Endometriosis may be generated by mimicking the ontogenetic development of the female genital tract

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Objective: To compare the expression of genes playing a decisive role during the embryonic development of the female genital tract (*WNT4, WNT5A, WNT7A, PAX8*) in the peritoneum of patients with endometriosis and control patients.
Design: Experimental study using real-time polymerase chain reaction and in situ hybridization.
Setting: University-based laboratory.
Patient(s): Patients with and without endometriosis undergoing surgery for benign indications.
Intervention(s): None.
Main Outcome Measure(s): Percentage of samples positive for gene expression by using real-time polymerase chain reaction, as well as relative expression values compared with housekeeping genes. Confirmation of results by in situ hybridization.
Result(s): Expression of *WNT7A* and *PAX8* was found in the normal peritoneum in approximately half of the patients with endometriosis in contrast to the controls. In patients with endometriosis *WNT7A* and *PAX8* in histologically normal peritoneum (with no evidence of endometriosis, endosalpingiosis, or other changes) were confirmed by in situ hybridization.
Conclusion(s): The expression of these genes in the normal peritoneum suggests that endometriosis can arise through metaplasia and can in the process make use of the developmental steps involved in the embryonic development of the female genital tract. (Fertil Steril® 2007;87:651–6. ©2007 by American Society for Reproductive Medicine.)

Key Words: WNT wingless-type MMTV integration site family, PAX8 paired box gene 8, peritoneum, endosalpingiosis, endometrium

Endometriosis is one of the most common gynecologic diseases. Some 10% of women of reproductive age and approximately 30%–55% of infertile women have endometriosis (1–3). In addition to sterility and dysmenorrhea, dyspareunia and cyclic lower abdominal pain are the most frequent symptoms. Although endometriosis has been the subject of numerous scientific investigations, the central elements involved in the pathogenesis of endometriosis are as yet unexplained. There are several theories regarding the development of endometriosis, each of which is supported by clinical observations and scientific results (4). However, none of these models is able to explain the various aspects of endometriosis fully, and none has been recognized as an ultimately valid explanatory model.

The various models can be traced back to two basic ideas. One group of theories is based on the hypothesis that endometriosis develops through endometrial cells that are transported by various routes (transtubal, hematogenous, or lymphogenous, or by disturbed uterine peristalsis) and implant in the peritoneum or affected organs. The best known and most widespread theory in this group is Sampson's transplantation theory, which is based on the assumption that endometrial cells enter the abdomen through retrograde menstruation (5). Less widely recognized are concepts according to which endometriosis develops through metaplastic processes in the peritoneum (the coelomic metaplasia theory, secondary müllerian system) or from scattered embryonic rests (6). The basic ideas underlying these theories were first formulated about 100 years ago. Because the pathophysiology of endometriosis is as yet unexplained, the only treatments available involve symptomatic rather than causal approaches to therapy.

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TABLE 1

Patients' characteristics.

	Diagnosis	No. of patients
Peritoneum control group[a]	Infertility	10
	Uterine fibroid	2
	Hydrosalpinx	1
	Paraovarian cyst	1
	Ovarian inclusion cyst	1
	Ovarian fibroma	1
	Pelvic pain (laparoscopy without pathologic findings)	1
	Tubal ligation	1
Peritoneum patients with endometriosis	Stage revised AFS I	6
	Stage revised AFS II	3
	Stage revised AFS III	3
	Stage revised AFS IV	3

[a] Patients without endometriosis = control group.

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During the development of the female genital tract from the müllerian (paramesonephric) duct in embryonic development, a number of genes that are activated in a specific sequence are decisively important. *WNT* signaling has a special role in the early steps of the developmental process (7, 8). In a fold of the coelomic epithelium that shows expression of *WNT4* (wingless-type MMTV integration site family gene member 4), the müllerian duct is found on the following day of embryonic development. The müllerian duct shows a high level of expression of *WNT7A* and paired box gene 8, *PAX8*. Studies in mice have shown that the interaction between *WNT7A* in the epithelium and *WNT5A* in the surrounding stroma is decisively important for the development of the uterine glands (9). *WNT7A* is thought to regulate both the unfolding of the epithelium and also the development of the smooth musculature in the surroundings of the endometrial tissue, and in the adult endometrium it is thought to regulate the expression of additional endometrial differentiation markers such as the homeobox (*HOX*) genes and ERα (9–11). In knockout mice, the loss of *WNT4* leads to complete absence of the müllerian duct and its derivatives, and the absence of *WNT7A* or *WNT5A* leads to the development of a hypoplastic uterus without endometrial glands (7–9).

The present study investigates the expression of these genes, which provide the prerequisites for the development of the female genital tract and in particular of the endometrium, in macroscopically normal peritoneum in patients with endometriosis and control patients.

MATERIALS AND METHODS

Materials

Endometrial and peritoneal biopsy samples were taken from patients undergoing hysterectomy, dilation and curettage, and other procedures for benign gynecologic disease. Peritoneal biopsy specimens were taken from the lower anterior abdominal wall in all patients. Samples from endometriotic lesions were obtained from patients undergoing laparoscopy for endometriosis. Patient characteristics are given in Table 1. The study was approved by the local ethics committee.

Analysis of Messenger RNA Expression by Real-time Polymerase Chain Reaction

Total RNA from human endometrial and peritoneal tissues was isolated with the guanidinium isothiocyanate method, as described elsewhere (12), in combination with affinity purification (Rneasy; Qiagen, Hilden, Germany). Complementary DNAs (cDNAs) were generated by randomly primed reverse transcription (ProSTAR cDNA Synthesis Kit; Stratagene, La Jolla, CA). Real-time polymerase chain reaction (PCR) analyses were carried out with the ABI 7700 sequence detection system (PE Applied Biosystems, Foster City, CA) as previously described (13) by using "assays on demand" for hs*WNT4* (acc. NM_030761), hs*WNT7A* (acc. NM_004625), hs*WNT5A* (acc. NM_003392), and hs*PAX8* (acc. NM_013992), respectively. All of the assays were located in the same region as the riboprobes used for in situ hybridization, allowing comparison of in situ hybridization and real-time PCR data. VIC fluorophore-labeled glyceraldehyde-3-phosphate dehydrogenase (GPDH) TaqMan probes served as internal quantification markers in multiplex PCR reactions. Each quantitation was reproduced three times and normalized by GPDH.

Preparation of Digoxigenin-labeled cRNA Probes

Polymerase chain reaction was used to obtain template cDNAs of hs*WNT4* (acc. NM_030761), hs*WNT7A* (acc.

NM_004625), hs*WNT5A* (acc. NM_003392), and hs*PAX8* (acc. NM_013992). The primer sequences were as follows; T7 promoter sites are underlined:

WNT4-U3 CCAGGTGGGTCACGCACTG
WNT4-T7-L4 TAATACGACTCACTATAGGGAGACTGTTTAAATTATCGGC
WNT5A-U2 TCGCCATGAAGAAGTCCATTG
WNT5A-T7-L2 TAATACGACTCACTATAGGGCCGATGTACTGCATGTGGTC
WNT7A-U3 TGGGCCACCTCTTTCTCAGC
WNT7A-T7-L2 TAATACGACTCACTATAGGG CGGAACTGAAACTGACACTC
PAX8-U5 GGCAATGCCTATGGCCACAC
PAX8-T7-L3 TAATACGACTCACTATAGGGTTCAGCATGGCATGGTTCTC

Before cRNA probe synthesis, the PCR products were purified by ultrafiltration with use of Millipore UFCTTK30 filters (Millipore, Billerica, MA). An amount of 1 μg DNA was used to prepare antisense and sense single-strand RNA probes labeled with digoxigenin with a digoxigenin RNA labeling kit (Roche Diagnostics Ltd., Mannheim, Germany) in accordance with the manufacturer's specifications. In brief, 10 μL of master mix for each probe template was prepared to contain final concentrations of 1 mmol/L adenosine triphosphate, cytidine triphosphate, and guanosine triphosphate; 0.65 mmol/L uridine triphosphate; 1× transcription buffer; 1 U/μL ribonuclease (RNase) inhibitor; and 20 U/μL T7 RNA polymerase. The reactions were incubated at 37°C for 2 hours. Two microliters of 10 U/μL RNase-free deoxyribonuclease (DNase) I was added to each sample, which was then incubated at 37°C (15 minutes) to remove the DNA template. Reactions were stopped by adding 2 μL of 0.2 mol/L ethylenediaminetetraacetic acid. Gel electrophoresis was used to control cRNA products. Probe concentrations were adjusted by spotting dilutions on nylon filters and analyzing them with the digoxigenin detection kit. The probes were used at a final concentration of 50 ng/mL hybridization mix.

In Situ Hybridization

In situ hybridization was carried out on samples quick-frozen in liquid nitrogen. Tissue sections of 8 μm were cut, mounted directly on Superfrost Plus slides (MICROM International GmbH, Walldorf, Germany), and heated for 2 minutes at 50°C to immobilize RNA. The sections were then air-dried for 30 minutes. Tissue sections were subsequently fixed by incubation in phosphate-buffered saline solution/4% paraformaldehyde (pH 9.5) for 60 minutes (14) and washed three times with phosphate-buffered saline and once with 2× SSC for 10 minutes, followed by prehybridization for 60 minutes at 50°C in 100 μL hybridization buffer (4× SSC, 10% dextran sulfate, 1× Denhardt's solution [0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin] (Sigma-Aldrich, Munich, Germany), 2 mmol/L ethylenediaminetetraacetic acid, 50% deionized formamide, and 500 μg/mL herring sperm DNA). The prehybridization buffer was removed, and the section was covered with 100 μL hybridization buffer containing the freshly denatured probe (10 minutes at 70°C). After hybridization for 16 hours, the sections were washed under high stringency for 3 × 5 minutes with 60% formamide in 0.2 × SSC at 37°C and 2 × 5 minutes with 2 × SSC at room temperature. The digoxigenin detection procedure was carried out in accordance with the manufacturer's protocol.

Statistical Analyses

All reported *P* values are two-sided. We considered *P* values of $<.05$ to indicate a significant result. Fisher's exact test was used to test for associations between expression of markers and the subgroup of patients. All analyses were performed with the use of SPSS 11.0 (SPSS Inc., Chicago, IL).

RESULTS

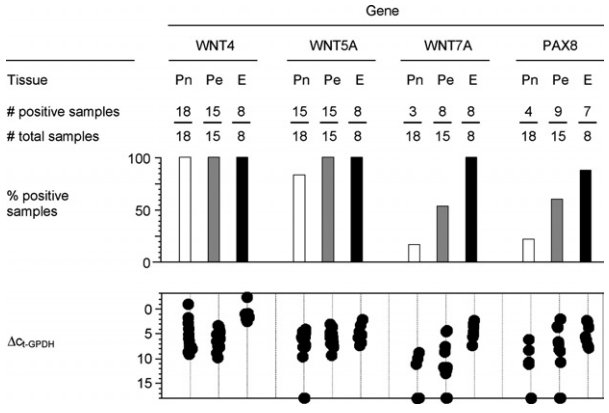
Analysis of the *WNT* Signaling Pathway in Patients With Endometriosis Using Quantitative Real-time PCR

Quantitative reverse transcription–based PCR (qRT-PCR) was used to compare the messenger RNA (mRNA) expression of several key components of the *WNT* signaling cascade in biopsy specimens of normal endometrium and endometriosis tissue, as well as peritoneum from patients without endometriosis, and of peritoneum without pathologic findings from patients with endometriosis. Table 1 gives a compilation of the clinical characteristics of the patients. All peritoneal samples were obtained from corresponding sites to ensure highest comparability.

As shown in Figure 1, expression of *WNT4*, *WNT5A*, and *WNT7A* was detectable in all of the endometrial and endometriosis biopsy samples investigated, whereas only one of eight samples was negative for *PAX8*. *WNT4* and *WNT5A* were also expressed in nearly all of the peritoneal biopsy specimens investigated. However, the frequency of *WNT7A*-positive and *PAX8*-positive samples was markedly lower in peritoneum from control patients (n = 3/18 and n = 4/18, respectively). Intriguingly, when *WNT7A* and *PAX8* were compared in peritoneal tissue from patients with endometriosis and from control patients, a higher frequency of samples positive for both *WNT7A* (n = 8/15 vs. n = 3/18, $P=.061$, Fisher's exact test) and *PAX8* (n = 9/15 vs. n = 4/18, $P=.038$, Fisher's exact test) was evident in peritoneum without pathologic findings from patients with endometriosis. In addition, when the levels of *WNT7A* and *PAX8* mRNA expression as revealed by Δct values were analyzed (Fig. 1), a clear trend toward higher levels of expression was observed when peritoneum from control patients and patients with endometriosis was compared. No significant correlation of *WNT7* and *PAX8* expression and the staging of patients with endometriosis was detected.

FIGURE 1

Messenger RNA expression in the *WNT* pathway components as analyzed by qRT-PCR. Real-time PCR analysis was carried out with use of TaqMan probes for *WNT4*, *WNT5A*, *WNT7A*, and *PAX8* with cDNA from endometrium, as well as endometriosis tissue samples (*E*); peritoneum without pathologic findings from patients without endometriosis (*Pn*); and peritoneum from patients with endometriosis (*Pe*). Expression levels were normalized as Δct values in comparison with endogenous GPDH mRNA. *Upper panel:* Bar graph of the percentage of samples positive for expression derived from the peritoneum of patients without endometriosis (*white bars, Pn*), the peritoneum of patients with endometriosis (*gray bars, Pe*), and endometrium as well as endometriosis tissue (*black bars, E*). The absolute number of positive samples, compared with the total number of samples, is given above the respective bars. *Lower panel:* Scatter plot of Δct_{GPDH} values for all samples from the bar graph in the upper panel to show the distribution of relative expression values.



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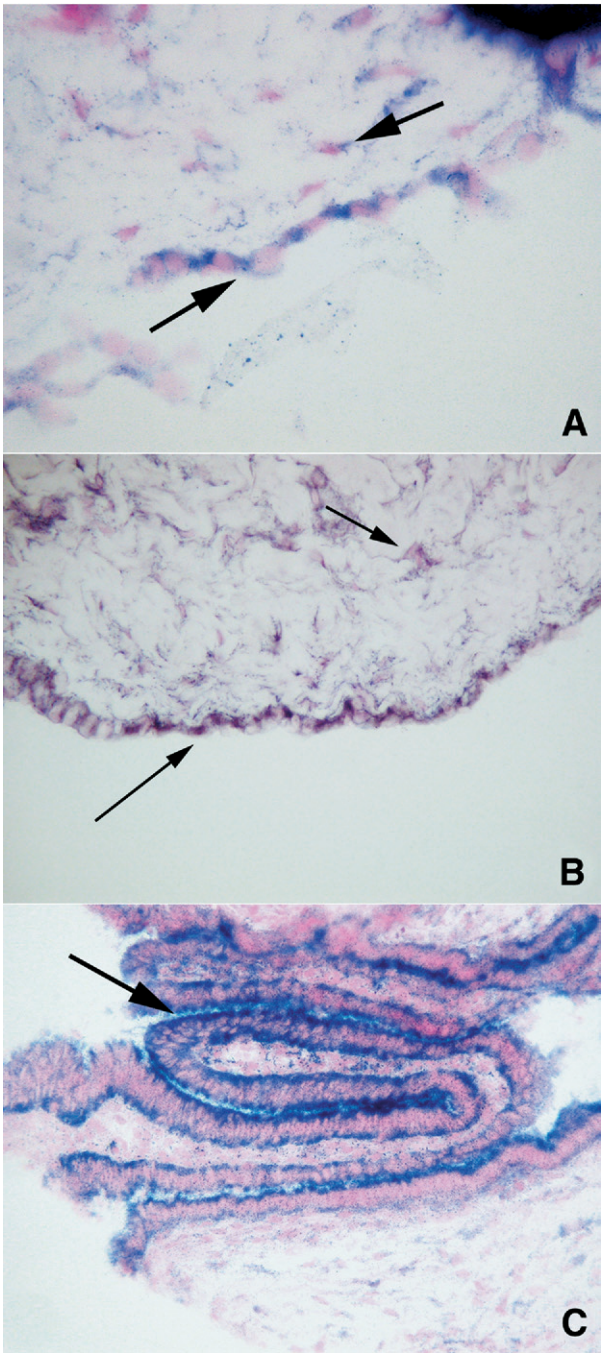
Analysis of the *WNT* Signaling Pathway in Patients With Endometriosis Using In Situ Hybridization

In situ hybridization was used to identify those specific cells in the peritoneal samples that are the source for the altered expression of *WNT7A* and *PAX8*. In endometrium and endometriosis, *WNT4*, *WNT7A*, and *PAX8* were detected in luminal and glandular epithelial cells. *WNT5A* was found in epithelial and stroma cells, with stronger expression of *WNT5A* in endometriotic stroma cells in comparison with the endometrium. Epithelial cells in endosalpingiosis showed strong expression of *WNT4*, *WNT5A*, *PAX8*, and *WNT7A* (as exemplified in Fig. 2C). In the surrounding tissue, expression of *WNT5A* was found in a few cells. In contrast, hybridization with sense probes as negative controls revealed no staining. Having

FIGURE 2

Messenger RNA expression in the *WNT* pathway, as analyzed by in situ hybridization. Histologically normal-appearing peritoneum from a patient with endometriosis, with *WNT5A* (**A**) and *WNT7A* (**B**). The mesothelial and stroma cells (*arrow*) show positive staining. The infolded epithelium of a sample of endosalpingiosis tissue shows *WNT7A* expression (**C**). Specimens are counterstained with nuclear fast red.



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established the in situ hybridization for *WNT*s and *PAX8* we next analyzed peritoneal tissues. In the peritoneum without pathologic finding from patients with endometriosis, expression of *WNT4*, *WNT5A*, and also *WNT7A* and *PAX8* was detected in mesothelial cells. *WNT5A* and *WNT7A* were also expressed in stroma cells. Figure 2 exemplifies results obtained with *WNT5A* (Fig. 2A) and *WNT7A* (Fig. 2B) showing expression in both mesothelial and stromal cells.

DISCUSSION

The present study has shown that expression of the genes that enable the development of the female genital tract and endometrium can be demonstrated in the peritoneum. *WNT4* and *WNT5A* are expressed ubiquitously in the peritoneum, whereas *WNT7A* and *PAX8* are observed in a higher percentage of cases in endometriosis than in control individuals. Because macroscopically normal-appearing peritoneum is found to have endometriosis in up to 13% of cases on histopathologic examination, and, as the results of the present study show, *WNT4*, *WNT5A*, *WNT7A*, and *PAX8* are found both in the endometrium and also in endometriosis and endosalpingiosis, in situ hybridization was required to demonstrate that the expression of *WNT7A* and *PAX8* is not due to previously existing endometriosis or endosalpingiosis (15, 16). In situ hybridization identified *WNT7A* and *PAX8* in the peritoneum without metaplastic or pathologic changes.

Experiments with knockout mice (9–11) have shown that the expression of *WNT4*, *WNT5A*, and *WNT7A* is of central importance for the development of the uterus and a normal endometrium, whereas *PAX8* deletion does not induce defects in the female reproductive tract, possibly because of rescue of function by other paired box genes (17). During embryonic development of the female genital tract, *WNT7A* has to be expressed in the epithelial cells and *WNT5A* in the stroma cells to ensure normal development. In mice with a *WNT7A* mutation, *WNT5A* expression in the adult stroma is lost but is limited to the epithelium. In heterozygotic mice, however, *WNT5A* is found in both stroma and epithelium. By contrast, in the wild type, *WNT5A* is mainly found in the stroma. *WNT7A* thus appears to play a decisive role in the regulation of *WNT5A* expression via a gene-dose effect.

The interaction between *WNT5A* and *WNT7A* appears to play the crucial role in the development of endometrial tissue. Heterozygotic animals with *WNT5A* expression in the stroma and in the epithelium show increased formation of endometrial glands (9). In the peritoneum of patients with endometriosis, the present study identified evidence of *WNT7A* expression in the epithelial cells and expression of *WNT5A* in the epithelium and in the stroma. This consequently demonstrates that the decisive "tools" for the development of tissue with müllerian differentiation can be induced in the peritoneum.

In the peritoneum of control individuals in whom no endometriosis was found, expression of *WNT7A* or *PAX8* was also found in approximately one fifth of the cases. Earlier studies have shown that in women who have no macroscopic evidence of endometriosis, endometriosis can nevertheless be detected in blind peritoneal biopsies in up to 6% of cases (18). This means that endometriosis may already have developed even when it is not clinically manifest. It is therefore not surprising that at the molecular biologic level, the changes that are associated with the development of endometriosis are found in several cases in the control group. *WNT7A* and *PAX8* expression was detected in more than half of the peritoneal biopsy samples of patients with endometriosis. Changes associated with the development of endometriotic lesions irrespective of the pathogenetic mechanism may not be expected all over the peritoneal cavity but should be focal events. The fact that markedly stronger expression of *WNT7A* and *PAX8* is detected in the peritoneum in the group of patients with endometriosis than in the control group points to a role of these genes in the development of endometriosis.

It is known that diseases that are associated with increased retrograde menstruation show an elevated risk for the development of endometriosis, and endometriosis can also develop when there is no uterus and thus no uterine endometrium present. The hypothesis that endometriosis is generated by mimicking the ontogenetic development of the female genital tract is supported by evidence of induction of WNT genes in the peritoneum. However, it is not clear which stimuli lead to the incidental expression of *WNT7A* and *PAX8* in the peritoneum. It may be speculated that substances that enter the abdominal cavity through retrograde menstruation could play a decisive role here. This view would combine the most important known risk factors for the development of endometriosis derived from the clinical disease pictures and the concepts involved in the metaplastic theory into a single hypothesis.

So far as we are aware, this is the first study concerned with molecular-genetic parallels between female genital development and endometriosis. Expression of *WNT7A* and *PAX8* in the normal-appearing peritoneum suggests that endometriosis can arise through metaplasia by engaging those developmental steps that are involved in the ontogenesis of the female genital tract.

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