# FSH promotes progesterone synthesis and output in human granulosa cells without luteinization

**by**

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This is to certify that I have examined this copy of a master's thesis by

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and have found that it is complete and satisfactory in all respects,
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**ABSTRACT**

In the modern era of assisted reproductive technologies, pituitary suppression with gonadotropin releasing hormone (GnRH) analogues has drastically reduced the incidence of premature luteinizing hormone (LH) surge to less than 2% per stimulated in vitro fertilization (IVF) cycle. [1] Nevertheless, subtle elevations in serum progesterone (P) level during late follicular phase may still occur without LH surge in up to 35% and 38% of the stimulated IVF cycles with GnRH agonist and antagonist protocols, respectively. [2-5]

The premature rise in serum P before ovulation trigger may reduce the success of pregnancy in stimulated IVF cycles by advancing endometrial histology and impairing endometrial receptivity when fresh embryo transfer (ET) is performed. [5-9] It is well-documented that serum P level at the time of human chorionic gonadotropin (hCG) administration is significantly correlated with the magnitude of ovarian response to stimulation (antral follicle count ≥ 14mm, estradiol level at the time of hCG administration and the number of eggs retrieved). [5, 6, 10-12]

Even though the underlying mechanism of this event is obscure its almost exclusive occurrence in stimulated but not in natural IVF cycles raises a fundamental question as to whether FSH stimulation itself might be responsible for P output from granulosa cells without luteinization. To address this issue we designed an experimental study and investigated 1) whether FSH stimulates granulosa cell progesterone output by upregulating the expression of 3β-hydroxysteroid dehydrogenase (3β-HSD); the enzyme that converts pregnenolone to progesterone, and 2) whether FSH induces proportional increases in the expression of ovarian steroidogenic enzymes (StAR, SCC, 3β-HSD, 17α-OH, 17β-HSD and aromatase).

To this end, non-luteinizing, mitotic and FSH-responsive human granulosa cells (HGrC1) and human ovarian tissue samples were stimulated with recombinant FSH at different concentrations and time points. To mimic the ovarian micro-environment in the ovary in stimulated IVF cycles, FSH was used at concentrations corresponding to its endogenous levels in the sera of IVF patients undergoing ovarian stimulation with rec-FSH. [13-15] FSH induced changes in the enzymatic activity of 3β-HSD were measured, and the expression of the ovarian steroidogenic enzymes were analyzed by quantitative real-time PCR and western blot. FSH responsiveness of these enzymes

was further investigated after FSH receptor was up-regulated with activin-A, and down-regulated with shRNA technology.

This thesis provides a molecular explanation for the largely unexplained phenomenon of P rise during the follicular phase of gonadotropin stimulated IVF cycles prior luteinization. Presented findings may pave the research to uncover potential mechanisms for preventing premature P rise that appears to be associated with inferior outcomes in women undergoing IVF.

**ÖZET**

Günümüz üremeye yardımcı tedavi (ÜYTE) uygulamalarında hipofiz bezini baskılamak için kullanılan GnRH analogları prematür LH piki görülme sıklığını IVF sikluslarında %2'nin altına düşürmüştür. [1] Bununla birlikte, geç foliküler fazda LH piki öncesi serum progesteron seviyelerinde artış görülme sıklığı GnRH agonist ile stimüle edilen IVF sikluslarında %35'i ve GnRH antagonist ile stimüle edilen sikluslarda da %38'i bulmaktadır. [2-5]

Serum progesteron düzeylerinde ovülasyon öncesi görülen prematür artış IVF sikluslarında endometriyal histolojiyi ve endometriyal gen ekspresyonlarını değiştirerek reseptiviteyi azaltabilmektedir. Bunun sonucunda da taze embryo transferlerinde düşük implantasyon ve klinik gebelik oranlarıyla karşılaşılmaktadır. [5-9]

Prematür progesteron yükselmesinin doğal sikluslara oranla stimüle sikluslarda görülmesi ve stimülasyonun derecesiyle kuvvetli korelasyon göstermesi bu prematür yükselmeden FSH stimülasyonun direkt olarak sorumlu olduğunu düşündürmektedir (hCG günü estradiol düzeyi, antral folükül sayısı ≥ 14mm ve elde edilen oosit sayısı). [5, 6, 10-12] Bu hipotezi araştırmak amacıyla düzenlenen deneysel dizaynda cevap aranılan sorular şunlardır: 1) FSH uygulaması granüloza hücrelerinde progesteron üretimini 3β-HSD geninin ekspresyonunu yükselterek mi stimüle etmektedir? 2) FSH uygulaması ile ovaryan seks steroidogenez enzimlerinin ekspresyonlarında görülen yükselme korelasyon göstermekte midir?

Bu amaçla lüteinze olmayan mitotik granüloza hücreleri ve over kortikal örnekleri DMEM-F12 kültür medyumu kullanılarak mililitre başına 12.5, 25 ve 50 mIU olmak üzere üç farklı FSH dozu ile farklı zaman aralıklarında inkübe edilmiştir. İnkübasyon periyotları sonunda steroidogenik enzimlerde FSH'a bağlı meydana gelen değişimler mRNA düzeyinde qRT-PCR ile ve protein düzeyinde western blot ile incelenmiştir. *In vitro* $E_2$ ve Progesteron düzeylerini elektrokemiluminesans (ECLIA) methoduyla ölçülmüştür. Son olarak da 3β-HSD'nin protein ekspresyonunun yanı sıra işlevini de gösterebilmek için spektrofotometrik method ile enzimatik aktivitesine bakılmıştır.

Bu tez, gonadotropin stimülasyonu uygulanan sikluslarda foliküler faz sırasında açıklanamayan P yükselmesine moleküler bir açıklama sağlamaktadır. Sunulan bulgular, IVF tedavisi gören kadınlarda prematür P artışını önlemeye yönelik potansiyel mekanizmaları ortaya çıkartmak için yapılacak çalışmalara örnek oluşturabilir.

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**NOMENCLATURE**

| | |
|---|---|
| 3β-HSD | 3β-Hydroxysteroid dehydrogenase |
| 17β-HSD | 17β-Hydroxysteroid dehydrogenase |
| ATP | Adenosine triphosphate |
| ART | Assisted Reproductive Techniques |
| cAMP | Cyclic Adenosine Monophosphate |
| COS | Controlled ovarian stimulation |
| CYP11A1 / P450Scc | Cholesterol side-chain cleavage enzyme |
| CYP19 / P450Aromatase | Aromatase |
| CYP17A1 / 17α-OH | 17α-Hydroxylase |
| ECLIA | Electrochemiluminesence |
| ET | Embryo transfer |
| $E_2$ | Estradiol |
| FSH | Follicle Stimulating Hormone |
| FSH-R | Follicle Stimulating Hormone Receptor |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GnRH | Gonadotropin Releasing Hormone |
| GPCR | G Protein Coupled Receptors |
| hCG | Humon Chorionic Gonadotropin |
| HGrC1 | Human non-mitotic luteinized granulosa cells |
| HPO | Hypothalamus – Pituitary – Ovary |
| IVF | In vitro fertilization |
| LH | Luteinizing Hormone |
| NADH | Nicotinamide adenine dinucleotide |
| P | Progesterone |
| PKA | Protein Kinase A |
| qRT-PCR | Quantitative real time polymerase chain reaction |
| rec-FSH | Recombinant FSH |
| shRNA | Short Hairpin RNA |
| StAr | Steroidogenic acute regulatory protein |
| ÜYTE | Üremeye yardımcı teknikler |

*To mom and dad…*

# Chapter 1 – REVIEW OF LITERATURE

## 1. FOLLICULOGENESIS

Folliculogenesis corresponds to maturation of ovarian follicle. This continuous event begins as early as embryonic development and ends with menopause when primordial follicle reserve depletes. At each menstrual cycle, a cohort of follicles are recruited for further growth. Among this, only one follicle is selected as dominant one. Dominant follicle development follows the order of primordial follicle, primary follicle, antral follicle and ovulatory Graafian follicle (Figure 1).

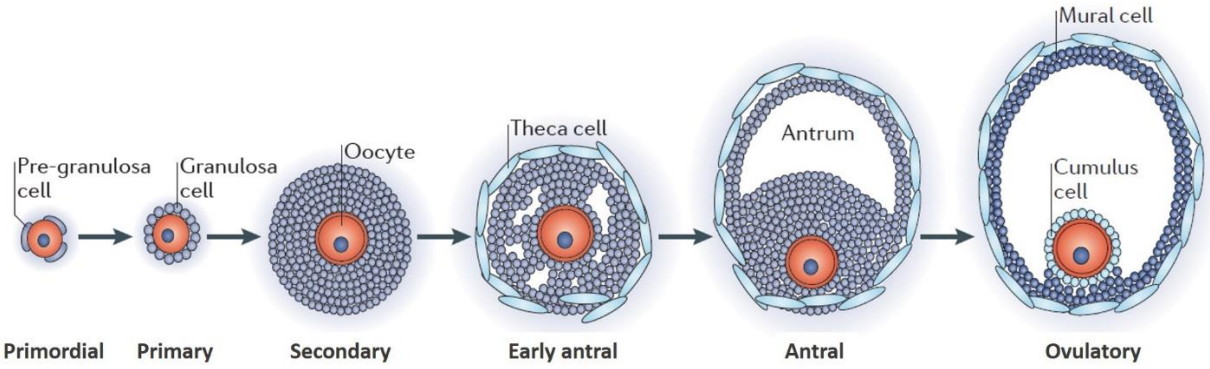



**Figure 1:** Follicle developmental stages. [22]

Primordial follicle pool develop within the ovaries during fetal life from migrated primordial germ cells and it determine the reproductive life span of women. They are characterized by diplotene arrested oocytes with 30 – 60 micrometers in size. [17] Flattened single layer pre-granulosa cells surround the diplotene oocytes. Until puberty, primordial follicles rest without any developmental activity. During puberty, with the increased pulsatile secretion of GnRH by hypothalamus, FSH and LH levels increase in the body which in turn stimulates ovaries to break the dormant state of primordial follicles.

As primordial follicles develop into primary follicles, flattened pre-granulosa cells become one-to-two layers of cuboidal granulosa cells with basement membrane surrounding them. [18] The oocytes in the primary follicles grows further than 60 micrometer in diameter size and zona pellucida begins to form. Even though FSH receptors are present on primary follicles, this stage of follicle growth is gonadotropin independent. [19]

Primary follicles develop into secondary and antral follicles. Follicle diameter increases from 60 micrometers up to 120 – 150 micrometers at the early antral stages. [17] When diameter size reaches to 200 micrometers follicle growth proceeds into antral stage. Zona pellucida formation and multilayer granulosa cell proliferation follows oocyte enlargement. [20] Stromal cells condenses around basal lamina for theca cell layer formation. The fluid-filled spaces in the early antral stages will coalesce to form a single antral cavity in the antral follicle.

Following antral development, both granulosa and theca cell layer proliferate along with oocyte growth. Highly vascularized theca externa provides systemic endocrine factors to follicles. [18] Follicle development after this stage becomes gonadotropin dependent.

Follicle development ends with ovulatory/Graafian follicle formation. [18] Graafian follicles are characterized by fully developed granulosa and theca cells and they can be found up to 25 mm diameter size. In the ovulatory follicle, the granulosa cells differentiate into two types; cumulus and mural granulosa cells. Mural granulosa cells surround the follicle while cumulus granulosa cells stay in a close interaction with growing oocytes. [17] After LH surge the oocyte inside the Graafian follicles is released from the ovary during ovulation and the remained ruptured follicle is called corpus luteum which will become the major progesterone source during luteal phase. [21]

## 2. MENSTRUAL CYCLE

Cyclic maturation of follicles is called menstrual cycle. Even though folliculogenesis is a continuos event of this cycle it is not the only one. Rather, menstrual cycle is the result of coordinated events that takes place in hypothalamus, pituitary, ovaries and endometrium to regulate follicle development in the ovaries (Figure 2). [23, 24] The cycle corresponds to time interval between two menses and it has been indicated to have a range between 25 – 35 days with a median length of 28 days. [23, 25]

Menstrual cycle can be divided into three phases as follicular phase, ovulation and luteal phase – however some sources accept ovulation as a transition event rather than a phase. Follicular phase starts with the first day of the menses and includes the recruitment of follicle cohort and selection and growth of dominant follicle. [21] Endometrial proliferation follows folliculogenesis with respect to increasing ovarian hormone levels. Following LH surge and ovulation of dominant

follicle, luteal phase begins during which empty follicle takes the form of corpus luteum to secrete progesterone in order to prepare endometrium for a possible pregnancy. Menses occurs in the absence of pregnancy, endometrial lining is shed and ovaries are prepared for new cycle afterwards.

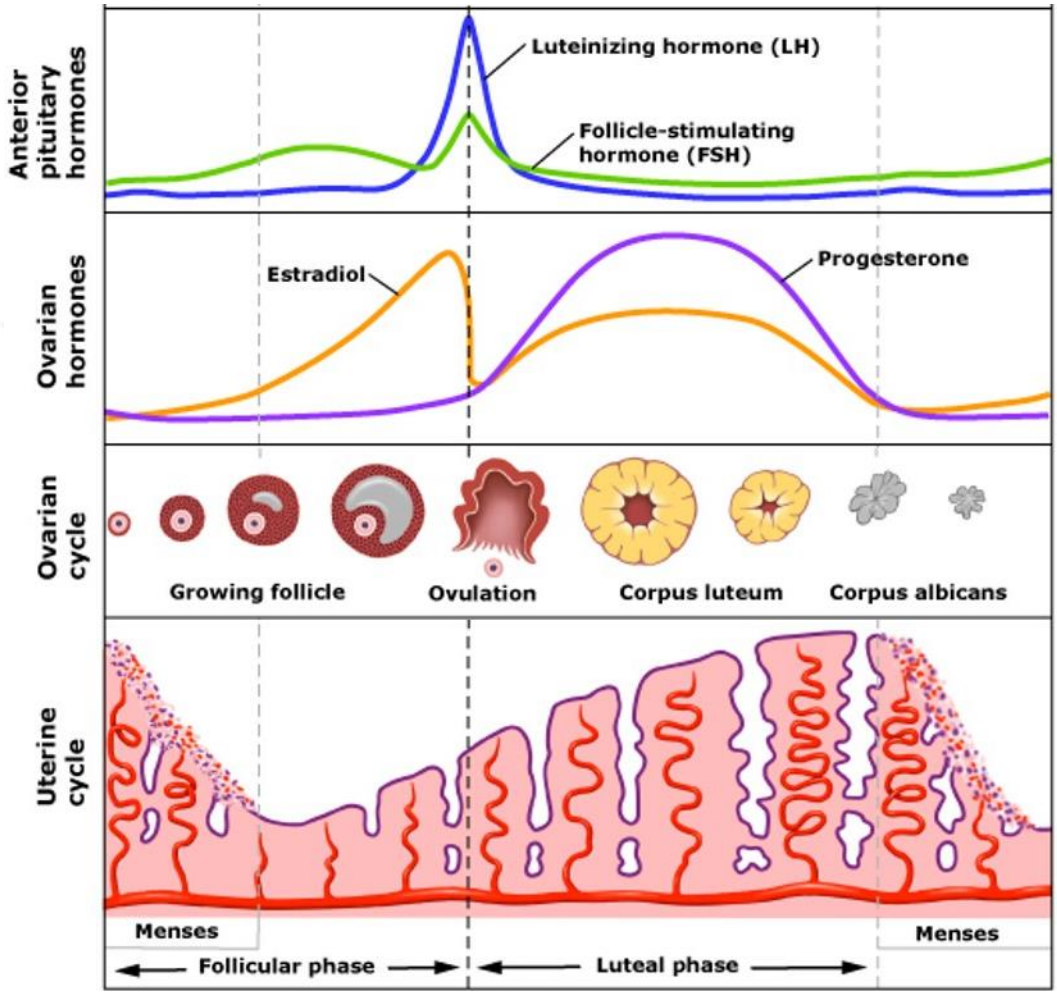



**Figure 2:** Menstrual cycle diagram. [21]

### 2.1 Hypothalamus – Pituitary –Ovary (HPO) axis during menstrual cycle

One of the required elements of menstrual cycle is an intact hypothalamus – pituitary – ovary (HPO) axis whose coordinated act and activation is also the onset of female puberty. [26] The first menstrual bleeding, menarche, results from the increasing secretion of gonadotropin-relasing hormone (GnRH) from hypothalamus.

During childhood, supressed GnRH release from hypothalamus results in minimal secretion of FSH and LH from pituitary, which in turn causes very low circulating levels of these hormones. [27] By

transition into puberty, pulse frequency and amplitude of GnRH secretion increase and activate HPO axis. [28]

Hypothalamic GnRH stimulates anterior pituitary to release FSH and LH. Pulsatile secretion of GnRH increase is followed by gradually increased circulating levels of FSH and LH also which in turn stimulate ovaries for follicle maturation (Figure 3). [27] While early follicle development is gonadotropin independent, follicules become more sensitive to gonadotropins as they grow further into antral stage. [17] Dominant follicle possesses more FSH receptors which makes it more sensitive to FSH, thus, it does not get affected by the falls in FSH levels during mid-follicular phase. [29] As the dominant follicle enlarges, granulosa cells produce estradiol and progesterone which are responsible for proper endometrial lining proliferation. Gonadotropin dependency of follicles changes from FSH to LH during the course of folliculogenesis when dominant follicle starts to acquire more LH receptor. Increased sensitivity to LH enables dominant follicle to proceed its growth and hormonal output during late follicular phase. [30, 31]

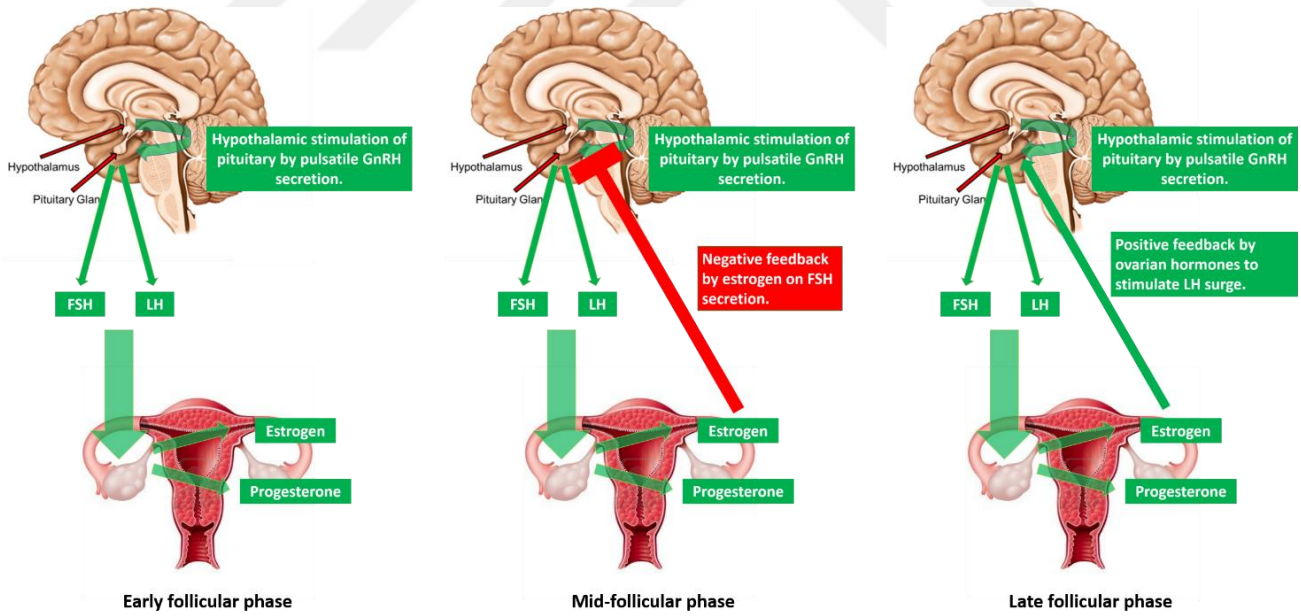



**Figure 3:** HPO Axis during menstrual cycle.

Estradiol ($E_2$) is the main director of the ovarian feedback mechanism on HPO axis both negatively and positively. During early follicular phase, estradiol concentrations in circulation increase in response to ovarian stimulation by FSH and LH. Mid-luteal phase is characterized by a fall in FSH

levels which is caused by estradiol negative feedback mechanism. By lowering the circulating FSH, estradiol helps dominant follicle selection which is based on FSH sensitivity; when there is lower FSH in circulation, only the most sensitive follicle will be able to continue its follicular growth. As dominant follicle progress into ovulatory Graafian stage, both theca and granulosa cell numbers increase. At that point, increased estradiol secretion from ovaries along with progesterone exert a positive feedback on pituitary for FSH and LH secretion which causes a peak in LH hormone secretion. This is called LH surge and ovulation occurs in response to LH surge. [32-36]

Luteal phase follows ovulation, during which remaining granulosa cells enlarge and acquire lutein. [37] Thus, follicle in this stage is called corpus luteum. During early luteal phase corpus luteum becomes the primary place for progesterone secretion. Increased progesterone secretion from corpus luteum is followed by decreased LH pulse and this inverse correlation stays still during luteal follicular transition. [38]

Progesterone secretion prepares endometrium for embryo implantation. If pregnancy occurs, syncytiotrophoblasts secrete hCG to support corpus luteum. Otherwise, corpus luteum deforms into a scar form, leading to a decrease in progesterone synthesis which destabilizes endometrial lining and cause menses. [37]

### 2.1.1 Two-cell-two-gonadotropin theory

Two gonadotropins FSH and LH stimulates two different cells of ovary. While FSH stimulates granulosa cells, LH found to be acting on theca cells (Figure 4). [39-43] Theca layer in the antral follicle produces androgens with exposure to LH. [24, 44] LH stimulation is responsible for ovulation and luteinisation. [45] On the other hand, during the dominant follicle selection process, FSH stimulation of granulosa cells induces both cell proliferation and aromatase expressions. Aromatase, a member of cytochrome P450 superfamily, converts androgens into estrogen in granulosa cells. Thus, FSH stimulation of granulosa cells further drives more estrogen synthesis. Elevated estrogen levels creates negative feedback loop to decrease levels of FSH.

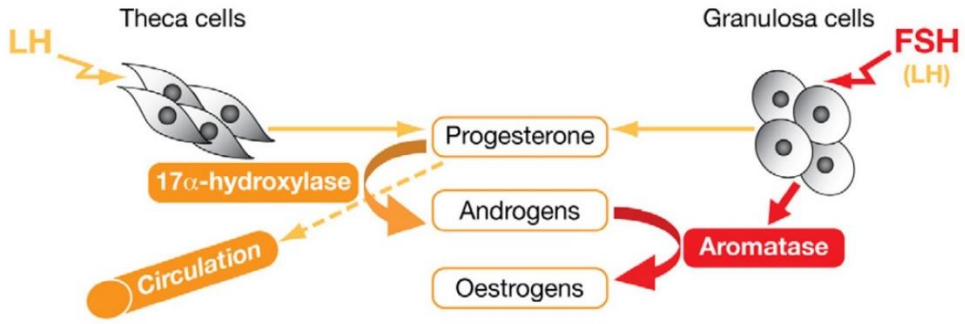



**Figure 4:** Two-cell-two-gonadotropin theory. [46]

### 2.1.2 Ovarian Steroidogenesis

The process of converting cholesterol into biologically active steroid hormones is called steroidogenesis and it takes place in mitochondria.

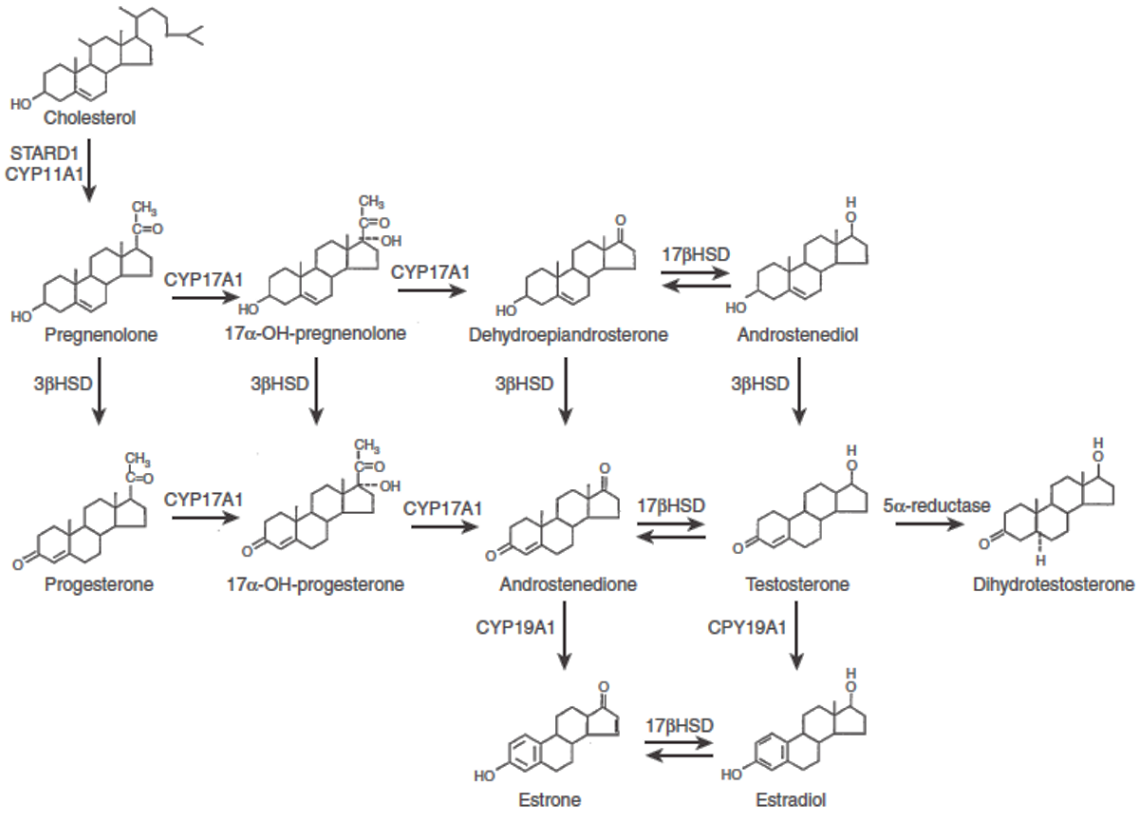



**Figure 5:** Ovarian steroidogenesis pathway. [21]

Steroidogenic acut regulatory protein (StAR) plays the first act by transferring cholesterol from the outer mitochondrial membrane to inner mitochondrial membrane. [47, 48] Within the mitochondria there are two classes of enzymes: cytochrome P450s (CYPs) and hydroxyl steroid dehydrogenases (HSDs). CYPs are oxidative enzymes which absorb light at 450 nm. HSDs can both reduce or oxidize molecules using cofactors nicotinamide adenine dinucleotide/phosphates (NADH/NADHP).

The first step of all steroid hormones synthesis is regulated by side-chain cleavage (P450scc / CYP11A1) enzyme. [49] Cholesterol is taken up from inner mitochondrial membrane and converted into pregnenolone by P450scc. [50] The end-product, pregnenolone can follow two ways: either be converted into 17α-hydroxypregnenolone or progesterone. 3β-hydroxysteroid dehydrogenase (3β-HSD) catalyzes the second conversion by an irreversible oxidation reaction during which $NAD^+$ is reduced into NADH (Figure 6). [51]

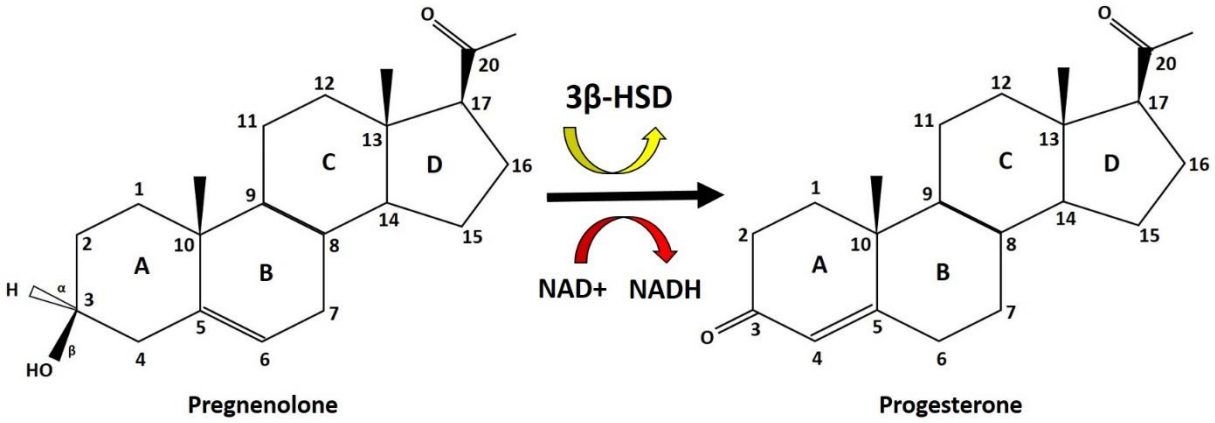



**Figure 6:** Chemical structures of pregnenolone and progesterone and schematic description of the conversion reaction.

Besides converting pregnenolone into progesterone, 3β-HSD mediates androgen synthesis in theca cells, and androgens are converted into estrogens by the actions of P450aro (aromatase – CYP19A1) in granulosa cells.

P450c17 (CYP17A1) enzyme can catalyze both 17α-hydroxylase and 17,20-lyase in gonads yielding 17α-OH Pregnenolone and 17α-OH Progesterone. The conversions of androstenedione into

testosterone in theca cells and estrone into estradiol in granulosa cells are mediated by 17β-hydroxysteroid dehydrogenase (17βHSD) enzyme. [50]

## 3. ASSISTED REPRODUCTIVE TECHNOLOGY IN A BLINK OF EYE: HOW DOES IT WORK?

Assisted reproductive techniques (ART) are used for the treatment of couples that failed to conceive by natural ways. During ART manipulations, gametes are retrieved from patients and after being fertilized outside the body, the embryo is transferred into uterus. For a successful ART cycle, available gametes should be sufficient enough both in quality and quantity. The efficiency of naturally occurring menstrual cycle is not enough for success since only one dominant follicle is ovulated during each cycle. Therefore, ovaries are stimulated for multifollicular development during each ART cycle with a procedure called controlled ovarian stimulation.

### 3.1 Controlled Ovarian Stimulation (COS)

As the quantity of available gametes gain importance for ART applications, more research has been done in obtaining multifollicular development in females. Baird suggested “FSH window” model which explains follicular development beyond 2 – 4 mm depends on exceeding threshold levels of FSH. [52] According to this model as long as threshold level is sustained during folliculogenesis, multifollicular development is possible. In other means, as long as the FSH window is widened i.e. by increasing the exposure time of ovaries to FSH, more follicles will be obtained for ART.

The way to widen FSH window during ART stimulations is to administrate exogenous FSH into female body. Once the circulating FSH levels reach supraphysiological doses via exogenous contribution, ovaries’ response can be observed as multifollicular development. Urinary products of menopausal women were used for exogenous gonadotropin treatments previously. However, they are later replaced with recombinant FSH (rec-FSH), produced in Chinese Hamster Ovary since urinary FSH might also include LH.[53]

While widened FSH window results in multifollicular development, high estradiol secretion from ovaries creates a problem via its positive feedback mechanism. During natural menstrual cycle, rising estradiol levels in the late follicular phase inform hypothalamus about the completion of folliculogenesis (also follicular phase), and hypothalamus responds to this message by stimulating pituitary for LH secretion so that menstrual cycle could proceed into luteal phase via LH peak.

During COS, supraphysiological doses of gonadotropins could cause premature LH peak which directly effects the oocyte pick-up procedure. GnRH analogs, either GnRH agonist or antagonist, are used during COS cycles to prevent premature LH peak by suppressing pituitary gonadotropin secretion either by desensitization and receptor down-regulation (GnRH agonist) or competitive binding to GnRH receptors (GnRH antagonist). [54, 55]

Eventhough premature LH peak can be prevented by using GnRH analogs, COS can still cause some other issues. Elevated serum progesterone levels during late follicular phase without luteinization is one of the most reported problems by several studies. [45] Both serum, follicular fluid and circulating progesterone levels were found to be increased during late follicular phase and showed strong correlation with increased ovarian response to exogenous gonadotropins. [56, 57] In a study involving 4000 patients, higher serum progesterone levels during hCG administration day has been found to be inversely correlated with ongoing pregnancy rates regardless of used GnRH analog type. [58]

Success of the ART cycles comes from live birth rates which is strongly correlated with successfull implantation for which a synchrony between endometrium and follicular development is necessary. Elevated late follicular phase progesterone level following COS has been thought to influence endometrial development and causing an asynchrony between endometrium and embryo. [46, 59] Moreover, advanced endometrial development has been associated with controlled ovarian stimulation. [60]

Based on current evidences, daily FSH doses, number of retrieved oocytes and estradiol showed a strong correlation with elevated serum progesterone levels which is inversely correlated with ongoing pregnancy rates. Even though the underlying mechanism behind this event is unknown, its relation with COS raises question of whether FSH stimulation’s itself is responsible for progesterone output from granulosa cells of ovary without luteinisation.

To address this issue we designed a translational experimental study to investigate;

- whether FSH stimulatation of granulosa cells upregulates the expression of 3β-HSD, the enzyme that converts mitochondrial pregnenolone reversibly into progesterone, for progesterone output

- whether FSH induces proportional increases in the expressions of ovarian steroidogenic enzymes (StAR, P450scc, 3β-HSD, CYP17A, 17β-HSD and Aromatase).

# Chapter 2 - MATERIALS AND METHODS

## 1. Chemicals, Drugs and Reagents

DMEM-F12 culture media, fetal bovine serum (FBS) and Penicillin-Streptomycin Amphotericin B (PSA) Solutions were purchased from Life Technologies (Thermo Fisher Scientific Inc., MA, USA). Gonal-F© (follitropin alpha, Merck Serono, Turkey) was used for recombinant FSH (rec-FSH) treatment. Activin-A was purchased from GFH6, Cell Guidance System. Western blotting gels, membranes, buffers and other supplies were obtained from BioRad. LightCycler 480 SYBR Green I Master was, from Roche Molecular Systems, Inc was used for qRT-PCR. HGrC1 cell line was a kind gift from Dr. Akira Iwase (Nogoya University, Japan). Primary antibodies for western blotting were purchased from Santa Cruz: sc-100466 for 3β-HSD, sc-53423 for Progesterone, sc-376719 for 17β-HSD and sc-376711 for CYP17A1. Aromatase antibody (MCA2077S) was purchased from AbD Serotec. Progesterone antibody LC-C194163, used in immunoprecipitation assay, was purchased from LifeSpan BioSciences, Inc. Enzymatic assay materials NAD (NAD100-RO), NADH (10107735001), Potassium hydrogen phthalate (P1088), Phenazine methosulfate (P9625) and 5-Pregnen-3β-ol-30-one (P9129) were all purchased from Sigma-Aldrich. Nitro Blue Tetrazolium (NSC 27622) was obtained from Cayman Chemical.

## 2. Cell and Tissue Culture

DMEM-F12 culture medium supplemented with 10% FBS and 1% PSA was used during cell and tissue culture. Cells were seeded onto 10 cm culture plates at the density of $1x10^6$ cells/plate and cultured at 37°C with 5% $CO_2$. Ovarian tissue samples were cultured in 24-well plates using 1 ml culture medium at the same incubator settings. Treatment with rec-FSH was performed at the doses of 12.5 mIU/ml, 25 mIU/ml and 50 mIU/ml for 24 hours and 48 hours.

### 2.1. Human mitotic non-luteinized granulosa cells (HGrC1)

HGrC1 is a mitotic, non-luteinized and immortalized human granulosa cell line. Cells were obtained from 35-years-old female and immortalized by lentivirus mediated transfection of several genes. [61] They express steroidogenic enzymes StAR, P450Scc and Aromatase. They can be stimulated by gonadotropin FSH and produce estradiol and progesterone in response to stimulation however they cannot undergo luteinization. Their growth characteristics resemble of mitotic granulosa cells of growing follicles.

### 2.2. Human ovarian cortical tissue

Ovarian cortical samples were obtained from 15 patients undergoing laparoscopic removal of benign ovarian tumors at early follicular phase of their menstrual cycle for dermoid cyst (n=12), and endometrioma (n=3). Obtained cortical samples were embedded to cyst wall and therefore their removal was accidentally. The samples were minced into equal sizes of 0.5x0.5 cm and cultured under mentioned conditions (Figure 7).

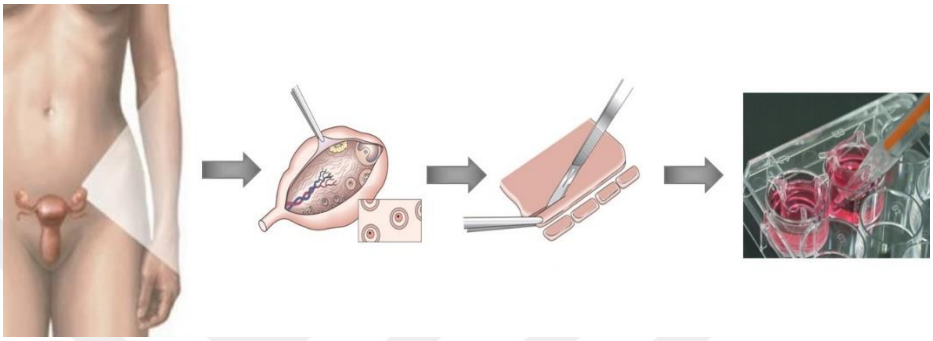


**Figure 7:** Schematic diagram demonstrating preparation of human ovarian cortical samples. [62]

### 3. cAMp-Glo™ Assay

The cAMP-Glo™ Assay (Promega Corporation, Maddison, WI) is a bioluminescent assay that is used to measure cAMP levels within the cells. Intercellular cAMP activity is regulated when the test compound stimulates G protein-coupled receptors (GPCRs). Increased cAMP stimulates protein kinase A (PKA) phosphorylation by ATP. The cAMP-Glo™ assay measures remained ATP level by combining it with luciferin in the presence of oxygen and the effects of the test compound on the cells can be determined by luminescence output (Figure 8). The ATP measure is inversely proportional with cAMP concentrations.

Gonadotropin action is transmitted into the nucleus via adenylate cyclase through cAMP production; and FSH stimulation of granulosa cells stimulates cAMP production.[63] Therefore, rec-FSH was used as the test compound in this assay.

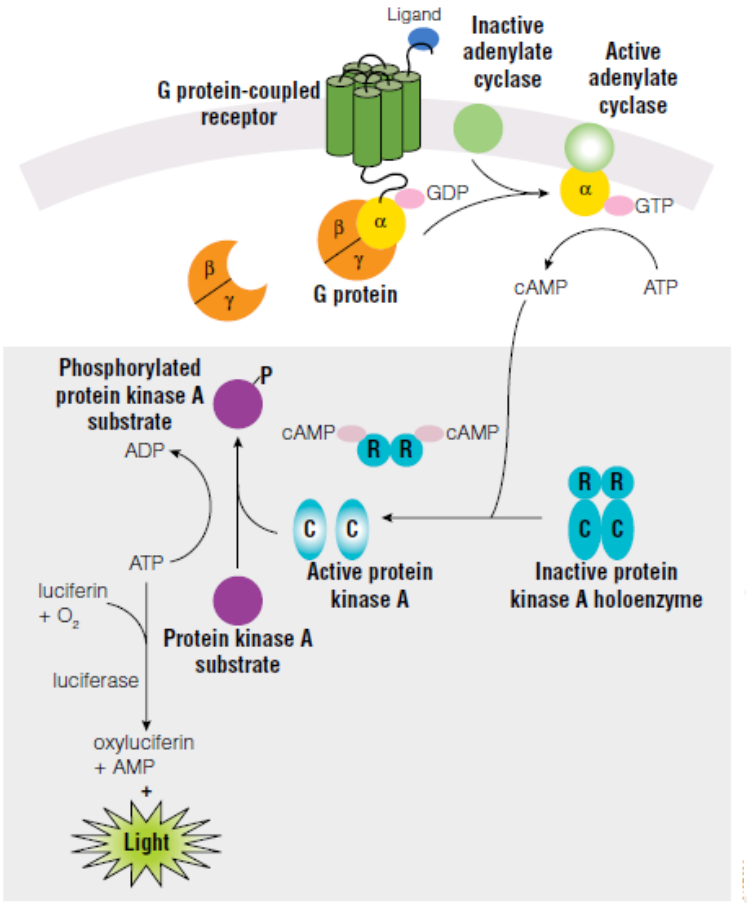



**Figure 8:** Illustration of FSH stimulated cAMP activation.

Cells were seeded to 96-well, poly-D-lysine coated, white, and clear bottom plate at the density of 5,000 cells/well and incubated overnight. During assay, medium was changed with 20 µl serum free medium containing 12.5, 25 and 50 mIU/ml rec-FSH (test compound) in induction buffer and cells were incubated for 10 minutes. Following user's manual, cells were lysed with 20 µl lysis buffer to release intreacellular cAMP. Detection solution containing PKA was added into the cells and incubated at room temperature for 20 min. Following incubation, 80 µl Kinase-Glo Reagent was added to all wells to terminate the reaction and the remaining ATP was measured with a plate-reading luminometer.

A standard curve of luminesence for various cAMP concentrations was also prepared by using diluting assay's cAMP stock.

### 4. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

RNA isolation was performed with Quick-RNA MicroPrep Kit (Zymo Research) following manufacturer's instructions. After isolation, RNA was quantified with spectrophotometric read at 260 nm by Nanodrop 2000 (Thermo Scientific) and 1000 ng cDNA was synthesized by using M-MLV Reverse Transcriptase (Invitrogen). Quantitative real time expressions of mRNAs were detected and compared by using Light Cycler 480 SYBR Green I Master. The genes used in the study are shown below in the table with their sequences.

| Gene | Primer Sequence | |
|---|---|---|
| **GAPDH** | Forward | ATGGAAATCCCATCACCATCTT |
| | Reverse | CGCCCCACTTGATTTTGG |
| **StAR** | Forward | AAACTTACGTGGCTACTCAGCATC |
| | Reverse | GACCTGGTTGATGATGCTCTTG |
| **CYP11A1** | Forward | CAGGAGGGGTGGACACGAC |
| | Reverse | AGGTTGCGTGCCATCTCATAC |
| **3β-HSD** | Forward | GCCTTCAGACCAGAATTGAGAGA |
| | Reverse | TCCTTCAAGTACAGTCAGCTTGGT |
| **17β-HSD** | Forward | TGGGGTCCACTTGAGCCTGAT |
| | Reverse | TGCTGTGGGCGAGGTATTGG |
| **CYP17A1** | Forward | GTTTCAGCCGCACACCAACT |
| | Reverse | ACTCACCGATGCTGGAGTCA |
| **CYP19A** | Forward | GGTCACCACGTTTCTCTGCT |
| | Reverse | GCAAGCTCTCCTCATCAAACCA |
| **FSH-R** | Forward | TTGAACTGAGGTTTGTCCTCACCA |
| | Reverse | GGCCTCAGGGTTGATGTAGAGC |

**Table 1:** List and sequence of primers used in qRT-PCR.

For each well of 96-well qRT-PCR plate; 1000 ng CDNA, 10 µl SYBR Green and 2 µl 10 µM primer mix (containing reverse and forward primers) were mixed and the final volume was completed to 20 µl with nuclease free water.

#### 4.1. Delta delta Ct (ΔΔCt) Method

ΔΔCt method was used in quantitation and comparison of RT-PCR results. In this method, relative expression rate of target sequence is calculated by using a Ct value of reference/normalizer sample. Usually housekeeping genes are used as normalizers since they have abundant expressions within the cell (thus their Ct values will be lower).

Ct is the threshold cycle during which fluorescent absorption reaches to a certain level. At the first step of calculations, Ct value of target gene (sample) was subtracted from the Ct value of normalizer (ΔCt). This step was calculated for each sample in the experiment. In the case of duplicates and/or triplicates, the mean of the first delta step was calculated among groups. Then, ΔCt value of one of the samples was chosen as baseline. Other sample's expressions was calculated in response to baseline by subtraction again (ΔΔCt).

PCR programs run with the idea of doubling at each cycle. By assuming there is 100% efficiency in the doubling process at each cycle, the comparative expression levels of target genes should be calculated by placing ΔΔCt values to the power of 2.

When there are more than one measurements for Ct values, error calculation is necessary. Standard error calculation guides us in estimating the precision of mean. Error bars were calculated using standard error within the desired confidence interval and informed us about the spread of the data.

GAPDH was used as a normalizer and the control group of the first time point was used as baseline in calculations.

**5. Western Blot Analysis**

Equal number of HGrC1 cells ($1x10^6$) were plated to 10 cm plates and incubated with three different doses of FSH (12.5, 25 and 50 mIU/ml) for two different time points (24 hours and 48 hours). At the end of each time period, cells were harvested by trypsin treatment and centrifuged at 1500 x rpm for 5 minutes. Pellets were washed once with ice cold 1X PBS and centrifuged again. After supernatant was discarded, cells were lysed with RIPA (Radio-Immunoprecipitation Assay) cell lysis buffer including 1% 1X protease inhibitor coctail and 1% 1X phosphotase inhibitor coctail (Sigma-Aldrich). Lysates were incubated at +4 $^oC$ for 20 minutes and then centrifuged at 14,000 x rpm for 20 minutes at +4 $^oC$ to obtain total cell extract.

Proteins were seperated by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membrane by Trans-Blot® Turbo™ RTA Mini PVDF Transfer Kit (#170-4272, Biorad, USA). Membranes were blocked with 5% non-fat dry milk in TBST for one hour at room temperature.

Primary antibodies were added and membranes were incubated overnight at +4 $^{o}$C with gentle rocking.

Primary antibodies 3β-HSD, Progesterone, 17β-HSD, CYP17A1 and aromatase were used for immunoblotting at their maximum dilutions given in datasheets. Anti-vinculin antibody was used at 1:10,000 dilution. Goat anti-mouse IgG-HRP was used as secondary antibody at the dilution of 1:2000 (Santa Cruz; sc-2005). Quantification of protein within membranes was done by using Clarity™ Western ECL Substrate (#170-5061, Biorad, USA). Chemiluminescence detections were performed by ChemiDoc XRS+ Imaging Sytem (Biorad, USA).

### 5.1. Immunoprecipitation

Immunoprecipitation assay was performed for protein pull-down of 3β-HSD from lysates and Progesterone from both lysates and spent culture media.

Cells were harvested as explained above. After adding RIPA, 20 µl of cell lysate was saved for protein amount control and used for Vinculin detection. The rest of the lysates were incubated overnight with primary antibodies 3β-HSD and Progesterone at +4 $^{o}$C with gentle rocking. Each primary antibody was used at 1:50 dilution. Following incubation overnight, 10 µl Protein-G agarose beads (Cell Signaling, #37478) were added to pull-down antigen – primary antibody conjugate and incubation continued for 3 more hours at the same settings. Antigen – primary antibody conjugate was washed three times with 500 µl 1X cell lysis buffer. After each wash, tubes were centrifuged at 14,000 x g for 30 seconds and supernatants were discarded. At the end, pellets were resuspended with 15 µl 4X SDS sample buffer and protein expressions were analyzed by western blotting.

200 µl spent culture media was used for immunoprecipitation. 4 µl primary antibody was added onto supernatants for protein pull-down and same methodology was followed.

## 6. Enzyme Activity Assay

3β-HSD catalyzes the convertion of pregnenolone into progesterone in the presence of NAD. As a result of this enzymatic activity NADH is also produced along with progesterone. To measure the

enzymatic activity of 3β-HSD a spectrophotometric method is developed from a previous reference. [64]

HGrC1 cells were treated with FSH 25 mIU/ml and 50 mIU/ml for 24 and 48 hours. Lysates were prepared by harvesting cells with 1600 µl 0.15 M Tris-HCl (with the help of a scraper) and centrifuging at 12,000 x g for 10 minutes at 4$^{o}$C. Supernatants, containing the enzyme, were saved after centrifuge and kept at +4$^{o}$C until assay procedure. For enzyme activity assay 0.75 ml 0.15 M Tris-HCl, 0.75 ml 400 µM NAD and 0.1 ml 400 µM Pregnenolone mixture were prepared for each tube and 200 µl supernatant was added onto the mixtures. Enzyme was omitted from the control group and 200 µl 0.15 M Tris-HCl was added instead. Tubes were incubated at 37 $^{o}$C for 45 minutes. At the end of the incubation period, 500 µl color reagent was added onto the tubes and after color formation 2 ml phthalate buffer was added to end the reaction. Absorbance was read with plate reader.

- Color reagent: 50 mg nitroblue tetrazolium, 15 mg phenazine methosulfate and 1 ml Tween 20 were dissolved in 50 ml distilled water.

- Phthalate buffer: 3.57 g potassium hydrogen phthalate was dissolved in 50 ml 0.15 M HCl and 3 ml Tween20. pH was adjusted to 4.25 and volume was completed to 300 ml with distilled water afterwards.

- Standard Curve: Various concentrations of NADH were prepared; ranging from 0 nm to 150 nm. 1 ml of corresponding concentration was reacted with 0.5 ml color reagent. After color formation 2 ml phthalate buffer was added to each tube.

## 7. FSH-Receptor (FSH-R) Expression Manipulations

### 7.1. Short hairpin RNA (shRNA) knockdown

FSH-R was knocked-down in HGrC1 cells by using FSH-R shRNA Plasmid by Santa Cruz (sc-35415-SH). shRNA Transfection Protocol of manufacturer's was followed during transfection. Cells were seeded into 6-well tissue culture plates at the density of 3x10$^{5}$ cells/well and cultured with antibiotic-free DMEM-F12 medium supplemented with 10% FBS until they reach 70% confluency. Transfection Solution was prepared with 1 µg FSH-R shRNA plasmid DNA, 1 µl Transfection Reagent (sc-108061, Santa Cruz) and 189 µl Transfection Medium (sc-108062, Santa Cruz) for a single well

of 6-well tissue culture plate. Control transfection was performed with 1 µg Control shRNA Plasmid-A DNA by Santa Cruz (sc-108060). Transfection solutions were incubated at room temperature for 30 minutes. Before transfection, cells were washed with 1 ml Transfection Medium twice and 800 µl Transfection Medium was added to each well. After incubation period, 200 µl Transfection Solution was added onto wells dropwise.

Cells were incubated for 6 hours under normal culture conditions and after 6 hours 1 ml 2X culture medium (containing two times of normal serum and antibiotics concentration) was added to cells.

Each shRNA Plasmid constructs carry puromycin resistance gene for the selection of successfully transfected cells. Puromycin selection was done 48 hours post transfection with 1 ug/ml puromycin in fresh culture medium. For the efficiency of transfection, every 2-3 days, culture media was replaced with selective media containing puromycin.

### 7.2. Activin-A treatment

HGrC1 cells were treated with 60 ng/ml Activin-A for 24 hours before FSH treatment. After incubation with activin, cells were treated with 50 mIU/ml FSH for 24 hours and 48 hours.

## 8. Hormone Assays

Estradiol and progesterone hormone levels were determined from the spent culture media by using electro-chemiluminescence immunoassay (ECLIA; Elecsys and cobas e immunoassay analyzers, Roche Diagnostics, USA). Culture media was stored at –80$^{o}$C until hormone measurements. Specific kits for estradiol (Elecsys® Estradiol II, Cobas) and progesterone (Elecsys® Progesterone II, Cobas) measurements were used following manufacturer's instructions. Lower detection limits of estradiol and progesterone were 5.00 pg/ml (18.4 pmol/ml) and 0.030 ng/ml (0.095 nmol/ml), respectively.

## 9. Statistical Analysis

mRNA and protein levels of the steroidogenic enzymes and hormone levels ($E_2$ and P) were continuous variables and expressed as the mean ± SD. Comparison of the change in the mRNA and protein levels over the baseline was made using an independent Student's t-test or Mann–Whitney test, according to the distribution of their values. Multiple comparison posthoc test was conducted

for intergroup comparisons. Significance level was set at 5% ($P < 0.05$). SPSS statistical program (version 22) was used to analyze the data.

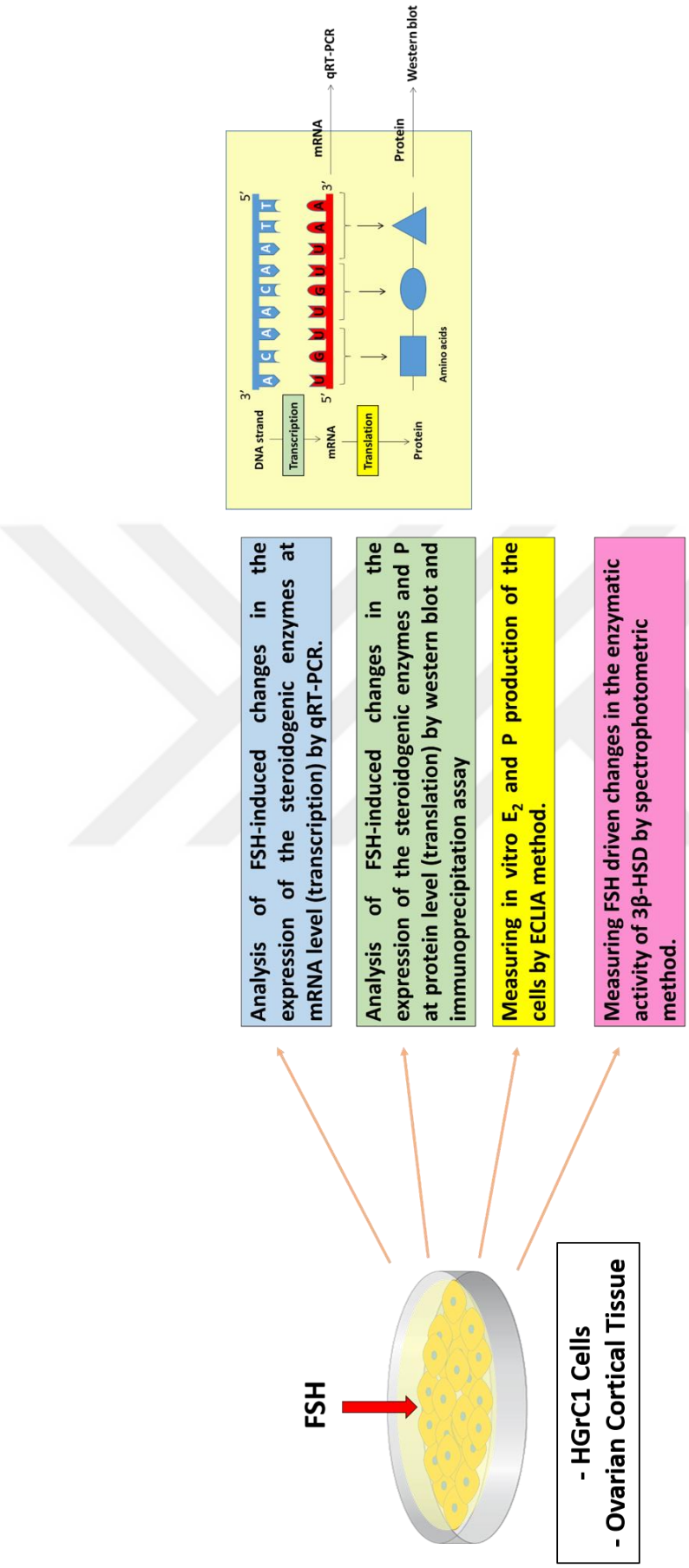



**Figure 9:** Overview of experimental methodology.

# Chapter 3 - RESULTS

## 1. FSH Responsiveness of HGrC1 Cell Line

The presence of FSH receptor in HGrC1 cell line is validated in the samples using qRT-PCR. Responsiveness of the FSH-R to exogenously administered rec-FSH was monitored with cAMP-Glo™ assay.

### 1.1 FSH receptor (FSH-R) expression

QRT-PCR results revealed that, treatment of HGrC1 cells with three different rec-FSH doses (12.5 mIU/ml, 25 mIU/ml and 50 mIU/ml) for 24 hours increased FSH-R expression 2.5, 3.7 and 4.1 folds with respect to control (Figure 10).

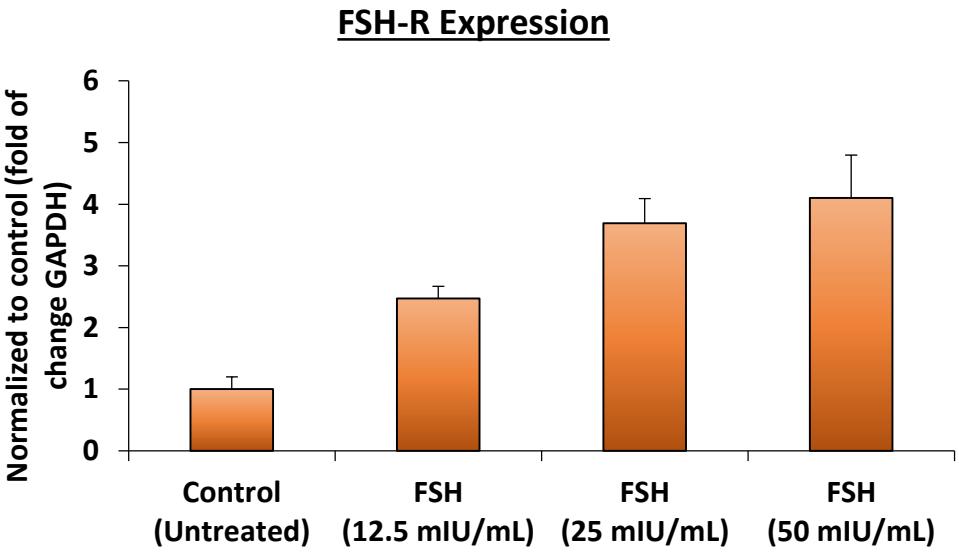



**Figure 10:** Changes is FSH-R expression 24 hours after rec-FSH treatment. FSH-R expression was increased with 24 hours rec-FSH stimulation in a dose dependent manner.

### 1.2 cAMP-Glo™ assay

For cAMP-Glo assay, HGrC1 cells were treated with same rec-FSH doses. Since cAMP response within the cell is immediate, incubation period was determined as 10 minutes. Changes in the cAMP concentrations were measured with plate reader.

The robust increase in the cAMP concentrations from baseline level of 2.2 nM to 4.9 nM, 7.9 nM and 14.1 nM was observed, respectively (Figure 11).

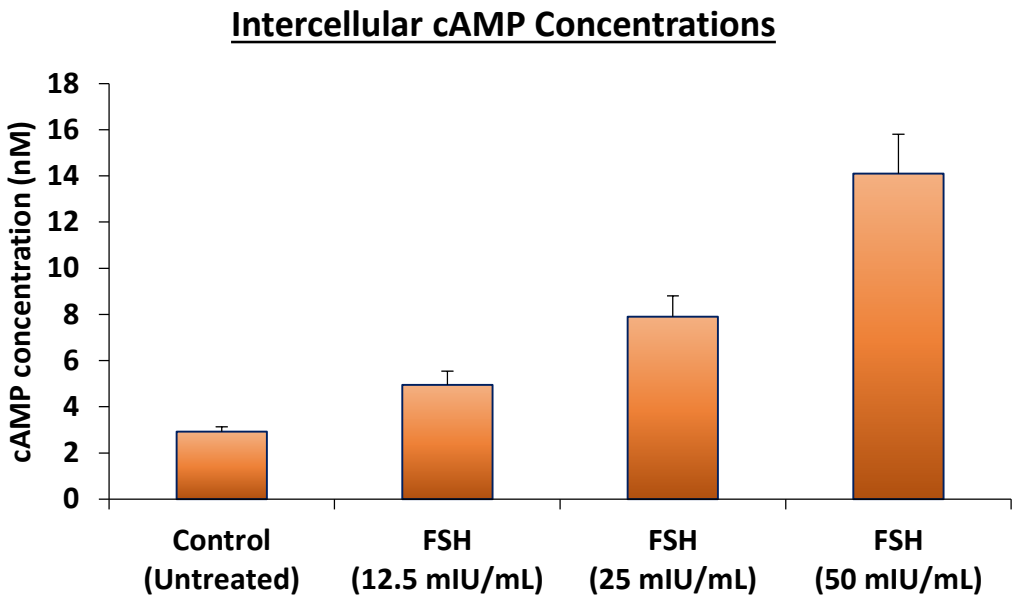



**Figure 11:** cAMP concentrations measured via cAMP-Glo™ assay. cAMP concentrations after 10 minutes of rec-FSH treatment were increased in dose-dependent manner.

## 2. FSH Induced Changes in the Expression of 3β-HSD and Other Steroidogenic Enzymes

### 2.1 HGrC1 cell line

#### 2.1.1 mRNA level analysis by qRT-PCR

Cells were analyzed at mRNA level with qRT-PCR for changes in steroidogenic enzymes' expressions. Treatment was performed with 12.5, 25 and 50 mIU/ml rec-FSH for 24 and 48 hours. FSH treatment caused a dose dependent increase in the expressions of 3β-HSD in the granulosa cells (Figure 12). The increase in 3β-HSD expression was much more evident at 48 hours (1.78, 2.1 and 2.6 folds, respectively) compared to the levels at 24 hours (1.18, 1.21 and 1.24 folds over basal levels, respectively) after stimulation with FSH at 12.5 mIU/ml, 25 mIU/ml and 50 mIU/ml concentrations (Figure 12).

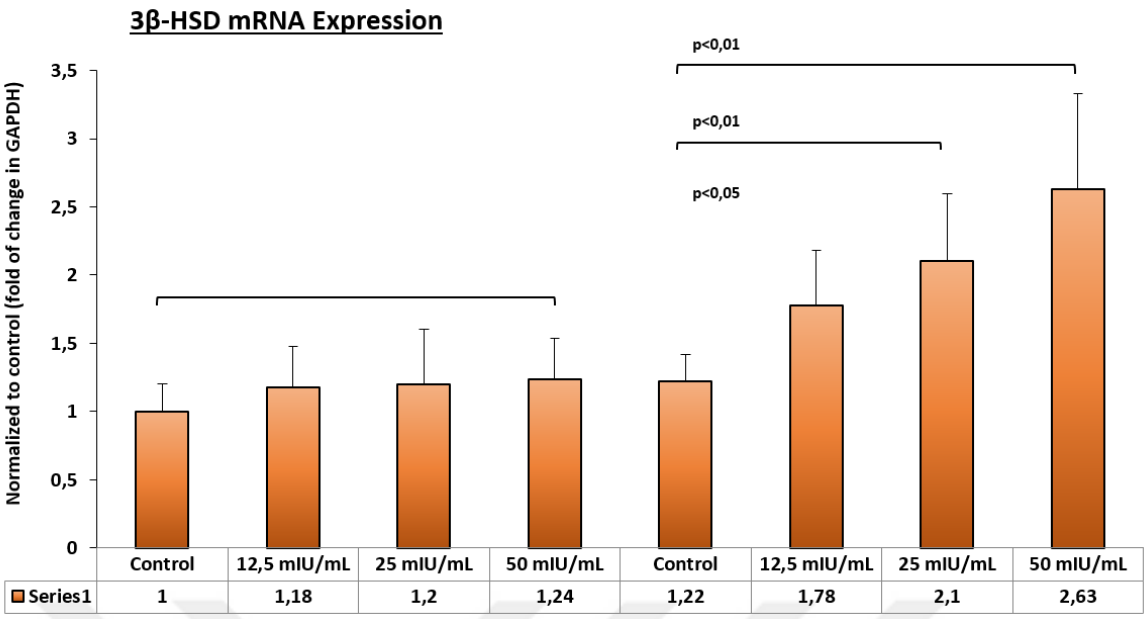



**Figure 12**: 3β-HSD gene mRNA expression in HGrC1 cell line after rec-FSH treatment. FSH stimulation resulted in a dose-dependent increase in the expression of 3β-HSD gene after 48 hours of treatment.

The expressions of the other steroidogenic enzymes were also increased following FSH stimulation in dose-dependent manner (Figure 13). The most robust increase was observed in the aromatase expression (Figure 26). Overall, when all time points and FSH doses were analyzed collectively, FSH significantly increased the expression of its own receptor (3.73 fold, $p<0.001$), StAR (1.7 fold, $p<0.01$), P450Scc (1.75 fold, $p<0.01$), aromatase (4.49 fold, $p<0.001$), 3β-HSD (1.68 fold, $p<0.01$) 17β-HSD (2.16 fold, $p<0.01$) except for 17α-OH (1.03 fold $p>0.05$).

17α-OH was one notable exception because its expression did not change considerably after FSH stimulation. It is overall expression was 1.03 fold over control ($p>0.05$) which was not significant.

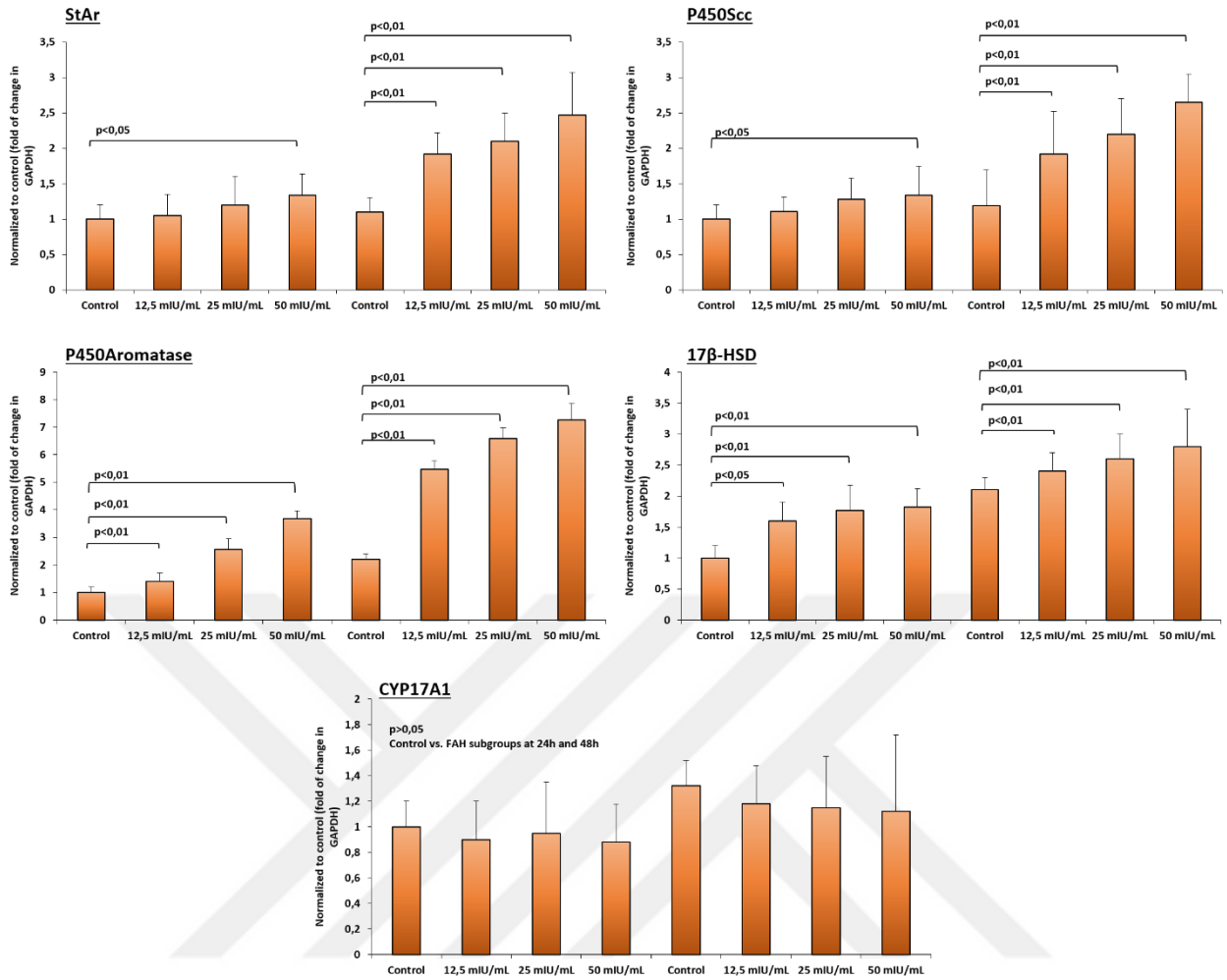



**Figure 13:** mRNA expressions of StAR, P450Scc, P450Aromatase, 17β-HSD and 17α-OH. Expressions of steroidogenic enzymes were increased in a dose-dependent manner following FSH stimulation. The most drastic increase was observed in the expression of aromatase. 17α-OH levels did not change significantly by FSH stimulation.

### 2.1.2 Protein level analysis by Western Blot

The protein expressions of the steroidogenic enzymes in the granulosa cells at baseline and 24 hours and 48 hours after stimulation with FSH at 12.5, 25 and 50 mIU/mL concentrations are shown as a western blot image in the figure 14. FSH did not cause any notable change in 3β-HSD and P expressions after 24 hours stimulation. However protein levels of 3β-HSD and P were markedly increased with continued stimulation after 48 hours in a dose dependent fashion. 17β-HSD and aromatase expressions were also increased with rec-FSH treatment. By contrast 17α-OH level was almost undetectable and did not respond to FSH stimulation.

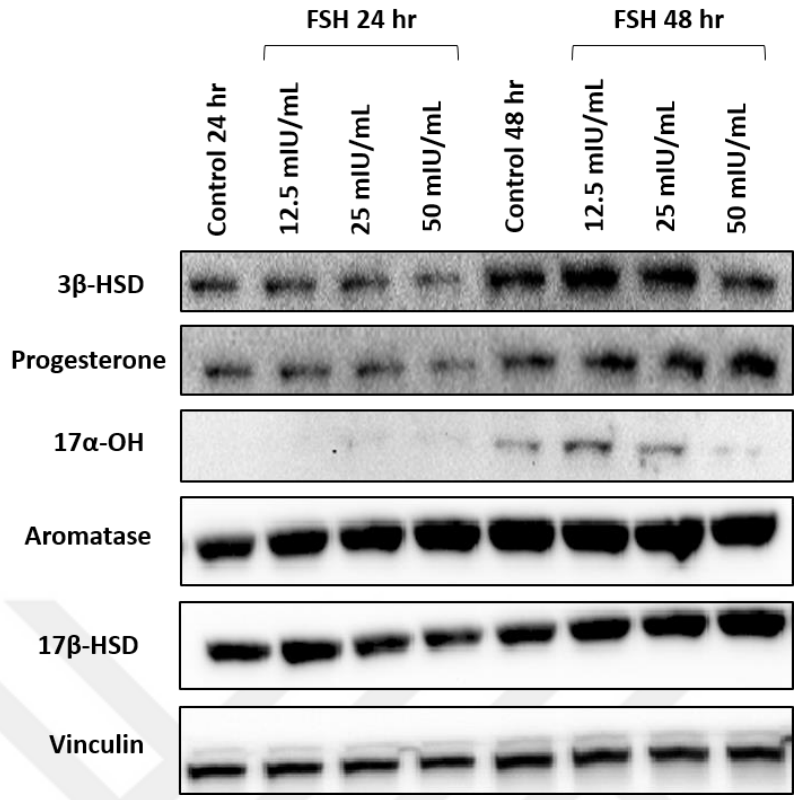



**Figure 14:** Western blot analysis of steroidogenic enzymes at HGrC1 cell line. Protein expressions of 3β-HSD and P were increased after 48 hours stimulation with recombinant FSH. Aromatase and 17β-HSD expressions were also up-regulated with the treatment. However there was not any notable change in 17α-OH protein levels.

Western blotting of protein pull down by immunoprecipitation showed the similar results. The protein expressions of 3β-HSD and Progesterone were increased in dose dependent manner after 48 hours treatement with rec-FSH (Figure 15).

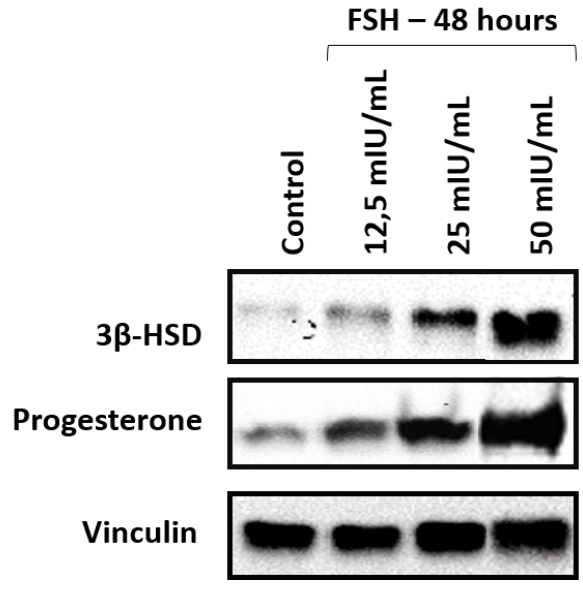



**Figure 15:** Protein expressions of 3β-HSD and P after immunoprecipitation at HGrC1 cells. Western blot analysis results were confirmed with protein pull-down with immunoprecipitation. 3β-HSD and P protein expressions showed a dose dependent increase after 48 hours of stimulation with recombinant FSH.

### 2.1.3 Hormone assays

In order to see increased production of P from the granulosa cells, $E_2$ and P levels were measured by ECLIA from spent culture media after treatments. Twenty-four hours FSH stimulation showed a discordance between $E_2$ and P production of the cells (Figure 16). While FSH stimulation resulted in a dose-dependent increase in $E_2$ production, there was not any notable increase in the P production at 24 hours. However, P levels began to rise with fourty-eight hour stimulation, along with $E_2$. Thus, measurements from ECLIA showed a correlation with western blot.

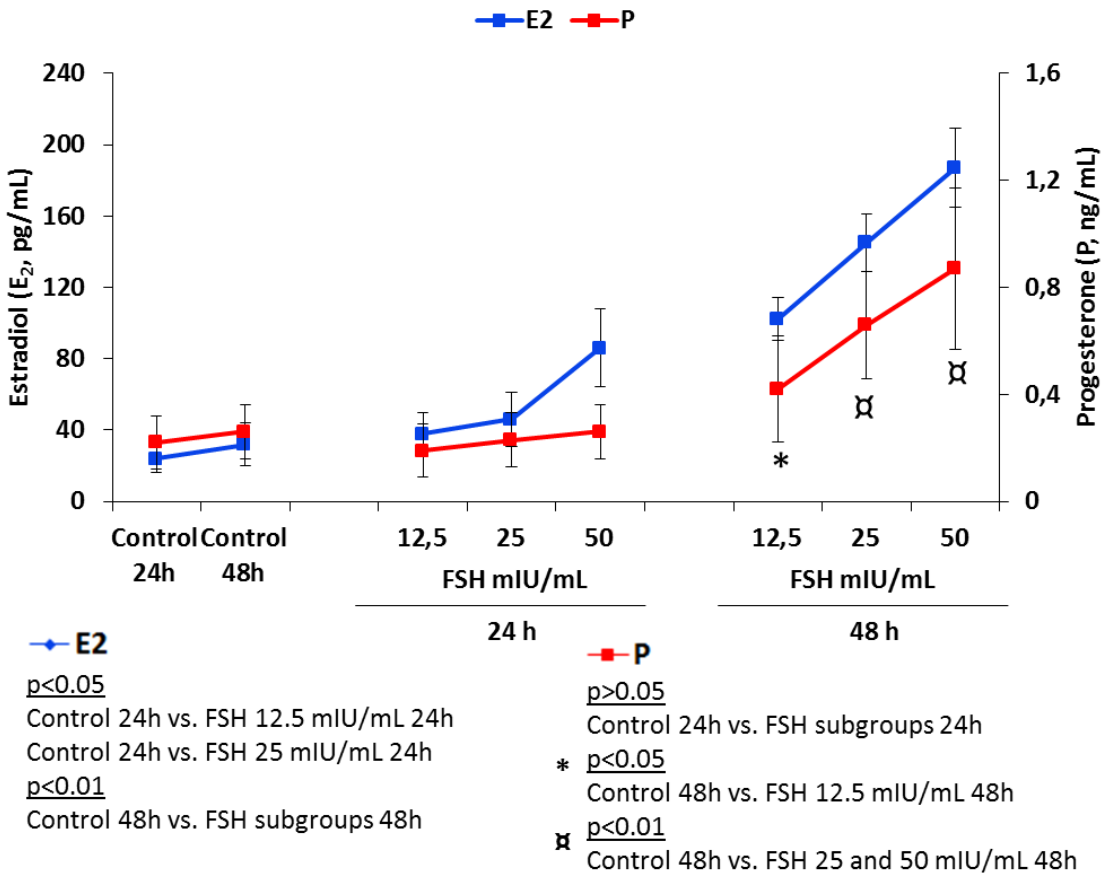



**Figure 16:** $E_2$ and P levels in the spent culture media of HGrC1, measured with ECLIA method. In line with findings of qRT-PCR and western blotting $E_2$ and P productions of cells increased significantly following FSH stimulation.

### 2.2 Human ovarian cortical tissue

FSH induced changes were analyzed at ovarian cortical samples by using same methods.

#### 2.2.1 Protein level analysis by Western Blot

*In vitro* stimulation of ovarian tissue samples with FSH at 12.5, 25 and 50 mIU/mL concentrations resulted in a dose-dependent increase in the protein expression of 3β-HSD, P, aromatase and 17β-HSD whereas such an increase was not observed in 17α-OH expression (Figure 17). Quantification of the intensity of the signals revealed that FSH stimulation significantly increased the expression of 3β-HSD (1.6 fold, $p<0.01$), 17β-HSD (1.9 fold, $p<0.01$), and aromatase (3.2 fold, $p<0.001$) but 17α-OH (1.12 fold, $p>0.05$) compared to controls.

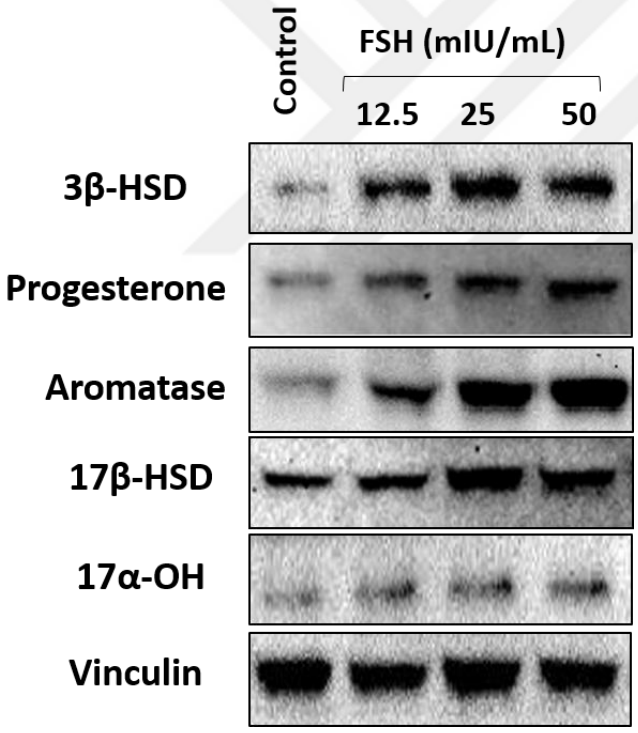
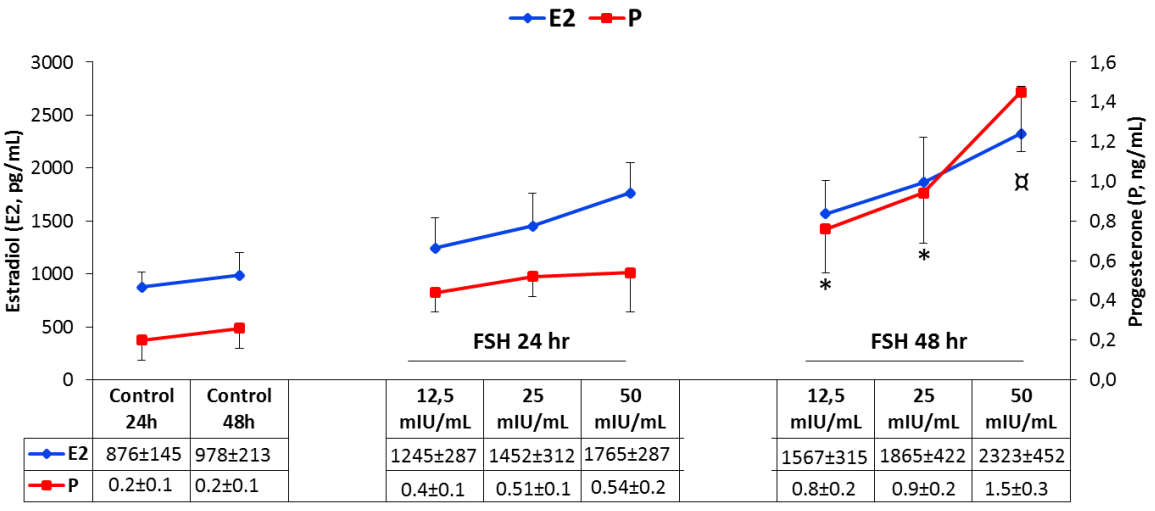



**Figure 17:** Changes in steroidogenic enzymes' expressions in ovarian tissue samples following FSH stimulation. Stimulation of ovarian tissue samples with FSH for 48 hours significantly up-regulated the protein expressions of 3β-HSD, P, 17β-HSD and aromatase in a dose-dependent manner. 17α-OH expression was not changed after FSH.

### 2.2.2 Hormone assays

When *in vitro* hormone productions of these samples were measured by ECLIA it appeared that P output from FSH stimulated samples was increased at 48 hours along with $E_2$ in a dose-dependent fashion (Figure 18).




| | Control 24h | Control 48h | 12,5 mIU/mL | 25 mIU/mL | 50 mIU/mL | 12,5 mIU/mL | 25 mIU/mL | 50 mIU/mL |
|---|---|---|---|---|---|---|---|---|
| E2 | 876±145 | 978±213 | 1245±287 | 1452±312 | 1765±287 | 1567±315 | 1865±422 | 2323±452 |
| P | 0.2±0.1 | 0.2±0.1 | 0.4±0.1 | 0.51±0.1 | 0.54±0.2 | 0.8±0.2 | 0.9±0.2 | 1.5±0.3 |

**E2**

p<0.05
Control 24h vs. FSH subgroups 24h
p<0.01
Control 48h vs. FSH subgroups 48h

**P**

p>0.05
Control 24h vs. FSH subgroups 24h
* p<0.05
Control 48h vs. FSH 12.5 and 25 mIU/mL 48h
¤ p<0.01
Control 48h vs. FSH 50 mIU/mL 48h

**Figure 18:** $E_2$ and P levels in the spent culture media of ovarian tissues, measured with ECLIA method. *In vitro* stimulation of ovarian tissue samples with FSH resulted in a dose-dependent increase in P output along with $E_2$. This effect of FSH became evident at 48 hours of stimulation.

## 3. FSH Receptor (FSH-R) Expression Manipulations

### 3.1 shRNA transfection

FSH receptors in the granulosa cells were knocked-down using shRNA technology. Changes in the expression of the steroidogenic enzymes were analyzed with qRT-PCR to observe cells' response to rec-FSH when the receptor is knocked-down (Figure 19). The mRNA level of FSH-R was significantly decreased confirming that FSH-R was adequately silenced.

Analysis of the expression of the steroidogenic enzymes in these cells revealed that knocking down FSH receptor is associated with a significant reduction in the expression of 3β-HSD, 17β-HSD and

aromatase. However, the expression of 17α-OH did not decrease considerably after FSH receptor was knocked-down.

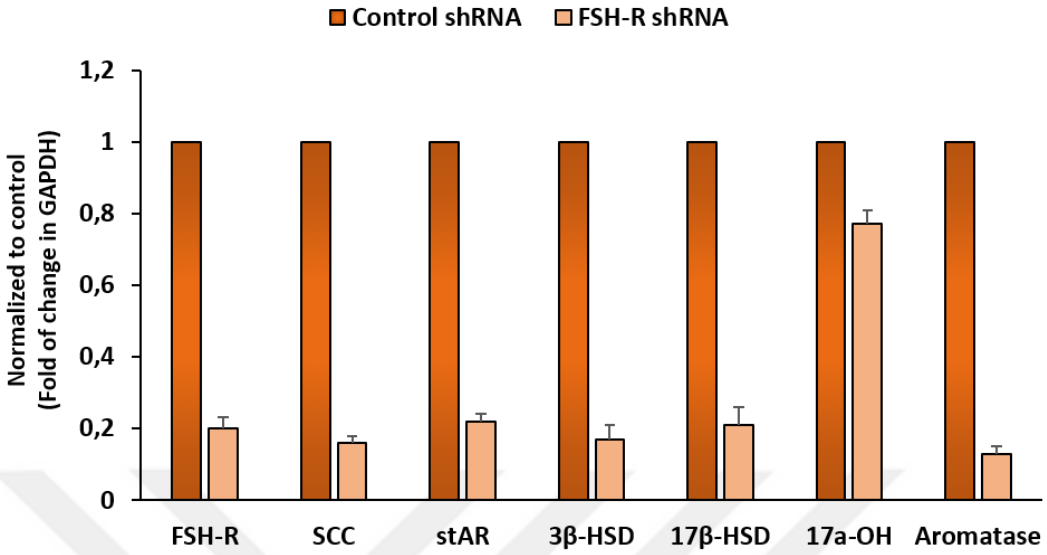



**Figure 19:** mRNA expressions of steroidogenic enzymes in the HGrC1 cell line after shRNA knockdown of FSH-R. Silencing of FSH-R gene expression with shRNA technology significantly decreased the expression of FSH-R. Analysis of the steroidogenic enzymes' expression profiles revealed thah knocking down FSH-R is associated with significant reduction of SCC, StAR, 3β-HSD, 17β-HSD and aromatase. However, that degree of reduction was not observed in the expression of 17α-OH.

Furthermore, cAMP productions of the cells in response to FSH (25 mIU/mL) stimulation was significantly blunted compared to control shRNA (Figure 20).

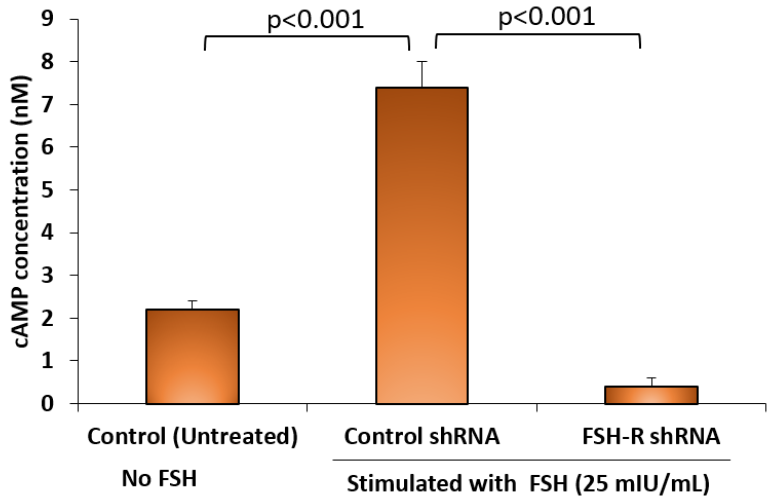



**Figure 20:** cAMP concentration within HGrC1 cells, after shRNA knockdown of FSH-R. cAMP productions of the cells in response to 25 mIU/ml FSH stimulation was significantly decreased after shRNA transfection.

### 3.2 Activin-A treatment

Activin-A has mitogenic effects on the granulosa cells and it increases follicle growth both in vivo and *in vitro* conditions. It also up-regulates the expression of aromatase and FSH receptor, and the response of the granulosa cells to FSH stimulation. [17, 65-67] Thus, in another set of experiments the HGrC1 granulosa cells were stimulated with recombinant activin-A (60 ng/mL) alone or with FSH (25 mIU/mL) for 48 hours to analyze how activin-A induced-increase in FSH responsiveness changes the expression of 3β-HSD and other steroidogenic enzymes.

The expressions of FSH-R (4 folds), 3β-HSD (2.1 folds), 17β-HSD (2 folds) and aromatase (4 folds) were increased significantly in comparison to control cells when stimulated with rec-FSH. The increase in 17α-OH (1.1 fold) expression was not significant. Activin-A treatment alone markedly up-regulated the expression of FSH-R (2 fold), 17β-HSD (1.3 fold) and aromatase (3.2 fold); but did not change the expressions of 3β-HSD (1.1 folds) and 17α-OH (1.04 fold) when compared to control cells (Figure 21).

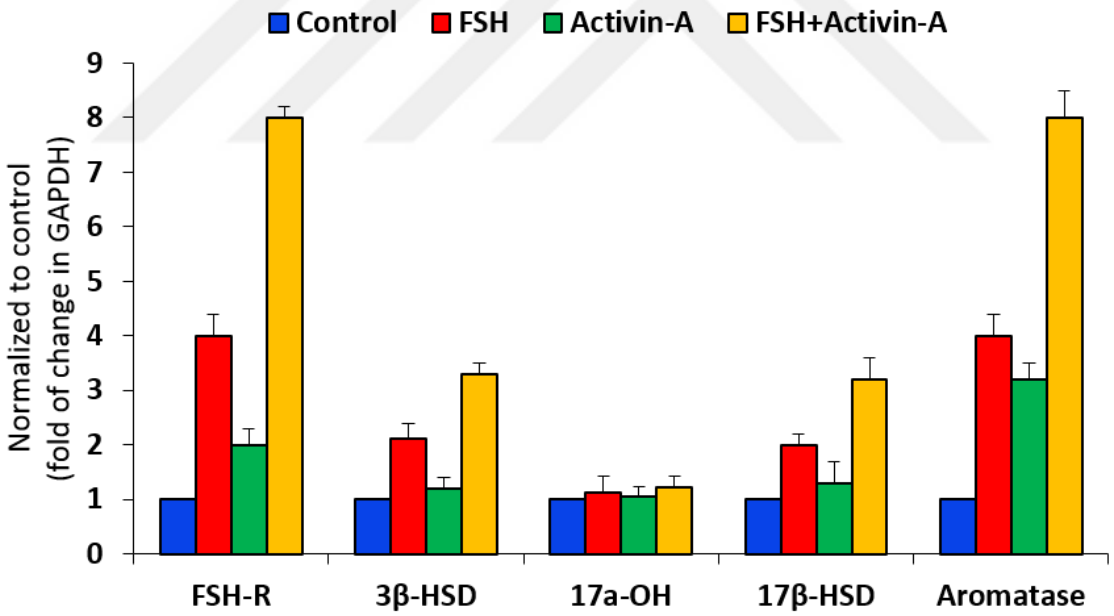



**Figure 21:** Gene expression patterns in the cells after FSH and Activin-A treatement. Activin-A treatment together with FSH has up-regulated the expression of FSH-R. As a result of this increase 3β-HSD, 17β-HSD and aromatase expressions were increased in the experiment group that has received Activin-A and FSH treatment together.

FSH and Activin-A stimulation together further increased the expression of FSH-R (8 vs 4 folds; respectively), 3β-HSD (3.3 vs 2.1 folds, respectively), 17β-HSD (3.2 vs 2 folds; respectively) and aromatase (8 vs 4 folds; respectively). 17α-OH expression did not change considerably between cells stimulated with FSH alone or together with Activin-A (1.12 vs 1.23 folds; respectively).

Protein expressions of 3β-HSD and P were further increased in the granulosa cells when they were stimulated with FSH and Activin-A combination compared to FSH only stimulation (Figure 22).

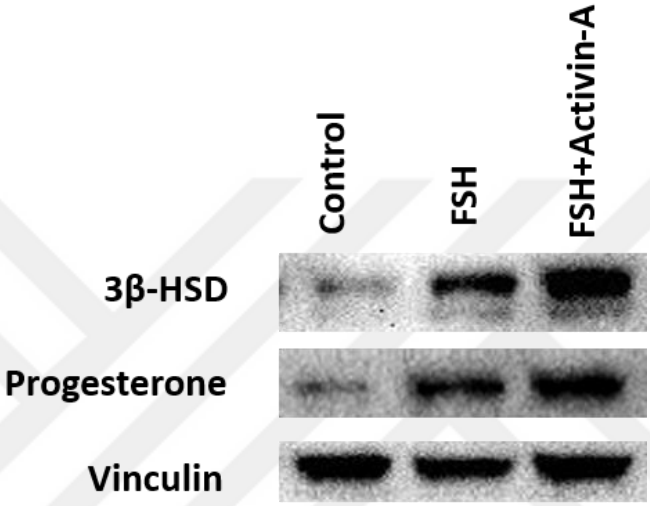



**Figure 22:** Western blot analysis of protein expressions of 3β-HSD and P after activin treatement.

### 4. 3β-HSD Enzymatic Activity Assay

Stimulation of the granulosa cells with FSH at 12.5, 25 and 50 mIU/mL concentrations significantly increased enzymatic activity of 3β-HSD in the HGrC1 granulosa cells at 48 hours (Figure 23). As a result of this, the conversion of the substrate pregnenolone to progesterone was significantly increased along with the generation of NADH as another indicator of the presence of enzymatic activity of 3β-HSD. This stimulatory effect of FSH on the 3β-HSD activity was dose-dependent.

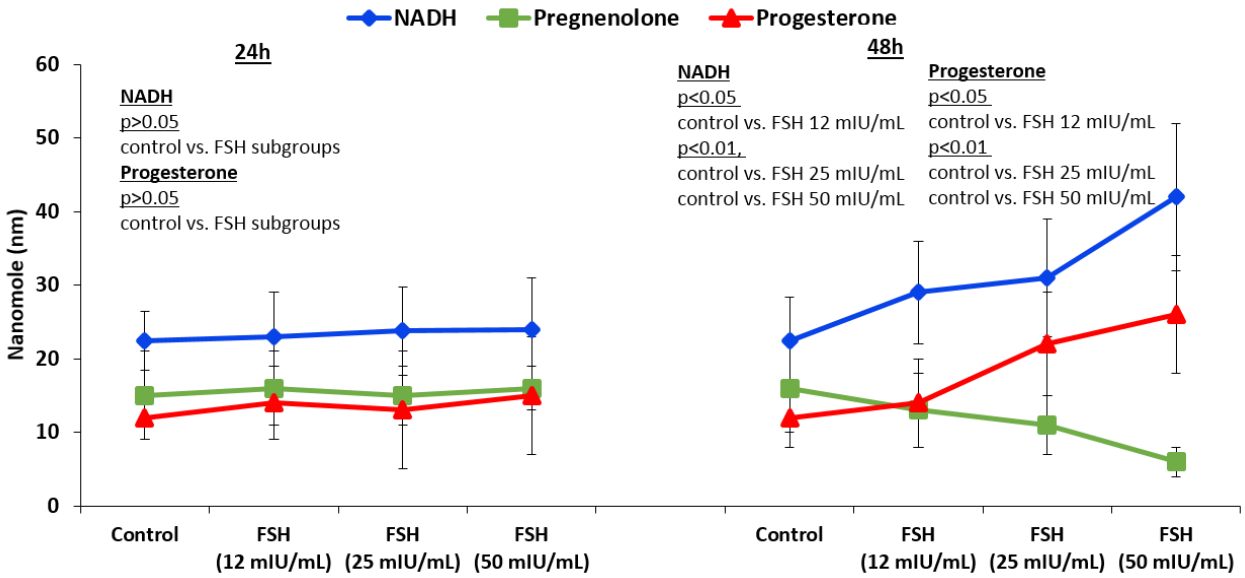



**Figure 23:** Changes in the NADH, Pregnenolone and Progesterone concentrations in the samples during 3β-HSD enzymatic activity assay. FSH stimulation significantly increased the enzymatic activity of 3β-HSD, which is indicated by increased production of NADH and the conversion of pregnenolone to progesterone in the granulosa cells. The stimulatory effect of FSH on the enzymatic activity of 3β-HSD particularly evident at 48 hours after stimulation

## Chapter 4 - DISCUSSION

A well-developed endometrium is the first condition of a successful pregnancy and endometrial development is directed during menstrual cycle by hormonal fluctuations. Progesterone is the main hormone that supports endometrial development and its synthesis is directed primarily by corpus luteum during luteal phase. However serum P levels may prematurely rise before ovulation trigger. This premature rise reduces pregnancy rates during fresh embryo transfers by impairing endometrial receptivity in stimulated IVF cycles. [5]

Since the rise in P precedes hCG administration and is not associated with premature LH surge it does not reflect a true luteinization event. It is typically seen in stimulated IVF cycles and is significantly correlated with the intensity of ovarian stimulation; hence patients with more follicles and oocytes have higher P levels. [5, 6, 9, 11, 12, 68] These findings suggest that gonadotropin stimulation and/or the degree of ovarian stimulation might play a pivotal role in premature rise of serum P level before ovulation trigger at the late follicular phase during multi-follicular development in stimulated IVF cycles.

We have shown in this study that FSH stimulates 3β-HSD expression and P biosynthesis in human granulosa cells and ovarian tissue samples in addition to its stimulatory effect on the expression of other steroidogenic enzymes required for estrogen synthesis. FSH had a direct stimulatory effect on the enzymatic activity of 3β-HSD and increased the conversion of pregnenolone to progesterone in the HGrC1 granulosa cells. As a result of this, P output from the samples stimulated with FSH was increased along with $E_2$ in a dose-dependent fashion. This effect of FSH was amplified and reversed when FSH receptor expression was up-regulated by activin-A, and down-regulated with shRNA, respectively. Taken together these results provide molecular evidence in the human for a direct stimulatory action of FSH on P production from human granulosa cells via upregulation of 3β-HSD expression and increasing its enzymatic activity.

As another important finding we also showed in this study that the expression of 17α-OH did not increase after FSH stimulation in HGrC1 granulosa cells and ovarian tissue samples. These results support the findings of the previous studies on ovarian steroidogenesis and the two cell two gonadotropin theory in human. [69-71] According to this theory, in order to be converted into

androgens, pregnenolone and progesterone produced by granulosa cells must enter the theca cells where they undergo 17-hydroxylation and side-chain splitting reactions by the actions of the enzymes 17α-OH and 17, 20 lyase respectively. These reactions are mainly driven by the actions of LH. [72]. 17α-OH and 17,20 lyase are encoded by the same gene CYP17A1. Therefore the weak baseline expression of 17α-OH and its unresponsiveness to FSH stimulation as shown by us and other previous studies not only help suppress androgen synthesis in the granulosa cells but also provide a biological basis for two cell two gonadotropins theory. [69]

What represses 17α-OH in the granulosa cells remained a mystery until 2009 when a study revealed that the AP-1 transcription factor, c-fos, was responsible for 17α-OH repression and hence suppression of androgen production in human granulosa cells. [73] By contrast, the activity of 17α-OH is already high in the rat granulosa and cannot be further amplified by FSH or LH, indicating that granulosa cells can produce estrogen from progesterone and do not require androgen precursors from the theca and/or interstitium. [74, 75] It therefore does not support the two-cell two-gonadotropin theory at least in the rats.

FSH stimulates 3β-HSD activity and promotes progesterone synthesis in a dose dependent manner in cultured rat granulosa cells. [76, 77] Concomitant treatment with GnRH decreased the FSH stimulation of enzyme activity. [75] A similar synergistic effect on progesterone synthesis was observed when the cells were treated with FSH and EGF [76]; and FSH and estradiol combinations. [78] Therefore, the stimulatory action of FSH on 3β-HSD activity and P synthesis in the rat granulosa cells is to provide precursors for direct estrogen synthesis without the use of androgens.

In the primate model FSH treatment of granulosa cells isolated from immature follicles stimulated both progesterone production and aromatase activity. [79] But these studies received little or no clinical attentions since clinical implications of FSH induced P elevations were not recognized at that time.

Unresponsiveness of 17α-OH to FSH stimulation in human granulosa cells not only supports the two-cell theory of human ovarian estrogen synthesis but may also provide a plausible explanation why FSH stimulation promotes P output from granulosa cells without luteinization. In the absence of LH support the activity of 17α-OH cannot be enhanced with FSH stimulation only. This may delay

the conversion of P into androgens in theca cells leading to their accumulation and leak into systemic circulation. When this effect is combined with direct stimulatory effect of FSH on 3β-HSD and P biosynthesis, high input precursor steroids generated during multi-follicular development under the influence of tonically elevated FSH level in stimulated IVF cycles may exceed the ability of the ovary to effectively convert them into estrogen pathway, creating a relative shunting at 17 hydroxylation step that diverts these precursors into progesterone pathway for conversion to androgenic substrates for final estrogen synthesis.

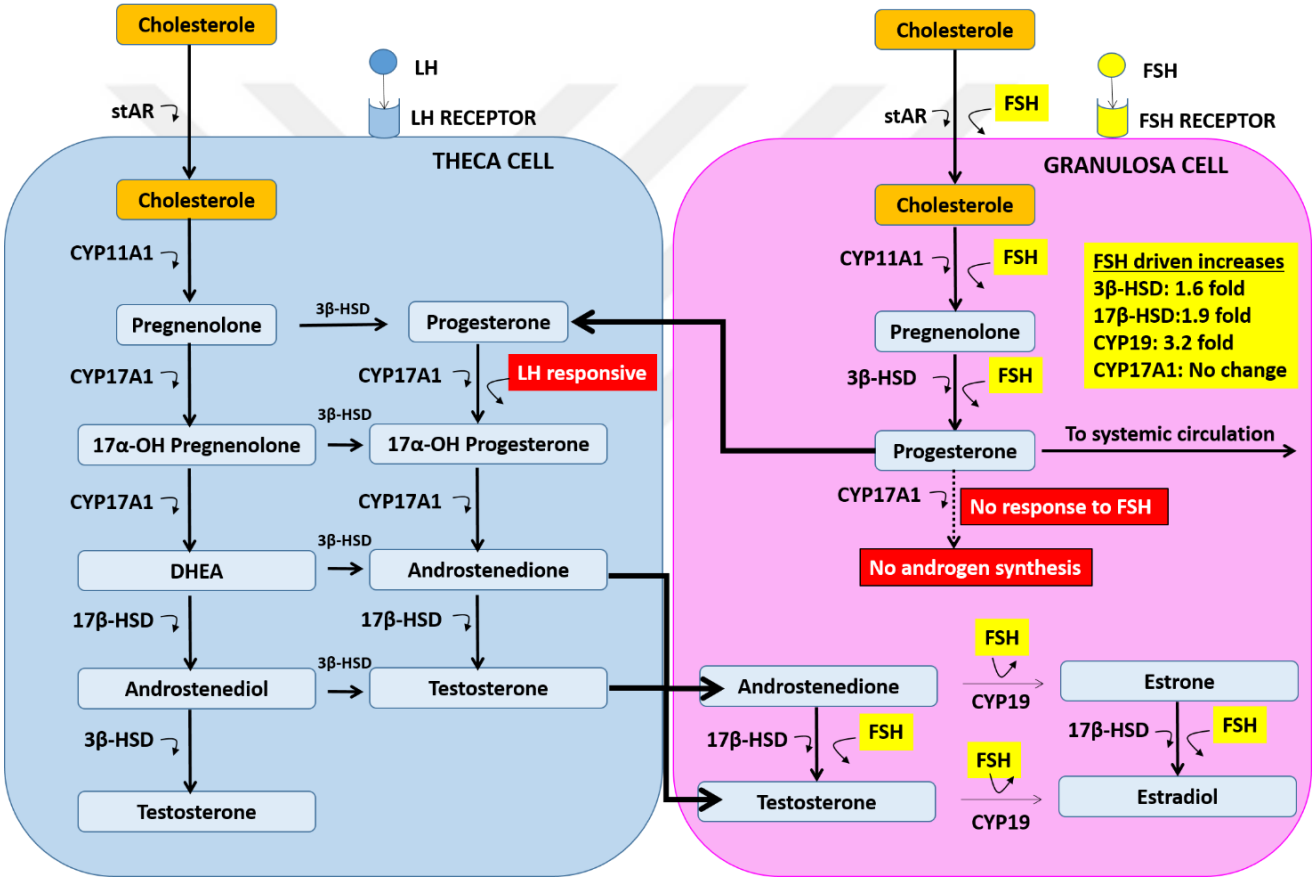



**Figure 24:** Proposed model for two-cell-two-gonadotropin theory. According to the two cell two gonadotropins theory P produced by granulosa cells enter theca cells to be converted into androgens because CYP17A1 enyzmes (cytochrome P450 enzymes; 17α-hydroxylase and 17,20 lyase),are not responsive to FSH, therefore no further reactions take place downstream in the granulosa cells. When this effect is combined with direct stimulatory effect of FSH on 3β-HSD and P biosynthesis, accumulated P may leak into systemic circulation.

**CONCLUSION**

This study shows that FSH has a direct stimulatory effect on 3β-HSD and P production in human granulosa cells, providing a molecular explanation why serum P level is elevated prior to ovulation trigger in stimulated IVF cycles. Given its relevance to the clinical practice in assisted reproduction technologies and direct impact on the pregnancy rates this issue deserves more attention at molecular level.

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## Vita

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