The department of structural biology is involved in studies to understand the structure-function relationship of proteins involved in reproductive processes and of clinical significance. Studies to gain insight into the molecular mechanisms involved in the interaction of gonadotropin with the extracellular domain of its receptor have been carried out using multifaceted approaches like synthetic peptides, antipeptide antibodies and bioinformatics. Studies have also been initiated to understand the functional role of the extracellular loops of the gonadotropin receptor. Identification of structural and functional domains of such proteins would help in designing antagonists of hormone action with potential application in contraceptive development. Identification of mutations and polymorphisms in the gonadotropin receptor genes are being undertaken with an aim to delineate the importance of specific amino acid residues necessary for active conformation of the protein. Studies to understand the functional significance of particular amino acid residues in proteins of clinical importance are also been carried out.

8.1 Immunology of the Peptides Corresponding to the Ligand Binding Regions from the Extracellular Domain of Rat Follicle Stimulating Hormone Receptor

Principal Investigator: Smita Mahale

Project Associates: J.D. Ghosalkar, Tarala Nandedkar, Dhanashree Jagtap, Leena Tendulkar and Vaishali Nakhwa

Duration: 2002-2006

Follicle stimulating hormone receptor (FSHR) has a large extracellular domain (ECD), a transmembrane domain and a cytoplasmic domain. Though the detailed mechanism of binding of FSH to its receptor is not well understood, it is well established that the interaction is through the discrete, discontinuous epitopes from the ECD of the receptor. Using predictive algorithms and synthetic peptide approach, we have delineated three different regions from the ECD (9-30, 216-235 and 285-309) that may be involved in hormone binding or signal transduction or both.

Detailed analysis of the region 9-30, identified a small peptide corresponding to the region 20-30 as a potent hormone binding inhibitor (Annual Report 2004-05, p173-174). Further investigation to understand the mechanism
of inhibition revealed it to be mixed type of inhibition as both $K_m$ and $V_{max}$ were altered. The effect of the peptide on FSH induced signal transduction was studied in HEK-293 cells expressing FSHR, and it inhibited the cAMP production with an $IC_{50}$ value of $0.579 \times 10^{-6} \text{ M}$ (Fig. 110). Thus, we have identified a smaller region from the N-terminus of the receptor which appears to be important for both hormone binding and signal transduction.

Peptide 216-235 was synthesized and antibodies raised in rabbit and characterized. The antibodies were able to detect the full length receptor as seen by Western blot (Fig. 111) and were found to be specific. This peptide and its antibody did not show any effect on binding of FSH to the receptor but were able to inhibit FSH induced cAMP production (Fig. 110 and Fig. 112). These results indicate that the region 216-235 is not directly involved in hormone binding. However, it is possible that upon binding of hormone to the receptor, conformational changes induced in the receptor structure expose this region.

The third region 285-309 was reported to be a bioneutralizing epitope (Annual Report 2005-06, p. 189-191). Further studies on this region identified a smaller region 295-309 to be crucial for hormone binding as well as signal transduction. The inhibition with this peptide was found to be mixed type and the $IC_{50}$ value in signal transduction assay was $0.426 \times 10^{-6} \text{ M}$ (Fig. 110).

The observations made in our study, clearly provide experimental evidence that these different regions from FSHR-ECD are crucial for receptor activity and support the X-ray crystallographic data of FSH complexed with the partial hormone binding domain of the receptor reported in the literature. Further studies will be undertaken with these small active peptides to design more potent antagonists of FSH action, which will have application in development of fertility regulating agents.

![Fig. 110: Effect of peptides 20-30 and 295-309 on FSH induced cAMP production, seen in HEK-293 cells expressing FSHR.](image-url)
Fig. 111: Western blot analysis showing the binding of antibodies against peptide 216-235 to the receptor. Lane 1: HEK-FSHR + Pre-immune sera, Lane 2: HEK-FSHR + antiserum against peptide 216-235 pre-incubated with 5µg peptide, Lane 3: HEK-FSHR + antiserum against peptide 216-235, Lane 4: HEK-FSHR + antiserum against peptide 216-235 pre-incubated with 10µg peptide.

Fig. 112: Effect of antipeptide antibody against peptide 216-235 on FSH induced cAMP production, seen in HEK-293 cells expressing FSHR.
8.2 Mutational Analysis of Gonadotropin Receptor Gene and its Implications in Physiology and Pathophysiology of Pituitary Gonadal Function

Principal Investigator: Smita Mahale
Project Associates: Swati Achrekar, Pervin Meherji, Vrinda Khole, Zareen Patel and D. N. Modi
Project Collaborator: Sadhana Desai, Consultant Gynaecologist, Mumbai.
Duration: 2002-2007

FSH receptor plays crucial role in determining the physiological response of FSH which in turn is very important for fertility of an individual. Alterations in the genotype of the FSH receptor may contribute towards differential receptor activity. Inactivating mutations in FSH receptor gene have been linked to clinical cases of infertility in women where these mutations result in altered protein structure and result in the impairment of the FSH receptor function. Incidences of known FSH receptor mutations in infertile patients with high FSH levels seem to be very rare in our population.

As FSH receptor gene is known to be polymorphic at two sites in exon 10 resulting in the change in the amino acid at position 307 (T/A) and 680 (N/S), we have screened the control group comprising of normogonadotropic proven fertile women and the normogonadotropic ovulatory women with infertility caused due to male factor or tubal factor undergoing IVF treatment. Frequency distribution of these polymorphisms showed no significant difference when subjects undergoing IVF were compared with the controls (Annual Report 2005-06, p192-193).

We have studied the correlation between these polymorphisms at position 307/680 and ovarian response to FSH, wherein various clinical and endocrinological parameters of the women undergoing IVF treatment was evaluated based on polymorphism at both these sites. The patients were independently segregated based on the genotype obtained for position 307 and 680. The age of the patients, basal FSH and LH levels, progesterone levels, number of preovulatory follicles, number of retrieved oocytes and the pregnancy rates showed no statistically significant differences among the three groups. This observation indicates that the treatment was equally successful and independent of the FSH receptor variants. However, the amount of exogenous FSH required for the ovulation induction and the peak estradiol levels before and on the day
of hCG administration for achieving successful treatment was significantly different among the FSH receptor variants (AA) at the position 307. Also, it was observed that the rate of developing OHSS was significantly high in the patients with the AA variant (Table 12).

Table 12: Clinical and endocrinological parameters of the women undergoing ART program segregated based on polymorphism at the position 307

Values expressed as mean ± SEM
One way ANOVA test for analysis of variance p < 0.05 is considered statistically significant

<table>
<thead>
<tr>
<th>No</th>
<th>Clinical parameter</th>
<th>TT (n=12)</th>
<th>TA (n=31)</th>
<th>AA (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Amount of FSH required for ovulation induction (IU)</td>
<td>3225.33±274.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2652.65±144.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2503.86±208&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.</td>
<td>Estradiol levels before hCG administration (pg/ml)</td>
<td>1934.17±303.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1872.13±183.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3060.29±428.1&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.</td>
<td>Estradiol levels on the day of hCG (pg/ml) administration (pg/ml)</td>
<td>2234.33±359.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2337.65±221.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3534.43±578.4&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.</td>
<td>No. of subjects developing OHSS</td>
<td>3 (25%)</td>
<td>6 (19%)</td>
<td>6 (86%)</td>
</tr>
</tbody>
</table>
8.3 Delineation of the Role of the Extracellular Loops of Follicle Stimulating Hormone Receptor in Ligand Mediated Signaling

Principal Investigator: Smita Mahale

Project Associates: Madhavi Dupakuntla, J. D. Ghosalkar, Leena Tendulkar, Vaishali Nakhawa

Duration: 2006-2010

The N-terminal extracellular domain (ECD) of follicle stimulating hormone receptor (FSHR) has been shown to be a major determinant in hormone binding and discrete regions within the ECD involved have been identified. Whether the three extracellular loops (ELs) of the transmembrane domain have any role in hormone recognition or receptor activation is not well understood. Whether FSH binds to these loops directly or the FSH bound to the ECD induces signals through these loops needs to be addressed.

The present study uses synthetic peptides and their corresponding antipeptide antibodies to the ELs of FSHR to delineate the role of these loops in hormone binding and subsequent signal transduction. The antipeptide antibodies would also be used as probes to check for the accessibility of the ELs and to monitor changes in the conformation of FSHR as a result of hormone binding.

The peptide corresponding to the EL3 region 581-591 of FSHR was synthesized, purified and characterized. The molecular mass determined by MALDI-TOF mass spectrometry was 1181.7 as against the expected mass of 1180. The peptide was conjugated to DT and antibodies were raised in rabbits. IgG was purified and peptide specific antibodies obtained by subjecting it to affinity chromatography using a column of CNBr Sepharose-4B tagged to the EL3 peptide. Binding of the purified antibody to the peptide as well as to DT is shown in Fig. 139. The ability of the antipeptide antibodies to detect the native receptor was studied by PAGE and Western blot (Fig. 140). The purification of peptide EL1 and EL2 are in progress. These peptides and their corresponding antisera will be tested in FSH radio receptor assay and FSH induced signal transduction assay.
Fig. 113: Binding of antipeptide antibodies against EL3 peptide of FSHR to the corresponding peptide and to DT as seen by ELISA.

Fig. 114: Binding of antipeptide antibodies against EL3 peptide of FSHR to the receptor expressed on the HEK-293 cells. Lane 1: probed with pre-immune serum; lane 2: probed with affinity purified EL3 antibody and lane 3 and 4 probed with EL3 antibody pretreated with 250 µg or 500 µg of the corresponding peptide.
8.4 Studies on β-Microseminoprotein: Biochemical, Molecular and Bioinformatic Approaches

Principal Investigator: Smita Mahale

Project Associates: V.D. Dighe, Dhanashree Jagtap,
Leena Tendulkar, Vaishali Nakhwa,
Pervin Meherji, Jyotsna Gokral and
Geeta Vanage

Collaborator: M. J. Swamy, University of Hyderabad

Duration: 2003-2010

β-Microseminoprotein (β-MSP) is a prostate secretory protein of 94 amino acids present in very high concentrations in the seminal plasma. Corresponding protein from the human seminal plasma was isolated and characterized at the institute and was referred to as human seminal plasma inhibin. Though a number of functions have been predicted for this protein, the exact biological function still needs to be elucidated. Multiple sequence analysis of this protein from different species reveals the presence of ten highly conserved cysteine residues which are present in the form of five disulphide bonds in human β-MSP. The present project deals with structural and functional characterization of β-MSP using different approaches.

To understand the importance of the five disulphide bonds present in human β-MSP, reduction and modification of all the ten cysteine residues was carried out. Changes occurring in the conformation of the native protein following reduction of disulphide bonds (modified β-MSP) was studied using the immunological and biophysical approaches (Annual Report 2003-04, p174-175 and Annual Report 2004-05, p193-195). The fluorescence quenching studies with native and modified β-MSP in the presence of fluorescence quenchers namely acrylamide, iodide ion and cesium ion were carried out and data analyzed using Stern-Volmer plots (Fig. 115 and Table 13). Results for acrylamide indicate that it is the most effective quencher and that the quenching occurs by a combination of static and dynamic mechanisms for both native and modified β-MSP. Quenching by iodide and cesium ion show opposite quenching results for native and modified β-MSP which indicate that reduction and modification of the cysteine residues results in the movement of negatively charged side chains of Asp20 and Glu31 closer to the indole side chain of the Trp32 or Trp92.
β-MSP has been reported to be involved in selective suppression of circulating levels of FSH in adult male rat model. We tested both native as well as the modified protein in the adult male rats, but could not detect any effect on FSH levels in treated animals. As the sequence of β-MSP is identical to a protein referred as immunoglobulin binding factor, we studied the ability of β-MSP and its modified form to bind to human IgG (Fig. 116). Results indicate that both the forms of β-MSP are able to bind to human IgG suggesting that the sequential epitopes of β-MSP are involved in IgG binding. The binding of human IgG was specific as rabbit IgG showed significantly lower binding. BSA and coating buffer when used as negative control, exhibited very low binding to both native as well as modified β-MSP. The significance of this IgG binding property needs to be addressed. As the sequence of β-MSP is not well conserved, functional studies in a homologous system have been initiated to obtain corresponding rat protein by using the recombinant approach for studies in rats.
Fig. 116: Binding of human IgG to native and modified β-MSP. Both native β-MSP as well as modified β-MSP bind to human IgG as observed in ELISA. (a: native β-MSP + human IgG; b: modified β-MSP + human IgG; c: native β-MSP + rabbit IgG; d: modified β-MSP + rabbit IgG; e: BSA + human IgG and f: buffer + human IgG).

Table 13: Summary of parameters obtained from the intrinsic fluorescence quenching of β-MSP and modified β-MSP.

<table>
<thead>
<tr>
<th>Quencher</th>
<th>% Quenching</th>
<th>$K_{sv1}$ (M$^{-1}$)</th>
<th>$K_{sv2}$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-MSP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrylamide</td>
<td>86.4</td>
<td>9.05</td>
<td>_</td>
</tr>
<tr>
<td>Iodide ion</td>
<td>80.5</td>
<td>8.05</td>
<td>_</td>
</tr>
<tr>
<td>Cesium ion</td>
<td>38.0</td>
<td>1.20</td>
<td>_</td>
</tr>
<tr>
<td>Modified β-MSP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrylamide</td>
<td>89.1</td>
<td>10.44</td>
<td>_</td>
</tr>
<tr>
<td>Iodide ion</td>
<td>69.4</td>
<td>4.46</td>
<td>_</td>
</tr>
<tr>
<td>Cesium ion</td>
<td>54.4</td>
<td>2.34</td>
<td>1.86</td>
</tr>
</tbody>
</table>