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Cancer Research

Agr2 Mediates Paracrine Effects on Stromal Fibroblasts That Promote Invasion by Gastric Signet-Ring Carcinoma Cells

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Abstract

Agr2 is a disulfide isomerase residing in the endoplasmic reticulum (ER), which physiologically regulates protein folding and mediates resistance to ER stress. Agr2 is overexpressed in adenocarcinomas of various organs, where it participates in neoplastic transformation and metastasis, therefore acts as a pro-oncogenic protein. Besides its normal localization in the ER, Agr2 is also found in the serum and urine of cancer patients, although the physiological significance of extracellular Agr2 is poorly understood. In this study, we demonstrated that extracellular Agr2 can activate stromal fibroblasts and promote fibroblast-associated cancer invasion in gastric signet-ring cell carcinoma (SRCC), where Agr2 is highly expressed. Agr2 secreted from SRCC cells was incorporated by the surrounding gastric fibroblasts and promoted invasion by these cells. In turn, activated fibroblasts coordinated the invasive behavior of fibroblasts and cancer cells. Our findings suggested that Agr2 drives progression of gastric SRCC by exerting paracrine effects on fibroblasts in the tumor microenvironment, acting also to increase the growth and resistance of SRCC cells to oxidative and hypoxic stress as cell autonomous effects. *Cancer Res; 75(2); 356–66. ©2014 AACR.*

Introduction

Gastric cancers are histologically classified as intestinal or diffuse type of adenocarcinomas (1). The latter type comprises poorly differentiated cancers, including variant subtypes such as signet-ring cell carcinoma (SRCC; refs. 2, 3). SRCCs are mucus-producing adenocarcinomas that represent approximately 15% of all primary carcinomas of the stomach (4, 5). In SRCC cells, the mucus is retained in the cytoplasm, resulting in a characteristic cell morphology in which a large vacuole full of mucin displaces the nucleus to the periphery. When gastric SRCC metastasizes, it tends to disseminate to the peritoneum and develop lymphatic invasion. Although SRCC has a variable prognosis, it is frequently accompanied with scirrhous gastric cancer upon progression, which is associated with abundant fibrosis in the cancer tissue. Therefore, the interaction of gastric SRCC cells with stromal fibroblasts may provide the microenvironment suitable for the progression of SRCC.

Anterior gradient 2 (Agr2), which belongs to the protein disulfide isomerase family member, which contains a single cysteine thioredoxin-like motif (6–8). Agr2 is physiologically localized in endoplasmic reticulum (ER), and it regulates the expression of components of the ER-associated degradation signaling and plays a pivotal role in resistance to ER stress (9–11). In addition, Agr2 acts as an ER chaperone for the intestinal mucins MUC2, MUC1, and MUC5 (10, 12, 13). Among them, gastric SRCC cells express MUC1, which is a membrane-bound mucin that stimulates dysregulated cell proliferation by increasing receptor-mediated signal transduction (14–16). Although expression of Agr2 promotes growth and transformed phenotypes of cancer cells (17–19), the significance of Agr2 expression in SRCC, a mucus-producing adenocarcinoma, is not well understood.

Agr2 has a unique carboxyl-terminal motif, KTEL. This motif interacts with the receptor in ER membrane that binds proteins with the terminal KDEL ER retention sequence, leading to ER localization of Agr2 (20). In some types of cancer, however, Agr2 is also present in the extracellular space, serum, and urine (21, 22). Although Agr2 is known to exert angiogenic effect (23), the functions of extracellular Agr2 are not as well characterized as the protein's roles in the ER.

In this study, we investigated the novel functions of Agr2 in gastric SRCC using two cell lines, Tu-katoIII and HSC-39. Our findings revealed that Agr2 secreted from these SRCC cells is incorporated by the surrounding stromal fibroblasts and activates invasive properties in those cells, which in turn promotes the

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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coordinated invasion by cancer cells and fibroblasts. In addition, Agr2 directly activates gastric SRCC cells by stimulating cell proliferation and increasing resistance to oxidative stress and hypoxia. Thus, Agr2 contributes to progression of gastric SRCC via cell-autonomous functions in cancer cells and paracrine effects on stromal fibroblasts. Together, these effects create a suitable microenvironment for cancer spreading. Consequently, extracellular Agr2 may be a suitable therapeutic target for preventing progression of gastric SRCC.

Materials and Methods

Cell culture

Cancer-associated fibroblasts (CAF) from the tumoral gastric wall and normal fibroblasts from the nontumoral gastric wall were established (24) and cultured in DMEM containing 4,500 mg/mL glucose, 1 mmol/L sodium pyruvate, and 10% FBS. The gastric cancer cell line Tu-katoIII was established by culturing cancer cells isolated from mouse tumors following implantation of KATO III cells and subsequent subcutaneous injection of the cultured cells into nude mice (25). Gastric cancer cell line HSC-39 was established previously (26). 44As3 and 58As9 were derived from patients with scirrhous gastric carcinoma (27), and MKN-28 and MKN-74 were derived from patients with intestinal type gastric carcinoma (28). KATO III, MKN-28, and MKN-74 were obtained via the Health Science Research Resources Bank. All cancer cells were cultured in RPMI1640 containing 10% FBS. To produce viral particles, recombinant lentiviral plasmids were cotransfected along with packaging vectors into 293T cells. After viral infection, Tu-katoIII and HSC-39 cells stably expressing Agr2 miRNA and MKN-74 cells expressing Agr2HA were established by selection in medium containing puromycin (1 μg/mL). Selected cells were collected and used in bulk for most experiments. Stable add-back of Agr2 to cells expressing Agr2 miRNA was established through hygromycin selection (400 μg/mL).

3D gel invasion assay

Gel invasion assays were performed as described previously (29). Briefly, gel containing type IP-collagen (Nitta Gelatin) and Matrigel (BD Biosciences) was overlaid onto the top chambers of Transwells in 24-well plates. Fibroblasts and cancer cells were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyllindo-carbocyanine perchlorate (DiI) and 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO), respectively (Invitrogen). Cells were mixed (1.5×10^4 cells each), and placed on the gels in medium containing 0.2% FBS. The bottom compartments were filled with medium containing 10% FBS. After incubation for 7 days, the gels were fixed and vertically cut into 200-μm slices using LinearSlicer (Dosaka EM). Labeled cells were visualized using a confocal microscope (LSM510, Zeiss). The area of invading cells was quantitated using the ImageJ software (NIH, Bethesda, MD; ref. 29). Invasion index (I) was calculated as the ratio of the area of the tested cells to the area of the control cells.

In vivo tumor transplantation

All protocols for animal experimentation were approved by the Committee for Ethics of Animal Experimentation, and the experiments were conducted in accordance with the guidelines for animal experiments at Akita University. Tu-katoIII cells (1.5×10^6) were injected into the subcutaneous tissue of 6-week-old BALB/c nude mice (CLEA Japan, Inc.). The mice were sacrificed 15 days after subcutaneous injection. Peritoneal dissemination of tumors was tested by intraperitoneal injection of Tu-katoIII cells (5×10^6) suspended in 300 μL of medium. The mice were sacrificed 60 days after injection. Invasion into the gastric wall of tumors was tested by submucosal injection of mixtures of DiO-labeled tumor cells and DiI-labeled fibroblasts (2×10^5 each), suspended in 30 μL of medium, into 6-week-old BALB/c nude mice (29). Ten mice were used for each group, and stomachs were resected 14 days after injection. The area of invading cells was detected using a fluorescence dissection microscope (Olympus) and quantitated using the ImageJ software.

Specimens from patients with cancer

SRCC specimens were obtained from 30 patients who had undergone resection of primary gastric tumors. None of the patients had undergone preoperative radiation or chemotherapy. All samples diagnosed as SRCC were collected from the surgical pathology files at Akita University Hospital (Akita, Japan), between 2008 and 2013 and tissues were obtained with the informed consent of the patients. Clinicopathologic findings from these patients are summarized in Supplementary Table S1. Pathologic diagnoses and classification followed the International Union Against Cancer tumor–node–metastasis classification (30), and the Japanese Classification of Gastric Carcinoma (31).

Immunohistochemical analysis

Tumor tissues of nude mice were fixed and embedded in paraffin. Paraffin blocks were sectioned and subjected to immunohistochemical staining using the Envision reagent (Dako). Antigen retrieval was performed using Target Retrieval Solution (Dako). As more than 80% tumor cells were positively stained in all cases, immunoreactivity was classified according to the intensity (Low, equal, or weaker than the intensity of noncancerous mucosa in the same patient; high, stronger than the noncancerous mucosa). Representative cases are shown in Supplementary Fig. S1.

Statistical analysis

Statistical significance was calculated using the Student *t* test. To assess the association between Agr2 expression levels and clinicopathologic parameters, Fisher exact tests were performed using the GraphPad Prism version 6.0 for Windows (GraphPad Software). Values of $P < 0.05$ were considered to represent statistically significant differences.

Apoptosis assays

Details are described in Supplementary Materials and Methods.

Results

Agr2 is highly expressed in gastric signet-ring cell carcinoma

To study the role of Agr2 in SRCC, we initially examined the expression of Agr2 in archives of human gastric carcinoma diagnosed as SRCC, using immunohistochemistry. Expression of Agr2 was detected in SRCC cells in all cases examined ($n = 30$). Agr2 staining was more intense in cancer tissue relative to areas of noncancer within the same patient, in which corpus neck or base of the antral glands was stained (67%, $n = 30$, Fig. 1A and B). Agr2 expression was significantly high in T3/4 stage ($P = 0.029$), lymphatic invasion ($P < 0.002$), and venous invasion ($P < 0.002$; Supplementary Fig. S1; Supplementary Table S1). In contrast, there was no significant correlation between Agr2 expression and lymph node metastasis and clinical stage.

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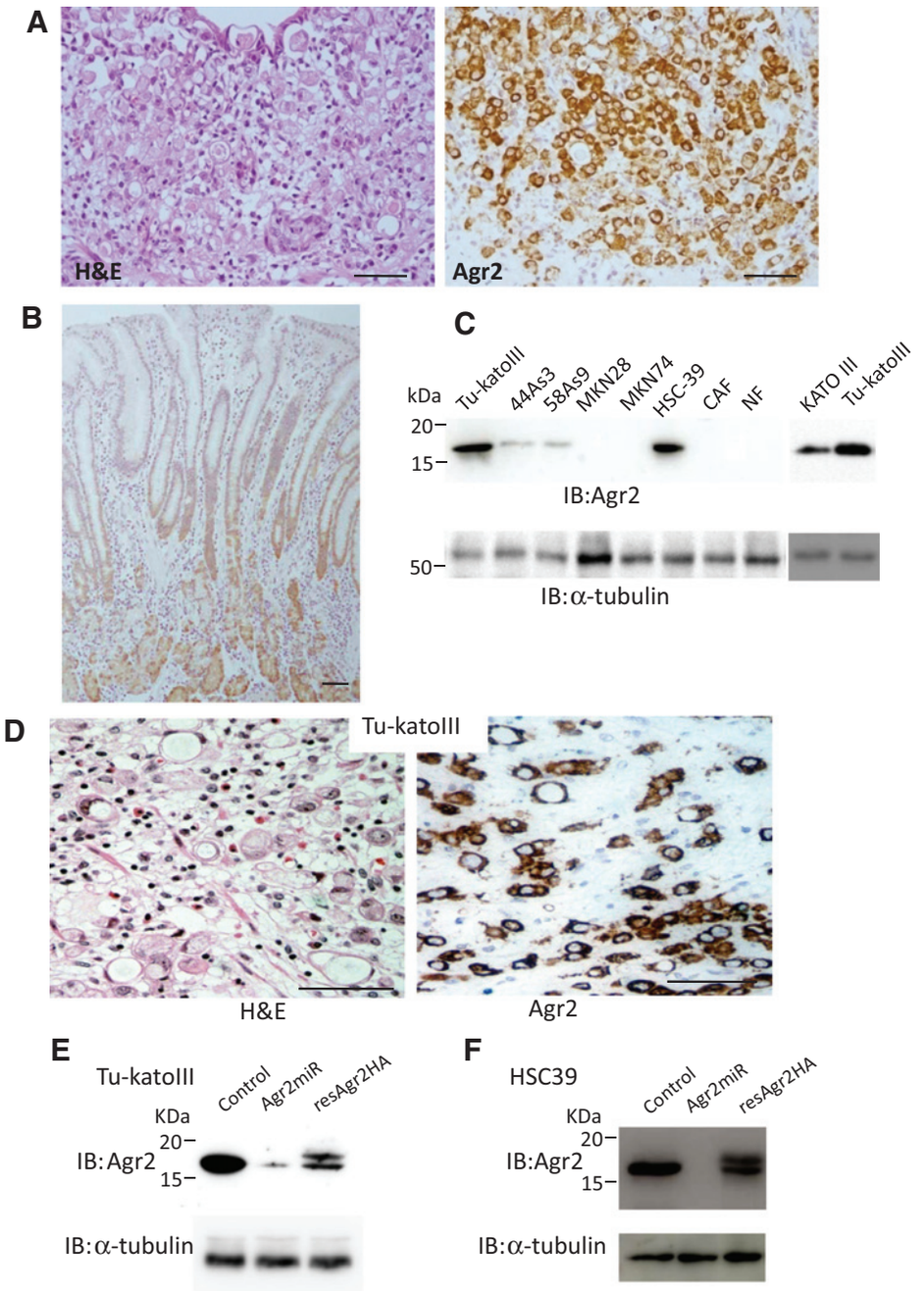

Figure 1.
A and B, Agr2 expression in gastric signet-ring cell cancer. Specimens of gastric SRCC were immunostained with rabbit anti-Agr2 antibody. B, lesion containing normal gastric mucosa. C, total cell lysates were prepared from various gastric cancer cell lines. CAF and normal fibroblasts (NF) were from the same patient. Lysates were subjected to immunoblotting with anti-Agr2 (mouse) and anti-α-tubulin antibodies. D, subcutaneous tumor of Tu-katoIII cells in nude mice, immunostained with anti-Agr2 antibody. Bar, 50 μm. E and F, Western blots of Agr2 in control Tu-katoIII cells, Agr2-knockdown cells (Agr2miR), and Agr2 knockdown cells in which HA-tagged Agr2 was reexpressed (resAgr2HA; E) and HSC-39 (F).

Among gastric cancer cell lines, Agr2 was expressed at high levels in Tu-katoIII and HSC-39, which are derived from SRCC, and low levels in several cell lines established from scirrhous gastric cancer (Fig. 1C). Tu-katoIII was established from parent KATO III cells, and has a high tumorigenic potential (24). The expression level of Agr2 was evidently higher in Tu-katoIII cells than in the parental KATO III cells (Fig. 1C). Tumors formed by Tu-katoIII cells in nude mice reflected typical features of human gastric SRCC (Fig. 1D).

To assess the effect of Agr2 on cancer progression, we generated Tu-katoIII and HSC-39 cell lines in which Agr2 was knocked down by a miRNA (Agr2miR). We then rescued the knockdown by transfection with miRNA-resistant Agr2, tagged with HA at the C-terminus (resAgr2HA; Fig. 1E and F). To generate miRNA-resistant Agr2, silent mutations were introduced into the Agr2 cDNA. The gross appearance of Tu-katoIII and HSC-39 cells did not change significantly as a function of Agr2 expression level.

Agr2 promotes growth and oxidative stress resistance of gastric SRCC cells

Next, we examined the biologic effects of Agr2 on Tu-katoIII and HSC-39 cells. First, we assessed whether Agr2 promotes the growth of these cells *in vitro*. Reduction of Agr2 expression in both

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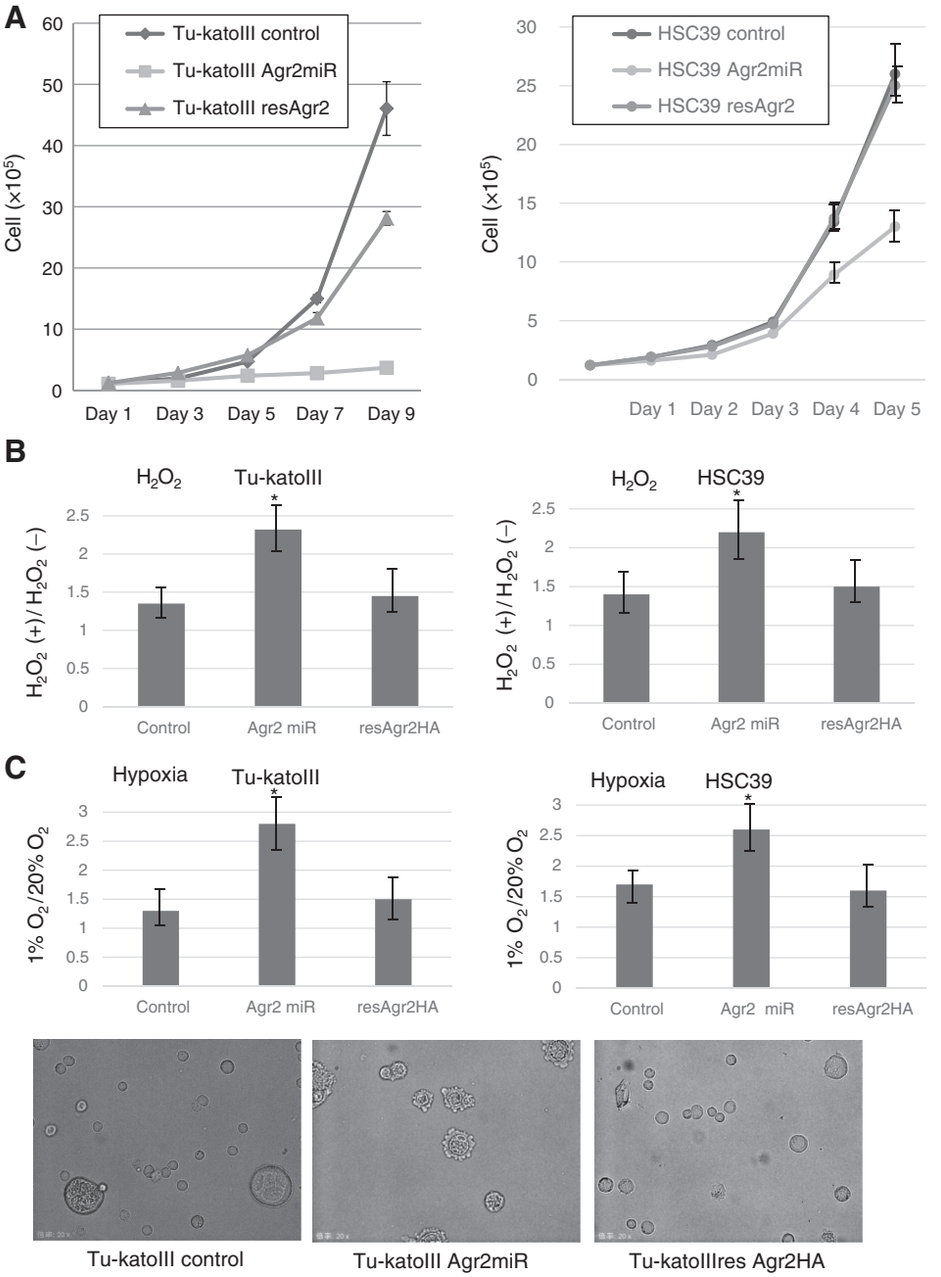

Figure 2.
Agr2 promotes growth and stress resistance of Tu-katoIII and HSC-39 cells. A, *in vitro* proliferation of Tu-katoIII or HSC-39 cells expressing various levels of Agr2 was evaluated by counting the cells under standard culture conditions. Data points indicate the average results from three dishes. B and C, apoptosis of control, Agr2miR, and resAgr2HA cells (Tu-katoIII or HSC-39) induced by treatment with hydrogen peroxide (B; 1 mmol/L H_2O_2, 14 hours) or hypoxic conditions (C; 1% O_2, 2 days). Relative apoptosis level was evaluated as the ratio relative to the untreated sample of the same cell line. The results from three independent experiments are shown as means ± SD. *, $P < 0.05$ by the Student *t* test. C, pictures of Tu-katoIII cells after exposure to hypoxia are shown at the bottom.

cell lines decreased cell growth, which recovered at least partially upon reexpression of Agr2 (Fig. 2A).

Agr2 acts as a disulfide isomerase and contains a thioredoxin-like motif, which suggests that it has redox activity (6). Furthermore, expression of Agr2 is regulated by the hypoxia-induced factor-1 (HIF1) transcriptional complex, and Agr2 mRNA levels are elevated in hypoxic condition (23). Therefore, we next investigated whether Agr2 affects the ability of cells to survive oxidative stress or hypoxia. Hydrogen peroxide–induced apoptosis of Tu-katoIII cells and HSC-39 cells was increased by knockdown of Agr2, but diminished by restoration of Agr2 expression (Fig. 2B). Similarly, apoptosis of Tu-katoIII cells and HSC-39 cells under hypoxic conditions was increased by knockdown of Agr2, but blocked by restoration of Agr2 expression (Fig. 2C). In cells exposed to 1% O_2, many blebs protruded from the cell membrane, indicative of apoptotic changes in Tu-katoIII Agr2miR cells, but not in control or Tu-katoIII resAgr2HA cells (Fig. 2C, bottom).

Agr2 is secreted from gastric SRCC cells and incorporated in fibroblasts

Because Agr2 is present in the serum and urine of some patients with cancer (21, 22), it must be secreted into the extracellular space. We detected Agr2 in conditioned medium of Tu-katoIII and HSC-39 cells, but not in the conditioned medium of Agr2miR cells (Fig. 3A). In addition, Agr2 was present in the CD81-positive

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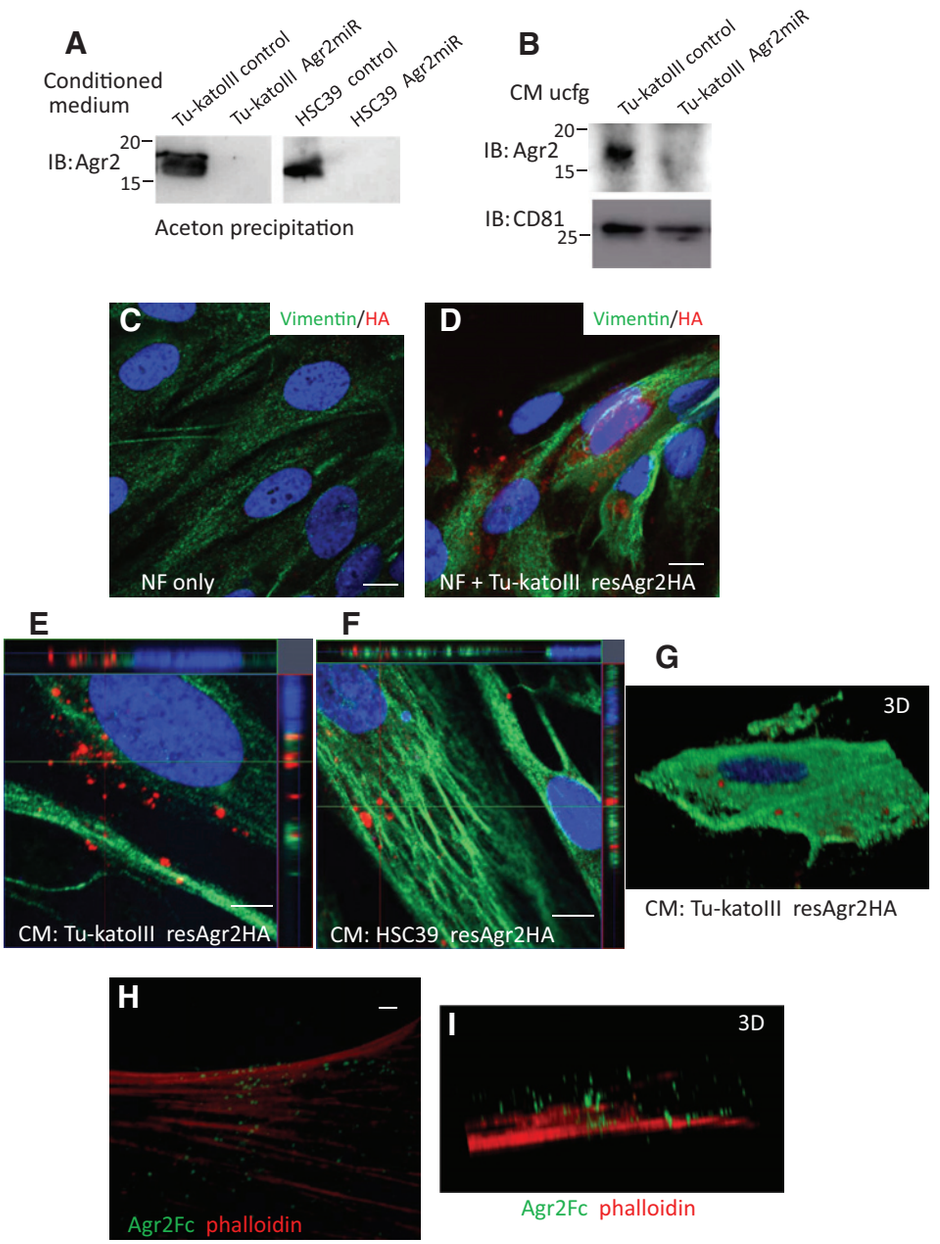

Figure 3.
Agr2 is secreted from gastric SRCC cells and incorporated into fibroblasts. A and B, cells were cultured in serum-free medium for 24 hours. A, protein in the conditioned medium was precipitated with acetone, and then subjected to immunoblotting with anti-Agr2 antibody. B, conditioned medium (CM) of Tu-katoIII cells was ultracentrifuged (ucfg) at 1×10^5 g for 2 hours. Precipitant was dissolved in sample buffer and immunoblotted with anti-Agr2 and CD81 antibodies. C and D, normal fibroblasts (NF) were cultured alone (C) or cocultured with Tu-katoIII resAgr2HA cells (D) for 8 hours. Cells were washed extensively, fixed, and immunostained with anti-HA (red) and vimentin (green) antibodies along with DAPI (blue). Cells were visualized by confocal microscopy. E–G, normal fibroblasts were incubated in CM of Tu-katoIII resAgr2HA cells (E and G) or HSC-39 resAgr2HA cells (F) for 8 hours. Normal fibroblasts were washed and fixed for immunostaining as in C and D. The corresponding computer-reconstructed vertical sections in *x*–*z* and *y*–*z* planes are added in E and F. C–F, bar, 5 μmol/L. G, computer-processed 3D image of an normal fibroblast cell is shown. H and I, normal fibroblasts were incubated with Agr2-Fc (4 μg/mL) for 6 hours and then fixed for immunostaining with Alexa Fluor 488-conjugated anti-mouse IgG Fc antibody (green). F-actin was stained with phalloidin (red). Bar, 2 μm. I, computer-processed 3D image rotated around 90° (lateral view of the cell) is shown. Particles of Agr2-Fc were incorporated and distributed at various depths in the normal fibroblast cell.

pellet fraction following ultracentrifugation of Tu-katoIII conditioned medium (Fig. 3B). These results suggest that Agr2 is secreted extracellularly, either as a soluble protein or as a cargo in membrane-coated microvesicles.

SRCC is often accompanied by scirrhous gastric cancer, which is characterized by prominent fibrosis of the stomach lesion. Therefore, to assess the effect of extracellular Agr2 on stromal cells, we focused on gastric fibroblasts. When Tu-katoIII cells expressing HA-tagged Agr2 (Tu-katoIII resAgr2HA) were cocultured with normal fibroblasts from the stomach, Agr2HA was detected in the cytoplasm of vimentin-positive normal fibroblasts (Fig. 3C and D). Agr2HA was also present in normal fibroblasts after they were incubated with conditioned medium from Tu-katoIII or HSC-39 cells expressing Agr2HA (Fig. 3E–G). Incorporation of Agr2 within fibroblasts was confirmed by images of vertical sections in the *x*--*z* or *y*–*z* planes (Fig. 3E and F). In three-dimensional (3D)-rendered images of normal fibroblast cells, particles containing HA-tagged Agr2 were detected in the cytoplasm network of vimentin-positive intermediate filaments (Fig. 3G). Moreover, when soluble recombinant Agr2, constructed by fusing Agr2 with the Fc region of mouse IgG2b, was added to the culture medium of normal fibroblast cells, Agr2Fc attached to fibroblasts and was incorporated in the cytoplasm (Fig. 3H and I). These results indicate that Agr2 secreted from cancer cells is incorporated by the surrounding fibroblasts. Stromal staining of Agr2 was also detected in some human SRCC specimens (Supplementary Fig. S2A). When such specimens were immunofluorescence stained with the antibodies against Agr2

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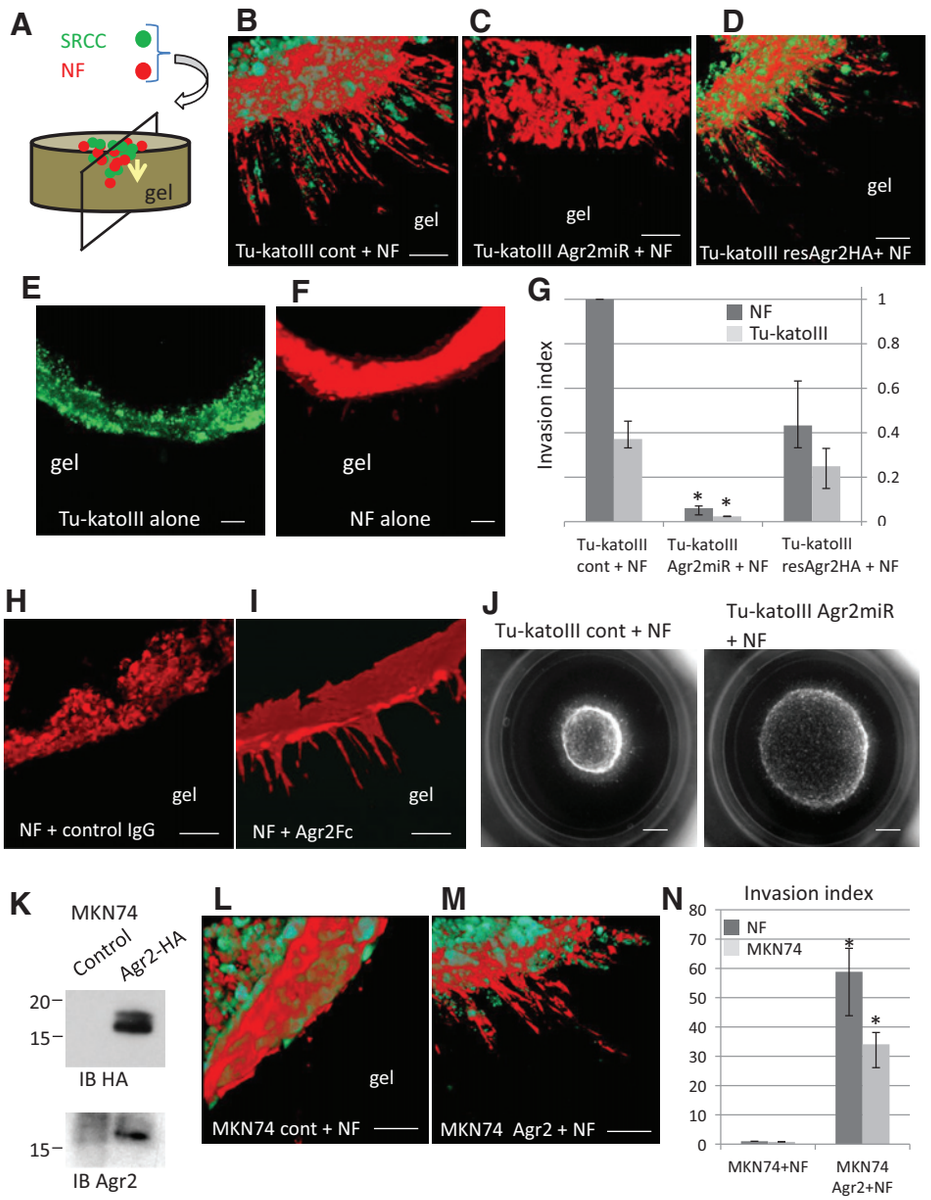

Figure 4.
Agr2 activates fibroblasts and causes coordinated invasion by fibroblasts and cancer cells. A, schematic representation of the experiment. Normal fibroblast (NF) and SRCC cells were labeled with DiI (red) and DiO (green), respectively, and subjected to 3D-gel invasion assay. B–D, normal fibroblasts, along with control (B), Agr2miR (C), or resAgr2HA (D) Tu-katoIII cells were plated on the gel and incubated for 7 days. E and F, only control Tu-katoIII cells (E) or normal fibroblasts (F) were assayed. Representative images of vertical sections of the gels. G, invasion index of each experiment is shown. The invading cells were quantitated as described previously (29). Invasion index was calculated as the ratio of the area of the tested cells to that of the control cells. The results from three independent experiments are shown as means ±SD. *, $P < 0.01$ by the Student t test. H and I, normal fibroblasts were treated with clustered control Fc or clustered recombinant Agr2-Fc (10 μg/mL). J, gels viewed from above. Pictures were taken 7 days after cells were seeded. K, Western blot analysis of control and Agr2HA-overexpressing MKN-74 cells with anti-HA and Agr2 antibodies. L and M, 3D gel-invasion assay of MKN-74 cells with normal fibroblasts as described above. The invasion index of each experiment is shown (N). *, $P < 0.01$ by the Student t test. Bar, 100 μm (B–F, H, I) or 1 mm (J).

and vimentin, Agr2-containing particles were scattered in the surrounding areas of vimentin-positive stromal fibroblasts (Supplementary Fig. S2B).

Extracellular Agr2 activates stromal fibroblasts and promotes coordinated invasion by fibroblasts and cancer cells

We next assessed the biologic function of extracellular Agr2. To understand the effects of Agr2 on invasion by fibroblasts and cancer cells, we performed 3D-gel invasion assays. In these experiments, we labeled normal fibroblasts and Tu-katoIII cells with distinguishable fluorescent dyes, and placed them on top of gels composed of extracellular matrix (Fig. 4A). Vertical sections of gels in which mixtures of normal fibroblasts and control Tu-katoIII cells were seeded revealed chains of cells protruding from the clumps of mixed cells and invading the gel (Fig. 4B). In contrast, mixtures of normal fibroblasts and Tu-katoIII Agr2miR cells did not invade the gels (Fig. 4C). Restoration of Agr2 expression in Tu-katoIII Agr2miR cells increased the invasiveness of both cancer cells and normal fibroblasts (Fig. 4D). When the cell lines were plated alone, neither normal fibroblasts nor Tu-katoIII invaded the gels (Fig. 4E and F). Similar results were obtained with another SRCC cell line, HSC-39 (Supplementary Fig. 3A–C). The area of protrusions in the gel was measured, and the mean invasion index is summarized. Fibroblasts were more invasive than cancer cells of both the Tu-katoIII and HSC-39 lines; within the mixtures, invasion by fibroblasts preceded invasion by cancer cells (Fig. 4G and Supplementary Fig. S3D). On the other hand, 44As3 and 58As3 cells, which express Agr2 at very low level, did not invade into the gel when they were mixed with normal fibroblasts (Supplementary Fig. S3E). These results indicate that a certain

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level of Agr2 is required to induce the fibroblasts to promote invasion.

Next, we investigated whether recombinant Agr2 activates normal fibroblasts. Incubation of normal fibroblasts with Agr2-Fc increased the invasion of normal fibroblasts into the gel, whereas control Fc protein did not (Fig. 4H and I). These results indicate that Agr2 secreted from Tu-katoIII cells activates fibroblasts, leading to coordinated invasion by fibroblasts and cancer cells.

The activation of normal fibroblasts by Tu-katoIII cells was also evident when the gels were viewed from above. Mixtures of normal fibroblasts and control Tu-katoIII cells contracted the gel, whereas such contraction was prevented by Agr2 knockdown in Tu-katoIII (Fig. 4J). As we observed previously (29), the activated fibroblasts contracted the gel surface and collected at the center of the gel, and the contractive ability of fibroblasts largely correlated with their invasive properties.

We then investigated whether overexpression of Agr2 in cancer cells accelerates invasion of surrounding fibroblasts. MKN-74, a gastric cancer cell line derived from intestinal type adenocarcinoma, does not express Agr2 (Fig. 1C). We stably overexpressed Agr2 with a carboxyl-terminal HA (Agr2HA) in MKN-74 cells, and subjected these cells to 3D-gel invasion assays (Fig. 4K). As expected, mixtures of normal fibroblasts and MKN-74 expressing Agr2HA exhibited more invasion by both normal fibroblasts and cancer cells than the mixture of normal fibroblasts and wild-type MKN-74 (Fig. 4L–N). On the other hand, expression of Agr2HA did not significantly increase the invasion by MKN-74 cells when the cancer cells were plated alone on the gel (Supplementary Fig. S4A). Taken together, these results suggest that Agr2 secreted from cancer cells promotes invasion of normal fibroblasts, which in turn leads to coinvasion by normal fibroblasts and tumor cells.

Because either coculture of normal fibroblasts with Tu-katoIII cells or addition of recombinant Agr2 protein activated the invasion of normal fibroblasts, we investigated whether expression of α-smooth muscle actin (α-SMA), a marker of activated fibroblasts or CAFs, is induced in normal fibroblasts by Agr2. In addition, we tested the effect of Agr2 on fibroblast proliferation. Expression of α-SMA was not induced in normal fibroblasts, and cell growth of normal fibroblasts was not affected after incubation with either Agr2-Fc or conditioned media from control Tu-katoIII cells (data not shown). Moreover, proliferation of MKN-74 cells was not statistically affected by overexpression of Agr2 (Supplementary Fig. S4B).

Agr2 promotes tumor growth and dissemination of gastric SRCC

To assess whether Agr2 affects the cell growth of gastric SRCC *in vivo*, we injected control, Agr2miR, or resAgr2HA Tu-katoIII cells subcutaneously into nude mice. The mice were sacrificed on day 15 after injection, and the tumors were resected and compared. The mean diameter of subcutaneous tumors derived from Tu-katoIII Agr2miR was reduced to one-eighth of that from control Tu-katoIII, and tumorigenicity was restored in the Agr2miR cells by re-expression of Agr2 (Fig. 5A–F). Histologic examination revealed that the density of cancer cells was reduced in tumors derived from Tu-katoIII Agr2miR (Fig. 5G–I).

Next, we assessed the effect of Agr2 on the progression of peritoneal dissemination of Tu-katoIII. When Tu-katoIII cells expressing various levels of Agr2 were injected intraperitoneally in nude mice, large tumors were observed in the abdominal walls of mice injected with control Tu-katoIII cells or Tu-katoIII resAgr2HA cells, whereas tumor size in the abdominal wall was reduced in mice injected with Tu-katoIII Agr2miR cells (Fig. 5J–L). The number of mice bearing tumor nodules larger than 2 mm in diameter were 10 of 10 (control Tu-katoIII), 0 of 10 (Agr2miR Tu-katoIII), and 8 of 10 (resAgr2HA Tu-katoIII). These observations indicate that Agr2 promotes the growth of SRCC *in vivo* and activates peritoneal carcinomatosis.

We next examined invasion of the gastric wall by mixtures of cancer cells and fibroblasts. Mixtures of Tu-katoIII cells and normal fibroblasts, labeled with distinguishable fluorescent dyes, were orthotopically injected into the submucosal space of mouse stomachs (29), and local spreading of cancer cells and fibroblasts in resected stomachs was evaluated by fluorescence microscopy. Spreading of Tu-katoIII Agr2miR cells was significantly reduced, and in some cases, undetectable (Fig. 6A, C, and E). Notably, the area of normal fibroblasts coinjected with Tu-katoIII Agr2miR cells was reduced compared with the area of normal fibroblasts coinjected with control or resAgr2HA Tu-katoIII cells (Fig. 6B, D, and F). These results suggest that invasion and spreading of normal fibroblasts was modified by Agr2 expression in coinjected cancer cells.

To confirm that Agr2 of cancer cells regulates the invasion of fibroblasts and leads to the expansion of tumor area *in vivo*, we examined tumor invasiveness of MKN-74, with or without overexpression of Agr2. When MKN-74 cells were injected alone in gastric wall, expression of Agr2 did not evidently affect their invasiveness (Fig. 6G and H). Mixtures of normal fibroblasts and control MKN-74, which does not express Agr2, did not significantly increase the spreading of cancer cells and fibroblasts (Fig. 6I and J). On the other hand, coinjection of normal fibroblasts with Agr2-overexpressing MKN-74 cells increased the invasiveness of both normal fibroblasts and cancer cells (Fig. 6K and L). Taken together, these results suggest that expression of Agr2 by cancer cells promotes fibroblast invasion *in vivo*, leading to the expansion of the tumor area consisting of cancer cells and fibroblasts.

Discussion

Agr2 is overexpressed in various cancer cells, and aberrant Agr2 expression in several tumor types predicts worse clinical outcomes (32–37). Previously, however, the functions of secreted Agr2 and its significance in the progression of gastric SRCC had not been extensively examined.

Agr2 contains a carboxyl terminal ER localization motif, KTEL, which is required for promotion of cancer cell growth, suggesting that Agr2 exerts its tumor-promoting effects from the ER (20). However, Agr2 is also present in the serum and urine of patients with cancer, suggesting that secreted Agr2 may also play roles in cancer progression. In our gel-invasion assay, expression of Agr2 in SRCC cells increased invasive properties of cocultured normal fibroblasts. Furthermore, recombinant soluble Agr2 (Agr2-Fc) increased invasion by normal fibroblasts. In turn, these fibroblasts led to coordinated invasion by fibroblasts and cancer cells.

Agr2 was detected in protein extracts of pellets following ultracentrifugation of Tu-katoIII conditioned medium, in which CD81, a marker of microvesicles, was also present. Therefore, extracellular Agr2 may be secreted as a soluble protein or released from cells in membrane-coated microvesicles. In this study, gel invasion by Tu-katoIII and HSC-39 did not depend on the direct effects of Agr2 on cancer cells, because neither control nor

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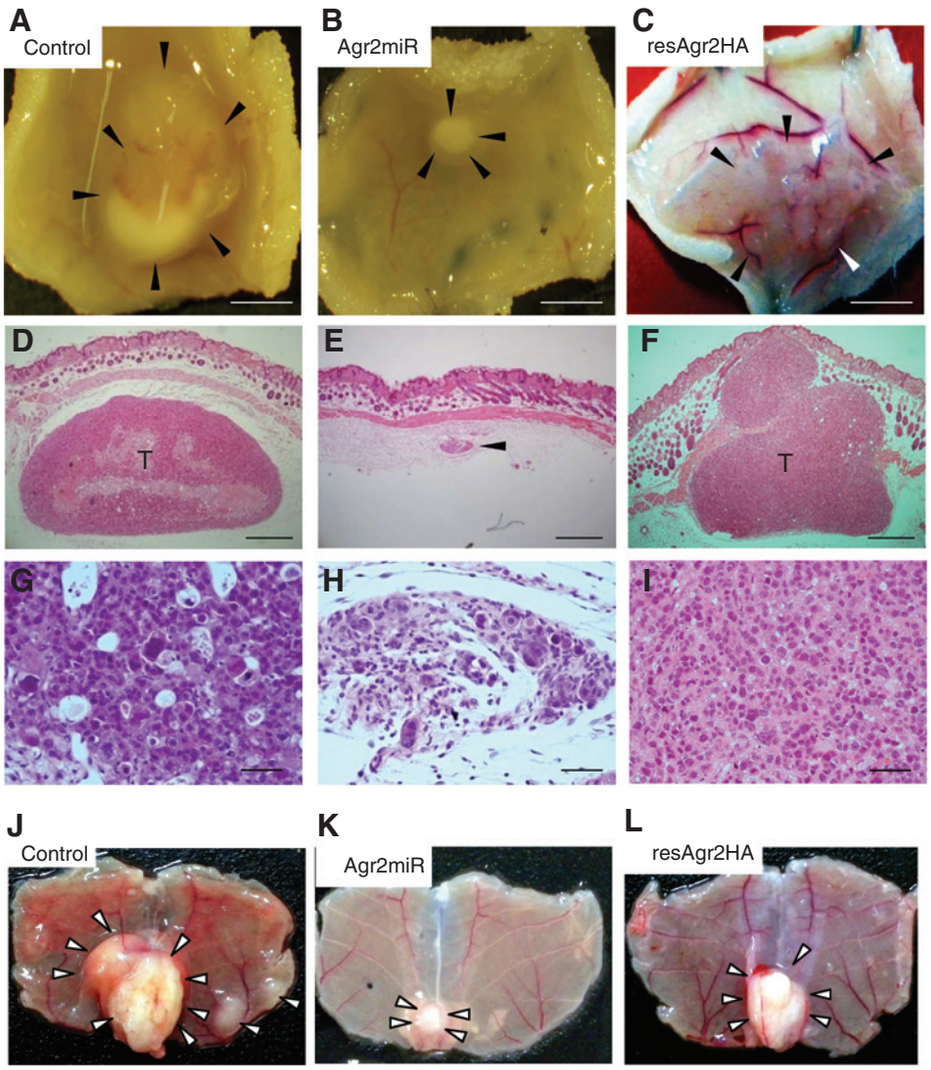

Figure 5.
Agr2 promotes tumor growth and peritoneal metastasis of SRCC. Control (A, D, G), Agr2 miR (B, E, H), or resAgr2HA (C, F, I) Tu-katoIII cells were injected subcutaneously in nude mice (1.5×10^6 cells). Ten mice for each group were analyzed, and representative tumors 15 days after injection are shown. A–C, bar, 1 mm. D–I, tumors were sectioned and subjected to H&E staining. Bar, 500 μm (D–F), 50 μm (G–I). J–L, Tu-katoIII cells expressing various levels of Agr2 (5×10^6 cells each) were injected into mouse peritoneal cavity ($n = 10$ for each group). Representative images of dissected abdominal walls 60 days after injection.

Agr2miR SRCC cells invaded the gel when they were seeded alone. In addition, overexpression of Agr2 in MKN-74 did not increase the invasiveness of these cells on its own. Therefore, elevated invasion by SRCC cells cocultured with normal fibroblasts largely depends on active invasion by fibroblasts. Previously, in a similar assay, we observed coordinated invasion by gastric scirrhous carcinoma cells and CAFs. Invasion by CAFs frequently preceded invasion by cancer cells, with the CAFs ultimately guiding the cancer cells. Gastric SRCC cells also followed the preceding fibroblasts in the gel-invasion assay performed in this study, suggesting that fibroblasts activated by extracellular Agr2 may lead to cancer cell invasion. High expression of Agr2 is correlated with metastasis of some types of cancer (33, 38). Thus, in addition to the cell-autonomous effects of Agr2 on cancer cells, fibroblast-associated cancer invasion mediated by extracellular Agr2 may play a pivotal role in cancer metastasis.

Expression of Agr2 in SRCC cells also affected invasion by fibroblasts *in vivo*. Knockdown of Agr2 in Tu-katoIII cells greatly decreased not only the growth of Tu-katoIII itself, but also the spreading of coinjected fibroblasts in the gastric wall. To further evaluate the significance of Agr2 on fibroblasts *in vivo*, we examined another cancer cell line, MKN-74, in which overexpressed Agr2 did not significantly affect growth *in vitro*. Expansion of the area infiltrated by normal fibroblasts was clearly increased by coinjection with MKN-74 Agr2HA. Similarly, spreading of MKN-74 Agr2HA cells was further increased by coinjection with normal fibroblasts, relative to that of MKN-74 Agr2HA alone. Therefore, Agr2-expressing cancer cells and fibroblasts invade cooperatively, and elevated fibroblast invasion was essential for infiltration of cancer cells *in vivo*.

Cancer cells are believed to "educate" the surrounding stromal cells, forcing them to adapt in a manner that provides a comfortable environment for tumor growth; however, the molecular mechanisms underlying this education are not well understood. In this study, we showed that Agr2 plays pivotal roles in the progression of two gastric SRCC cell lines, Tu-katoIII and HSC-39, and that extracellular Agr2 induces CAF-like invasive properties in stromal fibroblasts. In addition, in cell-autonomous effects, Agr2 promotes cancer cell proliferation and resistance to cellular stresses, including ROS and hypoxia. During the process of peritoneal carcinomatosis, cancer cells are exposed to both hypoxic conditions and oxidative stress. Thus, Agr2 may protect cancer cells

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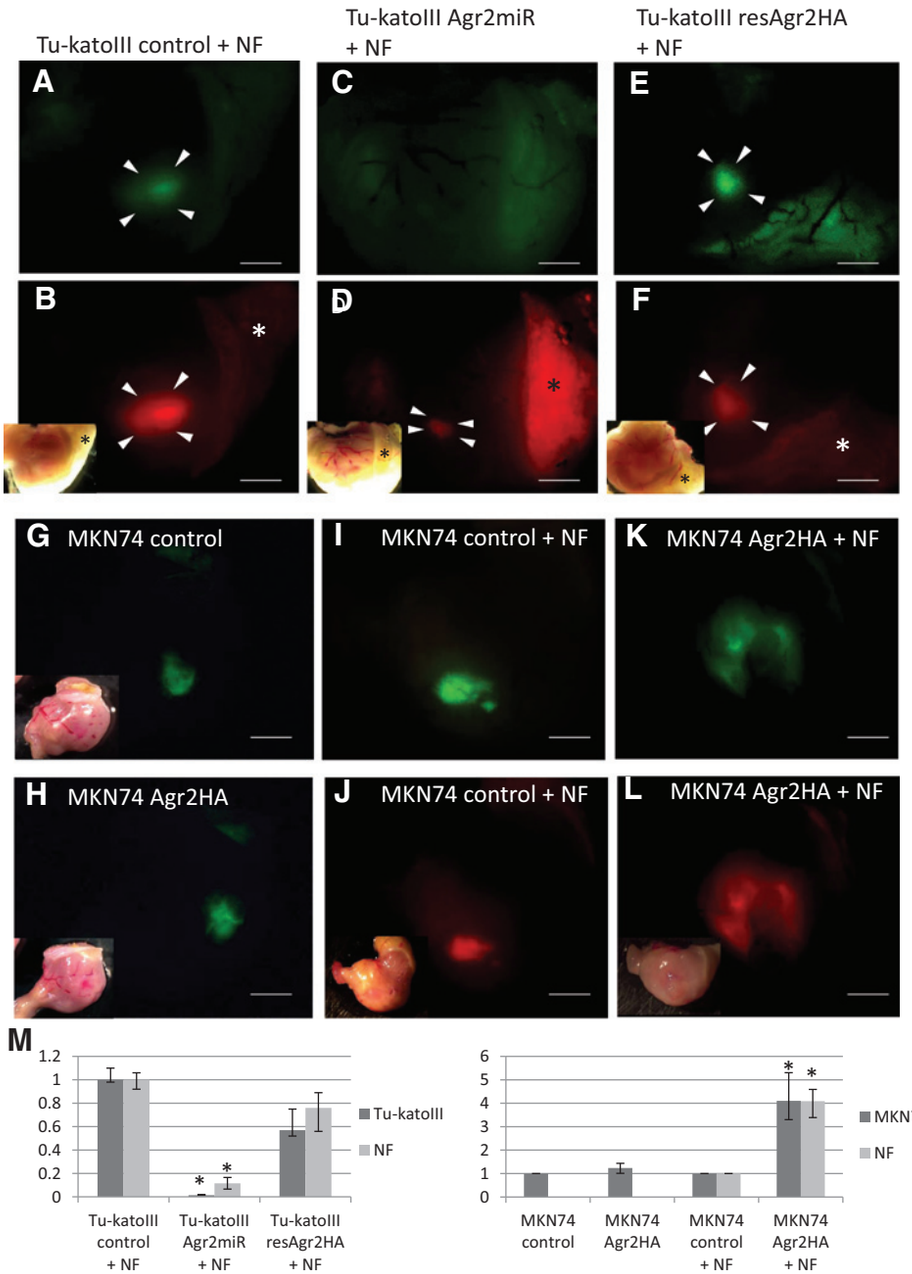

Figure 6.
Agr2 regulates coinvasion by fibroblasts and cancer cells *in vivo*. DiI-labeled normal fibroblasts (NF; red) and DiO-labeled cancer cells (green); Tu-katoIII cells (A–F) or MKN-74 cells (G–L) were mixed (2×10^5 cells each) and implanted into the submucosal layer of nude mouse stomachs. The stomachs were resected at day 14 and visualized using a fluorescence dissection microscope. Ten mice for each group were analyzed. Representative images are shown. Asterisk, position of the forestomach. Bar, 2 mm. M, the area of invading cells expressed as ratio of their area to that of control cells. Results are shown as means ±SD. *, $P < 0.05$ by the Student *t* test.

from both types of stress, thereby assisting in peritoneal dissemination.

It remains unclear why Agr2 overexpression did not increase the proliferation of MKN-74 cells. Indeed, the effect of Agr2 on cell proliferation is controversial. For example, overexpression of Agr2 in NIH3T3 cells promotes their proliferation (18), and Agr2 exerts a proproliferative effect in various cancers (17, 18, 39). However, proliferation of stem or progenitor cells in the gastric mucosa is elevated in Agr2-deficient mice (40). The ultimate effect of Agr2 on cell growth may depend on differences in the levels of Agr2 modulators or effector proteins in each cell, and the effects of Agr2 on cell proliferation and invasion may depend on separate signaling pathways. The N-terminal region, 21–40, is responsible for the ability of Agr2 to promote cell adhesion (6). Although we did not observe clear differences between control or Agr2miR Tu-katoIII cells in adhesion to normal fibroblasts, invasion by the mixtures of normal fibroblasts with MKN74 Agr2 Δ21–40, which lacks aa 21–40, was weaker than the mixtures of normal fibroblasts with MKN74 expressing wild-type Agr2 (Supplementary Fig. S5). Therefore, N-terminal region of Agr2 may be important for stimulation of cell invasion, and peptides or small molecules that interfere N-terminal function of Agr2 may become therapeutic. We cannot answer at present whether dimerization of Agr2 is critical to promote invasion of fibroblasts. As the peptide containing the dimerization motif 60-EALYK-64 of Agr2 destabilizes the oligomer *in vitro* (41), further examination of treatment of SRCC

cells with this peptide may be attractive. It is also important to investigate whether Agr2 can serve as a biomarker in serum or urine in patients with gastric SRCC.

Because Agr2 is expressed mainly in adenocarcinomas of various organs, it is possible that Agr2 secreted from cancer cells affects the biologic activity of stromal cells in general. Considering that signet-ring cells frequently exist in scirrhous-type gastric cancer, which is accompanied by marked fibrosis, Agr2 of SRCC cells may stimulate reacting fibroblasts and thereby establish the cancer microenvironment. Our findings elucidate a novel function of extracellular Agr2 as an activator of stromal fibroblasts that promotes fibroblast-associated cancer invasion. Therefore, extracellular Agr2 may represent a therapeutic target molecule for the development of drugs aimed at manipulating the cancer microenvironment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Tsuji, R. Satoyoshi, D. Maeda, A. Goto, M. Yashiro, M. Tanaka
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