Chapter 1

The Ovary: A General Overview of Follicle Formation and Development

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1.1 Introduction

In the context of reproduction, the key functions of the ovary are to provide an environment that supports the maturation of oocytes for ovulation alongside adequate production of sex steroids to prepare for and sustain a potential pregnancy. These functions are not mutually exclusive, as each depends on follicle and oocyte growth and maturation progressing in a coordinated and timely manner. Our current understanding of how the ovary is formed, with its limited supply of follicles, and how individual follicles develop and achieve these functions of oocyte support and steroidogenesis are summarised briefly this chapter.

1.2 Sex Determination and Early Gonadal Development

In humans, male and female embryos are morphologically similar until around week 6 of development when activation of the molecular programme from sex chromosomes allocated at fertilisation sets in motion the formation of a sex-specific gonadal phenotype. Prior to this, a small cluster of pluripotent primordial germ cells (PGCs), first identified in the posterior yolk sac endoderm, begins to proliferate and migrate along the hindgut, through the dorsal mesentery, to eventually settle on either side of the developing aorta in the emerging gonads, or 'gonadal ridges'. This migration of PGCs, driven by chemotactic signals (e.g. KIT/KITLG), occurs alongside proliferation and inward migration of somatic cells to create a bulge on the ventromedial aspect of each mesonephros called the gonadal ridge. The gonadal ridges, covered by coelomic epithelium, develop alongside the two adjacent embryonic ducts, the Wolffian (also known as the mesonephric) and Müllerian (also known as the paramesonephric) ducts, which all together constitute a bipotential reproductive system.

Subsequent differentiation of the gonads is generally dependent on the genetic constitution of the embryo. In males, brief and timely expression of the sex-determining region on the Y chromosome (*SRY* gene) in somatic cells, together with other transcription factors (e.g. SF1 and WT1), drives expression of SRY-box transcription factor 9 (SOX9). This transcription factor in turn activates the expression of an array of genes that drives a pre-Sertoli cell phenotype, including cues that amplify and propagate the molecular programme along adjacent somatic and germ cells (e.g. FGF9 and PTGDS), leading to the differentiation of the fetal testis. PGCs, now called spermatogonia, undergo further proliferation before arresting in mitosis. The production of testosterone from newly differentiated Leydig cell precursors stimulates the Wolffian ducts to develop into components of the male reproductive tract – the vas deferens, epididymis and seminal vesicles – while the production of anti-Müllerian hormone (AMH), driven by SOX9 in pre-Sertoli cells, causes regression of the Müllerian ducts.

Female XX embryos lack *SRY*, and therefore SOX9 expression remains low in somatic cells within the developing gonad. These cells, thought to be derived from the mesenchyme or coelomic epithelium overlying the gonadal ridge, additionally express factors that actively repress SOX9 and its targets. Such factors, including RSPO1, WNT4, CTNNB, FST and FOXL2, are necessary to promote and maintain the differentiation of these cells into 'pre-granulosa' cells. Adequate expression of these proteins is fundamentally important for maintaining the phenotype of this lineage, even into adulthood, with experimental examples of mutations shown to precipitate a partial gonadal sex reversal or the development of an ovotestis phenotype. Secreted signals from the pre-granulosa

cells are important for (1) stimulating PGCs (now called oogonia) to arrest in meiosis (see Section 1.3), and (2) promoting differentiation of nearby somatic cells, from an alternative lineage, into 'pre-theca' cells. Thus, the pre-granulosa cells initiate a series of developmental events to define the tissue that will eventually become the ovary. In comparison with the fetal testis, the relatively low levels of testosterone and AMH cause the Wolffian ducts to regress and the Müllerian ducts to persist, the latter of which will form the precursor structures to the oviduct, uterus and upper vagina in the female [1].

1.3 Follicle Formation: Establishment of the Ovarian Reserve

As the fetal ovary is being established, the germ cells – now called oogonia – continue to proliferate by mitosis to form dense oogonial cell clusters; however, cell division is incomplete, and many of the oogonia remain connected to each other with a shared cytoplasm. Retinoic acid, which is produced by the somatic cells of the ovary and the adjacent mesonephros, binds to stimulated by retinoic acid 8 (STRA8) receptors on oogonia to initiate the process of meiosis, while at the same time inhibiting further proliferation of these cells. Oogonia proceed relatively slowly through the early stages of meiosis to arrest in the diplotene stage of prophase I, at which point they are called oocytes. In humans, arrested oocytes are identifiable in fetal ovaries between 10 and 24 weeks of gestation. It is during this time that primordial follicle formation occurs, a process whereby pre-granulosa cells invade and interpose between the germ cells in their clusters to envelop individual oocytes. Germ cells that fail to enter meiosis or become completely encapsulated in pre-granulosa cells degenerate by apoptosis [2].

It is generally believed that the net effect of these mitotic and apoptotic processes throughout this period leads to the final establishment of the ovarian reserve. Based on a relatively small number of histological studies, the average number of follicles in the human ovarian reserve (i.e. in both ovaries) is estimated to be 500,000–1,000,000 at the time of birth [3, 4]. Evidence for *de novo* synthesis of oocytes after this time is still a matter of debate, although recent studies have identified a small population of 'oogonial stem cells' that persist into adulthood, and these can be isolated and differentiated into oocyte-like cells in vitro [5]. More recent studies in mice have shown that adult somatic cells of non-ovarian lineages can be 'reprogrammed' in vitro to generate new oocytes capable of fertilisation and generation of offspring [6, 7]. Thus, research efforts are being directed towards developing novel fertility preservation strategies through artificial amplification of the ovarian reserve, although the medical potential of these findings is still far from being realised.

1.4 Follicle Development

1.4.1 Primordial Follicles

Primordial follicles, situated in the cortex of the ovary, constitute the most numerous population of follicles at any one time. Each primordial follicle consists of a small, primary oocyte (~20 μm in diameter) arrested in prophase I of meiosis, surrounded by a layer of squamous pre-granulosa cells and enveloped by a basement membrane. Within the oocyte, the nucleus, also known as the germinal vesicle, contains chromosomes in the dictyate state, a configuration conducive for active gene transcription. Both the oocyte and pre-granulosa cells are tightly connected by junctional complexes to allow for bidirectional communication and preservation of a viable, yet relatively quiescent phenotype. Thus, although primordial follicles are often referred to as 'dormant', it is important to note that they are still intrinsically active, with the oocyte and surrounding pre-granulosa cells undergoing normal cellular metabolism and homeostasis.

From the time the reserve is established within the fetal ovary, the number of primordial follicles progressively decreases; in other words, the ovarian reserve begins a steady trajectory of decline. For most females, the main reason why the ovary eventually runs out of follicles is because they are continuously recruited to grow. In adults, this continuous activation eventually leads to the menopause, a natural event that occurs in women at an average age of 51 years, when fewer than 1,000 viable primordial follicles remain. Interestingly, it has been proposed that the initial size of the ovarian reserve formed during fetal development is a predictor of the timing of menopause, as there is a strong association between the rate of follicle loss and the advancement of chronological age [3]. Genetic variation, which can influence the initial size of the ovarian reserve but also

the rate of primordial follicle activation, can also impact the timing of menopause, which, if it occurs before the age of 40, is termed premature ovarian insufficiency. Regardless of genetic influence, there is a continuous departure of primordial follicles from the ovarian reserve as they activate and enter a trajectory of irreversible growth. The remaining non-growing primordial follicles may be retained in a relatively quiescent but potentially vulnerable state for up to 40–50 years before being activated. However, this protracted period of suspended animation makes them particularly susceptible to chronic and acute exposures to environmental toxicants. Products of cigarette smoking, diet and alcohol consumption are all lifestyle factors reported to affect the viability or rate of decline of the ovarian reserve. Therefore, it is not only the quantity of primordial follicles that becomes diminished with age but also the quality. This is important in the context of female fertility, especially now that the age of first-time parents has steadily increased over the past 40 years [8].

The question of why some primordial follicles are activated to grow while others stay arrested is a major area of interest in reproductive science and medicine. Numerous molecular signals have been implicated in a range of models; however, an intricate balance of stimulatory and inhibitory factors (e.g. KL, BMP4, BMP7, bFGF, LIF and KGF) along with spatial access to these factors are likely to be important. Transgenic mouse models have established that adequate expression of key transcription factors (e.g. Nobox, Sohlh1, Sohlh2, Foxo3a and Lhx8) are essential for oocyte activation. Studies have also found that AMH, produced by developing preantral follicles, exerts an inhibitory influence on the primordial pool, while factors that activate the PI3K/AKT/mTOR signalling pathways in pre-granulosa cells and oocytes have a stimulatory effect on growth. Identifying how these pathways are regulated in this context is now the subject of many research groups. In most mammalian species, an increase in the rate of pre-/granulosa cell division, accompanied by morphological changes in shape of these cells from a squamous to a cuboidal form, as well as a relatively abrupt increase in oocyte growth, are all morphological features characteristic of follicle activation [9, 10].

1.4.2 Preantral Follicles

Follicles committed to activate and grow eventually establish a tightly packed single layer of cuboidal granulosa cells. Once this layer is complete, these primary-stage follicles require adequate expression of oocyte-derived factors for further development, specifically growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), members of the transforming growth factor β (TGFβ) family. These molecular signals act on surrounding somatic cells, which in turn signal back to the oocyte – possibly by KIT/KITL – to ensure both cell types develop in synchrony. The oocyte also develops a glycoprotein-rich zona pellucida (ZP) coat, which remains throughout the life of the oocyte. The ZP is important for fertilisation and pre-implantation development and is only shed just prior to implantation. Despite the presence of this relatively thick barrier, granulosa cells develop long thread-like processes, called transzonal projections, which extend through the ZP to the surface of the oocyte where they connect to gap junctions. The existence of gap junctions between the oocyte and granulosa cells, and also between adjacent granulosa cells, is vital for allowing bidirectional molecular communication.

It is also during the primary stage when stromal stem cells begin to differentiate into a layer of theca cells and associate with the basement membrane. As preantral follicle development progresses, granulosa and theca cells continue to proliferate under the influence of local growth factors – principally originating from the oocyte (e.g. GDF9, BMP15) but also from the surrounding cells and tissues. Several signalling pathways play key roles at this stage, including the TGFβ (e.g. activin), insulin-like growth factor (IGF) and epidermal growth factor (EGF) pathways; however, many others are also implicated. The combination of these mitogenic signals causes multilayering of the somatic cells and further growth of the oocyte, leading to overall expansion of the follicle into a secondary-stage or multilayered preantral follicle [11].

Importantly, these early stages of preantral follicle development occur independently of gonadotrophins and steroid hormones. As such, early follicle development occurs throughout pre-pubertal life – even in the fetal ovary – although fetal preantral/small antral follicles will never develop much further due to insufficient levels of follicle-stimulating hormone (FSH). In the post-pubertal ovary, FSH from the pituitary binds to functional FSH receptors expressed on granulosa cells of preantral follicles. FSH augments the actions of local growth factors to mainly stimulate

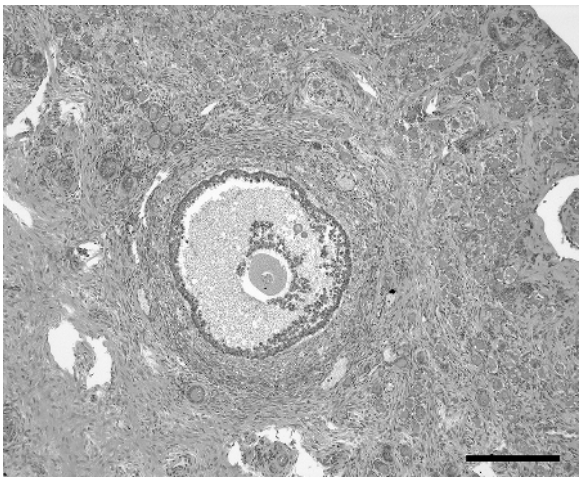

Figure 1.1 The human neonatal ovary. Numerous primordial follicles are embedded in the stroma, and a small antral follicle measuring approximately 400 µm in diameter is evident (centre). Although the antral follicle appears well developed, it is destined to undergo atresia due to insufficient levels of gonadotrophins in early life. Scale bar = 200 µm. A black and white version of this figure will appear in some formats. For the colour version, refer to the plate section. Image kindly provided by Dr Suzannah Williams and Briet Bjarkadottir (University of Oxford).

the rate of follicle growth by promoting cell proliferation and, with further development, stimulates the differentiation of follicle cells to become steroidogenic (see Section 1.4.3.2). Most of the large preantral/small antral follicles that develop when FSH levels are low – for example, before puberty (Figure 1.1), or during the luteal phase of the menstrual cycle – will perish, a process known as atresia. However, in the post-pubertal ovary, if the timing is right, around 7–10 follicles per ovary per cycle will be selected for further development to the antral stage.

1.4.3 Antral Follicles

In the human ovary, the developmental journey from a small primordial (0.03 mm/30 µm diameter) to a large preantral follicle (0.2 mm/200 µm) occurs over several months. By comparison, antral follicle development (>200 µm) occurs rapidly over a 6-week time frame, with significant expansion (from 2 to 25 mm) during the final 2 weeks corresponding to the follicular phase of the menstrual cycle. During antral follicle growth, a series of developmental events ensures that the ovary communicates effectively with the hypothalamus and pituitary to set in motion a feedback mechanism with the gonad that regulates the production of sex steroids to prepare the reproductive tract and coordinate this with ovulation. The following sections provide a brief summary of the developmental changes that occur in the oocyte and somatic compartments as gonadotrophin-dependent follicles develop towards the ovulatory stage.

1.4.3.1 Morphological Changes

As large preantral follicles become increasingly responsive to gonadotrophins, fluid rich in proteins, mostly derived from granulosa cell secretions and serum transudate, begins to appear. These discrete pockets of fluid eventually coalesce into a single, large antrum, which allows unimpeded diffusion of growth factors and molecules. This is important because, while the theca layer is highly vascularised, blood vessels never penetrate the basement membrane of the follicle until ovulation occurs; therefore, granulosa cells and the oocyte are sustained in a nutrient-rich environment despite their relative distance from the vasculature. Granulosa and theca cell proliferation occurs at an increased rate due to the powerful actions of steroid and peptide hormones and local growth factors, although the rapid expansion of the antral follicle throughout the follicular phase of the menstrual cycle occurs mostly as a consequence of the increased antral volume. Within the antral follicle, the granulosa cells differentiate into two specialised types: (1) the cumulus cells surrounding and in direct contact with the fully grown oocyte, and (2) the mural granulosa cells at the periphery of the follicle. Likewise, the theca cells differentiate into two specialised layers: (1) the vascularised theca interna, which is in direct contact with the basement membrane, and (2) the more peripheral theca externa, containing fibroblasts and smooth muscle-like cells, providing structural integrity to the expanding follicle.

1.4.3.2 Steroidogenesis and Atresia

The mural granulosa cells, along with the theca interna cells, are dependent on gonadotrophins for survival and steroidogenesis. The 'two-cell, two-gonadotrophin hypothesis' proposed in the 1970s refers to luteinising hormone (LH) binding and activating the LH receptors (LHRs) expressed on theca interna cells, stimulating production of androgen, while FSH signals via the FSH receptors (FSHRs) on mural granulosa cells to convert androgen to oestrogen [12]. FSHRs and LHRs are G protein-coupled cell-surface receptors that activate second

messengers – cyclic adenosine monophosphate (cAMP), which drives protein kinase A (PKA), as well as other kinase pathways – which each signal to the nucleus to drive transcription of target genes. Such targets are varied but include inhibitors of apoptosis, such as the BCL-2 family, which are important for follicle maintenance and survival. Inadequate gonadotrophin signalling in the antral follicle leads to somatic cell death and loss of basement membrane integrity, allowing leukocyte infiltration, collapse of the antrum and eventual oocyte demise. This degenerative process – otherwise known as atresia – is a discrete mechanism resulting in rapid clearance of follicles that are no longer destined for further development. Although atresia can occur at any stage of follicle development, it is predominantly a feature of antral follicles and is more easily recognisable microscopically in larger follicles due the relatively high number of cells. It is estimated that, in humans, atresia accounts for the loss of over 99.9% of the follicles from the original reserve at birth. Therefore, in order for antral follicles to survive, they need to express functional gonadotrophin receptors, particularly for FSH, during the early stages, and LH in the later stages to coincide with the cyclical rise in systemic gonadotrophins.

Of the small proportion of follicles that remain responsive to adequate levels of gonadotrophins (i.e. during the follicular phase of the menstrual cycle), cAMP/PKA signalling also drives the expression of key enzymes involved in growth and steroidogenesis. In theca cells, key steroidogenic enzymes include cholesterol side change cleavage/cytochrome P450 (encoded by the *CYP11A1* gene), which converts cholesterol, derived from the circulation, to pregnenolone within the mitochondria (Figure 1.2). 3-β-Hydroxysteroid dehydrogenase (HSD3B1) then converts pregnenolone to progesterone. Under the action of 17α-hydroxylase/17,20-lyase enzyme (CYP17A1), both pregnenolone and progesterone can be modified to the 17α-hydroxy forms, which are the precursor substrates for the androgens. These are the first rate-limiting steps involved in steroidogenesis.

Progestagens, often referred as the grandparental steroids, are converted to the androgens dehydroepiandrosterone (DHEA) and androstenedione in the presence of CYP17A1. Importantly, 17β-hydroxysteroid dehydrogenase 1 (HSD17B1) converts these androgens into androstenediol and testosterone, respectively. Dihydrotestosterone (DHT), a potent, non-aromatisable androgen, is also produced from the activity of 5α-reductase 1 (SRD5A1) on testosterone. Additionally, both DHEA and androstenediol can be further converted by HSD3B1 to androstenedione and testosterone, respectively, both of which are

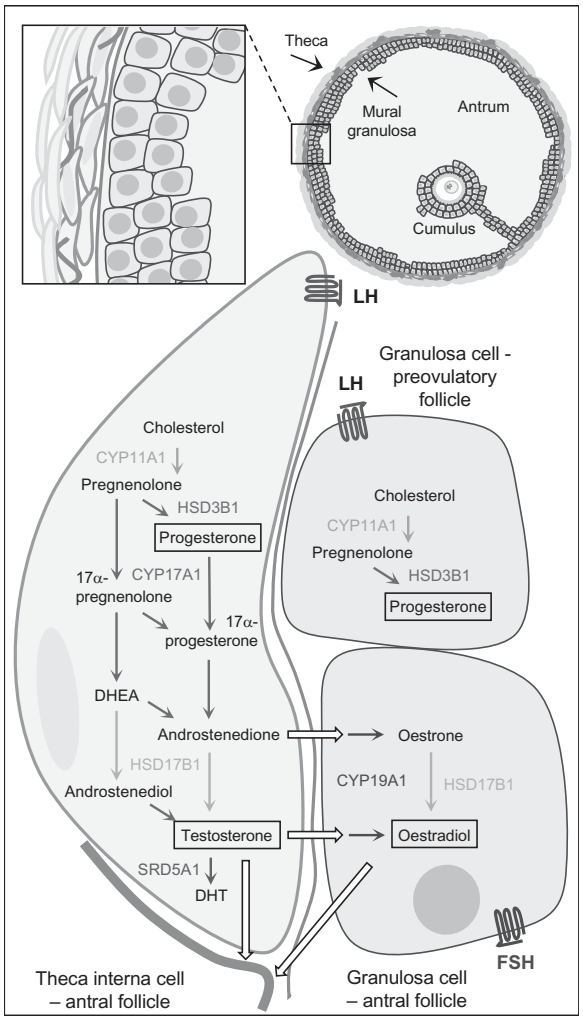

Figure 1.2 Steroid synthesis in antral follicles. During antral follicle development, luteinising hormone (LH) binds to the LH receptor on theca cells, which regulates the expression and activity of key steroidogenic enzymes, allowing the modification of cholesterol into androgens. Testosterone and androstenedione diffuse across the basement membrane where they are converted to oestrogens through the activity of aromatase (CYP19A1), which is regulated by follicle-stimulating hormone (FSH) signalling in granulosa cells. In pre-ovulatory follicles, LH receptor is also expressed in granulosa cells to further amplify progesterone synthesis. Note: steroidogenic enzymes are presented as protein symbols (refer to main text for protein name) with corresponding coloured arrows indicating enzyme activity. A black and white version of this figure will appear in some formats. For the colour version, refer to the plate section.

the precursor substrates for the oestrogens. These substrates, being lipophilic steroids, are able to readily diffuse through the basement membrane into the adjacent granulosa cells.

Although theca cells are capable of producing small amounts of oestrogens, granulosa cells fundamentally lack the enzyme (i.e. CYP17A1) required to produce the androgenic substrates. Instead, FSH/FSHR signalling in granulosa cells drives aromatase (CYP19A1) activity, which enables conversion of the theca-derived, aromatisable steroids androstenedione and testosterone to oestrogens. Here, oestradiol-17β is derived from aromatisation of testosterone, or through conversion of oestrone by HSD17B1. Oestradiol is the major form of oestrogen secreted back across the basement membrane into the theca interna where it enters the circulation through the large blood vessels [13, 14].

Although steroid hormones are fundamentally important for endocrine signalling throughout the body, both steroid and peptide hormones have important roles in further enhancing antral follicle development. Androgens from the theca, along with FSH, as well as oestrogens from the granulosa cells, all stimulate the further proliferation of granulosa cells. Androgens also promote aromatase activity, together creating a positive-feedback mechanism to significantly increase oestrogen output during the follicular phase of the cycle [15]. Other growth factors, such as IGF-1 and other TGFβ members (e.g. activins, inhibins and follistatin), are produced in response to gonadotrophin signalling and are also implicated in increasing the rate of granulosa and theca cell proliferation. FSH also causes elevated expression of inhibin B (inhibin α and βB heterodimer), while both FSH and LH promote the expression of inhibin A (inhibin α and βA heterodimer); thus, as follicle development proceeds, the elevated ratio of inhibin A: inhibin B along with rising oestrogen in the blood can be used as markers of follicle expansion. Inhibins and oestrogens are also important for supressing FSH, a feedback mechanism that causes a shift in hormonal support during the latter half of the follicular phase. In the human menstrual cycle, this results in regression of subsidiary follicles, allowing a single follicle to go on to ovulation (see Section 1.4.4).

1.4.3.3 Oocyte Development

Although the oocyte is arrested in the first prophase of meiosis throughout preantral and antral follicle development, it is far from inactive. Oocyte growth continues throughout the preantral stage to reach a maximum size of around 120 μm in small antral follicles. During this time, the oocyte synthesises and accumulates a vast amount of RNA – approximately 200× more relative to a somatic cell. Much of the mRNA is actively transcribed and polyadenylated for translation in a stage-dependent manner as the oocyte develops and becomes competent to resume meiosis; however, many mRNAs are also transcribed and deadenylated for degradation or storage towards the end of follicle development, a process indispensable for development into a viable embryo. During antral follicle development, the oocyte also begins to synthesise the machinery required for further meiotic progression. Maturation-promoting factor (MPF), a protein complex involving cyclin-dependent kinase (CDK) and cyclin B, is produced to enable meiotic progression but is held in abeyance by high levels of cAMP. The relationship between the oocyte and the adjacent granulosa cells is crucial for the maintenance of cAMP. This is because granulosa cells provide a source of cyclic guanosine monophosphate (cGMP) via gap junctions to inhibit the activity of phosphodiesterase (PDE3A), an enzyme that acts to degrade cAMP (see Section 1.4.4.2) [16].

1.4.4 The Ovulatory Follicle

In mono-ovulatory species such as humans, usually only one antral follicle will continue to develop to the pre-ovulatory stage. Although the mechanism for this selection is not yet fully understood, it is thought that follicles that produce comparatively high levels of IGF and the receptor IGFR1, in response to both FSH activity and oocyte signals, exhibit augmented steroidogenesis, which in turn supports a rapid advancement of follicle growth. The elevated levels of oestrogen produced by the pre-ovulatory follicle eventually promote LHR expression in the mural granulosa cells. Oestrogens entering the circulation also alter the activity of gonadotropin-releasing hormone-expressing neurons in the hypothalamus, resulting in a surge of LH secretion from the pituitary. These acutely high levels of circulating LH bind to LHR in the pre-ovulatory follicle to initiate a host of events in the lead up to ovulation 24–36 hours later. The following sections summarise the key changes that occur in the follicle and oocyte during that time.

1.4.4.1 Cumulus Expansion

Throughout antral follicle development, cumulus cells surrounding the oocyte are fairly closely packed, forming a unit known as the cumulus–oocyte complex (COC). Within a few hours of the LH surge, the cumulus cells surrounding the oocyte loosen and the COC undergoes rapid expansion. This process is precipitated by the elevation in LH signalling in mural granulosa cells, which drives expression of EGF-like factors (amphiregulin, epiregulin), which are secreted into the antrum to bind EGF receptors located on cumulus cells. These EGF-like signals, along with growth factors secreted from the oocyte (e.g. GDF9, BMP15 and others) provide a powerful cocktail of stimulants that promotes the upregulation of genes necessary for rapid proliferation and differentiation (e.g. *HAS2*, *PTGS2*, *PTX3* and *TNFAIP6*) of cumulus cells, which, in turn, secrete a gel-like matrix of hyaluronan and stabilisation factors causing expansion. This expanded cumulus is important for nourishment of the oocyte and the embryo during the peri-conception period, and also aids in oocyte pick-up by the Fallopian tube [15, 17].

1.4.4.2 Resumption of Meiosis

As the cumulus expands, the gap junctions/transzonal projections between the oocyte and surrounding cells close. This loss of cGMP source from the granulosa cells allows PDE3A activation and cAMP degradation in the oocyte. The consequential reduction in cAMP causes dephosphorylation of CDK1 and activation of MPF to enable meiosis to resume. Microscopically, the changes can be visualised by the breakdown of the germinal vesicle envelope (also known as germinal vesicle breakdown), formation and assembly of the meiotic spindle (enabling chromosomal segregation), formation of cortical granules towards the periphery of the oocyte and the subsequent extrusion of the first polar body following completion of the first meiotic division. A cytostatic factor then blocks MPF causing the oocyte to arrest again, this time in metaphase II. New proteins are then synthesised to prepare for the completion of meiosis to produce a haploid gamete for fertilisation [15, 16].

1.4.4.3 Ovulation

In the mural granulosa cells, as well as production of EGF-like factors, LHR signalling drives expression of steroidogenic enzymes (CYP11A1, HSD3B1) to enable the production of progesterone. The mural granulosa cells lose the ability to respond to oestrogens and instead the LH and progesterone stimulate mitogenesis and further progesterone synthesis. Although the elevation in progesterone is essential for ovulation to occur, it is also believed to depress the growth of less mature follicles, thus enabling dominance of a pre-ovulatory follicle. The actions of progesterone lead to considerable expansion of the pre-ovulatory follicle and cause it to bulge from the ovarian surface with a relatively thin layer of granulosa and theca cells. This thin layer, called the stigma, is avascular and is subject to the progesterone-stimulated activities of proteolytic enzymes such as matrix metalloproteinases (MMPs), tissue inhibitors of MMPs, plasminogen activator and cyclo-oxygenase/prostaglandins. The internal pressure of the follicle along with the contractile activity of the theca externa results in follicle rupture, leading to expulsion of follicular fluid and the COC around 24–36 hours after the LH surge [13].

1.5 Corpus Luteum

Following ovulation, the basement membrane of the follicle collapses and is breached by invading blood vessels. The loss of oocyte-derived factors along with the LH-induced promotion of steroidogenesis causes the remaining granulosa and theca cells to differentiate into large and small lutein cells, respectively. Both cells continue to produce progesterone, but androgens, oestrogens, oxytocin and inhibin A are also produced by the corpus luteum. The initial action of LH along with the paracrine activity of progesterone provides luteotrophic support to allow the structure to persist until pregnancy occurs, at which point human chorionic gondadotrophin from the developing conceptus replaces LH. If no pregnancy occurs, the loss of peptide hormone signalling and the progressive reduction in progesterone is associated with luteal regression towards the end of the menstrual cycle, with reabsorption of the corpus luteum [18].

1.6 Summary

The ovary is an incredibly dynamic organ: from the time it is formed in the fetus and right throughout adult reproductive life, there is constant remodelling (Figure 1.3). The activity of specific transcription factors drives the differentiation of somatic and germ cells, which become arranged into the basic germ cell units – the primordial follicles. From this precious reserve, a proportion of follicles are constantly being

Figure 1.3 Summary of germ cell development from follicle formation to ovulation. Key stage-specific events are indicated in the text boxes. CL, corpus luteum; FSH, follicle-stimulating hormone; LH, luteinising hormone; MII, metaphase II. A black and white version of this figure will appear in some formats. For the colour version, refer to the plate section.

activated to grow, a process driven by local cues and involving timely activity of cell-specific signals. Further development throughout the preantral stages depends on synchronous growth of the oocyte and somatic cells, which progressively become responsive and eventually dependent on the timely rise in gonadotrophins from the pituitary. Throughout the process, the developmental and maturational changes in the oocyte, granulosa and theca cells are critical determinants in ensuring the production of sex steroids and eventually the release of a mature metaphase II oocyte at the time of ovulation. The surrounding cells and structures, which have not been discussed here but include the vasculature, lymphatics and nerves, all set within stroma and encased by a surface epithelium, must also cope with the ongoing demands of internal restructuring. The complexity of these interactions in time and space is fundamental for ovarian function and female fertility.

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Chapter 2

Monitoring of Ovarian Stimulation

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2.1 Introduction

Monitoring of ovarian stimulation plays a key role in the recruitment of mature oocytes, which in turn leads to better fertilisation rates and embryo development.

The main aim of clinicians is to ensure that sufficiently mature oocytes are collected to achieve a pregnancy, while not overstimulating the ovaries, which can lead to the potentially fatal condition of ovarian hyperstimulation syndrome (OHSS). The emphasis for fertility clinics is controlled ovarian stimulation (COS).

Following the first successful in vitro fertilisation (IVF) cycle in 1978, original monitoring of ovarian stimulation was based on multiple daily urinary or serum oestrogen or pregnandiol levels. This method continued in the early years of IVF, moving more to oestradiol profiles, although when used alone this resulted in up to 40% of collections having only immature oocytes [1]. This was due to oestrogen levels varying for the number of follicles and not necessarily being associated with follicle maturity, leading to premature triggering and follicle aspiration.

Current practice for COS predominantly uses transvaginal ultrasonography with or without hormonal testing. This allows clinicians to adjust stimulation doses depending on the patient's response or even to cancel a cycle where the response has been inadequate or excessive. Regular monitoring of COS determines the appropriate timing for final trigger injection, and identification of those at risk of OHSS and implementation of interventions to reduce the likelihood of developing OHSS. Routine monitoring of COS cycles will normally begin anywhere from day 6 of gonadotrophin stimulation and will then be repeated every 2–3 days thereafter until significant follicular maturity has been achieved.

Oestradiol monitoring during ovarian stimulation can be used as an adjunct to ultrasound to help clarify decision making. Follicles less than 10 mm in mean diameter produce very small amounts of measurable oestradiol, whereas those that are greater than 10 mm will produce progressively more oestradiol as they enlarge and mature. Oestradiol levels will rise exponentially during COS with levels excreted from maturing follicles doubling every 2–3 days. In a natural ovulatory cycle, oestradiol levels peak at between 730 and 1,470 pmol/l, just before the luteinising hormone (LH) surge [2]. These levels can be transferred to COS cycles, and levels of oestradiol can be correlated against the number of follicles present. If levels of oestradiol remain below 730 pmol/l, the likelihood of pregnancy is low, and if levels remain static or are reducing, then this is an indication to consider cancelling the cycle. Levels between 1,835 and 5,506 pmol/l are when best results are achieved; however, levels may peak higher than this if there are more follicles. If levels of oestradiol exceed 11,000 pmol/l, this can act as an alert to the clinician of the risk of the patient developing OHSS. However, if a patient has polycystic ovaries, they may have higher baseline oestradiol levels and this needs to be taken into account when monitoring stimulation based on the patient's hormone profile.

Ultrasound monitoring throughout COS is essential for the management of most patients. Scanning at the beginning of the cycle helps to identify whether there are any pathologies that may hinder ovarian stimulation, such as ovarian cysts, and counting of small antral follicles can also allow adaptation of the initial dose of gonadotrophin to ensure a better response. Repeat monitoring can be commenced after 6 days of stimulation and can help determine the number of follicles recruited. Adjustment in gonadotrophin dosing can be implemented at this point if necessary; for example, if all follicles remain less than

10 mm in diameter, then an increase in the dose could be recommended and is done so in some treatment centres. However, some studies have shown that adjustment of gonadotrophin dose midway through the cycle results in poorer oocyte numbers and pregnancy rates, begging the question of whether it is necessary to make changes during a stimulation cycle and whether is it necessary to scan mid-stimulation if changes are not to be of benefit.

A day 6 scan can offer reassurance to a patient with regard to their progress and also makes them feel that they are getting additional/better care. However, this is another appointment for patients to arrange, at an additional cost to either the treatment centre or the patient themselves. Sometimes a balance needs to be struck between fewer patient visits and a belief of being better looked after.

Scans can otherwise be repeated at day 9 or 10 of stimulation and then every 2–3 days thereafter if the response is poor or excessive, depending on unit practice. Once follicles have reached a certain level of maturity (>17 mm in diameter), this is an indication for the maturation trigger injection. If follicles are less than 15 mm or greater than 24 mm, then the chance of obtaining good-quality oocytes and subsequently achieving a pregnancy is reduced [3]. Between 11 and 12 days of stimulation is more likely to yield more oocytes of optimal maturity; however, patient responses to gonadotrophins will inevitably vary, with some requiring only 9 days of stimulation and other patients requiring a more prolonged course of gonadotrophins. There is a reduction in pregnancy rates when stimulation is less than 8 days or more than 13 days (34% decrease in pregnancy rates at >13 days of stimulation), but those with polycystic ovaries are the exception, with a longer duration of stimulation resulting in pregnancies even after 18 days of stimulation [4].

Follicular measurements are best achieved by the mean of the two largest orthogonal measurements of the follicle. Single diameters of the follicles are more unreliable in a stimulated cycle, as the follicles are often deformed by adjacent follicular growth; therefore, a mean of the two diameters will give a more reliable result. It has been shown that there is only a slight difference between the mean of two diameters in comparison with three (–0.3 mm) and therefore it is more efficient to determine the mean size of the follicle based on the two measurements. However, with ultrasound, the reproducibility of follicle diameters is mediocre, with intraoperative variability of ±2 or ±3 mm highlighting the need for improvement in this area.

Current advances in ultrasound, particularly three-dimensional (3D) measurements of follicular size, may lead to quicker and easier determination of follicular maturity and reduce the likelihood of operator variability, which is inherent with two-dimensional mean diameter measurements. With most newer scan machines having the capability to perform 3D imaging and follicular measurements, this may become a tool used more frequently for ovarian stimulation monitoring and could potentially lead to an improvement in oocyte maturity and quality, although more studies involving 3D follicular monitoring are required to determine this.

The use of hormonal monitoring in COS is debatable and varies among units as to whether to include these additional tests. A meta-analysis reviewing differences between ultrasound monitoring alone versus ultrasound and hormonal monitoring failed to show any significant difference between oocyte numbers collected, clinical pregnancy rates and cases of OHSS [5]. However, this interpretation was limited by the imprecision of the findings and the overall low quality of the included studies [5].

Additional hormonal testing adds costs to both clinicians and patients, with potential delays on management decisions depending on the results of the tests. Performing additional hormonal testing may also require extra appointments, which may add to an already busy clinic workload, while patients may find additional appointments another stress in their treatment process when coordinating their appointment with their own working commitments.

However, recent studies have looked into the monitoring of progesterone levels in the late follicular phase of ovarian stimulation and its effect on assisted reproductive technology success rates. The review by Lawrenz et al. highlighted the issues that can occur with premature progesterone rises and the impact that these have on both endometrial receptivity and embryo development [6]. Gene receptivity and expression were monitored from endometrial biopsies and compared from natural and stimulated cycles. In comparison with the natural cycles, those that had a raised progesterone level showed increased upregulation and downregulation in genes thought to be involved with endometrial receptivity, and a number of studies in the review noticed reduced

pregnancy and implantation rates in those whose progesterone level was greater than 1 ng/ml, with more significant differences seen as the progesterone level increased. On reviewing embryo development, there was no difference between fertilisation rate and blastulation rate; however, the total number of top-quality blastocysts was significantly reduced in the group with raised progesterone.

Administration of corticosteroids during stimulation has been shown to prevent premature progesterone rises, as well as other considerations such as not prolonging ovarian stimulation, using a stepdown approach of stimulation with corifollitropin-α as opposed to daily follicle-stimulating hormone (FSH) injections or freezing all embryos created in cycles with a significant early progesterone rise. A minority of patients might see a significant LH fall on first commencing use of gonadotropin-releasing hormone (GnRH) antagonists mid-stimulation, but more work is needed to assess whether routine testing for this phenomenon is useful.

The review by Lawrenz et al. identified the significance of progesterone levels and the effect they have on IVF success rates [6]; however, more objective clinical evidence is required before this can be implemented into practice worldwide.

2.2 Criteria to Trigger

Ensuring the correct timing of maturation trigger injections for oocyte retrieval is vital to achieve the best quality and number of oocytes following gonadotrophin stimulation. If oocytes are too small, then the likelihood is that they will be too immature for successful fertilisation. If the oocytes are too big, then, similarly, they may be post-mature and equally lead to poor fertilisation rates. It is therefore essential that trigger injections for final maturation are given at the correct point in follicular stimulation.

It is generally understood that follicles between 16 and 22 mm in diameter are more likely to yield mature oocytes, whereas those above or below these measurements lead to poorer-quality oocytes (on the day of oocyte retrieval). As follicles will grow approximately 1.7 mm/day, you would expect that the follicle size on the day of trigger may be 3–4 mm smaller than they would be on the day of retrieval [7]. In the study by Abbara et al., follicles that measured 12–19 mm on day of trigger made the greatest contribution to the number of oocytes retrieved [7]. They also suggested that the sizes of follicles that contributed to the formation of embryos and high-quality embryos were comparable to those contributing to oocytes and mature oocytes.

It was also noted that when there is a higher ratio of follicles >17 mm:>10 mm, the chances of implantation, clinical pregnancy rate and live birth rate increased. Standard procedure is that once there are three or more follicles greater than 17 mm, then the trigger injection can be given.

Other criteria for determination of triggering can also be based on oestradiol levels; however, this is not routine in many clinics nowadays and is dependent on treatment centre preference. The trial by Hu et al. compared oestradiol with oocyte numbers and subsequent IVF success rates [8]. Mature ovum rate, fertilisation rate and good-quality embryo rate exhibited an increasing trend as the peak oestradiol level per oocyte increased, while pregnancy rate, implantation rate and live birth rate were found to be lower whenever the oestradiol:oocyte ratio exceeded 1,470 pmol/l per oocyte or was less than 370 pmol/l per oocyte [8].

Future determination of follicular maturity may be improved by the use of colour Doppler to detect vascularity of the ovarian stroma. Follicles that have more than 75% of their surface perfused, ovarian stromal peak systolic velocity of more than 10 cm/s and a resistance index of less than 0.4–0.48 have been shown to contain mature oocytes of satisfactory quality and result in a better grade of embryo. Follicles having a perifollicular blood flow of >50% have increased oocyte retrieval rates, with increased numbers of mature oocytes with higher fertilisation rates and lower triploidy rates, and lead to the formation of more higher-grade embryos [2]. The use of colour Doppler in the future has the potential for identification of better timing for final triggering of oocyte maturation; however, this has yet to be tested in comparison with mean follicle diameter, and further trials are needed to determine the effectiveness of this technique.

2.3 Timing between Trigger and Oocyte Retrieval

In a physiological state, LH surges will result in ovulation after 24–56 hours, with 32 hours being the mean time. However, during a COS cycle, there is an absence of natural endogenous LH surge. Therefore, an exogenous gonadotrophin is required

to replace the natural LH surge, most commonly human chorionic gonadotropin (hCG). Determining the timing when this trigger injection needs to be given is crucial for the final stages of oocyte development and maturity, and is still a debated topic for the optimal time for triggering.

Initially, oocyte retrieval was to be performed within 36 hours of the trigger injection to minimise the risk of ovulation and loss of oocytes, based on the duration between LH surge and ovulation that occurs in the natural physiological process.

The interval between trigger injection and oocyte retrieval is important, because a series of crucial processes, such as the start of luteinisation, expansion of cumulus cells and the resumption of oocyte meiosis, are accomplished during this time. Therefore, if oocyte retrieval is performed too soon, the oocytes will be immature, while too late there is a risk of postmaturity and ovulation leading to the loss of oocytes.

A meta-analysis of previous studies by Wang et al. looked at five randomised controlled trials to determine the optimal time delay between trigger injection and oocyte retrieval [9]. Their conclusion was that there was no significant difference between fertilisation, implantation or pregnancy rates between those whose oocyte retrieval occurred before or after 36 hours from their trigger injection. Of note, there were more mature oocytes in metaphase II in patients whose trigger was more than 36 hours before their oocyte collection in comparison with those who had oocyte collection less than 36 hours from their trigger.

Spontaneous ovulation from prolonged exposure to trigger injection is still something to be mindful of. However, several studies have reported first follicular rupture/ovulation occurring anywhere from 38 to 41 hours after trigger injection [10].

Aiming for oocyte retrieval between 36 and 38 hours after trigger injection would be the ideal target to ensure more mature oocytes while not risking follicular rupture and ovulation and potential cancellation of treatment cycles.

2.4 Type of Trigger Injection

In the normal menstrual cycle, the final maturation of the oocyte occurs with a surge in LH and to a smaller degree FSH. In IVF, the need to mimic this physiological process is essential to develop metaphase II oocytes that will subsequently be mature enough to inseminated and create embryos.

However, simply using recombinant LH to do this has proven not to be an effective method. LH administration has been thoroughly reviewed over the years and been found to be an inferior method for triggering final maturation of oocytes in COS cycles. Instead, hCG is used predominately as the triggering agent. hCG is very similar structurally to LH, and both of them act on the same receptor, eliciting similar but not identical physiological responses.

In comparison, hCG has a significantly longer half-life than LH, 2–3 days compared with 1–5 hours, respectively, with hCG also being five times more potent. The reduced half-life and potency of LH limits its duration of effect and consequently the maturation of oocytes. A multicentre study of LH administration versus hCG for final oocyte maturation found that there were similar success rates between the two groups [11]. However, much higher doses of LH (15,000–30,000 IU) were required for similar success rates compared with the hCG group (5,000 IU), raising serious cost versus efficacy concerns. A subsequent Cochrane review also found the evidence regarding LH performance to be very low, which strongly limits the ability to draw any conclusions regarding its use for triggering, even when ignoring cost issues [12].

The doses of hCG trigger injections range from 1,500 to 15,000 IU, with the most common dose being 5,000 or 10,000 IU. Abdalla et al. showed that doses of 2,000 IU in comparison with 5,000 or 10,000 IU had a reduced oocyte recovery rate, with no significant difference between the 5,000 and 10,000 IU dose [13]. An increase in pregnancy rates was not confirmed with higher doses of hCG [14]. With increased doses of hCG up to 10,000 or 15,000 IU, there was no difference in oocyte yield in comparison with a dose of 5,000 IU [14]. Patients with a raised body mass index (BMI) do have lower levels of circulating hCG following the trigger shot, but a study by Hoyos et al. showed no difference in clinical pregnancy rate with standard hCG dose irrespective of BMI [15]. When increasing the dose, however, this also increases the risk of OHSS, and patients who received 5,000 IU were less likely to develop OHSS in comparison with the 10,000 IU group.

The use of either recombinant or urinary preparations of hCG has also been debated. Historically, urinary hCG has been the sole preparation used, but there were questions with regard to variation in hormone levels, potential transmission of disease and antibody formation. The ability to create recombinant

hCG preparations has led to higher purity and a better safety profile in comparison with urinary hCG-based preparations, albeit at a greater production cost. This raised the question as to which would perform better clinically, with the result being that neither medication was significantly superior to the other.

The main issue of using hCG triggers is the risk of OHSS. hCG is considered important in OHSS due to its ability to upregulate vascular endothelial growth factor expression in granulosa cells, driving the hyperpermeability that causes OHSS to develop. In previous long cycles of stimulation, this was the only trigger injection that could be used, and in those who over-responded there was no option but to still trigger them with hCG, coast until the oestradiol levels fell or cancel the cycle. Introduction of the short GnRH antagonist cycle for stimulation has allowed the use of a GnRH agonist (GnRH-a) as a trigger for final oocyte maturation. Doses for GnRH-a triggers tend to be 0.5–2 mg for buserelin, 0.1–0.3 mg for triptorelin and 0.5–4 mg for leuprolide acetate. There has been no conclusive evidence that increasing the dose of the GnRH-a trigger improves outcomes with regard to oocyte numbers or maturity, although occasionally (in 1–2%) no eggs may be retrieved with lower doses. In this situation, it is recommended to stop collection after the first ovary, administer either 1,500 or 5,000 IU hCG depending on OHSS concerns, and collect from the second ovary 36 hours later.

GnRH-a significantly reduces the risk of OHSS, with the likelihood of patients developing moderate or severe OHSS being between 0% and 3% for a GnRH trigger in comparison with a 5% risk in those with an hCG trigger [16]. There is no difference in oocyte maturity, fertilisation rates or embryo development between hCG and GnRH-a triggers. However, the use of GnRH-a alone reduces the live birth rate, while increasing the risk of miscarriage. This is due to the lack of luteal support provided with a GnRH-a trigger compared with an hCG trigger, which has been attributed to the shorter half-life of the GnRH-a. Therefore, the use of a GnRH trigger alone should be reserved for those at risk of OHSS or those with planned elective freeze alls, such as those undergoing fertility preservation or oocyte donation.

Recently, there has been development of a 'dual-trigger' mechanism to achieve final oocyte maturation. The aim of this is to achieve decent oocyte maturation, embryo development and luteal support while reducing the risk of developing OHSS. A dual trigger means that GnRH-a and low-dose hCG (1,500 IU) are given at the same time and oocyte collection occurs 36 hours later. Recently, there have been promising results with administration of a dual trigger, but more research is required to determine its efficacy. There appear to be higher pregnancy rates with the dual triggers compared with GnRH triggers alone, but there is a higher chance of OHSS, although it is not as high as in those receiving a standard dose of hCG [17]. Luteal support during these studies was the same in both groups, and progesterone and oestrogen levels were monitored and doses of support were adjusted accordingly. There was no difference between the two groups in terms of their hormonal profile following dual or single trigger, despite being on the same luteal support.

Kisspeptin is another new trigger that has been developed recently. Kisspeptins are a group of hypothalamic arginine–phenylalanine amide peptides and act at the kisspeptin receptor on GnRH neurons in the hypothalamus to elicit endogenous GnRH release [14]. There have been a number of trials looking into the effect of kisspeptin and the dosing required. One of the first studies by Jayasena et al. showed that a single dose of kisspeptin given 36 hours prior to oocyte retrieval showed a dose-dependent mature oocyte yield (percentage of mature oocytes from follicles >14 mm on day of kisspeptin administration), with 36–49% at 1.6–3.2 nmol/kg, 76% at 6.4 nmol/kg and 103% at 12.8 nmol/kg [18]. The live birth rate per protocol was 19%. A subsequent study reviewed the benefit of an additional dose of kisspeptin 10 hours after the initial dose [14]. This increased the live birth rate to 39% in comparison with the single-dose group, whose live birth rate was 19%. Kisspeptin has also been used with patients at risk of OHSS. The study by Abbara et al. reviewed 60 women at risk of OHSS; only three developed mild OHSS, with none of them developing moderate to severe OHSS [14]. Kisspeptin is still new to assisted reproductive technology treatments, and further studies and comparisons with current ovulation triggers are required to determine its effectiveness and future role in reproductive medicine.

The serum profiles over time of the various triggers discussed are shown in Figure 2.1.

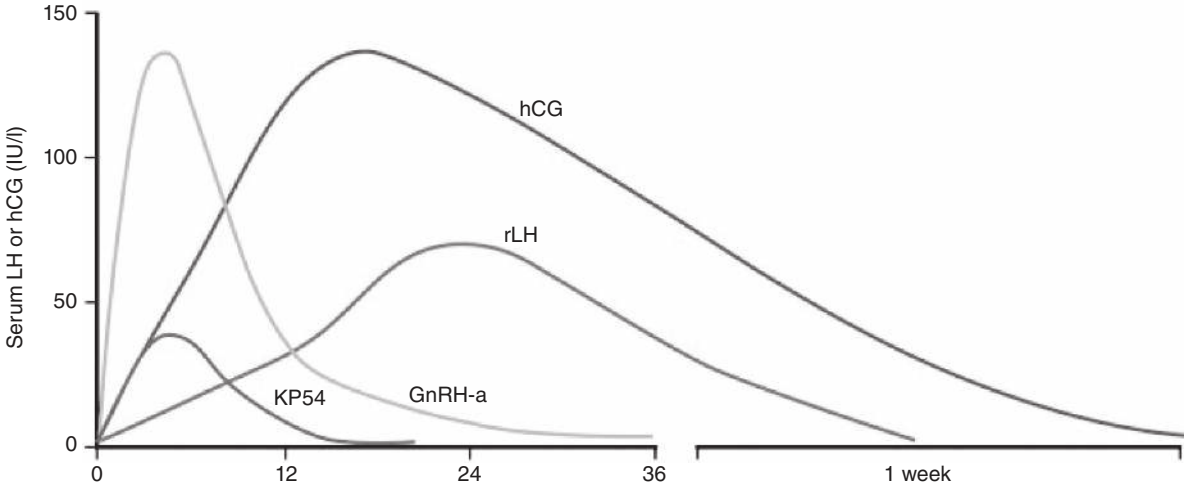

Figure 2.1 Serum profiles of inductors of oocyte maturation. The *x*-axis shows time (hours) after administration. hCG, human chorionic gonadotropin; rLH, recombinant luteinising hormone; GnRH-a, gonadotropin-releasing hormone antagonist; KP54, kisspeptin-54. A black and white version of this figure will appear in some formats. For the colour version, refer to the plate section. Reproduced from Abbara et al. [14].

2.5 Pre-operative Assessment for Oocyte Retrieval

Most oocyte retrievals are done using intravenous sedation, with a transvaginal approach being the most common. The benefit of sedation is that it is quick to administer, short acting, provides good analgesia and is associated with a good recovery profile. The transvaginal route is also the least invasive and traumatic approach to retrieve oocytes for the majority of IVF patients.

However, these approaches are not suitable for all patients, and good pre-operative assessment is essential in all patients about to undergo oocyte retrieval to ensure patient safety throughout the IVF process.

Understanding the capabilities of your specific unit is also necessary to determine whether you can take higher-risk patients and deal with them safely. Pre-operative assessment will help identify equipment, facilities and staffing capabilities required for more complex oocyte retrievals and aims to prevent procedures being performed in a setting that is unsuitable and unsafe for certain patients.

Most patients undergoing oocyte retrievals are women between 20 and 40 years of age. As a group, they tend to be relatively healthy, but consideration must be taken to review their past medical history. Causes for their subfertility such as raised BMI, cystic fibrosis, fibroids and endometriosis all have an impact on how you approach the oocyte retrieval. This approach needs to be considered particularly in those with raised BMI or multiple fibroids, and the transvaginal approach may not be the optimum route; therefore, an abdominal or laparoscopic approach may be necessary. Those with airway issues due to comorbidities such as raised BMI, poorly controlled asthma or cystic fibrosis may also require an anaesthetic review prior to retrieval to determine the anaesthetic technique required to ensure the best outcome for the patient. The above can all be determined at the pre-operative assessment and can allow appropriate planning prior to the procedure.

This is a group of patients who are at increased risk of venous thromboembolism, although the mechanism for this is unknown. One theory is that high oestrogen levels associated with ovarian stimulation may induce a pro-coagulant effect by increasing levels of coagulation factors such as von Willebrand factor, factor VIII, factor V and fibrinogen, while decreasing levels of the anticoagulants protein S and antithrombin. While ovulation induction itself is not an indication on its own to initiate thromboprophylaxis, if coupled with other risks factors, then low-molecular-weight heparin may be indicated.

2.6 Consent

When preparing a patient for any procedure, informed consent must be obtained. This allows them to make a decision fully knowing the risks and