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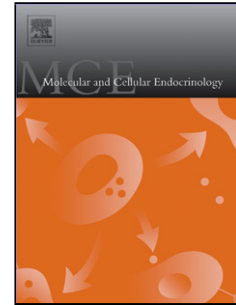
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The bioactivity of human bone morphogenetic protein-15 is sensitive to C-terminal modification: characterization of the purified untagged processed mature region

Minna M. Pulkki^{a*}, Samu Myllymaa^{a*}, Arja Pasternack^a, Stanley Lun^b, Helen Ludlow^c, Ahmed Al-Qahtani^c, Olexandr Korchynskyi^d, Nigel Groome^c, Jennifer L. Juengel^b, Nisse Kalkkinen^e, Mika Laitinen^f, Olli Ritvos^a and David G. Mottershead^{a,g}

^a Department of Bacteriology and Immunology, Haartman Institute, 00014 University of Helsinki, Helsinki, Finland.

^b AgResearch, Wallaceville Animal Research Centre, P.O.Box 40063, Ward Street, Upper Hutt 5140, New Zealand.

^c Centre for Proteins and Peptides, School of Life Sciences, Oxford Brookes University, Headington, Oxford, OX3 0BP.

^d Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands.

^e Protein Chemistry Research Group and Core Facility, Institute of Biotechnology, 00014 University of Helsinki, Finland.

^f Biotechvisions Ltd., Tukholmankatu 8B, 00290 Helsinki, Finland

^g Robinson Institute, School of Paediatrics and Reproductive Health, Medical School, The University of Adelaide, Adelaide 5005, Australia.

*Contributed equally to this study.

Corresponding author at: Department of Bacteriology and Immunology, Haartman Institute, 00014 University of Helsinki, Helsinki, Finland. Tel: +358-9-191 26848; Fax: +358-9-191 26675, Email: Minna.Pulkki@helsinki.fi (Minna M. Pulkki). Present address: Vactech Oy, Biokatu 8, FIN-33520 Tampere, Finland.

ABSTRACT

Oocyte-derived bone morphogenetic protein-15 (BMP15) is critical for the regulation of mammalian fertility. Previously we have found that a C-terminal His₆-tag destroys the bioactivity of growth differentiation-9 (GDF9, a homolog of BMP15). In this study we found that recombinant human BMP15 is produced by HEK-293T cells in an active form, but the bioactivity is lost by C-terminal modification, specifically, fusion to a Flag tag. After purification the mature BMP15wt is active in transcriptional reporter assays specific for Smad1/5/8 in human granulosa-luteal (hGL) and COV434 granulosa tumor cells, whereas BMP15 with a carboxy-terminal Flag tag remains inactive. Using these same cell models we found that treatment with purified mature BMP15wt causes a rapid phosphorylation of Smad1. The purified BMP15wt is a potent stimulator of rat granulosa cell DNA synthesis, which could be antagonized by the BMPRII ectodomain-Fc fusion molecule, whereas the BMP15C-Flag was completely inactive. Further, the BMP15wt form is a potent stimulator of inhibin B production in hGL cells. We found that the purified BMP15wt consists of P16 and -17, both of which are post-translationally modified forms. This is the first characterization of a purified untagged human BMP15 mature region, which is stable and highly bioactive in human and rodent granulosa cells and as such is of importance for studies on human fertility.

Keywords: BMP15, bone morphogenetic protein, granulosa cell, human

1. Introduction

Bone morphogenetic protein-15 (BMP15) also called growth differentiation factor-9B (GDF9B) is an oocyte-derived growth factor and a member of the transforming growth factor- β (TGF- β) superfamily discovered in 1998 by two independent research groups (Dube et al. 1998; Laitinen et al. 1998). BMP15 and its closest homolog growth differentiation factor-9 (GDF9)

(McPherron and Lee 1993; McGrath et al. 1995; Dube et al. 1998; Hayashi et al. 1999; Jaatinen et al. 1999) play crucial roles in the regulation of fertility (Gilchrist et al. 2004a; McNatty et al. 2005c). Mutations in these genes lead to reproductive defects in animals (McNatty et al. 2004) and with the recombinant forms of these growth factors the actions of the oocyte can be mimicked (Gilchrist et al. 2004b). Further, mutations in the human BMP15 or GDF9 genes are associated with polycystic ovary syndrome

(PCOS), premature ovarian failure (POF) or dizygotic twinning (Teixeira Filho et al. 2002; Di Pasquale et al. 2004; Dixit et al. 2005; Di Pasquale et al. 2006; Dixit et al. 2006; Laissue et al. 2006; Palmer et al. 2006). Furthermore, both proteins have been shown to regulate ovulation rate in sheep (Galloway et al. 2000; Juengel et al. 2004). One approach to the evaluation of the role of BMP15 on granulosa cells has been the use of unpurified wild type recombinant BMP15 produced in mammalian cells. Such studies show that the functions of BMP15 vary between species (McNatty et al. 2005b; McNatty et al. 2005a) and that BMP15 enhances oocyte developmental competence (Hussein et al. 2005; Hussein et al. 2006).

An alternative approach for the characterization of the effects of BMP15 on granulosa cells has utilized purified engineered recombinant forms of BMP15 (reviewed in Mottershead and Watson 2009). In particular, quite a number of studies have utilized a recombinant human BMP15 (rhBMP15) tagged with a Flag epitope at the C-terminus of the mature region and purified using anti-Flag M2 Affinity Gel (Otsuka et al. 2001a; Otsuka et al. 2001b; Otsuka and Shimasaki 2002b; Otsuka and Shimasaki 2002a; Moore et al. 2003; McMahan et al. 2008; Saito et al. 2008; Tibaldi et al. 2010). One of these recent studies using the C-terminally Flag tagged rhBMP15 showed that the 17 kDa rhBMP15 is O-glycosylated and the 16 kDa form is phosphorylated (Saito et al. 2008). Further, it was shown that the phosphorylation of the 16 kDa form is essential for rhBMP15 bioactivity (McMahan et al. 2008). In contrast to these reports, the recent study by Li et al. (Li et al. 2009) describes the production and isolation of recombinant hBMP15 tagged with a Flag epitope at the N-terminus of the mature region and purified using anti-Flag M2 Affinity Gel.

We have recently characterized recombinant human GDF9 (hGDF9) signaling in ovarian granulosa cells and found that the position of the affinity purification tag crucially affects GDF9 bioactivity (Mottershead et al. 2008). We found that a C-terminal His₆ tag destroys GDF9 bioactivity, the purified untagged mature region of recombinant human GDF9 being biologically active, exhibiting the ability to activate a Smad3/4 specific transcriptional reporter in human granulosa-luteal cells. Like other members of the superfamily, BMP15 and GDF9 are produced as precursor proteins with the biologically active portion of the protein residing in the C-terminus.

However, BMP15 and its closest homologue GDF9 are unusual within the TGF- β superfamily, as they lack the fourth conserved cysteine residue involved in dimer formation (Laitinen et al. 1998) found in most TGF- β family members (Daopin et al. 1992; Schlunegger et al. 1992; Griffith et al. 1996). In this current study we have characterized the purified recombinant human BMP15wt and – C-Flag (similar to the one in published literature (Otsuka et al. 2000)) mature regions and we find, that like in the case of GDF9, the bioactivity of rhBMP15 is crucially affected by the position of an affinity purification tag.

2. Materials and methods

2.1. Expression vector construction

To prepare human BMP15 expression constructs the coding region of hBMP15 was first amplified from human genomic DNA with following primers: the exon 1 encoding region 5'-AAATGGTCAGAGTGACGTCCCTT and 5'-AGGTACCTCTGTGAGGCCTTGCCAC and the exon 2 encoding region 5'-GGGCTGATTATAGCTATCAGTC and 5'-TCTCCTCCAGTTTCTGGGAAA. The amplified fragments were first subcloned into pGTE vector and thereafter transferred to the pEFIRES-P expression plasmid (Hobbs et al. 1998). Flag and His₆ tagged vectors were constructed by introducing the affinity tag into the pro- or mature region by PCR and by subcloning the pro- and the mature regions into the pEFIRES-P expression vector. Human BMP15 NFlag6H pro-region was amplified with following primers: (5' primer) 5' GAAGATCTCACACCATCACCATCACCATG AACACAGGGCCCAAATGG 3' and (3' primer) 5'GGACTAGTTTATCATCGACGCGCCGGA GAAGAGATTCCCT 3'. Human BMP15 C-Flag mature-region was amplified with following primers: (5' primer) 5' TAGATCTCGGCGCCGCGCAAGCAGATG GTATCTCA 3' and (3' primer) 5' AACTAGTTTATCACTTATCGTCGTCATCCT TGTAATCTCTGCATGTACAAGACTC 3'.

2.2. Protein expression and analysis

Development of a HEK-293T cell line expressing mGDF9wt protein has been previously described (Kaivo-Oja et al. 2003). Cell lines expressing epitope tagged processed human BMP15 were developed by a similar protocol and

were used as sources of recombinant hBMP15 proteins. The purified proteins were analysed on silver gels and Western blots. Reduced protein fractions (with 10 mM DTT) were run in 15% SDS-PAGE gels, and stained with silver nitrate and blotted onto a Hybond C nitrocellulose membrane as described previously (Kaivo-Oja et al. 2003). Blotted membranes were treated with BMP15 specific mAb-28 (1:10,000) and a secondary antibody, peroxidase-conjugated anti-IgG (Jackson ImmunoResearch Laboratories, Inc.; 1:20,000). Immunoreactive proteins were detected using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) or alkaline phosphatase based detection (Bio-Rad). The purified proteins were quantified by measuring absorbance at 280 nm divided by extinction coefficient of the protein.

2.3. Generation of monoclonal antibody for hBMP15

A synthetic peptide corresponding to a region near to the C- terminus of the human BMP15 was selected as the immunogen (prepared by Sigma-Genosys Ltd, UK). The sequence was S-A-E-V-T-A-S-S-S-K-H-S-G-P-E-N-N-Q-C. Five female outbred Tyler Original (T/O) mice (Southend on Sea, Essex, U.K.) were primed with BCG vaccine. Four weeks later, the mice were initially immunised with the immunogen (human BMP15 synthetic peptide/ PPD conjugate) in Freund's incomplete adjuvant. After that, the mice received 3x monthly subcutaneous boosts of 50 µg of the immunogen in Freund's incomplete adjuvant. The animals were killed and the spleens removed for fusion to Sp2/0 murine myeloma cells, following standard procedures. The hybridoma supernatants were initially screened by ELISA against the recombinant hBMP15 coated to Nunc immunoplates by standard protocol (Groome et al. 1990). Reactive clones were expanded and recloned by limiting dilution. These were then rescreened against recombinant BMP15 and the best reacting clones selected prior to expansion and isotyping. As each clone was found to be IgG1, all were purified on a protein G column using a high-salt protocol (Harlow and Lane 1988) before assessment.

2.4. Reagents and growth factors

TGF-β1 was purchased from R&D Systems (Minneapolis, MN). E. coli produced BMP2 was a kind gift from Dr. Peter Mace

(University of Otago, NZ). Fetal calf serum (FCS) was purchased from Euroclone Ltd. (Devon, UK). DMEM and Ham's F-12 were purchased from Invitrogen Life Technologies, Inc. (Gaithersburg, MD). Heparin (Fragmin) was purchased from Pharmacia & Upjohn (Stockholm, Sweden). BSA was purchased from Roche (Mannheim, Germany). Peroxidase-conjugated rabbit antimouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). BMPR2ecd-Fc was produced in mammalian cells and purified in house (Myllymaa et al. 2010).

2.5. Reporter gene constructs

The usage of the pGL3BRE-luciferase reporter plasmid (Korchynskyi and ten Dijke 2002) and generation of the recombinant adenovirus BRE-Luc has been previously described (Mottershead et al. 2008).

2.6. hGL cell cultures

hGL cells were obtained with informed consent from women undergoing *in vitro* fertilization (IVF) treatments. For each experiment, cells from one to six patients were pooled, enzymatically dispersed, and separated from red blood cells by centrifugation through Ficoll-Paque as previously described (Eramaa et al. 1993). Thereafter, hGL cells were plated at a density of 3–4x10⁴ cells/well on 24-well plates (Cellstar, Greiner Bio-one, Frickenhausen, Germany; final concentration, 3–4x10⁴ cells/ml). hGL cells were cultured in DMEM supplemented with 10% FCS, 2 mM l-glutamine, and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin-B; GIBCO). Cells were cultured 1-2 d before adenovirus infections and 2–3 d before ligand stimulation experiments.

2.7. Adenovirus infections

We have previously optimized the usage of a reporter BRE-Luc adenovirus (Mottershead et al. 2008) which was generated accordingly to provided protocol using AdEasy system as described previously (He et al. 1998). Viruses were amplified and titrated in transcomplemental 293A cells and purified with cesium chloride gradient ultracentrifugation as described previously (He et al. 1998). The use of recombinant adenoviruses in hGL cultures has been previously optimized (Bondestam et al.

2002). The hGL cells were infected by incubating the cells with viruses at 37° C in serum-free DMEM supplemented with l-glutamine and antibiotics for 45 min, and DMEM containing 2% FCS was added on top to stop the infection. The cells were then incubated for 24 h before continuing the luciferase-assay experiments.

2.8. Transient transfections and luciferase assays

COV434 cells were plated on 24-well plates and cultured in DMEM supplemented with 10% FCS, 2 mM l-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37° C in 5% CO₂. When the cells became 40-50% confluent, the transfections were performed in 0.5 ml medium with 100 ng/well of the BMP response element (BRE)-luciferase reporter construct (Korchynskiy and ten Dijke 2002) and 20 ng/well β-galactosidase reporter plasmid using the Fugene 6 transfection reagent (Roche Applied Science). Twenty-four hours later COV434 cells were starved 6-8 h in 0.2% FCS DMEM and treated with BMP2 or BMP15 for 24 h. The recombinant BMPRII receptor protein that was used for the neutralization assays consist of a human receptor extracellular domain (ECD) fused to the Fc region of human IgG via a polypeptide linker (receptor ECD/Fc chimera proteins). The purification of BMP2ecd-Fc is described in our recent study (Myllymaa et al. 2010). Ligand and receptor proteins were incubated in 0.2 FCS DMEM in RT for 45 minutes, before adding to the cells. Ad-BRE-luc infected hGL cells were treated with BMP2 or BMP15 in 2% FCS/DMEM for 24 h. The cells were then lysed into 1x passive lysis buffer, and luciferase activity was measured with luciferase assay reagent (Promega Corp., Madison, WI) and normalized to β-galactosidase activity. Data are the mean ±sem of triplicate determinations from representative experiments, relative to an adjusted value of 1.0 for the mean of the control wells. In each assay, each treatment was applied to 3 replicate wells. The bioassays were repeated at least 3 times with COV434 cells or with 3 separate pools of granulosa cells and the data are from representative experiments.

2.9. Analysis of Smad phosphorylation

COV434- and hGL cells were cultured on 24 well plates in DMEM supplemented with 10% FCS, 2 mM l-glutamine, and antibiotics (100 IU/ml penicillin, 100 µg/ml

streptomycin and additionally 0.25 µg/ml amphotericin-B for hGL cells; GIBCO). When COV434 cells reached 80% confluency and hGL cells were cultured 2-3 days, the COV434 cells were incubated with 0.2% FCS DMEM and hGL cells with 1% FCS DMEM for 4-6 hours before stimulations. Subsequent stimulation times varied from 15 to 90 minutes, and cells were thereafter recovered in ice-cold PBS and subsequently lysed in reduced (10 mM DTT) SDS-PAGE sample buffer. The samples were sonicated for 10 sec (amplitude, 5 µm) as described previously (Kumar et al. 2001), boiled 3 minutes, run in 10% SDS-PAGE gels, and blotted onto a Hybond C nitrocellulose membrane. The membranes were blocked for 30 minutes at RT in a 5% (wt/vol) nonfat dried milk in Tris-buffered saline/Nonidet P-40 [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Nonidet P-40] and incubated with the primary antibody in 5% milk in Tris-buffered saline/Nonidet P-40 overnight at 4° C (dilution, 1:1 000 for pS1). After washing, the membranes were incubated with the secondary antibody, a peroxidase conjugated anti-IgG (Jackson ImmunoResearch Laboratories, Inc.; 1:20,000), for 1 h in RT. Immunoreactive proteins were detected using enhanced chemiluminescence reagents (Pierce Biotech). Specificity of the antibodies for phospho-Smads has been previously reported (Persson et al. 1998).

2.10. Measurement of DNA synthesis

Determination of the effects of BMP15 on [³H]-thymidine incorporation of rat granulosa cells was performed as described (McNatty et al. 2005a). All experiments involving rats were approved by the Wallaceville Animal Ethics committee. Briefly, granulosa cells were collected from all surface visible follicles approximately 46 h after i.p. administration of 20 IU eCG (Intervet Ltd, Auckland NZ) to 23-26 day old Sprague-Dawley rats. Isolated cells (20,000 cells per well) were incubated in M199 (Earle's salts; Sigma) with 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 2mM GlutaMax-1 (Invitrogen), 0.23 mM sodium pyruvate (Sigma) 0.3 mg/ml polyvinyl alcohol (Sigma) purified hBMP15wt or -C-Flag tagged mature region at varying concentrations (0.2-400 ng/ml). After 18h of culture, methyl-[³H]-thymidine (Perkin Elmer) was added to each well and cells were harvested for determination of [³H]-thymidine incorporation

6 hours later. In each assay, each treatment was applied to 4 replicate wells. The bioassay was repeated with 3 separate pools of granulosa cells. The average cpm for each treatment was calculated as described for each pool of granulosa cells (McNatty et al. 2005a). Differences between controls and treatments or between treatments were analyzed using the paired t-test function of Microsoft Excel 2003. Data were transformed (natural log) prior to analyses.

2.11. Inhibin B ELISA Assay

We have previously developed an improved Inhibin B ELISA assay (Ludlow et al. 2008). The ELISA consists of monoclonal antibody '46A/F' (recognising the β B subunit) as the capture antibody and 'R1_FAb' (recognising the α subunit) as the detection antibody. Briefly, human granulosa cells were treated with growth factors in 24 well plates in triplicate and media was harvested after 48 hours or every 24 hours (24-96h) in time course assay. These samples were pipetted in duplicate to Nunc Maxisorp plates coated with antibody '46A/F' and incubated o/n. The following day the plates were washed and R1 FAb was added and incubated for 1 h. Plates were washed again, and streptavidin HRP was added for 1 h. Finally plates were washed again, and detection was carried out using TMB substrate, with colour development being detected with iEMS Microplate reader.

2.12. Reversed phase chromatography, in-gel digestion, protein sequencing and mass spectrometry

Protein separation by reversed phase chromatography was performed on a 1 x 150mm Jupiter C4, 5 μ m, 300Å (Phenomenex Inc, USA) column using a SMART™ System (Pharmacia Biotech, Uppsala, Sweden) instrument. Proteins were eluted with a linear gradient of acetonitrile (0-100% in 60min) in 0.1% trifluoroacetic acid. Flow rate was 50 μ l/min and detection at 214nm. For in-gel digestion the P16 and P17 protein bands from SDS-PAGE (Laemmli 1970) were separately in-gel digested with trypsin (Bamford et al. 2005) and the resulting peptides subjected to peptide mass fingerprint analysis by MALDI-TOF mass spectrometry.

N-terminal protein sequencing, after electroblotting from SDS-PAGE to PVDF

membrane (Matsudaira 1987) was performed using a Procise 494A Sequencer (Applied Biosystems, Foster City, CA, USA). MALDI-TOF mass spectrometry was performed in the reflector- or linear ion mode using an Ultraflex TOF/TOF instrument (Bruker-Daltonik GmbH, Bremen, Germany) equipped with a 337 nm UV nitrogen laser. Sinapic acid, α -cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid were used as the matrix. The instrument was externally calibrated using a standard mixture of proteins or peptides (Bruker-Daltonik). Electrospray mass spectrometry was performed using a Q-TOF instrument (Micromass Ltd, Manchester UK) externally calibrated with horse heart myoglobin (MW=16952Da).

3. Results

3.1. Human BMP15 produced by HEK-293T cells is sensitive to C-terminal modification

The different forms of recombinant BMP15 derived from the expression constructs shown in Fig. 1A were produced in stable HEK-293T cell lines. These epitope tagged and untagged versions of BMP15 were detected in medium conditioned by these cell lines utilizing monoclonal antibody 28 (mAb-28), which is specific for an epitope in the human BMP15 protein (see the materials and methods section). The specificity of this antibody for BMP15 was demonstrated via western blot analysis of various conditioned media containing either BMP15 or GDF9 (Fig. 1B). The western blot analysis in Fig. 1B shows that the form 1 is expressed at lower level than the wild type protein or form 2. The various forms of unpurified BMP15 in the conditioned media were tested for bioactivity via monitoring Smad1/5/8 activation. This was accomplished by transducing human granulosa luteal (hGL) cells with an adenovirus encoding the Smad1/5/8 responsive BRE-luciferase reporter containing BMP Smad binding sites derived from the Id1 promoter (Korchynskyi and ten Dijke 2002; Mottershead et al. 2008). Similarly to mouse GDF9 (mGDF9) (Mottershead et al. 2008), hBMP15 is produced by HEK-293T cells in an active form, but the bioactivity is lost by C-terminal modification (Fig. 1C). As shown in figure 1B, there is less form 1 than the wild type protein in the conditioned media. This is consistent with the bioactivity response of form 1

being lower than the response of the wild type protein (Fig. 1C).

3.2. The C-terminally Flag-tagged rhBMP15 mature region does not show any bioactivity as the purified mature region

Initially, an N-terminally His₆-tagged BMP15 mature region was produced and purified, which was found to activate the Smad1/5/8 responsive (BRE-luc) reporter in hGL cells in a dose dependent manner (data not shown). This is consistent with a recent study by Li et al. (Li et al. 2009). A preferable situation however, is to be able to purify the protein of interest (in this case hBMP15) without modification by the addition of any epitope tags. We determined in a previous study (Mottershead et al. 2008) that the purified untagged mature region of recombinant human GDF9 is biologically active. The approach used in that study was subsequently used to purify rhBMP15 from conditioned media. This approach was also used to purify a C-terminally Flag tagged rhBMP15 mature region for comparative purposes since previously in the literature a number of studies have used such a protein (Otsuka et al. 2001a; Otsuka et al. 2001b; Otsuka and Shimasaki 2002b; Otsuka and Shimasaki 2002a; Liao et al. 2003; Moore et al. 2003; McMahon et al. 2008; Saito et al. 2008; Tibaldi et al. 2010). The purified rhBMP15wt and -C-Flag mature regions appear as two distinct bands corresponding to approximately 16 and 17 kDa (further referred to as P16 and P17 respectively) on a silver stained SDS-PAGE gel or a Western blot probed with mAb-28 (Fig. 2A). The purified rhBMP15wt was found to activate the Smad1/5/8 signaling pathway in hGL cells in a dose dependent manner, whereas the C-terminally Flag-tagged rhBMP15 was inactive (Fig. 2 B). As the hGL cells have undergone a process of terminal differentiation in response to a luteinizing surge of gonadotropins, we wanted to characterize the effect of the purified rhBMP15 on granulosa cells which have not become terminally differentiated. An immortalized human granulosa cell line COV434 was chosen as it has retained the ability to produce estradiol upon FSH stimulation, thus demonstrating the presence of functional FSH receptors (Zhang et al. 2000). The COV434 cells were transiently transfected with the Smad1/5/8 responsive transcriptional reporter construct, BRE-luciferase (Korchynskiy and ten Dijke 2002) and treated with the purified rhBMP15wt mature

region, which activated BRE-luciferase reporter in a dose responsive manner (Fig. 2 C). The purified untagged hBMP15 was found to be very stable as storage at 4°C for up to four months did not have any effects on its activity in the COV434 transcription reporter (BRE-luc) assays (data not shown). Longer storage periods than four months were not tested.

3.3. Human BMP15wt activates the phosphorylation of Smad1

To further confirm the activation of the BMP signaling pathway by the purified rhBMP15 we investigated the time course of Smad activation via phospho-Smad western analysis (Fig 3). Lysates of hGL and COV434 cells stimulated with BMP2 or purified rhBMP15wt were subjected to SDS-PAGE and immunostained after transfer to membranes using phospho-Smad1 (pS1) antiserum that specifically recognises the phosphorylated C-tail of Smad1 (Persson et al. 1998). Following 90 minutes stimulation of hGL cells or 15 minutes stimulation of COV434 cells with rhBMP15wt (200 ng/ml), there was a pronounced increase in phospho-Smad 1, which was also observed in the lysates treated with BMP2 (100 ng/ml). Lysates from untreated (control) cells stimulated with DMEM containing 1% FCS (hGL) or 0.2% FCS (COV434) did not show detectable Smad 1 phosphorylation. All the membranes were probed with anti-β-actin antibody (Sigma-Aldrich) to show equal loading.

3.4. Human BMP15 stimulates rat granulosa cell [³H]-thymidine incorporation, which is antagonised by BMP2ectd-Fc.

Previously we have used the incorporation of [³H]-thymidine in primary cultures of rat granulosa cells as an additional *in vitro* model system for monitoring oocyte-derived growth factor effects in granulosa cells, and have reported that GDF9 is active in such a bioassay (Mottershead et al. 2008). We also find that our purified rhBMP15wt is highly active in this assay (Fig 4A). Consistent with the observed stimulation in DNA synthesis being due to the presence of the purified rhBMP15wt, we found that pre-incubation of the sample with the BMPRII ectodomain-Fc fusion molecule (BMP2ectd-Fc) caused a dose dependent inhibition of bioactivity (Fig. 4B). The purified rhBMP15-C-Flag protein was completely inactive in the rat granulosa cell [³H]-thymidine

incorporation assay (Fig. 4C) clearly demonstrating that the stimulation of DNA synthesis by the purified rhBMP15wt sample (Fig. 4A) was due to the presence of the rhBMP15wt protein and the epitope tag at the C-terminus of the rhBMP15 mature region destroys the bioactivity associated with this protein.

3.5. Human BMP15 stimulates inhibin B production in cultured human granulosa luteal cells

We have previously shown that GDF9 stimulates the production of inhibin B by human granulosa luteal cells (Kaivo-Oja et al. 2003; Roh et al. 2003; Kaivo-Oja et al. 2005). In this study, we find that the purified recombinant human BMP15wt protein also stimulates inhibin B production in human granulosa cells in dose responsive manner (Fig 5A). Further, a time course effect on the inhibin B production in human granulosa cells by rhBMP15wt protein was seen (Fig 5B).

3.6. Physio-chemical characterization of the purified rhBMP15 mature region.

Our purified BMP15 consists of two related peptide species P16 and P17 (Fig. 2A) and efforts to resolve these via reverse phase HPLC were not successful. For further characterization of the P16 / P17 mixture we subjected a sample to molecular mass determination by mass spectrometry. The results indicated the presence of essentially two peaks with molecular masses of 13917.0 Da and 14864.0 Da. Tryptic peptide mass fingerprint analysis of the in-gel digested P16 and P17 showed with a reasonable peptide coverage (>85%) that both proteins represent BMP15. In the mass fingerprint of P16 a unique peptide with a protonated $m/z = 1433.6$ Da corresponding to the blocked N-terminal tryptic peptide <QADGISA EVTASSSK (MW=1432.695 Da) was found. This peptide mass could not be found in the tryptic peptide mass fingerprint from P17 but instead a unique peptide with a protonated mass $m/z = 2380.9$ Da could be found which is suggested to represent the O-linked glycosylated N-terminal tryptic peptide of P17 (Saito et al. 2008). When analysing the tryptic peptides from P16 in negative ion reflector mode, a peptide mass $m/z = 1511.69$ Da corresponding to the phosphorylated N-terminal tryptic peptide was

detected in low amounts together with the corresponding non-phosphorylated peptide.

4. Discussion

In the current study we show that although BMP15 is produced by HEK-293T cells in an active form, the bioactivity is lost by C-terminal modification of the protein. The main purpose of this study was to produce and purify recombinant human BMP15 in sufficient quantities for subsequent characterization of the protein. First, we introduced the His₆ tag at the amino terminus of the BMP15 mature region. Specifically, the tag was inserted between the 4th and 5th amino acid after the furin consensus sequence with the aim of minimizing the impact of the tag on the processing of the protein (Wolfrain et al. 2002). Conditioned media containing this his6 tagged modified BMP15 and the respective purified form of the protein were both biologically active, indicating that N-terminal modification of the mature region of BMP15 does not overly impair bioactivity (data not shown), which is consistent with the recent study by Li et al. (Li et al. 2009). In a separate study we discovered that a C-terminal His₆ tag destroys GDF9 bioactivity, and although human GDF9 is produced by 293T cells in a latent form, the purified untagged mature region of human GDF9 is biologically active (Mottershead et al. 2008). Hence, in the current study we characterized untagged and C-terminally Flag-tagged BMP15 that were produced and purified via the same protocol that was previously used to obtain the purified GDF9. Both the purified and unpurified untagged BMP15 were potent activators of the Smad1/5/8 (BRE-luc) responsive transcriptional reporter in human granulosa cell bioassays. In contrast, both the purified and unpurified BMP15-C-Flag were inactive in the BRE-luc reporter assay.

In this article, we report that the bioactivity of rhBMP15 is lost by C-terminal modification, which is not in agreement with the published literature in the field, since part of the previous studies have been done using rhBMP15 Flag-tagged at the C-terminus of the mature region (Otsuka et al. 2000; Otsuka et al. 2001a; Otsuka et al. 2001b; Otsuka and Shimasaki 2002b; Otsuka and Shimasaki 2002a; Liao et al. 2003; Moore et al. 2003; McMahon et al. 2008; Saito et al. 2008; Tibaldi et al. 2010). It needs to be emphasized that the majority of the recombinant

BMP15 secreted from our 293T cells is as a non-covalent complex of the processed mature region bound to the corresponding proregion. This is also the case with the BMP15 produced by the Shimasaki group, however the two constructs, i.e. ours and that used by the Shimasaki group are not identical. In particular our BMP15 Form 2 expression plasmid (with a C-terminal Flag tag, see Fig. 1A) incorporates a Flag tag at both the N-terminus of the pro-region, as well as the C-terminus of the mature region. The Flag tag is highly negatively charged, and an explanation of the difference between previously published results and ours would be that in our case the N-terminal Flag tag repels the C-terminal Flag tag, which we suggest tends to cause separation of the pro-region from the mature region as the protein is secreted into the culture medium. Under these circumstances we suggest that the C-terminal Flag tags on the mature region cause separation of the mature region dimer, and hence inactivation of the protein. This scenario is consistent with the very poor yields we obtain of the C-Flag BMP15 compared to untagged BMP15 using the purification procedure of Biotechvisions, even though the C-Flag form is expressed at least as well as the untagged form.

In the case of the previously published C-Flag BMP15 (Otsuka et al. 2000; Otsuka et al. 2001a; Otsuka et al. 2001b; Otsuka and Shimasaki 2002b; Otsuka and Shimasaki 2002a; Liao et al. 2003; Moore et al. 2003; McMahan et al. 2008; Saito et al. 2008; Tibaldi et al. 2010), after purification the pro-region remains present with the mature region (Saito et al. 2008) and we suggest that the mature region bioactivity is stabilized by the presence of the pro-region. In contrast our purified BMP15 (both Forms 1 and 2) is free of pro-region, which in the case of Form 1 (no Flag tag) is biologically active, whereas Form 2, the Flag tagged form completely lacks bioactivity. Our studies highlight the effect of such a modification as a C-terminal Flag tag to the structural integrity of BMP15, and such modifications can not be recommended. Indeed, we show that they are not necessary as an untagged form of the human BMP15 mature region is possible to produce and purify, and is highly bioactive. Another recombinant form of BMP15 that has also appeared in the published literature is a BMP15 with a C-terminal Myc-His6 tag (Di Pasquale et al. 2004). Although this form has been shown to be biologically active (Di Pasquale et al. 2004; Fabre et al. 2006; Elis et al. 2007) it is likely that this form is also stabilised by

the presence of the pro-region, and may exhibit compromised bioactivity if the modified mature region alone was used.

In the present study, we monitored the effect of rhBMP15 on granulosa cell proliferation in primary rat granulosa cell cultures. It has been shown in previous studies, that rhBMP15 C-Flag directly stimulates the mitosis of rat GCs (Otsuka et al. 2000; Moore et al. 2003). The mitosis of rat GCs by rhBMP15 C-Flag has been shown to be antagonized by BMPR2ecd-Fc (Moore et al. 2003). However, we find in our study that the purified rhBMP15 C-Flag is completely inactive, whereas the wild type rhBMP15 is a potent stimulator of mitogenesis in rat granulosa cells and this effect is antagonized by BMPR2ecd-Fc. These results are all consistent with the above explanation of the basis for the difference between the Biotechvisions C-Flag BMP15 preparation and the previously published C-Flag BMP15 form.

Previous studies have shown that GDF9 (a close homolog of BMP15) stimulates inhibin B production in human granulosa cells (Kaivo-Oja et al. 2003; Roh et al. 2003; Kaivo-Oja et al. 2005; Huang et al. 2009; Shi et al. 2009a; Shi et al. 2009b). Here we are showing that rhBMP15wt protein stimulates inhibin B production in human granulosa cells in a dose responsive manner. We also find that TGF- β stimulates inhibin B production in human granulosa cells (Fig. 5A and Kaivo-Oja et al. 2005) and this response is seen at much lower levels of the ligand. However, it cannot be extrapolated from these results that TGF- β would be the more significant factor in vivo in the regulation of inhibin B levels as TGF- β is stored in vivo as an inactive complex. Hence to reveal the bioactivity of the TGF- β mature fragment in vivo activation of the complex must take place (Annes et al. 2003). Further, our observation that BMP15 stimulates inhibin B production is well in line with our previous report indicating that activation of both the activin and BMP Smad pathways results in significantly stimulated inhibin B production in human granulosa cells (Bondestam et al. 2002).

The calculated average molecular mass of the rhBMP15wt mature form starting from <Q(1) and ending with R(125) is 13923.9 Da. Our results indicated the mixture of P16 and P17 was 13917.0 Da and 14864.0 Da respectively. Assuming that rhBMP15wt contains 3 disulfide bridges (6 x Cys residues) the calculated molecular mass is 13917.9 Da. Thus P16

represents the unmodified polypeptide <Q(1)-R(125) without any modifications other than the three disulfide bridges. The larger mass 14864.0 Da then represents P17 with a modification (947 Da) which according to Saito et al. (Saito et al. 2008) is a mucin-type O-glycan present at a threonine (T10) residue. The presence of the O-glycan on the N-terminal tryptic peptide from P17 was further demonstrated as a $m/z=2380.914$ Da peptide from the tryptic peptide mass fingerprint. However, the physiological significance of the O-glycosylation is not known. Saito et al. (Saito et al. 2008) found that part of C-terminally tagged rhBMP15 P16 was modified by phosphorylation. Our peptide mass fingerprint analyses demonstrated the presence of low amounts of phosphorylation on the N-terminal tryptic peptide of P16. In our study only a minor part of P16 was phosphorylated as indicated by the lack of the mass of the phosphorylated protein in the direct mass spectrometric analysis. Despite the low level of phosphorylation of P16, it may well serve a regulatory role. Like the C-terminally Flag tagged rhBMP15 in McMahon et al. 2008 (McMahon et al. 2008), our untagged rhBMP15 mature region also stained using Pro-Q diamond phosphoprotein stain (data not shown). It can be suggested that part of the protein is phosphorylated and it might serve as a regulatory component for hBMP15 bioactivity as shown in previous studies (McMahon et al. 2008).

Clearly, different preparations of purified rhBMP15 have different characteristics. Thus, it has become apparent that the field of reproductive biology requires a consistent, accurate, and reliable source of recombinant rhBMP15 which can be used in studies by workers in this field. We conclude that the wild type rhBMP15 mature region protein is the most reliable form of rhBMP15 for biological studies.

In conclusion, our results demonstrate that the unpurified rhBMP15 wild type protein activates the Smad1/5/8 signaling pathway, but the bioactivity is lost by C-terminal modification. Similarly to GDF9 (Mottershead et al. 2008), the bioactivity of rhBMP15 is crucially affected by the position of an affinity purification tag. Understanding the molecular mechanisms behind the full biological activity of rhBMP15 will help clarify its functions in the ovary, having implications for fertility management and the treatment of ovarian diseases.

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Disclosure statement

The authors have nothing to disclose.

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Vitae

**Minna M Pulkki, MSc**

2008 - present **Research Scientist, Vactech Oy, Tampere**
2005 - present **PhD student, laboratory of Molecular reproductive medicine, Haartman Institute, Helsinki**
2006 - 2008 **R&D associate, Biotechvisions Ltd., Helsinki**
2004 - 2005 **Undergraduate student, Biomedicum Helsinki, Dept. of Bacteriology and Immunology, Program for developmental and reproductive biology, University of Helsinki**
2005 **Master of Science in cell biology (University of Jyväskylä, Department of biological and environmental sciences)**

**Samu Myllymaa, MSc**

2009 - present **Specialist of chemical risk assessment, REACHLaw Ltd**
2004 - present **PhD student**
2004 - 2008 **R&D associate, Biotechvisions Ltd**
2003 - 2004 **MSc student**
2001 - 2002 **Project scientist**
2001 **Junior researcher**
2007 **Master of Science (biotechnology)**
2002 **Bachelor of engineering**



Arja Pasternack, PhD

2007 - present senior scientist, University of Helsinki, Haartman Institute
 2004 - 2008 R&D scientist, Biotechvisions Ltd
 2004- 2006 Senior scientist, University of Helsinki, Developmental and Reproductive biology, Biomedicum Helsinki
 2000- 2003 Senior scientist, University of Helsinki, Institute of biotechnology
 1998- 2000 Scientist, University of Helsinki, Department of Biosciences
 1994-1997 Scientist, VTT Biotechnology
 1992-1993 Assistant, University of Joensuu
 2000 PhD University of Helsinki (Biochemistry).
 1992 MSc University of Joensuu (Biology).



Stanley Lun

2008 - present Technician, Mesynthes Ltd
 2001 - 2007 Research Associate, Agresearch, Wallaceville
 1980 - 2001 Technical Officer, Ministry of Agriculture and Fisheries Wallaceville
 1974 - 1980 Graduate Technologist, Dept of Medicine Christchurch Clinical School of Medicine



Helen Ludlow, MSc

2009 – present Molecular Scientist, Analytical Support, Syntaxin Ltd, Abingdon
 2009 – 2009 Research Associate, Institute of Women’s Health, UCL, London
 2005 – 2009 PhD student, School of Life Sciences, Oxford Brookes University, Oxford
 2005 – 2005 Senior Laboratory Technician, Oxford Immunotec Ltd, Milton Park, Abingdon
 2003 – 2005 Laboratory Technician, Oxford Immunotec Ltd, Milton Park, Abingdon
 2000 – 2003 Undergraduate student, BSc (hons), 2:1 – Cell and Molecular Biology, School of Life Sciences, Oxford Brookes University, Oxford



Ahmed Al-Qahtani

2009 - present CEO, Peak Medical (PeakMed), Riyadh, Saudi Arabia
2007 - 2008 Head of Medical Division, HSHC Ltd., Saudi Arabia
2002 – 2006 PhD student, Oxford Brookes University, UK
2000 - 2002 Graduate Student, Wolverhampton University, UK. Clinical laboratory Sciences
1997 – 2000 Biomedical Scientist, Pathology Department, King Abdulaziz Naval Base Hospital, Saudi Arabia
1992 – 1996 Undergraduate student, Prince Sultan College of Health Sciences, Medical Laboratory Sciences



Olexandr Korchynskyi, PhD

2008 - 2010 Research Scientist, Molecular Cell Biology, Leiden Univ. Medical Center, Leiden, The Netherlands.
2006 - 2007 Postdoc, Dept. of Orthopaedic Surgery, Virginia Commonwealth University, Richmond VA, U.S.A.
2005 - 2006 Visiting Scientist, Prof. M.Karin's lab, University of California @ San Diego, La Jolla, CA, U.S.A.
2002 – 2005 Postdoctoral Res. Associate, TARC, UNC-CH, NC, U.S.A.
2000 – 2002 Ph.D. Fellow, Dept.Cell. Biochem., The Netherlands Cancer Inst., Amsterdam, NL.
1992 – 2000 Senior Researcher - Junior Scientist - Scientist. Division Regulatory Cell Systems, Inst. Biochemistry, Natl.Acad.Sci.of Ukraine, Lviv, Ukraine
1989 - 1992 Post-graduate student
1993 PhD. in Biochemistry
1989 MSc in Biochemistry



Nigel Patrick Groome, Professor of Applied Immunology

1992 - present Professor of Applied Immunology, Oxford Polytechnic/Oxford Brookes University
 1985 - 1992 Reader, Oxford Polytechnic
 1978 - 1985 Senior Lecturer, Oxford Polytechnic
 1970 - 1978 Lecturer in biochemistry, Bromley College of Technology, Kent
 1978 MSc (part time) London University, Immunology
 1975 PhD (part-time) London University, Virology
 1970 MSc Birmingham University, Virology
 1969 BSc (honours) 2.1 Birmingham University, Biochemistry



Jennifer L. Juengel, PhD

2006 - present Section Manager, Reproductive Biology, AgResearch Limited, New Zealand
 2001 - 2006 Senior Scientist, Reproductive Biology, AgResearch Limited, New Zealand
 1998 - 2001 Scientist, Reproductive Biology Group, AgResearch Limited, New Zealand
 1992 - 1998 Postdoctoral Fellow, Department of Physiology, Colorado State University, USA
 1992 PhD Department of Animal Science, Reproductive Physiology, University of Missouri, USA
 1990 MS Department of Animal Science, Reproductive Physiology, University of Missouri, USA
 1987 BS Department of Animal Science, Michigan State University, USA



Nisse Kalkkinen, PhD

1992 – present Laboratory Director. Protein Chemistry Research Group, Institute of Biotechnology, University of Helsinki
 1991 Chief Engineer. Institute of Biotechnology, University of Helsinki
 1984 - 1990 Laboratory Engineer. Recombinant-DNA Laboratory and Institute of Biotechnology, University of Helsinki
 1975 - 1983 Research Assistant. Department of Biochemistry, University of Helsinki
 1981 Doctor of Philosophy (Biochemistry), University of Helsinki
 1978 Master of Science (Biochemistry), University of Helsinki



Mika Laitinen, PhD

2008 – present Clinical fellow, Helsinki University Central Hospital
 2001 - present CEO, Biotechvisions Ltd, Helsinki, Finland
 2004 Postdoc, University of Otago, Dunedin, New Zealand
 2000 - 2003 Postdoc, University of Helsinki
 2000 - 2003 Clinical fellow, Jorvi Hospital, Espoo, Finland
 2000 PhD, University of Helsinki
 1996 MD, University of Helsinki



Olli Ritvos, Professor

2008 - present Professor of Medical Biotechnology, University of Helsinki
 2008 Visiting professor, Karolinska Institutet, Stockholm, Sweden
 2006 - 2007 Senior professorial fellow, Academy of Finland, Helsinki
 2004 - 2006 Senior scientist, Finnish Cancer Institute, Helsinki
 2003 Visiting scientist, Ludwig Institute of Cancer Research, Uppsala, Sweden
 2002 Visiting professor, Karolinska Institutet, Stockholm, Sweden
 1997 - 2002 Senior scientist, Academy of Finland, Helsinki
 1995 - 1996 EMBO fellow, visiting scientist, University College London, U.K.
 1992 - 1997 Junior scientist, Academy of Finland, Helsinki
 1991 Junior post doc scientist, University of Helsinki
 1990 PhD, University of Helsinki
 1990 MD, University of Helsinki



David Mottershead, PhD, Docent

I received my BSc (Hons) in 1984 and my PhD in 1990, both from the University of Adelaide. My PhD studies focused on growth factors and autocrine growth of kidney cells. I worked for 4 years as a postdoc in Boston, studying retroviral superantigens. I worked from 1995-2008 in Helsinki, the last 5 years as the head of the R&D team of Biotechvisions working on oocyte-derived growth factors. At the beginning of 2009 I returned to Adelaide to join the Robinson Institute, at the University of Adelaide to establish my own research group.

Figures

Figure 1.

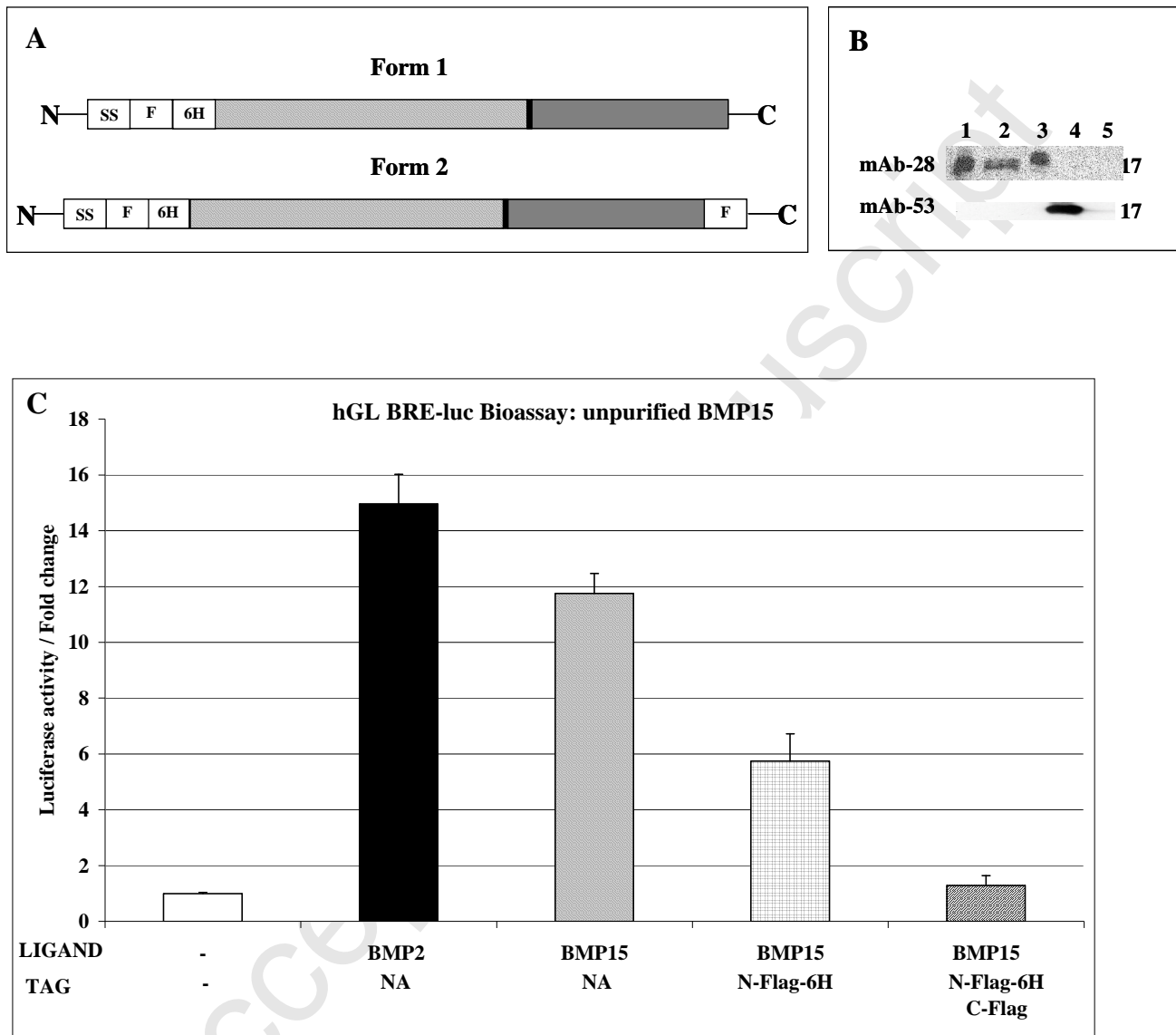
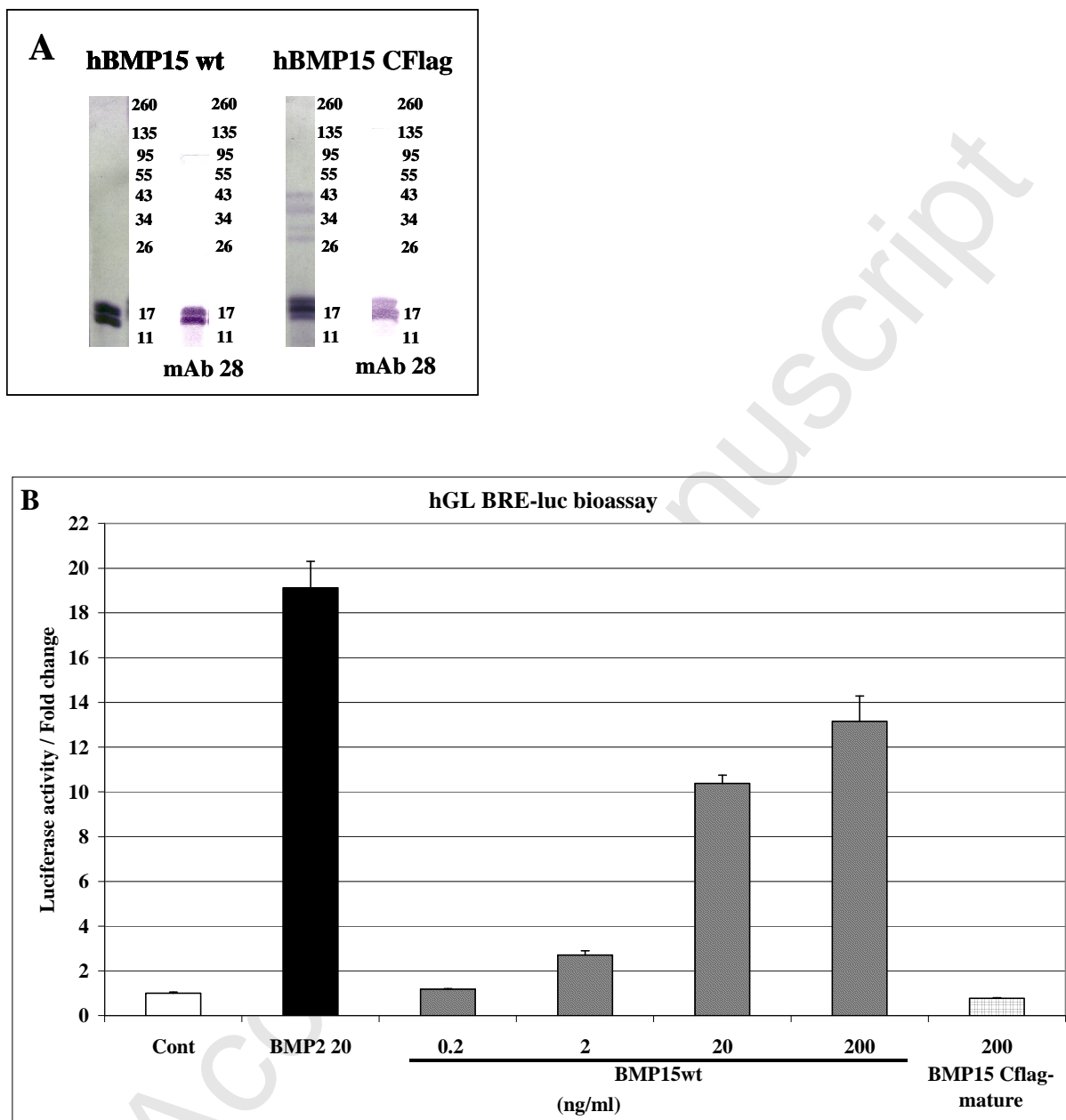


Figure 2.



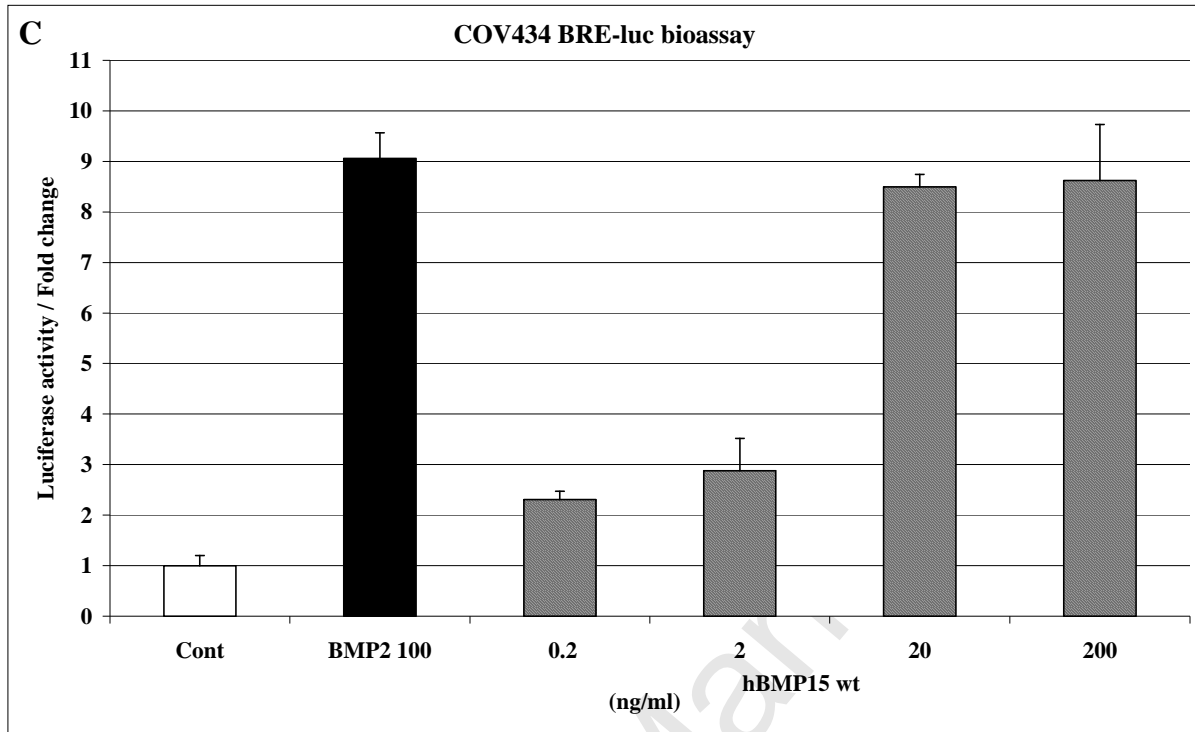


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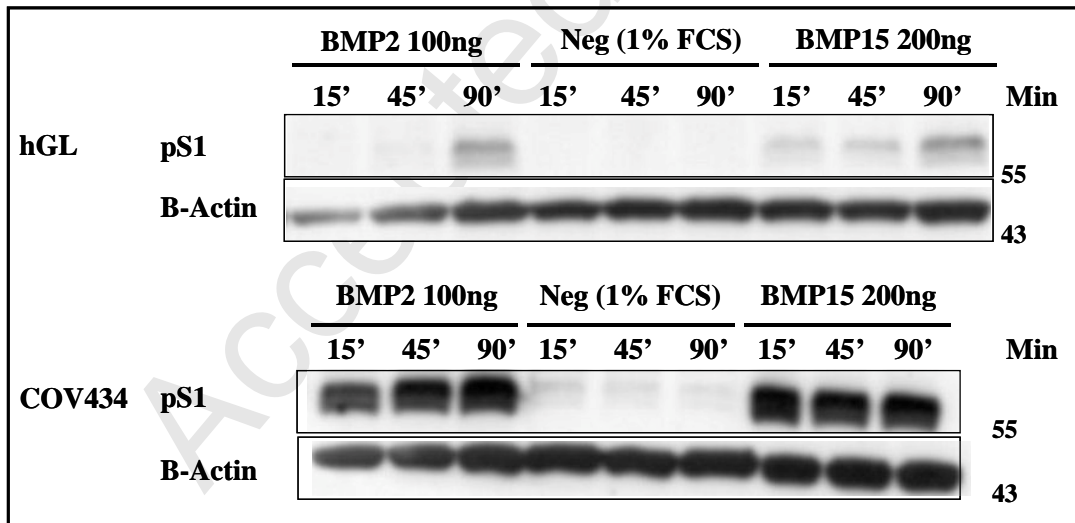


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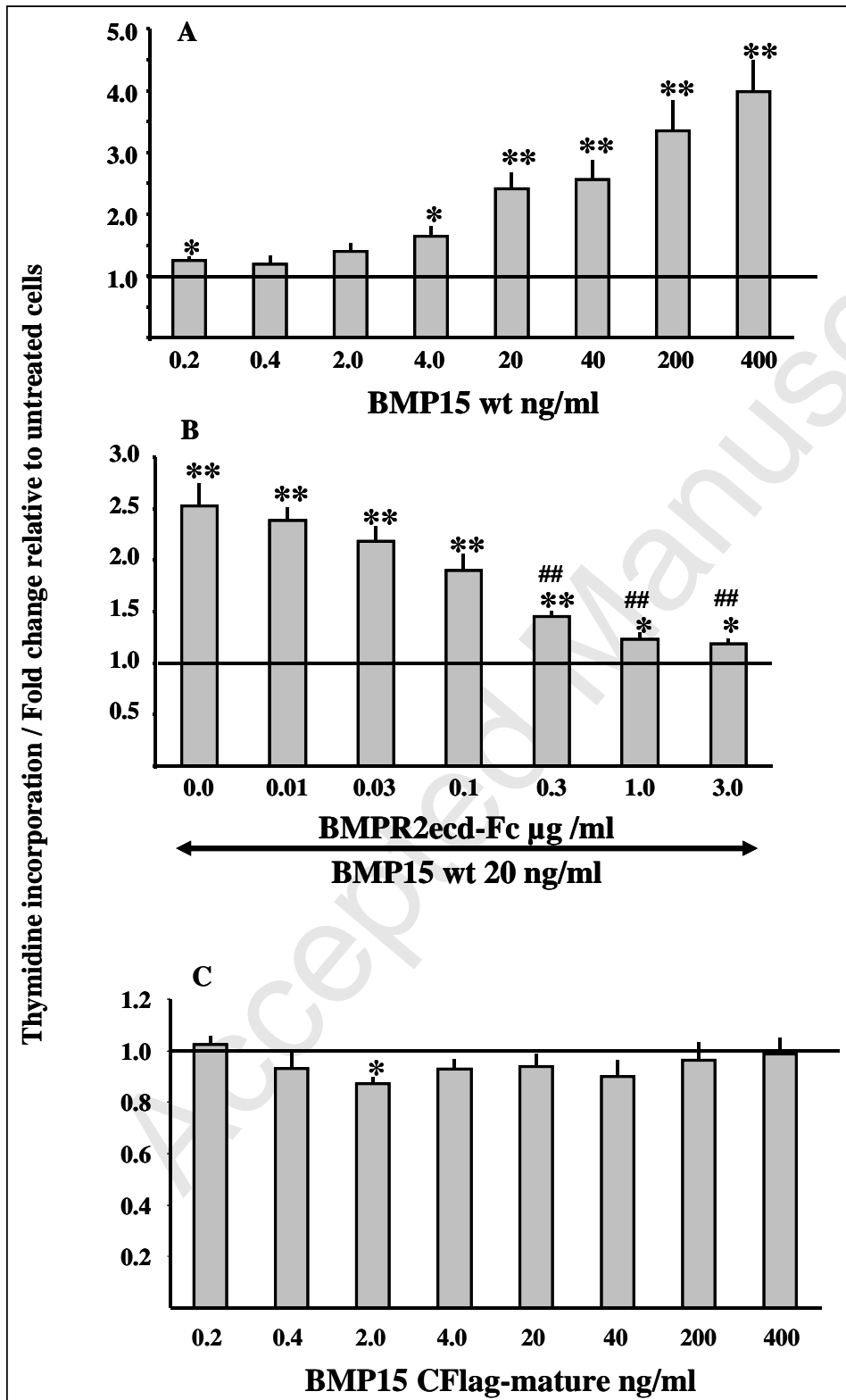


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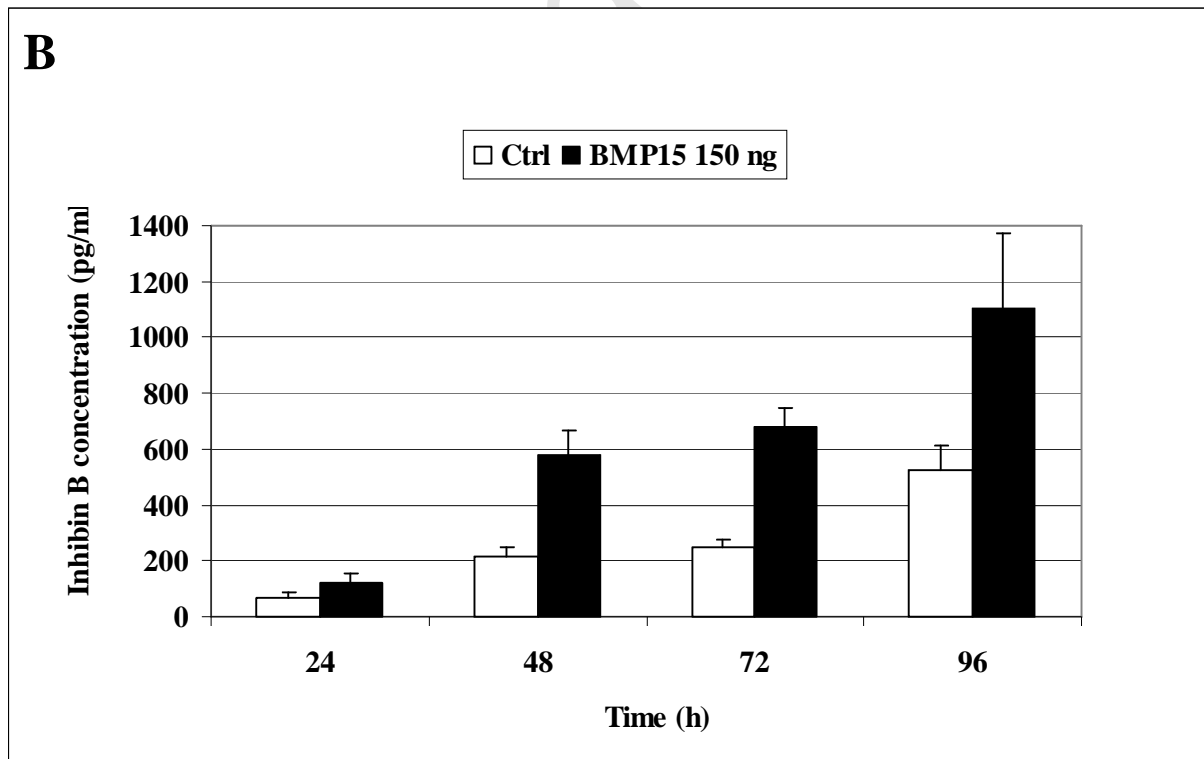
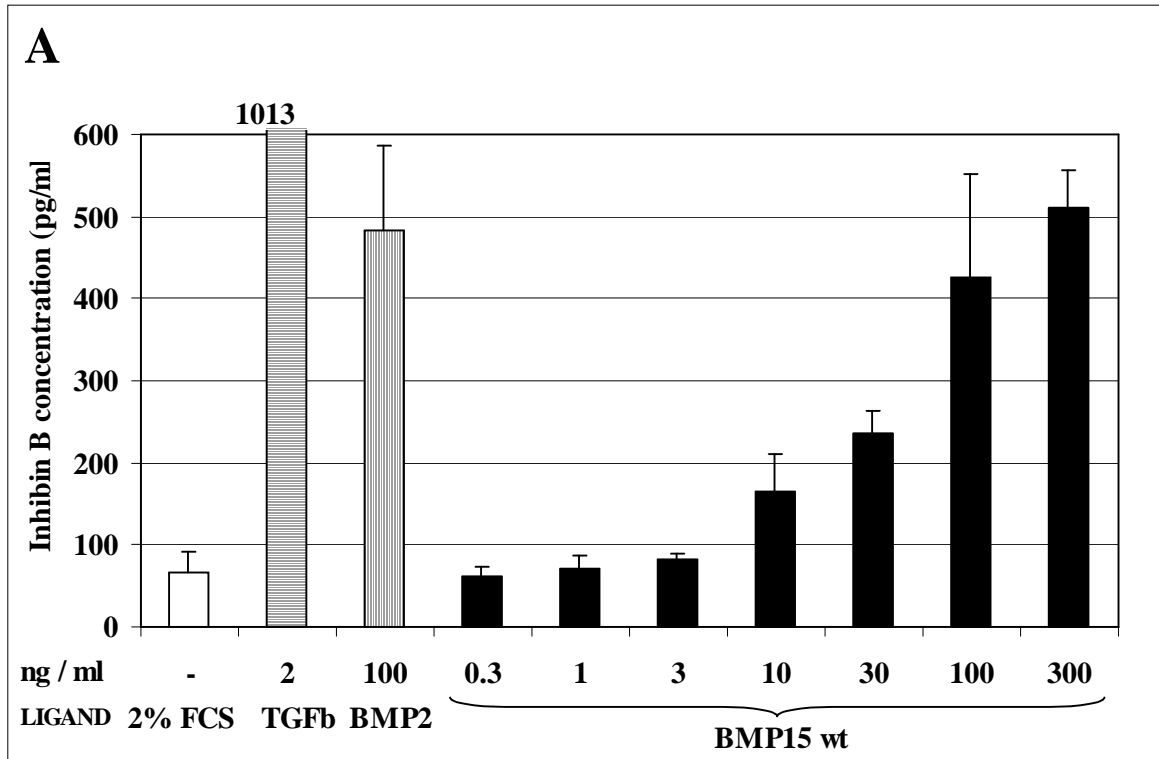


Figure legends

Fig. 1. Analysis of unpurified forms of recombinant BMP15. A. Schematic of the two epitope tagged proteins produced in this study: 1 has only the N-terminal Flag and His6 tags, whereas form 2 also has a C-terminal Flag tag (SS: signal sequence, diagonal lines: Pro-region, Black: Proteolytic processing site, 6H: His6-tag, F: Flag-tag, Grey: Mature region). B. Recombinant proteins produced from stable HEK-293T cell lines and subjected to SDS-PAGE immunoblotting (ECL). 1: BMP15wt, 2: BMP15 Form 1, 3: BMP15 Form 2 (with C-terminal Flag tag), 4: GDF9 wt (recognized by the GDF9 specific mAb-53 (Gilchrist et al. 2004b)), 5: TGF- β 200 ng. All samples were reduced with 10 mM DTT before running into the gels. The specific BMP15 mAb-28 reveals the 16-17 kDa mature human BMP15 protein. The mAb-28 does not cross-react with GDF9, showing the specificity of the antibody to BMP15. C. Bioactivities of unpurified recombinant BMP15 forms on human granulosa-luteal cells, transduced with an adenovirus encoding the Smad1/5 (BRE-luc) reporter, were incubated for 24 h in the absence (cont) or presence of BMP2 (50 ng/ml), BMP15 wild type conditioned medium (CM), BMP15 Form 1 CM or BMP15 Form 2 CM (with C-Flag). Enzyme activity was measured in the cell extract and is expressed as fold change compared with the control (an adjusted value of 1.0 for the mean of the control wells), *i.e.* the activity level in the absence of ligand. (N = N-terminus, F = Flag-tag, 6H = His6-tag, C = C-terminus.)

Fig. 2. Purified BMP15wt mature region activates the Smad1/5 pathway in ovarian granulosa cells in a dose dependent manner, whereas the C-terminally Flag-tagged rhBMP15 mature region does not show any bioactivity as the purified mature region. A. The purified human BMP15wt and -C-Flag mature regions were analyzed on a silver gel and on a western blot probed with the BMP15 specific mAb-28. The samples were reduced with 10 mM DTT before running into SDS-PAGE gels. B. hGL cells transduced with an adenovirus encoding the Smad1/5 (BRE-luc) reporter were incubated for 24 h in the absence (cont) or presence of BMP2 (20 ng/ml), rpHPLC purified BMP15wt or rhBMP15 C-Flag mature region. C. COV434 cells transfected with the Smad1/5 (BRE-luc) reporter were incubated for 24 h in the absence (an adjusted value of 1.0 for the mean of the control wells) or presence of BMP2 (100ng/ml) or various concentrations of rpHPLC purified rhBMP15wt mature region. Human BMP15 activated the Smad1/5 signaling pathway in hGL and COV434 cells in a dose dependent manner.

Fig. 3. Purified rhBMP15wt mature region phosphorylates Smad1 in human ovarian granulosa cells. Before Smad activation experiments, the primary human granulosa luteal (hGL) cells were first cultured 2-3 days or COV434 cells were cultured until they reached 80% confluency. After 4-6 h low serum starvation period (1% FCS DMEM for hGL and 0.2% FCS DMEM for COV434 cells), the cells were treated with BMP2 (100ng/ml) or purified rhBMP15wt protein (200ng/ml) for the indicated time points (minutes). The cell lysates from hGL and COV434 cells were subjected to SDS-PAGE immunoblotting using the pS1 antiserum. Treatment with purified rhBMP15wt protein caused a rapid phosphorylation of Smad1.

Fig. 4. Human BMP15wt stimulates rat granulosa cell [3 H]-thymidine incorporation, which is antagonised by BMPRII ectodomain-Fc fusion molecule (BMPR2ecd-Fc). A. Human BMP15wt protein stimulates rat ovarian granulosa cell [3 H]-thymidine incorporation in a dose dependent manner, B. which is neutralized with 1 μ g/ml of BMPR2ecd-Fc at the control level. C. The C-terminally Flag-tagged rhBMP15 is inactive. (* P<0.05, ** P<0.01 compared to untreated control; ## P<0.01 compared to 20 ng/ml BMP15wt with 0 ng/ml BMPR2ecd-Fc.)

Fig. 5. Human BMP15 stimulates inhibin B production in cultured human granulosa luteal cells. Three different cell pools were treated with 0.3-300 ng/ml hBMP15wt protein for 48 hours or with 150 ng/ml hBMP15wt protein for 24, 48, 72 or 92 hours and the spent media were harvested for the measurement of inhibin B concentrations with an inhibin B ELISA. Data was similar in three different human granulosa luteal cell pools (each pool contains cells from 2-6 women) and representative experiments are shown. A. Human BMP15wt protein stimulates human granulosa luteal cell inhibin B production in a dose dependent manner. B. Time course of hBMP15wt action on inhibin B production in human granulosa luteal cells. The

basal level of inhibin B production rises significantly upon stimulating the cells 96 hours with 2% FCS DMEM.

Potential referees

Aaron J.W. Hsueh, Ph.D.

E-mail: aaron.hsueh@stanford.edu

Department of Obstetrics and Gynecology, Program of Reproductive and Stem Cell Biology, Stanford University School of Medicine, Stanford, CA 94305-5317.

Professor Alan McNeilly

E-mail: a.mcneilly@hrsu.mrc.ac.uk

Medical Research Council Human Reproductive Sciences Unit, Centre for Reproductive Biology, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, UK.

Jenny Visser, Ph.D.

E-mail: j.visser@erasmusmc.nl

Department of Internal Medicine, Erasmus MC, PO Box 2040, 3000 CA Rotterdam, The Netherlands