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Full Length Article

Expression Patterns of Some Follicular Genes and their Association with Fertility Trait in Goat

Othman E Othman* and Nadia A Abo El-Maaty
Cell Biology Department, National Research Centre, Dokki, Egypt
*For correspondence: othmanmah@yahoo.com
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Abstract

The aim of this study was to assess the expression of some follicular genes and their association with fertility trait in goat using Qt-PCR. Two groups of Zaraibi goat breed; high (HFG) and low (LFG) fertility groups were used; each group has 15 animals. The animals from each group were sacrificed; all the ovarian follicles were mechanically collected, and the follicles were pulled together. RNA was extracted from ovarian follicles and cDNA was synthesized and subjected for QT-PCR using Sybr green and β-actin as House-Keeping genes. The expressions of twelve tested genes were increased in HFG comparing with LFG, which were *MCPH1*, *DACT1*, *DKKL1*, *PLCZ1*, *GADD45A*, *CST3*, *FOS*, *FUCA1*, *LGALS1*, *PNPLA3*, *ID3* and TPM2. On the other hand, six genes were expressed at a lower level in HFG than those in LFG, including *NEFM*, *INHBB*, *CD38*, *FST*, *APOE* and *RPS29*. Two genes were identified with expressions nearly at the same level in both fertility groups which were *LMX1A* and *STRBP*. It is concluded that the expressions of new-reported follicular genes assessed in this study and their associations with ovulation rate and fertility trait can help for selection the animals entering in reproductive breeding programs depending on these new fertility markers.

Keywords: Gene expression; Follicular genes; QRT-PCR; Domesticated goats

Introduction

Folliculogenesis is the crucial process in which the oocytes gain development and maturation. In this complex process, there are many interactions between follicles, oocytes and granulose cells that play important roles in determining fertility phenomena (Racowsky and Needleman 2018; Zhang *et al*. 2018). The interaction between oocytes and granulose cells during the folliculogenesis is complicated due to their regulatory function. The granulose cells pack up the meiotic process through the providing of some metabolites whereas the oocyte secretes many growth factors stimulating the differentiation and proliferation of granulose cells (Gilchrist *et al*. 2004; Wigglesworth*a et al*. 2013).

The identification of oocytes-specific genes and granulosa cell factors whose expressions affecting folliculogenesis is crucial for improvement the fertility trait especially in small ruminant including sheep and goat (Juenge *et al*. 2021). Recently, the discovery of the transcriptome patterns of genes related to oocytes and granulosa cells open the door toward the understanding of the folliculogenesis process (Biase and Kimble 2018) and the interactions between different factors and genes affecting follicular development, oocyte maturation and embryo quality (Chronowska 2014; D'Aurora *et al*. 2016). Transcriptome is the study related to the RNA content of the cell, but transcriptomics is the study of transcriptomes and their functions. It is considered as a tool to identify number of genes whose expressions are involved in different biological processes (Wang *et al*. 2019). Gene expression is a process in which genetic information is translated to functional products including proteins and functional RNAs. Gene expression levels are considered the essential keys for different biological activities in the single cell or a multicellular organism (Chen 2020).

Fecundity is one of the most important economic traits for goat meat production. The enhancing of goat fertility has economically important role in the increasing of goat meat production (Ahlawat *et al*. 2015). In Egypt, goat Zaraibi breed is breeding for milk production. The Zaraibi breed, also called Egyptian Nubian is considered one of the most important local goat breeds in Egypt because of its high prolificacy and milk production (Shaat and M€aki-Tanila 2009). The assessment of expressions for new-reported follicular genes and their associations with ovulation rate and fertility trait can help for selecting the animals entering in reproductive breeding programs depending on these new fertility markers to get animals with high fertility performance. The fertility phenomenon in goats depends on the ovulation rate (Lai *et al*. 2016), therefore this work was designed to validate the high

expression of some follicular genes and their association with fertility trait in goats using Qt-PCR.

Materials and Methods

Study design

All procedures performed in this study involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Committee of Animal Care and Welfare, Benha University, Egypt) with an approval number: BUFVTM 2019. Zaraibi breed is one of the indigenous goat breeds reared in Egypt, where it is domesticated since many years ago and it is economically important breed due to its highly fecundity trait. Thirty Zaraibi goats were used in the present study and classified into two groups: one of them included goats had three or more litter in one generation (high fertility, HFG) and the other included goats with one litter in four previous generations (low fertility, LFG). The animals from each group were sacrificed; all the ovarian follicles were mechanically collected and the follicles were pulled. RNA was extracted from the ovarian follicles for RNA sequencing (Data is not here) and for assessment of differential gene expression patterns of GCs in both HFG and LFG groups.

RNA extraction and cDNA synthesis

The extraction of RNA was conducted from ovarian follicles using the RNA purification kit according to its instructions. The concentration of extracted RNA and its purity assessed, and it was between 1.8 and 2.1. Moreover, RNA integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. The synthesis of cDNA was carried out from extracted RNA after treatment with DNase to remove any DNA residue.

Real-time polymerase chain reaction (Real-time PCR)

The assessment of gene expression was done using Real-time PCR. The reaction mixture contains SYBR Green PCR Master-Mix (12.5 μL), 0.5 μL of each forward and reverse primers (Table 1), 1 μL cDNA and RNase free water. The conditions of amplification included initial incubation and 40 cycles of amplification (denaturation, annealing and extension steps). Mean cycle threshold (Ct) values of triplicate samples are used for analysis.

Data analysis

The data of gene expressions was analyzed using chi-square test to evaluate the significant differences in gene expression The fold change of gene expression was calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schittgen 2001). The normalization of gene expression was done using *β-actin*.

Table 1: Primer sequences of follicle genes used in qRT-PCR analysis

Gene	Primer	References
NEFM	F: AGGAAGAGGAGGCTGAAGAA	ENSG00000104722
	R: TCTTTAAGTTCAGGCGCAGTAG	
LMX1A	F: TGTGGTTCAGGTGTGGTTC	ENSG00000162761
	R: CTGGGTGTTCTGCTGATCTT	
DACT1	F: ACCGTCAAGACAGACACTAA	ENSG00000165617
	R: GGACTAGGCTCAGAATGTAACC	
MCPH1	F: GTGTACAGTCCGACAGGTAAAG	ENSG00000147316
	R: CTGTTCAATCATCTGGGAGGAG	
CD38	F: CTAGGGACCAGTTCTGGTTAAG	ENSG00000004468
	R: GTGGATTAGCAGGGACCTATTT	
PNPLA3	F: CCTGTGATGTACGCAGAATGA	ENSG00000100344
	R: CACACCAAGAGTAGCTTCCAA	
DKKL1	F:GGAACTACCACCAAGAAGAGAAC	ENSG00000104901
	R: ATCACCACCTCTCCTGTCTT	
PLCZ1	F: GATAAGCATGGGCAGGTAGAG	ENSG00000139151
	R: AAATCACCCACTGGTTCTGG	
STRBP	F: GGCTGTAGACACCTATTCCAAG	ENSG00000165209
	R: CCAGCTCCAAGTCCATATCATC	
INHBB	F: CTGGACAATGCACACGTAGA	ENSG00000163083
	R: TGTGGCTGAGCTGCTATTT	
GADD45A	F: GCGCGTATGCAAATGAAGAG	ENSG00000116717
	R: CCACTAACCACGACACTAACTC	
CST3	F: CATGACCAGCCACATCTGAA	ENSG00000101439
	R: CAGGTGGATTTCGACAAGGT	
FOS	F: GAGCTGGTGCATTACAGAGA	ENSG00000170345
	R: GTGTGTTTCACGAACAGGTAA	
APOE	F: GCTGATGGAGGCTGGTTAC	ENSG00000130203
	R: CAACCCACAGAACCTTCATCT	
RPS29	F: TATGTGCCGCCAGTGTTT	ENSG00000213741
	R: TAAGGTTGGTGTGCTGGAT	
FST	F: GGCCTATGAGGGAAAGTGTATC	ENSG00000134363
	R: ACAGGCTCCTCAGACTTA	
FUCA1	F: CAAGCACATCCACCATCATTTC	ENSG00000179163
	R: GGAACACGAGGAGACCTTTATC	
ID3	F: CCCAACCTCATTGCTCAGTAT	ENSG00000117318
	R: CTCTTTAGGCCACCCATGTT	
TPM2	F: GCCCTCCAGAAGAAGCTAAA	ENSG00000198467
	R: CGATGACCTTCATTCCTCTCTC	
LGALS1	F: CGATTGGTCATCTCTGCTTCA	ENSG00000100097
	R: GCTTGATGGTTAGGTCTGTCTC	

Results

In the present work, the differential expression of ten newly reported follicle oocytes genes in high and low Zaraibi fertility groups was assessed. These genes are: Neurofilament Medium Chain (*NEFM*), LIM Homeobox Transcription Factor 1 Alpha (*LMX1A*), Disheveled Binding Antagonist of Beta Catenin 1 (*DACT1*), Microcephalin 1 (*MCPH1*), CD38 Molecule (*CD38*), Patatin Like Phospholipase Domain Containing 3 (*PNPLA3*), Dickkopf Like Acrosomal Protein 1 (*DKKL1*), Phospholipase C Zeta 1 (*PLCZ*1), Spermatid Perinuclear RNA Binding Protein (*STRBP*) and Inhibin Subunit Beta B (*INHBB*).

The results showed that four genes among them were highly expressed in high fertility groups compared to those in low fertility ones at the highly significant level ($P<0.001$) with different fold numbers, including *MCPH1* (6.1), *DACT1* (4.6), *DKKL1* (4.1) and *PLCZ1* (3.4), whereas *PNPLA3* was highly expressed in HFG by 2.1 folds at a significant level of $P<0.01$. On the other hand, three genes were expressed at

lower levels in HFG comparing with those in LFG, including *CD38* by 0.4 folds (P<0.001), *INHBB* by 0.5 folds as well as *NEFM* by 0.6 folds (P<0.01). Expression of Two genes, *LMX1A* and *STRBP*, were at similar levels in both tested groups indicating no association with fertility trait (Table 2 and Fig. 1).

The expression levels of ten granulosa cell genes were assessed in this work using two different fertility groups from Zaraibi breed. These genes were Growth Arrest and DNA Damage Inducible Alpha (*GADD45*A), Cystatin C (*CST3*), Fos Proto-Oncogene, AP-1 Transcription Factor Subunit (*FOS*), Apolipoprotein E (*APOE*), Ribosomal Protein S29 (*RPS29*), Follistatin (*FST*), Alpha-L-Fucosidase 1 (*FUCA1*), Inhibitor of DNA Binding 3, HLH Protein (*ID3*), Tropomyosin 2 (*TPM2*) and Galectin 1 (*LGALS1*).

The findings showed that five genes were highly expressed in HFG compared to those in LFG with high significant levels (P>0.001), including *GADD45*A, *CST3*, *FOS*, *FUCA1* and *LGAL* by 2.2, 3.8, 4.4, 3.1 and 2.4 folds, respectively. Also, there were 2 genes with enhanced expression in HFG, including *ID3* (1.7 folds; P<0.01) and *TPM2* (1.4 folds; P<0.05) (Table 3 and Fig. 2). On the contrary, expressions of three genes were decreased in HFG. These genes are RPS29 and APOE where they expressed by 0.6 and 0.5 folds, respectively in HFG comparing with LFG (P<0.01) and FST with insignificantly decreased by 0.7-fold.

Discussion

Reproductive efficiency is one of critical factors leading to the productivity improvement at special environmental conditions especially in small ruminants including sheep and goat where the reproduction performance is an indicator for their adaptation under harsh conditions (Atoui *et al*. 2018). Reproduction performance is measured as the ability of animals to produce viable offspring and this complicated trait was affected by numerous factors. The litter size trait is the most important trait for fecundity and it contributes 74–93% for fecundity. Many factors affect the litter size trait such as the ovulation rate, the uterine receptivity and quality of spermatozoa (Robertson *et al*. 2020). Therefore, it is meaningful to explore the mechanisms of the ovarian follicular development, atresia, oocyte maturation and ovulation rate (Ziadi *et al*. 2021).

Goats are distributed all over the world and it is one of the economically important farm animals in Egypt where it's number about 5 million heads. Goats play an important role in the production of meat, milk, and fiber in arid and semi-arid regions where they can adapt under hard environments easier than other livestock (Khalil *et al*. 2013). Zaraibi breed is considered one of the best dual-purpose breeds in Egypt combining high prolificacy with high milk production (Abd El-Hamid *et al*. 2017). In this study, the expression patterns of twenty follicular genes and their association with the fertility performance in this breed were validated using qRT-PCR amplification.

Table 2: Expression patterns of follicle oocytes genes in different fertility groups

Gene	Animal status	CT mean ± SD	ΔΔCt	No. of folds	Significant level
MCPH1	HFG	19.54 ± 0.98	-2.6	6.1↑	0.001***
	LFG	22.14 ± 0.76			
DACT1	HFG	21.67± 0.88	-2.21	4.6↑	0.001***
	LFG	23.88 ± 0.66			
DKKL1	HFG	22.99 ± 1.01	-2.04	4.1↑	0.001***
	LFG	25.03 ± 0.86			
PLCZ1	HFG	20.11 ± 0.99	-1.76	3.4↑	0.001***
	LFG	21.87 ± 1.11			
PNPLA3	HFG	20.66 ± 0.77	-1.09	2.1↑	0.01**
	LFG	21.75 ± 0.98			
LMX1A	HFG	22.11 ± 0.77	-0.17	1.1	N.S.
	LFG	22.28 ± 0.86			
STRBP	HFG	19.88 ± 0.59	-0.05	1.0	N.S.
	LFG	19.93 ± 0.64			
NEFM	HFG	21.62 ± 0.59	0.84	0.6↓	0.01**
	LFG	20.78 ± 0.64			
INHBB	HFG	23.47 ± 0.75	0.92	0.5↓	0.01**
	LFG	22.55 ± 0.87			
CD38	HFG	20.69 ± 0.79	1.31	0.4↓	0.001***
	LFG	19.38 ± 0.82			

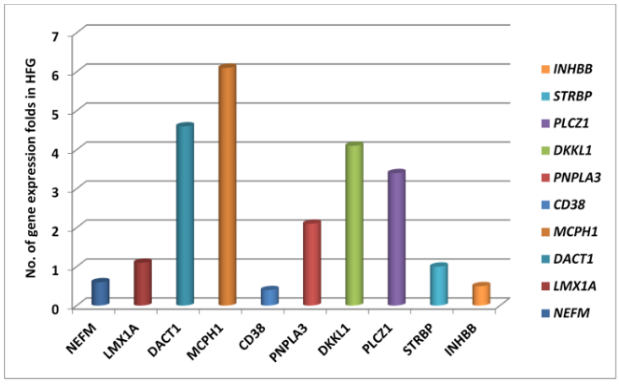

Fig. 1: No. of fold changes in expressions of follicle oocyte genes

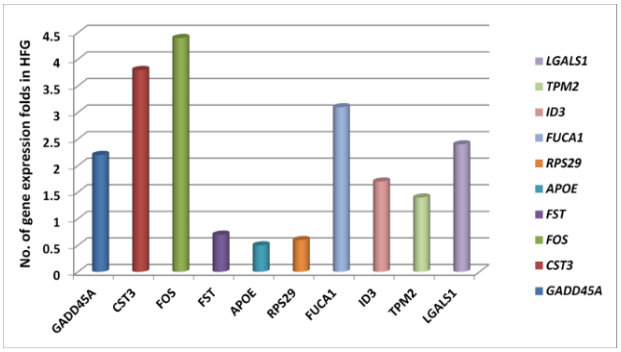

Fig. 2: No. of fold changes in the expressions of granulose cell genes

Li *et al.* (2021a) studied transcriptome profile of folliculogenesis in Chinese Ji'Ning grey through RNA-seq of follicle oocytes in different fertility populations. Their findings declared the association of transcriptome of many genes with the fertility performance; some of them with known fertility-related genes and others are newly reported.

Table 3: Expression patterns of granulosa cell genes in different fertility groups

Gene	Animal status	CT mean ± SD	ΔΔCt	No. of folds	Significant level
*GADD45*A	HFG	20.67 ± 0.82	-1.14	2.2↑	0.001***
	LFG	21.81 ± 0.77			
CST3	HFG	22.16 ± 0.98	-1.92	3.8↑	0.001***
	LFG	24.08 ± 0.87			
FOS	HFG	19.54 ± 0.95	-2.13	4.4↑	0.001***
	LFG	21.67 ± 0.77			
FST	HFG	20.61 ± 0.93	0.43	0.7↓	N.S.
	LFG	20.18 ± 0.84			
APOE	HFG	22.88 ± 0.74	0.91	0.5↓	0.01**
	LFG	21.97 ± 0.86			
RPS29	HFG	23.82 ± 0.99	0.75	0.6↓	0.01**
	LFG	23.07 ± 1.02			
FUCA1	HFG	20.13 ± 0.61	-1.62	3.1↑	0.001***
	LFG	21.75 ± 0.52			
ID3	HFG	19.87 ± 0.65	-0.74	1.7↑	0.01**
	LFG	20.61± 0.48			
TPM2	HFG	22.76 ± 0.69	-0.51	1.4↑	0.05*
	LFG	23.27 ± 0.85			
LGALS1	HFG	19.55 ± 0.55	-1.29	2.4↑	0.001***
	LFG	20.84 ± 0.67			

The increased expression of five from these newly reported genes was declared in our study, including *MCPH1, DACT1, DKKL1, PLCZ1* and PNPLA3.

The important physiological role of *MCPH1* in the development process of brain and gonads was reported by Liu *et al*. (2021). Also, the role of this protein in DNA repair and chromosome condensation was shown by Pulvers *et al*. (2015). The molecular characterization of *MCPH1* gene declared the involvement of this gene not only in brain development but also in the maintenance of genome integrity. Hou *et al*. (2015) discussed the important role of *DACT1* protein in placenta development through the activation of Wnt signaling and the role of *DACT1* in embryogenesis and organogenesis of the female reproductive tract in mouse models was proved by Xing *et al.* (2016).

DKKL1 gene is present only in mammals and this product is expressed in developing and in the trophectoderm/placental lineage suggesting its role in embryo development (Kohn *et al.* 2010; Yan *et al.* 2012). *PLCZ1* is considered a physiological stimulator for Calcium generating which has a role in activation of egg and early development of embryo in mammals (Saleh *et al.* 2020). *PLCZ1* was reported as a candidate marker in oocyte activation following fertilization and its mutation has a role in infertility (Escoffier *et al.* 2016). Mărginean *et al.* (2019) found an association between GNB3 polymorphism of *PNPLA3* gene with gestational weight gain in humans and rodents and reported the effect that weight gain on birth outcome. The highly expressions of these five recently reported genes in HFG from Zaraibi goats compared to those in LFG is in concordant with their fertility-associated backgrounds and their association with their roles in enhanced fertility performance.

During the study of transcriptomic patterns of granulosa cells in different follicle developmental stages using high-throughput single-cell RNA sequencing, Li *et al*. (2021b) identified that many GC marker genes were associated with fertility performance in Chinese Ji'Ning grey goat breed. The high expression of seven from these ten genes was reported in the present study, including *GADD45, CST3, FUCA1, ID3, TPM2* and *LGALS1*. The role of *GADD45* proteins in the differentiation of embryonic gonads and primary sex determination was showed in most mammals (Johnen *et al.* 2013). They reported that the deficient in *GAAD4*5 proteins – including *GADD45A* – influence testis development and male fertility. Cystatin C (*CST3*) is an inhibitor in seminal plasma and expressed in animal uteri. This protein preserves the fertilizing ability of sperms and facilitates their entry in the reproductive tract by enhancing the motility (Lee *et al*. 2018). Cui *et al*. (2019) found that the upregulation expression of *c-Fos/c-Jun* transcription factor-mediated poFUT1 promotes embryo adhesion. The evidence for the role of *fos-1* and *jun-1* in the ovulation control and regulation of rhythmic program in animals was confirmed by Hiatt *et al.* (2009). The regulation effect of some follicular genes including *FUCA1* expression on bovine oviduct genes was reported by Fontes *et al.* (2018). Carroll and Robaire (2012) provided the role of *ID3* in the reproductive system of male as well as healthy offspring. The role of regulated expression of some DNA binding proteins including *ID3* in fertility of pigs was discussed by Han *et al*. (2018). Many non-coding RNAs including *TPM2* are considered strong candidate markers related to spermatogenesis (Joshi and Rajender 2020). The different contributions of *LGALS1* and *LGALS3* in the homeostasis of fallopian tubes and pathophysiology of pathophysiology of ectopic pregnancy were reported by Zhu *et al.* (2016). Barrientos *et al.* (2014) declared the role of *LGALS1* in the regulation of development and maintenance of pregnancy and pointed out its essential role in pregnancy.

Conclusion

The assessment of expressions for these new-reported follicular genes evaluated in this study and their associations with ovulation rate and fertility trait can help in selection of the animals entering in reproductive breeding programs with high fertility performance.

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Competing Interest

There is absolutely no conflict of interest between the authors of this manuscript and any other scientists or producers.

Author Contributions

OEO: Planning, conducting the research, data analysis and manuscript preparation. NAA: Collecting samples, performing isolation, performing QTPCR test.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a request to the corresponding author.

Ethics Approval

Not applicable to this paper.

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