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## Dysregulation of Angiogenesis and Inflammatory Genes in Endometrial Mesenchymal Stem Cells and Their Contribution to Endometriosis

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### ABSTRACT

Endometriosis is a common, chronic, inflammatory disorder in women, characterized by the presence of endometrial tissue outside the uterus cavity. The disease affects ~10% of women during their reproductive age. There are some debates on the pathogenesis of endometriosis and its mechanism among scientists; therefore, different hypotheses have been suggested. According to Sampson's theory, a possible mechanism for seeding ectopic endometriotic lesions is a dysregulation of endometrial mesenchymal stem cells (eMSCs).

In the present study, we evaluated the expression of candidate genes in eMSCs obtained from endometriosis patients and compared them with non-endometriosis female patients. In addition, bioinformatic analysis was conducted to uncover the genes in the list of our co-expression gene network in endometriosis.

According to our results, the expression of vascular endothelial growth factor A, C-X-C-motif chemokine ligand 8, interleukin-6, and intercellular adhesion molecule-1 genes were up-regulated in the eMSCs isolated from endometriosis patients. There was no significant difference in the expression of the LaminB1 gene between the endometriosis and non-endometriosis patients. On the other hand, our bioinformatics analysis demonstrated that co-expressed genes were enriched in the cytokine signaling pathway.

Our study provides valuable insights into the gene expression dysregulation in eMSCs derived from endometriosis patients and suggests a possible function for co-expressed networks in the pathogenesis of endometriosis. To confirm the results, more investigations are required.

**Keywords:** Endometriosis; Gene expression; Inflammation; Mesenchymal stem cells

### INTRODUCTION

Endometriosis is a common, chronic, and inflammatory disorder in women, characterized by the presence of endometrial tissue outside the uterus

cavity, primarily in the pelvic region and even in the bowel and bladder.<sup>1</sup> The disease affects ~10% of women during their reproductive age,<sup>2</sup> and its clinical manifestations include dysmenorrhea, dyspareunia, pelvic pain, and infertility.<sup>3</sup>

The mechanism of the pathogenesis of endometriosis has been the subject of intense debate within the scientific community; therefore, different hypotheses have been suggested, such as retrograde

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menstruation, immune dysfunction, genetics, and malfunction of stem cells.<sup>4</sup> Previous studies have indicated that stem cells are present in the endometrium and mediate the regenerative capacity in this region.<sup>5</sup> These cells contribute to the pathogenesis of endometriosis through their retrograde shedding into the pelvic cavity after menarche.<sup>6</sup> The dysregulation of endometrial mesenchymal stem cells (eMSCs) has been proposed as a possible mechanism for seeding ectopic endometriotic lesions, possibly in concert with the Sampson theory.<sup>7</sup> During menstruation, eMSCs shed into the peritoneal cavity and subsequently proliferate, invade, and generate endometriotic implants. These cells could form endometrial stromal vascular tissues and support tissue growth and vascularization through the secretion of pro-angiogenic and growth-supporting factors.<sup>7</sup>

From another perspective, endometriosis is a chronic inflammatory disease in which macrophages and inflammatory responses play a detrimental role in the pathogenesis of the disease. Such a role regularly leads to increased oxidative stress in the endometrium. During retrograde menstruation, erythrocytes and apoptotic endometrial tissues can act as oxidative stress-inducing factors.<sup>8</sup> Reactive oxygen species can stimulate the expression of inflammatory cytokines through the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B).<sup>9</sup> The causal role of NF- $\kappa$ B in the pathogenesis of endometriosis has been documented.<sup>10</sup> Previous research showed that eMSCs might undergo senescence and reprogramming as a consequence of oxidative stress.<sup>11</sup> Such reprogramming and exhibiting the secretory phenotype contribute to the overexpression of pro-inflammatory cytokines.<sup>12</sup> There is some evidence that cytokines may play a role in the development and progression of endometriosis. Moreover, their aberrant expression could also induce the degradation of the extracellular matrix (ECM).<sup>13</sup> Taken together, it seems that angiogenesis, inflammation, oxidative stress, and ECM degradation are involved in the pathogenesis of endometriosis.

In this study, we compared the expression of candidate genes in eMSCs obtained from endometriosis patients with those afflicted with non-endometriosis disorders. There has been little quantitative analysis of gene expression in eMSCs; therefore, we conducted a bioinformatics analysis to understand the possible function and contribution of genes co-expressed with

our genes of interest in the pathogenesis of endometriosis. Our study provides insights into the pathogenesis of endometriosis by analyzing the expression of *VEGFA*, *CXCL8*, *IL6*, *LMNB1*, *ICAM1* genes.

## MATERIALS AND METHODS

### Sample Collection

Before the initiation of the experiment, ethical clearance was sought and granted from the Institutional Ethical Review Board Protocol of Tarbiat Modares University (IR.MODARES.REC.1397.204) and adhered to the tenets of the declaration of Helsinki. Humaneutopic endometrial tissues were obtained from 5 women with an age range of 27-45 (mean, 36.4 $\pm$ 5.5) who were in the proliferative phase and underwent hysteroscopy for the diagnosis of endometriosis. In addition, we recruited eutopic endometrial tissues from 5 premenopausal women with an age range of 25-36 years old (mean, 30.4 $\pm$ 3.5) who underwent hysterectomy for diagnosis of non-endometrial disorders. Written informed consent was obtained from all participants. None of the patients had taken medications or hormone treatments for the last three months before surgery, and they were diagnosed with endometriosis stage III/IV. The demographic characteristics of the patients were displayed in Table 1.

### Isolation of Endometrial Mesenchymal Stem Cells (eMSC)

We prepared a single-cell suspension using mechanically dissociating digested tissues for each collected specimen. Briefly, tissues were mechanically minced into 1mm<sup>3</sup> pieces in a medium containing penicillin-streptomycin antibiotic 1% and Dulbecco's Modified Eagle Medium F-12 (DMEMF12-Gibco, USA). Tissues were processed enzymatically with type 3 collagenase. Subsequently, cells were centrifuged, and their cell suspensions were filtered using 40 and 100 $\mu$  mesh to remove undigested tissues. The cells were cultured using DMEM/F12 medium containing 10% fetal bovine serum (FBS-Gibco, USA) and 1% penicillin-streptomycin antibiotic at 37°C in a 5% CO<sub>2</sub> incubator.

**Table 1. The demographic characteristics of patients**

	Patient ID	Age	Infertility	Stage	Location of endometriosis
<b>Endometriosis samples</b>	E1	27	Yes	III	Ovaries
	E2	32	Yes	IV	Ovaries and Intestines
	E3	38	Yes	IV	Ovaries and Fallopian tubes
	E4	45	No	III	Ovaries
	E5	40	Yes	IV	Ovaries, Fallopian tubes, and Intestines
<b>Non-Endometriosis control samples</b>	C1	27	Yes	NA	NA
	C2	29			
	C3	36			
	C4	25			
	C5	35			

NA, not applicable

### Flow Cytometry Analysis of eMSCs

Cultured cells were trypsinized, isolated, and centrifuged. The resulting precipitate was suspended in PBS with 5% FBS and incubated with specific monoclonal antibodies. Human monoclonal antibodies CD73, CD90, CD146 (BD Bioscience, USA), and CD105 (IMMUNOSTEP, Spain) were used as specific antibodies, while CD45 (BD Bioscience, USA) and CD34 (IMMUNOSTEP, Spain) was used as the negative control. Cells were evaluated with a FACS Calibur apparatus (Becton Dickinson, USA), and the obtained results were analyzed by FlowJo software.

### Invitro Differentiation of Endometrial Mesenchymal Stem Cells

Endometrial cells were seeded onto osteogenic and adipogenic differentiating media for 4 weeks. Control cells were also seeded onto a low-serum media containing DMEM/F12 with 1% FBS and incubated for 4 weeks, as well. After four weeks, to confirm the differentiation of cells into osteogenic and adipogenic cells, those differentiated cells were stained with alizarin red and Oil Red, respectively.

### Gene Expression Analysis

Total RNA was extracted from all samples using the TRIzol Reagent (Sigma-Aldrich, Germany). The concentration and integrity of the extracted RNA were confirmed by spectrophotometer and gel electrophoresis, respectively. We used 1 µg template

RNA for each specimen and a cDNA synthesis kit to obtain complementary DNA (cDNA) (Parstous, Iran). We used Primer3 (<https://bioinfo.ut.ee/primer3-0.4.0/>), SNPCheck(<https://genetools.org/SNPCheck/snpcheck.htm>), and NCBI BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) online tools to design highly specific and efficient exon-exon junction primers (Table S1). The RT-qPCR method was used to analyze the expression of selected genes; using the StepOne Real-time PCR instrument and SYBR Green PCR master mix. The efficiency of the PCR reaction was examined by the linReg software.<sup>14</sup> Raw data extracted from the qPCR instrument were normalized and adjusted by the expression of the *GAPDH* gene, as a reference gene, and then analyzed by the Pfaffl method.<sup>15</sup> Based on the non-parametric distribution of data the Mann-Whitney U-test was used to assess the differences between the two studied groups, and the *p*-value of less than 0.05 was statistically considered significant.

### Bioinformatics Analysis

The co-expression network of the selected genes expressed in mesenchymal stem cells and the uterus was examined, thereby the Search-Based Exploration of Expression Compendium (SEEK; <http://seek.princeton.edu/>) online tool. High-ranked co-expressed genes in datasets with a *p* value of less than 0.05 were selected from MSCs and uterine tissue, and then the overlapped genes between those datasets were

chosen for further analyses. The gene ontology-term analysis includes the biological process (BP), cellular component (CC), and molecular function (MF). The gene ontology function and Reactome pathway enrichment analysis were used for the evaluation of co-expressed genes using the Enrichr online tool (<https://amp.pharm.mssm.edu/Enrichr/>). The pathway analysis was carried out by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database ([www.genome.jp/kegg/pathway.html?sess=2764b8338258d6286de91bbebe6faf46](http://www.genome.jp/kegg/pathway.html?sess=2764b8338258d6286de91bbebe6faf46)) and Reactome online database (<https://reactome.org>). Upon the completion of the gene list shared between the datasets, the process of Gene Ontology (GO) enrichment analysis was conducted to identify genes with biological relevance and functional contributions in our genes of interest.

Functional interactions between co-expressed genes were analyzed using the protein-protein interaction (PPI) network analysis where appropriate. The analysis method was based on the framework provided by the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) search engine (<https://string-db.org/>), while the construction of PPI networks and visualization of them were performed by the Cytoscape software (<https://cytoscape.org/>). To characterize the densely connected regions in PPI networks, the Molecular Complex Detection (MCODE) plug-in of Cytoscape software was applied according to the following parameters; Degree cutoff 2, node score cutoff 0.2, k-core 2, and maximum depth 100. Subsequently, the KEGG pathway enrichment analysis was used for the analysis of co-expressed genes to unravel the most significant modules.

Genes encoding transcription factors were identified by the Animal Transcription Factors Database (AnimalTFDB, <http://bioinfo.life.hust.edu.cn/AnimalTFDB>) that encompasses genome-wide transcription factors (TFs), chromatin remodeling factors, and transcription co-factors. A total of 1665 human TFs were downloaded from this database. Subsequently, human TF-encoding genes intersected with the commonly expressed genes between MSCs, and the uterine dataset was applied to identify TFs involved in endometriosis using the Venn diagram.

## RESULTS

### Isolation and Characterization of Endometrial Stem Cells

To determine the endometrial stem cells were differentiated into adipogenic and osteogenic cells, the cells were incubated in a specific differentiation medium. The differentiation process was confirmed through lipid vacuole staining and calcium deposition by oil red and alizarin red, respectively (Figure 1, A, and B). The flow cytometry analysis confirmed the expression of mesenchymal cell markers, as well as lacking the expression of hematopoietic cell markers (Figure 1, C-H).

### Gene Expression Analysis

Gene expression analysis has revealed the up-regulation of *VEGFA* ( $p=0.0032$ ), *CXCL8* ( $p=0.009$ ), *IL6* ( $p=0.025$ ), and *ICAM1* ( $p=0.003$ ) genes in endometrial MSCs in comparison with cells derived from non-endometriosis patients (Figures 2A, B, C, and D). No significant difference was found in the expression levels of *LMNB1* between the endometrial MSCs of endometriosis and non-endometriosis patients (Figure 2E).

### Bioinformatics Analysis

#### Co-expressed Network

We constructed the co-expression network for the selected genes in MSCs and the uterus using the SEEK database. Generally, we discovered 41 and 7 co-expression datasets for MSC and the uterus, having a  $p$ -value of less than 0.05. Datasets and their features are summarized in Supplementary Tables S2 and S3. There were only 504 shared genes with a  $p<0.05$  in both datasets, which showed co-expressed genes in mesenchymal stem cells and the uterus. Figure 3 and Table S4 show an overview of the shared genes.

#### Enrichment Analysis

Enriched gene ontology terms were specified for the MF, BP, and CC. The results indicated that the overlapped co-expressed genes were mainly enriched in the cytokine-mediated signaling pathway. The analysis of molecular functions for overlapped genes showed that these genes were significantly enriched in the cytokine signaling, while a similar analysis for the cellular components demonstrated a significant contribution of those genes in a specific granule (Table 2).

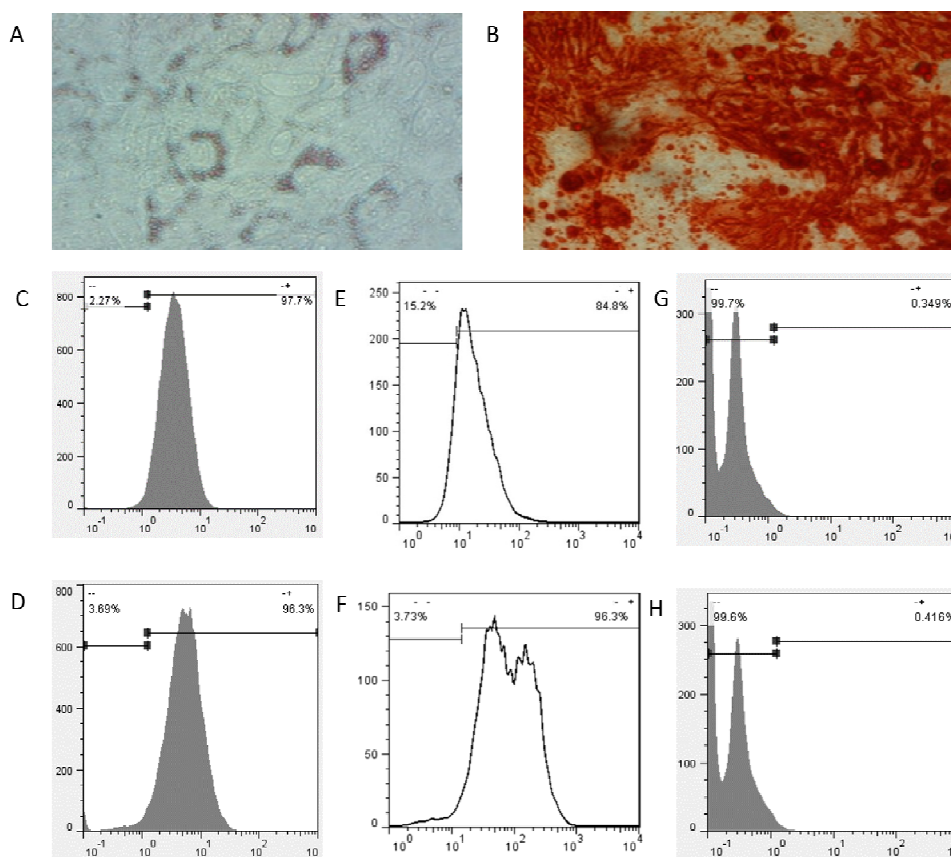
### The PPI Network Analysis and Pathway Identification

The PPI network was constructed based on the STRING database to better understand the role of those overlapped co-expressed genes. We established a network containing 504 genes. The network significantly possessed higher interactions than expectations, as 492 nodes and 2084 edges were identified, and the enrichment  $p$ -value of the PPI network was  $<1.0e-16$  (Figure 4). The cluster analysis of the PPI network using the MCODE plug-in method revealed the most significant module. The module is comprised of 27 nodes and 279 edges. Using the KEGG pathway-enrichment analysis of genes in the module, it was shown that most genes were enriched in the TNF- $\alpha$  and interleukin 17(IL-17) signaling pathways.

### Identification of TFs

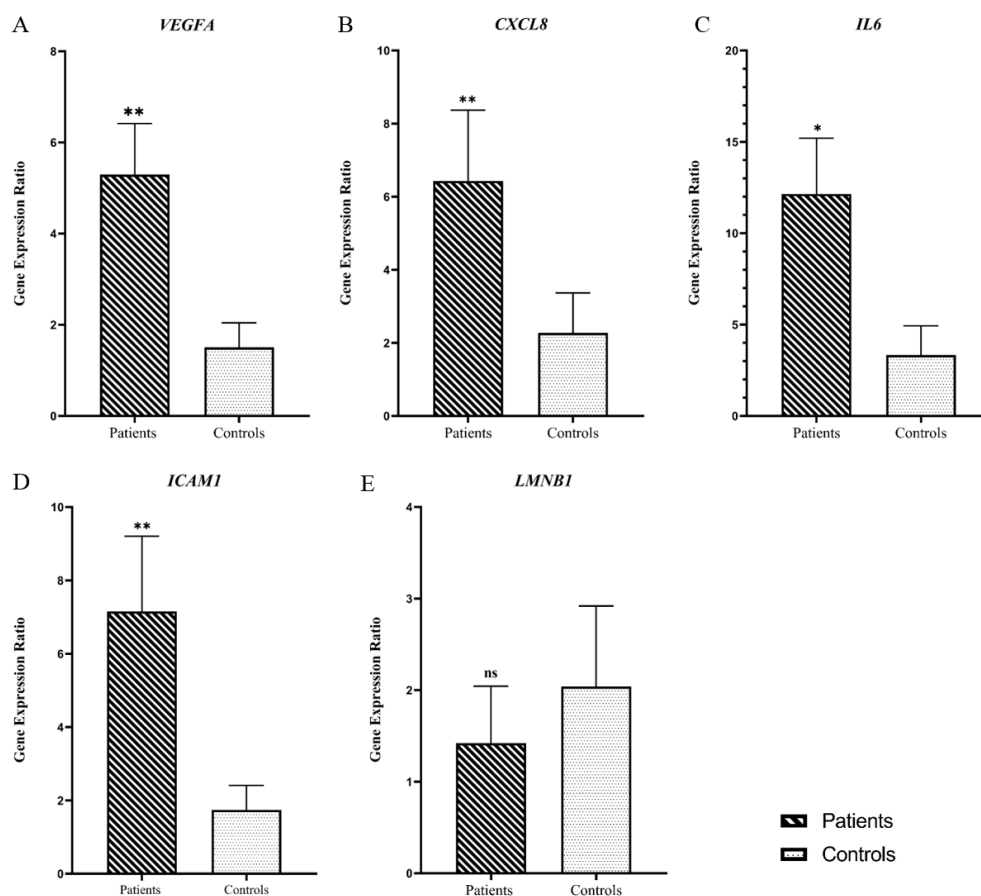
A total of 1665 known human TFs were downloaded from the Animal Transcription Factor Database(<http://bioinfo.life.hust.edu.cn/AnimalTFDB/>!). Amongst 504 common co-expressed genes, 47 genes act as transcription factors (Table S5). By analyzing these TFs with the help of the REACTOME database, it was indicated that these TFs are enriched in the cellular senescence pathway (Table 3).

Using analyzing the signaling pathways (Reactome database), we found cytokine signaling in the immune system as the most significantly enriched pathway. Thanks to the KEGG pathway enrichment analysis performed for overlapped genes, it was shown that most genes were enriched in the tumor necrosis factor-alpha (TNF- $\alpha$ ) signaling pathway (Table 4).

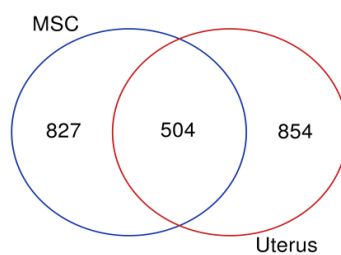


**Figure 1. Isolation and Characterization of endometrial mesenchymal stem cells (eMSCs). Adipogenic (A) and osteogenic (B) differentiation of isolated eMSCs. Flow cytometry analyses showed that endometrial MSCs positively expressed CD73(C), CD90 (D), CD146 (E), and CD105 (F) but negatively expressed CD45 (G) and CD34 (H).**

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**Figure 2.** The relative expression levels of vascular endothelial growth factor A (*VEGFA*) (A), C-X-C motif chemokine ligand 8 (*CXCL8*) (B), interleukin 6 (*IL6*) (C), LaminB1 (*LMNB1*) (D), and intercellular adhesion molecule 1 (*ICAMI*) (E) in endometrial mesenchymal stem cells (eMSCs) derived from patients with endometriosis (n=5) and non-endometriosis (n=5) (ns= not significant, \* $p < 0.05$ , \*\* $p < 0.01$ ).



**Figure 3.** The Venn diagram of overlapped co-expressed genes

**Table 2. The gene ontology analysis of overlapped co-expressed genes**

Name	<i>p</i>	Adjusted <i>p</i>
<b>Top 3 GO Biological Process</b>		
Cytokine-mediated signaling pathway	7.241e-21	3.695e-17
Inflammatory response	1.358e-15	3.466e-12
Response to lipopolysaccharide	1.986e-15	3.378e-12
<b>Top 3 GO Molecular Function</b>		
Cytokine activity	1.379e-13	1.587e-10
Transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding	7.583e-7	<0.001
Chemokine activity	<0.001	<0.001
<b>Top 3 GO Cellular Component</b>		
Specific granule	<0.001	0.3398
Specific granule lumen	0.004	1.000
Integral component of plasma membrane	0.007	1.000

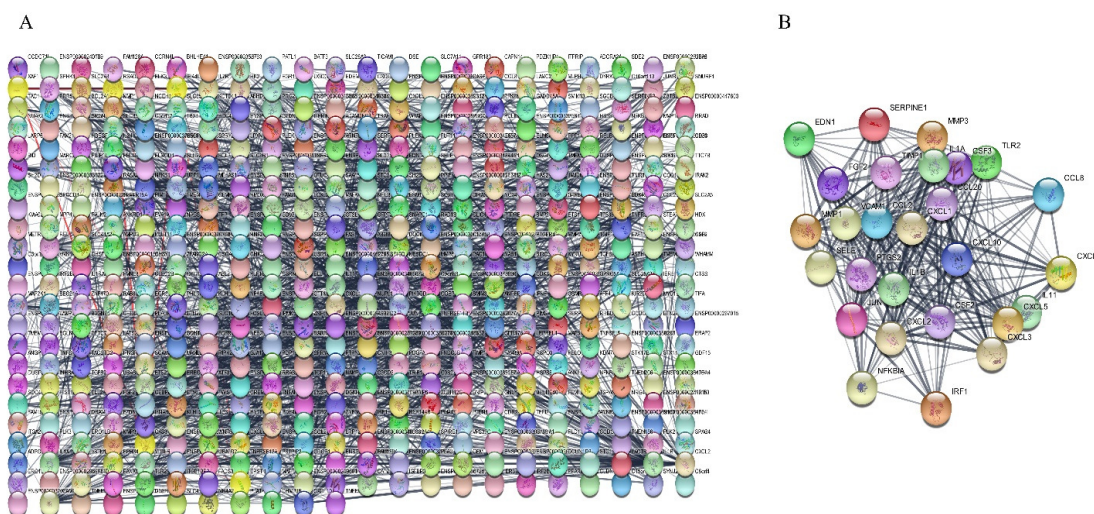
**Table 3. The Reactome pathways analysis for overlapped co-expressed genes that act as transcription factors**

Name	<i>p</i>	Adjusted <i>p</i>
Cellular Senescence	0.00003708	0.05673
CLEC7A (Dectin-1) signaling	0.00008556	0.06545
Interleukin-1 processing	0.0001127	0.05746

**Table 4. The Reactome and KEGG pathways analysis for the overlapped co-expressed genes**

Name	<i>p</i>	Adjusted <i>p</i>
<b>Top 3 Reactome Pathways</b>		
Cytokine Signaling in Immune system	1.500e-12	2.296e-9
Signaling by Interleukins	2.183e-8	0.00001670
Immune System	6.450e-8	0.00003289
<b>Top 3 KEGG Pathways</b>		
TNF signaling pathway	3.810e-24	1.173e-21
Cytokine-cytokine receptor interaction	4.240e-19	6.530e-17
IL-17 signaling pathway	1.225e-14	1.258e-12

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**Figure 4.** The PPI (protein-protein interaction) network of overlapped co-expressed genes was obtained from the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (A). The Molecular Complex Detection (MCODE) plug-in analysis of overlapped co-expressed genes shows the most significant module (B).

### DISCUSSION

The pathogenesis of endometriosis remains unclear to date. Several reports have shown the importance of endometrial mesenchymal stem cells in the pathogenesis of endometriosis.<sup>16</sup> This study aimed to assess and compare gene expression for some selected genes in eMSCs derived from endometriosis and non-endometriosis patients. Owing to the lack of comprehensive gene expression analysis for eMSCs, we conducted a bioinformatics analysis to unravel genes that are co-expressed in line with our selected genes and are involved in endometriosis pathology.

Previous studies demonstrate the significance of the maintenance and regulation of the stem cell microenvironment and emphasize its critical roles in normal stem cell development.<sup>17</sup> The dysregulation of gene expression in eMSCs can impair normal procedures and proliferate more aggressive cells. Such aggressive cells can migrate from the uterus cavity and establish ectopic endometriotic lesions.<sup>5</sup>

There is little information about the dysregulated gene expression in eMSCs. Most studies have focused on the dysregulation of gene expression in endometrial tissues, serum, or peripheral blood of endometriosis patients.<sup>18</sup> As mentioned in previous studies, endometriotic lesions stem from eutopic MSCs; therefore, the assessment of gene expression, such as

those selected in this study and estimation of their effects, may provide insight into the pathogenesis of endometriosis.

Previous studies have demonstrated the significance of angiogenesis as a key pathway in the development of endometriotic lesions. In this regard, some factors, such as *VEGFA* and *CXCL8*, play a critical role.<sup>19</sup> Pro-inflammatory cytokines synergistically promote the expression of *CXCL8* and *VEGF* proteins and induce angiogenesis in endometriosis patients.<sup>20</sup> Our results showed that these genes are upregulated in eMSCs of endometriosis patients. Following the present results, several studies have highlighted the upregulation of *VEGFA* in ectopic endometrial lesions when compared to eutopic specimens.<sup>21</sup> These results further support a possible role of eMSCs in angiogenesis, as well as the development of endometriotic lesions through the overexpression of *VEGFA*.

According to the literature, there is a marked correlation between *CXCL8* overexpression and the development of endometriosis through participation in chronic inflammation and adhesion processes.<sup>22</sup> In line with these studies, we showed that *CXCL8* and *IL6* inflammatory cytokines are overexpressed in eMSCs derived from endometriosis patients compared with non-endometriosis patients. Some investigations revealed the upregulation of these genes in serum and peritoneal fluid of women with endometriosis.<sup>23</sup>



A group of factors, such as IL6, can upregulate the expression of *ICAM1* and increase cell adhesion to the peritoneum.<sup>24</sup> Our findings showed that the expression of *ICAM1* was upregulated in eMSCs of endometriosis patients in comparison with non-endometriosis patients. These results are in agreement with the results of Kuessel et al, who showed the up-regulation of *ICAM1* in ectopic endometriotic lesions and peritoneal endometriosis patients.<sup>25</sup> The overexpression of this gene in eMSCs derived from endometriosis patients shows the ability of these cells to attach to the peritoneum and establish the endometriotic lesions. It seems that the microenvironment of ectopic lesions plays a significant role in the pathogenesis of endometriosis and could be a major topic for future studies.

The increase of inflammatory factors is capable of inducing oxidative stress,<sup>8</sup> which drives the senescence process in mesenchymal stem cells.<sup>11</sup> Such a process can explain the increment of estrogen concentration, which is considered as a pro-senescence factor in endometriosis.<sup>26</sup> It would be plausible that the overexpression of inflammatory cytokines, such as *IL6*, and *CXCL8*, contributes to the upregulation of *ICAM1* in eMSCs of endometriosis patients. This may induce oxidative stress, increase estrogen secretion and senescence. This cascade provides a possible explanation for mechanisms underlying the pathogenesis of endometriosis. To scrutinize the above-mentioned hypothesis, we analyzed the expression of the *LMNB1* gene, as a senescence-associated biomarker in eMSC.<sup>27</sup> In contrast to expectations, this study did not find a significant difference in gene expression of *LMNB1* between endometriosis patients and non-endometriosis patients, while several lines of evidence indicated the downregulation of this gene in endometriosis patients. Using in-silico analyses, we showed that transcription factors related to extracted co-expressed genes are enriched in the cellular senescence process, in contradiction with our investigations performed on the expression of the *LMNB1* gene. Similarly, Malvezzi et al identified a higher expression level of *LMNB1* in eutopic endometrium when compared with ectopic specimens, while they did not identify any differences between endometriosis patients and controls.<sup>18</sup> The inconsistency of our results with their results may be due to the difference in the nature of control samples or the type of samples.

We conducted a bioinformatics analysis to unravel the main function enriched in shared co-expressed genes in the uterus and MSCs. This analysis helps to find the probable gene expression profile in eMSCs. The KEGG pathway analysis has shown the TNF- $\alpha$  signaling pathway as an enriched pathway. According to the literature, the activation of peritoneal macrophages has been reported as a trigger factor for the local production of TNF- $\alpha$ . It has been reported that the level of TNF- $\alpha$  can be increased in the peritoneal fluid of endometriosis patients, and such levels are correlated with the number and size of active lesions as well as the disease severity.<sup>28</sup> Increased expression of TNF- $\alpha$  increases the possibility of cellular attachment to the peritoneal surface and ectopic growth of the endometrium via stimulating the proliferation and angiogenesis processes.<sup>29</sup> These pieces of evidence provide a strong background for our in-silico findings and explain a relatively good correlation between TNF- $\alpha$  and ectopic endometrium invasion.

Our bioinformatics analysis demonstrated the importance of the cytokine signaling pathway in MSCs isolated from the uterus. We also indicated the increased levels of cytokines (*IL6* and *CXCL8*) in eMSCs of endometriosis patients. Cytokines are small proteins involved in cell signaling, and numerous studies showed their role in the pathogenesis of endometriosis.<sup>30</sup> Our results support previous studies on the role of *IL6* and *CXCL8* in endometriosis.

Herein, we assessed the expression of key regulator genes relevant to angiogenesis, inflammation, and senescence pathways in eMSCs obtained from endometriosis and non-endometriosis patients. Our experiments confirmed the dysregulation of key genes in eMSCs in endometriosis patients and their possible critical roles in the establishment of endometriotic lesions through angiogenesis and production of cytokines. This study was limited by the absence of gene expression datasets related to eMSC. Despite this limitation, we implemented gene expression data in the uterus and MSCs separately. We found overlapped co-expressed genes in these two cell lines indicating the expression pattern of genes in eMSCs. Our analysis has shown the importance of cytokines in endometriosis with the help of databases. Regardless of the relatively limited samples, this study provides valuable insights into the function of mesenchymal stem cells in endometriosis pathogenesis. Further research should be

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carried out to establish the comprehensive gene expression profiles in eMSCs.

### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

### ACKNOWLEDGEMENTS

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