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, Tnfαp6 and Ptgs2), 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; Sugihara 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; Dube et al., 1998; Laitinen et al., 1998).

The Bmp15 gene is mapped to the X chromosome (Dube 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 synergistic 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–proregion–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) 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/6j/129SvJEv 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. Oligo-dT (GT)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 ZYMOC 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′-gagaaagcttgccgccaccatggtcctcctca g3′) and PB2 (5′-gagtgccagaccacaccatc-3′). The resultant amplicon, designated as fragment a (Fb), 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′-ggagaaagcttgccgccaccatggtcctcctca g3′) and PB4 (5′-gggccgccgttc ta gtgcgctgctgttcggagag-3′) were used to generate Fb, which was utilized as a template for primers PB3 and PB5 (5′-cttgctgctgctgctgttc ggagag-3′) to produce Fc. Then, Fd, which encodes the FLAG-tag and the mature domain, was derived using primers PB6 (5′-gccagaccgagcaaacacatc taggtgcgctgctgttcgag-3′) and PB7 (5′-gagtgccagaccacaccatc tgtgcgctgctgttc-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 XhoI 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
Production of bioactive human BMP15

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 nude mice for Brom15 (Bmp15+/−) (Yan et al., 2001) to produce the polyclonal antibody according to a protocol consisting of a primary injection and three following boosts. The sera from the mice were collected and tested by 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. 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) 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.

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 l volume using Taqman Universal PCR Master Mix and Taqman gene expression probes (Ptx3, Mm00477267_g1; Has2, Mm00515089_m1; Traf6, Mm00493736_m1; Ptg2, Mm00478374_m1; Fst, Mm00514982_m1; Inha, Mm03024204_s1; Inhba,
The von Willebrand factor (vWF) is a secreted glycoprotein that resembles those of the C-terminal FLAG-tagged rhBMP15. The predicted molecular weights (MWs) of the mature form and precursor of the N-terminal FLAG-tagged rhBMP15 were detected (Fig. 3A, lane 1) using anti-FLAG M2 antibody. The secretion of rhBMP15 by western blot. Two immunoreactive bands in HEK-293 cells. Note that furin, Pcsk2, Pcsk5, Pcsk6 and Pcsk7 mRNA are readily detectable in HEK-293 cells. Gapdh was used as an internal control for PCR. Ten microlitres of the PCR products from a 20 μl reaction volume were separated and visualized on 1% agarose gel containing ethidium bromide. The identities of the amplicons were verified by DNA sequencing. The PCR experiment was repeated twice. Pcsk, proprotein convertase subtilisin/kexin; + or −, RT with or without reverse transcriptase.

**Table I** Primers for amplification of proprotein convertases

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**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 (ggcgcacatgag) 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
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 mg/ml of rhBMP15 could be routinely produced. The purified rhBMP15 was quantified by immunoblot using FLAG-BAP standards (Fig. 3E).

**Figure 3** Expression and purification of N-terminal FLAG-tagged rhBMP15.

(A) Expression of rhBMP15 in mammalian cells under reducing or non-reducing conditions. Lane 1, The rhBMP15 was detected by anti-FLAG M2 antibody under reducing conditions as two bands of ~16 and 17 kDa in the conditioned medium (20 μl) of HEK-293T cells transfected with rhBMP15 expression vector, but was absent in medium from untransfected cells (data not shown). Lane 2, Detection of purified rhBMP15 (20 ng) using anti-FLAG M2 antibody under non-reducing conditions. Lane 3, Detection of purified rhBMP15 (20 ng) using anti-FLAG M2 antibody under reducing conditions. The purified rhBMP15 was obtained from conditioned medium produced by HEK-293 cells stably expressing rhBMP15. (B) Silver staining of purified rhBMP15. Lane 1, Conditioned medium (CM; 20 μl); lane 2, Purified rhBMP15 (100 ng). Note the major doublet bands around 15 kDa relative to the mature hBMP15 observed in western blot and a 34 kDa band corresponding to the size of the proregion. (C) Detection of rhBMP15 by anti-hBMP15 antibody. Recombinant hBMP15 was absent in medium (20 μl) from untransfected HEK-293T cells (lane 1), but present in the medium (20 μl) from pQL-rhBMP15 transfected cells (lane 2). Note the presence of non-specific high MW bands in both transfected and untransfected media. (D) Confirmation of the specificity of anti-hBMP15 body. Fifty nanograms of rhBMP15 were loaded per lane. Although the anti-hBMP15 antibody absorbed with control buffer readily detected the rhBMP15, absorption of the antibody (1:2000) with rhBMP15 (5 μg/ml) blocks its detection of the rhBMP15 signal. Re-probing of both blots with anti-FLAG antibody demonstrated the equal loading of rhBMP15. (E) Quantification of purified rhBMP15 by western blot. One microlitre of purified rhBMP15 (lane 4; arrow head) was analyzed under reducing conditions by western blot and quantified using FLAG-BAP standards (arrow) at 5 ng (lane 1), 10 ng (lane 2) and 20 ng (lane 3). All western blots/silver staining in A–D were repeated at least twice, and the image in E represents one quantification experiment of purified rhBMP15.

**Figure 4** Dose-dependent induction of Ptx3 mRNA by rhBMP15. mGCs were freshly prepared and treated with purified rhBMP15 at 0, 30, 50, 100 and 200 ng/ml. Cells were harvested after 5 h treatment, and total RNA was isolated. Real-time PCR was performed using a Taqman probe to quantify Ptx3 mRNA abundance. Gapdh was used as an internal control. Fold changes of the treated group relative to the control (0 ng/ml of rhBMP15) were calculated using ΔΔCT method. All PCR experiments were performed in duplicates. Data are mean ± SEM from four independent culture experiments. Bars without a common letter are significantly different at P < 0.05.

**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
Production of bioactive human BMP15

Figure 5 Activation of SMAD1/5/8 by N-terminal tagged rhBMP15.

The purified rhBMP15 (100 ng/ml) or untagged hBMP15 (R&D; 100 ng/ml) was preincubated with/without BMPR2 ECD (1 μg/ml) before adding to the mGC culture. mGCs treated with control buffer (control) were used as a negative control, whereas recombinant hBMP4 (50 ng/ml) was included as a positive control for SMAD1/5/8 activation. The cells were collected and proteins immediately prepared after 1 h of treatment. The proteins were quantified by BCA method, and 30 μg of total proteins was resolved by SDS–PAGE. Western blot demonstrated a single band of ~58 kDa, corresponding to the phospho-SMAD1/5/8 (p-SMAD1/5/8). The levels of p-SMAD1/5/8 in mGCs treated with rhBMP15, untagged hBMP15 (R&D) or rhBMP4 were elevated. Pre-incubation with BMPR2 ECD (1 μg/ml) attenuated the p-SMAD1/5/8 levels induced by the rhBMP15 or the untagged hBMP15 (R&D). All western blot experiments were performed at least twice and representative images from one experiment are presented.

As an initial step toward examining the activity of the N-terminal tagged rhBMP15, we analyzed its ability to induce gene expression in the mGC culture since GCS are targets of BMP15 action (Otsuka et al., 2000). BMPs can bind to the type 2 and type 1 receptors and activate SMAD signaling proteins, preferably SMAD1/5/8, to regulate gene transcription (Chang et al., 2002; Shimasaki et al., 2004). To ensure the maximal probability of responsive target identification, the gene candidates selected for the initial analysis of rhBMP15 bioactivity were ideally known targets of both BMPs and oocyte-derived factors such as GDF9. Thus, as the priority candidates for the gene induction assay, we selected several BMP4-induced genes (such as Ptx3, Has2, Peg2, Tnfaip6, Smad6, Smad7 and Grem1) from our previous microarray analysis (unpublished data), since all of these genes except Smad6 and Smad7 are also regulated by GDF9 in mGCs (Elvin et al., 1999a; Pangas et al., 2004; Li et al., 2008b). Additionally, a number of granulosa cell expressed genes of potential interest (Inhba, Inhbb, Inha, Fst and Kitl) were tested. mGCs are an abundant source that can be obtained from hormone-primed ovaries and have been used in the literature to examine the bioactivity and function of oocyte-produced factors (Elvin et al., 1999a; Otsuka et al., 2000; Pangas et al., 2004; McNatty et al., 2005). Moreover, GDF9 can induce cumulus expansion-related transcripts in mGCs (Elvin et al., 1999a; Li et al., 2008b), indicating that mGCs are capable of expressing 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 Peg2) (Fig. 6A–D). BMP antagonists (Fst and Grem1) (Fig. 6I and J), inhibitory SMADs (Smad6 and Smad7) (Fig. 6K and L) and activin/inhibin β subunits (Inhba and Inhbb) (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 (5 h) (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 (PFk) and lactate dehydrogenase A (Ldha)] in cumulus cells (Sugiuura et al., 2007) was verified in the presence of FGF8B [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 gene induction assays (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.
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), Tnfaip6 (C), Ptgs2 (D), Inha (E), Inhba (F), Inhbb (G), KIt (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), Tnfaip6 (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 ΔΔCT 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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