General and Comparative Endocrinology 178 (2012) 477–484

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Kinetics of GDF9 expression in buffalo oocytes during in vitro maturation and their associated development ability

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ARTICLE INFO

Article history:
Received 11 April 2012
Revised 30 June 2012
Accepted 6 July 2012
Available online 16 July 2012

Keywords:
Buffalo
Oocyte
GDF9

ABSTRACT

The capacity of fully grown oocytes to regulate their own microenvironment by secreted paracrine factors contribute to their developmental competence. In spite of growing evidence about the vital role of Growth Differentiation Factor 9 (GDF9) in determination of oocyte developmental competence, there is insufficient information about time dependent behavior of its expression during in vitro maturation (IVM) to have definite understanding about at what time point during IVM it plays most crucial role. The study reports the kinetics of GDF9 expression under four different IVM supplement conditions in buffalo oocytes and their concomitant development rate up to blastocyst. Oocytes matured under an ideal media condition with all supplements and those cultured with only FSH resulted in significantly higher cumulus expansion, nuclear maturation, cleavage and blastocyst rates. GDF9 expression at both mRNA and protein levels at different time points of IVM revealed that magnitude of mRNA abundance at 8 h of IVM was most important towards imparting development competence to buffalo oocytes. Appearance of GDF9 protein in maturing oocytes was found asynchronous with mRNA appearance in the time course of IVM suggesting possible posttranscriptional regulation of this gene under dynamic oocyte cumulus cell communication process. Abundance of mature GDF9 protein at 16 h was most consistently related with all oocyte development parameters.

1. Introduction

During the process of its maturation mammalian oocytes accumulate and store large amount of maternal mRNAs; an ordered regulation of which ultimately dictate the acquisition of developmental competence of oocytes and further embryonic development [50,58,33]. In a typical in vitro fertilization (IVF) procedure, in vitro maturation (IVM) of oocytes constitute the most challenging step because an orchestrated genes expression events during this time determines the efficiency of fertilization and subsequent embryonic division process leading to blastocyst formation and even successful implantation [49]. Aberrant degradation and/or maintenance of maternally inherited as well as *de novo* synthesized transcripts during oocyte maturation adversely affect the oocyte's ability to undertake an orderly development [52]. A suboptimum IVM support results in persistent alterations of the normal gene expression patterns and affect the developmental fate of IVF produced embryos very drastically [9,59,26]. Thus information on expression pattern of oocyte-expressed genes *vis-à-vis* their development fate is critical for deciding strategies for how more number of oocytes could be stimulated to gain optimum development competence eventually contributing to the success rate of various ART protocols in different livestock species [47].

GDF9 is an oocyte-specific paracrine factor which is expressed throughout most stages of folliculogenesis and persists after fertilization and play important role in bi-directional communication between oocytes and its surrounding cumulus cells [36,35]. GDF9 level in follicular fluid has been found significantly correlated with the nuclear maturation of the human oocytes and subsequent embryo quality [21]. Addition of exogenous GDF9 in oocyte maturation media has also enhanced subsequent development rate of oocytes in mice as well as bovine [60,25]. In spite of emerging evidence however, information about the kinetics of GDF9 expression during IVM remains incomplete and it is still not clear that at what stage of IVM GDF9 exerts most profound effect possibly in a species specific manner [7]. Buffaloes (*Bubalus bubalis*) in this regard remain a poorly understood species. Economic importance of this species as dairy animal in well documented and several ARTs have been successfully demonstrated [38,19] but, poor success rate of these procedures have kept pending their widespread adoption

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http://dx.doi.org/10.1016/j.ygcen.2012.07.001

[18,11]. The present study was planned to elucidate expression pattern of GDF9 gene in buffalo oocytes in course of in vitro maturation and their associated development competence. Appearance of GDF9 mRNA as well as its corresponding protein was tracked in buffalo oocytes at different time points of IVM to understand the dynamics of transcription and translation control of this gene and its relationship with development ability of oocytes [17,57,45].

2. Materials and methods

All media and chemicals were procured from Sigma Aldrich, St. Louis, MO, USA unless otherwise indicated. Disposable plastic wares used were from Falcon NJ, USA and Nunc, Denmark. Fetal bovine serum used was from Hyclone, Canada.

2.1. Production of buffalo embryos

Buffalo ovaries were collected at an abattoir, regardless of the estrous cycle and transported within 3–4 h to the laboratory in phosphate buffer saline (PBS) containing strepto-penicillin (0.05 mg/ml). Ovaries were washed several times in normal saline and cumulus oocyte complexes (COCs) were aspirated from visible ovarian surface follicles in hepes-buffered hamster embryo culture (HH) medium [27]. COCs were picked up under stereo zoom microscope and washed three times in HH medium. They were evaluated according to morphological criteria and separated according to their quality. Only excellent grade oocytes with more than five compact layers of cumulus cells and homogenous cytoplasm were used for IVM and IVF. A group of 25 COCs were put for in vitro maturation (IVM) in 4 different supplementation media viz. (1) All supplementation group (Henceforth called Control): TCM-199 with 0.005% streptomycin, 0.01% sodium pyruvate and 0.005% glutamine, 64 μg/ml cysteamine and 50 μl ITS as base medium and supplemented with 10% fetal bovine serum (FBS), 5.0 μg/ml porcine follicle stimulating hormone (pFSH), 10 μg/ml luteinizing hormone (LH), 1 μg/ml estradiol 17-β (E2) and 50 ng/ml epidermal growth factor (EGF), (2) FSH group: TCM-199 base medium containing PVA (1 mg/ml) and 5.0 μg/ml porcine follicle stimulating hormone (3) IGF1 group: same as media 2 where FSH was replaced with 100 ng/ml of IGF1 and (4) estradiol group: same as media 2 where FSH was replaced with 1 μg/ml of estradiol 17 β (E2). For IVM, oocytes were placed in drops of 100 μl maturation media and overlaid with mineral oil. Oocytes were allowed to mature at 38.5 °C in an atmosphere of 5% CO_2 in air. In vitro fertilization (IVF) was done in 100 μl droplets of BO medium [3] supplemented with 1% BSA (fatty acid free), 1.9 mg/ml caffeine sodium benzoate, 0.14 mg/ml sodium pyruvate and 0.01 mg/ml heparin. Prior to transfer in fertilization drops; matured COCs were washed thrice in BO medium. The frozen thawed buffalo semen was processed for in vitro capacitation as per the procedure described earlier [5] and 50 μl of the sperm suspension (at final concentration of 1×10^6/ml) was added to each fertilization drops having 15–20 COCs and incubated at 38.5 °C with 5% CO_2 for 14 h. Presumptive zygotes were removed from the fertilization drops after 14 h of insemination (HPI), adhered cumulus cells were mechanically removed by vortexing and washed five times in mCR2aa medium [30]. After washing, 15–20 presumptive zygotes were co-cultured with monolayers of granulosa cells in 100 μl drops of IVC-I medium (mCR2aa supplemented with 0.8% BSA, 1 mM glucose, 0.33 mM pyruvate, 1 mM glutamine, 1× MEM essential amino acid, 1× non-essential amino acid and 50 μg/ml gentamycin. After 48 h of insemination (HPI) zygotes were evaluated for evidence of cleavage. At 72 HPI all cleaved embryos were transferred to IVC-II medium (same as IVC-I except BSA replaced with 10% FBS) and maintained for 8 days at 5% CO_2 and 38.5 °C with replacement of medium after every 48 h.

2.2. Observations made on developing oocytes and embryos

All IVM and IVF experiments were repeated at least four times. After 24 h of IVM, levels of cumulus expansions was scored in a scale of 0–4 and the cumulus expansion index (CEI) was calculated as described before [13]. COCs with no cumulus expansion were scored as 0 and those with maximum degree of expanded cumulus mass were scored 4. Cumulus mass with intermediate expansions characterized by the detachment from oocytes or expansion of only the outer most layers was marked as 2 or 3 [54]. To determinate attainment of metaphase-II (M-II) denuded oocytes were stained with Hoechst 33342 using protocol described before [48] with slight modification. Briefly, denuded oocytes from all experimental groups were fixed in 4% paraformaldehyde solution (in PBS, pH 7.4) for 1 h at room temperature. After fixing and washing, groups of 50 oocytes were transferred to 200 μl drop of 10 μg/ml Hoechst 33342 dye solution for 20 min under dark condition. Stained oocytes were washed three times in PBS–PVP solution and placed on glass slides and mounted with Pro-Long mounting medium (Invitrogen, USA) and observed under the fluorescent microscope with UV filter (Olympus, Japan). Oocytes nuclei revealing two blue dots were considered as matured (M-II) oocytes. M-II% was calculated for each IVM groups for at least 250 oocytes. Cleavage and blastocyst stages of the embryos were recorded on day 2 and 7 post insemination, respectively, in all four experimental groups.

2.3. RNA isolation and cDNA preparation

Samples of a pool of 10 oocytes were used in 4 biological replicates for each experimental group. Total RNA was extracted from a fixed number of 10 oocytes were collected at different time intervals of maturation using the RNAqueous Micro Kit (Ambion) as per manufacturer's instruction. Total RNA was eluted in elution buffer and treated with RNase-free DNase I (Ambion) to remove any contaminating genomic DNA. Total RNA (10 μl) from each samples were reverse-transcribed using Revert-Aid Kit (Fermentas, USA) following the manufacturer's instructions using oligo-dT primers in a final volume of 20 μl. After termination of cDNA synthesis, each RT reactions were diluted with nuclease-free water to a final volume of 80 μl and stored at −80 °C till further use.

2.4. Quantitative real-time RT-PCR

Quantification of GDF9 transcript was carried out by real-time PCR using Maxima SYBR Green qPCR Master Mix (Fermentas, USA). Primers used for GDF9 amplification were forward: CTCAGCACAAGCAAGCTCCT and reverse: GGGAAGGGAAAAGAAATGGA designed using the Beacon Designer 7.0 (Premier Biosoft, International) and derived from buffalo specific GDF9 sequences FJ529501.2. RSP18 transcripts were amplified as an internal calibrator using primers forward: GAAAATTGCCTTTGCCATCACTGC and reverse: GATCACACGTTCCACCTCATCCTC (Designed using bovine sequence NM_001033614). A working primer concentration of 10 pmol was used to set a primer matrix experiment to optimize the primer concentrations for valid transcript quantification [2]. Real time PCR reaction mixtures were consisted of 10 μl of syber green qPCR mix, 2 μl of cDNA template, optimized primer quantities and nuclease free water to make the total reaction volume of 20 μl. Reactions were performed in duplicate for each samples using Mx3005P real time PCR System (Stratagene). PCR conditions used were 95 °C for 10 min, then 40 cycles consisting of denaturation at 95 °C for 30 s, annealing at 57 °C for 20 s and extension