Network-based bioinformatics analysis of HOX genes and their related genes in endometriosis

Fereshteh Chitsazian
Royan Institute for Reproductive Biomedicine
Raha Favaedi
Royan Institute for Reproductive Biomedicine
Masoumeh Golestan Jahromi
Shahid Sadoughi University of Medical Sciences and Health Services Yazd Research and Clinical Centre for Infertility
Masood Bazrgar
Royan Institute for Reproductive Biomedicine
Reza Aflatoonian
Royan Institute for Reproductive Biomedicine
Parvaneh Afsharian
Royan Institute for Reproductive Biomedicine
Abbas Aflatoonian
Shahid Sadoughi University of Medical Sciences and Health Services Yazd Research and Clinical Centre for Infertility
Maryam Shahhoseini (shahhoseini244@gmail.com)
Royan Institute

Research

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Abstract

Background: Dysregulation of some HOX genes are important for the development of endometriosis. Endometriosis is a complex gynecologic disorder affecting as many as 10-15% of premenopausal women. The pathogenesis of endometriosis, as the presence of endometrium-like tissue outside the uterine cavity, is largely unknown. HOX genes, encoding homeodomain transcription factors, are dynamically expressed in endometrium, in which they are necessary for endometrial growth, differentiation, and implantation. Few investigations have been studied in this vast range of HOX genes with a network-based view. Network theory allows for a holistic understanding of the role of genes in diseases. The purpose of this study is to identify genes that cause this disease by focusing on the HOX family genes and their regulatory genes. Materials & Methods: Fifteen samples of eutopic and ectopic endometrium collected from patients in proliferation phase. PCR array was performed on sample groups for 84 HOX genes and their related genes. The statistical significance was determined by using t-test. PCA and Hierarchical clustering were carried out between different groups. The genes co-expression networks were constructed based on correlation between normalized gene expression data. Gene function was annotated based on Gene Ontology, the intervention in other diseases and expression in other tissues using the DAVID to clarify the function of important genes and the mechanism of endometriosis. Results: Among the analyzed genes, 30, 40 and 35 significantly differentially expressed genes were in eutopic, ectopic compared with normal and ectopic with eutopic samples, respectively. Twenty-eight genes had no significant alteration in none of the sample groups. Conclusion: The most significant genes and the genes in co-expression networks are effective genes in development, metabolic and neural processes. Another important point is that the some non-differently expressed genes in networks have many interactions with the significant genes, indicating that these non-significant genes also are likely contributed in endometriosis. Keywords: endometriosis, HOX, PCR-array, Co-expression network

Introduction

Endometriosis is known as the presence of endometrial tissues inside an abnormal anatomical location other than those of the uterus. It is a common, estrogen-dependent gynecological disorder that can lead to multiple manifestations, including dysmenorrhea, pelvic pain, pelvic mass, infertility and malignant behaviors [1-4], with prevalence rates of 10-15% in women of reproductive age [5, 6]. Symptoms of endometriosis often affect social relationships, sexuality and mental health [7]. The understanding of the pathogenesis of endometriosis is poor. The combination of retrograde menstruation of endometrial tissue fragments through the fallopian tubes into the peritoneal cavity and the immune dysfunctions are the most accepted causes of the pathogenesis of endometriosis. The live endometrial fragments in retrograde menstruation can hold in the ectopic site through adhesion, aggression and angiogenesis process and then form the ectopic lesions; therefore, the inherent biological properties of eutopic endometrium may play an important role in the pathogenesis of endometriosis [8].

Although the association between endometriosis and infertility is supported in many literature [9-11], the involved mechanisms have not been figured out. Several factors have been reported, including distortion of pelvic anatomy, hormone secretion abnormalities, alterations in peritoneal fluid and fertilization disorders. Accordingly, changes in endometrial development in patients with endometriosis may help to infertility associated with endometriosis. It is believed that patients with endometriosis have reduced the incidence of implantation. Ultrastructural defects in endometrium in women with endometriosis have been reported. Molecular markers of the endometrial receptors undergo change in patients with endometriosis; the expression patterns of the endometrium in the native endometrium of women with endometriosis are aberrant. Also, changes in biochemical or molecular markers in the tissue have been pointed out [12].

The *Homeobox* [13] gene family operates through a conserved homeodomain as transcriptional regulators for controlling fetal morphogenesis. In addition, the lack of proper expression of the *HOX* gene results in developmental anomalies. As transcription factors, the *HOX* genes regulate other downstream target genes leading to proper endometrial development and receptivity in implantation and function in different tissues of the fetus to provide spatial and temporal coordination for each cell [14]. Initially, *HOX* genes were expressed during embryonic development. However, the stable expression of the *HOX* genes has been observed in the reproductive system. *HOX* genes are essential for endometrial growth, differentiation and receptivity by mediating some functions of the sex steroids during each reproduction period. 39 *HOX* genes have been identified to divide into four *HOX* loci [15], each localized on a different chromosome and containing 9 to 11 orthologous genes. Genes in the four clusters can be categorized into 13 paralogous groups. Individual genes of the *HOXA* cluster are assigned a distinctive identity for each part of the paramesonephric duct, leading to the development of the fallopian tubes (*hoxa9*), uterus (*hoxa10*), lower part of the uterus and cervix (*hoxa11*) and upper vagina (*hoxa13*) [16]. *HOXB* has been detected to be closely related to the self-renewal of the hematopoietic stem cells and effective proliferation of hematopoietic progenitor cells [17]. Studies show a collinear activation of the *HOXC* genes in the limb ectoderm besides, possible implication of *HOXC* genes in the nail/claw/hoof transition [18]. While all four *HOX* gene clusters are initially activated during embryonic development, both *HOXA* and *HOXD* clusters are then reactivated during the development of appendicular skeletons, involved in the building of the limbs [19]. The identification of molecular markers of endometriosis is likely to help understanding its course and eventually, to uncover novel genes to be targeted by drugs.

In the present survey, we compared gene expression using PCR-array in a collection of 15 ectopic and 15 eutopic endometrium samples with 15 normal endometrial samples, focusing on 84 genes of *HOX* family and their related genes. Such a PCR-based approach, in spite of relatively low throughput, has the advantage to directly examine the gene expression in a direct and robust analysis and identify mostly related with endometriosis. Gene Ontology (GO) was searched for the intervention of genes in other diseases and expression in other tissues using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; *https://david.ncifcrf.gov*) [20]. Co-expression gene network was constructed using correlation between genes expression data and visualized by Cytoscape software. In silico co-expression network construction can really provide useful information about pathogenesis of disease. Several parameters can be identified to help establish hierarchies between genes from a network or even belonging to preset remote networks. In general, the identification of new deregulated gene expression networks in endometriosis highlighted some of the interactions between the protein products of these genes and caused a significant reduction in infertility due to early detection of precursor lesions [21].

Material And Methods

Patients and Tissue Collection: This cross-sectional study was approved by the Ethics Committees of Clinical and Research Centers for Infertility, Yazd, and Royan Institute, Iran. This research was approved by the ethics committee of Royan Institute (IR.ACECR.ROYAN.REC.1396.238) and all methods were carried out in compliance with the approved guidelines. All participants, donating tissue for these studies signed an informed consent after being fully aware of all procedures.

Fifteen patients with endometriosis in stages III and IV, undergoing laparoscopic surgery, participated in the current survey (age: 29±1.25, ranged from 24 to 38 years old). Ectopic endometrium samples collected from them through surgery from ectopic sites in the abdomen, and eutopic endometrium samples were obtained by pipelle. None of the patients had endometrial hyperplasia, visible endometrial hyperplasia or neoplasia and inflammatory disease.

Fifteen patients, who suffered from secondary infertility, underwent diagnostic laparoscopy, considered as control group (age: 28±1.67, ranged from 22 to 36 years old). They had no evidence of endometriosis, polyps, myoma and inflammatory diseases of reproduction system with at least one child by natural pregnancy. Normal Endometrial samples were taken from these women during diagnostic laparoscopy.

All women participated in the present study had normal menstruation cycle and have not received hormone therapy since 3 months before the surgery. All patients were in the follicular phase of the menstrual cycle at the time of the surgical procedure. Endometrial samples, were placed immediately in RNA protection reagent, RNAlater (Ambion, Austin, TX), frozen in liquid nitrogen and stored at −80°C.

RNA extraction and cDNA synthesis: Five tissue samples in each ectopic, eutopic and normal group were pooled as three biological repeats, and all following analyses were performed on the nine sample groups.

RNA extraction of collected tissues was performed by using RNeasy microarray tissue mini kit (Qiagen, Cat.No: 73304) according to the manufacturer's instructions. RNA quality and concentration was measured by using Nanodrop 2000 spectrophotometer (Thermoscientific). Then cDNA synthesis was performed with the RT2 first strand kit (Qiagen, Cat.No: 330404).

PCR-Array: PCR array was conducted for *HOX* family genes by using RT2 profiler PCR array human homeobox (13) genes kit (Qiagen, Cat.No: PAHS-083Z) and RT2 SYBR green ROX qPCR mastermix (Qiagen, Cat.No: 330502). There were primers for 84 tests and housekeeping genes on mentioned HOX PCR array kit according to manufacturer's instructions. Cycling condition on step one plus real time PCR system (ABI), was included an initial denaturation at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. Results were expressed as values of cycle threshold (Ct) then normalized to *GAPDH* as housekeeping gene (control gene). To calculate fold changes in mRNA abundance $2^{-\Delta\Delta Ct}$ method was used.

Statistical analysis: The statistical significance of difference in expression levels between groups was analyzed using Student's t-test with two tails. P-value <0.05 was considered statistically significant. PCA (Principal Component analysis) [22] was carried out using the Minitab 16 statistical software. PCA was performed to simplify the large amount of data. PCA was applied algorithm on log2 of Differentially Expressed Genes (DEGs) and non-DEGs data to underlying cluster structures of endometrial samples.

Hierarchical clustering [23] was implemented by correlation coefficient and complete linkage of in Minitab 16 statistical software [24]. The methods were performed twice, using normalized gene expression data of DEGs and non-DEGs among sample groups in three clusters.

Construction of the gene co-expression network: The genes co-expression networks were constructed of the basis of the normalized gene expression data. Matlab was recruited to compute the Pearson correlation between each pair of genes , and then the significant correlation pairs ($p<0.05$) were imported into Cytoscape software [25] (version 3.6.1) for visualization, and the circle algorithms were used [26]. The co-expression networks were constructed of the normal tissues.

Coding gene functional analysis: Gene function was annotated based on the GO, the intervention in other diseases and expression in other tissues using the (DAVID; *https://david.ncifcrf.gov*) [20] to clarify the function of DEGs and the mechanism of endometriosis.

Results

PCR-array with primer sets targeting *HOX* genes and their associated genes used to evaluate the changes in their expression between ectopic and eutopic tissues with control tissues and ectopic with eutopic tissues. The genes expression which were significantly ($P<0.05$) altered (DEGs) in ectopic, eutopic with normal groups and ectopic with eutopic groups are illustrated in Table I. Among the analyzed genes, there are 33.33% (28) genes in non-DEG. The expression levels of 30, 41 and 35 genes were significantly altered in eutopic and ectopic compared with normal samples and ectopic rather than eutopic samples , respectively. Twelve genes were common between the three groups and 10 of 41 DEGs were exclusively related to ectopic versus normal tissue, 5 of 35 DEGs were solely related to eutopic versus normal tissue and and 3 of 35 DEGs were only related to ectopic versus eutopic tissue (Table I). One of the interesting points is that at least in one of the analysed groups, the expression out of five of the seven studied *HOXB* genes, were significantly up-regulated. Three genes of the *HOXA* cluster were present. Only *HOXA9* was significantly up-regulated in ectopic versus normal and eutopic samples. Seven genes of the *HOXC* cluster were studied and found that expression of five of them were significantly altered in three groups of analysis. Eight genes of the *HOXD* cluster were analyzed, and it was shown that expression of six of them underwent significant change in different studies (Fig. 1 and Table I).

DEGs were categorized into six groups based on venn diagram (Fig. 1). There are three analysis in the investigation: Ratio of expression levels of the target genes in ectopic tissue to their expression level in normal tissue (CtoN), ratio of expression level of the studied genes in eutopic tissue to their expression level in normal tissue (UtoN) and ratio of expression level of the studied genes in ectopic tissue to their expression level in eutopic tissue (CtoU). As shown in Table I, the expression levels of 12 genes were significantly changed in the all three above mentioned groups (CtoN, UtoN and CtoU), while 13 genes were

differentially expressed in CtoU and CtoN groups. Also there are 10, 3 and 5 genes which were differentially expressed only in CtoN, CtoU and UtoN, respectively.

For further investigation, hierarchical clustering was performed on the nine studied samples using DEGs (Fig. 3A) and non-DEGs (Fig. 3B) in the eutopic, ectopic samples and normal samples**.** Hierarchical clustering analysis showed a grouping according to PCA analysis based on the DEGs in ectopic, eutopic and normal samples. In the present study, three samples were properly *classified* into separate clusters on the basis of the DEGs**,** but non-DEGs could not classify the same samples together.

Seven co-expression networks were pointed out based on pairwise correlation of gene expression data and visualized using Cytoscape (Fig. 4). As pointed out above, expression about 64% (54/84) of the genes significantly changed between normal, eutopic and ectopic samples. All genes in the network B1 were significant genes in diseased tissues, and significant genes of ectopic tissues are abundant in the network A1 (71%) (Table II, Fig. 4).

The signaling pathways present in the KEGG and Reactome databases searched for genes in the networks (Table III, Fig. 4), have identified two pathways of " Signaling pathways regulating pluripotency of stem cells " and "Transcriptional misregulation in cancer" from the KEGG database and pathway of "activation of anterior HOX genes during the early embryogenesis" from the Reactome database. Fifteen of which were in these pathways and the MEIS1 were present in all three pathways.

Functional analysis

The DEGs and the genes in seven networks (Fig. 4) were mapped to the DAVID database to investigate the GO, pathways, interference with other diseases and expression in other tissues. DAVID is the most popular tool in the field of functional annotation (Tables IV, V and S1).

Analysis of interference with other diseases: According to analysis of interference with other diseases of genes, many DEGs in ectopic tissues play a role in developmental diseases class, such as cleft lip and clubfoot and metabolic diseases class like bone mineral density.

Many DEGs in eutopic tissues are also involved in neurological diseases class like parkinson's disease and autism in addition to developmental and metabolic diseases class. Many networks A1,2,3 and 4 and B2 genes affected developmental diseases. Most genes in the network C cause neurological diseases. (Table IV).

GO annotation analysis: On the basis of the GO annotation, the most of DEGs in the eutopic and ectopic tissues, and different networks were involved in the development or morphogenesis of various systems, especially skeletal system. Only most network C genes play roles in the development of the nervous system such as dopaminergic neuron and cerebellum (Table S1).

Expression in other tissues: Many DEGs in the patient tissues were also expressed in different tissue such as, craniofacial, prostate carcinoma, superior cervical ganglion, salivary gland and BM-CD33 + myeloid. Most genes in the networks were expressed in one or two tissues. Therefore, the most genes of A1, A3, A4, B1, B2, C and D networks appeared in embryo development, BM-CD33+Myeloid, superior cervical ganglion, spinal cord and ciliary ganglion respectively (Table V).

Discussion

The pathogenesis of endometriosis which was related to infertility remains unclear, but studies have shown that dysregulation of *HOX* genes are important for the development of endometriosis [2, 27-29]. The investigation of the molecular mechanism of the disease is critically important for the diagnosis, treatment and prognosis. The expression analysis by PCR-array can provide information about the expression differences. In this study, the role of *HOX* genes and their associated genes in the disease was investigated. The data shown 16 upregulated and 25 downregulated DEGs of ectopic to normal samples, 7 upregulated and 23 downregulated DEGs of eutopic samples to normal samples and 21 upregulated and 14 downregulated DEGs of ectopic samples to eutopic samples from 15 samples of endometriosis patients and 15 samples of normal women (Fig. 1).

Several studies have shown association of *Hox* genes with endometriosis and other diseases, especially cancers [15, 29, 30]. Differential expression of *HOXA1, A7* and *A9* genes which was detected in the current study, is in agreement with the previous reports on endometriosis [15, 28]. *HOXB2, B3, B4, B7* and *B9* genes which we observed their upregulation in association with endometriosis, was previously shown that are in contribution with endometriosis, and also are involved in progression and development of many cancers [27, 31-39]. About *HOXC6*, *C8, C9, C10, C11, C12* and *C13* genes which were analyzed here, no other studies have paid attention to the relationship between these genes and endometriosis, although their involvement with female reproductive tract are vastly shown [29]. Upregulation of *HOXD12* and *HOXD13* was observed in endometriotic lesions, while *HOXD1* and *D3* were downregulated in this study. This expression profile is detectable in some carcinomas [40-42], though we could not find a similar pattern in endometriosis.

The GO and related disease analysis of DEGs revealed that most of *HOXs* and their related genes with differential expression were significantly involved in development process especially skeletal system in ectopic and eutopic tissues as well as nervous system in eutopic tissues (Table S1, Table V).

PCA was performed for all the endometrial samples analyzed using their DEG and non-DEG expression levels. Each point in a PCA graph represents an endometrial sample and the distance between two points is proportional to the degree of similarity between the gene expression levels. The PCA plot was showed that similar endometrial samples clustered together in DEGs analysis. In addition, endometrial samples were subjected to hierarchical clustering analysis in order to generate a dendogram that clustered endometrial samples based on similarities in gene expression levels from DEGs and non-DEGs. The dendogram obtained displayed a striking segregation of similar samples into three major clustering branches in DEGs analysis. Thus using PCA and hierarchical clustering, it was observed that these DEGs were classified as expected samples into three ectopic, eutopic, and normal groups (Figs. 2, 3).

Evidence indicates the eutopic endometrium has greater ability in the formation of new blood vessels, cell metastasis and invasion, and the level of its gene expression is different compared to that of normal endometrium [8]. Therefore, given that the expression of the 12 genes was significantly different from that in normal and ectopic tissues, abnormal alterations of gene expression of eutopic endometrium might be the source of the pathogenesis of endometriosis. Although the ectopic lesions were established from eutopic tissues, evidence indicated that the properties of each are very distinctive [43]. Therefore, despite the fact that eutopic tissue is similar in appearance to normal endometrial tissue, since eutopic cells can transform into other cells, the magnitude of gene expression changes was high in these cells. The genes such as EN1 that are in both CtoU and UtoN groups, the expression of which have significantly altered in eutopic tissue to normal and ectopic endometrium, are probably important in the initiation of the disease. Also, the genes like ARX that are in both CtoU and CtoN groups, whose expression has significantly altered in ectopic endometrium to normal and eutopic endometrium, are probably important in creating of the ectopic tissue and spreading the disease.

The importance of *HOXB* genes in endometriosis were also pointed out [27]. In the current survey, five genes out of 7 genes of *HOXB* cluster were of DEGs in the eutopic, ectopic or both of them and most of them upregulated (Fig. 1). It is possibly due to their role in sensory perception of pain, hemopoiesis, hematopoietic stem cell differentiation and angiogenesis [17].

In this study, our main concern was about the pathogenesis process and relationships between the DEGs. Thus co-expression network construction with expression data of all studied genes in normal samples identified seven networks (Fig. 4). The most genes of six networks were involved in developmental and metabolic processes, but genes in the network C involved in neural processes. Of the six genes forming this network, there are three DEGs dependent on the eutopic tissues. Probably the neural processes in the eutopic endometrium mentioned above (Table S1) were associated with the network C genes.

Since all the nodes of the network B1 were DEGs and this network is a complete graph, it seems to be effective in the development of this disease. Two genes of this network plays a role in bone mineral density (Table IV**)**, which probably causes to calcification of endometriotic lesions [44]. This is true for the network A1, where 71% of its genes are DEGs of ectopic tissue and play a role in bone bone mineral density (Table IV). In addition, 81% of the genes in the network A3 were DEGs, also, 63% of them are expressed in BM-CD33+Myeloid *immune cell [45]*. Immunodeficiency may have been implicated in the development of endometriosis [8]. Thus, abnormal expression of genes in this network may be involved in the disease. Investigation of the tissues which express the genes of each network indicated that most genes in each network are expressed in a specific tissue, showing connections between the genes of each network together (Table V). Non-DEGs that were a minority in a network and had many connections with DEGs (Fig. 4) may also play a role in the development of endometriosis, but this role has not been shown a significant change in the expression of the genes. Hence, some non-DEGs genes like *SIX1* may be effective in creating ectopic tissue and *BARX1* genes which are likely to be effective in creating eutopic tissues.

In conclusion, our findings support the general notion, concerning the role of *HOX* family genes in development of endometriosis. In addition, the genes, such as *VAX2, PHOX2B, LMX1B, DLX1*and *SIX4*, have high significant alteration in gene expression of endometriosis tissue samples. Accordingly, the functional study showed that the significant genes engaged in developmental, neurological and metabolic pathways. Most of these genes have not previously been reported as endometriosis-related genes. In addition, since some non-DEGs have many connections to DEGs in co-expression networks, these non-DEGs also play important roles in endometriosis. There are two hypotheses regarding the cause of this nonintuitive finding: First, low and no significant expression changes of these non-DEGs may also cause phenotypic variation. Second, these non-DEGs may have a proper function due to factors such as post-translational modifications. Therefore, although they don't have significant expression change, they don't work well and probably these non-DEGs are underwent post-translational modifications by some of the DEGs that connected to them.

Abbreviations

cDNA: Complementary DNA; DAVID: Database for Annotation, Visualization, and Integrated Discovery; DEG: Differentially Expressed Gene; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; non-DEG: non- Differentially Expressed Gene; PCA: Principal Component Analysis; CtoN: Ratio of gene expression level in ectopic tissue to normal tissue; CtoU: Ratio of gene expression level in ectopic tissue to eutopic tissue; UtoN: Ratio of gene expression level in eutopic tissue to normal tissue

Declarations

Ethics approval and consent to participate

The study protocol was approved by Ethical Committee of Royan Institute for Reproductive Biomedicine. Informed consent was obtained from all the participants for being included in the study.

Consent for publication

Not applicable

Availability of data and material

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Competing interests

The authors declared no conflict of interest.

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Authors' contributions

F.C. statistical and bioinformatics analysis and interpretation of data, wrote the manuscript. R.F. the methodological design and performed the experiments. M.G.J. performed the experiments, collected samples. M.B. provided expertise. R.A. provided expertise. P.A. provided expertise. A.A. provided expertise and supervision and administrative support for collecting samples. M.S. provided expertise and supervision. revision of the content of the manuscript.

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Tables

Table I: 56 DEGs and the rate of gene expression changes between normal and patient tissues

Ectopic to Eutopic samples			Eutopic to Normal samples			Ectopic to Normal samples		
Gene symbol	Ratio*	p-value	Gene symbol	Ratio*	p-value	Gene symbol	Ratio*	p-value
12 DEGs common in all three groups								
DLX1	1.36	6.08E-04	*DLX1*	0.6	1.76E-04	*DLX1*	0.81	2.85E-03
EN2	1.88	2.41E-03	*EN2*	0.76	1.64E-02	*EN2*	1.43	2.55E-02
HHEX	0.74	7.02E-03	*HHEX*	0.88	4.14E-02	*HHEX*	0.65	1.09E-03
HOXB4	1.44	8.65E-04	*HOXB4*	1.23	2.36E-02	*HOXB4*	1.76	2.05E-04
HOXD3	0.89	3.29E-02	*HOXD3*	0.84	1.58E-02	*HOXD3*	0.75	5.98E-03
LBX1	2.97	7.94E-04	*LBX1*	0.28	6.09E-04	*LBX1*	0.83	9.93E-03
LMX1B	1.36	3.31E-04	*LMX1B*	0.6	9.80E-05	*LMX1B*	0.82	2.93E-03
MKX	0.34	7.45E-04	*MKX*	0.84	4.07E-02	*MKX*	0.28	1.12E-04
PITX3	1.53	6.64E-03	*PITX3*	0.54	3.28E-04	*PITX3*	0.83	1.76E-02
SHOX	1.39	2.24E-03	*SHOX*	0.6	5.94E-04	*SHOX*	0.84	7.14E-03
VAX1	1.86	9.63E-03	*VAX1*	0.44	3.35E-04	*VAX1*	0.82	1.40E-02
VSX1	1.64	1.85E-04	*VSX1*	0.52	2.86E-03	*VSX1*	0.86	3.06E-02
7 DEGs common in Ectopic to Eutopic samples group and Eutopic to Normal samples group						10 genes in only Ectopic to Normal samples group		
CUX1	0.5	4.79E-02	*CUX1*	1.68	4.97E-02	*MEOX1*	0.74	4.69E-02
EN1	2.23	2.49E-02	*EN1*	0.38	3.06E-02	*MIXL1*	0.79	3.72E-02
HOPX	0.46	4.51E-03	*HOPX*	1.34	5.97E-03	*NKX3-1*	0.89	3.41E-02
HOXC12	2.93	1.77E-02	*HOXC12*	0.26	5.69E-04	*PAX3*	0.84	4.65E-03
HOXC13	2.77	2.93E-04	*HOXC13*	0.33	1.47E-02	*POU5F1*	1.26	1.27E-02
ISL2	3.71	1.54E-03	*ISL2*	0.26	1.11E-03	*HOXD4*	0.75	4.92E-02
MEIS1	0.66	1.76E-02	*MEIS1*	1.63	3.45E-02	*HOXD9*	0.73	1.23E-03
						ALX4	1.32	2.10E-02
						HOXB3	1.73	1.82E-02
						HOXC6	0.57	8.86E-03
3 genes in only Ectopic to Eutopic samples group			6 DEGs common in Ectopic to Eutopic samples group and Eutopic to Normal samples group					
DMBX1	1.43	3.77E-02	*HLX*	0.71	2.34E-02	*HLX*	0.73	6.29E-03
LHX1	1.45	2.32E-02	*HOXB2*	1.38	3.12E-02	*HOXB2*	1.59	8.85E-03
OTP	1.3	2.03E-03	*HOXB7*	1.84	3.77E-02	*HOXB7*	2.11	5.04E-03
			PHOX2B	0.54	8.86E-05	*PHOX2B*	0.63	5.86E-04
			SIX4	1.62	1.89E-03	*SIX4*	1.43	1.91E-03
			VAX2	0.72	1.77E-03	*VAX2*	0.72	8.99E-04
13 DEGs common in Ectopic to Eutopic samples group and Ectopic to Normal samples group								
ARX	0.52	3.61E-02				*ARX*	0.4	1.43E-05
DLX3	1.43	9.78E-04				*DLX3*	1.43	8.92E-04
DLX5	1.62	3.53E-04				*DLX5*	1.51	4.10E-03
DLX6	1.86	7.44E-03				*DLX6*	2.65	1.62E-03
HOXA9	1.55	5.61E-03				*HOXA9*	1.49	7.78E-03
HOXC8	0.5	2.08E-02				*HOXC8*	0.44	3.90E-03
HOXC9	0.47	5.83E-04				*HOXC9*	0.44	1.39E-02
HOXD1	0.62	3.49E-03				*HOXD1*	0.6	1.25E-03
HOXD12	1.61	1.40E-02				*HOXD12*	1.28	3.05E-02
HOXD13	1.68	1.86E-03				*HOXD13*	1.31	8.12E-03
MSX1	1.63	1.05E-02				*MSX1*	2.34	8.20E-04
MSX2	1.95	6.90E-05	5 genes in only Eutopic to			*MSX2*	2.56	1.49E-04
PROX1	0.61	1.13E-03	Normal samples group			*PROX1*	0.6	6.79E-03
			DLX2	0.83	1.20E-02			

HOXB9	1.25	2.56E-02	
LMX1A	0.66	1.38E-02	
PITX2	0.78	1.05E-03	
TLX1	0.53	1.88E-02	

*The ratio of gene expression between different samples

Table II. The number of significant genes in the different tissues

Network Name	All*	Eu@	Ec$	Ec/Eu&	All DEGs
A1	7	2 (29.5%)	5 (71%)	0 (0%)	5(71%)
A2	7	1 (14%)	2 (29%)	0 (0%)	2(29%)
A3	27	13 (48 %)	17 (63%)	12 (44%)	22(81%)
A4	9	4 (44%)	4 (44%)	0 (0%)	5(56%)
B1	4	2 (50 %)	2 (50%)	2 (50%)	4(100%)
B2	12	5 (42 %)	5 (42%)	8 (67%)	8(67%)
C	6	3 (50 %)	0 (0%)	2 (33%)	3(50%)

* The total number of nodes

@ Number of nodes with significant changes in gene expression of the eutopic to normal tissues

$ Number of nodes with significant changes in gene expression of the ectopic to normal tissues

& Number of nodes with significant changes in gene expression of the eutopic to ectopic tissues

Table III. Pathways with genes in the constructed networks in the study.

Gene Symbol	HOXD4	HOXB3	DLX5	ISL1	HOXB4	HOXD1	HOXD3	HESX1	OTX1	SIX1	SIX4	HOXB2	POU5F1	MEIS1	HOXA
Network Name	A1	A2	A2	A2	A3	A3	A3	A3	A3	A3	A3	A4	B1	B2	C
Pathway Name	R	R	S	S	R	R	R	S	S	T	T	R	S	R,S,T	R

R: Activation of anterior HOX genes in hindbrain development during early embryogenesis
S: Signaling pathways regulating pluripotency of stem cells
T: Transcriptional misregulation in cancer

Table IV. Other diseases in which the DEGs and the genes in five networks interfere.

Category	Description	Count	P-value
	DEGs in Ectopic to Normal		
GAD_DISEASE_CLASS	DEVELOPMENTAL	25	1.14E-16
GAD_DISEASE	Bone Mineral Density	15	2.32E-13
GAD_DISEASE	Clubfoot	6	1.07E-08
GAD_DISEASE	Cleft Lip|Cleft Palate	15	3.81E-06
GAD_DISEASE	autism	6	1.68E-03
GAD_DISEASE_CLASS	METABOLIC	22	3.65E-03
GAD_DISEASE	Parkinson's disease	4	5.05E-03
GAD_DISEASE	Sleep Apnea, Obstructive	3	8.94E-03
	DEGs in Eutopic to Normal		
GAD_DISEASE_CLASS	DEVELOPMENTAL	13	1.18E-06
GAD_DISEASE	Parkinson's disease	5	8.64E-05
GAD_DISEASE	Cleft Lip|Cleft Palate	5	4.01E-03
GAD_DISEASE	neurodevelopmental psychiatric disorders	2	8.45E-03
GAD_DISEASE_CLASS	NEUROLOGICAL	11	1.33E-02
GAD_DISEASE	Autism	4	2.16E-02
GAD_DISEASE	Hirschsprung's disease	2	2.85E-02
GAD_DISEASE	Bone Mineral Density	4	3.11E-02
GAD_DISEASE	SIDS/sudden infant death syndrome	2	4.48E-02
	DEGs in Ectopic to Eutopic		
GAD_DISEASE	Bone Mineral Density	12	4.88E-11
GAD_DISEASE_CLASS	DEVELOPMENTAL	17	2.87E-10
GAD_DISEASE	Clubfoot	4	2.42E-05
GAD_DISEASE	Cleft Lip|Cleft Palate	10	4.33E-04
GAD_DISEASE	Parkinson's disease	5	1.23E-04
GAD_DISEASE	autism	6	4.24E-04
GAD_DISEASE_CLASS	METABOLIC	19	6.55E-04
GAD_DISEASE	talipes equinovarus	2	7.38E-03
GAD_DISEASE	hair thickness	2	2.38E-02
GAD_DISEASE_CLASS	NEUROLOGICAL	11	2.62E-02
	Genes in Network A1		
GAD_DISEASE	Bone Mineral Density	5	1.42E-05
GAD_DISEASE	Clubfoot	2	1.43E-02
GAD_DISEASE_CLASS	DEVELOPMENTAL	4	2.37E-02
GAD_DISEASE_CLASS	METABOLIC	6	4.36E-02
	Genes in Network A2		
GAD_DISEASE_CLASS	DEVELOPMENTAL	3	0.037103
	Genes in Network A3		
GAD_DISEASE_CLASS	DEVELOPMENTAL	14	3.13E-09
GAD_DISEASE	Bone Mineral Density	6	1.90E-04
GAD_DISEASE	Sleep Apnea, Obstructive	3	2.87E-03
GAD_DISEASE	Cleft Lip|Cleft Palate	11	2.29E-02
GAD_DISEASE	Parkinson's disease	6	2.44E-02
GAD_DISEASE	Clubfoot	2	4.22E-02
	Genes in Network A4		
GAD_DISEASE_CLASS	DEVELOPMENTAL	6	3.60E-04
GAD_DISEASE	Clubfoot	2	1.70E-02
	Genes in Network B1		
GAD_DISEASE	Bone Mineral Density	2	9.21E-02
	Genes in Network B2		
GAD_DISEASE_CLASS	DEVELOPMENTAL	5	8.53E-03
GAD_DISEASE	Cleft Lip|Cleft Palate	3	2.29E-02
	Genes in Network C		
GAD_DISEASE	Neurodevelopmental psychiatric disorders	2	1.16E-03
GAD_DISEASE_CLASS	NEUROLOGICAL	4	1.11E-02
GAD_DISEASE	Parkinson's disease	2	3.25E-02

Table V. Other tissues in which the DEGs and the genes in five networks are expressed.

Category	Description	Count	P-value
	DEGs in Ectopic to Normal		
U133A	Cerebellum	30	2.23E-03
U133A	Lymphomaburkittsraji	29	5.79E-03
U133A	Salivarygland	30	7.70E-03
U133A	Embryo_development	23	1.37E-02
U133A	Amygdala	13	1.66E-02
U133A	Brain	19	1.75E-02
UP_TISSUE	Craniofacial	2	2.13E-02
U133A	BM-CD33+Myeloid	27	2.51E-02
U133A	Superiorcervical ganglion	23	2.51E-02
U133A	Uterus	15	2.73E-02
U133A	Prostate	26	2.85E-02
UP_TISSUE	Osteoblast	2	3.09E-02
	DEGs in Ectopic to Eutopic		
UP_TISSUE	Craniofacial	2	1.33E-02
U133A	Lung	12	2.87E-02
U133A	BM-CD33+Myeloid	17	3.04E-02
SAGE	Prostate_carcinoma	5	4.25E-02
	DEGs in Eutopic to Normal		
UP_TISSUE	Craniofacial	3	1.07E-04
U133A	Superiorcervical ganglion	16	5.73E-02
U133A	Salivarygland	20	7.07E-02
U133A	Whole blood	11	8.47E-02
	Genes in Network A1		
UNIGENE_EST	Embryo_development	8	3.40E-3
U133A	Testis seminiferous tubule	5	1.5E-2
	Genes in Network A3		
UP_TISSUE	Craniofacial	2	1.33E-02
U133A	Lung	12	2.87E-02
U133A	BM-CD33+Myeloid	17	3.04E-02
SAGE	Prostate_carcinoma	5	4.25E-02
	Genes in Network A4		
UNIGENE_EST	Uterus	6	1.30E-2
U133A	Superior cervical ganglion	7	2.00E-2
	Genes in Network B1		
U133A	Spinalcord	3	2.20E-2
	Genes in Network B2		
U133A	Ciliary ganglion	7	9.40E-03
U133A	Whole blood	7	3.00E-02

Supplementary Table

Table S1. Annotation of the DEGs and the genes in five networks with GO

Category	Description	Count	P-value
	DEGs in Ectopic		
BP	Anterior/posterior pattern specification	18	2.10E-29
BP	Embryonic skeletal system morphogenesis	10	3.90E-16
BP	Multicellular organism development	13	4.50E-09
BP	Embryonic forelimb morphogenesis	5	1.30E-06
BP	Skeletal system development	6	2.50E-05
BP	Palate development	5	4.20E-05
BP	Embryonic skeletal system development	4	6.10E-05
BP	Muscle organ development	5	7.80E-05
BP	Definitive hemopoiesis	3	7.50E-04
BP	Dopaminergic neuron differentiation	3	1.40E-03
BP	Proximal/distal pattern formation	3	1.70E-03
BP	Neuron differentiation	4	1.80E-03
BP	Thyroid gland development	3	1.90E-03
BP	Stem cell differentiation	3	2.20E-03
BP	Embryonic hindlimb morphogenesis	3	2.30E-03
BP	Midbrain development	3	2.80E-03
BP	Neuron development	3	6.20E-03
BP	Skeletal muscle tissue development	3	7.50E-03
BP	Embryonic nail plate morphogenesis	2	7.70E-03
BP	Inner ear morphogenesis	3	7.80E-03
BP	Odontogenesis of dentin-containing tooth	3	8.70E-03
BP	Embryonic digit morphogenesis	3	9.00E-03
BP	Glossopharyngeal nerve morphogenesis	2	1.00E-02
BP	Olfactory placode formation	2	1.00E-02
BP	Epithelial cell differentiation	3	1.40E-02
BP	BMP signaling pathway involved in heart development	2	1.50E-02
BP	Dorsal spinal cord development	2	1.80E-02
BP	Morphogenesis of an epithelial sheet	2	2.00E-02
BP	Anatomical structure morphogenesis	3	2.30E-02
BP	Male gonad development	3	2.40E-02
BP	Hepatocyte differentiation	2	2.50E-02
BP	Anatomical structure formation involved in morphogenesis	2	2.80E-02
BP	Enteric nervous system development	2	2.80E-02
BP	Hematopoietic stem cell differentiation	2	3.00E-02
BP	Epithelial to mesenchymal transition involved in endocardial cushion formation	2	3.30E-02
BP	Nervous system development	4	3.70E-02
BP	Negative regulation of neuron apoptotic process	3	4.50E-02
BP	Pharyngeal system development	2	4.50E-02
BP	Positive regulation of branching involved in ureteric bud morphogenesis	2	4.80E-02
BP	Negative regulation of myeloid cell differentiation	2	4.80E-02
	DEGs in Ectopic		
BP	Anterior/posterior pattern specification	8	1.30E-10
BP	Dopaminergic neuron differentiation	5	7.80E-08
BP	Embryonic skeletal system morphogenesis	5	8.50E-07
BP	Neuron development	5	1.70E-06
BP	Negative regulation of neuron differentiation	5	3.70E-06
BP	Multicellular organism development	9	4.40E-06
BP	Anatomical structure morphogenesis	4	7.00E-04
BP	Proximal/distal pattern formation	3	9.50E-04
BP	Midbrain development	3	1.60E-03
BP	Dorsal/ventral pattern formation	3	1.70E-03
BP	Negative regulation of neuron apoptotic process	4	2.00E-03
BP	Cerebral cortex gabaergic interneuron fate commitment	2	3.80E-03
BP	Odontogenesis of dentin-containing tooth	3	4.90E-03
BP	Subpallium development	2	5.70E-03
BP	Regulation of transcription from RNA polymerase II promoter involved in forebrain neuron fate commitment	2	5.70E-03
BP	Locomotory behavior	3	1.10E-02
BP	Morphogenesis of an epithelial sheet	2	1.50E-02
BP	Embryonic brain development	2	1.50E-02
BP	Neuron migration	3	1.70E-02
BP	Negative regulation of oligodendrocyte differentiation	2	2.10E-02
BP	Enteric nervous system development	2	2.10E-02
BP	Lens morphogenesis in camera-type eye	2	2.30E-02
BP	Definitive hemopoiesis	2	3.00E-02
BP	Embryonic digestive tract morphogenesis	2	3.40E-02
BP	Positive regulation of branching involved in ureteric bud morphogenesis	2	3.60E-02
BP	Axon guidance	3	3.70E-02
BP	Thyroid gland development	2	4.70E-02
BP	Wnt signaling pathway	3	4.90E-02
BP	In utero embryonic development	3	4.90E-02
	Genes in Network A		
BP	Anterior/posterior pattern specification	5	6.30E-07
BP	Multicellular organism development	7	3.40E-06
BP	Negative regulation of myeloid cell differentiation	3	1.30E-04

BP	Definitive hemopoiesis	2	1.40E-02
BP	Proximal/distal pattern formation	2	2.10E-02
BP	Embryonic skeletal system morphogenesis	2	3.40E-02
BP	Embryonic limb morphogenesis	2	3.50E-02
BP	Neuron development	2	4.00E-02
BP	Negative regulation of neuron differentiation	2	4.90E-02
	Genes in Network D		
BP	Dopaminergic neuron differentiation	2	7.80E-03
BP	Cerebellum development	2	1.30E-02
BP	Anatomical structure morphogenesis	2	3.20E-02
	Genes in Network C		
BP	Anterior/posterior pattern specification	6	1.20E-10
BP	Embryonic skeletal system morphogenesis	4	6.40E-07
BP	Mammary gland development	2	1.00E-02
BP	Dorsal/ventral pattern formation	2	1.50E-02
BP	Multicellular organism development	3	2.40E-02
	Genes in Network B1		
BP	Anterior/posterior pattern specification	3	7.90E-04
BP	Dorsal spinal cord development	2	3.70E-03
BP	Regulation of transcription, DNA-templated	5	5.60E-03
BP	Nervous system development	3	9.70E-03
BP	Skeletal system morphogenesis	2	1.70E-02
BP	Embryonic skeletal system morphogenesis	2	2.10E-02
BP	Multicellular organism development	3	3.00E-02
BP	Hematopoietic progenitor cell differentiation	2	3.50E-02
	Genes in Network B2		
BP	Anterior/posterior pattern specification	7	1.60E-09
BP	Pharyngeal system development	3	3.20E-04
BP	Positive regulation of branching involved in ureteric bud morphogenesis	3	3.60E-04
BP	Dopaminergic neuron differentiation	3	4.80E-04
BP	Embryonic hindlimb morphogenesis	3	7.90E-04
BP	Multicellular organism development	6	9.00E-04
BP	Protein localization to nucleus	3	9.00E-04
BP	Embryonic skeletal system development	3	9.00E-04
BP	Midbrain development	3	9.60E-04
BP	Embryonic skeletal system morphogenesis	3	1.50E-03
BP	Skeletal muscle tissue development	3	2.60E-03
BP	Negative regulation of cell proliferation	5	2.60E-03
BP	Inner ear morphogenesis	3	2.70E-03
BP	Fungiform papilla morphogenesis	2	3.00E-03
BP	Positive regulation of mesenchymal cell apoptotic process	2	3.00E-03
BP	Odontogenesis of dentin-containing tooth	3	3.00E-03
BP	Embryonic nail plate morphogenesis	2	4.50E-03
BP	Regulation of branch elongation involved in ureteric bud branching	2	4.50E-03
BP	Myotome development	2	4.50E-03
BP	Positive regulation of ureteric bud formation	2	5.90E-03
BP	Olfactory placode formation	2	5.90E-03
BP	Regulation of synaptic growth at neuromuscular junction	2	5.90E-03
BP	Trigeminal ganglion development	2	7.40E-03
BP	Anatomical structure morphogenesis	3	8.20E-03
BP	Male gonad development	3	8.60E-03
BP	BMP signaling pathway involved in heart development	2	8.90E-03
BP	Generation of neurons	2	1.00E-02
BP	Neuron migration	3	1.10E-02
BP	Myoblast migration	2	1.20E-02
BP	Metanephric mesenchyme development	2	1.20E-02
BP	Enteric nervous system development	2	1.60E-02
BP	Positive regulation of organ growth	2	1.60E-02
BP	Negative regulation of neuron apoptotic process	3	1.60E-02
BP	Hematopoietic stem cell differentiation	2	1.80E-02
BP	Regulation of epithelial cell proliferation	2	1.80E-02
BP	Epithelial to mesenchymal transition involved in endocardial cushion formation	2	1.90E-02
BP	Embryonic digestive tract morphogenesis	2	2.60E-02
BP	Middle ear morphogenesis	2	2.90E-02
BP	In utero embryonic development	3	3.10E-02
BP	Thyroid gland development	2	3.70E-02
BP	Stem cell differentiation	2	3.90E-02
BP	Pattern specification process	2	4.10E-02
BP	Embryonic cranial skeleton morphogenesis	2	4.50E-02
BP	Positive regulation of BMP signaling pathway	2	4.50E-02
BP	Dorsal/ventral pattern formation	2	4.70E-02
BP	Embryonic forelimb morphogenesis	2	4.70E-02

Figures

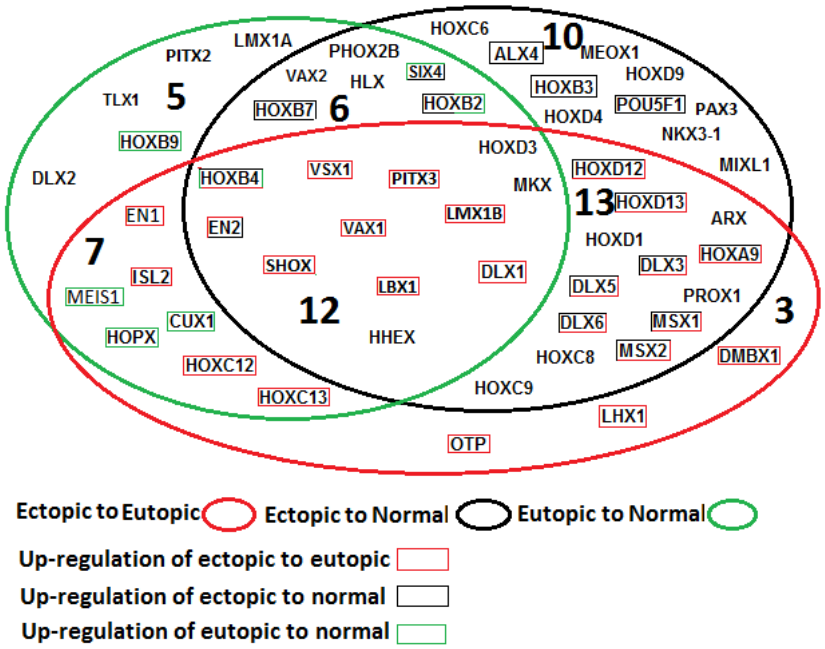

Figure 1

Venn diagram presentation of DEGs (P-value <0.05) in ectopic samples compared to normal samples (black elipse), eutopic samples in comparison to normal samples (green elipse) and ectopic samples compared to eutopic samples (red elipse). Genes that were significantly up-regulated are enclosed in a black, green and red rectangles according to the above mentioned agreements in the same legend. In multi-colored rectangles, each color represents the up-regulation in relevant analysis in the corresponding agreement.

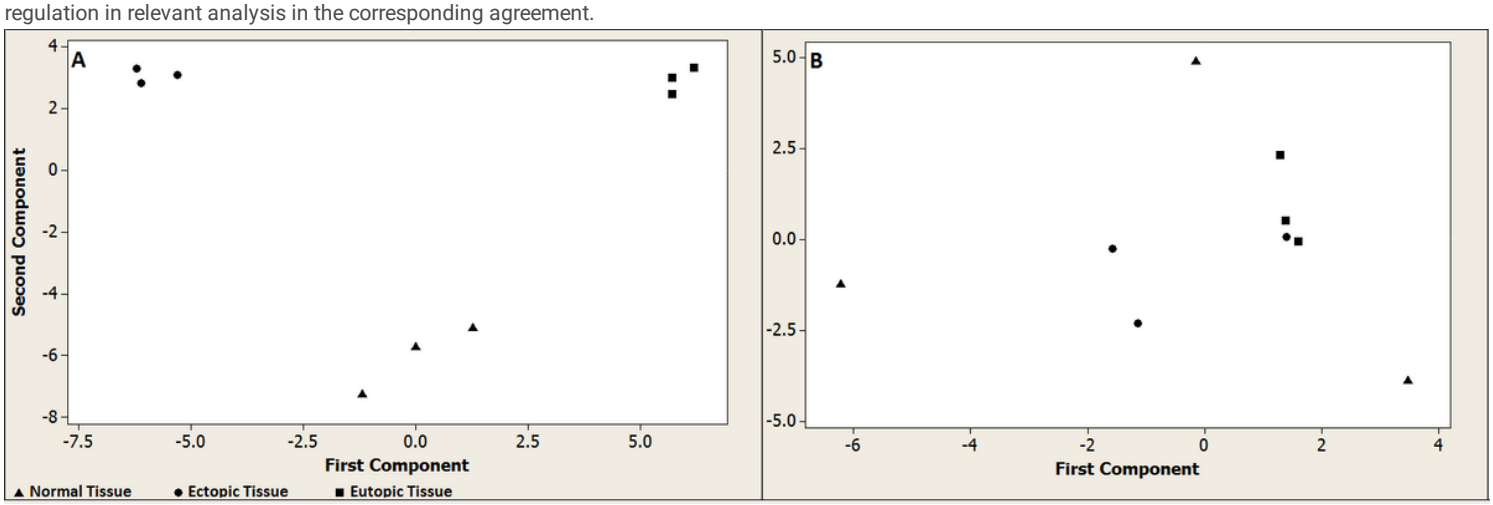

Figure 2

PCA analysis of gene expression in nine sample groups. Triangle, circle and square represents normal endometrial tissues of unaffected women, ectopic endometrial tissues of patients and eutopic endometrial tissues of patients, respectively. A: PCA of DEGs. B: PCA of non-DEGs. PCA analysis was performed using the normalized expression data of the DEGs and non-DEGs for all the analyzed endometrial samples. PCA analysis of the DEGs (Fig. 2a) revealed three distinct clusters of eutopic, ectopic and normal samples and the PCA analysis of the non-DEGs (Fig. 2b) didn't separate precisely these three groups. PCA graph represents the different endometrial samples to be separated according to the degree of correlation between the DEGs and non-DEGs.

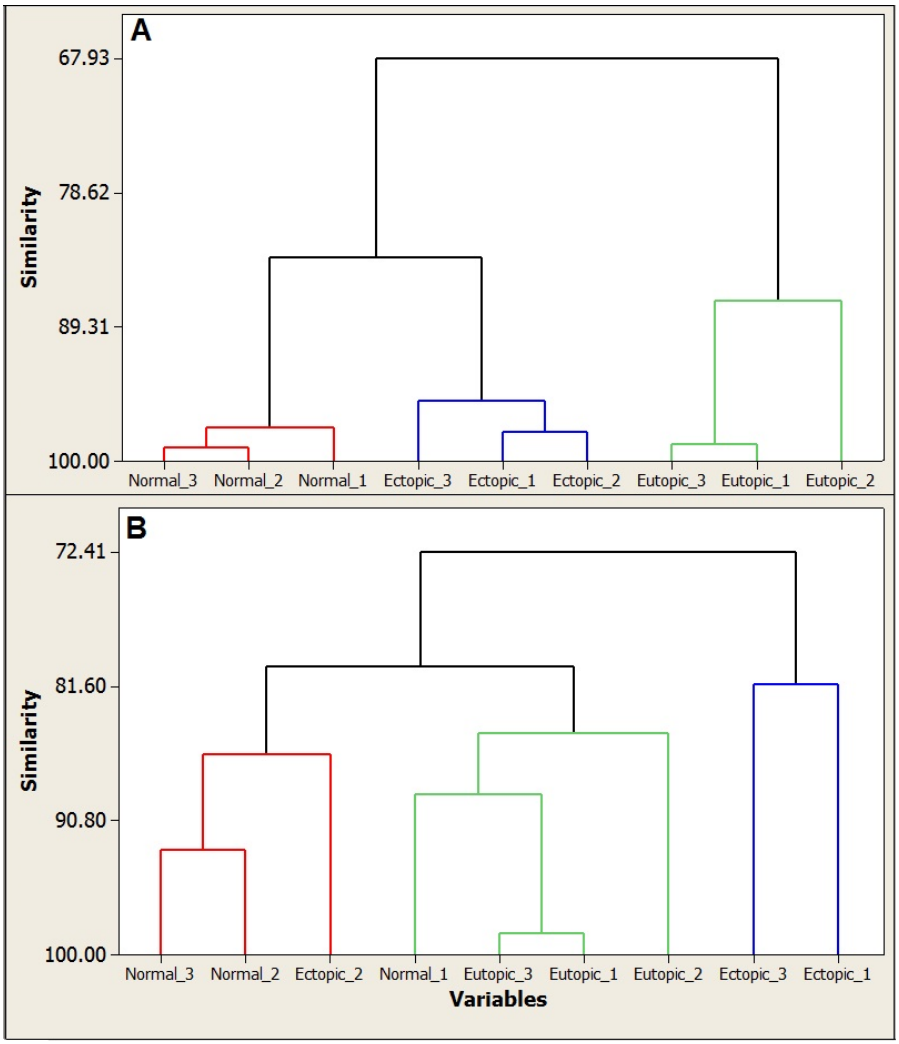

Figure 3

Hierarchical cluster analysis of nine samples into three clusters. A) based on DEGs; B) based on non-DEGs.

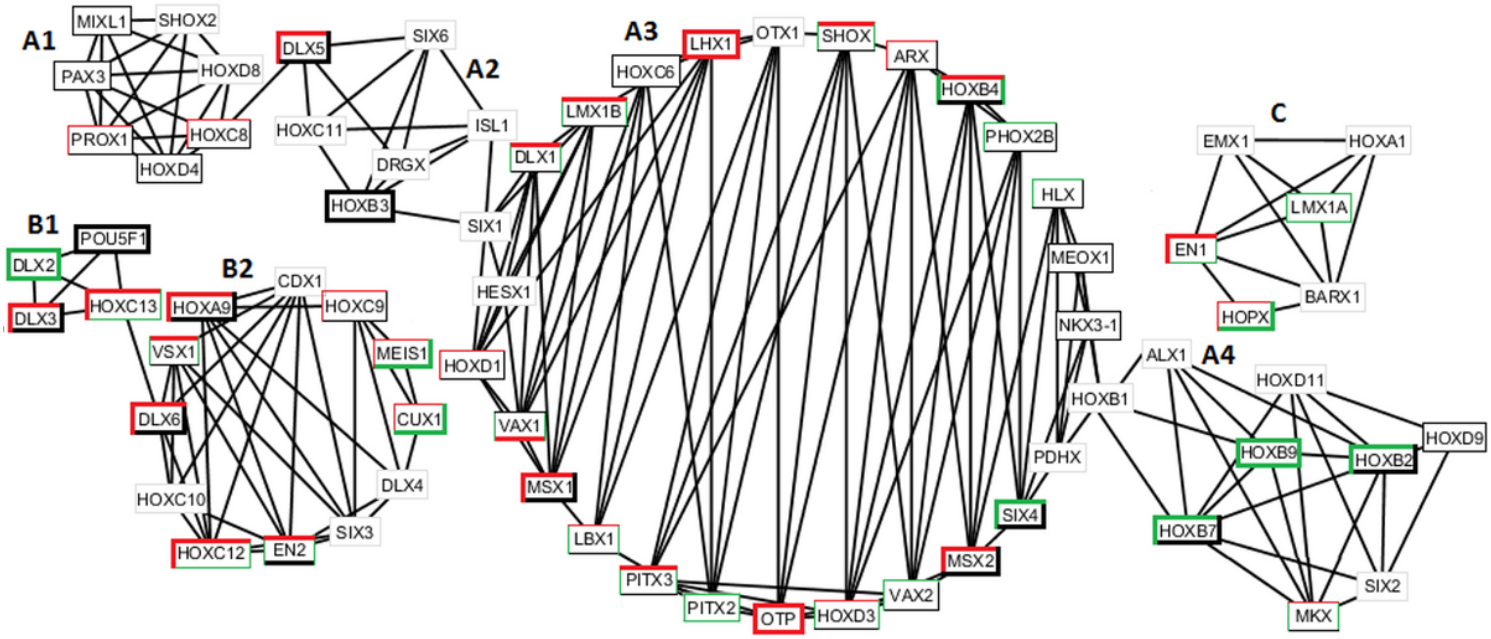

Figure 4

Co-expression gene network. The rectangle borderlines are colored based on the significant changes of gene expression of the different groups according to Figure 1. Thick lines represent up-regulation and thin lines show down-regulation .

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryData.docx