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# Amino Acid 72 of Mouse and Human GDF9 Mature Domain Is Responsible for Altered Homodimer Bioactivities but Has Subtle Effects on GDF9:BMP15 Heterodimer Activities[1]

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## ABSTRACT

**Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are oocyte-secreted paralogs of the transforming growth factor beta (TGFbeta) superfamily. In mammals, these two growth factors play critical roles in folliculogenesis. As previously reported, an arginine in the pre-helix loop of GDF5 defines the high binding specificity to its type 1 receptor. Interestingly, bioactive mouse GDF9 and human BMP15 share the conserved arginine in the pre-helix loop, but their low-activity counterparts (mouse BMP15 and human GDF9) have a glycine or a proline instead. To address the question of whether the arginine residue defines the different activities of GDF9 and BMP15 homodimers and their heterodimers in human and mouse, we used site-directed mutagenesis to change the species-specific residues in human and mouse proteins, and examined their activities in our in vitro assays. Although amino acid 72 of mature GDF9 is responsible for altered homodimer bioactivities, neither the corresponding BMP15 amino acid 62 nor the intact pre-helix loop is indispensable for BMP15 homodimer activity. However, amino acid 72 in GDF9 only has only subtle effects on GDF9:BMP15 heterodimer activity. Based on previous studies and our recent findings, we provide hypothetical models to understand the molecular mechanism to define activities of the homodimeric and heterodimeric ligands. The arginine residue in the pre-helix loop of GDF9 homodimer may prevent the inhibition from its pro-domain or directly alter receptor binding, but this residue in GDF9 does not significantly affect the heterodimer activity, because of suggested conformational changes during heterodimer formation.**

*follicular development, growth factors, ovary*

## INTRODUCTION

The transforming growth factor β (TGFβ) superfamily is the largest family of secreted proteins in mammals, and members of the TGFβ family are involved in most developmental and physiological processes [1, 2]. Oocyte-secreted GDF9 and BMP15 are closely related paralogs in the superfamily and play significant roles in ovarian follicular development [3]. However, the specific contributions of BMP15 and GDF9 differ among species. In mice, *Gdf9* is required for the transit from primary to secondary follicle [4], whereas *Bmp15* null mutants only exhibit slightly decreased ovulation rates and litter sizes due to defects in cumulus expansion [5]. In sheep, homozygous *BMP15* mutants ($FecX^I$ and $FecX^H$) are infertile because of a block of folliculogenesis at the primary stage, whereas sheep heterozygous for these BMP15 mutations have an increased rate of ovulation [6]. A naturally occurring point mutation in sheep GDF9 ($FecG^H$) produces a similar phenotype to the BMP15 mutants in the homozygous state [7].

Most members of the TGFβ family function as covalently linked homodimers or heterodimers to bind a heteromeric complex of type 1 and type 2 serine/threonine kinase receptors, leading to phosphorylation of downstream SMAD proteins. Unlike most TGFβ family ligands, GDF9 and BMP15 do not have the conserved cysteine that forms an intermolecular disulfide bond between the two monomers. Accordingly, previous studies have identified noncovalently linked homodimers and heterodimers of GDF9 and BMP15 by chemical crosslinking and immunoprecipitation [8]. Recently, our group has further demonstrated that mouse and human GDF9:BMP15 heterodimers have much higher activity compared with active homodimers [9]. These findings indicate the in vivo synergistic roles of GDF9 and BMP15 proteins in regulating ovarian functions, and explain diverse phenotypes caused by *GDF9* and/or *BMP15* among different animal models and humans.

GDF5, another TGFβ superfamily member, plays an important role in skeletal and joint development [10]. A single amino acid (R57) located in the pre-helix loop of GDF5 is responsible for its type 1 receptor specificity and biological activity [11]. Interestingly, bioactive mGDF9 and hBMP15 share the same pre-helix loop arginine (R72 and R62) as GDF5; however, their inactive counterparts (hGDF9 and mBMP15) contain a glycine or a proline at the same position (Fig. 1A). To identify how this residue defines the ligand

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A

| Ligand | Pre-helix Loop | Activity |
|---|---|---|
| GDF5 | 52 CEFPLRSHLEPT 63 | Yes |
| mGDF9 | 67 CPRAVRHRYGSP 78 | Yes |
| hGDF9 | 67 CPRAVGHRYGSP 78 | No |
| hBMP15 | 57 CLRVLRDGLNSP 68 | Yes |
| mBMP15 | 57 CTRVLPYGLNSP 68 | No |

B

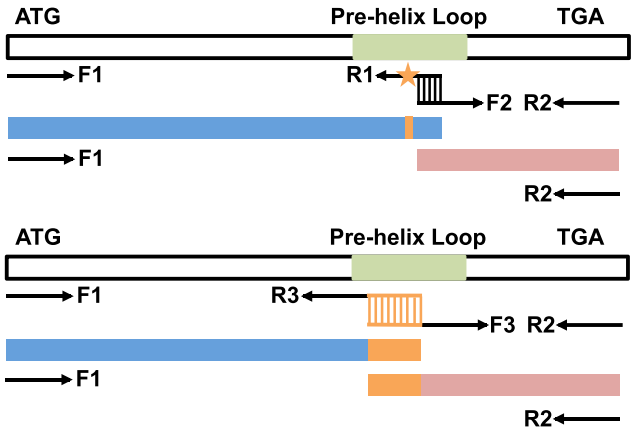


FIG. 1. Amino acid sequence alignment of the pre-helix loop and site-directed mutagenesis to generate mutant ligands. **A**) Bioactive mGDF9 and hBMP15 share the same pre-helix loop arginine (red) with GDF5, whereas low-activity ligands hGDF9 and mBMP15 contain a glycine (blue) or a proline (blue) at the same position. **B**) To mutate a single amino acid, a triple-nucleotide mutation (orange star) was designed in a reverse primer 1 (R1). Two PCR products were generated by two separate reactions with primer pairs F1 and R1 (blue box), or F2 and R2 (red box). There are 10 complementary nucleotides (black stripes) located at the 5′ ends of R1 and F2 to ensure the overlap extension PCR with F1 and R2. To mutate the intact pre-helix loop, the mutated complementary nucleotides (orange stripes) were designed at the 5′ ends of R3 and F3. Two PCR products were generated by two separate reactions with F1 and R3 (blue box), or F3 and R2 (red box). Overlap extension PCR with F1 and R2 was performed to obtain the full length of the mutant sequence.

bioactivity in GDF9 and BMP15 in different species, we used site-directed mutagenesis to generate mutant ligands and compared their activities with those of wild-type ligands in our in vitro mouse/human granulosa cell assays.

## MATERIALS AND METHODS

### Construction of Expression Plasmids

Plasmids containing recombinant GDF9 and BMP15 as described previously [9] were used as templates. Site-directed mutagenesis was used to generate mutant ligands (Fig. 1). For single-amino acid mutation in GDF9 and BMP15 (Fig. 1A), a triple-nucleotide mutation was designed in a reverse primer (R1). Two PCR products were generated by two separate reactions with primer pairs forward (F) 1 and R1 or F2 and R2. Ten complementary nucleotides located at the 5′ ends of R1 and F2 ensured the subsequent overlap extension PCR with F1 and R2. For the intact pre-helix loop switch in BMP15 (Fig. 1B), the mutated complementary nucleotides (from the first differing residue 58 to the last differing residue 63) were designed at the 5′ ends of R3 and F3. Two PCR products were generated by two separate reactions with F1 and R3 or with F3 and R2. Overlap extension PCR with F1 and R2 was performed to obtain the full length of the mutant sequence. FLAG-tagged mutant GDF9 and BMP15 sequences were cloned into pEFIRES-P to express homodimers. MYC-tagged mutant hGDF9 G72R and FLAG-tagged wild-type hBMP15 were cloned into pCEBud4.1 (Invitrogen) to express heterodimers. All sequences engineered in the aforementioned expression plasmids were confirmed by DNA sequencing.

### Transfection and Selection of Stable Cell Clones

HEK-293T cells were obtained from the tissue culture core at Baylor College of Medicine. All plasmids were transfected into HEK-293T cells using Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer's instructions. Two days after transfection, cells transfected with pEFIRES-P were selected with 5 μg/ml puromycin (Invitrogen), and cells transfected with pCEBud4.1 were selected with 500 μg/ml zeocin (Invitrogen). Individual puromycin- and zeocin-resistant cell colonies were isolated 2 wk after transfection.

### Ligand Purification and Quantification

Ligand purification and quantification were performed as described previously [9]. Briefly, FLAG-tagged wild-type and mutant homodimers were purified by anti-FLAG M2 affinity gel (Sigma-Aldrich) according to the manufacturer's protocol. The purified FLAG-tagged ligands were quantified by Western blot analysis using FLAG-bacterial alkaline phosphatase standards (Sigma-Aldrich) and mouse anti-FLAG M2 antibody (Sigma-Aldrich). Wild-type and mutant hGDF9 G72R (MYC-tagged):hBMP15 (FLAG-tagged) heterodimers were immunoprecipitated by the same method. The purified wild-type and mutant MYC-tagged hGDF9 were quantified using FLAG-mGDF9 standards with an anti-GDF9 monoclonal antibody.

### Mouse Granulosa Cell Isolation

Mice used in this study were maintained on a mixed C57BL/6/129S6/SvEv genetic background and handled according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. The isolation of mouse granulosa cells was described previously [9]. Briefly, 3-wk-old female mice were injected with 5 IU of equine chorionic gonadotropin, and ovaries were harvested 44–46 h after injection. Mouse granulosa cells were released by puncturing large antral follicles, and then filtered through a 40-μm nylon cell strainer to remove oocytes.

### Mouse and Human Granulosa Cell Assays

Mouse and human granulosa cell assays were described previously [9]. For gene induction analysis, mouse granulosa cells were treated with ligands for 5 h, and total RNA was extracted immediately after treatment. Real-time PCR was conducted to test the fold changes of *Ptx3*, *Has2*, and *Ptgs2*. For SMAD activation analysis, COV434 cells (immortalized human juvenile granulosa cells, a gift from Dr. Stephanie Pangas, Baylor College of Medicine) were treated with ligands for 1 h and lysed with RIPA buffer. SMAD2/3 phosphorylation was detected by Western blot analysis with anti-P-SMAD2/3 (Cell Signaling Technology). Actin was used as the internal control, detected by a monoclonal actin antibody (Sigma-Aldrich). For the inhibitor assay, COV434 cells were treated with ligands and coincubated with/without 100 nM ALK2/3/6 inhibitor LDN-193189 (gift from Dr. Randall Peterson, Harvard Medical School) or 1 μM ALK4/5/7 inhibitor SB-505124 (Sigma-Aldrich) for 1 h.

### Mouse Cumulus Expansion Assay

In vitro mouse cumulus expansion assays were performed as described previously [12]. Briefly, oocytes were removed from oocyte-cumulus cell complexes. The resulting oocytectomized (OOX) complexes consisted of the spherical zona pellucida surrounded by the cumulus cell mass without the oocyte. Ten OOX complexes were treated with ligands in the presence of 10 ng/ml epidermal growth factor (EGF). After 15 h of incubation, the cumulus expansion index was scored based on the degree of OOX cumulus cell expansion using a scale from 0 (no expansion) to 4 (full expansion).

### Statistical Analysis

All experiments presented in this study were repeated at least three times independently. Differences among groups were analyzed for statistical significance by using one-way ANOVA followed by Tukey multiple comparison test. The data represent the mean ± SEM, and $P < 0.05$ was considered to be statistically significant.

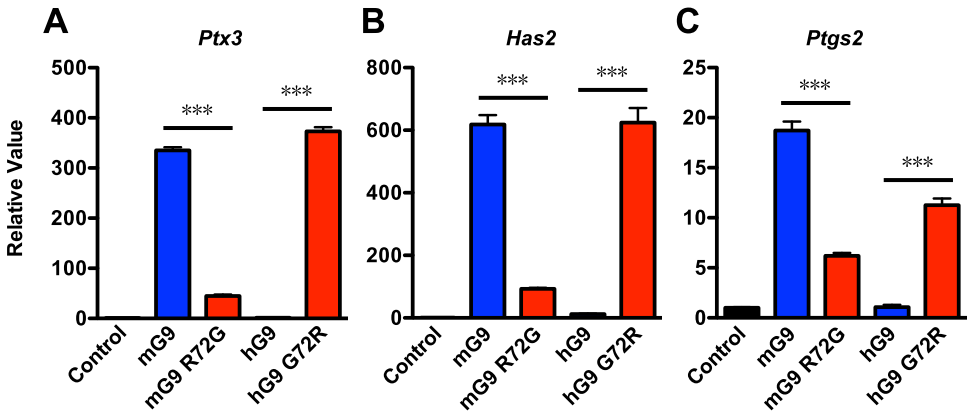


FIG. 2. Testing wild-type and mutant GDF9 activities in downstream ECM gene regulation. Mouse granulosa cells were collected and treated with no ligand (Control), mGDF9 (mG9) 100 ng/ml, mGDF9 R72G (mG9 R72G) 100 ng/ml, hGDF9 (hG9) 100 ng/ml, and hGDF9 G72R (hG9 G72R) 100 ng/ml for 5 h. Total RNA was extracted from treated granulosa cells, and *Ptx3* (**A**), *Has2* (**B**), and *Ptgs2* (**C**) were quantified by quantitative PCR. All panels show mean ± SEM (n = 3), ***$P < 0.001$. R72G, protein with an arginine (R)-to-glycine (G) mutation at amino acid 72 of the mature sequence. G72R, protein with a glycine (G)-to-arginine (R) mutation at amino acid 72 of the mature GDF9 sequence.

## RESULTS

### *The Arginine Residue in the GDF9 Pre-Helix Loop Is Responsible for Homodimer Activity*

In response to the ovulatory luteinizing hormone surge, cumulus cells expand and produce a complex extracellular matrix (ECM). This highly coordinated process is known as ''cumulus expansion'' [13, 14]. Several ECM genes expressed in granulosa cells are involved in cumulus expansion, including hyaluronan synthase 2 (*Has2*) [15, 16], pentraxin 3 (*Ptx3*) [17], and prostaglandin synthase 2 (*Ptgs2*) [18, 19]. In our mouse granulosa cell assay, we quantified activities of wild-type and mutant ligands according to their ability to regulate expression of the three aforementioned genes.

To identify the possible importance of the arginine in GDF9 pre-helix loop, we compared the activities of the mutant ligands with single-amino acid change (mGDF9 R72G and hGDF9 G72R) to wild-type m/hGDF9 in inducing *Ptx3*, *Has2*, and *Ptgs2* mRNAs (Fig. 2). mGDF9 potently upregulated transcription of the three ECM genes (*Ptx3*, *Has2*, and *Ptgs2*), whereas hGDF9 had minimal activity in the same assays. Introduction of a single-amino acid change (R72G) into mGDF9 dramatically decreased activity compared with wild-type mGDF9. Alternatively, introducing the G72R mutation into hGDF9 significantly increased its activity in stimulating expression of the three ECM genes. These results indicated that changing the pre-helix loop arginine to a glycine in mGDF9 resulted in significant reduction of bioactivity, whereas changing the pre-helix loop glycine to an arginine in hGDF9 led to a gain of activity. Thus, the single residue (R72) in the pre-helix loop of GDF9 is responsible for the homodimer activity.

### *The Corresponding Arginine in the BMP15 Pre-Helix Loop Is Dispensable for Homodimer Activity*

We next tested the corresponding residue in the pre-helix loop of BMP15 using the same assays (Fig. 3, A–C). mBMP15 showed no activity in regulating the transcription of the three ECM genes (*Ptx3*, *Has2*, and *Ptgs2*), whereas hBMP15 significantly upregulated the three gene transcripts. However, changing the single residue in the pre-helix loop of mBMP15 and hBMP15 did not decrease their activities compared to wild type. Actually, we observed slightly increased activity for the mutant hBMP15 in our *Ptx3* expression assay. Thus, mBMP15 P62R remained inactive, whereas hBMP15 R62P maintained activity similar to that of wild-type hBMP15 (Fig. 3, A–C).

The pre-helix loop is more conserved in GDF9 than in BMP15 (Fig. 1A). In GDF9, R72/G72 residue is the only nonconserved residue through the whole pre-helix loop. In contrast, BMP15 contains two additional unique amino acids besides P62/R62 in its pre-helix loop. To test if other residues in the pre-helix loop contribute to BMP15 activity, we switched the intact pre-helix loop between mBMP15 and hBMP15 and compared their activities to wild-type ligands in the same assays (Fig. 3, D–F). Changing the entire pre-helix loop between mBMP15 and hBMP15 did not alter their activities (Fig. 3, D–F). Unlike GDF9, neither the arginine residue nor the intact pre-helix loop in BMP15 is exclusively required for homodimer activity.

### *The Arginine Residue in the Pre-Helix Loop Only Has Subtle Effects on GDF9:BMP15 Heterodimer Activity*

Our previous studies demonstrated that human GDF9 and BMP15 form a noncovalently linked heterodimer, and this heterodimer is far more biopotent than the active hBMP15 homodimer [9]. To test if the pre-helix loop arginine contributes to heterodimer activity, we generated mutant human heterodimer protein with the G72R single-residue change in the hGDF9 monomer, and examined its activity in dose-response mouse granulosa cell assays (Fig. 4, A–C). We already demonstrated that human heterodimer is 1000 ~ 3000-fold more biopotent than hBMP15 in our reported results [9]. Consistent with the previous findings, here we observed that 0.1 ng/ml hGDF9:BMP15 had activity comparable to that of 100 ng/ml hBMP15 homodimer in upregulating the three cumulus expansion-related transcripts. Furthermore, 0.03 ng/ml mutant human heterodimer had activity similar to 0.1 ng/ml wild-type human heterodimer in the same assays, suggesting that there is approximately 3-fold increased activity between the mutant and wild-type heterodimers.

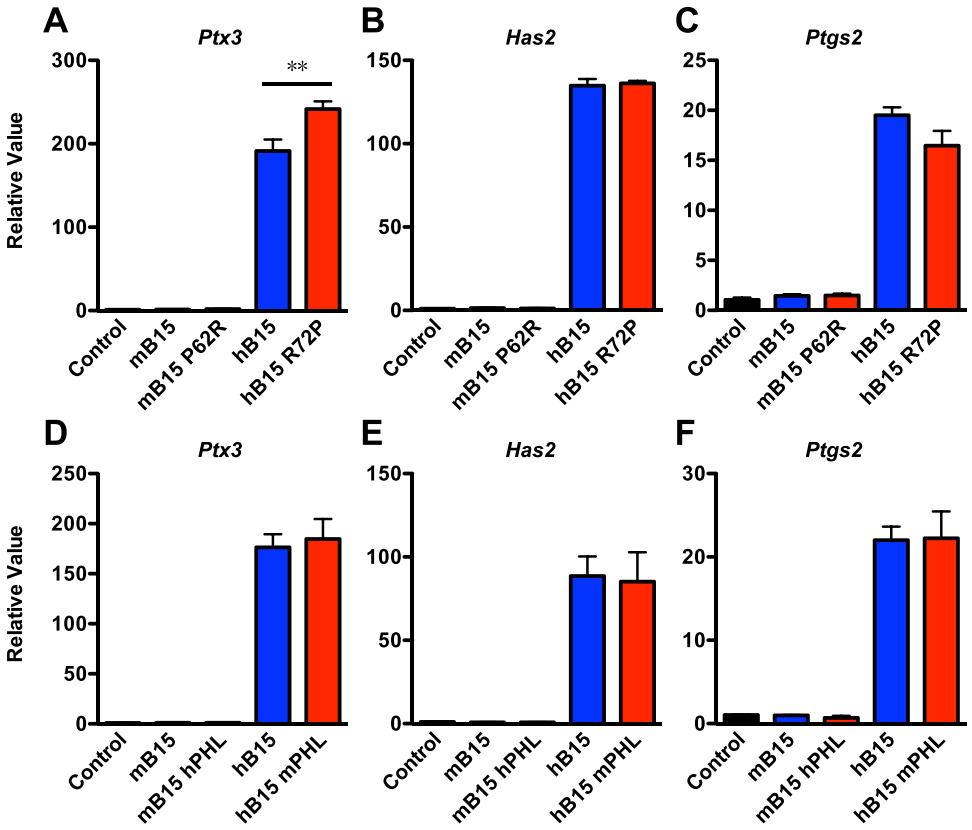


FIG. 3. Testing wild-type and mutant BMP15 activities in downstream ECM gene regulation. Mouse granulosa cell were collected and treated with no ligand (Control), mBMP15 (mB15) 100 ng/ml, mBMP15 P62R (mB15 P62R) 100 ng/ml, hBMP15 (hB15) 100 ng/ml, hGDF9 R62P (hB15 R72P) 100 ng/ml, mBMP15 hPHL (mB15 hPHL) 100 ng/ml, and hBMP15 mPHL (hB15 mPHL) 100 ng/ml for 5 h. Total RNA was extracted from those granulosa cells, and *Ptx3* (**A** and **D**), *Has2* (**B** and **E**), and *Ptgs2* (**C** and **F**) were quantified by qPCR. All panels show mean ± SEM (n = 3), $^{**}P < 0.01$. P62R, protein with a proline (P)-to-arginine (R) mutation at amino acid 62 of the mature sequence. R62P, protein with an arginine (R)-to-proline (P) mutation at amino acid 62 of the mature BMP15 sequence. PHL, pre-helix loop.

### *Mutant Ligand Signaling in OOX Cumulus Cell Expansion Assays Is Similar to Signaling in Mouse Granulosa Cell Assays*

We further tested the activities of the mutant ligands to promote the full process of mouse cumulus expansion by in vitro OOX cumulus cell expansion assays. In the presence of EGF, OOX cumulus cells were treated with wild-type and mutant homodimers, or a 1:10 serial dilution of wild-type and mutant heterodimer. As opposed to our findings in mouse granulosa assays, mGDF9 R72G exhibited similar ability to stimulate OOX cumulus cell expansion compared with wild-type mGDF9. Because in vitro mouse cumulus expansion requires at least two major signaling pathways—cumulus-expansion enabling factors secreted from the oocyte, and EGF or follicle-stimulating hormone [12]—the low bioactivity of the mutant mGDF9 in simulating ECM gene expression could be compensated by another growth factor. Alternatively, the limited ECM gene expression upregulated by the mutant mGDF9 could be sufficient to stimulate cumulus expansion. Consistent with our previous observation (Fig. 2), hGDF9 G72R was far more potent when compared with wild-type hGDF9 (Fig. 4D). Dose-response experiments revealed no significant differences between wild-type and the mutant human heterodimers among the five concentration gradients (Fig. 4E).

### *Human Granulosa Cell Assays Confirmed the Activities of Mutant Ligands and Defined Their Signaling Pathways*

To validate our conclusions in human granulosa cells, we tested the activities of mutant ligands in stimulating downstream SMAD2/3 phosphorylation in an immortalized human juvenile granulosa cell line (COV434). mGDF9 R72G had very low activity in its ability to trigger SMAD2/3 phosphorylation, whereas hGDF9 G72R was biopotent and comparable to that of mGDF9 (Fig. 5A). In dose-response experiments, wild-type and mutant human heterodimer showed similar activity in inducing SMAD2/3 phosphorylation among the three concentration gradients (Fig. 5B). We further investigated the signaling pathway(s) used by our mutant ligands by examining the type 1 receptor(s) that are involved in triggering downstream SMAD signaling. Inhibitors specific to particular type 1 receptor subgroups were coincubated with our ligands in the human granulosa cell assays (Fig. 5, C and D). LDN-193189 is an inhibitor of ALK2/3/6 [20, 21], and SB-505124 is an inhibitor of ALK4/5/7 [22]. SB-505124 abolished the activities of mGDF9, hGDF9 G72R, hGDF9:BMP15, and hGDF9 G72R:BMP15 in inducing SMAD2/3 phosphorylation (Fig. 5C). In contrast, LDN-193189 had no effect on the ability of these ligands to phosphorylate SMAD2/3 (Fig. 5D). This result indicated that mutant human GDF9 and GDF9:BMP15 heterodimer signal via the same type 1 receptor as wild-type ligands.

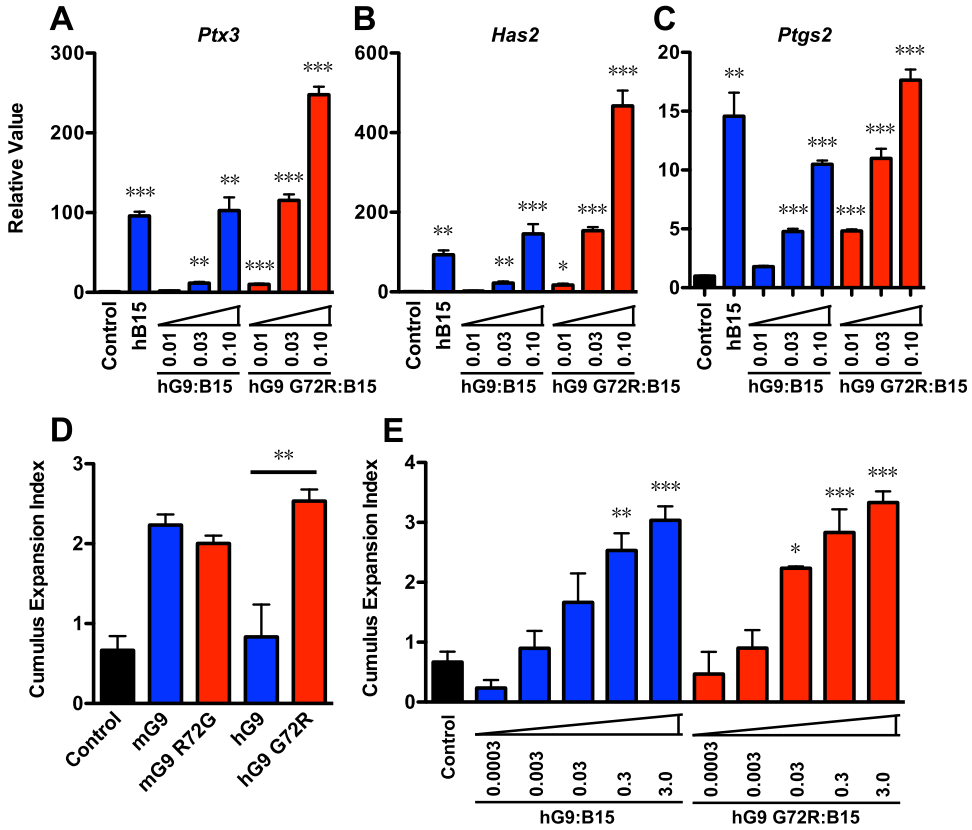


FIG. 4. Wild-type and mutant GDF9:BMP15 dose-dependent effects in downstream ECM gene regulation and OOX cumulus cell expansion. Mouse granulosa cells were collected and treated with no ligand (Control), 100 ng/ml hBMP15 (hB15), or a 1:3 serial dilution (0.01, 0.03, and 0.1 ng/ml) of hGDF9:BMP15 (hG9:B15) and hGDF9 G72R:BMP15 (hG9 G72R:B15) for 5 h. Total RNA was extracted from those granulosa cells, and *Ptx3* (**A**), *Has2* (**B**), and *Ptgs2* (**C**) were quantified by quantitative PCR. In the presence of 10 ng/ml EGF, OOX cumulus cells were treated with 30 ng/ml wild-type and mutant homodimers (**D**), or a 1:10 serial dilution (0.0003, 0.003, 0.03, 0.3, and 3.0 ng/ml) of hGDF9:BMP15 (hG9:B15) and hGDF9 G72R:BMP15 (hG9 G72R:B15; **E**) to test their activities in OOX cumulus cell expansion. All panels show mean ± SEM (n = 3), $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared to control. R72G, protein with an arginine (R)-to-glycine (G) mutation at amino acid 72 of the mature GDF9 sequence. G72R, protein with a glycine (G)-to-arginine (R) mutation at amino acid 72 of the mature GDF9 sequence.

## DISCUSSION

Oocyte-secreted GDF9 and BMP15 are essential regulators of ovarian folliculogenesis in mammals. In mouse and human, only one of the two ligands shows bioactivity in the current and previous in vitro studies [9]. An arginine reside (R57) in the pre-helix loop of GDF5 confers its receptor specificity and bioactivity [11]. Although the corresponding arginine residue was found in both mGDF9 and hBMP15, we demonstrate herein that it is only responsible for the high bioactivity of mGDF9 homodimer and is dispensable for hBMP15 homodimer. Furthermore, we also examined the effect of the arginine residue on a more potent ligand, human GDF9:BMP15 heterodimer, and conclude that its contribution is subtle in this human heterodimer. The mutant human heterodimer only has slightly increased activity to upregulate ECM genes, but there is no significant difference between the wild-type and mutant human heterodimers to promote the full process of mouse cumulus expansion.

In the TGFβ superfamily, ligands are first synthesized as pre-pro-peptide precursors and secreted as inactive dimeric pro-peptides, where pro-domains are removed from mature domains by furin or furin-like protease [1]. Even after proteolytic processing, the pro-domains of many TGFβ ligands noncovalently associate with the mature domains to form latent complexes. Previous studies have already demonstrated that the pro-domains of TGFβ [16], myostatin [23], and BMP2 [24] shield the ligands from recognition by their receptors and alter their conformations. In the case of GDF9, hGDF9 can also be secreted as a latent complex with its pro-domain to block its binding with receptors [16]. Changing a single residue in the pre-helix loop of hGDF9 results in increased affinity to its signaling receptors, confirming that the single residue confers the bioactivity of hGDF9 [25], similar to our studies.

The distinct activities of GDF9 and BMP15 homodimers and heterodimers could be based on direct interactions of the pre-helix loop with the receptor. However, consistent with a previously proposed model [25], a model could be proposed that includes a role for the pro-domain to explain the importance of the pre-helix loop arginine residue in these various ligands (Fig. 6). In mGDF9, the pre-helix domain arginine prevents the interaction between the ligand and its pro-domain to maintain the high activity (Fig. 6A). When the arginine is replaced by a glycine, the mutant mGDF9 shows high affinity to the pro-domain to form a latent complex (Fig. 6B). hGDF9 has a glycine in the pre-helix loop, and it is trapped by the pro-domain to inhibit its activity (Fig. 6C). When the glycine is changed to an arginine, the mutant hGDF9 is released from the pro-domain and gains high activity (Fig. 6D). However, the formation of the human heterodimer could be in a different conformation, which attenuates the inhibition from the pro-domain and increases affinity to its unique signaling receptor complex (including ALK4/5/7, ALK6, and BMPR2; Fig. 6E). Alternatively, it could be that the pro-

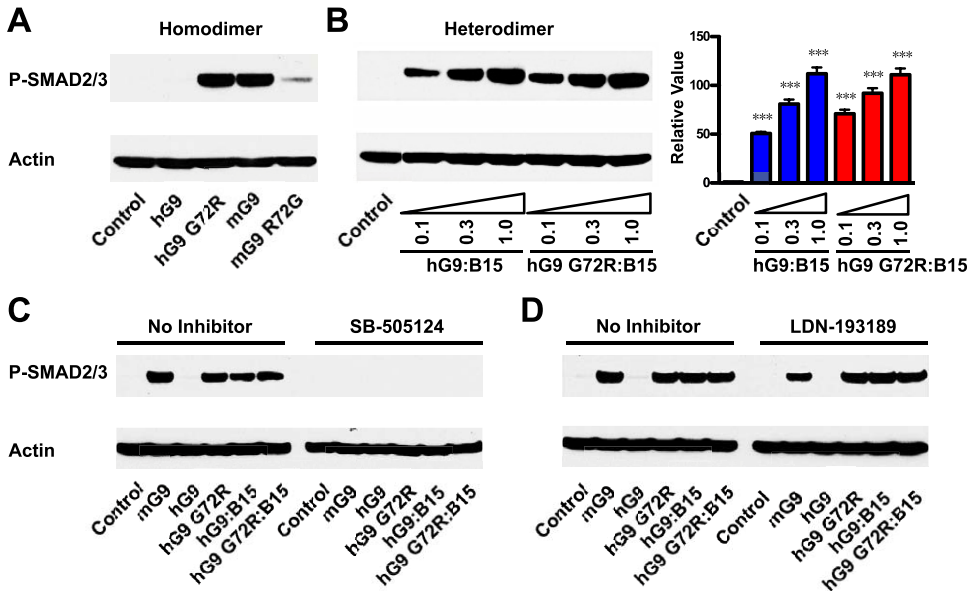


FIG. 5. Identification of mutant GDF9 and GDF9:BMP15 SMAD signaling pathway in COV434. **A**) COV434, an immortalized human juvenile granulosa cell line, was treated with 100 ng/ml hGDF9 (hG9), mutant hGDF9 (hG9 G72R), mGDF9 (mG9), or mutant mGDF9 (mG9 R72G) for 1 h. Anti-P-SMAD2/3 was used to detect the P-SMAD2/3 signaling pathways. **B**) COV434 was treated with a 1:3 serial dilution (0.1, 0.3, and 1.0 ng/ml) of wild-type or mutant hGDF9:BMP15 to test the dose-dependent effect on SMAD2/3 phosphorylation. Western blots were quantified by ImageJ, and the data are shown as the mean ± SEM (n = 3), $***P < 0.001$ compared with no-ligand control. **C** and **D**) A total of 100 nM LDN-193189 (ALK2/3/6 inhibitor) or 1 μM SB-505124 (ALK4/5/7 inhibitor) was coincubated with the ligands to test whether the induction of SMAD phosphorylation was abolished compared with no-inhibitor controls. Actin was used as the internal control. R72G, protein with an arginine (R)-to-glycine (G) mutation at amino acid 72 of the mature GDF9 sequence. G72R, protein with a glycine (G)-to-arginine (R) mutation at amino acid 72 of the mature GDF9 sequence.

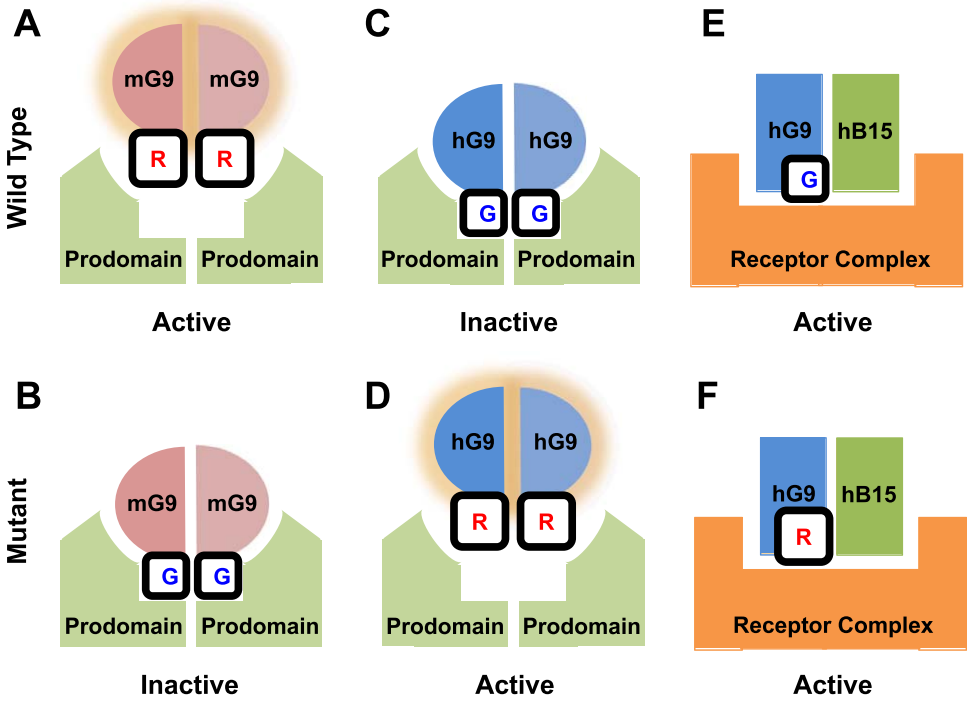


FIG. 6. Models of pro-domain-ligand complexes for BMP15 and GDF9 homodimers and GDF9:BMP15 heterodimers. **A**) The pre-helix domain arginine could prevent the interaction between mGDF9 (mG9) and the pro-domain to maintain the high activity of the homodimer. **B**) When the arginine is replaced by a glycine, the mutant mGDF9 binds to the pro-domain to form a latent complex. **C**) hGDF9 (hG9) has a glycine in the pre-helix loop, and it interacts with the pro-domain to form a pro-domain-ligand complex. **D**) When the glycine is replaced by an arginine, the mutant hGDF9 is released from the pro-domain to gain activity. **E**) The formation of hGDF9:BMP15 heterodimer (hG9:hB15) changes the conformation of the ligand, which blocks the inhibition from the pro-domain and also increases affinity to its unique signaling receptor complex. **F**) Changing the glycine to an arginine in hGDF9:BMP15 does not significantly affect its activity, because the interaction between the heterodimer and the pro-domain has already been disrupted by the conformational change of the heterodimer. Alternatively, the heterodimer is more active because the GDF9:BMP15 ligand binds to the ALK4/5/7-ALK6-BMPR2 complex in an optimized state for phosphorylation of downstream SMAD2/3 proteins.

domain of hGDF9 does not interact well with the hBMP15 monomer of the heterodimer, because it is expected for the dimeric pro-domain to interact with the two adjacent monomers of the homodimer. Thus, switching the glycine to an arginine in hGDF9:BMP15 only has subtle effects on its activity because the interaction between the heterodimer and the pro-domain is largely disrupted by the conformational change of the heterodimer (Fig. 6F).

In our previous study, we demonstrated that mouse and human GDF9:BMP15 heterodimers signal via a unique receptor complex that includes the type 2 receptor BMPR2, an ALK4/5/7 type 1 kinase receptor, and an ALK6 type 1 coreceptor. A number of heterodimeric ligands in the TGFβ superfamily have novel functions or enhanced potency compared with the homodimers [1]. In zebrafish, BMP2 and BMP7 homodimers and BMP2:BMP7 heterodimers are found in the embryo, but only the heterodimers can stimulate BMP signaling to mediate embryonic dorsoventral patterning [26]. In the same study, two classes of type 1 receptors, ALK3/6 and ALK8 (ortholog of ALK2 in mammals), were identified in the receptor complex for BMP2:BMP7 heterodimers. Although none of the heterodimeric ligand-bound receptor complexes in the superfamily have been structurally determined, it is reasonable to speculate that these heterodimeric ligands have higher affinity to their unique signaling receptor complexes in order to achieve higher or novel bioactivity.

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