Review

Differential actions of FSH and LH during folliculogenesis

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Abstract

In the gonadotrophin-dependent stage of follicular development, FSH- and LH-signalling pathways play an obligatory role in follicle differentiation, selection and survival. Under the effect of LH the theca–interstitial cell layer acts as an androgen producer. Thus, androgen diffusing into the mural granulosa cell layer represents the substrate for FSH-induced aromatase for follicular oestradiol synthesis. This is the landmark 'two cell-two gonadotrophin' concept in the physiology of ovarian function in mammals. The increase in plasma FSH during luteo–follicular transition is the basis for follicle selection. The rise of FSH to the threshold concentration represents a critical condition for the growth of the most sensitive follicle in a given time frame of the last 14 days of the dominant follicle odyssey. The gonadotrophin-induced follicular oestradiol secretion inhibits pituitary secretion of FSH, which in turn causes the concentration of FSH in the developing cohort follicles to drop below threshold concentrations and the arrest of the development of the less FSH-sensitive follicle (FSH threshold and window concept). In the gonadotrophin-dependent phase of follicular development, LH also seems to acts within a critical window of the hormone concentration framed between the minimal threshold and a ceiling for the normal functions of the follicle unit

Keywords: *folliculogenesis, FSH, LH, ovary, steroidogenesis*

Introduction

The ovary has two fundamental functions: to produce fertilizable and developmentally competent oocytes and to secrete the steroid hormones necessary for preparing the reproductive tract for fertilization and embryo implantation. Ovarian follicles are the functional units of the female gonads, and their maturation involves several sequential stages defined as initiation, growth, selection, ovulation and luteinization. Although many factors are involved in, and critically affect, follicle development, the two pituitary glycoproteins FSH and LH have a central role in the complex and delicate endocrine mechanisms regulating the biology of the ovary. Evidence supporting the existence of two gonadotrophins was provided more than 70 years ago (Fevold *et al*., 1931), and since that time, a vast number of basic and clinical studies have been undertaken. Understanding the molecular structure of the gonadotrophins and their receptors and the mechanisms governing their function have been the fundamental basis for the manufacture of pharmaceutical compounds and their use in ovulation induction and in the induction of multiple follicular development in clinical assisted reproductive technologies. This paper will review the basic biological processes involved in adult ovarian follicular development and discuss the role of the key molecules and pathways, in order to outline the scientific background behind successful clinical practice in reproductive endocrinology.

FSH, LH and their receptors

FSH and LH are the two anterior pituitary hormones that control gonadal function. Both hormones are synthesized and secreted by the same pituitary cells, the gonadotrophs, localized in the lateral portion of the pituitary gland, and

responsive to the pulsatile stimulation of the hypothalamic hormone gonadotrophin-releasing hormone (GnRH). Each hormone contains two subunits, α and β. The structure of the α subunits of all pituitary glycoproteins is identical, while the β chains are unique, and after linkage to the α chain, determine specific hormone function. The three-dimensional structure of each subunit is maintained by internally cross-linked disulphide bonds. Ten and 12 cysteine residues present in the mature α and β subunits respectively, are involved in the formation of disulphide bonds, essential for maintaining the subunits in the active conformation (Moyle and Campbell, 1996). FSH and LH are glycoproteins with molecular weights of approximately 30,000 Da, and contain fructose, mannose, galactose, acetylglucosamine and N-acetylneuraminidase as carbohydrate moieties. The sialic acid content varies widely among the glycoprotein hormones. These differences are largely responsible for the variations in biochemical properties and biological activities of the hormones isolated from various sources. The higher the sialic acid content, the longer the biological half-life. Moreover, oligosaccharides attached to the α and β chains play a critical role in determining the specific assembly of the subunits as well as the particular carbohydrate processing that occurs after dimer formation (Matzuk and Boime, 1988). The common α subunit, with a molecular weight of 14,600 Da, is composed of 92 amino acid residues in the same sequences, with five disulphide bonds, as well as two carbohydrate moieties. In humans, the mature α subunit is encoded by a single gene, comprising four exons on chromosome 6, in the 6q12–q21 location (Fiddes and Goodman, 1981).

FSH

The FSH β subunit is composed of 111 amino acids, with a molecular weight of 15,400 Da. It contains six disulphide bonds and, like the α subunit, carbohydrate moieties are N-linked at two asparagine residues. The FSH β subunit is encoded on chromosome 11, in the 12p13 location (Watkins *et al*., 1987). The FSH β gene is regulated by two dimeric proteins, inhibin and activin, by a single chain polypeptide follistatin, and by GnRH. Inhibin, activin and follistatin are produced by the gonads as well as by a variety of extragonadal tissue. Inhibin is considered as a selective suppressor of FSH synthesis and secretion. Activin increases the synthesis of FSH β chains and the secretion of FSH, whereas follistatin acts indirectly by binding to and neutralizing the effects of activin (Winters *et al*., 1997). Once synthesized and secreted, the FSH molecule has a plasma half-life averaging 149 min (Bogdanove and Gay 1969; Bogdanove *et al*. 1975), the liver being the major site of clearance. The relatively slow metabolic clearance rate of FSH concentrations *in vivo* means that they can neither increase nor decrease as rapidly as those of LH which has a half-life of ~30 min. This difference between the two gonadotrophins may explain why GnRH-mediated release of both hormones from the gonadotrophs is incapable of eliciting pulsatile FSH *in vivo*. However, although a pulsatile GnRH stimulus is required for FSH β chain gene expression and FSH secretion, the patterns of synthesis and secretion of this gonadotrophin differ from those of LH, which are also under GnRH control. Whereas fast GnRH pulse frequencies tend to favour LH synthesis and secretion, slower GnRH frequencies favour FSH (Dalkin *et al*., 1989). Besecke *et al*. (1996) suggested that the action of pulsatile GnRH stimulus upon FSH β chain gene expression might be exerted indirectly through changes in activin and follistatin tone.

Heterogeneity of FSH

On the basis of differences in the carbohydrate moiety structure of these glycoproteins as well as the number of incorporated terminal sialic acid residues, different forms (isoforms) of FSH are synthesized and secreted by the anterior pituitary. Up to 20 isoforms have been characterized for human FSH. Heavily sialylated (acidic) FSH has exhibit a reduced receptor binding and in-vitro bioactivity, whereas circulating half-life of these forms is longer. In contrast, basic isoforms are more biopotent *in vitro* (2- to 5-fold), whereas the circulating half-life is significantly reduced. Fluctuations were found during the normal menstrual cycle, as well as after menopause. More basic isoforms were described to be present at mid-cycle (Padmanabhan *et al*., 1988; Wide and Bakos, 1993; Zambrano *et al*., 1995). However, estimates of changes in FSH heterogeneity, as assessed by in-vitro bioassays, during the menstrual cycle are contradictory (Jia *et al*., 1986; Padmanabhan *et al*., 1988) and appear to be dependent on the assay used. It has been speculated that ovarian follicles are recruited in the early follicular phase by more acidic FSH isoforms, whereas follicle selection and rupture, during the follicular phase is dependent mainly on more basic FSH isoforms.

LH

LH is a heterodimer with a molecular weight of approximately 29,400 Da that consists of two non-covalently linked α and β subunits. The β subunit of LH has 114 amino acids and a molecular weight of 14,000 Da. The LH β subunit gene is composed of three exons, and it is located on chromosome 19, in the location 19q13.32. As previously mentioned, LH synthesis and secretion is under the control of the hypothalamic nuclei secreting the decapeptide GnRH. In a series of landmark experiments in the late 1970s, Ernest Knobil demonstrated the importance of GnRH pulsation and pulse frequency on gonadotrophin release (Knobil, 1980). Normal menstrual cycles require the maintenance of the pulsatile release of GnRH within a critical range of frequency and amplitude. Pulsatile rhythmic activity is an intrinsic property of GnRH neurons, and the effect of various hormones and neurotransmitters must be viewed as modulating actions (Stjilkovic *et al*., 1994). The pulsatile release of LH from the pituitary is the epiphenomenon of GnRH pulsatile secretion (Reame *et al*., 1984). Pulsatile LH secretion is more frequent but lower in amplitude during the follicular phase compared with the luteal phase. The slowing of GnRH pulse frequencies in the late luteal phase is an important change, favouring FSH synthesis and secretion, allowing the rise in FSH essential for the second phase of follicular recruitment.

LH heterogeneity

LH isoforms differ in the complexity and proportions of their glycosylation, sialylation and sulphation modifications (Sairam and Fleshner, 1981; Rosa *et al*., 1984; Sardanons *et al*., 1987; Smith *et al*., 1990; Fiete *et al*., 1991). Like FSH, such properties also control in-vitro LH bioactivity, which can vary by as much as 10-fold, and the in-vivo retention and tissue actions of LH, which can vary by 30-fold (Tsuruhara *et al*., 1972a,b; Dufau *et al*., 1976a,b; Wehmann and Nisula, 1979; Baenziger and Green, 1988; Bishop *et al*., 1995; Burgon *et al*., 1996). The synthesis of different glycoforms of LH is under multifactorial

endocrine control, oestradiol, testosterone and GnRH being important regulatory factors (Chen *et al.*, 1982; Weise *et al.*, 1983; Veldhuis *et al.*, 1989; Clarke *et al.*, 1990). The degree of sialylation and sulphation appear to determine the LH half-life, and thus influence in-vivo bioactivity more remarkably than in-vitro biopotency. Plasma bioactivity in healthy women varies within the menstrual cycle, and rises dramatically after the menopause (Dufau *et al.*, 1983; Veldhuis *et al.*, 1984). It has been reported that LH biological/immunological (B/I) ratios decline in the luteal phase of the menstrual cycle compared with those in the early follicular phase and at mid-cycle (Suginami *et al.*, 1982). Preferentially more alkaline LH products with higher in-vitro B/I ratios tend to be predominant in young oestrogen-enriched women. In contrast, acidic (long-lived) isoforms of LH tend to circulate in post-menopausal individuals (Lucky *et al.*, 1980a; Marut *et al.*, 1981; Veldhuis *et al.*, 1984; Reiter *et al.*, 1987; Fauser *et al.*, 1992; Imse *et al.*, 1992).

FSH and LH receptors

The gonadotrophin receptors are members of the rhodopsin-like G protein-coupled receptor family. Structurally, they exhibit a classical serpentine region with seven transmembrane helices and have a large N-terminal ectodomain of 350–400 residues, which is responsible for the high affinity and selective binding of the receptor with its ligand. In humans, FSH and LH receptor genes are located on chromosome 2p21–16. They are composed of 11 and 10 exons respectively where the first 10 or nine exons encode the extracellular domain, while the seven transmembrane segments and the G protein-coupling domain are encoded by the last exons (Segaloff and Ascoli, 1993; Simoni *et al.*, 1997). Both the glycoproteins of the β subunits and their receptors are encoded by genes with large shared sequences (Vassart *et al.*, 2004). Thus, the amino acid sequences and structural organization of the gonadotrophin receptors are highly homologous. They belong to the large family of G protein-coupled receptors, all having a transmembrane domain that consists of seven plasma membrane-traversing α-helices connected by three extracellular and three intracellular loops, responsible for interaction of G protein and signal transduction (Segaloff and Ascoli, 1993; Simoni *et al.*, 1997). A distinctive characteristic of the LH and FSH receptors (and thyroid-stimulating hormone receptors) is that they possess an unusually large extracellular domain at the N-terminus, responsible for binding of the hormone ligand. The extracellular ligand-binding domain of the gonadotrophin receptors is connected to the transmembrane signalling domain by a hinge region. This region is followed by the transmembrane domain with seven membrane-spanning α-helices, connected by three extracellular and three intracellular loops. This region is responsible for the interaction with the G protein and signal transduction. Facilitatory and inhibitory binding determinants are supposed to present in the binding region of gonadotrophin receptors (Ji and Ji, 1995). The former are responsible for the high-affinity binding to the proper ligand (leucine-rich repeats 1–8 in the case of LH; repeats 1–11, in the case of FSH). With regard to the role of the specific amino acids within the primary structure in the signal transduction, LH and FSH receptors are generally coupled to G_s, the G protein that activates the various adenyl cyclases, with resulting elevation of intracellular cAMP concentrations. Moreover, both gonadotrophin receptors are also able to activate other signal transduction pathways, involving increased phosphatidylinositide turnover and inositol triphosphate (IP_3) production, elevated Ca^{2+} and activation of mitogen-activated protein kinases (Themmen and Huhtaniemi, 2000). Thus, tonic stimulation of immature granulosa cells by FSH via the FSH receptor (FSHR) stimulates intracellular cAMP formation and activation of genes required for proliferation and differentiation. Late during preovulatory follicular growth, the response to FSH includes expression of the LH receptor (LHR), also coupled to protein kinase A (PKA; Abell *et al.*, 1998). Prior to onset of the mid-cycle LH surge that triggers ovulation, tonic stimulation of mature granulosa cells by LH via LHR mimics the action of FSH. Higher concentrations of LH dramatically up-regulate PKA signalling, also increasing inositol lipid hydrolysis and activation of protein kinase C (PKC), altering the expression panel of other genes that co-ordinate final stages of follicular development and ovulation (Robker and Richards, 1998a).

Gonadotrophin–gonadotrophin receptor interaction and the 'two cell–two gonadotrophin' concept

The post-receptor process that transmits gonadotrophin action into the cell nucleus rests mainly on adenylate cyclase, cAMP production, and activation of PKA (Richards, 1994; Richards *et al.*, 1998). Stimulation of immature granulosa cells by FSH via FSHR stimulates intracellular cAMP formation and activation of genes for proliferation and differentiation. Late during preovulatory follicular growth, the response to FSH includes expression of LHR, also coupled to PKA (Abell *et al.*, 1998). At the advanced follicular development, prior to the mid-cycle LH surge that triggers ovulation, stimulation of mature granulosa cells by LH mimics the action of FSH. Higher concentrations of LH intensely up-regulate PKA signalling, also increasing inositol lipid hydrolysis and activation of PKC, altering the expression panel of other genes that co-ordinate final stages of follicular development and ovulation (Robker and Richards, 1998b). With regard to the transcription regulators that mediate cellular responses to these signals, it seems that the CCAAT/enhancer-binding protein-β (C/EBPβ) (belonging to the family of transcriptional regulators) is a critical downstream target of G protein-coupled LHR signalling (Pall *et al.*, 1997; Sterneck *et al.*, 1997). Post-receptor signalling pathways that impact on gonadotrophin action include the serine/threonine kinase mothers against decapentaplegic-related protein (MAD) cell signalling pathway (Li *et al.*, 1997), which is activated by members of the transforming growth factorβ (TGFβ) superfamily (Massague, 1998). According to the two cell–two gonadotrophin theory, LH receptors are primarily located at the plasma membrane of the internal theca cells, while FSH receptors are expressed by the granulosa cells. Both cells and both gonadotrophins are crucial to oestrogen synthesis. The onset of follicular oestrogen secretion reflects a functional interplay between the two major steroidogenic cell types in the follicle, granulosa and thecal, regulated by FSH and LH respectively (**Figure 1**). In the preovulatory follicle, the vasculature of the theca is well developed and the mural layer of the granulosa cells are in contact with adjacent blood vessels. Therefore, these cells are well placed to respond to circulating hormone variations and to release the produced oestrogens directly into the venous effluent of the preovulatory follicles (Ravindranath *et al.*, 1992). Theca and granulosa cells in individual follicular units synthesize both androgens and oestrogens. The steroidogenic enzymes

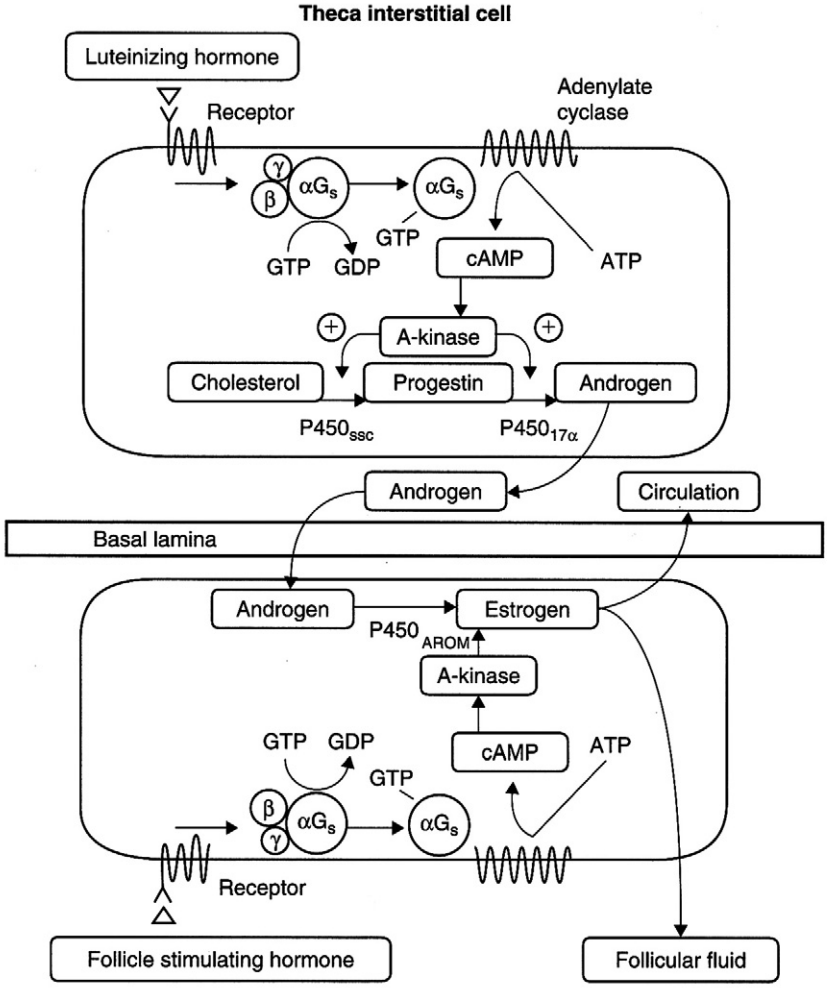

Figure 1. The two cell-two gonadotrophin concept of oestradiol production (reproduced from Erickson and Shimasaki, 2001, with permission from Elsevier).

expressed in the theca and/or granulosa cells vary with the menstrual cycle, through the regulation and modulation of gene expression of the steroidogenic enzymes (Yong *et al.*, 1994; Thiboutot *et al.*, 2000). In the follicular phase, theca cells express steroidogenic acute regulatory protein (StAR), the enzyme complex cytochrome P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase-II (3β-HSD-II), and P450c17 to produce androstenedione, which is the major androgen secreted by the human preovulatory follicle. Androstenedione is partially converted to testosterone by 17β-hydroxysteroid dehydrogenase-V (17β-HSD-V). The majority of ovarian C-19 steroids are produced from dehydroepiandrosterone (DHEA), the key precursor of androgens and oestrogens. The key enzyme in DHEA production is P450c17, which catalyses both 17α-hydroxylation and 17,20-lyase activities. The 17,20-lyase in human P450c17 strongly favours the Δ^5 pathway, so that most human androgens and oestrogens derive from DHEA (Miller *et al.*, 2006). Thecal cells abundantly express P450c17 (Sasano *et al.*, 1989), which is the rate-limiting steroidogenic enzyme in androgen synthesis, which is positively regulated by LH. Most thecal steroids diffuse to the granulosa cell, but some are secreted into the circulation. Increasing thecal androstenedione production and increased expression of 17β-HSD-I and P450arom in the granulosa cells results in abundant oestradiol production by the preovulatory follicle (Whitelaw, *et al.*, 1992; Teerds and Dorrington, 1993; Ghersevich *et al.*, 1994). Theca cells are unable to synthesize oestrogen *de novo* (Whitelaw, *et al.*, 1992) because P450arom is minimally expressed in these cells. During the luteal phase theca cells continue to provide androstenedione to the granulosa cells, while the granulosa cells undergo granulosa–lutein transformation and express StAR, P450scc, and 3βHSD-II, but not P450c17 (Voutilainen *et al.*, 1986). In granulosa cells of smaller follicles, only the FSH receptor is expressed, although both gonadotrophin receptors are expressed in follicles >8 mm diameter. In granulosa cells, FSH has been shown to stimulate low-density lipoprotein receptors (LDLr) concentrations, P450scc and P450arom activity. During the follicular phase, LH increases the theca cell expression of LH receptors, StAR, P450scc, 3β-HSD-II and P450c17, whereas FSH increases granulosa cell expression of aromatase and 17β-HSD-I. As a consequence, LH stimulates progesterone secretion from luteal cells and androgen secretion

from theca cells, whereas FSH stimulates progesterone and oestradiol secretion from granulosa cells (Voutilainen *et al.*, 1984). Around the time of selection of the dominant follicle, LH receptors and 3β-HSD mRNA expression can be detected in granulosa cells (Webb *et al.*, 1999, 2004), supporting the concept that the dominant follicle can utilize LH to support its continued growth even when circulating FSH concentrations are declining. In addition, during follicular growth, LH-produced thecal androgens are either converted to oestrogens or bound to the androgen receptors present in granulosa cells. In this way, androgens have been shown to increase the sensitivity of the follicle to the FSH via up-regulation of FSH receptor (Luo and Wiltbank, 2006). However, at an early stage of follicular development, an amplification of FSH-stimulated cAMP-mediated post-receptor signalling will induce the arrest of granulosa cell proliferation (Hillier *et al.*, 1991). During the 36–38 h of periovulatory interval of both spontaneous (Weik *et al.*, 1973; Hoff *et al.*, 1983) and stimulated (Chaffin *et al.*, 1999) menstrual cycle, follicular steroidogenesis shifts from predominantly oestrogen and androgen to progesterone secretion. This shift appears to be regulated by the LH/human chorionic gonadotrophin modulated patterns of enzymes associated with cholesterol metabolism (LDL-r and StAR) and steroidogenesis (P450scc, 3β-HSD, P450arom) in granulosa cells by shifting the ratio 3β-HSD:P450c17 in favour of progesterone synthesis (Conley *et al.*, 1995; Chaffin *et al.*, 2000).

Classification of ovarian follicles

The basic functional unit in the ovary is the ovarian follicle that is composed of somatic cells and the developing oocyte. The two primary somatic cell types in the ovarian follicle are the theca cells and granulosa cells. These two somatic cell types are the site of action and synthesis of a number of hormones that promote a complex regulation of follicular development. The proliferation of these two cell types is in part responsible for the development of the antral ovarian follicle. Granulosa cells are an actively differentiating cell with several distinct populations. In humans, the classification for ovarian follicles according to morphological criteria of the developmental stage has been proposed by Gougeon (1996). The primordial follicle is defined as a primary oocyte surrounded by flattened granulosa cells. When a few of the flattened cells become cuboidal the follicle is classified as transitionary or intermediary; these follicles are still considered to belong to the resting pool. A primary follicle is characterized by a full cuboidal granulosa cell layer surrounded by a basement membrane. Primary follicles are the first stage belonging to the growing pool. Preantral follicles can be identified as a growing primary oocyte surrounded by several granulosa cell layers. Theca cells are recruited from the interstitial stromal cells and can be recognized as individual cells on the basement membrane in part of the primary follicles (Hirshfield, 1991; Lundy *et al.*, 1999; Parrott and Skinner, 2000). As soon as the follicle reaches the secondary stage (two layers), a distinct theca cell layer is formed in all follicles (Gougeon, 1996; O'Shaugnessy, 1997; Lundy *et al.*, 1999). Preantral follicle growth can be divided into two phases: a vascular and an avascular phase. After seven to eight doublings of the number of granulosa cells, mammalian follicles reach a diameter of 200 μm. Fluid-filled patchy spaces appear within the granulosa cells and the follicles are termed early antral. Follicles are termed antral when the fluid-filled spaces have coalesced into a large crescent-shaped cavity; granulosa cells then differentiate into mural and cumulus cells. Other morphological milestones can be used to describe the developmental stages. Formation of the zona pellucida during transformation of the primordial follicle into a primary follicle is another distinct reference point. The zona pellucida and theca interna layer are formed when the follicles are at the primary stage (Braw-Tal and Yossefi, 1997; O'Shaugnessy *et al.*, 1997; Lundy *et al.*, 1999). In a further stage of preantral follicle development, a theca externa is formed as a highly vascularized layer, providing the follicle with systemic endocrine factors that permit its exponential volumetric expansion. In its development during the primary and secondary follicle stage, the oocyte acquires meiotic and developmental competence (Volarcik *et al.*, 1998; McLay *et al.*, 2002). The collection of fluid between the granulosa cell layers of the secondary follicle is the most evident morphological characteristic of the antral stage of follicle development. This process requires rapid influx of water, enabled by active ion transport by granulosa cells into the developing antrum. Granulosa cells from the antral wall are called mural cells and express the greatest steroidogenic activity and the highest concentration of LH receptors. The granulosa cells surrounding the oocyte compose the cumulus cells and have a lower density of LH receptors compared with the mural granulosa cells (Lawrence *et al.*, 1980). The final stage of antral follicle development is the Graafian follicle, with a diameter ranging from 15 to 25 mm. At ovulation, the ovum is released from the Graafian follicle having resumed meiosis and the granulosa and theca cells will differentiate into luteinized cells under the influence of LH surge.

Initial versus cyclic follicular recruitment and the FSH threshold/window concept for follicular development

At the fourth month of fetal development the ovaries contain some 6–7 × 10^6 oogonia that develop into oocytes by entering the first meiotic division, after which they become arrested at the diplotene stage of the prophase (Baker, 1963; Byskov, 1986). In humans, oocytes remain in the resting phase for many decades until meiosis is resumed by exposure to the mid cycle LH peak. Follicles are present in the ovary at different stages of development, and large numbers of follicles of different sizes can be observed at any given point of the menstrual cycle (Gougeon, 1986). Resting primordial follicles continuously enter the growing pool throughout life and the rate of consumption of the primordial follicle pool is dependent on age, being the most pronounced during fetal development. Approximately 0.5 × 10^6 primordial follicles are present at menarche. Thereafter, loss of follicles occurs at a fixed rate of around 1000 per month, being more marked beyond the age of 35 (Richardson *et al.*, 1987; Faddy *et al.*, 1992, 1995). Of the 1–2 × 10^6 follicles present at birth, approximately 400 will eventually develop into an ovulating dominant follicle. Thus, ~99.9% of the original follicle reserve will never complete their development and will undergo the apoptotic process called atresia (Hsueh *et al.*, 1994). Although the follicle development is a continuum, the process of follicular development can be divided into three successive phases: initial follicle development, FSH-dependent progression, and LH-responsive maturation (Hillier, 2001).

Initial follicle development

The dormant primordial follicles are recruited into the growing follicle pool in a continuous manner, whereas increases in circulating FSH during each reproductive cycle recruit a cohort of antral follicles. As stated before, when primordial follicles enter the growth phase (primordial–primary transition), the oocytes increase in size and granulosa cells proliferate. Transition into the secondary follicle stage involves alignment of stroma around the basal lamina and the development of an independent blood supply. The stroma subsequently differentiates into a theca externa and a theca interna layer. Theca interna cells express LH receptors early on (Channing and Kammerman, 1973). When an antral cavity develops, follicle size is approximately 100–200 μm). During early preantral follicle development, FSH receptors also become detectable on granulosa cells (Channing and Kammerman, 1973; Roy *et al*., 1987). The degree to which early stages of follicular development are influenced by FSH remains unclear. In the transgenic mouse model, some studies suggest that gonadotrophins may be involved in the activation of resting follicles (Meredith *et al*., 1986; Flaws *et al*., 1997). However, in human FSHR mRNA is only expressed from the primary follicle onward. Studies in women with mutated FSH β-subunit have shown follicular growth up to the stage of secondary follicles (Barnes *et al*., 2002). During initial recruitment, intraovarian factors stimulate some primordial follicles to initiate growth, whereas the rest of the follicles remain quiescent for months or years. The transition of primordial follicles into growing follicles is a process in which growth differentiation factor-9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) are involved. GDF-9 is produced by the oocyte and is considered an obligatory signal for further development beyond the primordial stage (Aaltonen *et al*., 1999; Knight and Glister, 2003) It promotes granulosa cell proliferation and differentiation and the formation of the theca cell layer of the primary follicle, together with kit ligand produced by granulosa cells (Erickson *et al*., 1985; Elvin *et al*., 2000; Eppig, 2001; Nilsson *et al*., 2004). BPM-15 plays a comparable role in early follicle development by promoting mitosis of granulosa cells and the initiation of the theca cell layer formation (Otsuka *et al*., 2000; Otsuka and Shimasaki 2002; Moore *et al*., 2003). Initial recruitment is believed to be a continuous process that starts just after follicle formation, long before pubertal onset. After initial recruitment, oocyte growth is a prominent feature of the growing follicles, but these oocytes remain arrested in the prophase of meiosis.

Cyclic follicular recruitment and FSH threshold/window concept

In contrast to early follicle development, stimulation by FSH is an absolute requirement for development of large antral preovulatory follicles. Duration and magnitude of FSH stimulation will determine the number of follicles with augmented aromatase enzyme activity and subsequent oestradiol biosynthesis. High FSH concentrations, usually occurring during the luteo–follicular transition, give rise to continued growth of a limited number (cohort) of follicles. Subsequent development of this cohort during the follicular phase becomes dependent on continued stimulation by gonadotrophins. Oocytes in these follicles have already completed their growth, acquired a zona pellucida, and are competent to resume meiosis (Trounson *et al*., 1998). In the human, only a single follicle from the cohort is selected to gain dominance and ovulate every cycle. Remaining cohort follicles enter atresia due to insufficient support by reduced FSH concentrations. The time interval between a primary and an early antral follicle in the human is 6–8 months (Gougeon, 1996). Subsequent stages from early antral to preovulatory follicles exhibit clear morphological characteristics, and the time interval is assessed to be approximately 3 months. Due to the regression of corpus luteum function during the late luteal phase of the menstrual cycle, oestradiol, inhibin A, and progesterone concentrations dramatically decline. This results in an increased frequency of pulsatile GnRH from the hypothalamus, inducing rising FSH concentrations at the end of the luteal phase (Hall *et al*., 1992; le Nestour *et al*., 1993; Miro and Aspinall, 2005). Although each follicle has the potential to reach full maturation, only those antral follicles that are at a more advanced stage of maturation (and therefore more sensitive to FSH) at the intercycle rise in FSH gain gonadotrophin dependence, undergoing the secondary recruitment as opposed to preceding gonadotrophin independent phase (primary recruitment) (McGee and Hsueh, 2000). In the subsequent luteal–follicular transition phase, the recruited follicles start to grow more rapidly under the influence of the initial selective rise of FSH beyond the critical threshold concentration (van Santbrink *et al*., 1995) able to open the so-called 'FSH window', allowing the entrance of the most sensitive (selected) antral follicles. Thereafter, the FSH concentration gradually declines by the negative feedback determined by follicular inhibin B (Groome *et al*., 1996), and oestradiol (Baird, 1987), closing the FSH window as soon as it reaches the concentrations under the critical threshold for follicular selection. This time interval of FSH concentration defining the FSH windows is considered to be critical for the selection of the single dominant follicle from the recruited cohort (van Santbrink *et al*., 1995). Thus, as FSH concentrations fall, all but the follicle with the increased sensitivity to FSH (dominant) arrest their development and become atretic (Fauser and Van Heusden, 1997; Maklon and Fauser 2001) (**Figure 2**).

Intraovarian modulators of follicular function

A large number of factors produced in the ovary modulate follicular function and development via a paracrine/autocrine effect (for review, see Knight and Glister, 2003, 2006; Juengel and McNatty, 2005). The principal regulatory systems involve the insulin-like growth factor (IGF) system the epidermal growth factor (EGF) system (Tapanainen *et al*., 1987), and the transforming growth factors α (TGFα) and TGFβ systems (Mason *et al*., 1995). IGF-I has been shown to stimulate proliferation and aromatase activity of granulosa cells *in vitro*, both alone or in synergism with FSH (Poretsky *et al*., 1999). EGF and TGFα appear to stimulate granulosa cell proliferation (Tapanainen *et al*., 1987), but inhibit FSH-induced aromatase expression and oestradiol synthesis (Roy, 1993). Ovarian cells have been shown to produce the TGFβ subfamily (isoforms TGFβ1, TGFβ2 and TGFβ3). The expression of these molecules is first detected in preantral follicles and in the subsequent stages of follicular development. In humans (and rodents), TGFβ is produced by both theca and granulosa cells. Similar to activin A, TGFβ can stimulate FSH receptor expression (Dunkel *et al*., 1994), amplify FSH-induced aromatase activity,

inhibin production, progesterone production and LH receptor induction (Hutchinson *et al.* 1987; Zhang *et al.* 1988; Kim and Schomberg 1989; Drummond *et al.* 2000). Like activin A, TGFβ has also been shown to suppress thecal P450c17 expression and androgen production (Fournet *et al.*, 1996). Among the members of the TGFβ superfamily molecules, the anti-Müllerian hormone (AMH) is expressed in granulosa cells (Durlinger *et al.*, 2002) and seems to act only in the reproductive organs (Lee and Donahoe, 1993). It is considered as a negative regulator of the early stages of follicular development. So far, this is the only known negative regulatory factor for primordial to primary follicle transition (Themmen, 2005). AMH is produced in the early secondary follicles, the preantral follicles and antral follicles in experimental animals (Visser and Themmen, 2005) and humans (Weenen *et al.*, 2004). AMH produced by the developing follicles can inhibit primordial follicle development (Durlinger *et al.*, 1999, 2001, 2002; Gruijters *et al.*, 2003; Salmon *et al.*, 2004; Weenen *et al.*, 2004; Visser and Themmen, 2005). It has been shown that oocytes up-regulate AMH expression in granulosa cells in a fashion that is dependent upon the developmental stage of the oocyte (Salmon *et al.*, 2004). Thus, the oocytes in the pool of growing follicles might control the pool of primordial follicles by modulating the expression of the inhibiting factor AMH. Inhibins and activins, in addition to the regulatory effects on pituitary FSH release, also act as paracrine and autocrine regulators of ovarian follicle development (Roberts *et al.*, 1993).

The role of LH in follicular growth (the LH threshold dose and ceiling value concept)

Recent evidence points to a central role for LH in monofollicular selection and dominance in the physiological ovulatory cycle (Sullivan *et al.*, 1999; Filicori *et al.*, 2002). Although granulosa cells from early antral follicles respond only to FSH, those from mature follicles express LHR (FSH-induced), becoming also responsive to LH and progressively less dependent on FSH stimulus. Basic and clinical experimental evidence indicates that development of ovarian follicles requires a threshold of LH stimulation for adequate follicular development and maturation (Hillier, 1994, 2001; Shoham, 2002). The amount of LH required seems to be very low (1–10 IU/l), since only 1% of the LH receptors need to be occupied in order to induce the maximal steroidogenic response from theca cells (Chappel and Howles, 1991). Moreover, clinical experimental evidence produced with the administration of recombinant FSH and LH to hypogonadotrophic hypogonadal women, suggested the requirement of a ceiling concentration of LH for adequate follicular development beyond which LH inhibits normal preovulatory follicle development (Hillier, 1994). Thus, it appears that in the delicate hormone interplay of the gonadotrophin-dependent phase of follicular development, LH acts within a window of hormone concentration framed between the minimal threshold and the ceiling. During the second half of the follicular phase, as plasma FSH concentrations decline, the LH-dependent phase of preovulatory follicular development progresses normally if LH is at concentrations within the window. When the ceiling is exceeded at mid-cycle LH surge, granulosa cell division is inhibited and luteinization takes place (**Figure 3**).

Conclusion

Progress in knowledge of the physiology of FSH and LH, and the theories conceived to explain the complex hormonal interplay acting during follicular development (and post-ovulatory events), have been the basis for the development of diagnostic investigations and strategies for infertility treatment. Studies on evaluation of ovarian reserve (Mol *et al.*, 2006; Verberg *et al.*, 2007), sophisticated and individualized approaches to ovarian stimulation (Filicori and Cognini, 2001; Alviggi, 2006; Balasch and Fábregues, 2006; Smitz *et al.*, 2007), oocyte in-vitro maturation techniques (Yang *et al.*, 2005), and ovarian tissue culture (Smitz and Cortvrindt, 2002) are a few examples of the implications in clinical practice. However, it must be borne in mind that due to the ethical problems inherent in conducting research in human clinical practice, most of the basic knowledge has been obtained from research in animal models, and problems exist in extrapolating the information to humans. Developments in the area of genomics and pharmacogenomics are promising

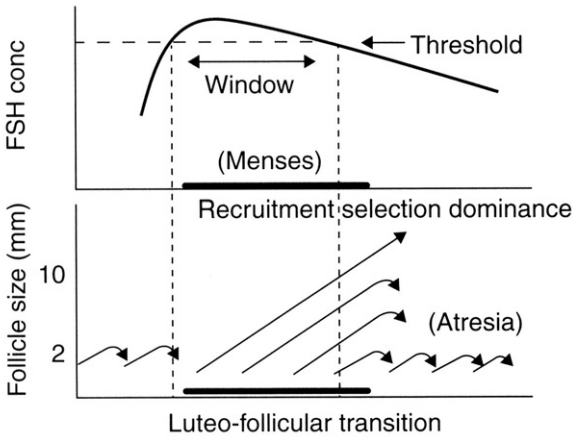

Figure 2. The FSH threshold/window concept of dominant follicle selection (reproduced with permission from Maklon and Fauser, 2001).

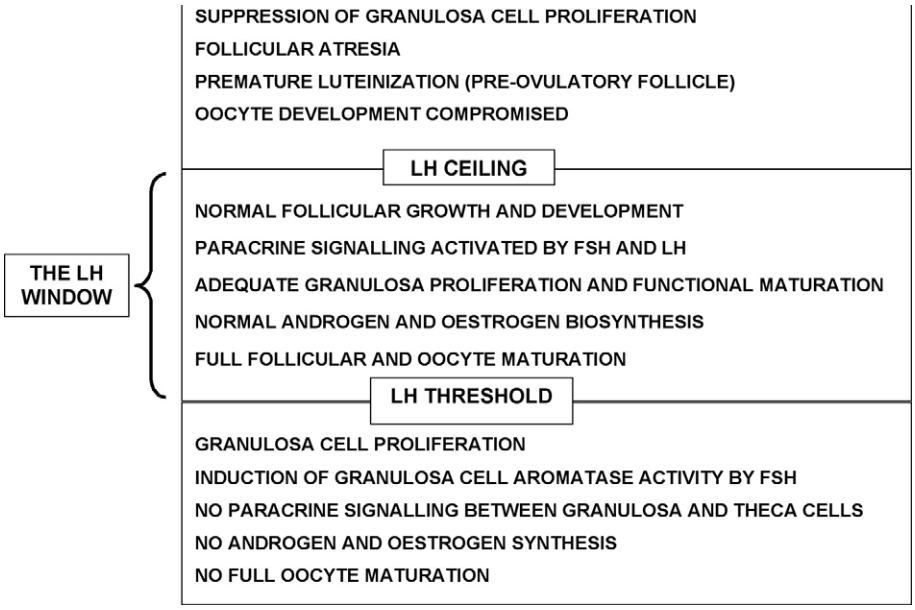

Figure 3. The LH window concept in follicular growth. Physiological (within the window) concentrations of LH are required for adequate follicular development. Above the ceiling and below the threshold LH concentration, various degrees of abnormal follicular development take place (reproduced with permission from Balasch and Fábregues, 2002).

horizons supporting new approaches in the understanding of ovarian physiology and treatment regimens. In this field, the challenge for the future will be to apply these tools in order to properly serve patients.

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