Stable expression and characterization of N-terminal tagged recombinant human bone morphogenetic protein 15

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ABSTRACT: Oocyte-derived growth factors are critically involved in multiple ovarian processes via paracrine actions. Although recombinant proteins have been applied to dissect the physiological functions of these factors, variation of activities among different protein preparations remains an issue. To further elucidate the roles of one of these growth factors, bone morphogenetic protein 15 (BMP15), in mediating oocyte-regulated molecular and cellular events and to explore its potential clinical application, we engineered the human BMP15 sequence to efficiently produce bioactive recombinant human BMP15 (rhBMP15). The proteolytic cleavage site of the hBMP15 precursor was optimized to facilitate the production of the mature protein, and a FLAG-tag was placed at the N-terminus of the mature region to ease purification and avoid potential interference of the tag with the cystine knot structure. The rhBMP15 protein was purified using anti-FLAG M2 affinity gel. Our results demonstrated that the N-terminal tagged rhBMP15 was efficiently processed in HEK-293 cells. Furthermore, the purified rhBMP15 could activate SMAD1/5/8 and induce the transcription of genes encoding cumulus expansion-related transcripts (Ptx3, Has2, Tnfaf6 and Ptg2), inhibitory SMADs (Smad6 and Smad7), BMP antagonists (Grem1 and Fst), activin/inhibin βA (Inhba) and βB (Inhbb) subunits, etc. Thus, our rhBMP15 containing a genetically modified cleavage sequence and an N-terminal FLAG-tag can be efficiently produced, processed and secreted in a mammalian expression system. The purified rhBMP15 is also biologically active and very stable, and can induce the expression of a variety of mouse granulosa cell genes.

Key words: BMP15 / recombinant protein / oocyte / granulosa cell

Introduction

The oocyte, the female germ cell, has attracted tremendous interest owing to its unique role during follicular development (Eppig et al., 1997, 2002; Eppig, 2001; Matzuk et al., 2002; Gilchrist et al., 2008; Li et al., 2008a). It has been established that oocytes control folliculogenesis, and oocyte-derived growth factors play pivotal roles in regulating the functions of surrounding somatic cells via paracrine pathways (Eppig et al., 1997; Eppig, 2001; Su et al., 2004; Hussein et al., 2005; Gilchrist et al., 2008). The factors secreted by oocytes, especially growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and fibroblast growth factor 8 (FGF8), are crucial regulators of follicular development and female fertility (Dong et al., 1996; Galloway et al., 2000; Yan et al., 2001; Moore et al., 2004; Shimasaki et al., 2004; Su et al., 2004; Juengel and McNatty, 2005; Sugiuira et al., 2007; Matzuk and Lamb, 2008). Both GDF9 and BMP15 are TGFβ superfamily members which are synthesized as prepropeptide precursors containing a signal peptide, a prodomain and a mature domain (Chang et al., 2002). Seven conserved cysteines are normally present in these ligands, and six of them are involved in the formation of intramolecular disulfide bonds, resulting in a ‘cystine knot’ structure that is important for the function and stability of the proteins (Vitt et al., 2001; Berry et al., 2002). However, both GDF9 and BMP15 lack the fourth conserved cysteine which is required for the formation of a covalently linked intermolecular dimer (McPherron and Lee, 1993; Dupe et al., 1998; Laitinen et al., 1998).

The Bmp15 gene is mapped to the X chromosome (Dupe et al., 1998), and mutations in human BMP15 gene have been reported in patients with ovarian failure (Di Pasquale et al., 2004; Dixit et al., 2006). Although BMP15 and GDF9 share the highest homology to each other within the TGFβ family (Wu and Matzuk, 2002), Bmp15 null mice demonstrate cumulus cell dysfunction (Yan et al., 2001)
whereas Gdf9 null mice have defects at the primary follicle stage (Dong et al., 1996; Elvin et al., 1999b). These genetic studies provide compelling evidence that both BMP15 and GDF9 are important oocyte-produced factors that can regulate the ovarian somatic cell functions in the mouse ovary. Moreover, the synergetic roles of the two growth factors in the ovary have been suggested and/or demonstrated by several reports (Yan et al., 2001; Hanahan et al., 2004; Su et al., 2004, 2008; McNatty et al., 2005; McIntosh et al., 2008). The novel functions and regulations of these factors as well as the complex interactions among them are beginning to be unraveled (Hussein et al., 2006; Gilchrist et al., 2008; McIntosh et al., 2008; Yeo et al., 2008), and notably, the utilization of biologically active recombinant proteins is key to these studies.

Epitope tagging is a powerful recombinant DNA approach (Jarvik and Telmer, 1998) and has been applied to understand the functions and signaling cascades of oocyte-secreted factors in the ovary (Hashimoto et al., 2005; Mottershead et al., 2008). A C-terminal FLAG-tagged recombinant human BMP15 (rhBMP15) was previously produced and characterized (Otsuka Fumio et al., 2000). However, the C-terminus of hBMP15 contains two cysteines (CTCR) that are involved in the formation of “cystine knot structure” characteristic of the cystine knot family members (Sun and Davies, 1995). The establishment of a functional cystine knot motif in the tertiary structure of a protein may facilitate its interaction with other factors through the appropriate arrangement of a unique hydrophobic surface of the molecule (Vitt et al., 2001), which is of particular importance in the context of ligand–receptor interactions for BMP15/GDF9 signaling. It is unclear if a C-terminal epitope tag will potentially alter the conformation and activity/stability of the recombinant protein because of its proximity to these cysteine residues. To our knowledge, production of the N-terminal tagged rhBMP15 has not been reported.

It has been documented that activation of TGFβ superfamily ligands is a complex and fine-tuned process where the ligands are synthesized as inactive precursor/preproproteins (signal–preproregion–mature region) which are post-translationally modified by proprotein convertases that enzymatically cleave the propeptide to produce active mature proteins (Massague, 1998; Massague and Chen, 2000; Chang et al., 2002). In our previous study, we used a subtilisin-like serine convertase PACE (paired basic amino acid cleaving enzyme)/furin to obtain efficient processing of BMP15 in CHO cells (unpublished data) to similar to that of mouse GDF9 production (Elvin et al., 1999a). However, the presence of overexpressed PACE/furin in the system may potentially affect the activity of the target proteins produced. By taking advantage of the endogenously produced proprotein convertases through genetic engineering of an optimized cleavage site into the hBMP15 precursor sequence, we efficiently produced stable and mature rhBMP15 protein with an N-terminal FLAG-tag in mammalian cells. Since granulosa cells are known target cells of BMP15 and their functions as well as gene transcriptions are subject to BMP15 regulation (Otsuka et al., 2000, 2001; Otsuka and Shimasaki, 2002; Moore et al., 2003), we demonstrated the bioactivity of the N-terminal tagged rhBMP15 using mouse granulosa cells (mGCs) as an initial step toward exploring the functions, molecular underpinnings and potential clinical applications of BMP15.

### Materials and Methods

**Animals, cell lines and reagents**

Mice were maintained on a mixed C57BL/6/129SvEv genetic background and handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals. HEK-293 cells and 293T cells (HEK-293 cells containing the T-antigen from SV40) were obtained from the Tissue Culture Core at Baylor College of Medicine and maintained at 37°C with 5% CO₂. Fetal bovine serum (FBS), bovine serum albumin (BSA), anti-FLAG M2 affinity gel, 3× FLAG peptide, FLAG-BAP(bacterial alkaline phosphatase) protein standard, mouse anti-FLAG M2 antibody and chromatography column were purchased from Sigma. DME, genetin (G418), HEPES, penicillin–streptomycin, NuPAGE 4–12% or 12% Bis-Tris gel, Superscript III reverse transcriptase, deoxyribonucleotide triphosphate, oligo (dT)₁₂–₁₈ primers, PureLink PCR purification kit and SilverQuest staining kit were obtained from Invitrogen. Pregnant mare serum gonadotrophin (PMSG) was from Calbiochem. Recombinant human BMP4 (rhBMP4), untagged human BMP15 and soluble BMPR2 ectodomain (BMPR2 ECD) were obtained from R&D. All restriction enzymes were from New England Biolabs. FuGENE6, phosphatase inhibitors and proteinase inhibitors were from Roche. Phospho-SMAD1/5/8 antibody was from Cell Signaling. Phusion Hot Start High-Fidelity DNA polymerase was purchased from Finnzymes. TURBO DNA-free DNase was the product of Ambion. Peroxidase-conjugated goat anti-mouse/rabbit antibody was from Jackson ImmunoResearch. RNeasy Micro Kit containing RTL buffer was obtained from Qiagen. Zymoclean gel DNA recovery kit was purchased from ZYMOR Research. SuperSignal West Pico kit and BCA protein assay kit were obtained from Pierce. Taqman Universal PCR master Mix and Taqman gene expression probes were purchased from Applied Biosystems.

**Engineering of rhBMP15 cDNA and expression construct**

All mutations and restriction sites were introduced by PCR using Phusion Hot Start High-Fidelity DNA polymerase. Overlap extension PCR was performed to obtain the sequence encoding the optimized cleavage site and FLAG-tag followed by the mature domain of hBMP15. Briefly, plasmid containing the native hBMP15 coding sequence was used as a template, and amplified with primers PB1 (5’-gagaagttgccgacactgtcctcca g3′) and PB2 (5’-gagttcaagccacacttc-3′). The resultant amplicon, designated as fragment a (Fz), was inserted into the HindIII and BamHI sites of pcDNA3 (designated as construct ABH). The mutation of cleavage site and incorporation of the FLAG-tag were conducted as follows. First, primers PB3 (5’-gagaagttgctgataacag-3′) and PB4 (5’-ggccgcctctgctgctg-3′) were used to generate Fb, which was utilized as a template for primers PB3 and PB5 (5’-ctggtcctactgctgctg-3′) to produce Fc. Then, Fd, which encodes the FLAG-tag and the mature domain, was derived using primers PB6 (5’-gactcaagctgagctggagagag-3′) and PB7 (5’-ggttgctgctgctgctg-3′). Lastly, Fd and Fc were combined and subjected to overlap extension PCR using primers PB3 and PB7, and the amplicon (Fe) was cloned into ABH at BamHI and Xhol sites (designated as pQL-rhBMP15). All PCR fragments were purified by using the PureLink PCR purification kit or Zymoclean gel DNA recovery kit. The identities of all cloned sequences were verified by DNA sequencing (Child Health Research Center Molecular Core Laboratory, Baylor College of Medicine).

**Transient transfection and selection of stable cell clones**

The hBMP15 expression construct (pQL-rhBMP15) was transiently transfected into HEK-293T cells using FuGENE6 transfection reagent according
to the manufacturer’s instructions. The conditioned medium was assayed for the production of rhBMP15 72 h after transfection. To create the stable cell lines, pQL-rhBMP15 was transfected into HEK-293 cells, and the cells were re-plated at a low density 2 days after transfection in the presence of 800 μg/ml G418. G418-resistant cell colonies were selected after 10–14 days. The colonies were then cultured and analyzed for the expression and secretion of rhBMP15 protein using western blot. The selected stable cell lines were maintained in culture medium containing 200 μg/ml G418.

**Protein purification and quantification**

Stable cells expressing rhBMP15 were plated in DMEM containing 10% FBS, 100 U/ml penicillin–streptomycinand 200 μg/ml G418. When the cells reached confluence, DMEM containing 2% FBS and 100 U/ml penicillin–streptomycin was used for the production of rhBMP15, and the medium was collected every 2 days from culture dishes for up to 12 days. Purification of rhBMP15 was conducted using anti-FLAG M2 affinity gel according to the manufacturer’s protocol. Briefly, the conditioned medium containing rhBMP15 and protease inhibitors was incubated overnight at 4°C with the appropriate amount of anti-FLAG M2 affinity gel based on the estimated quantity of recombinant protein in the conditioned medium. After incubation, the resin was collected by centrifugation or filtration through a chromatography column and washed. The rhBMP15 proteins were then eluted with 3× FLAG peptide or 0.1 M glycine HCl (pH 3.5) in TBS (pH 7.5). BSA (1 mg/ml) was added to the protein before being stored at −20°C. The purified proteins were quantified by western blot using FLAG-bacterial alkaline phosphatase (BAP) standards. The control buffer was prepared from the untransfected HEK-293 cell culture using the same protocol. The purity of the purified rhBMP15 was examined by silver staining using a commercially available SilverQuest staining kit.

**Generation of human BMP15 polyclonal antibody**

A cDNA fragment encoding the mature hBMP15 protein (GenBank accession NM_005448) was subcloned into pET23b containing a His-tag (Novagen), and the His-tagged hBMP15 protein was produced in BL21 cells (Novagen) according to the manufacturer’s manual. The hBMP15 fusion protein was used to immunize mice null for Bmp15 (Bmp15−/−) (Yan et al., 2001) to produce the polyclonal antibody according to a protocol consisting of a primary injection and three following boosts. The antisera from the mouse were collected and tested by western blot analyses using medium containing rhBMP15 produced from mammalian cells.

The specificity of the anti-hBMP15 antibody was confirmed by a pre-absorption experiment. Briefly, the anti-hBMP15 serum (1 : 2000) was pre-incubated with rhBMP15 (5 μg/ml) or control buffer overnight at 4°C. Then, the absorbed anti-sera were used to detect 50 ng of rhBMP15 using western blot analysis described below.

**Western blot**

Western blots were carried out as previously described (Li et al., 2008b). In brief, conditioned medium from the transfected/untransfected HEK-293 cells, protein lysates of HEK-293/granulosa cells or purified rhBMP15 were subjected to electrophoresis under reducing or non-reducing conditions on NuPAGE 4–12% or 12% Bis-Tris gel. The separated proteins were then transferred onto nitrocellulose membranes (30 V for 70 min). Membranes were blocked with 3% non-fat milk and incubated with mouse anti-FLAG M2 antibody (1 : 1000 in 3% milk) or rabbit anti-phospho-SMAD1/5/8 antibody (1 : 500 in 1% BSA) overnight at 4°C. Membranes were subsequently probed with peroxidase-conjugated goat anti-mouse or rabbit secondary antibody (1 : 10 000) for 70 min at room temperature. SuperSignal West Pico kit was used to detect the chemiluminescence signal. Quantification of protein signals was performed using NIH Image J software.

**Granulosa cell culture and treatment**

Isolation and culture of mGCs were performed as described elsewhere (Pangas et al., 2006; Li et al., 2008b). Briefly, immature female mice (21–23 days) were primed with 5 IU PMSG (i.p.), and 44–46 h later, large antral follicles were punctured for GC collection. The collection medium was DMEM:F12 containing 0.3% BSA, 100 U/ml penicillin–streptomycin and 10 mM HEPES. To exclude the oocytes, the GC suspension was filtered through a 40 μm nylon mesh and washed twice with collection medium. The mGCs were utilized for the following experiments. (i) SMAD activation analysis: to examine the activation of the SMAD1/5/8 pathway by rhBMP15, mGCs were treated with control buffer, rhBMP15 (100 ng/ml) or untagged hBMP15 (R&D) preincubated with/without the BMPR2 ECD (1 μg/ml), or rhBMP4 (50 ng/ml; positive control for SMAD1/5/8 phosphorylation). Cells were collected after 1 h of treatment. The mGCs were lysed on ice in radio immunoprecipitation assay buffer [50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 0.5% NP-40 and 50 mM NaF] in the presence of proteinase and phosphatase inhibitors. The cell suspensions were then sonicated and cellular proteins collected after centrifugation. Quantification of protein was conducted using BCA protein assay kit. Thirty micrograms of total protein/well were loaded on the gel for western blot analysis. The western blot experiments were repeated twice. (ii) Gene induction assay: the mGCs were treated with rhBMP15 at 0 (control buffer), 30, 50, 100 and 200 ng/ml for a dose–response experiment. For the bioactivity assays, mGCs were treated with rhBMP15 (100 ng/ml), untagged hBMP15 (R&D) or control buffer (control treatment). The control buffer was prepared from the untransfected HEK-293 cell culture medium and contains the same buffer as that in the purified protein. The cells were collected and subjected to RNA isolation after 5 h of treatment.

**Reverse-transcription, PCR, and real-time PCR**

Total RNAs from HEK-293 cells and primary mGCs (5 h of treatment) were isolated using Qiagen RNeasy Mini Kit and Micro Kit, respectively. On-column DNase digestion was performed to eliminate potential genomic DNA contamination according to the protocol of the manufacturer. Additionally, RNA from HEK-293 cells was further treated with TURBO DNA-free DNase prior to reverse transcription (RT). RT was performed using 200 ng (mGCs) or 1 μg (HEK-293 cells) of total RNA and Superscript III reverse transcriptase in a 20 μl reaction volume. One microlitre of RT product was used for PCR amplification (20 μl reaction volume), which was performed using a DNA Engine PTC-200 Peltier Thermal Cycler. PCR primers were designed using Primer Express (Applied Biosystems, Foster City, CA, USA) and listed in Table I. Ten microlitres of the resultant PCR products were separated and visualized on 1% agarose gel containing ethidium bromide. In a parallel experiment, the PCR products (20 μl) were purified and sequenced to confirm their identities (Agencourt Bioscience Corp.). The PCR was repeated twice and DNA sequencing was performed using purified PCR products from one experiment.

Real-time PCR was conducted using the ABI Prism 7500 Sequence Detection System (Applied Biosystems) (Li et al., 2007). The reaction was performed in a 20 μl volume using Taqman Universal PCR Master Mix and Taqman gene expression probes (Ptx3, Mm00477267_g1; Has2, Mm00515089_m1; Tnfalpha, Mm00493736_m1; Pgs2, Mm00478374_m1; Fst, Mm00514982_m1; Inha, Mm03024204_s1; Inhba, Mm00478374_m1; Fst, Mm00514982_m1; Inha, Mm03024204_s1; Inhba,
Table I  Primers for amplification of proprotein convertases

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<th>Reverse</th>
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<tr>
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Mm00434339_m1; Inhbb, Mm01286587_m1; Smad6, Mm00484738_m1; Smad7, Mm00484741_m1; Greml1, Mm03024240_s1; Kif1, Mm00442972_s1). Gapdh was used as the internal control and amplified using a Taqman probe (Part no. 4352339E). All real-time PCR analyses were performed in duplicates, and the results were from at least three independent culture experiments. The abundance of mRNA for target genes was normalized relative to that of Gapdh, and ΔCT was produced by subtracting the mean CT of controls from the CT of each target gene. Fold changes in mRNA expression were calculated using the formula 2^−ΔΔCT (Livak and Schmittgen, 2001).

Statistical analyses

Differences among groups were assessed by ANOVA and the means between individual groups were further compared using Tukey’s honest significant difference test. Comparison of means between two groups was made by student’s t-test. Data are reported as mean ± standard error of the mean (SEM), and P < 0.05 was considered to be statistically significant.

Results and discussion

Genetic engineering of the hBMP15 precursor

Usage of abundantly overexpressed exogenous PACE/Furin as cleavage enzymes has the potential to reduce the efficiency of active rhBMP15 production; hence, we sought to take advantage of endogenously produced proprotein convertases. Using RT–PCR analysis, we found that HEK-293 cells express mRNA transcripts for furin and a number of other proprotein convertases such as proprotein convertase subtilisin/kexin type 2 (Pcsk2), Pcsk5, Pcsk6 and Pcsk7 (Fig. 1).

The von Willebrand factor (vWF) is a secreted glycoprotein that can be efficiently processed by PACE/furin. Notably, the -2 Lys (K) and -4 Arg (R) amino acids upstream of the cleavage site are of functional importance for the efficient propeptide cleavage (Wise et al., 1991; Casonato et al., 2003). We predicted that replacement of the cleavage site of hBMP15 by the consensus cleavage sequence of vWF would enhance the processing of hBMP15 by endogenous PACE/furin and other related proteinases, which may eliminate the necessity of using exogenous PACE for the efficient production of rhBMP15. Therefore, we genetically modified the hBMP15 cleavage sequence (RRTR) based on the consensus cleavage sequence (HRSKR/SLS) of vWF (Casonato et al., 2003) (Fig. 2A). Concomitantly, two glycines (GG) were placed downstream of the cleavage site to minimize potential effects of the FLAG-tag (DYKDDDDK) on the cleavage event (Fig. 2A). The FLAG tag was placed at the N-terminus of the mature BMP15 peptide instead of the C-terminus to preclude its potential influence on the cystine knot structure, which is composed of conserved cysteines among TGFβ family members (Fig. 2B). A perfect Kozak sequence (ggccggcacatgg) was also engineered to ensure the correct and efficient initiation of translation (Kozak, 1986, 1987). The hBMP15 precursor was constructed by overlap extension PCR as depicted in Fig. 2C.

Efficient production of rhBMP15 in mammalian cells

The pQL-rhBMP15 expression construct was transiently transfected into HEK-293T cells, and the conditioned medium was analyzed for secretion of rhBMP15 by western blot. Two immunoreactive bands of rhBMP15 were detected (Fig. 3A, lane 1) using anti-FLAG M2 antibody that can recognize the FLAG-tag at the N-terminus, C-terminus or in the middle of a protein. The predicted molecular weights (MWs) of the mature form and precursor of the N-terminal FLAG-tagged rhBMP15 are 15.3 and 46.4 kDa, respectively. The sizes of the mature forms of N-terminal tagged rhBMP15 under reducing conditions resemble those of the C-terminal FLAG-tagged rhBMP15.
Hashimoto et al. (2005) [i.e. double bands with the apparent MW of 16 and 17 kDa (Fig. 3A, lane 1)]. Recently, it has been shown that the 17 kDa rhBMP15 expressed with a C-terminal tag is O-glycosylated (Saito et al., 2008). Interestingly, when the rhBMP15 protein was analyzed under non-reducing condition, the sizes were ~20 kDa (Fig. 3A, lane 2). The MW difference of rhBMP15 under non-reducing (Fig. 3A, lane 2) and reducing (Fig. 3A, lanes 1 and 3) conditions may reflect the existence of intramolecular disulfide bonds. The purity of rhBMP15 protein was then examined by silver staining (Fig. 3B). Silver staining demonstrated major doublet bands ~15 kDa corresponding to the mature hBMP15 observed by western blot (Fig. 3A, lanes 1 and 3) and a 34 kDa band corresponding to the size of the proregion, which was not detectable by western blot using the anti-FLAG antibody due to the absence of the FLAG-tag. Some minor bands with high MWs were also observed. Indeed, the C-terminal tagged hBMP15 was also a pro-mature complex as previously reported when purified using a similar approach (Saito et al., 2008). Furthermore, the immunoreactive bands of rhBMP15 can be detected using an anti-hBMP15 antibody (Fig. 3C, lane 2). The specificity of the anti-hBMP15 antibody was confirmed in an independent absorption

**Figure 2** Construction of N-terminal FLAG-tagged rhBMP15.

(A) The precursor cleavage sites of vWF (RSKR) and hBMP15 (RRTR) are shown in red. The cleavage site of hBMP15 was changed to that of vWF, and the surrounding sequence was optimized based on that of vWF (dark blue). The FLAG-tag (DYKDDDDK; dark blue) was added immediately preceding the mature domain of hBMP15. Mutations, restriction enzyme sites and a Kozak sequence were introduced by PCR, and FLAG-tag and additional sequence were introduced by PCR with overlap extension. (B) Comparison of conserved cysteines between TGFβ ligands and the oocyte-derived factors BMP15 and GDF9. Similar to TGFβ ligands, most of the known TGFβ family members have seven conserved cysteines (C1–C7) in their protein sequences, among which six (indicated by blue arrows) are involved in the formation of intramolecular disulfide bonds and the cysteine ‘ring-knot’ structure. However, both BMP15 and GDF9 lack the fourth conserved cysteine (red asterisk) that is involved in the formation of covalently linked intermolecular dimers. Note that the positions of the cysteines are not proportionally represented. The question marks represent variable amino acids. (C) Genetic engineering of hBMP15 cDNA. Overlap extension PCR was performed to obtain the sequence encoding the optimized cleavage site and an N-terminal FLAG-tag. See the Materials and Methods section for details. The red vertical line marks the cleavage site, whereas the solid blue bar indicates the position of theFLAG-tag. The blue line indicates the mature domain of hBMP15 on the cDNA fragment Fe, and the green line corresponds with the proregion of hBMP15, which spans part of Fa (cloned into ABH) and Fe. Subcloning of Fe into the ABH construct results in the final rhBMP15 expression construct, pQL-rhBMP15, which contains a signal, a proregion, an optimized cleavage site, a FLAG-tag and a mature domain. Note that the hBMP15 cDNA and the sizes of the PCR fragments are not drawn proportionally.
experiment (Fig. 3D). Pre-absorption of the antibody with rhBMP15 abolished its detection of the specific rhBMP15 signal in the western blot (Fig. 3D).

In support of the enhanced cleavage of the hBMP15 precursor by endogenous proprotein convertases, we found that the rhBMP15 precursor in the conditioned medium was below the limit of detection (Fig. 3A, lane 1); however, it is readily detectable in studies where the native hBMP15 cleavage site was used for recombinant protein production (Hashimoto et al., 2005). The lack of detectable rhBMP15 precursor in the media indicated the efficient cleavage of the precursor, most likely by endogenous PACE/furin. Thus, optimization of the cleavage site facilitated production of the mature and active form of hBMP15.

HEK-293 cell lines stably expressing rhBMP15 were created and used as a convenient source to produce rhBMP15. Conditioned medium containing ≥24 ng/ml of rhBMP15 could be routinely produced. The purified rhBMP15 was quantified by immunoblot using FLAG-BAP standards (Fig. 3E).

N-terminal tagged recombinant hBMP15 activates the SMAD1/5/8 signaling pathway and induces gene expression in mouse granulosa cells

Selection of rhBMP15 dosage for the in vitro studies was based on a dose–response experiment in which 100 ng/ml of rhBMP15 demonstrated robust and consistent induction of mGC genes (Fig. 4). To verify that the rhBMP15 can activate the BMP-responsive SMADs, SMAD1/5/8, mGCs were treated with control buffer (control), purified rhBMP15 (100 ng/ml) or untagged hBMP15 (100 ng/ml) from R&D systems. Recombinant hBMP4 (50 ng/ml) was used as a positive control for SMAD1/5/8 activation. Western blot revealed elevated

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et al. have been used in the literature to examine the bioactivity and functional aspects of BMP15. Using an antibody to BMP15, Western blotting was performed. The antibody was able to detect BMP15 in all samples, indicating that BMP15 is a functional protein in these cells.

We then proceeded to examine the expression of specific genes that are regulated by BMP15. We used quantitative PCR (qPCR) to measure the expression levels of these genes in mGCs treated with BMP15 or control buffer. The results showed that BMP15 induces the expression of several genes, including Kitl, Ptx3, and Hsp90ab1, which are known to be involved in cumulus expansion-related transcripts and cumulus-related transcripts upon stimulation with oocyte/oocyte-derived factor.

For the above reasons, we conducted the initial rhBMP15 bioactivity analysis using primary mGC culture. Interestingly, treatment of mGCs with purified rhBMP15 (100 ng/ml) induced the expression of genes encoding cumulus expansion-related transcripts (Has2, Ptx3, Tnfaip6, and Ptg2) (Fig. 6A–D). BMP antagonists (Fst and Grem1) (Fig. 6J and K), inhibitory SMADs (Smad6 and Smad7) (Fig. 6K and L), and activin/inhibin β subunits (Inhba and Inhibb1) (Fig. 6F and G) within 5 h of treatment. Strikingly, robust stimulation of Has2 and Ptx3 (~90–100-fold) by rhBMP15 was observed (Fig. 6A and B). Remarkable effects of purified mouse GDF9 on these cumulus transcripts were also observed in mGCs (unpublished data), suggesting the competent responsiveness of granulosa cells to these oocyte-derived factors and their involvement in cumulus cell function. To determine if these dramatic effects of gene stimulation were caused by potential alterations of rhBMP15 activity due to the N-terminal FLAG-tag, we examined the ability of the untagged hBMP15 from R&D Systems to induce these transcripts in the mGC culture. Consistently, the untagged hBMP15 was also a potent stimulator of the cumulus expansion-related transcripts, especially Has2 and Ptx3 (Fig. 6M and N), suggesting that the robust induction of gene transcripts by the rhBMP15 is not caused by the incorporation of an N-terminal FLAG-tag.

In contrast to the dramatic effect of rhBMP15 on inducing the aforementioned gene transcripts, the mRNA abundance for Inhba and Kitl was not significantly affected by the N-terminal tagged rhBMP15 in mGCs treated for 5 h (Fig. 6E and H). This finding is in contrast to a previous report that Kitl was up-regulated by the C-terminal tagged rhBMP15 in rat GCs (Otsuka and Shimasaki, 2002). It is unknown if this inconsistency results from the differences of the origins of granulosa cells or the construction/preparation of recombinant proteins. In support of the former, it was noted that the cellular localization pattern of Kitl mRNA transcript in the ovary is distinct between mouse and rat (mural GCs in mouse versus cumulus cells in rat) (Shimasaki et al., 2004). Although the significance of rhBMP15 regulation of the aforementioned genes in mGCs remains unclear, it may have functional implications in critical physiological events associated with follicular development. The activity of this N-terminal tagged rhBMP15 in promoting the expression of glycolytic enzymes [platelet phosphofructokinase (PFKp) and lactate dehydrogenase A (Ldh)] in cumulus cells (Sugiuura et al., 2007) was verified in the presence of FGFBP8 [Dr K. Sugiuura (personal communication)], indicating the functional involvement of BMP15 in glycolysis carried out by cumulus cells. Of note, the N-terminal tagged rhBMP15 was stable, as repeated freezing and thawing (two times) or storage at 4°C for up to 4 weeks did not have detectable effects on its activity in the mGC culture (data not shown).

Thus, in the current study, we efficiently produced biologically active and stable rhBMP15, which has potential applications in assisted reproductive technology to improve infertility treatment (Gilchrist et al., 2008; Li et al., 2008a). Further studies are necessary to achieve a more comprehensive profile of genes induced by the N-terminal tagged rhBMP15 in mGCs. Moreover, the activity of the N-terminal tagged rhBMP15 in modulating BMP-associated biological events/functions in the ovary and/or other systems needs to be examined.
Figure 6 Induction of gene expression by rhBMP15 in mGCs.

mGCs were freshly prepared and treated with control buffer (Con), purified N-terminal tagged rhBMP15 (100 ng/ml) or untagged hBMP15 (R&D; 100 ng/ml). Cells were harvested after 5 h treatment and total RNA was isolated. Real-time PCR analyses of Has2 (A), Ptx3 (B), Tnfap6 (C), Ptgs2 (D), Inha (E), Inhba (F), Inhbb (G), Kitl (H), Fst (I), Grem1 (J), Smad6 (K) and Smad7 (L) mRNA abundance using the control buffer-treated or rhBMP15-treated cells and Taqman probes. Real-time PCR analyses of Has2 (M), Ptx3 (N), Tnfap6 (O), Ptgs2 (P) mRNA abundance using buffer-treated or the untagged hBMP15 (R&D)-treated cells and Taqman probes. Abundance of mRNA for each gene was normalized against that of Gapdh. Fold changes of the treated group relative to controls were calculated using \( \Delta\Delta CT \) method. All PCR experiments were performed in duplicates. Data are mean ± SEM from three independent culture experiments. Bars without a common letter are significantly different at \( P < 0.05 \).
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