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Fibroblast growth factor 9 (FGF9) regulation of cyclin D1 and cyclin-dependent kinase-4 in ovarian granulosa and theca cells of cattle

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ABSTRACT

To determine the mechanism by which fibroblast growth factor 9 (FGF9) alters granulosa (GC) and theca (TC) cell proliferation, cell cycle proteins that regulate progression through G1 phase of the cell cycle, cyclin D1 (CCND1) and cyclin-dependent kinase-4 (CDK4; CCND1's catalytic partner), were evaluated. Ovaries were obtained from a local abattoir, GC were harvested from small (1–5 mm) and large (8–22 mm) follicles, and TC were harvested from large follicles. GC and TC were plated in medium containing 10% fetal calf serum followed by various treatments in serum-free medium. Treatment with 30 ng/mL of either FGF9 or IGF1 significantly increased GC numbers and when combined, synergized to further increase GC numbers by threefold. Abundance of *CCND1* and *CDK4* mRNA in TC and GC were quantified via real-time PCR. Alone and in combination with IGF1, FGF9 significantly increased *CCND1* mRNA expression in both GC and TC. Western blotting revealed that CCND1 protein levels were increased by FGF9 in TC after 6 h and 12 h of treatment, but CDK4 protein was not affected. A mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway inhibitor, U0126, significantly reduced FGF9-induced *CCND1* mRNA expression to basal levels. For the first time we show that *CCND1* mRNA expression is increased by FGF9 in bovine TC and GC, and that FGF9 likely uses the MAPK pathway to induce *CCND1* mRNA production in bovine TC.

1. Introduction

The fibroblast growth factor (FGF) family consists of 22 polypeptides that act primarily as paracrine signaling molecules and bind to one or more of the FGF receptors (FGFRs) that include: FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3c, and FGFR4 (Chaves et al., 2012; Laestander and Engström, 2014). Members of the FGF family are further divided into seven sub-families based on their receptor affinities; these families include FGF1 (includes FGF1 which binds all FGF receptors and FGF2 which binds to FGFR1c and -2c), FGF4 (includes FGF4, -5, and -6 which binds to FGFR1c and -2c), FGF7 (includes FGF7, -3, -10, and -22 which bind to FGFR2b and -1b), FGF8 (includes FGF8, -17, and -18 which bind to FGFR3c, 4, and 1c), FGF9 (includes FGF9, -16, and -20 which primarily bind to FGFR3c, -2c, and -4), FGF19 (includes FGF19, -21, and -23; endocrine factors that weakly bind to FGFR1c and -2c), and FGF11 (includes FGF11, -12, -13, and -14 which do not activate FGFRs) (Ornitz et al., 1996; Pownall and Isaacs, 2010; Laestander and Engström, 2014). However, not all FGFs or FGFRs are present in every cell type of every species.

In ovarian tissue of cattle, FGF2 is localized to granulosa cells (GC) and theca cells (TC) (van Wezel et al., 1995), FGF7 is present in TC (Parrott and Skinner, 1998), FGF8, -9, -10, and -17 are expressed in both GC and TC (Buratini et al., 2005, 2007; Drummond et al., 2007; Machado et al., 2009; Schreiber et al., 2012), and FGF18 is present in TC (Portela et al., 2010). Furthermore, FGFR2b, -2c, -3b and -3c are expressed in bovine GC (Parrott and Skinner, 1998; Berisha et al., 2000, 2004; Buratini et al., 2005), while FGFR2c, -3c and -4 are expressed in bovine TC (Berisha et al., 2004; Buratini et al., 2005). Thus, it should be expected that GC and TC would respond differently to the various FGFs.

FGF9 gene expression was first discovered in the rat ovary by Drummond et al. (2007) and later in the bovine ovary by Grado-Ahuir et al. (2011) who found that *FGF9* mRNA in GC was down-regulated in ovarian follicular cysts compared to normal-follicles. Subsequent studies found that FGF9 treatment significantly increases bovine GC and TC proliferation while inhibiting steroidogenesis in vitro (Schreiber and Spicer, 2012; Schreiber et al., 2012).

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Furthermore, *FGF9* mRNA is present in both TC and GC, with cells from small follicles having greater relative abundance compared to cells from large follicles (Schreiber and Spicer, 2012; Schreiber et al., 2012), and GC having a greater abundance of *FGF9* mRNA than TC (Schreiber et al., 2012) indicating that FGF9 may be playing both a paracrine and an autocrine role in growing follicles.

An increase in cell proliferation is mediated by increased speed of the cell cycle which is divided into four phases: gap 1 (G_1), synthesis (S), gap 2 (G_2), and mitosis (M) (Lim and Kaldis, 2013). Each of these phases are regulated by different proteins at specific checkpoints, insuring that the cell cycle progresses in the correct order, and only when the cell is healthy. Checkpoints include the restriction (R) point within G_1, G_1/S, S/G_2, G_2/M and M (Nigg, 2001; Lim and Kaldis, 2013). The most important protein families for cell cycle regulation consist of cyclins (CCNs), cyclin-dependant kinases (CDKs), and cyclin-dependant kinase inhibitors (CDKIs) (Bendris et al., 2015). Cyclins are the regulatory subunit of CDKs and once they are bound together, the dimer can activate its respective checkpoint to allow progression of the cell cycle; however, dimers can also be inactivated by CDKIs (Bendris et al., 2015). Each checkpoint is activated by specific proteins as follows: R point, CCND and CDK4 or -6; G1/S phase, CCNE and CDK2; S/G2 phase, CCNA and CDK2; G2/M phase, CCNA and CDK1; and M phase, CCNE and CDK1 (Nigg, 2001; Alberts et al., 2002; Lim and Kaldis, 2013). Therefore, we hypothesized that FGF9 increases cell proliferation by altering specific cell cycle proteins. Previous studies with ovarian endometrioid adenocarcinomas (Schwartz et al., 2003), human uterine endometrial stromal cells (Wing et al., 2005) and endometrial cancer tissues (Chan et al., 2012) have shown a link between FGF9 and CCND1. Therefore, our objectives were to characterize the change in *CCND1* and *CDK4* mRNA after FGF9 treatment of bovine GC and TC, and to determine which intracellular pathway mediated this change.

2. Materials and Methods

2.1. Reagents and hormones

Reagents used during cell culture included: Ham's F-12, Dulbecco modified Eagle medium (DMEM), gentamicin, sodium bicarbonate, streptomycin/penicillin, TRI reagent, trypan blue, protease, collagenase, hyaluronidase, and deoxyribonuclease from Sigma-Aldrich Chemical Company (St. Louis, MO); and fetal calf serum (FCS) from Atlanta Biologicals (Atlanta, GA).

The hormones and inhibitors used during cell culture included: ovine FSH (175 × NIH-FSH-S1 U/mg) and ovine LH (NIADDK-NIH-26; AFP5551B) from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA); testosterone from Steraloids (Newport, RI); recombinant human IGF1 and FGF9 (without carrier protein) from R&D Systems, Inc. (Minneapolis, MN); LY294002 (a PI3K inhibitor), H89 (a PKA inhibitor), U0126 (a MAPK/ERK inhibitor) and wortmannin (a PI3K inhibitor) from Enzo Life Sciences Inc. (Farmingdale, NY).

2.2. Cell culture

Ovaries were collected from cattle at a local abattoir, and follicular fluid was aspirated from small (1–5 mm) and large (8–22 mm) follicles, and GC and TC were isolated as previously described (Langhout et al., 1991; Lagaly et al., 2008; Spicer et al., 2002, 2009). Isolated GC and TC were resuspended in medium containing 1.5 mg/mL of collagenase and 0.5 mg/mL of DNase to prevent clumping as previously described (Lagaly et al., 2008; Spicer et al., 2009). Cell viability was determined by trypan blue exclusion method on a 0.1 mm deep hemocytometer (American Optical Corporation, Buffalo, NY). Viability of bovine GC from small and large follicles and TC from large follicles averaged 69%, 61%, and 92%, respectively.

Cells were plated on 24-well Falcon multi-well plates (Becton Dickinson, Franklin Lakes, NJ) in 1 mL of medium (1:1 DMEM and Ham's F12 with 2.0 mM glutamine, 0.12 mM gentamicin, and 38.5 mM sodium bicarbonate) or in 60 mm Falcon culture dishes (Becton Dickson) in 4 mL of medium with and average plating density of 2.0×10^5 (range $0.8–4.4 \times 10^5$) or 8.8×10^5 viable cells per well or plate, respectively, and cultured at 38.5 °C with 5% CO_2. Cells were cultured in the presence of 10% FCS until they reached 80% confluency (i.e., 48 h–96 h) with a medium change every 24 h. Cells were then washed twice with 0.5 mL of serum-free medium followed by treatment with various hormones, administered in serum-free medium, for 6 h, 12 h, 24 h, and/or 48 h depending on the experiment.

2.3. Experimental design

Experiment 1 was conducted to evaluate the interaction between FGF9 and IGF1 on cell proliferation. Granulosa cells from small- and large-follicle were cultured for 48 h in 10% FCS, and then GC were washed with serum-free medium and treated with 30 ng/mL of FSH and 30 ng/mL of IGF1, 30 ng/mL of FGF9 or both for an additional 48 h. GC were treated with FSH because the cell proliferation responses to insulin, IGF1 and FGF9 are greater in its presence than its absence (Spicer et al., 2002, 2011; Spicer and Aad, 2007; Schreiber and Spicer, 2012).

Experiment 2 was designed evaluate if FSH and IGF1 interact with FGF9 to regulate *CCND1* mRNA accumulation in small-follicle GC. After 48 h of culture in 10% FCS, GC were washed in serum-free medium and serum-starved for 24 h in order to synchronize cells in G1 of the cell cycle (Wing et al., 2005). Cells were then treated with either no addition (control), FSH (30 ng/ml) or IGF1 (30 ng/ml) with or without 30 ng/mL of FGF9 for 12 h and RNA was collected.

Experiment 3 was conducted to evaluate the effects of FGF9 on *CCND1* and *CDK4* gene expression in small- and large-follicle GC. After 48 h of culture in 10% FCS, GC were washed with serum-free medium and treated with 30 ng/mL of FSH and 30 ng/mL of IGF1 with or without 30 ng/mL of FGF9 and RNA was collected 24 h after treatment. GC were treated with both FSH and IGF1 because the cell proliferation response to FGF9 is greater in their presence than their absence (Schreiber and Spicer, 2012).

Experiment 4 was designed to determine the effect of LH on the *CCND1* and *CDK4* mRNA response to FGF9. After 72 h in 10% FCS medium, large-follicle TC were washed in serum-free medium and treated with IGF1 (30 ng/mL) and either no other additions (Control), LH (30 ng/mL), FGF9 (30 ng/mL), or both LH and FGF9; RNA was collected 12 h after treatment.

Experiment 5 was designed to determine which intracellular pathway(s) FGF9 uses to increase *CCND1* and/or *CDK4* mRNA expression. After 72 h in 10% FCS, large-follicle TC were washed with serum-free medium and treated with: 1) vehicle control; 2) 30 ng/mL of IGF1 and 30 ng/mL of FGF9; 3) FGF9 plus IGF1 and 20 μM of U0126, a MAPK/ERK inhibitor; 4) FGF9 plus IGF1 and 10 μM of H89, a PKA inhibitor; 5) FGF9 plus IGF1 and 200 nM of wortmannin, a PI3K/AKT inhibitor; or 6) FGF9 plus IGF1 and 10 μM of LY294002, a PI3K/AKT inhibitor. RNA was collected 24 h after treatment. The selected doses of inhibitors were based on previous studies (Asselin et al., 2001; Poretsky et al., 2001; Dewi et al., 2002; Laurich et al., 2002; Silva et al., 2006).

Experiment 6 was designed to determine the effect of U0126 on *CCND1* mRNA expression in IGF1- and FGF9-treated large-follicle TC. After 96 h in 10% FCS medium, large-follicle TC were washed in serum-free medium and treated with no addition (Control), IGF1