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Review Article

Multifaceted Approaches to Delineate the Structural and Functional Determinants of Follicle Stimulating Hormone Receptor

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The interaction of follicle stimulating hormone (FSH) with its cognate G-protein coupled receptor, the follicle stimulating hormone receptor (FSHR) is crucial for folliculogenesis in females and spermatogenesis in males. This necessitates the delineation of the hormone-receptor interaction in greater detail at the molecular level. Several strategies have been employed over last two decades to delineate the hormone-receptor interaction at structural, molecular and cellular level. Here, we have attempted to summarize the work carried out using synthetic peptides and anti-peptide antibodies corresponding to the predicted potential functional epitopes of FSHR, site-directed mutagenesis, naturally occurring mutations and single nucleotide polymorphisms. The collation of information from the methods discussed will presumably prove to be useful in designing modulators of FSH action and enable better understanding of the involvement of various residues or domains of the receptor in its function. The information will also possibly help in developing predictive markers of ovarian response in women undergoing assisted reproductive techniques.

Key Words: FSH Receptor; FSHR Gene; Synthetic Peptides; Antipeptide Antibodies; Site Directed Mutagenesis; Mutation; Polymorphisms

Introduction

Follicle-stimulating hormone (FSH) is a glycoprotein secreted by the anterior pituitary gland under the pulsatile regulation of gonadotropin releasing hormone (GNRH). It is one of the key hormones for mammalian reproduction and is involved specifically in the gonadal development, oocyte maturation at puberty and gamete production during the reproductive phase of life (Simoni and Nieschlag, 1995; Nieschlag *et al.*, 1999). The other members of the glycoprotein family include: luteinizing hormone (LH), chorionic gonadotropin (CG), and thyroid stimulating hormone (TSH). These heterodimeric glycoprotein hormones share a common α-subunit and differ in their unique β-subunit for a given species that determines receptor specificity (Pierce and Parsons, 1981). However, the biological activity is conferred only by the heterodimers.

FSH elicits its physiological action by binding to its cognate receptor (FSHR), expressed on the granulosa cells in the ovary and Sertoli cells in the testis (Sprengel *et al.*, 1990; Camp *et al.*, 1991; Kelton *et al.*, 1992) to bring about folliculogenesis (Richard and Midgley, 1976) in females and spermatogenesis (Nieschlag *et al.*, 1999) in males. FSHR, along with other glycoprotein hormone receptors (LHCGR/TSHR) form the subgroup of G-protein-coupled receptors (GPCR),which is characterized by the presence of unusually large extracellular domain (ECD) and a heptahelicle serpentine transmembrane domain (TMD) and a short cytoplasmic intracellular domain (ICD) (Dias and Van Roey, 2001).

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The putative FSHR DNA sequence was first reported in 1989 by Vassart's group, who discovered it while cloning the human TSH receptor (Parmentier *et al.*, 1989). In 1990, Sprengel *et al.* first cloned the cDNA encoding rat FSHR from testicular cells. Thereafter, the cDNA encoding FSHR from many species including human, monkey, mouse, ovine, bovine, equine and porcine has been isolated and sequenced from testis and ovarian tissues. These studies showed that each species has a single FSHR gene (Minegishi *et al.*, 1991; Kelton *et al.*, 1992; Gromoll *et al.*, 1993; Yarney *et al.*, 1993; Houde *et al.*, 1994; Remy *et al.*, 1995). Based on cDNA synthesis of granulosa and Sertoli cells in various species, the full length FSHR transcript is approximately 2.5 kb (Gromoll *et al.*, 1993).

Linkage analysis and *in situ* hybridization of chromosomes revealed that in humans, the *FSHR* gene is located on chromosome 2p21-p16 (Gromoll *et al.*, 1992; Rousseau-Merck *et al.*, 1993). It is interesting to note that other gonadotropin receptor gene, *LHR* gene, is also mapped to the same location. However, these two receptor genes share no common band within 1100 kb, suggesting rather large distance between the two genes (Gromoll *et al.*, 1992). The *FSHR* gene is 192 kb in size and comprises of ten exons ranging between 69-1234 bp and nine introns, ranging between 108 bp-15 kb. The exon-intron boundaries follow the GT-AG canonical splice consensus sequence conserved in all exons. The amino acids encoded by these junctions are mostly leucine or isoleucine (Gromoll *et al.*, 1994). The first nine exons of *FSHR* gene encode the N-terminal part of the extracellular domain (ECD). Exon 10 is large and encodes the C-terminal part of the ECD (hinge region), the trans membrane domain (TMD), and the intracellular domain (ICD) of the receptor.

The human FSHR protein is composed of 695 amino acids (aa), including a signal peptide of 17 aa, leading to a mature protein of 678 aa with a molecular weight of 75kDa (Minegishi *et al.*, 1991). FSHR is highly conserved among species with an overall homology of about 85%. The TMD shows highest homology. Compared to other gonadotropin receptors, the homology of TMD is still very high (~69%), but it drops to ~40% and 25% in the ECD and 'C' terminal tail, respectively. Binding of FSH to its receptor leads to conformational changes in the receptor protein, resulting in activation of the Gs protein and cyclic AMP (cAMP) production (Zhang *et al.*, 1991). Predominantly, the ICD is coupled to a Gs protein that is responsible for initiating a cascade of intracellular events. cAMP is the main second messenger of FSH action, although an increase in calcium ion influx (Gorczynska *et al.*, 1994) or inositol triphosphate has also been described (Quintana *et al.*, 1994 and Tena-Sempere *et al.*, 1999). The X-ray crystal structure of FSH complex with the ECD of FSHR (Fan and Hendrickson, 2005; Jiang *et al.*, 2012) have immensely contributed in understanding the interaction of FSH with its receptor at the molecular level. Yet certain important aspects of ligand-receptor binding and signal transduction remain unknown, most probably because of lack of structural data for the cryptic hinge region. Moreover, it does not provide information on ligand-induced activation of the receptor leading to steroidogenesis.

Studies with FSHβ (Kumar *et al.*, 1997) and FSHR female knockout mice have shown that these female mice are infertile with thin uteri and streak ovaries. But in both the cases the male mice were fertile, although there was a decrease in testicular size and partial spermatogenic failure. These findings clearly demonstrate a crucial function of FSH and its receptor in ovulation and reproduction.

The structure-function relationship of FSHR has been studied using different approaches such as chimeras, synthetic peptides, antipeptide-antibodies and mutagenesis. The use of synthetic peptides and antipeptide antibodies corresponding to the predicted surface exposed regions of FSHR have been used extensively for elucidating the key epitopes involved in hormone binding, signal transduction and bio-neutralizing epitopes. Additionally, the advances in molecular biology techniques have assisted the creation of chimeric receptors and ease of performing site directed mutagenesis with commercially available kits. This technique is currently being explored for analyzing the importance of single residues. Since the last decade, studies on naturally occurring mutations

and single nucleotide polymorphisms (SNPs) of *FSHR* gene have helped in understanding the genotype-phenotype correlation. The *in-vitro* functional characterization studies of *FSHR* gene mutations have elucidated the molecular events such as hormone binding, signal transduction and receptor trafficking in response to FSH action. Alternatively, spliced variants of *FSHR* gene also have been reported in literature and few of them are believed to affect fertility in females (Gerasimova *et al.,* 2010). The present review focuses on different approaches employed to delineate the structural and functional determinants of FSHR.

Synthetic Peptides and Antipeptide Antibodies

The use of synthetic peptides and antipeptide antibodies corresponding to various regions of a protein enable identification of functionally important epitopes. To study FSH-FSHR interactions at the molecular level as well as for designing modulators of FSH action, this strategy has been extensively employed. Most of these studies were performed before the crystal structure of FSH-FSHR (ECD) was published. The finding from studies where this approach was used has been summarized in Table 1.

The N-terminal extra cellular domain (ECD) has been demonstrated as the primary ligand binding domain and the serpentine transmembrane domain relays the signal downstream for bringing about specific biological effects by the glycoprotein hormone receptors FSHR, LH/CGR and TSHR (GPHRs). Owing to the large size of the receptors of GPHRs, the interaction of ligands FSH, LH/hCG and TSH with their cognate receptors, takes place at several sites in the ECD. The region corresponding to residues 9-30 in the extracellular domain of FSHR is FSHR specific i.e. there is no sequence homology with the other GPHRs. Dattatreyamurty *et al.* (1992) first reported that a synthetic peptide corresponding to residues 9-30 of FSHR specifically binds to FSH. Further, antipeptide antibodies raised against the region 9-30 were seen to inhibit FSH binding and also the ability of FSH to stimulate the conversion of androstenedione to estradiol in cultured immature rat Sertoli cells (Dattatreyamurty and Reichert, 1993). Immunization of prepubertal BALB/c male mice with phages displaying the FSHR specific decapeptide (amino acids 18-27) was shown to be an effective and reversible male contraceptive (Remy *et al.*, 1996).

Abdennebi *et al.* (1999) engineered filamentous phages displaying the peptides 18-27 (A), 25-34 (B) and 29-38 (C). They observed that Anti-C IgG had FSH-like agonistic activity and the peptidic vaccines A and B showed reversible inhibition of ovulation in ewes and impaired fertility in female mice. Further, peptide vaccine immunization (18-38) resulted in maintenance of sexual immaturity in male mice and bucks (Abdennebi *et al.*, 2003). Deletion mutant S^{9}-N^{30} of the mature rFSHR sequence showed impairment in FSH binding and folding due to the loss of cysteine 15, which forms a part of the cysteine cluster of rFSHR (Mann *et al.,* 2000). Immunization with peptide 19-29 in male rats suppressed spermatogenesis (Graf *et al.*, 1997).

Drop in sperm count in adult male bonnet monkeys actively immunized with the phages displaying the peptides 18-27, 25-34 and 27-38 was also reported (Rao *et al.*, 2004). Also, certain residues in the 9-30 stretch were indicated to play a role in hormone-receptor interaction by the partially solved crystal structure of FSH-FSHR complex (Fan and Hendrickson, 2005).

Since the FSHR specific region 9-30 was certainly crucial for FSH-FSHR interaction, overlapping peptides corresponding to the regions 9-19, 15-25 and 20-30 of rat FSHR were synthesized in order to identify a smaller immuno-dominant epitope within this region (Ghosalkar and Mahale, 2006). Epitope analysis carried out using antibody to 9-30 region showed that the peptide 20-30 displayed maximum binding to the antibody. Next, competitive ELISA was carried out and it was seen that out of the peptides tested, only the peptides 9-30 and 20-30 could inhibit the binding of antibodies against the region 9-30 to the corresponding peptide. Importantly, the inhibition occurred in a dose-dependent manner. Further, a radioreceptor assay (RRA) was carried out to detect the ability of the peptide to inhibit FSH binding. Here too, not only was the peptide 20-30 more

Table 1: Studies with synthetic peptide and anti-peptide antibodies indicating functionally crucial epitopes of FSHR

Peptide region of FSHR	Species	Significant findings	References
9-30	Human	Inhibited the binding of FSH to the receptor	Dattatreyamurty *et al.*, 1992, 1993
9-30, 9-19, 15-25, 20-30	Rat	Peptide 20-30 was more potent	Ghosalkar *et al.*, 2006
20-30 and its alanine analogs E^{22}, D^{26}, R^{29}	Rat	Charged residues responsible for the peptide activity	Dupakuntla *et al.*, 2009
18-27	Human	Effective and reversible contraception achieved in prepubertal BALB/c male mice injected with the phages displaying thepeptides	Remy *et al.*, 1996
19-29	Rat	Completely inhibited FSH binding. Moreover it was also observed that there was reduction in sperm counts in male rats immunized with the peptide	Graf *et al.*,1997
18-38	Human	Peptide vaccine immunization resulted in maintenance of sexual immaturity in male mice and bucks	Abdennebi *et al.*, 2003
18-27, 25-34, 27-38	Human	Drop in sperm count in adult male bonnet monkeys actively immunized with the phages and impairment in fertility in ewes and female mice	Rao *et al.*, 2004 Abdennebi *et al.*,1999
15-31, 79-89, 171-183, 184-195, 206-220, 216-235, 259-274, 285-300, 297-310, 327-341	Human	Region 285-300 identified to be crucial for both FSH binding and cAMP generation. Regions 285-300and 327-341seem to be the chief sites of FSH binding. Regions 15-31 and 216-235 seem to serve as ancillary FSH-binding sites	Kene *et al.*, 2004 and 2005
221-252	Human	Peptide brings about autologous biological response modification by associating with and altering the receptor conformation to an inactive state	Mahale *et al.*, 2001
265-296	Human	Antipeptide antibody inhibited FSH stimulated progesterone production	Liu *et al.*, 1994
285-309	Rat	Passive immunization in female rats with anti 285-309 antibodies resulted in infertility in 72% indicating that this could be a bioneutralizating epitope	Ghosalkar *et al.*2007
300-315	Rat	May constitute FSH binding site via interactions with FSH residues β 21-35 and 11-25. MAb 106-105 detecting this region showed reversible immunoneutralizing activity against FSHR	Leng *et al.*, 1995 Lindau-Shepard *et al.*, 2001
405-426 (EL1), 492-511 (EL2), 581-591 (EL3)	Human	EL1 and EL3 are surface accessible and probably act as secondary FSH binding sites	Dupakuntla*et al.*, 2010

effective (IC_{50} value of 0.59 $X10^{-4}$) than the parent peptide 9-30, a remarkable inhibition of more than 90% was achieved at the highest dose tested. The mode of inhibition was of mixed type. The other two peptides did not show any inhibition on FSH binding.

The peptide 20-30 was found to inhibit FSH induced cAMP signaling as well (Dupakuntla *et al.*, 2009). Further, alanine substitution analogs of FSHR specific charged residues E^{22}, D^{26} and R^{29} were unable to inhibit FSH binding and FSH induced cAMP response.

Thus, the charged residues seem to be essential for the antagonistic activity of FSHR peptide 20-30.

Analysis of a peptide corresponding to residues 221-252 which resembled a leucine-rich repeat domain of the FSHR ECD showed that it could inhibit FSH binding to FSHR as well as signal transduction (Mahale *et al.*, 2001). This peptide was proposed to bring about autologous biological response modification by associating with and altering the receptor conformation to an inactive state. Antibodies raised against peptide corresponding to the region 309-322 of human FSHR were shown to inhibit FSH-bioactivity but not LH-bioactivity (Zijlstra-Westhoff *et al.*, 1998). Antipeptide antibodies against FSHR R^{265}-S^{296} inhibited binding of radio-labeled hFSH to insect cell rat FSHR indicating the role of those residues in hormone binding (Liu *et al.*, 1994). Studies with a synthetic peptide corresponding to residues 300-315 of rat FSHR showed it to constitute a FSH binding site via interactions with FSH beta 21-35 and 11-25 residues (Leng N *et al.*, 1995). Lindau-Shepard *et al.* in 2001 reported reversible immuno-neutralization of FSHR with the help of a monoclonal antibody 106-105 whose epitope consisted of the region 300-315 of human FSHR.

Several peptides corresponding to the predicted surface oriented regions of ECD of hFSHR namely 15-31, 79-89, 171-183, 184-195, 206-220, 216-235, 259-274, 285-300, 297-310 and 327-341 were generated (Kene *et al.*, 2004). Competitive inhibition studies by radio receptor assay and cAMP assay were carried out using synthetic peptides corresponding to the above mentioned regions. The results revealed that the regions 285-300 and 297-310 of hFSHR are essential for FSH binding and the regions 15-31, 79-89, 184-195, 216-235 and 285-300 are essential for FSH-induced cAMP production. Thus, the region 285-300 was identified as a discriminatory epitope to be crucial for both FSH binding and cAMP generation. Anti-peptide antibodies to the peptides mentioned above showed that the regions 285-300 and 327-341 of hFSHR appear to be the chief sites of hormone binding whereas the regions 15-31 and 216-235 of hFSHR serve as ancillary FSH-binding sites. Antipeptide antibodies against regions 15-31, 216-235, 285-300 and 327-341 of hFSHR behaved as antagonists and inhibited the production of FSH-induced cAMP levels significantly in a dose dependent manner (Kene *et al.*, 2005a). Circular dichroism analysis revealed a β-sheet structure for peptide 285-300, 297-310 and 327-341 of hFSHR, consistent with their role in hormone binding. A α-helical structure was observed for peptides 15-31 and 216-235, which served as ancillary FSH-binding sites essential for signal transduction (Kene *et al.*, 2005b).

Since the above mentioned *in vitro* studies suggested the importance of the region 285-300 in hormone binding and signal transduction, further *in vivo* studies were carried out in a rat model. Antibodies to peptide 285-309 from rat FSHR detected FSHR on rat ovarian granulosa cells as seen by immuno-histochemistry (Ghosalkar *et al.*, 2007). Passive immunization of antibodies against the peptide 285-309 in female rats resulted in inducing infertility in 72% of them. Thus the epitope 285-309 was identified to be a bio-neutralizing epitope.

Binding of FSH occurs at the high affinity leucine rich repeats of the ECD followed by relaying the signal downstream by TMD. This process may involve contacts of the hormone-receptor complex with extracellular loops (ELs), which protrude outside the membrane. Determination of surface accessibility of ELs of FSHR was carried out using antipeptide antibodies against synthetic peptides corresponding to the FSHR-ELs. Flow cytometry experiments revealed that the antibodies against EL1 and EL3 of FSHR were able to detect the corresponding regions on FSHR expressing cells (Dupakuntla *et al.*, 2010). Antipeptide antibodies to EL1 and EL3 could inhibit the binding of the hormone to receptor as seen by RRA and also inhibit cAMP production. Thus, EL1 and EL3 probably serve as secondary FSH binding sites.

Site-Directed Mutagenesis Approach

Site-directed mutagenesis and chimeric receptor approach has led to the identification of residues or domains crucial for FSH-FSHR interaction. Furthermore, the residues involved in interaction between FSHR and adaptor proteins, residues

contributing to receptor oligomerization, functional specificity, cell surface trafficking, internalization and various signal transduction pathways could be mapped. Advances in computational modeling methods, availability of several databases like SSFA-GPHR(www.ssfa-gphr.de database for semi-quantitative Sequence-Structure- and Function-Analysis of GPHRs), GRIS (http://gris.ulb.ac.be/, glycoprotein-hormone receptor information system), GPCRDB (http://www.gpcr.org/7tm/,G protein-coupled receptors database), several bioinformatic tools and access to servers like Protein Interactions Calculator (PIC, http://pic.mbu.iisc.ernet.in/) that enable study of several types of interactions like ionic bonds, hydrophobic interactions, hydrogen and disulphide bonds, to name a few, have facilitated better understanding of the structure-function correlation of FSHR.

Braun *et al.*, in 1999, first demonstrated that the ECD of GPHRs confer hormone binding and signaling specificity to the receptor. Chimeric receptors of LRR1-11 of FSHR fused to the TMD of LH-CGR bound FSH and stimulated cAMP, but did not bind hCG. This suggested that the ECD determines the hormone binding specificity for GPCRs. By individual and collective substitutions of the residues, it was shown that N-linked glycosylation of Asn^{174} or $Asn^{276}$of ECD was essential for efficient folding of the nascent receptor protein into a conformation that allowed high affinity binding of hormone (Davis *et al.*, 1995). By chimeric receptor approach FSHR ECD/TMD and C-tail of *Drosophila melanogaster* LGR2 receptor chimeras were created which showed high basal cAMP levels (Nishi *et al.*, 2002). Constitutive activation of chimeric receptor was probably due to removal of constraint imposed by interaction of extra cellular loops with ECD, thus showing the importance of the extra cellular loops of FSHR.

To delineate the importance of a particular domain the approach employed was to swap the residues in the entire domain with the simplest amino acid alanine (Ala). If any loss-of-function was

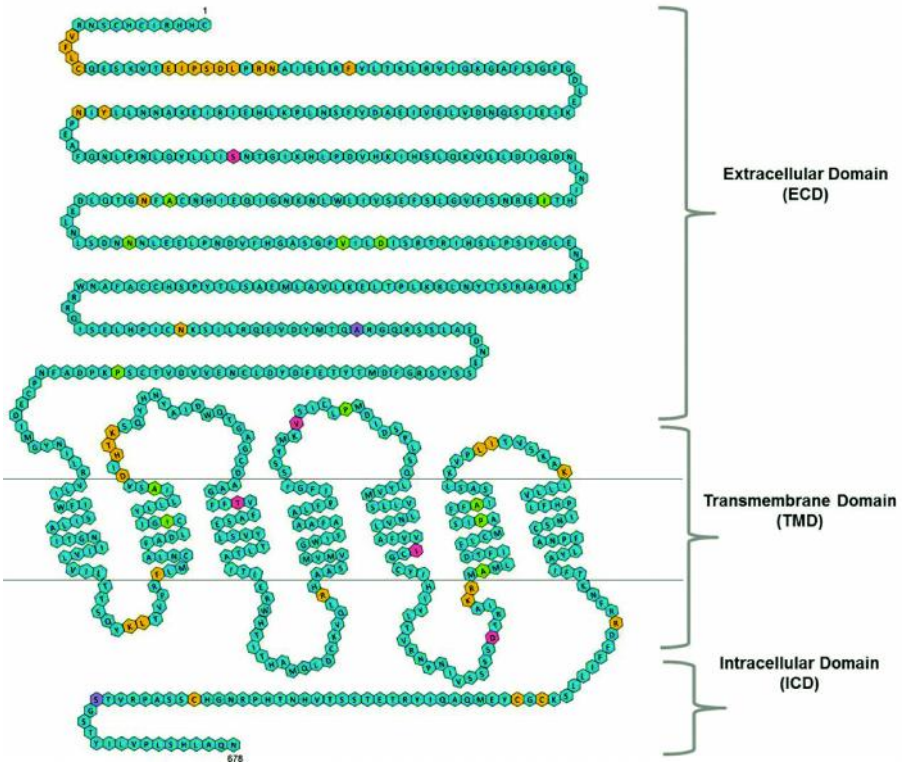

Fig. 1: Sequence of human FSHR. Naturally occurring inactivating mutations (■), activating mutations(■) and SNPs (■) are highlighted. Residues studied using site-directed mutagenesis approach (■) are also indicated. Explanation for each of these residues is provided in the text wherever applicable. The numbering was assigned according to the human sequence, assuming a signal peptide sequence of 17 amino acids

observed, further Ala scanning mutagenesis of individual amino acids was carried out in order to identify the contribution of side chains of the substituted residue. Deletion and Ala scanning and substitution mutations in the ECD region 9-30 of hFSHR indicated the residues to be essential for FSH binding and signal transduction (Nechamen *et al.*, 2000; Nechamen *et al.*, 2003). Individual substitution mutants $V^{12}A$ and $S^{25}A$ showed the ~80 kDa band corresponding to mature FSHR whereas $F^{13}A$, $L^{14}A$, $E^{22}A$, $I^{23}A$, $D^{26}A$, $L^{27}A$, $R^{29}A$ and $N^{30}A$ showed immature ~62 kDa form corresponding to FSHR trapped in the ER. Thus these residues in the ECD are crucial for cell surface trafficking of the receptor. The residues studied by site-directed mutagenesis approach are shown in Fig.1. Ala scan and substitutions of the first five residues in EL1 identified His^{407} to be important for FSH binding as seen by the decreased FSH binding affinity for His^{407}Ala mutant and Asp^{405}, Thr^{408} and Lys^{409} to be essential for cAMP production as the cAMP production was abolished in the Ala substitution mutants of these residues (Ji and Ji, 1995). Apart from ECD and EL1, residues in EL3 were shown to influence FSH binding and signal transduction. Binding affinity of Leu^{583}Ala or Ile^{584}Ala mutants was 4-6 fold higher than WT, whereas the cAMP production was abolished for the same (Ryu *et al.*, 1998). Substitution of EL3 residues with a panel of amino acids showed that substitutions/deletion of Leu^{583} or Ile^{584} enhanced FSH binding, whereas, substitutions at Leu^{583}, Ile^{584} and Lys^{590} abolished cAMP. Substitutions at Lys^{590} also abolished IP3 (Inositol 1,4,5-triphosphate) production (Sohn *et al.*, 2002; Sohn *et al.*, 2003). Our group has generated FSHR/LH-CGR extracellular loop chimeras, wherein, the FSHR specific residues from EL1, EL2 and EL3 were swapped with the respective residues from ELs of LH-CGR (Dupakuntla *et al.*, 2012). The EL2 residues in FSHR were found to be important for FSH-FSHR internalization and for FSH-induced cAMP response, whereas the EL3 residues were essential for recycling of FSH-FSHR complex.

The role of intracellular loops and C-tail has been discussed in several studies. Nakamura *et al.* (1998a and 1998b) showed that rFSHR is phosphorylated at Serine/Threonine residues at IL1 and IL3 but not on IL2 as seen by the decrease in phosphorylation of the mutants rFSHR-1L ($T^{369}I$, $S^{371}I$, $T^{376}N$ of IL1) and rFSHR-3L ($T^{536}A$, $T^{541}A$, $S^{544}A$, $S^{545}A$, $S^{546}A$, $S^{547}A$, $T^{549}A$ of IL3) but not of rFSHR-2L (Thr^{451}Ala, Thr^{453} Val of IL2) in response to FSH stimulation. Further, Krishnamurthy *et al.* in 2003a proved that association of arrestin-3 (β-arrest in 2) with FSHR is dependent on receptor phosphorylation and its over expression can rescue internalization of phosphorylation defective mutants $D^{389}N$ and $Y^{530}F$ (D and Y are highly conserved residues across GPCRs). IL2 residues (especially R^{467} which is present in the conserved ERW motif of GPHRs) were found to be essential for Gs coupling and cAMP production (Timossi *et al.*, 2002). Ala scan of IL1 residues revealed that these residues especially K^{376} are essential for interaction with APPL1 which brings about FSHR-mediated IP3 induction and FSH induced calcium signaling (Thomas *et al.*, 2011). IL3 was identified to be a site of FSHR ubiquitination by a yeast-based interaction trap (Cohen *et al.*, 2003).

Ala substitution of the reversed BBXXB motif (BXXBB) at the IL3 of the hFSHR showed it to be essential for Gαs coupling and cAMP production, whereas, the same motif in the C tail was seen to be more important for membrane expression as the mutations resulted in an immature form of the receptor (Timossi *et al.*, 2004). Confocal microscopy analysis showed that in contrast to the internalized WT receptors which localized only to endosomes, the internalized truncated mutants of C-tail of hFSHR and rFSHR localized to endosomes and lysosomes (Krishnamurthy *et al.*, 2003b). This study showed that most of the FSH-FHSR complex gets recycled back to the cell surface and truncation of eight residues from the C-tail re-route a substantial portion of the internalized FSH-FSHR complex to a degradation pathway. Uribe *et al.* showed in 2008 that the conserved Cys residues at C-tail ($C^{627/629/655}$) are important for palmitoylation since the triple mutant $C^{627/629/655}A$ does not show palmitoylation. Evidence was presented for the crucial role played by the Ser/Thr cluster in C-tail of rFSHR in phosphorylation and β-arrestin recruitment (Kara *et al.*, 2006). FSHR mutants (Ala substitution of the Ser/thr cluster $T^{638}A$, $T^{640}A$, $S^{641}A$, $S^{642}A$, $T^{644}A$) showed enhanced

cAMP production (due to its inability to get desensitized), impaired phosphorylation, β-arrestin recruitment and hence impaired internalization of FSH-FSHR. This study also revealed that FSH induced ERK phosphorylation occurred by two distinct pathways-Gαs/PKA-dependent or β-arrestin dependent.

Several interesting studies have unraveled previously unknown facets of FSHR. Thomas *et al.* in 2007 first demonstrated that FSHR forms oligomers and undergoes C-terminal proteolytic processing. Trans-activation of non-binding FSHR mutants by a functional FSHR ECD to bring about cAMP or IP production, but not both, was reported by Ji *et al.* in 2004. Rescue of function of mutants $R^{556}A$ (IL3) and $R^{618}A$ (C-tail) by co-transfection with WT fragments of TMD 5,6 or 7 and/or C-tail further substantiated that dimerization of FSHR indeed takes place (Zariñán *et al.*, 2010). Guan *et al.* (2010) attempted to identify the determinants of FSHR dimerization. BRET experiments revealed that constitutive homodimers of FSHR could be detected in both plasma membrane and ER. They also showed that residues in both the ECD and TMD contribute to homodimerization. Interestingly, BRET experiments using hFSHR-Rluc and hLHR-GFP^2, hLHR-Rluc and hFSHR-GFP^2 showed that heterodimerization of FSHR/LHR receptors takes place and these results in attenuation of signaling (Feng *et al.*, 2013). Further to this, a recent study by Mazurkiewicz *et al.* (2015) has been reported, wherein, Fluorescence Correlation Spectroscopy and Photon Counting Histogram studies showed the presence of freely diffusing FSHR homodimers on the surface of live cells. They also carried out FRET experiments and demonstrated that hFSHR-rLHR-cT (chimera of FSHR and extreme C-tail fluorescent fusion proteins: FSHR-LHRcT-YFP/FSHR-LHRcT-mCherrypairs) forms hetero-dimers/hetero-oligomers with LHR and this possibly occurs during granulosa cell differentiation.

Naturally Occurring Mutations of the FSHR Gene

Since 1995, several naturally occurring *FSHR* gene mutations have been reported in the literature, yet these are rare compared to the mutations reported in *LHR* and *TSHR* gene (Dufau *et al.*, 1995). The FSHR mutations leading to loss-of-function have been observed in women with ovarian dysgenesis (ODG), primary amenorrhea, and secondary amenorrhea. Such mutations are also reported in men with small testes and impaired spermatogenesis. On the other hand, the mutations causing gain-of-function, have been reported in women with ovarian hyperstimulation syndrome (OHSS) and in a single case of a hypophysectomized man. FSH exhibits its action by binding its receptor followed by signal transduction. These two events of hormone binding and signal transduction are generally studied by in-vitro experiments while characterizing the naturally occurring mutations of FSHR.

The activating and inactivating mutations of FSHR (Fig. 1) are known to provoke potential alterations in receptor function at different stages. Therefore, the functional studies on the naturally occurring mutations have gained importance in understanding the role of key residues involved in ligand interaction, activation and trafficking of the receptor.

Inactivating Mutations

The first inactivating FSHR mutation was found in several Finnish families after a genetic survey of women with hypergonadotrophic hypogonadism (Aittomaki *et al.*, 1995). Twenty-two of the 72 patients studied carried an identical homozygous loss-of-function $Ala^{189}Val$ mutation in the extracellular domain of the FSHR (The numbering was assigned according to the human sequence, assuming a signal peptide sequence of 17 amino acids). Segregation analysis confirmed the autosomic recessive mode of inheritance for the disease and it was mapped on chromosome number 2p, which corresponds to the locus for both FSHR and LHR. *In vitro* functional studies revealed that although the ligand-binding affinity of the receptor was not affected but its expression on the cell membrane might have been reduced as the MSC-1 cells expressing mutant receptor showed reduced ligand-binding ability. FSH-induced cAMP was observed to be reduced in the mutant receptor when

compared with the WT receptor. Amino acids from 189 to 193 (AFNGT) in FSHR are conserved across all the glycoprotein hormone receptors and also across all the species. N191 is the proposed glycosylation site and the mutations at position 189 may have an effect on glycosylation and probably on trafficking of mature receptor onto the membrane (Davis *et al.,* 1995).

Thereafter, almost five mutations located in ECD of FSHR, $Ile^{160}Thr$, $Asn^{191}Ile$, $Val^{221}Gly$, $Asp^{224}Val$, $Pro^{348}Arg$ have been reported (Beau *et al.,* 1998; Tapanainen *et al.,* 1997; Nakamura *et al.,* 2008; Touraine *et al.,* 1999; Allen *et al.,* 2003). It was interesting to note, that, in case of these mutations the ligand binding was mainly affected further leading to either decreased or completely abolished FSH induced cAMP production.

In 2003, Meduri *et al.* reported a novel mutation $Pro^{519}Thr$ in the EL2 of FSHR. This mutation has been observed in a homozygous condition in women with primary amenorrhea. This mutation completely abolished the ligand binding and cAMP production *in vitro*, due to the defect in the trafficking of receptor. These observations indicate that Pro^{519} is probably a pre-requisite for the FSHR trafficking and receptor function. Thus, along with ECD, ELs also seem to play an important role in ligand binding and receptor trafficking.

Apart from inactivating mutations identified in the ECD, a number of mutations have been reported in the helices of the TMD of FSHR. A mutation at $Ile^{411}Asn$ position was detected in women with polycystic ovary syndrome (PCOS; Orio *et al*., 2006). On the other hand, the mutation $Pro^{587}His$ was observed to lead to more adverse phenotypes such as primary amenorrhea (Kuechler *et al*., 2010). Recently, our group has also published a novel mutation in the 6^{th} TMD at position 575 leading to a substitution of Ala to Val (Achrekar *et al*., 2010a) presented by a primary amenorrhea subject. The surface localization of the mutant receptor 575 was observed to be lower as compared to WT receptor. Although the ligand binding ability of the receptor was reported to be decreased, the percentage of receptor internalized was remarkably higher as compared to WT receptor. These observations indicate defect in trafficking of the mutant receptor to the cell surface. Further, the cAMP levels were almost similar to basal levels even at higher doses of FSH stimulation. This suggests that along with reduced cell surface expression and rapid internalization, the mutation at position 575 resulted in reduced signaling activity of the receptor (Desai *et al.,* 2015). Such studies have helped in establishing a genotype-phenotype correlation and also have contributed in understanding the significance of affected amino acid in FSHR function.

Recently, a novel mutation at position 418 has been reported in a two POF subjects. Both these subjects were found to have a novel pathogenic variant in FSHR (c.1253T>G,p.Ile418Ser), inherited as autosomal recessive trait from heterozygous parents. This loss-of-function mutation is located in exon 10 of FSHR affecting the second trans-membrane helix of the FSHR protein. The trans-membrane domain of FSHR is highly conserved across species and is involved in signal transduction (Katari *et al.,* 2015).

Activating Mutations

Activating or gain-of-function mutations in the *FSHR* gene have been reported in both men and women. So far, only two constitutively activating FSHR mutations $Asp^{567}Gly$ and $Asn^{431}Ile$ in male/s have been reported (Casas-González P *et al.,* 2012). Interestingly, in case of Asn^{567} mutation was reported in a hypophysectomized male, who was under treatment with testosterone. The serum gonadotropin levels in this subject were undetectable; and the subject had normal testis volume and semen parameters. The subject had also fathered three children with testosterone treatment. The mutant receptor displayed three fold higher constitutive activities when studied *in vitro* and also responded to hCG and TSH treatment (Smits *et al*., 2003). The second mutation $Asn^{431}Ile$ was reported in an asymptomatic man. *In vitro* experiments demonstrated decreased cell surface expression of the mutant receptor. Further, the agonist-induced desensitization and internalization were found to be markedly altered. Both these mutants exhibited ligand-independent constitutive activity when tested *in vitro*.

All the activating mutations reported in women so far are associated with spontaneous ovarian hyperstimulation syndrome (sOHSS). These FSHR mutants have been observed to get activated upon hCG/TSH stimulation, suggesting that their ligand specificity could have been affected displaying constitutive activity of the receptor. So far many activating mutations have been reported in women with sOHSS; $Asp^{567}Asn$ (Smits *et al.*, 2003), $Thr^{449}Ile$ (Vasseur *et al.*, 2003), $Thr^{449}Ala$ (Montanelli *et al.*, 2004), $Ile^{545}Thr$ (De-Leener *et al.*, 2006) and $Ser^{128}Tyr$ (De Leener *et al.*, 2008).

Of these activating mutations, only one ($Ser^{128}Tyr$) is reported from the ECD (De-Leener *et al.*, 2008). In contrast to the other mutations, this mutation in the ECD did not show any constitutive activity but exhibited higher affinity and sensitivity toward hCG but not to TSH. Subsequently, the extensive site-directed mutagenesis study at position 128 demonstrated that the increase in the sensitivity to hCG selectively is not a consequence of the loss of serine residue, as some substitutions were found to be neutral (Ser to Ala/His), whereas some substitutions were observed to be sensitive to both hCG and TSH (e.g. Ser to Ile/Val).

So far, all these activating mutations of FSHR have been reported in the subjects who develop sOHSS during first trimester of the pregnancy. However, OHSS is usually a complication of gonadotropin stimulation during *in vitro* fertilization (IVF) protocol where it is known as iatrogenic OHSS (iOHSS). Recently, our group has published a novel mutation $Val^{514}Ala$ in IVF subject undergoing gonadotropin hyper stimulation for super-ovulation. This subject had developed iOHSS. This is the first report of FSHR mutation associated with iOHSS condition. It is noteworthy, that on administration of low amounts of exogenous FSH, the estrogen levels before and after hCG administration were drastically higher in this subject. Due to increased levels of estrogen, the number of pre-ovulatory follicles and retrieved oocytes were very high as expected, leading to development of aOHSS. The functional analysis of mutant receptor 514 was carried out to understand the impact of mutation on receptor activity (Desai *et al.*, 2015). It was observed that the cell surface expression of mutant receptor was very high as compared to the WT receptor. Further, it was observed that the binding of FSH to this mutant receptor was significantly higher as compared to WT receptor, which could be due to the increased cell surface expression of the mutant receptor. On the other hand, the internalization of hormone bound mutant receptor was lower as compared to the WT receptor, leading to significantly low percent internalization index. No distinct differences were observed in the ligand binding characteristics between the mutant and WT FSHR. The basal level of cAMP produced by the cells expressing mutant receptor 514 was higher as compared to WT receptor; however this difference was not significant.

FSHR Gene Polymorphisms and Ovarian Response

Although *FSHR* gene is highly polymorphic in nature; the three of the FSHR SNPs-29, 307 and 680 have been studied extensively in different ethnic populations both in male and female. The polymorphisms in the *FSHR* gene are being studied in relation to ovarian response in various populations. Currently, FSH is being used widely for therapeutic purpose in reproductive medicine and has pharmacogenetic potential for infertility treatment (Casarini and Simoni, 2014). Because of its crucial role in follicle development FSH is being used widely for ovarian stimulation during IVF. Although the protocols used for ovarian stimulations are similar, the ovarian response to exogenous FSH varies widely ranging from poor to hyper-responsive. Many parameters, such as age, diminished ovarian reserve (Klingman and Rosenwaks, 2001) and serum AMH levels (Nardo *et al.*, 2009), have been used as markers to predict the ovarian response. However, determining the dose of FSH to accomplish optimum response is one of the ongoing challenges in the field of infertility management in IVF clinics. Interestingly, *FSHR* gene polymorphisms have been observed to be associated with aberrant ovarian response.

A breakthrough finding was published in 2000 by Perez-Mayorga *et al.*, demonstrating the role of

FSHR SNP as a genetic marker to predict ovarian response when they demonstrated that the high serum FSH levels are associated with Ser^{680} genotype and poor ovarian response in women undergoing IVF. The *in-vitro* functional analysis of the Thr^{307}-Asn^{680} and Ala^{307}-Ser^{680} isoforms suggested that the FSH binding and cAMP production is similar for both these isoforms (Tilly *et al.,* 1992; Minegishi *et al.,* 1994; Simoni *et al.,* 1999; Sudo *et al.,* 2002; Nordhoff *et al.,* 2011; Zalewski *et al.,* 2013). Response of these isoforms to TSH stimulation was also characterized in HEK-293 cells and was observed to be alike (Ryan *et al.,* 2007). Therefore, the molecular mechanism by which both these polymorphisms might be altering the ovarian response is still unclear. In studies conducted for different ethnic groups, the significantly higher basal FSH levels and lower estradiol production upon hCG stimulation were associated with Ser680 allele (Sudo *et al.,* 2002; de-Castro *et al.,* 2004; Behre *et al.,* 2005; Greb *et al.,* 2005). de-Castro *et al.* (2004) reported that, other clinical parameters, such as peak E2 levels, number of follicles and oocytes, and amount of exogenous FSH, were observed to be similar in all the three genotypes at position 680 of FSHR although the percent of poor responders were significantly higher in case of women with Ser/Ser genotype.

Moreover, it was noted that in a systematic randomized control study, women with either Ser/Ser or Asn/Asn genotype with 150 or 225IU/day of FSH dose resulted in a similar number of follicles and retrieved oocytes, fertilization rate, and clinical pregnancy (Behre *et al.,* 2005). Furthermore, there are reports from different ethnic origins from Netherlands (Laven *et al.,* 2003; Klinkert *et al.,* 2006) and UK (Mohiyiddeen *et al.,* 2012), which did not report any association of the polymorphism at position 680 with poor ovarian response.

The systematic study of subjects with ODG, poor responders, and good responders demonstrated that subjects with Ser/Ser 680 genotype generate a significantly higher amount of estradiol and higher number of follicles and oocytes when compared with subjects with Asn/Asn genotype (Loutradis *et al.,* 2006). Daelemans *et al.* (2004) also reported that women with the Ser680 allele were at risk of showing hyper-response when compared with the Asn680 allele. Similarly, our group has observed that although not significant, 50% of the subjects (three of six subjects) with p.Ser680Ser genotype developed OHSS (Achrekar *et al.,* 2009a).

Recently, a meta-analysis carried out by Morón and Ruiz (2010), and Yao *et al.* (2011) suggested that the Ser680 allele is the only promising marker available to be used in clinical set-up to predict the ovarian response to FSH stimulation.

Our group recently published a novel observation indicating the association of polymorphism at position 307 with ovarian hyper-response in an Indian population. Almost 85% of the subjects (six of seven subjects) with Ala^{307}Ala genotype were found to be significantly associated with iatrogenic OHSS. These subjects developed OHSS when stimulated with significantly lower amount of exogenous FSH and demonstrated significantly higher levels of estradiol before and on the day of hCG stimulation (Achrekar *et al.,* 2009a). As mentioned earlier, Ala at 307 is linked with the Ser680 allele. Thus, it is more likely that the Ser680 allele along with the Ala307 allele may be associated with hyper-response to FSH stimulation.

The rate of pregnancy in an IVF cycle is very important as it is considered as a measure to determine the IVF outcome. Klinkert *et al.* (2006) reported that the Ser680 allele is more likely to give a higher pregnancy rate when compared with the Asn^{680} allele. Recently, a genome-wide analysis (GWAS) carried out by Boudjenah *et al.* (2012) reported that women with Ser/Ser genotype were more likely to have a high response than women with Asn/Asn genotype at position 680 (24 *vs.* 12% respectively, P=0.013). These observations suggest that there is a lack of consistency in the outcome of these association studies and there is a need for clarity in defining the poor ovarian response.

The level of FSHR expression also has an impact on the FSH action. Moreover, the reduced expression of FSHR on GCs has been shown to be associated with poor ovarian response (Cai *et al.,* 2007). The polymorphism at position -29 of the 5' UTR in the

FSHR gene has been studied with respect to its effect on receptor expression as this polymorphism may modulate the cETS-1 transcription factor binding site. The *in vitro* analysis carried out in CHO cells demonstrated that the A-29 allele expressed a significantly lower level of luciferase activity when compared with the G-29 allele of the FSHR gene (Nakayama *et al.*, 2006). Despite the fact that Wunsch *et al.* (2005) did not observe any association of the polymorphism at position -29 with ovarian response, the clinical parameters considered in this study were limited (basal FSH levels and E2 levels). Recently, we observed that subjects with AA genotype were significantly associated with a reduced number of follicles and retrieved oocytes when compared to subjects with GG genotype. The amount of FSH required for optimum response was also significantly higher in subjects with AA genotype when compared with GG genotype. Almost 66.67% (odds ratio 8.154; 95% CI 2.79 to 23.77; P<0.0001) subjects with AA genotype were poor ovarian responders (Achrekar *et al.*, 2009b; Desai *et al.*, 2011). Further, using granulosa cells (GCs) obtained from women undergoing IVF, we observed that subjects with AA genotype expressed significantly reduced FSHR expression both at transcript and protein level when compared with GG genotype (Desai *et al.*, 2011). This study provides the experimental evidence to suggest that reduced receptor expression observed in subjects with AA genotype could be the probable reason for the poor ovarian response to FSH stimulation observed in them. However, a greater number of subjects from various ethnic populations need to be analyzed to assess the usefulness of screening this polymorphism, which could be used as a biomarker to predict poor ovarian response.

FSHR Gene and Alternatively Spliced Variants

In humans, alternate splicing of exon 9 in normal testicular tissue has been reported, which suggests that this spliced variant does not affect the FSHR function in testis (Gromoll *et al.*, 1992). Deletion of exons 6 and 9 and insertion of part of intron 8 has also been identified in infertile men (Song *et al.*, 2002). Additionally, a number of spliced variants such as del exon 2, 6 and 9 and insertion of 102bp of intron 8 along with the WT FSHR in the granulosa cells obtained from women undergoing IVF has also been reported. The clinical parameters of these women suggested that deletion of exon 2 was associated with poor ovarian response whereas deletion of exon 6 was associated with a high response (Gerasimova *et al.*, 2010). In our study, in subjects undergoing IVF, we observed that out of a total of 20 subjects screened, only one subject was identified with insertion of a part of intron 8 (102bps) along with the WT-FSHR. However, this spliced variant did not seem to affect the various clinical parameters. To verify the role of these splice variants in ovarian response and female infertility, screening of these variants in large number of subjects is essential.

Association of FSHR SNPs and PCOS, POF and Amenorrhea

Polycystic ovary syndrome (PCOS) is known as a common endocrine disorder among women of reproductive age. The prevalence of PCOS is around 5-10 % and the clinical manifestation of the disease is diverse. The characteristics of PCOS mainly include oligomenorrhea or amenorrhea, hyperandrogenism and polycystic ovary morphology. The pathogenesis of PCOS is not fully understood. However, the influence of heredity and environment is considered to be the potential causative factors for the disease. In these subjects, LH:FSH ratio is generally increased and there is excessive production of androgen (Goodarzi *et al.*, 2011). So far, studies revealed that there are multiple susceptibility genes associated with PCOS, including growth hormone receptor exon 3 (Shen *et al.*, 2011); CYP11A1 (Zhang *et al.*, 2012) and GnRH (Valkenburg *et al.*, 2009). Recently, a study from Bahrain revealed the association of novel intron specific FSHR (rs11692782) SNP with PCOS, suggesting, that the differential association of FSHR variants with PCOS might be due to the racial/ethnic variation (Almawi *et al.*, 2015). A meta-analysis across the eight studies suggested that the Asp680 allele is associated with lower risk and the Ala307-Ser680 genotype is associated with an increased risk of developing PCOS (Du *et al.*, 2010).

Usually, treatment of infertility in cases with PCOS is based on increasing the level of FSH either by antagonizing the effect of estrogen by clomiphene citrate (CC) or by administrating recombinant FSH (rFSH). Recently, it was observed that subjects with Ser/Ser genotype at position 680 exhibited high resistance to CC treatment but showed the most favorable response to rFSH stimulation (Overbeek *et al.,* 2009). Likewise, in a study with PCOS women from Italy, Asn/Thr genotype at position 307 displayed higher response to FSH stimulation when compared with both the homozygous genotypes (Dolfin *et al.*, 2011).

Premature ovarian failure (POF) is also referred to as primary ovarian insufficiency (POI) and is characterized by low levels of gonadal hormones (estrogen, inhibin) and elevated levels of FSH with impaired ovarian folliculogenesis. The phenotype of the FSHR knockout mice was observed to be similar as observed in POF (Dierich *et al.,* 1998). Thus, FSHR could serve as a strong candidate gene responsible for the phenotype observed in the females with POF. Although there are no clear associations observed in FSHR gene polymorphism with POF, recently Kim *et al.* (2011) reported that epistasis between FSHR and CYP19A1 polymorphisms was significantly associated with POF.

In the case of subjects with amenorrhea, an association with the Ser680 allele has been observed (Sudo *et al.,* 2002). Recently, our group has investigated the association of polymorphisms at position -29, 307 and 680 in women with primary and secondary amenorrhea. We observed that in the subjects with primary amenorrhea, AA genotype at position -29 was significantly associated with increased basal serum FSH levels when compared with GG and GA genotype. We have also reported a novel homozygous inactivating mutation at position 575 (Achrekar *et al.,* 2010). Further, we have observed that the mutant receptor 575 showed lower cell surface expression and higher internalized hormone receptor complex. Additionally, a dose-dependent increase in the cAMP accumulation was not observed in the case of this mutant as compared with WT (Desai *et al.,* 2015).

FSHR Gene Polymorphisms and Male Infertility

Since the last decade, several reports have been published on the studies of FSHR gene SNPs and its association with the factors that are evaluated examining the male fertility such as sperm motility, sperm counts and testicular volume. Most of the reports showed no significant association of the polymorphisms at position -29, 307 and 680 with these parameters (Ahda *et al.,* 2005; Pengo *et al.,* 2006; Li *et al.,* 2011; Lindgren *et al.,* 2012). The recent three meta-analyses that summarized the seven original papers also suggest no association of these polymorphisms with reproductive abnormalities in men (Tüttelmann *et al.,* 2007; Lend *et al.,* 2010; Wu *et al.,* 2012). However, it was interesting to note that subjects with AA genotype at position -29 showed significantly smaller mean testicular volumes when compared with subjects with GG genotype (Lend *et al.*, 2010). Altogether, the reports from different populations suggest that FSHR genotype may not have a significant impact on male reproductive physiology.

FSHR Gene Polymorphisms and Gonadal Cancer

There are limited studies reported on FSHR gene SNPs and ovarian or testicular cancer. Contradicting observations with respect to the association of FSHR polymorphisms with low or high risk of forming gonadal cancers have been reported. Ala307/Ser680 allele alone or in combination with G-29/T-114 allele was observed to be associated with lower risk of developing testicular cancer (Ferlin *et al.,* 2008). On the other hand, homozygous Ala307/Ser680 alleles have been associated with higher risk and recurrence of developing ovarian cancer (Ludwig *et al.,* 2009). However, the mechanism by which these alleles could modulate the FSHR function in ovarian or testicular cancer cells still remains unknown and holds a great research interest.

Recently, in 2006 it was reported that, the two FSHR SNPs might affect the susceptibility of women to specific subtypes of ovarian cancer. Different types of ovarian cancer might adopt distinct carcinogenetic pathways. Such understanding may be important in selecting patients for ovulation induction therapy (Yang *et al.,* 2006).

Conclusion

In this review, we have discussed newer insights with reference to structure, function and molecular biology of FSHR. Specific emphasis was on approaches like synthetic peptides, antipeptide-antibodies, site-directed mutagenesis, naturally occurring mutations and polymorphisms. Studies on synthetic peptides have been gaining importance because of its potential in developing small molecules and structure-based design of agonists or antagonist. Antipeptide antibody studies have proved to be significant in identifying the bioneutralizing epitopes, in understanding the FSH-FSHR interactions at the molecular level as well as for designing modulators of FSH action. The naturally occurring mutation studies are helping to understand the functional significance of mutated residue. The *in-vitro* analysis for characterizing the mutants helps in understanding genotype-phenotype correlation. Studying the SNPs of the FSHR gene is evolving as an important support for pharmacogenomics and personalized medicine and needs to be explored further to optimize the dose of FSH required for super-ovulation and also to predict the ovarian response in women undergoing FSH treatment in the IVF set up.

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