

# Growth differentiation factor 9 signaling requires ERK1/2 activity in mouse granulosa and cumulus cells

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Accepted 25 May 2010

Journal of Cell Science 123, 3166–3176

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doi:10.1242/jcs.063834

## Summary

Ovarian folliculogenesis is driven by the combined action of endocrine cues and paracrine factors. The oocyte secretes powerful mitogens, such as growth differentiation factor 9 (GDF9), that regulate granulosa cell proliferation, metabolism, steroidogenesis and differentiation. This study investigated the role of the epidermal growth factor receptor (EGFR)–extracellular signal-regulated kinase 1 and 2 (ERK1/2; also known as MAPK3/1) signaling pathway on GDF9 action on granulosa cells. Results show that mitogenic action of the oocyte is prevented by pharmacological inhibition of the EGFR–ERK1/2 pathway. Importantly, EGFR–ERK1/2 activity as well as Rous sarcoma oncogene family kinases (SFK) are required for signaling through SMADs, mediating GDF9, activin A and TGFβ1 mitogenic action in granulosa cells. GDF9 could not activate ERK1/2 or affect EGF-stimulated ERK1/2 in granulosa cells. However, induction of the SMAD3-specific CAGA reporter by GDF9 in granulosa cells required active EGFR, SFKs and ERK1/2 as did GDF9-responsive gene expression. Finally, the EGFR–SFKs–ERK1/2 pathway was shown to be required for the maintenance of phosphorylation of the SMAD3 linker region. Together our results suggest that receptivity of granulosa cells to oocyte-secreted factors, including GDF9, is regulated by the level of activation of the EGFR and resulting ERK1/2 activity, through the requisite permissive phosphorylation of SMAD3 in the linker region. Our results indicate that oocyte-secreted TGFβ-like ligands and EGFR–ERK1/2 signaling are cooperatively required for the unique granulosa cell response to the signal from oocytes mediating granulosa cell survival and proliferation and hence the promotion of follicle growth and ovulation.

**Key words:** Growth differentiation factor 9, Granulosa cells, Extracellular signal-regulated kinase 1/2, SMAD2/3, Cumulus cells, Rous sarcoma oncogene family kinases

## Introduction

Development and function of the ovary, and important structures therein, including ovarian follicles and oocytes, depends on a complex interplay between maternal endocrine hormones and locally produced growth factors. In sheep, during folliculogenesis, from the primordial to the periovulatory stage, granulosa cells will undergo 16–19 doublings under the influence of mitogens including follicle stimulating hormone (FSH) (McNatty et al., 2007). FSH interacts with a range of intraovarian growth factors including oocyte-secreted factors. It is widely accepted that transforming growth factor β (TGFβ) superfamily members play a crucial role in this process (Edson et al., 2009; Trombly et al., 2009).

TGFβ superfamily members can be broadly divided into two groups, (1) the bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs), and (2) the TGFβ-like ligands. The BMP ligands and some GDF ligands act by binding to the BMP type 2 receptor, and one of the BMP type 1 receptors ALK2, ALK3 or ALK6. Some BMP and GDF ligands have also been reported to bind to the activin type 2A and 2B receptors (Sebald et al., 2004). Activation of ALK2, 3, 6 (ALK2/3/6) leads to the phosphorylation of the conserved Ser-Ser-x-Ser motif at the C-terminal end of SMAD1, 5 and 8 (SMAD1/5/8) proteins, which associate with SMAD4 and activate a specific transcriptional response through BMP-responsive elements (Kaivo-oja et al., 2006; Korchynskyi and ten Dijke, 2002). TGFβ-

like ligands, such as TGFβ or activins, bind to their respective type 2 receptors (TGFβR2, ACTR2A) and activate the alternative type 1 receptors, ALK4/5/7, and intracellular transducing proteins, SMAD2 and SMAD3. GDF9 is a relatively newly discovered member of the superfamily and remains poorly understood. However, GDF9 is particularly important and unusual as its expression is largely restricted to gametes and it is essential for ovarian development and fertility (Dong et al., 1996). GDF9 signals through the unique combination of a BMP type 2 receptor and the TGFβ type 1 receptor ALK5 (Kaivo-Oja et al., 2005; Mazerbourg et al., 2004), and/or potentially ALK4/7, resulting in SMAD2/3 downstream signaling. Another layer of complexity is added to TGFβ signaling through the array of non-canonical pathways that may be activated, such as extracellular signal-regulated kinase 1/2 (ERK1/2; also known as MAPK3/1), MAPK14 (p38 MAPK) or Jun N-terminal kinases (JNKs) (Zhang, 2009). Oocytes secrete potent TGFβ family growth factors, including BMP15 and GDF9, to induce expression of cyclin D2 and stimulate the incorporation of [<sup>3</sup>H]thymidine in granulosa cells (Gilchrist et al., 2001; Gilchrist et al., 2006; Vanderhyden et al., 1992). This mitogenic activity can be fully inhibited by SB4315542, an ALK4/5/7 inhibitor, a BMP type 2 receptor extracellular domain, and partially inhibited by mAb-53 (a GDF9 neutralizing antibody) (Gilchrist et al., 2004; Gilchrist et al., 2006). It is clear that oocytes secrete mitogens necessary for

granulosa cell growth and survival, but current evidence does not indicate whether oocyte factors alone are sufficient for granulosa cell proliferation (Gilchrist et al., 2008).

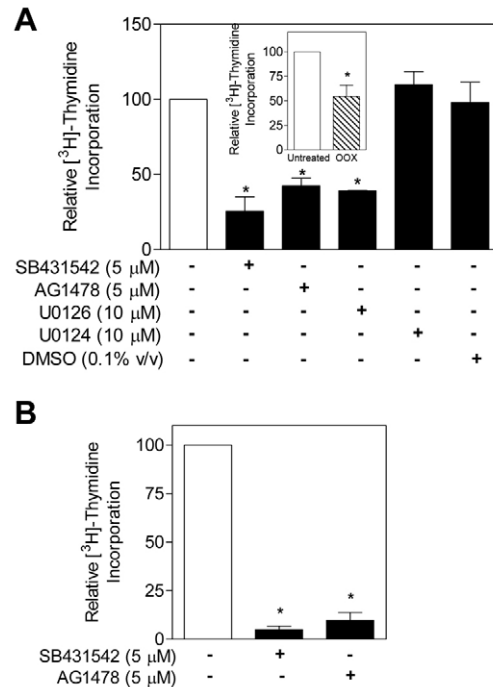
Epidermal growth factor (EGF) and related peptides have received an increasing amount of attention since the discovery of their fundamental role during late folliculogenesis and ovulation (Park et al., 2004). The EGF receptor (EGFR) is part of the receptor tyrosine kinase (RTK) family and is known to bind EGF-like growth factors (i.e. EGF, epiregulin, amphiregulin,  $\beta$ -cellulin) at the cell membrane. After ligand binding, autophosphorylation of multiple tyrosines of the EGFR intracellular domain transduces an array of signaling cascades. Mitogen-activated protein kinase kinase 1 (MEK1)–ERK1/2 is the best documented pathway activated by the EGFR. However, the EGFR has been shown to activate the phosphoinositide 3-kinase (PI3K)–protein kinase B (PKB) pathway as well (Linggi and Carpenter, 2006). Other activation mechanisms of the EGFR, apart from direct ligand binding, have been reported and are referred to as EGFR transactivation (for a review, see Wetzker and Bohmer, 2003). The activation of metalloproteinases by G-protein-coupled receptors that cleave membrane-bound EGF ligands is one of the better documented processes (Higashiyama et al., 2008). Alternatively, EGF shedding-independent mechanisms have been reported recently, especially implicating  $\text{Ca}^{2+}$ , SRC and ligand-independent tyrosine kinase PYK2 (Bohe et al., 2003). Inactivation of protein tyrosine phosphatases (PTP), which negatively control the phosphorylation level of RTKs such as the EGF receptor, was reported as another transactivation mechanism in many cell types (Wetzker and Bohmer, 2003) including granulosa cells (Hunzicker-Dunn and Maizels, 2006).

It is clear that ERK1/2 (Fan et al., 2009b) and SMAD2/3 signaling (Li et al., 2008) are both essential for normal ovarian function and fertility. There is also solid functional evidence in the ovary that there is some form of interaction between the two signaling pathways, for example for cumulus cell expansion (Diaz et al., 2007; Dragovic et al., 2007). A few reports have investigated the effect of TGF $\beta$ -like factors on EGF signaling (Diaz et al., 2006; Su et al., 2003; Su et al., 2002). Here we test the hypothesis that oocyte-secreted TGF $\beta$ -like ligands and EGFR–ERK1/2 signaling are cooperatively required for the unique granulosa cell response to the signal from oocytes that mediates granulosa cell survival and proliferation, and hence promotes follicle growth and ovulation. Special emphasis is given to GDF9 because it plays the dominant role in mammalian folliculogenesis, reproduction and development.

## Results

### The role of the EGF receptor–ERK1/2 pathway on oocyte and GDF9 mitogenic activity in granulosa cells at different stages of folliculogenesis

Isolated whole preantral secondary follicles, either intact or after microsurgical oocytectomy, were cultured with [ $^3\text{H}$ ]thymidine. Forty-six percent of the [ $^3\text{H}$ ]thymidine taken up was dependent on the presence of the oocyte (Fig. 1A inset). Interestingly, 5  $\mu\text{M}$  SB431542 (a TGF $\beta$ –GDF9 type I receptor ALK4/5/7 inhibitor), and also 5  $\mu\text{M}$  AG1478 (an EGF receptor inhibitor) and 10  $\mu\text{M}$  U0126 (a MEK1 inhibitor) reduced preantral granulosa cell proliferation in secondary follicles by between 60 and 70% (Fig. 1A). Endogenous mitogenic activity of cumulus–oocyte complexes (COCs) from mature (PMSG-treated) follicles was also inhibited by blocking ALK4/5/7 or the EGF receptor (Fig. 1B). As expected, SB431542 dose-dependently inhibited GDF9-stimulated



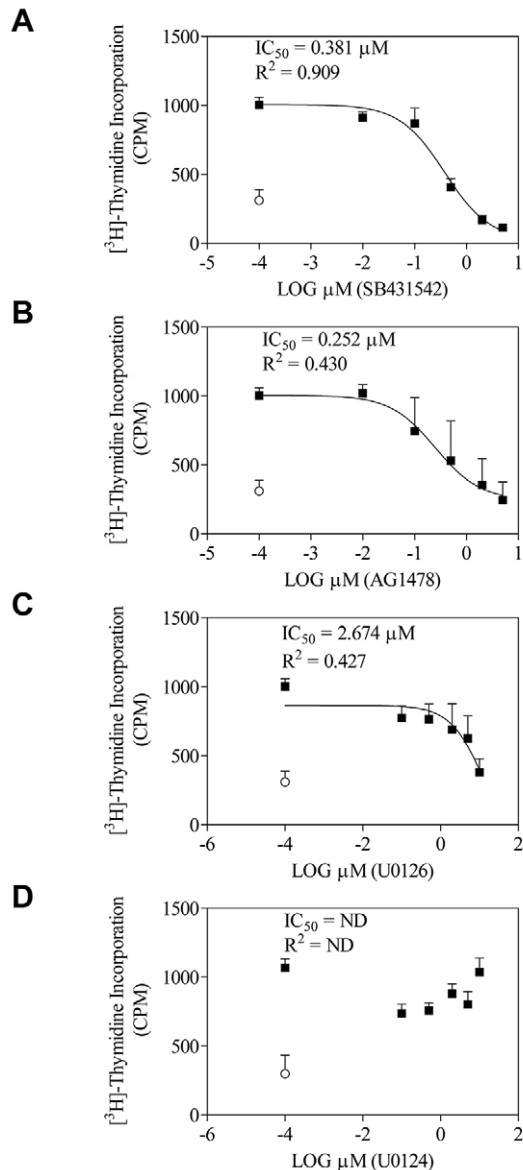
**Fig. 1. Granulosa cell proliferation at different differentiation stages.**

(A) [ $^3\text{H}$ ]thymidine incorporation of intact preantral secondary follicles for 6 hours after 18 hours of in vitro culture alone or in the presence of SB431542 (5  $\mu\text{M}$ ), AG1478 (5  $\mu\text{M}$ ), U0126 (10  $\mu\text{M}$ ), U0124 (10  $\mu\text{M}$ ) or DMSO vehicle. Inset: [ $^3\text{H}$ ]thymidine incorporation in preantral secondary follicles or in oocytectomized follicles (OOX). (B) [ $^3\text{H}$ ]thymidine incorporation of antral cumulus–oocyte complexes during in vitro culture alone or with SB431542 (5  $\mu\text{M}$ ) or AG1478 (5  $\mu\text{M}$ ). \*A statistically significant difference of the means compared with the untreated control, as determined by ANOVA ( $P < 0.05$ ).

[ $^3\text{H}$ ]thymidine incorporation by cultured granulosa cells with an  $\text{IC}_{50}$  (50% inhibitory dose) of 0.38  $\mu\text{M}$  in antral follicle mural granulosa cells (Fig. 2A). However, it was unexpected that the GDF9 effect was also inhibited by the EGF receptor kinase inhibitor AG1478 ( $\text{IC}_{50}$ : 0.25  $\mu\text{M}$ ; Fig. 2B) and by the MEK1 inhibitor U0126 ( $\text{IC}_{50}$ : 2.65  $\mu\text{M}$ ; Fig. 2C). An inactive analogue of U0126 (U0124) had no effect (Fig. 2D). These results (Figs 1, 2) suggest that the ALK4/5/7–SMAD2/3 and EGF receptor–ERK1/2 pathways are both required for oocyte- and GDF9-stimulated mitogenic activity in granulosa and cumulus cells.

### ALK4/5/7 and EGF receptor–ERK1/2 pathways are both required for TGF $\beta$ superfamily member stimulation of [ $^3\text{H}$ ]thymidine uptake

Oocyte- and GDF9-stimulated [ $^3\text{H}$ ]thymidine uptake by antral follicle granulosa cells was inhibited by SB431542 (5  $\mu\text{M}$ ) as well as by AG1478 (5  $\mu\text{M}$ ) and by U0126 (10  $\mu\text{M}$ ) (Fig. 2, Fig. 3A,B), but not by dorsomorphin (10  $\mu\text{M}$ ; a BMP type I receptor ALK2/3/6 inhibitor; Fig. 3A,B) or U0124 (Fig. 2D, Fig. 3A,B), the inactive analogue of U0126. Inhibition of ALK4/5/7, the EGF receptor and MEK1 also prevented the mitogenic activity of TGF $\beta$ 1 (Fig. 3C) and activin A (Fig. 3D) on mural granulosa cells, suggesting that the requirement of the EGF receptor–ERK1/2 pathway for mitogenic activity applies to other TGF $\beta$  superfamily members that signal through SMAD2/3.



**Fig. 2. Antral follicle granulosa cell proliferation induced by GDF9.** [<sup>3</sup>H]thymidine incorporation of mural granulosa cells, untreated (open circles) or stimulated with GDF9 (20 ng/ml; closed squares) and challenged with increasing doses of (A) SB431542 (5 μM), (B) AG1478 (5 μM), (C) U0126 (10 μM) or (D) U0124 (10 μM). The GDF9 only treatment is presented here 2 logs below the lowest inhibitor dose. The 50% inhibitory dose (IC<sub>50</sub>) and goodness of the fit (R<sup>2</sup>) were determined from the non-linear regression.

Possible transactivation of the EGFR by TGFβ-mediated induction of a metalloprotease activator of membrane-tethered EGF ligands has been reported in fetal rat hepatocytes (Murillo et al., 2005), but was excluded in our case because the metalloproteinase inhibitor GM6001 had no effect on GDF9, activin A or TGFβ1 mitogenic activity (Fig. 4A). An alternative route via TGFβ–BMP receptor activation of NF-κB through TAK1, has been proposed to carry the cell growth and mitogenic effects of GDF9 (Arsura et al., 2003; Juengel et al., 2008). However, in our granulosa cell model, the NF-κB inhibitor SN50 did not prevent GDF9-stimulated [<sup>3</sup>H]thymidine incorporation (Fig. 4B).

The rous sarcoma oncogene (SRC) family kinases (SFK) may act through phosphorylation of tyrosine residues on the EGF receptor to mediate ligand-independent EGF receptor transactivation (Bobbe et al., 2003). SFKs have also been shown to act downstream of the EGF receptor, by enabling EGF to activate the ERK1/2 pathway (Schauwienold et al., 2008). We found that SFK inhibition in granulosa cells dose-dependently inhibited GDF9-stimulated [<sup>3</sup>H]thymidine incorporation (Fig. 5A). SFK inhibition also significantly decreased secondary follicle and COC endogenous mitogenic activity (Fig. 5B,C). PP2 inhibited the basal level of ERK1/2 phosphorylation in granulosa cells (Fig. 5D) as well as prevented the increased ERK1/2 phosphorylation stimulated by EGF treatment (Fig. 5E).

### The oocyte-stimulated shift in granulosa cell gene expression profile is prevented by inhibiting ALK4/5/7 or the EGF receptor

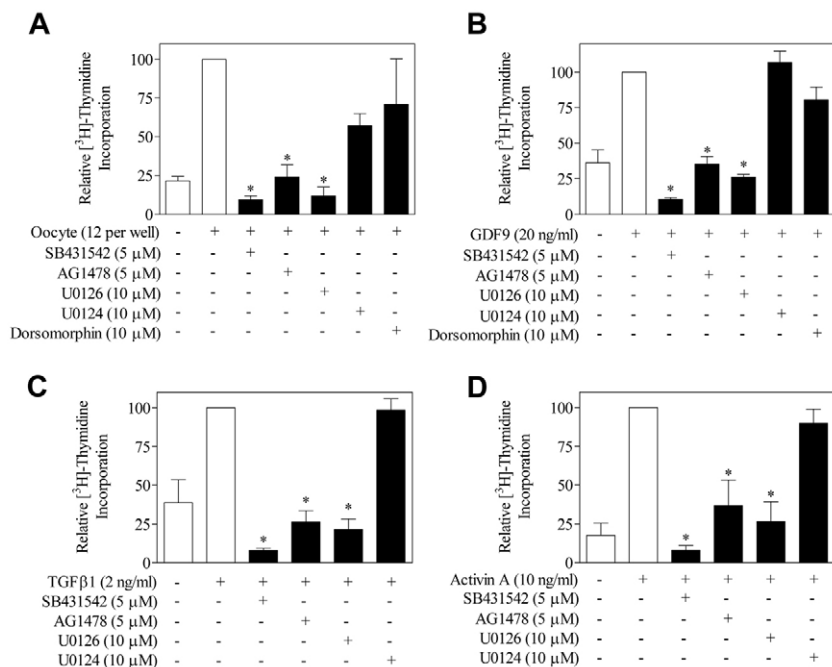
To further investigate the requirement for EGF receptor signaling in oocyte- and GDF9-stimulated granulosa cell functions, we co-cultured mural granulosa cells with denuded oocytes to induce them to adopt cumulus-cell-like characteristics. A recent report showed that oocyte-secreted factor BMP15 could induce cumulus-cell-like genes in mural granulosa cells (Li et al., 2009). After culture, granulosa cells co-cultured with oocytes had a significantly increased level of *Has2* and *Ptx3* mRNA compared with untreated cells (Fig. 6). The levels of *Has2* and *Ptx3* observed in oocyte-treated granulosa cells are comparable to the levels seen in cumulus cells derived from untreated COCs (data not shown), which suggests that oocyte-secreted factors induce a differentiation of the granulosa cells toward a cumulus cell phenotype. Inhibition of either ALK4/5/7 receptors or the EGF receptor prevented the oocyte-stimulated *Has2* and *Ptx3* increases, suggesting that the action of oocyte-secreted factors on the differentiation of follicular somatic cells is dependent on both GDF9–Smad2/3 and the EGF receptor–ERK1/2 pathways.

### The GDF9–ALK4/5/7 pathway does not affect ERK1/2 signaling

Immunodetection of phospho-ERK1/2 in mural granulosa cells showed that SB431542 does not affect either basal or EGF-stimulated ERK1/2 phosphorylation (Fig. 7A,B), in accordance with another study (Diaz et al., 2006). By contrast, both AG1478 and U0126 inhibited basal and EGF-stimulated ERK1/2 phosphorylation (Fig. 7A,B). These results suggest that ALK4/5/7 inhibition does not affect the EGF receptor–ERK1/2 pathway but that phospho-ERK1/2 is dependent on EGFR-mediated action.

To further determine whether there is any contribution of the GDF9–ALK4/5/7 pathway on the EGF receptor signaling cascade, EGF-stimulated activation of ERK1/2 was analyzed by immunoblotting at a number of time points. Fig. 8A shows that EGF stimulation markedly induced phosphorylation of ERK1/2 at 5 and 15 minutes, although this difference from the untreated granulosa cells was lost thereafter. Inhibition of ALK4/5/7 by SB431542 had no effect on EGF-stimulated ERK1/2 phosphorylation, whereas EGF receptor inhibition led to a severe decrease in phospho-ERK1/2 at all time points investigated (Fig. 8A–E). GDF9 treatment in addition to EGF did not change the ERK1/2 phosphorylation profile during culture (Fig. 8A–E).

Past evidence suggested that unpurified GDF9 (raw conditioned medium) could directly activate the ERK1/2 pathway in mouse



**Fig. 3. Sensitivity of granulosa cell proliferation stimulated by different mitogens to signaling pathway inhibition.** [ $^3$ H]thymidine incorporation of mural antral follicle granulosa cells was measured after stimulation with (A) denuded oocytes (12 per well), (B) GDF9 (20 ng/ml), (C) TGF $\beta$ 1 (2 ng/ml) or (D) activin A (10 ng/ml). For each mitogen, granulosa cell incorporation of [ $^3$ H]thymidine was challenged with an ALK4/5/7 inhibitor (SB431542, 5  $\mu$ M), an EGF receptor inhibitor (AG1478, 5  $\mu$ M), a MEK1/2 inhibitor (U0126, 10  $\mu$ M), its inactive analogue (U0124, 10  $\mu$ M) or an ALK2/3/6 inhibitor (dorsomorphin, 10  $\mu$ M). \*A statistically significant difference of the means compared with the ligand-treated control, determined by ANOVA ( $P < 0.05$ ).

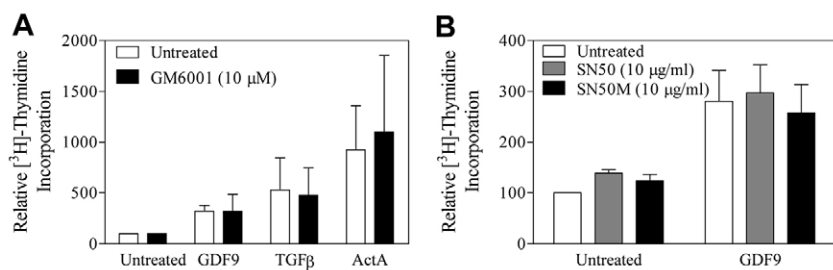
granulosa cells (Su et al., 2003). This study shows that the partially purified GDF9 does not trigger ERK1/2 phosphorylation in the dose range used in this study (Fig. 8F). Furthermore, a newly available purified GDF9 (from R&D Systems) was also unable to activate ERK1/2 phosphorylation (Fig. 8G). This evidence strongly suggests that GDF9 by itself does not activate the ERK1/2 pathway, which argues against the possibility that the inhibition of GDF9 mitogenic action, as shown in Figs 2 and 3, is due to a direct co-dependent activation of SMAD2/3 and ERK1/2 pathways by GDF9 in granulosa cells.

#### The EGF receptor–ERK1/2 pathway modulates GDF9 signaling through SMAD2/3

To determine if the EGFR–ERK1/2 pathway acts directly on GDF9–SMAD2/3 signaling or another pathway, a SMAD3 reporter construct was transfected into mouse granulosa cells followed by treatment with inhibitor and/or GDF9 (Gilchrist et al., 2006). As expected (Gilchrist et al., 2006), inhibition of the GDF9 type I receptor led to a dose-dependent decrease in SMAD3 promoter activation (Fig. 9A). Inhibition of the EGF receptor, MEK1 or SFK also significantly inhibited GDF9 activation of a SMAD3-dependent transcription (Fig. 9B–D). These results suggest that EGF receptor-dependent and SRC-dependent ERK1/2 phosphorylation is required for GDF9 signaling through SMAD2/3 in mouse granulosa cells.

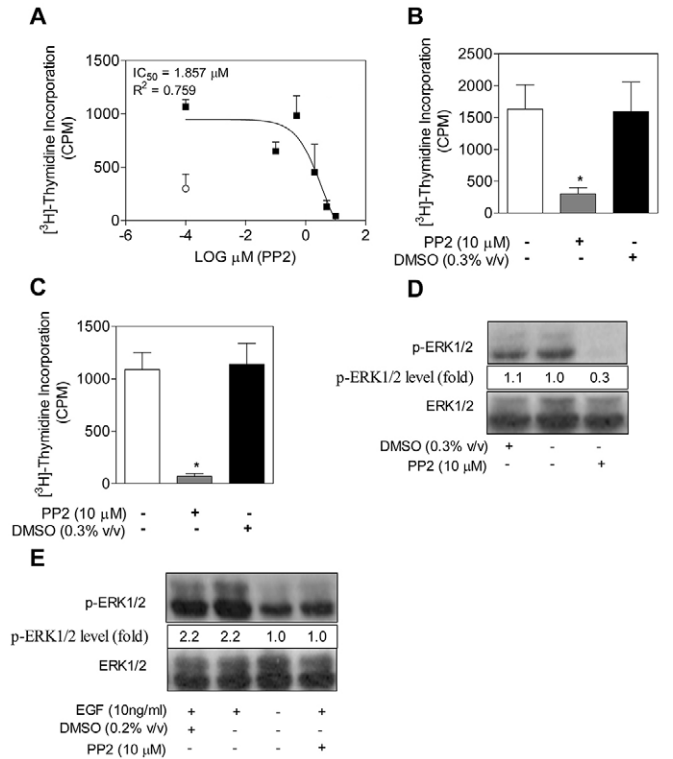
To further investigate this and to provide evidence of a functional interaction between the two pathways, phosphorylation of the

linker region of SMAD3 by ERK1/2 was examined. Serine residues in the linker region have been shown to increase or decrease the capacity of SMAD proteins to activate a SMAD-dependent transcriptional event, depending on the context (Wrighton et al., 2009). These phosphorylation sites are different from the residues phosphorylated on the SMAD2/3 C-terminus by the type I receptors ALK4/5/7. Previous studies have shown that the Ser208 is the best ERK1/2 phosphorylation site in Smad3 (Matsuura et al., 2005). This study therefore examined Ser208 phosphorylation as an indicator for Smad3 linker phosphorylation. To investigate whether this phosphorylation event could occur in vivo in response to the activation of ERK1/2, granulosa cells were recovered from mice 44 hours post-eCG (equine chorionic gonadotrophin) and at various times after an ovulatory hCG (human chorionic gonadotrophin) injection. Fig. 10 shows that the phosphorylation level of Ser208 of SMAD3 is increased 2.3-fold after 30 minutes and is maintained thereafter. Finally, to better understand the underlying mechanisms of Ser208 phosphorylation, similar experiments were performed in granulosa cells in vitro. The basal level of SMAD3 Ser208 phosphorylation in granulosa cells was transiently increased by EGF stimulation after 15 minutes (Fig. 11A), but no longer increased after 18 hours (Fig. 11B). The basal level of phosphorylation of SMAD3 Ser208 was decreased by inhibiting either the EGF receptor (AG1478, 5  $\mu$ M), MEK1/2 (U0126, 10  $\mu$ M) or SFK (PP2, 10  $\mu$ M) at both time points (Fig. 11). At 15 minutes and 18 hours, the inactive analogue of U0126 (U0124, 10  $\mu$ M) had no effect (Fig. 11). Inhibition of ALK 4/5/7 with



#### Fig. 4. Metalloproteinase and NF $\kappa$ B activity are not involved in the TGF $\beta$ family mitogenic activity.

(A) Incorporation of [ $^3$ H]thymidine by mural granulosa cells after treatment with GDF9 (20 ng/ml), TGF $\beta$ 1 (2 ng/ml) or activin A (ActA, 10 ng/ml) alone or with GM6001 (10  $\mu$ M). (B) [ $^3$ H]thymidine incorporation by granulosa cells untreated or after treatment with GDF9 in the presence of a specific NF $\kappa$ B inhibitor, SN50 (10  $\mu$ g/ml), or its inactive analogue SN50M.

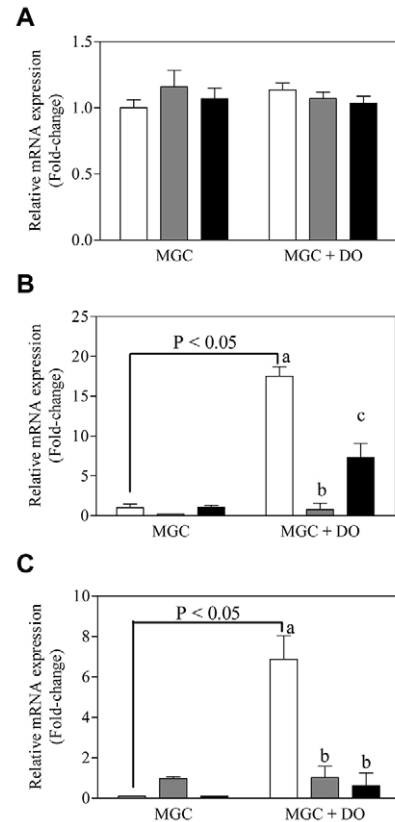


**Fig. 5. Involvement of SRC in GDF9 and EGF receptor signaling pathways.** (A) Incorporation of [ $^3H$ ]thymidine by mural granulosa cells, either untreated or after stimulation with GDF9 (20 ng/ml) and with increasing doses of a SRC inhibitor PP2 (0.1–10  $\mu M$ ). (B) Incorporation of [ $^3H$ ]thymidine by secondary ovarian follicles, either untreated (open circles) or in the presence of a SRC inhibitor (PP2, 10  $\mu M$ ; closed squares) or DMSO (0.001% v/v). (C) Incorporation of [ $^3H$ ]thymidine by cumulus–oocyte complexes, either untreated or in the presence of a SRC inhibitor (PP2, 10  $\mu M$ ) or DMSO (0.001% v/v). \*A statistically significant difference of the means compared with untreated controls, determined by ANOVA ( $P < 0.05$ ). (D) Mural granulosa cells were culture in vitro for 30 minutes alone, in the presence of DMSO (0.001% v/v) or a SRC inhibitor (PP2, 10  $\mu M$ ), and then phospho- and total ERK1/2 were detected by immunoblotting. (E) Mural granulosa cells were cultured in vitro for 15 minutes alone or with DMSO (0.001% v/v) or PP2 (10  $\mu M$ ) followed by the addition of EGF for 15 minutes, as indicated. Phospho- and total ERK1/2 were then immunodetected in each sample. The ratios of phospho- to total ERK1/2 relative to an untreated granulosa sample are reported between the blots in D and E, and are a mean of three independent experiments. A representative blot is shown.

SB431542 was used as a negative control and had minimal effect on SMAD3 Ser208 phosphorylation (mean fold-change of 1.2 at 15 minutes and 0.9 at 18 hours, data not shown). Together, these results suggest that ERK1/2 activity is required for GDF9 to signal via SMAD2/3 in mouse granulosa cells and that requirement could be mediated by the direct phosphorylation of the SMAD2/3 linker region by ERK1/2.

## Discussion

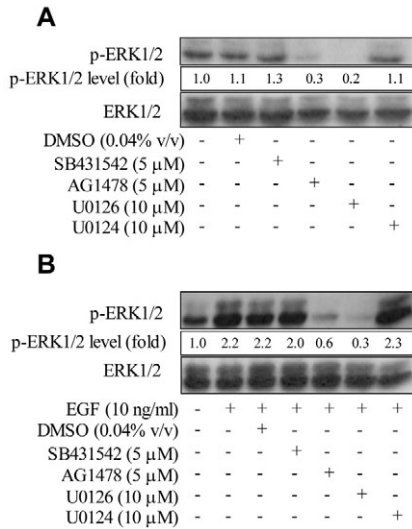
The present results are the first demonstration of a clear dependence of GDF9–SMAD2/3 signaling on the presence of phospho-ERK1/2. This study shows that transcription of a SMAD3 reporter required EGFR–ERK1/2 activity as well as the presence of a TGF $\beta$  ligand. Furthermore, cumulus genes, known to be dependent on oocyte-derived TGF $\beta$  ligands, were also dependent on EGFR–ERK1/2.



**Fig. 6. Oocyte-stimulated expression of cumulus cell markers in mural granulosa cells is prevented by inhibiting ALK4/5/7 or the EGF receptor.** Mural granulosa cells were cultured in vitro for 6 hours alone or in the presence of denuded oocytes (DO; 1 oocyte/ $\mu l$ ) and in combination with an ALK4/5/7 inhibitor (SB431542, 5  $\mu M$ ) or an EGF receptor inhibitor (AG1478, 5  $\mu M$ ). Extracted RNAs were analyzed by quantitative PCR for levels of *Ppia* (A), *Has2* (B) and *Ptx3* (C) mRNAs. Values are means  $\pm$  s.e.m. of four independent experiments and are presented relative to a geometric mean of three housekeeping genes (*Ppia*, *Actb*, *Gapdh*). Only *Ppia* is presented here. Different letters above the bars indicate a significant difference of the means determined by ANOVA ( $P < 0.05$ ). White bars, control; grey bars, SB431542; black bars, AG1478.

The results further suggest that this requirement is mediated by the direct phosphorylation of SMAD3 in its linker region. This region has been shown to promote transcriptional activity of SMAD3 in certain cell types (Engel et al., 1999; Funaba et al., 2002; Mori et al., 2004). Together, these observations indicate that transduction of the oocyte signal in follicular somatic cells requires both the interaction of GDF9 with its receptor and activation of ERK1/2 through EGFR that cooperatively promote SMAD2/3-mediated gene transcription.

It has been demonstrated that GDF9 signals through SMAD2/3 (Kaivo-Oja et al., 2005). However participation of non-canonical pathways has not yet been investigated. Apart from the classical SMAD2/3 signal transduction pathway, TGF $\beta$  has been shown to also activate a large number of parallel non-canonical signaling pathways, including ERK1/2 (MAPK3/1), MAPK14 (p38 MAPK), MAPK8/9/10 (JNK1/2/3) and RHOA (Zhang, 2009). TGF $\beta$  induces autophosphorylation of three tyrosine residues on its type 2 receptor (Y259, Y336, Y424), which enables the docking of GRB2 and SHC and the rapid activation of the ERK1/2 pathway (Gallier and

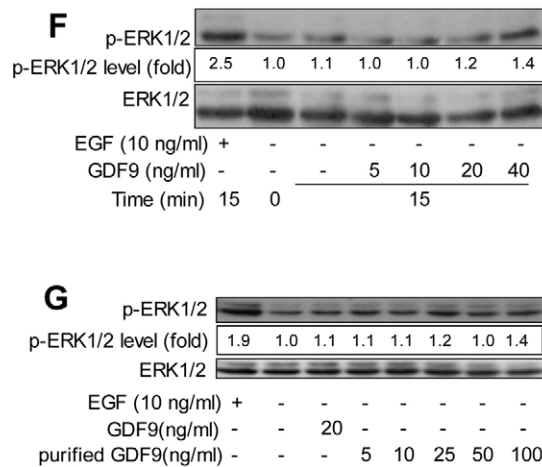
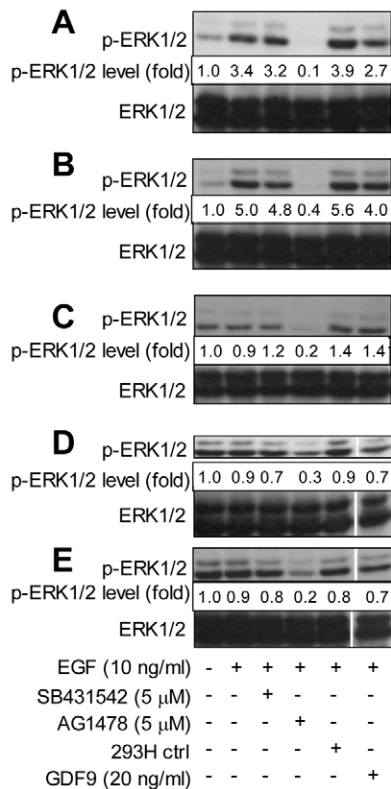


**Fig. 7. The effect of kinase inhibition on the basal (A) and EGF-stimulated (B) level of phospho-ERK1/2.** (A) Mural granulosa cells were cultured in vitro for 30 minutes alone or in the presence of DMSO (0.04% v/v), SB431542 (5 μM), AG1478 (5 μM), U0126 (10 μM) or U0124 (10 μM), and then phospho-ERK1/2 and total ERK1/2 were detected by immunoblotting. (B) Mural granulosa cells were cultured in vitro for 15 minutes with the inhibitors followed by incubation with EGF (10 ng/ml) for 15 minutes, as indicated. Phospho- and total ERK1/2 were immunodetected in each sample. The relative densitometric ratio between treatments of phospho- and total ERK1/2 are reported between the blots and are a mean of three independent experiments. A representative blot is shown.

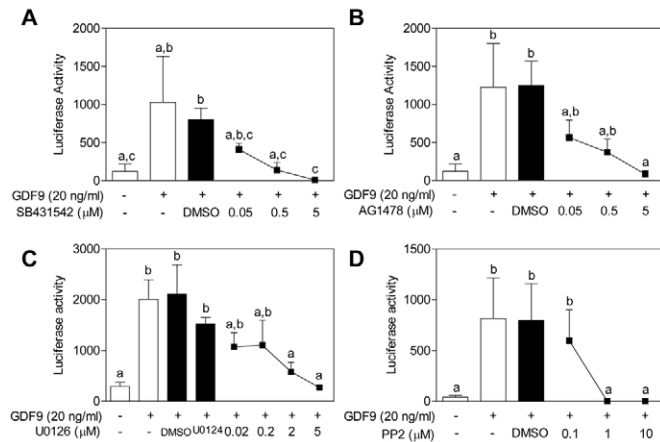
Schiemann, 2007). TGFβ and GDF9 share a common type 1, but not type 2 receptor: instead GDF9 binds BMPR2 (Vitt et al., 2002). Interestingly, some of those tyrosine residues located in the conserved ATP or substrate-binding motifs of the TGFβR2 are

present in the BMPRI2 also (data not shown). However, in our experiments using mural granulosa cells treated with two different recombinant GDF9 preparations, no ERK1/2 activation was observed, which is in opposition to the results reported by Su and collaborators using unpurified conditioned medium of GDF9-expressing 293HEK cells (Su et al., 2003).

There is already, in the literature, a clear precedence for some form of crucial interaction between GDF9 and ligands activating ERK. In the mouse, COC expansion is critically dependent on two signaling events: (1) activation of ERK by either FSH or EGF (Diaz et al., 2006; Su et al., 2002) and (2) activation of SMAD2/3 by oocyte-secreted paracrine factors including GDF9 (Dragovic et al., 2005; Dragovic et al., 2007; Li et al., 2008). This originally led



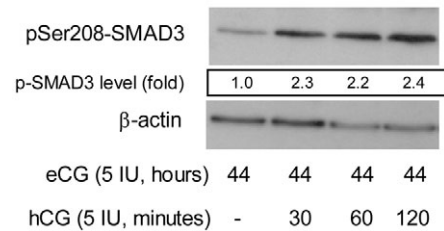
**Fig. 8. The effect of GDF9–ALK4/5/7 signaling on ERK1/2 phosphorylation by EGF.** Granulosa cells were culture in vitro alone or with SB431542 (5 μM), AG1478 (5 μM), 293H control medium or partially purified GDF9 (20 ng/ml) for 5 minutes followed by addition of EGF where indicated for (A) 5, (B) 15, (C) 60, (D) 120 or (E) 480 minutes. Cells were harvested, centrifuged and snap-frozen and phospho- and total ERK1/2 were detected by western blotting. (F) Granulosa cells were cultured in vitro alone, with EGF (10 ng/ml) or increasing doses of partially purified GDF9 (5–40 ng/ml) for 15 minutes, then harvested and then phospho-ERK1/2 and total ERK1/2 were detected by immunoblotting. (G) Granulosa cells were cultured in vitro alone, with EGF (10 ng/ml), increasing doses of partially purified GDF9 (20 ng/ml) or purified GDF9 (from R&D Systems; 5–100 ng/ml) for 15 minutes, and then phospho-ERK1/2 and total ERK1/2 were detected by immunoblotting. A blot representative of two replicates is shown with the densitometric analysis. A white space was left in blots to indicate samples that were run on the same gel but not next to each other.



**Fig. 9. The effect of EGF receptor, SRC and MEK1 inhibition on GDF9 capacity to activate a transfected SMAD3 reporter construct in granulosa cells.** Mural granulosa cells were transfected with a SMAD3 reporter construct and then cultured alone, with GDF9 (20 ng/ml) or with increasing doses of SB431542 (A), AG1478 (B), U0126 (C) or PP2 (D). After 18 hours of stimulation in the presence of inhibitors where indicated, quadruplicate samples of cell homogenate were assayed for luciferase luminescence. Values are the means of at least four independent experiments. Different letters above the bars indicate a statistical difference of the means ( $P < 0.05$ ) as determined by ANOVA followed by Tukey's multiple comparison post-hoc test.

to the hypothesis that the oocyte GDF9 paracrine signal enables the cumulus cells to respond to FSH or EGF (Buccione et al., 1990; Vanderhyden et al., 1990), i.e. that oocyte GDF9 activation of SMAD2/3 is required to enable the specific cumulus gene expression pattern in response to activation of ERK1/2. The current study allows us to revise this long held hypothesis and propose the reciprocal signaling interaction whereby activation of EGFR–ERK1/2 is a prerequisite for cumulus and granulosa cells to be able to respond to critical oocyte GDF9–SMAD2/3 paracrine signals. Results obtained indicate that EGFR and SFK maintain an ERK1/2-dependent basal level of phosphorylation of SMAD2/3 that is important for oocyte-secreted factor (including GDF9) signaling through BMPR2, ALK4/5/7 and SMAD2/3 (Fig. 12). It is conceivable, but remains to be determined, whether this is the main action of ERK1/2 or whether it independently maintains other key aspects of cumulus cell gene expression.

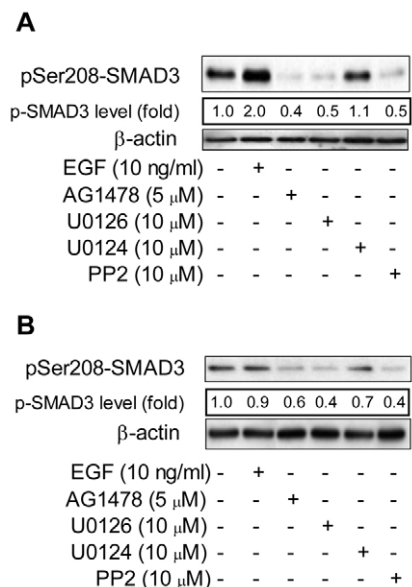
The oocyte and oocyte-secreted-factor mitogenic effects on granulosa cells have been studied extensively (Edwards et al., 2008; Gilchrist et al., 2001; Moore et al., 2003; Spicer et al., 2008; Vanderhyden et al., 1992; Vitt et al., 2000). The role of oocyte-secreted factors such as GDF9 and BMP15 during folliculogenesis has been demonstrated in the mouse (Dong et al., 1996; Yan et al., 2001), rat (Hayashi et al., 1999), sheep (Galloway et al., 2000) and pig (Hickey et al., 2005). However, their expression pattern alone does not fully explain the developmentally regulated mitogenic effect of the oocyte (Gilchrist et al., 2001). Even though GDF9 has been shown to be expressed from the primary follicle stage onwards (Dong et al., 1996), oocytes from secondary follicles have a notably lower mitogenic capacity compared with oocytes from antral follicles (Gilchrist et al., 2001). These studies raise the likelihood of other levels of control of oocyte-secreted-factor mitogenic effects, such as at the level of GDF9 secretion and/or post-translational processing, or alternatively maturation of the EGFR–



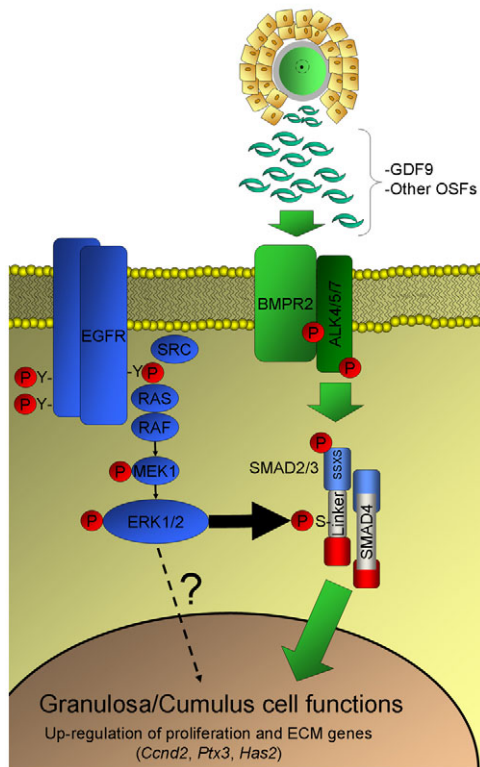
**Fig. 10. The temporal profile of phosphorylation of Ser208 of SMAD3 in vivo.** Granulosa cells were recovered from mice stimulated with eCG for 44 hours, or at various times after an additional administration of hCG. After washing, cells were snap-frozen and SMAD3 phospho-Ser208 and β-actin were analyzed by immunoblotting. A blot representative of four replicates is shown with the densitometric analysis of the ratio of SMAD3 phospho-Ser208 and β-actin.

ERK1/2 signaling system as folliculogenesis progresses, enabling variable responsiveness to GDF9.

In the present study, MEK–ERK1/2-dependent maintenance of SMAD3 linker phosphorylation on Ser208 was required for GDF9 to activate a SMAD3 transcriptional response. The overall physiological significance of this observation requires further investigation, although our results provide some potential insight. Our results (Fig. 10) show that SMAD linker phosphorylation occurs in vivo in mural granulosa cell after hCG stimulation, although the transcriptional activity of SMAD protein is unknown during that time. Our in vitro data in Fig. 9 show that SMAD3 transcriptional activity correlates with its linker phosphorylation



**Fig. 11. The effect of EGF receptor–ERK1/2 signaling on phosphorylation of Ser208 in SMAD3.** Granulosa cells were cultured in vitro alone, with EGF (10 ng/ml), DMSO (0.001% v/v), SB431542 (5 μM), AG1478 (5 μM), U0126 (10 μM), U0124 (10 μM) or PP2 (10 μM). Cells were harvested after 15 minutes (A) or 18 hours (B), snap-frozen and SMAD3 phospho-Ser208 and β-actin were analyzed by immunoblotting. A blot representative of four replicates is shown with the densitometric analysis of the ratio of SMAD3 phospho-Ser208 and β-actin.



**Fig. 12. A model illustrating that GDF9 actions on granulosa and cumulus cells require activation of the EGF receptor and ERK1/2 activity.** EGFR together with SRC family kinases maintain a basal level of activation of the RAS–RAF–MEK–ERK1/2 signaling cascade. Active ERK1/2 enables oocyte-secreted factor (OSF) and GDF9 action on granulosa cells at least partly by maintaining SMAD2/3 linker phosphorylation. OSFs, including GDF9, bind the BMP type II (BMPR2)–ALK4/5/7 receptor complex at the cell surface, which phosphorylates SMAD2/3 on the C-terminal end (SSxS). After association with SMAD4, the complex acts as a transcription factor and upregulates proliferation and ECM genes.

status, although the causality of the two events remains to be shown. Diaz and collaborators have provided a temporal pattern of ALK4/5/7-dependent C-terminal SSxS phosphorylation of SMAD2 in granulosa and cumulus cells during important steps of folliculogenesis. They demonstrated that SMAD2 phosphorylation was stable in granulosa cells during folliculogenesis, but that it was decreased 8 hours post-hCG, especially in cumulus cells (Diaz et al., 2007). Whether this decrease is due to a reduced capacity of the maturing oocyte to secrete TGF $\beta$ -like factors or to a lack of sensitivity of cumulus cells to those factors is unclear. Interestingly, it was shown previously that mouse oocytes gradually lose their mitogenic potential with hCG-stimulated nuclear maturation progression (Gilchrist et al., 2001). However, our current study opens up the possibility that granulosa and/or cumulus cells might regulate their responsiveness throughout folliculogenesis to oocyte factors such as GDF9 by modulating the basal or autocrine level of EGF receptor activity and resulting ERK1/2 phosphorylation. In support of the latter concept, Prochazka and collaborators (Prochazka et al., 2003) have shown that with progressive differentiation of pig cumulus cells through antral folliculogenesis, there is functional maturation of the EGFR. SFKs might play a key role in that context since it has been shown to act both upstream

and downstream of EGFR and to participate in its signal transduction (Bobe et al., 2003; Schauwienold et al., 2008). Wayne and collaborators have shown that amphiregulin-stimulated ERK1/2 phosphorylation was not affected by inhibiting SFK in rat granulosa cells, which does not agree with our findings (Wayne et al., 2007). The reason for that is unknown, although it could in part be due to the different EGF ligands and different species used in the two studies. Nevertheless, our results show that SFKs play a part in GDF9–SMAD2/3 signaling.

SMAD proteins are intracellular transducers of the TGF $\beta$  superfamily members. Although the key event is the C-terminal SSxS phosphorylation of SMAD proteins by the type I receptor, phosphorylation of the linker region can affect, positively or negatively, their transcriptional activity (for a review, see Wrighton et al., 2009). The entire phosphorylation profile of the SMAD3 linker region was not fully elucidated in the present study, although we have previously done this and shown that other linker region phosphorylation sites follow a Ser208 phosphorylation pattern in other cell types (Matsuura et al., 2005). EGF stimulation or hyperactive RAS was shown to induce an ERK1/2-dependent SMAD2 and SMAD3 phosphorylation in the linker region (Kretzschmar et al., 1999; Matsuura et al., 2005). Mutations of these ERK1/2 target sites resulted in an increased SMAD3 activity, suggesting that these phosphorylation sites were inhibitory (Matsuura et al., 2005). However, several studies have reported that MEK–ERK1/2 pathway activity leads to SMAD2 phosphorylation and transcriptional activity (Blanchette et al., 2001; Funaba et al., 2002). Interestingly, granulosa cells from mice with an ovarian double inactivation of ERK1 and ERK2 do not luteinize and continue to proliferate up to 16 hours post-hCG (Fan et al., 2009b). In addition, premature activation of a constitutively active Kras causes granulosa cell cycle arrest along with decreased levels of cyclin D2 and cyclin A1, suggesting that ERK might actually inhibit proliferation in some contexts (Fan et al., 2009a). Although the complete mechanism for this regulation remains to be elucidated, it appears that basal and EGF-stimulated ERK1/2 might play different role in granulosa proliferation. The function of phosphorylation of the linker regions of SMAD2/3 have not previously been investigated in ovarian granulosa cells. This study suggests that SMAD3 linker phosphorylation by basal ERK1/2 activity is stimulatory in mouse granulosa cells.

In conclusion, this study has shown that the EGF receptor–ERK1/2 pathway enables GDF9–SMAD3 signaling in granulosa and cumulus cells, possibly via the maintenance of SMAD linker phosphorylation. These results demonstrate a novel pathway crosstalk that provides new insight into how granulosa and cumulus cells modulate their responsiveness to TGF $\beta$  superfamily growth factors throughout folliculogenesis, thereby underpinning crucial events required for the appropriate development of the oocyte and the follicle.

## Materials and Methods

### Chemicals and reagents

Unless otherwise noted, all chemicals were bought from Sigma Chemical Co. (St Louis, MO, USA). All pharmacological inhibitors were stored in aliquots at  $-20^{\circ}\text{C}$  until use. Recombinant mouse GDF9 was produced and partially purified from culture media of human embryonic kidney (HEK) 293H cells stably transfected with a GDF9 expression vector as previously described (Gilchrist et al., 2004; Hickey et al., 2005). Culture media from control 293H cells underwent a mock partial purification and a dose equivalent to GDF9 was tested in all experiments to exclude non-specific effects. Purified recombinant mouse EGF and the mature region of GDF9 were purchased from R&D Systems (Minneapolis, MN). In all experiments, unless otherwise stated, 'in-house'-produced GDF9 was used.



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

Gene	GenBank accession no.	Forward primer	Reverse primer	PCR size	Reverse primer dilution
<i>Gapdh</i>	NM_008084	AGG TGA CAC TAT AGA ATA ATA CGG CTA CAG CAA CAG GG	GTA CGA CTC ACT ATA GGG AGC CTC TCT TGC TCA GTG TCC	142	1:250
<i>Has2</i>	NM_008216	AGG TGA CAC TAT AGA ATA AGA GGG GAG AAC CAA GCA TT	GTA CGA CTC ACT ATA GGG ACT CAT AGA CTC GTC CTC CGC	219	1
<i>Ptx3</i>	NM_008987	AGG TGA CAC TAT AGA ATA GTG GGT GGA AAG GAG AAC AA	GTA CGA CTC ACT ATA GGG AGG CCA ATC TGT AGG AGT CCA	228	1
<i>Ppia</i>	NM_008907	AGG TGA CAC TAT AGA ATA AGG GTG GTG ACT TTA CAC GC	GTA CGA CTC ACT ATA GGG AAA AGA CCA CAT GCT TGC CAT	238	1:10
<i>Actb</i>	NM_007393	AGG TGA CAC TAT AGA ATA TCG TGC GTG ACA TCA AAG AGA AGC	GTA CGA CTC ACT ATA GGG AAC AGC ACT GTG TTG GCA TAG AGG T	325	1:50

All primers are given in the 5' to 3' orientation.

### Tissue recovery and culture

COCs and mural granulosa cells were recovered from 21- to 26-day old 129/SV mice, 44–46 hours after administration of 5 IU of equine chorionic gonadotrophin (eCG, 5 IU; Folligon, Intervet, Castle Hill, Australia). Human chorionic gonadotrophin (hCG, 5 IU; Organon, Sydney, Australia) was used where indicated. Mice were maintained in accordance with the Australian code of practice for care and use of animals for scientific purposes and with the approval of the Adelaide University Animal Ethics Committee. Briefly, after recovery, ovaries were rinsed in Hepes-buffered tissue culture medium-199 (H-TCM-199; MP Biomedicals, Ohio, USA) supplemented with 0.03% (w/v) polyvinyl alcohol and cimetidine (1  $\mu$ M). Ovarian follicles were punctured using 27-gauge needles and COCs collected using a flame-pulled borosilicate Pasteur pipette under  $\times 15$  magnification. Mural granulosa cells and COCs were cultured in bicarbonate-buffered tissue culture medium-199 (B-TCM-199; MP Biomedicals) containing 0.03% polyvinyl alcohol, 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin sulfate, unless stated otherwise. Pre-antral follicles were recovered from the ovaries of 12-day-old female 129/SV mice. Follicles were released from the ovary by dissecting with 27 gauge needles in digestion buffer [H-TCM-199 with 0.03% (w/v) polyvinyl alcohol, DNase 1 (0.02% w/v) and collagenase (0.1% w/v)]. Oocytectomized cumulus–oocyte complexes and preantral follicles were generated by the microsurgical removal of the oocyte from the complexes using a micromanipulation apparatus on an inverted microscope. Pre-antral follicles were cultured in B-TCM-199 as described above.

### Mural granulosa cell [<sup>3</sup>H]thymidine incorporation assay

This mitogen bioassay was performed as previously described (Gilchrist et al., 2001; Gilchrist et al., 2004; Gilchrist et al., 2006). In brief, mural granulosa cells were recovered as described above. Denuded oocytes were generated by vortexing COCs for 5 minutes. Mural granulosa cells and oocytes were washed in B-TCM-199 with supplements (Gilchrist et al., 2001). Assays were conducted with 25,000 granulosa cells per well in a 96-well plate (Falcon; Becton Dickinson, Franklin Lakes, NJ) in a final volume of 125  $\mu$ l. Cells were cultured in an atmosphere of 37°C, 96% humidity in 5% CO<sub>2</sub> in air for 18 hours, followed by a further 6-hour pulse of 15.4 kBq [<sup>3</sup>H]thymidine (MP Biomedicals) under the same conditions. Following culture, mural GCs were harvested, and the incorporated [<sup>3</sup>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). Each treatment was performed in duplicate and whole experiments were repeated at least three times.

### Mural granulosa cell phospho-SMAD3 reporter assay

A luciferase reporter construct responsive specifically to phosphorylated SMAD3 was used to detect the activation of mural granulosa SMAD3 proteins by oocytes or by recombinant forms of TGF $\beta$  superfamily members (Gilchrist et al., 2006). Mural GCs were collected and processed as described above, except that cells were washed twice in bicarbonate-buffered Dulbecco's modified Eagle's medium [B-DMEM (MP Biomedicals) with 2% (v/v) fetal calf serum (FCS; Invitrogen Australia Pty Ltd, Mount Waverley, Australia)]. Granulosa cells (20,000 per well) were cultured in a 96-well plate (Falcon) in 250  $\mu$ l of B-DMEM + 2% FCS. After 4 hours in 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, the medium was aspirated and replaced with DMEM + 0.1% FCS. Cells were then treated with inhibitors 5 minutes before the addition of growth factors and cultured for a further 48 hours. Experiments were terminated by removing media from wells and freezing plates at  $-20^{\circ}$ C. Cells were ruptured by adding 100  $\mu$ l of lysis buffer (Promega, Madison, WI) to each well and plates were incubated at room temperature on a rocking platform for 20 minutes. 20  $\mu$ l of cell lysate was used for measurement of luciferase activity using a Galaxystar luminometer (GMB Labtechnologies, Offenburg, Germany).

### ERK1/2 immunodetection

Protein samples were prepared using hypotonic buffer containing protease inhibitors (Roche Diagnostics) followed by four freeze–thawing cycles. Protein samples were mixed with loading buffer containing 100 mM dithiothreitol and then loaded onto a 13.5% SDS–polyacrylamide gel for electrophoresis. Proteins were transferred to a Hybond-ECL membrane (GE Healthcare, Waukesha, WI) using a Mini Protean 3 Cell apparatus (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada). Membranes were blocked with 2% blocking reagent (supplied in an ECL Advance kit) diluted in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-Tw20). Membranes were incubated with the primary antibody, anti-phospho-ERK1/2 (Sigma, cat. no. M8159) diluted 1:10,000 at 4°C overnight, followed by incubation with a donkey anti-mouse peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) diluted 1:400,000. Total ERK1/2 was detected using an anti-ERK1/2 antibody (Sigma, cat. no. M5670, diluted 1:10,000), followed by a goat anti-rabbit peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) diluted 1:400,000. Binding was detected using the ECL Advance kit (GE Healthcare) and exposure to autoradiographic film (GE Healthcare).

### Phospho-Ser208 SMAD3 immunodetection

Protein samples were prepared as described above. Membranes were incubated with the primary antibody, anti-phospho-Ser208 SMAD3 (Matsuura et al., 2005) diluted 1:20,000 at 4°C overnight, followed by incubation with a goat anti-rabbit peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc.) diluted 1:200,000.  $\beta$ -actin was detected using an anti- $\beta$ -actin antibody (diluted 1:10,000), followed by a donkey anti-mouse goat anti-rabbit peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc.) diluted 1:400,000. Detection was performed as above. The phospho-Ser208 SMAD3 antibody does not cross react with the C-terminal SSxS motif on SMAD3, which are targeted by ALK4/5/7 (Matsuura et al., 2005).

### Quantitative PCR (QPCR)

QPCR primer design for the two candidate, three housekeeping and one internal control genes was performed using eXpress Designer software, part of the GenomeLab eXpress Profiler Suite from Beckman-Coulter (Fullerton, CA, USA). All gene PCR products were validated individually on mouse cumulus cells cDNA. A primer pair was considered valid if only one PCR product of less than one nucleotide differed from its predicted size after being run on the GenomeLab GeXP Genetic Analysis System (Beckman-Coulter). The list of genes and the primer pairs are given in Table 1. To account for the different scale of expression of each gene, the proportion of each reverse primer in the multiplex reverse transcription reaction was adjusted to obtain similar peak signals for each gene. The dilution factor of each reverse primer used in the QPCR is noted in Table 1. Reverse transcription was performed on 100 ng of RNA as a template according to the GenomeLab GeXP Genetic Analysis System protocol. QPCR amplification was done using a mix of the forward primers, and the resulting reactions were analyzed by capillary electrophoresis on the GenomeLab GeXP. The resulting data were analyzed using eXpress Analysis to calculate relative gene expression values, to verify data and to perform a first-pass data analysis.

### Statistical analysis

Statistical analyses were conducted using Prism 5.00 GraphPad for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was assessed by ANOVA followed by Tukey's multiple-comparison post-hoc tests to identify individual differences between means. The IC<sub>50</sub> for each inhibitor in Figs 2 and 6 were determined by fitting a non-linear regression of the results as a function of the log-transformed inhibitor doses. Probabilities of  $P < 0.05$  were considered statistically significant. All values are presented with their corresponding standard error of the mean (s.e.m.).

Support and laboratory infrastructure provided by Jeremy G. Thompson and the Robinson Institute are gratefully acknowledged. We thank Wayne Tilley for use of the luminometer. M.S. is supported by a Natural Science and Engineering Research Council of Canada (NSERC) post-doctoral fellowship. R.B.G. and D.L.R. are supported by the National Health and Medical Research Council of Australia with R. D. Wright Fellowships and through Project Grant no. 519235.

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