An Anti-Wnt5a Antibody Suppresses Metastasis of Gastric Cancer Cells *in vivo* by Inhibiting Receptor-Mediated Endocytosis

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Abstract

Wnt5a is a representative ligand that activates the β-catenin-independent pathway in Wnt signaling. It was reported that the expression of Wnt5a in human gastric cancer is associated with aggressiveness and poor prognosis, and that knockdown of Wnt5a reduces the ability of gastric cancer cells to metastasize in nude mice. Therefore, Wnt5a and its signaling pathway might be important targets for the therapy of gastric cancer. The aim of this study was to examine whether an anti-Wnt5a antibody affects metastasis of gastric cancer cells. One anti-Wnt5a polyclonal antibody (pAb5a-5) inhibited migration and invasion activities in vitro of gastric cancer cells with a high expression level of Wnt5a. Previously it was demonstrated that Wnt5a induces the internalization of receptors, which is required for Wnt5a-dependent activation of Rac1. pAb5a-5 inhibited Wnt5a-dependent internalization of receptors, thereby suppressed Wnt5a-dependent activation of Rac1. Laminin γ2 is one of target genes of Wnt5a signaling and Rac1 was involved in its expression. pAb5a-5 also inhibited Wnt5a-dependent expression of laminin γ2. In an experimental liver metastasis assay, gastric cancer cells were introduced into the spleens of nude mice. Laminin γ2 was required for liver metastatic ability of gastric cancer cells in vivo. Furthermore, intraperitoneal injection of pAb5a-5 inhibited the metastatic ability of gastric cancer cells. These results suggest that an anti-Wnt5a antibody was capable of suppressing Wnt5a-dependent internalization of receptors, resulting in the prevention of metastasis of gastric cancer cells by inhibiting the activation of Rac1 and the expression of laminin γ2.
Introduction

The Wnt protein family plays roles in embryogenesis and carcinogenesis (1). Wnt activates at least two intracellular signaling pathways, the β-catenin-dependent and β-catenin-independent pathways (2-4). Abnormal activation of the β-catenin-dependent pathway because of genetic alteration in β-catenin, adenomatous polyposis coli (APC), or Axin can be associated with human cancer (5). Recent evidence has clarified that the β-catenin-independent pathway is also involved in tumorigenesis (6, 7). Wnt5a is a representative of the Wnt protein family that activates the β-catenin-independent pathway, which primarily modulates cell movement and polarity (2, 6, 7). By binding to receptors, Frizzled (Fz) and receptor tyrosine kinase-like orphan receptor 2 (Ror2), Wnt5a induces the internalization of the receptors (8, 9), which is necessary for the Wnt5a-dependent activation of small GTP-binding protein Rac1 that plays important roles in the migration and invasion activities of cancer cells (10).

It has been shown that expression of Wnt5a is correlated with the aggressiveness of melanoma, breast cancer, lung cancer, gastric cancer, and prostate cancer (2, 6, 11-16), suggesting that Wnt5a has oncogenic properties. In experiments in vivo with nude mice, metastasis from the spleen to the liver was suppressed significantly in Wnt5a knockdown cells (15), strongly suggesting that Wnt5a is involved in gastric cancer invasion or metastasis and that Wnt5a is a good molecular target for gastric cancer therapy.

In addition to the activation of Rac1, several possible mechanisms by which Wnt5a regulates cell migration and invasion have been proposed. Wnt5a controls cell polarity and directional migration in melanoma cells by recruiting actin and myosin IIB to melanoma cell adhesion molecule (17). Wnt5a signaling has also been shown to cooperate with the integrin signaling pathway to regulate cell migration and adhesion (18). In this model Wnt5a, Fz2, and integrin β1 colocalize to the leading edge of polarized migrating cells, and this signal is
transmitted to focal adhesion kinase (FAK) and paxillin through the binding of Dvl and APC, respectively (18). Moreover, Wnt5a induces the expression of laminin γ2 and matrix metalloprotease 1 in gastric and prostate cancer cells (15, 16), which are known to be involved in invasion and metastasis of cancer. Thus, Wnt5a has different roles in cell migration and invasion in a cell or tissue context-dependent manner.

Wnt1 and Wnt2, which activate the β-catenin-dependent pathway, have been shown to be overexpressed in hepatoma, malignant melanoma, colorectal cancer, non-small cell lung cancer, breast cancer, and sarcoma (19-22). Blockade of Wnt signaling using anti-Wnt1 and anti-Wnt2 antibodies induced apoptosis and inhibit proliferation probably by inhibiting the β-catenin-dependent pathway in these cancer cells. Although how these antibodies inhibit Wnt signaling is unclear, interference with the β-catenin-dependent pathway would provide a potent and selective therapeutic strategy for cancers that overexpress Wnt1 or Wnt2. However, it has been never clarified whether an anti-Wnt5a antibody has anti-tumor effect through suppression of the β-catenin-independent pathway. Here we show that an anti-Wnt5a antibody inhibits metastasis of gastric cancer cells in vivo by suppressing the Wnt5a-dependent internalization of receptors.
Materials and Methods

Cell lines and cell culture

Gastric cancer cell lines, KKLS, MKN-1, MKN-45, and TMK-1 cells, were provided by Dr. W. Yasui (Hiroshima University, Hiroshima, Japan) in August 2006 (no authentication was done by the authors). These gastric cancer cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and were maintained at 37°C in humidified air with 5% CO2. More information is described in Supplementary data.

Cell migration and invasion assays

To measure cell migration and invasion activities, Transwell assays were done using a modified Boyden chamber (tissue culture treated, 6.5 mm in diameter, 10-μm thick, 8-μm pores; Transwell, Costar, Cambridge, MA) and a matrigel-coated modified Boyden chamber (BD Biosciences, San Jose, CA), respectively, as described previously (14, 15). The details are described in Supplementary Data.

Animals and implantation of tumor cells

Six-week old male BALB/cAnN Crj-nu mice (Charles River Laboratory Japan, Inc., Kanagawa) were anesthetized with a combination of Medetomidine (0.3 mg/kg body weight), Midazolam (4 mg/kg), and Butorphanol (5 mg/kg), and then KKLS (2.5 x 10^5), MKN-45 (2.5 x 10^6), or TMK-1 (1.0 x 10^6) cells in 50 μl Hanks’ balanced salt solution (HBSS) were injected into the spleen through a 27-gauge needle (day 0). pAb5a-5, anti-glutathione-S-transferase (GST) antibody (10 μg/g body weight), or phosphate buffered saline (PBS) was injected into the intraperitoneal cavity twice weekly for 5 weeks (at days -2, 1, 5, 8, 12, 15, 19, 22, 26, and 29). Five weeks later, under deep anesthesia with pentobarbital, hepatectomy was performed. Then, formalin-fixed samples were sectioned and the number of metastatic nodule was counted macroscopically. The protocols used for
all animal experiments in this study were approved by the Animal Research Committee of Hiroshima University and Osaka University.

**Receptor internalization assay**

Internalization of FLAG-Fz2 by Wnt5a and Wnt3a was examined as described (8) and its details are described in Supplementary Data. When the internalization of Ror2 or low density lipoprotein-like receptor protein 6 (LRP6) at endogenous levels was examined, KKLS cells were preincubated with 25 μg/ml pAb5a-5 or anti-GST antibody and then stimulated with 50 ng/ml Wnt5a or Wnt3a. The cells were incubated with 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL) at 4°C for 30 minutes (23, 24). After quenching of excess biotin with 50 mM NH₄Cl, the cells were lysed in 0.2 ml of TNE buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 5 mM EDTA-NaOH [pH 8.5] containing 0.4% sodium deoxycholate, 1% Triton X-100, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μM phenylmethylsulfonyl fluoride). The lysates were precipitated with NeutrAvidin agarose resin (Pierce) for 2 hours at 4°C, and the precipitates were probed with anti-Ror2 or anti-LRP6 antibody.

**Statistical analysis**

The incidence of metastasis was compared using the Mann-Whitney U-test. A P value less than 0.05 was considered statistically significant. Other experiments were performed at least three times and the results were expressed as means ± SE.
Results

Generation of anti-Wnt5a polyclonal antibodies

Using different synthetic peptides that corresponded to amino acid residues 87-103, 165-181, and 275-290 of human Wnt5a, we generated rabbit polyclonal anti-Wnt5a antibodies (Supplementary Fig. 1A). Among them, an anti-Wnt5a antibody, named pAb5a-1, was used for immunohistochemical analyses in human gastric and prostate cancer samples (14-16). However, this antibody did not affect cell migration of gastric cancer cells in vitro (data not shown). pAb5a-2 recognized both Wnt5a and Wnt5b, while pAb5a-5 reacted with Wnt5a only (Supplementary Fig. 1B). These anti-Wnt5a antibodies did not react with Wnt3a (Supplementary Fig. 1B).

Among gastric cancer cell lines Wnt5a mRNA levels were relatively higher in MKN-1, TMK-1, and KKLS cells compared with those in MKN-45 cells (14) (Supplementary Fig. 2A). Consistent with mRNA levels, Wnt5a protein was detected in lysates of MKN-1, TMK-1, and KKLS cells at endogenous levels but not in that of MKN-45 cells (Supplementary Fig. 2B). pAb5a-5 detected endogenous Wnt5a, which was secreted from MKN-1 and KKLS cells and attached to the extracellular matrix (ECM) fraction (Supplementary Fig. 2C).

An anti-Wnt5a antibody suppresses gastric cancer cell migration and invasion in vitro

Knockdown of Wnt5a suppressed migration and invasion activities of MKN-1, KKLS, and TMK-1 cells in vitro (14, 15) and the metastatic ability of KKLS and TMK-1 cells in vivo (15). Therefore, migration and metastatic abilities of these gastric cancer cells depend on Wnt5a expression. To test whether anti-Wnt5a antibodies affect these functions of gastric cancer cells, cell migration activity was measured using a Boyden chamber. When KKLS, MKN-1, and TMK-1 cells were treated with pAb5a-5, their ability to migrate was decreased, but pAb5a-2 did not affect it (Fig. 1A-C). The invasive potential of the cells was measured
using a matrigel-coated modified Boyden chamber. pAb5a-5 but not pAb5a-2 inhibited invasion activity of these three gastric cancer cells in vitro (Fig. 1A-C). The inhibitory effect of pAb5a-5 on KKLS cells was a dose-dependent manner (Supplementary Fig. 3).

To determine the specificity of pAb5a-5, MKN-45 cells, which show a low expression level of Wnt5a, were used. Unlike the effects on KKLS, MKN-1, and TMK-1 cells, pAb5a-5 did not affect migration and invasion activities of MKN-45 cells (Fig. 1D). Expression of Wnt5a in MKN-45 cells enhanced migration and invasion activities, and pAb5a-5 inhibited the activities of Wnt5a-expressing MKN-45 cells (Fig. 1D). Wnt5a was also shown to be involved in cell-to-substrate adhesion and to enhance adhesion-dependent activation of FAK (14, 18). pAb5a-5 inhibited adhesion activity in KKLS and MKN-1 cells (Fig. 1E). pAb5a-5 did not affect adhesion activity of MKN-45 cells but inhibited that of MKN-45 cells expressing Wnt5a (Fig. 1E). Furthermore, pAb5a-5 also suppressed adhesion-dependent activation of FAK in KKLS and Wnt5a-expressing MKN-45 cells (Supplementary Fig. 4).

Taken together, pAb5a-5 inhibited cell migration, invasion, and adhesion activities of gastric cancer cells expressing Wnt5a. However, pAb5a-5 did not affect the growth of KKLS and MKN-1 cells in vitro (Fig. 1F), which was consistent with our previous findings that knockdown of Wnt5a did not affect proliferation of gastric cancer cells in vivo and in vitro (14, 15).

pAb5a-5 inhibits Wnt5a-dependent activation of Rac1 and expression of laminin γ2

Wnt5a activated Rac1 (8, 14), which plays important roles in cell migration and adhesion (10). Consistent with inhibitory effects of pAb5a-5 on migration and invasion, the antibody inhibited Wnt5a-dependent activation of Rac1 in KKLS cells (Fig. 2A). Furthermore, the inhibitory effect of pAb5a-5 on invasion activity of KKLS cells was restored by the expression of a constitutive active form of Rac1 (Rac1V12) (Supplementary Fig. 5), suggesting that the antibody affects Wnt5a signaling upstream of Rac1.
It has been reported that Wnt5a induces the expression of laminin γ2 (15), which is suggested to be involved in invasion activity of gastric cancer cells (25). To examine whether laminin γ2 is necessary for metastatic ability of gastric cancer cells in vivo, wild-type TMK-1 or laminin γ2-knockdown TMK-1 cells were inoculated into the spleen of nude mice (Fig. 2B and Supplementary Fig. 6). Knockdown of laminin γ2 indeed reduced metastatic nodules in the liver (Fig. 2B).

To examine whether Rac1 is involved in Wnt5a-dependent expression of laminin γ2 gene (LAMC2), LAMC2-Luc, a reporter gene containing the LAMC2 promoter and a luciferase gene, was transfected into MKN-1, TMK-1, and MKN-45 cells. In these gastric cancer cells, Rac1V12 increased luciferase activity and a dominant negative form of Rac1 (Rac1N17) suppressed Wnt5a-dependent luciferase activity (Fig. 2C), suggesting that Wnt5a induces the expression of laminin γ2 through the activation of Rac1. Wnt5a increased protein levels of laminin γ2 in MKN-45 and MKN-1 cells although basal expression levels were different (Fig. 2D). pAb5a-5 suppressed protein and mRNA levels of laminin γ2 in MKN-1 cells and Wnt5a-treated MKN-45 cells (Fig. 2D). Immunocytochemical analyses confirmed that pAb5a-5 inhibits Wnt5a-dependent expression of laminin γ2 in MKN-1 cells (Fig. 2E and Supplementary Fig. 7). These results suggest that pAb5a-5 has a potential ability to suppress migration and invasion activities of Wnt5a-expressing cells by inhibiting the activation of Rac1 and the expression of laminin γ2.

**pAb5a-5 inhibits Wnt5a-dependent receptor internalization**

How pAb5a-5 inhibits Wnt5a signaling was examined. Wnt5a bound to the extracellular domain of its receptors, Fz2 and Ror2, in vitro (8, 26). Secreted frizzled-related protein 2
(sFRP2) is a secreted protein that is able to bind to Wnt ligands, thereby suppressing their binding to receptors (27). pAb5a-5 did not inhibit the interaction between Wnt5a and Fz2 under conditions where sFRP2 inhibited their binding (Fig. 3A). Wnt5a has been shown to induce phosphorylation of Ror2 in HeLaS3, HEK293, and NIH3T3 cells (28). Consistent with these results, Wnt5a induced a mobility shift of Ror2 in KKLS cells on SDS-PAGE, which indicated the phosphorylation of Ror2 (Fig. 3B). pAb5a-5 did not affect the mobility shift of Ror2 under conditions where sFRP2 reduced it (Fig. 3B). Taken together, these results suggest that pAb5a-5 does not affect the binding of Wnt5a to its receptors, Fz2 and Ror2.

Wnt5a has been shown to induce the internalization of FLAG-Fz2 through clathrin-mediated endocytic route and that this process is necessary for Wnt5a-dependent Rac1 activation in HeLaS3 and HEK293 cells (8). FLAG-Fz2 was internalized in KKLS cells in response to Wnt5a (Fig. 3C). Treatment of cells with pAb5a-5 increased numbers of cells expressing FLAG-Fz2 on the cell surface and decreased numbers of cells having intracellular punctuate structures of FLAG-Fz2, suggesting that pAb5a-5 inhibits Wnt5a-dependent internalization of FLAG-Fz2 (Fig. 3C). Endogenous Ror2 was also found to be internalized by Wnt5a in HeLaS3 cells in a clathrin-dependent manner (9). Wnt5a induced the internalization of endogenous Ror2 in KKLS cells in time- and dose-dependent manners, and pAb5a-5 suppressed it (Fig. 3D). Wnt3a induced the internalization of Fz2 and LRP6, and their internalization is required for Wnt3a-dependent activation of the β-catenin-dependent pathway (8, 23, 24). Unlike the effects on Wnt5a-dependent internalization of Fz2 and Ror2, pAb5a-5 did not affect Wnt3a-dependent internalization of Fz2 and LRP6 (Fig. 3E).
Furthermore, knockdown of clathrin suppressed Wnt5a-dependent increase in LAMC2 mRNA of TMK-1 and MKN-45 cells (Fig. 3F), suggesting that receptor-mediated endocytosis through a clathrin-dependent route is necessary for the expression of laminin γ2. Taken together, these results suggested that pAb5a-5 inhibits Wnt5a-dependent internalization of its receptors, thereby suppressing Wnt5a-dependent activation of Rac1 and expression of laminin γ2.

**pAb5a-5 recognized Wnt5a released from KKLS cells in 3D culture**

Next we tested whether pAb5a-5 recognizes Wnt5a secreted from cells. To observe Wnt5a secreted from cells, KKLS cells were embedded into 3D-matrigel. KKLS cells formed amorphous aggregates in 3D culture, and endogenous integrin β1 was enriched in the cell surface (Fig. 4). However, endogenous Wnt5a was hard to detect using pAb5a-5 in 3D culture conditions (Fig. 4A). When Wnt5a was expressed in KKLS cells transiently, it was recognized by pAb5a-5 in the area surrounding cells (Fig. 4B). This detection was specific, because pAb5a-2 and anti-GST antibody did not recognize Wnt5a produced from the cells (Fig. 4C, D). These results suggest that secreted Wnt5a is localized to the immediate vicinity of KKLS cells probably because of the interaction with matrigel proteins or heparan sulfate proteoglycans (HSPG) (29). Therefore, it was expected that pAb5a-5 is able to react with a native form of Wnt5a, which is secreted from cells and affects Wnt5a-producing cells *in vivo*.

**pAb5a-5 inhibits metastasis in vivo**

Finally, the effects of pAb5a-5 on metastatic ability *in vivo* of gastric cancer cells were examined using a mouse model. KKLS or MKN-45 cells were inoculated into the spleen of
nude mice. Injection of pAb5a-5 significantly inhibited the numbers of liver metastatic foci compared with that of PBS or anti-GST antibody (Fig. 5A). However, \textit{in vivo} metastasis of MKN-45 cells was not affected by treatment with pAb5a-5 (Fig. 5B). No obvious toxicity, including weight loss, during the treatment and no histological damage of liver or spleen were observed in any of the control mice administrated with pAb5a-5 (data not shown).

A polyclonal antibody contains multiple antibodies against different epitopes on the antigen even though a synthetic peptide was used as an antigen. Therefore, the neutralizing activity could vary among five different rabbit-generated pAb5a-5 antibodies. All of the antibodies suppressed migration and invasion activities of KLLS cells \textit{in vitro} although the degree of inhibition varies (Fig. 5C). Antibodies inhibiting migration well had a tendency to suppress invasion strongly. Furthermore, all pAb5a-5 from five different rabbits suppressed metastatic ability of KKLS cells \textit{in vivo} (Fig. 5D). Antibodies reducing invasion \textit{in vitro} well had a tendency to suppress metastasis strongly \textit{in vivo} (Fig. 5D). Taken together, these results suggested that the effects of pAb5a-5 on gastric cancer cell migration and invasion activities \textit{in vitro} reflect those of the antibody to suppress metastatic ability \textit{in vivo}.
Discussion

The molecular mechanisms by which pAb5a-5 inhibits invasion and metastasis

In this study, we have, for the first time, demonstrated that an anti-Wnt5a antibody (pAb5a-5) inhibits invasion and metastasis of gastric cancer cells. The inhibitory activity of pAb5a-5 was specific for gastric cancer cells expressing Wnt5a, because it did not affect migration, invasion, adhesion, and metastasis of MKN-45 cells, which express Wnt5a little. Wnt5a was shown to be involved in the activation of Rac1 and the expression of laminin γ2 (14, 15). Wnt5a induced the internalization of Fz2 and Ror2 through a clathrin-dependent manner and the internalization was necessary for Wnt5a-dependent activation of Rac1 (8) and expression of laminin γ2 (this study). Our present results revealed that pAb5a-5 suppresses Wnt5a-dependent receptor internalization, although pAb5a-5 does not inhibit the binding of Wnt5a to its receptors, thereby inhibiting the Wnt5a-dependent activation of Rac1 and expression of laminin γ2. Because an anti-FLAG antibody was internalized into cells with FLAG-Fz2 in response to Wnt5a (8) and pAb5a-5 did not affect Wnt3a-dependent internalization of Fz2 and LRP6, the effect of pAb5a-5 to inhibit the internalization of Wnt5a receptors could be specific. Taken together, it is likely that pAb5a-5 suppresses invasion and metastasis of gastric cancer cells by inhibiting Wnt5a-dependent receptor internalization.

Currently we do not know the mechanism by which pAb5a-5 blocks Wnt5a-dependent internalization of receptors. The interaction of Wnt5a with its receptors and other factors including HSPG and secreted proteins is highly complex. One possibility is that pAb5a-5 may inhibit the interaction of Wnt5a and components other than receptors. For example, a transmembrane type HSPG, syndecan (SDC), has been reported to regulate Wnt5a signaling. Expression of SDC1 and SDC4 correlates with Wnt5a expression in melanoma cell lines and knockdown of SDC1 and SDC4 decreases cell invasion activity, which is restored by Wnt5a (30). SDC4 has also been shown to be required for Wnt5a-dependent internalization of Fz7
in Xenopus embryos (31). In addition, another type of HSPG, glypican-4, enhanced Wnt5a-dependent activation of Rac1 (9). Therefore, it is intriguing to examine effects of pAb5a-5 on the formation of a complex between Wnt5a and HSPG.

**Possible effects of pAb5a-5 on other functions of Wnt5a**

It has been suggested that Wnt5a has tumor suppressive actions, because Wnt5a heterozygous mice develop B cell lymphoma (32). Furthermore, Wnt5a reduced the proliferation, migration, and invasiveness in thyroid tumor cells and inhibited the migration activity of colorectal cancer cells (6). The mechanism of the tumor suppressive effects of Wnt5a might be because of its inhibitory activity for the β-catenin pathway. It has been shown that Wnt5a antagonizes the β-catenin pathway by inhibiting either of three points; β-catenin-dependent transcriptional activation, the stabilization of β-catenin, or the binding of Wnt that activates the β-catenin pathway to Fz (7, 8, 33). In the first case, Ror2 functions as a receptor (33), and this inhibitory mechanism may require the internalization of Ror2. Therefore, if Wnt5a suppresses tumorigenesis through this mechanism, pAb5a-5 may exacerbate cancer progression. The third inhibitory mechanism of Wnt5a does not require the internalization of receptors but compete with other Wnt ligand for the binding to receptors (8). Therefore, pAb5a-5 is likely to show no effects on tumors when Wnt5a exerts this inhibitory action. At present it is not known whether receptor-mediated endocytosis is involved in the second inhibitory mechanism.

It has also been reported that Wnt5a activates the β-catenin pathway when Fz5 or Fz4 and LRP5 are overexpressed (7, 33), suggesting that Wnt5a promotes cell growth in cancers with Fzs and LRP5 overexpression. Whether the internalization of these receptors in response to Wnt5a is required for the activation of the β-catenin pathway is not known. However, it has been reported that Wnt3a induces the internalization of LRP6 and Fz2 or Fz5 through a caveolin-mediated route and the internalization is necessary for the activation of the β-catenin
pathway (8, 23, 34). Therefore, it is possible that by inhibiting receptor internalization pAb5a-5 suppresses cell growth in certain types of cancers in which Fzs and LRP5 are overexpressed and Wnt5a stimulates cell proliferation.

Our results demonstrated that anti-Wnt5a polyclonal antibodies generated from different rabbits suppress migration and invasion activities of gastric cancer cells in vitro to various extents. However, the inhibitory effects on metastasis in vivo were almost parallel with those on invasion in vitro. Therefore, in vitro invasion assays are useful for screening to find anti-Wnt5a antibodies capable of suppressing metastatic ability of cancer cells in vivo. It will be important to obtain anti-Wnt5a monoclonal antibody for further investigation how the antibody suppresses Wnt5a signaling and inhibit metastases of cancer cells for the clinical management of cancers with overexpression of Wnt5a.
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References


Legends to Figures

Figure 1  Anti-Wnt5a antibody inhibits migration, invasion, and adhesion of gastric cancer cells.  A, B, and C, Inhibition of migration (a) and invasion (b) of KKLS (A), MKN-1 (B), or TMK-1(C) cells by anti-Wnt5a antibody.  (a) Cells were placed in a Transwell chamber for the migration assay in the presence of 25 μg/ml anti-Wnt5a (5a-2 or 5a-5) or anti-GST antibody in the upper chamber.  (b) Cells treated with anti-Wnt5a or anti-GST antibody for 24 hours were applied to a matrigel-coated modified Boyden chamber for the invasion assay.  Relative migration or invasion activity was expressed as a percentage of migrated or invading cell numbers of anti-GST antibody-treated cells.  Results are shown as means ± SE of three independent experiments.  *, P < 0.05.  D, Migration (a) and invasion (b) activities of MKN-45/control cells or MKN-45 cells stably expressing Wnt5a (MKN-45/Wnt5a) were examined in the presence of pAb5a-5 or anti-GST antibody.  *, P < 0.05.  E, KKLS (a), MKN-1 (b), and MKN-45 (c) cells were subjected to the adhesion assay in the presence of pAb5a-5 or anti-GST antibody.  *, P < 0.05; **, P < 0.01.  F, KKLS (a) and MKN-1 (b) cells were cultured in the presence of pAb5a-5 (closed circles) or anti-GST antibody (open circles).

Figure 2  pAb5a-5 inhibits activation of Rac1 and expression of laminin γ2 in gastric cancer cells.  A, KKLS cells were stimulated with the indicated concentrations of Wnt5a for 1 hour in the presence or absence of 25 μg/ml pAb5a-5.  The cells were incubated with GST fusion Cdc42/Rac-interacting binding domain (CRIB) immobilized on glutathione-Sepharose.  The total lysates (total Rac) and precipitates (active Rac) were probed with anti-Rac1 antibody.  Band intensities were quantified using NIH image software.  Values in control cells without Wnt5a stimulation were set to 100%.  Results are shown as means ± SE of three independent experiments.  B, TMK-1/control (n=4) and TMK-1/laminin γ2-knockdown (KD) (n= 5) cells were injected into the spleens of nude mice.  Photographs representing liver metastases (a)
and numbers of metastatic nodules per mouse (b) are shown. Scale bars, 5 mm. *, $P < 0.05$. C, LAMC2-Luc was transfected into MKN-1 (a), TMK-1 (b), and MKN-45 (c) cells with pEXV/Rac1V12 or pEXV/Rac1N17 in the presence or absence of 150 ng/ml Wnt5a. Relative laminin $\gamma_2$ promoter activity was expressed as fold increases compared with the luciferase induction in control cells. D, MKN-1 (a) or MKN-45 (b) cells were treated with 150 ng/ml Wnt5a for 8 (left panels) or 4 (right panels) hours in the presence or absence of pAb5a-5 or anti-GST antibody. Left panels, ECM fractions from cells were probed with anti-laminin $\gamma_2$ antibody. The lysates of these cells were probed with anti-$\beta$-actin antibody. Right panels, LAMC2 mRNA level was quantified. Relative mRNA levels were expressed as percentages of LAMC2 mRNA levels in anti-GST antibody-treated cells. *, $P < 0.05$. E, MKN-1 cells were treated with 150 ng/ml Wnt5a for 8 hours in the presence of pAb5a-5 or anti-GST antibody and then stained with anti-laminin $\gamma_2$ antibody. Scale bars, 20 $\mu$m.

Figure 3 pAb5a-5 inhibits receptor-mediated endocytosis. A, FLAG-Fz2 was incubated with 200 ng/ml Wnt5a for 2 hours in the presence of 25 $\mu$g/ml pAb5a-5 or anti-GST antibody or 480 ng/ml sFRP2, and then the complexes were precipitated. The precipitates were probed with pAb5a-5. Results are representative of three independent experiments. B, KKLS cells were stimulated with 25 ng/ml Wnt5a for 1 hour in the presence of pAb5a-5, anti-GST antibody, or sFRP2, and then the lysates were probed with anti-Ror2 antibody. C, KKLS cells expressing FLAG-Fz2 were treated with 50 ng/ml Wnt5a for 30 minutes in the presence of pAb5a-5 or anti-GST antibody. Left panel, confocal images; right panel, quantification of internalized FLAG-Fz2. Internalization of FLAG-Fz2 was estimated as described in Supplementary Data. Scale bars, 20 $\mu$m. D, (a) KKLS cells were treated with 50 ng/ml Wnt5a for the indicated periods in the presence of pAb5a-5 or anti-GST antibody. After cell surface biotinylation, the lysates were precipitated with NeutrAvidin agarose resin. Left panel, the precipitates (cell surface Ror2) and lysates (total Ror2) were probed with anti-Ror2 antibody. Right panel, the amounts of cell surface Ror2 were quantified using NIH image software. Values at zero time in the presence of anti-GST antibody were set to 100%. (b) KKLS cells were treated with the indicated concentrations of Wnt5a for 15
minutes in the presence of pAb5a-5 or anti-GST antibody. Values at no Wnt5a in the presence of anti-GST antibody were set to 100%. E, (a) KKLS cells expressing FLAG-Fz2 were treated with 50 ng/ml Wnt3a for 15 minutes in the presence of pAb5a-5 or anti-GST antibody. (b) KKLS cells were treated with the indicated concentrations of Wnt3a for 30 minutes in the presence of pAb5a-5 or anti-GST antibody. After cell surface biotinylation, the lysates were precipitated with NeutrAvidin agarose resin. The amounts of cell surface LRP6 were quantified. F, MKN-45 and TMK-1 cells, which were treated with control or clathrin siRNA, were stimulated with or without 150 ng/ml Wnt5a for 4 hours, and then LAMC2 mRNA levels were quantified. Relative mRNA levels were expressed as fold increases compared with LAMC2 mRNA levels in control cells without Wnt5a stimulation. *, P < 0.05. KD, knockdown.

Figure 4 pAb5a-5 recognizes Wnt5a secreted from KKLS cells. KKLS cells transfected with (B-D) or without (A) pPGK2-neo/Wnt5a were grown in the matrigel for 36 hours. After the cells were incubated with anti-Wnt5a (A and B, pAb5a-5; C, pAb5a-2) or anti-GST (D) (green) and anti-integrin β1 antibodies (A-D) (red), the cells were viewed using a confocal microscope. Scale bars, 40 μm.

Figure 5 pAb5a-5 inhibits metastases in vivo. A, KKLS cells (2.5 x 10⁵ cells) in 50 μl HBSS (-) were injected into the spleen of nude mice. (a) PBS, pAb5a-5, or anti-GST antibody (10 μg/g) was injected into intraperitoneal cavity twice weekly for 5 weeks. Results are shown as means ± SE of seven to fourteen experiments. *, P < 0.05. (b) Photographs representing liver metastases (left, anti-GST antibody treatment; right, pAb5a-5 treatment). Scale bars, 5 mm. B, MKN-45 cells (2.5 x 10⁶ cells) in 50 μl HBSS (-) were injected into the spleen of nude mice. (a) The numbers of metastatic liver tumors were counted. Results are averages of two experiments. (b) Photographs representing liver metastases. C and D, Relative migration (C, a), invasion (C, b), or metastatic (D) activity of
KKLS cells treated with pAb5a-5 generated by five different rabbits (#1-#5) was expressed as percentage of KKLS cells treated with anti-GST antibody. In migration and invasion experiments \textit{in vitro}, results are shown as means ± SE of three independent experiments for each antibody. In metastatic experiments \textit{in vivo}, results are shown as averages of two or three independent experiments for each antibody except for #4 and #5. ND means no metastatic nodule in the liver. PBS treatment, n=14; anti-GST antibody treatment, n=7; pAb5a-5 treatment, n=10 (#1 (n=2); #2 (n=3); #3 (n=3); #4 (n=1); #5 (n=1)).
Fig. 2. Hanaki, H. et al.

A

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>pAb5a-5</th>
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</thead>
<tbody>
<tr>
<td>Wnt5a (ng/ml)</td>
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<td>25</td>
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<tr>
<td>Active Rac</td>
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<td></td>
</tr>
<tr>
<td>Total Rac</td>
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</tbody>
</table>

B

a

TMK-1/control  TMK-1/ laminin γ2 KD

b

Metastatic nodules numbers

C

a

MKN-1

Relative luciferase induction (Fold increase)

b

TMK-1

Relative luciferase induction (Fold increase)

Rac1 V12  Rac1 N17

Rac1 V12  Rac1 N17

Rac1 V12  Rac1 N17

Rac1 V12  Rac1 N17

D

a

MKN-1

PBS  GST Ab  pAb5a-5

Wnt5a  -  +  +

Laminin γ2  mRNA levels (%)

β-actin

b

MKN-45

PBS  GST Ab  pAb5a-5

Wnt5a  -  +  +

Laminin γ2  mRNA levels (%)

β-actin

E

MKN-1

Laminin γ2  Laminin γ2  Laminin γ2

(-) Wnt5a  GST Ab  pAb5a-5  (+) Wnt5a
**Fig. 5.** Hanaki, H. et al.

**A.**

- **a.** KKLS
- **b.**
  - GST Ab
  - pAb5a-5

**B.**

- **a.** MKN-45
- **b.**
  - GST Ab
  - pAb5a-5

**C.**

- **a.**
  - GST Ab
  - pAb5a-5

**D.**

- **a.**
  - GST Ab
  - pAb5a-5

**Relative migration (%)**

- **b.**
  - GST Ab
  - pAb5a-5

**Relative invasion (%)**

- **D.**
  - GST Ab
  - pAb5a-5

**Metastatic nodule numbers**

- **b.**
  - GST Ab
  - pAb5a-5

**Scale bars:**
- GST Ab
- pAb5a-5

**Notes:**
- GST Ab: Glutathione S-transferase
- pAb5a-5: Antibody against protein
- KKLS: Cell line
- MKN-45: Cell line
- Relative migration and invasion percentages are shown for different samples.
- ND: Not determined.
Molecular Cancer Therapeutics

An Anti-Wnt5a Antibody Suppresses Metastasis of Gastric Cancer Cells in vivo by Inhibiting Receptor-Mediated Endocytosis

Hideaki Hanaki, Hideki Yamamoto, Hiroshi Sakane, et al.

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