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REVIEW ARTICLE

Unique bioactivities of bone morphogenetic proteins in regulation of reproductive endocrine functions

Fumio Otsuka · Kenichi Inagaki

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Abstract Remarkable progress has been made in understanding the mechanism by which growth factors and oocytes can regulate the development and function of granulosa cells. Insufficiency of two oocyte-specific growth factors, growth differentiation factor-9 and bone morphogenetic protein (BMP)-15, cause female infertility. Expression of mRNA and/or protein for the BMP system components, including ligands, receptors and intracellular signal transduction factors, was demonstrated in cell components of growing preantral follicles, and biofunctional experiments have further revealed many important roles of the BMP system in regulation of reproductive function. In this review, recent advances in studies on biological actions of BMPs in ovarian folliculogenesis and in related endocrine tissues are discussed.

Keywords Bone morphogenetic protein · Folliculogenesis · Ovary · Reproduction and steroidogenesis

Introduction of BMPs in reproductive endocrinology

Accumulated evidence has established a concept that bone morphogenetic proteins (BMPs) formulate a multifunctional regulator system in various biological processes in vertebrates as well as invertebrates [1]. The expression of BMPs is known to be abundant in the kidney, lung, small intestine, heart, limb bud and teeth, in which BMPs regulate cellular homeostasis by paracrine mechanisms [2]. In addition to regulatory roles in bone formation and/or bone differentiation [3, 4], various BMP actions in many endocrine tissues, including the ovary, pituitary, thyroid, adrenal and cardiovascular tissues, have been gradually uncovered [5]. BMP ligands and receptors appear to play tissue-specific roles as fundamental regulators of endocrine functions. Recent research has demonstrated that the BMP system is a critical component of the local regulatory system in the ovary [6, 7]. Further research in this field will greatly advance our understanding of pathophysiology of systemic endocrine regulation and will lead to novel targets for wide-ranging clinical regimens aimed at controlling female reproduction, steroidogenesis and tumorigenesis in endocrine tissues.

In the reproduction field, much attention has been paid to oocyte-derived growth factors, based on the concept of bidirectional communication between oocytes and surrounding somatic cells that is critical for normal follicular development in the ovary. It has been hypothesized that oocytes continue to influence the behavior of surrounding granulosa cells via the production of oocyte-secreted factors with maintenance of the follicular microenvironment [8, 9]. Throughout folliculogenesis, oocytes play active roles in the regulation of development and function of granulosa cells. In this process, local factors, including several members of the transforming growth factor (TGF)-β superfamily, are involved in the transition of follicles from the primary stage to secondary stage and the subsequent follicle growth to late preantral and antral stages. It appears that oocytes play an organizing role in cell-to-cell communication, in which the rate of follicular development is regulated by a developmental program directed by oocytes. Oocytes achieve this by secreting soluble growth factors that act on neighboring follicular cells to regulate a broad range of granulosa cell functions.

F. Otsuka (✉) · K. Inagaki
Endocrine Center of Okayama University Hospital,
2-5-1 Shikata-cho, Kitaku, Okayama 700-8558, Japan
e-mail: fumiotsu@md.okayama-u.ac.jp

There is currently a great deal of interest in growth factor biology including the ovarian BMP system. In the past decade, remarkable progress has been made in understanding the mechanism by which oocytes can regulate the development and function of granulosa cells. Two remarkable studies have shown that insufficiency of two oocyte-specific growth factors, growth differentiation factor (GDF)-9 and BMP-15, causes female infertility [10, 11]. Expression of mRNA and/or protein for the BMP system components, including ligands, receptors and intracellular signal transduction factors, was clearly demonstrated in cell components of growing preantral follicles [12, 13], and biofunctional experiments have further revealed many important roles of the BMP system in regulation of ovarian function. In this review, recent advances in studies on biological actions of BMPs in ovarian folliculogenesis and in related endocrine tissues are discussed.

Characteristics of BMP molecules and BMP receptor signaling

BMPs were originally isolated from bone tissues as proteins that cause induction of bone and cartilage formation in ectopic extraskeletal sites in vivo [14]. The amino acid sequences from the corresponding cDNAs revealed that BMP ligands are structurally classified as TGF-β superfamily members. More than 30 members of the TGF-β superfamily have been identified to date [15, 16] and it has been well established that BMPs regulate multiple biological processes including cell proliferation, apoptosis, differentiation and morphogenesis. Seven type-I (ALK-1, -2, -3, -4, -5, -6 and -7) and five type-II (ActRII, ActRIIB, AMHRII, BMPRII and TβRII) receptors for TGF-β superfamily members have been characterized in mammals. Both type-I and type-II receptors are structurally similar and possess Ser/Thr kinase domains in their intracellular domains (Fig. 1). Dimeric TGF-β superfamily members bind to a heterotetrameric complex of two sets of type-I and type-II receptors. Type-I receptors have a Gly/Ser-rich GS domain in the transmembrane region. TGF-β and activins initially bind type-II receptors and corresponding type-I receptors are subsequently recruited into the ligand-receptor complex. In cases of BMP receptor signaling, both type-II and type-I receptors independently have a certain affinity for the ligands and the receptor complex further achieves higher affinity for binding [17].

Following the binding of BMP ligands to the corresponding receptors, phosphorylated type-I receptors activate downstream signaling molecules, Smads (Fig. 1). The pathway-restricted Smads (Smad1/5/8) are phosphorylated by type-I receptors, and then they interact with a common-mediator Smad (Smad4) to form a hetero-oligomeric

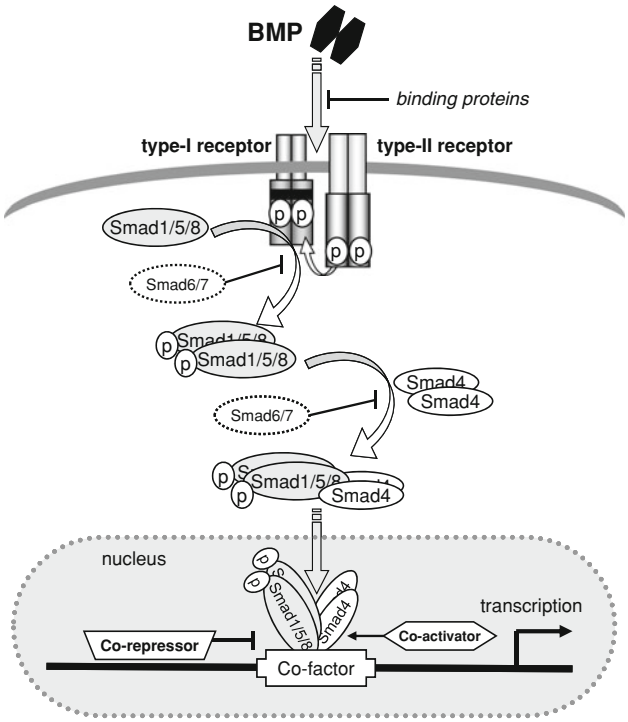

Fig. 1 BMP receptor signaling pathway. BMP ligands bind to type-I receptors first and then recruit type-II receptors or directly bind to preexisting complexes of type-I and type-II receptors. Once BMP ligands are bound to the receptor complex, the type-II receptor transphosphorylates the type-I receptor, which then transphosphorylates the intracellular signaling proteins Smad1/5/8. The phospho-Smad1/5/8 interacts with Smad4, and the complex translocates into the nucleus, where it interacts with transcription co-factors and regulates expression of target genes in a cell-type-specific manner. BMP action/signaling can be controlled at multiple levels, in which BMP binding to receptors can be inhibited by BMP-binding proteins and Smad 6/7 inhibits phosphorylation of Smad1/5/8

complex with Smad1/5/8. The complex is then translocated into the nucleus, where it binds directly or indirectly to target DNA and induces specific gene transcription [18]. Other signaling pathways may also be co-activated with Smad signaling such as TGF-β-activated kinase (TAK-1), a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family and members of the Ras or Rac families of small GTP-binding proteins. Extracellular signal-regulated kinase-1/2 (ERK1/2) and stress-activated protein kinase (SAPK)/Jun-N-terminal kinase (JNK) have also been linked to TGF-β signal transduction in some cell types.

Expression and functions of the ovarian BMP system

In the ovary, the expression of BMPs within different compartments of the antral follicle has been demonstrated in a variety of species including rodents, humans and

ruminants [6, 12, 19, 20]. Expression of BMP-2, -3, -3b, -4, -6, -7, -15 and GDF-9 and expression of BMP receptor type-IA (BMPRIA; also called ALK-3), type-IB (BMPRIB/ALK-6) and type-II (BMPRII) have been found in the ovaries of various mammals [12]. Various BMP ligands are expressed in cell-specific expression patterns in ovarian cells that undergo dynamic changes during follicular development and corpora luteal morphogenesis (Fig. 2) [6]. It is therefore possible that the developmental processes of folliculogenesis (recruitment, selection and atresia), ovulation, and luteogenesis (luteinization and luteolysis) are accompanied by dramatic changes in the spatial and/or temporal expression patterns of BMP system genes.

With regard to functional roles of the ovarian BMP system, there seem to be some overlapped actions among BMP ligands despite the unique role of each BMP in regulation of follicle development (Fig. 3). BMP-4 and BMP-7 are expressed in theca cells and regulate steroidogenesis in granulosa cells by increasing FSH-induced estradiol and suppressing progesterone production (Fig. 2) [21]. BMP-7 also promotes the recruitment process of primordial follicles into the growing follicle pool while inhibiting ovulation and progesterone production [22]. Similar to BMP-4 and -7, BMP-6 inhibits FSH-induced progesterone synthesis by suppressing adenylate cyclase activity in granulosa cells (Fig. 3) [23]. BMP-6 is expressed in oocytes and granulosa cells of healthy Graafian follicles (Fig. 2) [12]. Since BMP-6 expression in granulosa cells rapidly decreases when the dominant follicle is selected [12], BMP-6 may be a key factor for the selection process for dominant follicles. Unlike BMP-7 action, which induces granulosa cell proliferation, BMP-6 has no significant effect on granulosa cell mitosis. BMP-7 actions on FSH-induced estradiol production occur, in part, through suppression of ERK1/2 downstream of FSH receptor signaling (Fig. 3) [24]. Likewise, BMP-2 and -4 enhance FSH-induced p38-MAPK phosphorylation, leading to an increase of FSH-induced estradiol production (Fig. 3) [25]. Thus, BMP-2, -4, -6 and -7 differentially regulate FSH-induced steroidogenesis by granulosa cells via ligand-dependent mechanisms.

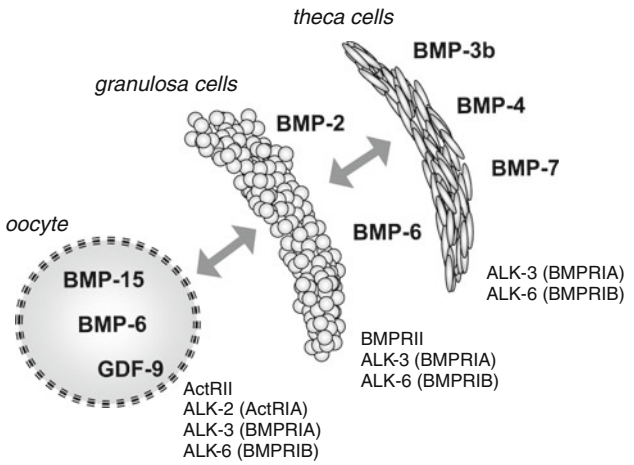

Fig. 2 Expression of the follicular BMP system in the ovary. BMP-6, -15 and GDF-9 are expressed by oocytes and exhibit biological activities in granulosa cells expressing BMPRII, ALK-3 and -6 and theca cells expressing ALK-3 and -6. Theca cells express BMP-3b, -4 and -7, which exhibit biological activities in granulosa cells and oocytes. Granulosa cells express BMP-2 and -6, which act on oocytes and theca cells. In addition to these paracrine actions, BMPs also have autocrine effects in the ovary. The BMP system acts to regulate cell proliferation as well as differentiation during folliculogenesis

Histological analysis has demonstrated exclusive expression of BMP-15 in oocytes, with its expression increasing in relation to follicle growth and development (Fig. 2) [26]. The two paralog genes *bmp15* and *gdf9*, each composed of two exons, are specifically expressed by oocytes in a similar pattern during folliculogenesis [6]. In rodents, BMP-15 and GDF-9 expression is observed in oocytes during folliculogenesis starting from primary-staged follicles [12, 27–29]. In mono-ovulatory species, such as ewes, cows and humans, GDF-9 expression is also observed in primordial follicles [12, 30, 31]. BMP type-I and type-II receptors are also expressed by granulosa cells in early stages of follicular development. BMP-15 signaling involves BMPRIB (ALK-6) and BMPRII and subsequent phosphorylation of the Smad1/5/8 pathway [32], in contrast to the finding that GDF-9 signaling involves interaction with TGFβRI (ALK-5) and BMPRII followed by Smad2/3 activation on the surface of target cells including granulosa and theca cells [33]. Expression of these receptor subtypes has been confirmed in granulosa cells from the primordial to primary stages, consistent with responsiveness to BMP ligands [34]. It is likely that BMP-15 and GDF-9 initially bind BMPRII, with a type-I receptor being subsequently recruited or already having formed a complex with BMPRII [35].

Biological functions of BMP-15 in regulation of folliculogenesis

In general, TGF-β superfamily ligands are initially synthesized as large precursor molecules, which are dimerized and cleaved by proteolytic processing to produce mature proteins (Fig. 3). BMPs are distinguished from other members by having seven, rather than nine, conserved cysteine residues in the mature region. Six of the seven common cysteines in the mature protein are linked within the subunit to form a rigid structure called a cysteine knot. The remaining cysteine is necessary for dimerization through a disulfide bond. BMP-15 and GDF-9 differ from

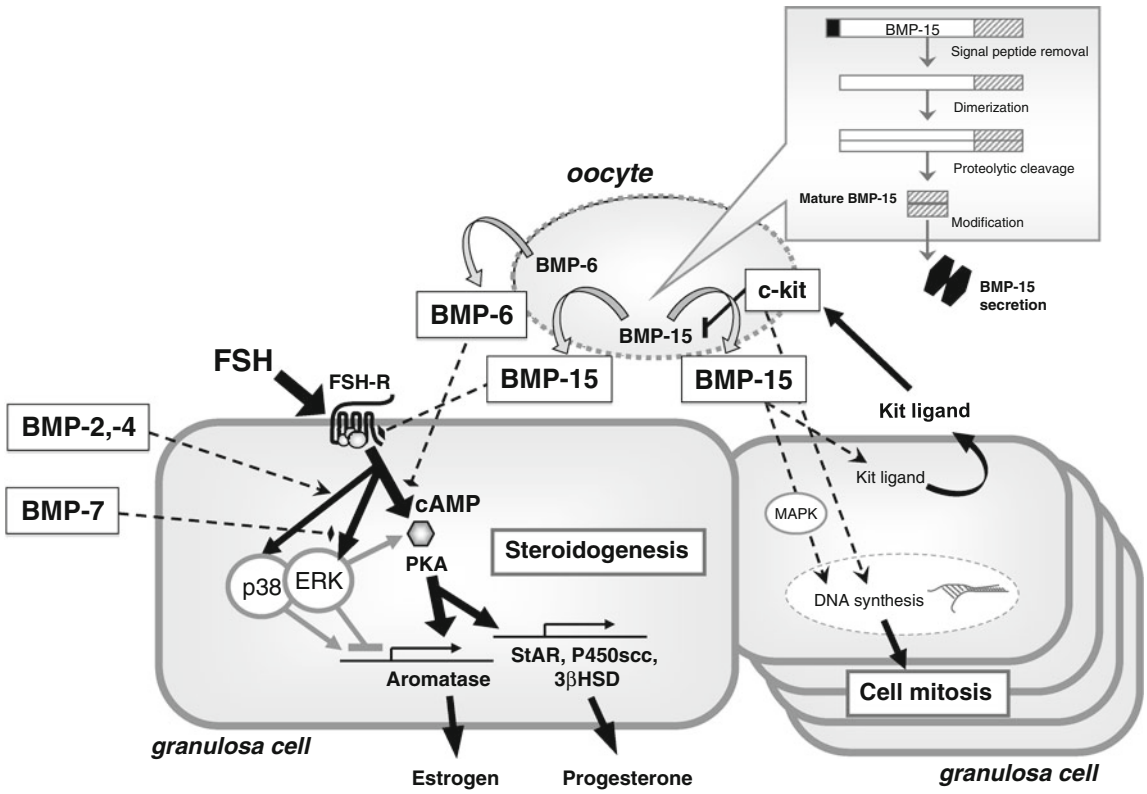

Fig. 3 Roles of the BMP system in steroidogenesis and mitogenesis in growing follicles. FSH activates estradiol and progesterone production through the cAMP-to-PKA pathway in granulosa cells in the ovary. FSH simultaneously stimulates MAPKs such as ERK and p38 pathways, leading to pathway-specific modulation of FSH-induced steroidogenesis. In oocytes, BMP-15 ligands are synthesized as large precursor molecules, which are dimerized and cleaved by proteolytic processing to produce mature BMP-15 proteins. BMP-15 inhibits FSH receptor expression, while BMP-6 suppresses adenylate cyclase activity, both of which result in reduction of progesterone production. BMP-2 and -4 activate FSH-induced p38, leading to stimulation of estradiol production. BMP-7 inhibits FSH-induced ERK1/2 phosphorylation, leading to upregulation of estradiol production since ERK1/2 activation is directly linked to inhibition of estradiol. In addition to the regulation of steroidogenesis by granulosa cells, oocytes and/or oocyte-derived factors facilitate FSH-to-MAP kinase and BMP-to-Smad signaling activity through oocyte-granulosa cell communication. Oocyte-derived BMP-15 also stimulates granulosa cell mitosis and Kit ligand expression. Kit ligand acts through c-kit on the surface of oocytes to inhibit BMP-15 expression, forming a negative feedback loop. Kit ligand-to-c-kit signaling also increases granulosa cell mitosis, presumably by stimulating oocytes to secrete some unidentified mitogens

other TGF-β superfamily members in that their mature regions lack the fourth cysteine residue responsible for inter-subunit disulfide bonding [27, 28]. Therefore, BMP-15 dimers are thought to be formed by non-covalent bonds between the mature subunits [36].

In vitro studies using recombinant human BMP-15 first revealed that BMP-15 stimulates proliferation of undifferentiated rat granulosa cells in an FSH-independent manner (Fig. 3) [26]. On the other hand, BMP-15 inhibits FSH actions by suppressing FSH receptor expression of rat granulosa cells [37]. Given the finding that FSH-induced progesterone synthesis is potently inhibited by BMP-15, BMP-15 can be categorized as a luteinization inhibitor [21, 38]. Importantly, BMP-15 stimulates mitotic activity of rat granulosa cells [26] with augmentation of kit ligand (KL) expression in granulosa cells (Fig. 3) [39]. Furthermore, BMP-15 and KL form a negative feedback loop between oocytes and the surrounding granulosa cells, in which KL-to-c-kit signaling inhibits BMP-15 expression in oocytes and BMP-15 in turn activates KL expression in granulosa cells, leading to the effective induction of granulosa cell proliferation (Fig. 3) [39]. These BMP-15 actions are extracellularly regulated by the binding protein follistatin [40]. Follistatin is strongly expressed in dominant follicles, while its expression level is very low or undetectable in atretic follicles. Since BMP-15 is an inhibitor of FSH receptor expression, regulation of BMP-15 actions by follistatin might be important for normal folliculogenesis. BMP receptor-to-Smad signal activities are also modulated by FSH receptor signaling, leading to fine-tuning of the mutual sensitivity of BMPs and FSH [41].

BMP-15 is also involved in the regulation of cumulus cell apoptosis. When oocytes are removed from cumulus–

oocyte complexes in bovine ovaries, cumulus cells become apoptotic. The apoptotic process can be rescued by treatment with BMP-15 but not by treatment with GDF-9 [42]. A similar response is also observed when cumulus cells are treated with BMP-15 in combination with FSH. BMP-15 may decrease the incidence of apoptosis within cumulus–oocyte complexes until initiation of the ovulation process. BMP-15 also plays a key role in stimulating cumulus expansion [43], which is associated with enhanced expression of epidermal growth factor (EGF)-like growth factors in cumulus cells. Given the finding that reduced EGF receptor expression in oocytectomized cumulus complexes is restored by treatment with either GDF-9 or GDF-9 plus BMP-15, both GDF-9 and BMP-15 are crucial for the process of cumulus cell expansion through EGF receptor induction [44].

The biological interrelationship between BMP and fibroblast growth factor (FGF) signals is also involved in ovarian folliculogenesis. FGF-8 is expressed specifically in oocytes of mouse and bovine tissues, and FGF receptors are substantially expressed in granulosa cells of human, mouse, rat and bovine follicles [45–47]. Since mammalian oocytes are not capable of carrying out glycolysis, the products of glycolysis that are necessary for oocyte development must be transmitted into oocytes by companion cumulus cells. It has been revealed that BMP-15 and FGF ligands commonly derived from oocytes cooperate to promote glycolysis in cumulus cells [48]. In this process, BMP-15 and GDF-9 also integrate cumulus cell metabolism, particularly in the steps of glycolysis and cholesterol biosynthesis before the preovulatory LH surge. Cholesterol synthesized *de novo* is downregulated in both BMP-15/GDF-9 double mutant cumulus cells and in wild-type cumulus cells after removal of oocytes from cumulus–oocyte complexes [49], suggesting that oocytes regulate cumulus cholesterol biosynthesis as a compensation for the deficiencies in cholesterol production in oocytes [49]. The interaction between FGF and the BMP system is important for the maintenance of FGF receptor signaling itself in granulosa cells [50].

Post-translational modification of mature BMP-15 protein is also critical for the biological activities [51]. Namely, N-terminal pyroglutamic acid structure and C-terminal Lys truncation are present in mature peptides. Phosphorylation at the 6th Ser and O-glycosylation at the 10th Thr have also been revealed [51]. The phosphorylation modification is essential for the bioactivity of recombinant BMP-15 and GDF-9, and de-phosphorylation of BMP-15 and GDF-9 abolishes the bioactivity of BMP-15, GDF-9 and BMP-7 but not that of activin, suggesting that the phosphorylation state of BMP-15/GDF-9 is a determinant of these biological activities [52].

Roles of BMP-15 in folliculogenesis revealed by animal studies

The discovery of the naturally occurring strain of sheep called Inverdale was a major breakthrough in the reproductive biology of BMP-15 [11]. The major gene involved in Inverdale was found to be X-linked; homozygous carrier ewes of the Inverdale gene were sterile due to ovarian hypoplasia, reflecting failure of ovarian follicles to progress beyond the primary stage of follicle development. Causal mutations of the Inverdale phenotype were identified in the *bmp15* gene, in which two independent point mutations were discovered and named Inverdale ($FecX^{I}$) and Hanna ($FecX^{H}$) [11]. The Inverdale mutation ($FecX^{I}$) is a substitution of V31D of the mature BMP-15 protein, while the Hanna mutation ($FecX^{H}$) replaces Glu by a stop codon at amino acid residue 23 of the mature domain of BMP-15, resulting in the synthesis of a short inactive peptide. Homozygous carriers of the Inverdale and Hanna *bmp15* mutations are infertile with streak ovaries showing arrested folliculogenesis at the primary stage. Heterozygous carriers of point mutations in the *bmp15* gene exhibit a higher ovulation rate than that of the wild type [11], i.e., heterozygotes that carry inactivating mutations in single copy of the *bmp15* gene show an increased ovulation rate in sheep.

Another important mutation that is responsible for the phenotype of hyperprolific Booroola ewes (FecB) was identified [53–55] in the coding sequence of BMPRIB (ALK-6), a type-I receptor for BMP-15 [32] consisting of a non-conservative Q249R substitution. The importance of BMP-15 in sheep fertility was further demonstrated by the identification of two additional *bmp15* point mutations, $FecX^{G}$ (truncation) and $FecX^{B}$ (S99I), in Cambridge and Belclare sheep that resulted in the same phenotype as that in Inverdale and Hanna ewes [56]. Another mutation ($FecX^{R}$) of the *bmp15* gene was also discovered in Rasa Aragonesa sheep; the mutation has a deletion of 17 bp in exon 2, leading to a premature stop codon before the coding region of BMP-15 mature protein [57, 58]. Similar to the phenotype in Inverdale and Hanna ewes, the $FecX^{R}$ mutation was also associated with high prolificacy or sterility, depending on its presence in the heterozygous or homozygous state, respectively. Another mutation in the *bmp15* gene bearing a C53Y mutation in the mature peptide of BMP-15 was identified in highly prolific Lacaune sheep [59]. This mutation was associated with an increased ovulation rate and sterility in heterozygous and homozygous animals, respectively. In vitro studies showed that the C53Y mutation was responsible for impairment of the maturation process of the BMP-15 protein, resulting in defective secretion of both precursor and mature BMP-15 peptides [59].

Furthermore, both the infertile and superfertile phenotypes of the BMP-15 mutant ewes were reproducible by immunizing wild-type ewes against BMP-15 proteins [60, 61]. Ewes immunized with entirely mature BMP-15 exhibited at least one estrous cycle, and the ovulation rate in these cycles was higher than that in control ewes, similar to the condition observed in the heterozygous Inverdale and Hanna ewes. Further studies on antisera for BMP-15 and GDF-9 provided evidence that antibodies generated against the N-terminal region of BMP-15 or GDF-9 potently inhibit the paracrine actions of these oocyte-secreted factors in vivo and in vitro [62]. Sheep BMP-15 is likely to play an important role in promoting the transition of follicles through the early stages of folliculogenesis while restraining the transition of follicles to the dominant preovulatory stage and also to be a central player in the determination of ovulation quota and litter size in ewes [6].

In contrast to the characteristic phenotypes of GDF-9 null female mice showing infertility due to arrested folliculogenesis at the primary stage [10], *bmp15* knockout mice showed unexpectedly minimal phenotypes regarding follicle development and fertility [63]. BMP-15 null males were fertile with normal testis size, whereas BMP-15 homozygous female mutants in both hybrid and inbred strains displayed a consistent subfertility with a reduced litter size compared with that of the heterozygous mutants. BMP-15 null ovaries showed all stages of follicle development and multiple corpora lutea. Nevertheless, BMP-15 null ovaries occasionally showed very few follicles and increased zona pellucida remnants with lower response to ovulation stimuli and larger oocytes accompanying less cumulus cells. Follicular recruitment and the number of preovulatory follicles were almost normal in BMP-15 null mice, while the oocytes appeared to be trapped within the follicles. Major defects in female mice lacking BMP-15 were found in the ovulation process, supporting the observation that functional mature protein of mouse BMP-15 is almost undetectable in oocytes in vivo until after the LH surge [43].

Mice with BMP-15 overexpression have further provided interesting findings regarding the roles of BMP-15. Since mouse BMP-15 proprotein is not directly processed into functional mature peptides [64], a chimeric protein consisting of the human proregion, human cleavage site, and mouse mature region (termed hhmBMP-15), which can be processed into the mature protein, was utilized in these transgenic mice [64]. BMP-15 protein was detected exclusively in oocytes from the primary follicle stage and throughout folliculogenesis in BMP-15 transgenic mice [65]. Interestingly, granulosa cells derived from the ovaries of BMP-15-overexpressing mice displayed an increased mitotic potential [26] and decreased FSH receptor expression [37] as shown in in vitro studies using recombinant BMP-15. Although the formation of primordial follicles and the transition from primordial to primary follicles occurred normally in BMP-15 transgenic mice, a significant decrease in primary follicles with a concomitant increase in secondary follicles was observed, possibly due to the excess of granulosa cell mitosis in primary follicles. Despite the increase in secondary follicles during the FSH-independent stages in BMP-15-overexpressing mice, many of the developing follicles undergo atresia, possibly due to the decreased FSH receptor signaling. Given that BMP-15 transgenic mice showed an early onset of acyclicity, the suppression of BMP-15 actions during early folliculogenesis is also important in restraining acceleration of follicle development to prevent premature loss in the follicle reserve.

Hence, the biological importance of BMP-15 in regulation of folliculogenesis appears to be different in sheep and mice. In contrast to the multi-functional in vitro activities of BMP-15, mice with null mutations in the *bmp15* gene are unexpectedly subfertile with less impact of ovarian phenotypes [63] compared with the phenotypes of GDF-9 null mice [10]. On the other hand, ewes homozygous for inactivating BMP-15 mutations are completely infertile with follicle development arrested at the primordial stage, similar to GDF-9 knockout mice [56, 60]. The species variation is evident in the role of oocyte-derived BMP-15 in early preantral follicle development. Accumulating evidence has revealed that BMP-15 has critical effects on granulosa cells of primordial and/or primary follicles that are essential for further follicle development in mono-ovulatory mammals rather than in poly-ovulatory mammals.

BMP-15 mutations discovered in humans

Indispensable roles of BMP-15 in female fertility have been finally demonstrated in humans. The discovery of human mutations of BMP-15 in female patients with reproductive defects provided the possibility of translation from basic knowledge to clinical application of BMP-15. Di Pasquale et al. [66] first identified a BMP-15 mutation in women that is associated with hypergonadotropic ovarian failure due to ovarian dysgenesis. The mutation is an A–G transition at position 704 of the *bmp15* gene and results in a non-conserved substitution of Y235C of the proregion of BMP-15 proprotein, which acts as a dominant negative by altering BMP-15 processing [66]. The patients with the BMP-15 mutation were sisters, who inherited the mutation from their father. Interestingly, women suffering primary amenorrhea were heterozygous carriers of the mutation and their streak ovaries resembled the ovarian phenotypes reported in homozygous mutant ewes.

Premature ovarian failure (POF)/primary ovarian insufficiency (POI) is a common pathology that leads to human infertility. POF/POI may manifest as primary amenorrhea without menarche or secondary amenorrhea after pubertal development, depending on the age of the affected patient. POF/POI has been proposed to appropriately express the condition reflecting the progression towards the cessation of ovarian function [67]. POF/POI represents a public health concern since it affects approximately 1% of women under the age of 40 years. In humans, relatively rare variants of the *bmp15* gene were shown to contribute to the pathogenesis of POF/POI [66, 68–71]. Among the variants described in the human *bmp15* gene, mutants including Y235C, E211X, R68W, L148P and R61W may be substantially linked to the POF/POI phenotype since these mutants were absent in normal cases. These mutations are located at conserved amino acid positions in mammalian species and have been suggested by in silico analyses to have deleterious effects [66, 70, 71]. Although all human BMP-15 mutations are heterozygous, one homozygous mutation, c.631C>T, located in a propeptide lacking the C-terminal region containing the mature region, has also been observed in an Indian patient [70]. The effect of this drastic variant may explain the severity of the phenotype, mainly characterized by primary amenorrhea.

Additionally, mothers of spontaneous dizygotic (DZ) twins (MODZT) carry rare deletions and missense mutations in the coding regions of BMP-15 and GDF-9, which are significantly associated with the occurrence of twinning [72, 73]. The mutations with BMP-15 (R76C and R206H) and GDF-9 (K67E and P103S), which occur with a high incidence in POF/POI patients, have not been identified in normal cases [69, 70, 74]. A mutation of GDF-9 (P103S) was also identified in MODZT with a significantly higher frequency than that in controls [73]. These mutations resulted in impaired post-translational processing of the proproteins, which caused reduced production of mature BMP-15 and GDF-9 proteins [75]. Therefore, these BMP-15 and GDF-9 mutations may have enhanced fertility, leading to the increased opportunity of DZ twins and/or rapid exhaustion of the ovarian reserve leading to POF/POI [25]. Nevertheless, no significant relationship between the haplotype frequencies and DZ twinning phenotype was found in a study in which 35 SNPs of the *bmp15* gene in DZ twinning families were screened [76]. Further accumulation of results of phenotype-genotype analysis will be necessary to reach a conclusion regarding the biological impact of human BMP-15 mutations in the occurrence of DZ twinning.

Taken together with the phenotypes in various mutants including those in humans and sheep, it can be concluded that reduced levels of BMP-15 are potentially linked to increased ovulation rate and litter size and also that proper post-translational processing of the BMP-15 proprotein is a critical aspect of female fertility in sheep as well as in humans.

Central regulation of gonadotropins by BMPs

It is very interesting that BMPs also play a critical role in the differentiation of pituitary gonadotropes [77, 78]. During normal mouse pituitary development, BMP-4 is expressed in the ventral diencephalon, while BMP-2 and the BMP-binding protein chordin are expressed in the ventral condensing mesenchyme. BMP ligands and receptors are expressed in the adult pituitary gland. Specifically, BMP-6, -7 and -15 mRNAs are expressed in the pituitary of mice [79, 80], GDF-9 mRNA is expressed in the pituitary of humans [81], BMP-15 and GDF-9 mRNAs are expressed in the pituitary of brushtail possums [82], and BMP-15 mRNA is expressed in the pituitary of sheep [11]. With regard to BMP receptors, BMPRII, ActRII, ALK-2 and ALK-3 mRNAs are expressed in mice pituitaries [80], ALK-6 mRNA is expressed in sheep pituitaries [55], and ActRII and ActRIIB mRNAs are expressed in rat pituitaries [83]. Of potential physiological importance are the findings that BMP-6, -7 and -15 can act directly on pituitary gonadotropes to regulate FSH synthesis and secretion.

The pituitary BMP system also acts as a regulator of transformation and tumorigenesis of differentiated pituitary cells. Human gonadotropinomas have reduced expression of follistatin, an activin/BMP-binding protein, compared with the expression in non-functioning tumors [84]. In addition, PPARγ activation is functionally linked to the inhibition of BMP receptor signaling in gonadotrope LβT2 cells [85]. It is interesting that BMP-4 is overexpressed in various lactotrope tumor models and human prolactinomas [86], with molecular interaction between BMP-4, Smad4 and estrogen receptor (ER) involved in regulation of prolactin (PRL)-promoter activity [87]. Thus, the pituitary BMP system is likely to act as a regulator not only for pituitary differentiation but also for the transformation of differentiated pituitary cells [88, 89].

Our recent study has shown a new regulatory role of ovarian BMPs in conditions with high concentrations of systemic PRL levels, namely hyperprolactinemia [90]. Elevation of serum PRL level is associated with various physiological and/or pathological conditions and is a major cause of amenorrhea. PRL exerts a direct inhibitory effect on gonadotropin actions in the ovary [91], although it is recognized that PRL directly inhibits secretion of gonadotropins from the anterior pituitary. In granulosa cells, PRL inhibits estradiol production [92] and stimulates progesterone production [93] by activating distinct signaling pathways in a differentiation-dependent manner [94]. The

mechanism by which excess PRL inhibits FSH-induced estradiol secretion in a variety of preovulatory follicle models has been reported to be reduction of aromatase activity [92, 93]. PRL also interferes with FSH action by suppressing LH receptor expression at sites downstream of cAMP synthesis in granulosa cells [95]. PRL regulates many functions in diverse target tissues through multiple PRL receptor isoforms of membrane-bound receptors [96]. It was revealed that PRL upregulates activity of the endogenous BMP system including oocyte-derived BMP-15. BMP actions in growing follicles play a key role in antagonizing PRL receptor signaling, which may prevent immature ovulation caused by increased progesterone but reduced estrogen levels in growing follicles exposed to hyperprolactinemia [90].

Hypothalamic GnRH synthesis and release are tightly regulated by gonadal steroids, which maintain control through a negative feedback system [97]. In females, estrogens act directly or indirectly on the GnRH neuronal network to modulate the final output of GnRH into the median eminence [98]. An immortalized GnRH-producing GT1-7 cell line is a valuable tool for studying the biology of GnRH neurons because of the expression of functional ER [99]. Interaction between BMP receptors and ER is involved in controlling hypothalamic GnRH production and secretion in GT1-7 cells [100]. In this system, BMP-2 and BMP-4 repress ER-induced downregulation of GnRH transcription by attenuating ER-MAPK signaling. BMP-6 and BMP-7, in turn, increase GnRH transcription and secretion by stimulating expression of the R-type calcium channel. Thus, BMPs are also involved in regulation of the pituitary–gonadal endocrine axis. Understanding the neural mechanisms through which estradiol regulates GnRH neurons is the key for elucidating reproductive control of the hypothalamo-pituitary-ovary (HPO) axis. Actually, the expression of some BMP receptors in the hypothalamus has been detected. BMP type-II receptors such as ActRII and ActRIIB were found in areas for neuroendocrine regulation including the suprachiasmatic, supraoptic, paraventricular and arcuate nuclei in the rat hypothalamus [83]. The interaction of BMPs and ER may be involved in the control of hypothalamic GnRH production and secretion by an autocrine/paracrine mechanism. The BMP system may play a key role in regulating not only in ovarian folliculogenesis by regulating gonadotropin sensitivity but also in modulating hypothalamic GnRH secretion leading to fine-tuning of gonadotropin secretion from pituitary gonadotropes.

Future direction and perspectives

The reproductive consequences of mutations and deletions in the *bmp15* gene coupled with the biological functions of BMP-15 in follicular cells have established the importance of the BMP system for normal fertility in females. The future direction in the field is translational studies to bring the basic science discoveries regarding BMPs to the bedside in the form of novel fertility treatments, contraceptive paradigms and diagnostic capabilities.

POF/POI occurs through two major mechanisms: "follicle dysfunction" and "follicle depletion". Follicle dysfunction is mainly caused by a pathologic process that prevents normal follicular function despite the presence of follicles in the ovary. In contrast, follicle depletion refers to the condition in which no primordial follicles are present in the ovary due to defects in the initial pool of primordial follicles. Follicle depletion leads to accelerated expenditure and/or destruction of follicles. Considering the roles of BMP-15 in promoting early stages of follicle growth and restraining the number of dominant preovulatory follicles leading to determination of litter size, it is reasonable to propose that administration of endogenous BMP-15 could provide new therapeutic regimens for preventing the "follicle dysfunction" of POF/POI. The timing of administration or induction of BMP-15 would likely be important for effective treatment of infertility. It is notable that each of the naturally occurring BMP-15 mutations appear to be primarily manifested through defects in specific processes involved in the post-translational processing of the proprotein. Accordingly, it is possible that therapeutic strategies focusing on the proprotein processing may provide novel approaches to modulate endogenous BMP-15 function.

Since BMP-15 is secreted by oocytes in the ovary, the diffusion-dependent transfer of BMP-15 from oocytes to surrounding cells appears to be an important aspect of the morphogen effects. Different follicular cell types are presumably exposed to very different mixtures of many signaling molecules, depending on their relative position and/or developmental stage. Experimental evidence indicates various autocrine/paracrine roles of granulosa-derived activins and BMP-6, oocyte-derived BMP-15/GDF-9 and BMP-6, and theca-derived BMP-4 and BMP-7 in promoting granulosa cell proliferation and/or modulating FSH-dependent follicle function [6, 38]. Interaction between signaling molecules other than BMP-15 would also need to be taken account to induce efficacious delivery of BMP-15 to growing follicles. Moreover, mutual communication between oocytes and surrounding granulosa cells and theca cells is obligatory for normal follicle recruitment and development [8]. Delicate balance of the bidirectional communication between oocytes and their surrounding follicular cells is crucial for maintaining the follicular unit in order to ensure normal ovulation, which is further fine-tuned by gonadotropins secreted from the anterior pituitary and estrogens produced by ovarian follicles.

Potential genetic causes of non-syndromic POF/POI have also been established by genetic linkage analysis of familial cases or by screening of mutations in candidate genes based on animal models. If a genetic alteration is found in a woman, it can be informative for family counseling because it can predict the female relatives who have a higher risk for POF/POI and fertility loss at a young age. Sequence variants in oocyte-specific BMP-15/GDF-9 are strong candidates for genetic factors of POF/POI pathophysiology. In addition to currently available biochemical markers such as FSH/LH, estrogens, inhibin B and antimullerian hormone (AMH), determination of BMP-15 and GDF-9 levels in follicular fluid, tissues and/or serum by sensitive methods could also be clinically useful for confirming a diagnosis of occult POF/POI.

Collectively, the elucidation of genetic differences in species-dependent ovulation rate has provided a novel insight regarding target molecules for manipulating female reproduction. Utilizing the BMP-15 system would be a powerful strategy for treatment of women with reproductive defects including POF/POI, by altering fertility at the ovary level regulating FSH receptor sensitivity and at the pituitary level modulating gonadotropin secretion.

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