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Unbiased analysis of pancreatic cancer radiation resistance reveals cholesterol biosynthesis as a novel target for radiosensitisation

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Background: Despite its promise as a highly useful therapy for pancreatic cancer (PC), the addition of external beam radiation therapy to PC treatment has shown varying success in clinical trials. Understanding PC radioresistance and discovery of methods to sensitise PC to radiation will increase patient survival and improve quality of life. In this study, we identified PC radioresistance-associated pathways using global, unbiased techniques.

Methods: Radioresistant cells were generated by sequential irradiation and recovery, and global genome cDNA microarray analysis was performed to identify differentially expressed genes in radiosensitive and radioresistant cells. Ingenuity pathway analysis was performed to discover cellular pathways and functions associated with differential radioresponse and identify potential small-molecule inhibitors for radiosensitisation. The expression of FDPS, one of the most differentially expressed genes, was determined in human PC tissues by IHC and the impact of its pharmacological inhibition with zoledronic acid (ZOL, Zometa) on radiosensitivity was determined by colony-forming assays. The radiosensitising effect of Zol *in vivo* was determined using allograft transplantation mouse model.

Results: Microarray analysis indicated that 11 genes (FDPS, ACAT2, AG2, CLDN7, DHCR7, ELFN2, FASN, SC4MOL, SIX6, SLC12A2, and SQLE) were consistently associated with radioresistance in the cell lines, a majority of which are involved in cholesterol biosynthesis. We demonstrated that knockdown of farnesyl diphosphate synthase (FDPS), a branchpoint enzyme of the cholesterol synthesis pathway, radiosensitised PC cells. FDPS was significantly overexpressed in human PC tumour tissues compared with healthy pancreas samples. Also, pharmacologic inhibition of FDPS by ZOL radiosensitised PC cell lines, with a radiation enhancement ratio between 1.26 and 1.5. Further, ZOL treatment resulted in radiosensitisation of PC tumours in an allograft mouse model.

Conclusions: Unbiased pathway analysis of radioresistance allowed for the discovery of novel pathways associated with resistance to ionising radiation in PC. Specifically, our analysis indicates the importance of the cholesterol synthesis pathway in PC radioresistance. Further, a novel radiosensitiser, ZOL, showed promising results and warrants further study into the universality of these findings in PC, as well as the true potential of this drug as a clinical radiosensitiser.

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Pancreatic cancer (PC) is the fourth leading cause of cancer death in the United States and has a 5-year survival rate of 6% (Siegel *et al*, 2013). Patients with PC are usually diagnosed late in the cancer's progression, and therefore only 15–20% of patients are eligible for curative-intent surgery. For patients diagnosed with resectable or locally advanced disease, accounting for ~40% of PC patients, external beam radiation therapy (EBRT) is considered a staple of therapy to either help prevent local failure or shrink the tumour to the point of surgical resectability, respectively (Callery *et al*, 2009; Hazard, 2009; Ryan and Mamon, 2009). Clinical trials that have tested the ability of radiation to prolong progression-free survival, increase overall survival, or improve quality of life in PC patients, either alone or in combination with chemotherapy, have shown mixed results (reviewed in Hazard (2009); Goodman and Hajj (2013)). Further, only 20% of pancreatic primary tumours show any significant response to radiation (Roldan *et al*, 1988; Hazard, 2009).

This lack of clinical efficacy of PC irradiation, combined with the substantial benefit that efficacious PC EBRT could provide, mandates exploration of methods to overcome radiation resistance in PC. Successful PC radiosensitisation could provide significant benefit to patients diagnosed with resectable disease, as an estimated 21% suffer solely from local failure post resection, whereas an additional 20% fail both locally and distantly (Oettle *et al*, 2007; Asiyanbola *et al*, 2009). To understand how to overcome PC's radioresistance and offer potential methods for therapeutic radiosensitisation, the mechanisms underlying the lack of radiation-induced cytotoxicity in PC must be elucidated.

The molecular pathways contributing to apparent resistance to ionising radiation (IR) in PC remain poorly understood (Brunner *et al*, 2005; Kimple *et al*, 2010; Fokas *et al*, 2012; Engelke *et al*, 2013). We employed a novel *in vitro* model of PC radiation resistance to determine the global transcriptional differences between radiosensitive and radioresistant PC cells. Several genes were identified and validated, including many in the cholesterol synthesis pathway, whose differential expressions significantly correlated with *in vitro* PC radioresponse. Further, through these methods, a putative radiosensitiser for PC was tested, zoledronic acid (ZOL, Zometa, Novartis, East Hanover, NJ, USA), currently used clinically for non-IR-related purposes. Finally, tumour-specific EBRT was performed using a linear accelerator for treatment of a subcutaneous allograft model of PC, testing whether ZOL could radiosensitise *in vivo*.

MATERIALS AND METHODS

Cell culture and materials. The human PC cell lines Panc-1 and BxPC3 were obtained from the American Type Culture Collection (Manassas, VA, USA). The L3.6pl human PC cell line was a kind gift from Dr Michel Ouellette (UNMC). The mouse PC cell line UN-KPC-961 was established in our laboratory from a primary tumour generated in a spontaneous mouse model of PC, the LSL-$Kras^{G12D/+}$;LSL-$Trp53^{R172H/+}$;Pdx-1-Cre (KPC) mouse model (Torres *et al*, 2013). Cell lines were cultured per established lab protocol (Singh *et al*, 2004). ZOL was purchased from Sigma (St Louis, MO, USA) and was dissolved in sterile water. Clinical grade ZOL (4 mg per 5 ml concentrate) was received from the oncology pharmacy (Nebraska Medical Center, Omaha, NE, USA).

***In vitro* irradiation.** *In vitro* irradiation was accomplished via a linear accelerator in the Department of Radiation Oncology at UNMC. Briefly, cells in exponential growth phase were plated at 40% confluence 24 h before irradiation. Flasks were placed on 10 cm of solid water (phantom material used for radiation beam calibration) positioned in the centre of the 40 cm × 40 cm radiation field and irradiated with 6 MV X-rays at a rate of 2.73 Gy min^{-1} from the posterior direction, with the media being 100 cm from the X-ray target. The dose to the media was verified with MOSFET detectors (Best Medical Canada, Ottawa, ON, Canada).

Analysis of radiosensitivity of PC cell lines. Cellular radioresponse was determined by colony survival assay (CSA) using standard protocol (Boothman *et al*, 1987). On the day of staining, cells were washed, fixed in MeOH, and stained using 0.4% crystal violet in 25% MeOH. Colonies containing >50 cells were counted for each well and the number of colonies corresponding to each radiation dose averaged. Surviving fraction at each dose was determined by the formula (number of surviving colonies in dose X)/(number of cells seeded for dose X × (average colonies arising from the non-irradiated cells (0 Gy)/number of non-irradiated cells seeded)) (Kimple *et al*, 2010). Log (surviving fraction) was plotted against dose for each condition. The linear quadratic model ($Y = e\text{^-}[\alpha^*X + \beta^*X^2]$) was fit to the clonogenic survival curve for each condition, where the parameters α and β were calculated according to the method of Fertil (Fertil *et al*, 1984). The survival curve and linear quadratic fit were plotted using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) according to a least-squares fit, weighted to minimise the relative distances squared as described previously (Kimple *et al*, 2010). The mean inactivation dose (equal to the area under the survival curve) was also calculated according to the method of Fertil (Fertil *et al*, 1984).

The radiation enhancement ratio (ER) was calculated as the ratio of the mean inactivation dose under control conditions divided by the mean inactivation dose of experimental conditions (that is, another cell line, or siRNA or drug treatment), as described previously (Morgan *et al*, 2008). A value significantly >1 indicates radiosensitisation.

Generation of radioresistant PC cell lines. Selection for radiation-resistant subpopulations within parental PC cell lines was attempted originally for both Panc-1 and BxPC3, chosen due to their primary tumour origins as well as their poorly and moderately differentiated status, respectively. Radioselection was also performed in the L3.6pl cell line (derived from COLO 357, which was established from a lymph node metastasis and has well-differentiated status) for validation of results. Cells were subjected to 2 Gray (Gy) radiation daily for 2 weeks, taking weekends off (giving a total of 10 fractions). Throughout the irradiation process and recovery time, cells were kept at 40–70% confluency to ensure potential for exponential growth. After completion of the second week of radiation, cells were maintained in media containing 10% FBS, being washed and re-fed daily for the first 7 days and then every third day thereafter. Radioselected radioresistance was verified by comparing the radiosensitivity of the radiation-selected cells (after a recovery period) with their respective parental cell lines by CSA as described above.

RNA extraction and cDNA synthesis from PC cell lines. Total RNA was extracted using the Qiagen RNAeasy RNA isolation kit (Qiagen, Valencia, CA, USA). RNA was converted to cDNA using 2 μg of total RNA, oligo$(dT)_{18}$ primer, and the SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA).

Global gene expression analysis. Global genome microarray analysis was performed by the UNMC microarray core facility. Phalanx whole-genome cDNA microarray platforms were used containing 30 275 features probing for ~22 000 unique genes. To ensure valid comparison across cell lines, all samples were normalised against a universal human reference (Stratagene, Cat: 740000, Cedar Creek, TX, USA).

Discovery of radioresistance-associated pathways. Radioresistance-associated pathways were determined by comparative gene expression analysis of radioresistant cell line (Panc-1RR) with that

of parental cell line exhibiting relative radiosensitivity (Panc-1). On completion of unbiased discovery of radioresistance-associated gene differential expression, ingenuity pathway analysis was performed to identify pathways and cellular functions most implicated in our radioresistance model, as well as to determine potential targets for radiosensitisation.

Quantitative real-time PCR validation of microarray transcriptional analysis. After analysis of microarray results for radioresistance-associated differential expression, altered transcription of 12 selected genes associated with PC radioresistance was validated using quantitative real-time PCR (qRT-PCR) using SYBR green-based chemistry. qRT-PCR was performed using 1 μl of a 1 : 5 dilution of first-strand cDNA using the SYBR Premix Ex Taq kit (Takara Bio, Madison, WI, USA) and specific primers (sequence in Supplementary Table 4) on a Roche Light Cycler 480 system (Roche Diagnostics, Mannheim, Germany). Fold change in gene expression was determined by the delta-delta C_T method as described previously (Baine *et al*, 2011).

RNA interference and transient transfection. Farnesyl diphosphate synthase (FDPS) siRNA pool of three target-specific 19–25 nt siRNAs was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (sc-75011). For transient transfection, recipient cells were seeded in a six-well plate 24 h before transfection. Following serum starvation for 4 h, the cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's protocol. Four hours following transfections, the cells were replenished with serum-containing media and protein was isolated from the cells 48 h post transfection.

Whole-cell lysate preparation. Cells were rinsed with PBS and then lysed on culture dishes using RIPA buffer containing protease and phosphatase inhibitors (Moniaux *et al*, 2004) and stored at -20°C. Thawed samples were passed through a 27-gauge needle and syringe, and clarified by centrifugation at 14 000 r.p.m. at 4 $^{\circ}$C for 20 min. Protein concentration in lysates was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

SDS–PAGE and western blot analysis. Equal amounts of protein were separated on 12% SDS–polyacrylamide gels and blotted onto polyvinylidene fluoride membranes (EMB Millipore, Billerica, MA, USA) and developed per lab protocol (Andrianifahanana *et al*, 2005). Horseradish peroxidase-conjugated appropriate secondary antibodies were diluted at 1 : 3000 in PBS and incubated for 1 h at room temperature. After three washes in PBST, membranes were developed using the Pierce ECL Western Blotting Substrate (Thermo, Rockford, IL, USA) and blots were exposed to ECL-sensitive film (MIDSCI, St Louis, MO, USA). The films were digitally scanned, and figures were assembled using Microsoft PowerPoint.

Immunohistochemistry. Tissue arrays comprised of normal pancreas (from donors for pancreas transplant) and PC patient samples were obtained from the Rapid Autopsy Program of the University of Nebraska Medical Center (PI: Michael Hollingsworth, IRB# 091-01-FB). The tissues were stained with anti-FDPS polyclonal antibody (ab38854, Abcam, Cambridge, MA, USA) using an antibody dilution of 1 : 100. The immunostaining technique was performed according to the standard laboratory procedure (Moniaux *et al*, 2008). The FDPS staining was scored by a pathologist using the criteria described in Supplementary Methods. Representative photographs were taken under a Nikon light microscope with a Q-capture Micropublisher 5.0 camera (Leeds Precision Instruments, Minneapolis, MN, USA) by utilising the Q-capture suite software package (QImaging, Surrey, BC, Canada).

Evaluation of pathway-directed pharmaceuticals for cell growth inhibition and radiosensitisation. MTT assay was performed with 10 nM–100 μM ZOL to evaluate toxicity. Subsequently, cell lines were exposed to ZOL in conjunction with radiation and assessed by colony forming assay as described above. Concentrations of 10 nM–10 μM of ZOL were used with radiation, as these concentrations have been previously shown to inhibit pathways of interest (Chen *et al*, 2002; Skerjanec *et al*, 2003; Almubarak *et al*, 2011). Cells were treated with ZOL either before and after radiation or only after as indicated. Once cells were switched to media containing 5% FBS, all media given to the cells was drug free.

Tumour implantation. All animal studies were performed under an approved protocol by the University of Nebraska Medical Center Institutional Animal Care and Use Committee (IACUC). Subcutaneous tumour growth was evaluated with the UN-KPC-961 mouse PC cell line. Cells (5×10^6) suspended in 50 μl sterile PBS were injected in two areas (one to be irradiated and one unirradiated) in immune-competent mixed Sv129/C57BL/6 background mice on the right and left posterior lateral thorax caudal to the axilla. Before implantation, mice were shaved at the site of injection. Tumours were allowed to grow for 2 weeks before randomisation. Tumour growth was monitored by measuring with vernier calipers.

Radiation treatment planning. Planning CT scans (Sensation Open; Siemens Healthcare, Eschborn, Germany) at a slice thickness of 0.6 mm were performed in anaesthetised mice that were implanted with pancreatic tumours. Before scanning, mice were anaesthetised with xylazine/ketamine, and restrained in the prone position. Gross tumour volume and the organs at risk such as lung and spinal cord were contoured on axial CT slices of all mice. A planning target volume expansion of 3 mm was used. The iPlan (Brainlab AG, Feldkirchen, Germany) system was used by a radiation oncologist and radiation physicist to generate 3D conformal plans, providing a steep dose fall-off in normal tissue away from the target to be irradiated (Supplementary Figure 1A). The prescribed target dose was 35 Gy in 7 Gy daily fractions. At least 95% of the prescribed dose encompassed 100% of the planning target volume and at least 95% of the planning target volume received 100% of the prescribed dose.

Radiation treatment. Radiation was delivered 2–3 days after planning CT scans. One hour before irradiation, mice were randomised to receive clinical grade ZOL (2 mg kg^{-1}, 50 μl of a 4 mg per 5 ml solution for a 20 mg mouse; equivalent to a 10-mg human dose calculated based on body surface area (Reagan-Shaw *et al*, 2008)) or vehicle control by intraperitoneal injection.

The Novalis accelerator (Brainlab AG) was used to deliver stereotactic radiation therapy. It incorporates stereotactic X-ray capabilities for verifying target position (ExacTrac; Brainlab AG). For soft-tissue targets, the system is designed to be used with radio-opaque metal markers placed near the target. These markers can be observed within the field of view of the X-ray localisation system at the time of treatment. In detail, each mouse was anaesthetised and restrained. A metal bb was placed on the tumour, and the locating lasers in the x–y–z planes were placed directly on the bb/dot. Each mouse was imaged with ExacTrac, and the mouse spine from the ExacTrac image was superimposed onto the mouse spine from the pre-treatment CT image (Supplementary Figure 1B shows before and after ExacTrac). The couch position was adjusted accordingly. Radiation treatment consisted of 35 Gy in 7 Gy daily fractions delivered in 5 consecutive days with 3–5.5-mm multileaf collimators (Novalis) and 6 MV photons with a dose rate of 480–650 cGy min^{-1}. No animal shielding was used, and no obvious radiation toxicity was observed for subcutaneous tumour-targeted radiation.

Measurement of treatment responses. Tumour volumes were measured with calipers immediately before irradiation, and every 3–5 days after that. Tumour volume was calculated using the formula: volume = [length × (width^2)]/2.

Histological analysis of radiated and ZOL-treated tumours. Pathologic responses were scored by a pathologist. Necrosis was measured in percentages at 10% increments. Fibrosis was measured as described in Supplementary Methods.

Statistical analysis. CSAs were performed no less than on two separate dates for each cell line. Mean surviving colonies and s.e. were calculated for each radiation dose and compared between cell lines. Colony survival was considered a continuous variable and cell lines were compared using two-tailed Student's *t*-test with *P*-values <0.05 being considered statistically significant. All CSA data were analysed using the MedCalc version 9.3.0.0 software (MedCalc Software bvba, Ostend, Belgium) and GraphPad Prism v.5.0.

Microarray analysis was accomplished through $\log_2$ transformation of all ratios followed by normalisation to 'centre' each array using lowess smoother through BRB ArrayTools (Simon *et al*, 2007). Genes in which the percent of spots missing or filtered out exceeded 50% were excluded. Genes whose expression differed by at least 1.5-fold from the median in at least 20% of the samples were retained, genes not meeting this criteria were excluded due to low variability across the samples. Univariate analyses were performed comparing each utilised cell line and cell line derivative using a two-sample *t*-test using the random variance model. *P*-values <0.001 were considered significant.

The non-parametric Wilcoxon rank sum test was used to compare protein levels with radiation and chemotherapy status. *P*-values <0.05 are statistically significant.

Response to treatment of tumour allografts was analysed with a two-sample *t*-test, with *P*-value <0.05 considered significant.

RESULTS

Natural radiosensitivity varies across PC cell lines. CSAs of PC cell lines revealed variation in their radiosensitivity (Figure 1A). Of note, Panc-1 and BxPC3 revealed a significant difference in natural radiosensitivity, with BxPC3 being more radioresistant than Panc-1 at doses of 3 Gy and above. These results indicate that an average of 1.6-fold radiation dose is required to produce equivalent cell death in BxPC3 as in Panc-1. UN-KPC-961 was the most resistant line used. The UN-KPC-961 cell line was derived and characterised in our laboratory (Torres *et al*, 2013) from a primary tumour developing in KPC (LSL-Kras$^{G12D/+}$;Trp53$^{R172H/+}$;Pdx-1-Cre) genetically engineered mouse model of PC (Hingorani *et al*, 2005). It was observed that the immortalised human pancreatic ductal epithelial cell line (HPDE) was highly sensitive to radiation, with no colonies observed with a 2 Gy treatment and consequently, could not be included in CSA analysis. Summary of cell line mutations is presented in Supplementary Table 1.

Generation of radioresistant cell lines. For the generation of radioresistant cell lines, cells were exposed to 10 fractions of 2 Gy X-rays and then allowed to recover for 4 weeks (6–10 passages; Figure 1B). After this recovery period, the Panc-1 subline that had been exposed to 10 fractions of 2 Gy had a significant increase in radioresistance as compared with parental Panc-1, with CSAs indicating that Panc-1RR requires a 1.6-fold higher IR dose as compared with parental Panc-1 to elicit equivalent cell death (Figure 1C). This Panc-1 subline with confirmed induced radioresistance was subsequently referred to as Panc-1RR. Importantly, this increased radioresistance was found to be stable, with similar results obtained 6 and 18 passages after completion of the fractionated IR regimen. No difference was observed in BxPC3 after fractionated IR exposure (BxPC3-RR *vs* BxPC3), potentially indicating that BxPC3 cells are either more homogenous in their radiosensitivity or are incapable of becoming more radioresistant (Figure 1D).

Global expression analysis of PC cell lines. Microarray analysis comparing global expression levels across cell lines revealed notable differential expression profiles. A total of 54 genes were found to be differentially expressed ($P \leqslant 0.001$), with fold differences ranging from six-fold higher (FDPS) to five-fold lower (LOC146481) in Panc-1RR as compared with Panc-1 parental cell line (Supplementary Table 2). In contrast, only a few genes were differentially expressed between BxPC3-RR and BxPC3, with expression levels of only 8 genes found to be differentially expressed between the two ($P \leqslant 0.001$), ranging from three-fold higher (RASSF8) to four-fold lower (ASIP) in BxPC3-RR (Supplementary Table 3).

Ingenuity pathway analysis of the differentially expressed genes in Panc-1RR compared with Panc-1 indicates an array of cellular functions that have a role in PC radioresistance, with the two most associated cellular functions being lipid metabolism ($P = 2.66\text{E}-08$ to $6.83\text{E}-03$) and small molecule biochemistry (that is, synthesis of cholesterol and lipids; $P = 2.66\text{E}-08$ to $6.83\text{E}-03$). Further, the canonical pathways exhibiting association with the differentially expressed genes are those of the superpathway of cholesterol biosynthesis ($P = 4.47\text{E}-11$) (Figure 2A; the top eight canonical pathways are involved in cholesterol/mevalonate synthesis) and fatty acid biosynthesis ($P = 4.43\text{E}-3$).

qRT-PCR validation of radioresistance-associated genes. To validate the results of the microarray analysis, 11 genes (FDPS, ACAT2, AG2, CLDN7, DHCR7, ELFN2, FASN, SC4MOL, SLC12A2, SQLE, and IDI1) found to be associated with PC radioresistance were validated by qRT-PCR for differential expression between Panc-1RR and Panc-1. From this analysis, 100% validation of microarray results was observed (Table 1).

Cell line expression of FDPS and siRNA knockdown for radiosensitisation. As FDPS was the top differentially upregulated gene in our microarray and because FDPS is a major branchpoint enzyme of the cholesterol synthesis pathway, we further investigated its role in radioresistance. Western blotting revealed that FDPS is expressed in all PC cell lines tested. Marginal increases in FDPS protein expression could be seen in the Panc-1RR cells compared with parental Panc-1, and in the recently induced radiation-resistant subline L3.6pl-RR compared with parental L3.6pl (Figure 2B).

To determine whether FDPS contributes to radiation resistance, Panc-1RR cells were transfected with pooled siRNA to specifically knockdown FDPS expression. Panc-1RR cells were successfully transfected with either FDPS-specific siRNA or nonspecific control scrambled siRNA, with knockdown of FDPS seen at 48 h after transfection (Figure 2C). Radiosensitivity was examined at this 48 h time point after transfection. Knockdown of FDPS increased radiosensitivity, with an ER of 1.34 (Figure 2D).

Immunohistochemistry of FDPS in PC patient samples. As knockdown of FDPS resulted in radiosensitisation in PC cells, we wanted to explore the presence of FDPS in human PC samples. PC patient tissue array samples were obtained from the Rapid Autopsy Program at the UNMC. Remarkably, staining with anti-FDPS antibody revealed low expression of FDPS in normal pancreas samples ($n = 8$) from donors for pancreas transplant, but was significantly overexpressed in 83% (24 out of 29) of primary pancreatic adenocarcinoma patient samples (Figure 2E and F). Tumour samples had a median composite score of 7.5 (range 0–12, with 12 being the maximum score possible), while the mean composite score of normal pancreas ranged from 0 to 1 (Figure 2G). No significant association between FDPS composite

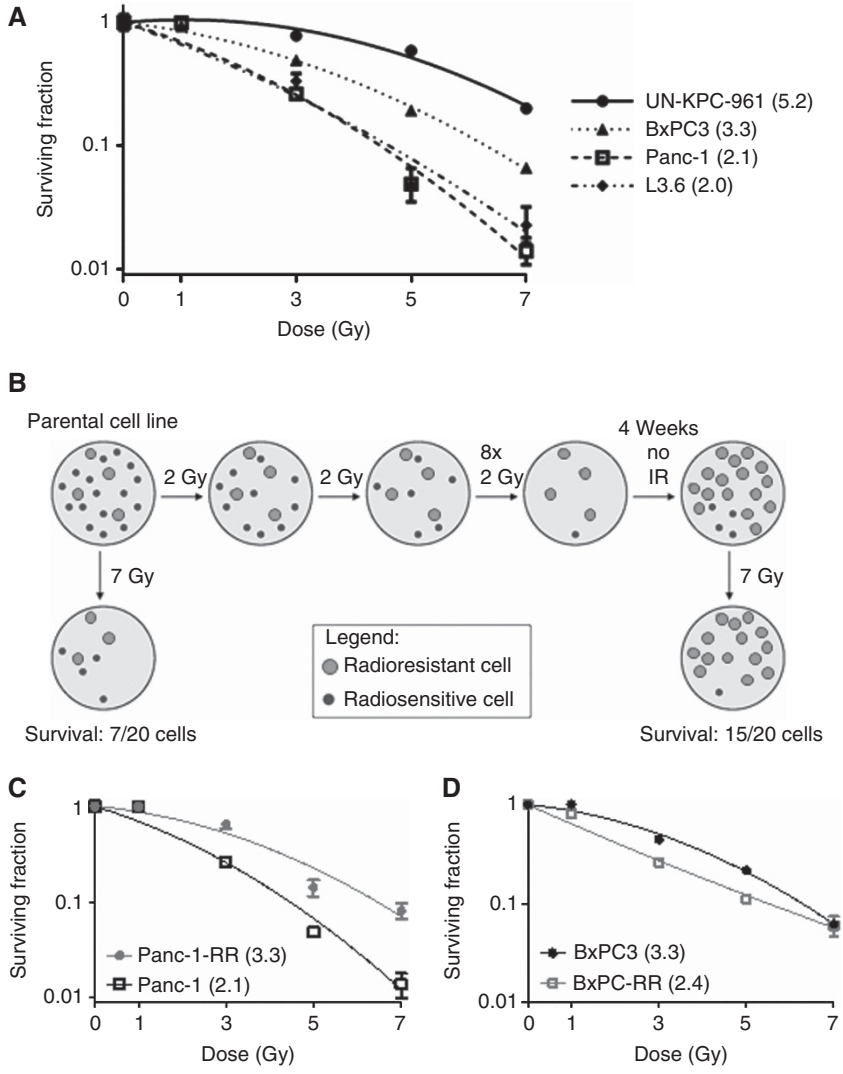
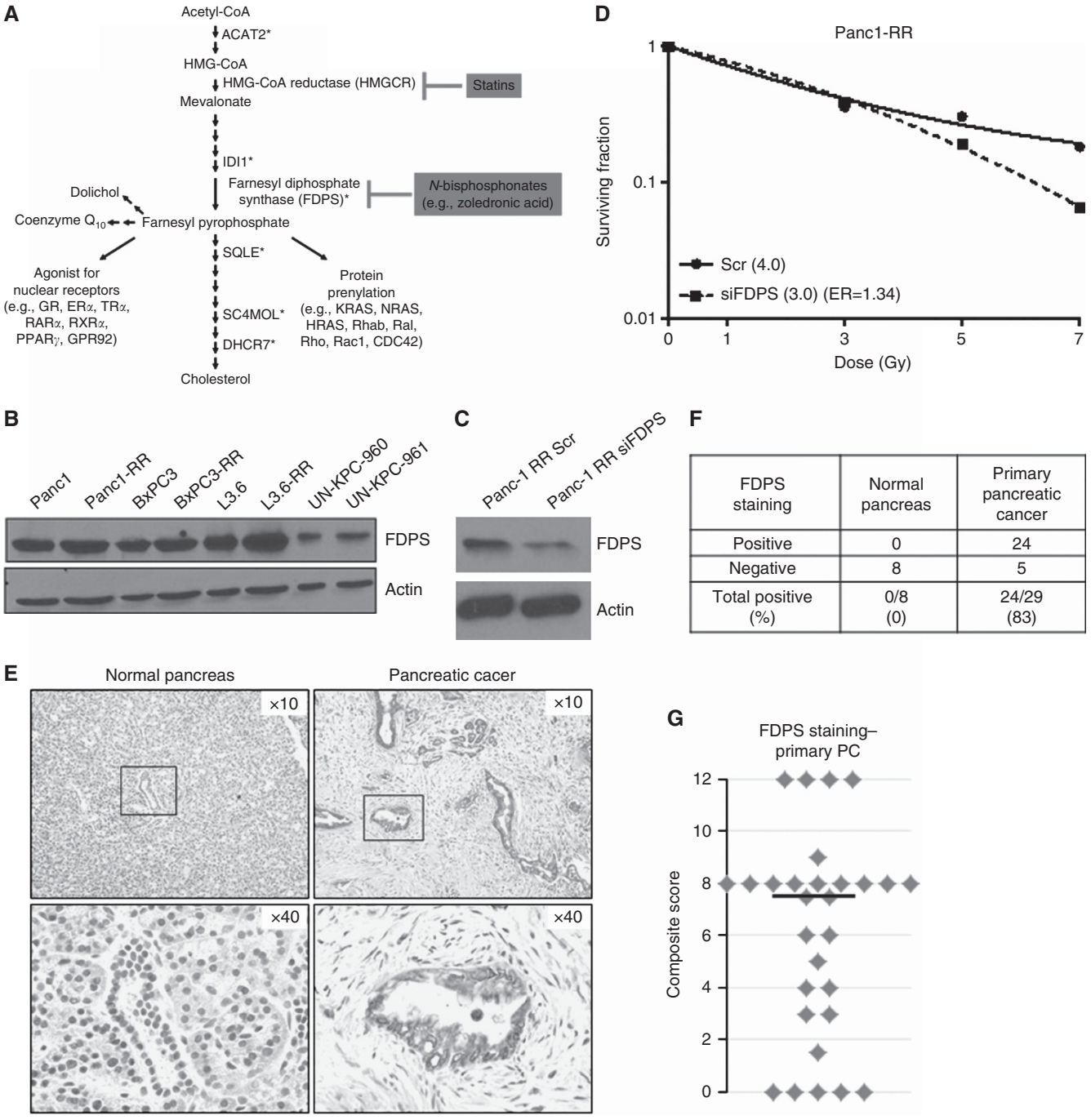


Figure 1. Response of PC cell lines to radiation and generation of radiation-resistant sublines. (**A**) Colony formation assays of PC cell lines exposed to the indicated dose of radiation reveal a heterogeneous response. It was observed that the immortalised human pancreatic ductal epithelial cell line (HPDE) was highly sensitive to radiation, with no colonies being observed with 2 Gy treatment (not shown). Cell line names are given with the mean inactivation dose, (QUOTE), given in parentheses. Mean inactivation dose has units of Gy. (**B**) Schematic of the generation of radiation-resistant sublines. Cell lines were exposed to 10 fractions of 2 Gy irradiation over a 2-week period and then allowed to recover. Subsequently, colony formation assays were done following exposure to varying doses of radiation to compare the sensitivity between the parental and radioresistant sublines. (**C**) Colony formation assay of Panc-1RR *vs* Panc-1. The Panc-1 subline exposed to fractionated radiation required 1.6-fold higher IR dose to elicit equivalent cell death as the parental Panc-1, and was thus referred to as radioresistant, Panc-1RR. The mean inactivation dose, (QUOTE), is given in parentheses. (**D**) Colony formation assay of BxPC3-RR *vs* BxPC3. No difference was observed between the naturally radioresistant parental line BxPC3 and the BxPC3 subline exposed to fractionated radiation (BxPC3-RR).

score and patient exposure to treatment with radiation or chemotherapy was observed, as assessed by Wilcoxon rank sum test (not shown).

ZOL radiosensitises PC cells. ZOL inhibits FDPS and thus, was used for further study as a potential radiosensitiser. On analysis, it was found that 10 μM ZOL acts as a potent radiosensitiser of Panc-1RR, BxPC3, and BxPC3-RR cell lines, but not the naturally sensitive Panc-1, with radiation ER of 1.26, 1.41, and 1.33 for BxPC3, BxPC3-RR, and Panc-1RR, respectively (Figure 3). In MTT assays, ZOL at 10 μM exhibited mild toxicity (data not shown); however, this toxicity was accounted for in CSAs, as surviving fraction is normalised to unirradiated control for each concentration of ZOL used. ZOL was also found to radiosensitise the radioselected L3.6-RR cells and the naturally radioresistant UN-KPC-961 cell line (Figure 3).

***In vivo* radiation treatment and ZOL radiosensitisation.** To determine whether ZOL treatment could radiosensitise PC cells *in vivo*, UN-KPC-961 tumours were established subcutaneously on the right (to be irradiated) and left (unirradiated control) posterior lateral thorax of Sv129/C57BL/6 mixed background mice. Each tumour-bearing mouse received a planning CT scan and 3D conformal radiation treatment plan, generated within 2 days of the start of radiation treatment (Supplementary Figure 1A). Mice bearing tumours of at least 100 mm^3 were randomly assigned to receive a single dose of either ZOL (2 $mg\,kg^{-1}$) or vehicle by intraperitoneal injection 1 h before radiation. Tumours on the right lateral chest received five fractions of 7 Gy (total 35 Gy) on 5 consecutive days. Even without shielding of the mice, no obvious radiation toxicity could be seen in mice receiving 35 Gy total dose targeted to subcutaneous tumours. Tumour volume was measured with



FDPS staining	Normal pancreas	Primary pancreatic cancer
Positive	0	24
Negative	8	5
Total positive (%)	0/8 (0)	24/29 (83)

Figure 2. Cholesterol (mevalonate) synthesis pathway genes are upregulated in radiation-resistant cells. (**A**) Simplified cholesterol biosynthesis pathway. Asterisks (*) mark upregulated genes in Panc-1 radiation-resistant (Panc-1RR) cells. FDPS is a branchpoint enzyme in the pathway, producing farnesyl pyrophosphate that is used for cholesterol synthesis, post-translational modification of small GTPases, including KRAS, termed farnesylation or geranylgeranylation (collectively called prenylation), which helps traffic proteins to membranes, and recently discovered to be an agonist for several nuclear receptors. ZOL (Zometa) inhibits FDPS, whereas the cholesterol-lowering statins inhibit HMG-CoA reductase (HMGCR), the rate-limiting enzyme of the cholesterol pathway. (**B**) Immunoblot of FDPS protein expression in various PC cell lines. (**C**) Immunoblot of Panc-1RR cells transfected with control scrambled (Scr) siRNA and FDPS siRNA indicating efficient knockdown at 48 h. (**D**) Colony formation assay comparing Panc-1RR cells transfected with scrambled control (Scr) or FDPS siRNA. Cells were irradiated 48 h after transfection, and then seeded for colony assay. siFDPS cells were sensitised to radiation, with ER being 1.34. Surviving fraction is expressed relative to non-irradiated cells in the respective treatment group. Graph shows the mean ± s.e. of triplicate samples of a representative experiment. (**E**) Immunohistochemical analysis of normal pancreas (left) and PC tissue (right) for FDPS. Normal pancreas samples were from donors for pancreas transplant while PC specimens were from the Rapid Autopsy Program at the University of Nebraska Medical Center. Samples were mounted as tissue arrays and all were stained at the same time. Scoring was done by one pathologist. (**F**) Table summarising the incidence of FDPS overexpression in pancreatic tumours. (**G**) Scatter plot indicating the distribution of composite score for FDPS staining in pancreatic tumours. The median composite score (black bar) was 7.5.

vernier calipers during the course of treatment to determine response compared with the first day of radiation.

Unirradiated tumours in the mice that received a single injection of ZOL were seen to shrink in the first week after treatment, but then grew at the same rate as untreated tumours, and by day 17 were not significantly different in mean percent change in tumour volume. Tumours receiving radiation alone had maximal tumour volume shrinkage by day 10, but in most mice it exhibited increased size by day 17. Tumours receiving a single injection of ZOL plus radiation had maximal tumour volume shrinkage at day 17. Treatment response at day 17 was plotted and compared by waterfall plot (Figure 4). Although 63% (5 out of 8) of animals showed response with radiation alone, ZOL plus radiation produced a response rate of 80% (8 out of 10). The mean percent change in tumour volume for tumours receiving ZOL plus radiation was not seen to be statistically significant ($P = 0.404$) compared with tumours receiving radiation alone. Histological comparison of tumours revealed increased areas of fibrosis with less tumour cell density in tumours receiving radiation, but no significant differences were observed for fibrosis score or tumour cell dropout in radiated tumours receiving ZOL compared with radiation alone (Supplementary Figure 2).

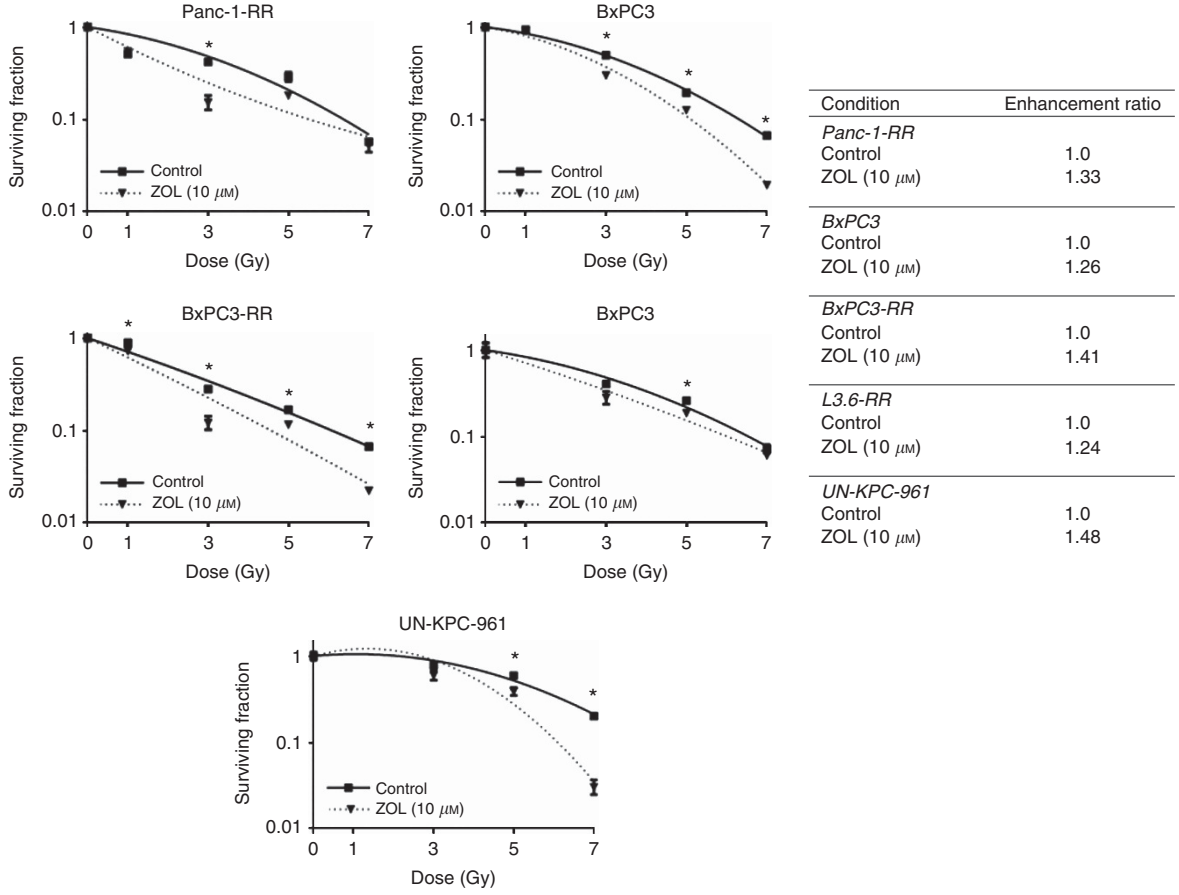
Table 1. Validated genes differentially expressed in radioselected Panc-1RR *vs* parental Panc-1 cell lines (fold change ratio shown)

	Panc-1RR *vs* Panc-1	
Gene name	**qRT-PCR**	**Microarray**
FDPS	7.14	5.93
ACAT2	2.49	3.03
AG2	1.87	2.59
CLDN7	0.07	0.49
DHCR7	1.15	2.96
ELFN2	0.01	0.34
FASN	1.97	2.62
SC4MOL	2.83	3.57
SLC12A2	1.49	1.99
SQLE	2.86	2.5
IDI1	7.23	4.15

Abbreviation: qRT-PCR = quantitative real-time PCR.

DISCUSSION

Successful radiosensitisation has significant potential to provide improved prognosis in PC patients. Increasing radiotherapy effectiveness would greatly improve outcome for patients with locally advanced disease, as a greater proportion of patients would be successfully downstaged to resectable disease, thereby improving



Condition	Enhancement ratio
Panc-1-RR	
Control	1.0
ZOL (10 μM)	1.33
BxPC3	
Control	1.0
ZOL (10 μM)	1.26
BxPC3-RR	
Control	1.0
ZOL (10 μM)	1.41
L3.6-RR	
Control	1.0
ZOL (10 μM)	1.24
UN-KPC-961	
Control	1.0
ZOL (10 μM)	1.48

Figure 3. Inhibition of FDPS radiosensitises PC cells. (Left) CSAs of radioselected Panc-1RR, BxPC3, fractionated BxPC3-RR, UN-KPC-961, and radioselected L3.6-RR cells following radiation treatment in the absence (control) and presence of indicated concentration of ZOL. ZOL treatment was 24 h before and 72 h after radiation treatment for Panc-1RR, BxPC3, fractionated BxPC3-RR, and 72 h after radiation treatment for UN-KPC-961 and radioselected L3.6-RR cells. Surviving fraction is expressed relative to non-irradiated cells in the respective treatment group. Graph shows the mean ± s.e. of triplicate samples of a representative experiment. Asterisks indicate *P*-value <0.05. (Right) ER for each cell line following ZOL treatment. A value significantly >1 indicates radiosensitisation.

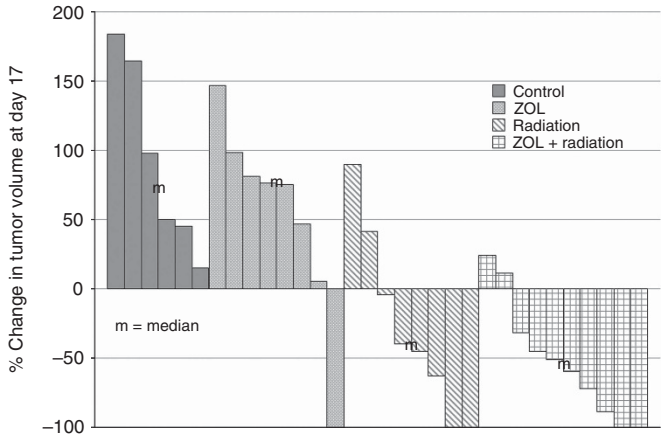


Figure 4. Effect of ZOL on radiation therapy of subcutaneous mouse allograft tumours. Immune-competent mice were injected s.c. with UN-KPC-961 cells in two areas – one to be irradiated and one as unirradiated control. Half of the mice were given ZOL (2 mg kg^{-1}) 1–2 h before the first radiation dose. Each mouse was aligned to its CT scan by ExacTrac and then stereotactically irradiated on 5 consecutive days with 7 Gy fractions. Waterfall plot showing percentage change in tumour volume at day 17 as observed by caliper measurement of tumours for all mouse tumours examined in the study. Similar results were observed with volume measured by CT imaging. Median of each treatment group is marked with 'm'.

their 5-year survival rate from 8.6 to 21.5% and providing them with access to the only PC therapy with reasonable curative potential (SEER; National Cancer Institute, 2011). This study determined pathways associated with PC radioresistance through comparison of natural and radioselected radioresistant cell lines with their differentially radiosensitive counterparts. These methods allow for the discovery of genes common to both PC cell lines with inherent radioresistance and the radioresistant subpopulations of PC cell lines with relative radiosensitivity, providing comprehensive insight into genes associated with PC's evasion of IR-induced cell death.

Such methods for isolating radioresistant subpopulations from cell lines have been previously reported using breast (Li *et al*, 2001), oesophageal (Fukuda *et al*, 2004), and lung cancer (Xu *et al*, 2008; Lee *et al*, 2010) cell lines. Unbiased analysis of radioresistance in PC has been attempted only once before (Ogawa *et al*, 2006), with some major differences with our study. Ogawa *et al* used high-dose fractions and high total dose (10 Gy fractions with 14-day recovery period between each fraction for total dose 150–180 Gy), whereas we used 10 fractions of 2 Gy in a 2-week period (total dose 20 Gy). Ogawa *et al* used a different microarray platform and used only one cell line in common with our study, Panc-1, but were unable to establish a radioresistant subline from it. These differences may explain why none of the differentially expressed genes were common between their study and ours. Ogawa *et al* found 73 upregulated genes common to radioresistant sublines, including the growth factor amphiregulin, and genes associated with cell proliferation (MAPKAPK2), intracellular calcium signalling (regucalcin) and disruption of vascularisation (angiopoietin 2). Downregulated genes included genes associated with apoptosis (caspase 8) and others. Encouragingly, siRNA screens are now being used to identify radiosensitisation targets, such as PPP2R1A, a scaffolding protein of phosphatase 2A (Wei *et al*, 2013). Of the 69 confirmed hits from this screen done in MiaPaCa-2 cells, suspected (Chk1, ATR, MCL1) and novel genes (PLA2G4B, GPR2, MTMR3, RAD9A, KLC2L, KIFAP3, KLF3, DVL2, LZTR1, SIAH2, PLCD1) were identified as targets for radiosensitisation. More cell lines require testing with these screens to capture the heterogeneities in PC radioresponse.

Such unbiased studies are necessary as previous attempts at determining the causes of PC radioresistance relied primarily on specific pathways related to apoptosis, cell survival, and cell cycle progression while overlooking other pathways and cellular processes that are considered to likely result in increased radioresistance, such as DNA damage recognition and repair (DiBiase *et al*, 1999; Satyamoorthy *et al*, 2000; Macaulay *et al*, 2001; Munshi *et al*, 2004; Kim *et al*, 2007; Hui *et al*, 2009; Wang *et al*, 2009; Romano *et al*, 2010). In addition, despite the few pathways currently implicated in PC radioresistance, none has yet been effectively targeted to elicit therapeutic radiosensitisation clinically.

The results of this study indicate the importance of cholesterol and fatty acid biosynthesis in PC radiation resistance. Such implication has only been indicated once before in a directed study showing that fatty acid synthase (FASN) is associated with PC radioresponse (Yang *et al*, 2011). Importantly, increased expression of FASN was found to be strongly associated with both natural and radioselected radioresistance in our study as well, validating both our methods as well as the observations of this previous study. We specifically found that FDPS, an important branchpoint enzyme of the cholesterol synthesis pathway, is directly involved in radioresistance (Figure 2C and D). Moreover, FDPS was expressed in all PC cell lines tested (Figure 2B) and is overexpressed in human PC tissues (Figure 2E–G). Low-to-moderate expression in normal pancreas as observed in our study has also been reported earlier (Romanelli *et al*, 2009). Although FDPS has been seen to be overexpressed in multiple solid cancers by gene expression microarray (Pilarsky *et al*, 2004), to our knowledge, this is the first report to determine FDPS protein levels in human cancer tissues. Although elucidation of the molecular mechanisms underlying the association of FDPS, FASN, as well as the nine other radioresistance-associated genes, with PC radioresistance has not yet been undertaken in this or previous works, previous studies indicating the necessity for cholesterol biosynthesis in the membrane association and activity of both mutant and wild-type KRAS in PC may explain our observations (Ura *et al*, 1994). To this point, ZOL and cholesterol-lowering statins (that is, HMG-CoA reductase inhibitors) have been shown to decrease Ras prenylation or Ras protein levels in cells with mutant Ras (Tassone *et al*, 2003; Marten *et al*, 2007; Wasko *et al*, 2011).

Our studies indicate the potential of targeting cholesterol biosynthesis for radiosensitisation of PC through the successful synergy of siRNA knockdown of FDPS and IR *in vitro*, and ZOL and IR *in vitro* and *in vivo*. Currently, ZOL, a potent inhibitor of FDPS, is used in the treatment of patients with breast and prostate cancers that have metastasised to the bone, to prevent treatment-associated bone loss in breast cancer, as well as to slow the progression of osteoporosis (Brufsky *et al*, 2007; Gnant *et al*, 2007). ZOL has also undergone clinical trials as an adjuvant to standard of care for breast cancer, showing mixed efficacy results (Brufsky *et al*, 2007; Gnant *et al*, 2007; Coleman *et al*, 2011). ZOL has been seen to radiosensitise fibrosarcoma (Koto *et al*, 2013), osteosarcoma (Ryu *et al*, 2010), oesophageal squamous cell carcinoma (You *et al*, 2014), and breast cancer cells (Ural *et al*, 2006) in preclinical studies. Importantly, ZOL has recently undergone preclinical study as a chemosensitiser for PC, specifically as an add-on therapy to enhance the efficacy of gemcitabine, with promising results (Zhao *et al*, 2012). A phase II clinical study of ZOL with gemcitabine in advanced PC patients ($n = 35$ enroled) showed the combination to be well-tolerated, although final results have not been published (ASCO 2006 abstract; Cov *et al*, 2006). An additional phase I trial ($n = 15$) for neoadjuvant ZOL treatment for patients with resectable PC also was well tolerated (Sanford *et al*, 2013). To date, however, no study has examined ZOL specifically as a radiosensitiser in PC. Our results indicate that ZOL treatment can significantly radiosensitise PC cell lines *in vitro* (Figure 3). The ER of 1.3–1.5 is comparable with that reported for known

radiosensitisers, such as gemcitabine (Pauwels *et al*, 2005) and cisplatin (Skov and MacPhail, 1991), and for other reported radiosensitisers, such as dual prenyl transferase inhibitors (Brunner *et al*, 2005), the HIV protease inhibitor nelfinavir (Kimple *et al*, 2010), and Chk1 and PARP inhibitors (Vance *et al*, 2011).

ZOL treatment also provided a trend towards radiosensitisation of allograft tumours of a radioresistant murine cell line derived from a genetically engineered KPC mouse tumour (Figure 4). The dose of ZOL used ($2\,mg\,kg^{-1}$) is equivalent to a 10 mg human dose calculated based on body surface area (Reagan-Shaw *et al*, 2008), and it should be noted that the standard clinical dose of ZOL, administered as an infusion every 3–4 weeks in the treatment of cancer-induced bone disease, is 4 mg, which yields a peak plasma concentration (C_{max}) of 1–2 μM (Rogers and Holen, 2011). This dose was chosen because it is within the range of ZOL used in clinical trials (4–16 mg) and because it is well below the highest reported non-lethal single dose ($10\,mg\,kg^{-1}$) in mice (Zometa package insert, 2006). Possible reasons for not reaching significance in the *in vivo* studies could be single dose administration of ZOL to more accurately reflect clinical dosing, use of a highly radioresistant cell line, or a sample size of mice too small to reach significance. Further study is needed to determine whether multiple doses of ZOL may enhance radiosensitisation.

Using a clinical CT scanner, clinical treatment planning software, and linear accelerator, we were able to accurately and precisely irradiate subcutaneous tumours at high doses without shielding. Although most preclinical studies use small-dose fractions (3–10 fractions of 1–2 Gy; Morgan *et al*, 2008; Kimple *et al*, 2010; Wei *et al*, 2013), we were able to deliver high-dose fractions (five fractions of 7 Gy), which are being investigated in stereotactic hypofractionated regimens for PC at UNMC and other institutions. By using immune-competent mice in our study, we better modeled the effects of radiation on the immune system, clearance of radiated cancer cells by the immune system, and possible immunogenicity of radiated tumours (abscopal effect). This also provides for added anti-tumour effects of ZOL from a competent immune system, such as through activation of gamma delta ($\gamma\delta$) T cells (Marten *et al*, 2007), repolarisation of macrophages (Coscia *et al*, 2010; Rogers and Holen, 2011), and impairment of myeloid-derived suppressor cells (Porembka *et al*, 2012).

Despite the novelty and positive results of this work, several pitfalls remain. Currently, the only *in vivo* model available for PC radiation research consists of subcutaneous transplantation that has proven a poor indicator of clinical efficacy for both chemo- and radiotherapy. In addition, only two cell lines and two cell line derivatives were utilised in the gene expression microarray profiling in this study (although more were used to check FDPS expression and to test ZOL), potentiating that our results may not remain consistent across all PC cells lines or in the complexities of PC patients. It is further important to note that the radioresisant phenotype was diminished beyond 18 passages in the absence of continued radiation exposure. Also, we are yet to determine whether ZOL can radiosensitise normal organs involved in radiation toxicity, such as small intestine. Biochemical tests are also needed to determine tumour uptake of ZOL, as bisphosphonates accumulate highly in bones. However, this study describes comprehensive pathway analysis from the context of PC radiation resistance and opens avenues for future studies both in PC as well as in other cancers with low radiation efficacy. For example, several interesting upregulated genes in our microarray could be investigated for their role in radioresistance, including the chemokine CCL2 and the splicing factor SFPQ (PSF), which is associated with DNA repair and RNA biogenesis and mediates radioresistance of HeLa cells (Ha *et al*, 2011). Downregulated genes in our microarray, such as the pro-apoptotic BNIP3 and galectin-3 (LGALS3), could prove to be important radioresponse genes.

On the basis of our results, several future studies are warranted. Microarray analysis and correlation of gene expression with radioresistance, both natural and radioselected, should be undertaken in a greater number of cell lines to determine the universality of the identified genes across all PC. This approach could also be used to compare the gene expression profiles of radiation responder *vs* non-responder human tumours. For example, unbiased analysis of tumour samples taken pre-irradiation could be correlated with treatment response. In addition, ZOL should be further studied as a radiosensitiser using either spontaneous genetically engineered mouse models or directly in phase I trials. Further, unbiased studies such as this should be undertaken in other cancers such as renal cell carcinoma and melanoma, in which radiotherapy has a significant role in patient treatment but lacks general efficacy, to determine novel pathways and targets for radioresistance.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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