Characterization of *WNT7A* expression in human endometrium and endometriotic lesions

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Objective: To characterize the expression of *WNT7A* in human eutopic and ectopic endometrium.
Design: Experimental study using real-time polymerase chain reaction, laser microdissection, in situ hybridization, and immunofluorescence.
Setting: University-based laboratory.
Patient(s): Patients with and without endometriosis undergoing surgery for benign indications.
Intervention(s): None.
Main Outcome Measure(s): Relative expression values compared with housekeeping genes using real-time polymerase chain reaction. Detection of positive cells by immunofluorescence and in situ hybridization.
Result(s): In endometriosis, statistically significant higher *WNT7A* mRNA expression was observed compared with eutopic endometrium. Expression of *WNT7A* was found in the luminal and glandular epithelial cells as well as stroma cells in endometrium and endometriosis by immunofluorescence, in situ hybridization, and polymerase chain reaction of laser microdissected tissue.
Conclusion(s): The results of the present study suggest that *WNT7A* plays a role in the pathophysiology of endometriosis. (Fertil Steril® 2007;88:1534–40. ©2007 by American Society for Reproductive Medicine.)

Key Words: WNT wingless-type MMTV integration site family, endometrium, endometriosis

The *WNT* genes are a large family of highly conserved genes that encode secreted signaling glycoproteins. The *WNT* genes have been shown to play an important role in embryogenesis, cell proliferation, cell differentiation, and epithelial–mesenchymal communication. In mice, *WNT7A* signaling is essential for the development of the uterus, and in particular for endometrial gland formation (1, 2). Mice who are *WNT7A*-mutant have stratified luminal epithelium surrounded by a small stromal layer lacking endometrial glands and hyperplastic myometrium. In contrast, the wild-type uterus consists of simple columnar luminal epithelium and uterine glands lined with simple columnar epithelial cells embedded in the stromal cell layer. It has been suggested that *WNT7A* is secreted by epithelial cells and that it has paracrine effects on stromal cells (2).

Although it has been known for considerable time that *WNT7A* plays a decisive role in the embryonic development of the endometrium and endometrial glands, there have only been a few studies so far investigating the role of the *WNT* family in the adult endometrium and the development of endometriosis (3, 4). During the hormonally controlled menstrual cycle, the endometrium undergoes numerous changes. After the shedding of the stratum functionale during menstruation, reepithelialization and reconstruction of the endometrium takes place, with elongation of the glandular tubes. Preparation for implantation occurs during the ovulatory cycle, and with secretory transformation, further growth of the glands follows. The decisive function of the *WNT* genes in the embryonic development of the endometrium and endometrial glands suggests that the *WNT* glycoproteins, which are also expressed in adult tissue, may also have a corresponding role in the re-formation of the endometrium during the menstrual cycle. The fact that *WNT* expression is regulated by sex steroids in vivo and in vitro provides further support for this (5–9). To date, investigations of the expression of the *WNT* genes in the endometrium have been carried out using evidence of RNA via quantitative polymerase chain reaction (PCR) or in situ hybridization (ISH). As far as we know, the present study is the first to investigate the expression of *WNT7A* using immunostaining and real-time PCR of endometrial cells isolated using laser-capture microdissection.

MATERIALS AND METHODS

Tissue Samples

Endometrial biopsy samples were taken from patients undergoing hysterectomy, dilatation and curettage, and other procedures for benign gynecologic disease. Peritoneal biopsy specimens were taken from the anterior abdominal wall in all patients. Samples from endometriotic lesions were obtained from patients undergoing laparoscopy for

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endometriosis. Patient characteristics are given in Table 1. The study was approved by the local ethics committee.

Analysis of mRNA Expression by Real-Time PCR

Total RNA from human endometrial and peritoneal tissues was isolated using the guanidinium isothiocyanate method, as described elsewhere (10), in combination with affinity purification (RNeasy; Qiagen, Hilden, Germany). We generated cDNAs by randomly primed reverse transcription (ProSTAR cDNA Synthesis Kit; Stratagene, La Jolla, CA). Real-time PCR analyses were carried out using the ABI 7700 sequence detection system (PE Applied Biosystems, Foster City, CA) as previously described elsewhere (11). “Assays on demand” were used for hs*WNT7A* (acc. NM_004625) mRNA detection. All of the assays were located in the same region as the riboprobes used for ISH, allowing comparison of ISH and real-time PCR data. The VIC fluorophore-labeled glycerol 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

We used PCR to obtain template cDNAs of hs*WNT7A* (acc. NM_004625). The PCR primers were used to introduce T7 promoter sites into the amplified fragments. The primer sequences were as follows (T7 promoter sites are underlined):

WNT7A-U3 TGGGCCACCTCTTTCTCAGC
WNT7A-T7-L2
TAATACGACTCACTATAGGGCGGAACTGAAACTGA CACTC

Cytokeratin ISH was used as a control. The following primers were applied to generate CK sense and antisense cRNA probes.

CK-up1 AGCTGGCGCTCAAGGATGCTC
CK-T7-low3 TAATACGACTCACTATAGGGCTTGCGGTA GGTGGCGATC
CK-T7-up1 TAATACGACTCACTATAGGGAGCTGGCGC TCAAGGATGCTC
CK-low3 GCTTGCGGTAGGTGGCGATC

Before cRNA probe synthesis, the PCR products were purified by ultrafiltration using Millipore UFCTTK30 filters. We used 1-μg DNA to prepare antisense and sense single-strand RNA probes labeled with digoxigenin (DIG) with a DIG 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 mM adenosine-5′-triphosphate (ATP), cytidine-5′-triphosphate (CTP), guanosine-5′-triphosphate (GTP), 0.65 mM uridine-5′-triphosphate (UTP), 1 × transcription buffer, 1 unit/μL RNase inhibitor, and 20 unit/μL T7 RNA polymerase. The reactions were incubated at 37°C for 2 hours. Two microliters of 10 unit/μL RNase-free DNase I were 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 M ethylenediaminetetraacetic acid (EDTA). Gel electrophoresis was used to control cRNA products. Probe concentrations were adjusted by spotting dilutions on nylon filters and analyzing them with the DIG 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 (Erie Scientific Company, Portsmouth, NH), 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 PBS/4% paraformaldehyde (pH 9.5) for 60 minutes (12) and washed three times with PBS and once with 2 × SSC for 10 minutes, followed by prehybridization for 60 minutes at 50°C in 100 μL of hybridization buffer (4 × SSC; 10% dextran sulfate; 1 × Denhardt’s solution [0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin]; 2 mM EDTA; 50% deionized formamide; 500 μg/mL herring sperm DNA). The prehybridization buffer was removed, and the section was covered with 100 μL of 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 DIG detection procedure was carried out in accordance with the manufacturer’s protocol.

Immunofluorescence

Immunofluorescence was carried out on samples quick-frozen in liquid nitrogen. Tissue section of 8 μm were cut and mounted directly on Superfrost Plus slides. Tissue sections were fixed by incubation in absolute methanol for 10 minutes (−20°C), air-dried, and subsequently incubated after of washing in PBS/0.1% Tween 20 with primary antibody (1:100 dilution) at room temperature for 1 hour. After three rounds of washing with 1 × PBS/0.1% Tween 20 secondary antibody was added at a dilution of 1:100 and incubated for 1 hour at room temperature. For double immunostaining staining, the specimens were fixed thereafter for a second time, and the staining procedure was repeated with the second primary antibody. Fluorescence was detected using Zeiss Axiovert microscope (Carl Zeiss, Göttingen, Germany). Antibodies directed against *WNT7A* (*WNT-7a (Q12*) goat polyclonal IgG) and Cytokeratin (Monoclonal Mouse, Clone *MNF116*) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and DakoCytomation (Glostrup, Denmark), respectively. Secondary FITC-conjugated anti-goat antibody and CY3-conjugated goat anti-mouse antibody were purchased from Dianova (Hamburg, Germany).

TABLE 1

Patient characteristics.

Clinical diagnosis	Patients (n)
PCR Endometrium endometriosis patients	
Age	40.4 ± 6.0
Endometriosis rAFS I	1
Endometriosis rAFS III/IV	2
Adenomyosis uteri	2
PCR Endometrium patients without endometriosis	
Age	38.4 ± 9.3
Intraepithelial cervical dysplasia	3
Uterine fibroids	2
Infertility	2
Chronic pelvic pain	1
PCR Peritoneal endometriosis	
Age	32.5 ± 6.7
Endometriosis rAFS I	6
Endometriosis rAFS III/IV	4
Endometriosis rAFS I + ovarian cyst	1
In situ hybridization eutopic endometrium	
Age	45.5 ± 7.2
Intraepithelial cervical dysplasia	2
Uterine fibroids	2
In situ hybridization ectopic endometrium	
Age	38.8 ± 5.0
Endometriosis rAFS I	2
Endometriosis rAFS I + uterine fibroids	2
Immunofluorescence eutopic endometrium	
Age	40.9 ± 8.2
Intraepithelial cervical dysplasia	5
Uterine fibroids	5
Paraovarian cyst	1
Bleeding disorder	4
Adenomyosis uteri	1
Endometriosis rAFS I/II	2
Endometriosis rAFS III/IV	1
Endometriosis rAFS I + uterine fibroids	1
Immunofluorescence ectopic endometrium	
Age	34.5 ± 5.9
Endometriosis rAFS I/II	7
Endometriosis rAFS III/IV	2
Endometriosis rAFS I + uterine fibroids	2
Microdissected endometrium	
Age	41.8 ± 8.8
Intraepithelial cervical dysplasia	3
Paraovarian cyst	1
Endometriosis rAFS I/II + uterine fibroids	1

Note: rAFS: Revised American Society for Reproductive Medicine classification of endometriosis: 1996. Fertil Steril 1997;67:817–21.

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Laser Microdissection

A laser microdissection and pressure catapulting device from P.A.L.M. Microlaser Technologies GmbH (Bernried, Germany) was used to isolate stromal and epithelial cells of endometrial tissues. In brief, 5 μm of frozen sections were mounted on PALM MembraneSlides (pen-membrane covered, 1 mm) and incubated with hematoxylin containing RNase inhibitor (200 U/mL; Roche) for 4 minutes. The samples were then washed in water for 2 minutes and subsequently incubated in 2% eosin for 15 seconds. After washing, the slides were dried for 30 minutes at 37°C and subjected to the ultraviolet laser beam–assisted microdissection. Dissected tissue areas were catapulted into vials containing 20 μL of Purescript lysis buffer (Biozym, Hamburg, Germany). Total RNA from approximately 800 cells was isolated by using a Gentra Total RNA preparation kit, Biozym (Gentra Systems, Minneapolis, MN). The RNA precipitation was enhanced by the addition of glycogen. The cDNA synthesis was done as previously described. Half of the reactions were employed in real-time PCR to detect GPDH and *WNT7A*, respectively.

Statistical Analyses

All reported *P* values are two-sided, and $P<.05$ was considered statistically significant. The Mann-Whitney *U* test was used for metric differences in *WNT7* expression between two sample groups, and the Kruskal-Wallis test was applied when more than two groups were compared. The chi-square test was used to test for associations between nominal variables. All analyses were performed using SPSS 11.0 (SPSS Inc., Chicago, IL).

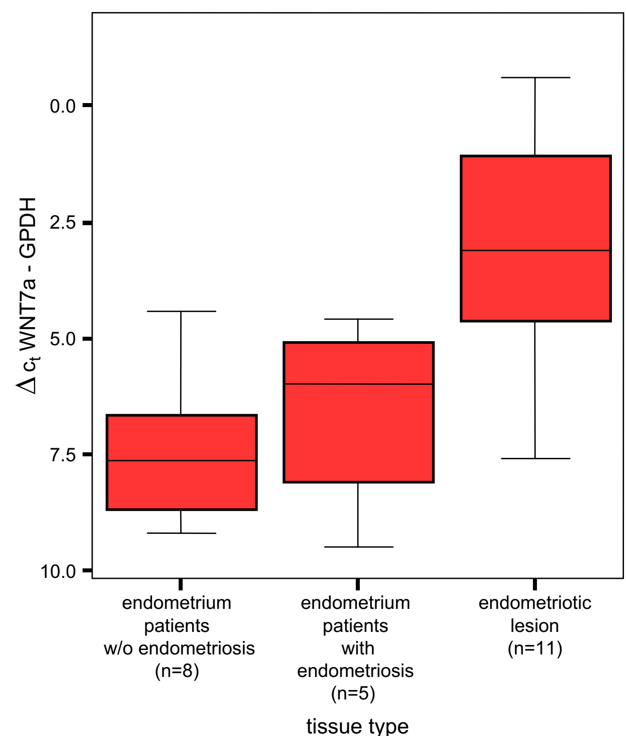
RESULTS

Analysis of *WNT7A* Expression in Human Eutopic and Ectopic Endometrium Using Quantitative Real-Time PCR

Quantitative reverse transcription–based PCR (qRT-PCR) was used to compare *WNT7A* in biopsy samples from endometriotic tissue and eutopic endometrium. The eutopic endometrium was derived both from patients without endometriosis and from patients with endometriosis. Table 1 lists the patients' clinical characteristics. Expression of *WNT7A* was detectable in all of the endometrial and endometriosis biopsy tissues investigated. As shown in Figure 1, when the levels of *WNT7A* mRNA expression as revealed by Δc_t values were analyzed, a significant difference was found among the three tissue types ($P=.003$; Kruskal-Wallis test). Although the stronger expression of *WNT7A* in endometriotic tissue compared with endometrium samples was highly statistically significant ($P=.001$ for all endometrium samples and $P=.015$ for endometrium from endometriosis patients, respectively; Mann-Whitney test), the increased expression of *WNT7A* in the endometrium samples from patients with endometriosis compared with patients without endometriosis was not statistically significant ($P=.56$; Mann-Whitney test).

FIGURE 1

Analysis of *WNT7A* mRNA expression in human endometrium and endometriosis by quantitative real-time polymerase chain reaction (PCR). *WNT7A* mRNA expression in the different tissues as revealed by quantitative real-time PCR is presented as box plots of the Δc_t values normalized to *GPDH*. A statistically significant difference was found among the three tissue types ($P=.003$; Kruskal-Wallis test) and when endometriotic tissue was compared with endometrium samples ($P=.001$ for all endometrium samples and $P=.015$ for endometrium from endometriosis patients, respectively; Mann-Whitney test). The difference between endometrium samples from patients with and without endometriosis was not statistically significant ($P=.56$; Mann-Whitney test).



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Next, we tested for a confounding influence on these results of several clinical variables: stage of endometriosis, age of the patient, and menstrual cycle phase. Using the revised American Fertility Society's staging system (13), a larger portion of the older patients had a diagnosis of stage III/IV endometriosis (five of seven samples from patients aged $\geq$35 years vs one out of seven samples from younger patients); however, this number was not statistically significant ($P=.1$; chi-square test). In contrast, we observed no differences in *WNT7A* expression when the patients were analyzed according to their age ($P=.7$; Mann-Whitney test), their stage of disease (I/II vs III/IV, $P=.8$; Mann-Whitney

test), or the menstrual cycle phase (proliferative vs secretory, $P=.2$; Mann-Whitney test).

Analysis of *WNT7A* Expression in Human Endometrium using Immunofluorescence and in Situ Hybridization

Immunostaining for *WNT7A* was detected in glandular (Fig. 2E) and luminal (Fig. 2D) epithelial cells from all of the endometrial samples investigated. Endometrial stroma cells showed *WNT7A* expression in almost all cases (Fig. 2D, E). Ectopic endometrium showed similar immunostaining to that of eutopic endometrium (Fig. 2F). These results of a positive staining both in the epithelial cells and in the stromal cells of the endometrium are in contrast with the previous view that *WNT7A* expression is restricted to luminal epithelial cells (3). To allow a better comparison between the results of the our study and the data that have been reported in the literature, the expression patterns of *WNT7A* were confirmed by using ISH (Fig. 2A–C). In eutopic endometrium, *WNT7A* was found in both luminal and glandular epithelial cells and also in stroma cells, with the stroma cells showing weaker staining in comparison with the epithelial cells (Fig. 2A, B). Ectopic endometrium also showed expression of *WNT7A* in the epithelial cells and the cytogenic stroma (see

FIGURE 2

WNT7A mRNA and protein expression in human endometrium and endometriosis. *WNT7A* mRNA and antigen expression were analyzed by (**A–C**) in situ hybridization and (**D–F**) immunofluorescence (red staining) of eutopic endometrium (A, B, D, E) and endometriosis tissue (C and F). Cytokeratin (green) was used for counterstaining of epithelial cells in D, E, and F. The luminal (→) and glandular epithelial cells (▶) as well as stroma cells (*S*) show positive staining.

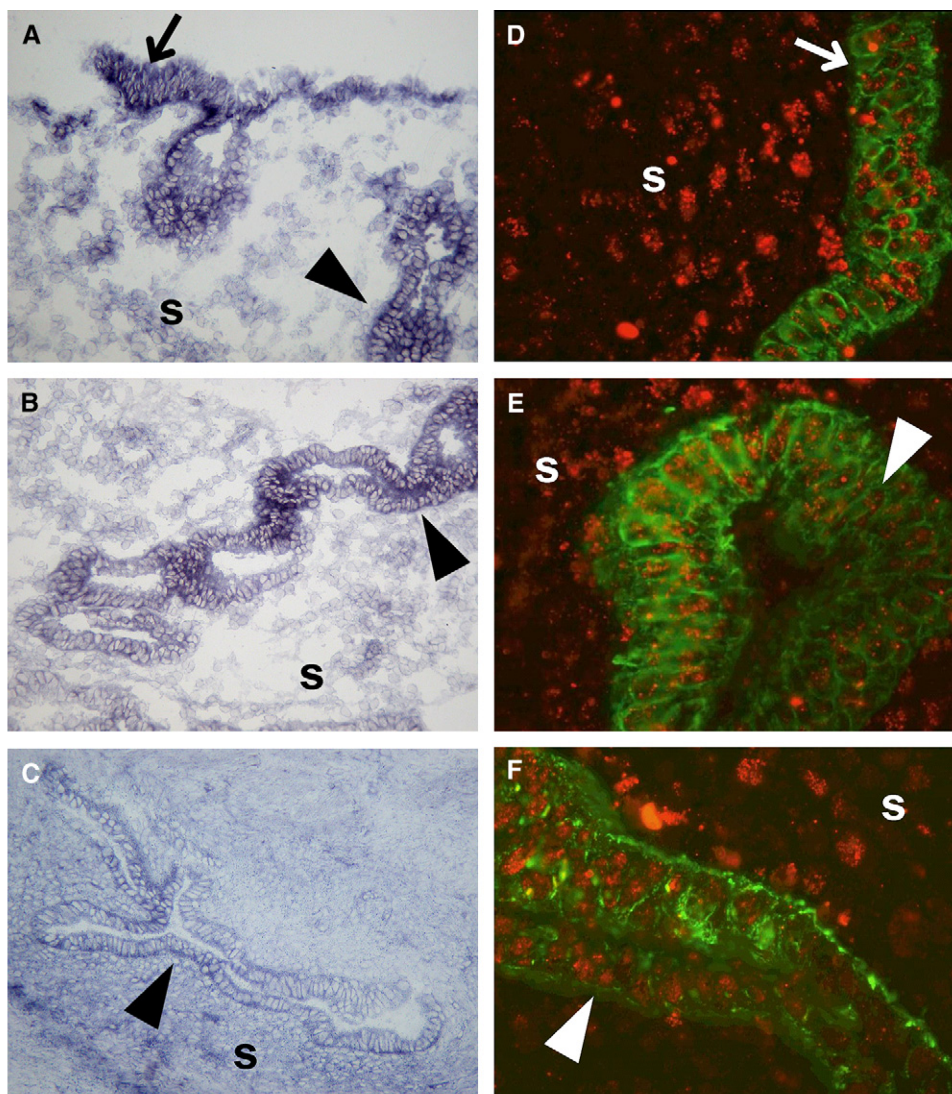

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TABLE 2

Analysis of *WNT7A* antigen expression in glandular and stroma cells in human endometrium using immunofluorescence.

Histology	Glandular epithelial cells (n)[a]		Stroma cells (n)[a]	
	Positive	Total	Positive	Total
Eutopic endometrium				
Proliferative phase	7	7	7	7
Secretory phase	8	8	7	8
Atrophic endometrium	2	2	2	2
Hyperplasia	3	3	3	3
Ectopic endometrium	8	8[b]	11	11[b]

[a] The number of specimens with positive immunostaining and the total number analyzed are given.
[b] In three of the 11 samples analyzed, endometrial epithelium was not found in the sections used for immunofluorescence; however, endometriosis was diagnosed histologically in all of these lesions.

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Fig. 2C). No relationship was found among the menstrual cycle phase, endometrial histology, or the patient's diagnosis and the staining pattern of the endometrial samples (Table 2).

Laser-capture Microdissection

As the expression patterns of immunostaining and ISH for *WNT7A* differed from the ISH results that had been previously reported in the literature, the observation of stromal *WNT7A* expression was verified using laser-controlled microdissection of glandular epithelial cells and stromal cells, followed by real-time PCR. Microdissection of glandular epithelial cells and stromal cells was performed successfully in all seven samples investigated, and sufficient RNA for real-time PCR was obtained from five of the samples. In all five samples, expression of *WNT7A* was demonstrated in the glandular epithelial cells (Table 3). In four of the five samples, however, the stromal cells demonstrated *WNT7A* mRNA expression as well, even though at slightly lower levels compared with the epithelial cells. Thus, the results of our immunostaining and ISH correlated closely with the real-time PCR data from the microdissected tissue.

TABLE 3

Analysis of *WNT7A* in microdissected endometrial glandular and stroma cells using real time polymerase chain reaction (PCR).

Glandular epithelial cells (n)[a]		Stroma cells (n)[a]	
PCR positive	Total	PCR positive	Total
5	5	4	5

[a] Only samples with positive immunostaining were used for the analyses.

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DISCUSSION

Although the role of *WNT7A* in the embryonic development of the endometrium has been well documented, only speculative suggestions are available regarding its function in the adult endometrium (1–3). A *WNT7A* knock-out leads to a loss of *HOXA10* and *HOXA11*, which are up-regulated during deciduation in the stroma (2). This led to the hypothesis that *WNT7A* has a function in implantation and deciduation. Other studies investigating *WNT7A* regulation in vitro have speculated that it may play a role in the development of endometrial neoplasia (8, 14). During embryonic development, the expression of *WNT7A* is one of the decisive steps involved in adequate development of the endometrial glands (1, 2). In addition, *WNT7A* expression is a prerequisite for the normal development of the myometrium and for the maintenance of *WNT5A* and *HOXA* expression in the endometrium (2). Moreover, the interaction between *WNT5A* expressed in the stroma and *WNT7A* expressed in the epithelial cells is crucial for development and correct differentiation (15). It is assumed that in the adult endometrium *WNT7A* expression in the epithelium influences the hormonally regulated interaction between the stroma and epithelium. The presumption of an exclusive expression of *WNT7A* in the epithelial cells led to the hypothesis that *WNT7A* has a paracrine effect (2, 15). However, the results of the present study demand a more detailed examination of the role of the *WNT* signaling pathway in the adult endometrium. The view that there is a clear spatial separation of *WNT7A* expression between the compartments of the stromal cells and epithelial cells is no longer sustainable. Accordingly, new interpretations and considerations of the function of *WNT7A* and its signaling pathway in the adult endometrium are needed. The lack of modulation of *WNT7A* in the human endometrium during the menstrual cycle does not

provide an indication of its precise function, but the consistent expression in our heterogeneous study population supports the hypothesis of an intrinsic role of *WNT7A* in the adult endometrium.

Consistent with results that had been obtained in the mouse, Tulac et al. (3) used ISH to demonstrate that *WNT7A* expression was restricted to the luminal epithelial cells of human endometrium. By contrast, the results of our study using both ISH, real-time PCR of laser microdissected tissue, and immunofluorescence reveal that *WNT7A* is expressed not only in the luminal epithelial cells, but also in the glandular epithelial cells and in the stromal cells. Because these findings were consistently observed independent of the phase of the cycle, histologic findings, clinical symptoms, and the diagnosis in the patients concerned, it is unlikely that the conflicting results are due to different patient groups analyzed. Because ISH is a variation-prone method, the differences may be more likely explained by technical differences in the procedure, with different detection thresholds for *WNT7A* mRNA. However, confirmation by three different methodologic approaches validated the results of our analysis.

In agreement with the literature, no correlation was found between *WNT7A* expression and the menstrual cycle (3). During embryonic development, estrogen exposure both in murine and ovine endometrium leads to down-regulation of *WNT7A* expression (6). In human endometrium, however, hormonal regulation of *WNT7A* has so far only been demonstrated in vitro (7, 8).

In an earlier study (4) we showed that in patients with endometriosis *WNT7A* was expressed at a significantly greater frequency in histologically normal peritoneum in comparison with controls, leading to the hypothesis that *WNT7A* along with other factors might be involved in the development of endometriosis. This would be in line with the special role of *WNT* for signaling in the early embryonic development of the female genital tract from the müllerian duct, where the interaction of *WNT7A* in the epithelium and *WNT5A* in the surrounding stroma is crucial for the development of the uterine glands (1, 2, 15). In our present study, we observed elevated levels of *WNT7A* mRNA in ectopic endometrium compared with eutopic endometrium. This further supported the hypothesis of a role for *WNT7A* in the formation and differentiation of ectopic endometrial tissue. It is interesting that, in the *WNT7A*-heterozygotic mouse, reduced *WNT7A* copy number leads via altered *WNT5A* expression to an increased development of endometrial glands, pointing to the necessity of a balanced expression of *WNT7A* for normal uterus development (2). Although these knock-out experiments in mice demonstrate the importance of *WNT7A* for endometrial gland formation, the consequences of increased *WNT7A* expression has not yet been investigated. Because we observed no histomorphologic differences between *WNT7A*-overexpressing and normal tissues, it might be possible that *WNT7A* influences cell survival, promoting an improved implantation of scattered endometrial cells and hence an increase in formation of endometriosis lesions. Further research is needed to explain the way in which the increased expression of *WNT7A* in ectopic endometrium is involved in the pathogenesis of endometriosis.

The results of our present study suggest that *WNT7A* plays a role both in the remodeling of the endometrium during the menstrual cycle and in the development of endometriosis.

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