

The role of bone morphogenetic proteins in ovarian function

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Bone morphogenetic proteins (BMPs) represent the largest subclass of growth factors in the transforming growth factor β (TGF- β) superfamily. BMPs have proven to be multifunctional regulators of a wide variety of biological processes in numerous types of cell and tissue. The role of inhibins, activins and TGF- β s (which also belong to the TGF- β superfamily) in reproduction has been studied extensively over the last 15 years. However, there were no reports on the role of BMPs in the mammalian ovary until 1999 when we reported an intrinsic ovarian BMP system replete with BMP ligands, receptors and novel biological functions. Since this report it has become clear that the BMP system plays an important role in the regulation of ovarian function, evidenced by the ability of BMPs to control granulosa cell proliferation and cytodifferentiation, as well as oocyte development. The physiological relevance of the BMP system has recently been highlighted by the discovery that genetic mutations in the BMP-15 ligand or the BMP type IB receptor lead to critical aberrations in folliculogenesis and ovulation. This review provides a current overview of the rapidly emerging field of the BMP system in mammalian ovarian function.

Introduction

TGF- β superfamily: the bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) were originally isolated from bovine and human bone as proteins that induce bone and cartilage formation in ectopic extra-skeletal sites *in vivo* (Wozney *et al.*, 1988). The deduced amino acid sequences from the corresponding cDNAs revealed that these proteins are structurally related to transforming growth factor β (TGF- β). To date, more than 30 members of the TGF- β superfamily have been identified in various species, including sea urchins, frogs, *Drosophila*, chickens, mice, rats and humans (Griffith *et al.*, 1996; Kawabata and Miyazono, 2000). Most of these members were identified by a homology-based PCR cloning strategy and designated simply as related to the name of the cDNA clone from which PCR primer sequences were selected. Therefore, overlapping names have been given to the same molecule (for example BMP-7/OP-1, BMP-6/Vgr-1, BMP-3b/GDF-10, BMP-13/GDF-6, BMP-15/GDF-9B). In addition, there is no direct evidence

that all molecules designated as BMPs can induce cartilage and bone formation; thus, the nomenclature is misleading and a proper nomenclature for these molecules is required. Indeed, it is well established that the BMPs regulate biological processes that are as diverse as cell proliferation, apoptosis, differentiation, determination of cell fate and morphogenesis.

Common structure of TGF- β superfamily members

All TGF- β superfamily members are synthesized as large precursor proteins that are approximately three times larger than the mature protein (Massagué *et al.*, 1994). The precursors are first dimerized and then undergo proteolytic processing at RXXR sites to yield mature active dimeric proteins composed of the carboxy terminal segments of the precursors. The BMPs are distinguished from other members by having seven, rather than nine, conserved cysteines in the mature protein region. Crystallography of the mature molecule, such as TGF- β 2 (Schlunegger and Grütter, 1992) and BMP-7 (Griffith *et al.*, 1996), showed that six of the seven common cysteines are closely linked within the subunit to a rigid structure known as a cysteine knot, and the remaining cysteine residue is necessary for disulphide bonding of the two monomers into a dimer (Vitt *et al.*, 2001). BMP-15 and growth differentiation factor 9 (GDF-9) are two closely related molecules which form a distinct subset of the TGF- β superfamily (Dube *et al.*, 1998). A major structural distinction of BMP-15 and GDF-9 from other TGF- β superfamily members is that they lack the cysteine residue involved in inter-subunit disulphide bonding (McPherron and Lee, 1993; McGrath *et al.*, 1995; Dube *et al.*, 1998). It is not known whether BMP-15 and GDF-9 exist as monomers or form non-covalent dimers.

Receptors of TGF- β superfamily members

Receptors for TGF- β superfamily members comprise a family of two closely related cell surface receptor subtypes: the type I and type II receptors (Miyazono *et al.*, 2001). In mammals, seven type I and five type II receptors have been characterized to date. Both types of the receptor are structurally similar and possess serine/threonine kinase domains in their intracellular domains. The type II receptor kinases are constitutively active without ligand stimulation. The dimeric TGF- β superfamily members bind to a heterotetrameric complex of two type I and two type II receptors. Type I receptors, but not type II receptors, have a region rich in glycine and serine residues (GS domain) in the juxtamembrane domain. In the case of TGF- β s and activins, it is thought that the ligand first binds specific type II receptors followed by the recruitment of type I receptors into the complex. The BMP receptor system is somewhat different in that both type II receptors and type I receptors independently have low affinity for the ligand, but together they can achieve high affinity binding (Liu *et al.*, 1995). Once activated, all type II receptors phosphorylate type I receptors at five serine and threonine residues located in a cluster in the GS domain. The phosphorylated type I receptors subsequently trigger downstream signalling cascades.

Signal transduction of TGF- β superfamily members

The signalling pathways of the TGF- β superfamily members have been studied extensively (Hogan, 1996; Heldin *et al.*, 1997; Miyazono *et al.*, 2001). In the signalling pathway, the phosphorylated type I receptors interact with and phosphorylate downstream signalling molecules known as Smads, namely Smad1, Smad2, Smad3, Smad5 and Smad8. Different members of the Smad family play different roles in signalling. For example, overexpression of Smad1 in *Xenopus* embryos mimics the effects of BMP-4 (ventral mesoderm induction) (Graff *et al.*, 1996; Thomsen, 1996), whereas overexpression of Smad2 mimics the effects of Vg-1

(dorsal mesoderm induction) (Graff *et al.*, 1996). As such, these members of the Smad family are known as 'pathway-restricted Smads' and are present in the cytoplasm as homotrimers. After pathway-restricted Smads are phosphorylated by type I receptors, they interact with another Smad molecule termed Smad4. Smad4 functions as a 'common-mediator Smad' to form a hetero-oligomeric complex with one of the pathway-restricted Smads. The complex is then translocated to the nucleus, where it binds either directly or in complex with other component(s) to the target DNA, and affects transcription of specific genes. More than 40 proteins are known to interact with Smads in various types of cell (Miyazono *et al.*, 2001). These Smad partner proteins can regulate membrane anchoring of Smads, ubiquitin-dependent protein degradation and transcription by Smads. As the number of type I and type II receptors and Smad proteins is limited, and yet a wide range of biological activities are elicited by a given member of the TGF- β superfamily, the role for Smad partner proteins in the signalling pathway must be physiologically important. It is also known that Smad6 and Smad7 prevent the phosphorylation and signalling activity of pathway-restricted Smads by binding to the type I receptors (Miyazono *et al.*, 2001). Thus, these Smads act as inhibitors of the bioactivity of TGF- β superfamily ligands.

Although Smads are key signalling molecules of the TGF- β superfamily members, other signalling pathways are present concurrently (Miyazono *et al.*, 2001). Examples include TGF- β -activated kinase (TAK-1), a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family, which is activated by BMP-4 or TGF- β , and members of the Ras or Rac families of small GTP-binding proteins, which also have been implicated in TGF- β signalling. Moreover, the extracellular signal-regulated kinase-1 (ERK1) and -2 (ERK2) and stress-activated protein kinase (SAPK)/Jun-N-terminal kinase (JNK) have also been linked to TGF- β signal transduction in some types of cell. Thus, there is certainly crosstalk between different signalling pathways in the TGF- β superfamily.

BMPs in non-ovarian tissues

Besides regulating bone formation *in vivo*, studies have demonstrated that BMPs are multi-functional regulators in a wide array of biological processes in both vertebrates and invertebrates (Kingsley, 1994; Hogan, 1996). In addition to bone, there is evidence that expression of BMP genes is high in the kidney (BMP-3, -4 and -7), lung (BMP-3, -4, -5 and -6), small intestine (BMP-3 and -7), heart (BMP-2, -4, -6 and -7), limb bud (BMP-2, -4, -5 and -7) and teeth (BMP-3, -4 and -7) and that they can regulate the cellular homeostasis by paracrine mechanisms (Lyons *et al.*, 1989, 1990).

Expression of BMP ligands and receptors in the ovary

To date, the mRNAs encoding BMP-2, -3, -3b, -4, -6, -7, -15 and GDF-9, and BMP receptor type IA (BMPRI-A), type IB (BMPRI-B) and type II (BMPRII) have been identified in the ovary of various mammals by northern blot or *in situ* hybridization analysis. The growing number of BMP ligands and receptors identified in ovarian cells is indicative of a role of BMPs in controlling ovarian function.

BMP-2, BMP-3 and BMP-3b

Expression of BMP-2, -3 and -3b mRNAs were first demonstrated in the adult rat ovary by northern blot analysis, and highest expression was of BMP-2 mRNA (Takao *et al.*, 1996). In human ovaries, BMP-3 mRNA is strongly detectable by northern blot analysis, whereas expression of BMP-2 mRNA is very weak and BMP-3b mRNA is undetectable (Hino *et al.*,

1996). BMP-3 mRNA expression was also identified in human granulosa–luteal cells (Jaatinen *et al.*, 1996). During *in vitro* culture of human granulosa–luteal cells, the basal steady-state expression of BMP-3 mRNA increased spontaneously, whereas treatment of these cells with hCG markedly suppressed BMP-3 mRNA (Jaatinen *et al.*, 1996). In addition, 8-bromo-cAMP (8-Br-cAMP) and 12-*O*-tetradecanoylphorbol-13-acetate decreased BMP-3 mRNA expression in these cells, indicating that BMP-3 mRNA expression can be regulated by pathways mediated by both protein kinase A (PKA) and protein kinase C (PKC) in these cells (Jaatinen *et al.*, 1996).

BMP-4 and BMP-7

Studies on the expression of mRNA encoding BMP-4 and -7 by *in situ* hybridization in rat ovaries revealed that BMP-4 and -7 co-localize to the theca interstitial cells and that the expression changes during folliculogenesis (Shimasaki *et al.*, 1999). Interestingly, the BMP-4 and -7 mRNAs are strongly expressed in theca interstitial cells of dominant follicles but are much less abundant in theca interstitial cells of atretic follicles. This finding underscores the potential importance of the regulated co-expression of BMP-4 and -7 in follicle development and demise. BMP-4 and -7 are also expressed in the surface epithelium and in some corpora lutea (Shimasaki *et al.*, 1999), indicating that these BMPs may also regulate functions in these tissues.

BMP-6

In the adult mouse ovary, mRNA encoding BMP-6 is highly expressed in immature and mature oocytes, and in lower amounts in the granulosa cells (Lyons *et al.*, 1989; Elvin *et al.*, 2000a). BMP-6 mRNA has also been found in adult rat oocytes, granulosa cells, surface epithelium and some corpora lutea (G. F. Erickson and S. Shimasaki, unpublished). The BMP-6 mRNA signal is extremely strong in the oocytes and granulosa cells of healthy Graafian follicles. It should be noted that the intense expression of BMP-6 mRNA begins in preantral follicles at the secondary stage. With respect to granulosa cells, BMP-6 mRNA appears to be uniformly present in all granulosa cells, that is, membrana, periantral and cumulus granulosa cells.

BMP-15

The expression of BMP-15 is confined exclusively to the oocyte in mice, rats and humans (Dube *et al.*, 1998; Laitinen *et al.*, 1998; Aaltonen *et al.*, 1999; Jaatinen *et al.*, 1999; Otsuka *et al.*, 2000). *In situ* hybridization studies in the mouse revealed that BMP-15 mRNA expression starts in oocytes of primary follicles and is strongly expressed in oocytes throughout the preantral (gonadotrophin-independent) and antral (gonadotrophin-dependent) stages of folliculogenesis (Dube *et al.*, 1998). Otsuka *et al.* (2000) demonstrated that BMP-15 mRNA and protein are co-expressed in oocytes, beginning in primary follicles throughout the preovulatory stage. Further studies by Otsuka and Shimasaki (2002) found that kit ligand inhibits BMP-15 mRNA expression in oocytes, whereas BMP-15 stimulates kit ligand mRNA expression in granulosa cells. This was the first demonstration of a paracrine negative feedback loop between the oocyte and granulosa cells.

GDF-9

As for BMP-15, the oocyte is the unique site of ovarian GDF-9 mRNA and protein expression in a variety of species including humans, rats, mice, sheep and cows (McGrath *et al.*, 1995; Dong *et al.*, 1996; Fitzpatrick *et al.*, 1998; Aaltonen *et al.*, 1999; Bodensteiner *et al.*, 1999; Elvin *et al.*, 1999a; Erickson and Shimasaki, 2000; Elvin *et al.*, 2000a). GDF-9 mRNA and

protein are co-expressed in oocytes at all stages of follicular development, except for primordial follicles in mice, rats and humans (McGrath *et al.*, 1995; Dong *et al.*, 1996; Aaltonen *et al.*, 1999; Elvin *et al.*, 1999a). However, in ovine and bovine ovaries, GDF-9 mRNA could be detected as early as the primordial follicle stage (Bodensteiner *et al.*, 1999). GDF-9 mRNA expression in human oocytes closely resembles that of BMP-15 mRNA but begins slightly earlier (at small primary follicles) than that of BMP-15, the expression of which begins at the large primary follicle stage (Aaltonen *et al.*, 1999).

BMP receptors

The first comprehensive investigation of BMP type I and type II receptors in the ovary revealed that this tissue is the main site of expression of these receptors (Shimasaki *et al.*, 1999), supporting the hypothesis that BMPs are important to ovarian physiology. The mRNA for BMP receptor IA (BMPR-IA) is expressed in granulosa cells and oocytes in adult rat ovaries (Shimasaki *et al.*, 1999). A relatively high BMPR-IA mRNA content can be detected in oocytes, whereas the content in granulosa cells is relatively low in the same follicles of healthy and atretic follicles. In addition, rat ovaries express BMPR-IB mRNA in the oocytes and in all the granulosa cells of healthy follicles after the primary stage. The expression of BMPR-II mRNA was detected exclusively in granulosa cells, beginning in the secondary follicle, that is, when there are two layers of granulosa cells. During the stages of preantral and antral follicle development, very high amounts of BMPR-II mRNA are present in all granulosa cells, that is, membrana, periantral and cumulus cells (Shimasaki *et al.*, 1999).

Baird and colleagues investigated the expression of BMP receptor proteins in the sheep ovary by immunohistochemical analysis (Souza *et al.*, 2002). All three BMP receptors (BMPR-IA, -IB and -II) are expressed strongly in the granulosa cell layer of follicles from the primary to the late antral stages of development. These receptors are also expressed in the oocytes, corpora lutea and ovarian surface epithelia, whereas they are expressed at a lesser extent in the theca cells of antral follicles (Souza *et al.*, 2002).

Role of BMPs in regulating follicle growth and development

BMP-4 and BMP-7

A major concept of ovarian physiology is that FSH stimulates the granulosa cells of growing follicles to produce increasing amounts of oestradiol during the follicular phase of the ovarian cycle. However, progesterone synthesis in response to FSH is inhibited until the periovulatory period when it increases suddenly. In contrast to the steroidogenic properties of granulosa cells *in vivo*, granulosa cells from early antral follicles cultured *in vitro* in the presence of FSH secrete copious amounts of both oestradiol and progesterone. These findings led to the concept that there is a luteinization inhibitor *in vivo* which selectively inhibits FSH-dependent follicular progesterone but not oestradiol production by granulosa cells. The elucidation of the biological effects of BMP-4 and -7 in the ovary identified these factors as the first candidate molecules the biological activities of which are consistent with the long sought luteinization inhibitor (Shimasaki *et al.*, 1999).

The addition of BMP-4 and -7 to cultures of rat primary granulosa cells from early antral follicles had no effect on steroid production when added alone. Interestingly, the addition of both BMP-4 and -7 led to an increase in FSH-induced oestradiol production, but decreased FSH-induced progesterone production (Shimasaki *et al.*, 1999). Thus, BMP-4 and -7 were identified as the first molecules that can differentially modulate FSH action in a manner that reflects normal follicle steroidogenesis during the oestrous cycle. Examination of the

cellular mechanisms by which BMP-7 differentially regulates FSH action in steroidogenesis demonstrated that BMP-7 enhances P450 aromatase (P450arom), but suppresses steroidogenic acute regulatory protein (StAR) mRNA expression induced by FSH (Lee *et al.*, 2001). However, BMP-7 did not change FSH-induced amounts of mRNA P450 side-chain cleavage (P450scc) or 3 β -hydroxysteroid dehydrogenase (3 β -HSD). These findings indicate that BMP-7 stimulation and inhibition of P450arom and StAR mRNA expression, respectively, may play a role in the mechanisms underlying the differential regulation of oestradiol and progesterone production. BMP-7 was also found to exhibit mitotic properties on granulosa cells, demonstrated by increases in granulosa cell thymidine incorporation and the number of cells (Lee *et al.*, 2001).

Lee *et al.* (2001) investigated the effects of BMP-7 on folliculogenesis and ovulation in the rat to establish the physiological relevance of BMP-7 function *in vivo*. Administration of BMP-7 into the ovarian bursa caused a decrease in the number of primordial follicles and, simultaneously, an increase in the number of primary, secondary and antral follicles. These findings indicate that BMP-7 may act to facilitate the recruitment of primordial follicles into the growing follicle pool. Importantly, administration of BMP-7 caused a pronounced decrease in plasma progesterone concentrations, confirming the relevance *in vivo* of the *in vitro* effects of BMP-7 on progesterone production. Furthermore, a significant decrease in the number of ovulated oocytes was observed in the BMP-7-treated ovaries as compared with the untreated contralateral control ovaries. Therefore, these findings could target theca-derived BMP-7 in the promotion of recruitment and thereby in determining ovary reserve.

Dooley *et al.* (2000) used human ovarian theca-like tumour (HOTT) cells which express BMPR-IA, BMPR-IB and BMPR-II to examine the impact of BMP-4 on theca cell function. In this system, BMP-4 inhibited forskolin-stimulated 17 α -hydroxylase expression without changing the expression of StAR, P450scc or 3 β -HSD induced by forskolin. This selective regulation of BMP-4 is likely to lead to an alternation of steroid production induced by forskolin resulting in increased progesterone accumulation and decreased 17 α -hydroxyprogesterone and androstenedione production, although the addition of BMP-4 alone (without forskolin) exhibited only a modest decrease in androstenedione secretion (Dooley *et al.*, 2000). Thus, BMP-4 led to the production of more progesterone and less androgen by HOTT cells. The mechanism by which BMP-4 selectively regulates forskolin-induced 17 α -hydroxylase remains to be elucidated.

BMP-2

Studies on the biological activities of BMP-2 in cultures of primary granulosa cells from sheep ovaries by Souza *et al.* (2002) led to the proposal that this factor may be involved in preventing spontaneous luteinization, similar to the roles of BMP-4 and -7 uncovered in the rat model (Shimasaki *et al.*, 1999). Specifically, BMP-2 augmented FSH-induced oestradiol synthesis and inhibin A production in granulosa cells isolated from ovine follicles that were 1–3 mm in diameter. In contrast to the effects of BMP-7 in rats, BMP-2 did not promote mitosis in the ovine granulosa cells (Souza *et al.*, 2002).

BMP-6

BMP-6 was also shown to exhibit biological activities in rat granulosa cells (Otsuka *et al.*, 2001b). Similar to BMP-4 and -7, BMP-6 was found to be potent in the attenuation of FSH-induced progesterone production; however, in contrast to BMP-4 and -7, BMP-6 did not alter FSH-induced oestradiol production. Consistent with its steroidogenic properties, BMP-6 inhibited FSH-induced stimulation of StAR and P450scc mRNA expression, without affecting

the expression of P450arom mRNA. Interestingly, BMP-6 also inhibited forskolin-induced progesterone production and the corresponding expression of StAR and P450scc mRNA, but failed to inhibit progesterone synthesis and steroidogenic enzyme expression induced by 8-Br-cAMP (Otsuka *et al.*, 2001a). A similar inhibition of the expression of mRNA encoding a battery of other FSH-responsive genes including the inhibin-activin subunits (α , β A and β B) and the LH receptor by BMP-6 was observed when stimulated by forskolin but not when stimulated by 8-Br-cAMP. These findings indicate that the inhibitory effect of BMP-6 on FSH action is caused by suppression of adenylate cyclase activity. We also found that BMP-6 lacks mitotic activity on rat granulosa cells. Together, this study provides the first insight into the biological function of BMP-6 in the ovary and demonstrates its unique regulatory mechanism of FSH action.

BMP-15

Studies using recombinant BMP-15 in rat primary granulosa cells revealed unique and important functions of BMP-15 in ovarian physiology (Otsuka *et al.*, 2000, 2001c; Otsuka and Shimasaki, 2002). First, BMP-15 is a potent stimulator of granulosa cell proliferation (Otsuka *et al.*, 2000). Importantly, the mitotic activity of BMP-15 is independent of FSH, indicating that BMP-15 is directly involved in controlling mitosis in the granulosa cells of pre-antral follicles during the FSH-independent stages of early folliculogenesis. Second, BMP-15 is a potent inhibitor of FSH receptor expression in granulosa cells (Otsuka *et al.*, 2001c). By inhibiting FSH receptor expression, BMP-15 suppresses the FSH-induced expression of a battery of FSH responsive genes including StAR, P450scc, 3β -HSD, LH receptor and inhibin-activin subunits (α , β A and β B). Although BMP-15 has a similar effect on FSH-induced steroidogenesis to BMP-6 (that is, suppressing progesterone but not oestradiol production), the effects of BMP-15 were due to a reduction in the expression of the FSH receptor in the granulosa cells (Otsuka *et al.*, 2001c), and not to the inhibition of adenylate cyclase, as is the case for BMP-6 (Otsuka *et al.*, 2001b). Thus, we suggest that BMP-15 functions as a negative regulator of granulosa cell cytodifferentiation induced by FSH, but as a positive regulator of granulosa cell replication. Third, oocyte-derived BMP-15 and granulosa cell-derived kit ligand form a negative feedback loop in regulating granulosa cell growth (Otsuka and Shimasaki, 2002). Specifically, BMP-15 stimulates kit ligand expression in granulosa cells, whereas kit ligand inhibits BMP-15 expression in oocytes. Interestingly, the blockage of c-kit (kit ligand receptor) signalling in oocytes suppressed BMP-15-induced granulosa cell mitosis, indicating that the oocyte plays a role in the events that govern granulosa cell responses to BMP-15. Therefore, we propose that the mitotic activities of BMP-15 and kit ligand on granulosa cells may depend upon this novel oocyte-granulosa cell communication system. As kit ligand is concomitantly expressed with BMP-15 in the early stages of follicular development, together with the established importance of these two factors in the regulation of preantral follicular development, this novel gametic-somatic cell feedback system may be important in the mechanisms controlling normal folliculogenesis.

We also found that the biological activities of BMP-15 are blocked by follistatin. This finding is based on the observation that follistatin attenuates the BMP-15 stimulation of granulosa cell proliferation and reverses the BMP-15 inhibition of FSH receptor mRNA expression and subsequent progesterone production (Otsuka *et al.*, 2001a). The antagonistic action of BMP-15 and follistatin was confirmed by using a surface plasmon resonance biosensor. Thus, the emerging scenario is that the precise regulation of the bioavailability of BMP-15 in the follicular microenvironment may be determined by the novel BMP-15 binding protein, follistatin.

GDF-9

Although GDF-9 shares the highest homology with BMP-15 and exhibits a similar oocyte-specific expression pattern to BMP-15, studies, mainly by the Hsueh, and Matzuk and co-workers, have demonstrated that the biological activities of GDF-9 are distinct from those of BMP-15. In rat whole ovarian follicle cultures, GDF-9 was first shown to be effective in stimulating preantral follicle growth (Hayashi *et al.*, 1999). Cultures of rat granulosa cells collected from early antral and preovulatory follicles were used to show that GDF-9 also promotes granulosa cell proliferation, and inhibits FSH-induced steroidogenesis and LH receptor expression (Vitt *et al.*, 2000a). On the basis of the finding that GDF-9 affects neither cAMP production nor steroidogenesis induced by forskolin, GDF-9 inhibition of FSH action was postulated to occur as a result of the modulation of FSH receptor content or FSH receptor coupling to Gs protein (Vitt *et al.*, 2000a). In isolated mouse ovarian cells, GDF-9 was shown to regulate diverse processes and gene expression during the preovulatory stage including the enhancement of FSH-induced cumulus cell expansion, the expression of hyaluronan synthase 2 (HAS2) and cyclooxygenase 2 (Cox2) as well as the inhibition of LH receptor and urokinase plasminogen activator expression (Elvin *et al.*, 1999b). In contrast to the inhibition of FSH-induced steroidogenesis, GDF-9 alone increased progesterone production in cumulus granulosa cells by stimulating the expression of an intrinsic prostaglandin E₂-EP₂ receptor signalling pathway (Elvin *et al.*, 2000b).

Another important function of GDF-9 involves its action on kit ligand expression. In contrast to the stimulatory action of BMP-15 observed in rats (Otsuka and Shimasaki, 2002), GDF-9 suppresses the steady-state mRNA content of kit ligand in mouse granulosa cells (Joyce *et al.*, 2000). These data corroborate earlier findings that in GDF-9 null mice there is an increase in ovarian kit ligand (Elvin *et al.*, 1999b). Intriguingly, studies on mice demonstrated that the addition of partly grown oocytes to granulosa cell cultures caused an increase in kit ligand mRNA expression, whereas the addition of fully grown oocytes had the opposite effect (Joyce *et al.*, 1999). Thus, the effect of oocytes on granulosa cell kit ligand expression depends on the developmental stages of the oocytes. Although both GDF-9 and BMP-15 mRNA and protein are detectable in oocytes throughout folliculogenesis, nothing is known about the temporal secretion pattern of the biologically active forms of these growth factors. On the basis of the opposing effects of GDF-9 and BMP-15 on kit ligand expression, coupled with the opposing effects of partly and fully grown oocytes on kit ligand expression, it can be hypothesized that the partly grown oocytes secrete primarily BMP-15 (a kit ligand stimulatory factor), whereas fully grown oocytes secrete primarily GDF-9 (a kit ligand-inhibitory factor) (Otsuka and Shimasaki, 2002).

Vitt *et al.* (2000b) were the first to demonstrate the effect of GDF-9 in folliculogenesis *in vivo*. Intraperitoneal injections of GDF-9 into immature rats promoted the progression of primordial and primary follicles to small preantral follicles and caused an increase in the expression of CYP17, a theca cell marker. Therefore, GDF-9, similar to BMP-7, has the potential to be used as a therapeutic agent to stimulate early follicular development. The clinical relevance of this idea is emphasized by organ culture experiments in which GDF-9 promoted recruitment and early preantral follicle growth in human ovaries (Hreinsson *et al.*, 2002).

Our recent study indicated that aberrant expression of GDF-9 may be associated with pathological ovarian dysfunction in women (Teixeira Filho *et al.*, 2002). In this study, the expression of GDF-9 and BMP-15 was investigated by *in situ* hybridization using sections of ovaries from polycystic ovary syndrome (PCOS) and polycystic ovary (PCO) patients. These studies revealed that the expression of GDF-9 mRNA is delayed and reduced in the ovaries of PCOS and PCO patients during the growth and differentiation phase. In contrast, there

was no observable difference in the expression pattern of BMP-15 mRNA in PCOS and PCO ovaries as compared with normal ovaries. Further investigations will be required to determine whether the reduced GDF-9 mRNA causes the follicular arrest in PCOS/PCO or whether they are simply an epiphenomenon of another primary abnormality.

Aberrant BMP system in female reproduction

These *in vivo* and *in vitro* studies clearly indicate that the BMP system appears to be of primary importance in early follicle recruitment. The recent elucidation of the reproductive phenotypes of animals that have mutations (either naturally occurring mutations or 'gene knockouts' by targeted deletion) of the genes encoding various components of the BMP system has provided significant breakthroughs in our understanding of the physiological importance of the BMP system in ovarian function. Of particular interest are mutations that result in phenotypic abnormalities which are, for the most part, restricted to female reproductive function. There are four examples of BMP system gene mutations that have ovary-specific phenotypes included: (i) GDF-9 knockout mice, (ii) Inverdale and Hanna ewes which have point mutations in the *bmp15* gene, (iii) BMP-15 knockout mice and (iv) Booroola ewes which have a mutation in the *bmprlb* gene.

GDF-9 null mice

Dong *et al.* (1996) established a line of mice that are deficient in the gene encoding GDF-9 and provided the first insight into a BMP-related gene that displays selective effects on early ovarian folliculogenesis. Male mice that were heterozygous and homozygous for the deletion of the *gdf9* gene were viable and fertile with no clear phenotype, as were heterozygous females. In contrast, female homozygous GDF-9 null mice were viable but, importantly, they were completely infertile due to a block in folliculogenesis at the primary follicle stage.

The pathological features of these homozygous GDF-9 null mice have been studied extensively and these studies have provided insight into the role of GDF-9 *in vivo*. Circulating concentrations of FSH and LH in GDF-9 deficient female mice are high (three- and twofold, respectively) compared with controls (Dong *et al.*, 1996). Follicles in GDF-9 deficient ovaries fail to progress beyond the primary stage as a result of impaired proliferation of the granulosa cells, but also fail to undergo apoptosis. It also appears that the follicles in GDF-9 deficient mice fail to develop a theca cell layer (Elvin *et al.*, 1999b). Expression of kit ligand and inhibin α is high in ovaries from GDF-9 deficient mice indicating that GDF-9 may play a role in suppressing these important ovarian factors.

Inverdale and Hanna ewes

Domestic sheep normally ovulate one to two oocytes giving rise to single and twin pregnancies. A few strains of sheep that consistently have an increased ovulation rate have been identified (Montgomery *et al.*, 1992). Two of these strains are called Inverdale (FecX^L) and Hanna (FecX^H) ewes. Genetic analysis of these unrelated families of sheep demonstrated that the phenotype of these prolific ewes was determined by a single putative gene on the X chromosome (Davis *et al.*, 1991). Females that carry one copy of the gene have increased ovulation and lambing rates, whereas females with two copies of the gene are infertile with 'streak' ovaries (Davis *et al.*, 1992). Male carriers of the gene are unaffected. Further genetic analysis revealed that both the Inverdale and Hanna phenotypes were correlated with single point mutations in the *bmp15* gene. Inverdale ewes have a T to A nucleotide substitution that results in a substitution of the valine with an aspartic acid at position 31 of mature BMP-15

(Galloway *et al.*, 2000). The region of the BMP-15 protein affected by this substitution is highly conserved between species. It was proposed by these workers that the substitution of the hydrophobic valine with a negatively charged aspartic acid may inhibit the ability of the BMP-15 subunits to form dimers, thus making the BMP-15 inactive. As discussed above, it is not yet known whether BMP-15 forms non-covalent dimers; therefore, this hypothesis has not been confirmed. In Hanna ewes, there is a C to T nucleotide substitution that results in a premature stop codon at amino acid residue 23 of the mature BMP-15 protein (Galloway *et al.*, 2000).

The finding that sheep lacking intact BMP-15 (FecX^L/FecX^L, FecX^H/FecX^H and FecX^L/FecX^H) are infertile with a block at the primary stage of folliculogenesis demonstrates the necessity of this growth factor for folliculogenesis in sheep. The increased ovulation rates in heterozygous Inverdale and Hanna mutants led to the proposal that BMP-15 affects fertility in a biphasic manner, and low doses increased fertility and the absence of BMP-15 caused infertility (Galloway *et al.*, 2000). Our *in vitro* experiments with recombinant BMP-15 provide some potential insight into understanding the mechanisms of the Inverdale–Hanna phenotype (Otsuka *et al.*, 2000, 2001c; Otsuka and Shimasaki, 2002). The phenotypic features of these BMP-15 mutant ewes and the correlating biological actions of BMP-15 that may be impaired or abolished in the heterozygous or homozygous mutants, respectively, are summarized (Table 1). In the homozygous mutants, the lack of the proliferative properties of BMP-15 (Otsuka *et al.*, 2000) could prevent the division of granulosa cells, which is necessary for the transition from primary to secondary follicles. In addition, the lack of BMP-15 stimulation of kit ligand expression may also cause impaired growth of primary follicles (Otsuka and Shimasaki, 2002) because kit ligand plays important roles in oogenesis and folliculogenesis during early follicular development. In contrast, the finding that BMP-15 attenuates FSH-induced granulosa cell differentiation by inhibiting FSH receptor expression (Otsuka *et al.*, 2001c) is consistent with the increased numbers of follicles selected for ovulation in the heterozygous sheep, which presumably have lower amounts of bioactive BMP-15. This hypothesis is supported by the following physiological features of the heterozygous Inverdale and Hanna ewes reported by Shackell *et al.* (1993): (i) there are more healthy oestrogenic follicles than in wild-type ewes (which is consistent with increased differentiation of granulosa cells than would be expected in a low BMP-15 environment); (ii) these developing follicles are smaller than those of wild-type ewes owing to fewer granulosa cells (which could be expected to be caused by the reduction of granulosa cell proliferation in a low BMP-15 environment); (iii) the follicles develop LH responsiveness at smaller sizes (which is probably due to increased FSH receptor expression in a low BMP-15 environment); and (iv) the corpora lutea of the heterozygous mutant sheep are smaller than those in wild-type ewes (which could occur because smaller follicles ovulate due to higher FSH sensitivity caused by lower BMP-15).

BMP-15 null mice

The establishment of BMP-15 null mice by targeted deletion of the *bmp15* gene provided the role of BMP-15 in female reproduction in mice (Yan *et al.*, 2001). Similar to the Inverdale and Hanna sheep, male BMP-15 null mice are fertile with no clear abnormal phenotype. Female mice heterozygous for *bmp15* gene deletion also have normal fertility. Females homozygous for deletions in the *bmp-15* gene, although fertile, had consistently fewer pups per litter and numbers of litters per month. These mice also developed histopathological ovarian defects including multiple oocytes in a single follicle. Female mice homozygous for deletions in BMP-15 had only decreased fertility, which is different from homozygous Inverdale and Hanna ewes which are infertile. This finding demonstrates the difference

Table 1. Characteristics and physiological basis of the aberrant phenotype of bone morphogenetic protein 15 (BMP-15) mutant ewes (Inverdale and Hanna) and the correlating biological activity of BMP-15

Features of phenotype	Physiological basis of features	Correlating biological activity of BMP-15
Homozygotes		
Infertility due to arrest of follicular development at the primary stage	Loss of GC mitosis	BMP-15 is a granulosa cell mitogen and stimulates kit ligand (a granulosa cell mitogen) expression
Heterozygotes		
Lower number of granulosa cells in follicles of component stages of developing follicles	Reduced granulosa cell mitosis	BMP-15 stimulates granulosa cell mitosis
Higher number of oestrogenic follicles	Increased sensitivity to FSH	BMP-15 suppresses FSH receptor expression
Higher LH responsiveness	Higher FSH-induced expression of LH receptors due to increased FSH sensitivity	BMP-15 suppresses FSH receptor expression
Increased ovulation rate	Increase in FSH-induced differentiation of follicles due to increased FSH sensitivity	BMP-15 suppresses FSH receptor expression
Smaller corpora lutea	Ovulation of oocytes from smaller follicles due to increased FSH sensitivity	BMP-15 suppresses FSH receptor expression

between sheep and mice with respect to the importance of BMP-15. When using these animals as models to understand fertility, it is important to note that mice are polyovulatory, whereas sheep are generally mono-ovulatory. Given that BMP-15 seems to play a central role in follicle recruitment and ovulation quota, the differences of the responses to the lack of BMP-15 between sheep and mice may be involved in defining the normal ovulation rate in these species.

The double mutant mice that are homozygous deficient in BMP-15 and heterozygous deficient in GDF-9 have more severe reductions in fertility compared with mice which are only homozygous deficient in BMP-15 (Yan *et al.*, 2001). This finding, when taken together with the similar spatial and temporal expression patterns and the sequence homology between GDF-9 and BMP-15, led to the proposal that there are physiologically important interactions between these two factors. These interactions could involve the formation and secretion of GDF-9–BMP-15 heterodimers from the oocyte or interactions with the same receptors on granulosa cells.

Booroola ewes

The Booroola, similar to the Inverdale and Hanna, is a strain of sheep that has been maintained as a result of its high fecundity. On the basis of Mendelian genetic analysis, it was proposed that the Booroola phenotype was caused by a single putative gene (*Fec^B*) and that ewes that carry one copy of the gene (heterozygotes) have increased ovulation and lambing rates, whereas those that carry two copies of the gene (homozygotes) have ovulation and lambing rates that are even higher than the heterozygotes (Montgomery *et al.*, 1992). Recently, three laboratories independently identified that the Booroola phenotype

Table 2. Characteristics and physiological basis of the aberrant phenotype of ALK-6 mutant ewes (Booroola) and the correlating biological activity of bone morphogenetic protein 6 (BMP-6)

Features of phenotype	Physiological basis of features	Correlating biological activity of BMP-6
Higher progesterone, but not oestradiol, induced by FSH and LH	Selective inhibition of progesterone synthesis	BMP-6 decreases progesterone synthesis without affecting oestradiol synthesis
Increased FSH-induced responsiveness to cAMP	Enhanced FSH activity	BMP-6 inhibits adenylate cyclase activity
Granulosa cells are more sensitive to FSH without a change in FSH B_{max} and K_d values	Enhanced FSH activity without altering FSH receptor number and affinity	BMP-6 inhibits adenylate cyclase activity without changing FSH receptor expression
Earlier onset of pre-ovulatory follicular maturation	Increased FSH activity and subsequent increased sensitivity to LH (precocious follicle maturation)	BMP-6 inhibits adenylate cyclase activity
Normal number of granulosa cells in preantral follicles	Normal mitotic index of granulosa cells	BMP-6 does not affect granulosa cell mitosis

corresponds to a single point mutation in the intracellular kinase domain of the BMPR-IB gene (Mulsant *et al.*, 2001; Souza *et al.*, 2001; Wilson *et al.*, 2001). The mechanisms by which this mutation causes an increase in fertility and the endogenous ligands that are involved have not yet been established. Nonetheless, BMP and BMP-related growth factors that are expressed in the ovary, including BMP-6, BMP-7, BMP-4 and GDF-5, have been shown to bind the BMPR-IB in various types of cell (Ebisawa *et al.*, 1999; Miyazono, 2001). Although the receptors for BMP-15 and GDF-9 have not yet been identified, the BMPR-IB receptor is a candidate for these ovarian factors.

On the basis of the biological activities of recombinant proteins *in vitro*, we propose that the phenotype of the Booroola ewe is most consistent with the ablation of the biological effects of BMP-6 (Otsuka *et al.*, 2001) as summarized (Table 2). Aspects of the Booroola phenotype that support this hypothesis include the following. First, when cultured for 48 h, granulosa cells from Booroola ewes produce higher amounts of progesterone but not oestradiol when compared with wild-type granulosa cell cultures (McNatty *et al.*, 1986). Second, Booroola follicles are more sensitive to FSH, but this response is not caused by increases in FSH binding capacity to granulosa cells (McNatty *et al.*, 1989). These findings are consistent with the evidence that BMP-6 inhibits FSH sensitivity by inhibiting adenylate cyclase activity, but has no effect on FSH receptor expression. In addition, consistent with the inability of BMP-6 to stimulate granulosa cell mitosis, it seems that the Booroola mutation in the BMPR-IB does not prevent granulosa cell mitosis. The finding that supports this hypothesis is that homozygous Booroola ewes have high ovulation rates in contrast to homozygous Inverdale and Hanna ewes which have a block in folliculogenesis at the primary stage.

Conclusion

The recent investigations of the expression and biological activities of various BMP ligands and the receptor signalling pathway in the ovary of a variety of mammals have uncovered numerous important roles for an intrinsic BMP system in the regulation of folliculogenesis and female fertility. A careful comparison of individual BMPs in a particular species reveals that there are many overlapping biological effects of the BMPs. However, each BMP does seem to have a

unique role in regulating follicle development. An emerging common theme is that the BMPs in the ovary are of paramount importance in regulating follicle growth and development. Furthermore, the ovary-specific phenotypes of animals with mutations in various components of the BMP system (GDF-9 knockout mice, BMP-15 knockout mice, Inverdale ewe, Hanna ewe and Booroola ewe) make the BMP system an attractive target for therapeutic regimens designed to alter fertility at the levels of granulosa cell proliferation and FSH responsiveness.

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