MicroRNAs as Potential Agents to Alter Resistance to Cytotoxic Anticancer Therapy

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Abstract

Tumor cells use preexisting prosurvival signaling pathways to evade the damaging and cytotoxic effects of anticancer agents. Radiation therapy is a primary form of cytotoxic anticancer treatment, but agents that successfully modify the radiation response *in vivo* are lacking. MicroRNAs (miRNA) are global gene regulators that play critical roles in oncogenesis and have been found to regulate prosurvival pathways. However, there is little understanding of how cellular miRNA expression affects the response of a cancer to cytotoxic therapy and ultimately outcome. The *let-7* family of miRNAs regulates expression of oncogenes, such as *RAS*, and is specifically down-regulated in many cancer subtypes. In fact, low levels of *let-7* predict a poor outcome in lung cancer. Here, we report that the *let-7* family of miRNAs is overrepresented in a class of miRNAs exhibiting altered expression in response to radiation. More strikingly, we also can create a radiosensitive state when the select *let-7* family of miRNAs is overexpressed *in vitro* in lung cancer cells and *in vivo* in a *Caenorhabditis elegans* model of radiation-induced cell death, whereas decreasing their levels causes radioresistance. In *C. elegans*, we show that this is partly through control of the proto-oncogene homologue *let-60/RAS* and genes in the DNA damage response pathway. These findings are the first direct evidence that miRNAs can suppress resistance to anticancer cytotoxic therapy, a common feature of cancer cells, and suggest that miRNAs may be a viable tool to augment current cancer therapies.

Introduction

Radiation therapy is one of the three primary modalities used in cancer treatment. Although radiation has been in practice for over a century, the global genetic response necessary for tissues to survive radiation-induced injury remains largely unknown. This has limited the ability to develop meaningful routes to minimize normal tissue toxicity while enhancing tumor eradication. Although single-protein targeting strategies have shown moderate success

in preclinical models, few have been successful in human trials. A failure to identify radiation modulators may be due to the complex genetic cellular response to radiation, as indicated by microarray studies showing significant changes in the expression of at least 855 genes (>1.5-fold) within 4 h of radiation (1). This suggests that regulatory molecules capable of affecting expression levels of a large number of target genes in a rapid manner may be required to affect the radiation response. One such class of potential regulators is microRNAs (miRNA; ref. 2).

miRNAs are small noncoding RNAs found in plants and animals that control gene expression by binding to complementary sites on target mRNA transcripts. The founding members of the miRNA family, *lin-4* and *let-7*, were identified in *Caenorhabditis elegans* (3, 4), where they were found to play critical roles in development and progenitor cell differentiation. The *lin-4* and *let-7* miRNAs are evolutionarily conserved in higher animals, including humans, and recent studies from our laboratory and others have shown roles for these (and other miRNAs) in human cancers (see ref. 2 for review). *let-7* is a member of a small family of miRNAs, including *mir-84* and *mir-48*, in *C. elegans* and *let-7a* through *let-7h* in humans (5, 6), which are down-regulated in lung cancer (7), a finding associated with a poor outcome for these patients (8). The *let-7* family of miRNAs has been shown to regulate the human *RAS* oncogene (7), the overexpression of which is commonly found in human tumors. *RAS* overexpression in tumors is considered a poor prognostic feature and is believed to be involved in the response to cytotoxic therapy (9). Recently, *RAS* signaling was shown to be critical for protection from radiation-induced reproductive cell death (10), the primary form of radiation-induced target cell death (11). Unfortunately, strategies directly targeting *RAS* or its upstream and/or downstream effectors have not successfully altered the radiation response *in vivo* (12). Because miRNAs target *RAS* as well as hundreds of additional genes (13), we hypothesized that their manipulation might more successfully alter the radiation response.

Materials and Methods

miRNA microarrays

Total RNA was collected from cells using the mirVana kit from Ambion (per manufacturer's instructions). A total of 10 µg was used for miRNA microarray by LC Sciences. To confirm the quality of the RNA, a UV test was performed and the samples were enriched for miRNAs by using a cutoff filter (um100 from Microcon-modified procedure). The microRNAs were then labeled and hybridized to a microarray chip with multiple repeat regions and a miRNA probe region, which detects miRNA transcripts listed in Sanger miRBase release 8.2. This consists of 440 human miRNA sequences. Multiple control probes were included in each chip. The control probes were used for quality controls of chip production, sample labeling, and assay conditions. For the in-depth data analysis of our time point experiments, LC Sciences performed multiarray normalization, ANOVA, and clustering analysis. The ANOVA and clustering analysis were performed on ratio data of individual arrays (with the multiarray normalization) instead of the often used intensity data of individual samples. They found this necessary to reveal the rather small miRNA variations among the samples of different time points. Because there was only one sample

for each time point, they used repeating probe sets of the arrays to have constructed "groups" that were needed for ANOVA analysis.

Real-time PCR

Quantification of levels of *let-7a, let-7b*, and *let-7g* was performed using the Taqman microRNA PCR system (ABI, per standard protocol). Levels were normalized to the 0 time point to determine changes in expression levels after irradiation.

Clonogenic assays

A549 cells were transfected with 90 nmol/L of the pre-*let-7* or control *pre-miR*. The transfection method was optimized using a luciferase reporter construct sensitive to *let-7* levels (*luc* fused to the *NRAS* 3′-untranslated region), and we chose the method with the least toxicity and most efficient transfection (X-tremeGENE, Roche; data not shown). Twenty-four hours after transfection, cells were treated with increasing doses of radiation and then plated at different dilutions and grown without being disturbed. Colonies were counted after 2 weeks. Experiments were done in quadruplicate for each dose and each miRNA, and all experiments were repeated at a minimum of two times. Stratified *t* tests were performed to analyze significance for all cases. The *P* value was based on a two-tailed evaluation of the data that were pooled for each experiment across all doses as is standard procedure and is shown in the figures.

C. elegans work

Methods for culturing, handling, and genetic manipulation of *C. elegans* were as described by Brenner unless otherwise indicated. The animals referred to here as wild-type (WT) *C. elegans* correspond to the Bristol strain N2. Strains used in this study were obtained from the *C. elegans* Genetics Center unless otherwise noted. *let-7*–overexpressing strains were generated as described (4, 7, 14). For synchronization, gravid hermaphrodites were treated and isolated embryos were treated with radiation as described previously (15). Dose-response curves were generated at the first S-phase radioresistance peak [determined as described previously (15)]. For each dose, a minimum of 100 animals was treated and scored per experiment, and experiments were repeated two to four times.

For statistical analysis, each of the mutant strains was compared against the WT using a stratified two-sample Wilcoxon rank sum test. Stratified *t* tests were performed to analyze significance for all cases. The *P* value was based on a two-tailed evaluation of the data and is shown in the figures or in the figure legends.

Determining the genetic basis of the *let-7* response

RNA interference (RNAi): after synchronization, animals were placed on plates with the appropriate bacterial strain containing the plasmid that overexpresses double-stranded RNA (dsRNA) from the gene of interest and grown until the appropriate time for radiation. After irradiation, animals were placed on plates with the same bacterial strain and grown until the phenotypic analysis was performed.

For analysis of alteration of mammalian gene levels in response to changing *let-7* levels, *let-7* downstream genes were analyzed by Western blot analysis using standard protocols.

Results and Discussion

Despite strong evidence that miRNAs are associated with cancer and are potential biomarkers for outcome, little is known about how they affect the response of a tumor to cytotoxic treatment. To determine whether miRNAs are involved in the cellular response to cytotoxic therapy, we used miRNA microarrays to compare the relative levels of cellular miRNAs before and after radiation. We irradiated a lung cancer cell line, A549, in which *let-7* levels are low (7) and *RAS* is activated (16). The levels of 81 miRNAs significantly changed after irradiation (Supplementary Figs. S1 and S2). Significant changes in expression of most miRNAs were observed as early as 2 h after irradiation, with most of these early affected miRNAs returning to their baseline expression levels by 24 h. The same microarray analysis was performed in a normal lung epithelial cell line, CLR2741. The levels of most miRNAs, including all members of the *let-7* family, were significantly different between these two cell types before radiation (data not shown). However, both the normal and tumor cells exhibited similar patterns of miRNA expression changes in response to radiation (Supplementary Figs. S1 and S2).

Interestingly, each member of the *let-7* family of miRNAs, barring one (*let-7g*), decreased significantly by 2 h after irradiation in both cancerous and normal lung epithelium (Fig. 1*A* and *B*). Of the 23 miRNAs with decreased expression after irradiation, 7 (30%) were members of the *let-7* family, a 17-fold enrichment over their representation on the array (1.8%; 8 of 440 miRNAs on the array). Real-time PCR confirmed the microarray findings for *let-7a* and *let-7b* (data not shown). In addition, the radiation experiments were repeated in a separate replicate of A549 cells as well as in two additional lung cancer cell lines, A427 (carcinoma) and H4441 (adenocarcinoma). Levels of *let-7a, let-7b*, and *let-7g* were evaluated by real-time PCR, and consistent with the microarray findings, *let-7a* and *let-7b* were significantly down compared with their baseline at 8 h after irradiation, whereas *let-7g* was significantly elevated by 24 h after irradiation in all of the lung cell lines studied (Fig. 1*C*).

The similarity between the miRNA response after irradiation in multiple cancerous and a normal lung epithelial cell lines suggests that a highly conserved global miRNA response exists in lung cells after irradiation (Fig. 1C; Supplementary Fig. S2). In addition, these findings suggest that perhaps miRNAs are components of the cellular response to cytotoxic insult. While this would indicate that miRNA expression patterns might be a biomarker for radiosensitivity, it further prompted us to test the hypothesis that altering miRNA levels could be an efficacious approach to alter the cellular radiation response.

let-7 overexpression in A549 cell lines causes defects in proliferation (8) but does not cause apoptosis in these cells (17). Clonogenic cell survival assays measure all forms of cell death and are the recognized standard for radiation sensitivity assays. Therefore, we used this assay to test the effect of altering *let-7* levels on the radiation response and cell survival. Specifically, we chose to evaluate the effect of *let-7b, let-7a*, and *let-7g* on the radiation

response because (*a*) *let-7b* drops the most significantly after irradiation in A549 cells (Fig. 1); (*b*) *let-7a* levels have been implicated in predicting outcome in certain cancers, most notably lung cancer (8, 18); and (*c*) *let-7g* levels are up-regulated after irradiation and significantly changed only in the lung cancer cell lines (Fig. 1). To overexpress each of the *let-7* homologues of interest, A549 cells were transfected with synthetic pre-*let-7* molecules or control pre-miRNA containing scrambled sequences (Ambion) and clonogenic assays were performed. Significant radiosensitization was found in cells treated with pre-*let-7b* or pre-*let-7a* compared with control pre-miRNA (Fig. 2*A*; Supplementary Fig. S3). This effect on the radiosensitivity of cultured cells is comparable with other studies of transfected small interfering RNA–based radiosensitizers (19). In parallel experiments, anti-miRs were delivered to A549 cells to specifically decrease *let-7* miRNA activity (7). As expected from the effects of *let-7b* overexpression, anti-*let-7b* caused significant radioprotection (Fig. 2*C*). Consistent with the opposite direction of altered expression levels of *let-7g* after irradiation, a unique role for *let-7g* in the radiation response was identified; *let-7g* overexpression protected A549 cells from radiation (Fig. 2*B*), whereas anti-*let-7g* caused radiosensitization of A549 cells (Fig. 2*D*). We hypothesize that overexpression of *let-7a* and *let-7b* causes radiosensitization and overexpression of *let-7g* radioprotection in part by overcoming the innate requirement of the cell to down-regulate or up-regulate these miRNAs as part of the response to cytotoxic insult. However, the molecular mechanisms of miRNA function in the radiation response may also be related to alteration in the levels of their targets, such as components of the DNA damage response (DDR) pathway and *RAS* (see below; refs. 7, 17). The exact differences in the roles of *let-7g* and the other tested *let-7* family of miRNAs in the radiation response remain to be determined.

To confirm the ability of *let-7* miRNAs to alter the radiation response *in vivo*, we turned to a powerful *C. elegans*–based *in vivo* model of radiation-induced reproductive cell death ["Radelegans" (15)]. The tissue studied in Radelegans is the developing *C. elegans* vulva, in which multipotential vulval precursor cells (VPC) undergo three rounds of cell division and differentiate into the mature vulva following *RAS* signaling (20). VPCs represent tissue clonogens, considered the critical and determinant targets of radiation in tumors that die via reproductive cell death (15), and thus, this model is considered an *in vivo* representation of clonogenic assays and radiation-induced tumor target cell death. In addition, because VPCs are synchronized, and all experiments are performed at the same point in the cell cycle (15), this model eliminates cell cycle alterations as a mechanism for radiosensitization. Previously, radiation resistance in VPCs was shown to depend on *RAS* signaling and a normal DDR pathway (10). VPCs show specific expression of three *let-7* paralogues, *let-7*, *mir-48*, and *mir-84* (7, 14), which repress *RAS* expression in this tissue but do not alter the VPC cell cycle (4, 7, 21). On irradiation, VPCs in strains that overexpress either *let-7* or *mir-84* were significantly radiosensitive compared with WT animals (Fig. 3*A*), consistent with results of the *in vitro* analysis of *let-7a* and *let-7b* in lung cancer cells (Fig. 2*A*; Supplementary Fig. S3).

let-7 loss-of-function mutants could not be analyzed for radiosensitivity due to gross defects in vulval development (4). Instead, animals harboring a *mir-84* or a *mir-48* deletion were analyzed because they develop without obvious vulval abnormalities (7, 21). In dose-response experiments, *mir-84(tm1304)* and *mir-48(n4097)* animals exhibited significant

radioresistance across all radiation doses (Fig. 3*B*) compared with a WT strain, also consistent with the results of the *in vitro* analysis of *let-7b* (Fig. 2). These findings in Radelegans are therefore an *in vivo* confirmation of the *in vitro* cell line studies, supporting the potential of alteration of *let-7* levels as a tool to change cell survival after irradiation.

To understand how *let-7* alteration affects the radiation response, we tested the hypothesis that radioresistance in the *mir-84* mutant was due to overexpression of some of its recently identified targets, including the *let-60/RAS* oncogene (7) as well as genes of the DDR pathway (17), which are also known to be critical in the radiation response (10). RNAi was performed through feeding in the *mir-84* mutant for genes of interest (Fig. 4*A*). Indeed, *let-60/RAS(RNAi), rad-51/RAD51(RNAi), coh-1/RAD21(RNAi), fcd-2/FANCD2(RNAi)*, and *cdc-25.3/CDC25(RNAi)* each significantly suppressed the radioresistance in *mir-84(tm1304)* animals across the doses studied (Fig. 4*B*). These findings support the hypothesis that the *let-7* family of miRNAs modulates the response to cytotoxic anticancer therapy in an *in vivo* model by altering levels of prosurvival and DDR pathways. In addition, we evaluated the level of several of these genes after irradiation in A549 cells by Western blot analysis and also saw an increase in their levels within the time frame of *let-7* alteration, further validating this mechanism in mammalian systems (Supplementary Fig. S4).

Our work reveals a role for miRNAs in the immediate cellular response to cytotoxic anticancer agents, and we show that alteration of cellular miRNA levels, specifically the *let-7* family, is able to affect cellular survival after cytotoxic therapy. Many miRNAs change levels significantly after irradiation, including *mir-34*, as shown by others as well (22). The mechanism of *miR-34* up-regulation was shown to be due to altered transcription from a known radiation response gene, *p53* (22). Our work instead shows down-regulation of most members of the *let-7* family, and we further prove the significance of the *let-7* family in the radiation response by showing that altering *let-7* levels before radiation affects cellular survival. One could speculate that one possible mechanism is alteration in the levels of miRNA processing components (23), although this cannot explain how numerous miRNAs increase whereas others decrease.

Although the mechanism of *let-7* on cell survival could result from its effect on cell proliferation and cell cycle (17), this is unlikely based on its effect in Radelegans, where cell cycle is removed as a variable. We hypothesize that the effects of *let-7* are genetically more direct based on the reversal of the radiation resistance in *C. elegans* by genetically decreasing levels of prosurvival and DDR genes that are *let-7* targets and the increased levels of some of these targets in mammalian cells after irradiation. It is tempting to speculate that miRNAs, which are often misexpressed in cancer, can now be used as a novel tool to help battle cancer in conjunction with current cytotoxic therapies. Future work will attempt to gain an understanding of the role miRNAs play in response to other cytotoxic therapies, such as chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Acknowledgments

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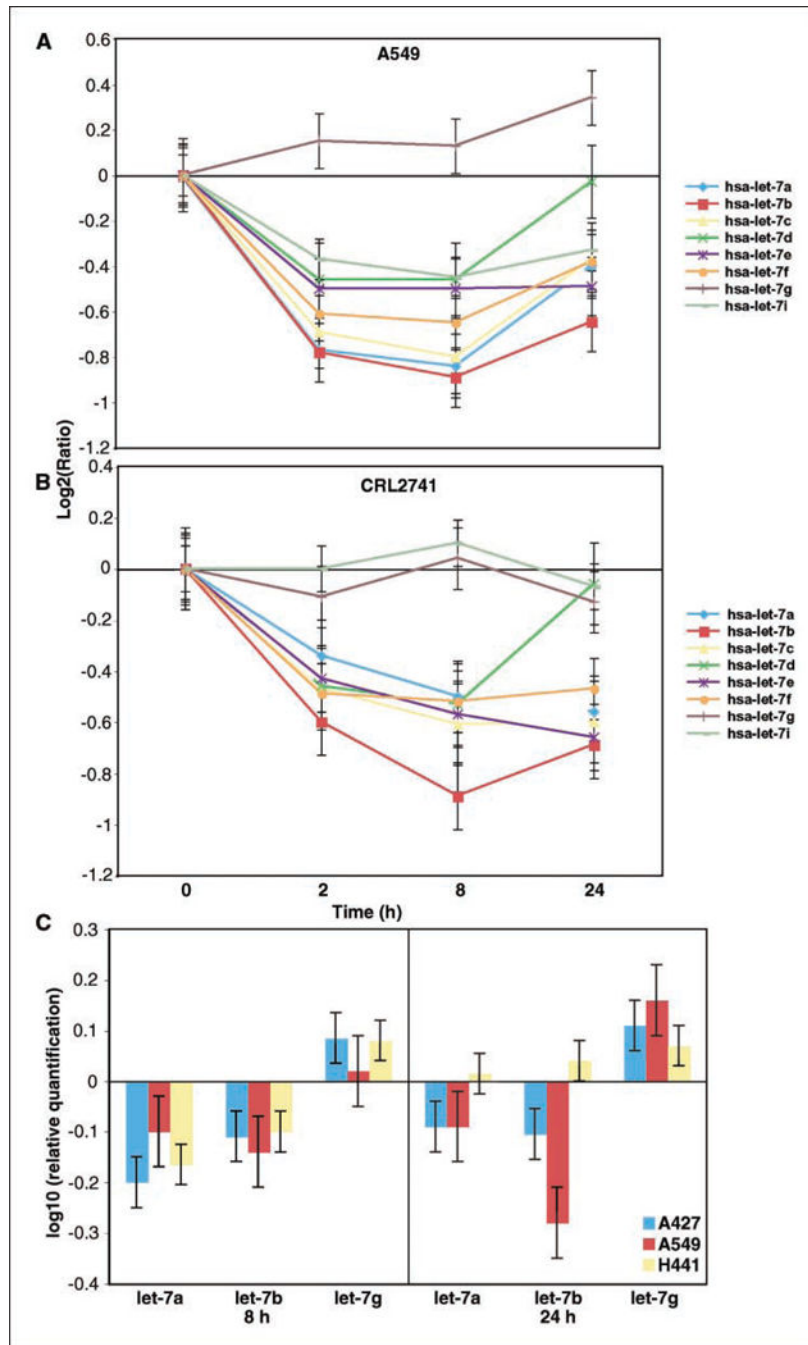
Figure 1.
Relative levels of the *let-7* microRNAs change significantly after irradiation. *A*, miRNA microarrays were performed on total RNA collected from irradiated A549 cells before and 2, 8, and 24 h after 2.5 Gy. The level of each member of the *let-7* family changed significantly over this time. *B*, levels of *let-7* miRNAs after irradiation in a normal lung epithelial line, CRL2741. *C*, real-time PCR results at 8 and 24 h of *let-7a, let-7b*, and *let-7g* from a separate A549 replicate and two additional lung cancer cell lines (A427, carcinoma; H441,
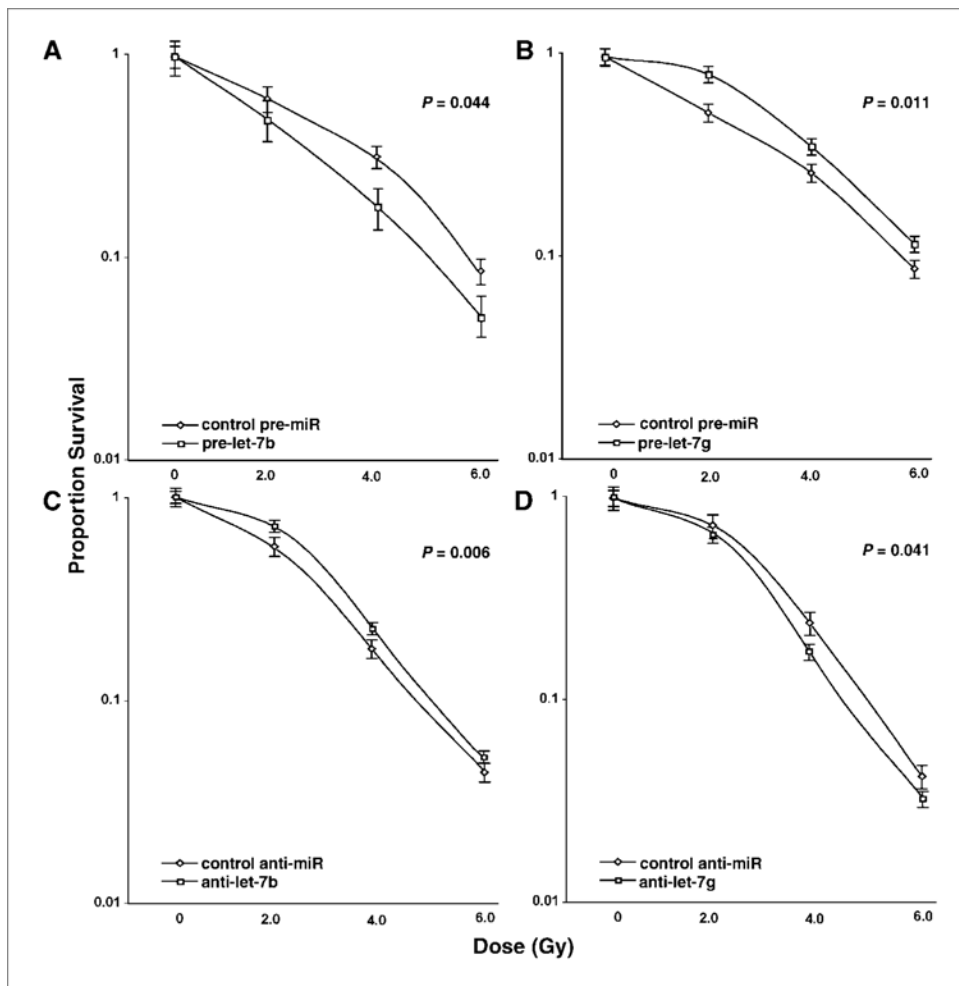
adenocarcinoma). *let-7a* and *let-7b* levels are reduced and *let-7g* levels are increased after irradiation. Ratio of *let-7* levels with the unirradiated 0 time point as the baseline. *Bars*, SD.
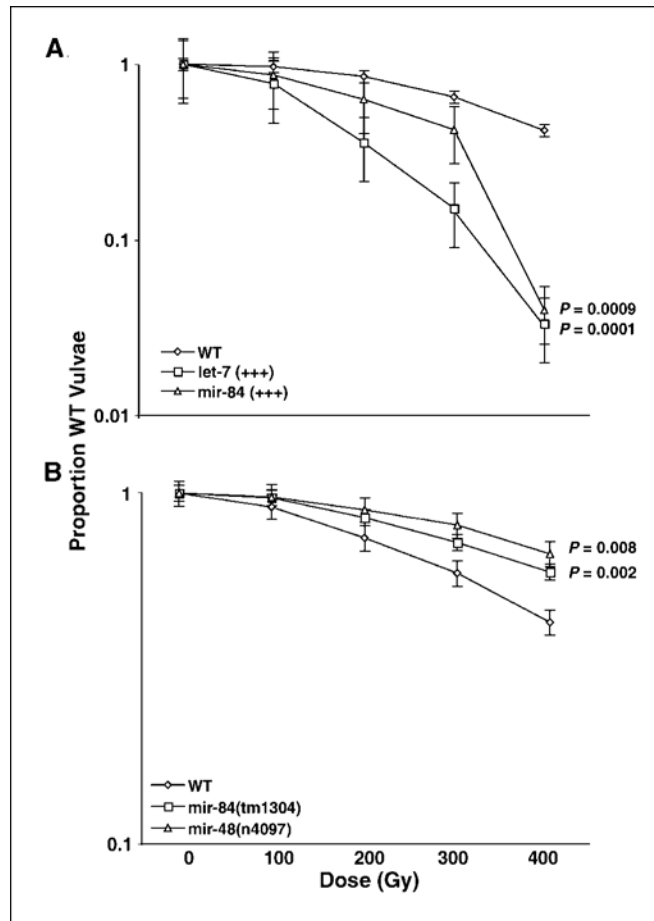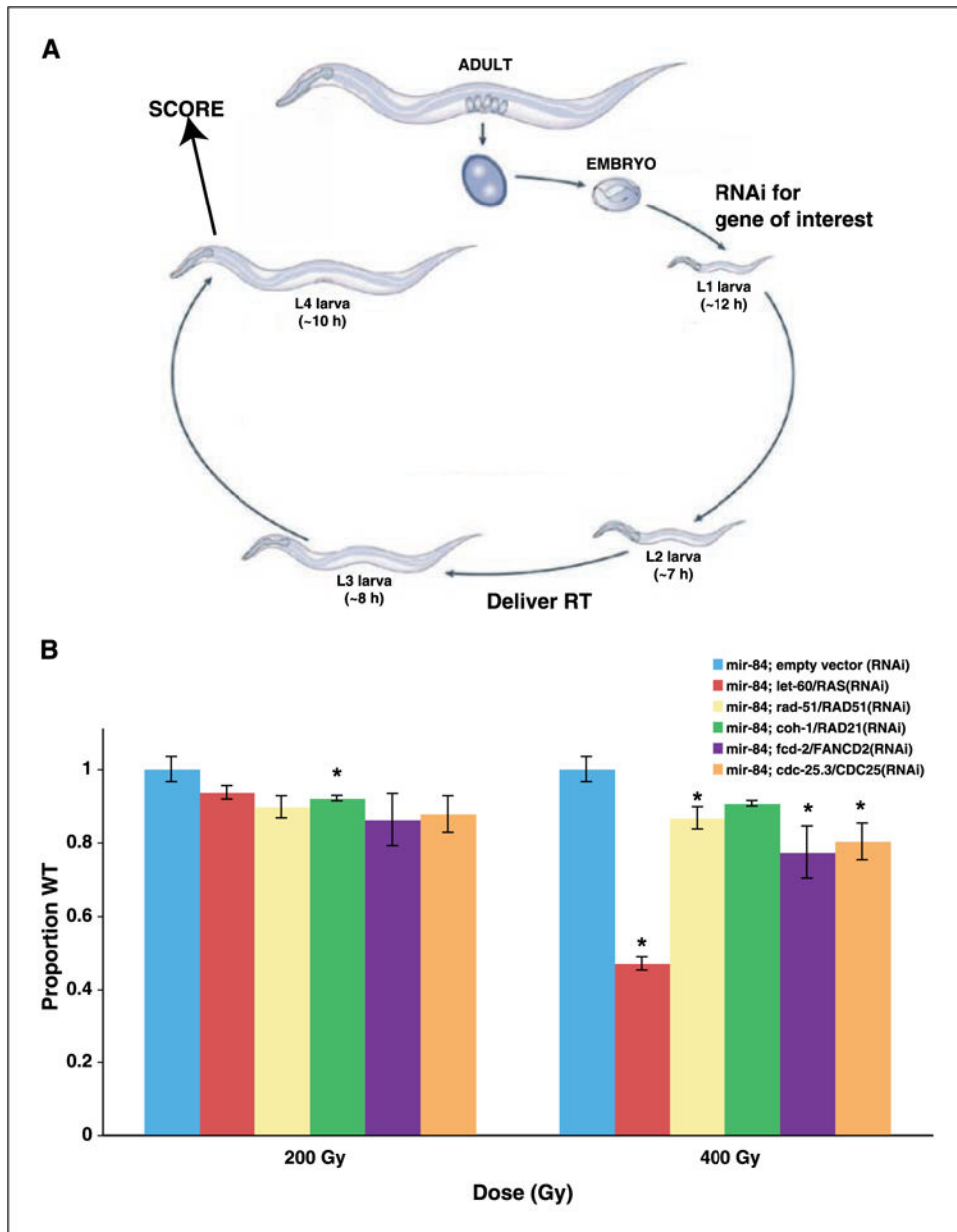
Figure 2.
Manipulating miRNA levels alters the radiation response in A549 cells. *A*, overexpression of *let-7b* causes significant radiosensitization. A549 cells were transfected with pre-*let-7b* and irradiated 24 h later with 2.0, 4.0, or 6.0 Gy and then analyzed by clonogenic assay. Results are depicted as dose-response curves. *B*, overexpression of *let-7g* causes significant radioprotection in A549 cells. Cells were transfected and irradiated as described above. *C*, decreasing *let-7b* causes significant radioprotection in A549 cells. Cells were transfected with anti-*let-7b*. *D*, decreasing *let-7g* causes significant radiosensitivity in A549 cells. Cells were transfected with anti-*let-7g* and treated as described above. *Bars*, SD.

Figure 3.
Manipulating miRNA levels alters the radiation response in *C. elegans*. *A*, overexpression of *let-7* and its homologue *mir-84* causes radiosensitization in a *C. elegans* radiation model. Results are depicted as dose-response curves, dose on the *X axis* and percent WT vulvas on the *Y axis. P* values are listed next to the curves they represent compared with WT animals. *B*, loss of the *let-7* homologues, *mir-84* and *mir-48*, causes radiation resistance in VPCs. *P* values represent the *mir-* strains compared with WT animals. *Bars*, SD.

Figure 4.
RNAi against *RAS* and *DDR* genes reverses the *mir-84* radiation-resistant phenotype. *A*, a
depiction of how the *C. elegans* RNAi experiments were performed. Synchronized embryos
are placed on plates with the appropriate bacterial strain containing the plasmid that
overexpresses dsRNA from the gene of interest and grown until appropriate time for
radiation. Radiation is delivered at the previously defined appropriate point in the life cycle
of the animal. *B*, RNAi for *RAS* and *DDR* genes leads to reversal of the radiation-resistant
phenotype of the *mir-84* (*KO*) strain. Results are normalized to *mir-84*. *Bars*, SD. Stratified *t*
tests were performed to evaluate significance, individual dose points that were significant are
marked with an asterisk, and *P* values based on a two-tailed evaluation of the data across
both doses were significant for all strains and are as follows: *let-60/RAS(RNAi), P* = 0.05;

rad-51/RAD51 (RNAi), P = 0.05; *coh-1/RAD21(RNAi), P* = 0.01; *fcd-2/FANCD2(RNAi), P* = 0.02; and *cdc-25.3/CDC25(RNAi), P* = 0.03.