Molecular basis of oocyte-paracrine signalling that promotes granulosa cell proliferation

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Accepted 16 June 2006
Journal of Cell Science 119, 3811-3821 Published by The Company of Biologists 2006
doi:10.1242/jcs.03105

Summary

Oocytes regulate follicle growth by secreting paracrine growth factors that act on neighbouring granulosa cells (GCs). Those factors identified to date are mainly members of the transforming growth factor-β (TGFβ) superfamily, but little is known about which specific receptor/signalling system(s) they employ. This study was conducted to determine the requisite pathways utilised by oocytes to promote GC proliferation. We used an established oocyte-secreted mitogen bioassay, where denuded mouse oocytes are co-cultured with mural GCs. Oocytes, growth differentiation factor-9 (GDF9), TGFβ1 and activin-A all promoted GC DNA synthesis, but bone-morphogenetic protein 6 (BMP6) did not. Subsequently, we tested the capacity of various TGFβ superfamily receptor ectodomains (ECD) to neutralise oocyte- or specific growth factor-stimulated GC proliferation. The BMP type-II receptor (BMPR-II) ECD antagonised oocyte and GDF9 bioactivity dose-dependently, but had no or minimal effect on TGFβ1 and activin-A bioactivity, demonstrating its specificity. The TGFβR-II, activinR-IIA and activinR-IIB ECDs all failed to neutralise oocyte- or GDF9-stimulated GC DNA synthesis, whereas they did antagonise the activity of their respective native ligands. An activin receptor-like kinase (ALK) 4/5/7 inhibitor, SB431542, also antagonised both oocyte and GDF9 bioactivity in a dose-dependent manner. Consistent with these findings, oocytes, GDF9 and TGFβ1 all activated SMAD2/3 reporter constructs in transfected GC, and led to phosphorylation of SMAD2 proteins in treated cells. Surprisingly, oocytes did not activate the SMAD1/5/8 pathway in transfected GCs although exogenous BMP6 did. This study indicates that oocyte paracrine factors primarily utilise a similar signalling pathway first identified for GDF9 that employs an unusual combination of TGFβ superfamily receptors, the BMPR-II and a SMAD2/3 stimulatory ALK (4, 5 or 7), for transmitting their mitogenic actions in GC. This cell-signalling pathway may also have relevance in the hypothalamic-pituitary axis and in germ-somatic cell interactions in the testis.

Key words: Oocyte-paracrine factors, Granulosa cell signalling, Growth-differentiation factor 9, Bone morphogenetic protein receptor-II, Activin-receptor like kinase, SMADs, Oocyte mitogen

Introduction

Mammalian oocyte growth and development is critically dependent on a functional two-way communication axis between the germ cell and its companion somatic cells, the ovarian granulosa cells (GCs). The cellular and molecular processes involved are poorly understood but it is evident that communication between the oocyte and somatic cells can be in the form of direct gap-junctional communication and/or paracrine signalling via soluble oocyte-secreted factors (OSFs). During the course of folliculogenesis a highly specialised subset of GCs differentiate in the immediate vicinity of the oocyte, called cumulus cells (CCs), and through gap-junctional communication, these cells establish intimate metabolic contact with each other and with the oocyte, forming the cumulus-oocyte complex. It has long been recognised that GCs and CCs transmit the endocrine and local ovarian signals responsible for nurturing growth and development of the oocyte. This communication is essential for oogenesis and acquisition of oocyte developmental competence. By contrast, it has only more recently become recognised that oocyte paracrine signalling to GCs/CCs is a fundamental process that regulates ovarian folliculogenesis and has profound effects on fertility if disturbed (Dong et al., 1996; McNatty et al., 2004).

Oocytes regulate folliculogenesis by modulating a broad range of GC and CC functions associated with somatic cell growth and differentiation, primarily achieved through the secretion of soluble growth factors acting locally on these cells, rather than by gap-junctional signalling (reviewed in Eppig, 2001; Gilchrist et al., 2004a). Some of the GC and CC functions regulated by OSFs include: promotion of cellular growth (Eppig et al., 2002; Gilchrist et al., 2001; Vanderhyden et al., 1992) and prevention of death (Hussein et al., 2005); modulation of steroidogenesis (Vanderhyden et al., 1993); CC expansion (Buccione et al., 1990; Salustri et al., 1990); inhibin, activin and follistatin synthesis (Glister et al., 2003; Lanuza et al., 1999); as well as modulation of kit-ligand (Joyce et al.,

2000), luteinizing hormone receptor (Eppig et al., 1997) and urokinase plasminogen activator expression (Canipari et al., 1995). By controlling the developmental pathway of its neighbouring somatic cells, the oocyte actively regulates its own microenvironment (Eppig et al., 1997; Li et al., 2000). Understanding oocyte control of GC/CC function and of folliculogenesis in general is important because altered expression of known oocyte paracrine factors can lead to sterility or increased fecundity in a species-dependent manner (McNatty et al., 2004).

The molecular basis of the paracrine communication axis from oocytes to GCs/CCs is still emerging as specific OSFs become identified and their signalling pathways in GCs/CCs are elucidated. One of the challenges that remains is the integration of this information into coherent physiological mechanisms. This study was undertaken to investigate the nature of the oocyte-GC/CC paracrine communication axis specific to regulation of somatic cell proliferation, a pivotal component of folliculogenesis. We hypothesised that this communication axis probably involves a transforming growth factor β (TGFβ) superfamily signalling system for the following reasons. Firstly, mutations in some oocyte growth factors belonging to this superfamily, namely growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), or in their receptors, have dramatic effects on female reproductive phenotype, which suggests that such signalling plays a critical role in ovarian folliculogenesis (Dong et al., 1996; Galloway et al., 2000; Juengel and McNatty, 2005). Interestingly, males carrying these same mutations have apparently normal fertility, even though these growth factors are also expressed in the hypothalamic-pituitary axis and in very high levels in testis (Fitzpatrick et al., 1998; Otsuka and Shimasaki, 2002). Secondly, certain TGFβ superfamily members, in particular TGFβ1, TGFβ2, GDF9 and BMP15, can mimic the actions of oocytes on the GC/CC functions outlined above, although it is now clear that this does not necessarily mean a certain growth factor accounts for the native OSF(s) normally mediating these processes (Dragovic et al., 2005; Gilchrist et al., 2003; Gilchrist et al., 2004b; Hussein et al., 2005; Vanderhyden et al., 2003). The essential growth-promoting molecules and signalling pathways utilised by oocytes remain to be determined.

The TGFβ superfamily receptor/signalling cascade is well characterised (for reviews, see Massague et al., 2000; Shimasaki et al., 2004). Extracellular ligands in the form of homodimers or heterodimers, bind to either a type-I receptor (commonly referred to as an activin receptor-like kinase; ALK) or a type-II receptor and subsequent receptor heteromerisation, leads to ALK phosphorylation followed by phosphorylation of intracellular receptor-regulated signal transducers called SMADs. Ligand-induced targeted gene transcription is mediated by a heterodimeric complex of receptor-regulated SMADs and receptor-independent co-SMADs, such as SMAD4. Intracellular signalling by TGFβ superfamily growth factors can be broadly divided into two distinct groups; those utilising the TGFβ/activin signalling pathway, and those using the BMP pathway. TGFβ/activin signalling typically involves binding to ligand-specific type-II receptors, recruitment and phosphorylation of ALK4 or ALK5, leading to activation of the SMAD2 and SMAD3 proteins. By contrast, BMP ligand binding to the BMP type-II receptor (BMPR-II) leads to phosphorylation of ALKs 2, 3 or 6, and subsequent activation of SMAD1, SMAD5 and/or SMAD8 molecules (Massague et al., 2000). Oocyte-derived BMP15 and BMP6 use the classic BMP pathway; binding BMPR-II and ALK6, and activating the SMAD1/5/8 pathway (Moore et al., 2003; Shimasaki et al., 2004). By contrast, oocyte-secreted GDF9, a homologue of BMP15, is an unusual member of the TGFβ superfamily in that it uses a hybrid of the two signalling systems; GDF9 binds BMPR-II (Vitt et al., 2002) but utilises the TGFβ type-I receptor, ALK5 (Kaivo-Oja et al., 2005; Mazerbourg et al., 2004), leading to activation of SMAD2 and SMAD3 signal transducers (Kaivo-Oja et al., 2003; Kaivo-Oja et al., 2005; Mazerbourg et al., 2004; Roh et al., 2003). Hence, even though GDF9 binds a BMP type-II receptor it can be thought that it induces a TGFβ-like intracellular response, in terms of SMAD activation. GDF9 may also activate the MAPK pathway, although at this stage it is unclear how this is mediated (Su et al., 2003).

Of the numerous TGFβ superfamily members, oocytes are known to express TGFβ1, TGFβ2, activins, GDF9, BMP15 and BMP6 (Juengel and McNatty, 2005), all of which, with the sole exception of BMP6, exert mitogenic effects when added in recombinant form to GCs in vitro. This study exploits a bioassay of primary mouse GCs aspirated from antral follicles and co-cultured with denuded oocytes, which enables scrutiny of the actual native oocyte-secreted growth factors and allows ablation of the signalling axis at various levels. Using this bioassay, we have previously demonstrated that immunoneutralisation of TGFβ1/2 has no effect on oocyte-stimulated GC proliferation, but immunoneutralisation of GDF9 eliminates approximately half of the mitogenic activity of oocytes, demonstrating that oocytes secrete multiple mitogens, one of which is GDF9 (Gilchrist et al., 2003; Gilchrist et al., 2004b). The current study was conducted to further investigate the nature of the paracrine signalling system(s) from germ to neighbouring somatic cells, including the receptors OSFs employ and the intracellular responses invoked in GCs to stimulate proliferation.

Results

OSFs stimulate GC proliferation – comparison with GDF9 and BMP6

Co-culture of oocytes with mural GCs led to a potent, dose-dependent, stimulation of GC DNA synthesis, as assessed by [^{3}H]thymidine incorporation (Fig. 1A). To confirm that increased GC ^{3}H-incorporation is associated with GC proliferation, we measured expression of the cell cycle transcript *Ccnd2* and found that mRNA levels were significantly (P<0.01) increased to sixfold that of controls by exposure to OSFs (Fig. 1B). GDF9 is known to be a potent GC mitogen and a dose of 60 ng/ml appeared to be the saturation point for this growth-promoting effect (Fig. 1A). We have previously shown that oocyte-secreted GDF9 accounts for some, but not all, of the oocyte mitogenic activity (Gilchrist et al., 2004b). As little is known about the function of oocyte-expressed BMP6, we compared the mitogenic effects of BMP6 to that of GDF9 and oocytes. Addition of BMP6 led to very modest changes in mural GC DNA synthesis; 3-fold increase at 400 ng/ml, compared with 40-fold for GDF9 at 60 ng/ml (Fig. 1A). Furthermore, a BMP6-specific neutralising antibody had no effect on the mitogenic effects of oocytes on mural GCs

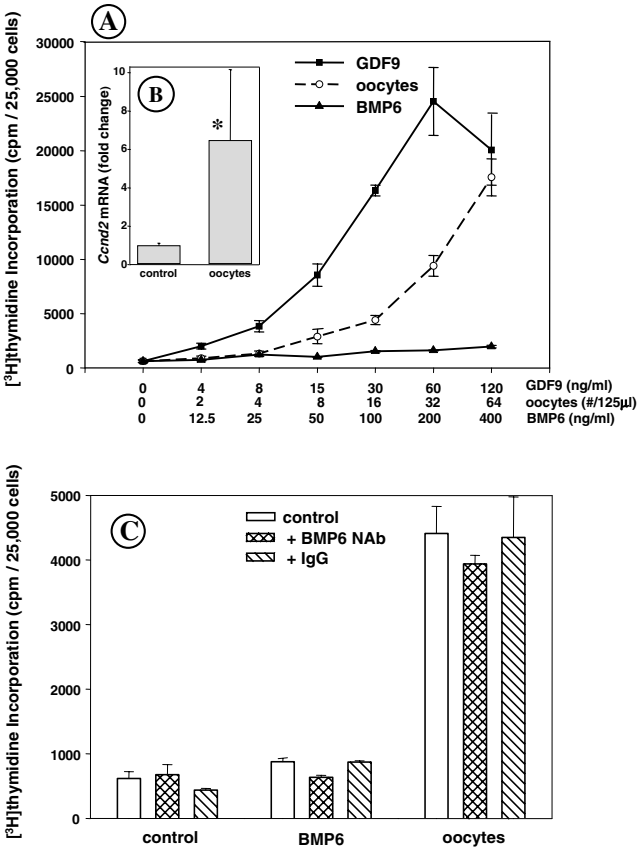

Fig. 1. Oocyte stimulation of granulosa cell DNA synthesis – comparison with GDF9 and BMP6. (A) Mural GCs were cultured with increasing numbers of oocytes per well or increasing doses of recombinant BMP6 or GDF9 and assessed for [^{3}H]thymidine incorporation after 24 hours. Points are means ± s.e.m. from triplicate wells and are representative of three replicate experiments. (B) Mural GCs were cultured alone or together with DOs (125 DOs/125-µl well) for 6 hours, RNA was extracted, reverse transcribed and subjected to real-time RT-PCR for *Ccnd2*. *mRNA levels significantly increased with oocyte co-culture. (C) Mural GCs were cultured alone, treated with BMP6 (50 ng/ml) or co-cultured with 16 oocytes per well, each with and without a neutralising dose (20 µg/ml) of a BMP6 monoclonal neutralising antibody (NAb) or a control IgG (20 µg/ml). Columns represent means ± s.e.m. from triplicate wells, representative of three replicate experiments. BMP6 NAb had no significant effect (P>0.05) on oocyte-stimulated GC DNA synthesis.

(Fig. 1C). Together these results provide evidence that oocyte-expressed BMP6 is unlikely to participate directly in the growth-promoting effects of oocytes on GCs.

Interactions between OSFs and TGFβ superfamily members

To examine possible interactions between GDF9 and other putative oocyte-secreted mitogens, in the regulation of GC DNA synthesis, mural GCs were treated with increasing concentrations of recombinant mouse GDF9 either alone, together with denuded oocytes (DOs), or together with TGFβ1, which shares a common type-I receptor but uses a different type-II receptor compared with GDF9 (Fig. 2). GDF9 was an exceptionally potent stimulator of cell proliferation (c.f.

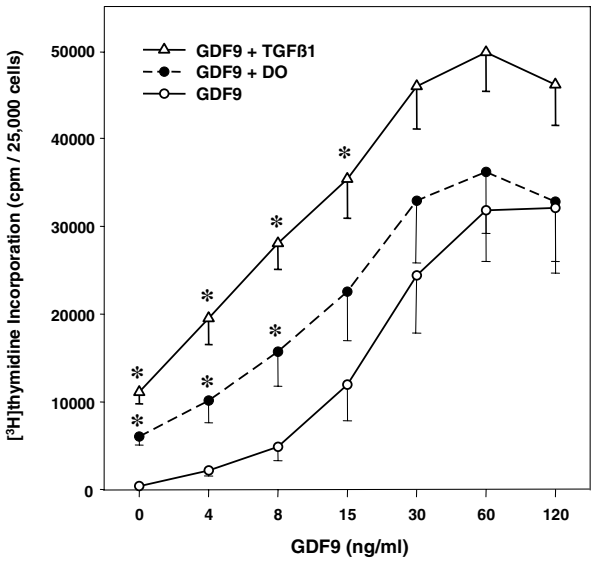

Fig. 2. Interactions between GDF9 and TGFβ1 or oocyte-stimulated granulosa cell DNA synthesis. Mural GCs were cultured with increasing doses of mouse GDF9 either alone or in the presence of DOs (16 DOs/125-µl well) or human TGFβ1 (0.5 ng/ml). After 24 hours of culture the labelled thymidine incorporated into cells was counted. Points are means ± s.e.m. from at least triplicate wells from five replicate experiments. The stimulatory effects of TGFβ1 and oocytes were additive to the stimulatory actions of GDF9 at low doses of GDF9 [means with an asterisk are significantly different to GDF9 alone at that dose (2-way ANOVA; P<0.01)], whereas this additive effect was lost at higher doses of GDF9 (>8 ng/ml for DOs; >15 ng/ml for TGF-β1; dose GDF9 X treatment interaction, P<0.001).

TGFβ1 or activin A; Fig. 3A), inducing 10-100-fold increases in mural GC [^{3}H]thymidine incorporation in a dose-dependant manner (2-way ANOVA main effect, P<0.001; Fig. 2). Denuded oocytes alone or TGFβ1 alone also significantly increased mural GC [^{3}H]thymidine incorporation (post-hoc Tukey test; P<0.001), as expected, and this constant dose of additional mitogen also augmented the effects of GDF9 at low doses (Fig. 2). However, a significant GDF9 × additional mitogen interaction was observed (2-way ANOVA; P<0.001). Although the effects of DOs or TGFβ1 were additive to GDF9 when at low doses, this additivity was lost at maximum and near-maximum doses of GDF9 (>30 ng/ml). This was most apparent with GDF9 + DO, where the additive effects of oocytes were lost at just 15 ng/ml GDF9 or greater (P>0.05). While TGFβ1 and GDF9 may appear to be additive at all doses (Fig. 2), this was not statistically significantly at the highest doses of GDF9. A similar result to GDF9 + TGFβ1 was observed with GDF9 + activin A (data not shown). These results indicate that a common receptor/signalling system is employed by both GDF9 and oocyte-secreted factors, and suggest that the type-II GDF9 receptor BMPR-II may be the rate-limiting component of the signalling system used by oocytes to promote mural GC proliferation.

OSFs signal through BMPR-II to promote granulosa cell proliferation

To test whether BMPR-II is a rate-limiting component of

oocyte mitogenic signalling, we examined the capacity of a known GDF9 antagonist, BMPR-II ECD, to neutralise the mitogenic activity of oocytes (Fig. 3). The specificity of the antagonistic action of the BMPR-II ECD was examined by comparing its effectiveness against related TGFβ superfamily mitogens: GDF9, activin A and TGF-β1. As expected, the BMPR-II ECD had no effect on TGFβ1-stimulated mural GC DNA synthesis ($P>0.05$), was partially antagonistic against activin A ($P<0.05$) and was a potent inhibitor of GDF9 ($P<0.001$; Fig. 3A), as previously reported (Moore et al., 2003; Vitt et al., 2002). Interestingly, this reagent also dramatically reduced oocyte-stimulated mural GC DNA synthesis ($P<0.01$; Fig. 3A), and this occurred in a dose-dependent manner that mirrored that observed for GDF9 (Fig. 3B). In both cases, [^{3}H]thymidine incorporation was approximately halved at 0.13 μg/ml BMPR-II ECD ($P<0.001$) and reduced to control levels at 2 μg/ml. The results in Fig. 3 demonstrate that >90% of oocyte mitogenic activity is mediated through BMPR-II.

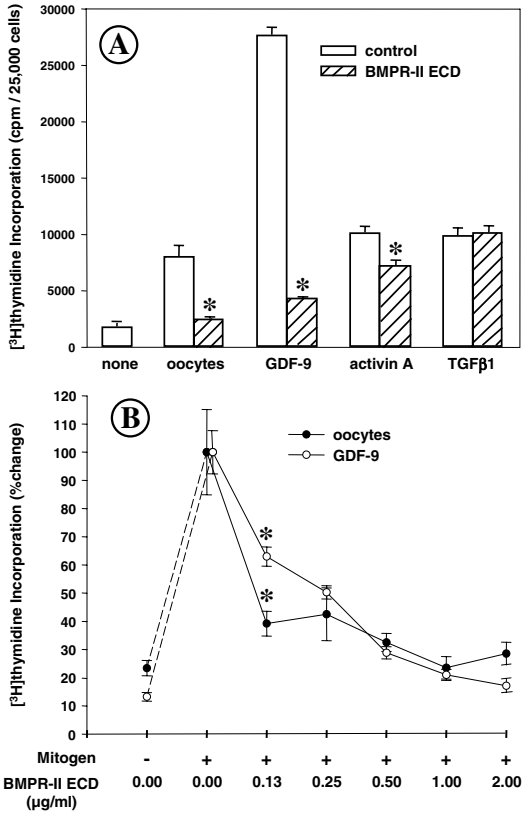

Fig. 3. Specificity and potency of the BMP receptor-II ectodomain at neutralising oocyte- and GDF9-stimulated mural GC DNA synthesis. (A) Mural GCs were cultured with DOs (12 DOs/125-μl well), mouse GDF9 (40 ng/ml), human activin A (20 ng/ml), and human TGFβ1 (0.5 ng/ml), in either the absence or the presence of a maximum neutralising dose of BMP receptor-II ectodomain (BMPR-II ECD; 2 μg/ml). Bars represent means ± s.e.m. from at least triplicate wells and are representative of three replicate experiments. Asterisks represent significant neutralisation ($P<0.01$) caused by the ECD. Control is mural GC alone or with mitogen. (B) Mural GCs were cultured either with or without DOs (12 DOs/125-μl well; ●) or 40 ng/ml mouse GDF9 (○), in either the absence or the presence of increasing doses of BMPR-II ECD. Points are means ± s.e.m. from at least triplicate wells, expressed as a fraction of the control (mitogen with no ECD), and are representative of three replicate experiments. An asterisk represents the lowest dose of BMPR-II ECD that is significantly less than the 100% control ($P<0.001$).

Because it is known that there is a large degree of redundancy within the superfamily of TGFβ receptors, we went on to examine whether oocyte-secreted molecules may also use other known type-II receptors, by testing a range of TGFβ superfamily type-II receptor ECDs in our bioassay. Fig. 4A-C illustrates that ECDs of TGFβR-II, ActR-IIA and ActR-IIB are all effective at specifically antagonising the bioactivity of their respective ligands ($P<0.05$), but neutralise <20% of oocyte-stimulated activity ($P>0.05$; Fig. 4D), whereas, consistent with the results in Fig. 3A, ~90% of oocyte bioactivity was neutralised by the BMPR-II ECD ($P<0.05$). Collectively, these experiments demonstrate that BMPR-II is the main type-II TGFβ superfamily receptor transmitting the oocyte paracrine signals that promote GC growth.

OSFs signal through BMPR-II to promote cumulus cell proliferation

Because paracrine factors secreted by oocytes appear to establish a morphogenic gradient in the ovarian follicle (Hussein et al., 2005), presumably the main target cells for OSFs are the granulosa cells immediately surrounding the oocyte. In secondary follicles, these cells are the preantral GCs, although oocytes do not appear to posses mitogenic activity at this stage of development (Gilchrist et al., 2001), and in tertiary follicles, these are the cumulus cells (CCs). To ensure that oocyte paracrine signalling via BMPR-II has relevance in CCs, we examined whether CCs express *Bmpr2*. Using real-time RT-PCR, we found that CCs express levels of *Bmpr2* mRNA equivalent to those of MGCs (Fig. 5A). We also tested whether the mitogenic effects of oocytes on CCs is mediated through BMPR-II. CC DNA synthesis was stimulated by co-culture with oocytes ($P<0.05$) and this response was completely ablated by additional treatment with the BMPR-II ECD (Fig. 5B), which demonstrates that, as for mural GCs, this is the key receptor for the transmission of oocyte paracrine effects in CCs.

OSFs activate the TGFβ/activin intracellular SMAD signalling pathway

Transduction of signals through BMPR-II requires formation of a heteromeric complex with a type-I receptor(s), an ALK, following ligand binding. Classical BMP signalling through BMPR-II involves interaction with ALK2, ALK3 or ALK6 leading to activation of SMADs 1/5/8 (Shimasaki et al., 2004). However, in the case of GDF9, signal transduction probably requires the interaction of the BMPR-II with ALK5, leading to SMAD 2/3 activation (Kaivo-Oja et al., 2005; Mazerbourg et al., 2004). To determine the type-I receptor and intracellular signalling molecules activated by oocytes, we co-cultured oocytes with mural GCs and examined activation of mural GC SMADs by use of SMAD-specific luciferase reporter constructs and western blot analysis of phospho-SMAD proteins. The CAGA promoter is activated by phosphorylated SMAD3 upon cellular stimulation of ALKs corresponding to the TGFβ/activin pathway, and the BMP-responsive element (BRE) promoter is activated by phosphorylated SMAD1 or 5, stimulated by BMPs (Mazerbourg et al., 2004). Treatment of transfected mural GCs with TGFβ1, as expected, stimulated

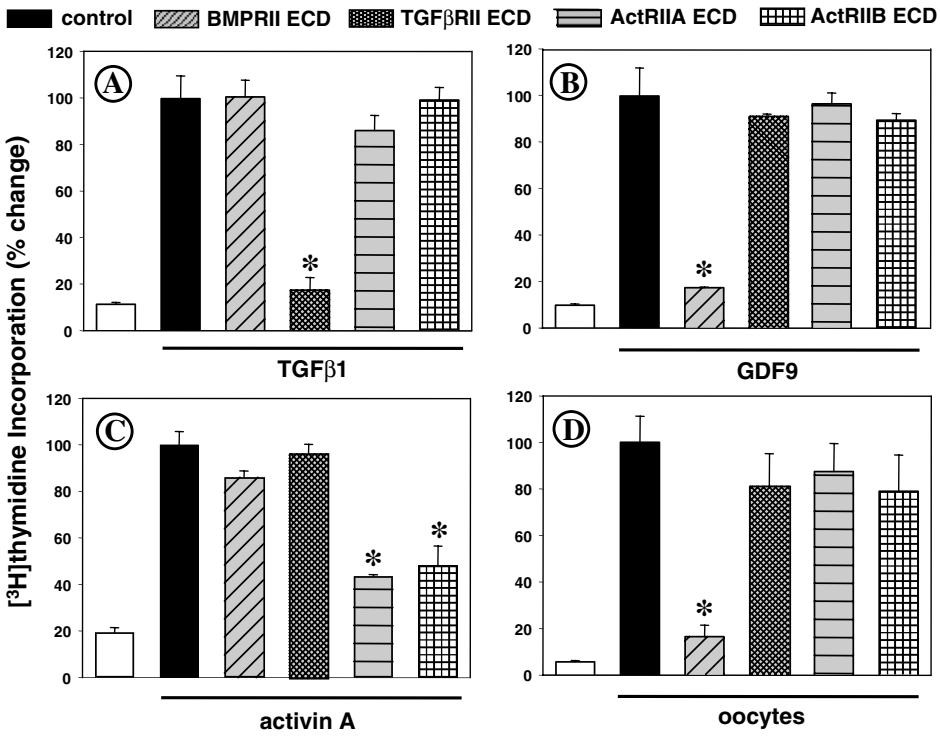

Fig. 4. Specificity of oocyte-secreted factors for BMPR-II within the superfamily of TGFβ type-II receptors. Mural GC thymidine incorporation was stimulated with either (A) human TGFβ1 (0.5 ng/ml), (B) mouse GDF9 (40 ng/ml), (C) human activin A (10 ng/ml) or (D) 12 oocytes/well. Each mitogen was also treated separately with four different soluble receptor ectodomains; BMPR-II, TGFβR-II, ActR-IIA and ActR-IIB, each at 2 μg/ml. Bars are means ± s.e.m from triplicate wells, expressed as a fraction of the positive control (mitogen with no ECD), and are representative of four replicate experiments. An asterisk represents significant antagonism relative to the positive control (P<0.05).

the luciferase reporter driven by the CAGA promoter, and this was also dose-dependently stimulated with GDF9 (Fig. 6A), which demonstrates that primary mouse GCs respond intracellularly to GDF9 in the same manner as rat (Mazerbourg et al., 2004) and human GCs (Kaivo-Oja et al., 2005). Accordingly, treatment of mural GCs with GDF9 also induced phosphorylation of SMAD2 molecules, as detected by western blot (Fig. 6B). Consistent with oocyte-secretion of GDF9, co-culture of mural GCs with oocytes also induced mural GC SMAD2 phosphorylation (Fig. 6B).

To further examine the TGFβ superfamily signalling pathways activated by oocytes, mural GCs transfected with CAGA or BRE reporter constructs were co-cultured with oocytes or treated with various TGFβ superfamily ligands. As expected, CAGA-luciferase was stimulated by TGFβ1 and GDF9 (P<0.01) but not by BMP6 and, in direct contrast, BRE-luciferase was stimulated by BMP6 (P<0.01) but not by TGFβ1 or GDF9 (Fig. 7). Consistent with the western blot results, oocytes also activated the TGFβ/activin/GDF9 signalling pathway, as evidenced by stimulation of CAGA-luciferase activity (P<0.01; Fig. 7A) but, surprisingly, oocytes at the same concentration did not stimulate BRE-driven luciferase activity (P>0.05; Fig. 7B). Only upon dramatically

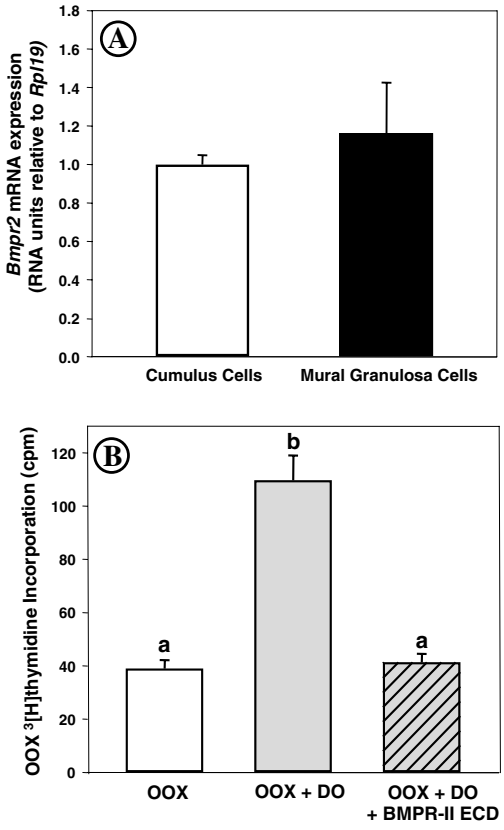

Fig. 5. Expression of BMP receptor-II mRNA in cumulus cells and BMP receptor-II ectodomain (BMPR-II ECD) neutralisation of oocyte-stimulated cumulus cell DNA synthesis. (A) Mural granulosa cells and cumulus-oocyte complexes were collected from antral follicles, cumulus cells were generated by denuding cumulus-oocyte complexes, RNA was extracted, reverse transcribed and subjected to real-time RT-PCR. There was no significant difference between cell types in the level of expression of BMPR-II mRNA.
(B) Oocytectomised complexes (OOX) were generated by microsurgically removing the oocyte contents from cumulus-oocyte complexes. OOX were cultured for 24 hours either alone, together with 30 DOs/well, or with oocytes and 2 μg/ml of BMPR-II ECD. Columns are means ± s.e.m. from three replicate experiments. Columns with different superscript letters are significantly different (P<0.001).

increasing oocyte density from 60 to 240 DOs per well was a small but significant (P<0.05) increase in BRE-driven luciferase observed. Hence, OSFs do not appear to readily activate the classic BMP signalling pathway.

OSFs promote GC and CC proliferation via the TGFβ/activin signalling pathway

We next exploited an ALK 4/5/7 kinase inhibitor to provide another line of evidence that oocytes stimulate GC and CC growth by activating the type-I receptors characterised for TGFβ/activin/GDF9 signalling. The inhibitor SB431542 specifically antagonises the kinase activities of ALKs 4, 5 and 7, without affecting the activities of ALKs 1, 2, 3 or 6 (Inman et al., 2002). Accordingly, in our system, SB431542 dose-dependently inhibited TGFβ1-stimulated mural GC CAGA-luciferase (Fig. 8A), but had no effect on BMP6-stimulated BRE-luciferase (Fig. 8B). Once this specificity was established in our system, we went on to show that SB431542 also completely abolished CAGA-luciferase activity stimulated by either GDF9 or oocytes in transfected mural GCs (Fig. 8C).

Based on the results so far, we hypothesised that oocytes promote GC growth by acting primarily through a GDF9-like signalling pathway (i.e. BMPR-II + ALK 4/5 with SMAD 2/3 activation) and not through the BMP pathway. Therefore, ablation of this pathway using the ALK 4/5/7 inhibitor should prevent oocyte-stimulation of GC and cumulus cell DNA synthesis. The results in Fig. 9 provide evidence to support this hypothesis. Expression of cumulus cell *Ccnd2* mRNA was increased by treatment with GDF9 and oocytes (Fig. 9A); mural GC [^{3}H]thymidine incorporation was stimulated by GDF9, TGFβ1 and oocytes (but not by BMP6; Fig. 9B); and the effects of these mitogens were eliminated by treatment with SB431542. Furthermore, oocyte-stimulation of mural GC growth was dose-dependently inhibited with increasing concentrations of the ALK inhibitor, in a pattern similar to inhibition of GDF9-stimulated growth, and completely eliminated proliferation (P<0.001) at doses as low as 1 μM (Fig. 9C).

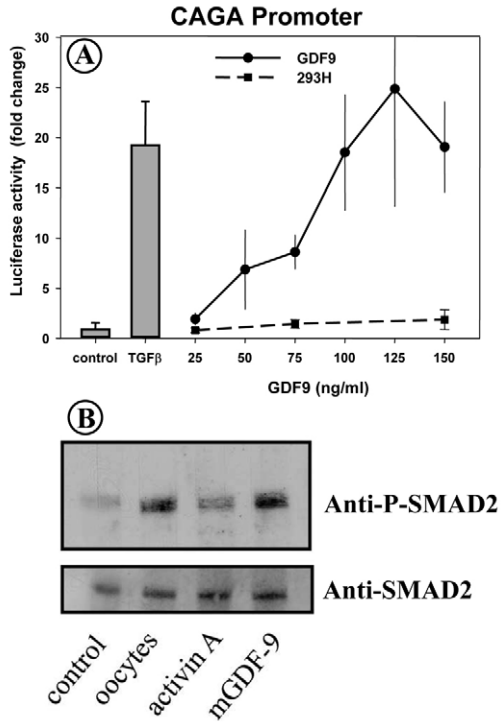

Fig. 6. Activation of granulosa cell SMAD2 and SMAD3 transducer molecules by GDF9 and oocytes. (A) Mural GCs were transiently transfected with a SMAD3-responsive CAGA-luciferase plasmid, and subsequently left untreated, treated with 0.5 ng/ml TGFβ1, or treated with increasing doses of GDF9 (or 293H: the negative v/v control of conditioned medium from untransfected 293H cells) for 48 hours, and then relative luciferase activity was measured from cell extracts. (B) Mural GCs were cultured for 90 minutes either alone (control), co-cultured with oocytes (500 DOs/1-ml well), with human activin A (50 ng/ml), or mouse GDF9 (40 ng/ml), then extracted granulosa cell proteins were subjected to 10% SDS-PAGE and western blotting using SMAD2 and phospho-SMAD2 antibodies. Mural GC SMAD2 was phosphorylated by treatment with mGDF9, oocytes and, to a lesser extent, activin A.

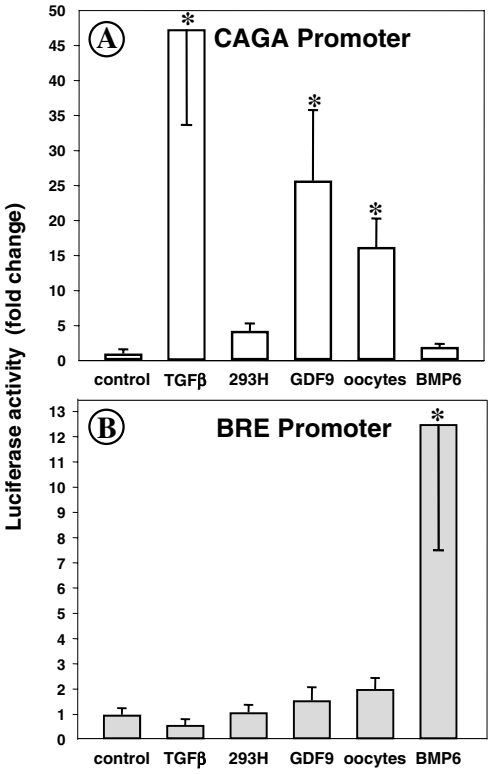

Fig. 7. Oocyte-paracrine factors signal through the TGFβ/activin intracellular pathway, and not through the BMP pathway. Mural GCs were transiently transfected during culture with either a SMAD3-responsive CAGA-luciferase plasmid (A) or a SMAD1-responsive BRE-luciferase reporter plasmid (B), then treated with various TGFβ superfamily growth factors or were co-cultured with 60 oocytes/250-μl well. GDF9 was used at 40 ng/ml. TGFβ1 (0.5 ng/ml) is a positive control for CAGA (and negative control for BRE), and BMP6 (50 ng/ml) is a positive control for BRE (and negative controls for CAGA). 293H is the GDF9 negative control: an equivalent v/v of conditioned medium from untransfected 293H cells. Columns represent means ± s.e.m. from four replicate experiments. Oocytes significantly (P<0.01) increased mural GC CAGA-luciferase, but not BRE-luciferase activity (P>0.05); asterisks indicate significantly different to control (P<0.01).

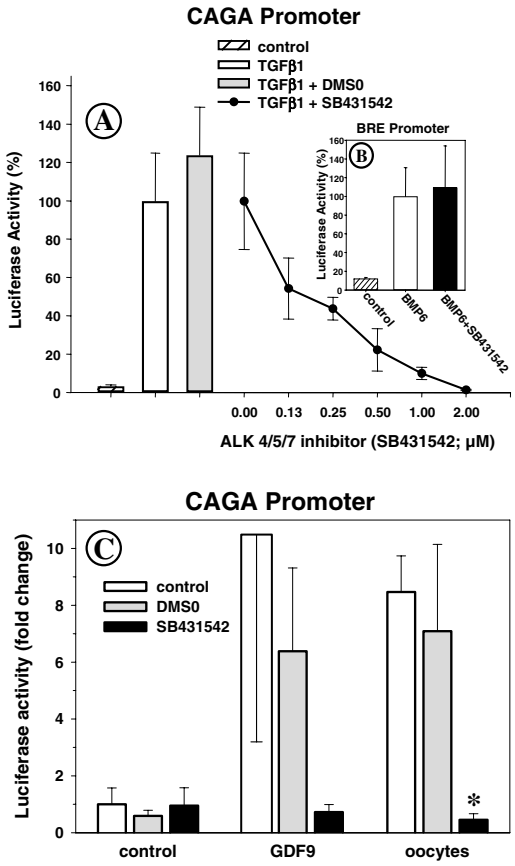

Fig. 8. Inhibition of oocyte- and GDF9-stimulated SMAD3 activation using the ALK4/5/7 kinase inhibitor, SB431542. Mural GCs were transiently transfected during culture with either a SMAD3-responsive CAGA-luciferase or a SMAD1-responsive BRE-luciferase reporter plasmid. (A) CAGA-luciferase was stimulated by TGFβ1, which in turn was dose-dependently antagonised by treatment with SB431542. The carrier, DMSO, at a v/v dose equivalent to 0.5 μM of SB431542, did not affect TGFβ-induced mural GC CAGA-luciferase activity. (B) BMP6 (50 ng/ml)-stimulated BRE-luciferase activity was not affected by a maximum dose of 2 μM SB431542. (C) Treatment of mural GCs with 0.5 μM SB431542 completely antagonised GDF9-activation (60 ng/ml) and oocyte-activation (60 oocytes/well) of SMAD3-responsive CAGA luciferase activity; asterisk indicates significantly different to control (oocytes alone; $P<0.05$).

Discussion

Germ cell regulation of ovarian and testicular somatic cell function is an essential component of normal gonadal function, and elucidating the cellular and molecular mechanisms of this newly recognised communication axis could have important implications for the understanding and regulation of fertility and developmental biology of the early embryo. The collective results of this study indicate that the TGFβ superfamily growth factors and receptor/signal transduction system are responsible for mediating OSF stimulation of GC proliferation. BMPR-II appears to be an indispensable receptor for the transmission of mitogenic signals from the oocyte to somatic GCs, which firmly establishes members of the TGFβ superfamily that bind this receptor as being the primary mediators of oocyte-stimulated GC proliferation. However, we have also

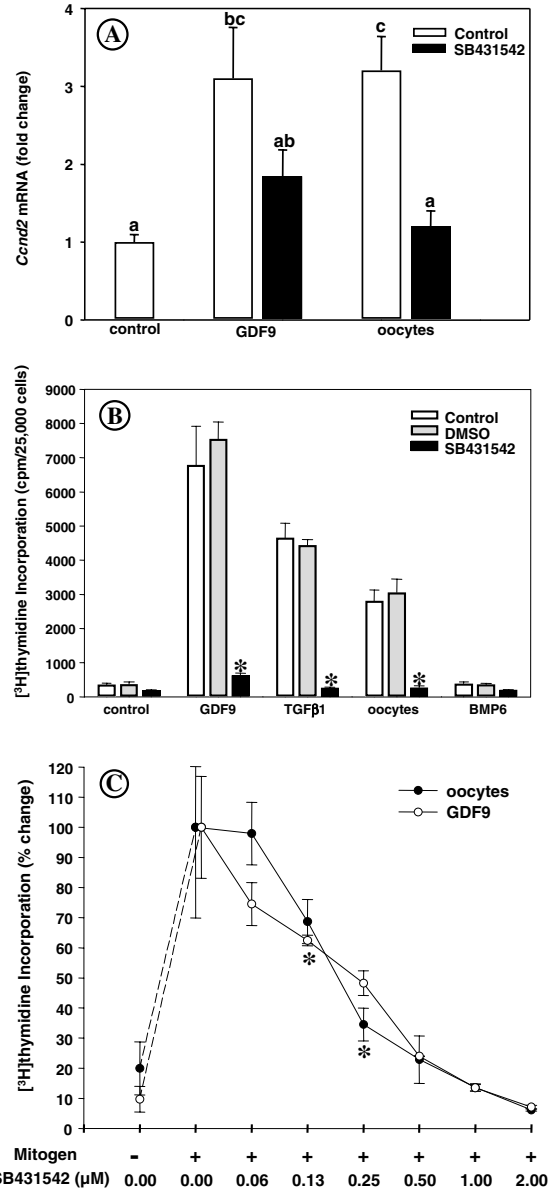

Fig. 9. Inhibition of oocyte- and GDF9-stimulated CC and mural GC proliferation using the ALK 4/5/7 kinase inhibitor, SB431542. (A) Cumulus cells (OOX) were treated with GDF9 (250 ng/ml) or DOs (0.8/μl), with or without 4 μM SB431542 and cultured for 6 hours, and *Ccnd2* mRNA levels were examined using real-time RT-PCR. Columns are means ± s.e.m. from six replicate experiments and columns with different superscripts are significantly different ($P<0.05$). (B) Mural GCs were cultured for 24 hours and thymidine incorporation was stimulated with either 20 ng/ml GDF9, 0.5 ng/ml TGFβ1, 16 DOs/well or 50 ng/ml BMP6, all of which, in turn, were antagonised by SB431542 at 1.0 μM. An equivalent dose of the SB431542 carrier DMSO did not affect ligand-stimulated mural GC DNA synthesis. Columns are means ± s.e.m. from triplicate wells and are representative of three replicate experiments; asterisks indicate significantly different to control (mitogen alone; $P<0.001$). (C) Oocyte (14/well)- and GDF9 (20 ng/ml)-stimulated mural GC DNA synthesis were inhibited in a dose-dependent manner with increasing concentrations of SB431542. Points are means ± s.e.m. from triplicate wells and are representative of three replicate experiments. Asterisks represent the lowest dose of SB431542 that is significantly less than the positive control ($P<0.05$).

determined that OSFs do not employ BMPR-II to activate classic BMP signalling in the stimulation of GC proliferation, and ligands that do stimulate such signalling in GCs are not particularly mitogenic. Rather, mitogenic activity exerted by the oocyte relies on a hybrid of classic TGFβ/activin and BMP signalling, characterised by a receptor combination of BMPR-II and ALK4/5 followed by activation of the SMAD 2/3 intracellular cascade. This unique signalling hybrid can be stimulated by GDF9 (Kaivo-Oja et al., 2005; Mazerbourg et al., 2004; Roh et al., 2003), but this factor alone does not account for the total mitogenic capacity of a whole oocyte (Gilchrist et al., 2004b).

BMPR-II is expressed in GCs at early stages of folliculogenesis, in primordial ruminant follicles and primary rodent follicles, and continues to be expressed throughout subsequent stages of folliculogenesis (Juengel and McNatty, 2005). Previously, it was unclear whether GCs that differentiate into CCs continue to express BMPR-II as preliminary observations suggested expression levels may be low in this GC subtype (Erickson and Shimasaki, 2003). Our current results demonstrate that CCs from large antral mouse follicles express equivalent levels of BMPR-II mRNA as their companion mural GCs, which indicates that expression of this receptor is not lost upon CC differentiation. This finding is important because during the antral phase of folliculogenesis, when the bioactivities of OSFs are most potent (Gilchrist et al., 2004a), the primary recipients of OSFs are CCs and not mural GCs (Hussein et al., 2005), which largely accounts for their substantially higher mitogenic potential (Li et al., 2000).

The BMPR-II ECD is a known functional antagonist of the recombinant forms of the key known OSFs that utilise BMPR-II, namely GDF9 and BMP15 (Moore et al., 2003; Vitt et al., 2002), and may antagonise the many other BMP ligands utilising this receptor, depending on the binding affinity of the individual ligand. In a previous study (Dragovic et al., 2005), we showed that the BMPR-II ECD completely blocks CC expansion induced by GDF9, but only partly prevents oocyte-induced expansion. In the current study, addition of the BMPR-II ECD to oocyte-GC co-cultures led to a dose-dependent suppression of oocyte-induced GC DNA synthesis. The neutralisation profiles of oocyte- and GDF9-induced DNA synthesis generated by the BMPR-II ECD were highly comparable, in which mitogenic activity of both was completely eliminated at moderate doses of antagonist. This approach of neutralising oocyte activity using the BMPR-II ECD was equally effective whether using CCs or mural GCs. Together these results suggest that the totality of oocyte-secreted mitogenic activity depends upon binding to BMPR-II.

Consistent with the notion of BMPR-II being a key receptor in OSF signalling, is the finding that at sub-maximal doses of GDF9, oocytes and GDF9 have an additive effect on mural GC DNA synthesis, whereas this additivity is lost at saturating GDF9 concentrations. By contrast, TGFβ1 and GDF9, which use different type-II receptors but share a common type-I receptor (ALK5), have additive mitogenic effects, even at maximal GDF9 doses. Therefore, BMPR-II, and not ALK5, availability is rate-limiting, providing further support to our hypothesis that access to BMPR-II is a key determinant of OSF efficacy (Hussein et al., 2005). To examine whether OSFs may also bind other TGFβ superfamily type-II receptors, we compared a range of type-II receptor ECDs. The TGFβ and activin type-II receptor ECDs had marginal effects on oocyte mitogenic activity. However, the BMPR-II ECD was by far the most effective of the ECDs at antagonising oocyte mitogenic activity, providing a further line of evidence that BMPR-II is the key type-II receptor transmitting the paracrine actions of oocytes to GCs/CCs.

Based on the hypothesis that, at a minimum, GDF9, BMP15 and BMP6 are the key OSFs that could signal through BMPR-II, we next went on to examine activation by oocytes of the classic TGFβ superfamily signalling cascades in GCs. Interestingly, our luciferase and western blot results indicate that oocytes appear to activate primarily the TGFβ/activin pathway, one not stimulated by BMPs. Oocytes strongly stimulated CAGA-luciferase activity, which is activated by phosphorylated SMAD3, but at best marginally stimulated BRE-luciferase, which is SMAD1 responsive. Furthermore, oocyte stimulation of CAGA-luciferase activity was completely eliminated using the ALK4/5/7 kinase inhibitor, SB431542. Oocyte activation of the SMAD2/3 pathway is not surprising given oocyte-secreted GDF9 is a key GC mitogen (Gilchrist et al., 2004b), and it is now known that GDF9 utilises ALK5 to activate SMAD2/3 molecules (Kaivo-Oja et al., 2003; Kaivo-Oja et al., 2005; Mazerbourg et al., 2004; Roh et al., 2003). Oocytes also express TGFβ1 and TGFβ2, but it appears that biologically active forms of these molecules are not secreted by the immature or maturing oocyte and these may represent maternally stored transcripts to be translated post-fertilisation (Gilchrist et al., 2003). If mouse oocytes activate only the SMAD2/3 pathway and not the SMAD1/5/8 pathway in GCs (present study), then inhibition specifically of this signalling cascade should prevent oocyte-stimulated GC DNA synthesis. Indeed, the results from the current study demonstrate this to be the case; SB431542, which has no effect on BMP-activation of SMAD1/5/8, dose-dependently antagonised the growth-promoting effects of oocytes on GCs. These results support the conclusion that oocyte stimulation of GC DNA synthesis is mediated via the GDF9/TGFβ signalling system, that is, BMPR-II dimerising with ALK4 and/or -5 and activating the SMAD 2/3 pathway, and that BMP intracellular signalling plays a minor role in this communication axis.

This cell-signalling pathway may also be relevant in non-ovarian tissues. The receptors and SMAD signalling molecules examined here are expressed widely throughout the body; however, GDF9 and BMP15 expression is restricted to the gametes and to the hypothalamic-pituitary axis (Aaltonen et al., 1999; Fitzpatrick et al., 1998). A physiological role for GDF9 and BMP15 in the testis or hypothalamic-pituitary axis has not been described, possibly as males with mutations in these genes appear completely normal. BMP15 has been shown to stimulate selectively FSH expression by pituitary cells (Otsuka and Shimasaki, 2002). The paracrine signalling system described here between oocytes and their neighbouring somatic cells, could well have parallels in the testis as spermatocytes express very high levels of GDF9 (Fitzpatrick et al., 1998) and paracrine communication between developing spermatocytes and Sertoli cells is vitally important for testis function and male fertility.

The very weak or complete lack of activation of the BMP intracellular pathway by mouse oocytes is perhaps surprising because certain BMPs are regarded as key OSFs. Although it is now widely recognised that the regulation of ovarian

granulosa cell growth and differentiation by OSFs is a critical process in ovarian biology (for reviews, see Eppig, 2001; Gilchrist et al., 2004a; McNatty et al., 2004), the mechanisms by which this occurs, including the exact identities of the oocyte-secreted molecules mediating this process, are not yet fully understood (Gilchrist et al., 2004a; Vanderhyden et al., 2003). GDF9, BMP15 and BMP6 are currently regarded as the best candidate molecules responsible for oocyte regulation of most GC processes. GDF9 is an exceptionally potent GC mitogen (Elvin et al., 1999; Gilchrist et al., 2004b; Hayashi et al., 1999; Hickey et al., 2005; Vitt et al., 2000) and, based on immunoneutralisation experiments using an antibody against GDF9, we have previously proposed that oocyte-derived GDF9 accounts for approximately half of the total mitogenic activity of mouse oocytes (Gilchrist et al., 2004b). The identity of the remaining half is currently unknown although TGFβ1 or TGFβ2 are most unlikely to have a role (Gilchrist et al., 2003; Gilchrist et al., 2004b). Oocyte-secreted BMP6 also seems most unlikely as at best it is weakly mitogenic in GCs (Otsuka et al., 2001) and, furthermore, we show that it does not stimulate the mitogenic pathways oocytes utilise to promote GC proliferation (present study). However, BMP15 seems a likely candidate OSF as recombinant human BMP15 stimulates rat GC proliferation (Otsuka et al., 2000) and BMP15 is required for early follicular progression in sheep (Galloway et al., 2000).

Complete antagonism of oocyte-stimulated GC DNA synthesis by the BMPR-II ECD in the current study, which was not achieved using a GDF9-specific neutralising antibody in a previous study (Gilchrist et al., 2004b), suggests a key role for a BMPR-II-binding molecule other than GDF9. However, even though BMP15 utilises BMPR-II, a BMP15 homodimer is unlikely to be involved as this molecule signals through SMAD1/5/8 (Moore et al., 2003), and the current results demonstrate that oocyte-stimulation of GC DNA synthesis is exclusively mediated through SMAD2/3. This failure of mouse oocytes to activate the GC BMP signalling cascade is perhaps clarified by new evidence which demonstrates that fully processed mouse BMP15 cannot be secreted by cell lines as an intact homodimer (Hashimoto et al., 2005), which explains why bioactive recombinant mouse BMP15 has never been produced. Whether mouse oocyte secretion of a BMP15 homodimer is atypical within the TGFβ superfamily, in some manner, remains to be determined, although this would be compatible with our current results, whereby (1) mouse oocytes fail to mimic recombinant human BMP15 by activating the BMP signalling pathway, and (2) ablation of SMAD2/3 signalling completely eliminates the growth-promoting effects of oocytes. Perhaps an oocyte-secreted GDF9/BMP15 heterodimer constitutes an important component of the total mitogenic activity of oocytes, and this is achieved via signalling through the same receptor/signalling cascade as used by GDF9 homodimers. To date very little is known of GDF9/BMP15 heterodimer biology including how it signals; however, a heterodimer of recombinant proteins has been demonstrated (Liao et al., 2003) and, furthermore, recombinant GDF9 and BMP15 interact substantially to stimulate GC growth, as well as to regulate steroidogenesis and inhibin production in vitro (McNatty et al., 2005a; McNatty et al., 2005b). Further studies are required to investigate these hypotheses.

In conclusion, this study has determined the requisite signalling pathways by which oocyte paracrine signals stimulate ovarian granulosa cell proliferation. This signalling is primarily achieved through GDF9 and GDF9-related molecules using the GDF9/TGFβ receptor and intracellular signal transduction cascade. BMPR-II appears to be an indispensable, and possibly a rate-limiting, type-II receptor on GCs/CCs for transmission of the growth-promoting effects of oocytes. OSFs elicit a TGFβ-like intracellular response in GCs, and surprisingly, do not appear to activate the BMP pathway. Participation of the TGFβ/activin/GDF9 type-I receptors, ALK4 and/or 5, and activation of the SMAD2/3 pathway, are indispensable for oocyte-stimulation of GC proliferation. Although the exact composition of the oocyte-secreted molecules promoting GC growth is not yet fully resolved, results from this study suggest GDF9 plus a GDF9-related molecule(s) account for most, if not all, mouse oocyte mitogenic activity. Elucidation of the molecular and cellular processes by which germ cells regulate the function of their companion somatic cells is vitally important to our basic understanding of mechanisms of cell-to-cell communication in the ovary and testis, and, furthermore, is likely to have implications for fertility management and diseases of impaired folliculogenesis.

Materials and Methods

Oocyte-secreted factor bioassay: mural GC DNA synthesis

Experimental procedures used in this study for the bioassay of mouse oocyte mitogens, including the collection, preparation and co-culture of mural GCs with denuded oocytes (DOs), have been previously described (Gilchrist et al., 2003; Gilchrist et al., 2001; Gilchrist et al., 2004b). This study was approved by local Animal Ethics Committees. In brief, ovaries were collected from immature (21-26 days old) 129/SV mice 46 hours after priming with 5 IU of equine chorionic gonadotrophin (Folligon®, Intervet, Castle Hill, Australia). Mural GCs and cumulus-oocyte complexes were collected by puncturing large antral follicles and DOs were generated by vortexing cumulus-oocyte complexes for 3-4 minutes to dissociate cumulus cells. Mural GCs and DOs were washed twice in protein-free culture medium: bicarbonate-buffered tissue culture medium-199 with supplements (Gilchrist et al., 2001). Depending on the individual experiments, mural GCs (2×10^5 viable and non-viable cells/ml), DOs, hormones, reagents and media were added to wells of 96-well plates (Falcon) to give a final volume of 125 μl. Within each experiment, all treatments were carried out in at least duplicate wells and each experiment was replicated on at least three occasions. Cells were cultured in an atmosphere of 37°C, 96% humidity in 5% CO_2 in air for 18 hours, followed by a further 6 hour pulse of 15.4 kBq tritiated thymidine ([^{3}H]thymidine, ICN) under the same conditions. Following culture, mural GCs were harvested, and incorporated [^{3}H]thymidine was quantified using a scintillation counter as an indicator of the proportion of cells in S-phase, hence providing an indication of the level of mural GC DNA synthesis and proliferation (Lee et al., 2001).

Oocyte-secreted factor bioassay: cumulus cell DNA synthesis

To determine whether oocyte-secreted factors also stimulate cumulus cell DNA synthesis via BMPR-II, the contents of each oocyte were microsurgically removed from the cumulus-oocyte complex as previously described (Buccione et al., 1990; Li et al., 2000). The resulting oocytectomised complexes (OOX) consist of a hollow zona pellucida surrounded by several layers of cumulus cells. Groups of 20 OOX were assigned to the following treatments: (1) cumulus cell control (OOX alone), (2) cumulus cells co-cultured with DOs (OOX + DO), or (3) OOX + DO + BMPR-II ECD (as described below), and each treatment was carried out in duplicate wells of 125 μl. Cumulus cell culture and assessment of DNA synthesis was as described above.

Oocyte-secreted factor bioassay: mural GC phospho-SMAD reporter assay

Luciferase reporter constructs responsive to specific phosphorylated SMADs were used to detect the activation of mural GC SMAD proteins by DOs or by recombinant forms of putative OSFs. Mural GCs were collected and processed as described above, except cells were collected in HEPES-199 + 2% FCS (Trace Biosciences, Castle Hill, Australia) and were washed twice in bicarbonate buffered DMEM (MP Biomedicals, Seven Hills, Australia) + 2% FCS. Following the final wash, cells were

transferred to individual wells of a 96-well plate (Falcon) and cultured at 1.6×10^5 cells/ml. After 4 hours of culture, cells were transiently transfected with 50 ng of luciferase reporter construct DNA using Fugene 6 (Roche Diagnostics, Castle Hill, Australia). Eighteen hours after transfection, medium was aspirated from cells and replaced with DMEM supplemented with 0.1% FCS. Specific ligands were added to cells at this point (see below) and the culture period extended for a further 48 hours. Experiments were terminated by removing media from wells and freezing plates at –20°C. To harvest cells, 100 μl of lysis buffer was added to each well and plates were incubated at room temperature on a rocking platform for 20 minutes. 20 μl of cell lysate was used for measurement of luciferase activity using a Galaxystar luminometer (GMB Labtechnologies, Offenburg, Germany).

Bioassays: experimental reagents and treatments

Mural GCs were treated with a number of growth factors: TGFβ1, activin A, BMP6 (all recombinant human from R&D Systems, MN), as well as mouse GDF9 and DOs. Fully processed, bioactive recombinant mouse GDF9 was produced and quantified in house (Gilchrist et al., 2004b; Kaivo-Oja et al., 2003), in stably transfected human embryonic kidney-293H cells and partially purified using hydrophobic interaction chromatography (HIC), as recently described (Hickey et al., 2005). Control conditioned medium (labelled '293H') from untransfected 293H cells (Gibco Life Technologies, Paisley, UK) was produced and HIC-purified under conditions identical to those used for the production of GDF9.

In an attempt to identify GC receptors used by oocyte-secreted mitogens to promote proliferation, experiments were designed to counter the growth-promoting activity of the mitogens using solubilised forms of known TGFβ superfamily type-II receptors. The solubilised receptor ectodomains (ECDs; purchased from R&D Systems) are recombinant chimeric proteins consisting of the extracellular portion of a type-II receptor fused to the Fc region of human IgG. The BMPR-II ECD can antagonise the bioactivity of recombinant mGDF9, hBMP15 and hBMP7 on granulosa cells and it has only partial neutralising activity against activin-A (Moore et al., 2003; Vitt et al., 2002). In neutralisation experiments, BMPR-II ECD, TGFβR-II ECD, ActR-IIA ECD and ActR-IIB ECD were pre-incubated at 2 μg/ml with mitogen for 30 minutes in culture wells, prior to addition of mural GC.

To examine the TGFβ superfamily type-I receptors employed by oocyte paracrine factors, their intracellular signalling and mitogenic activity were antagonised by treatment with SB431542 (generously donated by GlaxoSmithKline, Stevenage, UK). This small molecule inhibitor acts as a competitive ATP-binding site kinase inhibitor that is highly specific for ALKs 4, 5 and 7, and has no effect on ALKs 1, 2, 3 or 6 or other cellular kinases when used at <10 μM (Inman et al., 2002). SB431542 antagonises the bioactivity of activin and TGFβ (Inman et al., 2002) but its effects on activity of other members of the TGFβ superfamily or on oocytes are currently unknown. A BMP6-neutralising antibody (R&D Systems) was also employed which, in this culture system, is effective at antagonising BMP6 suppression of FSH-induced progesterone production (Ritter and Gilchrist, 2004).

Immunoblot analysis of phospho-SMAD proteins

To generate protein extracts for SMAD analyses, mural GCs were cultured as described above with the following exceptions: cells were cultured at 1×10^6 cells/ml in 1 ml of medium in 48-well plates (Falcon, Franklin Lakes NJ), and oocytes were co-cultured with mural GCs at 500 DOs/ml. After 90 minutes of culture, oocytes were removed, mural GCs were collected, washed in PBS, lysed in Laemmli protein extraction buffer and sonicated before western blot analysis. This experiment was replicated three times. Techniques for immunoreactive analysis of SMAD proteins have already been described (Kaivo-Oja et al., 2003). Briefly, membranes were blocked in 5% milk in Tris-buffered saline in the presence of NP-40, and treated with primary antibodies (anti-SMAD2 and anti-phospho-SMAD2 [PS2], kindly donated by Peter ten Dijke, Amsterdam, The Netherlands) overnight at 4°C. Secondary antibody (Jackson's peroxidase-conjugated anti-IgG anti-rabbit) treatment was performed at room temperature for 1 hour. Immunoreactive proteins were detected using Amersham Biosciences' ECL reagents.

Detection and quantification of mRNA in cumulus and granulosa cells

Real-time reverse transcription PCR (RT-PCR) was used to determine whether OSFs increase Ccnd2 mRNA levels in cumulus and granulosa cells as well as to quantify the level of Bmpr2 mRNA expressed in cumulus cells relative to GCs. Pure populations of CCs and mural GCs were collected and OOX were generated, as described above. RNA was extracted using TriReagent for Bmpr2 (Sigma, St Louis, MO) or a Micro RNA isolation kit for Ccnd2 (Qiagen, Clifton Hill, Australia), quantified using a Ribogreen assay (Molecular Probes, Eugene, OR), and reverse transcribed to cDNA using Superscript-II (Life Technologies, Grand Island, NY). cDNA was amplified by PCR using specific primers for Rpl19 housekeeping control gene (Rpl19 control for Bmpr2, forward primer, 5′ GTG CTT CCG ATT CCA GAG TTC T 3′; reverse primer, 5′ TCG TGT TCC TGA GGG TGA AAG 3′; or Rpl19 control for Ccnd2, forward primer, 5′GAA AGG TGC TTC CGA TTC CA 3′; reverse primer, 5′ TGA TCG CTT GAT GCA AAT CC 3′) and genes of interest: Ccnd2 (forward primer 5′ TGC ATT TAC ACC GAC AAC TCT GT 3′; reverse primer 5′ CTT GCG AAG GAT GTG CTC AA 3′) and Bmpr2 (forward primer, 5′ GAA CTC ATG ATG ATA TGG GAG AGA AAC 3′; reverse primer, 5′TGG CAC ACG CCT ATT ATG TGA 3′). The quantity of PCR amplicons was detected in real time using an Applied Biosystems 5700 Real Time light cycler using sybergreen as the detection reagent. Ccnd2 mRNA levels were expressed relative to a calibrator and normalised to Rpl19 using the standard curve method as outlined by Applied Biosystems. Bmpr2 expression levels in CCs and mural GCs were also expressed relative to a calibrator and normalised to steady state Rpl19 housekeeping gene expression using the comparative Ct method according to Applied Biosystems.

Statistical analyses

Data were generally log transformed and treatment effects were examined using one-way or two-way ANOVAs and differences between treatment means tested using Tukey (pairwise multiple comparisons) or Dunnett's method (multiple comparisons versus a control) post-hoc comparisons. A P value of <0.05 was considered statistically significant unless otherwise stated.

This project was funded by Program Grant #250306 from the National Health and Medical Research Council (NHMRC) of Australia. R.B.G. was supported by the FTT Fricker Medical Research Associateship from the University of Adelaide. Support and laboratory infrastructure provided by Prof. Robert Norman and the Research Centre for Reproductive Health (Adelaide) are gratefully acknowledged. Thanks also to Samantha Schulz and Fred Amato for technical assistance, and Wayne Tilley and Aleksandra Ochnik for use of their luminometer. The work in Helsinki was supported by grants from the Academy of Finland, the Jalmari and Rauha Ahokas Foundation, the Jenny and Antti Wihuri Foundation and the Emil Aaltonen Foundation. The SMAD2 antibodies were kindly supplied by P. ten Dijke and GlaxoSmithKline generously donated SB431542.

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