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Glutamate increases pancreatic cancer cell invasion and migration *via* AMPA receptor activation and Kras-MAPK signaling

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Glutamate has been implicated in tumorigenesis through activation of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPAR). However, the function of a glutamate-to-AMPAR signal in pancreatic ductal adenocarcinoma (PDAC) has remained elusive. We now show that glutamate-mediated AMPA receptor activation increases invasion and migration of pancreatic cancer cells *via* activation of the classical MAPK pathway. Glutamate levels were increased in pancreatic cancer accompanied by downregulation of GluR subunits 1, 2, and 4. In pancreatic cancer precursor lesions, pancreatic intraepithelial neoplasia (PanIN), GluR1 subunit levels were strikingly and step-wise increased but its expression was rare in PDAC. Pharmacological inhibition or RNAi-mediated suppression of GluR1 or GluR2 did not affect cancer cell growth but significantly decreased invasion. In a K-ras wildtype cell line, AMPA receptor activation enhanced K-ras activity and—further downstream—phosphorylation of p38 and of p44/42. Preemptive blockade of AMPA receptors in a mouse model of pancreatic cancer inhibited tumor cell settling. AMPA receptor activation thus not only activates MAPK signalling but also directly increases activity of K-ras. Glutamate might serve as a molecular switch that decreases the threshold of K-ras-induced oncogenic signalling and increases the chance of malignant transformation of pancreatic cancer precursor lesions.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human malignancies, with exceptionally low survival rates (5-year survival rate less than 5%).[1] Although the cell of origin of PDAC is controversially discussed,[2] pancreatic intraepithelial neoplasias (PanIN) of different grades (1A, 1B, 2, 3) in which increasing numbers of molecular alterations are found (*i.e.*, mutations in the Kras, Smad4 and p53 genes), are believed to belong to the multistep progression model of pancreatic cancer.[3,4] Neurotransmitters and neuropeptides have been shown to play a role in pancreatic carcinogenesis[5–11] and in pancreas-associated diseases[8,12–16] however, the role of excitatory neurotransmitters and particularly of glutamate in pancreatic cancer is not well defined. Glutamate activates metabotropic receptors (mGluR; G protein-coupled receptors) and the ionotropic (iGluR) receptors *N*-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate[17,18] receptors. AMPA receptors are heteromeric and are assembled from the four subunits GluR1-4 in different combinations. The presence of GluR2 subunit determines AMPA receptor impermeability to Ca^{2+}.[17,19,20] Since AMPA receptor activation regulates differentiation, proliferation and migration of embryonic stem cells,[21–24] it has been hypothesized that modulation of AMPA receptor-mediated signals might be involved in carcinogenesis, particularly because it has also been shown that AMPA signals *via* the MAPK pathway.[25–27] This hypothesis has subsequently been proven for some tumor entities such as astrocytoma, glioblastoma, breast carcinoma, lung carcinoma, colon adenocarcinoma, and prostate carcinoma.[26,28–31] An extensive search of online available databases on gene expression in pancreatic cancer (www.oncomine.com; www.pancreasexpression.org; http://cgap.nci.nih.gov/SAGE) revealed de-regulation of GluR2 (gene: GRIA2) in cancer as compared to normal pancreas (NP). Since we have recently characterized expression and function of cannabinoids as exemplary inhibitory neurotransmitters in pancreatic diseases, we now set out to define the role of the glutamate system in pancreatic carcinogenesis with an emphasis on AMPA receptors.

Key words: pancreatic cancer, AMPA, AMPA receptor, glutamate
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Materials and Methods

Sym2206 and S−(−)-5′-fluorowillardiine were purchased from TOCRIS Cookson (Ellisville); AMPA, from Biomol (Hamburg, Germany); rabbit polyclonal anti-GluR1, mouse

monoclonal anti-GluR2 were purchased from Chemicon International (Temecula, CA) rabbit anti-GluR4 was purchased from Upstate, Lake Placid, NY; anti-p38 MAP kinase, anti-phospho p38-MAP kinase (Thr180/Tyr182), anti-p44/42 MAP kinase, anti-phospho p44/42 MAP kinase (Thr202/Tyr204) antibodies were purchased from Cell Signaling Technology (Danvers, MA); secondary antibodies—anti rabbit—and anti mouse–HRP-labelled polymer (ready to use), normal rabbit IgG (15 g/l), mouse IgG1, mouse IgG2a, DAB Chromogen System were purchased from DAKO, Hamburg, Germany; ECL-anti-rabbit IgG HRP-linked secondary antibody, from GE Healthcare, UK; ECL, from Amersham Life Science (Bucks, UK); cell culture medium—RPMI 1640, Penicillin/Streptomycin, 0.25%Trypsin-EDTA were purchased from GIBCO, Invitrogen GmbH (Karlsruhe, Germany) fetal bovine serum, from PAN Biotech GmbH (Aidenbach, Germany). Ethanol, Methanol, H_2O_2, Na^+-Citrate, BSA, EDTA, SDS, TRIS, NaCl, Formalin, Glycine, Tween-20, TritonX-100, Roticlear were purchased from Carl Roth GmbH (Karlsruhe, Germany). DMSO, Hepes and $CaCl_2$ were purchased from Sigma Aldrich (St. Louis, MO) Mayer's Hematoxylin was purchased from Merck (Darmstadt, Germany).

Patients and tissue sampling

Tissue samples were collected from patients during pancreatic resections for PDAC ($n = 60$) or CP ($n = 10$). Normal pancreatic tissue samples were obtained through an organ donor procurement program, whenever there was no suitable recipient for pancreas transplantation ($n = 10$). Pancreatic tissues were immediately snap frozen at $-80^{\circ}C$ or formalin-fixed and paraffin-embedded. The use of human tissue for the analysis was approved by the local ethical committee (University of Heidelberg, Germany) and written informed consent was obtained from the patients prior to surgery.

(Quantitative) real time polymerase chain reaction [(Q)RT-PCR]

mRNA and cDNA were prepared using reagents and equipment from Qiagen (Hilden, Germany), following the manufacturer's instructions. Real time PCR was carried out on a Mastercycler (Eppendorf) Quantitative RT-PCR was carried out on a LightCycler480 (Roche Diagnostics). QuantiTect primer assays (Qiagen) for GluR1-GluR4 subunits were used (for sequences see www.qiagen.com).

Glutamate concentration measurement

Gluamate concentration in the eight pancreatic cancer cell supernatants was determined according to the protocol as previously described.[32]

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections as previously described.[8,15,16,33] The PanIN tissue array contained sections from PanIN1, 2, and 3. Semi-quantitative analysis of PanIN staining was performed as previously described.[15] The antibodies and dilutions were as follows: rabbit polyclonal anti-GluR1 (200 μg/ml; 1:300), mouse monoclonal anti-GluR2 and rabbit monoclonal anti-GluR4 [1.15 mg/ml; 1:5000; 1:200; all from Chemicon International (Temecula CA)].

Semiquantitative evaluation of AMPA receptor levels in human tissue specimens

Immunoreactivity of AMPA receptors in PanIN structures, cancer cells and/or nerves was quantitatively evaluated according to intensity and area as previously described[15]: the staining intensity of PanIN structures and cancer cells was recorded as "no staining" (0), "weak staining" (1), moderate staining (2)" or "strong staining (3)". The area of stained cells was recorded as $<33\%$ (1), 33-66% (2), or $>66\%$ (3) of all cancer cells. These numbers were then multiplied resulting in a score of 0–9. Regarding intrapancreatic nerves, only the staining intensity (0–3, as described above) was analyzed due to the generally low number of nerves in pancreatic cancer tissue specimens.

RNAi

Synthetic siRNA oligonucleotides for GluRs were purchased from Qiagen (Hilden, Germany), prepared and stored according to the manufacturer's instructions. For silencing of GluR1/2 subunit two different RNAi molecules were tested and the higher effect RNAi was chosen. Human GluR1 RNAi (sense 5¢-CCAUGAAGGUGGGAGGUAATT-3¢, antisense 5¢-UUACCUCCCACCUUCAUGGTG-3¢) and GluR2 RNAi (sense 5¢-CGUAUGUUAUGAUGAAGAATT-3¢; antisense 5¢-UUCUUCAUCAUAACAUACGGA-3¢) were used. Control siRNA sequence was UUCUCCGAACGUGUCACGU. Cells were grown to 70% confluency under standard growth conditions. For siRNA transfections, HiPerfect transfection reagent (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions. The final concentration of the control and the specific oligonucleotides was 10 nM. The efficacy of the RNAi was analyzed by RT-PCR 24 hr after transfection.

Cell viability assay

Cell viability assays were performed as described previously.[33,34] In 96 well-plates, 5.000 cells/well were seeded, were grown for 24 hr at $37^{\circ}C$, $5\%CO_2$ humidified atmosphere and were exposed to Sym2206 at concentrations of 6.25, 12.5, 25, 50, 75, and 100 μM and to S–(-)-5'-fluorowillardiine at concentrations of 1, 10, 50, 75, and 100 μM for 24 hr. To evaluate the effect of GluR1 and GluR2 RNAi on cell growth, cells were reseeded into 96-well plates 24 hr after RNAi and grown for further 24-48-72-96 hr. Twenty-four hours after reseeding (and as indicated in the results part), some cells were treated with 100 μM of AMPA or the appropriate control. After the indicated time points, yellow tetrazolium salt 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml in PBS; Sigma Aldrich, St. Louis, MO)

was added (50 μg/well) and cells were incubated with MTT for 4 hr. The MTT was subsequently solubilized in acidic isopropanol and was quantified spectrophotometrically by measuring the optical density at 570 nm wave length. The GI_{50} (the concentration required to achieve 50% growth inhibition) for SYM or FW was calculated by using the formula $100 \times (T - T_0)/(C - T_0)$, where T is the optical density after X h of exposure to the drug, T_0 the adsorption at time zero, and C the control after X hr. Alternatively, the growth after RNAi was calculated as percentage of time zero. All experiments were performed in triplicates and repeated at least three times.

Immunoblot assays

Immunoblot assays were performed as previously described.[8,14] Briefly, Su86.86 cells were treated with the drugs according to the experimental procedures and were then lysed in an ice cold lysis buffer (5M NaCl, 1M Tris-HCl, 10%Triton X-100, 100 mM EDTA, 200 mM sodium orthovanadate, 200 mM sodium fluoride, 200 mM sodium pyrophosphate, glycerol) supplemented with an EDTA-free mini protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). The primary antibodies were used as follows: anti-p38 MAP kinase, anti-phospho-p38 MAP kinase (Thr180/Tyr182), anti-phospho-p44/42 MAP kinase (Thr202/Tyr204), anti-p44/42 MAP kinase diluted 1:1000 inTBS/5%BSA. The blots were subjected to densitometric analysis as previously described.[34]

Wound healing assay

Su86.86 cells were seeded into petri-dishes in RPMI medium containing 10% FBS and 1% penicillin/streptomycin, and were grown at 37°C, 5%CO_2 humidified conditions until 90–95% confluency was reached. A scratch was made with a sterile 10 μl pipette tip and washed twice with sterile PBS. The cells were treated with 10 μM of S–(–)-5¢fluorowillardiine or 10 μM of Sym2206 using NaOH (1:5000) or DMSO (1:10.000) as appropriate controls, respectively. The scratch was photographed using a digital camera (Carl Zeiss Axiocam MRm, Germany) at a magnification of 10× at 2 preselected time points (0 and 24 hr) to observe migration of the cells. Migrated cells were counted using computerized imaging analysis.

Invasion assay

To assess the effects of SYM and FW and to evaluate the influence of specific MAPK antagonists on Su86.86 invasion, BD BioCoat Matrigel Invasion Chambers (BD Biosciences, San Jose, CA) were prepared according to the manufacturer's instructions. In 500 μl RPMI culture medium containing 0.5% FBS and 10 μM of Sym2206 or control (DMSO 1:10.000), 5×10^4 cells/ml were seeded. For the specific inhibitor invasion assay, cells were pre-treated for 1 hr with specific p44/42 and p38 inhibitors (U0126 and SB203580 at 10 μM, respectively) alone or in combination and were subsequently stimulated with 10 μM of S–(–)-5¢-fluorowillardiine. Twenty-four hours after treatment, invaded cells were fixed, stained and counted under a light microscope. The invasion index was calculated as the ratio of the number of invaded cells to the number of invaded control cells. All assays were repeated 5 times.

ELISA

Equal amounts (4×10^5/well) of Su86.86 cells were seeded and grown in 6-well plates in 10% FBS-containing growth medium at 37°C, 5%CO_2, humidified conditions until adherent. Then, the cells were treated with 10 μM of Sym2006 in 0.5%FBS containing growth medium and were incubated for 24 hr at the same growth conditions. Thereafter, supernatants were analyzed by matrix metallo-proteinase-2 (MMP-2) ELISA according to the manufacturer's instructions (BD Bioscience, Heidelberg, Germany).

p44/42 and p38 inhibition assay

Su86.86 cells were seeded into 6-well plates in 10% FBS containing growth medium at 37°C, 5% CO_2, humidified conditions and grown until adherent. The growth medium was then replaced by 0.5% FBS containing growth medium and the cells were incubated at the same conditions with FW and/or SYM (10 μM) for 24 hr. When indicated, cells were preincubated (for 1 hr) with SB203580 (SB, p38 inhibitor, 5 μM (when used in combination with U0126) or 10 μM) and/or U0126 (p44/42 inhibitor 5 μM (when used in combination with SB) or 10 μM). After 24 hr incubation with the drugs, the supernatants were collected and the amount of MMP-2 was evaluated by ELISA according to the manufacturer's protocol. The cells were lysed and levels of p44/42/phospho-p44/42 and p38/phospho-p38 were evaluated by immunoblot analysis.

Ras activation assay

Su86.86 and BxPC3 cells were seeded into 6 cm petri-dishes at 70–80% confluency in complete growth medium and grown until adherent. Then, cells were treated with 100 μM of AMPA or appropriate control for 24 hr and subjected to a Ras activation assay using the Ras activation assay kit (Millipore, Temecula, CA) according to the manufacturer's instructions.

Pathway array

Su86.86 were treated with 100 μM of AMPA or the appropriate control for 12 hr. mRNA and cDNA were prepared using reagents and equipment from Qiagen (Hilden, Germany) and were subjected to the RT^2 Profiler PCR array (SABiosciences, Frederick) according to the manufacturer's instructions.

In vivo model of pancreatic cancer

All animal procedures were performed according to local ethical guidelines. In 200 μl of sterile PBS, 10^6 Su86.86 cells were resuspended and were injected into the subcutaneous

tissue bilaterally at the sites behind the anterior forelimb of 4-week old athymic nude mice. One day after the injection of tumor cells, treatment with the AMPA receptor antagonist SYM (5 mg/kg, $n = 5$; s.c. injection once per day during 2 weeks) or DMSO (1:100, control group, $n = 5$; same injection protocol as for SYM) was started. After 2 weeks, the mice were sacrificed and visible tumors were collected.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 4 Software (GraphPad Software). Experimental results are expressed as mean +/- SEM unless indicated otherwise. The level of significance was set at $p < 0.05$.

Results

Analysis of glutamate levels in fresh frozen human NP, chronic pancreatitis (CP) and PDAC tissues demonstrated a striking increase of glutamate in CP and PDAC samples (Fig. 1*a*, $p < 0.0001$). Because GluR2 receptor subunit was found to be de-regulated in PDAC, we hypothesized that the increase in glutamate levels induces a pro-invasive and anti-apoptotic signal *via* activation of AMPA receptors. To this end, we determined AMPA receptor expression in pancreatic tissues: while there was a trend toward increased GluR (1–4) expression levels in CP, GluR1, 2, and 4 were down-regulated in bulk pancreatic cancer tissues (Fig. 1*b*, $p = 0.0084$ for GluR1, $p = 0.0423$ for GluR2, and $p = 0.0025$ for GluR4). While in NP, acinar cells (which form more than 80% of the total cellular mass of the organ) expressed GluRs at a low to medium level, pancreatic cancer cells (which form less than 30% of the total tumor mass) were strongly immunopositive for GluR2. These findings might explain the discrepancies between RT-PCR/cDNA microarray (down-regulation of GluR2) and immunohistochemistry results.

To determine the "source" of the decrease in GluR subunit transcripts, we performed extensive immunohistochemical analyses on pancreatic tissue sections using antibodies against GluR1, GluR2, and GluR4 subunits. In NP, the GluR1 subunit was found to be expressed mostly in islets and some faint staining was rarely seen at the apical membrane of duct cells (Fig. 1*c* and inset, NP), whereas with an increasing PanIN grade, increasing staining intensities were found (Fig. 1*c* and insets, PanIN1, 2 and 3); we thus performed GluR subunit stainings on PanIN tissue microarrays and evaluated these semi-quantitatively, confirming the gradual increase in immunoreactivity (Fig. 1*c*, graph). In pancreatic cancer cells, GluR1 subunit expression was again rarely found with a presumed nuclear retention if at all visible (Fig. 1*c*, PDAC). GluR2 subunit staining was seen at the basolateral membrane of acinar cells of NP tissues (Fig. 1*d*, NP). Both in low grade and high grade PanINs, as well as in pancreatic cancer, strong GluR2 subunit expression was found on the membranes of the precursor and the malignant cells (Fig. 1*d*, PanIN1, PanIN2, and PDAC). While a few PanIN1 localized GluR2 to the basolateral membrane of the columnar-shaped cells, its expression was mostly seen on all membrane compartments (Fig. 1*d* PanIN1 and inset). Although in some PanINs, only a minority of the cells were GluR2-positive, nearly all of the cancer cells revealed strong GluR2 immunoreactivity. Thus, no semi-quantitative evaluation was performed.

The GluR4 subunit was found to be expressed in islets in the NP, CP, and PDAC tissue samples. Few CP tissue samples showed GluR4 subunit expression in stromal components (*i.e.*, fibroblasts), accompanied by GluR4 subunit expression in infiltrating immune cells. In PDAC tissue samples, a polymorphic GluR4 expression pattern is seen, with a trend towards increased expression in samples with acinar-to-ductal metaplasia as well as in less differentiated cancer samples (Fig. 1*e*).

Silencing of AMPA receptors as well as their pharmacological inhibition decreases invasion and migration of pancreatic cancer cells

To determine the function of glutamate-AMPA receptor signalling in advanced pancreatic cancer—as reflected by high genetic instability, epigenetic de-regulation and (re-)activation of developmentally important and active pathways—we chose eight well established and characterized pancreatic cancer cell lines (*i.e.*, known mutation status of K-ras and p53, morphology and culture conditions). These cells were subjected to enzymatic measurement of glutamate levels as well as to conventional RT-PCR using oligonucleotides for the amplification of mRNAs encoding GluR subunits 1–4. Glutamate was released by all cell lines with the highest amounts found in the supernatants of Su86.86 cells (Fig. 2*a*); while all GluR subunits (1–4) were expressed by the (K-ras mutated) cell line Su86.86, the other tested cell lines expressed fewer or no transcripts of these receptors (Fig. 2*b*). Thus, for the subsequent experiments, Su86.86 cells were used (unless otherwise indicated).

Interestingly, the amounts of secreted glutamate in cancer cell supernatants were much lower compared to the freshly prepared pancreatic cancer tissue. This might probably be explained by the tissue celularity content. Other cells than tumor cells (fibroblast, nerves) may also contribute to the overall glutamate levels.

Furthermore, we hypothesized from these experiments that there might be an autocrine loop of glutamate-to-AMPAR signalling in Su86.86 cells. Thus, we suppressed GluR1 or GluR2 receptor subunit expression in Su86.86 cells using specific RNAi. In contrast to what would be expected from the known function of Glu receptors, neither silencing of the GluR1 nor the GluR2 receptor subunit had any effect on cancer cell growth (Fig. 2*c*, black *vs*. green dots/lines); in addition, incubation of the cells with the AMPA receptor ligand AMPA did not affect proliferation (Fig. 2*c*, red *vs*. blue dots/lines). However, both GluR1 and GluR2 RNAi reduced the invasiveness of Su86.86 cells in a Boyden chamber assay by ~50% (Fig. 2*d;* white *vs*. black bar); accordingly,

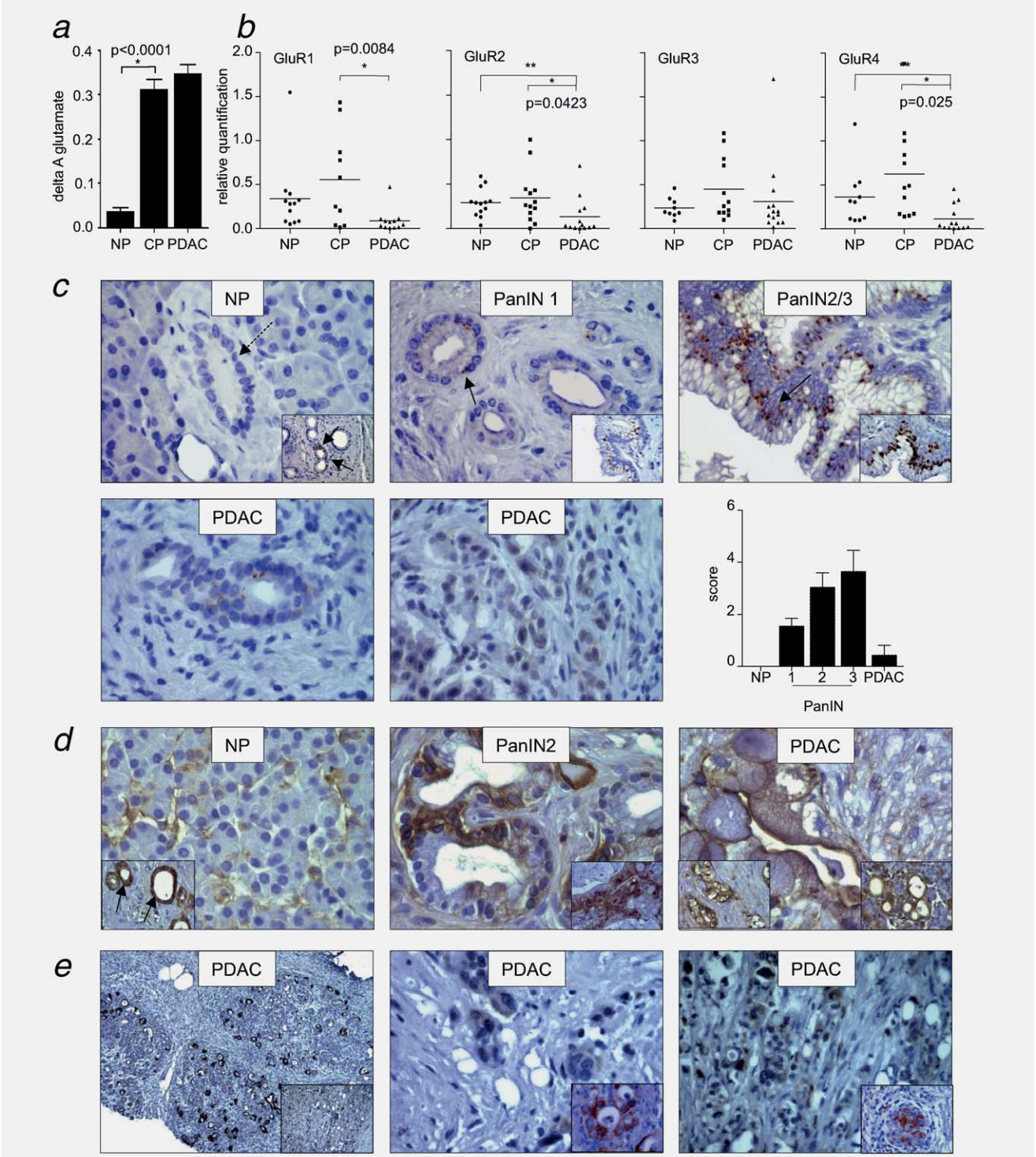

Figure 1. Glutamate levels and GluR1-4 expression in normal and diseased pancreas. (*a*) Levels of glutamate in human pancreatic tissues [normal pancreas, NP ($n = 10$), chronic pancreatitis, CP ($n = 10$) and pancreatic ductal adenocarcinoma, PDAC ($n = 10$)] were determined using an enzymatic assay as previously described.[32] (*b*) RT-PCR (RT-PCR) for GluR1-4 in NP, CP and PDAC tissues was carried out as described previously.[34] Gene expressions were normalized to the human housekeeping gene GAPDH. (*c–e*) Immunohistochemical analysis of GluR1, GluR2, and GluR4 was performed on paraffin-embedded sections of NP ($n = 10$), CP ($n = 10$), and PDAC ($n = 60$) as previously described.[8,16,33] Immunoreactivity of GluR subunits in PanIN structures, cancer cells and/or nerves was quantitatively evaluated according to intensity and area as previously described : the staining intensity of PanIN structures and cancer cells was recorded as "no staining (0)," "weak staining (1)," moderate staining (2)," or "strong staining (3)." The area of stained cells was recorded as <33% (1), 33–66% (2), or >66% (3) of all cancer cells. These numbers were then multiplied resulting in a score of 0–9.

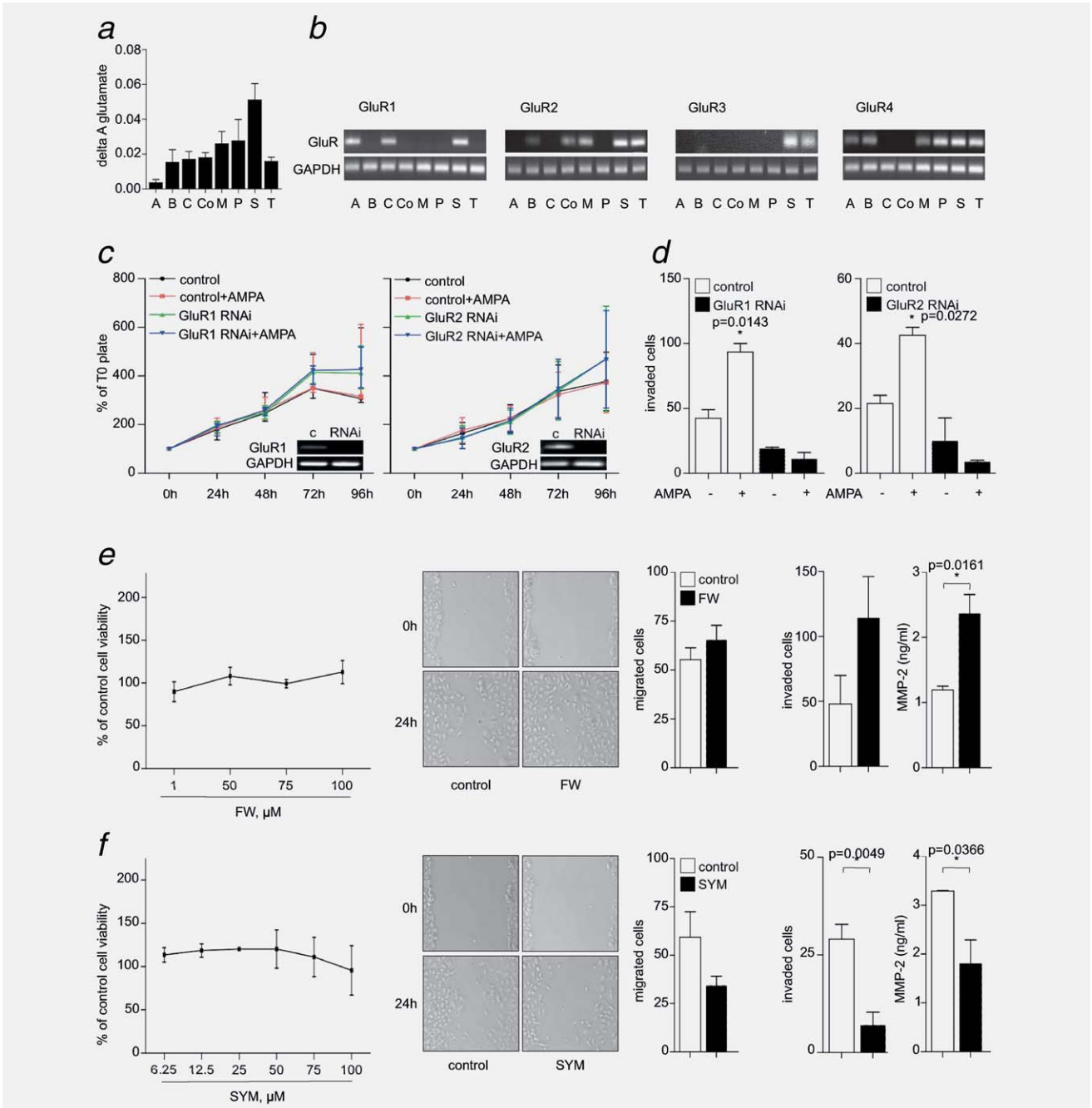

Figure 2. GluR silencing and blockade suppress cancer cell migration and invasion. (*a*) Levels of glutamate in supernatants of 8 pancreatic cancer cell lines (A, AsPC1; B, BxPC3; C, Capan1; Co, Colo357; M, MiaPaCa2; P, Panc1; S, Su86.86; T, T3M4) were determined as described in Figure 1*a*. (*b*) RT-PCR for GluR1-4 in 8 pancreatic cancer cell lines was carried out as described in Figure 1*b*. Fifteen microliter of each reaction product were loaded on an agarose gel; the product was visualized using ethidium bromide. The amplicon sizes of the GluR genes were as follows: GluR1–103bp, GluR2–115bp, GluR3–89bp, GluR4–88bp). (*c*) Effect of GluR1-2 RNAi on cell proliferation. RNAi procedures and cell viability assays were performed as previously described[33] using the HiPerFect transfection reagent. The final concentration of the control and the specific oligonucleotides was 10 nM. Efficacy of the RNAi was analyzed by RT-PCR 24 hr after transfection. For the assessment of the effect of GluR1 and GluR2 RNAi on proliferation, cells were reseeded 24 hr after RNAi cells into 96-well plates. Following incubation for the indicated time points, MTT assays were performed as previously described.[8] Growth curves were calculated as percentage growth of time zero. Experiments were performed in triplicates and repeated at least three times. (*d*) GluR1/2 RNAi decreases cancer cell invasiveness. BD BioCoat Matrigel Invasion Chambers (BD Biosciences, San Jose, CA) were used to determine invasiveness 24 hr after RNAi. The invasion index was calculated as the ratio of the number of invaded cells to the number of invaded control cells. The assays were repeated 5 times. (*e* and *f*) Effect of AMPAR activation/blockade on cell proliferation, migration and invasion. Cell viability and invasion assays were performed as described in the Materials and Methods section. For cell viability assays, cells were exposed to SYM at concentrations of 6.25, 12.5, 25, 50, 75, and 100 μM and to FW at concentrations of 1, 10, 50, 75, and 100 μM for 24 hr. For the invasion assays, FW and SYM (as well as the appropriate controls) were used at concentrations of 10 μM. To evaluate MMP-2 production, supernatants of FW- or SYM-treated Su86.86 cells (24 hr) were subjected to ELISA using a commercially available assay (BD Biosciences Inc., Heidelberg, Germany). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

stimulation with AMPA doubled the number of invaded cells (Fig. 2*d*; $p = 0.0143$ and $p = 0.0272$, respectively; white bars). Furthermore, knockdown of either the GluR1 or GluR2 receptor subunit expression was sufficient to inhibit the pro-invasive capacity of AMPA (Fig. 2*d*, black bars), which underlines the specificity of AMPA toward its receptor [receptor subunit(s)]. To corroborate these findings and to further confirm the specificity of the glutamate-to-AMPAR signal, we used the small molecule AMPA receptor agonist S−(–)-5′-fluorowillardiine (FW) and the AMPA receptor antagonist Sym2206 (SYM). Comparable to the experiments using AMPA and/or RNAi, neither FW nor SYM had any effect on proliferation of Su86.86 cells (both in MTT assays (Fig. 2*e* and 2*f*, left panels) and as determined by Annexin/PI stainings for apoptotic cells (data not shown); however, FW slightly increased migration (scratch assay analysis: (Fig. 2*e* middle panel) and doubled the number of invaded cells in the Boyden chamber assay (Fig. 2*e*, right panel). Accordingly, AMPA receptor antagonism reduced cancer cell migration and invasion (Fig. 2*f* middle and right panels, $p = 0.0049$). Because invasion through the "basement membrane-like" matrigel-coated chamber is—among others—mediated by increased levels of the MMP-2, we determined its concentration in cell culture supernatants of FW- and SYM-treated Su86.86 cells. In line with the results from the invasion assays, FW increased whereas SYM reduced MMP-2 protein levels (Fig. 2*e* and 2*f* right panels, $p = 0.0161$ and $p = 0.0366$, respectively).

AMPA receptor activation increases K-ras activity and signals into the MAPK pathway

To determine the effector pathways activated by the AMPA receptors, Su86.86 cells were stimulated with AMPA for 12 hr. The isolated mRNA was subjected to a "signal transduction" PCR array revealing downregulation of "stress" pathway genes (Fig. 3*a*, green bars) but upregulation of TGFbeta, Jak-Stat and Ca^{2+}/PKC pathway genes (Fig. 3*a*, red bars). These results lead us to hypothesize that—apart from the presumed MAPK pathway induction mediated by GluR activation (see below)—there might also be a direct signal into ras-GTP induction. This assumption is supported by recent reports on AMPA signal transduction in neurons, which have demonstrated that AMPA activation leads to activation of small GTPases of the ras family and also to Ca^{2+}-mediated Ras-GRF/Ras-GRP activation which again induce ras-GTP[35,36]; thus, K-ras activity was determined in a not K-ras-mutated cell line (BxPC3; expressing GluR2). Consistent with our hypothesis, K-ras was strikingly activated following stimulation with AMPA (Fig. 3*b*) in K-ras nonmutated cells. At the same time, phosphorylation of p44/42 was induced following stimulation with FW (Fig. 3*c* and densitometry), which was reversible by pre-incubation with SYM (Fig. 3*c*; $p = 0.0319$). These results demonstrated a direct effect of a glutamate-to-AMPAR receptor signal into the K-ras-MAPK pathway in non-Kras-mutated pancreatic cancer cells.

Inhibition of p44/42 and p38 pathways abrogates GluR-mediated pancreatic cancer cell invasion

To substantiate the finding that the MAPK pathways are crucial for AMPA receptor-mediated cancer cell invasion, we performed invasion assays using FW and specific inhibitors: U0126 (p44/42 inhibitor) and SB203580 (SB, p38 inhibitor). Comparable to the findings above, incubation of Su86.86 cells with FW enhanced p38 and p44/42 phosphorylation (Fig. 3*d*, left panel; completely abrogated by preincubation with the respective inhibitors or a combination hereof) and significantly increased the number of invaded cells (Fig. 3*d*, right panel) as well as the amount of secreted MMP-2 (Fig. 3*e*). Following pre-incubation with either U0126 or SB, these increases were completely blocked; additionally, preincubation of the cells with a combination of U0126 and SB suppressed the number of invaded cells and the amount of secreted MMP-2 far below the base-line (Fig. 3*e* right panels).

AMPA receptor antagonism inhibits pancreatic tumor growth *in vivo*

Having thus found *in vitro* evidence that a glutamate-AMPA signal decreases pancreatic cancer cell invasiveness and migration, we subcutaneously injected athymic nude mice with PDAC cells (Su86.86) and performed "tumor prevention" experiments. Two weeks after tumor cell injection and treatment with SYM and DMSO as a control, subcutaneous tumors were found in four mice in the control group ($n = 5$) (size 1 × 2 mm–2 × 2 mm), whereas in the SYM treated group ($n = 5$), no tumors were visible (Fig. 3*f*). This is of importance because it demonstrates that abrogation of glutamate-AMPA signalling seems to inhibit the settling of the injected tumor cells in the subcutaneous mouse tissue, which might be due to inhibition of an autocrine glutamate signal.

Discussion

Taken together, our results unravel the glutamate-AMPA axis as an important signal transducer towards a more aggressive and invasive pancreatic cancer phenotype. *In vivo*, both autocrine and paracrine mechanisms may contribute to the perpetuation of a glutamate signal, which could also be responsible for the immune response and cytokine release observed in pancreatic cancer. This assumption would be in line with the findings from our pathway array where "intrinsically," AMPA-induced upregulation of, that is, IL-1a, PECAM and myc also suggested an AMPA-induced stress response. Importantly, the functional and morphological findings of this study suggest that in contrast to the NP, the increased tissue glutamate directly activated AMPA receptors on PanIN and/or cancer cells to switch on invasive and migratory programs probably *via* activation of the K-ras/MAPK cascade (Fig. 4). This is of particular interest since it has been shown by a number of reports that there is a link between AMPA and K-ras signalling,[36–38] however, in this case, the link does not seem to be calcium-dependent because RNAi of both a

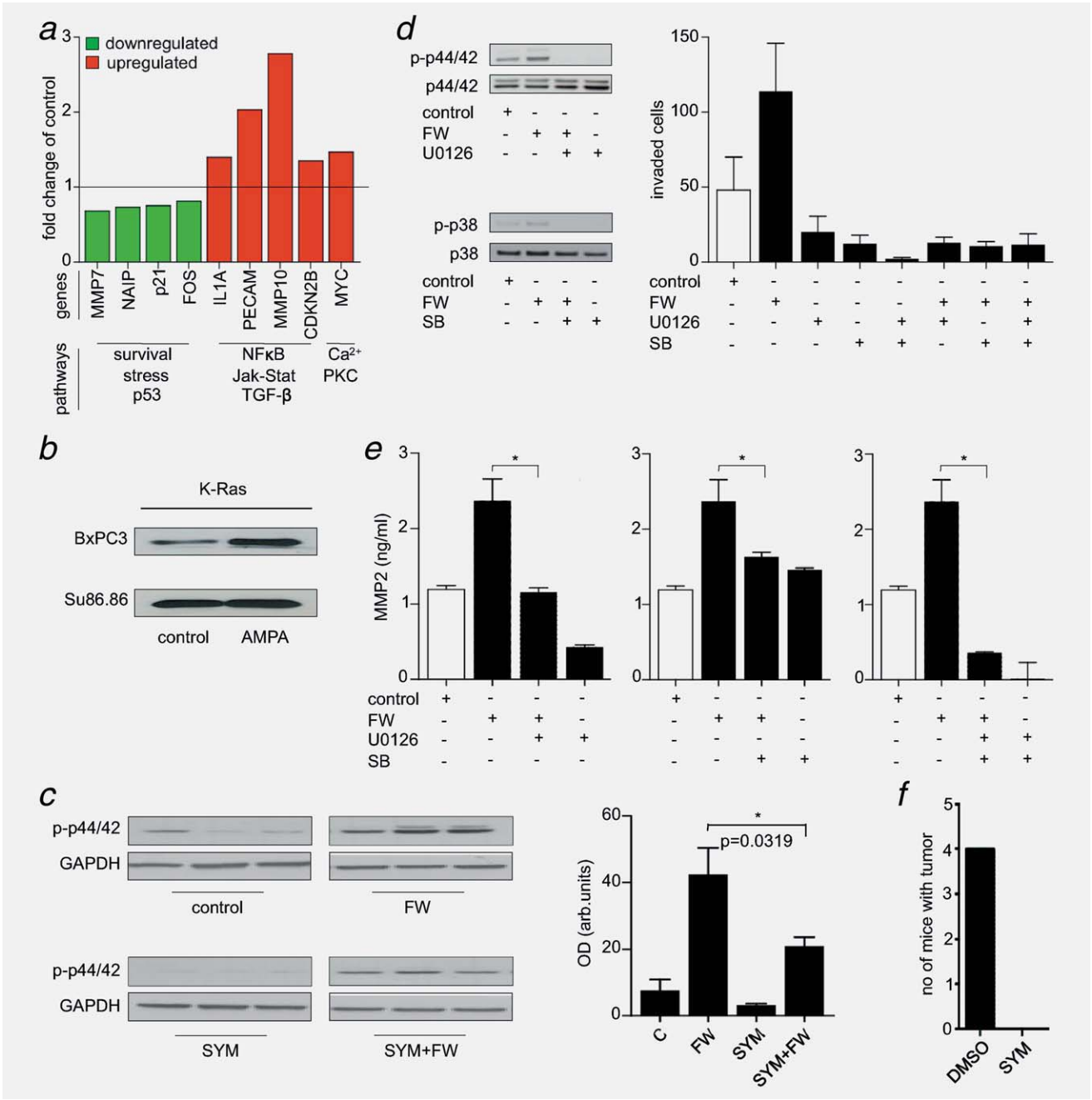

Figure 3. AMPAR activation signals into the K-ras/MAPK pathway. (*a*) AMPA-activated pathways. To evaluate signal transduction following AMPA receptor activation, Su86.86 were treated with 100 μM of AMPA 12 hr. mRNA and cDNA were prepared as described above and were subjected to an RT^2 Profiler PCR array (SABiosciences, Frederick). Genes whose expression varied more than 20% are shown. (*b*) Ras activity assay. Su86.86 and BxPC3 cells were treated with AMPA (100 μM) for 24 hr and were subjected to a ras activity assay (Millipore, Temecula, CA). (*c–e*) p42/44 and p38MAPK signal transduction following AMPAR activation/blockade. To assess downstream effectors following AMPAR activation/blockade, Su86.86 cells were preincubated with SB203580 (SB, p38 inhibitor, 5 μM (when used in combination with U0126) or 10 μM) and/or U0126 [p44/42 inhibitor 5 μM (when used in combination with SB) or 10 μM]. After 24 hr incubation with the drugs, levels of p44/42/phospho-p44/42 and p38/phospho-p38 in the cell lysates as well as levels of MMP-2 in the supernatant were evaluated. (*e*) *In vivo* model of pancreatic cancer. 10^6 Su86.86 cells were injected into the subcutaneous tissue bilaterally at the sites behind the anterior forelimb of 4-week old athymic nude mice. One day after the injection of tumor cells, treatment with the AMPA receptor antagonist SYM (5 mg/kg, $n = 5$; s.c. injection once per day during 2 weeks) or DMSO (1:100, control group, $n = 5$; same injection protocol as for SYM) was started. Tumors/tumor size was evaluated after 2 weeks. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

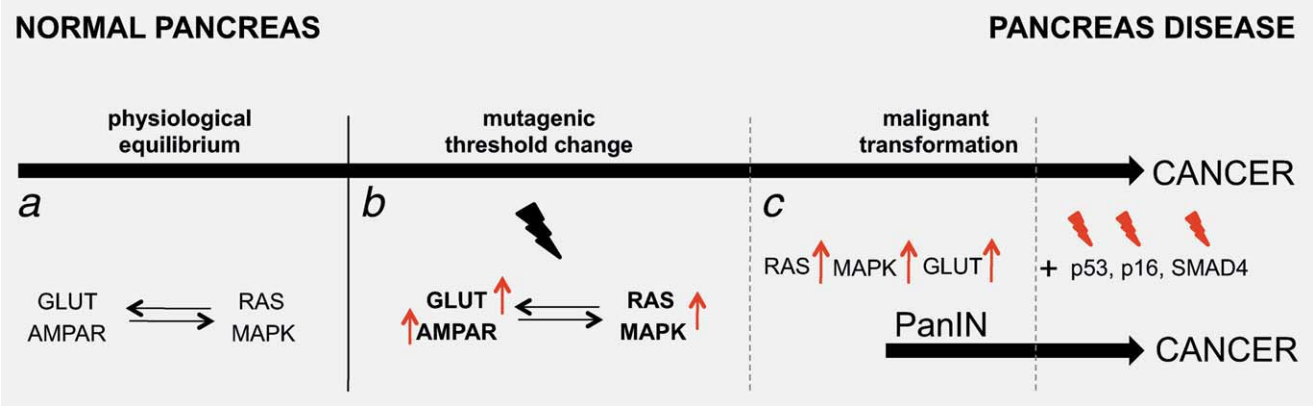

Figure 4. Schematic hypothesis of glutamate-to-AMPAR induced malignant transformation. (*a*) In the NP, there is an equilibrium between glutamate (GLUT)/AMPAR levels, and RAS–MAPK activity. (*b*) This situation changes when levels of GLUT/AMPAR increase, contributing to RAS-MAPK overactivity. (*c*) Mutagenic changes lead to malignant transformation and may contribute to PanIN and later pancreatic cancer development. The cancer cells gain "independency" from the initial mutagenic changes (*i.e.*, K-ras independency) and become more mesenchymal. In this situation, AMPA receptor expression is reduced and seems less important for the transmission of the invasive/migratory program, which is initially induced by increased glutamate levels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

calcium-permeable (GluR1) and calcium-impermeable (GluR2) AMPA subunit was associated with significantly decreased invasiveness. Furthermore, the direct increase of K-ras activity by glutamate-mediated AMPA receptor activation may contribute (at least in not K-ras-mutated cancer cells or in precursor lesions) to a lowering of the mitogenic threshold necessary for malignant transformation (Fig. 4). First of all, this is particularly important in the "early" steps of carcinogenesis in which the level of K-ras activity seems to determine whether or not a cell may undergo malignant transformation.[39] Secondly and because it has recently been demonstrated that inflammation might be instrumental in pancreatic carcinogenesis and progression[40–42] or, depending on the mouse model used, was even a prerequisite for K-ras-dependent oncogenic transformation,[43] the (over-)activation of pathways (*i.e.*, inflammation response signals) which directly increase K-ras activity might contribute to the loss of epithelial homeostasis and integrity. AMPA receptor activation may constitute such a molecular switch through increases in glutamate levels since these were found "already" in CP tissues, where, in contrast to PDAC, AMPA receptor expression was as high as and partially even higher than in the NP. Thus, AMPA receptor activation might be important in the transformation of PanIN to cancer and in the growth of K-ras wild type tumors while in later stages, K-ras independent (but mutated) cancers, high K-ras activity levels may probably not be further increased (as also demonstrated in Panc1 cells, Fig. 3*b*). This assumption is supported by a recent report demonstrating that one class of pancreatic cancer cells required K-ras to maintain viability while others were K-ras-independent.[44] The more "mesenchymal" the cancer cells were, the less they depended on K-ras; at the same time, K-ras-independency correlated with resistance to apoptosis following K-ras inhibition. This observation supports the conclusion that in "later stage" (*i.e.*, more mesenchymal) cancers, a pro-proliferative capacity is exchanged for a more robust invasive/migratory phenotype. Such a supposition would be in line with our finding (which is in contrast with reports from other tumors) that AMPA receptor activation does not increase proliferation of (probably "late" stage, because *in vitro* cultured/passaged) pancreatic cancer cells but significantly enhanced their invasion and migration. However, our findings are derived from *in vitro* experiments and may thus only be an imperfect picture of the *in vivo* situation. This is particularly important when taking into consideration the completely different effects of activation of calcium-permeable versus –impermeable AMPA receptors (*i.e.*, those containing a GluR2 subunit).[45] An adequate dissection of the contribution of the GluR subunits to the observed effects on pancreatic cancer cells and to putative effects in carcinogenesis would only be possible using conditional gene targeting in mice (*i.e.*, using the well-established genetically engineered mouse model of pancreatic cancer which uses expression of an oncogenic Kras from its endogenous locus specifically in the pancreas; plus/minus deletion of the respective GluR subunits). Such experiments will have to be a major part of further analyes of the glutamate/AMPA system in pancreatic diseases.

Conclusion

In conclusion, the glutamate-to-AMPAR signal shown in this report might serve as a molecular switch that decreases the threshold of Kras-induced oncogenic (in this case, invasive/migratory; Figs. 2 and 4) signalling and thus increases the chance of malignant transformation of pancreatic cancer precursor lesions.

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