# Sorafenib inhibits growth, migration, and angiogenic potential of ectopic endometrial mesenchymal stem cells derived from patients with endometriosis

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**Objective:** To characterize the proliferation, migration, and angiogenic properties of mesenchymal stem cells (MSC) from ectopic and eutopic endometrial tissue and to investigate the effect of the tyrosine kinase inhibitor sorafenib.
**Design:** In vitro studies.
**Setting:** University hospital and research center.
**Patient(s):** Patients receiving surgical treatment of endometriosis (n = 4) and control patients without endometriosis (n = 2) undergoing surgery for benign gynecologic diseases.
**Intervention(s):** Mesenchymal stem cell lines were isolated from ectopic and eutopic endometrial tissue, and sorafenib was administered to them.
**Main Outcome Measure(s):** Proliferation, migration, invasion of endometrial MSC, and expression of ezrin, vascular endothelial growth factor, and hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$) were measured.
**Result(s):** Ectopic endometrial MSC from patients with endometriosis showed a higher proliferation, migration, and angiogenic ability than eutopic MSC from the same patient or control MSC from patients without endometriosis. Sorafenib reduced the proliferation, motility, ezrin phosphorylation, vascular endothelial growth factor release, and HIF-1$\alpha$ expression of ectopic MSC.
**Conclusion(s):** The increased proliferative, migratory, and angiogenic phenotype of ectopic MSC may be reverted by treatment with sorafenib. Targeting of the MSC population involved in sustaining the ectopic lesions might be useful in eradicating endometriotic implants. (Fertil Steril® 2012;98:1521–30. ©2012 by American Society for Reproductive Medicine.)
**Key Words:** Endometriosis, mesenchymal stem cells, sorafenib, VEGF, ezrin, HIF-1$\alpha$

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Endometriosis is a common gynecologic disorder characterized by the growth of endometrial tissue outside the uterine cavity (1). It affects 6%–10% of all women and 35%–50% of women with pelvic pain and infertility (2). Many hypotheses have been made about the histologic origin of endometriosis. First, Sampson's theory postulated that endometriotic implants may arise from retrograde menstruation of endometrial tissue through the fallopian tubes into the peritoneal cavity (3, 4). In addition, genetic and immunologic factors seem to play important roles in the pathogenesis of endometriosis, because they may be involved in the survival of cells inside the ectopic lesions (5–7). In the last decade, cell

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populations displaying stem cell markers/properties were identified in the *basalis* layer of endometrium (8–17), adding new perspectives to the pathogenesis of endometriosis (18). Endometrial stem cells were concordantly isolated and characterized as cells with mesenchymal origin and multidifferentiative properties (13–17) and were implicated in cyclic endometrial regeneration (9, 10, 15–17). Stem cells with a mesenchymal phenotype were also isolated from ectopic endometrial implants (19). It was proposed that endometrial stem cells, shed through the fallopian tube during menstruation, could be responsible for the establishment of endometrial implants (18). However, the biological mechanisms triggering the characteristics of mesenchymal stem cells (MSC) in the eutopic and ectopic endometrium still deserve further investigation.

Endometriosis is not a cancer; however, it is characterized by some of the adaptive properties of tumor cells, such as migration, invasion, and angiogenesis. Indeed, survival and proliferation of endometrial lesions strictly depend on the formation of new blood vessels, which provide oxygen and nutrient supply (19–21). Accordingly, antiangiogenic treatment and vascular-disrupting agents could represent a possible therapeutic strategy against this pathology (22, 23).

In the present study, we isolated MSC from ectopic endometrial tissue to evaluate their characteristics in terms of proliferation, migration, ezrin phosphorylation, hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$), and vascular endothelial growth factor (VEGF) expression with respect to MSC obtained from eutopic tissue of the same patient. Subsequently, we tested the effect of sorafenib, a multi-tyrosine kinase inhibitor (24), as a possible tool to modulate their growth, migration, and angiogenic properties.

## MATERIALS AND METHODS

### MSC Isolation and Culture

The cell lines were obtained from four patients receiving surgery for treatment of ovarian and peritoneal endometriosis and from two control patients undergoing polypectomy and myomectomy (Supplemental Table 1, available online) in the Department of Obstetrics and Gynecology, University of Torino. Two samples were collected from the same patient with endometriosis—one of eutopic endometrium and one of ectopic implant. This study was approved by the Ethics Review Board of Ospedale Infantile Regina Margherita Sant Anna (Torino). Preoperative informed consent was obtained from each patient. Tissues were processed enzimatically with 0.1% type I collagenase (Sigma-Aldrich) for 30 minutes in a 37°C incubator. Later, cell aggregates were filtered through 60-$\mu$m and 120-$\mu$m meshes. Cells were seeded at a density of 1.0–1.5 $\times$ $10^5$ viable cells (80% viable cells determined by trypan blue) per $cm^2$ in endothelial cell basal medium with an EGM-MV kit (Lonza; containing epidermal growth factor, hydrocortisone, bovine brain extract, and 5% fetal calf serum [FCS]) previously described for MSC isolation (25). Dead cells were poured off 72 hours later and, after 5–7 days, cell clones were typically observed. Cells were passaged at confluence and after 2–3 days in the subsequent passages. The endometrial MSC obtained (eutopic MSC, n = 4; ectopic MSC, n = 4; control MSC, n = 2) were cultured for at least 20 passages to test the self-renewal ability typical of MSC. Bone marrow–derived MSC were cultured in mesenchymal stem cells basal medium (Lonza).

### Flow Cytometric Analysis

Cytometric analysis was performed using FACScan (Becton Dickinson) as previously described (25). Details are provided in Supplemental Materials and Methods (available online).

### Real-Time Polymerase Chain Reaction Analysis

Gene expression was performed by quantitative real time reverse transcription-polymerase chain reaction (RT-PCR) with Applied Biosystems StepOne, as previously described (26). Details are provided in Supplemental Materials and Methods.

### Immunofluorescence and Immunohistochemistry

Immunofluorescence was performed on chamber slides (Sigma) on which cells were fixed in 4% paraformaldehyde containing 2% sucrose for 15 minutes at 4°C, permeabilized with 0.1% Triton X-100 (Sigma) for 8 minutes at 4°C, and then incubated overnight at 4°C with the appropriate antibodies. Anti–pan-cytokeratin (Biomeda), anti-vimentin (Sigma), mouse anti–E-cadherin (DakoCytomation), and rabbit anti-estrogen receptor (ER) (clone SP1; Ventana-Diapath) antibodies were used. Primary antibodies were detected using anti-mouse or rabbit secondary antibodies conjugated with Alexa Fluor 488 or Texas Red (Molecular Probes). Hoescht 33258 dye (Sigma) was added for nuclear staining, and imaging was performed using an LSM 5 Pascal confocal microscope (Carl Zeiss International). Substitution with an unrelated rabbit serum or mouse IgG served as negative control. For immunohistochemistry, cell lines were harvested by cell scraper before reaching confluence, washed, and fixed in 4% neutral-buffered formalin (Histo-Line Laboratories) at room temperature. Afterward, cell pellets were processed to paraffin embedding with an automatic processor (ASP 300; Leica Microsystems). One section from each block was stained with hematoxylin and eosin. Immunohistochemistry was performed on additional sections using an automated slide-processing platform (BenchMark AutoStainer; Ventana) with prediluted anti-ER monoclonal antibody (clone SP1; Ventana-Diapath).

### In Vitro Differentiation

The MSC lines were seeded in differentiating conditions, as previously described (25). For in vitro osteogenic differentiation, cells were cultured (50,000 cells per well) in osteogenic medium (Lonza) for 2 weeks, and differentiation was analyzed by staining with alizarin red (Lonza) for 20 minutes. For epithelial differentiation, cells were seeded at 50,000 cells per well with Roswell Park Memorial Institute 1640 medium (Lonza) with addition of 10% FCS, 20 ng/mL hepatocyte growth factor, 10 ng/mL fibroblast growth factor-4, and $10^{-9}$ M dexamethasone (all from Sigma). After 2 weeks of differentiation, cells were subjected to immunofluorescence. Expression of epithelial markers was analyzed