Extra-ovarian expression and activity of growth differentiation factor 9

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Abstract

Growth differentiation factor 9 (GDF9) produced within the ovary plays an essential role during follicle maturation through actions on granulosa cells, but extra-ovarian expression, signalling and actions of GDF9 are less well characterised. The present studies confirm GDF9 expression in the mouse testis, pituitary gland and adrenocortical cancer (AC) cells, and establish its expression in L β T2 gonadotrophs, and in mouse adrenal glands, particularly foetal and neonatal cortical cells. AC, L β T2, TM3 Leydig and TM4 Sertoli cells express the requisite GDF9 binding signalling components, particularly activin receptor-like kinase (ALK) 5 and the bone morphogenetic protein (BMP)/GDF type II receptor, BMPRII (BMPR2). We therefore compared GDF9 activation of these potential extra-ovarian target cell types with its activation of granulosa cells. Recombinant mouse GDF9

stimulated expression of activin/transforming growth factor- β -responsive reporters, pGRAS-luc or pAR3-lux, in TM4 and AC cells (IC₅₀ = 145 ng/ml in the latter case), and two granulosa cell lines, KGN and COV434. The ALK4/5/7 inhibitor, SB431542, blocked GDF9 activity in each case. By contrast, GDF9 lacked specific effects on TM3 cells and rat primary pituitary and mouse L β T2 gonadotrophs. Our findings show that GDF9 regulates the expression of R-SMAD2/3-responsive reporter genes through ALK4, 5 or 7 in extra-ovarian (adrenocortical and Sertoli) cells with similar potency and signalling pathway to its actions on granulosa cells, but suggest that expression of BMPRII, ALK5 (TGFR1) and R-SMADs 2 and 3 may not be sufficient for a cell to respond to GDF9.

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Introduction

The transforming growth factor- β (TGF- β or TGFB), superfamily of growth and differentiation factors are dimeric proteins that display a wide range of context-dependent local actions within diverse tissues (Massague 1998, Chang *et al.* 2002). Agonists of this superfamily generally signal by bringing distinct combinations of type I and type II serine/threonine kinase receptors together to form heteromeric complexes (Massague 1998, Piek *et al.* 1999). Amongst the type II receptors, ActRII and ActRIIB (listed as ACVR2B in MGI Database) mediate signals for activins and some bone morphogenetic proteins (BMPs), and BMPRII (listed as BMPR2 in MGI Database) mediates signals for most BMP and growth/differentiation factor (GDF) isoforms, whereas T β RII (listed as TGFR2 in MGI Database) is exclusively used by TGF- β isoforms (Massague 1998, Piek *et al.* 1999, de Caestecker 2004). Each agonist ligand promotes the pairing of its preferred type II receptor(s) with matching subsets of type I activin receptor-like kinase (ALK) receptors (listed as ACVR

in MGI Database), leading to downstream phosphorylation of pathway-specific intracellular receptor-activated (R-) SMADs. Signals from ALK1, 2, 3 and 6 are transduced by R-SMADs 1, 5 and 8, whereas those from ALK4, 5 and 7 are transduced by R-SMADs 2 and 3.

GDF9 is a TGF- β superfamily agonist noted for its high and selective expression by oocytes within the ovary (McGrath *et al.* 1995), where its signals to surrounding cumulus granulosa cells are required for folliculogenesis to progress beyond the primary follicle stage (Gilchrist & Thompson 2007). In the GDF9 null mouse, granulosa cells stop proliferating but cannot undergo apoptosis, and display an altered differentiation pattern (Dong *et al.* 1996, Elvin *et al.* 1999, Hanrahan *et al.* 2004). *Gdf9* mRNA expression has also been identified in extra-ovarian sites, including the pituitary, hypothalamus, placenta, testis and adrenal, and in mouse adrenocortical cancer (AC) and Leydig (I-10) cell lines (Fitzpatrick *et al.* 1998, Pennetier *et al.* 2004, Faure *et al.* 2005, Farnworth *et al.* 2006b, Lee *et al.* 2007a), but studies of GDF9 actions have focused almost exclusively on the ovary.

By manipulating the cellular expression or interaction of various receptor pairs, Hsueh *et al.* deduced that GDF9 signals through an unusual combination of BMPRII and ALK5, the classical type I receptor for TGF- β , to R-SMADs 2 and 3 (Vitt *et al.* 2002, Mazerbourg *et al.* 2004), and findings in the physiological context of granulosa cells are consistent with this suggestion (Kaivo-Oja *et al.* 2003, Roh *et al.* 2003, Gilchrist *et al.* 2006). This pathway is atypical for the BMP/GDF family of ligands, nearly all of which signal via R-SMADs1/5/8 (de Caestecker 2004). We previously found that AC cells, L β T2 gonadotrophs, TM3 Leydig cells and TM4 Sertoli cells express mRNA encoding the requisite binding/signalling proteins for GDF9 (Farnworth *et al.* 2007). Taken together, these findings raised the possibility that GDF9 acts locally in the adrenal cortex, pituitary gland and testis. AC and L β T2 cells remarkably express ALK5 in the absence of T β RII, potentially making them ideal models for dissecting GDF9 actions without interference from endogenous TGF β (Ethier *et al.* 2002, Farnworth *et al.* 2007).

In the present study, we have confirmed and extended the identification of extra-ovarian tissues and cell types that express GDF9, examined the ontogeny of GDF9 expression in the adrenal, and compared GDF9 activation of extra-ovarian cell types that express the identified GDF9 binding/signalling species with its activation of human granulosa KGN and COV434 cells as examples of established GDF9 target cells. The results support the proposal that GDF9 signals via BMPRII, ALK5 and R-SMADs2/3 to activate AC and TM4 Sertoli cells. However, contrary to predictions, gonadotrophs and TM3 Leydig cells did not respond specifically to GDF9 for several measured endpoints.

Materials and Methods

Materials

The mouse TM3 and TM4 (Mather 1982), L β T2 (Pernasetti *et al.* 2001), AC (Rilianawati *et al.* 1998, Rahman *et al.* 2001, Farnworth *et al.* 2006b), COV434 (Zhang *et al.* 2000) and KGN (Nishi *et al.* 2001) cell lines were obtained as previously described (Farnworth *et al.* 2006b, 2007, Chand *et al.* 2007). Rats and mice were obtained from Central Animal House, Monash University (Clayton, Victoria, Australia). Adult mice and male rats were maintained under standard conditions, with free access to food and water, whereas foetal and neonatal mice were killed by cervical dislocation on the day they were obtained. The studies were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and procedures were approved by the Monash Medical Centre Animal Experimentation Ethics Committee. Purified recombinant human inhibin A (31 kDa monoglycosylated form) and inhibin B (mixture of 31 and 34 kDa forms) were isolated as previously described, and both preparations were well characterised with respect to potency in the classical rat

pituitary cell culture bioassay (Makanji *et al.* 2007). Isoforms of recombinant human activin (A, B, AB) and BMP(-6, -7) were obtained from R&D Systems (Minneapolis, MN, USA), TGF- β isoforms (1 and 2) were obtained from PeproTech Inc. (Rocky Hill, NJ, USA). A partially purified preparation of recombinant mouse GDF9 that was generated from a transfected human embryonic kidney 293H cell line, and a matching conditioned medium preparation lacking GDF9 (293H control), were prepared as previously described (Gilchrist *et al.* 2004, 2006, Hickey *et al.* 2005). The bioactivity of the mouse GDF9 preparation has previously been established in rat granulosa cell proliferation and reporter expression assays (Gilchrist *et al.* 2004, 2006). The concentrations of mouse GDF9 were determined by immunoanalysis using a rat GDF9 preparation as the standard (Gilchrist *et al.* 2004). A GDF9 immunoactivity of 50 ng/ml approximately corresponds to 2 nM protein dimer. SB431542, a synthetic inhibitor of ALK 4, 5 and 7 (Inman *et al.* 2002), was from Tocris Cookson Ltd (Northpoint, Fourth Way, Avonmouth, UK). Reagents and materials for RNA extraction and real-time RT-PCR amplification were as previously described (Farnworth *et al.* 2006b). Primers for gene amplification were made commercially (Sigma-Genosys). Goat anti-mouse GDF9 IgG (cat. no. NR-01-0095; a/b#1) was from RayBiotech, Inc. (Norcross, GA, USA), as was mouse anti-human BMP-15 IgG (cat. no. NR-01-0018; a/b#2). Rabbit anti-human GDF9 IgG (raised against N-terminal peptide sequence; cat. no. ab38544; a/b#3) was from Abcam plc (Cambridge, UK), and goat anti-human GDF9 (C-18) IgG (raised against C-terminal peptide sequence; cat. no. sc-12244; a/b#4) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The 3 $\times$ GRAS-PRL-lux (pGRAS-luc) reporter construct (Ellsworth *et al.* 2003) was kindly provided by Dr Buffy S Ellsworth (Colorado State University, Fort Collins, CO, USA). This and additional luciferase reporter constructs and co-factors (pAR3-lux, containing an activin and TGF- β response element from the *Xenopus Mix.2* gene (Hayashi *et al.* 1997); FAST2, required for pAR3-lux expression in many cell types) were employed as previously described (Ethier *et al.* 2002, Gilchrist *et al.* 2006, Chand *et al.* 2007).

Culture and treatment of cell lines and rat primary pituitary and adrenal cells

Primary cultures of adult (60–90-days old) male rat anterior pituitary and adrenal cells were prepared and maintained as previously described (Farnworth *et al.* 1988, 2006b). Primary cultures and all cell lines were cultured in a 1:1 (vol:vol) mixture of DMEM:F12 media buffered with sodium bicarbonate, and containing non-essential amino acids for MEM, and supplementary glutamine (2 mmol/l final). Medium was supplemented with 10% FBS for passaging and pre-incubations and, for AC cells, also included HEPES buffer (10 mmol/l). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air.

Following plating, cells were pre-incubated for 1 day in FBS-containing medium, then culture medium was changed to a chemically defined medium containing antibiotics, and 10% artificial serum (AS) that includes insulin, transferrin and BSA, as previously described (Farnworth *et al.* 2006b, 2007). Unless otherwise indicated, treatments or matching vehicle(s) were applied to cells overnight under standard culture conditions in this chemically defined AS-containing medium prior to the analyses. Activin, BMP, TGF- β and inhibin isoforms and GDF9 were tested at single concentrations that were maximally active in other assays in our laboratory (≥ 2 , ≥ 2 , 0.4 and 0.05 nM, and 50 ng/ml respectively; Farnworth *et al.* 2006a, 2007, Gilchrist *et al.* 2006), unless otherwise indicated.

Immunoassay of rat and mouse FSH

After a 3-day treatment period, the conditioned media samples from rat primary pituitary cell cultures were collected, the cells were lysed, and both medium and lysate samples were subsequently assayed for FSH by RIA as previously described (Mason *et al.* 1996), using goat secondary antibody precipitation with the following reagents, kindly provided by the NIDDK: rat FSH-RP-2 (Lot #AFP-4621B) as standard; rat FSH-I-8 (Lot #AFP-11454B) for preparing the tracer, which was iodinated using iodogen, and rabbit anti-rat FSH-S-11 (Lot #AFP-C0972881) as the primary antiserum. The mouse FSH levels in L β T2 cell-conditioned media samples were measured by ELISA as previously described (Farnworth *et al.* 2006b, Mankanji *et al.* 2007).

mRNA purification and real-time PCR analyses

Total RNA extracted from whole dissected pituitary and adrenal glands (or pools of glands in the cases of foetal and neonatal mouse adrenal), a portion of dissected testis or cell monolayers (2×10^6 cells/5 ml medium/well in 6-well (3.5 cm diameter) cluster dishes) using UltraSpec RNA reagent was purified using standard procedures, and cDNAs were synthesised from 2 μ g RNA by oligo(dT) priming using 50 U of Expand Reverse Transcriptase as previously described (Farnworth *et al.* 2006a, 2007). In the foetal and neonatal mouse adrenal samples, 0.6 μ g RNA was used. Primers for amplification of specific cDNA products were as follows: mouse GDF9 (forward primer, 5'-AACCCAGCA-GAAGTCACCTC -3'; reverse primer, 5'-AGGGGCT-GAAGGAGGGAGG-3'), yielding a 337 bp product; rat GDF9 (forward primer, 5'-GGCTCCCAGCAACCAGA-TGACA-3'; reverse primer, 5'-TGGCGCTCTTG-GGGTAGCCTTG-3'), yielding a 208 bp product. Primers for amplification of mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as a housekeeping gene were as previously described (Drummond *et al.* 2000, Farnworth *et al.* 2006b, 2007).

Real-time PCR amplification assays were performed in a Rotor-Gene RG-3000 (Corbett Research, Mortlake, NSW,

Australia; Farnworth *et al.* 2007). The amplification and assessment run for mouse (or rat) GDF9 consisted of: 1) denaturation at 95 °C for 8 (6) min to activate the Taq polymerase, 2) amplification and quantification for 40 cycles of 15 s at 95 °C for denaturation, 5 s at 62 (69) °C for annealing, 17 (10) s at 72 °C for extension, and a single fluorescence measurement at 72 °C for quantitation, 3) melting curve assessment between 57 and 95 °C at a temperature transition rate of 0.2 °C per s with a continuous fluorescence measurement, and 4) cooling to 40 °C. Under these conditions, a GDF9 transcript of the expected size was amplified in each case. Procedures for assay validation, and qualitative assessment of real-time RT-PCR products have been previously described (Farnworth *et al.* 2006b, 2007).

Immunohistochemical analyses of GDF9 expression in mouse tissues

Ovary (adult), pituitary gland (adult, both male and female) and adrenal glands from mice of various ages (foetal (14.5 and 16.5 days post-coitum), neonatal, 15-days old and adult) were excised, immersion-fixed in Bouin's solution and processed for light microscopy as described elsewhere (Nicholls *et al.* 2009). The primary antibody preparation was goat anti-human GDF9 (C-18) IgG from Santa Cruz Biotechnology, Inc. (see above for details) used at a final concentration of 0.20 μ g/ml. Specificity of staining was examined by substituting the primary antibody with an equivalent dilution of pre-immune goat immunoaffinity-purified IgG, and by co-incubation of sections with primary antibody and a GDF9 blocking peptide, as previously described (Nicholls *et al.* 2009).

Transient transfections and assay of luciferase activity

Each cell type was transiently transfected with several activin/TGF- β -responsive reporter constructs to examine its responsiveness to activin A and/or TGF- β as positive controls. After determining the optimal reporter for each cell line, AC, TM3, L β T2 and KGN cells were routinely transfected with the pGRAS-luc reporter construct (800 ng DNA per well), and TM4 and COV434 cells were transfected with a mixture of pAR3-luc reporter (700 ng DNA per well) and FAST2 transcription co-factor (100 ng DNA per well) using Lipofectamine 2000. Cultures of KGN cells (90% confluent in 75 cm² flasks) were transfected with liposomes generated between 8 μ g reporter construct DNA and 20 μ l Lipofectamine in a total volume of 2 ml serum- and antibiotic-free medium overnight. In all other cases, cells in suspension (0.15 – 0.3×10^6 cells/620 μ l FBS-containing medium per well) were mixed with the liposome solution at a DNA:lipofectamine ratio of 1:4 or 1:5 (mass:volume) at 80 μ l per well, then seeded in 24-well plates and incubated for 24 h. The transfection medium was subsequently replaced with fresh medium containing 10% FBS and antibiotics, and incubated overnight. Transfected cultures were then

incubated for 6 h in medium containing 10% AS and antibiotics, with or without treatments (see table and figure legends for details). Preparation of cell lysates and assay of luciferase levels were performed as previously described (Ethier *et al.* 2002).

Statistical analyses

Concentration–response curves were fitted, maximum/minimum effects and median effective/inhibitory concentrations (EC50/IC50) were determined by non-linear regression, and statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., CA, USA). Treatment effects on luciferase levels were replicated in separate experiments (see table and figure legends for details). After establishing homogeneity of variance across the dataset for each endpoint, mean data were compared using one-way ANOVA followed by post-hoc Neuman–Keuls Multiple Comparison Test, or unpaired Student's *t*-test where appropriate, and $P < 0.05$ was considered significant.

Results

Pituitary, adrenal and gonadal expression of *GDF9* and its signalling components

Gdf9 mRNA was detected by PCR in rat ovary (as the positive control) and rat primary pituitary cells in culture (Fig. 1A), confirmed by sequencing of the predicted 208 bp product. Assays with primers specific for mouse *Gdf9* mRNA showed expression in adult mouse ovary (o), testis (t) and, to a minor extent, pituitary (p; Fig. 1B, lanes 2–4 respectively), confirmed by sequencing of the predicted 337 bp product. *Gdf9* mRNA was also detected in pooled adrenal glands obtained from female mouse foetuses at 14.5 days post-coitum (dpc; a_f , Fig. 1B, lane 5), and adrenal tissue from neonatal and juvenile (5-day old) female mice (a_n and a_j respectively; Fig. 1B, lanes 6 and 7), but was on the threshold of detection in adult female (a_{af}) or male (a_{ad}) mouse adrenal (Fig. 1B, lanes 8 and 9 respectively). GAPDH cDNA was nevertheless amplified from samples that were negative for *Gdf9* (lower panel of Fig. 1B). *Gdf9* mRNA was readily detected in the L β T2 gonadotroph and AC cell lines, whereas expression by TM3 Leydig and TM4 Sertoli cells was minor (Fig. 1C). No product was obtained from any of these samples after the 40 cycles of amplification in the absence of reverse transcriptase (e.g. Fig. 1C). TM3, TM4, AC and L β T2 cell lines all express the mRNA species that encode BMPRII, ALK5 and SMADs 2 and 3, the previously identified signalling components for GDF9, along with the requisite signalling components for activin and BMP (Farnworth *et al.* 2007).

A commercially available affinity purified IgG directed against C-terminal peptide GDF9 (a/b #4) was used to examine the expression of GDF9 protein in the mouse pituitary and adrenal glands, with the adult mouse ovary as a

Figure 1 Presence of mRNA encoding GDF9 in rat and mouse tissues and cells. (A) Rat GDF9 products (208 bp) were obtained by RT-PCR from total RNA of whole ovary (o) and a primary pituitary cell culture (p) from adult rats. (B) RT-PCR primers for mouse GDF9 yielded products (337 bp; upper panel) from the purified mouse GDF9 cDNA standard derived from ovarian RNA (St, lane 1), and from total RNA extracted from adult mouse ovary (o, lane 2), testis (t, lane 3) and pituitary gland (p, lane 4), adrenal glands obtained from foetal (day 14.5 post-coitum; a_f , lane 5), neonatal (day 0; a_n , lane 6), juvenile (day 5; a_j , lane 7) and adult female (a_{af} , lane 8) mice, and the adult male mouse (a_{ad} , lane 9). Primers for mouse GAPDH yielded products (560 bp; lower panel) from all of these tissues. (C) RT-PCR primers for mRNA species encoding mouse GDF9 yielded 337 bp products from mouse Leydig TM3, Sertoli TM4, L β T2 gonadotroph, and adrenocortical cancer (AC) cells. +RT and –RT signify amplification yields after the inclusion and omission respectively, of reverse transcriptase. Molecular weight markers (Mr) were run in the first lane of each ethidium bromide-stained 1.7% agarose gel.

positive control. Low magnification photomicrographs of ovary sections show intense staining of oocytes within pre/antral follicles (Fig. 2A), and this was abolished by co-incubation with competitor peptide (Fig. 2B). In the adult male anterior pituitary, high magnification photomicrographs show strong staining of clusters and larger aggregates of cells in sections in which approximately two-thirds of the cells were positive for GDF9 (Fig. 2C). This staining was cytoplasmic, mainly punctate or granular in distribution (Fig. 2C), and was greatly attenuated by the competitor peptide (Fig. 2D, in which the inset additionally shows the lack of staining with a non-specific IgG applied at a matching concentration). Figure 2E shows a section of adult female pituitary with strong cytoplasmic staining within the majority of the anterior pituitary lobe, similar to the picture in the male, while the neurointermediate lobe shows more diffuse staining. Staining in both lobes of the pituitary was attenuated by the blocking peptide (e.g. Fig. 2F).

At 14.5 dpc, the foetal male adrenal was mainly composed of cells that showed strong cytoplasmic staining for GDF9, interspersed with unstained cells (Fig. 2G), and the staining was reduced by the GDF9 competitor peptide (Fig. 2H). Primordial cortical and medullary zones were evident in the male adrenal gland at 16.5 dpc: medullary cells were unstained, whereas intense cytoplasmic staining was present in cortical cells, especially those adjacent to the medulla

Figure 2 Immunohistochemical localisation of GDF9 in mouse tissues. (A) Adult ovary (magnification $\times 20$) as positive control shows intense cytoplasmic staining of oocytes (o) within preantral/antral follicles. (B) Such staining was blocked by inclusion of a GDF9 peptide competitor. (C) Anterior lobe (AL) of adult male pituitary gland ($\times 100$) shows dark cytoplasmic staining of at least half the cells, most of which are in clusters between unstained cells. (D) Application of the competitor peptide blocked staining in the anterior lobe. The inset to panel D shows the lack of staining when the primary antibody was replaced by pre-immune goat serum IgG. (E) A section of adult female pituitary ($\times 40$) shows intense cytoplasmic GDF9 staining of many anterior lobe cells, and more diffuse staining of cells within the neurointermediate lobe (NIL). (F) Staining of cells in both lobes was greatly reduced by the competitor peptide ($\times 40$) and pre-immune IgG provided no staining (inset to panel F). (G) The foetal male adrenal gland at 14.5 days post-coitum ($\times 100$) included unstained cells and cells with strong cytoplasmic staining for GDF9. (H) Such staining was attenuated by the competitor peptide. (I) By 16.5 days post-coitum, the adrenal ($\times 40$) showed segregation of unstained medullary zone (m) cells from GDF9-positive cortical cells (c), with strongest staining in the juxtamedullary cells of the X-zone. (J) Staining of these cortical cells was reduced by competitor peptide. (K) Cortical cells in the 15 days postnatal female adrenal gland ($\times 100$) showed staining for GDF9, in contrast to the medulla. (L) The competitor peptide displaced some cortical staining. The inset to panel L ($\times 40$) shows absence of staining of cortical and medullary adrenal cells in the absence of the primary antibody. The bar in each panel represents either 200 μm (A,B,E,F,I,J) or 50 μm , as indicated. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-08-0563>.

(Fig. 2I), and that staining was reduced by the competitor (Fig. 2J). Adrenocortical cells of female adrenal glands at postnatal day 15 showed a small amount of specific staining for GDF9, but the medullary cells were again negative (compare Fig. 2, panels K and L), and a similar pattern was observed in the adult adrenal glands from both sexes (data not shown).

Comparison of luciferase reporter responses to GDF9 in granulosa and extra-ovarian cell types

Human COV434 granulosa cells were transiently transfected with the pAR3-lux reporter, and KGN granulosa cells were transiently transfected with pGRAS-luc to validate the

responses of these reporters to GDF9 in its established target cell type. In COV434 granulosa cells, luciferase expression was significantly stimulated 3.4- and 2.4-fold by treatment for 6 h with activin A (2 nmol/l) and GDF9 (50 ng/ml) respectively, whereas the GDF9 control preparation derived from 293H cell-conditioned medium (293H control) was without significant effect (Table 1). A similar pattern was observed in KGN cells (Table 1).

GDF9 significantly increased pAR3-lux expression in TM4 cells by 2.5-fold, similar to stimulation elicited by activin A (3.3-fold increase), and by activins B and AB (average increases in 3.0- and 3.8-fold respectively; Table 1). The pattern of responses mimicked that seen in the granulosa

Table 1 Effects of transforming growth factor (TGF)-β superfamily members on the level of pAR3-lux or pGRAS-luc expression in granulosa and extra-ovarian cell types. Cells that had been transiently transfected with either reporter were treated 1 day later with medium alone (control), growth/differentiation factor (GDF)-9, its conditioned medium control (293H), or the indicated isoforms of activin or transforming growth factor (TGF)-β for 6 h, after which luciferase levels were determined in duplicate for replicate wells. Results (fold matching untreated control) from (n) replicate experiments are mean ± S.E.M., except where indicated

Reporter	pAR3-lux		pGRAS-luc			
Cell line	COV434	TM4	KGN	LβT2	AC	TM3
Treatment						
Control	1.00	1.00	1.00	1.00	1.00	1.00
293H	0.95 ± 0.05 (3)	1.16 ± 0.07 (3)	1.05 (2)	0.94 ± 0.03 (3)	1.08 ± 0.02 (3)	1.4 (2)
GDF9 (50 ng/ml)	2.4 ± 0.2*† (3)	2.5 ± 0.2* (3)	1.9 (2)	1.09 ± 0.07 (3)	15.9 ± 1.5*† (3)	1.3 (2)
Activin A (2 nM)	3.4 ± 0.5* (3)	3.3 ± 0.1* (4)	3.2 ± 0.4* (5)	1.7 ± 0.4 (3)	15.5 ± 2.6* (6)	4.8 ± 0.7* (4)
Activin B (2 nM)	ND	3.0 (2)	1.4 (1)	1.3 (1)	13.9 ± 0.4* (3)	2.2 ± 0.6 (3)
Activin AB (2 nM)	ND	3.8 (2)	4.1 (1)	1.3 (1)	21.6 ± 5.7* (3)	4.0 ± 0.8 (3)
TGF-β1 (0.4 nM)	ND	11.8 ± 2.8* (3)	6.7 (1)	ND	1.15 (2)	8.8 ± 1.9* (4)
TGF-β2 (0.4 nM)	ND	12 (2)	ND	ND	1.1 (1)	7.6 (2)

ND, not determined. *†Signify results that significantly differ from the untreated control group (=1), and the matching 293H control treatment group, respectively (P<0.05).

cell lines (Table 1). Both TM4 and KGN cells also displayed robust responses to TGF-β (Table 1). In contrast to the granulosa and Sertoli cell models, LβT2 gonadotrophs responded poorly to GDF9, although activin A significantly stimulated pGRAS-luc expression (Table 1).

AC cells transfected with pGRAS-luc were tested for responses to 6 h treatment with TGF-β superfamily agonists. GDF9 increased luciferase expression 16-fold above that in the untreated control, showing similar activity to activins A, B and AB (16-, 14- and 22-fold increases respectively (Table 1), whereas the 293H control sample, BMP-6 (2 nmol/l) and BMP-7 (4 nmol/l; data not shown) had no significant effects. These results for AC cells confirmed the specificity of pGRAS-luc responses to activators of R-SMADs 2 and 3. Note that the failure of AC cells to respond to R-SMAD 2/3-activating TGF-β isoforms (each 0.4 nmol/l; Table 1) reflects their lack of TβRII expression (Farnworth *et al.* 2007), and provides additional negative controls in this experiment.

GDF9 did not elicit any greater response than the 293H control in TM3 cells (Table 1), indicating that there was no specific response to the agonist. Activins A, B and AB nevertheless increased TM3 cell expression of pGRAS-luc by 2.2- to 4.8-fold, and TGF-β isoforms increased expression of the reporter by almost 10-fold (Table 1). The latter results provided evidence of functional ALK5 receptors in TM3 cells, and indicated their capacity to respond to R-SMAD 2/3-activating ligands other than GDF9.

GDF9 potency, specificity and signalling pathway in extra-ovarian cell types

GDF9 stimulated pGRAS-luc expression in AC cells in a concentration-dependent manner (EC50 = 145 ± 42 ng/ml; mean ± S.E.M., n = 3; Fig. 3A), similar to the pattern seen for stimulation of CAGA-luc expression by the same GDF9

preparation in transiently transfected mouse granulosa cells (EC50 = 80 ng/ml by analysis of data from (Gilchrist *et al.* 2006); computed curve shown for comparison in Fig. 3A). Matching high concentrations of the 293H control lacked effect in the pGRAS-luc assay (Fig. 3A).

The affinity purified IgG (4 µg/ml) directed against recombinant mouse GDF9 abolished the stimulation of pGRAS-luc expression by the mouse GDF9 preparation (used at 25 ng/ml, to clearly show the dependence of immunoneutralisation on antibody concentration) in AC cells (Fig. 3B, a/b #1). At 4 µg/ml, the antibody was equally effective against 50 ng/ml GDF9 (data not shown). However, this antibody did not significantly affect luciferase expression that was induced by activin isoforms (each 1 nmol/l) under similar conditions (Fig. 3C). An affinity purified polyclonal antibody raised against a human GDF9 N-terminal sequence showed only minor immunoneutralising activity (a/b #3 in Fig. 3C), whereas the antibodies raised against a C-terminal sequence of human GDF9 (data not shown) and recombinant human BMP-15 (a/b #2 in Fig. 3B) lacked immunoneutralising activity. Thus, the actions of the partially purified GDF9 preparation were fully accounted for by the specified ligand.

The stimulation of pGRAS-luc expression by GDF9 and activin in AC cells was abolished by pre- and concurrent treatment of the cells with the ALK4/5/7 inhibitor, SB431542 (20 µM; Fig. 4A), consistent with the proposed signalling pathways for these agonists. Neither the 293H control (Fig. 4A) nor BMP-6 alone (data not shown) significantly stimulated luciferase expression, and dimethyl sulfoxide, the vehicle for SB431542, added at a matching concentration did not modify any of the responses (Fig. 4A). GDF9 and activin isoform induction of luciferase reporter expression in TM4 Sertoli cells were likewise antagonised by SB431542 (Fig. 4B), as were reporter responses to these agonists in COV434 and KGN granulosa cells (data not shown).

Figure 3 Characterisation of pGRAS-luc reporter responses to GDF9 in AC cells. (A) To determine the concentration dependence of GDF9 action, AC cell cultures that had been transiently transfected with pGRAS-luc, and maintained overnight in medium containing 10% FBS, were subsequently treated for 6 h with logarithmically graded concentrations of GDF9 or matching dilutions of the 293H control in 0.25 ml medium containing 10% AS. Data at each point for the pGRAS-luc curve represent the average result $\pm$ s.d. obtained from replicate amplifications of single samples obtained in one experiment, to conserve stocks of GDF9. A similar pattern was obtained in a second experiment. These data for GDF9 induction of pGRAS-luc expression in AC cells were compared with similar data for the induction of CAGA-luc expression by GDF9 in transiently transfected mouse mural granulosa (gr.) cells (previously published data from Figure 6 of (Gilchrist *et al.* 2006)) during 48 h. For each sigmoidal curve, the luciferase response data have been normalised to the matching maximum level of expression, estimated by nonlinear regression analysis using GraphPad Prism software. This panel also shows the lack of effect by matching dilutions of the 293H control in the AC cells. (B) To immunoneutralise GDF9 activity, GDF9 (50 ng/ml) or a matching dilution of its 293H control preparation was mixed 1:1

Lack of GDF9 effects on FSH secretion by rat and mouse gonadotrophs

Although mouse L β T2 gonadotroph expression of pGRAS-luc was not specifically induced by GDF9 (see above), it was recently suggested (Safwat *et al.* 2005) that SMAD-independent signalling plays a significant part in activin induction of FSH production by the gonadotroph, which represents a more physiological endpoint. We therefore examined the potential for GDF9 to display either 1) autocrine actions on FSH synthesis and secretion by gonadotrophs in the manner of activin that is subject to antagonism by inhibin (Corrigan *et al.* 1991, Li *et al.* 1998) or 2) synergism with activin to stimulate FSH production in the manner of BMP (Lee *et al.* 2007b, Nicol *et al.* 2008). However, GDF9 (1–50 ng/ml) did not significantly increase FSH secretion (Fig. 5) or FSH cell content (data not shown) in gonadotroph-containing primary cultures of adult male rat anterior pituitary cells in either the absence or presence of FSH-suppressing concentrations of inhibin A or B (each 0.05 nmol/l) over a three-day period (Fig. 5A). GDF9 also did not block or enhance the twofold stimulation of FSH secretion by either activin A or B (each 0.5 nmol/l) in such cultures (Fig. 5B). Moreover, GDF9 alone at concentrations up to 50 ng/ml failed to modify FSH secretion or storage by L β T2 gonadotrophs in the presence of 0.2% FBS (data not shown). Progressive increases in FSH secretion from rat gonadotrophs that occurred in response to concentrations of GDF9 above 50 ng/ml

(vol:vol) with culture medium (control), increasing dilutions of a polyclonal antibody (a/b) preparation directed against a recombinant mouse GDF9 protein (a/b #1; 0.063–4 μ g/ml final concentration in culture medium), or a matching dilution of a polyclonal a/b preparation directed against a recombinant human BMP-15 protein (a/b #2; 4 μ g/ml final concentration), and mixtures were left standing for 60 min at 37 °C. Replicate cultures of AC cells were then treated overnight with the GDF9 (25 ng/ml final concentration; filled bars) or its 293H control (open bar), in the absence or presence of a/b, after which luciferase levels were determined in duplicate in triplicate samples for each treatment. Plotted data (average $\pm$ s.d.) were obtained after normalising results of treatments against the level of luciferase observed in untreated cultures (= 1, indicated by the broken horizontal line). Results labelled with a common letter (a, b or c) are not significantly different ($P > 0.05$). (C) To examine the specificity of action of the GDF9 immunoneutralising antibody, activins A, B and AB (each 2 nmol/l), GDF9 (50 ng/ml) or a matching dilution of its 293H control preparation was mixed 1:1 (vol:vol) with culture medium (control) (No antibody), open bars), GDF9 immunoneutralising antibody (a/b #1; 4 μ g/ml final concentration in culture medium; black bars), or a matching dilution of a polyclonal a/b preparation directed against the N-terminal sequence of human GDF9 (a/b #3; 4 μ g/ml final concentration; grey bars), and mixtures were left standing for 60 min at 37 °C. Replicate cultures of AC cells were then treated overnight with activin isoform (1 nmol/l final concentration), GDF9 (25 ng/ml final concentration) or its 293H control, in the absence or presence of a/b. Other details are as for B above. a signifies results that significantly differ from those obtained for the matching untreated (control) and 293H control-treated cells, respectively ($P < 0.01$). b signifies results that significantly differ from those obtained for the matching antibody-free treatment group ($P < 0.05$).

Figure 4 Antagonism of GDF9 activity by SB431542 in extra-ovarian cell types. (A) AC cells that had been transiently transfected with the reporter, pGRAS-luc, were stabilised overnight in medium containing 10% FBS, incubated for 24 h with no treatment, the ALK4/5/7 inhibitor, SB431542 (20 μ mol/l), or its vehicle, dimethyl sulfoxide (DMSO, 0.08%), in medium containing 10% AS, then duplicate cultures from each pre-treatment group were incubated a further 24 h under the same conditions in fresh medium with or without inhibitor/vehicle that additionally contained no agonist (control), activin B (2 nmol/l), GDF9 (50 ng/ml), or a matching dilution of its 293H control, after which luciferase levels were determined. Plotted data (average $\pm$ s.d.) were obtained after normalising results of treatments against the level of luciferase observed in untreated cultures (= 1, indicated by the broken horizontal line). (B) TM4 cells that had been transiently transfected with the reporter, pAR3-lux, were pre- and concurrently treated with SB431542 as above, and additionally treated with no agonist (control), activin A (2 nmol/l), GDF9 (50 ng/ml), or a matching dilution of its 293H control. Other details are as for A. In each panel, a, b and c signify results that significantly differ from those obtained for the matching medium-treated (no antagonist) or vehicle-treated (DMSO control) cells (a), ligand + DMSO-treated cells (b), and 293H + DMSO-treated cells (c), respectively ($P < 0.01$) in each case.

(log(GDF9) = 1.7) were also elicited by the 293H control at matching dilutions in these assays, indicating non-specific effects (Fig. 5C). These results confirmed the insensitivity of the gonadotroph to GDF9 stimulation.

Discussion

The best characterised site of GDF9 expression in mammals is the ovary where, depending on the species, this TGF- β superfamily member is principally or exclusively expressed by the oocyte (McGrath *et al.* 1995, Chang *et al.* 2002, Juengel &

McNatty 2005, Gilchrist & Thompson 2007). Secreted GDF9 provides essential inputs to the follicular granulosa cells, particularly the cumulus granulosa cells that envelop and support the oocyte (Dong *et al.* 1996, Hanrahan *et al.* 2004, Gilchrist & Thompson 2007, Su *et al.* 2008). Although GDF9 is sometimes referred to as an oocyte-specific factor, it is also expressed outside the ovary, most notably for the present studies in the testis (mouse, rat, cow and human), pituitary (sheep and human) and adrenal cells (mouse and human; Fitzpatrick *et al.* 1998, Pennetier *et al.* 2004, Faure *et al.* 2005, Farnworth *et al.* 2006b, Nicholls *et al.* 2009). Regulatory elements have been identified in the mouse GDF9 gene promoter that allow tissue-specific control of its expression (Yan *et al.* 2006). Whether GDF9 shows activity in extra-ovarian cell types had not been explored until recently (Nicholls *et al.* 2009). The present studies identify several cell types, but particularly L β T2 and AC cell lines that model gonadotroph and foetal adrenocortical cells respectively, as potential sources of GDF9. The AC, L β T2 gonadotroph, TM3 Leydig and TM4 Sertoli cell lines all express mRNA encoding the binding/signalling proteins thought necessary for conferring responsiveness to GDF9 (Farnworth *et al.* 2007). The cell types that are modelled by these cell line therefore represent potential targets of local (autocrine/paracrine) GDF9 action. In partial agreement with this proposal, GDF9 increases the expression of activin/TGF- β -responsive reporters in AC and TM4 cells. Surprisingly, GDF9 poorly activates the gonadotroph and Leydig cell types for the endpoints that were measured.

GDF9 signalling differs from that by other BMPRII-binding agonists, which activate R-SMADs 1, 5 and/or 8, because GDF9 instead recruits ALK5, and consequently activates R-SMADs 2 and/or 3 (Roh *et al.* 2003, Mazerbourg

Figure 5 Effects of GDF9 on secreted FSH levels in rat primary pituitary cell cultures. Cultures prepared from adult male rat anterior pituitary glands were treated with the indicated levels of recombinant mouse GDF9 for 3 d in serum-free medium containing 10% AS. FSH levels in the pituitary cell-conditioned media were measured by RIA. (A) GDF9 was applied in the absence (control, ■, dotted line) or presence of FSH-suppressing levels of inhibin A (∇ , broken line) or B ($\blacklozenge$, full line; each 0.05 nmol/l). (B) GDF9 was applied in the absence (control, ■, dotted line) or presence of FSH-stimulating levels of activin A (Δ , full line) or B ($\blacklozenge$, broken line; each 0.5 nmol/l). (C) The effects of GDF9 ($\blacksquare$, dotted line) were compared with those of matching dilutions of the 293H control ($\square$, full line). Results (ng FSH/ml medium) from triplicate wells are mean $\pm$ s.d. of data obtained by RIA, and curves were fitted by nonlinear regression using GraphPad Prism software.

& Hsueh 2006). GDF9 activation of R-SMAD 2/3-responsive reporters in AC and TM4 cells confirmed predictions that were based on the expression of mRNA encoding these R-SMADs, BMPRII and ALK5 by both cell lines (Farnworth *et al.* 2007). GDF9 activates pGRAS-luc reporter expression in AC cells with similar potency to its induction of CAGA-luc expression by mouse mural granulosa cells (Gilchrist *et al.* 2006). Responses of the latter reporter are selectively mediated by R-SMAD 3, but not R-SMAD 2, in combination with co-SMAD 4 (Dennler *et al.* 1998), whereas the pGRAS-luc responses to activin/TGF- β require FoxL2, a member of the forkhead family of DNA binding proteins, and AP-1, in addition to the SMADs (Ellsworth *et al.* 2003). On the other hand, responses to pAR3-lux reflect the activation of its three activin-responsive elements by a complex of phosphorylated R-SMAD 2 and a forkhead-containing DNA-binding protein (either FAST1 or FAST2; Hayashi *et al.* 1997). TM4 cells accordingly required co-transfection of pAR3-lux with FAST2 to give strong responses to agonists. It is notable that R-SMAD 3 acts as an inhibitor in this system (Labbe *et al.* 1998, Nagarajan *et al.* 1999). The ligand and R-SMAD specificities of the reporter responses, and ablation of GDF9 actions by SB431542, in AC and TM4 cells show that GDF9 stimulates these extra-ovarian cell types via ALK4, 5 or 7 activation of R-SMADs 2/3, as previously established using granulosa cells (Kaivo-Oja *et al.* 2003, Roh *et al.* 2003, Gilchrist *et al.* 2006) and confirmed with human granulosa cell lines in the present work. The observation that TGF- β isoforms elicit responses from the pAR3-lux reporter gene in TM4 cells provides independent evidence for their expression of functional ALK5 receptors, but similar evidence could not be obtained with TGF- β in AC cells because they lack T β RII. However, both cell lines also express ALK4, and AC cells express ALK7, so more work is required to completely define the extra-ovarian signalling pathway(s) used by GDF9.

The stimulation of pGRAS-luc expression by our partially purified mouse GDF9 preparation in AC cells is immunoneutralised by a commercially available GDF9 antibody, whereas other antisera are ineffective. Moreover, the active antibody does not suppress reporter responses to activin isoforms. Together with the data showing lack of action of the matched 293H control preparation, and the previous immunoneutralisation of this GDF9 preparation with a different antibody, mAB-GDF9-53 (Gilchrist *et al.* 2004), the current immunoneutralisation results confirm that the responses to the partially purified preparation of GDF9 in extra-ovarian cell types reflect specific actions of the hormone.

The demonstration by RT-PCR that AC cells express *Gdf9* mRNA confirms our previous observation using microarray analysis (Farnworth *et al.* 2006b). The AC cell line is believed to be derived from the X-zone cells of the foetal adrenal cortex, based in part on its expression of *Cyp17* mRNA and metabolism of progesterone substrate to 17 α -hydroxyprogesterone (Kananen *et al.* 1996, Farnworth *et al.* 2006b). It is therefore notable that foetal and neonatal mouse adrenal glands express *Gdf9* mRNA much more

abundantly than do adult male and female adrenal tissues. Immunohistochemical analysis of the ontogeny of GDF9 expression in the mouse adrenal gland established that expression is confined to cortical cells, especially those in the juxtamedullary X-zone of the foetal adrenal, and that the levels of specific staining for GDF9 expression in the adrenal cortex decline after birth. These results reinforce the foetal adrenocortical nature of the AC cell model. More importantly, they suggest that GDF9 may play a local role in the adrenal during foetal life, and hence the actions of GDF9 on AC cells as a model of the foetal adrenal are the subject of ongoing studies in our laboratory. Ontogeny of GDF9 expression in human extra-ovarian tissues has not been investigated. However, it is notable that low level GDF9 expression was found in a pool of human adrenal mRNA representing multiple individuals and covering a wide age range (Fitzpatrick *et al.* 1998).

Rat pituitary cells in culture and mouse pituitary glands were found to express mRNA encoding GDF9, confirming and extending the previously observed expression of *Gdf9* mRNA by the human and sheep pituitary (Fitzpatrick *et al.* 1998, Faure *et al.* 2005), but the pituitary cell type(s) responsible could not be identified in these circumstances. Our immunohistochemical studies showed that cells of particularly the anterior lobe, but also the neurointermediate lobe, of the mouse pituitary express GDF9 protein. With respect to the anterior lobe, our finding that L β T2 cells strongly express *Gdf9* mRNA suggests that gonadotrophs are a potential source of GDF9, which may act locally within the pituitary gland. However, gonadotrophs form a small subpopulation (below 20%) of cells within the anterior pituitary. They are therefore unlikely to be the only cell types that produce GDF9, since the immunohistochemical data revealed that more than 50% of cells within the mouse anterior pituitary are sources of GDF9 protein.

FSH secretion by L β T2 gonadotrophs and gonadotroph-containing primary cultures of adult male rat pituitary cells is stimulated by activin isoforms, reflecting the induction of FSH β -subunit transcription, which is at least partly mediated by ALK4 through the activation of R-SMADs 2 and 3 (Dupont *et al.* 2003, Bernard 2004). GDF9 was therefore expected to act similarly by activating R-SMADs 2 and/or 3 in the BMPRII/ALK5-expressing gonadotrophs. However, GDF9 surprisingly does not modify FSH synthesis/secretion by rat gonadotrophs in primary culture, or the expression of pGRAS-luc or secretion of FSH by mouse L β T2 gonadotrophs. This pattern nevertheless accords with a recent study in which GDF9 had minimal effects on FSH secretion in primary pituitary cell cultures prepared from sheep (Young *et al.* 2008). By contrast, FSH secretion is stimulated by the closely related ligand, BMP-15 (Otsuka & Shimasaki 2002), which instead signals via R-SMADs 1/5/8 (Moore *et al.* 2003). Apart from R-SMADs, additional signalling molecules are activated by activin in gonadotrophs (Dupont *et al.* 2003, Safwat *et al.* 2005), and such SMAD-independent signalling pathways can play major or supplementary roles in mediating

TGF- β superfamily agonist actions (Piek *et al.* 1999, Moustakas & Heldin 2005). For instance, TAK1 (listed as MAP3K7 in MGI Database), a TGF- β activated MEKKK, is reported to mediate the stimulation of FSH β gene transcription by activin in L β T2 gonadotrophs (Safwat *et al.* 2005). It remains to be determined whether differential activation of TAK1 by activin, but not GDF9, might be the basis for the relative insensitivity of gonadotrophs to bioactive GDF9.

Mouse and rat testes express *Gdf9* mRNA (this study; (Fitzpatrick *et al.* 1998, Nicholls *et al.* 2009)). *Gdf9* mRNA is localised to germ cells, including the more mature (pachytene) spermatocytes in the mouse, and round spermatids in both rat and mouse, but little or no evidence was obtained for expression by Leydig or Sertoli cells. Little *Gdf9* mRNA was evident by real-time RT-PCR in TM3 Leydig and TM4 Sertoli cell lines, and previous examination of TM3 cell expression of *Gdf9* by northern blot analysis was negative (Fitzpatrick *et al.* 1998). Although unlikely to be sources of GDF9, TM3 and TM4 cells were nevertheless expected to respond to GDF9, activin and TGF- β because they express mRNA encoding the requisite receptors and signalling molecules for all of these agonists. In addition, Sertoli cells perform an analogous role in the testis to that played by ovarian granulosa cells, and might therefore be expected to respond to any GDF9 made by the germ cells. GDF9 accordingly activates R-SMAD2/3 signalling via ALK4, 5 or 7 in TM4 cells in a similar way to its activation of the granulosa cell lines, KGN and COV434. This supports and extends our recent evidence that rat Sertoli cells co-express BMPRII and ALK5, and that GDF9 modifies Sertoli cell functions *in vitro* using a signalling pathway that is blocked by SB431542 (Nicholls *et al.* 2009). In contrast to TM4 cells, TM3 cells do not respond specifically to GDF9, although their robust responses to TGF- β and activin isoforms provide indirect evidence that TM3 cell expression of mRNA for ALK5 receptor and R-SMADs 2 and 3 (Farnworth *et al.* 2007) gives rise to functional proteins. As with gonadotrophs, there seems to be some characteristic of TM3 Leydig cells that prevents their activation by GDF9.

We conclude that GDF9 is expressed in foetal and neonatal mouse adrenal, as well as AC cells and pituitary gonadotrophs. Recombinant mouse GDF9 stimulates expression of R-SMAD 2/3-responsive luciferase reporter constructs in cell lines that model foetal adrenocortical and Sertoli cells, demonstrating a potential for autocrine/paracrine stimulation of these cell types by GDF9 *in vivo*. The lack of specific GDF9 actions in TM3 cells, and two different gonadotroph preparations, raises the possibility that expression of BMPRII, ALK-5, and R-SMADs 2 and 3 are necessary (Mazebourg & Hsueh 2006), but may not be sufficient, for a cell to respond robustly to GDF9. These studies nevertheless establish that GDF9 acts on receptive extra-ovarian cell types through a pathway involving ALK4/5/7 and R-SMAD2/3, as it does in its classical target, the ovarian granulosa cell.

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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