

Pancreatic Stellate Cells Radioprotect Pancreatic Cancer Cells through β 1-Integrin Signaling

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is characterized by a strong desmoplastic reaction where the stromal compartment often accounts for more than half of the tumor volume. Pancreatic stellate cells (PSC) are a central mediator of desmoplasia. There is increasing evidence that desmoplasia is contributing to the poor therapeutic response of PDAC. We show that PSCs promote radioprotection and stimulate proliferation in pancreatic cancer cells (PCC) in direct coculture. Our *in vivo* studies show PSC-dependent radioprotection in response to a single dose and to fractionated radiation. Abrogating β 1-integrin signaling abolishes the PSC-mediated radioprotection in PCCs. Furthermore, this effect is independent of PI3K (phosphoinositide 3-kinase) but dependent on FAK. Taken together, we show for the first time that PSCs promote radioprotection of PCCs in a β 1-integrin-dependent manner. *Cancer Res*; 71(10); 3453–8. ©2011 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the major malignancies in the Western world (1). It is characterized by a late-stage diagnosis, rapid development of metastasis (1) and a pronounced fibrotic reaction known as desmoplasia (2). This enrichment and activation of the stromal compartment surrounding and infiltrating the cancer is believed to have a detrimental effect on the response to chemotherapy and radiotherapy (3, 4). Nevertheless, radiotherapy was shown to be effective especially in the treatment of locally advanced disease as recently shown in a randomized trial (ECOG-4201) and even complete pathologic treatment response was reported (5).

In recent years, pancreatic stellate cells (PSC) gained interest, as these are believed to drive the desmoplastic reaction. PSCs differentiate from a quiescent state into activated myofibroblast-like cells in response to oxidative stress as well as secreted growth factors and cytokines from tumor cells (3). Activated PSCs produce extra cellular matrix (ECM) proteins that modulate the stroma and stimulate fibrosis (6–8). Moreover, PSCs express various growth factors, cytokines, and matrix metalloproteinases involved in stimulating proliferation, migration, and invasion of pancreatic cancer cells (PCC;

refs. 9, 10). Altogether, it is becoming increasingly clear that PSCs contribute to the malignant phenotype of PDAC.

Cell adhesion to the ECM is crucial for the regulation of tissue homeostasis and cell fate and is mediated through the integrin family of transmembrane surface receptors (11). Integrins are known to modulate the cellular response to genotoxic injury, and β 1-integrin in particular is implicated in mediating cell survival in response to radiation in different cancer cell lines (12–14). Hence, desmoplasia-induced changes in composition of the ECM surrounding PDAC are likely to impact on PCCs through integrin signaling.

In this study, we investigated the effects of radiation on PCCs in the presence of a stromal component, the PSC, *in vitro* and *in vivo*. In both conditions, PSCs mediated radioprotection of PCCs.

Materials and Methods

Cell culture

Panc-1 and MiaPaCa-2 were obtained from American Type Culture Collection, and PSN-1 through an MTA from Merck & Co., Inc. LTC-14 was kindly provided by Dr. G. Sparmann (Rostock, Germany). The human pancreatic stellate cell line hPSC and cell culture conditions are described in Supplemental Figure S1.

Clonogenic survival assay

For coculture assays, hPSCs were allowed to attach overnight before plating of PCCs in fresh medium. For LTC-14–PCC cocultures, both cell lines were seeded simultaneously. All mono- and cocultures were incubated for 5 hours prior to radiation (XRT) in a cesium source irradiator (IBL 637; CIS Bio International) at a dose rate of 0.98 Gy/min. The β 1-integrin blocking antibody MAB17781 (R&D Systems) or 20 μ mol/L LY294002 (Calbiochem) was added to cells 2 hours prior to radiation. Colonies were stained with crystal violet (Pro-Lab

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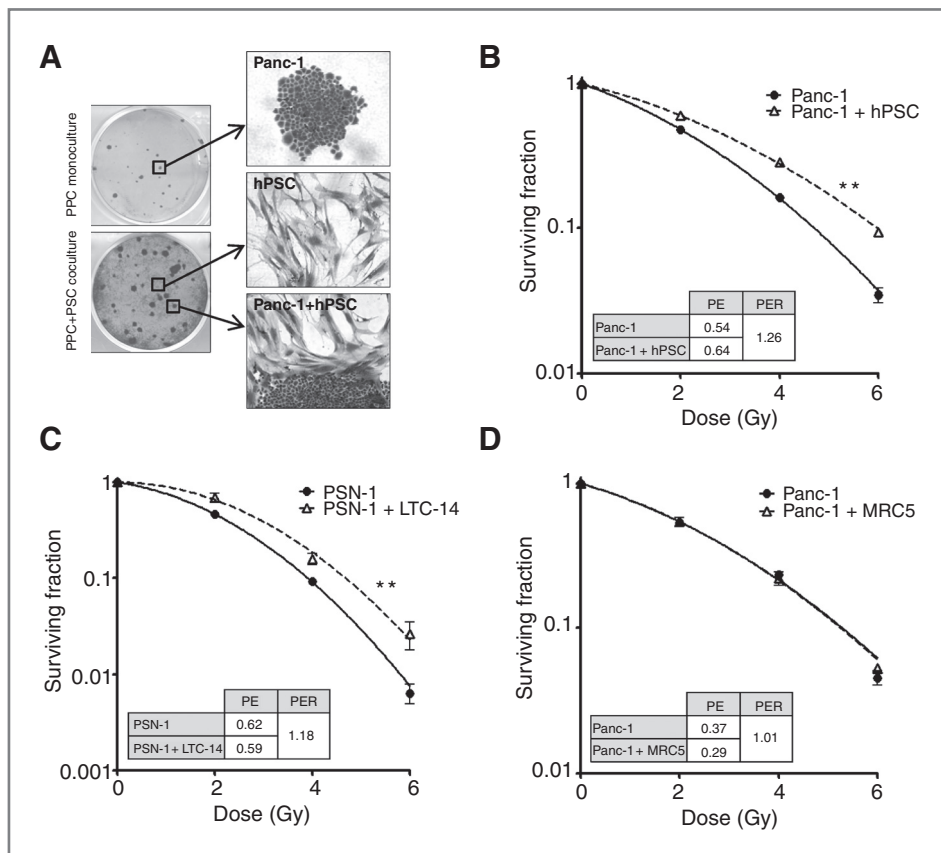


Figure 1. A, tumor cell colonies from a clonogenic survival assay with PCCs in monoculture and PCCs in coculture with PSCs. B, clonogenic survival curves of Panc-1 coculture with hPSCs; **, *F*-test, $P = 0.001$. C, PSN-1 coculture with LTC-14; **, *F*-test, $P = 0.001$. D, Panc-1 coculture with MRC5 fibroblasts. Graphs are representative of at least 3 independent experiments. Error bars are contained within the points. Inserts: PE, plating efficiency; PER, protection enhancement ratio.

Diagnostia) 10 to 14 days later and counted. The surviving fraction was calculated as described previously (15). The protection enhancement ratio was used to quantify radiosensitization and was calculated as described in Supplementary Figure S4.

In vivo experiments

All animal procedures were carried out in accordance with current U.K. legislation under an approved project license. Female nude mice were divided into 2 groups receiving injections subcutaneously into the flank with 1×10^6 PSN-1 with or without 4×10^6 LTC-14. Animals were assigned randomly to receive a 6-Gy single dose in one experiment or 3.5 Gy on 3 consecutive days in another experiment under anesthesia, when the tumors had attained a volume of 50 mm^3 . Tumor growth was measured regularly by callipers. In the single-dose experiment, one animal in the PSN-1 control group was terminated at day 5 because of gait restraint. The tumor growth curve was extrapolated to day 7 by fitting to the linear growth equation ($y = ax + b$) according to the trend of the other 5 growth curves in this group.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism (GraphPad Software, Inc.). Comparisons were made between whole clonogenic survival curves by use of the *F*-test. For all other significance testing a 1-way ANOVA with Tukey's multi-

ple comparison posttest was used. Statistical difference was denoted as follows: *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. All data are presented as mean \pm standard error.

siRNA transfection

Cells were transfected with $\beta 1$ -integrin and/or FAK siGENOME SMARTpool or control siRNA (Dharmacon). Dharmafect 4 (Dharmacon) and siRNA were used according to the manufacturer's recommendation. Cells were added to the transfection mix and incubated for 48 hours before seeding for clonogenic assays or lysed for Western blotting.

Western blotting

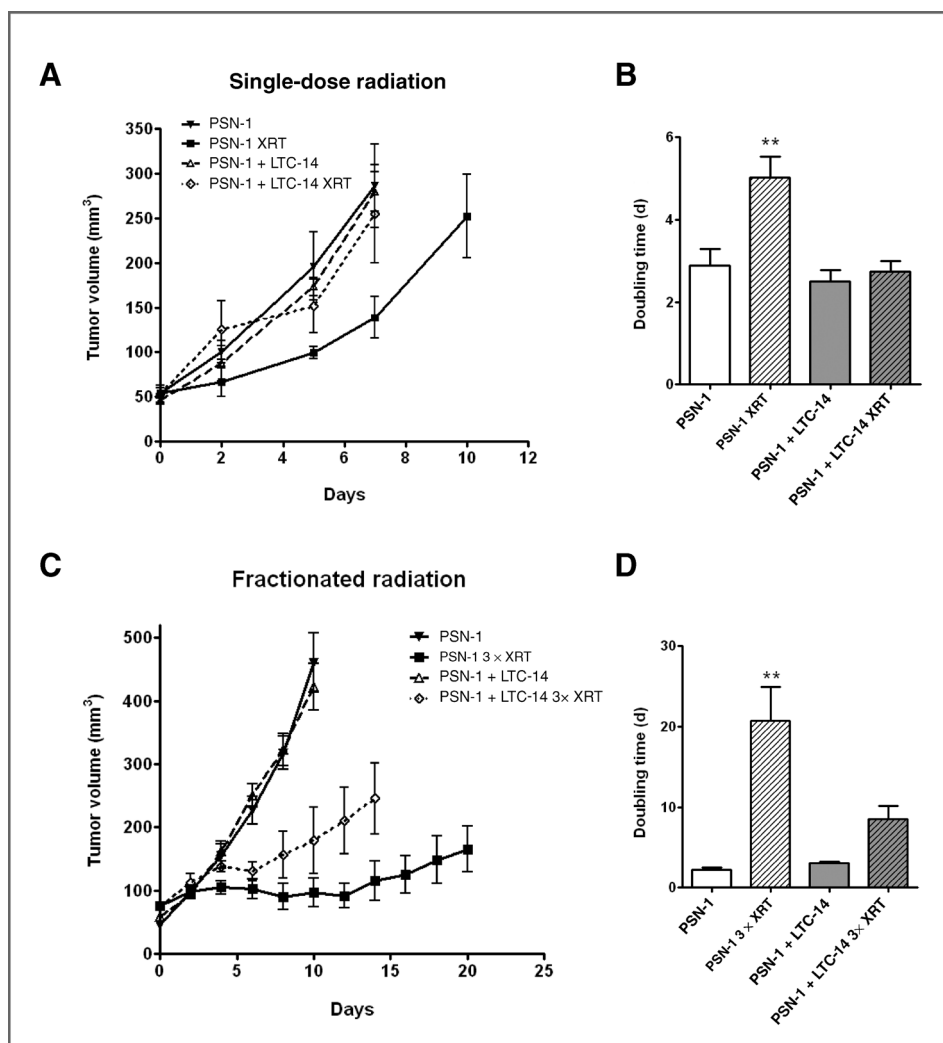
As described previously (15), antibodies used were $\beta 1$ -integrin (ab52971; Abcam), Akt, phosphorylated Akt (p-Akt), and FAK (#9272, #9271, and #3285; Cell Signaling), phosphorylated FAK (p-FAK; Invitrogen), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β -actin (Sigma-Aldrich).

Results

Coculture of tumor and stellate cells

We set up a clonogenic survival assay for PCCs directly cocultured with PSCs, which were grown as a monolayer before seeding of PCCs. The PCC colonies grown on top were clearly distinguishable from the PSC monolayer by means of crystal violet staining (Fig. 1A) and by immunofluorescence

Figure 2. Tumor growth was measured in mice injected with PSN-1 alone or PSN-1 + LTC-14. The animals were randomized and radiated when tumor volume reached 50 mm³. A, single dose of 6 Gy (XRT); *n* = 6 animals per group. B, tumor doubling time. **, ANOVA, *P* < 0.001. C, three 3.5 Gy doses given on consecutive days (3× XRT); *n* = 4 or 5 animals per group. D, as in B.



staining with PSC- and PCC-specific markers (Supplementary Fig. S1A). The larger size of PCC colonies in cocultured dishes indicated increased PCC proliferation (Fig. 1A), which was confirmed by flow cytometric analysis showing fewer cells in G₁ (Supplementary Fig. 1B). This observation is in agreement with previous reports showing increased PCC proliferation in response to PSC conditioned medium (9, 10).

Radiosensitivity studies

The radiosensitivity of different PCCs was investigated in monoculture and in coculture with PSCs. We discovered that PSCs increased the clonogenic survival of PCCs, an observation that applied to several different PSCs (hPSC and LTC-14) and PCCs (Panc-1, PSN-1 and MiaPaCa-2) (Fig. 1B and C; Supplementary Fig. S2A). The clonogenic survival curves of PSCs alone are shown in the Supplementary data (Supplementary Fig. S2B). Conditioned PSC medium did not radioprotect PCCs, but a PSC feeder layer created by giving a lethal dose of radiation indeed could do so, suggesting that a direct

contact between the cells is required (Supplementary Fig. S2C–F). The increased PCC survival was specific to PSCs, as the human fibroblast cell line MRC5 did not change the response to radiation (Fig. 1D). Together these data show that the survival of PCCs after radiation is enhanced by direct coculture with PSCs and that this response is specific to PSCs rather than a general mesenchymal cell response.

Tumor response to radiation *in vivo*

We investigated the effect of PSCs on PCCs *in vivo* and observed a faster tumor development in animals coinjected with PSN-1 and LTC-14 than with PSN-1 alone. PSN-1 tumors on average took 23 days to reach 50 mm³ versus 15 days for PSN-1 + LTC-14 tumors (*t*-test, *P* = 0.008). Importantly, PSCs alone did not form tumors during a 6-month period. A single-dose radiation induced a growth delay only in the PCC tumors (Fig. 2A). Fractionated radiation induced a growth delay in both groups, but the response was less pronounced in the PCC + PSC group (Fig. 2C). A significant increase in the tumor

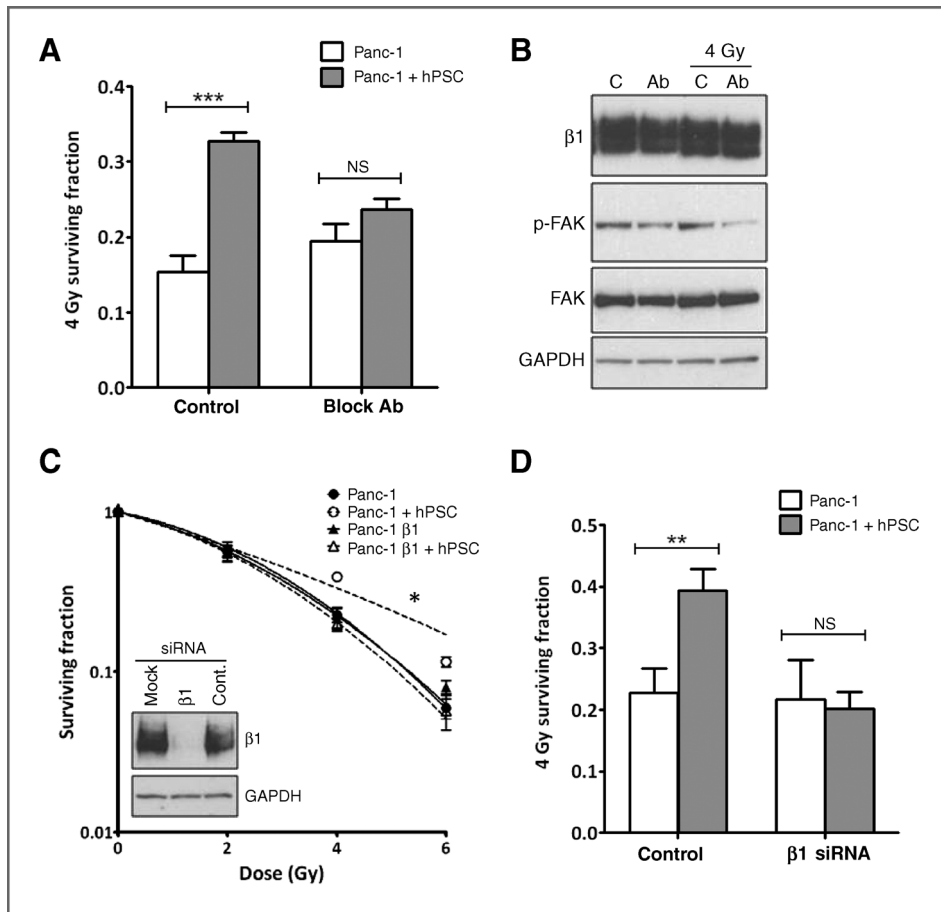


Figure 3. Blocking $\beta 1$ -integrin signaling. A, the 4 Gy surviving fraction of mono- versus cocultured Panc-1 cells treated with $\beta 1$ -integrin blocking antibody. ***, ANOVA, $P < 0.0001$. B, Western blotting of Panc-1 exposed to blocking antibody (Ab) for 2 hours (2 left lanes) and for 2 hours surviving fraction from C; followed by 16 hours of incubation (2 right lanes). C, no antibody control. C, Western blotting of $\beta 1$ -integrin siRNA-transfected Panc-1 and clonogenic survival assay of transfected cells cocultured with hPSCs; *, F -test, $P = 0.039$. D, 4 Gy surviving fraction from C; **, ANOVA, $P < 0.001$; NS, not significant. Plating efficiency (PE) and protection enhancement ratio (PER) values are given in Supplementary Figure S4.

doubling time was observed in the PSN-1 + XRT group only in both experiments (Fig. 2B and D). These findings are in agreement with our tissue culture observations (Fig. 1) and confirm that PSCs have a radioprotective effect on PCCs both *in vitro* and *in vivo*.

Integrin signaling

PSCs produce ECM proteins known to activate signaling through $\beta 1$ -integrins (16). We therefore treated mono- or cocultured PCCs with a $\beta 1$ -integrin blocking antibody prior to radiation and the PSC-mediated radioprotective effect was significantly reduced (Fig. 3A). p-FAK, a downstream adaptor protein of $\beta 1$ -integrin, was reduced after exposure to the blocking antibody (Fig. 3B). The effect of blocking $\beta 1$ -integrin in PCCs only was investigated by siRNA knockdown (Fig. 3C). When cocultured with untransfected hPSCs, Panc-1 cells were sensitized to radiation (Fig. 3C and D). The overall survival of monocultured Panc-1 remained unchanged in response to both blocking antibody and $\beta 1$ -integrin siRNA (Fig. 3A and D). We conclude that $\beta 1$ -integrin in PCCs is involved in the PSC-mediated radioprotection. This observation could not be attributed to changes in surface expression of $\beta 1$ -integrin in PCCs and PSCs in monoculture and coculture (Supplementary Fig. S5).

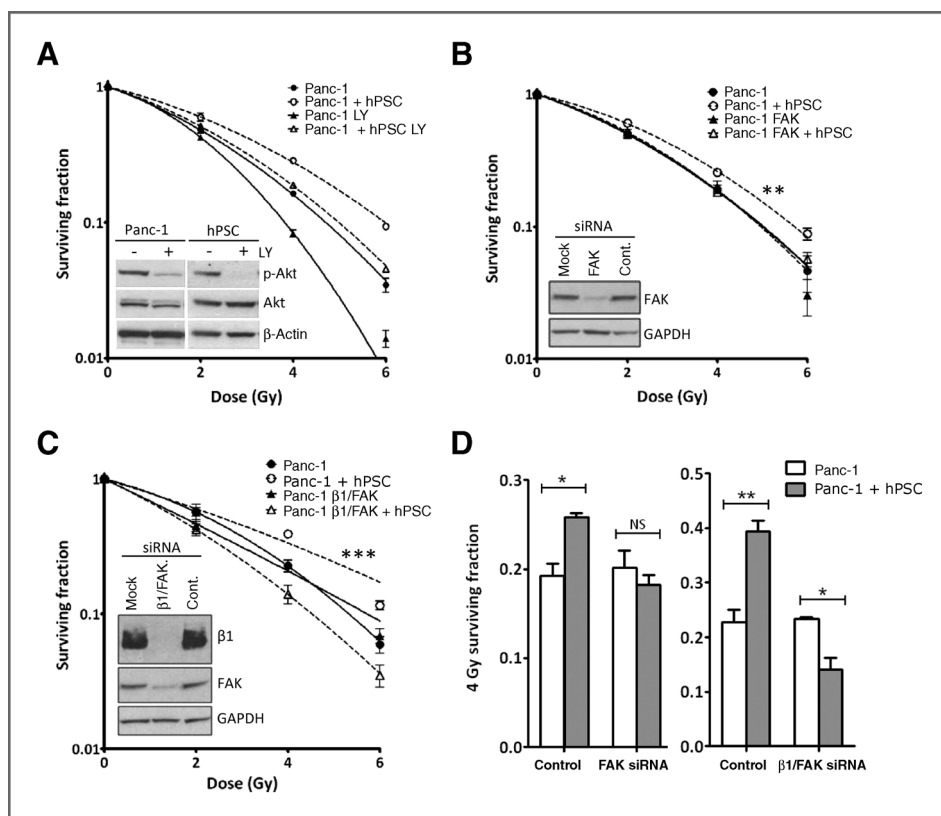
Downstream signaling from $\beta 1$ -integrin

To determine the intracellular signaling involved in this response, we blocked 2 downstream kinases of $\beta 1$ -integrin, Akt and FAK. The phosphoinositide 3-kinase (PI3K) inhibitor LY294002 reduced p-Akt levels in both Panc-1 and hPSCs (Fig. 4A). LY294002 sensitized monocultured and cocultured Panc-1 to radiation (Fig. 4A) but did not affect the radiosensitivity of the hPSC (data not shown). Blocking PI3K did not impact on the radioprotective effect of PSCs, precluding Akt as the downstream kinase of $\beta 1$ -integrin mediating the stroma response in the tumor cells. Knockdown of FAK had no effect on the radiation response of Panc-1 but prevented PSC-mediated radioprotection (Fig. 4B and D). $\beta 1$ -Integrin/FAK double knockdown likewise caused loss of the PSC-mediated radioprotection without a significant effect on the radiosensitivity of Panc-1 in monoculture (Fig. 4C and D).

Discussion

The tumor microenvironment is an important component when studying PCCs and their response to therapeutics. It is now accepted that PSCs stimulate the growth of PCCs, and vice versa, hence contributing to the malignant phenotype of PCCs (3, 9, 10), which was confirmed in our study. We are the

Figure 4. Signaling downstream of β 1-integrin. Clonogenic survival curves of Panc-1 cells in response to 2 hours LY294002 treatment (A); FAK siRNA knockdown (B), **, *F*-test, $P = 0.0004$; and, β 1-integrin/FAK double knockdown (C), ***, *F*-test, $P < 0.0001$. Graphs are representative of at least 2 independent experiments. Inserted Western blots show response to the given treatments. D, 4 Gy surviving fractions from B (*, ANOVA, $P < 0.05$; NS: not significant) and C (**, ANOVA, $P < 0.001$; *, $P < 0.05$).



first to report increased radiation survival of PCCs in coculture with PSCs in clonogenic assays, the gold standard to measure radiosensitivity, as well as *in vivo* using a single-dose and fractionated radiation.

Conditioned medium from radiated PSCs was reported to enhance proliferation of PCCs (17), but clonogenic survival of PCCs was not altered by conditioned media from PSCs in our hands. Furthermore, PCCs were protected from postradiation (100 Gy) apoptosis by conditioned media from PSCs (9). The significance of these data is however limited because proliferation does not predict survival after radiotherapy (18) and apoptosis is not a predominant type of cell death after radiation in solid tumors (19). We investigated whether radiation-induced DNA damage was repaired more efficiently in PCCs in the presence of PSCs, as this could improve survival. Residual 53BP1 foci in PCCs were counted up to 24 hours after radiation of monocultures and cocultures (Supplementary Fig. S3). Our findings revealed no differences in DNA repair kinetics, concluding that they do not form part of this radioprotective response.

Our *in vivo* data showed that the doubling time of PCC tumors increased significantly in response to single-dose radiation compared with that of PCC + PSC tumors. Importantly, the observed radioprotective effect of PSCs on PCCs could neither be attributed to a significant contribution of the PSC to the tumor volume at the time of randomization (Supplementary Fig. S6) nor be attributed to a higher metabolic demand on the tissue due to the difference of total cell

number injected (Supplementary Fig. S7). Fractionated radiation further increased the observed difference, and this is clinically important because, typically, patients have fractionated radiotherapy over 5 to 6 weeks. In addition, radiotherapy is known to enhance desmoplasia in PDAC (17). Therefore, inhibition of the radioprotective effect of PSCs is predicted to increase the efficacy of radiotherapy for patients with PDAC throughout the entire course of therapy.

We aimed to identify elements of heterotypic signaling, which are specifically relevant for this form of microenvironment-mediated therapeutic resistance. We showed that β 1-integrin signaling in tumor cells is required for radioprotection by PSCs. Integrins feed information to cancer cells from both ECM adhesion and soluble factors and is to be interpreted in the full context as a checkpoint for cell fate (11). The malignant phenotype of PCCs grown on collagen I *in vitro* is enhanced and α 2 β 1-integrin signaling is involved in this response (6, 16). These results point toward integrins as crucial components in PSC-PCC communication.

We further identified FAK as a downstream effector kinase in this response. Interestingly, dual β 1-integrin-FAK knockdown further radiosensitized PCCs in the presence of PSCs whereas it did not in PCCs only. This implies that β 1-integrin and FAK act not only purely within the same cascade but also in parallel and that dual inhibition seems to block a rescue pathway. The mechanism behind this response will be investigated further in future *in vivo* experiments.

Altogether, we provide evidence for PSC-mediated radio-protection of PCCs through β 1-integrin–FAK signaling. Targeting this pathway is predicted to enhance radiosensitivity of PCCs and may be successful to enhance the effects of radiotherapy in patients with PDAC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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