Transcriptional profiling of endometriosis tissues identifies genes related to organogenesis defects

Article *in* Journal of Cellular Physiology · September 2013

DOI: 10.1002/jcp.24358 · Source: PubMed

9 authors, including:

Stefania Crispi
Italian National Research Council
68 PUBLICATIONS **964** CITATIONS
SEE PROFILE

Aldo Donizetti
University of Naples Federico II
48 PUBLICATIONS **349** CITATIONS
SEE PROFILE

Raffaele Calogero
Università degli Studi di Torino
285 PUBLICATIONS **5,363** CITATIONS
SEE PROFILE

Alfonso Baldi
Università della Campania "Luigi Vanvitelli", Caserta, Italy
458 PUBLICATIONS **11,793** CITATIONS
SEE PROFILE

Original Research Article

Transcriptional profiling of endometriosis tissues identifies genes related to organogenesis defects†

Stefania Crispi[1], Maria Teresa Piccolo[1], Alfredo D'Avino[2], Aldo Donizetti[1], Rosa Viceconte[3], Maria Spyrou[3], Raffaele A. Calogero[4], Alfonso Baldi[2,3], Pietro G. Signorile[3]

[1]Gene Expression & Human Molecular Genetics Laboratory, Institute of Genetics and Biophysics "A.B.T" CNR, Naples, Italy; [2]Department of Enviromental, Biological and Pharmaceutical Sciences and Technologies, Second University of Naples, Italy; [3]Fondazione Italiana Endometriosi, Rome, Italy; [4]Bioinformatics and Genomics Unit, MBC Centro di Biotecnologie Molecolari, University of Turin, Italy;

^*Corresponding authors:* Alfonso Baldi and Pietro Giulio. Signorile, Fondazione Italiana Emdometriosi, Via Emilio Longoni 81, 00155 Rome, Italy. Tel +390635306066, Fax: +39062255261; email: research@endometriosi.it

Running Head : Developmental failure triggers deep endometriosis

Keywords: Endometriosis, Deep Endometriosis, Endometrium, Endocrine Disruptors, Gene expression, Embryonic development

Abbreviations:
ICC: Integrative correlation analysis; qRT-PCR: quantitative real-time polymerase chain reaction; IHC: Immunohistochemistry; rAF: Revised American Fertility Society, SERM : Selective Estrogen Receptors Modulator

Additional Supporting Information may be found in the online version of this article.

Received 16 February 2013; Accepted 20 February 2013
Journal of Cellular Physiology

DOI 10.1002/jcp.24358

Abstract

Endometriosis is a common benign pathology, characterised by the presence of endometrial tissue outside the endometrial cavity with a prevalence of 10–15% in reproductive-aged women. The pathogenesis is not completely understood, and several theories have been proposed to explain the etiology. Our group has recently described the presence of ectopic endometrium in a consistent number of human female foetuses analysed by autopsy, reinforcing the hypothesis that endometriosis may be generated by defects during the organogenesis of the female reproductive trait. Herein, in order to identify, at molecular level, changes involved in the disease, we compared the transcriptional profiling of ectopic endometrium with the corresponding eutopic one. Statistical analyses lead us to identify some genes specifically deregulated in the ectopic endometrium, that are involved in gonad developmental process or in wound healing process. Among them, we identified BMP4 and GREM1. BMP4 was never associated before to endometriosis and is involved in the mesoderm-Müllerian duct differentiation. GREM1 is needed for the initial step of the ureter growth and perhaps could possibly be involved in Müller ducts differentiation. These molecules might be related to the endometriosis etiology since we showed that their expression is not related to the menstrual cycle phase both at RNA and at protein levels. These data support the theory that embryological defects could be responsible of the endometriosis generation.

Introduction

Endometriosis is a chronic benign disease present in 10-15% of fertile-aged women (Bulun, 2009; Giudice, 2010), characterised by the presence of endometrial glands and stroma outside the uterine cavity. This pathology is frequently associated with infertility and pelvic pain, with a more severe pelvic pain in the deep infiltrating endometriosis of rectovaginal septum, which is characterised by lesions with very high activity (Baldi et al., 2008; Signorile et al., 2009a).

The pathogenesis of endometriosis is not completely understood, and different theories have been proposed. The retrograde menstruation theory claims that endometrial cells can be deposited, via blood vessel, in other pelvic regions where they adhere and develop (Sampson, 1927). The coelomic metaplasia theory, hypothesizes that endometriosis is caused by spontaneous metaplasia of mesothelial cells of the coelomic epithelium (Matsuura *et al.,* 1999). This theory is supported either by the ability of coelomic epithelium to differentiate in several histological cell types, and by the fact that mesothelium and embryonic precursors of endometrium (the Müllerian ducts) both have the same embryologic origin, so it is possible to correlate endometriosis to spontaneous mesothelium metaplasia. The endometrial stem cell theory proposes that the pathology is generated by endometrial stem/progenitor cells naturally located in the endometrium or by circulating stem cells deriving from bone marrow (Chan et al., 2004; Du and Taylor, 2007).

Another theory, explaining the presence of endometriosis along the migration pathway of the embryonic reproductive trait, hypothesizes that endometriosis is due to alterations in the fine tuning of female genital structures organogenesis (Signorile and Baldi, 2010). Briefly, cells committed to become endometrial, migrate at 8-10 weeks in the embryo following the female reproductive tracts (migrating uterus). Cells that are separated from embryonic development can generate ectopic endometrium that would remain quiescent until puberty, when the hormonal inputs would determine its re-activation and the onset of the symptoms of endometriosis. In support of this theory, we described the presence of ectopic endometrium in a consistent number of human female foetuses analysed by autopsy (Signorile *et al.,* 2009b; Signorile *et al.,* 2010b; Signorile *et al.,* 2012a). Further supporting the role of alteration during the fine tuning of the female reproductive system in the pathogenesis of endometriosis, is our observation that pre-natal exposition to the Selective Estrogen Receptors Modulator (SERM) bisphenol in mice, is able to induce in the pups an endometriosis-like phenotype (Signorile *et al.,* 2010c). Nevertheless, endometriosis is a multifaceted disease, so it is probable that complex interactions between genotype and environment could account for the disease onset.

In this study we analysed the transcriptome of the endometriosis lesions, in order to elucidate the molecular mechanisms underlying this phenomenon. Specifically, we compared the transcriptome of the ectopic and eutopic endometrium of the same patients with respect to endometrium from unaffected samples. Statistical analyses of the microarray data highlighted the presence of up-regulated genes in the endometriotic lesions, whose expression was confirmed by immunohistochemistry. The potential clinical relevance of these data is discussed.

Materials and Methods

Patients

Clinical samples of eutopic or ectopic endometrial tissues were both collected from women affected by endometriosis that underwent surgery for infertility, pelvic pain symptoms (including dysmenorrhea, deep dyspareunia and no-menstrual pain) or adnexal masses between 2009 and 2011 at the “Centro Italiano Endometriosi” in Rome. The diagnosis of deep infiltrating endometriosis was made during the diagnostic and therapeutic phase of laparoscopic surgery and was based on the macroscopic appearance of the lesions, using the following criteria: a) palpable and visible nodule or induration and retraction of peritoneum in the posterior and lateral area of cervix, at the level of the uterosacral ligaments and medial broad ligaments, and rectovaginal septum; b) dark blue nodule visible at the posterior vaginal wall at speculum examination.

The 27 cases used in this study were characterised by the presence of endometriotic glands confirmed histologically, excluding those who previously have had endometriosis surgery and women no longer menstruating. Main patients' characteristics are summarized in Table 1 and for each case both ectopic and eutopic endometrium was analized. Eutopic endometrial tissues were collected from healthy women in the proliferative phase of the menstrual cycle (days 3-13) undergoing laparoscopy or laparotomy for benign pathologies and were used as control samples. All the biopsies were collected in RNA Later (Ambion, Life Technologies, NY USA) before either formaldehyde-fixation for determination of histological endometriosis grade, or Trizol (Invitrogen, Life Technologies CA, USA) addiction for RNA extraction. Each patient gave a written informed consent in accordance with the Italian law. This study was submitted and approved by the Ethic Committee of Fondazione Italiana Endometriosi.

GeneChip array sample preparation and Data analysis

Total RNA was extracted from all the specimens using Trizol reagent. For each affected woman, two biopsies were obtained for the eutopic and ectopic endometrium respectively, while only one biopsy from the healthy eutopic endometrium was collected. RNA quality was assessed using an Experion RNA StdSens Kit (Bio-Rad Laboratories, CA USA). Biotinylated cRNAs targets were generated for 6 healthy women and 8 endometriotic patients. All samples were in the proliferative phase of the menstrual cycle. cRNAs were produced from both ectopic and eutopic endometria starting from 300 ng of total RNA using the Message AmpIII kit (Ambion) according to the manufacturer's instructions. Each cRNA was then fragmented to a length of 20–200 bp before hybridization to Genechip HGU133A 2.0 arrays (Affymetrix, CA, USA). Hybridization, washing, staining and scanner procedures were done using a Genechip Affymetrix station (Fluidics station 450, GeneChip Scanner 3000) as recommended by manufacturer. Laser scanning generated digitized image data files and CEL file that were used for the subsequent statistical analysis.

Probe set intensities were obtained by means of RMA method (Irizarry *et al.,* 2003) and normalization was done according to quantiles method (Bolstad *et al.,* 2003). Dataset complexity was reduced applying an interquartile filter (IQR>0.25) and retaining probe sets with expression signal ≥ log2(100)=6.64 in at least 25% of the arrays (von Heydebreck *et al.,* 2004).

Transcripts differentially expressed between ectopic and eutopic endometrium in the affected women and healthy specimens were detected using the so-called rank product non-parametric method (Breitling et al., 2004). This method addresses the multiple comparison problem and performs p-value correction by false-discovery-rate (FDR), comparing the true rank product distribution with a random one defined permutating gene labels in each of the arrays under analysis. Here, we have used 500 permutations and a threshold of percentage of false positive predictions (pfp) of 0.05.

Expression similarity between ectopic and eutopic endometrium in the affected women versus the control endometrium was evaluated using the integrative correlation methods (Parmigiani *et al.,* 2004). Parmigiani has devised an integrative correlation coefficient (ICC) for quantification of the extent to which independent studies can be reliably analysed together for the systematic comparison of microarray profiles. ICC ranges between zero and one; values closer to one indicate a strong correlation between the two groups. Specifically, we generated ICC for ectopic versus normal and eutopic versus normal. These pair-wise correlations were plotted between each other and 179 probe sets

characterised by divergent behaviour between ectopic and eutopic with respect to normal samples were selected (Figure 1, Supplemental Table 1). OneChannelGUI graphical interface package was used to run any step of the described analysis (Sanges et al., 2007). Ingenuity Pathway Analysis (IPA7.0, Ingenuity System®, http://www.ingenuity.com/) was used to functionally annotate probe sets according to biological processes and canonical pathways and to identify genes potentially associated to embryological development. To this aim, we analysed in detail molecules belonging to the functional categories showing a robust statistical significance (p-value ≤ E-09) (Table 3).The obtained list was further reduced selecting only genes with a fold change concordant with the predicted IPA activation state. These genes were biologically validated (see below). Hierarchical clustering analyses were done using Tmev tool (http://www.tm4.org/mev.html). Microarrays data reported in the manuscript were described in accordance with MIAME guidelines. Microarray data were deposited on GEO database: GSE25628 (http://www.ncbi.nlm.nih.gov/projects/geo/).

Quantitative Real-Time PCR analysis

Total RNA (300 ng) from each sample was converted to cDNA using High- Capacity cDNA Reverse transcription kit (Applied Biosystem, Life Technologies CA, USA) under conditions described by the supplier. Gene specific primers for the selected genes reported in Table 2, were designed using Primer Express 2.0 software (Applied Biosystem). GAPDH was used as internal control. Quantitative PCRs were done on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The entire procedure for qRT-PCR analysis - primer design, reactions, amplicon specificity and determination of gene target expression levels - was performed as previously described (Crispi *et al.,* 2009).

Immunohistochemistry

Immunohistochemistry was performed on 15 cases (5 for each of the menstrual phase) for a total of 30 specimens (15 eutopic and 15 ectopic endometria samples). Sections from each specimen were cut at 5 mm, mounted on glass and dried overnight at 37°C. All sections were then deparaffinised in xylene, rehydrated through a graded alcohol series and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antibody dilution. Endogenous peroxidase activity was blocked by 5% hydrogen peroxide. For immunohistochemistry, tissue sections were heated twice in a microwave oven for 5 min each at 700 W in citrate buffer (pH 6.0). Slides were then

incubated at 4°C overnight at 1:100 dilution with the following antibodies: mouse monoclonal to BMP4 (Abcam, Cambridge, UK; cat. # ab93939); mouse monoclonal to Thrombospondin 2 (Santa Cruz, Santa Cruz, USA, cat. # sc-136238); mouse monoclonal to Follistatin (US Biological, Swampscott, USA, cat. # F9090-03); and rabbit polyclonal to Serpine 2 (Proteintech, Manchester, UK, cat. # 11303-1-AP). After three washes in PBS to remove the excess of antiserum, the slides were incubated with diluted goat anti-rabbit or anti-mouse biotinylated antibody (Vector Laboratories, Burlingame, CA, U.S.A.) at 1:200 dilution in PBS-3% non-fat dry milk for 1 h. All the slides were then processed by the ABC method (Vector Laboratories) for 30 min at room temperature. Diaminobenzidine (Vector Laboratories) was used as the final chromogen and hematoxylin was used as the nuclear counter stain. Negative controls for each tissue section were prepared by leaving out the primary antiserum. All samples were processed under the same conditions. The intensity of expression per field (20X original magnification) using light microscopy was calculated and compared in different specimens by two separate observers (A.B. and A.D.) in a double blind fashion and described as: score 0 (absent); score 1 (low); score 2 (moderate); score 3 (intense). An average of 22 fields was observed for each specimen. The percentage of concordance between the two observers was 93% (28 over 30); in the remaining specimens the final score was reached after collegial revision.

Statistics

Statistical analysis was performed using GraphPad Prism 5.0H statistical software (GraphPad Software Inc., La Jolla, CA). Paired t test was used for comparison of two paired groups. Multiple comparisons were performed by the repeated measures ANOVA test with the Bonferroni correction for multiple.

Results

Genome-wide profiling analyses of ectopic vs eutopic endometrium leads to the identification of genes related to embryological developmental failure

To better understand the molecular pathways involved in the delocalization and growth of the ectopic endometrium, we have analyzed the transcriptomes of ectopic and eutopic endometrium with the aim to identify specific molecules that could account for a primordial and incorrect endometrium delocalization. The analysis was performed collecting ectopic

and eutopic endometrium from each affected woman. As control samples, we analyzed endometrium from healthy women.

The clinical samples characteristics are reported in Table 1. Subjects selected for the microarray analysis were 8 ectopic endometria and 8 matched eutopic endometria from the same patients compared to 6-coupled control patients.

Transcription profiling was performed using HGU133A 2.0 GeneChips and data were analysed using the oneChannelGUI Bioconductor package (Sanges *et al.,* 2007). Specifically, we compared ectopic endometrium and eutopic endometrium versus the healthy samples (see materials and methods). The complexity of the data set was reduced removing the non-significant probe sets (i.e. those not expressed and those not changing).

The complexity of the data set was reduced removing the non-significant probe sets (i.e. those not expressed and those not changing). Filtering procedures were used to reduce microarray data complexity allowing to improvement of the statistical power of the dataset. In our analysis the number of genes evaluated was reduced by applying an interquartile filter (IQR>0.25) retaining probe sets with expression signal ≥log2(100)=6.64 in at least 25% of the arrays (von Heydebreck et al., 2004). This filtering procedure resulted especially useful in the case of genome-wide arrays as often a minor of all genes are expressed at all in the cell type under consideration and allowed to reduce the initial set of 54675 probe sets to 10420 probes sets. Differential expression between ectopic and eutopic endometrium was detected using Rank Product (Breitling et al., 2004). Using a false positive predictions threshold < 0.05 we detected a total of 1429 probe sets that were used for further analysis.

ICC (Parmigiani et al., 2004) was used to identify the group of probe sets characterised by a divergent behaviour between ectopic and eutopic samples. With respect to other statistics ICC offers the possibility to detect the differences between the two tissues limiting the risk of underestimating gene expression differences that could due to high sample variance and low numerosity of the dataset.

Specifically, ICC for ectopic and eutopic endometrium versus the control endometrium was calculated and the most divergent probe sets were selected (Figure 1, Supplemental Table 1).

From the above-mentioned filtering approach a total of 150 genes (179 probe sets) resulted with a clear opposite behaviour between ectopic and eutopic samples (Figure 2). These 150 genes were filtered using a 1.2 fold change cut-off in order to eliminate non-significant genes. The resulting 120 genes were functionally classified through an IPA

Core Analysis (IPA7.0, Ingenuity System®). IPA associates molecules under analysis with known biological functions and diseases. The link between a category and the associated molecules is defined by a p-value. In general more molecules are involved more likely the association is not due to random chance, and thus more significant is the p-value. The analysis showed that most of our 120 genes had a strong association with Tissue and Organ Development, as functional category, and with Reproductive System disease, as main disorder category.

The molecules of our dataset associated to these biological functions were analyzed to select genes potentially involved in developmental processes and deregulated in ectopic endometrium. IPA functional categories describe the link between a category and the corresponding molecules involved and, in general, more molecules are involved more likely the association is not due to random chance, and thus more significant is the p-value. Interestingly the p-value levels of the categories reported in Table 3 indicate a strong likelihood of a tight association with the molecules of our dataset. Furthermore most of them showed an expression concordant with the IPA predicted activation state.

A detailed analysis of the molecules belonging to the categories listed in Table 3 allowed us to obtain a smaller list of genes involved in gonad organogenesis or in wound healing processes common to all of them (Table 4).

To find functional connections among these genes we performed an IPA pathway analysis, that adding relationship between molecules that met different criteria (type of molecules, tissues and cells, diseases etc.). As shown in Figure 3 all these genes were connected by a functional relationship.

To independently validate the transcriptional signatures, the expression of some representative genes was confirmed using quantitative real-time polymerase chain reaction (qRT-PCR) (Table 4).

The differential gene expression defined by the microarray analysis is independent from the phase of the menstrual phase

The genes validated by qRT-PCR are all associated to gonad development and tissue remodeling, so their deregulation might be associated, during the migration phase, to the erroneous localization of the cells that will be involved in uterus formation thus generating the ectopic endometrium.

To support this hypothesis we analysed the expression of most of the selected genes (Figure 3) in all the samples (Table 1) comparing the expression between the eutopic and ectopic and confirmed their expression levels. In addition, to assess a relationship between differential expression in ectopic and eutopic samples, we performed an agglomerative hierarchical clustering that separates the ectopic from the eutopic and clearly shows that the expression values did not correlate with the menstrual phase. (Figure 4).

Finally, we performed immunohistochemical analysis in order to detect protein expression of the selected genes in the endometriotic tissues in all menstrual phases; in detail, we selected 5 patients for each phase of the menstrual cycle, having a total of 15 patients analysed. Indeed, we were able to demonstrate the expression of all the selected genes in the endometriotic tissues at the different menstrual phases (both in the epithelial and in the stromal compartment). In Figure 5, some representative immunostainings are depicted. Interestingly, when we compared the expression levels of these proteins in the eutopic endometrium with respect to the ectopic endometrium, we found that all the proteins were significantly higher expressed in the endometriotic lesions, confirming the data obtained at transcriptional level.

Discussion

Endometriosis is defined as the presence of endometrial glands and stroma at extra-uterine sites. It is a multi-factorial disorder responsible for infertility and pelvic pain that affects approximately 2%–10% of women of reproductive age (Giudice, 2010). The multi-factorial aetiology allows taking into consideration different theories on its pathogenesis, that is currently still not completely understood.

In the last years, genome wide analysis based on microarrays have provided a powerful method to analyse simultaneously expression levels of thousands of genes. This approach has mainly been used in endometriosis studies, to identify differentially expressed gene in ectopic endometrium versus the corresponding eutopic counterpart or the healthy endometrium. The majority of these studies aimed to identify molecular biomarkers useful for non-invasive diagnosis or for development of new-targeted therapies. These papers were often referred to ovarian endometriosis samples or performed to detect genes involved in the implantation failure correlated to endometriosis. Nevertheless, several endometriosis studies based on microarray analysis have used

samples related to menstrual cycle phase (Burney et al., 2007; Eyster et al., 2007) or eutopic endometrium from affected and unaffected women (Burney et al., 2007; Sha et al., 2007). To the best of our knowledge, very few studies compared ectopic and eutopic lesions analyzing only some of the different cell type contained in the lesion (Matsuzaki et al., 2006; Wu et al., 2006).

Recent findings reported by us demonstrated the presence of ectopic endometrium in female fetus (Signorile *et al.,* 2009b; Signorile *et al.,* 2010b; Signorile *et al.,* 2012a) and the possibility to generate an endometriosis-like phenotype in mice, after pre-natal exposition to the SERM bisphenol (Signorile *et al.,* 2010c), thus suggesting that etiology could be due to embryogenesis defects. Furthermore, we have also demonstrated that pre-natal exposition to bisphenol was able to cause a severe impairment of the ovaries in term of number of primordial and developing follicles (Signorile *et al.,* 2012b). Interestingly, the observation that in our experimental setting, the effects on the ovarian follicles were variable and not all the treated animals displayed an endometriosis-like phenotype, further sustains the hypothesis of a complicated mechanism underlying the effects of SERMs, that includes genetic-epigenetic-neuroendocrine pathways.

To support molecularly this theory and to achieve new insights into the pathogenesis of this multifaceted disease, we have analyzed the gene expression patterns of the ectopic endometrium. This study has analyzed deep endometriotic lesions and their eutopic counterpart in comparison with normal endometrium applying a methodology, the integrative correlation (Parmigiani et al., 2004), which is particularly suitable to identify dissimilarities between transcription profiles. Subsequently, we observed that *Tissue Development* and *Organism Development* were the main biological categories associated to genes depicted by ICC method. Genes showing a different expression in ectopic endometrium were further analysed with the aim to detect molecules involved in gonad organogenesis. Their expression could support the hypothesis that endometriosis might be originated by alterations in the fine-tuning of the female genital structures organogenesis.

A small list of genes associated to these specific functions was generated and the expression was validated (Table 4) by q-PCR and immunohistochemistry both in ectopic and eutopic endometria. Interestingly their expression was not related to the samples menstrual cycle phase.

Among the validated genes some are involved in developmental processes. LEFTY2, and BMP4 are two members of the transforming growth factor-beta (TGF-beta) super-family that includes growth and differentiation proteins (Hinck, 2012). LEFTY2, is a gene

requested in the vertebrate development for a correct left-right embryonic body plan, determining the right asymmetrical position of the internal organs (Hamada *et al.,* 2002). It is expressed in the endometrium, where it has a role in the remodelling of the extracellular matrix (Cornet *et al.,* 2002). LEFTY2 has also been associated to infertility, since its incorrect expression can block the decidualization process (Tang *et al.,* 2010).

BMP4 regulates different developmental processes and has a role at various differentiation stages. It is involved in the gonadal development promoting the formation of the precursor of gametes, the primordial germ cells (Ying and Zhao, 2001). The BMP4 deregulation is particularly intriguing since it has never associated before to endometriosis. BMP4 is involved in the vagina differentiation from the Müllerian duct: a process that consists in the invagination of the coelomic epithelium followed by an elongation step. BMP4 expression, indeed, increases in the vagina primordium where it acts inducing the mesoderm-Müllerian duct differentiation (Cai, 2009).

GREM1 instead belongs to the BMP antagonist family, that acts binding the BMP receptors (Walsh *et al.,* 2010). The incorrect expression ratio between BMP and BMP antagonists has been associated to different diseases. Deregulated expression of GREM1 was associated to osteoarthritis progression and it has been shown to be up-regulated in different tumours. Also the expression of GREM1 was reported to show higher levels in serum in women affected with endometriosis (Sha et al., 2009). Interestingly during the development GREM1 is requested for the mesenchimal-metanephric transition that is the initial step of the ureter growth (Michos *et al.,* 2004). Considering the fact that in congenital urogenital anomalies, frequently ureteric ectopia is associated to uterus abnormalities, it could be possible to hypothesize a role for GREM1 also in the correct Müllerian duct formation (Michos et al., 2004).

FST (Follistatin) is another BMP antagonist originally known for its role in inhibiting activin A, a TGF beta member, in the ovary. It has been reported to have different biological role being involved not only in follicle maturation but also in muscle development (Matzuk *et al.,* 1995) and in wound healing process (Wankell *et al.,* 2001). Recent studies have reported an up-regulation of FST in ovarian endometriosis and the possibility to consider it as a diagnostic bio-marker (Florio *et al.,* 2009).

Other genes in the pathway are linked - directly or indirectly - to tissue remodelling processes. THBS2 and THBS1 belong to the same class of glycoprotein with a role in cell-cell interaction. They are involved in wound healing and inflammation, resulting up-regulated only in tissues with cyclical remodelling. Mice mutants for both genes have been shown to have delayed healing processes (Agah *et al.,* 2002).

SERPINE1 and SERPINE2 belong to the serine protease inhibitor (SERPIN) family that are inhibitors of tissue plasminogen activator and are involved in tissue remodelling. SERPINE1 expression was found to be increased in ovarian cancer in women with concomitant endometriosis with a role in the invasion and metastasis (Komiyama *et al.,* 2011). SERPINE2 has been reported up-regulated in human endometrium mainly in the secretory phase suggesting a role in implantation (Lee *et al.,* 2011).

Finally, we investigated the presence of other functional categories already associated to endometriosis such as inflammation, cell proliferation and tissue remodelling (Aghajanova and Giudice, 2011; Eyster et al., 2007; Hull et al., 2008). Immunological factors and angiogenesis play an important role in the pathogenesis of endometriosis characterized by alterations in the function of peritoneal macrophages and other cells of the immune system (natural killer cells and lymphocytes). The increased activation of the peritoneal macrophages is also accompanied by the production of various growth factors that stimulate endometrial cell proliferation and enhance the remodelling and regeneration of ectopic endometrium. Inflammation can promote tissue remodelling in the healing phase through the activation of several molecules as integrins, ECM (extra cellular matrix) and cytoskeleton proteins. In agreement with other studies, we found deregulation of molecules related to inflammation as prostaglandin synthase, interleukin receptor and chemokines and to cell proliferation such as fibroblast growth factors and their relative receptors.

We also checked for the concordance of our data with the two previous studies that analysed paired eutopic and ectopic samples either from ovarian endometriosis or from peritoneal endometriosis (Eyster et al., 2007; Hull et al., 2008). Overall, our results were concordant with both studies at least for gene function since in a comparison analysis we found "*top disease*" functions related to Inflammatory Response, Reproductive System Disease and Cancer (data not shown). Comparing our data with these studies, we were able to find a similar gene dis-regulation, at least for the molecules associated to the cyclic cellular processes of endometriosis and to the main functional categories already associated to the disease such as cell proliferation, inflammation and tissue remodelling.

From all the three studies, it is evident that endometriosis involves an enhanced immune response and several pathways related to the growth and maintenance of endometrium in the ectopic sites.

The novelty of our study is that we have evidenced some molecules potentially involved in the pathology onset, and expressed independently from the usual hormonal stimuli in the ectopic tissue.

Conclusion

The results of this analysis add another small piece contributing to the definition of the complex puzzle, that is the pathogenesis of endometriosis. The fact that several genes involved in embryogenesis are differentially expressed in endometriotic tissues and that this expression pattern is independent from the menstrual and hormonal phase, supports the hypothesis that endometriosis might be generated by a remodelling in gene expression during embryogenesis, at least in part due to the action of SERM substances, that causes an alteration of the fine-tuning of the female genital structures organogenesis. This is substantially in accordance with the recent finding of foetal endometriosis in women and with the reported ability of bisphenol, when administrated pre-natally, to induce an endometriosis-like phenotype in a murine model.

Acknowledgements

This work was supported by Fondazione Italiana Endometriosi. M.T.P. was supported by an I.N.B.B. fellowship. We thank Anna Maria Aliperti for help in the revision of the manuscript.

REFERENCES

Agah A, Kyriakides TR, Lawler J, Bornstein P. 2002. The lack of thrombospondin-1 (TSP1) dictates the course of wound healing in double-TSP1/TSP2-null mice. Am J Pathol 161(3):831-839.

Aghajanova L, Giudice LC. 2011. Molecular evidence for differences in endometrium in severe versus mild endometriosis. Reprod Sci 18(3):229-251.

Baldi A, Campioni M, Signorile PG. 2008. Endometriosis: pathogenesis, diagnosis, therapy and association with cancer (review). Oncol Rep 19(4):843-846.

Bolstad BM, Irizarry RA, Astrand M, Speed TP. 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19(2):185-193.

Breitling R, Armengaud P, Amtmann A, Herzyk P. 2004. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Lett 573(1-3):83-92.

Bulun SE. 2009. Endometriosis. N Engl J Med 360(3):268-279.

Burney RO, Talbi S, Hamilton AE, Vo KC, Nyegaard M, Nezhat CR, Lessey BA, Giudice LC. 2007. Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis. Endocrinology 148(8):3814-3826.

Cai Y. 2009. Revisiting old vaginal topics: conversion of the Mullerian vagina and origin of the "sinus" vagina. Int J Dev Biol 53(7):925-934.

Chan RW, Schwab KE, Gargett CE. 2004. Clonogenicity of human endometrial epithelial and stromal cells. Biol Reprod 70(6):1738-1750.

Cornet PB, Picquet C, Lemoine P, Osteen KG, Bruner-Tran KL, Tabibzadeh S, Courtoy PJ, Eeckhout Y, Marbaix E, Henriet P. 2002. Regulation and function of LEFTY-A/EBAF in the human endometrium. mRNA expression during the menstrual cycle, control by progesterone, and effect on matrix metalloprotineases. J Biol Chem 277(45):42496-42504.

Crispi S, Calogero RA, Santini M, Mellone P, Vincenzi B, Citro G, Vicidomini G, Fasano S, Meccariello R, Cobellis G, Menegozzo S, Pierantoni R, Facciolo F, Baldi A, Menegozzo M. 2009. Global gene expression profiling of human pleural mesotheliomas: identification of matrix metalloproteinase 14 (MMP-14) as potential tumour target. PLoS One 4(9):e7016.

Du H, Taylor HS. 2007. Contribution of bone marrow-derived stem cells to endometrium and endometriosis. Stem Cells 25(8):2082-2086.

Eyster KM, Klinkova O, Kennedy V, Hansen KA. 2007. Whole genome deoxyribonucleic acid microarray analysis of gene expression in ectopic versus eutopic endometrium. Fertil Steril 88(6):1505-1533.

Florio P, Reis FM, Torres PB, Calonaci F, Abrao MS, Nascimento LL, Franchini M, Cianferoni L, Petraglia F. 2009. High serum follistatin levels in women with ovarian endometriosis. Hum Reprod 24(10):2600-2606.

Giudice LC. 2010. Clinical practice. Endometriosis. N Engl J Med 362(25):2389-2398.

Hamada H, Meno C, Watanabe D, Saijoh Y. 2002. Establishment of vertebrate left-right asymmetry. Nat Rev Genet 3(2):103-113.

Hinck AP. 2012. Structural studies of the TGF-betas and their receptors - insights into evolution of the TGF-beta superfamily. FEBS Lett.

Hull ML, Escareno CR, Godsland JM, Doig JR, Johnson CM, Phillips SC, Smith SK, Tavare S, Print CG, Charnock-Jones DS. 2008. Endometrial-peritoneal interactions during endometriotic lesion establishment. Am J Pathol 173(3):700-715.

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. 2003. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res 31(4):e15.

Komiyama S, Aoki D, Saitoh E, Komiyama M, Udagawa Y. 2011. Biological significance of plasminogen activator inhibitor-1 expression in ovarian clear cell adenocarcinoma. Eur J Gynaecol Oncol 32:611-614.

Lee RK, Fan CC, Hwu YM, Lu CH, Lin MH, Chen YJ, Li SH. 2011. SERPINE2, an inhibitor of plasminogen activators, is highly expressed in the human endometrium during the secretory phase. Reprod Biol Endocrinol 9:38.

Matsuura K, Ohtake H, Katabuchi H, Okamura H. 1999. Coelomic metaplasia theory of endometriosis: evidence from in vivo studies and an in vitro experimental model. Gynecol Obstet Invest 47 Suppl 1:18-20; discussion 20-12.

Matsuzaki S, Canis M, Pouly JL, Botchorishvili R, Dechelotte PJ, Mage G. 2006. Differential expression of genes in eutopic and ectopic endometrium from patients with ovarian endometriosis. Fertil Steril 86(3):548-553.

Matzuk MM, Lu N, Vogel H, Sellheyer K, Roop DR, Bradley A. 1995. Multiple defects and perinatal death in mice deficient in follistatin. Nature 374(6520):360-363.

Michos O, Panman L, Vintersten K, Beier K, Zeller R, Zuniga A. 2004. Gremlin-mediated BMP antagonism induces the epithelial-mesenchymal feedback signaling controlling metanephric kidney and limb organogenesis. Development 131(14):3401-3410.

Parmigiani G, Garrett-Mayer ES, Anbazhagan R, Gabrielson E. 2004. A cross-study comparison of gene expression studies for the molecular classification of lung cancer. Clin Cancer Res 10(9):2922-2927.

Sampson JA. 1927. Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation. Am J Pathol 3(2):93-110 143.

Sanges R, Cordero F, Calogero RA. 2007. oneChannelGUI: a graphical interface to Bioconductor tools, designed for life scientists who are not familiar with R language. Bioinformatics 23(24):3406-3408.

Sha G, Wu D, Zhang L, Chen X, Lei M, Sun H, Lin S, Lang J. 2007. Differentially expressed genes in human endometrial endothelial cells derived from eutopic endometrium of patients with endometriosis compared with those from patients without endometriosis. Hum Reprod 22(12):3159-3169.

Sha G, Zhang Y, Zhang C, Wan Y, Zhao Z, Li C, Lang J. 2009. Elevated levels of gremlin-1 in eutopic endometrium and peripheral serum in patients with endometriosis. Fertil Steril 91(2):350-358.

Signorile PG, Campioni M, Vincenzi B, D'Avino A, Baldi A. 2009a. Rectovaginal septum endometriosis: an immunohistochemical analysis of 62 cases. In Vivo 23(3):459-464.

Signorile PG, Baldi F, Bussani R, D'Armiento M, De Falco M, Baldi A. 2009b. Ectopic endometrium in human foetuses is a common event and sustains the theory of mullerianosis in the pathogenesis of endometriosis, a disease that predisposes to cancer. J Exp Clin Cancer Res 28: 49.

Signorile PG, Baldi A. 2010a. Endometriosis: new concepts in the pathogenesis. Int J Biochem Cell Biol 42: 778-780.

Signorile PG, Baldi F, Bussani R, D'Armiento M, De Falco M, Boccellino M, Quagliuolo L, Baldi A. 2010b. New evidence of the presence of endometriosis in the human fetus. Reprod Biomed Online 21: 142-147.

Signorile PG, Spugnini EP, Mita L, Mellone P, D'Avino A, Bianco M, Diano N, Caputo L, Rea F, Viceconte R, Portaccio M, Viggiano E, Citro G, Pierantoni R, Sica V, Vincenzi B, Mita DG, Baldi F, Baldi A. 2010c. Pre-natal exposure of mice to bisphenol A elicits an endometriosis-like phenotype in female offspring.Gen Comp Endocrinol 168: 318-325

Signorile PG, Baldi F, Bussani R, Viceconte R, Bulzomi P, D'Armiento M, D'Avino A, Baldi A. 2012a. Embryologic origin of endometriosis: analysis of 101 human female fetuses. J Cell Physiol 227: 1653-1656.

Signorile PG, Spugnini EP, Citro G, Viceconte R, Vincenzi B, Baldi F, Baldi A 2012b. Endocrine disruptors in utero cause ovarian damages linked to endometriosis. Frontiers in Bioscience 4:1724-1730.

Tang M, Naidu D, Hearing P, Handwerger S, Tabibzadeh S. 2010. LEFTY, a member of the transforming growth factor-beta superfamily, inhibits uterine stromal cell differentiation: a novel autocrine role. Endocrinology 151(3):1320-1330.

von Heydebreck A, Huber W, Gentleman RC. 2004. Differential expression of the Bioconductor Project. Bioconductor Project Working Papers Working Paper 7.

Walsh DW, Godson C, Brazil DP, Martin F. 2010. Extracellular BMP-antagonist regulation in development and disease: tied up in knots. Trends Cell Biol 20(5):244-256.

Wankell M, Munz B, Hubner G, Hans W, Wolf E, Goppelt A, Werner S. 2001. Impaired wound healing in transgenic mice overexpressing the activin antagonist follistatin in the epidermis. Embo J 20(19):5361-5372.

Wu Y, Kajdacsy-Balla A, Strawn E, Basir Z, Halverson G, Jailwala P, Wang Y, Wang X, Ghosh S, Guo SW. 2006. Transcriptional characterizations of differences between eutopic and ectopic endometrium. Endocrinology 147(1):232-246.

Ying Y, Zhao GQ. 2001. Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse. Dev Biol 232(2):484-492.

Figure Legends

Figure 1. Integrative correlation analysis. ICC of ectopic versus normal and eutopic versus normal are plotted. Genes shown in red are those showing the greater dissimilarity between ectopic and eutopic (see Supplemental Table 1).

Figure 2. Hierarchical agglomerative clustering of the transcripts showing a divergent behaviour between ectopic and eutopic samples. For each gene the expression (red up-regulated, green down-regulated) is shown.

Figure 3. Functional relationship between deregulated genes belonging to the tissue and organ development categories. For each gene the relationship and the expression (red up-regulated) are shown. Arrows indicate the direction of the relationship.

Figure 4. Hierarchical agglomerative clustering of gene expression levels in the ectopic and eutopic samples at different cycle phase. For each gene the expression (red up-regulated, green down-regulated) is shown. Numbers are referred to the different phases: 1-13 proliferative; 14-19 secretive; 20-27 menstrual. Samples were statistical analyzed using a one-way ANOVA ($P<0.0001$) to confirm differential expression.

Figure 5. Representative immunohistochemistry staining of Serpin (A), BMP4 (B), Follistatin (C) and Thrombospondin 2 (D) in ectopic endometrium tissue. Samples were statistical analysed using a one-way ANOVA ($P<0.0001$) to confirm differential expression. (ABC, original magnification X 20).

Table 1. Patient clinical sample characteristics

Case no.	Age (years)	rAFS stage	Menstrual phase	Array	q-PCR	IHC
1	46	IV	proliferative		X	
2	29	IV	proliferative		X	
3	22	II	proliferative		X	
4	31	IV	proliferative		X	
5	39	IV	proliferative		-	
6	31	IV	proliferative	X	X	X
7	30	IV	proliferative	X	X	X
8	32	II	proliferative	X	X	X
9	35	IV	proliferative	X	X	X
10	30	IV	proliferative	X	X	X
11	30	IV	proliferative	X	X	
12	22	IV	proliferative	X	-	
13	42	II	proliferative	X	X	
14	34	IV	secretive		-	X
15	31	IV	secretive		X	X
16	35	IV	secretive		X	X
17	30	IV	secretive		X	X
18	32	IV	secretive		-	X
19	33	IV	secretive		X	
20	33	IV	menstrual		X	X
21	36	IV	menstrual		X	X
22	36	IV	menstrual		X	X
23	34	III	menstrual		X	X
24	38	II	menstrual		X	X
25	46	IV	menstrual		X	
26	34	IV	menstrual		X	
27	41	IV	menstrual		X	

Case no., paired ectopic and eutopic samples
rAF, Revised American Fertility Society
q-PRC, real time validation
IHC, Immunohistochemistry

Table 2. RT-PCR primer sequences and exon junction of the related target genes

BMP4	Forward	ACCACGAAGAACATCTGGAGAAC	3-4
	Reverse	AGGTTAAAGAGGAAACGAAAAGCA	
FST	Forward	TGCATCCCCTGTAAAGAAACG	2-3
	Reverse	TGTTCATTCGGCATTTTTTCC	
GREM1	Forward	GACAAGGCCCTGCATGTGA	1-2
	Reverse	GCTGGGTTTTGCACCAGTCT	
LEFTY2	Forward	TATGGAGCTCAGGGCGACTGTGACCCTGAAGCACCAA	4-5
	Reverse	CCCACTTCATCCCCTGCAGGTCAATGTACATCTC	
SERP2	Forward	AAAATAACAACAGGGTCAGAAAACCT	6-7
	Reverse	TCTTCACTGACTTCAATTTTTGCTTT	
THBS2	Forward	AGACGGAAAAGCAAGTGTGTGA	13-14
	Reverse	TGTGGCAGTTGTGTGTCTTGTC	
GAPDH	Forward	GGAGTCAACGGATTTGGTCGTA	2-3
	Reverse	GAATTTGCCATGGGTGGAAT	

Table 3. Top bio functions related to ectopic deregulated genes

Category	Functions Annotation	p-Value	Predicted Activation State	Molecules
Tissue Development	tissue development	8,44E-12	Increased	61
	development of organ	2,95E-09	Increased	44
Cellular Development	differentiation	4,75E-10	Increased	49
	differentiation of cells	5,29E-10	Increased	47
Organ Development	development of organ	2,95E-09	Increased	44
Organismal Development	development of organ	2,95E-09	Increased	44
Embryonic Development	development of organ	2,95E-09	Increased	44

Table 4. Common genes belonging to the selected top functional categories

Gene	Description	Fold Change	Pathway associated	q-PCR	IHC
BMP4	bone morphogenetic protein 4	1.217	Yes	Yes	Yes
EGR3	early growth response 3	1.183	No	-	-
FST	follistatin	1.300	Yes	Yes	Yes
FGF7	fibroblast growth factor 7	1.959	Yes	-	-
GATA6	GATA binding protein 6	4.556	No	-	-
GREM1	gremlin 1	2.213	Yes	Yes	-
LEFTY	left-right determination factor 2	1.998	Yes	Yes	-
SERPINE1	serpin peptidase inhibitor, member 1	1.405	Yes	-	-
SERPINE2	serpin peptidase inhibitor, member 2	1.215	Yes	Yes	Yes
THBS1	thrombospondin 1	1.391	Yes	-	-
THBS2	thrombospondin 2	1.327	Yes	Yes	Yes

q-PCR, real time validation

IHC, Immunohistochemistry

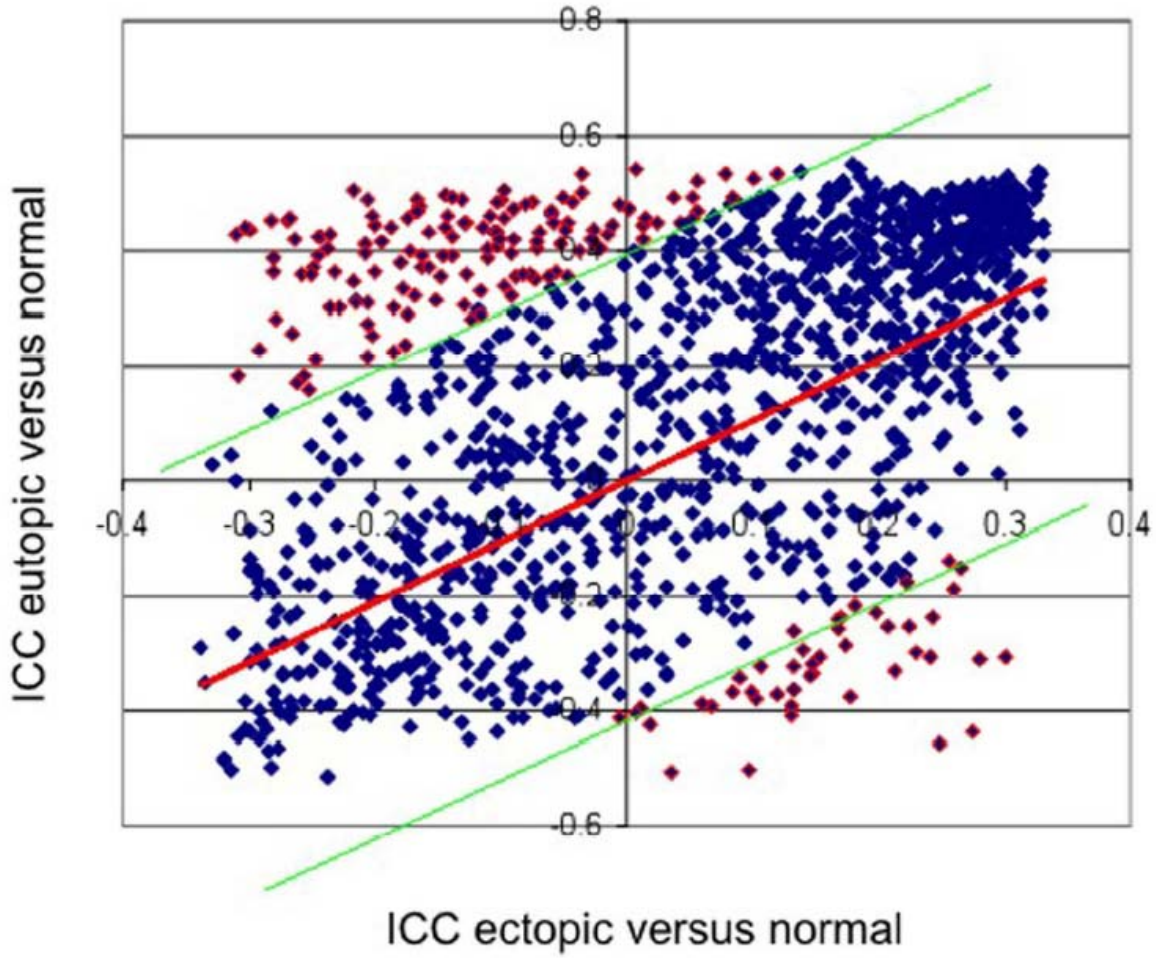

Figure 1

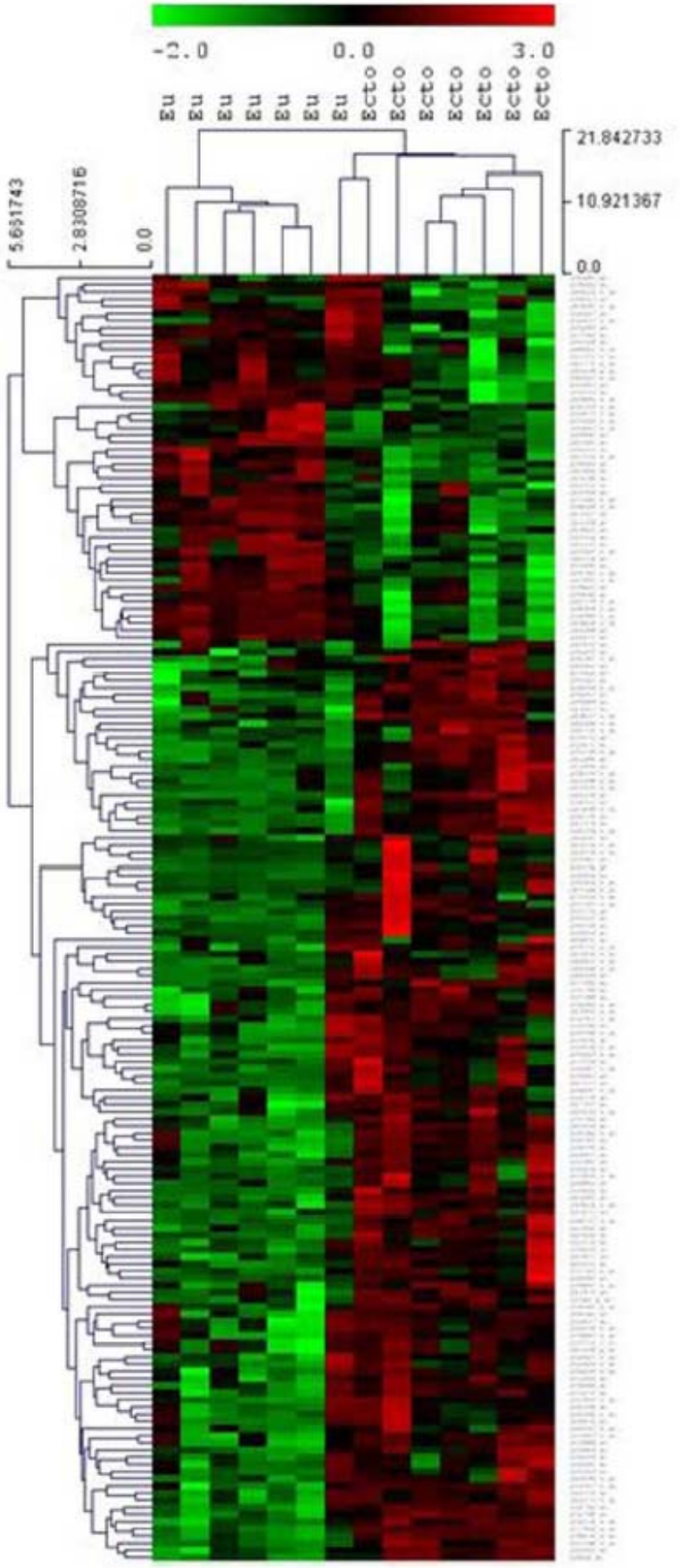

Figure 2

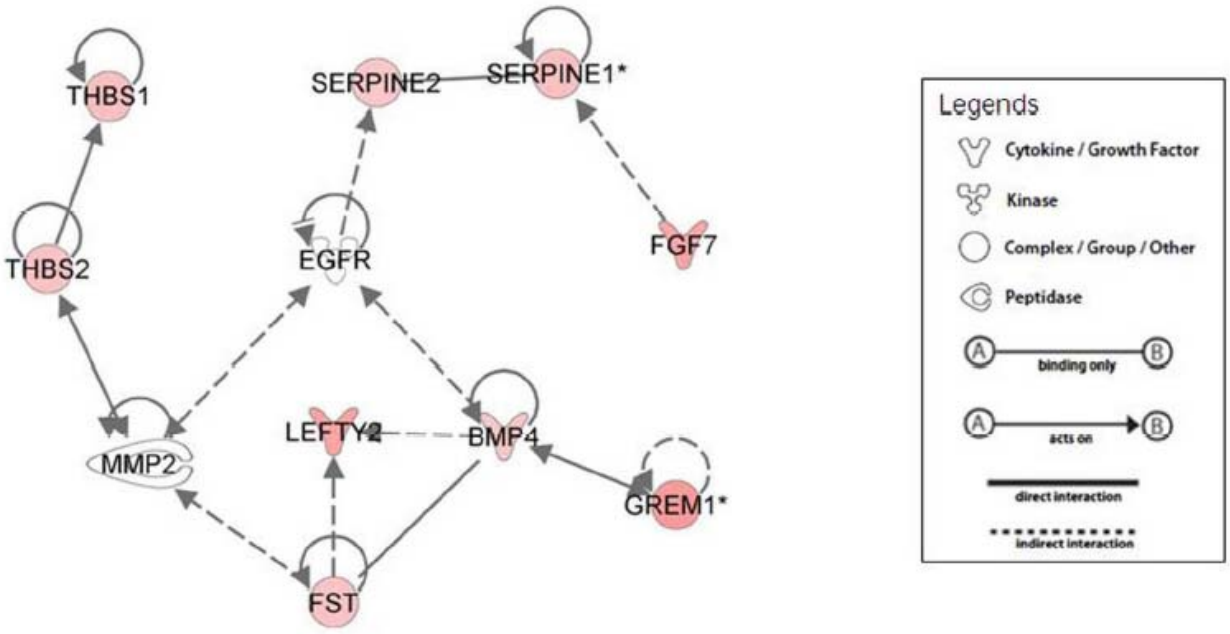

Figure 3

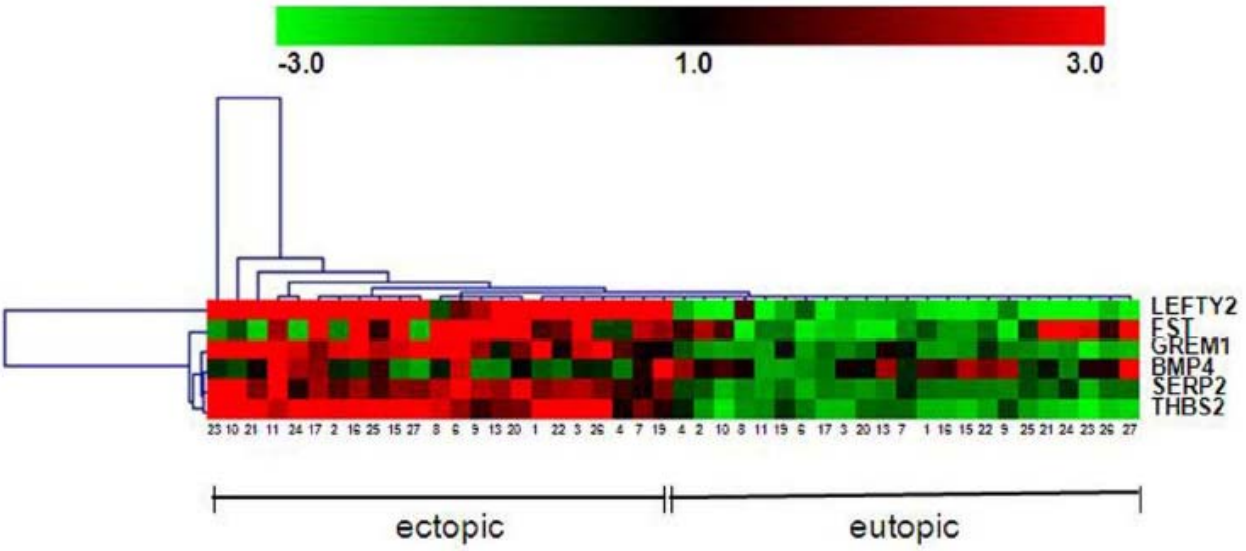

Figure 4

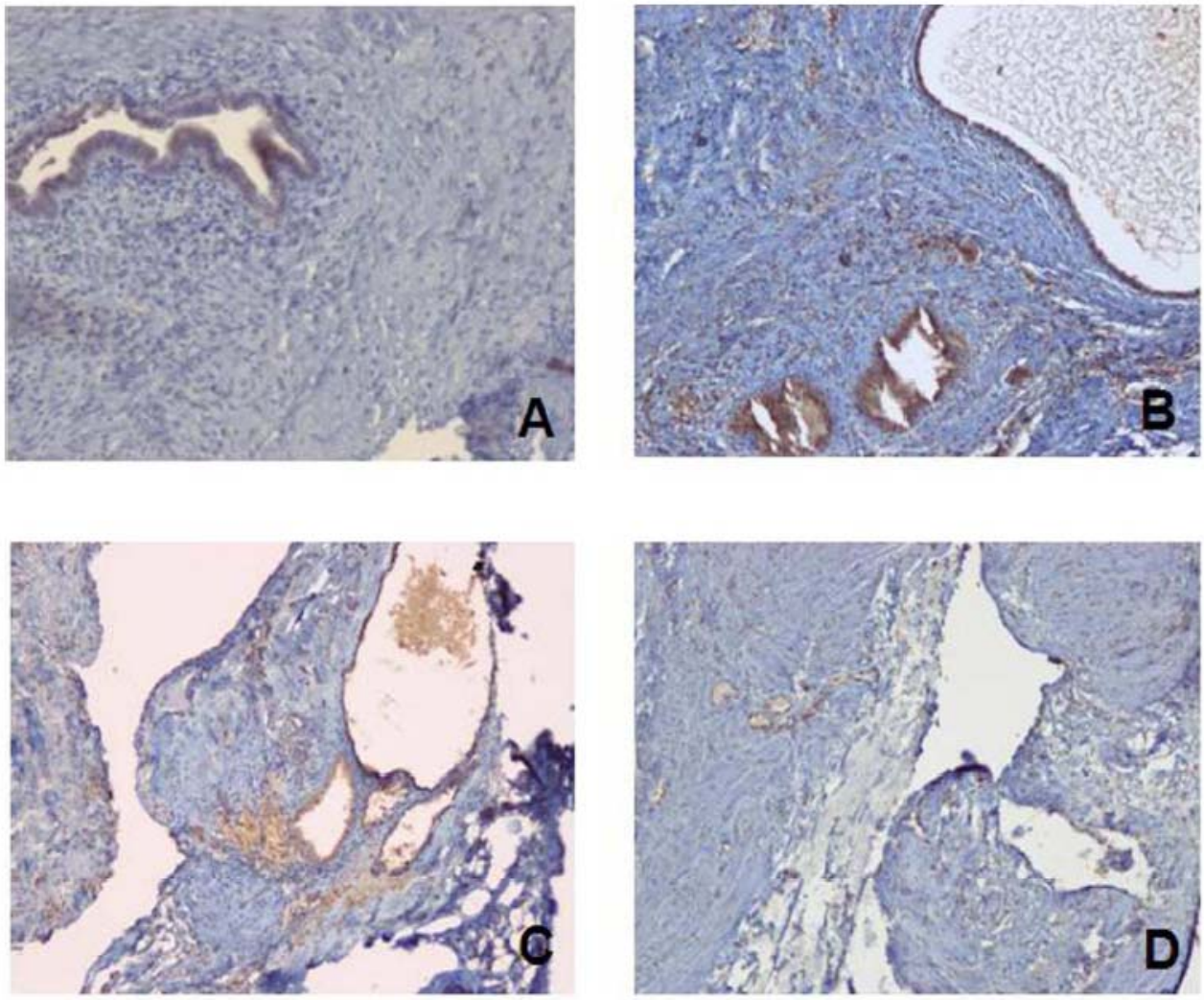

Figure 5