# REGULATED EXPRESSION OF FOLLICLE STIMULATING HORMONE RECEPTOR TYPE III IN CANCER CAUSING MOUSE OVARIAN SURFACE EPITHELIAL CELLS

**by**

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# Abstract

Follicle stimulating hormone (FSH) is known as the key hormone capable of causing proliferation of granulosa cells in the ovary. The classical receptor belongs to the G protein-coupled superfamily and is designated FSHR-1. A variant in the FSH receptor has been shown to be functional in mouse ovaries. The variant receptor is designated as FSHR-3, and when bound by FSH activates a pathway that shares similar characteristics to the growth factor type I receptor pathway, with no increase in cAMP. The FSHR-3 variant activates MAPK upon binding to FSH, and causes proliferation of cells on which it is known to be expressed. For example ID8 mouse ovarian surface epithelium cells (MOSEC), a cell line that when introduced in immunocompetent mice causes tumors similar to human ovarian cancer and which also express FSHR-3, proliferated in response to FSH. The present study explored the potential for decreasing expression of FSHR-3 protein. The RNA interference (RNAi) technique was used to insert small inhibitory RNA(siRNA) segments corresponding specifically to the R3 variant of the FSH receptor in ID8 MOSEC. Transfected cells were lysed and FSHR-3 protein was visualized using SDS Page and Western blotting analysis. A reduction in expression of FSHR-3 was observed in two of the transfection groups, with the greatest down-regulation of FSHR-3 being 30.1%. From these preliminary results we conclude that the FSHR-3 is expressed on ID8 cells, and that siRNA may be useful to reduce its expression. Thus, it may be possible to slow the growth of FSH-responsive tumors using siRNA to target the FSHR-3 receptor.

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# Chapter 1

# Literature Review

## *Introduction*

The dynamics of the ovary have been studied by scientists for years. The number of ovarian follicles is predetermined early in a female's life, and depletion of the follicle pool leads to reproductive senescence. Thus, it is extremely important for maximal reproduction efficiency in animal agriculture to understand factors involved in controlling follicular development.

Follicle stimulating hormone (FSH) is one such factor that will determine if a follicle is ovulated or not. Follicle stimulating hormone is an oligomeric glycoprotein hormone with several variants (Touyz et al., 2000). However, some FSH activities seem to be consistent throughout all reproductive cycles including: cell proliferation, inhibition of apoptosis, cellular differentiation, and stimulation of steroidogenesis (Touyz et al., 2000). The core role of FSH is to cause growth and development of follicles on the ovary.

In order to activate, FSH must initially bind to its receptor within the ovary. Since the FSH receptor was first cloned and sequenced it was believed FSH worked through a single G protein coupled-receptor (GPCR). The GPCR for FSH has seven transmembrane sections, and when bound by FSH it activates adenylate cyclase through a G protein (Gs), resulting in an increase in cAMP.

## *Ovarian Structure*

The bovine ovary houses the female gametes. These gametes or oocytes, can be found inside the ovary in a small but concentrated pool of resting follicles ready for development upon

hormonal stimulation. When activated, follicular development happens in a wave-like developmental dynamic (Evans et al., 2001). In a given cycle, there can be up to three follicular waves occurring before a dominant follicle is finally ovulated for fertilization. Before that dominant follicle is released it has to start at the primordial follicle stage which is essentially an undifferentiated tissue.

Within ovaries, four different types of structures can be identified: 1.) the germinal (ovarian) epithelium, 2.) tunica albuginea, 3.) cortex and 4.) medulla. Separately these structures are incapable of producing a fully function follicle, but together they provide a unique atmosphere from which thousands of follicle will be produced.

The germinal (ovarian) epithelium consists of cuboidal epithelium cells that surround the ovary and provide a barrier between the developing follicles and the body cavity. Once believed to be the origin of oocytes, it was so named germinal and remained labeled until which time oocytes were found to have originated elsewhere (Developmental Biology (6th Edn) Gilbert, Scott F. Sunderland (MA): Sinauer Associates, Inc.; c2000).

The second structure located just under the epithelium is the tunica albuginea. The tunica albuginea is made of condensed connective tissue and is located between the germinal (ovarian) epithelium and the cortex.

The third structure found in the ovary is classified as the cortex and is identified as loose connective tissue. Oocytes originate from the cortex and will continue to grow from the cortex until which time they are ovulated or become atretic.

The final structure located at the center of the ovary is the medulla. The medulla houses all large blood vessels which allow for proper function of the ovary. Along with housing of blood vessels the medulla is home to many nerves found in the ovary. It is the combining of

these layers in the ovary that provide an environment from which developing follicles will be held until such time they are ovulated or reabsorbed by the body.

***Follicle Dynamics***

The follicle's best case scenario within the ovary is to be ovulated. For fertilization to be successful, follicular development must proceed correctly through several stages. The stages through which the follicle passes through are initial recruitment, selection, deviation and dominance.

***Initial Recruitment of a Follicle***

Mechanisms for controlling initial growth of follicles have been difficult to determine due to the slow growth of follicles over a prolonged period of time (Hirshfield et al., 1989). However, there is clearly a role for FSH at some point in early follicular growth. For example, when FSH concentration was increased, a group of follicles, all of similar size, emerged as a cohort (as reviewed by Richards (1980)). In rats, this cohort grows from a size of 0.4-0.5 mm during metestrus and from 0.8-1.0 mm during proestrus; this stage of development happens at the start of the estrous cycle, days 0-3 (Fortune., 1994). Those that are not recruited to enter into the initial growing phases are thought to be under some type of inhibitory influence keeping these follicles from growing (Hirshfield et al., 1989).

One possible mechanism for hindrance may be that not all follicles possess enough gonadotropin receptors to initiate growth. With the need for FSH to stimulate growth of follicles, it is thought that if the concentration of FSH is not high enough or the number of receptors on the follicle is not inadequate, then the follicle will not enter into development (as reviewed by McGee et al., (2000)).

A blend of high FSH concentration, high FSH receptors and low concentration of LH/hCG is what leads a select number of follicles, in each wave, to be recruited into the initial stages of development. From this cohort a single follicle will be selected to grow further in pursuit of ovulating while the others become atretic. For the rest of the follicular pool, growth will remain halted until selected for initial recruitment and potential ovulation.

***Mechanisms Involved in Early Follicular Development and Differentiation***

Relatively more is known about rescuing of follicles than about factors that cause their initial growth; however, several challenges remain.

One challenge is to understand the importance of developing granulosa cells in primordial follicles. As primordial follicles develop into primary follicles, over a period of time the granulosa cells change as well. Granulosa cells start as a single flat layer of cells; as the follicle continues to grow granulosa cells begin to divide into several layers and upon the follicle reaches the secondary stage. Along with increasing in number of layers, the granulosa cells also change in shape. The once flat cells will proceed through morphological changes until they become the cuboidal shape found in primary follicles (as reviewed by McGee et al., 2000).

A second challenge involves the oocyte and whether it is possible that the order by which follicles are recruited is determined by the order in which the oocytes entered into meiosis, with the first ones to be recruited being lost during the infantile period (Edwards et al., 1977). One determining factor that specifies in what order the oocyte resumes meiosis might be the ability of the oocyte and the granulosa cells to communicate. Activation of pathways between the oocyte and granulosa cells seems to trigger the primordial follicle into the recruitment stage (as reviewed by McGee et al., (2000)). The kit ligand has been found to be expressed functionally on granulosa cells while c-kit, tyrosine kinase receptor is located on the oocyte (as reviewed by

McGee et al., (2000)). The binding of the kit ligand to the c-kit, tyrosine kinase receptor allows the oocyte to communicate with the granulosa cells. Furthermore, if the kit ligand is nonfunctional in the granulosa cell due to a mutation, the follicle will never reach the primary follicle stage even with the oocyte still developing (Huang et al., 1993).

***Selection of Follicles***

Once the cohort of follicles has been established the selection process is initiated, at the end of which a dominant follicle will be selected. The entire selection process is believed to be made up of three parts, including the ability of preantral follicles to respond to gonadotropins, production of inhibin and estrogen from a dominant follicle, and feedback between dominant follicles and the pituitary (Ireland., 1987). Initially, preantral follicles have low amounts of gonadotropin receptors (Ireland., 1987). As a result, any decrease in gonadotropin receptor concentrations decreases the ability of the preantral follicle to respond to FSH, thus making it less able to continue development (Ireland., 1987).

Suppression of FSH leads to a negative feedback loop where inhibin and estradiol from the follicle feedback to the hypothalamus, suppressing FSH secretion and causing other developing follicles to regress through lack of FSH binding to the granulosa cells.

***Future Dominant Follicles during Recruitment and Selection***

During follicular waves a follicle(s) is selected from the growing cohort of follicles to continue growing. As the follicle grows, the follicle also differentiates functionally in ways to prepare it for ovulation as well as help prepare the female for a potential pregnancy. The mechanisms are numerous as to why and how the follicle is selected from the cohort of follicles to become dominant while the others become atretic.

In a normal follicular wave, a rise in FSH is seen during the initial recruitment stage. It is documented that if the rise in FSH does not happen the wave will be delayed until the FSH surge occurs (Mihm et al., 2003). At the point of the FSH surge the follicles emerge as a cohort and begin to develop. However, after the surge, FSH availability begins to decline due mostly to the developing follicles producing inhibin. As the FSH concentration continues to decrease some follicles will not be able to continue growing due to a lower sensitivity of FSH receptors expressed, making these growing follicles less responsive to FSH concentrations (Mihm et al., 2003).

What remains unclear is the exact mechanism that is used to select the future dominant follicle for continued growth, even during periods of decreased concentrations of FSH. A possible outcome as to how the dominant follicle is able to survive during this time is by its ability to convert androgens to estradiol. As reviewed by Fortune., 1994, dominant follicles show an increased ability to convert androgens to estradiol within the granulosa cells in response to LH. This ability is continuous throughout the development of the dominant follicle, as compared to subordinate follicles, which appear to convert androgens to estradiol at a lower rate.

Along with the ability to convert androgens to estradiol at a higher efficiency, the follicle that is selected to become dominant also seems to show a higher concentration of mRNA for LH and FSH receptor (Fortune., 1994). Increased LH and FSH receptor mRNA is seen even before the dominant follicle is selected (Fortune., 1994). This higher concentration of LHR mRNA, which leads to more LH receptors expressed, may explain how the follicle is able to escape atresia when FSH is at basal levels. Because of this gonadotropin receptor, a higher concentration of 17$\alpha$-hydroxylase and aromatase mRNA are also found which in turn allows the follicle to produce estradiol by converting androgens to estradiol (Fortune., 1994).

Estradiol production may ultimately select the dominant follicle from other subordinates. Estradiol then feeds back to the hypothalamus and pituitary and diminishes the circulating FSH. In mammals only a single follicle is able to achieve this task of dominance and moves forward in development. With a continued decrease in FSH the dominant follicle increases production in LH receptor mRNA in both theca and in granulosa cells, allowing it to maintain its dominance over the subordinates (Mihm et al., 2003).

***Deviation of Dominant Follicle***

Dominant follicles develop due to endocrine control of the gonadotropins originating from the pituitary. The gonadotropins travel to the ovary to cause steriodogenic activity in the granulosa and theca cell layers ( Ireland., 1987). Typically, a higher concentration of estradiol is observed in dominant follicles which allows the follicle to trigger all the physiological events required for ovulation and reproduction (Ireland., 1987). The mechanism by which estradiol causes this event has been observed in cultured granulosa cells from hypophysectomized rats. These cells, when given FSH, grew at a higher rate than cells deprived of FSH. This outcome suggested that FSH is required for follicles to produce estradiol which ultimately feeds back to suppress FSH.

In cattle the process of deviation begins upon a single follicle reaching an average size of 8.5 mm and can range from 8 to 9 mm depending on breed (Ginther et al., 2000). As a follicle reaches the desired size for deviation to begin, the other follicles found in this cohort will not continue to grow. The process that causes the subordinate follicles to stop growth and begin atresia may be caused by the selection of the dominant follicle (Ginther et al., 2000). As the dominant follicle begins the deviation process, it begins the negative feedback loop with the

pituitary, causing an increase in inhibin and estradiol production which leads to a reduction of FSH. Therefore, decreasing FSH results in atresia of subordinates.

As the future dominant follicle progresses through deviation, the follicle no longer seems to be dependent on FSH for continued growth. As the FSH concentrations fall and subordinate follicles begin to regress, the future dominant follicle acquires the ability to produce receptor for LH. By expressing this receptor the follicle now can use the LH circulating in the follicular fluid as a means of sustaining its growth, separating it from the subordinates until ovulation (Ginther et al., 2000).

***Factors Associated with the Dominant Follicle***

Several factors found early in a follicle's development may cause critical changes that ensure the follicle's dominance. Initially the future dominant follicle emerges 6-7 h earlier in humans than the rest of the cohort (Hess et al., 2000). The added time for the follicle in this growth phase allows it to develop to a higher extent than the other follicles before the process of deviation begins. Yet before the cohort reaches initiation of deviation, an increase in mRNA of $P450_{scc}$ and $P450_{aromatase}$ can be seen in granulosa cells as early as 12 h after emergence (Rhodes et al., 2001). This increase in P450 mRNA leads to a higher conversion of androstenedione to estradiol in the granulosa cells. The production of estradiol causes granulosa cells to increase synthesis of LH receptors, providing the pathway in which to initiate further estradiol production.

Locally-produced factors may also influence survival of the dominant follicle; one such factor may be insulin-like growth factor-I (IGF-I). The paracrine-autocrine activities of IGF-I seem to assist the dominant follicle by increasing estradiol production while enhancing the sensitivity of the granulosa cells to FSH (Spicer et al., 1995). However, in comparing the

concentration of IGF-I of early dominant and subordinate follicles there appears to be no difference (Spicer et al., 1996). Instead, the mechanism that allows IGF-1 to increase the sensitivity in the dominant follicle and not the subordinates appears to involve the concentration of insulin-like growth-factor binding proteins (IGFBP) which, when present, inhibit the growth of the developing follicles. Concentrations of IGFBP are found to be similar between the two largest follicles as they near deviation. Once the future dominant follicle reaches a diameter of 8-9 mm the concentration of IGFBP begins to decline (Spicer et al., 1995). A higher concentration of IGFBP in subordinates causes a decrease in the follicle's ability to bind IGF-1 due to IGFBP binding to the IGF-I receptors.

Within the dominant follicle, theca cells also participate in setting the follicle apart from subordinates. The theca cells as early as day 3 after emergence produce a large amount of mRNA for LH receptors and 17α-hydroxylase (Rhodes et al., 2001). These androgens are essential for the follicle to produce more estradiol, since the androgens are the precursor the granulosa cells use to produce estradiol.

An additional mechanism for dominant follicles to use in order to survive is through the production of the steroidogenic acute regulatory protein (StAR). When produced by theca cells StAR is believed to participate in assisting the dominant follicle to produce higher concentrations of LH (Xu et al., 1995 a,b). The regulatory protein StAR works by regulating acute transport of cholesterol from the outer mitochondrial membrane to the inner membrane. Once transported, the cholesterol is then converted in the theca cells by $P450_{scc}$ to androgens (Xu et al., 1995 a,b).

Follicles that were collected at 48-96 hrs after the onset of estrous showed an increase in StAR mRNA but no differences between follicles (Xu et al., 1995 a,b). The difference was noticeable just prior to an increase in estradiol–17β production, signifying a dependency on

StAR for increased cholesterol transportation. This led to the hypothesis that the expression of StAR mRNA in theca cells is required for the continued production of LH, from the time of selection of follicles until ovulation (Xu et al., 1995 a,b).

***Structural Organization of FSH***

The pituitary glycoprotein hormone family is made up of four structurally similar proteins with very different physiological functions. Included in the family are Thyroid Stimulating Hormone (TSH), LH, as well as FSH. Each of these proteins has a common α-subunit. The α-subunit is required for the protein to function properly when bound to its individual receptors. It is the β-subunit that gives each protein its unique ability to activate specific receptors in a given target tissue. With the α-subunit being common among pituitary glycoproteins hormones, the 92 amino acids can be found throughout many cell types in the pituitary (Themmen et al., 2000). However, with the β-subunit defining the protein, the subunit is restricted to certain cell types depending on the glycoprotein produced.

The linking of the α and β-subunits is what leads to a stable tertiary structure which is crucial for the protein to function properly when bound to its receptor. The addition of glycosylated side chains adds protection from enzymatic degradation, thus increasing the half-life of the protein while also causing proper folding, proper assembly, and help with secretion into the extracellular space (Olijve et al., 1996). With glycosylation, the protein achieves a conformation capable of binding properly to its receptor.

***FSH G Protein Coupled Receptor***

Upon proper processing and secretion, FSH travels to the gonad and binds to a specific receptor. It is likely that FSH has at least three different receptors, which may be different functions. The first of these receptors to be characterized was identified as follicle stimulating

hormone receptor I (FSHR-1). The FSHR-1 form of the receptor belongs to the G protein-coupled receptor family and is characterized by seven hydrophobic helices which reside in the plasmalemma. Additionally, the G protein-coupled receptor is recognizable by both the intracellular and extracellular portions that can differ, depending on the type of glycoprotein.

The G protein-coupled FSH receptor is made up of 691 amino acids, which includes 17 amino acids that encode for a hydrophobic signal peptide (Sprengel et al., 1990). The cDNA sequence can range from 75 to 76.5 kDa, depending on species (Sprengel et al., 1990). The extracellular domain is composed of 348 amino acids followed by the transmembrane domain composed of 264 amino acids. The amino acids for the transmembrane domain are 90%, followed by a slight decrease in the extracellular domain at 85% for between-species (as reviewed by Simoni et al., (1997)).

The FSHR-1 gene expression is highly tissue specific (as reviewed by Simoni et al., 1997)). The unique aspect of the promoter region is that it lacks some typical promoter elements. There is no TATA box or CCAAT boxes. And the GC box motifs are not found in the FSHR-1 promoter (but are found to be present in the LH receptor promoter) (Atger et al., 1995). The FSHR-1 gene consists of 10 exons and 9 introns, with the exons only differ by a few nucleotides, and span a region of 84 kbp on the DNA (Gromoll et al., 1996). Expression of the FSHR-1 has been found to be present only in granulosa cells of the ovary in females. The acquisition of FSHR-1 is vital for the development of follicles, and for differentiation of granulosa cells.

Expression of FSHR-1 varies depending on the time and maturity of the developing follicle. In domestic species, FSHR-1 presence has been detected in all follicles, starting with a single layer of granulosa cells. Expression of FSHR-1 continues to rise as the follicle matures,

until the LH surge, once the LH surge occurs, the receptor begins to be down-regulated (as reviewed by Simoni et al., (1997)). Initially, after the surge, full length FSHR-1 transcripts are still detectable at day +1 and by day +3 full transcripts are no longer detectable (Rajapaksha et al., 1996). The decline in FSHR-1 is similar to the initiation of the LH receptor found in primordial stage follicles.

Binding of FSH to the FSHR-1 is a process that involves high affinity as well as high selectivity. Within the extracellular domain of the receptor, several leucine-rich repeats (LRR) are located between the N-terminus and the hinge region (Sohn et al., 2003). As FSH begins to bind to the receptor, it makes contact with the LRRs, the N-terminus, and also the hinge region. Upon binding, the receptor then reconfigures the 7 transmembrane sequences in order to partially internalize the FSH. By doing so, the intracellular portion of the receptor changes configuration, allowing activation of the stimulating G protein.

The activation of the FSHR-1 results in separation of the $\alpha$ subunit of the Gs from the heterotrimeric complex. The $\beta$ and $\gamma$ subunits remain attached to the receptor while the $\alpha$ subunit is attached to GTP, which the subunit uses for energy in activating additional pathways. Separation from the G protein*complex provides the $\alpha$ subunit with the opportunity to activate the membrane-bound adenylate cyclase. Upon activation, adenylate cyclase binds to three heptahelical G protein-coupled receptors including PAC1, VPAC1, and VPAC2 (Guirland et al., 2003). The binding of any three of these receptors brings about cAMP production. Increased production of cAMP in granulosa cells causes increased production of estrogen, allowing the follicle to potentially dominate over all others.

***Variants of the FSH Receptor***

Like other G protein-coupled receptors, genes for the FSHR-1 are large and complex. The increase in complexity and size brings an increase in splice variants. With some of the actions being mediated through the FSHR-1 variant, there continues to be quantifiable actions taking place that are not accounted for by FSHR-1. One such action is the influx of $Ca^{2+}$ into granulosa cells, which occurs independently of cAMP, indicating the possibility of receptor variants that are capable of receiving and responding to circulating FSH (Babu et al., 2000).

***Structure***

Two variant forms of the FSH receptor have recently been described, and appear to be functional. One such variant (termed FSHR-3) appears to take on the intriguing form of a Growth Factor Type I receptor. The first 8 exons for the FSHR-3 are identical to those of the FSHR-1. However, the 36 residues found after the amino acid number 223 differ due to alternative splicing (Touyz et al., 2000).

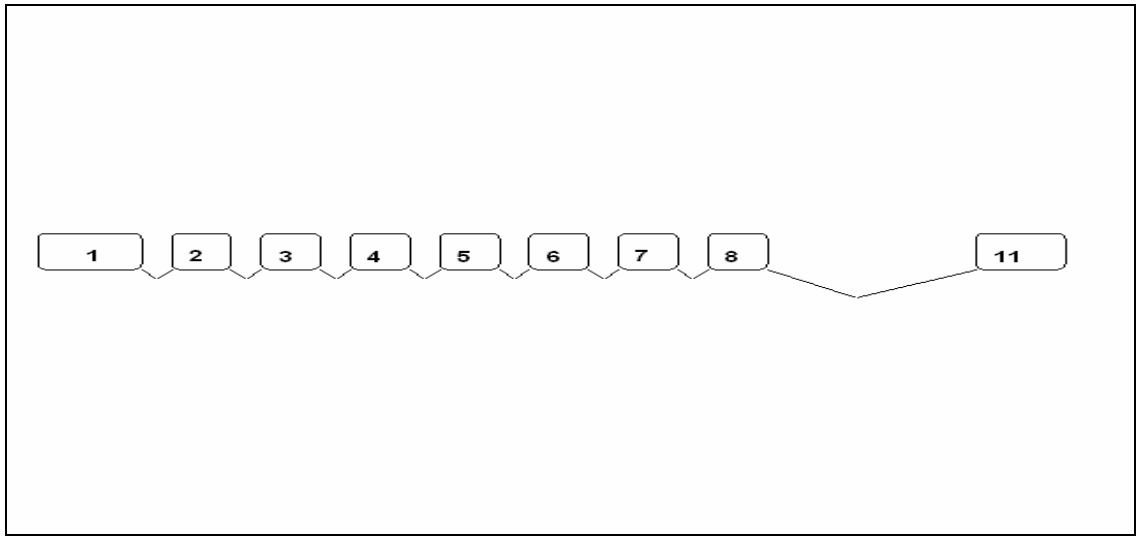



**Figure. 1.** Model for exon-intron configuration of FSHR-3 (Touzy et al., 2000). Exons 9 and 10 are removed by splice variation in this form of the receptor, and thus the exon which codes for the seven transmembrane and intracellular domains of the G protein coupled form (FSHR-1) is missing. The result is a receptor protein that can bind to FSH with high affinity but has only one transmembrane domain.

The major difference between the FSHR-1 and FSHR-3 variant is the number of transmembrane segments found bound to the membrane. Unlike the FSHR-1 variant, FSHR-3 has only one transmembrane segment available for coupling, causing alternative second messenger pathways to be activated (Touyz et al., 2000). The single transmembrane segment found within the FSHR-3 variant is typical of growth factor type I receptors and when activated causes activation of MAPK pathways (Touyz et al., 2000).

***FSHR-3 Pathway***

Because of the exclusive structure, the FSHR-3 variant is able to utilize pathways that are not directly dependent upon cAMP. Growth promoting events are mediated through activation of channels, such as MAPK, and $Ca^{2+}$. Touzy et al, (2000) documented an increase in $Ca^{2+}$ within 30 sec after application of only 1.0 nM FSH to an established cell line. The FSH was applied to human embryonic kidney cells (HEK) transfected to express the FSHR-3, FSHR-2, or FSHR-1 variant of the FSH receptor respectively. When compared to the other variants of the receptor, FSHR-3 showed a 3 fold increase in proliferation of the HEK cells, with regards to $Ca^{2+}$ response, unlike the FSHR-1 transfected HEK cells which showed only a 1 fold and FSHR-2, which did not increase at all. The ability of the FSHR-3 to cause a large influx of $Ca^{2+}$ provides a pathway for cells to acquire molecules required for cell proliferation in a very short amount of time, providing the cells with a special opportunity.

Besides causing a $Ca^{2+}$ influx, the FSHR-3 variant of the FSHR has also been found to directly activate the MAPK pathway in granulosa cells (Babu et al., 2000). By use of an alternative pathway FSHR-3 caused an influx of $Ca^{2+}$ which led to the activation of protein kinase C (PKC) followed by activation of MAPK (Babu et al., 2000). Phosphorylation of

MAPK leads to proliferation in the granulosa cells but the exact mechanism is yet to be determined (Babu et al., 2000).

***Dominant Negative FSH Receptor, FSHR-2***

In addition to the G protein-coupled receptor as well as the growth factor type-1, there appears to be an additional FSH variant. This variant was the second form of the FSHR to be identified and thus labeled FSHR-2. FSHR-2 has been deemed the dominant negative for the reason that it prevents FSH signaling even in the presence of the FSHR-1 form of the receptor (Yarney et al., 1996). The possibility of a variant receptor causing negative feedback would add to the already diverse pathways in which FSH appears to function, potentially providing a pathway for FSH to both stimulate and inhibit follicle growth.

***FSHR-2 Structure***

Like the other variants of the FSH receptor the FSHR-2 form differs from the FSHR-1 structure due to truncated exons with no change in affinity for FSH binding. When measuring the size of the FSHR-2 variant the mRNA sequence appears to be 25 amino acids shorter than the FSHR-1, even though the receptor does contain the seven transmembrane segments (Touzy et al., 2000). The difference in amino acids comes from truncated exons in this variant structure, which ultimately leads to a shortened C terminus. These shortened exons could allow the FSHR-2 receptor, when bound by FSH, to potentially create an environment where granulosa cells may become atretic. The truncated exons could cause the receptor to lose the ability to cause phosphorylation possibly of PKC, this condition could potentially be involved in the mechanism of atresia. Besides the possible decrease in phosphorylation sites, cysteines may also be increased in the C terminus, making a total of 23 for the entire receptor, potentially making the terminus more hydrophobic (Yarney et al., 1997).

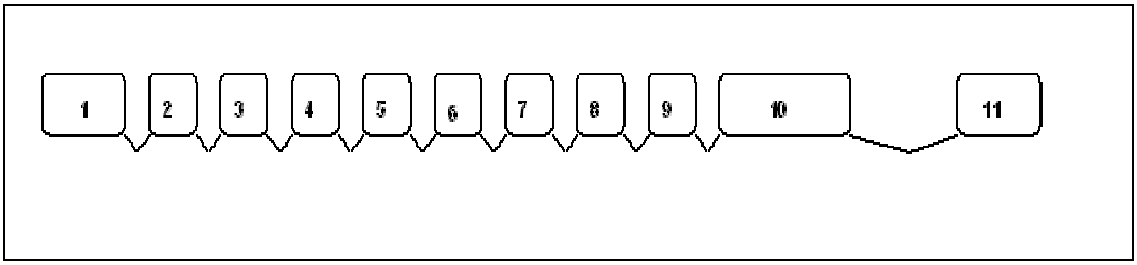



**Figure. 2.** Model of the arrangement for FSHR-2 exons and introns (Touzy et al., 2000). The truncated exon 10 is caused by a splice variant in this form of the FSH receptor and may be the reason it encodes for the seven transmembrane sections but with less phosphorylated sites, specifically for PKC.

***FSHR-2 Pathway***

When present, the FSHR-1 variant of the FSHR causes an increase in cAMP production when bound by FSH. However, if present the FSHR-2 variant could potentially inhibit cAMP production in granulosa cells, (Yarney et al., 2000). The dominant negative role for FSHR-2 was developed from cotransfection experiments with the FSHR-1 variant in culture. HEK cells were transfected to express FSHR-1 and FSHR-2, the cells were then placed in OPTI-MEM medium before having FSH applied for 1 hr at 37$^{o}$C. After radioimmunoassay, when administered with FSH, the FSHR-2 dominates over the FSHR-1 variant and caused an inhibition of cAMP activity (Yarney et al., 1997). The exact mechanism by which this receptor variant uses to cause this inhibition of cAMP remains to be identified. The FSHR-2 receptor may be able to bind a higher concentration of circulating FSH than other variants. This ability to bind a higher percentage could be what causes follicles to become atretic instead of ultimately ovulating. Whatever the outcome more experiments must be conducted before any defining statement about FSHR-2 can be made.

***Cultured Cell Line***

The idea that the FSH receptor may be expressed on cells other than granulosa cells is an interesting concept. Work done by Li et al, (2007) showed that a tumor causing cell line, derived from ovary surface epithelial cells, showed a response to exogenous FSH after only 24 hrs post treatment. The possibility of an FSH receptor being present on cancer cells helps in furthering our understanding of how ovarian cancers may become FSH responsive.

Mouse ovarian surface epithelial cells (MOSEC) were collected from adult female C7BL6 mice. Medium containing epithelial cells were allowed to grow 2-3 weeks at 37$^{o}$C and 5% $CO_2$ (Robey et al., 2000). While in culture, the epithelial cells grew in a cobblestone morphology and ceased growth upon contact with other cells. However, during later passages the cells did not exhibit cobblestone shapes and continued to grow, even after coming into contact with other cells, suggesting some type of change had occurred.

These later passages proved to be of special interest when injected into immunocompromised mice, where these cells caused tumors similar to the ones found in human ovary tissue. Time of death for injected mice was on average 33 days post injection. Tumors were observed primarily in the peritoneal cavity especially in the kidneys, pancreas, stomach, and spleen (Robey et al., 2000). Upon analysis of cells within these tumors, multiple chromosomes were gained or lost, depending on the type of tissue. These changes in chromosomes were found to be similar to those chromosome changes observed in human epithelial ovarian carcinoma (Robey et al., 2000). With these notable changes the MOSEC cell lines have become a model which gives researchers the ability to study ovarian cancer. A novel aspect to these cells is their ability to grow and cause tumors, even in mice with intact immune systems. Currently, these cells are the only ones capable of growing in the presence of a normal

immune system which provides researchers with critical information as to how normal cancer cells are able to escape attack by natural killer and other tumorolytic cells (Robey et al., 2000).

# Chapter 2

# Regulated expression of Follicle Stimulating Hormone Receptor Type III in Cancer causing Mouse Ovarian Surface Epithelial Cells.

***Introduction***

The drive to further understand how cancer cells differ from normal cells increases with every case that result in death or disability. Ovarian cancer is the eighth leading cause of death among women in the US, and thus reducing the number of cases will require an increased understanding of cancer cell growth mechanisms. The American Cancer Society estimates that about 22,430 new cases of ovarian cancer will be diagnosed in the United States during 2007, which accounts for 3% of all cancers in women. Of these new cases, approximately 15,280 will result in death (American Cancer Society, 2007). Recent work done by Li et al, (2007) showed that Mouse Ovarian Surface Epithelial Cell (MOSEC) lines, documented for causing tumors in mice, are responsive to FSH. The idea that FSH may impact ovarian cancer cell proliferation provides a new avenue to focus research efforts, provided these cells express the FSH receptor, and provided that the types of FSH receptor and control of its expression can be characterized.

*FSH Receptor*

Follicle Stimulating Hormone has long been believed to work through a G protein-coupled receptor (GPCR). This GPCR for FSHR-1 is characterized by seven hydrophobic helices and causes an increase in cAMP after binding to FSH. However, not all activities can be explained by the GPCR for FSH. Touzy et al, (2000) described a variant form of the FSH

receptor that may account for such activities as influx of $Ca^{2+}$ or cell proliferation in granulosa cells treated with FSH. This receptor variant FSHR-3 has been shown to have structural similarities to the growth factor type I class of receptors (Touyz et al., 2000). Not surprisingly, then, when bound to FSH, the FSHR-3 causes activation of the MAPK signaling pathway through *jak/stat*, with no increase in cAMP production (Touzy et al., 2000). Thus, it is likely that cells will proliferate in response to FSH when theFSHR-3.

Recently FSHR-3 was documented as being present in the ovarian epithelial tissue of mice (Li et al., 2006). These researchers found that mouse ovarian surface epithelial cells proliferated in response to FSH. Therefore the hypothesis has been developed that by down-regulating FSHR-3 in these cells, one could slow their growth rate in response to FSH. By extension, then, it may be possible to reduce the growth rates of FSH-responsive tumors in women.

*RNAi*

In the past several years RNA inhibition (RNAi) has become widely used in the area of gene knockdown. While RNAi is a relatively new procedure, it may hold the key to possible solutions for cancer patients. RNAi is a naturally occurring process originally believed to have arisen for protection from viruses and transposons. It was first observed in plants and invertebrates (Fire et al., 1998).

In mammalian systems, in order for the RNAi mechanism to target the correct sequence, synthetic small inhibiting RNA (siRNA) sequences must first be introduced (Brummelkamp et al., 2002). The designs of these siRNA sequences are complimentary in nucleotide sequence to their desired targets. However, slight mismatches in siRNA complimentarity still produced down-regulated effects, depending on where the mismatch was located (Bantounas et al., 2004).

Using RNAi with siRNA designed to target portions of FSHR-3 may therefore be effective in controlling expression of this FHSR variant. As a result, it may be possible to control cancer cell growth by causing a decrease in the number of growth promoting receptors such as FSHR-3.

***Materials and Methods***

*Reagents*

Antibodies were prepared specifically against FSHR-3 (Gallus Immunotech, Inc, Ontario, CA) based on the published amino acid sequence from the sheep testis and targeted for the C-terminus of the receptor (Babu et al., 1999). Actin antibody and all secondary antibodies were ordered from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Transfection reagent Lipofectamine 2000 was purchased from Invitrogen Corp. (Carlsbad, CA). Other reagents and chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO), unless otherwise specified.

*Cell Culture*

A clonal cell line of mouse ovarian surface epithelial cells (MOSEC) was transformed after repeated passages *in vitro*, and caused tumors when injected into in immunocompent mice (Roby et al., 2000). The MOSEC were provided by Dr. K. Roby from the University of Kansas Medical Center, Center for Reproductive Sciences (Kansas City, KS). The ID8 cells were one of ten clonal cell lines established from the original MOSEC, and were cultured in DMEM enhanced with 4% FBS, 100.0 IU/ml penicillin, 100.0 *u*g/ml streptomycin, 5.0 *u*g/ml insulin, 5.0 *u*g/ml transferrin and 5.0 ng/ml sodium at 37$^{o}$C and 5% $CO_2$.

The ID8$^{TaxR}$ cells were developed as previously reported (Taylor, C, 2003) from the ID8 cell line originally developed by Roby, and cultured using the same complete media as the ID8 cell line in the presence of 1.0 umol/L of Paclitaxel (Sigma, St. Louis, MO). Paclitaxel belongs to a group of drugs known as mitotic inhibitors and is used in chemotherapy treatments for

ovarian and breast cancer. Paclitaxel works by stabilizing the polymerization of microtubules in cancer cells, thus rendering the cell incapable of proliferating (Coley et al., 2006).

Upon presenting the ID8 cells with Paclitaxel, the surviving cells were collected and cultured before being presented again with Paclitaxel (Taylor, C, 2003) . This process was continued until a stable drug resistant cell line was established and labeled $ID8^{TaxR}$. The $ID8^{TaxR}$ cell line was cultured in complete medium, which included DMEM, 4% FBS, 100.0 IU/ml penicillin, 100.0 *u*g/ml streptomycin, 5.0 *u*g/ml insulin, 5.0 *u*g/ml transferrin and 5.0 ng/ml NaCl, with the addition of 1umol/L of paclitaxel.

*Assessment of Protein Concentrations*

Both the MOSEC ID8 (ID8) and the drug-resistant MOSEC $ID8^{TaxR}$ ($ID8^{TaxR}$ ) cells were seeded in six-well plates at a concentration of $5 \times 10^4$ cells/ml in complete medium. For the ID8 cells, four treatment groups were designed to test the efficiency of an siRNA pool designed to specifically knock-down FSHR-3. Each treatment group utilized one six-well plate per group, with ID8 groups defined as follows: 1.) FSHR-3-specific siRNA 48-hour single transfection; 2.) Nontarget siRNA 48-hour single transfection; 3.) FSHR-3-specific siRNA 24-hour double transfection; 4.) Nontarget siRNA 24-hour double transfection. The $ID8^{TaxR}$ cells were separated into two groups as follows: 1.) $ID8^{TaxR}$ Nontarget siRNA 24-hour double transfection; and 2.) $ID8^{TaxR}$ FSHR-3 siRNA 24-hour double transfection (results from preliminary experiments had indicated that double-transfections were more effective at increasing siRNA introduction into ID8 cells, and thus single transfection treatment groups were not included for $ID8^{TaxR}$ cells). "Double" and "single" transfections are as described below.

*Transfection*

For the single transfection treatment groups, ID8 cells were seeded as previously stated and allowed to grow until the well reached 30-40% confluency, which took approximately 8 hrs. Complete medium was then removed and replaced by 2.0 ml of OPTIUM for 0.5 hr. While in the OPTIUM medium, 5.0 $\mu$l of the FSHR-3 siRNA (1.0nmol/$\mu$l) or Nontarget siRNA (1.0nmol/ $\mu$l) was added to 95.0 $\mu$l of OPTIUM medium, then incubated at RT for five min. In a separate tube, 5.0 $\mu$l of Lipofectamine 2000 was added to 95.0 $\mu$l of OPTIUM medium and incubated for 5 min at RT. Following the 5 min incubation, Lipofectamine-2000 was added to the FSHR-3 siRNA to bring the amount per tube to 200 $\mu l$. The solution was incubated for 20 min with occasional mixing. This process was repeated for each well. OPTIUM medium was removed from the wells and replaced with 200.0 $\mu l$ of the appropriate siRNA/Lipofectamine-2000 mixture, and 800.0 $\mu l$ of OPTIUM medium was then added to each well to bring the total volume to 1.0 ml. The plates were then placed in the incubator at 37$^{o}$C and 5% $CO_2$. Sixteen hours after adding the siRNA solution, media were removed and replaced with 2.0 ml of complete medium, and cells were allowed to grow for 48 hours before collection, solubilization of total protein and assessment of FSHR-3 protein expression.

The double transfection treatment groups were treated the same as the single transfection groups, with the exception that, at 48 hours, each well within the treatment group was trypsinized and cells were reseeded into new plates. After reseeding, the cells were allowed to attain 30-40% confluency before the transfection procedure was repeated as described for single-transfection treatment groups. Transfection media were removed and replaced with complete media after 16 hours of transfection and the cells were incubated for an additional 24-30 hours at

37°C and 5% $CO_2$ before being collected for solubilization of total protein and assessment of FSHR-3 protein expression.

**Table 1.** Control and FSHR-3-targeting siRNA oligonucleotide sequences. Oligonucleotides used for RNAi were purchased from Dharmacon (Chicago, IL). Positive control siRNA oligonucleotides should cause reduced Lamin A/C expression after transfection. Negative control siRNA should cause activation of the RISC complex, including dicer, to ensure that any measured decreases in protein levels were not due solely to activation of the RNAi pathway. The FSHR-3 siRNA pool contained 4 oligonucleotides, each of which specifically targeted unique regions of FSHR-3. Thus, addition of FSHR-3 pool siRNA should cause formation of RISC and specific cleavage of FSHR-3 mRNA, and a subsequent reduction in FSHR-3 protein.

| **Standard Controls** | **Reagents** | **Sequences** |
|---|---|---|
| Positive Control | Lamin A/C | **Seq.**: GGUGGUGACGA UCUGGGCU |
| Negative Control | Nontarget | **Seq. 1**: UAGCGACUAAACACUCAA<br>**Seq. 2**: UAAGGCUAUGAAGAGAUAC |
| FSH Pool | | **Seq. 1**: PUAAAGCAGCAUGCCAUUAGUU<br>**Seq. 2**: PUAUGAUUUAGCUUCCUCUUUU<br>**Seq. 3**: PUUUGCUGUCAGAGCUAGUCUU<br>**Seq. 4**: PCAAGGAAGAAUGAUUGUCAUU |

*Protein Analysis*

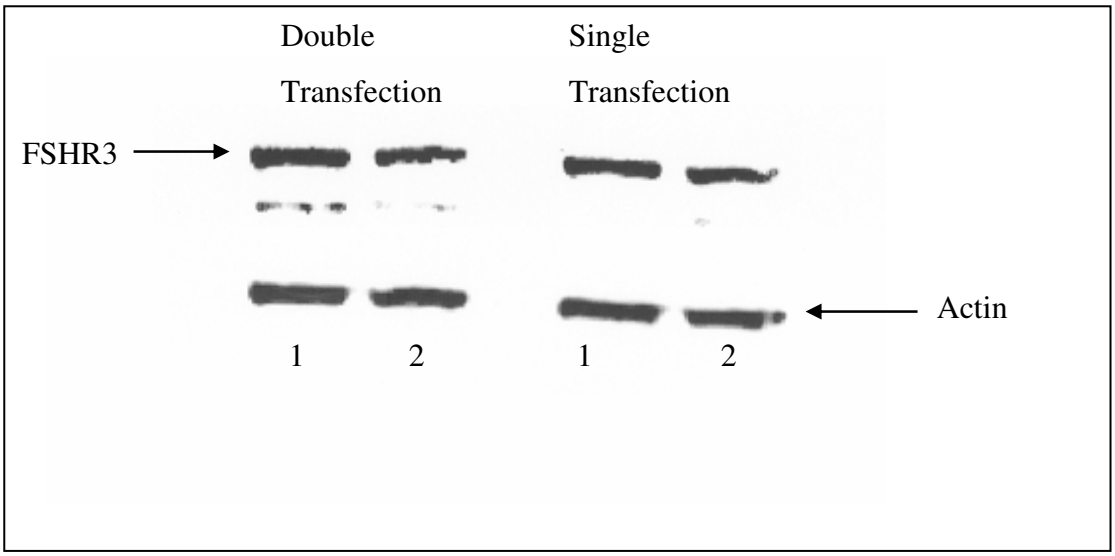
Lysates were made from MOSEC ID8 cells in RIPA buffer (20.0 mM Tris-HCl (pH 7.5), 150.0 mM NaCl, 1.0 mM $Na_2EDTA$, 1.0 mM EGTA, 0.01 NP-40, 0.01 sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1.0 mM beta-glycerophosphate, 1.0 mM $Na_3VO_4$, 1.0 µg/ml leupeptin and protease inhibitor solution (Sigma P-8340)). A Micro BCA protein assay kit (Pierce, Rockford, IL) was used to assess protein concentrations from extracts. A total of 15.0 *u*g of protein was loaded onto SDS-PAGE gradient gels (4-12% NuPAGE Novex Bis-Tris with MOPS running buffer, Invitrogen, Carlsbad, CA). Protein bands following electrophoresis were transferred to a nitrocellulose membrane. The membrane was then blocked with TBS containing 0.1% Tween-20, 10% nonfat dry milk and 10% glycerol for 1 hour at RT and then incubated in primary antibody (Chicken anti-FSHR-3, diluted 1:1000 in blocking solution) overnight at 4°C.

Following the primary antibody, the membrane was washed in TBS containing 0.01% Tween-20 and incubated in goat anti-chicken IgY antibody (Santa Cruz, CA) (diluted 1:2000 in blocking solution) for 1 hour at RT.

Primary antibody binding was observed using West Pico Chemiluminescent detection system (Kodak Imaging). Equal loading was visualized by stripping FSHR-3 primary and secondary antibodies and immunoblotting for actin using donkey polyclonal primary antibody (1:1000) and anti-donkey secondary antibody (1: 3000). Membrane stripping was accomplished using Restore Stripping Buffer (Pierce) to reprobe for actin.

***Results***

Transfection of siRNA sequences into target cells was confirmed by use of the positive control siRNA targeted for Lamin A/C. Lamin A/C protein was analyzed using western blotting techniques, and protein levels showed a 50% reduction after densitometric analysis of Lamin A/C from cells transfected with siRNA versus untransfected cells. The siRNA sequences used as a negative control activated the Dicer complex providing evidence that any reduction in protein was not due to activation of the RISC complex, while allowing for verification of transfection into the MOSEC line. After transfection of the FSHR-3 targeting siRNA pool into MOSEC, there was an average 18.2%, ± 4% decrease in FSHR-3 protein remaining at 48 hrs after transfection. Results were obtained by densitometric analysis of FSHR-3 protein on Western blots from cells transfected with FSHR-3 siRNA versus cells transfected with non-targeting siRNA (Figure 3).



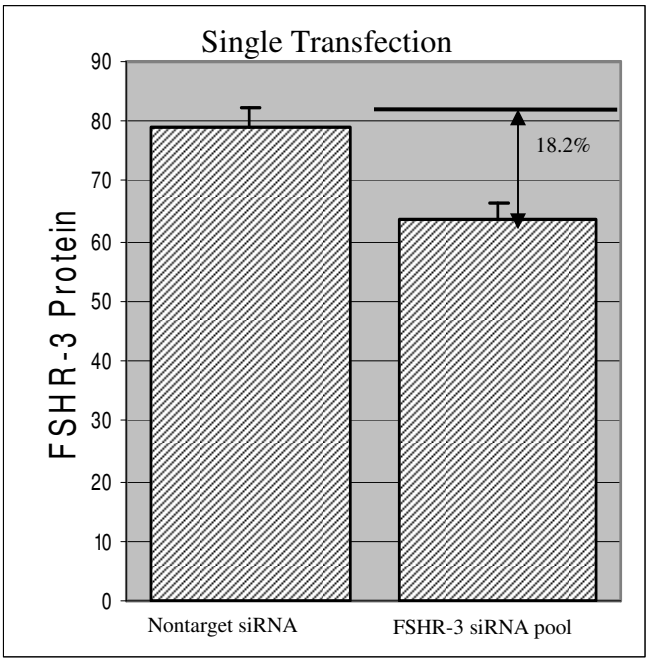




**Figure 3**. Reduction in FSHR-3 protein after single transfection of FSHR-3 targeting siRNA oligonucleotides. Proteins were collected from MOSEC ID8 cells collected at 64-hours post transfection and the amount of FSHR-3 protein was compared via densitometric analysis of Western blots between cells transfected with a non-targeting siRNA versus cells transfected with an anti-FSHR-3 siRNA pool. Shown in the upper panel are Western blot results of FSHR-3 (high band) or actin protein (lower band) after double (lanes 1 and 2) or single (lanes 3 and 4) transfection with siRNA. Lanes 1 and 3 show results of transfection with non-targeting siRNA verses transfection with a pool of 4 anti-FSHR-3 siRNA oligonucleotides (lanes 2 and 4). Data are expressed as mean $\pm$ SE from at least two experiments. Under conditions of equal protein loading, expression of FSHR-3 protein appeared to be reduced on Western blots after

introduction of a siRNA pool that targeted FSHR-3. Shown in the lower panel are percent decreases in FSHR-3 based protein expression, as determined by densitometric analysis.

After 48 hours post transfection the double transfection group was transfected a second time with the anit-FSHR-3 siRNA pool. Cells were allowed to grow for 24-30 hrs before collecting protein for Western blot analysis. Results for the double transfection showed a larger decrease in FSHR-3 protein with an average of 29.8%, ± 5% (Figure 4). The larger decrease in FSHR-3 protein provides evidence that multiply transfections may be required for larger percent knockdown in these cells.

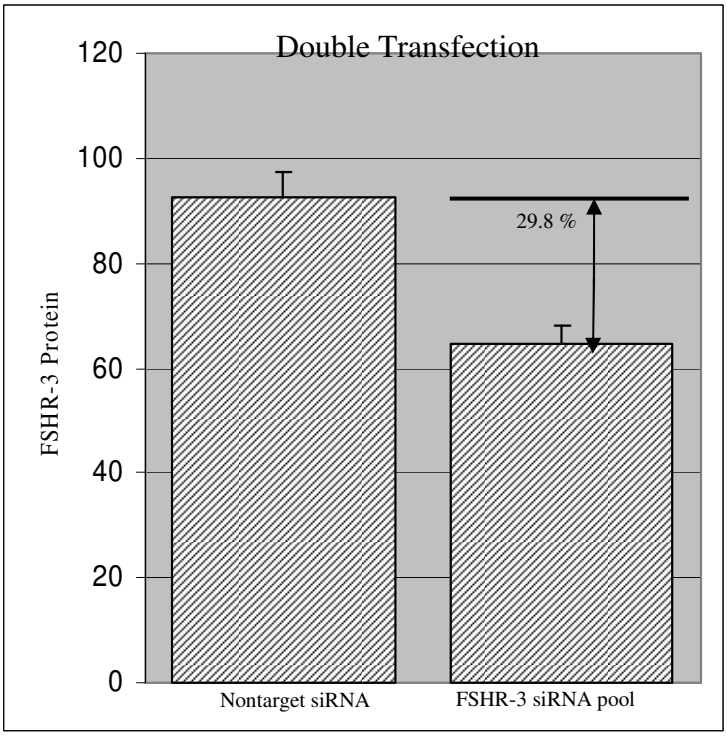



**Figure 4**. Reduction in FSHR-3 protein after double transfection of FSHR-3 targeting siRNA oligonucleotides. Proteins were collected from MOSEC ID8 cells collected at 72-hours post transfection and the amount of FSHR-3 protein was compared via densitometric analysis of Western blots between cells transfected with a Non-targeting siRNA versus cells transfected with an anti-FSHR-3 siRNA pool. Shown in Figure 1 are Western blot results of FSHR-3 (high band) or actin protein (lower band) after double (lanes 1 and 2) or single (lanes 3 and 4) transfection with siRNA. Lanes 1 and 3 show results of transfection with non-targeting siRNA verses transfection with a pool of 4 anti-FSHR-3 siRNA oligonucleotides (lanes 2 and 4). Data are

expressed as mean $\pm$ SE from at least two experiments. Under conditions of equal protein loading, expression of FSHR-3 protein appeared to be reduced on Western blots after introduction of a siRNA pool that targeted FSHR-3 into ID8 cells. Shown in Figure 3 are percent decreases in FSHR-3 protein expression, as determined by densitometric analysis after transfection of siRNA as described.

When $ID8^{TaxR}$ was compared to the nontarget siRNA control the $ID8^{TaxR}$ using densitometric analysis from Western blotting (Figure 4) nontarget siRNA showed over expression of the FSHR-3 variant. The results were confirmed for equal loading and proved that indeed an average of 19%, ± 2% over expression of the FSH receptor was being expressed in the resistant cell line as shown by Western blotting in Figure 5.

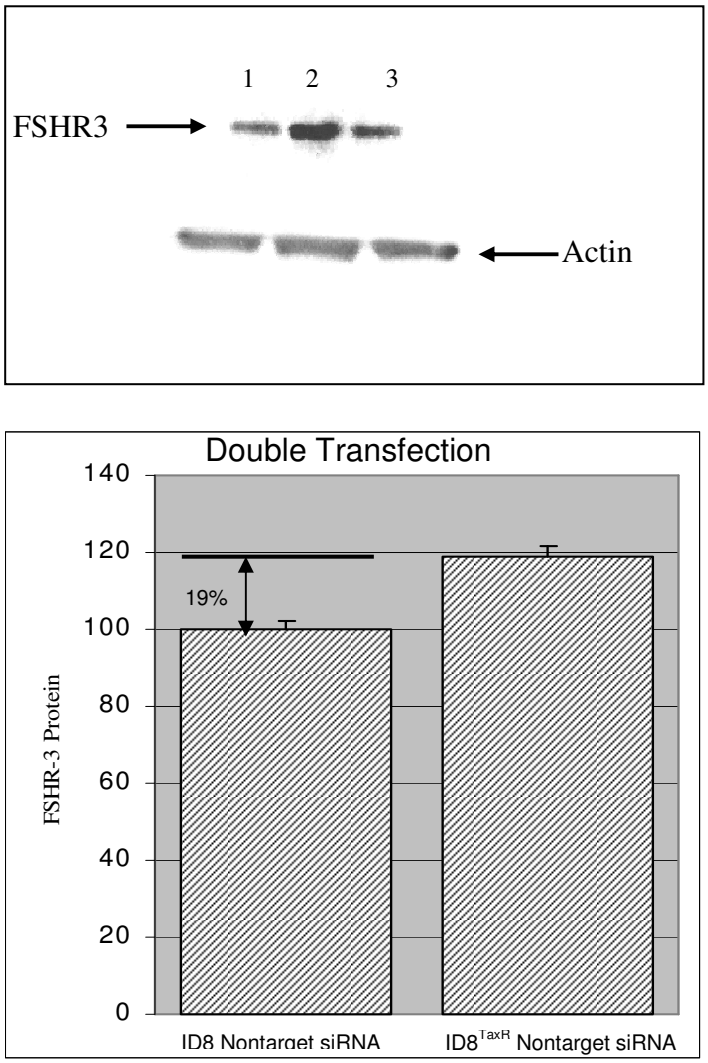



**Figure 5**. Overexpression in FSHR-3 protein after double transfection of FSHR-3-targeting siRNA oligonucleotides. Proteins were collected from MOSEC ID8 cells and ID8$^{TaxR}$ cells at 72-hours post siRNA transfection and the amount of FSHR-3 protein was compared via densitometric analysis of Western blots between cells transfected with a non-targeting siRNA versus cells transfected with an anti-FSHR-3 siRNA pool. Shown the upper panel are Western blot results of FSHR-3 (high band) or actin protein (lower band) after double (lanes 1 and 2). Shown in lane 1 are protein bands resulting from transfection with non-targeting siRNA while shown in lane 2 are protein bands resulting from transfection with a pool of 4 anti-FSHR-3 siRNA oligonucleotides. Representative results are shown from one experiment, and experiments were duplicated at least once. Under conditions of equal protein loading, expression of FSHR-3 protein appeared to be over-expressed on Western blots after introduction of a siRNA pool that targeted FSHR-3. Shown in the lower panel are percent increases in FSHR-3 protein expression, as determined by densitometric analysis.

***Discussion***

Follicle stimulating hormone, though clearly important for follicular development, may also have an important role in the proliferation of ovarian cells involved in certain types of cancer. In a clonal MOSEC line it has been documented that FSHR-3 is present and, when treated with FSH, these cells proliferated from $2.5x10^5$ to $3.5x10^5$ cells in 24 hrs (Li et al., 2007). However, Sairam et al. (2007) reported that any FSHR variants within ovaries that decreased early in life lead to tumor formation in aged mutant mice, suggesting that ovarian tumor cells all lack expression of FSHRs and proliferate through alternative pathways. Differences in results between this study and Sairam's can be attributed to the use of cultured cells instead of *in vivo* within knockout mice. However, in support of Li et al, (2007) the results obtained herein show that these tumor causing MOSEC express the R-3 variant form of the FSH receptor, which provided a means for proliferation in response to FSH through the MAPK pathway. As described by Babu et al. (2000), FSH caused activation of PKC of the MAPK pathway which lead to proliferation of these ovarian cells.

In the present study, both the FSHR-3 single siRNA transfection group and double transfection group showed a decrease in FSHR-3 protein when compared to the nontarget siRNA transfection groups. The positive control siRNA group showed a reduction in Lamnin A/C expression which provided evidence that the siRNA procedure was functional. The negative control siRNA group provided no reduction of expression due to the nonspecific activation of the RNAi pathway. Thus, it appears that assay and transfection conditions used in the present experiments were valid.

The use of siRNA in ID8 cells has been previously reported (Chen et al., 2005). These investigators transfected ID8 cells with siRNA sequences targeted for caspase-9 receptor and

analysed protein expression at 48 hours after transfection. They observed a 90% knockdown in caspase-9 receptor protein at this time (Chen et al., 2005). Based on our use of a similar protocol, we anticipated knockdown of FSHR-3 protein in ID8 cells. Due to Lipofectamine 2000's ability to insert siRNA sequences into suspended and adherent cells we believed by introducing a second siRNA sequence (see double transfection protocol), expression of any new receptors produced by the developing cells would also be reduced. Upon completion of the single and double transfection experiments, results for receptor knock down were not 90% as predicted even though the double transfection group did provide a larger percentage knockdown. Possible explanations for achieving lower silencing results than expected could include removing transfection agents after only 16 hrs of transfection, or using minimal concentration of siRNA sequence in solution for the single and double transfections. Future use of dose-response curves for siRNA transfection is definitely warranted.

An interesting finding from this study was an apparent over-expression of FSHR-3 observed in $ID8^{TaxR}$ cells when compared to ID8 cells. It is possible that these cells attain an increased drug resistance through increased ability to respond to circulating FSH concentrations through increased expression of FSHR-3, and thus to receive more gonadotropin support to increase their survivability. It is intriguing to note that the variant of the FSH receptor known to act through a growth-factor type I second messenger signaling pathway appears to be elevated in a cell line that is also capable of surviving chemotherapeutic agents such as Paclitaxel. It may be possible to speculate that increased growth factor support within tumor cells generally decreases responsiveness to chemotherapeutics.

In conclusion, we observed that the MOSEC line labeled as ID8 does express the FSHR-3 variant, a finding that correlates with earlier results in which ID8 cells proliferated in response

to FSH (Li et al., 2007). This study also showed an over expression of the FSHR-3 variant in the chemotherapeutic resistant cells, which may provide preliminary evidence that certain types of cancer cells escape killing from chemotherapeutics by increasing responsiveness to growth factors such as FSH. Moreover, we conclude that siRNA used in a pool targeted for a specific FSH receptor protein appears to be a valid experimental approach to study receptor expression and function. Eventually, the use of specific gene knockdown tools such as siRNA may be used to enhance the effectiveness of chemotherapeutics in cancer treatment.

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# APPENDIX

NuPAGE Bis-Tris Gel Instruction

## I. Preparing Samples

1. Grab a 'lane sheet' and fill it out completely
   - List lane sheet the order of lanes (put comparison lanes next to each other), concentration of sample etc,
   - You want to load 15 *u*g total protein per lane. Determine volume ($\mu$l) of sample needed to load X ug protein.
   - Add water, or RIPA, or 1X PBS to bring the volume up to 16 $\mu$l
   - Add 2.5 $\mu$l 10X reducing agent
   - Add 6.5 $\mu$l 4X LSB.
   - Total volume should be 25 $\mu$l
   - Consider using one marker lane per anti-body per gel: if you probe with two different antibodies and you will cut your membrane in half instead of running two gels, use two ladder lanes.
2. Thaw protein samples on ice (if frozen).
3. Get out one rack per gel (to avoid confusion)
4. Get out one 0.5 ml tube per lane (except marker). Label tubes according to lanes.
5. Centrifuge protein samples: 3m @ 14rcf to bring aggregates to bottom (we take protein from top).
6. Fill tubes with appropriate amounts of 6.5 $\mu$l LSB, 2.5 $\mu$l Reducing agent, and water.
7. Load protein into tubes, vortex to mix. [when done w/protein put back on ice].
8. Place tubes in thermocycler, close top, heat to 95$^{o}$C for 5 m, remove immediately.

## II. Running the gel

9. Working quickly (cover the next 4 steps in 10 min), get out gel from sliding glass fridge. Cut open package with scissors and remove the gel. Rinse gel cassette in $dH_2O$. Peel the tape from the bottom of cassette. In one smooth motion, remove comb from cassette.
10. Use a disposable pipette to rinse the sample wells with 1X SDS-Running Buffer to remove air bubbles and storage solution. Invert the cassette and shake to remove the buffer, repeat two more times.
11. Get out the XCell Mini-Cell, and orient the gel in the Mini-Cell so that the dam on the other side of the core, and lock the gel into place with the tension wedge.
12. Set aside 800 ml of the 1X NuPAGE SDS Running Buffer for use in the lower buffer chamber. Approximately 600 ml of 1X Buffer will fill the lower chamber. Immediately prior to run, prepare the remaining 200 ml for the upper chamber by adding 500 $\mu$l of the NuPAGE Antioxidant per 200 ml 1X NuPAGE SDS Running Buffer. Mix thoroughly.
13. Check for seal tightness in the mini-cell by filling the upper chamber with a small amount of reduced buffer. If there are no leaks, continue to fill the upper chamber so that the level of buffer exceeds the wells.

14. Using gel-loading tips, load samples into wells. Tip: Set pipette to just over total volume to get everything in tube.
15. Fill the lower buffer chamber with 600 ml of 1X NuPAGE SDS Running Buffer.
16. To run a single gel with MOPS buffer: 200V for 50 min. Use the BioRad Powerpack 200. Running times are different for MES buffer.
17. During the run prepare Transfer Buffers
    Prepare Transfer Buffer as follows:

| | |
|---|---|
| 20X NuPAGE Transfer Buffer | 40ml |
| Methanol | 80ml |
| $dH_2O$ | 680ml |

18. 10 min before end of run, get out XCell Trans Blot Module.
19. Use about 600 ml of 1X Transfer buffer to soak two blotting pads until saturated. Remove any air bubbles by squeezing the pads while they are submerged in buffer.
20. Cut filter paper and Nitrocellulose membrane to dimensions of the gel. Briefly soak the filter paper in transfer buffer immediately prior to use. In a separate Tupperware soak membrane in transfer buffer for several minutes.
21. After run is complete, shut off power, disconnect electrodes, and remove gel from mini-cell. Dump running buffer.
22. Place saran wrap on work area.
23. Separate each of the three bonded sides from the cassette. The notched "well" side of the cassette should face up. Carefully remove and discard the top plate, allowing the gel to remain of the bottom (slotted) plate.

**III. Transfer the gel**

24. Remove wells from gel with razor blade.
25. Place a piece of pre-soaked filter paper on top of the gel, and lay just above the slot in the bottom of the cassette, leaving the "foot" of the gel uncovered. Keep the filter paper saturated with transfer buffer. Using a pipette as a roller, make sure all air bubbles are removed.
26. Turn the cassette over, so the gel and filter paper are facing downwards. Push in on the "foot" to remove the gel from the plate. Cut the foot off the gel with a razor.
27. Wet the gel with transfer buffer, and position the membrane on the gel, ensuring all air bubbles are removed.
28. Place another pre-soaked filter paper on top of the membrane, remove any trapped air bubbles.
29. Open core of the transfer module so the dark side is facing down. Place pre-soaked blotting pads on the dark side. Place the gel/membrane & filterpaper another pre-soaked pad on top of the sandwich. Close the cassette and lock it with the white latch.
30. Hold the cassette together and slide it into the guide rails into the tank.

31. Fill the tank with transfer buffer until the gel/membrane sandwich is covered in transfer buffer. You do not fill all the way to the top: this will only generate extra conductivity and heat.
32. Fill outside chamber with dd$H_2O$.
33. Place lid on unit
34. Transfer gel in the fridge, to help keep temperature down. Place the tank on the magnetic stirrer. Transfer at 30V for 1 hour.

WESTERN BLOTTING

1. Get out 2 Tupperware containers and fill with 1X Semidry Transfer Buffer.

2. Remove gel from box

    i. Cut edges of gel with razor blade and cut out wells
    ii. Cut off any strings on sides of gel

3. Place gel with plate into Tupperware containing 1X Transfer Buffer
    i. Carefully remove gel plate from gel and discard
    ii. Incubate gel for 10-15 min in 1X Transfer Buffer

4. Get Blocking Buffer out
    i. 10% Blocking buffer
        1. 25 ml 10X TBS
        2. 25 ml Glycerol
        3. 25 g Milk Powder
        4. 500 $\mu$l Tween 20
        5. Bring volume to 250 ml with d$H_2O$
        6. Stir for 30 min
        7. Filter

5. Place gel in container with d$H_2O$ and incubate for 1 hour at RT.

6. Place membrane into Blocking Buffer; incubate for 1 hour at RT on rocker.

7. Gel-dump out d$H_2O$ and add 5-6 ml Blue Stain Reagent and stain 1 hour, then rinse with d$H_2O$ a few times.

8. After membrane has been blocked for 1 hour, add the primary antibody to the membrane, place on rocker at 4$^oC$ for an O/N incubation

9. After O/N incubation, get small Tupperware container and fill with 1X TBS+Tween. Remove membrane from container and place in container with TBS+Tween.

    i. Rock and wash 5 X at 6 min each

10. During last wash, spin down secondary antibodies for 3 min @ 14 rcf.

11. Make up secondary antibody, usually 1: 2000 concentration blocking buffer to antibody.

12. When final wash is complete add secondary antibody to Tupperware and put lid on. Place on rocker at RT for 1 hour.

13. Once secondary ab incubation is over, dump out secondary ab into sink,

    i. Rinse membrane with 1X TBS+Tween for 5 min
    ii. Repeat 6 times

14. Develop blot using Kodak imager
    i. Femto
        1. Put saran wrap on work area, place blot on saran wrap
        2. Mix 1.5 ml each of Femto reagents 1 and 2 in tube mix thoroughly.
        3. Using pipette, add Femto to top of membrane, covering it completely
        4. Let incubate for 5 min at RT
        5. Use tweezers to lift membrane into a plastic sheet protector.
        6. Develop membrane at 3 min and 4 min.

STRIPPING ANTIBODIES FROM MEMBRANE

1. Wash membrane 3x with 1X TBS+Tween; 5 min each

2. Strip membrane with Restore Stripping Buffer. Place membrane into Tupperware container and add enough Stripping Buffer to container to cover membrane well.

    a. Incubate 20 min at 37$^{o}$C
    b. 20 min RT on rocker

3. Wash membrane for 6 min with 1X TBS+Tween, repeat 5 X.

4. Prepare antibodies as stated previously for primary and secondary. Develop after secondary ab incubation as stated.

## Nupage data sheet

Date Gel type Project Running buffer

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| sample | | | | | | | | | | | | |
| Date of sample prepared | | | | | | | | | | | | |
| Conc. Of sample (ug/ul) | | | | | | | | | | | | |
| Vol. of sample makes ug/lane | | | | | | | | | | | | |
| $H_2O$ (ul) | | | | | | | | | | | | |
| Reducing agent (10x) | | | | | | | | | | | | |
| Loading buffer (4x) | | | | | | | | | | | | |
| Total vol. (ul) | | | | | | | | | | | | |
| 1° antibody | | | | | | | | | | | | |
| 2° antibody | | | | | | | | | | | | |

Comments:

Running buffer: 50ml 20x buffer + 950ml dd $H_2O$, 200ml 1x buffer +0.5 ml antioxidant (for reducing PAGE)
Transfer buffer: 40ml 20x buffer + 80ml methanol + 680ml dd$H_2O$ (+0.8ml antioxidant for reducing samples)