Progestrone Inhibition of Wnt/β-Catenin Signaling in Normal Endometrium and Endometrial Cancer

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Abstract

Purpose. Wnt signaling regulates the fine balance between stemness and differentiation. Here, the role of Wnt signaling to maintain the balance between estrogen-induced proliferation and progesterone-induced differentiation during the menstrual cycle, as well as during the induction of hyperplasia and carcinogenesis of the endometrium, was investigated.

Experimental Design: Endometrial gene expression profiles from estradiol (E2) and E2 + medroxyprogesterone acetate–treated postmenopausal patients were combined with profiles obtained during the menstrual cycle (PubMed; GEO DataSets). Ishikawa cells were transfected with progesterone receptors and Wnt inhibitors dickkopf homologue 1 (DKK1) and forkhead box O1 (FOXO1), measuring Wnt activation. Expression of DKK1 and FOXO1 was inhibited by use of sequence-specific short hairpins. Furthermore, patient samples (hormone-treated endometria, hyperplasia, and endometrial cancer) were stained for Wnt activation using nuclear β-catenin and CD44.

Results: In vivo, targets and components of the Wnt signaling pathway (among them DKK1 and FOXO1) are regulated by E2 and progesterone. In Wnt-activated Ishikawa cells, progesterone inhibits Wnt signaling by induction of DKK1 and FOXO1. Furthermore, using siRNA-mediated knockdown of both DKK1 and FOXO1, progesterone inhibition of Wnt signaling was partly circumvented. Subsequently, immunohistochemical analysis of the Wnt target gene CD44 showed that progesterone acted as an inhibitor of Wnt signaling in hyperplasia and in well-differentiated endometrial cancer.

Conclusion: Progesterone induction of DKK1 and FOXO1 results in inhibition of Wnt signaling in the human endometrium. This Wnt inhibitory effect of progesterone is likely to play a rate-limiting role in the maintenance of endometrial homeostasis and, on its loss, in tumor onset and progression toward malignancy. (Clin Cancer Res 2009;15(18):5784-93)

The female sex hormones estradiol (E2) and progesterone play rate-limiting roles in the cyclical renewal of the inner layer of the uterus (endometrium). In the first half of the regular menstrual cycle, the proliferation phase, E2 is required to expand the endometrial layer by inducing cell proliferation. In the second half of the menstrual cycle, the secretory phase, progesterone levels increase, which antagonizes the proliferative activity of E2 by inducing differentiation of epithelial and stromal cells of the endometrium (1). Thus, inhibition of E2-induced proliferation by progesterone is crucial for the maintenance of homeostasis in the endometrium.

Increased estrogen signaling often underlies endometrial hyperplasia and is a well-established risk factor for endometrial cancer (2). Because progesterone inhibits estrogen-induced endometrial proliferation, progesterone has been used in its synthetic form [i.e., medroxyprogesterone acetate (MPA)] in palliative treatment of advanced and recurrent endometrial cancer with modest though significant response rates (15-25%; ref. 3). Progesterone has also been used as a primary treatment for endometrial carcinoma confined to the endometrial layer of the uterus, for example, in premenopausal women determined to preserve fertility. Response rates in these women can be up to 60% (4, 5), indicating that progesterone signaling in well-differentiated endometrial cancer is a potent inhibitor of endometrial carcinogenesis.

Wnt/β-catenin signaling has a central function in the maintenance and control of stem cell compartments where it regulates...
Translational Relevance
Enhanced or unopposed estrogen signaling results in endometrial hyperplasia and is one of the leading causes for development of endometrial cancer. It is known that from well-differentiated endometrial cancers at least 30% display the hallmark of activated Wnt/β-catenin signaling. Here we describe that inhibition of early endometrial carcinogenesis can be accomplished by progesterone-induced inhibition of Wnt/β-catenin signaling. We also describe that failure of progesterone to inhibit Wnt/β-catenin signaling and to inhibit endometrial cancer growth is observed because of loss of progesterone receptors. Based on the findings of the present study, it is warranted to consider treatment of endometrial cancer patients whose disease has become progesterone resistant with inhibitors of the Wnt/β-catenin signaling pathway that are acting downstream of progesterone signaling.

the fine balance between stemness (Wnt-On) and differentiation (Wnt-Off). This central role in homeostasis of adult stem cell niches is reflected by the frequent association of Wnt/β-catenin signaling defects with different cancer types (e.g., breast, colon, stomach, liver, ovary, uterine, skin, etc.). Central in canonical Wnt signaling is the so-called “destruction complex.” This complex consists of the scaffold proteins adenomatosis polyposis coli (APC), AXIN1 or AXIN2 (conductor), β-catenin (CTNNB1), and the two kinases, casein kinase I and glycogen synthase kinase 3β. In the absence of Wnt, ligand formation of the destruction complex induces phosphorylation of β-catenin, which results in its degradation. On Wnt signaling, the destruction complex is not formed and, consequently, intracellular β-catenin accumulates and translocates to the nucleus. In the nucleus, β-catenin interacts with members of the T-cell factor/lymphoid enhancer binding factor (TCF/LEF) family of transcription factors, thus modulating the expression of a broad spectrum of Wnt downstream target genes (6). The latter include genes encoding for proteins with central roles in proliferation and survival, such as cyclin D1 (CCND1) and IGF-I (insulin-like growth factor 1), as well as others relevant for migration and invasion (e.g., CD44). Constitutive activation of the Wnt pathway was first observed in familial adenomatous polyposis where individuals carrying a germ-line APC mutation developed intestinal polyps on somatic inactivation of the wild-type APC allele. Notably, in the vast majority of sporadic colorectal cancers, Wnt signaling is constitutively activated as the result of somatic APC mutations. In the remaining cases, either oncogenic β-catenin mutations or alterations in genes encoding secreted proteins that are able to inhibit Wnt signaling such as dickkopf homologue 1 (DKK1; ref. 7) are found.

In endometrial cancer, gene mutations leading to constitutive activation of canonical Wnt/β-catenin signaling are an early event (8). Well-differentiated endometrioid adenocarcinomas show nuclear β-catenin accumulation in at least 30% of the cases (31%; ref. 9; 85%; ref. 10). Accordingly, oncogenic β-catenin mutations have been identified in 15% to 40% of endometrial carcinomas (11, 12), whereas loss of heterozygosity of APC was reported in 24% of cases with nuclear β-catenin staining (13). In addition, the APC A1 promoter was found to be hypermethylated in 47% of endometrial cancers with nuclear β-catenin (13) often in correlation with microsatellite instability (14). APC mutation analysis revealed truncating mutations in 10% of all endometrial cancers (15).

In the endometrium, Wnt/β-catenin signaling is involved in a number of developmental processes. For example, it has been shown that Wnt4 is required for Mullerian duct initiation (16), Wnt7A for subsequent differentiation (17), and Wnt5A for posterior outgrowth of the female reproductive tract (18). Furthermore, Wnt/β-catenin signaling has also been implicated in regulation of the normal menstrual cycle (19, 20). Nuclear β-catenin staining, for example, is observed during the proliferative phase of the menstrual cycle (21). Likewise, Nei et al. (21) showed the absence of nuclear β-catenin staining during the second half (differentiation phase) of the menstrual cycle. Hence, inhibition of Wnt/β-catenin signaling may correlate with the inhibition of E2-induced proliferation. Hou et al. (22) showed that estrogen-induced endometrial proliferation was effectively inhibited by the Wnt/β-catenin inhibitor SFRP2, and Jeong et al. (23) showed that correct β-catenin expression is vital for normal uterine function. As progesterone counteracts the proliferative effects of E2 during the menstrual cycle, it may do so by inhibiting E2-induced Wnt/β-catenin signaling.

Based on the above data, we postulate that progesterone counteracts the proliferative effects of E2 during the normal menstrual cycle, during hyperplasia, and during early endometrial carcinogenesis by inhibiting Wnt/β-catenin signaling. According to this hypothesis, sex hormones may modulate Wnt/β-catenin signaling in the normal and aberrant endometrium to maintain the balance between proliferation (Wnt-On) and differentiation (Wnt-Off).

Materials and Methods
Patients and patient samples
Postmenopausal hormone-treated patients. A description of the inclusion and exclusion criteria and of the histologic and molecular findings in the endometrium was documented earlier (1, 24). The study groups were control group (8 subjects, no hormonal treatment), E2 group (7 subjects, 2 mg of E2 administered orally everyday, starting 21 d before surgery), and E2 + MPA group (6 subjects, 2 mg E2 + 5 mg MPA administered orally everyday, starting 21 d before surgery). After treatment, endometrial tissue was dissected out, RNA was isolated and processed, and gene expression was measured using the Affymetrix U133plus2 GeneChips containing 54,614 probe sets, representing ∼47,000 transcripts (Affymetrix).

Premenopausal patients during different stages of the menstrual cycle. A description of the inclusion and exclusion criteria and of the histologic and molecular findings in the endometrium was documented earlier (25). The study groups were PE, proliferative endometrium (4 subjects); ESE, early-secretory endometrium (3 subjects); MSE, mid-secretory endometrium (8 subjects); and LSE, late-secretory endometrium (6 subjects). Endometrial tissue was dissected as described above, and isolated RNA was used on the same microarrays as described above (Affymetrix U133plus2 GeneChip).

Endometrial hyperplasia and tumor samples. In total, 10 well-differentiated tumor samples were stained for progesterone receptor and CD44. From one representative sample, hyperplasia as well as tumor was available. Paraffin-embedded samples were obtained from the Erasmus University Medical Center Department of Pathology. The histopathologic diagnoses of all samples were reviewed by our pathologist, Patricia C.Ewing, M.D., Ph.D. Descriptions of the samples are given in Results and in the legends to the figures.
Gene expression data analysis

All raw gene expression data have been posted at the GEO DataSets option in PubMed and are freely available to the scientific community. Raw data of both studies (24, 25) were normalized as a group using robust multiarray analysis normalization (26). From the Talbi data (25), we only used gene expression data from well-characterized tissue samples (Table 1 from ref. 25, with the exception of samples 455 and 562, which were not made available to the GEO DataSets option in PubMed). Statistical analysis of microarray (SAM) was done to identify significant differentially regulated genes between different groups (E2, E2 + MPA, PE, ESE, MSE, and LSE; ref. 27). The median false discovery rate was set to 1% for these comparisons. Genes with differential expression due to difference in tissue source, rather than hormonal effects, were identified and excluded from subsequent analysis. Cluster analysis was done as described by Hanifi-Moghadam et al. (24). Pathway analyses were conducted using Ingenuity Pathway software.6

Cell lines and transfections

Ishikawa cell lines were maintained as described earlier (28). During the transfaction, infection, and hormone stimulation experiments, the cells were cultured in DMEM/F12 supplemented with 5% dextran-coated charcoal-treated FCS in the presence of penicillin and streptomycin. Transfection was done 24 h after seeding of the cells (5,000 per well in a 24-well plate); 4 h after transfection, hormones were added to the cells (concentrations are indicated in the figures). At 48 h after transfection, the cells were harvested to perform reporter assays. The stable progesterone receptor–transfected cell lines used in the short hairpin RNA (shRNA) experiments were generated as described earlier (28); transient transfections were done as described earlier (29). The TOPflash and FOPflash vectors were obtained from Millipore (Upstate). DKK1 expression vector (cloned in pcDNA3.1) was obtained from Dr. X. He (Department of Neurology, Division of Gynecologic Oncology, Northwestern University, Chicago, IL; ref. 32); and dnTCF4 expression vector (cloned in pcDNA3.1) was a gift from Dr. X. He (Department of Neurology, Division of Gynecologic Oncology, Northwestern University, Chicago, IL; ref. 31); and dnTGF4 expression vector (cloned in pcDNA3.1) was a gift from Dr. H. Clevers (Hubrecht Institute, Developmental Biology and Stem Cell Research, University Medical Center, Utrecht, the Netherlands; ref. 32).

The production of lentivirus and the generation of stable cell line were done as described before (33). The shRNAs specific for hDKK1 (n = 5) and hFOXO1 (n = 6), nontarget shRNA control vector, and TurboGFP control vector were obtained from The MISSION TRC shRNA libraries from Sigma-Aldrich. The packaging plasmids were provided by the Naldini lab, Vita-Salute San Raffaele University, Milan, Italy.

Immunohistochemistry and Western blotting

Immunohistochemistry and Western blotting were done essentially as described previously (1, 29). The dilutions of antibody used in immunohistochemistry were rat monoclonal CD44 antibody, 1:1,000 (BD Pharmingen); rabbit monoclonal β-catenin antibody, 1:4,000 (Epicomics); and mouse progesterone receptor antibody, 1:50 (Ab-8 cocktail, Neomarkers). The dilutions in Western blotting were goat anti-hDKK1 antibody, 1:500 (AF1096, R&D Systems); rabbit polyclonal anti-hFOXO1 antibody, 1:5,000 (A300-297A, Bethyl Laboratories); and rabbit anti–β-tubulin, 1:1,000 (Ab6046, Abcam).

Results

Progestagens inhibit estrogen signaling during the regular menstrual cycle. SAM analysis between the endometrial gene expression profiles obtained from untreated postmenopausal women in comparison with women treated for 21 days with E2 or E2 + MPA indicated that 5,932 probe sets (representing ~4,500 genes) were significantly up-regulated or down-regulated (P < 0.00015).

Cluster analysis of these significantly up-regulated or down-regulated genes reveals three distinct dendrogram branches: one cluster encompassed all endometrial gene expression profiles from E2-treated women; the second contained profiles from untreated controls; and the third contained profiles from both E2 + MPA–treated women and untreated controls (Fig 1A). Ten genes were investigated using quantitative real-time reverse transcription-PCR in a previous study to validate differential expression (24). In Fig 1B, highly E2-regulated genes are shown to be counteracted (compensated for) by the addition of MPA. In total, 438 genes were identified as being significantly E2 regulated (i.e., >3-fold up-regulated or down-regulated; Fig 1B, filled columns). For 377 (233 + 144) of these genes, this E2 regulation was counteracted (compensated for) either in part or fully by simultaneous MPA administration (Fig 1B, open columns). These analyses show that treatment with E2 alone has a profound effect on endometrial gene expression, and that this effect is greatly diminished on addition of MPA to the E2 treatment.

We next integrated our profiling results with previously published gene expression data obtained during the main phases of the normal menstrual cycle (25). SAM analysis revealed a total of 11,866 probe sets (representing ~9,000 genes) significantly regulated between any of the six indicated groups [the E2- and E2 + MPA–treated endometria from the present study and four different phases of the menstrual cycle: proliferative endometria (PE), early-secretory endometria (ESE), mid-secretory endometria (MSE), and late-secretory endometria (LSE); ref. 25]. These differentially expressed genes were used for cluster analysis (Fig 1C). Three clusters were recognized: cluster 1, the samples from E2-treated patients and those from women in proliferative phase; cluster 2, early- and mid-secretory phase specimens; and cluster 3, the gene expression files from the E2 + MPA–treated patients and from women in late-secretory phase. These results indicate that E2 signaling is a very important factor during the proliferative phase of the menstrual cycle (Fig 1C, Cluster 1). Furthermore, the addition of MPA to the E2 treatment creates an endometrium with obvious similarities to the late-secretory phase of the menstrual cycle (Fig 1C, Cluster 3).

Progestosterone inhibition of Wnt/β-catenin signaling involves both DKK1 and FOXO1. The gene expression data from Fig 1C were subsequently used in a pathway analysis.7 It was observed that a number of pathways were significantly regulated by one of the treatments or in different phases of the menstrual cycle. For example, when we compared the proliferative endometrium to the early- or mid-secretory endometrium, “cancer,” “reproductive system disease,” and “gastrointestinal disease” were all significantly regulated (P < 0.006). It is interesting to note that gastrointestinal disease and cancer were both found to be significantly regulated, which could point to involvement of Wnt/β-catenin signaling. On assessing the involvement of “canonical pathways,” it was found that “Wnt/β-catenin signaling” was also significantly regulated (P < 0.003) in both the early-secretory and the mid-secretory endometrium compared with the proliferative endometrium.

Because of our interest in the Wnt/β-catenin signaling pathway, we decided to perform a more thorough pathway analysis for either downstream targets or integral parts of the Wnt/β-catenin signaling pathway8 (refs. 34, 35; Supplementary Table S1). On

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6 http://www.ingenuity.com
7 http://www.ingenuity.com
8 http://www.stanford.edu/~rnusse/wntwindow.html
performing this analysis, a large number of differentially expressed genes \((n = 228)\) were recognized. Because our investigations center on the mechanism through which progesterone counteracts the proliferative effects of \(E_2\) during the menstrual cycle and during early endometrial carcinogenesis, we have focused on the progesterone-induced Wnt/\(\beta\)-catenin signal inhibitor DKK1 \((36)\) and the alleged inhibitor FOXO1 \((37, 38; \text{Fig. 2})\).

To investigate the molecular mechanisms underlying progesterone-driven inhibition of Wnt/\(\beta\)-catenin signaling, we used the well-differentiated Ishikawa endometrial cancer cell line \((39)\). As shown in Fig. 3A, TOP/FOPflash reporter assay analysis indicates that Wnt/\(\beta\)-catenin signaling is constitutively active in Ishikawa cells. Furthermore, we could clearly show that Wnt/\(\beta\)-catenin signaling is inhibited on MPA treatment \((\text{Fig. 3A})\). The concentration of MPA necessary to fully inhibit Wnt/\(\beta\)-catenin signaling is \(~0.1\ \text{nmol/L},\) and this is close to the reported dissociation constant for both progesterone receptor isoforms \((40)\). Using this ligand concentration \((0.1\ \text{nmol/L})\), molecular excess of the antiprogestagen Org-31489 could fully reverse Wnt/\(\beta\)-catenin signaling inhibition. Furthermore, it was shown that both activated progesterone receptors \((\text{progesterone receptor A and progesterone receptor B})\) could inhibit Wnt/\(\beta\)-catenin signaling, although progesterone receptor B did so at lower MPA concentrations \((\text{Supplementary Fig. S1})\).

As shown in Fig. 2, the expression of the Wnt/\(\beta\)-catenin signaling inhibitors DKK1 and FOXO1 is elevated during the
differentiated endometrium carcinomas, and 3 of these showed
immunohistochemistry on histologic sections from 10 well-
named) display the hallmark of Wnt signaling activation,
at least 30% of early estrogen-associated tumors (type I endo-
It has been reported that
endometrium and endometrial cancer.

To further substantiate that induction of DKK1 and FOXO1 by
progesterone is central to inhibition of Wnt/β-catenin signaling,
expression of both DKK1 and FOXO1 was reduced by the use of
lentiviral sequence-specific shRNAs. Five DKK1 and six FOXO1
targeting lentiviruses were used to infect Ishikawa cells. As shown
in Fig. 4A, partial inhibition of progesterone induction of DKK1
and FOXO1 expression was achieved by infection with combina-
tions of all available specific DKK1 or FOXO1 shRNAs. Based on
these results, Ishikawa cells infected with either DKK1- or FOXO1-
specific shRNAs were then used to measure progesterone inhibi-
tion of Wnt signaling. MPA inhibition of TOPFlash Wnt/β-catenin
reporter system was partly circumvented by the lentiviral shRNAs
directed against DKK1 and FOXO1 (Fig. 4B). Furthermore,
prevention of MPA activity was observed when DKK1 and FOXO1
shRNAs were combined. In conclusion, progesterone-induced
up-regulation of DKK1 and FOXO1 is responsible, in part, for
Wnt/β-catenin signaling inhibition in the endometrium.

Progesterone inhibition of Wnt/β-catenin signaling in normal
dometrium and endometrial cancer. It has been reported that
at least 30% of early estrogen-associated tumors (type I endo-
metrioid) display the hallmark of Wnt signaling activation,
namely, nuclear β-catenin accumulation (9, 13). We performed
immunohistochemistry on histologic sections from 10 well-
differentiated endometrium carcinomas, and 3 of these showed

nuclear β-catenin staining. To confirm that intracellular β-catenin
accumulation was accompanied by up-regulation of known
Wnt/β-catenin downstream targets, CD44 immunohistochemical
analysis was also done on these three nuclear β-catenin-
positive tumors (41). In Fig. 5A, squamous morules are seen
staining strongly for nuclear β-catenin, and CD44 is shown in

Fig. 2. Endometrial expression of DKK1 (gray columns) and FOXO1
(black columns) during steroid hormone treatment (Con, E2, and E2 + MPA)
during the menstrual cycle. Microarray data of DKK1 and FOXO1 are plotted. The FOXO1 data represent the average of the data obtained from four different probe sets (see Supplementary Table S1). Y-axis, normalized expression levels; X-axis, different experimental groups. Con, E2, and E2 + MPA data are from ref. 24; PE, ESE, MSE, and LSE data are from ref. 25. *, P < 0.05, between Con and hormone treatments. **, P < 0.05, between PE treatment and other stages of the menstrual cycle.

Fig. 3. Progesterone, DKK1, and FOXO1 inhibit Wnt/β-catenin signaling in
Ishikawa cells. Ishikawa cells were transected with the TOPFlash Wnt/
β-catenin reporter construct and several other constructs as indicated in the
figure (plasmids are depicted in nanograms per well of a 24-well culture plate).
Furthermore, the cells were cultured for 48 h in the presence or
absence of MPA or the antiprogestagen Org-31489 (amounts are indicated
in the figure; ref. 62). dnTCF represents a dominant-negative inhibitor of
Wnt signaling (32). All experiments are corrected for transfection efficiency
by using the tk-renilla vector. The experiments done in A to C have all been
repeated at least three times and are independently done from each other.
*, P < 0.05, between no treatment and treatments and/or transfections.
adenocarcinoma showing nuclear β-catenin accumulation (data not shown).

In agreement with our hypothesis that E2 induces Wnt/β-catenin signaling to trigger proliferation of the endometrium, it was observed that expression of the Wnt target gene CD44 in the endometrium of the seven E2-treated women was higher than that in the six E2 + MPA–treated women (Fig. 5B). Furthermore, when CD44 expression was measured during hyperplasia, CD44 expression became markedly reduced on treatment with progestagens (Supplementary Fig. S3).

This particular patient underwent hysterectomy because of the development of a well-differentiated endometrial carcinoma. Immunohistochemical staining for CD44 in this tumor showed a heterogeneous staining pattern. In some fields, it was clear that staining for CD44 was positive in areas of carcinoma, whereas adjacent areas of hyperplasia were negative (Fig. 6A). Endometrial tumors often progress to become progestagen independent, and therefore progesterone receptor expression was also assessed by immunohistochemistry and compared with CD44 staining (Fig. 6B). Epithelial CD44 staining was minimal in areas where expression of the progesterone receptor was positive (Fig. 6B, filled arrows). Conversely, in regions devoid of progesterone receptor signaling, CD44 levels were clearly enhanced (Fig. 6B, open arrows). To further substantiate our findings, we reviewed 10 cases of well-differentiated endometrial cancer. In all these cases, when the progesterone receptor was present, CD44 levels were low or undetectable, and in those regions of the tumor where the progesterone receptor was lost, CD44 expression was high (Supplementary Fig. S4). These data suggest that Wnt/β-catenin signaling can be inhibited by progesterone in estrogen-associated endometrial hyperplasia and cancer, but only in the presence of progesterone receptors (Figs. 5 and 6).

**Discussion**

Sustained or unopposed estrogen signaling in the endometrium results in hyperplasia (45), which is followed in ~25% of cases by tumor formation (2). Two groups of women are known to be at high risk for endometrial cancer because of sustained or unopposed estrogen-like activity: those who are significantly overweight (ref. 46; relative risk >4) and those receiving tamoxifen (a partial estrogen antagonist) for breast cancer treatment (ref. 47; 5-year usage relative risk >7). It has been estimated that up to 40% of all endometrial cancers may be related to obesity, a figure that is increasing as the prevalence of obesity increases.

It has been reported that at least 30% of these early estrogen-associated tumors (type I endometrioid) display the hallmark of Wnt/β-catenin signaling activation, namely, nuclear β-catenin accumulation (9, 13), suggesting a cause-effect relationship between sustained estrogen signaling and aberrant Wnt/β-catenin signaling. Accordingly, we found a significant number of targets and components of the Wnt/β-catenin signaling pathway among...
the genes differentially regulated in the endometrium of E2-treated women (Supplementary Table S1). This is indicative of the effect of estrogen signaling on this pathway. For example, the Wnt/β-catenin signaling target IGF-I, regarded as one of the most important endometrial growth factors (48), was found to be strongly up-regulated (12-fold) by E2 and inhibited after the addition of progesterone. In literature, there are also a number of reports implicating progesterone and E2 in regulation of Wnt/β-catenin signaling in the endometrium. Cloke et al. (49), for example, showed that knockdown of the progesterone receptor resulted in activation of Wnt/β-catenin signaling in differentiating human endometrial stromal cells. In agreement with this, Satterfield et al. (50) could show that progestagens, administered to pregnant sheep, induced a transient decline in Wnt/β-catenin signaling activity. Seemingly in contrast to these finding, Rider et al. (51) showed that estrogens activated Wnt/β-catenin signaling in stromal cells, but only after initiation by progestagens. Finally, Katayama et al. (52) showed that administration of an estrogen agonist to immature female rats resulted in down-regulation of Wnt7A and up-regulation of Wnt4 in the uterus, whereas Catalano et al. (53) showed up-regulation of Wnt5A in the endometrium by antiprogestagen treatment in healthy volunteers. These reports and our own observations led us to hypothesize that Wnt/β-catenin signaling is stimulated by estrogens and inhibited by progestagens during the menstrual cycle.

Fig. 5. Wnt/β-catenin signaling in different endometrial samples. A, β-catenin and CD44 staining of an area of endometrial hyperplasia in a uterus containing a well-differentiated adenocarcinoma. Arrowheads, a region with cytoplasmic β-catenin staining, which is devoid of CD44 staining. The higher-power image shows a squamous morule, where it is recognized that nuclear β-catenin staining is particularly intense (42–44, 63). CD44 also stains intensely in this area. B, CD44 staining of the endometrium after 21 d of E2 or E2 + MPA treatment [1].
To investigate steroid regulation of Wnt signaling during the menstrual cycle, the possible involvement of the progesterone-induced Wnt/β-catenin signaling inhibitors DKK1 and FOXO1 was investigated by use of the well-differentiated endometrial cancer cell line Ishikawa. For DKK1, there is good evidence that this protein can bind to the Wnt coreceptors LRP5 and LRP6, thus inhibiting Wnt/β-catenin signaling (54). For FOXO1, the evidence is scarcer. Essers et al. (55) reported that FOXO1 directly binds to β-catenin, and Almelda et al. (38) could show that this binding results in inhibition of Wnt/β-catenin signaling. In the endometrial cancer cell line Ishikawa, where we could show that Wnt/β-catenin signaling was constitutively active, progesterone effectively inhibited Wnt/β-catenin reporter activity (Fig. 3A). Furthermore, on progesterone treatment, there was up-regulation of DKK1 and FOXO1 expression (Fig. 4; ref. 31), an observation in keeping with the profound up-regulation of the expression of both genes during the secretory phase of the menstrual cycle (Fig. 2; ref. 20). Interestingly, Tulac et al. (20), as well as Kane et al. (56), show that progesterone induced DKK1 expression specifically in stromal cells of the endometrium. In analogy to Wnt5A, which is produced by stromal cells and acts on uterine glands (18), it is possible that in vivo stroma-produced DKK1 is specifically important in inhibiting Wnt/β-catenin signaling in glandular and luminal epithelial cells of the endometrium.

On transfection of Ishikawa cells with vectors encoding either DKK1 or FOXO1, Wnt/β-catenin signaling was clearly inhibited, thus indicating that both proteins can serve as Wnt/β-catenin signal inhibitors in response to progesterone. Indeed, inhibition of Wnt/β-catenin signaling by progesterone treatment was partly circumvented by shRNA-driven down-regulation of DKK1 or FOXO1 expression.

By using the Wnt/β-catenin downstream target CD44 as a marker for Wnt signaling, it was shown that E2 treatment seems to result in enhanced Wnt/β-catenin signaling. CD44, hyaluronic acid receptor, in this respect is an interesting marker because it has been implicated in the progression and metastasis of a
number of different cancers (57). E2-induced CD44 expression was counteracted (compensated for) by MPA treatment (Fig. 6B). Furthermore, estrogen-induced endometrial hyperplasia (45) also displayed increased CD44 expression, which was shown to be inhibited by progesterone treatment (Supplementary Fig. S3). Notably, in an endometrial carcinoma arising during treatment of endometrial hyperplasia with progesterone, areas with enhanced Wnt/β-catenin signaling (CD44 positive) generally corresponded to areas of carcinoma, whereas regions that displayed reduced Wnt/β-catenin signaling (CD44 negative) tended to correspond to areas with the morphol of hyperplasia. Staining the same regions for progesterone receptor expression indicated that the presence of progesterone receptors correlated with a decrease in Wnt/β-catenin signaling, whereas its absence coincided with enhanced Wnt/β-catenin signaling. Similar observations were reported in other well-differentiated endometrial tumors (Supplementary Fig. S4) and several years ago by Hanekamp et al. (58), who could also show a clear correlation between the absence of progesterone receptors and the presence of CD44.

In endometrial hyperplasia and cancer, we have thus observed that when progesterone receptor signaling is intact, progesterone indeed seems to be able to inhibit Wnt signaling (inhibit CD44 expression). This inhibitory effect of progesterone most likely limits cancer progression, which is observed in 15% to 25% of patients with metastatic endometrial cancer who respond to progesterone therapy (3, 59). Progesterone treatment, however, only renders a temporary relief, and the disease often becomes progesterone insensitive (59). Based on the findings of the present study, it seems warranted to consider treatment of endometrial cancer patients whose disease has become progesterone resistant with inhibitors of the Wnt/β-catenin signaling pathway (60) that are acting downstream of progesterone signaling. Endostatin has been shown to inhibit Wnt/β-catenin signaling through inhibition of LEF1 (61). LEF1 is an important part of the Wnt/β-catenin pathway, which we have shown to be up-regulated by estrogens during the proliferative phase of the menstrual cycle and down-regulated by progesterogens during the secretory phase of the menstrual cycle (ref. 25; Supplementary Table S1). Endostatin may thus be a potential therapeutic option in progesterone-insensitive metastatic endometrial cancer.

In summary, our results provide support for the hypothesis that during the proliferative phase of the menstrual cycle, increased E2 levels induce Wnt/β-catenin signaling to enhance proliferation, whereas during the secretory phase, progesterone levels inhibit Wnt/β-catenin signaling thereby counterbalancing E2-induced proliferation and enhancing differentiation. Furthermore, progesterone also seems to be able to inhibit Wnt/β-catenin signaling during endometrial carcinogenesis, thus inhibiting the disease. In addition, we provide mechanistic evidence that the Wnt/β-catenin signaling inhibitors DKK1 and FOXO1 are acting downstream of the progesterone receptor to trigger inhibition of Wnt/β-catenin signaling.

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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