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DKK3 Overexpression Increases the Malignant Properties of Head and Neck Squamous Cell Carcinoma Cells

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DKK3, a member of the dickkopf Wnt signaling pathway inhibitor family, is believed to be a tumor suppressor because of its reduced expression in cancer cells. However, our previous studies have revealed that DKK3 expression is predominantly observed in head and neck/oral squamous cell carcinoma (HNSCC/OSCC). Interestingly, HNSCC/OSCC patients with DKK3 expression showed a high rate of metastasis and poorer survival, and siRNA-mediated knockdown of DKK3 in HNSCC-derived cancer cell lines resulted in reduced cellular migration and invasion. From these data, it was hypothesized that DKK3 might exert an oncogenic function specific to HNSCC. In the present research, the DKK3 overexpression model was established, and its influences were investigated, together with molecular mechanism studies. The DKK3 expression profile in cancer cell lines was investigated, including HNSCC/OSCC, esophageal, gastric, colorectal, pancreatic, prostatic, and lung cancers. DKK3 overexpression was performed in HNSCC-derived cells by transfection of expression plasmid. The effects of DKK3 overexpression were assessed on cellular proliferation, migration, invasion, and in vivo tumor growth. The molecular mechanism of DKK3 overexpression was investigated by Western blotting and microarray analysis. DKK3 overexpression significantly elevated cellular proliferation, migration, and invasion, as well as increased mRNA expression of cyclin D1 and c-myc. However, reporter assays did not show TCF/LEF activation, suggesting that the increased malignant property of cancer cells was not driven by the Wnt/β-catenin pathway. For the investigation of the pathways/molecules in DKK3-mediated signals, the Western blot analyses revealed that phosphorylation of Akt (S473) and c-Jun (Ser63) was elevated. The application of a PI3K kinase inhibitor, LY294002, on HSC-3 DKK3 cells significantly decreased tumor cell proliferation, migration, and invasion. From these results, we demonstrated that DKK3 might contribute to cellular proliferation, invasion, migration, and tumor cell survival in HNSCC cells through a mechanism other than the canonical Wnt signaling pathway, which might be attributed to PI3K–Akt signaling.

Key words: Head and neck squamous cell carcinoma (HNSCC); Oral cancer; DKK3; Wnt signaling

INTRODUCTION

Wnt signaling plays a crucial role in embryogenesis and cancer. Wnt ligands bind to its receptor complex, Frizzled and low-density lipoprotein receptor-related protein (LRP) 5/6, and trigger a signaling cascade. DKK3 belongs to the dickkopf Wnt signaling pathway inhibitor family (DKK), which consists of four main members (DKK1, 2, 3, and 4) and a unique DKK3-related protein Soggy (SGY-1 or DKKL1)[1]. It is reported that DKK family members can inhibit canonical Wnt/β-catenin signaling by antagonizing

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Wnt-mediated β-catenin stabilization, resulting in the negative regulation of Wnt target genes. DKKs will interfere with LRP5/6 and Kremen coreceptors and antagonize Wnt signaling by mediating internalization of LRP. However, DKK3 does not seem to antagonize Wnt signaling because DKK3 does not bind to either LRP or Kremen[2]. Therefore, the biological role of DKK3 in Wnt signaling is unclear.

On the other hand, in the cancer research field, DKK3 is known to have a reduced expression in cancer (REIC), which is frequently downregulated in several kinds of malignancies[3]. Moreover, adenovirus-mediated DKK3 overexpression results in reduced cancer cell proliferation or apoptosis induction[4–6]. Thus, DKK3 is believed to be a tumor suppressor that may affect a wide range of malignancies.

Interestingly, our previous report demonstrated that DKK3 protein expression was widely observed in head and neck squamous cell carcinoma (HNSCC) and its precursor lesions[7,8] and that patients with DKK3 expression showed shorter disease-free and metastasis-free survival[7]. In addition, knockdown of DKK3 in HNSCC-derived cell lines resulted in reduced cellular migration and invasion[9]. From these results, we hypothesized that DKK3 may exert an oncogenic function specific to HNSCC. In the present study, we validated this hypothesis using functional analyses together with molecular mechanism studies, which were performed in DKK3 overexpression models.

First, we measured the DKK3 expression status in several kinds of malignancies and demonstrated that DKK3 was selectively expressed in squamous cell carcinoma (SCC) cell lines of the head and neck and esophagus, and of pancreatic ductal adenocarcinoma cells. Overexpression of DKK3 by transfection of expression plasmids into an HNSCC-derived cell line resulted in increased cellular proliferation, migration, and invasion. Moreover, DKK3-overexpressing HNSCC cells formed a larger tumor mass when subcutaneously injected into nude mice. Our data suggested that DKK3 may not function as a tumor suppressor in HNSCC and may be a therapeutic target for cancer invasion/metastasis prevention in HNSCC/oral squamous cell carcinoma (OSCC).

MATERIALS AND METHODS

Cell Lines and Reagents

The following cancer cell lines were used in the study: HNSCC (HSC-2, HSC-3, HCS-4, Ca9-22, UT-SCC-16A, 16B, 60A, and 60B), esophageal SCC (TE-1, TE-10, and TE-14), gastric (HGC-27, Kato III, KE-39, and H-111-TC), colorectal (CW-2, Colo-320, LoVo, Colo205, and HCT116), pancreatic (PANC-1, BxPC3, and MIA Paca2), prostatic (DU145, PC-3, and LNCap FGC), and lung (SCC: EBC-1 and LK-2; adenocarcinoma: PC-9, HLC-1, and RERF-LC-KJ). Four of the HNSCC cell lines (UT-SCC-16A, 16B, 60A, and 60B) were kindly provided by Dr. Reidar Grenman of Turku University, Turku, Finland. The other cell lines were purchased from RIKEN BRC (Tsukuba, Japan). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA), supplemented by 10% FBS (Nichirei, Tokyo, Japan). A PI3K kinase inhibitor, LY294002 (Cell Signaling Technology, Danvers, MA, USA), was added in 50 μM in order to assess the involvement of PI3K–Akt signaling in DKK3 transfectants.

Expression Plasmids and Transfection

A full-length DKK3 expression plasmid was kindly provided by Dr. Toshiya Tsuji (Okayama University)[3]. Using this as a template, plasmid expressing DKK3 with hemoagglutinin (HA) tag in the C-terminal end, and restriction enzyme site of *Bam*HI (5′ side) and *Xba*I (3′ side) was prepared by PCR using the following primers: CCG GAT CCA TGC AGC GGC TTG GGG CCA CCC TGC TG (forward) and GGT CTA GAA TCA AGC GTA ATC TGG AAC ATC GTA TGG GTA CGC AAT CTC TTC CCC TCC CAG CAG TGC AGC GGC GGC AGC CGC AGG CTC CCC CAG CGC CAT C (reverse). PCR was done with KOD-Plus-Ver2 (TOYOBO, Osaka, Japan). The PCR products were electrophoresed in agarose gel, extracted by MinElute™ Gel Extraction Kit (QIAGEN, Valencia, CA, USA), and cloned into DH5α cells by TOPO® TA Cloning® Kit (Thermo Fisher Scientific, Waltham, MA, USA). DNA was extracted from selected colonies by QiAprep® Spin Miniprep Kit (QIAGEN). After checking the sequences, the DNA was cut with *Bam*HI and *Xba*I and recombined into pCS2$^+$ plasmid.

The plasmids were transfected into the cells with Turbofectin 8.0™ (ORIGENE, Rockville, MD, USA). As a control for transfection, Venus GFP/pCS2$^+$ was used. The empty vector of pCS2$^+$ was used instead of GFP. The next day, the cells were used for analyses.

Real-Time Quantitative PCR (RT-qPCR)

The cells were cultured in 100-mm diameter dishes or six-well plates, and total RNA was extracted from cells using ISOGEN (NIPPON GENE CO., Tokyo, Japan) or Nucleospin RNA® (Macherey-Nagel GmbH & Co. KG, Düren, Germany). cDNA was synthesized by ReverTra Ace® (TOYOBO). The RT-qPCR was done using THUNDERBIRD® qPCR Mix (TOYOBO) and Step One Plus™ (Thermo Fisher Scientific). Gene expression was quantified and then normalized against the RPL30 housekeeping gene expression[10]. The primer sequences were as follows: endogenous DKK3, CAG GCT TCA CAG TCT GGT GCT TG (forward) and ACA TTG TTT CCA TCT CCT CCC CTC (reverse)[11]; DKK3-HA-Tag, AGG AAC TGA TGG AGG ACA CG (forward) and CTT CTG CCT TCT TCG TCT CC (reverse); RPL30, ACA GCA TGC GGA AAA TAC TAC (forward) and AAA GGA AAA

TTT TGC AGG TTT (reverse); cyclin D1, CTT CCT CTC CAA AAT GCC AG (forward) and AGC GTG TGA GGC GGT AGT AG (reverse); and c-myc, TCC TCG GAT TCT CTG CTC TC (forward) and GTT GTG CTG ATG TGT GGA GA (reverse). The PCR conditions were 95°C for 1 min followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. Absolute quantification was used for analysis as previously described[12].

Western Blotting

Cancer cell lines were maintained until they became confluent. Then the cell lysate was harvested in IP buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100]. The amount of protein was quantified by DC™ Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), and 10 μg of protein was used. Cell extracts were boiled in Laemmli's buffer for 3 min. Each protein sample was loaded in an e-PAGEL® precast gel (ATTO, Tokyo, Japan) and then blotted onto polyvinylidene difluoride (PVDF) membranes. Following the blockade of nonspecific binding by soaking the PVDF membranes in PVDF Blocking Reagent for Can Get Signal® (TOYOBO) in room temperature for 1 h, the membrane was treated with primary antibodies at 4°C, overnight. Primary antibodies used in the study were DKK3 (Abcam, Cambridge, MA, USA), β-catenin, p-β-catenin (S675), non-phospho (active)-β-catenin, GSK-3β, p-GSK3 (Ser9), Akt, p-Akt (Ser473), c-Jun, p-c-Jun (Ser63), p-Smad1/5(Ser463/Ser465), Smad2, p-Smad2 (S465/467), β-actin (Cell Signaling Technology), Smad6 (Abnova, Taipei, Taiwan), and Smad7 (R&D, Minneapolis, MN, USA) at 1:500–1:1,000 dilution. The membranes were then rinsed in TBST, followed by secondary antibody (Jackson ImmunoReaserch Inc., West Grove, PA, USA) for 1 h at room temperature. Antibodies were diluted in Can Get Signal® (TOYOBO). Proteins were visualized using the ECL prime Western blotting detection system (GE Healthcare, Buckinghamshire, UK).

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed in order to quantify the amount of DKK3 in the cell culture medium using RayBio® Human DKK-3 ELISA Kit (RayBiotech, Inc., Norcross, GA, USA) according to the manufacturer's protocol.

Immunocytochemistry

Cells were fixed in PBS containing 4% paraformaldehyde. After three washes with PBS, cells were immunohistochemically stained using anti-DKK3 (Abcam) at 1:200 dilutions. After washing, cells were incubated with a 1:500 dilution of secondary Alexa Fluor® 594-conjugated goat anti-rabbit antibodies (Thermo Fisher Scientific). Cell nuclei were stained with 1 μg/ml of DAPI (Dojindo, Kumamoto, Japan). Fluorescent images were taken using an All-in-One Fluorescence Microscope BZ-X700 (Keyence, Osaka, Japan).

Apoptosis Assay

To assess the influence of DKK3 overexpression on the cancer cells, an apoptosis assay was done using APOPercentage™ apoptosis assay (Biocolor Life Science Assays, Carrickfergus, UK) according to the manufacturer's protocol. HNSCC-derived HSC-3 cells and prostatic cancer-derived PC-3 cells were used in the study. For positive control of apoptosis induction, cells were incubated with 20 mM H_2O_2 for 2 h.

Cell Proliferation Assay

In order to assess the effect of DKK3 overexpression on cell proliferation, MTT assay was performed using TACS® Cell Proliferation Assays (TREVIGEN, Gaithersburg, MD, USA). HSC-3 and HSC-3 with GFP or DKK3 transfectants (HSC-3 GFP, HSC-3 DKK3, respectively) were suspended in 1.0×10^3 cells/100 μl/well and plated into a 96-well microplate and cultured for 24 h. The MTT reagent was added and incubated for 4 h to form formazan crystals, and a detergent agent was added 4 h later. The absorbance at 570 nm was then measured. Data were acquired on days 1, 3, 5, and 7.

Migration Assay

The cell migration assay was performed using an Ibidi culture insert (Ibidi GmbH, Munich, Germany). HSC-3, HSC-3 GFP, and HSC-3 DKK3 were cultured and were suspended in DMEM with 10% FBS (5.0×10^5 cells/ml). Cell suspension (70 μl) was transferred to the well of the culture insert on a six-well plate and then removed using sterilized tweezers after 24 h of incubation in a 5% CO_2 incubator. Time-lapse photography was taken using BZ-X700 (Keyence). The area was measured using ImageJ software (http://rsb.info.nih.gov/ij/).

Invasion Assay

Calbiochem® InnoCyte™ Cell Invasion Assay Kit (EMD Millipore, San Diego, CA, USA) was used for the invasion assay. Cells were harvested and resuspended in serum-free DMEM into 1.0×10^6 cells/ml, and then 300 μl of cell suspension was applied into the upper chamber. After cell invasion (24 h), the cells were removed from the top chamber and treated with cell treatment solution. Relative cellular invasion was calculated according to the manufacturer's protocol.

Xenograft and Histological Evaluation

Cells were resuspended in 5.0×10^6 cells/150 μl of PBS and injected subcutaneously in 5-week-old BALB/cAJcl-nu/nu nude mice (CLEA Japan, Tokyo, Japan). The number of animals used was 30 in total ($n = 10$ for each experimental group). Tumor volume (V) was measured

and calculated by the following formula: $V = 4/3\pi \times L/2 \times (W/2)^2 = L \times W^2 \times 0.5236$ (L is the longest diameter, and W is the diameter perpendicular to L).

Four weeks after injection, the animals were sacrificed, and the tumors were extirpated for histological evaluation. This study was performed in accordance with the Guidelines for Animal Experiments at Kawasaki Medical School. The animal protocol for this experiment was approved by the Animal Care and Use Committee of Kawasaki Medical School (No. 15-052, 2015). Tissues were fixed with 10% neutral-buffered formalin and embedded in paraffin, then sectioned at 5-µm thickness, stained by hematoxylin and eosin (H&E), and used for immunohistochemistry for Ki-67 (DAKO, Glostrup, Denmark). Ki-67$^+$ cells were counted, and Ki-67 labeling index was calculated.

TCF Reporter Assays

In order to confirm TCF activity change by DKK3 transfection, a TOP/FOP Flash assay was performed as previously described[13]. HSC-3 cells were seeded into 24-well plates at 1.0×10^5 cells/well and cultured for 24 h. Cells were then cotransfected with 0.2 µg of DNA constructs, 0.2 µg of reporter plasmid, and 0.2 µg of internal control pRG-TK, then cultured for a further 24 h. Luciferase activity was measured and normalized for transfection efficiency using a Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA). Graphs show the average of three independent experiments with normalized transfection efficiency using *Renilla* luciferase. As a positive control, cells were stimulated by 100 ng/ml of rhEGF (R&D) for 24 h[14]. To confirm the TCF reporter assay, one more experiment was done using Cignal™ Reporter Assay Kits (QIAGEN). As a control, cells were stimulated by 50 mM LiCl for 24 h.

Microarray Analysis

Expression profiles were examined under the following conditions: HSC-3 versus HSC-3 GFP, and HSC-3 GFP versus HSC-3 DKK3. Labeling, hybridization, scanning, and data processing were carried out with Toray 3D-Gene® (Toray, Tokyo, Japan). Minimum information about a microarray experiment (MIAME)-compliant array data including raw data are deposited in the Gene Expression Omnibus (GEO) at NCBI with accession number GSE84725.

Statistical Analysis

Significant differences were determined by the two-tailed multiple Student's *t*-test with Bonferroni correction following Dunnett's test. All computations were performed using PASW® Statistics 18 (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was considered to be statistically significant. All the experiments were done at least three times.

RESULTS

DKK3 mRNA and Protein Expression Was Observed in a Tissue-Specific Manner

DKK3 protein expression was observed in all of the HNSCC-derived cell lines, two esophageal SCC cell lines (TE-10 and TE-14), and one pancreatic ductal adenocarcinoma-derived cell line (PANC-1) (Fig. 1A). DKK3 expression was rarely observed in gastric, colorectal, prostatic, and lung adenocarcinomas. Interestingly, cells derived from SCC in the lung did not show DKK3 expression. DKK3 mRNA expression was concordant with protein expression (Fig. 1B). HSC-3, a tongue SCC-derived cell line, was used in the following experiments.

Evaluation of DKK3 Plasmid Transfection

Transfection of DKK3 with HA tag expression plasmid significantly elevated DKK3 expression both in the protein (Fig. 2A) and mRNA levels in HSC-3 cells (Fig. 2B). As DKK3 is a secreted protein, the amount of DKK3 in the cell culture media was quantified. ELISA confirmed that the DKK3 secretion level was also significantly increased (Fig. 2C). The localization of DKK3 protein was confirmed by immunocytochemistry. Overexpression of DKK3 was observed as an increase in cytoplasmic DKK3 (Fig. 2D). Because previous reports demonstrated that adenovirus-mediated DKK3 overexpression caused cellular apoptosis[4–6], we performed an apoptosis assay to confirm whether DKK3 expression plasmid would cause apoptosis. Transfection of the DKK3 expression plasmid did not cause apoptosis either in HNSCC-derived HSC-3 cells or in prostatic adenocarcinoma-derived PC-3 cells, whereas DKK3 expression was significantly elevated in the mRNA and protein levels (Fig. 3E and F).

DKK3 Overexpression and its Effects on Cellular Proliferation, Migration, Invasion, and In Vivo Tumor Growth

The effects of DKK3 overexpression were investigated with regard to cellular proliferation, migration, and invasion. The MTT assay showed significantly increased cellular proliferation in HSC-3 DKK3 cells compared with control cells (Fig. 3A). Cellular migration of HSC-3 DKK3 cells was significantly increased compared to that of control HSC-3 cells ($p = 0.016$) and that of HSC-3 GFP cells ($p = 0.0480$) (Fig. 3B). Moreover, cellular invasion was significantly increased in HSC-3 DKK3 cells ($p = 0.0030$) (Fig. 3C). Namely, DKK3 overexpression in HSC-3 cells increased the malignant property of cells in vitro.

To confirm the surprising results of the in vitro experiments, HSC-3, HSC-3 GFP, and HSC-3 DKK3 cells were subcutaneously injected into nude mice. Tumor masses were observed from day 7 after injection and gradually

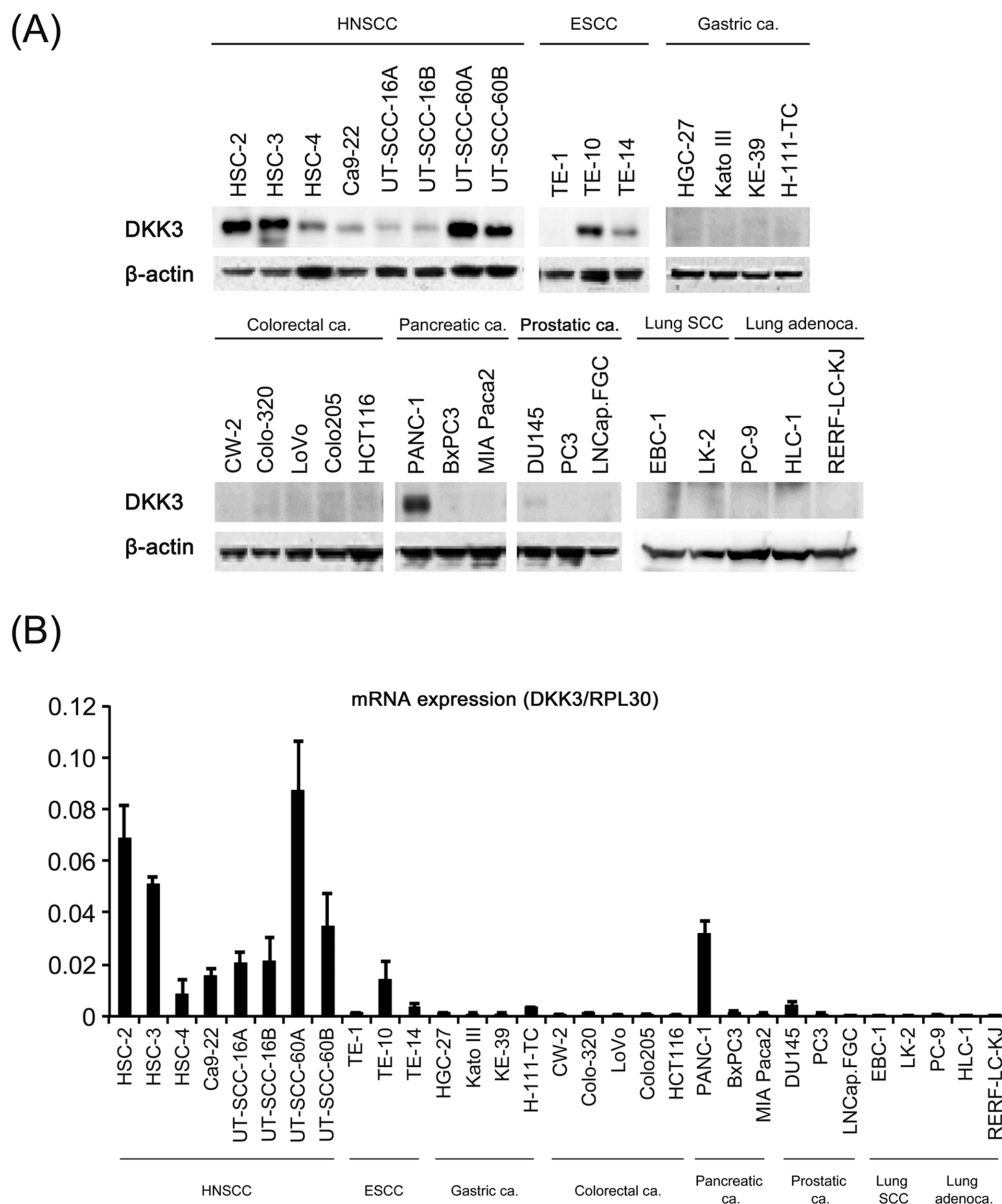

Figure 1. Expression of DKK3 protein and mRNA was assessed by Western blotting and real-time quantitative PCR (RT-qPCR). Western blotting revealed that DKK3 protein expression was observed in all of the head and neck squamous cell carcinoma (HNSCC)-derived cells and esophageal SCC (ESCC)-derived cells, except for TE-1 and PANC-1 pancreatic ductal adenocarcinoma-derived cells. Gastric, colorectal, prostatic, and lung cancer cells did not express DKK3 protein (A). RT-qPCR results were compatible with the Western blotting results (B).

increased in size. On day 18, the tumor masses of HSC-3 DKK3 cells became significantly larger than those of control HSC-3 cells ($p=0.009$) and those of HSC-3 GFP cells ($p=0.014$) (Fig. 3D). This difference in tumor size was conserved to day 28, the endpoint of the experiments (day 21: vs. HSC-3, $p=0.006$, vs. HSC-3 GFP, $p=0.018$; day 24: vs. HSC-3, $p=0.023$, vs. HSC-3 GFP, $p=0.011$; day 28: vs. HSC-3, $p=0.021$, vs. HSC-3 GFP, $p=0.031$, respectively).

On histological analysis, H&E sections did not show any differences in cancer cell morphology or invasive behavior. The injected cells formed a local tumor mass and did not show distant or lymph node metastasis. The Ki-67 index was significantly high in tumors from

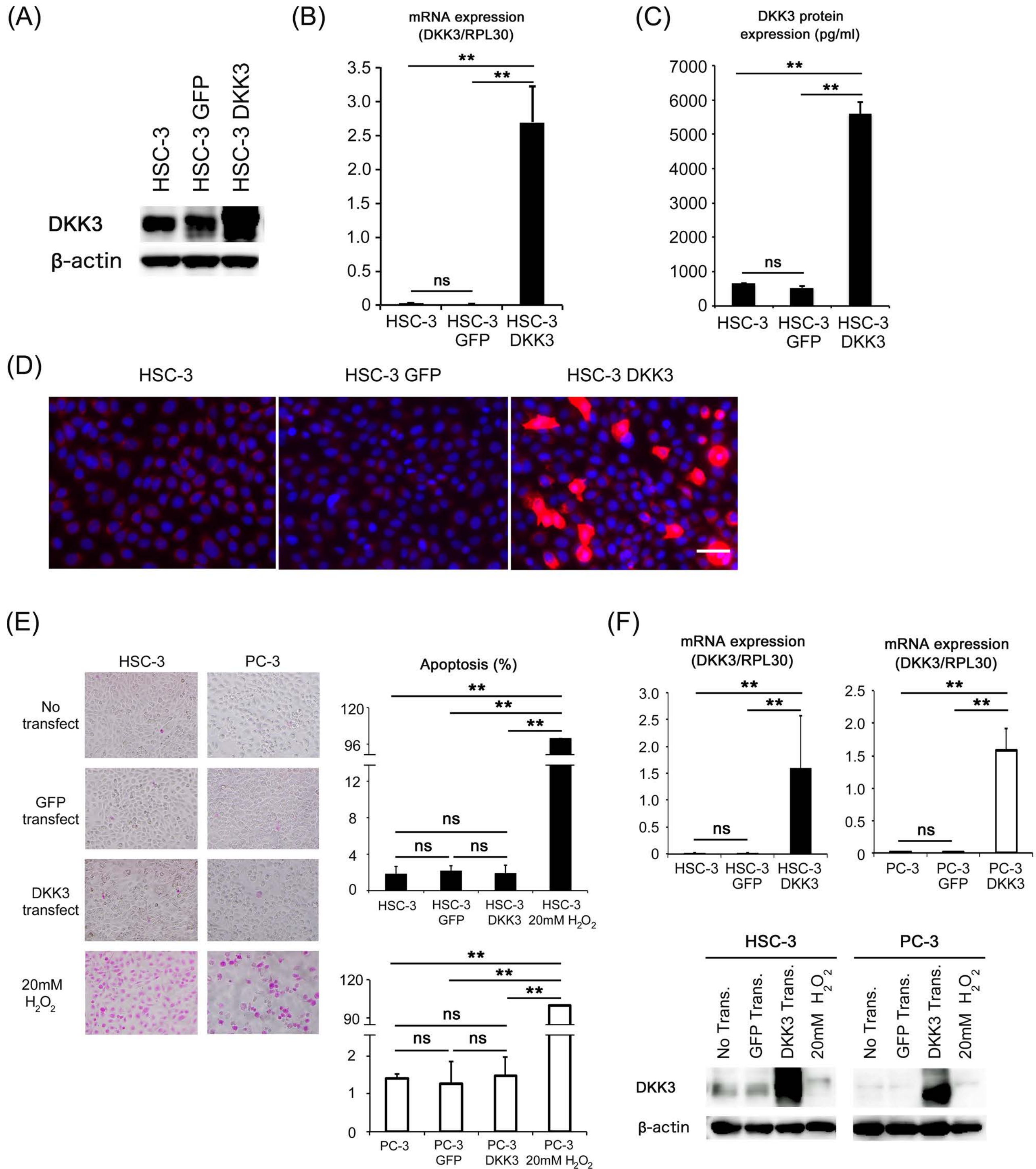

Figure 2. DKK3 overexpression in HSC-3 cells was confirmed by Western blotting, RT-qPCR, enzyme-linked immunosorbent assay (ELISA), and immunocytochemistry. DKK3 protein expression was elevated compared with control HSC-3 cells and HSC-3 GFP cells (A). RT-qPCR revealed significant DKK3 mRNA expression (B), and secretion of DKK3 protein was also significantly augmented by DKK3 transfection (C). In immunocytochemistry, DKK3 expression was observed in HSC-3 cells and HSC-3 GFP cells, and HSC-3 DKK3 cells showed intense cytoplasmic DKK3 expression (D). The apoptosis assay revealed that DKK3 overexpression did not induce apoptosis either in HSC-3 cells or in PC-3 cells (E). The DKK3 mRNA and protein expression changes are shown in (F). Scale bar: 50 μm. ns, not significant. $^{**}p<0.01$.

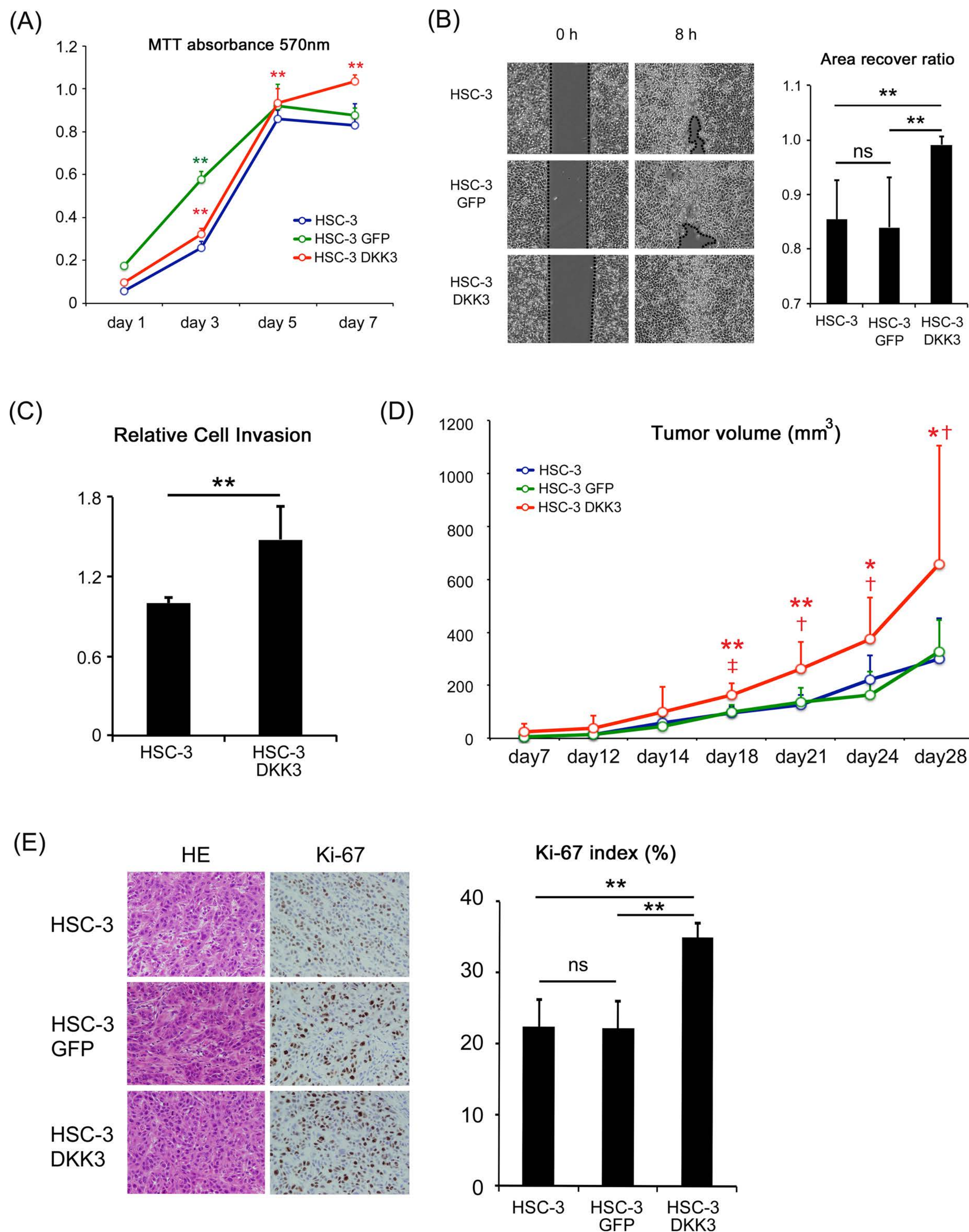

Figure 3. Effects of DKK3 overexpression on cellular proliferation, migration, invasion, and in vivo tumor growth were assessed. The MTT assay showed that DKK3 overexpression increases cellular proliferation (A) (** in red indicates HSC-3 vs. HSC-3 DKK3, and ** in green indicates HSC-3 vs. HSC-3 GFP). Migration and invasion assays revealed that DKK3 overexpression significantly increased cellular migration and invasion (B, C). In the xenograft model, HSC-3 DKK3 cells show a larger tumor mass compared with HSC-3 (*,**) and HSC-3 GFP (†,‡) (D). Histological evaluation showed a higher Ki-67 index in HSC-3 DKK3 cells (E). **,‡$p<0.01$; *,†$p<0.05$.

HSC-3 DKK3 cells compared to both HSC-3 control cells ($p = 0.0002$) and HSC-3 GFP cells ($p = 0.00017$) (Fig. 3E).

DKK3 Overexpression and the Wnt Signaling Pathway

To examine whether the Wnt signaling pathway mediates increased malignancy by DKK3 overexpression, RT-qPCRs for Wnt target genes and TCF/LEF reporter assay were performed. Interestingly, DKK3 overexpression significantly elevated cyclin D1 and c-myc mRNA expression (cyclin D1: vs. HSC-3, $p = 7.56 \times 10^{-8}$ and vs. HSC-3 GFP, $p = 4.52 \times 10^{-7}$, respectively; c-myc: vs. HSC-3, $p = 0.0034$ and vs. HSC-3 GFP, $p = 0.0054$, respectively) (Fig. 4A). This result suggested activation of the Wnt/β-catenin signal in HSC-3 DKK3 cells. Nevertheless, the TCF/LEF reporter assay did not show TCF activation (Fig. 4B and C).

The expression and phosphorylation status of the Wnt/β-catenin signaling molecules and other signaling including Akt, c-Jun, and Smads were then investigated. Western blotting exhibited expression of β-catenin, non-phospho (active)-β-catenin, GSK-3β, Smad2, Smad6, and Smad7, and phosphorylation of β-catenin (S675), GSK-3β (Ser9), Smad1/5 (Ser463/465), and Smad2 (S465/467) were not changed. Phosphorylation of Akt (Ser473) seemed to be slightly elevated, and notably c-Jun expression was decreased in HSC-3 DKK3, and consequently phosphorylation of c-Jun (Ser63) was increased (Fig. 4D). This implied that increased malignancy of HSC-3 cells by DKK3 overexpression might be driven by a certain signaling pathway separate from the Wnt/β-catenin signaling pathway.

LY294002 Significantly Decreased Cellular Proliferation, Migration, and Invasion in HSC-3 DKK3 Cells

To give a valid explanation for an elevated malignant property, we focused on the elevated phosphorylation of Akt in HSC-3 DKK3 cells. A PI3K kinase inhibitor, LY294002, was applied at 50 μM into HSC-3 DKK3 cells, which resulted in reduced phospho-Akt (S473), without affecting DKK3 overexpression (Fig. 5A). The MTT assay revealed that HSC-3 DKK3 + LY294002 showed significantly reduced cellular proliferation ($p < 0.01$) compared with HSC-3, HSC-3 GFP, and HSC-3DKK3 (Fig. 5B). Cellular migration and invasion were also significantly decreased ($p < 0.01$) compared with both HSC-3 cells and HSC-3 DKK3 cells (Fig. 5C and D).

Microarray Analyses and Gene Expression Changes by DKK3 Overexpression

Microarray analyses revealed that 89 genes and 54 genes were upregulated in HSC-3 DKK3 compared with the control HSC-3 and with HSC-3 GFP, respectively, and that 173 genes and 75 genes were downregulated in HSC-3 DKK3 compared with the control HSC-3 and with HSC-3 GFP, respectively. To exclude background nonspecific dispersion, genes were specified by the criteria that global normalization is higher than 100. Eighteen and nine genes were upregulated in HSC-3 DKK3 versus HSC-3 control and HSC-3 GFP, respectively. All nine genes upregulated in HSC-3 DKK3 versus HSC-GFP were included in the set of 18 upregulated genes (vs. the HSC-3 control). A total of three genes were downregulated both versus the HSC-3 control and versus the HSC-3 GFP groups. Finally, nine upregulated genes (NAB2, NAV3, ABHD14B, SMG6, DYRK4, AGR2, EPN3, RAD17, and RTKN) and three downregulated genes (BRI3BP, PDCD10, and SLC20A1) were identified.

DISCUSSION

DKK3 is a member of the dickkopf Wnt signaling inhibitor family. DKK family members antagonize Wnt signaling via binding to the membrane receptor, Kremen and LRP5/6. However, DKK3 has not been shown to bind to either of them; therefore, it is believed that DKK3 cannot regulate Wnt/β-catenin signaling[1,2]. To date, the receptor of DKK3 has not been identified[1], and the constitutive role of DKK3 is yet to be investigated.

On the other hand, in the cancer research field, DKK3 is predominantly known by its alternative name, REIC (reduced expression in immortalized cells or reduced expression in cancer[3]). As indicated by the name, DKK3 mRNA and protein expression are downregulated in many types of malignancies[15], including glioma[16], esophageal SCC[17], gastric adenocarcinoma[18], colorectal adenocarcinoma[19], hepatocellular carcinoma[20], non-small cell lung cancer (NSCLC, including SCC)[21–23], renal cell carcinoma[24], bladder cancer[25], prostatic carcinoma[26], breast cancers[27,28], cervical SCC[29], and malignant melanoma[30].

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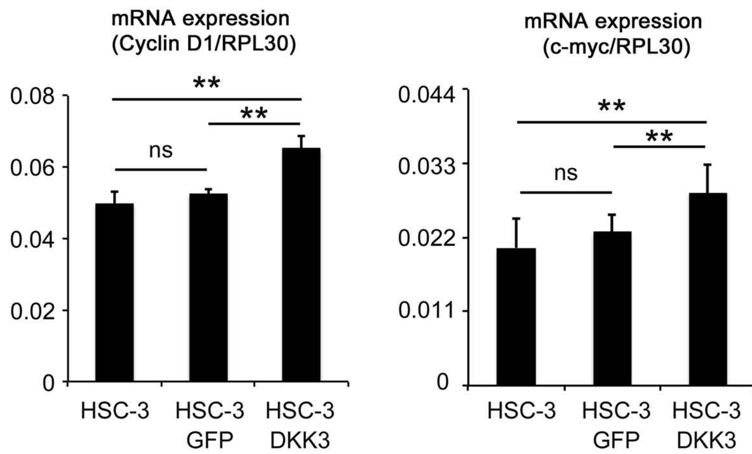
Figure 4. The expression of Wnt target genes, cyclin D1, and c-myc, was elevated in HSC-3 DKK3 cells (A), but neither the TOP/FOP reporter assay nor the TCF reporter assay showed TCF/LEF activation (B, C). Western blotting demonstrated that the expression and phosphorylation of Wnt/β-catenin signaling molecules or Smads were not altered in HSC-3 DKK3 cells, but that the phosphorylation status of Akt and c-Jun was changed (D). $^{**}p < 0.01$.


(A)
mRNA expression
(Cyclin D1/RPL30)
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ns
HSC-3
HSC-3 GFP
HSC-3 DKK3
mRNA expression
(c-myc/RPL30)
0.044
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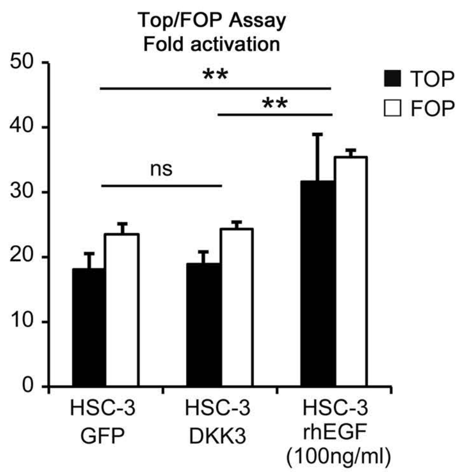
(B)
Top/FOP Assay
Fold activation
50
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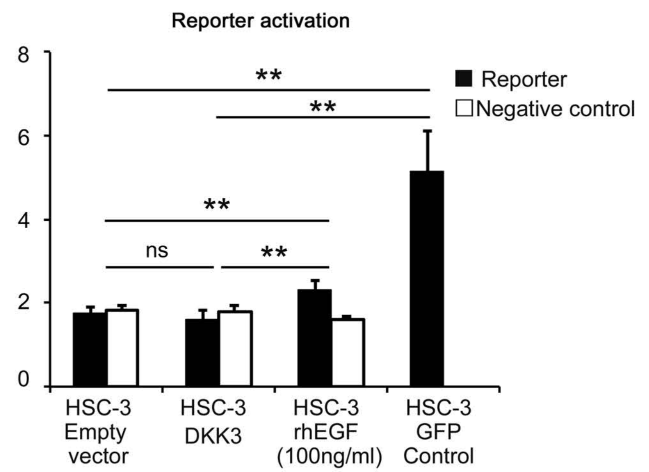
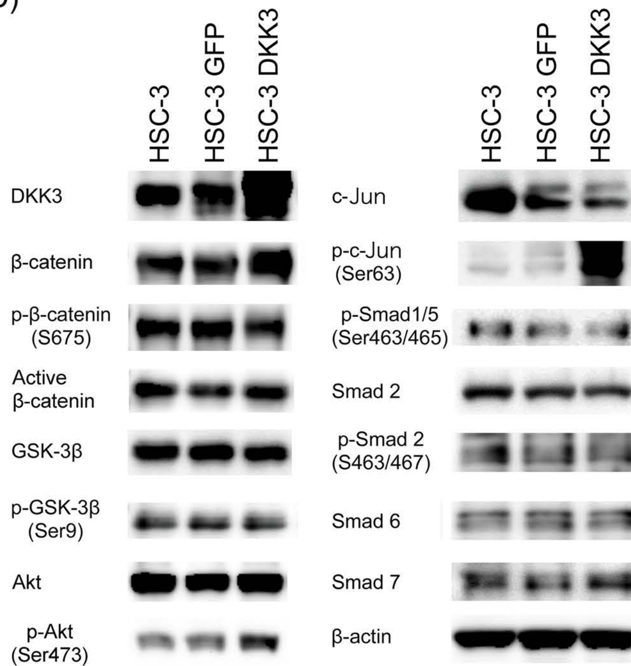
HSC-3 rhEGF (100ng/ml)


(C)
Reporter activation
8
6
4
2
0
**
**
**
ns
**
Reporter
Negative control
HSC-3 Empty vector
HSC-3 DKK3
HSC-3 rhEGF (100ng/ml)
HSC-3 GFP Control


(D)
HSC-3
HSC-3 GFP
HSC-3 DKK3
DKK3
β-catenin
p-β-catenin (S675)
Active β-catenin
GSK-3β
p-GSK-3β (Ser9)
Akt
p-Akt (Ser473)
c-Jun
p-c-Jun (Ser63)
p-Smad1/5 (Ser463/465)
Smad 2
p-Smad 2 (S463/467)
Smad 6
Smad 7
β-actin

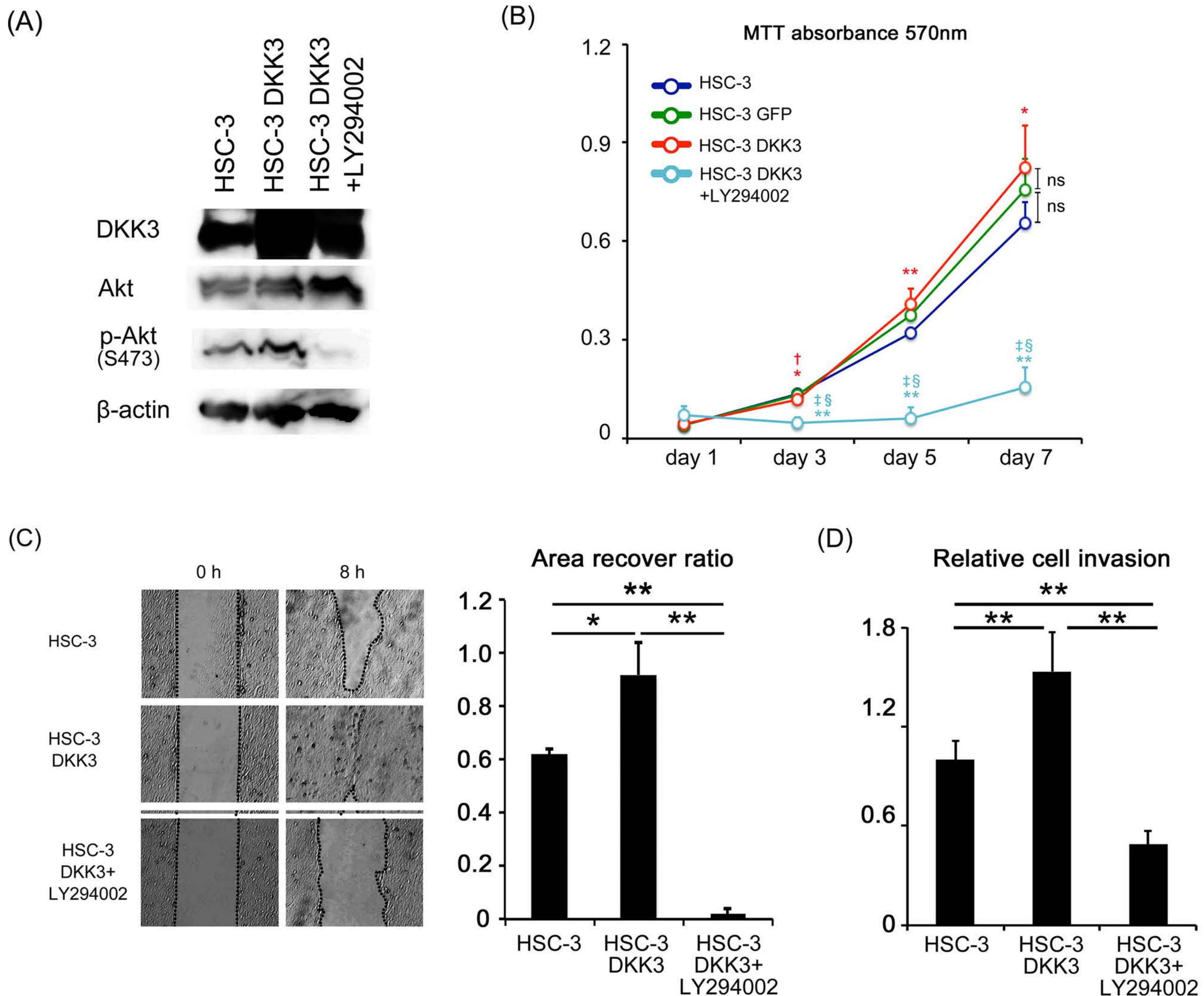


Figure 5. LY294002 (50 μM) decreased phospho-Akt (S473) without affecting DKK3 overexpression (A). Cellular proliferation in HSC-3 DKK3+LY294002 was significantly decreased (B). $**p<0.01$, $*p<0.05$ (vs. HSC-3), $‡p<0.01$, $†p<0.05$ (vs. HSC-3 GFP), and $§p<0.01$ (vs. HSC-3 DKK3). Red indicates HSC-3 DKK3, and pale blue indicates HSC-3 DKK3+LY294002. Migration and invasion were also decreased by LY294002 (C, D). Note that HSC-3 DKK3 showed significantly higher cellular proliferation, migration, and invasion than those of control HSC-3.

This downregulation or loss of expression was mainly caused by CpG island methylation[16–18,20,21,23,27,29]. Because of the widespread downregulation of DKK3 in cancer, DKK3 is considered to be a forcible tumor suppressor gene. Recent studies have demonstrated that adenovirus-mediated overexpression of DKK3 (Ad-REIC) induces cancer cell apoptosis; thus, DKK3 is thought to be attracting attention as a cancer therapeutic target. In fact, a phase I–IIa study of Ad-REIC gene therapy in prostate cancer is ongoing[31].

In contrast, our previous studies have demonstrated predominant DKK3 expression and its putative oncogenic function in HNSCC. We have shown that DKK3 is mostly expressed in HNSCC tissue samples and that patients with DKK3 expression showed significantly shorter disease-free and metastasis-free survival[7,8]. In addition, DKK3 knockdown by siRNA in HNSCC-derived cell lines resulted in reduced cellular migration and invasion[9]. These findings have led us to form the hypothesis that DKK3 may play an oncogenic role in HNSCC. In the present study, we compared the differential expression profiles of DKK3 in several kinds of cancer-derived cell lines and investigated the effect of overexpression in DKK3-expressing cell lines.

The key findings in the present study are as follows: (1) DKK3 expression was exclusively seen in HNSCC and ESCC; (2) DKK3 overexpression did not induce apoptosis in HSC-3 cells but increased cellular proliferation, invasion, migration, and in vivo tumor growth; and

(3) DKK3 overexpression resulted in elevated cyclin D1 and c-myc mRNA expression, which was not driven by Wnt/β-catenin signaling, and the malignant properties of DKK3-transfected HNSCC-derived cells were increased by augmentation of c-Jun phosphorylation and/or expression changes of the genes lured by DKK3 overexpression.

In accordance with the previous reports, DKK3 expression was not observed in gastric, colorectal, prostatic, or lung carcinoma-derived cell lines, whereas DKK3 was widely expressed in HNSCC and esophageal SCC. Oral mucosa and esophageal mucosa are both squamous epithelium, but the former derives from the ectoderm and the latter originates from the endoderm. Interestingly, cancer cells derived from lung SCC and those from adenocarcinomas did not express DKK3. Lung SCC arises from cells with squamous metaplasia in the lower respiratory tract, which originates from the endoderm (foregut), the same as the esophagus. Despite the difference in their origins, both oral and esophageal squamous mucosae express DKK3 protein under normal conditions[8,32,33]. DKK3 expression in these tissues is observed in the basal cell layer, suggesting its role in the maintenance and proliferation of squamous epithelia. Also, our findings were compatible with a previous report that DKK3 expression was upregulated in esophageal SCC and that DKK3 expression correlates with a poor prognosis[34].

As for pancreatic cancer, Gu et al.[35] demonstrated that PANC-1 cells did not show promoter methylation of DKK3, and its expression was conserved, whereas other cell lines (BxPC3, MIA Paca2, and AsPC-1) did not express DKK3. Interestingly, another study reported that PANC-1 cells express DKK3 protein to maintain the cells in dedifferentiation and proliferative states and that DKK3 knockdown resulted in the induction of $p15^{INK4b}$, $p21^{CIP1}$, and $p27^{KIP1}$ [36]. This supports our hypothesis that DKK3 may participate in oncogenic cellular proliferation in DKK3-expressing cancer cells.

In our experiments, DKK3 overexpression did not induce apoptosis either in HNSCC-derived HSC-3 cells or in prostatic cancer-derived PC-3 cells. Some researchers have pointed out that apoptosis induction might not be related to the function of endogenous DKK3 because the mechanism of apoptosis induction involves ER stress[37] and that antiapoptotic or antiproliferative effects of DKK3 overexpression might be in vitro artifacts caused by unfolded protein response[38]. In support of this, Romero et al.[37] established a DKK3 overexpression model by transfection of DKK3-expression plasmid without apoptosis induction in prostatic cancer-derived PC-3 cells. The response of the cells to DKK3 overexpression may differ by cell type, and it might be affected by the condition of gene transfer.

In the present study, we have demonstrated that DKK3 overexpression in HSC-3 cells increased the malignant properties with respect to proliferation, migration, invasion, and in vivo tumor growth. Initially, we thought that Wnt/β-catenin signaling might drive this response because Wnt/β-catenin signaling is also reported to be important for the malignant phenotype of HNSCC. High Wnt5A, Wnt7A, and Wnt 10B expression correlates with a poor prognosis[39]. Yang et al. have demonstrated that the Wnt/β-catenin signal might promote tumor cell migration and invasion[40]. However, the mRNA expression of Wnt target genes, cyclin D1, and c-myc, was upregulated, but the reporter assay for TCF/LEF activity denied this expectation. To seek an explanation for this, we investigated the expression and phosphorylation status of several signaling molecules. Western blotting results demonstrated that DKK3 overexpression slightly elevated phosphorylation of Akt (Ser473) and significantly increased c-Jun (Ser 63) phosphorylation, which may lead to proapoptotic resistance, cell cycle, and cell survival[41,42]. Importantly, these findings suggest the existence of a cell surface receptor for DKK3. In addition, phosphorylation of GSK-3β (Ser9) and the level of β-catenin (either phosphorylated or not) were not changed, which suggests that DKK3 might not be participating in the Wnt/β-catenin signal. β-Catenin expression is high in HNSCC cell lines and plays a role in cell survival, maintenance of cancer stemness, and self-renewal[43,44]. However, DKK3 did not seem to have an effect on the metabolism of β-catenin.

Activation of the PI3K–Akt signal also plays a role in tumor cell biology, including tumor cell growth and progression. Akt activation is commonly observed in HNSCC/OSCC[45,46], and it might modulate tumor cell aggressiveness[47]. For instance, phosphorylation of Akt (S473) correlates to local recurrence and a poorer prognosis[48] and an increase in the incidence of lymph node metastasis[49]. We then hypothesized that the increment of tumor aggressiveness in DKK3-transfectant HNSCC cells might be driven by Akt signaling. Application of LY294002, an inhibitor for Akt signaling to HSC-3 DKK3 cells, resulted in reduced phosphorylation of Akt (S473) and cancellation of the effects of DKK3 overexpression on cell proliferation, migration, and invasion. This suggests that DKK3 directly or indirectly modulates PI3K–Akt signaling. Zhang et al. reported that FRA1 (Fos-like antigen 1) might activate Akt, promote tumor growth, and enhance tumor cell migration by activating JNK/c-Jun in HNSCC[45]. There would be complex crosstalk between the PI3K–Akt signal and the JNK/c-Jun signal, and DKK3 might play a key integrator role in both signaling pathways.

Recently, the involvement of microRNAs (miRNAs) in cancer cell biology has been repeatedly reported. The miRNA that targets DKK3 has very recently been identified. Huo et al. demonstrated that miR-25 directly binds to the 3′-UTR region of DKK3 and negatively

regulates its expression in human melanoma-derived cells. Overexpression of miR-25 resulted in the reduction of DKK3 expression with elevated cellular proliferation, migration, in vivo tumor growth, and increased c-myc and cyclin D1 expression. In contrast, anti-miR-25 reduced cellular proliferation and migration[50]. In this context, DKK3 functions as a tumor suppressor in melanoma cells, as reported before[30], but what is important is that miR-25 targets DKK3. On the other hand, miR-25-3p expression is downregulated in OSCC (the sequence of miRNA corresponds to that in Huo et al.[50]). Upregulation of miR-25-3p in OSCC cell results in attenuated cellular proliferation with upregulated p21 (cip1) and p27 (kip1) expression. Moreover, cyclin D1, Akt, and FOXO1 expression was decreased, and phosphorylation of Akt and FOXO1 was inactivated in the miR-25-3p-transferred cells[51]. Taken together, these data might support our present results.

For further investigation, we compared the gene expression profiles among HSC-3, HSC-3 GFP, and HSC-3 DKK3 cells. Among the upregulated genes with DKK3 overexpression, anterior gradient protein 2 (AGR2) and SMG6 (also known as telomerase-binding protein EST1A) were thought to be important. AGR2 is a human homolog of secreted protein XAG-2 in *Xenopus laevis*, which is specifically expressed in the anterior region of the dorsal ectoderm during amphibian embryogenesis. Human AGR2 expression is reported to be upregulated in several malignancies, especially in hormone-dependent cancers (breast, prostate, and ovarian). AGR2 protein may interact with C4.4A and α-dystroglycan (DAG-1), which are related to metastasis formation. The role and regulating mechanism of AGR2 are not well described, but it is implied that some cellular signaling such as Her2/Her3, MAPK, and Akt may be its modulator[52]. In HNSCC, AGR2 plays a role in cancer cell self-renewal and epithelial–mesenchymal transition (EMT)[53]. SMG6 (EST1A) is a human counterpart of the "Ever Shorter Telomeres 1" gene in yeast *Saccharomyces cerevisiae*[54] and is important for telomere length homeostasis. In HNSCC, SMG6 is reported to be associated with the activation of telomerase[55]. For downregulated genes in DKK3 overexpression, apoptosis-related protein PDCD10 [also known as cerebral cavernous malformation 3 (CCM3)] was noted. PDCD10 is activated by oxidative stress and induces apoptotic cell death[56]. This may have an impact on the antiapoptotic features of DKK3 overexpression in cells.

In summary, our data strongly suggest an oncogenic role for DKK3 in HNSCC via activation of the Akt and c-Jun pathways and modulation of AGR2, SMG6, and PDCD10. Further studies are encouraged to clarify the exact role of DKK3 and discuss the possibility of clinical appreciation as a therapeutic target for cancer invasion and/or metastasis of HNSCC/OSCC.

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