Chapter 1

Introduction
epiblast cells, which involute through the posterior part of primitive streak during gastrulation. These cells proliferate near the endoderm of the yolk sac and migrate towards the genital ridge by amoeboid movement in the 5th gestational week of development. These germ cells, so forth called oogonia, increase in count to $6 \times 10^5$ in subsequent three weeks by vegetative divisions. Further, these oogonia can either undergo mitosis till 28th week of gestation or transform into primary oocytes by meiosis or die by programmed cell death termed as 'atresia'. The highest count of germ cells reaches to $7 \times 10^6$ by 20th gestational week. In later weeks, atresia dominates over the other two mentioned fates resulting in sharp down fall of germ cells. The germ cell count remains $2 \times 10^6$ at birth, $4 \times 10^5$ at puberty and just 1000 at menopause (Richardson et al. 1987). The primary oocytes, which enter meiosis, stay arrested at deplotene stage of meiosis I. This stage is retained until a follicle becomes fully-grown up and encounters LH surge. These cells either undergo atresia or upstage further into primordial ovarian follicles (PROFs). During later event, a single layer of squamous somatic cells called pre-granulosa cells surrounds each oocyte. These PROFs remain dormant in the ovaries until their recruitment for further growth. Recruited PROFs get first transformed into primary ovarian follicles (PRIFs). This transformation is very slow process, which may take 5-50 years and characterized by change in shape of pre-granulosa cells from squamous to cuboidal. This transition is essentially dependent on oocyte derived Growth Differentiation Factor 9 (GDF9) but independent of gonadotropin action. These surrounding granulosa cells divide and become multilayered. These follicles are now termed as pre-antral follicles or multi-
laminar follicles (MUFs). Peripheral ovarian stroma cells are called theca cells which begin formation of layers enclosing the granulosa cells. During this stage, oocyte undergoes rapid increase in size and begins differentiating. These growing oocytes secrete protective membrane, made up of glycoproteins, termed as ‘zona pellucida’. The continued divisions initiate a fluid filled cavity, termed as antrum, inside the multilayered granulosa cells. This stage is now called as antral follicles (ANFs). These follicles slowly become gonadotropin responsive due to gradually elevated expression of gonadotropin receptors. These follicles continue increasing in size and antrum. The further selection of these follicles depends on the gonadotropin surges leading to pre-ovulatory, mature and ovulatory follicle stages. Perceptibly, even subtle alterations in the process of folliculogenesis would threaten the fertility (van den Hurk et al. 2005).
Figure 1. Chronology of the process of folliculogenesis in human ovary; (From Gougeon A. 1986 Dynamics of follicular growth in the human: a model from preliminary results. Hum Reprod 1,81-87)

The underlying physiological mechanism of POF may possibly be gauged by one or more physiological events like lack of initial ovarian follicle pool, accelerated atresia, impaired follicle development and their recruitment (Christin-Maitre et al. 1998). POF can be divided into two histopathological types (i) the afoolicular form (ii) the follicular form (Kinch et al. 1965). The afoolicular form has total depletion of ovarian follicles possibly due to gonadal dysgenesis. The defective migration and differentiation of embryonic germ cells to gonadal ridges lead to streak and afoolicular ovaries. The follicular form can be subdivided into (a) oophoritis (inflammation of follicles) (b) ovaries with few follicles (c) ovaries with numerous primordial follicles.
follicles called as resistant ovary syndrome. Nevertheless, follicular form of POF may also enter into a follicular form. Apparently, FSH is required for recruitment of new follicle cohort during early follicular stage of each menstrual cycle. Constantly high FSH levels during consecutive early follicular phases result in chaotic follicle recruitment hence accelerated follicle depletion. These events represent premature menopausal symptoms and therefore deemed as reproductive aging.

A wide spectrum of genetic and non-genetic mechanisms has been anticipated for explanation of etio-pathogenesis of ovarian failure. The non-genetic mechanisms include immunological abnormalities, physical stress, nutrient deficiency, chemotherapy, radiation effects etc. Nearly 2-5% POF cases have been estimated to be associated with autoimmune disorders i.e. Addison's disease or adrenal autoimmunity. These cases have detectable titers of autoantibodies i.e. adrenal cytoplasmic antibodies (Cy-Ad-Abs) and steroid cell antibodies (St-C-Abs). Adrenal cytochrome P450 enzyme 21 Hydroxylase is a major auto-antigen recognized by these antibodies (Hoek et al. 1997). The effects of iatrogenic agents i.e. radiations, chemotherapy on ovarian function are dependant on dosage, time of exposure and individual's age (Waxman et al. 1983). Anthropometric factors like low socio-economic status; increased occupational burden; higher BMI, nutritional deficiencies and physical stress are other environmental factors for which care should be taken.

Based on the published reports, the ovarian failure has primarily been regarded as a polygenic disorder. Various comprehensive studies have been reported at the chromosomal as well as the gene level to elucidate the
etiology of ovarian failure but it still remains obscure (Schlessinger et al. 2002). Many reports have shown abnormalities in various regions of the X-chromosome in association with idiopathic ovarian failure (Schlessinger et al. 2002). Few recent chromosomal abnormalities reported in idiopathic ovarian failure are listed as Xq21 interstitial deletion (Rizzolio et al. 2006), Xq26.2-28 interstitial deletion (Fimiani et al. 2006) and Xp11.1-22.3 interstitial deletion (Rao et al. 2005) etc. These X-chromosomal abnormalities suggested the probable presence of few candidate genes on the X-chromosome viz. FMR1, DIA (Bione et al. 1998), ZFX (Luoh et al. 1997), FMR2 (Murray et al. 1999), XPNPEP2 (Prueitt et al. 2000) POF1 locus (Davison et al. 2000), POF2 locus, critical region etc (Zinn, 2001) but their role in ovarian dysfunction is not well defined.

The FMR1 (Fragile X Mental Retardation 1, Xq27.3) gene has been most studied among X-linked genes. Fragile X mental retardation is the most frequent type of inherited mental retardation in males and is caused by the expansion of polymorphic CGG repeat in the 5' untranslated region of the FMR1 gene. These patients have ≥200 CGG repeats in 5'UTR leading to methylation coupled gene silencing (Hagerman et al. 2004). The range of repeats has been classified into four stages namely (i) 'full mutation' (≥200 repeats), premutation (61-200 repeats), intermediate range (40-60 repeats) and normal range (≥40 repeats). Many articles correlating CGG repeats in the FMR1 gene and POF reported significant association of the disorder with the premutation range (Bodega et al. 2006, Machado-Ferreira et al. 2004). The presence of premutations in the FMR1 gene may probably lead to aberrant expression and X inactivation. The genetic association studies of
other above listed X-linked genes are not well supported in large scale/sporadic POF cases (Mumm et al. 2001, Schlessinger et al. 2002). Recently another important X-linked candidate gene the BMP15 has also been studied because of its crucial role in the oocyte-granulosa cell communications.

The genetic screening of the key molecules of the Hypothalamus-Pituitary-Ovarian axis (HPO) has been extensively studied from past many years. The axis begins with the release of gonadotropin releasing hormone (GnRH) from hypothalamus. The gonadotroph cells, in the anterior pituitary, present receptors for GnRH (GnRH-R) on their membrane. The ‘loss of function’ mutations in the GNRHR gene are strongly associated with the etiology of Idiopathic Hypogonadotropic Hypogonadism (IHH) in both males and females (Karges et al. 2005). These gonadotrophs release gonadotropins i.e. Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH). Both the gonadotropin hormones reach ovarian follicles. The granulosa cells, surrounding the oocyte, present receptors (FSHR) for FSH whereas theca cells, covering the antrum, present receptors (LHR) for LH. Extensive case-control studies have been carried out on the FSHβ (Matthews et al. 1993, Layman et al. 1993), LHβ (Takahashi et al. 2003, Liao et al. 1998), FSHR (Aittomaki et al. 1995) and LHR (Latronico et al. 1996) genes in various worldwide populations (Achermann et al. 2002). Very few variants have so far been reported in gonadotropins and corresponding receptors in cases with ovarian failure except the p.Ala189Val (c.566C>T) substitution in the FSHR gene. This missense variant in the extra-cellular ligand-binding domain (exon 7) of FSHR gene was reported in strong association with
ovarian failure in the Finnish population (Aittomaki et al. 1995). Functional analysis corroborated significant reduction in binding capacity and signal transduction of mutated receptor. The variations in the FSHR gene were uncommon in other world populations (Layman et al. 1998; Kohek MBF et al. 1998).

Few more genes have also been screened based on their ovarian expression and/or role in folliculogenesis. Noteworthy, many mutational studies of the FOXL2 gene have been reported. The FOXL2 protein is a member of the winged helix/forkhead transcription factor family. This protein is exclusively expressed in either the developing human eyelid or the mature ovary. Crisponi et al. (2000) reported the presence of truncated variants of the FOXL2 gene in few cases of familial Type 1 BPES (blepharophimosis-ptosis-epicanthus inversus syndrome, Drooping eye syndrome) cases where females inherit ovarian failure. Follow up association studies could reveal some variations in nonsyndromic POF but remained uncommon in diverse populations (De Baere et al. 2002, Harris et al. 2002, Bodega et al. 2004). Two more forkhead transcription factor family members FOXO3A and FOXO1A have also been screened among ovarian failure cases. Both the factors are largely expressed in ovary. The Foxo3a null mice exhibited similar phenotype as of POF. Genetic analysis of both factors could reveal some rare variants but significant association of any variant has not been established (Watkins et al. 2006). Zhao et al. (2005) screened the NOBOX gene in Japanese POF cases because of its high expression in the oocyte from primordial stage till matured follicles. The germline analysis of this gene could not reveal any variation in the coding regions. Deficiency in the
galactose-1-phosphate uridyl transferase (GALT) has commonly been considered in association with galactosemia etiology. Few decades earlier, the GALT deficiency was linked with increased galactose toxicity in the ovary (Fraser *et al.* 1987). This hypothesis remained obscure due to lack of any genetic appreciation for the GALT gene with ovarian failure (Mlinar *et al.* 2005, Kumar *et al.* 2005). Recently, mutations in the catalytic subunit of mitochondrial DNA polymerase gamma (POLG) were shown to segregate with POF in families with progressive external ophthalmoplegia (PEO) (Pagnamenta *et al.* 2006).

Present study is an endeavor to establish germline status of key regulators involved in inhibin mediated FSH down-regulation mechanism. Inhibin and activin have opposing regulatory action for FSH level modulation. Activin enhances FSH secretion while inhibin forms a negative feedback loop control of FSH level. Both the factors are produced from granulosa cells surrounding the oocyte. Production of both factors is positively regulated by FSH hormone. The production of inhibins is also positively regulated by two ‘oocyte derived growth factors’ i.e. BMP15 (Bone Morphogenetic Protein 15) and GDF9 (Growth Differentiation Factor 9). The primary function of these factors is to regulate granulosa cell proliferation and follicle development. Inhibins and activins, via blood stream, reach anterior pituitary where gonadotropin (FSH and LH) producing cells ‘gonadotrophs’ are located. The gonadotrophs present receptors for activins and inhibins. Most of the TGFβ members (including activins, inhibins, GDF9, BMP15) exert their signaling by binding their serine threonine type II receptor and this complex further recruits type I receptor which in turn
stimulates intracellular signaling. Similarly, activins bind their type II receptors (ActRIIA and ActRIIB) located on the gonadotroph cell surface. The activin/ActRII complex further recruits type I receptor ALK4. This activin/ActRII/ALK4 complex in turn activates Smad2/3 signaling and results in an elevated production of FSH. Conversely inhibins bind to a type III receptor betaglycan located on the gonadotrophs. This inhibin/betaglycan complex has an equal binding affinity for activin Type II receptors therefore efficiently compete the activins mediated signaling. Unlike activin/ActRII complex, the inhibin/betaglycan/ActRII complex fails to recruit ALK4 due to the lack of kinase activity in the cytoplasmic domain of betaglycan. This inhibin-mediated phenomenon is incompetent to initiate Smad signaling resulting in down-regulation of the FSH production (Lewis et al. 2000). Hence, the key factors of this above-mentioned mechanism are INHA (inhibin alpha), INHBA (inhibin beta A), INHBB (inhibin beta B), GDF9, BMP15, Betaglycan (TGFBR3) and ALK4 (ACVR1B). Mutational studies of these candidate genes have been aimed in this study to establish their germline status in Indian women with ovarian failure.