Inhibition of Wntless/GPR177 suppresses gastric Tumorigenesis

Jaesung Seo[1,#], Hyun Jung Kee[2,#], Hye Ji Choi[1,2], Jae Eun Lee[2], Soo-Yeon Park[1], Seung-Hyun Lee[1], Mi-Hyeon Jeong[1], Garam Guk[1], SooYeon Lee[1], Kyung-Chul Choi[3], Yoon Young Choi[2], Hyunki Kim[4], Sung Hoon Noh[2], Ho-Geun Yoon[1,] & Jae-Ho Cheong[1,2,*]*

[1]Department of Biochemistry and Molecular Biology, Brain Korea 21 PLUS Project for Medical Sciences, Yonsei University College of Medicine, Seoul 03722, [2]Department of Surgery, Yonsei University College of Medicine, Seoul 03722, [3]Department of Biomedical Sciences, University of Ulsan College of Medicine, Seoul 05505, [4]Department of Pathology, Yonsei University College of Medicine, Seoul 03722, Korea

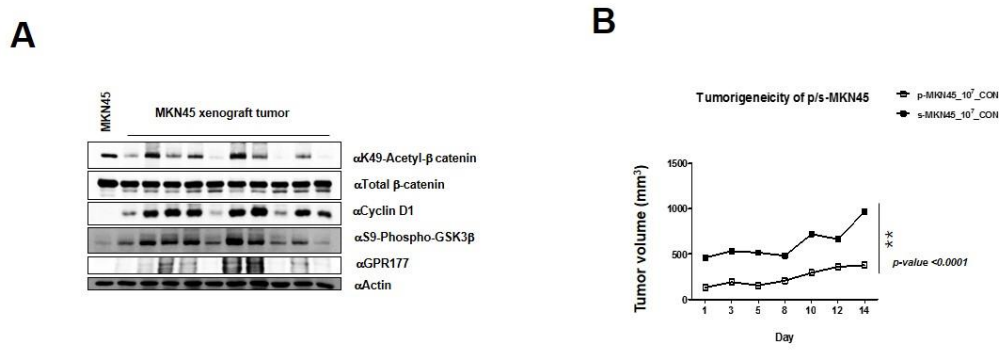
Supplementary Information

Supplementary Figure 1

A) Xenograft tumor from panel A was analyzed by western blotting. Total lysate (20 μg) was loaded on SDS-PAGE gel.

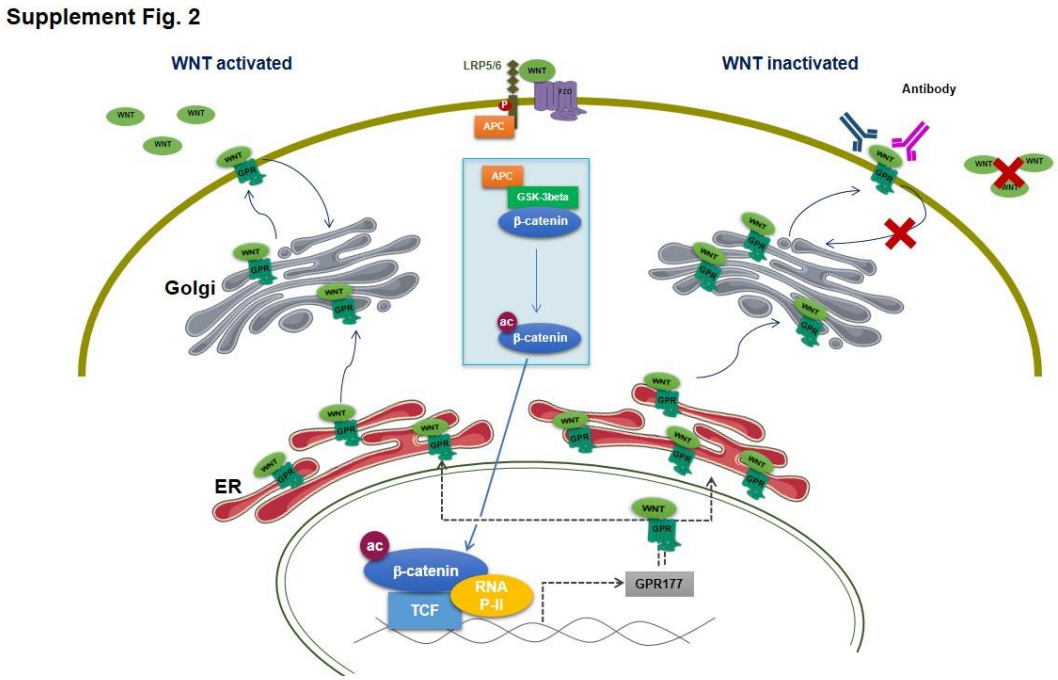
B) 10^7 cells of parental or selected MKN45 were subcutaneously injected BALB/C nude mouse and tumor volume was measured for 2 weeks.

Supplement Fig. 1



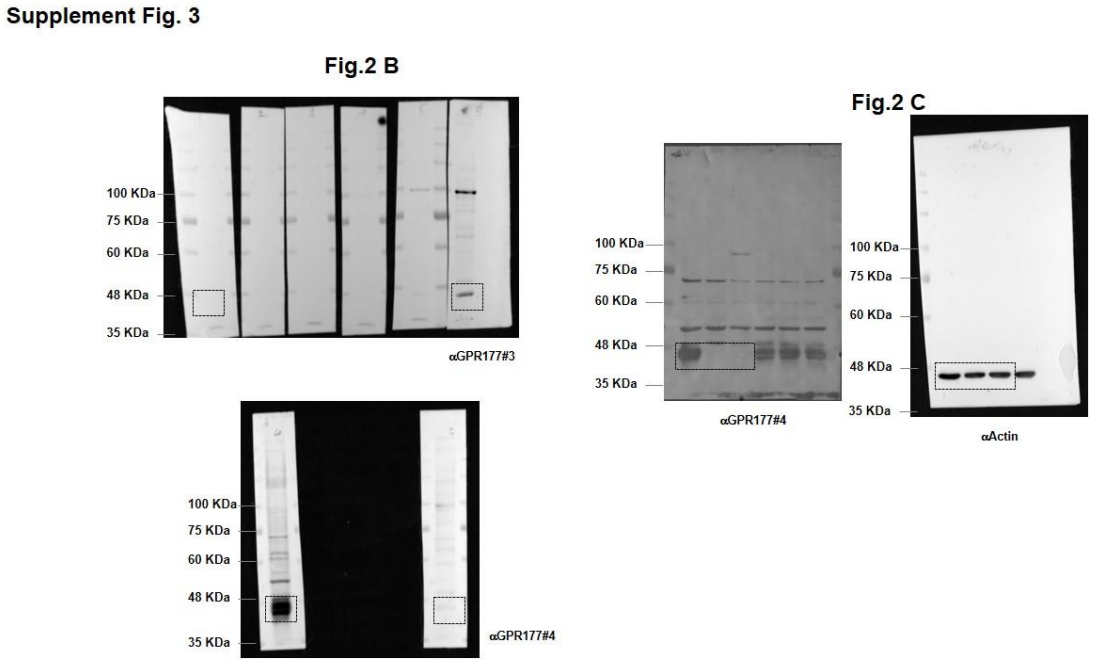
Supplementary Figure 2

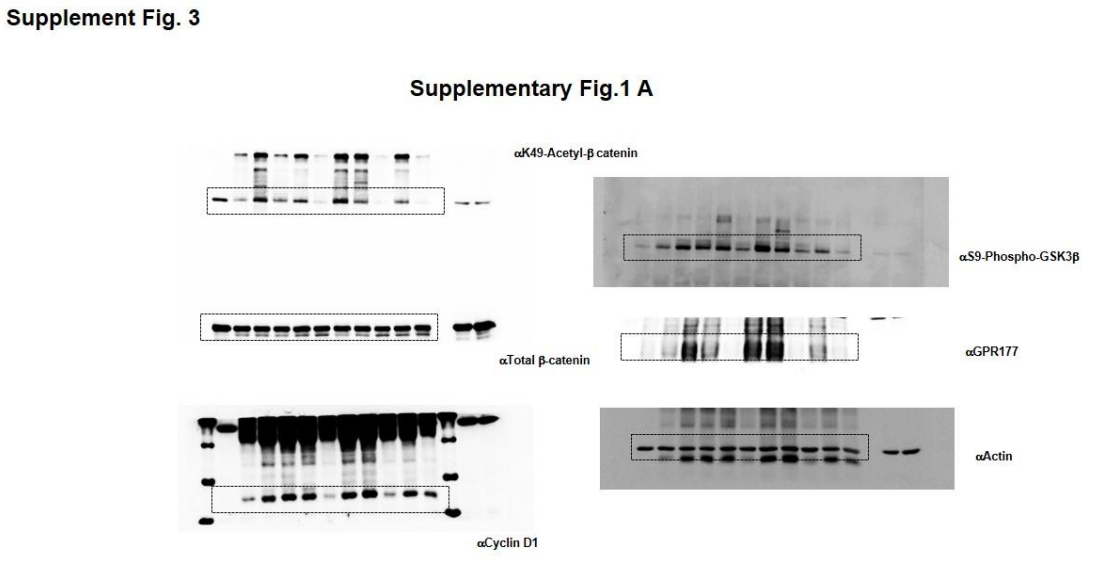
Schematic summary of functions of GPR177 and monoclonal antibody. WNT ligand stimulates WNT/β-catenin signaling resulting in trans-localization of β-catenin into the nucleus. Active β-catenin positively regulates transcription of GPR177 in both cell types. In WNT activated cells, WNT/GPR177 complex propagates further from ER to Golgi and cytoplasmic membrane where WNT/GPR177 complex are dissociated, confirming the WNT carrier function of GPR177. On the other hand, GPR177 monoclonal antibody treatment affects tumorigenicity of WNT secreting cells by inhibiting the secretion of WNT ligand.



Supplementary Figure 3

Full-length blots merged with protein size markers. All images were processed with provider's software. In Figure 2 C, siRNA-mediated knock down of GPR177 was evaluated in western blot and lysate which is efficiently inhibited by siGPR was further evaluated with anti-actin antibody as loading control.





Supplementary Methods

Western blotting

Cells were washed with cold phosphate-buffered saline (PBS) and collected. Cell extracts were prepared with lysis buffer (50 mmol/L Tris-Cl, pH 7.5, 150 mmol/L NaCl, 1% NP-40, 10 mmol/L NaF, 10 mmol/L sodium pyrophosphate, and protease inhibitors) and incubated on ice for 30 min. Lysates were centrifuged at 20,000*g* for 10 min at 4°C. Samples were denatured in 55°C water bath, separated on SDS-PAGE gels, and transferred to nitrocellulose membranes. Membranes were blocked for 2 h in PBS with Tween-20 (PBST) containing 5% nonfat milk. The blocked membranes were incubated with primary GPR177 antibody or anti-β-actin (Sigma-Aldrich) overnight at 4°C and then washed with PBST, incubated with the appropriate secondary anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibody (Thermo Scientific, Waltham, MA, USA) for 1 h, and bands visualized using the Vilber Solo S (Marne la Vallée, France) with an enhanced chemiluminescence detection reagent (Thermo Scientific). Acquired images were cropped and aligned using Vilber image processor software. Primary antibodies used in supplementary figure 1 are listed below. Anti-β-catenin and K48 acetyl-β-catenin, anti-GSK 3β (Cell Signaling Technology, USA), anti-Cyclin D1 (Santa Cruz Biotechnology, Dallas, Texas, USA)

Public cohort data and transcriptome analysis

Gene expression data from the Asian Cancer Research Group (GSE66229, n=300, Affymetrix Human Genome U133plus 2.0 Array) [PMID 25894828] and Singapore gastric cancer cohort (GSE15459, n=200, Affymetrix Human Genome U133plus 2.0 Array) [PMID 19798449] were downloaded from the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information. Each data set was processed according to the platform from which the data were obtained, using R software with Bioconductor version 3.4. Background

adjustment of raw data and normalization with log2 transformation were conducted with the gcrma package. Entrez Gene ID annotation, selection of Gene ID with largest variance for duplicating Gene IDs, filtering low variance and low Affymetrix quality-control probe sets were conducted with nsFilter function for gene filtering. GPR177 expression data were transformed into binary data as low and high expression with median cut-off of expression level in each data set.

Monoclonal antibody production and reagents

Mice were immunized with each antigenic peptides of human GPR177 WNT-binding motif (antigen 1, amino acids 118–137: IAFKLNNQIRENAEVSMDVS; antigen 2, amino acids 138–157: LAYRDDAFAEWTEMAHERVP; antigen 3, amino acids 146–165: AEWTEMAHERVPRKLKCTFT; antigen 4, amino acids 163–181: TFTSPKTPEHEGRYYECDV; antigen 5, amino acids 202–222: PVNEKKKINVGIGEIKDIRL). Most of the experiments was conducted with antibody 3(antibody#3) which is generated from antigen 3 if not mentioned. Monoclonal antibodies were produced by and purchased from ATgen(Seongnam-si, Korea). Cells were transiently transduced with 10 pM small interfering RNA (siRNA) targeting GPR177 (si#1, sense: 5’ GUCAUCUUCUUCAUCGUUAUU 3’, antisense: 5’ UAACGAUGAAGAAGAUGACUU 3’) using Lipofectamine RNAiMax (Life Technologies, USA) following the manufacturer’s protocol.

Quantitative PCR (qPCR)

Total RNA was extracted using TRIzol reagent following the standard protocol (Takara, Japan), and cDNA was prepared using oligo dT primers (Macrogen, Korea). PCR was performed using the following forward and reverse primers: human *GAPDH*, forward: 5’-GATGGCATGGACTGTGGTCA-3’ and reverse: 5’-GCAATGCCTCCTGCACCACC-3’;

human GPR177 (*WLS*), forward: 5'- CAGTCCAAGTGAACAGTGCC 3' and reverse: 5'-CTCCTGGGCCTCCTTGCG -3'; human *AXIN2*, forward: 5'-CTGGCTCCAGAAGATCACAAAG- 3' and reverse: 5'-ATCTCCTCAAACACCGCTCCA-3; human cyclin D1 (*CCND1*), forward: 5′- TGCTCCTGGTGAACAAGCTC -3′ and reverse: 5'- AGGACAGGAAGTTGTTGGGG -3'. cDNA concentration was normalized using GAPDH. qPCR analyses were performed using SYBR Green PCR master mix reagents and an ABI Prism 7700 sequence detection system (Applied Biosystems, USA). All reactions were performed in triplicate. Relative expression levels and standard deviations were calculated using the comparative method.

Immunocytochemistry and histology

Cells were cultured on coverslips, fixed in 4% paraformaldehyde for 30 min at 4°C or in 100% EtOH for 20 min at -20°C, and then treated with 0.3% Triton X-100 in PBS for 10 min at room temperature. Fixed and permeabilized cells were then incubated with anti-GPR177 antibody (#3 or #4) or peptide-blocked antibody at 37°C for 2 h, and then stained with goat anti-rabbit FITC-conjugated (Invitrogen, USA) at 37°C for 2 h. Nuclei were counterstained with Hoechst 33258. Cells were imaged and analyzed using a Zeiss LSM700 confocal microscope (Carl Zeiss, Germany).

Cell viability assay

Cell viability was determined with the conventional 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Cells were seeded in 96- or 24-well plates at 5×10^3–1×10^4 cells/well and incubated overnight. Twenty-four hours before addition of MTT solution (Sigma-Aldrich, USA) to wells, cells were exposed to anti-GPR177 monoclonal antibody at the indicated concentrations. Formazan formation was resolved with DMSO solution for 30 min with shaking. Absorbance was measured at 570 nm, and a

reference was measured at 630 nm with a microplate reader (Model 550, Bio-Rad, USA).

Fluorescence-activated cell sorting (FACS) analysis

Cells were harvested and apoptotic cells were detected by staining with ApoScan^{TM} Annexin V FITC Apoptosis Detection Kit(BioBud, Korea) according to the manufacturer's instruction. Propidium iodide- and annexin V-stained cells were analyzed with an LSRII flow cytometer (BD Biosciences, USA).

Xenograft models

AGS human gastric adenocarcinoma cells (10^6) stably expressing short hairpin RNA (shRNA) targeting GPR177, or control shCon cells, in 100 μL RPMI containing Matrigel (BD Biosciences, USA) were injected subcutaneously into the left flank of 5-week old male athymic BALB/c nu/nu mice (Orient, Korea). Each experimental group included five mice. Tumor size was measured every 2 d using a caliper. Mice were sacrificed, and tumors were harvested and photographed.

GC subjects and tissue microarray analysis (TMA)

Demographic and clinical information and tumor tissue samples were obtained from 909 GC patients who had undergone curative-intent gastrectomy between 2000 and 2003 at Yonsei University Severance Hospital, South Korea. Patient age, sex, tumor histology, Lauren classification, and pathologic tumor-node-metastasis (TNM) stages were evaluated as clinical parameters. The median follow-up time was 112 months (range, 1–163). Immunohistochemical analysis of sections of TMA blocks containing 909 gastric cancer tissue samples was carried out using a Ventana XT automated stainer (Ventana, USA) and anti-GPR177 antibodies. Sections were deparaffinized in EZ Prep solution (Ventana) and the CC1 standard solution (Ventana) was used for antigen retrieval. Sections were blocked in

inhibitor D (3% H_2O_2) for 4 min at 37°C. Slides were incubated with antibody for 40 minutes and then with a universal secondary antibody for 20 minutes, at 37°C. Streptavidin-horseradish peroxidase D was applied for 16 min, and substrate 3,3´-diaminobenzidine tetrahydrochloride and H_2O_2 were added for 8 minutes, followed by counterstaining with hematoxylin and bluing reagent at 37°C. Our study was approved and confirmed that all experiments were performed in accordance with relevant guidelines and regulations by the Institutional Review Board of Severance Hospital, Seoul, Republic of Korea (4-2015-0616) and all patients provided written informed consent.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Supplementary Table 1. Clinicopathological characteristics of human gastric cancer patients segmented by GPR 177 expression level

	Overall	GPR177 Negative (n=463)	GPR177 Positive (n=446)	*p*-value
Age (years)	57.19±11.94*	57.25±11.64	57.13±12.25	0.87
Sex				0.18
Male	598 (65.8%)∞	295 (63.7%)	303 (67.9%)	
Female	311 (34.2%)	168 (36.3%)	143 (32.1%)	
Histology				0.007
Differentiated	264 (29.0%)	116 (25.1%)	148 (33.2%)	
Undifferentiated	645 (71.0%)	347 (74.9%)	298 (66.8%)	
Lauren				0.382
Intestinal	457 (50.3%)	223 (48.2%)	234 (52.5%)	
Diffuse	406 (44.7%)	214 (46.2%)	192 (43.0%)	
Other	46 (5.0%)	26 (5.6%)	20 (4.5%)	
pTstage				0.419
pT1	4 (0.4%)	1 (0.2%)	3 (0.7%)	
pT2	168 (18.5%)	84 (18.1%)	84 (18.8%)	
pT3	135 (14.9%)	76 (16.4%)	59 (13.2%)	
pT4	602 (66.2%)	302 (65.2%)	300 (67.3%)	
pNstage				0.738
pN0	278 (30.6%)	147 (31.7%)	131 (29.4%)	
pN1	170 (18.7%)	81 (17.5%)	89 (20.0%)	
pN2	183 (20.1%)	95 (20.5%)	88 (19.7%)	
pN3	278 (30.6%)	140 (30.2%)	138 (30.9%)	
TNM stage				0.952

Stage I	104 (11.4%)	53 (11.4%)	51 (11.4%)
Stage II	259 (28.5%)	134 (28.9%)	125 (28.0%)
Stage III	546 (60.1%)	276 (59.6%)	270 (60.5%)

*mean±standard deviation; §n (%).

Supplementary Table 2. Multivariate Cox proportional hazard models

	GSE15459 (n=192)			GSE66229 (n=300)			TMA (n=909)		
	HR	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
Age (years)	1.008	0.992–1.024	0.301	1.031	1.105–2.151	**<0.001**	1.022	1.014–1.030	**<0.001**
Sex	0.728	0.452–1.174	0.193	0.879	0.624–1.240	0.463	1.049	0.869–1.267	0.616
Lauren*	0.958	0.624–1.471	0.845	1.599	1.184–2.158	**0.002**	1.235	1.065–1.433	**0.005**
TNM*	2.854	2.174–3.745	**<0.001**	2.355	1.917–2.892	**<0.001**	1.471	1.383–1.566	**<0.001**
GPR177	1.691	1.087–2.628	**0.020**	1.542	1.105–2.151	**0.011**	1.254	1.049–1.500	**0.013**

GSE, Gene Expression Omnibus (GEO) series; TMA, tissue microarray; HR, hazards ratio; CI, confidence interval.

*stage.