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Molecular forms of ruminant BMP15 and GDF9 and putative interactions with receptors

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Abstract

Bone morphogenetic factor 15 (BMP15) and growth differentiation factor 9 (GDF9) are oocyte-secreted factors with demonstrable effects on ovarian follicular development and ovulation rate. However, the molecular forms of BMP15 and GDF9 produced by oocytes remain unclear. The aims herein, using Western blotting (WB) procedures with specific monoclonal antibodies (mabs), were to identify the molecular forms of BMP15 and GDF9 synthesised and secreted by isolated ovine (o) and bovine (b) oocytes *in vitro*. The mabs were known to recognise the biological forms of BMP15 or GDF9 since they had previously been shown to inhibit their bioactivities *in vitro* and *in vivo*. Using recombinant variants of oBMP15 and oGDF9, including a cysteine mutant form of oBMP15 (S356C) and a human (h) BMP15:GDF9 heterodimer (cumulin), it was established that the mabs were able to identify monomeric, dimeric, promature and higher-molecular-weight forms of BMP15 and GDF9 and cumulin (GDF9 mab only). After using non-reducing, reducing and reducing + cross-linking conditions, the major oocyte-secreted forms of o and b BMP15 and GDF9 were the cleaved and uncleaved monomeric forms of the promature proteins. There was no evidence for dimeric or heterodimeric forms of either mature BMP15 or GDF9. From *in silico* modelling studies using transforming growth factor beta (TGFB), activin or BMP crystal templates, and both present and previously published data, a model is proposed to illustrate how the monomeric forms of BMP15 and GDF9 may interact with their type II and type I cell-surface receptors to initiate the synergistic actions of these growth factors.

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Introduction

The oocyte-secreted growth factors, bone morphogenetic protein 15 (BMP15) and/or growth differentiation factor 9 (GDF9), are essential for ovarian follicular growth and are key determinants of ovulation rate (McNatty *et al.* 2005*a*, Juengel *et al.* 2009, Monestier *et al.* 2014). In sheep, homozygous-inactivating mutations in *BMP15* or *GDF9* inhibit ovarian follicular growth, heterozygous-inactivating mutations in either of these genes lead to increases in ovulation rate and the effects of heterozygous-inactivating mutations in both growth factors lead to additive or multiplicative effects on ovulation rate (McNatty *et al.* 2006). A current hypothesis for the role of BMP15 and GDF9 is that the increased ovulation rate in mutant sheep is directly related to the extent of loss of function of either one or both of these genes and or a related receptor (McNatty *et al.* 2004, Fabre *et al.* 2006). In ruminants, *in vivo* immunisation studies demonstrate that BMP15 and GDF9 proteins act in a paracrine manner to influence cumulus and granulosa cell functions at most, if not all, phases of follicular growth including ovulation rate outcomes (Juengel *et al.* 2002, 2004, 2009). The protein forms that are present in ovarian follicular fluid have not been investigated in many species using validated and/or specific antibodies. However, in sheep, the predominant forms of BMP15 and GDF9 in follicular fluid, when tested by Western blotting (WB) under reducing conditions, were the monomeric promature proteins with no detectable levels of mature monomers, dimers or heterodimers (McNatty *et al.* 2006). In cattle, both promature and mature forms of BMP15 and GDF9 have also been observed in follicular fluid when tested by WB under reducing conditions (Behrouzi *et al.* 2016).

BMP15 and GDF9 are two closely related members of the transforming growth factor beta (TGFB) superfamily. In general, members of this superfamily are produced as promature proteins, with both the pro- and mature regions being enzymatically cleaved from one another to form, covalently linked mature dimers (Massague 1998). A characteristic 'cysteine knot' stabilising the structure of the mature growth factor domain is formed by six conserved cysteine residues with a seventh cysteine being involved in forming the covalent bonds between the mature proteins (McDonald & Hendrickson 1993). These dimers are thought to signal via a receptor–ligand complex following the binding of a dimeric proteins to two type I receptors and two type II receptors (Wrana *et al.* 1994). Upon formation of this receptor–ligand complex, the constitutively active type II receptor

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phosphorylates and activates a highly conserved glycine- and serine-rich region of the type I receptor, termed the GS domain (Wrana *et al.* 1994). In turn, the type I receptor recruits and phosphorylates intracellular signalling molecules (Miyazono 1998, Fujii *et al.* 1999).

In contrast to most TGFB family members, BMP15 and GDF9 belong to a small sub-family where this seventh cysteine has been substituted by a serine, so that dimers, if present, can only associate in a non-covalent manner. Moreover, current evidence suggests that both BMP15 and GDF9 are secreted partially in unprocessed forms as both cleaved (pro- and mature domains enzymatically cleaved but still associated non-covalently) and/or uncleaved (covalently bound) promature proteins along with some mature growth factor domains (McNatty *et al.* 2006, McIntosh *et al.* 2008, Lin *et al.* 2012, Mester *et al.* 2015). Although a dimeric form of recombinant mature ovine (o) and mouse (m) GDF9 has been identified (Edwards *et al.* 2008, McIntosh *et al.* 2008), mature oBMP15 domains have only been found as monomers *in vitro* or *in vivo*. However, Pulkki and coworkers (2012) have reported dimeric forms of recombinant human (h) BMP15 under cross-linking conditions.

Notwithstanding the synergistic effects of recombinant BMP15 and GDF9 reported in both *in vivo* (Yan *et al.* 2001, Hanrahan *et al.* 2004) and *in vitro* studies (McNatty *et al.* 2005*b*, Edwards *et al.* 2008, McIntosh *et al.* 2008, Reader *et al.* 2011, Mottershead *et al.* 2012, 2015, Peng *et al.* 2013), it remains to be established whether heterodimers of BMP15:GDF9 are present in biological fluids. Furthermore, the mechanism(s) to explain how oocyte-derived BMP15 co-operates with GDF9 and/or with its receptors to regulate somatic cell responses remain obscure.

The aims of this study, using WB procedures with specific monoclonal antibodies (mabs), were to identify the molecular forms of BMP15 and GDF9 synthesised and secreted by ovine and bovine oocytes *in vitro*. Moreover, by utilising recently published information on the crystal structures of BMP9, TGFB1 and Activin A, and using both the current as well as previously reported data, a hypothetical model was generated to explain how the predominant oocyte-secreted forms of BMP15 and GDF9 interact with their cell-surface receptors to produce a synergistic biological response.

Materials and methods

Oocyte collection

Ovine and bovine ovaries were collected at a local abattoir (Taylor Preston, Wellington, New Zealand) and transported to the laboratory at room temperature. Subsequently, the ovine ovaries were dissected free of extraneous tissues and rinsed twice in sterile phosphate buffered saline (PBS; 0.15M, pH7.4; Sigma). They were then dissected free of the medullary blood supply and cumulus cell-oocyte complexes (COC) were recovered by flushing surface visible follicles with dissection medium (M199 (Gibco, Life Technologies, Auckland, New Zealand) supplemented with 20 mM Hepes (Sigma)) using a 20G needle fitted to a 5 mL syringe. The bovine ovaries were rinsed twice in PBS but did not have any non-ovarian tissue removed prior to oocyte collection. Thereafter, the bovine COCs were collected by aspiration of follicular fluid into dissection medium using a 20G needle connected to a Venturi vacuum pump. The isolated ovine and bovine COCs were denuded of most of their cumulus cells by repeated passage through a 20G needle and then recovered and washed using a 10 µL micro-pipette and fresh dissection medium. Subsequently, both the bovine and ovine oocytes were washed once in incubation medium (M199 supplemented with $NaHCO_3$ (0.22% w/v; Sigma), Glutamax (2 mM; Life Technologies) and PenStrep (100 IU penicillin + 100 µg/mL streptomycin; Life Technologies)). Thereafter, pools of 100–130 oocytes were either immediately frozen at −20°C or incubated for 18 h in 50 µL incubation medium at 37°C in 5% CO_2 in air. The time taken from ovary collection to oocyte incubation was ≤4 h. Following incubation, the media and the oocytes were separated by centrifugation at 300 ***g*** for 2 min, with 50 µL of fresh incubation medium added back to the cultured oocytes and each stored at −20°C until analysed. Protease inhibitors (complete, EDTA-free protease inhibitor cocktail, Roche) were added to all samples prior to storage at −20°C.

Generation of BMP15 and GDF9 constructs

The ovine proregion-ovine mature *BMP15* wild-type (o/oBMP15) construct was prepared as previously described (McNatty *et al.* 2005*a*). The DNA sequence encoding the human proregion-ovine mature *BMP15 (S356C)* (i.e. mutant *h/oBMP15 (S356C)*) inserted into the cloning vector pGEM-T Easy was a kind gift from Dr DG Mottershead (The University of Adelaide, Australia). A human prodomain was used to increase the concentrations of BMP15 forms present in expression medium (Dr D M Mottershead, personal communication). The *h/oBMP15(S356C)* sequence is comprised of a rat albumin signal sequence, an *8His-tag*, a linker region, a human *BMP15* proregion and an ovine mature domain containing a mutation whereby the fourth cysteine has been reintroduced at amino acid position 356 (S356C). The *h/oBMP15 (S356C)* construct was subcloned into the expression vector *pEFIRES-P* (Hobbs *et al.* 1998) between the *XhoI* and *XbaI* sites and transfected into human embryonic kidney cells (HEK-293) with Fugene HD transfection reagent (Roche Diagnostics NZ Ltd, Auckland, NZ). The ovine proregion-ovine mature *GDF9* wild-type (*o/oGDF9*) construct was prepared as previously described (McNatty *et al.* 2005*a*).

Purification of o/oBMP15 and h/oBMP15 (S356C)

Expression medium (EM) containing o/oBMP15 or h/oBMP15 (S356C) was modified to include 5 mM imidazole, 300 mM NaCl and 1× complete EDTA-free protease inhibitor cocktail (Roche) at pH 7.4. Subsequently, the EM was incubated with nickel-nitrilotriacetic acid-agarose resin (Qiagen) on a rotary mixer at 4°C for 2 h, centrifuged at 200 ***g*** for 5 min and the supernatant was removed. The resin was washed further with 50 mM Tris–HCl, 300 mM NaCl and 20 mM imidazole, pH 7.4, and the His-tagged recombinant proteins were eluted

from the nickel-nitrilotriacetic acid-agarose using 15× 1 mL elution buffer (50 mM Tris–HCl pH7.4, 20 mM imidazole 300 mM NaCl, and 7 M Urea). The eluted material was loaded onto a reverse-phase HPLC column (Jupiter, 5 µM, C4, 300 Å; Phenomenix, Lane Cove, NSW, Australia) equilibrated with 0.1% v/v trifluoroacetic acid (TFA), and the different promature and mature forms were eluted via a linear gradient of acetonitrile. Before being interrogated further by WB, the HPLC fractions were evaporated to near dryness and made up to 40 µL with 4 mM HCl.

Validity of the antibodies for determining the molecular forms of BMP15 and GDF9

The production of HEK-293 expressed o/oBMP15 protein has been described previously (McNatty *et al.* 2005*a*). A similar protocol was used to generate a cell line producing h/oBMP15 (S356C) and o/oGDF9 proteins as well as a control cell line containing a *pEFIRES-P* expression vector lacking an insert for *BMP15* or *GDF9*. These cell lines were used as sources of recombinant proteins, which, along with oocyte lysates and oocyte incubation medium, were analysed by WB. Preliminary experiments using 13.5% native gels were performed. However, all detectable immunoreactive bands of BMP15 and GDF9 for both recombinant and oocyte-derived material remained as very-high-molecular-weight complexes close to the stacking/resolving gel interface. Consequently, this approach to examining any possible promature and lower molecular weight forms was discontinued. Thereafter, WB's were run on 13.5% w/v SDS-PAGE gels, under reducing, non-reducing or reducing + cross-linking conditions. Briefly, preparation of samples for cross-linking using the non-cleavable cross-linker bis(sulfosuccinimidyl)suberate (BS^3; ThermoFisher Scientific) involved expression media containing either o/oGDF9 o/oBMP15, h/oBMP15 (S356C), o/oGDF9 + o/oBMP15 or o/oGDF9 + h/oBMP15 (S356C) being incubated overnight at 4°C. Subsequently, the expression media, lysates of oocytes and oocyte incubation media were cross-linked by the addition of 7× BS^3 to a final concentration of 2 mM and incubated for 2 h at 4°C. Cross-linked samples were then mixed with 5× reducing sample buffer (20% v/v β-mercaptoethanol). Non-cross-linked samples were mixed with either 5× reducing sample buffer or 5× non-reducing sample buffer. The proteins were then blotted onto nitrocellulose membranes (Hybond C, GE Biosciences) as described previously (McIntosh *et al.* 2008).

Immunoblotting of the membranes was performed using a BMP15-specific monoclonal antibody mab61A (McNatty *et al.* 2005*a*) with detection of immunoreactive bands by chemiluminescence. Although mab61A has previously been used under reducing conditions to identify ovine (McNatty *et al.* 2005*a*), mouse (McIntosh *et al.* 2008) and rat (Lin *et al.* 2012) BMP15, it has not previously been validated for use in bovine oocytes. To test the ability of mab61A to recognise BMP15 proteins, mab61A was pre-adsorbed with *E. coli*-derived oBMP15 prior to use. Briefly, a 20× antibody solution was incubated overnight at 4°C with 20× mass of the *E. coli*-derived oBMP15. Immediately before use, the pre-adsorbed antibody was centrifuged at 2400 ***g*** for 5 min and the supernatant was diluted 1:20 with blocking buffer prior to being incubated with the nitrocellulose membrane. The epitope recognised by mab61A is located within the sequence SEVPGPSREHDGPES situated near to the N-terminus of the mature domain of oBMP15 (McNatty *et al.* 2005*a*). There is 100% homology across this region between ovine and bovine BMP15. As previous studies of recombinant oBMP15 were undertaken under reducing conditions without evidence that mab61A was capable of identifying homodimers, WB studies were undertaken with the recombinant h/oBMP15 (S356C), and with an *E. coli*-derived recombinant oBMP15, to determine if the antibody could recognise dimeric forms of recombinant BMP15. Additionally, the sensitivity of mab61A to detect monomeric and dimeric forms of mature BMP15 was determined by employing a dilution series of *E. coli*-derived recombinant oBMP15.

The anti-GDF9 IgG, mab37, has previously been validated for identifying GDF9 in ovine oocytes (Lin *et al.* 2012) as well as recombinant GDF9 in ovine and murine species (McNatty *et al.* 2005*a*, Edwards *et al.* 2008, McIntosh *et al.* 2008). Moreover, it has been used to identify dimeric GDF9 from recombinant oGDF9 and *E. coli*-derived oGDF9, but not heterodimers of recombinant oGDF9 + oBMP15 when both growth factors were added together to generate a synergistic response (Edwards *et al.* 2008). However, mab37A has not previously been validated for use in the bovine, and it remains uncertain as to whether it is capable of identifying heterodimers of GDF9 and BMP15. The epitope recognised by mab37A has not been determined definitively, but the recognition site lies within the first 32 amino acids of the N-terminal end of mature GDF9. Therefore, to establish that mab37A was able to recognise bovine GDF9, WB studies were undertaken using mab37A preabsorbed with 10 µg/mL *E. coli*-derived oGDF9 in a similar manner to that reported for mab61A. As mab37A is known to recognise recombinant hGDF9 (unpublished data), WB experiments were undertaken using an IMAC-purified recombinant human cumulin preparation (Mottershead *et al.* 2015), kindly supplied by Dr Jeremy Thompson and Lesley Ritter (The University of Adelaide, Australia), to determine whether heterodimeric forms of GDF9 and BMP15 could be identified. Mab61A does not recognise recombinant hBMP15 as the targeted ovine peptide sequence is different from that present in the human. Thus, after confirming this in preliminary studies, further WB studies using the mab61A antibody were not performed with the cumulin preparation.

An additional mab (mab53/1; supplied by Oxford Brookes University, UK) was used to test the molecular forms of human cumulin alongside those identified with mab37A. Mab53/1 had previously been used to identify the BMP15:GDF9 heterodimer by Mottershead and coworkers (2015).

Testing of biological activities of the recombinant proteins

Ovaries were collected from Sprague-Dawley rats (21–27 days old) with the approval of the Victoria University of Wellington Animal Ethics Committee in accordance with the 1999 Animal Welfare Act (Part 6) of New Zealand. The ovaries were dissected away from surrounding tissue and then rinsed

twice in dissection medium (DM; M199 media (Invitrogen) containing 20 mM Hepes (Sigma), 100 IU/mL penicillin and 100 mg/mL streptomycin). Granulosa cells were collected by syringe aspiration and suspended in DM. Cumulus cell–oocyte complexes, isolated oocytes and follicular debris were visualised using a dissecting microscope and most, if not all, were removed from the granulosa cells using a micro-pipette. The remaining cells were then washed twice in incubation medium (McCoys 5a containing 2 mM glutamax, 0.3 mg/mL poly (vinyl alcohol) (Sigma), 0.22% w/v $NaHCO_3$, 100 IU/mL penicillin and 100 mg/mL streptomycin) by centrifugation at 453 ***g*** for 5 min. The ^{3}H-thymidine incorporation assay was performed as previously described by McNatty and coworkers (McNatty *et al.* 2005*a*), modified such that the total assay volume was reduced to 55 µL. Thereafter, studies were undertaken to verify that the recombinant oBMP15 and oGDF9 generated for this study demonstrated either biological and/or synergistic activity. Likewise, the bioactivity of the mutant oBMP15 (S356C) expressed by 293HEK cells was also investigated using the ^{3}H-thymidine incorporation assay.

Molecular modelling of oBMP15 and oGDF9

Three-dimensional structural models of oBMP15 and oGDF9 were created using porcine TGFB1, mouse BMP9 and human Activin A as templates. The protein homology/anologY recognition engine v2 (Phyre^2) (Kelley & Sternberg 2009) was used to create models of oBMP15 and oGDF9 using pTGFB1 (Accession number 3RJR; (Shi *et al.* 2011*b*)), mBMP9 (Accession number 4YCG; (Mi *et al.* 2015)) and hActivin A (Accession number 5HLZ; (Wang *et al.* 2016)) as templates for the promature form and GDF5-BMPR1B complex (Accession number 3EVS; (Kotzsch *et al.* 2009)) as template for the receptor-bound form. Further details concerning the modelling approaches are included in the Supplementary Fig. 1 text for Supplementary Fig. 1 (see section on supplementary data given at the end of this article).

Subsequently, the UCSF Chimera package from the Resource for Biocomputing, Visualisation, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) (Pettersen 2004) was used to produce the molecular graphics images and to create models of the hetero-oligomeric signalling complexes comprising BMP15 or GDF9 dimers and their receptors. This was achieved by overlaying the oBMP15 and oGDF9 (modelled on mBMP9, hActivin A or pTGFB1) along with the extracellular domains of mouse BMPR1B (3EVS) and ovine BMPR2 (2HLR, (Mace *et al.* 2006)) onto a composite template comprising a BMP2 dimer/BMPR1A complex (1ES7, (Kirsch *et al.* 2000)), BMP2 associated with both BMPR1A and ACTR2 (2GOO, (Allendorph *et al.* 2006)) and GDF5–BMPR1B complex (3EVS). The creation of these models allowed the visualisation of the different molecular forms of BMP15 and GDF9 produced and/or secreted by oocytes and their potential interactions with each other and their receptors.

Statistical analysis

Results from the ^{3}H-thymidine incorporation bioassays were shown as Box and Whisker plots based on between 5 and 14 separate experiments with triplicate determinations for each treatment. To test whether or not the different recombinant preparations displayed bioactivity, the values were converted to fold-changes relative to the control media. The fold-changes for the treatments, relative to an assigned value of one for the control, were then subjected to REML analyses following natural log transformation. Three treatment blocks were analysed: (1) o/oGDF9, o/oBMP15 and o/oGDF9 + o/oBMP15, $n = 14$; (2) o/oGDF9, h/oBMP15 (S356) and o/oGDF9 + h/oBMP15 (S356), $n = 9$; (3) o/oGDF9, h/oBMP15 (S356) (high) and o/oGDF90 + h/oBMP15 (S356) (high), $n = 5$. The model included treatment as a fixed effect and replicate as a random effect. The effects of treatment were first examined to determine if they differed from the control treatment. If differences were observed, comparisons of the combined treatment were made to each growth factor alone (e.g. o/oGDF9 + o/oBMP15 to o/oGDF9 alone and to o/oGDF9 + o/oBMP15 to o/oBMP15) with values having $P < 0.05$ considered to be significantly different.

Results

Bioactivity of recombinant oBMP15, oBMP15 (S356C) and oGDF9

The effects of the different recombinant preparations on ^{3}H-thymidine incorporation relative to the control media

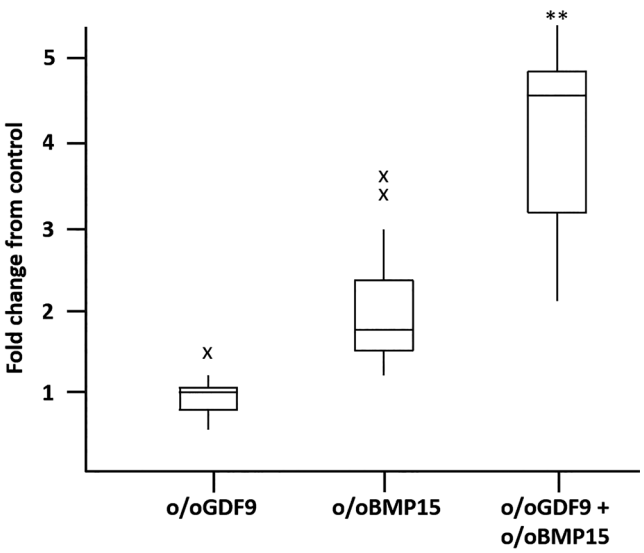

Figure 1 Effects of the recombinant proteins o/oGDF9 (50 ng/mL), o/oBMP15 (18 ng/mL), o/oBMP15 + o/oGDF9 (18 and 50 ng/mL respectively), on ^{3}H-thymidine incorporation by rat granulosa cells. Results are expressed as box and whisker plots of the fold-increases relative to that for the control medium for $n = 14$ independent rat granulosa cell pools. The symbol × refers to outliers. Based on log transformed fold-changes, o/oGDF9 is not significantly different from control (assigned a value of one), whereas both o/oBMP15 ($P < 0.01$) and o/oGDF9 + o/oBMP15 ($P < 0.001$) are different from control. **indicates o/oGDF9 + o/oBMP15 was significantly different from o/oBMP15 ($P < 0.01$).

for the o/oGDF9 (50 ng/mL) and o/oBMP15 (18 ng/mL) and o/oGDF9 (50 ng/mL) + o/oBMP15 (18 ng/mL) treatments are shown in Fig. 1. For these treatments, the o/oBMP15 ($P<0.01$) and o/oGDF9 + o/oBMP15 ($P<0.001$) but not the o/oGDF9 preparations showed significant fold-increases above that of the control. The o/oGDF9 (50 ng/mL) with or without the h/oBMP15cm preparations added at either 18 ng/mL or 90 ng/mL were not significantly different from the controls (data not shown). These results show that while o/oGDF9 alone was not biologically active, the addition of o/oGDF9 to o/oBMP15 significantly enhanced the effect of o/oBMP15 alone ($P<0.01$) with respect to ^{3}H-thymidine incorporation.

Recombinant forms of BMP15 and GDF9 recognised by mabs 61A and 37A

The epitopes recognised by mabs 61A (McNatty *et al.* 2005*a*) and 37A (unpublished data) lie within the N-terminal peptide sequences of their respective mature domains. Due to the flexible nature of this domain in the mature protein form, it was not possible to model this region. However, when in the promature conformation, these epitopes are exposed on the surface of the protein and would be expected to be freely available for interaction with mabs 61A and 37A (Supplementary Fig. 2).

Mab61A recognised mature and promature forms of BMP15 as monomers and dimers, under both non-reducing and reducing + cross-linking conditions (Supplementary Fig. 3A and C and Supplementary Fig. 3 text). Indeed, mab61A is capable of recognising multiple forms of rec o/oBMP15 and/or h/oBMP15 (S356C) including high-molecular-weight (i.e >100 Kd) and promature as well as dimeric and monomeric forms. Moreover, under the reducing conditions employed, mab61A was able to detect equivalents of *E. coli*-derived oBMP15 mature dimers at concentrations as little as 1.5 ng/mL (Supplementary Fig. 4). The most notable exception was that while mab61A recognised a h/oBMP15 (S356C) mutant dimeric form, it was unable to detect any homodimers of recombinant wild-type (o/o) BMP15. With respect to o/oGDF9, mab37A was able to detect wild-type dimers as well as high-molecular-weight, promature, monomeric and low-molecular-weight forms (Supplementary Fig. 3).

The inability of mab61A to detect o/oBMP15 dimers might possibly be due to the expression levels of the mature wild-type dimer being at concentrations below the threshold of the WB protocol. To investigate this further, highly purified and concentrated preparations of both h/oBMP15 (S356C) (Fig. 2) and o/oBMP15 (Fig. 3) were prepared, using IMAC chromatography followed by reverse phase HPLC. The level of enrichment of the HPLC-purified fractions on the WBs (Figs 2 and 3) was approximately 300× that of un-purified EM.

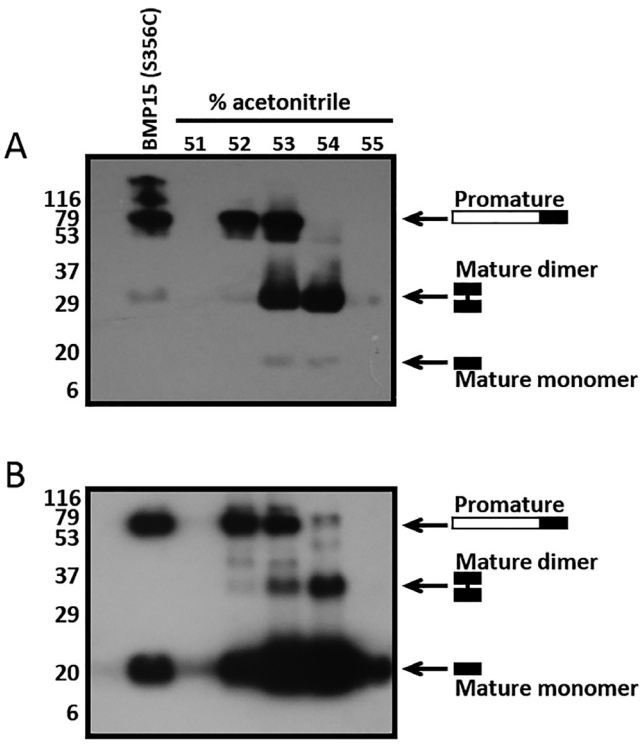

Figure 2 Molecular forms of HPLC-purified h/oBMP15 (S356C). WB's of fractions from HPLC under non-reducing (A) and reducing conditions (B). Lanes show the IMAC-purified h/oBMP15 (S356C) loading material (BMP15 (S356C)) and sequential fractions eluted at 51–55% v/v acetonitrile. The lanes for B are the same as those for A. The molecular size references (kDa) are shown to the left of the images.

Within these purified and concentrated preparations, h/oBMP15 (S356C) was found as promature forms as well as both dimeric and monomeric mature domains (Fig. 2). In contrast, the forms of o/oBMP15 consisted of bands corresponding to the monomeric promature and mature domains (Fig. 3). Homodimers of mature domains of oBMP15 were not observed for wild-type oBMP15 (Fig. 3).

Immunostaining for oBMP15 with mab61A showed the same pattern of immunostaining both in the absence and presence of oGDF9 (as observed in Supplementary Fig. 3C). Furthermore, increasing the concentration of mab61A by five-fold showed no change in the pattern of immunostaining either for o/oBMP15 alone or in the presence of o/oGDF9 (unpublished data). The staining patterns of h/oBMP15 (S356C) with, or without, the addition of o/oGDF9 were similar to that shown in Supplementary Fig. 3C. Likewise, mab37A detected no difference in the bands observed for o/oGDF9 in either the presence or absence of o/oBMP15 or h/oBMP15 (S356C) (see Supplementary Fig. 3G). Cumulin, a heterodimer of human BMP15 and GDF9, was used to confirm the ability of mab37A to detect heterodimers. Under

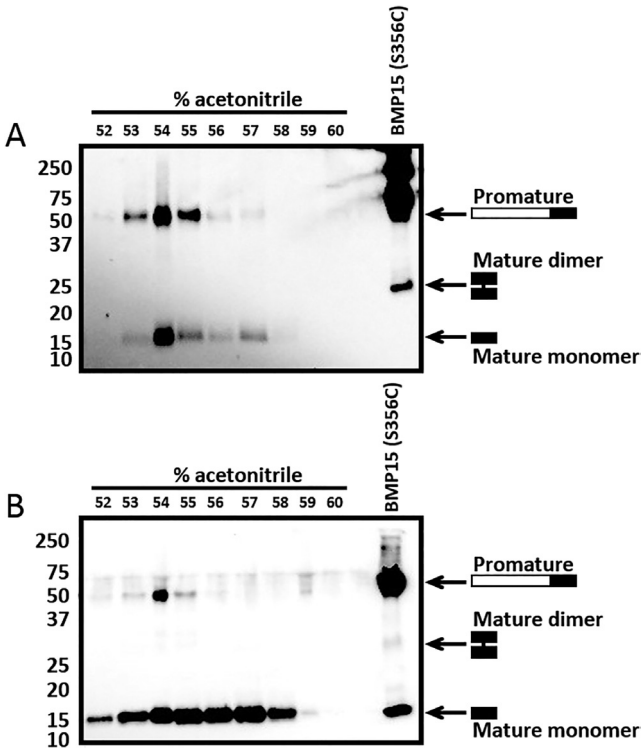

Figure 3 Molecular forms of HPLC-purified o/oBMP15. WB's of HPLC fractions under non-reducing (A) and reducing conditions (B). Lanes show fractions 42–50, eluted at 52–60% v/v acetonitrile respectively, along with h/oBMP15 (S356C) expression medium. The lanes for B are the same as for A. The molecular size references (kDa) are shown to the left of the images.

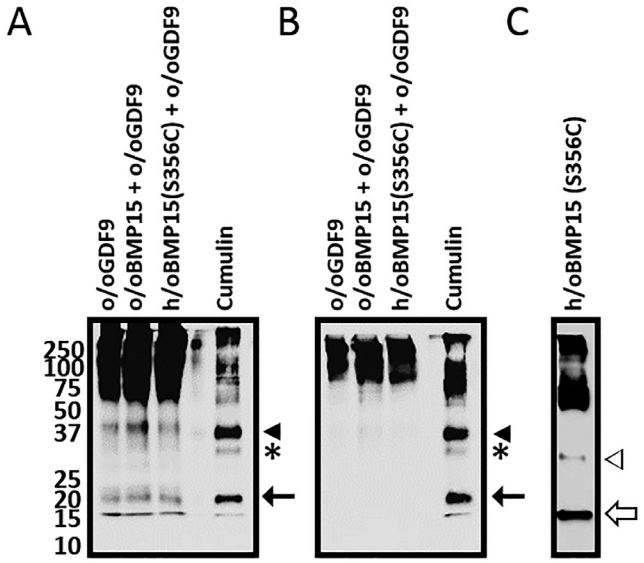

Figure 4 WB's under reducing + cross-linking conditions of ovine GDF9 alone or in the presence of o/oBMP15 or h/oBMP15 (S356C), human cumulin and h/oBMP15 (S356C). Immunostaining of molecular forms of GDF9 using mab37A (A) or mab53/1 (B) and BMP15 using mab61A (C). Location of oGDF9 mature monomer marked by (←) and dimer by (◀), the presumptive heterodimer is marked with (*) and the oBMP15 mature monomer and dimer are marked with (⇐) and (◁) respectively. The molecular size references (kDa) are shown to the left of the images.

reducing + cross-linking conditions, the same pattern of bands was observed for human cumulin as was observed for o/oGDF9 (Fig. 4A). Furthermore, mab37A showed the same pattern of immunostaining for cumulin as that for mab53/1 (Fig. 4A and B).

Native forms of BMP15 and GDF9 produced by ovine and bovine oocytes

The predominant form of BMP15 found in the lysates from freshly collected bovine oocytes (FCO) as well as the incubation medium (IM) and lysates of incubated oocytes (IO) was an uncleaved promature form (Fig. 5A). Additionally, a small amount of the fully processed mature domain was present following incubation (Fig. 5A, lane IO). The specificity of immunostaining of these forms of bBMP15 by mab61A was confirmed by the abolition of immunostaining when mab61A preabsorbed with *E. coli*-derived oBMP15-mature domains was used (Fig. 5A and B).

Likewise, the lysates from both freshly collected and incubated bovine oocytes showed the predominant form of bGDF9 was the uncleaved promature form. Also present, were fully processed mature domains, as well as additional higher and lower molecular weight forms (Fig. 5C). The IM contained mature domains. Preabsorbing mab 37A with *E. coli*-derived oGDF9-mature domains (Fig. 5C and D) blocked immunostaining to all molecular forms, demonstrated the specificity of mab37A for bovine oocyte-derived forms of GDF9.

Under non-reducing conditions, the only immunoreactive BMP15 form that was consistently detected in all replicate experiments from both bovine (Fig. 6A) and ovine (Fig. 6D) oocytes was a form(s) present at > 100 kDa. However, a ~40 kDa band was also observed in all oocyte lysates from FCO and, in three out of three ovine, and two out of three bovine lysates, from oocytes after 18 h of incubation. An additional band of 15 kDa, corresponding to the mature BMP15 monomer, was observed in two out of three freshly collected ovine oocyte lysates (data not shown), and in one out of three ovine oocyte lysates after an 18 h incubation (data not shown), but was not observed in any samples of the bovine oocyte lysates.

Under reducing conditions, the forms of immunoreactive BMP15 secreted by both ovine and bovine oocytes were predominantly bands corresponding to the unprocessed promature form and small amounts of mature monomer. The 40 kDa band was sometimes observed but only in pools of freshly collected or incubated ovine oocyte lysates (Fig. 6B and E).

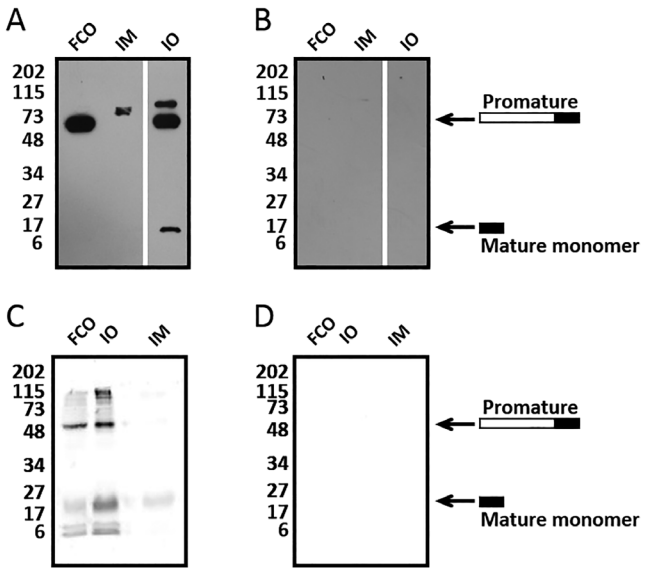

Figure 5 Immuno-blocking of mab61A and mab37A by *E. coli*-derived mature oBMP15 and oGDF9 respectively. WBs under reducing conditions (A, B, C and D) showing the immunostaining of bovine oocyte-derived forms of BMP15 and GDF9 from lysates of freshly collected oocytes (FCO) and the medium (IM) and lysates (IO) from incubated oocytes. Using mab61A (A), mab61A preabsorbed with *E. coli*-derived mature oBMP15 (B), mab37A (C) or mab37A preabsorbed with *E. coli*-derived mature oGDF9 (D). The immunoblot for Fig. A and C was chemically stripped (62.5 mM Tris–HCL + 2% w/v sodium dodecyl sulphate + 0.7% v/v 2-mercaptoethanol; 30 min @ 50°C) prior to immunostaining for Fig. B and D respectively. Molecular sizes are shown to the left of the images (kDa). Space between lanes indicates the removal of lanes not relevant to this study.

The cross-linking of proteins prior to their addition on a reducing gel revealed a similar pattern to that observed in non-cross-linked samples. Generally, for both the bovine and ovine oocyte lysates, BMP15 was located within bands corresponding to the promature and free mature monomer forms, with the ~40 kDa band being present in some, but not all, oocyte lysates (Fig. 6C and F).

Under non-reducing conditions, the forms of immunoreactive GDF9 present in lysates from both freshly collected and incubated bovine and ovine oocytes were at molecular sizes corresponding to the promature (3/3 replicates) and mature monomer (3/3 replicates) domains, as well as in bands of higher (>100 kDa; bovine 2/3 replicates, ovine 3/3 replicates) and lower (6–10 kDa; 3/3 replicates) molecular sizes (Fig. 7A and D). Secreted forms of GDF9 from bovine oocytes were consistently observed in high-molecular-weight complexes (Fig. 7A, lanes IMa-c). However, in one replicate, secreted bovine GDF9 was also found as promature and fully processed mature monomers (Fig. 7A, Lane IMa). Secreted forms of GDF9 from ovine oocytes were consistently found as fully processed mature monomers and, in two replicates, were also observed in high-molecular-size bands (>100 kDa) as well as a band corresponding to the promature domain (Fig. 7D).

Under reducing conditions, the predominant forms of immunoreactive GDF9 produced and secreted by both bovine (Fig. 7B) and ovine (Fig. 7E) oocytes were unprocessed promature and mature monomer forms, as well as an uncharacterised band at 6–10 kDa.

The oocyte lysates and IM subjected to cross-linking prior to their addition on a gel under reducing conditions resulted in more immunoreactive GDF9 being retained within the high-molecular-weight bands, compared to that in the samples that were not subjected to cross-linking. Oocyte lysates, both before (Fig. 7C and F, Lane FCO) and after (Fig. 7C and F, Lane IO) incubation showed GDF9 was present in a promature form and in high-molecular-weight complexes. Although not found in all replicate samples, the mature monomer form was also detected in oocyte lysates of both species before (Fig. 7C and F, Lane FCO) and after (Fig. 7C and F, Lane IO; not shown in the representative blot from bovine oocytes) incubation. Mature monomers in ovine and bovine oocyte lysates before incubation were detected in three out of three and two out of three replicates respectively. After incubation, mature monomers were detected in two out of three, and one out of three, ovine and bovine oocyte lysates respectively. High-molecular-weight complexes were consistently detected in media following the incubation period (Fig. 7C and F Lane IM). For both ovine and bovine oocytes, promature forms were detected in media in two of the three replicates. Mature monomers were only detected in the incubation media in two out of three ovine replicates but were not detected in media in which bovine oocytes were incubated.

Structural homology models

As the major forms of ovine or bovine BMP15 and GDF9 were the cleaved and/or uncleaved promature forms of monomeric protein, structural models were investigated to explore how these might interact with their type II and I receptors. The best-fit structural model for BMP15 and GDF9 was that based on the pTGFB1 crystal structure (see Supplementary Fig. 1 text). Using this template, a large conformational difference between the prodomain and receptor-bound forms of the mature domain of either BMP15 or GDF9 is evident (for further information see Supplementary Fig. 5). In this model, the major conformational change in the mature domain of BMP15 or GDF9 occurs at the 'wrist' with relatively small changes in the 'palm' and 'finger' regions. Evidence from the cysteine mutant forms for recombinant h/oBMP15 (S356C) indicates that dimeric promature forms of BMP15 may be present. If such a molecule of the wild-type was present, the model indicates that access to both the type II and I receptor-binding sites would be blocked.

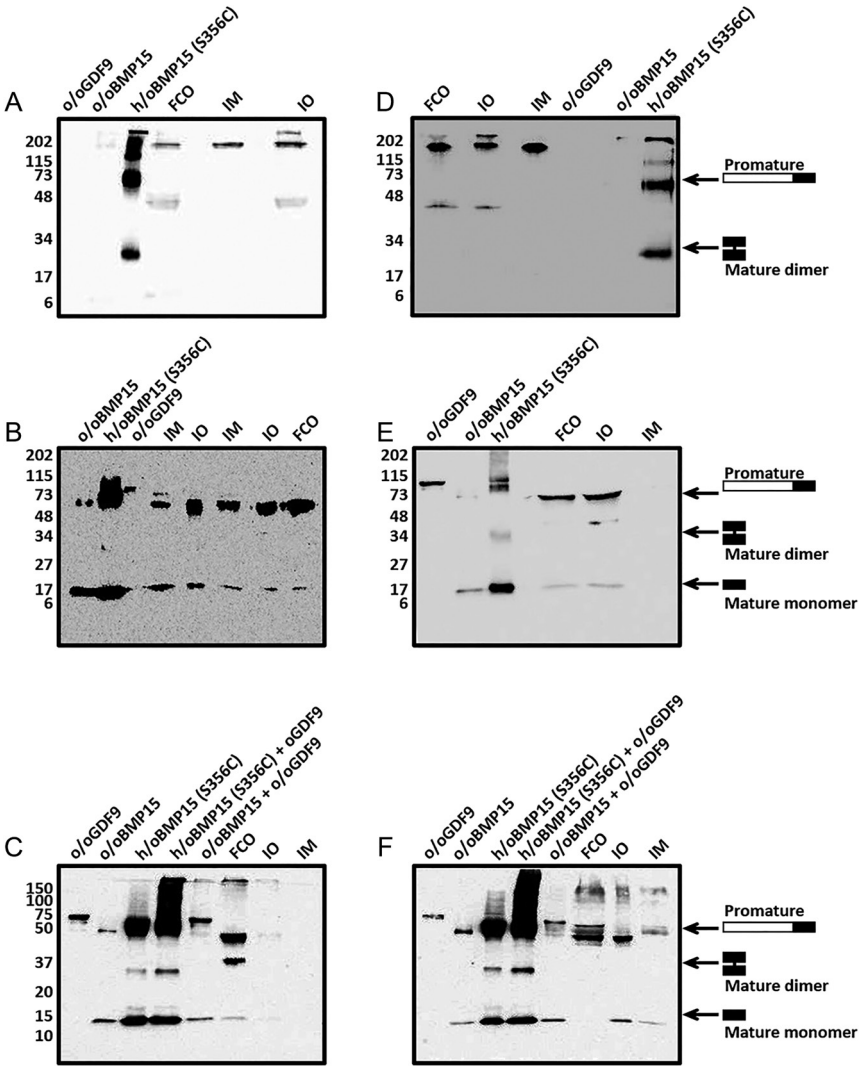

Figure 6 WBs under non-reducing (A and D), reducing (B and E) and reducing + cross-linking (C and F) conditions, using mab61A to show the molecular forms of BMP15 in lysates and incubation medium of bovine (A, B and C) and ovine (D, E and F) oocytes. The figures depict bands from recombinant o/oGDF9, o/oBMP15 or h/oBMP15 (S356C), either alone or in the presence of o/oGDF9, as well as lysates from freshly collected oocytes (FCO) and the medium (IM) and lysates (IO) from incubated oocytes. Dimeric promature form (dPm). Molecular sizes are shown to the left of the image (kDa).

However, if a monomeric promature form is present, only access to the type I binding site would be blocked (Supplementary Fig. 6). In the oocyte-derived media or with highly purified recombinant o/oBMP15, little or no dimeric promature BMP15 was present. Therefore, the relevant forms of BMP15 or GDF9 likely to interact with their receptors are the monomeric promature proteins. These interactions are explored in the discussion.

Discussion

The combined addition of recombinant o/oBMP15 and o/oGDF9 preparations to cultured granulosa cells produced a synergistic response with respect to ^{3}H-thymidine incorporation confirming the results of previous studies (McNatty *et al.* 2005*a*,*b*). In contrast, when the mutant h/oBMP15 (S356C) and o/oGDF9 were added together, no synergistic response was noted despite using a five-fold increase in concentrations of h/oBMP15 (S356C). The h/oBMP15 (S356C)-EM, unlike o/oBMP15, when incubated alone did not demonstrate biological activity. This is in agreement with results by Mottershead and coworkers (Mottershead *et al.* 2015), who also reported the hBMP15 (S356C) variant was unable to stimulate proliferation in mouse granulosa cells either with, or without, co-incubation with hGDF9. In contrast, the hBMP15 and hBMP15 (S356C) variants were equally able to stimulate the Smad 1/5/8 activity and inhibin B production in COV434 cells (Pulkki *et al.* 2012). The reasons for these differences are unknown. However, one explanation may be the different end points examined. In the previous study, Smad 1/5/8 activation and inhibin B production were investigated while the present study investigated granulosa cell proliferation, which is mediated via the Smad 2/3 pathway (Reader *et al.* 2011). An alternative and more likely explanation for an absence of synergism is that the covalent dimer of h/oBMP15 (S356C) was unable to dimerise or interact with GDF9.

A major objective in this study was to determine which molecular forms were detectable by the monoclonal antibodies used in these studies. It is

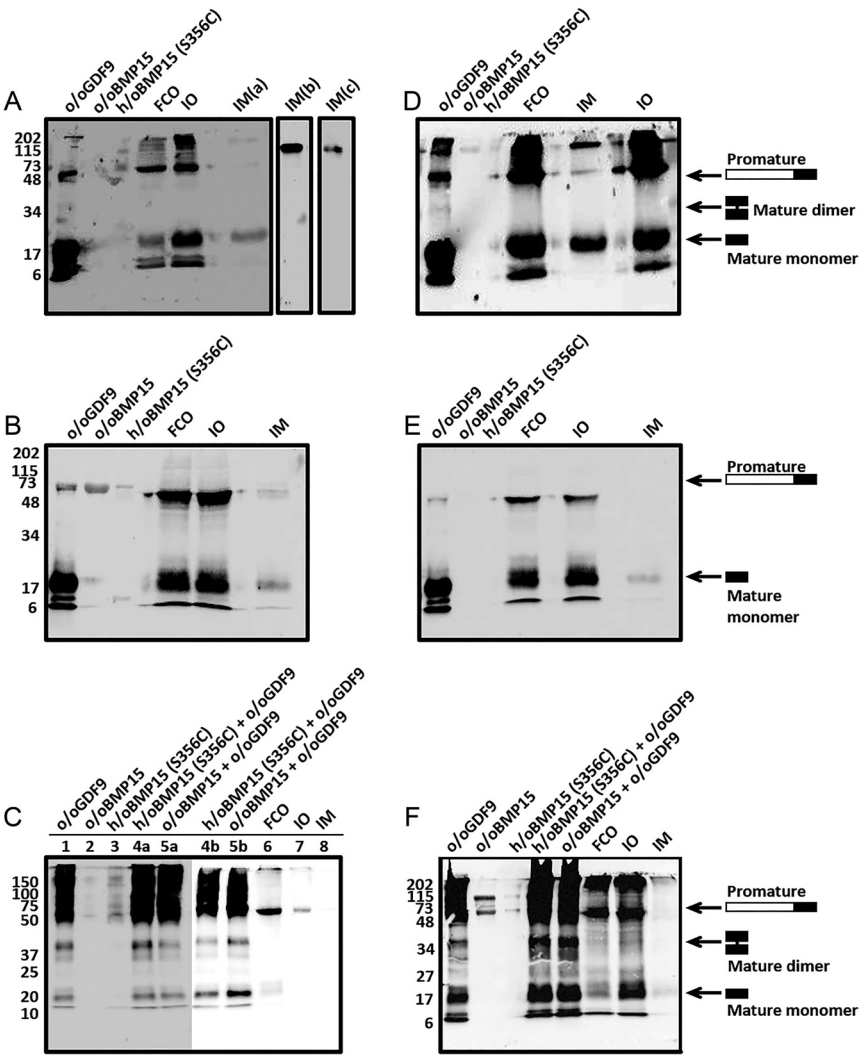

Figure 7 WBs under non-reducing (A and D), reducing (B and E) and reducing + cross-linking (C and F) conditions, using mab37A to show the molecular forms of GDF9 in lysates and incubation medium of bovine (A, B and C) and ovine (D, E and F) oocytes. The figures depict bands of recombinant o/oGDF9, o/oBMP15 or h/oBMP15 (S356C), either alone or in the presence of o/oGDF9, as well as lysates from freshly collected oocytes (FCO) and the medium (IM) and lysates (IO) from incubated oocytes. Lanes IMa-c show results of triplicate WBs. Lanes 1–5a (C; from a different WB than that for lanes 4b–8) were included to better illustrate the effect of cross-linking on recombinant proteins. Molecular sizes are shown to the left of the image (kDa).

important to note that mab61A and mab37A were raised against peptide sequences known to recognise biologically active forms of BMP15 or GDF9 and to inhibit their bioactivities *in vitro* (Lin *et al*. 2012) and *in vivo* (McNatty *et al*. 2007). As mentioned earlier, the human proregion was included in h/oBMP15 (S356C) to increase the production of BMP15 forms relative to o/oBMP15, thereby increasing the ability to detect dimeric forms. With respect to the recombinant forms of oBMP15, mab61A was shown to recognise mature forms of monomers and dimers as well as the cleaved and uncleaved promature proteins and higher molecular weight complexes. Moreover, this antibody recognised monomeric, dimeric, trimeric and tetrameric forms of *E. coli*-derived oBMP15 (Supplementary Fig. 3). Under reducing conditions, both with and without cross-linking, mab61A recognised a 70 kDa band in o/oGDF9 (Fig. 6B, C, E and F and Supplementary Fig. 3C). This band resides above that of the promature band and is also found in expression media from HEK cells transfected with an empty vector (data not shown). While the identity of this band is unknown, it is unlikely to be a form of GDF9. As mentioned earlier, the BMP15 peptide sequence recognised by mab61A differs from the corresponding sequence in hBMP15 and therefore was unable to recognise the recombinant forms of human cumulin. The GDF9 mab37A was shown to recognise mature monomer, mature dimer, cleaved and uncleaved forms of recombinant monomeric promature oGDF9 as well as higher and lower MW entities. The lower MW (6–10 kDa) bands of GDF9 recognised by mab37A were somewhat variable: this variability was also observed in the positioning of the low MW standards in this region of the gel. McIntosh and coworkers (McIntosh *et al*. 2008) reported that recombinant mGDF9 protein expressed from transfected HEK293 cells was also found in high-molecular-weight complexes comprising proregions, uncleaved promature forms with the majority as complexed mature domains; however, both monomeric and dimeric mature forms were also present.

Mab37A, which recognises a peptide sequence similar to that of hGDF9, also detected monomeric mature hGDF9, dimeric mature hGDF9, the potential hGDF9/BMP15 heterodimer (cumulin) as well as monomeric and dimeric promature hGDF9 and higher molecular weight forms. However, when recombinant oBMP15 or oBMP15 (S356C) were added together with recombinant oGDF9, no mature heterodimeric forms were identified either under non-reducing or reducing + cross-linking conditions. Similarly, under cross-linking conditions, WBs of the hBMP15:GDF9 heterodimer (cumulin) showed bands for GDF9 homodimers and the presumptive heterodimer (Fig. 4) at the same locations as those for oGDF9 alone (Figs 4A, 7C and 7F). However, based on relative densitometry, there is very little of the mature form of cumulin present. The band corresponding to the presumptive heterodimer comprised < 3% of all the forms present under reducing + cross-linking conditions. Either this presumptive heterodimer exists in which case mab37A, like mab 53/A, is able to detect heterodimers or alternatively this band does not depict a heterodimer. If the latter is correct, the possibility remains that mixtures of recombinant hBMP15 and hGDF9, like ovine, do not form detectable heterodimers in solution.

The cumulin used in this study was purified using IMAC chromatography to isolate a His-tagged hBMP15 complexed with a non-tagged hGDF9 (Mottershead *et al.* 2015). It is possible that this interaction is not mediated by non-covalent interactions between the mature domains but rather by the BMP15 prodomain. Similarly, Peng and coworkers (Peng *et al.* 2013) has also reported heterodimers between a MYC-tagged mGDF9 and a FLAG-tagged mBMP15. In the studies of Peng *et al.* (2013) and Mottershead *et al.* (2015), GDF9 mature domains when complexed with BMP15, were purified, at least in part, by affinity binding to the BMP15 promature domains. Furthermore, McIntosh and coworkers (McIntosh *et al.* 2008) reported interactions between mBMP15 prodomains and mGDF9 mature domains. Indeed such interactions can be observed (see Supplementary Fig. 3H), whereby in part, *E. coli*-derived oGDF9 was detected by binding to the membrane-bound promature form of BMP15. When h/oBMP15 (S356C) was examined under non-reducing, or reducing + cross-linking conditions, a dimeric mature form of oBMP15 was always observed. However, even under ideal conditions of having available a mutant oBMP15 (S356C) capable of forming covalently bound dimers and high concentrations of recombinant protein, the mature dimer only comprised 7% of all forms present under reducing + cross-linking conditions. Indeed, less than half of the available fully processed mature domains formed homodimers. Finally, all the observed forms were specific for each antibody as all WB bands were abolished or dramatically reduced when blocking experiments were undertaken. However, despite this study reporting the predominance of specific molecular forms, it needs to be acknowledged that the antibodies may not see every specific form equally.

Under *in vitro* conditions, BMP15 was secreted from both ovine and bovine oocytes, mainly in an unprocessed promature form. This is in agreement with the results from McNatty and coworkers (McNatty *et al.* 2006) where both oBMP15 and oGDF9 in follicular fluid were present in an uncleaved promature form. The result from the present *in vitro* studies is in contrast to that by Lin and coworkers (Lin *et al.* 2012), where the fully processed mature form was identified as the predominant form secreted by ovine oocytes. However, Lin and coworkers (Lin *et al.* 2012) only looked at molecular forms under reducing conditions and as such, this does not preclude the presence of cleaved, non-covalently associated, proregion and mature subunits being secreted. Mature BMP15 was identified in oocytes of both ovine and bovine cell lysates, both before and after 18 h of incubation, although not in all cases. The slight increase in the apparent size of bovine promature BMP15 (Fig. 5A; lanes IM & IO), relative to that in the oocyte lysate (FCO), suggests that some post-translational modification may be occurring immediately prior to secretion. Such translational modifications have previously been reported for human BMP15 (Saito *et al.* 2008). Additionally, a band intermediate in size to that of the promature and mature bands also appeared in some, but not all, bovine and ovine oocyte lysates. The size of this band correlates to that expected of a heterodimer of BMP15 and GDF9 although this band was only observed in oocytes lysates and was never found as a secreted material in the medium. And, importantly, this band was observed under reducing conditions (Fig. 6E), indicating this is likely to be a single non-cleavable polypeptide chain and therefore not a heterodimeric protein. If the presence of these bands were correlated with the health status of follicles, then the way in which these pools of oocytes were collected may provide a potential explanation of whether or not specific protein bands are present. Oocytes were retrieved from antral follicles without the health of these follicles first being assessed. Thus, it is likely that the proportion of oocytes from healthy follicles will vary between the different pools.

The prodomain of related TGFB family members have been shown to play several roles including: (1) conferring latency on the mature domain (TGFB) (Gentry & Nash 1990); (2) assembly and secretion (inhibin alpha) (Walton *et al.* 2009); (3) localisation to the extracellular matrix (BMP7) (Gregory *et al.* 2005) and (4), interaction with receptors (BMP2 (Hauburger *et al.* 2009); BMP7 (Sengle *et al.* 2008)). It therefore seems likely, that as with other members of the TGFB family, the continued association of pro- and mature growth factor domains of BMP15 and GDF9 are important in serving biological functions after secretion (McIntosh *et al.* 2012). Indeed, it has been suggested that the BMP15

prodomain may regulate cooperation between BMP15 and GDF9 (McIntosh *et al.* 2008). Despite the evidence that dimeric mature BMP15, dimeric mature GDF9 and dimeric GDF9/BMP15 heterodimers of recombinant protein are capable of being detected, no such forms were observed to be produced and/or secreted by either ovine or bovine oocytes under *in vitro* conditions. It is generally accepted that members of the TGFB family form covalent dimers, which then bind to their type II and I receptors. However, in the absence of oocyte-derived homo- or heterodimers, this sequence of events may not be employed by ruminant BMP15 and GDF9 when interacting with their receptors. An alternative explanation might be the formation of the required homo- and/or heterodimers at the level of the receptor. Disulfide-linked homodimers of BMPR2-extracellular domains (ECD), but not dimeric type 1 receptor-ECD or monomeric BMPR2-ECD or monomeric type 1 receptor-ECD, are required for inhibition of biological activity of recombinant GDF9 and BMP15 alone, as well as their synergistic effect on ^{3}H-thymidine incorporation by granulosa cells (Edwards *et al.* 2008). This suggests that stable dimers can form when both partners are bound to BMPR2. Likewise, ^{3}H-thymidine incorporation by rat granulosa cells in response to hBMP15 can also be blocked by a BMPR2-ECD (Moore *et al.* 2003). However, hBMP15 was able to associate with the BMPR1B-ECD. The most likely reason for this discrepancy is a species difference. Indeed, Al-Musawi and coworkers have identified two residues (Arg^{329} and Asp^{330}) within the pre-helical loop of hBMP15 that contribute to the affinity towards BMPR1B and subsequently the bioactivity of hBMP15. Substituting the two corresponding oBMP15 residues into hBMP15, reduced bioactivity almost 100-fold (Al-Musawi *et al.* 2013).

The RXXR site, where the promature form is cleaved, is encompassed within a very flexible region that was unable to be modelled in any of the templates (TGFB, BMP9 or Activin) used in this study. The RXXR site is likely on the surface of BMP15 and GDF9 and available to a furin-like proprotein convertase. However, it is not known if this site would still be accessible in the presence of BMPR2.

The presence of two conformations of BMPR2 has been reported, and it has been suggested that they represent the receptor in ligand bound and free states (Mace *et al.* 2006). This fits well with the idea that a cleaved promature form (complex of monomeric prodomain/mature growth factor domain) of BMP15 (Fig. 8A) or GDF9 (Fig. 8B) is presented concurrently to type II receptors on the cell surface causing conformational changes in both the receptor and mature growth factor domain of BMP15 (Fig. 8C) or GDF9 (Fig. 8D) followed by the release of the prodomains (Fig. 8E and F). In turn, this would result in the formation of a BMP15:GDF9 heterodimer at the level of the BMPR2 receptors (Fig. 8G). The release of the prodomains and the conformational changes to BMP15 and GDF9 would then allow full exposure of the type I ligand-binding domains for BMPR1B and TGFBR1 respectively (Fig. 8G) and the recruitment of the type I receptors (Fig. 8H) into an oligomeric receptor complex capable of intracellular signalling.

It is worth noting that purified mature domains of recombinant hBMP15, in the absence of a proregion, possess biological activity in cultured ovine granulosa

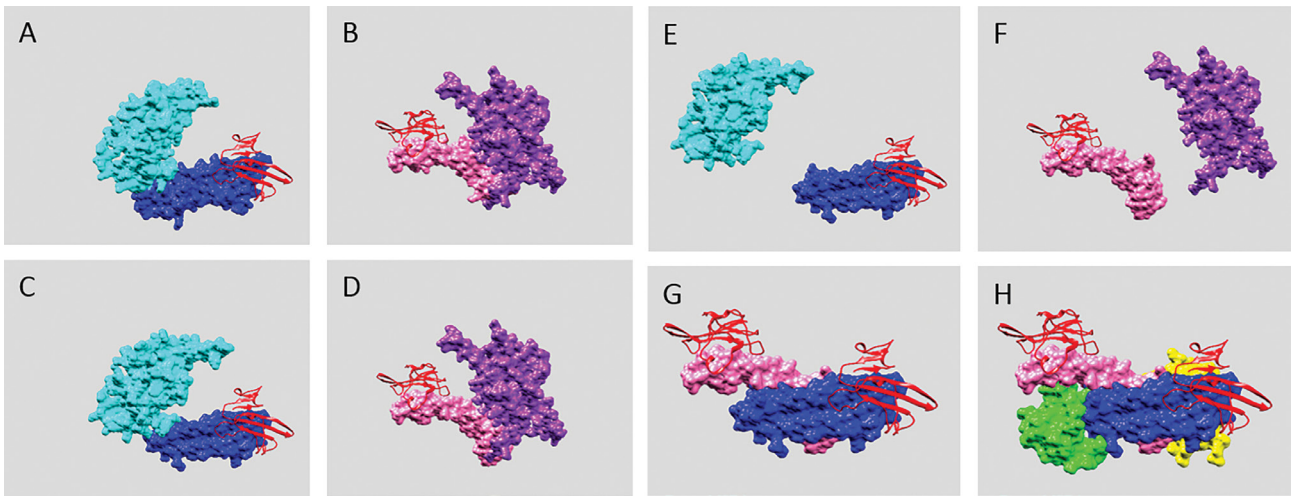

Figure 8 A proposed sequence of events involved in the formation of a BMP15:GDF9 heterodimer at the level of the receptor: a monomer of a cleaved promature BMP15 (A; cyan proregion and blue mature domain) or GDF9 (B; purple proregion and pink mature domain) binds to BMPR2 (red ribbon structure); this in turn, induces an allosteric change in the mature domain of BMP15 (C) or GDF9 (D) resulting in the release of the prodomain of BMP15 (E) or GDF9 (F) the concurrence of E and F results in the formation of a BMP15:GDF9 heterodimer (G) at the level of the receptor along with the formation of the complete type I binding sites allowing BMPR1B (green) and TGFBRI (yellow) to associate (H). This proposed sequence of events would then result in a functional heteromeric complex capable of activating intracellular signalling pathways.

cells (Pulkki *et al*. 2012, Mottershead *et al*. 2015). This indicates that the prodomain is not essential for a direct biological action of BMP15 *in vitro*, but it may have an upstream or indirect role as immunisation against the proregions of either mBMP15 or mGDF9 reduced litter size in the mouse (McIntosh *et al*. 2012). Disassociation of the non-covalently-bound prodomain from the mature growth factor domain may be sufficient to allow the mature protein conformations, with exposed binding sites for both the type II and type I receptors to form. This disassociation may be facilitated by chemical means during purification methodologies or by the presence of insertions such as a FLAG tag, which may decrease the strength of the non-covalent association between pro and mature domains.

Although no mature dimers of oocyte-derived GDF9 were observed, the existence of both monomeric and dimeric forms of mature oGDF9 in expression media raises the possibility that oGDF9 may associate with its receptors in more than one way. While monomeric oGDF9 may become receptor bound in a similar fashion to that postulated for BMP15 above, dimeric oGDF9 would associate with its receptors in much the same way as other traditional TGFB family members comprising covalently bound dimers.

If it holds true that the tightly controlled, species- and oocyte-specific *BMP15:GDF9* mRNA expression ratio is maintained at the protein level (Crawford & McNatty 2012), then the extent of heterodimeric BMP15:GDF9 synergistic interaction at the target cell relative to that for homodimeric or other interactions is likely to vary across species. Moreover, these species differences may be critical in determining the relative abundance of the different molecular forms in biological fluids.

Summary

Using monoclonal antibodies shown to recognise monomeric, dimeric, promature and higher-molecular-weight moieties of BMP15 and GDF9, the forms produced and/or secreted by bovine and ovine oocytes *in vitro* were established. Assessment of WB's under non-reducing, reducing and reducing + cross-linking conditions showed the major oocyte-secreted forms of ovine and bovine BMP15 and GDF9 was as the cleaved and uncleaved monomeric promature forms, with no evidence for homo- or heterodimeric forms of the fully processed mature domains. A theoretical model is proposed whereby these monomeric promature forms are presented to BMPR2, ultimately resulting in the formation of BMP15:GDF9 heterodimers at the level of the receptor.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-17-0188.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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