# FOLLICLE STIMULATING HORMONE-RECEPTOR EXPRESSION IN THE AGING HUMAN OVARY

A Thesis by

Karthik Asthi

Bachelor of Medicine and Surgery, Kilpauk Medical College, India, 2003

Submitted to the college of Liberal Arts and Sciences
and the faculty of the Graduate school of
Wichita State University in partial fulfillment of
the requirements for the degree of
Master of Science

December 2008

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I have examined the final copy of the Thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science with a major in Biological Sciences

_____
Jeffrey V May., Committee Chair

We have read this Thesis and recommend its acceptance:

_____
George R. Bousfield., Committee member

_____
William J Hendry III., Committee member

_____
James Bann., Committee member

# ACKNOWLEDGEMENTS

I sincerely thank my graduate advisor, Jeffrey V May PhD, for his professional support and guidance throughout my graduate studies, here at Wichita State University. Without his constant advice, it would have been an impossible task for me in doing research. My appreciation extends to my committee members: Drs Bousfield, Hendry and Bann. Thank you for your continuous support and insightful comments.

My special thanks to Dr. Shuai, for her valuable time in discussing about gene expression, oligonucleotide primers and PCR. My gratitude extends to Jennifer McCoy, for her suggestions on basic and complex strategies in Real time PCR. A special appreciation for Maria, for all the help she has done, regarding biology department.

I would like to thank the following friends: Jessica, Sowmya, Imala, Shane, Megan, Carrie, Ramesh, Monica, Anup, and too many to name, who have helped me professionally or personally over the course of my studies.

# ABSTRACT

The follicles in a human ovary gradually decline in number during infancy, adolescence and the reproductive years. However, during the perimenopausal period there is a more rapid decline in follicles. Older women have fewer granulosa cells in their follicles than younger women. The older ovary is less responsive to gonadotropins, especially FSH. It is hypothesized that age-related, decreased responsiveness to FSH is due to decreased FSH-receptor (FSH-R) levels and/or alternate splicing of FSH-R mRNA resulting in defective (impaired) receptors.

Human ovarian follicles of sizes ranging from 3 to 7 mm in diameter from 26-46 year-old women were isolated, snap frozen and stored at -80°C. RNA was isolated and real time PCR performed to measure the FSH-R mRNA levels in the follicles. Linear regression analysis was performed on FSH-R mRNA levels within follicle size categories as a function of age. The regression analysis was found to be non significant. Therefore, we reject the hypothesis, which stated that FSH-R mRNA levels within follicles decrease as the ovary ages.

To analyze alternate splicing of FSH-R mRNA, two oligonucleotide primer sets were designed. RT-PCR of total RNA from follicles of 26-46 year-old women was performed. PCR products were sequenced to determine the degree of homology for the FSH-R with the human FSH-R variant 1 published in Genebank. There was 99.7% sequence homology between the FSH-R PCR amplimers and human FSH-R variant 1. This suggests that impaired ovarian response is not due to alternate splicing of the FSH-R mRNA in older women, and is inconsistent with the hypothesis that alternate splicing of

FSH-R mRNA is the cause for decreased response to FSH in an aging human ovary. Accordingly, decreased response may be due to other factors, perhaps FSH/FSH-R post-receptor signaling mechanisms.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| 5'UTR | 5' Untranslated region |
| Ala | Alanine |
| AS | Alanine-Serine |
| Asn | Asparagine |
| Bcl-2 | B cell lymphoma 2 |
| BMP | Bone Morphogenic Protein |
| bp | Base pair |
| C | Centigrade |
| cAMP | cyclic Adenosine Mono Phosphate |
| cDNA | Complemenatry DNA |
| CHO | Chinese Hamster Ovary |
| COH | Controlled ovarian hyperstimulation |
| COOH | Carboxyl |
| CRE | cAMP Response Element |
| CREB | CRE binding protein |
| Ct | Cycle threshold |
| DEPC | Diethyl pyrocarbonate |
| DNA | Deoxyribo Nucleic Acid |
| ERα | Estrogen Receptor Alpha |
| ERβ | Estrogen Receptor Beta |
| FGF | Fibroblast Growth Factor |

## LIST OF ABBREVIATIONS (cont.)

| | |
|---|---|
| Figα | Factor in the germline alpha |
| FSH | Follicle-Stimulating Hormone |
| FSH-R | Follicle-Stimulating Hormone Receptor |
| GC | Granulosa Cell |
| GDF-9 | Growth Differentiation Factor-9 |
| GnRH | Gonadotropin Releasing Hormone |
| hCG | human Chorionic Gonadotropin |
| HOF | Human Ovarian Follicle |
| IGF-1 | Insulin like Growth Factor-1 |
| KL | Kit Ligand |
| LH | Luteinizing Hormone |
| Lhx9 | Lim homeobox 9 |
| MAPK | Mitogen Activated Protein Kinase |
| ml | Milliliter |
| mm | Millimeter |
| mRNA | messenger RNA |
| ng | Nanograms |
| $NH_2$ | Amino |
| PCR | Polymerase Chain Reaction |
| pg | Picograms |
| PKA | Protein Kinase A |
| PKC | Protein Kinase C |

## LIST OF ABBREVIATIONS (cont.)

| | |
|---|---|
| RNA | Ribonucleic acid |
| RT | Reverse Transcription |
| sec | seconds |
| Ser | Serine |
| SF-1 | Steroidogenic Factor-1 |
| SNP | Single Nucleotide Polymorphism |
| T | Thymine |
| TC | Theca Cell |
| Thr | Threonine |
| TN | Threonine-Asparagine |
| TSH | Thyroid Stimulating Hormone |
| Val | Valine |
| Wnt-4 | Wingless type-4 |
| WT-1 | Wilms Tumor-1 |

# INTRODUCTION

## Embryonic gonad

Formation of the gonad is critical to the development of the reproductive system. Genetic sex is determined at fertilization, however, the phenotypic gender is initially undetermined [1]. Even though the testis and ovary differ in terms of phenotype and genotype, their embryonic development is from the same progenitor tissues: the genital ridge and primordial germ cells (PGC) [2]. Both these progenitor tissues are developmentally bipotential [1].

The gonad originates from intermediate mesoderm at the end of 3$^{rd}$ week of gestation. The intermediate mesoderm further gives rise to the urogenital ridge, which develops three lineages: pronephros, mesoephros and metanephros. The mesonepros eventually differentiates into the embryonic indifferent gonad [3, 4].

The ovary has 2 basic cell lineages: germ cell and somatic cell. The germ cell lineage originates from primordial germ cells (PGC) which migrate and populate the sexually undifferentiated gonad [5]. Somatic cells arise from the mesonephros, coelomic epithelium and mesenchyme [6]. In the sexually differentiated gonad, the germ cell is finally termed an oocyte or spermatogonium in female and male, respectively. The supporting somatic cells in the female and male gonad are referred to as granulosa and Sertoli cells, respectively [4].

Endocrine factors, as well as autocrine/paracrine regulating factors are involved in modulating gonadal growth [7]. The following genes SF-1, Emx2, WT-1, Lim1, Lhx9,

SRY and GATA-4 are involved in the formation of indifferent gonad from the intermediate mesoderm [8]. Lack of any of these genes blocks gonadal development.

**Migration of PGC to Genital ridge**

In a human embryo, PGC's are first seen at 21-22 days of gestation. These originate from the endoderm of the dorsal wall of yolk sac, near the developing allantois [9, 10]. At 4-6 weeks of gestation, the PGC's migrate through the hindgut, into the mesonephric mesoderm and colonize the gonadal ridge [11, 12]. The migration is influenced by BMP [13, 14], chemotactic and adhesive peptides [15], integrins [16], and the c-kit/steel signaling pathway [17]. Interaction of the extracellular proteins fibronectin and laminin [18] also play a role in their migration. PGCs proliferate by via mitosis during migration, resulting in several thousand of these cells populating the indifferent gonad [19].

**Primordial follicle development**

The cells of coelomic epithelium and mesonephros proliferate to form cord-like structures called primary sex cords and cortical sex cords, respectively. The primary sex cords develop along the periphery (cortex) of an indifferent gonad, and as they grow, they penetrate interiorly (medulla). In the ovary, they finally degenerate and are replaced by cortical cords. The PGC then differentiate into primary oocytes. The cortical cords envelope the primary oocytes and differentiate into primordial follicles**.** Thus, the primordial follicle basic structure consists of a primary oocyte surrounded by a single layer of flat, pre-granulosa cells derived from supporting somatic cells [20].

Around the 20th week of gestation, there are approximately 7 million germ cells in each ovary. Autocrine/paracrine communication between the oogonia and granulosa cells is essential for primordial follicle formation [21]. Oogonia death (atresia) via apoptosis occurs if germ cells fail to associate with pre-granulosa cells. Bcl-2 and Bax regulate cell atresia via opposing actions. Increased Bcl-2 gene expression protects against atresia, whereas increased expression of Bax promotes germ cell death [22]. Mice null for factor in the germline α (Fig α), a transcription factor, are characterized by failure of primordial follicle development [23]. Gap junction proteins connexin 43 [24] and connexin 37 [25] also have a regulatory role in primodial follicle development.

**Folliculogenesis**

Folliculogenesis is a continuous process consisting of the following stages: primordial follicle formation, primordial follicle recruitment, preantral follicle development, antral follicle development, selection and growth of preovulatory follicle [26]. The change of flat, squamous-like granulosa cells of a primordial follicle, into a single layer of cuboidal granulosa cells, marks the formation of a primary follicle [27, 28]. The primary follicle is converted to a secondary follicle by: an increase in number of granulosa cell layers, formation of the zona pellucida and appearance of theca cells through recruitment of the surrounding stromal cells [29]. These changes define the preantral stage of follicular development.

The secondary follicle undergoes further growth changes to become a tertiary follicle. The early antral follicle is also referred to as a tertiary follicle. The hallmark of this stage is the formation of a fluid filled cavity called the antrum. Two theca cell

layers, the externa and interna, form on the outer surface of follicle [30]. Thecal layers developed from the ovarian stroma become highly vascularized, enabling the follicle to obtain a supply of circulating gonadotropins. In addition, the hormones secreted by follicle will be distributed to target organs including the pituitary, reproductive tract and mammary glands, to name a few.

Fully developed antral follicles become Graffian follicles. The granulosa cells of Graffian follicle differentiate into several subtypes: cumulus GCs that surround the zona pellucida, mural GCs that are interior to the basal lamina, and antral GCs that are adjacent to the antrum [29]. Development of the GC subtypes is due in part to the cell types and the matrix with which they interact.

FSH stimulation plays a major role in selection of dominant follicle from a cohort of tertiary follicles [31]. Increased uptake of gonadotropins by the dominant follicle, functions to drive proliferation of granulosa cells and differentiation of thecal cells. This creates a scenario wherein other tertiary follicles receive significantly less hormonal support/primarily FSH, resulting in their demise via atresia.

**Ovulation**

The expulsion of the oocyte from the ovary is called as ovulation. The mid-cycle LH surge is a key event that initiates ovulation [32]. Proteolytic enzyme release and protease inhibitor inhibiton results in localized degradation of underlying tissues, causing the surface epithelial cells of oocyte to detach from the basement membrane. The oocyte is expelled from the preovulatory follicle through an opening called the stigma. Post ovulation, the residual follicle cells (GC, TC, & endothelial cells) collapse and

differentiate into the corpus luteum. The corpus luteum is highly vascularized [33, 34], that is essential for its development and survival. Progesterone secreted by the corpus luteum supports early pregnancy following fertilization of the oocyte and implantation. Failure to develop an implantable embryo causes the corpus luteum to degenerate into a non-functional ovarian mass called the corpus albicans [35].

**Factors influencing folliculogenesis**

Ovarian follicular growth is regulated by gonadotropins, IGF-1 [36, 37], inhibin, activin [38], GDF-9 [39], BMP [40], FGF [41], estrogen, progesterone [42], androgen and Wnt-4. These factors act at defined stages of follicular growth. The importance of each factor has been documented by specific gene mutations. Follicular growth is impaired beyond the pre-antral stage in mice, null for FSH-R or FSH-β subunit. Mice null for IGF-1, show decreased follicular growth in the ovary. FSH action in granulosa cells is enhanced by IGF-1 [36].

The Wnt/Frizzled signaling pathway impacts gonadal development [43]. Wnt-4 is required for embryonic development of the ovary. Sex-reversed ovaries are observed in female mice null for Wnt-4. Estrogen receptor isoforms (ER)α and ERβ in granulosa cells are important for normal GC growth. Impairment of follicle growth and reversal of female phenotype is observed in mutations involving both estrogen receptors [44, 45].

Kit ligand [46], FGF, BMP-7, BMP-15 and GDF-9 regulate the transition of a primordial to primary follicle. KL and FGF have synergistic activities. BMP-7 increases granulosa cell proliferation [47]. BMP-15 inhibits FSH-mediated progesterone production in late-stage granulosa cells and stimulates pre-antral granulosa cells [48, 49].

In mice lacking GDF-9, single layers of primordial and primary follicles are seen, but further follicular development is blocked, resulting in infertility [39].

**Follicle-Stimulating Hormone (FSH)**

FSH is a gonadotropin secreted by the anterior pituitary gland [50]. FSH, TSH, LH and hCG belong to the glycoprotein hormone family [51]. These four hormones are heterodimers, having a common α subunit, and an unique β subunit [52]. Although the β subunit confers specificity in binding to its receptor [53], both subunits are essential to contact the receptor and drive post-receptor signaling [54]. Both subunits are noncovalently associated and are inactive individually.

FSH α subunit: The gene encoding the α subunit is located on chromosome 6 [55] and consists of four exons separated by three introns. The α subunit mRNA has 730-800 nucleotides, encoding a leader sequence of 24 amino acids followed by mature protein sequence of 92 amino acids [56, 57]. The α subunit gene structure has three domains [58]. The first domain is not involved in biological activity or dimer assembly of the hormone. The latter two domains show homology of 70-90% among mammals. The α subunit gene is transcriptionally activated through the cAMP/PKA pathway [59]. The cAMP/PKA response element (CRE) of the α subunit gene promoter binds to nuclear factor CRE-binding protein (CREB), activating gene transcription [60]. PKC and calcium-calmodulin protein kinase also activate transcription of the α subunit gene.

Glycosylation of FSH plays a major role in its bioactivity and clearance. Deglycosylation at $Asn^{52}$ impairs signal transduction, but not binding activity, indicating

that receptor binding and signal transduction are two different functions involving different residues [52]. Glycosylation at $Asn^{78}$ is vital for thermal stability of FSH [61].

FSH β subunit: The human β subunit of FSH is encoded by gene located on chromosome 11 and consists of three exons and two introns [62]. The β subunit mRNA encodes a sequence of 111 amino acids and both the nucleotide and amino acid sequences are highly conserved among species. The first exon contains the 5' UTR, $2^{nd}$ and $3^{rd}$ contain coding sequence. The FSH β subunit is unique from other glycoprotein hormones by having a very long 3' UTR and encoded by $3^{rd}$ exon [60, 63]. FSH β subunit gene expression is regulated by follistatin, inhibin, activin and GnRH [64-66]. Inhibin selectively suppresses FSH synthesis and secretion. Secretion of FSHβ subunit is increased by activin, while follistatin inhibits indirectly by binding to and bioneutralizing the effects of activin [67]. Low frequency pulsatile GnRH levels favor pituitary expression of activin, resulting in more FSH β mRNA. Studies have shown that pituitary adenylate cyclase-activating polypeptide suppresses FSHβ mRNA [68]. FSHβ glycosylation affects disulfide bond formation and the rate of secretion [52].

**Follicle-Stimulating Hormone Receptor (FSH-R)**

The FSH-R belongs to the G-protein coupled receptor family [50]. The FSH-R gene is located on chromosome 2p21 [69], spans a region of 54kbp and encodes 678-695 amino acid residues [70]. In humans, the FSH-R gene consists of 10 exons and 9 introns. The first 9 exons code for the $NH_2$ terminal, extracellular domain, and the $10^{th}$ exon codes for seven transmembrane spanning region and intracellular COOH terminus [71].

The binding of FSH to the extracellular domain of receptor, initiates a cascade of intracellular events, leading to specific biological responses to FSH. The intracellular terminus is coupled to a G-protein, which activates adenylyl cyclase to stimulate the synthesis of cAMP, which in turn activates Protein Kinase A [50]. Studies have shown other signal transduction pathways involved in FSH action. These include protein kinase B/phosphotidylinositol-3kinase pathway(PI-3k), Ras/Raf/MAPK pathway [72], and stimulation of intracellular calcium via phospholipase C [73].

Mutations, single nucleotide polymorphisms (SNP) and isoforms of FSH-R can influence the ovarian response to FSH stimulation. Female mice mutant for the FSH-R have small ovaries and are sterile because of a block in folliculogenesis [74]. The homozygous Ala307-Asn680 variant is associated with higher basal FSH levels and more FSH needed for ovarian stimulation in women undergoing assisted reproduction [75]. Alternate splicing of FSH-R mRNA potentially gives rise to different receptor isoforms [76]. Such isoforms could modify FSH action by interfering with intracellular signaling events or modifying hormone binding affinity. Two SNP's in exon 10 gave rise to allelic variants: Thr307-Asn680 (TN) and Ala307-Ser680 (AS). Recent studies showed a significant association between TN/AS genotype and polycystic ovary syndrome [75]. A C-T point mutation in nucleotide 566, resulting in Ala-Val substitution, leads to hypergonadotrophic ovarian dysgenesis due to decreased FSH-R [77].

**The Aging Human Ovary**

At 5 months gestation, the human ovary contains 6-7 million germ cells [78]. Most of these germ cells undergo apoptosis and numbers decrease to 1-2 million at birth.

Further, during infancy, childhood, adolescence and the reproductive years, follicle numbers are depleted at the same rate [79]. During perimenopause there is a rapid decline in follicle numbers [80]. Ovarian volume decreases as the woman ages. Studies have shown an association between deviations in reproductive hormone patterns and reproductive aging. Shortened follicular phase, increased follicular phase estrogen levels and reduced luteal phase progesterone levels have been reported [83].

Follicle growth rates are slower in older women than younger women [81]. Aging ovaries exhibit decreased responsiveness to gonadotropin stimulation [82]. Follicles of older women contained fewer granulosa cells than younger women, suggesting a decreased response to FSH. Any impairment in follicular response to FSH affects follicle maturation, the end result being anovulation. This impairment could be due to a decreased number of FSH-R in granulosa cells and/or alteration of FSH-R structure via alternate splicing of its mRNA. While there is strong evidence showing clinical and physiological ramifications of decreased ovarian function during the perimenopause, little is known at the molecular level. Our main goal is to investigate the decreased follicular response to FSH in an aging human ovary, at the FSH-R level.

**Preliminary studies**

Preliminary studies using data from clinical assisted reproductive procedures were undertaken to look for evidence of age-dependent decreased responsiveness of the human ovary. This was made possible through data provided by the Center for Reproductive Medicine, Wichita, KS. Initially, the subjects were treated with GnRH agonist at levels that suppressed endogenous gonadotropin secretion followed by the administration of

large doses of exogenous gonadotropin to stimulate follicle development. Follicle development was monitored by serum estradiol levels and ultrasonography. By employing controlled ovarian hyperstimulation (COH), we can rule out endogenous gonadotropin variability as a causative factor in decreased follicular response. Moreover the ovaries are maximally challenged by gonadotropin in terms of follicle development. Women undergoing COH were retrospectively monitored for number of oocytes retrieved (Figure 1), amount of exogenous gonadotropin required to stimulate the ovary to achieve follicle development (Figure 2), and the length of stimulation period (Figure 3). The results indicated that older women required greater amounts of exogenous gonadotropin, over a longer period of stimulation and produced fewer oocytes. These results supported those studies done by Jacob et. al. [82] and suggest that follicular response to gonadotropin declines with age.

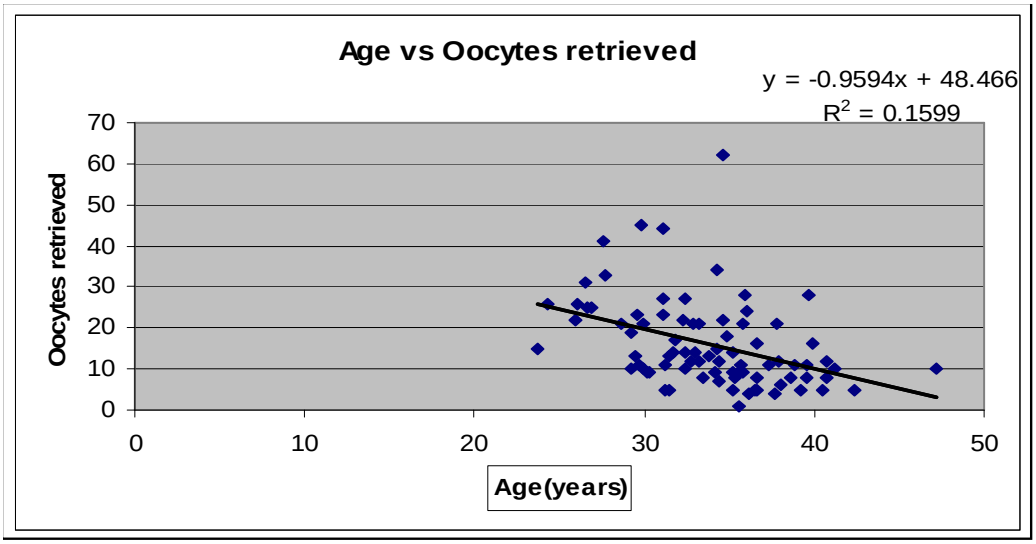


**Figure 1.** The effect of age upon the number of oocytes retrieved during COH for assisted reproductive procedures (p value =0.0002).

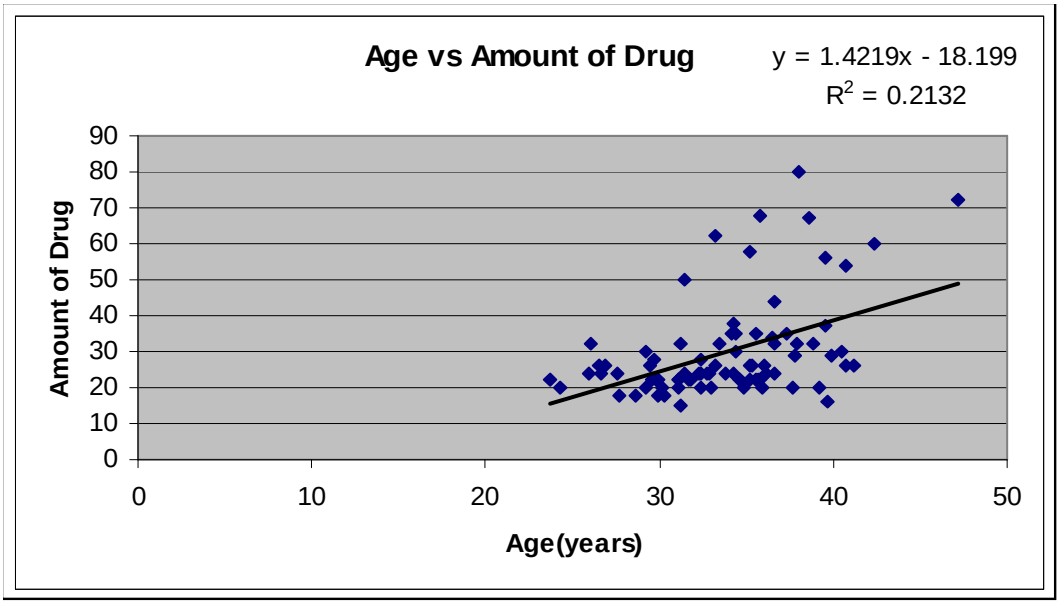


**Figure 2.** The effect of age upon the amount of exogenous gonadotropin used to stimulate the ovary for achieving follicle development (p value<0.0001)

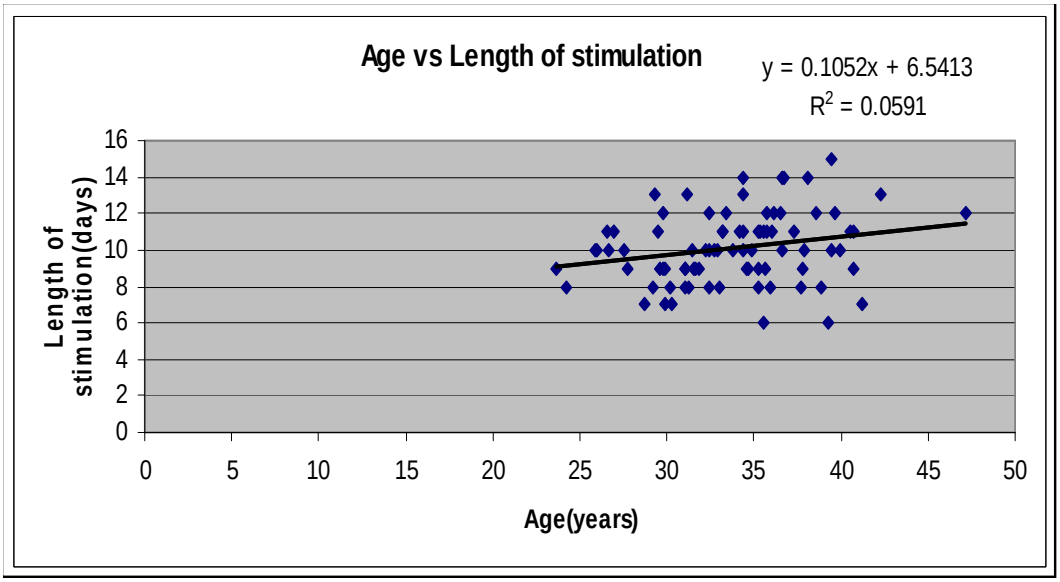


**Figure 3**. The effect of age upon the length of the stimulation period required to mature follicle for oocyte retrieval (p value=0.0277).

## AIM #1

DETERMINE THE EFFECT OF AGE UPON FSH-R mRNA EXPRESSION DURING HUMAN OVARIAN FOLLICLE DEVELOPMENT.

It is hypothesized that decreased ovarian responsiveness to FSH is mediated by decreased expression of FSH-R in an aging human ovary. To assess this, FSH-R mRNA levels will be measured using real time PCR and statistically analyzed by analysis of variance and regression analysis.

## AIM #2

DETERMINE THE EFFECT OF AGE UPON ALTERNATIVE SPLICING OF FSH-R.

FSH-R is known to exhibit alternate splicing, variations in receptor mRNA leading to modifications of the full length receptor. It is hypothesized that decreased ovarian responsiveness to FSH is mediated by alternate splicing of FSH-R in the aging human ovary. To assess this, RT-PCR was performed on samples of total RNA from human ovarian follicles (HOF), using the primer sets defined in Table 4. The PCR products were subjected to agarose gel electrophoresis, extracted from agarose gel and then submitted for sequencing.

## Methods and Materials

Human ovarian samples were obtained as fresh tissue from oophorectomies performed on women aged 26-50 years old. Ovaries not involved with ovarian cancer were used to provide follicles for analysis. Samples were obtained through an IRB-approved collaboration with Department of Pathology at Wesley Medical Center, Wichita, KS. Follicles were measured across their diameter on the ovary surface, bisected with a scalpel. The 'hemi-follicles" were teased out of the ovary, combined, snap frozen and stored at -80°C. These represent the archived material used in this study.

### Total RNA isolation

Total RNA was extracted from follicles using Ultraspec RNA reagent (Biotecx Inc, TX). Each follicle (i.e 2 hemifollicles) was homogenized in 1 milliliter of Ultraspec reagent with individualized microfuge tube homogenizing pestles which had been cleaned and autoclaved. DEPC-treated microfuge tubes containing homogenized follicles were incubated on ice for 5 minutes. Aliquots of 0.2 ml chloroform were added to each sample and the tubes incubated again for 5 minutes on ice with frequent shaking every 15-30 seconds. The tubes were then centrifuged at 12,000 rpm, at 4°C for 15 minutes. The aqueous supernatant was transferred to a fresh, DEPC-treated microfuge tube and an equal volume of cold isopropanol was added. The tubes were stored overnight at -20°C. Precipitated RNA was centrifuged at 12,000 rpm for 30 minutes at 4°C. The supernatant was discarded and the white RNA pellet washed twice using 1 ml of 75% ethanol in DEPC-treated water. Tubes were centrifuged at 7,500 rpm, 4°C for 5

minutes, following each wash. The RNA pellet was dried for 5-10 minutes using a Savant vacuum dryer. The dried RNA pellet was dissolved in 25 µl of nuclease free water. Total RNA content was determined via spectroscopy at 260/280 nm using an Eppendorf biophotometer. RNA samples were then stored at -80°C until utilized.

**Reverse transcription**

1µl of total RNA from samples was subjected to reverse transcription using a kit from Invitrogen (#18080-400). Samples were combined with reagents as shown in Table 1, and incubated at room temperature, 45°C and 94°C for 10, 30 and 5 minutes, respectively. The final RT product i.e, cDNA, was stored at -20°C until utilized.

Only for samples used to study Aim#2, 5 µg of total RNA was reverse transcribed instead of the usual 1µl. The same kit was employed and the protocol described above was followed.

**Table: 1**. Reverse transcription reaction components.

| Components | µl/reaction |
|---|---|
| 2X RT reaction mix | 10 |
| RT enzyme mix | 2 |
| RNA | 1 |
| Nuclease free water | 7 |
| Total volume | 20 |

**Real time PCR**

1µl of cDNA from human ovarian follicle (HOF) was subjected to real time PCR using a kit from Takara Bio (TAK#RR041A) using a Cepheid Smartcycler II. Human FSH-R specific primers were developed using Netprimer software

(http://www.premierbiosoft.com/netprimer/). The primers used are shown in Table 2. These primers exhibited neither intra- nor interchain dimerization nor stem loop structures. The primers amplify a 266 bp region of the extreme 5'end of the FSH-R extracellular domain mRNA sequence. The samples were mixed with reagents and primers as shown in Table 3. The protocol consisted of: 94°C melt for 10 seconds, followed by 35 cycles maximum of 94°C-6 sec, 64°C-30 sec, 72°C-15 sec, followed by a melt over a range of 60-95°C. The cycle threshold (Ct) values were noted at the end of the real time PCR reaction.

**Table 2.** Primers used for real time PCR.

| Name of primer | Direction of primer | Sequence |
|---|---|---|
| FSH-up | Sense | 5'-CCCTGCTCCTGGTCTCTTTGC-3' |
| FSH-dn | Antisense | 5'-CACATCTGCCTCTATCACCTCC-3' |

**Table 3.** Real time PCR reaction components.

| Components | µ l/reaction | Final concentration |
|---|---|---|
| 2X PCR mix | 12.5 | 1X |
| FSH-up primer | 1.5 | 0.3 µ M |
| FSH-dn primer | 1.5 | 0.3 µ M |
| cDNA | 1 | Variable |
| Nuclease free water | 7.5 | --- |
| Total | 25 | --- |

**FSH-R DNA Standard Curve**

Chinese Hamster Ovary (CHO) cells transfected with human FSH-R were obtained from Dr.George Bousfield, Department of Biological Sciences. Total CHO cell RNA was isolated, 1µg of RNA was reverse transcribed as shown in Table 1, and 5µl of cDNA was subjected to standard PCR as shown in Table 5, using primers shown in Table 2. PCR product was subjected to agarose gel electrophoresis, and the appropriate bands were extracted using QIAEXII gel extraction kit (Qiagen, CA). The DNA was measured using an Eppendorf biophotometer. Triplicate reactions of real time PCR were performed over a 10,000-fold range of the CHO cell hFSH-R PCR product DNA (100ng-10pg) and the cycle threshold (Ct) values were recorded. The arithmetic mean was calculated for Ct values from triplicate values. A Standard curve was generated from the Ct values obtained as shown in Figure 10. Unknown values of FSH-R cDNA (reflecting mRNA levels) from HOF samples were calculated using the standard curve and normalized on a whole follicle basis.

**Normalization of mRNA Levels on Whole Follicle Basis**

Follicle total RNA preps were resuspended in 25µl of nuclease free water. 1µl of total RNA was mixed with components as shown in Table 1, to obtain a final volume of 20µl and subjected to RT to obtain RT product/cDNA. Further 1µl of cDNA was subjected to realtime PCR and Ct values recorded. The Ct value of follicle sample was extrapolated on the standard curve to calculate the unknown FSH-R cDNA (reflecting mRNA levels). These FSH-R cDNA levels calculated using the standard curve,

represents the level values measured from utilizing only 1µl of total RNA (total volume = 25µl) and 1µl of cDNA (total volume = 20µl). To calculate the whole follicle mRNA levels, the following formula was used.

$$\text{Amount/1 µl} = X / 25 \times 20\text{µl}$$

The FSH-R mRNA levels obtained for follicle sizes 3-7 mm are illustrated in Figures 11-15, respectively. The Graphpad Instat V3.06 statistical program, Sandiego, CA, USA was used to perform ANOVA and linear regression analysis to analyze statistical significance.

**Standard PCR**

For Aim#2, standard PCR was performed using a kit from Takara Bio(TAK#RR041A) in a Thermolyne Amplitron II thermal cycler. The primers used in Aim#2 are shown in Table 4. 5µl of cDNA was mixed with the reagents and primers as shown in Table 5. The protocol consisted of: a 94°C melt for 1 min, followed by 35 cycles maximum of :94°C-30 sec, 60°C-30 sec, 72°C-2 minutes, followed by 72°C final extension for 5 minutes, and the thermal cycler reduced the temperature to 4°C until the run was stopped.

The goal was to produce overlapping PCR products and have them sequenced. The FSH-R mRNA would then be determined by aligning overlapping regions, assessing the sequence, and comparing the composite sequence to Genebank FSH-R sequences.

**Table 4**. Primers used for standard PCR.

| Primer | Direction | Sequence (5'-3') | Length of PCR product |
|---|---|---|---|
| FSH-up | Sense | CCCTGCTCCTGGTCTCTTTGC | 924 bp |
| P5 | Antisense | GACCCCTAGCCTGAGTCATAT | |
| P0 | Sense | ACCTGCTCTACATCAACCCTG | 1747 bp |
| P6 | Antisense | GAGGGACAAGTATGTAAGTGGAACC | |

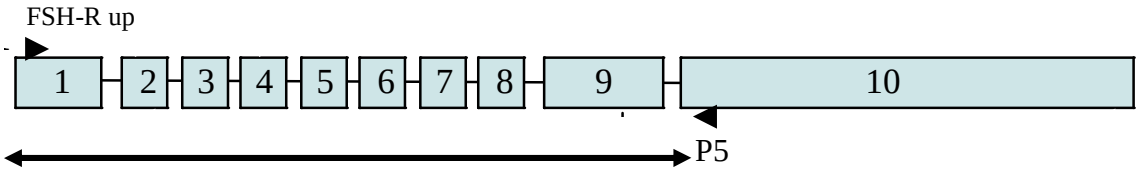


**Figure 4.** The numbers 1-10 denote the exons of FSH-R. FSH-R up and P5 indicates the sense and antisense primer set expected to amplify a 924 bp PCR product.

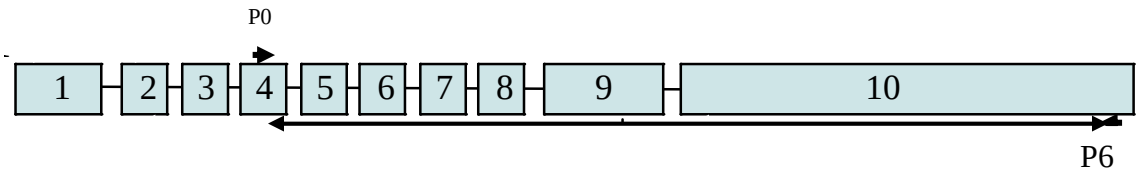


**Figure 5**. The primer set P0 and P6 amplify a PCR product of 1747 bp spanning exons 4-10.

**Table 5.** Standard PCR reaction components.

| Components | μ l/reaction | Final concentration |
|---|---|---|
| 2X PCR mix | 25 | 1X |
| Sense primer | 1 | 0.2 μ M |
| Antisense primer | 1 | 0.2 μ M |
| cDNA | 5 | Variable |
| Nuclease free water | 18 | --- |
| Total | 50 | --- |

**Gel electrophoresis and DNA sequencing**

PCR performed on samples used to investigate Aim#2 were subjected to gel electrophoresis on a 2% agarose gel containing ethidium bromide. A 1 kb DNA marker was employed to estimate size. Gel bands were visualized under UV light and photographed using a Kodak EDAS Photo Documentation System.

Extraction of PCR products from agarose gels was undertaken using QIAEXII gel extraction kit (Qiagen, CA). DNA content (PCR products) was determined using an Eppendorf biophotometer. 100 nanograms of extracted PCR product and 25 μl of primer pair (2 pmol/μl) were sent to Kansas University Medical Center Biotechnology Facility in Kansas City, KS for sequencing.

# RESULTS

The real time PCR raw data are essential for calculating mRNA levels. After performing real time PCR, we analyzed the melt curve and recorded the Ct values. Melt curve indicates a specific temperature (melting temperature) when the PCR product melts i.e, the two DNA strands separate, which is observed as a sudden decrease in PCR product fluorescence intensity. A single, sudden decline of fluorescence during a melt curve for a PCR product at a high temperature, indirectly indicates a specific amplicon being amplified during PCR run. Two or more fluorescence intensity declines for a PCR product in a melt curve suggests contamination, primer-dimer formation, etc.

Figures 6 and 7 illustrate representative melt curves for PCR products of CHO cell DNA and HOF cDNA, respectively. The melt curve of PCR for CHO cell DNA had to be analyzed before we proceed and further in testing our follicle samples. Real time PCR was performed over a 10,000 fold range of the CHO cell hFSH-R PCR product DNA.

Figure 6 clearly indicates a single and sudden decline in fluorescence at high temperature for each PCR product. This suggests that over a wide range of DNA template, a specific amplicon i.e only the target amplicon is amplified. The values of melting temperature range between 85.67- 85.93°C.

Figures 8 and 9 illustrate a representative Ct curve for PCR products of CHO cell DNA and HOF cDNA, respectively. The Ct values recorded are shown in Table 6 and 7. It was observed that the CHO cell DNA amount added in the PCR reaction and the Ct values obtained had an inverse relationship.

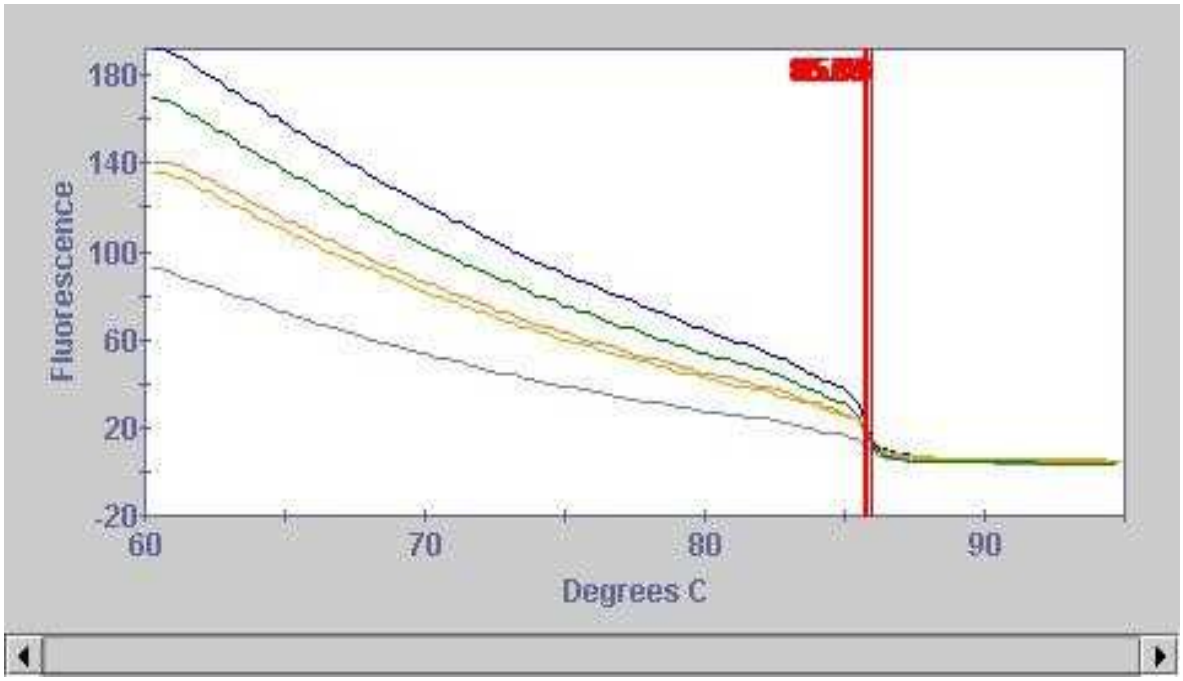


**Figure 6.** Fluorescence versus Temperature of melt curve for CHO cell DNA real time PCR products.

The above figure indicates the decline in fluorescence intensities of PCR products for CHO cell DNA. The closeness of the melting temperatures over a wide range of DNA template and the high melt temperature suggests that only target amplicon is amplified. The figure also clearly depicts there is no contamination, primer-dimer formation, etc. Refer to Table 6 for melting temperature values.

**Table 6**. Representative Ct and melting temperature values for CHO cell DNA real time PCR products.

| DNA | Ct value | Melting temperature(°C) |
|---|---|---|
| 100 ng | 19.14 | 85.68 |
| 10 ng | 21.72 | 85.73 |
| 1 ng | 25.63 | 85.81 |
| 100 pg | 29.16 | 85.67 |
| 10 pg | 31.75 | 85.93 |

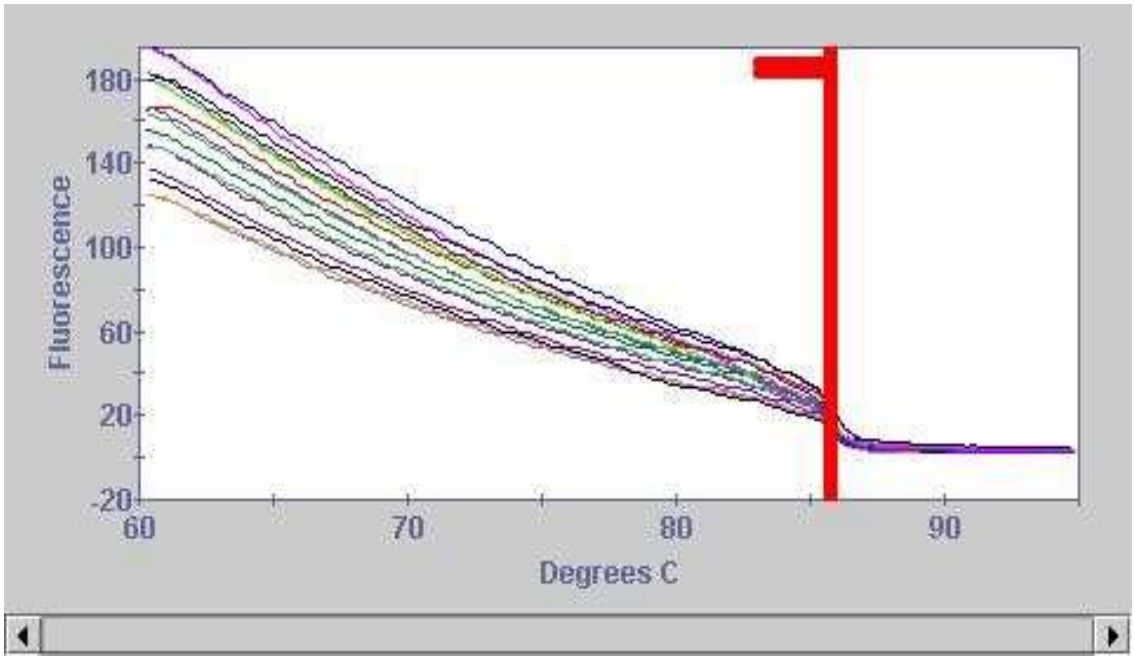


**Figure 7**. Fluorescence versus Temperature of melt curve for HOF cDNA real time PCR products.

In the above figure, sudden decline in the fluorescence intensities of the PCR products are similar. Refer to Table 7 for the melting temperature values.

**Table 7.** Representative Ct and melting temperature values for HOF cDNA.

| Sample | Ct value | Melting Temperature(°C) |
|---|---|---|
| 100 ng CHO cell DNA | 18.28 | 85.6 |
| 1 ng CHO cell DNA | 24.96 | 85.95 |
| 10 pg CHO cell DNA | 31.86 | 85.85 |
| HOF# 1 | 31.44 | 85.67 |
| HOF# 2 | 30.15 | 85.87 |
| HOF# 3 | 28.72 | 85.7 |
| HOF# 4 | 28.24 | 85.91 |
| HOF# 5 | 28.23 | 85.56 |
| HOF# 6 | 30.68 | 85.68 |
| HOF# 7 | 29.42 | 85.65 |
| HOF# 8 | 30.15 | 85.6 |
| HOF# 9 | 32.93 | 85.72 |
| HOF# 10 | 29.39 | 85.74 |
| HOF# 11 | 28.17 | 85.68 |
| HOF# 12 | 29.95 | 85.9 |
| HOF# 13 | 30.07 | 85.78 |

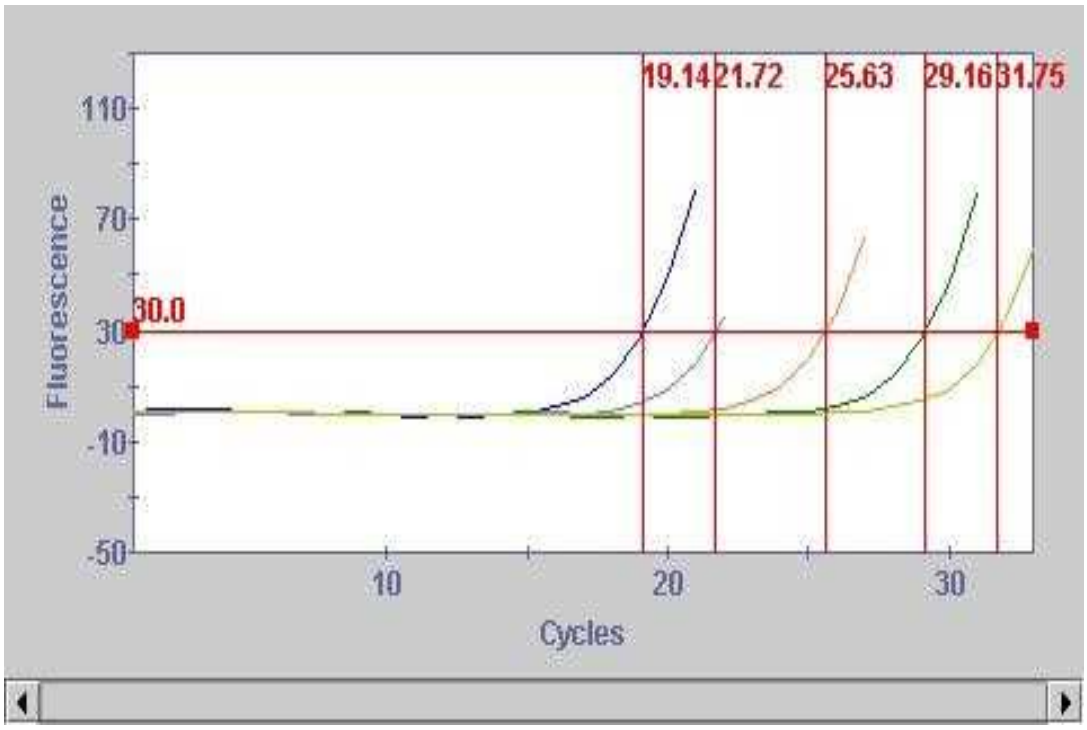


**Figure 8**. Fluorescence versus Ct for CHO cell DNA real time PCR products.

Each colored curve represents the PCR product amplification for a separate sample. The figure illustrates the exponential amplification of PCR products and the point they reach the threshold intensity level of 30, shown as the red horizontal line. The cycle of amplification curve, reaching the threshold is recorded as Ct value. Refer to Table 6 for DNA template levels and their corresponding Ct values. By analyzing the template levels and the Ct values, we can infer that as the template level decreases the Ct value increases.

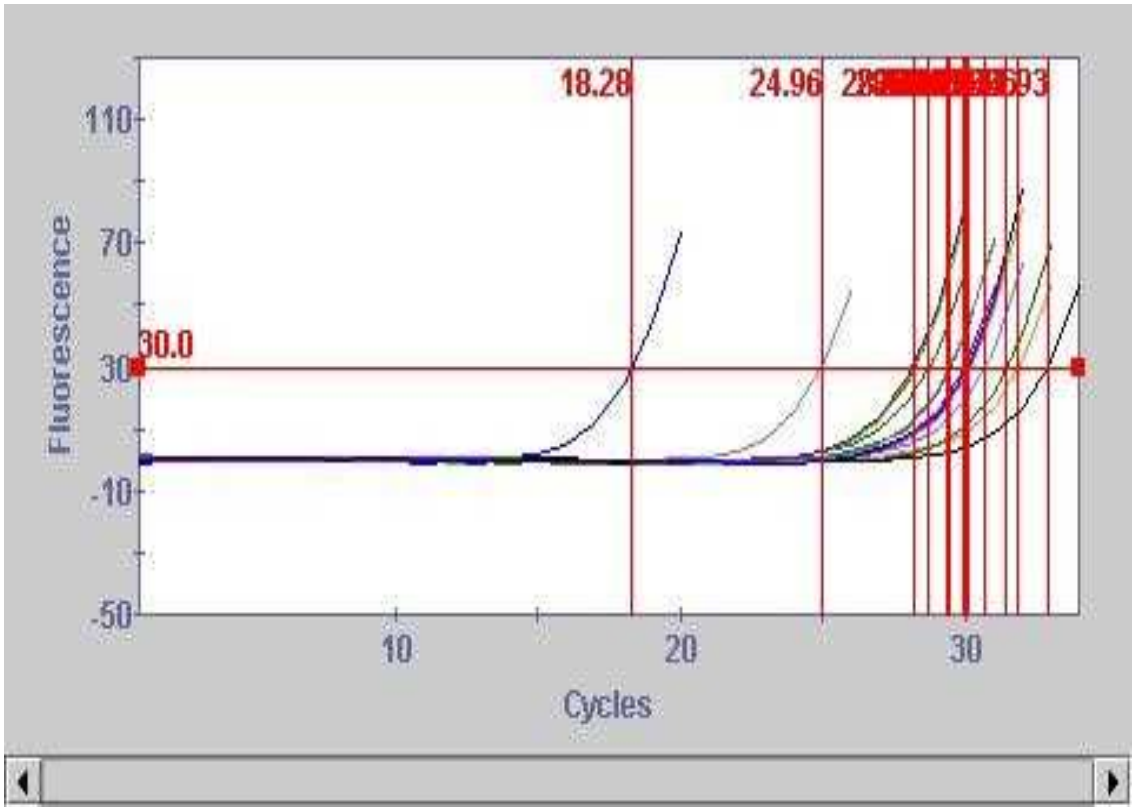


**Figure 9.** Fluorescence versus Ct for HOF cDNA real time PCR products.

Each curve in the figure illustrates fluorescence levels emitted by the PCR products during amplification. The Ct values for HOF samples are shown in Table 7. The Ct values recorded are then extrapolated on the standard curve.

The real time PCR standard curve generated using DNA made from CHO cells transfected with human FSH-R is shown in Figure 10.

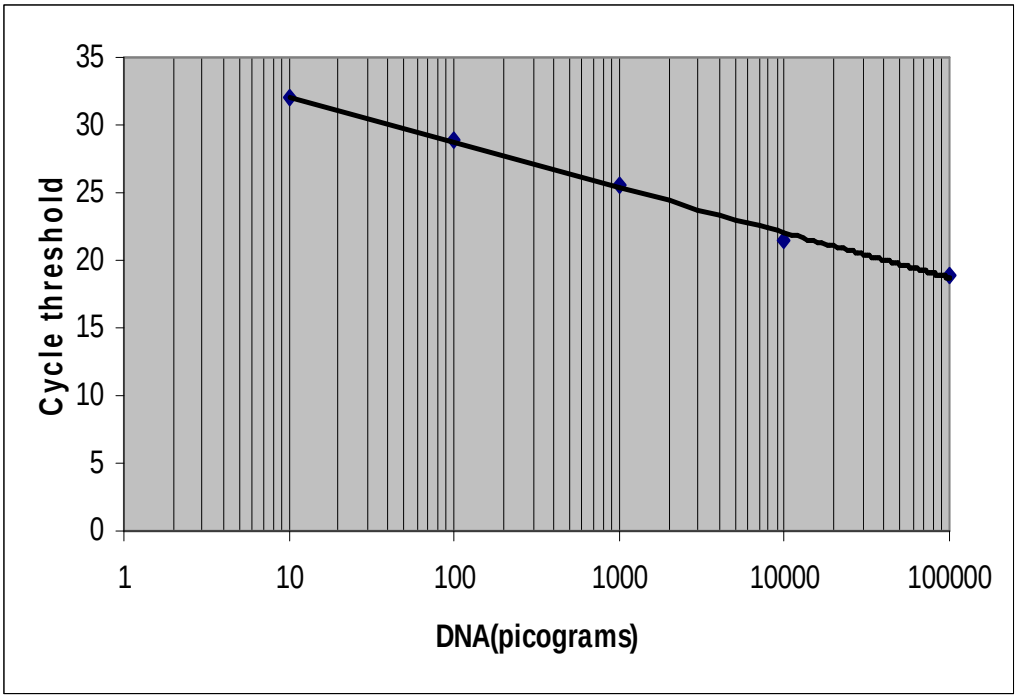


**Figure 10**. Real Time FSH-R DNA standard curve semi-log graph.

The above graph was plotted based on the Ct values (mean of 3 replicates) obtained from real time PCR for DNA levels of 10 picograms-100 nanograms. Linear regression analysis of the standard curve was significant ($p<0.0001$) indicating that it can be used to find the unknown values of FSH-R mRNA from the follicles tested.

Using the standard curve, the mRNA levels of 3-7 mm follicles were calculated. Further, the RNA values on a whole follicle basis were plotted as a function of each subject's age. The FSH-R mRNA levels from 3-7 mm follicles are plotted on figure 11-15 respectively.

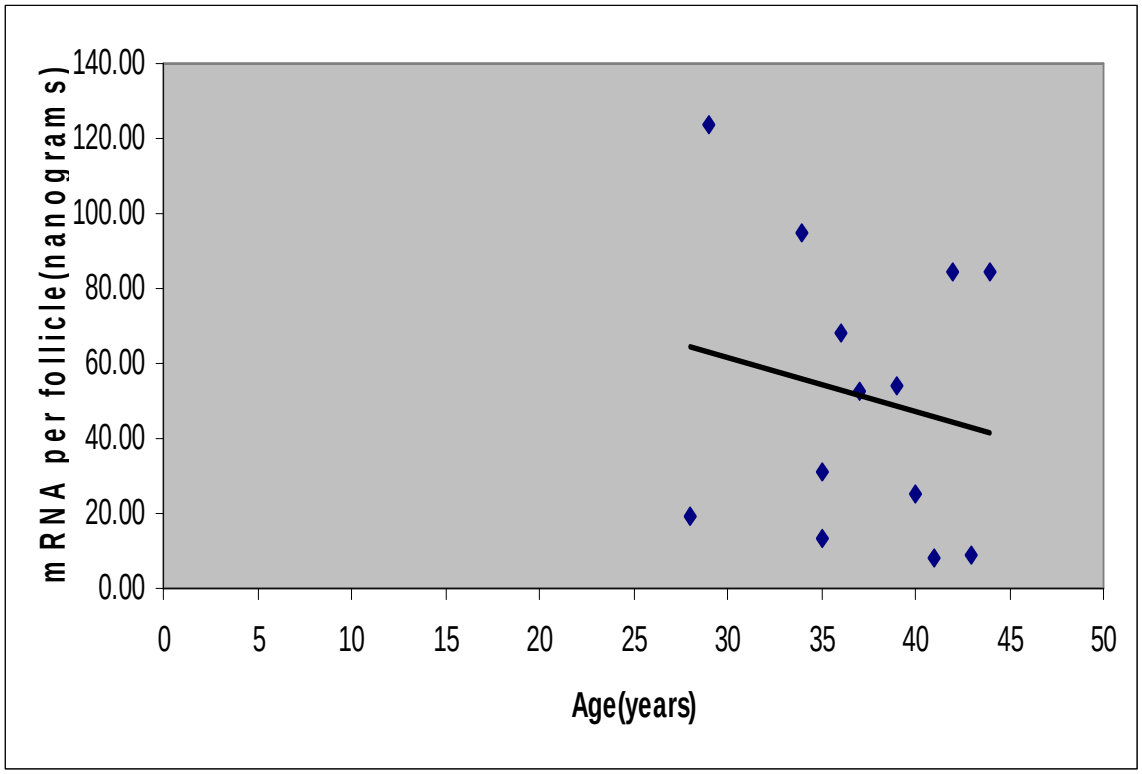


**Figure 11**. mRNA levels of FSH-R from 3 mm human ovarian follicles versus age of subject.

Thirteen samples were analyzed and the resulting mRNA levels on a whole follicle basis were plotted as a function of age of subject (p value=0.5388).

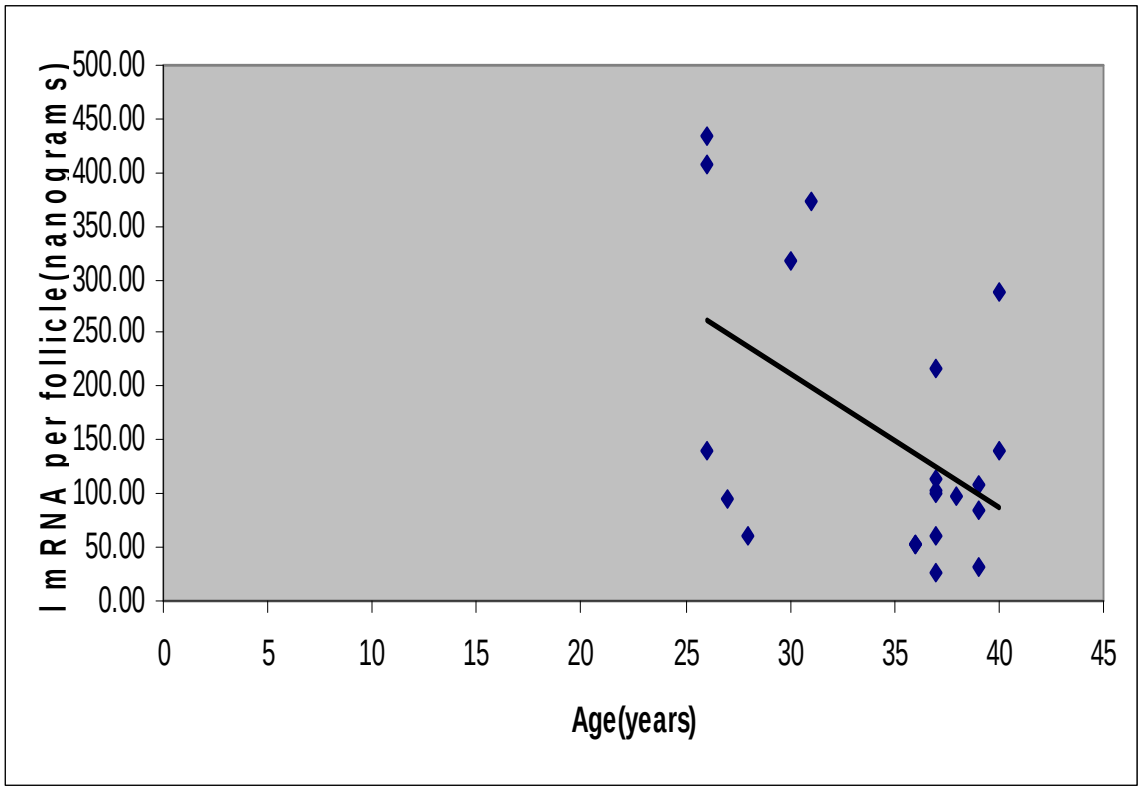


**Figure 12**. mRNA levels of FSH-R from 4 mm human ovarian follicles versus age of subject. Younger subjects showed generally higher FSH-R mRNA levels relative to older subjects (p value=0.0218).

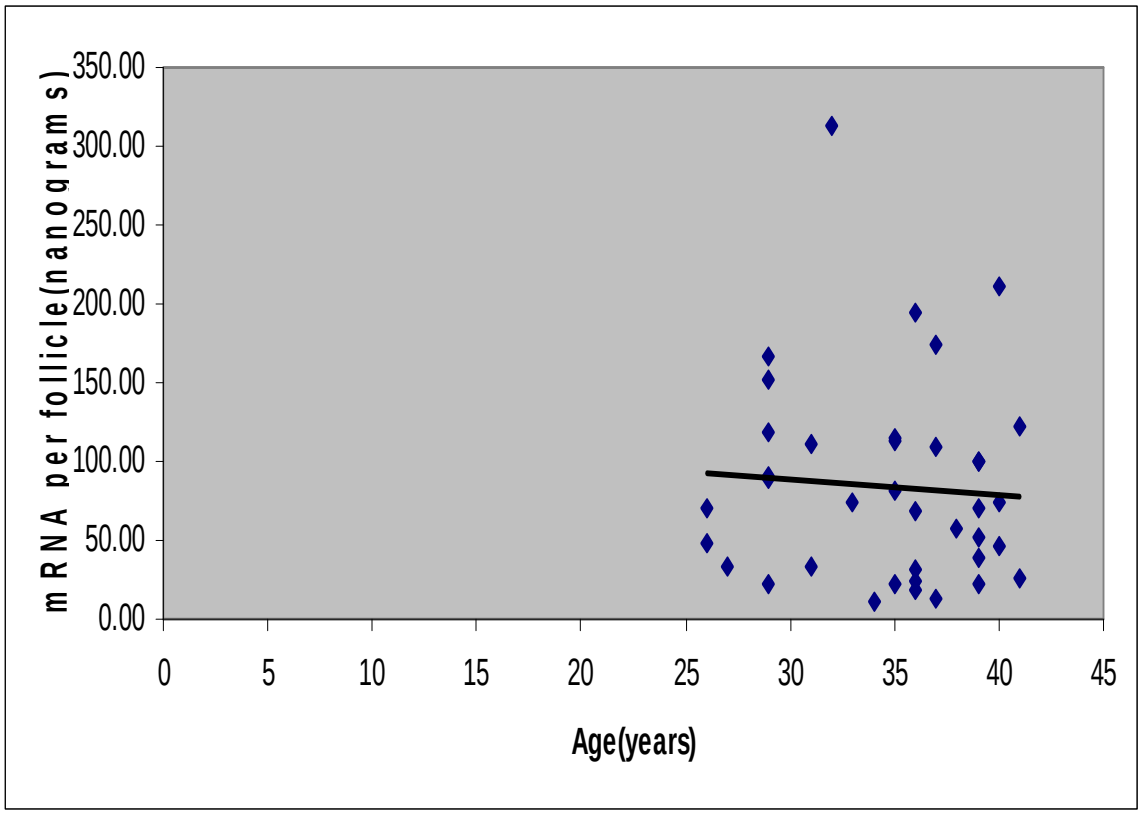


**Figure 13.** mRNA levels of FSH-R from 5 mm human ovarian follicles versus age of subject (p value=0.8534).

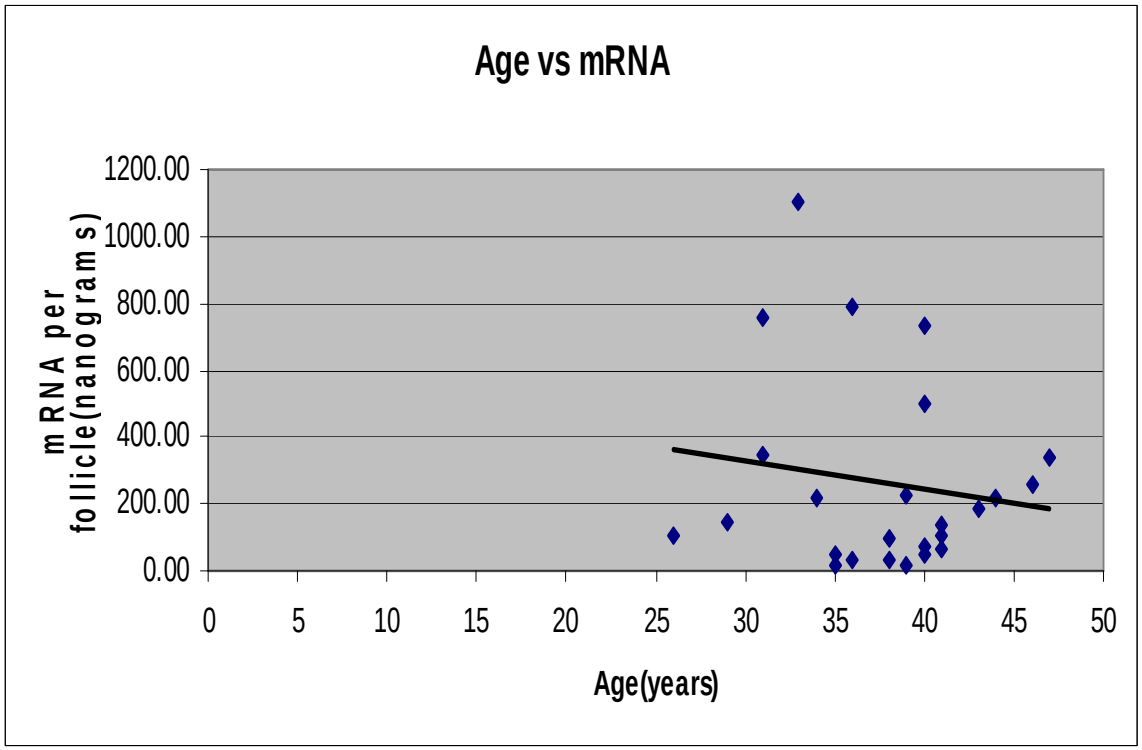


**Figure 14**. mRNA levels of FSH-R from 6 mm human ovarian follicles versus age of subject .

Subjects 35-45 years of age showed low, but variable levels of FSH-R levels (p value=0.4665).

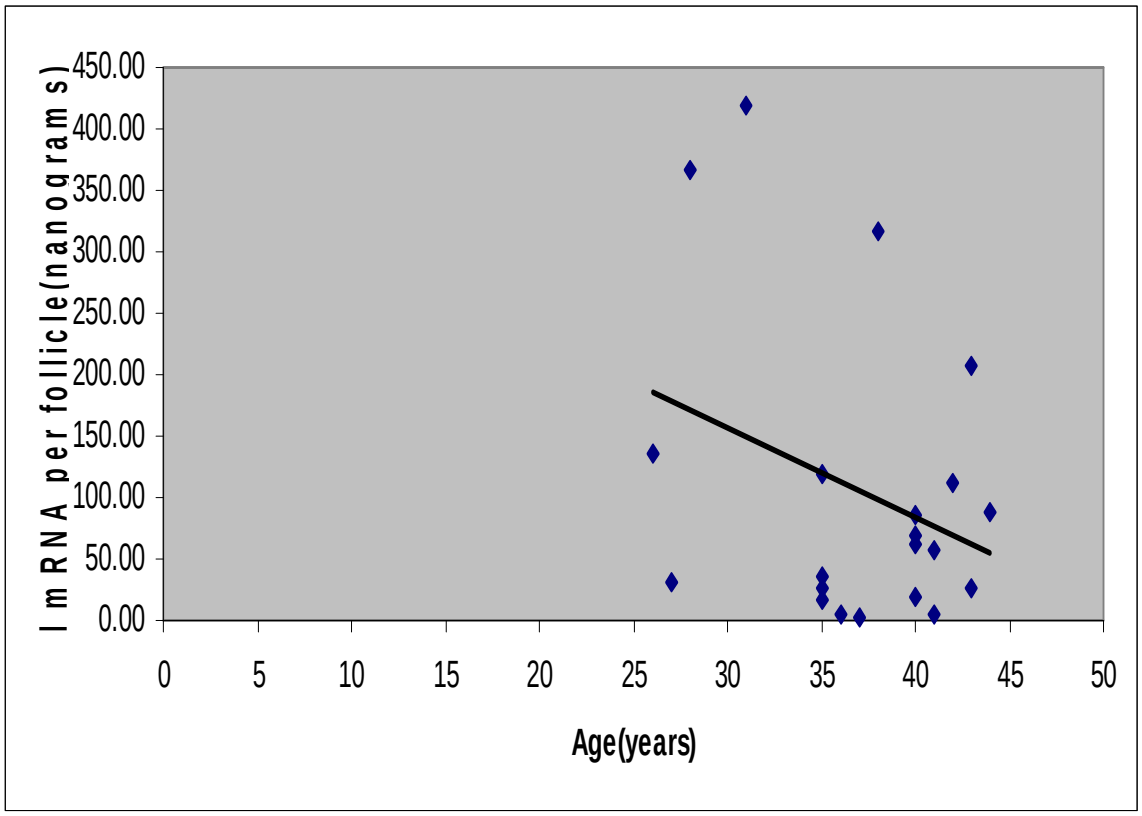


**Figure 15.** mRNA levels of FSH-R from 7 mm human ovarian follicles versus age of subject (p value=0.1578).

To determine if the FSH-R mRNA sequence changed with age via alternate splicing, standard PCR was performed on follicle samples using 2 primer sets. RNA from CHO cells and HOF were reverse transcribed and the cDNA subjected to PCR. The PCR products following electrophoresis and ethidium bromide staining are shown in Figure 16

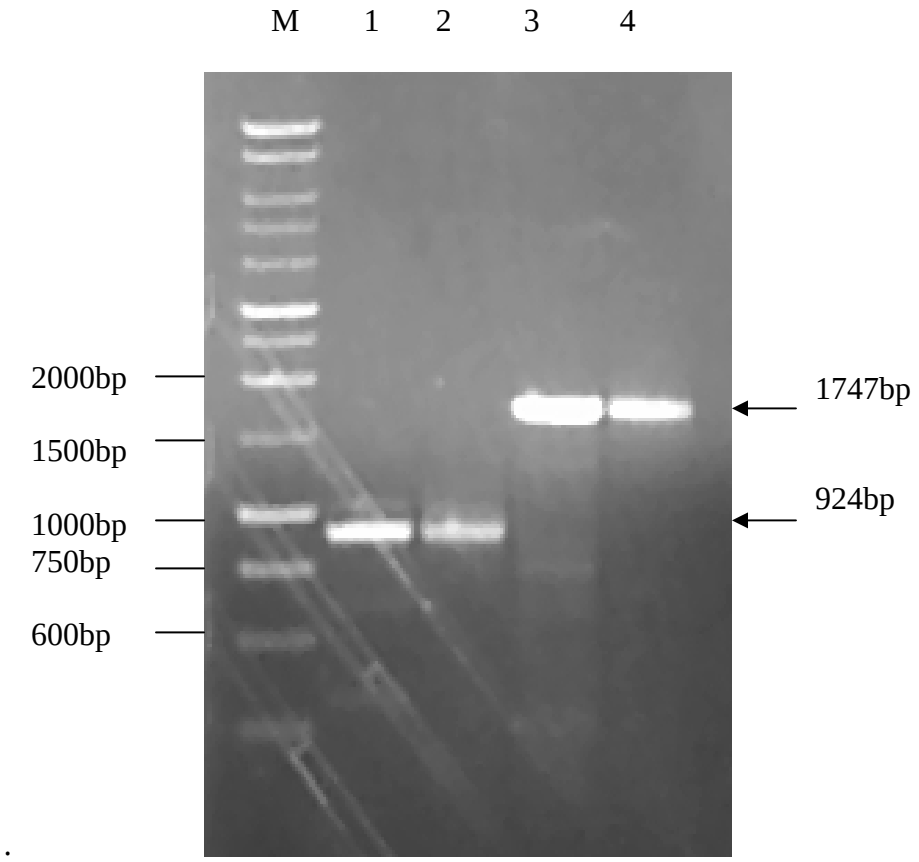


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**Figure 16**. RT/PCR of total RNA from HOF and CHO cells transfected with the hFSH-R cells using primer sets FSH-R up/P5 (lanes 1and 2) and P0/P6 (lanes 3 and 4). Lanes 1 and 3 are PCR products made from RNA of CHO cells. Lanes 2 and 4 are PCR products made from RNA from a 6 mm follicle sample of a 46 year old woman. PCR product sizes are indicated on the right margin and the numbers on left margin denote the sizes of DNA markers represented by lane M. The PCR product sizes were consistent with predicted estimates based upon the published FSH-R sequence.

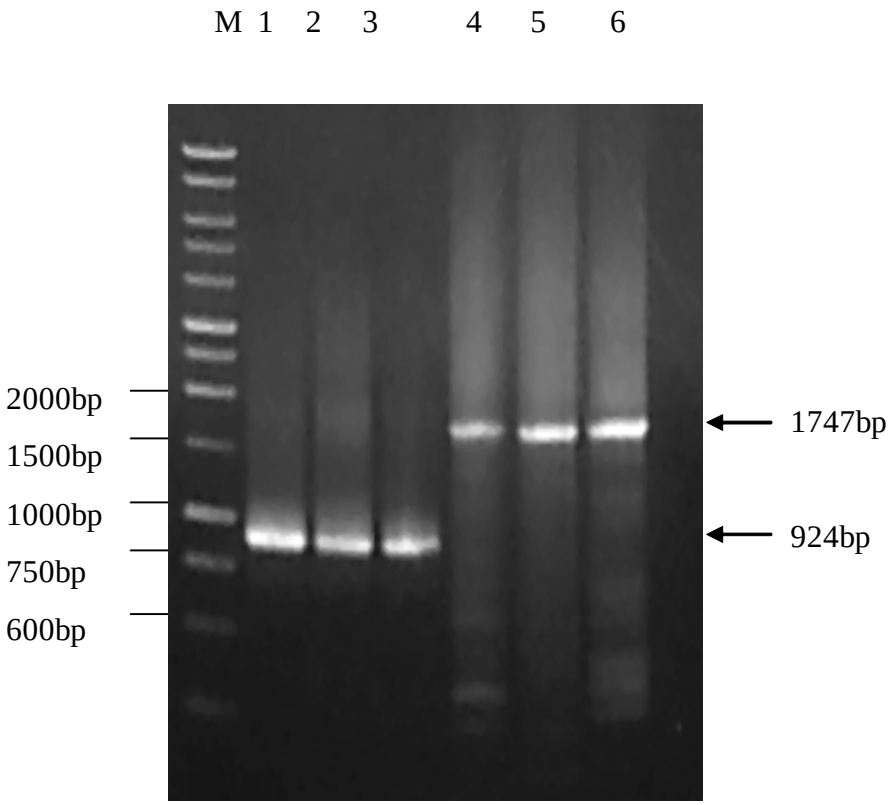


**Figure 17**. RT/PCR of RNA from HOF using primer sets FSH-R up/P5 (lanes 1, 2 and 3) and P0/P6 (lanes 4, 5 and 6). Lanes 1 and 4, 2 and 5, 3 and 6 represent PCR products made from RNA of 28, 36 and 40 year old women, respectively. PCR product sizes are indicated on the right margin and the numbers on left margin denote the sizes of DNA marker represented by lane M.

The above two figures clearly illustrate that the PCR products generated from total RNA from follicles of different aged ovaries were consistent with the expected size of 924 bp and 1,747 bp. This suggests that no major portion of FSH-R was deleted from the samples tested and that decreased ovarian responsiveness with age is likely not due to FSH-R alternative splicing.

# Discussion

We used only 1µl of total RNA from each sample to perform RT reaction and then used 1µl of cDNA to perform real time PCR. The unknown FSH-R mRNA levels extrapolated from the standard curve represent values obtained by using 1µl of cDNA. Finally the entire follicle FSH-R mRNA levels were determined by normalization on a "per follicle" basis for comparative analysis. The hypothesis stated that FSH-R mRNA levels decrease in an aging human ovary. FSH-R mRNA levels in 3 mm (13 samples), 4 mm (19 samples), 5 mm (41 samples), 6 mm (25 samples) and 7 mm (21 samples) diameter follicles from women aged 26-46 years were analyzed statistically by analysis of variance. Linear regression analysis was non-significant for 3-7 mm follicles as a function of age. This implies that no association was observed in FSH-R mRNA levels as a function of aging ovary. Therefore, based upon our data, we rejected the hypothesis that decreased responsiveness to FSH in an aging ovary was due to decreased FSH-R levels. That being said, the data we were able to obtain tended to support our hypothesis. It is possible with additional determinations, that statistical significance could be reached.

Alteration in the receptor length due to splicing could affect the responsiveness to FSH. A major variant of human FSH-R involves a deletion of exon 9. The primer pair FSH-R-up and P5 was expected to amplify a PCR product of 924 bp in length spanning the starting of exon 1 and into exon 10 as shown in Figure 4. Examining Figures 16 and 17 clearly indicated that PCR product of approximately 924 bp in size was amplified from RNA isolated from CHO cells and HOF of women aging 28, 36, 40 and 46 years old, indirectly implying that exon deletion was not seen in the aging human ovary.

The nucleotide sequence of 924 bp PCR product from 46 years old subject (blue letters) compared with human FSH-R variant1 (black letters) is shown in Figure 18.

5'-GGTCTCTTTGCTGGCATTCCTGAGCTTGGGCTCAGGATGTCATCATCGG**NN**CTGTCACTG
5'-GGTCTCTTTGCTGGCATTCCTGAGCTTGGGCTCAGGATGTCATCATCGG**AT**CTGTCACTG

CTCTAACAGGGTTTTTCTCTGCCAAGAGAGCAAGGTGACAGAGATTCCTTCTGACCTCC
CTCTAACAGGGTTTTTCTCTGCCAAGAGAGCAAGGTGACAGAGATTCCTTCTGACCTCC

CGAGGAATGCCATTGAACTGAGGTTTGTCCTCACCAAGCTTCGAGTCATCCAAAAAGGT
CGAGGAATGCCATTGAACTGAGGTTTGTCCTCACCAAGCTTCGAGTCATCCAAAAAGGT

GCATTTTCAGGATTTGGGGACCTGGAGAAAATAGAGATCTCTCAGAATGATGTCTTGGAGGT
GCATTTTCAGGATTTGGGGACCTGGAGAAAATAGAGATCTCTCAGAATGATGTCTTGGAGGT

GATAGAGGCAGATGTGTTCTCCAACCTTCCCAAATTACATGAAATTAGAATTGAAAAGGCCAA
GATAGAGGCAGATGTGTTCTCCAACCTTCCCAAATTACATGAAATTAGAATTGAAAAGGCCAA

CAACCTGCTCTACATCAACCCTGAGGCCTTCCAGAACCTTCCCAACCTTCAATATCTGT
CAACCTGCTCTACATCAACCCTGAGGCCTTCCAGAACCTTCCCAACCTTCAATATCTGT

TAATATCCAACACAGGTATTAAGCACCTTCCAGATGTTCACAAGATTCATTCTCTCCAA
TAATATCCAACACAGGTATTAAGCACCTTCCAGATGTTCACAAGATTCATTCTCTCCAA

AAAGTTTTACTTGACATTCAAGATAACATAAACATCCACACAATTGAAAGAAATTCTTTCGTG
AAAGTTTTACTTGACATTCAAGATAACATAAACATCCACACAATTGAAAGAAATTCTTTCGTG

GGGCTGAGCTTTGAAAGTGTGATTCTATGGCTGAATAAGAATGGGATTCAAGAAATAC
GGGCTGAGCTTTGAAAGTGTGATTCTATGGCTGAATAAGAATGGGATTCAAGAAATAC

ACAACTGTGCATTCAATGGAACCCAACTAGATGAGCTGAATCTAAGCGATAATAATAAT
ACAACTGTGCATTCAATGGAACCCAACTAGATGAGCTGAATCTAAGCGATAATAATAAT

TTAGAAGAATTGCCTAATGATGTTTTCCACGGAGCCTCTGGACCAGTCATTCTAGATA
TTAGAAGAATTGCCTAATGATGTTTTCCACGGAGCCTCTGGACCAGTCATTCTAGATA

TTTCAAGAACAAGGATCCATTCCCTGCCTAGCTATGGCTTAGAAAATCTTAAGAAGCTGAG
TTTCAAGAACAAGGATCCATTCCCTGCCTAGCTATGGCTTAGAAAATCTTAAGAAGCTGAG

GGCCAGGTCGACTTACAACTTAAAAAAGCTGCCTACTCTGGAAAAGCTTGTCGCCCTCAT
GGCCAGGTCGACTTACAACTTAAAAAAGCTGCCTACTCTGGAAAAGCTTGTCGCCCTCAT

GGAAGCCAGCCTCACCTATCCCAGCCATTGCTGTGCCTTTGCAAACTGGAGACGGCAAA
GGAAGCCAGCCTCACCTATCCCAGCCATTGCTGTGCCTTTGCAAACTGGAGACGGCAAA

TCTCTGAGCTTCATCCAATTTGCAACAAATCTATTTTAA**N**GCAAGAAGTTGATTATATGACTCA-3'
TCTCTGAGCTTCATCCAATTTGCAACAAATCTATTTTAA**G**GCAAGAAGTTGATTATATGACTCA-3'

**Figure 18**. Nucleotide sequence of PCR product amplified by primers FSH-R-up and P5.

The nucleotide sequence of DNA from our 924 bp PCR product of a 46 year old woman exhibited 99.7% homology, when a blast search was done with human FSH-R variant 1.

Figures 16 and 17 clearly indicate that a 1,747 bp PCR product was amplified using the primer set P0 and P6. However when the DNA from 1,747 bp PCR product of

46 year old woman was sent for sequencing, 298 bp showed up in the sequence as shown in Figure 19, indicating that only 17% of 1747 bp product was sequenced.

5'-CAGTGGCCATTCCTTGGATGGGTGTTGTGGACAGTGGATGAAGTTTCTGTCCTATAAATT
5'-CAGTGGCCATTCCTTGGATGGGTGTTGTGGACAGTGGATGAAGTTTCTGTCCTATAAATT

TGGGCTTGCATTTCATAGCAGCCACACTTGCTCAGCAGAATGAAGAAATCTCTGCGAAAG
TGGGCTTGCATTTCATAGCAGCCACACTTGCTCAGCAGAATGAAGAAATCTCTGCGAAAG

TTTTTGGTAAAGATGGCATAGAGGAAGGGGTTGGCACAGGAGTTGATGGGGTGAAACAGA
TTTTTGGTAAAGATGGCATAGAGGAAGGGGTTGGCACAGGAGTTGATGGGGTGAAACAGA

ACCAGCAGAATCTTTGCTTTGGACACAGTGATGAGGGGCACCTTGAGGGAGGCAGAAATG
ACCAGCAGAATCTTTGCTTTGGACACAGTGATGAGGGGCACCTTGAGGGAGGCAGAAATG

GCAAAGAAAGAAATGGGTGCCATGCA**N**AGGAAGTCAGTGAAGATGAGCATGGCCATGC-3'
GCAAAGAAAGAAATGGGTGCCATGCA**G**AGGAAGTCAGTGAAGATGAGCATGGCCATGC-3'

**Figure 19**. Nucleotide sequence of PCR product amplified by primers P0 and P6.

99.7% homology was seen when the 17% sequenced nucleotides (blue letters) were compared with human FSH-R variant 1 (black letters). Based on our agarose gel electrophoresis results, we did not see any decrease in the size of PCR products. Our nucleotide sequence results also indicated that major exon deletion was not seen in the samples tested suggesting strong evidence that alternate splicing is not seen in an aging human ovary.

The binding of FSH to the FSH-R, initiates a cascade of intracellular events leading to the various signal transduction pathways. The pathways discovered so far involve the activation of Protein Kinase A, Protein Kinase B, Ras/Raf/MAPK and intracellular calcium. Research information is very limited in regards to the changes in these pathways in an aging human ovary. One main reason for an aged ovary for being less responsive could be an alteration in the signal activation, which in turn fails to activate the necessary factors and/or genes involved in the pathway. This may eventually result in

an old follicle being less responsive for the hormonal stimulation. Researching about these pathway changes is beyond the scope of our study design.

**Limitations**

We used archived follicles, which might have not been as good as fresh samples. However analysis of RNA at 260/280 nm indicated good quality. Despite proper follicle storage at -80°C, the possibility of RNA degradation cannot be ruled out. The chronologic age of the follicle may not correspond with reproductive age i.e follicles from younger women may not be functioning well and follicles from older women might have full functioning capacity which will modify our results.

We had better reasons to design the study in measuring the levels of FSH-R mRNA instead of FSH-R protein. Analyzing FSH-R levels by western blot has its own practical difficulties. Unavailability of an antibody to probe human FSH-R makes the experiment difficult to perform. Also the ovarian follicle tissue being small may not result in adequate amounts of FSH-R protein that can be effectively detected by western blot. Decreased protein levels need not necessarily mean that mRNA levels were also decreased. So we had to analyze the FSH-R mRNA levels to study the changes at transcription level instead of studying the FSH-R protein at the translation level.

**Future directions**

The results could be improved by using follicles from freshly harvested ovaries. Sample size has to be increased. Including a reference gene/ endogenous control for the real time PCR might improve the outcome.

# REFERENCES

## LIST OF REFERENCES


1. Wilhelm, D., S. Palmer, and P. Koopman, *Sex Determination and Gonadal Development in Mammals.* Physiol. Rev., 2007. **87**(1): p. 1-28.

2. Pinon, R., *Biology of human reproduction*. 2002, Sanfrancisco: University science books. 5.

3. Swain, A. and R. Lovell-Badge, *Mammalian sex determination: a molecular drama.* Genes Dev., 1999. **13**(7): p. 755-767.

4. Loffler, K.A., Koopman, P, *Charting the course of ovarian development in vertebrates.* Int J Dev Biol, 2002. **46**(4).

5. Kurjak, A., *Ultrasound and the ovary*. 1994: Taylor and Francis.

6. Merchant, H., *Rat gonadal and ovarian organogenesis with and without germ cells.* Dev Biol, 1975. **44**(1): p. 1-21.

7. Capel, B., *The battle of sexes.* Mech Dev, 2000. **92**(1): p. 89-103.

8. MacLaughlin, D.T., J. Teixeira, and P.K. Donahoe, *Perspective: Reproductive Tract Development--New Discoveries and Future Directions.* Endocrinology, 2001. **142**(6): p. 2167-2172.

9. De Felici, M., et al., *Experimental approaches to the study of primordial germ cell lineage and proliferation.* Hum Reprod Update, 2004. **10**(3): p. 197-206.

10. Ginsburg, M., M.H. Snow, and A. McLaren, *Primordial germ cells in the mouse embryo during gastrulation.* Development, 1990. **110**: p. 521-28.

11. Rabinovici, J. and R.B. Jaffe, *Development and regulation of growth and differentiated function in human and subhuman primate fetal gonads [published erratum appears in Endocr Rev 1991 Feb;12(1):90]*. Endocr Rev, 1990. **11**(4): p. 532-557.

12. Francavilla S, C.G., Properzi G, Concordia N, Cappa F, Pozzi V, *Ultrastructure of fetal human gonad before sexual differentiation and during early testicular and ovarian development.* J Submicrosc Cytol Pathol, 1990. **22**(3): p. 389-400.

13. Fujiwara, T., N.R. Dunn, and B.L.M. Hogan, *Bone morphogenetic protein 4 in the extraembryonic mesoderm is required for allantois development and the localization and survival of primordial germ cells in the mouse.* Proceedings of the National Academy of Sciences, 2001. **98**(24): p. 13739-13744.

## LIST OF REFERENCES (cont)


14. de Sousa Lopes, S.M.C., et al., *BMP signaling mediated by ALK2 in the visceral endoderm is necessary for the generation of primordial germ cells in the mouse embryo.* Genes Dev., 2004. **18**(15): p. 1838-1849.

15. Godin, I., C. Wylie, and J. Heasman, *Genital ridges exert long-range effects on mouse primordial germ cell numbers and direction of migration in culture.* Development, 1990. **108**(2): p. 357-363.

16. Bendel, et al., *The origin and migration of primordial germ cells in the mouse.* Seminars in Cell and Developmental Biology, 1998. **9**: p. 393-400.

17. Buehr, M., *Proliferation and migration of primordial germ cells in mouse embryos.* Developmental Dynamics, 1993. **198**(3): p. 182-189.

18. Wiley, c., *Germ cells.* Curr opin Genet Dev, 2000. **10**(4): p. 410-30.

19. Tam P, P., and Snow MH., *Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos.* J Embryol Exp Morphol, 1981. **64**: p. 133-47.

20. Ottolenghi, C., et al., *Aging of Oocyte, Ovary, and Human Reproduction.* Ann NY Acad Sci, 2004. **1034**(1): p. 117-131.

21. Buccione, R., A.C. Schroeder, and J.J. Eppig, *Interactions between somatic cells and germ cells throughout mammalian oogenesis.* Biol Reprod, 1990. **43**(4): p. 543-547.

22. Boise, L., et al.,, *bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death.* Cell, 1993. **74**(4): p. 597-608.

23. John, J.E., *Intercommunication between mammalian oocytes and companion somatic cells.* BioEssays, 1991. **13**(11): p. 569-574.

24. Juneja, S.C., et al., *Defects in the Germ Line and Gonads of Mice Lacking Connexin43.* Biol Reprod, 1999. **60**(5): p. 1263-1270.

25. Simon, A.M., et al., *Female infertility in mice lacking connexin 37.* Nature, 1997. **385**(6616): p. 525-529.

26. Gougeon, A., *Dynamics of follicular growth in the human: a model from preliminary results.* Hum. Reprod., 1986. **1**(2): p. 81-87.

## LIST OF REFERENCES (cont)


27. Peters, H., et al., *Follicular growth: the basic event in the mouse and human ovary.* J Reprod Fertil, 1975. **45**(3): p. 559-566.

28. Eppig, J.J., *Oocyte control of ovarian follicular development and function in mammals.* Reproduction, 2001. **122**(6): p. 829-838.

29. Hunter, R.H.F., *Physiology of the Graafian Follicle and Ovulation*. 2003 New york: Cambridge University Press.

30. Erickson, G., F, etal, *The ovarian androgen producing cells: a review of structure/ function relationships.* End Rev, 1985. **6**(3): p. 371-99.

31. Chun, S.Y., et al., *Hormonal regulation of apoptosis in early antral follicles: follicle- stimulating hormone as a major survival factor.* Endocrinology, 1996. **137**(4): p. 1447-1456.

32. Stocco, C., C. Telleria, and G. Gibori, *The Molecular Control of Corpus Luteum Formation, Function, and Regression.* Endocr Rev, 2007. **28**(1): p. 117-149.

33. Bruce, N.W. and R.M. Moor, *Capillary blood flow to ovarian follicles, stroma and corpora lutea of anaesthetized sheep.* J Reprod Fertil, 1976. **46**(2): p. 299-304.

34. Davis, J., B. Rueda, and K. Spanel-Borowski, *Microvascular endothelial cells of the corpus luteum.* Reproductive Biology and Endocrinology, 2003. **1**(1): p. 89.

35. Lunn, S.F., H.M. Fraser, and H.D. Mason, *Structure of the corpus luteum in the ovulatory polycystic ovary.* Hum. Reprod., 2002. **17**(1): p. 111-117.

36. Zhou, J., et al., *Insulin-Like Growth Factor I Regulates Gonadotropin Responsiveness in the Murine Ovary.* Mol Endocrinol, 1997. **11**(13): p. 1924-1933.

37. Monget, P.a.C.B., *Importance of the IGF system in early folliculogenesis* Molecular and Cellular Endocrinology, 2000. **163**(1-2): p. 89-93.

38. Findlay, J.K., *An update on the roles of inhibin, activin, and follistatin as local regulators of folliculogenesis.* Biol Reprod, 1993. **48**(1): p. 15-23.

39. Dong, J., et al., *Growth differentiation factor-9 is required during early ovarian folliculogenesis.* Nature, 1996. **383**(6600): p. 531-535.

## LIST OF REFERENCES (cont)


40. Woo-Sik Lee, S.-J.Y.T.-K.Y.K.-Y.C.S.-H.L.S.S.S.L.K.-A.L., *Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary.* Molecular Reproduction and Development, 2004. **69**(2): p. 159-163.

41. Nilsson, E., et al., *Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis.* Mol Cell Endocrinol, 2001. **175**(1-2).

42. Couse, J.F. and K.S. Korach, *Estrogen Receptor Null Mice: What Have We Learned and Where Will They Lead Us?* Endocr Rev, 1999. **20**(3): p. 358-417.

43. Richards, J.S., et al., *Novel Signaling Pathways That Control Ovarian Follicular Development, Ovulation, and Luteinization.* Recent Prog Horm Res, 2002. **57**(1): p. 195-220.

44. Couse, J.f., etal.,, *Postnatal sex reversal of the ovaries in mice lacking estrogen receptors alpha and beta.* Science, 1999. **286**(5448): p. 2328-31.

45. Dupont, S., et al., *Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes.* Development, 2000. **127**(19): p. 4277-4291.

46. Parrott, J.A. and M.K. Skinner, *Kit-Ligand/Stem Cell Factor Induces Primordial Follicle Development and Initiates Folliculogenesis.* Endocrinology, 1999. **140**(9): p. 4262-4271.

47. Lee, W.S., etal.,, *Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary.* Molecular Reproduction and Development, 2004. **69**(2): p. 159-163.

48. Dube, J.L., et al., *The Bone Morphogenetic Protein 15 Gene Is X-Linked and Expressed in Oocytes.* Mol Endocrinol, 1998. **12**(12): p. 1809-1817.

49. Otsuka, F., et al., *Bone Morphogenetic Protein-15 Inhibits Follicle-stimulating Hormone (FSH) Action by Suppressing FSH Receptor Expression.* J. Biol. Chem., 2001. **276**(14): p. 11387-11392.

50. Ulloa-Aguirre, A. and C. Timossi, *Structure-function relationship of follicle-stimulating hormone and its receptor.* Hum Reprod Update, 1998. **4**(3): p. 260-283.

51. Pierce, J.G. and T.F. Parsons, *Glycoprotein Hormones: Structure and Function.* Annual Review of Biochemistry, 1981. **50**(1): p. 465-495.

## LIST OF REFERENCES (cont)


52. Fox, K.M., J.A. Dias, and P. Van Roey, *Three-Dimensional Structure of Human Follicle-Stimulating Hormone.* Mol Endocrinol, 2001. **15**(3): p. 378-389.

53. Godine, J.E., W.W. Chin, and J.F. Habener, *alpha Subunit of rat pituitary glycoprotein hormones. Primary structure of the precursor determined from the nucleotide sequence of cloned cDNAs.* J. Biol. Chem., 1982. **257**(14): p. 8368-8371.

54. Lapthorn, A.J., et al., *Crystal structure of human chorionic gonadotropin.* Nature, 1994. **369**(6480): p. 455-461.

55. Naylor, S.L., etal.,, *Chromosome assignment of genes encoding the alpha and beta subunits of gylcoprotein hormones in man and mouse.* Somat Cell Genet, 1983. **9**: p. 757-70.

56. Boothby, M., et al., *A single gonadotropin alpha-subunit gene in normal tissue and tumor- derived cell lines.* J. Biol. Chem., 1981. **256**(10): p. 5121-5127.

57. Gharib, S.D., et al., *Molecular biology of the pituitary gonadotropins.* Endocr Rev, 1990. **11**(1): p. 177-199.

58. Fiddes, J.C., Talmadge, K., *Structure, expression, and evolution of the genes for the human glycoprotein hormones.* Recent Prog Horm Res, 1984. **40**: p. 43-78.

59. Jameson, J.L., et al., *Transcriptional regulation of chorionic gonadotropin alpha- and beta- subunit gene expression by 8-bromo-adenosine 3',5'-monophosphate.* Endocrinology, 1986. **119**(6): p. 2560-2567.

60. Jameson, J.L., et al., *The gonadotropin alpha-gene contains multiple protein binding domains that interact to modulate basal and cAMP-responsive transcription.* J. Biol. Chem., 1988. **263**(20): p. 9879-9886.

61. Van Zuylen, C.W., Kamerling, J.P., and Vliegenthart, J.F ., , *Glycosylation beyond the Asn78-linked GlcNAc residue has a significant enhancing effect on the stability of the alpha subunit of human chorionic gonadotropin.* Biochem. Biophys. Res. Commun, 1997. **232**: p. 117-120.

62. Watkins, P.C., etal.,, *DNA sequence and regional assignment of the human follicle-stimulating hormone beta-subunit gene to the short arm of human chromosome 11.* DNA, 1987 **6**(3): p. 205-12.

63. Kim, K.E., Gordon,D.F., Maurer,R.A, *Nucleotide sequence of the bovine gene for follicle-stimulating hormone beta-subunit.* DNA, 1988. **7**(4): p. 227-33.

## LIST OF REFERENCES (cont)


64. Albanese, C., etal.,, *The gonadotropin genes: evolution of distinct mechanisms for hormonal control.* Recent Prog Horm Res, 1996. **51**: p. 23-58.

65. Besecke, L.M., et al., *Gonadotropin-releasing hormone regulates follicle-stimulating hormone- beta gene expression through an activin/follistatin autocrine or paracrine loop.* Endocrinology, 1996. **137**(9): p. 3667-3673.

66. Ying, S.Y., *Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone.* Endocr Rev, 1988. **9**(2): p. 267-93.

67. Krummen, L.A., et al., *Identification and characterization of binding proteins for inhibin and activin in human serum and follicular fluids.* Endocrinology, 1993. **132**(1): p. 431-443.

68. Winters, S.J., A.C. Dalkin, and T. Tsujii, *Evidence That Pituitary Adenylate Cyclase Activating Polypeptide Suppresses Follicle-Stimulating Hormone-{beta} Messenger Ribonucleic Acid Levels by Stimulating Follistatin Gene Transcription.* Endocrinology, 1997. **138**(10): p. 4324-4329.

69. Gromoll, j., *Localization of the human FSH receptor to chromosome 2 p21 using a genomic probe comprising exon 10.* J Mol Endocrinol, . 1994 **12**(3): p. 265-71.

70. Gromoll, j., *The Structure and Organization of the Human Follicle-Stimulating Hormone Receptor (FSHR) Gene.* Genomics, 1996. **35**(2): p. 308-11.

71. Simoni, M., J. Gromoll, and E. Nieschlag, *The Follicle-Stimulating Hormone Receptor: Biochemistry, Molecular Biology, Physiology, and Pathophysiology.* Endocr Rev, 1997. **18**(6): p. 739-773.

72. Gonzalez-Robayna, I.J., et al., *Follicle-Stimulating Hormone (FSH) Stimulates Phosphorylation and Activation of Protein Kinase B (PKB/Akt) and Serum and Glucocorticoid-Induced Kinase (Sgk): Evidence for A Kinase-Independent Signaling by FSH in Granulosa Cells.* Mol Endocrinol, 2000. **14**(8): p. 1283-1300.

73. Gorczynska, E., J. Spaliviero, and D.J. Handelsman, *The relationship between 3',5'-cyclic adenosine monophosphate and calcium in mediating follicle-stimulating hormone signal transduction in Sertoli cells.* Endocrinology, 1994. **134**(1): p. 293-300.

74. Dierich, A., et al., *Impairing follicle-stimulating hormone (FSH) signaling in vivo: Targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance.* Proceedings of the National Academy of Sciences, 1998. **95**(23): p. 13612-13617.

## LIST OF REFERENCES (cont)


75. Sudo, S., et al., *Genetic and functional analyses of polymorphisms in the human FSH receptor gene.* Mol. Hum. Reprod., 2002. **8**(10): p. 893-899.

76. Simoni, M., E. Nieschlag, and J. Gromoll, *Isoforms and single nucleotide polymorphisms of the FSH receptor gene: implications for human reproduction.* Hum Reprod Update, 2002. **8**(5): p. 413-421.

77. Aittomaki, K., et al., *Clinical features of primary ovarian failure caused by a point mutation in the follicle-stimulating hormone receptor gene.* J Clin Endocrinol Metab, 1996. **81**(10): p. 3722-3726.

78. Baker, T.G.,*A quantitative and cytological study of germ cells in human ovaries* Proc R Soc Lond B Biol Sci, 1963. **158**: p. 417-33.

79. Fulton, N., et al., *Germ Cell Proliferation and Apoptosis in the Developing Human Ovary.* J Clin Endocrinol Metab, 2005. **90**(8): p. 4664-4670.

80. Gougeon, A., R. Echochard, and J.C. Thalabard, *Age-related changes of the population of human ovarian follicles: increase in the disappearance rate of non-growing and early-growing follicles in aging women.* Biol Reprod, 1994. **50**(3): p. 653-663.

81. Santoro, N., et al., *Impaired Folliculogenesis and Ovulation in Older Reproductive Aged Women.* J Clin Endocrinol Metab, 2003. **88**(11): p. 5502-5509.

82. Jacobs, S.L., et al., *Effect of age on response to human menopausal gonadotropin stimulation.* J Clin Endocrinol Metab, 1990. **71**(6): p. 1525-1530.

83. Santoro, N., et al., *Characterization of reproductive hormonal dynamics in the perimenopause*. J Clin Endocrinol Metab, 1996. 81(4), 1495-1501