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Expression of the Wnt antagonist Dickkopf-3 is associated with prognostic clinicopathologic characteristics and impairs proliferation and invasion in endometrial cancer

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Abstract

Objective—Emerging evidence implicates the Wnt antagonist Dickkopf-3 (Dkk3) as a tumor suppressor and potential biomarker in solid tumors. We investigated whether Dkk3 plays an important role in the carcinogenesis of endometrial cancer (EC).

Methods—We analyzed Dkk3 mRNA expression via real-time RT-PCR in twenty-seven human primary EC tissues, and six matched normal endometrial controls. Dkk3 levels were correlated with various clinicopathologic characteristics. Additionally, enforced Dkk3 expression was examined in proliferation and tumorigenesis in vitro and in vivo, using MTT, soft agar assay, invasion assay, a xenograft mouse model, and a β-catenin-responsive SuperTopFlash luciferase assay.

Results—Compared with matched normal endometrial cases, Dkk3 was down-regulated in EC (p < 0.0001). Among cancer cases, Dkk3 expression was significantly reduced in patients with higher stage (p = 0.002), positive pelvic lymph nodes (p = 0.0004), non-endometrioid histology (p = 0.02), and cytology-positive ECs (p = 0.02). Enforced expression of Dkk3 in EC cell lines showed reduced proliferation (p < 0.0001), anchorage-independent growth (p = 0.005), invasion (p = 0.02), and reduced TCF activity (p = 0.04), confirming Dkk3 as a negative regulator of the β-catenin/Wnt signaling pathway. Tumor growth in Dkk3-injected mice was not statistically different, though did plateau towards the end, and was associated with increased lymphoid infiltration and tumor necrosis.

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Conclusion—Dkk3 gene expression is frequently downregulated in endometrial cancer, and is associated with poor prognostic clinicopathologic markers. The results also identify a role for Dkk3 as a tumor suppressor in EC, affecting both proliferation and invasiveness. These findings may prove to be important in the design of novel biomarkers and treatment modalities for advanced EC.

Keywords
Endometrial cancer; Canonical Wnt pathway; Wnt antagonist; Dickkopf-3; REIC/Dkk-3; Tumor suppressor

Introduction
Endometrial carcinoma (EC) is the most common gynecologic malignancy in the United States, with an estimated 46,470 cases expected to be diagnosed in 2011 [1]. It is a heterogeneous disease that can be largely classified into two major types: Type I ECs, the most common type, which are usually of endometrioid histology, and are often associated with obesity; versus Type II ECs, which are of non-endometrioid histology (e.g. papillary serous or clear cell), are not a result of unopposed estrogen, and usually carry a worse prognosis [2]. Despite a good prognosis in early-stage endometrial cancer, survival rates in advanced-stage cancer have been poor at just over 12 months for patients enrolled in chemotherapy trials [3]. Few effective treatment options are available once the disease has metastasized, and while recent Phase II trials have shown some promise with novel biologic agents (Mammalian target of rapamycin (mTOR) inhibitors, Bevacizumab), none have shown a response rate over 15% [4–6]. There is thus a dire need for a search for further treatment options in advanced EC. Importantly, the molecular pathogenesis of EC is understudied, and research in this field has lagged far behind breast, ovarian, and cervical cancer in terms of grant money allocation and progress. Despite a clinicopathologic model to predict prognosis based on a surgical pathology study carried out by the Gynecologic Oncology Group in the 1970s (GOG 33) [7], little is known about the molecular characteristics to predict who will recur, and who should receive what type of treatment (e.g. adjuvant radiation and chemotherapy). Moreover, the response to radiation, cytotoxic or hormonal therapy is difficult to predict. Therefore, identifying novel molecular biomarkers and therapeutic targets is imperative.

The Wingless-type (Wnt) signaling pathway regulates diverse developmental processes such as cell migration, adhesion, and proliferation. Dysregulation of the Wnt pathway has been implicated in a variety of human malignancies, but is best known for its role in colorectal cancer (CRC), where greater than 90% of CRCs carry an activating mutation in the canonical Wnt signaling pathway, most frequently in the form of a mutational inactivation of adenomatous polyposis coli (APC) [8]. The influence of Wnt signaling has expanded to other solid tumors, including melanoma, osteosarcoma, other gastrointestinal cancers, prostrate, breast, liver, lung, and ovarian cancer [9,10]. In these cancers, Wnt antagonists have been explored as potential tumor suppressors and biomarkers [11–15]. The role of Wnt signaling in EC has not been adequately elucidated. While early reports have shown that 10–45% of all ECs carry β-catenin mutations, with a slightly higher propensity in endometrioid ECs, no functional relationship or associated prognostic values have been assigned [16–26]. Recently, more emphasis has been placed on secreted Wnt antagonists, including members of the Dickkopf family [27–35]. The Dickkopf proteins are secreted Wnt inhibitors which induce removal of the Wnt co-receptor low-density lipoprotein receptor-related protein (LRP), and thus prevent Wnt signaling. Dkk3 is a member of the Dickkopf family, which has been suggested as a tumor suppressor [36]; initially, its gene was termed “REIC” (Reduced Expression in Immortalized Cells), reflecting its reduced expression in cancer.
cells [12]. Its overexpression suppresses tumor growth in vitro in osteosarcoma [37], though Dkk-3 knock-out mice have shown no enhanced tumor formation [38]. Much evidence exists to identify REIC/Dkk3 as a tumor suppressor and confirm its differential expression in many solid tumors [30,39]. Its reduced expression was first shown in lung cancer, in a study by Nozaki et al., which found reduced expression in 63% of lung cancer tissues compared to matched adjacent normal tissues [40]. Similar differential expression patterns were found in liver, prostate, testicular, colon, and breast cancers, confirming a significant role for Dkk3 in carcinogenesis [11,28,30,41,42]. Deregulation of Dkk3 expression appears to occur as a result of aberrant promoter hypermethylation [30,31,42–47]. In cervical cancers, Dkk3 was found to be frequently downregulated by microarray and real-time RT-PCR, when compared to normal cervical tissue [34]. In contrast, Jiang et al. reported that serum Dkk3 was increased in both endometrial and cervical cancer patients when compared to healthy subjects, with a stage-dependent pattern; however ovarian cancer patients exhibited reduced serum Dkk3 levels compared to healthy counterpart [48]. Why serum Dkk3 protein levels would be upregulated, in contrast to other reports revealing downregulation of the tissue Dkk genes, is unknown, and requires further study. Our knowledge of the role of canonical Wnt signaling in endometrial cancer is thus limited, and deserves further investigation to explore the mechanism and its importance [49]. In this study, we investigate the role of Dkk3 in the pathobiology of EC and its impact on known prognostic clinicopathologic factors. Our results show that Dkk3 is downregulated in EC, that this downregulation correlates with advanced stage and high risk clinicopathologic factors, and that forced Dkk3 expression in vitro can effectively reduce Wnt throughput, motility, proliferation, and invasiveness.

Materials and methods

Patient samples

Endometrial tissue samples were obtained from the University of California, Irvine, Medical Center (UCIMC) tissue bank and the Gynecologic Oncology Group (GOG) external tissue bank. Twenty-seven patients underwent total hysterectomy and bilateral salpingo-oophorectomy, with or without pelvic and para-aortic lymphadenectomy. All patients were staged according to the 1988 International Federation of Gynecologists and Obstetricians (FIGO) staging system for endometrial cancer. Endometrial tissue specimens were obtained as frozen tissues, after independent pathologic review at either the UCIMC or GOG tissue bank confirmed endometrial disease site, and distinguished between tumor and normal endometrium. Specimens included fourteen Stage I/II and thirteen Stage III/IV EC tissues. Additionally, six normal endometrial tissues (present adjacent to endometrial tumors), were obtained from six early stage EC patients, and considered as normal (matched) controls; these normal tissues were individually reviewed by either the GOG or UCI pathologist to determine lack of malignancy. Frozen samples were kept in liquid nitrogen until RNA isolation, at which point samples were placed in RNome-ICE (Ambion, Austin, TX) at −20 °C. This research did not qualify as human subjects research, due to the lack of personal health information, and thus was considered exempt from IRB approval at UCIMC, as evaluated by the UCIMC IRB board.

Cell lines and cell culture

The non-malignant immortalized human endometrial cell line T-HESC was kindly donated from the laboratory of Gil Mor (Yale University) [50]; the human endometrial cancer cell lines ECC-1, HEC-1A, and RL95-2 were obtained from the American Type Culture Collection (ATCC). The T-HESC and RL95-2, cell lines were maintained in DMEM-F12 with 10% fetal bovine serum (FBS) according to ATCC propagation guidelines. The ECC-1 and HEC-1A cell lines were maintained in RPMI 1640 and McCoy’s 5A media.
respectively, and supplemented with fetal bovine serum according to ATCC guidelines. All cell lines were cultured in a humidified incubator with 5% CO\textsubscript{2} at 37 °C.

**RNA isolation and reverse-transcriptional real-time PCR (RT-PCR)**

Total RNA was isolated from cultured cells and human endometrial tissues using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. Briefly, tissue specimens from six matched normal and endometrial cancer tissues from the same patients were minced with a scalpel and homogenized in Trizol via a rotor-run pestle (these instruments were disposed after each use, to avoid contamination among samples). Samples were purified using phenol chloroform extraction and isopropanol precipitation. RNA was washed with ethanol and resuspended in RNase-free water. cDNAs were synthesized with random primers via reverse transcription using High Capacity cDNA kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Gene expression was assessed by real-time PCR (RT-QPCR) using QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) and QuantiTect primer assays for Dkk3 in the Eppendorf RealPlex Silver Four detection system. Actin gene (Qiagen, Valencia, CA) was used as endogenous control to normalize expression data. Actin mRNA amounts in the samples did not show bigger variability than one threshold cycle. PCR conditions were as follows: 95 °C for 15 min, 50 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C. Relative mRNA expression was calculated from a calibration curve. The results are representative of three independent experiments. Data are shown as mean ± SE.

**Western blotting**

Whole-cell extracts were prepared in 1 × SDS [loading buffer: 50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol], and boiled for 5 min. Total protein was electrophoresed by SDS-PAGE and Western blotting was carried out using antibodies against Dkk3 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturers' protocols. Blots were exposed to secondary antibodies and visualized using the SuperSignal West Pico Chemiluminescent kit (Pierce). For loading control, membranes were stripped and reprobed with anti-β-actin.

**Transfections and Dkk3 expression**

A stable Dkk3-overexpressing ECC-1 cell line (ECC1-Dkk3) was established by transfecting the pcDNA 3.1 expression vector encoding Dkk3 cDNA (kindly provided by Dr. Xiaolin Zi). Cells were transfected at 50–70% confluence with Dkk3 expression vector or with empty vector control (pcDNA 3.1) using a liposome-mediated tranfection method (Lipofectamine 2000, Invitrogen). After 2 days, cells were trypsinized and replated at low density and maintained in G418-containing medium. Stable clones were selected and grown in combination to exclude cloning artifacts.

**SuperTopFlash luciferase assay**

Cells were transfected at 50–70% confluence with both constitutively produced Renilla luciferase expression construct and the β-catenin-responsive firefly luciferase plasmid Super8XTOPFlash (1:20), using a liposome-mediated tranfection method (Lipofectamine 2000, Invitrogen) according to the manufacturer's instructions. After 48 h, cells were trypsinized and plated in triplicates into 96-well plates in L-cell-conditioned media versus Wnt-3a-conditioned media (prepared according to Dr. Nusse's laboratory protocol, [http://www.stanford.edu/~rnusse/assays/W3aPurif.htm](http://www.stanford.edu/~rnusse/assays/W3aPurif.htm)), with or without exogenous Dkk3 from Dkk3-conditioned media (obtained from ECC1-Dkk3 cells). 24 h later, cells in 50 µl/well media were treated with 50 µl/well of Dual-Glo Firefly Luciferase substrate (Promega) according to the manufacturer's instructions, and activity, proportional to the Wnt pathway
throughput, was measured by a luminometer (Turner Biosystems Modulus Microplate multimode reader). The Stop & Glo reagent (50 µl/well) was then added to initiate constitutively expressed Renilla luciferase activity, and the ratio of firefly luciferase activity to Renilla luciferase activity was calculated to normalize the results for transfection efficiency and cell survival.

**MTT cell proliferation assay**

Cells were plated at 6000 cells per well in a 96-well plate and grown under G418 selection. After 48 h, cells were treated with 100 µl/well of MTT assay reagent (Sigma), and incubated for 2 h at 37 °C. Following centrifugation of the plate at 2500 rpm for 5 min, the medium was removed, and the MTT crystals were solubilized in DMSO. Spectrophotometric absorbance was measured via a plate reader at a wavelength of 570 nm.

**Soft agar colony formation**

A soft agar colony formation assay was done using 96-well plates. Cells were plated in triplicate in methylcellulose medium (MethoCult®, StemCell Technologies, Vancouver, BC, Canada). Cultures were maintained under standard culture conditions for five days, after which the number of colonies was determined by photographing the wells via microscopy, and colonies of ≥2.0 cm on the enlarged photographic image were counted.

**Invasion assay**

Dkk3-expressing ECC1 cells and control cells were analyzed for invasion/migration through Matrigel (BD Biosciences) according to the manufacturer’s protocol. Briefly, cells were starved without fetal bovine serum overnight, and then trypsinized and placed in Matrigel-containing inserts or control inserts at 1 × 10⁵ cells/ml in serum-free medium and were allowed to migrate for 6 days at 37 °C. Nonmigrating cells were removed from the top of the filter and cells that migrated were fixed with methanol, and stained with DAPI method for fluorescent microscopy according to the manufacturer's procedure (Invitrogen). The number of cells that migrated to the bottom side of the insert was counted manually by fluorescence microscopy. A normalizing cell motility assay was not technically feasible given the long time required for migration of these cells when Matrigel is absent in their microenvironment.

**Xenograft model**

NCR-nu/nu (nude) mice were obtained from Taconic (Germantown, NY). Cells from each stable line were suspended at 2.0 × 10⁶ in 200 µl PBS and injected subcutaneously into the right shoulder of each 4-week old female mouse. Once xenografts became established, their sizes were measured every 4 days. The tumor volume was calculated by the formula 1/6πab² (π = 3.14, a = long axis, and b = short axis of the tumor). Growth curves were plotted from the mean tumor volume ± SE from 10 animals in each group. All of the animal studies were approved by the Institutional Animal Care and Use Committee at the University of California (Irvine, CA). Tumors were harvested and hematoxylin and eosin slides were reviewed.

**Statistical analysis**

Statistical analyses correlating Dkk3 mRNA expression with various clinicopathologic characteristics were performed via the Student's t-test, Kruskal–Wallis test, and Wilcoxon signed-ranked test. Comparisons of cell density, number of colonies, invasion index, and relative levels of mRNA expression between the different transfections were conducted using Student's t test. All statistical tests were two sided, with a 95% confidence interval, and p < 0.05 was considered statistically significant. For tumor growth experiments,
repeated-measures ANOVA was used to examine the differences in tumor sizes among different transfections, time points, and transfection–time interactions.

**Results**

**Dkk3 is downregulated in human endometrial cancer tissues compared to normal endometrium**

To determine differential expression patterns of Wnt pathway genes in endometrial cancer (EC) and normal endometrium, we analyzed endogenous levels of Dkk3 mRNA by real-time RT-PCR in six human EC tissues and their matched normal counterparts. Dkk3 mRNA was downregulated in five of six EC tissue pairs (all p-values < 0.05, paired t-test), with ≥50% loss of Dkk3 expression in the EC samples (Fig. 1A). The mean of the Dkk3 mRNA levels of all EC samples was decreased compared to the mean of Dkk3 mRNA levels of all matched normal endometrial samples (p < 0.0001, unpaired t-test) (Fig. 1B).

**Dkk3 expression correlates with stage, histology, cytology, and pelvic lymph node status**

We then evaluated Dkk3 expression in the endometrial tumors of primary EC cases. Twenty-seven patients underwent total hysterectomy and bilateral salpingo-oophorectomy, with or without pelvic and paraaortic lymphadenectomy. The median age was 66 (41–93) years. Fifteen patients underwent bilateral pelvic lymphadenectomy with a median lymph node count of 19 (range 9 to 49), while three patients underwent a lymph node biopsy; nine patients did not undergo any lymphadenectomy. There were nineteen endometrioid, four clear cell, and four papillary serous histology cases; ten Grade 1, five Grade 2, and eleven Grade 3 cases. There were fourteen Stage I and II cases, versus thirteen Stage III and IV cases (FIGO 1988) (Table A, Supplemental data).

Dkk3 gene expression in EC was stage-dependent (p = 0.002), and correlated with several clinico-pathologic factors (Table 1). Dkk3 expression was on average four times higher in patients with negative pelvic lymph nodes than those with positive nodes (p = 0.0004). Its expression was higher in cytology-negative (p = 0.02) and endometrioid (p = 0.02) EC cases. There was a step-wise down-regulation in Dkk3 expression from intrauterine disease to pelvic metastatic disease to extrapelvic metastatic disease (p = 0.01). Patients with grade 1 and 2 disease had higher Dkk3 expression than those with grade 3 disease, though this was not quite statistically significant (p = 0.1).

**Loss of Dkk3 gene and protein expression in endometrial cancer cells lines**

Dkk3 expression was examined in several endometrial cancer cell lines (ECC1, HEC-1A, RL95-2), as compared to a benign endometrial cell line, HESC. ECC-1 is a well-established EC cell line derived from a patient with well-differentiated endometrial adenocarcinoma; this cell line is known to be steroid-responsive [51], and best represents a Type I EC cell line. HEC-1A is a cell line derived from a patient with a papillary serous endometrial adenocarcinoma, while RL95-2 is derived from a patient with adenosquamous EC; these two cell lines best represent Type II EC cells. HESC is a benign, immortalized, human tissue-derived endometrial cell line, derived from a patient with uterine myomas, and was used as the “normal” control. We determined the mRNA and protein expression of Dkk3 in ECC-1, HEC-1A, and RL95-2 EC cells, and compared these to the non-malignant HESC cell line. Fig. 2A shows the reduced Dkk3 mRNA expression in ECC-1, HEC-1A and RL95-2 cells as compared to HESC (p = 0.004, p = 0.02, p = 0.02). Protein expression of Dkk3 confirmed the above trend, with loss of Dkk3 expression in all three EC cell lines (Fig. 2B).
Dkk3 reduces canonical Wnt signaling in ECC-1 cells, in the absence or presence of Wnt3a

To determine the mechanistic effect of Dkk3 expression, we first determined whether our in vitro model, the ECC-1 cell line, is responsive to canonical Wnt signals. We thus transfected ECC1 cells with a beta-catenin-responsive luciferase vector (SuperTopFlash), which contains binding sites which are directly activated by the TCF/beta-catenin complex, and therefore resulting in luciferase activity. Upon addition of Wnt3a ligand, there was a 15 × fold surge in Wnt throughput, confirming that the ECC-1 cell line is responsive to canonical Wnt signaling (p<0.0005) (Fig. 3A).

We then set out to determine the effect of differential Dkk3 expression on endogenous Wnt signals, and measured Wnt throughput in the Dkk3-transfected cell line compared to GFP-transfected cell line. Fig. 3B shows that the Wnt throughput is significantly reduced in the Dkk3-transfected cell line compared to control ECC-1 cells (21-fold Wnt activation in control cells, compared to a 9-fold activation in Dkk3-expressing ECC-1 cells).

Additionally, considering that DKK3 conditioned medium (CM) was found ineffective in modulating Wnt pathway in osteoblasts [52], we compared the addition of CM from the cells transfected by pCMV as a vector only control and the cells with Dkk3 expression construct on ECC-1 cells. Addition of CM from pCMV-transfected cells does not result in inhibition of Wnt signaling (Fig. 3C, columns 1 and 2), as compared to L-conditioned media (L-CM: negative control), while CM from the cells transfected with Dkk3 expression construct suppresses Wnt throughput both in the absence (Fig. 3C, columns 2 and 3) and in the presence of Wnt ligand (Fig. 3C, columns 4 and 5). In summary, CM from Dkk3 expressing cells suppresses basal and Wnt3a driven Wnt signaling in endometrial cancer cells in vitro.

Dkk3 transfection decreases cell proliferation, anchorage-independent growth and invasiveness of ECC-1 cells

To examine the functional role of Dkk3 downregulation in EC, we utilized the EC cell line ECC-1 as in vitro model. To determine the effect of Dkk3 overexpression on proliferation and invasiveness, ECC-1 cells were stably transfected with an expression construct of Dkk3. Fig. 4A shows the increased mRNA expression of Dkk3 in the Dkk3-overexpressing ECC-1 cells (ECC1-Dkk3), as compared to parental ECC-1. The impact of Dkk3 overexpression on cell proliferation was then examined via MTT cell proliferation assay (Fig. 4B). ECC1-Dkk3 cells showed significantly reduced cell proliferation by 24.8%, when compared to ECC1-pCMV cells (p<0.0001). Anchorage-independent growth was suppressed in Dkk3-transfected ECC-1 cells by 30.6% compared to empty vector control cells, as shown by a reduced number of colonies formed on soft agar (p = 0.005) (Fig. 4C). Similarly, invasiveness was reduced by 58.1% in Dkk3-transfected ECC-1 cells (p = 0.02), as determined by Matrigel invasion assay (Fig. 4D).

Dkk3 expression in the xenograft mouse model results in increased lymphoid infiltrate and necrosis

To determine the ability of the secreted Wnt antagonist Dkk3 in the inhibition of tumor growth in nude mice, we performed xenograft experiments with Dkk3-expressing ECC-1 cells compared to cells transfected with vector only. There was no statistically significant difference in tumor volumes between control and Dkk3 producing ECC-1 xenografts (Fig. 5A). However, Dkk3 tumors appeared to have a growth plateau between 40 and 50 days (Fig. 5). Fig. 5B shows gross pictures of the tumors removed at day 50 from xenograft mice injected with Dkk3-expressing ECC-1 cells (DKK3), as well as tumors from mice injected with control ECC-1 cells at the same timepoint. Grossly, the tumors grown in Dkk3-injected mice more frequently showed necrosis and hemorrhage compared to control tumors. Representative H&E stains of tumor from Dkk3-expressing xenograft mice demonstrate...
increased amounts of lymphoid infiltrate, hemorrhage and necrosis (Fig. 5C, ii and iii) compared to controls (Fig. 5C, i).

Discussion

**Dkk3 downregulation in endometrial cancer**

While a large number of reports have implicated β-catenin mutations in 10–45% of endometrial cancers, the precise role of Wnt signaling in this disease has not been established. Wnt inhibitors play an important role in regulating the canonical Wnt pathway, and thus have been at the forefront of research efforts to investigate the mechanism of Wnt signaling in various solid tumors. To date, only one report has associated the Wnt inhibitor Dkk3 with EC, and in this report, serum Dkk3 was increased in both endometrial and cervical cancer patients, compared to serum Dkk3 levels in healthy controls, while ovarian cancer patients expressed lower serum Dkk3 protein levels [48]. This is in contrast to most other reports of Wnt inhibitor downregulation in solid tumors by immunohistochemistry or real-time RT-PCR of primary tumor tissues, such as described in cervical cancer [34]. Why serum protein Wnt inhibitor expression differs from tissue mRNA or protein expression, is subject to further investigation.

Our study findings of Dkk3 downregulation in both human primary EC tissues and EC cell lines confirm similar reports in gastrointestinal [11], breast [30], prostate[41], and renal carcinomas [53,54]. Importantly, our investigation compares primary human EC tissues with matched normal (adjacent) endometrial tissues, and revealed nearly uniform loss of Dkk3 expression in the malignant samples, by at least 50%. While the sample size is small, the matched properties of these two groups provide a strength to this study, and offer evidence for a potential role for Wnt signaling in the carcinogenesis of EC, as loss of Wnt inhibitor expression may result in increased Wnt activity and proliferation. This hypothesis was tested as well in several endometrial cancer cell lines (ECC1, HEC1A, RL95-2), at both the mRNA and the protein level. The results confirmed reduced endogenous Dkk3 mRNA expression compared to normal endometrial cells (HESC). The mRNA expression levels of all EC cell lines were minute, compared to an approximately 100-fold increased expression level in the normal endometrial cell line. At the protein level, this trend translated into an undetectable expression intensity for the EC cell lines, while the protein expression of HESC confirmed the mRNA expression detected on real-time RT-PCR. Thus, Dkk3 expression appears to be diminished or completely absent both in vivo and in vitro. These results support a hypothesis for the loss of Dkk3 as a carcinogenetic event in EC, leading to a reduction in Wnt inhibition, and subsequent increased Wnt signaling activity and proliferation. Further studies investigating the role of Dkk3 promoter hypermethylation in EC may elucidate the mechanism for the loss of Dkk3 expression shown above, as reported previously in other solid tumors, such as breast and lung cancer.

**Dkk3 expression correlates with stage, histology, pelvic lymph node status, and cytology**

A number of clinicopathologic characteristics predict clinical outcome in EC, including stage, grade, and histology. Additionally, depth of myometrial invasion, cytology, lymphovascular space invasion (LVSI), and pelvic lymph node status predict clinical behavior and direct adjuvant treatment options [55,56]. In our study, we demonstrate that Dkk3 expression is stage-dependent, as the Dkk3 mRNA levels in early stage EC tissues were frequently elevated, while expression in advanced stage EC tissues was reduced. This trend of higher Dkk3 expression in early stage EC mirrors the findings of stage-dependent Dkk1 expression in endometrial cancer, as reported by Yi et al. [35]. Though there was a significant variability in the degree of Dkk3 expression in early stage ECs in our study, with several tissues expressing high levels amidst other low-expressing tumor samples, advanced
stage tissues nearly uniformly showed low expression levels. The reduced expression levels in early stage cancer may reflect patients with poorer clinical outcomes, as there is quite a heterogeneity in prognoses in early stage cancers, though in the absence of survival data, this can only be hypothesized. However, the addition of other known prognostic clinicopathologic characteristics strengthens the predictive value of Dkk3 expression in EC, as Dkk3 levels were reduced in patients with positive nodes, extrapelvic metastases, non-endometrioid histologies, and positive cytology, all prognostic factors indicative of poorer clinical outcomes. Comparison between grade 1 and 2 disease and grade 3 disease did not yield a statistically significant difference, likely due to the small number of tissues used in this study, though there was a significant trend noted with lower Dkk3 expression in high grade tumors, with a p-value of 0.1. Despite these findings, an evaluation of Dkk3 expression linked to survival and recurrence in a higher-powered study would be of considerable value in this setting. Nonetheless, the role of Dkk3 as a potential prognostic marker is supported by prior reports in pancreatic cancer [33], where low Dkk3 expression in tumor endothelium is associated with a shorter survival, compared to patients with high Dkk3 expression (15 months vs. 7 months).

**Functional significance of Dkk3 in endometrial carcinoma**

We are the first to show that the endometrial cancer cell line ECC-1 is responsive to extracellular Wnt signaling, and that the Wnt inhibitor Dkk3 reliably reduces Wnt throughput, with or without exogenous Wnt ligand. This establishes the ECC-1 cell line as a useful model in the study of Wnt signaling in endometrial cancer. The ECC-1 cell line is a well-differentiated steroid-responsive endometrial cancer cell line with both estrogen and progesterone receptors [51]. Our data are consistent with prior studies which have demonstrated Wnt activation in Ishikawa cells (another well-differentiated endometrial adenocarcinoma cell line which bears estrogen and progesterone receptors [57]), with inhibition of Wnt activity by sFRP4 [58]. Importantly, we demonstrate that, in ECC1 cells, Dkk3-mediated inhibition of Wnt signaling is accompanied by decreased proliferation, reduced anchorage independent growth and decreased invasiveness, consistent with similar reports of soluble Wnt inhibitors in model systems for other malignancies [59,60].

The role of Dkk3 as a tumor suppressor has been suggested by many other authors [11–13,37,61]. In osteosarcoma cells, Hoang et al. [15] demonstrated that Dkk3 transfected Saos-2 cells have a reduction in invasive capacity and cell motility correlating with beta-catenin down-regulation in the nucleus. Tsuji et al. showed that Dkk3 inhibited Saos-2 cell growth [61] and Abarzua et al. showed that Dkk3 overexpression results in induction of apoptosis in human prostate cancer [41], noticing detachment of prostate cancer cells from the plastic of culture vessels after the treatment with Dkk3. We did not detect such Dkk3-induced detachment in endometrial cancer cell line (data not shown). We hypothesize that the mechanism of tumor suppression by Dkk3 in the ECC1 cell line is regulated through the Dkk3-induced Wnt-beta-catenin pathway down-regulation.

Prior studies have examined the therapeutic effects of Dkk3 in mouse models [62,63]. Edamura et al. showed that intratumoral injection with adenoviral vectors encoding for the Dkk3 gene, using an orthotopic mouse prostate cancer model, resulted in inhibited tumor growth, reduced lymph nodemetastasis, and prolonged survival [62]. Given our promising in vitro data, we examined the effects of Dkk3 expression in a xenograft mouse model by injecting mice with Dkk3-expressing ECC1 cells and comparing growth characteristics to pCMV-transfected ECC1 cells. We show that Dkk3-expressing xenograft mice exhibited large amounts of lymphoid infiltrate and necrosis in the setting of moderate to poorly differentiated adenocarcinoma, as compared to minimal to no necrosis and lymphoid infiltrate in pCMV-transfected tumors. Tumor volumes however were similar between the two groups, though the Dkk3-expressing tumors appear to have a growth plateau after 40
days, while the control tumors continued to grow. Unfortunately, continued observation was not possible due to increasing symptoms from the tumor burden, though we speculate that continuation of the experiment may have shown tumor suppression in the Dkk3 group compared to the control group. Additionally, the enhanced lymphoid infiltrate may have resulted from the release of tumor antigens because of tumor cell necrosis and apoptosis that may have been processed by dendritic cells and other antigen presenting cells in the tumor microenvironment. The lack of volume reduction in the Dkk3-expressing tumors compared to control may be a result of increased infiltration with lymphoid cells and tumor hemorrhage.

Conclusions

To date, a number of studies have suggested a role for Wnt signaling in endometrial carcinogenesis. Despite the limited literature associating Wnt signaling with endometrial carcinogenesis, this field deserves further study, especially in light of the inadequate treatment options which currently exist for women with advanced and recurrent EC. Our data demonstrate that Dkk3 expression is downregulated in endometrial cancer both in vivo and in vitro. The Wnt inhibitor Dkk3 is a stage-dependent predictor of disease, with low expression levels correlating with clinico-pathologic factors which predict poor prognosis, including histology, pelvic lymph node positivity, cytology, and stage. Larger studies are needed to validate these findings and correlate these with survival outcomes. In vitro, Dkk3 suppresses Wnt pathway throughput, tumor growth and tumor invasiveness. These effects are recapitulated in an in vivo animal model where Dkk3-expressing tumors exhibit significantly increased necrosis and inflammatory infiltrate. Our studies suggest that Dkk3 plays a role in EC as a tumor suppressor, and may be a candidate as a novel biomarker and therapeutic target, and suggest the importance of further studies to target the Wnt signaling pathway as novel targeted therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References


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Fig. 1.
Dkk3 expression is downregulated in endometrial cancer tissues. A, mRNA levels of Dkk3 in normal endometrial tissues and matched endometrial cancer tissues were determined by real-time RT-PCR. Percentage of loss of Dkk3 mRNA expression from normal endometrium to EC for each matched tissue pair is shown below the figure. B, mRNA levels in all normal endometrial tissues compared to all endometrial cancer tissues. C, Individual Dkk3 mRNA expression in all Stage I/II and Stage III/IV EC tumors. D, Dkk3 mRNA expression is reduced in Stage I/II compared to Stage III/IV EC tissues, as determined by real-time RT-PCR. All experiments were determined by real-time RT-PCR and performed in triplicates. * denotes p < 0.05, ** denotes p < 0.005, and *** denotes p < 0.0001.
Fig. 2.
Dkk3 mRNA and protein expression are decreased in the endometrial cancer cell lines, ECC-1, HEC1A, and RL95-2, as compared to the benign endometrial cell line, HESC. A, mRNA levels of Dkk3 in HESC, ECC-1, HEC1A, and RL95-2, were determined by real-time RT-PCR. B, Expression of Dkk3 protein was determined by Western blotting with anti-Dkk3 antibodies in the same cell lines. All experiments were performed in triplicates. * denotes p < 0.05.
Fig. 3.
Wnt3a activates β-catenin/TCF signaling in the endometrial cancer cell line ECC-1, but is inhibited by addition of exogenous or endogenous Dkk3. A, Addition of Wnt3a-conditioned medium results in a 15-fold increase in Wnt throughput in ECC-1, as determined by SuperTopFlash (STF) dual-luciferase assay. B, Cells endogenously producing Dkk3 following transient transfection exhibit a diminished response to Wnt3a. C, Exogenous Dkk3 CM reduces Wnt throughput in the presence and absence of Wnt3a. All experiments were performed in triplicates via SuperTopFlash dual-luciferase with Renilla normalization. * denotes p < 0.05.
Fig. 4.
Dkk3 expression is decreased in the ECC-1 cell line, and Dkk3 transfection decreases proliferation, invasion and anchorage-independent growth in ECC-1 cells. A, mRNA levels of Dkk3 in stably-transfected ECC-1 cells (ECC1-Dkk3) were determined by real-time RT-PCR and compared to wild-type ECC-1 cells (wt-ECC1). B, Dkk3 reduces proliferation in ECC-1, as determined by cell proliferation assay. C, Dkk3 reduces anchorage-independent growth in ECC-1 cells, as determined by soft agar colony formation assay. D, Dkk3 decreases invasion in ECC-1 cells, as determined by Matrigel invasion assay. All experiments were performed in triplicate. * denotes p < 0.05, and *** denotes p < 0.0001.
Fig. 5.
Xenograft mouse studies. A, Tumor volumes. Dkk3 expression in the xenograft mouse model results in no statistically significant difference in tumor volumes. B, Gross tumors showing necrosis and hemorrhage in Dkk3-expressing xenograft mice, compared to controls. C, Representative H&E stains of tumor from pCMV and Dkk3-expressing xenograft mice. i, Representative H&E stains of tumor from pCMV-ex show an endometrial adenocarcinoma with no necrosis and some glandular differentiation. ii, Representative H&E stains of tumor from Dkk3-expressing xenograft mice show an endometrial adenocarcinoma with large amounts of lymphoid infiltrate and necrosis. iii, Representative
H&E stains of tumor from Dkk3-expressing xenograft mice show an adenocarcinoma with increased necrosis and hemorrhage.
**Table 1**

Dkk3 expression by clinicopathologic characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n</th>
<th>Dkk3 mRNA expression (mean ± SEM)</th>
<th>p-Value</th>
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<tr>
<td><strong>Age</strong></td>
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<tr>
<td>&lt;50</td>
<td>3</td>
<td>6.480 ± 2.314</td>
<td>ns&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>50–70</td>
<td>12</td>
<td>2.115 ± 0.4444</td>
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<tr>
<td>&gt;70</td>
<td>12</td>
<td>0.6040 ± 0.09660</td>
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</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Endometrioid</td>
<td>19</td>
<td>2.344 ± 0.4500</td>
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<tr>
<td>Papillary serous</td>
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<td>0.9143 ± 0.1963</td>
<td></td>
</tr>
<tr>
<td>Clear cell</td>
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<td>0.2548 ± 0.1092</td>
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<tr>
<td><strong>Stage</strong></td>
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<td></td>
<td>0.002&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>I and II</td>
<td>14</td>
<td>2.422 ± 0.5308</td>
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<tr>
<td>III and IV</td>
<td>13</td>
<td>0.7654 ± 0.1348</td>
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<tr>
<td><strong>Grade</strong></td>
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<td>0.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>1 and 2</td>
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<td>2.424 ± 0.5561</td>
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<tr>
<td>3</td>
<td>11</td>
<td>0.9261 ± 0.1573</td>
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<td>ns&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>2.828 ± 0.6122</td>
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<td><strong>LVSI</strong></td>
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<tr>
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<td>14</td>
<td>2.589 ± 0.5599</td>
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<tr>
<td>Present</td>
<td>12</td>
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<tr>
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<tr>
<td>Negative</td>
<td>17</td>
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<tr>
<td>Positive</td>
<td>7</td>
<td>0.6216 ± 0.1386</td>
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<td><strong>Pelvic LNs</strong></td>
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<td>0.0004&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<tr>
<td>Positive</td>
<td>5</td>
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<tr>
<td>Pelvic mets</td>
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<td>0.8418 ± 0.2508</td>
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<tr>
<td>Extrapelvic mets</td>
<td>8</td>
<td>0.3794 ± 0.05635</td>
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</table>

DOI = Depth of invasion; LVSI = Lymphovascular space invasion; LNs = lymph nodes; ns = not statistically significant.

<sup>a</sup>Kruskal–Wallis test.

<sup>b</sup>Wilcoxon signed-ranked test.