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Signalling pathways involved in the synergistic effects of human growth differentiation factor 9 and bone morphogenetic protein 15

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Abstract. Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) act synergistically to regulate granulosa cell proliferation and steroid production in several species. Several non-*Sma* and mothers against decapentaplegic (SMAD) signalling pathways are involved in the action of murine and ovine GDF9 and BMP15 in combination, with the pathways utilised differing between the two species. The aims of this research were to determine if human GDF9 and BMP15 also act in a synergistic manner to stimulate granulosa cell proliferation and to identify which non-SMAD signalling pathways are activated. Human GDF9 with BMP15 (GDF9 + BMP15) stimulated an increase in ^{3}H-thymidine incorporation ($P < 0.001$), which was greater than the increase with BMP15 alone, while GDF9 alone had no effect. The stimulation of ^{3}H-thymidine incorporation by GDF9 + BMP15 was reduced by the addition of inhibitors to the SMAD2/3, nuclear factor-$_{K}$B (NF-$_{K}$B) and c-Jun N-terminal kinase (JNK) signalling pathways. Inhibitors to the SMAD1/5/8, extracellular signal-regulated kinase mitogen-activated protein kinase (ERK-MAPK) or p38-MAPK pathways had no effect. The addition of the BMP receptor 2 (BMPR2) extracellular domain also inhibited stimulation of ^{3}H-thymidine incorporation by GDF9 + BMP15. In conclusion, human GDF9 and BMP15 act synergistically to stimulate granulosa cell proliferation, a response that also involves species-specific non-SMAD signalling pathways.

Additional keywords: follicle, granulosa cells, oocyte, ovary, proliferation.

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Introduction

The oocyte-secreted, transforming growth factor-beta (TGF-β) superfamily members, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) play an essential role in regulating ovarian follicular development and ovulation rate in several species (Moore *et al.* 2004; Shimasaki *et al.* 2004; McNatty *et al.* 2005*c*). In many species, GDF9 and BMP15 are co-expressed during follicular development. In addition, GDF9 and BMP15 have been shown to act synergistically to influence proliferation and steroidogenesis in granulosa cells and the expression of cumulus expansion-related genes (McNatty *et al.* 2005*a*, 2005*b*; Mottershead *et al.* 2012; Peng *et al.* 2013). Interestingly, several laboratories have observed synergism when the growth factors were expressed separately and combined in culture (McNatty *et al.* 2005*a*, 2005*b*; Mottershead *et al.* 2012). However, others have shown that co-expression is required for the synergistic action (Peng *et al.* 2013). Whether the observed differences from these laboratories are due to the origin of species of the growth factors or differences in the preparation of the proteins is unclear.

In vivo evidence of species differences in the actions of these factors is provided by the different phenotypes exhibited by mice, sheep and humans with inactivating mutations in *GDF9*, *BMP15* or both. Mice lacking active BMP15 have only minor changes in follicular growth whereas sheep homozygous for inactivating mutations in *BMP15* exhibit primary ovarian failure, while those with a heterozygous mutation have increased fertility (Juengel and McNatty 2005). Humans with a heterozygous non-conservative mutation in *BMP15* exhibit primary ovarian failure (Di Pasquale *et al.* 2004). Both mice and sheep

with inactivated GDF9 are infertile, while aberrant expression of GDF9 in humans is associated with ovarian failure and polycystic ovary syndrome and a rare deletion mutation in the human *GDF9* gene resulted in spontaneous dizygotic twinning (Teixeira Filho *et al.* 2002; Montgomery *et al.* 2004; Dixit *et al.* 2005). Under *in vitro* conditions these factors appear to have different functions depending not only on the species of origin of the granulosa cells but also, surprisingly, on the species of origin of the growth factor (Reader *et al.* 2011; Lin *et al.* 2012).

GDF9 signalling has been shown to be mediated by the Type I receptor TGF-β receptor 1 (TGFBR1) and the Type II receptor BMP receptor 2 (BMPR2) to activate the *Sma* and mothers against decapentaplegic (SMAD)2/3 pathway (Vitt *et al.* 2002; Mazerbourg *et al.* 2004; Kaivo-Oja *et al.* 2005). The extracellular signal-regulated kinase mitogen-activated protein kinase (ERK-MAPK) signalling pathway is also involved in GDF9 stimulation of human granulosa cell proliferation (Huang *et al.* 2009). There is evidence that BMP15 activates the SMAD1/5/8 pathway and co-immunoprecipitates with BMPR1B, and its interaction with granulosa cells can be blocked by the extracellular domain (ECD) of BMPR2 (Moore *et al.* 2003). Furthermore, recent chemical crosslinking results confirm that BMPR1B and BMPR2 form a cell surface complex with BMP15 in a human granulosa cell line (Pulkki *et al.* 2012). However, siRNA knockdown of *BMPR1A*, and not *BMPR1B*, in human granulosa cell lines, abolished BMP15-induced SMAD1/5/8 phosphorylation (Chang *et al.* 2013). The cooperative effects of ovine GDF9 with BMP15 (GDF9 + BMP15) on granulosa cell proliferation were blocked by the ECD of BMPR2 and by the TGFBR1/activin receptor 1B and 1C (ACVR1B/ACVR1C)-SMAD2/3 inhibitor SB431542, while none of the Type I receptor ECDs tested had any effect (Edwards *et al.* 2008; McIntosh *et al.* 2008). Further studies have shown that stimulation of rat granulosa cell ^{3}H-thymidine incorporation by ovine GDF9 + BMP15 is mediated by the nuclear factor-$_{K}$B (NF-$_{K}$B), p38-MAPK and SMAD2/3 signalling pathways (Reader *et al.* 2011). Murine GDF9 + BMP15-stimulated ^{3}H-thymidine uptake, however, appeared to act via the ERK-MAPK, c-Jun N-terminal kinase (JNK) and SMAD2/3 signalling pathways (Reader *et al.* 2011) providing further evidence of species differences in the actions of these factors. There are no published studies examining the cooperative effects of human (h)GDF9 with hBMP15 on granulosa cell proliferation; however, hBMP15 has been tested in combination with mouse (m)GDF9 (Sugiura *et al.* 2010; Mottershead *et al.* 2012). In the Mottershead *et al.* (2012) study, stimulation of mouse granulosa cell ^{3}H-thymidine incorporation by a combination of mGDF9 and hBMP15 was inhibited by SMAD2/3 and ERK-MAPK inhibitors while inhibition of the NF-$_{K}$B pathway had no effect.

The objectives of this study were to: (a) determine if hGDF9 and hBMP15 act cooperatively to stimulate ^{3}H-thymidine incorporation in rat and mouse granulosa cells and confirm that mGDF9 also synergises with hBMP15 to produce similar effects and (b) to identify the second-messenger pathways involved in the stimulation of proliferation of rat granulosa cells by hGDF9 + hBMP15. Rodent granulosa cells were used for two reasons; first, to be able to compare the signalling pathways involved with previously published results from murine and ovine GDF9 + BMP15 and second, because rodent granulosa cells are more readily available from animals with normal fertility than human granulosa cells, which can only readily be obtained from women undergoing fertility treatment and are typically luteinised by ovarian stimulation regimes.

Materials and methods

Rat and mouse granulosa cell ^{3}H-thymidine incorporation bioassay

All animal protocols were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes or the 1999 Animal Welfare Act (Part 6) of New Zealand and were approved by the appropriate local Animal Ethics Committees. Rat and mouse mural granulosa cells were collected for ^{3}H-thymidine incorporation bioassays as previously described (Gilchrist *et al.* 2001, 2006; Reader *et al.* 2011). Briefly, granulosa cells were collected from ovaries from 21- to 26-day-old 129/SV mice or 21- to 24-day-old Sprague Dawley rats 46 h after administration of 5 IU (mouse) or 20 IU (rat) equine chorionic gonadotrophin (Folligon; Intervet, Castle Hill, Australia or Auckland, New Zealand). These were cultured in Medium-199 supplemented with 2 mM GlutaMAX-I (Invitrogen, Auckland, New Zealand), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.23 mM sodium pyruvate (Sigma, Auckland, New Zealand) and 0.3 mg mL^{-1} polyvinyl alcohol (Sigma). Cells (20 000 total cells per well, mouse; 20 000 viable cells per well, rat) were cultured in a total volume of 125 μL supplemented Medium-199 with the treatments described below, and incubated at 37°C with 5% CO_2 in air. After 18 h, 0.4 μCi methyl-^{3}H-thymidine (MP Biomedicals, Cleveland, OH, USA or Amersham, GE Healthcare, Auckland, New Zealand) was added to each well and the cells were incubated for a further 6 h. Following incubation, the cells were harvested onto a filtermat and the incorporated ^{3}H-thymidine determined using a liquid scintillation counter.

Experiment 1: dose response to mGDF9, hGDF9, hBMP15 and GDF9 + BMP15 combinations

To test the effect of treatment on ^{3}H-thymidine incorporation, mouse mural granulosa cells were cultured in the presence of increasing concentrations of mGDF9, hGDF9, hBMP15 or combinations of hGDF9 + hBMP15 or mGDF9 + hBMP15 for 24 h as described above. The concentrations of each treatment were 0 (control), 6.2, 12.5, 25 and 50 ng mL^{-1}. Treatments were performed in triplicate wells in four separate bioassays. Mouse GDF9 mature protein was purchased from R&D Systems Inc. (Minneapolis, MN, USA). For human GDF9 and BMP15, expression cassettes encoding the full-length cDNA sequence were synthesised (Genscript USA, Inc., Piscataway, NJ, USA), incorporating the rat serum albumin signal sequence at the 5′ end followed by a His8 tag and a Strep II epitope tag at the N-terminus of the respective pro-region. These were transferred to the pEFIRES expression vector. Stable HEK-293T cell lines were established expressing the respective proteins, which were purified as previously described (Mottershead *et al.* 2008; Pulkki *et al.* 2011). The lyophilised proteins were resuspended in 4 mM HCl with 0.1% bovine serum albumin (BSA).

Experiment 2: effect of BMPR2 on GDF9 + BMP15-stimulated ^{3}H-thymidine incorporation

To determine if BMPR2 ECD could block the stimulation of ^{3}H-thymidine incorporation in mouse granulosa cells by hGDF9 + hBMP15 or mGDF9 + hBMP15 at 12.5 ng mL^{-1} each, increasing doses of BMPR2 ECD (R&D Systems Inc.) between 0 and 5 μg mL^{-1} were pre-incubated with the GDF9 and BMP15 proteins for 30 min before culturing with the granulosa cells as described above. This dose of GDF9 and BMP15 was chosen as it produced a close to maximal response in ^{3}H-thymidine incorporation without having excess GDF9 or BMP15 in the culture medium. Bioassays were performed on five separate dates and treatments added in triplicate wells.

Experiment 3: signalling pathways of hGDF9 + hBMP15

The purpose of this experiment was to test the effect of hGDF9 and hBMP15 on ^{3}H-thymidine incorporation in rat granulosa cells and to determine which signalling pathways are involved. Rat granulosa cells were used for these experiments to allow a direct comparison with previously published results on the signalling pathways involved in murine and ovine GDF9 and BMP15-stimulated ^{3}H-thymidine incorporation (Reader *et al.* 2011). Full-length hGDF9 and hBMP15 were expressed in HEK-293T cell lines as described previously (Mottershead *et al.* 2008; Pulkki *et al.* 2011) and purified preparations of the mature region of these proteins were kindly supplied by Biotechvisions Ltd (Helsinki, Finland). These were reconstituted in 10 mM HCl and then Medium-199 supplemented with 2 mM GlutaMAX-I, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin (Invitrogen) and 0.2% fetal calf serum (FCS; Invitrogen).

Rat granulosa cells were cultured with the following treatments: hGDF9 (4 ng mL^{-1}), hBMP15 (4 ng mL^{-1}), hGDF9 + hBMP15 in combination (4 ng mL^{-1} each) and no treatment (control). Initial studies had shown that when hGDF9 + hBMP15 were added together at a concentration of 3, 30 or 300 ng mL^{-1} each, ^{3}H-thymidine incorporation was stimulated 4.5-fold relative to the control but there was no significant difference between the different concentrations (results not shown). Therefore, the dose of 4 ng mL^{-1} each of GDF9 and BMP15 provided a maximal response in ^{3}H-thymidine incorporation without having excess GDF9 or BMP15 in the culture medium. Each treatment was incubated with inhibitors to different signalling pathways: SMAD2/3 (SB431542; 1 μM; Tocris, Avonmouth, UK); SMAD1/5/8 (dorsomorphin; 1 μM; Sigma); NF-$_K$B (SN50; 10 μg mL^{-1}; Calbiochem, Merck, Auckland, New Zealand); JNK (TAT-TI-JIP$_{153–163}$; 5 μM; Calbiochem); p38-MAPK (SB239063; 5 μM; Tocris); ERK-MAPK (U0126; 5 μM; Calbiochem) and a dimethyl sulfoxide (DMSO) control, as the inhibitors were reconstituted in DMSO. The doses of inhibitors used for these bioassays were based on studies using the inhibitors with ovine and murine GDF9 + BMP15-treated granulosa cells (Reader *et al.* 2011). SN50 was stable for up to 6 months at −70°C after reconstitution while all other inhibitors were either used fresh or stored at −70°C and used within 2 weeks of reconstitution. Treatments were performed in quadruplicate wells with a minimum of five independent pools of granulosa cells and three independent bioassays.

Statistical analysis

For all experiments the counts from each of the replicate wells were averaged and expressed as a ratio of the control for each experiment, which was then log-transformed. Data from Experiment 1 was analysed by two-way analysis of variance with treatment, dose and day of bioassay fitted as main effects with the interactions of treatment-by-dose and treatment-by-day. The data was back-transformed and the bias-corrected mean standard errors were calculated (Neyman and Scott 1960). Individual comparisons were done using Tukey's test. The combined effect of either hGDF9 or mGDF9 with hBMP15 was compared with either the sum of the individual effects or the product of the individual effects using Tukey's test to determine significance. Data from Experiment 2 was analysed first by two-way analysis of variance with treatment-by-dose and day-of-bioassay as fixed effects. To determine if the dose response curves differed, residual maximum likelihood analysis was performed with treatment, dose and their interaction as fixed effects and random effects of date, cubic smoothing spline for dose (Verbyla *et al.* 1999) and treatment-by-dose interaction. Experiment 3 data was analysed by residual maximum likelihood (Patterson and Thompson 1971) since treatment allocation was not balanced across plates. Plate-within-granulosa cell and pool-within-date were assigned as random effects and DMSO concentration-plus-treatment (the factorial interaction of inhibitory and stimulatory treatments) as fixed effects. There was no significant effect of DMSO concentration at the control level of stimulatory treatment ($P > 0.05$). Standard errors were calculated using the Taylor's series approximation for the variance of a function.

Results

Experiment 1: dose response to mGDF9, hGDF9, hBMP15 and GDF9 + BMP15 combinations

All treatments at all concentrations tested gave a significant increase in ^{3}H-thymidine incorporation in mouse granulosa cells compared with the control ($P < 0.01$), except for hGDF9, which had no effect on its own at any of the doses (Fig. 1). Mouse GDF9 gave a strong dose response while increasing concentrations of hBMP15 produced only a weak increase in response. When hGDF9 + hBMP15 were added in combination they had a synergistic effect on ^{3}H-thymidine uptake at 6.2, 12.5 and 25 ng mL^{-1} that was greater than the product of the factors on their own ($P < 0.01$; Fig. 1). The response of hGDF9 + hBMP15 (50 ng mL^{-1} for each) appeared to be greater but not significantly different from either the additive or multiplicative effects of both factors on their own ($P > 0.05$). The effect of mGDF9 + hBMP15 on ^{3}H-thymidine incorporation was significantly greater than the additive effects of mGDF9 and hBMP15 on their own at the lowest three doses ($P < 0.01$, 6.2 and 12.5 ng mL^{-1}; $P < 0.05$, 25 ng mL^{-1}) but not different from the product of the individual effects ($P > 0.05$). The increase in ^{3}H-thymidine uptake stimulated by mGDF9 + hBMP15 (50 ng mL^{-1} for each) was equal to the sum of the two factors alone but was not different from the effect of mGDF9 on its own ($P > 0.05$).

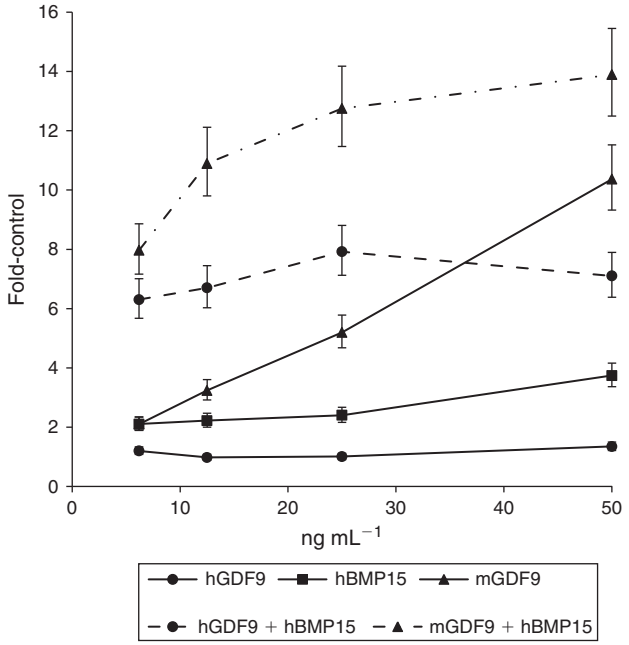

Fig. 1. The effects of increasing doses of mGDF9, hGDF9, hBMP15, mGDF9 + hBMP15 and hGDF9 + hBMP15 on ^{3}H-thymidine incorporation by mouse granulosa cells. Values represent fold-change ± s.e.m. relative to control (set at 1.0). Note: solid lines represent GDF9 or BMP15 added to the cultures alone, and dashed lines when GDF9 and BMP15 were combined.

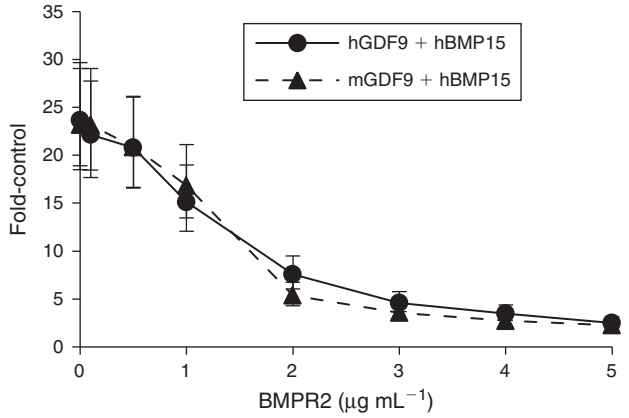

Fig. 2. Addition of increasing doses of BMPR2 ECD inhibits ^{3}H-thymidine incorporation stimulated by either hGDF9 + hBMP15 or mGDF9 + hBMP15 (12.5 ng mL^{-1} each) in mouse granulosa cells. Values represent fold-change ± s.e.m. relative to control (set at 1.0).

Experiment 2: effect of BMPR2 ECD on GDF9 + BMP15 ^{3}H-thymidine incorporation

When BMPR2 ECD was co-cultured at increasing concentrations with either mGDF9 + hBMP15 or hGDF9 + hBMP15 at 12.5 ng mL^{-1}, there was a significant effect of dose on ^{3}H-thymidine incorporation in mouse granulosa cells ($P < 0.001$; Fig. 2). Both mGDF9 + hBMP15- and hGDF9 + hBMP15-stimulated ^{3}H-thymidine uptake was inhibited in an identical manner by BMPR2 (Fig. 2).

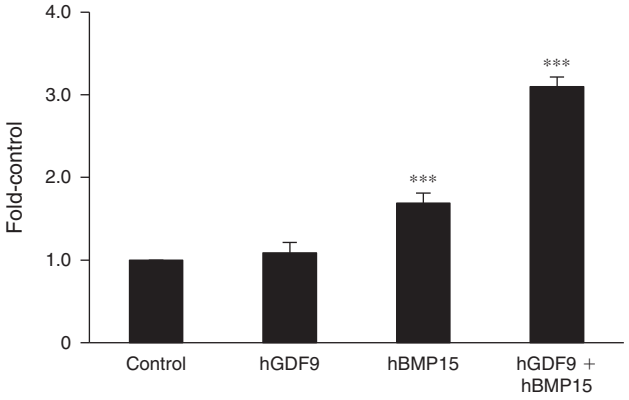

Fig. 3. The effects of addition of hGDF9 (4 ng mL^{-1}), hBMP15 (4 ng mL^{-1}) and hGDF9 + hBMP15 (4 ng mL^{-1} each) on ^{3}H-thymidine incorporation by rat granulosa cells. Values represent fold-change ± s.e.m. relative to control (set at 1.0). *** $P < 0.001$ compared with control.

Experiment 3: signalling pathways of hGDF9 + hBMP15

Human BMP15 at 4 ng mL^{-1} stimulated a 1.7-fold increase in ^{3}H-thymidine incorporation in rat granulosa cells when compared with the control ($P < 0.001$) while hGDF9 had no significant effect on ^{3}H-thymidine uptake. When hBMP15 and hGDF9 were added in combination they stimulated a 3.1-fold increase ($P < 0.001$) in ^{3}H-thymidine incorporation (Fig. 3). Similarly to the inhibition observed when adding BMPR2 ECD to hGDF9 + hBMP15 in the mouse granulosa cell ^{3}H-thymidine bioassay above, BMPR2 ECD completely inhibited the synergistic effects of hGDF9 + hBMP15 in rat granulosa cells (data not shown).

Because the inhibitors to the SMAD2/3 and ERK-MAPK pathways significantly reduced ^{3}H-thymidine incorporation in the controls (i.e. cells with no growth factor), the results are expressed as the effect of the inhibitor with treatment (i.e. control or growth factors) relative to treatment without inhibitor (i.e. with DMSO; dashed line; Fig. 4). The SMAD2/3 pathway inhibitor reduced ^{3}H-thymidine incorporation by the granulosa cells between 42 to 53% when cultured alone (control) and with either hGDF9 or hBMP15 ($P < 0.001$). However, when this inhibitor was cultured with hGDF9 + hBMP15 it caused a 63% decrease in ^{3}H-thymidine uptake ($P < 0.001$), which was significantly greater ($P < 0.05$) than the effect of the inhibitor with control (Fig. 4). Addition of the SMAD1/5/8 pathway inhibitor had no effect on any of the treatments (Fig. 4). When the NF-κB inhibitor was cultured with hGDF9 + hBMP15, it suppressed thymidine incorporation by 49% ($P < 0.001$), which was significantly different ($P < 0.01$) from the effect of the inhibitor with control (Fig. 4). This inhibitor also reduced thymidine uptake stimulated by hBMP15 ($P < 0.05$) when compared with hBMP15 alone, but in this case it was not significantly different from the effect of the inhibitor with control (Fig. 4). The JNK pathway inhibitor caused a 60% reduction in thymidine uptake ($P < 0.01$) stimulated by hGDF9 and hBMP15 in combination and had no significant effect on any other treatment. Addition of the p38-MAPK pathway inhibitor caused ~30% reduction in thymidine incorporation with all treatments but these were not

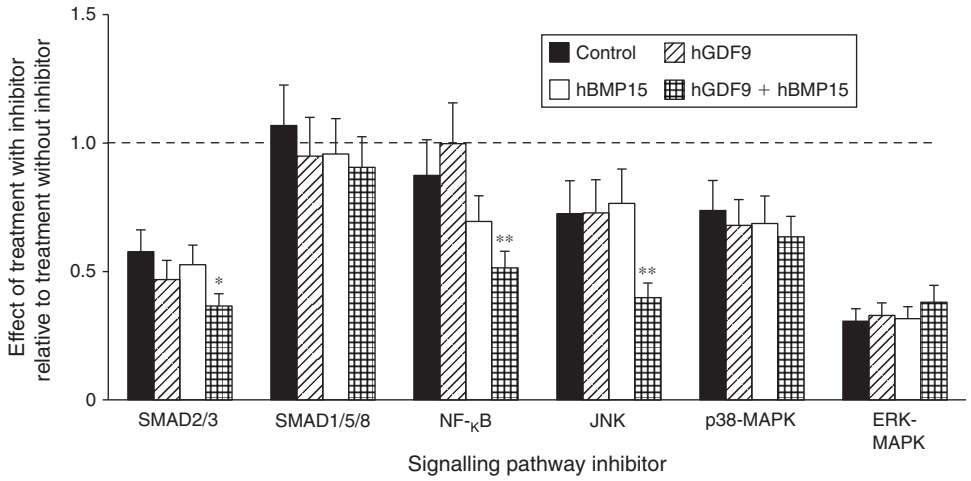

Fig. 4. The effects of inhibitors to the signalling pathways SMAD2/3, SMAD1/5/8, NF-$_K$B, JNK, p38-MAPK and ERK-MAPK cultured without growth factors (control) or with hGDF9 (4 ng mL^{-1}), hBMP15 (4 ng mL^{-1}) or hGDF9 + hBMP15 (4 ng mL^{-1} each) on ^{3}H-thymidine incorporation by rat granulosa cells. Values represent fold-change ± s.e.m. of treatment with inhibitor relative to treatment without inhibitor (dashed line). Significant differences between the treatment with inhibitor and control with inhibitor are represented by * $P < 0.05$ or ** $P < 0.01$.

significantly different from the effect of the inhibitor on its own (Fig. 4). The ERK-MAPK pathway inhibitor strongly suppressed ($P < 0.001$) ^{3}H-thymidine uptake for all treatments including the control.

Discussion

These results show clearly that purified mature hGDF9 and hBMP15 act synergistically to increase ^{3}H-thymidine uptake in mouse and rat granulosa cells as previously observed with unpurified preparations of ovine and murine GDF9 + BMP15 and purified mGDF9 + hBMP15 (McNatty *et al.* 2005*a*, 2005*b*; Mottershead *et al.* 2012). This synergistic effect is not dependent on the factors being synthesised together as a heterodimer, although, as GDF9 and BMP15 form non-covalent homodimers, there remains the possibility of a heterodimer forming *in vitro* (Mottershead *et al.* 2013). Peng *et al.* (2013) did not observe a synergistic effect on the expression of cumulus expansion genes when homodimers of GDF9 and BMP15 were added to mouse granulosa cells, only when GDF9 and BMP15 were co-expressed, whereupon the authors suggest the proteins form heterodimers. This lack of effect with the combination of homodimers may have been due to the presence of N-terminal FLAG- or MYC-tags, as any modification of the mature region of a TGF-β family member with an epitope tag risks altering bioactivity of the resultant protein (Mottershead *et al.* 2008; Pulkki *et al.* 2011). In previous reports, the synergistic effects of GDF9 + BMP15 were observed with unpurified preparations of the full-length proteins and it has been proposed that the cooperative action may involve the pro-regions (McIntosh *et al.* 2008). However, in the present study, and others, the combinations of purified mature regions of the proteins have been shown to have the same synergistic effect as unpurified preparations (Mottershead *et al.* 2012). The synergistic effect of hGDF9 + hBMP15 appeared to be at a maximum at the lowest dose tested while treatment with mGDF9 + hBMP15 increased with increasing dose. This, however, may be due to the fact that mGDF9 can stimulate thymidine incorporation on its own in a dose-responsive manner while hGDF9 cannot. There was no synergistic effect of mGDF9 and hBMP15 at the maximum dose tested.

As previously observed with murine or ovine GDF9 + BMP15 (Edwards *et al.* 2008; McIntosh *et al.* 2008), BMPR2 is essential for the proliferation of either mouse or rat granulosa cells stimulated by hGDF9 + hBMP15. This is also in agreement with the study by Peng *et al.* (2013), which demonstrated that BMPR2 ECD decreased the effect of human or mouse GDF9 + BMP15 in SMAD2/3 phosphorylation assays.

Previous studies have shown that the signalling pathways involved in the cooperative actions of GDF9 and BMP15 depend on the species of origin of the factors (Reader *et al.* 2011; Mottershead *et al.* 2012) and the present study extends these findings. The different signalling pathways involved in the stimulation of ^{3}H-thymidine incorporation in granulosa cells by murine, ovine or human GDF9 + BMP15 are summarised in Table 1. The SMAD2/3 pathway has been shown to be essential in the co-operative signalling of GDF9 + BMP15 from all species studied to date (McIntosh *et al.* 2008; Reader *et al.* 2011; Mottershead *et al.* 2012; Peng *et al.* 2013) and the results from this study support this. However, in the present study, addition of the SMAD2/3 pathway inhibitor to the hGDF9 + hBMP15 combination only suppressed thymidine uptake by 63%, compared with 80% with mGDF9 + mBMP15 or activin, and complete inhibition with oGDF9 + oBMP15 (Reader *et al.* 2011). Since activation of this pathway is thought to be dependent on GDF9 (Reader *et al.* 2011; Mottershead *et al.* 2012), the reduced effect of this inhibitor with hGDF9 + hBMP15 could be due to the lower concentration of hGDF9 used in this

Table 1. A summary of the signalling pathways involved in ^{3}H-thymidine incorporation in granulosa cells stimulated by GDF9 + BMP15 from different species

Pathway inhibitor decreased (↓), increased (↑) or had no effect on (×) ^{3}H-thymidine incorporation. Pathway inhibitor not tested (–). Murine (m), ovine (o) and human (h) GDF9 (9) + BMP15 (15). Data from Reader *et al.* (2011), Mottershead *et al.* (2012) and present study

Signalling pathway	Species of growth factor					
	m9 + m15	o9 + o15	h9 + h15	m9 + h15	m9 + o15	o9 + m15
BMPR2	↓	↓	↓	–	–	–
SMAD 2/3	↓	↓	↓	↓	–	–
SMAD 1/5/8	×	×	×	×	–	–
NF-κB	×	↓	↓	×	×	↓
JNK	↑	×	↓	–	↑	↓
p38-MAPK	×	↓	×	–	–	–
ERK-MAPK	↓	×	×	↓	–	–

study: 4 ng mL^{-1} compared with 25 ng mL^{-1} used in the murine and ovine study (Reader *et al.* 2011). The lack of effect on thymidine incorporation following addition of the SMAD2/3 pathway inhibitor to hBMP15 on its own further supports the idea that the activation of this pathway is mediated by GDF9 alone.

There was no effect of the SMAD1/5/8 pathway inhibitor on stimulation of thymidine incorporation with either hBMP15 alone or hGDF9 and hBMP15 in combination despite reports that BMP15 acts via the BMPR1B receptor (Moore *et al.* 2003). The same result was observed in the murine and ovine study (Reader *et al.* 2011). Although hBMP15 alone was able to phosphorylate SMAD1/5/8, there was no synergistic response in activation of this pathway by mGDF9 with hBMP15 (Mottershead *et al.* 2012). It is possible that the BMPR1B receptor and the SMAD1/5/8 pathway are involved in the activation of cell functions other than proliferation.

Addition of the NF-κB inhibitor reduced thymidine uptake stimulated by hGDF9 + hBMP15 but not to same degree as that observed with oGDF9 + oBMP15 where thymidine incorporation was completely blocked (Reader *et al.* 2011). Again, this difference could be due to the lower concentration of hGDF9 used in this study. Interestingly, the NF-κB inhibitor had no effect on thymidine incorporation stimulated by mGDF9 + mBMP15 and mGDF9 + hBMP15 (Reader *et al.* 2011; Mottershead *et al.* 2012). When combinations of mouse and ovine GDF9 + BMP15 were cultured with the NF-κB inhibitor, the suppressive effect of the NF-κB inhibitor on thymidine incorporation was only observed when oGDF9 was present, indicating that the activation of this pathway is mediated by oGDF9 but not mGDF9. The fact that there was no significant effect of this inhibitor on thymidine incorporation stimulated by hBMP15 alone, and that human and ovine GDF9 do not stimulate proliferation, suggests that signalling of the NF-κB pathway requires the presence of both BMP15 and GDF9 molecules.

Inhibition of the JNK pathway has been shown to cause an increase in thymidine uptake stimulated by mGDF9 + mBMP15, which suggests that this pathway is pro-apoptotic in rat granulosa cells (Reader *et al.* 2011). However, in the present study, JNK pathway inhibition reduced granulosa cell incorporation of ^{3}H-thymidine stimulated by hGDF9 + hBMP15, which is similar to the effect observed with oGDF9 + mBMP15 (Reader *et al.* 2011). In other studies, activation of the JNK pathway has been shown to be either pro- or anti-apoptotic depending on the duration of activation, the cell type and the activity of other signalling pathways (Lin 2003). How the different species of GDF9 and BMP15 could be activating different downstream responses in the same signalling pathway is not known.

There was no specific effect of inhibition of either the p38-MAPK or ERK-MAPK pathways on ^{3}H-thymidine incorporation stimulated by hGDF9 + hBMP15. This differs from the results observed with oGDF9 + oBMP15, which appeared to stimulate thymidine incorporation via the p38-MAPK signalling pathway, and mGDF9 + mBMP15 or mGDF9 + hBMP15, which operated via the ERK-MAPK signalling pathways (Reader *et al.* 2011; Mottershead *et al.* 2012). Human GDF9 activation of human granulosa cell proliferation has been shown to be mediated via the ERK-MAPK pathway (Huang *et al.* 2009). However, a much higher dose of GDF9 was used (200 ng mL^{-1}) compared with the present study, the GDF9 in question was bacterially produced (the risks of using bacterially produced GDF9 or BMP15 have been discussed in Mottershead and Watson (2009)) and the cells were luteinised human granulosa cells.

There is some evidence that the BMP15 pro-region plays a role in controlling the bioactivity of GDF9 and BMP15 in combination (McIntosh *et al.* 2008) and the lack of pro-region in the purified hGDF9 and hBMP15 preparations could explain the slightly different results observed in the human treatments compared with the ovine and murine factors. However, the mature regions of the proteins are still able to stimulate granulosa cell proliferation and activate some of the signalling pathways tested. Moreover, differences in the signalling pathways utilised were also observed between the unpurified murine and ovine factors (Reader *et al.* 2011). The same pathways were confirmed to be as important for purified mature mGDF9 + hBMP15 (Mottershead *et al.* 2012) as for unpurified mGDF9 + mBMP15 (McIntosh *et al.* 2008; Reader *et al.* 2011). These were SMAD2/3 and ERK-MAPK but not SMAD1/5/8 or NF-κB. Further work is needed to determine whether activation of the non-SMAD signalling pathways by GDF9 + BMP15 involves

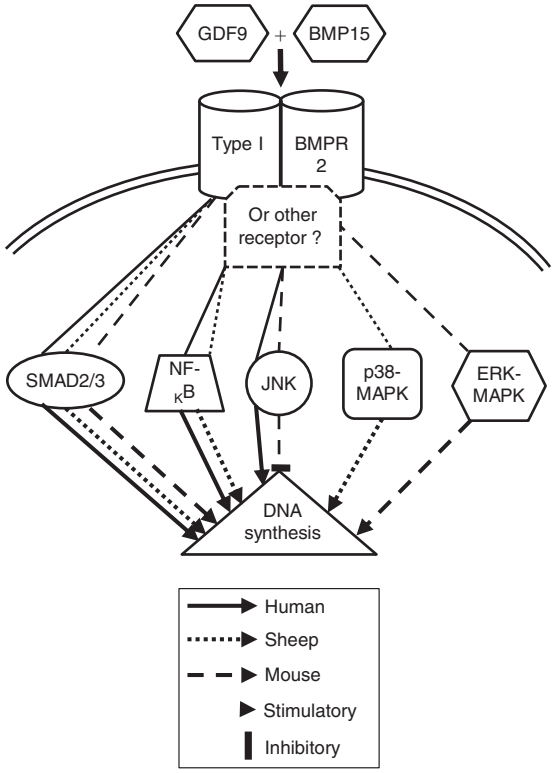

Fig. 5. Summary of the signalling pathways involved in stimulation of granulosa cell proliferation by human, sheep or mouse GDF9 + BMP15, adapted from Reader *et al.* (2011). The SMAD2/3 pathway activation is mediated by the BMPR2 receptor and either of the Type 1 receptors; TGFBR1, ACVR1B or ACVR1C. The non-SMAD signalling pathways (NF-κB, JNK, p38-MAPK and ERK-MAPK) could be activated by the same receptor complex, unknown receptor/s or via downstream release of other ligands.

the direct activation of the BMPR2–Type 1 receptor complex or whether another receptor is involved. Signalling may also be occurring indirectly via the downstream release of other ligands.

In summary, hGDF9 in combination with hBMP15 has been shown to act synergistically to stimulate thymidine incorporation in rat and mouse granulosa cells and this is dependent on BMPR2. This stimulation is mediated by the SMAD2/3 signalling pathway and not the SMAD1/5/8 pathway. The NF-κB pathway was also shown to mediate signalling by hGDF9 + hBMP15, which is in accord with the results obtained for the ovine factors but not mGDF9 + mBMP15 (Fig. 5) or mGDF9 + hBMP15. Inhibition of the JNK signalling pathway reduced thymidine incorporation stimulated by hGDF9 + hBMP15, which was the opposite effect to that observed with mGDF9 + mBMP15 and different from the lack of response seen with oGDF9 + oBMP15 (Fig. 5). It therefore appears that the different effects of GDF9 and BMP15 on granulosa cell function in different species could be due to the activation of divergent non-SMAD signalling pathways.

Declaration of interest

R. B. G. is a consultant to Cook Medical. The University of Adelaide holds a patent on applications of GDF9 and BMP15 (R. B. G. is an inventor). Otherwise 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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References

Chang, H. M., Cheng, J. C., Klausen, C., and Leung, P. C. (2013). BMP15 suppresses progesterone production by down-regulating StAR via ALK3 in human granulosa cells. *Mol. Endocrinol.* **27**(12), 2093–2104. doi:10.1210/ME.2013-1233

Di Pasquale, E., Beck-Peccoz, P., and Persani, L. (2004). Hypergonadotrophic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. *Am. J. Hum. Genet.* **75**(1), 106–111. doi:10.1086/422103

Dixit, H., Rao, L. K., Padmalatha, V., Kanakavalli, M., Deenadayal, M., Gupta, N., Chakravarty, B., and Singh, L. (2005). Mutational screening of the coding region of growth differentiation factor 9 gene in Indian women with ovarian failure. *Menopause* **12**(6), 749–754. doi:10.1097/01.GME.0000184424.96437.7A

Edwards, S. J., Reader, K. L., Lun, S., Western, A., Lawrence, S., McNatty, K. P., and Juengel, J. L. (2008). The cooperative effect of growth and differentiation factor-9 and bone morphogenetic protein (BMP)-15 on granulosa cell function is modulated primarily through BMP receptor II. *Endocrinology* **149**(3), 1026–1030. doi:10.1210/EN.2007-1328

Gilchrist, R. B., Ritter, L. J., and Armstrong, D. T. (2001). Mouse oocyte mitogenic activity is developmentally coordinated throughout folliculogenesis and meiotic maturation. *Dev. Biol.* **240**(1), 289–298. doi:10.1006/DBIO.2001.0451

Gilchrist, R. B., Ritter, L. J., Myllymaa, S., Kaivo-Oja, N., Dragovic, R. A., Hickey, T. E., Ritvos, O., and Mottershead, D. G. (2006). Molecular basis of oocyte–paracrine signalling that promotes granulosa cell proliferation. *J. Cell Sci.* **119**(Pt 18), 3811–3821. doi:10.1242/JCS.03105

Huang, Q., Cheung, A. P., Zhang, Y., Huang, H.-F., Auersperg, N., and Leung, P. C. K. (2009). Effects of growth differentiation factor 9 on cell cycle regulators and ERK42/44 in human granulosa cell proliferation. *Am. J. Physiol. Endocrinol. Metab.* **296**(6), E1344–E1353. doi:10.1152/AJPENDO.90929.2008

Juengel, J. L., and McNatty, K. P. (2005). The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. *Hum. Reprod. Update* **11**(2), 143–160.

Kaivo-Oja, N., Mottershead, D. G., Mazerbourg, S., Myllymaa, S., Duprat, S., Gilchrist, R. B., Groome, N. P., Hsueh, A. J., and Ritvos, O. (2005). Adenoviral gene transfer allows SMAD-responsive gene promoter analyses and delineation of Type I receptor usage of transforming growth factor-beta family ligands in cultured human granulosa luteal cells. *J. Clin. Endocrinol. Metab.* **90**(1), 271–278. doi:10.1210/JC.2004-1288

Lin, A. (2003). Activation of the JNK signalling pathway: breaking the brake on apoptosis. *Bioessays* **25**(1), 17–24. doi:10.1002/BIES.10204

Lin, J. Y., Pitman, J., Bibby, A. H., Hudson, N. L., McIntosh, C. J., Juengel, J. L., and McNatty, K. P. (2012). Effects of species differences on oocyte regulation of granulosa cell function. *Reproduction* **144**, 557–567. doi:10.1530/REP-12-0267

Mazerbourg, S., Klein, C., Roh, J., Kaivo-Oja, N., Mottershead, D. G., Korchynskyi, O., Ritvos, O., and Hsueh, A. J. (2004). Growth differentiation factor-9 signalling is mediated by the Type I receptor, activin receptor-like kinase 5. *Mol. Endocrinol.* **18**(3), 653–665. doi:10.1210/ME.2003-0393

McIntosh, C. J., Lun, S., Lawrence, S., Western, A. H., McNatty, K. P., and Juengel, J. L. (2008). The pro-region of mouse BMP15 regulates the cooperative interactions of BMP15 and GDF9. *Biol. Reprod.* **79**(5), 889–896. doi:10.1095/BIOLREPROD.108.068163

McNatty, K. P., Juengel, J. L., Reader, K. L., Lun, S., Myllymaa, S., Lawrence, S. B., Western, A., Meerasahib, M. F., Mottershead, D. G., Groome, N. P., Ritvos, O., and Laitinen, M. P. (2005*a*). Bone morphogenetic protein 15 and growth differentiation factor 9 co-operate to regulate granulosa cell function. *Reproduction* **129**(4), 473–480. doi:10.1530/REP.1.0511

McNatty, K. P., Juengel, J. L., Reader, K. L., Lun, S., Myllymaa, S., Lawrence, S. B., Western, A., Meerasahib, M. F., Mottershead, D. G., Groome, N. P., Ritvos, O., and Laitinen, M. P. (2005*b*). Bone morphogenetic protein 15 and growth differentiation factor 9 co-operate to regulate granulosa cell function in ruminants. *Reproduction* **129**(4), 481–487. doi:10.1530/REP.1.00517

McNatty, K. P., Smith, P., Moore, L. G., Reader, K., Lun, S., Hanrahan, J. P., Groome, N. P., Laitinen, M., Ritvos, O., and Juengel, J. L. (2005*c*). Oocyte-expressed genes affecting ovulation rate. *Mol. Cell. Endocrinol.* **234**(1–2), 57–66. doi:10.1016/J.MCE.2004.08.013

Montgomery, G. W., Zhao, Z. Z., Marsh, A. J., Mayne, R., Treloar, S. A., James, M., Martin, N. G., Boomsma, D. I., and Duffy, D. L. (2004). A deletion mutation in GDF9 in sisters with spontaneous DZ twins. *Twin Res.* **7**(6), 548–555. doi:10.1375/1369052042663823

Moore, R. K., Otsuka, F., and Shimasaki, S. (2003). Molecular basis of bone morphogenetic protein-15 signalling in granulosa cells. *J. Biol. Chem.* **278**(1), 304–310. doi:10.1074/JBC.M207362200

Moore, R. K., Erickson, G. F., and Shimasaki, S. (2004). Are BMP-15 and GDF-9 primary determinants of ovulation quota in mammals? *Trends Endocrinol. Metab.* **15**(8), 356–361. doi:10.1016/J.TEM.2004.08.008

Mottershead, D. G., and Watson, A. J. (2009). Oocyte peptides as paracrine tools for ovarian stimulation and oocyte maturation. *Mol. Hum. Reprod.* **15**(12), 789–794. doi:10.1093/MOLEHR/GAP085

Mottershead, D. G., Pulkki, M. M., Muggalla, P., Pasternack, A., Tolonen, M., Myllymaa, S., Korchynskyi, O., Nishi, Y., Yanase, T., Lun, S., Juengel, J. L., Laitinen, M., and Ritvos, O. (2008). Characterisation of recombinant human growth differentiation factor-9 signalling in ovarian granulosa cells. *Mol. Cell. Endocrinol.* **283**(1–2), 58–67. doi:10.1016/J.MCE.2007.11.007

Mottershead, D. G., Ritter, L. J., and Gilchrist, R. B. (2012). Signalling pathways mediating specific synergistic interactions between GDF9 and BMP15. *Mol. Hum. Reprod.* **18**(3), 121–128. doi:10.1093/MOLEHR/GAR056

Mottershead, D. G., Harrison, C. A., Mueller, T. D., Stanton, P. G., Gilchrist, R. B., and McNatty, K. P. (2013). Growth differentiation factor 9:bone morphogenetic protein 15 (GDF9:BMP15) synergism and protein heterodimerisation. *Proc. Natl. Acad. Sci. USA* **110**(25), E2257. doi:10.1073/PNAS.1303459110

Neyman, J., and Scott, E. L. (1960). Correction for bias introduced by a transformation of variables. *Ann. Math. Stat.* **31**, 643–655. doi:10.1214/AOMS/1177705791

Patterson, H. D., and Thompson, R. (1971). Recovery of inter-block information when block sizes are unequal. *Biometrika* **58**, 545–554. doi:10.1093/BIOMET/58.3.545

Peng, J., Li, Q., Wigglesworth, K., Rangarajan, A., Kattamuri, C., Peterson, R. T., Eppig, J. J., Thompson, T. B., and Matzuk, M. M. (2013). Growth differentiation factor 9:bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions. *Proc. Natl. Acad. Sci. USA* **110**(8), E776–E785. doi:10.1073/PNAS.1218020110

Pulkki, M. M., Myllymaa, S., Pasternack, A., Lun, S., Ludlow, H., Al-Qahtani, A., Korchynskyi, O., Groome, N., Juengel, J. L., Kalkkinen, N., Laitinen, M., Ritvos, O., and Mottershead, D. G. (2011). The bioactivity of human bone morphogenetic protein-15 is sensitive to C-terminal modification: characterisation of the purified untagged processed mature region. *Mol. Cell. Endocrinol.* **332**(1–2), 106–115. doi:10.1016/J.MCE.2010.10.002

Pulkki, M. M., Mottershead, D. G., Pasternack, A. H., Muggalla, P., Ludlow, H., van Dinther, M., Myllymaa, S., Koli, K., ten Dijke, P., Laitinen, M., and Ritvos, O. (2012). A covalently dimerised recombinant human bone morphogenetic protein-15 variant identifies bone morphogenetic protein receptor Type 1B as a key cell surface receptor on ovarian granulosa cells. *Endocrinology* **153**(3), 1509–1518. doi:10.1210/EN.2010-1390

Reader, K. L., Heath, D. A., Lun, S., McIntosh, C. J., Western, A. H., Littlejohn, R. P., McNatty, K. P., and Juengel, J. L. (2011). Signalling pathways involved in the cooperative effects of ovine and murine GDF9+BMP15-stimulated thymidine uptake by rat granulosa cells. *Reproduction* **142**(1), 123–131. doi:10.1530/REP-10-0490

Shimasaki, S., Moore, R. K., Otsuka, F., and Erickson, G. F. (2004). The bone morphogenetic protein system in mammalian reproduction. *Endocr. Rev.* **25**(1), 72–101. doi:10.1210/ER.2003-0007

Sugiura, K., Su, Y.-Q., Li, Q., Wigglesworth, K., Matzuk, M. M., and Eppig, J. J. (2010). Oestrogen promotes the development of mouse cumulus cells in coordination with oocyte-derived GDF9 and BMP15. *Mol. Endocrinol.* **24**(12), 2303–2314. doi:10.1210/ME.2010-0260

Teixeira Filho, F. L., Baracat, E. C., Lee, T. H., Suh, C. S., Matsui, M., Chang, R. J., Shimasaki, S., and Erickson, G. F. (2002). Aberrant expression of growth differentiation factor-9 in oocytes of women with polycystic ovary syndrome. *J. Clin. Endocrinol. Metab.* **87**(3), 1337–1344. doi:10.1210/JCEM.87.3.8316

Verbyla, A. P., Cullis, B. R., Kenward, M. G., and Welham, S. J. (1999). The analysis of designed experiments and longitudinal data by using smoothing splines. *J. R. Stat. Soc. Ser. C Appl. Stat.* **48**(3), 269–311. doi:10.1111/1467-9876.00154

Vitt, U. A., Mazerbourg, S., Klein, C., and Hsueh, A. J. (2002). Bone morphogenetic protein receptor Type II is a receptor for growth differentiation factor-9. *Biol. Reprod.* **67**(2), 473–480. doi:10.1095/BIOLREPROD67.2.473