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Regulation and action of early growth response 1 in bovine granulosa cells

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Abstract

Fibroblast growth factors (FGF) modify cell proliferation and differentiation through receptor tyrosine kinases, which stimulate the expression of transcription factors including members of the early growth response (EGR) family. In ovarian granulosa cells, most FGFs activate typical response genes, although the role of EGR proteins has not been described. In the present study, we determined the regulation of EGR mRNA by FGFs and explored the role of EGR1 in the regulation of FGF-response genes. Addition of FGF1, FGF2, FGF4 or FGF8b increased *EGR1* and *EGR3* mRNA levels, whereas FGF18 increased only *EGR1* mRNA abundance. No mRNA encoding *EGR2* or *EGR4* was detected. Overexpression of EGR1 increased *EGR3* mRNA levels as well as the FGF-response genes *SPRY2*, *NR4A1* and *FOSL1* and also increased the phosphorylation of MAPK3/1. Knockdown of EGR3 did not alter the ability of FGF8b to stimulate *SPRY2* mRNA levels. These data demonstrate the regulation of *EGR1* and *EGR3* mRNA abundance by FGFs in granulosa cells and suggest that EGR1 is likely an upstream component of FGF signaling in granulosa cells.

Reproduction (2017) **154** 547–557

Introduction

Many growth factors, including members of the epidermal, platelet-derived, vascular endothelial and fibroblast growth factor families (EGF, PDGF, VEGF and FGF, respectively), stimulate cells by activating transmembrane receptor tyrosine kinases (RTK) (Schlessinger 2000). Ligand activation of RTK leads to the activation of downstream pathways including mitogen-activated protein kinases (MAPK), protein kinase C (PKC), and protein kinase B (PKB)/phosphatidylinositol-3-kinase (PI3K) in a ligand and cell context-specific manner (Schlessinger 2000).

There are numerous examples of growth factor–RTK signaling in the ovarian follicle that regulate follicle activation, growth and ovulation (Park *et al.* 2004, Sirotkin 2011, Pepling 2012). The FGF family has been implicated in follicle development, and there is evidence that numerous FGFs stimulate proliferation of granulosa cells (Gospodarowicz & Bialecki 1979, Parrott & Skinner 1998, Roberts & Ellis 1999) and inhibit granulosa cell differentiation as shown by reduced steroidogenesis (Vernon & Spicer 1994, Parrott & Skinner 1998, Buratini *et al.* 2007, Portela *et al.* 2010).

Downstream signaling by FGFs in granulosa cells is consistent with classical FGF action, as activation of PLC, PKB and MAPK3/1 have been demonstrated (Peluso *et al.* 2001, Jiang *et al.* 2011). These signaling pathways stimulate the expression of transcription factors including the nuclear receptor 4A (NR4A) and early growth response (EGR) families in diverse tissues (Damon *et al.* 1997, Cholfin & Rubenstein 2008, Lammi & Aarnisalo 2008); in granulosa cells, FGF signaling increased *NR4A1* and *EGR1* mRNA levels (Jiang *et al.* 2011, 2013).

The EGR family comprises four zinc-finger containing transcription factors, EGR1, EGR2, EGR3 and EGR4. These proteins bind to GC-rich DNA recognition sites and stimulate the expression of a variety of genes in different tissues (reviewed in Thiel *et al.* 2014). In the ovary, *EGR1* mRNA levels are rapidly and transiently increased in granulosa cells by LH (Espey *et al.* 2000, Sayasith *et al.* 2006), FSH (Russell *et al.* 2003) and FGFs (Jiang *et al.* 2013) and in luteal cells by prostaglandins (Hou *et al.* 2008). Mice null for *Egr1* are infertile owing to pituitary and ovarian defects (Topilko *et al.* 1998). Less is known about expression and function of other EGRs in the ovary; *EGR2* mRNA has been detected in mouse granulosa cells and in the KGN human granulosa cell line and is regulated by gonadotrophins (Carletti & Christenson 2009, Jin *et al.* 2017), and *Egr3* mRNA was detected in mouse oocytes (Shin *et al.* 2014) and

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ISSN 1470–1626 (paper) 1741–7899 (online)
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were upregulated in bovine granulosa cells in response to mycotoxins (Guerrero-Netro *et al.* 2015). Global knock out of *Egr3* in mice is perinatal lethal (Tourtellotte & Milbrandt 1998) although *Egr4*-null female mice are fertile (Tourtellotte *et al.* 1999). All four *EGR* mRNAs have been detected in porcine granulosa cells (Wang *et al.* 2014).

Although EGR1 is associated with cell proliferation and differentiation in many tissues, it has been linked to apoptosis in several cell types including melanoma, epidermal, nerve and, more recently, granulosa cells (Muthukkumar *et al.* 1995, Catania *et al.* 1999, Thyss *et al.* 2004, Yuan *et al.* 2016). The role of EGR family members in ovarian function has not been investigated. We therefore hypothesized that EGR proteins are mediators of the actions of FGFs in granulosa cells. The objectives of this study were to determine which EGR genes are regulated by FGFs in bovine granulosa cells and to determine the effect of modulating EGR1 and EGR3 levels on the expression of typical FGF target genes.

Materials and methods

Cell culture

All materials were obtained from Life Technologies Inc. unless otherwise stated. Bovine ovaries were obtained from adult cows at a local abattoir, irrespective of stage of the estrous cycle and transported to the laboratory in phosphate-buffered saline (PBS) containing penicillin (100 IU/mL) and streptomycin (172 mmol/L). Granulosa cells were collected from follicles 2–5 mm diameter and the cell suspension was filtered through a 150 mesh steel sieve (Sigma-Aldrich). Cell viability was assessed by Trypan blue dye exclusion.

Granulosa cells were cultured under well-established serum-free conditions that maintain estradiol secretion and responsiveness to physiological concentrations of FSH (Gutiérrez *et al.* 1997, Silva & Price 2000). Cells were seeded into 24-well tissue culture plates (Sarstedt Inc., Newton, NC) at a density of 0.5×10^6 viable cells in 0.5 mL MEM containing sodium bicarbonate (10 mmol/L), Hepes (20 mmol/L), sodium selenite (23 nmol/L), bovine serum albumin (BSA) (0.1% w/v; Sigma-Aldrich), penicillin (100 U/mL), streptomycin (172 mmol/L), transferrin (2.5 µg/mL), nonessential amino acid mix (1.1 mmol/L), bovine insulin (10 ng/mL), androstenedione ($10-^{6}$ mol/L) and bovine FSH (1 ng/mL starting on day 2; AFP5346D; National Hormone and Peptide Program, Torrance, CA). The cells were cultured at 37°C in a 5% CO_2 atmosphere, 95% air for 5 days, with 70% medium being replaced on days 2 and 4.

Experimental design

The effect of FGF on EGR mRNA levels was measured by treating cells on day 5 of culture with 10 ng/mL human recombinant FGF1, FGF2, FGF4, FGF8b and FGF18 (PeproTech, Rocky Hill, NJ) or vehicle (PBS) for 0, 1, 2, 4 or 8 h. Cells were lysed in RNA Lysis Solution (Life Technologies) and stored at −20°C.

To assess the effect of EGR1 on granulosa cells, an adenoviral overexpression model was used. Cells were exposed to virus on day 4 of culture, and cells recovered 12 h after removal of virus for extraction of mRNA or were lysed in 100 µL/well cold RIPA buffer (25 mmol/L Tris–HCl pH 7.6, 150 mmol/L NaCl, 1% v/v NP-40, 1% w/v sodium deoxycholate, 0.1% w/v SDS, 1 mmol/L sodium orthovanadate, and protease inhibitor cocktail) for Western blotting. Using additional culture wells, steroid secretion was measured in spent medium and cells were lysed in 1 mol/L NaOH to measure total cell protein.

The effect of EGR3 on FGF target genes was determined by knocking down EGR3 with small-interfering RNA. On day 4 of culture, the medium was replaced with Opti-MEM containing 80 mM siRNA (target sequence, 5′-TGACCATGAGCAGTTTGCTAAACCA-3′; negative control RNA, Silencer Negative Control #1; Ambion) premixed with transfection reagent (DharmaFECT1; GE Dharmacon) for 6 h, after which the transfection mixture was replaced with fresh culture medium. Preliminary tests indicated that this EGR3 target sequence was the most efficient of five tested, and pooling did not further increase knockdown efficiency. Twenty-four hours later, cells were challenged with FGF8b (10 ng/mL) for 2 h. Negative control cells were not transfected and not treated with FGF8b, and positive control cells were not transfected but were treated with FGF8b. Cells were recovered for RNA extraction.

The specific MAPK inhibitor PD98059 (Sigma-Aldrich) was also used to assess the impact of MAPK3/1 signaling on FGF target genes stimulated by EGR1. Cells were pretreated for 1 h with 50 µmol/L PD98059 dissolved in DMSO before virus infection, and the inhibitor was included in the infection and post-infection media.

Adenovirus infection

The adenoviruses containing constitutively active rat EGR1 (Ad-EGR1; NAB-insensitive EGR1, I293F) and green fluorescent protein (Ad-GFP) have been described (Ehrengruber *et al.* 2000). Both virus vectors were amplified in 293T cells, and cells were harvested and lysed through three cycles of freeze/thaw to obtain viral supernatants. Granulosa cells were infected at a multiplicity of infection of 200 in culture medium containing 21 nmol/L polybrene (Sigma-Aldrich), which was replaced after 6 h with medium containing FSH (1 ng/mL). The Ad-GFP treatment was a virus control, and an additional control consisted of polybrene treatment without virus.

Total RNA extraction and real-time RT-PCR

Total RNA was isolated from granulosa cells using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was quantified by absorbance at 260 nm. Reverse transcription was performed on 200 ng total RNA with SuperScript VILO cDNA Synthesis Kit. Real-time PCR was performed in a 15 µL reaction volume with 0.5 µL cDNA and 2× Power SYBR Green PCR Master Mix on a CFX-96 Real-Time PCR Detection System (Bio-Rad Laboratories Ltd.). The bovine-specific primers used are given in Table 1, and amplification efficiencies were between 98% and 102%, depending on

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Table 1 Bovine-specific sequences of PCR primers used for real-time PCR.

Gene	Forward primers (5′-3′)	Reverse primers (5′-3′)	Source
H2AFZ	GAGGAGCTGAACAAGCTGTTG	TTGTGGTGGCTCTCAGTCTTC	Portela *et al.* (2010)
SPRY1	AGAGAGAGATTCAGCCTGCTGCTA	TCTTGTCTTGGTGTGGTTCGAGGA	Jiang *et al.* (2011)
SPRY2	CATGGGCGTCATGTCCCATAGT	GCCTGTTCACCCGGTCATAA	Jiang *et al.* (2011)
SPRY3	TGCCAGCTCAATGTCCCATAGT	TTTCAGAGCACCGTCAGCCTTT	Jiang *et al.* (2011)
SPRY4	ACAAGCACTTCTTGCTGTGCGA	ATGCAGGTGCCATAGTTGACCA	Jiang *et al.* (2011)
GADD45B	TACGAGTCGGCCAAGCTGAT	GTCCTCCTCTTCCTCGTCGAT	Jiang *et al.* (2011)
NR4A1	GCTTCCTGCCAGCATTATGG	CAGGCAGATGTACTTGGCATTTT	Jiang *et al.* (2011)
NR4A2	AGTATGGGTCCTCGCCTCAA	TGTATTCTCCCGAAGAGTGGTAACT	Jiang *et al.* (2011)
NR4A3	TCAACGCCCAGAGATCTTGAT	TGGAGGCTGTTAGAAGGTTGTAGA	Jiang *et al.* (2011)
BAX	AACATGGAGCTGCAGAGGAT	CAGTTGAAGTTGCCGTCAGA	Jiang *et al.* (2011)
BCL2	ATGACTTCTCTCGGCGCTAC	CTGAAGAGCTCCTCCACCAC	Jiang *et al.* (2011)
FOS	ATGGGTTCTCCCGTCAATGC	GGTCGAGATGGCAGTCACTGT	Jiang *et al.* (2013)
BAMBI	TCGCCACTCCAGCTACATCTT	TGGGCTGCATCACAGTAGCA	Jiang *et al.* (2013)
FOSL1	AGTGCAGGAACCGGAGGAAA	TCTCTCGCTGCAGTCCAGATT	Jiang *et al.* (2013)
XIRP1	CAAACAAGAGGAACCGACAGA	GGCATTGGCCATCCTTCT	Jiang *et al.* (2013)
PLK2	GAACCCTTGGAACACAGGAGAA	TTCACAGCCGTGTCCTTGTTT	Jiang *et al.* (2013)
EGR1	AAGCGAGCAGCCCTACGA	GCAGCCGGGTGGTTTG	Jiang *et al.* (2013)
EGR2	ACCATTTTCCCCAATGCCA	TGATCATGCCATCTCCGGC	
EGR3	AGCGCGCTTCAACCTCTTTT	GGTCAGACCGATGTCCATC	
EGR4	GAAGCTACCACTGTAGCCG	GGCTATCCGGCAGGTCATT	
XIAP	GAAGCACGGATCATTACATTTGG	CCTTCACCTAAAGCATAAAATCCAG	(Ferreira *et al.* 2011)
MDM2	TCAGGCAGGCGAGAGTGATA	GGGAGGATTCATTTCATTGCA	Portela *et al.* (2015)
TP53	GAATGAAGCGCCCAAAT	CTCCGTCATGTGCTCAAC	
CYP19A	CCACGATGGCACTTTCATAA	TCACCAAGCATCTGGACAG	
STAR	CCCAGCAGAAGGGTGTCATC	TGCGAGAGGACCTGGTTGAT	(Orisaka *et al.* 2006)

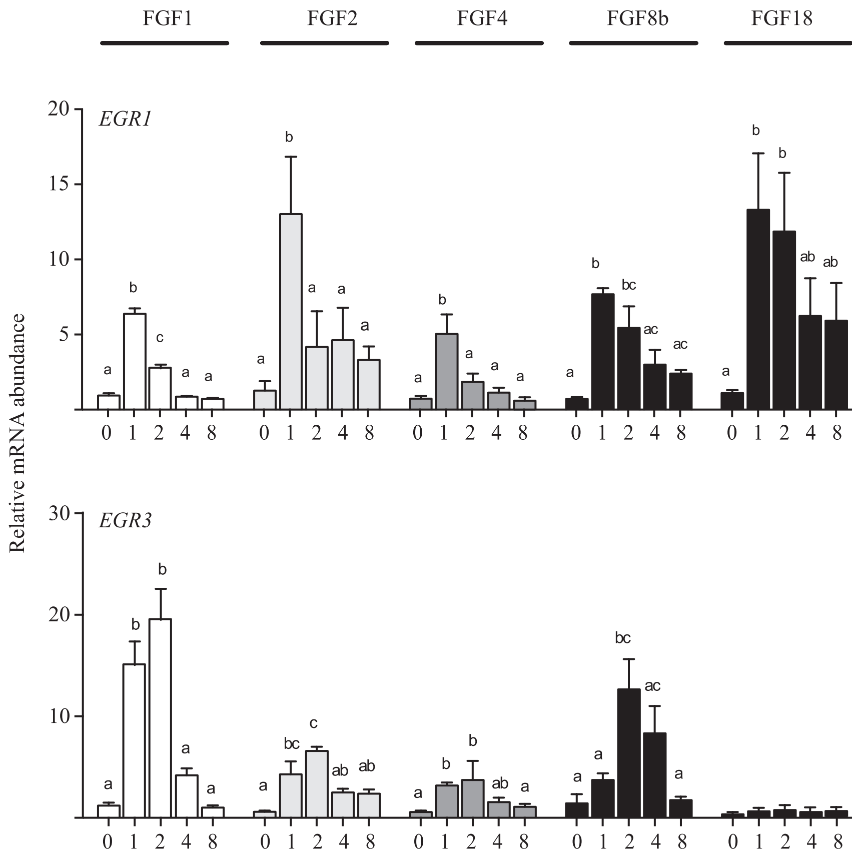

Figure 1 Regulation of *EGR1* and *EGR3* mRNA levels by FGFs. Bovine granulosa cells were cultured in serum-free conditions, and challenged for the times given with 10 ng/mL FGF1, FGF2, FGF4, FGF8b or FGF18. Relative gene expression was determined by real-time PCR and data are means ± S.E.M. of three replicate cultures. Bars without common letters are significantly different (Tukey–Kramer HSD; $P < 0.05$).

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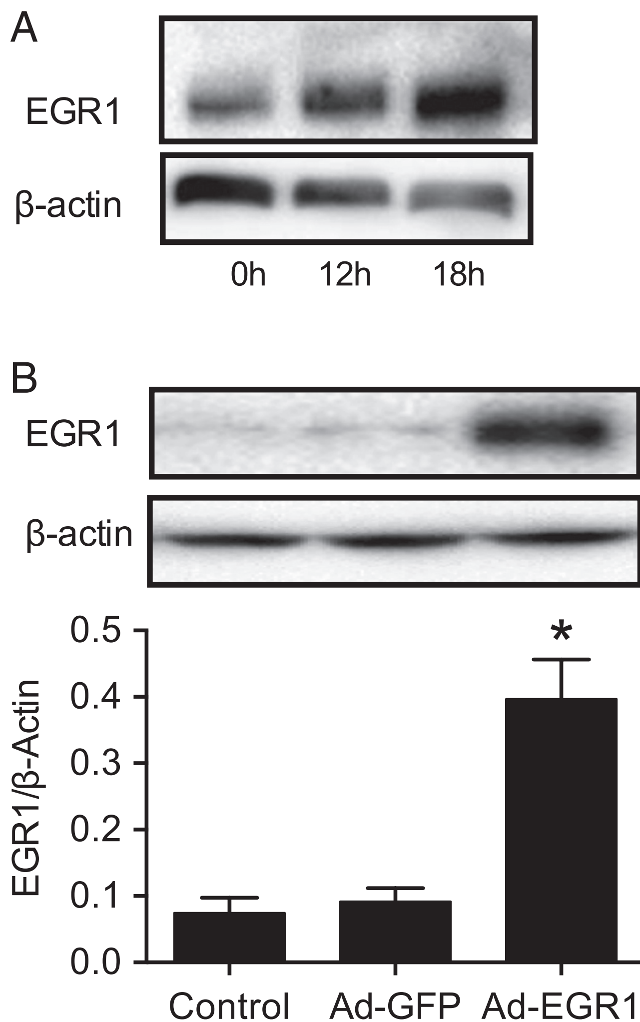

Figure 2 Increased EGR1 protein levels following exposure of granulosa cells to Ad-EGR1 virus. (A) Protein levels in cell lysates were monitored by Western blot at 0, 12 and 18 h after addition of viruses to cells. (B) Quantification of protein levels at 18 h after infection in control, Ad-GFP- and Ad-EGR1-infected cells. Data are means ± s.e.m. of three replicate cultures. Asterisk denotes a mean different from control (Dunnett's test; $P < 0.05$).

primer pair. The reference genes used, *H2AFZ* and *GAPDH*, are stable under serum-free conditions as determined by geNorm software (Ramakers *et al.* 2003); the Cq values did not differ under the treatments reported in this study.

The thermal cycling conditions were 3 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. Melting curve analyses were performed to verify product identity, and amplicons from novel primers were sequenced to establish identity. Samples were run in triplicate and data were normalized relative to the geometric mean of the reference genes using the $2^{-\Delta\Delta}$Cq method (Pfaffl 2001). All replicates of each experiment were run in the same plate, and data for all samples expressed relative to the control sample of the first replicate. Average coefficients of variation were 0.1% and 15% for Cq and ddCq values respectively.

Western blotting

Samples were resolved on 10% SDS–PAGE gels in a 'micro-blot' format, loading 3 µg total protein per lane in 3 × 0.75 mm wells (Naito *et al.* 1999); preliminary experiments with serial dilutions of total protein determined that 3 µg was within the linear range of detection of target proteins. Resolved proteins were transferred onto PVDF membrane in a Bio-Rad wet Blot Transfer Cell apparatus (transfer buffer: 39 mmol/L glycine, 48 mmol/L Tris-base, 20% v/v methanol, pH 8.3). After 2 h of transfer at 4°C, the membranes were blocked in 5% w/v non-fat dry milk in TTBS (10 mmol/L Tris–HCl, 150 mmol/L NaCl, 0.1% v/v Tween-20, pH 7.5) for 1 h. Membranes were incubated overnight with the primary antibodies against MAPK3/1 (1:1000, #9102, Cell Signaling Technology), phospho-MAPK3/1 (1:1000, #9101, Cell Signaling Technology), AKT (1:1000, #9272, Cell Signaling Technology), phospho-AKT (1:1000, #SC-101629, Santa Cruz Biotechnology) diluted in TTBS or EGR1 (1:2000, SC-189X, Santa Cruz Biotechnology) in TTBS-5% w/v non-fat milk. After three washes with TTBS, membranes were incubated for 1 h at room temperature with 1:10 000 anti-rabbit HRP-conjugated IgG (GE Healthcare) diluted in TTBS. After four washes in TTBS, protein bands were revealed by chemiluminescence (ECL, Bio-Rad Laboratories Ltd.). Analysis of pixel density within equal sized regions of interest was performed with Image Lab software (Bio-Rad), and data were expressed as the ratio of target to ACTB protein abundance or of phospho to total target protein abundance. All samples from each experiment were loaded onto the same gel to avoid between-gel variation.

Apoptosis analysis

Flow cytometry was performed to analyze apoptosis of granulosa cells after overexpression of EGR1 protein using the Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich). Briefly, cells were washed two times with DPBS and resuspended in binding buffer, and 500 µL aliquots incubated with 5 µL Annexin V-FITC and 10 µL propidium iodide solution for 10 min. Fluorescence was determined with a FACSVantage SE (BD Biosciences, Oakville, ON, Canada) flow cytometer and analyzed with Cell Quest Pro software (BD Biosciences).

Steroid assay

Estradiol concentrations in conditioned medium were measured in duplicate by RIA with an antibody raised in rams (Jiang & Price 2012). Intra- and inter-assay coefficients of variation (CV) values were 3 and 7%, respectively. Progesterone concentrations were measured by RIA in duplicate as described (Price *et al.* 1995) with mean intra- and inter-assay CV values of 2 and 8%, respectively. Steroid concentrations were corrected for cell number by expressing per unit mass of cell protein. The sensitivity of these assays was 10 pg and 4 pg per tube for estradiol and progesterone.

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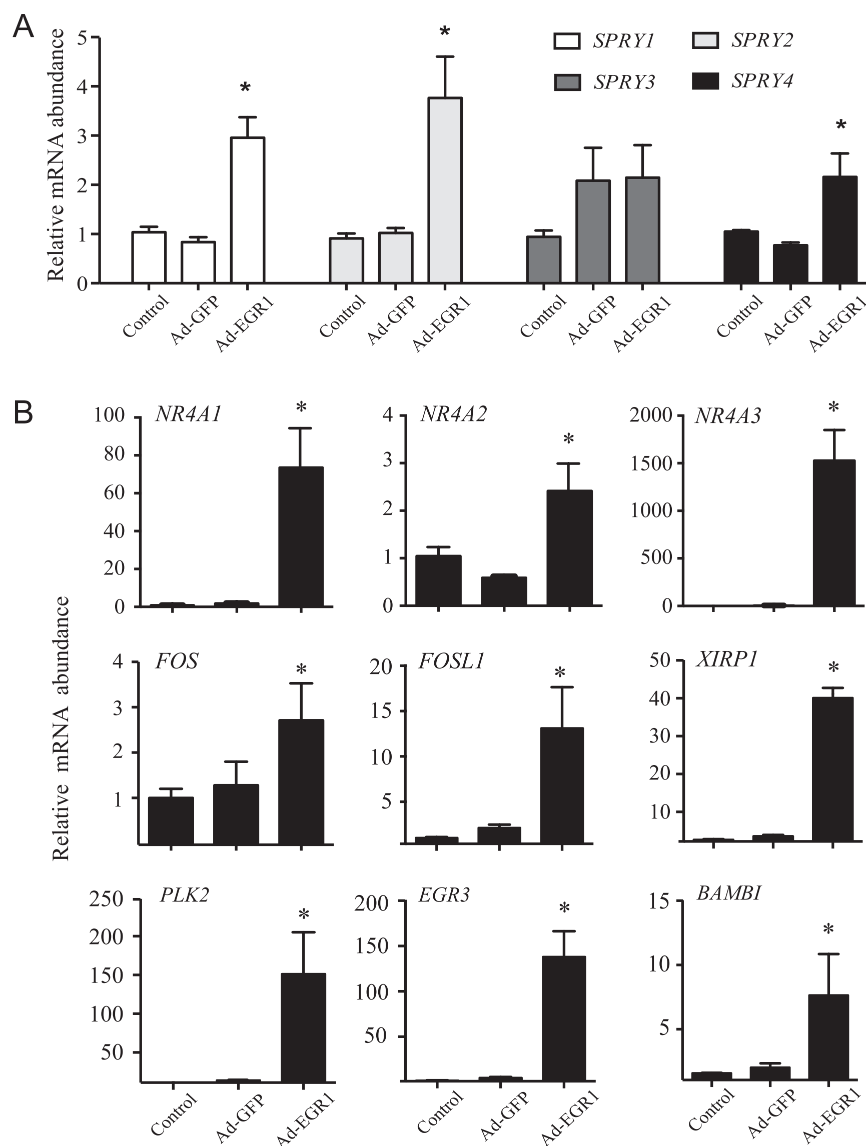

Figure 3 Overexpression of EGR1 increased abundance of mRNA encoding SPRY family members (A) and of other typical FGF-response genes (B). Granulosa cells in serum-free medium were exposed to Ad-GFP, Ad-EGR1 or polybrene alone (Con) for 6 h and cells recovered after 18 h to measure mRNA abundance by real-time PCR. Data are means ± s.e.m. of three replicate cultures. Asterisk denotes a mean different from Ad-GFP (Dunnett's test; $P<0.05$).

Statistical analysis

All statistical analyses were performed with JMP software (SAS institute, Cary, NC). ANOVA was used to test for main effects of treatments and culture replicate was included as a random effect. Multiple comparisons were tested using the Tukey–Kramer honestly significant difference (HSD) test, and comparisons of treatments with controls were performed with Dunnett's test. Means were considered statistically significant at $P<0.05$. The data are presented as least squares means ± s.e.m.

Results

Effect of FGFs on EGR expression in granulosa cells

Messenger RNA encoding EGR2 and EGR4 were readily detected in extracts of bovine fetal brain obtained from an abattoir (average Cq of 24 and 28, respectively) but were not detected in cultured granulosa cells after 40 cycles of PCR. Addition of FGF1, FGF2, FGF4 or FGF8b rapidly and transiently increased levels of *EGR1* and *EGR3* mRNA in granulosa cells (Fig. 1). Addition of FGF18 increased the abundance of mRNA encoding EGR1 but had no effect on levels of *EGR3* mRNA (Fig. 1). The maximum level of *EGR1* mRNA was consistently noted at 1 h after treatment and that for *EGR3* mRNA was at 2 h after treatment.

Effect of EGR1 overexpression on granulosa cells

Infection with Ad-EGR1 increased EGR1 protein abundance fivefold at 12 and 18 h after the start of infection (6 and 12 h after removal of virus; Fig. 2A), and 18 h was chosen as the time point to measure the

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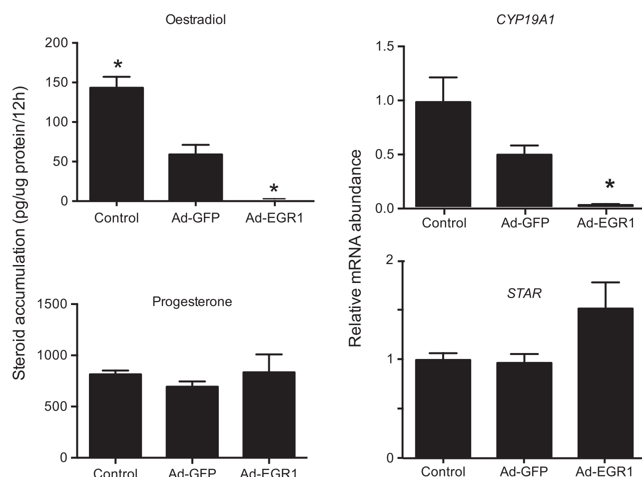

Figure 4 Granulosa cell steroidogenesis after overexpression with EGR1. Granulosa cells in serum-free medium were exposed to Ad-GFP, Ad-EGR1 or polybrene alone (Control) for 6 h and cells recovered after 18 h to measure mRNA abundance by real-time PCR. For steroid measurement, medium from the last 12 h culture (after removal of virus) was collected for RIA and total cell protein was used to correct for cell number. Data are means ± S.E.M. of three replicate cultures. Asterisk denotes a mean different from Ad-GFP (Dunnett's test; $P<0.05$).

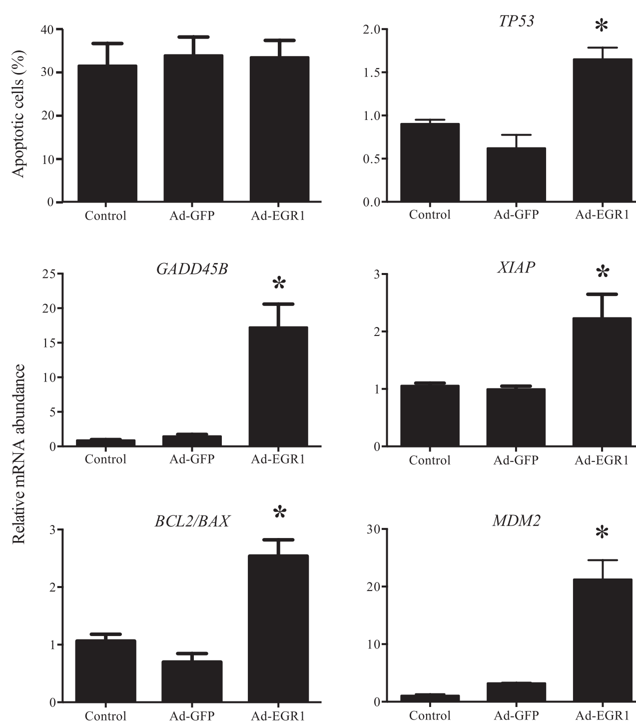

Figure 5 Effect of EGR1 on apoptosis and abundance of mRNA encoding proteins active in apoptotic pathways. Granulosa cells in serum-free medium were exposed to Ad-GFP, Ad-EGR1 or polybrene alone (Control) for 6 h and cells recovered after 18 h to measure mRNA abundance by real-time PCR. Cell apoptosis was assessed by annexin V flow cytometry after 18 h of exposure to viruses. Data are means ± S.E.M. of three replicate cultures. Asterisk denotes a mean different from Ad-GFP (Dunnett's test; $P<0.05$).

expression of typical RTK-induced genes. Infection with Ad-GFP did not alter EGR1 protein levels (Fig. 2B).

Overexpression of EGR1 protein increased *SPRY1*, *SPRY2* and *SPRY4* mRNA levels relative to Ad-GFP-infected cells, whereas there was no increase in *SPRY3* mRNA levels (Fig. 3A). EGR1 overexpression also increased mRNA encoding members of the NR4A family, with 100-, 3- and 2400-fold increases in *NR4A1*, *NR4A2* and *NR4A3* mRNA levels respectively (Fig. 3B). Infection with Ad-GFP did not alter levels of mRNA encoding any of these genes relative to control cultures without addition of virus (Fig. 3). Overexpression of EGR1 also increased the abundance of *FOS, FOSL1, BAMBI, XIRP1, PLK2* and *EGR3* mRNA compared with Ad-GFP controls (Fig. 3B).

The effects of EGR1 on cell function were measured. Overexpression significantly reduced E_2 secretion and *CYP19A1* mRNA levels compared with Ad-GFP ($P<0.001$); although Ad-GFP did not significantly reduce *CYP19A1* mRNA levels, it did decrease E_2 secretion relative to non-virus controls (Fig. 4). EGR1 did not alter progesterone secretion or *STAR* mRNA levels (Fig. 4).

Abundance of mRNA encoding GADD45B, XIAP TP53 and MDM2, proteins involved in cell health/apoptosis, increased following EGR1 overexpression (Fig. 5) and the ratio of *BCL2* to *BAX* mRNA was significantly elevated by EGR1 (Fig. 5). FACS results showed that EGR1 overexpression had no effect on the proportion of apoptotic cells (Fig. 5).

Overexpression of EGR1 in granulosa cells increases MAPK3/1 phosphorylation

To determine if the effect of EGR1 on target genes is a direct effect or mediated by downstream intracellular signaling, we measured MAPK3/1 phosphorylation by Western blot; overexpression of EGR1 increased MAPK3/1 protein phosphorylation compared with infection with Ad-GFP at 18 h after infection and had no effect on AKT phosphorylation (Fig. 6).

To determine the role of MAPK3/1 activity on EGR1 target gene mRNA abundance, cells were exposed to adenovirus and cotreated with the MAPK3/1 inhibitor PD98059, which successfully blocked the ability of EGR1 to phosphorylate MAPK3/1 after 18 h (Fig. 7). Overexpression of EGR1 stimulated the abundance of mRNA encoding *EREG*, a MAPK3/1 target gene, which was reduced in the presence of PD98059 (Fig. 7). Inhibition of MAPK3/1 activation significantly abrogated the ability of EGR1 to stimulate *NR4A1* mRNA levels (Fig. 8), but had no significant effects on the other mRNA species measured. Neither Ad-EGR1 nor cotreatment with MAPK3/1 inhibitor altered granulosa cell apoptosis (Fig. 8).

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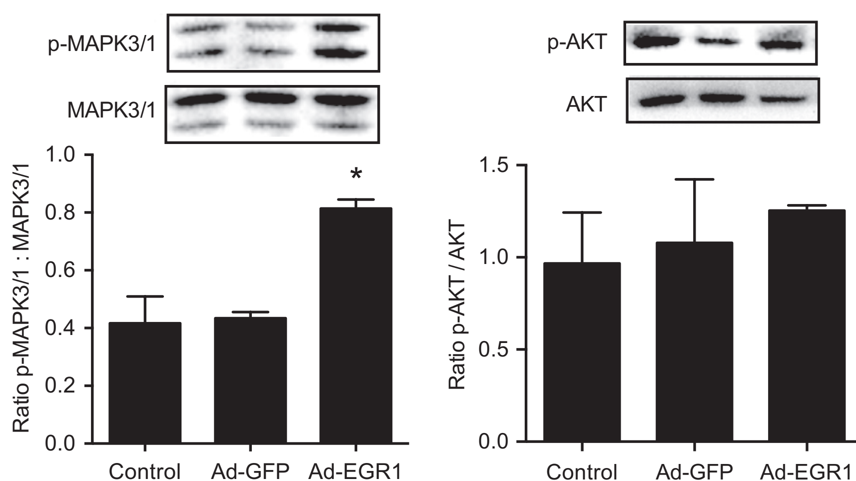

Figure 6 Activation of intracellular signaling pathways by EGR1. Granulosa cells cultured in serum-free medium were exposed to Ad-GFP, Ad-EGR1 or polybrene alone (Control) for 6 h and cells lysed into RIPA buffer after 18 h for Western blotting. Data are means ± S.E.M. of three replicate cultures, and representative blots are shown above each graph. The asterisk denotes a mean different from Ad-GFP (Dunnett's test; $P < 0.05$). Where there are no asterisks, there was no effect of treatments.

Effect of knocking down EGR3 on FGF target genes

As overexpression of EGR1 also resulted in increased endogenous *EGR3* mRNA levels, we determined the role of EGR3 by RNA knockdown in cells stimulated with FGF. Challenge with FGF8b significantly increased *EGR1*, *EGR3* and *SPRY2* mRNA levels, and transfection with negative control siRNA had no effect on mRNA levels (Fig. 9). The most efficient knockdown achieved was 53%, which increased abundance of *EGR1* mRNA compared to transfection with scrambled RNA and had no effect on *SPRY2* mRNA levels (Fig. 9).

Discussion

The expression of EGR1 has been detected in granulosa cells, where it is inducible by the preovulatory LH surge in rodents and cattle (Espey *et al.* 2000, Sayasith *et al.* 2006) and by cAMP/FSH in rats (Yoshino *et al.* 2002, Russell *et al.* 2003). In bovine granulosa cells, *EGR1* mRNA abundance was increased by FGF8b, FGF18 (Jiang *et al.* 2013), FGF1, FGF2 and FGF4 (present study). An increase in *EGR1* mRNA therefore appears to be a common response to FGF signaling in granulosa cells. We also demonstrate an increase in *EGR3* mRNA levels in response to FGF1, FGF2, FGF4 and FGF8b, and in granulosa cells, these FGFs have previously been shown to increase levels of mRNA of the typical FGF-response genes *SPRY1, SPRY2, SPRY4, NR4A1* and *NR4A3* among others (Jiang *et al.* 2011, 2013, Jiang & Price 2012), suggesting that EGR1 and EGR3 may be important components of these pathways. To our knowledge, there are no other reports on the regulation of EGR3 mRNA or protein in the ovary. Curiously, we could not detect *EGR2* or *EGR4* mRNA in cultured granulosa cells, although both have been detected in cultured porcine luteinizing granulosa cells (Wang *et al.* 2014) and *Egr2* mRNA levels are increased by gonadotrophins in mouse granulosa cells in vivo (Carletti & Christenson 2009, Jin *et al.* 2017). This was not likely to be a technical issue as we readily detected amplicons in fetal bovine brain, but may be a species or cell context (luteinized vs non-luteinized) difference.

Overexpression of EGR1 increased the abundance of mRNA encoding the typical FGF-response genes *SPRY1, SPRY2, SPRY4, NR4A1* and *NR4A3* and also increased *EREG* and *AREG* mRNA levels, which is consistent with the role of EGR1 in ovulation (Espey *et al.* 2000, Sayasith *et al.* 2006). Overexpression of EGR1 also increased *EGR3* mRNA levels as previously reported in myoblast and neuroblastoma cell lines (Ehrengruber *et al.* 2000). Thus, these data suggest that the typical response of cells to FGFs is mediated at least in part by EGR1 (or EGR1 and EGR3).

EGR1 is a transcription factor and binds to the promoters of target genes. In keratinocytes, EGR1 binds to and activates the *GADD45B* promoter (Thyss *et al.* 2004) and in silico analysis predicts the presence of EGR1 recognition sites on *SPRY2, NR4A1, EREG* and *EGR3* promoters (http://tfbind.hgc.jp/) (Tsunoda & Takagi 1999); this is consistent with the present study in which the abundance of mRNA of these genes was increased by forced expression of EGR1 in granulosa cells. One of these genes, *EREG*, encodes a protein that signals through the EGF receptor and MAPK3/1 signaling (Park *et al.* 2004), and this likely accounts for the increase in MAPK3/1 phosphorylation observed after EGR1 overexpression. Pharmacological suppression of MAPK3/1 activity did not cause significant reductions in EGR1 target gene expression, with the exception of *NR4A1*, suggesting that this pathway was not a major contributor to the cellular response to EGR.

Although most FGFs tested here increased the abundance of *EGR1* and *EGR3* mRNA, there was one notable exception, FGF18, which increased *EGR1* but

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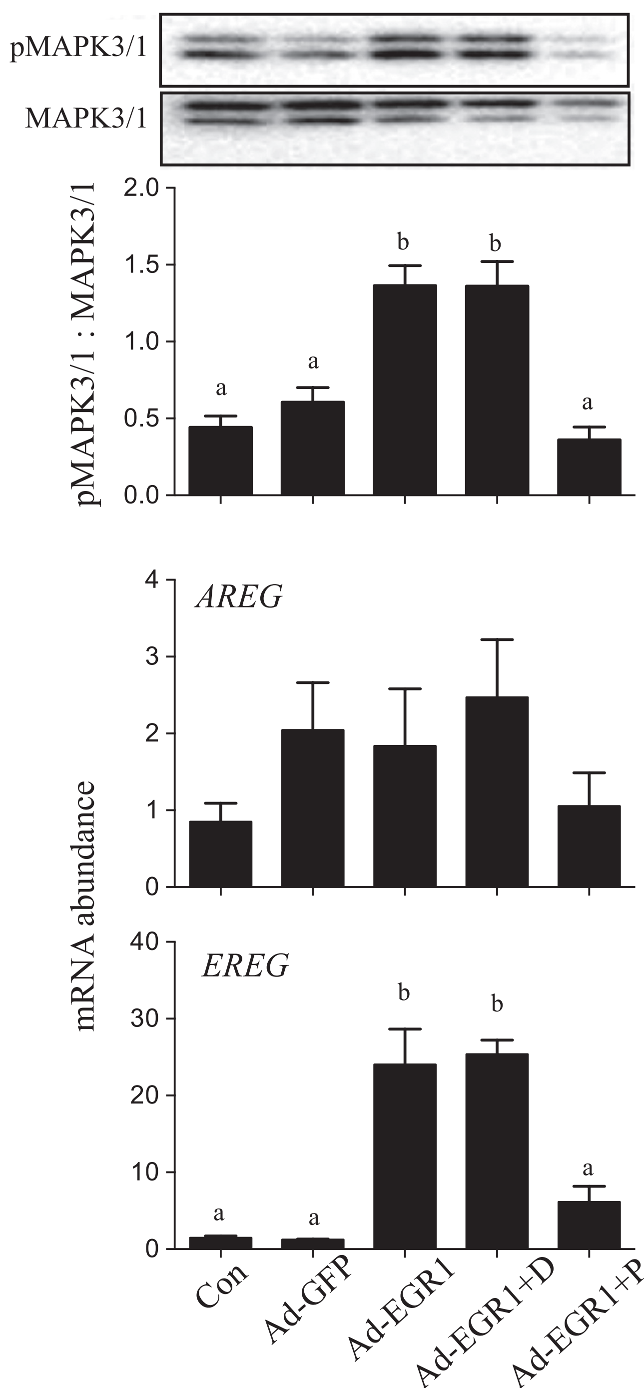

Figure 7 Role of MAPK activity in EGR1 stimulation of EGF signaling pathway. Granulosa cells cultured in serum-free medium were exposed to polybrene alone (Con), Ad-GFP, Ad-EGR1, Ad-EGR1 plus DMSO (Ad-EGR1+D) or Ad-EGR1 plus the MAPK3/1 inhibitor PD98059 (Ad-EGF1+P). Infections were performed for 6 h and cells were harvested after 18 h for Western blotting or RNA extraction. The inhibitor was added 1 h before infection and included in infection and post-infection media. A representative blot of phospho- and total MAPK3/1 are shown above the means for the ratio of phospho:total MAPK3/1. Messenger RNA levels were measured by real-time PCR. Data are means ± S.E.M. of three replicate cultures, and bars without common letters are significantly different (Tukey–Kramer HSD; $P<0.05$).

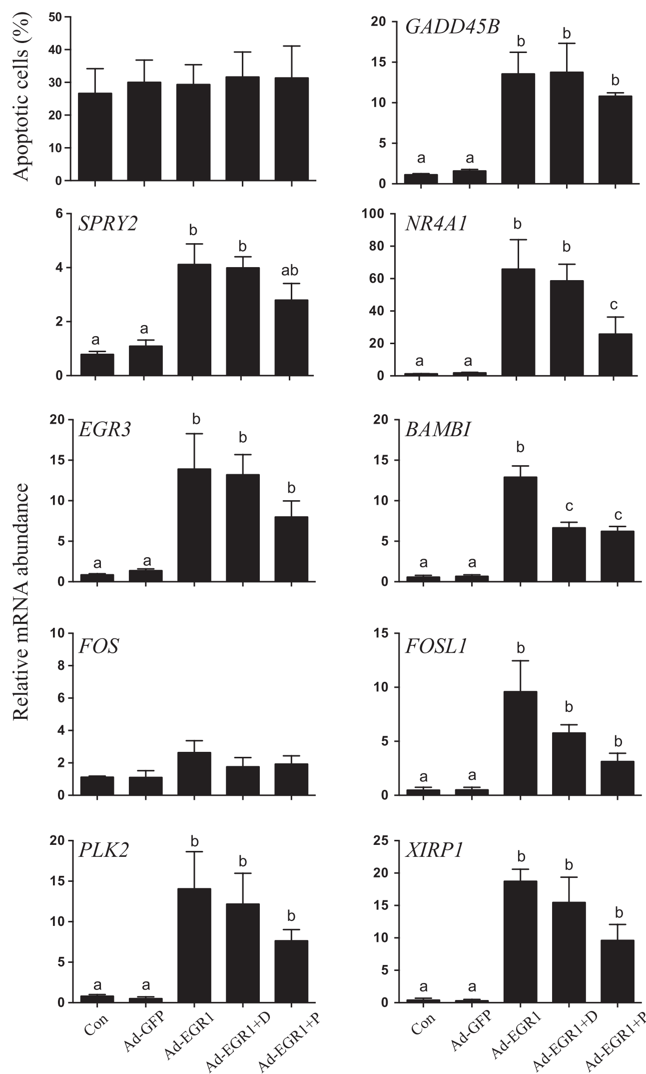

Figure 8 Effect of EGR1 on early response genes. Granulosa cells cultured in serum-free medium were exposed to polybrene alone (Con), Ad-GFP, Ad-EGR1, Ad-EGR1 plus DMSO (Ad-EGR1+D) or Ad-EGR1 plus the MAPK3/1 inhibitor PD98059 (Ad-EGF1+P). Infections were performed for 6 h and cells were harvested after 18 h for RNA extraction or assay of apoptosis by flow cytometry. The inhibitor was added 1 h before infection and included in infection and post-infection media. Messenger RNA levels were measured by real-time PCR. Data are means ± S.E.M. of three replicate cultures, and bars without common letters are significantly different (Tukey–Kramer HSD; $P<0.05$).

not *EGR3* mRNA levels. As overexpression of *EGR1* increased *EGR3* mRNA levels, it is not clear how FGF18 increased *EGR1* and not *EGR3* mRNA abundance in granulosa cells. One explanation may be the duration of the rise in EGR1 protein levels in the two cell models: forced overexpression likely resulted in an increase of greater amplitude and duration compared to a more transient rise expected *in vivo*, and this may have led to abnormal stimulation of *EGR3* expression. In this scenario, endogenous EGR1 alone may be insufficient

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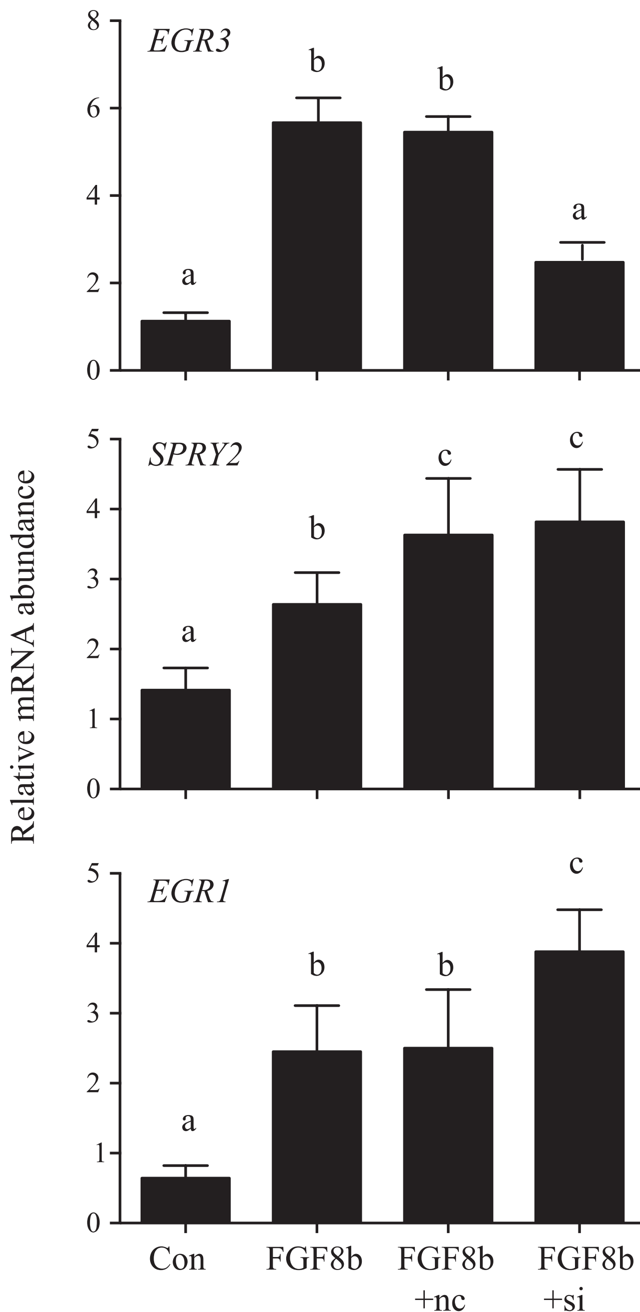

Figure 9 Knockdown of *EGR3* mRNA levels does not alter *SPRY2* mRNA levels but increases *EGR1* mRNA abundance. Granulosa cells were cultured in serum-free medium for 4 days and then transfected with EGR3 siRNA (+si) or negative control RNA (+nc). Twenty-four hours later, they were challenged with FGF8b for 2 h. Unchallenged cells were not treated with FGF8b (Con). Messenger RNA levels were measured by real-time PCR. Data are means ± s.e.m. of three replicate cultures, and bars without common letters are significantly different (Tukey–Kramer HSD; $P<0.05$).

to increase *EGR3* mRNA abundance, and specific signaling pathways are required to increase *EGR3* mRNA levels that may not be activated by FGF18. An alternative explanation is that FGF18 induces the expression or activity of a factor that suppresses or delays the expression of *EGR3*.

The inability of FGF18 to increase *EGR3* mRNA levels may be linked to its known inability to increase the abundance of mRNA of typical FGF-response genes, including *SPRY2* (Jiang *et al.* 2013). Therefore, we determined if knocking down *EGR3* mRNA levels would reduce the ability of a typical FGF (FGF8b in this case) to stimulate *SPRY2* mRNA levels; it did not. However, reducing *EGR3* mRNA levels resulted in an increase in *EGR1* mRNA levels, and it is possible that increased EGR1 compensated for reduced EGR3 in the stimulation of *SPRY2* mRNA levels. The relationship between *EGR1* and *EGR3* expression appears to be cell context specific, as overexpression of *Egr3* increased *Egr1* mRNA in mouse fibroblasts (Fang *et al.* 2013) but inhibited *Egr1* expression in T cells (Collins *et al.* 2008). Collectively, the present data suggest that EGR3 does not play a principal role in mediating the expression of FGF-response genes.

Overexpression of EGR1 (and increased EGR3) increased the abundance of mRNA encoding survival signals (MDM2 and GADD45B) and the ratio of *BCL2:BAX* mRNA, which is consistent with the effects of FGF2 on *GADD45B* mRNA previously observed (Portela *et al.* 2015). However, overexpression of EGR1 increased *TP53* mRNA levels, and although TP53 is a pro-apoptotic protein, there was no impact on the incidence of apoptosis. This is in contrast to a recent study, which demonstrated increased apoptosis in mouse granulosa cells transfected with EGR1 (Yuan *et al.* 2016); these two studies differed in overexpression strategy (infection vs transfection) and duration of exposure (18 h vs 48–72 h), which may have led to the different effects on apoptosis.

In summary, the present data demonstrate that the typical granulosa cell response to FGF is increased expression of the transcription factors EGR1 and EGR3. These factors likely account for downstream regulation of FGF-response genes including members of the SPRY and NR4A families. However, the atypical FGF18 remains an enigma, as it stimulated *EGR1* but not *EGR3* mRNA levels. Knocking down *EGR3* mRNA levels did not reduce the ability of FGF8b to stimulate *SPRY2* mRNA levels; therefore, we conclude that the pattern of *EGR1* and *EGR3* expression do not determine the cell response to FGF18.

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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Funding

This work was supported by the Natural Science and Engineering Research Council (NSERC), Canada, and P H was supported by a scholarship from the China Scholarship Council.

Acknowledgements

The authors thank Drs Markus Ehrengruber and John S Davis for providing Ad-EGR1 and Ad-GFP and Dr Parlow for making highly purified FSH available.

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Received 22 April 2017
First decision 15 May 2017
Revised manuscript received 26 June 2017
Accepted 21 July 2017

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