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The Bone Morphogenetic Protein System In Mammalian Reproduction

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Using molecular, cellular, and genetic approaches, recent studies examining the role of the bone morphogenetic protein (BMP) family of growth factors in the reproductive system have led to significant breakthroughs in our understanding of mammalian reproduction and fertility. Gene expression studies have revealed that key components of the BMP system (ligands, receptors, signaling molecules, and binding proteins) exhibit coordinated spatial and temporal expression patterns in fundamental cell types throughout the reproductive system. Availability of recombinant BMPs has enabled functional studies that have demonstrated important biological activities of BMPs in controlling cellular proliferation, differentiation, and apoptosis in reproductive tissues. The physiological importance of the BMP system for mammalian reproduction has been further highlighted by the elucidation of the aberrant reproductive phenotypes of animals with naturally occurring mutations or targeted deletions of certain BMP family genes. Collectively, these studies have established the concept that the BMP system plays a crucial role in fertility in female and male mammals. The purpose of this article is to review the evidence underpinning the importance of the BMP system in mammalian reproduction. (*Endocrine Reviews* 25: 72–101, 2004)

Abbreviations: ActR-II, Activin type-II receptor; ALK, activin receptor-like kinase; AMH, anti-Müllerian hormone; BAMBI, BMP and activin membrane bound inhibitor; BMP, bone morphogenetic protein; BMP-15^{I31D}, BMP-15 with an I31D substitution; BMPR-II, BMP type-II receptor; 8-Br-cAMP, 8-bromo-cAMP; CDMP, cartilage-derived morphogenetic protein; CTGF, connective-tissue growth factor; ECA, extracellular domain; FKBP, FK506 binding protein; GDF, growth and differentiation factor; hCG, human chorionic gonadotropin; 3β-HSD, 3β-hydroxysteroid dehydrogenase; KL, kit ligand; MIS, Müllerian inhibiting substance; OP, osteogenic protein; P450arom, P450 aromatase; P450scc, P450 side-chain cleavage enzyme; PDF, prostate-derived factor; PGC, primordial germ cell; PLAB, placental bone morphogenetic protein; R-Smads, receptor-regulated Smads; SMURF, Smad ubiquitination regulatory factor; StAR, steroidogenic acute regulatory protein.

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I. Introduction

THE NAME BONE morphogenetic protein (BMP) was first given in 1965 by Urist and colleagues (1–3) to the active components in demineralized bone and bone extracts that are capable of inducing bone formation at ectopic sites. In 1988, the first BMPs were isolated, and their cDNAs were cloned by Wozney *et al*. (4). In this report, the full-length cDNAs designated as BMP-1, BMP-2, BMP-2B (now called BMP-4; Table 1), and BMP-3 were described. The deduced amino acid sequences of BMP-2, -3, and -4 closely resemble the members of the TGF-β family, which at that time was composed of TGF-β1 (5), TGF-β2 (6), inhibin/activin subunits (α, βA, and βB) (7), decapentaplegic (8), Vgl (product of the *Xenopus* transcript present in the vegetal pole of eggs) (9), and Müllerian inhibiting substance (MIS) [also called anti-Müllerian hormone (AMH)] (10). Together, these factors represented the founding members of the TGF-β superfamily, which now consists of more than 35 members (11). Analysis showed that BMP-1 does not share structural homology with the BMPs. Therefore, BMP-1 is not included in the TGF-β superfamily. Subsequent studies showed that BMP-1 is identical to procollagen C-proteinase, which can cleave procollagen I, II, and III into mature monomers (12). With regard to bone formation, BMP-1 can also cleave the BMP binding protein, chordin, rendering it inactive. Therefore, it is believed that the bone-inducing activities of BMP-1 are manifested through its ability to cleave chordin and thereby eliminate the ability of endogenous chordin to antagonize endogenous BMP activities.

TABLE 1. Alternative names for members of the BMP family

Ligand	Alternative names
BMP-2	BMP-2A
BMP-3	Osteogenin
BMP-3b	GDF-10
BMP-4	BMP-2B
BMP-6	Vgr-1
BMP-7	OP-1
BMP-8a	OP-2
BMP-8b	OP-3
BMP-9	GDF-2
BMP-12	GDF-7, CDMP-3
BMP-13	GDF-6, CDMP-2
BMP-14	GDF-12
BMP-15	GDF-9B
BMP-16	Nodal
GDF-3	Vgr-2
GDF-5	CDMP-1
GDF-15	PLAB, MIC-1, PDF, PTGF-β
MIS	AMH

Vgr, Vg-related protein; MIC, macrophage inhibiting cytokine; PTGF-β, placental TGF-β.

Shortly after the cloning of the first BMPs, a number of new BMP family genes were identified (13–18). Because different research groups discovered the same or similar genes simultaneously, different names for BMP-related gene families arose, such as osteogenic protein (OP), cartilage-derived morphogenetic protein (CDMP), and growth and differentiation factor (GDF). This situation was further confounded by the fact that certain BMP-related genes were given different names, *e.g.,* BMP-7/OP-1, BMP-6/Vg-related protein-1, BMP-3b/GDF-10, BMP-13/GDF-6, CDMP-1/GDF-5, and BMP-15/GDF-9B (Table 1). Furthermore, because many subsequent BMP family genes were identified by homology-based cDNA cloning, the osteoinductive activity, if any, of many BMPs remains to be established. Consequently, the BMP nomenclature is confusing, and there is a need to develop an appropriate BMP terminology for this family. For the purposes of this review, we have chosen to define the BMP family of ligands to include the BMPs and the GDFs based on the high homology in their amino acid sequences (11). Recent research has suggested that MIS can use BMP-specific type-I receptors and activate BMP intracellular signaling pathways (19–22). It appears, therefore, that MIS has properties that resemble the BMP/GDF family. However, we have chosen not to focus on MIS in this review because excellent previous reviews have provided a detailed discussion of the MIS field (23–26).

As the tissue expression patterns of the BMP family became known and the recombinant BMPs became available for research, the conclusion emerged that the BMPs regulate growth, differentiation, and apoptosis in a wide variety of tissues in addition to bone (27, 28). After a few key papers that reported the expression of components of the BMP system in mammalian reproductive tissues (16, 29–32), genetic and experimental studies were quickly performed that demonstrated crucial roles of the BMP system in the regulation of reproductive processes, particularly in the gonads (32–42). The continued investigation of the roles of the BMP system in reproductive physiology has provided new insight into our understanding of the physiological regulation of key reproductive processes. Indeed, this information is beginning to provide answers to some of the long-sought questions in the field of reproductive physiology and endocrinology.

In this report, we present a comprehensive review of the literature as it applies to the roles of the BMP system in regulating mammalian reproduction. In a broad sense, we will focus our attention on three main areas: 1) the spatiotemporal expression pattern of BMP ligands, receptors, signaling molecules and regulatory factors in reproductive tissues; 2) the specific biological activities of BMP ligands in regulating reproductive processes; and 3) the genetic studies that have provided particular insight into the role of the BMP system in regulating mammalian fertility.

II. The BMP System

A. BMP ligands

The BMP family is the largest within the TGF-β superfamily of growth factors. A distinguishing structural feature of the TGF-β superfamily is the presence of seven conserved cysteines, which are involved in folding the molecule into a unique three-dimensional structure called a cystine knot (43–45). The one conserved cysteine residue that is not involved in cystine knot formation makes a single disulfide bridge between the two subunits. This results in the formation of a covalently linked dimer, which is critical for biological activity (46–48). Members of the TGF-β and activin families are distinguished from those of the BMP family by having two extra conserved cysteines. Studies using recombinant protein expression systems revealed that when coexpressed, BMP-2, -4, -5, -6, and -7 can form heterodimers. An interesting feature of the BMP heterodimers is that they can exhibit greater biological activity than their corresponding homodimers (49–52). The observation that different BMPs isolated from bone often copurify suggests that BMPs may exist as heterodimers under physiological conditions *in vivo* (4). Interestingly, GDF-9 and BMP-15 have only six of the seven conserved cysteines; both lack the fourth cysteine that is required for the intersubunit-disulfide bridge (16, 30, 31). This unusual structure raises the question: do BMP-15 and GDF-9 exist as monomers or as noncovalently linked dimers? Recent studies by Liao *et al.* (53) have demonstrated that both BMP-15 and GDF-9 do indeed form noncovalently linked homodimers, and when coexpressed can also form heterodimers. This finding indicates that the formation of a disulfide bridge may not be essential for dimerization of BMP subunits.

The posttranslational processing of BMPs is important for the secretion of biologically active molecules. All TGF-β superfamily members are translated as large preproproteins composed of a signal peptide, prodomain, and mature domain. After removal of the signal peptide, the proproteins undergo dimerization. As processing proceeds, specific proteolytic enzymes cleave the dimerized proprotein at the RXXR site, resulting in the generation of the biologically active dimeric mature protein. Some TGF-β superfamily members are secreted as a complex consisting of the mature dimer noncovalently bound to the prodomain. In the case of TGF-β, this complex is biologically inactive and can be ac-

tivated under chaotropic conditions such as low pH (54). There is also evidence that mature BMPs can be secreted as a complex with their prodomains (46–48, 53, 55); however, the physiological role and biological activities of such BMP complexes are poorly understood.

B. BMP receptors

Crosslinking studies have revealed two major types of membrane-bound receptors for the ligands of the TGF-β superfamily, type-I and type-II receptors (56, 57). The first of the TGF-β superfamily receptors to be cloned and characterized was an activin receptor (58). It consists of an extracellular ligand binding domain, a single membrane-spanning domain, and an intracellular domain containing a predicted serine/threonine kinase region. Activin A, activin B, and inhibin A, but not TGF-β1, bind to this receptor. This receptor was designated as the activin type-II receptor (ActR-II). After the cloning of the ActR-II cDNA, other related type-II receptor cDNAs were cloned, predominantly by homology-based PCR strategies. To date, five mammalian type II receptors have been identified: ActR-II (58), ActR-IIB (59), AMHR-II (MIS/AMH type-II receptor) (60, 61), BMPR-II (BMP type-II receptor) (62–65), and TβR-II (TGF-β type-II receptor) (66). In mammals, seven type-I receptors termed activin receptor-like kinase (ALK)-1 to -7 have also been cloned (67–72).

The ligand-receptor relationships between various BMP ligands and their cognate receptors are not exclusive (Table 2). Originally, ActR-II and ActR-IIB were identified as activin receptors; however, it is now clear that they can also act as receptors for BMP-6 (73), BMP-7 (74), and GDF-5 (75). Conversely, BMPR-II appears to bind exclusively to BMP ligands, including BMP-2 (63), BMP-4 (64, 65), BMP-6 (73), BMP-7 (63, 64), BMP-15 (76), GDF-5 (75), and GDF-9 (77). With respect to type-I receptors, ALK-2 (also called ActR-IA), ALK-3 (BMPR-IA), and ALK-6 (BMPR-IB) have been identified as BMP type-I receptors (73–75, 78–82), and overexpression of constitutively active forms of these type-I receptors activates BMP-specific cellular signaling (82–84). The complexity of the BMPR-ligand interactions is compounded by the fact that there is much cross-reactivity among different BMP ligands and the type-I receptors (Table 2). Furthermore, it has been shown that a particular BMP ligand binds different type-I receptors in different cell types. For example, in MC3T3-E1 and C2C12 cells, BMP-6 strongly binds to ALK-2 and also binds to ALK-3 with lower affinity, whereas in ROB-C26 cells BMP-6 binds most efficiently to ALK-6 and less efficiently to ALK-2 and -3 (73). Nonetheless, some general trends have emerged from a number of studies that have identified preferences of certain BMP ligands for certain BMP type-I receptors (82): BMP-2, BMP-4, and GDF-5 preferentially bind to ALK-3 and/or ALK-6 (75, 78, 82); BMP-6 and -7 most readily bind to ALK-2 and/or ALK-6 (73, 74, 78, 80, 82); BMP-15 most efficiently binds to ALK-6 with very little affinity for ALK-3 (76). Although GDF-9 has been found to bind to BMPR-II (77), the type-I receptor for GDF-9 has yet to be identified.

Given the promiscuity between the BMP ligands and BMP receptors, it has been difficult to determine the relative involvement of a given type-I receptor in the signal transduction of a particular BMP ligand. This dilemma has been highlighted by the search for the functional type-I receptors for MIS (26). There is a consensus that MIS most likely utilizes

TABLE 2. The relationships between ligands, receptors (R), and Smads in the TGF-β superfamily

Ligands	Type II-R	Type I-R	Smads	Refs.
BMP-2 BMP-4	BMPR-II	ALK-3 (BMPR-IA) ALK-6 (BMPR-IB)	Smad 1/5/8	63–65, 71, 78, 101, 102
GDF-5	BMPR-II ActR-II ActR-IIB	ALK-3 (BMPR-IA) ALK-6 (BMPR-IB)	Smad 1/5/8	75, 82
BMP-6 BMP-7	BMPR-II ActR-II ActR-IIB	ALK-2 (ActR-IA) ALK-6 (BMPR-IB)	Smad 1/5/8	63, 73, 74, 78, 80, 82, 105
BMP-15	BMPR-II	ALK-6 (BMPR-IB)	Smad 1/5/8	76
GDF-9	BMPR-II	?	Smad 2	77
MIS/AMH	AMHR-II	ALK-2 (ActR-IA) ALK-3 (BMPR-IA) ALK-6 (BMPR-IB)	Smad 1/5/8	19–22, 60, 61
Activin	ActR-II ActR-IIB	ALK-4 (ActR-IB)	Smad 2/3	58, 59, 70, 91, 100
TGF-β	TβR-II	ALK-1 ALK-5 (TβR-I)	Smad 2/3	66, 70, 96, 97

The ligand-receptor-signaling combinations that are listed above are the best representation of the preponderance of the published literature. Due to the promiscuous nature of the interactions of TGF-β superfamily members and their receptors, there are reports of specific ligand/receptor interactions, which may differ from those listed above, in certain cell types and overexpression systems. ?, A type-I receptor for GDF-9 has not yet been identified.

one of the BMP-specific type-I receptors, *i.e.*, ALK-2, -3, and/or -6; however, there appear to be conflicting results as to which one is actually responsible for the biological action of MIS (19–22). Thus, these findings suggest that under different conditions or in different cell types, a particular response of the BMP ligand may be mediated by different type-I receptors.

The pattern of receptor oligomerization for activin and TGF-β is well known. These ligands first bind to the type-II receptors, which in turn, leads to the recruitment of the type-I receptors. This pattern of receptor oligomerization is supported by the finding that neither activin nor TGF-β has an affinity for the type-I receptors alone (85), but they do have an affinity for the type-II receptors. Although the pattern of BMP receptor oligomerization is not well understood, there is evidence to suggest that it may be different from that of activin and TGF-β. This conclusion is based on the fact that BMP ligands have little, if any, affinity for BMPR-II, but they do have an affinity for type-I receptors. However, if BMPR-II is overexpressed together with the appropriate type-I receptor, the affinity of BMP ligands for BMPR-II is dramatically increased (63, 65, 73, 78, 79, 81). These data suggest that BMPR-II and the appropriate type-I receptor may act together to form a high-affinity complex for the BMP ligands (63, 76, 86). Alternatively, it has been proposed that BMP ligands may first bind to type-I receptors, followed by the recruitment of BMPR-II (63, 76, 86). If true, then the process of BMP ligand-receptor binding would be opposite of that for the activins and TGF-βs.

C. BMP signal transduction

Upon binding of the BMP ligand (Fig. 1), the type-II receptor transphosphorylates the type-I receptor at an intracellular juxtamembrane site termed the GS domain, which is rich in glycine and serine residues (68, 87). The phosphorylated type-I receptor, in turn, transphosphorylates a set of intracellular substrate signaling proteins called Smads (84, 88–90). The specific Smad proteins whose activities depend on phosphorylation by type-I receptors are called receptor-regulated Smads (R-Smads). These R-Smads include Smad1, 2, 3, 5, and 8. Once activated, the R-Smad molecules interact with another Smad molecule, termed Smad4, which is a common partner for all R-Smads and thus called common Smad (Co-Smad) (91–93). This Smad complex then translo-

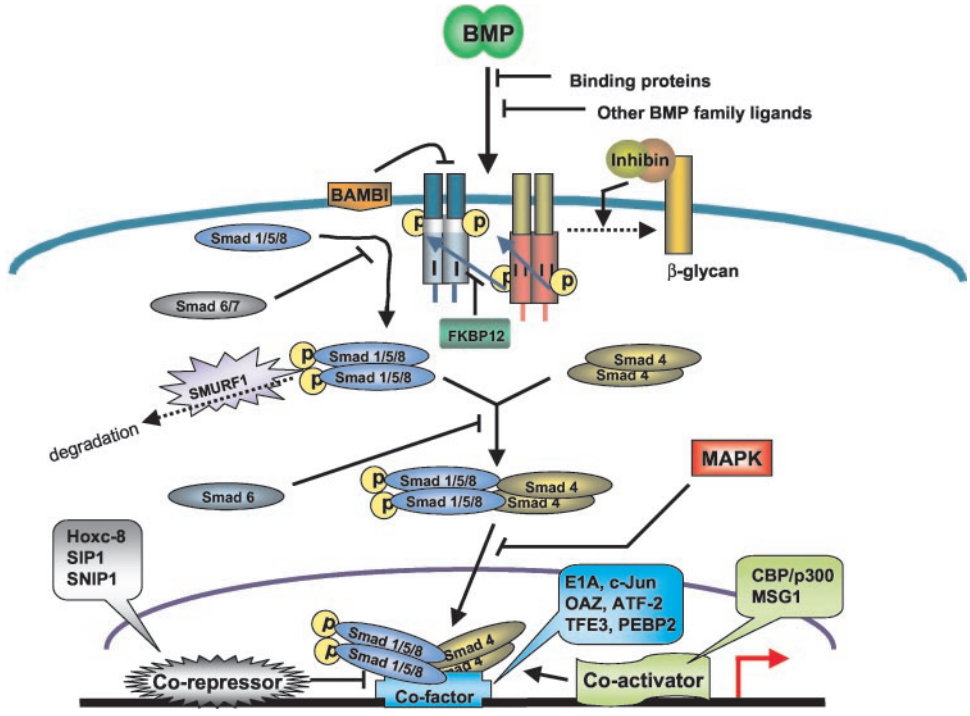

FIG. 1. BMP signaling pathways. BMP ligands bind to type-I receptors first and then recruit type-II receptors or directly bind to preexisting complexes of type-I and type-II receptors. Both type-I and type-II receptors have a Ser/Thr kinase domain. The type-II receptor is constitutively phosphorylated. 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 cofactors and regulates expression of target genes in a cell type-specific manner. BMP action/signaling can be controlled at multiple levels in the pathway: BMP binding to receptors can be inhibited by BMP binding proteins as well as other competitive BMP family members; inhibin inhibits BMP action by binding to β-glycan followed by sequestering the type-II receptors; BAMBI inhibits dimerization of type-I receptors, thereby inhibiting BMP signaling; FKBP12 inhibits type-I receptor phosphorylation; Smad 6/7 inhibits phosphorylation of Smad1/5/8; SMURF1 facilitates degradation of Smad1/5/8; Smad6 inhibits the association of Smad1 and Smad4; MAPKs such as ERK and JNK inhibit nuclear translocation of the Smad complex. In the nucleus, the Smad activation of the target gene is enhanced by coactivators and repressed by corepressors. ATF, activating transcription factor; CBP, CREB-binding protein; E1A, early region 1A; Hoxc, homeobox gene c; MSG, melanocyte-specific gene or mad-supporting gene; OAZ, Olf1/EBF associated zinc finger; PEBP, polyomavirus-enhancer-binding protein; SIP, Smad-interacting protein; SNIP, Smad nuclear interacting protein; TFE, transcription factor μ E3.

cates to the nucleus to interact with specific transcription factors to regulate the expression of target genes.

There is a distinct divergence in the Smad signaling pathways between the BMPs and the activins/TGF-βs (94, 95). The intracellular signals for the activins and TGF-βs are mediated through the phosphorylation of Smad2 and 3 (91, 96–100), whereas the phosphorylation of Smad1, 5, and/or 8 mediates the signaling of the majority of BMP ligands, including BMP-2, -4, -6, -7, and -15, GDF-5, and MIS (19, 73, 76, 93, 99, 101–105) (Table 2). This evidence, together with the fact that both the BMPs and the activins can bind to ActR-II and ActR-IIB, supports the hypothesis that the specificity of Smad signaling is determined by the type-I receptors, rather than the type-II receptors (106). Further support comes from the finding that overexpression of constitutively active forms of BMP-specific type-I receptors results in the activation of Smad1/5/8, which in turn, evokes BMP-specific biological responses (78, 80, 82–84, 103, 104). ALK-2 was originally identified as having an affinity for activin (70); however, later studies found that ALK-2 binds BMP ligands and transduces Smad signaling of BMPs but not activin (80, 107) (Table 2). Although there is evidence that individual BMP ligands may differentially activate Smad1, 5, and/or 8 (73, 82), little is currently known about the precise roles of a specific Smad protein in the mechanisms of different BMP ligand signaling (94). Uniquely, there are recent reports demonstrating that GDF-9 phosphorylates Smad2 but not Smad1/5/8 in rat and human granulosa cells (108, 109).

Although most research on BMP signaling has focused on the Smad pathway, there is increasing evidence that other signaling pathways may also be involved in mediating BMP action (86, 110–113). One line of evidence is that there can be cross-talk in the BMP signal transduction pathway between the Smads and the MAPK family of signaling molecules, *i.e.*, ERK1/2, p38, and stress-activated protein kinase/Jun N-terminal kinase (114, 115). Activated MAPK molecules have been shown to either positively transduce BMP signals (76, 113, 116–118) or act as inhibitors of Smad signaling (113, 119) (Fig. 1). The identification of the specific roles of other signaling pathways in the regulation of BMP biological activity is likely to progress rapidly.

D. Regulation of BMP action

The biological actions of BMPs can be regulated by 1) the extracellular regulation of ligand access to the receptors by BMP binding proteins as well as other BMP family ligands, and 2) the intracellular modulation of BMP signal transduction in the target cells (Fig. 1).

1. Extracellular regulation. There are a number of TGF-β superfamily binding proteins that can influence ligand access to receptors. The prototype of these binding proteins is follistatin, which was first isolated from ovarian follicular fluid as an inhibitor of pituitary FSH secretion, similar to inhibin (120, 121). Subsequent cloning studies, however, revealed that follistatin has no sequence homology with the inhibins (122–124). Several years later, follistatin was found to be an activin binding protein (125), and this property of follistatin accounts for much of its biological activity in the pituitary (126). Thereafter, the role of follistatin was extended to include the regulation of the activities of BMP-4, -7, -4/7 heterodimers, and BMP-15 (74, 127, 128). Therefore, it is not clear whether a given *in vivo* effect of follistatin is caused by the inhibition of the activins and/or the BMPs. It is interesting that male and female transgenic mice overexpressing follistatin exhibit numerous defects in reproduction: males exhibit Leydig cell hyperplasia, defective spermatogenesis, and seminiferous tubule degeneration; females exhibit small ovaries due to a block in folliculogenesis (129). These phenotypes are different from those seen in ActR-II knockout mice (130) and mice deficient for the activin subunits (131, 132). One possible interpretation of these results is that the phenotype of the follistatin-overexpressing mice may be caused by the inactivation of additional factors, quite possibly the BMPs. Another binding protein, follistatin-related protein, can also bind both BMPs and activins and inhibit their biological activity (133–135).

Interestingly, a recent study showed that follistatin can act to augment certain BMP-7 biological actions while inhibiting other BMP-7 biological activities (136). Low concentrations of BMP-7 induce muscle growth during chick embryogenesis through the up-regulation of Pax-3 expression. By contrast, high concentrations of BMP-7 induce apoptosis and muscle loss. It is interesting that the addition of follistatin inhibits the induction of apoptosis and muscle loss by BMP-7, yet augments BMP-7-stimulation of Pax-3 expression and muscle growth. Based on these data, it was proposed that the role of follistatin on BMP-7 action is to escort BMP-7 to the surface of myogenic cells and present it to the receptors at a concentration optimal for the stimulation of embryonic muscle growth (136).

Another interesting study in the field of BMP binding proteins is the recent discovery that connective-tissue growth factor (CTGF) has an affinity for both BMP-4 and TGF-β (137). An intriguing aspect of this finding is that CTGF inhibits BMP-4 biological activity while enhancing the biological activity of TGF-β. The underlying mechanism of this differential regulation by CTGF is not yet understood. However, because CTGF has a higher affinity for BMP-4 (K_d = 5 nM) than for TGF-β (K_d = 30 nM) it was suggested that CTGF may block BMP-4 but facilitate TGF-β binding to their cognate receptors. Although the impact of this novel finding on reproductive function has not yet been elucidated, there is accumulating evidence that suggests a role for CTGF in ovarian folliculogenesis (138–141).

There are other binding proteins with specific affinities for BMPs. The prototype of the BMP-specific binding proteins is noggin, which was first identified as a factor regulating normal dorsal development in *Xenopus* embryos (142). Later studies demonstrated that noggin is an inhibitory binding protein for BMP-2, -4, -7, and -14 and GDF-5 (143–146). The examination of the crystal structure of the complex of the noggin dimer bound to BMP-7 revealed that noggin blocks the epitopes in which BMP-7 binds to both the type-I and type-II receptors, demonstrating a mechanism for the inhibitory effects of noggin (145). Another binding protein, chordin, exhibits similar BMP-antagonistic properties as noggin in that both of these factors bind BMP-2 and -4 with high affinity, bind BMP-7 with lower affinity, and do not bind

activin or TGF-β (147, 148). A protein that is structurally similar to chordin, thus called chordin-like, binds and antagonizes BMP-4, -5, and -6, with the highest affinity for BMP-6. Although chordin-like does not bind activin, it does have a weak affinity for TGF-β1 and -2 (149). Gremlin, DAN and cerberus form a family of secreted BMP-binding proteins that share a region of structural homology called a can domain, which closely resembles the cystine knot motif of the TGF-β superfamily (150, 151). At present, nothing is known about the functional significance of these BMP antagonists in the mammalian reproductive system.

Inhibin, a member of the TGF-β superfamily, has been known to block activin actions in a number of cell types (152, 153). The mechanism underlying this block is still controversial (154); however, currently available information suggests that inhibin binds to the membrane-bound binding protein, β-glycan, and the complex then associates with ActR-II or ActR-IIB. In so doing, inhibin sequesters the type II activin receptors, thus preventing activin action (155). A recent study by Wiater and Vale (156) has extended the role of inhibin to include antagonism of BMP-2 and -7 activity. In this study inhibin A was shown to inhibit BMP actions by competitively sequestering ActR-II and ActR-IIB as well as BMPR-II (Fig. 1). The inhibition of BMP action by inhibin A is highly dependent on β-glycan. It will be interesting to investigate the significance of inhibin A inhibition of BMP action with respect to reproductive physiology.

2. Intracellular regulation. BMP actions can also be governed by the intracellular regulation of BMP signaling in the target cells (Fig. 1). Smad6 and 7, unlike other members of the Smad family, are inhibitors of TGF-β superfamily signaling. Hence, they are called inhibitory Smads or I-Smads (157–159). This inhibition occurs through the ability of Smad6 and 7 to compete with the R-Smads for binding to the activated type-I receptors (160). Smad6 has also been shown to inhibit BMP signaling by forming a complex with Smad1, and thereby competing with Smad4 binding (94). Although both Smad6 and 7 can inhibit the activin/TGF-β and BMP signaling pathways, it is generally believed that Smad6 preferentially inhibits BMP signaling (113). Administration of TGF-β superfamily ligands can induce expression of the I-Smads, suggesting that these Smads act as part of an autocrine negative feedback loop (161).

BMP and activin membrane bound inhibitor (BAMBI) has structural features that resemble type-I receptors, except that it lacks the intracellular serine/threonine kinase domain. There is evidence that BAMBI can compete with the type-I receptors for ligand binding and inhibit the signaling of the TGF-βs, activins, and BMPs (162, 163), suggesting that BAMBI might function as a dominant negative receptor. FK506 binding protein (FKBP)-12 binds TGF-β superfamily type-I receptors in the GS domain and prevents transphosphorylation by the type-II receptors (164). Smad ubiquitination regulatory factor-1 (SMURF1) is an intracellular signaling inhibitor that targets Smad1 and 5 for ubiquitination and proteosomal degradation (165). This factor appears to specifically target BMP signaling as evidenced by its failure to affect Smad2 and 4. SMURF1 is unique because it reduces the intracellular level of Smads, independent of BMP ligand stimulation (165). The roles of these inhibitory factors in reproductive function have not yet been established. However, as the importance of the BMPs in reproductive physiology becomes better understood, it is likely that the intracellular control of BMP action in target cells will prove to be of importance for reproductive processes.

III. Expression of the BMP System in Reproductive Tissues

The development and physiological functions of basic structures in the mammalian reproductive system are influenced by the tissue-specific expression of members of the BMP family. In this section, we will focus our attention on 1) the manner in which the specific components of the BMP system are expressed within the reproductive system, and 2) hypotheses that, when tested, could significantly advance our understanding of reproductive biology and medicine. Particular emphasis will be placed on the BMP ligands, receptors, and Smad signaling molecules listed in Table 2.

A. Primordial germ cell (PGC) formation

The establishment of the germ line is a fundamental aspect of reproduction in all animals. In mice, germ cell determination is induced in epiblast cells by the extraembryonic ectoderm (166, 167), and is not acquired through the inheritance of preformed germ plasma, as it is in nonmammals (168). There is compelling evidence that BMP-4 and -8b play a central role in determining PGC formation in the mouse embryo. The genes encoding BMP-4 and -8b have overlapping expression in the extraembryonic ectoderm before gastrulation, *i.e.*, before PGCs are seen (166, 169). Most BMP-4 knockout mice die in early gastrulation; however, some survive long enough to show that no PGCs develop (170). Thus, PGC formation in mice requires BMP-4 expression. There is also evidence from knockout mice that BMP-8b is required for PGC formation (169, 171). Interestingly, BMP-4 cannot rescue the defects in PGC formation in BMP-4-null mice, whereas the PGC defects can be rescued by BMP-8b (169, 171).

B. The pituitary

There is increasing evidence that locally produced BMPs play a critical role in the differentiation of the pituitary gonadotrope (172, 173). During normal mouse pituitary development, BMP-4 is expressed in the ventral diencephalon, whereas BMP-2 and the BMP binding protein, chordin, are expressed in the ventral condensing mesenchyme. Although the targeted deletion of the *Bmp4* gene in mice is embryonic lethal, the analysis of these embryos indicates a failure of the invagination of Rathke's pouch, the progenitor tissue for the anterior pituitary. According to this evidence, it has been concluded that BMP-4 plays an important role in pituitary development. In another study, the targeted expression of the BMP antagonist, noggin, to Rathke's pouch caused the arrest of pituitary development resulting in the absence of almost all endocrine cell types, including the gonadotropes that produce FSH and LH (174). These data suggest that the patterning and histogenesis of Rathke's pouch depend on the

tissue-specific expression and regulated action of BMP-2, BMP-4, and chordin.

It has become evident that BMP ligands and receptors are expressed in the adult pituitary gland. In terms of ligands, BMP-6, -7, and -15 mRNAs are expressed in mice (175, 176); GDF-9 mRNA in humans (177); BMP-15 and GDF-9 mRNAs in brushtail possums (178); and BMP-15 mRNA in sheep (38). In terms of receptors, BMPR-II, ActR-II, ALK-2, and ALK-3 mRNAs are expressed in mice (176); ALK-6 mRNA in sheep (40); and ActR-II and -IIB mRNAs in rats (179). 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 (175, 176).

C. The hypothalamus

Northern blotting experiments have identified GDF-9 mRNA in the hypothalamus of the mouse and rat (177). Presumably, the GDF-9 is expressed in neurons; however, the cellular site of GDF-9 expression in the hypothalamus remains to be determined. There is also evidence for the expression of ActR-II and -IIB mRNAs in the rat hypothalamus; these receptors are found in areas involved in neuroendocrine regulation including the suprachiasmatic, supraoptic, paraventricular, and arcuate nuclei (179). In terms of reproduction, this evidence begs the question: to what extent might a hypothalamic BMP system contribute to the regulation of the GnRH neuron?

D. The female reproductive organs

1. The ovary. Research performed in many laboratories in the last few years has provided important information about the expression of the BMP system in the mammalian ovary. The most comprehensive study on the expression of BMPs in the ovary has been reported recently using adult cycling rats (180) (Table 3). This section reviews the spatiotemporal expression patterns of the BMP system in the mammalian ovary with special emphasis on their implications for folliculogenesis and luteogenesis. It should be stressed that the pattern of BMP expression in the rat ovary may not necessarily apply to other species. Furthermore, given the importance of the mouse as a genetic model system, a thorough understanding of the tissue-specific expression of the BMP system in the mouse could help integrate the information on the functional roles of BMPs collected from experiments in other animals with the information that can be collected from genetic studies using the mouse model.

a. Folliculogenesis. Folliculogenesis involves a series of sequential steps in which a growing follicle either develops to the ovulatory stage or dies by apoptosis. The major steps in folliculogenesis include the primordial/primary transition, the primary/secondary transition, selection, and atresia. The concept to emerge is that folliculogenesis is accompanied by a precise spatial and temporal pattern of expression of the BMP family.

i. Primordial/primary transition. The initial event in folliculogenesis is the activation of a dormant primordial follicle to begin growing, a process termed the primordial/primary transition (181). The first indication that a primordial follicle has been activated is that the granulosa cells begin to transform from a squamous to a cuboidal shape. As this occurs, the granulosa cells begin to proliferate, and follicular growth is initiated. When more than 90% of the granulosa cells are cuboidal, there is an increase in RNA synthesis in the immature oocyte.

The mechanisms of the primordial/primary transition are poorly understood, but there is evidence that BMPs are involved. In rat primordial follicles, *in situ* hybridization and immunohistochemistry studies have demonstrated the expression of ALK-2, -3, -6, BMPR-II, ActR-II, ActR-IIB, β-glycan, Smad5, and Smad8 in oocytes and low variable expression of ALK-3 and -6 in the surrounding squamous granulosa cells (179, 180, 182) (Table 3). In sheep primordial follicles, the expression of ALK-6 has also been reported in oocytes, and BMPR-II is detectable in both oocytes and granulosa cells (40, 183). It should be emphasized that there appear to be species differences in the expression of BMP system components in primordial follicles. For example, unlike the rat and sheep, no ALK-6 is detected in any cell types of mouse primordial follicles (184), and no ActR-II is detectable in human primordial follicles (185).

Collectively, these results suggest that primordial follicles are a target tissue for BMPs. What are the functional ligands for these BMP receptors? Lee *et al.* (186) have shown that BMP-7 can activate primordial follicles in the rat ovary. Because rat primordial follicles do not express BMP-7 (180), the BMP-7 ligand must originate from another site. The finding that BMP-7 is expressed in the theca, secondary interstitial cells, and sex cords (180) suggests that BMP-7 produced by these tissues may promote the primordial/primary transition in the rat. Evidence supporting a role for GDF-9 in this process has also been presented by Vitt *et al.* (187). Because primordial follicles are activated to grow in GDF-9-deficient animals (33), it can be concluded that GDF-9 is not essential for this basic developmental event in mice. It is perhaps noteworthy that, in the rat, primordial follicles do not appear to express BMP-2, -3, -3b, -4, and -6 (180). GDF-9 and BMP-15 are predominantly expressed in the oocytes of growing follicles (*i.e.*, primary follicles throughout folliculogenesis); however, it appears that in some species mRNA transcripts of these genes can be detected in primordial follicles. In particular, there is accumulating evidence that in cattle, sheep, and humans, expression of GDF-9 precedes that of BMP-15 and can be detected in oocytes of primordial follicles, whereas BMP-15 expression can first be detected in oocytes of primary follicles (38, 188–191). This pattern of expression appears to be different from the mouse and the rat, in which neither GDF-9 nor BMP-15 expression can be detected in oocytes of primordial follicles and the onset of expression occurs during the primary stage of folliculogenesis (30, 31, 35–37, 189, 192–194). In the brushtail possum, both GDF-9 and BMP-15 transcripts are detectable in oocytes of primordial follicles (178). The significance of the species differences in the expression of these genes remains to be elucidated.

ii. The primary/secondary transition. A major event in mammalian folliculogenesis is the development of a second layer of granulosa cells. Among vertebrates, this step, termed the

TABLE 3. Cellular localization and intensity of mRNAs for the family of BMP ligands, receptors, and the binding protein in ovaries of adult cycling rats

Tissue	BMP-2	BMP-3	BMP-3b	BMP-4	BMP-6	BMP-7	BMP-15	ALK-3	ALK-6	BMPR-II	Follistatin
Oocyte											
Primordial	−	−	−	−	−	−	−	+	++/+	+/−	−
Primary	−	−	−	−	−	−	+	++	+++/++	+	−
Secondary	−	−	−	−	+++/+	−	++++/++	+++	++++	+/−	−
Tertiary	−	−	−	−	+++	−	++++	+++	+++	+/−	−
Dominant	−	−	−	−	+++	−	++++/+++	++	+++	+/−	−
Atretic	−	−	−	−	+++/++	−	++/+	+++/++	++	+/−	−
Granulosa cells											
Primordial	−	−	−	−	−	−	−	+/−	+/−	−	−
Primary	+/−	−	−	−	−	−	−	+	+/−	+	−
Secondary	++/−	−	−	−	+++/−	−	−	++/+	++++	+++/++	++
Tertiary	+++/−	−	−	−	+++/−	−	−	++	++++	+++	++++
Dominant	+++/−	−	−	−	+/−	−	−	++	++++	+++	++++
Atretic	++++	−	−	−	+++	−	−	++	++++	+++	+/−
Theca interstitial cells											
Primordial	−	−	−	−	−	−	−	−	−	−	−
Primary	−	−	−	+	−	−	−	−	−	−	−
Secondary	+/−	−	−	++/−	−	++/−	−	+	+	−	−
Tertiary	+/−	+/−	+	+++/−	−	+++/−	−	++/+	+	−	−
Dominant	+/−	+/−	++++/−	++++/−	−	+++/−	−	++/+	++	−	+/−
Atretic	+/−	−	+/−	+/−	−	+/−	−	+	+	−	−
Theca externa	−	−	++++/−	++++/−	−	−	−	+	++/−	−	−
Corpora lutea - healthy											
Granulosa lutein	+/−	−	−	+/−	++/+	−	−	++/+	+	++	++++
Theca lutein	+/−	+/−	+/−	+/−	−	++/−	−	++/+	+	+/−	+++
Vascular endothelial	−	−	−	−	−	−	−	+/−	+/−	−	
Theca externa	−	−	++/+	+++	−	−	−	+/−	++/+	−	
Corpora lutea - luteolytic	+++/−	+/−	−	++	+/−	−	−	+	+++/−	+/−	−/++++
Vascular system											
Endothelium	−	−	−	+++	+++/−	−	−	+/−	−	+/−	−
Smooth muscle	−	−	−	−	−	−	−	+/−	−	−	−
Tunica adventitia	−	−	−	−		−	−	+/−	++	−	−
Secondary interstitial cells	++/+	−	−	−	−	+/−	−	+	++/+	−	−/++++
Surface epithelium	−	−	+++/−	+++/−	+++	−	−	+/−	−	−	−
Sex cords	−	−	++++	+++	−	+++	−	+	+++	+/−	−

The intensities of the hybridization signals indicated above represent a subjective consensus estimated on a scale of − to ++++: +, silver grains sparse, but positive hybridization; ++, silver grains are numerous but do not cover the cell type in question; +++, silver grains are very numerous and begin to merge in some places; ++++, silver grains are very dense and form a near uniform mass above the cell type in question. The *slashes* represent the variability in signal intensity caused by the temporal (cycle-dependent or developmental), or spatial (heterogeneity within or between the histological unit) expression pattern, which is fully described in the original article (180). For example, BMP-2 mRNA expression in the granulosa cells in dominant follicles is heterogeneous (+++/−) with positive (+++) cells appearing in the membrana cells but no signal (−) seen in the cumulus cells (different spatial pattern of expression); follistatin mRNA expression in the secondary interstitial cells is undetectable (−) over most of the cycle except at proestrus 2000 h when it is strongly (++++) expressed (different temporal pattern of expression). [Reproduced with permission from G. F. Erickson and S. Shimasaki: *Reprod Biol Endocrinol* 1:9, 2003 (180).]

primary-to-secondary transition, is unique to mammals (195). Although the mechanisms involved in this basic event remain unknown, several lines of evidence indicate a functional role for BMPs. Studies in GDF-9-deficient mice (33) have demonstrated that folliculogenesis is blocked at the primary/secondary follicle transition stage in the absence of GDF-9. Therefore, the concept that oocyte-derived GDF-9 is essential for eliciting the primary/secondary transition in mice is clear. In sheep (38), but not mice (196), BMP-15 plays an essential role in evoking the primary/secondary transition. Considering this striking species difference in the physiological role of BMP-15 in the primary/secondary transition, much caution is needed in extrapolating the findings of BMP research from one species to another. An important question is how the BMPs regulate the primary/secondary transition. In the rat, the expression of BMP-6, BMP-15, and GDF-9 in the oocyte, BMP-2, -6, ALK-3, -6, and BMPR-II in the granulosa cells, and BMP-4 and -7 in the theca cells increases significantly during the primary/secondary transition (Table 3). GDF-9 and BMP-15 are expressed in human oocytes during the primary/secondary transition stage (189, 191). Questions concerning the role of BMPs in this process are of interest in light of the paper by Teixeira *et al.* (191) showing that the expression of GDF-9 mRNA is abnormally low in oocytes at the primary/secondary transition in women with polycystic ovary syndrome.

iii. Theca development. After the primary/secondary transition, the basal lamina of the follicle becomes surrounded by mesenchymal cells that will eventually develop into an inner theca interna and an outer theca externa (197). The terminal differentiation of the theca externa is characterized by the expression of contractile proteins (*i.e.*, actin and myosin) typical of smooth muscle cells. The terminal differentiation of the theca interna is characterized by the development of theca interstitial cells that produce androgens. Understanding theca development is important because theca-derived androgens are obligatory precursors for follicle estrogen production and have been implicated in atresia (197).

Genetic studies in mice have provided evidence that oocyte-derived GDF-9 may be necessary for attracting prospective theca cells to the basal lamina of developing preantral follicles (33, 198). Further evidence supporting a role of GDF-9 in theca development comes from the finding that recombinant GDF-9 stimulates the expression of a theca interstitial cell marker, CYP17, in the rat (187). Thus, rat theca interstitial cells appear to be targets for GDF-9. Analysis of theca development in adult cycling rats has revealed that the theca interstitial cells express BMP-3b, -4, -7, ALK-6, and follistatin (Table 3). One intriguing observation is that the theca interna of healthy follicles appears to be composed of two distinct populations of theca interstitial cells: one group, which expresses BMP-4, is present as an outer layer of cells juxtaposed to the theca externa; and another, which expresses BMP-7, is present at the proximal side of the theca interna near the basal lamina of the follicle (180). These differences are found throughout folliculogenesis, beginning during the secondary stage. This is intriguing because it implies, for the first time, that there is functional heterogeneity among the theca interstitial cells during follicular growth and development.

iv. Selection and follicle dominance. A hallmark of estrous and menstrual cycles is the selection of a dominant follicle that will ovulate a fertilizable egg and then develop into a corpus luteum that secretes progesterone. A fundamental concept in ovary physiology is that selection is critically dependent on the secondary rise in plasma FSH. Despite considerable effort, the mechanism by which FSH causes selection remains poorly understood.

There is increasing evidence that the development of dominant follicles in mammals is accompanied by the tissue-specific expression of the BMP system, replete with ligands, receptors, and binding proteins (Fig. 2 and Table 3). In a broad sense, the studies of a variety of species (31, 32, 179, 182–184, 191, 194, 199, 200), most notably the rat (201), indicate the following: the oocytes of Graafian follicles express BMP-6, -15, GDF-9, ALK-2, -3, -6, and ActR-II; the granulosa cells express BMP-2, ALK-3, -6, BMPR-II, and follistatin; and the theca cells express BMP-3b, -4, -7, ALK-3, and -6. In the rat, one particularly interesting observation is the dramatic loss of BMP-6 mRNA in the granulosa cells at or about the time the dominant follicle is selected during the normal estrous cycle (180). This observation suggests that a rapid loss of BMP-6 expression could be an incipient marker for the newly selected dominant follicle in the rat. Because BMP-6 can prevent FSH action (39), one could speculate that the rapid loss of BMP-6 expression in the granulosa cells may be required for FSH to exert its obligatory functions during the selection process. Because FSH induces the loss of BMP-6 mRNA in rat granulosa cells (G. F. Erickson and S. Shimasaki, unpublished data), one might predict that the secondary rise of FSH may down-regulate BMP-6 expression and this change may have a central role in selection. Finally, with regard to FSH action, follistatin has been shown to neutralize the bioactivity of BMP-15, which inhibits FSH receptor expression (128). It is possible, therefore, that the strong expression of follistatin by the dominant follicle (Table 3) may play a role in inhibiting BMP-15 action such that sufficient FSH receptors are expressed in the granulosa cells to permit FSH action during follicle development.

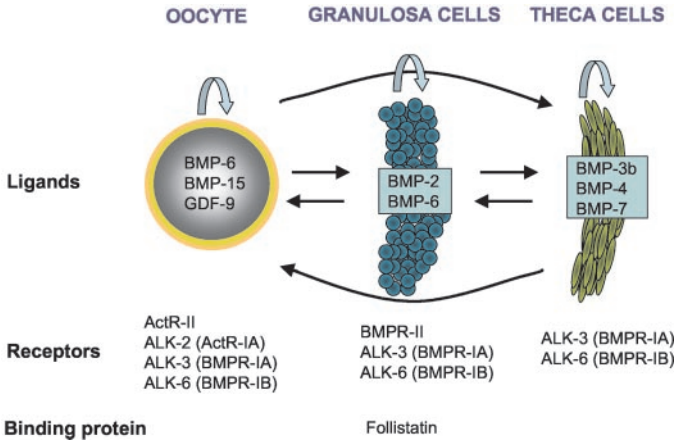

FIG. 2. Summary of the BMP system in the ovarian follicle. Normal folliculogenesis is controlled by the interplay of endocrine as well as autocrine/paracrine factors in the ovary. Accumulating evidence suggests that there is a bidirectional communication between follicular cells (258, 259, 261). In mammalian ovaries *in situ*, the spatiotemporal pattern of expression of BMP ligands and receptors suggests that the BMP system constitutes an important part of this intrafollicular communication network (30, 32, 179, 192, 199). Specifically, BMP-6, -15, and GDF-9 are expressed by oocytes and exhibit biological activities in granulosa cells that express BMPR-II, ALK-3, and -6 receptors and possibly theca cells that express ALK-3 and -6 receptors. Theca cells express BMP-3b, -4, and -7, which exhibit biological activities in granulosa cells and possibly oocytes. Granulosa cells express BMP-2 and -6, which may act on oocytes and theca cells. In addition to the paracrine actions of the BMP ligands, there is evidence that BMPs may also exhibit autocrine actions in the ovary. Follistatin, which is expressed by granulosa cells, may serve as a local regulator of BMP action. Collectively, the BMP system acts to coordinate cell proliferation, differentiation, and apoptosis during folliculogenesis.

v. Atresia. The vast majority of follicles die by atresia. The rapid cessation of mitosis and the onset of apoptosis in the granulosa cells is the sinequanone of follicle atresia (202–204). Recent evidence in adult rat ovaries suggests that BMPs may have a role in atresia (180). A striking feature of atresia in the rat ovary is a dramatic decrease in the expression of theca cell BMP ligands (BMP-3b, -4, and -7). The cause and consequence of these changes are unknown. In this connection, an interesting finding is that BMP-7 stimulates DNA synthesis in rat granulosa cells (186), as it does in human osteoblasts (205). In addition to its mitotic effects, it is known that BMP-7 inhibits apoptosis in several tissues (206, 207). Therefore, one might hypothesize that theca-derived BMP-7

is a part of the mechanisms that promote follicle survival through its ability to enhance granulosa proliferation and suppress apoptosis. In contrast to the theca BMP system, the granulosa cells of atretic follicles exhibit relatively high expression levels of BMP-2, -6, ALK-3, -6, and BMPR-II (Table 3). Indeed, the highest levels of BMP-2 expression in the rat ovary are found in the granulosa cells of atretic follicles. These findings, together with the fact that atresia is accompanied by a dramatic decrease in follistatin expression (Table 3), have led to the proposal that atresia might be governed by the high activity of BMP-2 and -6.

b. Corpus luteum formation. After ovulating the oocyte-cumulus complex, the dominant follicle transforms into a corpus luteum by a process termed luteinization. As luteinization proceeds, the corpus luteum secretes large quantities of progesterone. If embryo implantation does not occur, the corpus luteum stops synthesizing progesterone and degenerates, a process termed luteolysis. Despite enormous effort, the mechanisms underlying luteogenesis remain unclear. The idea that intrinsic BMPs might be involved is gaining support.

i. Luteinization. The classic view of luteinization is deeply rooted in the principle of luteinization inhibitors (208, 209). In this principle, oocyte-derived regulatory molecules, luteinization inhibitors, act within the follicle to inhibit premature luteinization and limit progesterone biosynthesis (208, 210–212). In terms of the oocyte, three BMPs with luteinization inhibitor activity in the granulosa cells have been identified: BMP-6 (39), BMP-15 (37, 213), and GDF-9 (214). Therefore, it is reasonable to conclude that the release of the oocyte at ovulation plays a prominent role in activating luteinization by virtue of the loss of these luteinization inhibitors. Further insight into the role of the BMP system in the rat corpus luteum has been reported recently (180). One key finding is that the level of expression of BMP ligands and receptors is quite low in the corpus luteum during the process of luteinization. Most notably, BMP-2 expression is rapidly down-regulated after ovulation, and there is no detectable BMP-2 in the corpus luteum during luteinization (Table 3). Importantly, luteinization is accompanied by a high level of follistatin expression. Taken as a whole, these data fit the hypothesis that a loss of BMP responses may have an important role in evoking and maintaining luteinization and progesterone production by the rat corpus luteum. These basic findings rest at the foundation of our present hypothesis that BMPs may be the long-sought luteinization inhibitors (32).

ii. Luteolysis. The spatiotemporal pattern of expression of the BMP system in the rat corpus luteum undergoes important changes during luteolysis (180). In a broad sense, these changes are consistent with an increase in the expression of the BMP system, suggesting the interesting hypothesis that the increased expression and action of BMPs may be a part of the physiological mechanism of luteolysis. There are three main lines of evidence to support this hypothesis. The first is that the expression of the *Bmp2* gene is reactivated at the time of luteolysis, being strongly expressed in clusters of endothelial cells throughout the corpus luteum (180). The abundant evidence linking BMP-2 to the induction of apoptosis in other cell types (111, 215–218) raises the question: does BMP-2 play a role in luteolysis by promoting apoptosis and in inhibiting progesterone production? The second is that follistatin, which is strongly expressed in healthy corpora lutea, becomes undetectable in the corpus luteum during early luteolysis (180) (Table 3). The loss of follistatin could be physiologically relevant because it may serve to allow BMP-2 to function during early luteolysis. It should be noted that follistatin mRNA remains undetectable in the corpus luteum until proestrus 2000 h when few lutein cells appear strongly positive (Table 3). The functional significance of this follistatin expression during the late luteolytic phase is unknown. A third line of evidence is the reactivation of ALK-6 expression during luteolysis. When luteolysis is initiated, the ALK-6 expression increases in the corpus luteum, being most abundant in the theca externa and vascular endothelial cells. Interestingly, a functional link between the expression of ALK-6 and luteolysis was provided by loss-of-function studies showing that the corpus luteum of the cycle continues to secrete progesterone in the absence of ALK-6 (184). The mechanisms by which the increase in BMP system activity may cause luteolysis have not yet been elucidated. The general principle to emerge from this study is that the expression of the BMP system in the rat corpus luteum appears to decrease in association with luteinization and increase during luteolysis.

c. Secondary interstitial cells. During atresia, the oocyte and granulosa cells undergo apoptosis, whereas the theca remains in the stroma as islands of secondary interstitial cells that continue to exhibit the potential for androgen synthesis (197). The physiological role of the secondary interstitial cells is not understood in any species. The secondary interstitial cells of the adult rat ovary express BMP-2 and -7, the receptors ALK-3 and -6, and the BMP binding protein, follistatin (Table 3). A particularly interesting feature of this system is the temporal pattern of follistatin mRNA expression in the secondary interstitial cells during the preovulatory period. Follistatin mRNA is absent in all the secondary interstitial cells during most of the cycle except at proestrus 2000 h when it is very strongly expressed (see footnote in Table 3) (180). Because secondary interstitial cells are target cells for LH (197), it is not unreasonable to propose that the preovulatory surge of LH during proestrus may be involved in the burst of follistatin gene expression in these cells at proestrus 2000 h. Based on the antiluteinization properties of the BMP system (32), it is possible that this burst of follistatin expression by the secondary interstitial cells may be involved in neutralizing ovarian BMP activities, which in turn, leads to the preovulatory surge of progesterone production, resulting in inducing ovulation and mating behavior in the rat. If this hypothesis proves correct, then the follistatin expression in the secondary interstitial cells in the rodent would be raised to a new level of prominence.

d. The ovarian surface epithelium. The mammalian ovary is covered by a layer of epithelial cells affixed to a basement membrane. A characteristic feature of the ovarian surface epithelium is the potential to undergo cyclic apoptosis and

regeneration, respectively, during the ovulatory and luteal phases of the cycle. In normal cycling rats, the genes encoding BMP-3b, -4, and -6 are expressed in the surface epithelium (180). The cells appear to express BMP-3b and -6 constitutively over the cycle, a finding consistent with a maintenance-type role for these BMPs. Notably, the expression of BMP-4 is cell- and cycle-specific. Namely, *Bmp4* gene expression occurs in a subset of epithelial cells covering the freshly ovulated follicle and the newly formed corpus luteum on estrus and diestrus (180). Therefore, the spatiotemporal pattern of BMP-4 expression is tightly correlated with the replenishment of the surface epithelium that is exfoliated from the ovulating follicle. This raises the interesting hypothesis that inducible and cell-specific BMP-4 expression may have a role in the regeneration of the ovarian surface epithelium during postovulatory repair. Evidence from epidemiological studies indicates that the risk of epithelial ovarian cancer is decreased by factors that suppress ovulation, such as pregnancy, breast-feeding, and the oral contraceptive pill (219, 220). Considering the relationship between ovulation and the induction of *Bmp4* gene expression, it will be interesting to explore a possible link between the risk of ovarian cancer and the ovulation-inducible and epithelial cell-specific *Bmp4* gene expression.

e. The sex cords. The sex cords are vestiges of the cranial portion of the mesonephric tubules and ducts, and in the adult ovary they appear as a cluster of blind tubules in the hilar region. Although the physiological importance of ovarian sex cords is unknown, they have been linked to the genesis of ovarian cancer through distinct TGF-β signaling pathways (221, 222). Furthermore, a role for follistatin in the genesis of this cancer has been established (129). In addition, the ovarian sex cords in adult rat ovaries express an intrinsic BMP system replete with ligands (BMP-3b, -4, and -7) and receptors (ALK-3, -6, and BMPR-II) (180) (Table 3). Because BMPs are potent growth factors and follistatin can modulate their bioactivity, it is possible that this intrinsic BMP system could be involved in the pathogenesis of sex cord tumors. The fact that sex cords are sometimes found juxtaposed to developing follicles, blood vessels, and secondary interstitial cells (180) suggests that the sex cord-derived BMPs may have paracrine actions on these tissues.

2. The uterus. The uterus is prepared for embryo implantation by estrogen and progesterone. Implantation involves a complex pattern of cellular changes resulting in placenta formation. These changes occur in the uterine epithelium, stroma, and embryonic trophoblasts. The general course of events involves cell proliferation, cytodifferentiation, and apoptosis. There is increasing evidence for a role of the BMP family in this important process.

a. The nonpregnant uterus. The mRNAs encoding a number of BMP family members have been identified in the nonpregnant uterus of both rodents and humans. In normal cycling mice, BMP-6 (223), BMP-7 (224), GDF-10 (225), and ALK-6 (184) are expressed in the uterus. *In situ* hybridization studies revealed that BMP-6 and -7 are coexpressed in the endometrial epithelial and subjacent stromal cells (223, 224), and ALK-6 mRNA is restricted to the epithelial and glandular cells (184). In mice, BMP-7 expression in the uterus is abolished in response to estrogen treatment (224). Because endometrial cells proliferate in response to estrogen, the reduced BMP-7 expression might be involved in the mechanism for estrogen-induced endometrial cell proliferation. In humans, but not rodents, GDF-9 mRNA has been identified by Northern blotting in the nonpregnant uterus (177). Evidence that the BMP system might have a functional role in the uterus comes from studies of ALK-6-deficient mice (184). In the absence of ALK-6, the uterine linings are thin, and endometrial glands are absent. This suggests that ALK-6 has an important role in the development of mouse endometrial glands.

b. Decidualization. In the mouse, the deciduum develops from uterine stromal cells and eventually completely surrounds the implanted embryo. During the decidual reaction, the adjacent endometrial stromal cells undergo hyperplasia and hypertrophy as they convert from mesenchymal to fully differentiated decidual cells. In addition, some cells undergo apoptosis, thereby creating space for the growing embryo. There is evidence from *in situ* hybridization studies that members of the BMP family are expressed during decidualization. The first is that BMP-7 mRNA is lost from the uterine epithelium shortly after implantation; however, it continues to be expressed in the decidualizing stromal cells, being distributed in a gradient with the highest expression in the stromal cells nearest the uterine epithelium (226). Secondly, when embryo attachment to the epithelium occurs, BMP-2 expression is induced in the subjacent stromal cells (227, 228). After implantation, BMP-2 continues to be expressed in the dividing decidual cells farthest from the embryo. A similar pattern of expression has been reported for BMP-8a (29). The loss of BMP-8a does not alter female fertility (229); thus, BMP-8a is not required for decidualization in mice. Thirdly, several other BMP family members have been identified in and around the mouse deciduum. BMP-4 mRNA and Smad1 are predominantly expressed in the vascular endothelial cells, suggesting a role of these components of the BMP system in the angiogenesis that accompanies decidualization (226). BMP-5 mRNA shows low levels of expression in the stroma close to the myometrium and in the myometrial connective tissue (228). There is also evidence that several BMP antagonists colocalize with these BMP ligands in mouse decidual tissue, including follistatin (230), noggin (228), and DAN/Dante (228).

c. The placenta. As in the deciduum, there is evidence for an intrinsic BMP system in the mouse and human placenta. In mouse embryos, the expression of BMP-4 in the epiblast-derived tissues has been implicated in the development of the vascular connection between the placenta and embryo (231). This could be of primary importance in maintaining the viability of the embryo. In the mouse placenta, BMP-8b is expressed in trophoblasts in the labyrinthine region (29), BMP-4 is expressed in the spongiotrophoblasts, and BMP-7 is highly expressed in the giant trophoblasts (224). Thus, the hypothesis has emerged that the expression of BMP-4, -7, and -8a in the mouse placenta may be involved in trophoblast proliferation differentiation. In humans, BMP-7 can act on

cultured trophoblasts to inhibit the secretion of human chorionic gonadotropin (hCG) (232). This could be clinically relevant because an appropriate level of hCG production is critical for maintaining early pregnancy in women. Little is known about the expression of the BMP family in the human placenta. In this regard, GDF-15, also called placental bone morphogenetic protein (PLAB) or prostate-derived factor (PDF) (Table 1), is highly expressed in the terminal villae of the human placenta (233, 234). Thus, GDF-15 may be involved in regulating functions in the human placenta. It should be noted that placental calcification commonly increases with gestational age during pregnancy and is often observed in placentas associated with preeclampsia and/or fetal growth restriction (235).

3. The mammary gland. Studies conducted in mice indicate that BMP-2 and -4 are expressed within the developing mammary gland (236). In the early stages of mammary gland development, BMP-2 and -4 are expressed in the epithelium and underlying mesenchymal cells, respectively (236). BMP-2 and -4 often show this tissue-specific pattern of epithelial-mesenchymal expression during morphogenesis and primary induction. Consequently, it has been proposed that the localized expression of these BMPs play a role in the induction and invagination of the mammary epithelium. In the postnatal mammary gland, both BMP-2 and -4 mRNAs are expressed within the cells of the stromal fat pad. Therefore, the transition of the mammary gland from fetal to postnatal development involves the transition of *Bmp2* gene expression from the epithelium to a zone of mesenchymal cells in which the mammary fat cells ultimately differentiate. There is evidence for the expression and function of BMPs in breast cancer. For example, BMP-2 is expressed in primary breast tumor tissue, and BMP-2 can act on the breast cancer cells to regulate growth (237). The question of the involvement of the BMP system in mammary tumorigenesis is a focus of current research.

E. The male reproductive organs

1. The testis. In situ hybridization studies have established that BMP receptors and ligands are expressed in testicular germ cells during spermatogenesis. In murine seminiferous tubules, ALK-3 (238) and ActR-II (179) are expressed in the pachytene spermatocytes and round spermatids (179). There is no detectable ALK-6 in the murine testis (238). A variety of BMP ligands have also been identified in male germ cells. Before puberty, both *Bmp8a* and *8b* genes are expressed at low levels in mouse spermatogonia and primary spermatocytes (29). After puberty, BMP-8a and -8b expression is shut off in spermatogonia, but high levels of expression continue in stage 6–8 round spermatids. The observation that there is an inverse relationship between the level of BMP-8a and -8b expression and germ cell apoptosis suggests that these BMPs may function to inhibit germ cell degeneration. Indeed, gene knockout studies have demonstrated that BMP-8b is required for germ cell survival and fertility in adult male mice (34). In addition to BMP-8a and -8b, GDF-9 has been shown to be strongly expressed in the mouse testis, being present in pachytene spermatocytes and early round spermatids (177). Because GDF-9-deficient male mice are fertile (33), one can conclude that testicular GDF-9 is not essential for normal spermatogenesis and male fertility in this species. It is notable that BMP-15 mRNA is not detectable in mouse, human, and sheep testes by Northern blot hybridization (30, 189); however, BMP-15 transcripts have been detected in human (189) and fetal ovine (38) testes by RT-PCR followed by Southern blot hybridization.

2. The epididymis. Mammalian sperm acquire the capacity for forward motility, which is essential for male fertility, during passage through the epididymis. This is accomplished through functional interactions between spermatozoa and the epididymal epithelial cells. *In situ* hybridization studies have demonstrated an intrinsic BMP system in the mouse epididymis. BMP ligands (BMP-7 and -8a), BMP receptors (ALK-3 and -6, BMPR-II), and Smad1 have been identified in the epithelial cells of the mouse epididymis (29, 34, 229, 238–240). The BMP ligands are coexpressed in the initial segment or caput (229), whereas the receptors and Smad1 are ubiquitously expressed throughout the epithelium of the epididymis (229). There is evidence that the cell-specific expression of BMP-7 is developmentally regulated. During early postnatal development, BMP-7 transcripts are expressed uniformly in the epithelial cells throughout the epididymis (240). As the mice age, BMP-7 expression becomes gradually restricted to the initial segment with an ascending gradient of expression in the epididymal epithelial cells in the transition from the cauda epididymal tubules to the vas deferens.

3. The prostate. The prostate is composed of alveoli and ducts embedded in fibromuscular tissue. Its secretion together with that of the seminal vesicle serves as a diluent and vehicle for transport of sperm to the female. The prostate is of great medical interest because 1) prostatic hyperplasia is a common disease in the aging male; and 2) prostate cancer is the most common malignant tumor in the elderly male in the United States (241).

In mice, BMP-4 and -7 are expressed in the prostate (242). BMP-7 mRNA is significantly decreased after orchidectomy and increased by testosterone and dihydrotestosterone treatment, indicating that BMP-7 expression in the mouse prostate is androgen dependent; this is in contrast to BMP-4 that is constitutively expressed (242). The authors postulate that the expression of BMP-7 in the mouse prostate may be involved in the stimulation of bone formation and osteosclerosis observed in metastatic prostate adenocarcinomas.

In humans, the prostate expresses BMP-4 mRNA as do the human prostate cancer cell lines, PC-3 and DU-145. Additionally, PC-3 cells also express BMP-2 and BMP-3 in fairly large amounts (243). By comparison, normal and neoplastic rat prostate tissue expresses predominantly BMP-3 mRNA. In humans and rats, the cellular sites of expression and the physiological importance of these BMPs in the prostate gland are unknown. Three possible functions of these BMPs in the prostate have been proposed: 1) normal prostate growth and morphogenesis; 2) neoplastic prostate cell behavior; and 3) the capacity of prostate cancer cells to stimulate new bone formation at metastasis tumor sites in bone. In this regard,

Paralkar *et al.* (234) found that GDF-15, also called PDF or PLAB (Table 1), is strongly expressed in the epithelium of the normal human prostate and induces ectopic cartilage formation and the early stages of endochondral bone formation. Studies in rats have established the concept that prostate PDF expression is regulated by androgens.

Another study has investigated the expression of BMP-6 mRNA and protein in normal and malignant rat and human prostate tissues (244). In the rat, BMP-6 was found at similar levels in both normal and malignant prostate tissues, and the level of BMP-6 expression appeared androgen-independent. In humans, BMP-6 mRNA and protein are expressed in normal and malignant prostate tissues, but interestingly, its expression is higher in prostate cancer as compared with adjacent normal prostate tissue. Taken together, these results have suggested that BMP-6 may contribute to prostate tumorigenesis.

IV. Reproductive Functions of BMPs

Studies demonstrating the gene expression of the BMP system provide basic information about the tissue-specific patterns of BMP expression but little about regulatory and functional processes involved. In this regard, important new information has come to light concerning how specific members of the BMP system regulate cellular functions in reproductive organs. In the following section, we discuss the recent advances in our understanding of the functions of BMP ligands in reproductive target cells. The majority of studies to be discussed in this section involve the actions of BMPs in female reproductive tissues and cell types. Our lack of discussion of the roles of BMPs in the male reproductive system is due to the paucity of research in this field.

A. The ovary

1. BMP-4 and BMP-7. The presence of a functional BMP system in the mammalian ovary was first reported by Shimasaki *et al.* (32) in 1999. In this study, BMP-4 and -7 mRNAs were identified predominantly in theca cells, and BMPR-II, ALK-3, and ALK-6 predominantly in granulosa cells of rat ovaries. This finding led to the obvious question: what is the functional role of the intrinsic ovary BMP system? Using primary cultures of rat granulosa cells grown in serum-free medium, recombinant BMP-4 and -7 were found to modulate FSH signaling in a way that promotes estradiol production (aromatization) while inhibiting progesterone biosynthesis (32). It is well established that granulosa cells in growing follicles respond to FSH stimulation *in vivo* by synthesizing estradiol, but not progesterone until the preovulatory period. On the other hand, when granulosa cells are cultured *in vitro* they synthesize both estradiol and progesterone in response to FSH stimulation. These results suggested that a selective inhibitor of progesterone synthesis, *i.e.*, a luteinization inhibitor, must act *in vivo* to modulate FSH bioactivity in a way that reflects normal follicle steroidogenesis during the estrous cycle (208, 209). The challenge has been to identify the nature of this putative luteinization inhibitor. In this regard, theca cell-derived BMP-4 and -7 are the first factors identified with biological activities that are consistent with the long-sought luteinization inhibitor (32). Notably, BMP-4 and -7 do not affect granulosa cell steroidogenesis in the absence of FSH in the rat (32). In a separate study, BMP-4 was also shown to inhibit FSH-stimulated progesterone synthesis by granulosa cells collected from sheep ovaries; however, in contrast to the rat model, BMP-4 also inhibited basal progesterone synthesis in sheep granulosa cells in the absence of FSH (41).

Progress has been made in our understanding of how BMP-7 differentially regulates FSH action. In rat granulosa cells, BMP-7 augments FSH-induced expression of P450 aromatase (P450arom) mRNA, while inhibiting FSH-induced expression of the mRNA encoding the rate-limiting factor in steroidogenesis, steroidogenic acute regulatory protein (StAR) (186). BMP-7 had no effect on the levels of FSH-stimulated P450 side-chain cleavage enzyme (P450scc) and 3β-hydroxysteroid dehydrogenase (3β-HSD) mRNAs. These results argue that the ability of BMP-7 to differentially regulate FSH-dependent estradiol and progesterone production is mediated by differential expression of P450arom and StAR.

Mice with null mutations in the *Bmp4* and *Bmp7* genes die either before birth (245) or shortly after birth, respectively (206). Therefore, knockout mouse technology has failed to provide direct information on the role of these BMPs in the ovary *in vivo*. To address this question, Lee *et al.* (186) injected recombinant BMP-7 into the rat ovarian bursa and characterized changes in folliculogenesis, ovulation, and steroidogenesis. These experiments revealed that BMP-7 decreases the number of primordial follicles but increases the number of primary, secondary, and antral follicles. Thus, BMP-7 can promote the recruitment of primordial follicles into the growing follicle pool. The finding that BMP-7 stimulates granulosa cell mitosis suggests a possible mechanism by which BMP-7 stimulates this process. Because BMP-7 is not expressed in rat primordial follicles, it is possible that BMP-7 derived from adjacent, larger follicles is responsible for this action of BMP-7 *in vivo*. In this study, administration of BMP-7 was also shown to inhibit ovulation and progesterone production (186). Given that progesterone is essential for ovulation (246–253), the inhibition of progesterone by BMP-7 could be causally connected to the mechanisms of ovulation inhibition.

The results of studies using human ovarian theca-like tumor (HOTT) cells have provided additional evidence for a role of BMP-4 in regulating steroidogenesis (254). These cells, which express ALK-3, -6, and BMPR-II, were established from an ovarian tumor that produces excessive androgens. Although HOTT cells do not respond to LH, they do exhibit increases in steroidogenesis in response to stimulation by forskolin and dibutyryl cAMP (255). In this system, BMP-4 inhibits forskolin-stimulated increases of CYP17 levels without changing the levels of StAR, P450scc, and 3β-HSD. One possible interpretation of this result is that BMP-4 may be involved in switching the androgen-secreting theca cell into a progesterone-secreting theca lutein cell.

2. BMP-2. Studies with cultured sheep granulosa cells have shown that recombinant BMP-2 can amplify FSH-induced estradiol and inhibin A production (183). To what degree BMP-2 modulates FSH-dependent progesterone production

is unknown. In another study, BMP-2 was found to stimulate the expression of inhibin βB subunit mRNA and the secretion of dimeric inhibin B by cultured human granulosa-lutein cells (256). These stimulatory effects of BMP-2 were inhibited by cotreatment with hCG. Similar results were obtained when these human cells were challenged with BMP-6, but not BMP-3 or BMP-3b.

3. BMP-6. Unique biological functions of BMP-6 have been identified in the ovary using primary cultures of rat granulosa cells (39) (Fig. 3). Like BMP-4 and -7, BMP-6 is effective in inhibiting FSH-induced progesterone synthesis. However, whereas BMP-4 and -7 augment FSH-induced estradiol production, BMP-6 has no effect on FSH-induced estradiol production. Consistent with its steroidogenic regulation by granulosa cells, BMP-6 inhibits FSH stimulation of StAR and P450scc mRNA expression without affecting the expression of P450arom mRNA. Furthermore, BMP-6 inhibits progesterone synthesis and the corresponding expression of StAR and P450scc induced by forskolin. In contrast, BMP-6 has no effect on these steroidogenic parameters induced by 8-bromo (Br)-cAMP (39). A similar inhibition by BMP-6 of the expression of mRNAs encoding other FSH-responsive genes, including the inhibin/activin subunits (α, βA, and βB) and the LH receptor, is observed when stimulated by forskolin and FSH, but not by 8-Br-cAMP. As depicted in Fig. 3, these data together with the fact that BMP-6 decreases FSH- and forskolin-induced cAMP production suggest that BMP-6 inhibits FSH action by suppressing adenylate cyclase activity. The failure of BMP-6 to influence granulosa cell mitosis is notably different from the mitotic effects of BMP-7. In terms of physiology, it should be noted that BMP-6 mRNA expression is rapidly decreased at the time the dominant follicle is selected during the normal estrous cycle in the rat (Table 3). The loss of the FSH action inhibitor, BMP-6, may be linked to the mechanism by which dominant follicles are selected in the rat.

4. BMP-15. A major field of interest in reproductive research concerns the role of the oocyte in regulating folliculogenesis (212, 257–259), in particular the identification of factors secreted by oocytes that can modulate follicular growth and development (194, 258, 260–262). In this context, Otsuka *et al.* (37, 213, 263) produced recombinant human BMP-15 and discovered the following important biological actions of BMP-15 in rat granulosa cells.

First, BMP-15 is a potent stimulator of granulosa cell proliferation as determined by BMP-15-induced increases in tritiated thymidine incorporation and cell number (37). The mitotic activity of BMP-15 is FSH-independent, suggesting that BMP-15 can stimulate granulosa cell mitosis in preantral follicles during the FSH-independent stages of early follicular growth. Whether BMP-15 regulates granulosa cell proliferation during Graafian follicle development is not known.

Second, one of the most interesting and perhaps important actions of BMP-15 is its ability to inhibit FSH receptor expression (213). As a result, the FSH-induced expression of StAR, P450scc, 3β-HSD, LH receptor, and inhibin/activin subunits (α, βA, and βB) are all inhibited by BMP-15 (Fig. 4). The finding that FSH-induced StAR, P450scc, and progesterone synthesis are blocked by BMP-15 demonstrates that BMP-15, like BMP-4, -6, -7, and GDF-9, is a luteinization

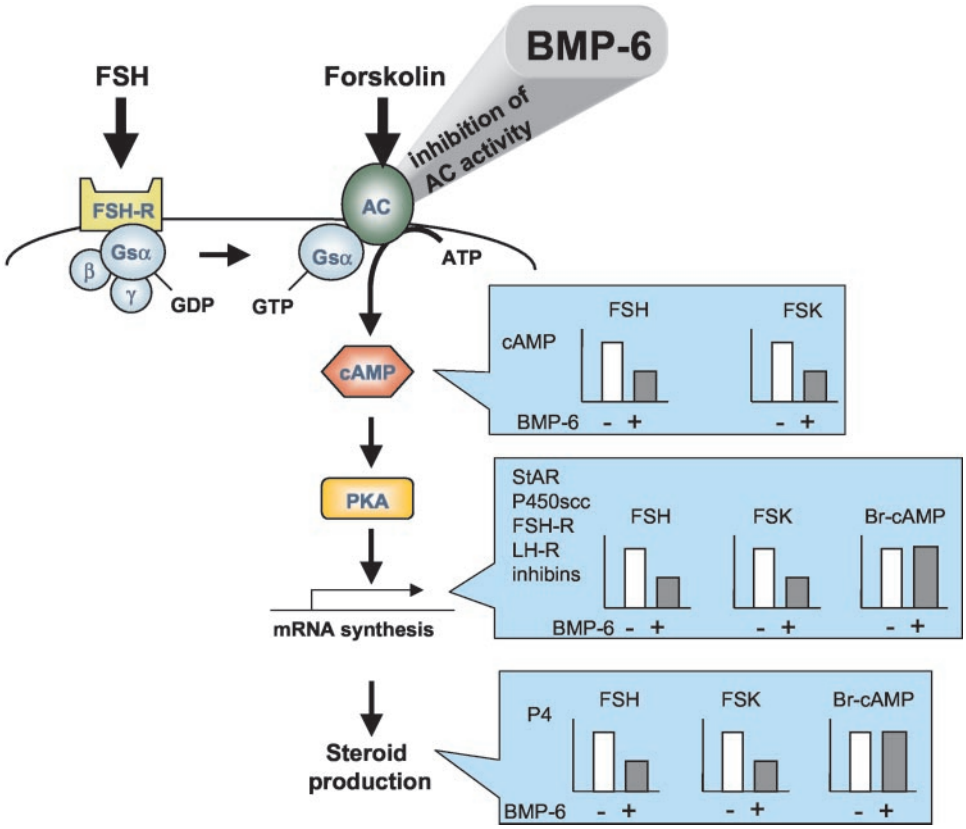

FIG. 3. Cellular mechanism of BMP-6 suppression of FSH activities in rat granulosa cells. BMP-6 suppresses FSH-induced cAMP production, FSH-induced expression of StAR, P450scc, FSH receptor (FSH-R), LH receptor (LH-R), and the inhibin subunits and FSH-induced progesterone synthesis (39). BMP-6 also inhibits the actions of forskolin (FSK) on all these parameters. However, BMP-6 has no effect on the actions of Br-cAMP. These data are consistent with BMP-6-inhibiting FSH action at the level of adenylate cyclase (AC). PKA, Protein kinase A.

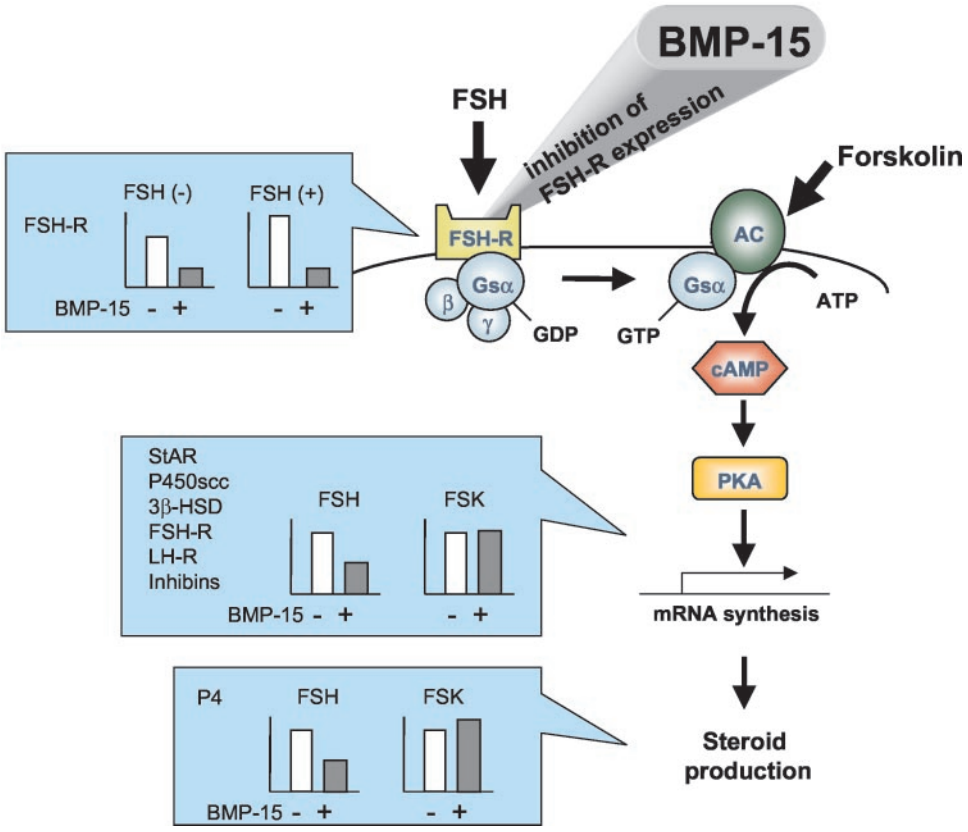

FIG. 4. Cellular mechanism of BMP-15 suppression of FSH activities in rat granulosa cells. BMP-15 suppresses FSH-induced expression of StAR, P450scc, 3β-HSD, FSH-R, LH-R, and the inhibin subunits as well as FSH-induced progesterone synthesis (213). In contrast, BMP-15 does not inhibit these actions induced by forskolin (FSK). Because BMP-15 inhibits FSH-R expression in the presence and absence of FSH, the suppression of FSH activity by BMP-15 is manifested through the suppression of FSH-R expression. PKA, Protein kinase A.

inhibitor. The suppression of FSH-induced steroidogenesis and gene expression by BMP-15 is quite similar to that of BMP-6; however, BMP-6 inhibits FSH signaling by suppressing adenylate cyclase activity, whereas BMP-15 inhibits FSH signaling by suppressing FSH receptor expression. Regarding BMP-6 and BMP-15 action, it may be particularly significant that neither ligand inhibits the stimulatory action of FSH on P450arom mRNA expression and estradiol production. In the *in vitro* cell culture experiments, androstenedione was added to the medium to serve as a substrate for P450arom. Interestingly, in the absence of androstenedione, BMP-15 inhibits FSH stimulation of P450arom mRNA expression (213). A similar result has been obtained using BMP-6 (F. Otsuka and S. Shimasaki, unpublished data). Given the fact that *in vivo*, FSH stimulates the expression of P450arom mRNA and estradiol production in dominant follicles replete with androstenedione in the follicular fluid, neither BMP-6 nor BMP-15 would be expected to affect FSH-dependent estradiol production *in vivo*. This action of the oocyte-derived BMP-15 is clearly different from that of theca-derived BMP-4 and BMP-7, both of which enhance FSH-induced estradiol production. Nonetheless, an important consensus is that all of these BMPs are selective inhibitors of progesterone synthesis induced by FSH.

A third major finding is that BMP-15 stimulates kit ligand (KL) expression in rat granulosa cells (263). *In vivo*, KL is highly expressed by granulosa cells during the early stages of folliculogenesis (264–266). Female mice with naturally occurring mutations in KL are infertile due to developmental abnormalities in oogenesis and folliculogenesis (267). The biological importance of the KL system in female reproduction is further demonstrated by the finding that naturally occurring mutations in the oocyte KL receptor, c-kit (268–270), result in a similar infertile phenotype (271–276). Interestingly, when KL is added to rat granulosa/oocyte cocultures, the expression of BMP-15 mRNA is significantly decreased (263). Thus, oocyte-derived BMP-15 and granulosa-derived KL form a novel gamete-somatic cell negative feedback loop that is functionally linked to granulosa cell proliferation (Fig. 5). There is evidence that partly grown oocytes isolated from immature mouse ovaries enhance KL expression when cocultured with granulosa cells (277, 278). Therefore, it is possible that the KL-inducing factor secreted by partly grown oocytes is BMP-15. The functional relevance of the BMP-15/KL feedback loop in the induction of follicular growth is demonstrated by two important findings: 1) the addition of KL to oocyte/granulosa cell cocultures stimulates granulosa cell mitosis; and 2) inhibition of the c-kit signaling reduces BMP-15 stimulation of granulosa cell mitosis (263).

The dependence on the oocyte for the maximum effects of different TGF-β superfamily members on granulosa cell mi-

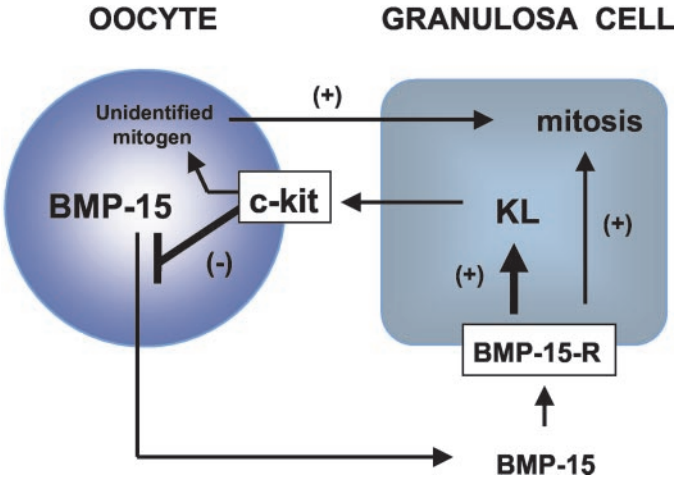

FIG. 5. Interaction of BMP-15 and KL in the regulation of granulosa cell mitosis. Oocyte-derived BMP-15 acts through its receptors (BMP-15-R) on granulosa cells to stimulate mitosis and KL expression. KL, in turn, acts through c-kit on the surface of the oocyte to inhibit BMP-15 expression, forming a negative feedback loop. KL also causes an increase in granulosa cell mitosis, presumably by stimulating the oocyte to secrete an unidentified mitogen (263).

tosis was further tested by comparing mitosis of granulosa cells cultured with and without oocytes. These experiments revealed that BMP-15, as well as activin A and BMP-7, cause higher increases in granulosa cell mitosis in the presence of oocytes (263). These results suggest that the mitotic effects may have a functional dependence on the oocyte. However, it appears that the dependency of activin A and BMP-7 on oocytes is significantly higher than that of BMP-15 (263). This difference could be explained by the localization of ActR-II to the oocyte (58, 59) and BMPR-II to the granulosa cells (32). In the rat granulosa/oocyte coculture system, the activin A and BMP-15 stimulation of granulosa cell mitosis is predominantly inhibited by the recombinant protein containing extracellular domain (ECD) of ActR-II (ActR-II-ECD) and BMPR-II-ECD, respectively (76). Therefore, it seems likely that activin A relies heavily on ActR-II on the surface of oocytes for the stimulation of granulosa cell mitosis, whereas BMP-15 acts primarily through BMPR-II on the surface of the granulosa cells for the direct stimulation of granulosa cell mitosis. The fact that the BMP-7 stimulation of granulosa cell mitosis is inhibited by both ActR-II-ECD and BMPR-II-ECD with similar effectiveness is supported by the ability of BMP-7 to utilize both ActR-II and BMPR-II.

With respect to the regulation of BMP-15 function, follistatin has been shown to inhibit BMP-15 actions on granulosa cell proliferation, FSH receptor expression, and progesterone production (128). As stated above, follistatin is strongly expressed in dominant follicles, whereas its expression in atretic follicles is very low or undetectable in rat ovaries. Given that BMP-15 is an inhibitor of FSH receptor expression, it can be predicted that follistatin regulation of BMP-15 is important for normal folliculogenesis *in vivo*.

5. GDF-9. GDF-9 has high amino acid homology and a similar oocyte-specific ovarian expression pattern to BMP-15 (30). GDF-9 has received much attention by reproductive biologists after the finding by the Matzuk laboratory (33) that deletion of the *Gdf9* gene in mice causes a block in folliculogenesis at the primary stage, thereby causing female infertility. Studies using recombinant protein have demonstrated that GDF-9 has diverse functions in the granulosa and theca cells during folliculogenesis (194, 260, 279).

The Hsueh laboratory has produced recombinant rat GDF-9 and discovered specific biological functions of GDF-9 in the rat model. First, GDF-9 is a stimulator of granulosa cell mitosis. This was demonstrated by the evidence that GDF-9 causes an increase in granulosa cell proliferation and DNA synthesis *in vitro* (214) and increases the diameter and protein content of cultured preantral follicles (35). Second, GDF-9 is a stimulator of inhibin production. This was demonstrated by the evidence that GDF-9 stimulates inhibin α-subunit synthesis by cultured neonatal ovaries (35) and stimulates inhibin A and B production by cultured granulosa cells (108, 109). Third, GDF-9 is a stimulator of steroidogenesis. This was demonstrated *in vitro* by the evidence that GDF-9 stimulates basal estradiol synthesis in differentiated and undifferentiated granulosa cells (214) and stimulates basal progesterone synthesis in differentiated, but not undifferentiated, granulosa cells (214). Fourth, GDF-9 is an inhibitor of FSH actions, as evidenced by the finding that GDF-9 inhibits FSH-dependent LH receptor expression, cAMP production, and estradiol and progesterone synthesis (214). Because GDF-9 does not affect the ability of forskolin to stimulate these parameters, it was concluded that the FSH-suppressive effects of GDF-9 occur upstream of adenylate cyclase, possibly at the level of FSH receptor expression and/or the coupling of the FSH receptor to the Gs-protein (214). However, the finding that GDF-9 and FSH act synergistically to stimulate inhibin A and B production by granulosa cells (108) is paradoxical in this context, in particular, when the suppressive effect of GDF-9 on FSH-induced cAMP production (214) is considered. In a study using human granulosa cells, the same GDF-9 was shown to inhibit the progesterone production stimulated by Br-cAMP (280) suggesting that the mechanisms underlying the inhibition of progesterone synthesis by GDF-9 are different between rat (214) and human granulosa cells (280).

Using a mouse model, Elvin *et al.* (36) showed that, in the absence of FSH, recombinant mouse GDF-9 stimulates progesterone synthesis by cultured mouse granulosa cells from preovulatory follicles. Further studies showed that this stimulation of progesterone synthesis by GDF-9 is dependent upon the ability of GDF-9 to stimulate the prostaglandin E2/EP2-receptor pathway (281). Another effect of GDF-9 in this model is to suppress FSH-induced expression of the LH receptor. They also demonstrated that GDF-9 can mimic the effects of oocytes in stimulating cumulus cell expansion. The mechanism underlying this GDF-9 effect involves the stimulation of the expression of hyaluronan synthase 2, which is important in the formation of the matrix necessary for cumulus expansion. The GDF-9-inducible gene product, pentraxin 3, may also play a role in the molecular mechanism by which GDF-9 promotes cumulus cell expansion (282).

Another important function of GDF-9 in mouse granulosa cells is the suppression of KL expression in granulosa cells (283). This evidence fits the earlier finding that the expression of ovarian KL is increased in GDF-9 null mice (198). These

data are also consistent with the earlier results of Joyce *et al.* (278) that the steady-state mRNA levels of KL are regulated by oocytes in a developmental stage-dependent manner. In this study, using a mouse granulosa cell/oocyte coculture system, it was shown that the addition of partly grown oocytes increases KL mRNA expression in granulosa cells, whereas addition of fully grown oocytes causes a decrease in KL mRNA expression. The fact that GDF-9 inhibits whereas BMP-15 stimulates KL expression has led to the hypothesis that secretion of BMP-15 from partly grown oocytes could explain the stimulatory effect of growing oocytes on KL expression (263), whereas the inhibitory effect of full-grown oocytes on KL expression may be caused by their secretion of GDF-9 (263). BMP-15 and GDF-9 mRNA and protein are detected in oocytes from primary follicles throughout folliculogenesis. However, it is possible that in addition to the regulation of gene expression, there may also be mechanisms that allow for the specific regulation of the secretion of the biologically active forms of BMP-15 and GDF-9 from the oocyte. In this regard, it was found that the level of secretion of the mature (bioactive) forms of BMP-15 and GDF-9 do not necessarily correspond with the expression levels of their mRNAs and proproteins (53). Therefore, the differential secretion of the bioactive forms of these molecules from the oocyte may be determined by the degree of processing of the proproteins. More advanced immunological assay techniques will be needed to verify the differential secretion of bioactive forms of BMP-15 and GDF-9 into the follicular fluid. It will also be important to determine the influence of the BMP binding proteins and receptor binding competitors in modulating the bioavailability of GDF-9 and BMP-15 under normal physiological conditions *in vivo* (Fig. 1).

It is also possible that the differences in the action of GDF-9 and BMP-15 on KL expression may be attributed to species differences between rats and mice. There have been no studies demonstrating the effects of BMP-15 on the expression of KL in mice. However, a study using rat GDF-9 in cultures of ovarian explants from neonatal rats found that GDF-9 stimulates KL expression (284), a result opposite of that reported for mice (283). Interestingly, there is evidence that KL is selectively expressed in mural, but not cumulus, granulosa cells in mouse antral follicles (264, 285). An opposite pattern of expression of KL is seen in rat ovaries in which the primary site of KL expression is in cumulus, not mural granulosa cells (265). Based on these data, it is possible that both BMP-15 and GDF-9 stimulate KL expression by cumulus granulosa cells in rats, whereas they inhibit KL expression by cumulus granulosa cells in mice. Given the established importance of KL, BMP-15, and GDF-9 in ovarian function, a further elucidation of the reciprocal relationships of these factors is warranted.

The GDF-9 knockout mice have provided considerable insight into the physiological role of GDF-9 in early folliculogenesis *in vivo*. Because the follicles of GDF-9 null mice fail to progress past the primary stage (33, 198), the requirement of GDF-9 at other stages of folliculogenesis cannot be determined in this model. In this regard, Vitt *et al.* (187) injected rat GDF-9 into the peritoneal cavity of immature rats and found increases in ovarian weight, primary follicle number, and CYP17 expression. Therefore, GDF-9 can promote primordial follicle recruitment and development of theca interstitial cells. The potential clinical relevance of this finding became apparent when it was reported that administration of GDF-9 to cultured human ovarian tissue also promoted early aspects of folliculogenesis (286).

The suggestion that GDF-9 may exhibit biological actions in theca cells is supported by *in vitro* studies in which GDF-9 was demonstrated to modulate steroidogenesis in both human and rat theca cell models. Interestingly, in both rat primary and immortalized theca cells, GDF-9 augments basal and LH- or forskolin-stimulated androstenedione production (287), whereas in human primary theca cell cultures, GDF-9 inhibits forskolin-stimulated production of progesterone, 17α -hydroxyprogesterone, and dehydroepiandrosterone (280). The opposite effects of GDF-9 on steroidogenesis in the rat and human theca cells suggest that GDF-9 may have species-specific effects in mammals (280).

6. BMP signaling pathways in the ovary. The use of recombinant proteins has revealed that the different BMP ligands expressed in the ovary exhibit overlapping, yet unique, biological activities. This phenomenon is represented by the specific steroidogenic and mitotic activities of different BMPs in undifferentiated rat granulosa cells (Fig. 6). Although the specific biological activities and physiological roles of the BMP ligands have received much recent attention, there have been only a few studies that have investigated the intracellular BMP signaling pathway in ovarian cells. BMP-2, -6, -7, and -15 have been found to stimulate the phosphorylation (thus activation) of Smad1/5/8 in human and rat granulosa cells (76, 288). However, nothing is known about the role of specific Smad molecules in generating a BMP response in granulosa cells. Although the Smad pathway is the most thoroughly studied signal transduction pathway for BMP ligands, other signaling molecules have been implicated in BMP signaling. In this regard, administration of inhibitors of ERK1/2 phosphorylation have been shown to inhibit granulosa cell mitosis induced by BMP-15 (76) and cumulus cell expansion induced by GDF-9 (118). ERK1/2 molecules have been shown to play important roles in FSH-induced steroidogenesis in granulosa cells (289–292), yet inhibition of ERK1/2 phosphorylation does not reverse the suppressive effects of BMP-15 on FSH-induced progesterone production by rat primary granulosa cells (76). Given the established

	Steroidogenesis				Mitosis		
	Estradiol		Progesterone		DNA	Cell	
FSH	-	+	-	+	Synthesis	Number	References
BMP-4	⇨	⇧	⇨	⇩	?	?	32
BMP-6	⇨	⇨	⇨	⇩	⇨	⇨	39
BMP-7	⇨	⇧	⇨	⇩	⇧	⇧	32, 185
BMP-15	⇨	⇨	⇨	⇩	⇧	⇧	37
GDF-9	⇧	⇩	⇨	⇩	⇧	⇧	213

⇧, stimulate; ⇩, inhibit; ⇨, no change; +, response after treatment with FSH; -, basal response

FIG. 6. Biological actions of BMP molecules in rat granulosa cells from early antral follicles. In the rat granulosa cell model, different BMP molecules play unique and specific roles with respect to the regulation of steroidogenesis and cell division. The biological action common to each of these BMP molecules in this model is the suppression of FSH-induced progesterone synthesis. The complexity of these activities of BMPs is indicative of the precision of the BMP network in governing follicular growth and development.

importance of BMPs in the ovary (293), the further elucidation of specific BMP signaling pathways in granulosa cells should provide insight into the mechanisms of granulosa cell function.

B. The pituitary

The production of FSH and LH by pituitary gonadotropes is regulated by a number of hormones and autocrine/paracrine factors (294). One of the hormones that controls gonadotropin production is GnRH, which stimulates both FSH and LH secretion. In contrast, activins selectively stimulate FSH synthesis by an autocrine/paracrine mechanism (295, 296).

There is evidence that BMPs may also be involved in regulating pituitary function. Huang *et al.* (175) reported that BMP-6 and -7 stimulate FSH synthesis and secretion in the gonadotrope cell line, LβT2 cells. In contrast, Yamashita *et al.* (74) reported that BMP-7 does not regulate FSH secretion in rat primary pituitary cell culture. Further work is necessary to resolve this apparent discrepancy. Otsuka and Shimasaki (176) have recently found that BMP-15 is expressed in LβT2 cells and mouse pituitary tissue. Interestingly, BMP-15 selectively stimulates FSH biosynthesis and secretion by rat primary pituitary cells without regulating LH biosynthesis and secretion (176). Furthermore, BMP-15 does not regulate GnRH receptor expression in LβT2 cells. A fundamental question in reproductive biology and medicine is how the monotropic rise in FSH occurs in females. In this regard, a central feature of BMP-15 action in the gonadotrope is that it causes the selective activation of FSHβ transcription without affecting LHβ and GnRH receptor transcription (176). Although the physiological relevance of this process is not yet clear, these data are consistent with a role for BMP-15 in specifying FSH, but not LH, production by the pituitary gonadotrope (176). Taken together with the evidence that BMP-15 is a negative regulator of FSH receptor expression in the ovary (213), the concept that emerges is that BMP-15 may be a central autocrine/paracrine player controlling both FSH responses and FSH bioavailability at multiple levels of the pituitary-gonadal axis in female mammals.

V. Genetic Mutations of the BMP System That Cause Specific Reproductive Phenotypes

Embryonic stem cell technology for generating knockout and transgenic mice has provided valuable tools for the identification of physiological roles of a particular gene of interest. Unfortunately, many mice with targeted deletions of BMP genes die before the reproductive system has developed (11, 297). Thus, the biological role of these genes in the reproductive system could not be determined using this technology. Some examples include: 1) the deficiency of BMP-2 in mice is embryonic lethal, with major abnormalities occurring in amnion/chorion and cardiac development (298); 2) the deficiency of BMP-4 is embryonic lethal due to a lack of mesoderm induction (245); 3) the deficiency of BMP-7 is perinatal lethal due to abnormalities in kidney development (206, 207); 4) deficiency of the ActR-IIB is perinatal lethal causal to numerous cardiac defects (299); 5) the deficiency of ALK-3 is embryonic lethal due to the lack of mesoderm induction (300); and 6) the deficiency of BMPR-II is embryonic lethal due to the arrest at gastrulation (301).

There are, however, a number of mice with targeted deletions in BMP genes that are viable, yet they exhibit reproductive defects. Additionally, genetic studies in ewes that exhibit abnormal fertility have revealed that they carry mutations in BMP-related genes. Thus, animals with these gene mutations or deletions have proven to be a valuable tool to better understand the role of these components of the BMP system in reproduction (Table 4).

A. GDF-9 knockout mice

The creation of a line of mice deficient in GDF-9 by targeted deletion was of paramount significance to the field of reproductive biology. Dong *et al.* (33) have established GDF-9 knockout mice and demonstrated that homozygous females are infertile because folliculogenesis becomes arrested at the primary stage. This is in contrast to homozygous GDF-9 knockout males and heterozygous GDF-9 knockout females, both of which are phenotypically normal. Further characterization of the GDF-9 knockout homozygous females provided information about the influence of GDF-9 on the func-

TABLE 4. Genetic mutations in the BMP system that cause ovary-specific phenotypes

	Type of mutation	Ovarian phenotype	Refs.
GDF-9 knockout mouse	Targeted deletion of the *Gdf9* gene	Infertility due to a block in folliculogenesis at the primary stage	33, 198
Inverdale ewe	Naturally-occurring V31D substitution in mature BMP-15	Infertility in homozygotes due to a block in folliculogenesis at the primary stage Increased ovulation rate in heterozygotes	38, 303
Hanna ewe	Naturally-occurring stop codon at amino acid 23 of mature BMP-15	Similar phenotype as Inverdale ewes	38, 306
BMP-15 knockout mouse	Targeted deletion of the *Bmp15* gene	Reduced fertility due to defects in ovulation and postfertilization embryo development	196
Booroola ewe	Naturally-occurring Q249R substitution in the kinase domain of ALK-6	Super fertility in heterozygotes, which is further increased in homozygotes	40–42, 310
ALK-6 knockout mice	Targeted deletion of the *Alk6* gene	Infertility due to defects in cumulus expansion and fertilization	184

tion of granulosa cells and oocytes (198, 302). In GDF-9 knockout female mice, the ability of the granulosa cells to divide mitotically is lost at the end of the primary stage when the oocyte is surrounded by a single layer of cuboidal granulosa cells. A role for GDF-9 in the promotion of atresia is also implied based on the absence of granulosa cell apoptosis in GDF-9 knockout mice.

These studies have provided evidence that differentiation of follicle cells (granulosa, theca, and oocyte) is also influenced by GDF-9. The major evidence to support this is as follows. First, the expression of KL and the inhibin α-subunit in granulosa cells of GDF-9-deficient mice is abnormally high (198). The implication of this observation is that GDF-9 normally acts as a negative regulator of these important genes. The question of how the GDF-9 regulation of KL and the inhibin α-subunit fits into the overall mechanism of normal follicle development remains to be answered. Second, the recruitment of theca cell precursors is also impaired in GDF-9 knockout ovaries as determined by the lack of expression of the mRNAs encoding CYP17, LH receptor, and c-kit in the theca compartment (198). This observation supports a functional link between oocyte-derived GDF-9 and theca development during the early stages of folliculogenesis. Third, an interesting finding in GDF-9 knockout mice is that granulosa cells in the immature primary follicles with degenerated oocytes express the specialized characteristics typical of dominant preovulatory follicles, namely LH receptor, P450arom, and P450scc (198). This result suggests that the expression of the terminal differentiated state of the granulosa cells is under the negative control of oocyte-derived GDF-9. And fourth, the development of the oocyte is abnormal in GDF-9-deficient ovaries (302). The types of abnormality found in GDF-9-knockout oocytes include: 1) they grow faster than control oocytes; 2) they exhibit perinuclear organelle aggregation and unusual peripheral Golgi complexes; and 3) they fail to develop cortical granules.

Collectively, the concept emerging from the GDF-9-deficient female mice is that oocyte-derived GDF-9 plays a central role in the mechanisms of folliculogenesis by regulating granulosa cell proliferation and cytodifferentiation, theca cell formation, and oocyte development, all of which are critical functions for female fertility.

B. Inverdale and Hanna ewes

A strain of highly prolific Romney ewes was the subject of much investigation in the early 1990s. The majority of these studies were based on the offspring of a single foundation ewe, and careful progeny testing involving 359 female progeny led to the conclusion that the increased prolificacy was due to a single X-linked gene (303). The gene causing this phenotype was given the name "Inverdale," and the locus of the gene was named FecX by the Committee on Genetic Nomenclature of Sheep and Goats in 1990 (303). Further genetic studies demonstrated that males are unaffected by the Inverdale gene and that the increased prolificacy occurs in ewes that are heterozygous carriers of the Inverdale gene. Interestingly, it was also found that ewes homozygous for the Inverdale gene are completely infertile, with "streak ovaries" containing normal numbers of germ cells but having no follicles past the primary follicle stage (304, 305). In 1993, another strain of sheep, which was unrelated to the Inverdale, was found to have the same X-linked phenotype (high prolificacy in the heterozygotes and infertility in the homozygotes). These sheep were named Hanna (306), and the gene locus was given the designation $FecX^H$ (the name of the Inverdale gene locus was later designated $FecX^I$ to distinguish it from the Hanna). Although there were a number of studies describing aberrant physiology of these animals, it was not until 2000 that the gene mutations responsible for causing the phenotypes of the Inverdale and Hanna ewes were identified (38). In this study the $FecX^I$ locus was restricted to a 10-cM region at the center of the sheep X chromosome, a region that is orthologous with human Xp11.2–11.4. The genetic linkage studies found that the *Bmp15* gene is most likely a candidate for the $FecX^I$ phenotype, and subsequent DNA sequencing revealed a mutation in the *Bmp15* gene: a T to A transversion at nucleotide position 92 of the mature domain of *Bmp15* that results in the substitution of an aspartic acid for the valine at amino acid residue 31 of the mature peptide (V31D). It is noteworthy that all members of the TGF-β superfamily from a wide range of species (human, sheep, mouse, rat, sea urchin, worm, cattle, zebrafish, and chicken) possess only the conserved hydrophobic amino acids of valine, leucine, or isoleucine at the 31st amino acid or the corresponding amino acid position. Thus, the substitution with an acidic amino acid is a critical change in a highly conserved position.

BMP-15 from Hanna ewes was also sequenced. It was found that these sheep have a C to T transition at nucleotide position 67 of the mature coding region of *Bmp15* that generates a premature stop codon at amino acid no. 23 of mature BMP-15. Because the length of the mature domain of wild-type BMP-15 is 125 amino acids, it is highly unlikely that the truncated Hanna BMP-15 is capable of exhibiting the same biological activity as intact BMP-15. Because the phenotypes of the Inverdale and Hanna ewes are the same (306), it is predicted that Inverdale BMP-15 most likely also lacks biological activity. This concept is supported by the finding that when Hanna and Inverdale heterozygotes are crossed, offspring that carry both the Hanna and Inverdale mutations exhibit the same infertile phenotype of the homozygous mutants (38).

The identification of the *Bmp15* gene as the cause of the ovarian phenotype in the Inverdale and Hanna ewes establishes the importance of oocyte-derived BMP-15 in female fertility. A retrospective analysis of the reports describing the physiology of the homo- and heterozygous Inverdale and Hanna ewes together with an evaluation of the *in vitro* biological activities of recombinant BMP-15 on granulosa cell function gives particular insight into the putative roles of BMP-15 in the ovary *in vivo*. In homozygous mutant ewes, the impaired granulosa cell proliferation that leads to the cessation of folliculogenesis at the primary stage corresponds to the finding that BMP-15 is a granulosa cell mitogen (37) and stimulates the expression of KL (263), which is essential for normal folliculogenesis (267). Thus, the primary cause of infertility in the homozygous ewes could be attributed to the lack of bioactive BMP-15, which is necessary for granulosa cell mitosis and subsequent folliculogenesis.

Furthermore, the phenotype of the heterozygous mutant ewes can be explained by the ability of BMP-15 to reduce the sensitivity of granulosa cells to FSH by inhibiting the expression of the FSH receptor. The ovulation rate (as determined by the number of corpora lutea) is increased in the heterozygous ewes from 2.0 ± 0.2 in the wild-type ewes to 2.7 ± 0.2 ($P < 0.05$); however, there are no differences in the levels of FSH, LH, and ovarian steroids (307). There are also no differences in the levels of FSH and LH in ovariectomized heterozygous and wild-type ewes, suggesting that the defects in the heterozygous Inverdale ewes are restricted to the ovary and that the function of the hypothalamic-pituitary axis may remain intact. In the ovary, the size of the corpora lutea is smaller in the heterozygous ewes, leading to the conclusion that the ovarian follicles in the heterozygotes gain competency to ovulate at an earlier stage of folliculogenesis than the wild-type ewes. Indeed, there are more estrogenic follicles in the heterozygotes, and the granulosa cells of the follicles of the heterozygotes are more sensitive to LH than those of the wild-type ewes. Because there are no differences in circulating FSH, it is most likely that a primary cause of the increased ovulation rate in the Inverdale ewes is a result of precocious maturation of small follicles due to increased FSH sensitivity. A major biological function of BMP-15 *in vitro* is the suppression of FSH receptor expression (213). Therefore, it can be further postulated that the decrease in bioactive BMP-15 in the heterozygous ewes allows for increased FSH receptor expression by granulosa cells of small follicles. If true, one would predict an increase in FSH action, which in turn accelerates follicle development and causes precocious ovulation.

C. BMP-15 knockout mice

The Inverdale and Hanna mutations in the *Bmp15* gene have dramatic consequences on follicle development in sheep. In striking contrast, the targeted deletion of the entire second exon of the *Bmp15* gene in mice has little effect on folliculogenesis (196). Nonetheless, female BMP-15 knockout mice are subfertile due to defects in the ovulation process and the ability of oocytes to develop into normal embryos.

The underlying cause of the differences in the phenotypes of the BMP-15 mutant ewes and BMP-15 knockout mice is not yet understood. It has been proposed that the relative importance of BMP-15 *vs.* GDF-9 in regulating events of folliculogenesis may differ between sheep and mice. Moreover, these differences may be involved in the mono- *vs.* polyovulatory nature of these animals (196, 308). However, as suggested by Liao *et al.* (53), it is also possible that the differences are caused by the nature of the mutations in the *Bmp15* gene, *i.e.*, single point mutations in the sheep *vs.* the deletion of the entire second exon in the mouse. In this study, recombinant human BMP-15 with a I31D substitution (BMP-15^{I31D}), which mimics the V31D mutation found in Inverdale sheep (38), was produced. Interestingly, when BMP-15^{I31D} is singly expressed, it is successfully processed, and mature dimeric BMP-15^{I31D} is secreted. In contrast, when BMP-15^{I31D} is coexpressed with GDF-9, the proteolytic processing and secretion of BMP-15^{I31D} is abolished, and the proteolytic processing and secretion of GDF-9 is also severely impaired (53). It was, therefore, proposed that the aberrant processing and secretion involves the formation of BMP-15^{I31D}/GDF-9 heterodimers that are not susceptible to proteolytic cleavage and thus degrade in the cells, as shown in Fig. 7. Given the negative impact of the BMP-15^{I31D} mutation on the secretion of bioactive GDF-9, together with the similarity between the phenotypes of homozygous Inverdale ewes and GDF-9 knockout mice, it is reasonable to propose that the decrease in the secretion of GDF-9 could be involved in the aberrant phenotype of the homozygous Inverdale ewes. According to this model, the deletion of the entire second exon of the *Bmp15* gene in the mouse would not be expected to inhibit GDF-9 processing and secretion because the peptide made by the first exon is too short to form a heterodimer with GDF-9. If true, this could explain the relatively normal folliculogenesis observed in the BMP-15 knockout mouse.

A recent study by the McNatty laboratory has suggested, however, that the bioavailability of both BMP-15 and GDF-9 is necessary for normal folliculogenesis in the sheep (309). In this study, the authors were able to cause infertility in ewes by immunizing them against specific peptides derived from BMP-15 or GDF-9, or against entire mature BMP-15. Subsequent histological analysis of the ovaries of the treated ewes revealed that there were few, if any, follicles that had developed past the primary stage of folliculogenesis in most of the ewes immunized against BMP-15 or GDF-9 peptides. Interestingly, ewes immunized with entire mature BMP-15 exhibited at least one estrous cycle, and the ovulation rate in these cycles was higher than in control ewes, similar to the condition observed in the heterozygous Inverdale and Hanna ewes. Collectively, this study suggests that, unlike the

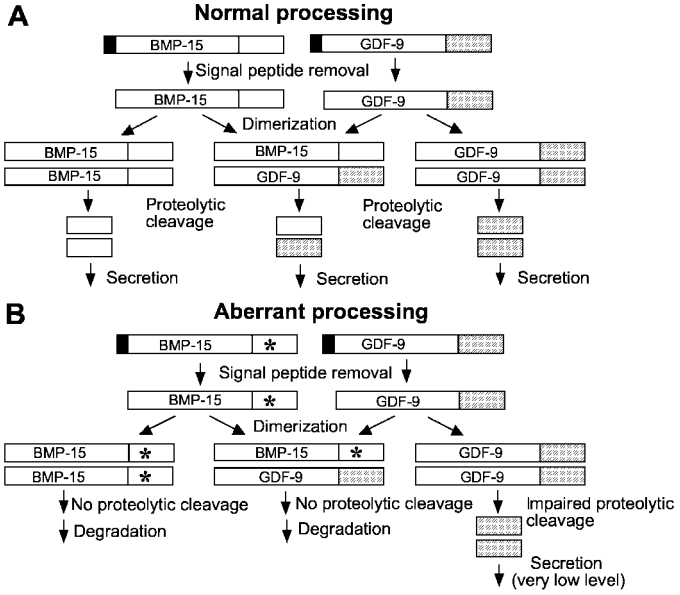

FIG. 7. Normal and aberrant processing and secretion of human BMP-15 and GDF-9 in cotransfected cell lines. A, Coexpression of wild-type BMP-15 and GDF-9 in 293T cells results in the secretion of mature BMP-15 homodimers, BMP-15/GDF-9 heterodimers, and GDF-9 homodimers. B, Coexpression of the mutant BMP-15, BMP-15^{I31D}, and wild-type GDF-9 results in severely reduced secretion of mature GDF-9 homodimers and the failure to secrete BMP-15^{I31D} homodimers and BMP-15^{I31D}/GDF-9 heterodimers. The I31D mutation in the mature domain of BMP-15 is represented by an *asterisk*.

mouse in which early folliculogenesis appears normal in the absence of BMP-15, both BMP-15 and GDF-9 are necessary for normal folliculogenesis in the sheep. Because BMP-15 and GDF-9 form heterodimers (53), however, it cannot be excluded that such heterodimers predominantly exist in the sheep ovary. If that is the case, then immunization against either BMP-15 or GDF-9 should cause the same phenotype in the ewes. Further studies are necessary to elucidate the precise role of BMP-15 and GDF-9 in the promotion and governance of folliculogenesis and ovulation quota in mice and sheep.

D. Booroola ewes

A highly prolific strain of Merino ewes has also given significant insight into the role of the BMP system in ovarian function. These sheep carry an autosomal gene, designated as Booroola (the gene locus, FecB), that causes increased ovulation rates and litter sizes (310). Unlike the Inverdale and Hanna in which homozygous mutant ewes are infertile, homozygous Booroola ewes exhibit ovulation rates that are even higher than the heterozygotes. Three independent groups reported the same point mutation in the *Alk6* gene that is associated with the Booroola phenotype (40–42). The mutation is an A to G transition at nucleotide 830 that results in a Q249R substitution in the regulatory serine/threonine kinase domain of the ALK-6 protein. Although the mechanism by which this mutation causes alterations in sheep fertility is not known, there is evidence that granulosa cells from Booroola ewes are less sensitive to BMP-4 and GDF-5 with respect to inhibition of progesterone biosynthesis (41, 311). The simplest interpretation of this result is that the Q249R mutation reduces the BMP-dependent function of the ALK-6 receptor.

Despite the relatively widespread expression of the ALK-6 receptor in sheep (40), the aberrant phenotype caused by the Booroola mutation is, for the most part, restricted to female reproduction (312). The most striking features of Booroola ewes can be observed in the ovary. Booroola follicles mature and ovulate at smaller sizes than those in wild-type ewes. The follicles in Booroola ewes exhibit increased responsiveness to FSH as evidenced by increased cAMP content and progesterone production by cultured granulosa cells (313). The increase of FSH responses most likely occurs downstream of the FSH receptor because FSH binding capacity is normal. Although BMP-2, -4, -6, -7, and -15 are expressed in mammalian ovaries and can bind to ALK-6 (Table 2), the relative degree to which each individual BMP ligand is involved in the Booroola phenotype has not been directly determined. Of the candidate ligands, a decrease in BMP-6 signaling activity would be expected to be quite consistent with the aberrant phenotype of the Booroola ewe. Specifically, the increased production of FSH-induced progesterone, but not estradiol, synthesis by granulosa cells from Booroola ewes is opposite of BMP-6, which specifically inhibits FSH-induced progesterone production (39). Furthermore, the inhibition of adenylate cyclase by BMP-6 is consistent as a cellular mechanism in which the Booroola granulosa cells are less responsive to FSH with no changes in FSH binding capacity (39). Based on the similarity of the Booroola ewes and the Inverdale and Hanna heterozygous ewes, it has been proposed that BMP-15 may also be a ligand that is involved in the phenotype of the Booroola ewes (40). This notion is supported by the recent finding of Moore *et al.* (76), showing that, of the candidate BMP type-I receptors, BMP-15 has the highest affinity for ALK-6. Further support of the role for BMP-15 in the phenotype of the Booroola was provided by the finding that ewes carrying both the Booroola and Inverdale genes have multiplicatively higher ovulation rates than those with each of the genes alone; this would be expected if ALK-6 is a receptor for BMP-15 (314, 315).

E. ALK-6 knockout mice

As is the case for the Inverdale and Hanna ewes and the BMP-15 knockout mouse, the ALK-6 knockout mouse does not display the same phenotype as the Booroola ewe (184). In striking contrast to the increased fertility in Booroola ewes, ALK-6 knockout mice are nearly infertile; however, the deletion of the *Alk6* gene, like the *Bmp15* gene, does not cause major defects in ovarian follicle development. ALK-6 mutant mice ovulate normal numbers of oocytes in response to mating with fertile males as well as to exogenous administration of gonadotropins; therefore, the infertility observed in these mice is due to postovulatory defects (184). Consistent with this, it was found that the oocytes from the mutant mice are capable of fertilization *in vitro*, but not *in vivo*. This fertilization defect appears to be caused by the failure of normal cumulus cell expansion in ALK-6 null mice (184). Also, in contrast to the Booroola ewes that exhibit relatively few nonreproductive defects, ALK-6 knockout mice exhibit defects in chondrogenesis resulting in defects in skeletal development (316, 317). The comparison of the ALK-6 knockout mice to the Booroola ewes again raises the question of whether the phenotypical differences are due to the monoovulatory or polyovulatory nature of these two species, or whether the Q249R mutation in Booroola ewes has different physiological consequences from that of the *Alk6* gene deletion.

F. BMP-8a and -8b knockout mice

Studies in *Bmp8a* and *Bmp8b* knockout mice have demonstrated that these factors are important in male reproduction. In the testis, both genes are expressed primarily in the germ cells. In the absence of BMP-8b, the germ cells undergo apoptosis, which in turn, leads to Sertoli-only seminiferous tubules and male infertility (34). In early puberty, the germ cells in BMP-8b-deficient males show a marked reduction in proliferation and differentiation. In adults, there is a significant increase in spermatocyte apoptosis. Thus, BMP-8b is critical for male germ cell proliferation in early puberty and for germ cell survival and fertility in the adult. In contrast to the BMP-8b knockout mice, targeted deletions of the *Bmp8a* gene do not display obvious germ cell defects in the initiation of spermatogenesis. However, during aging, some BMP-8a-deficient males became sterile causal to germ cell degeneration and severely compromised spermiogenesis. These observations suggest an age-related function of BMP-8a in germ cell survival and fertility in certain males (229) and indicate

no redundant roles for BMP-8a and BMP-8b in the male germ cells. Finally, in the absence of BMP-8a, the epithelium in the distal caput and cauda regions of the epididymis degenerates, which in turn, results in granuloma formation and sterility. Thus, BMP-8a is involved in the survival of the epididymal epithelium (229). This finding has potential implications for BMP-8a signaling in posttesticular sperm maturation.

VI. Perspectives and Future Directions

The past several years have been an exciting time for reproductive biology due in large part to the discoveries and rapid advancement of research on the roles of the BMP system in mammalian reproduction. The general concept to arise from these studies is that the BMP system is essential for female and male fertility. However, the field is still young and undoubtedly there will be more discoveries made in the future. We predict that these advances made in the laboratory will be translated to clinical and agricultural applications in the regulation of mammalian fertility and treatment of infertility.

One of the basic questions in reproductive biology is how ovulation quota and litter size are controlled. The discovery in sheep that naturally occurring mutations in BMP-15 (38) and ALK-6 (40–42) influence dramatically the number of ova ovulated provides definitive evidence that the ovarian BMP system plays a central role in the mechanisms governing ovulation quota and litter size. These observations are reinforced by the demonstration that BMP-7 and GDF-9 also regulate folliculogenesis and ovulation rate in rodents (186, 187). This work has led to the concept that normal ovulation quota requires the regulated expression of such BMP genes. The search for other possible mutations in the BMP system may be productive in identifying other physiological functions of the BMPs in reproduction. Furthermore, because the deletion of many BMP system genes in mice results in the death of the animals before the onset of reproductive competency, the establishment of mice with conditional deletions of these genes may provide insight into their roles in female fertility.

Genetic screening of mutations in the *Bmp15* and *Alk6* gene in sheep has already materialized as a method to optimize profitability of sheep farmers. The use of the mutant sheep as a paradigm to find genetic markers of prolificacy in genes of the BMP system in different domestic animals may lead to the enhancement of breeding practices in agricultural enterprises. Because studies have shown that manipulation of the BMP system can affect ovulation rate, the development of regimens that affect the function of BMPs could allow for the direct manipulation of litter size of domestic species. Indeed, the recent study in which sheep were immunized against BMP-15 and GDF-9 demonstrated the ability to either increase or decrease ovulation rate depending on the immunization conditions (309). Further development of technologies attributed to the BMP system could lead to novel strategies for revolutionizing reproductive management programs in agricultural settings.

As with the female, exciting new insights into the functional significance of the BMP system in male reproduction are emerging. In mice, it is clear that the BMP system is essential for spermatogenesis and epididymal function. Considering that these processes are critical for male fertility, it is not unreasonable to speculate that BMPs could be of importance for future progress in male contraceptive research. In this regard, there is a resurgence of interest in the development of new male contraceptives (318). However, before it is possible to accurately project the potential applications of the BMP system in male reproduction, a further evaluation of the expression patterns and biological roles of the BMP system in the male reproductive system is necessary.

Finally, it should be apparent that the understanding of BMPs in reproduction could have considerable clinical relevance for human fertility and infertility. Research to date has only scratched the surface of the role of BMPs in human reproduction. A few components of the BMP system have been identified in the human ovary, but whether the BMP system influences the function of human reproduction is unknown. However, based on the importance of the BMP system in regulating ovarian functions in other animals, it seems likely that disorders in the BMP system could be involved in human ovarian diseases such as polycystic ovary syndrome, premature ovarian failure, luteal phase defects, and ovarian cancer. The findings that the deletion of the *Gdf9* and *Bmp15* genes in mice and mutations in the *Bmp15* and *Alk6* genes in sheep cause phenotypes that are defective, for the most part, only with respect to ovarian function make these factors tremendously attractive targets for clinical applications. Accordingly, based on the roles of the BMP system in the ovary, targeted manipulation of these components of the BMP system may allow for the specific clinical modulation of early follicle recruitment, granulosa cell mitosis, ovarian steroidogenesis, luteinization, and ovulation with a low risk of nonovarian side effects. These properties of the BMP system provide the potential for a unique approach to the development of pharmacological regimens for regulating ovarian function, including: 1) novel fertility treatments aimed at enhancing fertility at the level of early follicular growth; 2) the development of new nonsteroidal contraceptives; and 3) treatments designed to delay the depletion of the ovarian reserve of follicles, and thus delay the onset of menopause.

In conclusion, we can see that the BMP system is of paramount importance in controlling mammalian reproduction. There seems little doubt that BMP research will continue to flourish in the years to come. A clearer understanding of the expression and actions of the BMP system in humans may help clinicians find a role for the BMPs in the diagnosis and treatment of reproductive disorders that affect human fertility.

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