TITLE PAGE

Title

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

Running title

SFRP2 and WNT signaling in endometriosis

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ABSTRACT

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 indicates 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 analyzed, 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 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), and 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 is 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.

Trial registration number: Not applicable.

KEY WORDS

Endometriosis, Extraovarian endometriosis, SFRP2, Canonical WNT signaling, CD10

INTRODUCTION

Endometriosis is a common, benign proliferative disease affecting approximately 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). Endometriosis subtypes differ in symptoms, recurrence and response to treatments (Giudice 2010, Guo 2009). 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 that typically persist after discontinuing the therapy, and surgical intervention is the most effective long-term treatment (Dunselman et al. 2014, Vercellini et al. 2014). The diagnosis of endometriosis also relies on invasive measures, such as laparoscopy combined with histopathological confirmation (Dunselman et al. 2014). The high recurrence rate of endometriosis (up to 50 %) remains a problem, 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 (Aghajanova et al. 2010, Eyster et al. 2007, Matsuzaki et al. 2010, Matsuzaki and Darcha 2013, Talbi et al. 2006, Wu et al. 2006, 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)/adenomatosis polyposis coli (APC) complex, which promotes degradation of CTNNB1. The stabilized CTNNB1 enters the nucleus and interacts with transcription factors promoting target gene expression (Anastas and Moon 2013, Klaus and Birchmeier 2008). Furthermore, two non-canonical WNT pathways exist, which do not operate through CTNNB1: the WNT/planar cell polarity and the WNT/Ca^{2+} pathways stimulated by, for example, WNT5A and WNT11 (Anastas and Moon 2013, Klaus and Birchmeier 2008).

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 favor 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 (Oh et al. 2013, Tanwar et al. 2009) and to be activated by estradiol in

ovarian endometriosis (Zhang et al. 2016a, Zhang et al. 2016b). Activating mutations of CTNNB1 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 endometriosis lesions.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Joint Ethics Committee of Turku University and Turku University Central 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, 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

187 women were collected. We also collected peritoneum samples from 24 control women and from 28
188 patients. Patient characteristics are presented in Table I. Tissue samples were snap-frozen and
189 stored in liquid nitrogen until used, or immediately processed for cell culture studies. An aliquot
190 was fixed in formalin and embedded in paraffin for histological analysis.
191
192 **RNA isolation**
193 For microarray analysis and quantitative reverse transcription PCR (RT-qPCR), total RNA was
194 isolated using Trizol reagent (Thermo Fisher Scientific, USA), further purified with RNeasy
195 columns (Qiagen, Netherlands), and treated with DNase (RNase-free DNase Set, Qiagen,
196 Netherlands; or DNase I, Invitrogen, Thermo Fisher Scientific, USA). For the siRNA experiment,
197 TRIsure reagent (Bioline, UK) was used according to manufacturer's instructions. The RNA
198 concentrations were measured using Nanodrop ND-1000 spectrophotometer (Thermo Fisher
199 Scientific, USA).
200
201 **Gene expression analysis by microarray**
202 The quality of the isolated RNA was controlled by Experion analysis (Bio-Rad Laboratories, USA).
203 The microarray analysis was performed using 177 endometriotic lesions (72 peritoneal, 77 deep and
204 28 ovarian endometriosis lesions), 63 endometrial biopsies and 28 peritoneum samples from 103
205 endometriosis patients. RNA from 41 endometrial biopsies and 24 peritoneum samples collected
206 from 47 healthy control women was also included. The study subjects provided a variable number
207 of samples per subject. Only data from patients without hormonal medication were included, unless
208 specifically mentioned. Microarray sample information is provided in Table II. The gene expression
209 profiles were measured using the Sentrix® Human Illumina 6 V2 Expression BeadChips (Illumina,
210 USA). Normalization and analyses were performed using the R package limma (http://www.r-
211 project.org).
212

Pathway and correlation analysis

Subjects using hormonal medication (n=12 controls and 44 patients), 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 (Clevers et al. 2014, Green et al. 2014, Niehrs 2012). The 54 human CTNNB1 target genes were selected based on human genes listed on the WNT home page (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/) and in the literature (Herbst et al. 2014) and analyzed using bioinformatics. We used Canberra 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 dysmenorrhea, the polyserial 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 I. Ribosomal protein L19 (RPL19) and HPRT were used as reference genes. The expression of selected WNT signaling pathway genes was analyzed by RT-qPCR from ovarian, deep and peritoneal lesions and endometrium tissue (n = 4-12).

Western blotting

Total protein lysates were analyzed 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 amount of total lysate was 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 (1.49v) 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 7 proliferative and 9 secretory phase endometrium samples and in 5 proliferative and 5 secretory phase extraovarian endometriosis lesions. For CTNNB1 staining, endometrial and extraovarian endometriosis samples from 3 study subjects in the proliferative and 5 subjects in the secretory phase were analyzed. For the lesion border analysis, extraovarian endometriosis lesions from 20 patients were evaluated of which 7 were in the proliferative, 6 in the secretory and 2 were in the menstruating phase, and 5 were using hormonal medication (progestin only n = 2, combined estrogen and progestin n = 1, GnRH analog n = 1, progestin only + combined estrogen and progestin 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 3 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 3 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 1 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 3 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 2 deep lesions and 1 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 the there was still fluorescent siRNA present in each patient sample, despite that 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 analyzed 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 ≥ 1.4) is shown in Supplemental Table II. 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). The cluster 2 further divided into two subclusters (2a, 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 CTNNB1 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 analyzed for the genes shown in Fig. 1B. Eight genes out of 30 (26.7 %) showed differential expression in proliferative vs. secretory endometrium,

as shown in Supplemental Figure 1, while 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 II) 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-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, 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-H).

We then correlated the *SFRP2* microarray gene expression data with our recently published hormone data from the same samples (Huhtinen et al. 2012, Huhtinen et al. 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 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-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 analyzed 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 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

393 extraovarian endometriosis. The staining intensity of the nuclei was scored, showing significantly
394 more nuclear CTNNB1 staining in endometriosis compared with control and patient endometrium
395 tissue in both the epithelium and stroma (Fig. 5A-F). Because no difference was observed between
396 control and patient endometrium samples, they were analyzed as one group. Also the membranous
397 CTNNB1 staining was increased in extraovarian endometriosis. To further confirm the increased
398 canonical WNT signaling pathway activation in endometriosis, we analyzed the expression of
399 known human CTNNB1 target genes. Of the 54 genes known to be upregulated upon CTNNB1
400 activation, 27 (50 %) were upregulated in extraovarian endometriosis compared with control and
401 patient endometrium samples (analyzed as one group). The microarray expression patterns for the
402 selected CTNNB1 target genes are shown in Fig. 5G. The full list of upregulated targets with their
403 microarray expression differences is shown in Supplemental Table III. We then analyzed the
404 hierarchical clustering pattern of the human CTNNB1 target genes, and as expected, these genes
405 formed similar clusters as those observed by analyzing the WNT pathway genes (Supplemental Fig.
406 2). Three out of 8 (37.5 %) CTNNB1 target genes (*CLDN1*, *JUN* and *VEGFB*) shown in Fig. 5G
407 were differentially expressed in proliferative vs. secretory endometrium tissues, while none of them
408 showed menstrual cycle-dependent regulation in endometriosis tissue and none showed differences
409 between control and patient endometrium samples (Supplemental Fig. 3).
410
411 **SFRP2 is a canonical WNT signaling agonist in endometriosis**
412 Primary cultured extraovarian endometriotic cells from 4 human lesions from 3 patients were
413 exposed to *SFRP2* siRNA-mediated knockdown, reducing *SFRP2* mRNA expression by 72 % ($p <$
414 0.05) on average and protein expression by 60 % ($p < 0.01$) compared with the control siRNA (Fig.
415 6A-C). SFRP2 knockdown significantly reduced cell proliferation (48 %, $p < 0.05$; Fig. 6D), and
416 cell proliferation and *SFRP2* mRNA expression showed a strong positive correlation in all the
417 samples in the siRNA experiment ($r = 0.732$, $p < 0.01$; Fig. 6G). Western blot analysis showed, on
418 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, F). In Supplemental Fig. 4, we summarize our findings. The data indicate suppression of SFRP2 expression by progesterone in the endometrium that is diminished in extraovarian endometriosis, 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 (Cao et al. 2015, Guo 2009, Rizk et al. 2014), identifying of lesion borders to aid endometriosis surgery has clinical value. A technique where methylene 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

445 the currently applied marker CD10 (Sumathi and McCluggage 2002), indicating that SFRP2 and
446 CTNNB1 are novel endometriosis lesion border markers.
447
448 The WNT pathway is active during uterine growth, implantation and cyclic remodeling of the
449 endometrium, as evidenced by the fact that some WNT pathway components are expressed in a
450 cycle-dependent and cell-specific manner in the human endometrium. In the proliferative phase,
451 estradiol enhances WNT signaling, whereas in the secretory phase, progesterone has an inhibitory
452 effect (Wang et al. 2010). For a few WNT pathway genes, cycle-dependent variations have been
453 reported (Talbi et al. 2006, Tulac et al. 2003, Wang et al. 2009, Wang et al. 2010), and direct
454 regulation by sex steroids has been shown for some of these genes (Banerjee et al. 2003, Wang et
455 al. 2009, Zhang et al. 2016a, Zhang et al. 2016b). Our data showed that endometrial epithelial
456 SFRP2 expression was cycle-dependently regulated, whereas the cycle-dependent regulation was
457 lost in extraovarian endometriosis. Our expression analysis of WNT pathway components and
458 CTNNB1 target genes showed that endometrial samples clustered separately from endometriosis
459 and further subclustered into proliferative and secretory phase endometrium samples. No clustering
460 according to the menstrual cycle phase was observed in endometriosis samples, which further
461 clustered into subclusters separating ovarian and extraovarian endometriosis. In line with these data,
462 we have shown that cycle-dependent changes in estradiol and progesterone concentrations observed
463 in the endometrium are lost in endometriosis tissue (Huhtinen et al. 2012, Huhtinen et al. 2014), and
464 the loss of cycle phase-specific expression patterns of WNT genes is likely to reflect the disturbed
465 hormonal environment of endometriosis. Furthermore, a negative correlation between *SFRP2*
466 mRNA expression and the intratissue progesterone concentration in extraovarian endometriosis
467 suggests that *SFRP2* expression is negatively regulated by progesterone in endometriosis, supported
468 by the presence of two strong PREs in the *SFRP2* promoter. Recent studies have shown that
469 estradiol directly upregulates *CTNNB1* expression by binding to the estrogen-response element in
470 the *CTNNB1* promoter in ovarian endometriosis (Zhang et al. 2016a, Zhang et al. 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 (Bovolenta et al. 2008, Esteve et al. 2011, Fontenot et al. 2013, Roth et al. 2000). 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). Tens of therapeutic approaches are currently being developed for diseases associated with abnormal WNT signaling (Lu et al. 2016, Rey and Ellies 2010), including approaches targeting SFRP2 (Fontenot et al. 2013), and the treatments under development could provide novel and effective opportunities to treat endometriosis.

AUTHOR'S ROLES

TH, MG, LK, KH and EK carried out experiments, TH, MG, LK, PS, TA, AP and MP conceived experiments, TH, MG, LK, PA, KH, TDL, EK, AM, PS, HK, TA, AP and MP 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.

ACKNOWLEDGEMENTS

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

FUNDING

This study was funded by the Academy of Finland (grant number 277624) and Tekes: Finnish Funding Agency for Innovation (grant numbers 40250/12 and 40279/14).

CONFLICT OF INTEREST

The authors have nothing to disclose.

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FIGURE LEGENDS

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 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.

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

635 analyzed by RT-qPCR (n = 4-12) to validate the microarray data. Statistically significant expression
636 changes were seen for *SFRP2*, *FRZB*, *DKK3*, *FZD7* and *WISP2*. *SFRP2* showed the highest
637 expression increase between endometrium and extraovarian endometriosis (183.3-fold increase in
638 extraovarian endometriosis), while the increase was much less pronounced (5.4-fold) for ovarian
639 endometriosis. *E = endometrium, OV Endo = ovarian endometriosis, EO Endo = extraovarian*
640 *endometriosis. Statistical analyses used: one-way ANOVA or Kruskal-Wallis multiple comparison*
641 *tests.*
642
643 **Figure 3. SFRP2 protein expression in endometrium and endometriosis tissue.** Western
644 blotting analysis showed that SFRP2 protein expression was substantially increased in extraovarian
645 endometriosis, while the increase was much less pronounced in ovarian endometriosis compared
646 with the endometrium. (A) A representative Western blot. (B) Protein intensities measured in the
647 endometrium, ovarian endometriosis and extraovarian endometriosis, each group containing 3
648 proliferative and 3 secretory phase samples. SFRP2 expression was normalized to TUBA1A
649 expression. No cycle-dependent effect was observed in the Western blot analysis (data not shown).
650 (C) A more detailed immunohistochemical scoring analysis showed that in the endometrium (n =16,
651 of which 7 were proliferative and 9 were secretory), SFRP2 was downregulated during the secretory
652 phase in the epithelium compared with stroma, whereas downregulation was not observed in
653 extraovarian endometriosis (n = 10, of which 5 were proliferative and 5 were secretory) (D). (E)-(F)
654 Representative pictures at two magnifications (34x and 100x, respectively) from the secretory phase
655 of the menstrual cycle are shown for endometrium tissue with apparent epithelial SFRP2
656 downregulation. (G)-(H) In extraovarian endometriosis, equal staining intensity in both the
657 epithelium and stroma was observed in the secretory phase (34x and 100x magnifications,
658 respectively). (I) A negative control for SFRP2 staining. (J) A correlation plot showing a negative
659 correlation between SFRP2 mRNA and the intratissue progesterone concentration in extraovarian
660 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 6.2x magnification. (B) Higher magnification (26.8x) of the staining observed in the regions outlined by black boxes in the adjacent pictures on the left. (C) Higher magnification (66x) 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 (100x) inserts in panel (D)], both SFRP2 and CTNNB1 staining showed a secondary lesion border with milder staining [gray lines in the full-sized 10x magnification pictures of panel (D)] that extended beyond the primary lesion border. (E)-(F) Negative controls for CTNNB1 and CD10 IHC staining, respectively, (negative control for SFRP2 shown in Fig. 3). (G)-(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 color comes from DAPI staining. All images were

686 taken in the secretory phase of the menstrual cycle, and the immunofluorescent pictures were taken
687 at 20x magnification. *EP = epithelium, S = stroma.*
688
689 **Figure 5. Increased nuclear CTNNB1 and target gene expression.** The intensity of nuclear
690 CTNNB1 immunohistochemical staining was scored in endometrium and extraovarian
691 endometriosis samples (n = 8 per group, of which 3 were in proliferative and 5 were in secretory
692 phase). There was significantly more nuclear CTNNB1 in extraovarian endometriosis than in the
693 endometrium both in the epithelial (A) and stromal (B) compartments. (C)-(D) Representative
694 images taken in the secretory phase of the menstrual cycle for CTNNB1 staining in the
695 endometrium show mainly cytoplasmic and membranous staining (magnification 34x and 100x,
696 respectively), while staining of the extraovarian endometriosis tissue (E)-(F) shows a high nuclear
697 CTNNB1 level (magnification 34x and 100x, respectively). (G) CTNNB1 target gene expression
698 analysis of the microarray data showed altered expression for numerous CTNNB1 target genes in
699 endometriosis compared with the endometrium (n = 66 for endometrium and 55 for extraovarian
700 endometriosis tissues). The expression patterns of *GREM1*, *CYR61*, *CLDN1*, *JUN*, *VEGFB*, *FST*,
701 *CTLA4* and *BMP4* are shown as examples. *E = endometrium, OV Endo = ovarian endometriosis,*
702 *EO Endo = extraovarian endometriosis, EP = epithelium, S = stroma. Statistical analyses used:*
703 *Student's t-test for (A) and (B), unpaired nonparametric Mann-Whitney test for (G).*
704
705 **Figure 6. SFRP2 siRNA knockdown.** SFRP2 expression was knocked down in primary cultured
706 extraovarian endometriotic cells (n = 3-4). (A) The knockdown resulted in a 72 % reduction in the
707 *SFRP2* mRNA level on average and (B) in a 60 % reduction in the SFRP2 protein level, as
708 indicated by intensity measurements of the Western blot signals. (C) A representative Western blot
709 shown. (D) The knockdown resulted in severely reduced cell proliferation and (E) to decreased
710 CTNNB1 protein expression as indicated by intensity measurements of the Western blot. (F) A
711 representative Western blot is shown. (G) Cell proliferation and *SFRP2* mRNA expression were

strongly and positively correlated over all experimental samples (SFRP2 siRNA A, SFRP2 siRNA B, non-targeting siRNA and untreated cells from all 4 lesions). *Statistical analyses used: RM-one way ANOVA or Friedman's test for (A) and (C), paired t-test for (B), Student's t-test for E and Spearman's correlation analysis for (G).*

SUPPLEMENTARY FIGURE LEGENDS

Supplemental Figure 1. WNT gene expression in the endometrium of controls and patients. Of the 30 WNT pathway genes driving the microarray data clustering (shown in main Fig. 1B), 8 (26.7 %) genes (*DKK3*, *SDC1*, *PPP3CB*, *SERPINF1*, *RUVBL1*, *FZD3*, *LGR4* and *SOX17*) were differentially expressed in the endometrium during the menstrual cycle. *2-way ANOVA.*

Supplemental Figure 2. Hierarchical clustering of CTNNB1 target gene expression. Gene expression profiling from control and patient endometrium samples (n = 66) and ovarian (n = 19) and extraovarian endometriosis (n = 55) lesions showed that CTNNB1 target genes clustered similarly to the WNT pathway genes. Two major clusters, endometrium (Cluster 2) and endometriosis (Cluster 1), were formed. The endometrium cluster further formed two subclusters, the proliferative phase samples (Cluster 2a) and the secretory phase samples (Cluster 2b). The endometriosis cluster subdivided into ovarian (Cluster 1a) and extraovarian (Cluster 1b) endometriosis, but no clustering according to the menstrual cycle phase was detected. B) A heatmap showing the expression of the human CTNNB1 target genes is shown.

Supplemental Figure 3. CTNNB1 target gene expression in the endometrium of controls and patients. Of the 8 CTNNB1 target genes with highest expression change between the endometrium and extraovarian endometriosis samples (shown in main Fig. 5B), 3 (37.5%) genes (*CLDN1*, *JUN*

736 and *VEGFB*) were differentially expressed in the endometrium during the menstrual cycle. *2-way*
737 *ANOVA*.

738 **Supplemental Figure 4. Role of SFRP2 in endometriosis as identified in the present study.**
739 Progesterone downregulates SFRP2 expression, while the reduced amount of the intratissue
740 progesterone and the progestin resistance in endometriosis strongly upregulate SFRP2 expression in
741 the extraovarian lesions. SFRP2 activates the canonical WNT signaling pathway, and as a
742 consequence, cell proliferation is increased, resulting in lesion growth and dysmenorrhea symptoms
743 in the patients.
744

Table I. Clinical characteristics of study subjects

STUDY SUBJECTS			
CONTROLS	**n**	**PATIENTS**	**n**
Healthy controls	47	Endometriosis patients	103
Control age ± SD	39 ± 4	Patient age ±SD	32 ± 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

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

Fig 1.

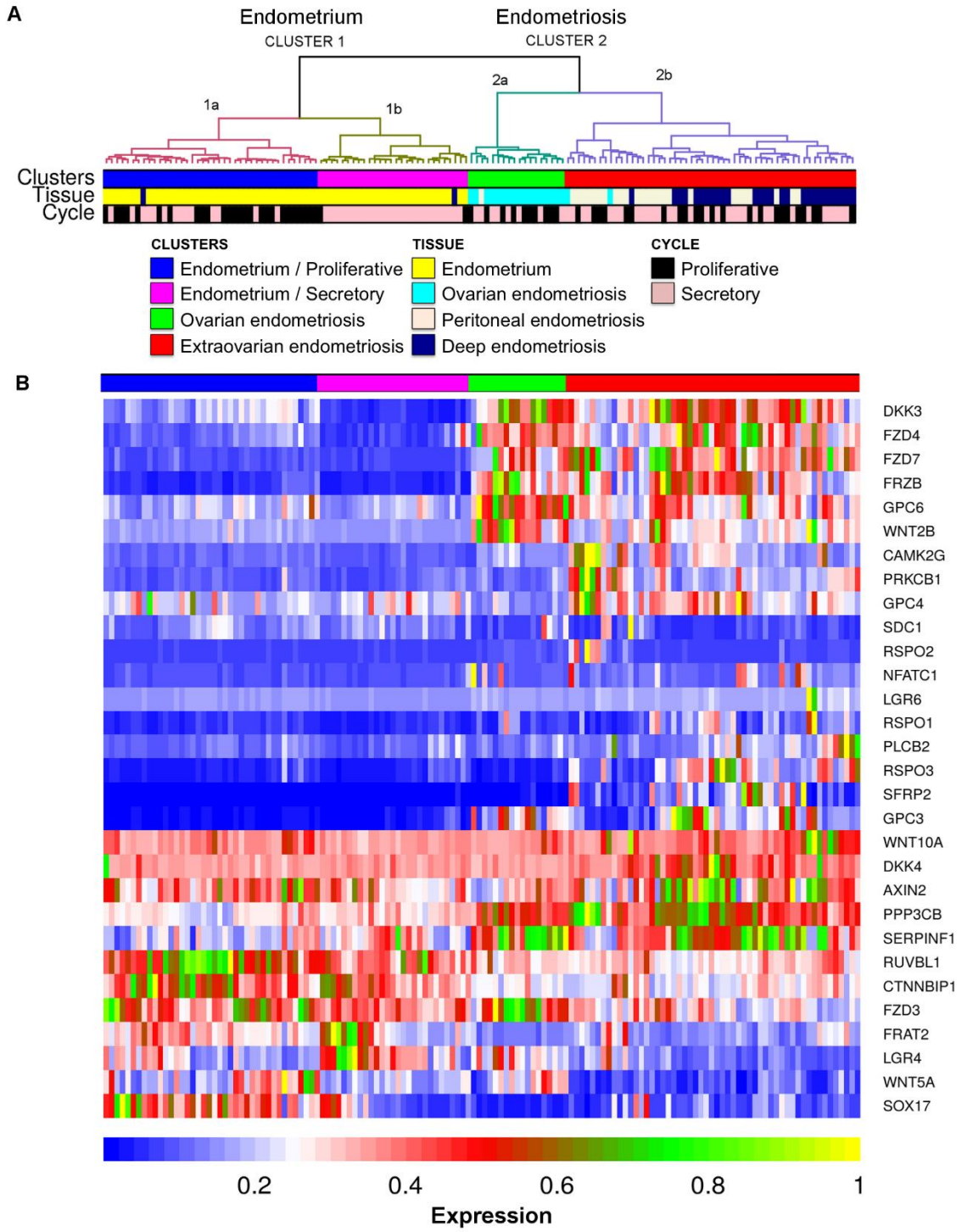

A
Endometrium
CLUSTER 1
Endometriosis
CLUSTER 2
1a
1b
2a
2b
Clusters
Tissue
Cycle
CLUSTERS
Endometrium / Proliferative
Endometrium / Secretory
Ovarian endometriosis
Extraovarian endometriosis
TISSUE
Endometrium
Ovarian endometriosis
Peritoneal endometriosis
Deep endometriosis
CYCLE
Proliferative
Secretory
B
DKK3
FZD4
FZD7
FRZB
GPC6
WNT2B
CAMK2G
PRKCB1
GPC4
SDC1
RSPO2
NFATC1
LGR6
RSPO1
PLCB2
RSPO3
SFRP2
GPC3
WNT10A
DKK4
AXIN2
PPP3CB
SERPINF1
RUVBL1
CTNNBIP1
FZD3
FRAT2
LGR4
WNT5A
SOX17
0.2
0.4
0.6
0.8
1
Expression

Fig 2.

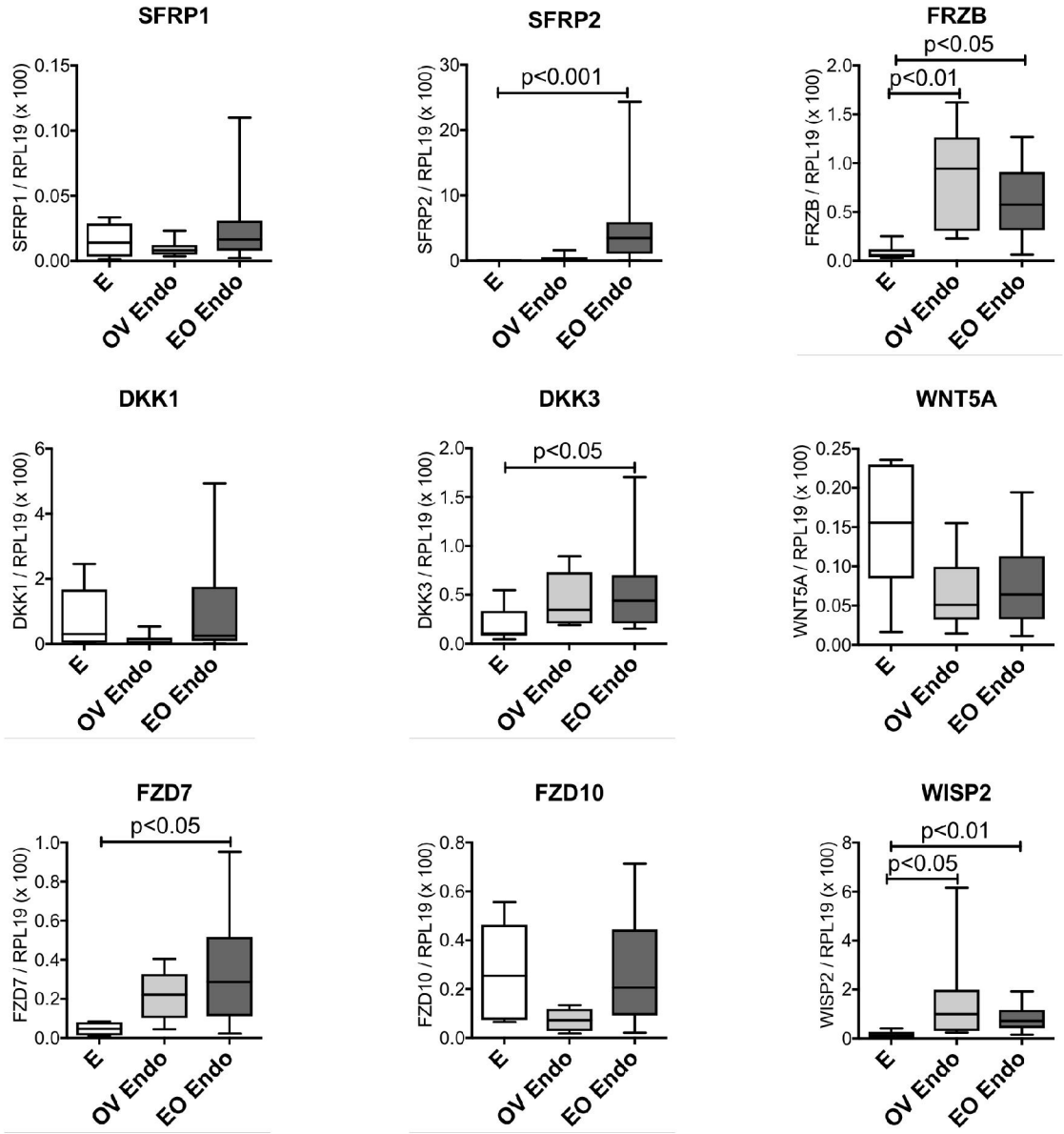

SFRP1
SFRP1 / RPL19 (x 100)
0.15
0.10
0.05
0.00
E
OV Endo
EO Endo
SFRP2
SFRP2 / RPL19 (x 100)
p<0.001
30
20
10
0
FRZB
FRZB / RPL19 (x 100)
p<0.05
p<0.01
2.0
1.5
1.0
0.5
0.0
DKK1
DKK1 / RPL19 (x 100)
6
4
2
0
DKK3
DKK3 / RPL19 (x 100)
p<0.05
2.0
1.5
1.0
0.5
0.0
WNT5A
WNT5A / RPL19 (x 100)
0.25
0.20
0.15
0.10
0.05
0.00
FZD7
FZD7 / RPL19 (x 100)
p<0.05
1.0
0.8
0.6
0.4
0.2
0.0
FZD10
FZD10 / RPL19 (x 100)
0.8
0.6
0.4
0.2
0.0
WISP2
WISP2 / RPL19 (x 100)
p<0.01
p<0.05
8
6
4
2
0

Fig 3.

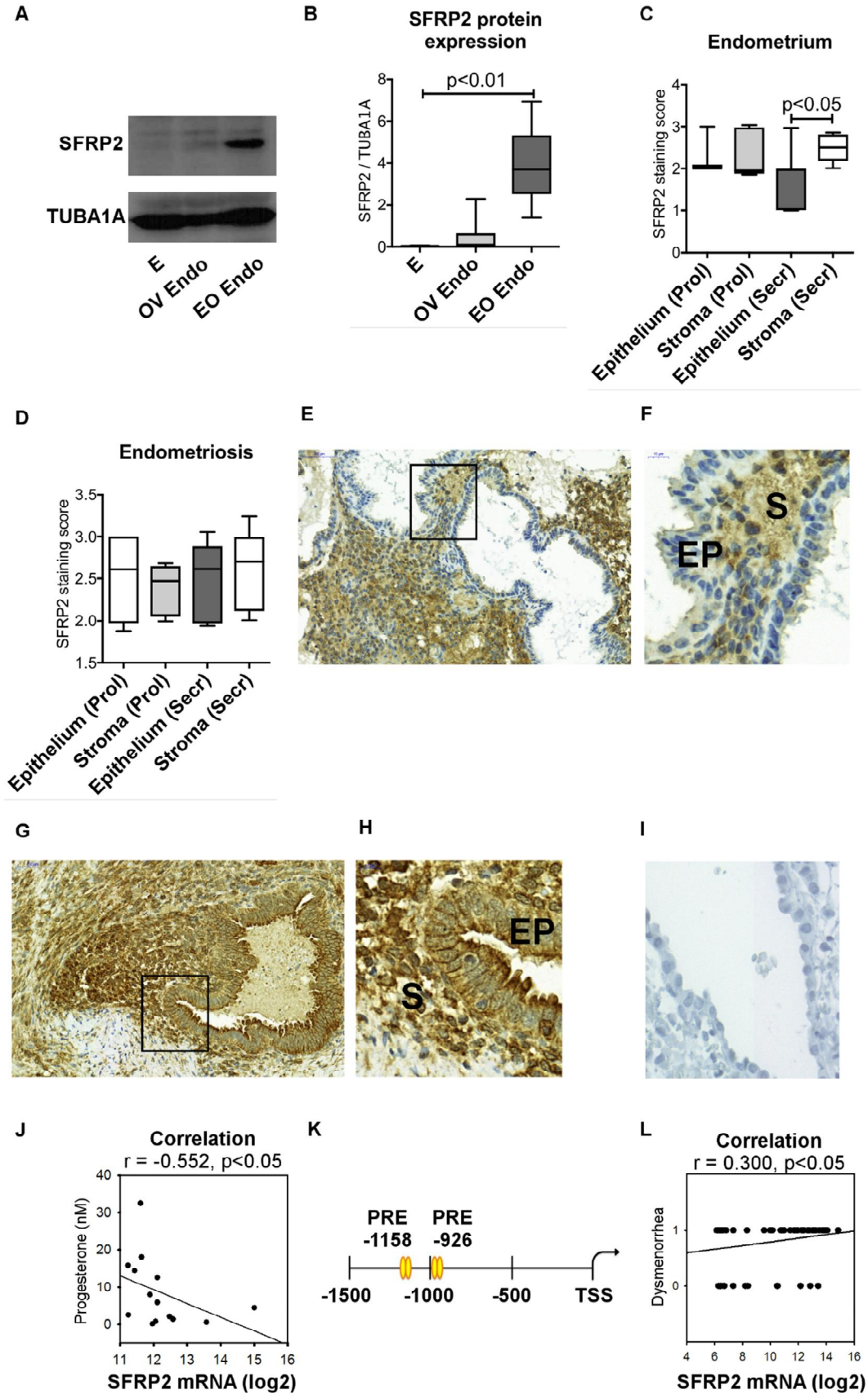

A
SFRP2
TUBA1A
E
OV Endo
EO Endo
B
SFRP2 protein expression
p<0.01
SFRP2 / TUBA1A
E
OV Endo
EO Endo
C
Endometrium
p<0.05
SFRP2 staining score
Epithelium (Prol)
Stroma (Prol)
Epithelium (Secr)
Stroma (Secr)
D
Endometriosis
SFRP2 staining score
Epithelium (Prol)
Stroma (Prol)
Epithelium (Secr)
Stroma (Secr)
E
F
S
EP
G
H
EP
S
I
J
Correlation
r = -0.552, p<0.05
Progesterone (nM)
SFRP2 mRNA (log2)
K
PRE -1158
PRE -926
-1500
-1000
-500
TSS
L
Correlation
r = 0.300, p<0.05
Dysmenorrhea
SFRP2 mRNA (log2)

Fig 4.

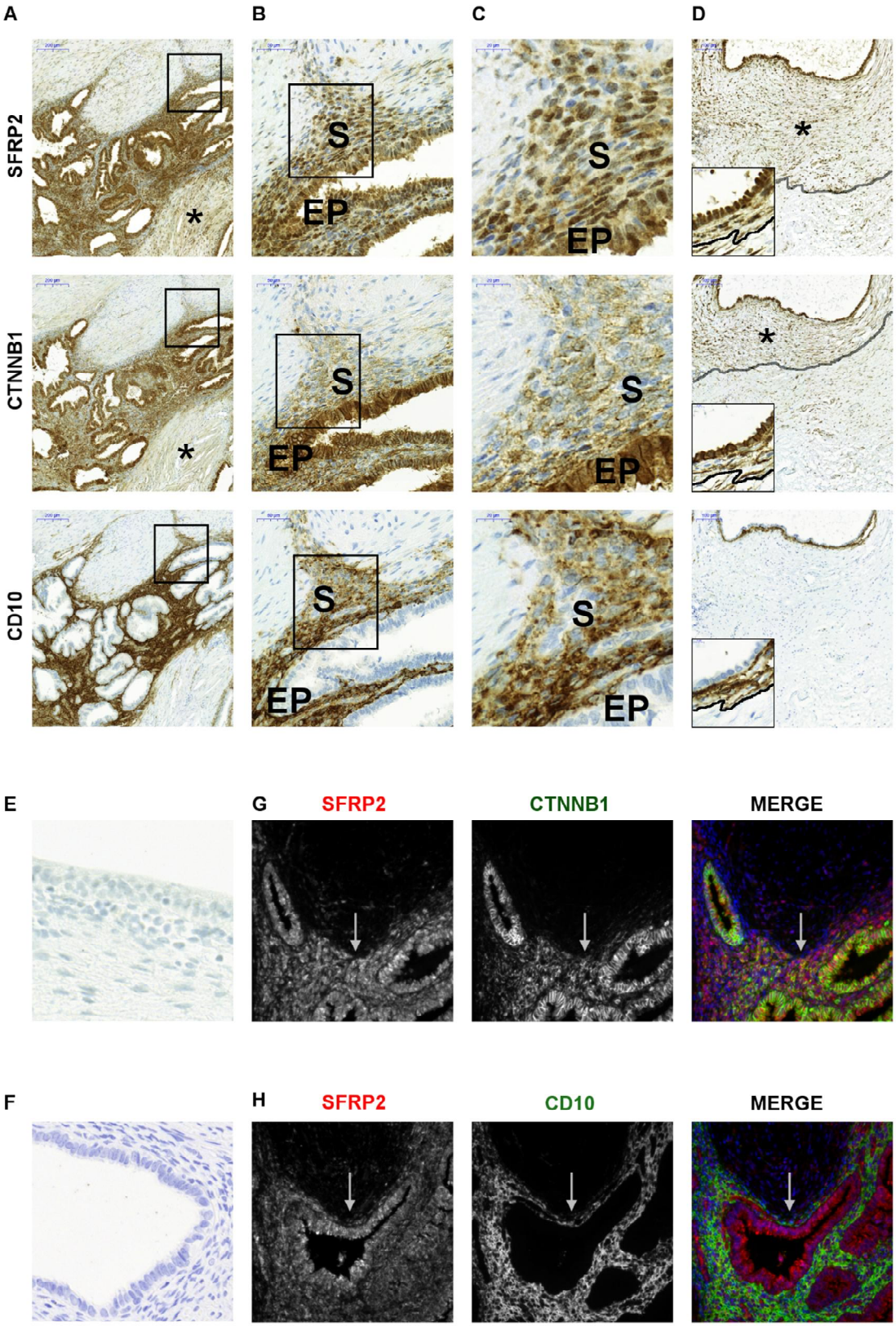

A
B
C
D
SFRP2
CTNNB1
CD10
S
EP
*
E
F
G
H
SFRP2
CTNNB1
MERGE
SFRP2
CD10
MERGE

Fig 5.

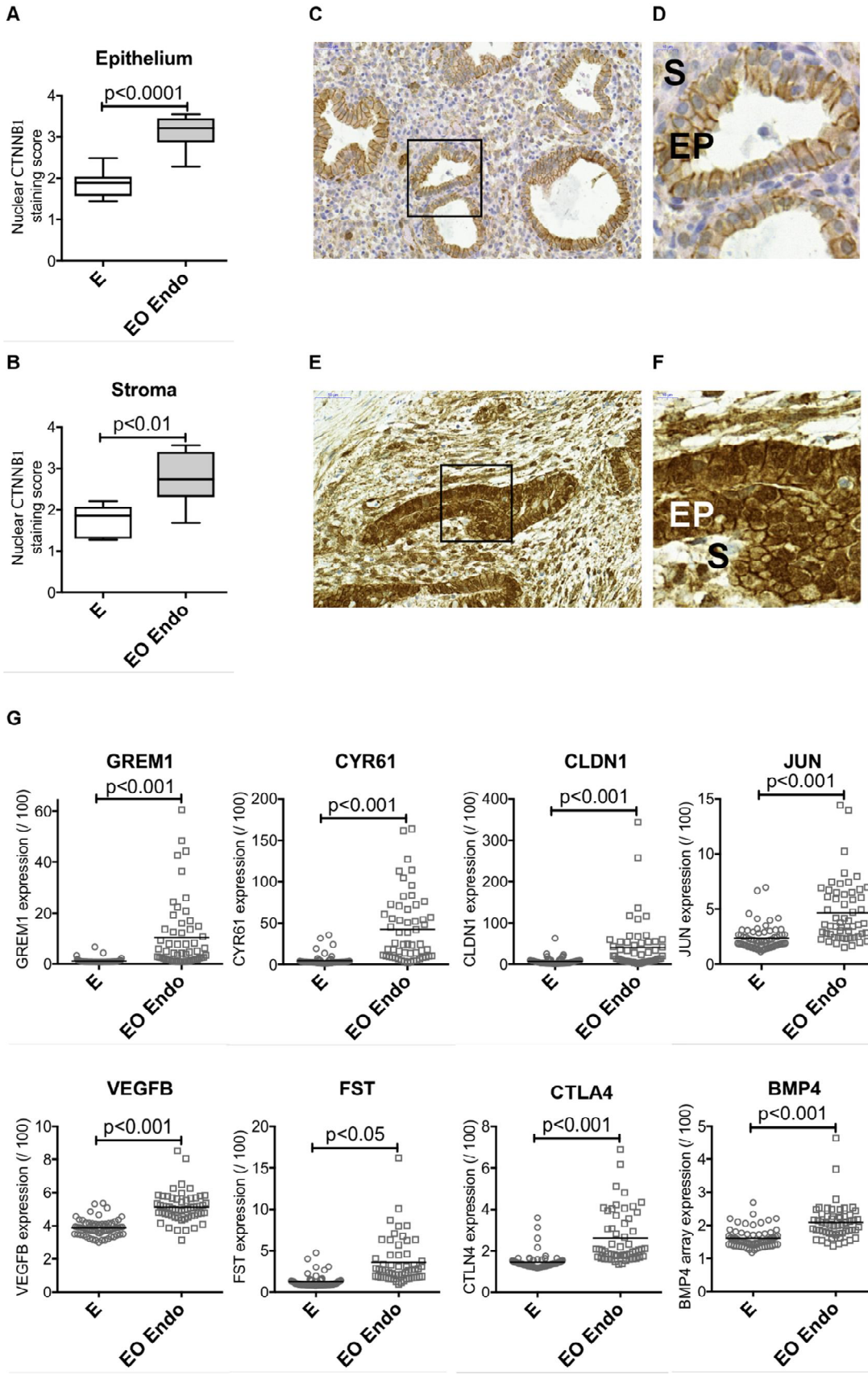
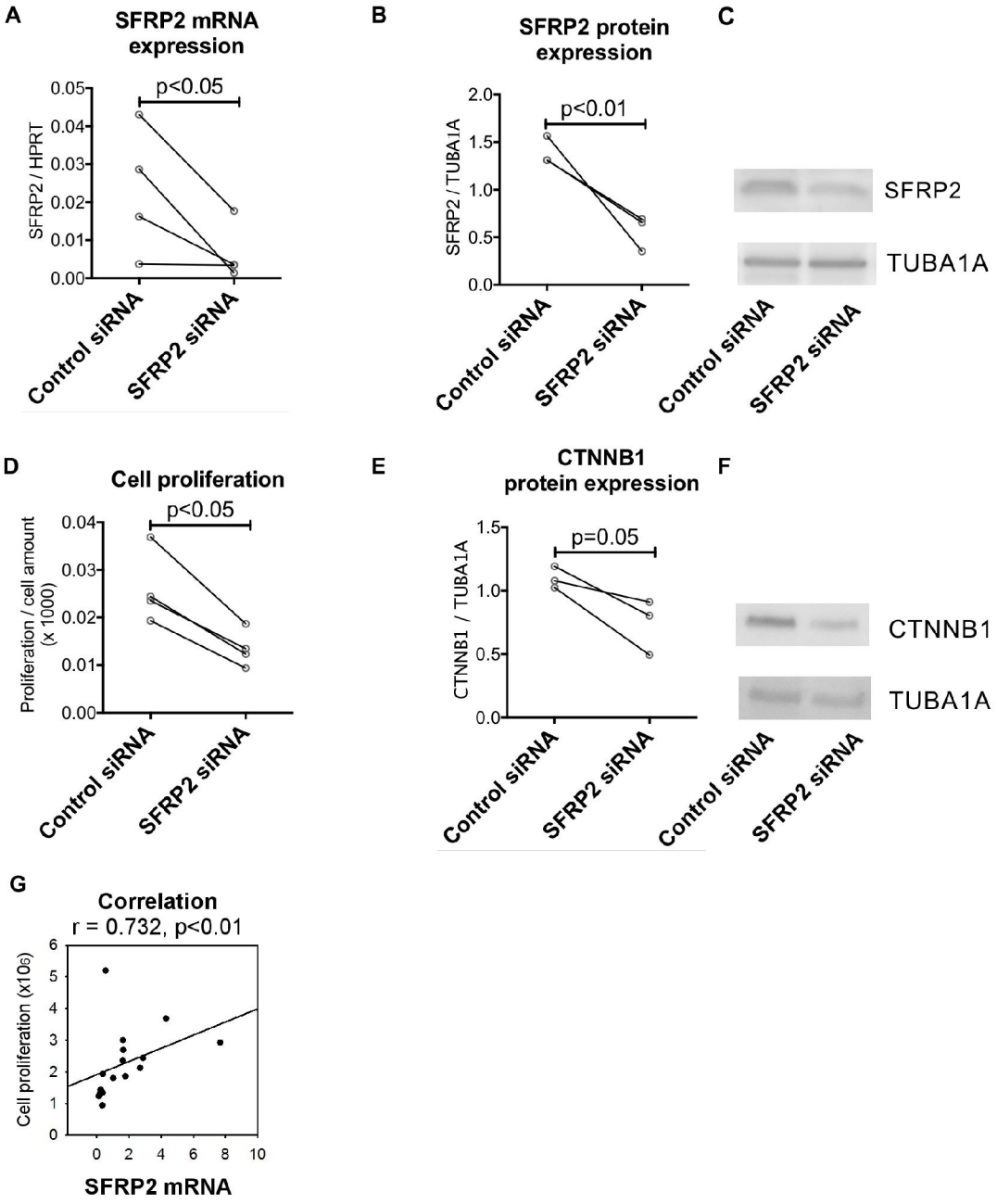
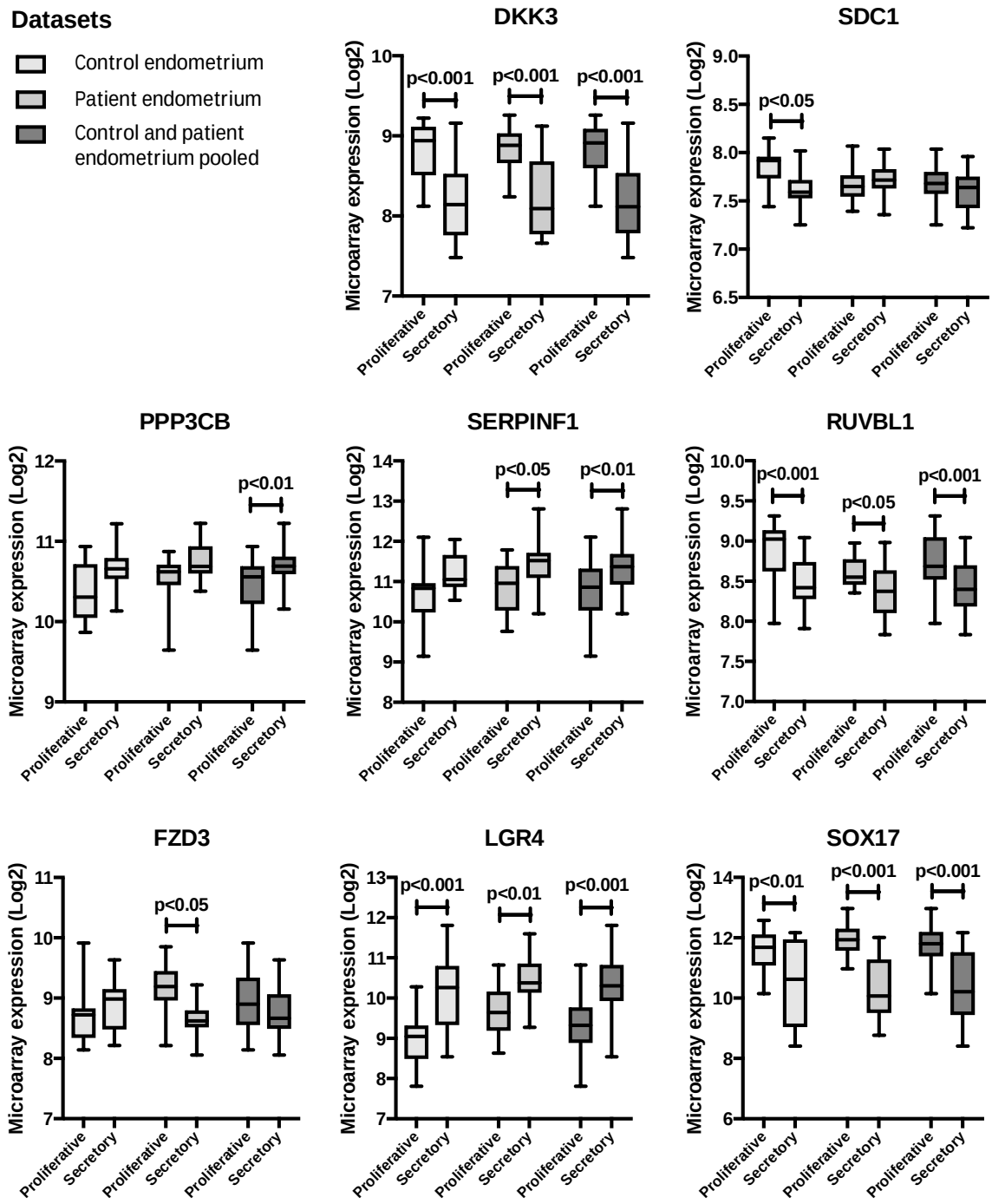

A
Epithelium
p<0.0001
Nuclear CTNNB1 staining score
E
EO Endo
B
Stroma
p<0.01
Nuclear CTNNB1 staining score
E
EO Endo
C
D
S
EP
E
F
EP
S
G
GREM1
p<0.001
GREM1 expression (/ 100)
CYR61
p<0.001
CYR61 expression (/ 100)
CLDN1
p<0.001
CLDN1 expression (/ 100)
JUN
p<0.001
JUN expression (/ 100)
VEGFB
p<0.001
VEGFB expression (/ 100)
FST
p<0.05
FST expression (/ 100)
CTLA4
p<0.001
CTLN4 expression (/ 100)
BMP4
p<0.001
BMP4 array expression (/ 100)
E
EO Endo

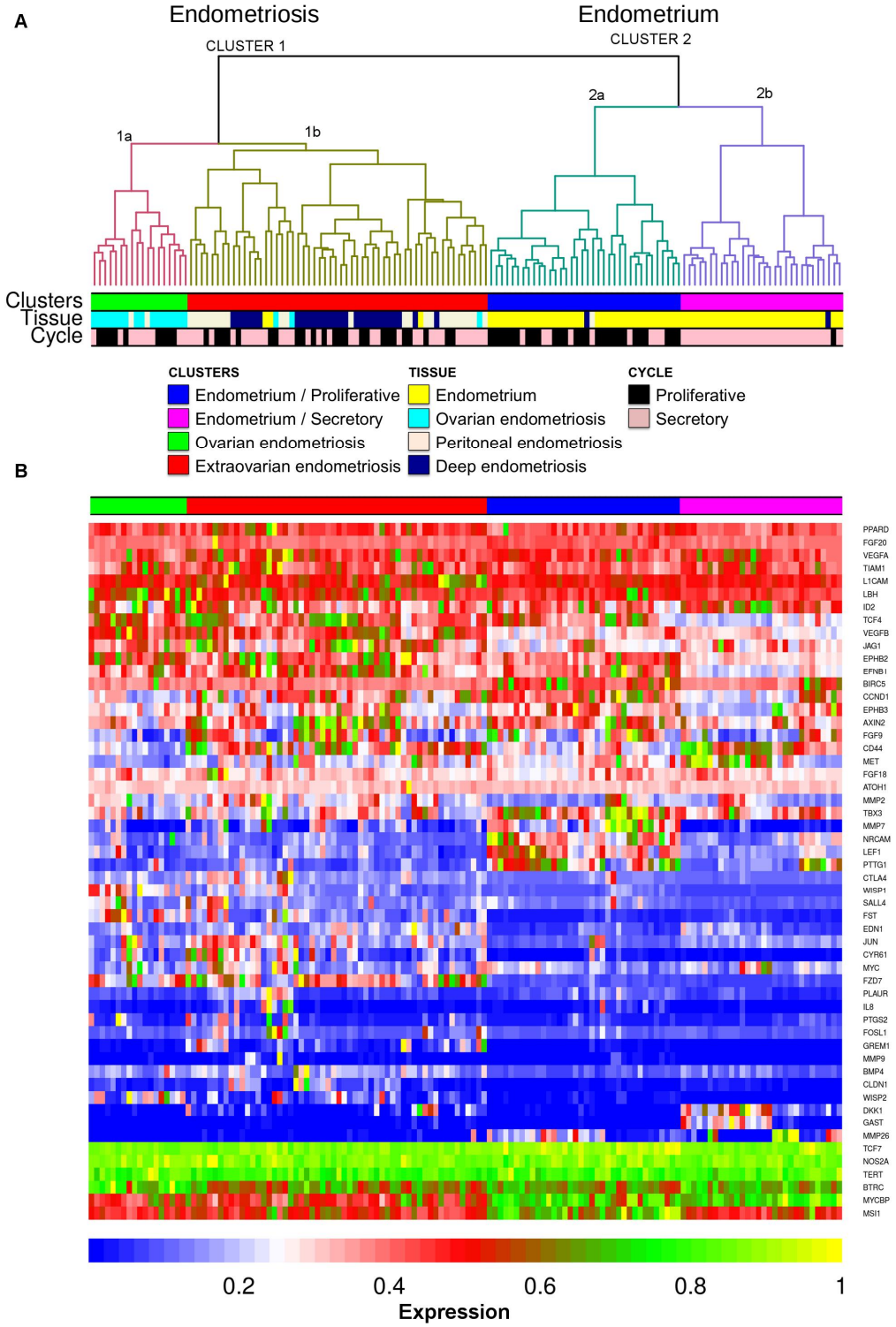
Fig 6.


A
SFRP2 mRNA expression
p<0.05
SFRP2 / HPRT
0.05
0.04
0.03
0.02
0.01
0.00
Control siRNA
SFRP2 siRNA
B
SFRP2 protein expression
p<0.01
SFRP2 / TUBA1A
2.0
1.5
1.0
0.5
0.0
Control siRNA
SFRP2 siRNA
C
SFRP2
TUBA1A
Control siRNA
SFRP2 siRNA
D
Cell proliferation
p<0.05
Proliferation / cell amount (x 1000)
0.04
0.03
0.02
0.01
0.00
Control siRNA
SFRP2 siRNA
E
CTNNB1 protein expression
p=0.05
CTNNB1 / TUBA1A
1.5
1.0
0.5
0.0
Control siRNA
SFRP2 siRNA
F
CTNNB1
TUBA1A
Control siRNA
SFRP2 siRNA
G
Correlation
r = 0.732, p<0.01
Cell proliferation (x10⁶)
6
5
4
3
2
1
0
0
2
4
6
8
10
SFRP2 mRNA

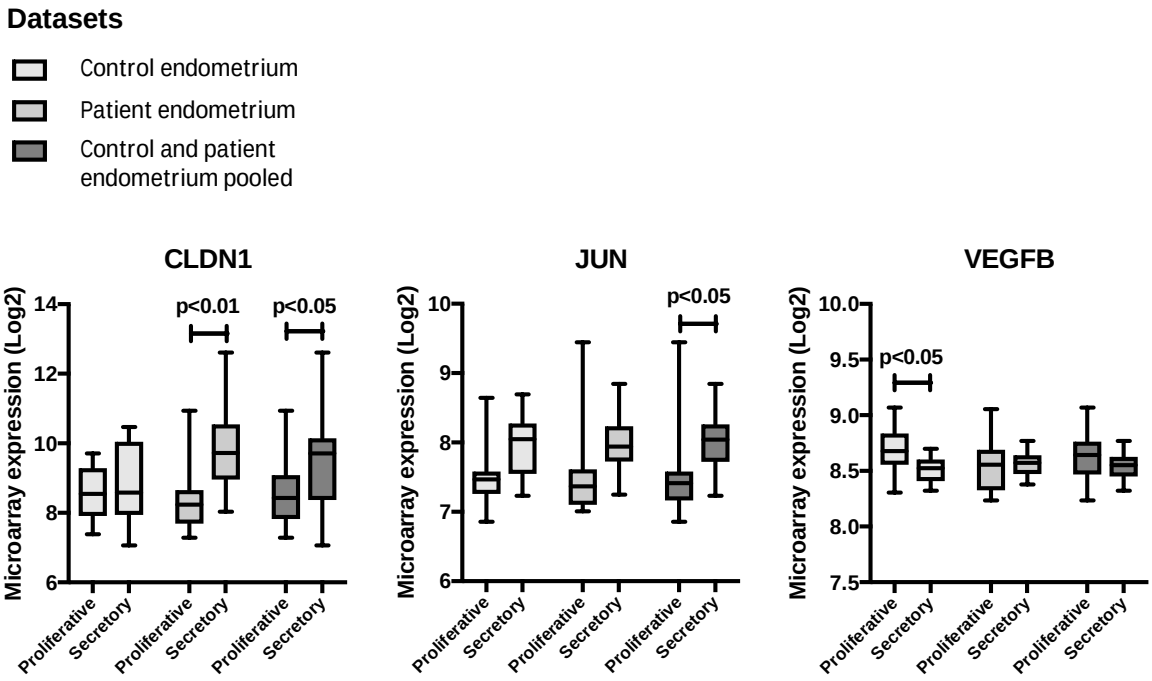
Supplemental figure 1.



Supplemental figure 2.



Supplemental figure 3.



Supplemental figure 4.