Downregulation of iNOS/NO Promotes Epithelial–Mesenchymal Transition and Metastasis in Colorectal Cancer

Qiang Du[1], Silvia Liu[2], Kun Dong[1,3], Xiao Cui[1,4], Jing Luo[1,5], and David A. Geller[1]

ABSTRACT

Metastasis is the major cause of cancer-related death in patients with colorectal cancer. Although inducible nitric oxide synthase (iNOS) is a crucial regulator of cancer development and progression, its roles in epithelial–mesenchymal transition (EMT) and the pathogenesis of metastatic colorectal cancer have not been fully investigated. Primary colorectal cancer and liver metastatic tissue specimens were analyzed showing 90% of liver metastatic colorectal cancer with reduced expressions of iNOS compared with 6% of primary colorectal cancer. The Cancer Genome Atlas database analyses via cBioPortal reveal that mRNA expression of iNOS negatively correlated with selected EMT markers in colorectal cancer in a cancer type–dependent manner. The transcriptomic profiling (RNA sequencing data) indicates that iNOS knockdown in SW480 colorectal cancer cells induced an EMT program with upregulated expression of selected stem-cell markers. iNOS knockdown did not alter E-cadherin mRNA expression but re-localized it from membrane to cytoplasm through iNOS-GATA4-Crb2-E-cadherin pathway. iNOS knockdown induced a change in cell morphology, and promoted cell invasion and migration *in vitro*, and metastasis *in vivo*.

Implications: iNOS downregulation-induced pathway networks mediate the EMT program and metastasis. As an EMT inducer, the reduced-iNOS may serve as a potential therapeutic target for patients with colorectal cancer.

Introduction

Colorectal cancer is the third leading cause of cancer-related deaths and also the third most common cancer in the United States. About 60% of patients diagnosed with colorectal cancer will develop colorectal cancer liver metastases (CRLM). The process of epithelial–mesenchymal transition (EMT) is important in the pathogenesis of CRLM as it can convert epithelial cells into a mesenchymal phenotype and acquire the ability to migrate during embryogenesis. Cancer cells can reactivate EMT programs, which induce subpopulation of cancer stem cells (CSC), invasiveness, drug resistance, and metastasis (1). EMT also gives rise to a variety of intermediate states between the epithelia and the mesenchyme (called hybrid EMT; refs. 1, 2). Colorectal cancer cells undergoing EMT increase its motility with invasion and cell resistance to apoptosis, and initiates metastasis (3).

EMT is activated in response to signals that induce its transcription factors (EMT-TFs) including SNAIL1/2, TWIST1/2, ZEB1/2, and others, which down- or upregulate transcription of epithelial and mesenchymal markers, respectively (4). Normally, each EMT-TF selectively regulates the expression of its target genes, such as, E-cadherin, N-cadherin, and others (5). However, the physiological roles of EMT-TFs are common in embryogenesis, organism development, and recurrence in cancer cells (5). Expression of EMT-TFs can overlap and form networks (4, 6). Hence, EMT is not simply a single, linear process, but is highly variable and modular (7). In cancer, EMT-TFs are often activated partially and transiently, creating a metastable window for the induction of key cancer-relevant traits (6). The transcription of EMT markers is regulated by multiple signaling pathways (5).

The inducible nitric oxide synthase (iNOS, also called *NOS2*) transcript or protein does not appear to be present under normal conditions in most cells. iNOS induction and the subsequent production of gaseous free radical nitric oxide (NO) fights certain infections and has antitumor effects in innate immunity (8, 9). In many tumors, iNOS expression is high; however, the role of iNOS during tumor development is complex with both promoting and inhibiting carcinogenesis (10). Despite considerable progress in iNOS/NO biological research, the role of iNOS/NO on EMT and metastasis still needs to be clarified. NO can promote or inhibit tumor metastasis, depending on the concentration, lesion stage and organs involved (9, 11–13). The objective of this study is to determine whether iNOS/NO intrinsically regulates EMT and metastasis in colorectal cancer. Our results demonstrate that iNOS expression was reduced in the tissues of colorectal metastatic cancers. The Cancer Genome Atlas (TCGA) database analysis and RNA sequencing (RNA-seq) indicate that iNOS inversely correlates with the expression of select EMT markers. Knockdown of iNOS induced a hybrid EMT in part through the activation of iNOS-GATA4-CRB2-E-cadherin pathway. Downregulation of iNOS is essential for colorectal cancer cells to mediate EMT, invasion, dissemination, and metastasis.

Materials and Methods

Cell culture and reagents

The human cancer cell lines SW480, (ATCC, catalog no: CCL-228, RRID:CVCL_0546) SW620, (ATCC, catalog no: CCL-227, RRID:

[1]Department of Surgery, Thomas E. Starzl Transplant Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. [2]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. [3]Department of Pediatric Surgery, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, China. [4]Department of Surgery, The Second Hospital of Anhui Medical University, Hefei, Anhui, China. [5]Department of Surgery, The Second Xiangya Hospital of Central South University, Changsha, Hunan, China.

Corresponding Author: David A. Geller, University of Pittsburgh, Pittsburgh, PA 15213. Phone: 412-692-2001; E-mail: gellerda@upmc.edu

Mol Cancer Res 2023;21:102–14

doi: 10.1158/1541-7786.MCR-22-0509

This open access article is distributed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) license.

©2022 The Authors; Published by the American Association for Cancer Research

CVCL_0547) DLD1, (ATCC, catalog no: CCL-221, RRID: CVCL_0248) RKO, (ATCC, catalog no: CCL-2577, RRID:CVCL_0504) HT-29, (ATCC, catalog no: HTB-38, RRID:CVCL_0320) HCT116, (ATCC, catalog no: CCL-247, RRID:CVCL_0291) and HEK 293T (ATCC, catalog no: CCL-3216, RRID:CVCL_0063) cells were purchased from ATCC. SW480 cells were derived from a patient with primary colorectal cancer, while SW620 cells were obtained from lymph node metastatic cancer of the same patient. SW480, SW620, and 293T cells were cultured in DMEM medium as described in (14). HT-29 and HCT116 cells were cultured in McCoy's 5A medium. DLD1 and RKO cells were cultured in RPMI1640 and Eagle Minimum Essential Medium, respectively. Bulk frozen stocks of SW480, SW620, DLD1, RKO, HT-29, HCT116, and 293T cells were used after authentication by morphologic inspection and confirmed *Mycoplasma* negative by PCR ELISA Kit (Roche). iNOS inhibitor, L-NIL [L-N6-(1-iminoethyl)-lysine], and NO donor, SNAP (s-nitroso-n-acetyl penicillamine) were obtained from Cayman Chemical.

Western blot

Western blot procedure was performed according to the method as previously described (14). The primary antibodies E-cadherin (BD Biosciences, catalog no: 610181 RRID:AB_397581), N-cadherin (BD Biosciences, catalog no: 610920 RRID:AB_398236), Vimentin (BD Biosciences, catalog no: 550513 RRID:AB _393716), iNOS (BD Biosciences, catalog 610332, RRID:AB_397722), β-actin (Sigma-Aldrich, catalog no: MAB1501 clone C4; catalog no: A2066 RRID:AB_476693), Cleaved caspase-9 (Cell Signaling Technology, catalog no: 9502 RRID: AB_2068621); (Cell Signaling Technology, catalog no: 7237 RRID: AB_10895832), Fibronectin [Cell Signaling Technology, catalog no: 26836 Fibronectin/FN1 (E5H6X) Rabbit mAb], Gata4 (Cell Signaling Technology, catalog no: 36966 RRID:AB_2799108), Lamin A/C (Cell Signaling Technology, catalog no: 4777, RRID:AB_10545756), Snail2 (Cell Signaling Technology, catalog no: 9585, RRID: AB_2239535), Twist1 (Cell Signaling Technology, catalog no: 69366, RRID:AB_2891135), Zeb2 [Santa Cruz Biotechnology, catalog no: sc-271984 (E-11)], CD45 (Miltenyi Biotec, catalog no: 130–115–938, RRID:AB_2751284), EpCam (Invitrogen, catalog no: MA5–13917 RRID:AB_11001308); (Novus Biologicals, catalog no: NBP2–27107), CRB2 (ThermoFisher Scientific, catalog no: PA5–25628, RRID:AB_2543128).

qRT-PCR and RT-PCR

Total cellular or tissue RNA was isolated with TRIzol Reagent (Invitrogen, Carlshbad, CA) or RNeasy Kit (Qiagen) and reversely transcribed into cDNA using Sprint RT Complete Products kit (Clontech, Mountain View, CA). RT-PCR was analyzed by using TITANIUM one-step RT-PCR kit (BD Biosciences). qRT-PCR was analyzed by using StepOnePlus Real-Time PCR System using SYBR-Green Mastermix Kit (Applied Biosystems; ref. 15). Each sample was tested in triplicate. PCR product specificity was confirmed by a melting-curve analysis. GAPDH was used as an endogenous control. The fold change of mRNA expression was calculated by using $2^{-\Delta\Delta Ct}$ method. For qRT-PCR, human Snail2 primers sense, 5′-ATCTGCGGCAAGGCGTTTTCCA-3′ antisense 5′-ATCTGCGG-CAAGGCGTTTTCCA-3′; human iNOS primers Sense 5′-ACAAG CTGGC CTCGC TCTGG AAAGA-3′ Antisense: 5′-TCCAT GCAGA CAACC TTGGG GTTGA AG-3′ human GAPDH: sense 5′-GGGAA GCTTG TCATC AATGG-3′, antisense 5′-CATCG CCCA CTTGA TTTTG-3′; RT-PCR was analyzed by using TITANIUM one-step RT-PCR Kit (BD Biosciences). For RT-PCR, human iNOS 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′. The primers were manufactured from Invitrogen.

Immunofluorescence staining

Cancer cell lines were cultured on coverslips, washed twice with cold PBS, fixed with 2% paraformaldehyde in PBS for 30 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 iNOS, E-cadherin, N-cadherin, TWIST1, SNAIL2, and GATA4. Vimentin, Fibronectin, CD45, Epcam, and ZEB2. The nuclear DNA was stained with Hoechst dye (bis-benzimide; blue). Immunofluorescence staining was conducted according to the procedures described previously (14).

RNA-seq, data analysis, and cBioPortal TCGA data analysis

Total RNA from 1×10^6 SW480-shiNOS1/2 or SW480-shControl cells were collected for by using TRIzol (Invitrogen, Carlshbad, CA) following the manufacturer's instructions. RNA libraries were prepared for sequencing using standard Illumina protocols on Next-Seq 500 sequencer. RNA-seq was performed by the University of Pittsburgh Health Sciences Core Research Facilities (HSCRF) shared Genomics Research Core. Ingenuity Pathway Analysis (IPA) was used for pathway analysis.

RNA-seq data per library was processed by the following steps: quality control by FastQC, adapter sequences and low-quality read trimming by Trimmomatic, alignment to hg38 reference and gene quantification by STAR aligner. Eventually read counts per gene per sample were collected for downstream statistical analysis. Differential expression analysis was performed by R package DESeq2 (16) and differentially expressed genes (DEG) were defined by FDR = 5% and fold-change ≥ 1.5. DEGs were applied into IPA to detect the enriched the pathways. Significant pathways were defined by FDR = 5%.

The adenomatous polyposis coli (*APC*) mutation and the correlations between the mRNA expression of iNOS and representative EMT markers were analyzed via cBioPortal (http://www.cbioportal.org) using TCGA databases included colorectal adenocarcinoma, 524 samples; skin cutaneous melanoma, 363 samples; stomach adenocarcinoma, 407 samples; liver hepatocellular carcinoma (HCC), 348 samples; pancreatic adenocarcinoma, 168 samples and breast invasive carcinoma, 994 samples. Genomic profiles included mutation, copy-number and mRNA expression were selected.

Short hairpin RNA knockdown assay

Short hairpin RNA (shRNA)-iNOS versus its control RNA construct were purchased from (Qiagen, catalog no: KH00173N). The Pre-Designed shRNA plasmids specifically knockdown the expression of hiNOS gene by RNA interference. shRNA sequence for iNOS is GCAGGTCGAGGACTATTTCTT and control sequence for nonspecific 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 cells separately using Lipofectamine 2000 (Invitrogen) for 72 hours. The stably transfected cells were selected with G418. Four hairpins targeting human iNOS were screened and one that reduced mRNA and protein levels by >80% was identified (14).

Cell invasion, migration, and wound-healing assays

Cell invasion, migration and wound-healing assays were conducted using SW480-shiNOS1/2 and SW480-shControl cell lines according to the published article (17). Briefly, invasion and migration assays were performed using Boyden chambers (BD Biosciences) and 8-μm pore size membrane with Matrigel (for invasion assays) or without Matrigel (for migration assays). Cells (1×10^5 cells/well) were seeded in the top chamber of the trans-well. After 4-hour incubation, cells that had completely migrated to the bottom chamber were counted.

SW480 cell lines (1×10^6 cells/well) were grown in 6-well plates for 24 hours. A wound on the monolayers was scratched with a 10-μL pipette tip. Images were captured using an inverted digital camera at 0, 18, and 36 hours after the wound generated. Using Image J software, cell migration was quantified by measuring the number of cells that migrated into the wound area at each time point. Each assay was replicated 3 times.

In vivo tumorigenesis and metastasis assays

Female SCID mice (strain no: 001303, RRID:IMSR-JAX:001303; 6 weeks) purchased from Jackson Laboratory were used in these studies, and all protocols were approved by the University of Pittsburgh Committee on Animal Care and Use Committee, and experiments were carried out in adherence to the NIH Guidelines for the Use of Laboratory Animals. Animals were raised in plastic cages under specific pathogen-free conditions. Animals were fed a standard diet and had free access to water in an animal facility of the University of Pittsburgh. Mice were anesthetized with isoflurane. SW480-shiNOS2 or SW480-shControl cells (2×10^6 cells/mouse) in PBS, were injected into spleen or inner canthus. The mice were sacrificed 4-week post-injections. The organs were collected, frozen with OCT, and 5-μm sections cut for hematoxylin and eosin (H&E) stains. Established metastatic colon and rectal tumors in lymph nodes, stomachs, and other organs as previously described (18).

Circulating tumor cells isolation and culture

For specific isolation of peripheral blood circulating tumor cell (CTC), a Ficoll-Paque density gradient was used as previously described (19) with modifications. Briefly, whole blood with PBS buffer (total volume should be exactly 30 mL) was gently added to 10 mL the Ficoll-Paque solution to obtain two clearly defined layers. After centrifugation at 930 *g* for 25 minutes, four layers can be discerned: red blood cells at bottom, followed by Ficoll solution, white blood cells, and blood plasma and CTCs. The CTCs were recovered and washed twice with PBS (pH 7.4) by centrifugation at 800 *g* for 15 minutes, and finally were suspended in 10 mL DMEM medium, then seed to the culture plate for growth.

Tissue specimens of primary and metastatic colon cancer

Fifty tissue specimens of patients with primary colon cancer were collected from two tissue arrays, Abcam (178133; Cambridge, MA) and Novus Biologicals (NBP2–42074; Centennial, CO). Fifty tissue specimens of CRLM and 5 paired tissue specimens of primary colorectal cancer and normal tissues were obtained from patients who underwent surgical resection at the UPMC Liver Cancer Center and the tissue bank of the department of pathology, respectively with institutional review board (IRB) approval.

Statistical analysis

Data are presented as the mean ± 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 by using Graphpad Prism (RRID:SCR_002798) versions 8 and 9. χ^2 test was analyzed by using an online χ^2 calculator $P<0.05$ was considered statistically significant.

Ethics approval and consent to participate

The ethics for animal research were reviewed and approved by the Animal Care and Use Committee of the University of Pittsburgh (Protocol 18012053 and protocol 21018530). Human tissue samples were obtained in accordance with the University of Pittsburgh IRB approved protocol (No. MOD08010372/PRO08010372).

Availability of data and materials

The RNA-seq data generated in this study are publicly available in Gene Expression Omnibus (GEO) at GSE178862. Raw sequencing and read count data can be downloaded from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE178862. Script for differential expression analysis was available at Code Ocean: https://codeocean.com/capsule/5406760/tree.

Results

Downregulation of iNOS/NO is related to EMT and metastasis in colorectal cancer

To explore the impact of iNOS expression on colorectal cancer metastasis, we examined iNOS expression in primary colon cancer SW480 and metastatic colorectal cancer SW620 cell lines. Immunofluorescence staining showed higher iNOS expression in SW480 than in SW620 cells (**Fig. 1A**). qRT-PCR and Western blot further demonstrated iNOS mRNA and protein higher expression in SW480 than SW620 cells (**Fig. 1B**). Moreover, representative immunofluorescence staining of clinical tissues showed that iNOS strongly expressed in primary colorectal cancer tumors compared with CRLM which exhibited low iNOS expression (**Fig. 1C**). Next, we examined primary colorectal cancer and CRLM tissue specimens from patients and found that 90% of CRLM showed relatively low iNOS expression, whereas only 6% of the primary colorectal cancer had low iNOS expression ($P = 0.0034$; **Fig. 1D**). Hence, the primary colorectal cancer tumors had high iNOS expression, but the CRLM did not. Another study has shown that iNOS expression is decreased with increasing tumor stage (Dukes' A-D) and was lowest in colon metastases to liver and lung (20). In addition, we analyzed tissue specimens from 5 additional patients comparing iNOS expression in the primary colorectal cancer, CRLM, and background normal colon. iNOS mRNA expression was very low in CRLM, undetectable in normal colon, and high in the primary colorectal cancer tumor (**Fig. 1E**). Many studies have indicated that cancer metastasis is driven by EMT program which provides the ability of migration and invasion to cancer cells and mediates cancer cellular plasticity during cancer progression (1). These results led us to hypothesize that iNOS downregulation might be essential for triggering colorectal cancer metastasis via activation of EMT. To determine correlations between the mRNA expressions of iNOS and 11 selected canonical-EMT markers among 7 types of cancers, TCGA databases were analyzed with cBioPortal. The selected 11 EMT markers were *SNAI1, SNAI2, TWIST1, TWIST2, ZEB1, ZEB2, GSC, CDH1, CDH2, VIM*, and *FN1*. The results of correlation analyses are shown in **Fig. 1F** including colorectal cancer, stomach, and lung cancer with 3 EMT markers, and more detailed analytic results of 7 types of cancers with 11 EMT markers displayed in Supplementary Table S1. The TCGA databases showed

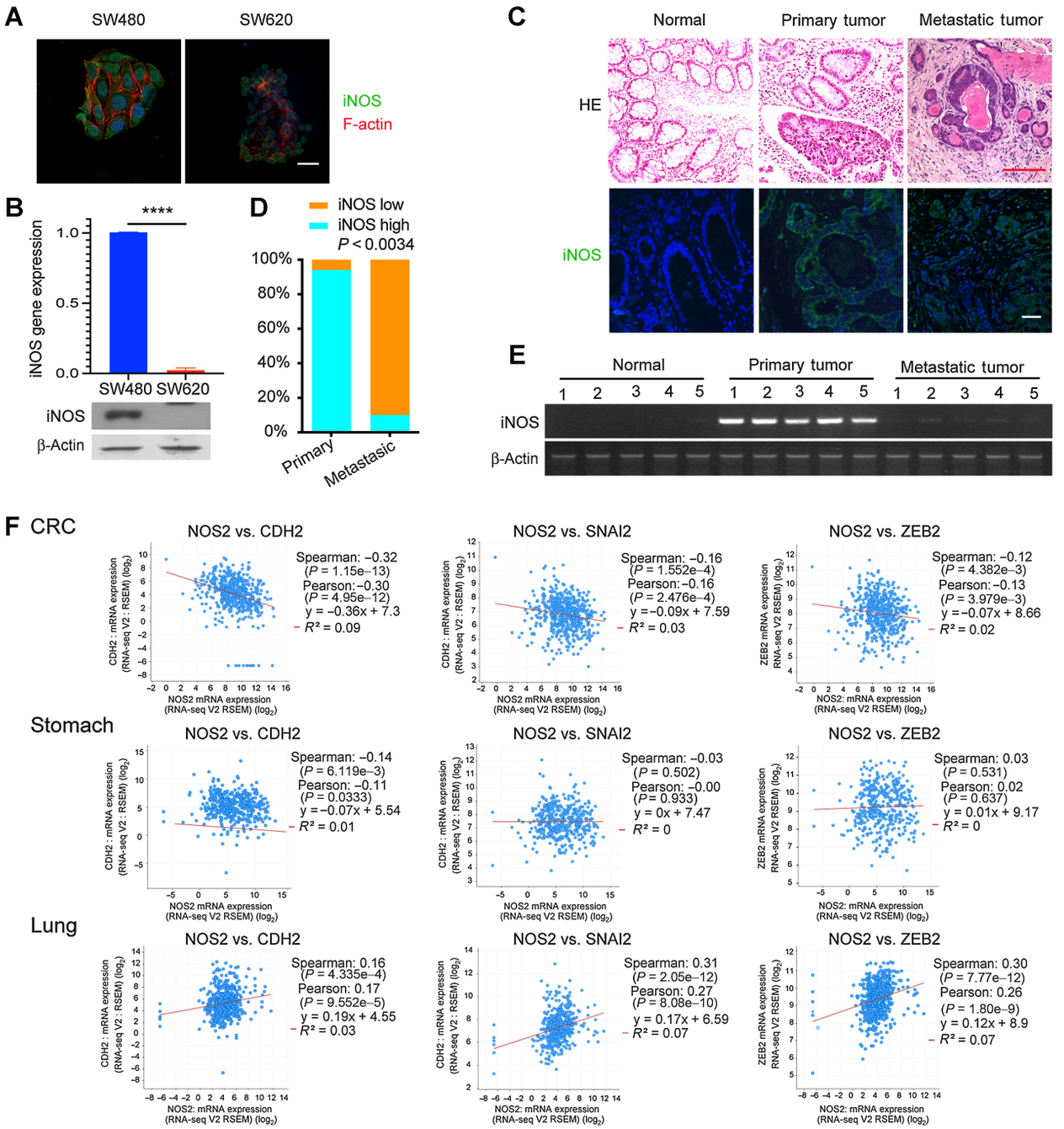


Figure 1.

iNOS expression was downregulated in patients with metastatic colorectal cancer and cell line and correlated with the selected EMT markers in colorectal cancer. **A,** representative images of immunofluorescence staining are shown to compare iNOS expression between the SW480 and SW620 cells. Green and red signal represents the staining of iNOS and F-actin, respectively (scale bars, 20 μm). **B,** total RNAs and protein collected from cultured SW480 and SW620 cells were analyzed for the expression of iNOS by qRT-PCR: ****, $P < 0.0001$ (top), and Western blot (bottom), respectively. **C,** tumor tissues were collected from patients with primary and metastatic colorectal cancer. H&E staining is shown (top; scale bars, 50 mm). Representative images of Immunofluorescence staining are shown to compare iNOS expressions between the primary and the metastatic colorectal cancer. Green signal represents the staining of iNOS (bottom; scale bars, 50 μm). **D,** immunofluorescence staining analyses of the tissue specimens from 50 patients with primary and equal metastatic colon cancer were performed to compare the iNOS expression. Immunofluorescence staining scores for the iNOS expression were generated and χ^2 test was used, $P = 0.0034$. **E,** total RNAs collected from normal, primary, and metastatic colorectal cancer tissues were subjected to RT-PCR for iNOS expression normalized to β-actin. **F,** the representative images of the cBioPortal TCGA database analysis showed the correlations of the mRNA expressions between iNOS and selected EMT markers in the indicated cancer types.

that only in colorectal adenocarcinoma iNOS negatively correlated with 10 EMT markers except *CDH1*. In contrast to colorectal cancer, lung adenocarcinoma, liver HCC, and breast invasive carcinoma, iNOS positively correlated with 9, 8, and 9 of the selected EMT markers, respectively (Supplementary Table S1). While iNOS expression had no correlation with 9 EMT markers except for *CDH2* and *GSC* with negative correlations in stomach adenocarcinoma (**Fig. 1F**; Supplementary Table S1). For pancreatic adenocarcinoma, iNOS positively correlated with 6 of the EMT markers and no-correlation with the others. Whereas in skin cutaneous melanoma, iNOS did not correlate with 6 EMT markers, but positively with 4 and negatively with 1 EMT marker (Supplementary Table S1). Together, these findings indicate that iNOS downregulation is associated with colorectal cancer metastasis, and the cBioPortal analysis of TCGA support that iNOS gene expression negatively correlates with selected-EMT markers in colorectal cancer. Thus, iNOS downregulation might play critical roles in induction of EMT and metastasis in colorectal cancer.

Knockdown of iNOS regulated the expressions of EMT markers in SW480 cells

To further determine the roles of iNOS in colorectal cancer EMT and metastasis, we chose human SW480 cells (harboring *APC* mutation) with iNOS gene knockdown as our cell models. The iNOS knockdown with > 80% efficiency at protein and mRNA levels was confirmed by Western blot and qRT-PCR, respectively with strong knockdown of iNOS expression using SW480-shiNOS2 transduction (**Fig. 2A**, top and bottom).

Next, transcriptomic profiling was analyzed by RNA-seq to discover the alteration of gene expressions in SW480-shiNOS2 versus SW480-shControl cells. No significant differences in reference genes, including *GAPDH*, *ACTB*, *B2M*, and *LDHA* were found (Supplementary Table S2). We selected some of EMT markers from RNA-seq data by volcano plot (**Fig. 2B**). The gene expressions of iNOS and epithelial markers, such as *CK8* (cytokeratin 8), *CK20*, *CLDN3*, (Claudin 3), *CLDN4*, *CLDN19*, *CTNNA1* (α-catenin), *JUP* (γ-catenin) and the others were decreased with Log2 fold change ≤ −1.5, and the adjusted *P* value (Adj-*P*) < 0.05 (**Fig. 2B**; Supplementary Table S3). More importantly, the reduced co-expression of *CK8* and *CK20* indicates an EMT representing a critical step in the development of more aggressive colorectal cancers (21). For mesenchymal markers, the gene expressions of *CDH2* (N-cadherin), *CDH12* (cadherin 12), *MMP16*, *COL3A1* (α1 type3 collagen), *COL4A1* (α1 type4 collagen), *COL4A5* (α5 type4 collagen), *COL4A6* (α6 type4 collagen), and others were upregulated with Log2 fold change ≥ 1.5, and Adj-*P* < 0.05 (**Fig. 2B**; Supplementary Table S3). EMT-TFs are key components in initiation and regulation of EMT (22), and therefore we examined the role of iNOS/NO in the regulation of EMT-TFs. RNA-seq data indicated that iNOS knockdown upregulated EMT-TFs and EMT inducers, including *SNAIL2*, *ZEB2*, *TWIST1*, *ALX1*, *E2–2* (TCF4), *FOXC1* (23), *GSC* (gooscoid), *ID1*, *KLF8*, *SIX1*, *TWIST2*, and other EMT inducers, *GATA4*, *GATA6* (22) and *TUSC3* (**Fig. 2B**; ref. 24). These findings elucidate that iNOS knockdown regulated EMT mainly through activating EMT-TFs and their networks. Heatmap (**Fig. 2C**) shows the components involved in the regulation of iNOS knockdown-induced EMT pathway, which supports that iNOS knockdown induces EMT through activating EMT pathways by targeting the expression of EMT-TFs, N-cadherin, and others. Moreover, it also suggests that iNOS knockdown can intrinsically trigger EMT by activating mitogenic growth factors and their receptors signaling pathway, especially FGFs and EGFs through autocrine stimulation loops.

To confirm iNOS knockdown-induced changes in gene expression result in cellular changes, we experimentally observed the transition of mesenchymal cell features. As expected, iNOS knockdown altered cell morphology from more epithelial features of SW480-shControl to more mesenchymal pattern of SW480-shiNOS1/2 (**Fig. 2D**). The cellular architecture was reorganized, a spindle-shaped mesenchymal morphology and motility might be acquired in the SW480-shiNOS1/2 cells undergoing EMT. Together, iNOS downregulation, as an EMT-inducing signal is essential to regulate epithelial–mesenchymal plasticity (EMP) in SW480 cells through the EMT-TFs networks representing by EMT markers which define and constitute various epithelial and mesenchymal cell characteristics (22).

Downregulation of iNOS induced EMT in a cell type–dependent manner and stem-cell properties

Next, we confirmed the expression of the representative EMT-TFs by Western blot, qRT-PCR, and immunofluorescence staining in our cell models. iNOS knockdown increased the expressions of SNAIL2 and TWIST1 at mRNA and nuclear protein levels (**Fig. 3A** and **B**). To further detect iNOS/NO involved in the regulation of EMT-TFs, we treated DLD1 and HT-29 colon cancer cells with NO donor SNAP, or iNOS inhibitor L-NIL and analyzed expression of SNAIL2 by Western blot and qRT-PCR. The results indicate that SNAP decreased, but L-NIL increased the nuclear SNAIL2 (**Fig. 3C**, top) and the mRNA expression in DLD1 and HT-29 cells, both harboring *APC* mutation (**Fig. 3C**, bottom). To determine whether iNOS downregulation induced EMT-TFs is cell type–dependent, we selected HCT116 and RKO cell lines, both with *APC* wild-type. Surprisingly, addition of the NO donor upregulated and iNOS inhibitor downregulated SNAIL2 protein (**Fig. 3C**, top) and mRNA expression (**Fig. 3C**, bottom) in these HCT116 and RKO cells. Hence, NO-mediated downregulation of EMT-TF SNAIL2 occurs in an APC-dependent manner. To further confirm these findings, we treated HT-29 (APC-mutant) and HCT-116 (APC-WT) cells with different doses of SNAP and found that SNAP decreased SNAIL2 expression in HT-29 cells, but increased SNAIL2 expression in HCT116 cells in a dose-dependent manner (**Fig. 3D**). Moreover, the analysis of TCGA database found *APC* with different mutation rates among the selected cancer types (**Fig. 3E**), and only colorectal cancer with a higher *APC* mutation rate (76.34%) compared with the other cancer types (6.74%–20.94%) negatively correlated with the expressions of selected-EMT markers (**Fig. 1G**; Supplementary Table S1). Collectively, these results indicate that iNOS downregulation promoted EMT at least by activating EMT-TFs and implies that iNOS/NO regulates cancer transformation, EMP, and metastasis dependent of *APC* mutation status in colorectal cancer.

Emerging data shows that EMT is linked to stemness of normal and CSCs and tumor cells, with the highest stem-cell capabilities residing in a hybrid E/M state (1). As mentioned above, iNOS downregulation is required for EMT, so we tested whether iNOS downregulation influences stemness associating signals in colorectal cancer. Among the selected CSC markers (25, 26), our RNA-seq data indicate *LGR5* (leucine-rich-repeat-containing G-protein-coupled receptor 5), *MSI1* (musashi-1), *LIN28B*, *VAV3*, and *HOPX* (the homeobox only protein homeobox) were upregulated (**Fig. 3F**; Supplementary Table S4), which revealed the acquisition of some CSC-like characteristics in SW480 cells with iNOS knockdown signature. The mechanisms of iNOS knockdown promoted cancer cell stemness might be provided by the downregulation of *FAT1* gene expression (**Fig. 3G**), as recent data show, *FAT1* gene deletion enhanced hybrid EMT, tumor stemness and metastasis (27). Because *LGR5* is a well-known intestinal stem-cell

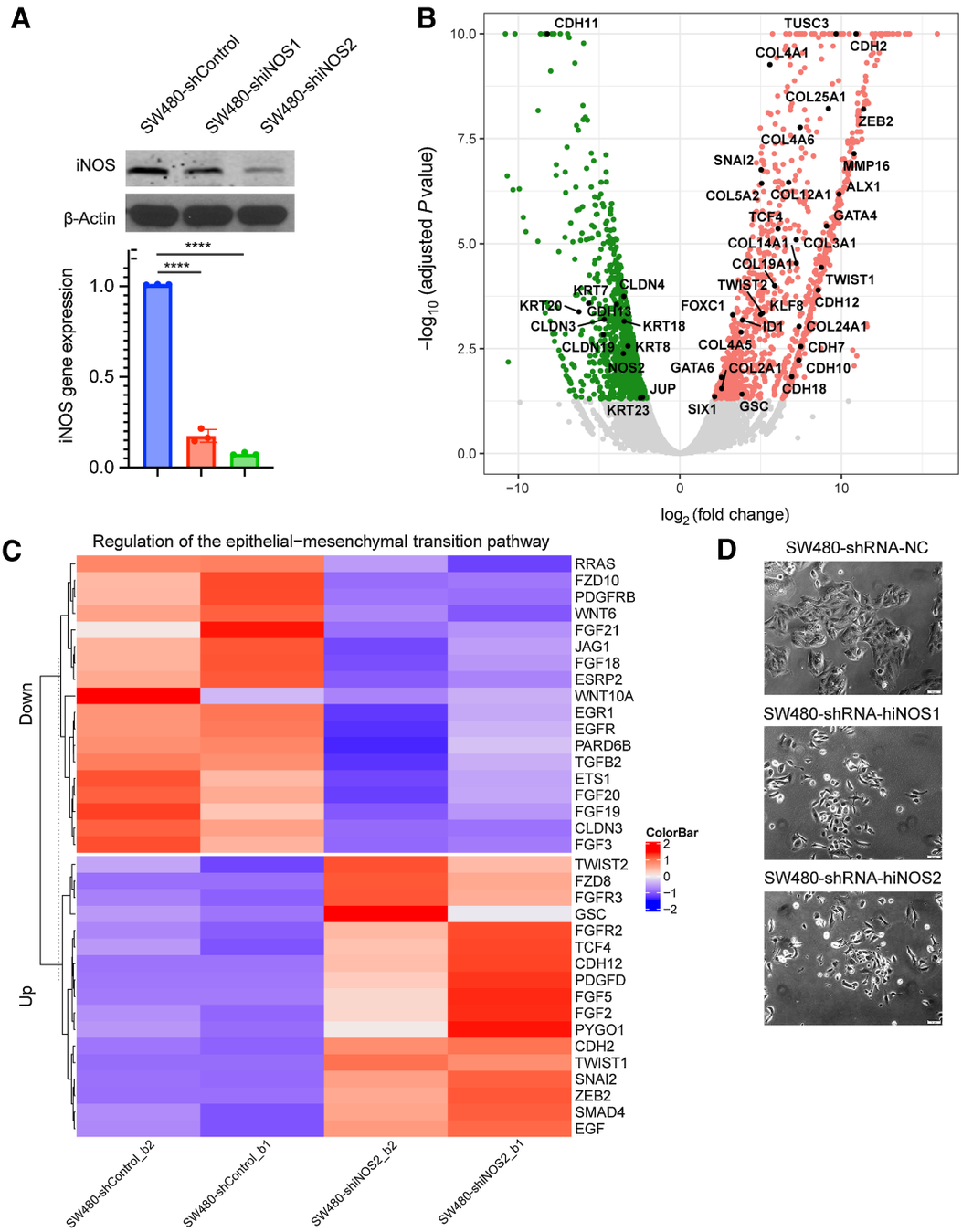


Figure 2.

iNOS knockdown mediated EMT in SW480 cells. **A,** SW480-shiNOS and SW480-shControl cells were cultured for 36 hours. iNOS expressions at mRNA and protein level between SW480-shiNOS and SW480-shControl cells were evaluated by Western blot (**A,** top) and qRT-PCR (**A,** bottom): ****, $P < 0.0001$. **B,** the RNA-seq data were analyzed and the gene expressions of the selected EMT markers demonstrated in volcano plot. **C,** the RNA-seq data were analyzed by using IPA, heatmap shows that EMT-pathway genes were mediated by iNOS knockdown ($n = 2$). **D,** SW480-shiNOS and SW480-shControl cells were cultured for 48 hours, the representative images were taken (Scale bars, 50 μm).

marker and its positivity defines stem-like cells in colorectal cancer (28, 29), we further analyzed our RNA-seq data and found at least two putative mechanisms involved in upregulating *LGR5* by downregulating HNF4A and upregulating GATA6 expression (**Fig. 3G**; refs. 30, 31). Together, these results show that iNOS knockdown promotes the expressions of some key stem-cell markers and suggests that iNOS downregulation may be an intrinsic inducer of stem cell–like features in colorectal cancer cells.

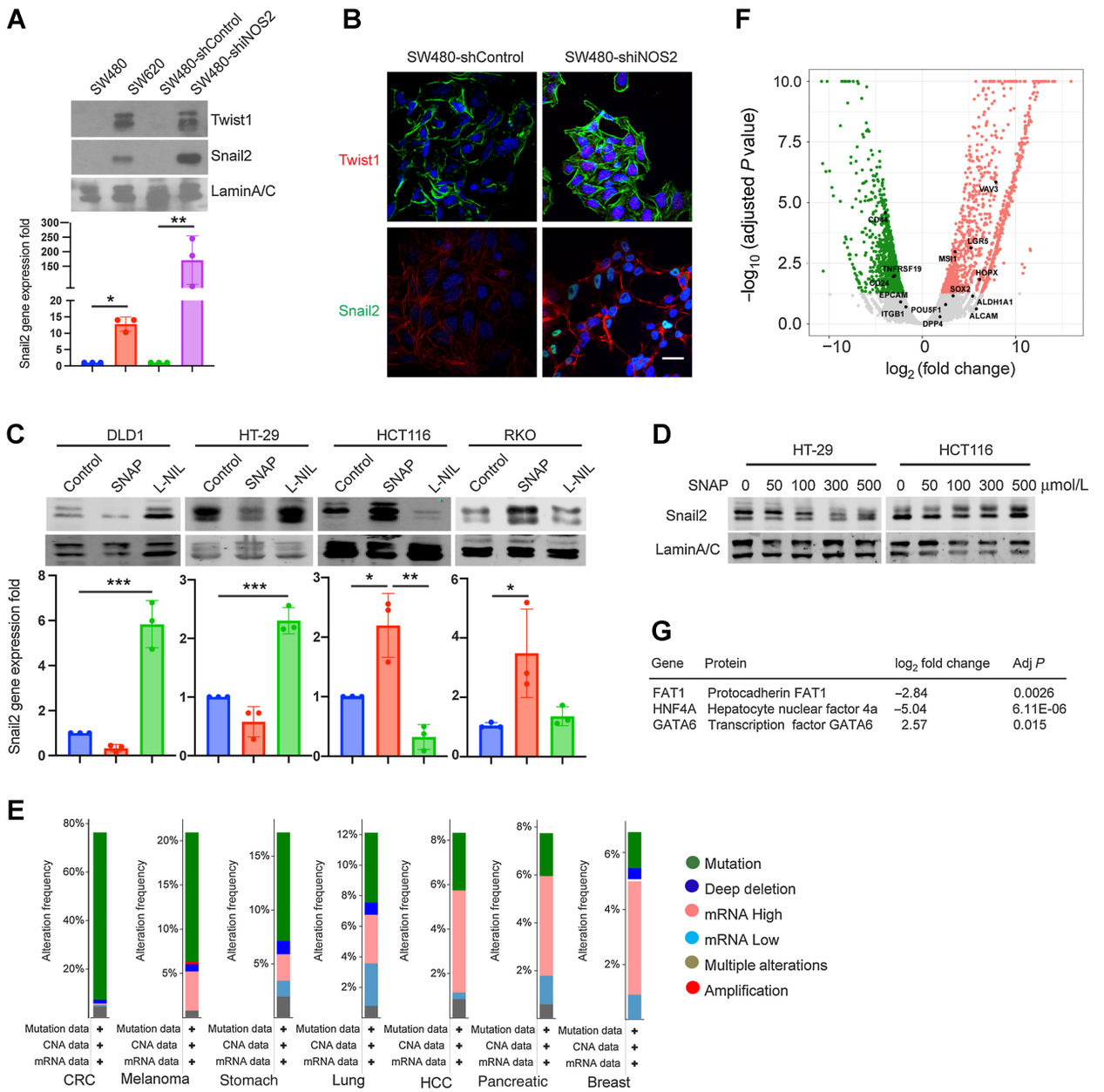


Figure 3.

iNOS knockdown regulated the expressions of EMT-TFs and enhanced stem-cell properties. **A** and **B,** SW480-shiNOS and SW480-shControl cells were subjected to culture for 36 hours, and nuclear proteins were harvested for Western blot with the indicated antibodies, Lamin A/C as loading control (top); and the total RNAs were extracted, and *SNAI2* expression was analyzed by qRT-PCR: *, $P < 0.05$; **, $P < 0.01$ (bottom); the cells for Immunofluorescence staining (**B**), the corresponding color represents the staining of TWIST1 and SNAIL2 as indicated. (Scale bars, 20 μm). **C,** DLD1, HT-29, HCT116 and RKO cells were subjected to seed for 24 hours, and treated with SNAP, a NO donor (100 μmol/L), or L-NIL, an iNOS inhibitor (100 μmol/L) for 24 hours. Nuclear proteins were harvested for Western blot with the indicated antibodies (top). Total RNA was extracted for qRT-PCR: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$ (bottom). **D,** HT-29 and HCT116 cells were subjected to seed for 24 hours, and treated with SNAP (0, 50, 100, 300, and 500 μmol/L) for 24 hours. Nuclear proteins were harvested for Western blot with the indicated antibodies. **E,** *APC* mutation rates among the selected cancer types based on the analyses of cBioPortal TCGA databases. **F,** the RNA-seq data were analyzed and the expressions of the selected stem-cell markers displayed in volcano plot. **G,** RNA-seq indicates that knockdown iNOS regulated the gene expressions of promoting hybrid EMT, cancer stemness, and *LGR5* expression.

Knockdown of iNOS induced E-cadherin translocation by activating GATA4-CRB2 pathway

Although *CDH1* (E-cadherin gene) expression was not altered in RNA-seq (Supplementary Table S3), Western blot analysis showed the increased expression of E-cadherin in cytoplasm in SW480-shiNOS1/2 cells (**Fig. 4A**). As expected, iNOS knockdown increased N-cadherin expression (**Fig. 4A** and **B**). The representative images of immunofluorescence staining demonstrates that iNOS

knockdown promoted E-cadherin translocation from membrane to cytoplasm (**Fig. 4B**). Cytoplasmic re-localization of E-cadherin implies an early feature of EMT initiation in various EMT models (22, 32, 33) and the targeting cells might undergo a hybrid EMT (31). Human *GATA4* and *GATA6* can also induce an epithelial to nonpolarized migratory cell transition by downregulation of apical-protein crumbs2 (*CRB2*), and similarly this occurs in an E-cadherin transcription independent manner in a *Drosophila*

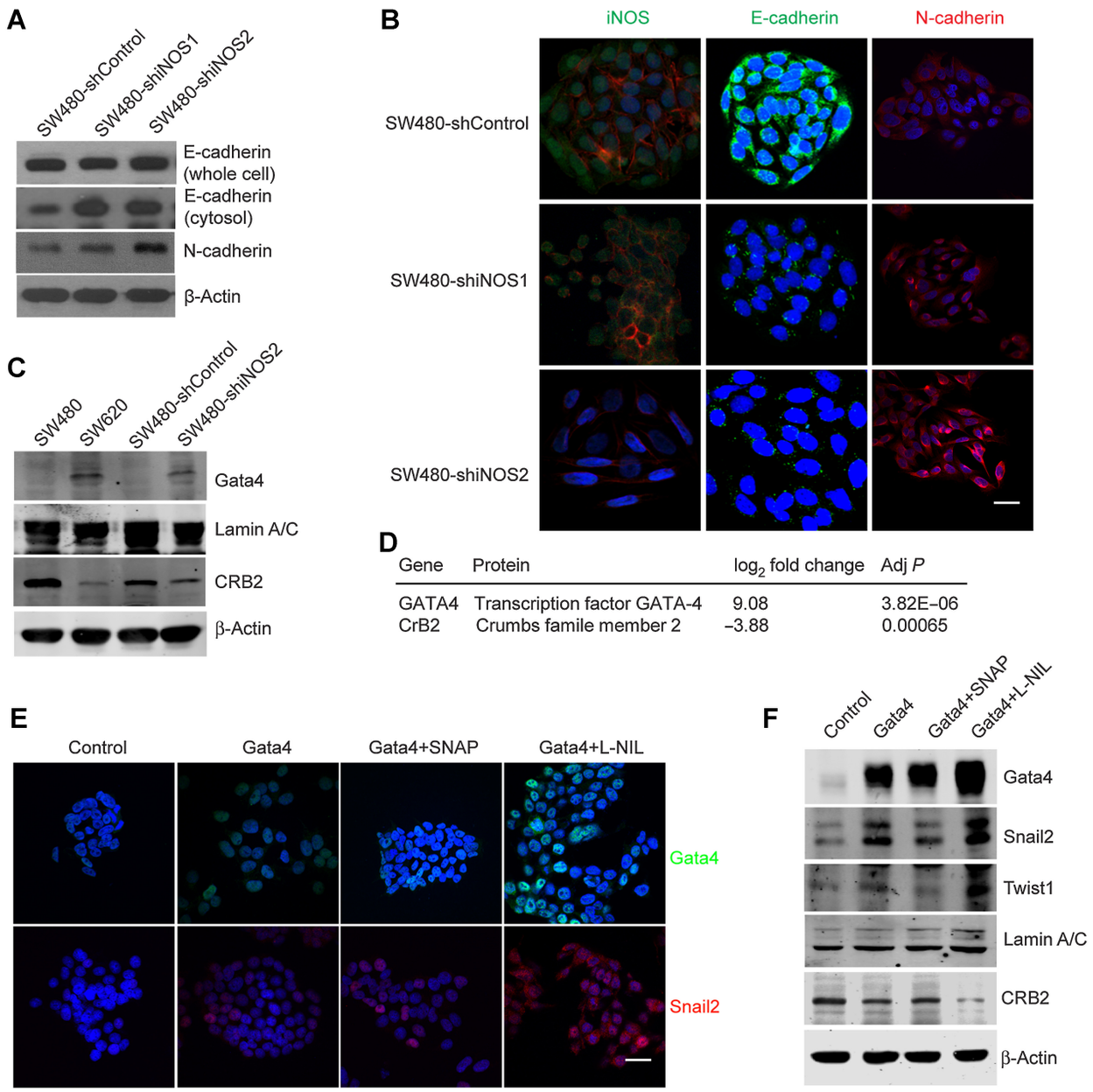


Figure 4.

iNOS knockdown induced E-cadherin translocation from membrane to cytoplasm via iNOS-GATA4-Crb2-E-cadherin pathway. **A,** SW480-shiNOS and SW480-shControl cells were cultured for 36 hours, Western blots show the change of E-cadherin localization and N-cadherin expression. **B,** SW480 cells treated as in **A**, immunofluorescence staining was conducted with the indicated antibodies. The representative images show the corresponding color signal representing the staining of iNOS, E-cadherin, and N-cadherin as indicated in the SW480-shiNOS cells compared with their controls (Scale bars, 25 μm). **C,** SW480, SW620, SW480-shiNOS, and SW480-shControl cells were cultured for 36 hours, nuclear or total protein was extracted for Western blot analysis with the indicated antibodies. **D,** RNA-seq indicates that the listed gene expressions were regulated by iNOS knockdown. **E,** 293 cells was transfected with GATA4 gene (2 μg) for 6 h in 35-mm dish, then the cells treated with SNAP (100 μmol/L) or L-NIL (100 μmol/L) for 24 hours. Representative Immunofluorescence staining images show the green and red signal representing the staining of GATA4 and SNAIL2, respectively. (Scale bars, 25 μm). **F,** 293 cells were treated as in **E**, nuclear or total proteins were collected for Western blot.

endodermal-EMT study model (32). Our Western blot analyses indicate that iNOS downregulation upregulated nuclear *GATA4* and downregulated *CRB2* expression in SW620 and SW480-shiNOS2 cells, but not in SW480 and SW480-shControl cells (**Fig. 4C**). Similarly, iNOS knockdown transcriptionally upregulated *GATA4* and *GATA6*, and downregulated *CRB2* by RNA-seq (**Figs. 3G** and **4D**). To further confirm the relationship between iNOS/NO and GATA4, SNAIL2, TWIST1, and CRB2, we overexpressed *GATA4* gene in 293 cells. The enforced *GATA4* can at least induce SNAIL2 and TWIST1 expressions. This observation implies that iNOS downregulation might induce SNAIL2, TWIST1 and probably the other EMT-TFS through the activation of GATA4 pathway. We then treated the overexpressed *GATA4* cells with SNAP or L-NIL. SNAP decreased GATA4, SNAIL2, and TWIST1, but increased CRB2 expression, while L-NIL increased GATA4, SNAIL2, and TWIST1, but decreased CRB2 expression by immunofluorescence staining (**Fig. 4E**) and Western blot (**Fig. 4F**). These results imply that iNOS knockdown induced *GATA4* and *GATA6* might depend upon transcriptional repression of *CRB2* expression, leading to disruption of the apico-basal polarity complex and allowing the E-cadherin translocation from membrane to cytoplasm (32). The role of specific GATA factors as EMT inducers are characterized by affecting E-cadherin localization rather than transcription, thus, iNOS downregulation might induce a hybrid EMT in SW480 cells (33).

Downregulation of iNOS prevented colorectal cancer cells from anoikis and promoted cell migration and invasion

Cancer cells undergoing EMT and inducing expression of stemness markers acquire mechanisms to develop anoikis-resistance and metastatic potential (34). SW480-shiNOS2 and SW480-shControl cells were cultured in a suspension culture system and cleaved caspase-9 was used as a marker to evaluate anoikis-induced cell death (35). To determine the role of iNOS knockdown in preventing anoikis, we examined for cleaved caspase-9 expression. The detached SW480-shiNOS2 cells lost the ability to express cleaved caspase-9 compared with control cells at 72 hours (**Fig. 5A**). These results demonstrate that iNOS downregulation-induced EMT might be one mechanism for colorectal cancer cells to alter cell-fate, resist anoikis, and enhance aggressive ability.

Next, we explored the characteristics of colorectal cancer cell movement driven by iNOS/NO in SW480-shiNOS1/2 and SW480-shControl cells. Unidirectional migration was observed in wound-healing assay. Typical wound healing at different time points are shown. iNOS knockdown SW480 cells more likely migrated compared with their controls in the wound-healing assay with a time-course manner (**Fig. 5B**). iNOS knockdown also promoted migration (**Fig. 5C** and **D**) and invasion (**Fig. 5C** and **E**) of SW480 cells in Boyden Chamber culture system. Collectively, these results suggest that iNOS downregulation not only prevents colorectal cancer

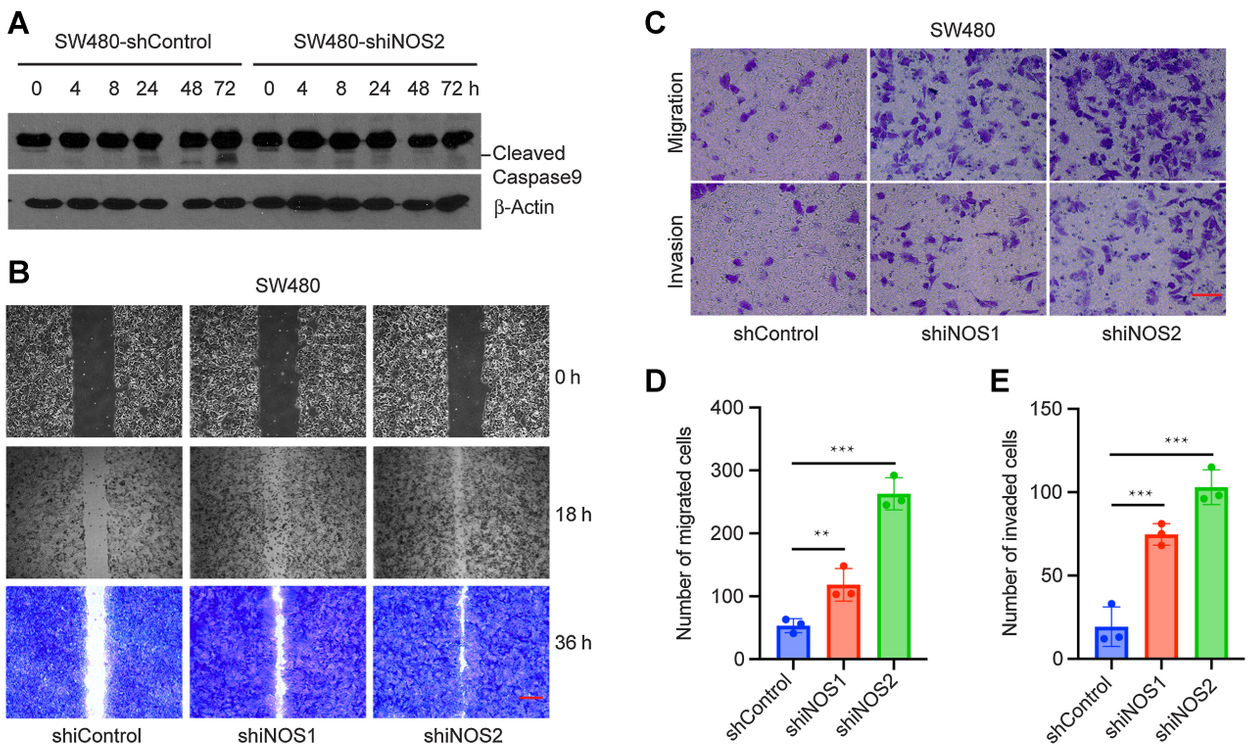


Figure 5.

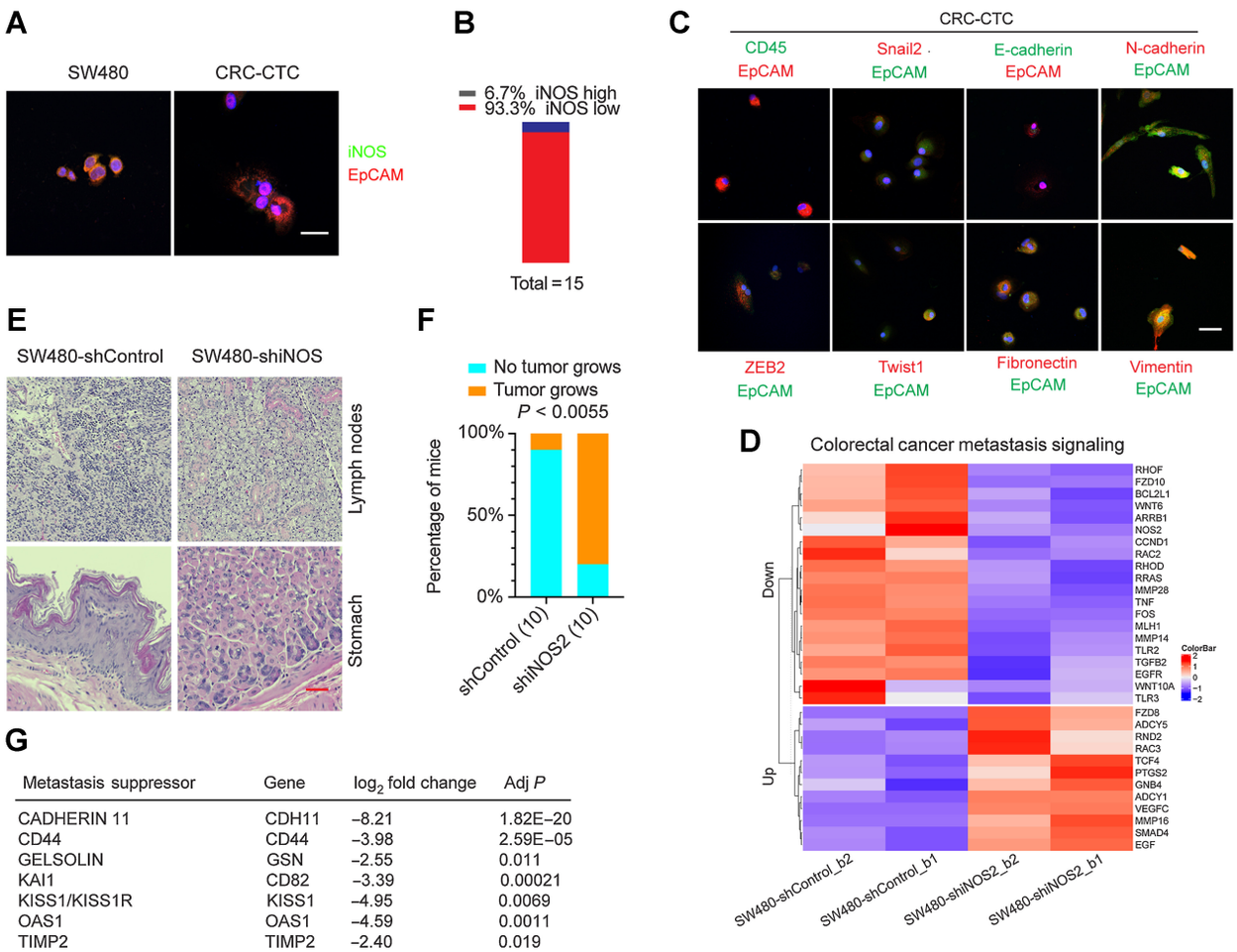
iNOS Downregulation prevented SW480 cells from anoikis and promoted cell migration and invasion. **A,** SW480-shiNOS and SW480-shControl cells were subjected to suspended culture with shaking for 0, 4, 8, 24, 48, and 72 hours, and harvested for Western blot with the indicated antibodies. β-actin as loading controls. **B,** confluent monolayers of SW480-shiNOS versus SW480-shControl cells were wounded as shown for 0, 18, and 36 hours to compare the cell motility (scale bars, 25 μm). **C,** colon cancer cell migration and invasion were regulated by downregulated iNOS gene. The representative images of the migration and invasiveness of SW480-shiNOS versus SW480-shControl cells, which were cultured using Boyden chambers for 4 hours (Scale bars, 200 μm). **D** and **E,** the numbers of cell migration and invasion: **, $P < 0.01$ and ***, $P < 0.001$ in a comparison of the SW480-shiNOS treated groups with SW480-shControl. This representative experiment was selected from three with the similar results.

cell death but promotes cell mobility. These characteristics acquired from downregulation of iNOS may be beneficial for colorectal cancer metastasis.

Downregulation of iNOS related to induction of EMT and tumor metastasis *in vivo.*

Following local invasion, tumor cells can enter the vasculature and become CTCs. To determine the relationship between iNOS and EMT markers in CTCs, we collected and analyzed the CTCs samples from 15 patients with CRLM. Immunofluorescence staining show the expression of EpCAM (epithelial cell adhesion molecule) on the surfaces of CTCs compared with SW480 cells (**Fig. 6A**), which reveals that the CTCs derived from epithelial cells (36). Interestingly, CTCs also expressed lower iNOS than SW480 cells (**Fig. 6A**). Of 15 CTC specimens, 14 expressed lower iNOS (**Fig. 6B**), which indicates that relatively low iNOS expression might be required for CTCs. Moreover, we detected the expressions of representative EMT markers. CTCs with the EpCAM signature, CD45-negative and reduced-iNOS ($CTC^{EpCAM+/CD45-/iNOS-low}$) upregulated SNAIL2, TWIST1, ZEB2, vimentin, fibronectin, and N-cadherin (**Fig. 6C**). These results suggest concurrent presence of EMT signature, which might be conferred stem-cell properties (1), and the relatively low iNOS in the CTCs implied that iNOS downregulation was also required for the formation of CTCs and tumor cell migration. As mentioned above, SW480 cells with iNOS knockdown induced an EMT and acquired stem-cell properties, colorectal cancer cells in patient might rely on the same mechanisms to acquire the potential with highly invasive and metastatic competency to lymph nodes and other organs.

The results of IPA show the transcriptomic profiling of the components of metastatic pathway including downregulation of iNOS in heatmap (**Fig. 6D**). Importantly, this analysis displays iNOS knockdown correlating with the increased expression of



G

Metastasis suppressor	Gene	log_2 fold change	Adj *P*
CADHERIN 11	CDH11	−8.21	1.82E−20
CD44	CD44	−3.98	2.59E−05
GELSOLIN	GSN	−2.55	0.011
KAI1	CD82	−3.39	0.00021
KISS1/KISS1R	KISS1	−4.95	0.0069
OAS1	OAS1	−4.59	0.0011
TIMP2	TIMP2	−2.40	0.019

Figure 6.

iNOS Downregulation promoted EMT and metastasis *in vivo.* **A,** SW480 cells and CTCs were cultured on slides for 36 hours and followed Immunofluorescence staining. The green and red signal represents the staining of iNOS and EpCAM, respectively (Scale bars, 25 μm). **B,** the percentages of iNOS gene expression among the CTC samples, $n = 15$. **C,** cells treated as in **A**, the representative images are shown for immunofluorescence staining with the indicated antibodies. EpCAM and CD45 are the epithelial and immune cell marker, respectively. The corresponding color signal represents the staining as indicated (Scale bars, 25 μm). **D,** the RNA-seq data were analyzed by using IPA, heatmap shows the transcriptomic profiling of colorectal cancer metastatic signaling induced by iNOS knockdown. **E,** representative H&E images of section from SCID mouse lymph node and stomach bearing metastatic colon cancer of SW480-shiNOS ($n = 10$) and SW480-shControl cells ($n = 10$). (Scale bars, 50 μm). **F,** the statistics of the metastatic tumors between the two groups of experimental mice, $P < 0.0055$ by Fisher exact test. **G,** RNA-seq shows the selected metastasis suppressors were downregulated by iNOS knockdown.

vascular endothelial growth factor C (*VEGFC*) and COX2 (*PTGS2*). The significance of activating these pathways is considered to promote angiogenesis, lymphangiogenesis, and metastasis by orthotopic colorectal tumors in mice (37, 38). To determine whether primary colon cancer cells with iNOS gene knockdown (SW480-shiNOS2) triggered colorectal cancer metastasis, we generated SCID mice models with iNOS knockdown by injecting SW480-shiNOS2 or SW480-shControl cells. The lymph nodes, stomach, and other organs were examined 4 weeks after injection. The representative images of H&E stains of the lymph nodes and stomach from SW480-shiNOS2 injected mice showed more metastatic colon cancers than the control mice, $P = 0.0055$ (**Fig. 6E** and **F**). These results imply that SW480 cells with iNOS knockdown undergoing EMT and acquiring colorectal cancer stem-cell properties are capable of initiating tumors when inoculated into mice.

Metastasis suppressors are recognized by their ability to inhibit cancer metastasis (39). RNA-seq confirmed iNOS knockdown-reduced the expressions of a group of metastasis suppressors, such as *CD82* (KAI1), *CD44*, *CDH11* (cadherin 11), *GSN* (gelsolin), *KISS1*, *OAS1*, and *TIMP2* (**Fig. 6G**). Together, these results demonstrate that iNOS downregulation-induced EMT promotes colorectal cancer cells to acquire more features of CSC to facilitate local tissue invasion and distant organ metastasis through not only activating metastasis-promoting processes, but also inhibiting metastasis suppressors (**Fig. 7**).

Discussion

Our data with gene expression profiling and other experiments highlight the following key findings. First, iNOS gene expression is reversely correlated with colorectal cancer EMT markers in a cancer type–dependent manner. To our knowledge, this relationship has not been previously described. Second, downregulation of iNOS induces a hybrid EMT at least through regulating E-cadherin by iNOS-GATA4-CRB2-E-cadherin pathway, rather than abolishing *CDH1* expression. Cancer cells undergo a hybrid EMT by the progressive acquisition of intermediate aspects of EMT which is controlled by distinct transcriptional and signaling pathways (40, 41). Metastatic ability peaks at specific late-hybrid EMT states, which are aggressively selected from a predominately epithelial ancestral pool (42). Third, downregulation of iNOS increases cancer cell stemness by inducing CSC-like marker expression. iNOS knockdown induced-hybrid EMT programs may link to cancerous epithelial stem cells and allow colorectal cancer cells to acquire stem cell–like properties (1). *LGR5* is an important intestinal stem-cell marker gene (28). Human $LGR5^{+}$ colorectal cancer cells serve as CSCs in growing cancer tissues (29) and reveal self-renewal and differentiation capacity (43). CSC is also called tumor initiating cell (TIC), and every cancer cell can become a TIC, which is critical for metastasis formation (44). Fourth, downregulation of iNOS promotes colorectal cancer metastatic seeding, tumor formation, and metastatic growth through upregulating EMT-inducing networks, which may play crucial roles in not only promoting colorectal cancer stemness and metastasis, but also contributing to cell death resistance.

It is interesting that colorectal cancer cells with *APC* mutation expressed nuclear-SNAIL2 under the conditions of iNOS inhibition, but not in colorectal cancer cells with *APC* wild-type. However, under the treatment with NO donor, colorectal cancer cells with *APC* wild-type expressed nuclear-SNAIL2, but not in the colorectal cancer cells with *APC* mutation. On the basis of these analyses we proposed iNOS downregulation-induced EMT in an *APC* mutant cell type–dependent manner. *APC* mutation plays a key role for the activation of Wnt/β-catenin pathway for tumorigenesis (45). TCGA database analysis indicated that *APC* inactivating mutations have been found in 76.34% primary colorectal cancer samples, compared with 6.74% to

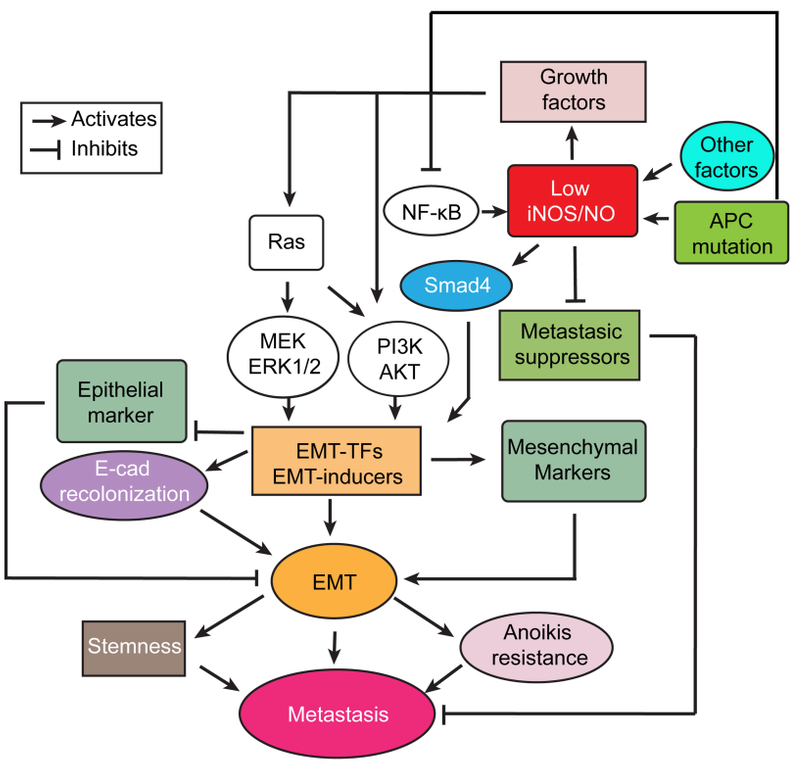


Figure 7.
iNOS Downregulation promotes colorectal cancer EMT and metastasis. Schematic mechanisms of iNOS downregulation promotes colorectal cancer EMT and metastasis based on the current study findings and the pathway analysis of the RNA-seq data by IPA. In this chart, the arrow indicates activation, while flat-end line shows inhibition in signaling pathways. Prior studies showed that *APC* mutation induced Wnt/β-catenin pathway was key for inhibit NF-κB signaling and maintain iNOS/NO at a low level Low iNOS expression activates growth factor pathways (as IPA indicated, the results published by others are shown in the white background) and Smad4 to upregulate the expression of EMT-TFs and EMT inducers leading to activate EMT. EMT plays key roles for the induction of cancer cell stemness and anoikis resistance, all which contribute to cancer metastasis. Meanwhile, low iNOS/NO also inhibits metastatic suppressors, which removes the brake on metastases.

20.94% in other types of cancers (**Fig. 3E**). iNOS negative correlation with gene expression of the selected EMT markers has been showed in colorectal cancer, but not in the other selected cancer types. Apparently, *APC* genotype reflecting the phenotypic heterogeneity of these various hybrid E/M states is seen within the originating primary tumors. Concerning the relationship between iNOS and *APC*, our previous study shows that cytokines did not induce iNOS expression of SW480 cells, however, reintroducing a wild-type *APC* (full length) into SW480 cells, iNOS expression was able to be induced by cytokines (46). These results imply that *APC* mutation activated Wnt/β-catenin pathway, which inhibits NF-κB activity may be one of the mechanisms contributing to downregulation of iNOS in colorectal cancer (9, 46). Therefore, we propose that *APC* mutation-related downregulation of iNOS induces cancer EMT in a cell- and/or cancer type–dependent manner.

Gene expressions of epithelial and mesenchymal markers were likely regulated by EMT-TFs (4). Expression profiling revealed that at least 14 EMT-TFs and EMT inducers were highly induced following iNOS knockdown (**Fig. 2B**). The activated EMT-TFs play oncogenic roles (40) and compose their networks thereby allowing cells to acquire mesenchymal characteristics, which involves the simultaneous decrease or re-localization of membrane E-cadherin and enhanced expression of N-cadherin (47). This cadherin switch disrupts adherens junctions and alters cadherin-associated signaling pathways (48). Alterations in E-cadherin localization and the expression of other epithelial and mesenchymal markers allow cells to invade, migrate to the front of cancer and enter the blood circulation, and eventually settle at distant metastatic sites. The pathway analysis by using IPA shows that growth factors, especially FGFs/FGFRs signaling pathway exerted a role in activating EMT-TFs and their pathway networks to regulate the initiation of EMT and its related CSC-like features, anoikis resistance and metastasis in colon cancer cells undergoing downregulation of iNOS. NO has been shown to attenuate several EMT transcription factors, growth factors and other signaling molecules which maintain the epithelial morphology and suppress cell migration and invasion (12). When iNOS is downregulated, the functions of these signaling components are restored. RNA-seq conferred the gene expression profiles of iNOS knockdown induced-EMT outline the intrinsic molecular mechanisms for the initiation of EMT and metastasis at the gene expression level (**Fig. 7**), although some types of EMP stimuli are derived from tumor microenvironment, cell–cell interactions, therapeutic agents, and the others are from tumor cells, such as tumor metabolism and genomic or epigenomic factors (49). However, EMT-induced by a spectrum of therapeutic agents and modalities had consequences for treatment resistance (49), downregulation of iNOS-induced EMT might be synergized with these therapeutic reagents to further increase therapeutic resistance. Collectively, this study provides a genomic landscape of downregulation of iNOS promoting colorectal cancer EMT, stemness, invasion, and metastasis. The implication of this study suggests that NO donors might be beneficial to inhibit EMT and metastasis in APC-mutant colorectal cancer tumors. Another study indicated that local nitric oxide (NO) production by tumor-infiltrating myeloid cells is important for adoptively transferred $CD8^+$ cytotoxic T cells to destroy tumors. These myeloid cells are phenotypically like iNOS- and TNF-producing dendritic cells (DC), or Tip-DCs (50). Also, depletion of immunosuppressive colony-stimulating factor 1 receptor (CSF-1R)-dependent arginase 1+ myeloid cells enhanced NO-dependent tumor killing. Tumor elimination via NOS2 required the CD40-CD40 L pathway (50).

Authors' Disclosures

No disclosures were reported.

Authors' Contributions

Q. Du: Conceptualization, data curation, validation, investigation, methodology, writing–original draft. **S. Liu:** Data curation, software, methodology. **K. Dong:** Data curation, investigation, methodology. **X. Cui:** Data curation, investigation, methodology. **J. Luo:** Data curation, investigation, methodology. **D.A. Geller:** Conceptualization, supervision, funding acquisition, writing–review and editing.

Acknowledgments

This work was supported by NIH grants HHSN276201200017C (D.A. Geller), P30DK120531–01 (D.A. Geller) and The Victor and Anna Mae Beghini Charitable Foundation (D.A. Geller), and in part by the University of Pittsburgh Center for Research Computing through the resources provided. We thank Nichol Martik and Taylor Austin for preparation of human tissue samples.

The publication costs of this article were defrayed in part by the payment of publication fees. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Note

Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Received June 29, 2022; revised September 28, 2022; accepted October 26, 2022; published first October 28, 2022.

References

1. Lambert AW, Weinberg RA. Linking EMT programs to normal and neoplastic epithelial stem cells. Nat Rev Cancer 2021;21:325–38.
2. Dongre A, Weinberg RA. New insights into the mechanisms of epithelial–mesenchymal transition and implications for cancer. Nat Rev Mol Cell Biol 2019;20:69–84.
3. Gurzu S, Silveanu C, Fetyko A, Butiurca V, Kovacs Z, Jung I. Systematic review of the old and new concepts in the epithelial–mesenchymal transition of colorectal cancer. World J Gastroenterol 2016;22:6764–75.
4. de Craene B, Berx G. Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer 2013;13:97–110.
5. Kang E, Seo J, Yoon H, Cho S. The posttranslational regulation of epithelial–mesenchymal transition-inducing transcription factors in cancer metastasis. Int J of Mol Sci 2021;22:3591.
6. Stemmler MP, Eccles RL, Brabletz S, Brabletz T. Nonredundant functions of EMT transcription factors. Nat Cell Biol 2019;21:102–12.
7. Cook DP, Banderhyden BC. Context specificity of the EMT transcriptional response. Nat Commun 2020;11:2142.
8. Bian K, Ghassemi F, Sotolongo A, Siu A, Shauger L, Kots A, et al. NOS-2 signaling and cancer therapy. IUBMB Life 2012;64:676–83.
9. de Oliveira GA, Cheng RYS, Ridnour LA, Basudhar D, Somasundaram V, McVicar DW, et al. Inducible nitric oxide synthase in the carcinogenesis of gastrointestinal cancers. Antioxid Redox Signal 2017;26:1059–77.
10. Vannini F, Kashfi K, Nath N. The dual role of iNOS in cancer. Redox Biol 2015;6:334–43.
11. Baritaki S, Huerta-Yepez S, Sahakyan A, Karagiannides I, Bakirtzi K, Jazirehi A, et al. Mechanisms of nitric oxide-mediated inhibition of EMT in cancer: inhibition of the metastasis-inducer Snail and induction of the metastasis-suppressor RKIP. Cell Cycle 2010;9:4931–40.
12. Khan FH, Dervan E, Bhattacharyya DD, McAuliffe JD, Miranda KM, Glynn SA. The role of nitric oxide in cancer: master regulator or not? Int J Mol Sci 2020;21:9393.
13. Monteiro HP, Rodrigues EG, AR AKC LLS Jr, Ogata FT, Moretti AIS, et al. Nitric oxide, and interactions with reactive oxygen species in the development of

melanoma, breast, and colon cancer: a redox signaling perspective. Nitric Oxide 2019;89:1–13.
14. Du Q, Zhang X, Liu Q, Zhang X, Bartels CE, Geller DA. Nitric oxide production upregulates Wnt/β-catenin signaling by inhibiting Dickkopf-1. Cancer Res 2013; 73:6526–37.
15. Du Q, Luo J, Yang MQ, Liu Q, Heres C, Yan YH, et al. iNOS/NO is required for IRF1 activation in response to liver ischemia-reperfusion in mice. Mol Med 2020; 26:56.
16. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.
17. Zhao T, Jiang W, Wang X, Wang H, Zheng C, Li Y, et al. ESE3 inhibits pancreatic cancer metastasis by upregulating E-cadherin. Cancer Res 2017;77:874–85.
18. Lee WY, Hong HK, Ham SK, Kim CI, Cho YB. Comparison of colorectal cancer in differentially established liver metastasis models. Anticancer Res 2014;34: 3321–8.
19. Justin GA, Zhang Y, Cui XT, Bradberry CW, Sun M, Sclabassi RJ. A metabolic biofuel cell: conversion of human leukocyte metabolic activity to electrical currents. J Biol Eng 2011;5:5.
20. Ambs S, Merriam WG, Bennett WP, Felley-Bosco E, Ogunfusika MO, Oser SM, et al. Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression. Cancer Res 1998;58:334–41.
21. Knösel T, Emde V, Schlüns K, Schlag PM, Dietel M, Petersen I. Cytokeratin profiles identify diagnostic signatures in colorectal cancer using multiplex analysis of tissue microarrays. Cell Oncol 2006;28:167–75.
22. Yang J, Antin P, Berx G, Blanpain C, Brabletz T, Bronner M, et al. Guidelines and definitions for research on epithelial–mesenchymal transition. Nat Rev Mol Cell Biol 2020;21:341–52.
23. Han B, Bhowmick N, Qu Y, Chung S, Giuliano AE, Cui X. FOXC1: an emerging marker and therapeutic target for cancer. Oncogene 2017;36:3957–63.
24. Gu Y, Wang Q, Guo K, Qin W, Liao W, Wang S, et al. TUSC3 promotes colorectal cancer progression and epithelial–mesenchymal transition (EMT) through WNT/β-catenin and MAPK signaling. J Pathol 2016;239:60–71.
25. Gonzalez-Villarreal CA, Quiroz-Reyes AG, Islas JF, Garza-Treviño EN. Colorectal cancer stem cells in the progression to liver metastasis. Front Oncol 2020; 10:1511.
26. Ong BA, Vega KJ, Houchen CW. Intestinal stem cells, and the colorectal cancer microenvironment. World J Gastroenterol 2014;20:1898–909.
27. Pastushenko I, Song Y, de Cock F, Meeusen B, Swedlund B, Impens F, et al. Fat1 deletion promotes hybrid EMT state, tumor stemness and metastasis. Nature 2021;589:448–55.
28. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 2007;449:1003–7.
29. Hirsch D, Barker N, McNeil N, Hu Y, Camps J, McKinnon K, et al. LGR5 positivity defines stem-like cells in colorectal cancer. Carcinogenesis 2014;35: 849–58.
30. Shin JH, Jeong J, Choi J, Lim J, Dinesh RK, Braverman J, et al. Dickkopf-2 regulates the stem-cell marker LGR5 in colorectal cancer via HNF4alpha1. iScience 2021;24:102411.
31. Tsuji S, Kawasaki Y, Furukawa S, Taniue K, Hayashi T, Okuno M, et al. The miR-363-GATA6-Lgr5 pathway is critical for colorectal tumorigenesis. Nat Commun 2014;5:3150.
32. Campbell K, Whissell G, Franch-Marro X, Batlle E, Casanova J. Specific GATA factors act as conserved inducers of an endodermal-EMT. Dev Cell 2011;21: 1051–61.
33. Aiello NM, Maddipati R, Norgard RJ, Balli D, Li J, Yuan S, et al. EMT subtype influences epithelial plasticity and mode of cell migration. Dev Cell 2018;45: 681–95.
34. Huang RY-J, Wong MK, Tan TZ, Kuay KT, Ng AHC, Chung VY, et al. An EMT spectrum defines an anoikis-resistant and spheroidogenic intermediate mesenchymal state that is sensitive to E-cadherin restoration by a src-kinase inhibitor, saracatinib (AZD0530). Cell Death Dis 2013;4:e915.
35. Maamer-Azzabi A, Ndozangue-Touriguine O, Bréard J. Metastatic SW620 colon cancer cells are primed for death when detached and can be sensitized to anoikis by the BH3-mimetic ABT-737. Cell Death Dis 2013;4:e801.
36. Balzar M, Winter MJ, de Boer CJ, Litvinov SV. The biology of the 17–1A antigen (Ep-CAM). J Mol Med 1999;77:699–712.
37. Tacconi C, Correale C, Gandelli A, Spinelli A, Dejana E, D'Alessio S, et al. Vascular endothelial growth factor C disrupts the endothelial lymphatic barrier to promote colorectal cancer invasion. Gastroenterology 2015;148: 1438–51.
38. Hidalgo-Estévez AM, Stamatakis K, Jiménez-Martínez M, López-Pérez R, Fresno M. Cyclooxygenase 2-regulated genes an alternative avenue to the development of new therapeutic drugs for colorectal cancer. Front Pharmacol 2020;11:533.
39. Marino N, Collins JW, Shen C, Caplen NJ, Merchant AS, Gökmen-Polar Y, et al. Identification and validation of genes with expression patterns inverse to multiple metastasis suppressor genes in breast cancer cell lines. Clin Exp Metastasis 2014;31:771–86.
40. Puisieux A, Brabletz T, Caramel J. Oncogenic roles of EMT-inducing transcription factors. Nat Cell Biol 2014;16:488–94.
41. Pastushenko I, Brisebarre A, Sifrim A, Fioramonti M, Revenco T, Boumahdi S, et al. Identification of the tumor transition states occurring during EMT. Nature 2018;556:463–68.
42. Simeonov KP, Byrns CN, Clark ML, Norgard RJ, Martin B, Stanger BZ, et al. Single-cell lineage tracing of metastatic cancer reveals selection of hybrid EMT states. Cancer Cell 2021;39:1150–62.
43. Shimokawa M, Ohta Y, Nishikori S, Matano M, Takano A, Fujii M, et al. Visualization and targeting of LGR5 + human colon cancer stem cells. Nature 2017;545:187–92.
44. Dieter SM, Glimm H, Ball CR. Colorectal cancer-initiating cells caught in the act. EMBO Mol Med 2017;9:856–58.
45. Nusse R, Clevers H. Wnt/β-catenin signaling, disease, and emerging therapeutic modalities. Cell 2017;169:985–99.
46. Du Q, Zhang X, Cardinal J, Cao Z, Guo Z, Shao L, et al. Wnt/beta-catenin signaling regulates cytokine-induced human inducible nitric oxide synthase expression by inhibiting nuclear factor-kappaB activation in cancer cells. Cancer Res 2009;69:3764–71.
47. Gravdal K, Halvorsen OJ, Haukaas SA, Akslen LA. A switch from E-cadherin to N-cadherin expression indicates epithelial to mesenchymal transition and is of strong and independent importance for the progress of prostate cancer. Clin Cancer Res 2007;13:7003–11.
48. Huang H, Wright S, Zhang J, Brekken RA. Getting a grip on adhesion: cadherin switching and collagen signaling. Biochim Biophys Acta Mol Cell Res 2019;1866: 118472.
49. Williams ED, Gao D, Redfern A, Thompson EW. Controversies around epithelial–mesenchymal plasticity in cancer metastasis. Nat Rev Cancer 2019;19: 716–32.
50. Marigo I, Zilio S, Desantis G, Mlecnik B, Agnellini AH, Ugel S, et al. T cell cancer therapy requires CD40-CD40L activation of tumor necrosis factor and inducible nitric oxide synthase—producing dendritic cells. Cancer Cell 2016; 30:377–90.