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Supplemental Information

miR-577 Regulates TGF-β Induced Cancer Progression through a SDPR-Modulated Positive-Feedback Loop with ERK-NF-κB in Gastric Cancer

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Supplemental Figure Legends

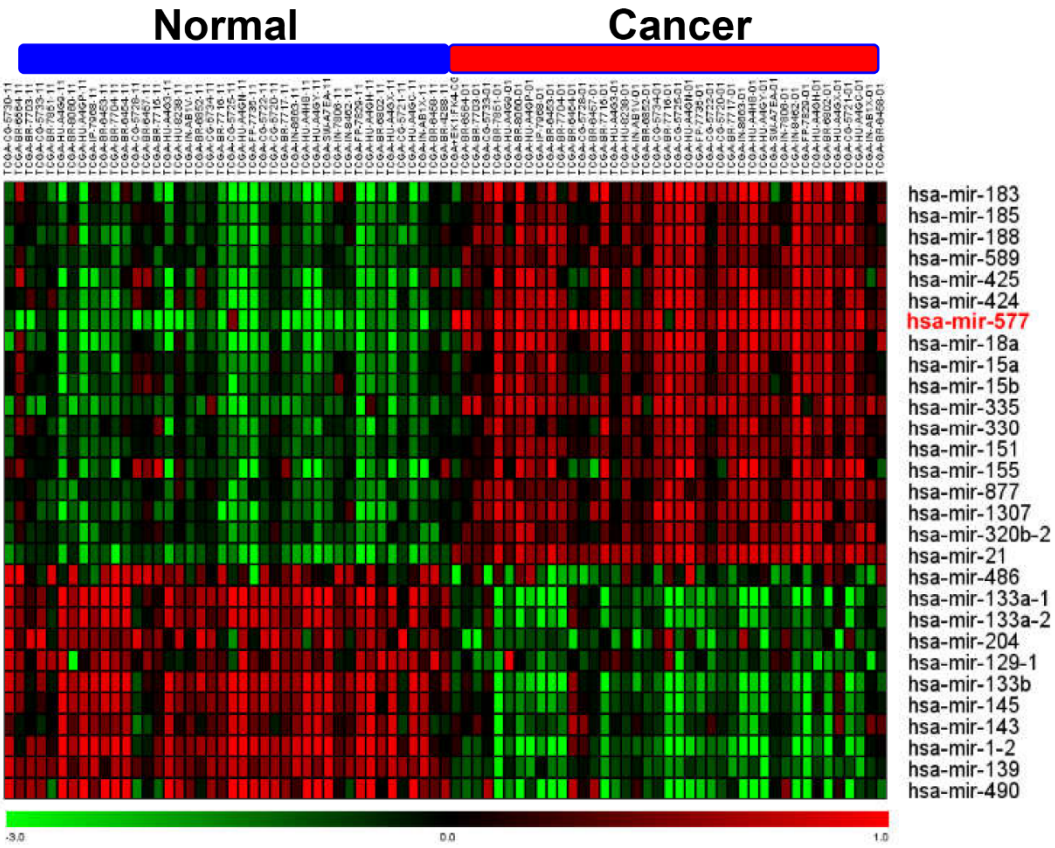

Figure S1. TCGA microarray analysis of microRNAs in gastric cancer. The hierarchical cluster heat map was based on 29 differentially expressed genes ($P < 0.001$) derived from TCGA microarray data.

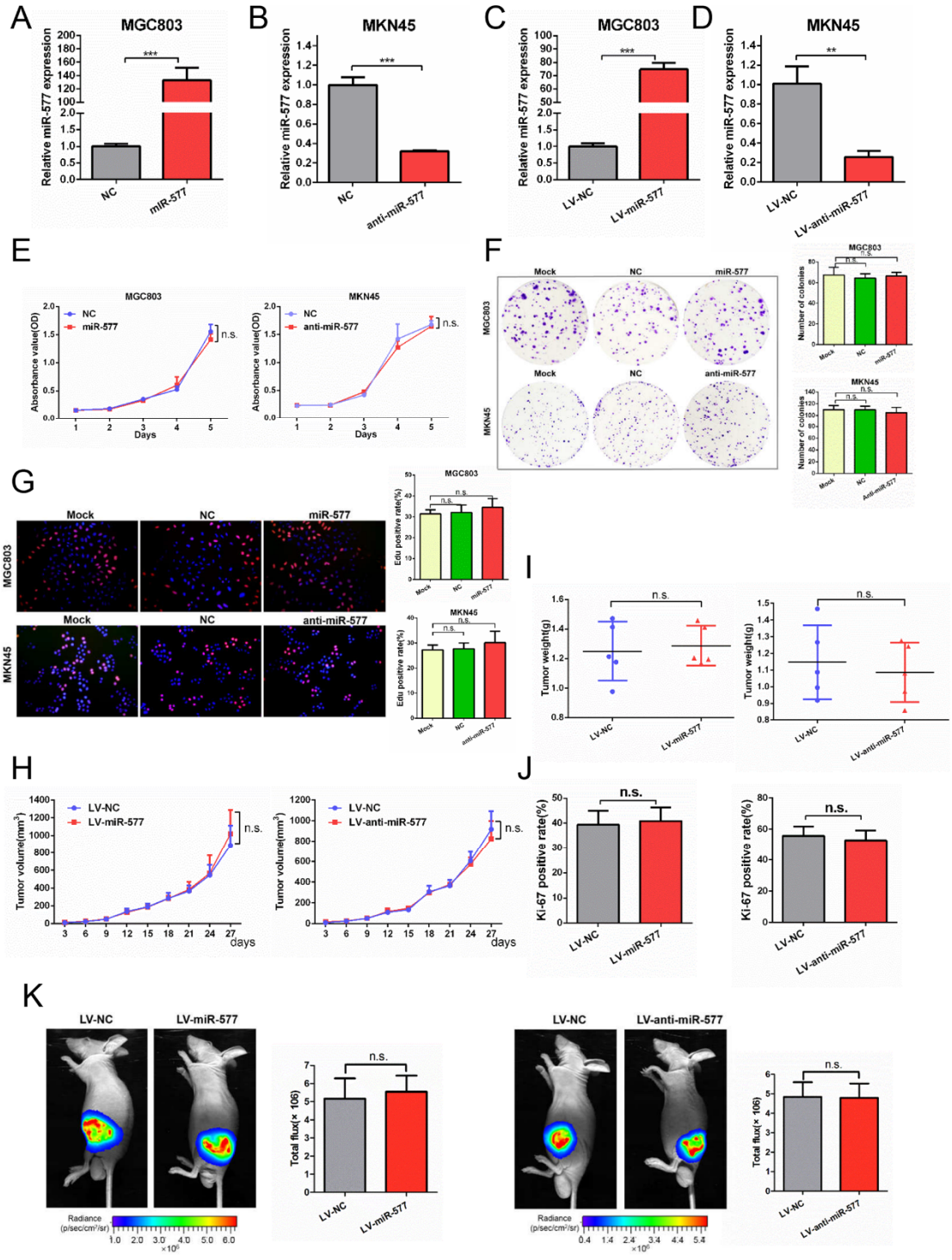

Figure S2. Effect of miR-577 on gastric cancer (GC) proliferation. (A-D) qRT-PCR analysis was performed for detection of the efficiency of miR-577 expression in MGC803 and MKN45 cells transfected with miR-577 AgomiR, AntagomiR, LV-miR-577 or LV-anti-miR-577. (E-G) MTT assays(E), colony formation assays (F), and EdU incorporation assays(G) were performed to determine the proliferation of GC cells transfected with control, miR-577 AgomiR, or miR-577 AntagomiR. (H) Subcutaneous tumor growth curves in mice with cells transplantation (n = 5). (I) The tumor weights derived from indicated cells at 30 d after subcutaneous implantation. (J) Representative Ki-67 immunohistochemical (IHC) staining of subcutaneous tumors of mice injected with indicated cells.

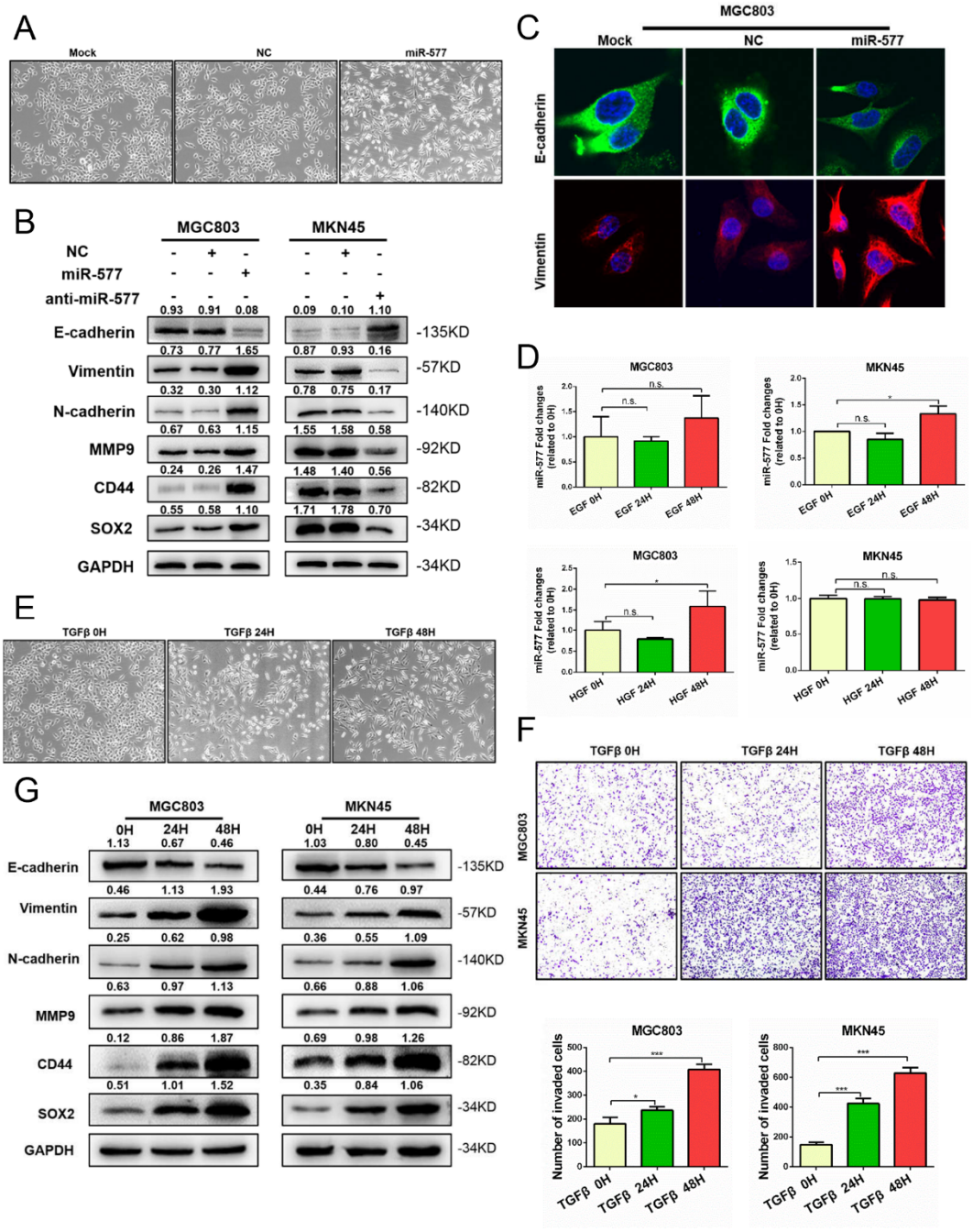

Figure S3. Impact of miR-577 on growth factors-induced EMT in gastric cancer (GC) cells. (A) Effect of miR-577 on the morphology of MGC803 cells observed using inverted microscopy. Scale bars: 50 μm. (B)Western blot of EMT markers (E-cadherin, vimentin, N-cadherin, and MMP9) and stemness markers (CD44 and SOX2) expression levels in MGC803 and MKN45 cells transfected with miR-577 AgomiR/AntagomiR. GAPDH served as a loading control. (C) Immunofluorescence assays of E-cadherin and vimentin proteins in MGC803 cells transfected with miR-577 AgomiR, as indicated. Representative figures are shown. (D) Real-time PCR analysis of miR-577 expression in EGF (100 ng/ml) or HGF (20 μg/ml) treated MGC803 and MKN45 cells for 24 and 48 h. Transcript levels were normalized to U6 expression. *$P < 0.05$. (E) The morphology of cultured MGC803 cells observed under an inverted microscope. (F) The ability of GC cell migration tested by transwell assays. (G) Western blot of EMT markers (E-cadherin, vimentin, N-cadherin, and MMP9) and stemness markers (CD44 and SOX2) were performed after GC cells were treated with TGFβ (20 ng/ml) for 24 and 48 h.

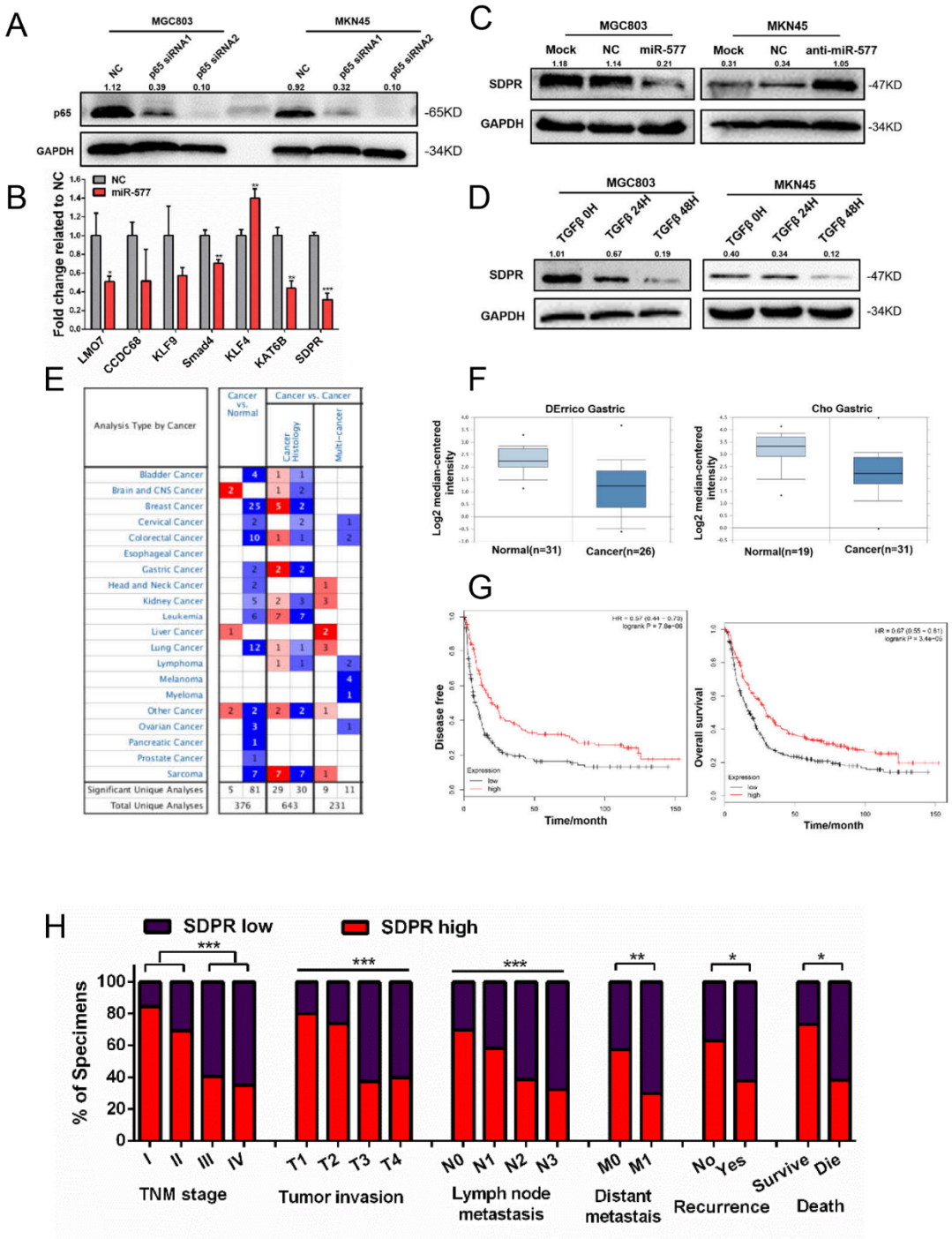

Figure S4. Preliminary analysis the role of SDPR in gastric cancer (GC). (A) Western blot of p65 protein expression level in MGC803 and MKN45 cells transfected with si-p65 (#1–2). GAPDH served as a loading control. (B) Expression of seven candidate genes were analyzed by qRT-PCR in MGC803 cells transfected with miR-577 AgomiR. GAPDH served as an internal control. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (C and D) Western blot of SDPR expression level in MGC803 and MKN45 cells treated with miR-577 AgomiR, AntagomiR, or TGFβ. (E) Oncomine database analysis regarding SDPR being down-regulated in multiple cancers included GC. (F) SDPR expression in different type of GC and normal tissues. (G) Representative analysis of Kaplan–Meier plots for SDPR expression in association with disease-free survival and overall survival by Kaplan-Meier Plotter. (H) Frequency of low and high miR-577 expression categorized by TNM stage, tumor invasion, lymph node metastasis, distant metastasis, recurrence, and death. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

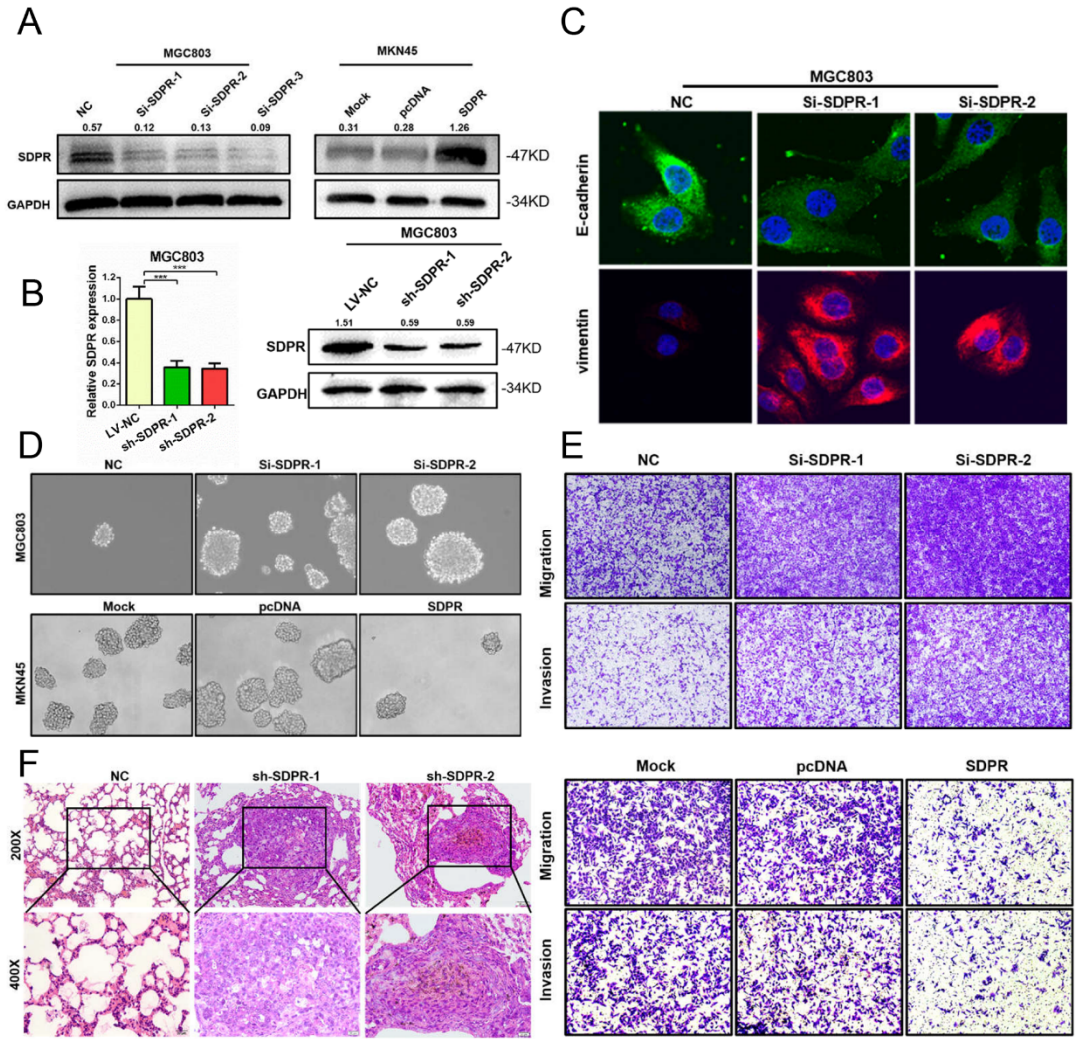

Figure S5. Effect of SDPR on EMT in gastric cancer(GC).

(A) Efficiency of si-SDPR (#1–3) and SDPR overexpression plasmid in transfected MGC803 and MKN45 cells were verified by Western blot analysis. (B) Efficiency of SDPR stable knockdown in lentivirus infections. (C) Immunofluorescence assays of E-cadherin and vimentin proteins in MGC803 cells transfected with SDPR siRNAs(#1-2), as indicated. Representative figures are shown.(D) Representative figures of SDPR on tumorsphere-formation ability. (E) Representative figures of SDPR on cell migration and invasion. (G) Representative figures of SDPR on lung homing capacity.

Supplemental Tables

Table S1: Correlation between miR-577/SDPR and clinicopathological features

Characteristics	n(%)	miR-577 expression			SDPR expression		
		miR-577 high	miR-577 low	P-value	SDPR high	SDPR low	P-value
Age（years）							
≥55	92(60.13)	50	42		40	52	
<55	61(39.87)	33	28	0.976	32	29	0.276
Gender							
Male	96(62.75)	57	39		45	51	
Female	57(37.25)	26	31	0.099	27	30	0.953
TNM stage							
I	13(8.50)	4	9		11	2	
II	26(17.00)	11	15		18	8	
III	57(37.25)	30	27		23	34	
IV	57(37.25)	38	19	0.022	20	37	0.000
Tumor invasion							
T1	15(9.80)	5	10		12	3	
T2	19(12.42)	8	11		14	5	
T3	56(36.60)	29	27		21	35	
T4	63(41.18)	41	22	0.019	25	38	0.000
Lymph node metastasis							
N0	33(21.57)	11	22		23	10	
N1	31(20.26)	17	14		18	13	
N2	39(25.50)	23	16		15	24	
N3	50(32.67)	32	18	0.027	16	34	0.000
Distant metastais							
M0	96(62.75)	43	53		55	41	
M1	57(37.25)	40	17	0.002	17	40	0.001
Tumor differentiation							
Well	24(15.69)	11	13		14	10	
Moderate	70(45.75)	34	36		32	38	
Poor	59(38.56)	38	21	0.132	26	33	0.475
Recurrence							
No	35(36.46)	12	23		22	13	
Yes	61(63.54)	35	26	0.029	23	38	0.017
Overall survival							
Survive	15	6	9		11	4	
Die	42	30	12	0.03	16	26	0.019

Table S2 : Sequence information used in this study

Name	Sequence
LV-NC	**5'-UUCUCCGAACGUGUCACGU-3'**
LV-miR-577	5'-TAGATAAAATATTGGTACCTG-3'
P65 NC	**5'-UUCUCCGAACGUGUCACGU-3'**
P65 siRNA1	5'-CCUGAGCACCAUCAACUAU(dTdT)-3'
P65 siRNA2	5'-GCGACAAGGUGCAGAAAGAdTdT-3'
SDPR NC	**5'-UUCUCCGAACGUGUCACGU-3'**
SDPR siRNA1	5'-GGGACAACUCACAGGUGAATT-3'
SDPR siRNA2	5'-UCCUCCGACGCAACCAUUUTT-3'
SDPR siRNA3	5'-GCAGUGAGCAGAUGCCAAATT-3'
has-miR-577 probe	**5'- CAGGTACCAATATTTTATCT -3'**

Table S3 : The qPCR primers used for this study

Gene	Primer Sequence		GC(%)	Tm(℃)
LMO7	Forward	5’-GTGGGTTGGCTGTATCTCA-3’	52.63	56.74
	Reverse	5’-TACGGGTTGCTTTGTGC-3’	52.94	55.33
CCDC68	Forward	5’-TTCACTCCCAACATCAG-3’	47.06	50.45
	Reverse	5’-AAACACCTTCGGTCTTC-3’	47.06	51.25
KLF9	Forward	5’-AACTGCTTTTCCCCAGTGTG-3’	50.00	58.60
	Reverse	5’-TCCCATCTCAAAGCCCATTA-3’	45.00	56.18
Smad4	Forward	5’-TGCCTCACCACCAAAACGG-3’	57.89	60.83
	Reverse	5’-CCAAACAAAAGCGATCTCCTCC-3’	50.00	59.84
KLF4	Forward	5’-CCCCGTGTGTTTACGGTAGT-3’	55.00	59.68
	Reverse	5’-GAGTTCCCATCTCAAGGCAC-3’	55.00	58.26
KAT6B	Forward	5’-GTCAGCCTTCTACCCCATGA-3’	55.00	58.80
	Reverse	5’-GCCACAATCTGCACAAGAGA-3’	50.00	58.47
SDPR	Forward	5’-CTCCGACGCAACCATTT-3’	52.94	55.09
	Reverse	5’-CTTTCTTGAGGCTATCCACTT-3’	42.86	55.30
GAPDH	Forward	5’-GGAGCGAGATCCCTCCAAAAT-3’	52.38	59.86
	Reverse	5’-GGCTGTTGTCATACTTCTCATGG-3’	47.83	59.38

Table S4: Information on antibodies used for this study

antibody	Cat. No.	WB	IP	IHC	IF	Specificity	Source
GAPDH	60004-1-Ig	1:5000				Mouse	Proteintech
Caspase3	#9662	1:500				Rabbit	Cell Signaling Technology
Cleaved Caspase3	#9664	1:500				Rabbit	Cell Signaling Technology
Caspase7	#9494	1:500				Mouse	Cell Signaling Technology
Cleaved Caspase7	#9491	1:500				Rabbit	Cell Signaling Technology
E-cadherin	#14472	1:1000			1:50	Mouse	Cell Signaling Technology
Vimentin	60330-1-Ig	1:1000			1:200	Mouse	Proteintech
N-cadherin	#14215	1:1000				Mouse	Cell Signaling Technology
MMP9	#13667	1:500				Rabbit	Cell Signaling Technology
CD44	#3570	1:500				Mouse	Cell Signaling Technology
SOX2	66411-1-Ig	1:500				Mouse	Proteintech
SDPR	ab103230	1:100				Rabbit	Abcam
SDPR	12339-1-AP		1:500		1:400	Rabbit	Proteintech
ERK1/2	66192-1-Ig	1:500	1:1000		1:100	Mouse	Proteintech
P-ERK1/2	#4370	1:1000				Rabbit	Cell Signaling Technology
P-p65	ab86299			1:500		Rabbit	Abcam
P-p65	#3033	1:800				Rabbit	Cell Signaling Technology
p65	#6956	1:1000			1:800	Mouse	Cell Signaling Technology
P-IKKα/β	#2697	1:1000				Rabbit	Cell Signaling Technology
IKKβ	#2678	1:1000				Rabbit	Cell Signaling Technology
P-IKBα	#2859	1:800				Rabbit	Cell Signaling Technology
IKBα	#4814	1:1000				Mouse	Cell Signaling Technology
Ki-67	ab15580			1:500		Rabbit	Abcam

Table S5: The PCR primers used for this study

Primer-name	Sequence	
p65-A	Forward	5’-GCAAAATAAAGCAGTG-3’
	Reverse	5’-CTTGGGTGCCTGAAAC-3’
p65-B	Forward	5’-GGGGTTTGGAGTAAGG-3’
	Reverse	5’-TTTGGAGGGTGACAGG-3’
p65-C	Forward	5’-AAATAGACCACTTACCAATT-3’
	Reverse	5’-ACTCCAAACCCCTCAC-3’

Table S6 : databases and their links in this study

Database Name	Links
TCGA	https://cancergenome.nih.gov/
TargetScan	http://www.targetscan.org/
UCSC	http://genome.ucsc.edu/index.html
PROMO	http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3
PITA	http://genie.weizmann.ac.il/pubs/mir07/mir07_dyn_data.html
RNA22	http://www.mybiosoftware.com/rna22-v2-microrna-target-detection.html
MiRDB	http://www.mirdb.org/
Oncomine	https://www.oncomine.org/
Kaplan-Meier Plotter	http://kmplot.com/analysis/

Supplemental Materials and Methods

Quantitative Real-time PCR（qRT-PCR）

Total RNA for cultured cells and fresh tissues were extracted with using Trizol Reagent (Takara Bio, Inc., Shiga, Japan). The mRNA expressions were detected by the PrimeScript RT Reagent Kit and SYBR Premix Ex Taq (Takara Bio, Inc., Shiga, Japan). GAPDH was used as control. All the primers designed for qPCR were listed in Supplemental Table 3. All-in-One microRNA qRT-PCR Detection Kits (GeneCopoeia, Inc., Maryland, USA) were used to detect miRNA expression with its specifically designed and synthesized qPCR primers for miR-577 and U6 used as a control (Cat#hmiRQP0678, GeneCopoeia, Inc.,USA). Every experiment was repeated 3 times according to the manufacturer's protocol. Final data were analyzed with the $2-\Delta\Delta Ct$ method.

Western blotting

Separate proteins by 10% SDS-PAGE, transfer to nitrocellulose membrane (Bio-Rad), block in 5% BSA, incubate with special primary antibodies included anti-Caspase3, Cleaved Caspase3, Caspase7, Cleaved Caspase7, E-cadherin, Vimentin, N-cadherin, MMP9, CD44, SOX2, SDPR, ERK, P-ERK, P-p65, p65, P-IKKα/β, P-IKBα and IKBα, incubate with secondary anti- Mouse/Rabbit IgG. The details of these antibodies were listed in Supplemental Table 4. Finally, the images with associated molecular weights indicated for each antibody were collected with Tanon-5200 Chemiluminescent Imaging System (Tanon, China).

MTT, EdU cell proliferation assays and Colony formation assays

MTT：Cells were seeded in 96-well plates (1000cells/mL) with 200uL cell culture medium in each well and incubated for 7 days. The viable cells were detected by methyl thiazolyl tetrazoliym (MTT) assay by adding 20 ul of MTT (5 mg/mL; Promega, Madison, WI) for 4 hours until the purple precipitate generated completely. Absorbance at 570 nm was measured in 10min after 150 ul DMSO was added into each well to dissolve the precipitates. *EdU cell proliferation assays* were performed to determine cell proliferation ability with Cell-Light™ EdU In Vitro Imaging Kit(RiboBio, Guangzhou, China) according to the standard protocol. Images were captured by Fluorescence Inversion Microscope System ((IX71, Olympus, Tokyo, Japan). For *colony formation assays*: About 800 cells per well were seeded into a 6-well culture plate and incubated at 37°C for 14 days. After washing with PBS twice, cells were fixed with 4% paraformaldehyde for 15 min and then stained with Giemsa solution. Only colonies ≥ 50 cells were counted under a microscope. Each experiment was repeated 3 times.

Cell Migration and Invasion Assays

After cells were resuspended in serum-free RPMI-1640 medium, 6×10^4 cells were incubated in the boyden chambers with 8-μm pores (Corning Costar, Corning, NY, USA). For invasion assays, cells were seeded into chambers coated with Matrigel (BD Biosciences, Boston, MA, USA). Then, RPMI-1640 with 10% FBS was added to the lower compartment to form the gradient as a chemotactic factor. After incubation for about 2 days at 37℃, using cotton swabs to remove the cells on the upper surface of the chambers. Cells Stuck to the low surface were fixed and stained with hematoxylin. Then, these stained cells were counted under a microscope (IX71, Olympus, Tokyo, Japan) in 5 random visual fields. At least three independent experiments were operated.

***In situ* hybridization**

The ISH Kit (Exiqon, Vedbaek, Denmark) was used to detect the expression of miR-577 in 153 fresh primary GC specimens and paired noncancerous gastric tissues with specific designed probe for miR-577 (Exiqon) (Supplemental Table 2). After the tissue sections were dewaxed with xylene, a series different gradient ethanol were used to rehydrate these sections. Then the sections were treated with 4% paraformaldehyde for 10 min and treated with a concentration of 15 μg/mL proteinase K for 10 min at 37 ℃, washed with PBS. Hybridization with the specific and scrambled control probes was performed at a concentration of 40nM at 50℃ for 5 hours. After hybridization, Sections were washed strictly According to the following procedures: 5 × SSC, at 50 ℃ for 10min, 1 × SSC, at 50 ℃ for 10min, twice, 0.2 × SSC, at 50 ℃ for 10min, twice, 0.2 × SSC, at RT for 5 min. After the procedures were finished, the sections were treated with 1x blocking reagent (Roche, Basel, Switzerland) at RT for 30min and then incubated with anti-digoxigenin antibody conjugated with rhodamine (Roche, Basel,

Switzerland)) diluted 1:150 at RT for 1 h. Afterword, Using running water carefully rinsed for 10 min, dehydrated and mounted with neutral balsam.

Immunohistochemically Staining

With the paraffin sections prepared from patients and in vivo experiments, IHC were performed to detect proteins expression included SDPR, Ki-67, E-cadherin, Vimentin, CD44 and SOX2 using Detection Kit (PV-9001/2, Zsgb Bio, Beijing, China) according to the manufacturer's standard protocol. The information of antibodies was listed in (Supplemental Table 4). Scoring was measured by three pathologists separately as: 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining. According to the staining score, 0-1 was considered as low expression and 2-3 was considered as high expression.

Supplemental Reference

1. Lin, L.*, et al.* MACC1 supports human gastric cancer growth under metabolic stress by enhancing the Warburg effect. *Oncogene* **34**, 2700-2710 (2015).
2. Zhang, F.*, et al.* MicroRNA-187, a downstream effector of TGFbeta pathway, suppresses Smad-mediated epithelial-mesenchymal transition in colorectal cancer. *Cancer Lett* **373**, 203-213 (2016).
3. Xia, J.*, et al.* Voltage-gated sodium channel Nav 1.7 promotes gastric cancer progression through MACC1-mediated upregulation of NHE1. *Int J Cancer* **139**, 2553-2569 (2016).
4. Wang, L.*, et al.* Metastasis-associated in colon cancer-1 upregulation predicts a poor prognosis of gastric cancer, and promotes tumor cell proliferation and invasion. *Int J Cancer* **133**, 1419-1430 (2013).