Increased expression of antimüllerian hormone and its receptor in endometriosis

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Objective: To evaluate antimüllerian hormone (AMH) and AMH receptor II (AMHRII) mRNA and protein expression in endometrium and in ovarian or deep lesions of women with endometriosis.
Design: Prospective study.
Setting: University hospitals in Italy and Brazil.
Patients: Patients with endometriosis (n = 55) and healthy women (n = 45).
Interventions: Specimens of endometrium obtained by hysteroscopy from patients with endometriosis and from healthy control subjects; specimens of ovarian endometriosis (n = 29) or of deep endometriosis (n = 26) were collected by laparoscopy. Serum samples were collected in some endometriotic patients (n = 23) and healthy control subjects (n = 20).
Main Outcome Measure(s): AMH and AMHRII mRNA levels were evaluated by quantitative reverse-transcription polymerase chain reaction and protein localization by immunohistochemistry. AMH levels in tissue homogenates and in serum were assessed by ELISA.
Result(s): Endometrium from women with endometriosis showed higher AMH and AMHRII mRNA levels than control women, with no significant differences between proliferative and secretory phases. Specimens collected from ovarian or deep endometriosis showed the highest AMH and AMHRII mRNA expression. Immunolocalization study confirmed the high AMH and AMHRII protein expression in endometriotic lesions. No difference of serum AMH levels between the groups was found.
Conclusion(s): The increased AMH and AMHRII mRNA and protein expression in endometrium and in endometriotic lesions suggests a possible involvement of AMH in endometriosis. (Fertil Steril® 2014;101:1353–8. ©2014 by American Society for Reproductive Medicine.)
Key Words: AMH, AMHRII, TGF-β superfamily, endometriosis, ovarian and deep endometriosis

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Endometriosis is a benign gynecologic disease characterized by the presence of endometrial cells outside the uterine cavity; it affects 6%–10% of women of reproductive age and is associated with pelvic pain and infertility (1, 2). Altered E_2 and P receptor activity and impaired local growth factor and cytokine expression induce proliferation of endometrial cells, peritoneal adhesion, and inflammation of endometriotic lesions (3). Among growth factors, an involvement of transforming growth factor β (TGF-β) superfamily in cell proliferation, immune function, and apoptosis in endometriosis has been shown (4–6). Antimüllerian hormone (AMH), also known as müllerian inhibiting substance (MIS), is a member of TGF-β superfamily (7), playing an essential role in sexual differentiation. In the female embryo, the absence of AMH allows the development of the Müller ducts and their differentiation into fallopian tubes, uterus, cervix, upper vagina, and ovarian surface epithelium (8). In adult female reproductive organs, the biologic functions of AMH is defined only in the ovary. In adult women, AMH is produced by granulosa cells in preantral and antral follicles (9), and the main physiologic role is the

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inhibition of the early stages of follicular development and follicular recruitment (10). Recently, serum AMH has been recognized as a useful diagnostic and prognostic tool as a reliable marker of the ovarian reserve and predictor of the ovarian response to controlled ovarian hyperstimulation (11), as well as an early indicator of relapse of ovarian granulosa cell tumors (12).

As a member of the TGF-β superfamily, AMH exerts its effect by binding to a heterodimeric transmembrane serine/threonine kinase cell surface receptor complex, consisting of its specific type II receptor (AMHRII), which then binds to a type I receptor of the activin-like kinase family (ALK2, ALK3, and ALK6) (13). The heterodimeric type I–type II receptor complex triggers a downstream signaling cascade of phosphorylation, activating Smads 1, 5, and 8 (14). The Smad signaling complex then enters the nucleus and interacts with transcription factors to induce gene expression for apoptosis (15), specifically for regression of müllerian ducts in the male embryo (13).

Glandular and stromal endometrial cells express mRNA and protein for AMH and AMHRII, resulting in measurable AMH in culture media (16); in agreement with the proapoptotic effect (13, 15), when AMH is added to the cultured human endometrial stromal cells, an increase of caspase 3/7 occurs, causing a decrease of cell viability (16). An effect of AMH on cell proliferation and cell death has been found also in endometrial/cervical cancer cells (17, 18), and even more pronounced in cultures of endometrial cells from endometriotic patients, where AMH was found to induce significant increase of annexin V (17).

Because of the involvement of the AMH/AMHRII pathway in modulating endometrial proliferative and invasive behavior, and because this is a typical characteristic of both endometrium and ectopic lesions (19), we aimed to evaluate the expression of AMH/AMHRII in eutopic and ectopic endometrium of endometriosis. In particular, we tested the hypothesis that: (1) endometrial AMH and AMHRII mRNA and protein expression in endometriotic patients is different from that in healthy women; (2) ovarian (endometrioma) and extraovarian (deep) endometriotic lesions express a specific AMH/AMHRII pattern (the two sites were also selected to exclude that the expression in endometrioma may depend by a possible contamination of ovarian tissue); and (3) the different expression of AMH in endometrium and lesions does not affect circulating AMH secretion of endometriotic patients.

MATERIALS AND METHODS

Patients and Tissue Collection

The study population consisted of women with regular menstrual cycles (28–32 days) undergoing gynecologic surgery (age range 21–39 years) and was divided into:

Endometriosis group: ovarian endometrioma (n = 29) and deep endometriosis (n = 26) undergoing laparoscopic treatment (for pain and/or infertility).

Nonendometriosis group: women without endometriosis (n = 45; control subjects) undergoing laparoscopy for tubal sterilization.

For each subject, a complete medical history was obtained and physical examination performed. Patients with hormonal treatment in the past 3 months were excluded from the study. Each of the patients gave written informed consent. The study was approved by the local Human Investigation Committee.

In all of the women, a specimen of endometrium was collected by hysteroscopy and separated into proliferative (days 5–14) and secretory phases (day 15 onward), according to the last day of menstruation, transvaginal ultrasound scan, and histologic criteria. In the endometriosis group, all patients showed stage III or IV endometriosis according to the American Society for Reproductive Medicine classification (20). Specimens of endometriotic lesions were collected during laparoscopic surgery. Ovarian endometriotic tissues (cyst diameter measured by ultrasound ranged from 38 to 72 mm) were carefully stripped from the inner cyst wall avoiding contamination with ovarian tissues. Deep endometriosis specimens were collected from cases with bowel or rectovaginal (n = 23) or bladder (n = 3) involvement. Serum samples were also collected in endometriotic (n = 23; 11 with endometriosis and 12 with deep endometriosis) and healthy (n = 20) women 1 week before surgery.

All specimens were immediately frozen and stored in liquid nitrogen for RNA and protein extraction or paraffin embedded for immunohistochemistry; they were all analyzed at the same time.

RNA Extraction and cDNA Preparation

Total RNA was extracted with the use of the SV Total RNA Isolation System (Promega), according to manufacturer's instructions. RNA was quantified by ultraviolet absorption and RNA integrity checked before downstream analysis with the use of the Flashgel System (Lonza Group). For cDNA synthesis, one microgram of total RNA was reverse transcribed with the use of Improm-II Reverse Transcriptase (Promega).

Reverse Transcription Polymerase Chain Reaction

Endometrial AMH and AMHRII expression was analyzed by quantitative reverse-transcription (RT) polymerase chain reaction (PCR). AMH and AMHRII mRNA levels, normalized for the transferrin receptor (TFRC) as housekeeping gene were measured in triplicate with the use of the 2 × Sybr Select Master Mix for CFX (Applied Biosystems) according to the manufacturer's protocol, on a CFX Connect 96 (Bio-Rad Laboratories) real-time PCR system.

Gene-specific primers used for PCR were chosen according to the published sequences of human AMH (Genbank accession no. NM_000479.3), AMHRII (Genbank accession no. NM_002192.2), and TFRC (Genbank accession no. NM_003234.2). The AMH forward primer was 5′–TCC GAG AAG ACT TGG ACT GG–3′, the reverse primer was 5′- TCC TCC AGG TGT AGG ACC AC–3′, and the expected size of the amplified fragment was 296 bp; the AMHRII forward primer was 5′–GAG ATC ATC ACG TTT GCC GAG–3′, the reverse primer was 5′- GAA GAG CCA GAC TTC TGC ACG–3′, and the expected size of the amplified fragment was 258 bp; the TFRC forward primer was 5′–ACC GGC ACC

ATC AAG CT–3′, the reverse primer was 5′- TGA TCA CGC CAG ACT TTG C–3′, and the expected size of the amplified fragment was 134 bp. All of the RT-PCR primer pairs used in this study span exon-exon junctions or are located on different exons. In selected experiments, PCR products were run on 3% agarose gel to check molecular size. For each RNA specimen, a negative control sample was prepared by omitting the reverse transcriptase.

Amplifications were carried out at 95°C for 5 minutes, followed by 10 seconds at 95°C and 30 seconds at 60°C for 45 cycles, with fluorescence detection at the end of each extension step. For each run, melting-curve analysis was used to confirm the specificity of the amplified products and the absence of primer-dimer formation. Quantitative RT-PCR was carried-out against standard curves created with 10–10^6 copies of AMH, AMHRII, and TFRC cDNA. For each sample, the amount of AMH, AMHRII, and TFRC mRNA was determined from the calibration curves. The target number of copies was divided by those of TFRC to obtain a normalized value.

Immunohistochemical Analysis

Endometrial and endometriotic specimens were fixed in 10% neutral-buffered formalin for 24 hours at room temperature. Sections (4 μm) were deparaffinized and rehydrated in alcohol. For immunohistochemistry, after dewaxing, sections were incubated overnight at 4°C with an anti–human AMH antibody (AF1737; R&D Systems), and an anti-AMHRII antibody (AP7111c; Abgent). Slides were washed three times with phosphate-buffered saline solution and endogenous peroxidase blocked with 3% hydrogen peroxide for 30 minutes at room temperature. Finally, incubation was carried out with the EnVision + System-HRP (Dako) for 45 minutes at room temperature. Slides were stained with diaminobenzidine, counterstained with Mayer hematoxylin, and mounted. In negative control samples, incubation with the primary antibodies was omitted. For positive control samples, ovarian sections containing follicles at different stage of development were used.

AMH Assay

AMH content in homogenates of endometriotic lesions or in serum samples was measured with the use of a commercial AMH ELISA kit according to the manufacturer's instructions (USCN Life Science; catalog no. CEA228-Hu). The detection range was 37.0–3,000 pg/mL, with a sensitivity of <13.2 pg/mL. The intra- and interassay coefficients of variation were <10% and <12%, respectively.

Statistical Analysis

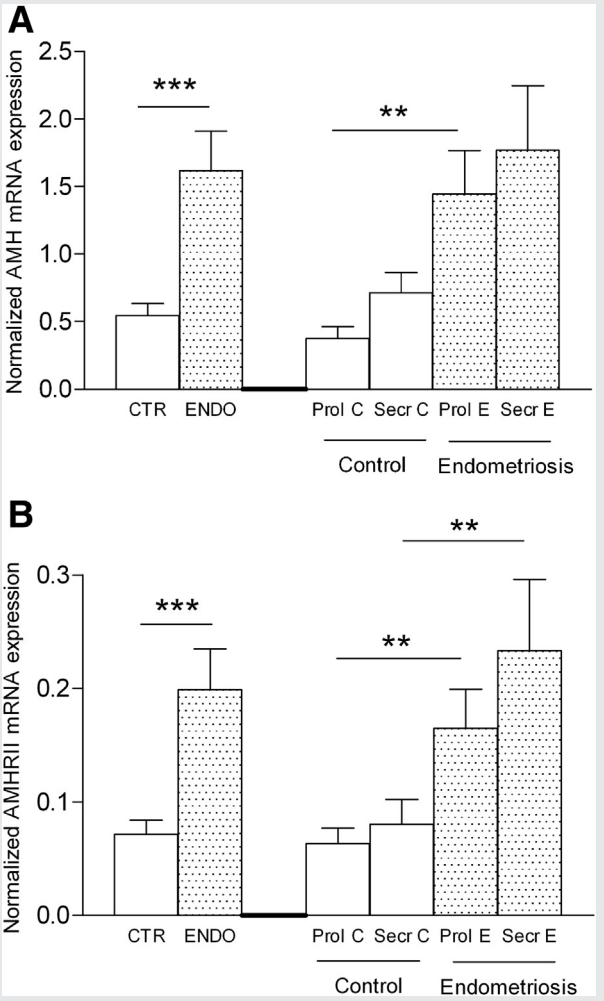
All data were assessed for normal distribution with the use of the Shapiro-Wilk test and Sigmaplot 12.0 software (Systat Software). Data not normally distributed were analyzed with a nonparametric test (Kruskal-Wallis one-way analysis of variance), followed by the Dunn post hoc test for multiple comparisons. Normalized RNA values in healthy and endometriotic specimens and corresponding control samples at different phases of the menstrual cycle, as well as serum levels, were compared with the use of a Student *t* test for unpaired data. Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was set for *P* values of < .05.

RESULTS

AMH and AMHRII mRNA Expression in Endometrium of Endometriosis and Control Subjects

Quantitative RT-PCR showed that AMH and AMHRII mRNA expression was significantly higher in endometrium of patients with endometriosis than in control subjects (AMH 3.4-fold: $P<.001$; AMHRII 2.8-fold: $P<.001$), without showing a significant change according to menstrual cycle (Fig. 1). AMH mRNA levels both in proliferative (3.9-fold:

FIGURE 1



(A) Antimüllerian hormone (AMH) and (B) AMH receptor type II (AMHRII) mRNA expression in human eutopic endometrium of healthy women (CTR) and of women with endometriosis (ENDO). On the right side of the graphs are the mRNA expression levels during proliferative (Prol) and secretory (Secr) phases of menstrual cycle. Fold change (*y* axis) represents mRNA expression normalized to transferrin receptor (TFRC). **$P<.01$; ***$P<.001$.

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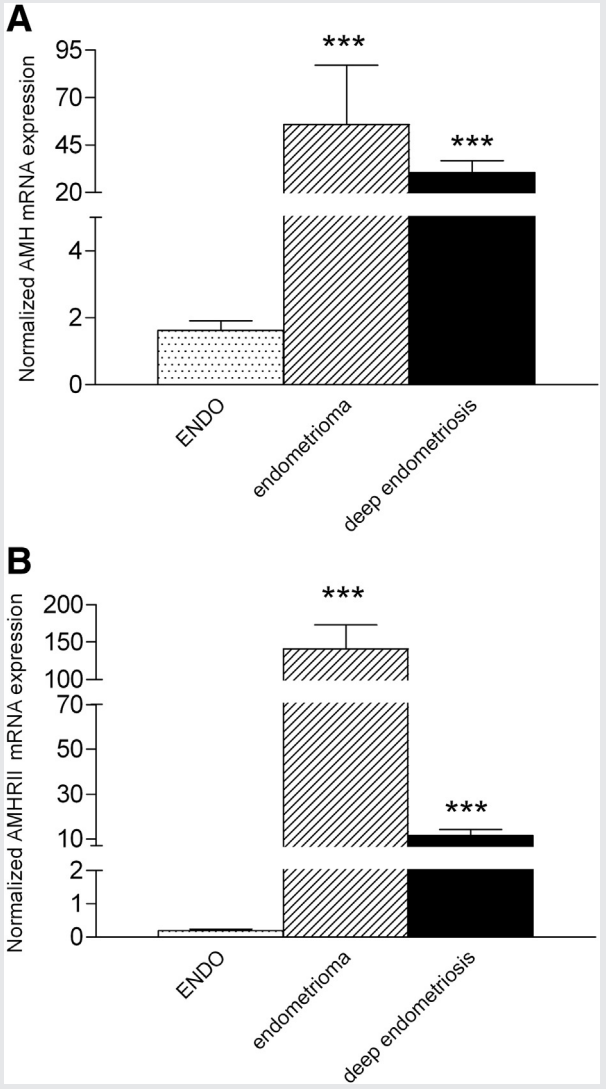
$P<.001$) and in secretory endometrium (2.5-fold: $P=.05$) of endometriosis were significantly higher than in control samples (Fig. 1A); similarly, endometrial AMHRII mRNA levels in endometriosis were significantly in both proliferative (2.61-fold: $P<.01$) and secretory (2.9-fold: $P<.01$; Fig. 1B) phases.

AMH and AMHRII mRNA and Protein Expression in Endometriotic Lesions

AMH mRNA expression analysis showed significantly higher levels in endometrioma and deep endometriosis compared with eutopic endometrium (endometrioma 34.2-fold: $P<.001$; deep endometriosis 18.8-fold: $P<.001$; Fig. 2A).

FIGURE 2



(A) AMH and (B) AMHRII mRNA expression in eutopic endometrium of women with endometriosis (ENDO), in ectopic endometrium of women with ovarian endometriosis, and in ectopic endometrium of women with deep endometriosis. Fold change (*y* axis) represents mRNA expression normalized to TFRC. ***$P<.001$. Abbreviations as in Figure 1.

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AMHRII mRNA expression pattern was similar, with significantly higher levels in both endometrioma (704-fold: $P<.001$) and deep endometriosis (57-fold: $P<.001$) than in endometrium (Fig. 2B).

Immunohistochemical localization of AMH and AMHRII showed the presence of both proteins in endometrial glandular epithelium and stromal cells (Fig. 3A and B), with an intense staining in endometrioma (Fig. 3C and D) and deep endometriosis (Fig. 3E and F). Immunohistochemical staining in positive control ovarian sections confirmed AMH and AMHRII antibody specificity, and negative control samples did not stain at all (data not shown).

When AMH concentration was measured by ELISA, high protein levels were detected in both endometrioma (44 ± 7.2 pg/μg of protein; $n = 7$) and deep endometriosis (85.6 ± 10.3 pg/μg of protein; $n = 7$). Blood concentrations of AMH were in the normal range without any significant difference between healthy control subjects (mean 2.13 ± 0.38 ng/mL) and endometriotic patients (mean 1.75 ± 0.43 ng/mL).

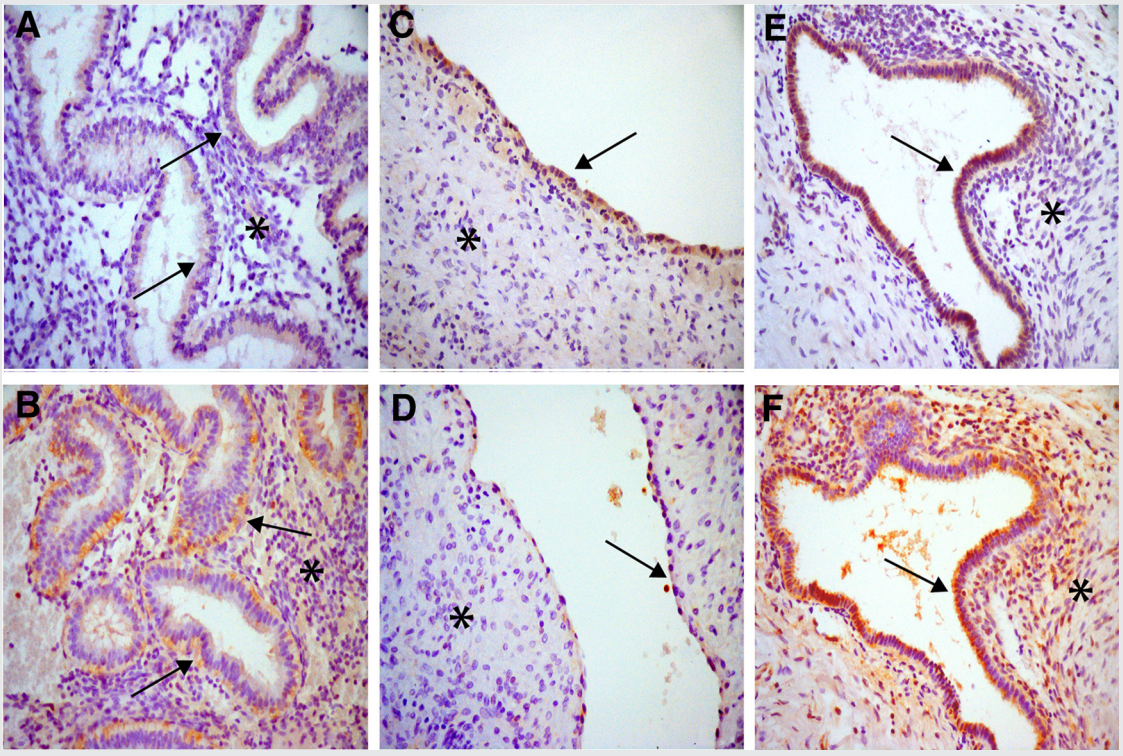
DISCUSSION

The present study showed that: 1) endometrial AMH and AMHRII mRNA and protein expression are elevated in endometrium of women with endometriosis; 2) endometriotic lesions express even higher levels of AMH and AMHRII mRNA and protein (and the elevated AMH expression in deep endometriosis lesions clearly rules out a possible contamination from ovarian tissues); and 3) the unchanged levels of serum AMH in women with endometriosis exclude a secretion from endometriotic lesions into bloodstream.

The present endometrial findings support the concept that endometrium is a source and a possible target of AMH and TGF-β–related factors. Cell growth and differentiation (21, 22), injury repair (23), immune response, and extracellular matrix remodeling (21, 22) are the main mechanisms regulated by TGF-β–related factors in endometrium. In particular, activin A (24, 25), bone morphogenetic proteins (BMPs), and growth differentiation factors (26) modulate cell differentiation and proliferation, apoptosis, and tissue remodeling, and AMH in endometrial cell cultures increases apoptosis (16). The effect of AMH on proliferation and cell death was even more pronounced in cultured endometrial/cervical cancer cells (18, 27), as well as in endometrium of patients with endometriosis (16, 17). The lack of significant changes in AMH/AMHRII mRNA levels throughout the menstrual cycle reported in the present study, suggests a sex hormone–independent expression like that of BMP-4, another TGF-β family member whose expression is dysregulated in endometrium of women with endometriosis (28). The increased expression of AMH in the endometrium of endometriotic patients is consistent with increased expression of TGF-β1, TGF-β2, TGF-β3 (5), and activin A (6) in endometrium of endometriosis, supporting the hypothesis that TGF-β family members may contribute to impaired endometrial functions and infertility in endometrium, owing to their effects on stromal-epithelial interactions and local inflammatory events.

The present study is the first to show increased AMH/AMHRII expression in lesions from endometrioma or deep

FIGURE 3



Immunohistochemical analysis of AMH and AMHRII expression in endometriosis. Immunohistochemistry was performed with the use of an indirect peroxidase technique (see Methods). AMH expression in (A) control endometrium, (C) ovarian endometriosis, (E) and deep endometriosis; expression of AMHRII in (B) control endometrium, (D) ovarian endometriosis, and (F) deep endometriosis. Original magnification 100×. Positivity is shown by brown staining. Arrows indicate epithelial cells; asterisks indicate stroma cells. Abbreviations as in Figure 1.

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endometriosis in terms of gene and proteins and opens up the question of the biologic significance. In endometrioma and deep endometriosis lesions, chronic inflammation and fibrosis represents a common finding and TGF-β signaling is critically involved in the fibrotic reaction (29, 30), enhancing the expression of a fibrosis marker (31). TGF-β family members and AMH act through serine/threonine kinase receptors and Smad effectors, and the regulate Smad expression and phosphorylation in endometrial epithelial and stromal cells (13, 14, 32, 33). In particular, AMH gains access to the Smad system through its specific type II receptor, sharing some biologic action with other TFG-β family members (34). Earlier studies showed that TGF-β/Smad signaling is activated in endometrioma lesions (35), as well as via the activin/crypto pathway (6), and is impaired in peritoneal endometriosis (36). Therefore, the increased AMH/AMHRII pathway in endometriotic lesions may have an impact on the development of the disease, probably affecting inflammation and apoptosis.

The evidence that serum AMH levels are unaffected by increased AMH mRNA and protein expression in endometriotic lesions excludes possible secretion in general circulation and its possible use as a marker of endometriosis and underscores a local autocrine/paracrine action of endometrial AMH. Moreover, our findings support earlier studies on infertility in women with endometriosis (37, 38), excluding a possible direct effect of ectopically produced AMH on ovarian reserve of women with endometriosis.

In conclusion, the present study showed an increased expression of AMH and AMHRII in endometrium and endometriotic lesions, suggesting a possible involvement of AMH in the pathogenic development of endometriosis.

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