FUNCTIONAL ANALYSIS OF CUMULUS CELLS ASSOCIATED GENES RELATED TO THE QUALITY OF *IN VITRO* FERTILIZED CAPRINE EMBRYOS

M. Elanchezhian, S. Gautham, D. Reena[1], D. Gopikrishnan[2] and A. Palanisammi[1]

Centralized Embryo Biotechnology Unit,
Department of Animal Biotechnology,
Tamil Nadu Veterinary and Animal Sciences University
Madhavaram Milk Colony, Chennai-51

As successful embryonic development is dependent on a time- and site-specific expression of appropriate genes, the studies on differential gene expression in cumulus oocyte complexes (COCs) could potentially elucidate the signaling pathways involved in the crosstalk between the oocyte and its somatic associates during maturation and the developmental processes (Mourad *et al*., 2008). Communication between the oocyte and the cumulus ells is accomplished mainly through the gap junction communications and the cumulus cells were thought to be the mediators of oocyte paracrine signals and developmental potential. Hence, transcriptomic analysis of cumulus cells could be a non- invasive method to assess developmental competence of the oocyte. Certain genes in cumulus cells are predicted to be expressed differentially that would predict the quality of oocyte, its developmental competence and also the final embryo quality.

1. Department of Animal Biotechnology,
Madras Veterinary College, Chennai – 600 007
2. Resident Veterinary Services Section,
Madras Veterinary College, Chennai – 600 007
[1]Corresponding Author e-mail id:
reena.d@tanuvas.ac.in

Growth differentiation factor 9 (GDF9), a member of the transforming growth factor-β superfamily, was the first oocyte-specific factor shown to cause cumulus expansion (Elvin *et al*. 1999). GDF9 functions as an oocyte-secreted paracrine factor that regulates several key granulosa cell enzymes involved in cumulus cell expansion and creates a microenvironment optimal for acquisition of oocyte developmental competence (Pangas and Matzuk, 2005). The expression levels of GDF9 downstream target genes in the cumulus cells may reflect GDF9 activity and could ultimately predict oocyte health (McKenzie *et al*., 2004) and the grade of the resulting embryos (McKenzie *et al*., 2004, Zhang *et al*., 2005). Cyclo-oxygenase 2 (COX2) / prostaglandin endoperoxide synthase 2 (PTGS2), gremlin1 (GREM1) and hyaluronic acid synthase 2 (HAS2) are all downstream GDF9 target genes found in cumulus cells and have been evaluated as markers for oocyte developmental competence and the subsequent embryonic development (Cillo *et al*., 2007). Analysis of the above mentioned genes might help us to evaluate the developmental competence of the oocytes thus determining the quality of embryos. Based on this hypothesis the

present study was undertaken to identify the target genes in cumulus cells associated with developmental competence of caprine oocytes.

Goat ovaries were obtained from the slaughter house and transported to the Centralized Embryo Biotechnology Unit, Department of Animal Biotechnology Unit II, TANUVAS, Chennai – 51, in 0.9 per cent normal saline containing penicillin (100 IU/ml) and streptomycin (50 mg/ml) at 30-35° C in a thermos flask within 2 h of slaughter.

Cumulus oocyte complexes (COCs) retrieved by slicing of ovaries were screened and graded as A, B, C, D and E based on their cumulus cells investment and ooplasm homogeneity. Only COCs of grades A and B were washed in TCM 199 + 10 per cent fetal bovine serum (FBS - GIBCO: Invitrogen, USA) and finally in *in vitro* maturation (IVM) medium, composed of TCM-199 supplemented with 10 per cent FBS, 1 µg/ml of Folltropin (FSH), 0.02 IU/ml of Luteinizing Hormone (LH), 1 µg/ml of estradiol and 10 ng/ml of epidermal growth factor (EGF). A group of ten to fifteen COCs were transferred to a 50 µl droplets of maturation medium in a 35 mm petridish, pre-equilibriated with the IVM medium for 2h at 38.5°C under 5 per cent CO_2 in air and cultured for 27 hrs at 38.5°C in a humidified atmosphere of 5 per cent CO_2 in air. After 27 hours of incubation, the COC's were observed under stereo-zoom microscope (Nikon, Japan). Maturation rate was evaluated based on degree of cumulus expansion and extrusion of the first polar body (Degree 2- cumulus cells homogeneously expanded, Degree 1-cumulus partially expanded with few clustered cells, Degree 0 - No morphological change).

The Degree 2 and Degree 1 COC's were washed separately in OCM in 35mm petri dishes by vigorous pipetting. This process detached the cumulus cells from the oocytes. Pipetting was carried out until all the cumulus cells were removed. The degree 1 and degree 2 cumulus cells were collected. They were washed in PBS twice and were stored in PBS in 1.5 ml microfuge tubes at -20°C for RNA isolation.

Sperms were extracted from the cauda epididymis of buck testes from the slaughter house. The motile sperms were separated by swim up method. Concentration of the final sperm pellet was determined with a haemocytometer and the sample was diluted with spTALP (sperm tyrode's albumin lactate pyruvate) to yield a concentration of $1\text{-}2 \times 10^6$ sperm/ml. The fertilization droplets of 75 µl of *in vitro* fertilization (IVF-TALP) medium supplemented with heparin (10µg/ml) in 35 mm petridish overlaid with sterile mineral oil was pre-equlibrated at 38.5˚C under 5 per cent CO_2 in air. The matured COCs were washed in pre-equilibriated spTALP and oocytes were washed in IVF TALP medium and transferred to the pre-equilibrated IVF droplets such that each droplet contained 10-15 oocytes degree 2 and degree1 matured oocytes separately. The motile sperm suspension obtained by swim up technique were inseminated into the IVF droplets containing oocytes to achieve the final concentration of 2 million sperm/ml and co-incubated for 18-24h at 38.5°C in a humidified atmosphere of 5 per cent CO_2 in air.

After 18 to 24 hours the plate was checked for fertilization process. The oocytes were removed from the droplets and were washed to remove the dead sperms from them. The cleaved cells were placed in SOF culture media drops covered with sterile mineral oil in 35mm petri dish till the morula stage. The culture media were changed once in 48 hours.

The Degree 2 and Degree 1 cumulus cells were thawed. They were centrifuged in a cooling centrifuge at 4°C, 7,500xg for 5 minutes. 0.25ml of sample (containing pellet) was used from each tube by discarding the supernatant. 0.75ml of Trizol reagent was added to 0.25ml of sample and mixed by pipetting. The samples were incubated at RT for 5 mins. 0.2 ml of chloroform was added to the above mixtures and mixed well. It was then incubated at RT for 2 to 3 minutes. After incubation, the samples were centrifuged at 4°C, 12,000xg for 15 minutes. The aqueous phases from the samples were transferred to fresh 1.5ml microfuge tubes. 0.5ml of isopropanol was added to each tube containing aqueous phases. The mixtures were incubated at RT for 10 minutes. Then they were centrifuged at 4°C, 12,000xg for 10 minutes. The pellet was washed with 75% ethanol at 7500xg for 5 minutes and the pellet air dried. The pellets were re-suspended in DEPC treated water/nucleus free water.

The extracted RNA was used for cDNA synthesis using a high capacity cDNA Reverse Transcriptase kit (Applied Biosystems Inc, USA) following the manufacturer's instructions. The cDNA obtained from RNA were used for predicting the expression of PTGS2, HAS2 and GREM1 genes in caprine cumulus cells by PCR amplification along with the β-actin as marker gene.

Primer Sequences

Gene Name	Primer Sequence	Annealing Temperature (° C)	Product Size
GREM1	5'-AACAGCCGTACCATCATCAAC-3'	55	65bp
	5'-TTCAGGACAGTTGAGAGTGACC-3'		
COX2	5'-CATGGGTGTGAAAGGGAGGAAAGA-3'	58	304bp
	5'-CCTTAGTGAAAGCTGGTCCTCGTT-3'		
HAS2	5'-ATAAATGTGGCAGGCGGAAGAAGG-3'	60	182bp
	5'-GTCTTTGTTCAAGTCCCAGCAGCA-3'		

Cycling conditions for PCR

Primer	Steps				
	1	2	3	4	5
GREM1	95°C/10 min	95°C/15 sec	55°C/45 sec	72°C/30 sec	72°C/10 min
HAS2	95°C/10 min	95°C/15 sec	60°C/45 sec	72°C/30 sec	72°C/10 min
COX2	95°C/10 min	95°C/15 sec	58°C/45 sec	72°C/30 sec	72°C/10 min

Step 2 to 4 was repeated for 50 cycles

After the run, the amplified products were analyzed by agarose gel electrophoresis

To date, non-invasive embryo selection had been based mainly on morphological and developmental criteria performed during *in vitro* development (Scott, 2003). The quality of the embryos depends on the oocyte quality which implies that with careful assessment of the oocyte quality, the quality of the embryos produced could be manipulated. The hindrance in assessing the oocyte quality might be overcome by the additional parameters that would support morphological and metabolic evaluation of the oocyte in order to appropriately select those that have the greater chance of fertilization and development. Given the essential nature of the interaction between cumulus cells and the maturing oocyte and that many aspects of cumulus function are regulated by the oocyte (Eppig, 2001, Matzuk *et al*., 2002), the analysis of cumulus cells appear a logical potential approach to the non-invasive assessment of oocyte developmental competence (Li *et al*., 2008). Growth differentiation factor 9 (GDF9), a member of the transforming growth factor-β superfamily, associated genes and their expression levels were considered as positive markers of developmental competence of oocytes.

In the present study, the expression of GDF9 associated genes (GREM1, HAS2 and COX2) was studied separately in grade A and grade B oocytes and compared with the cleavage rate and development of embryos. The cleavage rate/morula rate of grade A and grade B oocytes where found to be 44.29±1.31 / 28.60±1.78 and 34.83 ± 2.72 / 16.19 ± 1.79, respectively and all the three genes were expressed in both grades of oocytes. The results of the present study was in accordance with McKenzie *et al*. (2004), who stated that the expression of genes HAS2, PTGS2 and GREM1 could be correlated to morphological and physiological characteristics and might provide a novel approach to predict embryo development. In spite of their expression, the cleavage rate and morula rate were significantly different between grade A and grade B oocytes which might be due to the difference in the level of expression of genes (differential expression) between the two grades of oocytes. According to Anderson *et al*. (2009) genes HAS2 and PTGS2 had great level of expression in mature oocytes than immature oocytes with 4.7 fold increases in PTGS2 gene in mature cumulus. They also reported fertilization and early embryo

cleavage showed a positive relationship with PTGS2 and GREM1 expression stating that the quality of the embryos were high (graded by graduated embryo score (GES)) from oocytes in which these genes were differentially expressed.

From the present study it can be concluded that the expression of the candidate genes (HAS2, PTGS2 and GREM1) helps to predict the quality of the oocytes selected. Ultimately, the analysis of the level of expression of these genes would bring about a clear view in the determining the criteria for the selection of good quality embryos.

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