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 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 (Apcmin/C0) mice. Nitric oxide 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 RNA interference-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 nitric oxide pathway promotes cancer by unleashing Wnt/β-catenin signaling. These findings further the evidence that nitric oxide promotes human cancer and deepens insights in the complex control Wnt/β-catenin signaling during carcinogenesis. Cancer Res; 73(21); 1–12. ©2013 AACR.

Introduction

Both the Wnt/β-catenin and the inducible nitric oxide synthase (iNOS)/nitric oxide (NO) pathways have important roles in carcinogenesis (1–4), and both have been shown to be dysregulated 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 VEGF in mouse models (11, 12). Min mice with adenomatous polyposis coli (Apc) mutation, Apcmin/+ (13), that had chronic iNOS inhibition or double knockout mice with Apcmin/+iNOS−/− showed fewer tumors in the intestines (14). These results imply that iNOS-derived nitric oxide contributes to tumor formation and promotes carcinogenesis. Our previous studies show 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 nitric oxide can cause DNA damage (18) and nitric oxide 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 patients with estrogen receptor-negative breast cancer (8), melanoma (21, 22), glioma (23), and colon cancer (6, 24). Nitric oxide activates EGF receptor 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, 3, 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 coreceptor lipoprotein receptor-related protein 5/6 (LRP5/6) forming a ternary complex that stabilizes β-catenin. DKKs bind and modulate Wnt coreceptor 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 I transmembrane proteins and function as Wnt coreceptor, 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 feedback 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. Because 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 nitric oxide 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 Culture Collection (ATCC). They were cultured at 37°C in 5% CO₂ in media containing 10% FBS (Clontech), 100 U/mL penicillin, 100 μg/mL streptomycin, and 15 mmol/L Hepes (pH 7.4). SW480, DLD1, HeLa, HepG2, MCF7, and 293T cells were cultured in Dulbecco’s Modified Eagle Medium (Invitrogen Life Technologies), HCT116 in McCoy’s 5A, and NCIH727 in 1640 medium (Invitrogen Life Technologies). ATCC tests 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 on the basis of the protocol of NIH Liver Tissue and Cell Distribution System. The hepatocytes were used immediately following receipt and cultured in Hepatocyte Maintenance Medium (LONZA) with 5% newborn bovine serum. Unless indicated, cells were stimulated with a cytokine mixture consisting of 1,000 U/mL human TNF-α (R&D Systems), 100 U/mL interleukin (IL)-1β (R&D Systems), and 250 U/mL human IFNγ (R&D Systems) that were purified recombinant proteins. All reagents were from Sigma unless otherwise indicated. L-NIL, SNAP, and 1400 W were obtained from Cayman Chemical. BYK-191023 was obtained from Santa Cruz Biotechnology.

Human tissue specimen acquisition

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

Mice and animal experiments

Female C57BL/6-J-Apc<sup>min/+</sup> 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 × 10⁶ MCF7 cells containing 200 μL growth factor-depleted Matrigel (BD Biosciences) subcutaneously into the lower flank. Tumors were measured every 2 days, and tumor volume was calculated by the following formula: \(W^2 \times L \times 0.5\) (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 carried out in adherence to the NIH 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). The 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.

Short hairpin RNA 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 nonspecific and off-target effects is GGAATCTCATTCGATG-G.

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) 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 was 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 \( G \) method. Quantitative reverse transcriptase PCR (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 AATCC CAACG-3', antisense 5'-GCAT CCTCG TCTCT TG-3'; human GAPDH: sense 5'-GGGAA GCTTG TCATC AATGG-3', antisense CATCG CCCA CTTG TTTTG-3'; human p21 primers (PH00211E) purchased from Qiagen. RT-PCR was analyzed by using TITAN-UM one-step RT-PCR kit (BD Biosciences). For RT-PCR, human DKK1 primers: sense 5'-TCTCA AGGTG AC AAGAC ACTGG-3', antisense 5'-CCAAG AATTC CAACG-3'; hiNOS primers: sense 5'-ACAAG GCTGC TCTGG TG-3'; antisense 5'-ATGGA TGATG ATATC GCCGC-3'; human \( \beta\)-actin: sense 5'-ATGGA TGATG ATATC GCCGC-3', antisense 5'-GACAT GATGT AGATG AACAG-3'; mouse DKK1: sense 5'-GAGAAA AAGCAG CAGC-3', and antisense 5'-GAGAACAAGGCGAATGACC-3'. The primers were manufactured from Invitrogen.

### Western blotting analysis

SDS-PAGE was conducted 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-\( \beta\)-catenin monoclonal (Sigma), DKK1 antibody (R&D Systems), DKK1, c-MYC, VEGF (Santa Cruz Biotechnology), 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 minutes, permeabilized with 0.1% Triton X-100 and 10% FBS in PBS for 30 minutes at room temperature, and incubated with the specific primary antibodies for \( \beta\)-catenin, DKK1, LS-A2867 (Lifespan Biosciences), and iNOS. Immunofluorescence staining was conducted 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 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 \( \mu \)g/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% \( \text{CO}_2 \) 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 conducted by fluorescence-activated cell sorting (FACS) 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 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 conducted on an LSRII (BD Biosciences), and 1 to 2.5 \( \times 10^6 \) events were acquired per sample. Data were analyzed by using FlowJo software.

### Nitric oxide production assessment

Cell-cultured supernatants were collected and assayed for nitrite, the stable end products of nitric oxide oxidation, using the Greiss reaction as described (15).

### Statistical analysis

Data are presented as the mean \( \pm \) SD. Experiments were carried out in duplicate or triplicate, and each was conducted a minimum of three times. Data were analyzed by the Student \( t \) test or ANOVA where appropriate. \( \chi^2 \) 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 \( \beta\)-catenin translocation in human cancers**

To define the relationship between iNOS, DKK1, and \( \beta\)-catenin, we examined the localization and expression of these proteins in human colon carcinoma in vivo. Immunofluorescence staining for \( \beta\)-catenin, hiNOS, and DKK1 expression was conducted in colon cancers from 4 patients along with background adjacent normal colon. In each case, the background normal colon showed membrane-bound cytosolic \( \beta\)-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 \( \beta\)-catenin, strong hiNOS expression, and decreased DKK1. Routine hematoxylin and eosin staining of these colon adenocarcinoma tumors is shown in Supplementary Fig. S1A. hiNOS mRNA expression was significantly increased in the same 4 patients with primary colon cancer tumors (T) compared with adjacent normal (N) colon tissue determined by RT-PCR (Fig. 1B). Consistent with the immunofluorescent protein staining, DKK1 protein (Fig. 1C, bottom), and mRNA levels (Fig.
1C, graph) were decreased in the colon cancer tumors compared with the normal tissue determined by Western blot analysis and quantitative real-time PCR, respectively. Likewise, the tumor suppressor gene P21CIP1/WAF1 mRNA was downregulated in the colon cancer tumors compared with 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, whereas 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, whereas 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 expression in the colon cancers. Fourteen of 15 patients showed strong iNOS expression in the tumors along with decreased DKK1 expression compared with 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 DKK1 expression (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 whether this inverse correlation between iNOS and DKK1 expression was observed in other cancer types, we conducted immunofluorescence staining for β-catenin, hiNOS, and DKK1 in human breast cancer (n = 10) and primary hepatocellular carcinoma tumors (n = 15) along with their respective background normal tissues (Supplementary Table S1). In ten breast cancer tumors, nine showed nuclear β-catenin positivity, and all nine of these were DKK1 negative, whereas eight of nine 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 (hepatocellular carcinoma) tumors (P = 0.5; Supplementary Table S1). Only four of 15 (26%) hepatocellular carcinoma tumors exhibited nuclear β-catenin staining, which is consistent with approximately 30% of hepatocellular carcinoma 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<sup>min/+</sup> mice

To further examine the inverse relationship between iNOS and DKK1 gene expressions in vivo, we used the Apc<sup>min/+</sup> 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, top). In contrast, small intestinal adenomas from the Apc<sup>min/+</sup> mice had increased iNOS expression, decreased DKK1 expression, and increased β-catenin protein staining (Fig. 2A, bottom). Furthermore, small intestinal adenomas from 3 different Apc<sup>min/+</sup> mice exhibited high levels of endogenous iNOS protein in the adenomas, whereas iNOS was not detected in the intestine from normal mice (Fig. 2B, top). Conversely, endogenous DKK1 protein was constitutively expressed in the normal intestine, but was markedly downregulated in the adenomas that expressed iNOS (Fig. 2B, top). Likewise iNOS mRNA levels were induced in intestinal adenomas, whereas constitutive DKK1 mRNA was inhibited (Fig. 2B, bottom). Together, our current findings of an inverse correlation between iNOS and DKK1 expression in primary tumors from patients and Apc<sup>min/+</sup> mice led us to further hypothesize that induced nitric oxide synthesis may 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 nitric oxide 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 analysis confirmed strong constitutive expression of hiNOS protein in the stable transformed hiNOS cells, whereas there was low-level endogenous hiNOS expression in the GFP-transduced cells (Fig. 3A). The stable hiNOS-transduced cells resulted in significant nitric oxide synthesis compared with the GFP-transduced cells, the increased nitric oxide synthesis was blocked by the iNOS inhibitor L-NIL, as expected (Fig. 3A, bottom). 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 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 pTOP 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 with the control SW480-GFP cells (Fig. 3C). However, whether this resulted in changes in TCF4-regulated gene expression, such as c-MYC (36), remains unclear. SW480-hiNOS 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 whether cytokine-induced nitric oxide synthesis inhibited DKK1 in primary human cells, we stimulated freshly isolated human hepatocytes with cytokine mixture. As we have previously shown, cytokine mixture strongly induced human hepatocyte iNOS protein expression (Fig. 3E; ref. 37). Cytokine mixture 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 qRT-PCR (Fig. 4A) and DKK1 protein levels by Western blot analysis (Fig. 4B) compared with 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 approximately 8-fold increase of induced nitric oxide synthesis (Fig. 3A), multiple Wnt signaling genes were either upregulated (red) or downregulated (green; Supplementary Fig. S2). The top 16 genes that are upregulated in the SW480-iNOS cells compared with the SW480-GFP cells are shown in Supplementary Table S2, whereas the downregulated genes are shown in Supplementary Table S3. Those genes that showed a change of more than 1.5-fold increase are shown in red, whereas those that were decreased by more 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 with 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 nitric oxide synthesis as has been shown for nitric oxide-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 cytokine mixture to induce endogenous iNOS expression (Fig. 4D). Furthermore, the nitric oxide 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. Because 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). Cotransfection of a DKK1 expression plasmid antagonized the Wnt3a-stimulated β-catenin transcription, whereas addition of the hiNOS plasmid in the cotransfections 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 with negative control shRNA-NC. Likewise, hiNOS protein levels were also knocked down in a similar manner (Fig. 5A and B, Western blot analyses). Silencing of hiNOS expression with shRNA-hiNOS decreased iNOS mRNA and protein levels, and simultaneously increased DKK1 protein expression (Fig. 5A and B, Western blot analyses). 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 and B). Knockdown 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 with 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 nitric oxide synthesis decreased cancer growth

The ability of iNOS-selective inhibitors BYK191023 or 1400 W to inhibit iNOS-generated nitric oxide was confirmed in human 293T cells that were transiently transduced to express hiNOS (Fig. 6A). The effect of iNOS inhibition on tumor cell growth was shown in MCF7 breast cancer cells that exhibit spontaneous hiNOS expression (Fig. 5B). Treatment with iNOS-selective inhibitors BYK191023 or 1400 W markedly inhibited MCF7 cell growth compared with 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 BYK191023 twice daily for 2 weeks had reduced tumor volumes compared with vehicle controls (Fig. 6C). Furthermore, staining of these tumors confirmed that iNOS inhibitor BYK191023 increased DKK1 expression and decreased β-catenin nuclear translocation in the xenograft tumors (Fig. 6C, bottom). These findings suggest that induced nitric oxide synthesis is important for cell growth and proliferation in MCF7 cancer cells. iNOS/NO plays 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 whether 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 showed that overexpression of hiNOS gene markedly decreased DKK1 expression, and increased expression of nuclear c-MYC (purple) in 293T-pCDNA3-hiNOS cells (Fig. 7A).

Because DKK1 plays a central role in inhibiting Wnt/β-catenin signaling, and because a previous report showed a low level of endogenous DKK1 expression in the SW480 cell line (38), we generated a stable transformed SW480-DKK1 cell 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. 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 overexpressing both hiNOS and DKK1 (Supplementary Fig. S3D). Overexpressed DKK1 decreased Wnt/β-catenin target gene c-MYC expression (Fig. 7B), whereas 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), whereas hiNOS overexpression further increased c-MYC protein levels (Supplementary Fig. S3E, lane 4). It is interesting to note that when both hiNOS and DKK1 were coexpressed, 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. Because 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 the four cell lines: HeLa (endometrial), HCT116 (colon), NCI-H727 (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). Although we have previously shown that the hiNOS gene is a target gene activated by Wnt/β-catenin (15, 16), very little is known about the effect of iNOS expression and induced nitric oxide 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: (i) an inverse relationship between iNOS and DKK1 was identified in human colon and breast cancer tumors; (ii) iNOS and DKK1 protein expression were inversely correlated in small intestine adenoma tumors in the Apcmin/Dkk1 protein expression were inversely correlated in small intestine adenoma tumors in the Apcmin/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.

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 nitric oxide 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 during carcinogenesis.

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The major and novel findings in this study are: (i) an inverse relationship between iNOS and DKK1 was identified in human colon and breast cancer tumors; (ii) iNOS and DKK1 protein expression were inversely correlated in small intestine adenoma tumors in the Apcmin/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.
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 nitric oxide synthesis inhibits DKK1, which prevents the constitutive downregulation of β-catenin. Hence, the repression of β-catenin by DKK1 is unleashed because of nitric oxide-mediated inhibition of DKK1. The end result is a positive feedback loop where β-catenin drives iNOS transcription, which feedbacks via nitric oxide 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 nitric oxide-mediated inhibition of DKK1. For example, nitric oxide may directly influence other transcription factors controlling DKK1 gene expression, or nitric oxide may elicit epigenetic modifications including promoter methylation as has been shown for E-cadherin (42).

Induced nitric oxide 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 feedback loop between Wnt/β-catenin and DKK1 (26). Like other Wnt signaling inhibitors, sFRPs and WIF1, DKK1 is 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 P21CIP1/WAF1 and functions as a downstream effector of tumor suppressors including P53, BRCA1, WT1, and TGFβ (47). In addition, P21CIP1/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 P21CIP1/WAF1 deficiency (49). In this study, we also found that P21CIP1/WAF1 mRNA expression was decreased in human cancer tissues (Supplementary Fig. S1B) and also inversely correlated with iNOS gene expression. Because c-MYC has been shown to repress the P21CIP1/WAF1 promoter (50) and P21CIP1/WAF1 expression (47), these data are consistent with our observations that P21CIP1/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 P21CIP1/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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception 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.): Q. Du, C. Bartels
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Q. Du, X. Zhang, Q. Liu
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Zhang, C. Bartels, D.A. Geller
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References
29. Chamorro MN, Schwartz DR, Vonica A, Bravaniou AH, Cho KR, Varmus HE. FGF-20 is transcriptional targets of beta-catenin and
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