Multicenter cohort molecular evidence of the presence of endometriosis-inducing factor (mir-130a) as a potent regulator of gene expression in endometriosis

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Objective **To study the role of endometriosis-inducing factor on the differentiation of cultured stem cells into endometrial cells.**

Methods **This was a multicenter prospective cohort case-control study where 323 women were examined, but only 134 infertile women were recruited and compiled with the inclusion criteria. The study group comprised 64 women in whom laparoscopy showed endometriotic lesions, and the control group included 70 women free of endometriotic implants. The women's sera were cocultured with mesenchymal stem cells, which were followed up weekly by quantitative real-time PCR to examine the expression of *SPARC* (secreted protein, acidic, cysteine-rich) and *MYC* (myelocytomatosis) genes using the $2^{-\Delta\Delta C_t}$ method.**

Results **By coculturing the serum of women with endometriosis and the control group with stem cells, none of the cultures of the sera of women within the control group showed any changes in the normal expression of either *SPARC* or *MYC* gene levels. Furthermore, the stem cells were normally multiplying in the same cell line during the entire duration of the study. Nevertheless, stem cells that were cocultured with sera from women with endometriosis showed upregulation of the *SPARC* gene mRNA with mean respiratory quotient of 3.534 ± 1.129, whereas the *MYC* gene mRNA was downregulated with a mean respiratory quotient of 0.488 ± 0.104.**

Conclusion **The sera of women with endometriosis were able to induce transformation of mesenchymal stem cells into endometrial-like cells on a molecular basis. This evidence supports the endometriosis-inducing factor theory of endometriosis and may have tremendous effect on the therapeutic implications of this debilitating condition. *Med Res J* 13:1–5 © 2014 Medical Research Journal.**

Medical Research Journal 2014, **13**:1–5

Keywords: endometriosis, endometriosis-inducing factor, myelocytomatosis, secreted protein, acidic, cysteine-rich

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Received 12 March 2014 **accepted** 5 May 2014

Background

Endometriosis is a benign chronic inflammatory disease characterized by the survival of endometrial tissue outside the uterine cavity [1]. It affects 6–10% of women during the reproductive age [2]. Endometriosis can be classified as pelvic or extrapelvic according to its location. None of the well-known theories have been able to explain the development of endometriosis in all sites successfully, and the definitive cause is still unclear. The most commonly accepted mechanism for the development of endometriotic lesions is the Sampson theory claiming the adhesion and growth of endometrial fragments deposited into the peritoneal cavity through retrograde menstruation. However, endometriosis is reported to occur in locations that do not communicate with the peritoneal cavity [3]. Recently, the stem cell theory was introduced to explain how endometriosis can be found remote from the peritoneal cavity, how it resists some treatments, and recurs occasionally even after hysterectomy. We have proved in a previous study that an endometriosis-inducing factor (EIF) does exist in the sera of women suffering from endometriosis [4]. The *SPARC* (secreted protein, acidic, cysteine-rich) gene is located on chromosome 5 (5q31.3–q32) and is involved in extracellular matrix synthesis and promotion of changes to the cell shape. The gene product has been associated with tumor suppression, but it has also been correlated with metastasis on the basis of changes to the cell shape that can promote tumor cell invasion [5]. The *MYC* (myelocytomatosis) gene is located on chromosome 8 and is believed to regulate the expression of 15% of all genes. It also plays a very important role in regulating cell growth, apoptosis, differentiation and stem cell self-renewal [5]. The aim of this study was to assess the role of the EIF on the differentiation of cultured stem cells into endometrial cells.

Materials and methods

This was a multicenter prospective cohort case-control study in which 323 women undergoing diagnostic laparoscopy for infertility (whether primary or secondary) work out were included. Only 134 infertile women were recruited and compiled with the inclusion criteria. Women undergoing diagnostic laparoscopy for infertility

2090-6242 © 2014 Medical Research Journal DOI: 10.1097/01.MJX.0000450147.80067.f1

work out were included. The study was approved by both the Ethical Committee of Obstetrics and Gynecology Department, Faculty of Medicine, Ain Shams University, and the bioethical committee of the National Research Center. The Islamic conference guidelines on stem cell research were also insured. Written informed consents were obtained from women enrolled in this study. Sixty-four women had mild to moderate endometriosis on the basis of laparoscopic findings according to the Revised American Fertility Society scoring for endometriosis [6]. Moreover, biopsy samples from the lesions were acquired and pathological confirmation of the diagnosis was substantiated. The other 70 women served as a control group in whom the diagnostic laparoscopy showed no endometriotic implants. No women had a history of immunological diseases or received any type of hormonal therapy in the last 6 months before the diagnostic laparoscopy procedure. Also, women with pelvic inflammatory disease were excluded.

Stem cell preparation

Human umbilical cord blood (UCB) was collected in a cord blood bag after a full-term delivery. UCB was harvested in sterile tubes containing 100 mmol/l EDTA as anticoagulant at 22°C. Stem cells were obtained from the low-density mononuclear cells that were isolated using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Then, the cells were cultured in a growth medium (Dulbecco's modified Eagle medium – low glucose) with 10% fetal bovine serum, 2 mmol/l L-glutamine and 0.1% penicillin-streptomycin (Gibco-BRL, Carlsbad, California, USA). Cultures were incubated in a CO_2 incubator at 37°C. The mesenchymal stem cells (MSCs) derived from UCB were propagated and characterized by fluorescence-activated cell sorting analysis. Venous whole blood sample of 5 ml was obtained from the infertile women by venipuncture under complete aseptic conditions. These samples were left to clot at 37°C, centrifuged and sera were separated and kept in sterile tubes at −20°C in the freezer until further use. Sera samples of 10 µl was obtained from the study and the control groups were added to the stem cell culture medium at 37°C [7]. The cultures were observed weekly by real-time PCR for expression of the *SPARC* and the *MYC* genes.

Real-time quantitative PCR

Total RNA was extracted from cocultured cells using the RNeasy Purification kit (Qiagen, Valencia, California, USA) in accordance with the manufacturer's instruction. RNA quality and quantity were determined using the RNA 6000 Labchip/Agilent 2100 Bioanalyzer (Agilent Technologies, Germantown, Maryland, USA), and then a sample (1 µg) was reverse transcribed (RT) with AMV reverse transcriptase (Invitrogen, Carlsbad, California, USA) in the presence of oligo-dT primer in accordance with the manufacturer's instructions. All real-time PCR reactions were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, California, USA). Also, the entire primers and probes of the two target genes (*SPARC*-Hs00277762-m1and *MYC*-Hs00153408-m1) and an internal control gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*- Hs99999905-m1) were designed by Applied Biosystems (Applied Biosystems). A reaction mix was prepared using *TaqMan* gene expression master mix according to the manufacturer's protocol, in triplicate (Applied Biosystems). The thermal cycling conditions included an initial denaturation step at 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 45 s.

Analysis of gene expression using the $2^{-\Delta\Delta C_t}$ method

Details of the $2^{-\Delta\Delta C_t}$ method were described previously by [8,9]. In brief, the mean of the gene expression level for the two mRNA measurements was calculated. Thereafter, the $2^{-\Delta\Delta C_t}$ method was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments. In the present study, data are presented as the fold change in target gene expression in stem cells treated by women's sera normalized to the internal control gene *GAPDH* and relative to the normal sera treated stem cells (normal control as a calibrator). Results of the real-time PCR data were represented as C_t values, where C_t is defined as the threshold cycle number of PCR at which the amplified product was first detected. The average C_t was calculated for both the target genes and *GAPDH*, and the ΔC_t was determined as (the mean of the triplicate C_t values for the target gene) minus (the mean of the triplicate C_t values for *GAPDH*). The $\Delta\Delta C_t$ represented the difference between the paired tissue samples, as calculated by the formula $\Delta\Delta C_t = (\Delta C_t$ of women's treated stem cells $- \Delta C_t$ of normal sera treated stem cells). The *N*-Fold differential expression in the target gene of a patient sample compared with the normal sample counterpart was expressed as $2^{-\Delta\Delta C_t}$ or respiratory quotient (RQ) [8,9].

Statistical analysis

Data were analyzed using IBM SPSS Advanced Statistics version 20.0 (Armonk, New York, USA). The *t*-test was used to study the correlation between RQ of *SPARC* and RQ of *MYC* with age, parity, and BMI.

Results

The mean age of women was 27.88 ± 1.94 years and their parity was 0.68 ± 0.08. while their BMI was 23.13 ± 2.01 kg/m^2. There is no correlation between either *SPARC* or *MYC* gene levels with age, parity, and the BMI (Tables 1 and 2).

Both the study and the control groups were examined for mRNA expression of the two specified genes using quantitative real-time PCR. It was evident that none of the cultures of the sera of the control group showed any changes in the normal expression of either *SPARC* or *MYC* gene levels. Furthermore, the stem cells were normally dividing in the same cell line. Nevertheless the stem cells that were cocultured with sera from women with endometriosis showed upregulation of the *SPARC* gene mRNA with a mean RQ of 3.534 ± 1.129 as shown in Fig. 1, whereas the *MYC* gene mRNA was downregulated with a mean RQ of 0.488 ± 0.104 as shown in Fig. 2.

Discussion

To date, there is still no single, unifying theory to explain the existence of endometriosis in all its various forms.

Although retrograde menstruation and implantation is the most widely accepted theory, the presence of endometrial cells in the abdominal cavity is frequently observed in women during menses. Nevertheless, it is not conceivable as to why some women develop endometriosis whereas others do not. Recently, it was demonstrated that MSCs could lead to the expression of endometriosis in a mouse model. Du and Taylor [10] removed the uterus of a mouse model so that endometriosis could not arise from endometrial cells (either through retrograde menstruation or through hematogenous, or lymphatic dissemination), and still stem cells populated endometriotic implants, leading to disease progression. Our earlier work has proved that EIF exits in the serum of women suffering from endometriosis [11]. EIF in women with endometriosis can transform these endogenous stem cells, leading to the formation of endometriotic lesions, much more commonly in the pelvis or in any ectopic site outside the pelvis [12]. Azmy and Elgarf [13] found that this EIF is miR-130a. miR-130a appears to be a potent regulator of gene expression in endometriosis, raising the prospect of using blood miRNAs as biomarkers and therapeutic tools in endometriosis.

In this research, we studied the role of EIF in the differentiation of MSCs *in vitro* to endometrial-like cells. Our results had convincingly proved that by adding the serum of women with endometriosis to MSC for 4 weeks, both *SPARC* and *MYC* gene expressions were detected in these cells by real-time PCR, where the *SPARC* gene mRNA was upregulated, whereas the *MYC* gene mRNA was downregulated.

Table 1 Correlation between the respiratory quotient of *SPARC* with age, parity, and BMI

	N	Correlation	Significance
RQ of *SPARC* and age	65	−0.003	0.984
RQ of *SPARC* and parity	65	0.012	0.925
RQ of *SPARC* and BMI	65	−0.198	0.113

RQ, respiratory quotient; SPARC, secreted protein, acidic, cysteine-rich.

Table 2 Correlation between the respiratory quotient of *MYC* with age, parity, and BMI

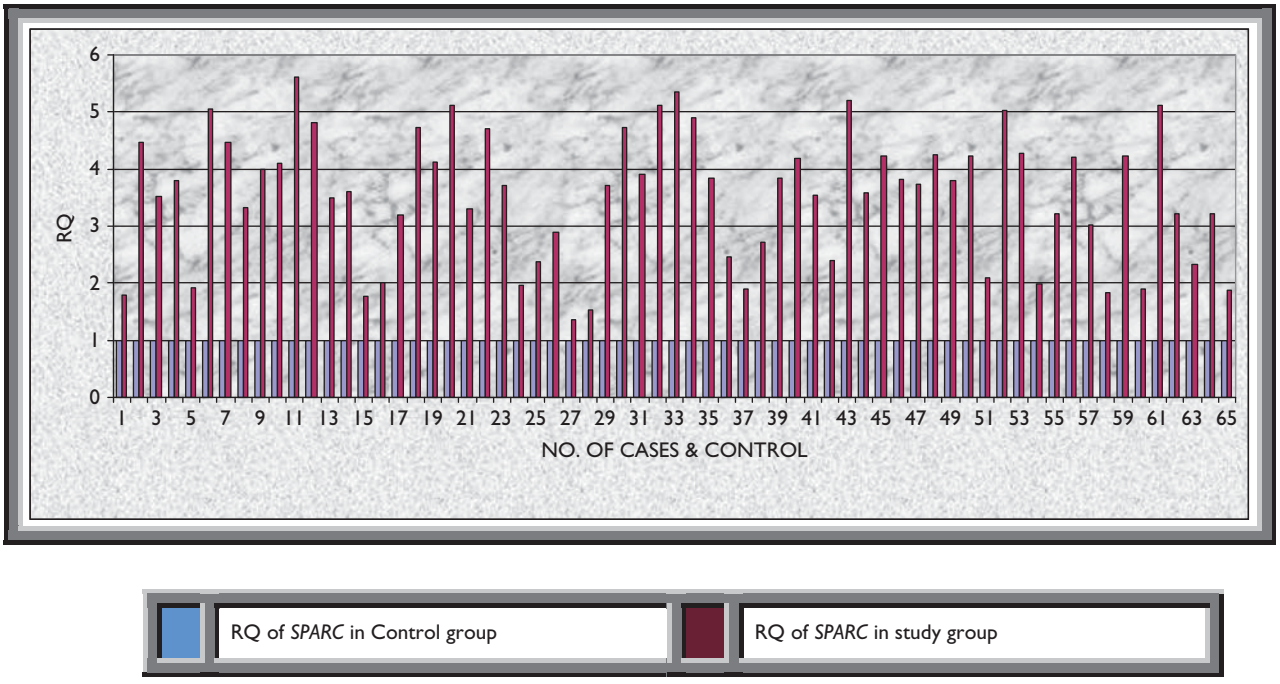
	N	Correlation	Significance
RQ of *MYC* and parity	65	−0.197	0.116
RQ of *MYC* and age	65	−0.110	0.385
RQ of *MYC* and BMI	65	−0.174	0.165

MYC, myelocytomatosis; RQ, respiratory quotient; SPARC, secreted protein, acidic, cysteine-rich.

Conclusion

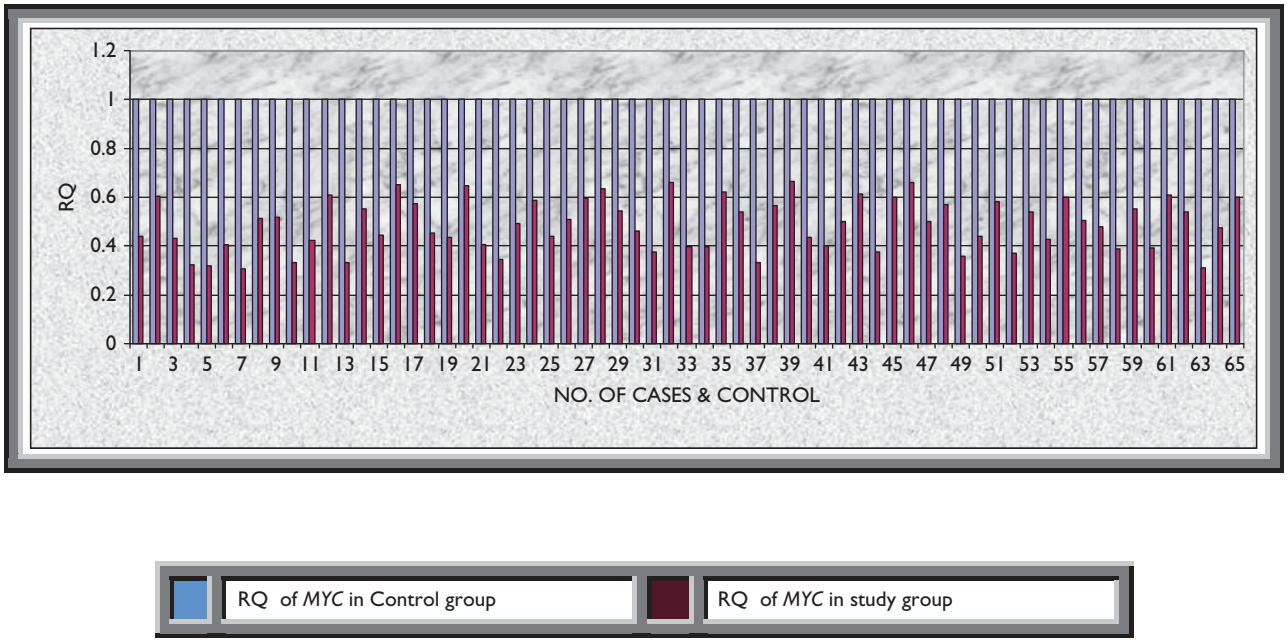
We conclude that EIF (miR-130a) is a potent regulator of gene expression in endometriosis leading to enhanced transformation of MSCs into endometrial-like cells, which is proved by the upregulation of the *SPARC* gene and the downregulation of the *MYC* gene. This finding supports the stem cell theory of endometriosis and may have tremendous effect on the therapeutic implications of this debilitating condition and opens up a new era in its management and therapy.

Fig. 1



The distribution of mRNA expression of the *SPARC* gene among the study group. There is an overexpression of the *SPARC* gene compared with the control group with a mean RQ of 3.534 ± 1.129. RQ, respiratory quotient; *SPARC*, secreted protein, acidic, cysteine-rich.

Fig. 2



The distribution of mRNA expression of the *MYC* gene within the study group. There is a decrease in the expression of the *MYC* gene compared with the control group with a mean RQ of 0.488 ± 0.104. *MYC*, myelocytomatosis; RQ, respiratory quotient.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

References

1 Donnez J. Endometriosis: enigmatic in the pathogenesis and controversial in its therapy. *Fertil Steril* 2012; **98**:509–510.
2 Signorile PG, Baldi F, Bussani R, D'Armiento M, De Falco M, Baldi A. Ectopic endometrium in human foetuses is a common event and sustains the theory of müllerianosis in the pathogenesis of endometriosis, a disease that predisposes to cancer. *J Exp Clin Cancer Res* 2009; **28**:28–49.
3 Yi D, Jin-hua L, Jing-he L, Xiao-yan L, Jun-ji Z. Anatomical distribution of pelvic deep infiltrating endometriosis and its relationship with pain symptoms. *Chin Med J* 2012; **125**:209–213.
4 Du H, Taylor HS. Stem cells and female reproduction. *Reprod Sci* 2009; **16**:126–139.
5 Gearhart J, Pashos EE, Prasad MK. Pluripotency Redeux – advances in stem-cell research. *N Engl J Med* 2007; **357**:1469–1472.
6 Buttram VC Jr. Evolution of the revised American fertility society classification of endometriosis. *Fertil Steril* 1985; **43**:347–350.
7 Mizuno N, Shiba H, Ozeki Y, Mouri Y, Niitani M, Inui T, *et al*. Human autologous serum obtained using a completely closed bag system as a substitute for foetal calf serum in human mesenchymal stem cell cultures. *Cell Biol Int* 2006; **30**:521–524.
8 Applied Biosystems Manual: Relative quantitation of gene expression. User bulletin No. 2. ABI Prism 7700 Sequence Detection System. PE Applied Biosystems; 1997.
9 Livak MJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 – ΔΔCT method. *Methods* 2001; **25**:402–408.
10 Du H, Taylor H. Contribution of bone marrow derived stem cells to endometrium and endometriosis. *Stem Cells* 2007; **25**:2082–2086.
11 Rasheed K, Atta H, Taha TF, Azmy O, Sabry D, Selim M, *et al*. A novel endometriosis inducing factor in women with endometriosis. *J Stem Cells Regen Med* 2010; **6**:157–164.
12 Sasson I, Taylor H. Stem Cells and the pathogenesis of endometriosis. *Ann N Y Acad Sci* 2008; **1127**:106–115.
13 Azmy O, Elgarf W. MiRNA-130a, a potential endometriosis-inducing factor. *Med Res J* 2012; **11**:40–47.

الملخص العربي

دراسة جزيئية عن تمييز الخلية الجذعية إلى خلية بطانة الرحم بواسطة العامل المحفز لمرض بطانة الرحم المهاجرة في مصل الدم

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مرض بطانة الرحم المهاجرة مرض مزمن حميد يصيب السيدات ويتميز بوجود آثار لأنسجه وغدد جدار الرحم خارج تجويف الرحم .يهدف هذا البحث إلى دراسة دور العامل المحفز لمرض بطانة الرحم المهاجرة في تحور الخلية الجذعية لخلية بطانة الرحم بواسطة استخدام جينات **SPARC and MYC** كدليل على هذا التحور ولقد شملت هذه الدراسة **130** مريضه بالعقم خضعن لجراحه منظار البطن التشخيصي تم تشخيص مرض بطانة الرحم المهاجرة في نصفهم وصنفوا كمجموعه دراسة .النصف الاخر لم يشخص فيهم المرض واستخدموا كمجموعه تحكم، تم سحب ٥ مل من دم جميع المرضي، ثم تم تحضير مزرعة للخلايا الجذعية المستخلصة من دم الحبل السري البشري ومن ذلك الدم تم استخراج الخلايا الجذعية وتمت تنميتها وإعداد مزرعة لها ثم أضيف دم المرضي من مجموعتي الدراسة والتحكم لمزرعة الخلايا الجذعية وتم فحص الخلايا أسبوعيا لمده ٤ أسابيع لاحتمال تحورها إلى خلايا مشابهه لخلايا جدار الرحم. وقد تبين من هذه الدراسة أن دم السيدات المصابات بمرض بطانه الرحم المهاجرة يحتوي علي عامل ما أحدث تحورا في الخلايا الجذعية التي اضيف إلي مزرعتها دم السيدات المصابات بهذا المرض بعد ٤ أسابيع من بداية المزرعة بينما لم يحدث هذا التحور في الخلايا المنزرعة مع دم السيدات من مجموعه التحكم ولقد أنتجت الخلايا الجذعية المتحورة كذلك دلالات لتحورها إلي خلايا جدار الرحم متمثلة في بروتين جينات **SPARC** و **MYC**الذي تم إثبات وجوده في الخلايا الجذعية المنزرعة مع دم السيدات المصابات بالمرض بواسطة **(Real time PCR)** بينما لم توجد هذه البروتينات في الخلايا المنزرعة مع دم سيدات من مجموعة التحكم. إن العامل المحفز لمرض بطانة الرحم المهاجرة عباره عن **MiRNA-130a** ولقد تبين ان **MiRNA-130a** هو محفز لتحور الجينات في مرض بطانة الرحم المهاجرة وأثبتت هذه الدراسة وجود هذا العامل المحفز في دم السيدات المصابات بهذا المرض مما يقدم تفسيرا جديدا لسبب حدوث المرض وبالتالي يترك للأبحاث المستقبلي وضع خطط جديده لاكتشاف العلاج المناسب لهذا المرض.