CHAPTER 1

INTRODUCTION
1.1 SEX-HORMONAL REGULATION OF GENE-EXPRESSION

Male and female mammals begin life phenotypically indistinguishable, despite an XY or XX genotype. During late embryonic development male and female developmental pathways diverge. At this point, the sex-determining region on the Y-chromosome (SRY), a HMG box transcription factor, is expressed, which ultimately leads to the production of testosterone and the development of the bipotential gonad into the testis. The female developmental pathway is determined by the absence of SRY, resulting in estrogen production and ovary development. Thus gonadogenesis is guided by key transcription factors resulting in different sex hormonal outputs, which produce different chemical environments in males and females that persist into adulthood [1].

Sex hormones are steroids (lipids) with important regulatory functions. They exert their main regulatory activities by entering target cells and associating with nuclear hormone receptors that, in turn, act as transcriptional regulators (activators/repressors) of steroid hormone-responsive genes by binding to specific hormone response elements (HREs) on the cis-regulatory regions of target genes [2]. This receptor binding to DNA is promoted by sequences adjacent to receptor binding sites and also involves the binding of other factors. Although a consensus HRE has been associated with transcriptional regulation by the androgen/glucocorticoid/progesterone/mineralocorticoid receptor subfamily of nuclear receptors [3-5], it is not clear how the specificity of these individual receptors is determined. Moreover, prostate cells express AR, GR, and PR, yet the actions of these hormones and their specificity for activating or repressing gene expression differ considerably [3]. Apart from the above described transcriptional (genomic) effects, steroid hormones can also act via rapid nongenomic processes, which do not involve de novo transcription. Such nongenomic effects have been extensively reviewed elsewhere [6] and will not be discussed further here.

1.1.1 Androgenic effects on gene expression mediated by androgen receptor

The androgen testosterone, synthesized and secreted mainly by the testis in male, and its metabolite, dihydrotestosterone, affect diverse responses in a variety of tissues. Androgens diffuse through the plasma membrane or are internalized along with steroid-hormone-binding-proteins from the plasma [7] and mediate their effects through a ligand-dependent nuclear transcription factor, the androgen receptor (AR). Apart from its classical genomic effects via the intracellular androgen receptor, androgen effects may also be mediated in some target cells via the aromatization of testosterone (the main blood androgen) to
estradiol-17β and subsequent involvement of estrogen receptors. The androgen metabolite DHT is however non-aromatizable and cannot be transformed to estradiol. As mentioned above, androgens can also act via nongenomic processes, which are characteristically rapid effects [8].

A 110-kDa androgen receptor encoded by a single gene is known, which in mammals, whenever investigated, was found to be located on the X-chromosome. Typical AR gene is composed of eight exons encoding three major functional domains: a central DNA binding domain (DBD), which is highly conserved among nuclear receptors, a variable N-terminal domain containing a transactivation function (AF-1), and a C-terminal ligand binding domain (LBD) [9] (Figure 1.1).

![Figure 1.1 Schematic of major functional domains of androgen receptor (AR). The AR consists of two functional domains, a variable N-terminal domain containing a transactivation function (AF-1), and a C-terminal ligand-binding domain (LBD). AF-2 domain of AR interacts with other proteins through LXXLL motif, in a strictly ligand dependent manner. (From Powell et.al, 2004 Endocrine-Related Cancer) [9].](image)

In addition to ligand binding, the LBD is involved in receptor-dimerization, nuclear localization, interaction with heat shock proteins, and it contains a ligand dependent transactivation function (AF-2). According to the current model of action of androgens, upon ligand binding, the AR changes its conformation leading to, dissociation of heat shock proteins/chaperones, phosphorylation, receptor dimerization, binding to specific DNA sequences (androgen response elements, ARE) in target genes and interactions with various coactivators that facilitate transcription by the general transcriptional machinery, which finally results in the modulation of the transcription of androgen-dependent genes [10].

The AR is not only involved in positive regulation of gene expression but also in negative regulation [11,12]. Activation or repression of transcription by AR is regulated by a number
of cellular proteins that interacts with the receptor. On the whole, negative regulation by the AR is far less characterized than positive regulation. Nevertheless, from the limited information available, negative regulation by the AR can be classified into two groups: (1) regulation that requires AR-DNA interactions and (2) regulation that is dependent upon protein–protein interactions (Figure. 1.2) [13].

By analogy to transactivation, it is possible that putative corepressors associate with the DNA-bound receptor to bring chromatin into the repressed state through a deacetylation reaction. However, as yet, no such corepressors have been identified in association with the negative action of the AR. Therefore, it is likely that the negative regulation occurs through binding of the receptor to other proteins required for gene expression (activation) [14]. The AF1 domain of AR has recently been shown to be a target for corepressor proteins, which may be required for anti-androgens to have their repressive effects on the gene expression. A group of proteins that interact with AR and mediate transrepression, includes, AP-1, NFκB and TR4 (testicular orphan receptor 4) [12,15-19].

Androgens show rich pleiotropic effects and play a critical role in both normal physiology and a spectrum of human disease including cancer. Prostate cancer is the most well studied cancer where malignant cells are stimulated by androgens. Lowering androgen levels can usually make prostate cancers shrink or grow more slowly. Thus, antiandrogen therapy is the most important treatment in patients with advanced prostate cancer where the aim of treatment is to reduce testosterone or block the body's ability to use any androgens [20]. The anti-androgens (shown in Figure 1.3) cyproterone acetate (steroidal), flutamide and bicalutamide (both non-steroidal), act by blocking the binding of dihydrotestosterone to its receptor thereby preventing androgen action [21]. Cyproterone acetate (CPA) due to its
progestational activity also reduces gonadotrophin release from the hypothalamus, which results in reduced androgen synthesis.

![Testosterone and Dihydrotestosterone](image1)

![Flutamide, Bicalutamide, Cyproterone acetate](image2)

**Figure 1.3** Chemical structures of androgens (testosterone and dihydrotestosterone) and anti-androgens (flutamide, bicalutamide & cyproterone acetate).

### 1.1.2 Estrogenic effects on gene expression mediated by estrogen receptor

Estrogen, one of the main female sex hormone, (the other being progesterone) mainly synthesized and secreted by ovaries in females, has a wide range of physiologic activities, including the control of development, reproduction and metabolism as well as effects on cell growth and differentiation [22,23]. Recent studies have revealed the existence of two distinct estrogen receptors: ER-α and ER-β, encoded by two genes localized in different loci.

![Schematic diagram of the two estrogen receptors, ERα and ERβ](image3)

**Figure 1.4** Schematic diagram of the two estrogen receptors, ERα and ERβ. Both receptors consist of functional domains like the DNA-binding domain (DBD), the ligand-binding domain (LBD), the ligand-independent activation function AF-1, and the ligand-dependent activation function AF-2. The percentage identity between the two receptors is indicated. (From Wenlin and Myle 2004 Breast Cancer Research).
chromosomes [24,25]. The ER-β (54 kDa) is smaller than the ER-α protein (67 kDa) [26] due to a shorter N-terminal but possesses the modular structure of distinct functional domains, characteristics of the superfamily of nuclear receptors (Figure 1.4) [27].

Although ER-α and ER-β have high amino acid identity in their DNA binding domains (~96%), there are significant differences between the ligand binding domains of the two subtypes (~59% identity). Nevertheless, estradiol (E2) binds with relatively similar affinity to ER-α and ER-β. However, due to this divergence in ligand binding domain sequences, the two ER subtypes bind some synthetic or naturally occurring ligands with quite different affinities [28,29]. Structures of some of the ER ligands are given in Figure 1.5. Tissue and cell type specific discrimination between estrogen agonists and antagonists (selective estrogen receptor modulator; SERM) is well known [30,31]. In order to treat breast and uterine cancer genesis and progression, as well as for hormone replacement therapy in postmenopausal women, many tissue-and ER subtype-specific SERMs are being designed and investigated. Tamoxifen, raloxifene and ICI 164,384 (commonly used chemotherapeutic SERMs; see Figure 1.5) have an ER-α selective partial agonist/antagonist function but a pure antagonist effect through ER-β.

![Figure 1.5 Chemical structures of estradiol-17β (estrogen) and tamoxifen & raloxifene (anti-estrogens; selective estrogen receptor modulators; SERMs)](image)

Although, first designed and used as an anti-estrogenic chemotherapeutic agent for breast cancer treatment, tamoxifen was later found to act as an estrogen-like ligand in uterine tissue (agonist activity) increasing the risk of uterine cancer [32]. Contrary to this, raloxifene has been reported to retain the anti-estrogen properties of tamoxifen in breast tissue and show minimal estrogen effects in the uterus. Additionally it has been shown to have estrogen-like effects in non-reproductive tissues such as bone and cardiovascular tissue [33,34]. Recently, ER subtype-selective agonist ligands had been developed to provide an
alternative, complementary approach to the use of receptor knockouts, to investigate the independent and possible co-modulatory actions of both the ER subtypes [28,29]. Two such potential compounds are propyl pyrazole triol (PPT) and diarylpropionitrile (DPN) (Figure 1.6). The compound PPT is a potent ER-α agonist, which does not activate ER-β. In contrast, the compound DPN is a selective agonist for ER-β [28,29]. It is proposed that the differences in the N-terminal regions of ER-α and ER-β are responsible for the difference in their responses to various ligands [35].

![Figure 1.6 Chemical structures of PPT (ER-α-specific agonist) and DPN (ER-β-specific agonist)](image)

While the expression of the two ERs may overlap in some target tissues, they are differentially expressed in others [28,35,36]. ER-α is expressed primarily in the uterus, liver, kidney, and heart, whereas ER-β is expressed primarily in the ovary, prostate, lung, gastrointestinal tract, bladder, hematopoietic and central nervous systems. ER-α and β are, however, coexpressed in a number of tissues including the mammary gland, epididymis, thyroid, adrenal, bone, and certain regions of the brain. It should be noted that both ER subtypes may be expressed in the same tissue, but they might not be expressed in the same cell type. The specific function for each ER subtype is sometimes difficult to evaluate, particularly in cases in which the two receptors oppose the action of each other [22,33].

ERs (α and β) belong to the common nuclear receptor family with common structural architecture as described above for androgen receptor. Binding of estrogen to ER triggers conformational changes in the receptor and this leads, via a number of events, to changes in the rate of transcription of estrogen-regulated genes [35]. These events, and the order in which they occur in the overall process, are not completely understood, but they include dissociation of ER from heat-shock proteins in the cytoplasm, receptor dimerization, nuclear localization, receptor-DNA interaction, recruitment of and interaction with coactivators and other transcription factors, and formation of a pre-initiation complex [22,23,27]. Like the other steroid hormone nuclear receptors, ER-α and ER-β homodimerize on hormone binding, and interact with specific palindromic DNA sequences termed estrogen response
elements (EREs) located in target genes [4,5]. In addition, ER-α and ER-β can form heterodimers at ERE-binding sites [37-39].

Transactivation function mediated via ERs is well characterized but transrepression mediated by ER is not well understood. Transactivation requires the recruitment of coactivators, such as SRC-1, that possess histone acetyltransferase activity or can recruit a histone acetyl transferase. This complex can decompact the chromatin, enabling a transcription initiation complex to form. On the other hand, silencing (transrepression) involves the recruitment of corepressors, such as SMRT, and histone deacetyltransferases. Although some estrogen responsive genes contain palindromic estrogen responsive elements (EREs) in their promoter regions to which the ligand-bound-ER binds directly, recent evidences have highlighted the complexity of ER transactivation and transrepression [40]. Ligand-bound ER can also regulate transcription at genes that contain non-consensus EREs or ERE half-sites. More importantly, ER is also able to alter transcription at particular sites without directly binding to DNA. At these sites ER exerts its effects by tethering to another transcription factor, such as AP-1 or Sp-1 (Figure 1.7) [33,41,42]. Likewise, ER can transrepress gene expression by inhibiting the DNA interaction or activity of other factors such as NFκB, which appears to underlie its inhibition of the interleukin-6 (IL6) gene (Figure1.7) [43-46].

![Figure 1.7 Model representing the various modes through which estrogen receptors can modulate transcription of genes.](image)

In the first panel is depicted the classical interaction of the activated receptor with estrogen response elements (EREs) on DNA. In the other three panels are representations of the indirect effects of estrogen receptors on transcription interactions. This occurs through protein-protein interactions with the Sp1, AP1, and NFκB proteins. (From Nilsson et.al. 2001 Physiological Reviews) [35].
Interestingly, the pharmacology of ER at a given target gene can vary depending on the ER subtype or nature of the ligand [28,29]. It has been shown that ER-α and ER-β when complexed with estradiol affect the transcription of genes in opposite ways from an AP1 site [33]. However, repression of genes mediated by ER involving protein-protein interaction of activated ER-complex (estrogen bound dimerized ER complex) is less well characterized [47,48]. The differences in the mode of action, tissue-distribution and ligand-binding properties of ER-α and β makes some of the SERMs ER antagonists or agonists in different tissues.

1.1.3 Sexual dimorphism in non-reproductive tissues
The term sexual dimorphism refers to any morphological, histochemical, biochemical or physiological differences between the sexes. The different hormonal stimuli between the sexes, have well documented developmental effects on the reproductive tissues e.g. uterus, mammary gland (in females) and prostate, seminal vesicle (in males). However, less is known about the identity of resulting genes that are differentially expressed in each sex in somatic/non-reproductive tissues, eg., liver [1,49-52], brain [1,53,54], kidney [55,56], salivary [57], lacrimal [58-61], Harderian [62] and meibomian glands [63]. This information will provide important insights into the different physiologies of mammalian sexes such as disease susceptibility, behavior and life span [1]. For example, differences in the prevalence of autoimmune disease between the sexes, with women 10 times more affected than men, are well known [64]. Similarly, sex differences in the prevalence of neurodegenerative disease, cardiovascular disease, susceptibility to certain cancers and many other diseases are known and sex hormones are thought to play a major role in these differences [64,65].

Apart from the disease related sex-differences, differences in behavior are also well known between the two sexes in different species. This is mainly believed to be due to the sex-differences in brain which is claimed to be the most sexually dimorphic somatic organ in the body [1,53,54]. Sexual dimorphisms in the secretions of many non-reproductive tissues e.g. ectodermal exocrine glands and liver, which are directly secreted to the exterior or into blood and excreted in urine, are believed to be also responsible for eliciting sexually dimorphic behavior, as well as in modulating behavior in general, in con-specifics [60,61,66-77]. Recently, microarray and proteomic studies have shown the immense number of genes which are up-regulated or down-regulated in different non-reproductive tissues, including ectodermal exocrine glands (e.g. lacrimal, salivary, and meibomian glands) and their secretions, in both sexes, indicating a need to investigate the regulation of sexually
dimorphic expression and function of these genes [1,49,50,57,59-61,78,79]. The sexual dimorphism of lacrimal and salivary glands and their secretions are discussed below.

1.2 LACRIMAL GLANDS

Lacrimal glands are seromucus glands located slightly below and just in front of the ear, in rodents and in the outer corner of the eye socket, immediately above the eye ball, in humans. In rodents, the major exorbital lacrimal gland (henceforth called as LG or ELG) and the closely located accessory infraorbital lacrimal gland (ILG) are histologically and biochemically indistinguishable and are connected by a common duct, which leads their secretions to the eye. The main function of the lacrimal gland (LG) is the synthesis and secretion of tears, an aqueous fluid containing proteins, electrolytes and nutrients that protects, nourishes, and lubricates the ocular surface [80,81]. The lacrimal glands are tubuloacinar glands, composed mainly of acinar, ductal and myoepithelial cells. Proteins in tears are mainly synthesized by the secretory acinar cells, which constitute >80% of this gland. Tear secretion by the mammalian lacrimal gland is regulated by the autonomic nervous system with the sympathetic nervous system playing an especially important role. Stimulation of lacrimal gland by physiological adrenergic agonists (such as epinephrine or norepinephrine), synthetic β-adrenergic agonists (isoproterenol) or α-adrenergic agonist (phenylepinephrine) results in increased tear flow [82,83]. Both nerves and growth factors are two important regulators of lacrimal gland secretions. Nerves provide rapid secretions in response to reflexes from the ocular surface and optic nerve. Growth factors like EGF and vasoactive intestinal peptide induce a slower response involving activation of genes and protein synthesis [82,84]. Additionally, as will be discussed later, sex hormones can have profound effects on lacrimal gland secretory activity and function. The secretions of the lacrimal glands form the major component of the pre-ocular tear film, which also contains lipid secretions from the meibomian gland (modified sebaceous gland embedded in eye lids) and the Harderian gland (a retro-orbital modified sebaceous gland present in rodents) [76,77,81,85-87].

1.2.1 Tear film

The pre-ocular tear film lubricates and protects the integrity of ocular surface, preserves visual acuity and defends against microbial challenge. Tears are also suspected to contain pheromones and odorant-/pheromone-binding proteins suggesting an unusual and additional role in chemical communication [66,72,85,87-91][60,61,76,86]. The tear film has a tri-
laminar structure, predominantly consisting of a middle aqueous phase (containing proteins and electrolytes secreted by lacrimal glands) overlying an innermost mucin layer (secreted by conjunctival goblet cells) and a thin outermost lipid layer secreted by meibomian glands of eyelids and additionally by the Harderian glands (in rodents) [76,81,85-87]. The electrolytes in the middle aqueous layer are largely responsible for the osmolality of tears and act as a buffer to maintain pH. The pH of tears is slightly alkaline (7.4) in humans, while a more alkaline estimate (~8.0) was obtained for hamsters (De P.K., unpublished observation). The lacrimal proteins in the aqueous phase of human tears include lysozyme, lactoferrin, transferrin, IgA, secretory component, lipid-binding lipocalins, cytokines, peptide growth factors, defensins, phospholipase A2, peroxidase etc [80,81,92]. These are believed to be essential to the health of the ocular surface or have antimicrobial functions. The superficial lipid layer of the tear film, comprised of wax-esters, sterol esters, hydrocarbons, triglycerides and phospholipids, serves as an evaporative barrier, provides a smooth optical surface for the cornea, reduces the surface tension of tears and is probably a site for production of pheromones in many species[60,61,72,76,85-87].

1.2.2 Sexual dimorphism of lacrimal and Harderian gland

Lacrimal gland: Significant sex-related differences in the anatomy and histomorphology of lacrimal glands have been shown in a number of species. Depending upon the species, distinct sex- and age-related differences may occur between the mean area and density of acinar complexes and the position, size and shape of acinar cell nuclei [93,330]. Moreover, sex differences are also seen in various aspects of lacrimal gland activity like transcription of genes, the production, amount, activity and phosphorylation status of various proteins, enzymes and receptors, the expression of secretory immunity, and response to neural stimulation, pharmaceutical agents and secretion of specific proteins (reviewed in [94,95]). Male rat lacrimal glands and tears have higher levels of IgA [96], secretory component [97], cystatin-related protein [88], α-2u globulin [72] and transforming growth factor-β [94] than females, and the levels of these were decreased upon castration and restored to male levels upon androgen treatment. Thus, until recently, all lacrimal gland sex differences were believed to be due to androgens and mediated by androgen receptor, which has been found to be present in acinar cells of lacrimal glands of several species including, rat, mice, hamster, rabbit and human [98-101]. Moreover, it was generally believed that androgens have only an inductive effect on gene expression in lacrimal glands [88,93,94,96-98,102-107]. However, studies from this laboratory have shown that androgens (and also
estrogens) can markedly repress expression of a major 20-kDa protein in the hamster lacrimal gland [89,91,108].

For a long time it was believed that estrogens do not have any effect on lacrimal glands [88,93,94,96-98,102-107]. Now, evidences are accumulating, which suggest that estrogens can have considerable effects on these glands [89,91,95,99,108]. In support of this proposition are reports that ovariectomy or anti-estrogen treatment can cause necrosis of acinar cell and lymphocytic infiltration in rabbit lacrimal glands, which was prevented by estrogen (and also androgen) treatment [95]. Moreover, studies in our laboratory also showed that ovariectomy induced the levels of a major 20-kDa protein in hamster lacrimal gland, which was repressed by estrogen (and also androgen) treatment [89,91,108,109]. Although, earlier studies could not identify presence of estrogen receptor in lacrimal gland, subsequent studies have clearly demonstrated presence of estrogen receptors in rat, mice, human and rabbit lacrimal glands [110,111].

Very recent reports on the microarray analysis of male and female lacrimal glands of mice showed sex differences in expression of almost 500 genes. Of these, 191 genes were expressed in higher levels in male lacrimal gland and 301 genes were expressed in higher levels in female lacrimal gland. The genes affected, were found to be involved in a wide range of biological processes, molecular functions and cellular components [58]. A follow up microarray study, by the above group, investigating lacrimal glands of intact and gonadectomized male mice with and without androgen treatment showed that androgens regulate the expression of over 2000 genes in lacrimal gland, of which, 1075 genes were significantly up-regulated and 1036 genes were down-regulated by androgen treatment. However, their results surprisingly showed significant down-regulation of only 321 genes and up-regulation of 499 genes in males after castration. Gene ontologies found to be most affected in this latter study by androgen treatment included those related to cell growth, proliferation and metabolism, cell communication and transport, nucleic acid binding, signal transduction and receptor activities [59].

**Harderian gland:** Dramatic sex differences have been reported in the content of free porphyrins and composition of certain unusual lipids, secreted in tears by hamster Harderian gland (HG) [62,85,112,113]. Male hamster HG has negligible levels of porphyrins while female hamster HG is the richest known source of porphyrins (~1000 fold higher than males)! This sex difference was found to be due to repressive effects of androgens on porphyrin biosynthesis in males [62,76,85,86,113]. The sex difference in lipid composition
was also found to be androgen regulated and males had a female like lipid composition after castration while profiles were unaltered in females after ovariectomy [86]. The significance of sex difference in porphyrins and lipids in hamster tear and their function in tears is unclear. Porphyrins are claimed to cause photo-oxidative damage [113], and are also believed to act as pheromones [85]. Interestingly, it has also been proposed that the unusual sex-specific lipids in tears of female hamsters may function as pheromones, particularly for pups, during lactation [76,86]. However, in the HG of rat, no sex differences in porphyrin or lipids have been found.

1.2.3 LG and tear film disorders, relation with sex, age and altered hormonal states

Dry eye (or dry eye syndrome) is a very common disorder caused by insufficient or altered composition of tears secreted by lacrimal glands [114]. This leads to intractable desiccation of the corneal epithelium, ulceration and increased incidence of infection ultimately resulting in complete visual impairment. Severe dry eye is also seen in Sjögren's syndrome, which is a chronic, multi-organ, autoimmune disorder resulting in destruction of secretory cells of the lacrimal gland [114,115]. Non-Sjögren's dry eye is mostly seen in aged people and in altered hormonal states and is ~3 times more prevalent in females while dry eye due to Sjögren's syndrome is about 9 times more prevalent in women. These indicate hormonal and age-related influences on the tear film and lacrimal gland [95,114-116].

1.3 SALIVARY GLANDS

Saliva is produced in and secreted by salivary glands. The three major salivary glands are submandibular or submaxillary glands (located under the floor of the mouth, close to the mandible), parotid glands (located subcutaneously, below and in front of the ear) and sublingual glands (located anterior to the submandibular glands). Salivary glands consist of highly specialized epithelial-derived cells with two fairly well defined morphological and functional segments, that is, the end pieces (acini and intercalated ducts) and a system of glandular ducts of varying complexity [117-120]. The submandibular glands (SMG) produce a mixed serous and mucous secretion, the parotid glands produce a serous, watery secretion and the sublingual glands secrete saliva that is predominantly mucous in character [121]. Secretion of saliva is controlled by the autonomic nervous system via signal transduction systems that couple receptor stimulation to ion transport and protein secretory mechanisms. The volumes of saliva produced vary depending on the type and intensity of stimulation, the largest volumes occurring with cholinergic stimulation [121].
1.3.1 Saliva

Saliva contains water, electrolytes, mucus and proteins. Saliva secretion is generally accepted to be a two-stage process, with initial secretion of an aqueous plasma-like primary fluid by the acinar cells and its subsequent modification during passage through the water-impermeable ductal cell system. Although the primary secretion is a plasma ultrafiltrate (that is, isotonic), energy-dependent reabsorption of Na⁺ and Cl⁻ in the ductal system results in a markedly hypotonic final secretion. Bicarbonate in saliva allows buffering, while calcium and phosphate help in maintenance of tooth mineral integrity [121]. Saliva contains a wide variety of proteins which are unique to this fluid or which have functions of particular importance to oral health. These include, antimicrobial enzymes like lysozyme and peroxidase; antimicrobial proteins like lactoferrin, slgA, histatins and cystatins; buffering enzymes like carbonic anhydrase; proteins which help in bone mineralization, statherins and proline rich proteins; digestive enzymes like amylase and lingual lipase and lubricants like the highly glycosylated mucins etc [121].

The main functions of saliva are, lubrication and binding food into a bolus, antimicrobial effect and preservation of oral hygiene, initiating digestion of starch, pH control by providing alkaline buffering and maintenance tooth enamel integrity [121]. Relatively less studied and a not so obvious function of saliva could be as a carrier of odor/pheromonal cues in many species [66-68,74,109,122,123]. The best-studied examples are in pig [73] and a very recent report in mice [66].

Mammalian salivary glands are commonly used models of exocrine secretion. However, there is now substantial experimental evidence showing the physiological existence of endocrine secretory pathways in these tissues [124,125]. Thus, in recent years, there is considerable research on salivary glands as possible target tissues for systemic (as well as oral) applications of gene therapeutics [124,125]. Salivary glands present numerous advantages for this purpose, including being well encapsulated, which limits extra-glandular dissemination of the viral vector and also having the luminal membranes of almost all parenchymal cells accessible via intraoral delivery of different kinds of viral vectors through the main excretory ducts. Salivary gland gene transfer has been used for two potential clinical goals: (i) the repair of hypofunctional gland parenchyma, and (ii) the production of secretory transgene products for either systemic or upper gastrointestinal tract pharmaceutical use. Secretory deficiency of the salivary gland leading to dry mouth disease is seen in Sjogrens syndrome, which results in autoimmune destruction of the secretory cells of salivary glands. Dry mouth diseases resulting from Sjogren's syndrome are
predominantly seen in women suggesting sex hormonal influences on the development of this disease [121,126].

1.3.2 Sexual dimorphism in salivary glands
Lacassagne (1940) was the first to report sexual dimorphism in SMG [117,119,120]. He noted by histology that granular ducts (GD; also called granular tubules) were more prominent in male mice SMG while acinar cells were more prominent in female SMG. Thus proportion of GD to acini (T/A ratio) in terms of relative space occupied by each in a lobule, showed marked sex difference in mice. It is now well known that development and maintenance of granular duct cells in mice are under hormonal (androgenic) control [117,119,120,127]. Thus, castration reduces GD cell size and this is prevented by androgen treatment. Post-natally, GD cells of mice SMG begin to show histological sexual dimorphism at 20 days age and at this age activities of several enzymes and biologically active polypeptides (amylase, proteases, EGF, NGF), begin to show sexual dimorphism in their levels [119,120]. Histological sexual dimorphism of SMG has been reported for several other species including rat, cotton rat, gerbil, vole, pig, rabbit, bovine and even in monkey [119,120]. In the hamster, histochemically, higher levels of sialomucins and acid phosphatase [119,120,128,129] were reported in SMG but a subsequent study by others did not confirm these and on the contrary, a complete absence of any obvious histological or histochemical sexual dimorphism was noted for hamster SMG [129]. For the sublingual glands, although some histological sexual dimorphism was noted in mouse and tree shrew there have been no such reports in any other species [119,120]. Finally, there are no reports of any histological/histochemical sexual dimorphism in parotid glands of any species [119,120].

Recent microarray analysis of salivary glands of mice revealed over 700 genes differentially expressed between male and female SMG with much fewer sex differences in the sublingual (~150 genes) and parotid glands (~100 genes). In SMG 413 genes were upregulated in males and 304 were upregulated in females [57]. However, prior to this study there were several reports of biochemical sexual dimorphism of salivary gland in many species. A number of proteins, which are processed to biologically active polypeptides, such as epidermal growth factor, nerve growth factor, rennin as well as several proteases are expressed at higher levels in male mice than in female mice SMG [117-119,130-133]. The sexually dimorphic expression of all the above in the SMG are regulated mainly by testosterone. Moreover, in mouse SMG, these proteins are all synthesized in the granular
convoluted tubular (GCT) cells and they are all secreted in saliva [123,127]. Androgen regulated male-specific polypeptides generated from the alternate splicing of VCSA mRNA, in rat SMG [68,123,127,134] have been reported, which is hypothesized to have a role in behavior modulation. A male-specific lipocalin in boar SMG and saliva is known which binds steroidal compounds known to have pheromonal effects on sow [73,74,135]. In the hamster a lipocalin called aphrodisin has been shown to be female specifically expressed in the parotid gland and secreted in saliva [122]. Aphrodisin purified from hamster vaginal discharge has been shown earlier to have aphrodisiac activity on the male [136]. This laboratory has shown earlier the male-specific expression of abundant lipocalins in the SMG of hamster [109,137]. This protein constitutes ~40% of total soluble proteins of the male SMG extract and such high levels of sex-specific proteins are not known in any mammalian non-reproductive tissue.

1.4 LIPOCALINS

The lipocalin (lipos=fat; calyx=cup) protein superfamily consists of abundant, secretory, usually acidic pi carrier proteins of Mr~20 kDa [138-140]. Lipocalins usually show low sequence similarity but whenever investigated, have a conserved 3D structure with a binding pocket for hydrophobic ligands. Lipocalins share sufficient similarity, in the form of short characteristic conserved sequence motifs, like-GXW, to form the basis of a useful definition of family memberships. However, membership to the lipocalin family is usually defined largely on the basis of sequence or structural, similarity, which includes shared lipocalin fold. This common structure of lipocalin protein fold is a highly symmetrical all-β structure dominated by single eight-stranded anti-parallel β-sheet closed back on itself to form a continuously hydrogen bonded β-barrel. The eight β-strands of the barrel, labeled A-H (see Figure 1.8) are linked by a succession of +1 connections giving it the simplest possible β-sheet topology. The β-barrel encloses a ligand-binding site composed of both an internal cavity and an external loop scaffold. This conserved three-dimensional structure results in shared molecular recognition properties; the binding of small, hydrophobic molecules [139]. The diversity of cavity and scaffold gives rise to a variety of different binding modes each capable of accommodating ligands of different size, shape and chemical character. Lipids, odorants, pheromones, retinoids, porphyrins, siderophores, steroids are some of the ligands bound by different lipocalins [135,138-142]. Although, members of lipocalin family are known to do different functions, their main function is as transport proteins or to bind to specific receptors. Thus they are usually found in body fluids such as blood, tears, saliva, urine, uterine fluid, semen etc.
Some of the lipocalsins, their properties and their endogenous or experimentally shown ligands are listed in Table 1.1.

Table 1.1 Members of the lipocalin superfamily (partial list) are shown with a summary of their physical and chemical properties; the proteins listed are divided between kernel and outlier lipocalsins. Molecular masses are given in kDa. Where a property, such as glycosylation, has been shown to be present experimentally this is indicated by a +, shown to be absent by a -; otherwise, where this is unknown the value is denoted by a ?. M=monomer; D=dimer; C=forms complex; Exp=experimental; End=endogenous. (This table is adapted from lipocalin web site: http://www.jenner.ac.uk/Lipocalin/REVIEW/Table%201.htm; also reviewed in [138]; only a partial list is shown)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Subunit molecular mass</th>
<th>pl</th>
<th>No. of residues</th>
<th>Oligomeri c state</th>
<th>Glycosylation</th>
<th>Number of disulph-ides</th>
<th>Ligand binding</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kernel lipocalsins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha_2u$-Globulin</td>
<td>18.7</td>
<td>5.7-6.7</td>
<td>162</td>
<td>M</td>
<td>+</td>
<td>1</td>
<td>Exp. Retinol, 2,2,4-trimethylpentane, decalin, JP-5, isophorone, 1,4-dichlorobenzene, limonene etc.</td>
</tr>
<tr>
<td>$\alpha_1$-Microglobulin</td>
<td>26.0-33.0</td>
<td>4.3-4.8</td>
<td>183</td>
<td>M/D + C</td>
<td>+</td>
<td>1</td>
<td>End: retinol.</td>
</tr>
<tr>
<td>$\beta$-Lactoglobulin</td>
<td>18.3</td>
<td>5.4</td>
<td>162</td>
<td>D/M</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
Lipocalins are generally divided into two subgroups called kernel lipocalins and outlier lipocalins. Kernel lipocalins have three conserved sequence motifs corresponding to the three structurally conserved regions. Outlier lipocalins, have not more than two of these diagnostic motifs [139]. Lipocalins are believed to have evolved from a common ancestral gene, which most likely must be an outlier lipocalin (as no kernel lipocalin has been found in bacteria and plants) [140]. Whenever investigated, lipocalins are usually found as clusters in the genome as shown for human, rat and mouse genomes [143,144]. In "Homo sapiens", for instance, many lipocalin genes were found at bands 32–34 of chromosome 9 long arm (9q), whereas in "Mus musculus" two clusters were found on chromosome 2 (band B to band...
C1, and bands F3 to G) and on chromosome 4 (bands B to C). In some cases, a biological significance has been attributed to gene clustering as it holds functionally related proteins within the same locus. In human, the so-called "immunocalins" (A1gp/a1-acid glycoprotein, Almg/a1-microglobulin, Gd/glycodelin, NGAL/neutrophil gelatinase-associated lipocalin and C8GC/complement factor 8γ chain) illustrate this concept: this subset of proteins, all encoded by the q32–34 region of chromosome 9, groups lipocalins that all share anti-inflammatory properties [145]. Additionally, it is also seen that lipocalins in clusters share common gene structure with conserved exon/intron distribution and arrangement. The cluster organization, together with the gene arrangement found in different phyla, also indicates that the lipocalins probably evolved by successive rounds of tandem gene duplications [140].

A recent phylogenetic analysis of the lipocalin super-family evidenced several evolutionarily related clades subsequently used to make a phylogenetic tree rooted with the eubacterial lipocalins [146,147]. However, such phylogenetic arrangement and chromosomal localization do not always position lipocalins with similar tissue distribution, expression pattern and proposed or determined function. For instance, in the phylogenetic analysis mentioned above, a clade was obtained which encompasses prostaglandin D synthase (PGDS), NGAL and the quiescence-specific p20K, whose functions and tissue distributions seem to be unrelated [146,147]. This supports the idea of positive selection acting on at least some of the duplicated genes so that they can have different expression pattern and different function.

1.4.1 Functions of Lipocalins

By definition lipocalins are secretory proteins. Thus, they are usually present in body fluids and excretions where they are believed to bind different hydrophobic ligands and help in their transport to specific locations within the body or to the exterior. Only a partial list of lipocalins relevant to this study, their tissue distribution and known or hypothesized functions are shown in Table 1.2.

<table>
<thead>
<tr>
<th>Lipocalin</th>
<th>Tissue distribution</th>
<th>Secreted into</th>
<th>Endogenous ligand</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinol binding protein</td>
<td>Liver, kidney, retina etc</td>
<td>Plasma</td>
<td>all-trans-retinol</td>
<td>Retinol transport</td>
</tr>
<tr>
<td>Epididymal Retinoic acid binding protein Rat/mouse</td>
<td>Epididymis</td>
<td>Semen</td>
<td>Retinoic acid</td>
<td>Sperm maturation (?)</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>Mammary gland</td>
<td>Milk</td>
<td>Long-chain fatty acids and triglycerides</td>
<td>Transport hydrophobic molecules</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------------</td>
<td>------</td>
<td>----------------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Glycodein</td>
<td>Endometrium, Seminal vesicles</td>
<td>Uterine fluid, Semen</td>
<td>(?)</td>
<td>Morphogenic, angiogenic, immuno-suppressor, apoptotic, contraceptive in uterus. In semen (?)</td>
</tr>
<tr>
<td>α1-Microglobulin</td>
<td>Liver</td>
<td>Plasma, extracellular matrix, urine</td>
<td>Complexed with monomeric IgA and other plasma proteins</td>
<td>Acute phase protein, immunosuppressive properties</td>
</tr>
<tr>
<td>Tear lipocalin</td>
<td>Lacrimal gland von Ebner's gland</td>
<td>Tears, saliva</td>
<td>Fatty acids, fatty alcohols, phospholipids, glycolipids, cholesterol</td>
<td>Lipid binding protein in tears, maintain surface tension in tear, physiological scavenger</td>
</tr>
<tr>
<td>Apolipoprotein D</td>
<td>Adrenal gland, kidney, pancreas, brain and many other tissues</td>
<td>Plasma</td>
<td>Cholesterol (?)</td>
<td>Transport and metabolism of cholesteryl esters (?)</td>
</tr>
<tr>
<td>Prostaglandin D synthase</td>
<td>Central Nervous System (CNS)</td>
<td>Plasma</td>
<td>PGH₂ and PGD₂</td>
<td>Synthesis of PGD₂</td>
</tr>
<tr>
<td>Mouse major Urinary protein</td>
<td>Liver, submandibular gland, lacrimal gland</td>
<td>Plasma, urine</td>
<td>2-sec-butyl-4,5-dihydrothiazole; dehydro-exo-brevicomicin</td>
<td>Chemical communication</td>
</tr>
<tr>
<td>Rat α2u-globulin</td>
<td>Liver, submandibular gland, lacrimal gland</td>
<td>Plasma, urine</td>
<td>(?)</td>
<td>Chemical communication (?)</td>
</tr>
<tr>
<td>Rat OBP</td>
<td>Nasal gland</td>
<td>Nasal secretions</td>
<td>(?)</td>
<td>Chemical communication (?)</td>
</tr>
<tr>
<td>Hamster aphrodisin</td>
<td>Vaginal epithelium, uterus, Bartholin's gland, parotid</td>
<td>Vaginal discharge, saliva</td>
<td>1-hexadecanol, 1-octadecanol (?)</td>
<td>Chemical communication</td>
</tr>
<tr>
<td>Boar SAL 1</td>
<td>Submandibular gland</td>
<td>Saliva</td>
<td>5 α-androst-16-en-3-one 5 α-androst-16-en-3 α -ol</td>
<td>Chemical communication</td>
</tr>
<tr>
<td>Bovine Bos d2</td>
<td>Skin</td>
<td>Dander</td>
<td>(?)</td>
<td>Chemical communication (?)</td>
</tr>
<tr>
<td>Probasin</td>
<td>Prostate</td>
<td>Seminal fluid (?)</td>
<td>(?)</td>
<td>(?)</td>
</tr>
</tbody>
</table>

For convenience lipocalins can be classified as follows, based on their proposed or determined function. Only a few examples are discussed below.

1.4.1.1 General transport and binding lipocalins

1. Retinol binding protein (RBP) is expressed in liver and secreted into blood where it helps in sequestering and transport of all-trans retinol to different tissues [148].

2. Epididymal retinoic acid binding protein is expressed in epididymis and binds retinoic acid. It is believed to play a role in the maturation of sperm [149].
3. β-lactoglobulin is a well-characterized example of transport lipocalin expressed in mammary tissue of many mammals (especially ruminants). It is secreted into milk and serves as a carrier for many micronutrients [150].

4. Apolipoprotein D is expressed in adrenal glands, kidney and in many other tissues and is believed to function, as a component of high density lipoprotein complex, in transport of cholesterol [151].

5. Human tear lipocalin expressed in lacrimal glands and secreted into tears is believed to bind tear lipids, scavenge harmful lipophilic compounds, microbial siderophores etc., and also stabilizes the tear film [141,152,153].

1.4.1.2 Immunocalins

6. LCN2/NGAL is expressed mainly in neutrophils but also detectable in uterus, lung etc., and is shown to bind bacterial siderophores and combat bacterial infection [154]. Recently they have been also shown to promote cell survival [155].

7. Glycodelin is expressed in both sexes in different tissues (uterus, endometrium etc, in females; seminal vesicles in male) and secreted into endometrial fluid and semen. In females it has been shown to induce morphogenesis, angiogenesis, immunosuppression, contraception etc., but in semen of males its function is not clear [156-159]

1.4.1.3 Sex-specific lipocalins with role in chemical communication

Apart from the above examples lipocalins expressed in other tissues can also show sex-specificity due to hormonal regulation. Incidentally, many of these lipocalins are believed to play a role in chemical communication, as described below.

8. Rat α-2u globulins and mouse major urinary proteins are male-specifically expressed in the liver and abundantly excreted in urine [160]. This male-specific expression is due to induction of their expression by androgens. Apart from liver, they are shown to be expressed in the SMG, LG, preputial gland etc., under multi-hormonal control [161,162]. These urinary lipocalins are believed to act as pheromone carriers and in mouse they may be used in marking territories. MUPs of male mouse urine have been also shown to hasten puberty in immature female mice [163-165].

9. Probasin a lipocalin of unknown function is expressed in prostate of rat and mouse [166] and in hamster (Srikantan et. al. unpublished data). Probasins have been proposed to have a role in chemical communication.
10. SAL1, male-specifically present in saliva of boar is abundantly expressed in the salivary glands and is shown to bind steroidal pheromones [73,74,135].

11. Aphrodisin of hamster vaginal discharge is abundantly expressed in the vaginal epithelium and other parts of female reproductive tract. The purified aphrodisin protein has been claimed to have aphrodisiac activity on male hamsters and recently, some natural ligands have been claimed to be bound to it [136,167,168].

1.4.1.4 Odorant-/pheromone-binding lipocalins
Some of the sex-specific lipocalins discussed above are believed to function as odorant-/pheromone-binding lipocalins. Apart from these, some lipocalins expressed in the nasal glands of both sexes and present in nasal secretions in many species are also believed to act as odorant-binding proteins as discussed below.

12. Odorant-binding lipocalins expressed in nasal glands and epithelium are believed to transport small hydrophobic odorants across the aqueous nasal mucus to and from their receptors on the main olfactory epithelium [169-175].

1.5 SEX-SPECIFIC PROTEINS IN SMG AND LG OF SYRIAN (GOLDEN) HAMSTER

In adult Syrian (golden) hamster (Mesocricetus auratus) submandibular gland (SMG), our lab had earlier reported the expression, regulation and partial cDNA of an abundant male-specific protein (MSP), expressed as N-glycosylated (~24-kDa) and non-glycosylated (~20.5-kDa), products of a single gene [109,137]. Although MSP expression was undetectable in adult females, lactating female hamsters surprisingly expressed male levels of MSP in their SMG. Additionally, in the exorbital LG of hamster, a major female-specific lacrimal protein of Mr 20-kDa (hereafter referred to as FLP) was found to crossreact with MSP antiserum [89,91,109]. MSP protein sequence, deduced from its partial cDNA, showed that it is a lipocalin [109]. However, the identity of FLP, its exact relationship with MSP, human tear lipocalin or any other protein in hamster or rat was unknown.
1.6 OBJECTIVES OF THE PRESENT STUDY

From the above, it is clear that the identity of FLP and its exact relationship with MSP is not known. Moreover, the localization of MSP and FLP in SMG and LG, their tissue distribution in hamsters and the identity and tissue distribution of their orthologues in other species is also not known. Since, MSP and FLP have male- and female-specific expression pattern with obvious influences of sex hormones, the detailed investigation of their regulation by sex hormones was required. Moreover, since hormonal levels change during development and lactation, their effects on FLP and MSP expression also needs to be checked. Finally, since the function(s) of these abundantly and sex-specifically expressed proteins are unclear this needed investigation. With these points in mind the following aims were set for this thesis:

a. Purification, characterization and identification of the MSP-like protein (FLP) of hamster LG. (Chapter-3)

b. Cloning, characterization and comparison of the full-length cDNA and chromosomal genes of FLP and MSP and identification of their orthologues in other species. (Chapters-3 & 7)

c. Detailed characterization of the lactational expression of MSP. (Chapter-6)

d. To study the expression pattern of FLP and MSP during development and their regulation in immatures. (Chapters-4 & 5)

e. Investigations of possible functions of FLP and MSP in hamster. (Chapter-8)