Chapter 1 Literature review

1.1 General introduction

The length of time a mammalian female is reproductive and fertile is dependent on the size of the primordial follicle pool produced during fetal life and on the depletion rate of primordial follicles (Macklon & Fauser, 2000). The primordial follicles occupy the ovarian cortex and serve as the store of oocytes for the reproductive lifetime. The initial transition from the primordial to the primary follicle is known as primordial follicle recruitment and begins when dormant follicles are selected from the primordial pool and actively start to grow, joining the cohort of developing follicles (Gougeon, 1996). Generally, at each reproductive cycle, a few follicles successfully reach the preovulatory stage, while the rest of the follicles undergo a process called atresia, an hormonally controlled apoptotic process distinguished by morphological changes in the nucleus and cytoplasm (Byskov, 1974). Follicular growth and atresia are under strict regulation of endocrine signaling from the pituitary gland and autocrine-paracrine signaling from the ovary (Gougeon, 1996).

The pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are required for terminal follicular development and regulating the number of ovulations (McNeilly *et al*., 1992). Secretion and release of gonadotropins are under the control of gonadotropin-releasing hormones and feedback loops from gonadal steroids. LH and FSH act together with the hypothalamic-pituitary axis through negative inhibitory feedback to regulate ovarian follicle development. In sheep and cattle, there is a positive association between cyclic FSH surges and follicular wave appearance (Adams, 1999), and at

the time of follicle selection, LH appears to be essential for further follicle development (Campbell *et al*., 1995).

Genetic studies using the mouse gene knockout model and mutant sheep have shown that ligands of the transforming growth factor-β (TGF-β) super family act in a coordinated manner for successful completion of follicle development and the regulation of ovulation rate (Barnett *et al*., 2006; Wilson *et al*., 2001). The TGF-β super family is comprised of over 35 proteins with common structural features (all members of the TGF-β family are cysteine knot proteins) (De Caestecker, 2004; Massagué, 1990; Massagué, 2000).

1.2 Transforming growth factor β super family (TGF-β)

Transforming growth factor-β (TGF-β) super family is a large group of more than 35 structurally related proteins that regulate a number of developmental processes ranging from embryonic development to cellular homeostasis, and the disruption of their actions has been implicated in a range of human diseases including developmental confusion and cancer (Epstein *et al*., 2000; Massagué *et al*., 2000). The TGF-β super family can be grouped into subfamilies including: activins, inhibins, growth differentiation factors (GDFs), anti Mullerian hormone (AMH), and bone morphogenetic proteins (BMPs) (Cate *et al*., 1986; Massagué, 1990; Massagué, 2000).

The term TGF-β refers to the products of different genes which have been identified by cDNA cloning or protein isolation that exist in at least five isoforms (Messagué, 1990). TGF-β1 was initially isolated from human and porcine blood platelets (Assoian *et al*., 1983), while TGF-β2 was found in porcine blood platelets (Cheifetz *et al*., 1987) and bovine bone (Seyedin *et al*., 1987). Both TGF-β1 and TGF-β2 have been identified according to their ability to

induce cartilage activity, and originally they were termed as CIF-A and CIF-B respectively, prior to being renamed as TGF-β1 and TGF-β2 (Seyedin *et al*., 1985). Human TGF-β3 was identified firstly at the cDNA level and then expressed in recombinant form (Ten Dijke *et al*., 1988). TGF-β4 and TGF-β5 were identified as a cDNA from *Xenopus laevis* and chick embryos (Kondaiah *et al*., 1990; Jakowlew *et al*., 1988). Mammalian TGF-β4 and TGF-β5 have not been identified so far.

The ligands of the TGF-β family can be divided into two groups depending on their signaling components, namely the TGFβ/activin group and the BMP/MIS group (Massagué & Chen, 2000). TGF-β super family members transduce their signals by a common mechanism in which ligands bind two pairs of cell surface receptors of serine/threonine kinase receptors named receptor type I (activin like kinase(ALK) and type II receptors (TβRII) (Heldin *et al*., 1997). Type II receptors bind ligands in the absence of type I receptors, however they require their respective type I receptors for signaling, but type I receptors require their respective type II receptors for ligand binding (Rosenzweig *et al.*, 1995; Massagué & Gomis, 2006). After ligand binding, heterotetrameric receptor complexes are formed which activate intracellular messenger proteins named SMADs. The SMADs translocate into the nucleus and modulate the transcription of the objective gene (Kretzschmar *et al.*, 1997). Furthermore, there are a growing number of TGF-β super family co-receptors that regulate ligand binding and indirectly transduce signals (Kirkbride *et al*., 2005). For example, the TGF-β super family co-receptors, betaglycan (TRβIII) mediate the function of TGF-β ligands (López-Casillas *et al*., 1993), inhibin (Lewis *et al*., 2000) and BMP (Kirkbride *et al*., 2008) via binding to these ligands, enhancing their interaction with other TGF-β superfamily signaling receptors (Kirkbride *et al*., 2008; López-Casillas *et al*., 1993).

1.2.1 Activin and inhibin subfamily

Inhibins and activins are a family of dimeric proteins which belong to the TGF-β super family. Inhibins are heterodimers of two subunits: the α unit which links to either βA to form inhibin A or links to βB to form inhibin B. Dimerization of the β subunit alone gives three types of activins: activin A, activin B, and activin AB (Ling *et al.*, 1985; Sallon *et al.*, 2010; Zhu *et al.*, 2008). Like other members of the TGF super family, activins and inhibins start their biological actions via simultaneously binding to two types of receptors with serine/threonine kinase activity (Mathews & Vale, 1992). There are two kinds of activin receptors which are designated as ActR-1A and ActR-1B (type I receptors) and ActR-2A and ActR-2B (type II receptors). The first step of activin action is their binding to a type II activin receptor which leads to the phosphorylation and activation of a type I receptor (Attisano *et al*., 1993). Once phosphorylated by the type II receptor and thereby activated, the type I receptor recruits and phosphorylates regulated Smad2 and Smad3. Upon phosphorylation, Smads form a heteromeric complex with the Smad4 (Co-Smad) and are translocated to the nucleus where they regulate the expression of activin target genes (Massagué & Weisa-Garcia, 1996).

Inhibins and activins were first recognized for their capability to modify FSH secretion *in vitro*; inhibins are feedback inhibitors of pituitary FSH secretion, whereas activins stimulate the secretion of FSH (Carroll *et al*., 1989). Later studies show that activin acts mainly as a local paracrine and autocrine factor of the reproductive and non-reproductive organs (Mather *et al*., 1997). Activins exhibit a wide range of biological activities in a wide range of cell types including wound repair (Werner & Alzheimer, 2006), hair follicle development (McDowall *et al*., 2008), and stem cell growth and differentiation (Watabe & Miyazono, 2008). In the ovary, activins stimulate oocyte maturation (Sadatsuki *et al*., 1993), regulate

granulosa cell (GC) proliferation and differentiation (Rabinovici *et al*., 1990), and increase FSH receptor mRNA expression in cultured rat GCs (Nakamura *et al*., 1995).

ActR-2A and ActR-2B have been found to play vital roles in skeletal patterning and organ development in mice. Some embryos homozygous for AcvR-2A knockout mice *(Acvr2a*$^{-/-}$) or AcvR-2b lacking mice (*Acvr2b*$^{-/-}$) exhibited abnormalities in the skeletal system and face although most lack these defects and developed into maturity (Matzuk *et al*., 1995; Song *et al*., 1999). However, mice carrying both receptor mutation *Acvr2a*$^{-/-}$*; Acvr2b*$^{-/-}$ fail to form mesoderm, and *Acvr2a*$^{-/-}$*; Acvr2b*$^{+/-}$ showed truncated brain development (Song *et al*., 1999). A targeted null mutation in activin receptor type II (ActR-2A) leads to a significant suppression in FSH plasma concentrations which most likely indicates that this receptor may regulate the synthesis and secretion of pituitary FSH (Matzuk *et al.*, 1995).

1.2.2 Anti-Mullerian hormone (AMH)

Anti-Mullerian hormone (AMH), also termed Mullerian inhibiting substance (MIS), was identified according to its ability to stimulate the regression of Mullerian ducts during development of the male embryo (Blanchard & Josso, 1974), and was afterwards identified as a dimeric glycoprotein related to the TGF β superfamily (Cate *et al*., 1986). AMH is produced by adult mammalian granulosa cells in females and by the Sertoli cells of fetal and adult testis in males (Blanchard & Josso, 1974; Vigier *et al.*, 1984). Transgenic female mice chronically over-expressing AMH showed ovarian degeneration, while transgenic male mice over-expressing AMH displayed feminization and regression of the external genitalia. These observations suggest that AMH might promote gonadal morphogenesis in addition to Mullerian ducts regression (Behringer *et al.*, 1990).

Recently, AMHR-2 has been isolated from rat Sertoli cell and rabbit fetal ovaries (Baarends *et al*., 1994; Di Clemente *et al*., 1994). The type 2 receptor of AMH (AMHR-2) is expressed in the mesenchymal cells surrounding the Mullerian ducts of males and females at day 15 of embryonic development in rats (Baarends *et al*., 1994). Generation of AMHR-2 knockout mice showed that this type 2 receptor is vital for AMH signaling, as proved by the lack of Mullerian duct regression in AMHR-2 knockout male mice (Mishina *et al*., 1996). The relevance of AMHR-2 to AMH action was additionally confirmed by the detection of mutations in the AMH or AMHR-2 genes of patients with persistent Mullerian duct syndrome, which is characterized by the persistence of Mullerian derivatives in phenotypic males (Imbeaud *et al*., 1996). These mutations include a receptor deletion (AMHR-2) in around 25% of cases with persistent Mullerian duct syndrome (Imbeaud *et al*., 1996). *In vitro* and organ culture studies using dominant-negative and antisense strategies revealed that ALK2 is involved in AMH-induced signaling during Mullerian duct regression (Visser *et al*., 2001).

1.2.3 BMP family ligands

BMPs are intercellular signaling molecules that belong to the TGF-β super family and have many functions in growth and differentiation in both vertebrates and invertebrates (Table 1-1) (Gelbart, 1989; Hogan *et al*., 1994). The BMPs were first discovered by Urist (1965) on the basis of their ability to promote both bone and cartilage formation at ectopic sites in rodents. BMPs can be categorized into subgroups depending on similarities in structure and function. BMP-2 and BMP-4 form one subgroup (the BMP-2/BMP-4 group); BMP-5, BMP-6, BMP-7 and BMP-8 form the second group (the OP-1 group); BMP-12, BMP-13 and growth and

differentiation factor 5 form the third group (the GDF5 group); while BMP-9 and BMP-10 form the fourth group (the BMP-9 group) (Kingsley, 1994).

Although the effect of BMPs as bone inducing proteins was first discovered by Urist (1965), the proteins responsible for bone induction were not identified until the purification and cloning of human BMPs in the 1980s. BMP-2, BMP-2B (now called BMP-4) and BMP-3 were the first BMPs to be isolated and cloned (Wozney *et al.*, 1988). Thereafter, new members of the BMP family that were cloned and identified included osteogenic protein 1 (BMP-7) (Ozkaynak *et al.*, 1990), BMP-5, BMP-6 (Celeste *et al.*, 1990), osteogenic protein 2 (BMP-8a) (Ozkaynak *et al.*, 1992) and GDF-3 (McPherron & Lee, 1993).

Table 1-1: Function of BMP members. Modified from Rengachary, 2002

BMP	Function	References
BMP-2	Osteoinductive and osteoblast differentiation	(Wang *et al.*, 1990; lee *et al.*, 2003)
BMP-3 (Osteogenin)	Endochondral bone formation	(Hiraki *et al.*, 1991)
BMP-4	Osteoinductive, lung development, kidney development and follicle development	(Weber *et al.*, 2008; Bellusci *et al.*, 1996; Tanwar *et al.*, 2008)
BMP-5	Chondrogenesis	(Sampath *et al.*, 1993; Ho *et al.*, 2008)
BMP-6	Chondrogenesis and osteoblast differentiation	(Ebisawa *et al.*, 1999; Gitelman *et al.*, 1994).
BMP-7 (OP-1)	Kidney and eye development, and osteoinductive	(Chubinskaya *et al.*, 2007; (Dudley *et al.*, 1995; Luo *et*

		al., 1995).
BMP-8	Induces bone development.	(Reddi, & Cunningham, 1993)
BMP-9	Nervous system, hepatic reticuloendothelial system and hepatogenesis	(López-Coviella *et al*., 2000; Miller *et al*., 2000; Bidart *et al*., 2012)
BMP-10	Cardiac development	(Chen *et al*., 2004)
BMP-11(GDF-8)	Patterning mesodermal and neuronal tissues	(Gamer *et al*., 1999)
BMP-12 (GDF-7)	Induces tendon –iliac tissue formation	(Lou *et al*., 2001)
BMP-13 (GDF-6)	Induces tendon and ligament-like tissue formation	(Wolfman *et al*., 1997)
BMP-14 (GDF-5)	Chondrogenesis, enhances tendon healing and bone formation	(Francis-West *et al*., 1999; Aspenberg & Forslund, 1999)
BMP-15	Modifies follicle – stimulating hormone activity	(Otsuka & Shimasaki, 2002b; Yan *et al*., 2001)

The presence of seven conserved cysteine is a structural characteristic of the TGF-β super family, and allows the folding of the molecule into a unique three-dimensional arrangement known as a cysteine knot (Vitt *et al.*, 2001). The crystal structure of the mature region of TGF-β shows that six of the cysteine residues form three disulfide bonds within each monomer subunit. The seventh cysteine residue is involved in the formation of an interchain bridge that links two monomers into a dimer (Schlunegger & Grutter, 1992; Vitt *et al.*, 2001).

The formation of linked dimers is important for the protein's biological activity and disruption of this link after reduction results in a loss of biological activity (Luyten *et al.*, 1989; Wang *et al.*, 1990).

It has been found that BMP-1 has a dissimilar structure to the other BMPs and consequently BMP-1 is not included in the TGF-β super family. Unlike other proteins in the TGF-β super family, neither GDF-3 nor GDF-9 have the fourth conserved cysteine residue that is necessary to form the inter subunit link found in other TGF-β family members (Dube *et al.*, 1998; McPherron & Lee, 1993). However, GDF-3 and GDF-9 can form non-covalent homodimers, as well as heterodimers when both are co-expressed (Liao *et al.*, 2004). The co-expression of recombinant BMP-2, BMP-4 and BMP-7 can lead to the formation of heterodimers that have stronger biological actions than homodimers (Aono *et al.*, 1995; Kusumoto *et al.*, 1997).

1.2.3.1 Receptor in the TGF-β super family

The biological activity of TGF-β super family members is potentiated through interactions with two major types of membrane-spanning serine/threonine kinase receptors, namely type I and type II receptors (Cheifetz *et al.*, 1990; Cheifetz *et al.*, 1987) (Figure 1-1). The type I and type II receptors are of about 55 and 70 kDa with core polypeptide of about 500 to 570 amino acids including signaling sequence (Rosenzweig *et al.*, 1995; Massagué, 1998).

The activin type II receptor (ActR-2A) was the first identified receptor in the TGF-β super, and was comprised of an intracellular kinase domain with predicted serine/threonine residues, a single membrane-spanning domain, and an extracellular ligand-binding domain (Mathews & Vale, 1991). Afterwards, other type II receptors with structural characteristics similar to the activin receptor have been cloned and identified. In mammals, there are five mammalian type

II receptors that have been identified, known as: activin receptor 2 (ActR-2) (Mathews & Vale, 1991), active receptor 2B (ActR-2B) (Attisano *et al.*, 1992), AMHR-2 (Baarends *et al.*, 1994), BMPR-II (Baarends *et al.*, 1994; Rosenzweig *et al.*, 1995), and TGF-β receptor II (Lin *et al.*, 1992). In addition, there are seven type I receptors named activin receptor-like kinases (ALK) (ALK-1 to ALK-7), that have been identified in mammals so far (Tendijke *et al.*, 1993; Yamaji *et al.*, 1994). Both receptors are required for signal transduction, yet type I receptors can only transmit a cellular signal when co-expressed with type II receptors (Attisano *et al.*, 1993; Ebner *et al.*, 1993).

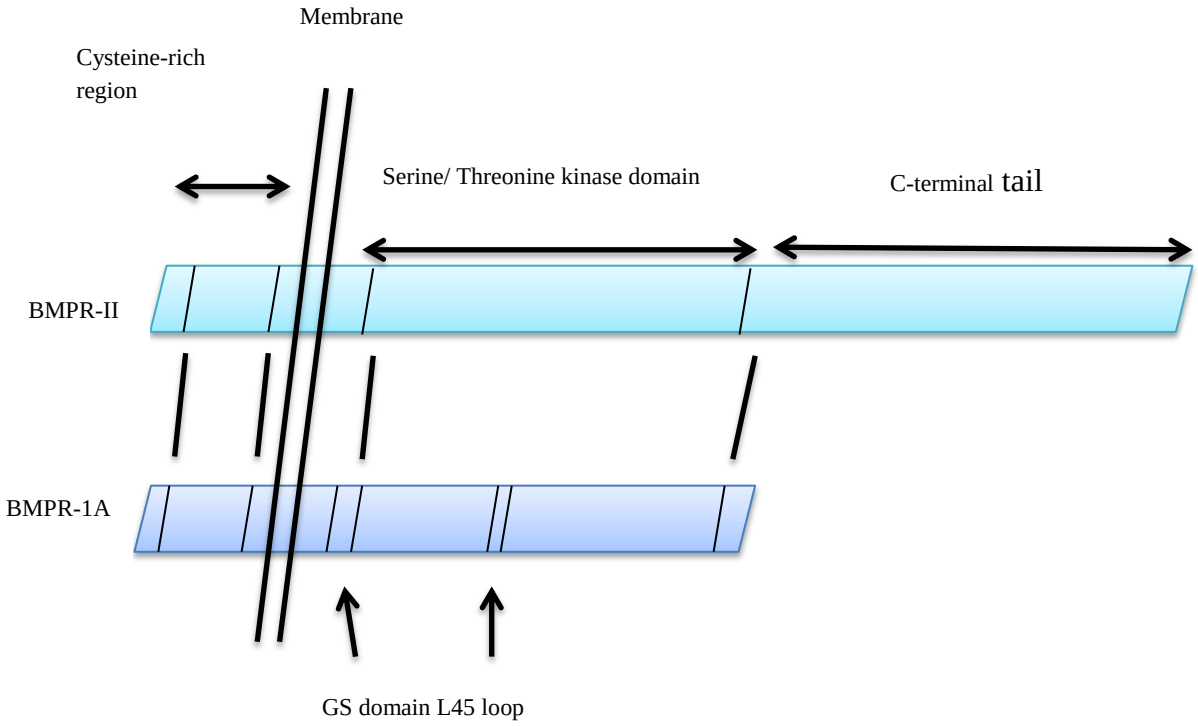

Figure 1-1: Structure of BMPR 2 and BMPR1A. Modified from Miyazono, 2008.

BMP family members can bind to different receptors in different cell types. For instance, during osteoblast differentiation, BMP-6 binds strongly to ALK-2 and with lower affinity to ALK-3 in mouse osteoblastic cells, while it binds strongly to ALK-3 and with less affinity for

ALK-2 in rat osteoprogenitor-like cells *in vitro* (Ebisawa *et al.*, 1999). Different BMPs appear to have a common tendency to bind with specific receptors. For example, BMP-2, BMP-4 and GDF-5 bind to ALK-3 and/or ALK-6 (Aoki *et al.*, 2001; Nishitoh *et al.*, 1996; Tendijke *et al.*, 1994), BMP-6 and BMP-7 bind to ALK-2 and/or ALK-6 (Ebisawa *et al.*, 1999; Tendijke *et al.*, 1994), while BMP-15 was found to effectively bind with ALK-6 (Moore *et al.*, 2003).

The ligand/receptor interactions between different BMP ligands and their receptors are not exclusive. Although ActR-2A and ActR-2B have been initially identified as activin type I receptors, it has been shown that they can also work as receptors for BMP-6, GDF-5, and BMP-7 (Ebisawa *et al.*, 1999; Nishitoh *et al.*, 1996; Yamashita *et al.*, 1995). Inversely, three type I receptors (ALK-2, ALK-3 and ALK-6) (Koenig *et al.*, 1994; Macias-Silva *et al.*, 1998; Tendijke *et al.*, 1994), and one type II receptor (BMPR-2) act mainly as receptors for BMPs, including BMP-2 (Liu *et al.*, 1995), BMP-4 (Rosenzweig *et al.*, 1995), BMP-6 (Ebisawa *et al.*, 1999), BMP-7 (Yamashita *et al.*, 1995), BMP-15 (Moore *et al*., 2003), GDF-5 (Nishitoh *et al*., 1996), and GDF-9 (Vitt *et al*., 2002).

Dragon (RGMb) has been identified by Samad *et al.* (2005) to be a co-receptor for BMPs. It is a 436-amino-acid, glycosylphosphatidylinositol-anchored protein and is part of the repulsive guidance molecule family (RGM) (Samad *et al*., 2004). Dragon is expressed in the embryonic nervous system (Samad *et al*., 2004), the kidneys' epithelial cells, the ovaries, and the testes (Samad *et al*., 2005; Xia *et al*., 2005). Recently, Dragon (RGMa) and the other two RGM family members, repulsive guidance molecule (RGMb) and hemojuvelin (RGMc), have all been identified as co-receptors that enhance BMP signaling via increased utilization of BMP type II receptor (ActR-2A) by BMP-2 and BMP-4 (Samad *et al*., 2005; Xia *et al*., 2005; 2007; 2008).

Bmpr2 mutant mice (homozygote) arrest at the egg cylinder phase and fail to form the mesoderm, which emphasizes the importance of BMPR-2 and demonstrates the need for BMP signal transductions in initiation of the mesoderm (Beppu *et al.*, 2000). Similarly, mice deficient *Bmpr1b* showed irregular oestrous cycles and displayed a pseudo pregnancy response, suggesting that BMP signaling via this receptor is vital for normal reproductive development in mice (Yi *et al*., 2001).

The levels of BMPR-1A and BMPR-2 mRNA in hamster ovaries are radically higher than BMPR-1B during ovarian development, and the levels of BMPR-1A and BMPR-2 mRNA are significantly increased on embryonic day 14 before declining through to postnatal day 5 and 6. Furthermore, the level of BMPR-2 mRNA was high during postnatal day 7 as well, while the level of BMPR-1A mRNA increased during postnatal day 8 with primordial follicle formation (Wang & Roy, 2009). BMPRs proteins expression were low in oocytes and somatic cells during embryonic development (day 13), yet they gradually increase throughout postnatal growth. The presences of previous pattern of BMP receptors expression throughout perinatal ovarian growth indicate a regulatory role of BMP family during the formation of primordial follicles (Wang & Roy, 2009).

1.2.3.2 BMP intracellular signaling

BMP family members exert their signals by binding to two kinds of receptors (type I and type II); in both cases this binding is crucial for ligand signaling. A heteromeric complex forms after BMP members bind to their receptors, after which the type II receptors phosphorylate the type I receptors. The type I receptors then transduce the signal through phosphorylating a group of proteins called SMADs (Heldin *et al.*, 1997). According to their structure and

function, SMAD proteins can be categorized into three groups known as: receptor regulator Smads (R-Smads), common partner Smads (Co-Smads), and inhibitory Smads (I-Smads). R-Smads can be classified into two subclasses: namely Smad2 and Smad3, which are activated by TGF-β and activin receptors (Macias-Silva *et al*., 1996), and Smad1, Smad5 and Smad8, which are activated by BMP receptors (BMPR-1A or BMPR-1B) (Nishimura *et al*., 1998; Kawai *et al*., 2000). SMAD proteins have two highly conserved regions, an N-terminal Mad Homology1 domain (MH1) and a C terminal Mad Homology2 domain (MH2), with a less conserved linker region separated from the two highly conserved regions (Kawabata *et al*., 1998).

In vitro, SMAD proteins exist as monomers and have the ability to form homotrimers, or heterotrimers. The formation of Smad oligomers is important for signal transduction of the BMP family members (Kawabata *et al*., 1998). After receptor activation and phosphorylation of R-Smads, R-Smads interact with the Co-Smad (Smad4) to form a hetero-oligomer complex (Kretzschmar *et al.,* 1997). The hetero-oligomer complex then translocates into the nucleus and modulates the transcription of the objective gene. Figure (1-2) illustrates the BMP signaling pathway.

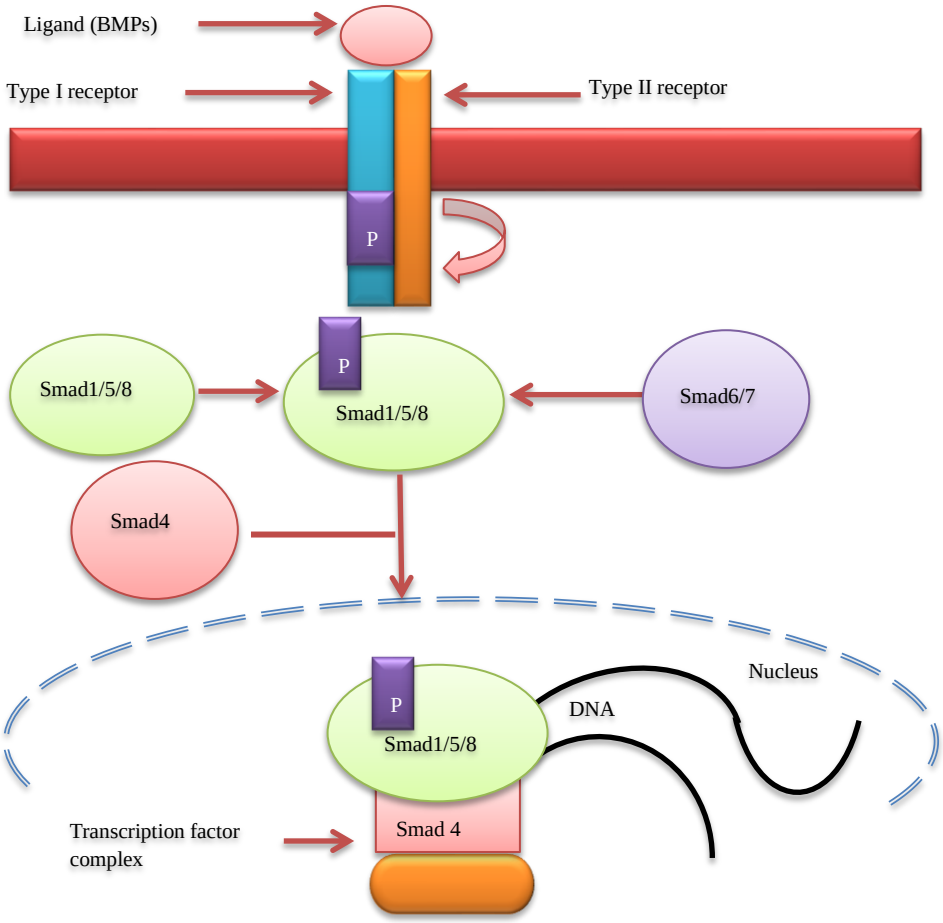

Figure 1-2: Diagram showing the BMP signalling pathway. BMP ligands bind to its receptors and stimulate Smad1/5/8 phosphorylation. Activated Smads (Smad1/5/8), form heteromeric complexes with Smad4 and regulate the expression of target gene.

The BMP signaling pathway is regulated by different molecules, including inhibitory Smads, some of which (Smad6 and Smad7) antagonize the BMP/TGF-β signaling pathway by various mechanisms at several levels. First, both Smad6 and Smad7 interact with the activated type I receptors to prevent the phosphorylation of R-Smads (Imamura *et al.*, 1997; Nakao *et al.*, 1997). Second, Smad6 has been shown to have another mechanism for blocking the BMP/Smad signaling by competing with Smad4 for binding to Smad1, which forms an inactive Smad1–Smad6 complex (Hata *et al.*, 1998). In addition, the N-terminal domain of

Smad7 interacts with its MH2 domain and inhibits the signaling of the TGF-β super family (Hanyu *et al.*, 2001), and finally, Smad7 induces degradation of the BMP type I receptor via recruitment of Smurfs (Ebisawa *et al*., 2001). Smurf1 and Smurf2 are two closely related factors that have been identified as Smad ubiquitination mediator factors (Zhu *et al*., 1999; Zhang *et al*., 2001a). This group of enzymes, which contains HECT catalytic domains characteristic of E3-ubiquitin ligases, regulate BMP singling by selectively interacting with the activated type I receptors (Ebisawa *et al*., 2001), or by interacting with R-Smads and inducing the degradation and hence their inactivation (Zhu *et al.*, 1999).

In addition to inhibitory Smads (Smad6 and Smad7), there is another intracellular inhibitory factor for the BMP signaling pathway, termed BAMBI. BAMBI is a transmembrane glycoprotein that, co-expressed with BMP-4 in *Xenopus* embryogenesis, can negatively regulate the BMP signaling pathway by acting as a pseudo-receptor and binding to different BMP ligands without forming receptor complexes necessary for cell signaling (Onichtchouk *et al.*, 1992).

Ski, SnoN, and Tob are other inhibitory proteins that can negatively regulate BMP signaling. Ski is a nuclear protein that is required for the successful growth of skeletal muscles (Berk *et al.*, 1997; Nagase *et al.*, 1990). Homozygous Ski deficient mice showed a reduction in skeletal muscle mass, yet this defect did not affect reproductive function (Berk *et al.*, 1997). Ski could block BMP signaling in *Xenopus* and mammals in several ways, firstly by interrupting the complex of Smad1 and Smad4 by binding to the MH2 domains. Additionally, Ski might inhibit Smad1 by stimulating the expression of the inhibitory Smad6 or Smad7 (Wang *et al*., 2000). Finally, Ski has the ability to interfere directly with Smad2, Smad3, and Smad4 on a TGFβ-responsive promoter element and inhibit their function to activate

transcription signals by recruiting the nuclear co-repressor N-CoR and its associated histone deacetylase complex (Luo *et al*., 1999).

The SnoN protein has also been shown to have the same function as Ski. SnoN might work in a negative feedback control of the TGF-β- signaling by simultaneously interacting with the R-Smads (Smad2 and Smad3) and with the Co-Smad (Smad4), blocking their transactivation ability and inhibiting TGF-β signal transduction (Stroschein *et al*., 1999; Wu *et al*., 2002).

Tob is one of the emerging family of anti-proliferative proteins consisting of Tob, Tob2, BTG1, BTG2 in humans, (TIS21 in mouse and PC3 in rat), and ANA (BTG3 in mouse) (Bradbury *et al*., 1991; Fletcher *et al*., 1991; Rouault *et al*., 1992; Matsuda *et al*., 1996; Rouault *et al*., 1996; Guehenneux *et al*., 1997; Yoshida *et al*., 1998; Ikematsu *et al*., 1999). An earlier study of Tob protein activities has shown that Tob can interact with BMP receptor-regulated Smad1, Smad5 and Smad8, as well as with Smad4 (Yoshida *et al*., 2000). An alternative mechanism whereby Tob can antagonize the BMP signaling is by interacting with Smad6 and Smad7 and enhancing the interaction of inhibitory Smad6 with the activated BMP receptor type I to inhibit BMP signaling in *Xenopus* embryos and in cultured mammalian cells (P19 cells) (Yoshida *et al*., 2003).

1.2.3.3 Healing roles of BMPs

A large body of research has shown that the BMP family has a regulatory function in bone and cartilage stimulation, maintenance and repair. Recombinant human BMP-2 implantation in rats induced both cartilage and bone formation, with bone formation being observed after five days when BMP-2 was used in high doses (Wang *et al*., 1990). Both BMP-2 and BMP-6

have a similar expression in hypertrophic cartilage, and BMP-2 may compensate for the absence of BMP-6 function in BMP-6 knockout mice (Solloway *et al*., 1998).

It has been proposed that BMP-6 could play a crucial role in endochondral bone formation (Ebisawa *et al.*, 1999; Gitelman *et al.*, 1994). BMP-6 signaling pathway is involved in osteoblast differentiation as shown by a strong induction of alkaline phosphatase activity in mouse osteoblast cell lines including undifferentiated mesenchymal cells (C2C12) and osteoblastic or osteoprogenitor cell lines (MC3T3-E1 cells and ROB-C26 cells) (Ebisawa *et al.*, 1999). Similarly, BMP-3 and BMP-4 have the ability to stimulate the formation of cartilage *in vivo* (Wozney *et al*., 1988). Furthermore, when Sprague-Dawley rats were immunized with BMP-9, all animals showed endochondral bone formation within three weeks of treatment (Varady *et al.*, 2001). *In vivo*, BMP-7 has the ability to repair cartilage in different models of articular cartilage degradation including chondral defects, focal osteochondral defects, and osteoarthritis, which suggests that BMP-7 has potential as a therapeutic for cartilage repair (Chubinskaya *et al.*, 2007). Likewise, there was greater repair of tibial fractures when BMP-7 was implanted with a type 1 collagen carrier in human clinical cases (Friedlaender *et al*., 2001).

The capability of BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, and BMP-7 to stimulate DNA synthesis in bone cell cultures was compared in periosteal cells and epiphyseal and sternal chondrocytes of embryonic chicks. BMP-2 and -4 showed the highest specific activity, while BMP-7 exhibited the lowest specific activity in these three tested cell types. BMP-5 and BMP-6 activity was temperately reduced in periosteal cells and significantly reduced in epiphyseal and sternal chondrocytes (Mayer *et al*., 1996).

1.3 TGF-β Ligands and the female reproductive system

1.3.1 Ovarian follicle development

The critical function of the ovary is the release of the mature oocyte for fertilization, and the production of steroid hormones that allows the development of female secondary sexual characteristics and helps the reproductive tract to support pregnancy (Peters, 1980). The ovarian follicle is the basic functional unit in the ovary, and is composed of the developing oocyte and somatic cells; the future of each follicle within the ovary is regulated by endocrine and paracrine factors (Gougeon, 1996).

Ovarian follicle development can be divided into three development phases based on their developmental stage and gonadotropin dependence. The first phase (gonadotropin independent phase) begins with the recruitment of primordial follicles, which contain oocytes surrounded by the flattened GCs; this stage continues with follicles growing until they become secondary follicles (McGee & Hsueh, 2000). The second phase starts when follicles progress from the preantral to early antral follicle stages (gonadotropin responsive phase), although the growth of these follicle does not require gonadotropins, and is enhanced by the presence of FSH (Kumar *et al*., 1997). The third phase is a continual progress from the early antral stage to ovulatory stage, which includes follicle recruitment, selection, and ovulation (gonadotropin dependent phase) (Fauser & Heusden, 1997). In cows and sheep, these phases of follicular development can be divided into five types, based on the number of GCs surrounding the follicle. Type 1 (primordial) follicles have one layer of flattened GCs, with type 1a (transitory) referring to follicles with one layer of a mixture of flattened and cuboidal GCs; type 2 (primary) follicles have one or two layers of cuboidal GCs; type 3 (preantral) follicles have two or three layers of GCs; type 4 (preantral) follicles have four to six layers of

GCs; and type 5 (early antral) follicles have more than five layers of GCs supported by the formation of an antrum (McNatty *et al*., 1999).

1.3.2 Factors affecting the development of primordial to primary follicle

The recruitment and growth of primordial follicles are significant features of female reproduction, and the progression from arrested primordial follicles to developing primary follicles is a vital process in ovarian biology. Mammalian ovaries have a pool of primordial follicles; every follicle has an oocyte arrested at the prophase stage 1 of the meiosis phase surrounded with a single layer of flattened GCs (Peters, 1980). The primordial pool either develops through fetal life in some species, such as primates and ruminates, or develops through the early stages of neonatal life, as can be seen in rodents and rabbits (Peters, 1980). Once the growth of the primordial follicles begins, it continues until the follicles reach one of two stages, ovulation or atresia (Skinner, 2005).

Earlier studies have proposed that there is a multiparty system of cells – cell exchanges that are required to organize the alteration of primordial to primary follicles stages. Kit ligand (KL also called stem cell factor (SCF)) (Parrott & Skinner, 1999), basic fibroblast growth factor (BFGF) (Nilsson *et al.*, 2001), leukemia inhibitory factor (LIF) (Nilsson *et al*., 2002), BMP (Lee *et al*., 2001; Nilsson & Skinner, 2003), keratinocyte growth factor (KGF) (Kezele *et a*l., 2005), and AMH (Durlinger *et al.*, 2002) have all been shown to regulate primordial follicle development (Figure 1-3).

1.3.2.1 Kit ligand (KL)

Kit ligand, also called stem cell factor is expressed on granulosa cells, its receptor tyrosine kinase c-kit is expressed on oocyte and theca cells (Zsebo *et al*., 1990; Nilsson & Skinner, 2004). KL has a broad range of effects on a variety of cell types including cell proliferation, cell migration and cell survival (Besmer, 1991). KL has been found to stimulate the initiation and development of primordial follicles. When four-day-old rat ovaries were treated with KL for 4 and 15 days, primordial follicle recruitment and development into primary and preantral follicles increased (Parrott & Skinner, 1999). In the early stage of follicle formation, the KIT-receptor-KL interaction has been shown to control the growth of oocytes and theca cells, as well as regulate the ability of oocytes to go through cytoplasmic maturation in large antral follicles. Furthermore, the KIT receptor-KL interaction is also required for the formation of an antral cavity (Driancourt *et al*., 2000).

It has been shown that the physiological roles of KIT receptor and KL may not have the same effect in different species, as recombinant mouse KL in ovarian cell culture promoted oocyte growth in both rabbits and mice and stimulated the growth of primordial follicles in mice but not in rabbits. KL had no effect on the follicle survival in mice or rabbits (Hutt *et al.*, 2006). In pig primordial oocyte culture, KL did not enhance primordial follicle development but did enhance primordial follicle survival (Moniruzzaman & Miyano, 2007).

Carlsson *et al.* (2006) showed that in humans KL is expressed in the GCs of primary follicles, while c-kit is expressed in GCs of primary follicles, secondary follicles, and oocytes. Carlsson *et al.* (2006) examined the effect of c-kit and KL on early human follicle development by adding a c-kit antibody (ACK2) in human ovarian tissue culture. They found a significant increase in atretic follicles when ACK2 was added to the culture, whereas adding KL to the

culture did not change the proportion of atretic follicles nor did it influence the developmental stages of the ovarian follicle. Blocking c-kit function leads to disturbed initiation of primordial follicle growth and the formation of follicular fluid in preantral follicles (Yoshida *et al*., 1997). The c-kit mRNA has been detected in all types of goat ovarian follicles, and the addition of KL to cultured preantral follicles in the absence of FSH promoted follicle survival and antral cavity formation (Lima *et al*., 2011). Magamage *et al.* (2011) showed that in primordial follicles cultured in media supplemented with KL, KL promoted follicle survival but had no effect on follicular development.

1.3.2.2 Basic fibroblast growth factor (bFGF)

The basic fibroblast growth factor (bFGF) gene has been expressed in rat ovaries during follicle growth and the expression of BFGF is cell specific, as no bFGF mRNA was detected in granulosa cells (Koos & Olson, 1989). A study by Ergin *et al*. (2008) found that bFGF protein is readily detected in the oocytes of young and adult rats (Ergin *et al.*, 2008) and bFGF mRNA is clearly detectable in bovine follicles during the final growth to preovulatory follicles (Berisha *et al.*, 2000). bFGF has also been shown to be present in follicular and peritoneal fluids of women; however, the concentration of bFGF in these fluids did not change around ovulation or in the presence of endometriosis (Seli *et al*., 1998). When two-cell murine embryos are treated with bFGF, basic FGF has an inhibitory effect on embryonic growth because it reduces the rate of blastocyst development and embryo hatching (Seli *et al.*, 1998).

bFGF has been found to stimulate the growth of primordial to primary follicles; the addition of bFGF to cultured rat ovaries increased the number of developing follicles compared with untreated ovaries (Nilsson *et al.*, 2001). Recently, it has been demonstrated that bFGF

improves the growth and survival rate of individual human early follicles after 8 days in culture. The percentage number of primordial and primary follicles was significantly lower in the presences of 200 ng bFGF/ml, although a significant increase was reported in the percentage of follicles in the pre-antral stage in the same group than in the group without bFGF (Wang *et al*., 2014). Moreover, the addition of bFGF in cultured rat ovarian tissue has been shown to inhibit follicle apoptosis (Tilly *et al*., 1992). The number of developing follicles in human ovaries cultured in media with BFGF was significantly higher than uncultured ovaries and the addition of a neutralizing antibody for bFGF significantly reduced estradiol (E2) secretion (Garor *et al.*, 2009), which indicates that basic FGF may have a modulatory role in the E2 production of early antral follicles.

1.3.2.3 Leukemia inhibitory factor (LIF)

Leukemia inhibitory factor is a glycoprotein which was initially identified based on its ability to enhance differentiation and inhibit proliferation of the murine myeloid leukemia cell lines M1 (Hilton *et al*., 1988). The expression of LIF has been identified in many species such as mice and humans during implantation and the LIF receptor is expressed in the uterine endometrium tissue, oocytes, and blastocysts (Lass *et al.*, 2001; Cullinan *et al*., 1996). LIF expressed by theca cells is necessary to promote embryo implantation as well as uterus decidualization, since neither of these occur in the absence of LIF (Stewart & Cullinan, 1997). In addition, it has been found that LIF improved oocyte nuclear and cytoplasmic maturation subsequent embryonic development in dose-dependent manner during *in vitro* bovine oocyte development (Mo *et al*., 2014).

Immunocytochemical studies showed higher levels of LIF protein in the GC and surrounding somatic cells of primordial and primary follicles in contrast to the oocyte; however, later pre-antral and antral stage follicles exhibited LIF expression mainly in the oocyte of rats (Nilsson *et al*., 2002). LIF has been also found to be present in both preovulatory and growing follicles and its level changes during different stages of follicular maturation. LIF concentrations have been shown to be higher in preovulatory follicles compared with growing follicles in human follicular fluid (Coskun *et al.*, 1998).

LIF stimulates the transition from primordial to primary follicles in four-day-old rat ovarian culture (Nilsson *et al*., 2002), and LIF treatment increases the mRNA expression of KL in the cultured GCs which might indicate that it interacts with KL to stimulate primordial follicle development (Nilsson *et al*., 2002). Likewise, an *in vitro* study by da Nóbrega *et al*. (2011) showed that LIF stimulates primordial follicle development, GC formation, and differentiation and maintenance of preantral follicle survival for seven days in culture, using goat ovaries as a model (da Nóbrega *et al.*, 2011). *In vitro,* the combination of LIF and stem cell factor (SCF) positively influences the survival of primordial germ cells (PGCs) in mouse cultured ovaries (Morita *et al*., 1999) and has a significant effect on the maturation and cleavage rate of mouse oocytes (Amiri *et al.*, 2009).

1.3.2.4 BMP family

Locally produced factors such as BMP-4 and BMP-7 are reported to influence the transition of primordial follicles to primary follicles (Nilsson & Skinner, 2003; Lee *et al*., 2004). Glister *et al.* (2004) indicate that both BMP-4 and BMP-7 can be expressed by theca cells in the ruminant ovary and the theca cell layer of rat follicles (Shimasaki *et al.*, 1999). The level of

BMP-4 and BMP-7 mRNAs changes during follicle development in ovaries of adult cycling rats, a weak BMP-4 signal was first seen in primary follicles, while in secondary follicles, BMP-4 mRNA was detectable at moderate levels in the cells of theca externa and in theca interstitial cells, then the expression of BMP-4 mRNA reached high levels in theca cells of developing dominant follicles (Erickson & Shamasaki, 2003). However, the mRNA expression of BMP-7 was first seen in theca cells of developing follicles when the second layer of granulosa cells appeared; BMP-7 mRNA was expressed at high levels in the theca cells of developing dominant follicles (Erickson & Shamasaki, 2003). In the reproductive system of mice, BMP-4 has been observed to be expressed during all follicle stages in the uterus and oviductal epithelium (Tanwar & McFarlane, 2011). In addition, both BMP-4 and BMP-7 and their receptors (BMPR-1A, BMPR-1B and BMPR-2) have been found to be expressed in the oogonia /oocytes and stroma cells of fetuses and adult human ovaries (Abir *et al.*, 2008).

Exogenous BMP-4 increased the number of developing primary follicles in neonatal rat ovarian tissue (Nilsson & Skinner, 2003). Moreover, BMP-4 has been shown to maintain the transition of primordial to primary follicles and thus be a crucial factor for oocyte survival (Nilsson & Skinner, 2003). In support of these findings, Tanwar *et al*. (2008) found a decrease in the number of primary follicles and an increase in the number of primordial follicles after seven days of immunization against BMP-4 in mice (Tanwar *et al*., 2008). The *in vivo* injection of recombinant BMP-7 into the ovarian bursa of rats increased the number of primary, preantral, and antral follicles, but decreased the number of primordial follicles (Lee *et al*., 2001). Additionally, *in vitro,* BMP-7 stimulates the development of follicles in neonatal mouse ovaries after four days in the presence or absence of FSH (Lee *et al*., 2004).

Using postnatal rat ovaries in culture, Nilsson and Skinner (2002) have shown that GDF-9 stimulates the progression of early primary follicle development; however, it did not affect primordial follicle development. In contrast, the *in vivo* study by Vitt *et al*. (2000a) found that GDF-9 induced the transition of primordial and primary follicles to further stages in immature female rats. GDF-9 is expressed in the oocyte of follicles from different mammalian species, such as humans, pigs and mice (Aaltonen *et al.*, 1999; Bodensteiner *et al.*, 1999; Shimizu *et al.*, 2004). There is no progress beyond the primary follicle stage in GDF-9 null mice (Dong *et al.*, 1996) or ewes homozygous for GDF-9 mutation (Galloway *et al*., 2000). Moreover, knockout mice lacking GDF-9 are infertile (Yan *et al.*, 2001). An *in vitro* study by Wang and Ray (2004) provided direct evidence that GDF-9 stimulates the formation of primordial follicles during perinatal development and their subsequent growth in hamster ovaries in the presence of FSH and SCF. Further, this study indicates that FSH plays an essential part in the differentiation of somatic cells into GCs by regulating the production of GDF-9 and SCF by oocytes and somatic cells, respectively (Wang & Ray, 2004).

1.3.2.5 Keratinocyte growth factor (KGF)

Keratinocyte growth factor (KGF), also well-known as fibroblast growth factor-7, is a mesenchymal-derived growth factor (Rubin *et al*., 1995). KGF is a 28-kDa protein that belongs to the heparin-binding fibroblast growth factor family, which has been found to induce epithelial cell proliferation (Finch *et al.*, 1989) and promote ovarian follicular development (Parrott & Skinner, 1998). Further, it has been shown that KGF, hepatocyte growth factor, and KL are all crucial for growth of the ovarian surface epithelium (Parrott *et al.*, 2000). KGF is expressed by GCs, theca cells, stroma cells and oocytes in all follicle developmental stages in goat ovaries (Faustino *et al.*, 2011). It has also been implicated as a

positive regulator of the primordial to primary follicle transition, and exerts its effects by up-regulated KL mRNA expression in GCs in four-day-old rat ovary organ cultures (Parrott & Skinner, 1999; Kezele *et al*., 2005). A novel positive feedback loop between GCs and theca cells are mediated by KL and KGF. KGF has the ability to stimulate GC-derived KL expressions which support primordial follicle development, and KL can stimulate KGF expression (Kezele *et al*., 2005). This model of action for these growth factors is supported by a previous behavior of KL as a theca cell organizer that stimulates thecal cell growth and differentiation (Parrott & Skinner, 2000), and theca cells have been found to produce KGF that stimulates the primordial to primary follicle transition (Parrott & Skinner, 1999).

1.3.2.6 Anti Mullerian hormone (AMH)

As one of many local growth factors that control follicular differentiation, Mullerian inhibiting substance (MIS), also termed anti-Mullerian hormone (AMH), is a member of the TGF-β super family (Cate *et al*., 1986). In females, AMH is expressed by GCs of preantral and early antral follicles. Modi *et al.* (2006) found that AMH is expressed at high levels in a small number of oocytes/oogonia of human fetal ovaries. AMH mRNA is expressed at low levels in the primordial follicles, which increases in the primary and secondary follicles in adult human and monkey ovaries (Modi *et al.*, 2006). The highest expression of AMH has been found in GCs of secondary, preantral, and small antral follicles, but this expression slowly disappeared in large antral follicles (Weenen *et al.*, 2004). The high expression of AMH in GCs after ovulation might indicate that it plays a regulatory role in follicle development. Correspondingly, a high concentration of AMH has been found in the follicular fluid of women with polycystic ovary syndrome (PCOS) as compared with normal controls. High levels of AMH in PCOS patients are most likely due to the increased secretion of AMH

by individual follicles rather than an increase in small antral follicles (Das *et al.*, 2008). Recently, it has been hypothesized it is suggested that the high AMH concentrations present in women with PCOS play an essential role in causing anovulation due to its inhibitory effect on the actions of FSH which generally stimulates follicular development from the small antral to the ovulatory stage (Homburg & Crawford, 2014).

In women with premature ovarian failure (POF) the expression of AMH is decreased in early antral follicles, which may suggest a defect in the development of antral follicles (Meduri *et al.*, 2007). In the marmoset ovary, the expression of AMH protein decreases in early preantral follicles in the presence of gonadotropins antagonist (Thomas *et al.*, 2007). This indicates the role of gonadotropins in the regulation of AMH expression in growing follicles.

AMH has an inhibitory role on the initiation of primordial follicle development. In cultured mouse ovaries, there is a reduction in the number of developing follicles compared with control ovaries in the presence of AMH (Durlinger *et al.*, 2002). It has been shown that AMH also inhibits the imitation of follicle development in bovine ovaries (Gigli *et al.*, 2005). Intriguingly, in a whole-ovary culture system, it has been found that treatment with Gremlin 1 (GREM1) and GREM2 (endogenous BMP inhibitors) reverse the inhibitory effect of AMH on primordial to primary follicle transition in rat ovary. GREM1 and GREM2 treatment also led to insignificant numbers of differentially expressed genes, proposing that the direct effects of GREM2 or GREM1 seem to be at the level of protein–protein interactions, rather than immediate effects on the cells (Nilsson &Skinner, 2014).

Using an *in vitro* follicle culture system, FSH-stimulated growth of preantral follicles was inhibited by the presence of AMH in a time dependent manner and there was no effect of AMH on follicle survival rate (Durlinger *et al.*, 2001). This result has been subsequently supported by an *in vivo* study that showed an increased number of growing follicles in

response to exogenous FSH in immature AMH-deficient females compared with their wild type mice (Durlinger *et al.*, 2001).

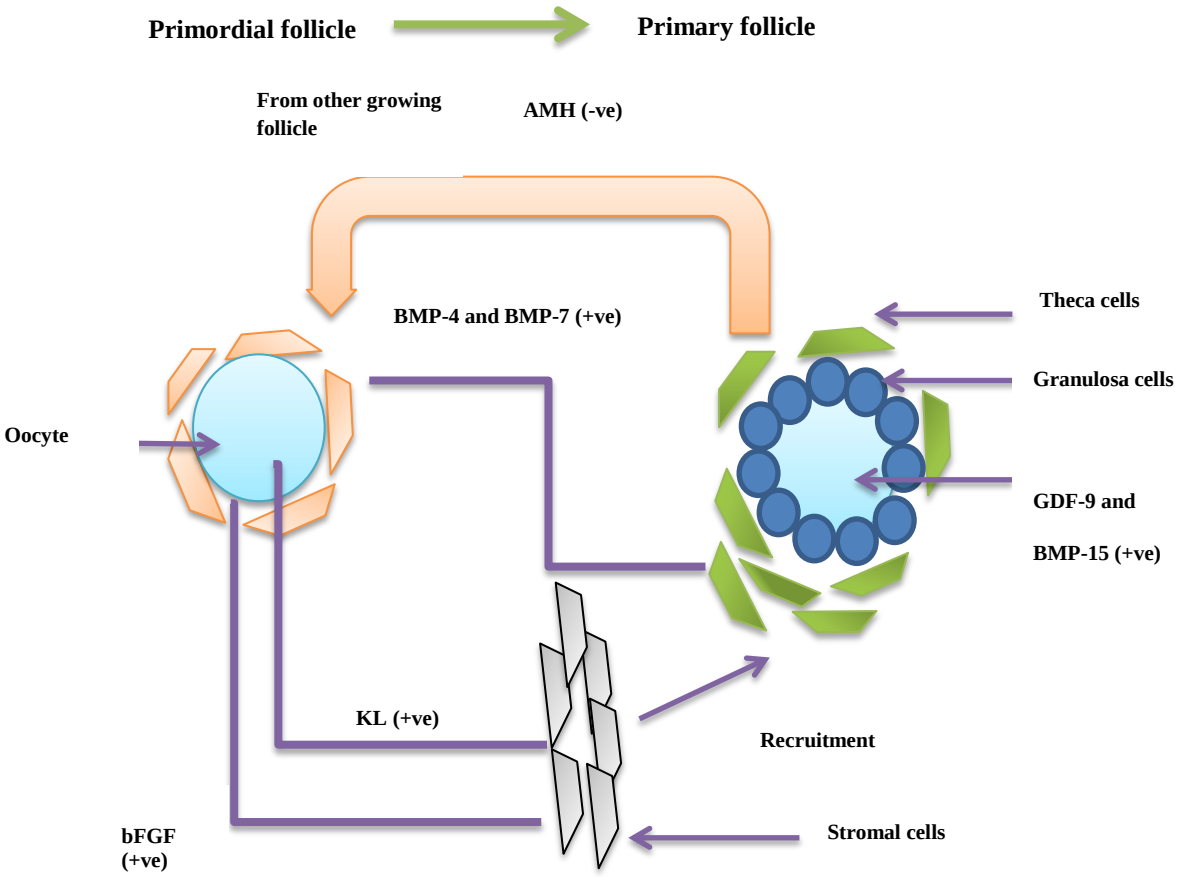

Figure 1-3: Possible signaling interactions involved in the transition of the primordial-to-primary follicle. Modified from Knight & Glister, 2006.

The growth of the primary follicles to late preantral follicle stage occurs through the growth of oocytes, creation of the zona pellucida, expansion of GCs, formation of a basal lamina, and a reduction of stromal cells around the basal lamina to create an enclosing theca layer (Knight & Glister, 2006). In addition, there is an enlargement of a fluid-filled space that slowly joins together to form a signal antral cavity. As the preantral follicles develop, there is also an

increase in oocyte size and a continuation of the proliferation of GC and theca cells (Knight & Glister, 2006).

Follicular growth beyond the late-preantral/small-antral stage depends on the pituitary gonadotropin stimulating hormone (FSH) and luteinizing hormone (LH), which are secreted by the pituitary gland (McNeilly *et al*., 1992). The regulation of LH and FSH secretion depends on hypothalamic gonadotropin releasing hormone (GnRH) (Dalkin *et al*., 1999). FSH and LH exert their effects on ovarian cells by binding particular receptors (FSHR, LHR), the membrane receptor for FSH is expressed on GCs from primary to preovulatory stages of a follicle's growth (McNatty *et al*., 1999), while the LH receptor is expressed on the theca interna at the tertiary stage of expansion (Richards, 1994). In LH receptor knockout mice (7-12 weeks old) follicles are able to develop up to the early antral stage (Zhang *et al.*, 2001b). The addition of recombinant LH in an *in vitro* culture system of mouse preantral follicles significantly enhances the oocyte's meiotic maturation and the formation of the antral cavity (Cortvrindt *et al*., 1998). Similar to the LH receptor knockout, follicle development stopped at the preantral stage in FSH receptor knockout mice (Abel *et al.*, 2000).

The peak of the cyclic FSH surge occurs when the diameter of the dominant follicle reaches 4mm, and an initial decline in FSH concentration occurs when the diameter of the dominant follicle reaches approximately 6mm (Ginther *et al.*, 1996). Dominant follicle selection ends during the period when FSH concentration declines, which occurs between days three and five of the oestrous cycle in heifers (Mihm *et al.*, 1997). Furthermore, the decline in FSH also causes dramatic changes in FSH dependent growth factors and hormones within the preselected follicle cohort. During this period, the intrafollicular estradiol increased > 5 and total insulin-like growth factor I (IGF-I) increased 1.3 fold in dominant follicles. Simultaneously, the total inhibin-A decreased 1.8 fold (Mihm *et al.*, 1997) and the exogenous

FSH administered on days two and three of the oestrous cycle delayed selection of the dominant follicle and atresia of subordinates follicles and inhibited most of the abovementioned alterations in hormones and growth factors, suggesting that the decline in FSH concentration has a vital role in follicle selection by generating follicle dominance (Mihm *et al.*, 1997).

Other factors involved in the regulation of FSH production include steroid hormones, inhibin, activin, follistatins, and BMPs (Knight & Glister, 2001; Otsuka & Shimasaki, 2002b). Inhibin and follistatins suppress FSH secretion while activin enhances it (Carroll *et al.*, 1989; De Kretser *et al.*, 2000; Knight, 1996). Inhibins and activins were first isolated from ovarian follicular fluid and are known by their ability to regulate FSH secretion from pituitary cells (Ling *et al*., 1985), but now they are also known to have a wide range of effects in a wide variety of cell types, including cell proliferation, differentiation, and endocrine function (Phillips, 2005). Lu *et al.* (2009) showed that the addition of inhibin- A to cultured rat GC can suppress FSH by inhibiting FSH-induced FSHR mRNA level. In addition, BMP-4 can reduce FSHβ mRNA expression and FSH levels in ewe pituitary cell culture and inhibit the stimulatory effect of activin on FSH secretion *in vitro* (Faure *et al.*, 2005). Follistatin is another FSH-suppressing protein, isolated from ovarian follicular fluid, which has been shown to bind activin with high affinity, thereby bio- neutralizing the FSH stimulatory actions of activin (Nakamura *et al*., 1990).

Though it is evident that ovarian function is regulated generally by the pituitary gonadotropins FSH and LH and their receptors (FSHR, LHR), it is also proven that locally produced factors such as peptides, steroid hormones, and growth factors have regulatory roles in ovarian follicular development (Fortune, 1994). One of these growth factors is GDF-9, which has been shown to be expressed in GCs but not in theca cells, and GDF-9 mRNA

appears to be high in GCs of small rather than large follicles (Spicer *et al*., 2008). It has been suggested that bovine GCs are targets for GDF-9 and that GDF-9 has stimulatory roles in promoting the proliferation of GCs throughout follicle development (Spicer *et al*., 2006). Further, the proliferation of theca cells is stimulated by GDF-9 in the presence or absence of insulin-like growth factor 1(IGF-1), and the stimulatory effects of GDF-9 are more obvious in theca cells from large follicles than theca cells derived from small follicles. GDF-9 increased theca cell numbers in the presence of both LH and IGF in small theca cell culture (Spicer *et al*., 2008). The injection of GDF-9 gene fragments into pig ovaries increased the number of primary, secondary, and tertiary follicles (Shimizu *et al.*, 2004).

GDF-9 plays an important role in stimulating follicular growth from the preantral to early antral period via the up-regulation of follicular androgen biosynthesis (Orisaka *et al.*, 2009). *In vitro* studies have displayed that androgens stimulate the primary/secondary follicle transition in bovine, the preantral follicle growth and granulosa cell mitosis in mice, and ovarian tissue survival in humans (Murray *et al*., 1998; Otala *et al*., 2004; Yang & Fortune, 2006). A positive interaction between androgens and FSH effects in primate follicle development has been established by Weil *et al*. (1999). Androgens stimulate the FSH action in the follicles by increasing FSH receptor expression, FSH-induced GC aromatase activity and proliferation, and follicular growth in adult rhesus monkeys (Weil *et al*., 1999). Moreover, GDF-9 enhances FSH-induced preantral follicle growth in follicles cultured from 14-day-old rats and treatment with GDF-9 in the presence of FSH inhibited the apoptosis in follicular culture (Orisaka *et al*., 2006).

BMP-15 is an oocyte specific growth factor that can promote GC proliferation (Otsuka *et al.*, 2000), by working together with GDF-9 to regulate gonadotropin production, which stimulates the differentiation of GCs in mammalian ovaries. When ovine BMP-15 and GDF-9

(murine and/or ovine) are added together into rat GC culture, the production of FSH-stimulated progesterone is inhibited. However, neither murine GDF-9 nor BMP-15 (ovine) can regulate FSH-stimulated progesterone production individually. Furthermore, the level of α-inhibin from GCs increases more than 15 fold when ovine BMP-15 and GDF-9 (either murine or ovine) are added together compared with the control (McNatty *et al*., 2005).

The development and maturation of preantral follicles in culture is enhanced also by the presence of LIF (Haidari *et al.*, 2006). In preantral follicle culture, LIF increases the diameter of follicles without increasing the rate of survival (Haidari *et al.*, 2008). In addition, an increase in the expression of KL and c-kit in GCs of preovulatory and small antral follicles of mouse ovaries in response to KL treatment has been demonstrated. The treatment of cultured cumulus-oocyte complexes with KL improves the extrusion of the first polar body; furthermore, this has been associated with an increase in cyclin B1 synthesis, which is essential for the sequence of meiotic maturation. In contrast, KL has no effect on cytoplasmic maturation in cultured preovulatory follicles (Ye *et al.*, 2009).

Another factor is vascular endothelial growth factor (VEGF), which is expressed by GCs and has been found to increase slowly during follicle growth (Greenaway *et al.*, 2005). The short-term injection of VEGF antibodies during the late follicle stage in monkeys causes a significant decline in inhibin-B levels which propose an arrest in the development of the group of recruited antral follicles. Antibody treatment also significantly lengthened the follicular phase compared with the control (Zimmermann *et al*., 2002). Hormonal therapy consisting of a combination of equine chorionic gonadotropin, human chorionic gonadotropin, and VEGF gene fragment injections into the rat ovary increased the number of large antral and preantral follicles. Moreover, the direct injection of VEGF gene fragments stimulates the

formation of thecal vasculature (Shimizu *et al.*, 2007). Collectively, these studies indicate that VEGF may have a regulatory role during follicle development.

1.3.3 Reproductive function of TGF-β family ligands

Reproduction is an essential process necessary for the survival of all species. BMP members play a vital role in the regulation of reproductive processes, and different BMPs have been found to be expressed in male and female reproductive tissues. The functional role of BMPs in the reproductive system was first documented by Shimasaki *et al.* (1999), who identified BMP-4 and BMP-7 mRNAs in the theca cells as well as the mRNAs of BMPR-2, BMPR-1A and BMPR-1B in the GCs of rat ovaries. In addition, the mRNAs encoding BMP receptors are expressed in the uterus of non-pregnant rats. BMPR-1A, BMPR-1B and BMPR-2 are expressed in the epithelium and BMPR-1A and BMPR-2 are expressed in the periluminal stroma and smooth muscle cells (Erickson *et al*., 2004). However, BMP-2 and BMP-7 are expressed in the periluminal stroma of the rat uterus and the expression of BMP-2, but not BMP-7, which has been shown to vary during the oestrous cycle (Erickson *et al*., 2004). BMP-7 is also expressed by the human placenta (Martinovic *et al.*, 1996). Furthermore, the mRNAs of BMP-2, BMP-4, BMP-6, BMP-7, and BMP-15 have also been found to be expressed in mouse and sheep pituitary glands (Faure *et al.*, 2005; Otsuka & Shimasaki, 2002b). BMP-5 is expressed by rat antral follicle GCs that enhance their proliferation, which was associated with an increase in cyclin D2 protein accumulation by 1.5 fold and a decrease in the expression of steroidogenic acute regulatory protein in GCs *in vitro*; the biological effects of BMP-5 on GCs can be inhibited by follistatin (Pierre *et al.*, 2005).

The ability of BMP-6 and BMP-7 to induce FSHβ synthesis was initially discovered using pituitary cell culture derived from transgenic mice carrying the ovine FSHβ promoter (oFSHβLuc). This transgene has been found to be expressed specifically in pituitary gonadotropes and in pituitary cultures (Huang *et al*., 2001a; Huang *et al.*, 2001b). The addition of BMP-6 and BMP-7 caused a six-fold increase in oFSHβ gene transcription and FSH secretion in primary pituitary cultures and transformed gonadotropes (LβT2 cells) (Huang *et al.*, 2001b). FSH synthesis was inhibited when rat and sheep pituitary cultures were treated with antibodies against BMP-7 (Huang *et al.*, 2001b). Similarly, BMP-15 was found to be expressed in the gonadotrope cell line LßT2 and treating mice cells with BMP-15 stimulated transcription of FSHβ subunit in LßT2 cells with no effect on the LHβ and GnRH receptor (Otsuka & Shimasaki, 2002b).

BMP-4 and BMP-7 have been found to act directly on GCs and modulate FSH signaling in positive and negative ways. Specifically, the BMPs enhanced estradiol production and attenuated progesterone production in the presence of FSH in rat GC culture, and these effects were dose and time dependent (Shimasaki *et al.*, 1999). In ovine GC culture, BMP-4 inhibited progesterone secretion by suppressing steroidogenic acute regulatory protein (StAR) expression at the mRNA and protein levels (Pierre *et al*., 2004). Similarly, an *in vitro* study using GCs obtained from Booroola sheep ovaries showed that BMP-4 inhibited progesterone synthesis (Mulsant *et al.*, 2001). Further, it has been suggested that BMP-4 can regulate activin and/or GnRH pathways, with BMP-4, GnRH, and activin working in a synergistic way to up-regulate FSHβ mRNA and FSH secretion. BMP-4 inhibited GnRH and/or activin-induced up-regulation of LHβ and reduced GnRH receptor mRNA, while it stimulated activin/GnRH-induced stimulation of FSHβ mRNA and had no effect on follistatin mRNA (Nicol *et al.*, 2008). Also, the addition of BMP-7 to cultured human granulosa cells increased

FSH receptor gene expression; however, BMP-7 decreased LH receptor gene expression and increased activin-βA and activin-βB gene expression (Shi *et al*., 2010).

BMP-6 has the same biological effect as BMP-4 and BMP-7, inhibiting FSH-stimulated progesterone synthesis (Otsuka *et al*., 2001a). Unlike BMP-4 and BMP-7, however, BMP-6 had no effect on estradiol production. Both BMP-4 and BMP-6 have been shown to decrease FSH release and FSHβ mRNA expression from ewe pituitary cells in a dose-dependent manner. By contrast, there are no inhibitory effects of BMP-4 and BMP-6 on LH secretion (Faure *et al.*, 2005). *In vitro* BMP-6 stimulates gene expression of FSH receptors, inhibin/activin β subunits, and AMH in human GCs, where BMP-6 was strongly present in the GCs of tertiary follicles (Shi *et al*., 2009a).

The inhibitory effects of BMP-2, BMP-4 and BMP-6 on FSH secretion from primary pituitary cells collected from Booroola ewes $FecX^{1}\backslash FecX^{1}$ was significantly higher compared with wild type animals (Young *et al.*, 2008). In addition, BMP-6 decreased FSH mRNA level and the co-treatment of cultured rat GCs with BMP-6 and forskolin caused a significant increase in cAMP production, which suggests that BMP-6 inhibits FSH activity by suppressing the action of adenylate cyclase (Otsuka *et al.*, 2001a).

BMP-15 has been found to reduce the levels of FSH receptor expression as well as to inhibit the LH receptor and inhibin/activin subunits in rat GCs (Otsuka *et al*., 2001b). The activity of BMP-15 is FSH independent, which means that it is involved in stimulating GC mitosis in preantral follicles during the FSH-independent phase of follicle development (Otsuka *et al.*, 2000). Another function of BMP-15 is stimulating KL expression in GCs, while KL inhibits the expression of BMP-15 in oocytes. This suggests that both BMP-15 and KL can form a negative feedback loop (Otsuka & Shimasaki, 2002a). Follistatin can also block BMP-15, thereby inhibiting its bioactivity and reducing GC proliferation (Otsuka *et al*., 2001b).

Another ligand that has a similar structure to BMP-15 is GDF-9, which has been found to enhance basal steroidogenesis in cultured rat GCs; in addition, GDF-9 suppressed FSH-dependent LH receptor expression and production (Vitt *et al*., 2000b). Unlike BMP-15, GDF-9 has been found to inhibit the expression of KL in GCs (Joyce *et al.*, 2000). Shi *et al.* (2009b) reported that BMP-9 increased the response of human granulosa-lutein cells to activin A by modulating the signaling pathway of activin and increasing the secretion of inhibin. In rat preantral follicle culture, GDF-9 increased preantral follicle growth by increasing follicular androgen biosynthesis. BMP-9 is also an important factor for CYP17A1 mRNA expression throughout the development of preantral to the early antral follicles (Orisaka *et al*., 2009).

1.3.4 TGF-β ligands and female infertility

It has been well documented that the BMP family ligands play an important role in follicle development and ovulation (Dong *et al*., 1996; Galloway *et al*., 2000; Takebayashi *et al*., 2000). Any defect in the TGF-β family function and their receptors results in many reproductive deficiencies, including infertility. For instance, the number of preantral and small antral follicles was reduced in 13-month old AMH null mice (Durlinger *et al*., 1999). Similarly, the lack of ovarian activin in mice leads to infertility (Pangas *et al.*, 2007), and the lack of *Bmpr1b* in mice also results in irregular oestrous cycles and a pseudopregnancy response. Although *Bmpr1b* mutants can produce oocytes that have the ability to be fertilized *in vitro*, the defects in cumulus cell development impede fertilization *in vivo* (Yi *et al*., 2001).

GDF-9 and BMP-15, which are expressed in oocytes, are necessary for female fecundity, as they play a significant role in the regulation of ovarian folliculogenesis and ovulation. GDF-9

deficiency in female mice leads to sterility as a result of a block in follicular development beyond the primary stages (Dong *et al.,* 1996). Neutralization of either BMP-9 or BMP-15 in sheep resulted in unusual ovarian morphology that included the presence of abnormally enlarged oocytes and abnormal changes in GC numbers or morphology (Hanrahan *et al.,* 2004; Juengel *et al.,* 2002). Furthermore, *in vivo* immunization against GDF-9 and BMP-15, alone or together, reduced ovarian follicular development and altered ovulation rates in cattle (Juengel *et al.,* 2009).However, in cultured media, it has been found that the continuing exposure to BMP15 (48-72 hour) caused a reduction in follicle size and atresia with increased granulosa cell apoptosis in mice preantral follicles. Interestingly, the atretogenic effect of BMP15 was refuted in the presence of GDF9 and a stimulatory impact on follicle growth was detected (Fenwick *et al.,* 2013). Collectively, these findings support the fact that both BMP-9 and BMP-15 are important for normal ovarian follicular development.

In sheep, mutations in BMP-15 or GDF-9 cause either sterility or increased ovulation rate depending on the number of altered alleles at each locus (McNatty *et al.,* 2004). Homozygous mutant ewes $FecX^{I}\backslash FecX^{I}$ result in a blockade of follicle development in the primary stage (Braw-Tal *et al.,* 1993), whereas heterozygous mutant ewes $FecX^{I}\backslash FecX^{+}$ increase the ovulation rate (Davis *et al.,* 1992). Recently, natural mutation in the *BMP15* gene in the Rasa Aragonesa breed has been identified, which consists of a deletion of 17 bp that leads to either infertility in homozygotes or high prolificacy in heterozygotes (Martinez-Royo *et al.,* 2008).

Mutations in both GDF-9 and BMP-15 have been hypothesized to contribute to human diseases such as PCOS and premature ovarian failure (POF) (Kovanci *et al.,* 2007; Takebayashi *et al.,* 2000). PCOS refers to ovulation, sterility and menstrual abnormalities in women (Hull, 1987), while POF is used to describe gonadal failure before 40 years of age (Davis, 1996). A natural mutation in human BMP-15 is linked with hyper-gonadotrophic

ovarian failure as well as with reduced GC growth (Di Pasquale *et al*., 2004). The level of GDF-9 mRNA but not BMP-15 mRNA is reduced in PCOS oocytes during their growth and differentiation phase compared with normal cyclic women (Teixeira Filho *et al.*, 2002). Zhao *et al.* (2009) found that GDF 9 expression in cumulus GCs was considerably lower in PCOS patients. In addition, a mutation in GDF-9 has been found in Caucasian woman who developed POF at age 22 (Kovanci *et al.*, 2007).

BMP-6 has been reported to be important for supporting the growth of ovarian follicles. Although BMP-6 is expressed in GCs of tertiary follicles of human ovaries, it is absent in atretic follicles (Shi *et al*., 2009a). BMP-6 has been reported to induce the expression of the inhibin/activin β subunit receptor, FSH, and AMH in human GCs (Shi *et al*., 2009a). The direct infusion of BMP-6 in ewe ovaries shows that the BMP-6 increases the secretion of inhibin A, androstenedione, and estradiol, yet has no effect on ovulation rate (Campbell *et al*., 2009). Finally, *Bmp6* knockout mice cause a reduction in litter size and ovaries appear to be less reactive to LH and hCG (Sugiura *et al*., 2010), which suggests that BMP-6 is essential for normal fecundity in female mice. BMP-7 is also involved in the reproduction system and there is evidence that it has an essential role in the developmental transition of primordial to primary follicles (Biyikli *et al*., 2005; Lee *et al*., 2004). *Bmp5* and *Bmp7* double deficient mice died at 10.5 days post-coitum and showed development defects in tissues known to co-express both BMP-5 and BMP-7 (Solloway & Robertson, 1999).

1.4 The effects of BMP mutation in sheep

Generally, during the menstrual or oestrous cycle, only one follicle is chosen to ovulate in humans, cattle, and many breeds of sheep. However, it has been discovered that high

prolificacy in some strains of sheep results from mutations in major genes (Table 1-2). The first main gene recognized for productivity in sheep was the fecundity Booroola gene (*FecB*), the effect of which was subsequently shown to be the result of a single amino acid substitution replacing a glutamine with an arginine at position 249 (Q249R) in the coding sequence of the BMPR-1B on ovine chromosome 6 (Wilson *et al*., 2001; Mulsant *et al.*, 2001). The genotypes of Booroola ewes are categorized as homozygous carriers $FecB^{B}/FecB^{B}$ with five or more ovulations per cycle, heterozygous carriers $FecB^{B}/FecB^{B+}$ with three or four ovulations and homozygous non-carriers $FecB^{B+}/FecB^{B+}$ with an ovulation rate of one or two (Davis *et al*., 1982).

The Booroola is one of the most popular fertile sheep breeds in the world and was acquired from a commercial sheep property known as 'Booroola,' near Cooma, New South Wales in 1958 (Bindon, 1984). The Indian Garole sheep that carry the *FecB* mutation are believed to be the source of the Australian Booroola mutation (Davis *et al*., 2002). The significant effects of *FecB* gene include an increase in ovulation rate of 0.9-1.8 ova and an increase in litter size of up to 1.0 lamb born per ewe lambing (Willingham & Waldron, 2000). In addition, the most reliable characteristics reported for Booroola ewes are that preovulatory follicles in homozygous (BB) and heterozygous (B+) carrier ewes have smaller diameters (i.e. 3–5 mm) compared with the wild type (++) ewes (> 5 mm diam.) (McNatty & Henderson, 1987). Follicles from Booroola ewes show an increase in their responsiveness to FSH, which is supported by augmented cAMP and progesterone production from cultured GCs (McNatty *et al*., 1986).

In Booroola ewes, the concentration of FSH is high during early life and during the oestrous cycle, while there is a lower inhibin concentration compared with Merino ewes (Cummins *et al.*, 1983; Davis, 2005). The expression levels of BMP-15, but not GDF-9, in heterozygous

ewes have been found to be significantly lower in all non-atretic follicles compared with homozygous ewes. This suggests that the high ovulation rate in heterozygous ewes is due at least in part to the lower levels of BMP-15 mRNA in oocytes which is linked to the mutation in the BMPR-1B gene compared with those in homozygous ewes (Crawford *et al.*, 2011).

The $FecB^{B}$ gene exerts its effect by increasing the ovulation rate by raising ovarian sensitivity to gonadotrophic stimulation rather than increasing the level of the gonadotrophic hormones, and this is characterized by 'precocious' maturation of ovarian follicles (Souza *et al.*, 2003). Therefore, there is a decrease in the diameters of small and medium follicles (< 3.5 mm and < 3.5-4.5 mm, respectively) and an increase in the large follicles (>4.5 mm) compared with wild type follicles (Campbell *et al.*, 2003), and this is linked with precocious ovarian GC differentiation (Fabre *et al.*, 2003). No differences were found under *in vitro* conditions between Booroola and wild type pituitary cells in their capability to stimulate the secretion of FSH in response to GnRH, BMP-2, BMP-4, BMP-6, and GDF-9. All these hormones have a tendency to suppress FSH release from ovine pituitary cells, and this inhibitory effect on FSH secretion is more obvious in pituitary cells collected from homozygous carriers in comparison with wild type animals (Young *et al.*, 2008).

Davis *et al.* (1991) provided evidence for a new key gene (the Inverdale gene (*FecX*)) that affect the ovulation rate in the Inverdale strain of sheep, a mutation in an oocyte-derived growth factor, BMP-15, improved the ovulation rate in sheep (Galloway *et al.*, 2000). The autosomal gene for BMP-15 is located on the X chromosome and the symbols for the alleles are I and +, so the homozygous female carriers are signified $FecX^{1}$ $FecX^{1}$, the heterozygous female carriers are signified $FecX^{1}$ $FecX^{+}$, and the non-carrier females are signified $FecX^{+}$ $FecX^{+}$. The ovulation rate of heterozygous ewes is twice as high as that of non-carrier ewes, while it has been found that homozygous ewes have non-functional “streak” ovaries and are

infertile (Davis *et al*., 1992). The other mutation in BMP-15 gene is the Hanna mutation ($FecX^H$) at nucleotide position 871 in the BMP-15 coding sequence (Galloway *et al*., 2000).

The effect of both Booroola and Inverdale mutations on heterozygous animals results in high ovulation rates – higher than the individual effect of each gene (Davis *et al*., 1999). It is thought that the interaction between the Inverdale and Booroola mutation might be a result of BMPR-1B being involved with BMP-15 signaling (Wilson *et al*., 2001). It has been found that in primary cultures of GCs from homozygous $FecB^+$ and $FecB^B$ ewes, the mutation of BMPR-1B, particularly the mutation of Q249R, is capable of modifying the BMPR-1B signaling pathway (Fabre *et al.*, 2003).

After identification of the Booroola and Inverdale mutations, a number of known prolific strains carrying putative major genes have been screened for the presence of the fecundity Booroola gene (*FecB*) using DNA testing (Davis *et al*., 2002). These breeds include the Javanese (Bradford *et al*., 1986), Woodlands (Davis *et al*., 2001), Cambridge (Hanrahan & Owen, 1985), Thoka (Jonmundsson & Adalsteinsson, 1985), Lacaune (Bodin *et al*., 1998), Olkuska (Martyniuk & Radomska, 1991), and Belclare (Hanrahan, 1991). The *FecB* mutation was found in Garole and Javanese breeds excluding Woodlands, Olkuska, Lacaune, Belclare, and Cambridge strains (Davis *et al*., 2002).

Later, another two novel mutations in the BMP-15 gene have been identified in Belclare and Cambridge strains (Hanrahan *et al.*, 2004), the added mutation in BMP-15 called $FecX^G$ (Galway) and $FecX^B$ (Belclare) are C to T and G to T changes at nucleotide 718 and 1100, respectively (Hanrahan *et al.*, 2004). $FecX^G$ causes a premature stop codon in the place of glutamic acid at amino acid residue 239 of the non-mature protein, and $FecX^B$ causes changes in the serine residue with an isoleucine at amino acid 99 of the mature active protein (Hanrahan *et al.*, 2004). Female sheep that were heterozygous carriers of $FecX^G$ or $FecX^B$

exhibit similar increases in ovulation rate to sheep heterozygous for *FecX*^{I} while homozygous carriers of *FecX*^{G} or *FecX*^{B} are sterile (Hanrahan *et al.*, 2004).

The other identified mutation that increases the ovulation rate has been reported in the Lacaune sheep (French), the Lacaune gene (*FecL*^{L}) has been mapped on sheep chromosome 11 (Lecerf *et al.*, 2002) and it has been suggested that additional mutation is segregating in this strain. Recently, a new mutation known as *FecX*^{L} identified in the BMP-15 gen different from *FecX*^{I}, *FecX*^{H}, FecX^{G} and *FecX*^{B}; it is a G to A change at nucleotide 1196 replacing a cysteine with a tyrosine at position 53 of the mature protein (Fabre *et al.*, 2006).

Although a high ovulation rate is observed in ewes that have GDF-9 and BMP-15 mutations, it has been found that homozygous ewes are infertile (Hanrahan *et al.*, 2004). Immunization against GDF-9 and BMP-15 in cattle reduces follicle size to less than 25% of controls (Juengel *et al.*, 2009). By contrast, short-term immunization against GDF-9 and BMP-15 in sheep increased the ovulation rate without affecting fertilization and fetal development (Juengel *et al.*, 2004). It could be concluded that the BMP system is imperative for the regulation of ovarian folliculogenesis and ovulation rate.

Table 1-2 : Known main genes affecting ovulation rate in sheep. Modified from Davis, 2005.

Gene	Name	Allele symbol	Chromosome	Founder breed	References
BMPR-1B	Booroola	*FecB*^{B}	6	Merino, Garole and Javanese	(Davis *et al.*, 2006; Muslant *et al.*, 2001; Wilson *et al.*, 2001; Davis *et al.*, 2002)
BMP-15	Inverdale	*FecX*^{I}	X	Romney	(Galloway *et al.*, 2000)
BMP-15	Hanna	*FecX*^{H}	X	Romney	(Galloway *et al.*, 2000)

BMP-15	Belclare	*FecX*^{B}	X	Belclare	(Hanrahan *et al.*, 2004)
BMP-15	Galway	*FecX*^{G}	X	Belclare and Cambridge	(Hanrahan *et al.*, 2004)
GDF-9	High Fertility	*FecG*^{H}	5	Belclare and Cambridge	(Hanrahan *et al.*, 2004)
-	Woodlands	*FecX2*^{W}	X	Coopworth	(Davis *et al.*, 2001)
	Lacaune	*FecL*^{L}	11	Lacaune	(Lecerf *et al*., 2002)
-	Thoka	*FecI*^{I}	-	Icelandic	(Jonmundsson & Adalsteinsson, 1985)
-	-	-	-	Olkuska	(Martyniuk & Radomska, 1991)
-	-	-	-	Belle-Ile	(Malher & Le Chere, 1998)

1.5 BMPs and male reproductive system

Spermatogenesis is a unique and complex process that takes place in the testes, within the seminiferous tubules. Spermatogenesis can be divided into three different phases: spermatogonial proliferation, meiosis, and the maturation of the spermatids by a process called spermiogenesis (Zirkin, 1993). Various BMP family members have been identified in mice testes. For example, *Bmp7* and *Bmp8a* have been shown to be expressed in the same initial segment of the epididymis and in the seminiferous tubules, while *Bmp8b* are not detectable in the epididymis (Zhao *et al*., 2001; Zhao & Hogan, 1996).

Bmp7 homozygous mutant mice do not survive, while the heterozygous mutant mice show no defects in the testes and epididymis (Zhao *et al*., 2001). In contrast, the *Bmp8b* homozygous

mutant mice show different degrees of germ-cell deficiency and sterility. Germ cells from homozygous mutants display either an obvious reduction in proliferation or failure to proliferate throughout early puberty. In addition, there is a significant increase in germ cell apoptosis which starts in the primary spermatocyte stage and ultimately leads to infertility (Zhao *et al*., 1996).

BMP-4 has been shown to be expressed in the testes and epididymis of mice; *Bmp4* heterozygous males display compromised fertility as a result of germ cell degeneration, reduced sperm count, and reduced sperm motility (Hu *et al.*, 2004). The BMP-4 receptor and Smad5 are expressed in proliferating PGCs and in postnatal spermatogonia. The addition of BMP-4 to spermatogonia culture can regulate Kit expression by activating Smad4 and Smad5, which in turn modulate the expression of c-kit in germ cells (Pellegrini *et al.*, 2003).

BMP-7 has been shown to be expressed in both male and female gonads and appears to be necessary for germ cell proliferation (Ross *et al.*, 2007). Both BMP-2 and BMP-7 have stimulatory effects on the proliferation of spermatogonia and sertoli cells, which suggests that the BMP family has a regulatory role during the early stage of spermatogenesis (Puglisi *et al.*, 2004).

1.6 The effects of BMPs during embryonic development

BMPs play significant roles during early embryonic life and in later developmental stages during gestation. Recent genetic advances, particularly those using the mouse gene knockout system, have shown that BMP signaling is involved in early developmental patterning. BMPs have been found to be essential for the formation and patterning of different kinds of tissues and different kinds of organs, including the formation of the skeletal system, the central

nervous system, heart, kidney, eyes, teeth and liver (Dudley *et al*., 1995; Li *et al*., 1998; Luo *et al*., 1995; Rossi *et al.*, 2001; Shi *et al*., 2000; Wozney *et al*., 1988).

In mice, BMPs are essential for the formation of the mesoderm and PGCs (Hogan, 1996; Ying *et al.*, 2000). There is a positive relationship between the level of BMP-9 in the follicular fluid, oocyte maturation, and embryo quality (Gode *et al.*, 2011). It has been shown that BMP signaling is required for the patterning of the dorsal neural tube cells and in neural crest cell formation (Stottmann & Klingensmith, 2011).

BMP-4 and BMP-8b are essential factors in the production of PGCs in mouse embryos. Mice embryos that have a mutation in BMP-8b exhibit an absence of PGCs compared with non-mutant mice, *Bmp4* homozygous embryos lack PGCs, while the heterozygous embryos have fewer primordial cells compared with normal mice embryos (Lawson *et al*., 1999). Similarly, mice embryos that are double heterozygotes for *Bmp8b* and *Bmp4* mutations show the same defects in PGC number as the BMP-4 heterozygote (Ying *et al.*, 2000). BMP-5 knockout mice have some developmental defects in different tissues, especially the skeletal system (King *et al.*, 1994). Mutations in the mechanisms of the BMP signaling pathways in humans can lead to a variety of diseases, such as cancer, vascular diseases, gastrointestinal neoplasia, and Cowden syndrome (Waite & Eng, 2003a). A mutation in the tumor suppressor gene PTEN, located on the human chromosome 10q23, has been reported to cause Cowden syndrome (Li *et al.*, 1997). Recently, mutations in BMPR-1A have been found in families with Cowden syndrome, indicating that there may be an association between BMP signaling and PTEN (Waite & Eng, 2003b).

BMPs play essential roles in a range of developmental processes and their roles during early embryonic development have been widely studied. For example, BMPs regulate the differentiation of neural crest cells in rodents, dorsal–ventral patterning in *Xenopus* embryos,

and epithelial–mesenchymal interactions during the beginning of tooth development (Jones *et al.*, 1992; Shah *et al.*, 1996; Vainio *et al.*, 1993). In addition, the expression of BMP-7 during human fetal development suggests it may have a regulatory role in human pregnancy by regulating reproductive hormones, since exogenously added recombinant BMP-7 to human placental cultures has been found to decrease the secretion of chorionic gonadotropin and progesterone (Martinovic *et al.*, 1996).

BMPR-2 homozygous mutant embryos were smaller than the wild type embryos. The growth of mutant embryos were further delayed, and unlike their normal littermates they were unable to go through normal gastrulation and they contained no mesoderm (Beppu *et al.*, 2000). Furthermore, *Bmp5* and *Bmp7* double mutant embryos show striking defects, mostly affecting tissue such as the rostral neural tube, allantois, heart, branchial arches, ventral body wall, and somites (Solloway & Robertson, 1999). *Bmp7* null mice show a rapid loss in nephron progenitors, implying that BMP-7 is a very important factor for nephron development. BMP-7 acts biologically through the JNK signaling circuitry (Blank *et al.*, 2009). The over expression of BMP-15 during early *Xenopus* embryo development leads to an ectopic head embryo. In addition, BMP-15 has been found to have an inhibitory effect on the Smad1, Smad5, and Smad8 pathway and the Wnt pathway during early embryogenesis (Di Pasquale & Brivanlou, 2009).

The addition of BMP-4 to human embryonic stem cell to trophoblast culture improved differentiation (Xu *et al.*, 2002). The effects of exogenous BMP-4 or BMP-2 on oocytes maturation were studied during *in vitro* maturation (IVM) of bovine oocytes, and it has been found that the addition of BMP-2 and BMP-4 to IVM medium does not affect the maturation of the oocyte nucleus or the formation of blastocysts following IVF (Fatehi *et al.*, 2005).

1.7 Aim of this project

The recruitment and growth of primordial follicles are essential features of female reproduction. Recent studies have demonstrated an important role for BMP-4 in follicle development, especially in the transition of primordial to primary follicles. The recruitment and activation of dormant primordial follicles and their subsequent growth and development into primary follicles is a vital process in ovarian biology, especially considering the ovaries of humans and other mammals have a fixed pool of primordial follicles and exhaustion of that stock leads to menopause or infertility.

Recent studies of the involvement of BMPs in the reproductive system have improved our knowledge and understanding of reproduction and fecundity. The essential role of the BMP system in mammalian reproduction has been further highlighted by the discovery of unusual reproductive phenotypes of animals with normally occurring mutations or targeted alteration of specific BMP family genes. Together, these studies demonstrate the importance of BMP proteins for fecundity in both male and female mammals. Notwithstanding, significant gaps in our understanding still remain with regard to how BMPs function in reproductive tissues and cells. The objective of this research is to clarify the role of the BMP system in mammalian reproduction, especially the role of BMP-4 and BMPR-1B in follicle development using mice and sheep as experimental models.