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Nitric Oxide Production Upregulates Wnt/β-catenin Signaling By Inhibiting Dickkopf-1

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Abstract

Nitric oxide signaling plays complex roles in carcinogenesis, in part due to incomplete mechanistic understanding. In this study, we investigated our discovery of an inverse correlation in the expression of the inducible nitric oxide synthase (iNOS) and the Wnt/β-catenin regulator Dickkopf-1 (DKK1) in human cancer. In human tumors and animal models, induced nitric oxide (NO) synthesis increased Wnt/β-catenin signaling by negatively regulating DKK1 gene expression. Human iNOS (hiNOS) and DKK1 gene expression were inversely correlated in primary human colon and breast cancers, and in intestinal adenomas from Min ($Apc^{min/+}$) mice. NO production by various routes was sufficient to decrease constitutive DKK1 expression, increasing Wnt/β-catenin signaling in colon and breast cancer cells and primary human hepatocytes, thereby activating the transcription of Wnt target genes. This effect could be reversed by RNAi-mediated silencing of iNOS or treatment with iNOS inhibitors, which restored DKK1 expression and its inhibitory effect on Wnt signaling. Taken together, our results identify a previously unrecognized mechanism through which the NO pathway promotes cancer by unleashing Wnt/β-catenin signaling. These findings further the evidence that NO promotes human cancer and deepens insights in the complex control Wnt/β-catenin signaling during carcinogenesis.

Keywords

iNOS; nitric oxide; DKK1; Wnt/β-catenin; cytokine

Introduction

Both the Wnt/β-catenin and the inducible nitric oxide (NO) synthase (iNOS)/NO pathways have important roles in carcinogenesis (1–4), and both have been shown to be dysregulated

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No potential conflicts of interest were disclosed.

Authors' Contributions
Conceptions and design: Q. Du, X. Zhang, D.A. Geller
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Acquisition of data (provided animals, acquired and managed patients provided facilities, etc.): D.A. Geller, Q. Du, Q. Liu
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in colon and breast carcinomas (5–9). Genetic disruption of iNOS reduces the incidences of gastric carcinogenesis induced by *Helicobacter pylori* (10) and urethane-induced lung tumor formation and lower vesicular endothelia growth factor (VEGF) in mouse models (11, 12). Min mice with adenomatous polyposis coli (*Apc*) mutation, $Apc^{min/+}$, (13) that had chronic iNOS inhibition or double knock-out mice with $Apc^{min/+}$/$iNOS^{-/-}$ showed fewer tumors in the intestines (14). These results imply that iNOS-derived NO contributes to tumor formation and promotes carcinogenesis. Our previous studies demonstrate that the human iNOS (hiNOS) gene is a transcriptional target of Wnt/β-catenin signaling, and Wnt/β-catenin signaling regulates hiNOS gene expression through effects on NF-κB (15–17).

High levels of NO can cause DNA damage (18) and NO activates diverse signaling pathways to regulate gene expression (19) and proliferation (20). Recent findings have shown that iNOS expression is correlated with tumor growth and poor prognosis in estrogen receptor-negative breast cancer (8), melanoma (21, 22), glioma (23), and colon cancer patients (6, 24). NO activates epidermal growth factor receptor (EGFR) signaling pathway (25), and P53 and VEGF to promote angiogenesis of tumors (5).

The DKK family encodes at least 4 members of secreted proteins in vertebrates. DKKs are glycoproteins, DKK1, 2 and 4 regulate Wnt signaling through binding the same effectors (26). Among this gene family DKK1 was firstly identified as a Wnt antagonist and embryonic head inducer in *Xenopus* (27). Wnts activate canonical pathway through binding their receptor of the frizzled seven transmembrane class and co-receptor lipoprotein receptor-related protein 5/6 (LRP5/6) forming a ternary complex that stabilizes β-catenin. DKKs bind and modulate Wnt co-receptor of the LRP5/6 class, which are indispensable for routing the Wnt signaling to β-catenin pathway (28). LRP5 and LRP6 are closely related type 1 transmembrane proteins and function as Wnt co-receptor, whose activity is modulated by DKKs (28). Moreover, DKK1 is transcriptionally targeted by Wnt/β-catenin signaling and involved in an autocrine-loop for a negative feed-back mechanism that fine-tunes the regulation of Wnt/β-catenin signaling in cancer (29–31).

The genetic control mechanisms for the negative regulation of DKK1 have not been defined. Since DKK1 is an endogenous antagonist of Wnt/β-catenin, and the relationship between iNOS/NO and DKK1 has not been established, we tested our hypothesis that induced NO synthesis inhibits DKK1 expression, thereby abrogating the negative feedback of DKK1 on Wnt/β-catenin signaling. The net effect is a more powerful oncogenic Wnt/β-catenin pathway that promotes carcinogenesis.

Materials and Methods

Cell Lines and Reagents

The human cancer cell lines SW480, DLD1, HCT116, NCIH727, HepG2, MCF7, HeLa, and 293T were obtained from American Type of Culture Collection (ATCC). They were cultured at 37 °C in 5% CO_2 in media containing 10% fetal bovine serum (Clontech), 100 units/mL penicillin, 100 μg/mL streptomycin, and 15 mM Hepes (pH 7.4). SW480, DLD1, HeLa, HepG2, MCF7 and 293T cells were cultured in DMEM (Invitrogen Life Technologies), HCT116 in McCoy's 5A, and NCIH727 in 1640 medium (Invitrogen Life Technologies). ATCC test the authenticity of these cell lines using short tandem repeat analyses. SW480, DLD1, HCT116, NCIH727, HepG2, MCF7, HeLa, and 293T cells were used immediately following receipt. Bulk frozen stocks of SW480 and MCF7 cells were prepared immediately following receipt and used within 3 months following resuscitation; during this period, cell lines were authenticated by morphologic inspection, and tested negative for mycoplasma by Mycoplasma PCR ELISA kit (Roche) in November 2012. The authenticity of human primary hepatocytes was validated based on the protocol of National

Institutes of Health (NIH) Liver Tissue and Cell Distribution System. The hepatocytes were used immediately following receipt and cultured in Hepatocyte Maintenance Medium (HMM) (LONZA) with 5% Newborn Calf Serum. Unless indicated, cells were stimulated with a cytokine mixture (CM) consisting of 1,000 units/mL human TNF-α (R&D Systems), 100 units/mL IL-1β (R&D Systems), and 250 units/mL human IFN γ (R&D Systems) which were purified-recombinant proteins. All reagents were from sigma unless otherwise indicated. L-NIL; SNAP and 1400W were obtained from Cayman Chemical. BYK191023 was obtained from SantaCruz.

Human Tissue Specimen Acquisition

All human tissues were acquired in accordance with the University of Pittsburgh Institutional Review Board (IRB) protocols, and partial of human tissue samples included tissue arrays (IMH-327; IMH337; IMH 326; IMH336) were purchased from IMGENEX.

Mice and animal experiments

Female C57BL/6J-$Apc^{Min/+}$ and wild-type C57BL/6 mice at 8 weeks of age were purchased from The Jackson Laboratory. All of the experimental mice were terminated at the age of 16 weeks with ether anesthesia. Athymic nude mice (J: NU, female, 4–6 weeks old, and 16–20 g) were purchased from The Jackson Laboratory. Nude mice were injected with 5×10^6 MCF7 cells containing 200 μL growth factor-depleted Matrigel (BD Biosciences) subcutaneously into the lower flank. Tumors were measured every two days, and tumor volume was calculated by the following formula: (W^2 x L) x 0.5 (W, width; L, length). Tumors were frozen with OCT, and 5 μM sections were cut. Animal Care and Use Committee of the University of Pittsburgh approved the Animal Protocols, and experiments were performed in adherence to the National Institutes of Health Guidelines for the Use of Laboratory Animals. All of the animals were raised in plastic cages under specific pathogen-free conditions. Animals were fed a standard diet for mice and had free access to water in an animal facility of the University of Pittsburgh.

Plasmid Constructs

pMSCV-GFP-iNOS plasmid: the hiNOS cDNA fragment (3.5 kb-Hind III and EcoRV fragment) was subcloned into the multicloning site of the retroviral vector MSCV-GFP (32). The reporter plasmids pTOP-FLASH and pFOP-FLASH were kindly provided by Dr. Bert Vogelstein (John Hopkins University, Baltimore, MD; ref. (33). pCDNA3.1/V5-His-hDDK1 plasmid was kindly provided by Dr. Kestutis Planutis (Mount Sinai School of Medicine, New York, NY). Human Wnt3a expression vector was purchased from OriGene Technologies Inc.

shRNA Knockdown Assay

SureSilencing shRNA plasmid for hiNOS was purchased from Qiagen. The SureSilencing™ Pre-Designed shRNA plasmids specifically knockdown the expression of hiNOS gene by RNA interference. Short hairpin RNA (shRNA) sequence for iNOS is GCAGGTCGAGGACTATTTCTT and control sequence for non-specific and off-target effects is GGAATCTCATTCGATGCATAC. The vector contains the shRNA under control of the U1 promoter and neomycin resistance gene. The shRNA-iNOS and control vectors were transiently transfected into SW480 and MCF7 cells separately. The stably transfected cells were selected with G418.

Microarray Expression Analysis

Human Wnt Signaling Pathway Array (OHS–043) purchased from SuperArray was used. The microarray data in a MIAME-compliant format have been deposited to the Gene Expression Omnibus at NCBI. The accession number is GSE50009.

Total cellular RNA was extracted from cells using TRIzol reagent (Invitrogen). RNA was quantified by using NanoDrop 2000 (Thermo). Using the True-Labeling AMP Linear RNA amplification kit (SuperArray), the mRNA was reversely transcribed to obtain cDNA and converted into biotin-labeled cRNA using biotin-16-UTP (Roche) by in vitro transcription. Before hybridization, the cRNA probes were purified with an ArrayGrade cRNA cleanup kit (SuperArray). The purified cRNA probes were then hybridized to the pretreated Oligo GEArray Human Wnt Signaling Pathway Arrays (OHS-043, SuperArray), which cover 114 Wnt-related genes plus controls. After washing steps, array spots binding cRNA were detected by the chemiluminescence method according to the manufacturer's procedure. Spots were then analyzed and converted into numerical data by using the manufacturer (SuperArray) software.

Analysis of mRNA levels by qRT-PCR and RT-PCR

Total cellular or tissue RNA isolated with TRIzol reagent (Invitrogen) or RNeasy kit (Qiagen) and reverse transcribed into cDNA using Sprint RT Complete Products kit (Clontech). Differences in expression were calculated using the Ct method. qRT-PCR was analyzed by using StepOnePlus™ Real-Time PCR System using SYBR-Green Mastermix (Applied Biosystems) and gene-specific primers as follows. For qRT-PCR, human DKK1 primers: sense 5′-CTCGG TTCTC AATTC CAACG- 3′, antisense 5′–GCACT CCTCG TCCTC TG- 3′; human GAPDH: sense 5′–GGGAA GCTTG TCATC AATGG- 3′, antisense CATCG CCCA CTTGA TTTTG-3′; human p21 primers (PPH00211E) purchased from Qiagen. RT-PCR was analyzed by using TITANIUM one-step RT-PCR kit (BD Biosciences). For RT-PCR, human DKK1 primers: sense 5′–TTCCA ACGCT ATCAA GAACC T-3′, antisense 5′–CCAAG GTGCT ATGAT CATTA CC-3′; hiNOS primers: sense 5′–ACAAG CTGGC CTCGC TCTGG AAAGA-3′, antisense 5′-TCCAT GCAGA CAACC TTGGG GTTGA AG-3′, and human β-actin: sense 5′–ATGGA TGATG ATATC GCCGC GCT-3′, antisense 5′–GACTC GATGC CCAGG AAGGA-3′; mouse DKK1: sense 5′-GCCAGAGACACTAAACCGACAG-3′, and antisense 5′-GAGAAACAAGGCAATGTACCAC-3′. The primers were manufactured from Invitrogen.

Western Blotting Analysis

SDS-PAGE was preformed according to Towbin's method as previously described (15). The specific antibodies used for Western blot analysis were rabbit anti-hiNOS polyclonal (BD Biosciences); mouse anti-β-catenin monoclonal (Sigma); DKK1 antibody (R&D); DKK1, c-MYC, VEGF (SantaCruz) and Cyclin D1 monoclonal (MS-210-P1) (Neo Markers).

Immunofluorescent Staining

Cancer cell lines were cultured on coverslips, washed twice with cold PBS, fixed with 2% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 and 10% FBS in PBS for 30 min at room temperature, and incubated with the specific primary antibodies for β-catenin, DKK1, LS-A2867 (Lifespan Biosciences) and iNOS. Immunofluorescence staining was performed according to the procedures described previously (15). Slides were viewed with Olympus Provis microscope and FV1000 confocal microscope (Olympus).

Retroviral transduction

pMSCV-GFP vector or pMSCV-GFP-iNOS was cotransfected into 293T cells using a calcium phosphate precipitation method with pKat, an amphotropic packaging plasmid, and pCMV-VSV-G, a plasmid encoding the vesicular stomatitis virus G-glycoprotein. Supernatants containing pseudo-typed retrovirus were collected at 48 and 72 hours and were used to infect SW480, and DLD1 cell lines. SW480, and DLD1 cells were washed in phosphate-buffered saline (PBS) (Sigma) and resuspended at the concentration of 1 to 2 $\times 10^5$/mL in 1 mL of 80% retroviral supernatant and 20% fresh complete medium plus Polybrene (final concentration, 10 μg/mL) (Sigma). Cells in suspension were placed in a 48-well plate, spinoculated at 1,700 revolutions per minute for 50 minutes, and incubated at 37°C 5% CO_2 for an additional 6 to 8 hours, washed, and resuspended in fresh medium overnight. A second and a third infection was conducted on the following days using an identical procedure. Infection efficiency was evaluated by GFP expression 4 days after the last infection. hiNOS overexpression was also determined by using an antibody against hiNOS. GFP-positive cell enrichment was performed by fluorescence-activated cell sorting in some experiments (FACS Vantage; Becton Dickinson).

Adenoviral Vectors

The Vector Core carries a variety of reagents in the adenovirus and the stock of adenoviruses of the hiNOS and its control were provided by the University of Pittsburgh Pre-clinical Vector Core Facility based on the published paper.

Transient Transfection Assay

DNA transfections of cells were carried out in 6-well plates (Corning) by using Lipofectamine plus (Invitrogen) and MIRUS Trans-IT reagent (Mirus) as previously described (15, 16)

FACS Analysis/Flow Cytometry

FACS BrdU flow kit was purchased from BD Biosciences. Immunofluorescent analysis was performed on an LSRII (BD Biosciences), and 1–2.5 × 10^6 events were acquired per sample. Data were analyzed by using FlowJo software.

NO Production Assessment

Cell-cultured supernatants were collected and assayed for nitrite, the stable end products of NO oxidation, using the Greiss reaction as described (15).

Statistical Methods

Data are presented as the mean ± standard deviation (SD). Experiments were performed in duplicate or triplicate, and each was conducted a minimum of three times. Data were analyzed by the Student *t* test or analysis of variance where appropriate. Chi-square (χ^2) test was used to analyze the correlation between iNOS and DKK1 gene expression. $P < 0.05$ was considered statistically significant.

Results

DKK1 expression inversely correlated with iNOS and β-catenin translocation in human cancers

To define the relationship between iNOS, DKK1, and β-catenin, we examined the localization and expression of these proteins in human colon carcinoma *in vivo*. Immunofluorescence staining for β-catenin, hiNOS, and DKK1 expression was performed in

colon cancers from four patients along with background adjacent normal colon. In each case, the background normal colon demonstrated membrane bound cytosolic β-catenin, minimal hiNOS expression, and constitutive expression of cytosolic DKK1 (Fig. 1A). In contrast, the matching colon cancer specimen from the same patient showed nuclear localization of β-catenin, strong hiNOS expression, and decreased DKK1. Routine HE staining of these colon adenocarcinoma tumors is shown in Supplementary Fig. S1A. Human iNOS mRNA expression was significantly increased in the same four patients with primary colon cancer tumors (T) compared to adjacent normal (N) colon tissue determined by RT-PCR (Fig. 1B). Consistent with the immunofluorescent protein staining, DKK1 protein (Fig. 1C, lower), and mRNA levels (Fig. 1C, graph) were decreased in the colon cancer tumors compared to the normal tissue determined by Western blot and, quantitative real-time PCR, respectively. Likewise, the tumor suppressor gene $P21^{CIP1/WAF1}$ mRNA was downregulated in the colon cancer tumors compared to normal colon (Supplementary Fig. S1B). Next, we expanded the immunofluorescence staining for β-catenin, hiNOS, and DKK1 to an additional 11 human colon cancers (n=15). Each tumor was scored as positive (+) or negative (−) for the respected protein; β-catenin positivity referred to nuclear staining, while iNOS and DKK1 positivity referred to cytosolic staining (Supplementary Table S1). The normal background colon tissue was negative for β-catenin or iNOS staining in all 15 cases, while 14 of 15 normal colons had constitutive DKK1 expression (DKK1+). In the colon cancer tumors, as expected, all 15 stained positive for nuclear β-catenin. Interestingly, an inverse correlation was observed between iNOS and DKK1 in the colon cancers. 14/15 patients showed strong iNOS expression in the tumors along with decreased DKK1 expression compared to the constitutive DKK1 expression in the normal colon tissue. In contrast, the one colon tumor that was negative for iNOS had preserved DKK1 expression. These results suggest that cytoplasmic iNOS expression is inversely correlated with DKK1expression (P=0.0001) in colon cancer, and led us to hypothesize that iNOS/NO signaling decreased DKK1 expression. Diminished DKK1 expression would favor activation of canonical Wnt signaling and carcinogenesis.

To determine if this inverse correlation between iNOS and DKK1 expression was observed in other cancer types, we performed immunofluorescence staining for β-catenin, hiNOS, and DKK1 in human breast cancer (n=10) and primary hepatocellular carcinoma (HCC) tumors (n=15) along with their respective background normal tissues (Supplementary Table S1). In ten breast cancer tumors, 9 showed nuclear β-catenin positivity, and all 9 of these were DKK1 negative, while 8/9 were iNOS positive (P=0.003). Representative staining for β-catenin, hiNOS, and DKK1 in a breast cancer tumor is shown in Supplementary Fig. S1C. All 10 of the background breast tissues were negative for nuclear β-catenin, and most (9/10) were negative for iNOS, and positive for constitutive DKK. In contrast, this inverse correlation was not observed in primary liver cancer (HCC) tumors (P=0.5) (Supplementary Table S1). Only 4/15 (26%) HCC tumors exhibited nuclear β-catenin staining, which is consistent with the ~30% of HCC tumors that contain Wnt signaling (34). These results implied that the inverse correlation might be involved in the regulation of Wnt/β-catenin signaling in some cancers.

iNOS reversely correlated with DKK1 in intestinal tumors from $Apc^{min/+}$ mice

To further examine the inverse relationship between iNOS and DKK1 gene expressions *in vivo*, we utilized the $Apc^{min/+}$ mice which have mutated Apc resulting in strong Wnt/β-catenin signaling activation and spontaneous intestinal adenomas (35). The normal small intestine tissue showed constitutive DKK1 protein expression in the villi, and minimal iNOS and β-catenin protein (Fig. 2A, upper). In contrast, small intestinal adenomas from the $Apc^{min/+}$ mice had increased iNOS expression, decreased DKK1 expression, and increased and β-catenin protein staining (Fig. 2A, lower). Furthermore, small intestinal adenomas from

3 different $Apc^{min/+}$ mice exhibited high levels of endogenous iNOS protein in the adenomas, while iNOS was not detected in the intestine from normal mice (Fig. 2B, upper). Conversely, endogenous DKK1 protein was constitutively expressed in the normal intestine, but was markedly downregulated in the adenomas that expressed iNOS (Fig. 2B, upper). Likewise iNOS mRNA levels were induced in intestinal adenomas, while constitutive DKK1 mRNA was inhibited (Fig. 2B, lower). Together, our current findings of an inverse correlation between iNOS and DKK1 expression in primary tumors from patients and $Apc^{min/+}$ mice led us to further hypothesize that induced NO synthesis might also promote carcinogenesis by inhibiting DKK1 which is an antagonist of Wnt/β-catenin signaling. If so, nitric oxide mediated activation of β-catenin signaling by inhibiting DKK1 would be a novel and previously unrecognized function for induced NO synthesis during inflammation-associated cancers.

HiNOS/NO activated Wnt/β-catenin signaling

To explore this hypothesis, we generated stable transformed human colon cancer cell lines DLD1 and SW480 overexpressing hiNOS by infection with retroviral-hiNOS or GFP control plasmid. Western blot confirmed strong constitutive expression of hiNOS protein in the stable transformed hiNOS cells, while there was low-level endogenous hiNOS expression in the GFP-transduced cells (Fig. 3A). The stable hiNOS-transduced cells resulted in significant NO synthesis compared to the GFP-transduced cells, the increased NO synthesis was blocked by the iNOS inhibitor L-NIL, as expected (Fig. 3A, lower). The stable transformed hiNOS DLD1 cells maintained their inflammatory phenotype because they still responded to further endogenous hiNOS protein induction in response to stimulation by the cytokine mixture (CM) of TNFα+IL-1β+IFNγ (Fig. 3B).

Next, to determine whether iNOS gene expression activates Wnt/β-catenin signaling, we transfected a β-catenin reporter pTOP and its negative control pFOP plasmid (33) into SW480-hiNOS and SW480-GFP cell lines, respectively. Constitutive iNOS gene expression significantly increased β-catenin/TCF4 regulated transcription (CRT activity) in SW480-hiNOS by 2-fold compared to the control SW480-GFP cells (Fig. 3C). However, whether this resulted in changes in TCF-4 regulated gene expression, such as c-MYC (36) remains unclear. SW480-iNOS and SW480-GFP cell lines were used to examine c-MYC expression by immunofluorescent staining. Overexpression of hiNOS upregulated c-MYC expression (red) in SW480-iNOS compared with control SW480-GFP cell line by confocal microscopy analysis (Fig. 3D). Next, to determine if cytokine-induced NO synthesis inhibited DKK1 in primary human cells, we stimulated freshly isolated human hepatocytes with CM. As we have previously shown, CM strongly induced human hepatocyte iNOS protein expression (Fig. 3E) (37). CM also inhibited basal DKK1 protein and increased nuclear β-catenin expression in the primary human hepatocytes, as well as increased CRT activity by 67% (Fig. 3E, graph).

Wnt/β-catenin signaling was increased by hiNOS via downregulating DKK1 in cancer cells

Using the stable transformed colon cancer SW480-iNOS cells or SW480-GFP control cells, we found that hiNOS expression significantly decreased DKK1 mRNA by real-time qRT-PCR (Fig. 4A) and DKK1 protein levels by Western blot (Fig. 4B) compared to GFP-transduced cells. To examine the global effect of iNOS on Wnt signaling, we profiled the effect of iNOS overexpression on 114 genes involved in Wnt/β-catenin signaling using the Oligo GEArray Human Wnt Signaling Pathway Microarray analysis (SuperArray Bioscience). Total RNA was isolated from the stable transformed SW480-iNOS or SW480-GFP cells and then hybridized to the Wnt signaling microarray panel. In the SW480-iNOS cells which exhibited ~8-fold increase of induced NO synthesis (Fig. 3A), multiple Wnt signaling genes were either up-regulated (red) or down-regulated (green) (Supplementary

Fig. S2). The top 16 genes that are upregulated in the SW480-iNOS cells compared to the SW480-GFP cells are shown in Supplementary Table S2, while the downregulated genes are shown in Supplementary Table S3. Those genes that showed a change of greater than 1.5-fold increase are shown in red, while those that were decreased by greater than 1.5-fold are shown in green (Supplementary Fig. S2). Noteworthy is that the microarray analysis confirmed that DKK1 gene expression was dramatically decreased in SW480-iNOS cells compared to SW480-GFP control cells (Supplementary Fig. S2). Also of interest is that multiple other genes in the Wnt/β-catenin pathway were influenced by induced NO synthesis as has been shown for NO-mediated effects on apoptosis and inflammatory molecule pathways. Likewise, using infection of the MCF7 breast cancer cell line with AdiNOS or AdLacZ, overexpression of hiNOS downregulated constitutive expression of DKK1 mRNA and DKK1 protein levels (Fig. 4C). DKK mRNA and protein expression were also downregulated in MCF7 human breast cancer cells stimulated with CM to induce endogenous iNOS expression (Fig. 4D). Furthermore, the NO donor SNAP decreased DKK1 mRNA in HCT116 colon cancer and MCF7 breast cancer cell lines by RT-PCR (Supplementary Fig. S3A).

This downregulation of DKK1 by hiNOS expression in the colon and breast cancer cell lines is consistent with our *in vivo* findings of an inverse relationship between hiNOS and DKK1 in the colon and breast cancer tumors. Since DKK1 is an antagonist of Wnt/β-catenin signaling, we next examined the effect of hiNOS overexpression on DKK1-mediated inhibition of Wnt/β-catenin transcriptional activation. Wnt3a protein activates the canonical Wnt signaling through binding to the Wnt3a receptor (2). Transfection of 293T cells with a Wnt3a expression plasmid significantly increased β-catenin reporter activity of the pTOP-luciferase plasmid (Fig. 4E). Co-transfection of a DKK1 expression plasmid antagonized the Wnt3a stimulated β-catenin transcription, while addition of the hiNOS plasmid in the co-transfections abrogated the inhibitory effect of DKK1 on β-catenin activation (Fig. 4E). Transfection of DKK1 alone (in the absence of Wnt3a) did not affect basal β-catenin reporter activity. To our knowledge, this is the first report that the hiNOS/NO pathway promoted Wnt/β-catenin signaling by decreasing DKK1 gene expression.

Knocking-down hiNOS upregulated DKK1 and downregulated Wnt/β-catenin signaling

To provide functional evidence that hiNOS regulates DKK1 and subsequent Wnt/β-catenin signaling in cancer cells, we used shRNA to specifically knockdown iNOS gene expression in SW480 and MCF7 cells. Stable expression of shRNA targeting hiNOS or negative control (NC) non-sense sequence were generated in SW480 and MCF7 cells using a vector with the U1 promoter and neomycin resistance gene. The stable transfected cells were selected with G418. Stable expression of shRNA-hiNOS in SW480 (Fig. 5A) and MCF7 (Fig. 5B) cells knocked down endogenous hiNOS mRNA which was confirmed in two different stable transformed colonies (shRNA-iNOS1 and shRNA-iNOS2) compared to negative control shRNA-NC. Likewise, hiNOS protein levels were also knocked down in a similar manner (Fig. 5A, 5B, Western blots). Silencing of hiNOS expression with shRNA-hiNOS decreased iNOS mRNA and protein levels, and simultaneously increased DKK1 protein expression (Fig. 5A, B, Western blots). Knocking-down hiNOS gene expression in the SW480 and MCF7 cells also significantly downregulated Wnt/β-catenin signaling by 60% and 52%, respectively analyzed by pTOP/pFOP luciferase reporter assay (Fig. 5A, B). Knock-down of hiNOS gene expression in the SW480 colon cancer cells was associated with diminished fraction of cells in S phase from 29.3% to 13.2% compared to the negative controls, and is consistent with cell growth inhibition (Fig. 5C). Likewise, shRNA-hiNOS exhibited similar results with S phase decreasing from 21.9% to 7.8% in MCF7 breast cancer cells (Fig. 5C). Hence, the inverse relationship between iNOS and DKK1 gene expression and their effect on regulating Wnt signaling and cancer cell proliferation was further confirmed by these

shRNA-iNOS studies. Taken together, the results indicate that endogenous iNOS/NO signaling in cancer cells suppresses DKK1 expression and in turn promotes Wnt/β-catenin signaling.

Inhibition of induced NO synthesis decreased cancer growth

The ability of iNOS-selective inhibitors BYK191023 or 1400W to inhibit iNOS-generated NO was confirmed in human 293T cells that were transiently transduced to express human iNOS (Fig. 6A). The effect of iNOS inhibition on tumor cell growth was demonstrated in MCF7 breast cancer cells which exhibit spontaneous hiNOS expression (Fig. 5B). Treatment with iNOS-selective inhibitors BYK191023 or 1400W markedly inhibited MCF7 cell growth compared to control cells (Fig. 6B). Next, we tested the effect of the iNOS inhibitor BYK191023 in nude mice bearing subcutaneous human breast cancer MCF7 xenografts. After an engraftment period, tumor-bearing animals were randomly assigned to treatment groups. Mice receiving 60 mg/kg of BYK190123 twice daily for two weeks had reduced tumor volumes compared to vehicle controls (Fig. 6C). Further, staining of these tumors confirmed that iNOS inhibitor BYK191023 increased DKK1 expression and decreased β-catenin nuclear translocation in the xenograft tumors (Fig. 6C, lower). These findings suggest that induced NO synthesis is important for cell growth and proliferation in MCF7 cancer cells. iNOS/NO play an important role in cellular homeostasis by maintaining a balance between proliferation and senescence via its regulation of DKK1 and Wnt signaling.

By inhibiting DKK1, iNOS/NO increased the expressions of Wnt/β-catenin signaling target genes

To determine if iNOS overexpression could activate Wnt/β– catenin target gene signaling, we overexpressed hiNOS in 293T embryonic kidney cells by transfecting pCDNA3-hiNOS or control pCDNA3-vector. Immunofluorescence staining demonstrated that overexpression of hiNOS gene markedly decreased DKK1 expression, and increased expression of nuclear c-MYC (purple) in 293T-pCDNA3-hiNOS cells (Fig. 7A).

Since DKK1 plays a central role in inhibiting Wnt/β-catenin signaling, and since a previous report showed a low level of endogenous DKK1 expression in the SW480 cell line (38), we generated a stable transformed SW480-DKK1cell line that overexpressed DKK1 to test whether DKK1 could inhibit endogenous Wnt/β-catenin signaling. Compared with its control cell line (SW480-vector), DKK1 protein was strongly expressed in SW480-DKK1 by Western blot analysis (Supplementary Fig. S3B). Moreover, DKK1 overexpression was able to inhibit Wnt/β-catenin signaling evaluated by pTOP/pFOP reporter assay. β-catenin/TCF4 regulated transcription (CRT) was decreased in SW480-DKK1 relative to its control cell line (Supplementary Fig. S3C). Similar to endogenous DKK1 effects, exogenously expressed DKK1 also inhibited canonical Wnt signaling, and its target gene, c-MYC expression (Fig. 7B).

In inflammation-associated cancers, the iNOS gene is often activated. However, it is unclear whether iNOS or DKK1 would have the dominant role in regulating Wnt/β-catenin signaling if both were expressed. Therefore, we generated stable transformed SW480 colon cancer cells over-expressing both hiNOS and DKK1 (Supplementary Fig. S3D). Overexpressed DKK1 decreased Wnt/β-catenin target gene c-MYC expression (Fig. 7B), while hiNOS overexpression increased c-MYC and Cyclin D1 as expected (Fig. 7D). This same relationship was observed in another human colon cancer cell line HCT116, where DKK1 overexpression decreased basal c-MYC protein levels (Supplementary Fig. S3E, lane 2), while hiNOS overexpression further increased c-MYC protein levels (Supplementary Fig. S3E, lane 4). It is interesting when both hiNOS and DKK1 were co-expressed, c-MYC and Cyclin D1 were dramatically inhibited (Fig. 7D). These findings indicated that exogenous

DKK1 reversed the effect of iNOS/NO on endogenous DKK1 and restored its inhibition on Wnt/β-catenin signaling.

Previous studies show that Wnt/β-catenin signaling pathway interacts with many oncogenic signaling, such as c-MYC, Cyclin D1, and VEGF. Wnt/β-catenin activation of these oncogenes is influenced by iNOS inhibition of DKK1. Since VEGF is a target gene of Wnt/β-catenin signaling, we overexpressed hiNOS by AdiNOS infection and this resulted in abundant hiNOS mRNA and increased VEGF protein, along with decreased levels of constitutive DKK1 mRNA in each of four cell lines: HeLa (endometrial), HCT116 (colon), NCIH727 (lung), and HepG2 (liver) cells (Fig. 7E). The same scenario was also observed in SW480-hiNOS cells where hiNOS overexpression induced VEGF protein expression (Fig. 7C). Collectively, these results support our notion that iNOS/NO inhibits DKK1 gene expression resulting in increased Wnt/β-catenin signaling to promote carcinogenesis.

Discussion

The canonical Wnt/β-catenin signaling is activated in many cancers, and has shown to be negatively regulated by DKK1 (26, 39). While we have previously shown that the human iNOS gene is a target gene activated by Wnt/β-catenin (15, 16), very little is known about the effect of iNOS expression and induced NO synthesis on β-catenin signaling. Therefore, we explored the potential interaction between iNOS/NO and DKK1 expression, and their effects on the Wnt/β-catenin oncogenic pathway.

The major and novel findings in this study are: 1) An inverse relationship between iNOS and DKK1 was identified in human colon and breast cancer tumors; 2) iNOS and DKK1 protein expression were inversely correlated in small intestine adenoma tumors in the $Apc^{min/+}$ mice. 3) iNOS overexpression or NO donor decreased DKK1 mRNA and protein in colon and breast cancer cell lines; 4) inhibition of DKK1 resulted in increased β-catenin transcriptional activation of oncogenic target genes c-MYC, CyclinD1, and VEGF; 5) cytokines known to induce iNOS also decreased endogenous DKK1 in breast cancer MCF-7 cells and in primary human hepatocytes; 6) knockdown (silencing) of hiNOS with shRNA abrogated the inhibition of DKK1 resulting in greater DKK1 protein and less β-catenin transcriptional activity; 7) iNOS blockade resulted in cancer cell growth inhibition and decreased breast cancer tumor burden in nude mice xenografts.

Our data identify a previously unrecognized mechanism by which the iNOS/NO pathway promotes cancer by inhibiting DKK1, which unleashes Wnt/β-catenin signaling leading to enhancing β-catenin target gene activation. Cytokine induction or overexpression of the hiNOS gene activated canonical Wnt signaling in cancer cells by downregulating DKK1 expression. Moreover, shRNA silencing of iNOS expression or biochemical iNOS inhibition decreased Wnt signaling by upregulating DKK1 expression, suggesting a pivotal role of the iNOS/NO pathway in negatively regulating DKK1 to promote Wnt/β-catenin signaling in cancer cells. These findings provide new insight into the complex control mechanisms of iNOS/NO, DKK1, and Wnt/β-catenin during carcinogenesis.

The exact molecular mechanism(s) by which iNOS/NO downregulates DKK1 expression is not known. Interestingly, the DKK1 promoter contains NF-κB response elements, and previously we and others have shown that NO inhibits NF-κB DNA-binding activity (40, 41). Additional mechanistic insight is explained by the positive and negative feedback loops for iNOS/NO, DKK1, and β-catenin signaling. DKK1 is a target gene for activation by β-catenin due to the presence TCF-binding elements (TBE) in the DKK1 promoter (31). A negative feedback loop occurs where β-catenin activates DKK1 transcription via TBE *cis*-acting motifs, and then DKK1 inhibits β-catenin in a negative feedback loop (27, 39).

Likewise, β-catenin transcriptionally activates iNOS by binding to two specific TBE in the hiNOS promoter (15). Induced NO synthesis inhibits DKK1, which prevents the constitutive downregulation of β-catenin. Hence, the repression of β-catenin by DKK1 is unleashed due to NO mediated inhibition of DKK1. The end result is a positive feedback loop where β-catenin drives iNOS transcription which feedbacks via NO to inhibit DKK1 and allows for greater β-catenin mediated transcriptional activity. C-MYC, CyclinD1, and VEGF are three β-catenin target genes that we have shown are induced as a result of iNOS expression and DKK1 inhibition. We acknowledge that additional mechanisms may account for NO-mediated inhibition of DKK1. For example, NO may directly influence other transcription factors controlling DKK1 gene expression, or NO may elicit epigenetic modifications including promoter methylation as has been shown for E-cadherin (42).

Induced NO synthesis upregulates β-catenin signaling by transcriptionally decreasing *DKK1* expression to maintain constitutively activated Wnt signaling. Wnt/β-catenin signaling works as a master switch in mediating cell proliferation, differentiation and apoptosis. DKK1 acts as an antagonist of Wnt/β-catenin signaling and plays a critical role in regulating Wnt/β-catenin signaling through a negative feed-back loop between Wnt/β-catenin and DKK1(26). Like other Wnt signaling inhibitors, sFRPs and WIF1, DKK1 are also involved in establishment of an autocrine loop, which mediates Wnt constitutive activation observed in breast, ovarian, lung and colon cancer cells (43–45). Canonical Wnt signals not only are essential for homeostasis of the intestinal epithelium (46), but also are required for onset and progression of carcinogenesis (11, 12, 14). We therefore, believe that cancer cells may take advantage of this mechanism to maintain their homeostasis within the network of cell proliferation, differentiation, and apoptosis. This phenotype prevents cancer cells from the dysregulation of their homeostasis, which is essential for cancer cell survival.

The iNOS/NO pathway controls Wnt signaling in malignant epithelial cells by directly decreasing DKK1 gene transcription. Through this regulatory loop iNOS/NO can upregulate Wnt signaling by blocking the DKK1 expression and thereby increasing TCF-4 target gene expression, such as c-MYC. C-MYC directly represses the Cyclin/CDK (CDK) inhibitor $P21^{CIP1/WAF1}$ and functions as a downstream effector of tumor suppressors including P53, BRCA1, WT1 and TGFβ (47). In addition, $P21^{CIP1/WAF1}$ controls cell cycle entry, and inhibits cellular growth in tissue culture and tumor xenograft formation (47, 48). Moreover, mammary gland targeted c-MYC-induced tumorigenesis was enhanced by $P21^{CIP1/WAF1}$ deficiency (49). In this study we also found that $P21^{CIP1/WAF1}$ mRNA expression was decreased in human cancer tissues (Supplementary Fig. S1B) and also inversely correlated with iNOS gene expression. Since c-MYC has been shown to repress the $P21^{CIP1/WAF1}$ promoter (50) and $P21^{CIP1/WAF1}$ expression (47), these data are consistent with our observations that $P21^{CIP1/WAF1}$ expression was decreased in our clinical colon cancers (Supplementary Fig. S1B). Following the inhibition of β-catenin/TCF4 activity by DKK1 overexpression, the decreased expression of c-MYC can allow for $P21^{CIP1/WAF1}$ transcription, which in turn mediates G1 arrest and differentiation (50). Our data support the notion that knockdown or inhibition of iNOS leads to downregulation of Wnt/β-catenin signaling and decreasing cancer cell proliferation and tumor growth. Blockage of Wnt signaling is an attractive strategy to overcome carcinogenesis. Inhibiting the iNOS/NO pathway may be a suitable therapeutic target in certain subpopulations of cancer cells where iNOS/NO contributes to the malignant phenotype (23). Alternatively, augmenting DKK1 expression might be an approach to block β-catenin mediated carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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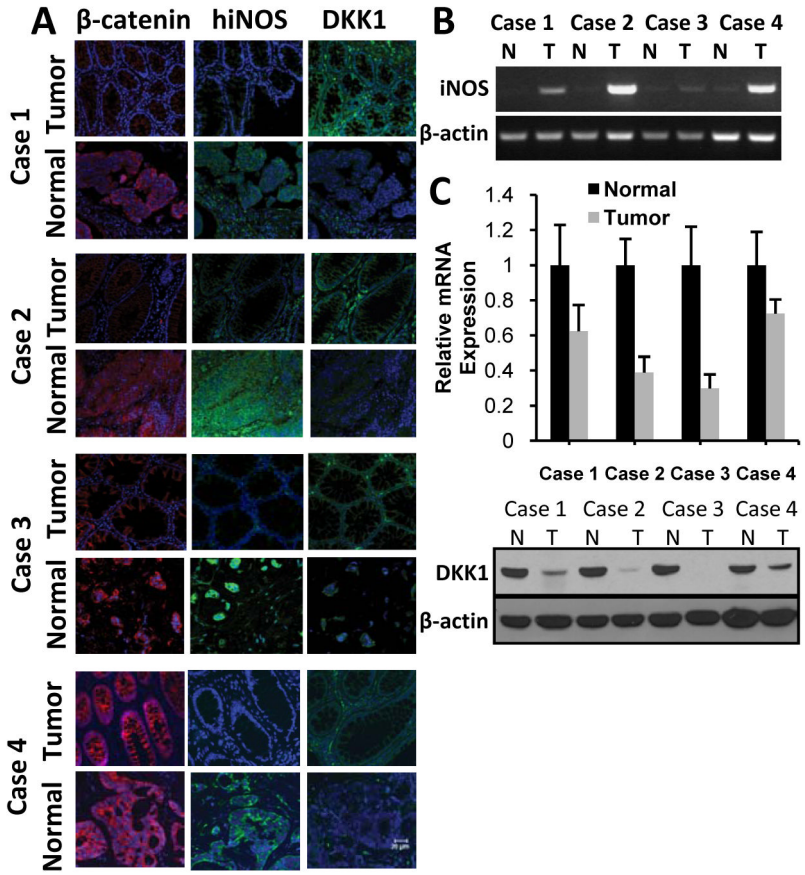

Figure 1.

iNOS expression correlated with the expression of DKK1 and β-catenin translocated to cytoplasm and nucleus in human tumors. A, Primary human colon cancer specimens were stained with the indicated antibodies specific to iNOS, DKK1 and β-catenin by immunofluorescent analysis. Scale bars, 20 μM. B, iNOS gene expression in colon cancer compared to the normal adjacent tissue was determined by RT-PCR. C, DKK1 gene expression in colon cancer tissues was compared with normal adjacent tissues determined by qRT-PCR (graph) and Western blot analysis.

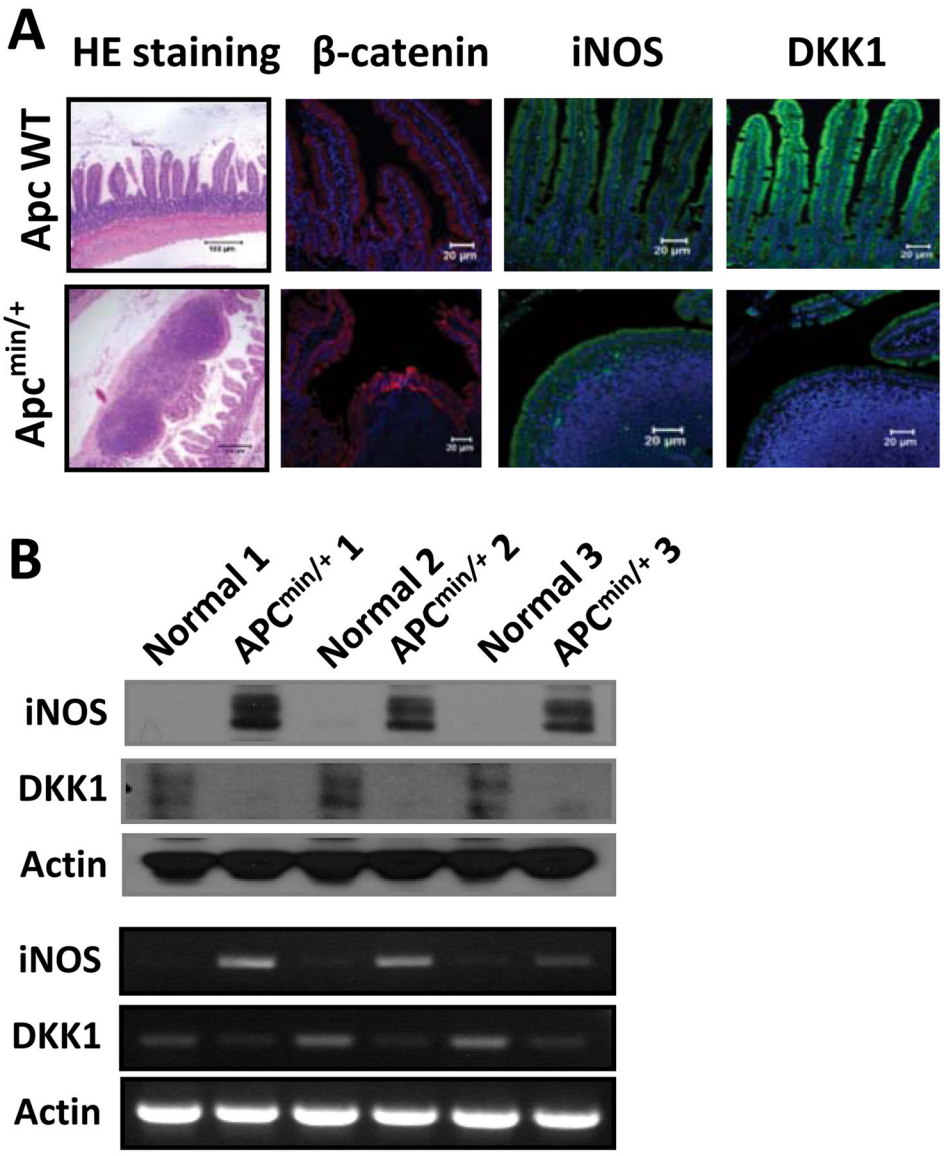

Figure 2.

hiNOS gene inversely correlated with DKK1 expression in $Apc^{min/+}$ mice. A, Normal and adenoma-bearing small intestinal specimens from APC wild type and $Apc^{min/+}$ mice, respectively were analyzed by immunofluorescence staining with the indicated antibodies. Scale bars, 20 μM. B, Normal and adenoma-bearing small intestinal specimens from *Apc* wild type and $Apc^{min/+}$ mice, respectively were analyzed by immunoblotting with the indicated antibodies (upper three blots), and RT-PCR with the indicated primers (lower 3 blots).

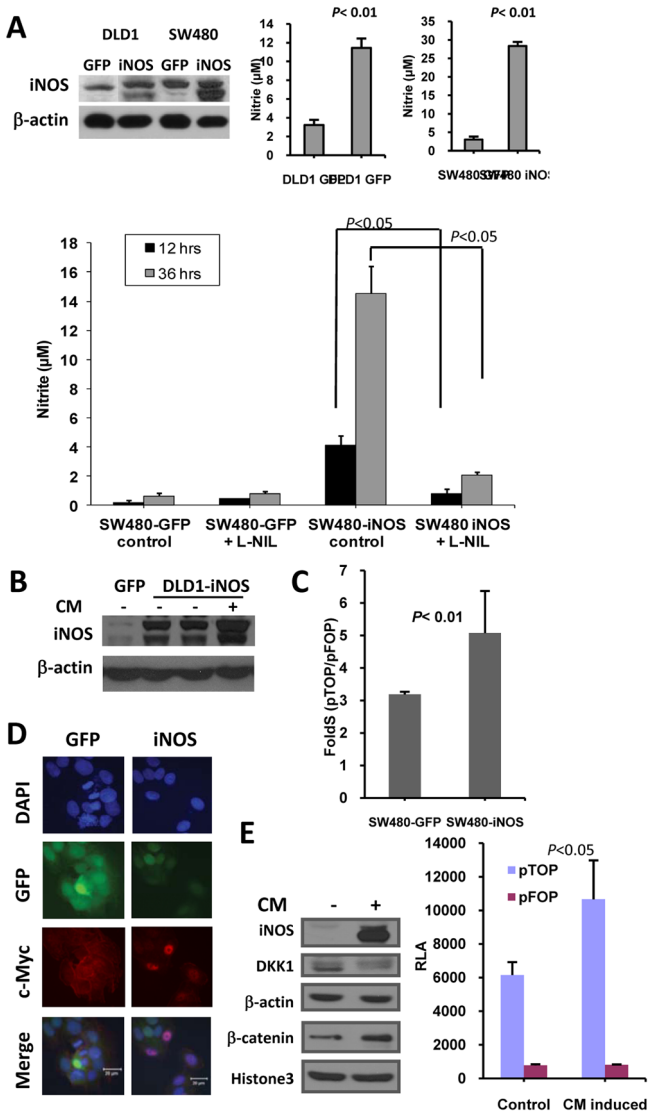

Figure 3.

hiNOS gene expression increased Wnt/β-catenin signaling. A, Stable expression of hiNOS in DLD1 and SW480 cells was established by infection with retroviral-hiNOS/GFP and retroviral-GFP. Whole cell lysates were collected after incubation of the cells for 24 hours and the basal levels of hiNOS protein expression in these cell lines were analyzed by immunoblotting compared with cells expressing GFP only. Nitrite production was detected after 48 hours in the media of the cell culture by Greiss assay. The cells expressing GFP were used as controls for the comparison. Also SW480-iNOS and SW480-GFP cell lines were incubated for 24 hours, and then treated with or without iNOS inhibitor, L-NIL (100μM) for 12 and 36 hours. The production of nitrite in the cultured media was detected by Greiss assay. B, DLD1 cells with stable expression of hiNOS or GFP were incubated for 24 hours and then treated with or without cytokine mixture (CM) for 8 hours. Whole cell lysates were collected for Western blot analysis with hiNOS antibody. C, SW480-hiNOS and SW480-GFP cells were transfected with pTOP/pFOP for 48 hours, and the cell lysates were used for luciferase reporter assay. HiNOS expression increased β-catenin/TCF4-regulated transcription (CRT) in SW480-hiNOS cells compared with control GFP. D,

SW480-iNOS and SW480-GFP cell lines were incubated for 24 hours followed by immunofluorescent staining with antibodies specific to c-MYC or iNOS by fluorescence microscopy analysis. Scale bars, 20 μM. E, Human hepatocytes were treated with CM for 8 hours. Total proteins were isolated for iNOS and DKK1 Western blot analysis, while nuclear proteins were isolated for β-catenin protein analysis. Human hepatocytes were also transfected with pTOP/pFOP for 24 hours, than CM treated for 8 hours, and the cell lysates were used for luciferase reporter assay.

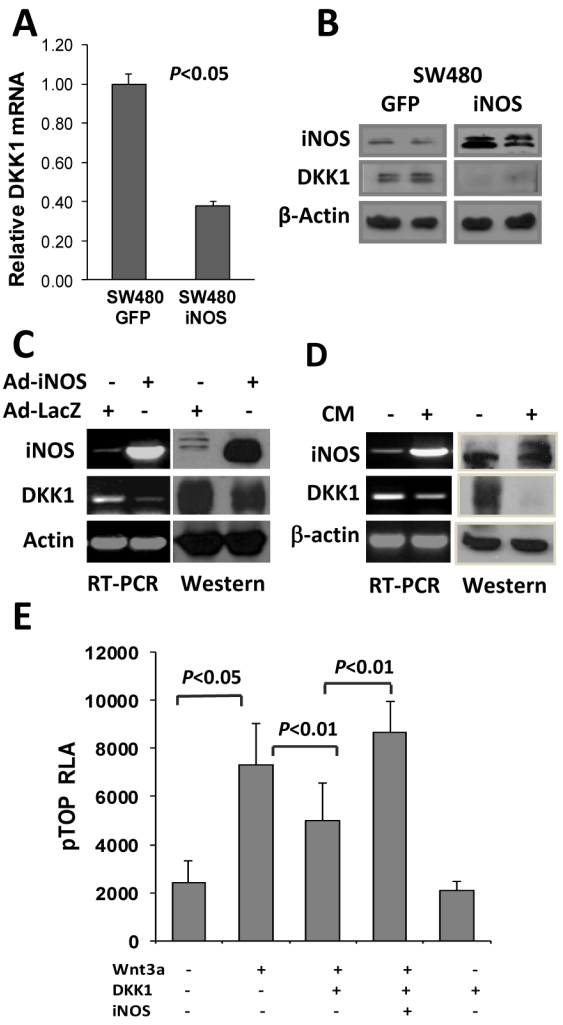

Figure 4.

hiNOS downregulated DKK1 expression. A, SW480-hiNOS and SW480-GFP cell lines were incubated for 36 hrs. Total RNA was isolated and DKK1 mRNA expression determined by qRT-PCR. B, SW480-hiNOS and SW480-GFP cell lines were incubated for 36 hours and Western blot was performed for DKK1 protein using whole cell lysates. C, MCF7 cells were infected with adenoviral-hiNOS or adenoviral-LacZ. After 24 hours, total RNA and proteins were isolated for the analysis of DKK1 mRNA expression by RT-PCR, and DKK1 protein expression by Western blotting. D, MCF7 cells were treated with CM for 8 hours. Total RNA and total proteins were isolated for RT-PCR and Western blotting analysis, respectively. E, 293T cells were transfected with pTOP FLASH (0.3 μg), Wnt3a (0.5 μg), DKK (0.5 μg) or iNOS (1 μg) and Wnt-luciferase reporter assay determined.

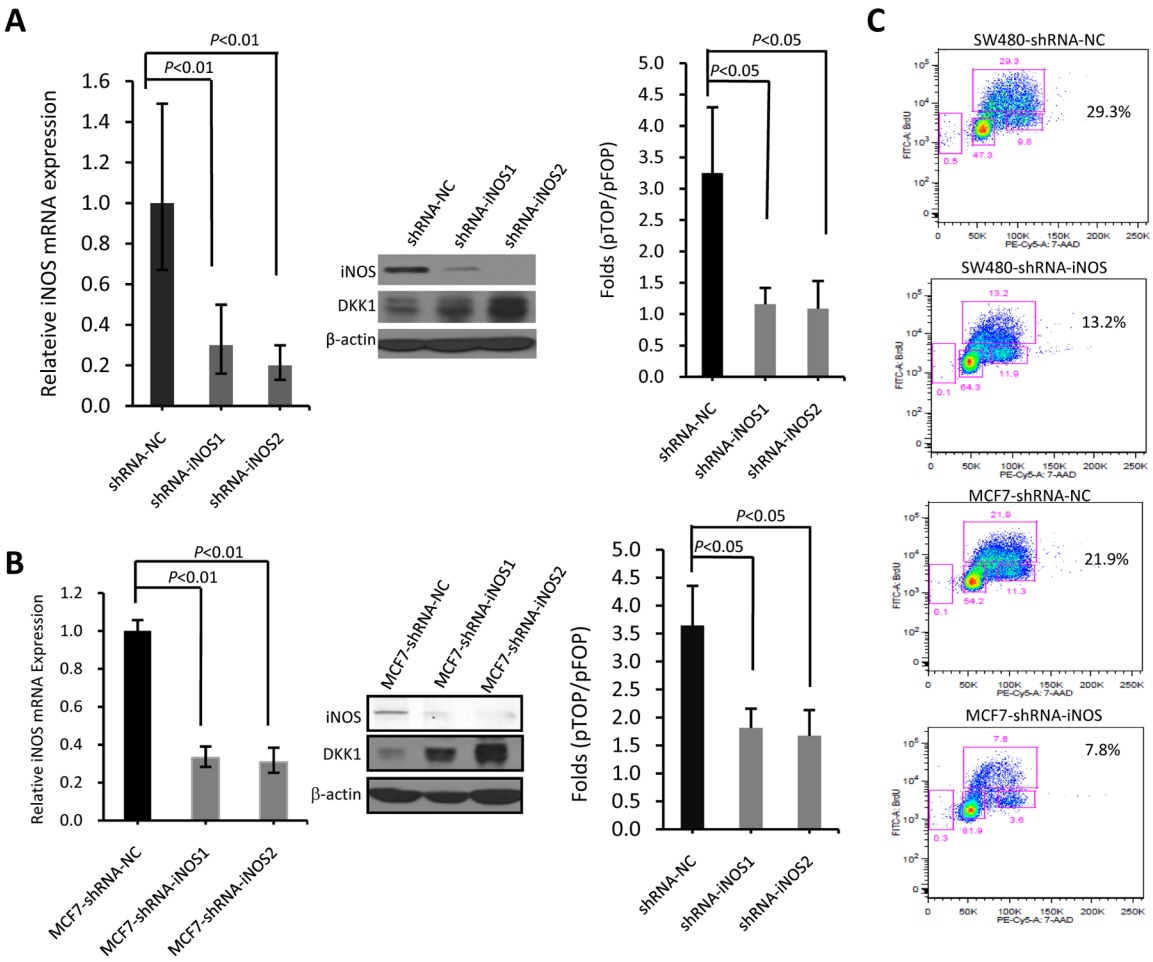

Figure 5.

Knock-down of the human iNOS gene with a specific shRNA in SW480 and MCF7cell lines led to increased DKK1 gene expression and decreased Wnt/β-catenin signaling. A, (left) SW480-shRNA-NC (NC) negative control and SW480-shRNA-hiNOS (cell lines 1 and 2) cell lines were incubated for 24 hours, and total mRNA was isolated for qRT-PCR. (middle) SW480-shRNA-NC and SW480-shRNA-hiNOS cell lines were incubated for 24 hours, and total proteins were isolated for Western blot analysis with the indicated antibodies. (right) SW480-shRNA-NC and SW480-shRNA-hiNOS cell lines were incubated for 24 hours. The cell lines were transfected with pTOP/pFOP plasmids for 36 hours followed by luciferase assay. B, (left) MCF7-shRNA-NC (negative control) and MCF7-shRNA-iNOS (cell lines 1 and 2) were incubated for 24 hours, and total mRNA was isolated for qRT-PCR. (middle) MCF7-shRNA-NC and MCF7-shRNA-iNOS were incubated for 24 hours; total proteins were isolated for Western blot analysis with the indicated antibodies. (right) MCF7-shRNA-NC and MCF7-shRNA-hiNOS cell lines were incubated for 24 hours. The cell lines were transfected with pTOP/pFOP plasmids for 36 hours followed by luciferase assay. C, Bromodeoxyuridine (BrdU) incorporation assay was employed to evaluate the effect of iNOS silencing on S phase cell cycle extent in SW480 colon (SW480-shRNA-iNOS) or MCF7 breast (MCF7-shRNA-iNOS) cells and compared to its negative control (-shRNA-NC) by FACS analysis.

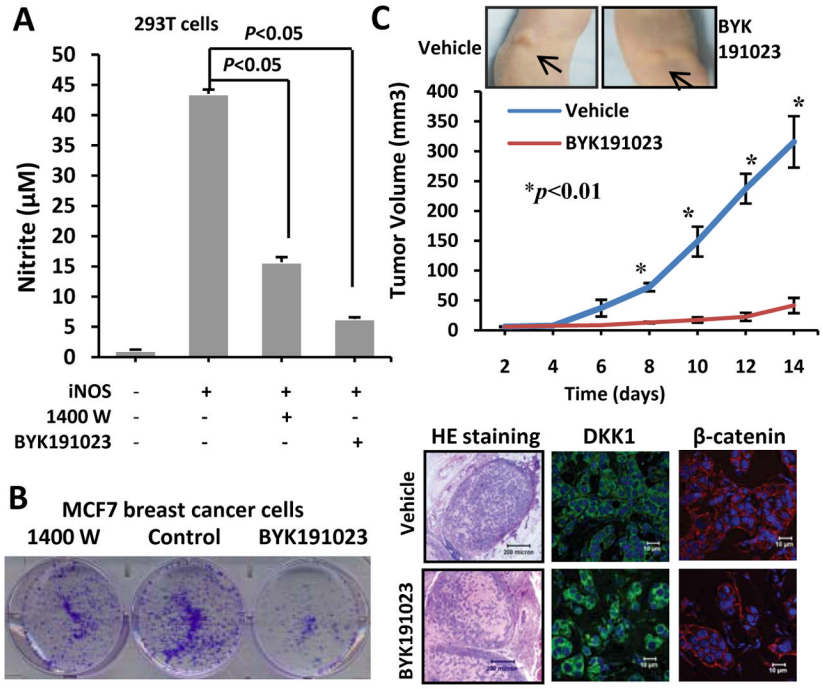

Figure 6.

Inhibition of iNOS decreased cancer cell growth. A, After 293T cells were transfected with hiNOS expression plasmid pCDNA3-hiNOS or empty vector pCDNA3 for 24 hours, the cells were treated with or without iNOS inhibitor (1400W 100 μM or BYK191023 100 μM) for 24 hours. The production of nitrite in the cultured media was detected by Greiss assay. B, Crystal violet staining of human MCF7 cells after 10 days with or without 1400W or BYK191023 is shown. C, (upper) Tumor volumes and gross images of MCF7derived subcutaneous xenografts treated with daily intraperitoneal vehicle (n=6) or BYK191023 (n=6). (lower) Vehicle and BYK191023 treated tumor were immunostained for DKK1 (green), β-catenin (red), or nuclei (blue) by confocal microscopy analysis. Scale bars, 10 μM.

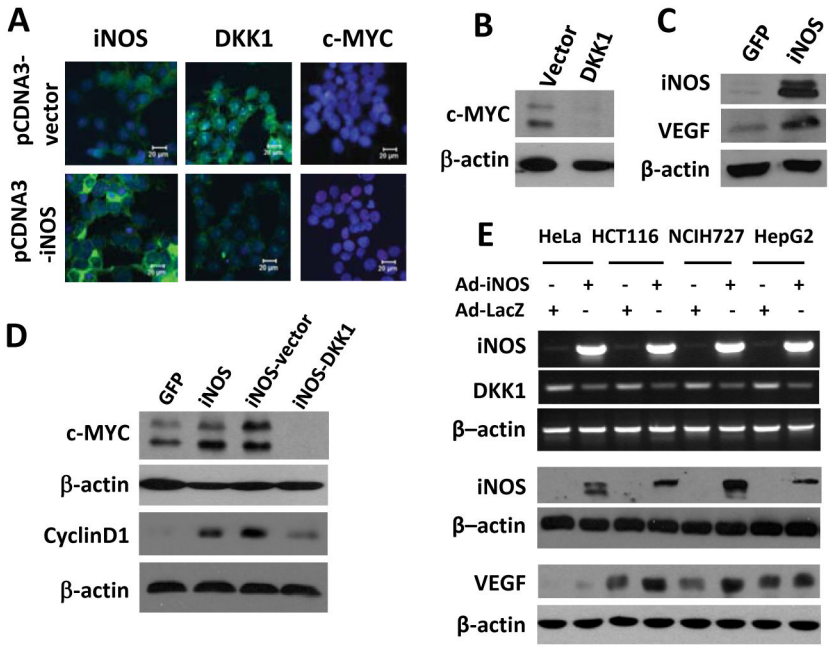

Figure 7.

iNOS/NO inhibited DKK1 and increased Wnt/β-catenin signaling target gene expressions. A, 293T cells were transfected with pCDNA3-hiNOS and pCDNA3-vector. Immunofluorescence staining with the indicated antibodies for hiNOS, DKK1, or c-MYC protein in 293T-pCDNA3-hiNOS cells, compared with its control cell line (293-pCDNA3-vector). The images were analyzed with confocal microscopy. Scale bars, 20 μM. B, SW480-DKK1/SW480-vector cell lines were incubated for 24 hours, and total proteins were collected for Western blot analysis with c-MYC antibody. C, SW480-iNOS and SW480-GFP cells were cultured for 24 hours. Total proteins were isolated for Western blot analysis with VEGF and iNOS antibodies. D, Two pairs of SW480-iNOS/SW480-GFP, and SW480-iNOS-DKK1/SW480-iNOS-vector cell lines were incubated for 24 hours, and total proteins were collected for Western blot analysis with c-MYC and Cyclin D1 antibody. E, The indicated cell lines were infected with Ad-iNOS or Ad-LacZ. After 24 hours, total proteins and mRNA were collected for Western blot and RT-PCR analyses with the indicated antibodies and primers.