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 (PanINs), PDACs, circulating tumour cells and metastases studied. Confocal microscopy and flow cytometry analyses indicated that AGR2 localized to the endoplasmic reticulum (ER) and the external surface of tumour cells. Furthermore, induction of AGR2 in tumour cells regulated the expression of several ER chaperones (PDI, CALU, RCN1), proteins of the ubiquitin-proteasome degradation pathway (HIP2, PSMB2, PSMA3, PSMC3, PSMB4) and lysosomal proteases (cathepsins B and D), 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 pro-invasive activity of AGR2 included CTSB and D 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.

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 malignancy. At presentation the disease has, in most cases, already spread locally and to distant organs and patients usually succumb within 3-6 months. Moreover, even after curative resection, the vast majority of patients relapse due to undetected disseminated tumour cells (DTC) that have spread prior to primary tumour
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 malignancy.

Anterior gradient 2 (AGR2) protein is up-regulated in multiple cancers, including breast [2,3], lung [4], ovarian [5], oesophageal [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 (PanIN1) to PDAC, 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 tumour model [10].

AGR2 has been shown to have structural characteristics of the protein disulphide 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 isomerisation of disulfide bonds, thereby facilitating the maturation of proteins in the endoplasmic reticulum (ER), and ensure correct folding and multimerisation of proteins targeted for the secretory pathway [11].

Park et al., 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 demonstrated that AGR2 mediates processing of the intestinal mucin MUC2 via formation of mixed disulphide 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 tumour cells of pancreatic cancer patients 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 post-transcriptional induction of two proteases, cathepsins B and D.

Materials and Methods

Tissues and cell lines

Three tissue arrays (Cleveland Clinic, Ohio and University of Washington, Seattle) comprising 42 normal, 48 PanIN and 84 PDAC cores from both familial and sporadic PDAC cases; eight primary PDAC and matched infiltrated lymph nodes (Department of Pathology, Osijek, Croatia), and ten cases of primary PDAC and nine matched liver and one lung metastases (GICRMDP, John Hopkins University, Baltimore, USA) were analysed. 30 cases of perineural invasion found within the PDAC tissues were also examined. All specimens were obtained with full ethical approval from the host institutions.

The HPDE cell line was obtained from Dr Ming-Sound Tsao, University of Toronto and grown as described previously [13]. Other cell lines, verified by STR 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, Praisley, UK) supplemented with 10% heat-inactivated fetal calf serum (Autogen Bioclear, Whiltshire, UK).

Establishment of stable cell lines

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

Gene silencing

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

RNA extraction and semi-quantitative real-time PCR

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

Real-time PCR was performed on a 7500 Real Time PCR System (Applied Biosystems, Warrington, UK) using SYBR Green dye (Thermo Fisher Scientific, Epsom, UK). The primers used were: S16, forward 5’ GTACGTGGCCCAGATTTAT 3’ and reverse 5’ TCTCCTTCTGGAAGCCTCA 3’; CTSB, forward 5’ CACTGACTGGGGTGACAATG 3’ and reverse 5’ GCCACCACTTCTGATTGAT 3’; CTSD, forward 5’ GCGAGTACATGATCCCCTGT 3’ and reverse 5’ CTCTGGGGACAGCTTGTAGC 3’. All samples were tested in three independent experiments. Relative changes of expression were expressed after normalization to the human ribosomal S16 gene.

Western blotting

Cell lysis was performed using NP40 buffer (1% NP40, 50 mMTris pH7.4, 150 mMNaCl) with protease inhibitors (Roche Diagnostics). For secretome analyses, cells were serum starved for 16h and culture supernatants centrifugated at 5000 rpm for 15 min at 4 °C. Secretome
samples were concentrated using Amicon Ultra Centrifugal filters Ultracel 3kDa (Millipore, Watford, UK). 25 µg of protein lysate or 5 µg of secretome proteins were analysed by SDS-PAGE as previously described [14]. Primary antibodies were rabbit anti-AGR2 1:250 (Abcam, Cambridge, UK), goat anti-actin 1:2000 (Santa Cruz Biotechnology, Heidelberg, Germany), mouse anti-cathepsin D 1:5000 and rabbit anti-cathepsin B 1:1000 (Abcam).

Immunofluorescence

Cells were seeded on coverslips (5 x 10^4/well in 24-well plate) and cultured for 48 hrs. After fixing in 4% paraformaldehyde, permeabilization with 0.1% Triton X and blocking in 2% BSA cells were incubated with mouse anti-AGR2 1:500 (Santa Cruz Biotechnology), rabbit anti-giantin 1:1000, 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 (1:2000; Invitrogen). DNA was stained with 50 µg/ml DAPI (Invitrogen), and imaging performed with LSM 710 confocal microscope (Zeiss, Herts, UK).

Immunohistochemistry

Staining was performed 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 (Illkirch, France). Counterstaining was performed with haematoxylin. 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 0–3, moderately positive 4–6, and strongly positive >6.
Flow cytometry

Subconfluent cells were harvested by trypsin/ethylene diamine tetraacetic acid (EDTA) (0.25% wt:vol, 5 mM) and resuspended in DMEM, 0.1% BSA, 0.1% sodium azide. AGR2 was detected with rabbit antibody (Santa Cruz Biotechnology, 1:10) on ice for 45 min. Bound antibodies were detected with Alexa Fluor-488-conjugated secondary antibody (Invitrogen). Labelled cells were scanned on a BD FACSARia II Cell sorter (BD Biosciences, Oxford, UK) and analyzed using Cellquest Pro software.

Functional assays

MTT, invasion and wound healing assays were performed as described previously [14]. For migration assays, Biocoat Cell Culture Inserts with 8 µm pores (BD Biosciences) were used. 750 µl DMEM media supplemented with 10% FCS was added to the lower chamber and 2.5 x 10^4 cells in 500 µl serum-free medium to the upper chamber. The assays were set up 48 hrs after transfection and cells were incubated for 24 hrs. 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 three individual experiments.

Protein expression profiling by 2D-DIGE/MS

MiaPaCa2 pCEP4 and pCEP4-AGR2 were lysed and labelled in triplicate with either NHS-Cy3 or NHS-Cy5 dyes and run in first and second dimension as described previously [14]. In total, six gels were run generating 18 images that were exported into DeCyder software v5.0 (GE Healthcare). Spots displaying a > 1.4 average-fold increase or decrease in abundance, matching across all images and having P values < 0.05 (Student’s t-test) were selected. Spot picking, tryptic digestion and protein identification using liquid chromatography-tandem mass spectrometry (LC-MS/MS) were performed as described previously [15].
electrospray ionisation-collision-induced dissociation MS/MS was performed on an Ultimate HPLC with a PepMap C18 75 µm inner diameter column (both Dionex, Surrey, UK) at a flow rate of 300 nl/min, coupled to a Q-TOF1 mass spectrometer (Micromass, Manchester, UK). 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 three 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 hrs dark/14 hrs light periods). The developing embryos were kept in an incubator at constant temperature.

Cancer cells in suspension were stained with 10 µM CMTMR or CMFDA (Invitrogen) and resuspended in serum free medium before injecting into 48 hrs old zebrafish embryos. Injections were performed using a manual injector (Picospritzer III, Parker Hannifin instruments, Pine Brook, NJ). Embryos were dechorionated and anesthetized with tricaine (Sigma-Aldrich) prior to injection. After injections, embryos were incubated at 35°C. Three separate experiments were performed 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 performed 24 hrs after injections; embryos were assessed using a Zeiss Axioplan epifluorescence microscope and disseminated cells counted under high magnification.

**Statistical analysis**

The statistical analysis was performed with the Student’s t-test using Prism software; a $P$ value of <0.05 was statistically significant.
**Results**

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

Immunohistochemistry confirmed that AGR2 is not expressed in normal pancreas ([Figure 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 [Figure 1 B-D](#). High levels of AGR2 expression were seen in almost all PDAC specimens (73/84, 87%) ([Figure 1E](#)) except the rare cases with squamoid differentiation which were negative ([Figure S1 A I](#)), and undifferentiated PDACs which showed low expression (data not shown). AGR2 expression was retained in perineural invasion ([Figure 1F](#)), in circulating tumour cells ([Figure 1G](#)) and all metastatic samples, namely lymph node, liver ([Figure 1H, I](#)) and lung metastases ([Figure S1 AII](#)). The data are summarized in [Table 1](#).

The immunochemistry 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 clinicopathological data. Furthermore, using a multi-organ tissue array, we have seen that AGR2 is expressed in a limited number of normal human tissues ([Figure S1 B, 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 ([Figure 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 (Figure 2B), showed that AGR2 is predominantly localized in a fine reticular network around the nucleus (Figure 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 (Figure S2A). Immunostaining of three non-permeabilized AGR2-expressing cell lines, PaTu 8988s, FA6 and CFPAC1 (Figure 2D), indicated that AGR2 also localized at the external surface of the plasma membrane. This finding was further confirmed on non-permeabilized cells by flow cytometry (Figure 2E I), which also permitted the physical isolation of cell surface AGR2-expressing cells (Figure 2E II). This was not observed in the MiaPaCa2 cell line, that does not express AGR2 endogenously and thus served as a negative control. Of note, similar surface expression was also seen on breast cancer cells (Figure S2B), indicating that AGR2 also might be a cell surface antigen in other tumour types. Furthermore, western blotting (Figure S2C) showed the presence of AGR2 in culture supernatants of PaTu 8988s and CFPAC1, but not in FA6 or BxPC3 that also express AGR2 (Fig 2B, D and E), suggesting that AGR2 might be shed from the cell surface rather than being actively secreted.

Taken together, our data indicate that in pancreatic tumour cells the PDI protein AGR2 has not only a conserved ER localization, but is also a cell surface marker.

**AGR2 regulates the invasiveness of PDAC cancer cells**

To assess the functional roles of AGR2 in pancreatic cells, long-term stable expression of AGR2 was achieved in MiaPaCa2 cells, an undifferentiated pancreatic cancer cell line. AGR2 expression was confirmed by western blot in MiaPaCa2-pCEP4 AGR2 cells compared to cells transfected with empty control vector (pCEP4) (Figure 3A I). The recombinant AGR2 protein had the same sub-cellular localization as endogenous AGR2, as it was detected in the ER, at the cell surface and in the culture supernatant of pCEP4 AGR2-transfected cells (Figure S3 A,
B and C, respectively). In parallel, a complete loss of AGR2 protein expression was seen in FA6 cells (which express high endogenous levels of AGR2) 48 hours after transfection with 50 nM of AGR2-specific siRNA (Figure 3A II). No morphological change was observed after alteration of AGR2 expression in these cells (Figure S4A). Moreover, no difference was observed in cell proliferation, wound healing, nor migration (Figure S4B, C and D, respectively) following over-expression or knock-down of AGR2. However, in invasion assays, a significant increase ($P < 0.05$) in the number of invading cells was observed in AGR2-expressing MiaPaCa2 cells, compared to vector-only controls (Figure 3B I) and a significant decrease ($P < 0.05$) in the number of invading cells was seen following siRNA-mediated knock-down of AGR2 in FA6 cells (Figure 3B II). The invasive capabilities of pancreatic cancer cells are therefore proportional to levels of AGR2 expression.

**AGR2 induces changes in cancer cell proteome**

To assess the molecular mechanisms underlying the pro-invasive activity of AGR2, proteomic analysis of AGR2-expressing MiaPaCa2 versus vector control-transfected cells was performed using two-dimensional difference in gel electrophoresis (2D-DIGE). A representative gel is shown in Figure 4A. We identified 15 up-regulated and 24 down-regulated proteins (represented by 15 and 36 spots, respectively) with a cut-off of 1.4 fold at $P < 0.05$ (Figure 4B).

Two ER chaperones, CALU and RCN1, were identified as the highest up-regulated proteins (both 2-fold), in addition to AGR2 (8.5-fold up-regulated). The archetypal cellular ER protein disulphide isomerase, PDI/P4HB, was also up-regulated, in addition to deregulation of several proteins of the ubiquitin-proteasome degradation pathway (HIP2, PSMB2, PSMA3, PSMC3, PSMB4) that play a role in ER-associated degradation of improperly processed proteins. The expression levels of a number of structural proteins (LMNA, VIM, KRT1, KRT8,
KRT18, and KRT19) were also altered. The functional interactions between the majority of these differentially expressed proteins mapped in a network are shown on Figure S5.

**AGR2 is a post-transcriptional regulator of cathepsin B and D**

Interestingly, the proteases CTSB and CTSD were both up-regulated (1.79- and 1.52-fold, respectively) in AGR2-expressing cells. As CTSB and CTSD are known to be frequently over-expressed and hyper-secreted in several cancers, including pancreatic [16,17], they were selected for further analyses.

CTSB/D over-expression was confirmed by western blotting (Figure 5A I; Figure 5A II shows densitometry quantification), while no change was detected at the mRNA level using real-time PCR (Fig 5A III). A large increase in the expression of the precursor isoforms, pro cathepsin B and D (pro CTSB/D) in AGR2-expressing cells was also observed. This was further confirmed in additional pancreatic cells: in AGR2-expressing FA6 cells when compared to normal human pancreatic ductal epithelial (HPDE) cells (Figure 5B I and III), and in PaTu 8988s compared to PaTu 8988t cells, which originate from liver metastases of the same patient [18]. Metastatic PaTu 8988s cells express AGR2 and higher levels of CTSB/D isoforms compared to non-metastatic PaTu 8988t cells that do not express AGR2 (Figure 5B II and IV).

These results indicate that AGR2 may be an important post-transcriptional regulator of CTSB and CTSD in PDAC cells.

As over-expression of cathepsins B and D is often associated with an increase in their secretion, the culture supernatants of PDAC cells were analyzed: higher levels of procathepsin D were detected in the supernatants of AGR2-expressing MiaPaCa2 compared to vector-only transfected cells, and inversely, silencing of AGR2 in FA6 cells strongly inhibited pro CTSD secretion (Figure 5C). AGR2 is thus an important regulator of pro CTSD secretion. We were not able to detect pro CTSB or CTSB/D in the supernatants, which might be due to their binding to the plasma membrane rather than being secreted [19].
**Cathepsins B and D are involved in AGR2-mediated dissemination of pancreatic cancer cells**

To examine whether cathepsins are involved in the AGR2-induced increase in cell invasion, CTSB and CTSD were silenced in AGR2-expressing MiaPaCa2 cells (Figure 5D I). About 50% ($P < 0.005$) and 80% ($P < 0.001$) reduction in the number of invading cells was observed after silencing of CTSB and CTSD, respectively (Figure 5D II), thus reducing the invasiveness of MiaPaCa2-pCEP4 AGR2 cells close to the level observed in control MiaPaCa2-pCEP4. The same dramatic effect was observed in FA6 cells after silencing of CTSB ($P < 0.05$) and CTSD ($P < 0.001$) (Figure 5D III and IV). AGR2 thus regulates the expression, and potentially secretion, of CTSB and CTSD, which in turn mediate the AGR2-induced invasiveness of pancreatic cancer cells.

To analyze whether AGR2, CTSB and CTSD are important regulators of the dissemination of pancreatic cancer cells *in vivo*, a zebrafish embryo xenotransplant model was employed. PaTu 8988s cells were stained *in vitro* with red or green fluorescent dyes and were co-injected into the yolk sack of 48 hours old zebrafish embryos (Figure 6A). After 24 hours, both red and green tumour cells were equally observed as disseminated into the tail of the embryo (Figure 6B I and III) or localized in the vicinity of the injection site in the yolk sack (Figure 6B II and IV). Silencing of AGR2 in PaTu 8988s cells significantly lowered the number of DTC compared to siRNA-control transfected cells ($P < 0.001$) (Figure 6C and D). A similar decrease of DTC was observed after silencing of CTSB ($P < 0.001$) or CTSD ($P < 0.001$) (Figure 6E and F, respectively). These results demonstrate that all three 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, where 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 employed for the detection of circulating tumour cells in the peripheral blood of pancreatic cancer patients, and could also be a novel tumour 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 disulphide bonds, (reviewed in [23,25]) where they can modulate the activity of membrane receptors (and thus activate and regulate signalling pathways) [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 up-regulation of several ER chaperones (PDI, CALU and RCN1), suggesting activation of the ER stress-response. This correlates with a previous report on Agr2−/− mice which demonstrated 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 tumour cells are known to induce ER stress [32], characterized by the up-regulation of ER chaperones. This enables cells to adapt to an unfavourable microenvironment and avoid ER stress-induced apoptosis. The main cellular protein disulphide isomerase, PDI/P4HB, whose up-regulation 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 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] which guides the transport of nascent presecretory proteins into the ER lumen. The regulation of these two ER chaperones by AGR2 could thus directly affect one of the very first steps in the protein secretion process [35].

Interestingly, Denoyelle et al. [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 setting suggests that activation of ER-stress response could be one of the earliest events in PDAC development and could provide a survival advantage to pre-malignant cells. While 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 tumour types [33,37], and we here provide similar evidence for the role of AGR2 in PDAC.

AGR2 expression induced an increase in the levels of cathepsins B and D, two disulphide-containing thiol proteases that have previously been reported to be up-regulated in pancreatic cancer [16,38,39] and are known to play a role in the dissemination of cancer cells [40,41,42]. 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 cathepsin B and D 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 since 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 post-translational 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 up-regulation. Identifying the formation of mixed disulfide bonds between AGR2 and cathepsin B and D 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 D 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 disseminated tumour cells 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 two cathepsins. Lastly, 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 pro-form [48]. Furthermore, increased level 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 ovarian cancer patients [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 cathepsins B and D 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 as well as development of novel immunotherapeutic strategies in pancreatic adenocarcinoma.

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**References**


Figure legends

Figure 1. AGR2 protein expression in pancreatic tissues.
Immunohistochemical analysis of AGR2 expression in normal pancreas (A), familial PanIN1, PanIN2, PanIN3, PDAC (B-E, respectively); perineural invasion, circulating tumour cells, lymph node and liver metastasis from sporadic pancreatic cancer samples (F-I, respectively).
Scale bars 50 µm.

Figure 2. Localisation 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. Co-immunostaining of AGR2 (green) and actin or calreticulin (red) in permeabilised PaTu 8988s
cells demonstrating that AGR2 is situated in the endoplasmic reticulum. D. Cell surface immunostaining for AGR2 (green) on nonpermeabilised PaTu 8988s cells. Scale bars: 10 µm. E. (I) Flow cytometry 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.

**Figure 3. Functional roles of AGR2.**

A. Western blot analysis of AGR2 expression in (I) MiaPaCa2 cells stably transfected with empty vector control (pCEP) or pCEP-AGR2 vector, and (II) in FA6 cells 48h after transfection with specific AGR2 siRNA or non-targeting (NT) control siRNA. Actin was used as a loading control. B. Invasion assays using (I) stably transfected MiaPaCa2 cells and (II) siRNA-transfected FA6 cells. Mean values of triplicate experiments are shown. * P<0.05.

**Figure 4. Proteomic profiling of MiaPaCa2 AGR2-expressing cells.**

A. Representative gel image displaying protein spots with AGR2-dependent changes in expression is shown (vector only sample (blue, Cy5), AGR2-overexpressing sample (red, Cy3)). Magnified regions showing several differentially expressed proteins. CALU, Calumenin; CTSB, cathepsin B; CTSD, cathepsin D; PDI, protein disulphide isomerase; RCN1, Reticulocalbin 1. B. Table listing the deregulated proteins identified by 2D DIGE analysis comparing MiaPaCa2-pCEP AGR2 to MiaPaCa2-pCEP Cells.

**Figure 5. Cathepsins B and D are functional downstream targets of AGR2.**

A. Western blot analysis (I) and densitometry quantification (II) showing increased expression of precursor and mature CTSB and D in MiaPaCa2-pCEP4 and pCEP4-AGR2 lysates. Actin was used as a loading control. (III) Semi-quantitative real-time PCR analysis of CTSB and CTSD gene expression in MiaPaCa2-pCEP4 and pCEP4-AGR2 demonstrating no
change in their transcript levels. S16 was used as a reference gene. **Western blot (I and II)** and densitometry (III and IV) showing increased protein levels of precursor and mature CTSB and D in FA6 pancreatic cancer cells in comparison with HPDE cells, and in metastatic PaTu 8988s in comparison with non-metastatic PaTu 8988t. C. Levels of pro CTSD in the culture supernatant of MiaPaCa2-pCEP4 and pCEP4 AGR2, and in FA6 cells 48h after transfection with AGR2 or non-targeting siRNA. **D.** After siRNA silencing of cathepsins B and D for 48 hours (I and III), decreased invasion 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.

**Figure 6.** AGR2 and cathepsins B/D are major regulators of pancreatic cancer cell dissemination in vivo.

A. 50 to 100 PaTu8988s cells stained with CMTMR or CMFDA dyes were injected into the yolk sack of 48h-old embryos and assessed by epifluorescence microscopy (I) of the whole embryo and confocal microscopy (II) of the injection area. **B.** 24 hours post-injection disseminated cancer cells were observed in the tail of the embryo (I) or in the yolk sack (II). Elongated cells imaged by confocal microscopy in the tail (III) and the yolk sack (IV) of the embryo. Scale bars 50 µm. C. Labelled PaTu 8988s cells transfected with AGR2 or non-targeting siRNA (I) were assessed by epifluorescence microscopy 24 hours after injection. The number of disseminated cells was statistically significantly decreased after AGR2 silencing (II-III). **D.** Representative image of decreased tumour cell dissemination in zebrafish tail after AGR2 silencing (I). The enlarged respective images of the two marked areas in tail are shown on D II and III. **E-F.** After silencing of CTSB/CTSD (I), a significant decrease in the number of disseminated cells was also seen (II, III), respectively. **P < 0.005, *** P < 0.0001.
Table 1. Summary of immunohistochemistry findings in human pancreatic tissues and pancreatic cancer metastases.
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<th>Positive cases (%)</th>
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<td>0 2 4 1</td>
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<tr>
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Figure 2

Dumartin et al.

A

B

C

D

E

Figure 2 Dumartin et al.

A

B

C

D

E

Figure 2 Dumartin et al.

A

B

C

D

E

Figure 2 Dumartin et al.

A

B

C

D

E

Figure 2 Dumartin et al.

A

B

C

D

E

Figure 2 Dumartin et al.

A

B

C

D

E

Figure 2 Dumartin et al.

A

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Figure 2 Dumartin et al.

A

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Figure 2 Dumartin et al.

A

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Figure 2 Dumartin et al.

A

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Figure 2 Dumartin et al.

A

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Figure 2 Dumartin et al.

A

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C

D

E

Figure 2 Dumartin et al.

A

B

C

D

E

Figure 2 Dumartin et al.

A

B

C

D

E

Figure 2 Dumartin et al.

A

B

C

D

E

Figure 2 Dumartin et al.

A

B

C

D

E

Figure 2 Dumartin et al.

A

B

C

D

E

Figure 2 Dumartin et al.
### Figure 4

#### A

![Image of Western Blot](image)

- **pH 3**
- **pH 10**
- **KDa**

#### B

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*Note: All data and images are hypothetically generated for demonstration purposes.*
Figure 5

Dumartin et al.

A

I

PCEN
PCEN AGR2

kDa
52
34
42
26
24
52

II

pro pre CTSD CTSD
pro CTSD
CTSD
CTSD
CTSD
CTSD

III

Transmembrane

leukocytes

Relative expression

B

I

pre-CETD
CTSD
CTSD
CTSD
CTSD

II

kDa
52
34
42
26
24
52

III

Relative expression

IV

pCEP4 pCEP4 AGR2

pCEP4

pCEP4 AGR2

pCEP4

pCEP4 AGR2

C

I

MiaPaCa2
FA6

II

supernatant
pro CTSD

D

I

MiaPaCa2-pCEP4 AGR2

II

Actin

III

Actin

IV

Actin

**p = 0.0007
p = 0.0027

***p = 0.0018
p = 0.015

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Figure 6

Dumartin et al.
AGR2 is a Novel Surface Antigen that Promotes the Dissemination of Pancreatic Cancer Cells through Regulation of Cathepsins B and D

Laurent Dumartin, Hannah J Whiteman, Mark E Weeks, et al.

Cancer Res  Published OnlineFirst September 26, 2011.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-11-1367

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2011/09/26/0008-5472.CAN-11-1367.DC1

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