Molecular Basis of Bone Morphogenetic Protein-15 Signaling in Granulosa Cells*

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Bone morphogenetic protein-15 (BMP-15), an oocyte growth factor belonging to the transforming growth factor-β (TGF-β) superfamily, has recently been shown to be necessary for normal female fertility in mammals. We have previously demonstrated that BMP-15 regulates granulosa cell (GC) proliferation and differentiation; namely, BMP-15 promotes GC mitosis, suppresses follicle-stimulating hormone (FSH) receptor expression, and stimulates kit ligand expression. Although the role of BMP-15 in female reproduction has progressively deserved much attention, there is nothing known to date about the signaling pathway and receptors for BMP-15. Using rat primary GCs and a human GC cell line, COV434, we have now found that administration of BMP-15 causes a rapid and transient phosphorylation, thus activation, of the MAPK pathway. BMP-15 also stimulates transcriptional activity of a selective BMP-responsive reporter construct, further demonstrating the stimulation of Smad2 phosphorylation by BMP-15. In contrast, BMP-15 stimulation of Smad1/5/8 phosphorylation was very weak.

To identify the receptors for BMP-15, we utilized recombinant extracellular domains of individual transforming growth factor-β superfamily receptors and found that activin receptor-like kinase-6 extracellular domain most effectively co-immunoprecipitates with BMP-15, whereas BMP receptor type II extracellular domain was most effective in inhibiting BMP-15 bioactivity on FSH-induced progesterone production and GC thymidine incorporation. We also investigated whether activation of the MAPK pathway is necessary for BMP-15 biological activity and found that the addition of U0126, an inhibitor of ERK1/2 phosphorylation, suppresses BMP-15 activity on GC mitosis but not on FSH-induced progesterone production, suggesting a selective signaling cascade in GC proliferation and differentiation.

Bone morphogenetic protein-15 (BMP-15), also called growth and differentiation factor-9B (GDF-9B), is a recently discovered oocyte-derived growth factor and a member of the transforming growth factor-β (TGF-β) superfamily (1, 2). BMP-15 has been shown to play integral roles in the regulation of ovarian folliculogenesis and follicular function (3). Our previous in vitro studies in rats using recombinant BMP-15 identified granulosa cells (GCs) as the predominant target cell type for BMP-15 (4). These studies also identified three major biological functions of BMP-15 in the ovary (4–6). (i) BMP-15 is a potent stimulator of GC mitosis; (ii) BMP-15 inhibits the expression of FSH receptor mRNA in GCs, resulting in the subsequent suppression of FSH-induced progesterone synthesis as well as FSH-induced expression of a battery of mRNAs in GCs; and (iii) BMP-15 stimulates the expression of kit ligand mRNA in GCs. Furthermore, the biological activity of BMP-15 was found to be inhibited by follistatin, which is highly expressed by GCs in developing follicles (7, 8). The importance of BMP-15 in fertility in sheep was exemplified by the demonstration that naturally occurring mutations in the bmp15 gene in Inverdale (FecX⁻) and Hanna (FecX⁺) ewes caused an increase in the ovulation rate in heterozygotes due, in part, to an increased FSH sensitivity of GCs but caused infertility in homoygotes due to a block in the primary stage of folliculogenesis (9). Mice with targeted deletions in the bmp15 gene are subfertile, demonstrating the importance of BMP-15 in fertility in the mouse as well (10).

The TGF-β superfamily consists of over 30 members, which are classified into the superfamily based upon structural homology (1, 11). A common feature of most of the members of the TGF-β superfamily is the presence of seven conserved cysteine residues, six of which form a characteristic “cysteine knot” structure and one of which forms a disulfide bridge between two subunits, making the molecule a covalently linked dimer (12). Interestingly, BMP-15 and its closest homologue, GDF-9, which is also an oocyte-derived factor, are unique among the TGF-β superfamily in that they lack the conserved fourth cysteine, which is responsible for the dimerization of the subunits (1). Thus, it is not known whether BMP-15 exists as noncovalently linked dimers or as monomers.

Despite the established importance of BMP-15 in female fertility, the receptors and signaling pathways for BMP-15 have yet to be identified. Receptors for the majority of TGF-β superfamily members consist of two closely related subsets of transmembrane serine/threonine kinase receptors, the type I and type II receptors (13). To date, seven type I and five type II receptors have been identified in mammals. Upon binding of dimeric TGF-β superfamily members to specific type I and type II receptors, the receptor complexes cause the phosphorylation of extracellular signal-regulated kinase 1/2; FSH, follicle-stimulating hormone; GC, granulosa cell; GDF, growth and differentiation factor; MAPK, mitogen-activated protein kinase; TGF-β, transforming growth factor-β; PBS, phosphate-buffered saline.
of intracellular signaling molecules called Smads, which then translocate to the nucleus and regulate transcription of target genes (14–16). The receptor-regulated Smads can be grouped into two subsets; Smad2 and Smad3 are activated by TGF-β and activin, whereas Smad1, Smad5, and Smad8 are activated by BMPs. For this signaling to occur, the ligand must bind both type I and type II receptors, but the specificity of the Smad signaling pathway, which is stimulated by a particular TGF-β superfamily ligand, is determined by the type I receptor (17). In addition to Smad signaling, recent studies have demonstrated that the receptor-regulated protein kinase (MAPK) family of signaling molecules can modulate the signal transduction of TGF-β superfamily members through cross-talk with the Smad pathway in certain physiological circumstances (18).

Currently, there is nothing known about the receptors or signaling pathway of BMP-15. Given BMP-15’s unique structure compared with other TGF-β superfamily members, it is possible that BMP-15 uses unique receptors and signaling; however, it has been speculated that BMP-15 may use one or more of the established activin, TGF-β, or BMP receptors and may stimulate a subsequent Smad signaling pathway (19–21). The presence of activin receptors, which include ActR-II, ActR-IIB, and activin receptor-like kinase 4 (ALK-4, also called ActR-IB) and BMP receptors, which include ActR-II, BMPR-II, ALK-3 (BMPR-IA), ALK-6 (BMPR-IB), and ALK-2 (ActR-IA), as well as Smad molecules have been identified in GCs in various mammalian species (19, 20, 22–29); however, there is a paucity of reports that demonstrate the functional activation of these pathways in any GC model. Of the MAPK molecules, extracellular signal-regulated kinase 1/2 (ERK1/2) are expressed in GCs and have been most closely associated with the regulation of GC function (30–34).

In the present study, we investigate whether BMP-15 can use previously identified TGF-β superfamily receptors and can activate specific Smad signaling pathways. We also investigate whether ERK1/2 signaling molecules play a role in mediating the biological activities of BMP-15. For these studies, we use two GC models, rat primary GCs from early antral follicles and a human GC cell line, COV434 (35). The rat primary GC model has been well established and used to elucidate the biological activity of recombinant BMP-15 by our laboratory (4–6). COV434 cells are an immortalized human GC cell line that has retained the ability to produce estradiol upon FSH stimulation, thus demonstrating the presence of functional FSH receptors (36).

**EXPERIMENTAL PROCEDURES**

**Reagents and Supplies—** Diethylstilbestrol was purchased from Sigma, and U0126, an inhibitor of ERK1/2 phosphorylation, was from Promega Corp. (Madison, WI). Female Sprague-Dawley rats were from Charles River Laboratories (Wilmington, MA). Recombinant extracellular domains (ECDs) fused to the Fc region of human IgG (receptor-ECD/Fc chimeras) of human BMPR-II, ActR-IA, ALK-3 (BMPR-IA), ALK-6 (BMPR-IB), and ALK-2 (ActR-IA) were purchased from R & D Systems (Minneapolis, MN). Recombinant human BMP-6 and BMP-7 were generously provided by Dr. Kuber Sampath. Recombinant rat BMP-6 and BMP-7 co-transfected with 500 ng of pXVent2-Luc (kindly provided by Dr. Joan Massague) and 50 ng of cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal) using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Basel, Switzerland). 12 h after transfection, the culture media were replaced with 1% fetal bovine serum medium, and BMP-15 was added to a final concentration of 100 ng/mL. After 24 h, the cells were washed with phosphate-buffered saline (PBS) and then lysed with 60 μl of cell culture lysis reagent (Promega Corp., Madison, WI). Luciferase activity in 20 μl of the cell lysate was measured with a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA) using Luciferase Assay Reagent (Promega Corp., Madison, WI). β-Galactosidase activity in 10 μl of the lysate was measured using the Galacto-Light Plus System (Applied Biosystems, Bedford, MA) and used to normalize transfection efficiencies. The data are shown as the ratio of luciferase to β-galactosidase activity and expressed as the -fold change as compared with non-BMP-15-treated (control) cells.

Co-immunoprecipitation of BMP-15 and Receptor ECD/Fc Chimera—BMP-15 (100 ng/mL) was mixed with the indicated receptor-ECD/Fc chimera proteins (2 μg/mL) in PBS to a total volume of 250 μl and incubated at 4 °C for 4 h. Anti-FLAG monoclonal antibodies conjugated to agarose beads (anti-FLAG M2 affinity gel; 10 μl of the cell lysate was measured with a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA) using Luciferase Assay Reagent (Promega Corp., Madison, WI). β-Galactosidase activity in 10 μl of the lysate was measured using the Galacto-Light Plus System (Applied Biosystems, Bedford, MA) and used to normalize transfection efficiencies. The data are shown as the ratio of luciferase to β-galactosidase activity and expressed as the -fold change as compared with non-BMP-15-treated (control) cells.

**Analysis of Steroid Production—** GCs (105 viable cells) were cultured in a 96-well plate with 200 μl of serum-free medium containing one or a combination of FSH, BMP-15, various receptor-ECD/Fc chimera, and U0126. After a 48-h culture, the culture media were collected and stored at −20 °C until assay. The levels of progesterone in the medium were measured by standard radioimmunoassay procedures (4).

Thymidine Incorporation Assay—GCs (2 × 106 viable cells) were cultured in 1.5-ml polypropylene tubes (containing 0.5 mL of saline) of serum-free culture medium. After a 24-h preincubation period, cells were incubated with 0.5 μCi/tube of [methyl-3H]thymidine either alone or with a combination of BMP-15 and individual TGF-β superfamily receptor-ECD/Fc chimera or U0126. After another 24 h of culture, the GCs were washed with PBS, centrifuged (2000 × g, 30 min), and incubated with 10% ice-cold trichloroacetic acid for 30 min at 4 °C. The cell pellet was solubilized in 0.2 M NaOH, and its radioactivity was measured (4).

**Statistical Analysis—** All results shown are mean ± S.E. of at least three separate experiments with triplicate determinations for each treatment. Differences between groups were analyzed for statistical significance using analysis of variance (SPSS Standard version 10.0.1, SPSS Inc., Chicago, IL). p values of <0.05 were accepted as statistically significant.
After culture for 1 h, cells were lysed and subjected to SDS-PAGE. We have previously demonstrated that recombinant BMP-15 exhibits specific biological activities in rat primary GCs (4–6). To investigate the signaling pathways of BMP-15, we first subjected lysates of rat primary GCs that were exposed to BMP-15, BMP-6, BMP-7, or activin-A to SDS-PAGE/immunoblotting analysis using PS-1 antiserum, which has been validated to specifically detect phospho-Smad1/5/8. Following a 1-h exposure of GCs to BMP-15 (100 ng/ml), there was a pronounced increase in phospho-Smad1/5/8, which was also observed in the lysates of GCs treated with 100 ng/ml BMP-6 and BMP-7 (Fig. 1A). In contrast, lysates from untreated (control) GCs and GCs treated with 100 ng/ml activin-A did not display any significant amount of phospho-Smad1/5/8. A similar stimulation of the Smad1/5/8 pathway by BMP-15, BMP-6, and BMP-7, but not by activin-A, was observed in a human GC cell line, COV434 (Fig. 1B). The same phospho-Smad1/5/8 response to these growth factors was also observed in CHO-K1 cells (data not shown), indicating that BMP-15 stimulation of this pathway is not restricted to GCs. To further characterize the BMP-15 response in COV434 cells, we analyzed the time course of the presence of phospho-Smad1/5/8 following BMP-15 administration. The presence of phospho-Smad1/5/8 was evident as early as 15 min after BMP-15 administration, increased at 30 min, and peaked at 1 h, and by 4 h it was barely detectable (Fig. 2).

The lysates from primary rat GCs and COV434 cells exposed to BMP-15, BMP-6, BMP-7, or activin-A were also subjected to SDS-PAGE/immunoblotting using the PS-2 antiserum, which is specific for phosphorylated Smad2. In the lysates of rat primary GCs treated with activin-A, a very weak PS-2 signal was detectable, whereas the PS-2 signals in cells treated with BMP-15, BMP-6, and BMP-7 were indistinguishable from that of untreated controls (data not shown). However, in the lysates from COV434 cells that were exposed to activin-A, there was a pronounced increase in phospho-Smad2, whereas treatment with BMP-15, -6, and -7 had little effect on levels of phospho-Smad2 (Fig. 3).

To further demonstrate the functional activation of the Smad1/5/8 pathway by BMP-15, COV434 cells were transiently transfected with a transcriptional reporter construct, pXVent2-Luc, which has been shown to specifically respond to BMP signaling in P19 cells, C3H10T1/2 cells, and Xenopus embryos (38–40). Treatment with BMP-15 at 100 ng/ml caused a 3.3-fold increase in XVent2-luciferase activity as compared with untreated controls (Fig. 4).

Of the TGF-β superfamily receptors identified to date, BMPR-II, ActR-II, ALK-3, ALK-6, and ALK-2 have been implicated in the activation of the Smad1/5/8 pathway (14); thus, we hypothesized that one or more of these receptors may be involved in BMP-15 activation of the Smad1/5/8 pathway. To characterize biochemical binding ability, we used receptor-ECD/Fc chimera proteins for co-immunoprecipitation studies with BMP-15. Using a monoclonal anti-FLAG antibody conjugated to agarose beads, we analyzed the ability of BMP-15 to bind each receptor-ECD. SDS-PAGE/immunoblot analysis of the receptor-ECD chimera before immunoprecipitation showed that there was a similar amount of immunoreactive ECD in each group and that the individual receptor-ECDs had unique SDS-PAGE migration patterns (Fig. 5A). The BMPR-II-ECD migrates at 65–75 kDa, ActR-II-ECD migrates at 50–60 kDa, ALK-3-ECD migrates at 55–65 kDa, and ALK-6-ECD and ALK-2-ECD migrate at 50–55 kDa. Co-immunoprecipitation studies with BMP-15 revealed that, of the five candidate receptor-ECD chimeras, ALK-6 co-immunoprecipitated was clearly higher than the other candidates (Fig. 5B). The relative ability of each receptor-ECD to co-immunoprecipitate with BMP-15 was found to be approximately as follows: ALK-6-ECD > ALK-2-ECD = ActR-II-ECD > BMPR-II-ECD > ALK-3-ECD. In order to ensure that the co-immunoprecipitation was due to the binding of the receptor-ECDs to BMP-15, and not directly to the anti-FLAG-agarose, the receptor-ECDs were immunoprecipitated in the absence of BMP-15. In these studies, there was no detectable immunoprecipitated receptor-ECDs, indicating that the observed co-immunoprecipitation was due to the specific binding of the receptor-ECDs to BMP-15.

We next used the receptor-ECD/Fc chimera to determine whether these proteins can inhibit the biological effects of BMP-15 in rat primary GCs. To this end, we first evaluated the effects of BMP-15 on FSH-induced progesterone production in the presence and absence of the receptor-ECD/Fc chimera (Fig. 6). The addition of BMP-15 (30 ng/ml) caused a 38% drop in FSH-induced progesterone production compared with FSH alone. Interestingly, the inhibitory effect of BMP-15 on FSH-induced progesterone synthesis was almost completely abolished by BMPR-II-ECD. ActR-II-ECD and ALK-6-ECD also reversed the effects of BMP-15; however, their effects were not pronounced increase in phospho-Smad2, whereas treatment with BMP-15, -6, and -7 had little effect on levels of phospho-Smad2 (Fig. 3).
as pronounced as BMPR-II-ECD. ALK-3-ECD had no effect on BMP-15 suppression of FSH-induced progesterone production. In addition, although not shown, ALK-2-ECD alone (in the absence of BMP-15) suppressed FSH-induced progesterone synthesis by 46% and had no observable effect on BMP-15 action; therefore, it was not possible to determine the effects of this receptor-ECD on BMP-15 steroidogenic properties.

To further evaluate the specific ability of the receptor-ECDs to inhibit BMP-15 biological activity, we performed a thymidine incorporation assay. In this analysis, similar to the steroid assay, BMPR-II-ECD was most effective in inhibiting BMP-15 biological activity (Fig. 7). To verify the specificity of the receptor-ECDs in this system, we examined the ability of these receptor-ECDs to inhibit thymidine incorporation induced by BMP-7 and activin-A. BMPR-II-ECD was most effective in inhibiting BMP-7-induced thymidine incorporation followed by ActR-II-ECD. The type I receptor ECDs were less effective than the type II receptor-ECDs. In contrast, ActR-II-ECD completely inhibited activin-A-induced thymidine incorporation, whereas the other receptor-ECDs had only a small effect. Since the type II receptor-ECDs were more effective than the type I receptor-ECDs in inhibiting thymidine incorporation induced by BMP-15, BMP-7, and activin-A, we compared the dose-response effects of these receptor-ECDs using the thymidine incorporation assays (Fig. 8). BMPR-II-ECD inhibited BMP-15 biological activity in a dose-dependent manner and was significantly more potent than ActR-II-ECD; BMP-7-induced thymidine incorporation was equally inhibited by BMPR-II-ECD and ActR-II-ECD; and ActR-II-ECD was significantly more effective in inhibiting activin-A biological activity than BMPR-II-ECD.

Since ERK1/2 signaling has been shown to act either as a positive transducer of BMP signaling or as an antagonist of BMP-induced Smad signaling, depending on the cell type (18), we investigated whether ERK1/2 molecules also play a role in BMP-15 signaling in GCs using U0126, a selective inhibitor of ERK1/2 phosphorylation. The addition of U0126 (3 μM) to rat primary GC cultures had no significant effect on basal levels of thymidine incorporation but did cause a reduction of BMP-15-induced thymidine incorporation when added at 1 and 3 μM (Fig. 9). We further determined whether inhibition of ERK1/2 phosphorylation also mediates the effects of BMP-15 on GC progesterone production. As previously reported (30), the addi-
tion of U0126 (3 μM) inhibited FSH-induced progesterone production, as did BMP-15 (100 ng/ml) (Fig. 10). However, U0126 had no effect on BMP-15 inhibition of progesterone synthesis.

DISCUSSION

The majority of TGF-β superfamily members use intracellular second messenger molecules called Smads as a major component of their signaling pathway. In this pathway, the TGF-β superfamily ligands first bind to specific serine/threonine kinase receptors, which then phosphorylate (activate) ubiquitously expressed “pathway-restricted” Smad molecules. These pathway-restricted Smads can be classified into two groups based on the subfamily of ligands that can activate specific Smad molecules. Smad2 and Smad3 are considered activin/TGF-β-responsive Smads, because the addition of the activins and the TGF-βs cause the activation of these Smads. Nodal, another TGF-β superfamily member, has also been shown to use Smad2/3 for its signaling (41). Smad1, Smad5, and Smad8 are considered to be BMP-responsive Smads, because most BMPs identified to date, including BMP-2, -4, -6, and -7, have been shown to activate this Smad pathway in a number of different cell types (17, 42, 43). Other ligands that use the Smad1/5/8 pathway include GDF-5 (44) and Müllerian inhibiting substance (40, 45, 46). Based on the fact that most TGF-β superfamily members can activate one of these signaling pathways, we investigated whether BMP-15 activates the Smad1/5/8 or the Smad2/3 pathway.

Treatment of both rat primary GCs and a human GC cell line, COV 434, with BMP-6, BMP-7, and also BMP-15 caused a clear increase in the levels of phosphorylated (activated) Smad1/5/8 as determined by immunoblotting using the PS-1 antiserum. These data represent the first demonstration of BMP-15 signaling in any cell type. Although the activation of the Smad1/5/8 pathway by BMP-6 and BMP-7 has been well documented in a number of cell types (17, 42, 43), this is also the first demonstration of the activation of this pathway by any ligand in a GC model. The rapid and transient time course of BMP-15 action on levels of phospho-Smads was consistent with a direct effect of BMP-15 and was similar to that reported for other BMPs in various cell types. To verify the functional activation of the Smad1/5/8 pathway by BMP-15, we transiently transfected COV434 cells with the BMP-responsive reporter, pXVent2-Luc. The administration of BMP-15 caused a significant increase in the luciferase activity by this reporter, indicating that the Smad proteins acti-
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Fig. 10. Effect of U0126 on BMP-15 suppression of FSH-induced progesterone production. Rat primary GCs were cultured with FSH (10 ng/ml) in the absence or presence of BMP-15 (100 ng/ml) and U0126 (3 μM). Following a 48-h culture, progesterone levels in the culture medium were measured by a specific radioimmunooassay. Bars with different letters indicate that group means are significantly different from each other at p < 0.05.

Fig. 11. Proposed mechanism of the interaction between BMP-15 and its receptors. A, in the absence of receptor-ECDs, BMP-15 binds to the highly expressed ALK-6 receptors on the surface of the GCs. After a conformational change in the ALK-6 receptor, the BMP-15/ALK-6 complex recruits BMPR-II, resulting in signal transduction (Smad1/5/8 phosphorylation). B, BMP-15 binds ALK-6-ECD in a GC-free system; however, in the presence of GCs, BMP-15 predominantly binds to cell surface ALK-6 receptors. C, BMPR-II-ECD effectively binds to the BMP-15/ALK-6 complex formed on the GC surface, which inhibits the recruitment of the endogenous BMPR-II receptors, thus inhibiting subsequent BMP-15 signaling and biological activity.

Analyzed by BMP-15 can transduce its signals to activate transcription of the target gene.

Of the various TGF-β superfamily members, ActR-II, BMPR-II, ALK-3, ALK-6, and ALK-2 have been shown to be directly involved in the stimulation of the Smad1/5/8 signaling pathway in many cell types (14). Given that BMP-15 activates this pathway in GCs and that each of these receptors is expressed in GCs, we hypothesized that it is likely that BMP-15 may have an affinity for one or more of these receptors. To identify biochemical binding ability, we utilized the ECD/Fc chimera of these receptors and attempted to co-immunoprecipitate with BMP-15. Of these ECDs, ALK-6-ECD was most efficiently co-immunoprecipitated with BMP-15. In contrast, ALK-3-ECD exhibited a very low affinity to co-immunoprecipitate with BMP-15. BMPR-II-ECD, ActR-II-ECD, and ALK-2-ECD all clearly co-immunoprecipitated with BMP-15, but the degree of co-immunoprecipitation was substantially less than that of ALK-6-ECD. BMPs can be grouped into subcategories based on their relative affinities for specific type I receptors. In this model, it has been shown that BMP-4 and GDF-5 preferentially bind ALK-3 and/or ALK-6, whereas BMP-6 and -7 preferentially bind ALK-2 and/or ALK-6 (17, 43, 47). Because of the low ability of BMP-15 to bind ALK-3, BMP-15 signaling probably more closely resembles BMP-6 and -7 than that of BMP-4 and GDF-5.

In contrast to the relatively low biochemical binding ability of the BMPR-II-ECD to BMP-15 identified by co-immunoprecipitation, the BMPR-II-ECD was most effective in inhibiting the effects of BMP-15 on both FSH-induced progesterone production and thymidine incorporation. A characteristic feature of the interactions of TGF-β superfamily ligands and their component receptors is that the choice of receptors for a given ligand depends on the presence and amount of type I and type II receptors available. As a result, the particular receptor subset that a ligand preferentially binds can differ, depending on the cell type (43). In this regard, a number of studies have shown that, when singly overexpressed on the cell surface, BMPR-II has very low or no affinity for BMP-2, -4, -6, and -7, whereas when co-overexpressed with the appropriate type I receptors, the affinity of these BMPs for BMPR-II increases dramatically. In contrast, BMPs do bind to type I receptors when singly overexpressed (45–51). According to this nature of the interactions between BMP ligands and their receptors, it would be expected that BMPs may first bind to the type I receptors followed by the recruitment of type II receptors (50, 51). Therefore, it seems likely that in the GC cultures, BMP-15 first binds to the highly expressed endogenous ALK-6 receptors on the surface of the GCs (23), which causes a conformational change in the ALK-6 receptor structure, which then facilitates the BMP-15/ALK-6 complex to recruit BMPR-II, consequently eliciting BMP-15 biological activity (Fig. 11A). Consistent with this model, our data in this study could be explained by the mechanism that BMP-15 preferentially binds the highly expressed intact ALK-6 on the surface of GCs, as compared with the exogenously added ALK-6-ECD (Fig. 11B). Also in accordance with this model, the ability of BMPR-II-ECD to inhibit BMP-15 biological activity could have been elicited by its ability to bind to the BMP-15/ALK-6 complex and disrupt the recruitment of the endogenous BMPR-II and subsequent signaling (Fig. 11C).

Although the Smad pathway is the most studied signaling pathway for BMP ligands, there are many recent reports identifying alternative signaling pathways that mediate biological actions of BMPs in various cell types (52). In particular, the MAPK family of signaling molecules, including ERK1/2, p38, and stress-activated protein kinases/Jun N-terminal kinases, have been shown to exhibit cross-talk with the Smad pathway in the intracellular transduction of BMP signals. Interestingly, activated MAPK molecules have been shown to either positively transduce BMP/Smad signals or to act as an inhibitor of Smad signaling. Of these MAPK molecules, ERK1/2 have recently been implicated in the regulation of GC function (30–33). To further characterize the signaling of BMP-15 in rat primary GCs, we evaluated whether a specific inhibitor of ERK1/2 phosphorylation, U0126, has any effect on BMP-15-induced thymidine incorporation and BMP-15 inhibition of progesterone synthesis. The inhibition of BMP-15-induced GC thymidine incorporation by U0126 demonstrates that ERK1/2 signaling is in fact necessary for this biological activity of BMP-15. A recent report by the Eppig laboratory (34) demonstrated that U0126 also inhibits cumulus GC expansion induced by GDF-9, the growth factor that most closely resembles BMP-15, suggesting that both growth factors may share the ERK1/2 signaling pathway in GCs to exert certain biological activities. In contrast to the inhibition of BMP-15 action on GC...
mitosis, U0126 had no effect on BMP-15 suppression of FSH-induced progesterone production, demonstrating that activation of the ERK1/2 pathway is not necessary for this action of BMP-15. These findings indicate that there is a divergence in the signal transduction pathways regulating steroidogenesis and mitosis by BMP-15.

In summary, the present studies identify that BMP-15 activates the Smad1/5/8 pathways in two GC models, rat primary GCs and mitosis by BMP-15. These findings indicate that there is a divergence in BMP-15 biological activities in rat primary GCs. Additionally, we show that activated ERK1/2 molecules are necessary for FSH-induced progesterone synthesis, suggesting that there are selective pathways for BMP-15 regulation of GC proliferation and differentiation.

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REFERENCES

1. Dube, J. L., Wang, P., Elvin, J., Lyons, K. M., Celeste, A. J., and Matzuk, M. M. (1998) Mol. Endocrinol. 12, 1809–1817
2. Laitinen, M., Vuojokiainen, K., Jaatinen, R., Ketola, I., Aalto, J., Lehtonen, E., Heikkinen, M., and Ritvos, O. (1998) Mech. Dev. 76, 135–149
3. Shimasaki, S., Moore, R. K., Erickson, G. F., and Otsuka, F. (2002) Reproduction, in press
4. Otsuka, F., Yao, Z., Lee, T. H., Yamamoto, S., Erickson, G. F., and Shimasaki, S. (2002) J. Biol. Chem. 277, 39523–39528
5. Otsuka, F., Yamamoto, S., Erickson, G. F., and Shimasaki, S. (2002) J. Biol. Chem. 276, 11387–11392
6. Otsuka, F., and Shimasaki, S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 8060–8065
7. Otsuka, F., Moore, R. K., Iemura, S.-I., Ueno, N., and Shimasaki, S. (2001) Biochem. Biophys. Res. Commun. 289, 961–966
8. Shimasaki, S., Koga, M., Buscaglia, M. L., Simmons, D. M., Kuppen, P. J. K., Lewis, K. A., Sawchenko, P. E., Chang, R. J., and Erickson, G. F. (1999) J. Cell. Physiol. 180, 363–369
9. Hata, A., Seoane, J., Lagna, G., Montalvo, E., Hemmati-Brivanlou, A., and Massague, J. (2000) Cell 100, 229–240
10. Clarke, T. R., Hoshaya, Y., Irj, S. E., Liu, X., Lyons, K. M., and Donahoe, P. K. (2001) Mol. Endocrinol. 15, 946–953
11. Kumar, A., Novoselov, V., Celeste, A. J., Wolfman, N. M., ten Dijke, P., and Kuehn, M. R. (2001) J. Biol. Chem. 276, 656–661
12. Tamaki, K., Souchelnytskyi, S., Itoh, S., Nakao, A., Sampath, K., Helin, C. H., and ten Dijke, P. (1998) J. Cell. Physiol. 177, 355–363
13. Ishihara, T., Tada, K., Kitajima, I., Tojo, K., Sampath, K. T., Kawabata, M., Miyazono, K., and Imamura, T. (1999) J. Cell Sci. 112, 3519–3527
14. Aoki, H., Fujii, M., Imamura, T., Yagi, K., Takeharu, K., Kato, M., and Miyazono, K. (2001) J. Cell Sci. 114, 1483–1489
15. Gouedard, L., Chen, Y. G., Thevenet, L., Racine, C., Borie, S., Lamarre, I., Issos, N., Massague, J., and di Clemente, N. (2000) J. Biol. Chem. 275, 27973–27979
16. Visser, J. A., Olaso, R., Verhoef-Post, M., Kramer, P., Themmen, A. P. N., and Ingraham, H. A. (2001) Mol. Endocrinol. 15, 936–945
17. ten Dijke, P., Yamashita, H., Sampath, K. T., Reddi, A. H., Esterve, M., Riddle, D. L., Ichijo, H., Helin, C. H., and Miyazono, K. (1994) J. Biol. Chem. 269, 16895–16898
18. Liu, F., Venturini, F., Doody, J., and Massague, J. (1995) Mol. Cell. 15, 3479–3486
19. Nohno, T., Ishikawa, T., Saito, H., Hoshikawa, K., Noji, S., Wolsing, D. H., and Rodwell, J. W. (1995) J. Biol. Chem. 270, 22522–22526
20. Gilboa, L., Nohe, A., Geissendorfer, T., Sebald, W., Henis, Y. I., and Knaus, P. (2002) J. Biol. Chem. 277, 5320–5328
21. Piek, E., Heldin, C. H., and Ten Dijke, P. (1999) FASEB J. 13, 2105–2124
