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Targeted inhibition of Wntless/GPR177 suppresses gastric tumorigenesis

Jaesung Seo

Department of Medical Science

The graduate School, Yonsei University

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Targeted inhibition of Wntless/GPR177 suppresses gastric tumorigenesis

Directed by Professor Ho-Geun Yoon

The Doctoral Dissertation
submitted to the Department of Medical Science
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Jaesung Seo

June 2017

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YONSEI UNIVERSITY

This certifies that the Doctoral Dissertation

of Jaesung Seo is approved.

Thesis Supervisor: Ho-Geun Yoon

Thesis Committee Member#1: Jae-Ho Cheong

Thesis Committee Member#2: Kyung Chul Choi

Thesis Committee Member#3: Yun-Han Lee

Thesis Committee Member#4: Ki-Taek Nam

The Graduate School

Yonsei University

June 2017

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Abstract

Targeted inhibition of Wntless/GPR177 suppresses gastric tumorigenesis

Jaesung Seo

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor **Ho-Geun Yoon**)

Wntless/GPR177 functions as WNT ligand carrier protein and activator of WNT/β-catenin signaling, however, its role in gastric tumorigenesis has remained elusive. We investigated the role of GPR177 in gastric tumorigenesis and provided the therapeutic potential of a clinical development

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of anti-GPR177 antibodies. GPR177 mRNA and protein expression were associated with unfavorable prognosis [log-rank test, GSE15459 (p=0.00736), GSE66229 (p=0.0142), and Yonsei TMA (p=0.0334)] and identified as an independent risk predictor of clinical outcomes {GSE15459 [hazard ratio (HR) 1.731 (95% confidence interval; CI; 1.103–2.715), p=0.017], GSE66229 [HR 1.54 (95% CI, 1.10–2.151), p=0.011], and Yonsei TMA [HR 1.254 (95% CI, 1.049–1.500), p=0.013]. Overall survival and multivariate prognostic significance of GPR177 expression were analyzed by Kaplan-Meier curves (log-rank test) and Cox proportional hazard regression models, respectively. GC cell lines were classified as WNT-secreting or non–WNT-secreting. Anti-GPR177 antibody reduced WNT secretion and viability in WNT-secreting cells by inhibition of WNT/β-catenin signaling. GPR177 alleviated ER–stress-induced apoptosis by ectopic localization to ER in non–WNT-secreting cells. Antibody treatment suppressed proliferation of WNT-secreting cells only; however, GPR177 knockdown sensitized non–WNT-secreting cells to ER–stress-induced apoptosis. Anti-GPR177 antibodies exhibited anti-cancer efficacy in xenograft and PDX models. GPR177 overexpression correlated with poor GC patient prognosis. Inhibition of GPR177 using monoclonal antibodies or short–hairpin–RNA-mediated knockdown suppresses in vitro

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and in vivo tumorigenesis in WNT-secreting GC cells and inhibits WNT/β-catenin signaling. Non–WNT-secreting cells were sensitized by GPR177 knockdown, but not by antibody treatment, via enhancement of ER stress, leading to cell death. Anti-GPR177 antibody treatment suppresses in vivo gastric tumorigenesis.

Key words: Wntless/GPR177/WLS, Gastric cancer, monoclonal antibody, WNT/β-catenin signaling, PDX

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I. INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies and the third leading cause of cancer death worldwide.[1] Many studies have attempted to elucidate the molecular mechanisms underlying gastric tumorigenesis and develop novel molecular targeted therapeutics.[2,3] Recently, anti-HER2

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receptor and anti-VEGFR2 receptor antibodies were developed and approved as targeted therapy for GC patients;[4-6] however, identification and validation of more candidate therapeutic targets are still required.[7]

Dysregulation of WNT/β-catenin signaling is commonly observed in cancer, including GC.[8-12] When WNT ligands are secreted and interact with their cognate receptors, β-catenin–dependent or –independent signaling cascades are transduced, resulting in cancer progression.[13-15] In the absence of WNT ligand, β-catenin is maintained at a low level through degradation by the β-catenin destruction complex.[16] Secreted WNT ligand binds to FZD-LRP receptors, triggering the WNT/β-catenin axis. As a consequence, AXIN complexes with DVL which inhibits β-TrCP, resulting in cytoplasmic accumulation of β-catenin, which then translocates into the nucleus where it binds to the TCF/LEF transcription complex, activating target genes transcription. In many types of cancer, mutations in CTNNB1, AXIN, and APC cause hyperactivation of WNT/β-catenin signaling, resulting in cancer progression even in the absence of WNT ligand stimulation.[17] Therefore, various small molecules and antibodies are under development as cancer therapeutics.[16,18] A recent report suggests that blocking of WNT secretion may be a useful means of treating colorectal cancer.[19-22]

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WNTs are secreted as glycosylated/lipid-modified proteins, mediated by the seven-pass transmembrane protein Wntless/GPR177.[21,23,24] GPR177 localizes to compartments of the secretory pathway, including the ER, Golgi apparatus, endosomes, and plasma membrane.[25-29] GPR177 is overexpressed in several cancer types, and GPR177 knockdown reduced cancer formation in a STAT3-dependent manner.[9] Growth of colon cancer cells harboring the β-catenin active mutant or APC mutant was inhibited in a GPR177-dependent manner. Thus, these studies suggest that blockade of GPR177 function may be a promising therapeutic target.[11]

In cancer cells, elevated protein synthesis in the ER can cause accumulation of misfolded proteins and activation of the unfolded protein response (UPR), which leads to chemoresistance or apoptosis.[30-32] However, crosstalk between ER-stress pathways and WNT/β-catenin signaling in cancer is poorly understood. A previous study showed that hypoxia-induced ER stress reduced WNT/β-catenin signaling due to WNT ligand misfolding following colon cancer cell death.[33] GPR177 transports lipid-modified WNT ligand from ER to plasma membrane.[21,23,34] Thus, dysregulation of GPR177 function in the ER may affect WNT ligand secretion, and in turn, cell viability. However, the role of GPR177 in the ER is unknown.

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Here, we demonstrate that overexpression of GPR177 mRNA and protein correlates with poor prognosis of GC patients. GPR177-expressing GC cells were classified as WNT-secreting or non–WNT-secreting. In WNT-secreting lines, WNT secretion and cell proliferation were inhibited by either anti-GPR177 monoclonal antibody treatment or GPR177 knockdown. In non–WNT-secreting lines, GPR177 was predominantly localized in the ER, not the Golgi, and suppressed ER–stress-mediated apoptosis. GPR177 knockdown, but not antibody treatment, sensitized non–WNT-secreting cells to ER–stress-induced apoptosis. Moreover, anti-GPR177 antibody exhibited anticancer efficacy in mouse and patient-derived xenograft (PDX) models.

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II. MATERIALS AND METHODS

1. Cell culture and reagents

Human gastric cancer cell lines AGS, Hs746T, KATOIII, SNU216, SNU484, SNU601, SNU638, SNU668, SNU719, SNU1, SNU5, SNU16, SNU520, SNU620, MKN1, MKN28, MKN45, MKN74, YCC6, YCC7, YCC16 were gifted from the Cheong's lab including Human oesophagus cell line SK4 and Human gastric mucosa cell line GES1.[35] All cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (Hyclone, Logan, UT, USA) at 37℃ under 5% CO_2 except 293FT, Hs746T and YCC cells in DMEM. Etoposide and cycloheximide were purchased from Sigma-Aldrich (St. Louis, MO, USA). MG-132 was purchased from Calbiochem (Darmstadt, Germany). Transient transfection was performed using TransIT 2020(Mirus, Madison, WI, USA).

2. 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

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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, Tucson, AZ, 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% H2O2) for 4 min at 37℃. Slides were incubated with antibody for 40 minutes and then with a universal secondary antibody for 20 min, at 37℃. Streptavidin-horseradish peroxidase D was applied for 16 min, and substrate 3,3´-diaminobenzidine tetrahydrochloride and H2O2 were added for 8 min, followed by counterstaining with hematoxylin and bluing reagent at 37℃. Our study was approved by the Institutional Review Board of Severance Hospital, Seoul, Republic of Korea (4-2015-0616).

3. Western blot analysis

Cells were lysed in lysis buffer (20 mM Tris-Cl, 150 mM NaCl, 1% Triton X-100, 1.5% $MgCl_2$, 1 mM EDTA, 1 mM Na_2VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail, pH 7.5). Lysates were briefly vortexed and cleared by centrifugation at 13,000

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rpm for 20 minutes at 4℃. The supernatants were collected and transferred to fresh tubes. Protein concentrations were determined by 660 nm protein assay reagent (Thermo Scientific, Rockford, IL, USA). Equal amount of protein extracts was subjected to electrophoresis on SDS-polyacrylamide gels and then transferred to Nitrocellulose transfer membranes (Whatman, Dassel, Germany). The membranes were blocked in Tris-buffer (pH 7.4) containing 0.1% (v/v) Tween 20 (Sigma-Aldrich) and 5% (w/v) nonfat Difco™ skim milk (BD Biosciences, Sparks, MD, USA) and probed with primary antibodies. The following antibodies were used: GPR177 (ATgen, Seongnam-si, Korea), HA tag, Calnxin, BiP, CHOP, PERK, phosphor S473 AKT1, AKT1, cleaved caspase-12, cleaved Caspase-3, acetyl K49 β-catenin (Cell signaling, Danvers, MA, USA), GM130 (Abcam), Cleaved PARP, β-catenin, phosphor S9 GSK3β (BD Biosciences), phosphor T981 PERK, CyclinD1 (SantaCruz), β-actin (Sigma-Aldrich). The signals were developed by substrate (Thermo Scientific) according to the manufacturer's protocol.

4. Immunoprecipitation

Cells were lysed in lysis buffer. After centrifugation, 500 μg of the clarified cell lysate was pre-cleared with G plus/protein A-agarose (Santa Cruz), by incubating for 30 minutes. The supernatant was collected and 1 μg

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of antibody was added. After overnight incubation, 30 μl of 50% slurry of G plus/protein A-agarose was added and the mixture was incubated for 2 hours. The agarose bead was centrifuged, washed thrice with ice-cold washing buffer, and suspended in electrophoresis sample buffer, and boiled for 5 min. The immunoprecipitated protein was further analyzed by Western blotting.

5. Immunocytochemistry and histology

Cells were cultured on coverslips, fixed in 4% paraformaldehyde for 30 min at 4℃ or in 100% EtOH for 20 min at -20℃, 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), anti-calnexin, anti-AKT, anti-phosphoserine473 AKT antibody (Cell Signaling Technology, Danvers, MA, USA), or anti-GM130 (Abcam, Cambridge, UK) antibody at 37℃ for 2 h, and then stained with goat anti-rabbit FITC-conjugated or goat anti-mouse rhodamine-conjugated secondary antibody (Invitrogen) at 37℃ for 2 h. Nuclei were counterstained with Hoechst 33258. Cells were imaged and analyzed using a Zeiss LSM710 confocal microscope (Carl Zeiss, Oberkochen, Germany).

6. Fluorescence-activated cell sorting (FACS) analysis

Cells were harvested and apoptotic cells were detected by staining with

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ApoScanTM 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, Franklin Lakes, NJ, USA).

7. Xenograft models

AGS human gastric adenocarcinoma cells (106) stably expressing short hairpin RNA (shRNA) targeting GPR177, or control shCon cells, in 100 μL RPMI containing Matrigel (BD Biosciences) were injected subcutaneously into the left flank of 5 week old male athymic BALB/c nu/nu mice (Orient, Seoul, Korea). Each experimental group included five mice. Tumor size was measured every 2 day using a caliper. Mice were sacrificed, and tumors were harvested and photographed. Tumor volume was estimated using the formula, volume = ½ × a × b2, where a and b represent the largest and smallest tumor dimensions, respectively. Animal studies were approved under the guidelines of the Institutional Animal Care committee of the Yonsei University College of Medicine.

8. PDX model

A PDX model was established as previously reported.[36] Briefly, a tumor was obtained from a surgical specimen (F0) immediately after gastrectomy.

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Formation of a tumor greater than 500 mm^3 in volume at the implant site was considered a successful engraftment in the case of subcutaneous implantation. The mouse was anaesthetized, and the tumor (F1) was removed for serial transplantation into the next generation (F2, F3). Mice were housed and fed according to the institutional guidelines for animal care. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Yonsei University College of Medicine (2014-0130). Six-week old female athymic nude mice (Japan SLC, Inc., Japan) and male NOG mice (NOD/Shi-scid, IL-2 Rγnull, Ciea, japan) were used for each PDX model. Before the experiments, the animals were acclimated for seven days to a 12-h light/12-h dark cycle. One case of a GPR177-positive tumor specimen (F0) that was successfully maintained through passages was subcutaneously implanted into a NOG mouse (passage 1 or F1). Two weeks later, the tumor was removed and inoculated into 20 nude mice (passage 2 or F2). These mice were divided into two groups.

9. 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 5×103–1×104 cells/well

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and incubated overnight. Twenty-four hours before addition of MTT solution (Sigma-Aldrich) to wells, cells were exposed to anti-GPR177 monoclonal antibody or tunicamycin (TM) 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, Hercules, CA, USA).

10. Quantitative PCR (qPCR)

Total RNA was extracted using TRIzol reagent following the standard protocol (Takara, Kyoto, Japan), and cDNA was prepared using random hexamer primers (Chromogen, Seoul, 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'-

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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, Carlsbad, CA, USA). All reactions were performed in triplicate. Relative expression levels and standard deviations were calculated using the comparative method.

11. Monoclonal antibody production and reagents

Mice were immunized with 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). 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’;

si#2, sense: 5’ GUAAGAUUUACUGUAUUAAUU 3’,

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antisense: 5’ UUAAUACAGUAAAUCUUACUU 3’) using Lipofectamine RNAiMax (Life Technologies, Grand Island, NY, USA) following the manufacturer’s protocol.

12. Statistical Analysis

Prognosis was evaluated by overall survival, from day of surgery to death by any cause. Correlation of gene expression with overall survival was estimated using Kaplan-Meier survival plots and log-rank tests. Multivariable Cox proportional hazards regression analysis was used to evaluate independent prognostic factors associated with overall survival, including age, sex, Lauren type, and TNM-stage groups as covariates. A two-sided P value <0.05 was considered statistically significant. All statistical analyses were conducted in the R language environment (http://www.r-project.org).

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III. RESULTS

1. GPR177 overexpression in GC is correlated with poor survival

To investigate the clinical significance of GPR177 in GC, we analyzed publicly available tumor transcriptome data sets and found that GPR177 mRNA level strongly correlated with poor overall survival in patients with GC (Fig. 1 A and B). To evaluate whether GPR177 mRNA level correlates with protein level while maintaining the clinical implications, we analyzed GPR177 protein expression in tissue microarrays (TMAs) prepared using tissue of GC patients (n=909) and a monoclonal antibody generated for this study. Patient age and sex, tumor histology, Lauren classification, and pathological tumor-node-metastasis (TNM) stage were analyzed as clinicopathological parameters in relation to GPR177 expression level. Intriguingly, all clinical and histological parameters were comparable between GPR177-negative (n=463) and GPR177-positive (n=446) GC patients, with the exception of tumor histology. In general, undifferentiated GC histology would correlate with unfavorable clinical outcomes; however, GPR177-positive tumors were significantly correlated with more highly differentiated histological subtypes, signifying that GPR177 expression may be

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independently associated with poor clinical outcomes. Indeed, positive GPR177 protein expression correlated significantly with poorer prognosis of GC patients. Furthermore, GPR177 expression at both the mRNA and protein levels was identified as an independent risk predictor of clinical outcome of GC patients even after adjusting the covariates as Lauren and TNM stages. Together, the clinical data suggest that GPR177 expression may be a reliable and legitimate predictor of unfavorable clinical outcome of GC patients, evidence that further investigation of molecular mechanisms in GC tumorigenesis is warranted.

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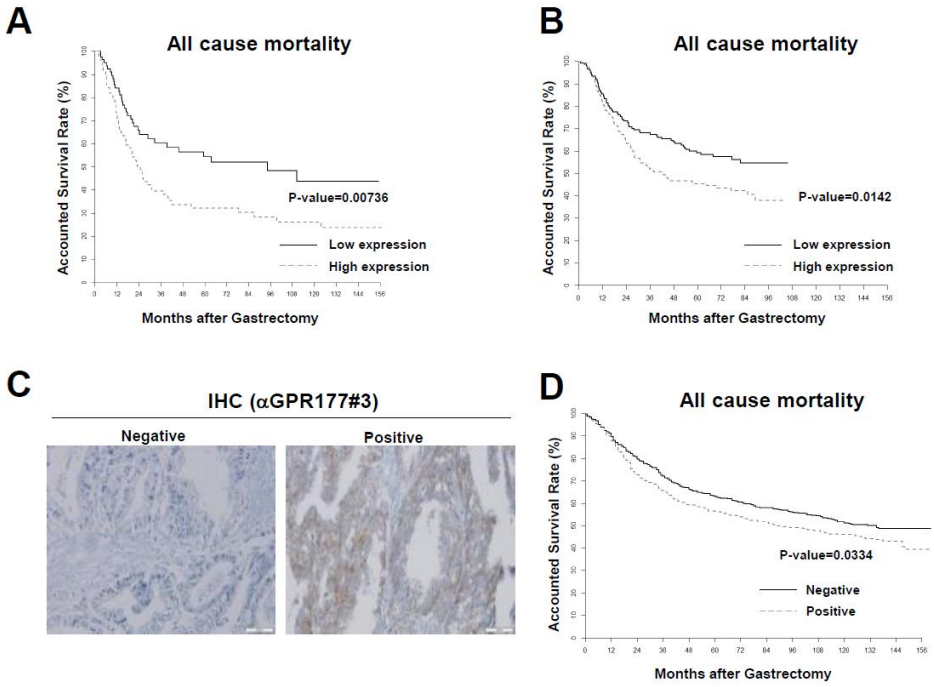

Figure 1. Clinical impact of GPR177 in gastric cancer (GC) patients. A and **B**) Correlation of GPR177 (*WLS*) mRNA expression with overall survival for patients (A, n=184; B, n=300). Solid and broken lines denote patients whose tumors expressed lower and higher levels, respectively, than the median value. **C**) Micrographs of immunohistochemical staining of GPR177 in human GC tumor samples. Representative images of negative (left) and positive (right) staining. Scale bar, 100 μm. **D**) Kaplan-Meier analysis of overall survival in a data set of 909 GC samples used in immunohistochemical analysis and classified as having positive (broken line) or negative (solid line) GPR177 expression.

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

*mean±standard deviation; §n (%).

	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%)	
	645 (71.0%)	347 (74.9%)	298 (66.8%)	
Undifferentiated				
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%)	

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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%)	

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2. Graphical domain of GPR177 and anti-GPR177 monoclonal antibody

GPR177 binds cytoplasmic WNT ligand and secretes it into the extracellular milieu.[37-39] GPR177 domains and their functions are described in Fig. 2 A. To analyze its biological function in GC cell lines, we raised monoclonal antibodies that recognize the WNT-binding motif of GPR177. Antibodies were raised against each of five antigens. The monoclonal antibodies raised against antigen 3 (antibody #3) and antigen 4 (antibody #4) were selected and specificity was verified by western analysis of GPR177-overexpressing and knockdown AGS cells in Fig. 2 B, C.

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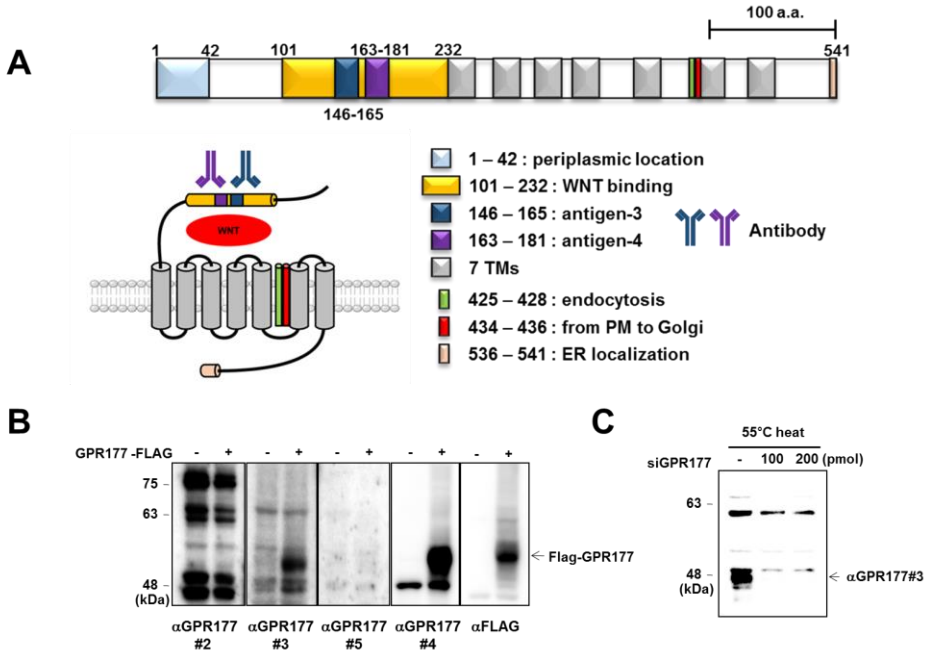

Figure 2. Clinical impact of GPR177 in gastric cancer (GC) patients. A) Schematic diagram of GPR177 structure and function. Upper: linear representation of GPR177. Lower: GPR177, with WNT cargo, spanning plasma membrane. The seven transmembrane domains consist of amino acids 233–252, 265–287, 302–319, 332–354, 379–401, 434–456, and 471–493. **B**) Validation of monoclonal antibody specificity. Left: AGS cells were transiently transfected with plasmid encoding GPR177-FLAG, and total lysates were analyzed by western blotting. **C)** AGS cells were transiently transfected with small interfering RNA (siRNA) targeting GPR177 as indicated.

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3. Screening of WNT secreting cells and non-WNT secreting cells

Considering heterogeneity of GC,[40] we evaluated GPR177 expression levels in multiple GC cell lines using qPCR and western blotting (Fig. 3 A, B). GPR177 expression levels showed a certain degree of variation across cell lines, including SNU484, SNU668, SNU1 and SNU620, considered GPR177 null cells. Among the GC cell lines, SNU5 and MKN28 expressed high levels of GPR177. Next, we examined the possibility that GPR177 is required for WNT secretion from GC cells.[10,23-25,27-29,41-43] GC cells transiently expressing HA-tagged WNT1 were cultured with or without anti-GPR177 antibody. 293FT served as a positive control. Levels of WNT ligand in medium were assessed by western blotting with anti-HA antibody and ELISA (Fig. 3 C, D). Cell lines AGS, MKN45, YCC6, YCC7, and YCC16 secreted WNT ligand, and secretion was inhibited by treatment with anti-GPR177 antibody. Although SNU5 and MKN28 cells showed the highest GPR177 expression levels among GC cells, both lines failed to secrete WNT ligand. Based on these observations, we classified the GC cell lines as WNT-secreting and non–WNT-secreting phenotype.

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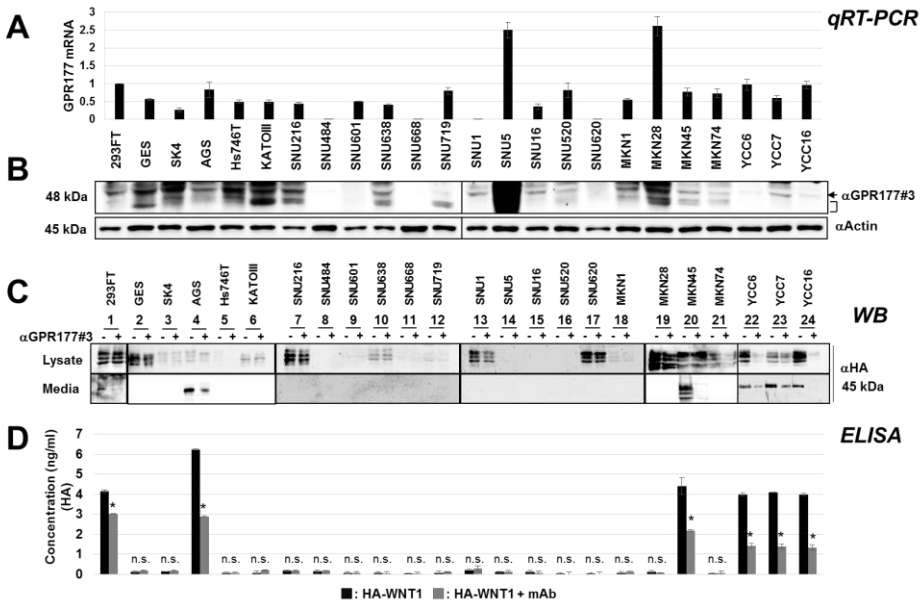

Figure 3. GC cell lines are classified into WNT secreting cell or WNT non-secreting cell. **A**) GPR177 mRNA expression was measured by qPCR. Twenty-three GC cell lines and 293FT cells were collected and analyzed. Compared to 293FT cells, SNU5 and MKN28 cells showed higher (2.5-fold) expression levels; SNU484, SNU668, and SNU620 cells showed no GPR177 expression; the remainder of cells showed expression levels similar to that of 293FT cells. **B**) GPR177 protein and mRNA levels in cell lines showed no significant differences. Arrow indicates GPR177 transcript variant 1; bar indicates variant 2. **C**) Western analysis of WNT-secreting cells. Twenty-four cultured cells transiently expressing plasmid encoding

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HA-WNT1 were cultured with or without GPR177 monoclonal antibody (1 μg/ml) for 24 h. Total lysate and culture medium were analyzed by western with anti-HA antibody. Medium harvested from AGS, MKN45, YCC6, YCC7, and YCC16 cultures contained HA-WNT1.

D) ELISA detection of secreted HA-WNT1. WNT secretion was reduced by monoclonal antibody treatment. *P<0.05.

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4. GPR177-mediated WNT secretion is inhibited by anti-GPR177 monoclonal antibody

We found that proliferation of WNT-secreting cells was inhibited by anti-GPR177 antibody in a concentration-dependent manner. However, antibody treatment had no effect on non–WNT-secreting MKN28 cells (Fig. 4 A, B). Whereas WNT overexpression increased the migration of AGS cells compared with control cells, this effect was decreased by monoclonal antibody treatment (Fig. 4 C). In the colony-forming assay, WNT-secreting, but not non–WNT-secreting cells, were sensitive to GPR177 antibody treatment (Fig. 4 D). It has been reported that MKN74 cells are capable of brain metastasis upon injection into mouse heart, and the brain metastatic (NI3-3) cells have been established in cell culture.[44] NI3-3 cells are sensitive to anti-GPR177 antibody treatment compared with the parental MKN74 cells and expressed high levels of GPR177 (WLS), AXIN2, and cyclin D1 (CCND1) mRNA (Fig. 4. E, F). Taken together, WNT-secreting, but not non–WNT-secreting, GC cells, are efficiently inhibited by treatment with anti-GPR177 monoclonal antibodies.

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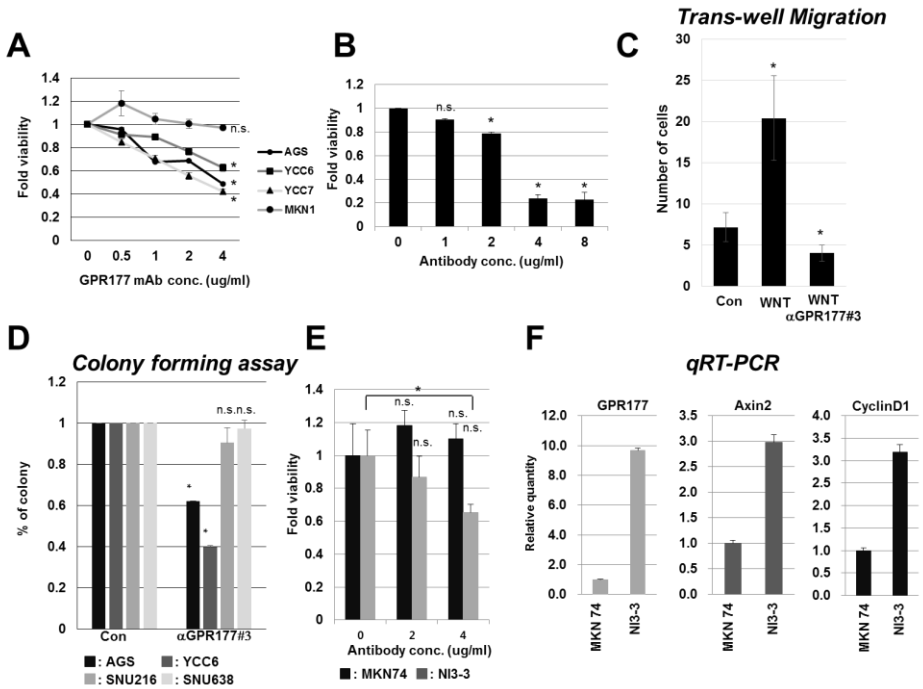

Figure 4. WNT-secreting, but not non–WNT-secreting, cells are sensitive to anti-GPR177 antibody treatment. **A**) GPR177 monoclonal antibody treatment reduced cell viability. Cells were treated with GPR177 monoclonal antibody at the indicated concentration for 24 hr. Only WNT-secreting cells showed reduced viability with antibody treatment. **B**) AGS cells were treated with monoclonal antibody at the indicated concentrations and for the indicated times to determine lethal dose (LD)50 . Viability was assessed by MTT assay. *P<0.05. **C**) Monoclonal

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antibody treatment reduced cell migration. AGS cells transiently overexpressing WNT and control cells were seeded on transwell plates, and migrated cells were counted. GPR177 monoclonal antibody suppressed cell migration (concentration 1 μg/ml). *P<0.05. **D**) WNT-secreting (AGS and YCC6) and non–WNT-secreting (SNU216 and SNU638) cells were seeded at 10^4/60-mm dish and treated with monoclonal antibody. Colonies were fixed and stained with crystal violet and counted under microscope. *P<0.05. **E**) MKN74 and NI3-3 cells were treated with 2 or 4 μg/ml monoclonal antibody for 24 h. Suppression was observed only in NI3-3 cells. *P<0.05. **F**) qPCR analysis showed that WNT/β-catenin signaling is upregulated in NI3-3 cells compared to MKN74 cells. GPR177 (*WLS*), *AXIN2*, and cyclin D1 (*CCND1*) mRNA levels were detected.

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5. WNT ligand stimulates translocalization of GPR177 from ER to Golgi in WNT-secreting GC cells

Prompted by the distinct responses between WNT-secreting and non-WNT-secreting cells to anti-GRP177 antibodies treatment, we investigated the subcellular localization of GPR177 to examine topographic regulation of GPR177 expression in GC cells. Using anti-GPR177 monoclonal antibodies, we detected endogenous GPR177 by immunofluorescence microscopy (Fig. 5 A). In accord with earlier results, GPR177 was upregulated and co-localized with overexpressed WNT ligand.[43] However, the localization pattern differed from that described in a report that GPR177 is transported from the ER to the Golgi and plasma membrane.[25-29] We found that GPR177 remained predominantly in the cytoplasm, and the cytoplasmic compartment was identified as the ER (Fig. 5 A, upper panel). In both WNT-secreting and non–WNT-secreting cells, GPR177 was localized in the ER but not in the Golgi. While residing in ER in WNT ligand absent conditions, GPR177 moved from ER to Golgi only in the WNT-secreting cells upon WNT ligand stimulation (Fig. 5 A). This observation may explain why only the WNT-secreting cells are sensitive to monoclonal antibody treatment. GPR177 carries WNT cargo from the ER to the Golgi and plasma membrane. Therefore, if an abnormality

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or malfunction occurs in the secretory pathway or process, intracellular trafficking of GPR177 might be disrupted.[45] Consequently, GPR177 may not be displayed at the plasma membrane to be available for recognition by cognate antibody, rendering the non–WNT-secreting cells insensitive to the anti-GPR177 antibody treatment. To further interrogate the precise localization of GPR177 inside the cell, WNT was transiently overexpressed in AGS cells followed by detection of WNT and ER marker calnexin or Golgi marker GM130. Upon WNT stimulation, GPR177 moved from the ER to the Golgi in WNT-secreting AGS cells, but not in non–WNT-secreting SNU638 and SNU216 (Fig. 5 B). Collectively, these data indicate that GPR177 localizes to the ER and is transported to the Golgi when the WNT signal is activated in WNT-secreting cells but not in non–WNT-secreting cells.

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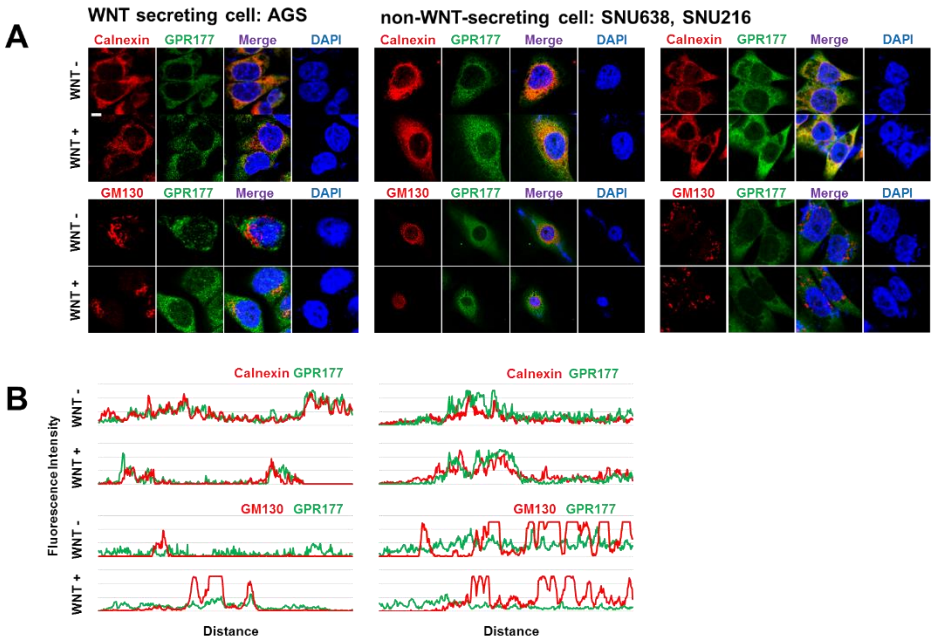

Figure 5. ER-localized GPR177 attenuates ER–stress-induced unfolded protein response (UPR). **A**) GPR177 localizes to ER and Golgi. Immunofluorescence analysis showed that GPR177 localized to the ER only in WNT-secreting (first row) and non–WNT-secreting cells (third row) in WNT absent condition. With HA-WNT1 overexpression, GPR177 localized to the Golgi only in WNT-secreting cells (second and fourth row). In non–WNT-secreting cells, Golgi structures were abnormally distributed, indicating nonfunctional Golgi-mediated secretion. Green: GPR177; blue: Hoechst; red (upper panel): ER marker calnexin; red (lower panel): Golgi marker GM130; purple: merged image. Scale bar: 10 μm. **B**) Graphical analysis of fluorescence intensity across the distances (30 μm). Intensity in

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a pixel (0.09×0.09 μm) was transformed in each image using Carl Zeiss Zen imaging software. Red (upper): calnexin; red (lower), GM130. Yellow arrows indicate differences in GPR177 localization in control and WNT-overexpressing cells.

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6. GPR177 attenuates ER–stress-induced UPR

As mentioned above, we found that GPR177 is primarily located in the ER in non–WNT-secreting GC cells even after WNT ligand treatment. This observation prompted us to speculate that GPR177 may have unknown functions in the ER. Cancer cells have faster doubling times than normal cells necessitating timely provision of a sufficient supply of macromolecules for cell division. These elevated metabolic demands, which require a nonphysiologically high rate of protein production, can induce ER stress or UPR.[30,31,46] Under these stressful conditions, cancer cells must cope with ER stress to survive in the presence of UPR.[30-33,46-49] A recent study showed that accumulated WNT ligand in the ER results in ER stress because of the hydrophobicity of WNT ligand.[50] Therefore, we hypothesized that GPR177 reduces ER stress upon UPR. To induce ER stress, GC cells were treated with TM, which inhibits protein glycosylation and triggers ER stress.[31,51] As shown in Figure 6 A, TM treatment induced UPR as confirmed by western analysis using antibodies against ER-stress markers Bip, CHOP, Ero1, PDI, IRE1, and PERK. Remarkably, the level of GPR177 was gradually reduced in a dose-dependent manner. Among these ER-stress markers, CHOP and PERK were significantly increased with siRNA-mediated GPR177 knockdown, indicating

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a significant role of GPR177 in regulation of ER stress. It is well known that PERK phosphorylation and CHOP induction lead to apoptosis.[52,53] Therefore, we examined expression of apoptosis markers cleaved caspase-3, cleaved PARP1, and cleaved caspase-12. GPR177 knockdown resulted in a marked increase in apoptosis compared to the negative control (Fig. 6 A). Conversely, GPR177 overexpression reduced TM-induced apoptosis (Fig. 6 B). Intriguingly, activation of WNT/β-catenin signaling was not observed when GPR177 was ectopically expressed in GC cells, suggesting that overexpression itself is insufficient to activate WNT/β-catenin signaling (Fig. 6 C). However, treatment of WNT-secreting, but not non–WNT-secreting, cells with anti-GPR177 antibody reduced WNT/β-catenin signaling (Fig. 6 D, E). Together, these findings suggest that GPR177 functions as an ER–stress-reliever upon WNT activation to sustain cancer cell fitness.

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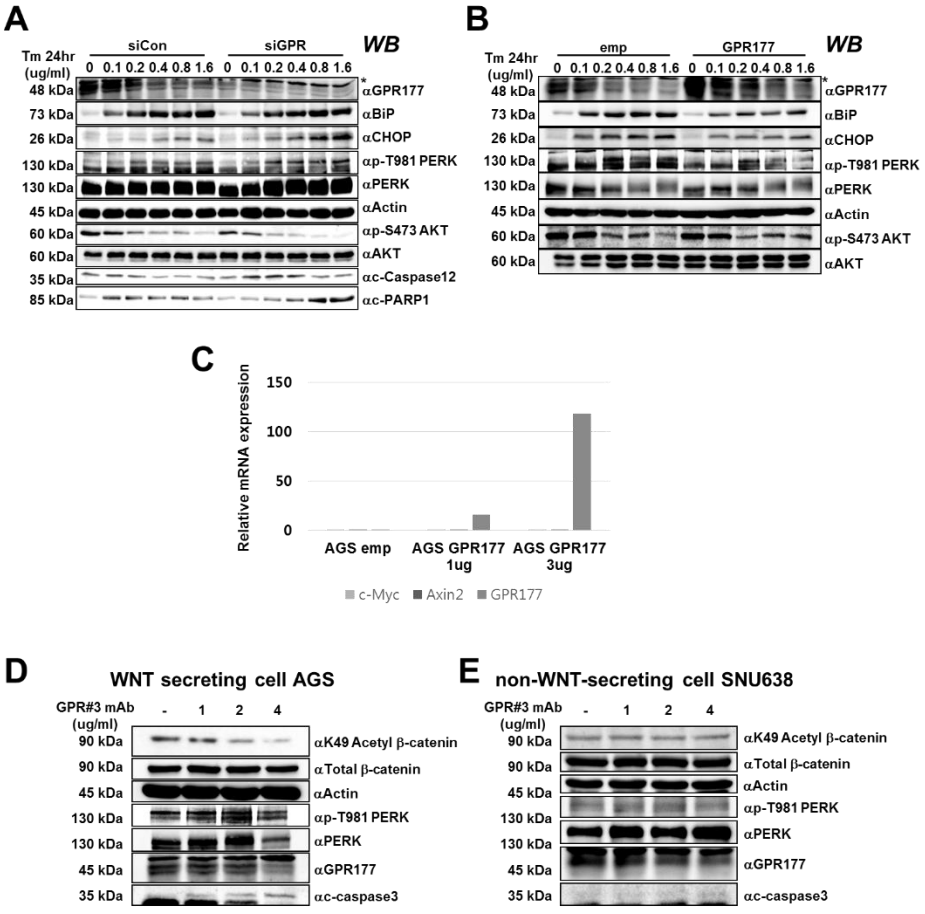

Figure 6. ER-localized GPR177 attenuates ER–stress-induced unfolded protein response (UPR). A) Non–WNT-secreting SNU638 cells were transiently knocked down with siRNA and stimulated with the indicated concentration of tunicamycin (TM) for 24 hr. Total lysates were analyzed by western blotting. GPR177 was degraded in a dose-dependent manner. UPR markers were also detected with antibodies. **B**) GPR177-overexpressing SNU638 cells were treated with the indicated

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concentrations of TM. UPR stress was induced at a lower level than in control cells. Asterisk; non-specific. **C**) GPR177 was transiently overexpressed in AGS as indicated. Relative expression of GPR177, Axin2 and C-myc were compared by qRT-PCR. **D**) Anti-GPR177 antibody treatment reduced WNT activation. AGS cells were treated with the indicated concentration of anti-GPR177 monoclonal antibody. Total lysates were analyzed by western blotting. Asterisk; non-specific. **E**) SNU638 cells were treated with the indicated concentration of anti-GPR177 monoclonal antibody. Total lysates were analyzed by western blotting.

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7. GPR177 co-localize with AKT1

Because knock down of GPR177 inhibited the activation of AKT, we verified the interaction between GPR177 and AKT (Fig. 7 A, B and C). Their interaction was ubiquitous in WNT secreting or non-WNT secreting-cells. Intriguingly, co-localization was reduced by AKT inhibitor treatment. In addition to that, AKT inhibitor treatment also shifted the localization of GPR177 from ER to cytosol (Fig. 7 D). On the contrary, WNT overexpression enhanced co-localization between GPR177 and AKT1. Strikingly, anti-GPR177 antibody treatment reduced not only β-catenin activation but also AKT activation, both of which are known to be essential to GC development and progression (Fig. 7 E).[2,15,54-57] Furthermore, GPR177 knockdown also inhibited AKT activation (Fig. 7 F). Tough these reciprocal regulation between GPR177 and AKT1 should be examined precisely, the molecular interaction suggests that GPR177 mediated regulation of UPR also could be controlled by AKT signaling pathway.[51] Together, these findings suggest that GPR177 functions as an ER–stress-reliever and AKT co-activator to sustain cancer cell fitness.

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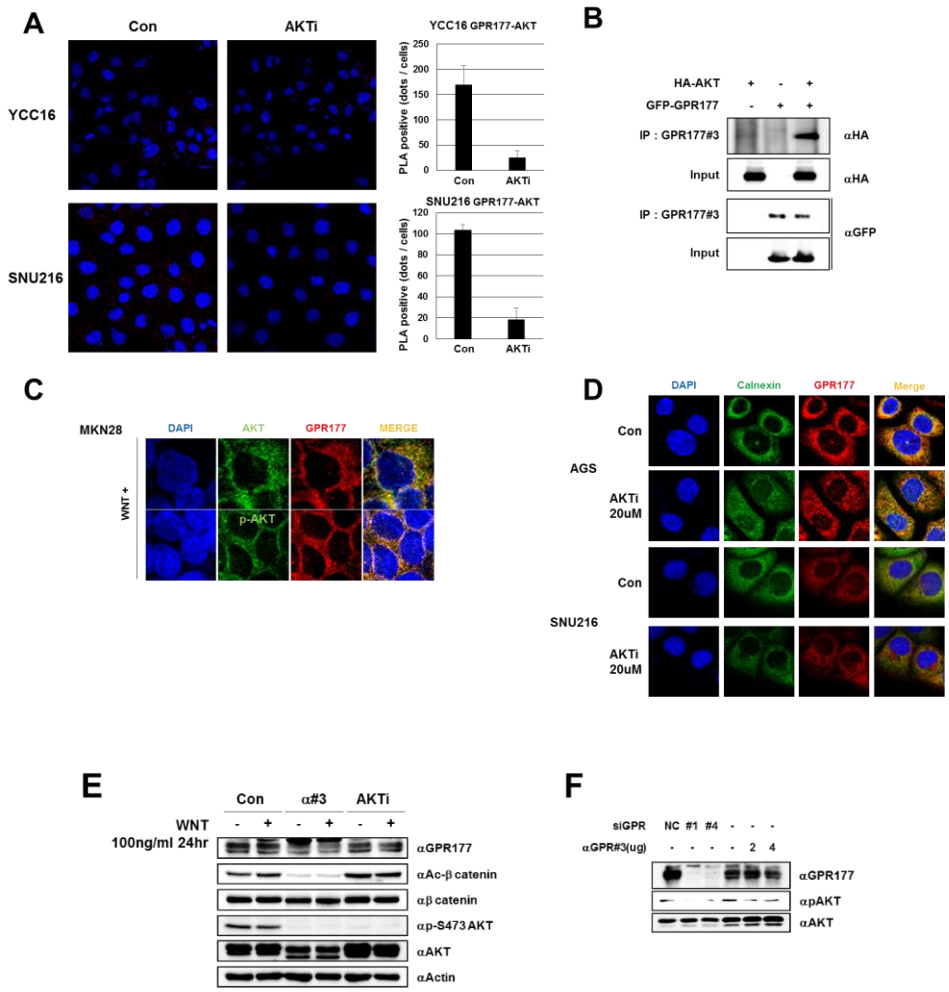

Figure 7. GPR177 co-localizes with AKT to sustain cell fitness. A) YCC16 and SNU216 cells were treated with vehicle control or AKT inhibitor for 20uM 24hr and the interaction between GPR177 and AKT was analyzed with Duolink PLA (sigma, DUO92101). Positive dots per cells were calculated with ImageJ. **B)**

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HEK293FT cells were transiently overexpressed as indicated. Total lysate was co-immunoprecipitated with GPR177 antibody and analyzed with western blotting. **C**) MKN28 cells were transiently expressed with HA-WNT1 and stained with anti-AKT (green upper panel) or anti-phospho-S437 (green lower panel) with anti-GPR177. (red) **D**) AGS and SNU216 cells were treated with vehicle control or AKT inhibitor for 20uM 24hr and stained with anti-Calnexin (green) and anti-GPR177 (red). **E**) AGS cells were treated with WNT3a ligand for 24hr with antibody treatment or AKT inhibitor 10uM (A6730 SIGMA). Total cell lysate was assayed in western blotting. Phosphorylation of serine 437 of AKT1 was dramatically reduced by antibody treatment. **F**) AGS cells were knocked down by siRNA (50pmol for 48hr) or treated with GPR177 antibody as indicated concentration. Total cell lysate was assayed in western blotting.

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8. Anti-GPR177 antibodies failed to enhance ER–stress-induced apoptosis in non–WNT-secreting cells

Given the finding that GPR177 attenuates ER–stress-induced UPR, we next examined the effect of GPR177 inhibition on ER stress-induced apoptosis in GC cells. Remarkably, we found that siRNA-mediated silencing of GPR177 enhanced TM-induced apoptosis (Fig. 6 A). Therefore, we next investigated whether cell viability is synergistically suppressed by GPR177 inhibition with TM treatment. GC cell lines were treated with TM (0.1 μg/ml) for 24 hr or antibody (1 μg/ml), or siRNA targeting GPR177 (siGPR177) alone or together with TM, and cell viability was assessed by MTT assay (Fig. 8 A–C). As shown in Figure 8 A, the viability of WNT-secreting, but not non–WNT-secreting, cells, was effectively decreased by antibody treatment. However, viability of non–WNT-secreting cells was suppressed by treatment with TM or siGPR177, but not with antibody treatment (Fig. 8 B). In addition, suppression of SNU1, SNU216, KATOIII, and MKN28 cell viability was additive with combinatorial treatment (Fig. 8 B). Although the viability of some GC cells was suppressed by TM, siGPR177, and antibody treatment, SNU484, SNU668, and Hs746T cells showed resistance to these treatments (Fig. 8 C). As SNU484, and SNU668 cells express low levels of GPR177, we

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speculated that these cells are taking advantage of other oncogenic signals to maintain viability and presume that either other inhibitors or an antagonistic antibody is needed for precise inhibition of cell viability (Fig. 8 C).[22,47,58,59] Since TM triggers apoptotic cell death, we performed propidium iodide and annexin V staining of cells (Fig. 8 D). Because higher concentrations of TM induced uncontrolled cell death, we treated cells with a low dose (0.1 μg/ml) that had previously been used to induce apoptosis. GPR177 knockdown consistently provoked a higher level of apoptosis in TM-treated cells (Fig. 8 D). In addition, anti-GPR177 antibody treatment enhanced apoptotic cell death in WNT-secreting, but not in non–WNT-secreting, GC cells. These results indicate that targeted inhibition of GPR177 suppresses GC cell viability via inhibition of WNT signaling as well as by promoting ER–stress-induced apoptosis.

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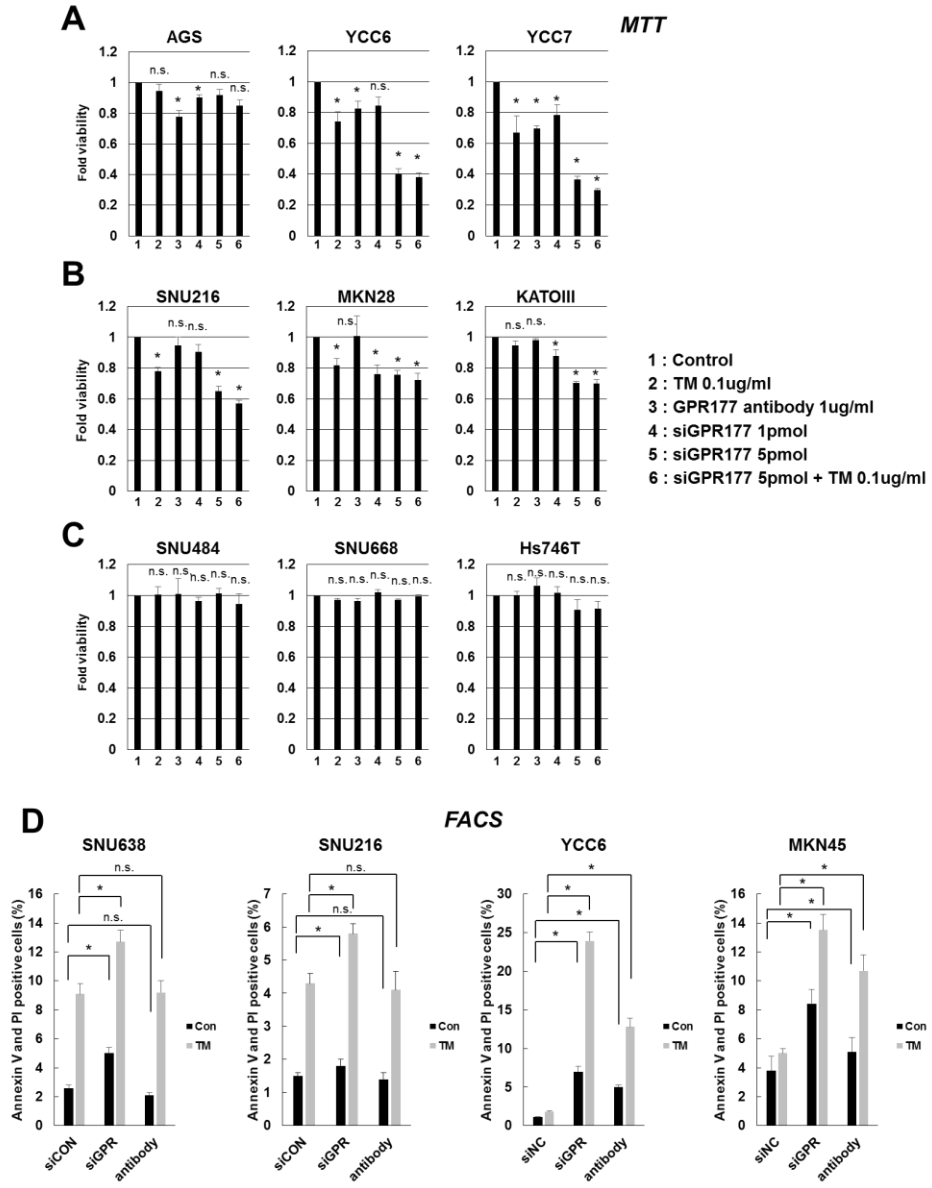

Figure 8. Anti-GPR177 antibody increases apoptotic cell death in WNT-secreting, but not non–WNT-secreting, GC cells. **A**) Cells sensitive to

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antibody-mediated inhibition. GC cells were treated with: lane 1, control; lane 2, 0.1 μg/ml TM for 24 h; lane 3, anti-GPR177 monoclonal antibody; lane 3, 1 μg/ml for 24 h; lane 4: 1 pmol GPR177 siRNA for 48 h; lane 5, 5 pmol GPR177 siRNA for 48 h; lane 6, 0.1 μg/ml TM and 5 pmol GPR177 siRNA for 48 h. Cells were treated with MTT for 1 h before analysis. *P<0.05. **B**) Cells sensitive to inhibition by TM and siRNA, but not by antibody treatment. *P<0.05. **C**) Cells resistant to inhibition by TM, siRNA, and antibody. **D**) Cells were treated with siRNA or antibody and then with 0.1 μg/ml TM. Apoptosis was assayed by FACS.

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9. Anti-GPR177 antibody treatment suppresses gastric tumorigenesis in vivo

To evaluate in vivo efficacy of anti-GPR177 monoclonal antibodies, MKN45 cells were injected into nude mice (n=10 each).[16] After two weeks of injection, anti-GPR177 antibodies were delivered intravenously every 2 days for 2 weeks, and tumor size was measured. Treatment with anti-GPR177 antibodies reduced tumor growth significantly (Fig. 9 A). Upon completion of experiments, tumors were removed from mice and further analyzed by western blotting. Intriguingly, we found that the WNT/β-catenin signaling pathway was upregulated in xenograft tumors compared to the parental MKN45 cells (Fig. 9 B). We hypothesized that this might be the result of selective evolutionary pressure imposed by the tumor environment, favoring stress-resistant molecular traits. Concordant with this, it has recently been reported that metabolic stress induces stem-like phenoconversion through the WNT pathway. To confirm this, we generated metabolic–stress-selected MKN45 (sMKN45) cell lines as previously reported.[48] Compared to parental MKN45 lines, sMKN45 cells showed a more aggressive tumor-forming phenotype. Next, sMKN45 cells were subcutaneously injected into nude mice to establish xenografts. Treatment with anti-GPR177 antibody substantially reduced tumor progression in terms of both volume and weight (Fig. 9 C, D). The sMKN45 cells showed increased GPR177 expression and sensitivity to anti-

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GPR177 antibody in vitro (Fig. 9 E). Previous studies have clearly demonstrated that loss of GPR177 function in other types of cancer suppresses tumor formation in xenograft models. Therefore, we tested whether GPR177 knockdown in GC cancer would inhibit tumor formation. Using shRNA targeting GPR177 (shGPR177), we generated AGS cell lines that stably express low levels of GPR177 mRNA and protein (Fig. 9 F). Control (shCON) and stable shGPR177 AGS cells were subcutaneously injected into BALB/C nude mice (n=5 mice/group). Two weeks later, xenograft tumors were measured with a caliper every 2 days and terminated when volume exceeded 800 mm3. Compared to control cells, shGPR177-expressing AGS cells showed retarded growth rate.

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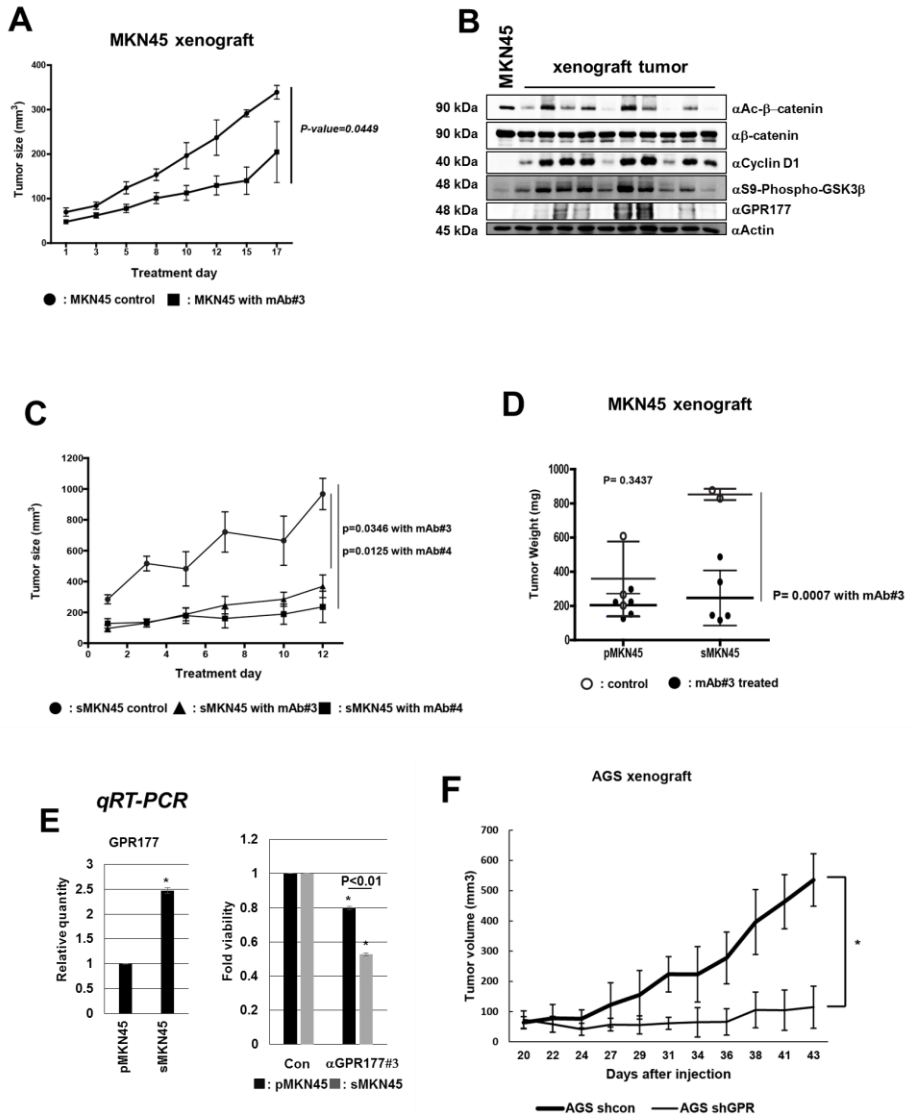

Figure 9. GPR177 monoclonal antibody inhibits tumor growth in GC xenograft assays. A) Nude BALB/C mice were injected subcutaneously with 10^5 MKN45 cells. Two weeks later, mice were randomly divided into two groups of 10

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mice each. One group was intravenously injected with anti-GPR177 monoclonal antibody #3 eight times over 3 weeks, approximately every 3 days. Control group was injected with vehicle PBS. Tumor size was measured with calipers. **B**) Xenograft tumor from panel A was analyzed by western blotting. Total lysate (20 μg) was loaded on SDS-PAGE gel.

C) Metabolic–stress-selected MKN45 (sMKN45) cells (10^5) were subcutaneously injected into nude mice. Two weeks later, mice were randomly divided into three groups of 5 each. One group was intravenously injected with anti-GPR177 monoclonal antibody #3 eight times over 3 weeks, and the other was treated with GPR177 antibody #4 or vehicle PBS. sMKN45 cells were also significantly inhibited by antibody treatment. P=0.0346 for monoclonal antibody #3, P=0.0125 for monoclonal antibody #4. **D**) Weight of xenograft tumors excised from mice shown in panels A and C. pMKN45, parental MKN45 cells; sMKN45, selected MKN45 cells. **E**) Metabolic stress induces GPR177 in sMKN45 cells. GPR177 mRNA levels in pMKN45 and sMKN45 cells were determined by qPCR. Monoclonal antibody treatment (2 μg/ml for 24 hr) reduced sMKN45 cell viability to a greater extent than pMKN45 cell viability determined by MTT assay. *P<0.05. **F**) Stable shRNA-mediated knockdown of GPR177 in AGS cells. Control cells were treated with control SRNA. Cells were subcutaneously injected into nude mice. Tumors were measured 2

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weeks later with calipers every 2 days. *P<0.05.

10. Anti-GPR177 antibody treatment suppresses gastric tumorigenesis in PDX model

Finally, we tested whether this tumor repression would be reproducible in a PDX model.[36,58,60,61] Among GC patients who had undergone gastrectomy, GPR177 expression level of tumor specimens was determined by immunohistochemistry and designated high, moderate, or low (Fig. 10 A, B). Case GA083 was expanded and divided randomly into two groups (Fig. 10 C). One group was injected intravenously with anti-GPR177 monoclonal antibody #3 every 2 days for 3 weeks, and the other group was injected with PBS as negative control. Tumor size was measured every 2 days and 2 days after the last antibody injection. Both tumor size and weight were significantly reduced by antibody treatment (Fig. 10 D). Taken together, these data show that targeted inhibition of GPR177 function suppresses gastric tumorigenesis in vivo.

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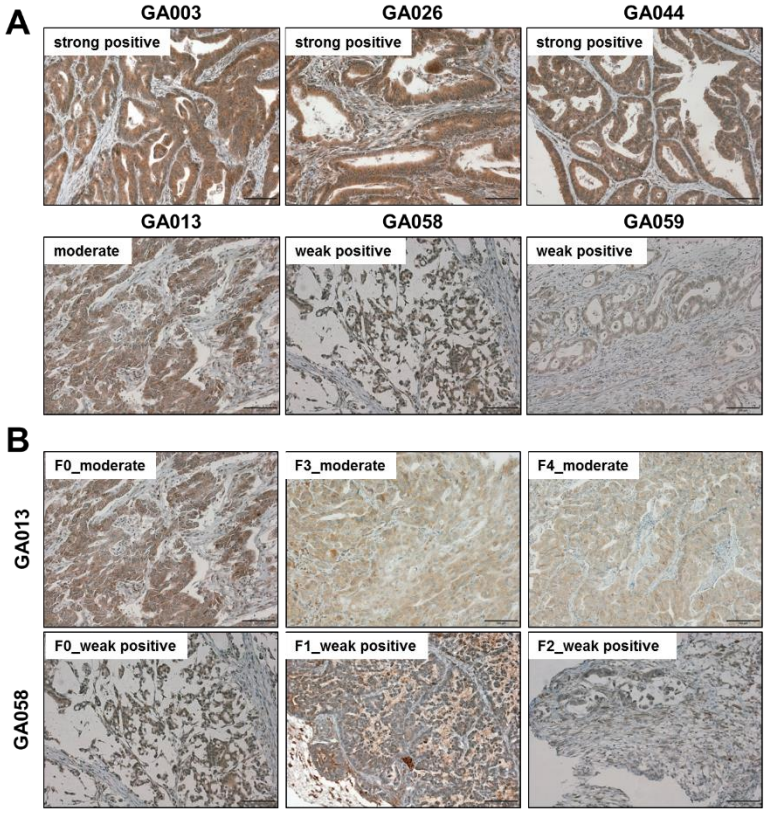

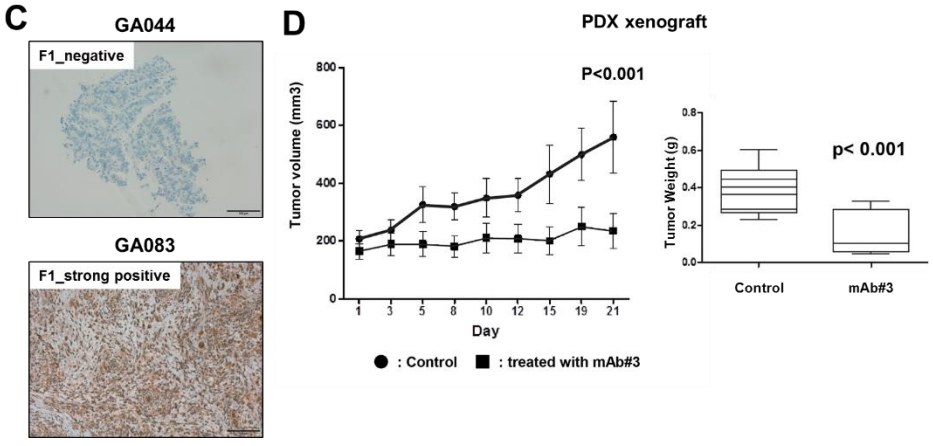

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Figure 10. GPR177 monoclonal antibody reduces tumor growth in PDX model. **A**) Patient specimens (F0) used for PDX model. GPR177 expression was detected by immunohistochemistry using anti-GPR177 antibody #3. Cases were classified based on expression level as strong positive, moderate, weak positive, and negative. **B**) Representative cases of successful engraftment as assessed by maintenance of GPR177 expression from generation to generation. **C**) PDX sample GA083 (F1), a strong-positive case, was used for serial transplantation to the next generation. Case GA044 was strong positive in F0 but GPR177 expression was not sustained through generations. **D**) GA083 F1 tumors were removed and transplanted into nude mice (n=20). After 2 weeks of engraftment, mice were randomly divided into two groups One group was intravenously injected with anti-GPR177 monoclonal antibody #3 eight times over 3 weeks. The control group was injected with vehicle PBS. Tumor size was measured with calipers. Two days after the last injection of antibody, mice were sacrificed and tumors were removed.

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IV. DISCUSSION

GPR177 is seven-pass transmembrane protein that binds cytoplasmic WNT ligand and secretes it to the extracellular space. Dysregulation of WNT secretion and uncontrolled activation of the WNT/β-catenin signaling pathway is associated with disease, such as cancer, and developmental abnormalities.[10,12,20,43,62,63] GPR177 knockout results in WNT loss-of-function phenotypes in species ranging from Drosophila to Mouse. In cancer, GPR177 upregulation enhances tumor progression, and downregulation has been proposed as a potential therapeutic target.[10,11]

Previous studies have shown that inhibition of GPR177 expression efficiently suppresses cancer progression. The cancer-promoting activity of GPR177 has been explained by two different mechanisms: (1) positive upregulation of the WNT/β-catenin signal in most cancer types and (2) regulation of the STAT3 signal in glioma.[9] In this study, we found that GPR177 activates AKT, as well as WNT/β-catenin, signaling in GC cells. It was previously reported that AKT signaling also mediates UPR stress in the ER. In accordance with the findings that GPR177 knockdown suppresses AKT activation and that GPR177 reduces UPR-mediated apoptosis, we

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speculated about the correlation between GPR177 function and AKT activation in the ER. Indeed, we observed a molecular interaction between GPR177 and AKT by co-immunoprecipitation analysis. Further studies are necessary to verify this hypothesis and more precisely define the molecular mechanism.

Firstly, we examined the role of GPR177 in the WNT-secreting pathway in 23 GC cell lines. We expressed exogenous WNT1 ligand and confirmed that WNT was secreted into the culture medium. We classified 23 GC cell lines as WNT-secreting cells (AGS, MKN45, YCCs) and non–WNT-secreting cells. To prevent WNT secretion, we treated GC cells with anti-GPR177 monoclonal antibody. Because the antibodies were raised against the middle of the WNT-binding motif, we presumed that they would block this function of GPR177. Antibody treatment reduced not only WNT secretion but also cell viability and migration. As antibody treatment was effective only on WNT-secreting cells, we speculated that, whereas expression level is important for cancer cell proliferation, the presence of intact WNT-secretory machinery, including the ER, Golgi, and cargo carrier systems, was likely a more critical factor in the case of antibody treatment of GC. Thus, we concluded that some

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GC cell lines secrete WNT ligand and only these cells are sensitive to monoclonal antibody treatment in vitro.

As a positive component of the WNT/β-catenin signaling pathway, GPR177 is upregulated when the signal is activated. More precisely, β-catenin, which is stabilized and dissociated from the destructive complex, is translocated into the nucleus and transcriptionally activated. Consequently, GPR177 transcription is induced and mRNA expression increases. However, it is still not clear that in this mode of positive feedback regulation, a cell is induced to produce more WNT ligand in an autocrine manner. Regardless of whether WNT ligand synthesis is induced in a WNT–ligand-dependent or –independent manner, increased GPR177 expression is followed by induction of WNT/β-catenin signaling. GPR177 was detected in the ER and translocated to Golgi when WNT1 was introduced into GC cells, particularly WNT-secreting cells. Notably, GPR177 also resides in the ER and is upregulated by WNT overexpression but was not transported to the Golgi in the case of non–WNT-secreting cells. Additionally, non–WNT-secreting cells exhibited abnormal distribution of the Golgi network. This might be the reason that these cells cannot secret WNT ligand and are insensitive to anti-GPR177 monoclonal antibody treatment.

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Next, we evaluated the function of GPR177 as an ER-stress reliever. Based on the knowledge that most GPR177 is located in the ER in the inactive condition, we sought to identify the role of GPR177 in ER stress. We demonstrated that GPR177 reduces ER–stress-induced apoptosis. GPR177 knockdown or treatment with anti-GPR177 antibody enhanced apoptosis in TM-induced ER stress. Once WNT ligand is synthesized in the ER, it is modified by addition of monounsaturated lipid, and the hydrophobic protein might induce ER stress if GPR177 is not properly transported through normal secretory pathway. In the absence of GPR177, cells showed a more severe response to ER–stress-induced apoptosis and increased phosphorylation of PERK and accumulation of CHOP; whereas cell death was attenuated by GPR177 overexpression. In previous studies, GPR177 knockdown reduced tumor-cell viability. Here, we confirm that inhibition of GPR177 function at the mRNA and protein levels also effectively suppressed tumor progression in in vivo xenograft and PDX models. According to our hypothesis, we need to further subdivide GC patients who are expressing high levels of GPR177. Because non–WNT-secreting cell lines were insensitive to antibody administration, we would expect that a patient with a non-WNT secreting phenotype would not respond to antibody treatment. However, if we use the

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PDX model and personalized genome sequencing, we can predict the therapeutic outcome of anti-GPR177 monoclonal antibody treatment as a novel therapy. We also demonstrated that induction of ER stress in GC patients may be a candidate cancer treatment in association with reduction of GPR177 level during ER stress. Taking these findings together, we also suggest that GPR177 relieves ER stress and suppresses ER–stress-induced cancer cell apoptosis in the non–WNT-secreting GC subtype.

Molecular-targeted therapy for GC has been pursued and many clinical trials have been conducted. As a result, anti-HER2 monoclonal antibody (trastuzumab) was approved for treatment of HER2-overexpressing GC patients. Recently, Cyramza (ramucirumab) was approved for treatment of gastric cancer as a monotherapy or in combination with other cancer medicines. However, due to the relatively small proportion of HER2-overexpressing patients and the risk of serious bleeding with Cyramza, additional molecular targets are needed for general therapeutic use for GC. Here we report for the first time that GPR177 is highly expressed in GC patients and that GPR177 expression level is positively correlated with poor patient prognosis. Importantly, TMA analysis using anti-GPR177 monoclonal

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antibody confirmed our findings that GPR177 is a promising therapeutic target for GC.

V. CONCLUSION

Many efforts, including classifying, subtyping, and specifying GC would enhance the overall understanding and treatment outcomes of GC. Our findings suggest GPR177 as a potential prognostic marker for GC patient survival and explain the rationale of GPR177 inhibition as a therapeutic target for GC treatment. In order to accomplish a precise outcome for this targeted therapy, we need further investigation of GPR177 from molecular biology to clinical approach.

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Abstract (in Korean)

Wntless/GPR177 단백질의 표적치료를 통한 위암형성 억제 기전 연구

<지도교수 윤 호 근>

연세대학교 의과대학 의과학과

서 재 성

위암은 여러 암 종 중에서 전이가 잘 되는 암중의 하나이며 전 세계적으로 위암으로 인한 사망률은 세 번째로 높다. 이러한 위암의 분자적인 발생원인과 그 치료법을 밝히기

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위한 연구들이 많이 있었다. 최근에는 HER2 유전자의 증폭으로 인한 위암의 치료법으로 HER2 특이적인 항체를 처리하는 연구가 진행된 바 있다. 또한 VEGFR2 수용체 단백질과 특이적으로 결합하는 항체를 이용한 치료법이 FDA의 승인을 받았다. 하지만 HER2 또는 VEGFR2 에 특이적으로 결합하는 항체를 이용한 표적치료의 한계로써 제시된 점은, 이러한 유전자형을 가지고 있는 위암환자의 수가 전체 환자 군에서 적은 비율로 발견된다는 점이다. 따라서 위암의 발생원인과 그로 인한 치료법의 폭 넓은 연구가 필요한 시점이다.

WNT/β-catenin 신호전달체계의 조절 장애는 위암에서 흔히 발견된다. WNT 리간드가 세포 수용체에 결합하게 되면 β-catenin 의존적이거나 비 의존적인 신호전달체계가 전도되게 된다. 이러한 신호체계의 활성화는 암세포의 세포성장을 촉진시키게 된다. WNT 리간드가 없을 때에는 β-catenin 단백질은 지속적으로 프로테아좀 의존적인 분해 신호를 받고 있다. 그러나 WNT/β-catenin 신호전달체계가 활성화 되면 β-catenin 단백질은 분해 복합체로부터 떨어져 나와 세포질에 축적되고, 축적된 β-catenin 단백질은

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핵속으로 이동하여 유전자의 발현을 촉진한다. 여러 암종에서 WNT/β-catenin 신호전달체계를 담당하는 유전자들의 돌연변이가 발견되며 이러한 돌연변이는 암을 유발하는 것으로 잘 알려져 있다. 따라서 이러한 유전자의 기능을 조절하여 암의 억제를 유도하는 여러 연구들이 있었다. 이 WNT/β-catenin 신호전달체계에서 작용하면서 암의 성장을 촉진하는 유전자로써 GPR177 단백질이 잘 알려져 있다.

WNT 단백질은 세포 내 수용성이 낮은 특징을 가지고 있으며 seven-pass transmembrane 단백질인 GPR177과 결합하여 세포 내 구획을 이동하게 된다. WNT 리간드와 GPR177 단백질은 ER에서 결합하여 ER, Golgi, 내포체 그리고 세포막으로 이동되는 분비전달체계를 거치게 된다.

GPR177의 발현이 몇몇 암 종에서 높게 발현되는 것으로 알려져 있는데 WNT/β-catenin 신호전달체계 의존적이거나 STAT3 의존적인 신호전달체계를 통해 암의 성장을 촉진한다고 알려져 있다. WNT/β-catenin 신호전달체계의 돌연변이에 의한 대장암에서 GPR177의 발현 저해를 통한 암 억제를 관찰한 사례가 잘 알려져 있다. 이러한 사실들은 GPR177단백질의 기능 저하를 통한 암 표적치료에 대한

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가능성을 제시한다.

암세포는 일반 세포에 비해 세포 분열속도가 빠른 것이 특징이다. 이러한 사실은 필연적으로 세포 내 단백질합성 속도를 증가시키는 신호를 증가시키며 이는 다시 세포 내 unfolded protein response (UPR)의 신호를 증가시킨다. 세포 내에서 정상적인 3차구조를 갖지 못한 단백질은 UPR 신호를 유발 시키며 이는 암세포의 성장을 촉진하거나 항암제 내성을 가지는 신호로 작용한다. WNT/β-catenin 신호전달체계와 UPR 신호전달체계의 상호작용은 아직 잘 알려져 있지 않으며 이 상호작용을 연구함으로써 새로운 항암제 표적을 제시할 수 있을 것이다.

이 연구에서 위암환자 그룹에서 높은 GPR177의 발현이 환자의 예후 판정인자로써 작용한다는 것을 확인하였다. 위암 세포주를 이용하여 WNT 리간드를 분비하는 그룹과 분비하지 못하는 그룹을 분류하였고, 오직 WNT 리간드를 분비하는 그룹만이 GPR177을 표적으로 하는 단일클론항체에 의해 세포의 성장이 저해되는 것을 확인하였다. WNT를 분비하지 못하는 세포에서는 GPR177 단백질이 주로 ER에 존재하면서 ER Stress 에 의한 세포사멸을 조절한다는 것을 확인하였다.

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또한 동물 모델을 이용하여 GPR177 단일클론항체의 처리가 *In vivo* 조건에서도 작용한다는 것을 확인하였고 patient-derived xenograft (PDX) 모델에서도 단일클론항체의 세포 성장 저해효과를 확인하였다.

핵심되는 말: Wntless/GPR177/WLS, Gastric cancer, monoclonal antibody, WNT/β-catenin signaling, PDX