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ORIGINAL RESEARCH

Signalling pathways mediating specific synergistic interactions between GDF9 and BMP15

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ABSTRACT: Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are two proteins selectively expressed in the oocyte which are essential for normal fertility. Both of these proteins are members of the transforming growth factor beta (TGF-β) superfamily and as such are produced as pre-proproteins, existing after proteolytic processing as a complex of the respective pro and mature regions. Previous work has shown that these two proteins interact both at the genetic and cellular signalling levels. In this study, our aim was to determine if the purified mature regions of GDF9 and BMP15 exhibit synergistic interactions on granulosa cells and to determine if such interactions are specific to these two proteins. We have used primary cultures of murine granulosa cells and [^{3}H]-thymidine incorporation or transcriptional reporter assays as our readouts. We observed clear synergistic interactions between the mature regions of GDF9 and BMP15 when either DNA synthesis or SMAD3 signalling were examined. GDF9/BMP15 synergistic interactions were specific such that neither factor could be replaced by an analogous TGF-β superfamily member. The GDF9/BMP15 synergistic signalling response was inhibited by the SMAD2/3 phosphorylation inhibitor SB431542, as well as inhibition of the mitogen-activated protein kinase or rous sarcoma oncogene (SRC) signalling pathways, but not the nuclear factor kappa B pathway. In this study, we show that purified mature regions of GDF9 and BMP15 synergistically interact in a specific manner which is not dependent on the presence of a pro-region. This synergistic interaction is targeted at the SMAD3 pathway, and is dependent on ERK1/2 and SRC kinase signalling.

Key words: synergism / GDF9 / BMP15 / TGF-β superfamily / SMAD signalling

Introduction

Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are two related oocyte-derived secreted proteins essential for normal fertility in mammals (Otsuka *et al.*, 2011). The level of importance of these two proteins for reproductive success differs across species, as BMP15 knockout mice exhibit a mild reduction in fertility (Yan *et al.*, 2001), whereas sheep homozygous for a mutant form of BMP15 are sterile (Galloway *et al.*, 2000; Hanrahan *et al.*, 2004). In contrast, both GDF9 knockout mice (Dong *et al.*, 1996) and sheep homozygous for a mutation in GDF9 (Hanrahan *et al.*, 2004) are sterile. In humans the limited available data to date indicate that both proteins are involved in various reproductive functions, as aberrant expression has been implicated in premature ovarian failure, twinning and possibly polycystic ovarian disease (Dixit *et al.*, 2006; Hoekstra *et al.*, 2008; Wei *et al.*, 2011).

Both GDF9 (McPherron and Lee, 1993) and BMP15 (Dube *et al.*, 1998; Laitinen *et al.*, 1998) are members of the transforming growth factor beta (TGF-β) superfamily of proteins, and hence are similar at a structural level. All the members of the TGF-β superfamily are produced as pre-proproteins which are processed during secretion, resulting in an amino terminal pro-region and a smaller carboxyl terminal mature region (Massague, 2000). The receptor-binding and hence bioactivity of the protein are attributes associated with the processed mature region. Recent studies indicate that for members of the TGF-β superfamily the proteolytically processed pro and mature region proteins remain as a non-covalently bound complex (Sengle *et al.*, 2011). This is also the case for recombinantly produced GDF9 (Mottershead *et al.*, 2008) and BMP15 (Pulkki *et al.*, 2011). GDF9 and BMP15 are structurally unusual as they lack a conserved Cys residue, associated within the TGF-β superfamily with the formation of a disulphide bond between the dimerized mature regions giving rise to a covalent dimer. Hence, GDF9 and BMP15 may theoretically act as monomers or non-covalent homodimers or heterodimers. The processed mature region of the secreted GDF/BMP protein signals via the formation of a complex with so-called type I and II cell surface Ser/Thr kinase receptors, which activate SMAD and other signalling pathways leading to the regulation of gene transcription (Schmierer and Hill, 2007). Work to date suggests that both GDF9 and BMP15 utilize the same type II receptor [the BMPRII (Vitt *et al.*, 2002; Moore *et al.*, 2003; Pulkki

et al., 2011), but different type I receptors, consistent with the known SMAD pathways activated by these two proteins. GDF9 appears to activate the TGF-β type I receptor (Alk5; Mazerbourg et al., 2004; Kaivo-Oja et al., 2005)], whereas BMP15 has been associated with the BMP type Ib receptor (Alk6; Moore et al., 2003). Hence, GDF9 elicits a TGF-β-like intra-cellular response activating SMAD2/3 (Kaivo-Oja et al., 2003, 2005), whereas BMP15 activates the BMP pathway characterized by SMAD1/5/8/ signalling (Moore et al., 2003; Pulkki et al., 2011).

An additional unique feature of GDF9 and BMP15 within the TGF-β superfamily is that protein expression is restricted essentially to just the gametes. In females, GDF9 and BMP15 are co-expressed in oocytes from the earliest stages of folliculogenesis, their ontogeny of expression is matched (in a species-specific manner), and the oocyte expresses exceptionally high levels of these growth factors throughout folliculogenesis (Juengel and McNatty, 2005). Furthermore, their target cells, the granulosa and cumulus cells, express the full complement of type I and type II receptors and intra-cellular transducer molecules, necessary to respond to both growth factors, throughout follicle development. Hence *in vivo*, GDF9 and BMP15 are likely to interact and it is perhaps unlikely that GDF9 and BMP15 would ever operate in isolation (McNatty et al., 2006).

The first indications of an interaction between GDF9 and BMP15 were provided by crossing heterozygote mutant $GDF9^{+/-}$ and $BMP15^{+/-}$ animals and analysing the reproductive phenotype of the resulting offspring. Such studies have been undertaken in both mice (Yan et al., 2001; Su et al., 2004) and sheep (Hanrahan et al., 2004; McNatty et al., 2006). The first indication of an interaction at the protein level came with the study of Liao et al. (2003), which showed at a biochemical level the formation of homo and heterodimers when GDF9 and BMP15 are co-expressed in human embryonic kidney-293T (HEK-293T) cells. This was followed up by the studies of McNatty et al. (2005a, b), which showed a clear co-operation at the protein level, indeed, a notable synergistic response to the combined action of GDF9 and BMP15 (McNatty et al., 2005a). These landmark studies used unpurified recombinant (both murine and ovine) GDF9 and (ovine) BMP15 as produced by HEK-293H cells and secreted into serum-free Dulbecco's modified Eagle's medium (DMEM)/F12-based culture media. The most clear synergistic effects were observed when using primary cultures of rat granulosa cells and monitoring either [^{3}H]-thymidine incorporation or inhibin production (McNatty et al., 2005a). Further studies by this group have shown that GDF9/BMP15 synergistic interactions are mediated by the BMPRII receptor (Edwards et al., 2008; McIntosh et al., 2008) and involve the pro-region of BMP15 (McIntosh et al., 2008). Most recently, again using unpurified recombinant GDF9 and BMP15 in HEK-293H cell conditioned media, the different signalling pathways involved in the synergistic interaction of GDF9 and BMP15 have been examined via the use of various chemical inhibitors (Reader et al., 2011).

The forms and pitfalls of the various 'in-house' recombinant GDF9 and BMP15 forms have recently been reviewed (Mottershead and Watson, 2009; Gilchrist, 2011). With a consistent commercial source of purified mouse GDF9 and human BMP15 proteins now available, we undertook this study to determine if these purified preparations, which consist of the mature protein regions only, exhibit synergistic interactions and to examine the specificity of this interaction. Specifically, synergism is being defined here as the combined action of two components to produce an effect greater than their individual effects. Further, given our recent demonstration of the dependence of GDF9 signalling on extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation of the SMAD3 linker (Sasseville et al., 2010), we have investigated the involvement of ERK1/2 and SRC signalling in GDF9/BMP15 synergistic interactions.

Materials and Methods

Unless specified, all chemicals and reagents were purchased from Sigma (St. Louis, MO, USA). The following human recombinant proteins were all purchased from R&D Systems Inc. (Minneapolis, MN, USA),TGF-β1, activin A, activin B, BMP6, BMP7 and BMP15, as was recombinant mouse GDF9.

Isolation of mouse granulosa cells and cumulus–oocyte complexes

Mice used in this study were maintained at the University of Adelaide animal house. The study was approved by the University's local Animal Ethics Committee and was conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Twenty one to 26 day-old 129/sv mice were injected with 5 IU equine chorionic gonadotrophin (Folligon; Intervet, Castle Hill, Australia), and ovaries collected 46 h later in HEPES-buffered tissue culture medium 199 (MP Biomedicals, Cleveland, OH, USA) supplemented with 0.03% w/v polyvinyl alcohol (H-TCM199/PVA) (granulosa cell and cumulus cell [^{3}H]-thymidine incorporation assay) or, for the SMAD luciferase reporter assay, in H-TCM199 supplemented with 0.3% bovine serum albumin (BSA). Large antral follicles were ruptured with a 27-gauge needle to liberate cumulus–oocyte complexes and mural granulosa cells. Only cumulus cells from cumulus–oocyte complexes with an intact and uniform covering of compact cumulus cells were used in this study.

Mural granulosa and cumulus cell [^{3}H]-thymidine incorporation assay

This bioassay was performed as previously described (Gilchrist et al., 2001, 2004, 2006). Briefly, isolated cumulus cell clumps were generated by mechanically denuding cumulus–oocyte complexes of their cumulus cells by vigorously pipetting complexes up and down with a P200 pipette. No chemical or enzymatic methods were used to aid isolation. Denuded oocytes were then removed and the remaining cumulus cells collected into a sterile 1.75 ml Eppendorf tube. Both granulosa cells and cumulus cells were washed once with H-TCM199/PVA and twice in culture medium (bicarbonate-buffered TCM199/PVA). Depending on the experiment, granulosa cells (2×10^5 cells/ml) or cumulus cells (1.6×10^5 cells/ml) were cultured in the presence of growth factors and inhibitors in a 96-well plate (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) in a final volume of 125 μl. Application of all inhibitors used in this study has previously been reported by us using the same bioassay (Gilchrist et al., 2006; Sasseville et al., 2010). SB431542 was generously donated by GlaxoSmithKline (Stevenage, UK); the nuclear factor kappa B (NF-κB) inhibitor SN50 and its inactive analogue SN50M was purchased from Biomol International LP, (Plymouth Meeting, PA, USA) and the MAPK/ERK kinase 1 (MEK1) inhibitor U0126, its inactive analogue U0124, and the SRC inhibitor PP2 were all purchased from Calbiochem (Nottingham, UK). In experiments where inhibitors were involved, cells were pre-treated for 10 min with the inhibitor before the addition of ligands. Cells were cultured in an atmosphere of 37°C, 96% humidity in 5% CO_2 in air for 18 h, followed by a further 6-h pulse of 15.4 kBq [^{3}H]-thymidine (MP Biomedicals) under the same conditions. Following

culture, mural granulosa cells and cumulus cells were harvested, and the incorporated [^{3}H]-thymidine was quantified using a scintillation counter. Each treatment was performed in duplicate and whole experiments were repeated at least three times.

Granulosa cell phospho-SMAD reporter assay

To determine the capacity of GDF9/BMP15 to activate SMAD signalling, a phospho-SMAD reporter bioassay was used as previously described (Gilchrist *et al.*, 2006). This assay utilizes primary mouse mural granulosa cells transiently transfected with luciferase reporter constructs responsive to either phosphorylated SMAD3 (Dennler *et al.*, 1998) or phosphorylated SMAD1/5/8 (Korchynskyi and ten Dijke, 2002). In brief, mouse granulosa cells were collected as described above, washed once in H-TCM199/BSA and twice in bicarbonate-buffered DMEM (Gibco; Invitrogen, Mulgrave, Australia) with 2% (v/v) fetal calf serum (FCS). Granulosa cells (1.6×10^5cells/ml) were cultured in a 96-well plate (Falcon, Becton Dickinson) in 250 μl of B-DMEM/FCS. After 4 h of culture, cells were transfected with 100 ng of luciferase reporter construct DNA using Fugene 6 (Roche Diagnostics, Castle Hill, Australia). Eighteen hours after transfection, the medium was aspirated and replaced with 125 μl of B-DMEM + 0.1% FCS. Cells were then treated with one of the following: (i) control media, (ii) 2 ng/ml TGF-β1, (iii) 50 ng/ml BMP6, or increasing doses (3.1–12.5 ng/ml) of (iv) GDF9, (v) BMP15 or (vi) a combination of GDF9 and BMP15, and then cultured for a further 48 h as described above. In experiments involving the ALK4/5/7 inhibitor SB431542, transfected granulosa cells were pre-treated with SB431542 for 10 min before the addition of ligands. Experiments were terminated by removing media from the wells and freezing the plates at −20°C. Cells were ruptured by adding 100 μl of lysis buffer (Promega; Madison, WI) to each well and plates were incubated at room temperature on a rocking platform for 20 min. Twenty microlitres of cell lysate was used for measurement of luciferase activity using a Galaxystar luminometer (GMB Labtechnologies, Offenburg, Germany). Each treatment was performed in quadruplicate and whole experiments were repeated at least three times.

Statistical analysis

As the objective of this study was to specifically examine synergism between GDF9 and BMP15, the reporting of results from statistical analyses focuses on the GDF9 × BMP15 interaction terms, as assessed by multivariate analysis of variance (ANOVA). The effects of GDF9 or BMP15 in isolation have previously been reported by us and others and so are not reported here. Data were log transformed when the normality test and/or equal variance test failed. Data in Figs 3 and 4 were subjected to one-way ANOVA and differences between individual means and the positive control (GDF9 + BMP15) were detected using the Holm–Sidak method (non-transformed data) or using Dunnett's method (transformed data). $P < 0.05$ was considered statistically significant.

Results

The proteins used in this study are the purified mature regions of mouse GDF9 and human BMP15 (R&D Systems Inc.). Both of these recombinant proteins have been produced in Chinese hamster ovary host cells and purified such that the associated pro-region has been separated from the receptor-binding bioactive mature region. As a previous study has indicated that the pro-region is involved in the regulation of GDF9/BMP15 synergism (McIntosh *et al.*, 2008), we investigated if such synergistic interactions can take place in the absence of the respective pro-regions. In order to examine this issue, we have utilized primary cultures of murine granulosa cells, an *in vitro* model system that we have extensive experience with in terms of granulosa cell responses to GDF9 and other oocyte-secreted factors (Gilchrist *et al.*, 2004, 2006; Sasseville *et al.*, 2010). In this model system GDF9 stimulates DNA synthesis, as assessed by [^{3}H]-thymidine incorporation, and we found that there is a clear synergistic effect upon the co-addition of low doses of GDF9 and BMP15 (Fig. 1A). Biological synergism can also be tested statistically by multivariate ANOVA analysis and we found a highly significant interaction term (three-way ANOVA; $P < 0.001$) between GDF9

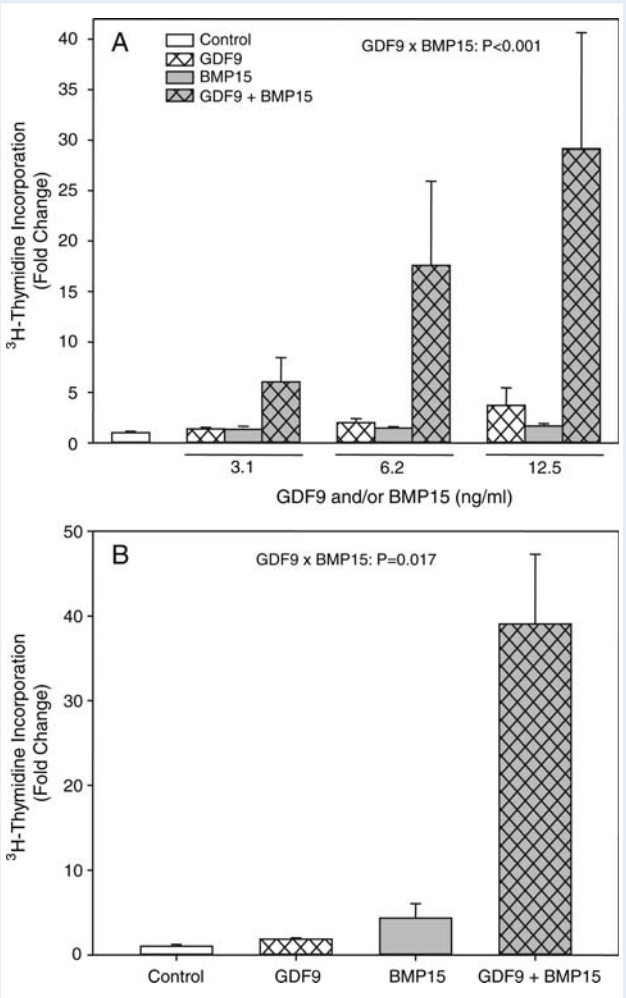

Figure 1 Synergistic activity of GDF9 and BMP15. Mouse granulosa cells (**A**) were either left untreated (control) or treated with one of the following: (i) an increasing dose of GDF9 (3.1–12.5 ng/ml), (ii) an increasing dose of BMP15 (3.1–12.5 ng/ml) or (iii) a combination of both GDF9 and BMP15 at a 1:1 ratio (3.1–12.5 ng/ml each). Mouse cumulus cells (**B**) were similarly left untreated (control) or treated with either 12.5 ng/ml of GDF9, 12.5 ng/ml of BMP15 or a combination of both GDF9 and BMP15 (both at 12.5 ng/ml). [^{3}H]-thymidine incorporation was measured following 24 h of culture. Bars represent means ± SEM from three replicate experiments expressed as fold change relative to the control. Data were analysed by multivariate ANOVA and only the GDF9 × BMP15 interaction term is reported in the figure.

and BMP15 (Fig. 1A). GDF9/BMP15 synergism was most notable at low doses of both proteins (<20 ng/ml) and the synergistic nature of the response decreased with higher doses of GDF9 (data not shown). It is worth noting that the level of stimulation reached by GDF9/BMP15 at 12.5 ng/ml is far in excess of GDF9 alone at 25 ng/ml (data not shown).

We wished to determine if this effect was also observed in cumulus cells, as in antral follicles these cells are in intimate contact with the oocyte and hence are the closest cell type to the source of oocyte-derived GDF9 and BMP15 *in vivo*. We chose the concentration of GDF9 and BMP15 (12.5 ng/ml) which gave a maximal synergistic response on the mural granulosa cells and applied this to cumulus cells, which in a similar fashion, showed a clear synergistic response (two-way ANOVA, GDF9 × BMP15; $P = 0.017$; Fig. 1B). For both mural and cumulus cells, when low-dose levels (<15 ng/ml) of GDF9 or BMP15 were added as separate factors, the effect on DNA synthesis was less than a 5-fold change at the maximum concentration used, however, when the two proteins were added simultaneously there was a 30–40-fold increase (Fig. 1).

As it is problematic to obtain large quantities of cumulus cells for experimental purposes, we pursued our further characterization of GDF9/BMP15 synergistic interactions using primary murine mural granulosa cells. As both GDF9 and BMP15 are known to activate SMAD signalling pathways in granulosa cells (Kaivo-Oja *et al.*, 2003, 2005; Moore *et al.*, 2003; Pulkki *et al.*, 2011) we investigated the comparative effects of the combined addition of these factors upon the SMAD3 (CAGA-luciferase (Dennler *et al.*, 1998)) or SMAD1/5/8 (BRE-luciferase (Korchynskyi and ten Dijke, 2002)) pathways. While these low GDF9 and BMP15 doses used in isolation had a minimal effect on activation of SMAD3-luciferase, the results in Fig. 2A show a substantial synergistic interaction (three-way ANOVA, GDF9 × BMP15; $P = 0.005$) between GDF9 and BMP15. In contrast, the SMAD1/5/8 pathway was sensitive to stimulation by BMP15 alone, but exhibited no synergistic response (three-way ANOVA, GDF9 × BMP15; $P > 0.05$) to additional stimulation with GDF9 (Fig. 2B). These results demonstrate that GDF9/BMP15 synergism is observed when monitoring the activity level of the SMAD3 pathway, and is not observed when monitoring the SMAD1/5/8 pathway which is traditionally associated with BMP signalling. The level of GDF9/BMP15 synergism as measured via SMAD3 activation (Fig. 2A) was quite comparable with that seen via DNA synthesis (Fig. 1A). Consistent with the synergistic actions of GDF9/BMP15 on activation of the SMAD3 pathway, we found that we could dose-dependently inhibit the activation of this pathway with the Alk4/5/7 inhibitor SB431542. Low dose inhibition was observed both via monitoring SMAD3 activation itself ($P < 0.05$ at doses >0.25 μM; Fig. 3A) or when monitoring DNA synthesis via [^{3}H]-thymidine incorporation ($P < 0.05$ at doses >0.13 μM; Fig. 3B).

GDF9 and BMP15 are not the only TGF-β superfamily members expressed in the ovary, or by the oocyte. TGF-β, activins and BMPs all have important functions within the ovary (Juengel and McNatty, 2005). Hence, we wished to determine if the very clear synergistic interactions we find between GDF9 and BMP15 are specific to these two ligands, or if they are a reflection of wider interactions between the members of this family of proteins. To test specifically for synergism, we co-incubated mural granulosa cell cultures with activin A, activin B, TGF-β1, BMP6 or BMP7 each, either alone or with either GDF9 or BMP15 (Fig. 4). While most combinations of ligands produced an additive effect of the response of the individual ligands (e.g. activin A + GDF9), no ligand when added to GDF9 or BMP15 exhibited a synergistic effect (two-way ANOVA, interaction term; $P > 0.05$), with the sole exception of when GDF9 and BMP15 were added together (two-way ANOVA, GDF9 × BMP15; $P < 0.05$). Most telling, there were no synergistic interactions ($P > 0.05$) between GDF9 and BMP6 or between BMP15 and TGF-β1, as in the past these were candidate oocyte-secreted factors (Gilchrist *et al.*, 2008).

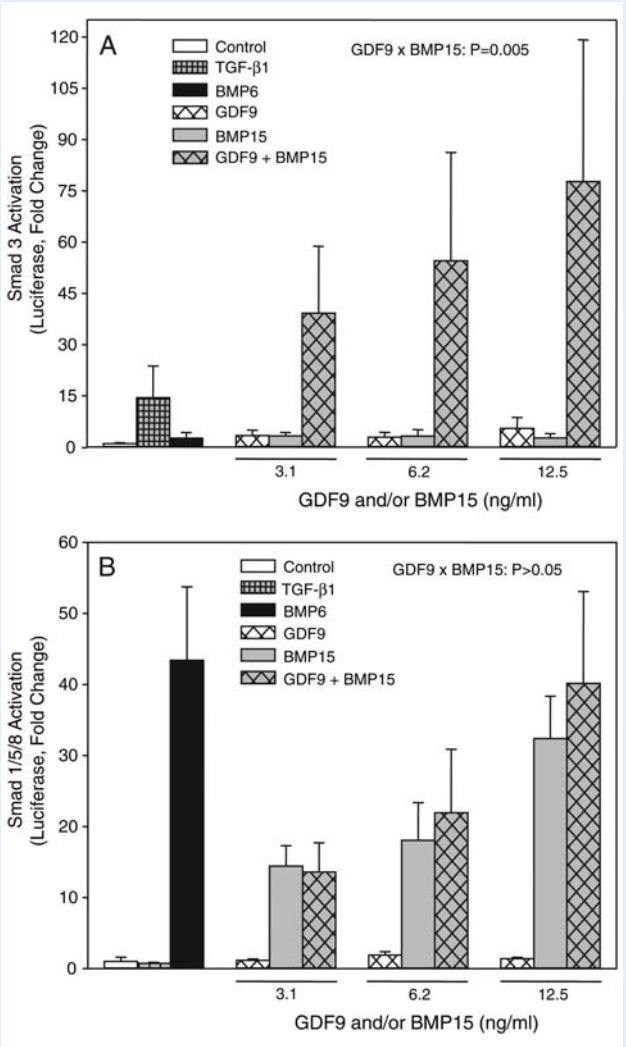

Figure 2 GDF9/BMP15 synergistic activity stimulates the SMAD3, but not the SMAD1/5/8 pathway. Mouse granulosa cells were transiently transfected during culture with either a SMAD3 responsive CAGA-luciferase plasmid (**A**) or a SMAD1 responsive BRE-luciferase plasmid (**B**). In both experiments cells were either left untreated (control) or treated with one of the following; TGF-β1 (2 ng/ml), BMP6 (50 ng/ml), GDF9 (3.1–12.5 ng/ml), BMP15 (3.1–12.5 ng/ml) or a combination of both GDF9 and BMP15 at a 1:1 ratio (3.1–12.5 ng/ml each). Luciferase activity was measured following a 48 h treatment period. Bars represent means ± SEM from four replicate experiments expressed as fold change relative to the control. Data were analysed by multivariate ANOVA and only the GDF9 × BMP15 interaction term is reported in the figure.

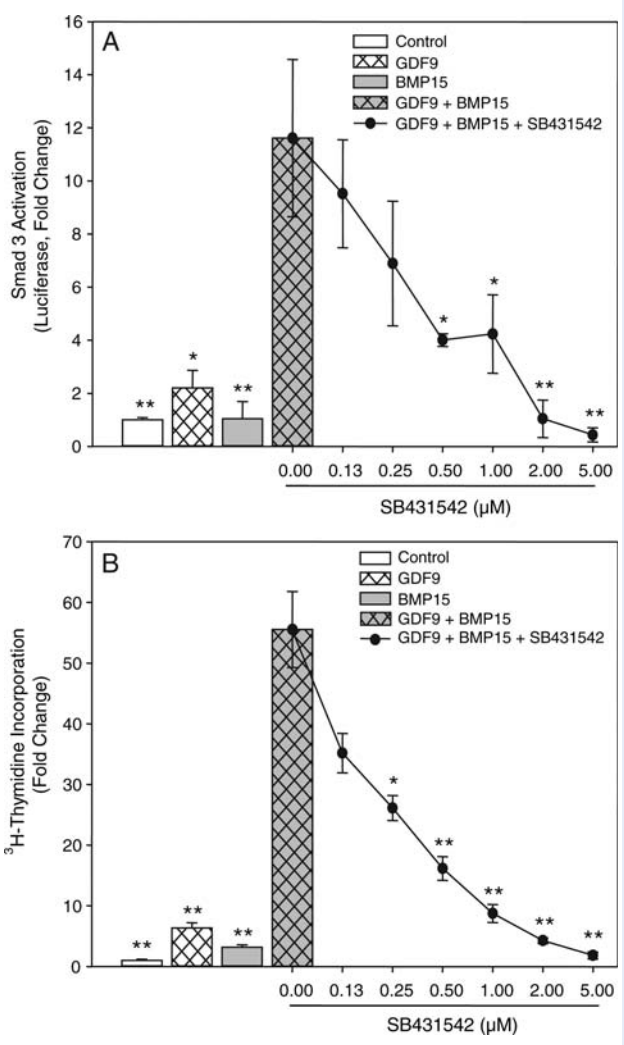

Figure 3 GDF9/BMP15 synergistic activity is inhibited by the Alk4/5/7 inhibitor SB431542. Mouse granulosa cells were transiently transfected during culture with a SMAD3 responsive CAGA-luciferase plasmid (**A**) or radio-labelled with [^{3}H]-thymidine (**B**). In both experiments, cells were left untreated (control) or treated with GDF9 (12.5 ng/ml), BMP15 (12.5 ng/ml), GDF9 + BMP15 (12.5 ng/ml each) or GDF9 + BMP15 + SB431542 (0.13–5 μM). Bars represent means ± SEM from three replicate experiments expressed as fold change relative to the control. Data were analysed by one-way ANOVA; *$P < 0.01$, **$P < 0.001$ designate significant differences relative to the GDF9 + BMP15 positive control.

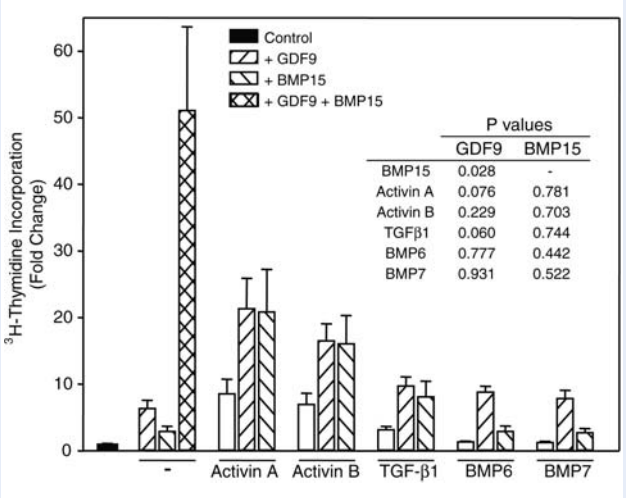

Figure 4 GDF9/BMP15 synergism is specific. Mouse granulosa cells were left untreated (control), or treated with the following growth factors; activin A, activin B, BMP6, BMP7 (all at 50 ng/ml) or TGF-β1 (2 ng/ml), each either alone (open bars), or in combination with GDF9 (12.5 ng/ml) or BMP15 (12.5 ng/ml). [^{3}H]-thymidine incorporation was measured following 24 h of culture. Bars represent means ± SEM from four replicate experiments expressed as fold change relative to the control. Potential synergistic interactions between growth factors were tested by two-way ANOVA and the *P* values for the respective interaction terms only are presented in the figure.

We have previously investigated the involvement of ERK1/2 activity in GDF9 signalling and found that basal or epidermal growth factor (EGF) receptor-activated ERK1/2-stimulated phosphorylation of the SMAD3 linker region is required (Sasseville *et al.*, 2010). From these previous results, we predicted that the MEK1 inhibitor U0126, which acts upstream of ERK1/2 activation, would inhibit the synergistic response of mural granulosa cells to GDF9/BMP15, and indeed this is what we observed ($P < 0.001$ at doses >0.13 μM; Fig. 5A). Further, U0124, which is an inactive analogue of U0126, was without an effect on the GDF9/BMP15 synergistic response ($P > 0.05$). We have also previously shown that this involvement of EGF receptor-activated ERK1/2 in GDF9 signalling requires the rous sarcoma oncogene (SRC) family kinases (SFK) (Sasseville *et al.*, 2010). In a similar manner, we found in the current study that SFK inhibition using PP2 in granulosa cells dose-dependently inhibited GDF9/BMP15-stimulated [^{3}H]-thymidine incorporation ($P < 0.05$ at doses >0.6 μM; Fig. 5B). Further, no specific effect of inhibition of the NF-κB pathway upon the GDF9/BMP15 response was detected. The active inhibitor (SN50) had no effect ($P > 0.05$) on the GDF9/BMP15-stimulated response, whereas the inactive variant of the inhibitor (SN50M) significantly ($P < 0.05$) decreased [^{3}H]-thymidine incorporation at all doses tested (Fig. 5C).

Discussion

Given that both GDF9 and BMP15 are critical for normal fertility in mammals (Otsuka *et al.*, 2011), and that both of these factors are produced by the oocyte with an overlapping ontogeny of expression, we propose that the synergistic nature of the interactions between these two proteins documented within this report will be of great importance for the fields of reproductive biology and reproductive medicine. Hence, it is important to determine the molecular mechanism of this synergism, and this report is our first step towards that goal. This is the first report documenting this synergistic interaction using purified proteins, as all previous studies (McNatty *et al.*, 2005a; Edwards *et al.*, 2008; McIntosh *et al.*, 2008; Reader *et al.*, 2011) have used whole HEK-293H cell conditioned media containing recombinant forms of

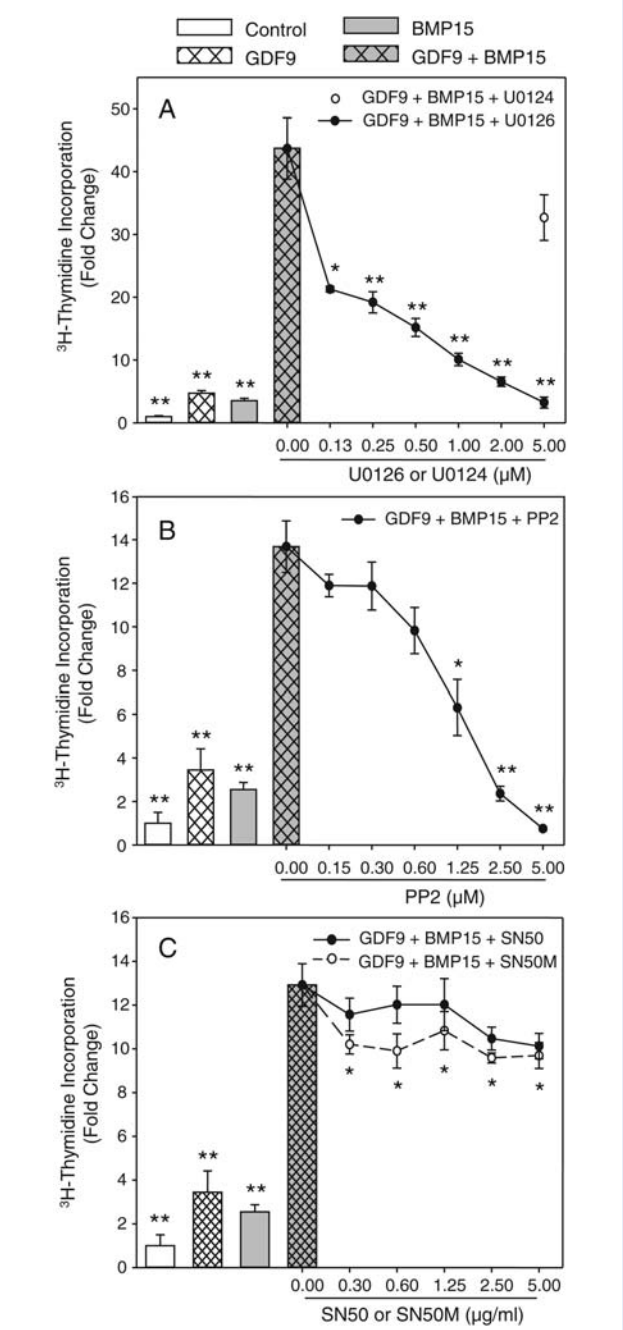

Figure 5 ERK1/2 and SRC but not NF-κB are required for GDF9/BMP15 synergistic activity. [^{3}H]-thymidine incorporation was measured in granulosa cells either left untreated (control) or treated with GDF9 (12.5 ng/ml), BMP15 (12.5 ng/ml), GDF9 + BMP15 (12.5 ng/ml each) or GDF9 + BMP15 plus one of the following inhibitors: U0126 (0.13–5 μM) or its inactive analogue U0124 (5 μM) (**A**), PP2 (0.15–5 μM) (**B**), or SN50 or its inactive analogue SN50M (both at 0.3–5 μg/ml) (**C**). Bars represent means ± SEM from three to four replicate experiments expressed as fold change relative to the control. Data were analysed by one-way ANOVA; *$P < 0.05$, **$P < 0.001$ designate significant differences relative to the GDF9 + BMP15 positive control.

GDF9 or BMP15. As such, we have avoided any complications which may arise from the use of conditioned media; for example, we know there are factors inhibitory to granulosa cell responses contained within such conditioned media (Hickey *et al.*, 2005). Further, we have shown that GDF9/BMP15 synergistic interactions clearly occur even in the absence of the respective pro-regions. Hence, all the structural information required for GDF9/BMP15 synergism is already present within the respective processed mature regions of these proteins. Although *in vivo* it is likely that GDF9 and BMP15 act as pro-mature complexes, it is important when establishing the mechanism of this synergism to know that this response also occurs when just the respective purified mature regions are added to granulosa cells *in vitro*.

Isolated GDF9 and BMP15 proteins share some common and distinct features in their respective signalling pathways. GDF9 and BMP15 share a common type II receptor, the BMPRII, however, because their putative type I receptors are distinct (ALK5 and ALK6, respectively), they appear to elicit discrete intra-cellular responses; activating the SMAD2/3 (Kaivo-Oja *et al.*, 2003, 2005) and SMAD1/5/8 pathways (Moore *et al.*, 2003; Pulkki *et al.*, 2011), respectively. However, given their common cell of origin, their matching expression ontogeny and their related structures, these two proteins are unlikely to operate in isolation in their regulation of granulosa/cumulus cell functions (McNatty *et al.*, 2006). Hence, the nature of interactions between GDF9 and BMP15 is likely to be highly physiologically relevant. Furthermore, evidence from genetic studies in mice and sheep, as well as evidence from *in vitro* studies, have demonstrated that cooperative and even synergistic interactions exist between GDF9 and BMP15. The molecular basis for these interactions is currently unclear, however a number of mechanisms can be proposed. Firstly, both GDF9 and BMP15 could be acting independently as homodimers, activating their respective signalling pathways. In this case, the activation of the BMP15 SMAD1/5/8 pathway at the same time as the GDF9 SMAD2/3 pathway would result in a much greater activation level of the SMAD2/3 pathway. An alternative mechanism could be the formation of a GDF9/BMP15 heterodimer (given that both of these proteins are not forming covalent dimers of their mature regions), which may recruit a unique combination of receptors resulting in a synergistic SMAD2/3 response. Clarification of the mechanism of GDF9/BMP15 synergism awaits further studies.

Although previous studies (McIntosh *et al.*, 2008; Reader *et al.*, 2011) have been suggestive of the involvement of the TGFβ/activin/nodal signalling pathway in the GDF9/BMP15 synergistic response, based upon the use of the Alk4/5/7 inhibitor SB431542, our current studies provide the most direct evidence to date of the involvement of this pathway. We quantified activation of the SMAD3 or SMAD1/5/8 pathways, and only found a synergistic interaction of GDF9/BMP15 upon the SMAD3 pathway. Furthermore, we are able to inhibit this response with the SB431542 inhibitor. These results still leave open the potential, indeed likelihood, that a generalized activation of the SMAD2/3 pathways is a result of GDF9/BMP15 synergistic interactions.

Our results are also consistent with the recently reported results of Reader *et al.* (2011) utilizing a range of chemical inhibitors. That report suggests that there are species differences in the signalling pathways

activated by the GDF9/BMP15 combination when comparing the mouse proteins with those of sheep. The effects seen in the Reader study, as well as in our current study, are indicative of the GDF9 ligand being the determinative partner in the signalling pathways being activated. Therefore, our results should be compared with that portion of the Reader study where mouse GDF9 and BMP15 were used, and consistent with those results we find an involvement of the ERK1/2 MAPK in GDF9/BMP15 signalling, as the specific inhibitor U0126 completely inhibits the GDF9/BMP15 response. However, given our previous results demonstrating the requirement for activation of ERK1/2 for GDF9 signalling (Sasseville *et al.*, 2010), we would suggest a different model to that of Reader *et al.* for the involvement of ERK1/2 in GDF9/BMP15 signalling. We suggest that, just as for GDF9 signalling, the GDF9/BMP15 synergistic response is dependent upon ERK1/2 activation and that this is the result of tyrosine kinase activation of the EGF receptor, and not a direct activation of ERK1/2 by a BMPRII/type I receptor complex. Our results also demonstrate that the SFK inhibitor PP2 dose dependently negated the effect of GDF9/BMP15 on thymidine incorporation in granulosa cells. However, the effects we observe of ERK1/2 or SFK inhibition on the GDF9/BMP15 response may simply reflect that these inhibitors target the GDF9 signalling pathway (Sasseville *et al.*, 2010). In our current studies we found no participation of NF-κB signalling in the GDF9/BMP15 synergistic response, consistent with our previous report of no effect of this pathway on GDF9 signalling (Sasseville *et al.*, 2010).

In support of our contention that GDF9/BMP15 synergism is of central importance to oocyte-somatic cell communication, we find that this response also occurs in cumulus cells and that the response is specific for the GDF9 and BMP15 proteins. Hence, other members of the TGF-β superfamily (e.g. TGF-β1, activin A, activin B, BMP6 or BMP7) are not capable of causing a synergistic biological response with either GDF9 or BMP15, even though they utilize the same intracellular signalling pathways. These results are consistent with those we previously reported whereby we observed no synergistic interaction between a partially purified preparation of recombinant mouse GDF9 and TGF-β1 (Gilchrist *et al.*, 2006). Together these provide solid support for the concept that GDF9 and BMP15 are the principal TGF-β superfamily members mediating the paracrine effects of oocytes on granulosa/cumulus cells (Gilchrist *et al.*, 2008). Further confirmation of species differences in the signalling pathways activated by GDF9/BMP15 will require purified proteins from a range of species. Given the contrasting importance of BMP15 for reproductive success for rodents compared with other species (e.g. sheep and humans), a comparison between purified mouse and sheep/human proteins would certainly appear warranted. With the principles of GDF9 and BMP15 synergistic interactions now well established, further studies are now required to interrogate the interactions between GDF9 and BMP15 *in vivo* within the complex context of their local microenvironment in the growing follicle and the cumulus–oocyte complex.

Authors' roles

D.G.M. and R.B.G. conceived the study and secured funding. D.G.M., L.J.R. and R.B.G. designed the experiments. L.J.R. performed all experiments. D.G.M., L.J.R. and R.B.G. analysed the data and L.J.R. prepared the figures with input from R.B.G. and D.G.M. R.B.G. carried out the statistical analyses. D.G.M. and R.B.G. wrote the manuscript with input from L.J.R. and review by all authors.

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