The expression pattern of fibroblast growth factor 10 and its receptors during buffalo follicular development

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Abstract: This study explored the expression and localization of fibroblast growth factor (FGF) 10 and its receptors (FGF receptor 1 and FGF receptor 2, FGFR1 and FGFR2) during buffalo follicular development, laying a foundation for the further study of FGF signaling pathways in follicular development and oogenesis. Granulosa cells and ovarian follicles were extracted from buffalo ovaries, and in vitro maturation culture of oocytes was conducted. Immunohistochemistry was performed to detect the expression of FGF10 and its receptors FGFR1 and FGFR2. In addition, immunofluorescence staining was used to detect the expression of FGF10 in buffalo cumulus oocyte complexes (COCs). Moreover, mRNA levels of FGF10, sub-types of FGFR1 and FGFR2 (FGFR1b and FGFR2b) were measured using qRT-PCR. Immunohistochemistry results showed that FGF10 and its receptors FGFR1 and FGFR2 appeared to have positive responses in buffalo primordial follicles, primary follicles, secondary follicles, and mature follicle oocytes and granulosa cells, and mature follicle basal membrane cells. However, no expression of FGF10 mRNA was detected in granulosa cells from follicles of different diameters, but immunofluorescence results showed that FGF10 could be detected in both cumulus cells and oocytes. With an increase in the in vitro maturation time of buffalo COCs, FGF10 and receptor sub-types FGFR1b and FGFR2b mRNA expression also gradually increased, and significantly higher than before maturation. In summary, FGF10 and its receptors may be involved in the process of buffalo follicular development and oocyte maturation.

Keywords: Buffalo, FGF10, FGFR, follicular development, oocyte

Introduction

Follicular development is a highly complex and precisely regulated process. A follicle is a functional organizational unit of the ovary, a place of development of oocytes and secreting sex hormones [1]. A large number of follicles at different stages are stored in the ovaries. According to the shape and developmental degree of the follicle, the follicle can be divided into the primordial follicle, the developing follicle, and the mature follicle, and the developing follicle is divided into primary follicle and secondary follicle. Follicles are composed of oocytes, granulosa cells, and theca cells. Ovarian follicular development starts from the generation of primordial follicles in which squamous somatic cells, often called pre-granulosa cells, encircle a primary oocyte arrested at the first meiotic prophase [2]. Therefore, oocytes are required from the very beginning of follicular development. Follicular cells and granulosa cells also play a role in follicular development. In the process of follicle growth and development, the granulosa cells, the theca cells, the oocytes and the stromal cells can also secrete a series of cytokines to promote and/or regulate the differentiation, development and maturation process of oocytes [2, 3].

Fibroblast growth factors (FGFs) play important roles in follicular development in a variety of mammals. FGFs were discovered in brain and pituitary extracts in the 1930s [4]. They were named for their ability to promote the growth of fibroblasts. However, the substance was not isolated and purified until 1974 [5]. FGFs are a family with a wide range of physiological func-
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tions, including initiating the transcription of downstream target genes by binding to FGFRs to activate signaling pathways [6-9]. FGFRs not only promote cell proliferation and differentiation, but they also play an important role in the formation and repair of various tissues and organs, the development of follicles and embryos, the occurrence and metastasis of tumors, and the metabolism of blood glucose and lipids [10-16]. The functional diversity of FGF family members is mainly due to the differences in the N- and C-terminal sequences on both sides of the core region. Most of the FGF family members are secreted extracellularly. FGF3-8, FGF10, FGF15, FGF17-19, and FGF21-23 have a typical signal secretion sequence at the N-terminus of approximately 20 amino acids, which can be secreted outside the cell and combined with receptors.

FGF10, which was first cloned from rat embryos in 1996, belongs to the FGF7 (FGF3/7/10/22) subfamily of 7 subfamilies [10], and it is an essential gene for embryonic development. FGF10 is involved in many life activities such as cell proliferation, cell migration, and development and differentiation [17]. It has been reported that FGF10 functions as a paracrine factor [18, 19]. In mice and bovines, FGF10 is expressed in oocytes, while its receptors are mainly expressed in cumulus and granulosa cells [20-22]. During fetal ovary development, primitive, primary and secondary oocytes and granulosa cells exhibit an FGF10 immune response at different stages. In the mouse ovary, FGF10 and its receptors are detected in the cytoplasms of the oocytes, and FGFR2 is also expressed in follicular cells. Also, the FGF-10 protein has been found in human oocytes and granulosa cells, and it may contribute to human preantral follicle development [23].

However, till now, to the best of our knowledge, there is no research about the expression and localization of FGF10 and its receptors in buffalo. Therefore, this experiment aimed to investigate the expression pattern of FGF10 and its receptors during buffalo follicular development and oocyte maturation.

Materials and methods

Buffalo ovaries and oocytes collection

Buffalo ovaries were obtained from slaughter house in Nanning, and placed in 37°C normal saline and quickly transferred to the laboratory. Appropriate ovarian tissue was selected and fixed in 4% paraformaldehyde (Sigma-Aldrich). Follicles were extracted from the ovaries to obtain cumulus and oocyte complexes. The present study was approved by the Animal Ethics Committee of Guangxi University.

Granulosa cell collection

The buffalo ovary was soaked with 75% alcohol for 20-30 s, then washed with sterile saline. We then withdrew the follicular fluid with a 10 ml syringe. The follicles were classified by their diameters: follicular diameter ≤ 2 mm, 2 < follicular diameter ≤ 4 mm, 4 < follicular diameter ≤ 6 mm, 6 < follicular diameter ≤ 8 mm, follicular diameter > 8 mm. The aspirated follicular fluid was collected in an EP tube and centrifuged at 3000 rpm for 10 minutes. The granule cells were washed with autoclaved PBS, followed by centrifugation at 3000 rpm for 5 min. Finally, 1 ml of Trizol was added to lyse the cells. The cell lysate was stored at -80°C until use.

In vitro maturation culture of oocytes

A 10 ml sterile syringe was used to extract ovarian follicles of 2 to 6 mm above the surface of the ovary. Then the cumulus oocyte complexes (COCs) with uniform cytoplasms and three or more than three cumulus cells were collected under the stereo-microscope. Subsequently, the COCs were washed 2-3 times in a washing medium (TCM-199 (Sigma-Aldrich) containing 2% FBS (Gibco) and 1.2 g HEPES (Sigma-Aldrich), aggregated into a maturation medium (TCM-199 containing 10% FBS and FSH 0.2 μg/ml (Sigma-Aldrich), and matured at 38.5°C, with 5% CO₂ and 100% humidity for 24 h. The oocytes were collected at 0 h, 12 h and 24 h, respectively, and washed 3 times in PBS. Five cells were transferred to an EP tube containing Cell-to-cDNA II cell lysis buffer (Beyotime Institute of Biotechnology) and stored at -80℃ for experiments.

QRT-PCR

Total RNA from the granulosa cells was extracted using the Trizol reagent (Takara, Japan) in accordance with the instructions supplied by the manufacturer. The RNA concentration was measured using the NanoDrop ND-1000. Then, total RNA was reverse transcribed into cDNAs
using SuperScript II Reverse Transcriptase (Takara Bio) following the manufacturer’s protocol. Then real-time quantitative PCR was performed to detect the mRNA expression of buffalo FGF10 and its receptors using the SYBR Green PCR Master Mix reagent (Applied Biosystems, USA) by the Applied Biosystems 7500 Real-time PCR System. The reaction system was 20 μl, including 1 μl cDNA, 0.8 μl primers (10 nM), 10 μl SYBR Premix Ex Taq (2X), 0.4 μl ROX Reference Dye II (50X), and 7.8 μl RNase-free water. The reaction conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 45 s. The primer sequences were listed as follows: FGF10-forward-5’-CCTCCTGTCGTCGTCGTTT-3’ and reverse-5’-AGCAAAAATCCTGTTTCA-3’; FGFR1b-forward-5’-ACGTCCTGGTAGCCGAGG-3’ and reverse-5’-CCGTCATACCTGTTGAGA-3’; FGFR2b-forward-5’-TGTGTTGAGGGATGATGT-3’ and reverse-5’-CGAGTGCTTCAGAACCTTG-3’; β-actin-forward-5’-ACCGCAAATGCTTCTAGG-3’ and reverse-5’-ATCCAACCGACTGCTGTC-3’. Relative gene expression was analyzed by the 2^-ΔΔCt method [24].

Immunohistochemistry

The ovaries were fixed for 24 hours in 4% paraformaldehyde (Sigma-Aldrich), then placed in different concentrations of alcohol and xylene, and paraffin of different melting points, and finally embedded in paraffin. The paraffin block was cut into 6 μm slices, fully spread and attached to a polylysine coated slide, and dried at 45°C. The slices were successively deparaffinized with xylene, alcohol, and water and placed in 3% H₂O₂ in methanol for 30 min to eliminate endogenous peroxidase. The preincubated sodium citrate buffer was used for antigen retrieval. The slices were placed in PBS containing 1% TritonX-100 (Sigma-Aldrich) and 0.3% BSA (Sigma-Aldrich) for 5 min each time to remove serum and other components. Then the cells were permeabilized with 1% TritonX-100 for 10 min, they were transferred into 1% BSA and incubated at room temperature for 1 h. The cells were then incubated overnight at 4°C with the rabbit-anti-human FGF10 (sc-7917, Santa Cruz Biotechnology) primary antibody diluted to 1:200. The cells were incubated with fluorescein-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature and washed three times for 5 min each time. After staining with 10 μg/ml PI for 10 min at room temperature, the cells were placed on glass slides, and an anti-quencher was added. The slides were then examined under a laser-scanning confocal microscope.

Statistical analysis

Data was analyzed using SPSS version 18.0 software. All data were presented as the mean ± SD of three independent experiments. The one-way ANOVA was used to determine statistical differences. Difference is considered statistically significant when P < 0.05.

Results

Immunohistochemical localization of FGF10 and its receptors in buffalo follicles

In this study, immunohistochemistry was used to detect the expression and localization of FGF10 and its receptors during follicular development in buffalo ovary. Brown indicated positive for immunohistochemistry, and blue-purple
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Figure 1. A. The immunohistochemical staining for the localization of FGF10 in buffalo ovary; B. The immunohistochemical staining for the localization of FGFR1 in buffalo ovary; C. The immunohistochemical staining for the localization of FGFR2 in buffalo ovary. P: Primordial follicle; F1: Primary follicle; F2: Secondary follicle; F3: Mature follicle; NC: Negative control; CC: Cumulus cell; GC: Granular cell; MC: Basal membrane cells; OC: Oocyte.
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indicated negative. The results showed that FGF10 and its receptors FGFR1, FGFR2 expressed in primordial follicles, primary and secondary follicles, granulosa cells of mature follicles, and oocytes, as well as the basal membrane cells of mature follicles (Figure 1A-C).

Buffalo follicles were classified by diameter (diameter ≤ 2 mm, 2 ≤ diameter ≤ 4 mm, 4 ≤ diameter ≤ 6 mm, 6 ≤ diameter ≤ 8 mm, and diameter > 8 mm), and the granulosa cells of all follicles were collected and used. QRT-PCR was used to detect the mRNA expression of FGF10 and its receptors. We found that FGF10 was barely detected in buffalo granulosa cells (Ct > 36). Both FGFR1b and FGFR2b were expressed in all grades of follicular granulosa cells, and there was a significant difference (P < 0.05). FGFR1b had the highest expression in granulosa cells from follicles with follicular diameters ≤ 2 mm and 6 < follicular diameter ≤ 8 mm. The expression level of FGFR1b was the lowest in granulosa cells from follicles with 2 < follicular diameter ≤ 4 mm (Figure 2A). The expression of FGFR2b was lowest in granulosa cells from follicles with 2 < follicular diameter ≤ 4 mm (P < 0.05); in addition, FGFR2b had the highest expression in granulosa cells from follicles with 4 < follicular diameter ≤ 6 mm, and then gradually decreas-
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Expression of FGF10 in buffalo cumulus-ooocyte complexes (COCs)

Buffalo COCs and denuded oocytes were collected. We performed immunofluorescence staining to detect the expression of FGF10. The results showed that the fluorescence signals of FGF10 expressed in the cumulus cells of buffalo COCs and in the cytoplasms of the denuded oocytes (Figure 3).

Expression of FGF10, FGFR1b and FGFR2b during the in vitro maturation process

We collected different maturation stages of buffalo COCs and performed qRT-PCR to detect the mRNA expression levels of FGF10, FGFR1b, and FGFR2b during in vitro maturation. With the increasing of the COCs maturation time, the expression of FGF10 gradually increased (Figure 4A). Furthermore, the expression of FGFR1b and FGFR2b in buffalo COCs was higher at 24 hours than it was at 0 hours during in vitro maturation (P < 0.05), and the increase of FGFR2b mRNA was the most significant (Figure 4B).

Discussion

In the present study, we found that FGF10 and its receptors FGFR1 and FGFR2 appeared as positive responses in buffalo primordial follicles, primary follicles, secondary follicles and mature follicle oocytes and granulosa cells, and mature follicle basal membrane cells. Also, as the in vitro maturation time of buffalo COCs increased, the expressions of FGF10 and its receptor sub-types FGFR1b and FGFR2b mRNA also gradually increased. The data from our current study indicated that FGF10 and its receptors may be involved in the process of buffalo follicular development and oocyte maturation.

FGF10 has been identified as being involved in ovarian development of mice, bovines, humans, and goats [20-22, 25]. It is also involved in the proliferation and differentiation of cells and regulates various biological responses [26-28]. In recent years, FGF10 has received increased attention from researchers. However, till now, there has been no research about the expression and localization of FGF10 and its receptors in buffalo. Therefore, we conducted the present study.

Previous researchers have mentioned that FGF10 and its receptors FGFR1b and FGFR2b are immunopositive in the oocyte cytoplasm of mouse ovaries [20, 21]. In this experiment, we found that FGF10, FGFR1, and FGFR2 were positively detected by immunohistochemistry in buffalo primordial follicles, primary follicles, secondary follicles, mature follicles, oocytes with luminal follicles, granulosa cells, basal membrane cells, and this result was basically consistent with a previous study [29]. However, there was no expression of FGF10 at the mRNA level, but FGFRs expressed and there were sig-
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significant differences during buffalo follicular development. In addition, we also found that the expression of FGF10 was not consistent with the results of immunohistochemical localization. The reason may be associated with the presence of FGFRs in granulosa cells, which combined with FGF10.

With the development of follicles, the occurrence of eggs is an important physiological activity. The maturation of oocytes in vitro directly relates to the development of the embryos. And with the increase of COCs’ in vitro maturation time, the expression of FGF10, FGFR1b, and FGFR2b mRNA increased gradually, and was significantly higher at 24 h after the vitro maturation culture than it was before the mature culture. It is worth noting that the expression level of FGFR2b mRNA is significantly increased after the vitro maturation culture compared with before the maturation, indicating that FGFR2b has a higher impact on FGF10 expression, indirectly demonstrating that it is a high-affinity receptor for FGF10.

Taken together, our current study indicated that FGF10 and its receptors may be involved in the process of buffalo follicular development and oocyte maturation.

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Disclosure of conflict of interest

None.

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