# LUTEINIZING HORMONE (LH) AND FOLLICLE STIMULATING HORMONE (FSH) IN MODULATION OF INSULIN GENE EXPRESSION FOR HEALTHY EGG PRODUCTION IN FEMALE

**(A REVIEW)**

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**A Thesis submitted**

**By**

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**Examination Roll No. MS-130707**

**Session: 2012-2013**

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**Biotechnology and Genetic Engineering Discipline,**
**Life Science School, Khulna University,**
**Khulna-9208, Bangladesh**
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*Dedicated*

*To*

***My Beloved Parents***

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**(A REVIEW)**

# DECLARATION

This review paper is the original research work of the author, except as otherwise stated. The material presented has not submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been / will not be published anywhere without the concurrence of the concerned supervisor.

Fatima-Tuz-Zohra
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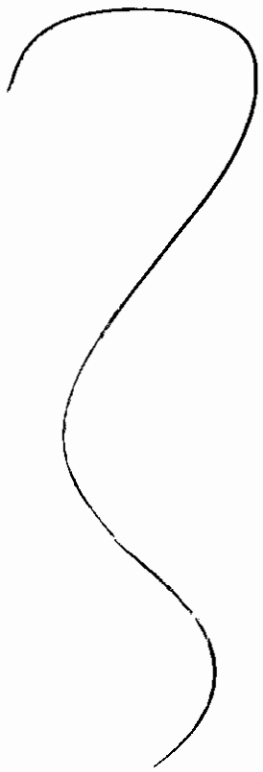

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The Author

# ABSTRACT

Egg health is one of the cornerstone of healthy fertility. To shield of a safe pregnancy, healthy egg production is ineluctable. Reproduction and metabolism are tightly connected and reciprocally regulated. Insulin plays a role in regulating reproduction and perform as a major signal linking metabolism and reproductive status. Insulin acts as a co-gonadotropin at the level of the ovary which enhances steroidogenic responses to gonadotropins. Most of the insulin pathways are active in the ovary and their ability to interact with each other and also with FSH and LH signaling pathways enables insulin to exert direct modulating influences on ovarian function. In this review, a modulatory effect of insulin gene expression on follicle stimulating hormone (FSH) and luteinizing hormone (LH) has been shown that could have been playing role on healthy fertility. The process is regulated by Akt pathway which is the signal transduction pathway of living cell in the ovary; where key proteins are Phosphatidylinositol 3 kinase (PI3K) and protein kinase B (Akt). Insulin signaling through Akt and FOXO1 can enhance FSH and LH stimulated steroidogenesis. FSH and LH acting via mTOR/ERK can enhance cell proliferation and protein synthesis. When insulin binds to its receptor, it activates b-subunit tyrosin kinase which enhance PI3K activation. Then DNA is synthesized through $p^{21}$ Ras and MAPK. Actually, Akt can not directly activate mTOR, only Rheb can. Inside the cell the Rheb is inactivated or turned off by Tsc1 and Tsc2. Akt inhibits this Tsc timers, then Rheb is free which regulate mTOR. Inositol glycans are also generated by insulin signaling pathway which mediate steroidogenic effects of insulin action. On the other hand, there are some negative regulators which are used the maintenance of dormancy of primordial follicles like PTEN, Tsc1, Tsc2, 4EBP1 etc. So there is a harmonic relationship among insulin, FSH and LH which guides to fabricate fertile follicular progress as well as manufacture of healthy egg.



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# LIST OF ABBREVIATION

| Abbreviated name | Full name |
|---|---|
| AGE | Advanced glycation end-product |
| AMPK | AMP-dependent protein kinase |
| AS160 | Akt substrate of 160 kDa |
| Bad | Bcl-2 associated death promoter |
| BMP | Bone morphogenetic protein |
| CDK | Cyclin dependent kinase |
| COF | Cystic ovarian follicle |
| CREB | cAMP response element binding |
| CYP | Cytochrome P450 |
| ERK | Extracellular signal regulated protein kinase |
| FOXO | Forkhead box O |
| FSH | Follicle stimulating hormone |
| GLUT | Glucose transporter |
| GnRH | Gonadotropin releasing hormone |
| GS | Glycogen synthase |
| GSK3 | Glycogen synthase kinase 3 |
| GV | Germinal vesicle |
| hCG | Human chorionic gonadotropin |
| IGF | Insulin like growth factor |
| IGFBP | IGF binding protein |
| IR | Insulin receptor |
| IRS | Insulin receptor substrate |
| IVF | In vitro fertilization |
| JAK2 | Janus kinase 2 |
| JNK | c-Jun N-terminal kinase |
| LH | Luteinizing hormone |
| MAPK | Mitogen activated protein kinase |
| MEK | MAPK/ERK kinase |
| mTOR | Mammalian target of rapamycin |

| Abbreviated name | Full name |
|---|---|
| mTORC | mTOR complex |
| NEB | Negative energy balance |
| OCT | Organic cation transporter |
| PCOS | Polycystic ovary syndrome |
| PDE3A | cAMP-phosphodiesterase 3A |
| PDK | Phosphoinositide dependent kinase |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PGC | Primordial germ cell |
| PKA | Protein kinase A |
| PKB | Protein kinase B |
| PKC | Protein kinase C |
| PI3K | Phosphoinositide kinase |
| PIP2 | Phosphatidylinisitol 4, 5-biphosphate |
| PIP3 | Phosphatidylinisitol 3, 4, 5-triphosphate |
| POF | Premature ovarian failure |
| PRAR | Peroxisome proliferator activated receptor |
| PPP | Pentose phosphate pathway |
| PTEN | Phosphate and tensin homolog |
| rpS6 | Ribosomal protein S6 |
| SH2 | Src homolog 2 |
| Shc | Src homology collagen |
| SHIP2 | SH2 domain containing inositol phosphatase 2 |
| S6K1 | $p^{70}$ ribosomal S6 kinase 1 |
| StAR | Steroidogenic acute regulatory protein |
| TGF | Transforming growth factor |
| TSC2 | Tuberous sclerosis complex 2 |
| VFA | Volatile fatty acid |

# CHAPTER 1

## INTRODUCTION

All living things reproduce. This is something that sets the living apart from non-living. Even though the reproductive system is essential to keeping a species alive, it is not essential to keeping an individual alive. There are the different parts of the female reproductive system: the organs involved in the process of reproduction, hormones that regulate a woman's body, the menstrual cycle, ovulation and pregnancy, the female's role in genetic division, birth control, sexually transmitted diseases and other diseases and disorders [1].

Reproduction can be defined as the process by which an organism continues its species. In the human reproductive process, two kinds of sex cells (gametes), are involved: the male gamete (sperm), and the female gamete (egg or ovum) [2].These two gametes meet within the female's uterine tubes located one on each side of the upper pelvic cavity, and begin to create a new individual. The female needs a male to fertilize her egg; she then carries offspring through pregnancy and childbirth.

So, to ensure a safe pregnancy and childbirth, healthy egg production is necessary. Egg health is one of the cornerstones of healthy fertility. The health of your eggs (ovum) can affect whether or not fertilization and implantation will occur as well as the viability of pregnancy. Protecting the eggs we currently have encouraging ovarian health diet, supplements and increased circulation of to the reproductive system is of the utmost importance [3]. During the cycle of an egg's journey towards ovulation there is a window of opportunity, a period of time, when certain factors can affect the health of the eggs that are preparing for ovulation.

Infertility, which is defined as not being able to conceive after one year of unprotected sex, is an ongoing problem estimated to affect 186 million people worldwide [1]; in some regions of the world, such as in developing countries, the percentage of infertility could reach an average of 30% [2]. Although male infertility contributes to more than half of all cases of global childlessness, infertility remains a woman's social burden [1]. The etiologies of female infertility include ovulation and tubal problems, endometriosis and in 20%–30% cases remain unexplained [3]. Recently, the effects of lifestyle on female reproductive health have received new attention [3,4,5]. Body weight, body composition, physical activity, and nutrients intake are all factors that could impact female fertility [4].

In female animals, reproduction and metabolism are tightly connected and reciprocally regulated [6,7]. During the reproductive period of life, the physiological activity of the gonads, with their cyclic production of sex hormones, ensures continuous regulation of energy metabolism [6,7]. On the other hand, in females, in particular in mammals, energy metabolism is tuned on reproductive needs: the energetic costs of puberty, pregnancy and lactation rely on female ability in saving oxidizable fuels [6,8]. Throughout evolution mechanisms have been developed to store energy in the case of food abundance and to prevent reproduction in nutrient poor environments [9,10]. When this physiological balance between reproduction and metabolism is disrupted problems occur. Pathologies associated with ovarian dysfunction, such as polycystic ovarian syndrome (PCOS) or Turner syndrome are generally more susceptible to developing metabolic disturbances [11,12], and physiologically, after the cessation of ovarian function, women are also at higher risk for developing metabolic and cardiovascular disorders [13]. On the other hand, in our obesogenic world, the gender-specific strategy developed throughout evolution to guarantee the survival of the species has negative effects on female health [9] and is now also impacting women's fertility [14].

We all know that, follicle stimulating hormone (FSH) stimulates follicular growth and ovulation and luteinizing hormone (LH) plays a key role in gonadal function. Thus normal follicular growth is the result of complementary action of FSH and LH. On the other hand, most of the insulin pathways are active in the ovary and their ability to interact with each other and also with FSH and LH signaling pathways enables insulin to exert direct modulating influences on ovarian function.

Given the tight interconnection between energy metabolism and reproduction, in this review, I discuss the impact that the metabolic state, and related hormones, proteins, genes that could have paly role on fertility. Moreover, we discuss the impact of LH and FSH which can modulate the insulin gene expression for healthy egg production in female. Finally, we report the beneficial effects of the healthy egg production and the pathways of insulin gene expression to produce healthy or fertile ovum.

So, from the above discussion, this research was designed with the following objectives:

- To identify the pathways that are being activated by Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) through the modulation of insulin gene expression.
- To find out the modulators like increasing steroidogenesis, protein synthesis, cell proliferation etc. and inhibition of apoptosis.

# Chapter 2

# REVIEW OF LITERATURE

## 2.1 Female Reproductive System Anatomy

The female reproductive system is framed to perform different functions. It creates egg cells that are essential for reproduction known as ova. The system is organized to deliver the ova to the region of fertilization. The egg fertilization takes place in the Fallopian tubes along with the sperm. The implanting in the walls of the uterus and initiating the stages of pregnancy is the next step of fertilized eggs. [15] Apart from these function, the reproductive system also creates female sex hormones to maintain the reproductive cycle.

The female reproductive system is composed of a pair of ovaries along with oviducts, vagina, cervix, uterus, and the external genitalia that are located in the pelvic region. These parts along with a pair of mammary glands that are integrated both functionally and structurally also support the process of ovulation, fertilization, birth and finally the child care.

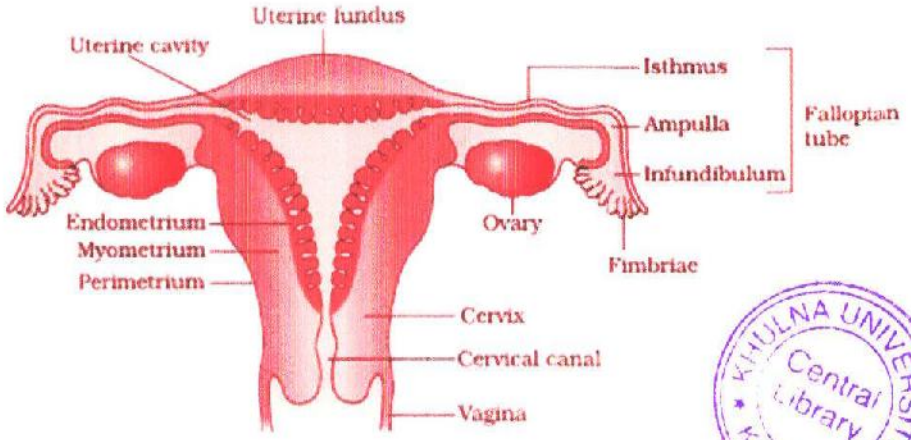


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**Figure 1: Female reproductive system**

**Ref:** Inhorn, M.C.; Patrizio, P. Infertility around the globe: New thinking on gender, reproductive technologies and global movements in the 21st century, 2015.

### 2.1.1 Ovaries

Ovaries act as the main female sex organs that produce the female gamete and various hormones. These organs are situated one on both the side of the lower abdomen. Each ovary measures about 2 to 4 cm in length which is then connected to the uterus and pelvic wall

through ligaments. The ovary is surrounded by a thin covering of epithelium, encloses the ovarian stroma and is divided into two zones – outer cortex and inner medulla.

The cortex consists of various ovarian follicles in different stages of development. The ovarian follicle is called as the basic unit of the female reproductive system. Each oviduct is divided into three anatomical regions- ampulla, isthmus, and infundibulum.

### 2.1.2 Uterus

A uterus is also called as the womb. It is a muscular, inverted pear-shaped organ of the female reproductive system.The walls of the uterus consist of three layers- the inner glandular layer, the middle thick layer, and the outer thin layer. These three layers are maintained by ligaments which are attached to the pelvic wall which then opens into the vagina from a narrow cervix. Cervical canal along with vagina creates the birth canal. The vagina is a muscular tube which starts at the lower end of the uterus to the outside.

### 2.1.3 Fallopian Tubes

Fallopian tubes are a pair of muscular tubes and funnel-shaped structures, extend from the right and left of the superior corners of the uterus to the edge of the ovaries. These tubes are enclosed in small projections called fimbriae that swipe over the ovaries to pick up released ova and deliver them to the infundibulum for supplying uterus. Each fallopian tube is covered by cilia that functions by carrying the ovum to the uterus.

### 2.1.4 Vagina

The vagina is a muscular and elastic tube that connects the cervix to the external body. It functions as the receptacle for the penis in sexual intercourse and delivers sperm to the fallopian tubes and uterus. It also acts as a birth canal by expanding to allow delivery of the fetus during childbirth.

## 2.2: Physiology of the female reproductive system

The reproductive cycle begins with the maturation of oocytes through the process of oogenesis, as well as concurrent follicle development that stimulates ovulation. Oogenesis starts with the process of developing oogonia via the transformation of primordial follicles into primary oocytes, a process called oocytogenesis [16]. Oocytogenesis is complete either before or shortly

after birth in humans. During the menstrual cycle primary oocytes complete maturation through further meiotic divisions.

Follicle development signals the beginning of the menstrual cycle. At the start of the menstrual cycle, some 12-20 primary follicles begin to develop under the influence of elevated levels of follicle-stimulating hormone (FSH) to form secondary follicles. The primary follicles form from primordial follicles, which develop in the ovary as a fetus during conception and are arrested in the prophase state of the cellular cycle [17, 18].

By around day 9 of the menstrual cycle, only one healthy secondary follicle remain. The rest are reabsorbed into the ovary. The remaining follicle, called the dominant follicle, is responsible for producing large amounts of estrogen during the late follicular phase.

On day 14 of the cycle, a luteinizing hormone surge is triggered by the positive feedback of estrogen. This causes the secondary follicle to develop into a tertiary follicle, which then leaves the ovary 24–36 hours later. An important event in the development of the tertiary follicle occurs when the primary oocyte completes the first meiotic division, resulting in the formation of a polar body and a secondary oocyte [19].The empty follicle then forms a corpus luteum which later releases progesterone to maintain a potential pregnancy.

Immediately after meiosis I, the haploid secondary oocyte initiates meiosis II. However, this process is also halted at the metaphase II stage until fertilization occurs. When meiosis II has completed, an ootid and another polar body is created.

Both polar bodies disintegrate at the end of meiosis II, leaving only the ootid, which eventually develops into a mature ovum [16]. The formation of polar bodies serves to discard the extra haploid sets of chromosomes that have resulted as a consequence of meiosis.

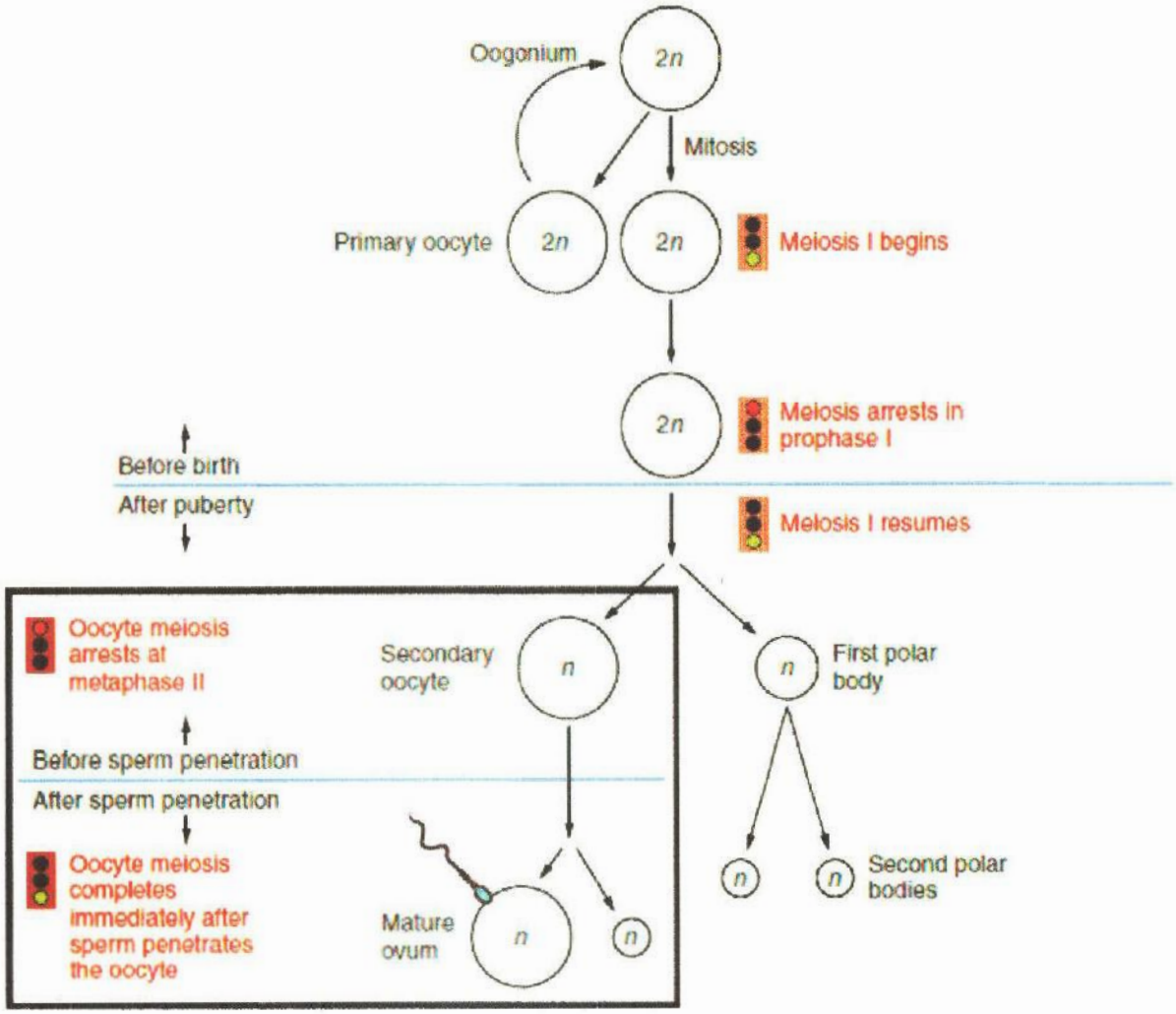


**Figure 2: The oogenesis process, diagram showing maturation of the ovum.**

**Ref:** Ombelet, W.; Cooke, I.; Dyer, S.; Serour, G.; Devroey, P. Infertility and the provision of infertility medical services in developing countries. *Hum. Reprod. Update* **2008**.

### 2.3: Ovarian cycle

The menstrual cycle is the physiological process that fertile women undergo for the purposes of reproduction and fertilization.

The menstrual cycle is the scientific term for the physiological changes that occur in fertile women for the purpose of sexual reproduction. The menstrual cycle is controlled by the endocrine system and commonly divided into three phases: the follicular phase, ovulation, and the luteal phase. However, some sources define these phases as menstruation, proliferative phase, and secretory phase. Menstrual cycles are counted from the first day of menstrual bleeding [20].

## 2.4: The follicular phase

The follicular phase (or proliferative phase) is the phase of the menstrual cycle in humans and great apes during which follicles in the ovary mature, ending with ovulation. The main hormone controlling this stage is estradiol. During the follicular phase, follicle-stimulating hormone (FSH) is secreted by the anterior pituitary gland. FSH levels begin to rise in the last few days of the previous menstrual cycle and peak during the first week of the follicular phase [21].The rise in FSH levels recruits five to seven tertiary-stage ovarian follicles (also known as Graafian or antral follicles) for entry into the menstrual cycle. These follicles compete with each other for dominance.

FSH induces the proliferation of granulosa cells in the developing follicles and the expression of luteinizing hormone (LH) receptors on these granulosa cells. Two or three days before LH levels begin to increase, usually by day seven of the cycle, one or occasionally two of the recruited follicles emerges as dominant. Many endocrinologists believe that estrogen secretion of the dominant follicle increases to a level that indirectly lowers the levels of LH and FSH [21, 22]. This slowdown in LH and FSH production leads to the atresia (death) of most of the recruited follicles, though the dominant follicle continues to mature.

These high estrogen levels initiate the formation of a new layer of endometrium in the uterus. Crypts in the cervix are also stimulated to produce fertile cervical mucus that reduces the acidity of the vagina, creating a more hospitable environment for sperm. [20] In addition, basal body temperature may lower slightly under the influence of high estrogen levels. Ovulation normally occurs 30 (± 2) hours after the beginning of the LH surge (when LH is first detectable in urine) [23].

## 2.5: Ovulation

Ovulation is the phase in which a mature ovarian follicle ruptures and discharges an ovum (also known as an oocyte, female gamete, or egg). Ovulation also occurs in the estrous cycle of other female mammals, which differs in many fundamental ways from the menstrual cycle [24].The time immediately surrounding ovulation is referred to as the ovulatory phase or the per ovulatory period.

## 2.6: The Luteal Phase

The luteal phase (or secretory phase) is the latter part of the menstrual or estrous cycle. It begins with the formation of the corpus luteum and ends in either pregnancy or luteolysis. The main hormone associated with this stage is progesterone, which is significantly higher during the luteal phase than in other phases of the cycle. Some sources define the end of the luteal phase as a distinct ischemic phase.

After ovulation, the pituitary hormones FSH and LH cause the remaining parts of the dominant follicle to transform into the corpus luteum. It continues to grow for some time after ovulation and produces significant amounts of hormones, particularly progesterone, and to a lesser extent, estrogen. Progesterone plays a vital role in making the endometrium receptive to implantation of the blastocyst and supportive of the early pregnancy. It also raises the woman's basal body temperature [20,23]. The hormones produced by the corpus luteum suppress production of the FSH and LH, causing the corpus luteum will atrophy. The death of the corpus luteum results in falling levels of progesterone and estrogen. This in turn causes increased levels of FSH, leading to recruitment of follicles for the next cycle. Continued drops in estrogen and progesterone levels trigger the end of the luteal phase, menstruation, and the beginning of the next cycle [22].

The loss of the corpus luteum can be prevented by implantation of an embryo. After implantation, human embryos produce human chorionic gonadotropin (hCG), which is structurally similar to LH and can preserve the corpus luteum. Because the hormone is unique to the embryo, most pregnancy tests look for the presence of hCG. If implantation occurs, the corpus luteum will continue to produce progesterone (and maintain high basal body temperatures) for eight to twelve weeks, after which the placenta takes over this function [18, 19].

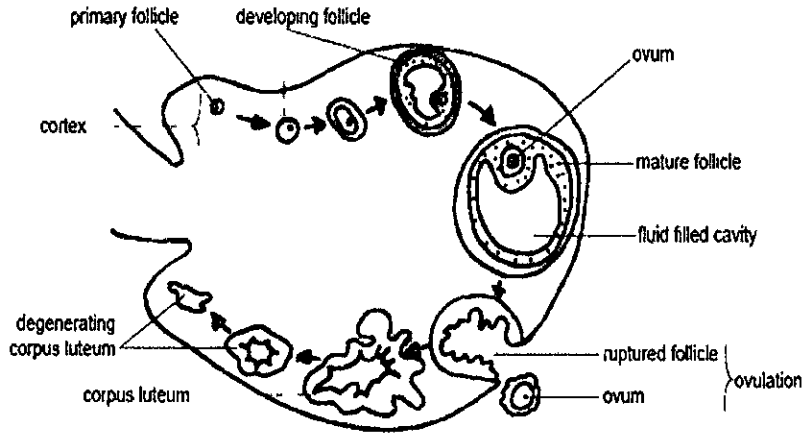

**Figure 3: The ovarian cycle: The ovarian cycle is the series of changes that occur in the ovary during the menstrual cycle that cause maturation of a follicle, ovulation, and development of the corpus luteum.**

**Ref:** Templeton, A. Infertility and the establishment of pregnancy—Overview. *Br. Med. Bull.* **2000.**

### 2.7: Uterine (Menstrual) Cycle

The uterine cycle describes a series of changes that occur to the lining of the uterus, or endometrium, during a typical menstrual cycle.

Several changes to the uterine lining (endometrium) occur during the menstrual cycle, also called the uterine cycle. The endometrium is the innermost glandular layer of the uterus. During the menstrual cycle, the endometrium grows to a thick, blood vessel-rich tissue lining, representing an optimal environment for the implantation of a blastocyst upon its arrival in the uterus [23, 24]. Menstrual cycles are counted from the first day of menstrual bleeding and are typically 28 days long.

During menstruation, the body begins to prepare for ovulation again. The levels of estrogen gradually rise, signalling the start of the follicular, or proliferation, phase of the menstrual cycle. The discharge of blood slows and then stops in response to rising hormone levels and the lining of the uterus thickens, or proliferates. Ovulation is triggered by a surge in luteinizing hormone [25]. The sudden change in hormones at the time of ovulation sometimes causes minor changes in the endometrium and light midcycle blood flow.

After ovulation, under the influence of progesterone, the endometrium changes to a secretory lining in preparation for the potential implantation of an embryo to establish a pregnancy. If a blastocyst implants, then the lining remains as the decidua [23, 25].This becomes part of the placenta and provides support and protection for the embryo during gestation.

If implantation does not occur within approximately two weeks, the progesterone-producing corpus luteum in the ovary will recede, causing sharp drops in levels of both progesterone and estrogen. This hormone decrease causes the uterus to shed its lining and the egg in menstruation. The cessation of menstrual cycles at the end of a woman's reproductive period is termed menopause. The average age of menopause in women is 52 years, but it can occur anytime between 45 and 55.

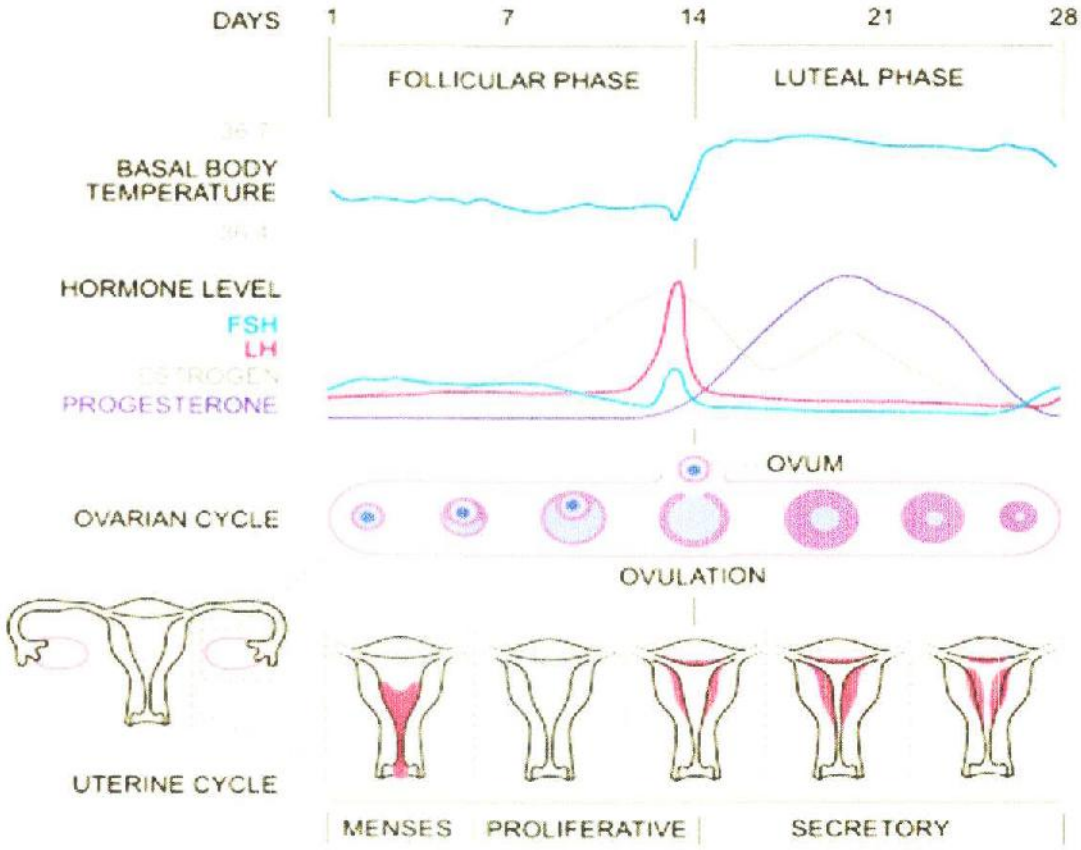


**Figure 4: The Uterine Cycle: High estrogen and progesterone levels stimulate increased endometrial thickness, but following their decline from a lack of implantation, the endometrium is shed and menstruation occurs.**

**Ref:** Templeton, A. Infertility and the establishment of pregnancy—Overview. *Br. Med. Bull.* **2000.**

Normal menstrual flow can occur although ovulation does not occur. This is referred to as an anovulatory cycle. Follicular development may start but not be completed although estrogen will still stimulate the uterine lining. Anovulatory flow that results from a very thick endometrium caused by prolonged, continued high estrogen levels is called estrogen breakthrough bleeding. However, if it is triggered by a sudden drop in estrogen levels, it is called withdrawal bleeding. Anovulatory cycles commonly occur before menopause and in women with polycystic ovary syndrome.

## 2.8: Hormonal regulation of the female reproductive cycle

The menstrual cycle is controlled by a series of changes in hormone levels, primarily estrogen and progesterone.

The menstrual cycle is the physiological change that occurs under the control of the endocrine system in fertile women for the purposes of sexual reproduction and fertilization.

### 2.8.1: Follicle stimulating hormone (FSH)

The amount and timing of FSH release by the anterior pituitary changes throughout the ovulatory cycle. This mechanism is influenced by many factors. With the sudden demise of the corpus luteum which immediately precedes menstruation, the negative feedback effects of estradiol, progesterone and inhibin A on FSH secretion are suddenly lost so that FSH is secreted in relatively large quantities during menstruation itself [18,20]. This rise in FSH concentrations stimulates the growth of antral follicles, granulosa cell proliferation and differentiation. It also encourages the action of the enzyme aromatase in the conversion of the basic androgens, androstendione and testosterone to estrogens. The sum total of these actions results in increasing estradiol and inhibin B concentrations, feedback mechanisms come into play and there is a consequent reduction of FSH concentrations. At mid-cycle, in tandem with the LH surge, there is a temporary increase in FSH secretion, more like a blip, whose significance is not clear [16, 17]. It may be a mere byproduct of the GnRH surge or may have a function in preparing a cohort of small antral follicles for the next cycle. With the formation of the corpus luteum and the outpouring of both estradiol and progesterone [23], the negative feedback mechanism comes into play and continues its suppression of FSH release until just before the next menstruation. The main undulations in FSH levels throughout the ovulatory cycle are very simply illustrated in Fig. 5

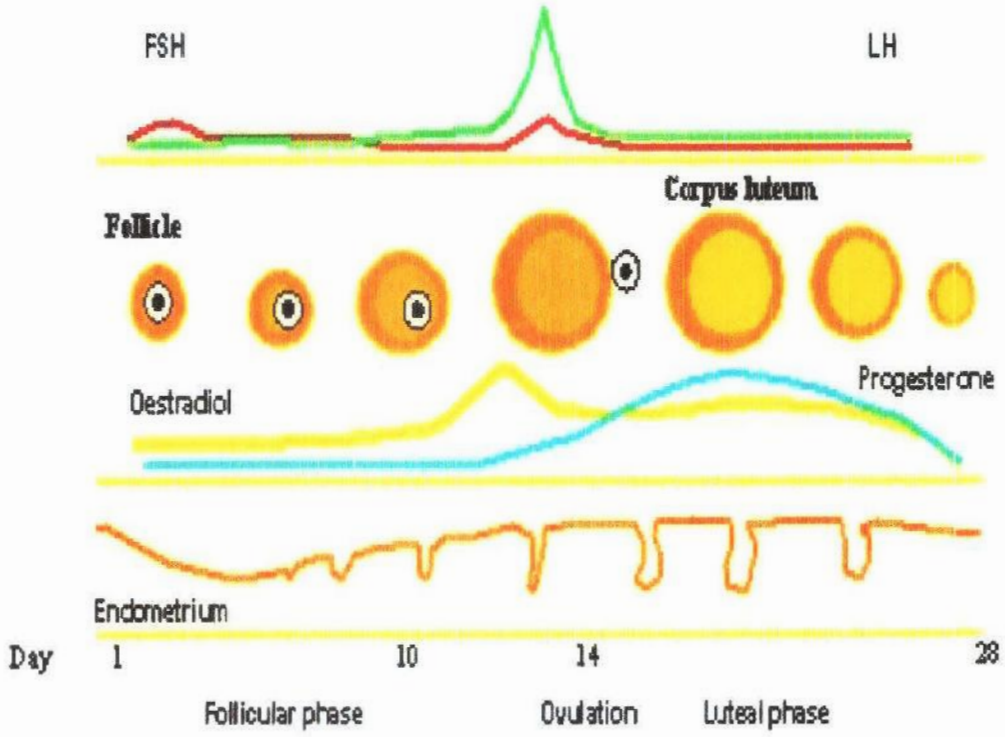


**Figure 5: Hormonal, follicular and endometrial changes across the phases of the ovarian cycle.**

**Ref:** Ciana, P.; Maggi, A. Amino acid-dependent activation of liver estrogen receptor alpha integrates metabolic and reproductive functions via IGF-1. *Cell Metab.* **2011.**

FSH is a hormone of many roles. It is a promotor of:

1. Granulosa cell proliferation and differentiation

2. Antral follicle development

3. Estrogen production

4. Induction of LH receptors on the dominant follicle

5. Inhibin synthesis

In addition to these functions, the decrease in FSH concentrations with rising estrogen concentrations is thought to play an important part in the selection of the dominant follicle. The declining secretion of FSH prevents multiple follicular development, as only the largest of the developing follicles stays above the FSH threshold, has the most FSH receptors, remains most sensitive to FSH and produces most estrogen. It is then less sensitive to the declining FSH concentrations and can continue to develop while others fade into atresia due to lack of enough FSH stimulation [22, 23]. The induction of LH receptors on the largest developing follicle(s) enables LH to take a part in the development of the dominant follicle in the late follicular phase and prepare it for the oncoming LH surge.

This basic knowledge of the mode of action of FSH, particularly regarding the FSH threshold for follicular growth, has influenced a change in ovulation induction regimes. This has become particularly important in the development of a chronic low-dose regimen for the induction of mono-follicular ovulation and the avoidance of multiple pregnancies and ovarian hyperstimulation syndrome [25].

### 2.8.2: Luteinizing hormone (LH)

During the early and mid-follicular phase, the secretion of LH is relatively quiet with pulses every 60–90 minutes and a fairly constant low concentration of circulating LH. However, this is the calm before the storm. An enormous climax is reached with the onset of the LH surge in the late follicular phase, the central event of the ovulatory cycle (Fig. 5) [24]. Concentrations of LH rise to 10–20 times their resting level during the rest of the cycle. The duration of the surge is 36–48 hours.

The LH surge, without which ovulation does not occur, is brought about by a combination of circumstances. Principally, there is a dramatic switch from a negative to a positive feedback action of estradiol at both the pituitary and hypothalamic level, triggered when persistently increasing estradiol concentrations reach a critical point. LH secreting pituitary gonadotrophs clearly become highly sensitive to GnRH stimulation, probably by increasing their numbers of GnRH receptors, a GnRH surge occurs and a small rise in progesterone levels in the late follicular phase may also have a triggering role.

The pre-ovulatory LH surge has a number of key functions:

1. Triggering of ovulation and follicular rupture about 36 hours after the surge.
2. Disruption of the cumulus–oocyte complex.
3. Induction of the resumption of oocyte meiotic maturation.
4. Luteinization of granulosa cells.

Following the formation of the corpus luteum, increasing concentrations of progesterone slow down the frequency of the LH (GnRH) pulses to one every 3 then one every 4 hours. Concentrations of LH once again dip down to baseline levels. It is therefore, not clear what kind of influence LH levels have on the maintenance of the corpus luteum [26]. This structure, which produces large quantities of hormones, seems to 'have a mind of its own' or a built-in program which terminates in a very constant manner after 14 days. The luteal phase is thus the constant part of the ovulatory cycle whereas the follicular phase is much more likely to be prone to changes in duration.

### 2.9: Phases of menstrual cycle

The menstrual cycle is divided into three stages: follicular phase, ovulation, and the luteal phase.

#### 2.9.1: Follicular phase

During the follicular phase (or proliferative phase), follicles in the ovary mature under the control of estradiol. Follicle-stimulating hormone (FSH) is secreted by the anterior pituitary gland beginning in the last few days of the previous menstrual cycle. Levels of FSH peak during the first week of the follicular phase. The rise in FSH recruits tertiary-stage ovarian follicles (antral follicles) for entry into the menstrual cycle.

Follicle-stimulating hormone induces the proliferation of granulosa cells in the developing follicles and the expression of luteinizing hormone (LH) receptors on these cells. Under the influence of FSH, granulosa cells begin estrogen secretion [27,28]. This increased level of estrogen stimulates production of gonadotropin-releasing hormone (GnRH), which increases production of LH. LH induces androgen synthesis by theca cells, stimulates proliferation and differentiation, and increases LH receptor expression on granulosa cells.

Throughout the entire follicular phase, rising estrogen levels in the blood stimulate growth of the endometrium and myometrium of the uterus. This also causes endometrial cells to produce receptors for progesterone, which helps prime the endometrium to the late proliferative phase and the luteal phase. Two or three days before LH levels begin to increase, one or occasionally two of the recruited follicles emerge as dominant. Many endocrinologists believe that the estrogen secretion of the dominant follicle lowers the levels of LH and FSH, leading to the atresia (death) of most of the other recruited follicles. Estrogen levels will continue to increase for several days [26].

High estrogen levels initiate the formation of a new layer of endometrium in the uterus, the proliferative endometrium. Crypts in the cervix are stimulated to produce fertile cervical mucus that reduces the acidity of the vagina, creating a more hospitable environment for sperm [28]. In addition, basal body temperature may lower slightly under the influence of high estrogen levels.

### 2.9.2: Ovulation

Estrogen levels are highest right before the LH surge begins. The short-term drop in steroid hormones between the beginning of the LH surge and ovulation may cause mid-cycle spotting or bleeding. Under the influence of the preovulatory LH surge, the first meiotic division of the oocytes is completed. The surge also initiates luteinization of theca and granulosa cells. Ovulation normally occurs 30 (± 2) hours after the beginning of the LH surge.

Ovulation is the process in a female's menstrual cycle by which a mature ovarian follicle ruptures and discharges an ovum (oocyte). The time immediately surrounding ovulation is referred to as the ovulatory phase or the periovulatory period [29]. In the preovulatory phase of the menstrual cycle, the ovarian follicle undergoes cumulus expansion stimulated by FSH. The ovum then leaves the follicle through the formed stigma. Ovulation is triggered by a spike in the amount of FSH and LH released from the pituitary gland [27, 29].

### 2.9.3: Luteal phase

The luteal phase begins with the formation of the corpus luteum stimulated by FSH and LH and ends in either pregnancy or luteolysis. The main hormone associated with this stage is progesterone, which is produced by the growing corpus luteum and is significantly higher during the luteal phase than other phases of the cycle. Progesterone plays a vital role in making

the endometrium receptive to implantation of the blastocyst and supportive of the early pregnancy. It also raises the woman's basal body temperature.

Several days after ovulation, the increasing amount of estrogen produced by the corpus luteum may cause one or two days of fertile cervical mucus, lower basal body temperatures, or both. This is known as a secondary estrogen surge [30]. The hormones produced by the corpus luteum suppress production of the FSH and LH, which leads to its atrophy. The death of the corpus luteum results in falling levels of progesterone and estrogen, which triggers the end of the luteal phase. Increased levels of FSH start recruiting follicles for the next cycle.

Alternatively, the loss of the corpus luteum can be prevented by implantation of an embryo: after implantation, human embryos produce human chorionic gonadotropin (hCG). Human chorionic

gonadotropin is structurally similar to LH and can preserve the corpus luteum [25, 27]. Because the hormone is unique to the embryo, most pregnancy tests look for the presence of hCG. If implantation occurs, the corpus luteum will continue to produce progesterone (and maintain high basal body temperatures) for eight to 12 weeks, after which the placenta takes over this function [29].

# Chapter 3

# LH and FSH in female reproduction

## 3.1: LH and FSH in the Ovary

The term ovulation refers to the release of a viable oocyte from the ovary. In the normal course of events, ovulation occurs once a month between the time of menarche and menopause [31]. The release of a mature, fertilizable oocyte from the dominant follicle is the culmination of a wonderfully integrated and synchronized succession of hormonal actions and morphological changes involving principally the anterior hypothalamus, anterior pituitary and ovaries. The major players in this system are gonadotropin releasing-hormone (GnRH), FSH, LH, estrogen and progesterone but essential fine-tuning is provided by a large number of other factors including inhibin, activin and growth factors [32].

Oocyte release is preceded by estrogen-induced distinctive changes that prepare the reproductive tract for possible conception as if conception was being anticipated every month. For example, towards the time of ovulation the pH in the vagina becomes less acidic, the cervical mucus becomes more copious and less viscous and the cervical or becomes more patulous, all of which favor the progress of motile sperm towards the released oocyte [33]. In addition estrogen serves as the 'growth factor' of the endometrium and prepares it for the transition of the endometrium from the proliferative to secretory stages by the post-ovulatory secretion of progesterone from the corpus luteum. The secretions of the corpus luteum, estrogen and progesterone prepare the uterus for the possible nidation of a fertilized egg. The corpus luteum is formed from the collapsed ovarian follicle, the ingrowth of capillaries and fibroblasts from the theca cell layer and luteinization of the granulosa cells. In the absence of pregnancy, ovulation defines the time of succeeding menstruation as the corpus luteum has a proscribed life span of 14 ± 2 days unless 'rescued' by human chorionic gonadotropin (hCG) from the trophoblast of the conceptus. An appreciation of the steps involved in the process of ovulation, necessitating the exact sequence of so many events, leaves one in awe of the ingenuity of the system and a little surprised that its breakdown, i.e. anovulation, does not occur much more frequently than is actually seen [34, 35].

## 3.2: Hypothalamic- Pituitary- Ovarian Axis

The normal functioning of this axis is dependent on the correct synchronization of the timing of release and the quantity of the hormones involved. These change dramatically throughout the

cycle as a result of the various feedback mechanisms involved. First, we will consider the individual hormones involved, their target organs and actions [34], before piecing together the mosaic of the feedback mechanisms to complete the hormonal profile of the normal ovulatory cycle. Fig. 1 provides a very simple representation of the origin, target organ and feedback mechanisms of the principal hormones involved in this axis.

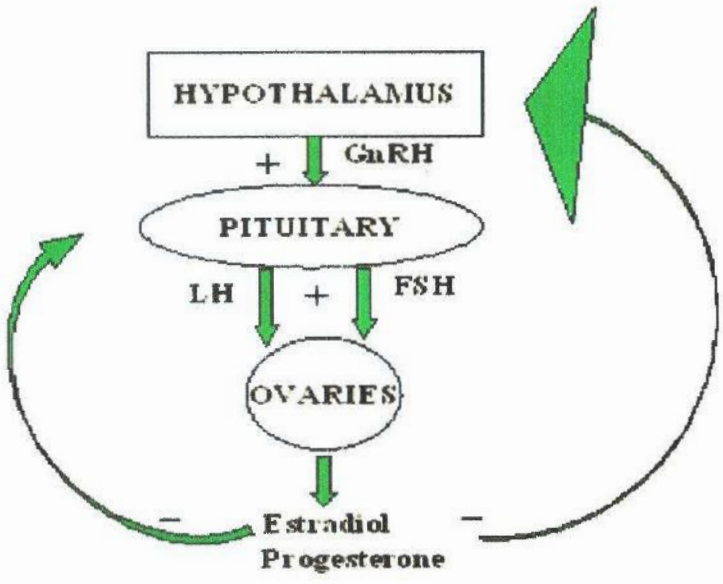


**Figure 6: A simple diagrammatic representation of the origins, target organs and feedback mechanisms of the principal hormones involved in the hypothalamic-pituitary-ovarian axis.**

**Ref:** Ciana, P.; Maggi, A. Amino acid-dependent activation of liver estrogen receptor alpha integrates metabolic and reproductive functions via IGF-1. *Cell Metab.* **2011.**

### 3.2.1: Gonadotropin releasing hormone (GnRH)

GnRH is a decapeptide which is synthesized and released by specific neuronal endings in the anterior and mediobasal hypothalamus. It is secreted into the portal vessels which run a very short course to the anterior pituitary. It is the compactness of the portal system which allows small quantities of GnRH to be concentrated enough to exert its action of gonadotropin release from the pituitary and explains why GnRH is undetectable in the peripheral circulation [35]. The discharge of the gonadotropins, FSH and LH, induces the production of estradiol and progesterone from the ovary which, in turn, through a feedback mechanism, influence the pattern of release of GnRH from the hypothalamus.

GnRH is released in a pulsatile fashion and it is the frequency and amplitude of these pulses, in addition to the sensitivity of the pituitary gonadotrophs, that dictate the pattern of the release of

the two gonadotropins. The GnRH pacemaker is principally influenced by the ovarian steroids but many other factors, including opiates, catecholamines, neuropeptide Y, etc., also play a role. If GnRH is released in a constant, non-pulsatile fashion, gonadotropin release is suppressed due to an apparent desensitization of the pituitary GnRH receptors [36]. Pulsatile release of GnRH and fluctuations in the pattern of this pulsatility are thus integral features in the normal functioning of the ovulatory cycle.

As GnRH cannot be detected in human peripheral circulation, we have relied on the correlation with LH pulsatile release for our information on variations of pulsatility through the ovulatory cycle and in pathological conditions [37]. Pulses of FSH are much more difficult to detect due to its longer half-life. In the follicular phase of a normal cycle, pulses of LH (reflecting GnRH) can be detected every 60–90 minutes.

Dramatic changes occur immediately preceding the pre-ovulatory LH surge. Hypothetically, the LH surge could be generated by an enormous discharge of GnRH or a temporary release from inhibition of pituitary LH discharge and a consequent increased pituitary sensitivity. Practically, both mechanisms are probably involved in creating the central event of the ovulatory cycle [38,39]. Speculation is rife surrounding the existence of a proposed gonadotropin surge attenuating factor, produced by granulosa cells, which inhibits pituitary LH discharge. Although its structure is not yet known, a substance with this property has been isolated.

Following ovulation, under the influence of rising progesterone concentrations, the frequency of the LH pulses gradually decreases from one every 2–4 hours in the early luteal phase to every 8–12 hours towards the end of the cycle. The amplitude of LH pulses in the luteal phase is significantly greater than in the follicular phase. The fluctuations in the frequency and amplitude of GnRH pulsatile release are central in dictating the pattern of release of FSH and LH and, in turn, the triggering of the ovulatory process and ovarian steroid production [40].

This knowledge of the basic physiology of the pattern of release and action of GnRH has brought with it many clinical implications. Induction of ovulation for women who have hypothalamic hypogonadrophic hypogonadism is very successful when GnRH is administered in a pulsatile fashion with one pulse every 60–90 minutes. This is an ideal example of pure substitution therapy. The search for an agonist to boost GnRH action proved to have exactly the opposite eventual effect due to desensitization of GnRH receptors. These compounds are now very widely used before and during ovarian hyperstimulation for IVF to prevent premature LH surges [39, 40].The use of GnRH antagonists is now also routine for use during controlled

ovarian stimulation for IVF as they do not induce an initial, fleeting gonadotropin release as do the agonists, but an immediate decrease in their concentrations.

## 3.3: Role of LH in physiology: The physiological hormonal interplay

### 3.3.1: Two cells – two gonadotropins

Outside the tumultuous events of the mid-cycle surge, the main function of LH is to encourage the production of androgens by theca cells. The androgens, androstendione and testosterone, are then 'passed on' to the granulosa cells. Here they meet aromatase (CYP19), whose function it is to convert them into estrogens, mainly estradiol but also estrone. Aromatase action, and therefore estrogen production, is controlled by FSH. Hence, the function of theca cells and granulosa cells are controlled by LH and FSH respectively [41]. There is some, however, as the LH receptors expressed by FSH on the granulosa cell membranes of developing follicles of >10mm diameter render LH capable of inducing estradiol production and follicular growth in the mid-late follicular phase.

The ovary comprises of two cellular components, which are stimulated independently by LH and FSH, leading to the production of ovarian steroids [8, 9]. Androgen production from cholesterol and release during folliculogenesis is dependent on the stimulation of the theca cells by LH and FSH .This is universally recognized as the key driver of ovarian follicle growth and maturation [10].

Ovarian steroidogenesis in the preovulatory follicle takes place through LH receptors on theca and FSH (possibly plus LH) receptors on granulosa cells.[11] The steroidogenic acute regulatory protein (StAR protein) is the primary regulator of production of androstenedione, which subsequently diffuses into granulosa cells to serve as an estrogen precursor. In the preovulatory follicle, cholesterol in theca cells arises from circulating lipoproteins and *de novo* biosynthesis [42,13].

FSH is responsible for follicular growth and estrogen formation. FSH may be crucial at an earlier stage of follicular development, perhaps earlier in the follicular phase, to induce the aromatase enzyme that converts androgen to estradiol [14]. During the later stages of follicular growth, activins and estradiol, the predominant estrogen in humans, enhance the actions of FSH [15].

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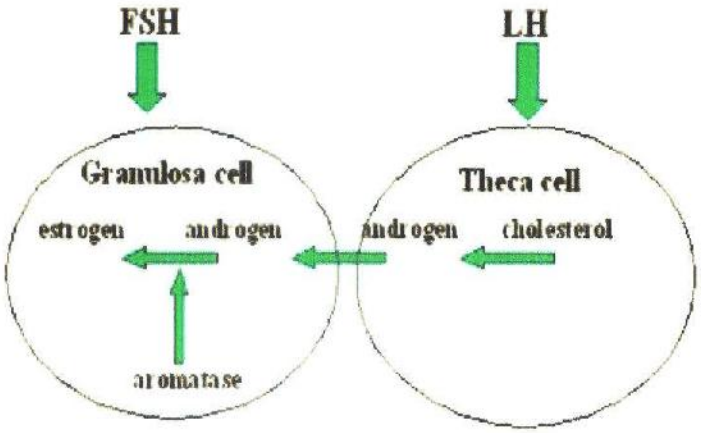


**Figure 7: The two-cell, two gonadotropin hypothesis.**

**Ref:** Ciana, P.; Maggi, A. Amino acid-dependent activation of liver estrogen receptor alpha integrates metabolic and reproductive functions via IGF-1. *Cell Metab.* **2011**

### 3.3.2: Concept of follicle stimulating hormone threshold and role of luteinizing hormone

The concept of the FSH "threshold" proposed by Brown postulated that in gonadotropin therapy, the ovary has a minimum requirement level (threshold requirement) for FSH below which follicular development does not occur [42]. More recent studies also confirm that follicular growth does not occur below the threshold levels.

Following optimum FSH stimulation, there is follicular recruitment, growth, selection and dominance. Subsequent development of this cohort during the follicular phase becomes dependent on continued stimulation by gonadotropins. Increasing FSH concentrations should surpass the threshold level to initiate the final gonadotropin-dependent phase of follicular growth [Figure 8] [43].

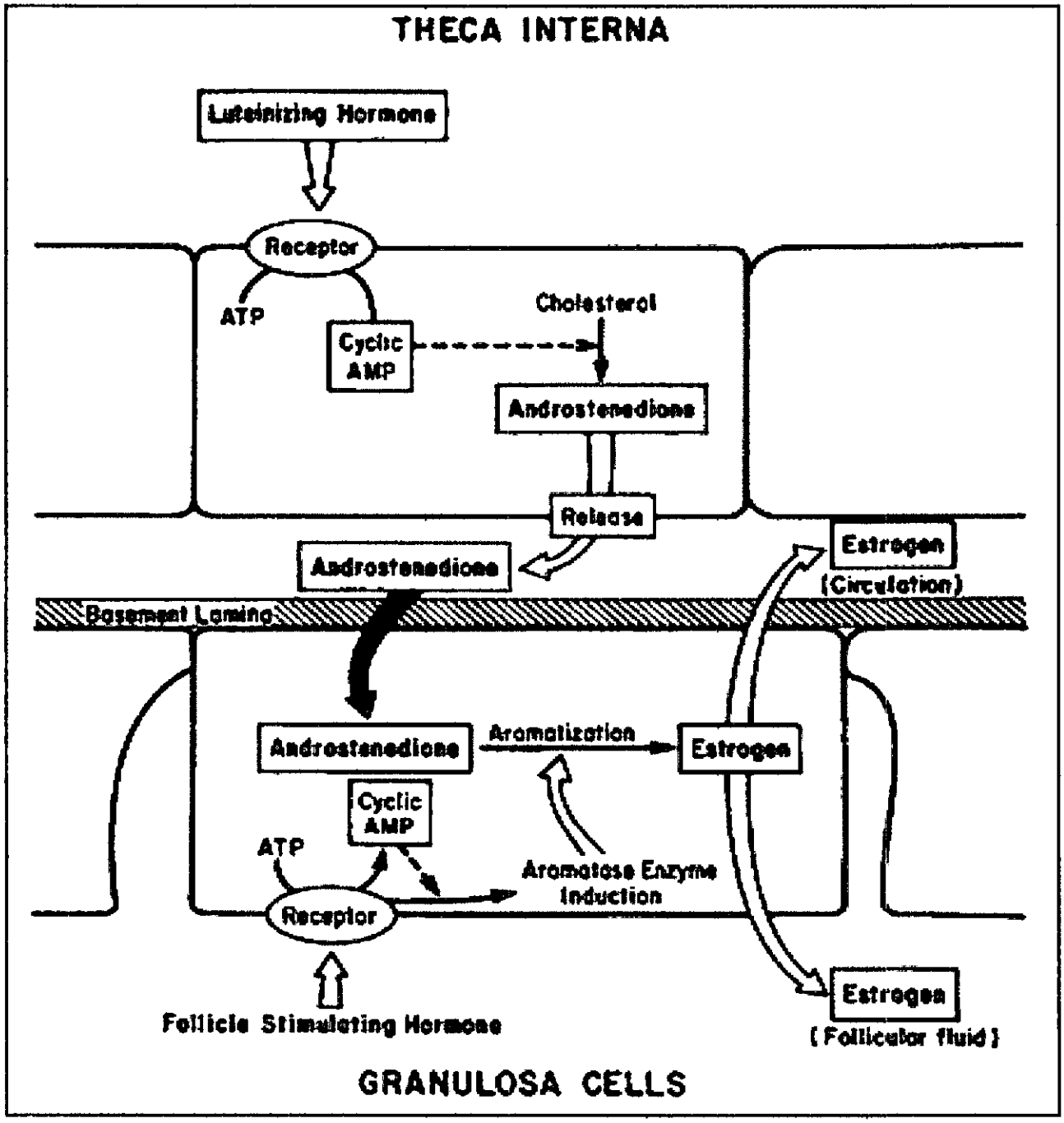


**Figure 8: Follicle stimulating hormone threshold and recruitment window**

**Ref:** Karsch FJ, Bowen JM, Caraty A, Evans NP, Moenter SM. Gonadotropin-releasing hormone requirements for ovulation. Biology of reproduction. 1997.

There is a secretion of increasing amounts of estradiol during this phase. The peripheral estradiol levels are increased with feedback inhibition of FSH secretion. The maturing follicle inhibits FSH secretion leading to a fall in its levels below threshold, thus stopping less mature follicles from maturing [44].

Further, it has been shown that FSH threshold is not fixed for any given follicle, but depends on the developmental stage and varies over time [45, 46]. The follicles exhibit different degrees of FSH sensitivity at the time of recruitment; highest need for FSH is at the early antral stage and declines in the late antral stage. The follicle with the highest sensitivity will benefit most from increasing FSH levels and will subsequently gain dominance [46].

The suggested reasons for the response of ovarian follicles to certain FSH level than to a specific dose are fluctuating levels of the endogenous production of gonadotropin,[44] and up-regulation of its receptors due to FSH administration [45].

Although FSH can induce follicular growth even without LH, there is evidence that the follicles may have developmental deficiencies like abnormally reduced estradiol production and lack of ability to luteinize and rupture, following hCG stimulus [46]. Hence, a certain amount of LH exposure is necessary for optimal follicular development.

Another possibility is that FSH stimulates the production of progesterone by driving cholesterol conversion into the steroid pathway [44, 45, 46, 47, 48]. Early increased exposure to progesterone can advance the endometrium, leading to asynchrony of embryo development to endometrial development and the reduction of implantation. LH stimulates the conversion of progesterone into androgens, which can be further aromatized to estrogens. The addition of LH may benefit the endometrium by decreasing the risk of a premature progesterone increase and therefore improve the likelihood of implantation and clinical pregnancy [45, 46].

### 3.3.3: Concept of luteinizing hormone therapeutic window

The concept of the LH therapeutic window has been explained in brief in Figure 9. Though studies support the use of r-hLH in addition to r-hFSH in GnRH antagonist protocols in ovarian follicular development, these studies are fewer in number. There is also no clear cut guideline regarding the optimum levels of serum LH and timing of its supplementation are fewer in number. This is an area that warrants further research [6]. Studies have shown that serum LH levels should be between 1.2 IU/L and 5.0 IU/L,[8] for optimal development follicle in cycles where endogenous LH is suppressed [6,19].

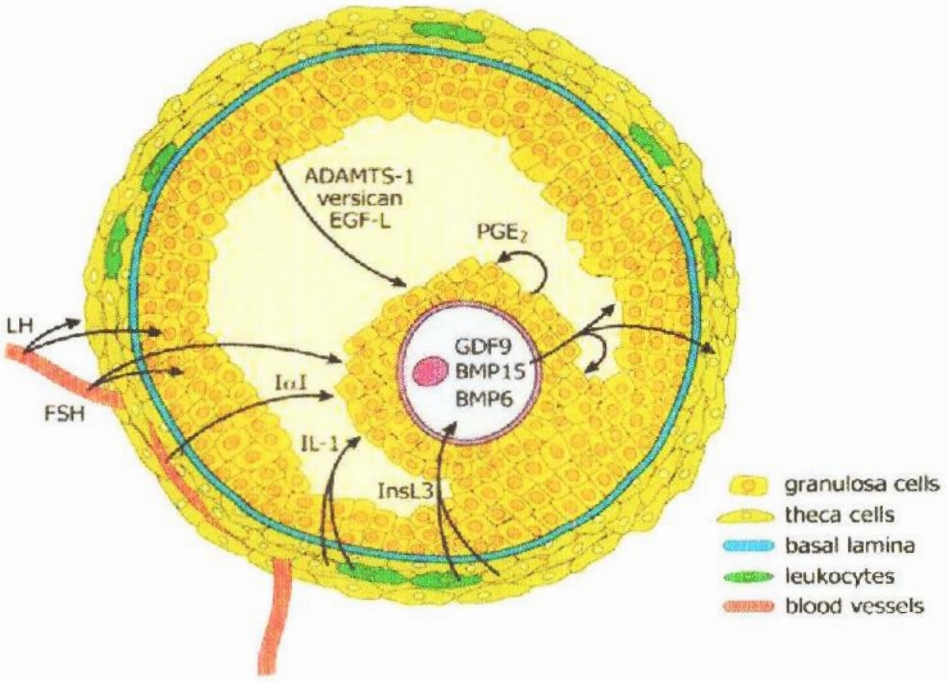


**Figure 9: Luteinizing hormone therapeutic window**

**Ref:** Karsch FJ, Bowen JM, Caraty A, Evans NP, Moenter SM. Gonadotropin-releasing hormone requirements for ovulation. Biology of reproduction. 1997.

Some of the recent studies suggest that the indicators for adding LH to an ART cycle are mid follicular (day 6) hypo-response to long GnRH agonist, no follicles > 10 mm, E2 < 200 pg/ml, endometrial thickness < 6 mm and baseline serum LH < 1.2 IU/ml on day 6. [8, 44]

A recent meta-analysis of seven randomized controlled trials (RCTs) done by Hill *et al.* on the use of LH in ART in advanced patient age group concluded that five RCTs were in favor of adding LH in ART therapy in patients of advanced age group [26]. However, it is critical that add-back LH is administered in appropriate patients as an excess of LH can cause suppression of granulosa cells and follicular atresia.

## 3.4: Estradiol

Estrogens are the basic female hormones and estradiol is the most important as far as the ovulatory cycle is concerned. The synthesis of estradiol by granulosa cells is a function of the action of FSH. FSH stimulates the enzyme aromatase (CYP19) to convert the substrate of basic androgens, androstendione and testosterone, to estradiol in granulosa cells. The production of this vital hormone thus requires the availability of the androgen substrate, whose production in theca cells is promoted by LH, and then the action of FSH.

The key functions of estradiol in the ovulatory cycle are:

1. As a cog in a negative feedback mechanism suppressing the secretion of FSH and so aiding in the selection of the dominant follicle and preventing multifollicular development in the mid-late follicular phase.
2. Triggering of the LH surge in mid-cycle by initiating a positive feedback mechanism when its concentrations rise to a critical level.
3. As a 'growth hormone' for the development of the endometrium.

Estradiol concentrations are at their lowest during menstruation. The FSH induced follicular development brings about rapidly rising estradiol production in the mid-follicular phase. When estradiol levels attain a persist

ently high critical concentration in the late follicular phase, they induce the LH surge. Following ovulation, estradiol concentrations dip temporarily but are revived by corpus luteum activity. With the demise of the corpus luteum, estradiol concentrations sink rapidly to their lowest levels and invoke the FSH rise immediately preceding menstruation (Fig. 9).

A wonder of nature, hypogonadotrophic hypogonadism, in which both FSH and LH secretion are essentially missing, has provided a learning tool for the understanding of ovulatory physiology. The absence of FSH results in a lack of follicular development and estrogen production and the absence of LH in a lack of androgen substrate production [50]. When treatment with pulsatile GnRH is administered, pure substitution therapy, everything falls into place and ovulation can be successfully induced. If pure FSH is used to induce ovulation by direct stimulation of the ovaries, the lack of LH and therefore lack of production of androgen substrate, allows the growth of follicles but not estradiol production. Even if ovulation can be triggered by hCG or recombinant LH when a large follicle is obtained, implantation cannot occur due to the lack of estrogen stimulation on the endometrium [51, 52].

## 3.5: Progesterone

Progesterone is produced by luteinized granulosa cells. Large quantities are synthesized by the corpus luteum following ovulation. Progesterone concentrations rise to a peak 7–8 days following ovulation and fall rapidly with the failure of the corpus luteum (Fig. 2). The main function of progesterone from the corpus luteum is to fashion a secretory endometrium, capable of hosting the implantation of an embryo and to maintain this endometrium throughout the early weeks of pregnancy until trophoblastic/placental hormones take over this role [53, 54]. Under

the influence of progesterone the endometrial glandular structures increase greatly in numbers and become more tortuous. Progesterone also plays a role in the expression of genes needed for implantation at the level of the endometrium.

Together with estradiol, progesterone suppresses pituitary gonadotropin release during the luteal phase. The increasing concentrations of progesterone following ovulation gradually reduce the frequency of the GnRH/LH pulses and increase their amplitude. During this phase, FSH is synthesized and stored ready for release when freed from the inhibition imposed by progesterone and estradiol when the corpus luteum fails. The initial rise of progesterone concentrations immediately preceding the LH surge may play a role in the triggering of this surge [55].

## 3.6: Selection of the dominant follicle

Of the millions of primordial follicles that started life in the ovary, only about 400 will actually achieve ovulation during the reproductive life span. That means that more than 99.9% of follicles become atretic. At the beginning of each cycle, a group of the most mature follicles of 2–5 mm diameter are recruited for further growth, granulosa cell differentiation and multiplication. The follicles more sensitive to FSH rather than those less mature are selected at the time of the FSH inter-cycle rise for further development. The key to being chosen as the month's ovulatory follicle is sensitivity to FSH [27,41].The follicles most sensitive to FSH will utilize it to increase aromatase activity and produce estrogens and inhibin. As FSH concentrations fall in response to rising estrogen and inhibin B levels and become less available, only the most sensitive follicle to FSH, that with the lowest threshold for a response to FSH, can survive, continue to thrive and produce the most estrogen and LH receptors [51].The rest, starved of the possibility of FSH stimulation, become atretic. The selection of the dominant follicle is an example of survival of the fittest for which a good start in life is extremely important!

steps resulting in collagenolysis. The confusion is compounded by the suggested participation of various cytokines, oxygen free-radicals, nitric oxide and angiotensin II. More study is clearly needed to elucidate the complicated mechanism of follicular rupture.

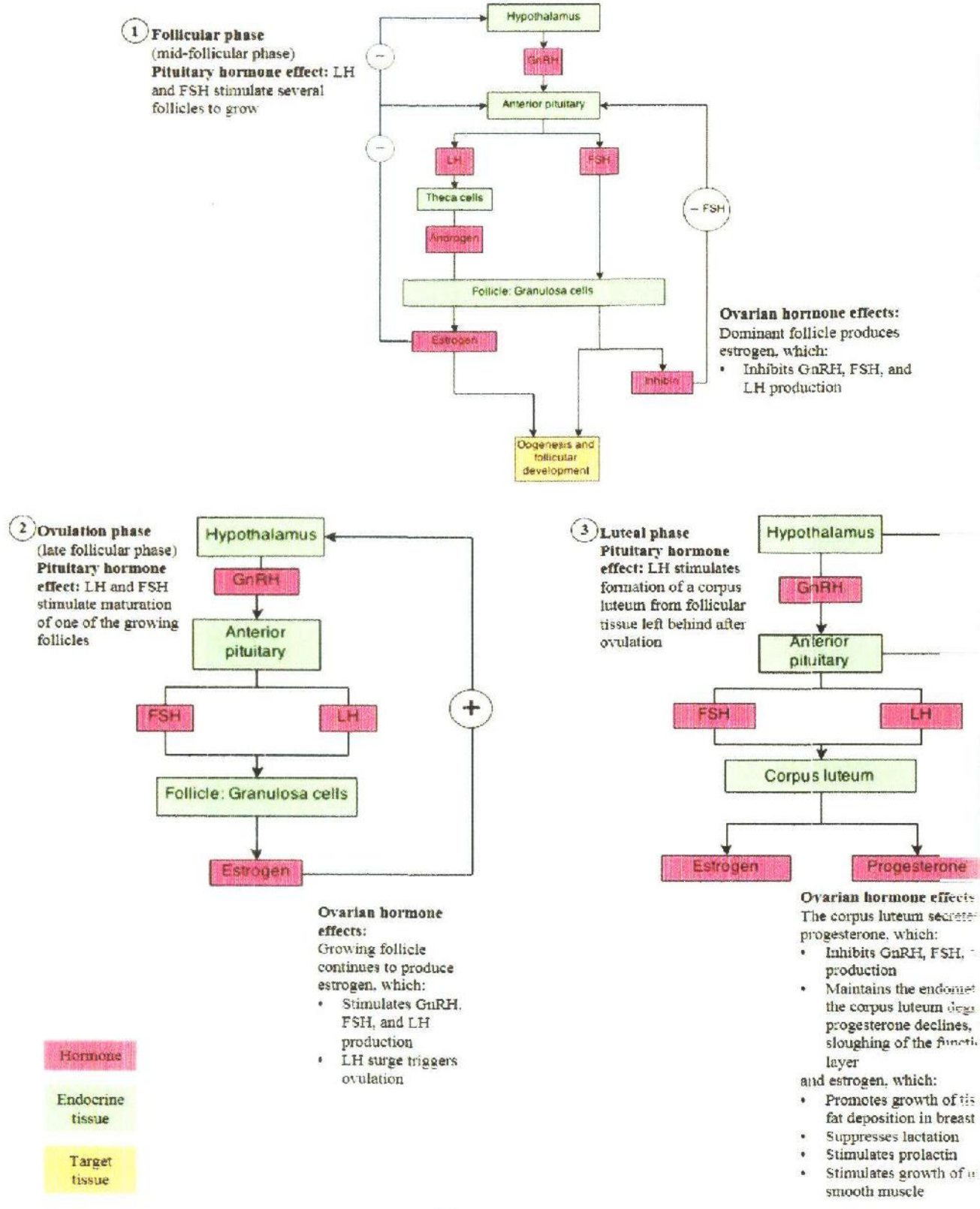

**Figure 12: Hormonal Regulation of Ovulation**

**Ref:** Clarke IJ, Cummins JT. The temporal relationship between gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) secretion in ovariectomized ewes. Endocrinology. 1982

## 3.8: Fine tuning

Such an intricate process as ovulation would not be complete without a fine-tuning system. This involves a large number of compounds, endocrine, autocrine and paracrine factors.

### 3.8.1: Inhibin

Inhibin is secreted by granulosa cells. Inhibin A and inhibin B are dimers which differ in their pattern of secretion. Inhibin A concentrations are low during most of the follicular phase but start to rise during its latest stages and peak in the mid-luteal phase. In contrast, inhibin B concentrations start rising early in the follicular phase, paralleling but later than the FSH rise [58]. Inhibin B negatively influences FSH concentration and also reflects the size of the follicle cohort. Estrogens and inhibin B are both inhibitory factors for the secretion of FSH.

### 3.8.2: Activin and follistatin

Activin is a promotor of many actions of FSH in that it increases FSH secretion, promotes ovarian follicular development and inhibits androgen production. Follistatin is an activin-binding protein that neutralizes activin bioactivity [57, 58].

### 3.8.3: Growth factors

Many growth factors form a network of interactions within the ovary and its compartments. The most well known are the insulin-like growth factors (IGFs) I and II which are very active and are counteracted by IGF binding proteins, six of which have been identified so far. Insulin, as well as binding to IGF receptors, has its own ovarian receptors and is known to promote androgen production. The transforming growth factor (TGF) family is also well represented in the ovary as is epithelial growth factor (EGF) [59].All play a passive role in the regulation of gonadotropin activity within the follicles.

### 3.8.4: Anti-Mullerian hormone (AMH)

AMH, a dimeric glycoprotein and member of the transforming growth factor-beta family, is produced by ovarian follicular granulosa cells in late pre-antral and small antral follicles. It seems to have a role in the regulation of folliculogenesis at the two extremes of this process: (a) by restricting the progression of development of primordial follicles; and (b) by an inhibition of the sensitivity of antral follicles to FSH and inhibition of aromatase activity during an ovulatory cycle. Production of AMH is not seen in a maturing pre-ovulatory follicle, possibly inhibited by rising estradiol levels, leaving uninhibited FSH to carry the process further forward. These roles of AMH suggest not only that it plays an important function in the regulation of folliculogenesis, [58, 59] but also that its concentrations are able to reflect ovarian reserve or, more practically, the number of small antral follicles in a cohort available for ovarian stimulation. The age-related decline in ovarian reserve is mirrored by serum AMH concentrations which can also reliably predict the number of oocytes collected following ovarian stimulation or, at the least, enable a prediction of low, normal and high responders [60].

## 3.9: Ovarian Steroidogenesis

The pathways of ovarian steroid production are diagrammatically illustrated in Fig. 13. The enzymes involved in these complicated processes are also signified. A basic knowledge of these actions is necessary not only for the understanding of normal ovarian physiology but especially for pathophysiological conditions such as polycystic ovary syndrome (PCOS) [53, 57].

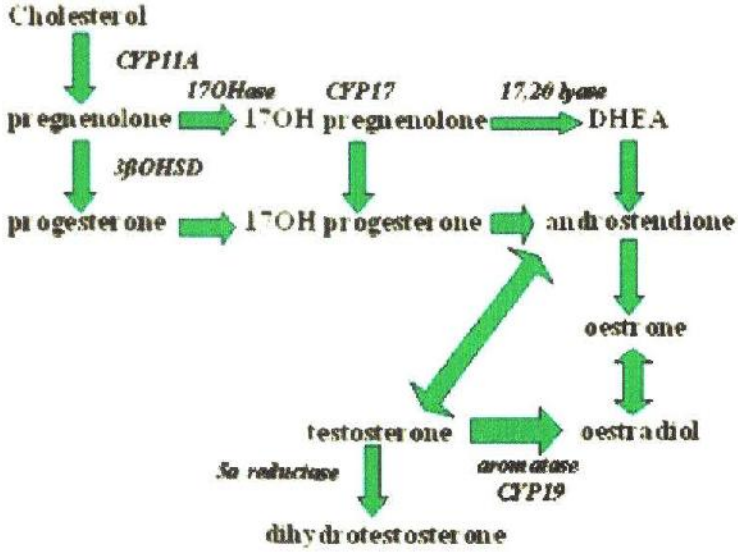

**Figure 13: Pathways of ovarian steroid production and some of the enzymes in steroidogenesis. DHEA, dehydroepiandrosterone**

**Ref:** Clarke IJ, Cummins JT. The temporal relationship between gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) secretion in ovariectomized ewes. Endocrinology. 1982

# Chapter 4

# Insulin in Female Egg Production

## 4.1: Reproduction and energy balance

The functional connection in health and disease Reproduction is a crucial function of the organism and is controlled by complex interactions between the hypothalamus, pituitary and gonads, the so-called hypothalamic-pituitarygonadal (HPG) axis [41] . However, reproduction and the survival of offspring is also an energetically demanding process, and the relationship between reproductive success and energy balance is well established. Energy is stored primarily as fat and glycogen, and together with glucose, allows organisms to grow and reproduce [42]. However, an animal's energy stores depend not only on the availability of energy sources (food), but also on energy expenditure. Pregnancy, parturition, lactation and maternal behavior are all energetically demanding states and in order to be successful in reproducing, the organism must be able to monitor energy status. Thus, negative energy balance either due to hypophagia (e.g. fasting, anorexia nervosa, and cachexia) or excessive energy expenditure (e.g. excessive exercize-induced amenorrhea and lactation) is linked to a suppression of reproductive function and ovarian cyclicity in a variety of species including humans [42, 43].

Since its discovery by Banting and Best in 1921 [44], insulin has been recognized as a key circulating signal mediating energy homeostasis. While major role of insulin is to maintain peripheral glucose homeostasis, via stimulation of glucose uptake, oxidation and storage [45], there is also strong evidence that insulin plays a role in regulating reproduction and may serve as a major signal linking metabolism and reproductive status. In this review we will focus on the role of insulin as an important factor in the control of reproduction, through actions occurring not only in the periphery but also in the central nervous system (CNS). We will review evidence primarily from animal studies demonstrating a role of insulin in the regulation of reproduction during adulthood, as well as during fetal development. In addition, we will examine evidence for potential CNS targets of insulin action specifically related to reproduction. Finally we will discuss the clinical relevance of the relationship between insulin and reproduction, with a specific focus on potential neural sites of action.

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## 4.2: Insulin as a signal in the metabolic control of adult reproduction

### 4.2.1: Insulin: its major role in controlling glucose homeostasis

In a simple sense, physiological maintenance of the regulation of blood glucose levels is the result of the coordinated function of three organs: the pancreas, which secretes insulin in response to increases in blood glucose; the liver, which decreases glucose production in response to raising levels of insulin; and skeletal muscle (and other tissues) that respond to insulin by increasing glucose uptake. In addition to this role, insulin also plays an important function in fat and protein metabolism, as it promotes the transport of amino acids from the bloodstream into muscle and other tissues/cells. Acting within cells, insulin increases the rate of incorporation of amino acids into protein and reduces protein breakdown. Moreover, insulin stimulates lipid (fat) synthesis from carbohydrate (in the process called lipogenesis) and decreases fatty acid release from tissue (in the process called lipolysis), leading to an increase in total body lipid stores [46].

### 4.2.2: Evidence for a role for insulin in the control of the HPG axis

Metabolism and reproduction are closely interlinked and a large body of research has focused on elucidating the mechanism by which signals from the periphery are conveyed to the HPG axis under various metabolic states. The master molecule for the control of the HPG system in mammals is the decapeptide, gonadotropin-releasing hormone (GnRH). GnRH is synthesized by a relatively small number of neurons, whose cell bodies are dispersed over an area that extends from the rostral ventral forebrain to the caudal hypothalamus and varies between species [47-49]. GnRH neurons send a major axonal projection to the median eminence [50-52], where GnRH is secreted into the pituitary portal system, and subsequently causes the release of luteinizing hormone (LH; [53]) from gonadotrophs of the anterior pituitary [54].

There are two major modes of GnRH secretion: the tonic or episodic secretion of GnRH that is seen in both males and females, and the preovulatory surge secretion of GnRH which is responsible for triggering ovulation and occurs only in females [54]. Both pulsatile and surge modes of GnRH secretion are sensitive to metabolic signals [55-60] and pathological situations which lead to acute and/or chronic hypo- or hyperinsulinemia are frequently coupled with disturbed GnRH/LH pulse and surge release patterns. The importance of insulin as a regulator of GnRH/LH pulses remains to be fully elucidated as results vary considerably between studies and the effects of insulin per se are difficult to tease apart from the role of accompanying peripheral signals and metabolites. For example, in diabetic male rats, there was a 50% reduction in LH pulse frequency and amplitude compared to non-diabetic controls [55].

Those deficits were completely reversed by twice daily insulin treatment [55]. Similarly, in Streptozotocin-induced (STZ-induced) diabetic male lambs, 24h withdraw from insulin supplementation decreased LH pulse frequency and acute re-supplementation reversed the inhibition [56]. However, in this study, a longer-term insulin withdrawal (96h) exaggerated the effects on LH (with a further reduction in LH pulses) during which insulin and glucose plasma concentrations remained constant. Therefore, other suppressors such as non-esterified fatty acids and ketone bodies cannot be ruled out [56]. By contrast, studies of other hypoinsulinemic models such as fasting yield different results. In adult (non-diabetic) male rhesus monkeys that underwent 24 h of fasting a profound suppression of LH was recorded, and rapid re-feeding reversed those deficits. To test the possible role of insulin, on the day of re-feeding, post-meal insulin secretion was partially suppressed by diazoxide (40-99%). However, this treatment did not block the LH increase observed after feeding [61] indicating that insulin alone was could not account for the observed inhibition. Similarly a central role of insulin in regulating GnRH/LH pulses remains controversial. Hileman et al. [62] reported that central injection of insulin (lateral ventricle) did not increase LH secretion in the growth-restricted, hypogonadotropic lamb. By contrast, Miller et al. [63] found that the infusion of insulin into the third ventricle stimulated pulsatile LH secretion in adult male sheep.

In a male diabetic sheep model, insulin infusion in the lateral ventricle reversed the decrease in LH pulse frequency but not to the same extent as peripheral insulin, providing further evidence that insulin alone cannot account for the diabetes-induced deficit in LH pulses. The reason for the discrepancy between these studies is not known, however, differences may be due to the type of model used (diabetic vs. fasting models), the doses, infusion site, and rate of insulin administered (pharmacological vs. supraphysiological; lateral vs. third ventricle; acute vs. chronic), the species (polygastric vs. monogastric animals) and the level of hypoinsulinemia [complete (diabetes) vs. partial (fasting)]. Despite variable results, the above studies taken together suggest that the peripheral and central actions of insulin are permissive rather than necessary for normal GnRH/LH pulsatile secretion. In addition to GnRH/LH pulses, the GnRH/LH surge is also sensitive to metabolic cues. Models of negative energy balance induced by fasting, caloric restriction and lactation are accompanied by a decrease in circulating insulin and disruption of estrous cyclicity in a number of species such as rats [64, 65], ewes [66], heifers [67] and monkeys [68]. Specifically, in adult female rats, short-term food deprivation blocks the LH surge [69, 70]. Short-term fasting during the luteal phase of the estrous cycle in sheep, increased serum concentrations of progesterone and delayed or diminished the preovulatory LH surge [71, 72]. Data on the role of insulin in these disruptions are lacking, however, insulin

replacement during lactational anestrous- a model of severe undernutrition did not restore estrous cyclicity [73].

Therefore, it is likely that other metabolic signals, such as hypoglycemia, leptin, or even the activation of the stress axis may be responsible for these disruptions [73, 74]. Experimental diabetes induced in female rats with STZ [75], a state of extreme hypoinsulinemia, results in impaired ovulation rates over an extended period of observation, disruption of the positive feedback effects of estradiol, and absent or delayed LH surges [57, 76, 77]. However, in this model, reproductive abnormalities are at least partially reversed after peripheral insulin administration [78]. These results are similar to those described above, in that effects on pulsatile and surge secretion are not reversed by insulin during negative energy balance but are at least partially reversed in diabetic models (hypoinsulinemia vs. extreme hypoinsulinemia). Another commonly used experimental model for metabolic stress is insulin-induced hypoglycemia (IIH).

This model mimics the detrimental effects of an acute decrease in energy availability, but also the effects of iatrogenic insulin overdose in diabetic patients. Even though the individual roles of supraphysiological amounts of insulin and the acute hypoglycemia are difficult to tease apart, there are several pieces of evidence that suggest that insulin does indeed contribute to the GnRH surge disruption during IIH. Studies carried out in ewes have determined that IIH during the activation, transmission or secretory phases of the GnRH surge mechanism [79, 80] initiates a sudden activation of the hypothalamicpituitary-adrenal (HPA) axis [81, 82] resulting in a delayed and reduced amplitude LH surge in the majority of treated ewes [80, 81, 83]. By contrast, there are reports of no effect of IIH on the LH surge of proestrous rats [44] and monkeys [85]; however, the doses of insulin used in these studies were significantly less than those used in the sheep experiments, suggesting that a specific threshold of hyperinsulinemia/hypoglycemia may need to be reached for deleterious effects to occur. Glucose replacement in the IIH sheep model reverses the effects of IIH on the timing of the surge [86] but not on its amplitude [80].

Therefore, even though the timing of the LH surge appears to be sensitive to glucose availability, surge amplitude does not, and it may be that hyperinsulinemia in this experimental model is responsible for this effect. Similar conclusions can be drawn from experiments in sheep that have been prenatally exposed to excess testosterone and exhibit metabolic and reproductive deficits similar to those seen in women with polycystic ovarian syndrome (PCOS) [87, 88]. Prenatal testosterone treated female ewes display hyperinsulinemia and insulin resistance [48], as well as defects in steroid feedback control of LH secretion, including delayed and reduced

amplitude LH surges [89]. Interestingly, in this model, restoration of cumulative plasma insulin levels with an insulin sensitizer, rosiglitazone, was able to restore the amplitude but not the timing of the LH surge [90]. Taken together, data from these two sheep models (IIH and prenatal testosterone exposure) imply that hyperinsulinemia does not abolish the LH surge but does reduce its amplitude. Whether this is a result of decreased GnRH release and/or reduced pituitary responsiveness to GnRH remains to be determined, however, there is evidence that both these sites are involved. For example, in women with PCOS pituitary response to GnRH is suppressed under a euglycemic, hyperinsulinemic clamp [91] and this may account for the reduced surge amplitude observed in the prenatal testosterone treated ewe model.

Similarly, substantial evidence suggests that insulin acts directly within the hypothalamus, and specifically via insulin receptor (IR) containing cells to influence GnRH excitability. The site(s) of action (neural vs pituitary) of insulin in regulating both pulsatile and surge secretion of GnRH/LH may be best addressed by future studies using the sheep model, where a specific advantage is the ability to repeatedly measure GnRH in portal blood in awake animals [92]. 3. Insulin: its potential role in prenatal programming of reproduction There is a growing body of both epidemiological and experimental evidence indicating that environmental factors can act early in the development to shape relationships between the regulation of energy status and reproduction later in life. The concept that early environmental factors can permanently organize or imprint physiological and behavioral systems is called fetal or early programming [93-96]. This hypothesis originated from studies indicating that low birth weight is associated with an increased biological risk for coronary heart disease in adult life [93]. Later studies performed by Philips and collaborators [97, 98] demonstrated a strong correlation between low birth weight, high cortisol levels and development of hypertension and Type 2 diabetes. There is now a good body of evidence, both from epidemiological data and experimental studies in animals, linking the intrauterine environment with the development of hypertension, diabetes, elevated blood cholesterol and PCOS in adulthood (for review see [93, 99, 100]). Several not mutually exclusive hypotheses have been developed to explain a link between a low body weight at birth and later adult risk of metabolic syndrome.

"The Fetal Insulin Hypothesis" states that pancreatic beta cell dysfunction can lead to defects in glucose stimulated insulin secretion, which in turn lead to reduced insulin mediated fetal growth and a low birth weight [101]. Those alterations at early stages would later in adulthood result in defects in beta cells and decreased insulin sensitivity, and thereby affect whole body glucose metabolism. Interestingly, however, Ng et al. [102], using a rat model of chronic high fat diet, reported that not only maternal but also paternal metabolic status could affect offspring

health. Specifically, they found that the female offspring of males fed a high fat diet in adulthood showed impaired glucose tolerance and insulin secretion. Moreover, the gene expression profile of the insulin-secreting pancreatic islet cells obtained from the daughters was abnormal, with changes in multiple gene networks and cellular pathways. The authors speculated that exposure to a high fat diet may have affected spermatogenesis in those fathers, re-programming the gametes possibly via epigenetic mechanisms.

Another explanation of the relationship between body weight at birth and adult metabolic syndrome comes from the "Thrifty Phenotype Hypothesis", according to which a fetus that endures poor nutrition during gestation, would spare the growth of vital organs, e.g. the brain, at the expense of tissues such as the muscle and the endocrine pancreas [103]. Thus, the fetus would adapt its metabolism to conditions of limited nutrition with permanent changes in insulin and glucose metabolism, increasing the risk of adult Type 2 diabetes and the metabolic syndrome [103]. In the light of evidence discussed above it would be of particular interest to identify the regions of the brain affected by early nutritional insults. We speculate that hypothalamus, where information about nutritional status is "read", and which plays a key role in governing reproduction, could be one such region. In support of this, recent studies using an intrauterine growth restriction rat model (maternal low protein restriction) found impaired insulin signaling in the hypothalamus in 20 days old pups [104]. Specifically, tyrosine phosphorylation levels of IRS2 and PIK3 p85α were impaired, changes which could potentially block insulin signal transduction.

However, it is not known if these hypothalamic changes in insulin signaling pathway components persist into puberty and adulthood, or whether they play a causal role in affecting later metabolic or reproductive function. Of particular interest for the current review are also findings suggesting that the adverse environmental factors are related to intrauterine growth retardation (IUGR) and low birth weight may predispose individuals to the later onset of development of metabolic syndrome, and that those individuals may also have reproductive system abnormalities. Based on those findings, a hypothesis termed the developmental origins of health and disease (DOHaD) has been developed [105], which states that an adverse perinatal environment programs or imprints the development of several tissues. In agreement with this concept, perinatal perturbations of the fetus/neonate nutrient supply might be a crucial determinant of individual programming of body weight set-point. The best-known example of the influence of negative metabolic status is the Dutch Famine Study, in which fetuses exposed to famine during early pregnancy had a higher energy intake and adiposity in adulthood [106, 107]. Importantly, prenatal growth restraint, followed by postnatal catch-up growth has been

associated with relative hyperinsulinism, increased visceral fat, FSH hypersecretion, development of exaggerated adrenarche with reduced uterine and ovarian size, reduced ovulation rate in adolescent girls and early post-menarche (for review see [108, 109]) as well as an advanced tempo of pubertal development and menarche [109]. Moreover, during the post-menarcheal period, girls born with low body weight have increased risk of developing PCOS, a disorder of androgen excess (in particular elevated free testosterone levels) as well as ovarian and metabolic dysfunctions [110-112].

Furthermore, women with PCOS demonstrated higher risk of developing of gestational diabetes mellitus (GDM) [113] and approximately 40% of PCOS women are insulin resistant [114]. Although PCOS manifests clinically during adolescence, the disease may originate in intrauterine life [115]. Importantly, experiments in sheep that have been prenatally exposed to excess testosterone lead to adult metabolic and reproductive deficits similar to those seen in women with PCOS [117, 118]. Thus, both epidemiological studies and animal models indicate that nutritional status during gestation has long-term effects on central and peripheral systems that regulate energy balance and reproduction in the developing offspring. Moreover, perinatal nutrition impacts susceptibility to developing metabolic disorders and plays a role in programming body weight set points (for an review see [116]). Those observations led to the hypothesis of metabolic imprinting, according to which a stimulus or insult occurring during a critical period of development has a long-term effect on the physiologic and metabolic responses of the offspring (for review see [117]). However, the role of altered neural organization in effects of prenatal programming by nutrients has not been studied in the same degree of detail as the role of peripheral organ function.

Insulin, which is increased in offspring of fatfed dams [118], and insulin-like growth factors, are thought to be pivotal to neuronal differentiation, as well as synapse formation and consolidation, in the hypothalamus [119] which plays a crucial role in regulation of appetite and food intake [120, 121]. As insulin and leptin are two important hormonal signals, which are secreted into the bloodstream in proportion to the amount of adipose tissue, they are often studied in the animal models discussed above [122]. Those hormones are blood-borne and cross the brain-blood barrier to act upon the brain, including the arcuate nucleus of the hypothalamus (ARC). Within the ARC, neuropeptide Y (NPY), agouti-related peptide (AgRP) and proopiomelanocortin (POMC) are synthesized and released [123-126]. NPY acts to stimulate food intake and reduce energy expenditure via interactions with receptors located in the paraventricular nucleus of the hypothalamus (PVN) and the lateral hypothalamus area (LHA). POMC neurons release alpha-melanocyte-stimulating hormone (α-MSH), which acts in the

PVN and LHA on melanocortin receptors to decrease intake and increase energy expenditure. AgRP released from ARC NPY/AgRP neurons acts as a functional antagonist of α-MSH at melanocortin receptors [124, 127]. In healthy organisms, adipocyte stores are correlated with a rise in the levels of insulin and leptin. Insulin and leptin, in turn, inhibit NPY/AgRP and stimulate POMC neurons, providing a feedback influence which acts to inhibit food intake [126]. However offspring of diabetic pregnant rats displayed increased hypothalamic insulin levels, and both at weaning [128] and as adults [119] had increased number of NPY-positive neurons in ARC.

Thus changes in hypothalamic appetite regulatory peptides may contribute to the development of obesity and metabolic disturbances in the offspring of diabetic female rats [88] although experimental manipulations are needed in this model to convincingly demonstrate this role. In summary, current evidence suggests that insulin may play a role in the programming of both metabolism and reproductive systems during development, and these early alterations could lead to peripheral and central abnormalities in both systems during puberty and adulthood. A possible target for early insulin action is the hypothalamus, where information about metabolic status is conveyed to the reproductive functions. Thus, in case of prenatal programming by nutrients, where insulin functions are impaired, the disruptions of reproductive system are also observed. Importantly both under- and over-nutrition could lead to obesity and diabetes, diseases associated with insulin abnormalities, in which secondary abnormalities including disruptions of reproductive functions are present.

## 4.3: Insulin and Adipokines: The Most Involved Molecular Players Linking Obesity and Reproductive Impairment

From what is said above, it appears clear that energy metabolism and female fertility are tightly connected and reciprocally regulated. Not surprisingly many peripheral signals report nutritional status to the CNS and to the ovary in order to coordinate reproduction. Undoubtedly, hormones derived from the gut, adipose tissue and pancreas (such as peptide YY, adipokines, insulin and others) were recognized as regulating human reproduction, as these tissues are ideally placed to sense and detect nutritional intake and status [70, 71]. In the sections below, we decided to focus our discussion on the signal of insulin and adipokines as they are largely described to mediate the negative impact that obesity has on fertility, highlighting their effects at the level of the HPG axis and their influence on steroidogenesis, as summarized in Figure 14.

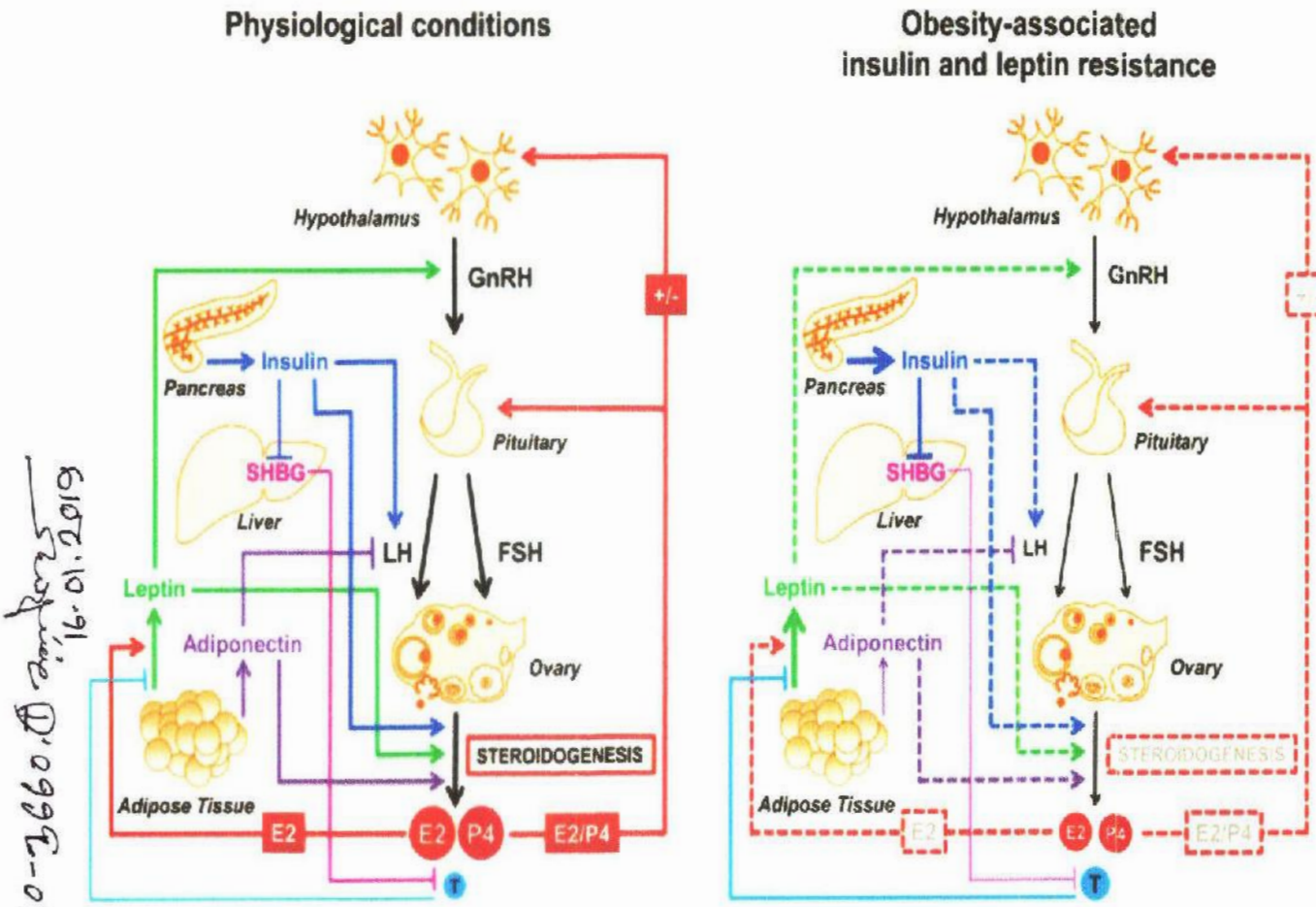


**Figure 14. Effects of insulin, leptin and adiponectin on the regulation of hypothalamic pituitary gonadal axis (HPG) and steroidogenesis under physiological and obesity-associated conditions. Under physiological conditions (left) the signaling pathways of insulin, leptin and adiponectin are able to sense the nutritional status, and, jointly with sex hormones sustain ovarian activity and reproduction. In obesity (right), the increased levels of insulin and leptin lead to insulin and leptin resistance that, together with the reduced adiponectin levels, contribute to a dysregulation of both the HPG axis and ovarian steroidogenesis, by further worsening the endocrine milieu (impaired E2 and P4 synthesis, increased T synthesis and free levels due to reduced SHBG synthesis) and impairing ovarian function and fertility success rate. In the figure the dotted lines represent impaired regulations. E2 = 17β-estradiol; P4 = progesterone; T = testosterone.**

**Ref:** Crown A, D. K. C., Steinera RA. Neuropeptide Signaling in the Integration of Metabolism and Reproduction. Neuroendocrinology. 2007

## 4.4: INSULIN ACTION IN THE FEMALE REPRODUCTIVE SYSTEM

### 4.4.1: Insulin and the Ovary

The insulin receptor is a heterotetramer consisting of 2 a subunits and 2 b subunits.8 Insulin binds to the extracellular a subunit. Subsequently, the b subunit becomes phosphorylated on tyrosine residues and acquires kinase activity. This phosphorylation initiates activation of a series of intracellular proteins, including insulin receptor substrate (IRS) proteins, phosphatidylinositide-3 kinase (PI3K), and mitogenactivated protein kinase (MAPK) [129, 130]. The activation of this signaling cascade acts as an effector for insulin's many metabolic effects in protein synthesis, lipogenesis, and carbohydrate metabolism. Perhaps the most important effect is facilitation of glucose transport through the plasma membrane mediated by translocation of intravesicular glucose transporter proteins (GLUTs) from the cytoplasm to the cell membrane. This process is mediated by the PI3K pathway. MAPK activation is thought to mediate the growth-promoting effects of insulin [129, 130].

An alternative signaling pathway for insulin action has been described. Insulin binding to the a subunit, independent of b-subunit activation, leads to the generation of inositol-glycan second messengers at the cell membrane. The role of this pathway in traditional metabolic effects of insulin has not been identified. It has been suggested that this alternative pathway may participate in regulation of ovarian steroidogenesis mediated by insulin. Definitive evidence for this, however, is lacking (Fig. 1) [130]. The classical target organs for insulin action are the muscle, liver, and adipose tissue. Demonstration of insulin receptor expression, however, in ovaries from both humans and animals supports a role for insulin action in the ovary. Insulin receptors are widely distributed throughout all ovarian components, including the granulosa, thecal, and stromal compartments [131, 132]. Studies of normal ovarian tissue have shown that insulin binds to its receptor in stromal and follicular components of the ovary. Complete inhibition of insulin binding by specific anti-insulin receptor antibodies and lack of such an effect with antibodies to the type 1 insulin like growth factor (IGF-1) receptor demonstrate that insulin binds to its ovarian receptors. Moreover, insulin receptors extracted from ovarian stromal tissues were shown to be autophosphorylated by incubation with insulin, similar to insulin receptor activation in classic target tissues.11

Insulin has also been shown to bind to stromal tissues in ovaries from women with PCOS. Complete inhibition by anti-insulin receptor antibodies demonstrates the specificity of this interaction. Insulin binding to ovarian cell membranes from the luteal phase is similar to that in the follicular phase.10 Although it was shown that insulin specifically binds to its own receptor

in the ovary, the question arose whether ovarian insulin receptor function and regulation were similar to those in classical target organs. Hyperinsulinemia is known to downregulate insulin receptor expression in classical target tissues [133–134]. In vitro, exposure of stromal ovarian tissue to high-dose insulin eliminates specific insulin binding, whereas termination of exposure leads to the recovery of this activity within 4 hours.12 In vivo, induction of hyperinsulinemia is accompanied by decreased insulin binding in the ovary, indicating the down-regulation of insulin receptors. Labeled IGF-1 binding was increased, suggesting up-regulation of IGF-1 receptors by hyperinsulinemia.15 Therefore, in settings of insulin resistance, alternate pathways may become important players in the ovary. In human studies, a correlation has been seen in insulin binding to ovarian stromal tissue and circulating leukocytes in postmenopausal women. This relationship was not consistent in premenopausal women [130, 133]. It is plausible that in premenopausal women, other factors, such as circulating gonadotropins, gonadal steroids, and autocrine regulators (such as IGFs), may influence insulin receptor expression. Insulin effects on ovarian steroidogenesis have been studied both in vitro and in vivo.

In vitro, insulin increases production of androgens, estrogens, and progesterone by granulosa and thecal cells [131,137]. Because, in some studies, hyperinsulinemia is required to stimulate steroidogenesis, it has been debated whether this action is modulated through IGF-1 receptors. The ability to block steroidogenesis from both granulosa cells and theca cells obtained from women with PCOS with anti-insulin receptor antibodies but not with antibodies against IGF-1 receptors, however, indicates that insulin mediates these actions through its own receptor [138, 139]. It is possible that insulin's effect on steroidogenesis is mediated through stimulation of aromatase activity [140].

Insulin in some studies has also been shown to activate 17a-hydroxylase and enhance forskolin stimulation of 3b-hydroxysteroid dehydrogenase [141, 142]. It has not been clearly demonstrated that insulin acutely stimulates steroidogenesis in vivo. In some studies of PCOS patients, insulin levels have been correlated with testosterone levels. Other studies, however, have not consistently supported this relationship [143-144]. Similarly, discrepant results in effect on androgen production have been noted with insulin infusion studies that have maintained euglycemic hyperinsulinemia for several hours [145-148]. Studies suggest that insulin acts as a co-gonadotropin at the level of the ovary. It enhances steroidogenic responses to gonadotropins both in vivo and in vitro [149,150–151] Insulin also enhances human chorionic gonadotropin (hCG)-induced ovarian growth and cyst formation in experimental animals [129]. In vivo, the degree of hyperinsulinemia in women with PCOS has been correlated with ovarian volume [152].

Moreover, with gonadotropin hyperstimulation, the increase in ovarian dimensions is greater in hyperinsulinemic PCOS patients than in normoinsulinemic PCOS women [152]. Insulin has been shown to decrease hepatic sex hormone–binding globulin (SHBG) production. This suppressive action may be an important player in increasing free testosterone in patients with hyperinsulinemia. An increase in SHBG can be seen in women with PCOS who are treated with insulin sensitizers [153, 154]. Another protein that is under the regulatory control of insulin is IGF binding protein (IGFBP)-1. Insulin inhibits production of this protein by the liver, thereby resulting in reduced circulating levels of IGFBP-1. Insulin also inhibits IGFBP-1 production by ovarian granulosa cells through activation of the insulin receptor. Decreased IGFBP-1 may increase circulating free IGF-1 levels [130].

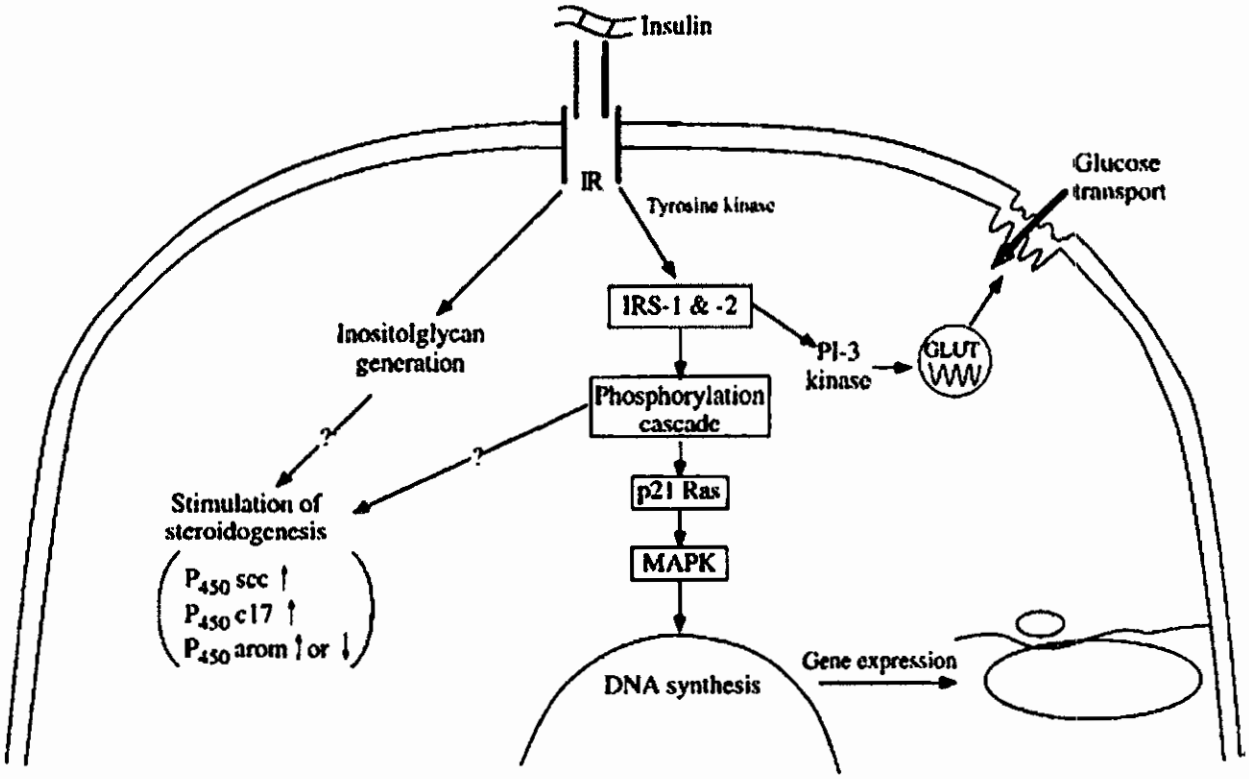


**Figure 15: Once insulin binds to its receptor, a signaling cascade is activated. The main pathway involves binding to the insulin receptor a subunit and activation of the b-subunit tyrosine kinase activity. This, in turn, leads to IRS-1 and IRS-2 phosphorylation and PI3K activation. Subsequently, GLUT are translocated to cell membranes to allow influx of glucose. An alternate pathway leads to generation of inositol glycans after insulin binding to its own receptor. This second pathway may be important in mediating steroidogenic effects of insulin action.**

**Ref:** Crown A, D. K. C., Steinera RA. Neuropeptide Signaling in the Integration of Metabolism and Reproduction. Neuroendocrinology. 2007

### 4.4.2: Reproductive Phenotypes with Altered Insulin Signaling

The importance of insulin action in female reproductive function is suggested by the preservation of this effect in various species. Mutations in the insulin and the related IGF-1 pathways have repeatedly resulted in alterations of the female hypothalamicpituitary- gonadal axis. In Caenorhabditis elegans, mutations in the insulin/IGF-1 receptornhomolog, DAF-2, induce developmental arrest in the dauer stage with reduced fertility [155]. Mutations in the Drosophila IRS protein, CHICO, cause female sterility accompanied by reduced growth and increased lipid storage [156]. In mammals, female IRS-2 null mice are sterile. They have small anovulatory ovaries. The ovaries are resistant to superovulation with gonadotropins, suggesting an intrinsic ovarian defect. In addition, there seem be central abnormalities, because the pituitary size is decreased and expression of LH is low in these animals [157]. Female mice with neuron-specific insulin receptor knockout have impaired follicular maturation secondary to altered hypothalamic control of LH secretion [157]. Reconstitution of the brain, liver, and pancreas insulin signaling in insulin receptor knockout mice rescues the mice from neonatal death, prevents diabetes, and restores reproductive function [158]. Despite this evidence that insulin/IGF-1 is important in the proper development and functioning of the reproductive system, the exact mechanism by which these pathways regulate gonadal function remains elusive.

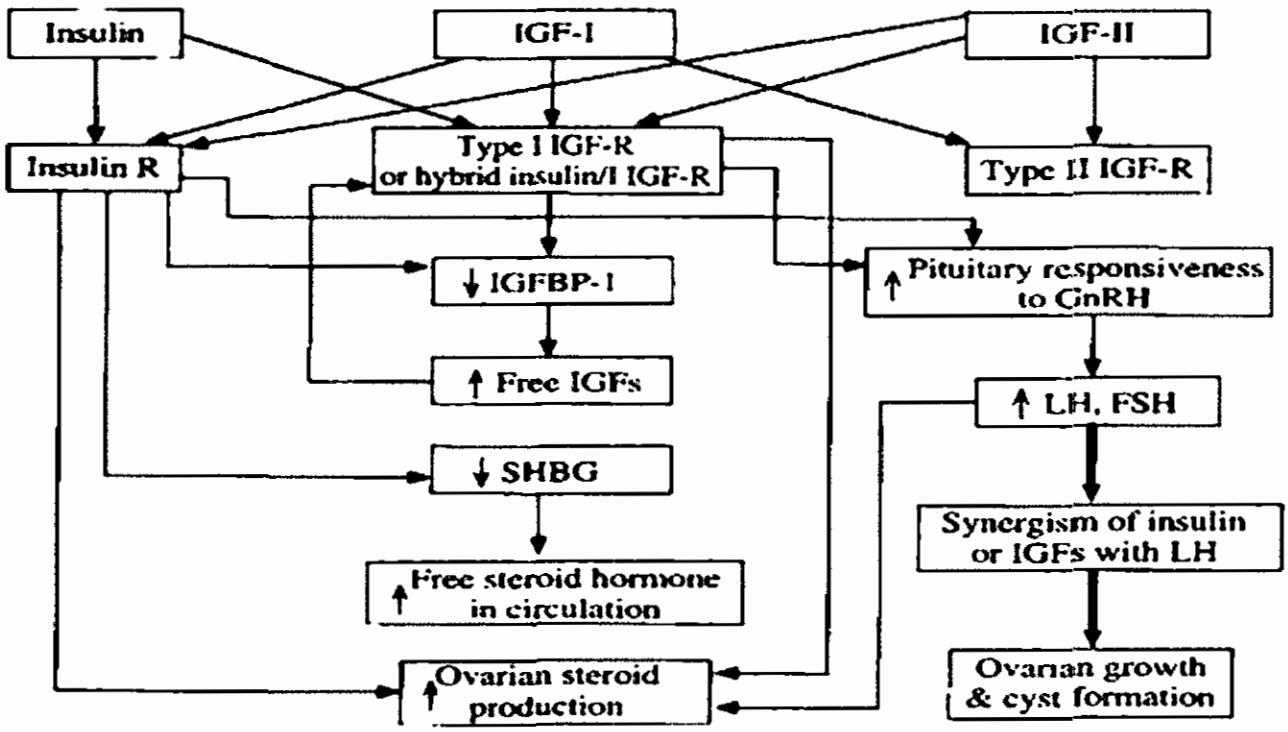

**Figure 16: The interactions between components of the insulin-related ovarian regulatory system, which involves insulin, IGF-I, IGF-II, and their receptors and IGFBPs.**
**Ref:** Crown A, D. K. C., Steinera RA. Neuropeptide Signaling in the Integration of Metabolism and Reproduction. Neuroendocrinology. 2007

## 4.5: GENERAL OVERVIEW OF INTRACELLULAR INSULIN SIGNALLING

The transduction of the insulin signal has been intensively studied principally using muscle, liver and adipose tissue, and these findings are summarized in Figure 1. Some, if not all, of these pathways appear to be functional in ovarian cells. Insulin exerts its pleiotropic effects after binding to the insulin receptor (IR), a member of the tyrosine kinase receptor family. Upon ligand binding, the IR undergoes conformational changes, which allow auto-phosphorylation of tyrosine residues located on the β subunits. Phosphorylated tyrosine residues serve as docking sites for various cellular substrates. In mammals, at least 11 intracellular substrates of the IR have been identified (reviewed in [28]). These include the family of IRS (insulin receptor substrate) and Shc (Src homology collagen) proteins. Four different IRSs have been reported in various mammals and two additional IRSs have been detected in humans (IRS5/DOK4 (docking protein 4) and IRS-6/DOK5 (docking protein 5)). These additional IRSs are involved in insulin signalling but are not involved in phosphoinositide kinase (PI3K) activation [159] and both are poor substrates for the IR [30]. The IRSs have different molecular masses (60–180 kDa) and show differential tissue distribution [31]. In mammals, Shc is also an IRS. There are 46, 52 and 66 kDa isoforms that arise from alternative splicing of the primary Shc transcript [162]. The 52 kDa and, to a lesser extent, the 46 kDa Shc isoforms are tyrosine phosphorylated in response to insulin. Most reports suggest that, in mammals, IRS-1 is the major substrate leading to stimulation of glucose transport in muscle and adipose tissue, whereas, in liver, IRS-1 and IRS-2 have complementary roles in insulin signalling and metabolism. In contrast, Shc does not appear to be directly involved in the metabolic signalling of insulin, but has a critical role in insulininduced mitogenesis [163]. In mammals, tyrosine phosphorylation of IRS and Shc makes these proteins docking sites for various signalling proteins containing an SH2 (Src homology 2) domain. These intracellular interactions lead to the activation of the two main IR signalling pathways: PI3K/Akt and MAPK (mitogenactivated protein kinase) [164,165].

Mammalian PI3K is a heterodimer [166] composed of a catalytic subunit of 110 kDa (p110) and a SH2-domaincontaining regulatory subunit of approximately 85 kDa (p85) the two are associated non-covalently. In mammals, PI3K has five isoforms of the regulatory subunit (in

three sizes) and three of the catalytic subunit. All four IRSs interact with all the regulatory subunits of PI3K, and thereby activate the catalytic subunit. Once docked to IRS, PI3K phosphorylates the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2), thereby producing phosphatidylinositol 3,4,5-trisphosphate (PIP3), which in turn acts as a second messenger to activate PDK1 (phosphoinositide-dependent kinase 1). The biological effects of the PI3K pathway can be negatively regulated at the level of PIP3 by phospholipid phosphatases, including PTEN (phosphatase and tensin homolog and SHIP2 (SH2-domain-containing inositolphosphatase-2, which inactivate PIP3 by de-phosphorylation [167,168]. Activated PDK1 can then phosphorylate Akt/PKB (protein kinase B). Akt comprises three isoforms each encoded by a separate gene and it is a key branching point (critical node) for insulin signalling.

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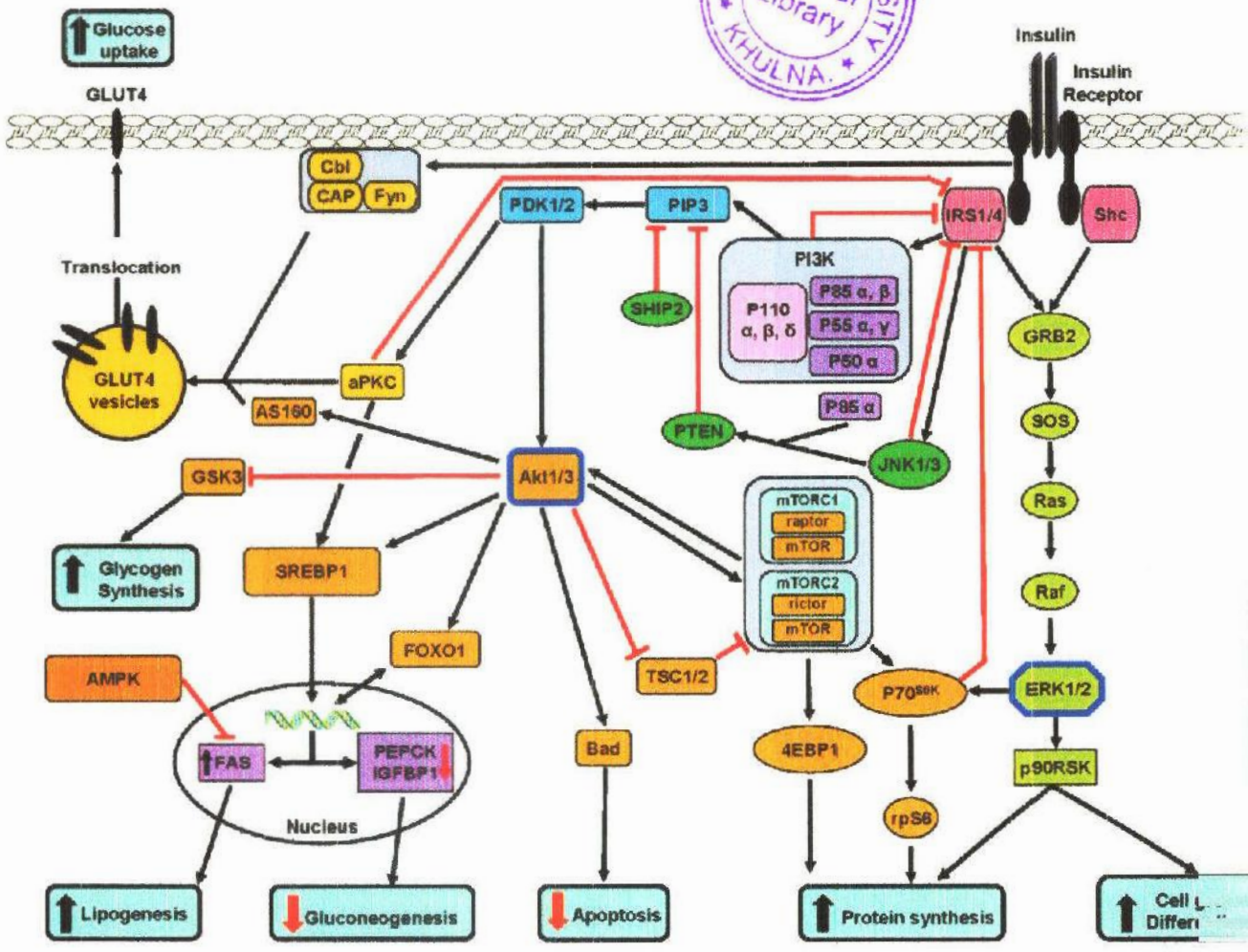


**Figure 17: Following insulin binding, the IR auto-phosphorylates and then phosphorylates its substrates including the IRSs and Shc**

**Ref:** Attia, G.R., Rainey, W.E. and Carr, B.R. (2001) Metformin directly inhibits androgen production in human thecal cells.

Following phosphorylation, specific domains in these substrates interact with other proteins. One of the major complexes interacting with IRSs is PI3K, which is formed of two interacting proteins: a regulatory subunit (p85) and a catalytic subunit (p110). There are several isoforms of both subunits: p85α, p55α, p50α, p85β and p55γ for the regulatory subunit and p110α, β and δ for the catalytic subunit. The binding of the regulatory subunit to IRS forms an active heterodimer which phosphorylates membrane phosphatidylinositol residues in the 3 position of PIP3 to form PDK1/2 (shown in blue). In turn, PDK1 phosphorylates (on threonine and serine residues) and thereby activates Akt/PKB (a serine/threonine kinase, with three isoforms). Downstream (orange boxes), Akt phosphorylates several kinases or transcription factors (some examples are shown) involved in most of the major metabolic effects of insulin: (i) glycogen synthesis through GSK3, which is inactivated by phosphorylation. The inactivation of GSK3 leads to the de-phosphorylation and activation of GS and hence to an acceleration of glycogen synthesis, (ii) inhibition of gluconeogenesis by turning off the PEPCK gene through inhibition of FOXO1 function (mainly in liver and adipose tissue), (iii) protein synthesis through mTOR and subsequent activation of p70S6K and 4EBP1 (eukaryotic translation initiation factor 4E-binding protein 1), (iv) the Akt pathway also stimulates cell growth and differentiation (not shown in the figure) and (v) exerts anti-apoptotic effects by phosphorylating the anti-apoptotic protein, Bad. The kinase, PI3K also activates aPKCs (atypical PKCs) (yellow boxes), which in concert with Akt (via AS160) exerts different effects according to the tissue: in muscle and adipocytes they initiate the translocation of intracellular GLUT4 on to the membrane and thereby stimulate glucose transport into the cell. Another important and well-documented signalling pathway is the Ras/ERK pathway (green boxes). The binding of Grb2 (growth factor receptor-bound protein 2) and Sos (son of sevenless) to tyrosine-phosphorylated IRS or Shc can also activate the small GTPase Ras cascade, Raf and ultimately ERK1 and ERK2 (green boxes). When activated, these serine kinases simulate protein synthesis through the activation of p90S6K and activate transcription factors and ultimately cell multiplication and differentiation. Some lipid phosphatases including PTEN and SHIP2 act on PIP3 to decrease or extinguish the insulin signal. Some of the insulin-stimulated serine kinases (aPKC, JNKs, p90RSK, ERKs and S6 kinase) may also exert negative effects on insulin sensitivity. Stimulatory connections are shown in black and inhibitory ones are shown in red.

Activated Akt directly phosphorylates and inactivates TSC2 (tuberous sclerosis complex 2) and GSK3 (glycogen synthase kinase-3), whereas it directly phosphorylates and activates Bad (Bcl-2-associated death promoter) and the forkhead transcription factor FOXO1 (forkhead box O1).

Phosphorylation of FOXO1 results in downregulation of transcriptional activity and TSC2 is a direct upstream negative regulator of mTORC1 that is involved in the regulation of protein synthesis. Mammalian target of rapamycin (mTOR) is a highly conserved protein kinase that is found in two structurally and functionally distinct protein complexes: mTOR complex1 (mTORC1) and mTOR complex-2 (mTORC2). In the first, mTOR is associated with raptor (regulatory associated protein of mTOR) and is a key regulator of cell growth, cell proliferation and mRNA translation, whereas in the second, mTOR is associated with rictor (rapamycin-insensitive companion of mTOR) and promotes actin cytoskeletal rearrangement, cell survival and cell cycle progression.

The two best characterized downstream targets of mTORC1, 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) and S6K1 (p70 ribosomal S6 kinase 1), regulate protein synthesis. Phosphorylation of 4E-BP1 by mTORC1 induces the release of eIF4E (eukaryotic translation initiation factor 4E) from 4E-BP1 and the subsequent formation of eIF4F (translation initiation factor 4F) complex. Activation of S6K1 also contributes to increased translation elongation via the phosphorylation of the eEF2 (eukaryotic elongation factor 2). In addition, phosphorylation of S6k1 negatively regulates insulin signalling by phosphorylating IRS-1 on serine residues. The inactivation of GSK3 leads to the de-phosphorylation and activation of glycogen synthase (GS) and hence to an acceleration of glycogen synthesis. The phosphorylation of FOXO1 by Akt in response to insulin leads to the exclusion of FOXO1 from the nucleus and consequently prevents the positive effect of FOXO1 on phosphoenolpyruvate carboxykinase (PEPCK) gene transcription. Akt also exerts anti-apoptotic effects by phosphorylating Bad.

In mammals, inhibition of the PI3K/Akt pathway blocks almost all of insulin's metabolic actions, including stimulation of glucose transport, glycogen synthesis and lipid synthesis [169]. Thus, this pathway has a pivotal role in the metabolic actions of insulin. Thirteen glucose transporter (GLUT)-like proteins have been identified and characterized [170] in mammals. One of them GLUT4, is a member of the facilitative GLUT family, characterized by preferential expression in muscle and adipose tissue, and it is responsible for insulin-stimulated glucose uptake (reviewed in [171]). The insulin-derived signalling pathways regulating the net gain in surface GLUT4 include activation of PI3K, PDK1/2, atypical PKC (protein kinase C) [172], Akt and AS160 (Akt substrate of 160 kDa) and a pathway (Cbl/CAP) that is independent of the PI3K/Akt pathway [173]. In mammals, the MAPK/extracellular-signal-regulated protein kinase 1/2 (ERK1/2) pathway is activated by insulin through IRSs or Shc tyrosine phosphorylation [158]. This pathway is composed of a set of three sequentially acting kinases. The activation of a MAPKKK (MAPK kinase kinase) (Raf) leads to the phosphorylation and activation of a

MAPKK (MAPK kinase) called MEK1/2 (MAPK/ERK kinase 1/2), which then stimulates ERK1/2 MAPK activity through dual phosphorylation on threonine and tyrosine residues of ERK1/2 MAPK to regulate the expression of genes including some in the PI3K pathway, controlling cell growth and differentiation [170]. It also positively controls S6K1 phosphorylation [172] and this pathway can be blocked at Shc, by cytokines acting through the JAK (Janus kinase)/STAT (signal transducer and activator of transcription) pathway (Figure 17).

## 4.6: AKT Pathway

Identification of the phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt) pathway and activating receptor tyrosine kinases (RTKs) began in earnest in the early 1980s through vigorous attempts to characterize insulin receptor signaling [177]. These humble beginnings led to the identification of the components and mechanism of insulin receptor signaling via insulin receptor substrate (IRS) proteins to PI3K and consequent PKB/Akt-mediated activation by 3-phosphoinositide-dependent protein kinase 1 (PDK1). With the discovery of the potent contribution of PI3K and PKB/Akt activation to tumorigenesis, intense research into the regulation of this pathway led to the discovery of the negative regulators, the protein phosphatase 2 (PP2A), phosphatase and tensin homolog (PTEN), and the PH-domain leucine-rich-repeat-containing protein phosphatases (PHLPP1/2). More recently, the elusive PKB/Akt hydrophobic motif kinases—i.e., the mammalian target of rapamycin (mTOR), when associated with the mTOR complex 2 (mTORC2), and the DNA-dependent protein kinase (DNA-PK)—were identified, as was the ability of Ras to affect the PI3K-PKB/Akt pathway via PI3K, completing our current model of the PI3K-PKB/Akt pathway.

The PI3K-PKB/Akt pathway is highly conserved, and its activation is tightly controlled via a multistep process. Activated receptors directly stimulate class 1A PI3Ks bound via their regulatory subunit or adapter molecules such as the insulin receptor substrate (IRS) proteins. This triggers activation of PI3K and conversion by its catalytic domain of phosphatidylinositol (3,4)-bisphosphate ($PIP_2$) lipids to phosphatidylinositol (3,4,5)-trisphosphate ($PIP_3$). [170] PKB/Akt binds to $PIP_3$ at the plasma membrane, allowing PDK1 to access and phosphorylate T308 in the "activation loop," leading to partial PKB/Akt activated pathway. [175]. This PKB/Akt modification is sufficient to activate mTORC1 by directly phosphorylating and inactivating proline-rich Akt substrate of 40 kDa (PRAS40) and tuberous sclerosis protein 2 (TSC) [176]. mTORC1 substrates include the eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), and ribosomal protein S6 kinase, 70 kDa, polypeptide 1 (S6K1), which, in

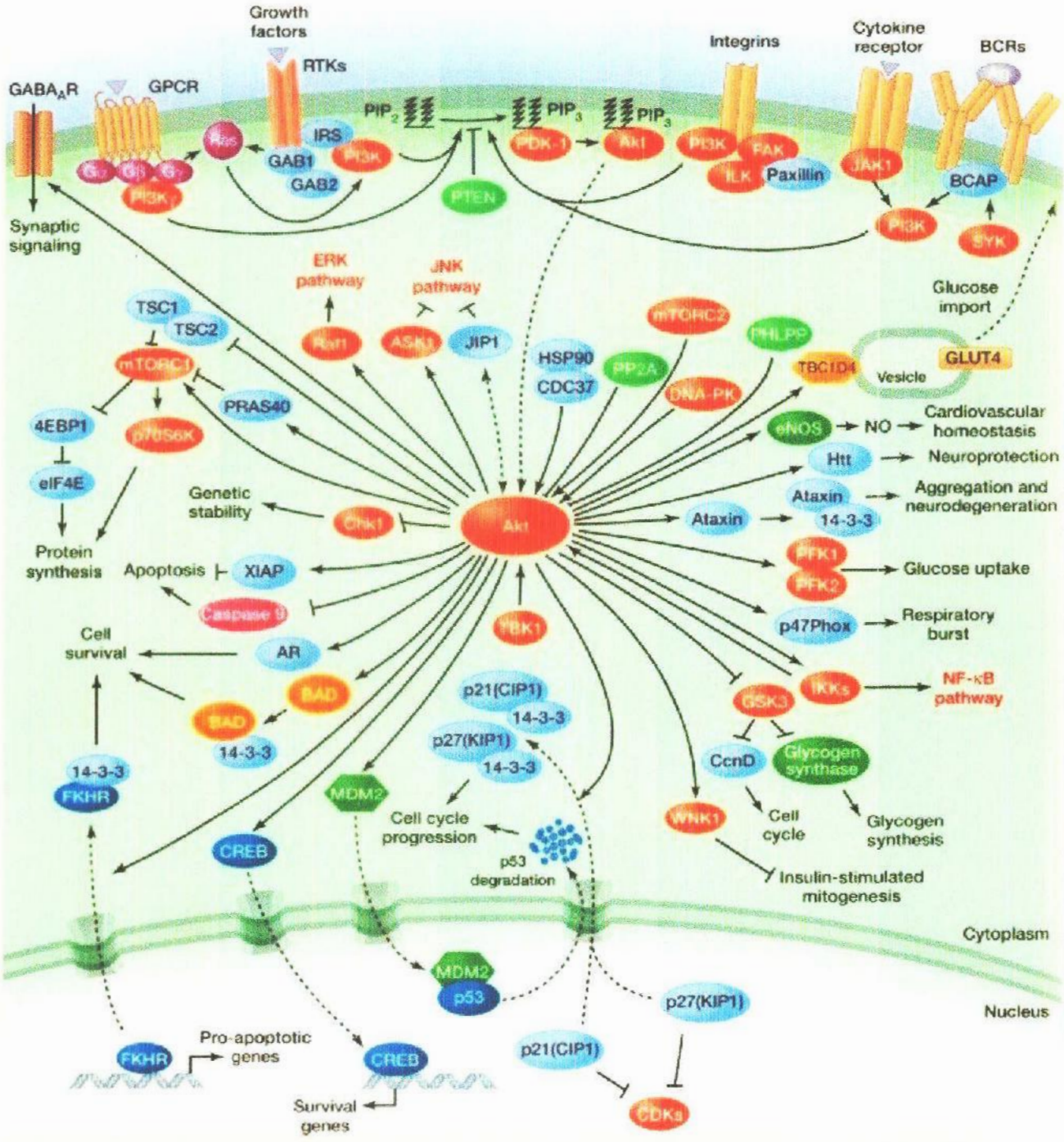


**Figure 19: Signalling events activating PKB/Akt and cellular functions regulated by PKB/Akt.**

**Ref:** Attia, G.R., Rainey, W.E. and Carr, B.R. (2001) Metformin directly inhibits androgen production in human thecal cells.

## 4.7: GLUCOSE

From a physiological viewpoint, glucose is an enigmatic molecule. On one hand, it is a critical source of metabolic energy for cellular metabolism and therefore for life itself, and on the other

hand it is potentially extremely harmful to protein function. Hypoglycaemia will quickly become life-threatening, whereas prolonged hyperglycaemia will lead to protein malfunction through the process of non-enzymic glycation of proteins and the formation of advanced glycation end-products (AGEs), thus altering their functional properties [166]. Consequently, mammals have evolved homoeostatic mechanisms that maintain blood and tissue concentrations of glucose within very narrow physiological limits. The AGEs have been implicated in reproductive conditions such as ovarian hyperstimulation syndrome and PCOS [167], and glycated proteins have been implicated in the fetal malformations that can accompany diabetic pregnancy [178,179].

### 4.7.1: Uptake of glucose by ovarian tissues and the role of the GLUTs

Glucose is a hydrophilic molecule and cannot permeate the plasma membrane. Consequently, its uptake is mediated by a number of facilitative sugar transporters that exhibit different substrate specificities, kinetic properties and patterns of tissue distribution [180]. Published data show that various GLUTs are present in ovarian tissues, but with considerable species and tissue differences in the pattern of expression. GLUTs 1 and 4 are expressed in follicles from sheep [181] and GLUTs 1, 3 and 4 mRNAs were all expressed in bovine corpora lutea and follicles [182]. In the rat, GLUTs1, 3 and 4 and their mRNAs are expressed in ovaries [183]. In a recent study [184], mRNAs for GLUTs 1, 3, 5, 8 and 13 were constitutively expressed, whereas GLUTs, 4, 6, 9, 10, 11 and 12 were differentially expressed, but GLUTs 2 and 7 were not expressed in human granulosa cells from patients with PCOS. The authors of some of these reports have also demonstrated that the expression of these GLUTs can be regulated by intraovarian factors involved in follicular development, maturation and ovulation, such as E2, IGF-I and interleukin-1β [185] as well as the classic regulators of ovarian function, gonadotropins [183]. In the ovary of the rat the mRNA for GLUT3 was increased following an ovulatory stimulus using human chorionic gonadotropin (hCG) [186] and in bovine follicles, highly significant negative correlations were observed between the concentrations of glucose in follicular fluid and the levels of GLUTs1 and 3 mRNAs in granulosa cells [182]. These data imply that the hormonal environment of the follicle can affect glucose uptake and that the ovary has reproductive mechanisms to regulate glucose uptake by ovarian cells.

### 4.7.2: Role of glucose in the ovary

There is considerable published data reporting that glucose is present in follicular fluid from humans [187], macaques [188], buffalos [189], sheep [190], cattle [191], pigs [192], camels [193] and horses [64], and follicular fluid is the presumed source of glucose for the avascular

membrana granulosa. Most authors report that the concentration of glucose in follicular fluid is lower than the concomitant concentration in serum or plasma and furthermore most report significant differences in the concentrations of glucose in follicular fluid of different follicles from the same animal, implying some local (hormonal) regulation of the movement of glucose into or its utilization by follicles. The concentration of glucose in follicular fluid was higher in larger follicles [190,195,196] and highest in pre-ovulatory follicles. Although intrafollicular glucose was increased following an ovulatory stimulus in the macaque, the mural granulosa cells were not able to utilize it, sparing the glucose for the cumulus–oocyte complex [198]. Thus, the intra-follicular concentration of glucose appears to be modulated to some extent by the hormonal environment of the follicle [190,195,196], and it is correlated positively with in vitro fertilization (IVF) outcome [197]. However, the existing data do not reveal a completely clear picture, with numerous contradictory reports describing the relationships between intrafollicular glucose, follicle size and follicle status. Further research is required to establish how the intra-follicular concentration of glucose is regulated during folliculogenesis and its significance to fertility, especially in relation to the hyperinsulinaemia and hyperglycaemia associated with metabolic disorders [198]. Glucose is an important energy substrate for the generation of ATP for the metabolic and physiological functions of the ovary, one of the most dynamic of the endocrine organs. It simultaneously nurtures the cyclic development of several follicles, the maturation and ovulation of the selected follicle(s) and subsequent formation and maintenance of a functional corpus luteum. There is a significant positive difference in the arteriovenous concentration of glucose in the blood flowing to and from the ovary [199], indicating the uptake of glucose by the ovary. In the bovine ovary, the predominant energy substrate appears to be glucose, and the whole ovarian respiratory exchange ratio was calculated as 0.95 [199]. Glucose can be metabolized along multiple pathways, including glycolysis, the pathway of glycogen synthesis, the hexosamine pathway and the pentose phosphate pathway (PPP). Regardless of the pathway, the first step is the metabolism of glucose to glucose 6-phosphate, a step mediated by hexokinase and glucose-6-phosphatase. Glucose 6-phosphate can enter glycolysis through the action of phosphohexose isomerase or PPP via glucose-6-phosphate dehydrogenase. The outcomes of these two pathways are ATP and NADPH and ribose sugars respectively. Early studies using the rat indicated that LH increased glucose uptake and lactate output, along with increased hexokinase activity [200,201], although relatively little subsequent work has been done on the metabolic fate of ovarian glucose. The exception to this is the cumulus–oocyte complex, where glucose metabolism by the cumulus cells has been shown to provide essential metabolites to the oocyte [202–204].

In sheep granulosa cells, glucose, metabolized under anaerobic conditions to lactate, is the preferred energy substrate to support the gonadotropin-induced differentiation of granulosa cells [205] and theca cells [206] cultured in vitro, and that fructose and pyruvate, but not galactose, are alternative energy substrates despite marked differences in the way that these substrates are metabolized. The existence of well-characterized energy-sensing mechanisms in cells raises the possibility that glucose, in addition to being a source of energy, may also function as a signalling molecule. The two glucose- or energy- sensing mechanisms that could potentially act as signalling mechanisms in follicular cells are via AMPK (AMP-dependent protein kinase) and the hexosamine pathway. These have been discussed in previous reviews [177,178]. Briefly, the serine/threonine kinase AMPK is an energy sensor that is activated by nutritional stress. It is present in the ovary and its activation leads to alterations in ovarian function. There is inhibition of P4 and E2 production [179] and of FSH-stimulated cell proliferation [180]. Glucose can enter the hexosamine nutrientsensing pathway which allows glucose to directly mediate gene expression [181, 182].

The end-product of this pathway is UDPN-acetylglucosamine which, in addition to being an important substrate for glycosylation, can also increase the expression of the genes for some growth factors such as fibroblast growth factor 2 (FGF2), transforming growth factor β (TGFβ) [182] and TGFα [183] that have reproductive effects, in particular TGFα suppressed E2 secretion from the ovary in a sheep ovarian auto-transplant model [84]. Glucose inhibited KATP channels in hypothalamic neurons, thus increasing cell excitability [185]. Glucose starvation stimulated AMPK in many cell types, including hypothalamic neurons [186]. In ovarian cells, on one hand, glucose deprivation activates AMPK and induces cell death though modulation of Akt [187], and, on the other hand, the infusion of glucose into sheep reduced the phosphorylation of AMPK in granulosa cell lysates [202,203].

## 4.8: ACTIONS OF INSULIN IN GRANULOSA, THECAL CELLS, LUTEAL CELLS AND OOCYTES

### 4.8.1: Critical cross-talk nodes

Earlier, it had been suggested that insulin had gonadotrophic actions in the ovary [208]. It now appears much more likely that the gonadotropin-like actions of insulin are mediated via interactions between their respective signalling pathways at critical nodes such as Akt or MAPK [208]. In the follicle, there are several lines of evidence strongly suggesting that insulin–FSH, insulin–LH and FSH–LH cross-talk takes place and that it has biochemical and physiological consequences in the follicle.

### 4.8.2: Insulin receptors in ovarian cells

The IR is found in granulosa and thecal cells of different species [189,190] and insulin receptor transcripts have been described in human, bovine and rat oocytes [191, 192]. The IR has two isoforms, IRA and IRB, which differ by the presence of 12 amino acids at the C-terminus of the α subunit [93]. Both isoforms can hybridize with each other as well as with the closely related IGF-IR (70% homology). The expression of IRA mRNA greater than that of IRB in normal mural granulosa and cumulus cells [94]. In the oocyte, IR is not required for oocyte growth, differentiation and maturation. Indeed, when the IR is ablated in oocytes, the female mice are fertile and exhibit normal oestrous cyclicity, oocyte development and maturation, parturition frequency and litter size [195]. Although GLUTs have been reported in the oocyte [156], it seems that they are non-functional. The oocyte obtains glucose via gap junctions from cumulus cells which take up glucose via GLUT-mediated mechanisms [196].

### 4.8.3: The in vivo effects of insulin on the ovary

The published in vitro data suggest that insulin affects follicular development in vivo. However, although most of the published in vivo data agree with this broad generalization there are numerous contradictions between data derived from in vitro and in vivo experimental approaches [197]. Data from the two models are not directly comparable, but there should be broad agreement. The main difference between these two experimental methodologies is that in vitro insulin appears to stimulate E2 production by granulosa cells, whereas, in vivo, insulin appears to inhibit the ovarian secretion of E2. Using immature rat ovaries in a perfusion system, Peluso et al. [198] demonstrated that insulin was able to stimulate mitogenic activity and simultaneously to suppress oestradiol secretion. Although the infusion of glucose or the feeding on glucogenic diets, all treatments which increased plasma glucose concentrations, increased the number of follicles and stimulated insulin secretion [200], it reduced the concentration of E2 and the follicular levels of CYP19a1 (cytochrome P450 19a1) [200, 202, 203]. Using an ovarian auto-transplant sheep model [199] in which the ovary was perfused in vivo with insulin without increasing the peripheral concentration of glucose, thus separating out the actions of insulin and glucose, insulin did not affect follicular steroidogenesis, and, when glucose was included in the perfusate, follicular steroidogenesis was inhibited [199]. Thus the in vivo effect of insulin on steroidogenesis appears to require the uptake of glucose and is associated with decreased phosphorylation of Akt, ERK and AMPK [202]. Finally, in the post-partum dairy

cow, glucogenic diets that stimulated the secretion of insulin enhanced follicular development and advanced the resumption of ovarian cyclicity but, they had detrimental effects on embryo survival [100–102].

### 4.8.4: The in vitro action of insulin in ovarian cells

In most in vitro systems, insulin stimulates both cell proliferation and steroidogenesis in cultured granulosa and theca calls. Insulin stimulated basal and FSH or LH-induced secretion of P4 and E2 and it was also able to stimulate basal and FSH-induced aromatase activity [103,104]. However, in bovine granulosa cells high doses of insulin did not stimulate aromatase activity [105], thus suggesting that a high concentration of insulin favours the secretion of P4 and androgen secretion over the secretion of E2. Insulin can also directly stimulate granulosa cell proliferation [106]. Finally, insulin increased glucose transport [107], GS activity [108], the intracellular accumulation of free fatty acids [109] and the expression of LH-induced low-density lipoprotein receptors [110]. Insulin-stimulated androgen secretion by human and animal theca cells [111–113] that provides substrates for the synthesis of E2 in granulosa cells [114,115]. In rat thecal cells, insulin-stimulated cell proliferation and the expression of the cell cycle regulatory proteins CDK4 (cyclin-dependent kinase 4), cyclin D3 and PCNA (proliferating-cell nuclear antigen) were all blocked by rapamycin implicating the mTORC1 signalling pathway in these actions of insulin [116]. Landau et al. [100] suggested that insulin may affect oocyte maturation in vivo because there were increased levels of insulin in preovulatory follicles compared with subordinate follicles. Insulin in supra-physiological concentrations had a stimulatory effect on the cleavage and maturation of oocytes in vitro [101], but prolonged hyperinsulinemia in vivo had a negative impact on the developmental competence of oocytes [117,118]. In addition, it was reported that hyperinsulinemia was associated with impaired oocyte quality in cattle [119].

## 4.9: INSULIN SIGNALLING PATHWAYS IN THE OVARY

The various insulin signalling pathways (Figure 1) in ovarian cells can function independently of FSH and LH to, for example, promote glucose transport, cell proliferation, cell differentiation and fatty acid synthesis and to inhibit apoptosis in gonadotropin-independent follicles. In gonadotropin-responsive and gonadotropin-dependent follicles, insulin can also interact with the gonadotropins to modulate hormone secretion and perhaps other differentiated functions. Studies of insulin signaling pathways in ovarian cells have been largely confined to granulosa cells and oocytes, with limited data available for the other cell types in the ovary .

### 4.9.1: GONADOTROPIN SIGNALLING PATHWAYS IN THE OVARY

Both FSH and LH signalling pathways in ovarian cells have been extensively studied [134–137]. The first reported, and probably the primary pathway, for both gonadotropins is the cAMP/PKA (protein kinase A) pathway. However, subsequently, several other signalling pathways that respond to gonadotropin stimulation have been reported in granulosa, thecal and luteal cells. These include the PI3K/PDK1/Akt pathway, the MAPK pathways and the PKC pathway.

### 4.9.2: Follicle-stimulating hormone in granulosa cells

Granulosa cells contain FSH receptors and, on ligand binding, adenylate cyclase produces cAMP which then phosphorylates PKA which then phosphorylates multiple downstream substrates along diverse pathways leading to gene transcription. Over 100 FSH-inducible genes have been identified in granulosa cells [134]. Additional FSH signalling pathways that have been identified in granulosa cells include the cAMP-independent pathways PI3K/Akt and PKC that result in cAMP-independent responses to FSH in granulosa cells. The MAPK pathways ERK, JNK (c-Jun N-terminal kinase) and p38MAPK can also be activated in granulosa cells by FSH via cAMP/PKA or independently. This array of FSH signalling networks has considerable overlap with the array of insulin signalling networks providing opportunities for cross-talk between insulin and FSH signalling pathways.

### 4.9.3: LH in granulosa and thecal cells

Thecal and luteal cells contain LH receptors and, similarly to FSH, ligand binding leads to the production of cAMP and phosphorylation of PKA. The signalling pathways for LH are very similar to those for FSH. Indeed, in the selected ovulatory follicle(s), LH can substitute for FSH to protect the selected follicle during the follicular phase from the damaging influence of the E2-driven decline in the circulating concentrations of FSH [77]. This action of LH providing an alternative to FSH to protect the follicle from atresia is probably mediated by the LH receptor. The principal pathway is the cAMP/PKA/CREB (cAMP-response-element-binding protein) pathway and other known pathways include the PI3K and ERK pathways.

## 4.10: INSULIN OOGENESIS AND FOLLICULOGENESIS

### 4.10.1: Primordial follicles

The formation of primordial follicles occurs during the latter half of pregnancy in humans and ruminants, but immediately after birth in rodents. The postnatal mouse ovary is mainly populated by primordial follicle each composed of a meiotically arrested primary oocyte surrounded by flattened pre-granulosa cells. Several lines of evidence suggest a close

relationship between KIT ligand (KL) and the PI3K/Akt pathway, and that both are necessary for oogenesis and the early stages of folliculogenesis [138]. Because of its chemoattractant action, KL is fundamental in the control of the migration of primordial germ cells (PGCs) into the genital ridges. In purified mouse PGCs isolated 11.5– 12.5 days post-coitum, KL stimulates the phosphorylation of Akt by either PI3K or Src kinases. The crucial role of Akt in promoting the survival and proliferation of PGCs has been confirmed by Kimura et al. [139] who found that apoptosis of PGCs was decreased and proliferation increased with the induction of Akt. The primordial to primary follicle transition is also controlled by KL signalling through induction of the PI3K/Akt pathway. The interaction between KL, which is expressed primarily by granulosa cells and KIT receptor, which is present in oocytes [140], stimulates the recruitment of PI3K from the cytoplasm to the cell membrane through the binding of the SH2 domain of the p85 subunit of PI3K to the phosphorylated tyrosine on KIT [138]. This binding promotes the activation of Akt and the subsequent phosphorylation of downstream target proteins. Activated Akt controls the phosphorylation of TSC2 that, in association with hamartin (TSC1), controls mTOR. In oocytes and granulosa cells, TSC1 is active and helps to regulate the pool of primordial follicles [141]. However, despite this evidence suggesting the importance of Akt for the survival and proliferation of PGCs, the effects of hyperinsulinemia on the formation of PGCs is not known.

**4.10.2: Oocytes**

The transformation of diploid oogonia into haploid oocytes involves many of the downstream targets of insulin signalling and thus is potentially influenced by insulin. Phosphorylated Akt controls the activity of the serine/threonine kinase, GSK3 and some of the FOXO family of transcription factors (FOXO1, FOXO3a and FOXO4). In mouse oocytes, granulosa cellderived KL regulates the PI3K/Akt pathway, because the specific PI3Kinhibitor LY294002 inhibits this process. The Akt1- mediated phosphorylation of GSK3α at Ser21 or GSK3β at Ser9 results in the inactivation of GSK3. In the bovine ovary, GSK3β is localized to cytoplasm of granulosa cells and oocytes throughout folliculogenesis. In MII (metaphase-II) oocytes, the kinase is present in a region between the oocyte and the first polar body. The use of specific inhibitors has suggested a role for GSK3β in the regulation of the MI to MII transition, probably participating in the activation of Aurora A kinase [142]. Also mouse oocytes contain GSK3 mRNA and protein, and its pharmacological inhibition causes an abnormal configuration and function of the meiotic spindle, chromatin organization and bivalent chromatin segregation. On the other hand, phosphorylated PKCζ inactivates GSK3β by phosphorylation on Ser9 to help maintain spindle stability during meiotic metaphase arrest [143]. The transcription factors

FOXO1, FOXO3a and FOXO4 are also involved in the control of folliculogenesis [144]. One of them, FOXO3a, has been detected in the nucleus of oocytes in primordial and early primary follicles, and its expression is dramatically down-regulated in oocytes of primary and growing follicles [120,145]. Although FOXO1 mRNA is expressed in mouse oocytes [120] and can regulate different functions, it is not expressed in sufficient quantities to substitute for FOXO3a. When oocytes are treated with KL, FOXO3 is phosphorylated and inactivated. In the oocyte, FOXO3a inhibits the production of bone morphogenetic protein (BMP-15) and possibly the activation of SMAD (homolog of Drosophila and C. elgans proteins) pathways that control the proliferation and differentiation of surrounding granulosa cells. It has been suggested that the only role of oocyte PI3K is to regulate FOXO3a activity [146].

### 4.10.3: Gonadotropin-independent follicles

In the mammalian ovary, FOXO3a controls follicular activation and early follicle development. In fact, it suppresses the initiation of follicular growth and controls the rate of activation of primordial follicles. Knockout experiments in mice have demonstrated the importance of FOXO3a but not GSK3 for the growth and development of gonadotropin-independent follicles. The functional consequences of FOXO3a inactivation is a rapid decline in fertility, an increased number of atretic follicle and a depletion of the primordial follicle pool. In these knockout mice, POF and infertility are established by 15 weeks of age [146]. The absence of oocyte-specific FOXO3 induces global activation of primordial follicles, as does deletion of PTEN [123]. However, in the absence of PTEN, PI3K is capable of directly stimulating Akt-dependent phosphorylation and nuclear export of FOXO3a, thereby triggering uncontrolled primordial follicle activation and POF. In contrast, overexpression of FOXO3a leads to female infertility due to impaired follicle development. In fact, in neonatal rat ovaries, the overexpression of FOXO3a and its downstream factors, Bim (B-cell lymphoma-2), FasL (Fas ligand) and the cell cycle inhibitor p27 (CDK inhibitor p27) stimulated apoptosis in naked oocytes. In addition, constitutively active FOXO3a dramatically affects BMP-15, connexin37 and connexin43, thereby inhibiting the growth of oocytes and follicles. Other specific target genes regulated by FOXO3 are p27 and the enzyme galactose-1-phosphate uridyltransferase (GALT), both involved in the control of follicular activation. In particular, FOXO3a enhances p27 expression and prevents its transfer from nucleus to ooplasm, thereby maintaining the growth-inhibitory function of this protein [147].

### 4.10.4: FSH-responsive and FSH-dependent follicles

#### 4.10.4.1: Granulosa cells

In granulosa cells, FSH regulates ovarian atresia via the PI3K/Akt/FOXO3a pathway. Indeed, in the porcine ovary, FSH inactivated FOXO3a and prevented its binding to the pro-apoptotic Bim promoter [148]. Thus, in granulosa cells, FSH-dependent activation of Akt regulates apoptosis [148] and cell differentiation [149]. In contrast, in cumulus cells, FSHstimulated phosphorylation of Akt and MAPK participate in the maintenance of cell survival (Figure 2) and the synthesis and retention of hyaluronic acid [150]. The expression and activity of FOXO1 are modulated by FSH and LH through the PI3K/Akt pathway [151] and FOXO1 expression is elevated in granulosa cells of growing follicles. Expression of a constitutively active nuclear form of FOXO1 in granulosa cells reduces cyclin D2, aromatase P450 (CYP19a1), FSH receptor, LH receptor and enzymes of the cholesterol biosynthetic pathway [147]. Following ovulation and luteinization, FOXO1 expression is reduced, and the most intensive FOXO1 staining is seen in the nucleus of granulosa cells of atretic follicles. This finding suggests a causal relationship between follicular atresia and increased nuclear FOXO1. In addition, FOXO1 is negatively regulated by Wnt signalling [152]. Unlike other FOXO members, FOXO4-null mice are viable and have no developmental defects or histological abnormalities suggesting functional redundancy in this subfamily. The PI3K/Akt pathway in the mammalian ovary is subject to regulation by the TGFβ superfamily which includes factors derived from granulosa (activin, BMP-2, -5 and -6) and theca cells (BMP2, -4 and -7) that are involved in the regulation of granulosa cell proliferation and follicle survival and apoptosis [120]. Recent data support a link between BMP signalling, PI3K/Akt and the small GTPase (Ras)/ERK pathways in the regulation of cell survival. In particular, it has been found that BMP-4 suppresses the apoptosis of granulosa cells by maintaining CAD protein (a 'fusion' protein encoding four enzymes of the pyrimidine pathway [152]) in the cytosol instead of the nuclei, where the protein triggers DNA digestion.

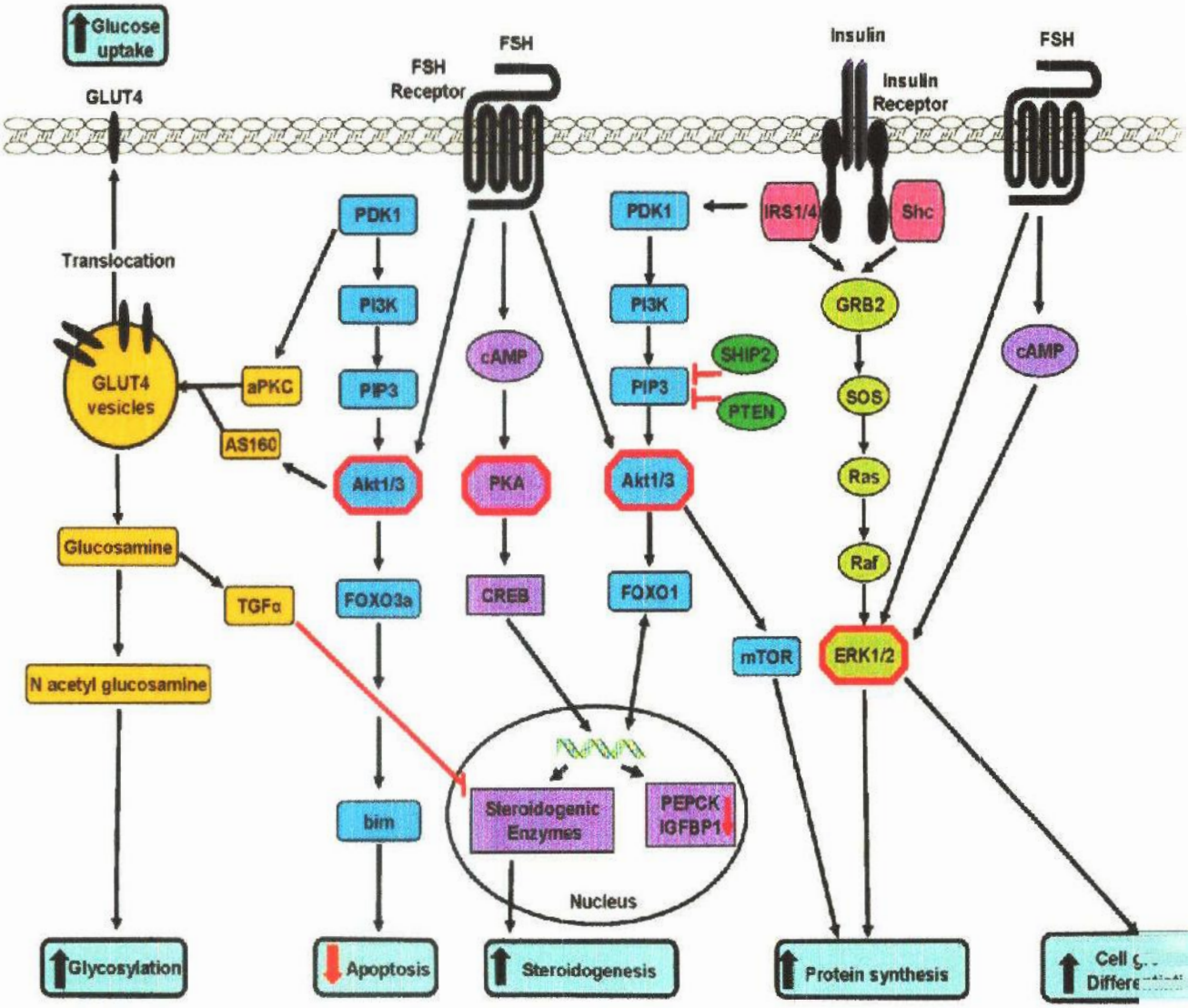


**Figure 20: Some possible sites of cross-talk (critical nodes) between the insulin and FSH signaling pathways in granulosa cells.**

**Ref:** Attia, G.R., Rainey, W.E. and Carr, B.R. (2001) Metformin directly inhibits androgen production in human thecal cells.

Insulin signalling through Akt1 and FOXO1 can enhance FSH-stimulated steroidogenesis and, similarly, FSH acting through Akt1 can enhance insulin-stimulated glucose transport leading to increased flux through the hexosamine pathway and to the inhibition of aromatase. Thus insulin can interact with FSH to stimulate P4 and inhibit E2 production. Likewise, FSH acting via mTOR and/or ERK can enhance cell proliferation and protein synthesis, cell proliferation and, acting via Akt1 and FOXO3a, it can inhibit apoptosis. The FSH-stimulated FOXO1 pathway appears to have stage -specific effects in follicles; in small follicles granulosa cell proliferation is stimulated, in antral follicles it is steroidogenesis that is stimulated and, at ovulation, atresia is stimulated in non-ovulatory follicles. Stimulatory connections are shown in black and inhibitory ones are shown in red.

#### 4.10.4.2: Theca cells

Because LH and insulin act physiologically via distinct intracellular signalling mechanisms, their synergistic enhancement of theca-cell steroidogenesis likely entails important interactions between these two respective pathways (Figure 3). Indeed, it has been shown that insulin significantly increased LH-stimulated cAMP accumulation in cultured porcine theca cells [153]. The insulin-stimulated increase in cAMP is probably induced through PI3K because the activation of adenylate cyclase by insulin was blocked with the use of non-specific inhibitors of PI3K in rat muscle. In addition, LH induced rapid activation of JAK2 in the rat ovary that then activated, through IRS-1, both the PI3K and the MAPK pathways [154]. Simultaneous stimulation with LH and insulin induced higher phosphorylation levels of these proteins compared with each hormone alone. Therefore, important cross-talk exists between the insulin and LH signalling pathways, such that increased activity of one pathway might also increase the responsiveness of the other pathway (Figure 3). A study on normal human theca cells demonstrated that specific blockade of PI3K, but not of MEK, inhibited the stimulating effect of insulin and LH on ovarian cytochrome P450, 17α-hydroxylase/17,20 lyase (CYP C17-lyase) [155]. Another study confirmed these results in theca cells from normal and PCOS women and also constitutively reduced levels of phosphorylated MEK and ERK in PCOS cells. These constitutive defects were correlated with increased androgen production, irrespective of the presence of insulin [156]. Infection with dominant-negative MEK1 increased CYP C17-lyase mRNA, whereas constitutively active MEK1 reduced CYP C17-lyase mRNA. This suggests that alteration of the MAPK pathway can induce androgen hyper-responsiveness to insulin in PCOS ovaries.

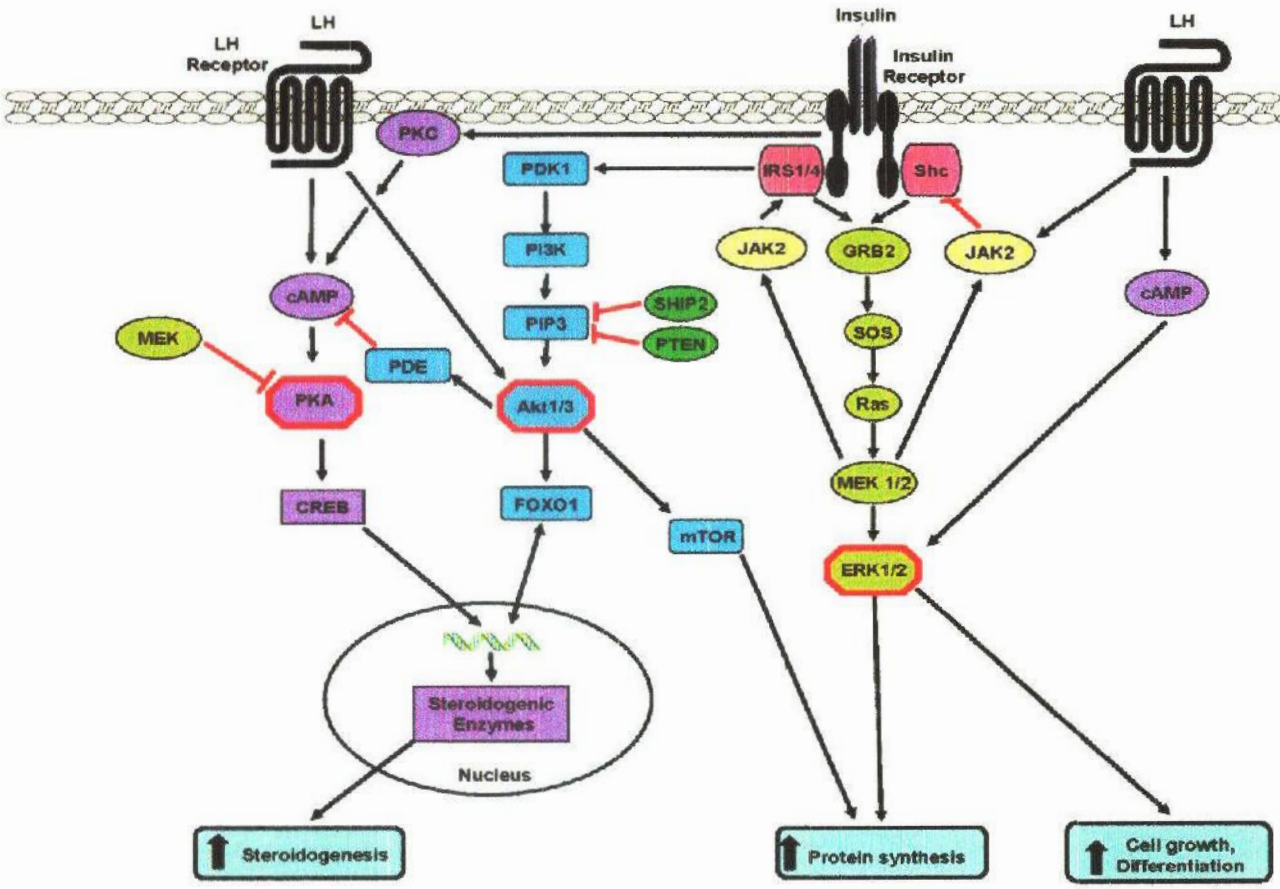


**Figure 21: Some possible sites of cross-talk (critical nodes) between the insulin and LH signaling pathways in theca cells.**

**Ref:** Attia, G.R., Rainey, W.E. and Carr, B.R. (2001) Metformin directly inhibits androgen production in human thecal cells.

Insulin signaling through Akt1 can stimulate PDE to inhibit the degradation of cAMP and so enhance LH-stimulated androgen production; similarly, insulin acting via Akt1 and FOXO1 can enhance LH-stimulated steroidogenesis. Acting via mTOR and/or ERK, LH can enhance cell proliferation and protein synthesis and it can inhibit apoptosis. LH acting directly or via cAMP can enhance insulin stimulation of ERK, whereas, at the same time, LH can stimulate JAK2 which inhibits Shc activation of ERK. The balance of these opposing actions can modulate insulin-stimulated cell proliferation and protein synthesis. Stimulatory connections are shown in black and inhibitory ones are shown in red.

## 4.11: Oocyte maturation

In rodents and humans, elevated levels of cAMP are necessary to maintain oocytes in meiotic arrest, and phosphorylated Akt and the mRNA for Akt have been detected in GV (germinal

vesicle) stage oocytes [157]. The processes of ovulation, luteinization and oocyte maturation are triggered by the LH surge when this gonadotropin induces some elaborate signalling cascades that activate specific intracellular substrates. The LH surge stimulates PI3K/Akt [158–160]. High activity of Akt is necessary to phosphorylate PDE3A (cAMP-phosphodiesterase 3A) that specifically degrades cAMP [161] during the early stages of oocyte maturation to activate either PDE3A [158] or the downstream mTOR pathway, which phosphorylates factors promoting protein synthesis [162]. Suppression of Akt decreased CDK1 and delayed the resumption of meiosis in mouse oocytes [162]. Following germinal vesicle breakdown, phosphorylated kinases are associated with microtubules of the first meiotic spindle [157,162]. In vitro experiments clearly indicated that inhibition of Akt inhibits MI itself, and, at MII, Akt controls the extrusion of polar body 2 and normal chromosomal alignment on microtubules [159]. This role was confirmed by treatment with LY294002 that decreased phosphorylated Akt-Thr308, altered localization of phosphorylated Akt-Ser473 during oocyte maturation and impaired extrusion of the polar body [159]. In cattle oocytes, Akt was involved in the regulation of meiotic MI–MII transition [160] and furthermore spindle migration and the asymmetric division during meiotic maturation are controlled also by mTOR [163]. Comparison of the levels of mRNA for Akt1 during in vivo maturation showed a significant decrease in Akt1 mRNA from GV to MII and the level of expression can be differentially modulated by FSH [157]. At MII, phosphorylated Akt is still expressed and its role may be to support oocyte survival, at least during the fertilization window. The findings that Akt 1, 2 and 3 mRNA levels are very low in oocytes retrieved from ampullae 29 h post hCG and that protein content was dramatically decreased by 33 h post hCG confirm this hypothesis [164]. These data confirm the importance of PI3K/Akt pathway in oocyte maturation and indicate key roles for FSH and IGF-I, but, apart from being theoretically possible, tell us little of the involvement of insulin in oocyte maturation.

## 4.12: Intracellular Signaling in Oocytes for Maintenance of Dormancy of Primordial Follicles

### 4.12.1: PTEN

Phosphatase and tensin homolog deleted on chromosome 10 is a negative regulator of PI3K. In the absence of PTEN, 3,4,5-triphosphate (PIP3) increase leads to protein kinase B (Akt) signaling. The role of PTEN in primordial follicles was elucidated by a study using oocyte-specific *Pten* deleted mice (*OoPten*$^{-/-}$mice) [205].

**tuberin/tuberous sclerosis complex 1,2; S6K1, p70 ribosomal protein S6 kinase 1; rpS6, S6K1-ribosomal protein S6; p27, cyclin-dependent kinase inhibitor $p27^{Kip1}$(Modified from Reddy et al. [22]).**

**Ref:** Attia, G.R., Rainey, W.E. and Carr, B.R. (2001) Metformin directly inhibits androgen production in human thecal cells.

### 4.12.2: TSC (tuberin/tuberous sclerosis complex)

Mammalian target of rapamycin (mTOR) complex 1 (mTORC1) is negatively controlled by a heterodimeric complex consisting of two molecules: Tsc1 (*hamartin*) and Tsc2 (tuberin).

TSC1 and TSC2 are products of *TSC1* and *TSC2* genes, respectively. The *TSC1* and *TSC2* gene mutations occur in autosomal dominant tumor syndrome, tuberous sclerosis [208]. Tsc 1 is considered to be important for maintenance of the dormancy of primordial follicles. In mice with Tsc1 deleted in oocytes ($OoTsc1^{-/-}$ mice), primordial follicles were prematurely activated, which also occurs in $OoTsc2^{-/-}$ mice [209]. These results imply that Tsc1/Tsc2 complex plays a role in regulating the quiescence and activation of follicles. In Tsc1 or Tsc2 deletion, mTORC1 increases and activates S6K1-rpS6 signaling (p70 S6 kinase-ribosomal protein), which promotes protein translation and ribosome synthesis (Figure 1) [210]. The phenotypes of over activation of primordial follicles in $OoTsc1^{-/-}$ mice and $OoPten^{-/-}$ mice are similar. Although both Tsc and PTEN regulate follicular activation by inhibiting S6K1-rpS6, Tsc inhibits phosphorylation of S6K1 at threonine 389, and PTEN inhibits the phosphorylation at threonine 229 [210]. Therefore, for the maintenance of the dormancy of primordial follicles, S6K1 should be suppressed by Tsc and PTEN, respectively.

### 4.12.3: *Foxo3a*

*Foxo3a* is expressed in the nucleus of oocytes of primordial follicles, and its expression decreases in primary or growing follicles. The decrease of *Foxo3a* initiates the activation of follicles and growth of oocytes. In *Foxo3a*-deleted mice ($Foxo3a^{-/-}$), follicles are depleted, which results in infertility, indicating that *Foxo3a* is important for maintaining the quiescence of primordial follicles [211]. $Foxo3a^{-/-}$ mice show a phenotype similar to that of $Pten^{-/-}$ mice. In the *Pten* deleted mice, constant Akt activation causes the suppression of *Foxo3a*.

Therefore, both PTEN and *Foxo3a* play important roles in maintaining the dormancy of primordial follicles and PTEN acts upstream of *Foxo3a* [212]. Akt inactivates *Foxo3a* through

an extracellular signaling pathway such as insulin signaling, so the effect of insulin for follicular activation is by the inhibition of *FOXO3* [213-218].

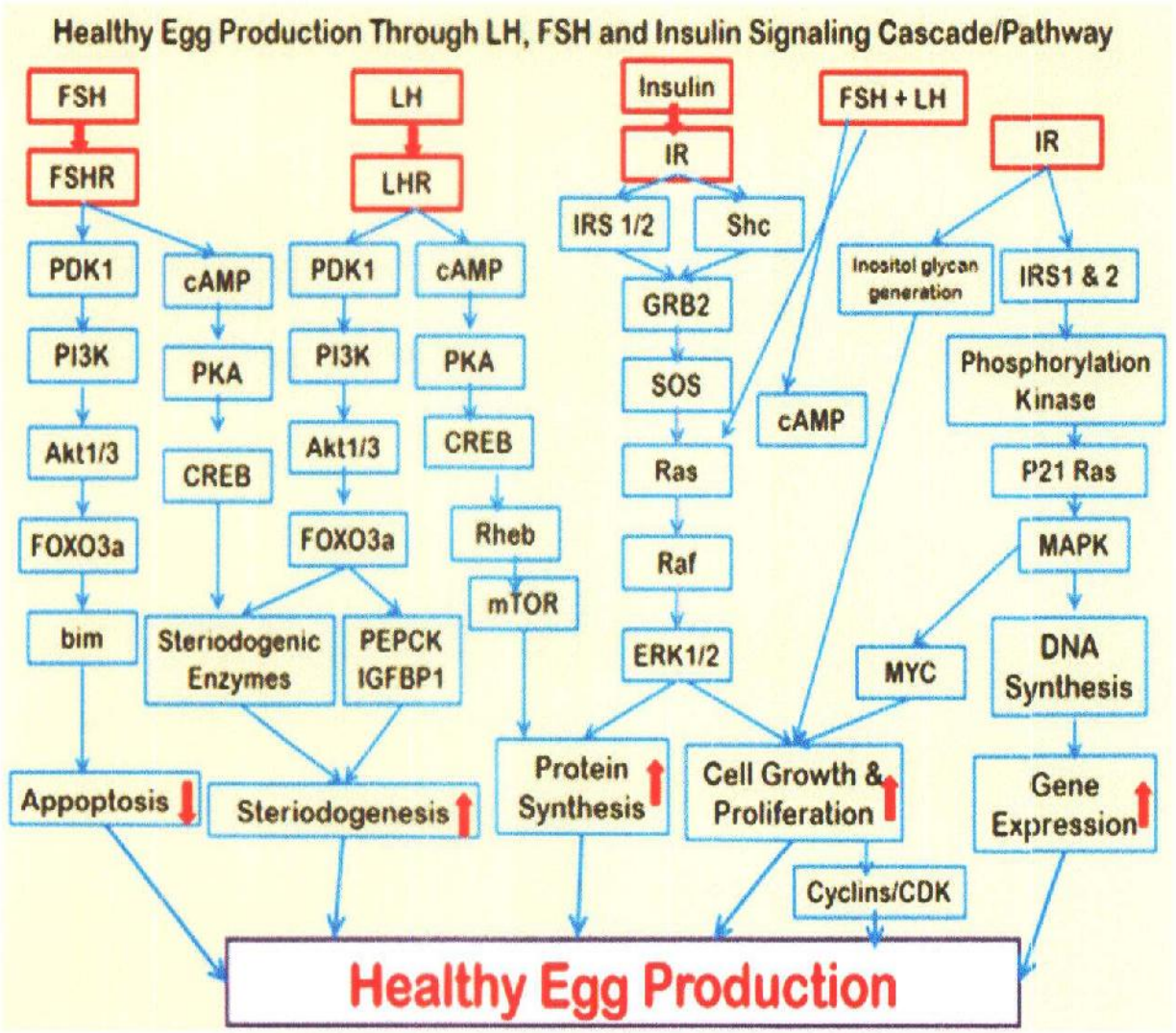


**Figure 23: Healthy egg production through LH, FSH and insulin signaling pathway**

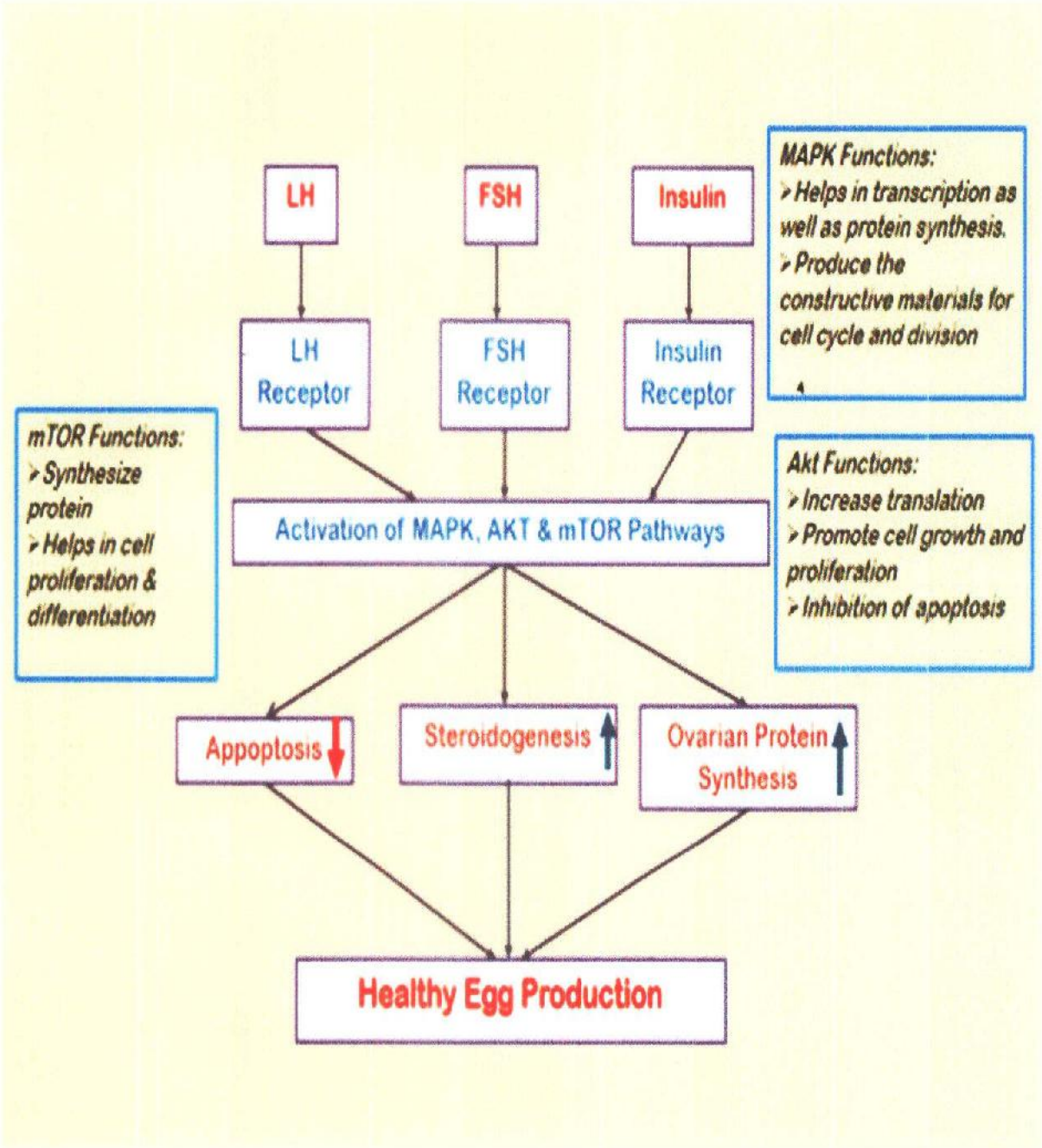


**Figure 24: An Overall view of healthy egg production**

# CHAPTER 5

# DISCUSSION AND CONCLUSION

While there is ample evidence to support insulin as a key regulator of reproductive function, current knowledge of its neural actions with respect to reproduction is in many instances incomplete and rudimentary. Insulin is clearly an important regulator of pulsatile and surge GnRH/LH secretion, but whether these effects of due to insulin, per se, or whether changes in accompanying peripheral signals and metabolites may be involved, [56]remains to be determined. Insulin appears to play a primarily permissive role in the control of pulsatile GnRH secretion, and those effects are due to different aspects (amplitude vs. timing) of the generation of the GnRH/LH surge responsible for ovulation.

There is much epidemiological and experimental evidence to suggest a role for insulin in fetal programming of the metabolic and reproductive axes, [58] but it is not known whether these long-term effects are due to primary actions on the developing brain. Recent preliminary evidence in the sheep suggests that there may be a convergence of insulin and gonadal hormones early in development responsible for programming of reproductive neuroendocrine circuitry. Specifically, co-treatment of insulin sensitizer blocked the effect of prenatal testosterone on arcuate AgRP cell number in female sheep hypothalamus suggesting that a common mediator involving both insulin and androgen signaling is responsible for the prenatal programming of this hypothalamic circuitry. However, again, whether these effects are due to primary actions of insulin on the developing brain, or due to effects on maternal or placental function, remains to be explored.

At a neural level, the specific brain targets of insulin have been examined which may be involved in relaying its influence in reproduction: these include GnRH neurons, the final common pathway mediating control of the hypothalamic-pituitary gonadal axis, as well as upstream neurons, such as those containing the neuropeptides, kisspeptin, AgRP, and POMC. While data is mixed as to whether insulin receptors are present in GnRH neurons, there is clear evidence of their presence in the other cell types. While kisspeptin neurons may not by themselves be critical components in insulin's effects on reproduction [146], insulin receptors in POMC neurons may be more important since deletion of IR leads to adult female reproductive deficits [150], and the contribution of AgRP neurons to this influence have yet to be specifically investigated. Multiple anatomical interconnections among these neuronal subpopulations, however, suggest that they may comprise a redundant network that mediates insulin's reproductive actions upon GnRH neuronal activity and neuroendocrine output [156].

Finally, clinical evidence clearly implicates insulin deficiency/resistance in adult human female fertility, but whether these effects are due to primary actions upon reproductive neuroendocrine circuitry, or are exerted at the level of the pituitary or gonads, is not known. Further, while there is growing consensus of the importance of insulin signaling in the central control of reproduction, well-defined experimental models are needed both in the adult and development nervous system to determine insulin's mechanisms of action independent of associated changes in metabolic signals. The ability to selectively manipulate components of insulin signaling in a cell-specific manner within defined neuronal subpopulations by transgenic approaches [138] presents such an opportunity, but will also need to coupled with careful and detailed physiological models of adult reproductive function in order to ensure the effective clinical translation of this knowledge in the future.

The action of insulin and insulin-mediated uptake of glucose on ovarian cells is of importance for animal production (dairy cattle, small ruminants and pigs) as well as to some fields of human reproductive medicine. It is evident from the available literature that the function of the ovary is subject to some form of local or direct modulation by the insulin and insulin/glucose systems. Although the gonadotropins, LH and FSH, are the primary regulators of terminal folliculogenesis, early folliculogenesis can proceed in the absence of the gonadotropins. Furthermore, in the later gonadotropin-dependent stages of development, this process can be modulated by numerous external influences, insulin being one of them. Likewise, the converse is also possible, that is, the gonadotropins can also modulate the action of insulin in follicular cells. Thus in granulosa cells, insulin signalling through Akt1 and FOXO1 can enhance FSH-stimulated steroidogenesis, and similarly, FSH acting through Akt1 can enhance insulin stimulated glucose transport leading to increased flux through the hexosamine pathway and to the inhibition of aromatase [177, 178]. Likewise,
FSH acting via mTOR and/or ERK can enhance cell proliferation and protein synthesis, and FSH acting via Akt1 and FOXO3a can inhibit apoptosis. In theca cells, insulin signalling through Akt1 can stimulate PDE to inhibit the degradation of cAMP and so enhance LHstimulated androgen production, similarly insulin acting via Akt1 and FOXO1 can enhance LH-stimulated steroidogenesis. Acting via mTOR and/or ERK, LH can enhance cell proliferation and protein synthesis and can inhibit apoptosis. LH acting directly or via cAMP can enhance insulin stimulation of ERK, while, at the same time, LH can stimulate JAK2 which inhibits Shc activation of ERK. The balance of these opposing actions of LH could modulate insulin-stimulated cell proliferation and protein synthesis in ovarian cells. Folliculogenesis is a dynamic process, and the effects of the gonadotropins on folliculogenesis are stage-dependent

and this stage-dependence is likely to also apply to the actions of insulin and to the efficacy of insulin-sensitizing treatments. For example, the FSH-stimulated FOXO1 pathway appears to have stage-specific effects in follicles: in small follicles, granulosa cell proliferation is stimulated; in medium-sized antral follicles, it is steroidogenesis that is stimulated; whereas, at ovulation, atresia

is stimulated in the atretic non-ovulatory follicles. However, the FOXO1 pathway is but one example and the picture is far from complete, and we neither know nor understand all of the regulatory mechanism involved in the stage-specific effects of insulin during the continuum of folliculogenesis.

All signaling pathways have on or off switch. Now, it is necessary to discuss the negative activation of the whole process. Akt activates mTOR. mTOR is a protein that helps in the activation of so many transcription factor (TF). TF sit on a promoter in the nucleus and then transcription of several target genes. Akt work together with mTOR along with some other accessory proteins like Tsc1 and Tsc2. Tsc1 and Tsc2 blocks another protein like Rheb which can activate mTOR. Akt can not directly activate mTOR only Rheb can. Tsc1 and Tsc2 play an inhibitory role in Rheb. Inside the cell Rheb is inactivated or turned off by Tsc1 and Tsc2. Akt inhibits this Tsc timers, Then Rheb is free. 4EBP1 is inhibiting the protein gene factor in transcription process. Action of 4EBP1 is halted by mTORC1.

Activation of RAS protein activates MAPK kinase. RAS is usually inactive when it is bounded with GDP. GRB2 with SOS can activate RAS by substituting the GDP with GTP. RAS is very important mediator; once RAS is activated all of the downstream mediators will be activated sequentially. After the activation of RAS there is a cycle of inactivation also. [203,204]The way is to hydrolysis of GTP into GDP, then inorganic phosphate is released. Then RAS attached with GDP which becomes inactivated. In RAS protein mutation, GTP can bind with RAS permanently, in that cases the RAS will not revert into the inactivate form. The whole process signaling pathway remain on, it can converts the cell into cancerous one [211].

So it is shown the overall process in the figure of the next page that , a modulatory effect of Luteinizing hormone (LH) and Follicle Stimulating Hormone (FSH) on the insulin gene expression that could have been playing role on healthy fertility.

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## It could be concluded that

- I have got MAPK, Akt, mTOR type of pathways that are being activated insulin through the modulation of FSH and LH

- I have got some effectors which take part in activating pathways leading to healthy egg production

## It should be recommended that

- Additional pathways can be identified which are involved in healthy egg production
- Supplementary effectors can be recognized which leads to produce healthy egg

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