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Investigation of the Functional Proteins Related to Fertility in Cattle's Endometrium by Protein-Protein Interactions Networks

Research Article

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ABSTRACT

Pregnancy loss is an important economic loss in cattle industry. This study was conducted to identify pre- and / or post-implantation genes and cellular algorithms. For this purpose, transcriptome data of endometrium tissue were analyzed. These data refer to three heifer categories: high fertile (HF), sub-fertile (SF) and infertile (IF). After gene detection, genes were divided into two groups: Up-expressed genes, which were up-regulated in every comparison of either favorable fertility cases or unfavorable fertility cases (HF *vs*. SF, HF *vs*. IF, and SF *vs*. IF), and down-expressed genes, which were down-regulated in the mentioned comparisons. String database was applied to construct protein-protein interaction (PPI) networks and clusterone plugin was used to determine the significant sub-network. Enrichment analysis, which involves the gene ontology and functional pathway, was performed to enrich the results. Our results suggested that over-expression of *SHCBP1*, *NOP14*, *PGM5*, and *DHX58* genes may have positive effect on the outcome of pregnancy, and down-expression of *IMP3*, *ATP5O*, and *RPL7* genes could help the reproductive efficiency. The results of the present study showed that the genes in up-regulated clusters could manipulate epithelial differentiation, fundamental biological role, glucose metabolism, and immune response, which led to reduced pregnancy loss. Also the genes in down-regulated clusters may participate in the improvement of pregnancy outcome by inducing anti-apoptotic processes. This study proposes the pregnancy-associated key genes and pathways to improve pregnancy success in cattle and other domestic animals.

KEY WORDS gene expression, major genes, molecular pathways, pregnancy loss.

INTRODUCTION

It is known that in dairy cattle industry the improvement of reproductive performance is essential for increasing milk production and economic efficiency. Reproductive diseases (such as retained placenta, metritis, and ovarian cysts), calving season, milk production, and genetics are the most important elements affecting fertility. Furthermore, the problems related to cattle health and management, such as impairment of immune function, poor estrous symptoms, increased non-ovulation period after calving, reducing pregnancy rate, and high rate of mortality, affect the reproductive performance (Walsh *et al.* 2011). In other words, fertility is affected by various factors, which all of those factors have slight, but not negligible, effect on this trait. Genetic improvement of this trait is more complicated due to its low heritability (Sheikhlou *et al.* 2018). Identifying the most important genetic regulators controlling the reproductive trait, and using them in genetic selection or selection index, is a difficult, complex and challenging task.

The reproductive genetic markers have been investigated in several studies. Walker *et al.* (2009) studied the expression profiling of endometrium tissue in order to evaluate the effect of genes on pregnancy. They also provided 10 molecular markers controlling the pregnancy establishment. The potential genes which cause embryo mortality, which describe infertility and subfertility phenotypes, were also investigated (Geary *et al.* 2016). These researchers also reported that biomarker genes related to uterine competency for pregnancy are involved in immune signaling pathway. In another study, endometrial transcriptome (6 days after artificial insemination) was used to screen the potential gene markers for pregnancy maintenance (Binelli *et al.* 2015).

Genome wide association analysis was implemented to provide quantitative trait loci (QTL) markers related to early pregnancy loss in cattle (Neupane *et al.* 2017). The gene expression profiling of peripheral white blood cells was applied to introduce the potential biomarkers of pregnancy status, and it is possible to use these biomarkers not only for physiological condition, but also for classifying the methods of pregnancy (artificial insemination or natural breeding) (Dickinson *et al.* 2018).

Bioinformatics is a powerful tool in identification of novel biomarkers related to the complex traits from OMIC data. One of the most favorite tools in this topic is protein-protein interactions (PPIs) networks. Biomarkers proposed by this approach, have more biological importance, because these networks consider all the interactions between significant markers of a given trait (Dehmer *et al.* 2013). PPIs were applied to recognize the most important regulatory pathways and genes involved in activation of immune system in weaning stress condition (Behdani and Bakhtiarizadeh, 2017).

Huang *et al.* (2018b) constructed gene regulatory network form disease-related gene sets, which was screened by meta-analysis.

They also detected biomarkers involved in human disease by identifying PPIs. PPIs network was applied to discover novel interactions and elicit similar functional gene sets (Tian *et al.* 2017).

Although studies have been conducted on the identification of molecular biomarkers related to pregnancy, but considering the importance of this issue on domestic animals, especially dairy cattle, further studies should be conducted to clarify signaling pathways controlling pregnancy in the endometrium and identify more gene markers regulating different aspects of this physiological condition. In this study, the most important signaling pathway and gene markers related to the endometrial ability to establish and maintain pregnancy in cattle were described by screening PPIs network.

MATERIALS AND METHODS

Data set

In this study, endometrium RNA-Seq data from the study conducted by Geary *et al.* (2016) were used to achieve fertility-related molecular markers and signaling pathways. These data referred to endometrium gene expression of three predominantly classified groups of Angus heifers: high fertile (HF), sub-fertile (SF), and infertile (IF). Heifers' ability to maintain pregnancy from embryo transfer was the basis of the fertility classification. Endometrium tissue was biopsied 14 days after the pregnancy. These data are available on NCBI database under the code of GSE81449 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE81449).

Gene detection

The quality control analysis of all samples reads was performed using FastQC (a quality control tool for high throughput sequence data). Then, low quality read filtering (quality score below 20) and adaptor sequence removing were performed by Trimmomatic software (Bolger *et al.* 2014). In the next analysis step, the rest of reads were mapped by Tophat2 software (v2.0.9) to bovine genome (UMD3.1) from Ensemble database (Trapnell *et al.* 2009).

HTseq (v0.6.1) was also used to detect the genes using the intersection-strict mode and the Ensemble annotation (version 3.1.78) of the bovine genome (Anders *et al.* 2015). In this step, in each given gene the number of reads which strictly matched to gene's sequence were determined. An expression matrix was constructed by merging HTseq output from each sample. Furthermore, the EdgeR Bioconductor package was used to differentially detect gene expression (Robinson *et al.* 2010). In order to increase the accuracy of differential gene expression analysis, low expression genes (rather 1 count per million in at least 3 sample) were removed from the expression matrix by this package (Labaj *et al.* 2011). In this study, it was attempted to classify genes into the following categories by fold change: up-expression genes and down-expression genes, in comparisons of HF *vs*. SF, HF *vs*. IF, and SF *vs*. IF.

PPIs network and significant sub-network

It was assumed that a given gene could be an important fertility biomarker depending on the up-expressed or down-expressed in comparisons of either favorable fertility cases or unfavorable fertility cases, such as HF *vs*. SF, HF *vs*. IF and SF *vs*. IF. String website (https://string-db.org/) was applied to survey the interactions of up-expressed and down-expressed genes (Szklarczyk *et al.* 2014). Clusterone cytoscape plugin was used to find significant sub-network (Maraziotis *et al.* 2008).

This plugin was applied to the network clustering and modules detecting based on graph topology and overlapping neighborhoods (Sahrawat and Bhalla, 2013; Shao-Jun, 2015).

Enrichment analysis

In order to predict biological gene pathways of significant sub-networks, which manage the animal's ability to pregnancy, comparative GO (www.comparativego.com) was used (Fruzangohar *et al.* 2013). Comparative GO is a web tool GO resource for gene classification, based on the cellular component, biological process, and molecular function. EnrichNet (www.enrichnet.org) (Glaab *et al.* 2012), which is a web-based application, was applied to peruse the functional network and KEGG (Kyoto Encyclopedia of Genes and Genomes) was the pathway of the significant sub-network.

RESULTS AND DISCUSSION

To recognize the cellular algorithm for pregnancy maintenance, the endometrium transcriptome data of three cattle category (HF, SF and IF) were analyzed (Geary *et al.* 2016). In this study, co-expressed genes were also considered, based on which all the genes were divided into two groups of up-expressed genes and down-expressed genes. Up-expressed genes were those with expression of a fold change above zero in all comparisons of desirable uterine capacity vs. undesirable uterine capacity (HF *vs*. SF, HF *vs*. IF, and SF *vs*. IF). Moreover, those genes with expression fold change under zero in all the same comparisons, were called down-expressed genes.

The results showed that 2517 genes were up-expressed (supplemental file S1: Co-expressed gene which up-regulated in every comparison of desirable uterine capacity vs. undesirable) and 2865 genes were down-expression (supplemental file S2: Co-expressed gene which down-regulated in every comparison of desirable uterine capacity vs. undesirable). The Search Tool for the Retrieval of Interacting Genesb (STRING 10.0, http://string.embl.de/) database was used to confirm the co-expressed relation of up-expressed and down-expressed genes and construct a co-expression network. Co-expression of up-regulated gene network consisted of 97 genes and 165 interactions (Figure 1), also the PPIs enrichment P-value was < 1.0e-16 (supplemental file S3: PPIs of co-expressed of up-regulated genes). Co-expression of 354 down-regulated genes was approved by STRING web-tool and based on co-expressed relation by 1648 interactions (Figure 2) and PPIs enrichment p-value was < 1.0e-16 (supplemental file S4: PPIs of co-expressed of down-regulated genes).

ClosterOne, which is a cytoscape plugin, was used to dissect co-expressed networks and achieve significant sub-network. Up-expressed co-expression network was analyzed to 4 clusters (P<0.001) (Table 1, detailed information on supplemental file S5: Clustering of PPIs of up-regulated co-expression genes), and down-expressed co-expression network was dissected to 3 sub-networks (P<0.001) (Table 3, detailed information on supplemental file S6: Clustering of PPIs of down-regulated co-expression genes).

To find functional roles of significant up- and down-expressed co-expression sub-networks, enrichment analysis was performed by comparative GO analysis for biological process related to fertility capacity and EnrichNet for pathway. The biological processes, which overexpress in up-expressed co-expression sub-networks, were cell proliferation, RNA processing, immune response, and gluconeogenesis, respectively (Table 5, detailed information on supplemental file S7: Biological processes of up-regulated co-expression gene clusters). The biological processes down-regulated by down-expressed co-expression sub-networks were ribosomal biogenesis, mitochondrial respiratory chain, and apoptotic process (Table 6, detailed information on supplemental file S8: Biological processes of down-regulated co-expression gene clusters). The analysis showed that the first up-expressed co-expression sub-networks activated cell cycle pathway and other up-expressed co-expression sub-networks did not affect any cellular pathways (Table 2). Second and third down-expressed co-expression sub-networks affected the oxidative phosphorylation and ribosome pathways (Table 4).

Geary *et al.* (2016) used differential gene expression analysis to find the genes effective in pregnancy maintenance. To achieve these findings, they obtained 26 and 12 differentially expressed genes respectively in comparison of HF to SF and SF to IF. They selected two thresholds (>2fold change and false discovery rate (FDR) P<0.10). In various studies, different levels of FDR and fold change were applied to select differentially expressed genes. In each levels of thresholds, it is possible to loss some effective genes, such as transcription factors, which express in basic levels but have unignorable roles in cellular functions and programs. Therefore, in this study, co-expressed genes were investigated to reduce the ignorance of potential effective genes related to uterine capacity to stablish and maintain pregnancy. Co-expressed genes are the up- or down-expressed genes in every applied comparison (HF *vs*. SF, HF *vs*. IF and SF *vs*. IF). In other words, in this study, it is assumed that a given up- or down-expressed gene, not only in comparison of HF *vs*. SF but also in HF *vs*. IF and SF *vs*. IF, potentially affected the animal's ability to maintain pregnancy.

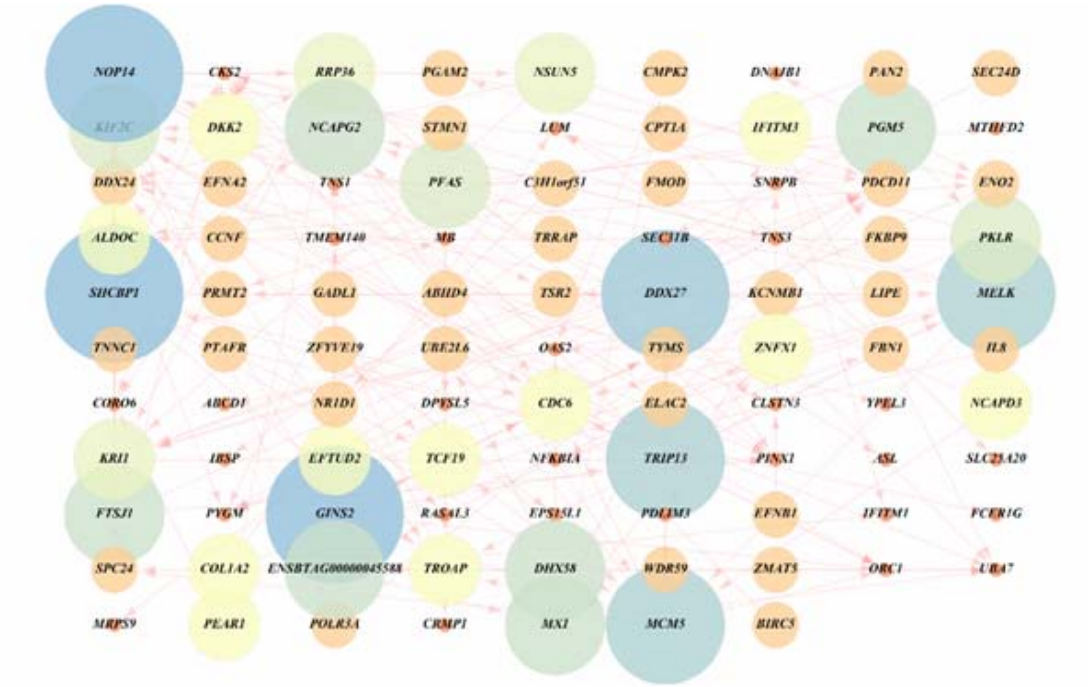

Figure 1 Pregnancy-related up-regulated PPI networks
The important regulatory genes are shown in large blue nodes
Nodes' regulatory role reduce, as they turn to red color and small size

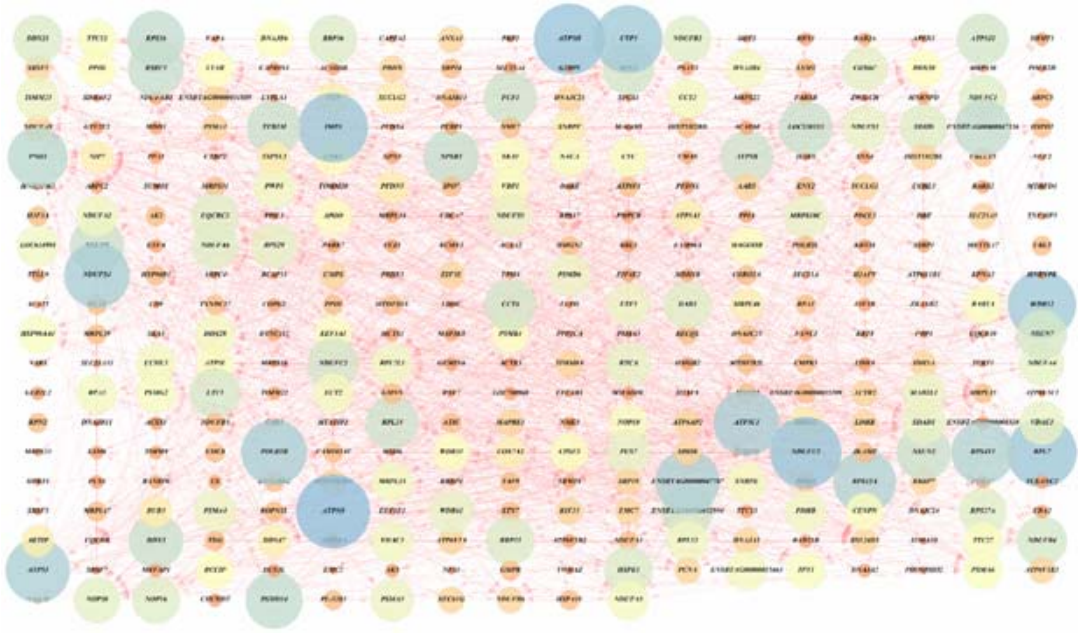

Figure 2 Pregnancy-related down-regulated PPI networks
The important regulatory genes are shown in large blue nodes
Nodes' regulatory role reduce, as they turn to red color and small size

Based on this, two gene categories were formed: up co-expressed genes and down co-expressed genes. Gene interactions of each category, searched by String database, constructed the up and down PPIs networks. ClusterOne, which is a cytoscape plugin, segregated up and down PPIs networks to achieve significant up and down sub-networks.

Network analysis by cytoscape showed that SHCBP1, NOP14, PGM5 and DHX58 were the most important regulator genes in up co-expressed clusters (supplemental file S9: Network analysis of up-regulated co-expression gene clusters). SHCBP1, which is the major regulator gene in first cluster, is a conserved gene in eukaryotes and plays

critical roles in T-cell proliferation (Peng *et al.* 2017) and carcinogenesis (Buckley *et al.* 2014). Various studies identified the important roles of SHCBP1 and its orthologue genes in spermatogenesis in cytokinesis step of vertebrates (Montembault *et al.* 2010; Liu *et al.* 2014). In a recent report by Huang *et al.* (2018b) RNA-Seq data illustrated that this gene expression was induced in endometrial tissue in hamster. *SHCBP1* gene was responsive to estrogen and progesterone by microarray analysis (Ren *et al.* 2015). The SHCBP1 up-regulated greater than five folds in endometrium, detected as functional molecule in maternal-fetal communications (Pavličev *et al.* 2017).

Biological processes and functional role of genes, involved in the first sub-network of up-regulated PPIs network, contributed to cell proliferation (Table 5). It is known that endometrial proliferation and vascular remodeling not only is necessary during pregnancy (Robb *et al.* 2007), but also is critical to embryo implantation (Tranguch *et al.* 2005; Egashira and Hirota, 2013). Our findings suggested that this sub-network had the major candidate genes affecting pregnancy maintenance through proliferation and differentiation of endometrial tissue and proper embryo–uterine communications.

Network analysis showed that in second significant up-regulated co-expressed sub-network, NOP14 had a critical biological role (supplemental file S9). NOP14 is a conserved protein and it is essential for proper maturation of 18S rRNA and ribosome biogenesis (Liu and Thiele, 2001). Exome sequencing using DNA indicated that homozygous variant of this gene led to pregnancy loss and fetal mortality (Suzuki *et al.* 2018).

It was previously reported that this gene contributes in different tissue development in zebrafish embryo (Burns *et al.* 2009). Fundamentally biological role of this gene in cell leads to embryonic lethal in mutation cases (Ching *et al.* 2010).

It was illustrated that NOP14 was down-regulated in hypoxia condition and it is related to pregnancy disorder (Chakraborty, 2013). The results of this study suggest that genes of the second significant up-regulated sub-network, are involved in ribosomal processing and affect pregnancy by fundamental cellular roles.

Based on this study, PGM5 was a considerable gene in the third cluster of up-expressed genes (Figure 3). Gene ontology also showed that carbohydrate metabolic pathways are significantly overexpressed (Table 5).

Table 1 Significant up-expressed co-expression sub-networks

Cluster	Size	Density	P-value
1	15	0.5333	1.03E-06
2	13	0.4615	1.17E-05
3	8	0.5	0.000181
4	10	0.5	0.000146

Table 2 Functional pathways of up-expressed co-expression sub-networks

Cluster	Pathway	Q-value
1	Cell cycle	0.0094
2	Not significant pathway	-
3	Not significant pathway	-
4	Not significant pathway	-

Table 3 Significant down-expressed co-expression sub-networks

Cluster	Size	Density	P-value
1	40	0.4936	0
2	34	0.5045	0
3	32	0.5101	1.04E-07

Table 4 Functional pathways of down-expressed co-expression sub-networks

Cluster	Pathway	P-value
1	Not significant pathway	-
2	Oxidative phosphorylation	2.8E-45
3	Ribosome	7.60E-14

The single blastocyst transcriptome analysis, which was performed by RNA-Seq, showed the participation of PGM5 in glucose metabolism and cell adhesion biological pathways (Chitwood *et al.* 2013). In another study, it was stated that homeostasis and transport of lipid and glucose were up-regulated in endometrial tissue at day 17 of pregnancy (Cerri *et al.* 2012). These results indicated that the genes of the third up-expressed cluster are the major markers in pregnancy maintenance, along with important biological role in glucose homeostasis. In the fourth significant sub-network of up-regulated co-expressed genes, DHX58 played the most important role in comparison with other genes (Figure 3).

DHX58 can identify pathogenic RNA and contribute in response to cytosolic double-stranded DNA (dsDNA) (Pollpeter *et al.* 2011; Zhu *et al.* 2014).

Previous studies have indicated that DHX58 induces antiviral immune responses by producing interferons (Satoh *et al.* 2010; Zhu *et al.* 2014).

Transcriptomic analysis of oviduct epithelium demonstrated that genes related to immune terms (such as CXCL6, DHX58, HERC5, ISG15, MX1, MX2, NKL, OAS2, and OASL) up-regulated and affected the equine embryo development (Smits *et al.* 2016).

According to these data, it was declared that this cluster contributes in the immune responses in endothelial tissue and helps to increase uterus capacity (Table 5).

In many studies, it was stated that endometrial lymphocytes and cytokines not only have significant effect on successful pregnancy (Red-Horse *et al.* 2004; Chakraborty, 2013), but also control the endometrial differentiation and implantation (Takabatake *et al.* 1997).

Table 5 Biological processes of up-expressed co-expression sub-networks

Sub-network	GO ID	GO name	P-value
1	278	Mitotic cell cycle	2.90E-07
	8283	Cell proliferation	0.001599
	6270	DNA replication initiation	4.61E-06
2	462	Maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	8.61E-06
	10501	RNA secondary structure unwinding	8.78E-05
3	61621	Canonical glycolysis	6.25E-10
	6006	Glucose metabolic process	3.70E-08
	5975	Carbohydrate metabolic process	1.71E-06
	6094	Gluconeogenesis	1.87E-06
4	6955	Immune response	0
	9615	Response to virus	3.03E-10
	35456	Response to interferon-beta	7.23E-10
	35455	Response to interferon-alpha	1.05E-09

Table 6 Biological processes of down-expressed co-expression sub-networks

Sub-network	GO ID	GO name	P-value
1	6351	Transcription, DNA-templated	1.50E-08
	42273	Ribosomal large subunit biogenesis	1.46E-05
	6364	rRNA processing	2.46E-05
2	22904	Respiratory electron transport chain	0
	32981	Mitochondrial respiratory chain complex I assembly	0
	15992	Proton transport	0.003936
	6915	Apoptotic process	0.014482
3	42274	Ribosomal small subunit biogenesis	3.46E-05
	70124	Mitochondrial translational initiation	0.000168
	6915	Apoptotic process	0.031914

Studies also revealed that cytokines and steroid hormones construct integrated pathways to control gene expression involved in the preparation of uterine for implantation (Li *et al.* 2001; Spencer *et al.* 2008).

Down co-expressed clusters IMP3, ATP5O and RPL7 were the most biologically important genes, controlling the gene expression of others (supplemental file S10: Network analysis of down-regulated co-expression gene clusters).

IMP3, which is the most important regulatory gene in first down-regulated sub-network (Figure 4), acts as RBPs (RNA-binding proteins) and it has biological and pathological roles in mRNA fate by regulating miRNA activity through imposing a stabilizing effect on the structure of mRNA targets (Degrauwe *et al.* 2016).

This gene is associated with cell migration and tumor invasion and it was reported in recurrent pregnancy loss (Luizon *et al.* 2017), oncofetal biomarker (Zheng *et al.* 2008), and endometrial cancer (Djordjevic *et al.* 2012). Furthermore, studies have indicated that IMP3 is a regulatory element for IGFII.

Also, IMP3 has a negative effect on IGFII signaling and it inhibits the differentiation and proliferation induced by this signaling pathway in central nervous system (Mori *et al.* 2001). These results suggest that down expression of IMP3 is necessary for pregnancy maintenance and its success. Also the first cluster of down-regulated network is involved in RNA processing and ribosome biogenesis.

It seems that considering the high regulatory effect of IMP3 in this cluster, these genes could cooperate in mRNA fate by interactions between miRNA and RBPs. Also, there are studies demonstrating the functional link between miRNAs and other important regulatory genes, such as WDR12 (Shen and Liu, 2017), POLR1B (Hu *et al.* 2012), and UTP3 (Gutierrez *et al.* 2010). In second down-expressed cluster, ATP5O is the most considerable regulatory element (Figure 4). ATP5O is involved in mitochondrial energy production processes and it is one of the genes introduced as biomarker of mitochondrial electron chain activity (Brüggemann *et al.* 2017). Overexpression of this gene was reported in the blastocyst and its development (Gad *et al.* 2011). It has been reported that ATP5O is one of the 35 genes down-regulated in normal endometrium tissue (Saxena, 2015). This gene was also induced in immature uterine of rats through estradiol and estrogenic compounds (Hong *et al.* 2006). Also, the ATP5O involvement in the implantation processes and endometrium modulation in mouse was indicated using LongSAGE method (Ding *et al.* 2013).

It was demonstrated that down-regulation of ATP5O and other mitochondrial electron transfer genes inhibits the apoptosis by reducing reactive oxygen species (ROS) and inducing oxidative stress pathway (He *et al.* 2006). It seems that down-expression of this gene help the survival and existence of endometrium cell.

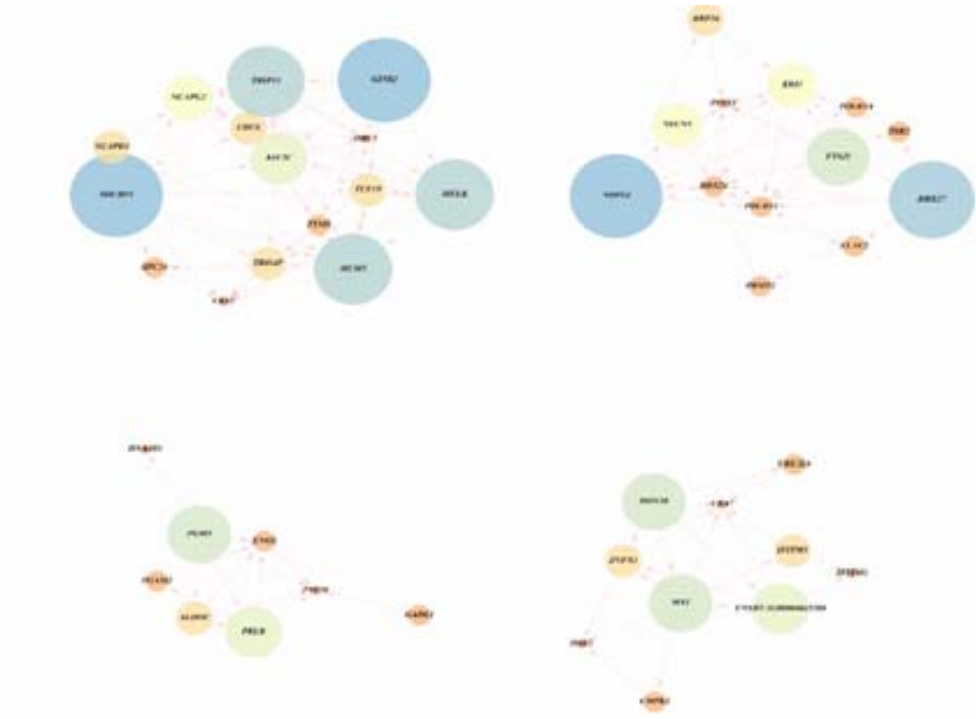

Figure 3 Significant up-regulated sub-networks associated with pregnancy maintenance
The important regulatory genes are shown in large blue nodes
Nodes' regulatory role reduce, as they turn to red color and small size

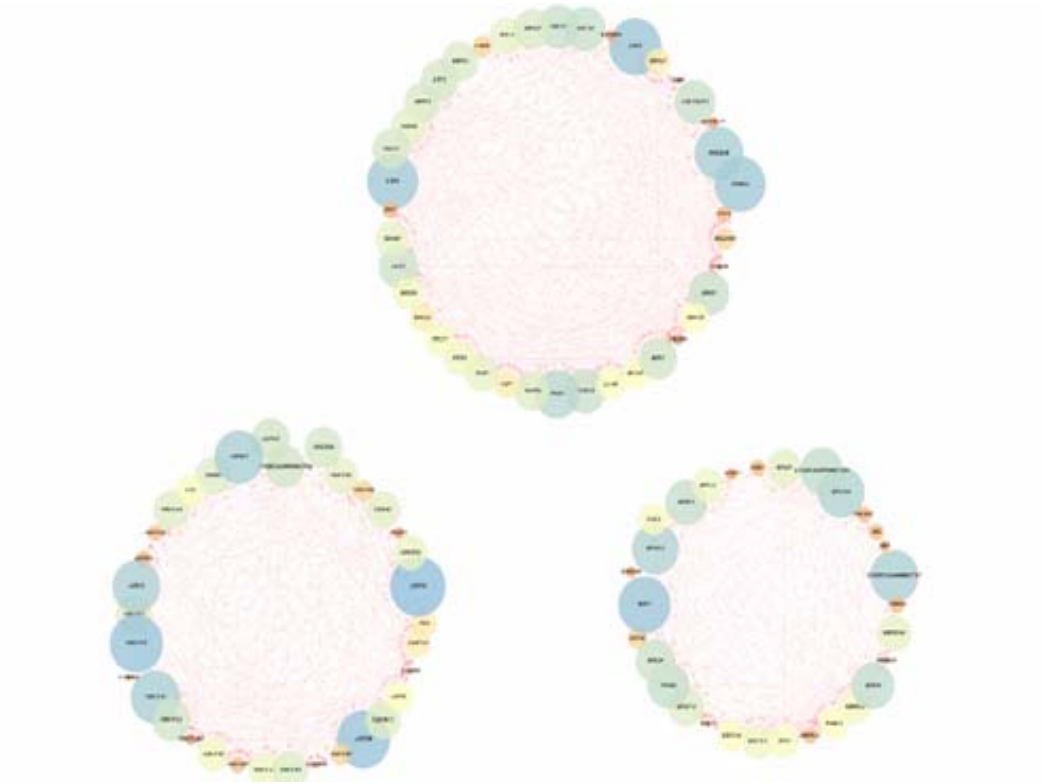

Figure 4 Significant down-regulated sub-networks associated with pregnancy maintenance
The important regulatory genes are shown in large blue nodes
Nodes' regulatory role reduce, as they turn to red color and small size

According to gene ontology results (Table 6), ATP5O is related the cluster participating in respiratory-related processes, which induce production of intracellular ROS and cell degeneration. The down-regulation of this cluster potentially provides possible regulatory manners for endometrial cell maintenance by inhibiting cellular oxidative stress condition.

Based on the statistical analysis of network, RPL7 is the major principal node in interactions of the third down-expressed clusters (Figure 4). This gene is a ribosomal protein and it is highly conserved across eukaryotes. RPL7 is a multifunctional protein involved in growth, transformation, and aging of cell (Hemmerich *et al.* 1993).

It was also proposed that RPL7 is one of significant genes in apoptosis and could regulate metastasis processes (Zucchini *et al.* 2008) and expression of this gene was reported to be in response to cellular oxidative stress (Grewal *et al.* 2004). In endometrial cell, down-expression of RPL7 potentially mediates cell aging and inhibits the apoptosis process. These results indicated that the reaction of mitochondrial oxidative phosphorylation and mitochondrial translation processes are the most significant biological overexpressed processes of the third down-expressed clusters (Table 6), which induce precocious aging. Therefore, this cluster induces uterine capacity by inhibiting the mitochondrial activity. There are several studies indicating mitochondrial oxidative reaction, mitochondrial protein expression interaction, and protein synthesis machinery construct complex pathways, which determine cell fate (Wadhwa *et al.* 2015; Ansoleaga *et al.* 2016).

CONCLUSION

The results of this paper highlight genes and molecular mechanisms, participating in the capability of endometrium cell and providing the necessary conditions for pre- and/or post-implantation of fetus. Our analysis proposes the potential biomarkers of the optimal endometrial tissue conditions in implantation, which may have positive effect on cell proliferation, gluconeogenesis, immune response, and anti-apoptotic processes.

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