Bone morphogenetic protein 15 and growth differentiation factor 9 co-operate to regulate granulosa cell function

Kenneth P McNatty, Jennifer L Juengel, Karen L Reader, Stan Lun, Samu Myllymaa¹, Steve B Lawrence, Andrea Western, Mohamed F Meerasahib², David G Mottershead¹, Nigel P Groome², Olli Ritvos¹ and Mika P E Laitinen¹

AgResearch, Wallaceville Animal Research Centre, Ward Street, PO Box 40063, Upper Hutt, New Zealand,

¹Program for Developmental and Reproductive Biology, Biomedicum Helsinki, and Department of Bacteriology and Immunology, Haartman Institute, 00014 University of Helsinki, Helsinki, Finland and ²School of Biological and Medical Sciences, Oxford Brookes University, Headington, Oxford, UK

Correspondence should be addressed to K P McNatty; Email: ken.mcnatty@agresearch.co.nz

Abstract

The oocyte-secreted polypeptide growth factors, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15, also known as GDF9B) have both been shown to be essential for ovarian follicular growth and function. The effects of murine (m) and ovine (o) GDF9 as well as oBMP15, alone or together, on ³H-thymidine uptake and progesterone and inhibin production by granulosa cells from rats were determined. Murine GDF9 stimulated thymidine incorporation by granulosa cells whereas oGDF9 and oBMP15 alone had no effect. However, oBMP15 given together with mGDF9 or oGDF9 was very potent in stimulating ³H-thymidine incorporation by granulosa cells with a greater than 3-fold stimulation compared with any growth factor alone. The synergistic effect of oBMP15 and oGDF9 was almost completely blocked by antibodies generated against these growth factors when administered either alone or in combination. While neither GDF9 (murine or ovine) nor oBMP15 were able to modulate FSH-stimulated progesterone production on their own, FSH-stimulated progesterone production by granulosa cells was potently inhibited when BMP15 and GDF9 were administered together. Immunoreactive α -inhibin levels increased more than 15-fold from granulosa cells when BMP15 and GDF9 were given together whereas consistent stimulatory effects of either growth factor alone were not observed. The effects of GDF9 and BMP15, when added together, were different than those observed for the growth factors alone. Therefore, we hypothesize that within the ovary, these oocyte-secreted growth factors co-operate to regulate proliferation and gonadotropin-induced differentiation of granulosa cells in mammals.

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Introduction

The oocyte-derived members of the transforming growth factor- β superfamily, namely growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15, also known as GDF9B), have both been shown to be essential regulators of follicular development in rodents, sheep and humans (Elvin *et al.* 2000, McNatty *et al.* 2003, Di Pasquale *et al.* 2004, Shimasaki *et al.* 2004). In addition, there is evidence that both proteins are involved in regulating ovulation rate in sheep (Davis *et al.* 1991, Galloway *et al.* 2000, Hanrahan *et al.* 2004, Juengel *et al.* 2004). Although it is evident that there are interactions between these closely-related growth factors with respect to follicular growth and ovulation rate (Yan *et al.* 2001, Hanrahan *et al.* 2004), the molecular mechanisms underlying the functions of GDF9 and BMP15 together on

follicular cell proliferation and function are not well understood. The objectives of the present studies were to examine the effects of ovine (o) GDF9 and oBMP15 alone and together on granulosa cell functions in rats. Since the effects of murine (m) GDF9 on granulosa cells of several species have been characterized, a comparison between the effects of oGDF9 and mGDF9 was also undertaken.

Materials and Methods

Generation of mGDF9, oGDF9 and oBMP15 constructs

The mGDF9 construct used for generating stably transfected cell lines has been described previously (Kaivo-Oja *et al.* 2003). Ovine GDF9 coding sequence was generated by PCR from ovine ovarian cDNA in two fragments to

facilitate the generation of constructs (Bodensteiner *et al.* 1999). The cDNA encoding the prepro part of the protein was amplified with primer pair oGDF9-1/oGDF9-2 (see Table 1 for listing of all primers used in generation of mammalian expression constructs) whereas the mature part was first amplified with primer pair oGDF9-3/oGDF9-4, subcloned to pGEM T-Easy vector and thereafter used as a template for second amplification with primer pair oGDF9-5/oGDF9-6. The prepro and mature protein encoding fragments generated were fused using the NarI site inserted into primers oGDF9-2 and oGDF9-5. Thereafter, the whole open reading frame (ORF) was transferred to pEFIREs-P expression vector (Hobbs *et al.* 1998) through EcoRI-XbaI sites.

The coding region of oBMP15 (Galloway *et al.* 2000) was amplified from ovine ovarian cDNA in two fragments using primers oBMP15-1/oBMP15-2 to obtain the pro-region and primers oBMP15-3/oBMP15-4 to obtain the mature fragment. The mature protein encoding cDNA was then re-amplified with primers oBMP15-5/oBMP15-6. Both fragments were subcloned into pGEM T-Easy vectors, fused through NarI site and transferred to pEFIREs-P vector through EcoRI-XbaI. The modified primer oBMP15-1 caused a V to A change in the second amino acid of the signal peptide. The twelfth amino acid in the signal peptide region changed from W to L based on the published sequence due to a PCR error or polymorphism.

The modified primers in the putative proteolytic cleavage site of oGDF9 and oBMP15 created four arginine residues (R-R-R-R) instead of the wild type sequence in oGDF9 (R-H-R-R) and in oBMP15 (R-R-A-R). As the four arginine residue motif perfectly matches to the furin protease consensus sequence (R-X-X-R) and the four arginine repeat is found naturally in mGDF-9 sequence, it is unlikely that these alterations affect the biological activity or the proteolytic cleavage efficiency of the respective proteins.

Expression of recombinant proteins

All the cDNAs were subcloned into a pEFIREs-P expression vector (Hobbs *et al.* 1998) and transfected into

Table 1 Primers used for creation of constructs for expression of ovine growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) in mammalian cells.

Primer name	Primer sequence
GDF9-1	5'ATCGATGCTAATTCTTCCAAGCCATG
GDF9-2	5'GCGGCGCCGGGACGATCTTACACCTCAGC
GDF9-3	5'CGGCGCCGACAGACGAGAGAGTGCC
GDF9-4	5'AGGAGTCTGTAAACGACAGGTACA
GDF9-5	5'ATCGATGCTAGCCTAGTGATTCGGCGCCGC
GDF9-6	5'ATCGATTCTAGAAGGAGTCTGTAAACG
BMP15-1	5'ATCGATCCAAGCCATGCGCTCCTGAGCATCCTAGA
BMP15-2	5'ACGGCGCCTCAAGAGAAGAGAAGGGTC
BMP15-3	5'CGGCGCCGCGCTCAAGCAGGCAGTATT
BMP15-4	5'CCTTTGCCGTACCTGCATGTGCA
BMP15-5	5'ATCGATGCTAGCCTAGTGATTCGGCGCCGC
BMP15-6	5'ATCGATTCTAGACTTTTGCCGTACCT

a human embryonic kidney cell line, HEK-293H (293H), by the Fugene 6 transfection reagent (Fugene 6, Roche, USA and Roche Diagnostics NZ Ltd, Auckland, NZ). Cells expressing high levels of the recombinant protein were selected with increasing concentrations of puromycin in DMEM (high glucose) supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (all supplies from Invitrogen, Auckland, NZ). The recombinant protein was produced into serum-free harvesting medium (DMEM/Ham's F-12, 1:1) supplemented with l-glutamine and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), 0.01% (w/v) BSA (Sigma, Auckland, NZ) and 100 µg/ml heparin (Sigma, Auckland, NZ).

The amounts of GDF9 or BMP15 in conditioned media were determined by Western blotting under reducing conditions using monoclonal antibodies against GDF9 and BMP15 and chemiluminescence. The generation of the GDF9 antibody has been described (Juengel *et al.* 2002) and the BMP15 antibody was generated in the same manner except that an *E. coli* expressed mature region of oBMP15 (Juengel *et al.* 2002) was used as antigen and media from 293H cells expressing oBMP15 was used for ELISA screening. Further testing of the resulting antibody indicated that it recognized the BMP15 peptide SEVPGPS-REHDGPES, which is near the N-terminus of the mature region of BMP15. The reaction of the 293H expressed GDF9 or BMP15 was compared with the reaction of a standard curve of *E. coli* expressed oGDF9 or oBMP15 (Juengel *et al.* 2002) using the Scion Image program (Scion Corporation, Frederick, MD, USA) to determine the concentration of the GDF9 or BMP15 in the media (Fig. 1). The mammalian expressed GDF9 and BMP15 were similar in size to those previously reported for mammalian expressed GDF9 and BMP15 (Elvin *et al.* 1999, Otsuka *et al.* 2000). The differences in size between the *E. coli* expressed and the mammalian expressed proteins most likely relates to the expression construct (*E. coli* proteins contain a histidine tag) and differences in secondary structure not resolved by the denaturing-reducing PAGE. The *E. coli* expressed proteins aggregate and these aggregates were not fully dissociated hence the presence of the higher molecular weight forms present in Fig. 1. The concentration of GDF9 in the media averaged 5.4 µg/ml (range 3.0–7.8 µg/ml) as assessed by comparison to an ovine *E. coli* expressed GDF9 mature region by Western blotting. The concentration of BMP15 in the media averaged 0.020 µg/ml (range 0.018–0.022 µg/ml) as assessed by comparison to an ovine *E. coli* expressed BMP15 mature region by Western blotting. The untransfected 293H cell conditioned media contained undetectable amounts of GDF9 (i.e. ≤0.001 µg/ml) or BMP15 (i.e. ≤0.005 µg/ml). It is important to note that the relative affinities of the antibodies for the *E. coli* expressed proteins versus the mammalian expressed proteins are unknown and this may account for the differences observed in the concentrations of mammalian BMP15 and GDF9.

Figure 1 Quantification of GDF9 and BMP15 by Western blotting using monoclonal antibodies against GDF9 (mab37; panel A), BMP15 (mab61a; panel B) or an ovine parasite antigen (Control; pab3; panel C). Lane 1, media from untransfected 293H cells; lane 2, media from 293H cells transfected with the mGDF9 construct; lane 3, media from 293H cells transfected with the ovine (o) GDF9 construct; lane 4, media from 293H cells transfected with the oBMP15 construct, lane 5–9 or 10 are the *E. coli* produced mature regions of the respective proteins. For GDF9, lanes 5–10 represent 50, 25, 12.5, 6.25, 3.125 and 1.56 ng respectively. For BMP15, lanes 5–9 represent 12.5, 6.25, 3.125, 1.56 and 0.78 ng respectively. Molecular weight markers are indicated on the right hand side of each panel. Arrowheads on the left hand side of each panel indicate the migration of the mammalian expressed protein.

Granulosa cell collection

Granulosa cells were collected from all surface-visible follicles from equine chorionic gonadotrophin (eCG)-treated rats to maximise the numbers of cells for the bioassay. Vitt *et al.* (2000) have shown previously that granulosa cells from small antral (i.e. diethylstilbestrol (DES)-treated) and preovulatory follicles (i.e. eCG treated) responded similarly to GDF9 treatment when examined for thymidine incorporation or follicle-stimulating hormone (FSH)-stimulated progesterone production. Ovaries were collected from Sprague-Dawley rats (23–26 day old; University of Otago, Dunedin, NZ) approximately 46 h after i.p. administration of 20 IU eCG (Intervet Ltd, Auckland, NZ). The ovaries were dissected away from surrounding tissue and were then rinsed twice in Leibovitz L-15 media (Invitrogen, Auckland, NZ) containing 0.1% (w/v) BSA, 100 U/ml penicillin and 100 µg/ml streptomycin. Granulosa cells

were collected by syringe aspiration and suspended in Leibovitz L-15 media. Oocyte–cumulus cell complexes, isolated oocytes and follicular debris were visualized using a dissecting microscope and most, if not all, were removed from the granulosa cells using a glass pipette. The remaining cells were recovered following centrifugation at 300 *g* for 5 min at room temperature after one wash in 5 ml Leibovitz L-15 media.

Culture of granulosa cells for determination of ³H-thymidine incorporation

Before culture, cells were washed in 5 ml M199 (Earle's, Sigma) with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM GlutaMAX-1 (Invitrogen), 0.3 mg/ml polyvinyl alcohol (Sigma) and 0.23 mM sodium pyruvate (Sigma) and resuspended using a syringe and needle in M199. Cell viability was determined using trypan blue exclusion. Granulosa cells (20 000 viable cells in a total volume of 125 µl per well) were cultured in M199 with varying amounts of conditioned media from control (untransfected), GDF9 expressing or BMP15 expressing 293H cells at 37 °C in a 5% CO₂ incubator. After 18 h of culture, methyl-³H-thymidine (Perkin Elmer, Boston, MA, USA; 20 Ci/mmol, 0.4 µCi per well) was added to each well and the culture continued for an additional 6 h after which cells were harvested with a cell harvester onto a thin filter mat. Incorporation of ³H-thymidine was determined using a Wallac Trilux MicroBeta 1450 liquid scintillation counter (Biolab, Auckland, NZ). Treatments included 1000 or 2000 ng/ml of mGDF9 or oGDF9, 4 or 8 ng/ml of oBMP15, or the combination of 1000 ng/ml mGDF9 with 4 ng/ml oBMP15, or 1000 ng/ml oGDF9 with 4 ng/ml oBMP15. These concentrations of GDF9 and BMP15 were the maximum that could be tested within the volume limits of the bioassay based on the use of a maximum of 40% of conditioned media. All control wells were treated with an identical volume of conditioned media from untransfected 293H cells. In addition, the effects of diluting oGDF9 (200, 100, 20 and 10 ng/ml) while oBMP15 concentrations were held constant at 4 ng/ml or diluting the oBMP15 (2, 0.8, 0.4, 0.08 and 0.04 ng/ml) while oGDF9 concentrations were held constant at 1000 ng/ml were determined. To determine specificity of the observed effect, the antibodies shown to have abilities to neutralize oGDF9 and oBMP15 *in vivo* (Juengel *et al.* 2002) were used to deplete oGDF9 and oBMP15 from culture media prior to adding to rat granulosa cells. Media (500 µl) containing 40 ng oGDF9 and 2 ng oBMP15 were preincubated at 4 °C for 2 h with 500 µg/ml of KLH, oGDF9, oBMP15 or an equal combination of GDF9 and BMP15 IgG. Antibody–antigen complexes were precipitated by adding 0.1 by volume of a slurry of 50% protein G sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden) in PBS and mixing for 2 h at 4 °C. Preadsorbed and non-preadsorbed media were collected after centrifugation at 12 000 *g* for 20 s and applied to wells of granulosa cells as described above to provide a final concentration of

16 ng/ml of oGDF9 and 0.8 ng/ml of oBMP15. Media not preadsorbed by antibodies (i.e. with 16 ng/ml oGDF9 + 0.8 ng/ml oBMP15 with no preabsorption and the untransfected 293H media) underwent all of the same treatments as listed for the preadsorbed media except they received no antibodies. The concentrations of 16 ng/ml GDF9 + 0.8 ng/ml BMP15 were chosen as representing a combination dose that caused a stimulation of thymidine incorporation by granulosa cells that was between 0.5 and 0.8 of the maximum effect. For all assays, all treatments were applied at least in triplicate with a minimum of three independent pools (range 3–10) of granulosa cells being tested. Within an assay, individual values outside 30% of the mean value for the treatment were discarded. Points in which at least two of the replicates were not within 30% of each other were regarded as missing data.

Culture of granulosa cells for determination of progesterone and inhibin production

Cells were washed a further time in 5 ml McCoys 5a media (Sigma) with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM GlutaMAX-1 and 0.1% BSA and resuspended using a syringe and needle in McCoys 5a media. Cell viability was determined using trypan blue exclusion. Granulosa cells (100 000 viable cells per well; 250 µl total volume) were added to McCoys 5a media containing 5 ng/ml sodium selenite (Sigma), 10 ng/ml bovine insulin (Sigma), 5 µg/ml holo-transferrin (Invitrogen), 30 ng/ml androstenedione (Sigma), 3 ng/ml ovine FSH (prepared in our laboratory, 1.4 × USDA-oFSH-19-SIAFP RP2), 1 ng/ml IGF-1 (Long-R3, Gro-Pep, Sydney, Australia) with varying amounts of conditioned media from control (untransfected), GDF9 expressing and/or BMP15 expressing 293H cells. Cells were cultured at 37°C in a humidified incubator with a 5% CO₂ in air gas phase. Every 48 h, 200 µl of media was removed from each well and replaced with 200 µl of warmed media that had been prepared at the start of the culture and stored at 4°C. Media samples from the last 48 h of treatment were collected on day 6 of treatment and frozen at –20°C for later determination of progesterone and inhibin concentrations by RIA. Treatments included the addition of 1000 or 2000 ng/ml of mGDF9 or oGDF9, 4 or 8 ng/ml oBMP15 or the combination of 1000 ng/ml mGDF9 with 4 ng/ml oBMP15, or 1000 ng/ml oGDF9 with 4 ng/ml oBMP15. In addition, the effects of diluting oGDF9 (200, 100, 20 and 10 ng/ml) while oBMP15 concentrations were held constant at 4 ng/ml or diluting oBMP15 (2, 0.8, 0.4, 0.08 and 0.04 ng/ml) while oGDF9 concentrations were held constant at 1000 ng/ml were determined. All treatments were applied at least in triplicate to a minimum of three independent pools (range 3–5) of granulosa cells. Within an assay, individual values outside of 20% of the mean value for the treatment were discarded. Points in which at least two of the replicates were not within 20% of each other were regarded as missing data.

Determination of progesterone and inhibin concentrations in media

Concentrations of progesterone in media were determined by RIA as described (Asher 1990). The sensitivity of the assay (90% maximum binding) was 17 pg/ml and the intra- and inter-assay coefficients of variations, averaged for a standard pool sample at approximately 20%, 50% and 80% binding, were overall 7.1% and 8.7% respectively. No samples were below the sensitivity of the assay. The concentrations of inhibin were determined by RIA as previously described (McNatty *et al.* 1992). The antibody to inhibin detects 31 kDa inhibin and the pro-αC subunit (Robertson *et al.* 1989). The intra- and inter-assay coefficients of variations were 9.4% and 13.1% respectively. The sensitivity of the assay was 13 IU/ml bINH R-90/1 standard: no samples were below the sensitivity of the assay.

Statistical analysis

The differences between treatments and control as well as those between pre-adsorbed media and non-preadsorbed media were determined using the two-tailed paired *t*-test function in Microsoft Excel 2003. The control samples always contained an equivalent amount of untransfected 293H conditioned media as the treated samples. For determining dose responsiveness of effects, all dilutions were made in conditioned media collected from untransfected 293H cells and thus all groups were treated with equivalent volumes of conditioned media. The values for ³H-thymidine incorporation and inhibin were transformed (natural log) prior to analyses. Differences in responses between treatments were determined using the two-tailed *t*-test function in Microsoft Excel 2003 using the ratio of control values. For clarity, all values have been converted to a ratio of the appropriate controls which were assigned a value of 1.00. The geometric means (and 95% confidence limits) for ³H-thymidine incorporation and inhibin production by granulosa cells treated with 293H control media were 198 (132, 296) c.p.m./well and 382 (331, 461) IU/well respectively. The mean ± S.E.M. for progesterone production by granulosa cells treated with 293H control media was 15 ± 2 ng/well.

Results

³H-thymidine incorporation

mGDF9 stimulated thymidine incorporation into rat granulosa cells whereas oGDF9 or oBMP15 had no effect (Fig. 2). The addition of either mGDF9 or oGDF9 with oBMP15 resulted in a synergistic effect with thymidine incorporation being at least 3-fold above either hormone alone (see Fig. 2). In the GDF9 and BMP15 serial dilution studies, the effects of both oGDF9 and oBMP15 were dose dependent (Fig. 3). The minimum effective dose (defined as a significant difference to media control) was 10 ng/ml for oGDF9 (lowest dose tested) and 0.4 ng/ml

Figure 2 The effects of addition of murine (m) or ovine (o) GDF9 and/or oBMP15 on ^3H -thymidine incorporation (top), progesterone production (middle) or inhibin production (bottom) in rat granulosa cells (numbers above each histogram refer to the number of independent pools of granulosa cells tested). * $P < 0.05$ versus conditioned media control, ** $P < 0.01$ versus conditioned media control, *** $P < 0.001$ versus conditioned media control.

for oBMP15. In the antibody neutralization studies, the antibodies directed against oGDF9 or oBMP15 were able to neutralize most (78–92%) of the activity whereas antibodies directed against KLH had no such effect (Fig. 4) indicating that the stimulation of ^3H -thymidine incorporation was specific for oGDF9 and oBMP15.

Progesterone production

Neither GDF9 (murine or ovine) nor oBMP15 alone affected the production of progesterone by rat granulosa cells (Fig. 2). The addition of GDF9 (murine or ovine) and oBMP15 together resulted in a significant suppression of progesterone production with the effect observed being more dramatic with oGDF9 than with mGDF9 ($P < 0.01$; Fig. 2). In the GDF9 and BMP15 serial dilution studies, the effects of both oGDF9 and oBMP15 were dose dependent (Fig. 3). The minimum effective dose (defined as a significant difference to media control) was 100 ng/ml for oGDF9 and 2.0 ng/ml for oBMP15.

Figure 3 The effects of varying concentrations of oGDF9 and oBMP15 on ^3H -thymidine incorporation (top), progesterone production (middle) or inhibin production (bottom) in rat granulosa cells. Varying concentrations of oGDF9 (solid line) were tested in the presence of a constant concentration of oBMP15 (4 ng/ml) and varying concentrations of oBMP15 (dashed line) were tested in the presence of a constant concentration of oGDF9 (1000 ng/ml). Values above (^3H -thymidine and inhibin) or below (progesterone) the dashed line are significantly different than conditioned media control values ($P < 0.05$). For inhibin, all values except the 10 ng/ml GDF9 were significantly greater than conditioned media controls.

Inhibin production

Murine GDF9 did not consistently affect inhibin production (Fig. 2). In addition, neither oGDF9 nor oBMP15 affected inhibin production. However, when added together, GDF9 (either murine or ovine) and oBMP15 stimulated inhibin production in a synergistic manner (Fig. 2) with a greater effect seen with oGDF9 than with mGDF9 ($P < 0.05$). In the GDF9 and BMP15 serial dilution studies, the effects of both oGDF9 and oBMP15 were dose dependent (Fig. 3). The minimum effective dose (defined as a significant difference to media control) was 100 ng/ml for oGDF9 and 0.04 ng/ml for oBMP15 (lowest dose tested).

Discussion

In the present study, mGDF9 stimulated thymidine incorporation in rat granulosa cells. This is consistent with previous findings showing that rat GDF9 stimulated thymidine incorporation in these cells (Vitt *et al.* 2000).

Figure 4 The effects of preadsorption of media containing oGDF9 and oBMP15 with antibodies directed against keyhole limpet haemocyanin (KLH), oGDF9, oBMP15 or oGDF9 + oBMP15 on ³H-thymidine incorporation in rat granulosa cells. * $P < 0.05$ versus conditioned media control, ** $P < 0.01$ versus conditioned media control, *** $P < 0.001$ versus conditioned media control; b, $P < 0.01$ to non-adsorbed media containing oGDF9 and oBMP15.

However, oGDF9 was not able to stimulate thymidine incorporation in rat granulosa cells suggesting that the bioactivity of GDF9 may be dependent upon the species of origin of this growth factor. In the present study, oBMP15 did not stimulate thymidine incorporation, which is in contrast to the finding that hBMP15 is a potent stimulator of thymidine incorporation in rat granulosa cells (Otsuka *et al.* 2000) and in human granulosa cells (Di Pasquale *et al.* 2004). The question as to whether this is due to the species of origin of the growth factor remains to be elucidated.

When ovine or murine GDF9 and oBMP15 were added together, potent stimulation of thymidine incorporation was observed in granulosa cells. This stimulation was inhibited by specific neutralizing antibodies to either GDF9 or BMP15 indicating that the effects were specific for both GDF9 and BMP15 and required both molecules. These data strongly support the hypothesis that GDF9 and BMP15 co-operate to regulate thymidine incorporation and thus cell proliferation in granulosa cells.

With respect to progesterone, neither mGDF9 nor oGDF9 alone affected FSH-stimulated production by rat granulosa cells. This finding for the rat is in contrast to that found by Vitt *et al.* (2000) where rat GDF9 caused a dose-dependent decrease in FSH-stimulated progesterone production in granulosa cells from DES or gonadotrophin stimulated rats and by Yamamoto *et al.* (2002) who showed a dose-dependent decrease in 8-bromo cAMP-stimulated production in human granulosa cells. However, the present results are similar to those by Elvin *et al.* (1999) who showed a lack of interaction between mGDF9

and FSH in regulating progesterone production by mouse granulosa cells. The observed differences in the various studies in the role of GDF9 in regulating progesterone production from granulosa cells seems most likely related to methodological differences possibly including the use of granulosa cells of different maturational age, different species, the origin of the growth factor used or different culture conditions. oBMP15 alone did not affect FSH-stimulated progesterone production by granulosa cells. This is in contrast to previous studies reporting that hBMP15 suppresses FSH-stimulated progesterone production by rat granulosa cells (Otsuka *et al.* 2000). Again, the reasons for the observed difference are unknown but could be related to methodological difference including the use of BMP15 of different species. When ovine or murine GDF9 and oBMP15 were added together, a significant suppression of progesterone production was observed whereas no effect was observed for either growth factor alone. Thus, similar to ³H-thymidine incorporation, it appears that GDF9 and BMP15 co-operate to regulate progesterone production.

In contrast to previous results showing an increase in inhibin production using rat GDF9 with rat (Hayashi *et al.* 1999, Roh *et al.* 2003) or human (Kaivo-Oja *et al.* 2003) granulosa cells, mGDF9 alone, in the present study, did not significantly increase inhibin production from rat granulosa cells. A large part of this may be due to differences in the assays used to measure inhibin as well as the culture conditions employed. In the present study the assay utilized an antibody that does not distinguish between dimeric inhibin and the free α -inhibin subunit to measure inhibin production. In previous studies, Otsuka *et al.* (2001) report that human BMP15 inhibited the syntheses of inhibin α and inhibin/activin β_A and β_B subunit mRNA in FSH-stimulated rat granulosa cells, whereas in the absence of FSH, human BMP15 had no effect. In contrast, the present data suggest that oBMP15 alone does not affect inhibin protein production by FSH-stimulated rat granulosa cells. However, when mGDF9 or oGDF9 were added to oBMP15, strong synergistic effects were noted suggesting that BMP15 and GDF9 co-operate to regulate inhibin production in the rat.

Clearly, GDF9 and BMP15 are co-operating to regulate granulosa cell function in the rat. This is consistent with the co-operative effects on follicular growth observed in sheep heterozygous for mutations in both genes and mice heterozygous for a mutation in GDF9 and lacking an active BMP15 gene (Yan *et al.* 2001, Hanrahan *et al.* 2004). Other members of this superfamily are known to exert their biological activity as dimers with homodimers as well as heterodimers shown to be biologically active (Chang *et al.* 2002, Shimasaki *et al.* 2004). Notably, heterodimers of other members of the TGF β superfamily have been shown to be more potent than their respective homodimeric forms (Israel *et al.* 1996).

The molecular basis behind these combined effects remains to be elucidated. Both GDF9 and BMP15 are

missing the highly conserved cysteine residue that is involved in dimer formation (Elvin *et al.* 2000, Chang *et al.* 2002, McNatty *et al.* 2003). Both GDF9 and BMP15 have been shown to produce homodimers and heterodimers when produced in the same cell in culture (Liao *et al.* 2002). Clearly, these proteins do not need to be produced by the same cell to elicit their co-operative effects as combining conditioned media containing GDF9 and BMP15 just prior to adding to the granulosa cells is sufficient to observe the effect. Thus, these growth factors could be acting on the granulosa cells as homodimers, heterodimers or even as monomers.

In conclusion, GDF9 and BMP15 appear to be co-operating, as the effects observed when both growth factors were added together were different from that when either growth factor was added separately. Together, these growth factors appear to stimulate proliferation but inhibit gonadotrophin-induced differentiation of granulosa cells as reflected by the biosynthesis of progesterone. Given that both GDF9 and BMP15 are likely to be present in the microenvironment of individual follicles throughout most stages of follicular growth, it is perhaps reasonable to consider these growth factors together as a functional signaling unit rather than as individual paracrine reagents acting independently of one another.

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