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Dkk3, downregulated in cervical cancer, functions as a negative regulator of β-catenin

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The Wnt/β-catenin signaling pathway is activated during the malignant transformation of keratinocytes that originate from the human uterine cervix. Dkk1, 2 and 4 have been shown to modulate the Wnt-induced stabilization of the β-catenin signaling pathway. However, the function of *Dkk3* in this pathway is unknown. Comparison of the *Dkk3* gene expression profiles in cervical cancer and normal cervical tissue by cDNA microarray and subsequent real-time PCR revealed that the *Dkk3* gene is frequently downregulated in the cancer. Methylation studies showed that the promoter of *Dkk3* was methylated in cervical cancer cell lines and 22 (31.4%) of 70 cervical cancer tissue specimens. This promoter methylation was associated with reduced expression of Dkk3 mRNA in the paired normal and tumor tissue samples. Further, the reintroduction of Dkk3 into HeLa cervical cancer cells resulted in reduced colony formation and retarded cell growth. The forced expression of Dkk3 markedly attenuated β-catenin-responsive luciferase activity in a dose-dependent manner and decreased the β-catenin levels. By utilizing a yeast two-hybrid screen, βTrCP, a negative regulator of β-catenin was identified as a novel Dkk3-interacting partner. Coexpression with βTrCP synergistically enhanced the inhibitory function of Dkk3 on β-catenin. The stable expression of Dkk3 blocks the nuclear translocation of β-catenin, resulting in downregulation of its downstream targets (VEGF and cylcin D), whereas knockdown of Dkk3 abrogates this blocking. We conclude from our finding that Dkk3 is a negative regulator of β-catenin and its downregulation contribute to an activation of the β-catenin signaling pathway.

Key words: *Dkk*3; methylation; β-catenin; βTrCP; cervical cancer

Despite the significant benefits conferred by screening for cervical cancer, cervical cancer is responsible for 190,000 deaths annually worldwide and the leading cause of cancer mortality in women in developing countries.[1] Thus, there is a great need for improvements in cervical screening and targeted therapies. Such improvements depend, however, on a better understanding of the molecular biology of cervical neoplasia.

One possible focus of attention is the Wnt/β-catenin signaling pathway. Deregulated stabilization of β-catenin in colon carcinoma has been well known to be mainly caused by inactivation mutations of the adenomatous polyposis coli tumor suppressor gene or by activating mutations in exon 3 of the *β-catenin* gene that includes the glycogen synthase kinase-3 β phosphorylation site.[2,3] These mutations protect β-catenin from ubiquitination-proteosome pathway, in which phosphorylated β-catenin-Axin-adenomatous polyposis coli complex by glycogen synthase kinase-3 β is followed immediately by βTrCP recognition, thereby promoting rapid ubiquitination and degradation.[4] *β-catenin* is rarely mutated in cervical cancer cells, but nevertheless aberrant accumulation of β-catenin in the cytoplasm and/or nucleus has been found in cervical carcinoma samples.[5–7] In addition, Wnt/β-catenin signaling pathway is activated during the malignant transformation of keratinocytes that originate from the human uterine cervix.[8] These findings implicate the Wnt/β-catenin signaling pathway in the development of cervical cancer. Much remains to be discovered about the regulation of this pathway and the activation of β-catenin without mutation in cervical carcinoma.

Of the Dickkopf (Dkk) proteins that appear to provide critical molecular signals in development, Dkk1, 2 and 4 have been shown to modulate the Wnt signaling pathway by binding Wnt coreceptors and affect the β-catenin signaling.[9–11] Dkk1 blocks Wnt signaling during early Xenopus embryogenesis, which is required for head induction,[9] whereas Dkk2 activates the pathway.[12] However, the function of Dkk3 protein in Wnt/ β-catenin signaling pathway has not been defined.

Dkk3 was first cloned as a mortalization-related gene,[13] which means that loss of *Dkk3* may be involved in bypass cell senescence, a potentially critical step in the neoplastic transformation of cells. Indeed, *Dkk3* is downregulated in some cancer cell lines and cancer tissues including cancers of the liver, kidney, lung and prostate as well as in melanoma, and the transcriptional silencing is partly due to the aberrant hypermethylation of the *Dkk3* promoter.[13–18] The Dkk3 protein was found to inhibit the invasion and motility of osteosarcoma cells and melanoma.[18,19] Cell growth inhibition induced by the ectopic expression of Dkk3 was observed *in vitro* and *in vivo*.[15,17,20] These findings collectively indicate that Dkk3 possesses a tumor suppressor property, but the mechanism has not been confirmed.

In this study, we discovered that Dkk3 mRNA is frequently downregulated in association with promoter methylation in cervical cancer and that the overexpression of Dkk3 reduces colony formation and the growth of cervical cancer cells. We also found that Dkk3 attenuates β-catenin protein expression and its transcriptional activity by interacting with βTrCP and blocks the translocation of β-catenin into the nucleus. We conclude from these finding that the downregulation of Dkk3 may contribute to the activation of the β-catenin signal in cervical cancer and thus that *Dkk3* could be exploited as a therapeutic gene targeting β-catenin.

Material and methods

Human tissues and cell lines

Cervical tissue and uterine leiomyoma tissue samples were obtained and snap frozen in liquid nitrogen at The Department of

Additional Supporting Information may be found in the online version of this article.

Abbreviations: βTrCP, β-transducin repeat-containing protein; APC, adenomatous polyposis coli; BSA, bovine serum albumin; *Dkk*3, dickkopf homolog 3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK-3β, glycogen synthase kinase-3β; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor.

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Gynecology at the Samsung Medical Center, Seoul, Korea, with the approval of the Institutional Review Board. The histology and cellular composition of tissues were confirmed before RNA extraction. Cells of the 293 embryonic kidney cell line and HeLa, CaSki and HT3 cervical cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in Eagle's minimum essential medium (293 cells), Dulbecco's minimum Eagle's medium (HeLa and CaSki cells) or McCoy's 5a medium (HT3 cells) supplemented with 10% fetal bovine serum. All cells were kept at 37°C in a humidified atmosphere with 5% CO_2. The media were routinely changed every 3 days.

Total RNA isolation, reverse-transcriptase polymerase chain reaction and cDNA microarray

Total RNA was isolated with the Trizol reagent (Life Technologies, Gaithersburg, MD) as described by the manufacturer. About 0.5–1 μg of total RNA was reverse transcribed into cDNA using the Superscript II enzyme (Invitrogen-Gibco, Carlsbad, CA) and oligo (dT) (Invitrogen-Gibco). The resulting cDNA was used as the template for PCR amplification. For the reverse-transcriptase polymerase chain reaction of full-length *Dkk3*, the sense primer 5′-GAG CGA GCA GAT CCA GTC-3′ and antisense primer 5′-AGC CAT GTA GAA CAA ACG GC-3′ were designed. PCR consisted of an initial denaturing step at 95°C for 1 min, followed by 30 cycles of 95°C 30 sec/60°C 1 min/72°C 1 min, and a final extension step at 72°C for 7 min. The resulting fragments were resolved by 1% agarose gel electrophoresis and stained with ethidium bromide. cDNA microarray analysis was performed using the DNA chip (Genetrack Human 17 K cDNA chip; Genomictree Products, Taejon, South Korea) as previously described.[21]

Quantitative real-time PCR

TaqMan PCR was done on the iCycler iQTM Real-Time PCR Detection System (Bio-Rad) by using TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression probes (Applied Biosystems). The relative expression of Dkk3 mRNA was normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase in the same cDNA by using the standard curve method described by the manufacturer.

Analysis of methylation-status at the promoter regions

To analyze the methylation status of the promoter of the *Dkk3* gene, we used previously reported primers.[14] DNA (250 ng) was incubated with 20 units of *Msp*I, *Hpa*II or dH2O in 1× buffer at 37°C for 2 hr. The enzymes were then inactivated by heating at 70°C for 20 min. PCR was performed with the DNA polymerase GC-007-0250 (GeneCraft, Germany) in the presence of 5% DMSO at 95°C for 10 min, followed by 40 cycles of 95°C 1 min/65°C 30 sec/72°C 30 sec.

Colony formation assay

For the colony formation assay, HeLa cells (2×10^5 cells per well) were seeded in six-well tissue culture plates and then 24 hr later transfected with 0.5 μg of either pcDNA3.1(+)-Dkk3 or pcDNA3.1(−)-Dkk3. Selection for G418 (500 μg/ml)-resistant colonies was started 48 hr after transfection. Two weeks after seeding, colonies were stained with 0.05% crystal violet containing 50% methanol and counted.

Stable clone establishment

To establish stable cell lines that overexpress Dkk3, we transfected HeLa cervical cancer cells with a pcDNA3.1 expression vector encoding *Dkk3* cDNA using FuGENE6 reagent (Roche Diagnostics Corporation, Indianapolis, IN). Transfected cells were subsequently selected in the presence of G418 (500 μg/ml) for 3 weeks. The expression of Dkk3 clones was determined from western blots of culture media using an anti-Dkk3 antibody (R&D Systems). Established stable cells were maintained with antibiotics.

Western blot analysis

For the western blot analysis, cells were lysed for 30 min on ice in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet-P-40, 0.1 mM Na_2VO_3 and 1 mM NaF) containing freshly added protease inhibitor cocktail tablets (Roche, Mannheim Germany), and the lysates were cleared by centrifugation at 14,000 rpm for 15 min. Total proteins were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro-transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Chicago, IL). The membrane was blocked with 3% bovine serum albumin in 1× phosphate-buffered saline (PBS) containing 0.11% Tween 20 for 4 hr and then incubated overnight at 4°C with a 1:1,000 dilution of indicated antibodies. The membranes were washed 3 times for 15 min each with washing buffer (1× PBS containing 0.1% Tween 20) and incubated with the appropriate secondary antibody at room temperature for 1 hr. After three 15-min washes in washing buffer at room temperature, the membrane-bound proteins were detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ). Western blot bands were quantified using the NIH ImageJ software (NIH image Processing and Analysis in Java).

MTT cell proliferation assay

For the MTT cell proliferation assay, cells (2,000–3,000) were seeded in 24-well plates in triplicate. After 24, 48 or 72 hr, 100 μl of MTT was added to each well of cells and the plate was incubated for 4 hr at 37°C. The medium was removed, and the MTT crystals were solubilized in DMSO, after which the spectrophotometric absorbance of each sample was measured at 570 nm using a fluorometer (Wallac Victor; Perkin-Elmer Life Sciences, Boston, MA).

Immunofluorescent staining and confocal microscopy

For immunofluorescent staining, 293 cells (10^5 cells/chamber) or HeLa cells (10^4 cells/chamber) were plated on 4-well chamber slides, fixed with 4% paraformaldehyde in PBS at room temperature for 30 min, and then permeabilized with 0.1% Triton X-100 in PBS at 4°C for 25 min. After this, the cells were washed in PBS at room temperature, blocked with 1% bovine serum albumin in PBS for 1 hr, and then incubated with appropriate primary antibodies (1:500 dilutions) at room temperature for 1 hr. The cells were rinsed with PBS and incubated with Alexa Fluor (1:1,000 dilutions) (Molecular Probes, Eugene, OR). The expression and localization of the proteins were observed under a confocal microscope (BioRad, Hertfordshire, United Kingdom).

Dual-luciferase assay

The dual-luciferase assay was performed using a dual-luciferase reporter assay kit (Promega, Madison, WI). Each experiment was performed in triplicate. Briefly, cells were harvested and dissolved in 40 μl of 1× passive lysis buffer (Promega, Madison, WI). Lysates were cleared by centrifugation at 14,000 rpm for 15 min, and 10 μl of each cell extract was transferred to a 96-well assay plate containing 50 μl/well of the provided Luciferase Assay Reagent II. The provided Stop and Glo Reagent (50 μl/well) was then added to initiate *Renilla* luciferase activity, and the ratio of firefly luciferase activity to *Renilla* luciferase activity was calculated.

Yeast two-hybrid analysis

For bait construction with human *Dkk3*, cDNA encoding full-length human *Dkk3* was sub-cloned into the *Eco*RI and *Xho*I restriction sites of the pGilda. The resulting plasmid pGilda-*Dkk3* was introduced into yeast strain EGY48 [*MATα, his3, trp1, ura3-52, leu2: pLeu2-LexAop6*/pSH18-34 (*LexAop-lacZ* reporter)] by a modified lithium acetate method.[22] The cDNAs encoding B42 fusion proteins were introduced into the competent yeast cells that already contained pGilda-*Dkk3*, and the tryptophan prototrophy (plasmid marker) transformants were selected for on a synthetic

medium (Ura$^-$, His$^-$, Trp$^-$) containing 2% glucose. We tested their interactions with pGilda-*Dkk3* on a medium containing 5-bromo-4-chloro-3-indolyl-*β*-D-galactoside as described. Then *β*-galactosidase activity was measured by adding 140 *μ*l of 4 mg/ml *o*-nitrophenyl *β*-D-galactopyranoside.[23] The *β*-galactosidase activity was calculated using the formula units= $[1{,}000 \times (A_{420} - 1.75 \times A_{550})]/(\text{time} \times \text{volume} \times A_{600})$.

Small interfering RNA (siRNA) transient transfection

The siRNA oligonucleotide sequence targeting *Dkk3* was purchased from Santa Cruz and then 100 nM of *Dkk3* siRNA was used to knockdown the expression of Dkk3 using an Oligofectamin reagent (Invitrogen) according to the manufacturer's instructions. The scrambled siRNA was used as the control. Silencing of Dkk3 expression was confirmed by western blot analysis with anti-Dkk3 antibody.

Subcellular fractionation

Subcellular fractionation was performed using the ProteoExtract™, Subcellular Proteome Extraction Kit from Calbiochem (Nottingham, United Kingdom) according to the manufacturer's directions.

Chemicals and antibodies

Leptomycin B and LiCl were purchased from Sigma Chemical (St. Louis, MO). Antibodies to the following proteins were used for the western blot analysis: anti-tubulin, lamin B, HA, His, VEGF, SOX9, GFP and cyclin D1, all from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Flag from Sigma; anti-β-catenin from Abcam; and anti-*Dkk*3 from R&D Systems.

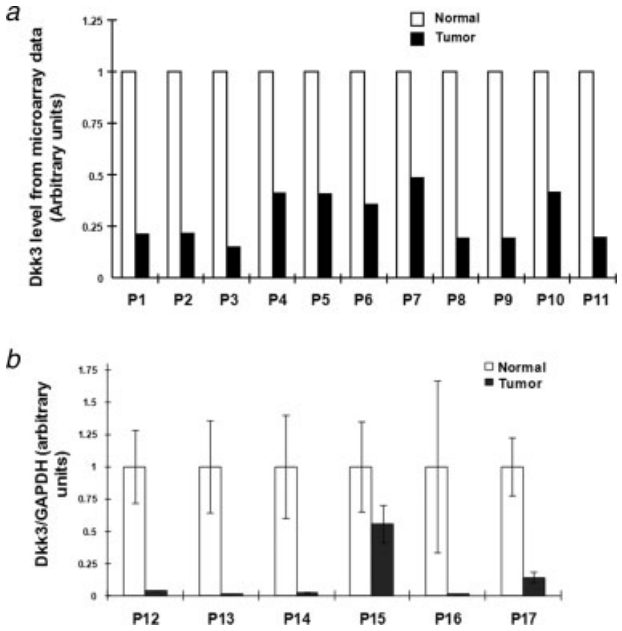

Figure 1 – Downregulation of Dkk3 mRNA in cervical cancer. (*a*) Eleven paired normal and cancer tissue specimens (P1–P11) were used in a cDNA microarray analysis. Dkk3 mRNA was markedly downregulated in all cervical cancer specimens compared with paired normal cervical tissue specimens. (*b*) Another 6 paired samples (P12–P17) were used in real-time PCR. Dkk3 mRNA was significantly downregulated in 5 of 6 cervical cancer tissues compared with normal cervical tissues.

Results

Dkk3 is frequently downregulated in cervical cancer

To identify the important genes involved in cervical cancer, we performed a cDNA microarray analysis of 11 paired samples of normal and cancerous cervical tissues. We found that *Dkk3* was markedly downregulated in all cervical cancer samples when compared with normal cervical samples (Fig. 1*a*). We then confirmed this finding by performing quantitative real-time PCR with *Dkk3*-specific primers in another 6 paired normal and cancerous cervical tissue samples. This showed that *Dkk3* was significantly downregulated in 5 of 6 cervical cancer samples when compared with normal cervical samples (Fig. 1*b*). These data thus showed frequent downregulation of *Dkk3* in cervical carcinoma when compared with normal cervical tissue.

Single-nucleotide polymorphism in codon 335 of Dkk3

We next checked whether *Dkk3* is repressed by intragenic mutations. For this study, we extracted genomic DNA from 3 cervical cancer cell lines and 70 cervical cancer specimens. We used previously reported primer pairs[11] for each coding exon and directly sequenced the PCR products of all coding exons. We did not find any intragenic mutations except a single-nucleotide change, GGG/AGG at codon 335 (exon 8), which resulted in an amino acid substitution from glycine to arginine. HeLa and CaSki cells were homozygous for guanine, and HT3 cells were heterozygous. Among 70 patient samples, 42 (60%) were homozygous for guanine, 3 (4.3%) were homozygous for adenine and 25 (35.7%) were heterozygous. The same findings were made in paired normal tissue specimens of 22 cancer samples, which indicate that the nucleotide change is a genetic polymorphism (data not shown). However, the genotype frequency of the polymorphism did not differ from that noted in the healthy population,[14] meaning that this single nucleotide polymorphism is not contributing factor to develop cervical cancer.

Methylation at the promoter region of Dkk*3 with transcriptional repression is frequent in cervical cancer*

Because the cervical cancer cell lines and tissues had no mutation, and because it has been widely documented that methylation in the promoter regions is a powerful mechanism of transcriptional repression and an alternative means for the inactivation of tumor-suppressor genes such as *retinoblastoma gene*, *von Hippel-Lindau gene* and *p16*,[24–26] we next investigated the methylation status of the promoter region of the *Dkk3* gene by combining the use of methylation-sensitive restriction enzymes and PCR.[27] We digested genomic DNAs either with *Msp*I, which cleaves the CpG regardless of the methylation status, or with *Hpa*II, which cleaves only unmethylated CpG. We then performed PCR with 3 pairs of primers designed for the promoter regions of the *Dkk3* gene.[14] The PCR amplification band from the *Hpa*II-cleaved DNA shows the methylation status. We also used *Msp*I-cleaved DNA as a negative control for detecting incomplete restriction and noncleaved DNA as a positive control.

We detected methylation at the promoter of *Dkk3* in HeLa and CaSki cells, which express *Dkk3* at very low levels, whereas we detected no methylation at this promoter in HT3 cells, which express *Dkk3* at high levels (Figs. 2*a* and 2*b*). We also found that *Dkk3* promoter was methylated in 22 of 70 (31.4%) tumor specimens from patients with cervical cancer (Fig. 2*c*). To determine whether promoter methylation is associated with the downregulation of *Dkk3*, we selected 9 patients showing methylation and 13 patients not showing methylation, for whom total RNA from normal or tumor tissues was reserved. Real-time PCR with *Dkk3*-specific primers showed that the reduction of Dkk3 mRNA in cancer when compared with normal is more significant in the patients showing methylation than in the patients not showing methylation ($p < 0.01$, Wilcoxon 2-sample test) (Fig. 2*d*). This result indicates that methylation of the promoter sequence accounts for the transcriptional repression of *Dkk3* in cervical cancer.

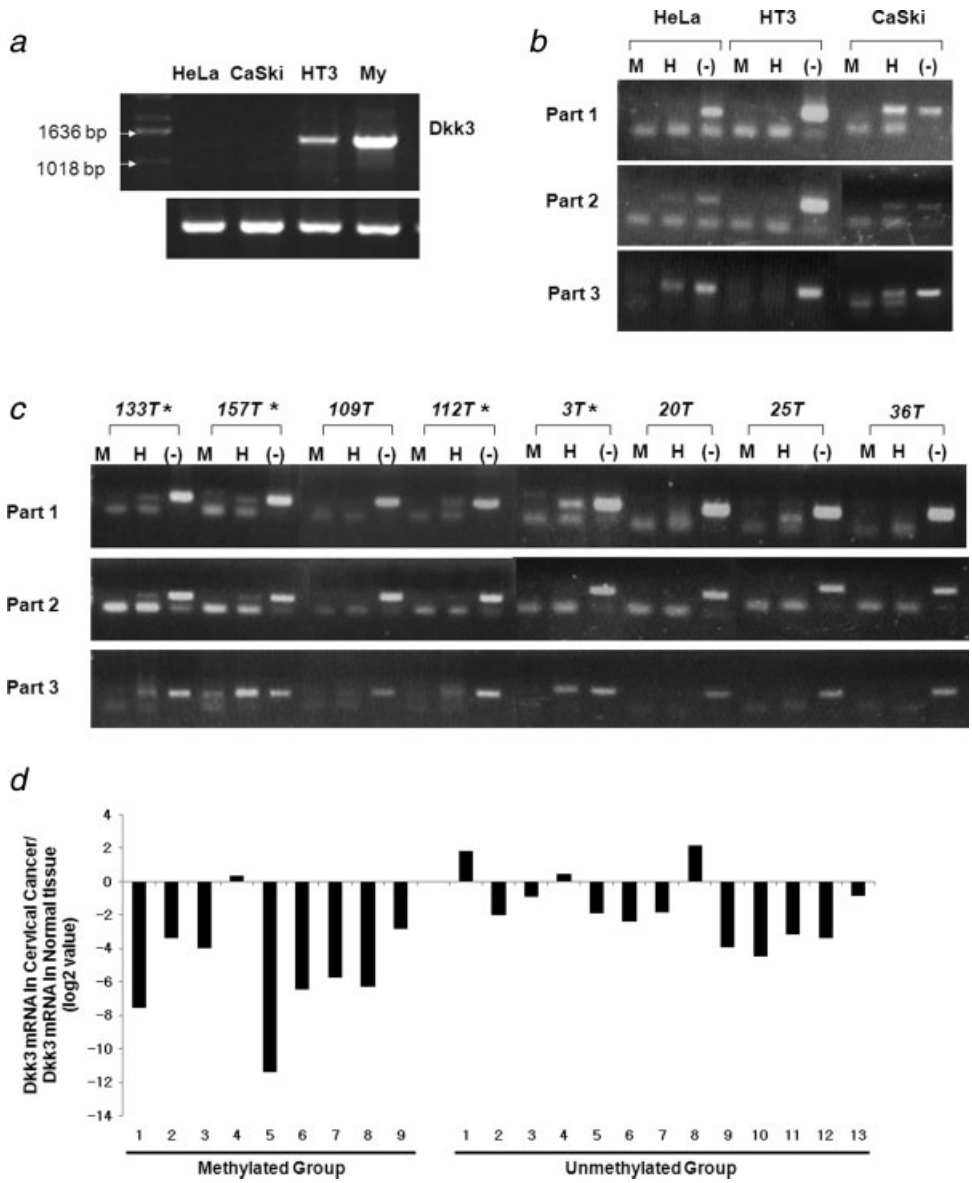

FIGURE 2 – Methylation at the promoter region of *Dkk3* is frequent and causes transcriptional repression in cervical cancer. (*a*) Endogenous expression of Dkk3 mRNA in 3 cervical cancer cell lines was assessed using RT-PCR with primers targeting the full sequence. Dkk3 mRNA was little expressed in HeLa and CaSki cells but relatively abundant in HT3 cells. Human normal uterine myometrial tissue (My) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used for a positive control and a loading control, respectively. (*b*) The combination of methylation-sensitive restriction enzymes and PCR with 3 paired primers (Parts 1–3) designed for the promoter region of *Dkk3* was used for the promoter methylation study. HeLa cells showed methylation at Parts 2 and 3, and CaSki cells showed methylation in all 3 parts of the *Dkk3* promoter region. However, no methylation was detected in HT3 cells. (M, *Msp*I-digested DNA; H, *Hpa*II-digested DNA; -, undigested DNA). (*c*) The methylation status of the *Dkk3* promoter region was determined in genomic DNA extracted from 70 cervical cancer tissue specimens. Twenty-two of 70 (31.4%) patients showed heavy methylation in the *Dkk3* promoter region. Shown are representative data from the methylation study in cervical cancer tissue specimens. (* means sample showing methylation). (*d*) Quantitative real-time PCR with *Dkk3*-specific primer was performed in 9 samples showing promoter methylation and 13 samples not showing promoter methylation, for which we had both normal and cancerous tissue specimens. The data shows that Dkk3 mRNA in cancerous cells was more reduced when compared with normal cells in the methylation group than in the unmethylation group (Wilcoxon 2-sample test, $p < 0.01$).

For more detailed methylation analysis, we prepared additional new 17 normal cervical tissues from the patients undertaken hysterectomy due to benign diseases such as leiomyoma and 28 cervical cancer tissues from advanced staged patients (Stage Ib2–IIb) to obtain enough samples for extracting both total RNA and genomic DNA with minimizing contamination from normal tissues. We performed pyrosequencing as described in legend of supplement Figure 2 and real time PCR. This result showed higher methylation status (Mann-Whitney test with Bonferroni's correction, $p < 0.005$ at CpG 1–4) along with lower expression of Dkk3 mRNA in cancer than that in normal tissue samples (Fig. S2). We did not find any correlation between the methylation status in the CpG sites analyzed in this study and mRNA expression of Dkk3 in cancer tissues samples.

Dkk3 overexpression leads to growth inhibition of cervical cancer cells

We next investigated whether Dkk3 acts as a tumor suppressor of cervical cancer cells by performing a colony formation assay.

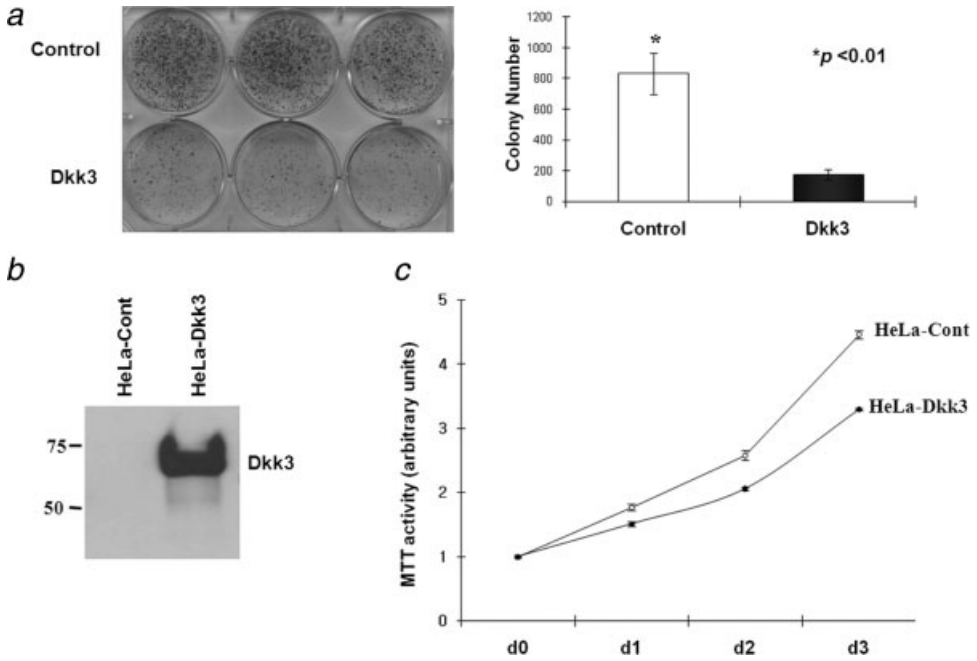

FIGURE 3 – Dkk3 has antiproliferative activity in cervical cancer cells. (*a*) Colony formation assay using HeLa cervical cancer cells showed that the colony formation activity of the Dkk3-transfected cells was markedly less (mean 133 colonies/dish) than that in the control vector-transfected cells (mean 832 colonies/dish) (*t*-test, $*p < 0.01$). (*b*) Western blotting shows Dkk3 protein expression in the culture medium from HeLa-Cont and HeLa-Dkk3 stable cells. Cells were plated in a 100-mm culture dish and incubated for 2 days (80–90% confluence). Medium was replaced with 5 ml of OPTI-MEM (serum free), after which the cells were incubated for 24 hr and immunoblotted using the anti-Dkk3 antibody. (*c*) Equal numbers of HeLa-Cont and HeLa-Dkk3 stable cells were plated in 6-well plates and the number of live cells was quantified by MTT assay for 3 days.

For this study, we used HeLa cervical cancer cells, which have little endogenous *Dkk3*, that had been transfected with either *Dkk3* or control vector. The result showed that the colony formation activity of the Dkk3-transfected HeLa cells was markedly lower (mean 133 colonies/dish) than that of the control vector-transfected HeLa cells (mean 832 colonies/dish) (Fig. 3*a*)

To confirm the colony formation assay findings, we next established a Dkk3-overexpressing stable HeLa cell line (HeLa-Dkk3) and control vector-stable HeLa cell line (HeLa-Cont) and examined whether the levels of Dkk3 protein secreted into the culture media were greater for the HeLa-Dkk3 cells than for the HeLa-Cont cells. This showed that indeed the culture media of HeLa-Dkk3 cells contained Dkk3, but not the media of HeLa-Cont cells (Fig. 3*b*). We did not observe any morphological changes in HeLa-Dkk3 cells when compared with HeLa-Cont cells. However, the MTT assay showed that the HeLa-Dkk3 cells showed growth retardation when compared with the HeLa-Cont cells (Fig. 3*c*). Together, these data showed that the Dkk3 protein suppresses the tumor growth.

Dkk3 attenuates the transcriptional activity of β-catenin

It is fairly well established that the Dkk proteins are involved in the regulation of the Wnt/β-catenin signaling pathway. To determine whether Dkk3 affects β-catenin, we used a dual-luciferase reporter assay kit with the β-catenin-responsive luciferase vectors pGL3-OT or pGL3-OF (containing wild-type or mutant TCF sites, respectively).[28] We then cotransfected an increasing amount of plasmids encoding *Dkk3* together with a constant amount of the β-catenin expression vector into 293 cells and measured the resulting luciferase activities. Our results revealed that Dkk3 significantly attenuated the transcriptional activity of β-catenin in a dose-dependent manner (Fig. 4*a*). To confirm this finding in HeLa stable cells, we induce endogenous β-catenin using LiCl, a phosphate inhibitor because basal level of β-catenin in HeLa cells was very low (data not shown). We first transfected pGL3 luciferase reporter vectors containing β-catenin-response promoter sequences into HeLa-Dkk3 and HeLa-Cont cells, treated the cells with LiCl for 12 hr and then measured the luciferase activity. We detected double the luciferase activity in the LiCl-treated HeLa-Cont cells than in the nontreated HeLa-Cont cells. In contrast, we did not observe any increase in the luciferase activity in HeLa-Dkk3 cells (Fig. 4*b*). This finding suggests that β-catenin is unlikely to have transcriptional activity in Dkk3-overexpressing cells. We conclude from these findings that Dkk3 negatively regulates the transcriptional activity of β-catenin.

Dkk3 reduces the protein expression of β-catenin

We then assessed whether Dkk3 affects the expression of β-catenin. In this experiment, we cotransfected Flag-Dkk3 with His-β-catenin into 293 cells and harvested the cells after 24 and 72 hr. Western blotting showed that the total level of expression of β-catenin was reduced by Dkk3 (Fig. 5*a*). The data from the fractionation of the cytoplasmic and nuclear compartments showed that this was markedly decreased in the cells transfected by Dkk3 (Fig. 5*b*). To assess whether Dkk3 is able to affect the endogenous β-catenin level, we induced the endogenous β-catenin with LiCl in the 293 cells and then fractionated the cytoplasmic, nuclear and cell membrane compartments. This showed that the Dkk3 reduced the cytoplasmic and nuclear levels of β-catenin but not the level in the cell membrane (Fig. 5*c*). We performed confocal microscopy to confirm this finding. We transfected the *Dkk3* plasmid into the 293 cells and then after 24 hr, treated the cells with LiCl for 12 hr. Coimmunostaining with anti-β-catenin and anti-Flag and subsequent confocal microscopy showed that β-catenin was not expressed in the Dkk3-transfected cells (Fig. 5*d*). In addition, we examined whether Dkk3 can reduce β-catenin expression by the paracrine mechanism of secreted Dkk3. We incubated HeLa cells for 24 hr with conditioned medium containing secreted Dkk3 from

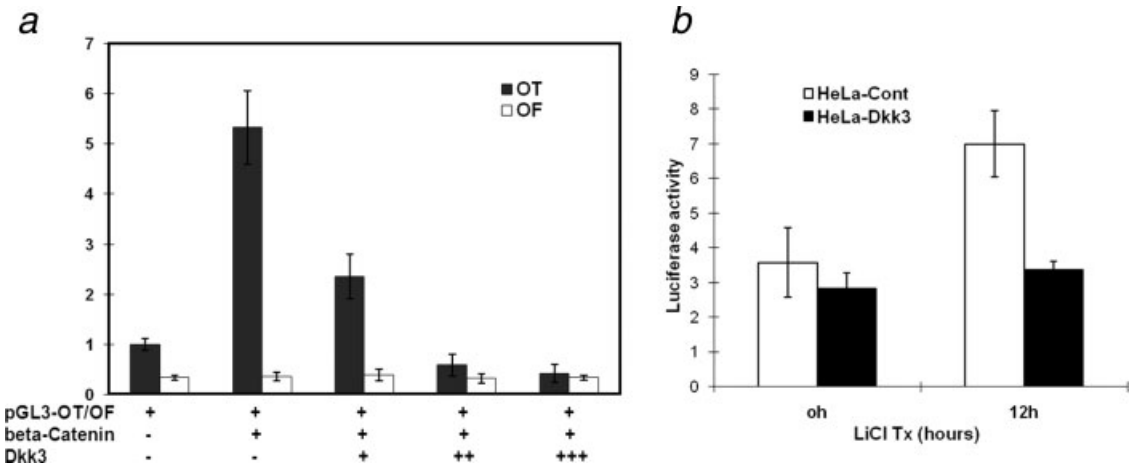

FIGURE 4 – Dkk3 attenuates the transcriptional activity of β-catenin. (*a*) The 293 cells were grown in 6-well plates and cotransfected with β-catenin (0.5 μg), Dkk3 (1.0, 2.0 and 3.0 μg), the pGL3-OT or pGL3-OF reporter plasmid (0.1 μg) and pRL containing the Renilla luciferase cDNA (0.05 μg). Twenty-four hours after transfection, the cells were harvested and the transcriptional activity measured using a dual-luciferase reporter assay kit. The Dkk3 attenuated the TCF-responsive luciferase activity in a dose-dependent manner without producing any change in the mutant pGL3-OF activity. (*b*) HeLa-Cont and HeLa-Dkk3 stable cells were plated in 6-well plates and cotransfected with β-catenin (1.0 μg), Dkk3 (1.0 μg), the pGL3-OT reporter plasmid (0.5 μg) and pRL (0.05 μg). Twenty-four hours after transfection, the cells were treated with LiCl for 12 hr and harvested. The luciferase activity was not changed in HeLa-Dkk3 cells after the activation of β-catenin by LiCl in contrast to HeLa-Cont cells, in which luciferase activity was doubled.

HeLa-Dkk3 cells, and then treated the cells with LiCl for 12 hr. The results showed that the cytoplasmic and nuclear levels of the β-catenin protein were dramatically reduced (Fig. 5*e*). Thus these results indicate that secreted Dkk3 also facilitates β-catenin evacuation from the cytoplasm and nucleus.

Dkk3 attenuates β-catenin by interacting with βTrCP

We screened a human ovary cDNA library using full-length *Dkk3* cDNA fused to the pGilda DNA-binding domain as bait to identify proteins that have direct interactions with Dkk3. From this screen, several independent clones containing fragment of βTrCP were identified. Positive clones detected in the primary screen were confirmed by β-galactosidase two-hybrid interaction assays (Fig. 6*a*). To further verify the interaction between Dkk3 and βTrCP that we observed in the yeast two-hybrid assays, we performed coimmunoprecipitation and confocal microscopic analysis. Flag-Dkk3 and HA-βTrCP expression plasmids were cotransfected into 293 cells using Fugene transfection reagent. Forty-eight hours after transfection, cells were harvested and lysed to yield the cell extract. Flag-Dkk3 was immunopurified from the cell extract using anti-Flag antibody, and the bound proteins were probed with anti-HA antibody. Flag-Dkk3 interacts with HA-βTrCP, whereas Flag alone did not (Fig. 6*b*). In addition, the confocal microscopy analysis revealed the colocalization of Dkk3 with βTrCP at cytoplasm (Fig. 6*c*). Because Dkk3 has been known to be at the Golgi apparatus in accordance with secreted protein,[29] we assessed whether Dkk3 is also present in the cytosolic fraction (Fig. S3). When we divided cell lysate into cytoplasmic and nuclear fraction, multiple forms of Dkk3 were detected in the cytoplasmic fraction. The upper band could be a glycosylated band because this form is secreted into medium.[29] When we further fractionated the cytoplasmic proteins into cytosolic and microsomal part, we found that the upper and middle bands are localized in the microsomal portion and lower band is localized in the cytosolic fraction. We thereby confirmed that Dkk3 is present in the cytosol, where Dkk3 could bind with βTrCP. These data collectively indicates that Dkk3 interacts with βTrCP *in vivo*.

Because the βTrCP is involved in β-catenin degradation, we examined whether Dkk3 affects β-catenin *via* interaction with βTrCP. Transient expression of Dkk3 and βTrCP synergistically inhibited β-catenin dependent transcription and also downregulated β-catenin expression suggesting the interaction of Dkk3 with βTrCP is relevant to the inhibition of β-catenin signaling (Fig. 6*d*).

Dkk3 blocks the nuclear transport of β-catenin

We also checked the protein level of β-catenin in the HeLa-Dkk3 and HeLa-Cont cells. In this experiment, we induced endogenous β-catenin expression with LiCl and then fractionated the cytoplasmic and nuclear compartments. Interestingly, cells expressing Dkk3 showed no detectable expression of the β-catenin protein in the nucleus in contrast with the control cells (Fig. 6*a*). We surmised from this that Dkk3 inhibited the nuclear transport of β-catenin. To test this hypothesis, we treated HeLa-Dkk3 and HeLa-Cont cells with LiCl and then with leptomicin B, a specific inhibitor of nuclear export.[24] Consistent with the western blotting results, we did not observe the nuclear accumulation of β-catenin in the HeLa-Dkk3 cells (Fig. 6*b*). To confirm that the β-catenin was not actually working in the Dkk3-overexpressing cells, we assessed the protein expression of *VEGF* and *cyclin D1*, target genes of β-catenin. Western blotting showed the marked downregulation of VEGF and cyclin D1 (Fig. 6*c*).

We then knocked down Dkk3 protein using specific *Dkk3* siRNA to confirm whether Dkk3 is directly involved in the inhibition of the nuclear translocation of β-catenin in cervical cancer cells. Downregulation of Dkk3 by siRNA could recover the capacity of nuclear transportation of β-catenin in HeLa-Dkk3 stable cells. Likewise, β-catenin induced by LiCl was not accumulated in the nucleus in HeLa-Dkk3 cells. In contrast, LiCl-induced β-catenin in the HeLa-Dkk3 cells transfected with siRNA was shifted to the nucleus (Fig. 7*d*). These findings therefore indicate that Dkk3 disables β-catenin by blocking nuclear transport and provide a clue to understand the observation of the aberrant nuclear accumulation of β-catenin in invasive cervical carcinoma samples.

Discussion

We have presented the findings that the *Dkk3* gene is frequently downregulated in cervical cancer and associated with hypermethylation of its promoter. We propose that the resultant downregulation of the *Dkk3* gene is responsible for the activation of the Wnt/

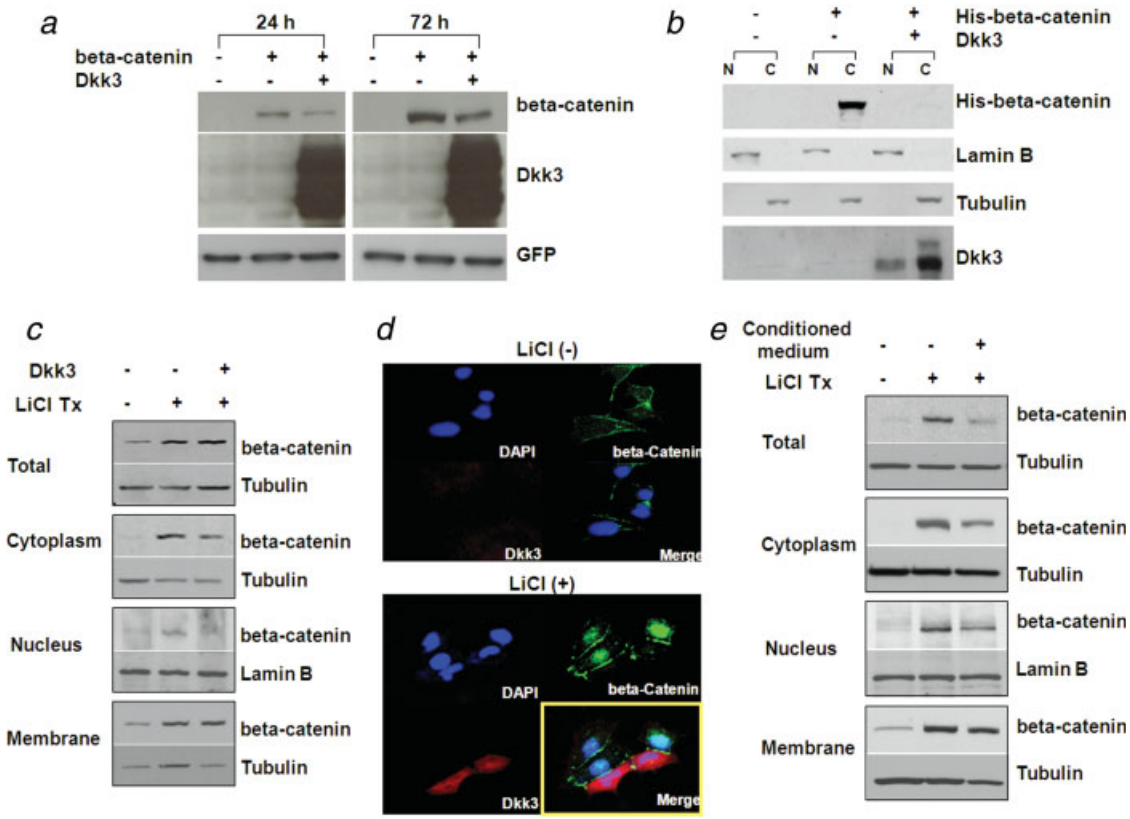

FIGURE 5 – Dkk3 reduces expression of the β-catenin protein. (*a*) The 293 cells were transiently transfected with plasmid encoding His-β-catenin, Flag-Dkk3, and GFP. The cells were harvested 24 and 72 hr after transfection. The protein extracts were resolved in 8% SDS-PAGE and immunoblotted using antibodies against His, Flag, and GFP. This showed that the total level of expression of β-catenin was markedly decreased in the cells transfected with Dkk3. GFP was used as a loading control. (*b*) The 293 cells were harvested 24 hr after transient transfection with the plasmid encoding His-β-catenin and pcDNA3.1(+)-*Dkk3* and fractionated into nuclear (N) and cytoplasmic (C) compartments. Protein extracts were resolved in 10% SDS-PAGE and immunoblotted using antibodies against His, lamin B, tubulin and Dkk3. Western blotting showed that the transfected β-catenin was mainly localized in the cytoplasm and that this was markedly decreased by Dkk3. Complete fractions of cytoplasmic and nuclear proteins were verified by western blotting with antibodies against tubulin and lamin B, respectively. (*c*) Around 293 cells were transfected with either the empty vector or the pcDNA3.1(+)-*Dkk3* vector. Twenty-four hours later, cells were treated with LiCl for 12 hr and harvested. Total, cytoplasmic, nuclear and cell membrane proteins were resolved in 8% SDS-PAGE and immunoblotted using antibody against β-catenin. The level of β-catenin in the cytoplasm and nucleus, but not in the cell membrane, was reduced by Dkk3. (*d*) Firstly, we stained 293 cells with the anti-β-catenin antibody to assess the endogenous β-catenin distribution. Another batch of 293 cells were transfected with Flag-Dkk3, incubated for 24 hr and treated with LiCl for 12 hr. Immunofluorescent staining and confocal microscopy showed that endogenous β-catenin is mainly distributed in the cell membrane, but in response to stimulation with LiCl, endogenous β-catenin is localized in the nucleus. However, Dkk3-expressing cells treated with LiCl have no β-catenin in the nucleus. (*e*) HeLa cells were treated with conditioned medium from HeLa-Dkk3 stable cells for 24 hr followed by treatment with LiCl for 12 hr and then prepared for subcellular fractionation. Total, cytoplasmic, nuclear and cell membrane proteins were immunoblotted using anti-β-catenin, anti-tubulin and anti-lamin B antibodies. Western blotting showed that the cytoplasmic and nuclear levels of the β-catenin protein were markedly reduced. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

β-catenin signaling pathway that contributes to the tumorigenesis of cervical cancer. Transcriptional inactivation of *Dkk3* associated with promoter hypermethylation has been observed in cancer tissues, including acute lymphoblastic leukemia, nonsmall-cell lung cancer, prostate cancer and bladder cancer,[14,30–32] indicating that the *Dkk3* gene may be a frequent target for methylation and silencing in cancer.

In this study, we did not find any correlation between the methylation status in the CpG sites analyzed by pyrosequencing and mRNA expression of Dkk3 in cancer tissues samples. This analysis has a limitation, in which we failed to compare Dkk3 mRNA expression between paired normal and tumor samples. Because of the expression variations in normal tissue samples across individuals shown in Fig S1, the comparison of an absolute level of Dkk3 mRNA expression among individual tumor samples, not between normal and tumor samples in each patient, was not possible to define correlation between methylation status and Dkk3 mRNA expression. Also there is another possibility that the methylation of CpG sites analyzed in this study may not be responsible to the transcriptional repression of Dkk3.

Dkk3 has different functions in various cancer cells. Our study showed that Dkk3 possesses antiproliferative activity against cervical cancer cells, similar to observations made in a part of prostate cancer cell lines[17] and in hepatoma cell lines.[15] This is contrary to the finding in the Mel Im melanoma cell line[18] and Saos-2 sarcoma cell line,[19] in which Dkk3 reduced cell migration and the invasion capacity but had no effect on proliferation. These findings indicate that Dkk3 has a tissue-specific function in human tumors but that it has an antagonistic effect in common in tumor cells, suggesting that *Dkk3* is a tumor suppressor gene.

So far, it is known that Dkk proteins play a role in a wide range of normal and pathological developmental processes, including cancer, by modulating Wnt signaling through its direct binding with Wnt coreceptors of the lipoprotein receptor-related protein 5/6 class.[33,34] However, because the identification of the involvement of Dkk3 in the Wnt/β-catenin signaling pathway as well as

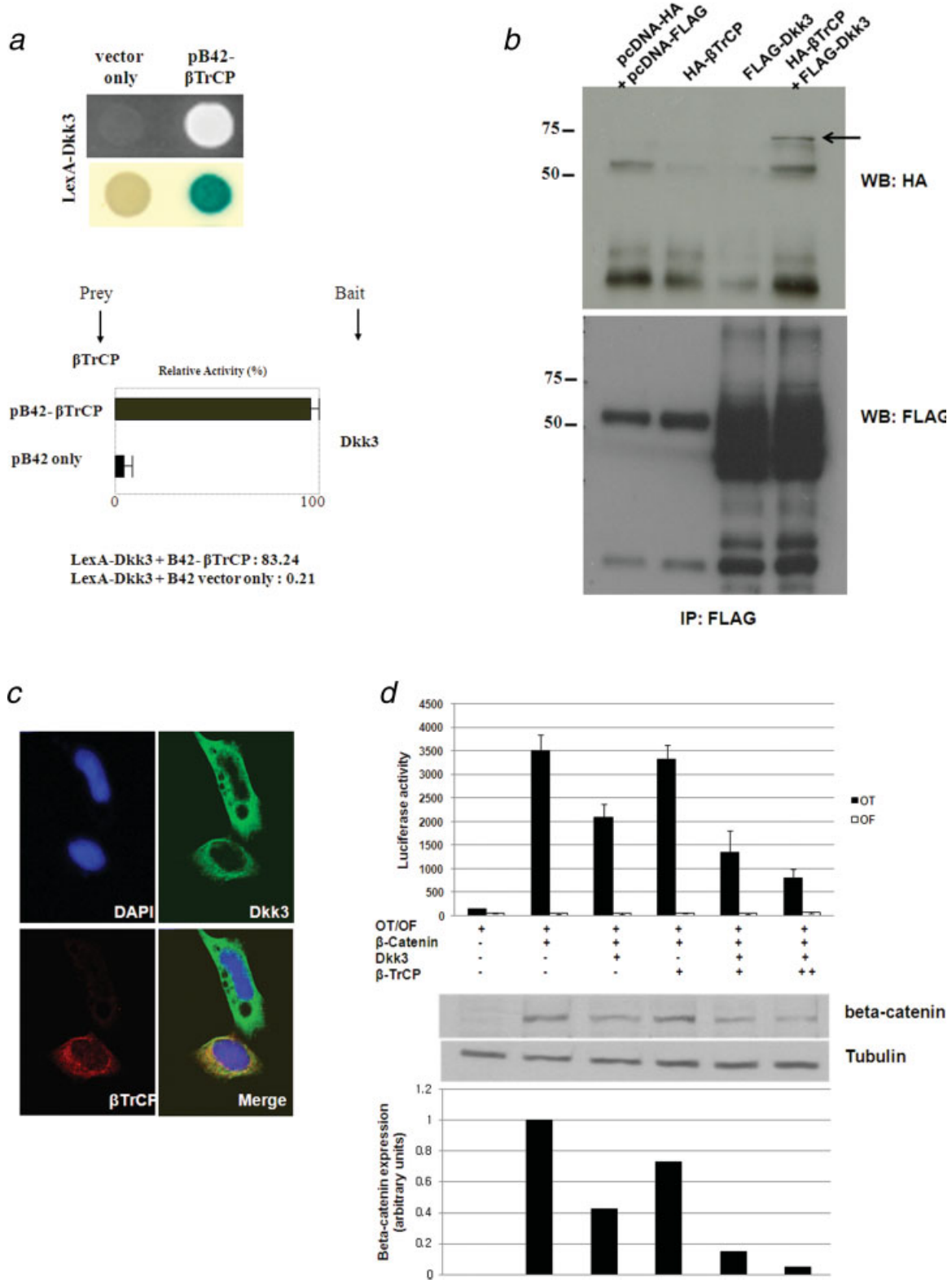

FIGURE 6 – Dkk3 interacts with βTrCP. (*a*) The full-length human *Dkk3* cDNA and either plasmid containing an empty vector or a full-length human *βTrCP* cDNA were cotransformed into EGY48 yeast cells and positive interactions were revealed by cell growth for 3 days at 30°C on medium lacking leucine as well as by the formation of blue colonies on medium containing X-gal (upper panel). Then, *β*-galactosidase activity was measured by adding ONPG (low panel). (*b*) Coimmunoprecipitation of Flag-Dkk3 and HA-*β*TrCP. Proteins immunoprecipitated with anti-FLAG antibody were analyzed by western analysis with anti-HA antibody (IP, immunoprecipitation; WB, immunoblotting). (*c*) Around 293 cells were transiently cotransfected with plasmid encoding FLAG-Dkk3 and HA-*β*TrCP. Immunofluorescent staining followed by confocal microscopic analysis revealed colocalization of 2 proteins in the cytoplasm. (*d*) The 293 cells were grown in 6-well plates and triply transfected with genes indicated. Twenty-four hours after transfection, the cells were harvested. The transcriptional activity and protein expression of *β*-catenin were measured using a dual luciferase reporter assay kit and western blot respectively. Dkk3 and *β*TrCP synergistically attenuates the luciferase activity and reduces *β*-catenin protein expression. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

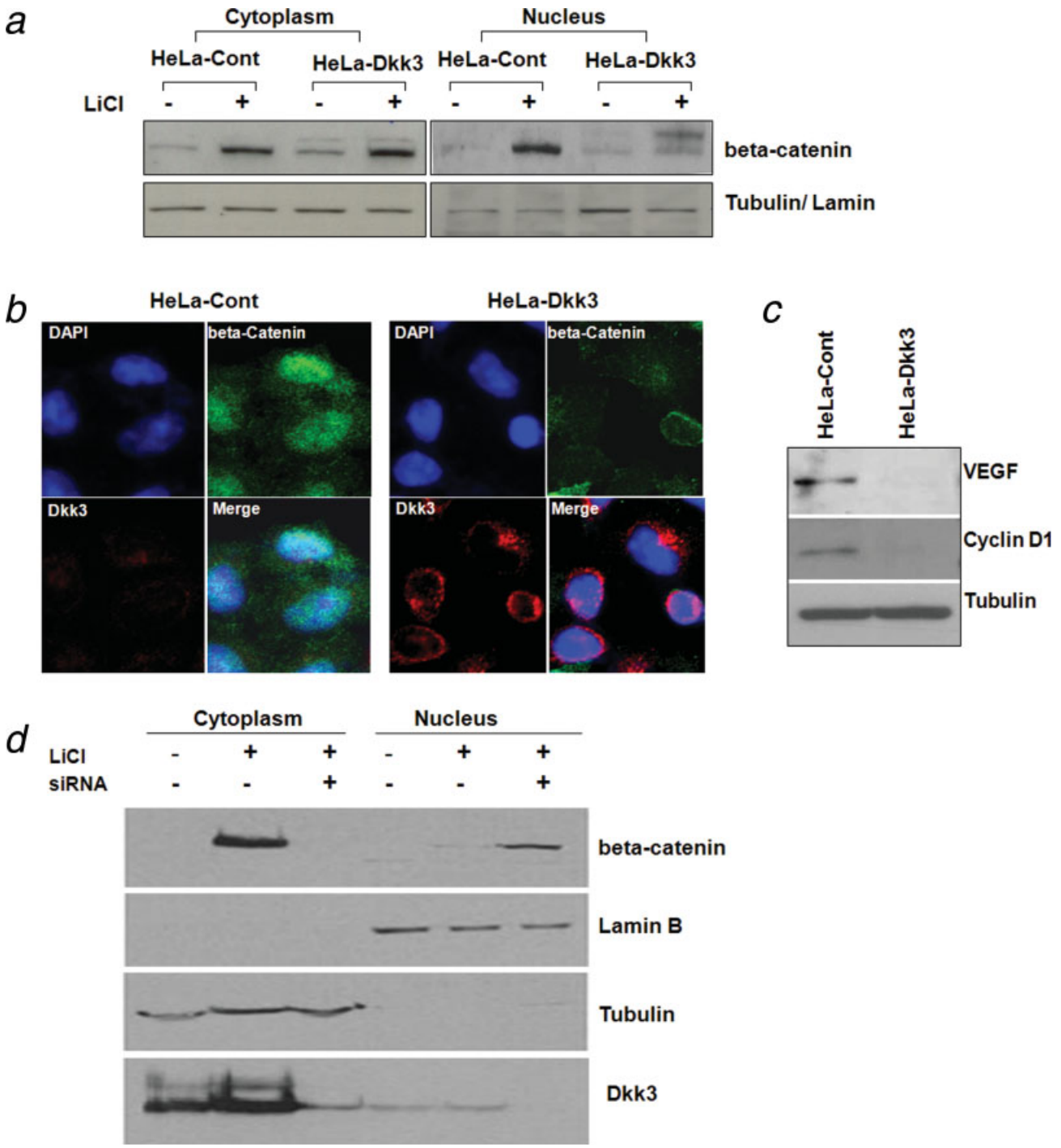

FIGURE 7 – Dkk3 blocks the nuclear translocation of β-catenin. (*a*) HeLa-Cont and HeLa-Dkk3 stable cells were treated with LiCl for 12 hr and then harvested. Cytosolic and nuclear fractions were prepared. Protein extracts were resolved by 8% SDS-PAGE and immunoblotted using the antibodies indicated in the figure. The endogenous level of β-catenin in the cytoplasm and nucleus of HeLa-Cont stable cells was induced by LiCl. However, HeLa-Dkk3 stable cells showed no detectable expression of the β-catenin protein in the nucleus. Tubulin and lamin B were used as a loading control for cytoplasmic and nuclear protein, respectively. (*b*) HeLa-Cont and HeLa-Dkk3 stable cells were treated with LiCl for 8 hr followed by treatment with leptomycin B for 3 hr, after which immunofluorescent staining followed by confocal microscopy was performed. Nuclear β-catenin was not observed in the HeLa-Dkk3 stable cells. (*c*) Western blotting showed that VEGF and cyclin D1, the proteins on the downstream pathway of β-catenin were downregulated in HeLa-Dkk3 stable cells. (*d*) SiRNA was transfected into HeLa-Dkk3 stable cells, 24 hr after transfection LiCl was treated for 12 hr and then cytoplasmic and nuclear compartments were fractionated. LiCl-induced β-catenin was observed in cytoplasm, not in nucleus of HeLa-Dkk3 cells, while β-catenin was accumulated in the nucleus of the HeLa-Dkk3 cells having knock-down of *Dkk3* with siRNA transfection. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

direct interaction between Dkk3 and these coreceptors have been failed, and it has been suggested that *Dkk3* is distinct from other *Dkk* family members.[11,34–36] Recently, a few pieces of evidence of a relationship between the Wnt signaling pathway and Dkk3 have been reported. For example, Dkk3 was found to have an antagonistic effect on WNT7A activity in a luciferase assay.[37] Dkk3 was also observed to induce the translocation of β-catenin, a gene activated by Wnts, into the cell membrane of sarcoma cells.[19] Further, involvement of C-jun kinase activation, which can be caused by the Wnt-triggered planar cell polarity pathway[38] was observed in the Dkk3-induced apoptosis of prostate cancer cells.[17] Although mostly its effect is inhibitory, the function of Dkk3 in the Wnt/β-catenin signaling pathway is not fully understood. We have added an important piece to the puzzle by showing a strong regulation of β-catenin by Dkk3 in cervical cancer. Based on our findings, we suggest the following working model to explain the role of Dkk3 as a negative regulator of Wnt/β-catenin pathway. Dkk3 binds to βTrCP in the cytoplasm and enhances the degradation of β-catenin and/or inhibits the nuclear translocation of β-catenin. Consequently, Dkk3-mediated downregulation of β-catenin led to decrease the β-catenin downstream targets such as VEGF, and cyclin D1. Further study would contribute to our understanding of the mechanism of Dkk3-mediated Wnt/β-catenin signaling in cervical cancer.

In summary, we showed that the *Dkk3* gene was significantly downregulated in human cervical cancer and Dkk3-mediated downregulation of β-catenin led to its antiproliferative activity in cervical cancer cells. These results strongly support Dkk3 as a useful therapeutic candidate for targeting β-catenin signaling.

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