Differences in the participation of TGFB superfamily signalling pathways mediating porcine and murine cumulus cell expansion

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Abstract

It is widely held that mammalian cumulus cell (CC) expansion requires oocyte-paracrine signalling, however in three of the four species studied to date, CC expansion occurs in the absence of the oocyte. This study was conducted to examine the paracrine and SMAD/MAPK intracellular signalling mechanism mediating porcine CC expansion, and to compare these to the mouse. Cumulus–oocyte complexes (COCs) and oocyte-free complexes (OOXs) from pigs and eCG-primed mice were treated in vitro with FSH and a broad range of TGFB superfamily antagonists. Expansion of porcine COCs and OOXs was unaffected by neutralisation of growth differentiation factor 9, TGFB, activin A, activin B and a broad spectrum bone morphogenetic protein antagonist. A SMAD-responsive luciferase reporter assay confirmed that porcine oocytes secreted factors that activate SMAD3 and SMAD1/5/8 in granulosa cells, but murine oocytes activated SMAD3 only. Treatment of COCs with a SMAD2/3 phosphorylation inhibitor (SB431542) partially inhibited porcine CC expansion and expression of TNFAIP6, but ablated murine CC expansion. SB431542 was equally effective at attenuating porcine CC expansion in the presence or absence of the oocyte. By contrast, a SMAD1/5/8 phosphorylation inhibitor (dorsomorphin) had no effect on porcine or murine CC function. Inhibition of ERK1/2 and p38 MAPK signalling pathways prevented porcine COC expansion and expression of most matrix genes examined. The activation of CC SMAD signalling by oocytes, and the requirement of SMAD2/3 signalling for expansion, is notably contrasted in pigs and mice. Nonetheless, porcine CC SMAD2/3 signalling is likely to be needed for optimal matrix formation, possibly by facilitating essential MAPK signals.

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Introduction

The final phase of oogenesis occurs in large antral ovarian follicles where the oocyte is closely surrounded by its support somatic cells, the cumulus cells (CCs), forming the cumulus–oocyte complex (COC). The preovulatory LH surge induces the resumption of oocyte meiosis and expansion of the COC leading to ovulation. During this phase, the ovulatory cascade induces CCs to express hyaluronan synthase 2 (HAS2), prostaglandin-endoperoxide synthase 2 (PTGS2), pentaxin 3 (PTX3), tumour necrosis factor α-induced protein 6 (TNFAIP6) and other factors, leading to the secretion of a mucinous hyaluronan-rich extracellular matrix within the COC. Mucification and expansion of the COC is required for ovulation and failure of these processes causes infertility (Russell & Robker 2007). CC expansion also aids COC pickup by the fimbria, processes of fertilisation and subsequent embryonic development.

Substantial progress has been made in recent years in elucidating the molecular signalling mechanisms mediating mouse oocyte maturation, CC expansion and ovulation. Compelling evidence from mouse studies has revealed that the ovulatory LH surge induces a rapid secondary cascade of the EGF-like peptides; amphiregulin, epiregulin and β-cellulin, in the somatic cells of the follicle, which lead to CC expansion, oocyte maturation and ovulation (Park et al. 2004, Hsieh et al. 2007). These peptides are initially produced by the mural cells and then by the CCs where they act on the EGF receptor. The EGF-like peptides are potent inducers of CC expansion and oocyte meiotic resumption via a mechanism requiring the activation of CC extracellular signal-related protein kinases 1/2 (ERK1/2; Su et al. 2003, Hsieh et al. 2007, Fan et al. 2009). When oocytes are matured in vitro for artificial reproductive technologies, oocyte maturation and CC expansion are typically stimulated by the addition of FSH and less commonly by EGF. The FSH second messenger cAMP activates protein kinases (including p38 MAPK and PKA) that induce CC expression of the EGF-like peptides and thereby indirectly activate ERK1/2 (Shimada et al. 2006, Yamashita et al. 2007). This new knowledge has been gained largely from mouse models and to date there is limited understanding of these processes in most other mammals.
The second area of ovarian biology where substantial progress has been made is in the knowledge that oocytes regulate granulosa/CC growth and differentiation through the production of soluble paracrine growth factors (Gilchrist et al. 2008). The two most important oocyte-secreted factors are growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15). GDF9 and BMP15 act locally on CCs through their receptors, BMP receptor type-II (BMPR2) and activin receptor-like kinases (ALKs), to activate Smad- and Mad-related (SMAD) intracellular signal transducers (Kaivo-oja et al. 2006). While oocyte-secreted GDF9 uses a combination of BMPR2 and ALK5 to signal through SMAD2/3, BMP15 utilises BMPR2/ALK6 to activate SMAD1/5/8 (Kaivo-oja et al. 2006). Aberrant expression or activity of any of these ligands, receptors or second messengers causes sterility, enhanced fecundity or ovarian disease in a highly species-specific manner (Dong et al. 1996, Galloway et al. 2000, McNatty et al. 2004, Li et al. 2008b).

CCs are at the intersection of the converging signals emanating from the oocyte and from the endocrine system via local mediators. In the mouse, CC expansion is critically dependent on activation of these two pathways: 1) activation of ERK1/2 by the LH-induced EGF cascade and/or FSH (Su et al. 2003) and 2) activation of SMAD2/3 signalling by oocyte paracrine factors (Dragovic et al. 2007). Ablation of either pathway prevents CC expansion in the mouse. Hence, FSH stimulates expansion of intact murine COCs, whereas complexes where the oocyte has been removed (oocyte-free complex, OOX) fail to expand (Buccione et al. 1990, Vanderhyden et al. 1990). However, this is notably contrasted by other species where rat, bovine and porcine CCs are capable of undergoing morphological expansion in response to FSH in the absence of the oocyte (Prochazka et al. 1991, Vanderhyden 1993, Ralph et al. 1995). This suggests substantial species differences in the fundamental signalling mechanisms regulating CC function and ovulation. However, it seems highly unlikely that oocyte-secreted factors have minimal or no role in cumulus expansion in these species, given their fundamental role in regulating CC differentiation and the complexity of interactions with other endocrine and local mediators of folliculogenesis (Gilchrist et al. 2008). In particular, the recent demonstration of intricate and critical crosstalk between GDF9/BMP15-activated SMAD2/3 signalling and EGF receptor/ERK1/2 signalling in CCs (Sasseville et al. 2010, Su et al. 2010), illustrates the high level of complexity required to coordinate ovolatory events. Hence, this study was conducted to examine the participation of oocyte paracrine and SMAD signalling and interactions with MAPK signalling, in porcine cumulus expansion. In some experiments, mouse oocytes were also studied or parallel experiments conducted with mouse COCs to highlight differing mechanisms.

Results

Participation of the oocyte and GDF9 in porcine CC expansion

Figure 1 confirms findings from previous studies that show that porcine CC expansion occurs independently of the oocyte (Prochazka et al. 1991, Singh et al. 1993). As GDF9 is the principal oocyte-secreted factor regulating mouse CC expansion (Diaz et al. 2007, Dragovic et al. 2007) and porcine CCs are reported to express GDF9 (Prochazka et al. 2004), we examined the effect of GDF9 antagonists on FSH-induced expansion of COCs and OOXs. Neither the GDF9 neutralising MAB (MAb53) nor the soluble ectodomain (ECD) of the BMP-type II receptor had any detectable effect on COC or OOX expansion (P>0.05; Fig. 1). We have previously shown that MAb53 and BMPR2-ECD antagonise recombinant GDF9 and oocyte-mediated events such as CC expansion and cumulus and granulosa cell proliferation (Gilchrist et al. 2004, 2006, Dragovic et al. 2005), at doses notably lower than those used in this study (80 and 5 μg/ml respectively). Furthermore, before commencing this study the antagonising activities of these reagents were re-confirmed and found to be effective at <5 μg/ml (date not shown).

Activation of SMADs by porcine oocyte-secreted factors

To determine if porcine oocytes secrete molecules that activate SMAD signalling, SMAD3- and SMAD1/5/8-responsive luciferase reporter bioassays were undertaken. As reported previously (Gilchrist et al. 2006), the effects of recombinant GDF9 and oocyte-secreted events such as CC expansion and cumulus expansion were assessed at 24 h. Data are represented as means±S.E.M. ±FSH

Figure 1 Requirement of GDF9 and BMPR2 for porcine cumulus cell (CC) expansion. Porcine COCs or OOXs cultured in the presence of FSH were treated with previously characterised GDF9 antagonists; either a GDF9 neutralising antibody (80 μg/ml) or a BMPR2 ECD (5 μg/ml). Negative control: COCs cultured without FSH. The degree of CC expansion was assessed at 24 h. Data are represented as means±S.E.M. from 28 to 30 complexes/treatment from three replicate experiments. Means with different superscript letters are significantly different (ANOVA, P<0.05).
SMAD3-responsive CAGA-luciferase activity was induced in transfected granulosa cells by treatment with recombinant TGFB1 and by coculture with denuded mouse oocytes, but not by BMP6 (Fig. 2A). Coculture with increasing numbers of denuded porcine oocytes dose-dependently increased CAGA-luciferase activity (Fig. 2A). Similarly, porcine oocytes also activated SMAD1/5/8-responsive BRE-luciferase in a dose-dependent manner (Fig. 2B), although by contrast mouse oocytes did not as previously reported (Gilchrist et al. 2006). As expected for a SMAD1/5/8-responsive reporter construct, BRE-luciferase was induced by BMP6 but not by TGFB1.

Effect of inhibition of TGFB/SMAD2/3 signalling on porcine CC expansion

The results from Figs 1 and 2 suggest that porcine CCs appear to expand in an oocyte-independent and GDF9-independent manner but that the oocyte secretes molecules that activate both major SMAD signalling pathways. Hence, experiments were conducted to further examine the participation of these pathways and other activating ligands in mediating expansion. Soluble ECDs of the activin RIIA, activin RIIB and TGFBR2, which antagonise their respective activating ligands in vitro (Gilchrist et al. 2006), all failed to have any appreciable effect on expansion of intact COCs or OOXs (Fig. 3A). By contrast, the small molecule ALK4/5/7 kinase (and hence SMAD2/3 phosphorylation) inhibitor, SB431542, which has been widely used to antagonise the actions of oocyte-secreted factors (Gilchrist et al. 2006, Diaz et al. 2007, Dragovic et al. 2007), significantly reduced CC expansion regardless of the presence or absence of the oocyte ($P<0.05$; Fig. 3B).

As the SMAD2/3 phosphorylation inhibitor, SB431542, reduced but did not prevent CC expansion (Fig. 3B), a further experiment incorporating a dose-range of SB431542 was conducted, as well as a parallel experiment using mouse COCs to allow effective dose comparisons.SB431542 eliminated FSH-stimulated mouse COC expansion at just $1 \mu$M (Fig. 4A), whereas $1 \mu$M of the inhibitor had no effect on porcine COC expansion (Fig. 4B). Expansion of porcine COCs was significantly reduced, but not eliminated, at higher doses ($P<0.05$; Fig. 4B). The SMAD1/5/8 phosphorylation inhibitor dorsomorphin had no effect on mouse COC expansion at any dose tested ($P>0.05$; Fig. 4B). In one experiment dorsomorphin did slightly attenuate ($P<0.05$) porcine COC expansion, although this was not significantly different to the DMSO vehicle control (Fig. 5B), and in a second experiment with larger numbers (Fig. 6A) it had no effect ($P>O.05$). To validate dorsomorphin antagonism of BMPs in porcine ovarian cells, we confirmed that BMP15-stimulated porcine granulosa cell proliferation was inhibited by dorsomorphin at $8 \mu$M ($P<0.05$; Fig. 5C).

Effect of inhibition of SMADs on porcine CC gene expression

To further explore the participation of SMAD signalling in CC expansion, we examined the effect of the SMAD inhibitors on porcine CC gene expression. Confirming the lack of effect of dorsomorphin on morphological CC expansion (Fig. 6A), this SMAD1/5/8 phosphorylation
A

(b) 

Cumulus expansion index

0 1 2 3 4

Control – 

Activin RIIA-ECD

Activin RIIB-ECD

TGFB RII-ECD

Murine

DMSO Control SB431542

SB431542 (µM)

A

Cumulus expansion index

0 1 2 3 4

Control –

SB431542

B

Cumulus expansion index

0 1 2 3 4

Control –

SB431542

Figure 4 Comparative effects of inhibition of SMAD2/3 phosphorylation on murine and porcine cumulus cell (CC) expansion. (A) Murine or (B) porcine COCs were treated with FSH and an increasing dose of SB431542 (0–8 µM) and CC expansion was assessed at 20 h (murine) or 24 h (porcine). The SB431542 carrier, DMSO, was used at a vol/vol dose equivalent to 8 µM. Bars and points are means ± S.E.M. of 20 COCs/treatment from two replicate experiments (A) or 30 COCs/treatment from three experiments (B). Means with no common superscript letters are significantly different (ANOVA, P<0.05).

Participation of MAPKs in porcine CC expansion and gene expression

New evidence is emerging from mouse studies of requisite crosstalk between SMAD and MAPK signalling for coordinated oocyte–somatic cell development (Sasseville et al. 2010, Su et al. 2010). The role of p38 and ERK1/2 MAPK pathways in COC function in non-rodent species is limited, although some information is available for the pig. Consistent with a previous report (Yamashita et al. 2009), we found that inhibition of ERK1/2 MAPK using U0126 prevented porcine COC expansion at 10 µM (Fig. 7A). Inhibition of this pathway ablated the expression of the major porcine CC genes involved in matrix formation; HAS2, PTGS2 and TNFAIP6 (Fig. 7B–D). The inactive analogue U0124

inhibitor had no effect on porcine CC expression of HAS2, PTGS2 or TNFAIP6 (Fig. 6B–D). By contrast, the SMAD2/3 phosphorylation inhibitor SB431542 substantially reduced (P<0.05) CC expression of TNFAIP6, tended to reduce HAS2 (P=0.08), but had no effect on PTGS2 expression (Fig. 6B–D).
Inhibition of phosphorylation of p38 MAPK using SB203580 also prevented CC expansion ($P<0.05$; Fig. 7A). This inhibitor prevented CC expression of PTGS2, but had no appreciable effect on HAS2 or TNFAIP6 expression (Fig. 7B–D).

**Discussion**

This study was conducted to examine the participation of oocyte paracrine and SMAD signalling and interactions with MAPK signalling in mediating porcine CC expansion and to compare and contrast these to the requirements for mouse CC expansion. Our results build on the knowledge that morphological porcine CC expansion can occur in the absence of the oocyte (Prochazka et al. 1991, Vanderhyden 1993) by extending this knowledge to demonstrate that important members of the TGFβ superfamily, including GDF9, appear to have a minimal role in FSH-stimulated CC expansion. This notably contrasts with the signalling mechanisms regulating CC expansion in the most widely studied species, the mouse. Nonetheless, the results from the SMAD-reporter assay confirmed that porcine oocytes secrete factors that activate both major SMAD signalling pathways in granulosa cells. Hence, SMAD signalling may facilitate some functions of FSH-activated MAPK signalling, which in contrast, is obligatory for porcine CC expansion. In support of this notion, inhibition of SMAD2/3 signalling inhibited the expression of HAS2 and TNFAIP6 CC matrix genes and attenuated CC expansion in intact COCs and in OOXs. However, inhibition of SMAD1/5/8 signalling had no effect on porcine CC gene expression or expansion or murine COC expansion.

In recent years, major progress has been made using the mouse model in elucidating the nature of oocyte paracrine and endocrine signalling in CCs and how these interact to coordinate oogenesis and folliculogenesis. In the mouse, cooperation of oocyte-secreted factor-activated signalling in CCs (mediated by SMAD2/3) (Dragovic et al. 2007) and FSH/EGF signalling (mediated by ERK1/2 MAPK) is obligatory for cumulus expansion and ovulation (Su et al. 2003). Hence, absence of the oocyte or genetic depletion of ERK1/2 prevents mouse CC expansion (Buccione et al. 1990, Vanderhyden et al. 1990, Fan et al. 2009). Whilst these concepts are now widely appreciated in the field, it is less well appreciated that the mouse appears to be the exception, as other mammals studied to date (rat, cow and pig) do not appear to require oocyte-paracrine signalling for CC expansion (Prochazka et al. 1991, Vanderhyden 1993, Ralph et al. 1995). Given this clear species divergence, studies of oocyte and follicular biology using other species as well as the laboratory mouse are important for the understanding of mammalian ovarian biology.
Paracrine and autocrine activation of CC SMAD2/3

On the basis of the knowledge that; 1) oocyte-secreted GDF9 and subsequent SMAD2/3 activation in CCs is crucial for mouse CC expansion (Dragovic et al. 2005, 2007), 2) porcine CC expansion can occur without the oocyte (Prochazka et al. 1991) and 3) porcine CCs have been reported to express GDF9 (Prochazka et al. 2004), we hypothesised that activation of SMAD2/3 in porcine CCs may occur via CC autocrine mechanisms rather than by oocyte paracrine signals. Hence, using oocyte-free CC complexes (OOXs) we attempted to ablate GDF9/TGFβ and SMAD2/3 signalling by various means. Both the GDF9 monoclonal neutralising antibody and the soluble ECD of BMPR2 failed to prevent expansion of OOXs. Both these reagents have previously been shown to antagonise GDF9 bioactivity (Vitt et al. 2002, Gilchrist et al. 2004), including GDF9-stimulated expansion of mouse OOXs (Dragovic et al. 2005). Likewise, antagonism of activin A, activin B and TGFβ also failed to prevent FSH-stimulated porcine COC and OOX expansion. This is perhaps not surprising as these TGFβ superfamily members probably play no role in oocyte regulation of CC/granulosa cell steroidogenesis, proliferation or expansion in other species (reviewed; Gilchrist et al. 2008). Together, these results may cast some doubt on the hypothesis that porcine CC expansion is achieved by CC autocrine expression of GDF9.

The SMAD2/3 phosphorylation inhibitor SB431542 has recently been widely used as a powerful experimental tool to antagonise a broad range of CC functions regulated by oocyte-secreted factors (Gilchrist et al. 2006, Diaz et al. 2007, Dragovic et al. 2007). Our results showing mild inhibition of FSH-stimulated COC expansion and notable reduction in HAS2 and TNFAIP6 expression after inhibition of SMAD2/3 phosphorylation are consistent with another recent report (Nagyova et al. 2011). These investigators were more effective at preventing COC expansion and hyaluronan retention in the COC using an alternative SMAD3-specific inhibitor, SIS3 at 20 μM (Nagyova et al. 2011). Our SB431542 dose–response experiment using mouse and pig COCs showed that in the optimal dose range for this inhibitor of <5 μM, the inhibitor completely abolished mouse COC expansion but only partially inhibited pig COC expansion. Notably, we also show that the CC response to inhibition of SMAD2/3 phosphorylation is equivalent with or without the presence of the oocyte, providing further evidence to support the hypothesis that any role of SMAD2/3 signalling in FSH-stimulated porcine CC expansion may not be dependent on oocyte-paracrine signalling, but more likely is CC in origin.

Participation of BMP signalling in CC expansion

The other major SMAD pathway is the BMP-activated SMAD1/5/8 pathway that, within a COC, is principally
activated by oocyte-secreted BMP15 (Gilchrist et al. 2008). The current results show that porcine oocytes secreted factors that activated the SMAD1/5/8-responsive BRE-luciferase reporter plasmid, but inhibition of BMP15 function and SMAD1/5/8 signalling using dorsomorphin had no effect on FSH-stimulated COC expansion or expression of the three matrix-related genes in CCs. The role of BMP15 in mammalian CC expansion is not yet clear. In general terms, BMP15 plays a relatively minor role in reproductive function in the mouse compared with its central roles in folliculogenesis and fecundity in ruminants (McNatty et al. 2004). The BMP15 knockout mouse has a mild reproductive lesion (Yan et al. 2001), whereas BMP15 deficient sheep exhibit a complete block in folliculogenesis and are sterile (Galloway et al. 2000). Porcine oocytes express BMP15 at high levels and in a developmentally regulated manner (Li et al. 2008, Paradis et al. 2009), although to date BMP15 has not been attributed a major role in porcine folliculogenesis or fertility. The BMP15 knockout mouse has a mild cumulus expansion defect, slightly reduced oocyte developmental competence, and interestingly reduced levels of phospho-SMAD2 and EGF receptor expression, relative to wild-type mice, and all these features are greatly amplified on the GDF9 heterozygote genetic background (Su et al. 2004, 2010). Specific preparations of recombinant human BMP15 have been shown to stimulate mouse CC expansion independent of FSH (Yoshino et al. 2006).

Taken together, BMPs and SMAD1/5/8 signalling in CCs may have a relatively minor role in regulating mammalian CC expansion that may be restricted to facilitating the important roles of GDF9/SMAD2/3.

**Role of porcine oocyte-secreted factors**

Even though porcine COCs from large antral follicles are capable of FSH-stimulated expansion independent of oocyte-paracrine signalling, nonetheless, we found that these oocytes produce soluble factors that are potent activators of both SMAD pathways. This is consistent with the observation that porcine oocytes secrete factors that stimulate mouse OOX expansion (Singh et al. 1993, Vanderhyden 1993). Regardless of whether activated by these paracrine signals from the oocyte or by CC autocrine means, SMAD2/3 signalling in CCs may have a relatively minor role in regulating mammalian CC expansion that may be restricted to facilitating the important roles of GDF9/SMAD2/3.

**Figure 7** Effect of inhibition of MAPK signalling on porcine cumulus cell (CC) expansion and gene expression. Porcine COCs cultured in the presence of FSH were treated with either the ERK1/2 MAPK inhibitor U0126 (8 μM) or its inactive analogue U0124, the p38 MAPK inhibitor SB203580 (8 μM), or a vol/vol equivalent of DMSO. Negative control: COCs cultured without FSH. (A) CC expansion was assessed at 20 h and bars represent means ± S.E.M. of 90 COCs from three replicate experiments. CC mRNA from (A) was examined by quantitative real-time RT-PCR for the expression of HAS2 (B), PTGS2 (C) and TNFAIP6 (D). All transcripts were normalised to the housekeeping gene cyclophilin and expressed relative to the positive control (COCs + FSH). (B–D) Bars are means ± S.E.M. of three replicate experiments. An asterisk denotes a mean significantly different to the + FSH positive control (ANOVA, P<0.05).
oocytes as demonstrated by increased FSH-stimulated COC progesterone production. Therefore, the production of potent SMAD-activating molecules by porcine oocytes from large antral follicles, may have less to do with the regulation of cumulus expansion, but rather seems more likely to be important for the continuing control of CC differentiation and appropriate functioning of CCs to support oocyte development and ovulation.

**Interactions between SMAD and MAPK signalling**

Recent studies in mice suggest a mutual dependency and probable crosstalk between ERK1/2 and SMAD2/3 signalling in CCs. Su et al. (2010) showed that oocyte-secreted GDF9 and BMP15 and subsequent activation of SMAD2/3 in CCs is required for expression of the EGF receptor, which is a principle activator of ERK1/2 in CCs. Our studies elucidated the reciprocal relationship, by demonstrating that EGF receptor/ERK1/2 activity is required to maintain phosphorylation of the SMAD3 linker region, required for downstream SMAD2/3 signalling and oocyte-regulated granulosa/CC functions (Sasseville et al. 2010). Together, these two studies suggest some form of feedback loop is operating in CCs between GDF9/BMP15-activated SMAD2/3 and EGF receptor/ERK1/2. This study shows that whilst SMAD signalling may have an equivocal role in porcine CC expansion, signalling through ERK1/2 and p38 MAPKs are essential for expansion and expression of key matrix genes. These MAPK results are largely consistent with the findings of Yamashita et al. (2009) who also demonstrated the key involvement of PKA in FSH-stimulated porcine COC expansion. The extent to which SMAD2/3 participates in or enables this crucial MAPK signalling in porcine COC expansion remains to be determined.

In conclusion, this study has confirmed that FSH-stimulated porcine COC expansion does not require oocyte-paracrine signalling but does require activation of MAPK. Porcine oocytes secrete factors that activate both SMAD2/3 and SMAD1/5/8 signalling pathways, however these signals appear to have a negligible role in FSH-stimulated CC expansion. Although CC autocrine activation of SMAD2/3 plays some role in matrix formation, this signal may not come from CC-expressed GDF9.

**Materials and Methods**

Unless specified, all chemicals and reagents were purchased from Sigma.

**Isolation of porcine granulosa cells and COCs**

Prepubertal pig ovaries were collected from the local abattoir and transported to the laboratory in warm saline (0.9% w/v; Baxter Healthcare Australia, Old Toongabbie, NSW, Australia) supplemented with antibiotics (50 IU/ml penicillin G and 50 μg/ml streptomycin sulphate). COCs were aspirated from large antral follicles (>4 mm) using an 18G needle, through which constant suction was applied. Aspirates were diluted with H-TCM199/BSA (HEPES-buffered tissue culture medium 199 (MP Biomedicals, Solon, OH, USA) supplemented with 50 μg/ml kanamycin and 0.3% (w/v) BSA) and COC with even, intact, compact CCs were collected. Mural granulosa cells were collected and cultured in a [3H]thymidine DNA synthesis assay as described previously (Hickey et al. 2004).

**Isolation of mouse granulosa cells and COCs**

Mice used in this study were maintained at the University of Adelaide animal house. The study was approved by the University’s local animal ethics committee and was conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Twenty-one to 26 days old 129/Sv mice were injected with 5 IU equine chorionic gonadotropin (Folligon; Intervet, Castle Hill, NSW, Australia), and ovaries collected 46 h later in H-TCM199/BSA (COC expansion assay) or, for the SMAD-luciferase reporter assay, in H-TCM199 with 2% (v/v) heat inactivated FCS (Invitrogen Australia). Large antral follicles were ruptured with a 27-gauge needle to liberate COCs and mural granulosa cells. In this study, only COCs with an intact and uniform covering of compact CCs were used.

**Phospho-SMAD reporter assay**

To determine the capacity of pig oocyte-secreted factors to activate SMAD signalling, a phospho-SMAD reporter bioassay was used as described previously (Gilchrist et al. 2006). This assay utilises primary mouse mural granulosa cells transiently transfected with luciferase reporter constructs responsive to either phosphorylated SMAD3 (CAGA-luciferase) or phosphorylated SMAD1/5/8 (BRE-luciferase). In brief, mouse granulosa cells were washed once in H-TCM199/FCS and twice in bicarbonate-buffered DMEM (B-DMEM; MP Biomedicals) with 2% (v/v) FCS. Granulosa cells (20 000 per well) were cultured in a 96-well plate in 250 μl of B-DMEM/FCS. After 4 h of culture, cells were transfected with 50 ng of luciferase reporter construct DNA using Fugene 6 (Roche Diagnostics). Eighteen hours after transfection, medium was aspirated and replaced with 125 μl of DMEM +0.1% FCS. Cells were then treated with one of the following: 1) control media, 2) 0.5 ng/ml TGFβ1 (R&D Systems, Minneapolis, MN, USA), 3) 50 ng/ml BMP6 (R&D Systems), 60 mouse denuded oocytes (DOs; 0.5 DO/μl), or an increasing dose of pig DOs (0.1–0.8 DO/μl) and cultured for a further 48 h. Experiments were terminated by removing media from wells and freezing plates at −20 °C. Cells were ruptured by adding 100 μl of lysis buffer (Promega) to each well and plates were incubated at room temperature on a rocking platform for 20 min. Twenty microlitre of cell lysate was used for measurement of luciferase activity using a Galaxystar luminometer (GMB Labtechnologies, Offenburg, Germany).


**COC expansion assay**

To examine the participation of paracrine growth factors and intracellular signal transduction pathways in the regulation of COC expansion, the widely used COC expansion assay was employed (Vanderhyden et al. 1990). To generate CC complexes lacking an oocyte (OOX), microsurgical removal of the oocyte from the COC was performed as described previously (Buccione et al. 1990). Porcine and murine COCs and OOXs were washed twice in bicarbonate-buffered TC199 supplemented with 50 μg/ml kanamycin, 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 μM cysteamine and 5% (v/v) FCS (B-TCM199/FCS), with or without 50 μM/ml recombinant human FSH (Pureogen; Organon, Oss, The Netherlands), before being transferred to culture drops under oil. Ten COCs or OOXs were cultured per 50 μl drop +/- various treatment reagents (see below) for either 20 h at 37 °C (mouse) or 24 h at 38.5 °C (pig) at 96% humidity in 5% CO2 in air. After culture, morphological cumulus expansion was scored and the cumulus expansion index calculated according to well-established criteria (Fagbohun & Downs 1990, Vanderhyden et al. 1990).

**Experimental reagents and treatments**

FSH-stimulated COCs and OOXs were treated with a number of solubilised ECDs of known TGFB superfamily type-II receptors; BMPR2, activin RIIA, activin RIIB and TGFBR2. We and others have previously demonstrated that these ECDs specifically antagonise the actions of their respective ligands in COCs and granulosa cells (Moore et al. 2003, Gilchrist et al. 2006). COCs and OOXs were treated with 5 μg/ml of ECD (all from R&D Systems). Application of the GDF9 neutralising MAB (MAb53; generously donated by Nigel Groome, Oxford Brookes University, Oxford, UK) using granulosa cells and COCs has been previously characterised (Gilchrist et al. 2004, Dragovic et al. 2005) and was used at 80 μg/ml in this study. SB431542 (generously donated by GlaxoSmithKline) is a small molecule ATP-binding site competitor on ALKs 4, 5 and 7 and SB431542 (generously donated by GlaxoSmithKline) is a small molecule ATP-binding site competitor on ALKs 4, 5 and 7 and thus prevents BMP-mediated SMAD1/5/8 phosphorylation (Yu et al. 2002). This agent has now been used in numerous studies as a broad spectrum oocyte-secreted factor inhibitor (Gilchrist et al. 2006, Dragovic et al. 2007). Dorsomorphin is a newly described small molecule inhibitor that selectively inhibits ALKs 2, 3, 6 and thus prevents BMP-mediated SMAD2/3 phosphorylation (Inman et al. 2005) and was used at 80 μg/ml in this study. Dorsomorphin is a newly described small molecule inhibitor that selectively inhibits ALKs 2, 3, 6 and thus prevents BMP-mediated SMAD2/3 phosphorylation (Inman et al. 2005). To validate use of this agent in ovarian biology we show that 8 μM dorsomorphin antagonises BMP15 actions on porcine granulosa cells (see Results).

**Quantitative RT-PCR**

Real-time RT-PCR was used to determine the effect of inhibition of SMAD and MAPK signalling (10 μM U0126, 10 μM SB203580, 8 μM SB431542 and 8 μM dorsomorphin) on the expression of various pig CC matrix gene transcripts. COCs were collected and cultured as described above. After 20 h of culture, COCs were mechanically stripped of their CCs by vigorous pipetting using a P200 pipette. CCs were collected and snap frozen in liquid nitrogen until the time of RNA extraction. RNA was extracted using a Micro RNeasy RNA extraction kit (Qiagen), quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC, Australia), and reverse transcribed using Superscript III (Invitrogen Australia Pty Ltd) and random primers (Invitrogen). cDNA was amplified using specific primers for the housekeeping gene cyclophilin A (PPIA), and CC matrix genes HAS2, prostaglandin synthase 2 (PTGS2) and TNFAIP6 (see Table 1 for primer sequences and accession numbers). Cyclophilin A was deemed to be an appropriate housekeeping gene as its expression was constant regardless of treatment (data not shown). The quantity of PCR products were detected using a Corbett Rotor-Gene 6000 (Qiagen) using Power SYBR Green (Applied Biosystems, Mulgrave, VIC, Australia). CC matrix gene expression was calculated by the standard curve method and expressed relative to a calibrator and normalised to PPIA.

**Statistical analysis**

Treatment effects on cumulus expansion, as scored by the cumulus expansion index, were tested by a Kruskal–Wallis one-way ANOVA on ranks, and differences between individual means were detected by a Tukey post hoc procedure (pairwise comparisons) or by Dunnett’s method (compared with a control). Real-time RT-PCR data were analysed by one-way ANOVA followed by post hoc comparisons by Dunnett’s method. P<0.05 was considered statistically significant.

**Table 1 Sequences of PCR primers used for quantitative RT-PCR.**

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Primer sequence (5’→3’)</th>
<th>Amplicon length (bp)</th>
<th>GenBank accession number</th>
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<tr>
<td>PPIA</td>
<td>F: CAT TGC CAC TGCCAA GAC TGA R: GGA CCC AAA GCG CTC CAT</td>
<td>103</td>
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<tr>
<td>HAS2</td>
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<td>TNFAIP6</td>
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<td>106</td>
<td>NM_001159607.1</td>
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</table>
Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References


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