Bone Morphogenetic Protein-15
IDENTIFICATION OF TARGET CELLS AND BIOLOGICAL FUNCTIONS*

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In developing ovarian follicles, the regulation of cell proliferation and differentiation is tightly coordinated. Precisely how this coordination is achieved is unknown, but recent observations have suggested that molecules emitted by the oocyte are involved in the process. The newly discovered oocyte-specific growth factor, bone morphogenetic protein-15 (BMP-15), is one such molecule. At present, nothing is known about the target cells and biological functions of BMP-15. To fill this gap in our knowledge, recombinant BMP-15 and its antibody were produced and used to determine BMP-15 expression and bioactivity. BMP-15 mRNA and protein were shown to be co-expressed in oocytes throughout folliculogenesis, supporting the idea that BMP-15 is a physiological regulator of follicle cell proliferation and/or differentiation. To test this, we used primary cultures of rat granulosa cells (GCs). We found that BMP-15 is a potent stimulator of GC proliferation, and importantly, the mitogenic effect was follicle-stimulating hormone (FSH)-independent. By contrast, BMP-15 alone had no effect on steroidogenesis. However, it produced a marked decrease in FSH-induced progesterone production, but had no effect on FSH-stimulated estradiol production. This result indicates that BMP-15 is a selective modulator of FSH action. In summary, this study identifies GCs as the first target cells for BMP-15. Moreover, it identifies the stimulation of GC proliferation and the differential regulation of two crucial steroid hormones as the first biological functions of BMP-15. Significantly, BMP-15 is the first growth factor that can coordinate GC proliferation and differentiation in a way that reflects normal physiology.

Much current research is directed toward identifying local regulatory molecules involved in ovarian physiology and identifying their target cells and biological responses. It is clear that some of these molecules are synthesized and secreted by the oocyte (1). The current theory is that some oocyte factors act as morphogens to control follicle growth and differentiation (2). Compelling evidence that this concept operates in vertebrates comes from the important work of Matzuk and co-workers (3–5), demonstrating that oocyte-derived growth differentiation factor-9 (GDF-9)1 is obligatory for normal folliculogenesis and female fertility.

In 1998, another oocyte-specific growth factor was discovered by two independent laboratories. This factor, which is a new member of the transforming growth factor-β (TGF-β) superfamily, was named bone morphogenetic protein-15 (BMP-15) (6) or GDF-9B (7, 8). The primary structure of mouse (6), human (6), and rat (9) BMP-15 most closely resembles that of GDF-9 (6, 9). An interesting structural feature of BMP-15 and GDF-9 is that they lack the fourth cysteine residue of the seven cysteines that are typically conserved in the more than 40 members of TGF-β superfamily (6). Because the fourth cysteine is responsible for interchain disulfide bond formation, it is possible that BMP-15 and GDF-9 may be monomers or perhaps linked noncovalently as hetero- and/or homodimeric proteins.

Despite its physiological significance, the target cells of BMP-15 have not been identified, and nothing is known about the biological functions of BMP-15 protein. To fill this gap in our knowledge, we report the temporal pattern of BMP-15 mRNA and protein expression during folliculogenesis and show that BMP-15 acts directly on follicular GCs to regulate their proliferation and cytodifferentiation. This is the first demonstration of the expression, the target cells, and biological function of BMP-15 protein.

MATERIALS AND METHODS

Reagents and Supplies—Ovine FSH (NIDDK-oFSH-S20) was provided by Dr. Parlow of the National Hormone and Pituitary Program (Rockville, MD). Dulbecco’s modified Eagle’s medium/F12, medium 199, and McCoy’s 5a medium were from Life Technologies Inc. (Rockville, MD). Anti-Flag M2 monoclonal antibody (anti-Flag mAb), anti-Flag mAB agarose, and diethylstilbestrol were from Sigma–Aldrich Co.

Construction of Expression Plasmids—The protein coding region of human BMP-15 cDNA was amplified by reverse transcription-PCR from ovarian total RNA with a primer set containing the amino- and carboxyl-terminal part of prepro-BMP-15 (GenBank® accession nos. AF082349 and AF082350). A PCR product of expected size was cloned in frame at the upstream site of Flag epitope tag (an amino acid sequence of Asp-Tyr-Lys-Asp-Asp-Asp-Lys) present in pCMV-Tag4A (Stratagene, San Diego, CA) and designated pBMP-15-Flag. Another expression plasmid, pBMP-15-GST, was constructed by inserting the DNA segment encoding only the mature domain of hBMP-15 in frame at the downstream site of GST present in the prokaryotic expression vector, pGEX-4T-1 (Amersham Pharmacia Biotech). DNA sequences of both plasmids were confirmed.

Construction of BMP-15 Probe Plasmid—A DNA segment of rat...
BMP-15 cDNA (nucleotides 406–814 in GenBank accession no. AJ132407) (9) was amplified by reverse transcription-PCR from ovarian total RNA. The product was gel-purified, cloned into pBluescript SK+ (Stratagene), and the DNA sequence confirmed. This plasmid, prBMP-15–409, which contained the pro-region of rat BMP-15 precursor showed high sequence homology with other known TGF-β superfamily members, including GDF-9.

Production of rhBMP-15-GST and Its Antibodies—Escherichia coli strain XL1-Blue (Stratagene) was transformed with phBMP-15-GST, and its production was induced by isopropyl-1-thio-β-d-galactoside (IPTG). The fusion protein was purified from whole cell lysate by glutathione-Sepharose affinity column. The purified protein was injected into rabbits to generate anti-rhBMP-15-GST polyclonal antibodies (anti-BMP-15 Ab). The IgG fraction was purified by protein A-Sepharose affinity column.

Production of rhBMP-15-Flag—Human embryonic kidney 293 cells were transfected with phBMP-15-Flag (10). Clonal cell lines permanently expressing rhBMP-15-Flag were selected using 0.4 mg/ml Geneticin (Life Technologies, Inc.) and maintained in Dulbecco’s modified Eagle’s medium/F12 containing 10% fetal bovine serum. When transfected cells (TF) reached ~90% confluence, they were recultured for 3 days in serum-free medium, after which the conditioned medium (CM) was harvested. As a control, CM from non-transfected cells (n-TF) was harvested. CM containing rhBMP-15-Flag was subjected to affinity column chromatography using anti-Flag mAb agarose.

Protein Analysis—Western immunoblotting was performed as described (11) under reducing conditions using the anti-Flag mAb (1:1,000 dilution) or anti-BMP-15 Ab (1:10,000 dilution). Immunofluorescent signals were detected by the ECL system (Amersham Pharmacia Biotech). N-Linked and O-linked glycosylation analyses were performed by treating the samples with peptide N-glycosidase F (New England Biolabs, Inc., Beverly, MA) or O-glycanase together with neuraminidase II, galactosidase III, and hexosaminidase I (Glyco Inc., Novato, CA), respectively. The density of each protein band stained by silver was determined for each treatment. Differences between groups were analyzed for statistical significance using analysis of variance (StatView 5.0 software, Abacus Concept, Inc., Berkely, CA). P values < 0.05 were accepted as statistically significant.

RESULTS

In situ hybridization (Fig. 1, a and b) revealed strong hybridization signals for BMP-15 mRNA in the oocytes. No hybridization signal was detected in other ovarian cell types. Hybridization with the sense BMP-15 probe showed only a low level of nonspecific background signal (data not shown). These results are in agreement with previous reports (6, 7).

To assess the relative changes in BMP-15 mRNA expression during folliculogenesis, the oocyte hybridization signals were subjected to quantification. Virtually all (99%) of the oocytes in primordial follicles showed no hybridization signal above background (Table I and Fig. 1d). In the majority (75%) of primary follicles, no BMP-15 mRNA was detectable (Table I). However, when the oocyte was surrounded by one complete layer of cuboidal GCs, a weak hybridization signal was observed (Table I and Fig. 1e). A positive hybridization signal was found in 97% of the oocytes in secondary follicles (Table I). As the number of GC layers increased, the number of silver grains over the oocyte increased (Fig. 1, e and f). In healthy Graafian follicles, 100% of the oocytes showed relatively strong labeling (Table I and Fig. 1g).

Whether BMP-15 mRNA in the oocytes is translated is unknown. To address this, we first developed an anti-BMP-15 Ab. Fig. 2A shows that bacterial lysates from rhBMP-15-GST transformants induced by IPTG exhibited a strong band at ~42 kDa, which was then purified by a glutathione-Sepharose affinity column (Fig. 2B). This 42-kDa protein exhibits the expected molecular weight (Mr) of rhBMP-15-GST (the calculated masses of GST, intervening amino acid chain between GST and BMP-15, and BMP-15 are 26, 1, and 14 kDa, respectively). The purified rhBMP-15-GST protein was injected into rabbit to generate the anti-BMP-15 Ab. Using the anti-BMP-15 Ab, we examined BMP-15 protein expression in immature rat ovaries by immunohistochemistry. Consistent with the mRNA data, the anti-BMP-15 Ab selectively stained oocytes, and no immunoreactivity was evident in other ovarian cell types (Fig. 1c). In control experiments, no immunoreactive cells were seen in control sections incubated with preimmune serum. Notably, the anti-BMP-15 Ab showed no cross-reactivity with 100 ng of rhGDF-9 when examined by Western immunoblotting (data not shown).

Quantitative analysis revealed that oocytes in most primordial follicles (71%) were not stained by the anti-BMP-15 Ab (Table I and Fig. 1A); however, it is interesting that a significant percentage (29%) appeared weakly positive for BMP-15 protein (Table I). The majority (55%) of the oocytes in primary follicles were stained by the anti-BMP-15 Ab (Table I and Fig. 1b), and the strength of the signal appeared related to the number of cuboidal GC, e.g., the highest signal (+2) appearing in those oocytes surrounded by a complete layer of cuboidal cells. When developing follicles reached the secondary stage, 100% of the oocytes appeared immunostained, and in the majority (54%) the intensity of the signal was high, +3 (Table I and Fig. 1f). A relatively high level of immunoreactivity was evident in all oocytes of healthy Graafian follicles (Table I and Fig. 1k).

For bioactivity studies, GCs (105 viable cells) were cultured in a 96-well plate with 200 μl of medium containing 100 nm 4-androstene-3,17-dione (substrate of P450-aromatase) either alone or together with the indicated concentrations of FSH and/or rhBMP-15-Flag. After 48 h of culture, the levels of progesterone (P4) and estradiol (E2) in the media were measured by a radioimmunoassay.

Bioactivity results shown are averages (mean ± S.E.) of at least four separate experiments, with triplicate determination for each treatment. Differences between groups for each treatment were analyzed for statistical significance using analysis of variance (StatView 5.0 software, Abacus Concept, Inc., Berkely, CA). P values < 0.05 were accepted as statistically significant.
To uncover the biological functions of BMP-15, 293 cells were transfected with a phBMP-15-Flag. A single colony of 293 cells producing high levels of rhBMP-15-Flag protein was selected by Geneticin and established as a permanent cell line. SDS-PAGE analysis of CM from n-TF and TF revealed numerous proteins (Fig. 3A). When gels were immunostained with anti-BMP-15 Ab under reducing conditions, two proteins migrating at 16 and 50 kDa were seen in the CM from TF, but not n-TF (Fig. 3B). It is likely that the small and large proteins are the mature rhBMP-15-Flag and its proprotei, respectively. Examination of the same samples with anti-Flag mAb also revealed the same two bands; however, this antibody identified an additional band, which runs at a slightly higher mass (17 kDa) than the 16-kDa protein in the CM from TF (Fig. 3C).

We next immunopurified the rhBMP-15-Flag from CM of TF using an anti-Flag mAb agarose column. Silver-staining of the immunopurified proteins revealed two small proteins (16 and 17 kDa) and several larger proteins in the 50–60-kDa range (Fig. 4A). In the smaller mass range, only the 16-kDa band was detected by anti-BMP-15 Ab (Fig. 4B) whereas the anti-Flag mAb detected both 16- and 17-kDa bands (Fig. 4C). In addition, both antibodies also detected a 50-kDa band, which is most likely the rhBMP-15-Flag proprotein. At this point, the nature of the 17-kDa band is unknown. However, its mass was not altered by the treatment with N-glycosidase or O-glycosidase together with neuraminidase II, galactosidase III, and hexosaminidase I, suggesting it is not a glycosylated protein.

Our in situ hybridization and immunohistochemistry results indicating increased BMP-15 expression during follicle growth and development (see Fig. 1) led us to hypothesize that BMP-15 might modulate GC proliferation and/or differentiation. Before testing this hypothesis, we first estimated the concentration of rhBMP-15-Flag protein in the immunopurified sample by comparing the intensity of the rhBMP-15-Flag protein on SDS-PAGE with that of known amounts of activin A (Fig. 5). Since the nature of 17-kDa protein is not clear, it was not used in the estimation of the concentration of rhBMP-15-Flag stock.

Fig. 6 shows DNA synthesis data derived from cultured rat GCs that had been stimulated with rhBMP-15-Flag. As shown in the inset, unpurified CM from TF produced an increase (−2-fold) in [3H]thymidine incorporation when compared with CM from n-TF. Treatment of the cells with purified rhBMP-15-Flag (200 ng/ml) produced a marked increase in thymidine incorporation, and the rhBMP-15-Flag effect was dose-dependent (ED50 = ~30 ng/ml). We also found that 30 ng/ml rhBMP-15-Flag increased the GC number by 1.5-fold, indicating rhBMP-15-Flag stimulates cell proliferation as well as DNA synthesis. The stimulatory effect of rhBMP-15-Flag on DNA synthesis was unaffected by co-treatment with a saturating dose (3 ng/ml) of FSH (Fig. 6).

Finally, we determined the effects of rhBMP-15-Flag on P4 and E2 production by cultured GCs. As observed earlier (15), FSH increased P4 and E2 production in a dose-dependent manner (Fig. 7). By comparison, rhBMP-15-Flag alone was unable to directly stimulate basal P4 and E2 production. However, co-treatment of FSH with rhBMP-15-Flag produced a marked inhibition (as much as 50%) of the FSH-induced P4 levels (ED50 of rhBMP-15-Flag = 10 ng/ml), whereas the rhBMP-15-Flag had no effect on FSH-induced E2 production.

DISCUSSION

Our experiments provide the first evidence for the target cells and biological activity of BMP-15 ligand. Experiments in which 10–5 M concentrations of BMP-15 were added to cultured GCs indicated that BMP-15 is a potent regulator of mitosis and a negative regulator of FSH-induced P4 production. These findings suggest two new principles; (i) GCs are target cells for BMP-15 ligand, and (ii) the BMP-15 receptor signaling pathways in GCs are functionally coupled to DNA replication/cell proliferation and the modulation of FSH action. We propose that the biological significance of oocyte-specific BMP-15 in vivo might be to promote follicle growth while preventing premature luteinization. Because human oocytes express BMP-15 (8), it will be interesting to determine whether these principles apply to women. It is noteworthy that the effects of BMP-15 on GCs are different from those of other members of the TGF-β superfamily, including inhibin (16, 17), activin (17–19), TGF-β (16, 20–23), BMP-4 (15), BMP-7 (15), and GDF-9.
As such, it would appear that the biological activity of BMP-15 in the GC is unique. Prior to this work, nothing was known about the cellular expression of BMP-15 protein. Our experiments now provide the first evidence for the oocyte-specific expression of BMP-15 protein and show that BMP-15 protein and mRNA are selectively co-expressed in the oocytes throughout the course of folliculogenesis. From a physiological perspective, these results fit the hypothesis that BMP-15 produced by the oocytes plays a role regulating cell proliferation and differentiation during follicle development. The presence of BMP-15 protein in some primordial follicles is of interest because it pushes the question of oocyte BMP-15 expression back to the beginning stages of folliculogenesis. This could suggest that oocyte BMP-15 plays a role in follicle recruitment. What mechanisms are responsible for the initiation and maintenance of BMP-15 mRNA and protein expression in the oocyte remains an important unanswered question.

**TABLE I**

| Signal strength | Primordial | Primary | Secondary | Graafian |
|----------------|------------|---------|-----------|----------|
| BMP-15 mRNA    |            |         |           |          |
| ND*            |            | 99 (99%)| 102 (75%) | 0 (0%)   |
| +1             | 1 (1%)     | 32 (24%)| 19 (25%)  | 0 (0%)   |
| +2             | 0 (0%)     | 1 (1%)  | 11 (14%)  | 8 (25%)  |
| +3             | 0 (0%)     | 0 (0%)  | 24 (31%)  | 14 (44%) |
| +4             | 0 (0%)     | 0 (0%)  | 21 (27%)  | 10 (31%) |
| BMP-15 protein |            |         |           |          |
| ND             | 39 (71%)   | 54 (45%)| 0 (0%)    | 0 (0%)   |
| +1             | 15 (27%)   | 56 (47%)| 0 (0%)    | 0 (0%)   |
| +2             | 1 (2%)     | 10 (8%) | 13 (32%)  | 5 (56%)  |
| +3             | 0 (0%)     | 0 (0%)  | 22 (54%)  | 4 (44%)  |

* ND, nondetectable.

**Fig. 2.** rhBMP-15-GST fusion protein expression and purification. Panel A, Coomassie Blue staining of bacterial protein after SDS-PAGE under reducing conditions. Lysate proteins from bacteria transformed with phBMP-15-GST before (-) and after (+) IPTG induction. Panel B, lysate proteins purified by glutathione-Sepharose affinity column before (-) and after (+) IPTG induction.

**Fig. 3.** SDS-PAGE analysis of the CM of 293 cells transfected with phBMP-15-Flag under reducing conditions. Panel A, silver staining of the medium proteins from n-TF and TF. Panel B, Western immunostaining analysis of the medium proteins from n-TF and TF by anti-BMP-15 Ab. Panel C, Western immunostaining analysis of the medium proteins of n-TF and TF by anti-Flag mAb.

**Fig. 4.** SDS-PAGE analysis of immunopurified rhBMP-15-Flag. CM from TF was subjected to an affinity column chromatography using anti-Flag mAb agarose. The immunopurified rhBMP-15-Flag was detected by silver staining (panel A) or by Western immunostaining with anti-BMP-15 Ab (panel B) or anti-Flag mAb (panel C).

**Fig. 5.** Estimation of the concentration of immunopurified rhBMP-15-Flag. Samples of rhBMP-15-Flag (9 μl) and different amounts of activin A (20, 40, and 80 ng/lane) were electrophoresed on SDS-PAGE (right panel). After silver staining (inset), the bands were scanned and their intensity (pixels) determined. The intensity of the activin A bands is graphed with closed circles (left panel). Intensity of the 16-kDa band of rhBMP-15-Flag is noted with an open circle.
Our results identify GC as the first target cells for BMP-15. From a functional point of view, nothing was known about the biological activity of BMP-15 until this study. We found that BMP-15 acts directly on GC to markedly stimulate mitosis. These results suggest that BMP-15 functions as a GC growth factor. An important point to emerge from these experiments is that the mitogenic activity of BMP-15 is FSH-independent. Although it is not clear what this means physiologically, it is possible that oocyte-derived BMP-15 is involved in controlling GC mitosis in preantral follicles during the FSH-independent or preantral period of folliculogenesis. Consistent with this idea, our in situ hybridization and immunohistochemistry results show strong BMP-15 mRNA and protein expression in oocytes in preantral follicles. Taken together, our results provide the new concept that BMP-15 is a mitogen for GCs.

Our studies also suggest that BMP-15 is likely to be involved in the regulation of GC steroidogenesis. A striking feature of the BMP-15 steroidealogenic response is that, in contrast to the proliferative response, it is FSH-dependent. The finding that BMP-15 produced a marked inhibition in FSH-induced P^4 biosynthesis, while having no effect on FSH-induced E_2 production, indicates that the biochemical pathways that eventually lead to P^4 synthesis in GCs are preferentially inhibited by BMP-15. The ability of BMP-15 to selectively inhibit the stimulation of P^4 production by FSH suggests a novel role for BMP-15 in the luteinization process. There is a large body of evidence supporting the concept that the oocyte secretes a putative regulatory factor that inhibits GC luteinization (1, 2, 25, 26). Although the nature of the putative oocyte steroid-regulating factor is unknown, our data suggest that BMP-15 is a possible candidate for the oocyte-derived luteinization inhibitor.

What do we know about the physiological relevance of BMP-15? Genetic studies in sheep have provided compelling evidence for the involvement of BMP-15 in folliculogenesis and fertility. The Inverdale (FecX^I) mutant has a single T to A transition at nucleotide position 92 in the mature BMP-15 coding region (27). In female homozygous mutants (FecX^I/FecX^I), follicular development is arrested at the primary stage and the animals are infertile. Interestingly, these female homozygous BMP-15 mutants (FecX^I/FecX^I) are superfertile, having increased ovulation rates and multiple pregnancies. A different BMP-15 mutant, Hanna (FecX^H), generates a premature stop codon at amino acid residue 23 in mature BMP-15. When FecX^I and FecX^H sheep are crossed, the resulting (FecX^I/FecX^H) females exhibit a phenotype indistinguishable from (FecX^H/FecX^H) infertile females (27). It seems clear from this work that BMP-15 molecules are central to ovarian function, regulating follicle development and ovulation quota in a very intriguing gene dosage manner. Based on our results, one would propose that the GC might mediate some of these BMP-15 effects.

Over the past few years, much attention has been focused on another oocyte growth factor, GDF-9 (2, 5). Both BMP-15 and GDF-9 are closely related structurally, and both proteins can stimulate GC mitosis with similar potency (24). Therefore, it is reasonable to assume that both of these oocyte growth factors might be directly involved in the regulation of GC proliferation and follicle growth. It will be important to determine the interaction of BMP-15 and GDF-9 in the context of GC mitosis. Regarding differentiation, GDF-9 itself can stimulate E_2 production and inhibit both FSH-induced E_2 and P^4 production by cultured GCs (24). These results, together with our findings, suggest that important differences exist between BMP-15 and GDF-9 in their ability to influence GC cytodifferentiation. If true, the data would support the view that BMP-15 and GDF-9 have different effects on follicle differentiation and in vivo. Under physiological conditions, FSH causes developing follicles to produce E_2 but not P^4 during the cycle (28). As such, the effects of BMP-15 on GC differentiation would appear to be more physiologically relevant than those reported for GDF-9. It will be of great interest to find out how specific BMP-15 and GDF-9 signaling pathways lead to the stimulation and inhibition of particular effector genes and ultimately to the specific biological responses that are critical for follicle growth and development. Given the oocyte-specific expression and structural similarity between BMP-15 and GDF-9, it will be of great interest to determine whether these molecules form heterodimers, which in turn might have yet to be discovered biological responses.

In summary, these studies identify the GCs as the first target cells for BMP-15 and provide the first insight into the expression and biological functions of BMP-15 protein. From a physiological perspective, our results led us to propose that BMP-15 is a unique oocyte-specific molecule that coordinates GC proliferation and FSH-dependent cytodifferentiation.
ing folliculogenesis. Because coordinated cell proliferation and differentiation is essential for preovulatory follicle development, our findings could have new implications for understanding fertility and infertility in the female.

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