

Selective Inhibition of Ras, Phosphoinositide 3 Kinase, and Akt Isoforms Increases the Radiosensitivity of Human Carcinoma Cell Lines

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Abstract

Ras activation promotes the survival of tumor cells after DNA damage. To reverse this survival advantage, Ras signaling has been targeted for inhibition. Other contributors to Ras-mediated DNA damage survival have been identified using pharmacologic inhibition of signaling, but this approach is limited by the specificity of the inhibitors used and their toxicity. To better define components of Ras signaling that could be inhibited in a clinical setting, RNA interference was used to selectively block expression of specific isoforms of Ras, phosphoinositide 3 (PI3) kinase, and Akt. Inhibition of oncogenic Ras expression decreased both phospho-Akt and phospho-p42/44 mitogen-activated protein (MAP) kinase levels and reduced clonogenic survival. Because pharmacologic inhibition of PI3 kinases and Akt radiosensitized cell lines with active Ras signaling, whereas inhibition of the MAP/extracellular signal-regulated kinase (ERK) kinase/ERK pathway did not, we examined the contribution of PI3 kinases and Akts to radiation survival. Selective inhibition the PI3 kinase P110 α + p85 β isoforms reduced Akt phosphorylation and radiation survival. Similarly, inhibition of Akt-1 reduced tumor cell radiation survival. Inhibition of Akt-2 or Akt-3 had less effect. Retroviral transduction and overexpression of mouse Akt-1 was shown to rescue cells from inhibition of endogenous human Akt-1 expression. This study shows that Ras signaling to the PI3 kinase-Akt pathway is an important contributor to survival, whether Ras activation results from mutation of *ras* or overexpression of epidermal growth factor receptor. This study further shows that selective inhibition of the PI3 kinase P110 α + p85 β isoforms or Akt-1 could be a viable approach to sensitizing many tumor cells to cytotoxic therapies. (Cancer Res 2005; 65(17): 7902-10)

Introduction

Ras activation and survival after DNA damage. Ras activation by mutation is frequent in certain human tumors (1, 2), whereas activation of Ras signaling by growth factor receptor deregulation or other mechanisms is common in other tumor types (3, 4). Activation of Ras signaling has been shown to increase the survival of tumor cells exposed to agents that cause DNA damage. Survival after irradiation of many transformed and tumor cell lines is

increased by expression of oncogenic Ras (reviewed in ref. 5). Overexpression of the *ras* proto-oncogene, or receptor-mediated activation of wild-type Ras signaling, has also been shown to promote radiation survival (6, 7). Ras activation also increases survival after DNA damage induced by chemotherapeutic agents. Early studies in mouse fibroblasts showed that expression of oncogenic H-, K-, or N-Ras conferred resistance to *cis*-platinum (8). Enhanced survival was shown in breast carcinoma cells expressing oncogenic Ras after exposure to *cis*-platinum (9), paclitaxel, doxorubicin, and 5-fluorouracil (10), and myeloma cells exposed to doxorubicin (11). Nooter et al. (12) showed enhanced resistance to doxorubicin in a rhabdomyosarcoma cell line transfected with the H-*ras* oncogene. Jansen et al. (13) showed melanoma xenograft resistance to *cis*-platinum after transfection with oncogenic N-*ras*. However, resistance has not been reported by all investigators. Choi et al. (14) found that resistance was imparted by oncogenic H-*ras*, but not K-*ras* transfection of rodent cells, and equivocal results or no resistance was seen in some other experimental models (15–17). However, the preponderance of evidence links Ras activation to enhanced survival of cells treated with DNA-damaging agents.

Disrupting Ras signaling has been shown to reverse the resistance imparted by Ras activation. Inhibition of Ras expression by antisense oligonucleotides has been reported to radiosensitize cells (18). Blocking posttranslational prenylation, which is required for Ras-mediated transformation, has also been shown to reduce radiation survival in a number of rodent and human tumor models that express oncogenic Ras (19–22). Gana-Weisz et al. (23) showed that disruption of Ras membrane anchorage increased SW480 colon carcinoma and Panc-1 pancreatic carcinoma cell sensitivity to gemcitabine. Both of these cell lines express oncogenic K-Ras. These findings point to Ras signaling as an important factor in the response of tumors to a range of cytotoxic therapies. This hypothesis is supported by the results obtained in several clinical studies that have examined Ras activation and tumor responses. These studies have reported K-*ras* mutation as an independent prognostic factor pointing to poor progression-free survival in patients with stage III non-small cell lung cancer that were treated with surgery, radiation, and chemotherapy (24); non-small cell lung cancer treated with single agent paclitaxel (25); pancreatic cancer treated with radiotherapy (26); and colon cancer treated with CPT-11 (27). Not all clinical reports, however, have shown an association between *ras* mutation and resistance (28–30).

Ras activation by epidermal growth factor receptor. Signals transmitted through wild-type Ras proteins by mutated or overexpressed tyrosine kinase receptors can also influence cell survival. The epidermal growth factor receptor (EGFR, also denoted as erbB1 or HER1) plays a pivotal role in the regulation of cell growth and differentiation. This receptor transmits growth regulatory

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signals on binding of EGF or transforming growth factor- α . A number of studies have shown a positive relationship between EGFR expression and tumor resistance to radiation (31). Conversely, EGFR signal inhibition enhances radiosensitivity. Milas et al. (32, 33) showed enhanced tumor radiosensitivity in head and neck carcinoma xenografts on mice after combined treatment with monoclonal anti-EGFR antibody (C225) and radiation. Bonner et al. (34) have shown that combining C225 and radiation results in enhanced apoptosis and decreased proliferation of squamous cell carcinoma lines. Harari and Huang (35, 36) have reported similar findings both *in vitro* and *in vivo*.

The mechanism by which EGFR signaling leads to altered radiosensitivity is only partially defined. EGFR receptors initiate cytoplasmic signaling through autophosphorylation of their intracellular domains (37). This signaling activates a number of effectors, including Ras and phosphoinositide 3 (PI3) kinase. Chakravarti et al. (38) showed that EGFR-mediated resistance to chemoradiation therapy in primary human glioblastoma was Ras dependent. Gupta et al. (7) showed that EGFR signaling in head and neck squamous cell carcinomas contributed to radiation survival, and that this could be reversed by inhibition of EGFR, Ras, or PI3 kinase. These results point to Ras signaling as a transducer of EGFR signals that are important for cell survival after DNA damage.

Ras activation and signaling to other proteins. Ras signaling activates both mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK and PI3 kinase/Akt pathways. Both of these pathways have been implicated in tumor cell survival after exposure to DNA-damaging agents. The MEK/ERK pathway has been implicated by some studies in mediating radiation survival in prostate cancer mammary carcinoma and glioma cells (38–40); however, this finding has not been observed in all tumor models (10, 41–45).

There are at least eight members of the PI3 kinase family that fall into three classes (46). The type I class comprises four members, p110 α , p110 β , p110 δ , and p110 γ , with their regulatory subunits (p85 α , p85 β , p55, p50, and, in the case of p110 γ only, p101). The type I class is associated with growth factor receptor signaling. p110 α , p110 β , and p110 δ are effectors of Ras signaling, whereas p110 γ transduces the signal from G-protein-coupled receptors. P110 α and p110 β are ubiquitously expressed, whereas p110 δ and p110 γ are expressed in hematopoietic lineage cells. The PI3 kinase isoforms seem to have differential roles in tumor growth and survival as determined using microinjection of blocking antibodies specific to the α , β , and γ isoforms. P110 β seems to promote DNA synthesis, whereas p110 α activity was shown to be antiapoptotic. Inhibition of P110 γ did not affect either parameter (47). Loss of either PI3 kinase α or β results in embryonic lethality, although the stage at which embryos die is different for α versus β knockouts (48, 49). Both PI3 kinase α and β show increased activity in human tumors. This increase was reported to be stage independent and resulted from both increased PI3 kinase expression and from increased activity (47). For these reasons, we focused on the p110 α and the p110 β isoforms and the p85 regulatory subunits.

PI3 kinase signals lead to activation of Akt/PKB (50). There are three major isoforms of Akt/PKB that have been found in mammalian cells; these are termed Akt1/PKB α , Akt2/PKB β , and Akt3/PKB γ , which are encoded by three separate genes with >85% sequence identity. Despite a high degree of sequence homology, Akt-1 and Akt-2 seem to serve distinct cellular functions with regard to cell survival and metabolism (51). Akt1 $^{-/-}$ mice are more sensitive to genotoxic stress and their thymocyte are more

sensitive to apoptosis induced by γ -irradiation, but these mice do not display a diabetic phenotype (52). In contrast, Akt-2 is more important in insulin signaling than Akt-1 (53). It thus seems that Akt isoforms have differing roles and may contribute differentially to the survival of cells after DNA damage. Activation of the PI3 kinase/Akt pathway has consistently been shown to enhance tumor cell survival (4, 7, 44, 45, 54–57) and reduce apoptosis (reviewed in ref. 58). Inhibition of PI3 kinase leads to enhanced susceptibility to killing by both cytotoxins and radiation (10, 42, 44, 57, 59–61).

In this report, we have investigated the contribution of the three Ras isoforms to survival promoted by EGFR activation. We also directly tested the contribution of oncogenic activation of H-Ras and K-Ras to radiation survival by targeting expression of these oncoproteins. Because both PI3 kinase and Akt participate in survival signaling, we tested the effects of knocking down expression of these proteins in cells with signaling activated by oncogenic Ras or by EGFR. In the case of Akt, we examined the three isoforms to determine their relative contributions to radiation survival. The approach used was to selectively reduce the expression of the various isoforms of these proteins using small interfering RNA (siRNA) to target their mRNA (62). The results show that selective inhibition of Ras signaling pathway components is effective in reducing tumor cell survival after DNA damage.

Materials and Methods

Cell culture. Cells were cultured at 37°C in water-saturated 5% CO₂. Cultures were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 units/mL penicillin, and 100 μ g/mL streptomycin (Life Technologies). Routine PCR testing was used to establish that cultures were *Mycoplasma*-free. The SW480 human colon carcinoma and T24 bladder carcinoma cell lines were obtained from the American Type Culture Collection (Rockville, MD). The SQ20B cell line (63) was derived from a patient with recurrent laryngeal carcinoma and was obtained from Dr. Ralph Weichselbaum (University of Chicago, Chicago, IL). Normal human fibroblast cells (POC cells) were derived in our laboratory and used at early passage.

Synthetic small interfering RNA. Synthetic siRNA were based on specific target sequences and analyzed for specificity by BLAST homology search. Oligonucleotides were purchased from Dharmacon Research (Lafayette, CO) and annealed according to the specifications of the manufacturer using annealing buffer and RNase-free water provided with the oligonucleotides. Scrambled II and nonspecific oligo VIII (Dharmacon) were used to control for nonspecific siRNA effects. The target sequences for the siRNAs used in this study were as follows:

K-Ras ^{V12}	GGAGCUGUUGGCGUAGGCAA
H-Ras ^{V12}	CCGUCGGUGUGGGCAAGAGU
PI3K-p110 α	AAGUAAUUGAACAGUAGGCA
PI3K-p110 β	AAUCGACCUAUGAUGCGGAGA
PI3K-p85 α	AAGCUCGUGGAAGCCAUUGAA
PI3K-p85 β	AAA GGACAGGGCUGGACAGCG
Akt-1	AAGGAGGGUUGGCGUCACAAA
Akt-2	AACUUCUCCGUAGCAGAAUUC
Akt-3	AACUGGAGGCCAAGAUACUUC

Pooled siRNA (SMART pools, Dharmacon), which combines four or more specific siRNA duplexes in a single pool, was also used to inhibit Akt-1, Akt-2, and Akt-3 where indicated.

Pharmacologic inhibitors. The PI3 kinase inhibitor LY294002 was obtained from Eli-Lilly Pharmaceuticals (Indianapolis, IN) and the MEK inhibitor U0126 was obtained from Sigma Chemical Co. (St. Louis, MO). Inhibitors were dissolved as concentrated stock solutions in DMSO, stored at -80°C , and diluted at the time of use in culture medium.

Control cells were treated with medium containing an equal concentration of DMSO.

Transfection of small interfering RNA and clonogenic survival determination. One hundred thousand to 2×10^5 cells were plated into each well of six-well tissue culture plates. The next day (when the cells were 30–40% confluent), the culture medium was changed with antibiotic-free medium. Thirty to 60 μL of each annealed oligo duplex (25 mmol/L concentration) in 235 μL of reduced serum medium (Opti-MEM, Life Technologies) was transfected into cell using 15 to 30 μL of Oligofectamine (Life Technologies) according to the protocol of the manufacturer. In some experiments, sequential transfections separated by 48 hours were done to achieve more complete inhibition of targeted gene expression. This is indicated in the figure legends. Transfection efficiency was monitored by fluorescence microscopy and fluorescence-activated cell sorting analysis for intracellular uptake of Cy-5-labeled oligo at the initial part of this study and was consistently over 80%.

Two to 4 days after transfection of siRNA, cells were trypsinized, diluted to the appropriate cell density, and plated in 60 mm dishes for irradiation and colony formation. To test inhibition of MEK and PI3 kinase, inhibitor treatment was done on cells from log growth cultures treated 2 to 4 hours before irradiation with 10 $\mu\text{mol/L}$ of inhibitors (U0126 or LY294002, respectively). Controls were treated with equal concentrations of drug carrier (DMSO). Prenyltransferase inhibition was initiated 24 hours before replating for irradiation and clonogenic survival. Cells were irradiated with a Mark I cesium irradiator (J.L. Shepherd, San Fernando, CA) at a dose rate of 1.6 Gy/min. Colonies were stained and counted 14 to 21 days after irradiation. Plating efficiency in the absence of radiation was monitored for all inhibitor and siRNA treatments. Each point on the survival curves represents the mean surviving fraction from at least three dishes.

Radiation dose-modifying factors for siRNA or inhibitor treatment were calculated at 10% clonogenic survival from linear-quadratic curve equations used to plot the curves shown in the figures. The dose-modifying factors reported are the root values for control curves divided by the values for treated curves in each instance.

Western blot analysis. Cells were harvested using lysis buffer with phosphatase inhibitors (1 mol/L sodium fluoride, 0.1 mol/L sodium pyrophosphate, 0.1 mol/L sodium orthovanadate, 0.1 mol/L Tris, 10% SDS, 10% glycerol). Samples were boiled, sonicated, and clarified by centrifugation at 14,000 rpm and stored at -20°C until analyzed. The protein concentration in the samples was determined by Amido Black staining (64). Samples containing equal amounts of protein were separated on a 12.5% or 2% to 20% gradient polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. Membranes were blocked in PBS containing 0.1% Tween 20 and 5% powdered milk before the addition of primary antibody. Membranes were probed with monoclonal antibody directed against H-*ras* (Quality Biotech, Camden, NJ) or with monoclonal antibody directed against c-K-*ras* or N-*ras* (Oncogene Research Product, Cambridge, MA). Polyclonal anti-phospho-MAP kinase (MAPK; phospho-Thr²⁰²-Tyr²⁰⁴), pan-MAPK, polyclonal anti-phospho-Akt (Ser⁴⁷³), and polyclonal pan-Akt were obtained from Cell Signaling (Beverly, MA) and used at a dilution of 1:1,000. Polyclonal anti-Akt-1 (Upstate Biotechnology, Lake Placid, NY) was used at a dilution of 1:1,000. Polyclonal anti-Akt-2 (65) was generated in Dr. Birnbaum's laboratory (University of Pennsylvania, Philadelphia, PA) and was used at a dilution of 1:5,000. Detection of antibody binding was done using the enhanced chemiluminescence detection kit from Amersham (Piscataway, NJ) using the appropriate secondary antibody supplied with the kit.

Northern blot analysis. Total RNA was isolated from siRNA-transfected cells using Trizol (Life Technologies) according to the method recommended by the manufacturer. RNA was separated by electrophoresis and transferred to a Duralon-UV membrane (Stratagene, Cedar Creek, TX). Blots were prehybridized in Zeta probe hybridization solution (10% dextran sulfate, 5 \times SSC, 4 \times Denhardt solution, 10 mmol/L Tris, and 1% SDS) for 3 hours. Membranes were probed with the coding portion of Akt-3. Probes were labeled with ³²P using Ready-To-Go DNA labeling beads (Amersham Biosciences). Hybridization was done in Zeta probe hybridization solution containing 10⁶ cpm/mL ³²P-labeled probe at 42 $^\circ\text{C}$ overnight. Blots were

then washed to a stringency of 2 \times , 0.5 \times , 0.1 \times SSC (3 mol/L NaCl, 0.3 mol/L sodium citrate)/0.1% SDS for 20 minutes each at 65 $^\circ\text{C}$ and exposed to X-ray films for 1 to 14 days.

Retroviral transduction of mouse Akt-1. Ecotropic BOSC cells were transiently transfected with pVSV G and pCgp, pantropic retroviral packaging constructs, and retroviral vector (pMIGR1-Akt1/PKB α). Cell-free viral supernatants were harvested at 24 hours and used to infect SW480, T24, and SQ20B cells. The pMIGR1-Akt/PKB α construct contains the Akt kinase as well as green fluorescent protein (GFP) separated by an internal ribosomal entry site; thus, relative expression can be assessed by GFP expression. To avoid cross-reactivity with siRNA targeted to human Akt-1, cDNA for mouse Akt-1 was used in this retroviral construct. The murine mRNA is mismatched with the human sequence at two internal C nucleotides within the siRNA sequence (AAGGAGGGCTGGCTGCACAAA). SW480, T24, and SQ20B cells were incubated with viral supernatant for 2 days. Infection efficiency was almost 100% judged by green fluorescence under fluorescent microscopy.

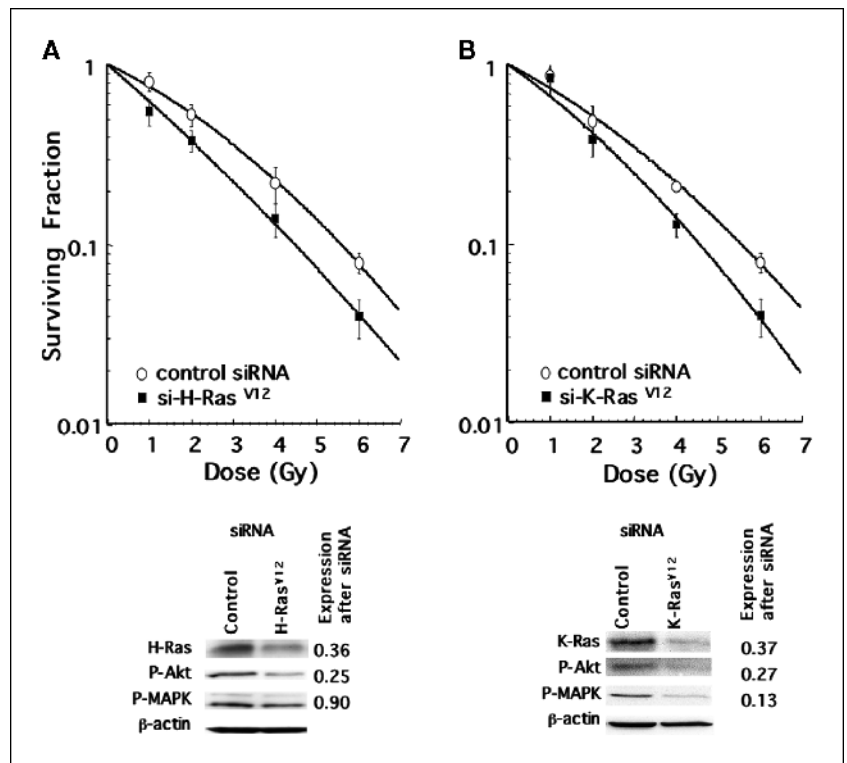
Results

Selective inhibition of oncogenic Ras isoforms reduces tumor cell radiation survival. To directly show whether oncogenic H- and K-Ras activation contributes to cellular radiation resistance, we examined the radiation sensitivity of T24 and SW480 cells treated with siRNA specific for the mutant *ras* allele expressed in each cell line. Equivalent inhibition of H-Ras^{V12} K-Ras^{V12} expression was obtained using siRNA specific for each isoform (Fig. 1). Inhibition of oncogenic Ras expression was accompanied by reduced phosphorylation of Akt that was similar in both cell lines, whereas phosphorylation of MAPK was reduced to a greater extent in SW480 than in T24 cells, where inhibition was minimal. Inhibition of oncogenic Ras expression also decreased radiation survival in both cell lines to a similar degree. H-Ras inhibition in T24 resulted in a radiation dose-modifying factor of 1.25 at 10% clonogenic survival. Inhibition of K-Ras in SW480 cells resulted in a dose-modifying factor of 1.22.

Differential effects of PI3 kinase and MEK activity in tumor cell survival. As shown above, inhibition of Ras expression can down-regulate both MAPK and Akt phosphorylation. To better define which of the signal transduction pathways that activate these proteins had the greatest effect on radiation survival, we used pharmacologic inhibitors of MEK and PI3 kinase and tested for the effect of this inhibition on radiation survival. Inhibition of PI3 kinase with LY294002, but not MEK with PD98059, was previously shown to sensitize T24 cells (41, 44). Here, we tested whether inhibiting PI3 kinase and MEK would have the same effects on clonogenic survival in SW480 and SQ20B cells. SQ20B cells are relatively radioresistant as a result of wild-type Ras activation due to EGFR overexpression. Inhibition of PI3 kinase activity with LY294002 greatly reduced Akt phosphorylation in both SW480 and SQ20B cell lines (Fig. 2A and B) and caused a similar reduction in clonogenic survival (Fig. 2). The dose-modifying factor for LY294002 treatment was 1.28 in both cell lines. In contrast, although inhibition of MEK using the U0126 inhibitor was almost complete at the time of irradiation as determined by loss of MAPK phosphorylation, this inhibition had little effect on radiation survival in either cell line. The dose-modifying factor for U0126 treatment was below 1.1 in both cell lines. Thus, similar to cells with oncogenic H-Ras, in cells expressing oncogenic K-Ras and cells expressing wild-type Ras activated by EGFR overexpression, radiation survival seems to be influenced primarily by PI3 kinase pathway activity.

Inhibiting PI3 kinase expression reduces tumor radiation survival. Because pharmacologic inhibition of PI3 kinase activity reduced the clonogenic survival of irradiated cells, we next tested

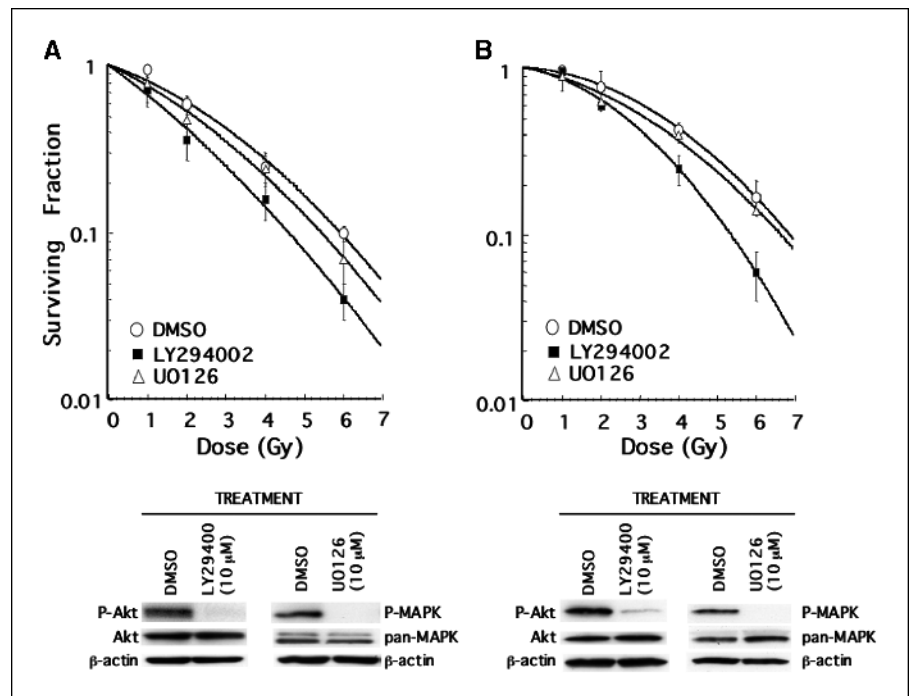
Figure 1. Effects of selectively inhibiting oncogenic Ras isoforms on tumor cell radiation survival and signaling. **A**, T24 cells were treated with siRNA to H-ras^{V12} (■) or treated with a nonspecific control siRNA (oligo VIII, ○). Transfections were done twice at 48-hour intervals. Two days after the last transfection, cells were plated for survival and proteins harvested for analysis of Ras expression and MAPK and Akt phosphorylation status. The plating efficiencies of unirradiated siRNA-treated cells were 0.36 for oligo VIII and 0.26 for siRNA to H-ras. **B**, SW480 cells were treated as in (A), except that an siRNA specific to K-ras^{V12} was used. Plating efficiencies of unirradiated siRNA-treated SW480 cells were 0.20 for control siRNA and 0.38 for siRNA to K-ras. In all experiments, Ras expression and protein phosphorylation status were determined at the time of irradiation. Points, mean surviving fraction from at least three dishes. β-actin was used to control for loading on Western blots. Experiments were repeated a minimum of three times.



whether selective inhibition of individual PI3 kinase isoforms would have the same effect. Using siRNA, we selectively inhibited expression of either the p110α or p110β or the p85α or p85β subunits of PI3 kinase. These isoforms were chosen on the basis of their ubiquitous expression. Inhibition of individual chains showed only modest decreases in radiation survival in T24, SW480, and SQ20B cells (not shown). However, combining inhibition of p110α or p110β expression with inhibition of p85β expression resulted in consistent reductions in both clonogenic survival (Fig. 3A-C) and phospho-Akt

(Fig. 3D). The radiation dose-modifying factor was greatest with inhibition of the p85β subunit. In SQ20B cells, combining inhibition of p85β with inhibition of either p110α or p110β resulted in a dose-modifying factor of 1.28. In T24 cells, inhibition of p110α and p85β gave a dose-modifying factor of 1.19 and inhibition of p110β and p85β gave a dose-modifying factor of 1.28. The dose-modifying factor in SW480 cells after inhibition of p110α and p85β was 1.22 and after inhibition of p110β and p85β was 1.15. Combinations of siRNA to p110α or p110β with p85α had less effect on clonogenic survival.

Figure 2. Survival effects of inhibiting PI3 kinase or MEK signaling pathway in cells with mutant K-Ras or EGFR-activated Ras signaling. Cells from log growth cultures of SW480 (A) or SQ20B (B) were trypsinized, plated for clonogenic assay, and treated for 2 to 4 hours with LY294002 (10 μmol/L) or U0126 (5 μmol/L) or with equal concentration of drug carrier (DMSO). Cultures were then irradiated and allowed to grow for 14 to 21 days, after which colonies were stained and counted. Points, mean surviving fraction from at least three dishes. Experiments were repeated thrice. The plating efficiencies for unirradiated SW480 cells were 0.65, 0.76, and 0.56 for DMSO- (controls), LY294002-, and U0126-treated cells, respectively. The plating efficiencies for SQ20B cells were 0.30 for controls, and 0.25 and 0.31 for LY294002 and U0126-treated cells, respectively. Phosphorylation states of ERK and Akt were determined by sampling of replicate inhibitor-treated and control cultures at the time of irradiation.



The influence of Akt isoforms on radiation survival. Akt proteins have been implicated in cell survival after DNA damage and are activated by PI3 kinase. To determine whether Akt proteins have a role in enhanced radiation survival in cells with activated Ras and PI3 kinase signaling, and if so, which isoforms of Akt contribute to survival, we used siRNA to inhibit specifically each of the three Akt isoforms (Fig. 4). Specific siRNA to Akt-1 or Akt-2 selectively down-regulated the target isoform, as shown by Western blot. The inhibition by siRNA of Akt-3 expression was shown by Northern blot due to the absence of a specific antibody for this protein. Selective inhibition of Akt-1 or inhibition of all three Akt isoforms resulted in the most consistent down-regulation of total phospho-Akt (Fig. 4).

The effect on clonogenic survival of inhibiting the three Akt isoforms individually and in combination was also assessed (Fig. 5). Of the three isoforms of Akt, selective inhibition of Akt-1 had the greatest effect on radiation survival, although cotransfection of Akt-1, Akt-2, and Akt-3 siRNA further decreased radiation survival in all three lines. In contrast to treatment with siRNA to Akt-1, selective inhibition with siRNA to Akt-2 or siRNA to Akt-3 alone generally had little effect on survival. We monitored whether the radiosensitization observed in the siRNA-treated cells was associated with cell cycle redistribution occurring as a result of siRNA treatment. No changes in cell cycle distribution were noted at 48 hours after inhibition of Akt-1 or combined inhibition of Akt-1, Akt-2, and Akt-3. Subsequent to irradiation, however, 60% to 66% of siRNA-treated SQ20B and SW480 cells accumulated in G₂ at 12 hours compared with 34% to 35% in control siRNA-treated cells (not shown). Thus, although siRNA treatment did not lead to cell cycle redistribution, it affected radiation-induced G₂-M accumulation.

An important question in any approach that potentiates tumor cell killing by radiation is whether the treatment also affects normal cell survival. Although Akt-1 inhibition sensitized the tumor cells, it had no effect on the radiosensitivity of normal human fibroblasts, which have a lower level of Akt phosphorylation than the tumor cell lines (Fig. 6). Simultaneous inhibition of all three isoforms of Akt did have a modest effect on the clonogenic survival of these normal cells; however, the dose-modifying factor was only 1.09. Results obtained in MR4-immortalized rat embryo fibroblasts (not shown) were similar with a dose-modifying factor of 1.05 after inhibition of all three Akt isoforms.

Validation that siRNA inhibition of Akt-1 expression was responsible for radiosensitization was obtained by transducing SW480, T24, and SQ20B cell lines with retroviral vectors encoding mouse Akt-1. Mouse Akt-1 is not susceptible to knockdown by the siRNA used in these experiments due to differences in nucleotide sequence in the siRNA target region. Transduction caused a marked increase in the expression of Akt-1 (both the mouse and human form are recognized by the antibodies used for blotting Fig. 7A). Phospho-Akt was also markedly increased. Overexpression of Akt-1 did not enhance radiation survival over that observed in cells expressing endogenous levels of this protein. However, cells transduced with the murine Akt-1 vector and overexpressing this protein were not radiosensitized by siRNA to human Akt-1. In contrast, specific inhibition of endogenous (human) Akt-1 with siRNA radiosensitized cells transduced with a control vector, consistent with the observations in nontransduced cells (see also Fig. 5). These results show rescue from siRNA inhibition of endogenous Akt-1 by exogenous Akt-1 that was not susceptible to the human gene-specific siRNA. The results also show that there is a finite limit to the radiation survival advantage provided by Akt that cannot be surpassed by overexpression of the protein or its activated form.

Discussion

The success or failure of cancer therapy can be influenced by the sensitivity of the tumor target and the limits imposed on treatment by the sensitivity of the normal surrounding tissues. In this report, we have investigated possible points in Ras signaling that could be targeted to selectively enhance the sensitivity of tumors to therapy.

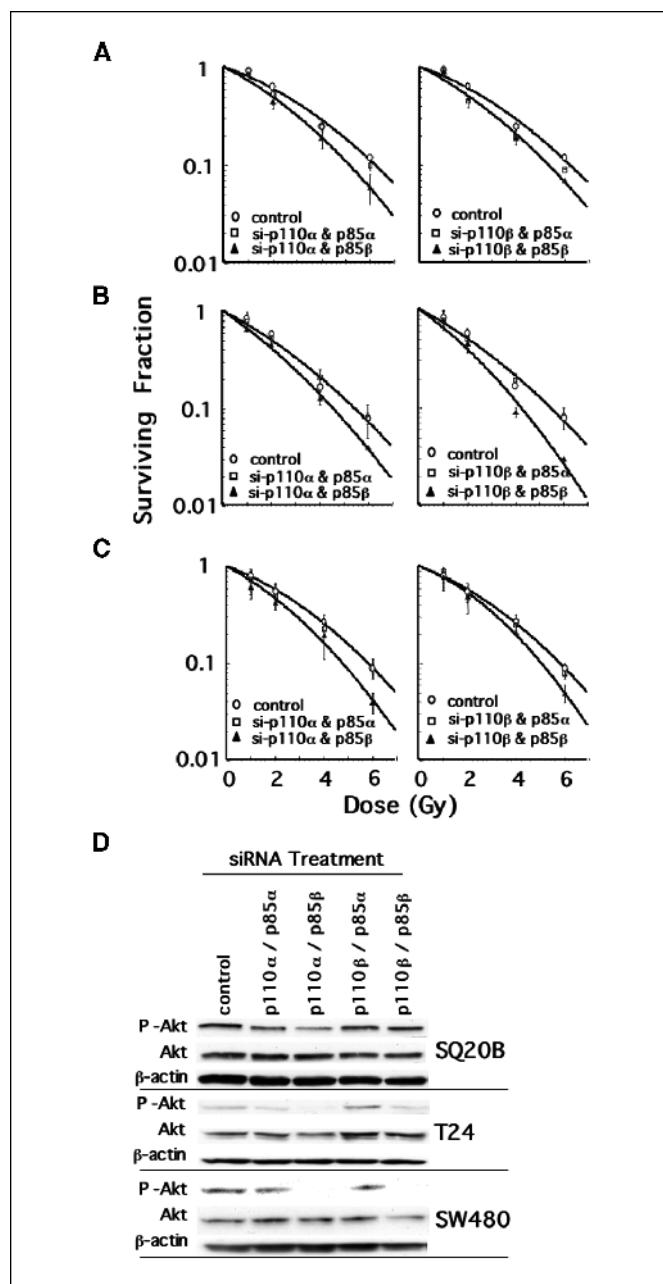
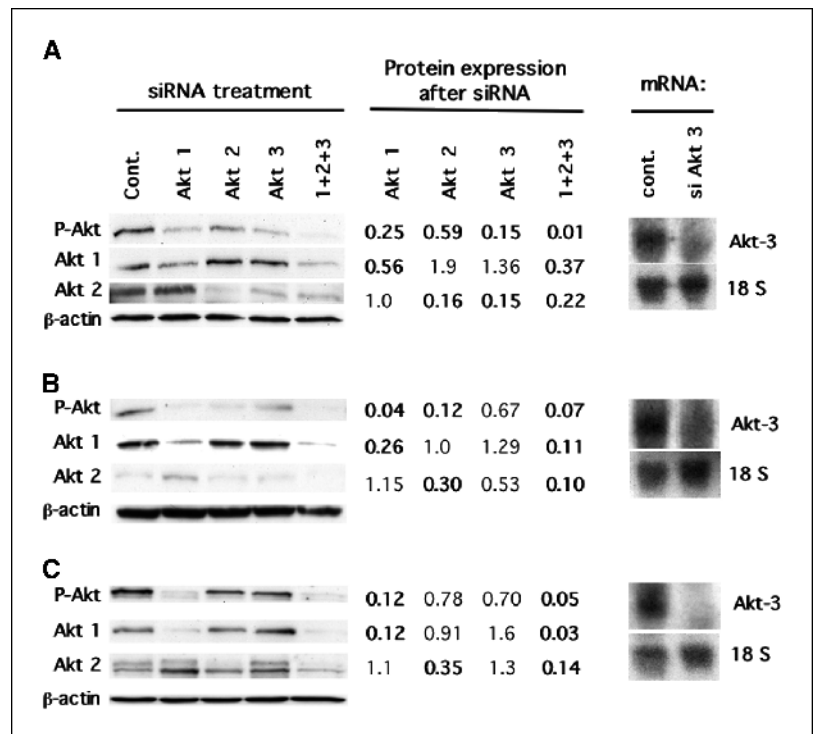


Figure 3. Inhibition of catalytic and regulatory subunit of PI3 kinase using siRNA. The effects on clonogenic survival of inhibiting PI3 kinase p85 α or p85 β combined with inhibition of either p110 α or p110 β subunits were tested in SQ20B (A), T24 (B), and SW480 (C). The plating efficiencies for unirradiated cells in clonogenic survival studies were 0.35 for control siRNA-treated SQ20B and between 0.13 and 0.14 for those treated with siRNA to PI3 kinase subunits. In T24, control cells had a plating efficiency of 0.19 and PI3 kinase siRNA-treated cells had plating efficiencies between 0.20 and 0.33. In SW480, control cells had a plating efficiency of 0.24 and PI3 kinase siRNA-treated cells had plating efficiencies between 0.17 and 0.42. Cell lysates from each cell line and treatment were prepared from replicate plates at the time of irradiation and probed for Akt phosphorylation (D).

Figure 4. Selective inhibition of Akt isoform expression. siRNA was used to inhibit selectively the expression of Akt-1, Akt-2, or Akt-3. Down-regulation of each isoform was shown by Western blot analysis (Akt-1 and Akt-2) or Northern blot analysis (Akt-3). Experiments were carried out with both single-sequence siRNA and with SMART pool siRNAs. The experiments shown for SQ20B (A) were done with single-sequence siRNA. The experiments shown for T24 (B) and SW480 cells (C) were done with SMART pool siRNAs. Experiments were done a minimum of three times in each cell line.



The Ras survival pathway identified in prior studies (44, 45) and further defined in the present work is comprised of tyrosine kinase receptor (EGFR), Ras, PI3 kinase, and Akt. This pathway is an attractive target for molecularly targeted therapies as it is frequently activated by mutation of Ras or components of the pathway, and by deregulated growth factor receptor signaling to Ras.

Ras activity can contribute to the survival of tumor cells exposed to radiation or chemotherapy. The current report further defines the role of Ras in cell survival by examining the contribution of the different Ras isoforms to the survival of tumor cells after DNA damage induced by irradiation. We have shown that the three Ras isoforms, H-Ras, K-Ras, and N-Ras, can contribute to radiation survival when activated by oncogenic mutations. This had previously been implied, not only by a series of studies using *ras* oncogene transfection (cited above), but also by the results of studies into prenyltransferase inhibitor-mediated radiosensitization (reviewed in ref. 66). In these studies, inhibition of prenylation, which is required for Ras activity, resulted in tumor radiosensitization of tumors with oncogenic Ras (20, 21). The interpretation of these studies, however, as well as those utilizing lovastatin inhibition of Ras processing (19), is complicated by the large number of prenylated proteins affected by these inhibitors (67–69). Our results also accord with prior findings from our group that the presence of oncogenic *K-ras* and *N-ras* alleles is associated with enhanced radiation survival in DLD-1 colon and HT1080 sarcoma lines, respectively (70). In these studies, the presence of oncogenic *ras* alleles was the only variable, but it was still formally possible that the derivation of tumor cell clones lacking activated *ras* could have engendered other changes affecting radiosensitivity. In the current report, specific and transient down-regulation of oncogenic *ras* allele expression resulted in radiosensitization.

The findings reported here further define the role of wild-type Ras in transmitting survival signals initiated at the EGFR. EGFR activation is clearly implicated as a contributor to radiation survival

(33, 35, 71) through activation of Ras (38). Furthermore, there is evidence that the EGFR forms part of an endocrine loop for Ras activation (72). EGFR is the target of inhibition by antibodies and kinase inhibitors currently in clinical trials (reviewed in ref. 73). The work presented here shows that the pathways that promote survival after DNA damage in cells with oncogenic Ras activation also affect survival in SQ20B cells, which overexpress EGFR. This finding shows that using a common approach to the inhibition of Ras signaling is valid both for cells with Ras mutation and cells with Ras activation due to receptor tyrosine kinase activation.

Several approaches in targeting Ras signaling have been tested (reviewed in ref. 74), but the results to date in clinical trials have not been as good as was expected (75, 76). For this reason, we investigated not only Ras, but also other potential contributors to radiation survival whose activity is promoted by Ras signaling. Radiosensitization of tumor cells after inhibition of *ras* expression with siRNA was accompanied by decreased Akt phosphorylation in cells with mutations in *H-ras* and *K-ras* and by decreased MAPK phosphorylation in cells with mutations in *K-ras*. This decrease indicates reduced signaling through these proteins. Prior studies have proposed a role for both MAPK (40, 77) and PI3 kinase/Akt (10, 56, 61, 78) signaling in cell survival. We found that pharmacologic inhibition of PI3 kinase/Akt signaling in cells with *ras* oncogene expression or EGFR amplification reduced radiation survival irrespective of the mechanism of Ras activation or, in the case of oncogenic N-Ras or K-Ras, the activated allele of *ras*. In contrast, inhibition of MEK/ERK pathway had little effect in any of the cells tested here. The results of inhibiting MEK/ERK and PI3 kinase in T24 cells that express oncogenic H-Ras have been previously reported (41, 44). These results agree with the findings in the present report. Together, these studies point to the PI3 kinase pathway as a primary mediator of enhanced survival.

Because PI3 kinase signaling promotes survival, and Akt is activated by this signaling and has also been implicated in survival

(reviewed in ref. 50), we probed the contribution of Akt to radiation survival. In these studies, siRNA-mediated specific inhibition of expression of the three Akt isoforms was used to probe for their contribution to radiation survival. The results show that survival signaling from all three Ras isoforms is in large part transmitted through Akt-1. These findings imply that Akt-1 is a reasonable target for radiosensitization strategies. Because Akt-2 is more important for insulin signaling (53), selective inhibition of Akt-1 may avoid toxicities from inhibition of insulin signaling that would be expected with inhibitors targeting all three Akt isoform. The observation that overexpression of Akt-1 in cells that already expressed active Akt signaling did not further promote radiation survival implies that there is a limit to the survival benefit that signaling from this protein can impart, and thus to the degree of radiosensitization that can be obtained through its inhibition. In accord with our findings, it has been shown that down-regulation of Akt-1 protein by antisense oligonucleotides induced apoptosis selectively in tumor cells but not in normal cells (79). Our findings are also in accord with those of Liang et al. (78), who showed that constitutively active Akt-1 and PI3 kinase-dependent activation of Akt-1 each increased cellular resistance to radiation in MCF7

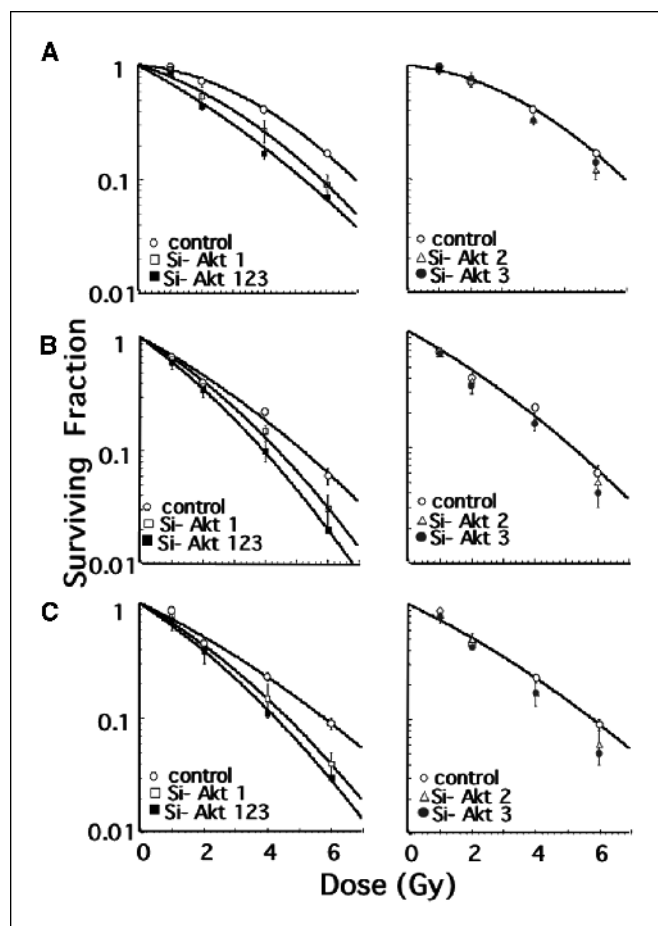


Figure 5. Radiation survival after selective inhibition of Akt isoform expression. Replicate cultures of SQ20B (A), T24 (B), and SW480 cells (C), from siRNA treatments shown in Fig. 4 were tested for clonogenic survival after irradiation. Experiments were done a minimum of three times in each cell line. The plating efficiencies for all SQ20B treatment groups (0.2-0.29) and for all T24 treatment groups (0.4-0.47) were equivalent. For SW480, the plating efficiency was between 0.2 and 0.28 for all groups except those treated with siRNA to Akt-3, which had a plating efficiency of 0.47.

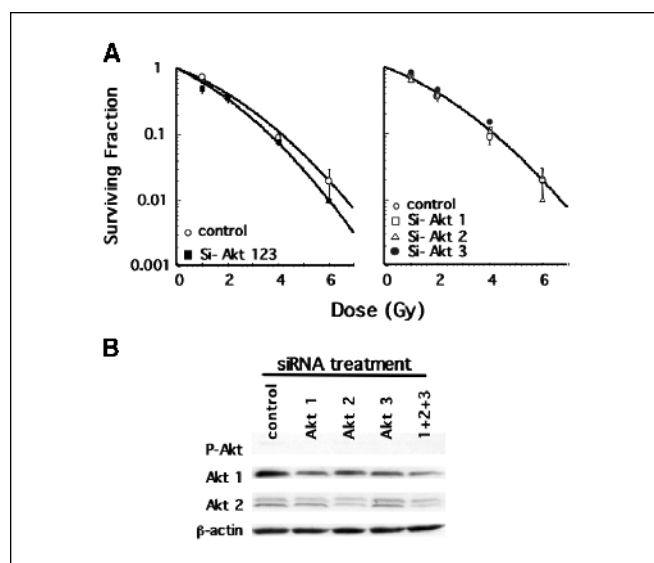


Figure 6. Akt knockdown and radiation survival in normal human fibroblasts. Normal human fibroblasts were transfected with single-sequence siRNA to Akt-1, Akt-2, or Akt-3 or a combination of all three. Clonogenic survival (A), and western analysis of Akt activation and expression changes was carried out on replicate dishes. Data are representative of repeated experiments. The plating efficiency for all groups was between 0.03 and 0.04 irrespective of treatment.

breast cancer cells. Taken together, these studies present a strong case for the role of Akt-1 alone in inducing cellular resistance to ionizing radiation in cancer cells. Our findings are also in accord with those of Jetzt et al. (80), who recently reported that expression of an Akt-1 kinase-dead mutant led to selective induction of apoptosis in tumor cells expressing activated Akt and had minimal effect on normal and tumor cells expressing low levels of activated Akt. In our study, all three carcinoma cell lines with activated Akt showed increased radiosensitivity after Akt-1 or Akt-1, Akt-2, and Akt-3 inhibition. In contrast, primary human fibroblasts with low level of Akt activation did not show radiosensitization by selective inhibition of Akt-1 alone and only modest sensitization by inhibition of all three isoforms. The demonstration that normal cell survival is not affected by specific inhibition of Akt-1 is of importance to the potential application of this approach to therapy.

The results presented here imply that survival signaling initiated at the cell surface at EGFR or at the level of Ras can be blocked either at Ras or at Akt. The levels of radiosensitization obtained by inhibiting different points in this survival-signaling pathway seem similar, as evidenced by the similarity in the dose-modifying factors obtained by inhibiting expression of the different components in this pathway. Future experiments will determine whether additive effects can be obtained by combining signaling inhibition at different points in the pathway and whether inhibitors of Akt activity currently under development can be applied in the context of radiation treatment.

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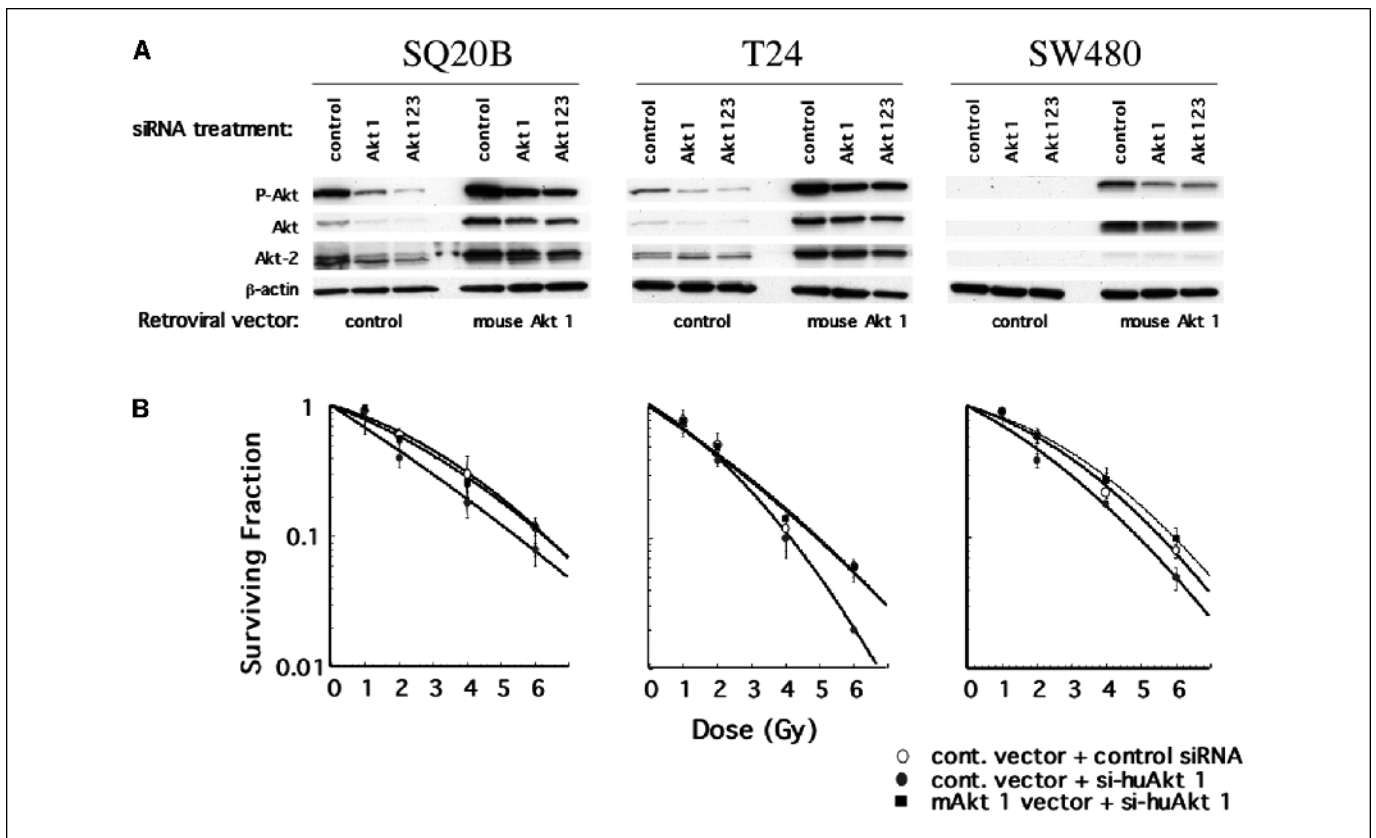


Figure 7. Rescue of retroviral mouse Akt-1 cDNA transfectant from selective inhibition of Akt-1 using siRNA. SQ20B, T24, and SW480 cells were transduced with retroviruses encoding mouse Akt-1 or a control vector containing only GFP as described in Materials and Methods. Transduced cells were then transfected with the siRNA to Akt-1 or combined siRNA to Akt-1, Akt-2, and Akt-3 and assessed for protein expression and radiation survival 48 hours later. Western blot exposures were timed for presentation of transduced cell Akt expression. Longer exposures (not shown) showed expression of Akt and phospho-Akt in SW480 cells. In each survival panel, circles represent GFP control vector transduced cells. Squares represent mouse Akt-1 transduced cells. Open symbols were treated with control siRNA. Closed symbols were treated with siRNA to Akt-1. The survival of mouse Akt-1-transduced cells treated with control siRNA (not shown) overlapped with the curves of control vector + control siRNA-treated cells. Experiments are representative of three replicates in SQ20B and T24 cells and were duplicated in SW480 cells. The plating efficiencies of SQ20B cells were between 0.36 and 0.31 for all groups transduced with control retroviral vector, 0.54 for control, and 0.36 for Akt-1 siRNA-treated cells transduced with mouse Akt-1. For T24 cells transduced with control vectors, the plating efficiencies were 0.21 for control siRNA and 0.46 for Akt-1 siRNA-treated cells. For T24 cells transduced with mouse Akt-1 vectors, the plating efficiencies were 0.10 for control siRNA and 0.17 for Akt-1 siRNA-treated cells. For SW480 cells transduced with control vectors, the plating efficiencies were 0.83 for control siRNA and 1.0 for Akt-1 siRNA-treated cells. For SW480 cells transduced with mouse Akt-1 vectors, the plating efficiency was 0.30 for both control siRNA and Akt-1 siRNA-treated cells.

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Selective Inhibition of Ras, Phosphoinositide 3 Kinase, and Akt Isoforms Increases the Radiosensitivity of Human Carcinoma Cell Lines

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