

Secreted frizzled-related protein 2 (SFRP2) expression promotes lesion proliferation via canonical WNT signaling and indicates lesion borders in extraovarian endometriosis

T. Heinosalo^{1,†}, M. Gabriel^{1,2,†}, L. Kallio¹, P. Adhikari¹,
K. Huhtinen^{1,3,4}, T. D. Laajala^{5,6,7}, E. Kaikkonen¹, A. Mehmood^{1,8},
P. Suvitie², H. Kujari^{3,4}, T. Aittokallio^{5,6,7}, A. Perheentupa^{1,2},
and M. Poutanen^{1,7,9,*}

¹Institute of Biomedicine, Research Centre for Integrative Physiology and Pharmacology, University of Turku, 20014 Turku, Finland ²Department of Obstetrics and Gynecology, University of Turku and Turku University Hospital, 20014 Turku, Finland ³Institute of Biomedicine, Research Center for Cancer, Infections and Immunity, University of Turku, 20014 Turku, Finland ⁴Department of Pathology, Turku University Hospital, 20521 Turku, Finland ⁵Department of Mathematics and Statistics, University of Turku, 20014 Turku, Finland ⁶Institute for Molecular Medicine Finland (FIMM), University of Helsinki, 00014 Helsinki, Finland ⁷Turku Center for Disease Modeling (TCDM), University of Turku, 20014 Turku, Finland ⁸Turku Centre for Biotechnology, University of Turku and Åbo Akademi, Turku, Finland ⁹Institute of Medicine, Sahlgrenska Academy, 405 30 Gothenburg University, Gothenburg, Sweden

*Correspondence address. University of Turku, 20014 Turku, Finland. Tel: +358-2-333-7571; E-mail: matti.poutanen@utu.fi

Submitted on September 30, 2017; resubmitted on December 28, 2017; accepted on January 24, 2018

STUDY QUESTION: What is the role of SFRP2 in endometriosis?

SUMMARY ANSWER: SFRP2 acts as a canonical WNT/CTNNB1 signaling agonist in endometriosis, regulating endometriosis lesion growth and indicating endometriosis lesion borders together with CTNNB1 (also known as beta catenin).

WHAT IS KNOWN ALREADY: Endometriosis is a common, chronic disease that affects women of reproductive age, causing pain and infertility, and has significant economic impact on national health systems. Despite extensive research, the pathogenesis of endometriosis is poorly understood, and targeted medical treatments are lacking. WNT signaling is dysregulated in various human diseases, but its role in extraovarian endometriosis has not been fully elucidated.

STUDY DESIGN, SIZE, DURATION: We evaluated the significance of WNT signaling, and especially secreted frizzled-related protein 2 (SFRP2), in extraovarian endometriosis, including peritoneal and deep lesions. The study design was based on a cohort of clinical samples collected by laparoscopy or curettage and questionnaire data from healthy controls and endometriosis patients.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Global gene expression analysis in human endometrium ($n = 104$) and endometriosis ($n = 177$) specimens from 47 healthy controls and 103 endometriosis patients was followed by bioinformatics and supportive qPCR analyses. Immunohistochemistry, Western blotting, primary cell culture and siRNA knockdown approaches were used to validate the findings.

MAIN RESULTS AND THE ROLE OF CHANCE: Among the 220 WNT signaling and CTNNB1 target genes analysed, 184 genes showed differential expression in extraovarian endometriosis ($P < 0.05$) compared with endometrium tissue, including SFRP2 and CTNNB1. Menstrual cycle-dependent regulation of WNT genes observed in the endometrium was lost in endometriosis lesions, as shown by hierarchical clustering. Immunohistochemical analysis indicated that SFRP2 and CTNNB1 are novel endometriosis lesion border markers,

[†]Equal contribution.

complementing immunostaining for the known marker CD10 (also known as MME). SFRP2 and CTNNB1 localized similarly in both the epithelium and stroma of extraovarian endometriosis tissue, and interestingly, both also indicated an additional distant lesion border, suggesting that WNT signaling is altered in the endometriosis stroma beyond the primary border indicated by the known marker CD10. SFRP2 expression was positively associated with pain symptoms experienced by patients ($P < 0.05$), and functional loss of SFRP2 in extraovarian endometriosis primary cell cultures resulted in decreased cell proliferation ($P < 0.05$) associated with reduced CTNNB1 protein expression ($P = 0.05$).

LIMITATIONS REASONS FOR CAUTION: SFRP2 and CTNNB1 improved extraovarian endometriosis lesion border detection in a relatively small cohort ($n = 20$), although larger studies with different endometriosis subtypes in variable cycle phases and under hormonal medication are required.

WIDER IMPLICATIONS OF THE FINDINGS: The highly expressed SFRP2 and CTNNB1 improve endometriosis lesion border detection, which can have clinical implications for better visualization of endometriosis lesions over CD10. Furthermore, SFRP2 acts as a canonical WNT/CTNNB1 signaling agonist in endometriosis and positively regulates endometriosis lesion growth, suggesting that the WNT pathway may be an important therapeutic target for endometriosis.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded by the Academy of Finland and by Tekes: Finnish Funding Agency for Innovation. The authors have no conflict of interest to declare.

Key words: endometriosis / extraovarian endometriosis / SFRP2 / canonical WNT signaling / CD10

Introduction

Endometriosis is a common, benign proliferative disease affecting ~5–10% of women at reproductive age and is characterized by the presence of functional endometrium in ectopic locations (Bulun, 2009; Giudice, 2010). The lesions are classified as peritoneal, deep or ovarian diseases, all with poorly understood etiology (Nisolle and Donnez, 1997). There are various theories regarding endometriosis pathogenesis, including endometrial tissue or stem cell implantation during retrograde menstruation, Müllerian remnant abnormalities and coelomic metaplasia (Vercellini et al., 2014). Recently, a unifying hypothesis regarding the misplacement of stem cells due to altered gene expression patterns during embryonic development has been proposed (Lagana et al., 2017). The diagnosis of endometriosis also relies on invasive measures, such as laparoscopy combined with histopathological confirmation (Dunselman et al., 2014). Endometriosis subtypes differ in symptoms, recurrence and response to treatments (Guo, 2009; Giudice, 2010). Currently, hormonal approaches, such as contraceptives, anti-progestagens, GnRH agonists and antagonists, and aromatase inhibitors, are used to treat endometriosis. These treatments often relieve the symptoms although they typically persist after discontinuing the therapy, and surgical intervention is the most effective long-term treatment (Dunselman et al., 2014; Vercellini et al., 2014). However, the high recurrence rate of endometriosis (up to 50%) remains a problem, with one major cause being incomplete surgical removal of the lesions (Guo, 2009). Endometriosis reduces quality of life by causing pain and infertility and results in treatment costs comparable with those of diabetes (Simoens et al., 2012).

Aberrant wingless-type MMTV integration site family (WNT) signaling is an early event in carcinogenesis in several tissues (Anastas and Moon, 2013), and there is evidence that WNT signaling also plays a role in the endometrium and endometrial diseases (Talbi et al., 2006; Wu et al., 2006; Eyster et al., 2007; Aghajanova et al., 2010; Matsuzaki et al., 2010; Matsuzaki and Darcha, 2013; Zhang et al., 2016a). WNT family proteins are secreted signaling glycoproteins with functions in multiple normal cellular processes and human diseases. In canonical

WNT/beta-catenin (CTNNB1) pathway, WNTs interact with Frizzleds (FZD) and low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/LRP6) and activate dishevelled (DVL) proteins. DVLs inhibit the axin/glycogen synthase kinase 3beta (GSK3B)/adenomatous polyposis coli (APC) complex, which promotes degradation of CTNNB1. The stabilized CTNNB1 enters the nucleus and interacts with transcription factors promoting target gene expression (Klaus and Birchmeier, 2008; Anastas and Moon, 2013). Furthermore, two non-canonical WNT pathways exist, which do not operate through CTNNB1: the WNT/planar cell polarity and the WNT/Ca²⁺ pathways stimulated by, e.g. WNT5A and WNT11 (Klaus and Birchmeier, 2008; Anastas and Moon, 2013).

The WNT pathway involves numerous extra- and intracellular regulator proteins belonging to two functional classes. Class 1 members primarily bind to WNTs and include the various secreted frizzled-related protein (SFRP) family proteins, WNT inhibitory factor 1 (WIF1), Cerberus and Sclerostin. Class 2 members comprise the Dickkopf (DKK) family proteins, inhibiting WNT signaling by binding to LRP5/LRP6 (Kawano and Kypta, 2003). Therefore, these proteins only inhibit the canonical WNT pathway, while the SFRPs interact with both WNTs and FZDs and affect all types of WNT signaling (Bovolenta et al., 2008). Many of these regulator proteins, including SFRPs, have opposite activities in different tissues (Anastas and Moon, 2013). Different mechanisms have been proposed for SFRPs to positively or negatively regulate WNT signaling. For instance, SFRPs can prevent FZD-dependent WNT activation by blocking their interaction via binding to one of the interacting partners (Bovolenta et al., 2008). Furthermore, SFRPs can promote WNT pathway activation by inactivating themselves via binding to each other, or they can form complexes with both WNTs and FZDs that favour WNT–FZD interaction, and additional proteins can participate in these interactions (Bovolenta et al., 2008).

Canonical WNT signaling has been shown to directly cause the development of adenomyosis in an estrogen-dependent manner (Tanwar et al., 2009; Oh et al., 2013) and to be activated by estradiol

in ovarian endometriosis (Zhang *et al.*, 2016a, 2016b). Activating mutations of CTNNT1 are frequently found in endometriosis-associated ovarian cancers (Maeda and Shih, 2013). However, WNT pathway activation has not been systematically characterized during the menstrual cycle or in peritoneal and deep endometriosis, hereafter referred to as extraovarian endometriosis, and the aim of the present work was to characterize the WNT pathway changes during the menstrual cycle in the endometrium and in endometriosis lesions.

Materials and Methods

Ethical approval

The study was approved by the Joint Ethics Committee of Turku University and Turku University Hospital in Finland. Written informed consent was provided by all study subjects prior to sampling.

Patient samples

Samples of endometriosis and eutopic endometrial biopsies were collected from endometriosis patients, and as a control group, endometrial biopsies from healthy, endometriosis-free women undergoing laparoscopic tubal ligation were collected. Women with other significant disease or medication, suspicion of malignancy, pregnancy or acute infection were excluded. Endometriosis samples were collected during laparoscopy or laparotomy, and histological evaluation was performed to confirm the presence of normal endometrial histology in controls and patients and to diagnose endometriosis in patients. Laparotomy was only performed when the surgery carried an increased risk of major complication if carried out through laparoscopy. Three different endometriosis sample subtypes were collected, including 77 deep infiltrating lesions (rectovaginal, uterosacral, intestinal and bladder), 72 peritoneal lesions (red, black and white) and 28 ovarian endometriomas. Furthermore, 63 endometrium samples from patients and 41 endometrium samples from healthy women were collected. We also collected peritoneum samples from 24 control women and from 28 patients. Patient characteristics are presented in Table I. Tissue samples were snap-frozen and stored in liquid nitrogen until used or immediately processed for cell culture studies. An aliquot was fixed in formalin and embedded in paraffin for histological analysis.

RNA isolation

For microarray analysis and quantitative reverse transcription PCR (RT-qPCR), total RNA was isolated using Trizol reagent (Thermo Fisher Scientific, USA), further purified with RNeasy columns (Qiagen, Netherlands), and treated with DNase (RNase-free DNase Set, Qiagen, Netherlands; or DNase I, Invitrogen, Thermo Fisher Scientific, USA). For the siRNA experiment, TRIreagent (Bioline, UK) was used according to manufacturer's instructions. The RNA concentrations were measured using Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA).

Gene expression analysis by microarray

The quality of the isolated RNA was controlled by Experion analysis (Bio-Rad Laboratories, USA). The microarray analysis was performed using 177 endometriotic lesions (72 peritoneal, 77 deep and 28 ovarian endometriosis lesions), 63 endometrial biopsies and 28 peritoneum samples from 103 endometriosis patients. RNA from 41 endometrial biopsies and 24 peritoneum samples collected from 47 healthy control women was also included. The study subjects provided a variable number of samples per subject. Only data from patients without hormonal medication were included, unless specifically mentioned. Microarray sample information is provided in Table II. The gene expression profiles were measured using the Sentrix[®] Human Illumina 6 V2 Expression BeadChips (Illumina, USA). Normalization and analyses were performed using the R package limma (<http://www.r-project.org>).

Pathway and correlation analysis

Subjects using hormonal medication ($n = 12$ controls and 44 patients), those with unknown cycle or medication status ($n = 10$ controls and 13 patients), and subjects who gave a peritoneum sample only (11 controls and 3 patients) were excluded with the exception of correlation analyses of clinical features with SFRP2 expression, where data from all subjects were included. The WNT signaling pathway molecules listed in the human Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were included in the clustering analysis and were complemented with more recently identified WNT signaling genes (Niehrs, 2012; Clevers *et al.*, 2014; Green *et al.*, 2014). The 54 human CTNNT1 target genes were selected based on human genes listed on the WNT home page (<http://web.stanford.edu/group/nusselab/cgi-bin/wnt/>, date last accessed 2 January 2018) and in the literature (Herbst *et al.*, 2014) and analysed using bioinformatics. We used Canberra

Table I Clinical characteristics of study subjects.

Study subjects			
Controls	n	Patients	n
Healthy controls	47	Endometriosis patients	103
Control age \pm SD	39 \pm 4	Patient age \pm SD	32 \pm 7
Parous controls	44 (93.6%)	Parous patients	31 (30.1%)
Nulliparous controls	3 (6.4%)	Nulliparous patients	72 (69.9%)
Controls using hormonal medication	12 (25.5%)	Patients using hormonal medication	44 (42.7%)
Combined	7 (58.3%*)	Combined	33 (75.0%#)
Progestin only	5 (41.7%*)	Progestin only	8 (18.2%#)
GnRH agonist	0	GnRH agonist	3 (6.8%#)
Anti-progestagen	0	Anti-progestagen	0
Aromatase inhibitor	0	Aromatase inhibitor	0

*% from controls using hormonal medication, #% from patients using hormonal medication.

Table II Samples in gene expression profiling.

Samples					
Tissue type	Total (n)	Proliferative (n)	Secretory (n)	Hormonal medication (n)	Other (n)
Control endometrium	41	12	18	6	5
Patient endometrium	63	16	20	21	6
Ovarian endometriosis	28	10	9	7	2
Peritoneal endometriosis	72	14	15	35	8
Deep endometriosis	77	9	17	41	10
Lesion stages 1–2	49	5	13	27	4
Lesion stage 3	41	6	10	16	9
Lesion stage 4	90	20	19	45	6
Control peritoneum	24	3	6	9	6
Patient peritoneum	28	5	6	11	6

distance metric and Ward clustering algorithm for hierarchical clustering analysis. The microarray expression of WNT genes and CTNNB1 target genes was compared among different tissues using non-parametric unpaired Mann–Whitney test. Thresholds $r \geq 0.3$ and multiple comparison-adjusted P -value < 0.05 were considered as significant for correlation analyses. For dichotomous clinical variables such as disease status and fertility, the point-biserial correlation was calculated. For categorical variables such as menstruation length and the number of days with dysmenorrhoea, the polychoric correlation was calculated. For numerical variables such as pain strength, height and weight, Pearson correlation was calculated. Pearson correlation was also used to examine the association between the WNT gene expression and intratissue steroid concentrations (estradiol, testosterone and progesterone) using the rcorr method in R software. The SFRP2 promoter transcription factor binding site prediction analysis was done by the free software ALGGEN PROMO using 0% maximal dissimilarity rate (Messeguer et al., 2002).

RT-qPCR

For RT-qPCR analysis, 0.5 µg of RNA was converted to cDNA using the DyNAmo HS SYBR Green RT-qPCR kit (Finnzymes, Thermo Fisher Scientific, USA). The RT-qPCR reactions were carried out for 40 cycles with the primers presented in Supplemental Table S1. Ribosomal protein L19 (RPL19) and HPRT were used as reference genes. The expression of selected WNT signaling pathway genes was analysed by RT-qPCR from ovarian, deep and peritoneal lesions and endometrium tissue ($n = 4–12$).

Western blotting

Total protein lysates were analysed by Western blotting using a rabbit polyclonal antibody against human SFRP2 (0.12 µg/ml, HPA002652, Sigma-Aldrich, Merck, USA), a rabbit polyclonal antibody against human CTNNB1 (2 µg/ml, SC7199, Santa Cruz Biotechnologies, USA) or a mouse monoclonal antibody against human TUBA1A (0.02 µg/ml, MS-581-P, Thermo Fisher Scientific, USA). Total lysates (30 µg/well) from 6 endometrium, ovarian endometriosis and extraovarian endometriosis lesion samples from different patients were used (proliferative $n = 3$, secretory $n = 3$). From cell culture studies, equal amounts of total lysate were loaded from cells treated with SFRP2-siRNA and control-siRNA (10–19 µg per patient). The intensities of the protein bands were measured using the ImageJ (1.49 v) program.

Immunohistochemical staining

Immunohistochemical (IHC) staining was performed with primary antibodies against human SFRP2 (rabbit polyclonal, #HPA002652, Sigma-Aldrich, Merck, USA; 0.3 µg/ml for scoring analysis and 0.75 µg/ml for lesion border analysis; rabbit polyclonal, #sc-13940, Santa Cruz Biotechnology, USA, 1.3 µg/ml to verify lesion border analysis results), human CTNNB1 (mouse monoclonal, #610153, BD Transduction Laboratories, USA, 0.08 µg/ml, for scoring and lesion border analysis), and CD10 (also known as MME; mouse monoclonal, #NCL-L-CD10-270, Leica Biosystems, Germany, 0.75 µg/ml) for lesion border analysis. Antigen retrieval was performed in a pressure cooker (Retriever 1200) in Tris-EDTA (pH 9.0) for SFRP2 and CTNNB1, and in 10 mM sodium citrate buffer (pH 6.0) for CD10. Sections were scanned for analyses with the panoramic 250 Flash series digital slide scanner (3DHISTECH, Hungary). SFRP2 staining intensity was scored in seven proliferative and nine secretory phase endometrium samples and in five proliferative and five secretory phase extraovarian endometriosis lesions. For CTNNB1 staining, endometrial and extraovarian endometriosis samples from three study subjects in the proliferative and five subjects in the secretory phase were analysed. For the lesion border analysis, extraovarian endometriosis lesions from 20 patients were evaluated of which seven were in the proliferative, six in the secretory and two were in the menstruating phase, and five were using hormonal medication (progesterin only $n = 2$, combined estrogen and progesterin $n = 1$, GnRH analog $n = 1$, progesterin only + combined estrogen and progesterin $n = 1$). Two researchers evaluated all the analyses independently.

Double immunofluorescence staining (SFRP2 and CTNNB1; SFRP2 and CD10) was performed on extraovarian endometriosis samples from three patients in the secretory phase of the menstrual cycle using primary antibodies against human SFRP2 (rabbit polyclonal, #sc-13940, Santa Cruz Biotechnology, USA, 1.3 µg/ml), human CTNNB1 (mouse monoclonal, #610153, BD Transduction Laboratories, USA, 0.08 µg/ml) and CD10 (mouse monoclonal, #NCL-L-CD10-270, Leica Biosystems, Germany, 0.75 µg/ml). Antigen retrieval was performed with the pressure cooker (Retriever 1200) in Tris-EDTA (pH 9.0) for SFRP2 and CTNNB1 double staining, and in 10 mM sodium citrate buffer (pH 6.0) for SFRP2 and CD10 double staining. Tyramide signal amplification system detection kits (TSA™ Kit #41 with Alexa Fluor® 555 tyramide for SFRP2 and TSA™ Kit #2 with Alexa Fluor® 488 tyramide for CTNNB1 and CD10) were used according to the manufacturer's instructions (Thermo Fisher Scientific, USA). The nuclei were stained with DAPI. Analysis was done using the

Zeiss Axioimager M1 Epifluorescence and Brightfield Microscope with exposure times set so that negative controls without primary antibodies showed no signal.

Primary cell culture and siRNA knockdown experiments

Primary cells were isolated from surgically obtained extraovarian endometriosis samples from three patients and plated in serum-free DMEM F12 (Sigma-Aldrich, Merck, USA) supplemented with 1% penicillin/streptomycin and 1% L-glutamine (Gibco, Thermo Fisher Scientific, USA). After one passage to 24-well plates, siRNA treatments were carried out according to the manufacturer's instructions (Origene, USA) with minor modifications. Two different human SFRP2-specific siRNAs (A and B) were tested using three different concentrations (0.1, 1 and 10 nM), of which 10 nM siRNA A resulted in best knockdown level. After 24 h, equal amount of cells treated with SFRP2-siRNA and control siRNA was seeded into 96-well plates for a cell proliferation assay (WST-1; Roche, Switzerland), which was performed the next day according to the manufacturer's instructions, and into 24-well plates for RNA harvesting (p2). For Western blot analysis, the cells from two deep lesions and one peritoneal lesion in passage 1 were transferred to a 6-well plate, and within 1–2 days, the siRNAs were added to the cells, which were cultured until enough protein could be harvested (11–17 days in culture with the siRNAs). Fluorescence imaging at the day of protein harvesting indicated that there was still fluorescent siRNA present in each patient sample, although the non-targeting siRNA did not reduce cell proliferation.

Statistics

GraphPad Prism 6–7 software was used for statistical analysis. The data distribution was tested using D'Agostino & Pearson and Shapiro Wilk tests, and the appropriate test (parametric for normal distribution or non-parametric for non-normal distribution) was selected.

Results

The WNT pathway is dysregulated in endometriosis

We analysed gene expression changes between the endometrium of healthy women and endometriosis patients and different endometriosis lesions in a set of 14 control women and 43 patients after exclusions. The analysis revealed that WNT signaling was strongly affected in endometriosis. Of the 165 pathway genes listed in the KEGG pathway and selected based on literature analysis, 141 (85%) were differentially expressed ($P < 0.05$). The list of the significantly changed WNT pathway genes ($P < 0.05$, $FC \geq 1.4$) is shown in Supplemental Table SII. In a hierarchical clustering analysis of WNT signaling genes, two well-defined clusters were revealed (Fig. 1A). Cluster 1 included endometrium specimens from both controls and patients, whereas cluster 2 contained endometriosis specimens, suggesting that there are major differences in WNT pathway gene expression between the endometrium and endometriosis. Cluster 1 further fragmented into cluster 1a containing most of the proliferative phase endometrium samples (26 out of 28), and cluster 1b containing most of the samples in the secretory phase (26 out of 38). Cluster 2 further divided into two subclusters (2a and 2b), separating ovarian and extraovarian endometriosis, respectively. Thus, strong clustering based on menstrual cycle phase was evident both in the control and patient

endometrium samples. However, this cycle-dependent regulation was completely lost in endometriosis tissue, and the clustering analysis further showed that WNT gene expression differed between ovarian and extraovarian endometriosis. The genes with the strongest contribution to the clustering are shown in Fig. 1B. The largest differences were observed between the endometrium and extraovarian endometriosis, and therefore, we focused our further analyses on extraovarian endometriosis. Furthermore, no differences in the expression of WNT genes between control and patient endometrium samples were observed in the microarray data. Additionally, there were no differences in WNT and CTNINB1 target gene expression between the control and patient peritoneum tissues (data not shown), and the peritoneum samples were excluded from further analyses. To better understand the hormonal regulation of the WNT pathway, the expression patterns in different menstrual cycle phases were analysed for the genes shown in Fig. 1B. Out of 30 genes, 8 (26.7%) showed differential expression in proliferative vs secretory endometrium, as shown in Supplemental Fig. S1; none of the genes showed menstrual cycle-dependent expression differences in endometriosis tissue, and none showed differences between control and patient endometrium samples.

We next validated the mRNA expression of selected WNT pathway genes by RT-qPCR (Fig. 2). The gene expression patterns were similar to those observed by the microarray profiling (Supplemental Table SII) for all the selected genes. Among these genes, *SFRP2* was one of the most upregulated genes in extraovarian endometriosis compared with the endometrium, in both the microarray and RT-qPCR analyses. Notably, the expression of *SFRP2* with RT-qPCR analysis was 183-fold higher in the extraovarian endometriosis than in the endometrium ($P < 0.001$). Only patient endometrium samples were used in RT-qPCR analyses, except for *SFRP1* and *SFRP2*, for which the analysis also included control endometrium samples showing no differences compared with patient endometrium samples.

High SFRP2 expression indicates extraovarian endometriosis lesion borders

Western blotting analysis from tissue homogenates showed significantly increased expression of SFRP2 in extraovarian endometriosis compared with patient endometrium and ovarian endometriosis samples ($P < 0.01$; Fig. 3A and B). Due to the variability of the tissue architecture in endometriosis specimens, we continued the analyses with IHC, enabling a more detailed analysis of cell type-specific expression. SFRP2 staining was observed in cells from both epithelial and stromal components in all samples. In the endometrium of controls and patients, SFRP2 staining was equally strong in epithelial and stromal cells during the proliferative phase, whereas in the secretory phase, SFRP2 staining was reduced, especially in the epithelial cells of the endometrium (Fig. 3C, E and F). In extraovarian endometriosis, the suppression was diminished, and strong SFRP2 staining was observed throughout the menstrual cycle in the epithelial and stromal components (Fig. 3D, G and H).

We then correlated the *SFRP2* microarray gene expression data with our recently published hormone data from the same samples (Huhtinen et al., 2012, 2014) and observed a negative correlation between *SFRP2* expression and the intratissue progesterone concentration in extraovarian endometriosis ($r = -0.552$, $P < 0.05$), while no

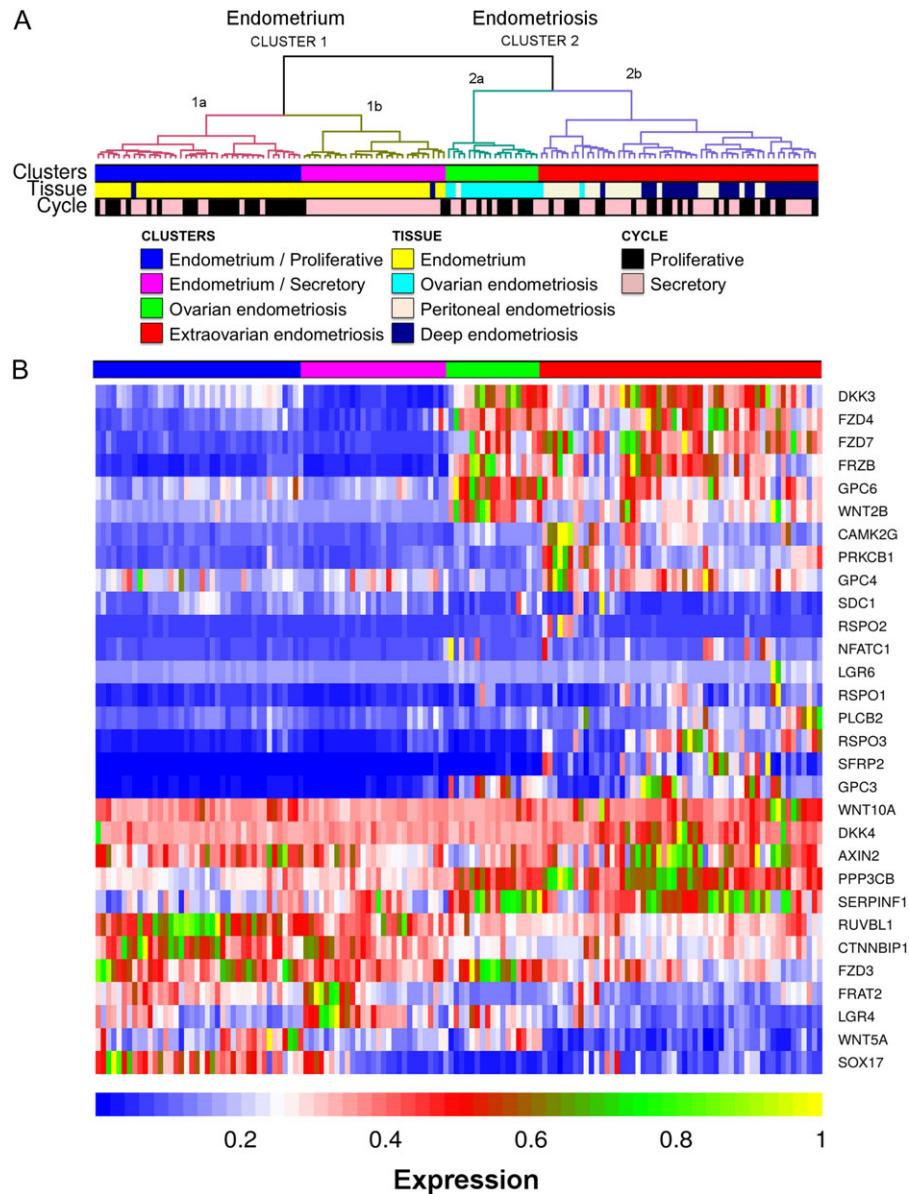


Figure 1 Hierarchical clustering of the WNT signaling pathway genes. Gene expression profiling data from proliferative and secretory control and patient endometrium samples ($n = 66$) and ovarian ($n = 19$) and extraovarian endometriosis ($n = 55$) lesions without hormonal medication showed that the WNT signaling pathway is strongly altered in extraovarian endometriosis. **(A)** The WNT pathway genes formed two major clusters in the hierarchical clustering analysis: cluster 1 containing endometrium samples and cluster 2 containing endometriosis samples. Cluster 1 further fragmented into two subclusters: 1a, which was mainly composed of proliferative phase endometrium samples, and cluster 1b, which contained secretory phase endometrium samples. Cluster 2 also formed two subclusters: 2a, composed of ovarian endometriosis samples, and cluster 2b, composed of extraovarian endometriosis samples. No subclustering based on menstrual cycle phase was seen for endometriosis samples in cluster 2. **(B)** A heatmap showing the expression of the WNT pathway genes most strongly driving the clustering.

correlations were found with estradiol and testosterone. Two strong progesterone response elements (PREs) in the *SFRP2* promoter were predicted to be present by computer analyses (Fig. 3J and K). Patient questionnaire data collected on the day of surgery further revealed that the expression of *SFRP2* mRNA positively correlated with the occurrence of abdominal menstrual pain, the main symptom of endometriosis ($r = 0.300$, $P < 0.05$), as shown in Table III and Fig. 3L.

A marked difference in the *SFRP2* IHC and immunofluorescence staining intensities were observed between extraovarian endometriosis lesions and the normal-like tissue surrounding the lesions (Fig. 4A–H). Since *SFRP2* is involved in WNT signaling and *CTNNB1* is a key mediator of the canonical WNT pathway, we analysed the expression of *CTNNB1* in extraovarian endometriosis, and remarkably similar expression patterns were observed for *SFRP2* and *CTNNB1* (Fig. 4A–H). Strong *SFRP2* and *CTNNB1* signals were consistently observed in the

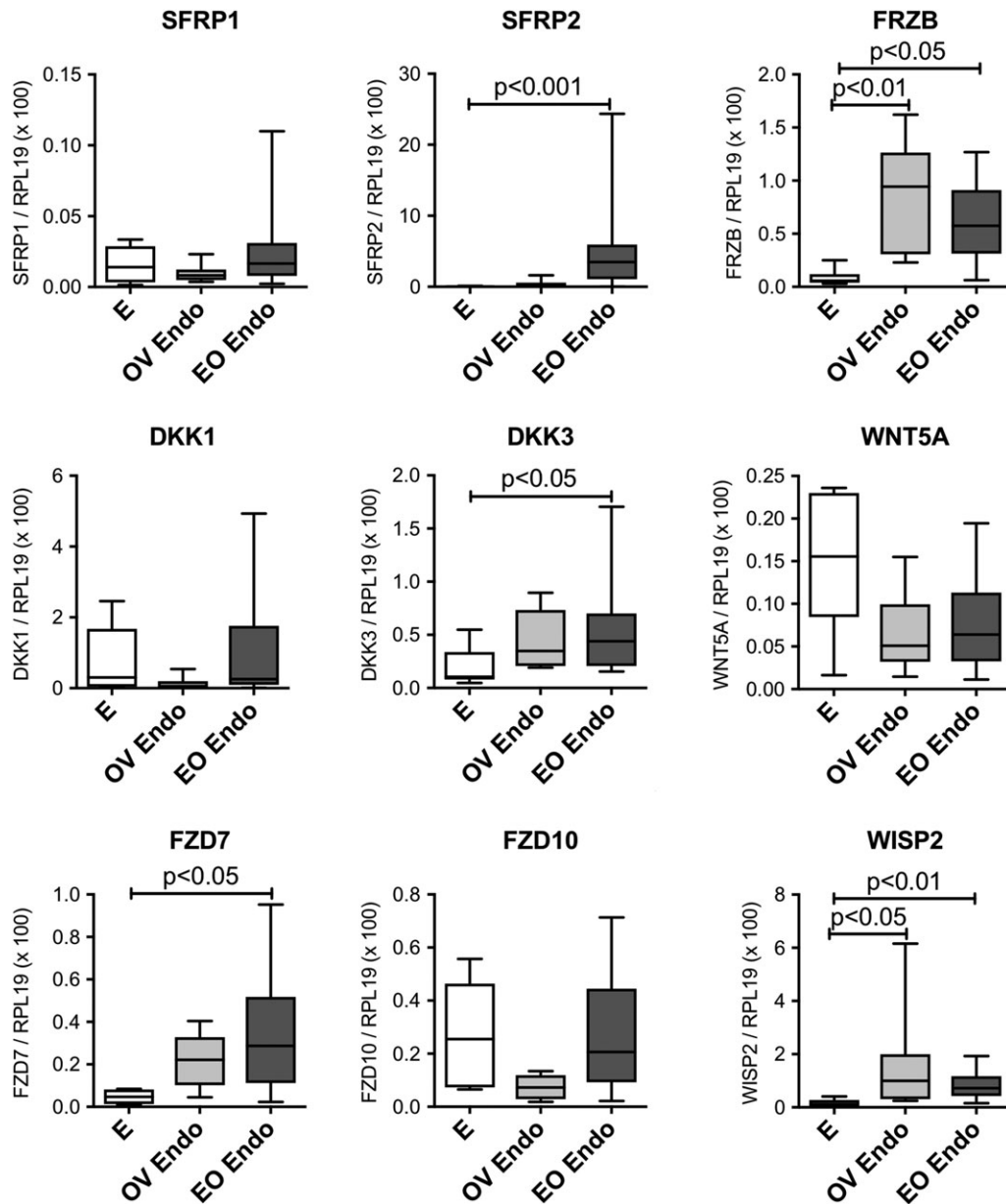


Figure 2 Validation of selected WNT pathway target gene expression by RT-qPCR. Expression of *SFRP1*, *SFRP2*, *FRZB*, *DKK1*, *DKK3*, *WNT5A*, *FZD7*, *FZD10* and *WISP2* was analysed by RT-qPCR ($n = 4-12$) to validate the microarray data. Statistically significant expression changes were seen for *SFRP2*, *FRZB*, *DKK3*, *FZD7* and *WISP2*. *SFRP2* showed the highest expression increase between endometrium and extraovarian endometriosis (183.3-fold increase in extraovarian endometriosis), while the increase was much less pronounced (5.4-fold) for ovarian endometriosis. E = endometrium, OV Endo = ovarian endometriosis, EO Endo = extraovarian endometriosis. Statistical analyses used: one-way ANOVA or Kruskal-Wallis multiple comparison tests.

same regions as CD10, which is used as a standard marker to identify the stroma surrounding the epithelium of endometriosis lesions (Sumathi and McCluggage, 2002). In contrast to CD10, SFRP2 and CTNNB1 staining was also found in the endometriosis epithelium. Furthermore, SFRP2 and CTNNB1 indicated a secondary, more distant border with less intense staining that was not detected with CD10 (Fig. 4D).

CTNNB1 protein expression is increased in extraovarian endometriosis

The nuclear localization of CTNNB1 is a hallmark of canonical WNT pathway activation. We therefore examined the subcellular localization of CTNNB1 with IHC in the endometrium and extraovarian endometriosis. The staining intensity of the nuclei was scored, showing

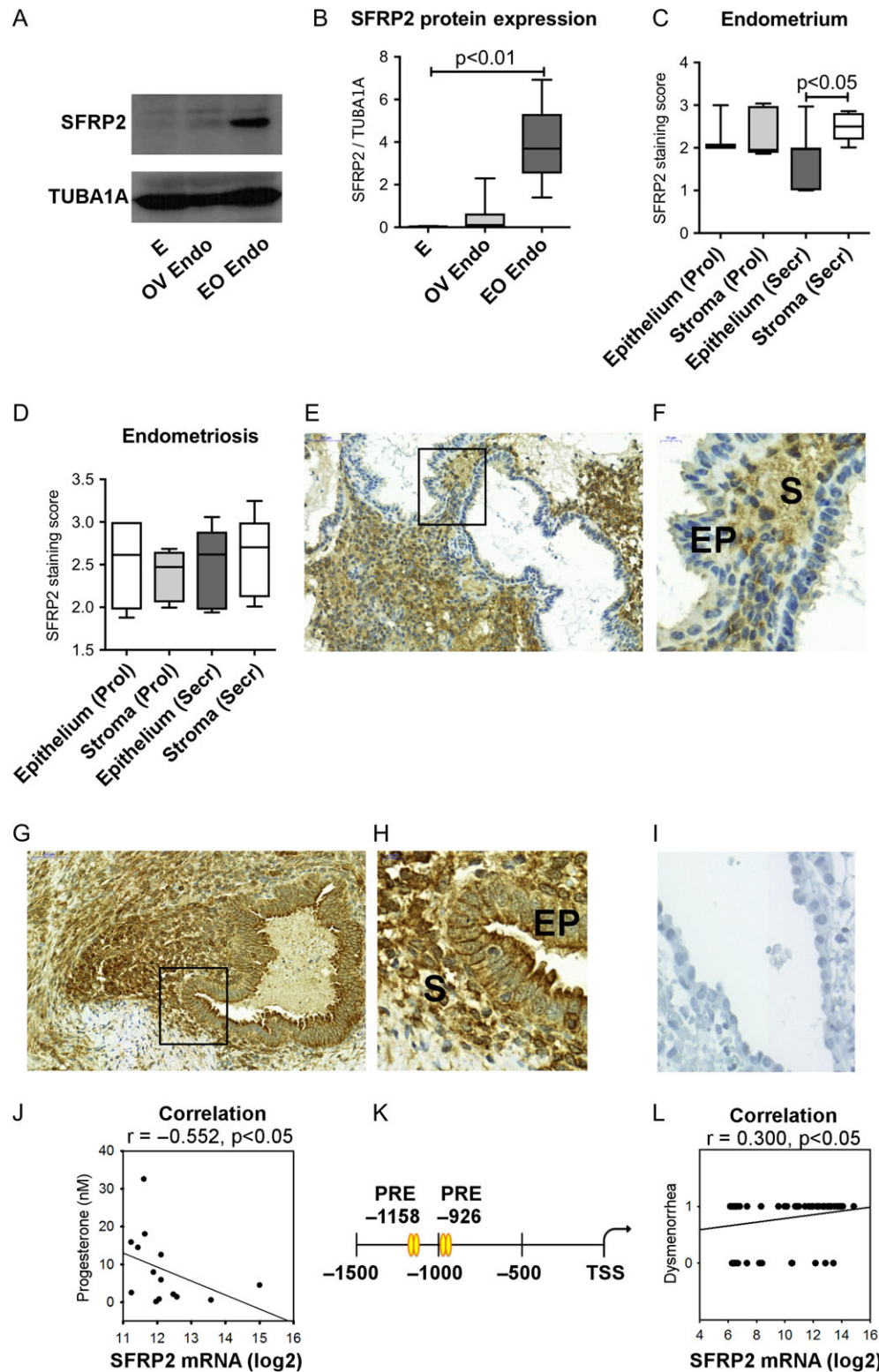


Figure 3 SFRP2 protein expression in endometrium and endometriosis tissue. Western blotting analysis showed that SFRP2 protein expression was substantially increased in extraovarian endometriosis, while the increase was much less pronounced in ovarian endometriosis compared with the endometrium. **(A)** A representative Western blot. **(B)** Protein intensities measured in the endometrium, ovarian endometriosis and extraovarian endometriosis, with each group containing three proliferative and three secretory phase samples. SFRP2 expression was normalized to TUBA1A expression. No cycle-dependent effect was observed in the Western blot analysis (data not shown). **(C)** A more detailed immunohistochemical scoring analysis showed that in the endometrium ($n = 16$, of which seven were proliferative and nine were secretory), SFRP2 was downregulated during the secretory

Table III Correlation of SFRP2 mRNA expression with clinical features of patients.

Clinical variable	Correlation coefficient	P-value
Abdominal pain occurrence	0.097	<0.001
Menstrual pain days	0.118	<0.001
Menstruation length	0.124	<0.001
Menstrual cycle length	-0.032	<0.001
Abdominal menstrual pain	0.300	<0.05
Menstrual pain strength	0.120	<0.05
Menstruation pain	0.165	<0.05
Pregnancy wish	0.129	<0.05
Intercourse pain	0.115	<0.05
Abdominal pain strength	0.107	0.054
Urination pain	0.110	0.064
Abdominal pain	0.097	0.081
Recurrence	-0.115	0.136
Urination pain strength	0.077	0.165
Defecation pain strength	-0.012	0.866
Intercourse pain strength	0.004	0.951

significantly more nuclear CTNNB1 staining in endometriosis compared with control and patient endometrium tissue in both the epithelium and stroma (Fig. 5A–F). Because no difference was observed between control and patient endometrium samples, they were analysed as one group. Also the membranous CTNNB1 staining was increased in extraovarian endometriosis. To further confirm the increased canonical WNT signaling pathway activation in endometriosis, we analysed the expression of known human CTNNB1 target genes. Of the 54 genes known to be upregulated upon CTNNB1 activation, 27 (50%) were upregulated in extraovarian endometriosis compared with control and patient endometrium samples (analysed as one group). The microarray expression patterns for selected CTNNB1 target genes are shown in Fig. 5G. The full list of upregulated targets with their microarray expression differences is shown in Supplemental Table SIII. We then analysed the hierarchical clustering pattern of the human CTNNB1 target genes, and as expected, these genes formed similar clusters as those observed by analysing the WNT pathway genes (Supplemental Fig. S2). Out of 8 (37.5%)

CTNNB1 target genes shown in Fig. 5G, three genes (*CLDN1*, *JUN* and *VEGFB*) were differentially expressed in proliferative vs secretory endometrium tissues, while none of them showed menstrual cycle-dependent regulation in endometriosis tissue and none showed differences between control and patient endometrium samples (Supplemental Fig. S3).

SFRP2 is a canonical WNT signaling agonist in endometriosis

Primary cultured extraovarian endometriotic cells from four human lesions from three patients were exposed to *SFRP2* siRNA-mediated knockdown, reducing *SFRP2* mRNA expression by 72% ($P < 0.05$) on average and protein expression by 60% ($P < 0.01$) compared with the control siRNA (Fig. 6A–C). *SFRP2* knockdown significantly reduced cell proliferation (48%, $P < 0.05$; Fig. 6D), and cell proliferation and *SFRP2* mRNA expression showed a strong positive correlation in all the samples in the siRNA experiment ($r = 0.732$, $P < 0.01$; Fig. 6G). Western blot analysis showed, on average, a 33% reduction in total CTNNB1 protein expression ($P = 0.05$) after *SFRP2* knockdown compared with treatment with a non-targeting siRNA (Fig. 6E and F).

Summary of results

In Supplemental Fig. S4, we summarize our findings. The data indicate suppression of *SFRP2* expression by progesterone in the endometrium that is diminished in extraovarian endometriosis, as evidenced by a negative correlation between *SFRP2* mRNA and the intratissue progesterone level. As a consequence, *SFRP2* is highly overexpressed in extraovarian endometriosis and activates the canonical WNT signaling pathway, resulting in increased cell proliferation, endometriosis lesion growth and abdominal menstrual pain symptoms experienced by patients.

Discussion

We show differential activation of the WNT signaling pathway in endometriosis compared with the endometrium, providing evidence for a central role of WNT pathway in endometriosis pathogenesis. The highest expression change was observed for *SFRP2* in extraovarian endometriosis, whereas its expression was less pronounced in ovarian endometriosis. Because incomplete removal of endometriosis lesions during surgery is a significant cause of recurrence (Guo, 2009; Rizk et al., 2014; Cao et al., 2015), identifying of lesion borders to aid endometriosis surgery has clinical value. A technique where methylene

phase in the epithelium compared with stroma, whereas downregulation was not observed in extraovarian endometriosis ($n = 10$, of which five were proliferative and five were secretory) (D). (E, F) Representative pictures at two magnifications ($\times 34$ and $\times 100$, respectively) from the secretory phase of the menstrual cycle are shown for endometrium tissue with apparent epithelial *SFRP2* downregulation. (G, H) In extraovarian endometriosis, equal staining intensity in both the epithelium and stroma was observed in the secretory phase ($\times 34$ and $\times 100$ magnifications, respectively). (I) A negative control for *SFRP2* staining is shown. (J) A correlation plot showing a negative correlation between *SFRP2* mRNA and the intratissue progesterone concentration in extraovarian endometriosis. (K) Promoter analysis in silico revealed two strong progesterone response elements (PREs) in human *SFRP2* promoter, 926 and 1158 bases upstream from the transcription start site (TSS). (L) *SFRP2* mRNA expression was positively correlated with the occurrence of abdominal menstrual pain over all the microarray samples and clinical patient questionnaire data (1 = abdominal menstrual pain, 0 = no abdominal menstrual pain). E = endometrium, OV Endo = ovarian endometriosis, EO Endo = extraovarian endometriosis, prol = proliferative cycle phase, secr = secretory cycle phase, EP = epithelium, S = stroma. Statistical analyses used: Kruskal–Wallis multiple comparison test and Spearman's and polyserial correlation analyses for (J) and (L), respectively.

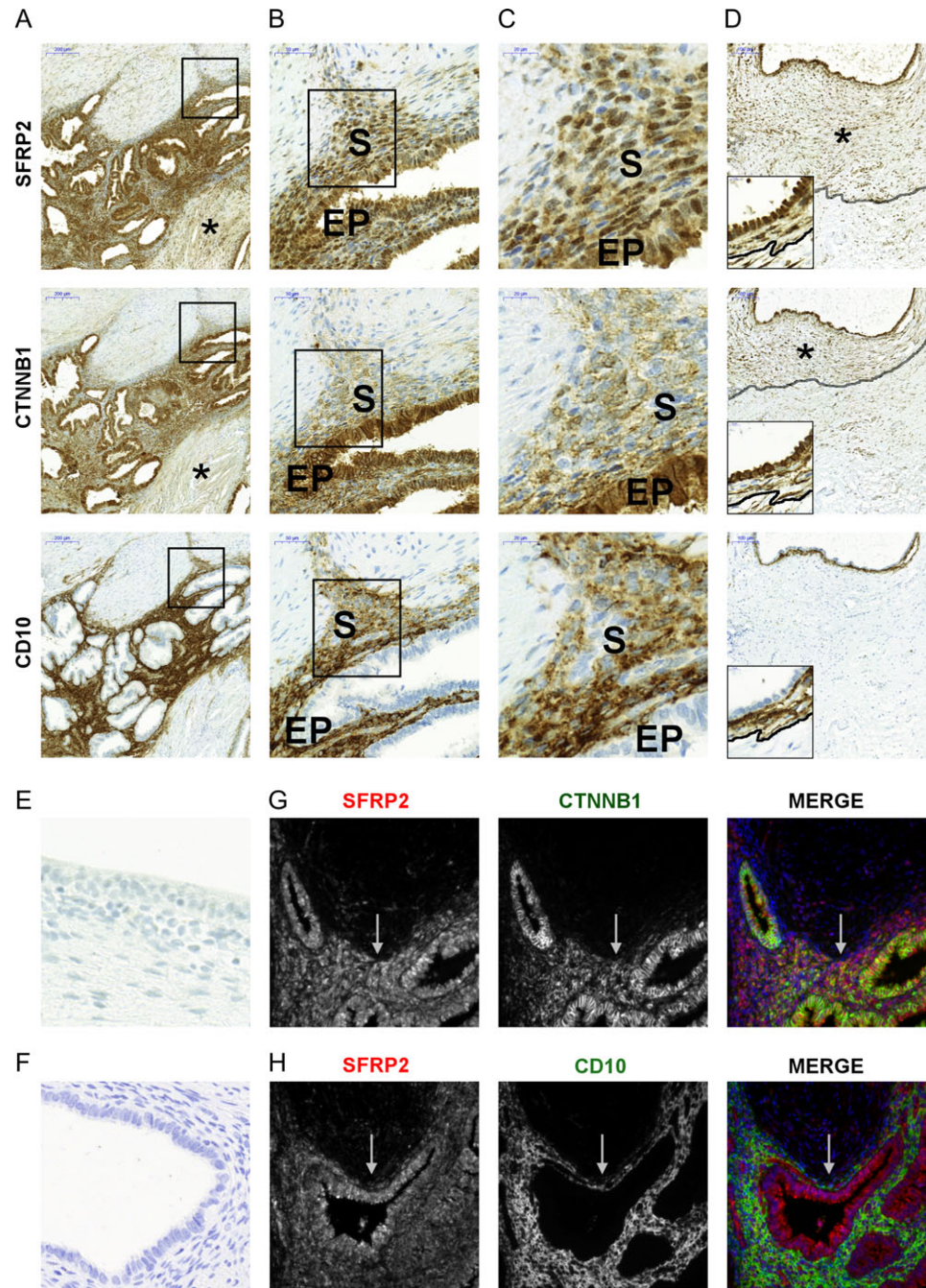


Figure 4 Lesion border staining with SFRP2, CTNNB1 and CD10. **(A)** Representative pictures of the immunohistochemical analysis of 20 extraovarian endometriosis lesions show that SFRP2 and CTNNB1 staining indicate lesion borders similar to the known endometriosis stromal marker CD10. Furthermore, less intense staining with SFRP2 and CTNNB1 antibodies was observed in an extended area surrounding the lesions [stars in (A) and (D)]. Images were taken at $\times 6.2$ magnification. **(B)** Higher magnification ($\times 26.8$) of the staining observed in the regions outlined by black boxes in the adjacent pictures on the left. **(C)** Higher magnification ($\times 66$) of the staining observed in the regions outlined by black boxes in the adjacent pictures on the left. **(D)** In addition to a primary lesion border [black lines in the high magnification ($\times 100$) inserts in panel (D)], both SFRP2 and CTNNB1 staining showed a secondary lesion border with milder staining [gray lines in the full-sized $\times 10$ magnification pictures of (D)] that extended beyond the primary lesion border. **(E and F)** Negative controls for CTNNB1 and CD10 IHC staining, respectively (negative control for SFRP2 shown in Fig. 3). **(G and H)** Double immunofluorescent staining with antibodies against (G) SFRP2 (red) and CTNNB1 (green) and (H) SFRP2 (red) and CD10 (green) confirmed that SFRP2 was expressed in the same region with both CTNNB1 and CD10. Primary lesion borders are indicated by gray arrows. The blue colour comes from DAPI staining. All images were taken in the secretory phase of the menstrual cycle, and the immunofluorescent pictures were taken at $\times 20$ magnification. EP = epithelium, S = stroma.

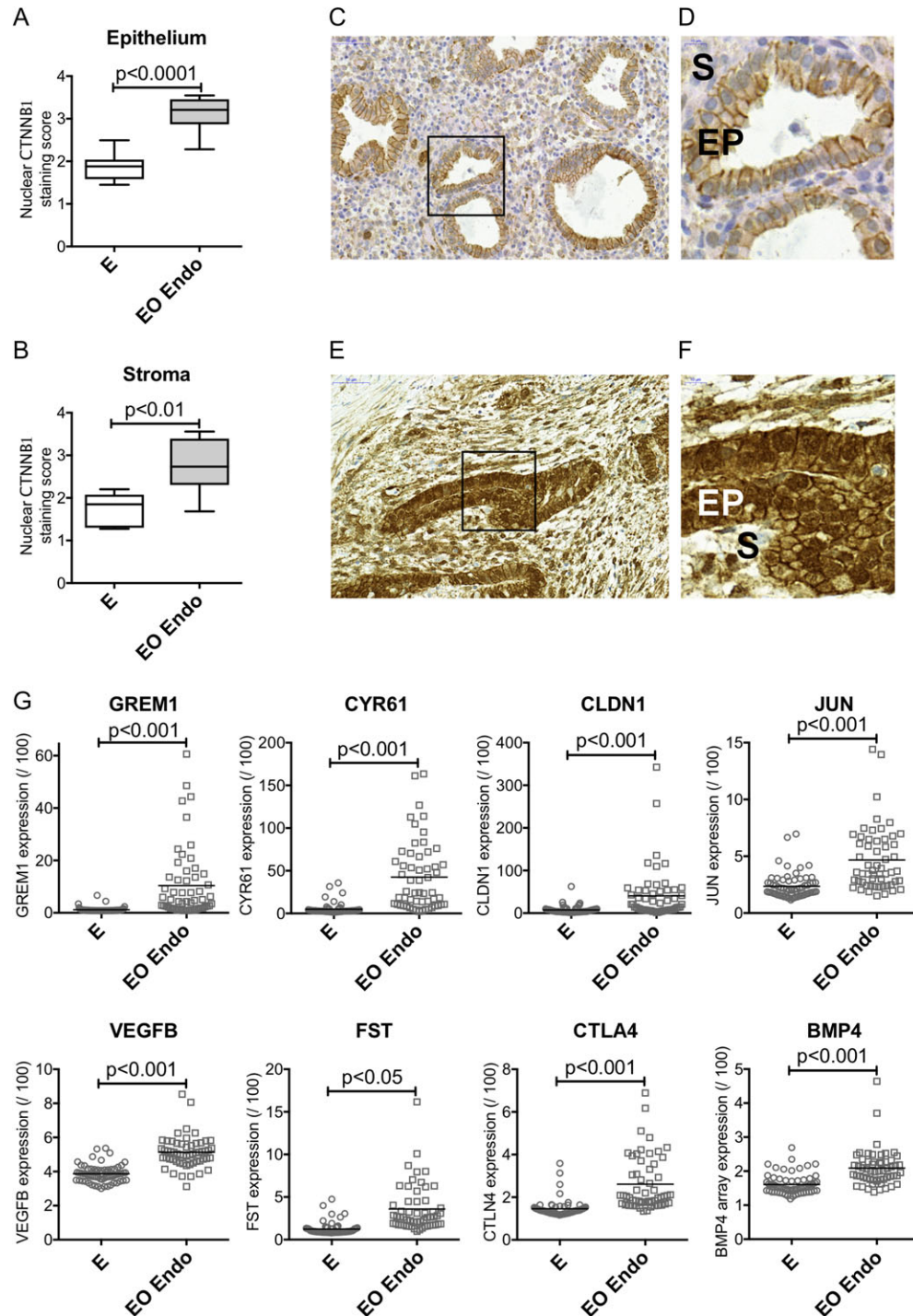
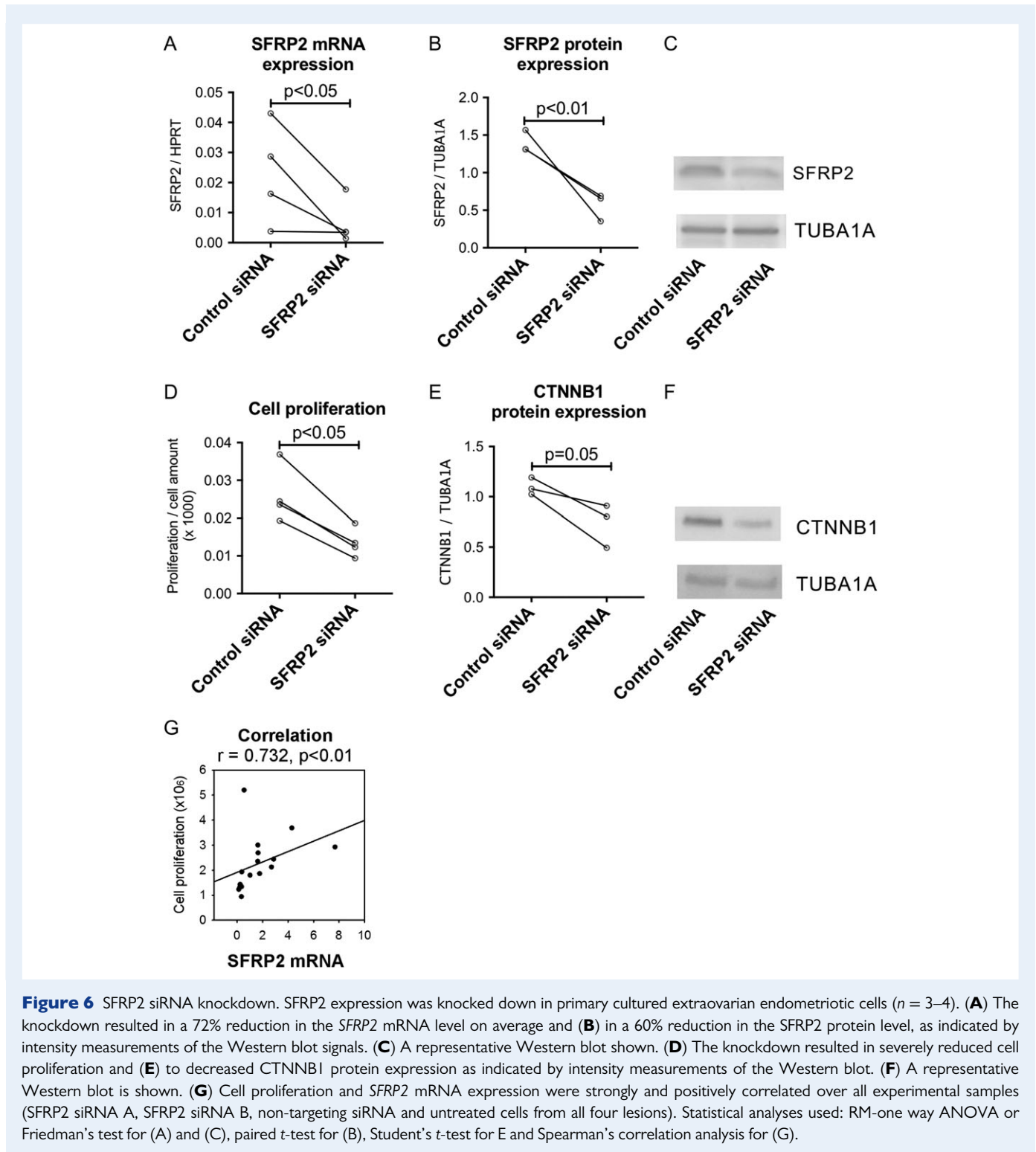


Figure 5 Increased nuclear CTNNB1 and target gene expression. The intensity of nuclear CTNNB1 immunohistochemical staining was scored in endometrium and extraovarian endometriosis samples ($n = 8$ per group, of which 3 were in proliferative and 5 were in secretory phase). There was significantly more nuclear CTNNB1 in extraovarian endometriosis than in the endometrium both in the epithelial (**A**) and stromal (**B**) compartments. (**C** and **D**) Representative images taken in the secretory phase of the menstrual cycle for CTNNB1 staining in the endometrium show mainly cytoplasmic and membranous staining (magnification $\times 34$ and $\times 100$, respectively), while staining of the extraovarian endometriosis tissue (**E** and **F**) shows a high nuclear CTNNB1 level (magnification $\times 34$ and $\times 100$, respectively). (**G**) CTNNB1 target gene expression analysis of the microarray data showed altered expression for numerous CTNNB1 target genes in endometriosis compared with the endometrium ($n = 66$ for endometrium and 55 for extraovarian endometriosis tissues). The expression patterns of *GREM1*, *CYR61*, *CLDN1*, *JUN*, *VEGFB*, *FST*, *CTLA4* and *BMP4* are shown as examples. E = endometrium, EO Endo = extraovarian endometriosis, EP = epithelium, S = stroma. Statistical analyses used: Student's *t*-test for (A) and (B), unpaired non-parametric Mann–Whitney test for (G).



blue stain is applied on peritoneal surfaces during surgery has raised awareness that endometriotic lesions extend beyond the most visible lesion area and that enhanced visualization of the lesions could help in identifying minimal endometriosis (Lessey et al., 2012). The use of indigo carmine staining was also recently introduced for this purpose (Rauh-Hain and Laufer, 2011). In the present study, we show that the

highly expressed SFRP2 indicates the active endometriotic epithelium and stroma and, thus, likely the endometriosis lesion borders, while it was evidently less expressed in the adjacent areas, including rectovaginal septum, uterosacral ligaments, bladder, intestine, peritoneum or ovarian tissue. Furthermore, the key canonical WNT signaling mediator CTNNB1 showed an expression pattern that was highly similar to

that of SFRP2, suggesting that their expression is interrelated. Importantly, SFRP2 and CTNNB1 indicated a secondary, more distant lesion border not shown by the currently applied marker CD10 (Sumathi and McCluggage, 2002), indicating that SFRP2 and CTNNB1 are novel endometriosis lesion border markers.

The WNT pathway is active during uterine growth, implantation and cyclic remodeling of the endometrium, as evidenced by the fact that some WNT pathway components are expressed in a cycle-dependent and cell-specific manner in the human endometrium. In the proliferative phase, estradiol enhances WNT signaling, whereas in the secretory phase, progesterone has an inhibitory effect (Wang *et al.*, 2010). For a few WNT pathway genes, cycle-dependent variations have been reported (Tulac *et al.*, 2003; Talbi *et al.*, 2006; Wang *et al.*, 2009, 2010), and direct regulation by sex steroids has been shown for some of these genes (Banerjee *et al.*, 2003; Wang *et al.*, 2009; Zhang *et al.*, 2016a, 2016b). Our data showed that endometrial epithelial SFRP2 expression was regulated in a cycle-dependent manner, whereas the cycle-dependent regulation was lost in extraovarian endometriosis. Our expression analysis of WNT pathway components and CTNNB1 target genes showed that endometrial samples clustered separately from endometriosis and further subclustered into proliferative and secretory phase endometrium samples. No clustering according to the menstrual cycle phase was observed in endometriosis samples, which further clustered into subclusters separating ovarian and extraovarian endometriosis. In line with these data, we have shown that cycle-dependent changes in estradiol and progesterone concentrations observed in the endometrium are lost in endometriosis tissue (Huhtinen *et al.*, 2012, 2014), and the loss of cycle phase-specific expression patterns of WNT genes is likely to reflect the disturbed hormonal environment of endometriosis. Furthermore, a negative correlation between SFRP2 mRNA expression and the intratissue progesterone concentration in extraovarian endometriosis suggests that SFRP2 expression is negatively regulated by progesterone in endometriosis, supported by the presence of two strong PREs in the SFRP2 promoter. Recent studies have shown that estradiol directly upregulates CTNNB1 expression by binding to the estrogen-response element in the CTNNB1 promoter in ovarian endometriosis (Zhang *et al.*, 2016a, 2016b), supporting our data indicating that steroid hormone action is a central upstream regulatory mechanism for WNT signaling in endometrium and endometriosis tissue.

In extraovarian endometriosis, we observed significantly more nuclear and membranous CTNNB1 staining, accompanied by increased target gene expression, compared with the endometrium and ovarian endometriosis, indicating increased canonical WNT signaling activity in extraovarian endometriosis. Reduced membranous CTNNB1 staining has been shown during the transformation from normal endometrium to cancer (Saegusa *et al.*, 2001), and degradation of membranous CTNNB1 is associated with cancer metastasis and invasion (Kudo *et al.*, 2004). Thus, CTNNB1 might have a dual role in endometriosis by promoting proliferation but at the same time controlling invasion and metastasis. Studies have shown a role for SFRP2 as either a WNT signaling agonist or antagonist (Roth *et al.*, 2000; Bovolenta *et al.*, 2008; Esteve *et al.*, 2011; Fontenot *et al.*, 2013). By using primary cultured extraovarian endometriosis cells, we showed that SFRP2 knockdown resulted in severely reduced cell proliferation and lower CTNNB1 protein expression, indicating that SFRP2 expression stimulates canonical WNT signaling and lesion growth upstream of CTNNB1 in extraovarian endometriosis.

Interestingly, we could not detect changes in WNT gene expression between the endometrium of control women and endometriosis patients, suggesting a non-endometrial origin of these changes, most likely gained after the endometrial tissue has escaped during retrograde menstruation, or reflecting the differential origin of the tissues, as suggested by the metaplasia- or stem cell-based endometriosis pathogenesis models (Vercellini *et al.*, 2014). Numerous therapeutic approaches are currently being developed for diseases associated with abnormal WNT signaling (Rey and Ellies, 2010; Lu *et al.*, 2016), including approaches targeting SFRP2 (Fontenot *et al.*, 2013), and the treatments under development could provide novel and effective opportunities to treat endometriosis.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Acknowledgements

We acknowledge Ms Anu Salminen and the TCDM Histology Unit for technical assistance.

Authors' roles

T.H., M.G., L.K., K.H. and E.K. carried out experiments, T.H., M.G., L.K., P.S., T.A., A.P. and M.P. conceived the experiments, TH, M.G., L.K., P.A., K.H., T.D.L., E.K., A.M., P.S., H.K., T.A., A.P. and M.P. contributed to data analysis. All authors contributed to drafting the article or revising it critically for important intellectual content, and gave a final approval of the version to be published.

Funding

Academy of Finland (Grant number 277624) and Finnish Funding Agency for Technology and Innovation (Grant numbers 40250/12 and 40279/14).

Conflict of interest

The authors have nothing to disclose.

References

- Aghajanova L, Horcajadas JA, Weeks JL, Esteban FJ, Nezhat CN, Conti M, Giudice LC. The protein kinase A pathway-regulated transcriptome of endometrial stromal fibroblasts reveals compromised differentiation and persistent proliferative potential in endometriosis. *Endocrinology* 2010; **151**:1341–1355.
- Anastas JN, Moon RT. WNT signalling pathways as therapeutic targets in cancer. *Nat Rev Cancer* 2013; **13**:11–26.
- Banerjee S, Saxena N, Sengupta K, Tawfik O, Mayo MS, Banerjee SK. WISP-2 gene in human breast cancer: estrogen and progesterone inducible expression and regulation of tumor cell proliferation. *Neoplasia* 2003; **5**:63–73.
- Bovolenta P, Esteve P, Ruiz JM, Cisneros E, Lopez-Rios J. Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease. *J Cell Sci* 2008; **121**:737–746.
- Bulun SE. Endometriosis. *N Engl J Med* 2009; **360**:268–279.

- Cao Q, Lu F, Feng WW, Ding JX, Hua KQ. Comparison of complete and incomplete excision of deep infiltrating endometriosis. *Int J Clin Exp Med* 2015;**8**:21497–21506.
- Clevers H, Loh KM, Nusse R. Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. *Science* 2014;**346**:1248012.
- Dunselman GA, Vermeulen N, Becker C, Calhaz-Jorge C, D'Hooghe T, De Bie B, Heikinheimo O, Horne AW, Kiesel L, Nap A et al. ESHRE guideline: management of women with endometriosis. *Hum Reprod* 2014;**29**:400–412.
- Esteve P, Sandonis A, Ibanez C, Shimono A, Guerrero I, Bovolenta P. Secreted frizzled-related proteins are required for Wnt/beta-catenin signalling activation in the vertebrate optic cup. *Development* 2011;**138**:4179–4184.
- Eyster KM, Klinkova O, Kennedy V, Hansen KA. Whole genome deoxyribonucleic acid microarray analysis of gene expression in ectopic versus eutopic endometrium. *Fertil Steril* 2007;**88**:1505–1533.
- Fontenot E, Rossi E, Mumper R, Snyder S, Siamakpour-Reihani S, Ma P, Hilliard E, Bone B, Ketelsen D, Santos C et al. A novel monoclonal antibody to secreted frizzled-related protein 2 inhibits tumor growth. *Mol Cancer Ther* 2013;**12**:685–695.
- Giudice LC. Clinical practice. Endometriosis. *N Engl J Med* 2010;**362**:2389–2398.
- Green J, Nusse R, van Amerongen R. The role of Ryk and Ror receptor tyrosine kinases in Wnt signal transduction. *Cold Spring Harb Perspect Biol* 2014;**6**. [10.1101/cshperspect.a009175](https://doi.org/10.1101/cshperspect.a009175).
- Guo SW. Recurrence of endometriosis and its control. *Hum Reprod Update* 2009;**15**:441–461.
- Herbst A, Jurinovic V, Krebs S, Thieme SE, Blum H, Goke B, Kolligs FT. Comprehensive analysis of beta-catenin target genes in colorectal carcinoma cell lines with deregulated Wnt/beta-catenin signaling. *BMC Genomics* 2014;**15**:74. [doi:10.1186/1471-2164-15-74](https://doi.org/10.1186/1471-2164-15-74).
- Huhtinen K, Desai R, Stahle M, Salminen A, Handelsman DJ, Perheentupa A, Poutanen M. Endometrial and endometriotic concentrations of estrone and estradiol are determined by local metabolism rather than circulating levels. *J Clin Endocrinol Metab* 2012;**97**:4228–4235.
- Huhtinen K, Saloniemi-Heinonen T, Keski-Rahkonen P, Desai R, Laajala D, Stahle M, Hakkinen MR, Awosanya M, Suvitie P, Kujari H et al. Intra-tissue steroid profiling indicates differential progesterone and testosterone metabolism in the endometrium and endometriosis lesions. *J Clin Endocrinol Metab* 2014;**99**:E2188–E2197.
- Kawano Y, Kypta R. Secreted antagonists of the Wnt signalling pathway. *J Cell Sci* 2003;**116**:2627–2634.
- Klaus A, Birchmeier W. Wnt signalling and its impact on development and cancer. *Nat Rev Cancer* 2008;**8**:387–398.
- Kudo Y, Kitajima S, Ogawa I, Hiraoka M, Sargolzaei S, Keikhaee MR, Sato S, Miyauchi M, Takata T. Invasion and metastasis of oral cancer cells require methylation of E-cadherin and/or degradation of membranous beta-catenin. *Clin Cancer Res* 2004;**10**:5455–5463.
- Lagana AS, Vitale SG, Salmeri FM, Triolo O, Ban Frangez H, Vrtacnik-Bokal E, Stojanovska L, Apostolopoulos V, Granese R, Sofo V. Unus pro omnibus, omnes pro uno: a novel, evidence-based, unifying theory for the pathogenesis of endometriosis. *Med Hypotheses* 2017;**103**:10–20.
- Lessey BA, Higdon HL 3rd, Miller SE, Price TA. Intraoperative detection of subtle endometriosis: a novel paradigm for detection and treatment of pelvic pain associated with the loss of peritoneal integrity. *J Vis Exp* 2012. pii: 4313. [doi:10.3791/4313](https://doi.org/10.3791/4313)
- Lu B, Green BA, Farr JM, Lopes FC, Van Raay TJ. Wnt drug discovery: weaving through the screens, patents and clinical trials. *Cancers (Basel)* 2016;**8**. [10.3390/cancers8090082](https://doi.org/10.3390/cancers8090082).
- Maeda D, Shih I. Pathogenesis and the role of ARID1A mutation in endometriosis-related ovarian neoplasms. *Adv Anat Pathol* 2013;**20**:45–52.
- Matsuzaki S, Darcha C. Involvement of the Wnt/beta-catenin signaling pathway in the cellular and molecular mechanisms of fibrosis in endometriosis. *PLoS One* 2013;**8**:e76808.
- Matsuzaki S, Darcha C, Maleysson E, Canis M, Mage G. Impaired down-regulation of E-cadherin and beta-catenin protein expression in endometrial epithelial cells in the mid-secretory endometrium of infertile patients with endometriosis. *J Clin Endocrinol Metab* 2010;**95**:3437–3445.
- Messeguer X, Escudero R, Farre D, Nunez O, Martinez J, Alba MM. PROMO: detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics* 2002;**18**:333–334.
- Niehrs C. The complex world of WNT receptor signalling. *Nat Rev Mol Cell Biol* 2012;**13**:767–779.
- Nisolle M, Donnez J. Peritoneal endometriosis, ovarian endometriosis, and adenomyotic nodules of the rectovaginal septum are three different entities. *Fertil Steril* 1997;**68**:585–596.
- Oh SJ, Shin JH, Kim TH, Lee HS, Yoo JY, Ahn JY, Broaddus RR, Taketo MM, Lydon JP, Leach RE et al. beta-Catenin activation contributes to the pathogenesis of adenomyosis through epithelial-mesenchymal transition. *J Pathol* 2013;**231**:210–222.
- Rauh-Hain JA, Laufer MR. Increased diagnostic accuracy of laparoscopy in endometriosis using indigo carmine: a new technique. *Fertil Steril* 2011;**95**:1113–1114.
- Rey JP, Ellies DL. Wnt modulators in the biotech pipeline. *Dev Dyn* 2010;**239**:102–114.
- Rizk B, Fischer AS, Lotfy HA, Turki R, Zahed HA, Malik R, Holliday CP, Glass A, Fishel H, Soliman MY et al. Recurrence of endometriosis after hysterectomy. *Facts Views Vis Obgyn* 2014;**6**:219–227.
- Roth W, Wild-Bode C, Platten M, Grimm C, Melkonyan HS, Dichgans J, Weller M. Secreted Frizzled-related proteins inhibit motility and promote growth of human malignant glioma cells. *Oncogene* 2000;**19**:4210–4220.
- Saegusa M, Hashimura M, Yoshida T, Okayasu I. beta-Catenin mutations and aberrant nuclear expression during endometrial tumorigenesis. *Br J Cancer* 2001;**84**:209–217.
- Simoens S, Dunselman G, Dirksen C, Hummelshoj L, Bokor A, Brandes I, Brodzsky V, Canis M, Colombo GL, DeLeire T et al. The burden of endometriosis: costs and quality of life of women with endometriosis and treated in referral centres. *Hum Reprod* 2012;**27**:1292–1299.
- Sumathi VP, McCluggage WG. CD10 is useful in demonstrating endometrial stroma at ectopic sites and in confirming a diagnosis of endometriosis. *J Clin Pathol* 2002;**55**:391–392.
- Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, Dosiou C, Le Shay N, Nezhat CN, Kempson R, Lessey BA et al. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. *Endocrinology* 2006;**147**:1097–1121.
- Tanwar PS, Lee HJ, Zhang L, Zukerberg LR, Taketo MM, Rueda BR, Teixeira JM. Constitutive activation of Beta-catenin in uterine stroma and smooth muscle leads to the development of mesenchymal tumors in mice. *Biol Reprod* 2009;**81**:545–552.
- Tulac S, Nayak NR, Kao LC, Van Waes M, Huang J, Lobo S, Germeyer A, Lessey BA, Taylor RN, Suchanek E et al. Identification, characterization, and regulation of the canonical Wnt signaling pathway in human endometrium. *J Clin Endocrinol Metab* 2003;**88**:3860–3866.
- Vercellini P, Vignani P, Somigliana E, Fedele L. Endometriosis: pathogenesis and treatment. *Nat Rev Endocrinol* 2014;**10**:261–275.
- Wang Y, Hanifi-Moghaddam P, Hanekamp EE, Kloosterboer HJ, Franken P, Veldscholte J, van Doorn HC, Ewing PC, Kim JJ, Grootegeed JA et al. Progesterone inhibition of Wnt/beta-catenin signaling in normal endometrium and endometrial cancer. *Clin Cancer Res* 2009;**15**:5784–5793.
- Wang Y, van der Zee M, Fodde R, Blok LJ. Wnt/Beta-catenin and sex hormone signaling in endometrial homeostasis and cancer. *Oncotarget* 2010;**1**:674–684.

- Wu Y, Kajdacsy-Balla A, Strawn E, Basir Z, Halverson G, Jailwala P, Wang Y, Wang X, Ghosh S, Guo SW. Transcriptional characterizations of differences between eutopic and ectopic endometrium. *Endocrinology* 2006; **147**:232–246.
- Zhang L, Xiong W, Xiong Y, Liu H, Li N, Du Y, Liu Y. Intracellular Wnt/Beta-Catenin signaling underlying 17beta-estradiol-induced matrix metalloproteinase 9 expression in human endometriosis. *Biol Reprod* 2016a; **94**:70.
- Zhang L, Xiong W, Xiong Y, Liu H, Liu Y. 17 beta-Estradiol promotes vascular endothelial growth factor expression via the Wnt/beta-catenin pathway during the pathogenesis of endometriosis. *Mol Hum Reprod* 2016b; **22**:526–535.