Differential actions of fibroblast growth factors on intracellular pathways and target gene expression in bovine ovarian granulosa cells

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Abstract

Several fibroblast growth factors (FGFs), including FGF1, FGF4 and FGF10, alter ovarian granulosa cell function. These ligands exhibit different patterns of receptor activation, and their mechanisms of action on granulosa cells remain unknown. The objective of this study was to identify the major pathways and target genes activated by FGF1, FGF4 and FGF10 in primary oestrogenic granulosa cells cultured under serum-free conditions. FGF1 and FGF4 increased levels of mRNA encoding Sprouty family members, SPRY2 and SPRY4, and the orphan nuclear receptors NR4A1 and NR4A3. Both FGF1 and FGF4 decreased levels of mRNA encoding SPRY3 and the pro-apoptotic factor BAX. FGF1 but not FGF4 stimulated expression of the cell cycle regulator, GADD45B. In contrast, FGF10 altered the expression of none of these genes. Western blot demonstrated that FGF4 activated ERK1/2 and Akt signalling rapidly and transiently, whereas FGF10 elicited a modest and delayed activation of ERK1/2. These data show that FGF1 and FGF4 activate typical FGF signalling pathways in granulosa cells, whereas FGF10 activates atypical pathways.

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Introduction

Fibroblast growth factors (FGFs) have been shown to play various roles in the regulation of reproductive processes. FGFs belong to a family of 22 closely related proteins (Itoh & Ornitz 2004), most of which signal through transmembrane receptor tyrosine kinases. In the ovary, the FGF that has been the attention of most research is FGF2. The FGF2 gene is predominantly expressed in the theca cell layer (Koos & Olson 1989, Stirling et al. 1991, Berisha et al. 2000) and acts upon granulosa cells. FGF2 has been demonstrated to promote granulosa cell proliferation, prevent apoptosis and decrease steroidogenesis in a number of species (Gospodarowicz & Bialecki 1992, Baird & Hsueh 1986, Yamoto et al. 1992, Lavranos et al. 1994, Vernon & Spicer 1994, Cao et al. 2006).

The mechanism of action of FGFs in the ovary has not been extensively explored. Studies from a variety of non-reproductive tissues and cell-lines have demonstrated that FGF receptor (FGFR) activation induces intracellular signalling through MAPKs, protein kinase C (PKC) and phosphatidylinositol-3-kinase (PI3K) and Akt activation (Dailey et al. 2005, Iwata & Hevner 2009). FGF signalling through MAPK appears to be ubiquitous whereas other pathways and actions of FGF vary between cell types (Dailey et al. 2005). The predominant pathways of FGF action in ovarian cells have received little attention; FGF2 activated PKC in rat granulosa cells (Peluso et al. 2001) and increased ERK1/2 and Akt phosphorylation in bovine granulosa cells (Jiang et al. 2011).

Activation of these pathways leads to expression of FGF target genes, including the Sprouty (SPRY) family of negative regulators of FGFR tyrosine kinase activity (Cabrita & Christofori 2008). In the ovary, FGF2 induced SPRY2 expression in human granulosa–luteal cells and mouse cumulus cells (Haimov-Kochman et al. 2005, Sugiuara et al. 2009) and increased levels of mRNA encoding both SPRY2 and SPRY4 in bovine granulosa cells (Jiang et al. 2011).

FGF2 is not the only FGF shown to act on granulosa cells. FGF1 is similar to FGF2 in that it is also expressed predominantly in theca cells in cattle (Berisha et al. 2004) and is mitogenic in granulosa and theca cells (Roberts & Ellis 1999). FGF1 has been shown to induce transient expression of SPRY2 in osteoblast cells (Yang et al. 2006), although its target genes in granulosa cells are unknown. Two other FGFs have been shown to alter SPRY expression in a reproductive tissue; FGF4 and
FGF10 increased SPRY2 mRNA abundance in human placenta (Anteby et al. 2005), but curiously neither FGF4 nor FGF10 increased SPRY2 mRNA levels in human luteal cells (Haimov-Kochman et al. 2005). Theca cells express FGF10 in cattle, and FGF10 inhibited oestradiol (E2) secretion from granulosa cells in vitro and in vivo (Buratini et al. 2007, Gasperin et al. 2007).

A comparison of FGF1, FGF4 and FGF10 could offer insight into differential FGF signalling, as they have different receptor (FGFR) activation patterns. There are seven FGFR proteins derived from four genes through alternative splicing events; each splice variant has specific ligand binding affinities (Zhang et al. 2006). Bovine granulosa cells express all receptors except FGFR2b (Zhang et al. 2006). FGF1 activates all six granulosa receptors, FGF4 activates the ‘c’ splice variants (FGFR1c, FGFR2c and FGFR3c), and FGF10 activates ‘b’ splice variants, predominantly FGFR2b (Zhang et al. 2006). The objective of this study was to determine whether FGF1, FGF4 and FGF10 have divergent signalling pathways and target genes in non-luteinizing bovine granulosa cells. The target genes studied were early response genes of the SPRY, ETV and NR4A families known to be regulated in granulosa cells (Jiang et al. 2011) as well as other cell types and growth and survival genes known to be regulated in various cancer cells (Karsan et al. 1997, Cosgrave et al. 2006).

Results

**FGF1 and FGF4 regulate gene expression in granulosa cells**

The addition of 10 ng/ml FGF1 increased the abundance of mRNA encoding SPRY2 and SPRY4 (P<0.01), decreased the abundance of mRNA encoding SPRY3 (P<0.05) and had no effect on SPRY1 mRNA levels (Fig. 1). The addition of 10 ng/ml FGF4 increased abundance of mRNA encoding SPRY2 and SPRY4 but had no effect on mRNA encoding SPRY1 or SPRY3 (Fig. 1). For both FGFs, the effect on SPRY2 mRNA levels was rapid, with a significant increase within 1 h, whereas the effects on SPRY4 were slower, not reaching significance until 4 h. In contrast, FGF10 had no effect on SPRY mRNA levels (Fig. 1). Addition of FSH also had no effect on SPRY mRNA levels (data not shown).

The effects of FGF1 and FGF4 on SPRY expression were dose dependent. When challenged for 2 h, both FGFs increased levels of mRNA for SPRY1, SPRY2 and SPRY4 and decreased those encoding SPRY3 (Fig. 2). Even at the highest dose tested, FGF10 did not alter SPRY mRNA abundance.

We then determined the effects of these FGFs on other FGF target genes, specifically members of the NR4A orphan nuclear receptors and the ETS transcription factor family. Addition of graded doses of FGF1 and FGF4 for 2 h caused significant increases in abundance of mRNA encoding NR4A1 and NR4A3 but had no effect on NR4A2 mRNA levels (Fig. 3). Even at the highest dose tested, FGF10 did not stimulate NR4A mRNA abundance.

FGF1 at 10 ng/ml caused a transient increase in ETV5 mRNA levels (Fig. 4) but did not alter abundance of mRNA encoding ETV1 or ETV4 (P>0.1; not shown). The addition of FGF4 did not affect abundance of mRNA encoding ETV1 or ETV4 (not shown) and almost doubled the abundance of ETV5 mRNA, but this did not reach significance (Fig. 4). FGF10 had no effect on abundance of mRNA encoding ETV1, ETV4 or ETV5.

The effects of FGFs on apoptosis and cell cycle genes were also measured. FGF1 stimulated abundance of mRNA encoding GADD45B and decreased BAX mRNA levels (Fig. 4), while having no effect on BIRC5 or BCL2.
mRNA levels (not shown). FGF4 decreased BAX mRNA levels without effect on GADD45B (Fig. 4), BIRC5 or BCL2 mRNA levels (not shown), and FGF10 did not alter abundance of mRNA of any of these genes.

To verify that the FGF10 was biologically active in our cell model, we cultured cells for 6 days and added 10 ng/ml FGF1 or FGF10 for the last 4 days of culture. FGF1 and FGF10 significantly reduced E2 concentrations compared with control (2.5 ± 0.3, 2.0 ± 0.3 and 8.0 ± 1.9 ng/ml respectively; P<0.05).

**Intracellular pathways activated by FGF in granulosa cells**

We further explored the discrepancy between the effects of FGF4 and FGF10. To verify whether both activate the MAPK pathway in granulosa cells, abundance of intracellular phosphorylated ERK1/2 was measured by western blot. FGF4 significantly increased the levels of pERK1/2 within 5 min (Fig. 5). In contrast, there was a weak gradual increase in pERK1/2 protein after challenge with FGF10 (Fig. 5); there was no main effect of time by ANOVA, but post hoc comparison of 120 and 240 min time points with the time 0 control (orthogonal contrasts) indicated a significant difference (P<0.05; Fig. 5).

As FGF4 increased ERK1/2 phosphorylation, we explored further intracellular signalling by FGF4. Inhibition of PI3K significantly inhibited basal SPRY1, SPRY2 and SPRY3 mRNA levels (Fig. 6). FGF4 increased SPRY1, SPRY2 and SPRY4 mRNA abundance, and cotreatment with PI3K inhibitor blocked the effect of FGF4 (Fig. 6A). As before, FGF4 did not stimulate SPRY3 mRNA levels. Pretreatment of cells with the PKC inhibitor GF109203X reduced basal SPRY expression, although this reached significance for SPRY1 only (Fig. 6B). Inhibition of PKC reduced FGF4-stimulated

**Discussion**

FGFs act on ovarian granulosa cells to inhibit steroid secretion, but the mechanisms involved are not clear. FGF2 activates the typical FGF pathway in granulosa cells, through pERK1/2 (Jiang et al. 2011), as do FGF1 and FGF4 (this study). However, the major finding of this study is that FGF10 signalling differs from that of the other FGFs and appears not to employ typical FGF signalling pathways.

Activation of ERK1/2 and increased expression of SPRY genes are generally considered to be typical responses to FGFs (Bottcher & Niehrs 2005, Dailey et al. 2005, Iwata & Hevner 2009), as observed for FGF2 in bovine granulosa cells (Jiang et al. 2011). In this study, FGF10 decreased E2 secretion from granulosa cells, as previously observed (Buratini et al. 2007, Gasperin et al. 2012), but did not result in rapid phosphorylation of ERK1/2 or acute upregulation of any SPRY gene studied. This in contrast to the activation of ERK1/2 by FGF10 observed in endometrial carcinoma cells, human and rat alveolar epithelial cells and in the developing mouse prostate bud (Taniguchi et al. 2003, Upadhya et al. 2005, Kuslak & Marker 2007), and the FGF10-induced increase in SPRY2 mRNA levels in the mouse lung epithelium and the human placenta (Tefft et al. 2002, Anteby et al. 2005, Natanson-Yaron et al. 2007). Thus, granulosa cells appear to be unusual in that the inhibitory effect of FGF10 on E2 secretion does not involve acute stimulation of ERK1/2 or the typical FGF
early-response genes, or that there is a species-specific difference in FGF10 signalling.

Addition of FGF1 and FGF4 to cultured granulosa cells resulted in rapid and dose-dependent increases in mRNA encoding SPRY2 and SPRY4 in a manner similar to that previously observed for FGF2 (Jiang et al. 2011). Higher doses of FGF1 were required to increase SPRY1 mRNA levels compared with those needed to stimulate SPRY2 or SPRY4, which is also compatible with the less robust regulation of SPRY1 observed for FGF2 (Jiang et al. 2011). The sixfold stimulation of SPRY2 mRNA by 50 ng/ml FGF4 observed here is in contrast with the lack of effect of the same dose of FGF4 reported in human luteal cells (Haimov-Kochman et al. 2005), and this may be explained by different patterns of FGFR expression in these cells; in cattle at least, granulosa cells express FGFR3c whereas luteal cells do not (Guerra et al. 2008). Of some interest is the inhibitory effect of FGF1 and, to a lesser extent, FGF4 on SPRY3 mRNA levels, which was not previously observed for FGF2 (Jiang et al. 2011). To our knowledge, the one other report describing regulation of SPRY3 expression comes from a study of Xenopus embryos involving brain-derived neurotropin factor and not FGF signalling (Panagiotaki et al. 2010).

Other putative FGF response genes are not well described in mammals. In lower vertebrates, FGF1 and FGF4 are expressed in a similar manner to FGF2, with little evidence for responsiveness to FGF5 (Guerra et al. 2008). This is in contrast to the more robust response of bovine granulosa cells to FGF2 and FGF4 (Jiang et al. 2011). In this study, addition of FGF1, FGF4 and FGF10 to cultured bovine granulosa cells resulted in rapid and dose-dependent increases in mRNA encoding the orphan nuclear receptors NR4A1, NR4A2 and NR4A3 in a manner similar to that previously observed for FGF2 (Jiang et al. 2011). Higher doses of FGF1 were required to increase NR4A1 mRNA levels compared with those needed to stimulate NR4A2 or NR4A3, which is also compatible with the less robust regulation of NR4A1 observed for FGF2 (Jiang et al. 2011). The sixfold stimulation of NR4A2 mRNA by 50 ng/ml FGF4 observed here is in contrast with the lack of effect of the same dose of FGF4 reported in human luteal cells (Haimov-Kochman et al. 2005), and this may be explained by different patterns of FGFR expression in these cells; in cattle at least, granulosa cells express FGFR3c whereas luteal cells do not (Guerra et al. 2008). Of some interest is the inhibitory effect of FGF1 and, to a lesser extent, FGF4 on NR4A1 mRNA levels, which was not previously observed for FGF2 (Jiang et al. 2011). To our knowledge, the one other report describing regulation of NR4A1 expression comes from a study of Xenopus embryos involving brain-derived neurotropin factor and not FGF signalling (Panagiotaki et al. 2010).

Figure 3 Effect of FGF1, FGF4 and FGF10 on abundance of mRNA encoding the orphan nuclear receptors NR4A1, NR4A2 and NR4A3 in bovine granulosa cells in serum-free culture. Cells were challenged on day 5 of culture for 2 h with the doses of FGF given. Data are mean ± s.e.m. of three independent replicates. For each treatment, means with common or no letters are not significantly different (P<0.05).

Figure 4 Effect of FGF1, FGF4 and FGF10 on abundance of mRNA encoding the transcription factor ETV5, the cell cycle regulator GADD45B and the apoptosis-associated protein BAX. Bovine granulosa cells were cultured in serum-free medium for 5 days and then challenged with 10 ng/ml FGF for the times given. Data are mean ± s.e.m. of three independent replicates. For each treatment, means with common or no letters are not significantly different (P<0.05).
FGF4 were shown to stimulate expression of ETV4 (McCabe et al. 2006, Eloy-Trinquet et al. 2009), but this was not observed in this study. FGF2 stimulated ETV5 mRNA levels in bovine granulosa cells (Jiang et al. 2011), and this study shows that FGF1 had a similar effect. FGF4 stimulated ETV5 expression in the chick embryo (Firnberg & Neubuser 2002), and a trend in this direction was observed in granulosa cells, but this did not reach significance. In this study, FGF1 and FGF4 stimulated levels of mRNA encoding the orphan nuclear receptors NR4A1 and NR4A3, in partial agreement with the effect of FGF2 on NR4A1 expression in bovine granulosa cells (Jiang et al. 2011). We are not aware of other reports describing regulation of NR4A family members by FGF1 or FGF4.

Some members of the FGF family are mitogenic and/or anti-apoptotic, including FGF1 (Roberts & Ellis 1999); therefore, we assessed the effects of FGF1 and FGF4 on select death/survival genes. FGF1 and FGF4 decreased abundance of mRNA encoding the pro-apoptotic protein BAX, whereas we previously showed that FGF2 did not alter BAX mRNA (Jiang et al. 2011), and FGF1 increased levels of mRNA encoding the cell cycle regulator GADD45B, consistent with a similar effect of FGF2 in granulosa cells (Jiang et al. 2011). Neither FGF1, FGF4 (this study) nor FGF2 (Jiang et al. 2011) altered abundance of mRNA encoding the cell survival genes BIRC5 and BCL2 in granulosa cells, which is in contrast to effects of FGF in cancer cells (Karsan et al. 1997, Cosgrave et al. 2006). Thus, the effects of FGF on survival/death genes appear to be cell type and ligand specific.

As FGF10 did not stimulate any of the target genes examined, we could not use inhibitors to explore the intracellular pathways activated by this growth factor. However, experiments with FGF4 indicated that inhibition of PI3K or PKC pathways completely inhibited the ability of FGF4 to stimulate SPRY1 and SPRY4 mRNA levels and attenuated the stimulation of SPRY2. These results are in agreement with the effects of FGF2 in granulosa cells (Jiang et al. 2011), consistent with the similar receptor activation pattern of these two ligands

Figure 5 FGF4 and FGF10 regulation of ERK1/2 phosphorylation. Bovine granulosa cells were cultured in serum-free medium for 5 days and then challenged with 10 ng/ml FGF4 or FGF10 for the times given. One representative western blot for phospho- and total ERK1/2 is shown. Data are mean ± S.E.M. of three independent replicates. Means without common letters are significantly different (P<0.05); asterisks denote difference from time 0 by orthogonal contrasts.

Figure 6 Involvement of the PI3K and PKC signalling pathways in FGF4 stimulation of SPRY mRNA abundance. Cells were challenged for 2 h with FGF4 (10 ng/ml) with and without pretreatment with PI3K inhibitor LY294002 (panel A) or PKC inhibitor GF109203X (panel B). Phosphorylation of Akt in response to FGF4 was measured by western blot (panel C). Data are mean ± S.E.M. of three independent replicates. For each mRNA, means with common or no letters are not significantly different (P<0.05); asterisks in panel C denote difference from time 0. C, control; L, LY294002; F, FGF4; G, GF109203X; FL, FGF4+LY294002; FG, FGF4+GF109203X.
(Zhang et al. 2006). These inhibitors also attenuated SPRY mRNA abundance in the absence of FGF, which we have previously observed (Jiang et al. 2011), and which suggests that PI3K and PKC pathways are active in these cells, although we cannot rule out potential non-specific effects of these inhibitors on these cells. Taken together, these data demonstrate that FGF1, which activates all FGFRs, and FGF4, which activates the ‘c’ splice variants, had similar actions on ovarian granulosa cells and activated typical FGF target genes. In contrast, FGF10, which activates only FGFR2b, did not activate the typical FGF target genes. The mechanism of action of FGF10 remains to be determined.

Materials and Methods

Cell culture

All materials were obtained from Invitrogen Life Technologies unless otherwise stated. Bovine granulosa cells were cultured in serum-free conditions under which E2 and progesterone secretion increase with time over 6 days and are responsive to FSH (Gutiérrez et al. 1997). FSH stimulates abundance of oestrogenic enzymes (Silva & Price 2000, Sahmi et al. 2004) through a predominantly PKA/CAMP pathway (Silva et al. 2006), and there is little evidence of luteinization as determined by the lack of increase in mRNA encoding STAR protein (Sahmi et al. 2004, Zheng et al. 2008). These cells respond to FGF2 and FGF10 with dose-dependent decreases in steroid secretion when added for 6 days of culture (Cao et al. 2006, Buratini et al. 2007).

Bovine ovaries were obtained from adult cows, irrespective of stage of the oestrous cycle, at an abattoir and transported to the laboratory at 30°C and 200 mmHg CO2, 95% air for 6 days, with treatment as appropriate for detecting increases in both SPRY2 and SPRY4 mRNA. As a negative control for SPRY expression, cells were also challenged with 10 ng/ml FSH for the same periods examined for FGFs. Specific signalling inhibitors were also used to dissect the major signalling pathway activated by FGF4. The inhibitors were GF109203X, an inhibitor of PKC (3 μmol; Sigma–Aldrich), and LY294002, a PI3K inhibitor (20 μmol). Inhibitors were dissolved in dimethyl sulfoxide (DMSO) and added directly to the medium. The cells were pretreated with inhibitors for 1 h before adding FGF4 for a further 2 h. Controls were treated with DMSO. Each pool of cells collected on a specific day constituted one replicate, and all experiments were performed with three independent pools of cells. For logistical reasons, treatments (FGF1, FGF4 or FGF10) were not applied to the same replicate.

To verify that FGF10 was biologically active, 10 ng/ml FGF10 was included in the medium added on days 2 and 4, and E2 concentrations in medium on day 6 were compared with cells treated with FGF1 (10 ng/ml) or with medium alone.

Total RNA extraction and real-time RT-PCR

After treatments, the culture medium was removed and total RNA was extracted using TRIzol according to the manufacturer’s instructions. Total RNA was quantified by absorbance at 260 nm. RT was performed on 1 μg DNase-treated total RNA in the presence of 1 mmol/l oligo(dT) primer and 4 U Omniscript RTase (Qiagen), 0.25 mmol/l dideoxynucleotide triphosphate (dNTP) mix and 19.33 U Rnase Inhibitor (GE Healthcare, Baie D’Urfé, QC, Canada) in a volume of 20 μl at 37°C for 1 h. The reaction was terminated by incubation at 93°C for 5 min.

Real-time PCR was performed on a 7300 Real-Time PCR system (Applied Biosystems, Streetsville, ON, Canada) with Power SYBR Green PCR Master Mix. The bovine-specific primers for target genes have previously been published (Jiang et al. 2011). Common thermal cycling parameters (3 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 59°C and 30 s at 72°C) were used to amplify each transcript. Melting curve analyses were performed to verify product identity. Samples were run in duplicate and were expressed relative to the histone H2AFZ as housekeeping gene. This gene is routinely used in our laboratory and shows similar stability to cyclophilin A, both of which were more stable in granulosa cells than glyceraldehyde-3-phosphate dehydrogenase as determined by geNorm Software (Ghent, Belgium) (Ramakers et al. 2003). Data were normalized to a calibrator sample using the ΔΔCt method with correction for amplification efficiency (Pfaffl 2001).

Western blotting

After challenge with FGF4 or FGF10, cells were washed with cold PBS and lysed in 100 μl/well cold RIPA buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate and protease inhibitor cocktail). The lysate was centrifuged at 6000 g for 5 min at 4°C. The resulting supernatant was retained and stored at −20°C. Protein concentrations were determined by BCA protein assay (Pierce; Rockford IL, USA).
E2 assay

E2 was measured in medium in duplicate by RIA. The antibody was raised in rams as described previously (Sanford 1987), and the tracer was E2(2,4,6,7,16,17-3H; Perkin Elmer, Montreal, QC, Canada). Free and bound tracers were separated by incubation with dextran–charcoal. This antibody crossreacted with oestrone (15%) but not with other steroids tested. The intra-assay coefficient of variation of the single assay used was <7%, and the sensitivity of the assay was 0.70 ng/ml medium.

Statistical analysis

All statistical analyses were performed with JMP Software (SAS Institute, Cary, NC, USA). Data were transformed to logarithms if they were not normally distributed (Shapiro–Wilk test). ANOVA was used to test the main effects of treatments, and culture replicate was included as a random variable in the F-test. Differences between means were tested with the Tukey–Kramer honestly significant difference (HSD) test. The data are presented as least square means ± s.e.m.

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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