miR-21-mediated Radioresistance Occurs via Promoting Repair of DNA Double Strand Breaks*

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MicroRNA-21 (miR-21) overexpresses in almost all types of human tumors (1, 2). Previously, we reported that up-regulating miR-21 in human non-tumorigenic cells could promote the development of the cells into tumorigenic cells (3), and other groups have used mouse models to show that up-regulating miR-21 promotes tumorigenesis (4–6). These results demonstrate that miR-21 is an onco-miR. In addition, miR-21 was involved in tumor cell resistance to ionizing radiation (IR)7, 8; however, the underlying mechanism remains unclear. IR-induced cell killing occurs mainly by generating DNA double strand breaks (DSB). Non-homologous end-joining (NHEJ) and homologous recombination repair (HRR) are the two main DNA DSB repair pathways in mammalian cells (9, 10). In general, any gene-mediated radioresistance should directly or indirectly promote NHEJ, HRR, or both. Therefore, the purpose of this study is to examine whether miR-21-mediated cell radioresistance occurs through promoting repair of DNA DSB, and if so, how miR-21 stimulates DNA DSB repair.

Glycogen synthase kinase 3 (GSK-3) is a serine/threonine protein kinase (11–13), and its function involves a number of diseases, including cancer (14). GSK-3 is encoded by two known genes: GSK-3α (GSK3A) and GSK-3β (GSK3B). GSK3B phosphorylates multiple proteins and promotes degradation, and the targets of GSK3B include CRY2 (15), cyclin D1 (16), and CDC25A (17). CRY2 can interact with PP5 and inhibit PP5 phosphate activity (18). PP5 is important for dephosphorylating DNA-PKcs and decreasing DNA-PKcs activity (19). Cyclin D1 promotes RAD51 recruiting to damaged DNA sites and thus facilitates HRR (20), but CDC25A inhibits cyclin D1 activity (21). CDC25A is a known target of miR-21 (22). In addition, CDC25A is a downstream factor of CHK1 that is an important checkpoint protein (23). CDC25A degradation induced by CHK1 phosphorylation plays an important role in response to IR-induced DNA damage (24), which in turn inhibits HRR (25, 26) and sensitizes cells to IR (27, 28). Thus, cyclin D1 and CDC25A have an opposite effect on promoting HRR and cell radioresistance. In this study, we identify GSK3B as a novel target of miR-21 and demonstrate that miR-21 up-regulation-mediated radioresistance occurs through promoting both NHEJ and HRR, which are involved in targeting GSK3B as well as CDC25A.

Results

miR-21-mediated Radioresistance Occurs through Promoting DNA DSB Repair—to confirm miR-21-mediated radioresistance, we examined the survival sensitivity of miR-21 knock-in mice (generated in our lab (29)) and miR-21 knock-out mice (obtained from Dr. Olson’s lab (30)) to IR. The miR-21 knock-out mice or mouse embryo fibroblast (MEF) cells derived from the mice did not show a detectable miR-21 level. The miR-21 levels in the miR-21 knock-in mice were shown to be 3–12-fold higher in different organs versus levels in the wild type counterparts. The miR-21 levels in the MEF derived from the miR-21 knock-in mice were shown to be 10–50-fold higher than that from the wild type mice. The survival results showed that when compared with the wild type counterparts, miR-21 knock-in mice or MEF cells were much more radioresistant and miR-
21−/− mice or their MEF cells were much more radiosensitive (Fig. 1, A and B). These results demonstrate that miR-21 up-regulation contributes to radioresistance in mice. To study whether miR-21-mediated radioresistance is involved in promoting DNA DSB repair, we examined the kinetics of γ-H2AX foci (DNA DSB marker) following IR exposure as we described previously (31) among wild type, miR-21 knock-in, or miR-21−/− MEF cells at different times following IR (2 Gy) (Fig. 1C). The results showed that there was no significant difference in the foci number (per cell) at 0.5 h after IR (Fig. 1D), indicating that miR-21 does not affect the IR-induced yield of DSB. However, the foci number among these cell lines had significant differences at 4 h (p < 0.05) and 8 h after IR (p < 0.01) (Fig. 1D), supporting that miR-21-mediated radioresistance occurs through promoting repair of DNA DSB.

To verify the effects of miR-21 on radioresistance in human cells, we initially compared the miR-21 levels in 30 pairs of human lung cancer tissue with their adjacent non-tumor tissue (NSCLC) cell lines, 3KT (obtained from Dr. Mina’s lab (32)), and NL20 (purchased from the American Type Culture Collection (ATCC)) using NanoString technology (33) (Fig. 2A). The results showed that the miR-21 levels were up-regulated in 24 of the 30 pairs of samples and 12 human NSCLC lines when compared with their non-tumor counterparts (Fig. 2B). Only H157 and A2780 cells demonstrated lower miR-21 levels than in 3KT and NL20 control cells (Fig. 2B). We then examined the effects of regulating the miR-21 level in human cell lines on cell radiosensitivity. The NL20 cell lines, miR-21-1 and miR-21-2, which are stably up-regulated with miR-21 (generated in our lab (34)), and four human NSCLC cell lines, A549, H460, H358, and H2792, which are down-regulated with miR-21 using a miR-21 inhibitor (antisense RNA) to block miR-21 function, were used for the radiosensitivity experiments. The results showed that when miR-21 was up-regulated in the normal cells (6-fold), the cells became more resistant to IR (Fig. 2C), and when miR-21 was blocked by its inhibitor, the radioresistance was abolished in the human tumor cells (Fig. 2D). These results further demonstrate that miR-21 is associated with radioresistance in human cells.

miR-21 Promotes Both NHEJ and HRR—To investigate which DNA DSB repair pathway, NHEJ or HRR, was promoted by miR-21, we used the reporter assay. Interestingly, up-regulating miR-21 in miR-21 knock-in MEF cells (Fig. 3A) or using miR-21 mimics in human cells (Fig. 3E, top panel) promoted NHEJ and HRR efficiency in both MEF (Fig. 3, B and C) and human cells (Fig. 3E, middle and bottom panels). To identify which NHEJ or HRR relative factors were affected by miR-21, we examined the major NHEJ factors including DNA-PKcs, Ku70, ligase IV, and XRCC4, and the major HRR factors including RAD51, RPA34, RPA70, XRCC2, and XRCC3, as well as cyclin D1. We did not find any change in the protein level for the tested NHEJ factors in MEF (Fig. 3D, left panel) or human cells (Fig. 3F, top panel), but found that DNA-PKcs autophosphorylation signal increased after up-regulating miR-21 in MEF (Fig. 3D, left panel) or human cells (Fig. 3F, top panel). Because DNA-PKcs autophosphorylation is essential for promoting NHEJ efficiency (35, 36), these results indicate that miR-21 promotion of NHEJ occurs through stimulating DNA-PKcs phos-
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Identifying GSK3B as a New Target of miR-21 That Can Stimulate DNA-PKcs Activity—To search for key factors that affect the cyclin D1 level or determine whether DNA-PKcs autophosphorylation could be targeted by miR-21, we focused on GSK3B because GSK3B is a known miR-21 target (22). To examine whether GSK3B is a real miR-21 target, we used the mature miR-21 sequence during

phosphorylation. On the other hand, we did not find any changes in levels of RAD51, RPA34, RPA70, XRCC2, or XRCC3 between miR-21 up-regulated MEF or human cells and their control counterparts (Fig. 3D, right panel, and Fig. 3F, bottom panel); however, we found that the levels of cyclin D1 increased after up-regulating miR-21 in MEF (Fig. 3D, right panel) and human cells (Fig. 3F, bottom panel). Because it is known that cyclin D1 promotes RAD51 to recruit to damaged DNA sites and thus facilitates HRR (20), these results suggest that miR-21 promoting HRR is linked to up-regulating cyclin D1. It is known that CDC25A is a miR-21 target; therefore, miR-21 targeting CDC25A might also contribute to miR-21-increased HRR and radiosensitivity.

Identifying GSK3B as a New Target of miR-21 That Can Stimulate DNA-PKcs Activity—To search for key factors that affect the cyclin D1 level or determine whether DNA-PKcs autophosphorylation could be targeted by miR-21, we focused on GSK3B because GSK3B is a known miR-21 target (22), up-regulating miR-21 resulted in increased CRY2 levels in both MEF (Fig. 4C and 4D) and human cells (Fig. 4F), which demonstrates that inhibition of GSK3B-stimulated DNA-PK activity and NHEJ efficiency (37) occurs through the CRY2/PP5 pathway. Cell survival data from MEF (Fig. 4E) and human cells (Fig. 4F) provide strong evidence to support that GSK3B as a novel target of miR-21 is involved in miR-21-mediated radiosensitivity.

miR-21-mediated Radioresistance Occurs through Targeting Both GSK3B and CDC25A—To examine whether miR-21-mediated radioresistance is involved in targeting both GSK3B and CDC25A, we examined the protein levels of CDC25A and cyclin D1 as well as the radiosensitivities of GSK3B−/− cells or cyclin D1−/− cells with or without knocking down cyclin D1 or CDC25A. The results showed that both cyclin D1 and CDC25A increased in the GSK3B−/− cells but GSK3B−/− cells were only mildly resistant to IR when compared with their wild type counterpart cells (Fig. 5A). Knocking down cyclin D1 sensitized GSK3B−/− cells to IR, but knocking down CDC25A made the cells more radioresistant when compared with the wild type cells treated with the control RNA (Fig. 5A). Because CDC25A is a known miR-21 target (22), up-regulating miR-21 reduces CDC25A levels, which decreases the CDC25A accumulation induced by targeting GSK3B (Figs. 4C and 5A). Next, we examined the DNA DSB repair activities in these cells in which some proteins were manipulated as described for MEF cells (Figs. 4C and 5A). GSK3B−/− MEF cells demonstrated increased NHEJ efficiency but no significant changes in HRR efficiency, and knocking down CDC25A promoted HRR in the cells (Fig. 5, A and B). Similar results were observed in human cells (Fig. 5, C and D). These results explain why GSK3B−/− cells did not promote HRR, which might be due to two reasons: (i) increased CDC25A in GSK3B−/− cells might inhibit the cyclin D1 activity because it is known that CDC25A has an inhibition effect on the cyclin D1 activity (21); and (ii) increased CDC25A in GSK3B−/− cells reduces the checkpoint response and thus reduces HRR efficiency, which neutralizes the increased cyclin D1-promoted HRR. Overexpressing GSK3B in miR-21 up-regulated cells abolished the increased NHEJ efficiencies but had a match search at the 3′-UTR of mouse or human GSK3B. We found that two conservative sequences at 3′-UTR of mouse or human GSK3B match miR21-5p and miR-21-3p, respectively (Fig. 4A). A luciferase reporter assay showed that a wild type sequence dramatically reduced the luciferase activity but mutation at the key sites could not (Fig. 4, A and B), indicating that miR-21 could bind to such sequences to inhibit a GSK3B expression level and confirming that GSK3B is a target of miR-21. Although it is known that inhibition of GSK3B stimulates DNA-PK activity and protects mouse hippocampal neurons from irradiated-induced damage (37, 38), the underlying mechanism remains unclear. To address this question, we compared the CRY2 level after up-regulating miR-21 because CRY2 is also a target for GSK3B phosphorylation-induced degradation (15) and CRY2 could interact with PP5 and inhibit PP5 phosphate activity (18), which is important for dephosphorylating DNA-PKcs (19). Up-regulating miR-21 resulted in increased CRY2 levels in both MEF (Fig. 4C) and human cells (Fig. 4D), which demonstrates that inhibition of GSK3B-stimulated DNA-PK activity and NHEJ efficiency (37) occurs through the CRY2/PP5 pathway. Cell survival data from MEF (Fig. 4E) and human cells (Fig. 4F) provide strong evidence to support that GSK3B as a novel target of miR-21 is involved in miR-21-mediated radiosensitivity.
There Is a Correlation between High miR-21 Levels and Low GSK3B Levels in Some Human Cancers—We discovered in this study that GSK3B as an important target of miR-21 requires miR-21-mediated radioresistance. Because most human tumors have a high level of miR-21 (1, 2), we wanted to see whether our discovery has any link to human tumor data. For this purpose, we searched The Cancer Genome Atlas (TCGA) database and found that miR-21 up-regulation in human tumors has a negative correlation with GSK3B down-regulation in different human tumors including pheochromocytoma/paraganglioma (Fig. 6, A and B), kidney tumor (Fig. 6 C), and testicular germ cell tumors (Fig. 6 D). These results indicate that our data pertaining to miR-21 targeting GSK3B have an important translational potential, and these data provide useful information for developing strategies to improve radiotherapy.

Our results reveal that miR-21-mediated radioresistance occurs through promoting NHEJ and HRR of DNA DSB. That
is, promoting NHEJ occurs because targeting GSK3B increases DNA-PKcs activity through the CRY/PP5 pathway; promoting HRR occurs through targeting GSK3B, thus increasing the cyclin D1 level, and targeting CDC25A neutralizes the effects of targeting GSK3B-induced accumulated CDC25A, which thus increases the checkpoint response (Fig. 7).

Discussion

In this study, we demonstrate that miR-21-mediated radioresistance occurs through promoting repair of DNA DSB, which is involved in targeting both GSK3B and CDC25A. Because whether radiation kills cells depends mainly on the generation of DNA DSB, any factor that affects cell radiation sensitivity should either increase the yield of DNA DSB or increase the cell ability to repair DNA DSB. miR-21-mediated cell resistance to IR-induced killing is not due to increasing the yield of DNA DSB, but is due to promoting DNA DSB repair. Previously, it was reported that miR-21 prevents cell apoptosis via targeting PTEN (39), which, as a phosphatase, is an apoptosis promoter and a tumor suppressor, suggesting that miR-21-mediated...
radioresistance may involve reducing apoptosis via targeting PTEN. However, knocking PTEN out does not affect cell sensitivity to radiation (40), which might be due to the fact that apoptosis is not a considerable factor affecting cell radiosensitivity (41). Therefore, targeting PTEN may not contribute to miR-21-mediated cell radioresistance.

miR-21 expression is stimulated by the IR-activated EGFR/STAT3 pathway (3) or by the IR-activated ATM (ataxia telangiectasia-mutated) pathway through phosphorylating KSRP (KH-type splicing regulatory protein) to stimulate global pri-miRNA biogenesis (42). After ATM is activated by IR, both ATM and its downstream target, CHK2, could phosphorylate CDC25A on serine 123, which promotes CDC25A degradation and results in an S phase checkpoint (43) to facilitate HRR. Thus, miR-21-mediated radioresistance not only depends on its endogenous expression level as well as its targets but also partially involves an IR-activated DNA damage response.

We show here that overexpression of miR-21 promotes both NHEJ and HRR, but the phenotype of cell radioresistance is not as significant as that of NHEJ- or HRR-deficient cells. We believe that such results occur mainly because of the following two reasons. (i) miRNA knockdown of a gene is not as efficient as siRNA knockdown (31) because miRNA occurs by partially matching the sequence in the 3′-UTR of the gene, and siRNA occurs by completely matching the sequence in the coding region of the gene. (ii) Most importantly, miR-21 targets so many factors (44) that some of the factors (both discovered and undiscovered) may indirectly neutralize the effects of miR-21 on promoting NHEJ or HRR, and thus, reduce the miR-21-mediated cell radioresistance. miR-21 overexpression is found

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**Figure 5.** miR-21-mediated radioresistance occurs through targeting both GSK3β and CDC25A. 

**A.** upper panel, whole cell lysates were prepared from wild type, GSK3β−/−, and cyclin D1−/− MEF cells that were treated with control RNA (CtRNA, lanes 1–3), cyclin D1 siRNA (lanes 4–6), or CDC25A siRNA (lanes 7–9). The protein levels were measured using a standard Western blotting assay. Actin was used as an internal loading control. Lower panel, cell survival fraction from 4 Gy irradiated cells. The data were mean ± S.D. from three independent experiments, **, p < 0.01. B, NHEJ or HRR efficiency was examined in MEF cells that were transfected with the reagents as described above for 24 h and then transfected with either the HRR or NHEJ reporter for an additional 24 h. The NHEJ or HRR efficiency assays were as described in the legend for Fig. 3. The data presented are the mean ± S.D. from three independent experiments. C, the protein levels were examined in human 293FT cells at 48 h after the cells were transiently transfected with GSK3β siRNA, CCND1 (cyclin D1) siRNA, or CDC25A siRNA. Actin was used as an internal loading control. D, after 293FT cells were transfected with the reagent for 24 h, the cells were transfected with the NHEJ or HRR reporter for an additional 24 h, and then the cells were collected to detect NHEJ or HRR efficiency. Data shown are the mean ± S.D. from triple seta of two independent experiments; ND, no significant difference.
in most types of human tumors; however, not all of these tumors demonstrated the same radioresistance. This is mainly due to the heterogenic features of human tumors; even different cells isolated from the same human tumor show dramatically different radiosensitivities due to different expressions of some DNA DSB repair factors (45).

Taken together, our results in this study reveal the mechanism underlying miR-21-mediated cell radioresistance, and can provide useful information for clinical consideration about how to treat miR-21-mediated resistance to radiotherapy in the near future.

**Experimental Procedures**

**Mice, Cell Lines, and Irradiation**—All animal experiments were conducted following an animal protocol (#2002753) approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University. C57BL/6J mice were purchased from The Jackson Laboratory. miR-21 knock-in mice with a C57BL/6J genetic background were generated in our lab as described (29). miR-21−/− mice were obtained from Dr. Olson’s lab (46). All mice were male, 6 weeks old, and either irradiated (whole body) with 8 Gy or sham-irradiated at the same time as described previously (47). The related mouse embryo fibroblast cells derived from the mice were generated in our laboratory. GSK3B−/− cells were obtained from Dr. Woodgett’s lab (12), and cyclin D1−/− cells were obtained from Dr. Chenguang Wang’s lab (48). Non-viral immortalized human bronchial epithelial cells (3KT) were obtained from Dr. Mina’s lab (Ramirez et al. (32)). Human 293FT cells, immortalized human bronchial epithelial cells (NL20), and human lung tumor cell lines A549, H460, H358, H522, Calu1, and Skemes-1 were purchased from the ATCC. Human lung tumor cell lines 95C, 95D, H226B, H1597, and H2797 are as described previously (49). All cell lines used in this study were checked for mycoplasma contamination. These cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. Irradiation of mice or cells was performed with an X-ray machine (X-RAD 320, North Branford, CT) at 320 kV, 10 mA, and the filtration was performed with 1.5-mm aluminum filter, 0.8-mm tin filter, and 0.25-mm copper filter for mice, and with 2-mm aluminum filter for cells in our laboratory. The dose rate was 1–2 Gy/min.

**Plasmid Construction**—The plasmids containing mouse GSK3B (pSP72 GSK3B) or the human GSK3B gene (HA-GSK3B wt pcDNA-3) were purchased from Addgene. The plasmid containing mouse CDC25A was purchased from OriGene Inc., and the human CDC25A was obtained from Dr. Jiri Bartek’s lab (43). The primers used to generate an expression plasmid from

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**FIGURE 6. A correlation of high miR-21 and low GSK3B expression in some human tumors.** A–D, opposite correlation of miR-21-5p/miR-21-3p and GSK3B in human pheochromocytoma/paraganglioma, kidney tumors, and testicular germ cell tumors.

**FIGURE 7. A model explains how miR-21 mediates cell resistance to radiation-induced killing.** The factors in red are promoting cell resistance to IR, and the factors in black are promoting cell sensitivity to IR (see detail under “Results”).

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**TABLE 1**

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<th>Primers</th>
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**References**


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