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Transcriptome profiling of bovine ovarian theca cells treated with fibroblast growth factor 9

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ABSTRACT

We reported previously that fibroblast growth factor 9 (FGF9) acts as an antidifferentiation factor, stimulating proliferation of granulosa cells (GCs) and theca cells (TCs) while suppressing hormone-induced steroidogenesis of these cells. How FGF9 acts to simultaneously suppress steroidogenesis and stimulate proliferation remains to be fully elucidated. Thus, this study was undertaken to clarify the effects of FGF9 on the TC transcriptome. Ovaries were obtained from beef heifers at a local abattoir, TCs were isolated from large antral follicles, and cultured with or without 30 ng/mL of FGF9 for 24 h in the presence of LH and IGF-1. After treatment, total RNA was extracted from TC and processed for microarray using Affymetrix GeneChip Bovine Genome Arrays (n = 4/group). Transcriptome analysis comparing FGF9-treated TC with control TC using 1.3-fold cutoff, and a $P < 0.05$ significance level identified 355 differentially expressed transcripts, with 164 elements upregulated and 191 elements downregulated by FGF9. The ingenuity pathway analysis (IPA) was used to investigate how FGF9 treatment affects molecular pathways, biological functions, and the connection between molecules in bovine TC. The IPA software identified 346 pathways in response to FGF9 in TC involved in several biological functions and unveiled interesting relationships among genes related to cell proliferation (eg, *CCND1*, *FZD5*, and *MYB*), antioxidation/cytoprotection (eg, *HMOX1* and *NQO1*), and steroidogenesis (eg, *CYP11A1* and *STAR*). Overall, genes, pathways, and networks identified in this study painted a picture of how FGF9 may regulate folliculogenesis, providing novel candidate genes for further investigation of FGF9 functions in ovarian follicular development.

1. Introduction

Fibroblast growth factors (FGFs) constitute a large family of single chain polypeptide factors present in both vertebrates and invertebrates [1,2]. Currently, 22 different members have been reported in mammals (FGF 1–23), binding to high affinity receptors and several cofactors to regulate a variety of biological processes in various tissues [3–5]. One of these, fibroblast growth factor 9 (FGF9), was originally isolated from human glioma cells and characterized as a mitogenic factor [6]. In the last 2 decades, research has shown that FGF9 plays diverse roles in many different tissues, including heart [7,8], cartilage [9], liver [10], and the reproductive system [11,12]. In these tissues, FGF9 binds to FGF receptors (*FGFR1c*, *FGFR2c*, *FGFR3c*, *FGFR3b*, and *FGFR4*) to activate specific tyrosine residues and downstream intracellular signaling pathways, including the mitogen activated protein kinase-like protein-mitogen activated protein kinase-like protein, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-serine/threonine kinase (AKT), phospholipase Cγ, and signal transducers and activators of transcription pathways, which regulate cell

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proliferation, survival, metabolism, and differentiation [13]. In fact, FGF9 has been shown to regulate sex determination [14], steroidogenesis [15–17], tissue development [18], and even mood disorders [19]. Moreover, the expression of FGF9 has been related to brain [20] and gastric [21] cancers.

In the ovaries, FGF9 was first described to be present in murine corpora lutea, stromal, and theca cells (TCs) and was observed to stimulate progesterone (P4) production by granulosa cells (GC) in a paracrine way [12]. In porcine GC, FGF9 in the presence of IGF-1 stimulated cell proliferation and steroidogenesis [22]. In cattle, FGF9 has been suggested to be an antidifferentiation factor by stimulating in vitro proliferation of TC and GC while suppressing P4 production by TC and GC and estradiol (E2) production by GC in the presence of IGF-1 [15,16]. Abundance of FGF9 messenger RNA (mRNA) in GC and TC is hormonally regulated [15,16] and changes according to the size and estrogenic status of follicles and days after ovulation in cattle [23]. Hence, it seems clear that FGF9 is an important regulator of ovarian function in mammals, but its role may differ between monotocous and polytocous animals. Nevertheless, detailed information about signaling mechanisms activated by FGF9 in the ovary is lacking.

The technology of microarray is a powerful tool for one to investigate how a specific cell type reacts to certain stimuli, enabling the simultaneous measurement of thousands of gene transcripts [24]. Therefore, we used this resource to investigate how bovine TC from large ovarian follicles respond to exogenous FGF9 in vitro. The present study unveils novel signaling pathways activated by FGF9 in TC and may provide valuable information to understand molecular aspects of cell proliferation, steroidogenesis, and apoptosis in a mono-ovulatory species such as cattle and humans.

2. Material and methods

2.1. Reagents and hormones

The reagents used in cell culture were Ham's F-12 (F12), Dulbecco's Modified Eagle's Medium (DMEM), gentamicin, glutamine, sodium bicarbonate, trypan blue, deoxyribonuclease (DNase), and collagenase from Sigma–Aldrich Chemical Co. (St. Louis, MO) and fetal calf serum (FCS) from Equitech-Bio, Inc. (Kerrville, TX). The hormones used in cell culture were recombinant human FGF9 and IGF-1 (R&D Systems, Minneapolis, MN; all carrier-free), and ovine LH (NIDDK-oLH-26; activity: 1.0× NIH-LH-S1 U/mg) from the National Hormone and Pituitary Program (Torrance, CA).

2.2. Cell collection and in vitro culture

Ovaries were obtained from beef heifers (n = 76) at a local abattoir and transported to the laboratory in 0.9% saline with 1% streptomycin/penicillin on ice. Theca cells were isolated from large antral follicles (8.1–22 mm in surface diameter) with adequate vascularity and moderately transparent follicular fluid as previously described [16,25]. Briefly, follicles were bisected, GCs were scraped free from the theca interna, and the theca interna tissue was removed via microdissection and enzymatically digested for 1 h at 37°C on a rocking platform. Nondigested thecal tissue was removed via filtration through a 149-μm mesh screen (Gelman Sciences, Ann Arbor, MI). Theca cells were then centrifuged at 50 × *g* for 7 min, washed twice in medium (1:1 DMEM and F12 containing 2.0-mM glutamine, 0.12-mM gentamicin, and 38.5-mM sodium bicarbonate), and resuspended in serum-free medium containing collagenase and DNase at 1.25 and 0.5 mg/mL, respectively, to prevent clumping of cells before plating [16,26].

Viability of TC was determined by trypan blue exclusion test, and 3×10^5 viable cells (per well) were transferred to Falcon 24-well multiwell plates (Becton Dickinson, Lincoln Park, NJ) with medium containing 10% FCS. Cells were cultured at 38.5°C in a humidified 95% air and 5% CO_2 environment for the first 48 h with a medium change at 24 h. Then, TCs were washed twice in serum-free medium and cultured in 1 mL serum-free medium containing 15 ng/mL of LH and 15 ng/mL of IGF-1 with or without 30 ng/mL of FGF9 for 24 h. Dose of *IGF1*, LH, and FGF9 was based on the previous studies [16,25].

2.3. RNA extraction, microarray and statistical analyses

Following treatment, TCs were lysed with 0.5 mL of TRIzol reagent (Life Technologies Inc., Gaithersburg, MD), and total RNA was extracted as previously described [26,27]. Affymetrix GeneChip Bovine Genome Arrays (Affymetrix, Santa Clara, CA) were used for the microarray as previously described [28]. This particular array is designed to monitor the expression of approximately 23,000 bovine transcripts through 24,072 probe sets. A total of 8 chips were hybridized with RNA extracted from 4 biological replicates of the same number of TC pools in a paired design for the 2 treatments (FGF9 or control). Each pool of TC was generated from 5 to 7 large follicles collected from 4 to 5 animals. The processing of RNA, including RNA purification and hybridization of microarray slides, was performed by the University of Tulsa Microarray Core Facility. Affymetrix GeneChip Operating Software (GCOS, ver. 1.1.1, Affymetrix, Santa Clara, CA) was used to quantitate each GeneChip. A false discovery rate (FDR) threshold was set at $P = 0.10$. Summary intensities for each probe were loaded into DNA-Chip Analyzer (dChip), version 1.3, for normalization, standardization, and analysis. Paired *t* tests were calculated using dChip to evaluate the significant differences between treatments as previously described [27,28].

2.4. Microarray functional data analysis

To explore the biological knowledge associated with the statistically significant probe sets from the microarray chips in addition to the annotation produced along with statistical comparisons in dChip, the QIAGEN's ingenuity pathway analysis (IPA; QIAGEN Redwood City, http://www.qiagen.com/ingenuity) was used. The analyses were performed to investigate how FGF9 treatment affects molecular pathways, biological functions, and the connection between molecules in bovine TC.