Chapter 1: General Introduction

Various reproductive processes and functions of a species are maintained by the complex interplay of hypothalamic, pituitary and gonadal factors. Several endocrine hormones and paracrine and autocrine factors act in a coordinated fashion for successful completion of a reproductive process. On the basis of various experimental and genetic data, there is growing evidence that the majority of these autocrine and paracrine factors belong to the transforming growth factor (TGF) β superfamily (Shimasaki *et al.*, 2004, Zhao, 2003). The TGF-β super family consists of more than 35 members and they are further subdivided into various subfamilies such as TGF-β family, the activin family, mullerian inhibiting substance (MIS) and bone morphogenetic proteins (BMPs) (Fig. 1.3) (Chang *et al.*, 2002, Shimasaki *et al.*, 2004). Almost all members of the TGF-β superfamily have six to nine conserved cysteine residues in their mature domain, which are involved in folding these molecules into three dimensional structures known as cystine knot (Chang *et al.*, 2002, Vitt *et al.*, 2001). The cysteine knot is a characteristic of the TGF-β superfamily (Vitt *et al.*, 2001).

The BMPs are a group of proteins, which were originally identified in extracts of demineralized bone on the basis of their ability to promote healing in osteomyelitic defects (Rengachary, 2002). The term BMPs was first coined by Urist (Urist, 1965) on the basis of their capacity to induce new bone formation at ectopic sites in rodents, but the identity of these proteins remained unknown until 1980s. In 1988, bovine BMP-3 was purified and sequenced from highly purified preparation of bovine bone (Wozney *et al.*, 1988). In same year, human BMP-1, BMP-2 and BMP-3 were also cloned (Wozney *et al.*, 1988).

Like other members of the TGF-β super family, BMPs are synthesized as a precursor peptide, containing hydrophobic secretive leader sequence as well as substantial pro-peptides, which is processed to a mature form and secreted as a dimer (Constam & Robertson, 1999, Cui *et al.*, 2001). BMPs are present in cytoplasm as large dimeric pro-proteins and they are cleaved by proteases during their secretion and the functional carboxyl region is released into extra-cellular compartment to bind to the specific membrane receptors (Constam & Robertson, 1999, Cui *et al.*, 2001, Degnin *et al.*, 2004). The mature portion of protein is located at the carboxy terminal of the precursor molecule. This carboxy terminal of all BMPs contains seven cysteine amino acids, of which six are involved in intra-chain disulphide bonds, while the seventh cysteine amino acid is involved in an inter-chain disulphide bond for the formation of dimer (Aono *et al.*, 1995, Hillger *et al.*, 2005). Most BMPs exist as homodimers in physiological conditions. Recently, recombinant heterodimers were synthesized by various laboratories (Aono *et al.*, 1995, Kusumoto *et al.*, 1997). BMP-2/7 and BMP-2/6 heterodimers are more potent than BMP-2 and BMP-7 in alkaline phosphatase induction assay (*in vitro*) as well as in induction of cartilage and bone formation (*in vivo*) (Israel *et al.*, 1996). Similarly, *Xenopus* BMP-4/7 heterodimer has been shown to have more potent mesoderm inducing capacity than BMP-4 and BMP-7 homodimers in the vertebrate embryo (Suzuki *et al.*, 1997). In contrast, recombinant human BMP-2 homodimer has more potent ectopic osteoinduction ability in *in vivo* than heterodimers (Kusumoto *et al.*, 1997). Similarly, BMP homodimer activity but not of BMP heterodimers are required for mesoderm induction in *Xenopus* embryo (Eimon & Harland, 1999). At present there is no physiological evidence of existence of these heterodimers in any organ system and further studies are required to elucidate their role in influencing different biological processes.

In general, the name BMP only describes one particular function of these proteins but they are involved in multiple cellular functions such as proliferation, differentiation, migration, organization and death (Shimasaki *et al.*, 2004). BMPs are similar to other growth factors such as TGF-β, insulin-like growth factor (IGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) in their mitogen stimulating activity, but quite different in their morphogenic activity as BMPs are able to transform connective tissue cells into osteoprogenitor cells (Rengachary, 2002). Almost all the member's of TGF- β super family signal through a group of transmembrane proteins called serine/threonine kinase or TGF- β receptor family, except glial cell derived neural cell factor (GDNF). GDNF signals through tyrosine kinase receptors but because of cysteine knot in its structure, it is included in the TGF-β superfamily (Massague, 1998).

1. 1. Following are some of the important members of the BMP family –

1.1. 1. BMP-1

BMP-1 was isolated with BMP-2A and BMP-3 from bovine bone protein extracts on the basis of their bone and cartilage inducing capacity (Wozney *et al.*, 1988). Unlike other BMPs, BMP-1 is not a member of the TGF β superfamily; instead it belongs to family of metalloproteases. It is a protein of 730 amino acids including 20 amino acids encoding a signal sequence. Signal sequence or leader sequence is usually a part of most of the cytokines, which help in their passage through intracellular membranes and also their export through the outer cellular membranes (Kessler *et al.*, 1996). BMP-1 cleaves the

BMP binding proteins like chordin and counteracts its dorsalizing effect on overexpression in *Xenopus* embryo (Scott *et al.*, 1999). It is involved in cartilage, bone (Wozney *et al.*, 1988) and skin (Amano *et al.*, 2000) development in various species but its role in reproduction is not fully explored yet.

1.1. 2. BMP-2 (BMP-2A or BMP-2- α)

BMP-2 was purified from demineralized bone on the basis of its capacity to induce cartilage formation at an ectopic site (Wozney *et al.*, 1988). It is involved in various physiological functions such as bone (Tabas *et al.*, 1991) and lung (Langenfeld *et al.*, 2003) development and was recently shown to be involved in regulation of ovarian functions (Souza *et al.*, 2002). BMP-2 gene deleted mice died early during embryogenesis indicating that BMP-2 is vital for various developmental processes (Table 1.1) (Zhang & Bradley, 1996). It is a highly conserved protein across species and even BMPs from non-vertebrate species share 70-87 % homology with human BMP-2. It shares 68 percent homology with *Drosophila* decapentaplegic A gene (dpp) protein and is functionally interchangeable in a mammalian bone induction assay (Abrams *et al.*, 2004). Chinese hamster ovary (CHO) cell lines expressing recombinant human BMP-2 by using methotrexate mediated gene amplification system secrete three major forms of BMP-2 : (1) 60 kDa precursor protein (2) 40-45 kDa propeptide (3) 30 kDa homodimer consisting of 18-22 kDa subunits (Israel *et al.*, 1992). Dpp is a gene isolated from the genome of *Drosophila melanogaster*, which can functionally replace both BMP-2 and BMP-4 in ectopic bone forming assay (Abrams *et al.*, 2004, Heller *et al.*, 1999).

1.1. 3. BMP-4 (BMP-2B or BMP-2-β)

It is a 408 amino acid (aa) propeptide and its gene located on chromosome 14 in mouse and humans (Dickinson *et al.*, 1990), and is composed of a 19 aa signal sequence,

a 273 aa pro-region and a 116 aa mature segment (Wozney *et al.*, 1988). Both the pro-region and mature segment contains two potential N-linked glycosylation sites. The mature region of human, mouse and rat BMP-4 have 98% aa sequence homology (Fig. 1.1) (Cui *et al.*, 2001).

BMP-4 is a vital regulatory protein and has an important role as a signaling molecule in embryonic tissues, including the development of central and peripheral nervous system, musculature and skeleton system (Sedohara *et al.*, 2002, Wilson *et al.*, 1997). BMP-4 gene deleted mice embryos form little or no mesoderm and die at the time of gastrulation (Table 1.1) (Winnier *et al.*, 1995). BMP-4 heterozygous mice are viable and have a variety of birth defects such as reduced number of primordial germ cells (PGC), polydactyly, and defective kidney, eye and craniofacial development (Chang *et al.*, 2001, Lawson *et al.*, 1999, Miyazaki *et al.*, 2000). Recent studies have shown that excessive production of BMP-4 also leads to birth defects. Gene knockout mice for BMP-4 antagonists (gremlin, noggin and chordin) have shown developmental defects in spinal chord, forebrain, somites, skeleton and kidney (Brunet *et al.*, 1998, Gong *et al.*, 1999, Khokha *et al.*, 2003, McMahon *et al.*, 1998). Moreover, in humans, mutations in the noggin gene are responsible for multiple synostoses syndrome, a genetic disease characterized by fusion of joints (Gong *et al.*, 1999). Similarly, strong correlation has been observed between over expression of BMP-4 and occurrence of fibrodysplasia ossificans progressive, a hereditary disorder in which ectopic bone forms throughout the body (Shafritz *et al.*, 1996). In mice, cooperation and interplay of BMP-4 and FGF is required for normal tooth development (Tucker *et al.*, 1998). In human ovarian theca-tumor cell culture model (HOTT), BMP-4 treatment decreased forskolin-stimulated HOTT cell secretion of androstenedione and 17 α hydroxyprogestrone, and increased production of progesterone (Dooley *et al.*, 2000). In another study on rat granulosa cell

culture, addition of BMP-4 decreased FSH-induced production of progesterone and increased FSH-induced estradiol production (Shimasaki *et al.*, 1999). These studies indicates that BMP-4 plays many different roles in different organ systems and strict regulation by various other factors including BMPs antagonist is required for controlling BMP-4 activity at multiple levels.

1.1. 4. BMP-5

BMP-5 was identified from demineralized bone extract on the basis of its ability to induce endochondral osteogenesis at extra-skeletal sites (Urist, 1965). On the basis of amino acid sequence homology BMP-5, BMP-6 and BMP-7 are recognized as a subfamily in BMP family. Sequence analysis studies have shown that 60 A gene of *Drosophila* is a dipteran homolog of this BMP subfamily (Hahn *et al.*, 1992). Presently, these proteins are categorized into 60 A subgroup of BMP family, also including BMP-6, BMP-7, BMP-8a and BMP-8b as members (Solloway & Robertson, 1999). It is a 138 amino acid protein and human and mouse peptide sequences share a 96 percent homology. BMP-5 is implicated in limb and bone development, dendritic growth in sympathetic neurons and in apoptosis (Arosarena & Collins, 2003, Beck *et al.*, 2001, Zuzarte-Luis *et al.*, 2004). BMP-5 gene knockout studies in mice have shown negligible effects on development except a few skeletal defects including short ears (Table 1.1) (King *et al.*, 1994). Recently, BMP-5 has been shown to be involved in granulosa cell proliferation and steroidogenesis in rat ovary (Pierre *et al.*, 2005a).

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BMP4 CHICK MIPGNRMLMVILLCQVLLGGTNHASLIPETGRKKVAELQGQAGSGRRSAQSHELLRGFET
BMP4 DAMDA MIPGNRMLMVVLLCQVLLGGATHASLIPETGKKKVAEIQGHAG-GRRSGQSHELLRDFEA
BMP4 HUMAN MIPGNRMLMVVLLCQVLLGGASHASLIPETGKKKVAEIQGHAG-GRRSGQSHELLRDFEA
BMP4 MOUSE MIPGNRMLMVVLLCQVLLGGASHASLIPETGKKKVAEIQGHAG-GRRSGQSHELLRDFEA
BMP4 RABIT MIPGNRMLMVVLLCQVLLGGASHASLIPETGKKKVAEIQGHAG-GRRSGQSHELLRDFEA
BMP4 RAT   MIPGNRMLMVVLLCQVLLGGATDASLIPETGKKKVAEIQGHAG-GRRSGQSHELLRDFEA
BMP4 XENLA MIPGNRMLMVILLSQVLLGGTNYASLIPDTGKKKVAADIQGGG--RRSPQSNELLRDFEV
           **********:**.******:. *****:**:****     .*  *** **:****.**.

BMP4 CHICK TLLQMFGLRRRPQPSKSAVIPSYMLDLYRLQSGEEEERS-LQEISLQYPERSASRANTVR
BMP4 DAMDA TLLQMFGLRRRPQPSKSAVIPDYMRDLYRLQSGEEEEEEQIQGIGLEYPERPASRANTVR
BMP4 HUMAN TLLQMFGLRRRPQPSKSAVIPDYMRDLYRLQSGEEEEE-QIHSTGLEYPERPASRANTVR
BMP4 MOUSE TLLQMFGLRRRPQPSKSAVIPDYMRDLYRLQSGEEEEEEQSQGTGLEYPERPASRANTVR
BMP4 RABIT TLLQMFGLRRHPQPSKSAVIPDYMRDLYRLQSGEEEEEEQMPSGGLEYPERPASRANTVR
BMP4 RAT   TLLQMFGLRRRPQPSKSAVIPDYMRDLYRLQSGEEEEEEQSQGTGLEYPERPASRANTVR
BMP4 XENLA TLLQMFGLRKRPQPSKDVVVPAYMRDLYRLQSAEEED--ELHDISMEYPETPTSRANTVR
           *********::*****..*:* ** *******.***:       .::*** .:*******

BMP4 CHICK SFHHEEHLESVPGPSEAPRIRFVFNLSSVPDNEVISSEELRLYREQVEEPSAAWERGFHR
BMP4 DAMDA SFHHEEHLENIPGTSENSAFRFLFNLS-IPENQVISTAELRDFREQVDQ-GPDWERGFHR
BMP4 HUMAN SFHHEEHLENIPGTSENSAFRFLFNLSSIPENEVISSAELRLFREQVDQ-GPDWERGFHR
BMP4 MOUSE SFHHEEHLENIPGTSESSAFRFLFNLSSIPENEVISSAELRLFREQVDQ-GPDWEQGFHR
BMP4 RABIT SFHHEEHLENIPGTSENSAFRFLFNLSSIPENEAISSAELRLFREQVDQ-GPDWERGFHR
BMP4 RAT   SFHHEEHLENIPGTSESSAFRFFFNLSSIPENEVISSAELRLFREQVDQ-GPDWEQGFHR
BMP4 XENLA SFHHEEHLENLPGTEENGNFRFVFNLSSIPENEVISSAELRLYREQIDH-GPAWDEGFHR
           *********.:**..*   :**.**** :*:*:.**: *** :***::. .. *:.****

BMP4 CHICK INIYEVMKPLSERSQ--AITRLLDTRLVHHNVTRWETFDVSPAVIRWTKDKQPNHGLVIE
BMP4 DAMDA INIYEVMKPPAEAVPGHLITRLLDTRLVHHNVTRWETFDVSPAVLRWTREKQPNYGLAIE
BMP4 HUMAN INIYEVMKPPAEVVPGHLITRLLDTRLVHHNVTRWETFDVSPAVLRWTREKQPNYGLAIE
BMP4 MOUSE INIYEVMKPPAEMVPGHLITRLLDTRLVHHNVTRWETFDVSPAVLRWTREKQPNYGLAIE
BMP4 RABIT INIYEVMKPPAEAVPGHLITRLLDTRLVHHNVTRWETFDVSPAVLRWTREKQPNHGLAVE
BMP4 RAT   INIYEVMKPPAEMVPGHLITRLLDTRLVRHNVTRWETFDVSPAVLRWTREKQPNYGLAIE
BMP4 XENLA INIYEVMKPITAN--GHMINRLLDTRVIHHNVTQWESFDVSPAIMRWTLDKQINHGLAIE
           ********* :       *.******:::****:**:******::*** :** *:**.:*

BMP4 CHICK VTHLHQAQTHQGKHVRISRSLPQGHGGDWAQLRPLLVTFGHDGRGHALTRR--ARRSPKH
BMP4 DAMDA VTHLHQTRTHQGQHVRISRSLPQGSG-DWAQLRPLLVTFGHDGRGHALTRHRRAKRSPKH
BMP4 HUMAN VTHLHQTRTHQGQHVRISRSLPQGSG-NWAQLRPLLVTFGHDGRGHALTRRRRAKRSPKH
BMP4 MOUSE VTHLHQTRTHQGQHVRISRSLPQGSG-DWAQLRPLLVTFGHDGRGHTLTRRR-AKRSPKH
BMP4 RABIT VTHFHHTRTHQGQHVRLSRSLLQGSG-DWAQFRPLLVTFGHDGRGHALTRRRRAKRSLKH
BMP4 RAT   VTHLHQTRTHQGQHVRISRSLPQGSG-NWAQLRPLLVTFGHDGRGHTLTRRR-AKRSPKH
BMP4 XENLA VIHLNQTKTYQGKHVRISRSLLPQKDADWSQMRPLLITFSHDGRGHALTRRS--KRSPKQ
           * *:::::*:**:***:****    . :*:*:****:**.******:***:   :** *:

BMP4 CHICK HG--SRKNKKNCRRHALYVDFSDVGWNDWIVAPPGYQAFYCHGDCPFPLADHLNSTNHAI
BMP4 DAMDA HPQRARKKNKNCRRHSLYVDFSDVGWNDWIVAPPGYQAFYCHGDCPFPLADHLNSTNHAI
BMP4 HUMAN HSQRARKKNKNCRRHSLYVDFSDVGWNDWIVAPPGYQAFYCHGDCPFPLADHLNSTNHAI
BMP4 MOUSE HPQRSRKKNKNCRRHSLYVDFSDVGWNDWIVAPPGYQAFYCHGDCPFPLADHLNSTNHAI
BMP4 RABIT HPQRARKKNKNCRRHALYVDFSDVGWNDWIVAPPGYQAFYCHGDCPFPLADHFNSTNHAI
BMP4 RAT   HPQRSRKKNKNCRRHSLYVDFSDVGWNDWIVAPPGYQAFYCHGDCPFPLADHLNSTNHAI
BMP4 XENLA Q--RPRKKNKHCRRHSLYVDFSDVGWNDWIVAPPGYQAFYCHGDCPFPLADHLNSTNHAI
           :   .**::*:****:**********************************:*******

    BMP4 CHICK VQTLVNSVNSSIPKACCVPTELSAISMLYLDEYDKVVLKNYQEMVVEGCGCR
    BMP4 DAMDA VQTLVNSVNSSIPKACCVPTELSAISMLYLDEYDKVVLKNYQEMVVEGCGCR
    BMP4 HUMAN VQTLVNSVNSSIPKACCVPTELSAISMLYLDEYDKVVLKNYQEMVVEGCGCR
    BMP4 MOUSE VQTLVNSVNSSIPKACCVPTELSAISMLYLDEYDKVVLKNYQEMVVEGCGCR
    BMP4 RABIT VQTLVNSVNSSIPKACCVPTELSAISMLYLDEYDKVVLKNYQEMVVEGCGCR
    BMP4 RAT   VQTLVNSVNSSIPKACCVPTELSAISMLYLDEYDKVVLKNYQEMVVEGCGCR
    BMP4 XENLA VQTLVNSVNSSIPKACCVPTELSAISMLYLDEYDKVVLKNYQEMVVEGCGCR
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Figure 1. 1. Comparision of the amino acid sequence of BMP-4 in Chicken (*Gallus gallus*), Dama dama (Fallow deer/ *Cervus dama*), Human (*Homo sapiens*), Mouse (*Mus musculus*), Rabbit (*Oryctolagus cuniculus*), Rat (*Rattus norvegicus*) and African clawed frog (*Xenopus laevis*). Identical aa are indicated with (*) while differences in aa are indicated with (.) or (:). (Human protein reference database www.hprd.org).

1.1. 5. BMP-6 (Vgr-1)

Like BMP-5, BMP-6 was also identified from demineralized bone extract (Urist, 1965) and belongs to a 60A subgroup of the BMP family (Hahn *et al.*, 1992). It is a 513 amino acids propeptide and has 139 amino acids in a mature protein. On the basis of sequence homology with *Xenopus* Vg-1(Vegetalising factor-1), BMP-6 was first identified from mouse embryonic cDNA library and was named Vgr-1. Subsequently, human and bovine homologues were isolated and were named as BMP-6 because of its similarities with other BMPs. It plays significant role in cartilage and bone formation (Ebisawa *et al.*, 1999, Yang *et al.*, 2003). Homozygous BMP-6 mutant mice are viable and fertile and show no defect in tissues and organs, which are already known to express BMP-6 mRNA including ovary (Table 1.1) (Solloway *et al.*, 1998). BMP-6 has similar expression as BMP-2 in hypertrophic cartilage indicating that BMP-2 might compensate for the loss of functions of BMP-6 in BMP-6-knockout mice (Solloway *et al.*, 1998). BMP-6 mRNA is expressed in mouse pituitary and LbetaT2 cell line (Huang *et al.*, 2001b). In addition, BMP-6 treatment to LbetaT2 cell culture increases the endogenous production of FSH (Huang *et al.*, 2001b). The LbetaT2 cell lines are derived from transgenic mice expressing ovine FSHbeta promoter linked to a luciferase reporter gene (oFSHbetaLuc) (Huang *et al.*, 2001a, Huang *et al.*, 2001b).

1.1. 6. BMP-7 (Osteogenic protein-1 or OP-1)

BMP-7 was first purified as a osteogenic protein (Ozkaynak *et al.*, 1990), but later studies showed that osteogenic protein consists of two protein subunits: a 18-kDa subunit, which is human equivalent of OP-1 gene (BMP-7) and a 16-kDa subunit, which is equivalent of human BMP-2A gene (BMP-2) (Sampath *et al.*, 1990). It is a 431 amino

acid propeptide and has 139 amino acids in the mature segment. The mature forms of BMP-5, BMP-6 and BMP-7 have 75 percent homology. Human and mouse proteins are 98 percent identical. Besides bone formation, it is also involved in kidney functions and reproduction (Biyikli *et al.*, 2005, Lee *et al.*, 2001, Lee *et al.*, 2004). Expression of BMP-7 in heart, proximal and distal forelimb, clavicle and scapula are positively correlated with Holt-Oram syndrome (Marker *et al.*, 1995). In mouse mesenchymal stem cell line and human osteoblastic cell line, BMP-7 induces all markers of osteoblast differentiation in pluripotential and mesenchymal stem cells (Cheng *et al.*, 2003). BMP-5 and BMP-7 are co-expressed in various tissues (Solloway & Robertson, 1999). BMP-5 null mutants are viable and physiologically normal, whereas, BMP5/BMP-7 double mutant mice died after 10.5 day post coital (dpc) and displayed developmental defects in various tissues (Table 1.1) (Solloway & Robertson, 1999). These studies indicates that loss of function of BMP-5 in BMP-5 gene deleted mice might be functionally compensated by BMP-7 (Solloway & Robertson, 1999).

1.1. 7. BMP-15 (GDF-9B)

BMP-15 was first identified from mouse embryo library database (129 SvEv genomic library, EST cDNA) by comparing with growth differentiation factor 9 (GDF-9) (Dube *et al.*, 1998). BMP-15 is also known as GDF-9B because of its similarities with GDF-9 including 52% homology in peptide sequence, co-expressions in oocyte and absence of seventh cysteine amino acid (Yan *et al.*, 2001). Mouse and human BMP-15 share 63 % amino acid homology and their mature protein peptides have 75% homology between amino acids (Dube *et al.*, 1998). BMP-15 is secreted as precursor protein, and biological active portion of protein is in the C-terminus of the molecule. The precursor protein consists of 393 amino acids and mature portion or biologically active region of

protein consists of 125 amino acids (Dube *et al.*, 1998). GDF-9 and BMP-15 both lack the seventh cysteine amino acid required for disulphide bond formation, which is the characteristic of the TGF-β superfamily and help in formation of biologically active dimmers (Dube *et al.*, 1998). The absence of the fourth of the seventh cysteine amino acid in these proteins indicates that formation of dimer might not be essential criteria for biological activity of these proteins as it is for other members of the TGF-β superfamily (Dube *et al.*, 1998, Laitinen *et al.*, 1998). Homodimer and heterodimer of GDF-9 and BMP-15 exist in transfected cell lines (Liao *et al.*, 2003). However, their existence in biological system is not known yet. In a recent study, molecular characteristics of mouse (polyovulatory) and human (monoovulatory) BMP-15 were compared in 293T cells transfected with mouse or human BMP-15 expression plasmids (Hashimoto *et al.*, 2005). It was found in this study that proregion of mouse BMP-15 resists cleavage and causes reduction in production of mature mouse BMP-15 protein (Hashimoto *et al.*, 2005). Similarly, fusion of mouse BMP-15 proregion with mature human BMP-15 region resulted in inhibition of production of human mature protein in transfected cell lines (Hashimoto *et al.*, 2005). On the basis of these studies, the authors concluded that BMP-15 might have different roles in monoovulatory and polyovulatory species.

In situ hybridization and immunohistochemical studies have revealed that BMP-15 expression first appears in oocyte with recruitment of primordial follicle for further follicular development and remained until ovulation (Dube *et al.*, 1998, Otsuka *et al.*, 2000). Recently, genetic analysis studies have shown that heterozygous mutation (A to G transition) in BMP-15 gene at base pair 704 is responsible for phenotype of female sibling of an Italian family affected with hypergonadotropic ovarian failure (streak ovaries and underdeveloped uterus), primary amenorrhea and hirsutism (Di Pasquale *et al.*, 2004).

1.1. 8. GDF-9

GDF-9 gene was identified from mouse 129SvEv genomic library by using mouse GDF-9 cDNA as a probe (Incerti *et al.*, 1994). The rat GDF-9 prepropeptide is 440 amino acid (aa) in length and the mature peptide is 135 amino acid long (Jaatinen *et al.*, 1999). Using *in situ* hybridization, GDF-9 expression was localized in oocyte of neonatal and mature mouse ovaries (McGrath *et al.*, 1995). In addition, human homologue of mouse GDF-9 was cloned by using a complementary DNA library (McGrath *et al.*, 1995). GDF-9 is closely related to Vg-1 (57%), human BMP-4 (34%) (McPherron & Lee, 1993) and BMP-15 (52%) (Yan *et al.*, 2001). Vg-1 gene was identified in the genome of *Xenopus laevis* (Asashima *et al.*, 1991). It acts as erythroid differentiation factor and its effects are synergized by epidermal growth factor (EGF) and TGF-β (Asashima *et al.*, 1991). Human and mouse GDF-9 share 96% homology (Dube *et al.*, 1998), whereas ovine GDF-9 shares 77% and 66% homology with human and mouse GDF-9, respectively (Bodensteiner *et al.*, 1999). Moreover, pig GDF-9 (mature protein) has 92.1%, 97.8%, 97%, 89.6%, 88.1% homologies with human, bovine, ovine, rat and mouse GDF-9, respectively (Shimizu *et al.*, 2004). GDF- 9 gene deleted female mice are infertile, but fertility of male counterparts is normal (Dong *et al.*, 1996). Histological studies on the ovaries of GDF-9 deleted mice revealed that oocytes from follicles of homozygous mutant mice ovaries grow faster than controls and follicular growth stops at primary follicular stage (Table 1.1) (Carabatsos *et al.*, 1998).

Table 1. 1. The mutant phenotype of some of the members of the TGF β superfamily

Ligands	Mutant phenotype
BMP-2	Embryonic lethality, delayed primitive streak, small allantois, lack of amnion, heart defects and decreased number of PGCs.
BMP-3	Viable, increased bone density in adult mice.
BMP-4	Embryonic lethality, lack of allantois and PGCs, posterior truncations, heart defects and lack of optic vesicle, heterozygote- cystic kidney, craniofacial malformations, microphthalmia, preaxial polydactyly of the right hind limb.
BMP-5	Short ear phenotype includes defects in skeleton lung and kidney.
BMP-6	Completely viable and fertile.
BMP-7	Perinatal lethal, skeletal defects, kidney agenesis, eye defects.
BMP- 8a	Viable, male infertility due to defects in spermatogenesis and epididymis.
BMP-8b	Viable, defects in PGC formation, testis cord formation and spermatogenesis.
BMP-12 (GDF- 7)	Viable, hydrocephalic abnormalities, infertility in males due to growth defects

	in seminal vesicles.
BMP-15	**Viable, infertile due to defects in oogenesis, interacting role with GDF-9 in ovarian physiology.**
GDF-9	**Viable, sterile due to defects in oogenesis, folliculogenesis arrested at one layer of primary follicle stage.**
Activin βa	**Lack whiskers and lower incisors and cleft palate**
Activin βb	**Defect in eyelid development and female reproduction**
Inhibin α	**Ovarian cancer, severe wasting syndrome**
MIS	**Pseudo hermaphrodites (female reproductive tract present in males), leydig cell hyperplasia, premature disappearance of primordial follicles in females.**
TGF-β1	**Hyperactive immunity and defects in angiogenesis**
TGF-β2	**Perinatal lethality due to multiple defects in heart, lung, limb, spinal column, eye, inner ear, and urogenital system**
TGF-β3	**Cleft palate and delayed lung development**

Modified from (Chang *et al.*, 2002, Zhao, 2003).

1. 2. BMP Signaling

All the members of the TGF-β superfamily except GDNF signal through serine/threonine kinase receptor superfamily (Massague, 1998). The serine/threonine kinase receptor superfamily consists of type 1 and type II receptors. There are five type II (TβR-II, ActR-II, ActR-IIB, BMPR-II, AMHR-II) and seven type I receptors (Activin Receptor-Like Kinases, ALKs 1-7) for signaling of more than 35 members of the TGF-β superfamily (Fig. 3) (Derynck & Zhang, 2003). In absence of ligand binding these receptors exist as homodimers and upon activation by ligand, form heterodimers. The binding of ligand with type II receptor allows formation of hetero-oligomeric complex, which subsequently leads to phosphorylation of GS domain of type I receptor by type II receptor (Fig. 1.2) (Massague, 1998). This newly formed complex in turn transphosphorylates intra-cytoplasmic signaling molecules called R-smads (receptor regulated smads). These activated R-smads are released from the receptor complex to form a new hetero-dimeric complex with common smad (co-smad) (smad-4) and translocate to the nucleus for smad mediated transcription (Derynck & Zhang, 2003). After entering into the nucleus, these smads can directly interact with DNA sequence by using their MH1 domain or with the help of various other factors such as Runx-2/Cbfa-1, FAST-1 (Canalis *et al.*, 2003, Lee *et al.*, 2000). Smad-2 and smad-3 are activated by MH2 domain of TβR-I and ActR-IB, whereas ALK-I, ALK-2, BMPR-IA/ALK-3 and BMPR-IB/ALK-6 activates smad-1, smad-5 and smad-8 (Fig. 1.3) (Massague, 1998). In C2C12 cell lines, BMP-4 and GDF-5 bind to BMPR-IA and BMPR-IB, whereas BMP-6 and BMP-7 preferentially bind to ALK-2 (Aoki *et al.*, 2001). In silk worm system and surface plasmon resonance studies, extracellular domain of type I receptor is sufficient for BMP-2

and BMP-4 ligand binding (Natsume *et al.*, 1997). In human fibroblast cells, BMP-4 specifically binds to BRK-3 (a type II receptor, identified and cloned from the human fibroblast cells (Nohno *et al.*, 1995)). Similarly, BMP-4 and BMP-7 binds to BMPR-II in transfected COS-1 cell lines (Rosenzweig *et al.*, 1995). In C2C12 cell culture, BMP-4 and GDF-5 induce osteogenic differentiation through activation of three receptor regulated smads (smad-1, smad-5 and smad-8), whereas BMP-6 and BMP-7 only use smad-1 and smad-5, but not smad-8 for inducing alkaline phosphatase activity in same cell line (Aoki *et al.*, 2001). In SW480.7 human colon carcinoma cells, amino terminal domain of smad-4 promotes binding of heteromeric complex to DNA and with carboxy terminal domain promotes activation of smad-1 or smad-2 for transcription (Liu *et al.*, 1997).

In addition to BMPs and TGF-β members, epidermal growth factor (EGF) and hepatocyte growth factor (HGF) also induce phosphorylation and transcription of smad-2 protein in COS7 cells, indicating that smads are also used for signaling of other proteins than members of the TGF-β superfamily (Fig. 1.2) (Derynck & Zhang, 2003). The tyrosine kinase receptor stimulated Erk mitogen activated protein kinase (MAPK) pathway phosphorylates MH1 domain of smad-1 and linker segments of smad-1, smad-2 and smad-3 (Derynck & Zhang, 2003). Additionally, oncogenenically activated Ras causes phosphorylation of smad-2 and smad-3 by using Erk MAP kinases and inhibits signaling of the TGF-β members by negative regulation of smad-2 and smad-3 in mammary and lung epithelial cells (Kretzschmar *et al.*, 1999). These studies indicated that the interaction of smad dependent and independent pathways is responsible for signal transduction of BMPs and other TGF β family members (Fig. 1.2) (Canalis *et al.*, 2003, Derynck & Zhang, 2003).

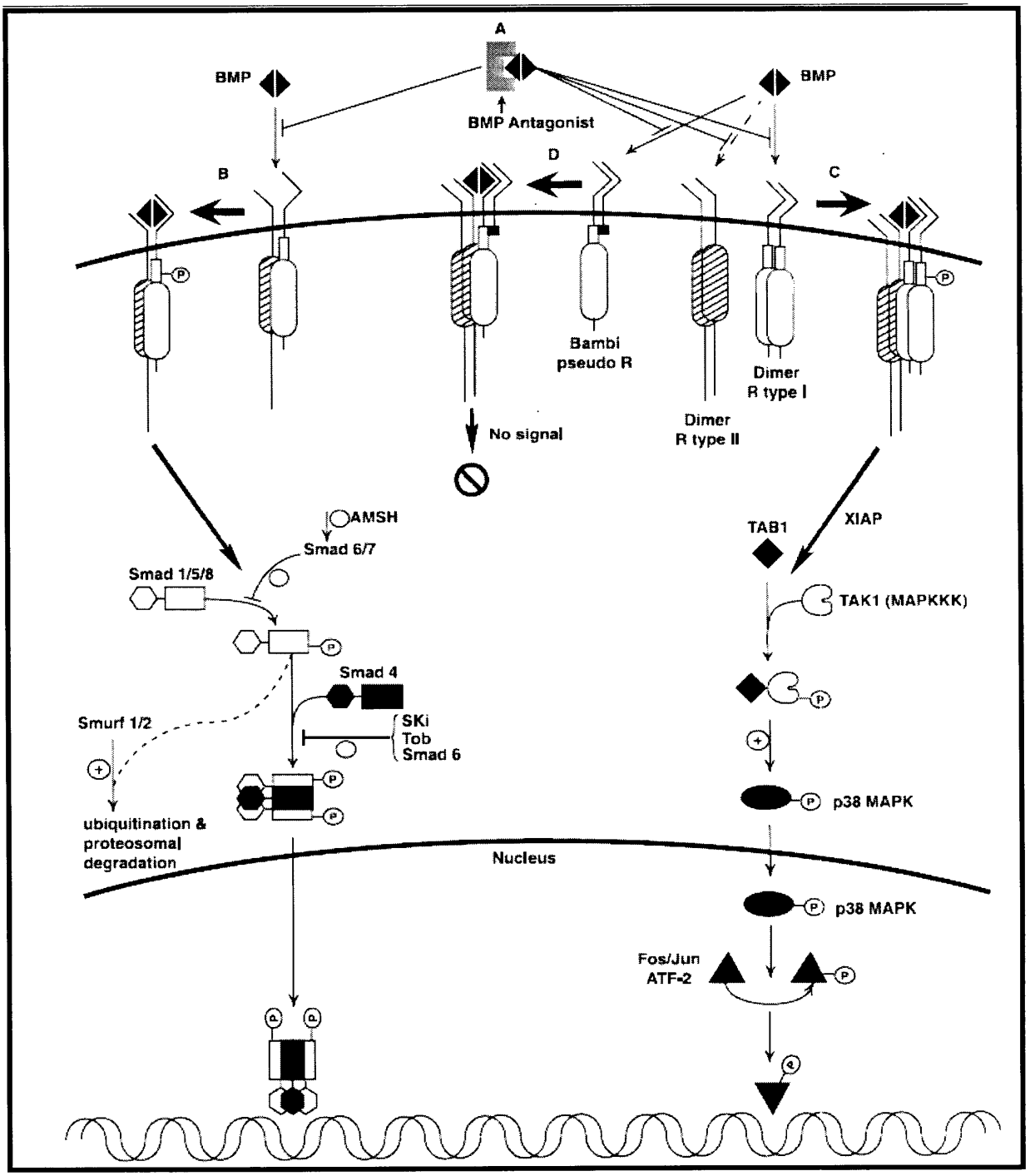

Figure 1. 2. Mechanism of BMP signaling revealing multiple levels of regulation, including : A) secreted extracellular antagonists that bind to their cognate BMPs and prevent receptor binding; B) signaling from preformed heteromeric complex of type I and type II BMP receptors in which ligand binding results in activation of smads-1/-5 pathway, which can regulated by inhibitory smad-6 and -7, smad binding proteins, Ski and Tob, and ubiquitination and degradation by smurf -1 and -2; C) signaling from ligand induced heteromeric complexes of type-I and type-II BMP receptor, which results in the activation of a p38 MAPK pathway; and D) nonsignaling BMP pseudoreceptors, BAMBI. Reproduced from (Canalis *et al.*, 2003).

1.2. 1. Receptors

The TGF- β receptor family is subdivided into two subfamilies known as type I and type II receptor families. Members having high similarity in their kinase domains and signaling activities come under type 1 receptor family, which is further divided into three groups: (a) T β R-I and ActR-IB & ALK-7 (b) BMPR-IA and BMPR-IB (c) ALK-I and ALK-2. Whereas, type II receptor subfamily mainly consists of five members, ActRII, ActRIIB, T β R-II, BMPR-II and AMHR (Anti-Mullerian hormone receptor) (Chang *et al.*, 2002). The main phenotypic expressions observed in TGF- β family receptor knockouts have been described in Table 3. Because these receptors were simultaneously identified by different laboratories they got different names and some of the receptors with their synonyms are described in following table (Table 1.2):

Table 1. 2. Mammalian TGF β super-family receptors and their alternative names

ALK1	**ACTVRL1**
ALK2	**ActRIA, ACVR1**
ALK3	**BMPR1A**
ALK4	**ActRIB**
ALK5	**Tβ RI**
ALK6	**BMPRIB**
ALK7	**N/A**
ACVR2	**ActRIIA**
ACVR2B	**ActRIIB**
AMHR2	**MISRII**
BMPR2	**BMPRII**
TGFBR2	**TβR2**

Modified from (Chang *et al.*, 2002).

Recently, Dragon (a co-receptor for BMPs) has been identified (Samad *et al.*, 2005). It is a 436 amino acid, Glycosylphosphatidylinositol (GPI) anchored protein and a member of the repulsive guidance molecule (RGM) family (Samad *et al.*, 2005). Dragon

was shown to enhance expressions of BMP responsive promoters (I-BRE-Luc, BRE-Luc, and Msx2-Luc) in BMP responsive cell lines such as LLC-PK1 (kidney epithelial cells), 10 T1/2 cells (mouse mesenchymal stem cells), and HepG2 cell line (Samad *et al.*, 2005). In immunoprecipitation and cell free binding assays, dragon directly and specifically binds to BMP-2 and BMP-4 (Samad *et al.*, 2005). In *Xenopus* embryo, co-injection of Dragon and smad-1 mRNA were able to induce expression of pan-mesodermal marker (Xbra) and two endodermal markers (mix and mixer) in dose dependent manner, whereas individually, neither dragon nor smad-1 elicited expression of these markers (Samad *et al.*, 2005). The effect of dragon on BMP signaling is reduced by noggin indicating that the activity of dragon is ligand dependent (Samad *et al.*, 2005). *In situ* hybridization and immunolocalization studies have shown expression of dragon in reproductive organs of mice and in cell lines derived from reproductive tissues *viz.* Ishikawa, Hela, LβT2, MCF-7 and JEG3 cells (Xia *et al.*, 2005). In immature testis, dragon is detected in gonocytes and spermatogonia. In adult testis, its expression is limited to spermatocytes and spermatid (Xia *et al.*, 2005). In the ovary, dragon is detected to the oocyte. In the pituitary, dragon is found to express in FSH expressing cells (Xia *et al.*, 2005).

Table 1. 3. The mutant phenotype of some of the receptors of the TGF β family

Receptors	Mutant phenotype
ALK1	**Defect in embryonic angiogenesis and arteriovenous malformations.**
ALK-2	**Defects in mesoderm formation as a result of defective visceral endoderm.**
BMPR-IA	**Defects in epiblast proliferation and no mesoderm induction in null mutants, impaired cardiac and limb development in conditional mutants**
BMPR-IB	**Viable, defects in seminal vesicle development, female reproduction and limb skeletal formation.**
BMPR-II	**Embryonic lethality, defects in gastrulation / lack of mesoderm.**
ActR-IIA	**Deficiency in reproduction due to suppressed FSH and mild defects in skeletal development.**
ActR-IIB	**Defects in axial patterning and left right symmetry**

Modified from (Chang *et al.*, 2002, Zhao, 2003).

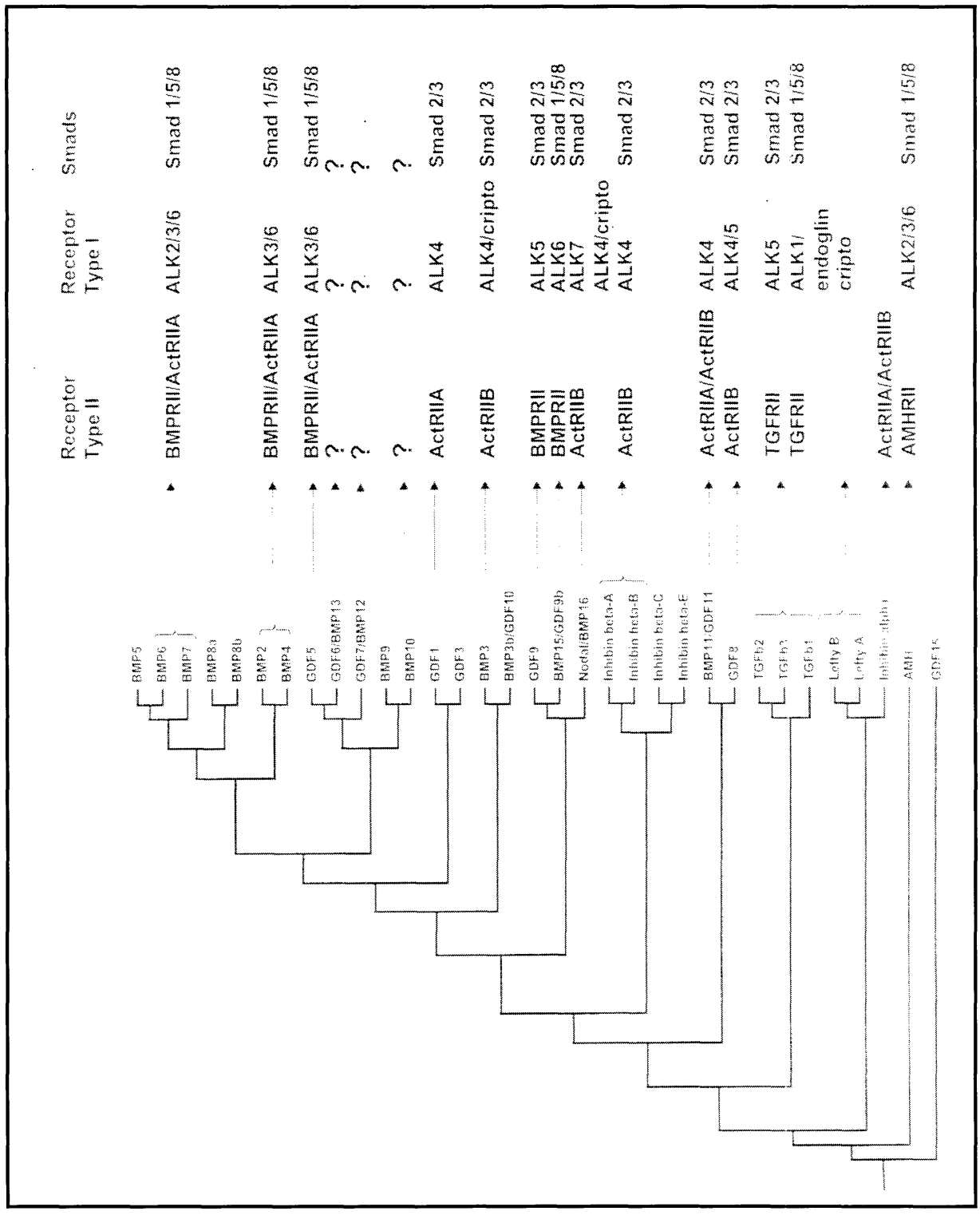

Figure 1. 3. Phylogenetic relationship of paralogous TGF-β/ GDF/ BMP ligands, as well as characterized receptors and signaling pathways for individual ligands. The alignment of 35 TGFβ-related ligands was performed using the C-terminal region containing the cystine knot structure, starting from the first invariant cysteine residue. Based on published literature, the type II and type I receptors as well as the intracellular signaling smad proteins for individual ligands are listed. Dashed lines indicate orphan ligands under investigation. Reproduced from (Mazerbourg *et al.*, 2005).

1.2.1. 1. Structure of receptors

The basic structure of serine/ threonine receptor superfamily members can be divided into three major domains *viz.* Extracellular domain, GS domain and the Kinase domain (Fig. 1.4) (Massague, 1998). The type I and type II receptors are of about 55 kDa and 70 kDa with core polypeptide of about 500 to 570 amino acids including signaling sequence. The extracellular domain of these receptors is made of about 150 amino acids and is involved in modulation of TGF-β responses (Franzen *et al.*, 1993, Lin & Wang, 1992, Lin *et al.*, 1992, Mathews & Vale, 1991). However GS domain, which is the region immediately preceding kinase domain, is made up of 30 amino acids. These 30 amino acids are highly conserved in type I receptors. GS domain is rich in glycine and serine and also contains SGSGSG sequence, which is a characteristic of GS domain. GS domain is important for the signaling of type I receptors as it contain phosphorylation site required for their phosphorylation by type II receptor. Mutation in GS domain inhibits both phosphorylation and signal transduction of type I receptor (Attisano *et al.*, 1996, Wieser *et al.*, 1995). Immediately after SGSGSG sequence, GS domain of type 1 receptor contains Leu-Pro sequence, which is binding site for FKBP12. FKBP12 is an immunophilin binding protein isolated from yeast and inhibits functions of type I receptors by binding to Leu-Pro sequence (Charng *et al.*, 1996, Chen *et al.*, 1997). The third domain, the kinase domain of type I and II receptor is quite similar to serine/ threonine protein kinase domain and it plays important role in phosphorylation of smad proteins (Massague, 1998). The nine-amino-acid L-45 loop region of kinase domain of type I receptor is involved in substrate recognition and determines intracellular signaling specificity as it directly interacts with L3 loop in the MH2 domain of R-smads, which indicates importance of kinase domain in downstream signaling (Feng & Derynck, 1997).

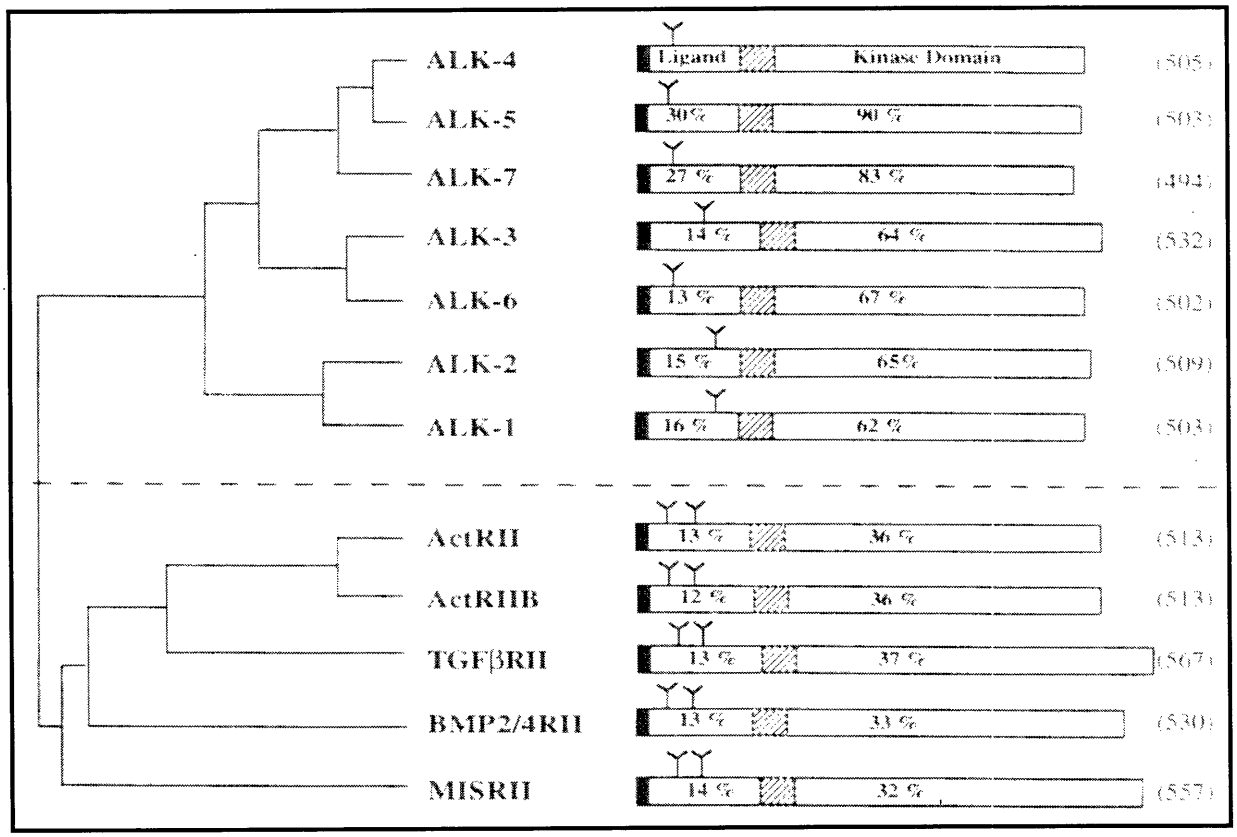

Figure 1. 4. Members of the activin/TGF-β receptor serine/threonine kinase receptor superfamily. Left: dendrogram representing the different type I and type II receptors. Right: graphic representation of the structure of the TGF-β receptor superfamily members. Number in parenthesis (right side) represents the number of amino acids for each receptor. The percentages of homology in the ligand binding domain and in the cytoplasmic kinase domain are indicated inside the sequence of the receptors and are relative to the ALK4/ ActR-IB. The signal peptide sequence and the transmembrane domain of the receptors are represented in shaded box, and the N-glycosylation sites on the extracellular domain are marked as follows (Y). The horizontal dotted line in the center separates the type I receptors from the type II receptors. MIS: mullerian inhibiting substance. Reproduced from (Lebrun *et al.*, 1996).

1.2. 2. SMAD proteins

Smad proteins are intracellular components of the TGF-β superfamily receptor system and were discovered through genetic studies in *Drosophila* and *Caenorhabditis elegans* (Massague, 1998). There are eight smad proteins in mouse and humans, four in Drosophila and three in *C. elegans* and they are involved in various cellular functions

including folliculogenesis and cell differentiation (Table 1.4.) (Chang *et al.*, 2002, Tomic *et al.*, 2004). The founding member of smad family Mad (mothers against dpp) was identified in *Drosophila* and then its three homologues were identified in *Caenorhabditis elegans* and got name sma1, sma2 and sma3 because their mutation caused small body size (Massague, 1998). Mammalian homologue of these proteins is known as "smad" (Massague, 1998). In humans, pancreatic carcinoma is associated with mutations in smad-4/ DPC 4 suggesting role of smads as tumor suppressing factors in *in vivo* conditions (Hahn *et al.*, 1996).

Broadly, smad proteins can be classified in three subclasses: Receptor regulated smads (R-smads), Common smads (Co-smads) and Inhibitory or antagonistic smads (I-smads) (Fig. 1.5) (Massague, 1998). R-smads are directly activated through carboxy terminal phosphorylation of kinase domain of different ligands. Smad-1, smad-5 and smad -8 are mediators of BMP signaling, while smad-2 and smad-3 are mediators of TGF-β and activin signaling (Fig. 1.3) (Derynck & Zhang, 2003). In the *Xenopus* embryos, overexpression of smad-1 mimics BMP-2 and BMP-4 activities, whereas smad-2 overexpression activates activin, Vgl and nodal like activities (Graff *et al.*, 1996). Smad-4 is only known co-smad in vertebrates, but two more co-smad, smad-4α and smad-4β, are also found in the *Xenopus* (Howell *et al.*, 1999). The final subclass of smad proteins is inhibitory smads and they inhibit the signaling of R-smads. Smad-6 and smad-7 are the members of this group in vertebrates, while madea and sma-4 are found in *Drosophila* and *Caenorhabditis elegans*, respectively (Miyazono, 2000). The smad-6 competes with smad-4 by forming complex with smad-1 and preferentially inhibit BMP signaling, while smad-7 can inhibit signaling of both TGF-β and BMPs (Shimasaki *et al.*, 2004).

Table 1. 4. The mutant phenotype of intracellular signaling molecules of TGF-β receptors family

Smads	Mutant phenotype
Smad-1	Perinatally lethal, defects in visceral endoderm, extra embryonic mesoderm and reduced number of or lack of PGCs.
Smad-2	Defects in visceral endoderm causing abnormal mesoderm formation and anterior posterior patterning. Mutant ES cells fail to contribute to gut endoderm; left-right patterning defects.
Smad-3	Colon cancer, impaired immune response, accelerate wound healing, abnormal hypertrophic chondrocyte differentiation and reduced fertility in females.
Smad-4 (DPC-4)	Gastrulation defects-lack of mesoderm formation; gastric polyposis/cancer in heterozygotes.
Smad-5	Impaired angiogenesis in yolk sac, reduced mesoderm, defects in PGC formation and left-right asymmetry.
Smad-6	Hyperplasia of the cardiac valves and defects in outflow tract septation.
Smad-7	Scleroderma-like phenotype (autoimmune).

Modified from (Chang *et al.*, 2002, Zhao, 2003).

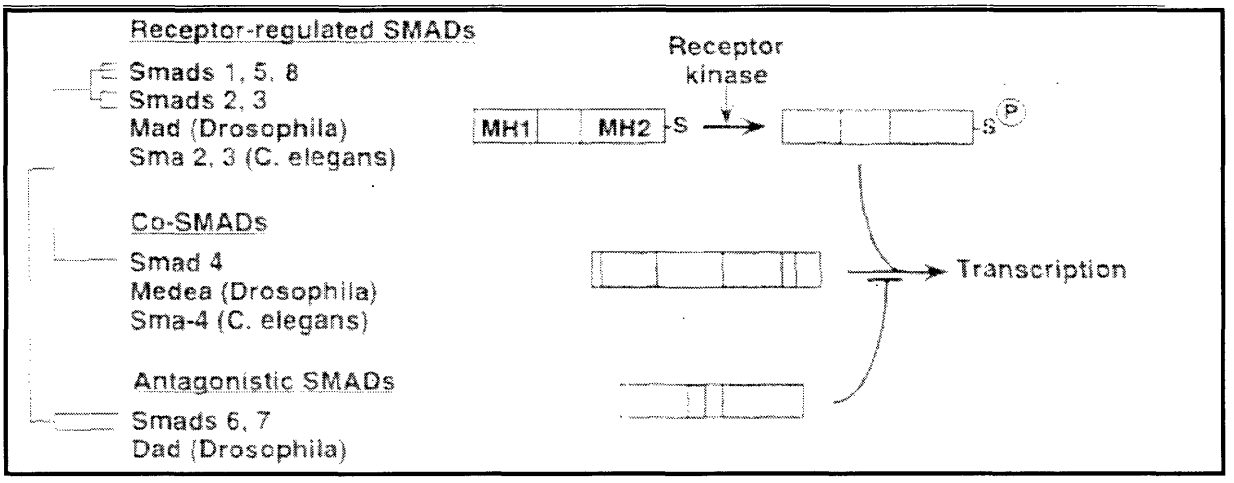

Figure 1. 5. The smad family. Listed members are from vertebrates unless otherwise indicated. Vertebrate smads are highly conserved between human and Xenopus. The dendrogram indicates the relative level of amino acid sequence identity between vertebrate smads. The highly conserved MH1 and MH2 are indicated. Receptor regulated smads are directly phosphorylated by TGF-β family type I receptors, and this phosphorylation allows association with a collaborating smad (co-smad). Antagonistic smads inhibit this smad activation process. Reproduced from (Massague, 1998).

1.2.2. 1. Basic structure of SMAD proteins

The smad proteins contain two highly conserved domains at N-terminal and C-terminal of protein known as MH1 (MAD homologous region 1) and MH2 (MAD homologous 2), respectively (Fig. 1.6) (Inman, 2005, Massague, 1998). MH1 domain is highly conserved in R-smads and Co-smad and has approximately 130 amino acids, while MH2 domain has 200 amino acids and contain phosphorylation site for R-smads (Kretzschmar *et al.*, 1997, Massague, 1998). In basal state, MH1 and MH2 domain inhibit functions of each other. However, all the functions of MH1 domain are not of inhibitory nature as MH1 domain of smad-4 promotes DNA binding of smad-2/smad-4/fast1 transcription complex and *Drosophila* Mad MH1 domain DNA binding is required for Dpp-induced activation of an enhancer with vestigial wing patterning gene (Kim *et al.*, 1997, Liu *et al.*, 1997). MH1 and MH2 domains of smad-2 and smad-4 physically interact

with each other and overexpression of any of the domain prevent association of smad-2 / smad-4 and subsequent TGF-β signaling (Hata *et al.*, 1997). However, activation of MH2 domain of smad-4 is required for active transcription of smad-1 and smad-2. In inhibitory smads, MH2 domain is sufficient for their antagonistic properties (Hata *et al.*, 1997, Liu *et al.*, 1997). These two domains are linked by a highly variable linker region which helps in formation of homo-oligomers of smads (Fig. 1.6) (Massague, 1998). In smad-1, the phosphorylation site is located in linker region and phosphorylation of this site leads to inhibition of nucleus translocation of smad-1 (Kretzschmar *et al.*, 1997).

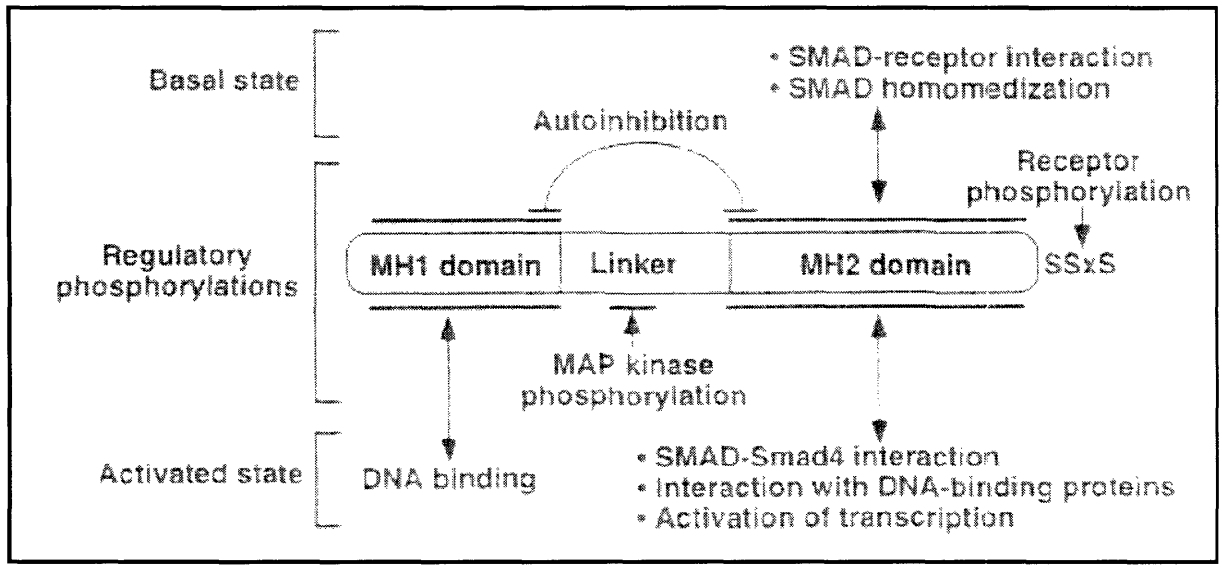

Figure 1. 6. Smad domains and their functions. In the basal state, smads form homo-oligomers and remain in an inactive state through an interaction between the MH1 and MH2 domains. Receptor-regulated smads interact with activated type I receptors via the MH2 domain and become activated by receptor mediated phosphorylation at the C-terminal SS(V/M)S motif. In the activated state, smads associate with smad-4 and with the DNA-binding protein by the MH2 domain. The MH1 domain of some smads also participates in DNA binding, and the MH2 domain participates in transcriptional activation. MAP kinases phosphorylate some smads in their linker region, inhibiting smad accumulation in the nucleus. Reproduced from (Massague, 1998).

1. 3. Factors affecting Bone morphogenetic proteins signaling

The signaling of BMPs is regulated at multiple levels by various extracellular and intracellular factors (Chang *et al.*, 2002, Miyazono, 2000). These factors affect BMP signaling in many ways such as acting as pseudoreceptor (BAMBI), blocking BMP signaling (inhibitory smads), binding to smad proteins (Ski and Tob), degradation of smad proteins (smurfs) and blocking BMPs binding to receptors (BMP binding proteins) (Fig 1.2) (Canalis *et al.*, 2003).

1.3. 1. Intracellular factors

BMP signaling is regulated by a number of intracellular factors. BAMBI (BMP and activin membrane bound inhibitor) was cloned in the *Xenopus* embryo as a transmembrane glycoprotein (Onichtchouk *et al.*, 1999). Extracellular domain of BAMBI is similar to type I receptor of BMPs but it lacks an intracellular kinase domain (Onichtchouk *et al.*, 1999). This absence of intracellular signaling domain is responsible for inhibitory effects of BAMBI as it can bind to different BMP ligands similar to type I BMP receptor without forming receptor complexes for further signaling (Onichtchouk *et al.*, 1999). BAMBI is co-expressed with BMP-4 during *Xenopus* embryogenesis and BMP-4 is required for its expression (Onichtchouk *et al.*, 1999). Its expression is also detected in granulosa and thecal cell of ovary and in spermatogonia, spermatocytes, round spermatids, sertoli cells of testis in rats (Loveland *et al.*, 2003).

Inhibitory smads (smad-6 and smad-7) interfere in BMP signaling by inhibiting phosphorylation of smad-1/5 and interfering in their heterodimerization with smad-4 as I-smads form a complex with smad-1 and compete for smad-4 binding (Hata *et al.*, 1998). I-smads lack a MH1 domain, which is required for DNA binding so antagonism of I-

smads only occurs at protein level. Both I-smads smad-6 and smad-7 are able to block signaling of BMPs, activin and other members of the TGF β superfamily. However, smad-6 preferentially block signaling of BMPs (Ishisaki *et al.*, 1999). Ski and Tob are another group of proteins found to affect BMP related activities in both *in vivo* and *in vitro* conditions (Wang *et al.*, 2000, Yoshida *et al.*, 2000). Ski, the transforming protein of the avian homolog of the Sloan-Kettering retrovirus (v Ski), is a nuclear oncogenic protein and has been implicated in cell growth and skeletal muscle differentiation (Berk *et al.*, 1997). Ski blocks BMP signaling in *Xenopus* embryo and in murine W-20-17 cells (Wang *et al.*, 2000). The transducer of Erb B-2 (Tob) is a member of the PC3/ BTG/ Tob family of genes and is involved in various cell functions (Yoshida *et al.*, 1997). They block BMPs signaling by binding to MH2 domain of smads before their heterodimerization during BMPs signal transduction (Yoshida *et al.*, 2000). These proteins are important regulators of BMPs signaling in bone formation. However, there are no reports of their involvement in reproductive process. Ski and Tob knockout mice showed no phenotypic defect related to reproduction (Berk *et al.*, 1997, Yoshida *et al.*, 2000). Smad ubiquitination regulatory proteins (smurf) are another group of proteins involved in intracellular regulation of BMP signaling. Ubiquitin is a small and highly conserved protein involved in targeted degradation of proteins by the proteasome (adenosine 5'-triphosphate-dependent protease) (Conaway *et al.*, 2002). Smurf 1 and smurf 2 are smad specific ubiquitin ligases. These proteins inhibit BMP signaling by interacting with R-smads and cause their smurf mediated degradation (Zhang *et al.*, 2001, Zhu *et al.*, 1999). Smurf 1 and smurf 2 have two WW domains in their structure, which help them in their interaction with proline rich PPXY motifs present in linker region of different smads. Smurfs are unique in their activity as they are able to control sensitivity of cells to different smads independent of ligand stimulation (Ebisawa *et al.*, 2001,

Kavsak *et al.*, 2000). These studies have shown that these proteins are important regulators of BMP signaling in both *in vivo* and *in vitro* models, and further research is required to understand the role of these interactions in different reproductive processes.

1.3. 2. Extracellular factors

Biological actions of BMPs are also regulated at the extracellular level by different BMP binding proteins and other BMP family ligands such as BMP3 antagonize functions of BMP-4 in the *Xenopus* embryos (Gamer *et al.*, 2005). These BMP binding proteins can inhibit BMP signaling by influencing the access of ligand to the receptors. There are numerous BMP binding proteins *viz.* follistatin and follistatin like protein, inhibin, members of the Dan / Cerberus family, chordin, noggin, gremlin, twisted gastrulation (Tsg) and ventropin (Canalis *et al.*, 2003, Groppe *et al.*, 2003, Ohyama *et al.*, 2001, Scott *et al.*, 2001, Shi *et al.*, 2001, Sudo *et al.*, 2004, Tsuchida *et al.*, 2000).

1.3.2. 1. Follistatin and Follistatin related protein

Follistatin was isolated from bovine and porcine ovarian follicular fluid and has been shown to suppress FSH release from pituitary cell culture (Robertson *et al.*, 1987, Ueno *et al.*, 1987, Ying *et al.*, 1987). Follistatin is a single chain, cysteine rich, glycosylated polypeptide with molecular weight of about 31 to 45 kDa, depending on the degree of glycosylation (Esch *et al.*, 1987, Ying *et al.*, 1987). Northern blot analysis has detected follistatin mRNA expression in almost all human tissues, with highest expression in adult ovary, pituitary, kidney and in fetal heart and liver (Tortoriello *et al.*, 2001). In the mouse, follistatin mRNA was first detected in the deciduum at embryonic day 5.5 and in hindbrain, somites, vibrissae, teeth, epidermis, and muscles in later stages of embryonic development (Matzuk *et al.*, 1995a). Follistatin deficient mice were growth retarded, had

decreased mass of diaphragm and inter-costal muscles, defects in development of hard palate and thirteenth pair of ribs and had abnormal skin, whisker and tooth development (Matzuk *et al.*, 1995a). The phenotypic defects observed in these mice were more widespread than those in activin deficient mice (Table 1.1) (Matzuk *et al.*, 1995a, Matzuk *et al.*, 1995b), indicating that follistatin is likely to regulate the functions of several other members of the TGF-β superfamily.

Follistatin is a high affinity activin binding protein and studies demonstrated that follistatin can also bind to other members of the TGF-β superfamily including BMPs (Fainsod *et al.*, 1997, Iemura *et al.*, 1998). It has been shown in surface plasmon resonance biosensor analysis that follistatin significantly bound to the BMP-4 homodimer and activin-A without binding to TGFβ-I (Iemura *et al.*, 1998). The affinity of follistatin for BMPs (Kd, ~23 nM) is lower than that of follistatins for activins (Kd = 540-680 pM) and that of BMPs for BMPR-IA (Iemura *et al.*, 1998). Follistatin also antagonizes all BMP related activities in *Xenopus* embryo (Iemura *et al.*, 1998). In mouse terato-carcinoma P19 cell line, BMP-4 inhibits follistatin induced neural differentiation of cells (Fainsod *et al.*, 1997). In rat fetal mandibular cells, follistatin inhibits BMP-2 induced osteoblast differentiation (Abe *et al.*, 2004). Similarly, follistatin inhibits dendrite-promoting activity of BMP-5 in cultured sympathetic neurons (Beck *et al.*, 2001). All these studies emphasize that follistatin may play an important role in regulation of BMPs related activities.

Follistatin has higher affinity for heterodimer of BMP-4 / BMP-7 than BMP-4 and BMP-7 homodimers (Iemura *et al.*, 1998). Studies have shown that binding of follistatin with BMP-4 is noncompetitive in nature, while other BMP antagonists (chordin or noggin) bind to BMPs in a competitive manner (Iemura *et al.*, 1998). This indicates that

follistatin has different underlying mechanism of antagonism for BMPs than other BMP antagonists. Interestingly, follistatin isoforms (FS-288 and FS-315) have different binding affinity to BMPs, FS-288 has greater affinity for BMP-4 than FS-315 (Yamamoto *et al.*, 2000). Recent studies have shown that FS-315 is a circulatory form of follistatin while FS-288 acts locally in different tissue compartments (ovarian follicle) because of its greater affinity for cell surface proteoglycans (Schneyer *et al.*, 2004). These studies suggest that follistatin isoforms have different regulatory roles for BMPs dependent activities in different organ system.

Follistatin inhibits the actions of BMP-5 in rat granulosa cell culture (Pierre *et al.*, 2005a), but has no effect on the action of BMP-2, BMP-4 and BMP-6 in ovine granulosa cell culture (Pierre *et al.*, 2005b). Differential display PCR studies have shown that recombinant BMP-2 increases the expression of activin βA and follistatin mRNA in mouse limb bud cell line (MLB13MYC clone 17) (Kearns & Demay, 2000). Presently, there is no published report regarding interplay of follistatin with BMPs in *in vivo* condition and further studies are required to understand the role of interaction of follistatin and BMPs in different reproductive processes.

Various proteins with follistatin domains in their structure are designated as members of follistatin family (Phillips & de Kretser, 1998). At present 14 proteins have been identified as members of the follistatin family including follistatin related protein (FSRP) (Schneyer *et al.*, 2001, Tsuchida *et al.*, 2000), TGF β stimulated clone (TSC-36) (Shibanuma *et al.*, 1993), SPARC (secreted protein acidic and rich in cysteine or osteonectin or BM-40) (Yan & Sage, 1999), agrin (Patthy & Nikolics, 1993) and matrix glycol protein SC1 (Tsuchida *et al.*, 2000). FSRP contains two follistatin domains instead of three follistatin domains and these domains act as growth factor binding motifs

(Tsuchida *et al.*, 2000). In northern blot analysis, mouse FSRP mRNA was localized in heart, lung, kidney and testis, while in humans, FSRP mRNA was detected in placenta, heart, aorta, testis, adrenal gland, lung and ovary (Tsuchida *et al.*, 2000). Like follistatin, FSRP also binds to activin A (KD, ~ 850 pm), but with a lower affinity than FS-288 (KD, ~ 47 pm) (Tsuchida *et al.*, 2000). In rat C6 glioma cells, overexpression of mFSRP cDNA inhibits BMP-2 induced signaling, whereas exogenous FSRP protein in rat astroglial cell line blocks activin-A induced and BMP-2 induced signaling (Tsuchida *et al.*, 2000). In a pull-down assay or in an ELISA binding assay, recombinant mouse FSRP binds to activin and BMP-2, but its binding affinity for BMP-2 is much lower than for activin A (Tsuchida *et al.*, 2000).

1.3.2. 2. Inhibin

The first evidence of the existence of inhibin came from the observation that irradiation of a testis leads to appearance of hypertropic 'castration cells' in the pituitary of rats (Mottram & Cramer, 1923). It was observed in following years that the administration of aqueous extract of bull testis caused the disappearance of the 'castration cells' in pituitary. The active constituent of watery extract was termed 'inhibin' (McCullagh, 1932). Despite all efforts inhibin was not isolated and purified until up to late 1970s. Then on the basis of inhibin ability to inhibit FSH secretion in anterior pituitary cell cultures, inhibin was fully or partially purified from human (Chari *et al.*, 1979), bovine (Robertson *et al.*, 1986, Robertson *et al.*, 1987, Sugino *et al.*, 1992), ovine (Dobos *et al.*, 1983) and porcine (Miyamoto *et al.*, 1985) follicular fluids. In knockout studies, inhibin deficient mice developed early sex cord tumors, showed severe wasting syndrome (similar to human cachexia syndrome of humans) and hepatocellular necrosis of liver (Table 1.1). These studies indicates that *in vivo* inhibin acts as a tumor suppressing factor (Matzuk *et al.*, 1992, Matzuk *et al.*, 1994).

Inhibin is a member of the TGF-β superfamily (Fig. 1.3) (Shimasaki *et al.*, 2004). It is a heterodimer glycoprotein and consists of an α subunit and a β subunit linked by disulphide bonds (Robertson *et al.*, 1986, Sugino *et al.*, 1992). Inhibin is endogenous antagonist of activin and binds to activin type II receptors in presence of betaglycan (Wiater & Vale, 2003). BMPs also use type II activin receptors for their signaling so it was hypothesized that BMPs and inhibin might compete for activin type II receptors and this may leads to inhibition of BMPs induced responses. In hepatocyte HepG2 cell line expressing a BMP-responsive promoter construct (BRE-Luc), BMP-2, BMP-7, BMP-9 and GDF-5 treatment stimulates expressions of BRE-Luc, whereas BRE-Luc expression is unaffected by treatment with activin-A or inhibin-A (Wiater & Vale, 2003). In contrast, co-treatment of inhibin and BMPs has no effect on BRE-Luc expressions; supporting the hypothesis that inhibin can antagonize BMP induced responses (Wiater & Vale, 2003). In HepG2 and mouse sertoli TM4 cell lines, BMP induced phosphorylation of smad-1 is inhibited by co-treatment with inhibin-A (Wiater & Vale, 2003). In addition, antagonism of inhibin to BMPs is betaglycan dependent as betaglycan transfection increased potency of inhibin-A blockade of BRC-Luc induction by BMP-7 in HepG2 cell lines (Wiater & Vale, 2003). In bovine granulosa cells, addition of BMP-2, BMP-4, BMP-6 and BMP-7 promotes accumulation of phosphorylated smad-1, but not smad-2, and enhanced basal and IGF stimulated secretion of estradiol (E2), inhibin-A, activin-A, and follistatin (Glister *et al.*, 2004). In human granulosa luteal cells, recombinant BMP-2 stimulates secretion of inhibin B in a dose dependent manner (Jaatinen *et al.*, 2002). These recent studies are able to shed new light on interaction of inhibin and BMPs and further studies are required to characterize the role of this interaction in different reproductive processes.

1.3.2. 3. Members of Dan / Cerberus family, noggin, chordin, and connective tissue growth factor (CTGF)

Differential screening-selected gene aberrative in neuroblastoma (Dan) is a cysteine knot family of glycoprotein's capable of antagonizing BMP functions. There are about seven members in this family including Dan, Cerberus, PRDC (Protein related to Dan and Cerberus), Gremlin, Dante, Caronte and Sclerostin (Hsu *et al.*, 1998, Minabe-Saegusa *et al.*, 1998, Pearce *et al.*, 1999, Piccolo *et al.*, 1996, van Bezooijen *et al.*, 2004). Cerberus is expressed in anterior endoderm and induces ectopic head formation in the *Xenopus* embryo (Silva *et al.*, 2003). Cerberus antagonizes activity of many growth factor including BMP, Nodal and Wnt in the *Xenopus* embryo (Piccolo *et al.*, 1999) and suppresses BMP-4 signaling in P-19 cells of mouse (Pearce *et al.*, 1999). Dan is a tumor suppressor glycoprotein belonging to Dan / Cerberus family and inhibits BMP-2 and BMP-4 signaling in animal cap assays in the *Xenopus* (Stanley *et al.*, 1998) and also inhibits GDF-5 action in the frog embryo assay (Dionne *et al.*, 2001).

Gremlin was cloned from the *Xenopus* ovarian library and has been shown to regulate limb patterning of mice by antagonizing BMPs (Bardot *et al.*, 2004, Gazzerro *et al.*, 2005, Hsu *et al.*, 1998, Khokha *et al.*, 2003). In lung organ culture, gremlin inhibits BMP-4 functions (Shi *et al.*, 2001) and antagonizes the effects of BMP-4 in mice granulosa cells (Pangas *et al.*, 2004). In mouse granulosa cells, BMP-4 and GDF-9 have been shown to increase expression of gremlin, but gremlin selectively inhibits the functions of BMP-4 (Pangas *et al.*, 2004). PRDC is another member of this family and was identified by gene trapping in embryonic stem cells (Minabe-Saegusa *et al.*, 1998). PRDC shares 65% homology with gremlin and like gremlin, it also inhibits BMP-2 and

BMP-4 signaling without having any effect on activin or any other member of the TGF-β superfamily (Sudo *et al.*, 2004). Co-precipitation assay studies have shown that direct interaction at protein level is required between PRDC and BMPs for antagonism of BMP activities, which is different from the mechanisms used by other BMPs antagonist (Sudo *et al.*, 2004). In rat granulosa cells, PRDC antagonize inhibitory effects of BMP-4 on FSH induced progesterone production (Sudo *et al.*, 2004). Interestingly, mRNA of PRDC has been detected in brain, ovary and spleen (Sudo *et al.*, 2004). In ovary, its expressions increase with gonadotrophin treatment (Sudo *et al.*, 2004).

Noggin is another BMP antagonistic protein and was identified as a factor involved in normal dorsal development in the *Xenopus* embryo (Smith & Harland, 1992). Recent studies have shown that it binds to BMP-2, BMP-4, BMP-7, BMP-14, GDF-5 and GDF-6 with varying degree of affinity (Aspenberg *et al.*, 2001, Zimmerman *et al.*, 1996). The examination of crystal structure of noggin-BMP-7 complex revealed that noggin inhibits BMP signaling by blocking the epitopes binding interface for type I and type II receptor of BMPs (Groppe *et al.*, 2002). Like Noggin, Chordin is another BMP antagonist protein. It is a 120 kDa protein, containing four small cysteine rich domains (CRs), which determine its biological activity including inhibition of BMP-4 activity in the *Xenopus* embryo (Larrain *et al.*, 2000). Chordin has higher affinity for BMP-2 and BMP-4 and lower affinity for BMP-7, but has no affinity for activin and TGF-β (Piccolo *et al.*, 1996). Chordin like protein is a protein of structural similarity with chordin and was isolated from mouse bone marrow stromal cells (Nakayama *et al.*, 2001). Like chordin, this protein has also been shown to bind and antagonize BMP-4, BMP-5 and BMP-6 activities in embryonic stem cells, but have no effect on the TGF-βs and activin actvity (Nakayama *et al.*, 2001). CTGF is another BMP binding protein and has affinity for BMP-4 and TGF-

β (Abreu *et al.*, 2002). It inhibits the biological activity of BMP-4 and enhances the activity of TGF-β (Abreu *et al.*, 2002). There are several other BMPs binding proteins, like Tsg, ventropin, showing varying degree of inhibition against BMPs in the *Xenopus* embryo (Ray & Wharton, 2001, Sakuta *et al.*, 2001) and further studies are required to signify their role in reproductive processes.

1. 4. Local Regulatory functions of BMPs in reproduction

1.4. 1. Pituitary

The development of anterior pituitary from oral ectoderm depends on various factors secreted by neural epithelium of ventral diencephalon such as BMP-4, Wnt5a (Wingless-type MMTV integration site family, member 5), fibroblast growth factor (FGF) 10 and FGF 8 (Cohen & Radovick, 2002, Rosenfeld *et al.*, 2000). During mouse pituitary development BMP-4 expression was found in the ventral diencephalon, while BMP-2 and chordin (BMP binding protein) were detected in the ventral condensing mesenchyme (Scully & Rosenfeld, 2002). In mice, BMP-4 gene deleted embryos fail to develop an ectodermal pouch placode, which is required for development of the Rathkes pouch (Takuma *et al.*, 1998). The Rathkes pouch is the future anterior and intermediate lobes of pituitary gland, while posterior or neural lobe of pituitary develops from a portion of neuron ectoderm or infundibulum (Dasen & Rosenfeld, 2001). In another study, Treier *et al.* used a transgenic mouse model created by linking regulatory sequence of the Ptx1 (a transcription factor known to express in oral ectoderm at very early stage of development) with the coding sequence of Noggin (antagonist of BMP-2, BMP-4 and BMP-7) to study role of BMPs in pituitary development. They reported that the Ptx-noggin transgenic mutant mice had arrested pituitary development after formation of a definitive pouch

(Treier *et al.*, 1998). These studies indicated that the BMP-4 plays a crucial role in pituitary organogenesis.

FSH is a key reproductive hormone involved in various gonadal functions like follicular growth, proliferation of granulosa cells, induction of aromatase in females and spermatogenesis in male (Fauser & Van Heusden, 1997, McLachlan *et al.*, 1995). Its secretion is regulated by various factors such as Gonadotrophin- releasing hormone (GnRH), FSH Releasing factor (FSH-RF) and various members of TGF β superfamily (Bilezikjian *et al.*, 2006, Fauser & Van Heusden, 1997). It is well established that inhibin and follistatin inhibit FSH secretion in pituitary cell culture, while activin stimulates secretion of FSH (Carroll *et al.*, 1989, Ling *et al.*, 1986a, b). Recent studies have indicated the role of various member of BMP family in regulation of gonadotrophins secretion. Northern blot analysis and PCR studies have shown mRNA of BMP-2, BMP-4, BMP-6. BMP-7 and BMP-15 in mouse and sheep pituitary (Faure *et al.*, 2005, Otsuka & Shimasaki, 2002, Souza *et al.*, 2003). In pituitary cell culture derived from transgenic mice carrying oFSHbetaLuc, BMP-6 and BMP-7 treatment increased oFSHbetaLuc expressions by 6 fold (Huang *et al.*, 2001b). In another experiment on transformed gonadotrope cell line, LβT2, BMP-6 or BMP-7 treatment caused 4-fold increase oFSHβLuc expression and 10 to 14 times increase in endogenous FSH production (Huang *et al.*, 2001b). Moreover, addition of bioneutralizing antibodies against BMP-7 (cross react with BMP-6) to the transgenic mouse pituitary cultures decreased oFSHβLuc expression and FSH secretion by 83-88 % and 47-48 %, respectively (Huang *et al.*, 2001b). In addition, treatment with bioneutralizing antibodies against BMP-7 of mouse, rat and sheep pituitary cell culture also resulted in inhibition of FSH synthesis (Huang *et al.*, 2001b). In a similar study, BMP-15 treatment has been shown to selectively stimulate transcription of FSH-β subunit in LbetaT2 gonadotrope cell line without affecting

expression of LH-β and GnRH receptors (Otsuka & Shimasaki, 2002). Furthermore, addition of BMP-15 to rat primary pituitary cell culture increased (~1.5 fold) secretion of FSH without affecting LH in dose dependent manner (Otsuka & Shimasaki, 2002). Interestingly, a recent study in sheep pituitary cell culture has shown that addition of BMP-4 and BMP-6 suppresses FSH production and expression of FSH-β mRNA in sheep pituitary cell cultures (Faure *et al.*, 2005). Additionally, BMP-4 has also been shown to antagonize and amplify the effect of activin and 17-β estradiol respectively, in sheep pituitary cell culture (Faure *et al.*, 2005). In folliculostellate cells (FS/D 1h), BMP-4 stimulates follistatin secretion (Bilezikjian *et al.*, 2006). In another study on rat and human pituitary prolactinomas, BMP-4 and 17-β estradiol have shown additive effect on cell proliferation (Paez-Pereda *et al.*, 2003). Furthermore, coimmunoprecipitation studies have detected physical interaction of BMP-4 stimulated smads (smad-1 and smad-4) with the estrogen receptor (Paez-Pereda *et al.*, 2003). Recently, it was demonstrated in pituitary derived LβT2 cell line that decrease in expression of smad-3 by using RNA interference caused abrogation of activin mediated stimulation of FSH-β transcription (Suszko *et al.*, 2005). Overall, these findings have shown presence of functional BMP system in pituitary and its role in regulation of FSH secretion in *in vitro* condition. Further studies are required to establish the physiological importance of BMP family members in regulation of pituitary functions.

1.4. 2. Ovary

The primary function of ovaries is to release a fertilizable egg in each estrous cycle and to secrete endocrine and paracrine hormones to prepare the accessory organs for pregnancy and parturition (Gougeon, 1996, McGee & Hsueh, 2000). Folliculogenesis is a continuous process of follicle development and it occurs even during the pregnancy

(Peters *et al.*, 1975). In recent years, several members of the TGF β superfamily including inhibin, activin and BMPs have been shown to regulate follicle development (Findlay, 1993, Findlay *et al.*, 2002). In rat ovary, *in situ* hybridization studies have detected BMP-2 mRNA in granulosa cells of atretic and graafian follicles, and BMP-4 mRNA in theca, corpus luteum, ovarian surface epithelium, stromal cells and endothelium of some blood vessels (Erickson & Shimasaki, 2003). In addition, BMP-6 mRNA is expressed in oocyte, granulosa cell, corpus luteum, blood vessels and ovarian surface epithelium, whereas BMP-7 mRNA is identified in the theca, corpus luteum and stromal cell of rat ovary (Erickson & Shimasaki, 2003). Moreover, expression of GDF-9 and BMP-15 are strictly limited to the oocyte of the follicles throughout follicular development (Aaltonen *et al.*, 1999, Erickson & Shimasaki, 2003, Juengel *et al.*, 2002, McGrath *et al.*, 1995).

In whole ovary culture system, BMP-4 increases the number of developing primary follicles and subsequently, decreasing the number of primordial follicles (Nilsson & Skinner, 2003). Furthermore, anti-BMP-4 antibodies decreased the size of ovaries associated with loss of oocytes, primordial follicles and ovarian tissue morphology and cellular apoptosis (Nilsson & Skinner, 2003). Similarly, addition of BMP-7 (100ng/ml) to the neonatal mouse culture increases the transition of primordial follicle to primary follicle (Lee *et al.*, 2004). In another study, injection of BMP-7 (1μg in a 20 μl heparin vehicle) in ovarian bursa of the female rat significantly decreased the number of primordial follicles and increased the number of primary, pre-antral and antral follicles suggesting that BMP-7 can act as facilitating agent in different stages of follicular development (Lee *et al.*, 2001). In RNase protection assay, BMP-7 upregulates P450 aromatase and downregulates STAR expression induced by FSH (Lee *et al.*, 2001). In rat granulosa cells, BMP-7 increases DNA synthesis and granulosa cell proliferation (Lee *et al.*, 2001). In another study, injection of GDF-9 (10 μg ip) in immature rats for 7-10 days

increased the weight of ovaries. In addition, numbers of primary and small preantral follicles increased by 30% and 60 %, respectively. However, number of primordial follicles decreased by 29% (Vitt *et al.*, 2000). Shimizu *et al.* conducted a similar study in prepubertal gilts and reported that injection of GDF-9 gene fragments in the ovaries of 2-month prepubertal gilts increases the number of primary, secondary and tertiary follicles and simultaneously, decreases the number of primordial follicles (Shimizu *et al.*, 2004).

The smad proteins are the family of proteins involved in downstream signaling of members of the TGFβ superfamily including BMPs (Derynck & Zhang, 2003, Massague, 1998, Massague & Chen, 2000). The smad-2 and smad-3 expressions are detected in unfertilized human oocyte and in 4-cell, 8 cell and blastocyst stage of embryo development (Osterlund & Fried, 2000). The expressions of smad-2, smad-3, smad-4 and smad-6 are also detected in oocyte, granulosa cells, luteal cells and theca interna in mouse ovary (Gueripel *et al.*, 2004), indicating that these cells can respond to BMP signaling. In smad-3 deficient mice, ovarian surface epithelium (OSE) was thick and with plump cuboidal cells, while wild type mice OSE was flat with thin cells (Symonds *et al.*, 2003). In addition, smad-3 deficient mice had less estradiol than the wild type mice, but there was no difference in progesterone concentration (Symonds *et al.*, 2003). Smad-3 deficient mice also had reduced fertility as compared to wild type mice (Tomic *et al.*, 2002). Smad-3 deficient animals had slow follicle growth indicated by small follicle diameter and low level of proliferating cell nuclear antigen (PCNA) and cell cycle genes (cyclin dependent kinase 4 and cyclin D2) expressions (Tomic *et al.*, 2004). Moreover, smad-3 deficient mice follicles had increased expression of ERα, and decreased expression of ERβ and inhibin α (Tomic *et al.*, 2004). In addition, smad-3 deficient mice were also shown to have higher FSH and lower estradiol than wild type mice (Tomic *et al.*, 2004). There were more atretic follicles and degenerated oocytes, and lower expression of bcl-2 in the smad-

3 deficient mice than in the wild type mice (Tomic *et al.*, 2004). Additionally, Smad-3 deficient ovaries were without corpus luteum and there was no ovulation even after exogenous treatment with gonadotrophins (Tomic *et al.*, 2004). These studies indicated that BMP signaling plays an important role in regulation of ovarian functions.

1.4. 3. Granulosa cells

The granulosa cells originate from the mesonephric cells during embryogenesis and their number increases with follicular growth by mitosis in response to estradiol and various intra-ovarian growth factors (Erickson *et al.*, 1985). According to the "two cell, two gonadotrophins" theory, aromatizable androgens are produce by theca interna cells (TICs) in presence of LH and these androgens are then transported to granulosa cells and converted to estrogens by aromatizing enzymes under the influence of FSH (Fig. 1.7) (Hillier *et al.*, 1994). The undifferentiated thecal cells are incapable of producing androgen even in presence of LH and with follicular development, TICs respond to LH with activation of several enzymes such as cholesterol side chain cleavage (P450scc), 17α-hydroxylase and 3β hydroxysteroid dehydrogenase (3βHSD) (Erickson *et al.*, 1985). During folliculogenesis granulosa cells are incapable of estradiol until theca cell differentiate in to androgen producing cells (Erickson *et al.*, 1985). In granulosa cells, 17β-hydroxysteroid dehydrogenase (17βHSD) transforms androstenedione (A) into testosterone (T) and aromatase (P450arom) transforms T to estradiol (Erickson *et al.*, 1985, Hillier *et al.*, 1994). Before the human mid-cycle gonadotrophin surge granulosa cells are estradiol producing cells and after gonadotrophin surge, granulosa cells predominantly secrete progestin (Erickson *et al.*, 1985, Hillier *et al.*, 1994). In granulosa cells, both FSH and LH enhance production of progesterone by increasing uptake of lipoproteins, liberating cholesterol from lipoproteins, mobilizing and converting

cholesterol into pregnenolone and then converting pregnenolone to progesterone by activating 3βHSD (Hsueh *et al.*, 1984).

In sheep ovaries, expression of BMPR-IA, BMPR-IB and BMPR-II has been detected in the granulosa cells of the primary follicle to the late antral stage follicles, ovarian surface epithelium and corpus luteum (Souza *et al.*, 2002). In rat ovaries, BMP-5 has been shown to express in granulosa cells of antral follicle (Pierre *et al.*, 2005a). In addition, expression of BMP-2, BMP-4, BMP-6, BMP-7 has been detected in granulosa and thecal cells of chicken ovary (Onagbesan *et al.*, 2003). Interestingly, expression of these proteins in chicken ovaries is different from rat ovaries (Shimasaki *et al.*, 1999) and bovine ovaries (Fatehi *et al.*, 2005), indicating that BMPs might have different roles during follicular development in different species of animals.

In ovine granulosa cells, inhibitory effects of the BMP/ TGF-β factors on progesterone production decreases with follicular growth as inhibitory effects were greater on granulosa cells collected from the small antral follicles than those from preovulatory follicles (Fabre *et al.*, 2003). The granulosa cells from homozygous carrier (BB) ewes ovaries were 3 to 4 fold times less responsive to GDF-5 and BMP-4 than from heterozygous carrier (B+) ewes (Fabre *et al.*, 2003). In contrast, TGFβ1 and activin showed similar effects on the progesterone production regardless of the source of granulosa cells, suggesting that the alteration in BMPR-IB is affecting steroidogenesis in Booroola follicles as both BMP-4 and GDF-5 use BMPR-IB for their signaling. In rat granulosa cell culture, BMP-4 and BMP-7 stimulated FSH induced estradiol and inhibited FSH induced progesterone production, but showed no effect on basal secretion of these hormones (Shimasaki *et al.*, 1999). However, in sheep granulosa cell culture, BMP-4 inhibits both basal and FSH induced production of progesterone (Mulsant *et al.*, 2001). In

bovine granulosa cells, BMP-4, BMP-6 and BMP-7 treatment have been shown to increase both basal and FSH induced production of estradiol, inhibin-A, activin-A and follistatin and inhibited both basal and IGF stimulated progesterone production (Glister *et al.*, 2004). In a similar study, BMP-2 has been shown to increase the production of estradiol and inhibin-A production in sheep granulosa cells (1-3mm follicle) (Souza *et al.*, 2002). In contrast, BMP-4 and BMP-7 stimulated basal, IGF and gonadotrophins induced progesterone production in chicken granulosa cells (Onagbesan *et al.*, 2003). In bovine granulosa cells, BMP-4, BMP-6 and BMP-7 increased cellular accumulation of smad-1 and caused up-regulation of cytochrome P450 aromatase enzyme (P450arom) activity (Glister *et al.*, 2004). In addition, BMP-6 and BMP-7 also increased viable cell number in bovine granulosa cell culture (Glister *et al.*, 2004). In a similar study, BMP-7 increased DNA synthesis and proliferation of rat granulosa cells (Lee *et al.*, 2001). Furthermore, addition of BMP-7 in the rat granulosa cell culture had no effect on the basal expression of mRNA's of P450 aromatase, STAR, P450scc and 3β-HSD, but decreased STAR and increased P450 aromatase expression in presence of FSH (Lee *et al.*, 2001). These findings suggests that effects of BMP-7 on estradiol and progesterone production might be because of its effects on P450 aromatase and STAR as P450 aromatase helps in conversion of androstenedione to estradiol and STAR helps in transport of cholesterol across mitochondrial membrane (Lee *et al.*, 2001). Similarly, BMP-4 increases the activity of smad-1, which leads to the inhibition of steroidogenic factor-1 dependent expression of STAR and P450scc, which ultimately decreases the progesterone production in ovine granulosa cells (Pierre *et al.*, 2004). In rat granulosa cell culture, BMP-5 inhibits both basal and FSH induced production of progesterone without affecting estradiol production (Pierre *et al.*, 2005a). The effect of BMP-5 on progesterone production has been correlated with decreased expression of STAR and CYP11A1 (Pierre

et al., 2005a). In addition, BMP-5 also stimulated proliferation of granulosa cells with increased expression of cyclin D2 (Pierre *et al.*, 2005a). The cyclins (D1, D2, D3) are a group of proteins involved in cell proliferation and differentiation (Mermelshtein *et al.*, 2005). Cyclin D2 knockout females were infertile owing to inability of granulosa cells to proliferate in the presence of FSH and their male counterparts had hypoplastic testes (Sicinski *et al.*, 1996).

In rat granulosa cell culture, BMP-15 increased proliferation and differentiation of granulosa cells, independent of FSH stimulation (Otsuka *et al.*, 2000). In addition, BMP-15 decreases FSH-induced production of progesterone without affecting estradiol production (Otsuka *et al.*, 2000). Moreover, BMP-15 also decreases FSH induced expression of STAR, P450scc, 3β-HSD, LH receptor (LH-R), inhibin/activin subunits and FSH receptor (FSH-R) without affecting forskolin stimulated expression of these factors (Otsuka *et al.*, 2001b), suggesting that BMP-15 exerts its inhibitory effect on FSH-induced expressions through up-regulation of cAMP signaling. In rat granulosa cell culture, BMP-15 decreases both basal and FSH-induced expression of FSH-R (Otsuka *et al.*, 2001b), indicating that BMP-15 is an important factor in regulation of FSH by acting on FSH-R or even by affecting different factors such as inhibin, activin and follistatin (Otsuka *et al.*, 2001b). In another study, follistatin has been shown to inhibit proliferation of rat granulosa cells in response to BMP-15 and it also antagonizes the suppression of mRNA expression of FSH-R by BMP-15, which indirectly affects FSH-induced progesterone production (Otsuka *et al.*, 2001c). GDF-9 is another member of BMP family and has been shown to co-express with BMP-15 in oocyte (Aaltonen *et al.*, 1999). Recombinant GDF-9 has been shown to increases the synthesis of hyaluronan synthase 2 (HAS2), cyclooxygenase 2 (COX-2), and STAR mRNA and suppresses urokinase plasminogen activator (uPA) and LH-R mRNA synthesis in mouse granulosa cell cultures

(Elvin *et al.*, 1999). In addition, GDF-9 also increases granulosa cell progesterone synthesis in the absence of FSH (Elvin *et al.*, 1999). The induction in the activity of HAS2 and suppression of the uPA activity are important steps required for formation of hyaluronic acid rich extracellular matrix for cumulus expansion in cumulus oocyte complex (Elvin *et al.*, 1999). In rat granulosa cells, GDF-9 showed dose dependent increase in production of inhibin-A and inhibin-B and these effects were synergistically increased with addition of FSH (Roh *et al.*, 2003).

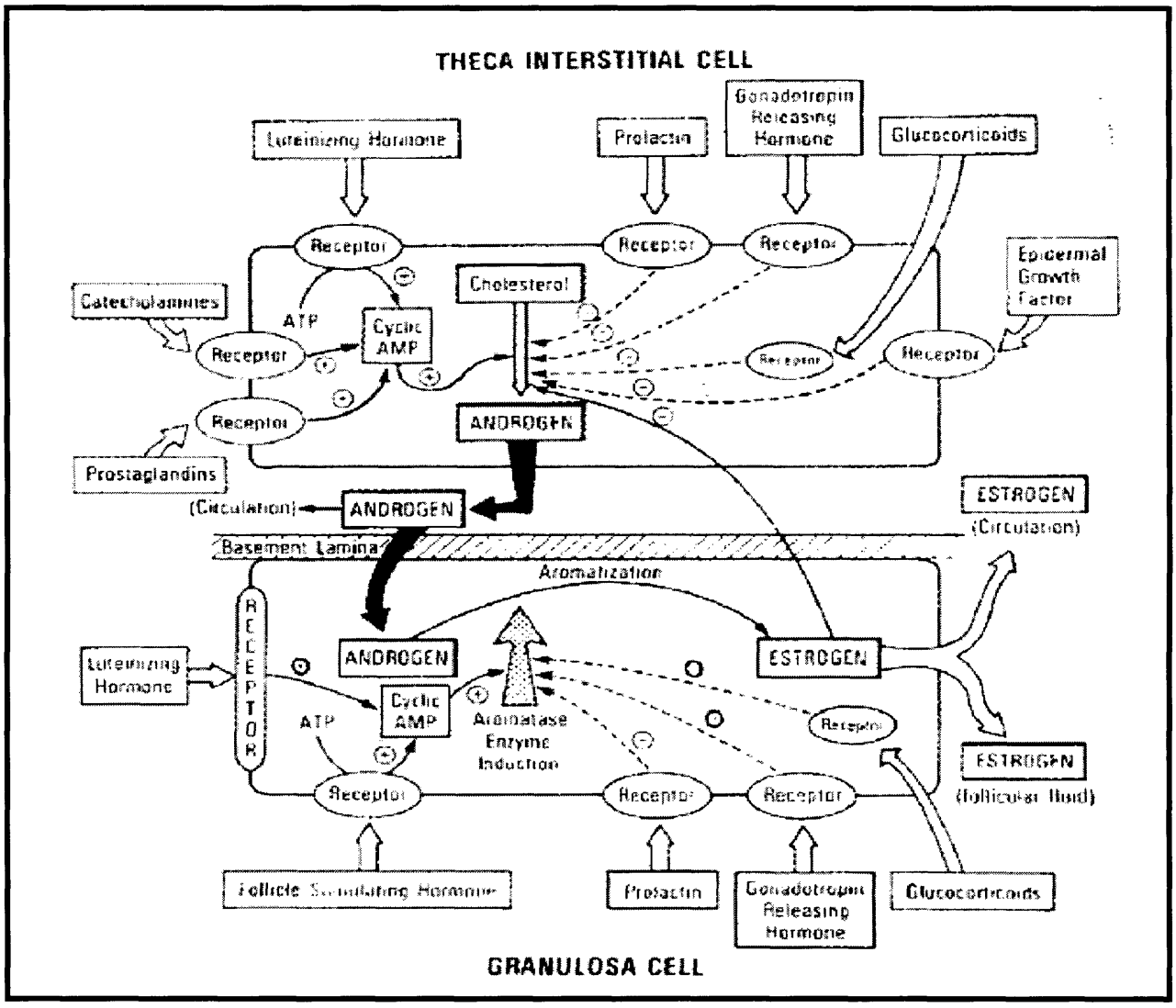

Figure 1. 7. A model of the two-cell, two gonadotrophin theory of follicular estrogen biosynthesis. The diagram combines known events taking place in the granulosa and theca-interstitial cells: (+) stimulatory; (-). Reproduced from (Erickson *et al.*, 1985).

1.4. 4. Thecal cells

At the secondary stage of follicular development, some stromal cells near the basal lamina become aligned to each other and form the thecal layer. With follicular development, theca cells differentiate into two parts. The outer part, the theca externa, similar to undifferentiated thecal cells and inner part, the theca interna, appeared as steroid secreting cells also known as epitheliod cells (Gougeon, 1996). *In situ* hybridization studies have shown expression of BMP-4 and BMP-7 mRNA in the thecal cells of rat ovary (Erickson & Shimasaki, 2003). In bovine ovary, BMP-4 protein is localized in theca interna and oocyte (Fatehi *et al.*, 2005). The presence of BMP-4 and BMP-7 in thecal cells indicated that these proteins might be involved in follicular steroidogenesis as estradiol synthesis required coordination of thecal and granulosa cells (as shown in figure 1.7). In HOTT cells, BMP-4 decreased the production of androgen by modulating CYP 17 and 3β HSD ratio, which ultimately leads to increase in production of progesterone and decrease in the production of androstenedione (Dooley *et al.*, 2000). In this study, HOTT cells were used because of easy availability and similar properties to the normal human thecal cells such as production of C19 steroids. In a similar study in bovine thecal cells, BMP-4, BMP-6 and BMP-7 decreased both basal and LH induced androgen production without having any significant effect on progesterone production (Glister *et al.*, 2005). In addition, all three BMPs significantly decreased expression of P450c17, P450scc, STAR and 3β HSD (Glister *et al.*, 2005). These finding are different from previous studies in regards to progesterone production and suppression of different enzymatic activities but, as both studies used different cell lines, this might be a contributing factor for these differences.

1.4. 5. Primordial germ cell (PGC)

PGCs are extragonadal in origin and sole source of adult germ cells. Basically, PGCs are responsible for the transfer of genetic information from one generation to the next generation (Wylie, 1999). The blastocyst stage of pre-implantation mouse embryo consists of an inner cell mass and trophoectoderm. After implantation, the inner cell mass gives rise to epiblast and extraembryonic endoderm, whereas trophoectoderm forms extraembryonic ectoderm (Okamura *et al.*, 2005). The epiblast further differentiates into embryonic germ layers and PGCs, while after gastrulation, extraembryonic endoderm develops as a yolk sac and extraembryonic ectoderm forms the placenta (Okamura *et al.*, 2005). The PGCs were first recognized in the mouse at E7.25 as a cluster of alkaline phosphatase positive cells (Ginsburg *et al.*, 1990). During embryogenesis, PGCs migrate through extraembryonic tissue of yolk sac and allantois (E8-E9), hindgut epithelium (E9-E10), dorsal mesentery of gut (E10-E12) and finally into developing gonads, either testis or ovary (E12-E13) (Wassarman & Albertini, 1994).

In cell lineage analysis, PGC precursors were identified in epiblast close to the extraembryonic ectoderm in pre-gastrulation as well as in early-streak stage of embryo (Lawson & Hage, 1994). In *in vitro* condition, epiblast isolated from E5.5 embryo do not form PGCs, but PGCs emerged from same epiblast when co-cultured with extraembryonic ectoderm indicating that some factors produced by extraembryonic ectoderm are responsible for inducing conditions required for formation of PGCs (Yoshimizu *et al.*, 2001). Knockout studies in mice have shown that BMP-4 is that elusive factor secreted by extraembryonic ectoderm, which is required for PGCs formation and for normal development of epiblast (Fujiwara *et al.*, 2001, Lawson *et al.*, 1999). BMP-4 gene deleted

homozygous embryos were without PGCs and heterozygous embryos had few PGCs, suggesting that BMP-4 plays a major role in PGCs formation (Lawson *et al.*, 1999).

BMP-2 and BMP-8b are found to express in extraembryonic ectoderm and visceral endoderm, respectively (Ying *et al.*, 2000). BMP-8b homozygous null embryos are deficient in PGCs and heterozygous embryos have fewer PGCs (Ying *et al.*, 2000). The phenotypic characters of BMP-8b null embryos are similar to BMP-4 deleted embryos indicating that BMP-4 and BMP-8b might have synergistic action on PGCs formation. BMP-4/ -8b double mutant mice are PGCs deficient, whereas heterozygote's for BMP-4 and -8b have few PGCs, which is similar to the phenotype of BMP-4 null embryos (Ying *et al.*, 2000). These findings have suggested that the effects of these two BMPs on PGCs formation are not additive and they act as a heterodimer or homodimer in PGCs formation (Lawson *et al.*, 1999, Ying *et al.*, 2000). Moreover, individually both, BMP-4 and BMP-8b, homodimers are unable to induce PGC formation and combination of both homodimers are able to induce PGC formation in epiblast culture (E6.0-E6.5) (Ying *et al.*, 2001). Interestingly, PGCs defect in BMP-8b mutant is easily overcome by BMP-8b homodimer, whereas BMP-4 homodimer is unable to overcome PGC defect in BMP-4 mutant embryos (Ying *et al.*, 2001). These findings strongly suggest that BMP-4 is required at multi steps of primordial germ fate determination whereas BMP-8b is mainly required at later stages. Overall, these studies have shown that BMP-4 protein is the prime factor required by epiblast cells from extraembryonic ectoderm to gain germ cell competency before synergistic action of BMP-4 and BMP-8b (Ying *et al.*, 2001).

BMP-2 is expressed in visceral endoderm at pregastrula and gastrula stage of mouse embryo (Ying & Zhao, 2001). BMP-2 gene deleted embryos have been shown to have shortened allantois and fewer PGCs (Ying & Zhao, 2001), suggesting that BMP-2 is

also required for PGCs formation. Furthermore, heterozygous double mutants of BMP-2 and BMP-4 have fewer PGCs than the embryos heterozygous for either BMP-2 or BMP-4, indicating that BMP-2 and BMP-4 have additive effect on PGCs formation (Ying & Zhao, 2001). Comparative studies of these double mutant embryos have shown that effects of BMP-4 and BMP-8b or BMP-2 and BMP-8b mutants on PGCs formation are not additive (Ying & Zhao, 2001). In another study, Hayashi *et al.* created smad-1 knockout mice by inserting lacZ gene into the exon of the smad-1 gene and reported that smad-1 homozygous mutant embryos contained no PGCs and have short allantois (Hayashi *et al.*, 2002). In a similar study, smad-5 homozygous mutants embryos were without PGCs (Chang & Matzuk, 2001). Recently, ALK-2 deficient mice embryos are also PGCs deficient (de Sousa Lopes *et al.*, 2004). These studies further indicate that the BMPs are important determinant of PGC formation as ALK-2, smad-1 and smad-5 are involved in downstream BMP signaling.

1.4. 6. Oocyte and oocyte derived factors

Folliculogenesis is the process of development of follicles, which includes growth and maturation of the follicles, their enlargement, ovulation, atresia and meiotic division of egg (Peters *et al.*, 1975). The primary goal of folliculogenesis is selection of an ovarian follicle capable of releasing an oocyte, which can be fertilized by sperm (Ireland, 1987, Richards, 1980). The word 'oogenesis' represents a biological process of development of fully mature unfertilized eggs from primordial germ cells. The ovary of adult mammals contains a pool of non growing and growing oocytes arrested in dictyate stage of first meiotic prophase and during each estrous cycle fully grown oocytes resume meiosis and ovulate (Wassarman & Albertini, 1994). The recruitment of an oocyte to the pool of growing oocytes seems to be under control of pituitary gonadotrophins, but recruitment also occurs in hypophysectomized animals, indicating that it is not totally under control of

gonadotrophins (Wassarman & Albertini, 1994). The meiotic maturation of an oocyte refers to conversion of fully grown oocyte, which is usually present in antral follicle, to an unfertilized egg just prior to ovulation following preovulatory surge of gonadotrophins (Wassarman & Albertini, 1994). In *in vitro*, mammalian oocyte has the ability to go into meiotic maturation spontaneously when released from the follicle. Some agents, such as dibutyryl cyclic adenosine mono-phosphate (dbcAMP), which increases the levels of cyclic adenosine mono-phosphate (cAMP), can easily block meiotic maturation of the oocyte in *in vitro* conditions (Schultz *et al.*, 1983). cAMP is an important regulator of meiotic maturation of oocyte, as decrease in intracellular cAMP concentration signals meiotic progression as well as intracellular level of cAMP goes down in the oocyte just before nuclear envelope (GV) breakdown, whereas cAMP level of follicle and cumulus cells increases during the meiotic maturation (Schultz *et al.*, 1983). The spontaneous maturation of mouse oocyte does not occur in culture medium in presence of membrane permeable analogs of cAMP *viz.* dbcAMP and 8-bromo-cAMP or inhibitor of nucleotide phosphodiesterase (PDE) *viz.* methylxanthine (IBMX) and theophylline (Cho *et al.*, 1974, Dekel & Beers, 1978). Similarly, some other agents such as forskolin, inhibit maturation of oocyte by increasing cAMP level by activating adenylate cyclase activity (Urner *et al.*, 1983). The treatment of cumulus oocyte complex with FSH results in increase in level of cAMP in cumulus cells, which leads to inhibition of germinal vesicle breakdown or oocyte maturation (Eppig *et al.*, 1983, Freter & Schultz, 1984). The various steroid hormones, which increase FSH concentration, inhibit the maturation of oocyte in cumulus oocyte complex (Eppig *et al.*, 1983, Freter & Schultz, 1984).

The process of folliculogenesis is controlled by interaction of pituitary gonadotrophins and various autocrine and paracrine factors such as members of the TGF-β superfamily (Findlay *et al.*, 2002). Several members of the TGF-β superfamily are

involved in follicle development and their expressions are detected in different follicle cells (Erickson & Shimasaki, 2003). BMP-6, BMP-15 and GDF-9 are expressed in oocyte and also known as oocyte derived factors (Aaltonen *et al.*, 1999, Erickson & Shimasaki, 2003). In granulosa cell culture, BMP-15 and GDF-9 inhibit FSH-induced progesterone production, expression of P450scc and LH-R mRNA and increase estradiol production and granulosa cell mitosis (Goldschmit *et al.*, 1989, Vanderhyden & Tonary, 1995). Similarly, BMP-6 decreases FSH-induced progesterone production, but has no affect on estradiol production and mitogenic activity of granulosa cells (Otsuka *et al.*, 2001a). In addition, BMP-6 decreases FSH and forskolin-stimulated cAMP production by down regulating adenylate cyclase activity, whereas BMP-15 regulates steroidogenesis by suppressing FSH receptors (Otsuka *et al.*, 2001a, Otsuka *et al.*, 2001b). It was observed in comparative studies of granulosa cell functions in *in vitro* and *in vivo* conditions that granulosa cells of follicles did not respond to FSH to induce progesterone production in *in vivo* conditions, but when these same granulosa cells were cultured *in vitro*, they produce both progesterone and estradiol in response to FSH (Wassarman & Albertini, 1994). In rabbit, *in situ* removal of oocyte from dominant follicle causes luteinization of follicular cells and leads to production of large amount of progesterone (el-Fouly *et al.*, 1970). Similarly, removal of oocyte from antral follicles culture caused luteinization of granulosa cells and granulosa cells cultured in proximity of oocyte appeared to maintain their non luteinized cell appearance (Nekola & Nalbandov, 1971). Moreover, the removal of full grown oocyte from antral follicle leads to the gonadotrophin independent meiotic maturation of the oocyte (Pincus & Enzmann, 1935, Pincus & Shapiro, 1940). These studies have indicated that oocyte secretes some inhibitory factor to affect steroidogenesis of the granulosa cells or to block the luteinization of follicular cells and follicular somatic cells also secrete some factor to maintain meiotic arrest of oocyte. Recently, several

laboratories have suggested that GDF-9 is that long sought elusive factor secreted by oocyte and is responsible for the interaction of oocyte and granulosa cells (Elvin *et al.*, 1999, Eppig, 2001, Gilchrist *et al.*, 2004, Vanderhyden *et al.*, 2003). In oocytectomized cumulus cell-oocyte complex, GDF-9 induces cumulus expansion in absence of oocyte (Elvin *et al.*, 1999). Similarly, GDF-9 inhibits progesterone production from cumulus cells (Vanderhyden *et al.*, 2003). In oocyte secreted mitogen assay, GDF-9 has been shown to be partially responsible for the bioactivity of oocyte, which raises the possibility of involvement of some unknown factor with GDF-9 for bioactivity of oocyte (Gilchrist *et al.*, 2004). However, GDF-9 knockdown oocytes are unable to exhibit cumulus expansion (Gui & Joyce, 2005), further supporting the role of GDF-9 as a key mediator of cumulus expansion in mice.

As mentioned previously GDF-9 and BMP-15 are co-expressed in oocyte of most of the animals studied so far (Bodensteiner *et al.*, 1999, Carabatsos *et al.*, 1998, Juengel *et al.*, 2004, Shimizu *et al.*, 2004, Wang & Roy, 2004). Interestingly, GDF-9 expression is also present in mural and cumulus granulosa cells of the primates, compared to other species in which GDF-9 expression is limited to the oocyte (Duffy, 2003). During follicular development, GDF-9 expression is detected in oocyte of primary and large follicle in mice (Dong *et al.*, 1996, Elvin *et al.*, 1999), rat (Hayashi *et al.*, 1999, Jaatinen *et al.*, 1999), pig (Shimizu *et al.*, 2004) and humans (Aaltonen *et al.*, 1999), whereas in ovine and bovine ovaries, GDF-9 expression is also present in oocyte of primordial follicles (Bodensteiner *et al.*, 1999). These differences in expression of GDF-9 in different species suggest that either GDF-9 plays different role in different species or GDF-9 expression in primordial follicle is too low to be detected except in ovine and bovine ovary. GDF-9 deleted mice have arrested follicular development at primary follicular stage (Dong *et al.*, 1996). Interestingly, BMP-15 knockout mice are fertile except some

females are sub-fertile showing decreased ovulation and impaired fertilization (Yan *et al.*, 2001). Furthermore, the double homozygous mutants of BMP15 -/- GDF-9-/- are similar to GDF-9 mutants and BMP-15 -/- GDF+/- heterozygous mutants have more severe defects in fertility as compared to other mutants, indicating a synergistic role of BMP-15 and GDF-9 during normal follicular development (Yan *et al.*, 2001).

1.4. 7. Sheep breeds with a high ovulation rate

In sheep different breeds or sometime within same breed, have different ovulation rates and litter size. Ovulation rate of breeds such as Romanov and Finnish-Landrace, Booroola merino, Javanese, Olkuska, Belclare, Cambridge, Inverdale, Woodlands and Lacaune is genetically regulated by different genes (Table 1.5) (Souza *et al.*, 2004). The Booroola merino is one of the most prolific breeds of the world. The first experimental flock of these sheep was acquired from the property called 'Booroola', at Cooma, New South Wales, which lead to their name as Booroola sheep (Bindon, 1984). Booroola ewe's have a few distinguishing characteristics from Merino ewe's such as high FSH level very early in life (Bindon *et al.*, 1985, Isaacs *et al.*, 1995) and during the estrous cycle (Robertson *et al.*, 1984, Xia *et al.*, 2003), small size of ovulating follicles with fewer granulosa cells, lower inhibin concentration in ovaries in comparison to merinos (Cummins *et al.*, 1983). The ram of both merino and Booroola have almost similar characteristics (Bindon, 1984, Hochereau-de Reviers *et al.*, 1997). When hypophysectomized Booroola ewes with or without fecundity gene (carrier and non-carrier), were injected with equal doses of PMSG (pregnant mare serum gonadotrophin) and hCG (human chorionic gonadotrophin), the mean ovulation rate was higher in carriers than non-carriers (Fry *et al.*, 1988). Similarly, no difference was observed in half life of FSH in both carrier and noncarrier ewes (Fry *et al.*, 1987). Furthermore, when ewes

(carriers and noncarrier) with ovarian auto-transplants (ewes with ovarian auto-transplants were used to assist in sample collection, to directly inject FSH into blood supply of ovaries and for ultrasonographic examination of ovaries) were treated with similar regimen of gonadotrophin treatment, follicular population and number of corpus luteum formed showed the same genetic difference (Campbell *et al.*, 2003). Interestingly, the concentrations of estradiol, androstenedione and inhibin-A were same in both of the treatment groups (Campbell *et al.*, 2003). In follicular population, noncarrier ewes had small (<3.5mm), medium (3.5-4.5mm) and large follicles (>4.5), whereas carrier ewes did not have any follicle larger than 4.5mm (Campbell *et al.*, 2003). In contrast, numbers of corpus luteum formed were much higher in carrier than noncarrier ewes (Campbell *et al.*, 2003). These studies indicated that effect of fecundity gene is more local / ovarian than central / on hypothalamus-pituitary axis. In contrast to this hypothesis, several laboratories reported that the differences in prolificacy of carrier and noncarrier ewes are because of differences in concentration of FSH (Bindon, 1984, Robertson *et al.*, 1984, Xia *et al.*, 2003). To test this hypothesis, Hudson *et al.* studied the effect of infusion of varying concentrations of FSH on hypothalamic pituitary disconnected ovary intact and GnRH agonist (Deslorelin) treated ewes and reported that ovulation rate in both carrier and noncarrier ewes increases with increase in FSH concentration (Hudson *et al.*, 1999). This study demonstrated that the FecB gene acts at both hypothalamic pituitary axis and ovary to stimulate ovulation rate in carrier ewe. Despite of all these studies to understand reason of higher prolificacy in Booroola ewe, none were able to provide the exact physiological mechanism of action of the FecB gene in Booroola ewes and further studies are required to elucidate the physiological mechanism of action of FecB gene.

In 1982, Piper and Bindon proposed that the exceptional fecundity of the Booroola sheep in part results from the action of a single major gene (Piper & Bindon, 1982). It was

found in segregation studies that Booroola phenotype is because of mutation in the autosomal gene named FecB (Davis & Kelly, 1982, Piper *et al.*, 1985). Recently, three laboratories independently reported that the Booroola phenotype is because of mutation (Q249R) in the highly conserved intracellular kinase signaling domain of BMPR-IB, which is a specific receptor for BMP-4 and GDF-5 (Fig. 1.3 and Table 1.5) (Mulsant *et al.*, 2001, Souza *et al.*, 2001, Wilson *et al.*, 2001). The kinase domain of BMPR-IB had two point mutations, one at base 746 which leads to change of glutamine in noncarrier to arginine in carrier ewes, while another mutation is at 1113 with no affect on amino acid sequence (Mulsant *et al.*, 2001, Souza *et al.*, 2001, Wilson *et al.*, 2001). Genetic analytical studies have revealed that FecB gene locus is on the 6q 23-31 ovine chromosome corresponding to human chromosome 4q22-23 (Mulsant *et al.*, 2001, Wilson *et al.*, 2001). Recently, DNA mutation tests have revealed the Booroola mutation is also found in Garole sheep of India and Javanese sheep of Indonesia (Table 1.5) (Davis *et al.*, 2002). In another study, Inverdale sheep has been shown to carry a X-linked mutation (FecXI) and the heterozygote's of this mutation (FecXI/Fec+) have higher ovulation rate, whereas the homozygote's (FecXI/FecXI) are infertile and have impaired follicular development after the primary follicular stage (Galloway *et al.*, 2000). The Hanna (FecXH) sheep, another unrelated strain of sheep, has a similar mutation (Galloway *et al.*, 2000). On the basis of homology with syntenic human chromosome region, mutation was located on coding region for BMP-15 (Galloway *et al.*, 2000). Moreover, further analysis of these mutations have shown that FecXI mutations leads to T to A transition at nucleotide position 92 results in the substitution of valine with aspartic acid in mature peptide of BMP-15 (Galloway *et al.*, 2000). In addition, in FecXH, C to T transition at nucleotide 67 of coding region of mature peptide leads to introduction of premature stop codon in its nucleotide (Galloway *et al.*, 2000). Hanrahan *et al.* reported that the mutation in GDF9

and BMP-15 is responsible for higher ovulation rate and sterility in Cambridge and Belclare sheep (Hanrahan *et al.*, 2004). The animals heterozygous for the mutation had higher ovulation rate and homozygous animals were sterile (Hanrahan *et al.*, 2004). Additionally, active immunization against either BMP-15 or GDF-9 leads to disrupted estrous cycle and anovulation in ewes (Juengel *et al.*, 2002). Histological examination of their ovaries revealed that folliculogenesis was stopped at the primary stage of follicular development (Juengel *et al.*, 2002). Similarly, passive immunization against GDF-9 at start of luteal regression resulted in fewer number of corpus luteum formed and disrupted luteal phase pattern of progesterone secretion (Juengel *et al.*, 2002). Furthermore, passive immunization against BMP-15 caused anovulation in a majority of the animals (Juengel *et al.*, 2002).

Although genetic mutations are responsible for higher ovulation rate in different breeds of sheep, there are no reports of ill affects of these mutations on physiological functions of sheep. Some laboratories have reported that the fetal development in Booroola ewes have some altered phenotype such as delay in development, lower body weight of fetus for gestation age, light ovaries and adrenal glands in females (day 95), retarded development of heart (day 28) and mesonephros (day 30-40), lesser number of oogonia (day 30-40), primordial follicle(days 75-90) and growing follicle(day 120 to birth) in ovaries, but none of these defects persist up to the postnatal stage except differences (higher ovulation rate) in ovaries (Bindon, 1984, McNatty *et al.*, 1995, Smith *et al.*, 1993, Souza *et al.*, 2003). However, male fetuses showed no difference in testicular growth and in total somatic cell contents (Hochereau-de Reviers *et al.*, 1997, Smith *et al.*, 1996).

Name	Gene (Chromosome)	allele	Mutation pro/mature protein	Founder breed
Inverdale	BMP-15 (X)	$FecX^{I}$	V299D / V31D	Romney
Hanna		$FecX^{H}$	Q291stop / Q23stop	Romney
Belclare		$FecX^{B}$	S367I / S99I	Belclare
Galway		$FecX^{G}$	T239stop / no	Belclare, Cambridge
Lacaune X-linked		$FecX^{L}$	C321Y / C53Y	Lacaune
High Fertility	GDF-9 (5)	$FecG^{H}$	S395F / S77F	Belclare, Cambridge
Booroola	BMPR-1B (6)	$FecB^{B}$	Q249R	Merino, Garole, Javanese, Hu, Han

Table 1. 5. Identified major genes affecting ovulation rate in sheep. Reproduced from (Fabre *et al.*, 2006).

1.4.7. 1. Mutations of type I BMP receptor BMPR-IB and female reproductive system

In mice, BMPR-IB expression has been detected in oocyte of maturing follicle and oocyte and granulosa cells of antral follicles (Yi *et al.*, 2001). No expression of BMPR-IB has been shown in granulosa cells of resting, primordial, developing or atretic follicles, corpus luteum and thecal cells (Yi *et al.*, 2001). BMPR-IB was also detected in uterine endometrium, but was absent in pituitary gland indicating that BMPR-IB has no direct role in regulation of FSH production (Yi *et al.*, 2001). BMPR-IB gene deleted

females were infertile showing irregular estrous cycles and an impaired pseudo pregnancy test (Yi *et al.*, 2001). Histological examination of ovaries of these animals revealed no differences in folliculogenesis, ovulation rate, and corpora luteum formation (Yi *et al.*, 2001). In BMPR-IB knockout mice, cumulus oocyte complex (COC) had fewer cumulus cells than COC of control animals (Yi *et al.*, 2001). As cumulus cell expansion helps in oocyte maturation and sperm penetration or fertilization, it was inferred that lack of cumulus cell expansion might be a possible reason for female infertility of BMPR-IB knockout mice (Yi *et al.*, 2001). The uterus of mutant mice had few uterine glands and very thin endometrial lining in comparison to control animals (Yi *et al.*, 2001). This study showed importance of BMPR-IB in mice reproduction, but phenotypic results in this study are in contrast to Booroola phenotype. Although, we have to consider that the Booroola mutation is a highly conserved point mutation compared to gene deletion in this study and its still unclear whether Booroola mutation is able to make the whole BMPR-IB receptor dysfunctional or not.

In humans the role of BMPR-IB in reproduction is still unclear because of fewer experimental studies and different physiological action of these proteins in different species. Recently, Demirhan *et al.* reported a clinical case of a 16 year old girl with homozygous BMPR-IB mutation of 8 bp (del 359-366) deletion in extracellular ligand binding domain of BMPR-IB, showing a severe form of limb malformation *viz.* aplasia of fibula, brachydactyly, ulnar deviation of hands and fusion of carpal and tarsal bones (Demirhan *et al.*, 2005). In addition to skeletal defects, she presented with genital anomalies and primary amenorrhea (Demirhan *et al.*, 2005). Ultrasonographic examination revealed the absence of ovaries and hypoplastic uterus (Demirhan *et al.*, 2005). The endocrinological examination confirmed hypergonadotrophic hypogonadism

syndrome, which indirectly points towards the ovarian malfunction (Demirhan *et al.*, 2005).

1.4. 8. BMPs and Testis

Spermatogenesis is a complex process. In mice it starts just after birth with resumption of male germ proliferation and differentiation. Basically, mice spermatogenesis is divided into two different phases on the basis of stage of development. The first phase is related to the initiation of spermatogenesis, which is first 6 weeks of postnatal development and the second phase is maintenance phase and this phase starts after 6 weeks of postnatal development (Kluin *et al.*, 1982, Setchell, 1978). Various members of the TGF-β superfamily *viz.* activin, follistatin, inhibin are found to express and play an important role in male reproductive system (De Kretser & McFarlane, 1996, de Kretser *et al.*, 2002). Recently, BMP family members have been shown to regulate spermatogenesis (Itman *et al.*, 2006). Knockouts of BMP-4, BMP-7, BMP-8a, and BMP-8b have been shown to have defects in spermatogenesis, PGC formation, testis cord formation and epididymis (Hu *et al.*, 2004, Zhao & Hogan, 1996, Zhao *et al.*, 1998). BMP-4 gene deleted heterozygous male mice have been shown to have compromised fertility because of degeneration of germ cells, reduced sperm count and sperm motility (Hu *et al.*, 2004). In addition, they also have defect in corpus region of epididymis (Hu *et al.*, 2004). In mouse testis, BMP-4, ALK-3 and Smad-5 pathways are active and have mitogenic and differentiatative effects in spermatogonia development (Pellegrini *et al.*, 2003). In addition, BMP-4 is also secreted by sertoli cells in very early stages of postnatal life and its expression decreases with development (Pellegrini *et al.*, 2003). In contrast, Hu *et al.* reported that BMP-4 expression increases after 2 weeks of postnatal life with maximum expression at pachytene spermatocyte stage of spermatogenesis (Hu *et al.*, 2004). BMP-4 also has a high level of expression in epididymis throughout postnatal

development (Hu *et al.*, 2004). At 2 wks of age, expression BMP-4 is higher in epididymis than testis, afterwards testis has more expression than the epididymis (Hu *et al.*, 2004). Interestingly, pachytene spermatocyte and expression of the smad-1 appeared in seminiferous tubules at same time at the age of 2 wks (Pellegrini *et al.*, 2003, Puglisi *et al.*, 2004, Steinberger & Steinberger, 1975). In another study, BMP-2 has been shown to increase spermatogonia proliferation in presence of FSH (Puglisi *et al.*, 2004). Similarly, BMP-7 has also been shown to increase proliferation of sertoli cells in presence of FSH (Puglisi *et al.*, 2004). In addition, expressions of ActR-IIB and BMPR-II are also detected in spermatogonia and sertoli cells, respectively (Puglisi *et al.*, 2004).

1.4. 9. Embryo development

In early embryonic development, BMPs are found to play a central role in development in the *Xenopus* and frog embryos (Yamamoto & Oelgeschlager, 2004). Gratsch *et al.* studied role of BMP-4 in post implantation embryo in mice by injecting short hairpin RNAs (sh RNA) prepared against BMP-4 in pregnant dam, just after implantation or very early stages of embryo development (Gratsch *et al.*, 2003). They reported that inhibition of BMP-4 caused defect in neural fold elevation and defects in cardiac and allantois development (Gratsch *et al.*, 2003). In addition, the defective embryos showed up-regulation in expression of BMP antagonist, noggin and chordin (Gratsch *et al.*, 2003). In another study, addition of various concentration of BMP-2 and BMP-4 to different maturation media of bovine COC and embryos showed no affect on oocyte quality and on blastocyst development (Fatehi *et al.*, 2005). The possible explanation for differences in these results might be because of adequate amount of BMPs are released from embryo during development so external addition of BMPs had no affect

on their development and further studies are required to clarify the role BMPs in embryonic development.

1. 5. Aim of the project

The BMPs are pleiotropic molecules shown to be involved in almost all physiological processes, but this review highlights the role of BMPs in reproduction. Since the identification of the Booroola mutation, responsible for high prolificacy in Booroola merino sheep, research has been focused on the role of BMPs in the biology of reproductive functions. Most of our knowledge about BMPs comes from *in vitro* experiments and very few reports exist regarding *in vivo* experimentation on BMPs. These *in vitro* experimental models are useful but they may not present the whole picture of the actions and functions of BMPs.

In recent years significant studies have revealed the important role BMP-4 plays in physiology of different reproductive process. Furthermore, interaction of BMP-4 with activin/follistatin and inhibin underlines its complex mechanism of action as a regulatory protein hormone. This project was aimed at investigating and elucidating the role of BMP-4 in folliculogenesis, estrus, and spermatogenesis with mice and sheep as experimental models. I hope that our serial studies presented in this thesis will be able to define more clearly the role of BMP-4 in different reproductive processes and will promote further research in this area.

Chapter 2: General Material and Methods

The protocols for techniques common to different chapters are described in this chapter. The methods specific to an experiments are described in the relevant sections of each chapter.

2. 1. Antiserum production

2.1. 1. Immunization of sheep

A synthetic peptide equivalent to amino acids 88-102 from mouse BMP-4 (Invitrogen Australia, Mount Waverly Vic 3149 Australia) was conjugated to diphtheria toxoid for the preparation of vaccine for active immunization against BMP-4 in sheep. Ewes were injected (i.m.) with 30 µg of BMP-4 peptide conjugate in 1ml of Freunds complete adjuvant (FCA). The animals were boosted after 4 weeks and after further 6-8 weeks using half the primary concentration of rbBMP-4 peptide in Freunds incomplete adjuvant (FIA). Blood samples were collected at regular intervals to measure antibody titre.

2.1. 2. Enzyme-linked immunoabsorbent assay (ELISA)

To determine antibody titer, plasma samples from actively immunized sheep were tested for reactivity with both BMP-4 and BMP-2. To do this we used the indirect ELISA technique as described below. Briefly, flat bottomed microtitre plates (Greiner Labortechnik, Austria) were coated with 30 ng/well BMP-4 in binding buffer (0.05 M bicarbonate, 0.02% sodium azide, pH 9.6). Excess binding sites were blocked with 200 µl 5 % skim milk in PBS for 1 hr at 37 ^{o}C. The plates were washed 5 times with 0.9% saline

and 0.05% Triton-X 100, using a Titertek Microplate washer (Labsystems, Helsinki, Finland). Serial double dilutions (1000 ng/ml → 0.48 ng/ml) of BMP-2 and BMP-4 peptide were prepared in phosphate buffer, pH 8.0, containing 0.5 M sodium chloride, 0.5% Tween-20 and 0.1% BSA (ELISA buffer) and were added to the ELISA plate in a volume of 100 µl. Then, biotinylated anti BMP-4 antibody (2 µg/50 µl) was added to the ELISA plate and incubated overnight at 37 ^{o} C. Following day, the plates were washed and streptavidin conjugated to alkaline phosphatase at dilution of 1:10000 was incubated for 1 hr at 37 ^{o} C. The plates were developed with p-nitrophenylphosphate disodium salt hexahydrate (NPP) and read at 405 nm using a Titertek Multiskan Plus microplate reader (Labsystems).

Results

The anti BMP-4 antibody showed specific binding to BMP-4 peptide and had no cross-reactivity with BMP-2 peptide (Fig. 2.1).

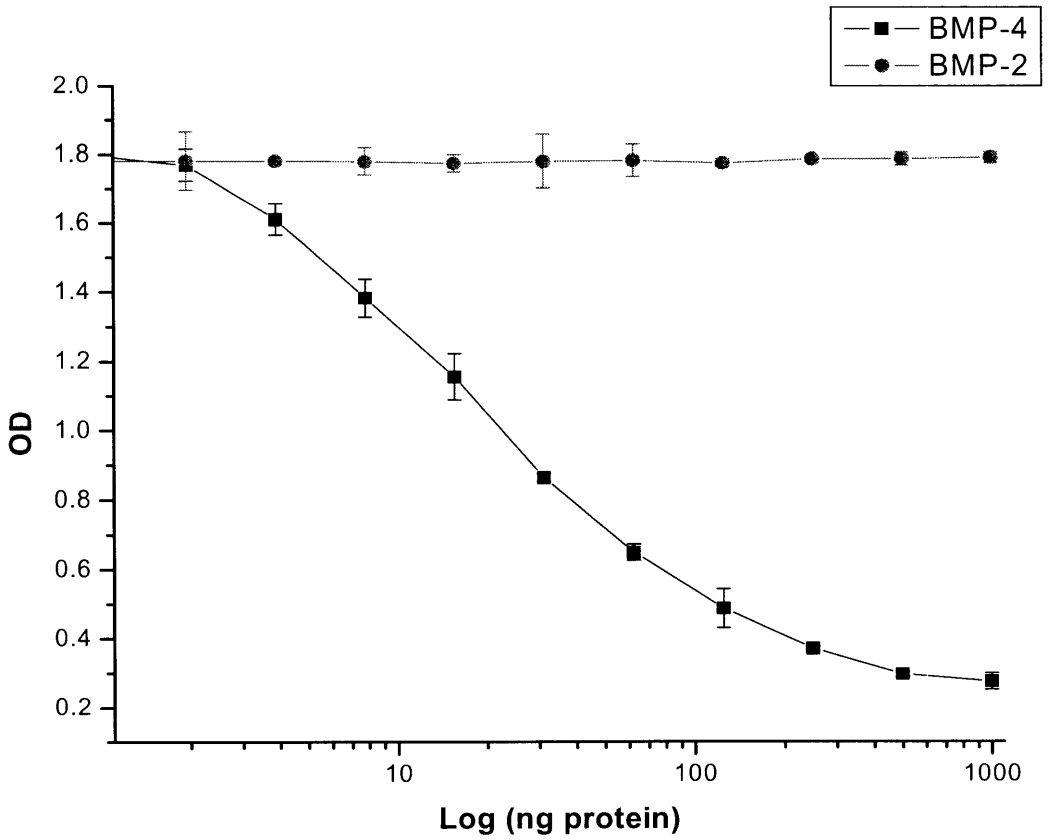

Figure 2. 1. This graph shows the binding of anti-BMP-4 antibody to BMP-4 peptide in competition with increasing amount of BMP-2 and BMP-4 peptide. The amounts of BMP-2 and BMP-4 peptide used to compete with BMP-4 peptide coated on plate are indicated on X-axis.

2.1. 3. Antibody purification from sheep plasma

Antibodies were purified from the plasma of the animals with highest titre as determined by indirect ELISA. The method we used for antibody purification was similar to that described in previous studies (Kauter *et al.*, 2000, McKinney & Parkinson, 1987). Plasma from sheep with high titre was diluted with 4 volumes acetate buffer (60 mM, pH 4). The pH was adjusted to 4.5 with 0.1 N NaOH and refrigerated overnight. The following day, total volume of the solution was measured and then caprylic (n-Octanoic) acid was slowly added to the solution with thorough mixing and the solution was further stirred for 30 minutes. The solution was then centrifuged at 6000 rpm for 30 minutes and the supernatant was separated from the solidified fat with a strainer. The supernatant was then mixed with 10x PBS (9 part of supernatant and 1 part of 10x PBS) and then pH was readjusted to 7.4 with 1 N NaOH. After that, supernatant was brought to 45% saturation with ammonium sulphate (0.277 g/ml) and mixed thoroughly for 30 minutes and then centrifuged at 6000 rpm for 15 minutes. The supernatant was discarded and pellet/s was resuspended with 1x PBS containing 0.02 % sodium azide, and dialyzed against 50-100 volumes of water. Dialyzed Ig was then concentrated using Amicon stirred cell concentrator (Amicon, Beverly, MA, USA) to 10 mg/ml before being stored at -20 ^{o} C.

2.1. 4. Biotinylation of antibody

Using the manufacturer's method of biotinylation (EZ-link ^{TM} sulpho-NHS-LC-Biotin, Pierce, Rockford, IL, USA), 2 mg of antibody was added to sodium bicarbonate buffer (pH 8.5) to make a total volume of 1 ml. Then, 75 µl of biotin solution (1 mg biotin/1 ml of dH_2O) was added to the antibody solution and incubated on ice for 2 hrs with occasional gentle mixing. Unreacted biotin was then removed by centrifugation at 6000

rpm for 30 minutes (Centricon Centrifugal Filters, Millipore Corporation, Bedford, MA, USA) with repeated wash with 1x PBS. Concentrated biotinylated antibody was then mixed with equal amount of glycerol and then with 50 % glycerol in PBS to make a total volume of 1 ml and stored at -20 °C.

2. 2. Immunohistochemistry

2.2. 1. Tissue preparation, embedding and sectioning

Tissues were immersion fixed in Bouin's fluid (75 ml saturated aqueous picric acid, 25 ml 40% formalin, 5 ml glacial acetic acid) for 6-12 hrs. The tissues were then transferred into 50 % alcohol, and stored until required.

The tissue samples were dehydrated in a graded ethanol series (50% ethanol for 2 hrs → 70% for 2 hrs → 80% ethanol – 2hrs → 90% ethanol- 1 hr → absolute ethanol 2x 1 hr → 1:1 ethanol/xylol- 1 hr → xylol I-1 hr → xylol II-2 hrs→ paraplast I-2 hrs→ paraplast II-3 hrs) and embedded in paraffin.

Prior to sectioning, slides were coated with 3-aminopropyltriethoxysilane (APS) (Sigma Chemical co., St. Louis, MO) to prevent the section dislodging during antigen retrieval. Embedded samples were sectioned on a microtome at 5 µm thickness. Each section was mounted on APS-coated slides and dried in hot air oven at 37 °C overnight.

2.2. 2. Immunostaining

Serial sections of tissues were deparaffinised with xylene and rehydrated with graded series of ethanol (absolute, 80% and 50%, respectively, and distilled water), followed by two washes of 5 minute each in PBS-T (0.05 M phosphate buffer saline containing

0.05% Tween 20; PH 7.4). Tissue sections were then incubated for 10 min in 3% (v/v) hydrogen peroxide in methanol to block the endogenous peroxidase activity. After a wash with PBS-T, antigen retrieval was performed by microwaving the sections in 0.01 M citrate buffer (pH 6) in a domestic microwave oven (750 W) at full power for 15 min and, the sections were kept in buffer until cool (20-30 min). Sections were then washed for 5 min in PBS-T and blocked at room temperature for 1 hr by using 10% (W/V) skim milk powder and 5% (V/V) NRS (normal rabbit serum) in PBS. Tissues sections were then incubated in humidified chamber for 2 hrs at room temperature, followed by overnight incubation at 4^0C with anti BMP-4 antibody diluted in 0.05 M PBS containing 5% NRS and 0.3% BSA. This antibody was raised against a synthetic BMP-4 peptide (aa 88-102, Invitrogen Australia, Mount Waverly Vic 3149 Australia) in sheep, and had previously been shown to be specific against BMP-4 (as above in fig. 2.1, and chapter 3 and 4) (Tanwar & McFarlane, 2005). Sections were subsequently washed with PBS-T and incubated at 37^0C for 1 hr with 1:800 dilution of biotinylated rabbit anti-sheep immunoglobulin in 0.05 M PBS containing 0.3% BSA. After a wash with PBS-T, sections were incubated at 37^0C for 1 hr with diluted alkaline phosphatase-conjugated streptavidin at 1:800 in PBS containing 0.3% BSA. Bound antibodies were visualized by incubation in BCIP/NBT one-step (Chemicon international, Temecula,CA) for 5-10 min. Sections were washed in distilled water, dehydrated through increasing concentration of alcohol and mounted with coverslips using DPX (BDH Laboratory Supplies, Poole, England). Negative controls were performed by replacing the primary antibody with normal sheep serum at similar protein concentration.

2. 3. Western analysis

Samples were diluted in non-reducing (0.125M Tris-HCl, 0.07% SDS, 0.6% Glycerol, 1.5 % Nonidet P-40 detergent, Bromophenol blue) and reducing sample buffer (0.125M Tris-HCl, 0.07% SDS, 0.6% Glycerol, 10% 2-mercaptoethanol, Bromophenol blue) and were heated at 95 ^{0}C for 10 min and then loaded on to a SDS polyacrylamide gel (12.5% resolving and 4.06% stacking gel) using a slab electrophoresis unit (Model SE 250-Mighty Small II, Hoefer, USA). The proteins were transferred from gel to polyvinylidiene fluoride membrane (Micron Separations Inc., Westborough, MA) by using the transfer electrophoresis unit (Model TE 22-Mighty Small, Hoefer, USA) in a transfer buffer (25mM Tris base, 200mM glycine and 15% methanol). After protein transfer, membrane was slightly stained with Ponceau S (0.2% Ponceau, 3% Tricholoacetic acid, and 3% Sulphosalisyclic acid) to see the successful transfer of protein and then Ponceau S was washed off by using Tris-buffer (20mM Tris-base, 0.15 M NaCl, 0.1% Triton-X 100, pH 8.2-8.4). The membrane was blocked for 1 hr with 10% skimmed milk powder in PBS-T. The membrane was washed for 5 min with Tris-buffer and then incubated overnight with anti BMP-4 antibody (0.5 µg/ml) diluted in high salt ELISA buffer. The following day membrane was incubated for 2 hours with biotinylated rabbit anti-sheep immunoglobulin (1:10000) diluted in high salt ELISA buffer. After washing, membrane was incubated with strepavidin alkaline phosphatase conjugate (1:10000) for one hour. The appearance of specific bands on membrane was detected by using BCIP/NBT one-step (Chemicon international, Temecula, CA). The specificity of bands was determined by using pre immune sheep immunoglobulin in place of primary antibody.

2. 4. Image processing

The images for western blotting were scanned by using Paint shop. Immunolocalization sections were photographed using a digital camera (Nikon Coolpix 950).

2. 5. Animal ethics

All animals used in this study were housed under standard animal housing conditions and all experimentation was approved by the animal ethics committee, University of New England, Armidale, NSW, Australia.

2. 6. Statistics

Two-way analysis of variance followed by a Student-Newman-Keuls multiple range tests were performed using the SAS computer software package (SAS Institute Inc., Cary, NC, USA). Data which was not normally distributed was log transformed. Analysis of discrete variable data such as ovulation rates were analysed by the chi-square test.