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Role of BCL2L10 in regulating buffalo (*Bubalus bubalis*) oocyte maturation

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ABSTRACT

It has been reported that BCL2L10 is abundantly and specifically expressed in adult human and mouse oocytes and played a very important role in oocytes maturation and early embryonic development. This study is to investigate the expression pattern of BCL2L10 in buffalo ovaries and its effect on the in vitro maturation of buffalo oocytes, so as to dissect mechanism of oocytes maturation and provide theoretical guidance for improvement of the in vitro maturation of buffalo oocytes. The results showed that *BCL2L10* gene was enriched in ovary and the expression of BCL2L10 was oocyte specific and up-regulated during oocyte maturation. BCL2L10 protein and mRNA were detectable in buffalo early embryos, upregulated at 2-cell to 8-cell stages and down-regulated in the later stages. Knockdown of BCL2L10 by RNA interference resulted in a significant decrease in the maturation rate (33.5%) and cleavage rate (37.52%) of buffalo oocytes coupled with up-regulation of apoptosis-related gene *Caspase-9*. We concluded that BCL2L10 is a candidate associated with buffalo oocyte maturation.

1. Introduction

Mammalian oocytes undergo a series of regulated events during follicular development in ovary and an oocyte reaches its full size at the germinal vesicle (GV) stage. When GV breakdown (GVBD) occurs, the nuclear material of oocyte was exposure to the cytoplasm, and following GVBD, chromatin condense and the first polar body is extruded. Afterwards, oocytes proceed to the metaphase II (MII) stage of meiosis and undergo ovulation [1]. In many species, oocytes synthesize and accumulate RNAs and proteins during oogenesis for later development, maturation, fertilization and further embryonic development [2]. Previously, our groups have obtained a group of differentially expressed proteins in immature (GV stage) and mature (MII stage) buffalo oocytes using two-dimensional electrophoresis couple to tandem mass spectrometry (2DE-MS/MS) [3] and liquid chromatography (LC-MS/MS) methods. We found that BCL2L10 protein was highly expressed in MII stage oocytes compared with GV oocytes (data not shown). BCL2-like 10 (BCL2L10) [4], also known as BCL-B [5], BOO [6], NRH [7], and DIVA [8], was identified as a novel anti-apoptotic member of BCL-2 family and characterized as a selective BAX partner [9]. Recent studies reported in zebrafish [10], mouse [11], and human [12,13] showed that *BCL2L10* gene is primarily expressed in ovary, oocytes, and early-stage embryos and is critical for oocyte maturation and early embryonic development. However, there has not yet been any direct report of BCL2L10 in buffalo ovary and oocytes.

Swamp or water buffalo (Bubalus bubalis) is of considerably economic and biological interest especially for tropical and subtropical regions, but have low reproductive efficiency compared to other large farm animals. Basic studies to elucidate the roles of BCL2L10 during oocyte developmental should help us have a better understand of buffalo reproductive performance, and will also be important for improving the in vitro maturation systems and reproductive biotechnologies.

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2. Material and methods

2.1. In vitro embryo production

2.1.1. Oocytes collection and in vitro maturation (IVM)

Buffalo ovaries were collected from local slaughterhouse and transported to the lab in sterile isotonic saline fortified with penicillin and streptomycin at 37 °C within 4 h. All procedures involving animal treatment and collection of ovaries to be used in the study were based on the Guiding Principles for animal use as described by the Council for International Organizations of Medical Sciences (CIOMS) http://www.cioms.ch/images/stories/CIOMS/IGP2012.pdf and approved by the Animal Experimentation Ethics Committee of Guangxi University, Nanning, China. Oocytes were aspirated from follicles (2–8 mm diameter) with an 18 gauge needle attached to a 10 mL syringe containing washing medium (TCM-199 + 20 mM HEPES + 5 mM sodium bicarbonate + 3% newborn calf serum + 0.06 mg/ml penicillin +0.1 mg/mL streptomycin). Cumulus oocyte complexes (COCs) having compact and unexpanded cumulus mass with ≥3 layers of cumulus cells and homogenous granular ooplasm were selected for in vitro maturation (IVM). The COCs were washed three times with the washing medium and then twice with IVM medium consisting of TCM-199 + 5 mM HEPES +26.2 mM sodium bicarbonate +10% oestrus calf serum (OCS) + 3% buffalo follicular fluid + 0.5 μg/mL FSH + 5 μg/mL LH + 1 μg/mL estradiol-17β + 0.1 mM cysteamine + 0.06 mg/ml penicillin +0.1 mg/mL streptomycin. Groups of 15–20 COCs were placed independently in 100 μL droplets of IVM medium covered with sterilized mineral oil and incubated for 22–24 h at 38.5 °C under 5% CO_2 atmosphere with maximum relative humidity.

2.1.2. In vitro fertilization (IVF)

After maturation, COCs were washed twice in IVF medium (modified Tyrode's medium supplemented with 2 mM caffeine, 20 μg/mL heparin, 0.6% BSA, 1 mg/mL glucose, 0.06 mg/ml penicillin and 0.1 mg/mL streptomycin) and cumulus cells were removed by gentle pipetting. Groups of 15–20 matured oocytes were transferred to 40 μL fertilization drops covered with sterilized mineral oil. Single frozen buffalo semen straw (0.25 mL) was thawed in a water bath at 38 °C for 30 s and spermatozoa was prepared using swim-up methods [14]. In brief, the thawed semen containing 2 mL IVF medium was put into a conical tube and incubated for 30 min at 38.5 °C in a humidified 5% CO_2 incubator. After swim-up, the spermatozoa were removed and pooled in a centrifugation tube and then centrifuged at 1500 rpm for 6 min. The supernatant was discarded and the sperm pellet was resuspended in IVF medium and number of spermatozoa was adjusted to 2×10^6/mL. Approximately 15 μL spermatozoa of the IVF medium were added to the fertilization drops containing oocytes and incubated in a humidified 5% CO_2 incubator at 38.5 °C for 24 h for IVF.

2.1.3. In vitro culture of embryos (IVC)

Following fertilization, presumptive zygotes were separated from fertilization drops and washed three times in IVC medium (modified Tyrode's medium supplemented with 36% TCM-199, 10% fetal bovine serum, 0.06 mg/ml penicillin and 0.1 mg/mL streptomycin) and then transferred to 100 μL culture drops. The culture medium was refreshed every 48 h by replacing half of original medium with similar volume. Zygotes were cultured for up to 7–8 days post insemination in a humidified 5% CO_2 incubator at 38.5 °C.

2.2. Quantitative real-time RT-PCR

Quantitative real-time RT-PCR analysis was accomplished as described previously [15]. The total RNA was extracted from buffalo various tissues (heart, liver, lung, kidney, intestines, stomach, muscle, ovarian and testis), oocytes (GV and MII stages) and early embryos (2 cell, 4 cell, 8 cell, morula and blastocyst) using RNAiso Plus (Takara) as per manufacturer's instruction. First-strand cDNA was synthesized using PrimeScript RT Master Mix (Takara, Dalian, China) according to the manufacturer's instructions and stored at −80 °C. Real-time PCR analysis was carried out using SYBR-Premix Ex Taq kit (Takara) detection on a LightCycler 480 Real-time PCR instrument (Roche, Basel, Switzerland). The detailed of primer sequences used in this study are summarized in Table 1. Relative levels of the target genes against the reference (β-actin) were determined using the formula: $2^{-\Delta\Delta CT}$.

2.3. Immunohistochemistry

Immunohistochemistry was performed on 4% paraformaldehyde-fixed ovaries. The paraffin-embedded ovarian sections were dewaxed and dehydrated, and endogenous peroxidase activity was quenched by methanol containing 3% H_2O_2 for 30 min. Sections were subjected to antigen retrieval in 0.01 M sodium citrate buffer (pH 6.0) using microwave heating. The sections were then incubated in blocking solution (5% BSA) for 1.5 h at room temperature and incubated overnight at 4 °C with primary antibody against BCL2L10 (Bioss technology, Beijing, China) at a dilution of 1:100. After washing twice in PBS-Tween-20, the sections were incubated with horseradish peroxidase-conjugated secondary antibodies (CWBIO, Beijing, China). Immunoreactive sites were visualized in brown after staining with diaminobenzidine (CWBIO) and the sections were counterstained with hematoxylin and mounted for observation using bright-field microscopy by Olympus IX73 inverted fluorescence microscope (Olympus, Japan). The negative controls were carried out by omitting the primary antibody.

2.4. Immunocytochemistry

Buffalo oocytes (GV and MII stages) and early embryos (2 cell, 4 cell, 8 cell, morula and blastocyst) were washed three times in PBS and then fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.1% Triton X-100 for 10 min. The oocytes and early embryos were then incubated in blocking solution consisting of 5% goat serum (BOSTER, Wuhan, Hubei, China) for 1 h at room temperature and then incubated with primary antibody against BCL2L10 (Bioss technology) overnight at 4 °C at a dilution of 1:50. After washing three times in PBS, the oocytes and early embryos were incubated with secondary antibody labeled with FITC (CWBIO, Beijing, China) for 1 h at room

Table 1
Primer sequences of genes used for quantitative real-time RT-PCR.

Primer	Primer sequence (5′–3′)	Annealing temperature	The fragment length
BCL2L10-F	GACCCGACGGCAGGAGAA	60 °C	170 bp
BCL2L10-R	CCCAAGATGGCTGGAATGAG	60 °C	
BAX-F	GCAAACTGGTGCTCAAGGC	60 °C	191 bp
BAX-R	GAGCACTCCAGCCACAAAGA	60 °C	
BCL-2-F	GGGACGCCTTTGTGGAGC	60 °C	123 bp
BCL-2-R	GATACGCACCCAGGCTGATG	60 °C	
Caspase-3-F	CGGCAAACCTCAGGGAAAC	60 °C	127 bp
Caspase-3-R	CTGCTCCTTCTGCTATGGTCTTC	60 °C	
Caspase-9-F	CTGCTGCGTGGTGGTCATC	60 °C	202 bp
Caspase-9-R	CCCGTGGTCTTTCTGCTCTC	60 °C	
β-actin-F	TGGCACCCAGCACAATGAA	60 °C	166 bp
β-actin-R	AAGCATTTGCGGTGGACG	60 °C	