

## Original Article

# Dickkopf-1 (DKK1) promotes tumor growth via Akt-phosphorylation and independently of Wnt-axis in Barrett's associated esophageal adenocarcinoma

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**Abstract:** Esophageal adenocarcinoma (EAC) is still associated with poor prognosis, despite modern multi-modal therapies. New molecular markers, which control cell cycle and promote lymph node metastases or tumor growth, may introduce novel target therapies. Dickkopf-1 (DKK1) is a secreted glycoprotein that blocks the oncogenic Wnt/ $\beta$ -catenin signaling and its aberrant expression has been observed in many malignancies, including EAC. In this study, we investigated the biological role of DKK1 and active  $\beta$ -catenin expression in human esophageal tissues confirmed a simultaneous DKK1-overexpression together with aberrant activation of  $\beta$ -catenin signaling in EAC in comparison with Barrett's and healthy mucosa. To elucidate the molecular role of DKK1, the OE33 adenocarcinoma cells, which were found to overexpress DKK1, were subjected to functional and molecular assays following siRNA-mediated DKK1-knockdown. At the functional level, OE33 cell viability, proliferation, migration and invasion were significantly attenuated by the absence of DKK1. At the molecular level, neither DKK1-knockdown nor application of exogenous recombinant DKK1 were found to alter the baseline  $\beta$ -catenin signaling in OE33 cells. However, DKK1-knockdown significantly abrogated downstream Akt-phosphorylation. On the other hand, the Wnt-agonist, Wnt3a, restored the Akt-phosphorylation in the absence of DKK1, without, however, being able to further stimulate  $\beta$ -catenin transcription. These findings suggest that the  $\beta$ -catenin transcriptional activity in EAC is independent of Wnt3a/DKK1 site-of-action and define an oncogenic function for DKK1 in this type of malignancy via distinct activation of Akt-mediated intracellular pathways and independently of Wnt-axis inhibition. Taken together, DKK1 may present a novel therapeutic target in EAC.

**Keywords:** Dickkopf-1, Wnt signaling, Barrett's adenocarcinoma, esophagus, Akt

## Introduction

The incidence of esophageal adenocarcinoma (EAC) has been rapidly increased in the western world over the last decades [1] and its development has been associated with Barrett's esophagus (BE), which is the replacement of normal squamous epithelium of the distal esophagus by metaplastic columnar mucosa in response to chronic gastroesophageal reflux disease (GERD) [2]. Radical surgical resection of the esophagus offers the best treatment option for EAC-patients while the addition of neoadjuvant chemo (radio) therapy has improved survival rates [3]. However, the prognosis of EAC still

remains unfavorable, with 5-year survival less than 15%, due to local recurrence and early regional or systemic metastases [4]. EAC-patients might further benefit from addition of other forms of therapies, such as molecular therapy, which specific targets biological markers that have a prominent role in cell cycle control progression, lymph node metastases and tumor progression. Identification of such novel targets requires understanding of the molecular mechanisms underlying the EAC tumor biology, which still remains largely undefined.

The Wnt-signaling cascade orchestrates a wide variety of biological processes during embryon-

ic development and tissue homeostasis, such as cell division, proliferation or differentiation, while its dysregulation has been tightly associated with cancer [5]. Wnt-signaling is either canonical or non-canonical based on the role played by the effector protein  $\beta$ -catenin [6]. Activation of the canonical Wnt/ $\beta$ -catenin signaling is marked by nuclear translocation of  $\beta$ -catenin in the presence of secreted Wnt glycoproteins or different mitotic signals. In the absence of Wnt-signal,  $\beta$ -catenin is sequestered in a “destruction complex”, consisting of glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), casein kinase 1 (CK1), adenomatous polyposis coli (APC) and Axin [7]. This complex forces the phosphorylation of  $\beta$ -catenin, which then is subjected to subsequent degradation by the ubiquitin-proteasome system [7]. Upon Wnt-signal,  $\beta$ -catenin is dephosphorylated and accumulates in the cytoplasm and subsequently to the nucleus, where it binds to members of the TCF/LEF-1 transcription factors and initiates transcription of target genes (Cyclin D1, Axin2, c-MYC, DKK1, etc.) [8].

During the neoplastic progression of BE towards EAC, an increased nuclear accumulation of  $\beta$ -catenin has been reported, signifying an aberrant activation of Wnt-signaling in EAC [9-11]. Intriguingly, the common genetic alterations of Wnt-signaling components, such as  $\beta$ -catenin, APC and Axin, are not frequently detected in EAC similarly to other human malignancies [12]. Instead, EAC is marked by changes, such as loss of the negative regulators of the pathway, such as the Wnt inhibitory factor 1 (WIF) or the secreted frizzled receptor proteins (SFRPs), along with induction of Wnt-2 expression, which together are expected to increase signaling along the WNT axis [12, 13]. However, the underlying mechanism of increased Wnt/ $\beta$ -catenin signaling in EAC and its impact in tumor biology needs to be explored.

Dickkopf-1 (DKK1) is a secreted glycoprotein, which specifically antagonizes the oncogenic canonical Wnt/ $\beta$ -catenin signaling, by binding onto the extracellular domain of the Wnt co-receptor LRP-6 (Low density lipoprotein receptor-related protein 6), thus preventing the Wnt-induced stabilization of  $\beta$ -catenin [14]. At the transcriptional level, DKK1 is a direct target of the  $\beta$ -catenin/T-cell factor (TCF) complex, suggesting a negative feedback loop in Wnt-sig-

nal regulation [15, 16]. Dysregulation in any step of the Wnt/ $\beta$ -catenin signaling cascade, including DKK1 mediated repression, leads to numerous developmental abnormalities and diseases, with the most widely studied being cancer [17]. Several studies reported downregulation or silenced DKK1-expression in intestinal cancers [15, 18], supporting that DKK1 functions as tumor suppressor, via inhibition of the oncogenic Wnt-signaling [19]. Then again, DKK1-overexpression has been detected and correlated with poor survival in diverse malignancies, such as multiple myeloma, non-small cell lung cancer, urothelial carcinoma, bladder cancer, gynaecological cancer and hepatocellular carcinoma (HCC), suggesting that DKK1 could promote tumor growth [17, 20]. Characteristically, DKK1-upregulation in gastric cancer correlated with tumor invasion [21]. In BE-dysplasia and mainly in EAC, elevated DKK1-expression has been previously reported [22, 23]. Interestingly, DKK1 was found to suppress  $\beta$ -catenin signaling in Barrett-metaplastic cells (CP-A) *in vitro*, supporting that an early perturbation of DKK1-mediated suppression of Wnt-axis may contribute to EAC-tumorigenesis [23]. In line with tumor progression, lymph node metastasis in EAC was found to correlate with high levels of DKK1 in the loco-regional lymph nodes of the primary tumor [24], proposing that DKK1 tumor secretion may orchestrate lymphatic metastasis. Taken together, these random observations so far propose a key role for DKK1 in the EAC, which merits further clarification.

The present study was undertaken to explore the molecular role of DKK1 in EAC tumor biology with regard to (a) tumor cell proliferation and invasion as well as (b) modulation of tumorigenic intracellular pathways, including the canonical Wnt/ $\beta$ -catenin signaling, the common intracellular tyrosine kinases activation and the epithelial-mesenchymal transition (EMT). By analyzing esophageal cell lines *in vitro*, we provide evidence supporting that DKK1 functions as a tumor promoter in EAC by inducing tumor cell proliferation and invasion. Intriguingly, this DKK1-mediated tumor promotion in EAC cells was found to be independent of the Wnt-axis, but via activation of Akt-mediated intracellular pathways. These novel findings underscore the significance of DKK1 as a potential therapeutic target in EAC and provide new insights into the

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molecular mechanisms of DKK1 in tumor progression.

## Materials and methods

### *Human esophageal tissues*

Human esophageal specimens were collected from patients with EAC following surgical esophagectomy without previous neo-adjuvant treatment. Studies were approved by the Human Research Review Committee of the Medical College of Wisconsin, and study participants gave written informed consent prior to their studies.

### *Cell culture*

Human squamous esophageal telomerase-immortalized cells EPC1-hTERT (EPC1) and EPC2-hTERT (EPC2) were the generous gifts of Dr. Hiroshi Nakagawa, Gastroenterology Division, University of Pennsylvania, USA and were grown as previously described [25]. The non-dysplastic columnar cell line CP-A (CRL-4027) and the high-grade dysplastic columnar cell line CP-B (CRL-4028) were obtained from the American Type Culture Collection (Rockville, MD) and were grown in MCDB-135 Basal Medium (Biochrom, Berlin, Germany) supplemented according to the manufacturer's protocol. Esophageal adenocarcinoma cell lines OE33 (ECACC-96070808) and OE19 (ECACC-96071-721) purchased from Sigma Aldrich (Taufkirchen, Germany) were adapted to RPMI 1640 growth medium (Gibco, Waltham, USA) supplemented with 10% bovine serum (Biochrom, Berlin, Germany). All cell lines were preserved optimally at 37°C in humidified air with 5% CO<sub>2</sub> until confluency reached 80% to 90%.

### *Reagents and antibodies*

Recombinant human DKK1 (rhDKK1), recombinant human Wnt3a (rhWnt3a), as well as recombinant human VEGF (rhVEGF) were obtained from R&D Systems Inc. (Minneapolis, MN). Primary antibodies were used against DKK1 (Abcam, Cambridge, MA), LRP6 (R&D Systems Inc.), p53 (Millipore, Billerica, MA), Caspase 3 (BD Transduction Laboratories Biosciences), E-Cadherin (Novus Biochemicals) and  $\beta$ -actin (Sigma Aldrich, St. Louis, MO). Other primary antibodies against  $\beta$ -catenin, Phospho-GSK3 $\beta$  (Ser9), GSK3 $\beta$ , Phospho-Akt (Ser473), Akt, Ph-

ospho-Erk1/2 (Thr202/Tyr204), Erk1/2 and Bcl-xL were obtained from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson Immuno Research Laboratories Inc. (West Grove, PA). All other reagents and chemicals were obtained either from Sigma Aldrich (Taufkirchen, Germany) or CarlRoth (Karlsruhe, Germany) unless otherwise indicated.

### *RNA isolation and cDNA synthesis*

RNA was isolated from cell cultures using Qiagen RNeasy Mini Kit (Valencia, CA) according to the manufacturer's protocol. cDNA preparation was performed with Revert Aid Kit purchased from Thermo Fisher Scientific (Waltham, MA). The cDNA probes were synthesized from one microgram of total RNA.

### *Quantitative polymerase chain reaction (qRT-PCR)*

RT-PCR was performed with DreamTaq Green PCR Master Mix (ThermoFisher Scientific, Waltham, MA) by adding 1  $\mu$ l primer, 0.5  $\mu$ l ( $\beta$ -actin primer) and 2  $\mu$ l (Dkk1 primer) of cDNA per 20- $\mu$ l reaction, respectively. Gel electrophoresis for fractionation of the nuclear acids was performed with 1.5% agarose gels. qRT-PCR was performed in 20- $\mu$ l reaction, 1  $\mu$ l primer and 1  $\mu$ l cDNA were added to the fluoresced SYBR-Green Master-Mix (Thermo Fisher Scientific) and gene expression was analyzed with RotorGene Anaysis Software (Qiagen Corbett Research). Relative quantification to reference gene  $\beta$ -actin was calculated with the  $\Delta\Delta$ Ct-method. The primers used for DKK1 [Forward-AGCGTTGTTACTGTGGAGAAG, Reverse-GTGTG-AAGCCTAGAAGAATTACTG],  $\beta$ -actin [Forward-CACTCTCCAGCCTTCCTTC, Reverse-GGTGTAA-CGCAACTAAGTCATAG], Axin2 [Forward-CCACT-GGCCGATTCTTCCTT, Reverse-TACCGGAGGATG-CTGAAGGC], Cyclin-D1 [Forward-GGCGATTG-GAAATGAACTT, Reverse-TCCCTCCAAAATGCC-AGAG] were obtained from Sigma Aldrich (Taufkirchen, Germany).

### *Western blot analysis*

Cell lysis, protein extraction and western blot analysis was performed as previously described [23].

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## ELISA

DuoSet Ancillary Reagent Kit and the corresponding Human Dkk-1 DuoSet ELISA were obtained from R&D Systems Inc. (Minneapolis, MN). Cell culture supernatants were diluted at a ratio 1:3 in 1% BSA in bovine serum and ELISA was performed according to the manufacturer's protocol.

## Dkk1 gene silencing

Transfection of OE33 cells (60%-70% confluence) was performed with specific short interfering RNAs (siRNAs) targeting either human DKK1 (target gene) (ON-TARGET plus SMART pool, Human-DKK1 (22943), Dharmacon, Lafayette, CO) or GAPDH-siRNA (positive control) (human ON-TARGETplus GAPDH Control siRNA, Dharmacon) with DharmaFECT transfection reagent. Non-targeting-siRNAs (ON-TARGETplus Non-targeting siRNA, Dharmacon) were used as a negative control. All reagents were used according to the manufacturer's protocol (Dharmacon), resulting in a final siRNA concentration of 25 nM.

## Cellular assays

*Proliferation* was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and <sup>3</sup>H-thymidine incorporation assay. For MTT assays, OE33 cells were seeded in 96-well plates. If not treated with rhDkk1 (500 ng/ml) or rhWnt3a (200 ng/ml), Dkk1 gene silencing was performed and MTT-measurements were conducted in six-fold replicates at the time 0 h, 48 h, 72 h, 96 h, 120 h and 144 h following siRNA transfection by adding MTT stock solution (5 mg/ml in bovine serum) to the wells. Thus, time of treatment represents 0 h. After 3 hours of incubation at 37°C, MTT stop-solution (sodiumdodecylsulfate (5.87 M) in 50% dimethylformamide solution) was added and absorption at 560 nm was measured after 24 hours by Spektramax M5 (Molecular Devices, Sunnyvale, CA). <sup>3</sup>H-thymidine incorporation assay was performed as previously described [25].

*Motility/migration* was determined by wound healing assays by placing OE33 cells into the two chambers of ibidi culture inserts (Madison, WI). Then, DKK1-gene silencing was performed, and cells were grown until confluency reached 90%. After inserts removal, OE33 cells were

separated through a 500 µm gap. The growth process over the gap was observed and documented under the microscope at certain times points as indicated. The gap width was quantified with ImageJ 1.48v (National Institute of Health, NY).

*Transmigration assay:* Cells were seeded into the upper chamber of special 24-well plates (BD Biosciences, San Jose, CA) following DKK1-Knockdown. After 48 hours of incubation, the cells were fluorescence stained with 4 µg/ml Calcein (Becton Dickson, Franklin Lakes, NJ) and fluorescence signal in the lower chamber was detected "from bottom" (405/595 nm) by Spektramax M5.

## Luciferase reporter assay

Luciferase assay using DNA plasmids of β-catenin-LEF/TCF sensitive (TOP-flash) and β-catenin-LEF/TCF insensitive (FOP-flash) reporter vectors (Addgene, Cambridge, MA), as well as Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was performed as previously described [23].

## Immunofluorescence & inverted microscopy

Zen lite 2012 software (Axiovert25, Zeiss, Oberkochen) was used for cell culture observation and photography (100-fold magnification). Immunofluorescence was performed as previously described [23].

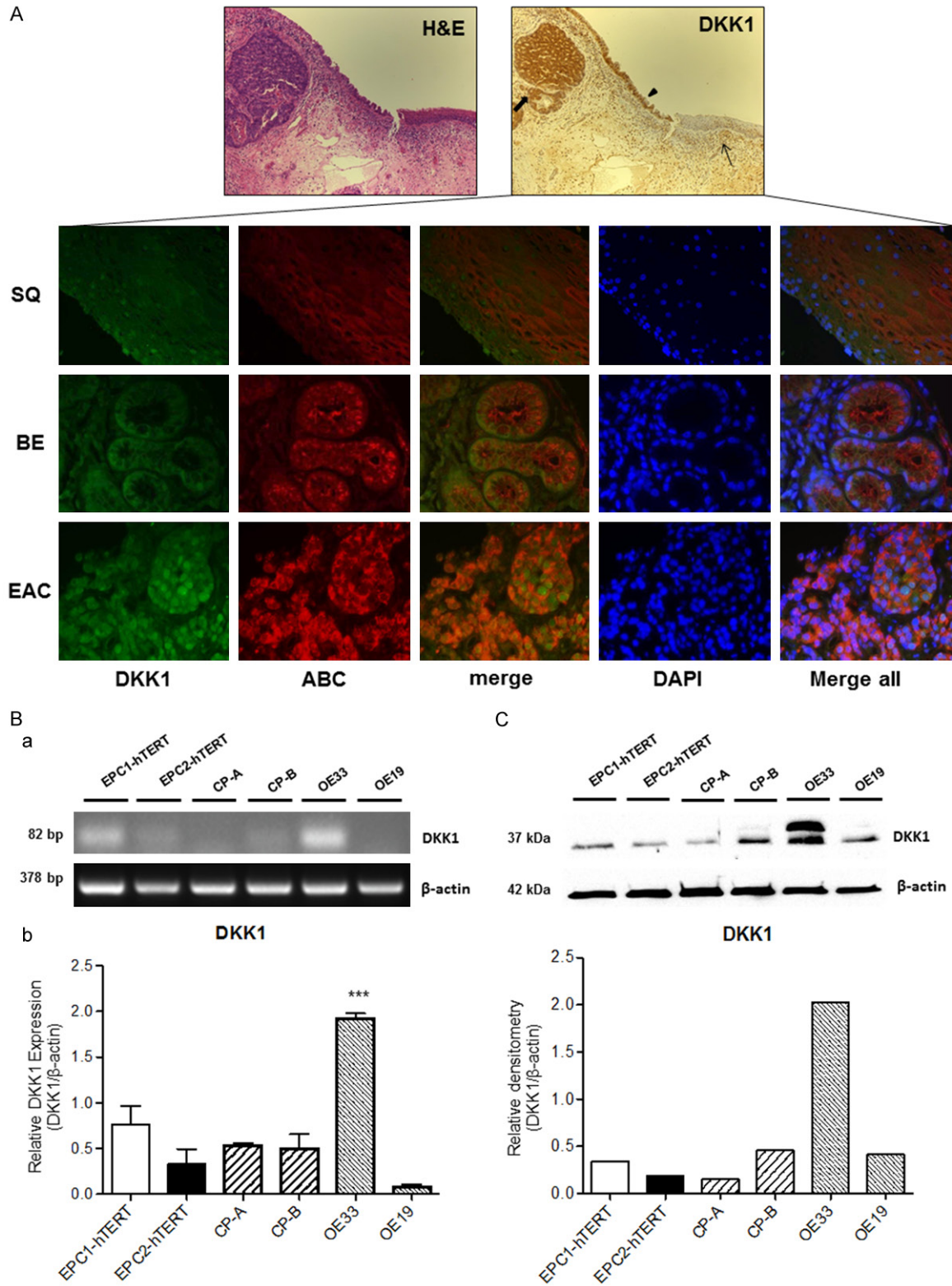
## Statistical analysis

Calculation was performed using GraphPad Prism 5.0 analysis software. All data were expressed as mean ± SEM. Dependent on the presence of a Gaussian distribution, Student's t tests or Mann-Whitney tests were used to evaluate significant differences. Cell culture experiments with three independent variables were tested with Two-Way-Anova (Post-hoc analysis: Bonferroni's Multiple Comparison Test). P-values less than .05 were considered statistically significant.

## Results

### *DKK1 is overexpressed in BE-associated esophageal adenocarcinoma cells*

We have previously reported that DKK1 expression is higher in BE and even higher in EAC, when compared to that of the corresponding



**Figure 1.** DKK1 expression in esophageal squamous mucosa, Barrett's metaplasia and esophageal adenocarcinoma: A. Immunofluorescence microscopy for expression and co-localization of DKK1 and ABC protein in squamous esophageal mucosa (SQ), non-dysplastic Barrett's metaplasia (BE) and esophageal adenocarcinoma (EAC). Representative tissue staining of at least three different tissues is presented. B. RT-PCR (a) and qRT-PCR (b) analysis of DKK1 mRNA expression in two squamous esophageal cell lines (EPC1/EPC2), two BE metaplasia cell lines (CP-A; nondysplastic/CP-B; high grade dysplasia), one barrett-associated esophageal adenocarcinoma cell line (OE33)

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and one EJC-adenocarcinoma cell line (OE19) in resting conditions. C. Western blot analysis of DKK1 protein expression in all cell lines in resting conditions.  $\beta$ -Actin serves as a loading control. Significantly elevated amounts of DKK1 were demonstrated in OE33 cells compared with the other cell lines (\*\* $P < .0001$ ; one way anova).

healthy squamous mucosa (SQ) in human esophageal specimens *ex vivo* by using immunohistochemistry [23]. Given that DKK1 counteracts the Wnt/ $\beta$ -catenin signaling as a specific Wnt-inhibitor, we were firstly interested to reveal, how DKK1-expression correlates with  $\beta$ -catenin signaling activation in EAC-tissue. By using a specific antibody that detects the amounts of the dephosphorylated  $\beta$ -catenin, specifically at Ser37/Thr41, which is not susceptible to ubiquitination and degradation, and its cytoplasmic/nuclear amounts are considered to be highly transcriptionally active (ABC) [26], we co-stained human esophageal specimens for DKK1- and ABC-protein expression. As shown by fluorescence microscopy (**Figure 1A**), DKK1-protein demonstrated a reverse pattern of expression with that of ABC in SQ, while high levels of DKK1-protein co-existed with elevated nuclear and cytoplasmatic ABC-expression in EAC compared to BE, favoring a failure of DKK1 to negatively regulate the transcriptionally active  $\beta$ -catenin in cancer cells as in normal squamous cells.

To further elucidate the molecular role of DKK1 in EAC, we utilized a panel of cell lines *in vitro*, representing the sequence of the esophageal squamous mucosa (EPC1-, EPC2-hTERT), the esophageal Barrett's metaplasia (CP-A) and dysplasia (CP-B) as well as the BE-associated esophageal adenocarcinoma (OE33) and the adenocarcinoma of gastric cardia/esophageal gastric junction (OE19) and characterized them on DKK1-expression. Compared to the rest of cell lines, OE33 cancer cells demonstrated significantly higher DKK1-mRNA levels ( $P < 0.0001$ ) (**Figure 1Ba, 1Bb**), as well as markedly elevated intracellular DKK1-protein levels ( $P < 0.001$ ) (**Figure 1C**), as shown by conventional PCR, qRT-PCR, and Western blotting respectively. In contrast, OE19 cells demonstrated lower levels of DKK1-mRNA and protein, equivalent to those of the dysplastic CP-B cells. These results verify an up-regulation of DKK1-expression along the progression of squamous epithelium towards EAC. Accordingly, we focused on OE33 cells in our following experiments in order to investigate the impact of DKK1 on cellular functions and molecular signaling pathways.

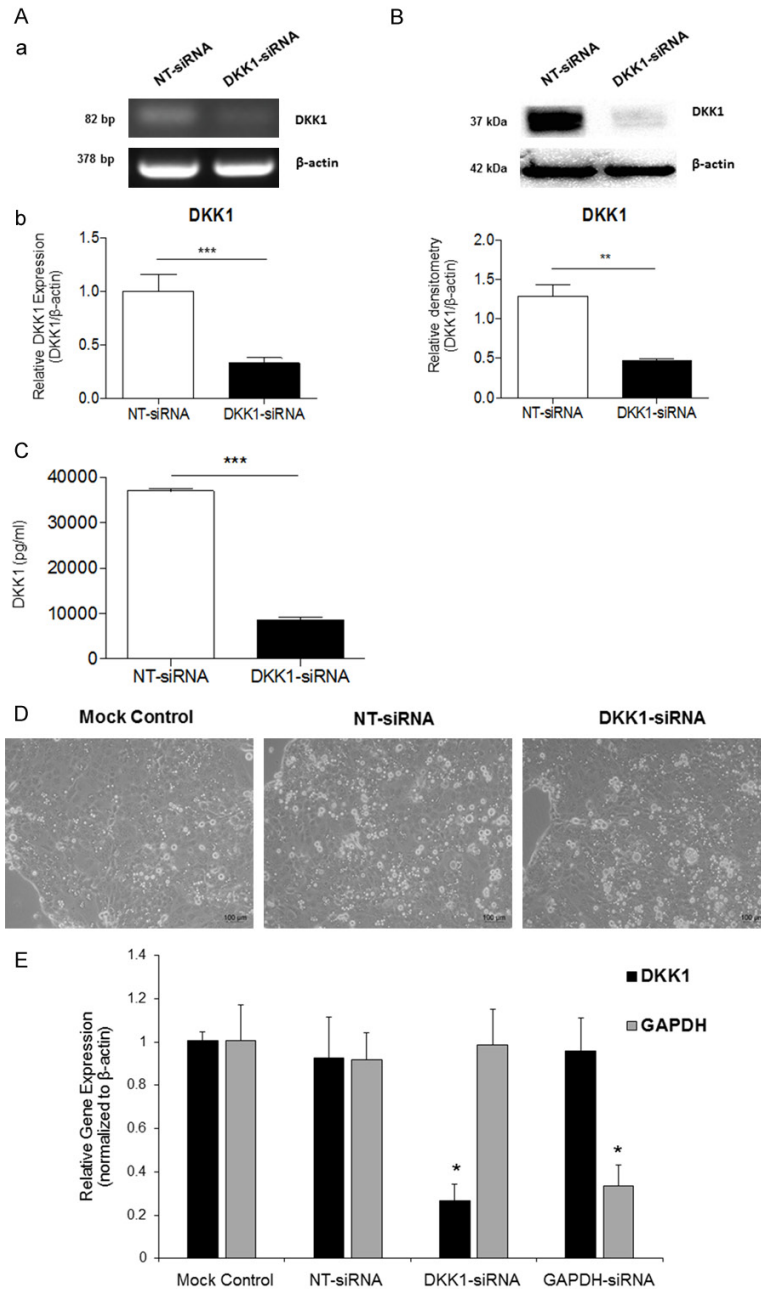
### *Efficient siRNA-mediated DKK1-knockdown in OE33 esophageal adenocarcinoma cells*

To explore the biological impact of DKK1-upregulation in EAC, we aimed to knockdown DKK1 in OE33 cells and seek for cell function alterations. Following transfection of resting OE33 cells with small-interfering RNA against DKK1 (DKK1-siRNA) for 48 hours, we demonstrated a significant decrease in DKK1-mRNA levels, over 70% of the baseline, either by conventional PCR (**Figure 2Aa**) or qRT-PCR (**Figure 2Ab**). The efficient DKK1-knockdown was further confirmed at the protein level. The intracellular levels and the secreted amounts of DKK1-protein were significantly attenuated following DKK1-siRNA transfection in OE33 cells, as shown by western blotting (**Figure 2B**) and ELISA (**Figure 2C**) respectively. The DKK1-knockdown did not alter the cell morphology (**Figure 2D**). Notably, siRNA-mediated DKK1-knockdown was sustained at the protein level even after 144 hours following siRNA-transfection, as demonstrated by western blotting (**Supplementary Figure 1A**), enabling us to perform additional experimentation over this time frame. The specific knockdown of DKK1 by DKK1-siRNA was demonstrated by using appropriate controls (GAPDH-siRNA as a positive control and nontargeted siRNA as a negative control) (**Figure 2E**). Thus, OE33 cells, following efficient DKK1-knockdown, were subjected to further functional and molecular assays.

### *DKK1-knockdown attenuated OE33 cell proliferation and migration*

We examined first the functional consequences of DKK1-knockdown in OE33 cell growth. Following DKK1-knockdown, cell viability was significantly decreased, as measured by MTT-assay up to 144 hours following DKK1-siRNA transfection (**Figure 3A**). To elucidate, whether the attenuated cell viability following DKK1-knockdown is a result of decreased proliferation, we proceeded with the  $^3\text{H}$ -thymidine incorporation assay for 24 hours following siRNA-transfection. VEGF-treatment increased OE33 cell proliferation and served as positive control (**Figure 3B**).  $^3\text{H}$ -thymidine uptake was markedly decreased in DKK1-silenced OE33 cells in

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**Figure 2.** DKK1 expression in OE33 cells following siRNA-mediated DKK1 gene silencing: A. RT-PCR (a) and qRT-PCR (b) analysis of DKK1 mRNA expression. B. Western blot analysis of DKK1 protein expression.  $\beta$ -Actin serves as a loading control. C. ELISA of DKK1 protein concentration (pg/ml) in cell culture medium. D. Microscopy of Mock Control as well as NT-siRNA and DKK1-siRNA transfected OE33 cells, 96 h after siRNA-mediated DKK1 gene silencing (\*\*P < .01; \*\*\*P < .0001; t test). E. qRT-PCR demonstrates the specific knockdown of DKK1 gene by DKK1-siRNA using GAPDH-siRNA as a positive and nontargeted siRNA as the negative controls.

comparison with control cells or cells transfected with NT-siRNA (**Figure 3B**), indicating that DKK1-overexpression promotes cell proliferation in wild type OE33 cells. This notion was further supported by a lower baseline proliferation

of resting OE19 cells, which were found to express less DKK1, compared to that of resting OE33 cells as measured by  $^3\text{H}$ -thymidine incorporation assay for 24 and 48 hours (**Figure 3C**).

The impact of DKK1-knockdown on cell motility and invasion of OE33 cells was investigated next. As shown by transmigration and wound scratch assays respectively, DKK1-knockdown resulted in significant attenuation of cell invasion (**Figure 3D**) and cell migration (**Figure 3Ea, 3Eb**), suggesting that DKK1 promotes the metastatic properties of OE33 cells.

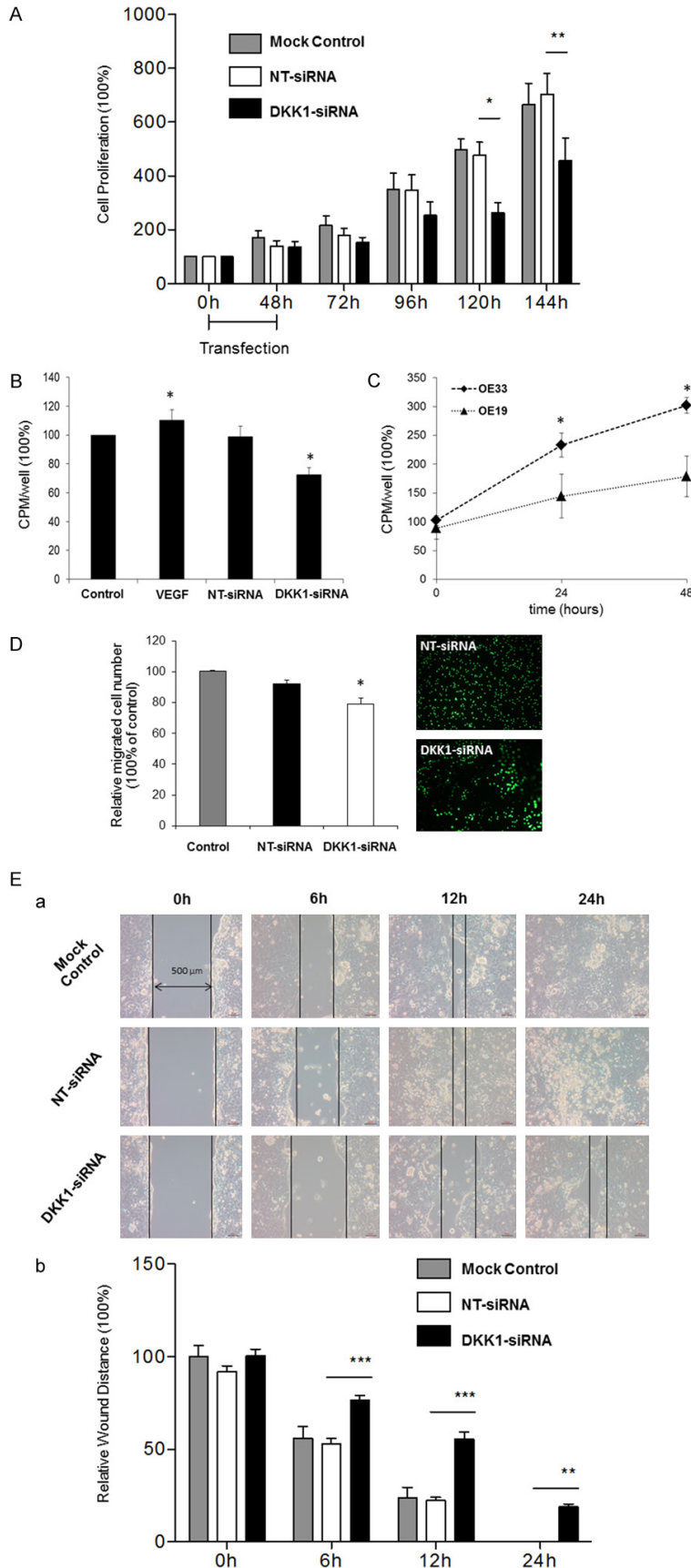
Taken together, these findings signify that the high amounts of DKK1 expressed by OE33 cancer cells induce cell growth and migration in an autocrine loop, and as such, DKK1 presents as a potential independent tumor-inducer in EAC.

*DKK1-overexpression does not regulate Wnt/ $\beta$ -catenin signaling negatively*

To determine the tumor promoting nature of DKK1, we tested next, how DKK1 regulates tumor-associated signaling pathways at the molecular level. We firstly focused on the oncogenic Wnt/ $\beta$ -catenin signaling. Given the previously reported downstream signaling activation in OE33 cells [23] together with the DKK1-upregulation, we wondered, whether DKK1 suppresses  $\beta$ -catenin transcriptional activity as expected in a negative

feedback mechanism [15]. As shown by western blotting, DKK1-knockdown resulted in slight stabilization of total  $\beta$ -catenin, suggesting potential downstream  $\beta$ -catenin signaling activation (**Figure 4A**). However, DKK1-knockdown

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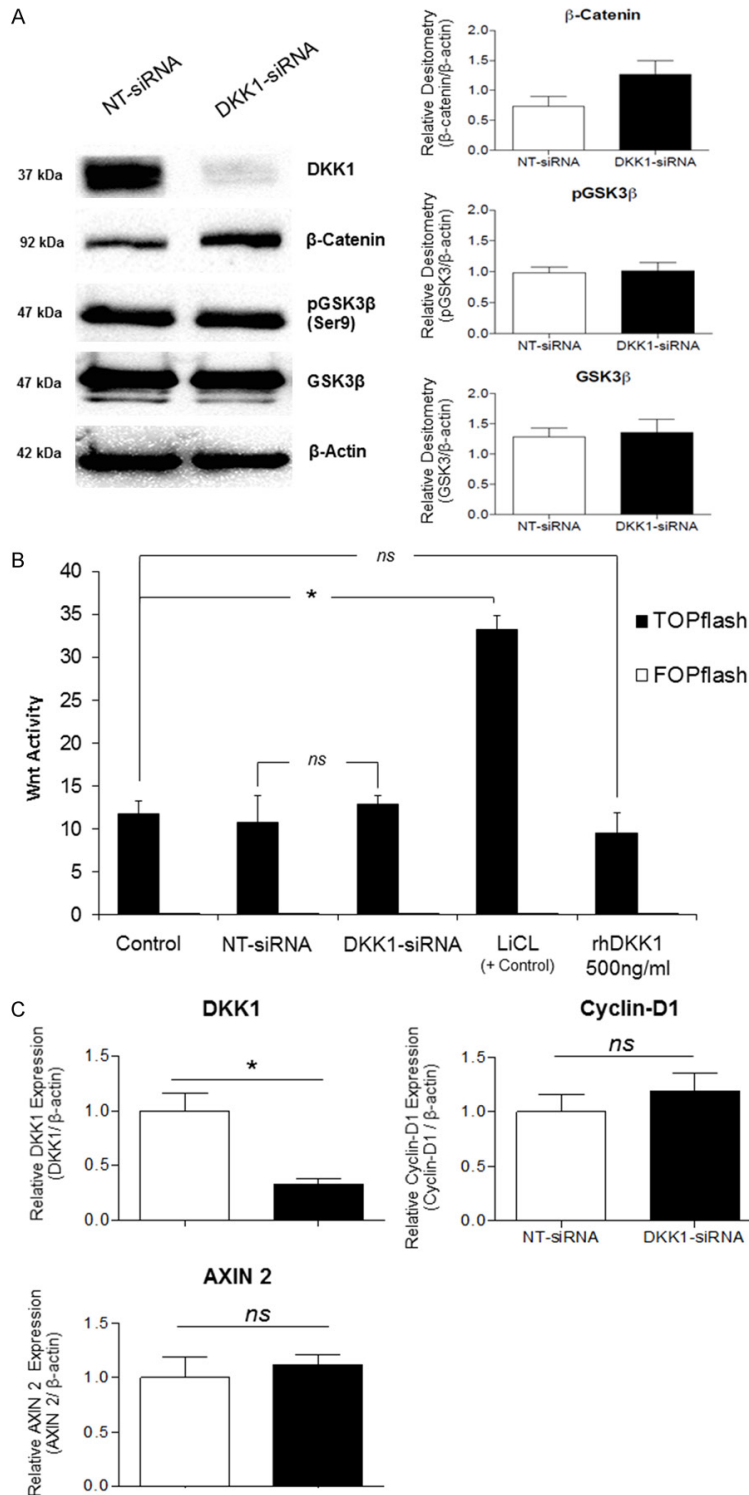


**Figure 3.** DKK1 promotes proliferation and migration in OE33 cells: A. MTT assay in OE33 cells following siRNA-mediated DKK1 gene silencing. Measurements were conducted 0, 48, 72, 96, 120 and 144 hours after siRNA transfection in sixfold replicates. Values are means  $\pm$  SE of 3 independent experiments and presented as percentage relative to time of treatment (0 h; 100%) (\* $P < .05$ ; \*\* $P < .01$ ; two way anova). B. <sup>3</sup>H thymidine incorporation assay in OE33 cells following siRNA mediated DKK1 gene silencing. VEGF treatment serves as a positive control. Values are means  $\pm$  SE of at least 3 replicates and presented as percentage of untreated controls (100%) (\* $P < .05$ ). C. <sup>3</sup>H thymidine incorporation assay in esophageal adenocarcinoma cells (OE33/OE19) in resting conditions with measurements after 24 and 48 hours. Values are means  $\pm$  SE of 3 replicates and presented as percentage relative to baseline proliferation (0 h; 100%). OE33 cells show significantly elevated proliferation compared with OE19 cells both after 24 and 48 hours (\* $P < .05$ ). D. Transmigration assay in OE33 cells following siRNA-mediated DKK1 gene silencing. Values are means  $\pm$  SE of 3 replicates and presented as percentage of untreated controls (100%) (\* $P < .05$ ). E. Wound healing assay in OE33 cells following siRNA-mediated DKK1 gene silencing. (a) Measurements of the gap distance were conducted after 0, 6, 12 and 24 hours via microscopic analysis. (b) Values are means  $\pm$  SE of 6 independent experiments and presented as percentage relative to the initial gap distance (\*\* $P < .01$ ; \*\*\* $P < .001$ ; two way anova).

had no impact on GSK3 $\beta$  phosphorylation at Serine 9 (Figure 4A), indicating absence of additional GSK3 $\beta$ -inactivation, which would be expected to enhance  $\beta$ -catenin de-phosphorylation.

To examine, whether DKK1-knockdown actually enhanc-





**Figure 4.** Wnt/ $\beta$ -Catenin signaling in OE33 cells following siRNA-mediated DKK1 gene silencing: A. Western blot analysis of DKK1,  $\beta$ -Catenin and pGSK3 $\beta$ /GSK3 $\beta$  protein expression.  $\beta$ -Actin serves as a loading control. Densitometry graphs depict the means  $\pm$  SE of 3 independent experiments. B. Luciferase assay (top) in OE33 cells following siRNA-mediated DKK1 gene silencing as well as stimulation with rhDKK1 (500 ng/ml) for 24 hours. LiCL-treatment serves as a positive control. C. qRT-PCR analysis of DKK1, AXIN 2 and Cyclin-D1 mRNA expression following DKK1 gene silencing. *ns*: nonsignificant (\* $P < .05$ ; t test).

es the  $\beta$ -catenin signaling in response to the slight  $\beta$ -catenin stabilization, we performed a luciferase assay. The levels of TOP-flash activity, which reflect actual  $\beta$ -catenin signaling activity, did not alter following DKK1-knockdown (Figure 4B), suggesting that the DKK1-overexpression exaggerates no inhibition on the downstream baseline  $\beta$ -catenin signaling in wild type OE33 cells. Accordingly, we investigated next, whether a molecular defect of the endogenous DKK1 could justify this absence of inhibition. Therefore, we treated OE33 cells with external, wild type, recombinant DKK1 protein (rhDKK1), seeking to observe downstream inhibition of  $\beta$ -catenin signaling. Instead, exogenous rhDKK1 (500 ng/ml) for 24 hours onto OE33 cells had no effect on the baseline TOP-flash activity, as shown by luciferase assay (Figure 4B). The treatment with lithium chloride (LiCL), an established chemical GSK3 $\beta$ -inhibitor, known to enhance Wnt/ $\beta$ -catenin signaling, increased TOP-flash activity and served as positive control (Figure 4B). In agreement with DKK1-failure to attenuate  $\beta$ -catenin signaling, DKK1-knockdown failed to alter the Cyclin-D1- and AXIN2-mRNA levels, the two main  $\beta$ -catenin target genes, as shown by qRT-PCR (Figure 4C).

In summary, these observations support that neither endogenous nor exogenous DKK1 can inhibit the downstream  $\beta$ -catenin signaling in OE33 cells, proposing a Wnt-independent mechanism of DKK1-mediated tumor promotion.

### *DKK1 induces tumor growth via induction of Akt-phosphorylation*

To enlighten the underlying molecular mechanism of DKK1-mediated tumor growth, we examined next, how DKK1 affects common downstream oncogenic signaling pathways. As shown by western blotting, DKK1-knockdown in OE33 cells significantly attenuated Akt-phosphorylation at Ser 473, whereas the MAPK-phosphorylation remained unaffected (**Figure 5A**), supporting a distinct DKK1-mediated tumor promotion via Akt-phosphorylation. To further support, whether Akt mediates the role of DKK1, we treated serum-starved OE33 cells (1% FCS) with different concentrations of exogenous rhDKK1 and showed that DKK1 significantly increased AKT-phosphorylation in a dose-dependent manner (**Figure 5B**). Furthermore, restoration of DKK1-protein levels after gene silencing by application of exogenous rhDKK1 (500 ng/ml for 4 hours) was able to rescue the AKT-phosphorylation, which was significantly attenuated by the elimination of endogenous DKK1 via siRNA gene silencing (**Figure 5C**). These findings support that AKT mediates the downstream signaling of DKK1 and provide a mechanism to address the observed DKK1-mediated tumor progression.

To elucidate whether Wnt-receptor interaction influences the DKK1-mediated Akt-activation, we subsequently determined the impact of Wnt3a-protein, the natural receptor-antagonist of DKK1, on the Akt<sup>ser473</sup>-phosphorylation following DKK1-knockdown. Interestingly, the recombinant Wnt3a (200 ng/ml rhWnt3a for 1 h) increased Akt<sup>ser437</sup>-phosphorylation only in OE33 cells following DKK1-Knockdown, and not in control NT-siRNA transfected cells (**Figure 5D**). This finding proposes a Wnt3a-receptor mediated downstream Akt-activation only in the absence of DKK1. To determine, whether this Akt-activation comes along with a further Wnt3a-driven  $\beta$ -catenin transcriptional activation, qRT-PCR of AXIN2 and Cyclin-D1 was performed. As shown in **Figure 5E**, transcription of both  $\beta$ -catenin target genes remained unchanged following Wnt3a-treatment even after DKK1-knockdown, suggesting that DKK1-knockdown did not sensitize cells to Wnt3a-mediated  $\beta$ -catenin activation.

The impact of DKK1-knockdown on the epithelial-mesenchymal transition (EMT) or the pro-apoptotic pathways was further examined by

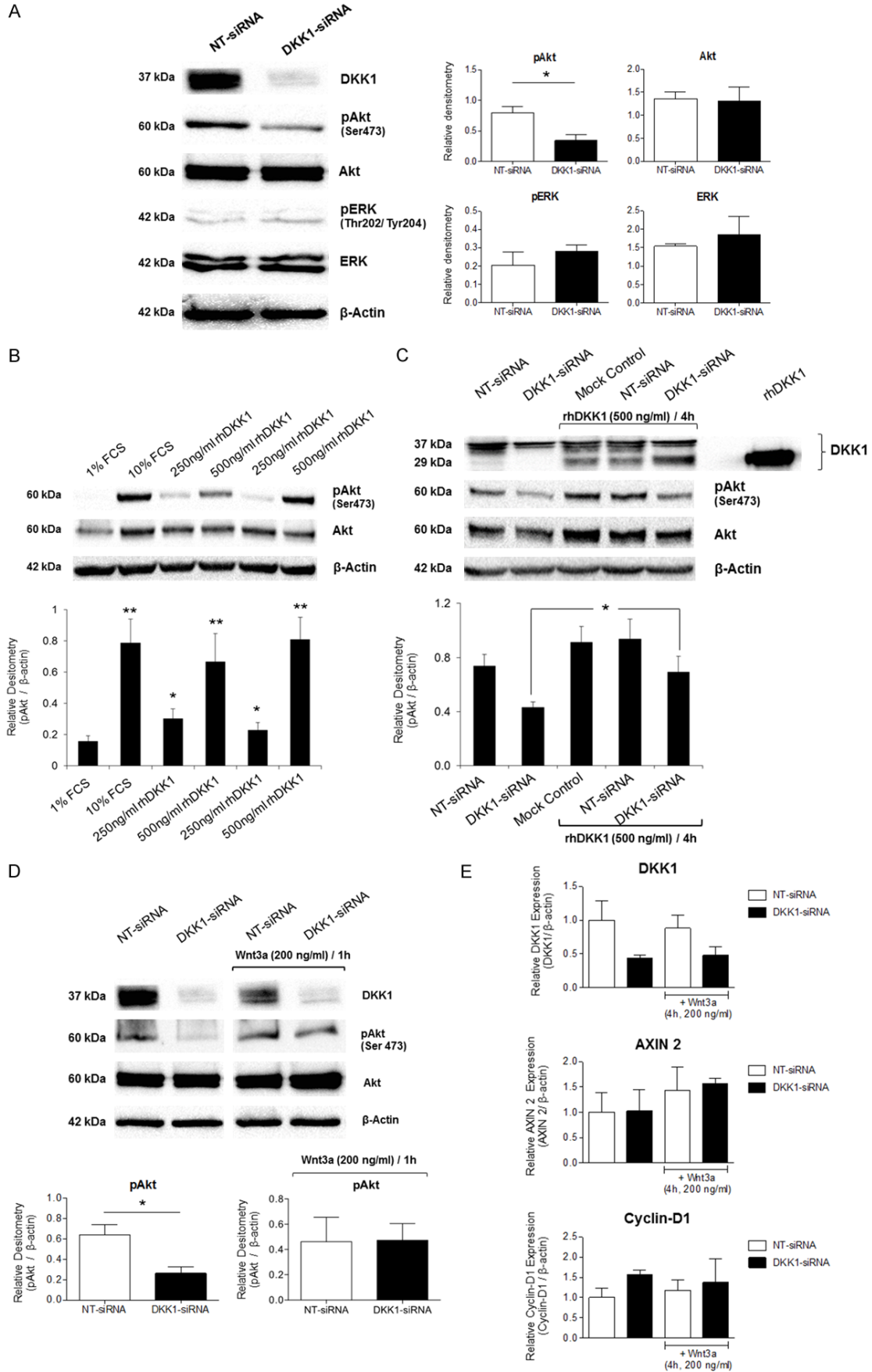
western blotting in OE33 cells on key pathway proteins. The expression of E-Cadherin as well as that of Caspase 3, p53 and BCL-xl remained unaffected by DKK1-knockdown, underscoring that DKK1-mediated tumor promotion in EAC is not associated with EMT-induction or inhibition of pro-apoptotic agents (**Figure 5F**).

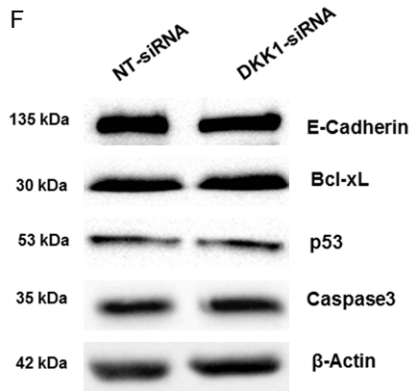
In total, the above pieces of evidence underscore that DKK1 promotes tumor growth in EAC via distinct downstream activation of Akt-mediator, dependent of the receptor context and presence of canonical Wnts.

### Discussion

In this study, we defined that the specific Wnt-antagonist, DKK1, which is highly expressed in EAC, functions as a tumor promoter via Akt-phosphorylation and independently of Wnt-axis. Human EAC-tissues were found to co-express highly DKK1-protein along with elevated levels of transcriptionally active  $\beta$ -catenin in comparison to the corresponding healthy and premalignant esophageal tissues. To elucidate the function of DKK1, the OE33 adenocarcinoma cells, which overexpress DKK1, were subjected to functional and molecular assays following siRNA-mediated DKK1-knockdown. At the functional level, OE33 cell viability, proliferation, migration and invasion were significantly attenuated by DKK1-silencing, suggesting that DKK1-overexpression induces tumor growth and survival. In favor of this notion, OE19 cells, which express less DKK1-protein, demonstrated lower proliferation rates. At the molecular level, neither DKK1-knockdown nor exogenous rhDKK1 were able to regulate the baseline  $\beta$ -catenin signaling in OE33 cells, suggesting that  $\beta$ -catenin transcriptional activity is independent of the DKK1 site-of-action. The tumor inducing properties of DKK1 were further investigated in conjunction with downstream oncogenic pathways. Here, DKK1-knockdown was found to significantly inhibit Akt-phosphorylation downstream, whereas MAPK-phosphorylation and the expression of EMT-Marker, E-Cadherin or pro-apoptotic agents remained unchanged, signifying a rather distinct Akt-mediated downstream mechanism for the DKK1-dependent tumor promotion. Intriguingly, the Wnt-agonist, Wnt3a, restored the Akt-phosphorylation only in absence of DKK1, without however being able to further stimulate  $\beta$ -catenin signaling. These findings define DKK1 as a tumor promoter in EAC and subsequently

# The biological role of DKK1 in esophageal adenocarcinoma





**Figure 5.** Expression analysis of molecular regulators of tumor growth in OE33 cells following siRNA-mediated DKK1 gene silencing and stimulation with rhDKK1 or rhWnt3a: (A) Western blot analysis of DKK1, pAkt/Akt and pERK/ERK protein expression in OE33 cells following siRNA-mediated DKK1 gene silencing. (B) Western blot analysis of pAkt/Akt protein expression in OE33 cells following serum starvation (1% FCS) followed by stimulation with different concentrations of rhDKK1 for 24 hours. Cells incubated with 10% FCS served as positive control. (C) Western blot analysis of DKK1 and pAkt/Akt protein expression in OE33 cells following siRNA-mediated DKK1 gene silencing and subsequent treatment with 500 ng/ml rhDKK1 for 4 hours. The cells stimulated with rhDKK1 demonstrated an extra band by 29 kDa of DKK1 protein, which depicts the intracellular trans-localization of the rhDKK1 protein, which has not been subjected to further post-translationally modification as in case of the endogenous DKK1 protein (37 kDa). On the very right panel, only rhDKK1 protein was loaded in order to confirm the molecular weight. (D) Western blot analysis of pAkt/Akt protein expression in OE33 cells following siRNA-mediated DKK1 gene silencing and treatment with 200 ng/ml rhWnt3a for 1 hour. (E) qRT-PCR analysis of DKK1, Axin2 and Cyclin-D1 mRNA expression in OE33 cells following siRNA-mediated DKK1 gene silencing and subsequent treatment with 200 ng/ml rhWnt3a for 4 hours. (F) Western blot analysis of E-Cadherin, Bcl-xL, p53 and Caspase 3 protein expression in OE33 cells following siRNA-mediated DKK1 gene silencing.  $\beta$ -Actin serves as a loading control (\* $P < .05$ ; t test). Densitometry graphs depict the means  $\pm$  SE of 3 independent experiments.

underscore its significance as well as that of its receptor as a potential therapeutic target in EAC.

The need to investigate the exact role of DKK1 was prompted by its aberrant upregulation in EAC compared to the premalignant esophageal specimens [23]. Given that DKK1 is an established direct target-gene of  $\beta$ -catenin, the reported activation of the canonical Wnt/ $\beta$ -catenin signaling in EAC could justify the DKK1-upregulation [15, 16]. However, induction of DKK1-transcription independently of Wnt-axis has also been reported in other malignancies and could apply in EAC as well [27]. Nevertheless, the simultaneous co-expression of high levels of DKK1-protein together with elevated levels of transcriptional active  $\beta$ -catenin in EAC-tissues favors the  $\beta$ -catenin-driven DKK1-upregulation. Yet, this notion raises questions on the role of DKK1 upon Wnt-signaling, as an established specific Wnt-antagonist functioning in a negative feedback-loop [16]. As such, the excess secretion of DKK1-protein in EAC should have been expected to suppress the Wnt-signaling in return. However, as we show here,

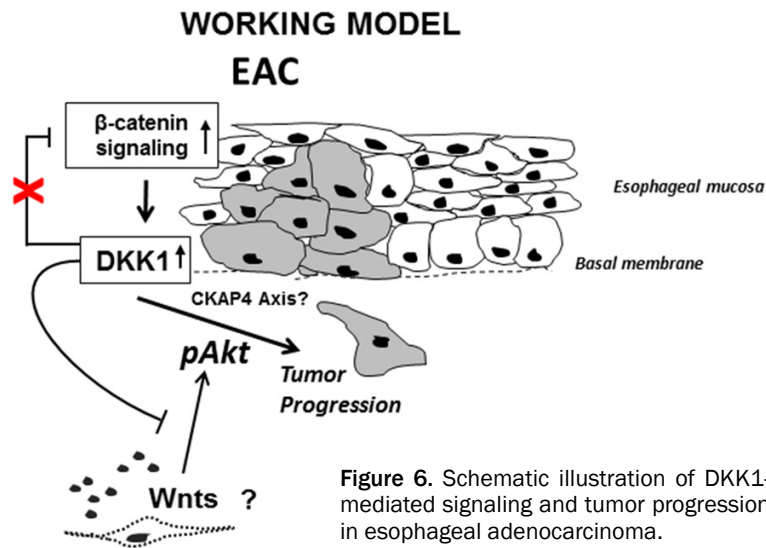
DKK1 failed to impact the  $\beta$ -catenin signaling. Neither DKK1-knockdown nor exogenous rhDKK1 could regulate the  $\beta$ -catenin transcriptional activity *in vitro*. These findings become highly important, given our previous report, which demonstrated DKK1-induced suppression of Wnt/ $\beta$ -catenin signaling in Barrett-metaplastic cells, suggesting that a possible early perturbation of the DKK1-mediated signaling suppression may trigger dysplasia and further EAC-tumorigenesis [23]. Why DKK1 fails to inhibit  $\beta$ -catenin signaling in OE33 cells similarly to BE-metaplastic cells, can possibly be explained either by (a) a defective negative feedback loop, caused by post-transcriptional DKK1 loss-of-function or by (b) an aberrant signaling activation downstream of the DKK1 site-of-action. Intriguingly, the inability of the rhWnt3a to stimulate  $\beta$ -catenin signaling activity, even following DKK1-silencing, allow us the assumption, that the aberrant oncogenic  $\beta$ -catenin signaling in EAC is independent of the Wnt-axis and is triggered downstream of the DKK1/Wnt3a site-of-action, the LRP6-receptor [28]. In the light of these findings, it may not be a perturbation of DKK1-function that prevents

Wnt-axis inhibition, but a rather, still unclear, neoplastic “switch on” of aberrant  $\beta$ -catenin transcriptional activation, downstream of DKK1 site-of-action. In favor, emerging evidence supports that more than one mechanism, also independent of Wnts, can induce the oncogenic  $\beta$ -catenin signaling activation towards tumor formation [29]. For example, tumor cells over-expressing EGF-receptor, as in case of OE33 cells [30], could display GSK-3 $\beta$ -independent activation of  $\beta$ -catenin signaling in response to EGF stimulation, without changes on  $\beta$ -catenin’s stability or phosphorylation levels [31]. Undoubtedly, similar growth factor-based mechanisms may underlay the aberrant  $\beta$ -catenin signaling seen in EAC and merit further clarification.

Enhanced DKK1-transcription elevates the amounts of the secretory protein, which antagonizes the oncogenic Wnt-signals by binding to the Wnt co-receptor LRP6, resulting to its internalization [28]. As such, DKK1 is mostly expected to act as a tumor suppressor. Indeed, DKK1 has been found downregulated in colorectal cancer and melanoma due to epigenetic inactivation (DNA-hypermethylation), while its loss-of-expression has been strongly correlated with advanced stages of carcinogenesis [15, 18, 32]. Accordingly, several reports have shown that DKK1 inhibits growth in various cancer cell lines, while it can also induce or increase sensitivity of cancer cells to apoptosis (summarized in [33]), supporting a role as tumor suppressor. However, several parallel reports have also defined a role for DKK1 as a tumor promoter. In various carcinomas including lung, prostate, pancreas and breast cancers, DKK1-expression has been found significantly elevated, while high DKK1-secretion has been associated with cancer progression [33]. In line with the latter, we defined an oncogenic role for DKK1 in EAC, based on (a) its overexpression in EAC malignant tissues, (b) its failure to suppress the downstream oncogenic  $\beta$ -catenin signaling activity and mainly (c) the observed attenuation of cell growth, metastasis and invasion of OE33 cells following DKK1-silencing *in vitro*. Similar DKK1-dependent cancer promotion has been proposed in different malignancies, which also originate from foregut-derivatives. For example, induced DKK1-expression increased the proliferation rate and invasion of squamous esophageal cancer cells [34], while its overexpression has been associated with tumor aggressive-

ness and chemotherapy-refractory phenotype in non-small cell lung cancer cells [35]. Notably, lymph node metastasis in EAC were recently reported to correlate with high DKK1-expression in the loco-regional lymph nodes of the primary tumor [24], suggesting that tumor DKK1-secretion may orchestrate the lymphatic metastasis in EAC. These observations together with our current findings point to a significant oncogenic role of DKK1 in EAC.

Although the molecular impact of DKK1 has been mainly examined in the context of Wnt-signaling [16], the evidence presented here suggests that DKK1-dependent cancer progression in EAC is independent of Wnt-axis. Therefore, the oncogenic function of DKK1 may require binding to a putative receptor along with activation of distinct downstream signaling cascades. Increased activity of growth factor-regulated kinases, up-regulated expression of anti-apoptotic proteins and endothelial-mesenchymal transition are reported to contribute to the EAC tumor phenotype [36] and were the subject of further investigation following DKK1-knockdown. A significant attenuation of Akt-phosphorylation was revealed following DKK1 loss-of-expression, while MAPK-kinases and pro-apoptotic or EMT-markers remained unaffected. Given that aberrant activation of PI3K/Akt pathway is an important driver of neoplastic progression [37], the inactivation of serine/threonine protein kinase Akt in OE33 cells following DKK1-knockdown justifies the detected phenotypical changes. Phospho-Akt, an indicator of active Akt-signaling, increases along the progression from normal esophagus to BE, dysplasia, and EAC and its overexpression has been associated with tumor progression and poor prognosis [38, 39]. However, this is likely due to increased upstream signaling, since activating mutations in Akt have not been reported in EAC [40]. Thus, DKK1-protein appears as a novel trigger of Akt-activation in EAC, with, however, still unclear mediator-receptor. Recently, the cytoskeleton-associated protein 4 (CKAP4) has been recognized as a novel receptor for DKK1 [27, 41], providing new insights in DKK1-dependent cancer promotion. DKK1-CKAP4 interaction is reported to activate Akt through phosphatidylinositol 3-kinase (PI3K) and as such to induce cellular proliferation [27]. Simultaneous overexpression of both, DKK1 and CKAP4, has been reported in pancreatic



and lung tumors accompanied by a poor prognosis [27], while DKK1-CKAP4 interaction induced proliferation of squamous esophageal cancer cells via Akt-activity [42]. Accordingly, the DKK1-CKAP4-Akt cascade appears attractive to present a novel signaling pathway for EAC tumor progression and is currently under investigation in our laboratory.

The rescue of Akt-phosphorylation by rhWnt3a, only in the absence of DKK1-protein, proposes a cross-talk between Wnt-axis and downstream Akt-phosphorylation. In support, Wnt3a has been reported to rapidly activate Wnt/β-catenin signaling and to promote Akt-phosphorylation via IRS/PI3K signaling in pancreatic NIT-1 beta cells, with these effects to be completely abrogated by DKK1 [43]. In our report, following DKK1-elimination, Wnt3a restored Akt-phosphorylation but failed to activate the actual downstream β-catenin signaling. These observations verify, on one hand, the Wnt-independent nature of the aberrant β-catenin signaling in EAC, but also, on the other hand, highlight an interesting downstream cross-talk between the Wnt and DKK1 common site-of-action, the LRP6-receptor, and downstream oncogenic signaling cascades. As such, the exact function of DKK1 in cancer progression may also be dictated by the context of Wnt-ligands in tumor microenvironment and the receptor's context of pre-malignant and malignant cells. In the absence of Wnt-ligands and upon expression of oncogenic receptors, such as CKAP4, DKK1 acts as a tumor promoter, while in the presence of Wnt-lig-

ands and the absence of oncogenic receptors it can act as a tumor suppressor, as previously reported in Barrett-metaplastic cells via inhibition of Wnt-axis [23]. Thus, our current model supports that DKK1-upregulation in EAC promotes tumor growth through distinct Akt-phosphorylation, highly likely by binding to a primitive receptor, possibly the CKAP4, while at the same time, maintains its ability to block Wnt-ligand-receptor interactions, and, as such, to abrogate downstream oncogenic cross-activations (Figure 6). The significance of such DKK1-mediated Wnt-inhibition, without, however, an actual suppression of β-catenin signaling, requires further investigation in accordance with the, rather still unclear, presence of canonical Wnts in EAC tumor microenvironment.

and lung tumors accompanied by a poor prognosis [27], while DKK1-CKAP4 interaction induced proliferation of squamous esophageal cancer cells via Akt-activity [42]. Accordingly, the DKK1-CKAP4-Akt cascade appears attractive to present a novel signaling pathway for EAC tumor progression and is currently under investigation in our laboratory.

An acknowledged limitation of our study is that the conclusions are based mostly on an *in vitro* model. However, the mechanistic approach utilized here, with functional and molecular assays following DKK1-knockdown in OE33 cells, uncovers a clear oncogenic role for DKK1, while it provides a sufficient interpretation of the, so far unexplained, DKK1-overexpression in EAC. Undoubtedly, the DKK1-failure to inhibit β-catenin signaling in combination with the induction of Akt-phosphorylation in those cells, justify the observed phenotypical changes indicative of tumor progression. Then again, it remains to be clarified, whether DKK1 promotes tumor growth also *in vivo*. Such studies could be performed using EAC patient-derived xenografts in mice. Possible correlations of tissue DKK1-expression or serum DKK1-levels with the tumor-stage in EAC-patients along with their predictive value are not addressed in this study. However, in malignancies like hepatocellular carcinoma and prostate cancer, in which DKK1-knockdown inhibited cell proliferation and migration *in vitro* assays [44, 45], metastatic sites demonstrated higher DKK1-expression in comparison with that of the primary tumors, and high DKK1-levels of tumor tissue and in blood serum were predictive for shorter overall survival [46, 47].

Particularly, in squamous esophageal cells EC-9706, a vector-induced DKK1-overexpression promoted cell growth and invasion *in vitro* [34], while DKK1-positive tumors were found to have poorer disease-free-survival (DFS) than those with negative tumors [48]. Similarly, based on the DKK1-driven tumor progression seen in OE-33 cells, the prognostic significance of DKK1-overexpression should be also anticipated in EAC-patients; however, the clinical relevance of DKK1 in EAC still remains ambiguous and needs to be proven.

In conclusion, in this study we define for the first time an oncogenic role for DKK1 in EAC. We attribute this DKK1-dependent tumor progression to a rather Wnt-independent mechanism, which involves distinct downstream Akt-activation. Given that  $\beta$ -catenin signaling was neither attenuated by DKK1 nor further elevated by Wnt3a in OE33 cells even following DKK1-silencing, signifies that the aberrant  $\beta$ -catenin transcriptional activity in EAC is triggered by growth factors, independently of Wnt-axis. This activation subsequently results in DKK1-overexpression, which, in return, can promote tumor growth and metastasis, based on the context of Wnt-ligands and specific receptors. These novel findings underscore the significance of DKK1 in EAC tumor biology and point to its possible usefulness as prognostic marker or novel therapeutic target for future tailored therapies for this malignancy.

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### Disclosure of conflict of interest

None.

### Abbreviations

EAC, esophageal Adenocarcinoma; DKK1, Dickkopf-1; BE, Barrett's esophagus; siRNA, small interfering RNA; NT-sRNA, Non targeting siRNA; GSK-3 $\beta$ , glycogen synthase kinase-3 $\beta$ , CK1, casein kinase 1; APC, adenomatous polyposis coli; LRP, Low density lipoprotein receptor-related protein; WIF, Wnt inhibitory factor 1; SFRPs, secreted frizzled receptor proteins; ABC, Active  $\beta$ -catenin; LiCl, lithium chloride.

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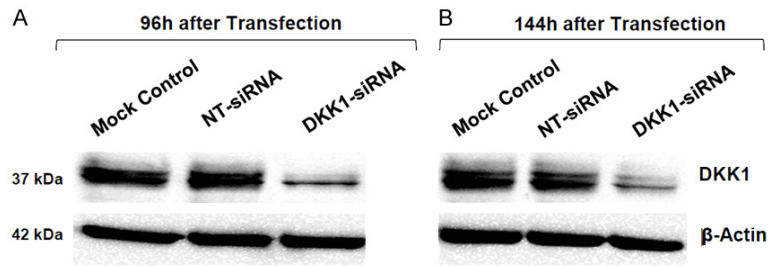
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**Supplementary Figure 1.** Western blot analysis of DKK1 protein expression in OE33 cells following siRNA-mediated DKK1 gene silencing, (A) 96 hours and (B) 144 hours after siRNA transfection.  $\beta$ -Actin serves as a loading control.