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ORIGINAL ARTICLE

Identification of the key regulating genes of diminished ovarian reserve (DOR) by network and gene ontology analysis

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Abstract Diminished ovarian reserve (DOR) is one of the reasons for infertility that not only affects both older and young women. Ovarian reserve assessment can be used as a new prognostic tool for infertility treatment decision making. Here, up- and down-regulated gene expression profiles of granulosa cells were analysed to generate a putative interaction map of the involved genes. In addition, gene ontology (GO) analysis was used to get insight intol the biological processes and molecular functions of involved proteins in DOR. Eleven up-regulated genes and nine down-regulated genes were identified and assessed by constructing interaction networks based on their biological processes. PTGS2, CTGF, LHCGR, CITED, SOCS2, STAR and FSTL3 were the key nodes in the up-regulated networks, while the IGF2, AMH, GREM, and FOXC1 proteins were key in the down-regulated networks. MIRN101-1, MIRN153-1 and MIRN194-1 inhibited the expression of SOCS2, while CSH1 and BMP2 positively regulated IGF1 and IGF2. Ossification, ovarian follicle development, vasculogenesis, sequence-specific DNA binding transcription factor activity, and golgi apparatus are the major differential groups between up-regulated and down-regulated genes in DOR. Meta-analysis of publicly available transcriptomic data highlighted the high coexpression of CTGF, connective tissue growth factor, with the other key regulators of DOR. CTGF is involved in organ senescence and focal adhesion pathway according to GO analysis. These findings provide a comprehensive system biology based insight into the aetiology of DOR through network and gene ontology analyses.

Keywords Diminished ovarian reserve · Granulosa cells · Biological processes · Molecular functions · Interaction network

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Introduction

Infertility is a prevalent disorder worldwide affecting 15–20 % of couples at the reproductive age [29]. Although infertility treatments have been improved through assisted reproductive technology (ART) over the past 30 years, the molecular reasons of infertility have not been fully identified [31].

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Women who respond well to fertility treatments are defined as having normal ovarian reserve (NOR). Ovarian reserve (OR) refers to the number and quality of the eggs that are produced by the ovaries in the follicular phase of the menstrual cycle and the eggs that are produced in the ovaries following injection of follicle stimulating hormone (FSH) for infertility treatment.

One of the challenging reasons for infertility is diminished ovarian reserve (DOR). This term refers to a clinical condition in which the ovary does not have as many oocytes as would be estimated using the woman's age. The aetiology is currently unknown, and no new treatment is available aside from the present standard fertility treatment. Furthermore, there is no unique screening test for young patients [31, 35]. Bidirectional paracrine oocyte-granulosa cell communication is necessary for normal follicular growth and oocyte development [22]. The maturation, development and quality of oocytes are provided through cumulus cells by growth factors, paracrine signals, secondary messenger and nutrient exchanges [3, 5, 17, 22, 37, 41]. In addition, cumulus cells are involved in extracellular matrix and apoptosis via microRNAs (MIR29a, MIR30d, MIR21, MIR93, MIR320a, MIR125a and the LET7 family) [7].

There are differences in hormonal levels throughout the cycle in DOR patients compared to patients with NOR at the same age, which suggests that granulosa cells are useful as target points for further investigation [31]. In addition, studies in mice, sheep and humans [22, 31] have revealed changes in ovulation and fertilisation rates due to alterations in the gene expression of granulosa cells. These finding also suggest a role of granulosa cell function in women who have DOR and in fertilisation.

Many research groups have investigated eggs and granulosa cell transcriptomes to find the regulatory mechanism of OR using several high-throughput techniques, such as SAGE, EST analysis, massively parallel signature sequencing and microarrays [1, 14, 32, 33]. These efforts have succeeded in identifying altered important genes that are involved in DOR, such as *CXXC5, FOXC1, CTGF, FSTL3, PTGS2* and *SOCS2, GREM1, PGRMC2, PTGER3, StAR, LHCGR, StARD4, IGF1R* and *IGF2R* [16, 19, 22, 31]. Advanced computational biology approaches such as network analysis, gene ontology (GO), and meta-analysis have been developed recently to discover functions of genes and protein structures and provide a comprehensive view on underlying molecular mechanisms of systems biology [2]. Dynamic protein interaction network analysis, in particular regulatory network analysis, is a strong well-developed system biology analytical tool in understanding of the final goal of gene/protein interactions, and selecting the central genes/proteins (hubs) [30]. Herein, protein interaction networks of up- or down-regulated DOR genes were examined to investigate the underlying biological processes that govern gene functions and operations and also to discover any possible novel interaction pathway.

GO of up-regulated and downregulated genes is an important analysis in interpreting the transcriptome and disease [9, 12, 13]. GO enrichment categorises the genes and proteins using the controlled universal vocabulary in three groups of Biological Process, Molecular Function, and Cellular Component which results in the same functional annotation understanding. GO analysis of a given sample verses GO distribution of whole genome (as reference) by hypergeometric test monitors the different pathways and functional groups.

The aim of this study was to apply a range of computational biology methods such as network construction, network discovery, and GO analysis to unravel regulatory components and mechanism of DOR.

Materials and methods

Up- and down-regulated gene lists (presented in Tables 1 and 2, respectively) of DOR were extracted based on microarray experiments from previous scientific publications [19, 21, 22, 31, 38]. The up- and down-regulated DOR genes (presented in Tables 1 and 2, respectively) were targeted for bioinformatics analysis to construct regulatory networks.

Protein interaction network construction

Interaction protein networks for the mentioned up- or down-regulated genes were constructed using Pathway Studio Web tool from Elsevier Life Science. Pathway Studio is a "biological decision support tool" that gathers the interaction information based on literature mining, microRNA prediction effect, transcription factor-promoter binding and helps the scientists understand disease mechanisms and predict putative functionality and target-drug interactions by analysing and visualising them in a biological context" (http://www.elsevier.com/online-tools/pathway-studio). This tool integrates data from several databases, namely, RESNET (http://www.ariadnegenomics.com/), KEGG [20], GO [6], DIP [40], ERGO (http://www.integratedgenomics.com/) and PathArt (http://jubilantbiosys.com/) [24]. The system provides computational algorithms that automatically extract data from the literature. In the present study, algorithms such as neighbourhoods of expansion, sub-network discovery by Gene Set Enrichment (GSE) gene ontology were used to construct the up- and down-regulated DOR gene networks in this study. In particular, GSE is a powerful tool which finds the putative significant networks generated buy up- and down-regulated DOR genes at $p = 0.05$ [4, 10].

To construct interaction pathways from the genes of interest (Tables 1, 2), one neighbourhood of the expansion algorithm was used. Both the upstream and downstream direction for each entity were chosen and set for the following parameters: group, functional class, complex, protein, pathway, cell process, cell object, treatment and diseases as well as the relation types of expression, regulation, mol-transport, protein modification, promoter binding, mol-synthesis, chemical reaction and direct regulation (Tables 3, 4, 8).

Moreover, one neighbour in the expansion algorithms was used to construct the pathway that is related to microRNAs entities. Following the selection of both the upstream and downstream directions for each entity, miRNAs that affect the relation types were nominated for further analysis. Protein interaction networks based on the mentioned entities were extracted to demonstrate their relations at the protein level.

To construct an interaction network based on small molecules and diseases with all of the pre-existing protein–protein interaction datasets, the algorithm was created using neighbour expansion between entities. Both the upstream and downstream directions for each entity were chosen and set for small molecules and diseases, to search relations with regulation and expression parameters.

Gene ontology (GO) analysis

GO analysis was performed to describe the gene products in three categories, namely, biological process, molecular function and cellular component [6, 12, 13].

Comparison of GO distribution of up/down expressed genes in DOR verses genome

GO distribution of up/down expressed genes in DOR were compared to the genome (as reference) to find the functional groups in biological process, molecular functions, and cellular components which are over/down represented in DOR altered genes based on hypergeometric method by Comparative GO web tool [9, 12, 13]. Also, Pathway Studio software was used to identify overrepresented GO terms and to find out whether the genes are functionally related, as well. Over-represented GO terms were nominated using corrected *p*-values at the significance level of $p < 0.05$ and the enrichment of three specific categories, namely, biological processes, molecular functions, cellular components.

Comparison of GO distribution of up/down expressed genes in DOR verses genome

Wilcoxon signed rank test and two-sample Kolmogorov–Smirnov test were used to compare GO distribution in biological process, molecular functions, and cellular components between up and down expressed genes in DOR.

Table 1 Selected up-regulated genes in diminished ovarian reserve (DOR)

Genes up-regulated in diminished ovarian reserve	Designation	Reference
CTGF	Connective tissue growth factor	[22]
FSTL3	Follistatin-like 3	[22]
PTGS2	Prostaglandin endoperoxide synthase 2	[22]
SOCS2	Suppressor of cytokine signaling 2	[22]
LHCGR	Luteinizing hormone receptor	[31]
PGTER3	Prostaglandin E receptor 3 (subtype EP3)	[31]
PGRMC2	Progesterone receptor membrane component 2	[31]
StAR	Steroidogenic acute regulatory protein	[31]
StARD4	StAR-related lipid transfer domain containing 4	[31]
CITED2	C-terminal domain 2	[22]
GAS-1	Growth arrest-specific 1	[22]

Table 2 Selected down-regulated genes in diminished ovarian reserve (DOR)

Genes down-regulated in diminished ovarian reserve	Designations	Reference
CXXC5	CXXC finger protein 5	[22]
FOXC1	Forkhead box C1	[22]
GREM1	Impaired gremlin1	[19]
CXXC5	CXXC finger protein 5	[22]
GBP2	Guanylate-binding protein 2	[22]
ZMIZ1	Zinc finger MIZ-domain containing 1	[22]
IGF1	Insulin-like growth factor 1	[19]
IGF2	Insulin-like growth factor 2	[19]
AMH	Anti-Müllerian hormone	[38] [21]

Table 3 Relation between small molecules and up-regulated proteins

Relation	Type	Connectivity
Melatonin —| PTGS2	Expression	2
Zn^{2+} –+> PTGS2	Expression	2
AMP –+> STAR	Expression	2
Ca^{2+}–+> STAR	Expression	2
AMH —| LHCGR	Expression	2
Lipid –+> CTGF	Expression	2
Glucocorticoids —> STAR	Expression	2
Steroids —| PTGS2	Expression	2
Estrogens –+> LHCGR	Expression	2
Progesterone —> PTGS2	Expression	2
Progesterone —| LHCGR	Expression	2
Estrogens —> PTGS2	Expression	2
D-glucose –+> PTGS2	Expression	2
Aldosterone —+> PTGS2	Expression	2
Steroids —> STARD4	Expression	2
Glucocorticoids –+>CTGF	Expression	2
Aldosterone –+> CTGF	Expression	2
Serotonin —> PTGS2	Expression	2
Tretinoin —| LHCGR	Expression	2
AMP –+> PTGS2	Expression	2
Lipid –+> PTGS2	Expression	2
Glucocorticoids –+> SOCS2	Expression	2
D-glucose –+> CTGF	Expression	2
Tretinoin –+>CTGF	Expression	2
Estrogens –+>SOCS2	Expression	2
Cholesterol —> STARD4	Expression	2
Testosterone –+> PTGS2	Expression	2
ATP —> PTGS2	Expression	2
Serotonin –+> CTGF	Expression	2
PGE2 –+> PTGS2	Expression	2
Cholesterol —| PTGS2	Expression	2
Glucocorticoids —| PTGS2	Expression	2
Tretinoin —> PTGS2	Expression	2
Ca^{2+}–+> PTGS2	Expression	2
Steroids —> LHCGR	Expression	2
Dopamine —| PTGS2	Expression	2
Testosterone —| STAR	Expression	2
Taurine —| PTGS2	Expression	2
PGE2 —> CTGF	Expression	2
Fe^{2+}–+> PTGS2	Expression	2
Mn^{2+}–+> PTGS2	Expression	2
D-aspartate –+> STAR	Expression	2

Network and GO enrichment analysis

Subnetwork analysis

For subnetwork discovery, Gene Set Enrichment [34] and Fisher's exact test was used. In short, highly possible subnetworks ($p \leq 0.05$) of up and down regulating genes in DOR with were identified by:

(1) Selection of an enriched database of gene, protein, and small RNA interactions of Pathway Studio web tool (Elsevier), called ResNet, as reference. This database includes 30,730 pathway collections and 6,566,957 relations of different types of interactions (such as promoter binding, microRNA effect, expression, etc.) and their references. The data is collected via text mining by Medscan tool [25] as well as entries from the major resources such as KEGG (http://www.genome.jp/kegg/) and mirBAse (http://www.mirbase.org/), etc. MedScan employs NLP(Natural Language Processing) algorithm and is extensively used for literature mining and relationship extraction [25].

(2) The obtained list of up and down regulated genes in DOR were used as a query against the pathways of small molecules, microRNAs, protein interaction networks, etc. to evaluate the existence of DOR genes in each of these pathways. Statistical presence, here called enrichment, of DOR related genes was measured using Fisher's exact test at $p \leq 0.05$.

GO analysis

We also used the enrichment concept to predict the functional meaning/aim of the DOR related genes in three terms of biological process, molecular function, and cellular component using Comparative GO tool, as previously described [9, 13]. As enrichment analysis needs a reference for GO enrichment comparison and calculating the p value, we used the GO enrichment distribution of human genome as reference. The difference between enrichment of DOR related genes, compared to GO enrichment of genome, in each GO category was measured by Fisher's exact test. This procedure is called hyper-geometric analysis of GO distribution [12]. It is expected that the differentially enriched GOs verses genome govern the induction of DOR describing the particular functions that the DOR gene list are involved.

Table 4 Sub-networks enriched with expression targets using up-regulated genes

Name	Total no. of neighbors	Overlap	Percent overlap	Gene set seed	Overlapping entities	p values
Neighbors of GDF9	17	3	16	GDF9	PTGS2,STAR,LHCGR	3.93454e−06
Neighbors of STAT5A	22	3	13	STAT5A	PTGS2,CITED2,SOCS2	8.49831e−06
Neighbors of prostaglandin F2alpha	30	3	9	Prostaglandin F2alpha	PTGS2,STAR,LHCGR	2.14041e−05
Neighbors of ADIPOQ	42	3	6	ADIPOQ	PTGS2,STAR,LHCGR	5.80889e−05
Neighbors of gonadotropin	49	3	6	Gonadotropin	PTGS2,STAR,LHCGR	9.16357e−05
Neighbors of MET	8	2	22	MET	PTGS2,CTGF	0.000117095
Neighbors of INHBA	58	3	5	INHBA	CTGF,LHCGR,FSTL3	0.000150673
Neighbors of HMGCR	12	2	15	HMGCR	PTGS2,CTGF	0.000252697
Neighbors of IGF1	191	4	2	IGF1	PTGS2,CTGF,STAR,LHCGR	0.000292064
Neighbors of PRKCZ	13	2	14	PRKCZ	PTGS2,LHCGR	0.000294519
Neighbors of CDC42	14	2	13	CDC42	PTGS2,CTGF	0.000339491
Neighbors of PRKCB	15	2	12	PRKCB	PTGS2,CTGF	0.000387604
Neighbors of BMP6	18	2	10	BMP6	STAR,LHCGR	0.000550686
Neighbors of SMAD7	19	2	10	SMAD7	CTGF,FSTL3	0.000611264
Neighbors of PTGER2	21	2	9	PTGER2	PTGS2,STAR	0.000741688
Neighbors of NPPA	23	2	8	NPPA	PTGS2,STAR	0.000884408
Neighbors of serotonin	24	2	8	Serotonin	PTGS2,CTGF	0.000960355
Neighbors of F2R	25	2	7	F2R	PTGS2,CTGF	0.00103935
Neighbors of EGF	271	4	1	EGF	PTGS2,CTGF,STAR,LHCGR	0.00110643
Neighbors of FSH	26	2	7	FSH	STAR,LHCGR	0.00112138
Neighbors of VEGFA	122	3	2	VEGFA	PTGS2,CTGF,GAS1	0.00131819
Neighbors of KNG1	30	2	6	KNG1	PTGS2,CTGF	0.00147967
Neighbors of JAK2	30	2	6	JAK2	PTGS2,STAR	0.00147967
Neighbors of glutathione RED	30	2	6	Glutathione RED	PTGS2,CTGF	0.00147967
Neighbors of PGR	32	2	6	PGR	PTGS2,LHCGR	0.0016768
Neighbors of RAC1	36	2	5	RAC1	PTGS2,CTGF	0.00210663
Neighbors of LPA	38	2	5	LPA	PTGS2,CTGF	0.00233919
Neighbors of sphingosine-1-P	39	2	5	Sphingosine-1-P	PTGS2,CTGF	0.00245985
Neighbors of LEP	152	3	1	LEP	PTGS2,CTGF,STAR	0.00247597
Neighbors of glucocorticoids	351	4	1	Glucocorticoids	PTGS2,CTGF,STAR,SOCS2	0.00290404
Neighbors of RHOA	43	2	4	RHOA	PTGS2,CTGF	0.00297148
Neighbors of VIP	46	2	4	VIP	PTGS2,STAR	0.00338538
Neighbors of cAMP	372	4	1	cAMP	PTGS2,CTGF,STAR,LHCGR	0.00359656
Neighbors of lipid	54	2	3	Lipid	PTGS2,CTGF	0.00461362
Neighbors of steroids	203	3	1	Steroids	PTGS2,LHCGR,STARD4	0.00561095
Neighbors of aldosterone	61	2	3	Aldosterone	PTGS2,CTGF	0.00583408
Neighbors of TGFB1	428	4	0	TGFB1	PTGS2,CTGF,STAR,LHCGR	0.00599532
Neighbors of TGFA	63	2	3	TGFA	PTGS2,CTGF	0.00620733
Neighbors of GNRH1	63	2	3	GNRH1	PTGS2,LHCGR	0.00620733
Neighbors of LIF	64	2	3	LIF	PTGS2,SOCS2	0.00639799
Neighbors of cholesterol	72	2	2	Cholesterol	PTGS2,STARD4	0.00801931
Neighbors of BMP2	76	2	2	BMP2	PTGS2,CTGF	0.00889307
Neighbors of IL4	252	3	1	IL4	PTGS2,CTGF,SOCS2	0.0102287
Neighbors of AMP	82	2	2	AMP	PTGS2,STAR	0.010281
Neighbors of PRL	93	2	2	PRL	LHCGR,SOCS2	0.0130612

Table 4 continued

Name	Total no. of neighbors	Overlap	Percent overlap	Gene set seed	Overlapping entities	p values
Neighbors of EDN1	99	2	2	EDN1	PTGS2,CTGF	0.0147029
Neighbors of cortisol	99	2	2	Cortisol	PTGS2,CTGF	0.0147029
Neighbors of ROS	99	2	2	ROS	PTGS2,CTGF	0.0147029
Neighbors of F2	100	2	1	F2	PTGS2,CTGF	0.014985
Neighbors of PGE2	102	2	1	PGE2	PTGS2,CTGF	0.0155562
Neighbors of HGF	111	2	1	HGF	PTGS2,CTGF	0.0182432
Neighbors of GH1	111	2	1	GH1	STAR,SOCS2	0.0182432
Neighbors of testosterone	111	2	1	Testosterone	PTGS2,STAR	0.0182432
Neighbors of EGFR	112	2	1	EGFR	PTGS2,CTGF	0.0185534
Neighbors of MAPK3	119	2	1	MAPK3	PTGS2,CTGF	0.0207881
Neighbors of MAPK8	133	2	1	MAPK8	PTGS2,CTGF	0.0255837
Neighbors of MAPK14	152	2	1	MAPK14	PTGS2,CTGF	0.0327557
Neighbors of estrogens	390	3	0	Estrogens	PTGS2,LHCGR,SOCS2	0.033061
Neighbors of IFNG	408	3	0	IFNG	PTGS2,CTGF,SOCS2	0.0371777
Neighbors of Ca^{2+}	163	2	1	Ca^{2+}	PTGS2,STAR	0.0372393
Neighbors of Ethanol	183	2	1	Ethanol	PTGS2,CTGF	0.0459772

Meta-analysis of the key regulating genes of DOR

To verify our conclusions, after the identification of the key regulating genes in diminished ovarian reserve (DOR) by network and GO analysis, the key up-regulated genes (PTGS2, CTGF, LHCGR, CITED, SOCS2, STAR and FSTL3) were used for meta-analysis based on available microarray and RNAseq-based expression data using COXPRESdb web tool [27] as previously described [10]. COXPRESdb has been developed for gene coexpression analysis of large amount of publicly available microarray and RNA-seq expression data which allows relative expression analysis of thousands of genes simultaneously [10, 27]. For meta-analysis of gene expression data, we used correlation ranking approach using Mutual Ranking (MR). MR is a reliable ranking correlation coefficient for measuring biological significance of gene coexpression [26]. In particular, the higher effectiveness of MR comparing to Pearson correlation coefficient is documented in large-scale microarray data of different organisms.

Control independent dataset for measuring the validity of network analysis

In addition to the above mentioned statistical analysis, a control independent dataset was generated and subjected to network analysis to show the validity of the performed analysis.

To this end, the following steps were performed:

(1) Whole list of human genes were downloaded from BioMart– Ensembl (http://www.ensembl.org/biomart/)
(2) One hundred random samples were taken from the all human genes by randomisation tool in Microsoft Excel. Each sample contained 50 genes.
(3) Regulatory subnetwork analysis was performed on each of random samples

If the random samples, does not announce the network of regulatory genes significant, this means that our enrichment analysis is robust and the networks of DOR genes in this study are reliable.

Results

General networks

Protein interaction (PI) networks were constructed based on the mentioned methods for up- and down-regulated genes to demonstrate their relations at the protein level. Supplementary 1 was constructed using one neighbourhood in the expansion algorithm, and it illustrates a regulatory network that is common to the up-regulated proteins of DOR. This network is comprised of 768 entities and 1128 relations. Different relation types in the PI networks were computed, including Expression (580 types), Regulation (315 types), Promoter Binding (88 types), Binding (71 types), Direct Regulation (30 types), miRNA Effect (18 types), Mol-Synthesis (17 types), Mol-Transport (13 types) and Chemical-Reaction (3 types). Among the different proteins in this network, PTGS2, CTGF, LHCGR, CITED, SOCS2, STAR and FSTL3 proteins were the key nodes

with the highest number of interactions (Supplementary). PTGS2 showed positive effects on reproductive functions such as decidualisation, follicular rupture, ovulation, fertilisation, cell growth and cell migration.

For down-regulated genes, 294 entities and 386 relations were extracted. Different relation types in the PI networks were observed, including Regulation (408 types), Expression (376 types), Binding (65 types), Mol-Transport (42 types), Promoter Binding (27 types), Direct Regulation (27 types), Mol-Synthesis (20 types), miRNA Effect (13 types) and Prot-Modification (4 types). Among the different proteins in this network, the IGF2, AMH, GREM1, FOXC1 proteins showed the highest number of interactions. IGF2 showed positive effects on reproductive functions such as pregnancy, oocyte maturation and steroid production. The excel files of these networks are provided in Supplementary materials.

Sub-networks

To examine the details of the aforementioned networks, various sub-networks were constructed, as follows:

miRNA Sub-networks

A total of 46 objects were checked, and the sub-network of the miRNA pathways of the nodes, including 16 miRNAs with 18 relations, was obtained. Some of the differentially expressed proteins, such as STAR, STAR4, LHCGR and GAS1, have not been implicated in the microRNA networks; on other hand, MIRN101-1, MIRN153-1 and MIRN194-1 serve as negative regulators of the SOCS2 protein, and MIRN101-1 and MIRN26A1 serve as negative regulators of the PTGS2 protein (Supplementary 2). In addition, PI networks were extracted to demonstrate microRNA relations at the level of th protein for down-regulated genes. A total of 34 objects were checked, and 12 entities with 13 relations were found. Our results showed that CXXC5 was inhibited by MIRN219-1, MIRN188 and MIRN199A1. Moreover, MIRN128-1, MIRN129, MIRN23A and MIRN27A suppressed GREM1; however, some proteins, such as AMH, IGF2 and GBP2, have not been affected by any microRNAs (Supplementary 2).

Small Molecule Sub-networks

We constructed an interaction network based on small molecules, cell process and diseases with the entire pre-existing protein–protein interaction dataset of up/down regulated genes and the relations in the PI networks that were found in the up/down regulated proteins of DOR; this network is demonstrated in Fig. 1, respectively. As demonstrated in Fig. 1, small molecules such as Zn^{2+}, Mn^{2+}, Fe^{2+} and Ca^{2+} have a positive effect on the expression of PTGS2; however, some small molecules, such as steroids, melatonin and taurine, have a negative effect on PTGS2 expression. Glucocorticoids have a positive effect on STAR and SOCS2 expression. As shown in Fig. 1, Zn^{2+} and Ca^{2+} have a positive effect on IGF1 expression, as well.

The PTGS2/COX2 (Prostaglandin-endoperoxide synthase or cyclooxygenase 2), up-regulated protein in DOR is located in the membrane. The COX2-derived PGI2 played a critical role in blastocyst implantation and decidualisation, and PGI2 mediates its function via PPAR delta receptor. Cox-2 deficit contributes to faulty ovulation, fertilisation, implantation, and decidualisation. The excel format of the networks are provided in the corresponding Supplementary 3.

Regulatory sub-networks

Sub-networks that were enriched with expression and regulation targets were generated based on the genes in Table 5 and Supplementary 5. According to the analysis, small molecules of glucocorticoids overlapped with PTGS2, CTGF, STAR and SOCS2 (Fig. 2), and cAMP overlapped with PTGS2, CTGF, STAR and LHCGR (Fig. 2). The gene set seed, IGF1 protein, overlapped with PTGS2, CTGF, STAR and LHCGR (Fig. 2). Interestingly, the network demonstrates that transforming growth factor-beta1 (TGF-beta 1) regulates an important sub-network that is enriched with expression entities. Relations of this network and its corresponding references are presented in Supplementary 5 and Fig. 2. As illustrated in, TGF-beta 1, as the central network protein, interacts with the up-regulated proteins DOR, including prostaglandin-endoperoxide synthase (PTGS2), CTGF, STAR and LHCGR. This protein causes the expression of PTGS2 and CTGF but inhibits the expression of STAR and LHCGR. PTGS2 expression has been demonstrated to be controlled by GDF-9. In addition, GDF9 showed a positive effect on PTGS2 and STAR expressions and inhibited LHCGR expression (Fig. 2 and Supplementary 4). As can be seen in Fig. 2, EGF protein has covered PTGS2, CTGF, STAR and LHCGR. Moreover, CTGF affects ovulation (Fig. 2 and Supplementary 5).

In addition, the other network indicates that IL-6 is an important regulating sub-network (Fig. 2). The relations of this network and its corresponding references are presented in Supplementary Table 4. In addition, IL6, as the central network protein, interacts with the up-regulated proteins PTGS2 and LHCGR in DOR Fig. 3.

Further predictive bioinformatics analyses were conducted using GO analysis to identify gene products in three categories: the biological process, molecular function and cellular component. Receptor binding and DNA binding

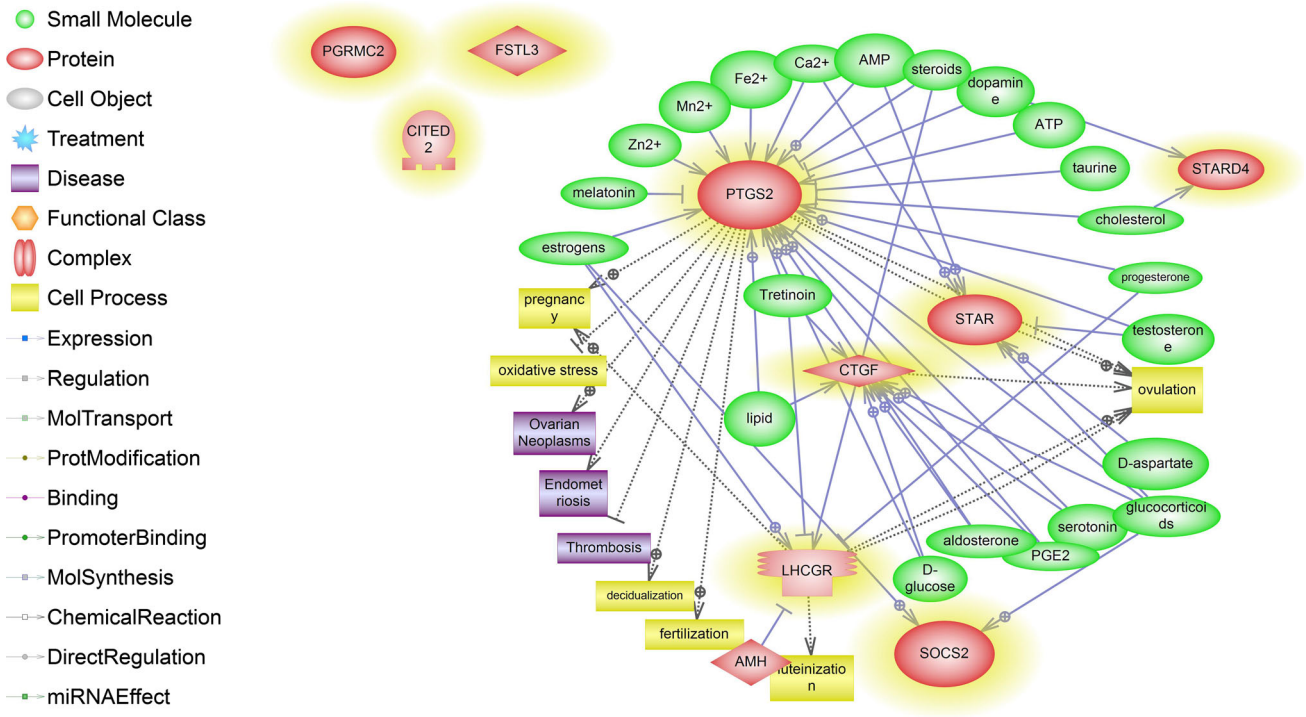


Fig. 1 Interaction network of small molecules and proteins in diminished ovarian reserve (DOR)

Table 5 Sub-networks enriched with regulation targets using up-regulated genes

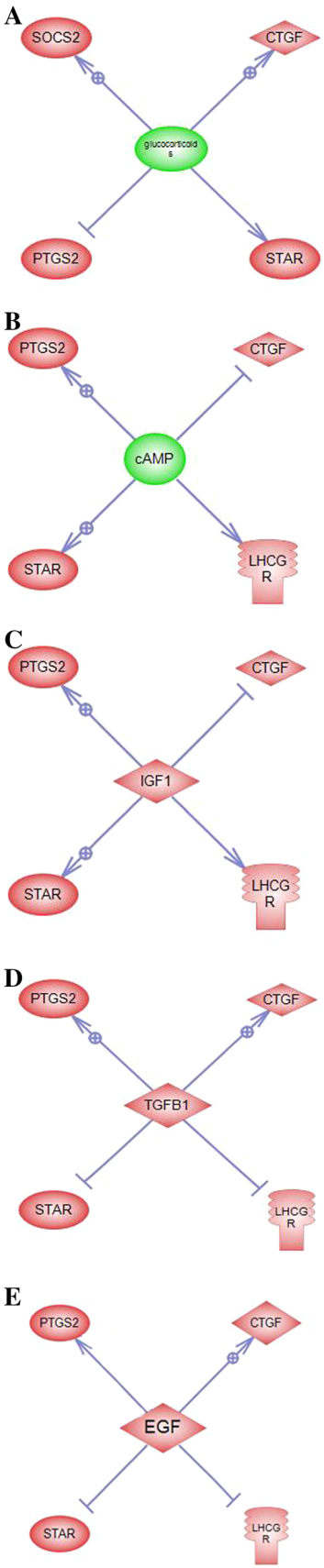
Name	Total no. of neighbor	Overlap	overlap percent	Gene set seed	Overlapping entities	p value
Neighbors of FGF9	10	2	18	FGF9	STAR,LHCGR	4.7311e-05
Neighbors of FSH	30	2	6	FSH	STAR,LHCGR	0.000396034
Neighbors of gonadotropin	51	2	3	gonadotropin	PTGS2,LHCGR	0.00111759
Neighbors of IL6	237	2	0	IL6	PTGS2,LHCGR	0.0216627
Neighbors of EGF	238	2	0	EGF	PTGS2,LHCGR	0.0218345

were overrepresented categories based on molecular function. Most of the represented data that was enriched in the GO terms were related to biological process, molecular function and cellular component, respectively (Supplementary data 9). As demonstrated in the supplementary data, GO analysis has provided the key role of up-regulated genes regulating the biological processes in the reproductive field. PTGS2 and CITED2 regulate decidualisation, and PTGS2 regulates ovulation and embryo implantation. The embryonic process involved in female pregnancy and embryonic development in utero are regulated by CITED2. Additionally, spermatogenesis and the luteinising hormone signalling pathway are regulated by FSTL3 and LHCGR, respectively.

Expression sub-networks

Sub-networks that were enriched with expression and regulation were constructed and analysed using the down-regulated genes (Supplementary 6). CSH1 has a positive effect on IGF1 and IGF2 expression (Supplementary 1), and BMP2 serves as a positive regulator of IGF2, IGF1 and GREM1. GH1 has both positive and negative effects on IGF1 expression and a positive effect on IGF2 expression. Supplementary data are provided in excel format (Supplementary data excel 10). We could not find statistically significant results regarding sub-networks that were enriched with chemical reactions.

Other predictive bioinformatics analyses were conducted for Anti-Mullerian hormone, which mediates male sexual differentiation. This hormone causes the regression of Mullerian ducts, which prevents their differentiation into the uterus and fallopian tubes. In addition, it is used as a marker in some ovarian tumours or ovarian reserve assessments in infertility cases. AMH expression is affected by oestrogen in mammals through E2-mediated inhibition of follicle growth. It reduces the amount of LH



◀**Fig. 2** Statistically significant regulatory sub-networks ($p \leq 0.05$) in in diminished ovarian reserve (DOR)

receptor messenger RNA (mRNA) in postnatal rat granulosa cells. Similarly, AMH decreases the FSH-dependent increase in aromatase activity and LH receptor expression in granulosa cell cultures (Supplementary 6).

GO analysis

Comparison of GO distribution of up/down regulated genes in DOR verses genome

GO distribution of up and down regulated genes compared to genome is presented at Tables 6 and 7, according to Fisher Exact test (hypergeometric test).

For up-regulated genes, GOs which were significantly altered ($p < 0.05$) in Biological Process were: male gonad development, response to fatty acid, positive regulation of cell–cell adhesion, response to estradiol, decidualization, positive regulation of gene expression, response to peptide hormone, response to estrogen, hematopoietic progenitor cell differentiation, and regulation of cell growth. GOs which were significantly altered ($p < 0.05$) in Molecular Function were: lipid binding and heme binding. GOs which were significantly altered ($p < 0.05$) in Cellular Component were neuron projection, Golgi apparatus, and extracellular space.

For up-regulated genes, GOs which were significantly altered ($p < 0.05$) in Biological Process were: exocrine pancreas development, positive regulation of glycogen biosynthetic process, artery morphogenesis, positive regulation of activated T cell proliferation, positive regulation of mitosis, skeletal system development, positive regulation of transcription from RNA polymerase II promoter, blood vessel remodelling, and collagen fibril organization. GOs which were significantly altered ($p < 0.05$) in Molecular Function were: insulin-like growth factor receptor binding, insulin receptor binding, hormone activity, and zinc ion binding. GOs which were significantly altered ($p < 0.05$) in Cellular Component were platelet alpha granule lumen, extracellular space, extracellular region, and cytoplasm.

Comparison of GO distribution of up regulated genes versus down regulated genes in DOR

According to Wilcoxon signed rank test with continuity correction and Two-sample Kolmogorov–Smirnov test, GO distribution of up regulated genes were different from down regulated genes at $p < 0.01$.

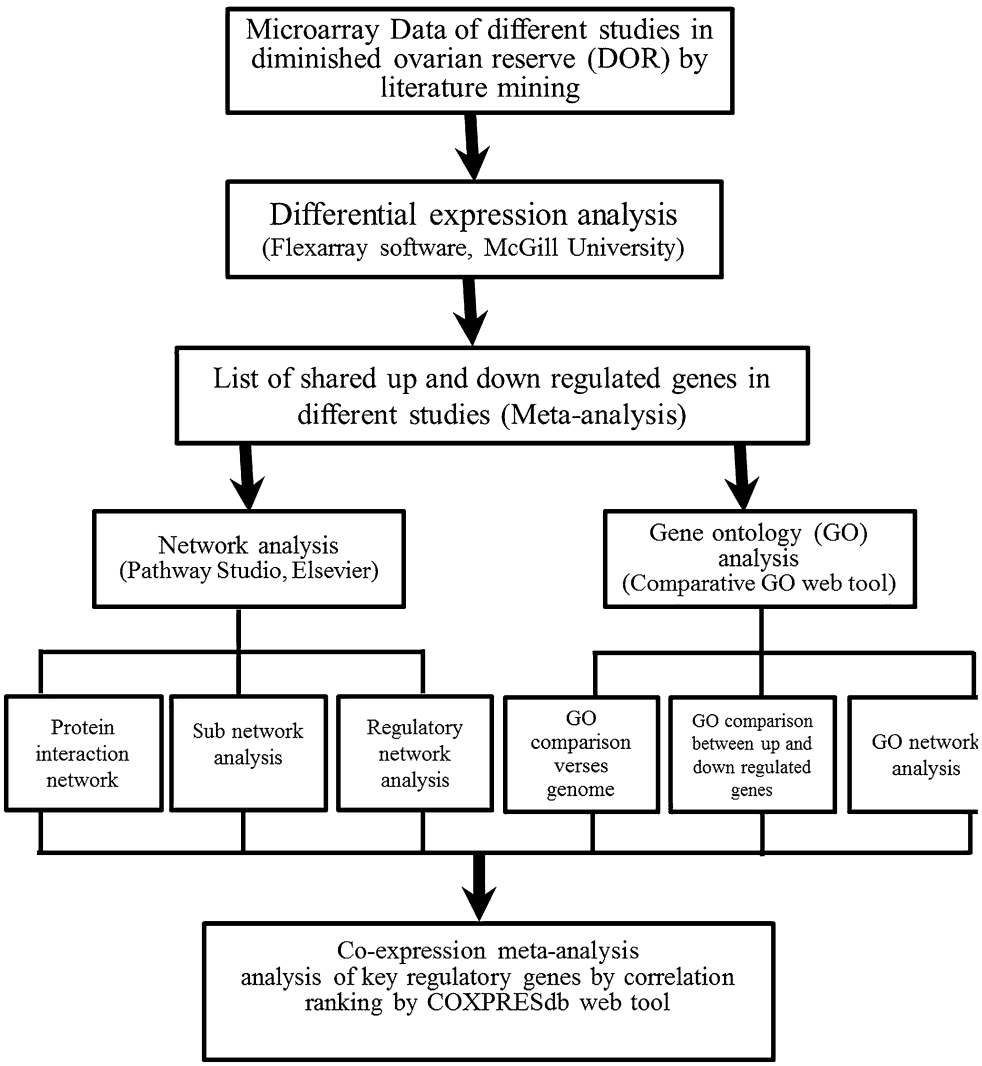


Fig. 3 Schematic representation of data analysis and computational tools used in this study

Comparative GO analysis of up and down regulated genes is presented at Supplementary 7, Supplementary 8, and Supplementary 9, respectively, for Biological Process, Molecular Function, and Cellular Component.

In Biological Process, GOs including ossification, ovarian follicle development, vasculogenesis, and transcription, DNA-templated are different between up regulated genes and down regulated genes. In Molecular Function term, sequence-specific DNA binding transcription factor activity (CITED2 and FOXC1), and insulin-like growth factor receptor binding (SOCS2, IGF1, and IGF2) are different. Golgi apparatus is the major differential group in cellular component.

Meta-analysis of the key regulating genes of DOR

The key up-regulated genes in interaction network of DOR, including, PTGS2, CTGF, LHCGR, CITED, SOCS2, STAR and FSTL3, were used for co-expression using correlation ranking by COXPRESdb. GO tool was used to predict the function of co-expressed genes. The microarray data in NCBI GEO repository (http://www.ncbi.nlm.nih.gov/geo/) employed for meta-analysis. The list of 100 co-expressed genes with each of PTGS2, CTGF, LHCGR, CITED, SOCS2, STAR and FSTL3 are presented in Supplementary 10.

The list of genes which were shared between two or more co-expressed list is presented at Supplementary 11. CTGF, connective tissue growth factor, had the highest number of shared co-expressed genes with the other co-expressed datasets (Supplementary 11) as 26 out of 100 coexpressed genes with CTGF were also coexpressed with the other datasets with the key up-regulated genes in DOR. Biological process (GO) of CTGF is organ senescence. According to KEGG, CTGF is linked to focal adhesion pathway coexpressed with MYL9, THBS1, ITGA5, and VEGFC.

Table 6 Significant up-regulated gene ontology verses genome in diminished ovarian reserve (DOR)

Biological process		Molecular function		Cellular component	
GO name	p value	GO name	p value	GO name	p value
Male gonad development	1.28E−09	Lipid binding	2.61E−06	Neuron projection	0.000248
Response to fatty acid	4.74E−08	Heme binding	0.000116	Golgi apparatus	0.024017
Positive regulation of cell–cell adhesion	1.08E−07	Metal ion binding	0.6018	Extracellular space	0.049973
Response to estradiol	2.60E−07			Cytosol	0.084198
Decidualization	5.47E−07			Extracellular region	0.094955
Positive regulation of gene expression	3.26E−06				
Response to peptide hormone	5.84E−06				
Response to estrogen	9.54E−06				
Hematopoietic progenitor cell differentiation	1.66E−05				
Regulation of cell growth	2.02E−05				
Ossification	2.41E−05				
Lung development	2.76E−05				
Negative regulation of gene expression	4.24E−05				
Negative regulation of apoptotic process	0.000290148				
Angiogenesis	0.00045005				
Small molecule metabolic process	0.01055518				
Positive regulation of transcription	0.01655691				

Control independent dataset of network analysis

The result of regulatory network analysis is presented at Supplementary 12. Most of random samples did not pick any regulator or significant subnetwork. Also, none of the regulatory genes is present as regulator of random samples. This shows that the performed analysis is robust in determining the specific regulators and networks of DOR genes.

Discussion

Schematic representation of data analysis and computational tools used in this study are presented in Fig. 3. Protein interaction networks of up- or down-regulated genes help to find the underlying biological processes that govern these genes' functions and operations. Network analysis of up-regulated genes in diminished ovarian reserve showed that some of the differentially expressed proteins, such as STAR, STAR4, LHCGR, and GAS1, did not resemble any connectivity to participants in the miRNA network; on the other hand, MIRN101-1, MIRN153-1 and MIRN194-1 inhibit the SOCS2 protein. In addition, MIRN153-1 inhibited both the CITED2 and SOCS2 up-regulated proteins. SOCS2, which is also known as a suppressor of the cytokine signalling (SOCS) family, has an important role in the Janus kinase (JAK) and STAT signalling pathways. In addition, it is a negative regulator of the insulin-like growth factor (Igf)-1 signalling cascade [23]. It has been shown that it has relations with follicular growth and granulosa cell proliferation in mice ovaries, and the absence of its expression contributes to poor reproductive performance [18, 22].

PTGS2/COX2 is an up-regulated protein in DOR that is located in the membrane. This protein is the main enzyme in prostaglandin biosynthesis, which is induced in inflammation and mitogenesis and is up-regulated in patients with DOR. Its expression affects cumulus cell expansion and maturation, differentiation, oocyte capability and embryo quality [8, 11, 15, 17, 36]. Interestingly, GO analysis of these proteins using the Pathway Studio package revealed that this protein is involved in biological processes and is responsible for the negative regulation of cell proliferation, synaptic transmission, dopaminergic and calcium ion transport as well as the positive regulation of cell proliferation, synaptic transmission, glutamatergic, apoptosis, smooth muscle cell proliferation, vasoconstriction, smooth muscle contraction and the regulation of cell cycle; it is also involved in different molecular functions (Supplementary data 7).

GDF9, an oocyte-secreted factor [8, 11], has a positive effect on PTGS2 and STAR (steroidogenic acute regulator protein) expression but inhibits LHCGR expression (neighbour of GDF9). GDF9 controls the expression of HAS-2 (hyaluronan synthase 2) and PTGS2 (COX-2) in the cumulus-*oocyte* complex (*COC*), thus contributing to expansion, growth, proliferation and differentiation of the cumulus cells (Supplementary data GDF9). GDF9 plays an important role in female fertility, and mutation in GDF9 is

Table 7 Significant down-regulated gene ontology verses genome in diminished ovarian reserve (DOR)

Biological process		Molecular function		Cellular component	
GO name	p value	GO name	p value	GO name	p value
Exocrine pancreas development	1.09E−08	Insulin-like growth factor receptor binding	3.43E−08	Platelet alpha granule lumen	2.11E−06
Positive regulation of glycogen biosynthetic process	3.93E−08	Insulin receptor binding	5.67E−08	Extracellular space	0.000225
Artery morphogenesis	2.39E−07	Hormone activity	1.20E−06	Extracellular region	0.007908
Positive regulation of activated T cell proliferation	2.93E−07	Zinc ion binding	0.0358	Cytoplasm	0.089425
Positive regulation of mitosis	3.79E−07				
Skeletal system development	5.80E−07				
Positive regulation of transcription from RNA polymerase II promoter	8.80E−07				
Blood vessel remodeling	8.82E−07				
Collagen fibril organization	1.41E−06				
Positive regulation of fibroblast proliferation	2.41E−06				
Positive regulation of MAPK cascade	5.37E−06				
Positive regulation of peptidyl-tyrosine phosphorylation	9.48E−06				
Platelet degranulation	1.07E−05				
Positive regulation of protein kinase B signaling	1.09E−05				
Positive regulation of NF-kappaB transcription factor	4.94E−05				
Positive regulation of cell proliferation	6.32E−05				
In utero embryonic development	0.000241				
Cell–cell signaling	0.000264				
Response to drug	0.000567				
Blood coagulation	0.001671				
Signal transduction	0.001721				
Positive regulation of transcription, DNA-templated	0.002347				

a novel cause of diminished ovarian reserve in young women [39].

In addition, TGF-beta 1 interacts with the up-regulated proteins in DOR, including PTGS2, CTGF, STAR and LHCGR. SMAD4 is an important factor of the TGFbeta superfamily signalling pathway. The findings of Pangas [28] demonstrate that the problem in SMAD4 in the ovarian granulosa cells results in the premature luteinisation of granulosa cells and premature ovarian failure; thus, the TGFbeta superfamily is important in growth and in differentiation control of granulosa cells. The TGFB family has a role in the creation of oocytes during embryogenesis followed by gathering and activation of the primordial follicle.

IL-6, as the central network protein, is interacting with up-regulated proteins such as PTGS2 and LHCGR in DOR. The results are consistent with the view that IL-6 may have a physiological role in the maturation of ovarian follicles by modulating the achievement of the LHR in granulosa cells. GO analysis of these proteins using the Pathway Studio package revealed that this protein is involved in biological processes and is responsible for the regulation of angiogenesis, negative regulations of cell proliferation, hormone secretion, apoptosis, chemokine biosynthetic process, and collagen biosynthetic process as well as the positive regulation of anti-apoptosis, B cell activation, T-helper 2 cell differentiation, and T cell proliferation. Additionally, IL-6 itself can also lead to the induction of COX-2. Furthermore, our analysis demonstrates that cAMP is important in the induction of PTGS2, STAR, LHCGR gene expression and CTGF inhibition.

Network analysis of down-regulated expressed genes in diminished ovarian reserve showed that proteins such as AMH, IGF2 and GBP2 did not find connectivity to participate in the microRNA network; on the other hand, MIRN128-1, MIRN129, MIRN23A and MIRN27A inhibit the GREM1 protein. In addition, MIRN129 inhibits both the IGF2 and GREM1 down-regulated proteins.

Table 8 Comparison of gene ontology distribution between up-regulated and down-regulated genes in diminished ovarian reserve (DOR)

Gene ontology	Up-regulated	Down-regulated	Average change	All genes
Biological process				
Ossification (1503)	2	3.75	1.875	CTGF|FSTL3|FOXC1
Ovarian follicle development (1541)	1	3.75	3.75	LHCGR|FOXC1
Vasculogenesis (1570)	2	1.25	0.625	CITED2|ZMIZ1
Transcription, DNA-templated (6351)	1	2.5	2.5	FSTL3|ZMIZ1|FOXC1
Sex determination (7530)	2	1.25	0.625	CITED2|AMH
Aging (7568)	2	1.25	0.625	SOCS2|AMH
Cell aging (7569)	2	1.25	0.625	CITED2|ZMIZ1
Positive regulation of cell proliferation (8284)	1	3.75	3.75	CTGF|GREM1|IGF2|IGF1
Positive regulation of gene expression (10,628)	6	1.25	0.208333333	STAR|CITED2|CTGF|AMH
Negative regulation of BMP signaling pathway (30,514)	1	2.5	2.5	FSTL3|GREM1
Response to estradiol (32,355)	4	2.5	0.625	PTGS2|SOCS2|CTGF|IGF2
Response to nicotine (35,094)	4	2.5	0.625	STAR|IGF2
Positive regulation of NF-kappaB import into nucleus (42,346)	2	1.25	0.625	PTGS2|GREM1
Response to drug (42,493)	6	3.75	0.625	STAR|PTGS2|IGF2|AMH
Camera-type eye development (43,010)	1	3.75	3.75	GAS1|FOXC1
Negative regulation of apoptotic process (43,066)	4	1.25	0.3125	CITED2|GAS1|SOCS2|IGF1
Response to ethanol (45,471)	4	2.5	0.625	STAR|IGF2
Positive regulation of transcription, DNA-templated (45,893)	1	2.5	2.5	CITED2|FOXC1|IGF1
Positive regulation of transcription from RNA polymerase II promoter (45,944)	2	11.25	5.625	FSTL3|CITED2|GREM1|FOXC1|IGF1|ZMIZ1|IGF2
Positive regulation of smooth muscle cell proliferation (48,661)	2	1.25	0.625	PTGS2|IGF1
Molecular function				
Sequence-specific DNA binding transcription factor activity (3700)	2	2.5	1.25	CITED2|FOXC1
Insulin-like growth factor receptor binding (5159)	1	2.5	2.5	SOCS2|IGF1|IGF2
Cellular component				
Extracellular space (5615)	2	13.75	6.875	CTGF|FSTL3|IGF1|IGF2|GREM1|AMH
Nucleus (5634)	3	7.5	2.5	PTGS2|FSTL3|CITED2|FOXC1|GBP2|ZMIZ1
Cytoplasm (5737)	10	6.25	0.625	SOCS2|PTGS2|CITED2|CXXC5|AMH|ZMIZ1|FOXC1
Golgi apparatus (5794)	2	1.25	0.625	CTGF|FSTL3|GBP2
Cytosol (5829)	6	1.25	0.208333	STAR|CTGF|SOCS2|GBP2
Membrane (16,020)	1	1.25	1.25	PGRMC2|GBP2
Intracellular membrane-bounded organelle (43,231)	1	1.25	1.25	CTGF|CXXC5

Furthermore, in unexplained infertility, primary elevated GH-BP by excessive GH-BP levels may prevent GH from binding to its receptor and therefore reduce intraovarian IGF-1 production.

In conclusion, the up-regulated PTGS2, CTGF, LHCGR, CITED, SOCS2, STAR and FSTL3 proteins were selected as the key nodes with the highest number of interactions; at the same time, the IGF2, AMH, GREM1, and FOXC1 proteins had the highest number of interactions in down-regulated networks. Three miRNAs (MIRN101-1, MIRN153-1 and MIRN194-1) inhibited SOCS2 protein expression, and TGF-beta 1, as a small molecule, regulated an important sub-network that was enriched with expression entities. CSH1 showed a positive effect on IGF1 and

IGF2 expression, while BMP2 exerted a positive effect on IGF2, IGF1 and GREM1 expression. GH1 had both a positive and negative effect on IGF1 expression and a positive effect on IGF2 expression.

This study employs meta-analysis and computational biology to find the candidate genes, regulators, and networks in DOR, opening a new avenue in this field. More mechanistic studies with in vitro validation need to be undertaken in future studies.

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