

Effect of Intracellular Interactions on the Processing and Secretion of Bone Morphogenetic Protein-15 (BMP-15) and Growth and Differentiation Factor-9

IMPLICATION OF THE ABERRANT OVARIAN PHENOTYPE OF BMP-15 MUTANT SHEEP*

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Bone morphogenetic protein-15 (BMP-15) and growth and differentiation factor-9 (GDF-9) are members of the transforming growth factor- β superfamily. Both molecules are closely related in their primary structures and share a nearly identical spatiotemporal expression pattern in the oocyte during folliculogenesis in mammals. Here we have established a series of cell lines, which express recombinant BMP-15, GDF-9, or both, and investigated whether they form homodimers and/or heterodimers. We demonstrate the first evidence that both BMP-15 and GDF-9 can form non-covalent homodimers when expressed individually, while when both are co-expressed BMP-15/GDF-9 heterodimers are produced. Interestingly, when GDF-9 and BMP-15 are co-expressed the processing of both proproteins are significantly impaired as compared with that of the singly expressed proproteins, suggesting that the proprotein heterodimer is less susceptible to proteolytic cleavage than the individual homodimers. Since BMP-15 mutant sheep, called Inverdale, exhibit severe defects in ovarian function we have also established stable transformants expressing the mutant BMP-15 (InvBMP-15) alone or together with GDF-9. Although InvBMP-15 was previously predicted to be unable to form homodimers, we show here that it does form non-covalent dimers; however, the processing efficiency of InvBMP-15 proprotein is significantly lower than wild-type BMP-15. Surprisingly, when GDF-9 is co-expressed, the processing and secretion of InvBMP-15 is abolished, and the processing of GDF-9 is also severely impaired, suggesting that the heterodimers of InvBMP-15/GDF-9 proproteins are not susceptible to proteolytic cleavage and thus degrade in the cells. Based on these findings we propose a novel hypothesis that a decrease in GDF-9 secretion may be involved in causing infertility in homozygous Inverdale ewes.

Two structurally similar and oocyte-derived growth factors termed growth and differentiation factor-9 (GDF-9)¹ (1, 2) and bone morphogenetic protein-15 (BMP-15, also called GDF-9B) (3, 4) have recently been identified by adapting a homology-based PCR cloning and *in silico* (data base search) cloning strategies using conserved amino acid sequences of BMP subfamily members of the transforming growth factor- β (TGF- β) superfamily. Like other members of the TGF- β superfamily, GDF-9 and BMP-15 are synthesized as preproteins comprised of a signal peptide, a prodomain and a mature (fully processed bioactive)-domain (1, 3, 4). Members of the TGF- β superfamily possess a proteolytic cleavage site(s) between the pro and mature domain where a specific protease binds and separates these two domains as an important component of the post-translational processing of these molecules (5). Importantly, all of the evidence to date shows that for the proteolytic cleavage of TGF- β superfamily member proproteins to occur, the proproteins must first dimerize (6). A characteristic feature of most TGF- β superfamily members is the presence of seven conserved Cys residues in the mature region, the fourth of which forms the intersubunit disulfide bond responsible for the covalent linkage of the two subunits (7). BMP-15 and GDF-9 are unique in that the fourth conserved Cys is substituted by a Ser, thus, it is not known whether these molecules exist as monomers or form non-covalent dimers.

The crucial role of GDF-9 in follicle development and female fertility has recently been demonstrated by loss-of-function studies in mice in which it was shown that female mice lacking GDF-9 are infertile due to a block in folliculogenesis at the one-layer primary follicle stage (8). In contrast, female mice lacking BMP-15 are subfertile and exhibit only minimal ovarian histopathological defects (9). Therefore, it seems that BMP-15 is a less critical factor for normal folliculogenesis than GDF-9 in mice.

The recent discovery of naturally occurring mutations in the *bmp15* gene in domestic sheep termed *Fecundity X Inverdale* (*FecX^I*) and *Hanna*, (*FecX^H*) has provided a new insight into understanding the physiological relevance of BMP-15 in female reproduction (10). In *FecX^I* ewes, a T-A transition at nucleotide 92 substitutes a Val with an Asp at residue 31 of the mature protein. In *FecX^H* ewes, a C-T transition in nucleotide 67 of the *bmp15* gene replaces a Glu by a stop codon at amino acid

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¹ The abbreviations used are: GDF-9, growth and differentiation factor-9; Ab, polyclonal antibody; BMP-15, bone morphogenetic protein-15; BS³, bis-sulfosuccinimidyl suberate; CDMP-1, cartilage-derived morphogenetic protein-1; CM, conditioned media; RT-PCR, reverse transcription-polymerase chain reaction; TGF- β , transforming growth factor- β ; mAb, monoclonal antibody.

residue 23 of the mature domain of BMP-15, thus resulting in the synthesis, if any, of a very short peptide, which is highly unlikely to be biologically active. Both mutant ewes exhibit increased ovulation and lambing rates in the heterozygotes, whereas the homozygous mutant ewes are infertile with a phenotype that closely resembles that of GDF-9 knockout mice (10).

Therefore, there is a distinct difference in phenotypes between mice and sheep having *bmp15* gene mutations. A major difference between mice and sheep with homozygous mutations in the *bmp15* gene is that in BMP-15 mutant sheep, GDF-9 is incapable of rescuing the defects in early follicular development, whereas the progression of the ovarian follicles through the component stages of folliculogenesis occurs quite normally in BMP-15 knockout mice, suggesting that, in mice, GDF-9 may be sufficient to compensate for the lack of BMP-15 in promoting folliculogenesis (8–10). There has been speculation as to how the differences in the phenotype of the BMP-15 mutant mice and sheep can be explained. One hypothesis is that by virtue of species differences GDF-9 plays a dominant and necessary role in mouse folliculogenesis with BMP-15 providing a dispensable supporting role, whereas in sheep BMP-15 is indispensable. It has been noted that these differences may be associated with the poly *versus* mono-ovulatory nature of mice and sheep, respectively (11). Here, we have developed an alternative hypothesis by focusing on the difference in the point mutations in the *bmp15* gene observed in sheep as compared with the deletion of the entire second exon of the *bmp15* gene in knockout mice. In this hypothesis we propose that the relative roles of intact GDF-9 and BMP-15 could be similar in mice and sheep, but that the *FecX^I* and the *FecX^H* mutations in the BMP-15 protein may be able to affect GDF-9 biosynthesis in a dominant-negative fashion, possibly manifested through BMP-15/GDF-9 heterodimer formation, whereas the deletion of the entire second exon of the *bmp15* gene in the knockout mouse has no effect on regulating GDF-9 biosynthesis; thus the differences in GDF-9 bioavailability may account for the differences in the phenotype caused by the single point mutations in BMP-15 in sheep *versus* the entire deletion of the second exon of the *bmp15* gene in mice. In the present study, as a first step in evaluating these hypotheses, we investigate the intracellular interactions of BMP-15 (wild-type and mutant) and GDF-9 and characterize the impact of these interactions on the processing and secretion of these growth factors.

MATERIALS AND METHODS

Reagents and Supplies—Fetal bovine serum, penicillin, streptomycin, L-glutamine, and zeocin were purchased from Invitrogen (San Diego, CA). Anti-FLAG M2 monoclonal antibody (anti-FLAG mAb), Anti-FLAG M2 affinity gel, and Triton X-100 were purchased from Sigma-Aldrich Co. Anti-c-Myc polyclonal antibody (anti-Myc Ab) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All restriction enzymes were purchased from New England Biolabs Inc. (Beverly, MA).

Construction of Expression Plasmids—Human GDF-9 cDNA was amplified by reverse transcription-PCR (RT-PCR) from ovarian RNA with a primer set encompassing the amino- and carboxyl termini of prepro-GDF-9 (GenBank™ accession no. NM_005260). A PCR product of expected size was cloned in-frame at the upstream site of the c-Myc epitope tag of the pcDNA3.1/Zeo(–) expression vector (Invitrogen). The GDF-9 cDNA with the Myc tag was then released by digestion with *NotI* and *KpnI*, subcloned into one of the multiple cloning sites of a dual expression vector, pBudCE4.1 (Invitrogen), to form a plasmid designated as phGDF-9M. Human BMP-15 cDNA fused with a FLAG epitope tag was released from the previously constructed phBMP-15-FLAG (12) by digestion with *SacII* and *PacI*, blunt-ended with T4 DNA polymerase and subcloned into the *ScaI* site of the other multiple cloning site of phGDF-9M. The resulting plasmid, designated as phBMP-15F/hGDF-9M, contains the full structure of human BMP-15 tagged with a FLAG epitope under the control of the cytomegalovirus promoter and human GDF-9 tagged with a c-Myc epitope under the control of the *EF-1 α*

promoter. We next performed standard PCR-based site-directed mutagenesis using phBMP-15F/hGDF-9M to make a plasmid that co-expresses intact GDF-9 and mutant BMP-15 having a single amino acid substitution of the Ile at position 31 of the mature region to an Asp (I31D), which mimics the mutation found in the Inverdale ewe. This plasmid was designated as phBMP-15F^{I31D}/hGDF-9M. To make plasmids that singly express BMP-15 or BMP-15^{I31D} we released the GDF-9 cDNA from phBMP-15F/hGDF-9M or phBMP-15F^{I31D}/hGDF-9M, respectively. The resulting plasmids were designated as phBMP-15F or phBMP-15F^{I31D}, respectively. The structures of all plasmids were confirmed by DNA sequencing.

Transfection and Cell Culture—Human embryonic kidney 293T cells (293T) were transfected with each expression plasmid using FuGENE 6 (Roche Molecular Biochemicals, Basel, Switzerland) according to the manufacturer's instructions. Cells containing the expression plasmid were selected under 1 mg/ml of zeocin and maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% fetal bovine serum, 100 μ g/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine (PSG), and 100 μ g/ml zeocin. When the cells reached 90–100% confluency, the medium was replaced by DMEM/F12 containing PSG without zeocin. After 3–4 days of the serum-free culture, the conditioned media (CM) were harvested.

SDS/PAGE Immunoblot Analysis and Chemical Cross-linking—For analysis of intracellular proteins, cells were washed with ice-cold phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride followed by lysis in radioimmune precipitation assay buffer (Upstate Biotechnology, Lake Placid, NY) containing Protease-Arrest (Geno Technology Inc., St Louis, MO), 1 mM EDTA, 2% SDS, and 4% β -mercaptoethanol. Cell lysates or CM in SDS sample buffer with 4% β -mercaptoethanol were then subjected to SDS/PAGE-immunoblotting using 12% polyacrylamide gels. Anti-FLAG Ab were used to detect BMP-15, and anti-Myc Ab were used to detect GDF-9. Where indicated, integrated relative densities of individual bands were digitized by multiplying the absorbance of the surface areas using the software package, NIH ImageJ 1.28.

For chemical cross-linking, a non-cleavable cross-linker, bis-sulfosuccinimidyl suberate (BS³) (Pierce, Rockford, IL) was added to CM at a final concentration which ranged from 0.125 to 5 mM, and incubated for 2 h on ice followed by SDS/PAGE immunoblotting analysis.

Immunoprecipitation—Anti-FLAG monoclonal antibodies conjugated to agarose beads (Anti-FLAG M2 affinity gel, 10 μ l) and Triton X-100 (0.1% final concentration) were added to 800 μ l of CM from BMP-15, BMP-15/GDF-9, or GDF-9 cell lines. After a 2-h incubation at 4 °C on a rotator, proteins bound to the agarose beads were eluted with SDS/PAGE sample buffer and subjected to SDS/PAGE immunoblotting analysis. For immunoprecipitation of intracellular proteins, BMP-15, BMP-15/GDF-9, or GDF-9 cells were grown to 100% confluency in 100-mm dishes followed by lysis in a buffer containing 1% Triton X-100, Protease-Arrest and 1 mM phenylmethylsulfonyl fluoride. Lysates were diluted 10-fold in phosphate-buffered saline, and immunoprecipitations were carried out as described above.

RNA Extraction and RT-PCR—RNA was extracted by guanidium acid-isothiocyanate-phenol-chloroform methods using TriZOL® (Invitrogen). After the treatment of RNA with DNase I (Promega, Madison, WI), cDNA was synthesized by reverse transcriptase (Invitrogen). PCR was performed using MgCl₂ (1.5 mM), dNTP (0.2 mM), and 2.5 units of Platinum TaqDNA polymerase (Invitrogen) under the following conditions: 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C (for GDF-9 and β -actin) or 60 °C (for BMP-15) for 30 s, and extension at 72 °C for 30 s.

Statistical Analysis—All SDS/PAGE immunoblotting data presented here are representative examples of at least three separate experiments. Differences in the ratios of the mature/protein in the CM are shown as mean \pm S.E. of at least three separate experiments. Differences between groups were analyzed for statistical significance using analysis of variance (SPSS Standard Version 10.0.1, SPSS Inc, Chicago, IL). A *p* value \leq 0.05 was considered to be statistically significant.

RESULTS

In order to evaluate whether there are intracellular interactions between BMP-15 and GDF-9 following translation (*i.e.* the formation of heterodimers), we established stable transformant 293T cell lines expressing either BMP-15 or GDF-9 alone or both BMP-15 and GDF-9 simultaneously. The pBudCE4.1 expression vector was used to ensure equal copy number of BMP-15 and GDF-9 genes in the co-transfected cell line be-

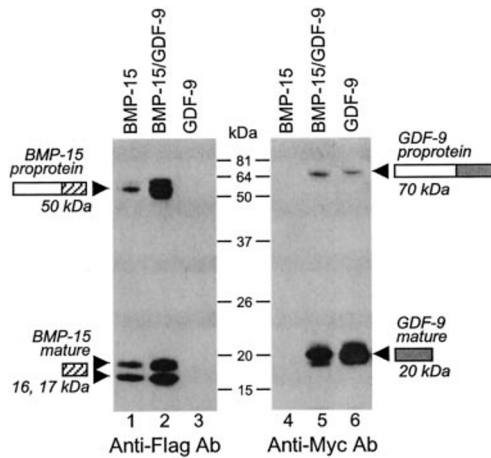


FIG. 1. **Production of recombinant BMP-15 and GDF-9 by BMP-15, GDF-9, and BMP-15/GDF-9 cell lines.** CM from 293T cells stably transformed with BMP-15, GDF-9 or both were subjected to SDS/PAGE immunoblotting analysis. Anti-FLAG Ab and anti-Myc Ab were used to detect BMP-15 (left panel) and GDF-9 (right panel), respectively.

cause this vector contains two separate cloning sites, each having an independent promoter. To verify the expression of BMP-15 and GDF-9 in the CM of each, the CM were subjected to SDS/PAGE immunoblotting analysis using an anti-FLAG Ab to detect BMP-15 and an anti-Myc Ab to detect GDF-9 (Fig. 1). Both cell lines that were transfected with phBMP-15F or with phBMP-15F/hGDF-9M secreted BMP-15 which was detected with the anti-FLAG Ab as three bands: the proprotein detected at ~50 kDa and two bands of the mature protein detected at 16 and 17 kDa as previously reported (12). On the other hand, the GDF-9 and the BMP-15/GDF-9 cell lines both secreted GDF-9, with the proprotein migrating at ~70 kDa and mature GDF-9 migrating at ~20 kDa.

To determine whether BMP-15 and GDF-9 can form homodimers we subjected the CM to chemical cross-linking using BS³ followed by SDS/PAGE immunoblotting analysis (Fig. 2). Cross-linking of BMP-15 revealed a homodimer of mature BMP-15 that migrated at ~34 kDa and cross-linking of GDF-9 revealed a homodimer of mature GDF-9, which migrated at ~40 kDa (Fig. 2, lanes 2 and 6). Both BMP-15 and GDF-9 homodimers were also clearly detectable in CM from the BMP-15/GDF-9 cells (Fig. 2, lanes 4 and 8). Additionally, there were also high molecular mass complexes detectable in cross-linked samples, which migrated at 65–180 kDa. These molecules most likely represent complexes of the mature domains bound to the prodomains, a phenomenon that is common in the TGF- β superfamily (13–15).

To determine whether these cells are also capable of producing BMP-15/GDF-9 heterodimers, we used anti-FLAG Ab conjugated to agarose beads to co-immunoprecipitate GDF-9 with BMP-15 from CM of the various cell lines followed by SDS/PAGE immunoblotting using anti-Myc Ab to detect GDF-9. Following co-immunoprecipitation from the CM of BMP-15/GDF-9 cells, mature GDF-9 was clearly detectable (Fig. 3A, lane 5), whereas no detectable GDF-9 was immunoprecipitated from the CM of GDF-9 cells (Fig. 3A, lane 6). The ability of GDF-9 to specifically co-immunoprecipitate with BMP-15 provides evidence that these molecules exist as heterodimers in the CM of co-transfected cells. The presence of GDF-9 in the CM of the respective cell lines before immunoprecipitation with anti-FLAG Ab agarose was verified (Fig. 3A, lanes 2 and 3), indicating that GDF-9 homodimers do not immunoprecipitate with anti-FLAG Ab agarose. We also attempted to co-immunoprecipitate GDF-9 with BMP-15 from the cell lysate of the various cell lines. In these studies we found that the GDF-9

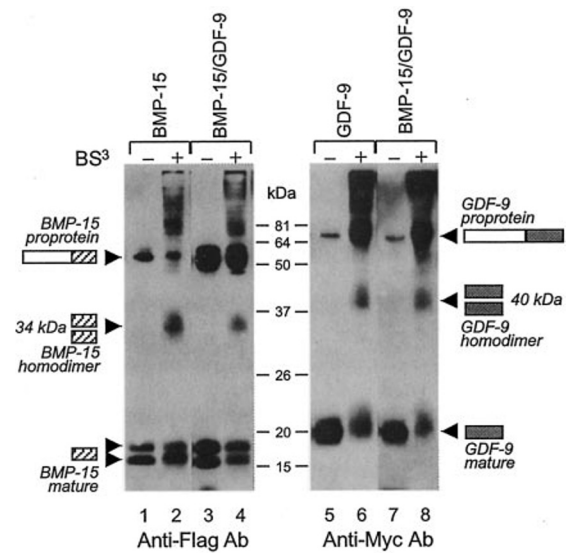


FIG. 2. **BMP-15 and GDF-9 form homodimers.** CM of indicated cell lines were subjected to chemical cross-linking with BS³ followed by SDS/PAGE immunoblotting analysis (lanes 2, 4, 6, and 8). Noncross-linked CM (control) were loaded in lanes 1, 3, 5, and 7. Immunoblotting of cross-linked samples with anti-FLAG Ab revealed homodimers of mature BMP-15, which migrated at ~34 kDa (lanes 2 and 4), and immunoblotting with anti-Myc Ab revealed homodimers of mature GDF-9, which migrated at ~40 kDa (lanes 6 and 8). Both BMP-15 and GDF-9 also formed large molecular mass molecules (65–180 kDa) upon cross-linking, which most likely represent complexes of the mature domains bound to the prodomains.

proprotein co-immunoprecipitates with BMP-15 in the BMP-15/GDF-9 cells, but not the GDF-9 cells (Fig. 3B, lanes 5 and 6), indicating that inside the cells, the proproteins of BMP-15 and GDF-9 form heterodimers. To further verify the existence of the BMP-15/GDF-9 heterodimer in the CM, we subjected the CM from the cell lines to cross-linking with BS³ followed by co-immunoprecipitation with anti-FLAG Ab agarose and detection by anti-Myc Ab. Again, there was no detectable GDF-9 co-immunoprecipitated from the CM of BMP-15 cells and GDF-9 cells (Fig. 3C, lanes 1 and 3). Co-immunoprecipitation of the cross-linked CM from BMP-15/GDF-9 cells; however, resulted in the detection of mature GDF-9 at ~20 kDa together with a new band at 37 kDa, which is the predicted size of the heterodimer of mature BMP-15/GDF-9 (Fig. 3C, lane 2). The mature GDF-9 is most likely derived from noncross-linked heterodimer of BMP-15/GDF-9 under these conditions. Collectively, these data provide clear evidence that BMP-15 and GDF-9 can heterodimerize.

To characterize the impact of the Inverdale mutation on the production and secretion of BMP-15, we used site-directed mutagenesis to create recombinant human BMP-15 with an Asp at residue 31 of the mature protein (replacing the normal Ile present in wild-type human BMP-15), which mimics the mutation identified in the Inverdale ewe (10). 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) contain only the conserved hydrophobic amino acids of either Val, Leu, or Ile at the 31st or its corresponding position of the molecules (10). The mutant BMP-15 (BMP-15^{I31D}) was successfully produced and secreted (Fig. 4, lane 3). Moreover, cross-linking studies revealed that, in disagreement with previous predictions (10), BMP-15^{I31D} does form homodimers, similar to wild-type BMP-15 (Fig. 4, lane 4).

We then investigated whether BMP-15^{I31D} can interact with GDF-9 in a similar fashion to wild-type BMP-15. A stable transformant cell line was established for this purpose that expresses both BMP-15^{I31D} and GDF-9 using the same tech-

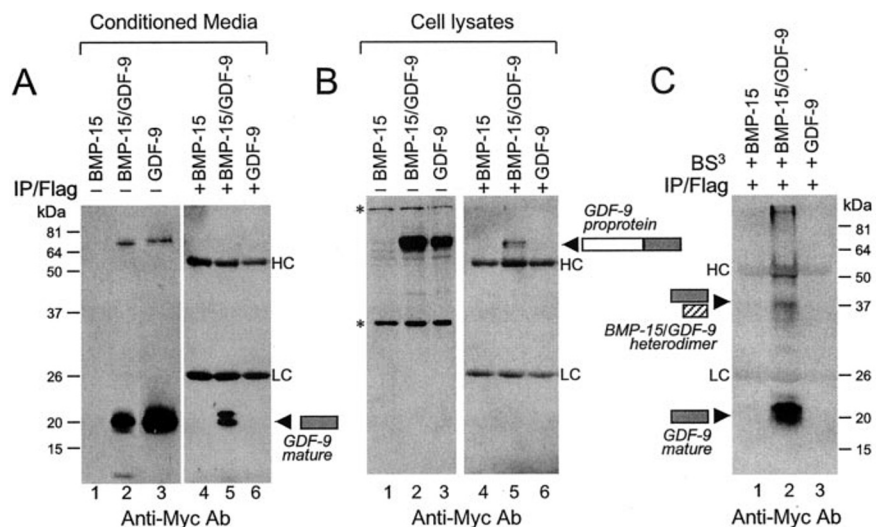


FIG. 3. **BMP-15 and GDF-9 form heterodimers.** A, CM from indicated cell lines were first subjected to SDS/PAGE immunoblotting with anti-My Ab to ensure expression of GDF-9 in the CM of the appropriate cell lines (lanes 2 and 3). Following immunoprecipitation of BMP-15 with anti-FLAG Ab conjugated to agarose beads (IP/Flag), precipitates were subjected to SDS/PAGE immunoblotting with anti-Myc Ab to detect mature GDF-9 that co-immunoprecipitated with BMP-15 (lane 5). B, lysates of indicated cell lines were treated in the same manner as described for the CM. Lane 5 shows that GDF-9 propeptides form heterodimers with BMP-15 in the cell lysates. Asterisks indicate nonspecific bands. C, CM from indicated cell lines were subjected to chemical cross-linking with BS³ followed by immunoprecipitation with anti-FLAG Ab agarose. SDS/PAGE immunoblotting of the precipitants with anti-Myc Ab revealed the cross-linked BMP-15/GDF-9 heterodimer, which migrated at ~37 kDa (lane 2). HC indicates IgG heavy chain, and LC indicates IgG light chain.

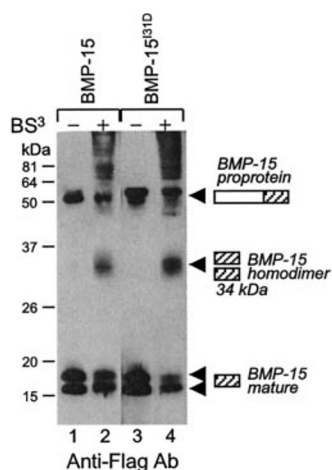


FIG. 4. **BMP-15^{I31D} is processed, secreted, and forms homodimers.** CM of indicated cell lines were subjected to SDS/PAGE immunoblotting analysis using anti-FLAG Ab before (lanes 1 and 3) or after (lanes 2 and 4) chemical cross-linking with BS³.

niques as described above. Unlike the BMP-15^{I31D} cell line that effectively produced and secreted BMP-15^{I31D} (Fig. 5A, lane 2), when BMP-15^{I31D} was co-transfected with GDF-9, the resulting cell lines were unable to produce and secrete detectable BMP-15^{I31D} into the culture media (Fig. 5A, lane 4). Indeed, we screened 52 individual BMP-15^{I31D}/GDF-9 cell lines, none of which produced any detectable amount of BMP-15^{I31D}. In addition to the lack of BMP-15^{I31D} secretion from BMP-15^{I31D}/GDF-9 cells, we also observed that the levels of mature GDF-9 secreted from the BMP-15^{I31D}/GDF-9 cells was lower than those of the BMP-15/GDF-9 cells and the singly-transfected GDF-9 cells (Fig. 5A, lanes 8–10). Because BMP-15^{I31D} was undetectable in the CM from BMP-15^{I31D}/GDF-9 cells, we attempted to detect the mRNA transcripts of BMP-15^{I31D} and GDF-9 derived from the transfected genes by semiquantitative RT-PCR analysis. The mRNA encoding BMP-15 or GDF-9 was not detected in GDF-9 nor BMP-15^{I31D} and BMP-15 cells, respectively (Fig. 5B, lanes 5–7), indicating that BMP-15 and GDF-9 mRNAs possibly transcribed from their intrinsic genes

(as compared with transfected genes) are under the detection level. However, either or both mRNAs were clearly detected in their corresponding transformants, and their levels were comparable among these cell lines (Fig. 5B), demonstrating that the differences in the levels of secreted proteins were not caused by differences in the levels of mRNA expression.

From the immunoblot data of the CM we also noted that there were variations in the relative levels of the propeptides secreted from the different cell lines, causing differences in the ratios of the mature/propeptide in the CM (Fig. 5C). Specifically, the ratios of the mature/propeptide of BMP-15^{I31D} or BMP-15 in the CM of the BMP-15^{I31D} cells or the BMP-15/GDF-9 cells, respectively, were significantly lower than that of the BMP-15 cells (Fig. 5C, left panel). This difference is most likely due to the decreased ability of the endopeptidases to cleave the propeptides to release the corresponding mature BMP-15. Additionally the cleavage of GDF-9 in the BMP-15/GDF-9 cells was also significantly impaired as compared with the singly transfected GDF-9 cells (Fig. 5C, right panel). When GDF-9 was co-transfected with BMP-15^{I31D} the cleavage of GDF-9 was severely impaired, to levels which were ~90% lower than GDF-9 cells.

Since it appeared that the I31D mutation in BMP-15 affects the cleavage of the BMP-15^{I31D} as well as GDF-9 propeptides, we further investigated whether the component propeptides and mature proteins were also altered in the lysates of respective cell lines (Fig. 6). BMP-15 propeptides and mature proteins were clearly detectable in the lysates from the BMP-15 cells, BMP-15^{I31D} cells and the BMP-15/GDF-9 cells. In contrast, mature BMP-15^{I31D} was not detectable in the BMP-15^{I31D}/GDF-9 cells yet the propeptide was clearly detected (Fig. 6, lane 4). Additionally, the cleavage of GDF-9 is nearly abolished in the BMP-15^{I31D}/GDF-9 cells (Fig. 6, lane 9), suggesting that the inhibition of the cleavage of the GDF-9 propeptide may be the mechanism that accounts for the low levels of mature GDF-9 secreted by the BMP-15^{I31D}/GDF-9 cells.

DISCUSSION

BMP-15 and GDF-9 form a unique subset of the TGF- β superfamily defined by their high amino acid homology, oocyte-

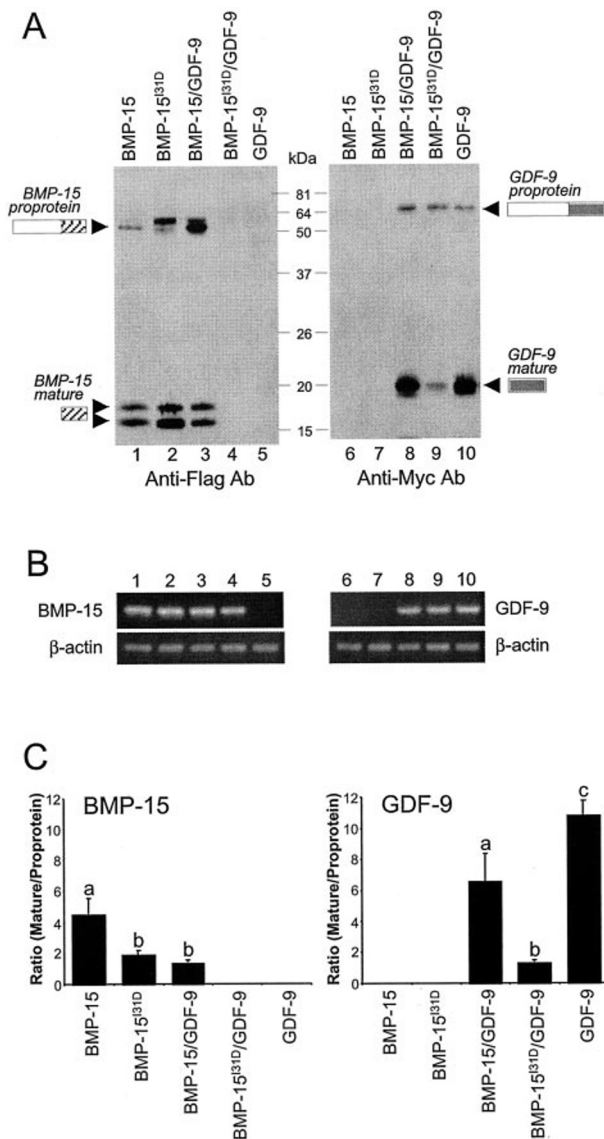


FIG. 5. Production of mutant BMP-15^{131D} and GDF-9 by singly and co-transfected cell lines. A, CM of indicated cell lines were subjected to SDS/PAGE immunoblotting analysis using anti-FLAG Ab to detect BMP-15 (left panel) and anti-Myc Ab to detect GDF-9 (right panel). Data shown are representative of at least three independent experiments. B, semiquantitative RT-PCR analysis was performed to verify comparable expression of BMP-15 mRNA (left panel) and GDF-9 mRNA (right panel) in the cell lines corresponding to those shown in A. C, ratios of the mature BMP-15 (left panel) or GDF-9 (right panel) to the corresponding proproteins secreted into the culture media were determined using densitometric analysis of the SDS/PAGE immunoblotting results shown in A. Bars represent the mean values of a minimum of three different gels for each group. Different letters denote significant differences at $p \leq 0.05$.

specific expression pattern and the fact that they both lack the fourth Cys residue of the seven Cys residues that are typically conserved in almost all members of the TGF- β superfamily (1, 2, 4, 7). Because the fourth Cys is responsible for intersubunit disulfide bond formation, there has been an argument as to whether BMP-15 and GDF-9 exist as monomers or homodimers. It has been predicted that GDF-9 is a monomer based on its molecular weight determined by SDS-PAGE under non-reducing conditions (15). However, since non-covalently linked dimers migrate as monomers in SDS-PAGE, even under non-reducing conditions, the stoichiometry of GDF-9 still remained unproven. In the present study, we demonstrate that both BMP-15 and GDF-9 do indeed form non-covalent ho-

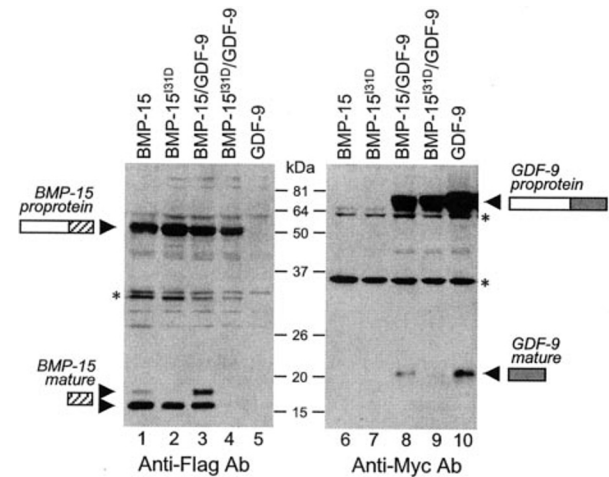


FIG. 6. Presence of BMP-15 and GDF-9 mature and proproteins in the lysates of singly and co-transfected cell lines. Lysates of indicated cell lines were subjected to SDS/PAGE immunoblotting analysis using anti-FLAG Ab to detect BMP-15 (left panel) and anti-Myc Ab to detect GDF-9 (right panel). Asterisks indicate nonspecific bands.

modimers. Furthermore, because of the high similarity in the structure of BMP-15 and GDF-9 as well as their nearly-identical spatiotemporal expression patterns in the oocyte, it has been speculated that BMP-15 and GDF-9 may form heterodimers (9). In this regard, the present study clearly demonstrates that BMP-15 and GDF-9 can also heterodimerize. The functional significance of the BMP-15/GDF-9 heterodimers remains to be elucidated, however, this finding reminds us of our previous discovery of activin-A (16, 17) and activin-AB (18) and of the discovery of activin-B by Nakamura *et al.* (19). Our current studies show that, like the activins, BMP-15 and GDF-9 form both homo- and heterodimers. At present we are unaware of the relative amounts of the BMP-15 and GDF-9 homodimers and heterodimers that are produced and secreted from the oocyte *in vivo*. To answer these questions specific assays for these molecules, similar to the specific assays developed for inhibins A and B utilizing two-site enzyme-linked immunosorbent methods (20, 21), need to be established.

It is well recognized that during the biosynthetic processing of TGF- β superfamily members, the proproteins have to first dimerize in order for the proteolytic separation of the mature bioactive protein and the pro-domain to occur (6). There is evidence that for some members of the TGF- β superfamily, low efficiency of this processing may serve as a possible mechanism of governing the biological activity of these molecules *in vivo*. For example, the proprotein of Vg-1 is localized to vegetal blastomeres in *Xenopus* embryos, and is abundant in vegetal cells, but the processed mature form is not readily produced and secreted (22, 23). Interestingly, the fusion of the prodomain of BMP-2 with the mature-domain of Vg-1 has been shown to promote production of mature Vg-1 (24). Thus, the prodomain may determine the rate and efficiency of dimer formation and proteolytic cleavage. An intriguing finding in the present study was that when BMP-15 was co-expressed with GDF-9, the processing of GDF-9 as well as BMP-15 proproteins was significantly impaired (Fig. 5C). Since the processing of both proproteins were efficient when they were expressed individually, these findings strongly suggest that GDF-9 and BMP-15 proproteins form heterodimers that are less susceptible to the proteolytic cleavage and probably undergo degradation more readily than the homodimeric proproteins of GDF-9 and BMP-15. The more drastic effect of defective processing of BMP-15 and GDF-9 was observed when BMP-15^{131D} was co-expressed with GDF-9. In these conditions, although the BMP-15^{131D}

proprotein was clearly detectable in the cell lysates, the mature BMP-15^{I31D} protein was undetectable in both the CM and the cell lysates. Additionally, the processing of GDF-9 and the levels of mature GDF-9 in the CM of BMP-15^{I31D}/GDF-9 cells was markedly lower than that of the BMP-15/GDF-9 cells. Thus, it seems likely that the heterodimers comprised of BMP-15^{I31D} and GDF-9 proproteins severely resist being processed by endoproteases and are rapidly degraded inside the cells. Given that the expression patterns of these two factors in the oocyte are nearly identical, we speculate that the levels of both mature BMP-15^{I31D} and GDF-9 proteins secreted from the oocytes of homozygous Inverdale ewes is markedly low. It has been shown that BMP-15 and GDF-9 mRNA expression levels in the oocyte of Inverdale ewes are indistinguishable from those in wild type ewes (10, 25). Our present data, however, predict that the mutation affects the post-translational processing of the proproteins of both BMP-15^{I31D} and GDF-9 and subsequent secretion of the mature molecules by the oocyte, which could not be evaluated by *in situ* hybridization of the transcripts as reported in the previous studies (10, 25).

Based on our previous findings on the *in vitro* biological effects of recombinant BMP-15, we have presented a model by which the levels of bioactive BMP-15 may account for the divergent phenotypes of the heterozygous and homozygous Inverdale ewes (12, 26, 27). In the heterozygotes of Inverdale ewes, the reduced level of bioactive BMP-15 results in higher levels of follicle-stimulating hormone (FSH) receptor in the granulosa cells which in turn leads to more developing healthy follicles with more luteinizing hormone (LH) receptors, resulting in precocious follicle growth and increased ovulation at earlier stages of follicular development. In the homozygotes, the entire absence of BMP-15-induced mitosis of and kit ligand expression by granulosa cells may cause the cessation of follicular development at the primary stage. This hypothesis based on the biological functions of BMP-15 determined by *in vitro* granulosa cell culture has strongly been supported by the facts observed in the ovaries of Inverdale ewes that, in the heterozygotes: (i) there are more healthy estrogenic follicles, (ii) the number of granulosa cells in these developing follicles is significantly smaller, (iii) these granulosa cells have a higher mean LH responsiveness at smaller follicle stages, and (iv) the corpora lutea are smaller than normal (28). In contrast, follicle development in the homozygotes is arrested at the primary follicle stage and thus they are infertile (29).

However, given that *gdf9*-null mice are infertile because of an arrest of follicle development at the primary stage, similar to the phenotype of Inverdale homozygotes, yet *bmp15*-null mice are fertile with only minimal ovarian defects, it is also possible that the decrease in the secretion of mature GDF-9, which would be predicted based on our present data, would be sufficient to cause infertility. Our present findings that the Inverdale mutation in BMP-15 inhibits the processing and secretion of GDF-9 support this possibility. According to this model, the deletion of the entire second exon (containing most of the proprotein domain) of the *bmp15* gene in the BMP-15 knockout mouse (9), may prevent the interactions of BMP-15 and GDF-9 proproteins; thus GDF-9 processing and secretion would remain unaffected, resulting in the lack of a severely disrupted ovarian phenotype. To further determine whether the phenotypes caused by the BMP-15 mutations differ due to the indispensability of either BMP-15 or GDF-9 in these two species, or if indeed the phenotype of the homozygous Inverdale ewe is primarily determined by the decrease in GDF-9 processing and secretion, a line of transgenic mice with the Inverdale mutation in the *bmp15* gene would need to be generated, which is currently in progress in our laboratory. Studies on BMP-15

with the Hanna mutation may also provide insight as to whether the reduction of GDF-9 is the primary cause of infertility in BMP-15 mutant sheep. Since the phenotype of the Hanna ewe is indistinguishable from the Inverdale ewe (30), if GDF-9 is a primary factor then it would be expected that FecX^H-BMP-15 would also inhibit GDF-9 processing. Because the premature stop codon in FecX^H-BMP-15 leaves the entire prodomain and a portion of the mature domain of BMP-15 intact, it is likely that FecX^H-BMP-15 proprotein is also capable of forming heterodimers with GDF-9 proprotein, and subsequently decreasing GDF-9 processing through a similar mechanism as Inverdale BMP-15.

An intriguing similar example of a point mutation in one TGF- β superfamily member causing the inhibition of the secretion of another TGF- β superfamily member has been reported by Thomas *et al.* (31) in which they identified a causative point mutation in the gene encoding cartilage-derived morphogenetic protein-1 (CDMP-1, also called as GDF-5) in chondrodysplasia Grebe type (CGT) in humans. CGT is an autosomal recessive disorder characterized by severe abnormality of the limbs and limb joints. The mutation substitutes a Tyr at amino acid 400 (counting from the amino terminus of the preproprotein) for the first of seven highly conserved Cys residues (C400Y) in the mature-domain of CDMP-1. Because of this mutation, CDMP-1^{C400Y} proprotein is not processed to separate the pro-domain and mature-domain, which prevents the secretion of CDMP-1 from the cells. Of particular interest is that CDMP-1^{C400Y} can form heterodimers with structurally related molecules such as BMP-2, BMP-3 and BMP-7 when they are co-expressed by the same cells. Intriguingly, the resulting heterodimer proproteins of CDMP-1^{C400Y} and BMPs are not secreted, thus, CDMP-1^{C400Y} acts as a dominant negative regulator of the associated intact BMPs by preventing their processing and secretion (31).

In summary we present the first evidence that BMP-15 and GDF-9 can form homo- and heterodimers and that the intracellular interaction of these molecules can affect the processing and secretion of the mature proteins. Importantly we show that BMP-15^{I31D} is secreted and can form homodimers when singly expressed, but when it is co-expressed with GDF-9, the processing and secretion of BMP-15^{I31D} is abolished and the processing and secretion of GDF-9 is also severely impaired. These findings suggest that levels of bioactive GDF-9 in homozygous Inverdale sheep may be markedly low, thus, insufficient GDF-9 could be the cause, at least in part, of infertility in homozygous Inverdale ewes. The present study, therefore, serves an important paradigm for the potential impact of how a mutation in one gene can influence related genes at the level of post-translational processing.

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