New Blocking Antibodies against Novel AGR2-C4.4A Pathway Reduce Growth and Metastasis of Pancreatic Tumors and Increase Survival in Mice

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Abstract

Anterior gradient 2 (AGR2) promotes cancer growth, metastasis, and resistance to therapy via unknown mechanisms. We investigated the effects of extracellular AGR2 signaling through the orphan glycosylphosphatidylinositol-linked receptor C4.4A in pancreatic ductal adenocarcinoma (PDAC). Proliferation, migration, invasion, and apoptosis were measured using colorimetric, Boyden chamber, and FACS analyses. We developed blocking mAbs against AGR2 and C4.4A and tested their effects, along with siRNAs, on cancer cell functions and on orthotopic tumors in nude mice. Extracellular AGR2 stimulated proliferation, migration, invasion, and chemoresistance of PDAC cell lines. AGR2 interacted with C4.4A in cell lysates and mixtures of recombinant proteins. Knockdown of C4.4A reduced migration and resistance to gemcitabine. PDAC tissues, but not adjacent healthy pancreatic tissues, expressed high levels of AGR2 and C4.4A. AGR2 signaling through C4.4A required laminins 1 or 5 and integrin B1. Administration of antibodies against AGR2 and C4.4A reduced growth and metastasis and caused regression of aggressive xenograft tumors, leading to increased survival of mice. These data support a model in which AGR2 binds and signals via C4.4A in an autocrine loop and promotes the growth of pancreas tumors in mice. Blocking mAbs against AGR2 and C4.4A may have therapeutic potential against PDAC. Mol Cancer Ther; 14(4); 1–11. ©2015 AACR.

Introduction

Anterior Gradient 2 (AGR2) is expressed in a wide variety of tumors formed in different tissues with diverse patterns of genetic alterations, including pancreatic ductal adenocarcinoma (PDAC: ref. 1) and cancers of the breast (2, 3), prostate (4), lung (5), and colorectum (6). AGR2 supports aggressive growth and metastasis of a variety of cancer cells (7–9). Hence, understanding the function of AGR2 may serve as useful therapeutic target. However, little is known of the mechanisms through which AGR2 functions.

AGR2 [also called hAG-2 (2) or Gob-4 (10)] is the human ortholog of the Xenopus laevis, XAG-2. XAG-2 is secreted and takes part in ectodermal patterning of the frog embryo and in amphibian limb regeneration by interacting with the receptor Prod-1 of Ly6 superfamily (11–13). However, there is no human homologue of Prod-1. It is unknown whether AGR2 functions through a receptor on the cell surface or functions within cells in humans. The tissue distribution of AGR2 in healthy adult humans indicates that it is restricted to organs possessing mucin-producing cells. A mouse genetic deletion model of AGR2 showed alterations in mucin synthesis (14). Other studies have supported the concept that AGR2 possesses sequence similarity to the protein disulfide isomerase (PDI) family (15–18). A member of the PDI protein family may catalyze formation, reduction, and isomerization of disulfide bonds, thereby stabilizing intermediate conformations during protein maturation in the endoplasmic reticulum (17). However, a role of AGR2 in protein synthesis in normal cells does not resemble its actions in amphibians and also does not well explain its observed roles in cancer.

Previously, it was reported that AGR2 could bind to dystroglycan-1 (DAG-1) and C4.4A based on yeast two-hybrid results (3). However, no evidence was provided to support the interactions of these molecules in cells or the biologic function of these interactions. In the current study, we identified C4.4A (LYPD3) as the functional cell surface receptor for extracellular AGR2. Similar to Prod-1, C4.4A belongs to the Ly6 family of receptors, including CD59 (13) and uPAR (19), and is associated with increased metastasis of PDAC (20, 21), melanoma (22), and non–small cell lung cancer (23). AGR2 protein levels are correlated with poor prognosis in breast cancer (23) and colorectal cancer (24, 25). Furthermore, like other glycosylphosphatidylinositol (GPI)-linked plasma membrane receptors, C4.4A lacks intracellular domains to mediate its downstream signaling. To date, the signaling mechanisms engaged by C4.4A have not been identified.
To support the idea that an AGR2–C4.4A autocrine loop may be a therapeutic target against cancer, we developed novel mouse monoclonal blocking antibodies against both AGR2 and C4.4A. In vivo treatment with these antibodies significantly reduced PDAC tumor weight and metastasis and prolonged survival. These results suggest that further investigation of AGR2–C4.4A as a potential target for cancer therapy is warranted.

### Materials and Methods

Cell lines

NIH 3T3, BxPC3, SW86.86, MiaPaCa-2, AsPC-1, and Capan-2 cells were obtained from the ATCC. Cell line identities were verified using DNA fingerprinting (Powerplex1.6 system; Promega). Cells were routinely cultured in DMEM containing 10% FBS and were maintained at 37°C in a humidified atmosphere of 5% CO₂.

### Antibodies and recombinant proteins

Antibodies were purchased for AGR2 (mouse polyclonal), DAG-1, CD59 (cat. no. ab56703, ab105304, ab9182; AbCam), C4.4A, uPAR (cat. no. AF5428, MAB807; R&D Systems), laminin 1, 5, ITG β1, a6, and b4 (sc-74417, sc-20145, sc-9936, sc-10730; Santa Cruz Biotechnology), p-ERK (cat. no. 9160; Cell Signaling), b-actin (cat. no. A2066; Sigma), and control IgG (cat. no. OB010701; Southern Biotech). Human and mouse AGR2 proteins have 96% homology (26); therefore, human recombinant (rAGR2) (ab64013; AbCam) was used for all studies. Recombinant C4.4A was purchased (5428-C4-050; R&D Systems).

### Transient transfection of siRNA

The following predesigned and prevalidated siRNAs were purchased from Qiagen: siControl (cat. no. 1027281), siAGR2 (cat. no. SI04274522), sciC4.4A (cat. no. SI00105700,707,714,721), scitPAR (cat. no. SI03033289), scitCD59 (cat. no. SI03052616), laminin1 (α1) (cat. no. SI02779511), laminin 5 (β3) (cat. no. SI02664116), integrin a6 (cat. no. SI02654078), integrin b1 (cat. no. SI0300573), integrin b2 (cat. no. SI03648848), and integrin b4 (cat. no. SI02664109). Cells were transfected with siRNAs (5 or 10 nmol/L) with HiPerFect transfection reagent (cat. no. 301705; Qiagen), and lysates were prepared after 72 hours.

### Coimmunoprecipitation studies

Commercial AGR2 Ab (Abcam; 2 μg) was used to immunoprecipitate AGR2 from SW86.86 lysate (100 μg) using a Pierce Coimmunoprecipitation Kit (cat. #26149), and Western blot was conducted. The same membrane was then probed for each antibody individually (Supplementary Fig. S2) and with pooled antibodies. In addition, rAGR2 and rC4.4A were suspended together (2 μg each) in lysis buffer, and coimmunoprecipitation was conducted. IgG (mouse) served as control. Western blot imaging and processing was done with an Odyssey imaging system (LI-COR Biosciences).

### Cell growth, migration, invasion, and apoptosis assays

Wild-type and siRNA-transfected PDAC cells were grown with rAGR2 (0–500 nmol/L) in the presence or absence of Abs (polyclonal commercial or newly developed mAbs; 1 μmol/L). The medium was refreshed daily. Cell numbers were estimated after 48 hours by MTS assay as described previously (27). Proliferation is shown as percentage of viable cells over the appropriate control. To make the scales similar for easy comparisons, as well to avoid the differences in basal values for each cell line, the data are presented as the percentage of viable cells compared with the appropriate controls. Migration and invasion assays were conducted at 24 hours, as described previously (27). Apoptosis assays were conducted 72 hours after adding Gemcitabine (Gem) to Gem-sensitive BxPC-3 cells (1 μmol/L) and Gem-resistant AsPC-1 cells (5 μmol/L; ref. 28), as described previously (1). Because siRNA transfection itself was observed to increase basal apoptosis, cells transfected with siRNAs were treated with a lower concentration of Gem (BxPC3, 0.5 μmol/L; AsPC-1, 1 μmol/L).

### Immunohistochemical staining

IHC was performed on tissue microarray (TMA) slides with mAbs (1:1,000) and developed using the Vectastain Universal Kit (Vector Laboratories), as described previously (1). Results were blindly evaluated by pathologist (H. Wang), and expression levels were categorized as positive or negative (cytoplasmic staining of >10% or <10% of the tumor cells, respectively) and staining intensity as strong, moderate, or no staining. Apoptotic cells were detected in paraffin sections by fluorescence-labeled TUNEL staining (Promega; Cat # G3250). Activation of the Mapk pathway was evaluated by analysis of the levels of p-ERK (Cell Signaling Technology; Cat # 9160; 1:1,000), and the proliferative index of the cancer cells was measured by Ki-67 staining (Thermo Scientific; Cat # RM-9106-S0; 1:200).

### Blocking mAb development

Mouse mAbs against AGR2 and C4.4A were developed in the Monoclonal Antibody Core of UT MDACC using unconjugated antigenic peptides. Antigenic peptide sequence for AGR2 falls between 25 to 125 amino acids, and for C4.4A between 240 to 340 amino acids. Several hybridoma colonies (2,400 for each Ab) were screened for target recognition by ELISA using KHL-conjugated peptides. Selected hybridoma colonies were then cloned and screened for ELISA again to select those with the highest affinity. Selected clones were subcloned and purified using protein A columns. Validation of antibody specificity, blocking ability, and purity was conducted by Western blotting against recombinant and cell lysates proteins, functional screening by apoptosis assay, binding screening by ELISA assay, and analysis of the purity of selected Abs by SDS-PAGE. In vitro validation experiments with the selected antibodies included inhibition of cancer cell migration and invasion and ability to increase in Gem-induced apoptosis. Top candidate antibodies with high affinity and functional blocking ability, one each against AGR2 and C4.4A, were purified and further used to conduct in vivo experiments. Final selected clones were 28B for AGR2 and 1A for C4.4A. Antibodies were subtyped as IgG1 for AGR2 and IgG2b for C4.4A. Purified antibodies for in vivo experiments were produced in the Monoclonal Antibody Core.

### In vivo studies

In vivo experiments were conducted with athymic nude mice (B6.Cg-Foxn1nu/J—female—age 9 weeks; NCI) according to the UT MDACC regulatory standards and IUCAC committee approval. Orthotopic tumors were developed with luciferase-labeled cells (0.25 × 10⁶). IgG (cat. no. OB010701; Southern Biotech) served as a control Ab.

**Model 1: AsPC-1 aggressive cell model**

Two weeks after the injection of the aggressive AsPC-1 cells, when the tumors weighed less than 0.5 g (as surgically confirmed...
from a parallel untreated group), mice \((n = 6)\) were treated with control or mAbs \(5 \text{ mg each of AGR2–C4.4A mAb in combination/kg/body weight/twice a week/i.p.}\) and with or without Gem \((100 \text{ mg/kg/body weight/once a week/i.p.})\) until all of the control mice had died \((7 \text{ weeks})\). Tumor weight and metastasis to liver and lung were compared between control and treated groups \textit{ex vivo} at the end of the experiment.

Model 2: CaPan-2 stromal model

Two weeks after the injection of stroma-forming Capan-2 cells, mice \((n = 7)\) were treated with 15 mg AGR2 or C4.4A Ab/kg/body weight/twice a week/i.p. Treatment was stopped after 15 weeks \((13 \text{ weeks of treatment})\), and tumor size in surviving animals was monitored by bioluminescence until 63 weeks.

Model 3: CaPan-2 regression study

Four weeks after the injection of Capan-2 cells, when tumors weighed more than 1 g \(\text{as surgically confirmed from a parallel untreated group}\), mice \((n = 5)\) were treated with AGR2 or C4.4A Abs \(15 \text{ mg/kg/body weight/twice a week/i.p. or with both Abs in combination} \((7.5 \text{ mg each})\). Treatment was stopped after 12 weeks. Bioluminescence was monitored on surviving animals until 18 weeks. Tumor growth and metastasis were measured weekly with the IVIS live animal bioluminescence imaging system after injecting luciferin substrate \(\text{(Xenogen)}\). The number of mice surviving was recorded each week and shown as the percentage of the original group size.

Statistical analysis

All \textit{in vitro} experiments were conducted in triplicate and carried out on three or more separate occasions. Data presented are mean of the three or more independent experiments \(\pm \text{ SEM}\). \textit{In vivo} experiments were conducted with groups of 7 to 10 mice. Statistically significant differences were determined by ANOVA analysis \(\text{(Newman–Keuls multiple comparison test)}\) and were defined as a \(P\) value of <0.05.

Results

Extracellular AGR2 stimulates PDAC aggressiveness and chemoresistance \textit{in vitro}

We previously reported that AGR2 is highly expressed and secreted by PDAC cells and contributes to chemoresistance \(\text{(1)}\). In the current study, we assessed whether extracellular AGR2 \(r\text{AGR2})\) could mimic the effects of AGR2 expression. Because PDAC cell lines are heterogeneous, we used multiple cell models—BxPc-3 \(\text{(epithelial phenotype, sensitive to Gem)}\), AsPC-1, and MiaPaCa-2 cells \(\text{(mesenchymal phenotype, highly resistant to Gem) ref. 28)}\). Results from the AsPC-1 cells are shown throughout the article; similar results obtained with BxPC-3 and MiaPaCa-2 cells are provided in Supplementary Fig. S1.

In AsPC-1 cells, treatment with \(r\text{AGR2}\) increased proliferation \(\text{(3-fold)}\), migration \(\text{(10-fold)}\), and invasion \(\text{(3-fold)}\) in a concentration-dependent manner \(\text{(Fig. 1A–C)}\). Similar effects were observed with BxPC-3 and MiaPaCa-2 cell lines \(\text{(Supplementary} \text{Fig. S1A–S1C)}\). To determine the effects of \(r\text{AGR2}\) on cancer cell resistance to therapeutic agents, we treated PDAC cells with Gem in the presence and absence of \(r\text{AGR2}\). Although AsPC-1 cells are highly resistant, a significant 3-fold increase in apoptosis was induced at a concentration of 5 \(\text{nmol/L Gem} \text{(Fig. 1D)}\). Simultaneous treatment with \(r\text{AGR2}\) reduced the effect of Gem to nearly the control level \(\text{(}>50\% \text{ reduction)}\), demonstrating a strong survival effect. AGR2 treatment had even larger effects with BxPc3 cells, which are more Gem sensitive \(\text{(Supplementary Fig. S1D)}\). Thus, extracellular recombinant AGR2 recapitulated the effects on PDAC cells previously observed with AGR2 expression \(\text{(1)}\).

C4.4A is the functional receptor for AGR2

Candidate receptors for AGR2 were selected from the literature and examined for importance in AGR2 functions. Ly6 receptor family members \text{uPAR, C4.4A, and CD59} communoprecipitated with AGR2 \(\text{(Fig. 2A)}\), whereas \(\text{DAG-1 does not communoprecipitate} \text{ (Supplementary Fig. S2A–S2C for individual} \text{)}\).
immunoprecipitations). To determine the functional importance of each receptor, they were silenced using siRNAs and significant silencing was confirmed (Fig. 2B; see Supplementary Fig. S2D–S2F for full blots). Only silencing of C4.4A significantly reduced basal and nearly completely abolished rAGR2-stimulated cell proliferation, migration, and invasion in AsPC-1 cells (Fig. 3A–C) and BxPC-3 cells (Supplementary Fig. S3A–S3C). On the other hand, silencing of CD59 and uPAR significantly increased AsPC-1 cell migration.

C4.4A silencing also blocked AGR2-mediated chemoresistance to Gem (Fig. 3D and Supplementary Fig. S3D). Silencing of C4.4A alone, and in combination with Gem, resulted in significantly increased rates of apoptosis (2-fold), which was a greater increase than that observed with Gem treatment of control cells (Fig. 3D). Importantly, the ability of AGR2 treatment to protect cells from Gem was abolished after C4.4A silencing. To control for off-target effects, we examined four siRNA sequences for C4.4A, each of which showed comparable results (Fig. 3E). These data support the idea that the effects of extracellular AGR2 are mediated by interaction with C4.4A.

To determine whether AGR2 and C4.4A interact directly or only by association in a complex, rAGR2 and rC4.4A were combined in the absence of other proteins, and coimmunoprecipitation was conducted. Direct interaction between rAGR2 and rC4.4A was indicated by the presence of an obvious band in this assay (Fig. 2C). We also examined nine PDAC cell lines for C4.4A mRNA and protein expression and observed that it was present in all lines (see Supplementary Fig. S2G–S2I for full-length gels).

C4.4A requires integrin β1 and laminins 1 and 5 for activity

In light of previously identified signaling complexes of uPAR, a member of this receptor family (29), we investigated surface receptors, including integrins and extracellular matrix components, that might be involved in C4.4A signaling. C4.4A was reported to bind laminins 1 and 5, although the functional consequences were unknown (30). Hence, candidate integrins and laminins 1 and 5 were silenced, and AGR2-mediated Gem-resistance effects were assessed. Silencing of laminin 1, laminin 5, or integrin β1 completely abolished the protective effects of AGR2, whereas silencing of integrin β2, β4, or α6 had no effect (Fig. 4A). Similarly, commercial blocking antibodies to laminins 1, laminins 5, and integrin β1 also abolished AGR2-mediated stimulation of proliferation and chemoprotective effects (Fig 4B and C). Unfortunately, our efforts to identify the participating integrin α subunits did not provide definitive answers. Similar results for the BxPC-3 cell line are shown in Supplementary...
Fig. S4A and S4B. Taken together, these data suggest that laminins 1 and 5 and integrin β1 are involved in the AGR2–C4.4A receptor complex.

Developed AGR2 and C4.4A mAbs are highly specific and block the binding of AGR2 to C4.4A

To further understand the roles of AGR2 and C4.4A in cancer, we wished to block their interactions using antibodies. Unfortunately, commercially available antibodies, while recognizing AGR2 (18 kD) and C4.4A (50 kD; Fig. 5A), did not block AGR2-induced cell migration (Fig. 5B) or Gem resistance (Fig. 5C). Therefore, we developed novel AGR2 and C4.4A mAbs, which recognized their respective antigens and blocked their interactions (Fig 5A). The novel mAbs were more specific than the commercial antibodies, as indicated by the lack of nonspecific bands in Western blots of pancreatic cancer cell lysates. We also observed that the novel mAbs blocked AGR2 stimulation of cell migration and resistance to Gem, whereas the commercially...
available antibodies were without effects (Fig 5B and C). Antibody binding, functional screening, and purity testing of lead antibodies have been added in Supplementary Fig. S5A–S5G. Validation of antibody specificity, blocking ability, and purity was conducted by Western blotting against recombinant and cell lysates proteins, functional screening by apoptosis assay, binding screening by ELISA assay, and analysis of the purity of selected Abs by SDS-PAGE.

**AGR2–C4.4A are widely expressed in pancreatic cancer**

The expression patterns of AGR2 and C4.4A were also assessed in patient tissues (TMA) using the mAbs developed.

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**Figure 4.** Effects of AGR2 are mediated by C4.4A interacting with integrin β1 and laminin 1 or laminin 5. A, in SiControl AsPC-1 cells, Gem stimulated apoptosis and addition of rAGR2 inhibited this effect. AsPC-1 cells were also transfected with siRNAs against ITG-β1, ITG-β2, ITG-β4, ITG-α6, laminin 1, and laminin 5. Only silencing of laminins 1 and 5 and integrin β1 increased Gem-stimulated apoptosis and abolished the survival effects of AGR2. B, silencing of laminins 1 and 5 and integrin β1 by siRNA significantly abolished AGR2-mediated proliferation of AsPC-1 cells. C, commercially available blocking antibodies to ITG-β1, ITG-β2, ITG-β4, ITG-α6, laminin 1, and laminins 5 showed similar results as the siRNA treatments with only Abs to laminins 1 and 5 and integrin β1 blocking AGR2-mediated survival effects. Data, mean ± SEM for 3 experiments (*, P < 0.05).
Both antibodies showed strong labeling of PDAC, but normal pancreas was not labeled. For AGR2, 105 of 140 (75%) were positive with respective staining of 46% (high), 29% (moderate), and 25% (no staining). High levels of AGR2 (91%) were positive with respective staining of 52% (high), 39% (moderate), and 9% (no staining). These data confirm that AGR2 and C4.4A are both highly expressed in advanced PDAC. Both molecules tend to be expressed together as the correlation between the expression of AGR2 and C4.4A in patients with PDAC was significant ($P < 0.0001$, correlation coefficient 0.74, Spearman r).

**Inhibition of the AGR2–C4.4A autocrine loop provides potential therapeutic benefits**

To evaluate the potential therapeutic benefits of inhibiting the AGR2–C4.4A autocrine loop, we tested the effects of treatments with the blocking mAbs in preclinical models. In the aggressive cell model (model 1; Fig. 6A), we used AsPC-1, a highly tumorigenic, metastatic, and Gem-resistant cell line. We tested the effects of the combination of both mAbs with and without Gem. Mice were injected orthotopically with luciferase-expressing AsPC-1 cells, and tumors were allowed to form for 2 weeks before the start of treatments. After 4 weeks of treatment (6 weeks total), all mice in the control Ab group had died, and the other mice were sacrificed to compare tumor weights and metastasis. At that time, 30% of the mice treated with the control Ab in combination with Gem, 100% of the mice with the combination of AGR2 and C4.4A mAbs, and 80% of the mice with the combination of mAbs and Gem remained alive. Compared with the control Ab, combined mAb treatment reduced tumor weight by 33% ($P < 0.03$) and incidence of metastasis by 66% ($P < 0.05$; Fig. 7A and B). Combining Gem with the mAbs did not have a significant advantage, as this combination resulted in a reduction in tumor weight by 40% ($P < 0.003$) and incidence of metastasis by 50% ($P < 0.05$). As no substantial benefits were obtained in combination with Gem treatment in model 1, Gem treatment was not considered in models 2 and 3. Tumor volume data for group mice and individual mice are included in Supplementary Fig. S6A. Tumor volume was estimated weekly by bioluminescence imaging. Combo IgG with and without Gem showed reduction in tumor volume as compared with Control IgG with and without Gem. We also noticed that treatment with the mAbs did not reduce the animal’s body weight as compared with control Ab–treated mice, suggesting a lack of systemic toxicity associated with blocking this pathway.
Figure 6.

In vivo treatment with AGR2-C4.4A antibodies reduced tumor volume and improved survival. Tumor growth and metastasis were measured weekly with the IVIS live animal bioluminescence imaging system after injecting luciferin substrate (Xenogen). The number of mice that survived until the end of the experiment was noted (percent surviving). A representative image of mice showing reduction/regression in tumor volume is also shown for each group. A, model 1 (AsPC-1 aggressive model), 2 weeks after the injection of the aggressive AsPC-1 cells, when the tumors weighed less than 0.5 g (as surgically confirmed from a parallel untreated group), mice (n = 6) were treated with control or mAbs (5 mg each of AGR2-C4.4A mAb in combination/kg/body weight/twice a week/i.p.) and with or without Gem (100 mg/kg/body weight/once a week/i.p.) until 7 weeks. All mice treated with combined mAbs survived for at least 6 weeks, whereas all control mice perished within 6 weeks. B, model 2 (CaPan-2 stromal model), 2 weeks after the injection of stromal-forming Capan-2 cells, mice (n = 7) were treated with 15 mg AGR2 or C4.4A Ab/kg/body weight/twice a week/i.p. Treatment was stopped after 15 weeks (13 weeks of treatment), and tumor size in surviving animals was monitored by bioluminescence until 63 weeks. Treatment with either mAbs individually showed 24 weeks improvement in survival as compared with control mice that died by 9 weeks. Forty-eight weeks after no treatment, mice were allowed to survive until they died or were severely morbid. Median survival (the point at which 50% of the animals survived) was 6 weeks for the control Ab, 9 weeks for the AGR2 Ab, and 10 weeks for the C4.4A Ab (P < 0.05). After week 63, one mouse was surviving in each of the AGR2 Ab and C4.4A Ab groups. After sacrifice, these animals were examined and no evidence of tumor was observed. Tumor volume data for group mice and individual animals were included in Supplementary Fig S6B. Mean tumor volume changes indicated that mice treated with either mAb showed slower growth. Both AGR2 and C4.4A Ab treatments reduced the tumor volume by 50% compared with the control Ab (P < 0.05). Some mice (1/7 of AGR2 Ab treated; 3/7 of C4.4A Ab treated) showed complete tumor regression as indicated by bioluminescence imaging and confirmed by surgical examination. In several mice, tumors were observed to disappear.

Regression studies (model 3) were conducted on mice beginning 5 weeks after cancer cell implantation when tumors were more than 1 g size (Fig 6C). In this study, all mice in the control Ab group died 3 weeks after initiation of treatment (8 weeks total). At that time, 60% of each mAb-treated groups survived. Treatment with mAbs was discontinued after 12 weeks and the mice were allowed to survive until they died or were severely morbid. Median survival times were 8 weeks for control Ab-treated animals, 12 weeks for AGR2- or C4.4A-treated animals, and 11 weeks for animals treated with the combination of AGR2 and C4.4A mAbs (P < 0.05). Tumor volume data for group mice and individual mice are included in Supplementary Fig. S6C. Reduction in tumor volume for each treatment group is shown as measured by bioluminescence imaging. Control group mice showed an increase in tumor volume, whereas AGR2–C4.4A–combo Ab-treated mice showed regression in tumor volume. One of the 5 mice treated with AGR2 mAb showed complete regression of its tumor. Analysis of the residual tumor in surviving animals indicated a high level of apoptotic cells in mAb-treated groups (Fig. 7C and D). Analysis of p-ERK levels indicated that activity of this pathway was completely abolished in antibody-treated groups (Fig 7C). Analysis of the proliferation indicator, Ki-67, showed no...
staining on mAbs-treated groups (Fig. 7C). Similar results were also observed in models 1 and 2.

Discussion

AGR2 is associated with poor outcomes in several tumor types (26), but the mechanisms remain unknown. AGR2 has been reported to be involved in protein maturation and folding (14–16, 31), to regulate cathepsins (32) and to modulate MUC-1 levels (14, 33). However, these roles of AGR2 do not explain its ability to act as an oncogene (34), or its ability to increase the aggressiveness of several types of cancer. It is therefore likely that this protein has multiple intracellular and extracellular functions. Potentially, its physiologic and pathologic roles differ. In our current study, extracellular addition of rAGR2 stimulated the proliferation, migration, invasion, and chemoresistance of PDAC cells. These actions required the presence of cell surface receptors. Thus, based on these data, it seems likely that the important role of AGR2 in cancer is mechanistically similar to its roles in amphibians, where it is a secreted signaling molecule that interacts with a specific receptor.

In amphibians, AGR2 promotes limb growth by interacting with Prod1 (12, 13), a GPI-linked receptor related to the Ly6 family of receptors in humans (19, 35). The Ly6 family includes uPAR, C4.4A, and CD59 (19, 13). Our current study indicated that Lys family receptors (uPAR, C4.4A, and CD59) were coimmunoprecipitated with AGR2, likely because of the structural homologies between these receptors (19). Nevertheless, only blocking the interaction of AGR2 with C4.4A by silencing or blocking antibodies reduced endogenous (basal) and extracellular rAGR2-stimulated PDAC cell functions. Though it was reported that in a yeast two-hybrid system, Dystoglycan-1 bound to AGR2, our coimmunoprecipitation study could not verify this interaction. Surprisingly, we observed that silencing of other two receptors, CD59 and uPAR, slightly increased the migration of PDAC cells. This observation was
unexpected, as a previous report suggested that silencing of uPAR inhibited PDAC cell migration (36). It is unclear what accounts for this difference, but it may be due to the studies being conducted in different cell lines. Nevertheless, the data showed here support a model in which AGR2 and C4.4A participate in an autocrine loop that activates survival mechanisms.

In our previous gene profiling studies, C4.4A was found to be highly expressed in pancreatic cancer but not in normal or chronic pancreatitis tissue (37). C4.4A is an orphan receptor described previously as a regulator of cancer cells metastasis (21, 38). We have now identified for the first time C4.4A as a functional cell surface receptor for AGR2. Silencing or antibody-mediated blocking of C4.4A eliminated the effects of extracellular AGR2, thus supporting AGR2 as the ligand for C4.4A. However, the mechanism of action of C4.4A had not previously been investigated. Hence, we further examined the signaling complex molecules that interact with C4.4A to identify a few specific molecules.

Like other GPI-linked receptors, C4.4A does not have an intracellular domain to mediate downstream signaling mechanisms. On the basis of homologies between C4.4A and uPAR, another member of the Ly6 family, these interactions likely include extracellular matrix proteins and specific integrin receptors. Recent studies suggest that C4.4A promotes migration by associating with α6β4 (39). C4.4A was also previously reported to bind laminins 1 and 5, although functional studies were not conducted (30). Laminins 1 and 5 are thought to interact primarily with integrin α3β1 (40, 41). Integrin α3β1 is expressed by pancreatic ductal cells (42). We observed that silencing of laminin 1 or 5 or integrin β1 abolished the effects of AGR2 treatments, thus suggesting their involvement in AGR2-mediated C4.4A receptor complex. Further studies will be required to fully understand the signaling mechanisms involved in the actions of C4.4A.

To examine the potential therapeutic benefits of blocking the AGR2–C4.4A autocrine loop, we developed blocking mAbs against the ligand (AGR2) and the receptor (C4.4A). Both Abs blocked basal and AGR2-mediated functions. Preclinical studies using the blocking mAbs in different types of preclinical models resulted in significant reductions in tumor weight and metastasis and improved survival. We found that treatment with the mAbs had to better therapeutic benefits compared to Gem, the clinical standard of care for PDAC. Partial or complete regression of tumors was observed in several mice after mAb treatment individually or in combination, and even several weeks after treatment, no tumor recurrence was observed.

In summary, this study indicated that AGR2 has extracellular functions to increase the aggressiveness of cancer cells and that C4.4A is the functional receptor of AGR2. The signaling complex of C4.4A likely includes laminins 1 and 5 and β1 integrin. Blocking mAbs developed against AGR2 and/or C4.4A significantly reduced tumor growth and metastasis and led to tumor regression, resulting in remarkably improved survival in preclinical mouse models. Thus, this study has provided valuable new insights into a poorly understood but clinically significant pathway and supports the further investigation of the AGR2–C4.4A autocrine loop as a potential target for cancer therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


33. Norris AM, Gore A, Balboni A, Young A, Longnecker DS, Koc M. AGR2 is a SMAD4-suppressible gene that modulates MUC1 levels and promotes the initiation and progression of pancreatic intrap epithelial neoplasia. Oncogene 2013;32:3867–76.


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