

Expression Pattern of Stemness-Related Genes in Human Endometrial and Endometriotic Tissues

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Endometriosis is a chronic disease characterized by the presence of ectopic endometrial tissue outside of the uterus with mixed traits of benign and malignant pathology. In this study we analyzed in endometrial and endometriotic tissues the differential expression of a panel of genes that are involved in preservation of stemness status and consequently considered as markers of stem cell presence. The expression profiles of a panel of 13 genes (*SOX2*, *SOX15*, *ERAS*, *SALL4*, *OCT4*, *NANOG*, *UTF1*, *DPPA2*, *BMI1*, *GDF3*, *ZFP42*, *KLF4*, *TCL1*) were analyzed by reverse transcription–polymerase chain reaction in human endometriotic ($n = 12$) and endometrial samples ($n = 14$). The expression of *SALL4* and *OCT4* was further analyzed by immunohistochemical methods. Genes *UTF1*, *TCL1*, and *ZFP42* showed a trend for higher frequency of expression in endometriosis than in endometrium ($P < 0.05$ for *UTF1*), whereas *GDF3* showed a higher frequency of expression in endometrial samples. Immunohistochemical analysis revealed that *SALL4* was expressed in endometriotic samples but not in endometrium samples, despite the expression of the corresponding mRNA in both the sample groups. This study highlights a differential expression of stemness-related genes in ectopic and eutopic endometrium and suggests a possible role of *SALL4*-positive cells in the pathogenesis of endometriosis.

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Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2009.00068

INTRODUCTION

Human endometrium undergoes cyclical processes of growth, differentiation, shedding, and regeneration as part of the menstrual cycle during the reproductive life of women (1).

Endometriosis is a multifactorial estrogen-dependent disease that affects 5% to 10% of women of reproductive age in the Western countries. Its defining feature is the presence of endometrium-like tissue in sites outside the uterine cavity, primarily on the pelvic peritoneum and ovaries (2).

Endometriosis can originate from anatomical or biochemical aberrations of uterine function. Theories on the histogenesis of endometriosis belong to five categories: coelomic metaplasia, retro-

grade menstruation, embryonic cell rest, induction, and lymphatic and vascular dissemination (3).

Many studies thus far have focused on the biomolecular and cellular characteristics of endometriosis compared to endometrium and with the possible molecular mechanisms at the basis of the development of endometriotic lesions. Among these investigations, of particular interest is a recent analysis revealing a list of 22 microRNAs differentially expressed in paired ectopic and eutopic endometrial tissues, which could contribute to endometriosis progression through their cognate target mRNAs (4). Other studies highlighted a differential expression of the genes *SF1* and estrogen receptor beta in endometriotic tissue compared with

endometrium. Results indicated that expression was primarily controlled by a methylation-dependent epigenetic mechanism (5,6). In addition, various chromosomal aberrations have been reported in endometriotic samples and in ovarian carcinoma (7).

Differences in stromal cell migration, inflammatory markers, and other pathways between eutopic and ectopic endometrial tissues have been also highlighted (8).

It should also be mentioned that endometriosis may have a genetic basis, because its incidence in relatives of affected women is much higher than the incidence in women without a family history (9).

Stem cells are increasingly becoming the focus of many areas of biomedical research. Stem cells are rare undifferentiated cells present in virtually all adult tissues and organs. These cells retain high proliferative, self-renewal, and differentiation potential. The number of stem cells in adult tissues is actively regulated through a strict balance between cell proliferation, cell differentiation, and cell death (10). Recent studies revealed the presence of

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Submitted May 27, 2009; Accepted for publication August 10, 2009; Epub (www.molmed.org) ahead of print August 10, 2009.

adult stem cells in endometrium. In particular, work by Chan *et al.* (11) revealed clonogenic stromal and epithelial cells in human endometrium, possibly indicating the presence of stem cells.

Results of two studies (12,13) using the label-retaining cell approach suggested the presence of stem cells in murine endometrium. Other studies, also conducted in murine models, demonstrated that stem cells in endometrium derive from bone marrow (14).

The presence of stem cells in endometrium has been demonstrated mainly through the analysis of their surface markers, their clonogenic properties, and their differentiation ability.

Endometriosis can evolve into ovarian cancer (3,15) and other malignant diseases in which stem cells could play a role, as recently demonstrated (16). The relationship of endometriosis and ovarian cancer has been demonstrated both by epidemiological studies and by common genetic alterations (3).

Studies on transcriptional profiling of stem cells allowed a preliminary identification of stemness-related genes actively involved in the control of stem cell properties, such as self-renewal ability and retention of an uncommitted state. Initially, genes that control stemness were identified in embryonic stem cells (17,18). In adult stem cells, some embryonal stemness genes are not expressed.

In this study we aimed to detect the expression of a panel of 13 genes considered as stem cell markers in eutopic endometrium and in endometriotic tissue, through analysis at the mRNA level for all the 13 genes and verification of the data at the protein level for 2 of them. The 13 genes were selected on the basis of data reported in the currently available literature.

Among these genes, *BMI1* (*BMI1 polycomb ring finger oncogene*) plays a central role in the inheritance of stemness. *BMI1* belongs to the polycomb group (PcG) genes and is involved in the maintenance of cellular memory through epigenetic chromatin modifications. Recent studies have implicated a role for PcG genes in

the self-renewal of stem cells, a process in which cellular memory is maintained through cell division (19). *ERAS* (*ES cell expressed Ra*) encodes a Ras-membrane protein involved in proliferation and tumorigenicity of embryonic stem cells (20). *TCL1* (*T-cell leukemia/lymphoma 1A*) is an oncogene involved in regulation of proliferation of embryonic stem cells and is a downstream gene of *OCT4* (*POU class 5 homeobox 1* [*POU5F1*, also known as *OCT4*]) (21). *UTF1* (*undifferentiated embryonic cell transcription factor 1*) encodes a tightly DNA-associated protein with transcriptional repressor activity and is expressed in embryonic pluripotent stem cells (22). All the other genes we analyzed, including *OCT4*, *SOX2* (*SRY [sex determining region Y]-box 2*), *SOX15* (*SRY [sex determining region Y]-box 15*), *NANOG* (*Nanog homeobox*), *SALL4* (*sal-like 4*), *DPPA2* (*developmental pluripotency associated 2*), *GDF3* (*growth differentiation factor 3*), *ZFP42* (*zinc finger protein 42 homolog*), and *KLF4* (*Kruppel-like factor 4*), code for transcription factors for genes involved in the preservation of stem cell pluripotency (see also Supplementary File 1 for additional references specific for stemness-related genes).

Our results highlight the expression of stem cell markers both in endometrial and endometriotic tissues, suggesting that stem cells may play a role in disease progression.

MATERIALS AND METHODS

Patients and Samples

Clinical samples of endometrial and endometriotic tissues were collected from 26 patients (endometrial tissues from $n = 14$ patients aged 29–58 years, mean 46.9 years; endometriosis samples from $n = 12$ patients, aged 24–46 years, mean 34.4 years) at the Department of Gynaecology, Obstetrics and Reproductive Medicine of the Second University of Naples. The patients were undergoing hysterectomy, laparoscopy, or laparotomy for benign pathologies. Informed written consent was obtained from each patient. Surgery was performed irrespec-

tive of the day of the patient's menstrual cycle. The patients had never received any hormonal treatment before surgery.

After surgery, endometrial biopsies and excised ovarian endometriotic lesions were formaldehyde fixed, and hematoxylin-stained cross sections were analyzed by experienced histopathologists for assessment of the grade of endometriosis (I–IV) and for determination of the stage of the menstrual cycle (proliferative or secretory), referring to established histological criteria (23). The clinical characteristics of the patients and samples are shown in Table 1.

The samples from each patient were either snap frozen and stored at -80°C or fixed in buffered formaldehyde 4% (Sigma-Aldrich, St. Louis, MO, USA) and embedded in paraffin using standard techniques for immunohistochemical (IHC) analysis.

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from frozen tissue samples using TRIzol (Molecular Research Center, Cincinnati, OH, USA) and from paraffin-embedded tissues (RNeasy minikit; Qiagen, Valencia, CA, USA) according to manufacturer's instructions. RNA was treated with DNase I (Ambion, Austin, TX, USA) to remove DNA contamination. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA integrity was verified by electrophoresis on denaturing 1% agarose gel.

Absence of residual genomic DNA was verified by polymerase chain reaction (PCR) on total RNA without reverse transcription (RT). Genomic human DNA was used as a positive control of PCR reactions.

cDNA was generated from 200 ng of each RNA sample. RT was done at 42°C for 1 h in the presence of random primers and Moloney-murine leukemia virus reverse transcriptase (Finnzymes, Espoo, Finland). GeneBank sequences for human mRNAs *SOX2*, *SOX15*, *ERAS*, *SALL4*, *OCT4*, *NANOG*, *UTF1*, *DPPA2*, *BMI1*, *GDF3*, *ZFP42*, *KLF4*, *TCL1* and Primer Ex-

Table 1. Patient clinical characteristics.*

Case no.	Age, years	Phase of menstrual cycle	Grade of endometriosis	Pathology
Endometrium 1	58	M		Uterus fibromatosis
Endometrium 2	29	PP		Uterine myoma
Endometrium 3	53	SP		Endometrial polyp
Endometrium 4	54	SP		Uterus fibromatosis
Endometrium 5	46	SP		Uterus fibromatosis
Endometrium 6	58	M		Cystocele
Endometrium 7	52	SP		Uterus fibromatosis
Endometrium 8	43	PP		Uterine myoma
Endometrium 9	41	PP		Uterus fibromatosis
Endometrium 10	37	SP		Ovarian cyst
Endometrium 11	51	M		Endometrial polyp
Endometrium 12	41	SP		Uterine myoma
Endometrium 13	52	SP		Uterus fibromatosis
Endometrium 14	41	PP		Uterus fibromatosis
Endometriosis 1	38	SP	II	
Endometriosis 2	39	SP	III	
Endometriosis 3	44	SP	IV	
Endometriosis 4	29	SP	II	
Endometriosis 5	26	SP	III	
Endometriosis 6	28	PP	III	
Endometriosis 7	31	PP	III	
Endometriosis 8	46	SP	IV	
Endometriosis 9	24	PP	III	
Endometriosis 10	42	PP	IV	
Endometriosis 11	33	PP	III	
Endometriosis 12	33	PP	I	

*PP, proliferative phase; SP, secretory phase; M, menopause.

press software (Applied Biosystems, Foster City, CA, USA) were used to design primer pairs for the genes and the house keeping gene *GAPDH*. Primer sequences are listed in Table 2. They were chosen to yield 100–150 bp. Each PCR was repeated for 35 cycles. PCR products were validated by running the PCR products on agarose gel to confirm a single band.

Each RT-PCR reaction was repeated at least three times. A semiquantitative analysis of mRNA levels was performed by the GEL DOC UV system (Bio-Rad, Hercules, CA, USA) on agarose gels containing the GelStar nucleic acid gel stain (Lonza, Basel, Switzerland), a highly sensitive fluorescent stain able to detect as little as 20 pg of DNA, with a four–sixteen-fold increase of sensitivity compared with ethidium bromide.

To determine the lowest number of molecules of a given mRNA in a pool that can be detected by RT-PCR, it is war-

ranted to know the percentage of that mRNA in the pool. In many cases, it is not possible to determine this percentage. Consequently we established an alternative method based on serial dilutions of total RNA, ranging from 1000 ng to 1 ng, used to carry out RT-PCR to detect high- (*GAPDH*), medium- (*HPRT*) and low-expressed (*E2F2*) mRNAs after 35 cycles.

Highly expressed mRNA was detected in all experimental conditions we used in the presence of GelStar, whereas 10 ng of total RNA was the lowest quantity to detect medium- and low-expressed mRNAs.

In the RT-PCR analysis in this study we used 200 ng of total RNA and 35 cycles for amplification, far above the limit of detection of low-expressed mRNAs.

When minimal differences in gene expression were detected by PCR, experiments were repeated using the real-time PCR assays, run on an Opticon 4 machine (Bio-Rad). Reactions were performed ac-

cording to the manufacturer's instructions using the SYBR Green PCR master mix (Stratagene, La Jolla, CA, USA). Relative quantitative RT-PCR was used to determine the fold difference for genes. Melting curves (65°C–94°C) were also generated to determine whether there were any spurious amplification products. The real-time PCR efficiency was calculated for each primer pair using a dilution series and MJ Opticon II analysis software.

Immunohistochemical Analysis

Tissue samples from patients were fixed in 4% buffered formaldehyde, dehydrated, and embedded in paraffin. Consecutive 5- μ m cross sections were placed on coated slides, deparaffinized through a series of xylene and ethanol washes, and used for IHC analysis of SALL4 and OCT4 expression. We verified the IHC signal for SALL4, using sections of mouse adult testis and heart as positive and negative controls, respectively (Supplemental Figure 1). We verified the IHC signal for OCT4 using sections of mouse embryo testis (E13.5) and mouse adult heart as positive and negative controls, respectively (Supplemental Figure 2).

Antigen retrieval was obtained through incubation in citrate buffer at pH 6.0 for 10 min followed by gradual cooling at room temperature for 20 min. After 1 h incubation in blocking solution (5% bovine serum albumin and 1% donkey serum), slides were incubated overnight at 4°C with SALL4 mouse monoclonal antibody (1:100, Abnova, Walnut, CA, USA) or OCT4 rabbit polyclonal antibody (1:250, Abcam, Cambridge, UK) diluted in blocking solution, according to manufacturers' instructions. In negative controls the primary antibodies were omitted.

After being washed, slides were incubated with biotinylated antimouse or antirabbit secondary antibodies for 30 min at room temperature. The slides were then washed again and incubated with streptavidin-peroxidase (HRP) (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Finally, specific hybridization of antibodies was

Table 2. Summary of RT-PCR primer sequences, position, annealing temperature, and chromosome mapping position of the stemness-related target genes.

Gene	Primer position	Primer sequence	Annealing temperature, °C	PCR product, bp	Chromosome mapping of the gene
GAPDH	472	5'-GCATCCTGCACCACCACCTG-3'	55	347	12p13
	799	5'-GCCTGGTTCACGACGTTCTT-3'			
SOX2	1563	5'-CCATCCACACTCACGCAAAA-3'	59	139	3q27
	1701	5'-TATACAAGGTCCATTCCCCCG-3'			
OCT4	1121	5'-TCCCATGCATTCAAACCTGAGG-3'	60	103	6p21,31
	1223	5'-CCAAAAACCCTGGCACAACCT-3'			
NANOG	1169	5'-TGGACACTGGCTGAATCCTTC-3'	59	142	12p13,31
	1310	5'-CGTTGATTAGGCTCCAACCAT-3'			
KLF4	1508	5'-CTGCGGCAAAACCTACACAA-3'	60	182	9q31
	1689	5'-GGTCGCATTTTTGGCACTG-3'			
ERAS	969	5'-AATGTAGACCTTCCCCAGGC-3'	58	135	Xp11,23
	1103	5'-AAAGCCCCTCACCAAGTGAA-3'			
GDF3	778	5'-AAAAGGAAGAGCAGCCATCCCT-3'	60	110	12p13.1
	887	5'-GCAATGATCCACTTGTGCCAA-3'			
SOX15	315	5'-GAACAGGTTGGAAGCAAAGGC-3'	59	127	17p13
	441	5'-GCGTCGATCCTGAAAATGGA-3'			
DPPA2	798	5'-AGCCATGTTGGCATCATGG-3'	58	108	3q13,13
	905	5'-GAGGCTTGCAAGCAAAAAGGC-3'			
SALL4	2394	5'-GCCAGATATCCTGAAAACCA-3'	60	115	20q13,13/13,2
	250	5'-TTCTCGGAGCTCTGTGCTTG-3'			
TCL1	667	5'-CTCGGCTTTTTCTCAGCTGGAT-3'	59	127	14q32,1
	793	5'-GGTGAATCGGCTGTGTTCTCA-3'			
ZFP42	953	5'-ATGACAGTCTGAGCGCAATCG-3'	60	133	4q35,2
	1085	5'-AACGCTTTCCACATTCCG-3'			
UTF1	876	5'-CGACATCGCGAACATCCTG-3'	64	117	10q26
	992	5'-AGAATGAAGCCCACGGCCA-3'			
BMI1	437	5'-AATGTCTTTCCGCCCCGCT-3'	59	139	10p11,23
	575	5'-ACCTCCACAAAGCACACACAT-3'			

highlighted through incubation with diaminobenzidine and HRP substrate buffer (Vector). The diaminobenzidine substrate solution gives a brown precipitate at the site of the target antigen recognized by the primary antibody. Nuclei were counterstained blue with Mayer's hematoxylin (Merck, Darmstadt, Germany). Dried slides were immersed in xylene solution and coverslips applied using ultramount.

Image screening and photography of serial cross sections were performed using a Leica IM 1000 System (Leica Microsystems, Wetzlar, Germany). Slides were analyzed by two blinded independent observers.

Statistical Analysis

The Multivariate Statistical Package (Kovach Computing Service, Isle of Anglesey, UK) was used for Ward's mini-

mum variance clustering method to evaluate gene expression variability among different samples.

Statistical analyses (Fisher exact test; Student *t* and Bonferroni tests) were evaluated using the GraphPad Software (Prism 4.0).

All supplementary materials are available online at www.molmed.org.

RESULTS

RT-PCR Analysis of Stemness-Related Genes

We analyzed by RT-PCR the expression of a set of 13 stemness-related genes (Table 1) in endometrial (*n* = 14) and endometriotic (*n* = 12) biopsy samples. Overall results are shown in Table 3. The histogram in Figure 1A shows the percentage of expression of each gene in the

endometrium and endometriotic sample groups and the histogram in Figure 1B reports the number of expressed stemness-related genes in endometrial and endometriotic samples.

Results indicated that *SOX2* mRNA was not expressed in any of the samples we analyzed (Table 3, Figure 1A). Conversely, *OCT4*, *KLF4*, and *BMI1* mRNAs were expressed in all the endometrium and endometriotic samples we examined (Table 3, Figure 1A).

Other genes, such as *DPPA2* and *SOX15*, were found to be expressed in the same percentage of patients in endometrial and endometriotic sample groups (Figure 1A).

ERAS, *NANOG*, and *GDF3* showed a slightly higher (but not statistically significant) frequency of expression in endometrial than in endometriotic samples (Figure 1A).

Table 3. Qualitative RT-PCR analysis of stemness-related genes in 14 endometrial tissues and in 12 endometriotic samples.*

Case no. (cycle phase or endometriosis grade)	Gene												
	<i>SOX2</i>	<i>DPPA2</i>	<i>GDF3</i>	<i>TCL1</i>	<i>ZFP42</i>	<i>UTF1</i>	<i>ERAS</i>	<i>SALL4</i>	<i>NANOG</i>	<i>SOX15</i>	<i>OCT4</i>	<i>KFL4</i>	<i>BMI1</i>
Endometrium 1 (M)	-	-	-	-	-	-	-	-	+	+	+	+	+
Endometrium 2 (PP)	-	-	-	-	-	-	-	+	+	+	+	+	+
Endometrium 3 (SP)	-	-	-	+	-	-	-	+	+	+	+	+	+
Endometrium 4 (SP)	-	-	-	-	-	+	+	+	+	+	+	+	+
Endometrium 5 (SP)	-	-	+	+	-	+	+	+	+	+	+	+	+
Endometrium 6 (M)	-	-	-	-	-	-	-	+	+	+	+	+	+
Endometrium 7 (SP)	-	-	+	-	-	+	+	+	+	+	+	+	+
Endometrium 8 (PP)	-	-	-	+	-	-	-	+	+	+	+	+	+
Endometrium 9 (PP)	-	-	+	-	-	+	+	+	+	+	+	+	+
Endometrium 10 (SP)	-	-	+	+	-	-	+	-	+	+	+	+	+
Endometrium 11 (M)	-	-	+	+	-	-	+	+	+	+	+	+	+
Endometrium 12 (SP)	-	-	-	-	-	-	+	-	-	-	+	+	+
Endometrium 13 (SP)	-	+	+	+	-	+	+	+	+	+	+	+	+
Endometrium 14 (PP)	-	+	+	+	-	+	+	+	+	+	+	+	+
Endometriosis 1 (II)	-	-	+	+	-	+	+	+	+	+	+	+	+
Endometriosis 2 (III)	-	-	+	+	-	+	+	+	+	+	+	+	+
Endometriosis 3 (IV)	-	-	-	+	+	+	+	+	+	+	+	+	+
Endometriosis 4 (II)	-	-	-	-	-	+	+	-	+	+	+	+	+
Endometriosis 5 (III)	-	-	-	+	+	-	-	+	+	+	+	+	+
Endometriosis 6 (III)	-	-	-	+	+	+	+	+	+	+	+	+	+
Endometriosis 7 (III)	-	-	-	+	-	+	-	+	-	+	+	+	+
Endometriosis 8 (IV)	-	-	+	+	-	+	-	+	-	+	+	+	+
Endometriosis 9 (III)	-	-	-	+	-	-	-	+	-	+	+	+	+
Endometriosis 10 (IV)	-	-	-	+	-	+	-	+	+	+	+	+	+
Endometriosis 11 (III)	-	+	-	-	-	+	+	+	+	-	+	+	+
Endometriosis 12 (I)	-	+	-	+	-	+	-	+	+	+	+	+	+

*Summary of the results on the presence (+) or absence (-) of gene expression for each patient. PP, proliferative phase; SP, secretory phase; M, menopause.

The remaining genes we analyzed (*SALL4*, *UTF1*, and *TCL1*) showed a different percentage of expression in endometrium and endometriotic sample groups, with a trend for higher percentages of expression in endometriotic samples than in endometrium samples. In more detail, *UTF1* (also known as undifferentiated embryonic cell transcription factor 1) showed a significantly higher frequency of expression in endometriotic samples than in endometrium (83% versus 43%, $P < 0.05$). Also *TCL1* showed a remarkable difference in the percentage of expression between endometrial and endometriotic samples (50% versus 83%), although the difference was not statistically significant. Of note, *ZFP42* was expressed in only 25% of endometriotic tissues (classified as III and IV grade) and in none of the endometrial biopsy samples.

The 12 endometriotic samples coexpressed a minimum of 6 to a maximum of 10 stemness-related genes (Figure 1B). Conversely, the 14 endometrial samples coexpressed a minimum of 4 to a maximum of 11 stemness-related genes (Figure 1B). No significant differences were observed in the number of expressed genes between the two groups of samples.

For this study we report only qualitative RT-PCR data about the expression of a panel of 13 stemness-related genes, because the endometrial and endometriotic biopsies were harvested during the last decade and in some cases the quality of RNA extracted from frozen or paraffin-embedded tissues did not allow us to obtain fully reliable quantitative RT-PCR data. Nevertheless, in some patients we found a correlation between the expression level of stemness-related genes and the grade of endometriosis, as well as a

trend (not statistically significant) for a higher expression level of some genes (for example, *SALL4*) in endometriotic tissues rather than in endometrium samples (data not shown).

The RT-PCR data concerning the presence or absence of gene expression in the 26 samples under analysis were used to carry out a minimum variance test to evaluate gene expression variability among different patients. Our goal was to obtain a minimum variance clustering based on a matrix constructed with the presence/absence of gene expression points, such that patients having similar patterns of expressed/not expressed genes fall in the same cluster and have more genetic homogeneity compared with those showing different expression patterns, which are then classified in distinct clusters. We did not find any correlation between the phase of the

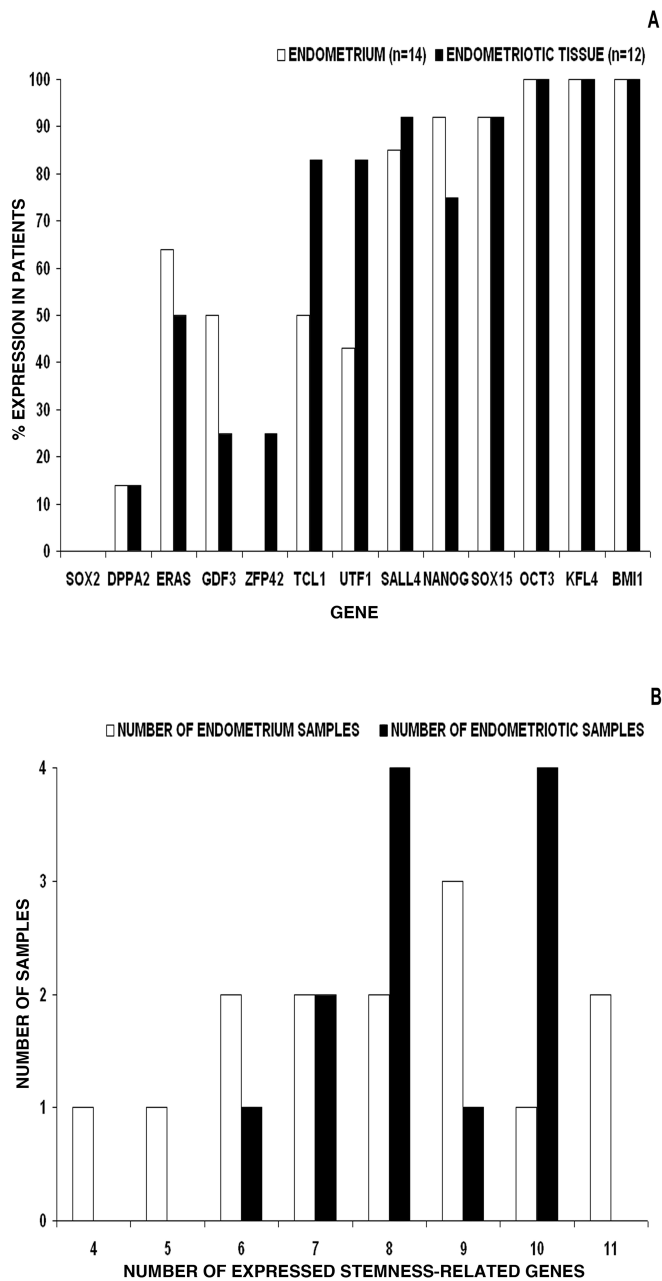


Figure 1. (A) The histogram shows the frequency of expression of stemness-related genes in endometrial tissues (white columns) and in endometriotic samples (gray columns). (B) The histogram shows the number of expressed stemness-related genes in endometrial tissues (white columns) and in endometriotic samples (gray columns).

menstrual cycle and the number of expressed stemness-related genes (Table 3). Similarly, no significant evidence was detected for a correlation between the grade of endometriosis and the number of expressed stemness-related genes (Table 3).

Immunohistochemical Detection of SALL4 and OCT4 Proteins in Endometrial and Endometriotic Samples

Endometrial (n = 14) and endometriotic (n = 12) samples embedded in paraffin were submitted to IHC-mediated

analysis of the expression of SALL4 and OCT4. We selected these two proteins for IHC analysis because these proteins play an important role in stemness preservation (24), because they may clarify possible misleading results deriving from RT-PCR analysis of OCT4 expression, and finally, because quantitative RT-PCR data indicated a trend for a higher expression level for their mRNAs in endometriosis samples rather than in endometrium, even though the difference was not statistically significant.

We analyzed at least five consecutive cross sections for each tissue sample. Only cross sections of endometrial and endometriotic tissues with markedly brown-stained cells, showing a clear structure, were scored positive for SALL4 and OCT4 protein expression.

Positive cells for SALL4 and OCT4 were detectable in different consecutive cross sections of the tissue samples we analyzed (Figures 2 and 3). The staining for both SALL4 and OCT4 showed nuclear localization.

Cells positive for SALL4 were found in all the endometriotic tissues we analyzed (Figure 2). None of the endometrial samples revealed cells positive for SALL4. To further confirm these data, IHC detection of SALL4 was also conducted on paired ectopic and eutopic endometrium from the same patient (sample endometriosis 8, Tables 1 and 3), revealing SALL4-positive cells only in endometriotic tissue.

Cells positive for OCT4 were found in the stroma of all the endometriotic tissues we analyzed. Stromal cells positive for OCT4 were also detected in the endometrial samples (Figure 3).

We observed only single stromal cells positive for OCT4 immunostaining both in endometrium and in endometriotic samples. Conversely, SALL4-positive cells in endometriotic tissues were also located in a periglandular position and in the stromal vasculature.

Control IHC reaction for SALL4 was positive on mouse adult testis and negative on mouse adult heart (Supplemental Figure 1). Control IHC reaction for OCT4 was positive on mouse embryo testis

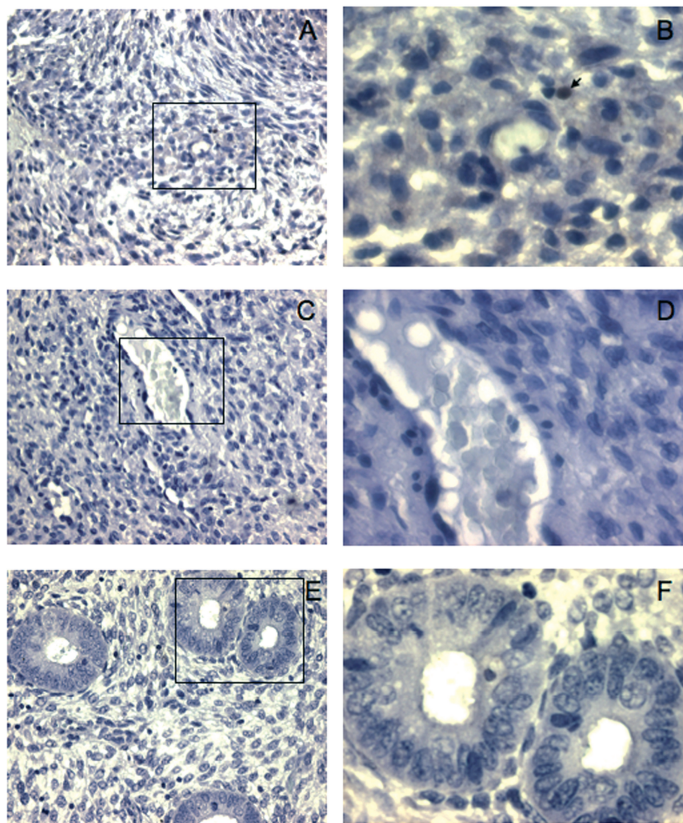


Figure 2. Representative IHC staining of SALL4 in human endometrium and in endometriotic tissue. Hematoxylin counterstaining. Endometriotic tissue (A, B) is compared with endometrial tissue (E, F). IHC staining of serial sections of the tissue used in A without primary antibody was done as negative control of the reaction (C, D). Black arrow in B indicates a representative SALL4 IHC-positive cell. Subparts (B, D, F) represent 100 \times magnification of the area enclosed in the black perimeter in A, C, E (40 \times magnification).

(E13.5) and negative on mouse adult heart (Supplemental Figure 2).

DISCUSSION

In this study, we have characterized at the mRNA level the expression of a panel of 13 embryonic stemness-related genes in two sets of human endometrium and endometriotic samples, together with the IHC verification for a subgroup of two factors, to evaluate which of them were present in endometrial and endometriotic tissues.

Various studies have highlighted the presence of stem cells in endometrium. In particular, Du *et al.* (14) demonstrated that lethally irradiated female mice receiving bone marrow transplantation from male donors show male-derived

cells incorporated into the endometrium. The presence of stem cells has also been demonstrated in women who received bone marrow transplants from mismatched donors (25). The bone marrow compartment can be subdivided into two interdependent spaces: the hematopoietic cell compartment and the stroma. The stroma is composed of mesenchymal stem cells, fibroblasts, adipocytes, nerves, and the bone marrow's vascular system. Mesenchymal stem cells are quite rare, comprising between 0.01% and 0.001% of nucleated cells in adult human bone marrow, depending on the age of individuals (26). Nonhematopoietic stem cells from bone marrow can potentially contribute to the preservation of multiple tissues. Some studies indicate that stem cells in

endometrium are of bone marrow origin and share many characteristics with mesenchymal stem cells, because they are able to differentiate into chondrocytes, osteocytes, and adipocytes and express peculiar antigens (27).

Other recent studies have revealed the presence of stem cells in the menstrual blood, characterized by a high proliferative rate *in vitro*, high differentiation ability, expression of a number of stemness-related nonhematopoietic markers (including OCT4), and production of matrix metalloproteases, cytokine growth factors, and angiogenic factors (28,29). Nevertheless, the presence of hematopoietic stem cells has also been demonstrated immunologically in endometrium (30). The endometrial stem cells, both of hematopoietic or nonhematopoietic nature, probably contribute to the *de novo* formation of stroma, glands, and vasculature in the reproductive cycle.

In this study, we highlighted the possible presence of stem cells in all the endometrium and endometriotic samples through the expression of 13 stemness-related genes.

Our RT-PCR data highlight a significantly higher number of endometriotic samples expressing *UTF1* mRNA compared to endometrial biopsy samples ($P < 0.05$). *UTF1* is highly and almost exclusively expressed during embryogenesis (31). In more detail, *UTF1* is specifically expressed in the inner cell mass and primitive ectoderm and is downregulated at early primitive streak stages (32). Of interest, it has been reported that *UTF1* expression is maintained in the primordial germ cells in developing embryos and in the gonads in adult animals (33).

ZFP42 (also known as *REX-1*) is expressed only in about 25% of endometriotic samples, classified as III and IV grade (Table 3). A recent study by Kristensen *et al.* (34) showed that *ZFP42* and *UTF1* are expressed throughout human testes development and in testicular germ cell tumors and in testicular carcinoma, showing similarities with pluripotent embryonic stem cells.

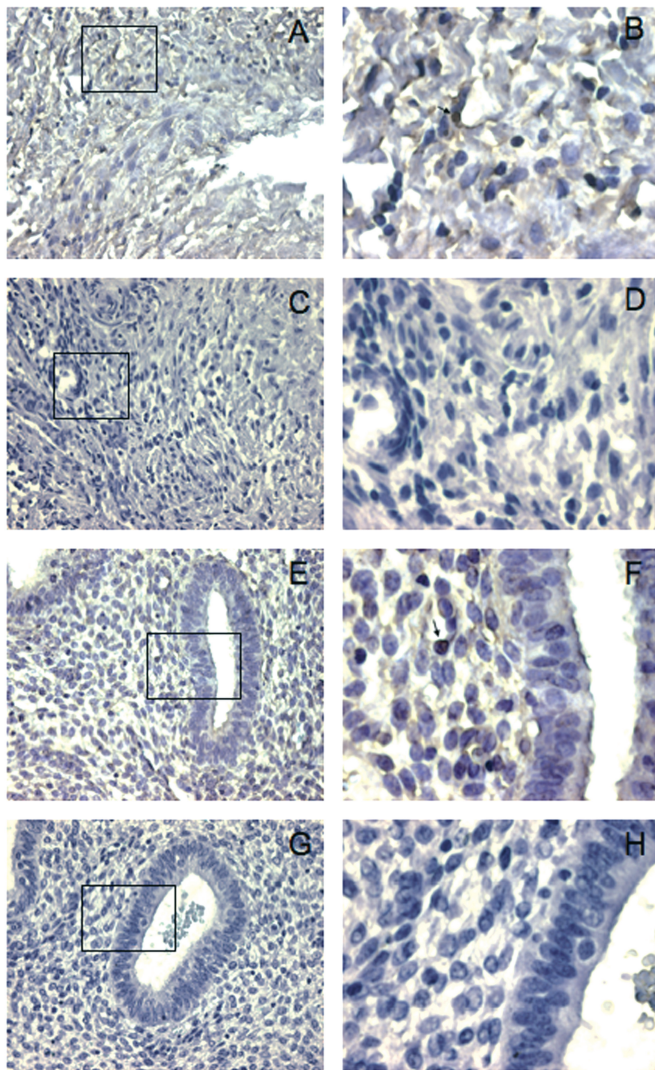


Figure 3. Representative IHC staining of OCT4 in human endometrium and in endometriotic tissue. Hematoxylin counterstaining. Endometriotic tissue (A, B) is compared with endometrial tissue (E, F). IHC staining of serial sections of the tissue used in A without antibody was done as a negative control of the reaction (C, D). Immunohistochemical staining of serial sections of the tissue used in E without antibody was done as a negative control of the reaction (H, G). Black arrows in B and F indicate representative OCT4 IHC-positive cells. Subparts (B, D, F, H) represent 100x magnification of the area enclosed in the black perimeter in A, C, E, G (40x magnification).

Promoter analysis indicated that the murine *UTF1* gene is transcriptionally regulated by OCT4 and SOX2 (35). Finally, a recent study indicated that UTF1 is a stably chromatin-associated transcriptional repressor protein involved in the initiation of embryonic stem cell differentiation, but not in embryonic stem cell self-renewal (36).

RT-PCR data also indicate a trend for a higher frequency of expression of *TCL1* in endometriotic samples. *TCL1* (also known as T cell leukemia 1) is a protooncogene highly activated in various human neoplastic diseases, whereas its physiological expression is tightly limited to early developmental cells as well as various developmental stages of immune cells (37).

One of the analyzed genes (*SOX2*) was detected in neither endometrial nor endometriotic tissue, whereas *DPPA2* was expressed only in samples from two patients for each group. This result is not surprising, because embryonic stem cells have broader stemness properties (self-renewal, pluripotency) compared with adult stem cells.

The analysis of minimum variance did not reveal any homogeneous clusters of samples on the basis of gene expression data, possibly because of the relatively low number of samples we analyzed or because of the heterogeneity of samples in relation to the number and type of cells they contain.

Recently, it has been discovered that rare cells in the endometrial stroma of about 44% of women are positive for OCT4 (also known as OCT3/4, OCT3 and POU5f1) (38), a protein member of the POU transcription factor family. OCT4 is expressed in pluripotent cells, and its downregulation is associated with loss of pluripotency. The results of the mentioned study are in agreement with our RT-PCR and IHC data, because we highlighted the expression of *OCT4* mRNA and protein in all the eutopic endometrium samples we analyzed.

The latest results about OCT4 isoforms reveal the presence of three alternative splice variants (OCT4-A, OCT4-B, and OCT4-B1) (39).

The PCR primers we used for *OCT4* mRNA analysis (Table 2) are both enclosed within the exon 5 sequence and cannot be used to distinguish among the variants OCT4-A, OCT4-B, and OCT4-B1 and the RNA transcribed by the two pseudogenes identified by the GeneBank numbers NG_005793 and NG_006104. For this reason, together with our observation that the OCT4 RT-PCR signal was higher in samples from patients with endometriosis samples than in samples from the endometrium group, we decided to further analyze the OCT4 expression in the two sets of human endometrial and endometriotic samples at the protein level. The antibody for OCT4 we used was obtained using a synthetic

peptide derived from within residues 300 to the C-terminus of human OCT4 as immunogene. The OCT4 splice variants OCT4-A and OCT4-B share an identical C-terminal domain, whereas the recently discovered OCT4-B1 lacks the C-terminal domain because of a stop codon in the cryptic exon 2b, and consequently the IHC data we obtained were potentially related to the OCT4-A and -B isoforms. Nonetheless, because we obtained a clear nuclear localization of the IHC signal for OCT4 (Figure 3), we can argue that it corresponds to the OCT4-A variant, as it has been reported that the OCT4-B variant is localized in the cytoplasm (40,41).

The variant *OCT4-B1* has been discovered very recently (39), and consequently all the currently available literature data concerning the expression of OCT4 protein involve the isoforms A and B, because the antibody specific for the putative truncated protein translated by the *OCT4-B1* splice variant is not available. The translation of the *OCT4-B1* mRNA variant identified by Atlasi *et al.* has not yet been demonstrated, and its putative role in stemness and in carcinogenesis has been only suggested, but not demonstrated experimentally (for example, through RNA interference assays). Moreover, nothing is known about the cellular localization (at the nuclear or cytoplasmic level) of the protein possibly expressed by the novel *OCT4* mRNA splice variant.

It should be underlined that OCT4 has been considered for a long time a reliable marker for stemness, but a recent study demonstrated the expression of OCT4 also in normal differentiated adult cells from human peripheral blood, thus suggesting that the presence of OCT4 alone can no longer be considered sufficient to define a cell as pluripotent (42). Nevertheless, in our experiments we supported the presence of OCT4 as a marker of stemness with the expression data of adjunctive 12 stemness-related genes.

Parallel experiments revealed the presence of *SALL4* mRNA both in eutopic

and ectopic endometrium samples, but revealed the presence of *SALL4* protein only in endometriotic samples.

It should be underlined that we were also able to analyze the *SALL4* expression in paired ectopic and eutopic endometrial tissue from the same patient (sample endometriosis 8 in Table 1), identifying *SALL4*-positive cells only in ectopic endometrium. The direct comparison between autologous ectopic and eutopic endometrium can exclude variables related to individual genetic variability and to various effects of hormonal stimulation during the menstrual cycle, and thus such comparison can further clarify the contribution of stem cells to the pathogenesis of endometriosis.

Nevertheless, it should be considered that this differential expression of *SALL4* protein between endometrial and endometriotic tissues could be related not necessarily to a translational mechanism of regulation of *SALL4* expression, but could be related to the very low expression of *SALL4* protein in endometrium.

The presence of OCT4- and *SALL4*-positive cells mainly in the stroma of endometrial and endometriotic samples is in agreement with results of other studies based on stem cell detection through the analysis of stemness markers (38,16). Nevertheless, we found some *SALL4*-positive cells also in the vasculature and in periglandular positions.

SALL4 and OCT4 work as essential stemness factors. Our choice to analyze at the protein level both *SALL4* and OCT4 relies also on experimental evidence that *SALL4* forms a crucial interconnected autoregulatory network with OCT4 in embryonic stem cells (43). It has also been demonstrated in mouse embryonic stem cells that *SALL4* is a transcriptional regulator of OCT4 and has a critical role in the maintenance of stem cell pluripotency by modulating OCT4 expression (44).

CONCLUSIONS

Our data indicating an increased presence of stem cell markers in endometriotic samples are in agreement with the recent studies revealing an increased ex-

pression of the adult stem cell marker Musashi-1 in endometriosis and endometrial carcinoma (16). Our preliminary results indicate that the percentages of single cells positive for *SALL4* and OCT4 we detected in the stroma of endometriotic tissues are comparable to those found by Gotte M *et al.* for Musashi-1-positive cells (data not shown). The contribution of stem cells to endometriosis has been hypothesized in many reports of studies and reviews (45,14).

If further verified, the presence of stem cells in ectopic and eutopic endometrium can provide new insights into the mechanisms at the basis of gynecological diseases related to cell proliferation, including endometrial carcinoma.

To our knowledge, this is the first study highlighting the expression of a panel of stemness-related genes in human endometrial and endometriotic samples, with a particular relevance for *UTF1* and *TCL1*. Moreover, we report for the first time the expression of *SALL4* and OCT4 proteins in endometriotic samples. Overall data obtained in this study suggest a possible role for stem cells in the pathogenesis of endometriosis, even if further data are warranted to support this hypothesis.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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