantilism, congenital webbed neck, and cubitus valgus. Endocrinology, vol. 23, pp. 566-574, 1938. Am J Obstet Gynecol. 1972;113(2):279.
153. Elsheikh M, Dunger DB, Conway GS, Wass JA. Turner's syndrome in adulthood. Endocr Rev. 2002;23(1):120-40.
154. Modi DN, Sane S, Bhartiya D. Accelerated germ cell apoptosis in sex chromosome aneuploid fetal human gonads. Mol Hum Reprod. 2003;9(4):219-25.
155. Tejada MI, Garcia-Alegria E, Bilbao A, Martinez-Bouzas C, Beristain E, Poch M, et al. Analysis of the molecular parameters that could predict the risk of manifesting premature ovarian failure in female premutation carriers of fragile X syndrome. Menopause. 2008;15(5):945-9.
156. Heitz D, Devys D, Imbert G, Kretz C, Mandel JL. Inheritance of the fragile X syndrome: size of the fragile X premutation is a major determinant of the transition to full mutation. Journal of medical genetics. 1992;29(11):794-801.
157. Tassone F, Hagerman PJ, Hagerman RJ. Fragile x premutation. Journal of neurodevelopmental disorders. 2014;6(1):22.
158. Conway GS, Kaltsas G, Patel A, Davies MC, Jacobs HS. Characterization of idiopathic premature ovarian failure. Fertil Steril. 1996;65(2):337-41.
159. Murray A, Webb J, Grimley S, Conway G, Jacobs P. Studies of FRAXA and FRAXE in women with premature ovarian failure. Journal of medical genetics. 1998;35(8):637-40.
160. Lu C, Lin L, Tan H, Wu H, Sherman SL, Gao F, et al. Fragile X premutation RNA is sufficient to cause primary ovarian insufficiency in mice. Hum Mol Genet. 2012;21(23):5039-47.
161. Rife M, Nadal A, Mila M, Willemsen R. Immunohistochemical FMRP studies in a full mutated female fetus. American journal of medical genetics Part A. 2004;124A(2):129-32.
162. Rosario R, Filis P, Tessyman V, Kinnell H, Childs AJ, Gray NK, et al. FMRP Associates with Cytoplasmic Granules at the Onset of Meiosis in the Human Oocyte. PLoS One. 2016;11(10):e0163987.

163. Waggoner DD, Buist NR, Donnell GN. Long-term prognosis in galactosaemia: results of a survey of 350 cases. *Journal of inherited metabolic disease*. 1990;13(6):802-18.
164. Fridovich-Keil JL, Gubbels CS, Spencer JB, Sanders RD, Land JA, Rubio-Gozalbo E. Ovarian function in girls and women with GALT-deficiency galactosemia. *Journal of inherited metabolic disease*. 2011;34(2):357-66.
165. Banerjee S, Chakraborty P, Saha P, Bandyopadhyay SA, Banerjee S, Kabir SN. Ovotoxic effects of galactose involve attenuation of follicle-stimulating hormone bioactivity and up-regulation of granulosa cell p53 expression. *PLoS One*. 2012;7(2):e30709.
166. Meirow D. Reproduction post-chemotherapy in young cancer patients. *Mol Cell Endocrinol*. 2000;169(1-2):123-31.
167. Kreuser ED, Hetzel WD, Billia DO, Thiel E. Gonadal toxicity following cancer therapy in adults: significance, diagnosis, prevention and treatment. *Cancer treatment reviews*. 1990;17(2-3):169-75.
168. Roness H, Gavish Z, Cohen Y, Meirow D. Ovarian follicle burnout: a universal phenomenon? *Cell cycle*. 2013;12(20):3245-6.
169. Forges T, Monnier-Barbarino P, Faure GC, Bene MC. Autoimmunity and antigenic targets in ovarian pathology. *Hum Reprod Update*. 2004;10(2):163-75.
170. Novosad JA, Kalantaridou SN, Tong ZB, Nelson LM. Ovarian antibodies as detected by indirect immunofluorescence are unreliable in the diagnosis of autoimmune premature ovarian failure: a controlled evaluation. *BMC women's health*. 2003;3(1):2.
171. Abalovich M, Mitelberg L, Allami C, Gutierrez S, Alcaraz G, Otero P, et al. Subclinical hypothyroidism and thyroid autoimmunity in women with infertility. *Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology*. 2007;23(5):279-83.
172. Poppe K, Glinoe D, Van Steirteghem A, Tournaye H, Devroey P, Schiettecatte J, et al. Thyroid dysfunction and autoimmunity in infertile women. *Thyroid : official journal of the American Thyroid Association*. 2002;12(11):997-1001.
173. Reato G, Morlin L, Chen S, Furmaniak J, Smith BR, Masiero S, et al. Premature ovarian failure in patients with autoimmune Addison's disease: clinical, genetic, and immunological evaluation. *J Clin Endocrinol Metab*. 2011;96(8):E1255-61.
174. Nelson LM. Clinical practice. Primary ovarian insufficiency. *N Engl J Med*. 2009;360(6):606-14.
175. Cooper AR, Baker VL, Sterling EW, Ryan ME, Woodruff TK, Nelson LM. The time is now for a new approach to primary ovarian insufficiency. *Fertil Steril*. 2011;95(6):1890-7.
176. Heller CG, Heller EJ. Gonadotropic Hormone: Urine Assays of Normally Cycling, Menopausal, Castrated, and Estrin Treated Human Females. *J Clin Invest*. 1939;18(2):171-8.
177. Albright F, Smith PH, Fraser R. A syndrome characterized by primary ovarian insufficiency and decreased stature: report of 11 cases with a digression on hormonal control of axillary and pubic hair. *American Journal of the medical sciences*. 1942;204(5):625-8.
178. European Society for Human R, Embryology Guideline Group on POI, Webber L, Davies M, Anderson R, Bartlett J, et al. ESHRE Guideline:

- management of women with premature ovarian insufficiency. *Hum Reprod.* 2016;31(5):926-37.
179. Rafique S, Sterling EW, Nelson LM. A new approach to primary ovarian insufficiency. *Obstetrics and gynecology clinics of North America.* 2012;39(4):567-86.
 180. Welt CK. Primary ovarian insufficiency: a more accurate term for premature ovarian failure. *Clin Endocrinol (Oxf).* 2008;68(4):499-509.
 181. Bidet M, Bachelot A, Bissauge E, Golmard JL, Gricourt S, Dulon J, et al. Resumption of ovarian function and pregnancies in 358 patients with premature ovarian failure. *J Clin Endocrinol Metab.* 2011;96(12):3864-72.
 182. Mendoza N, Julia MD, Galliano D, Coronado P, Diaz B, Fontes J, et al. Spanish consensus on premature menopause. *Maturitas.* 2015;80(2):220-5.
 183. Nelson LM. One world, one woman: a transformational leader's approach to primary ovarian insufficiency. *Menopause.* 2011;18(5):480-7.
 184. Nelson LM, Anasti JN, Kimzey LM, Defensor RA, Lipetz KJ, White BJ, et al. Development of luteinized graafian follicles in patients with karyotypically normal spontaneous premature ovarian failure. *J Clin Endocrinol Metab.* 1994;79(5):1470-5.
 185. Suzuki N, Yoshioka N, Takae S, Sugishita Y, Tamura M, Hashimoto S, et al. Successful fertility preservation following ovarian tissue vitrification in patients with primary ovarian insufficiency. *Hum Reprod.* 2015;30(3):608-15.
 186. Massin N, Gougeon A, Meduri G, Thibaud E, Laborde K, Matuchansky C, et al. Significance of ovarian histology in the management of patients presenting a premature ovarian failure. *Hum Reprod.* 2004;19(11):2555-60.
 187. Jewelewicz R, Schwartz M. Premature ovarian failure. *Bulletin of the New York Academy of Medicine.* 1986;62(3):219-36.
 188. Hubayter ZR, Popat V, Vanderhoof VH, Ndubizu O, Johnson D, Mao E, et al. A prospective evaluation of antral follicle function in women with 46,XX spontaneous primary ovarian insufficiency. *Fertil Steril.* 2010;94(5):1769-74.
 189. Nakano R, Hashiba N, Washio M, Tojo S. Ovarian follicular apparatus and hormonal parameters in patients with primary and secondary amenorrhea. *Acta obstetrica et gynecologica Scandinavica.* 1978;57(4):293-300.
 190. Fox H. The pathology of premature ovarian failure. *The Journal of pathology.* 1992;167(4):357-63.
 191. Nelson LM, Kimzey LM, White BJ, Merriam GR. Gonadotropin suppression for the treatment of karyotypically normal spontaneous premature ovarian failure: a controlled trial. *Fertil Steril.* 1992;57(1):50-5.
 192. Bidet M, Bachelot A, Touraine P. Premature ovarian failure: predictability of intermittent ovarian function and response to ovulation induction agents. *Current opinion in obstetrics & gynecology.* 2008;20(4):416-20.
 193. Kreiner D, Droesch K, Navot D, Scott R, Rosenwaks Z. Spontaneous and pharmacologically induced remissions in patients with premature ovarian failure. *Obstet Gynecol.* 1988;72(6):926-8.
 194. Taylor AE, Adams JM, Mulder JE, Martin KA, Sluss PM, Crowley WF, Jr. A randomized, controlled trial of estradiol replacement therapy in women with hypergonadotropic amenorrhea. *J Clin Endocrinol Metab.* 1996;81(10):3615-21.
 195. Keettel WC, Bradbury JT. Premature Ovarian Failure, Permanent and Temporary. *Am J Obstet Gynecol.* 1964;89:83-96.
 196. Van Campenhout J, Vauclair R, Maraghi K. Gonadotropin--resistant ovaries in primary amenorrhea. *Obstet Gynecol.* 1972;40(1):6-12.

197. Metcalf MG, Donald RA, Livesey JH. Pituitary-ovarian function in normal women during the menopausal transition. *Clin Endocrinol (Oxf)*. 1981;14(3):245-55.
198. Szlachter BN, Nachtigall LE, Epstein J, Young BK, Weiss G. Premature menopause: a reversible entity? *Obstet Gynecol*. 1979;54(3):396-8.
199. Fraser IS, Russell P, Greco S, Robertson DM. Resistant ovary syndrome and premature ovarian failure in young women with galactosaemia. *Clinical reproduction and fertility*. 1986;4(2):133-8.
200. De Baere E, Dixon MJ, Small KW, Jabs EW, Leroy BP, Devriendt K, et al. Spectrum of FOXL2 gene mutations in blepharophimosis-ptosis-epicanthus inversus (BPES) families demonstrates a genotype-phenotype correlation. *Hum Mol Genet*. 2001;10(15):1591-600.
201. Aittomäki K, Herva R, Stenman UH, Juntunen K, Ylostalo P, Hovatta O, et al. Clinical features of primary ovarian failure caused by a point mutation in the follicle-stimulating hormone receptor gene. *J Clin Endocrinol Metab*. 1996;81(10):3722-6.
202. Allen LA, Achermann JC, Pakarinen P, Kotlar TJ, Huhtaniemi IT, Jameson JL, et al. A novel loss of function mutation in exon 10 of the FSH receptor gene causing hypergonadotrophic hypogonadism: clinical and molecular characteristics. *Hum Reprod*. 2003;18(2):251-6.
203. Duncan M, Cummings L, Chada K. Germ cell deficient (gcd) mouse as a model of premature ovarian failure. *Biol Reprod*. 1993;49(2):221-7.
204. Pellas TC, Ramachandran B, Duncan M, Pan SS, Marone M, Chada K. Germ-cell deficient (gcd), an insertional mutation manifested as infertility in transgenic mice. *Proc Natl Acad Sci U S A*. 1991;88(19):8787-91.
205. Smith ER, Yeasky T, Wei JQ, Miki RA, Cai KQ, Smedberg JL, et al. White spotting variant mouse as an experimental model for ovarian aging and menopausal biology. *Menopause*. 2012;19(5):588-96.
206. Reddy P, Liu L, Adhikari D, Jagarlamudi K, Rajareddy S, Shen Y, et al. Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. *Science*. 2008;319(5863):611-3.
207. Castrillon DH, Miao L, Kollipara R, Horner JW, DePinho RA. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science*. 2003;301(5630):215-8.
208. Vandormael-Pournin S, Guigon CJ, Ishaq M, Coudouel N, Ave P, Huerre M, et al. Oocyte-specific inactivation of Omcg1 leads to DNA damage and c-Abl/TAp63-dependent oocyte death associated with dramatic remodeling of ovarian somatic cells. *Cell death and differentiation*. 2015;22(1):108-17.
209. Matzuk MM, Finegold MJ, Su JG, Hsueh AJ, Bradley A. Alpha-inhibin is a tumour-suppressor gene with gonadal specificity in mice. *Nature*. 1992;360(6402):313-9.
210. Jorgez CJ, Klysik M, Jamin SP, Behringer RR, Matzuk MM. Granulosa cell-specific inactivation of follistatin causes female fertility defects. *Mol Endocrinol*. 2004;18(4):953-67.
211. Ledent C, Demeestere I, Blum D, Petermans J, Hamalainen T, Smits G, et al. Premature ovarian aging in mice deficient for Gpr3. *Proc Natl Acad Sci U S A*. 2005;102(25):8922-6.
212. Zhang CX, Zhang Q, Xie YY, He XY, Xiang C, Hou XS, et al. Mouse Double Minute 2 Actively Suppresses p53 Activity in Oocytes during Mouse Folliculogenesis. *Am J Pathol*. 2017;187(2):339-51.

213. Wang W, Tang Y, Ni L, Kim E, Jongwutiwes T, Hourvitz A, et al. Overexpression of Uromodulin-like1 accelerates follicle depletion and subsequent ovarian degeneration. *Cell death & disease*. 2012;3:e433.
214. Gaytan F, Garcia-Galiano D, Dorfman MD, Manfredi-Lozano M, Castellano JM, Disen GA, et al. Kisspeptin receptor haplo-insufficiency causes premature ovarian failure despite preserved gonadotropin secretion. *Endocrinology*. 2014;155(8):3088-97.
215. Jiang ZZ, Hu MW, Ma XS, Schatten H, Fan HY, Wang ZB, et al. LKB1 acts as a critical gatekeeper of ovarian primordial follicle pool. *Oncotarget*. 2016;7(5):5738-53.
216. Saatcioglu HD, Cuevas I, Castrillon DH. Control of Oocyte Reawakening by Kit. *PLoS Genet*. 2016;12(8):e1006215.
217. Adhikari D, Zheng W, Shen Y, Gorre N, Hamalainen T, Cooney AJ, et al. Tsc/mTORC1 signaling in oocytes governs the quiescence and activation of primordial follicles. *Hum Mol Genet*. 2010;19(3):397-410.
218. Reddy P, Adhikari D, Zheng W, Liang S, Hamalainen T, Tohonen V, et al. PDK1 signaling in oocytes controls reproductive aging and lifespan by manipulating the survival of primordial follicles. *Hum Mol Genet*. 2009;18(15):2813-24.
219. Freiman RN, Albright SR, Zheng S, Sha WC, Hammer RE, Tjian R. Requirement of tissue-selective TBP-associated factor TAFII105 in ovarian development. *Science*. 2001;293(5537):2084-7.
220. Voronina E, Lovasco LA, Gyuris A, Baumgartner RA, Parlow AF, Freiman RN. Ovarian granulosa cell survival and proliferation requires the gonad-selective TFIID subunit TAF4b. *Dev Biol*. 2007;303(2):715-26.
221. Ju T, Cummings RD, Canfield WM. Purification, characterization, and subunit structure of rat core 1 Beta1,3-galactosyltransferase. *J Biol Chem*. 2002;277(1):169-77.
222. Ju T, Brewer K, D'Souza A, Cummings RD, Canfield WM. Cloning and expression of human core 1 beta1,3-galactosyltransferase. *J Biol Chem*. 2002;277(1):178-86.
223. Robertson MA, Etchison JR, Robertson JS, Summers DF, Stanley P. Specific changes in the oligosaccharide moieties of VSV grown in different lectin-resistant CHO cells. *Cell*. 1978;13(3):515-26.
224. Schlesinger S, Gottlieb C, Feil P, Gelb N, Kornfeld S. Growth of enveloped RNA viruses in a line of chinese hamster ovary cells with deficient N-acetylglucosaminyltransferase activity. *Journal of virology*. 1975;17(1):239-46.
225. Sauer B, Henderson N. The cyclization of linear DNA in *Escherichia coli* by site-specific recombination. *Gene*. 1988;70(2):331-41.
226. Williams SA, Xia L, Cummings RD, McEver RP, Stanley P. Fertilization in mouse does not require terminal galactose or N-acetylglucosamine on the zona pellucida glycans. *J Cell Sci*. 2007;120(Pt 8):1341-9.
227. Shi S, Williams SA, Seppo A, Kurniawan H, Chen W, Ye Z, et al. Inactivation of the Mgat1 gene in oocytes impairs oogenesis, but embryos lacking complex and hybrid N-glycans develop and implant. *Mol Cell Biol*. 2004;24(22):9920-9.
228. Varki A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology*. 1993;3(2):97-130.
229. Ercan A, Cui J, Chatterton DE, Deane KD, Hazen MM, Brintnell W, et al. Aberrant IgG galactosylation precedes disease onset, correlates with disease

activity, and is prevalent in autoantibodies in rheumatoid arthritis. *Arthritis and rheumatism*. 2010;62(8):2239-48.

230. Itoh N, Sakaue S, Nakagawa H, Kurogochi M, Ohira H, Deguchi K, et al. Analysis of N-glycan in serum glycoproteins from db/db mice and humans with type 2 diabetes. *Am J Physiol Endocrinol Metab*. 2007;293(4):E1069-77.

231. Jiang X, Dias JA, He X. Structural biology of glycoprotein hormones and their receptors: insights to signaling. *Mol Cell Endocrinol*. 2014;382(1):424-51.

232. Pankhurst MW, McLennan IS. Human blood contains both the uncleaved precursor of anti-Mullerian hormone and a complex of the NH₂- and COOH-terminal peptides. *Am J Physiol Endocrinol Metab*. 2013;305(10):E1241-7.

233. Hayashi M, McGee EA, Min G, Klein C, Rose UM, van Duin M, et al. Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. *Endocrinology*. 1999;140(3):1236-44.

234. Williams SA, Stanley P. Mouse fertility is enhanced by oocyte-specific loss of core 1-derived O-glycans. *FASEB J*. 2008;22(7):2273-84.

235. Williams SA, Stanley P. Oocyte-specific deletion of complex and hybrid N-glycans leads to defects in preovulatory follicle and cumulus mass development. *Reproduction*. 2009;137(2):321-31.

236. Grasa P, Kaune H, Williams SA. Embryos generated from oocytes lacking complex N- and O-glycans have compromised development and implantation. *Reproduction*. 2012;144(4):455-65.

237. Burda P, Aebi M. The dolichol pathway of N-linked glycosylation. *Biochim Biophys Acta*. 1999;1426(2):239-57.

238. Varki A, Lowe JB. Biological Roles of Glycans. In: Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, et al., editors. *Essentials of Glycobiology*. 2nd ed. Cold Spring Harbor (NY)2009.

239. Gill DJ, Clausen H, Bard F. Location, location, location: new insights into O-GalNAc protein glycosylation. *Trends in cell biology*. 2011;21(3):149-58.

240. Wang Y, Ju T, Ding X, Xia B, Wang W, Xia L, et al. Cosmc is an essential chaperone for correct protein O-glycosylation. *Proc Natl Acad Sci U S A*. 2010;107(20):9228-33.

241. Batista F, Lu L, Williams SA, Stanley P. Complex N-glycans are essential, but core 1 and 2 mucin O-glycans, O-fucose glycans, and NOTCH1 are dispensable, for mammalian spermatogenesis. *Biol Reprod*. 2012;86(6):179.

242. Mehta AE, Matwijiw I, Lyons EA, Faiman C. Noninvasive diagnosis of resistant ovary syndrome by ultrasonography. *Fertility and sterility*. 1992;57(1):56-61.

243. Shuster LT, Rhodes DJ, Gostout BS, Grossardt BR, Rocca WA. Premature menopause or early menopause: long-term health consequences. *Maturitas*. 2010;65(2):161-6.

244. Grasa P, Sheikh S, Krzys N, Millar K, Janjua S, Nawaggi P, et al. Dysregulation of follicle development in a mouse model of premature ovarian insufficiency. *Reproduction*. 2016.

245. Pedersen T, Peters H. Proposal for a classification of oocytes and follicles in the mouse ovary. *J Reprod Fertil*. 1968;17(3):555-7.

246. Knauff EA, Eijkemans MJ, Lambalk CB, ten Kate-Booij MJ, Hoek A, Beerendonk CC, et al. Anti-Mullerian hormone, inhibin B, and antral follicle count in young women with ovarian failure. *J Clin Endocrinol Metab*. 2009;94(3):786-92.

247. Byskov AG, Guoliang X, Andersen CY. The cortex-medulla oocyte growth pattern is organized during fetal life: an in-vitro study of the mouse ovary. *Mol Hum Reprod*. 1997;3(9):795-800.
248. Lunenfeld B, Kraiem Z, Eshkol A. The function of the growing follicle. *J Reprod Fertil*. 1975;45(3):567-74.
249. Pedersen T. Determination of follicle growth rate in the ovary of the immature mouse. *J Reprod Fertil*. 1970;21(1):81-93.
250. Da Silva-Buttkus P, Jayasooriya GS, Mora JM, Mobberley M, Ryder TA, Baithun M, et al. Effect of cell shape and packing density on granulosa cell proliferation and formation of multiple layers during early follicle development in the ovary. *J Cell Sci*. 2008;121(Pt 23):3890-900.
251. Zheng W, Zhang H, Gorre N, Risal S, Shen Y, Liu K. Two classes of ovarian primordial follicles exhibit distinct developmental dynamics and physiological functions. *Hum Mol Genet*. 2014;23(4):920-8.
252. Quattropani SL. Morphogenesis of the ovarian interstitial tissue in the neonatal mouse. *Anat Rec*. 1973;177(4):569-83.
253. Baker SJ, Spears N. The role of intra-ovarian interactions in the regulation of follicle dominance. *Hum Reprod Update*. 1999;5(2):153-65.
254. Durlinger AL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, et al. Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. *Endocrinology*. 2001;142(11):4891-9.
255. Durlinger AL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, et al. Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology*. 2002;143(3):1076-84.
256. Chakraborty P, Roy SK. Expression of FSH receptor in the hamster ovary during perinatal development. *Mol Cell Endocrinol*. 2015;400:41-7.
257. Dullaart J, Kent J, Ryle M. Serum gonadotrophin concentrations in infantile female mice. *J Reprod Fertil*. 1975;43(1):189-92.
258. Halpin DM, Jones A, Fink G, Charlton HM. Postnatal ovarian follicle development in hypogonadal (hpg) and normal mice and associated changes in the hypothalamic-pituitary ovarian axis. *J Reprod Fertil*. 1986;77(1):287-96.
259. Stiff ME, Bronson FH, Stetson MH. Plasma gonadotropins in prenatal and prepubertal female mice: disorganization of pubertal cycles in the absence of a male. *Endocrinology*. 1974;94(2):492-6.
260. Riasat F, Yamada K, Liu XW, Wang H, Yokota H, Kobayashi J, et al. Ovarian follicular differentiation with prepubertal gonadotropin surges and gonadotropin priming in mice. *Life sciences*. 2002;71(10):1187-97.
261. Hardy B, Danon D, Eshkol A, Lunenfeld B. Ultrastructural changes in the ovaries of infant mice deprived of endogenous gonadotrophins and after substitution with FSH. *J Reprod Fertil*. 1974;36(2):345-52.
262. Fukuda M, Fukuda K, Yding Andersen C, Byskov AG. Does corpus luteum locally affect follicular growth negatively? *Hum Reprod*. 1997;12(5):1024-7.
263. Buffler G, Roser S. New data concerning the role played by progesterone in the control of follicular growth in the rat. *Acta Endocrinol (Copenh)*. 1974;75(3):569-78.
264. Byskov AG. Cell kinetic studies of follicular atresia in the mouse ovary. *J Reprod Fertil*. 1974;37(2):277-85.
265. Peluso JJ, Steger RW. Role of FSH in regulating granulosa cell division and follicular atresia in rats. *J Reprod Fertil*. 1978;54(2):275-8.

266. Nakano R, Akahori T, Katayama K, Tojo S. Binding of LH and FSH to porcine granulosa cells during follicular maturation. *J Reprod Fertil.* 1977;51(1):23-7.
267. Eppig JJ. Intercommunication between mammalian oocytes and companion somatic cells. *Bioessays.* 1991;13(11):569-74.
268. Lundy T, Smith P, O'Connell A, Hudson NL, McNatty KP. Populations of granulosa cells in small follicles of the sheep ovary. *J Reprod Fertil.* 1999;115(2):251-62.
269. Hirshfield AN, DeSanti AM. Patterns of ovarian cell proliferation in rats during the embryonic period and the first three weeks postpartum. *Biol Reprod.* 1995;53(5):1208-21.
270. Takahashi N, Tarumi W, Ishizuka B. Involvement of hyaluronan synthesis in ovarian follicle growth in rats. *Reproduction.* 2014;147(2):189-97.
271. Dumaresq-Doiron K, Edjekouane L, Orimoto AM, Yoffou PH, Gushulak L, Triggs-Raine B, et al. Hyal-1 but not Hyal-3 deficiency has an impact on ovarian folliculogenesis and female fertility by altering the follistatin/activin/Smad3 pathway and the apoptotic process. *J Cell Physiol.* 2012;227(5):1911-22.
272. West DC, Kumar S. Hyaluronan and angiogenesis. *Ciba Found Symp.* 1989;143:187-201; discussion -7, 81-5.
273. Tempel C, Gilead A, Neeman M. Hyaluronic acid as an anti-angiogenic shield in the preovulatory rat follicle. *Biol Reprod.* 2000;63(1):134-40.
274. Maciel GA, Baracat EC, Benda JA, Markham SM, Hensinger K, Chang RJ, et al. Stockpiling of transitional and classic primary follicles in ovaries of women with polycystic ovary syndrome. *J Clin Endocrinol Metab.* 2004;89(11):5321-7.
275. Webber LJ, Stubbs S, Stark J, Trew GH, Margara R, Hardy K, et al. Formation and early development of follicles in the polycystic ovary. *Lancet.* 2003;362(9389):1017-21.
276. Young HT, Carr NJ, Green B, Tilley C, Bhargava V, Pearce N. Accuracy of visual assessments of proliferation indices in gastroenteropancreatic neuroendocrine tumours. *Journal of clinical pathology.* 2013;66(8):700-4.
277. Hirshfield AN. Heterogeneity of cell populations that contribute to the formation of primordial follicles in rats. *Biol Reprod.* 1992;47(3):466-72.
278. Mork L, Maatouk DM, McMahon JA, Guo JJ, Zhang P, McMahon AP, et al. Temporal differences in granulosa cell specification in the ovary reflect distinct follicle fates in mice. *Biol Reprod.* 2012;86(2):37.
279. Dorrington JH, McKeracher HL, Chan AK, Gore-Langton RE. Hormonal interactions in the control of granulosa cell differentiation. *J Steroid Biochem.* 1983;19(1A):17-32.
280. Aten RF, Kolodecik TR, Behrman HR. A cell adhesion receptor antiserum abolishes, whereas laminin and fibronectin glycoprotein components of extracellular matrix promote, luteinization of cultured rat granulosa cells. *Endocrinology.* 1995;136(4):1753-8.
281. Fujiwara H, Honda T, Ueda M, Nakamura K, Yamada S, Maeda M, et al. Laminin suppresses progesterone production by human luteinizing granulosa cells via interaction with integrin alpha 6 beta 1. *J Clin Endocrinol Metab.* 1997;82(7):2122-8.
282. Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M. EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science.* 2004;303(5658):682-4.
283. Russell DL, Robker RL. Molecular mechanisms of ovulation: co-ordination through the cumulus complex. *Hum Reprod Update.* 2007;13(3):289-312.

284. van de Lagemaat R, van Koppen CJ, Krajnc-Franken MA, Folmer BJ, van Diepen HA, Mulders SM, et al. Contraception by induction of luteinized unruptured follicles with short-acting low molecular weight FSH receptor agonists in female animal models. *Reproduction*. 2011;142(6):893-905.
285. Richards JS, Russell DL, Robker RL, Dajee M, Alliston TN. Molecular mechanisms of ovulation and luteinization. *Mol Cell Endocrinol*. 1998;145(1-2):47-54.
286. Sirard MA, Desrosier S, Assidi M. In vivo and in vitro effects of FSH on oocyte maturation and developmental competence. *Theriogenology*. 2007;68 Suppl 1:S71-6.
287. Robker RL, Richards JS. Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. *Mol Endocrinol*. 1998;12(7):924-40.
288. Eppig JJ, O'Brien MJ, Wigglesworth K, Nicholson A, Zhang W, King BA. Effect of in vitro maturation of mouse oocytes on the health and lifespan of adult offspring. *Hum Reprod*. 2009;24(4):922-8.
289. Eppig JJ, O'Brien M, Wigglesworth K. Mammalian oocyte growth and development in vitro. *Mol Reprod Dev*. 1996;44(2):260-73.
290. O'Brien MJ, Pendola JK, Eppig JJ. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod*. 2003;68(5):1682-6.
291. Spears N, Baker S, Srsen V, Lapping R, Mullan J, Nelson R, et al. Mouse ovarian follicles secrete factors affecting the growth and development of like-sized ovarian follicles in vitro. *Biol Reprod*. 2002;67(6):1726-33.
292. Baker SJ, Srsen V, Lapping R, Spears N. Combined effect of follicle-follicle interactions and declining follicle-stimulating hormone on murine follicle health in vitro. *Biol Reprod*. 2001;65(4):1304-10.
293. Axford J. The impact of glycobiology on medicine. *Trends in immunology*. 2001;22(5):237-9.
294. Sinclair AM, Elliott S. Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins. *Journal of pharmaceutical sciences*. 2005;94(8):1626-35.
295. Davidson B, Murray AA, Elfick A, Spears N. Raman micro-spectroscopy can be used to investigate the developmental stage of the mouse oocyte. *PLoS One*. 2013;8(7):e67972.
296. Hughesdon PE. Morphology and morphogenesis of the Stein-Leventhal ovary and of so-called "hyperthecosis". *Obstet Gynecol Surv*. 1982;37(2):59-77.
297. West ER, Xu M, Woodruff TK, Shea LD. Physical properties of alginate hydrogels and their effects on in vitro follicle development. *Biomaterials*. 2007;28(30):4439-48.
298. Xu M, West E, Shea LD, Woodruff TK. Identification of a stage-specific permissive in vitro culture environment for follicle growth and oocyte development. *Biol Reprod*. 2006;75(6):916-23.
299. Andersen MM, Kroll J, Byskov AG, Faber M. Protein composition in the fluid of individual bovine follicles. *J Reprod Fertil*. 1976;48(1):109-18.
300. Grimek HJ, Bellin ME, Ax RL. Characteristics of proteoglycans isolated from small and large bovine ovarian follicles. *Biol Reprod*. 1984;30(2):397-409.
301. Cristol P, Chabab A, Diafouka F, Flandre O, Bressot N, Cristol AM, et al. [Pregnancies by fertilization in vitro. Biochemistry of the follicular fluid]. *Pathologie-biologie*. 1985;33(3):155-61.

302. Mendoza C, Ruiz-Requena E, Ortega E, Cremades N, Martinez F, Bernabeu R, et al. Follicular fluid markers of oocyte developmental potential. *Hum Reprod*. 2002;17(4):1017-22.
303. Das M, Gillott DJ, Saridogan E, Djahanbakhch O. Anti-Mullerian hormone is increased in follicular fluid from unstimulated ovaries in women with polycystic ovary syndrome. *Hum Reprod*. 2008;23(9):2122-6.
304. Piomboni P, Focarelli R, Capaldo A, Stendardi A, Cappelli V, Cianci A, et al. Protein modification as oxidative stress marker in follicular fluid from women with polycystic ovary syndrome: the effect of inositol and metformin. *J Assist Reprod Genet*. 2014;31(10):1269-76.
305. Bender K, Walsh S, Evans AC, Fair T, Brennan L. Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows. *Reproduction*. 2010;139(6):1047-55.
306. Hodgson J. Fetal bovine serum revisited. A year ago serum regulations were a hodgepodge. Has anything changed? *Bio/technology*. 1993;11(1):49-53.
307. Kawamura K, Cheng Y, Suzuki N, Deguchi M, Sato Y, Takae S, et al. Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment. *Proc Natl Acad Sci U S A*. 2013;110(43):17474-9.
308. Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum Reprod Update*. 2008;14(2):159-77.
309. Parrott JA, Skinner MK. Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. *Endocrinology*. 1999;140(9):4262-71.
310. Durlinger AL, Visser JA, Themmen AP. Regulation of ovarian function: the role of anti-Mullerian hormone. *Reproduction*. 2002;124(5):601-9.
311. Eppig JJ, Wigglesworth K, Hirao Y. Metaphase I arrest and spontaneous parthenogenetic activation of strain LTXBO oocytes: chimeric reaggregated ovaries establish primary lesion in oocytes. *Dev Biol*. 2000;224(1):60-8.
312. Eppig JJ, Wigglesworth K. Development of mouse and rat oocytes in chimeric reaggregated ovaries after interspecific exchange of somatic and germ cell components. *Biol Reprod*. 2000;63(4):1014-23.
313. Friedrich G, Soriano P. Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev*. 1991;5(9):1513-23.
314. Kawamura K, Cheng Y, Sun Y-P, Zhai J, Diaz-Garcia C, Simon C, et al. Ovary transplantation: to activate or not to activate. *Human Reproduction*. 2015;30(11):2457-60.
315. Park MR, Choi YJ, Kwon DN, Park C, Bui HT, Gurunathan S, et al. Intraovarian transplantation of primordial follicles fails to rescue chemotherapy injured ovaries. *Sci Rep*. 2013;3:1384.
316. Takehara Y, Yabuuchi A, Ezoe K, Kuroda T, Yamadera R, Sano C, et al. The restorative effects of adipose-derived mesenchymal stem cells on damaged ovarian function. *Lab Invest*. 2013;93(2):181-93.
317. Akil M, Amos RS, Stewart P. Infertility may sometimes be associated with NSAID consumption. *British journal of rheumatology*. 1996;35(1):76-8.
318. Katz E. The luteinized unruptured follicle and other ovulatory dysfunctions. *Fertil Steril*. 1988;50(6):839-50.
319. Marik J, Hulka J. Luteinized unruptured follicle syndrome: a subtle cause of infertility. *Fertil Steril*. 1978;29(3):270-4.
320. Eppig JJ. Mouse oocyte development in vitro with various culture systems. *Dev Biol*. 1977;60(2):371-88.

321. Xiang C, Li J, Hu L, Huang J, Luo T, Zhong Z, et al. Hippo signaling pathway reveals a spatio-temporal correlation with the size of primordial follicle pool in mice. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*. 2015;35(3):957-68.
322. Cheng Y, Feng Y, Jansson L, Sato Y, Deguchi M, Kawamura K, et al. Actin polymerization-enhancing drugs promote ovarian follicle growth mediated by the Hippo signaling effector YAP. *FASEB J*. 2015;29(6):2423-30.
323. Stein IF, Cohen MR, Elson R. Results of bilateral ovarian wedge resection in 47 cases of sterility; 20 year end results; 75 cases of bilateral polycystic ovaries. *Am J Obstet Gynecol*. 1949;58(2):267-74.
324. Hajjarian Z, Nadkarni SK. Measurement of bulk mechanical properties of tissue using laser speckle rheology. *Conference proceedings : Annual International Conference of the IEEE Engineering in Medicine and Biology Society IEEE Engineering in Medicine and Biology Society Annual Conference*. 2011;2011:5746-8.
325. Conca Dioguardi C, Uslu B, Haynes M, Kurus M, Gul M, Miao DQ, et al. Granulosa cell and oocyte mitochondrial abnormalities in a mouse model of fragile X primary ovarian insufficiency. *Mol Hum Reprod*. 2016;22(6):384-96.
326. Diot A, Hinks-Roberts A, Lodge T, Liao C, Dombi E, Morten K, et al. A novel quantitative assay of mitophagy: Combining high content fluorescence microscopy and mitochondrial DNA load to quantify mitophagy and identify novel pharmacological tools against pathogenic heteroplasmic mtDNA. *Pharmacological research*. 2015;100:24-35.