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Biology Contribution

Therapy-Naive and Radioresistant 3-Dimensional Pancreatic Cancer Cell Cultures Are Effectively Radiosensitized by β1 Integrin Targeting

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Purpose: Pancreatic ductal adenocarcinoma (PDAC) is a cancer with unmet needs. The role of highly conformal radiation therapy is still under debate for PDAC. Owing to its desmoplastic nature, integrin-mediated interactions between PDAC cells and extracellular matrix (ECM) profoundly contribute to PDAC therapy resistance. In this study, we investigated the radiochemosensitizing potential of β1 integrin targeting in therapy-naive and radioresistant PDAC cell cultures grown in 3-dimensional (3D) ECM.

Methods and Materials: In a panel of 3D, ECM-based PDAC cell cultures, β1 integrin was inhibited by antibodies or siRNA-mediated knockdown. Together with x-ray irradiation and specific chemotherapies, we determined 3D colony formation capacity in therapy-naive and radioresistant PDAC cultures. We used kinome profiling, Western blotting, and immunofluorescence stainings to characterize these cell lines. Various siRNA screens were conducted to identify novel therapeutic targets.

Results: We found a significant radiosensitizing potential of β1 integrin inhibition both in therapy-naive and radioresistant PDAC cell cultures. Kinome profiling upon β1 integrin targeting identified a generally declined tyrosine and serine/threonine kinase activity, which presented less prominent in radioresistant than in therapy-naive PDAC cells. siRNA screens employing the top 34 deregulated kinases in combination with β1 integrin inhibition revealed less efficacy and less radiosensitization in radioresistant relative to therapy-naive PDAC cell cultures. Triple inhibition of β1 integrin, protein kinase D1, and rearranged during transfection turned out to be most effective in reducing 3D colony formation of radioresistant PDAC cells.

Conclusions: Our study clearly shows that β1 integrins are robust targets for overcoming radioresistance in PDAC. This seems to apply equally to therapy-sensitive and radioresistant cells. Concerning tumor heterogeneity, this dual therapy-sensitizing potential might be exploitable for a significant improvement of patient survival.

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Disclosures: none.

Research data are stored in an institutional repository and will be shared upon request to the corresponding author.

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Introduction

Despite progress in treatment, pancreatic ductal adenocarcinoma (PDAC) remains a disease with unmet needs.[1,2] PDAC is highly resistant to conventional and molecular therapies mainly resulting from late diagnosis at advanced, metastasized stages as well as its desmoplastic nature, mutational landscape, and intratumoral heterogeneity.[2,3] An additional therapeutic pillar, high conformal and precise radiation therapy, is still under debate for PDAC.[4,5]

Tailored strategies demand a profound characterization of the underlying molecular circuitry of a particular cancer. Owing to its desmoplastic nature, extracellular matrix (ECM) interactions have been intensively investigated in PDAC models.[6-8] Today, the cancer cell adhesion resistome controlling survival-advantaging mechanisms is widely accepted. Various cell adhesion molecules, with integrins being the largest family, fundamentally coregulate resistance to therapy.[9-12]

Rich in collagens and other ECM proteins, PDAC cells express high levels of the cognate integrin receptors.[13,14] Among these, the ubiquitously expressed β1 subunit is central to 12 out of the 24 integrin receptor combinations. Together with different α integrin subunits, β1 integrins serve as receptors for ECM proteins like collagens, laminins, and fibronectin.[15] In cancers, including PDAC, β1 integrins are frequently overexpressed compared with the corresponding normal tissue.[16,17] In PDAC, small interfering RNA (siRNA)−mediated silencing of β1 integrins and other focal adhesion proteins decreased tumor growth and progression and sensitized PDAC cells to x-rays and chemo- or targeted therapies.[7,18-21]

Regarding the potential of radiation therapy as pillar in the multimodal treatment strategy for PDAC and the lack of mechanistic knowledge, we undertook this study to explore the radiochemosensitizing potential of an antibody-mediated β1 integrin inhibition in both therapy-naive and radioresistant PDAC cell lines. For this, cells were cultured in 3D ECM-embedded conditions to avoid poorly physiological 2-dimensional culture conditions. We show strong radiosensitization but no chemosensitization by β1 integrin inhibition in our panel of human PDAC cell cultures and reveal how kinome profiling assists the identification of effective multitargeting approaches to overcome different levels of radioresistance in PDAC cells.

Methods and Materials

Antibodies

The β1 integrin-inhibitory monoclonal antibody AIIB2 was used as published.[22] Nonspecific rat IgG1 was purchased from Santa Cruz Biotechnology (Dallas, Texas). The β1 integrin-inhibitory monoclonal antibody mAb13 was obtained from Merck (Darmstadt, Germany) and nonspecific rat IgG2a was purchased from Invivogen (Toulouse, France). The β1 integrin-activating monoclonal antibody of β1 integrin TS2/16 was obtained from ThermoFisher Scientific (Erlangen, Germany) and nonspecific mouse IgG1 was purchased from R&D Systems (Wiesbaden, Germany). Both inhibitory and control antibodies were used at a concentration of 10 μg/mL. Primary antibodies for β1 integrin were purchased from Calbiochem (San Diego, California; immunofluorescence) and from Abcam (Cambridge, United Kingdom; Western Blotting). Secondary antibodies for Western Blotting antimouse IgG, HRP conjugated and antirabbit IgG, HRP conjugated, were purchased from Pierce (Bonn, Germany; Western Blotting). Immunofluorescence secondary antibody Alexa Fluor594 antimouse IgG was purchased from Life Technologies GmbH (Darmstadt, Germany).

Generation of radioresistant cell cultures

The radioresistant (RR) MiaPaCa-2 cell line (RR-MiaPaCa-2) was generated by 2-Gy x-ray irradiation applied 5 times per week for 4 weeks (40 Gy total dose). During the course of fractionated radiation, cells were passaged when a confluency of 70 to 80 % was reached. Acquired resistance was determined using the 3D colony formation assay.

Photon irradiation exposure

Cells were irradiated at room temperature using 2, 4, or 6 Gy single doses of 200-kVp x-rays (Yxlon Y.TU 320; Yxlon; dose rate ≈1.3 Gy/min at 20 mA) filtered with 0.5 mm Cu as published.[23] The absorbed dose was measured using a Semiflex ionization chamber (PTW Freiburg; Freiburg, Germany).

siRNA and SMARTpool siRNA transfection

siRNAs (siITGB1#1 5‘-GGAACCCUUGCACAAGUGAtt-3‘, siITGB1#2 5‘-GGAUAUUACUCAGAUCCAAtt-3‘, siITGB1#3 5‘-GGAAUGUUCCUAUUUUAACtt-3‘) for β1 integrin and Silencer Negative Control siRNA (5’-AAAACAGUUGCGCAGCCUGAAtt-3’) were purchased from MWG Eurofins (Ebersberg, Germany). ON-TARGETplus SMARTpool consisting of a mixture of 4 siRNAs (used for silencing ITGB1, EGFR, FGFR2, PRKD1 and rearranged during transfection (RET) as well as in the Cherry-pick library) and the ON-TARGET nontargeting pool were produced from Dharmacon and were obtained from Horizon Discovery (Cambridge, United Kingdom). siRNA transfection was performed as previously published.[24] Briefly, for 20 nM siRNA transfection 4 μl Oligofectamine and for 10 nM SMARTpool siRNA 2 μl Lipofectamine RNAiMAX was used. Transfections were carried out under serum-free conditions with Opti-MEM (Invitrogen, Karlsruhe, Germany). After 8 hours or Opti-

MEM plus 10 % FCS (transfection with Oligofectamine) were added to the transfected cells and, after 4 hours, one-third of transfection reagent was exchanged by complete DMEM (Lipofectamine RNAiMAX). Twenty-four hours post transfection cells were used for 3D colony formation assays.

Chemotherapies

Cells were treated with EC_{10} (cell line-dependent) of the chemotherapeutics cisplatin (Hexal AG, Holzkirchen, Germany), gemcitabine (Gemzar, Accord Health care GmbH, München, Germany), and taxol (Sigma Aldrich, Taufkirchen, Germany). For Cisplatin concentrations of 0.015 nM and 0.7 nM, EC_{10} for Colo357 and (RR-)MiaPaCa-2 were applied, respectively. Gemcitabine was used at 15 nM and 40 nM, EC_{10} for Colo357 and (RR-)MiaPaCa-2, respectively. For taxol, 0.9 nM and 1.5 nM, EC_{10} for Colo357 and (RR-)MiaPaCa-2 was applied, respectively. DMSO or complete DMEM was used as controls.

Kinome analysis

Kinome analysis was performed with PamGene technology as previously published.[24] In brief, 1.5 times 10^6 cells were cultured in 0.5 mg/mL lrECM and treated for 1 h with AIIB2 or control IgG after 4 days. To determine differences in the early events on the molecular level, whole cell lysates were harvested 1 hour posttreatment with 3 times kinase buffer (Cell Signaling, Frankfurt, Germany) containing HALT phosphatase and protease inhibitor cocktail (Thermo Scientific, Darmstadt, Germany). The samples were transferred in triplicates to Genomics and Proteomics Core Facility Microarray Unitcenter at DKFZ (Heidelberg, Germany) on dry ice. The phospho-tyrosine kinase (PTK) and serine/threonine kinase (STK) PamChip assays consist of 196 and 144 peptide sequences, respectively. Sample incubation, detection and analysis were performed using the PamStation 12 system. Briefly, after 2% bovine serum albumin in water for 30 cycles blocking the arrays were washed 3 times with protein kinase assay buffer. Phosphorylated peptides were measured using primary (PTK) and primary and secondary (STK) fluorescence labeled antibodies. Imaging of arrays was performed using the PamChip station with a 12-bit CCD camera. BioNavigator software (PamGene International BV) was applied for data analysis of the images obtained from the phosphorylated arrays. A list of significantly phosphorylated peptides was generated of AIIB2-treated to IgG-treated samples. Peptide phosphorylation data was analyzed with GeneGo, PhosphoSite among other databases and KinMap by means of various ranking and scoring methods to predict main down- or upregulated kinases.

Cell culture

Detailed description is provided in the Supplementary Materials and Methods section.

Total protein extraction and Western blotting

Detailed description is provided in the Supplementary Materials and Methods section.

Immunofluorescence staining

Detailed description is provided in the Supplementary Materials and Methods section.

3D colony formation assay

Detailed description is provided in the Supplementary Materials and Methods section.

Statistical analysis

Means $\pm$ standard deviation (SD) of at least 3 independent experiments were calculated. For statistical significance analysis of colony formation assay 2-sided Student's t test was performed with Excel (Microsoft) or one-way analysis of variance followed by post hoc analysis using Tukey's correction was executed in Prism 8 (GraphPad, San Diego, California) and a P value of < 0.05 was considered statistically significant.

Results

Targeting β1 integrins sensitizes 3D PDAC cell cultures to x-ray irradiation

PDAC shows a significant overexpression of β1 integrin mRNA compared with normal pancreatic tissues (oncomine.org) (Fig. 1A),[17,25] which correlates with poorer survival of PDAC patients (TCGA data set; www.oncolnc.org) (Fig. 1B).[26] Consequently, we commenced our study by antibody-mediated β1 integrin inhibition using AIIB2 combined with either x-ray irradiation, 3 different clinically applied chemotherapies or radiochemotherapy in a panel of human PDAC cell lines grown in 3D laminin-rich ECM (lrECM). The cell line panel presented varying β1 integrin protein expression and localization (Fig. 1C-E). Basal survival remained largely unaffected by AIIB2 in the tested cell models (Fig. 1G,H). All PDAC cell cultures were radiosensitized by AIIB2 in a cell line-dependent manner relative to nonspecific IgG controls (Fig. 1G,I; Table E1). Confirmatory data were generated using siRNA-mediated knockdown (Fig. E1). Total, mature and immature expression of

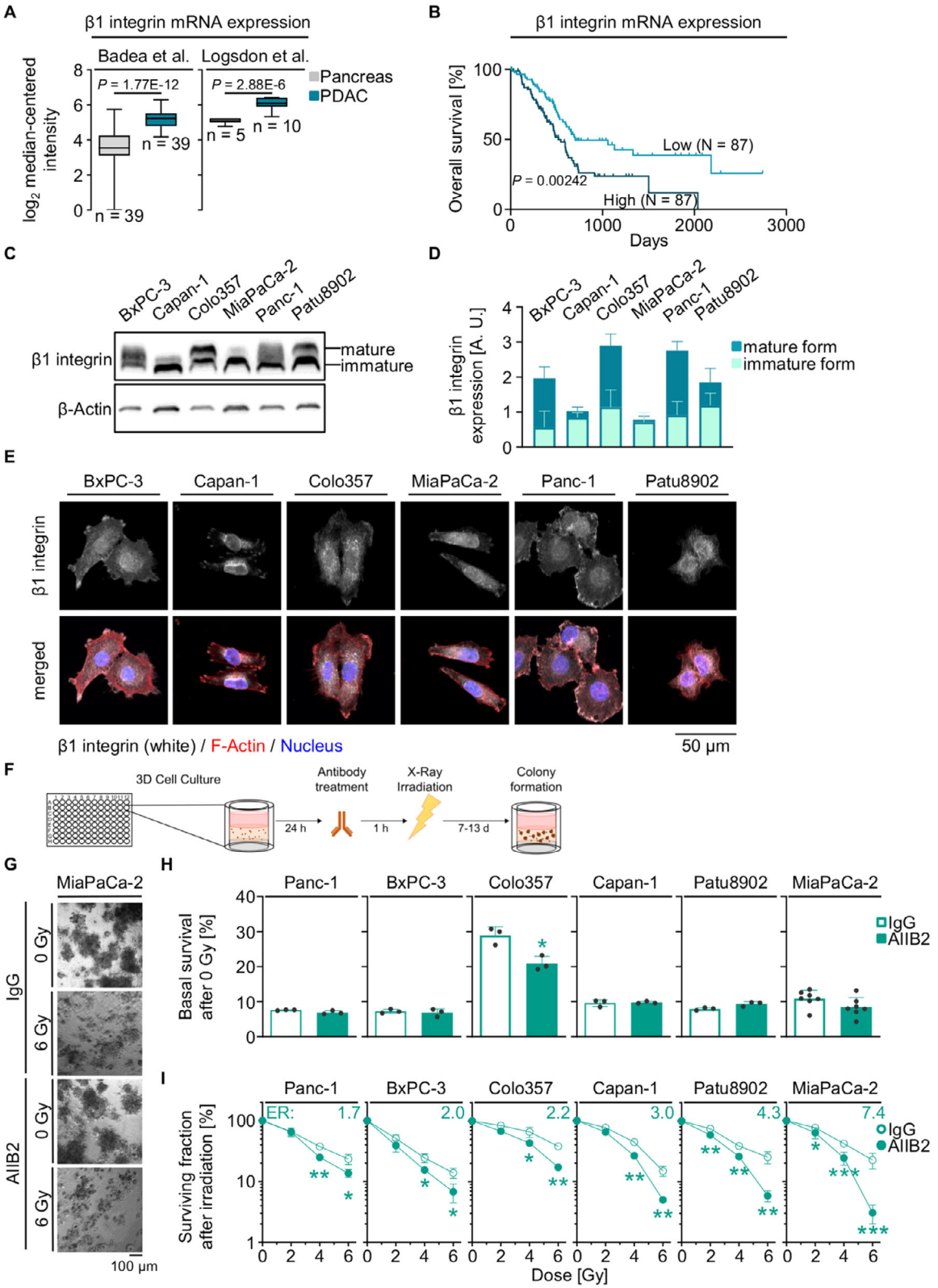

Fig. 1. β1 integrin mRNA is overexpressed in PDAC and its inhibition confers radiosensitization in therapy-naive 3D PDAC cell cultures. (A) Comparative analysis of β1 integrin mRNA expression between PDAC and normal pancreas using Oncomine database (www.oncomine.org). (B) Kaplan-Meier plot of patients with PDAC with tumors expressing low and

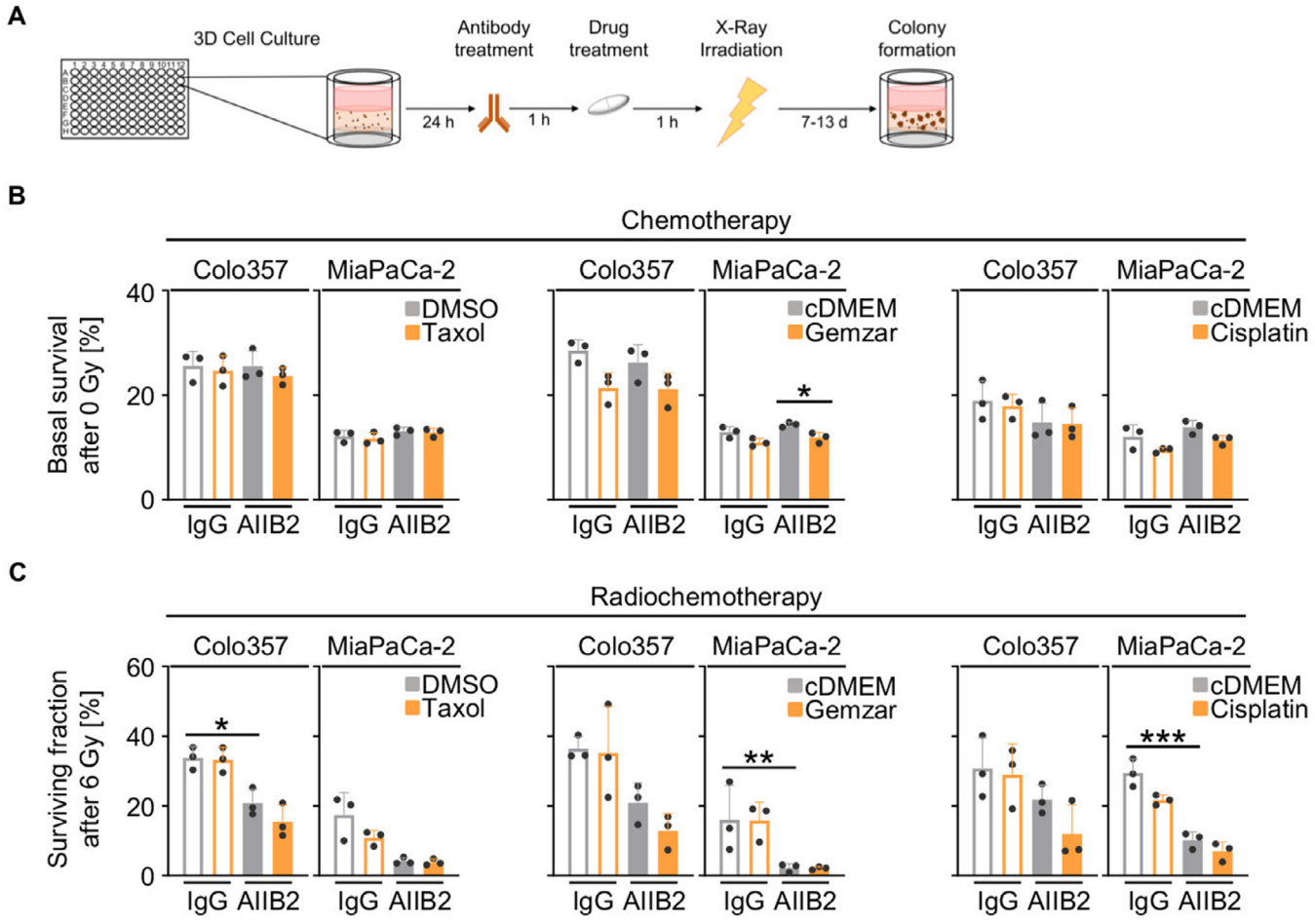

Fig. 2. β1 integrin targeting combined with irradiation is similarly effective as radiochemotherapy. (**A**) Experimental design. (**B**) Basal clonogenic survival of indicated cell lines 2-hour pretreated with AIIB2 and nonspecific IgG control and 1-hour pretreated with EC_{10} of indicated chemotherapies. (**C**) Surviving fraction of indicated cell lines upon 2-hour pretreatment with AIIB2 and nonspecific IgG control and 1-hour pretreatment with indicated chemotherapies combined with 6-Gy irradiation. All results show mean ± standard deviation (n = 3; 1-way analysis of variance followed by post hoc analysis using Tukey's correction; *$P < .05$; **$P < .01$; ***$P < .001$). EC_{10} = effective concentration inducing 10 % cell kill; lrECM = laminin-rich extracellular matrix.

β1 integrins correlated with radiosensitivity upon AIIB2 administration but not with intrinsic (IgG-treated) radiosensitivity or the enhancement of sensitization. (Fig. E2). However, AIIB2 did not change the sensitivity to the chemotherapies tested (Fig. 2A,C). For the combined radiochemotherapy-treated cell lines, there was also no significant difference between AIIB2 and controls treated with DMSO/AIIB2 (Fig. 2A,C). Taken together, our data

high β1 integrin mRNA levels, data provided by OncoLnc (www.oncolnc.org) using The Cancer Genome Atlas (TCGA) with PDAC patient survival data. (C) Immunoblots on whole cell lysates from the 3D cultured PDAC cell line panel showing immature and mature β1 integrin. Actin served as loading control. (D) Densitometry of normalized immature and mature forms of β1 integrin shown in C relative to total expression. (E) Confocal microscopy of immunofluorescence stainings of β1 integrin (white; Phalloidin for F-Actin (red), 4′,6-diamidino-2-phenylindole for nucleus (blue)) in a panel of PDAC cell lines. (F) Experimental design for 3D PDAC colony formation analysis. (G) Representative bright-field images of unirradiated and 6-Gy irradiated antibody-pretreated 3D PDAC colonies. (H) Basal clonogenic survival of unirradiated PDAC cell line panel 1-hour pretreated with AIIB2 and nonspecific IgG control. (I) Surviving fractions of PDAC cell line panel upon 1-hour AIIB2 and nonspecific IgG control plus irradiation. All results show mean ± SD (n = 3; 2-sided *t* test in D, E; *$P < .05$; **$P < .01$; ***$P < .001$). 3D = 3-dimensional; ER (6 Gy) = enhancement ratio after 6 Gy; lrECM = laminin-rich extracellular matrix; PDAC = pancreatic ductal adenocarcinoma.

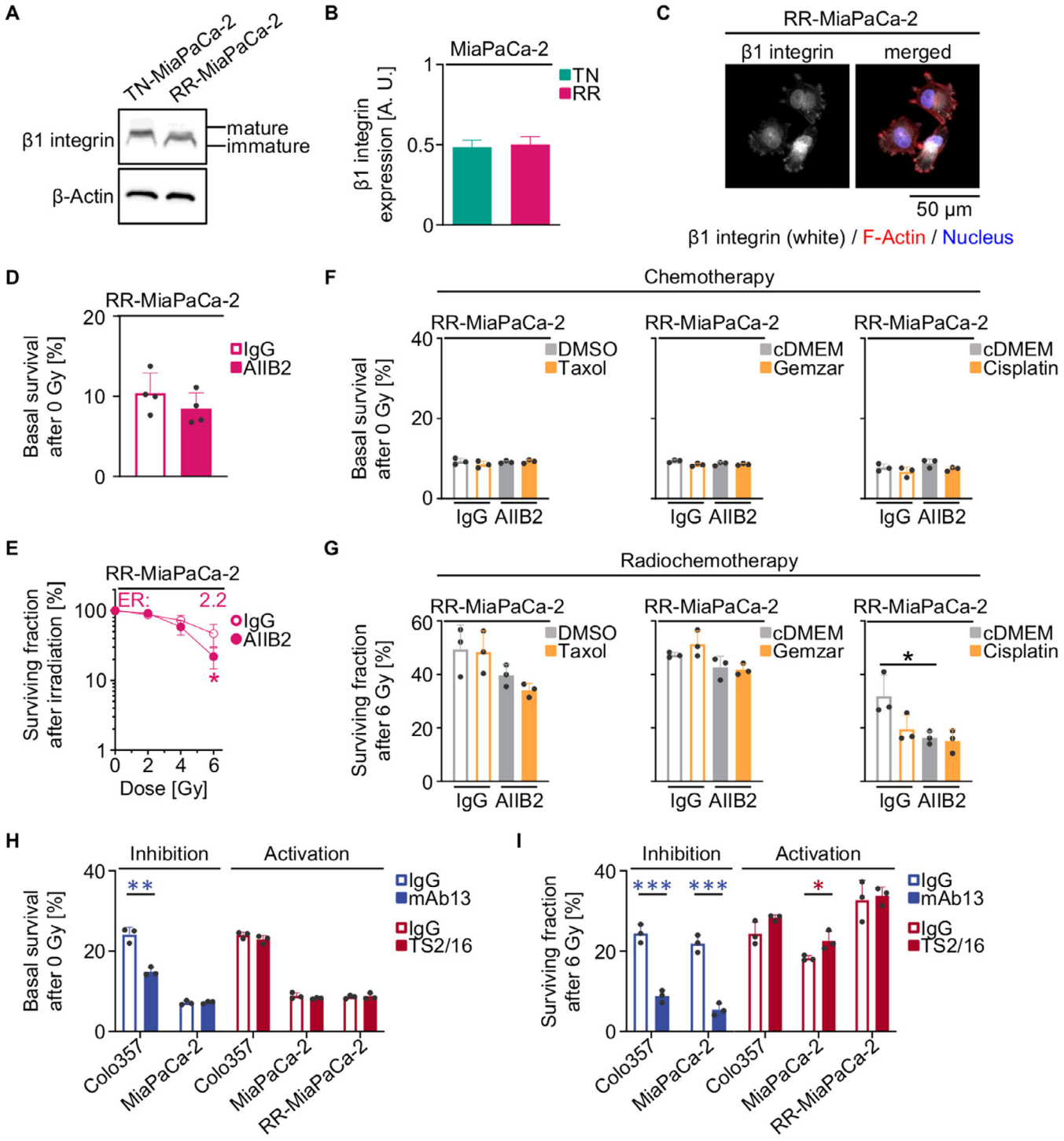

Fig 3. β1 integrin inhibition confers radiosensitization in therapy-naive and radioresistant 3D PDAC cell cultures. (A) Immunoblots on whole cell lysates from the 3D cultured TN- and RR-MiaPaCa-2 cells showing immature and mature β1 integrin. Actin served as loading control. (B) Densitometry of normalized immature and mature forms of β1 integrin shown in A relative to total expression. (C) Confocal microscopy of immunofluorescence stainings of β1 integrin (white; Phalloidin for F-Actin (red), 4′,6-diamidino-2-phenylindole for nucleus (blue) in RR-MiaPaCa-2 cells. (D) Basal clonogenic survival of unirradiated RR-MiaPaCa-2 cell cultures 1-hour pretreated with AIIB2 and nonspecific IgG control. (E) Surviving fraction of RR-MiaPaCa-2 cell cultures upon 1-hour AIIB2 and nonspecific IgG control plus irradiation. (F) Basal survival of unirradiated indicated cell lines 2-hour pretreated with AIIB2 and nonspecific IgG control and 1-hour pretreated with EC_{10} of indicated chemotherapies. (G) Surviving fraction of indicated cell lines upon 2-hour pretreatment with indicated antibodies and 1-hour pretreatment with indicated chemotherapies plus 6-Gy irradiation. (H) Basal clonogenic survival of unirradiated indicated cell lines 1-hour pretreated with mAb13 or TS2/16 and

strongly indicate a sensitizing potential of AIIB2 in 3D PDAC cell cultures exposed to irradiation that is not further enhanced by chemotherapy.

Radioresistant PDAC cell cultures are sensitizable by AIIB2-mediated β1 integrin inhibition

PDAC cell subpopulations present in the tumor before, during or after therapy may express different degrees of therapy resistance. To explore whether AIIB2 is able to sensitize radioresistant PDAC cells to a similar degree as observed for therapy-naive cells (see Fig. 1I), we employed MiaPaCa-2 cells made radioresistant by multiple x-ray irradiations, in which the β1 integrin expression and localization profiles remained unchanged compared with parental MiaPaCa-2 cells (Fig. 3A-C). Basal survival remained unaffected by AIIB2 (Fig. 3D). The colony formation assay showed a 2.1-fold increase in survival for the radiation-resistant PDAC cells at 6 Gy x-rays relative to their therapy-naive counterpart (Figs. 1I and 3E). AIIB2 was able to reduce the survival at 4 Gy (nonsignificant) and at 6 Gy (significant) (see Table E1) compared with IgG controls (Fig. 3E). The AIIB2 survival curve appears very similar to that of the parental, IgG-treated curve (see Fig. 1I). Similar to parental cells, radioresistant MiaPaCa-2 cell cultures failed to show chemosensitization (Fig. 3F,G). In our hands, although similar cell culture conditions were applied, there appeared an inexplicable intertrial variability between our testing of Taxol and Gemzar versus Cisplatin in combination with IgG, AIIB2 or irradiation (see Figs. 2C and 3G). However, the trend toward a higher cell kill upon AIIB2 remained detectable (see Figs. 2C and 3G). Generally, confirmatory data were generated by siRNA-mediated ITGB1 knockdown as well as a second β1 integrin-inhibiting (mAb13) and a β1 integrin-stimulating (TS2/16) antibody in therapy-naive and radioresistant PDAC cell cultures (Fig. 3H,I; also see Fig. E1). Our findings reveal that also radioresistant 3D PDAC cell subpopulations can be radiosensitized by antibody-mediated β1 integrin inhibition.

Kinome profiles of therapy-naive *versus* radioresistant PDAC cell cultures reveal great differences upon inhibition of β1 integrins

Given the radiosensitization by AIIB2 in therapy-naive and radioresistant 3D PDAC cultures to be qualitatively similar but quantitatively different, we sought to unravel the kinome upon AIIB2 exposure by means of PamGene technology. After a 1-hour AIIB2 incubation, PTK and STK measurements revealed less differences in peptide phosphorylation intensities (PPIs) in radioresistant than therapy-naive MiaPaCa-2 cell cultures relative to controls (Fig. 4A-C, Table E2) (illustrated by negative ΔPPI values). Concerning kinase activities bioinformatically deduced from phosphorylation changes of peptides, we found that affected kinases differed between AIIB2-pretreated therapy-naive and radioresistant MiaPaCa-2 cell cultures as well as the extent of downregulation (Fig. 4D,E; Fig. E3A, B; Table E3). We further focused our analysis on the top 34 candidates consisting of 23 PTK and 11 STK, defined by a high median final score (see Figs. 4E and E3A,B). Taken together, the downregulation of PTK and STK after inhibition of β1 integrin is less pronounced in radioresistant PDAC cell cultures than in their parental counterpart, which indicates different signaling pathways and associated mechanisms.

The degree of radiosensitization through simultaneous targeting of β1 integrins and protein kinases differs greatly between therapy-naive and radioresistant PDAC cell cultures

Subsequently, we took these 34 kinase candidates to decipher their potential to further enhance the marginal radiosensitization elicited by β1 integrin inhibition in radioresistant MiaPaCa-2 cell cultures. The knockdown of these 34 kinase candidates alone or in combination with β1 integrins demonstrated considerable similarity of the knockdown approaches without irradiation between therapy-naive and radioresistant cell models (Figs. 5A and E4A,B). In this setup, single knockdown of β1 integrin led to a 2-fold enhancement of the radiosensitivity of therapy-naive compared with a 1.3-fold enhancement in radioresistant MiaPaCa-2 cell cultures (Fig. 5A, Table E4). The simultaneous knockdown of a PTK or STK together with β1 integrins, however, pinpointed a fundamental discrepancy between these 2 cell populations (see Figs. 5A and E4A,B). Although radioresistant cells exhibited a response pattern with a maximum enhancement ratio (ER) of 1.6 (Figs. 5A, B and E4C), therapy-naive 3D MiaPaCa-2 cell cultures showed ER up to 3.7-fold upon double depletion approaches (see Fig. 5A,B and Table E4). In conclusion, our findings suggest that radioresistant PDAC cells undergo yet to-be-determined survival-promoting changes that make them resistant to both single and double inhibition of β1 integrin and protein kinases.

nonspecific IgG controls. (I) Surviving fraction of indicated cell lines 1-hour pretreated with mAb13 or TS2/16 and nonspecific IgG controls plus 6-Gy irradiation. All results show mean ± standard deviation (n = 3; 2-sided *t* test in H, I; 1-way analysis of variance followed by post hoc analysis using Tukey's correction in F, G; $*P < .05$; $**P < .01$; $***P < .001$). 3D = 3-dimensional; EC_{10} = effective concentration inducing 10 % cell kill; ER (6 Gy) = enhancement ratio after 6 Gy; PDAC = pancreatic ductal adenocarcinoma; RR = radioresistant; TN = therapy-naive.

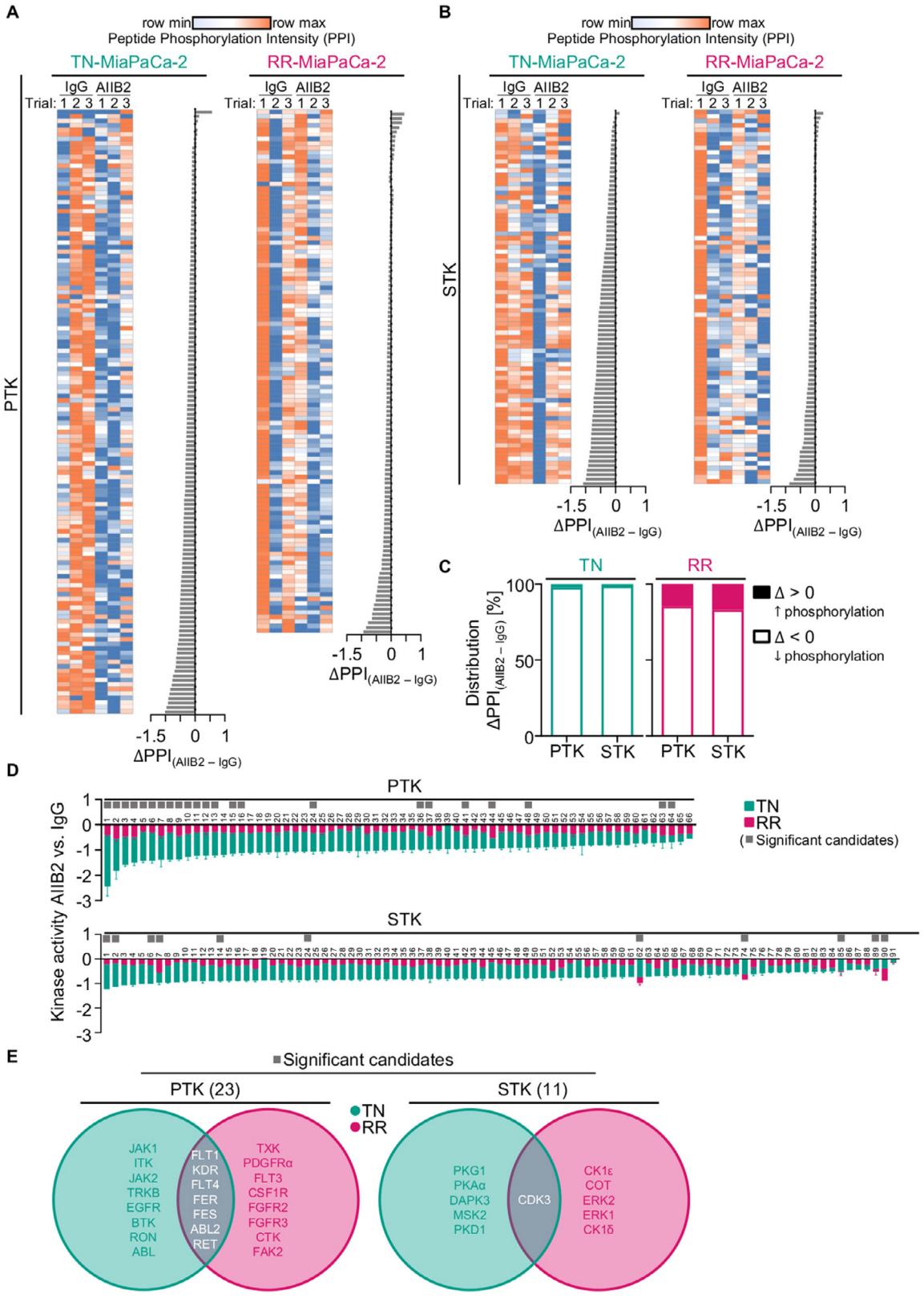

Fig. 4. β1 integrin inhibition decreases kinome activity both in therapy-naive and radioresistant 3D PDAC cultures. Peptide phosphorylation measurements by PamGene technology of (A) PTK array and (B) STK Array in 3D TN- and RR-MiaPaCa-2 cultures 1 h after AIIB2 or IgG administration depicted in heatmaps (generated with https://software.broadinstitute.org/morpheus/; raw data in n = 3; Table S2) and ΔPPI values (PPI_{AIIB2} - PPI_{IgG}) shown in waterfall blots. (C) Distribution of ΔPPI

Triple targeting of PKD1 and RET together with β1 integrin significantly mitigates survival of radioresistant 3D PDAC cell cultures

Next, we conducted a triple targeting approach to significantly increase the cell kill of radioresistant PDAC cell cultures. We limited the approach to candidates who showed an ER ≥1.3 in the double targeting (see Fig. 5B, blue rectangles). These top 14 kinase candidates underwent a pathway overrepresentation analysis. Predominantly growth factor receptor signaling associated with vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), and fibroblast growth factor (FGF) signaling was linked to these 14 kinases, complemented pathways indicated involvement in immune responses, vascular system and other noncancerous diseases or hormonal signaling (Fig. 5C). Based on the highest ER for FGFR2, PKD1, EGFR, FES, and RET (see Fig. 5A,B, magenta stars), we subsequently performed a triple targeting, which included depletion of β1 integrin and PKD1 (as most effective kinase targeting) plus depletion of either EGFR, FES, FGFR2, or RET (Figs. 5D and E4D). The triple combinations including EGFR, FES, or FGFR2 failed to enhance the radiosensitivity of radioresistant MiaPaCa-2 cells over the level observed for β1 integrin depletion alone (see Fig. 5D). In contrast, triple targeting of RET, PKD1, and β1 integrin resulted in an ER of 2.2 (see Fig. 5D). This ER surpassed the ER of 1.8 upon simultaneous β1 integrin/RET depletion in therapy-naive MiaPaCa-2 cells (see Fig. 5A and Table E4). Collectively, our data indicate a high degree of insusceptibility to protein kinase targeting for radiosensitization in radioresistant 3D PDAC cultures. Despite the fact that additional radioresistant PDAC cell models need to undergo analysis, a triple targeting approach of RET, PKD1 and β1 integrin successfully reduced the radioresistant phenotype of 3D MiaPaCa-2 cell cultures.

Discussion

Resistance mechanisms present multifaceted and highly variable among the different cancer types. Independent of their origin, either inherent or acquired, they account for an unforeseeable survival-advantaging behavior. Based on the desmoplastic nature of PDAC, cell-ECM interactions fundamentally contribute to the therapy-resistant phenotype. In the present study, we addressed 2 points in therapy-naive and radioresistant PDAC cell models: (1) the therapy-sensitizing potential of an antibody-mediated β1 integrin inhibition and (2) the exploitability of kinome changes induced by a β1 integrin targeting. We found that (1) β1 integrin mRNA is overexpressed in PDAC relative to normal pancreas and correlative to poorer patient survival, (2) β1 integrin targeting significantly radiosensitizes both therapy-naive and radioresistant 3D PDAC cell cultures, (3) β1 integrin inhibition results in an overall downregulation of kinase activities in both therapy-naive and radioresistant PDAC cultures, (4) a double targeting of kinases plus β1 integrin elicits strong radiosensitization in therapy-naive but not radioresistant 3D cultures kinase-dependently, and (5) a triple targeting of β1 integrins together with PKD1 and RET is an effective radiosensitizing strategy in radioresistant PDAC cells.

Intratumoral heterogeneity is known to modulate inherent and acquired therapy resistance in several cancer types, including PDAC.[27,28] The fact that all PDAC cell lines tested in this study were radiosensitized by β1 integrin targeting suggests that different levels of resistance, as we would expect from our knowledge of intratumoral heterogeneity, can be similarly addressed by this treatment approach. Our previous studies and the work of others in various cancer types originating from, for example, head and neck, breast, lung, prostate, chordoma, brain, demonstrated the cell death-inducing and therapy-sensitizing potential of β1 integrin inhibition mediated by either small interfering RNA or antibodies like AIIB2.[19-21,29-33] Here we show, for the first time to our knowledge, that not only therapy-naive but also radioresistant PDAC cell cultures are radiosensitized upon AIIB2-mediated β1 integrins inhibition.

Although a radiosensitizing efficacy of β1 integrin targeting can be found in different tumor entities, the underlying mechanisms of action differ markedly. Recent work documented the heterogeneous involvement of focal adhesion- and nonfocal adhesion-related kinases such as FAK, Src, c-Abl as well as c-Jun N-terminal kinase 1 (JNK1), Akt, EGFR, MEK1/2, respectively, in β1 integrin signaling in PDAC, head and neck cancer or glioblastoma.[29,34-36] There are even greater knowledge gaps in the field of network adaptation during and after a cancer therapy. This and the diversity in signaling networks among different cancer types prompted us to perform a comparative broad-spectrum kinome profiling in therapy-naive and radioresistant 3D MiaPaCa-2 cell cultures that express varying susceptibility toward AIIB2. Despite the fact that AIIB2 per se failed to mediate strong antisurvival effects, β1 integrin deactivation obviously modulated the response behavior to genotoxic stress, which translated into significant enhancement of PDAC cell radiosensitivity.

Our kinome data outline 3 important points: (1) a surprising lack of activated kinases upon β1 integrin inhibition, (2) β1 integrin targeting elicits more extensive

values from A and B. (D) Superimposed waterfall blots demonstrating PTK and STK activity in AIIB2-treated 3D TN- and RR-MiaPaCa-2 cultures (kinases in Table S3; gray rectangles = high significance and specificity). (E) Venn diagrams comparing kinases provided by median final score (high significance and specificity) in AIIB2-treated 3D TN- and RR-MiaPaCa-2 cultures. 3D = 3-dimensional; PDAC = pancreatic ductal adenocarcinoma; PPI = peptide phosphorylation intensity; PTK = phospho-tyrosine kinase; RR = radioresistant; STK = serine/threonine kinase; TN = therapy-naive.

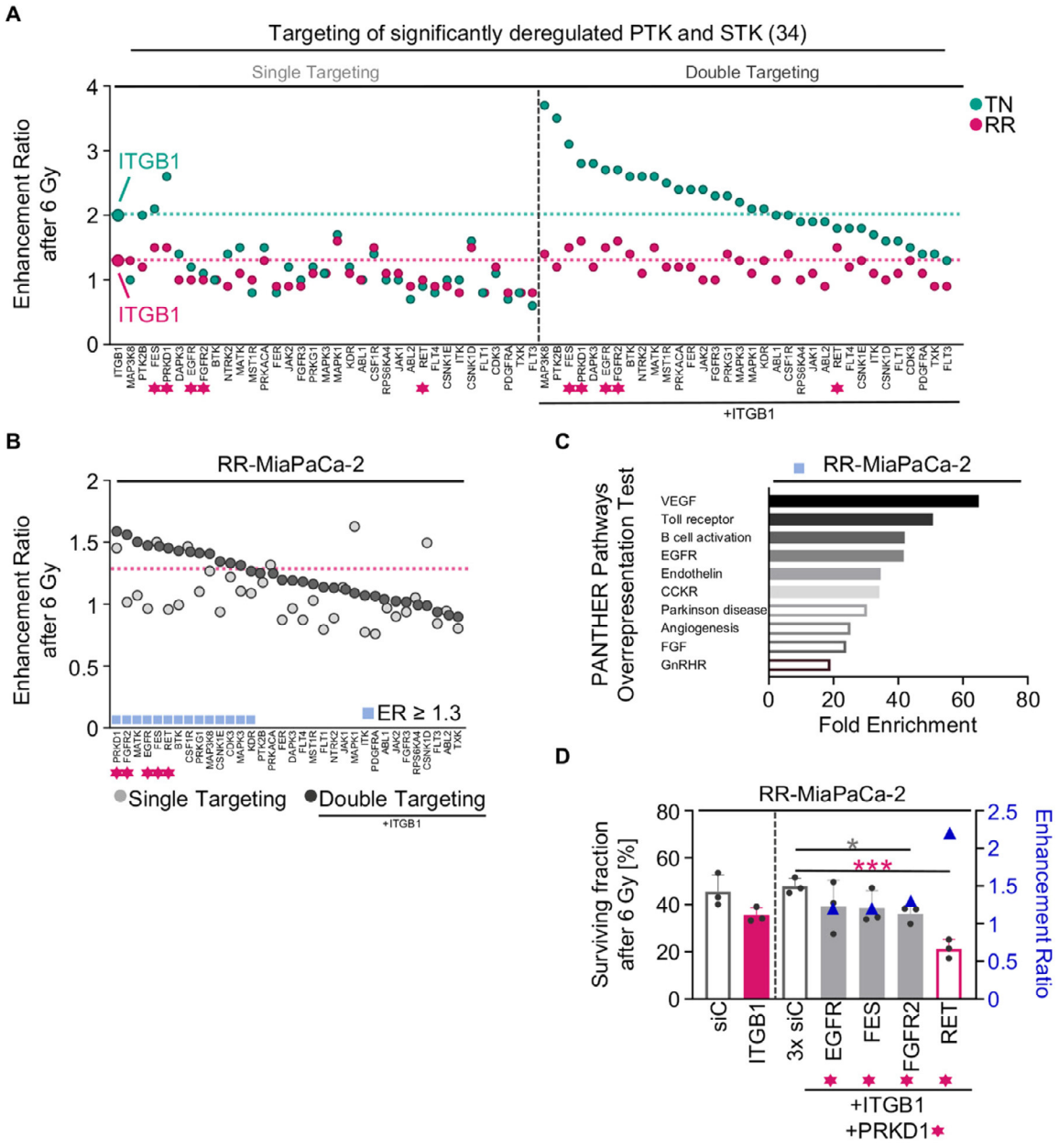

Fig. 5. Triple targeting of β1 integrin, PKD1 and RET is highly effective in overcoming the radioresistance phenotype of 3D PDAC cultures. (A) Enhancement ratios (surviving fraction upon siC and 6 Gy/surviving fraction upon knockdown and 6 Gy; see Fig. S4, Table S4) depicted as circles ordered by ER of double knockdown in TN-MiaPaCa-2 cell cultures. (B) Enhancement ratios in RR-MiaPaCa-2 cell cultures ordered by ER of double knockdown; blue rectangles mark ER ≥1.3. (C) Overrepresentation test of kinases with ER ≥1.3 when depleted in combination with β1 integrin in 3D RR-MiaPaCa-2 cell cultures (http://geneontology.org/). (D) Normalized clonogenic survival of 3D RR-MiaPaCa-2 cell cultures upon single and triple siRNA-mediated knockdown and 6-Gy irradiation and corresponding enhancement ratios. In all graphs gene names are given. All results show mean ± standard deviation (n = 3; 2-sided *t* test; $^{**}P < .01$; $^{***}P < .001$); stars mark targets presenting ER >1.3 in RR-MiaPaCa-2 cell cultures. 3D = 3-dimensional; C = control; CCKR = cholecystokinin receptor; ER (6 Gy) = enhancement ratio after 6 Gy; GnRHR = Gonadotropin-releasing hormone receptor; PDAC = pancreatic ductal adenocarcinoma; PKD1 = protein kinase D1; RET = rearranged during transfection; RR = radioresistant; TN = therapy-naive.

reductions of PTK and STK activity in therapy-naive than radioresistant cells, and (3) signaling networks in radioresistant PDAC cells seem to be less dependent on β1 integrin than therapy-naive cells. Obviously, β1 integrin inhibition failed to evoke activation of pro−survival signaling eliciting adaptation and bypass mechanisms. In contrast, blocking of β1 integrins in HNSCC resulted in EGFR hypersignaling as a bypass.[26] In pancreatic and lung cancer,

reports illustrated opposite resistance to EGFR inhibition induced through bypass mechanisms via β1 integrin signaling.[35,37] Lesniak et al revealed that trastuzumab efficacy was circumvented by β1 signaling in HER2-positive breast cancer cell lines.[38] In squamous cell lung carcinoma, an interaction of the soluble splice variant of VEGFR1-13 with β1 integrins exerted an activation of VEGFR1 and 2 signaling, thus, affecting the response to antiangiogenic drugs.[39] Moreover, simultaneous JNK inhibition profoundly amplified killing of glioblastoma cells refractory to β1 integrin targeting alone.[36]

Although therapy-naive PDAC cells indicated to be easily killed by numerous double targeting combinations, we continued our study by focusing on those kinases with the largest enhancement ratio from the double targeting approach in radioresistant cells. PANTHER-based pathway analysis predominantly revealed growth factor receptor-mediated signaling pathways associated with VEGF, EGFR and FGF. However, only the triple targeting of RET together with β1 integrin and PKD1 surpassed double efficacies and reached an ER similar to the one found in the β1 integrin-inhibited therapy-naive counterpart. Opposingly, specific inhibition of EGFR, FGFR or VEGFR has previously been reported to radiosensitize and induce proapoptotic mechanisms in 2-dimensional grown pancreatic cancer cell lines.[40-45] Cockburn et al observed that β1 integrin activity was affected by RET, and RET-mediated cell adhesion and migration depend on β1 integrins.[46] Although the importance of RET for radioresistance is novel in PDAC, Znati and colleagues showed RET inhibition with the small molecule inhibitor vandetanib against VEGFR and RET to radiosensitize hepatocellular carcinoma cells.[47] PKD1 is known to play a role in viability and clonogenicity of pancreatic cancer cells.[48] So far, no interrelation between β1 integrins or radiosensitization and PKD1 has been reported. Beyond the scope of our study, additional potential targets involved in resistance and bypass mechanisms require attention. For example, Wiechmann et al discovered the radiosensitizing potential of inhibiting the intrinsically enhanced FAK activity in radioresistant murine PDAC cell lines.[49] Likewise, acquired radioresistance is likely related to alterations in the DNA repair machinery. In radioresistant PDAC as well as breast cancer cells, pharmacologic inhibition of DNA repair and cell cycle regulators conferred radiosensitization.[49,50]

Conclusions

Our study clearly shows that β1 integrins are robust targets for overcoming radioresistance in PDAC. This seems to apply equally to therapy-sensitive as well as radioresistant cells. Concerning tumor heterogeneity, this dual therapy-sensitizing potential might be exploitable for a significant improvement of patient survival. Despite these promising results, which once again show the potential of radiation therapy in combination with molecularly targeted therapies, further investigations in more translational PDAC models are warranted.

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