AGR2 Is a Novel Surface Antigen That Promotes the Dissemination of Pancreatic Cancer Cells through Regulation of Cathepsins B and D

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most lethal cancers largely due to disseminated disease at the time of presentation. Here, we investigated the role and mechanism of action of the metastasis-associated protein anterior gradient 2 (AGR2) in the pathogenesis of pancreatic cancer. AGR2 was induced in all sporadic and familial pancreatic intraepithelial precursor lesions (PanIN), PDACs, circulating tumor cells, and metastases studied. Confocal microscopy and flow cytometric analyses indicated that AGR2 localized to the endoplasmic reticulum (ER) and the external surface of tumor cells. Furthermore, induction of AGR2 in tumor cells regulated the expression of several ER chaperones (PDI, CALU, RCN1), proteins of the ubiquitin-proteasome degradation pathway (HIP2, PSMB2, PSMA3, PSMC3, and PSMB4), and lysosomal proteases [cathepsin B (CTSB) and cathepsin D (CTSD)], in addition to promoting the secretion of the precursor form pro-CTSD. Importantly, the invasiveness of pancreatic cancer cells was proportional to the level of AGR2 expression. Functional downstream targets of the proinvasive activity of AGR2 included CTSB and CTSD in vitro, and AGR2, CTSB, and CTSD were essential for the dissemination of pancreatic cancer cells in vivo. Taken together, the results suggest that AGR2 promotes dissemination of pancreatic cancer and that its cell surface targeting may permit new strategies for early detection as well as therapeutic management. Cancer Res; 71(22): 7091–102. ©2011 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is almost invariably lethal and remains one of the most devastating cancers in man (1). Most deaths from pancreatic cancer are due to the silent and aggressive nature of this malignant disease. At presentation, the disease has, in most cases, already spread locally and to distant organs, and patients usually succumb within 3 to 6 months. Moreover, even after curative resection, the vast majority of patients relapse due to undetected disseminated tumor cells (DTC) that have spread prior to primary tumor diagnosis. A better understanding of the molecular mechanisms that promote dissemination of cancer cells is, therefore, essential for the development of novel detection and therapeutic strategies for this, at present, largely incurable malignant disease.

Anterior gradient 2 (AGR2) protein is upregulated in multiple cancers, including breast (2, 3), lung (4), ovarian (5), esophageal (6), and prostate cancers (7), and is associated with a metastatic phenotype and poor prognosis (2). It has been identified previously by gene profiling as a marker for DTC detection (8). In sporadic pancreatic cancer, AGR2 protein is present from the earliest precursor lesions [pancreatic intraepithelial precursor lesions (PanIN)] to PDACs but is not expressed in normal pancreas (9). Recent data also suggested that silencing of AGR2 in the pancreatic cancer cell line MPanc-96 results in fewer metastases in an orthotopic pancreas tumor model (10).

AGR2 has been shown to have structural characteristics of the protein disulide isomerase (PDI) family, including a carboxy-terminal endoplasmic reticulum (ER) retention signal KTEL and a single thioredoxin-like domain with a CXXS motif (11). PDI proteins catalyze formation, reduction, and isomerization of disulfide bonds, thereby facilitating the maturation of proteins in the ER and ensure correct folding and multimerization of proteins targeted for the secretory pathway (11).
Park and colleagues have recently shown that AGR2 localizes in the ER of normal intestinal epithelial cells and is essential for in vivo production of protective mucus. They showed that AGR2 mediates processing of the intestinal mucus MUC2 through formation of mixed disulfide bonds and that the absence of AGR2 resulted in a dramatic reduction of mucus production and secretion and an increased sensitivity to colitis in Agr2−/− mice (12). The precise molecular effects of AGR2 in cancers, however, remain largely unknown.

Here, we report that AGR2 is almost universally expressed in tumor cells of patients with pancreatic cancer in both sporadic and familial settings and that it localizes both in the ER and at the external surface of the plasma membrane. We reveal proteomic changes resulting from the induction of AGR2 expression and show that AGR2 promotes in vitro and in vivo dissemination of cancer cells through posttranscriptional induction of 2 proteases, cathepsin B (CTSB) and cathepsin D (CTSD).

Materials and Methods

Tissues and cell lines

Three tissue arrays comprising 42 normal, 48 PanIN, and 84 PDAC cores from both familial and sporadic PDAC cases (University of Washington, Seattle, Washington); 8 primary PDACs and matched infiltrated lymph nodes (Department of Pathology, KBC Osijek); and 10 cases of primary PDAC and 9 metastatic tumors and cell lines (Toronto, ON, Canada, and grown as described previously (13). Other cell lines, verified by short tandem repeat profiling (February 2010) were obtained from Cancer Research UK Cell Services (Clare Hall, Middlesex, UK) and cultured in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Autogen Bioclear).

Establishment of stable cell lines

The pCEP4 AGR2 vector was constructed by excising AGR2 from pcMV-SPORT6-AGR2 (MRC Geneservice) using KpnI and NotI. AGR2 cDNA was cloned into the KpnI/NotI site of pCEP4 (Invitrogen). MiaPaCa2 cells (2 × 10^6 cells per 10-cm plate) were transfected with FuGENE6 (Roche Diagnostics) in a 3:1 ratio with pCEP4 AGR2 or pCEP4. Stable cell lines were established following selection with 300 μg/mL hygromycin B (Merck) and single-cell clones isolated.

Gene silencing

Cells were seeded at 2 × 10^6 cells per well in a 6-well plate and transfected with 1, 5, 10, or 50 nmol/L of siGENOME ON-TARGETplus SMARTpool siRNA specific for human AGR2, CTSB, or CTSD gene or siGENOME Non-Targeting siRNA pool #2 (Dharmacon) using INTERFERin (PeqLab) according to manufacturer’s instructions.

RNA extraction and semiquantitative real-time PCR

Total RNA was extracted using RNAqueous RNA extraction kit (Ambion). First-strand cDNA was prepared from 1 μg of total RNA with Quantitect Reverse Transcription Kit (Qiagen).

Real-time PCR was carried out on a 7500 Real-Time PCR system (Applied Biosystems) using SYBR Green dye (Thermo Fisher Scientific). The primers used were S16, forward 5'-GTCACGTGCCCAGATTATAT 3' and reverse 5'-TCTCCCTCTT-GGAAAGCTCA 3'; CTSB, forward 5'-CAGTACTGGGTGTA- CAATG 3' and reverse 5'-GCCACACCTCCTGATCGAT 3'; and CTSD, forward 5'-GGAGATACATGATCCCTGT 3' and reverse 5'-CTCTGGGGACAGCTTGTAGC 3'. All samples were tested in 3 independent experiments. Relative changes of expression were expressed after normalization to the human ribosomal S16 gene.

Western blotting

Cell lysis was done using NP40 buffer (1% NP40, 50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl) with protease inhibitors (Roche Diagnostics). For secretome analyses, cell lines were serum starved for 16 hours and culture supernatants centrifuged at 5,000 rpm for 15 minutes at 4°C. Secretome samples were concentrated using Amicon Ultra Centrifugal filters Ultracel 3 kDa (Millipore). Twenty-five micrograms of protein lysate or 5 μg of secretome proteins were analyzed by SDS-PAGE as previously described (14). Primary antibodies were rabbit anti-AGR2 (1:250; Abcam), goat anti-actin (1:2,000; Santa Cruz Biotechnology), mouse anti-CTSD (1:5,000) and rabbit anti-CTSB (1:1,000; Abcam).

Immunofluorescence

Cells were seeded on coverslips (5 × 10^4 per well in 24-well plate) and cultured for 48 hours. After fixing in 4% paraformaldehyde, permeabilization with 0.1% Triton X, and blocking in 2% bovine serum albumin (BSA), cells were incubated with mouse anti-AGR2 (1:500; Santa Cruz Biotechnology), rabbit anti-CTSD (1:1,000), rabbit anti-calreticulin (1:200), and rabbit anti-LAMP1 (1:100; Abcam). Secondary antibodies were Alexa Fluor 568/488-conjugated anti-mouse or anti-rabbit IgG (1:1,000; Abcam) and 4',6-diamidino-2-phenylindole (DAPI; Invitrogen), and imaging done with LSM 710 confocal microscope (Zeiss).

Immunohistochemistry

Staining was done on 4-μm thick paraffin sections using rabbit anti-AGR2 antibody (Abcam) diluted 1:30 with DABMap kit, following protocols for the Ventana Discovery System. Counterstaining was done with hematoxylin. The intensity of immunoreactivity was graded on a scale from 0 to 3 and the extent according to the percentage of stained cells (0 points for no staining, 1 point, <20%; 2 points, 20%–50%; and 3 points, >50%). The total score was the product of intensity and extent of staining. Negative or weakly positive cases scored 0 to 0.5, moderately positive as 1 to 2, and strongly positive as more than 6.

Flow cytometry

Subconfluent cells were harvested by trypsin/EDTA (0.25% w/v, 5 mmol/L) and resuspended in DMEM, 0.1% BSA, and 0.1%
sodium azide. AGR2 was detected with rabbit antibody (Santa Cruz Biotechnology; 1:10) on ice for 45 minutes. Bound antibodies were detected with Alexa Fluor 488–conjugated secondary antibody (Invitrogen). Labeled cells were scanned on a BD FACSaria II Cell sorter (BD Biosciences) and analyzed using CellQuest Pro software.

**Functional assays**

MTT, invasion, and wound-healing assays were conducted as described previously (14). For migration assays, Biocoat Cell Culture Inserts with 8-μm pores (BD Biosciences) were used. Seven hundred and fifty microliters of DMEM media supplemented with 10% fetal calf serum was added to the lower chamber and 2.5 × 10^5 cells in 500 μL serum-free medium to the upper chamber. The assays were set up 48 hours after transfection and cells were incubated for 24 hours. Cells that moved through the pores were fixed in 100% methanol and stained with 1% Giemsa Blue (Sigma-Aldrich) before counting. All functional assays were carried out in triplicate in at least 3 individual experiments.

**Protein expression profiling by 2D-DIGE/MS**

MiaPaCa2-pCEP4 and pCEP4 AGR2 were lysed and labeled in triplicate with either NHS-Cy3 or NHS-Cy5 dyes and run in first and second dimension as described previously (14). In total, 6 gels were run generating 18 images that were exported into DeCyder software v5.0 (GE Healthcare). Spots displaying a greater than 1.4 average-fold increase or decrease in abundance, matching across all images, and having values of P < 0.05 (Student t test) were selected. Spot picking, tryptic digestion, and protein identification using liquid chromatography/tandem mass spectrometry (LC/MS-MS) were done as described previously (15). Nano-HPLC-electrospray ionization-collision-induced dissociation MS/MS was done on an Ultimate HPLC with a PepMap C18 75 μm inner diameter column (both Dionex) at a flow rate of 300 nL/min, coupled to a Q-TOF1 mass spectrometer (Micromass). Spectra were processed using MassLynx (Micromass) software and submitted to Mascot database search routines against the Human IPI database. Positive identifications were made when at least 3 peptide sequences matched an entry and MOWSE scores were above the significance threshold value (P = 0.05).

**Zebrafish embryo xenograft model**

Zebrafish (Danio rerio) were handled in compliance with local animal care regulations and standard protocols. Fish were kept at 28°C in aquaria with day/night light cycles (10-hour dark/14-hour light periods). The developing embryos were kept in an incubator at constant temperature.

Cancer cells in suspension were stained with 10 μmol/L CMTMR or CMFDA (Invitrogen) and resuspended in serum-free medium before injecting into 48-hour-old zebrafish embryos. Injections were done using a manual injector (Picospritzer III, Parker Hannifin Instruments). Embryos were dechorionated and anesthetized with tricaine (Sigma-Aldrich) prior to injection. After injections, embryos were incubated at...
Three separate experiments were carried out for each protein of interest. In total, 103 embryos were injected for AGR2 experiments, 109 for CTSB, and 107 for CTSD. Counting of disseminated cells was done 24 hours after injections; embryos were assessed using a Zeiss Axioplan epifluorescence microscope and disseminated cells counted under high magnification.

**Statistical analysis**

The statistical analysis was done with the Student t test using Prism software; a value of $P < 0.05$ was statistically significant.

**Results**

**AGR2 expression is induced from the earliest lesions of pancreatic neoplasia and is retained in all DTCs**

Immunohistochemistry confirmed that AGR2 is not expressed in normal pancreas (Fig. 1A) but is induced in all sporadic PanIN lesions. Here, we additionally show that AGR2 is similarly expressed in familial cases; representative images of familial PanIN1–3 lesions are shown on Fig. 1B–D. High levels of AGR2 expression were seen in almost all PDAC specimens (73 of 84, 87%; Fig. 1E) except the rare cases with squamoid differentiation, which were negative (Supplementary Fig. S1, A1), and undifferentiated PDACs, which showed low expression (data not shown). AGR2 expression was retained in perineural invasion (Fig. 1F), in circulating tumor cells (Fig. 1G) and all metastatic samples, namely, lymph node, liver (Fig. 1H and I), and lung metastases (Supplementary Fig. S1, AII). The data are summarized in Table 1. The immunohistochemistry therefore revealed that AGR2 is expressed in almost all *in situ* or disseminated cancer cells, suggesting that it may be implicated in all steps of PDAC development and spreading. As expected, due to the almost invariable expression of AGR2 across the cases tested, there was no correlation with any of the clinicopathologic data. Furthermore, using a multiorgan tissue array, we have seen that AGR2 is expressed in a limited number of normal human tissues (Supplementary Fig. S1 B and Supplementary Table S1). Of note, most adenocarcinomas of various organs show AGR2 expression, in contrast to squamous cell carcinomas.

**AGR2 is an endoplasmic reticulum and a cell surface antigen**

Both cytoplasmic and membranous immunoreactivity for AGR2 were noted in PDAC cells (Fig. 2A). The precise subcellular localization of AGR2 was further explored *in vitro* using confocal microscopy. Immunofluorescent staining in permeabilized PaTu 8988s pancreatic cancer cells, which express high levels of AGR2 (Fig. 2B), showed that AGR2 is predominantly localized in a fine reticular network around the nucleus (Fig. 2C). The extensive colocalization with calreticulin confirmed that AGR2 is localized in the ER in PDAC cells. However, AGR2 was not seen in the Golgi apparatus and lysosomes (Supplementary Fig. S2A). Immunostaining of 3 nonpermeabilized

<table>
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Figure 2. Localization of AGR2 in pancreatic cancer cells. A, immunohistochemical analysis of AGR2 in a representative human PDAC sample showing both cytoplasmic and membranous (arrows) immunoreactivity. B, Western blot analysis showing AGR2 expression in a panel of pancreatic cancer and normal HPDE cells. C, coimmunostaining of AGR2 (green) and actin or calreticulin (red) in permeabilized PaTu 8988s cells showing that AGR2 is situated in the endoplasmic reticulum. D, cell surface immunostaining for AGR2 (green) on nonpermeabilized PaTu 8988s cells. Scale bars, 10 μm. E, i, flow cytometric analysis of AGR2 cell surface expression on intact pancreatic cells in the same order (as in D) and (ii) sorting of AGR2-positive–gated cells. MiaPaCa2 cells that do not endogenously express AGR2 were used as a negative control.

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AGR2 in Pancreatic Cancer Invasion

A

B

C

D

E

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AGR2 is a posttranscriptional regulator of CTSB and CTSD

Interestingly, the proteases CTSB and CTSD were both upregulated (1.79- and 1.52-fold, respectively) in AGR2-expressing cells. As CTSB and CTSD are known to be frequently overexpressed and hypersecreted in several cancers, including pancreatic (16, 17) cancer, they were selected for further analyses.

CTSB/D overexpression was confirmed by Western blotting [Fig. 5A (i); Fig. 5A (ii) shows densitometry quantification], whereas no change was detected at the mRNA level using realtime PCR [Fig. 5A (iii)]. A large increase in the expression of the precursor isoforms, pro-CTSB and CTSD, in AGR2-expressing cells was also observed. This was further confirmed in additional pancreatic cells, in AGR2-expressing FA6 cells when compared with normal HPDE cells [Fig. 5B (i) and (iii)] and in PaTu 8988s compared with PaTu 8988t cells, which originate from liver metastases of the same patient (18). Metastatic PaTu 8988s cells express AGR2 and higher levels of CTSB/CTSD.
isoforms than nonmetastatic PaTu 8988s cells that do not express AGR2 [Fig. 5B (ii) and (iv)]. These results indicate that AGR2 may be an important posttranscriptional regulator of CTSB and CTSD.

As overexpression of CTSB and CTSD is often associated with an increase in their secretion, the culture supernatants of PDAC cells were analyzed: higher levels of pro-CTS B were detected in the supernatants of AGR2-expressing MiaPaCa2 than in vector-only transfected cells, and inversely, silencing of AGR2 in FA6 cells strongly inhibited pro-CTSD secretion (Fig. 5C). AGR2 is thus an important regulator of pro-CTSD secretion. We were not able to detect pro CTSB or CTSB/CTSD in the supernatants of AGR2-expressing MiaPaCa2 cells close to the level observed in vector-only transfected cells [Fig. 5D (ii)], thus reducing the invasiveness of invading cells was observed after silencing of CTSB and CTSD, respectively [Fig. 5D (iii)] and 80% (P < 0.001; Fig. 6C and D). A similar decrease of DTC was observed after silencing of CTSB (P < 0.001) or CTSD (P < 0.001; Fig. 6E and F, respectively). These results show that all 3 proteins are major regulators of the in vivo ability of PDAC cells to disseminate.

Discussion

AGR2 expression has previously been shown to be induced from the earliest precursor lesions of pancreatic cancer, PanINs, as well as in PDACs (9, 10, 20). Here, we provide a comprehensive analysis of AGR2 expression in both pancreas and extrapancreatic tissues, showing that it is widely expressed in both sporadic and familial PanINs, PDACs, and metastatic lesions. In contrast, AGR2 is not widely expressed in normal organs, in which it is mostly confined to mucin-secreting cells (21).

We show that in PDAC cells, AGR2 localizes to the ER, as observed in normal intestinal epithelial cells (12), suggesting that AGR2 could exert a PDI activity on presecretory proteins also in transformed cells. Furthermore, we provide the first evidence that AGR2 also localizes to the external surface of AGR2-expressing pancreatic cancer cells; accumulation at the cell surface has previously been shown for several ER proteins (22, 23). Therefore, AGR2 may be used for the detection of circulating tumor cells in the peripheral blood of patients with pancreatic cancer and could also be a novel tumor cell surface antigen for the development of antibody-targeting strategies (24). An increasing number of recent studies report that PDI proteins have important roles on the cell surface, as majority of
surface proteins contain disulfide bonds (reviewed in refs. 23, 25) in which they can modulate the activity of membrane receptors (and thus activate and regulate signaling pathways; ref. 26), adhesion molecules integrins (27), or even proteases such as ADAM17 (28). We and others show that AGR2 can also be found in cell culture supernatants (10) and pancreatic juice (29). It remains to be established whether AGR2 functions also at the cell surface or when secreted and whether it is immunogenic as shown for PDI proteins in renal cell carcinoma (30).

Protein profiling of AGR2-expressing MiaPaCa2 cells identified upregulation of several ER chaperones (PDI, CALU, and RCN1), suggesting activation of the ER stress response. This correlates with a previous report on Agr2−/− mice that showed that Agr2 is involved in the ER stress response as well as being itself induced by ER stress (31). Stressful conditions such as hypoxia, nutrient deprivation, and pH changes encountered by tumor cells are known to induce ER stress (32), characterized by the upregulation of ER chaperones. This enables cells to adapt to an unfavorable microenvironment and avoid ER stress–induced apoptosis. The main cellular PDI, PDI/P4HB, in which upregulation was seen here, is a well-established executor of the ER stress response and has been shown to protect melanoma cancer cells against ER stress–induced apoptosis.

Table 2. Deregulated proteins identified by 2D-DIGE analysis comparing MiaPaCa2-pCEP AGR2 with MiaPaCa2-pCEP cells

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cell death (33). CALU and RCN1 are EF-hand members of the CREC protein family (34) which is associated with various Ca\(^{2+}\)-dependent processes in the secretory pathway; in the ER, these proteins interact with the protein translocase (35) that guides the transport of nascent presecretory proteins into the ER lumen. The regulation of these 2 ER chaperones by AGR2 could thus directly affect one of the very first steps in the protein secretion process (35).

Interestingly, Denoyelle and colleagues (36) showed that the induction of ER stress chaperones is an early event in the initiation of melanoma. Similarly, the universal AGR2 expression in all precursor lesions in both sporadic and familial settings suggests that activation of ER stress response could be one of the earliest events in PDAC development and could provide a survival advantage to premalignant cells. Although in the present study, we report on the consequences of AGR2 induction in the pancreas, the nature of the initial trigger that causes early AGR2 induction is not known and currently under investigation as it could potentially lead to a better understanding of pancreatic cancer initiation.

The expression of PDI proteins and ER chaperones has also been correlated with cancer invasion and metastasis in several tumor types (33, 37), and we here provide similar evidence for the role of AGR2 in PDAC.

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**Figure 5.** CTSB and CTSD are functional downstream targets of AGR2. A, Western blot analysis (i) and densitometry quantification (ii) showing increased expression of precursor and mature CTSB and CTSD in MiaPaCa2-pCEP4 and pCEP4 AGR2 lysates. Actin was used as a loading control. III, semiquantitative real-time PCR analysis of CTSB and CTSD gene expression in MiaPaCa2-pCEP4 and pCEP4 AGR2 showing no change in their transcript levels. S16 was used as a reference gene. B, Western blot (i and ii) and densitometry (iii and iv) showing increased protein levels of precursor and mature CTSB and CTSD in FA6 pancreatic cancer cells in comparison with HPDE cells and in metastatic PaTu 8988s in comparison with nonmetastatic PaTu 8988t. D, levels of pro-CTSD in the culture supernatant of MiaPaCa2-pCEP4 AGR2 cells (ii) and FA6 cells (iv) was seen. Mean values of triplicate experiments are shown, *, \(P < 0.05\); **, \(P < 0.005\); ***, \(P < 0.0001\).
AGR2 expression induced an increase in the levels of CTSB and CTSD, 2 disulfide-containing thiol proteases that have previously been reported to be upregulated in pancreatic cancer (16, 38, 39) and are known to play a role in the dissemination of cancer cells (40–42). Our in vitro data indicated that AGR2-induced invasion was mediated through the action of these proteases rather than by increased cell motility, as no difference in migration and wound-healing assays was observed. The AGR2-induced increase in CTSB and CTSD levels could be the direct result of AGR2 PDI activity in the ER during the processing of pro-cathepsins, as previously reported for the production of MUC2 in enterocytes (12), especially because we also observed an increase in the levels of cathepsin precursor forms. This is also in accordance with a number of other reports of PDI activity in protein folding being a limiting factor for protein synthesis and secretion (43, 44). The concept of a posttranslational regulation of cathepsins as observed previously (45), and here reported to be mediated by AGR2, is additionally supported by the absence of change in cathepsins mRNA levels upon AGR2 upregulation. Identifying the formation of mixed disulfide bonds between AGR2 and CTSB and CTSD would confer a formal evidence of this mechanism in pancreatic cancer cells; however, the speed and the transient nature of this type of interaction potentiated by the presence of only 6 and 4 disulfide bonds in CTSB and CTSD, respectively (46), rendered such experimental confirmation challenging. Our in vivo studies using transparent zebrafish embryos, which provide an elegant short-term invasion assay and allows quantification of the DTCs with a single-cell resolution (47), faithfully recapitulated in vitro data and further substantiated that the role of AGR2 in increased invasion of pancreatic cancer cells is largely mediated by the 2 cathepsins.

Finally, we observed that AGR2 expression in PDAC cells is also involved in regulation of pro-CTSD secretion; overexpression of CTSD in cancer cells was reported previously to lead to the hypersecretion of its proteolytically inactive proform (48). Furthermore, increased levels of pro-CTSD have been found in the plasma of patients with metastatic breast carcinoma (49).
and antibodies recognizing pro-CTSD in the serum of patients with ovarian cancer (50). If a similar situation is established in PDAC, pro-CTSD might constitute a potential target for new detection strategies.

In summary, we provide new insights into the mechanisms of action of AGR2 and its role in dissemination of pancreatic cancer cells through regulation of CTSD and CTSD both in vitro and in vivo. In addition, we show that AGR2 can also be immunodetected at the surface of cancer cells, which could open new avenues for both the early detection and the development of novel immunotherapeutic strategies in pancreatic adenocarcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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