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Laminin γ2 Mediates Wnt5a-Induced Invasion of Gastric Cancer Cells

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BACKGROUND & AIMS: Wnt5a expression stimulates in vitro migration and invasion of cultured gastric cancer cells by an unknown mechanism and is also correlated with aggressiveness of gastric tumors. The aim of this study was to show that Wnt5a is involved in metastasis of gastric cancer cells in vivo and to explore the molecular mechanism by which Wnt5a regulates migration and invasion. **METHODS:** In an experimental liver metastasis assay, Wnt5a-knockdown gastric cancer cells were injected into the spleens of nude mice. Microarray analyses were used to compare expression patterns between mouse fibroblast L cells that stably express wild-type and a mutant form of Wnt5a to investigate Wnt5a-dependent gene expression. The expression of genes found to be regulated by Wnt5a was investigated in cultured gastric cancer cells. Immunohistochemical analyses were performed to measure levels of Wnt-regulated gene products in 153 gastric cancer samples. **RESULTS:** Knockdown of Wnt5a in gastric cancer cells reduced the number of liver metastases that formed in nude mice. Microarray analyses indicated that Wnt5a activity induced expression of the gene encoding laminin γ2, a subunit of the epithelial basement membrane protein laminin-5. Wnt5a induced the expression of laminin γ2 through the activation of protein kinase C and c-Jun-N-terminal kinase. The invasive activity of gastric cancer cells depended on laminin γ2; Wnt5a expression levels correlated with those of laminin γ2 in diffuse-scattered type gastric tumor samples from patients. **CONCLUSIONS: Wnt5a contributes to gastric cancer progression by increasing metastatic potential. Wnt5a up-regulates laminin γ2 to mediate gastric cancer cell aggressiveness.**

BASIC–ALIMENTARY TRACT

Gastric cancer (GC) is the fourth most common malignancy in the world and is the second leading cause of death after lung cancer.[1] Advances in diagnostic tools and treatments have led to excellent long-term survival for patients with early GC.[2] However, despite improvements in diagnostic and therapeutic strategies, the prognosis of patients with advanced GC with extensive invasion and metastasis remains poor. Several discrete steps can be discerned in the biological cascades of metastasis,[3] and several molecules have been suggested to be involved in the processes of aggressiveness in GC.[4]

It has been shown that frequent up-regulation of *Wnt5a* messenger RNA (mRNA) is detected in primary GC[5] and that expression of Wnt5a protein is associated with advanced T classification (depth of invasion) and N classification (degree of lymph node metastasis) and poor prognosis.[6] Wnt5a has also been reported to be involved in the aggressiveness of melanoma, lung cancer, and breast cancer.[7,8] Wnt5a, a member of the Wnt family of proteins, is a representative ligand that activates the β-catenin–independent pathway via mobilization of intracellular Ca^{2+} and the activation of protein kinase C (PKC), resulting in the stimulation of migration of several cultured cells, including cancer cells.[7–9] Using GC cell lines, a preceding study showed that overexpression of Wnt5a activates focal adhesion kinase and that knockdown of Wnt5a reduces the turnover number of paxillin at the focal adhesion.[6] However, whether Wnt5a is involved in invasion and metastasis of cancer cells in vivo has not yet been reported, and the molecular mechanism by which Wnt5a is involved in the aggressiveness of GC remains to be clarified.

Extracellular matrix proteins, including collagens, laminins, fibronectins, and proteoglycans, not only create tissue architecture but also regulate complex cellular functions by binding to specific cell-surface receptors, integrins. Basement membranes are thin sheets of specialized extracellular matrix proteins supporting epithelial cell layers. A major component of the epithelial basement membrane, laminin-5, has been implicated in cell migration and adhesion.[10,11] Laminin-5 promotes attachment, scattering, and migration of nontumorigenic epithelial cells through the interaction with integrins α3β1, α6β1, and α6β4 in vitro. It also stimulates human tumor cells to form marked lamellipodia, leading to enhanced cell migration and invasion.[12,13] Laminin-5 consists of 3 subunits, α, β, and γ, linked by disulfide bonds to form

Abbreviations used in this paper: AP-1, activator protein-1; ChIP, chromatin immunoprecipitation; CM, conditioned medium; Fz, Frizzled; GC, gastric cancer; JNK, c-Jun-N-terminal kinase; kb, kilobase; KD, knockdown; PCR, polymerase chain reaction; PKC, protein kinase C; siRNA, small interfering RNA; Tcf, T-cell factor.

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a cross-shaped structure. In particular, the laminin γ2 chain has been reported to be expressed at the invasive front in tumor cells such as colon, breast, pancreas, lung, and gastric cancers,[11,14,15] showing that laminin γ2 is one of the most specific invasion markers.

In this study, to further explore the previous results concerning the effects of Wnt5a on GC, Wnt5a knockdown (KD) GC cells were generated and injected into the spleens of nude mice. Here, it is shown for the first time that Wnt5a is involved in the invasion and metastasis of GC cells in vivo. In addition, gene expression changes in Wnt5a-overexpressing cells were explored. It is shown that Wnt5a induces the expression of laminin γ2 and that laminin γ2 is involved in the aggressiveness of GC with Wnt5a expression.

Materials and Methods

Animals and Implantation of Tumor Cells

After 6-week-old male Balb/cAnN Crj-nu mice (Charles River Laboratory Japan, Inc, Kanagawa, Japan) were anesthetized with pentobarbital (0.05 mg/g body wt), 2.5×10^5 KKLS cells or 1×10^6 TMK-1 cells in 50 μL Hanks' balanced salt solution were injected into the spleen through a 31-gauge needle. Five weeks later, under deep anesthesia with ether, hepatectomy and splenectomy were performed. The number of metastatic liver tumors was counted and tumor sizes were measured. For tumor growth assays, 1×10^6 KKLS cells in 50 μL Hanks' balanced salt solution were injected into the subserosa of the stomach orthotopically. Four weeks later, under deep anesthesia with ether, gastrectomy was performed and tumor weight was measured. The protocols used for all animal experiments in this study were approved by the Animal Research Committee of Hiroshima University.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assay was performed using a OneDay ChIP Kit (Diagnode, Philadelphia, PA) in accordance with the manufacturer's instructions. Cells were cross-linked with formaldehyde, lysed, and sonicated to shear DNA to small sizes. Sheared chromatin samples were incubated with anti-JunD or anti–c-jun antibody. The DNA fragments were purified from the immunocomplexes and used as a template for polymerase chain reaction (PCR). The PCR primers were designed for the fragment containing activator protein-1 (AP-1) sites of the promoter region of *LAMC2* (*laminin γ2* gene). The detailed method of ChIP assay was described in the Supplementary information (see Supplemental Material).

Tissue Samples

For immunohistochemical analysis, archival formalin-fixed, paraffin-embedded tissues from 153 patients who had undergone surgical excision for GC were used. Histologic classification was made according to the Lauren classification system.[16] In addition, diffuse-type GCs were further classified into diffuse-adherent and diffuse-scattered subtypes.[6] Tumor staging was according to the TNM staging system. The procedure to protect the privacy of patients was in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese government.

Immunohistochemistry

The samples were sectioned, deparaffinized, and stained with H&E to ensure that the sectioned block contained tumor cells. Adjacent sections were then immunohistochemically stained. Immunostaining for Wnt5a, β-catenin, and laminin γ2 was performed as described.[6,15] When more than 50% of cancer cells were stained, the immunostaining was considered positive. The detailed immunohistochemical analyses are described in the Supplementary information (see supplemental material online at www.gastrojournal.org).

Results

Wnt5a Is Involved in In Vivo Metastasis of GC Cells

To examine the in vivo relationship between expression of Wnt5a and metastasis in cancer cells, the present study generated 2 GC cell lines stably expressing small interfering RNA (siRNA) for Wnt5a: KKLS/Wnt5a KD cells and TMK-1/Wnt5a KD cells (Supplementary Figure 1*A*). KKLS cells were established from a lymph node metastasis of a patient with undifferentiated GC.[17] TMK-1 cells were established from a xenotransplantable tumor (SC-6-JCK) in nude mice, which originated from the lymph node metastasized tumor of human GC.[4] These Wnt5a KD GC cells showed reduced in vitro cell migration and invasion activities in Transwell assays as compared with control cells (Supplementary Figure 1*B*). However, reduction of Wnt5a did not affect the cell growth of these GC cells (Supplementary Figure 1*C*).

KKLS/Wnt5a KD cells, TMK-1/Wnt5a KD cells, and their control cells were injected into the spleens of nude mice to observe experimental liver metastasis. Although the metastatic liver tumor sizes were similar in KKLS/control and KKLS/Wnt5a KD cells, knockdown of Wnt5a in KKLS cells significantly decreased the numbers of the experimental liver metastatic nodules (Figure 1*A* and *B*) ($P = .0060$). When the cells were implanted in gastric subserosa, primary tumors were formed and their tumor weights in the stomach were statistically similar for control and Wnt5a KD cells ($P = .6098$) (Figure 1*C*). Similar results were observed in TMK-1 cells (Figure 1*D*). These are the first in vivo results showing that Wnt5a is required for invasion and metastasis rather than growth in GC cells. However, because in vivo metastatic experi-

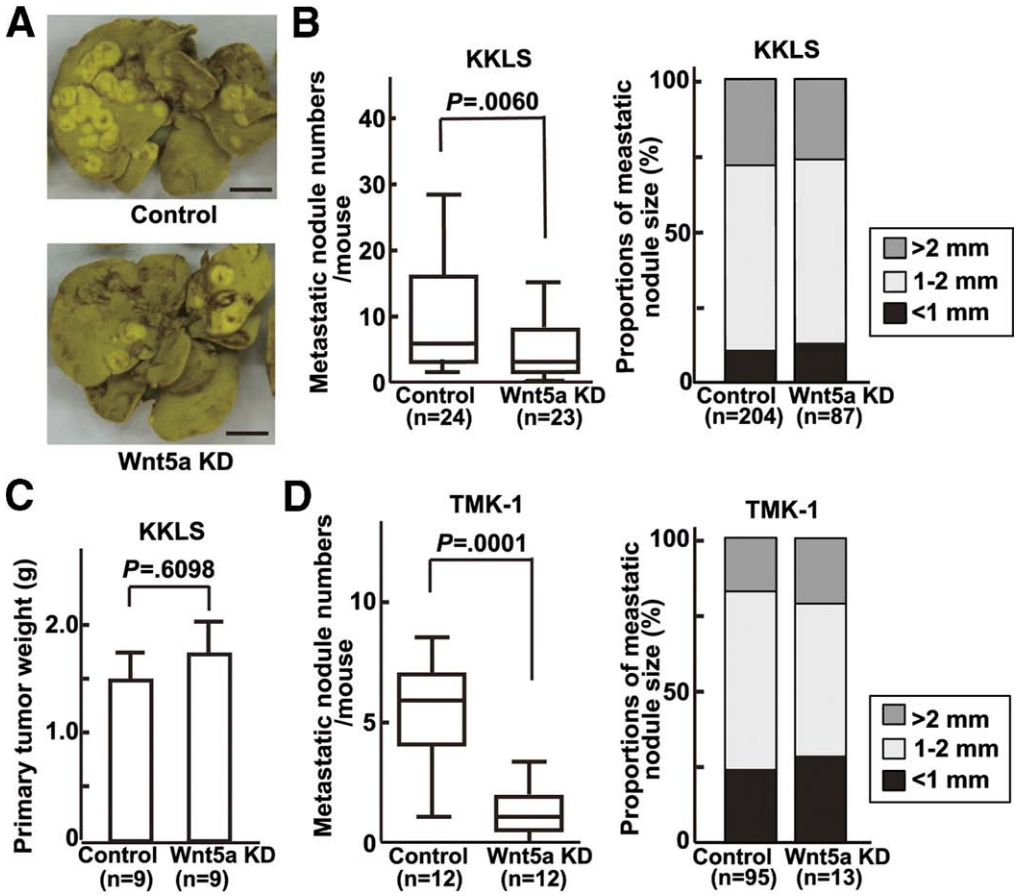

Figure 1. Wnt5a silencing reduces the metastatic potential of GC cells. (*A* and *B*) Liver tumor colonies were produced by the intrasplenic injection of KKLS cells. Photographs representing (*A*) liver metastases, (*B*, *left panel*) numbers of metastatic nodules per mouse, and (*B*, *right panel*) apparent sizes of each metastatic nodule are shown. *Scale bars* = 5 mm. n = 24 and n = 23 indicate the numbers of mice into which KKLS/control and KKLS/Wnt5a KD cells, respectively, were injected. n = 204 and n = 87 indicate the total numbers of metastatic nodules produced by KKLS/control and KKLS/Wnt5a KD cells, respectively. The statistical comparison was performed using Mann–Whitney *U* test. (*C*) Orthotropic implantation was performed by the injection of KKLS cells in the stomach. n = 9 indicates the number of mice into which KKLS/control or KKLS/Wnt5a KD cells were injected. The statistical comparison was performed using unpaired Student *t* test. (*D*) The numbers and sizes of liver tumor colonies produced by the intrasplenic injection of TMK-1 cells were measured. n = 12 indicates the number of mice into which TMK-1/control or TMK-1/Wnt5a KD cells were injected. n = 95 and n = 13 indicate the total numbers of metastatic liver nodules produced by TMK-1/control and TMK-1/Wnt5a KD cells, respectively. The statistical comparison was performed using Mann–Whitney *U* test.

ments with nude mice also reflect the ability of tumor cells to proliferate in a different organ (hepatic) microenvironment,[18] it is still possible that Wnt5a affects GC cell growth in a distant site.

Wnt5a Drives Changes in Gene Expression

Next it was attempted to analyze the mechanisms of aggressiveness of GC, commencing with a broad survey of the gene expression patterns affected by the expression of Wnt5a. To this end, mouse fibroblast L cells stably expressing Wnt5a or Wnt5a CA were used.[9] Wnt5a CA is a mutant that is not posttranslationally palmitoylated at Cys104 and loses its biological activity. Candidate gene lists were created by determining which molecules were overexpressed by ≥1.5-fold in L/Wnt5a cells but did not change in L/Wnt5a CA cells as compared with control cells (Supplementary Table 1). Laminin γ2 was further investigated among the candidate genes suggested to be involved in migration and invasion, because it was reported that laminin γ2 can be detected at the invasive front of various cancers.[11,14,15]

Laminin γ2 is a subunit of laminin-5, which is found in the epithelial basement membrane.[10] mRNA levels of *Wnt5a* and *LAMC2* were analyzed in 4 different GC cell lines: MKN-1, TMK-1, MKN-45, and KKLS cells. MKN-1 and MKN-45 cells were established from metastatic foci to lymph nodes and liver, respectively, of human GC.[4] mRNA levels of *Wnt5a* and *LAMC2* were variable, depending on the cell type. Although MKN-1, TMK-1, and MKN-45 cells showed expression of *LAMC2* mRNA, KKLS cells barely produced *LAMC2* mRNA (Figure 2*A*). The expression of *Wnt5a* mRNA in MKN-45 cells was lower than that of other cells (Figure 2*A*). TMK-1/Wnt5a KD cells reduced the mRNA level of *LAMC2* as compared with control cells (Figure 2*B*). When purified

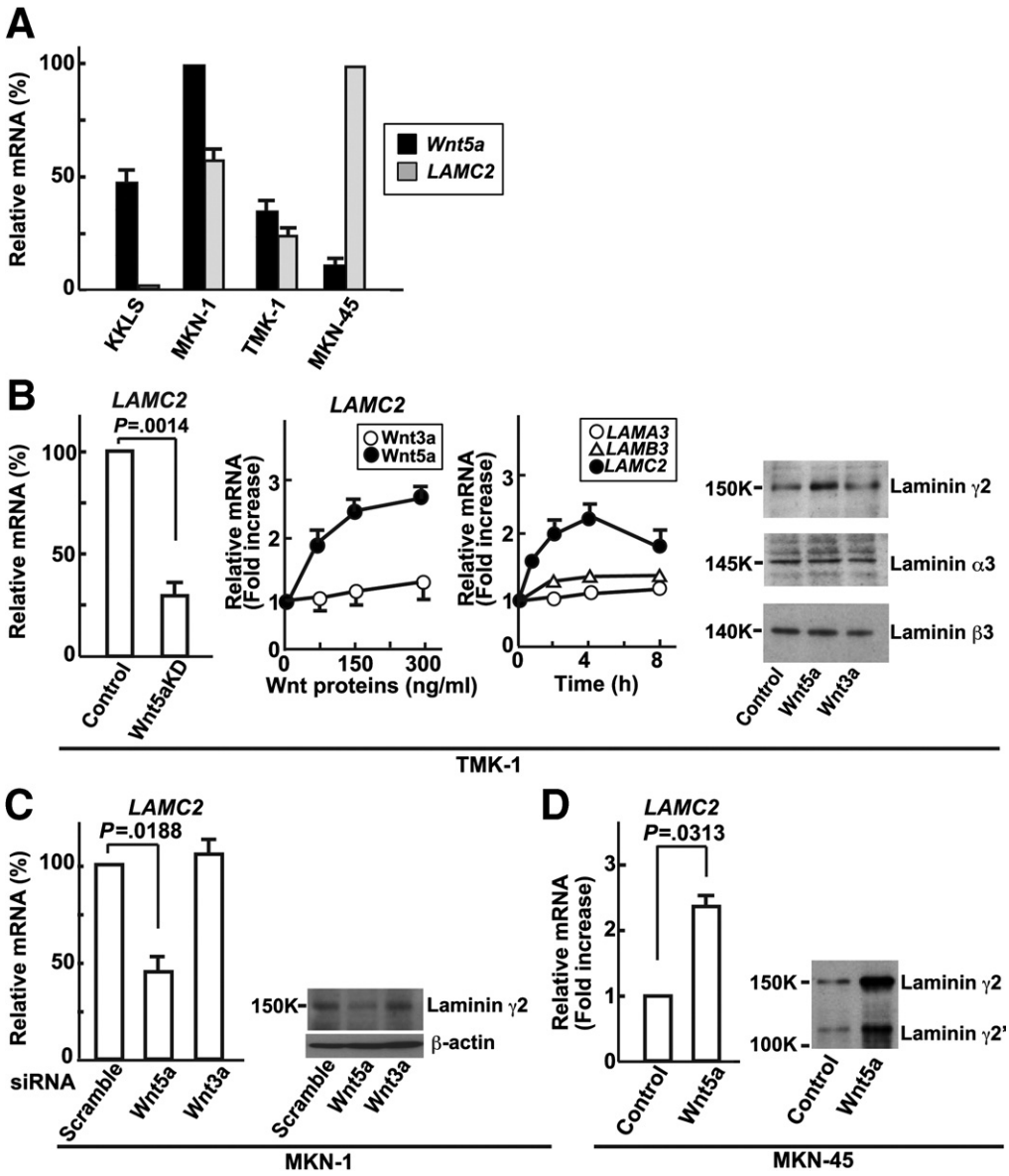

Figure 2. Wnt5a induces the expression of laminin γ2 in GC cells. (*A*) *Wnt5a* and *LAMC2* mRNA expression in various GC cell lines was quantified by quantitative reverse-transcription PCR. Relative mRNA levels in each cell line were expressed as percentages of *Wnt5a* mRNA in MKN-1 cells and *LAMC2* mRNA in MKN-45 cells. The results shown are the means ± SE of 3 independent experiments. (*B*) TMK-1 cells. (*Left panel*) *LAMC2* mRNA levels in TMK-1/control and TMK-1/Wnt5a KD cells were quantified. (*Second panel*) TMK-1 cells were treated with the indicated concentrations of Wnt5a (*closed circles*) or Wnt3a (*open circles*) for 4 hours, and then the *LAMC2* mRNA level was quantified. (*Third panel*) TMK-1 cells were treated with 100 ng/mL Wnt5a for the indicated periods, and then *LAMA3*, *LAMB3,* and *LAMC2* mRNA levels were quantified. (*Right panel*) TMK-1 cells were treated with 150 ng/mL Wnt5a or Wnt3a for 8 hours, and then extracellular matrix was collected and probed with anti-laminin α3, β3, and γ2 antibodies. (*C*) MKN-1 cells. (*Left panel*) *LAMC2* mRNA levels in MKN-1 cells transfected transiently with scramble (control), Wnt5a, or Wnt3a siRNA were quantified. (*Right panel*) Total cell lysates of MKN-1 cells transfected transiently with scramble, Wnt5a, or Wnt3a siRNA were probed with anti-laminin γ2 antibody. (*D*) MKN-45 cells. (*Left panel*) *LAMC2* mRNA levels in MKN-45/control and MKN-45/Wnt5a cells were quantified. Relative mRNA levels were expressed as fold increases compared with the *LAMC2* mRNA level in MKN-45/control cells. (*Right panel*) Extracellular matrix prepared from MKN-45/control and MKN-45/Wnt5a cells was probed with anti-laminin γ2 antibody. Laminin γ2′ is a 105-kilodalton processed form of laminin γ2.

Wnt5a and Wnt3a proteins were added to TMK-1 cells, Wnt5a but not Wnt3a increased *LAMC2* mRNA in a dose-dependent manner (Figure 2*B*). *LAMC2* mRNA started to increase at 2 hours after Wnt5a stimulation, reached a maximal level at 4 hours, and then declined gradually (Figure 2*B*). Laminin-5 is a trimmer of laminin α3, β3, and γ2 chains. mRNA levels of other components of laminin-5, *LAMA3* (*laminin α3* gene) and *LAMB3* (*laminin β3* gene), were not up-regulated by Wnt5a (Figure 2*B*). Consistent with these results, Wnt5a increased protein levels of laminin γ2 but not laminin α3 and laminin β3 (Figure 2*B*).

Knockdown of Wnt3a from MKN-1 cells did not affect *LAMC2* mRNA and laminin γ2 protein levels under conditions where knockdown of Wnt5a reduced them (Figure 2*C*). MKN-45 cells stably overexpressing Wnt5a (MKN-45/Wnt5a cells) increased the expression of *LAMC2* mRNA and laminin γ2 protein as compared with control cells (Figure 2*D*). Taken together, these results indicate that Wnt5a induces the expression of laminin γ2 in 3 different GC cell lines.

Wnt5a Activates the Promoter of Laminin γ2 Through the AP-1 Element

To examine how Wnt5a signaling induces the expression of laminin γ2, TMK-1 cells were pretreated

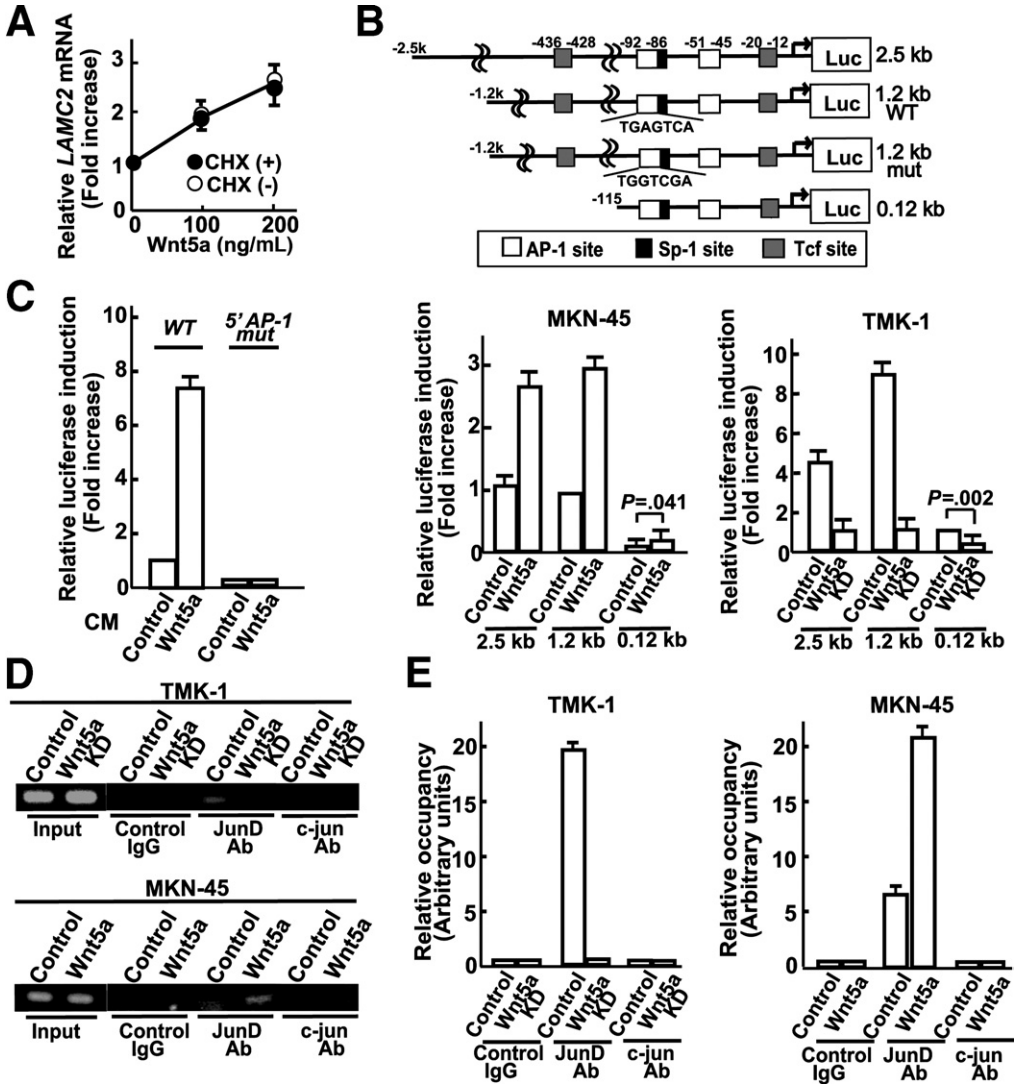

Figure 3. AP-1 is involved in Wnt5a-dependent expression of laminin γ2. (*A*) TMK-1 cells were treated with (*closed circles*) or without (*open circles*) 1 μg/mL cycloheximide (CHX) for 4 hours before stimulation with the indicated amounts of Wnt5a for 4 hours. *LAMC2* mRNA was quantified, and relative mRNA levels were expressed as fold increases compared with the *LAMC2* mRNA level in TMK-1 cells without Wnt5a stimulation. (*B*) (*Upper panel*) The *LAMC2*-luciferase constructs used in this study. (*Bottom left panel*) MKN-45/control or MKN-45/Wnt5a cells were transfected with the indicated pGL3/*LAMC2-Luc* constructs, and then the luciferase activities were measured. (*Bottom right panel*) TMK-1/control or TMK-1/Wnt5a KD cells were transfected with the indicated pGL3/*LAMC2-Luc* constructs, and then the luciferase activities were measured. Relative laminin γ2 promoter activity was expressed as fold increase compared with that of MKN-45/control cells transfected with pGL3/(1.2 kb) *LAMC2-Luc* or TMK-1/control cells transfected with pGL3/(0.12 kb) *LAMC2-Luc*. (*C*) After TMK-1 cells were transfected with pGL3/(1.2 kb) *LAMC2-Luc* with or without mutation in the 5′AP-1 site (*5′AP-1 mut* or *WT*, respectively), the cells were stimulated with control CM or Wnt5a CM for 24 hours. Luciferase activities were measured and expressed as fold increases compared with that of cells transfected with pGL3/(1.2 kb) *LAMC2-Luc* and treated with control CM. WT, wild type. (*D*) Chromatin from TMK-1/control, TMK-1/Wnt5a KD, MKN-45/control, and MKN-45/Wnt5a cells was immunoprecipitated with anti-JunD antibody, anti–c-jun antibody, or control immunoglobulin G, and the samples were analyzed by PCR for the *LAMC2* promoter region containing 2 AP-1 sites. (*E*) The amounts of precipitated DNA in *D* were quantified by real-time PCR with the same primers. The relative amounts of the DNA fragments containing AP-1 sites immunoprecipitated with anti-JunD or anti–c-jun antibody were expressed as arbitrary units compared with that with control immunoglobulin G. The results shown are means ± SE from 3 independent experiments.

with cycloheximide before Wnt5a stimulation. Cycloheximide did not affect Wnt5a-dependent expression of *LAMC2* mRNA (Figure 3*A*), suggesting that Wnt5a induces the expression of *LAMC2* mRNA without protein synthesis.

To evaluate the role of the upstream elements in controlling basal activity of the *LAMC2* promoter, a series of deletions of the *LAMC2* 5′-flanking region with the *luciferase* gene (*LAMC2-Luc*) was transfected into MKN-45/control cells. Consistent with previous observations in colon cancer cells,[19] the 2.5-kilobase (kb) and 1.2-kb *LAMC2-Luc* constructs showed luciferase activity, but deletion from position −1.2 kb to −0.12 kb reduced the luciferase activity to 10% (Figure 3*B*). Similar findings were observed in TMK-1 cells (Figure 3*B*). When 3 different *LAMC2-Luc* constructs were transfected into MKN-45/Wnt5a cells, the luciferase activity increased approximately 3-fold as compared with the control cells (Figure 3*B*). When Wnt5a was knocked down from TMK-1 cells, the *LAMC2-Luc* promoter activities were decreased (Figure 3*B*). These results suggest that the region between −1.2 kb and −0.12 kb was required for the basal activity of the *LAMC2* promoter and that the −0.12 kb region has a Wnt5a response element.

It has been reported that the 0.12-kb promoter fragment contains 2 AP-1 elements and that the 5′ AP-1 site is critical for phorbol ester–dependent activation.[19] As shown in Figure 3C, the basal reporter gene activity was decreased dramatically by introducing mutations in this area (*LAMC2(5′AP-1 mut)-Luc*), and Wnt5a did not stimulate it. Therefore, the 5′AP-1 site could be crucial for the basal activity of the *LAMC2* promoter as well as the upstream region.

The AP-1 protein containing JunD has been shown to bind to the 5′ AP-1 site in the *LAMC2* promoter using electrophoretic mobility shift assays.[19] In ChIP assays, PCR amplification of the anti-JunD antibody immunoprecipitants showed that the fragment of the *LAMC2* promoter containing 2 AP-1 sites exists in TMK-1/control cells but not in TMK-1/Wnt5a KD cells (Figure 3*D* and *E*). PCR using the fragment containing a T-cell factor (Tcf) binding site, which is located about 400 base pairs upstream of the AP-1 sites, showed no detectable bands in both samples (data not shown). In addition, the PCR fragment of the *LAMC2* promoter containing AP-1 sites in the anti-JunD antibody immunoprecipitants was increased in MKN-45/Wnt5a cells (Figure 3*D* and *E*). However, anti–c-jun antibody did not immunoprecipitate the PCR fragment (Figure 3*D* and *E*).

We also examined which molecules are involved in Wnt5a-dependent expression of laminin γ2. Consistent with previous results in other cells,[7,8,20] Wnt5a induced the phosphorylation of c-Jun-N-terminal kinase (JNK) and PKCα in MKN-45 and TMK-1 cells (Figure 4*A*), indicating the activation of JNK and PKCα. SP600125, a JNK inhibitor, and staurosporine, a PKC inhibitor, suppressed Wnt5a-dependent *LAMC2-Luc* activity (Figure 4*B*). Frizzled (Fz) family are known to function as Wnt receptors.[21] Fz2, Fz5, and Fz7 were expressed highly in MKN-1 cells (data not shown). Knockdown of Fz2 but not of Fz5 and Fz7 reduced Wnt5a-dependent expression of *LAMC2* mRNA (Figure 4*C*). Taken together, these results suggest that Fz2 is a Wnt5a receptor in this signaling of GC cells and that JNK and PKC mediate Wnt5a-dependent expression of laminin γ2 through the recruitment of JunD to the AP-1 site of the *LAMC2* promoter.

Laminin γ2 Mediates Wnt5a-Dependent Invasion

It has been reported that β-catenin activates *LAMC2* promoter function through 2 Tcf-biding ele-

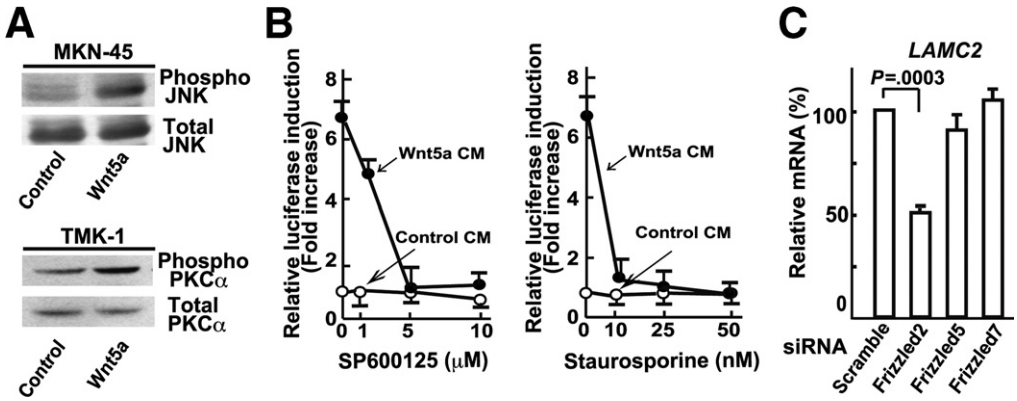

Figure 4. The mechanism by which Wnt5a induces the expression of laminin γ2. (*A*) MKN-45 cells were stimulated with 300 ng/mL Wnt5a for 1 hour, and then the cell lysates were probed with anti–phospho-JNK antibody. TMK-1 cells were stimulated with 300 ng/mL Wnt5a for 30 minutes, and then the cell lysates were probed with anti–phospho-PKCα antibody. (*B*) After TMK-1 cells were transfected with pGL3/(1.2 kb) *LAMC2-Luc*, the cells were stimulated with control (*open circles*) or Wnt5a (*closed circles*) CM for 24 hours in the presence of the indicated concentrations of SP600125 (*left panel*) or staurosporine (*right panel*). Luciferase activities were measured and expressed as fold increases compared with that of cells treated with control CM and without inhibitors. (*C*) After MKN-1 cells were transfected with the indicated siRNA, *LAMC2* mRNA levels were quantified.

ments, suggesting that laminin γ2 mediates β-catenin–dependent invasion by colon cancer cells.[22] Therefore, the possibility that β-catenin mediates Wnt5a-dependent expression of laminin γ2 was examined, because Wnt5a can activate the β-catenin–dependent pathway in a receptor context.[23] Expression of β-catenin and Tcf-4 in TMK-1 cells stimulated the promoter activity of *LAMC2*, but the activity was not affected by SP600125 (Figure 5*A*). Laminin γ2 protein levels were also increased by the expression of β-catenin and Tcf-4, although the increment was slight compared with the extent induced by Wnt5a stimulation but reproducible (Figure 5*B*). ΔTcf-4, which lacks the β-catenin–binding region, is known to act as a dominant negative form.[24] ΔTcf-4 did not affect the Wnt5a-dependent promoter activity of *LAMC2* (Figure 5*A*). Furthermore, Wnt5a and β-catenin stimulated the promoter activity of *LAMC2* additively (Figure 5*A*). These results suggest that Wnt5a induces the expression of laminin γ2 without the activation of the β-catenin–dependent pathway.

Knockdown of laminin γ2 in TMK-1 and MKN-1 cells clearly decreased invasion activities in the Transwell assays with Matrigel (Figure 5*C*). Furthermore, MKN-45/Wnt5a cells showed higher invasion activity compared with control cells, and knockdown of laminin γ2 decreased the activity (Figure 5*C*). Consistent with these observations, laminin γ2 was clearly expressed in TMK-1 cells metastasized to liver in nude mice (Figure 5*D*). Taken together, these results suggest that Wnt5a is involved in the expression of laminin γ2, which mediates Wnt5a-dependent invasion at least partly in a certain type of GC.

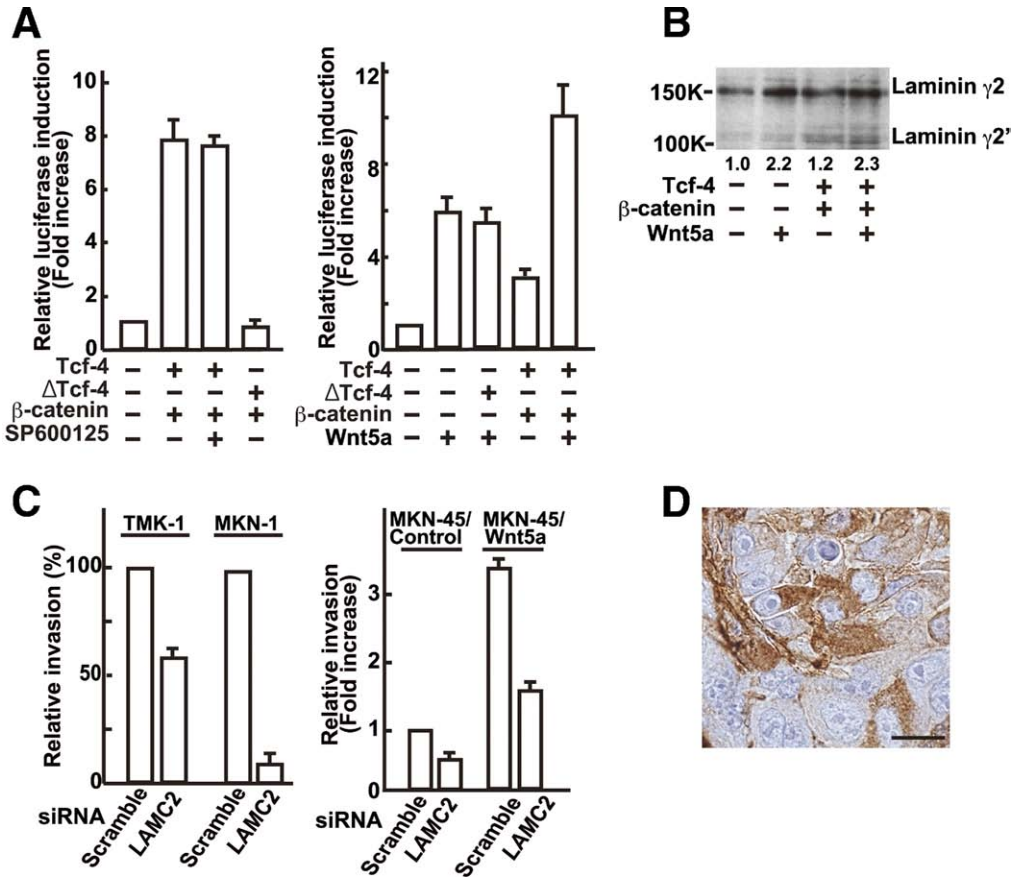

Figure 5. Laminin γ2 mediates Wnt5a-dependent cell invasion. (*A*) (*Left panel*) TMK-1 cells were transfected with pGL3/(1.2 kb) *LAMC2-Luc* and the indicated plasmids in the presence or absence of 10 μmol/L SP600125. At 48 hours after transfection, luciferase activities were measured. The relative laminin γ2 promoter activity was expressed as fold increase compared with that of the cells transfected with empty vectors and without treatment of SP600125. (*Right panel*) At 24 hours after TMK-1 cells were transfected with pGL3/(1.2 kb) *LAMC2-Luc* and the indicated plasmids, the cells were stimulated with control CM or Wnt5a CM for 24 hours. The relative luciferase activities were expressed as fold increases compared with that of cells transfected with empty vectors and stimulated with control CM. (*B*) TMK-1 cells were transfected with plasmids for β-catenin and Tcf-4 and then stimulated with 150 ng/mL Wnt5a as indicated. The extracellular matrix fractions of all these samples were collected and probed with anti–laminin γ2 antibody. The *numbers below the panel* show relative quantification values of laminin γ2 in each lane. (*C*) (*Left panel*) TMK-1 and MKN-1 cells were transfected with scramble or *LAMC2* siRNA, and then the cells were subjected to the invasion assay. Relative invasion activity was expressed as percentages of the invaded cell numbers of control cells. (*Right panel*) MKN-45/control and MNK-45/Wnt5a cells were transfected transiently with scramble or *LAMC2* siRNA, and these cells were subjected to the invasion assay. Relative invasion activity was expressed as fold increase compared with the invaded cell numbers in MKN-45/control cells transfected with scramble siRNA. (*D*) Liver tumors produced by the intrasplenic injection of TMK-1 cells were sectioned and stained with anti–laminin γ2 antibody. All the cells shown in this figure were metastasized GC cells. Laminin γ2 was stained in *brown*. *Scale bar* = 19 μm.

Coexpression of Wnt5a and Laminin γ2 in GC Cases

To investigate the induction of laminin γ2 expression by Wnt5a in GC cases, immunohistochemical analyses of Wnt5a and laminin γ2 in 153 GC cases were performed. Representative results of Wnt5a immunostaining of GC are shown in Figure 6*A* and Supplementary Figure 2 (see supplemental material online at www.gastrojournal.org). Wnt5a was detected in the cytoplasm of cancer cells. Consistent with the previous observations,[6] positivity for Wnt5a was associated with advanced T classification, N classification, and tumor stage (Supplementary Table 2).

Two laminin γ2 staining patterns (extracellular staining and cytoplasmic staining) have been reported in GC.[15] In diffuse-scattered type (so-called scirrhous type) GC cases, cytoplasmic laminin γ2 staining but not extracellular laminin γ2 staining was found (Figure 6*B* and Supplementary Figure 2). In other types (intestinal or diffuse-adherent type) of GC cases, both staining patterns were observed (Figure 6*C* and *D*). Because Wnt5a staining was detected in few cancer cells in the GC cases with extracellular laminin γ2 expression, the relationship between the expression of Wnt5a and cytoplasmic laminin γ2 was investigated.

In total, 35 (23%) of 153 GC cases were recognized as cytoplasmic laminin γ2 positive. Positivity for laminin γ2 was associated with advanced N classification ($P = .0037$) and tumor stage ($P = .0330$) (Table 1). Among 36 diffuse-scattered type GC cases, the frequency of laminin γ2

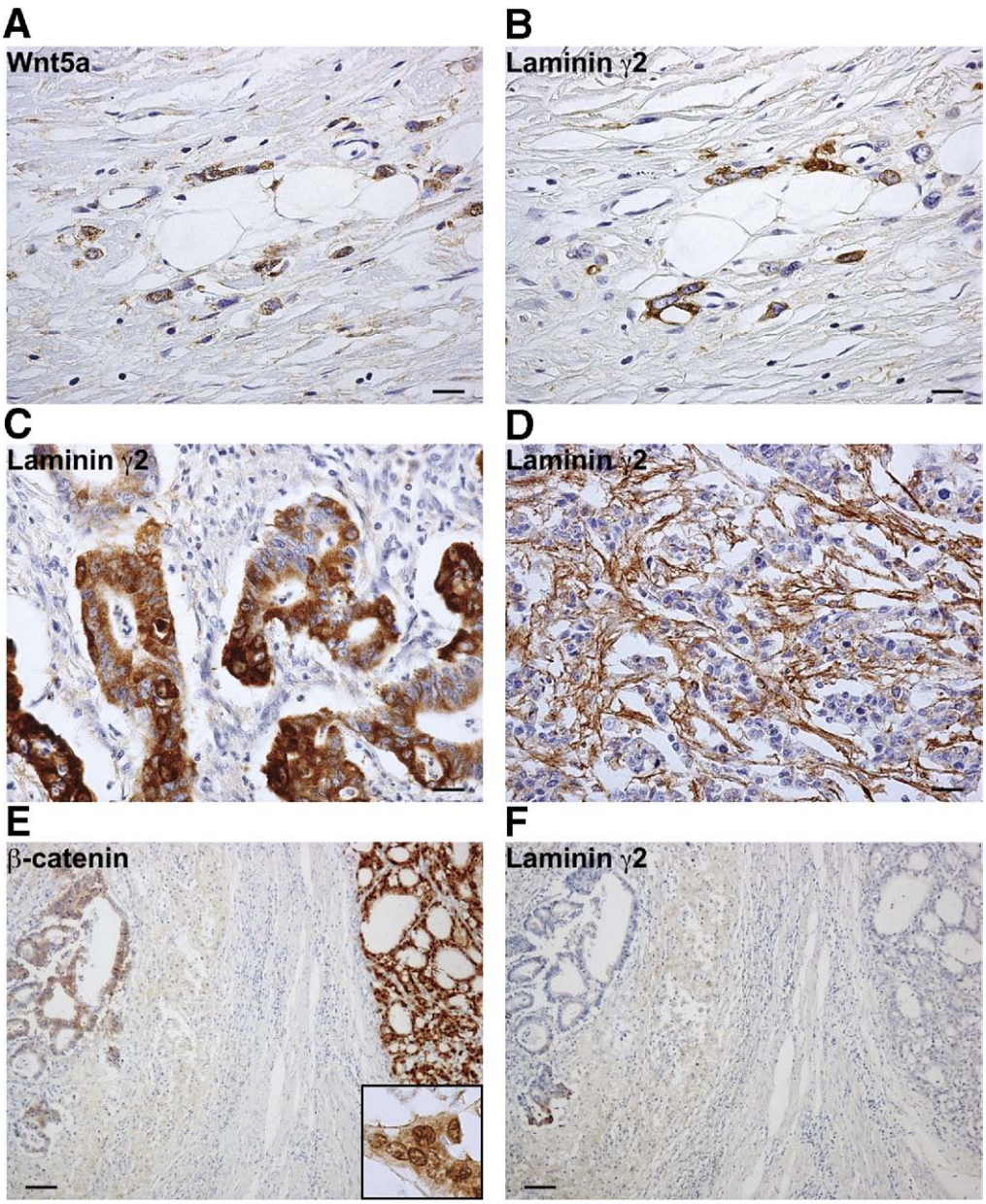

Figure 6. Coexpression of Wnt5a and laminin γ2 in scirrhous GC. (*A* and *B*) A sample of diffuse-scattered type GC was stained with (*A*) anti-Wnt5a and (*B*) anti-laminin γ2 antibodies. Cytoplasmic laminin γ2 was detected in Wnt5a-positive cancer cells. *Scale bars* = 19 μm. (*C* and *D*) Samples of other GC types were stained with anti–laminin γ2 antibody. Laminin γ2 was observed as (*C*) cytoplasmic expression in intestinal type GC and (*D*) extracellular expression in diffuse-adherent type GC patterns. *Scale bars* = 19 μm. (*E* and *F*) A sample of GC was stained with (*E*) anti–β-catenin and (*F*) anti–laminin γ2 antibodies. (*Inset*) High-magnification image of β-catenin staining. *Scale bars* = 75 μm.

Table 1. Relationship Between Laminin γ2 Expression and Clinicopathologic Characteristics in GC

	Laminin γ2		
	Positive	Negative	*P* value
T classification			
T1	9 (19%)	39	.5345
T2/3/4	26 (25%)	79	
N classification			
N0	8 (12%)	60	.0037
N1/2/3	27 (32%)	58	
Stage			
I/II	15 (17%)	75	.0330
III/IV	20 (32%)	43	
Histologic classification			
Diffuse-scattered type	10 (28%)	26	.4966
Other types	25 (21%)	92	

expression in Wnt5a-positive GC cases (10 of 24 cases; 42%) was significantly higher than that in Wnt5a-negative GC cases (0 of 12 cases, 0%; $P = .0146$) (Table 2). There was a tendency for laminin γ2 to be expressed in GC cells expressing Wnt5a (Figure 6*A* and *B* and Supplementary Figure 2). In contrast, among 117 other types of GC cases, the frequency of laminin γ2 expression in Wnt5a-positive GC cases (5 of 20 cases; 25%) was not different from that in Wnt5a-negative GC cases (20 of 97 cases, 21%; $P = .7649$) (Table 2). However, in 5 GC cases positive for both Wnt5a and laminin γ2, there was a tendency for GC cells stained by laminin γ2 to also be positive for Wnt5a (Supplementary Figure 2). These results suggest that Wnt5a is involved in laminin γ2 expression, at least in diffuse-scattered type GC.

To examine the relationship between expression of β-catenin and laminin γ2, further immunohistochemical analysis of β-catenin was performed on 153 GC cases. Cancer cells showing cytoplasmic or nuclear accumulation of β-catenin were not always positive for laminin γ2 (Figure 6*E* and *F* and Table 2).

Discussion

Wnt5a Induces Expression of Laminin γ2

The results of this study showed for the first time that Wnt5a is involved in the metastasis of GC cells in vivo using nude mice. It was also found that Wnt5a induces the expression of laminin γ2 in at least 3 different GC cell lines and that laminin γ2 mediates Wnt5a-dependent invasion. From the results in biochemical, reporter gene, and ChIP assays, the possible mechanism by which Wnt5a induces the expression of laminin γ2 in GC cells is that the binding of Wnt5a to Fz2 would stimulate the expression of laminin γ2 by the recruitment of JunD to the AP-1 binding site of the promoter region of the *LAMC2* gene through the activation of PKC and JNK. Immunohistochemical studies have shown that laminin γ2 is overexpressed at the invasive front of human cancers.[14,15] Taken together with previous clinicopathologic studies showing that the expression of Wnt5a is associated with aggressiveness and poor prognosis in GC,[6] these results suggest that laminin γ2 is involved in aggressiveness of GC in which Wnt5a is highly expressed.

Although the molecular mechanism by which laminin γ2 stimulates the migration of cancer cells is not understood, the proteolysis of laminin γ2 to 105-kilodalton and 70-kilodalton fragments increases cell motility activity but decreases its cell adhesion activity.[11] In addition, it has also been reported that domain III of laminin γ2 stimulates cell migration by binding to epidermal growth factor receptor[25] and that the 70-kilodalton fragment binds to syndecan-1 and disrupts the hemidesmosome-like structure.[26] The cleaved form of laminin γ2 was indeed produced in the extracellular matrix fraction from MKN-45 cells overexpressing Wnt5a (see Figure 2*D*). Therefore, production of proteases, such as metalloproteinases, from cancer cells may cooperate to enhance the effects of laminin γ2 on cancer cells.

β-Catenin activates laminin γ2 gene expression through the 2 Tcf-binding elements in colorectal cancer.[22] It was found that the activation of Wnt5a and β-catenin pathways induces the expression of laminin γ2 independently in cultured GC cells. It is notable that overexpression of Tcf-4 is required for β-catenin–dependent laminin γ2 expression in this assay. Because it was reported that Tcf-4E is expressed in colon cancer where β-catenin is up-regulated,[27] expression of β-catenin could induce the expression of laminin γ2 in colon cancer. However, it is unlikely that the β-catenin pathway stimulates the

Table 2. Relationship Between Laminin γ2 Protein Expression and Wnt5a or β-Catenin in GC

	Laminin γ2		
	Positive	Negative	*P* value
All cases (n = 153)			
Wnt5a			
Positive	15 (34%)	29	.0543
Negative	20 (18%)	89	
β-catenin			
Positive	4 (18%)	18	.7847
Negative	31 (24%)	100	
Diffuse-scattered type (n = 36)			
Wnt5a			
Positive	10 (42%)	14	.0146
Negative	0 (0%)	12	
β-catenin			
Positive	0 (0%)	2	1.0000
Negative	10 (29%)	24	
Other types (n = 117)			
Wnt5a			
Positive	5 (25%)	15	.7649
Negative	20 (21%)	77	
β-catenin			
Positive	4 (20%)	16	1.0000
Negative	21 (22%)	76	

expression of laminin γ2 in GC, because an immunohistochemical study suggested that accumulation of β-catenin in GC cases is not associated with laminin γ2 expression. The expression level of Tcf-4 may not change in GC cells overexpressing β-catenin.

Clinical Relevance of the Coexpression of Wnt5a and Laminin γ2 in GC

It was shown previously that Wnt5a is expressed in diffuse-scattered type (scirrhous type) GC more frequently than in other types of GC.[6] Scirrhous GC is characterized by extensive cancer cell infiltration and proliferation accompanied by abundant stromal fibrosis, and the prognosis of patients with scirrhous GC remains poor.[28] One reason for the poor prognosis is rapid infiltration of cancer cells. It has been reported that abnormalities in several molecules are involved in the pathogenesis of scirrhous GC. The present study found that the coexpression of Wnt5a and laminin γ2 in the same cancer cells is observed at a statistically significant rate in diffuse-scattered type GC but not in other GC types. Wnt5a stimulates cell migration by activating Rac and focal adhesion kinase.[6,9] In addition to this pathway, Wnt5a induces the expression of laminin γ2 by activating JNK (this study) and JNK also phosphorylates paxillin, resulting in stimulation of cell motility.[29] Therefore, the activation of these pathways by Wnt5a overexpressed in scirrhous GC may trigger invasion and metastasis by GC cells, resulting in poor prognosis. Among GC cell lines, KKLS cells barely expressed laminin γ2, although Wnt5a was required for in vivo metastasis and in vitro invasion. These results suggest that Wnt5a also activates other pathways that are involved in invasion and metastasis.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.02.003.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Information

Materials and Chemicals

Various *LAMC2* (*laminin γ2* gene) promoter constructs (pGL3/2.5 kb, 1.2 kb, and 0.12 kb *LAMC2-Luc*) and PUC/EF-1α/β-catenin^{SA} (a constitutively active form of β-catenin) were provided by Drs J. Olsen (University of Copenhagen, Copenhagen, Denmark)[1] and A. Nagafuchi (Kumamoto University, Kumamoto, Japan), respectively. Wnt5a was purified to homogeneity, and anti-Wnt5a polyclonal antibody was generated as described.[2,3] Wnt3a was purified to near homogeneity.[4] Control CM and Wnt5a CM were prepared as described previously.[3] Extracellular matrix was prepared from a culture dish (60-mm diameter) after the cells had been lysed and scraped in 250 μL Nonidet P40 buffer (50 mmol/L Tris-HCl, pH 8.0, 130 mmol/L NaCl, and 1% [vol/vol] Nonidet P40) supplemented with protease inhibitors. Extracellular matrix remaining on the dish was extracted at 100°C for 5 minutes in 375 μL of Laemmli sample buffer after washing with phosphate-buffered saline and Nonidet P40 buffer 3 times. Anti–laminin α3 (LSα3c4) antibody was provided by Dr K. Miyazaki (Yokohama City University, Yokohama, Japan).[5] Anti–laminin γ2 (D4B5) and anti–laminin β3 (kalininB1) antibodies were purchased from Chemicon (Temecula, CA) and BD Biosciences (San Jose, CA), respectively. Anti–β-actin antibody and anti-JunD (sc-74) and anti–c-jun (sc-45) antibodies were from Sigma (St Louis, MO) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-JNK1 ((C-17)-G) and anti–phospho-SAPK/JNK (Thr183/Tyr185) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology (Beverly, MA), respectively. Anti–phospho-PKCα (Thr638) and anti-PKCα (clone M4) antibodies were from Upstate (Temecula, MA). Transfection was performed using Lipofectamine 2000 or Oligofectamine (Invitrogen, Carlsbad, MA). pEF-BOS-HA/hTcf-4E and pEF-BOS-HA/hTcf-4E (Δ1-53) were constructed as described previously.[3,6] Other materials were obtained from commercial sources.

Cell Cultures

TMK-1, KKLS, MKN-45, and MKN-1 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. L cells were grown in DH10 supplemented with 10% fetal bovine serum. Expression of Wnt5a in KKLS and TMK-1 cells was stably suppressed using pSUPER RNAi System (Oligo Engine, Seattle, WA). KKLS and TMK-1 cells stably expressing pSUPER retro vectors were generated by selection with G418, of which concentrations were 400 μg/mL and 200 μg/mL, respectively. MKN-45 cells expressing stably Wnt5a were generated as described previously.[2] The concentration of G418 for the selection of MKN-45 stably expressing Wnt5a was 400 μg/mL. L cells stably expressing Wnt5a were generated by selection with 400 μg/mL of G418.

Cell Migration and Invasion Assays

To evaluate the cell migration activity, Transwell assays were performed using a modified Boyden chamber (tissue culture treated, 6.5-mm diameter, 10-μm thickness, 8-μm pores, Transwell; Costar, Cambridge, MA) as described previously.[7] The lower surface of filters was coated with 10 μg/mL fibronectin for KKLS cells and 10 μg/mL type I collagen for TMK-1 cells. These cells (2.5×10^4 cells in 100 μL) suspended in serum-free RPMI 1640 medium containing 0.1% bovine serum albumin were applied to the upper chamber. The same medium was added to the lower chamber. After the cells were incubated at 37°C for 4–8 hours, the number of cells that migrated to the lower side of the upper chamber was counted. The invasive potential of cells was analyzed using a Matrigel-coated modified Boyden chamber (Becton Dickinson and Company, Bedford, MA). RPMI 1640 medium containing 10% fetal bovine serum was added to the lower chamber. After incubation at 37°C for 24 hours, the number of cells that migrated to the lower side of the upper chamber was counted.

Immunohistochemical Analysis

Formalin-fixed, paraffin-embedded samples were sectioned, deparaffinized, and stained with H&E to ensure that the sectioned block contained tumor cells. Adjacent sections were then immunohistochemically stained. For immunostaining of Wnt5a, a Dako CSA Kit (Dako, Carpinteria, CA) was used according to the manufacturer's recommendations. Sections were pretreated by microwaving in citrate buffer for 30 minutes to retrieve antigenicity. After peroxidase activity was blocked with 3% H_2O_2-methanol for 10 minutes, sections were incubated with normal goat serum (Dako) for 20 minutes to block nonspecific antibody binding sites. Anti-Wnt5a antibody was incubated with tissue samples for 15 minutes at room temperature and detected by incubating for 15 minutes with biotinylated goat anti-rabbit immunoglobulin G, and the signal was amplified and visualized by the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. Wnt5a staining was classified according to the percentage of stained cancer cells. Expression was considered to be negative if less than 50% of cancer cells were stained. When at least 50% of cancer cells were stained, the immunostaining was considered positive.

For immunostaining of β-catenin and laminin γ2, a Dako LSAB Kit was used according to the manufacturer's recommendations. Sections were pretreated by microwaving in citrate buffer for 30 minutes to retrieve antigenicity for β-catenin or by Protease XXIV (Sigma, St Louis, MO) for 15 minutes at room temperature for laminin γ2. After peroxidase activity was blocked with 3% H_2O_2-methanol for 10 minutes, the sections were incubated with normal goat serum (Dako) for 20 minutes to block nonspecific antibody binding sites. The sections were

incubated with the antibody dilutions of mouse monoclonal anti–β-catenin antibody 1:50 (Transduction Labs, Lexington, KY) and mouse monoclonal anti-laminin γ2 1:50 (Chemicon, Temecula, CA) and then incubated with primary antibody for 1 hour at room temperature, followed by incubations with biotinylated anti-mouse immunoglobulin G and peroxidase-labeled streptavidin for 10 minutes each. Staining was completed with a 10-minute incubation with the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. The cytosomal or nuclear accumulation of β-catenin and laminin γ2 staining was classified according to the percentage of stained cancer cells. When more than 50% of cancer cells were stained, the immunostaining was considered positive.

ChIP Assay

Cells (2×10^6) were cross-linked with 1% formaldehyde for 10 minutes at room temperature. The cell pellets were lysed with sodium dodecyl sulfate lysis buffer and sonicated to shear DNA to a size range between 200 and 1000 base pairs. Sheared chromatin samples were diluted in ChIP buffer and incubated for 12 hours at 4°C with 6 μg of anti-JunD (sc-74) antibody, anti–c-jun (sc-45) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or negative control immunoglobulin G (Diagnode). Immunocomplexes were collected, and then the DNA fragments were purified using DNA purifying slurry. After incubation at 55°C for 30 minutes to reverse protein/DNA cross-links, the purified DNA was used as a template for PCR. Forward and reverse primers were as follows: fragment containing AP-1 sites, 5′-GGGAGCGATTTTCCAGCCCG-3′ and 5′-CTCGGTTGGTGGTTCTCACT-3′; fragment containing a Tcf site without AP-1 sites, 5′-CTCTCCGACTGACTTGT-3′ and 5′-CCTTTGCCTGGAATGTG-3′.

The occupancy of *LAM2* promoter by JunD or c-jun was quantified by real-time PCR for immunoprecipitants with anti-JunD or anti–c-jun antibody from the same cross-linked chromatin samples. The primers for the fragment containing AP-1 sites of *LAMC2* promoter were also used in the real-time PCR. The relative amounts of the DNA fragments containing AP-1 sites immunoprecipitated with anti-JunD or anti–c-jun antibody were expressed as arbitrary units compared with that with control immunoglobulin G.

Cell Proliferation Assay

KKLS and TMK-1 cells were seeded at densities of 1.0×10^5/mL and 0.5×10^5/mL, respectively. At 12 hours after plating, the medium was replaced with 0.5% serum medium for KKLS cells and 1% serum medium for TMK-1 cells. The cell number was counted every 24 hours for 4 days.

Reverse-Transcription PCR

Semiquantitative reverse-transcription PCR was performed as described previously.[8] Forward and reverse primers were as follows: *Wnt3a*, 5′-CCCACTCGGATACTTCT-3′ and 5′-ATCTCCACGTAGTTCCTG-3′; *Wnt5a*, 5′-CTTCGCCCAGGTTGTAATTGAAGC-3′ and 5′-CTGCCAAAAACAGAGGTGTTATCC-3′; *LAMA3*, 5′-GGGACTACATCGGCATGGCA-3′ and 5′-AAAATCAGGTGGGTAACCTCCA-3′; *LAMB3*, 5′-ACCTGACAGGACTGGAGAAGCG-3′ and 5′-ATTGGCTCAGGCTCAGCTGC-3′; *LAMC2*, 5′-ACCGAGTTCGGGATAC-3′ and 5′-TGCTCCATGTTACTGGC-3′; *Fz2*, 5′-GAGCGTGATTGTGCTG-3′ and 5′-GCTCTGGGTAGCGGAA-3′; *Fz5*, 5′-CTGTCGCTAAACTTTCCG-3′ and 5′-CGTCCAAAGATAAACTGCT-3′; *Fz7*, 5′-GACGTGCAAGAGCTATG-3′ and 5′-TGCTGCTGTGGCTAAGT-3′; *β-actin*, 5′-ACAATGAGCTGCGTGTG-3′ and 5′-ATCACGATGCCAGTGGT-3′; and *GAPDH*, 5′-CCTGTTCGACAGTCAGCCG-3′ and 5′-CGACCAAATCCGTTGACTCC-3′.

siRNA

In analysis with siRNA for *Wnt5a, LAMC2, Wnt3a, Fz2, Fz5,* and *Fz7*, the following target sequences were used. *Wnt5a*, 5′-CTGTGGATAACACCTCTGTTT-3′ and 5′-CTGTTCAGATGTCAGAAGTAT-3′; *LAMC2*, 5′-CAGAATACAGTGTCCATAAGA-3′; *Wnt3a*, 5′-GGAACTACGTGGAGATCAT-3′; *Fz2*, 5′-GCCCTCATGAACAAGTTCG-3′; *Fz5*, 5′- GGTCCTCTGCATGGATTAC-3′ and 5′-CTATTTTGCGTTTCTTACT-3′; and *Fz7*, 5′-TCACCTACCTGGTGGACAT-3′.

Reporter Gene Assay

MKN-45 or TMK-1 cells were transfected with pGL3/(2.5 kb, 1.2 kb, or 0.12 kb) *LAMC2-Luc* and pME18S/LacZ. At 48 hours after transfection, the cells were lysed, and the luciferase activity was measured with PicaGene reagent (Toyo Ink, Tokyo, Japan). When necessary, at 24 hours after transfection, the cells were cultured in serum-free medium for 12 hours and stimulated with Wnt5a CM or control CM for a further 24 hours, and then the luciferase activities were measured. β-Galactosidase activities were determined to normalize the transfection efficiency.

DNA Microarray Analysis

For the analysis of differential gene expression, RNA was isolated from 3 independent clones of L/control, L/Wnt5a, and L/Wnt5a CA cells and processed for array hybridization using BeadArray (Illumina, San Diego, CA). For gene expression comparison, analysis software GeneSpring (Agilent Technologies, Santa Clara, CA) was used. Microarray data were submitted to Moritex Corp (Kanagawa, Japan) under the experimental user ID 31U13A0028.

Among the target genes listed in Supplementary Table 1, mRNA levels of laminin γ2, cullin 4B, glycam 1, and sentrin 3 were increased in L/Wnt5a cells as

compared with wild-type L cells. Furthermore, knockdown of Wnt5a in TMK-1 cells reduced the mRNA levels of laminin γ2 and cullin 4B. Therefore, it was believed that laminin γ2 and cullin 4B are real targets for Wnt5a signaling in GC cells.

Statistical Analysis

Incidence of metastasis was compared using the Mann–Whitney *U* test. Orthotropic tumor weights were compared using unpaired Student *t* test. Statistical analyses for Tables 1 and 2 and Supplementary Table 2 were performed with Fisher exact test. A *P* value less than .05 was considered statistically significant. Other experiments were performed at least 3 times and the results were expressed as means ± SE. Statistical analysis was performed using StatView software (SAS Institute, Inc, Cary, NC).

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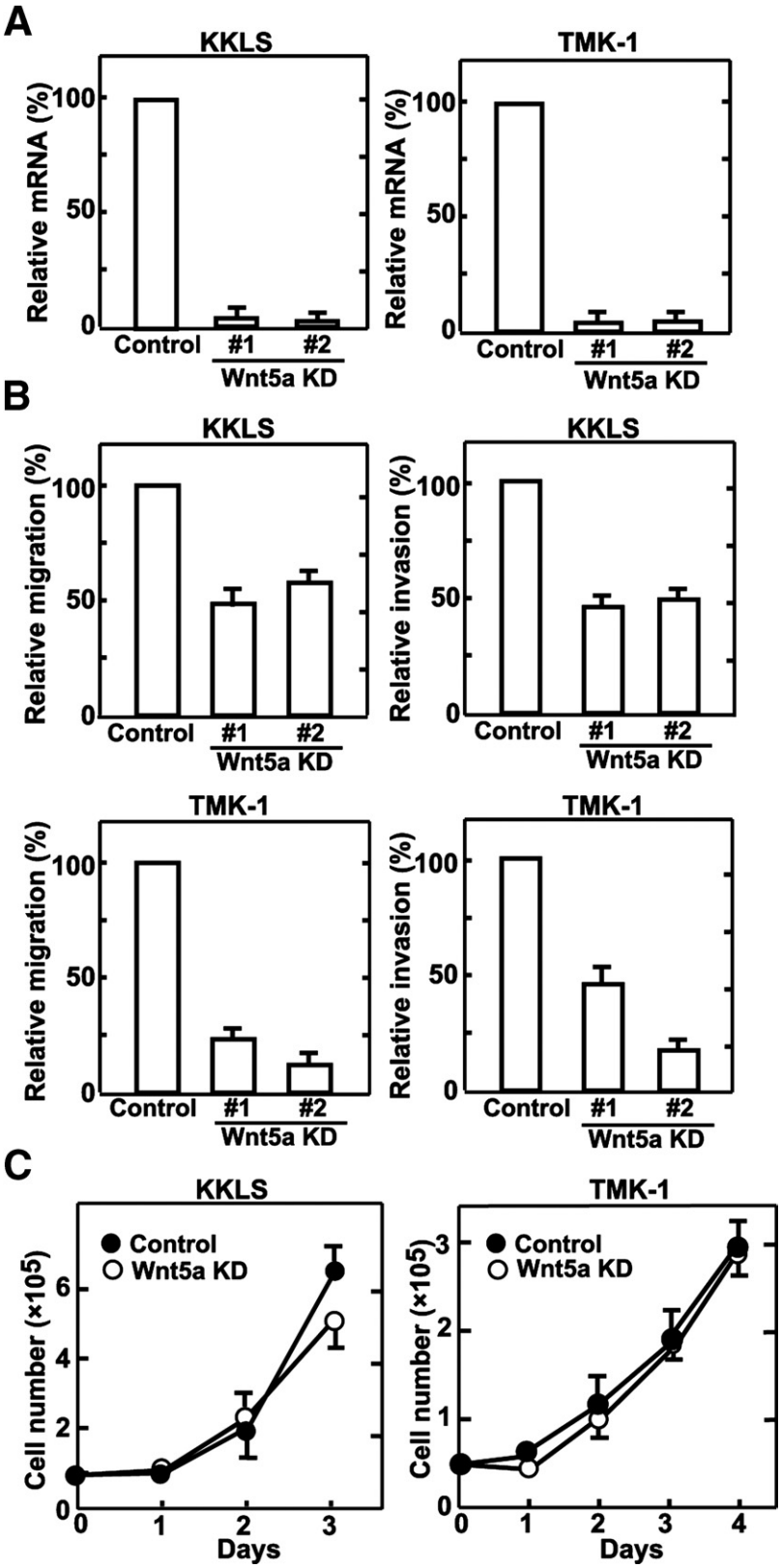
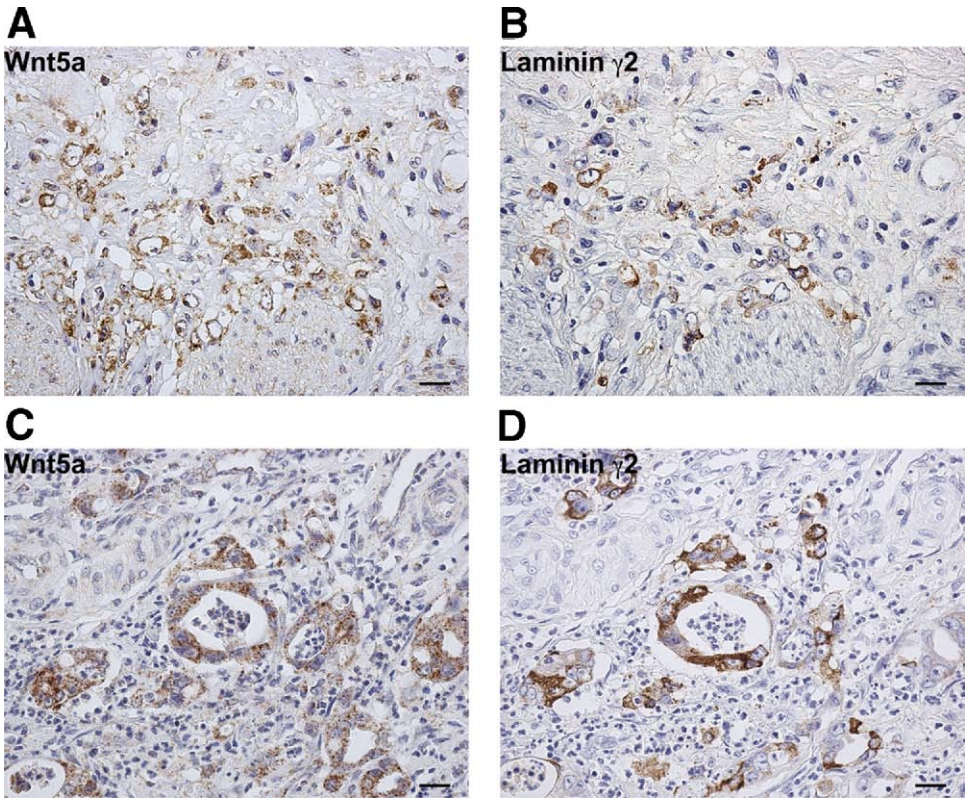


Supplementary Figure 1. Generation of Wnt5a knockdown cells. (*A*) The levels of Wnt5a mRNA in KKLS cells or TMK-1 cells stably expressing pSUPER siRNA empty vector (KKLS/control, TMK-1/control) or pSUPER siRNA vector targeting Wnt5a (KKLS/Wnt5a KD, TMK-1/Wnt5a KD) were measured by quantitative reverse-transcription PCR. Relative mRNA levels were expressed as the percentages of Wnt5a mRNA in control cells. #1 and #2 indicate the clone numbers of established cells. The results shown are means ± SE from 3 independent experiments. (*B*) KKLS/control, KKLS/Wnt5a KD, TMK-1/control, and TMK-1/Wnt5a KD cells were subjected to the migration assay or invasion assay. In 3 independent fields, 392 ± 33 KKLS/control cells and 112 ± 11 TMK-1/control cells transmigrated and 129 ± 25 KKLS/control cells and 40 ± 7 TMK-1/control cells invaded. Relative migration and invasion activities of Wnt5a KD cells were expressed as percentages of migrated and invaded cell numbers of control cells. (*C*) KKLS/control, KKLS/Wnt5a KD, TMK-1/control, and TMK-1/Wnt5a KD cells were cultured in the presence of 0.5% (for KKLS cells) or 1% (for TMK-1 cells) fetal bovine serum for the indicated periods of days, and then cell numbers were counted. *Closed circles*, KKLS/control or TMK-1/control cells; *open circles*, KKLS/Wnt5a KD or TMK-1/Wnt5a KD cells.

Supplementary Table 1. List of Candidate Genes Induced by Wnt5a

Gene symbol	L/Wnt5a fold change[a]	L/Wnt5a CA fold change[b]	Description
Sesn3	6.9431	1.1567	Sestrin3
Glycam1	3.7772	1.0149	Glycosylation dependent cell adhesion molecule 1
Runx2	3.6753	0.9363	Runt related transcription factor 2
Ifi204	2.9923	1.1577	Interferon activated gene 204
Akt1	2.8358	0.8954	Protein kinase B1
Cul4b	2.5080	1.0949	Cullin 4B
Anxa1	2.4059	1.0831	Annexin A1
Tob1	2.4049	1.1525	Transducer of ErbB-2.1
Fbxo30	2.1056	0.8623	F-box protein 30
Lamc2	2.0939	1.0645	Laminin γ2
Jun	2.0707	1.0274	Jun oncogene
Socs5	2.0341	1.0652	Suppressor of cytokine signaling 5
Sema5a	2.0071	0.9693	Semaphorin5A
Dlg7	1.9890	0.9325	Discs, large homolog 7
Rab8b	1.8769	1.1308	RAS oncogene family (Rab8b)
Nrp1	1.7876	1.0534	Neuorophilin1
Rab10	1.7825	1.1153	Ras oncogene family (Rab10)
Mapre2	1.7509	1.1343	Microtubule-associated protein, RP/EB family, member 2
Epn2	1.7366	1.0682	Epsin2
Timp2	1.7062	1.0931	Tissue inhibitor of metalloproteinase 2
Iqgap1	1.6510	1.0915	IQ motif containing GTPase activating protein 1
Nedd9	1.5555	0.9614	Neural precursor cell expressed, developmentally down-regulated gene 9

[a]L/Wnt5a compared with L/control.
[b]L/Wnt5aCA compared with L/control.



Supplementary Figure 2. Coexpression of Wnt5a and laminin γ2 in scirrhous GC. (*A* and *B*) A sample of diffuse-scattered type GC was stained with (*A*) anti-Wnt5a and (*B*) anti–laminin γ2 antibodies. Cytoplasmic laminin γ2 was detected in Wnt5a-positive cancer cells. *Scale bars* = 19 μm. (*C* and *D*) A sample of intestinal-type GC was stained with (*C*) anti-Wnt5a and (*D*) anti–laminin γ2 antibodies. Cytoplasmic laminin γ2 was detected in Wnt5a-positive cancer cells. *Scale bars* = 19 μm.

Supplementary Table 2. Relationship Between Wnt5a Expression and Clinicopathologic Characteristics in GC

	Wnt5a		
	Positive	Negative	*P* value
T classification			
T1	3 (6%)	45	<.0001
T2/3/4	41 (39%)	64	
N classification			
N0	6 (9%)	62	<.0001
N1/2/3	38 (45%)	47	
Stage			
I/II	11 (12%)	79	<.0001
III/IV	33 (52%)	30	
Histologic classification			
Diffuse-scattered type	24 (67%)	12	<.0001
Other	20 (17%)	97	