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

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This article has been withdrawn by Shuofeng Hu, Xiaomin Ying, Xiangming Zhang, and Ya Wang. Baocheng Hu, Xiang Wang, Ping Wang, Jian Wang, and Hongyan Wang could not be reached. In Fig. 1C, the DAPI and merged images for the no IR control were switched. The DNA-PKcs and actin immunoblots on the left appear to have been spliced. In Fig. 4C, the DNA-PKcs immunoblot appears to have been spliced. In Fig. 4D, lanes 1 and 5; lanes 2, 6, and 8; and lanes 3 and 7 of the DNA-PKcs immunoblots are the same. In the p-DNA-PKcs immunoblot, lanes 1 and 8, lanes 2 and 6, and lanes 3 and 7 are the same. In the CRY2 immunoblot, lanes 5 and 7 are the same. In the CDC25A immunoblot, lanes 3 and 8 are the same. In the GSK3B immunoblot, lanes 1 and 5 and lanes 3 and 7 are the same. Also in the GSK3B immunoblot, the upper GSK3B bands in lanes 6 and 8 are the same. Lanes 4 and 8 of the cyclin D1 immunoblot are the same. In Fig. 5A, the CDC25A immunoblot appears to have been spliced. Also in Fig. 5A, lanes 2-4 and lanes 6-8 of the CDC25A immunoblot are the same. Lanes 4-6 and 7-9 of the actin immunoblot are the same. In Fig. 5C, lane 1 of the CDC25A immunoblot was reused in lane 5, and lanes 3 and 4 were reused in lanes 7 and 8. In the GSK3B immunoblot, lanes 2-4 and lanes 6-8 are the same. Lane 1 of the cyclin D1 immunoblot was reused in lane 5, and lanes 3 and 4 were reused in lanes 7 and 8. Lanes 2-4 of the actin immunoblots were reused in lanes 6-8.

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**FIGURE 1.** miR-21 mediates radioresistance and promotes DNA DSB repair. A, mice (male, 6 weeks old, 14 mice/group) were exposed to X-rays (8 Gy). The survival (%) was p < 0.001 between groups. B, the different types of MEF cells were exposed to different radiation doses (2 Gy), and the H2AX foci in non-irradiated or irradiated cells, following a similar protocol as described in our previous publication (31). C, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. D, the miR-21 level in human cell lines on the survival rate of cells. E, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. F, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. G, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. H, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. I, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. J, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. K, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. L, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. M, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. N, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. O, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. P, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. Q, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. R, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. S, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. T, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. U, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. V, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. W, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. X, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. Y, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. Z, the image shows the effects of regulating the miR-21 level in human cell lines on the survival rate of cells. **WITHDRAWN**
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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 involved in decreasing both DNA-PKcs activity and cyclin D1 levels. To examine whether GSK3B is a real miR-21 target, we used the mature miR-21 sequence during 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 target sequence. GSK3B is a target of miR-21. Although it is known that inhibition of GSK3B stimulates DNA-PKcs activity and promotes 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 (12), and CRY2 could interact with PP5 and inhibit PP5 phosphatase activity (18), which is important for dephosphorylating DNA-PKcs (19). Up-regulating miR-21 resulted in increased CRY2 in both MEF (Fig. 4C) and human cells, which suggests that inhibition of GSK3B increases CRY2 and NHEJ efficiency (37). Cell survival data from miR-21 modulated radioresistance in human cells. A, illustration of NanoString measurement. miRs are specifically ligated to unique tags for downstream detection (top panel). Capture and reporter probes were measured using NanoString technology. The miR-21 levels in the normal control counterparts (Fig. 3, D), stably overexpressed NL20 cell lines, miR-21 (vector transfected) (Fig. 3, B), and miR-21 (vector transfected) (Fig. 3, bottom panel). The data are the mean ± S.D. and were obtained from three separate experiments. B, the sensitivities of two miR-21 stably overexpressed NL20 cell lines, miR-21-1 and miR-21-2) or without miR-21 (vector transfected) (Fig. 3, B). 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 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 radioresistance.

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 involved in decreasing both DNA-PKcs activity and cyclin D1 levels. To examine whether GSK3B is a real miR-21 target, we used the mature miR-21 sequence during 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 target sequence. GSK3B is a target of miR-21. Although it is known that inhibition of GSK3B stimulates DNA-PKcs activity and promotes 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 (12), and CRY2 could interact with PP5 and inhibit PP5 phosphatase activity (18), which is important for dephosphorylating DNA-PKcs (19). Up-regulating miR-21 resulted in increased CRY2 in both MEF (Fig. 4C) and human cells, which suggests that inhibition of GSK3B increases CRY2 and NHEJ efficiency (37). Cell survival data from miR-21 modulated radioresistance in human cells. A, illustration of NanoString measurement. miRs are specifically ligated to unique tags for downstream detection (top panel). Capture and reporter probes were measured using NanoString technology. The miR-21 levels in the normal control counterparts (Fig. 3, D), stably overexpressed NL20 cell lines, miR-21 (vector transfected) (Fig. 3, B), and miR-21 (vector transfected) (Fig. 3, bottom panel). The data are the mean ± S.D. and were obtained from three separate experiments. B, the sensitivities of two miR-21 stably overexpressed NL20 cell lines, miR-21-1 and miR-21-2) or without miR-21 (vector transfected) (Fig. 3, B). 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...
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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. 6C), and testicular germ cell tumors (Fig. 6D). 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...
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.

**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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| TABLE 1 | Primers used in the study |
|----------|---------------------------|
| Primer   | Sequences 5’ to 3’         |
| mGSK3β-F | GGGTACCATGTAGGCGGCACGCA   |
| mGSK3β-R | GCAGATCGACTGAGGACCTGGA     |
| mGSK3β-UTR-5p-F | TCTGACCATGATAAACTTATACCTGTC |
| mGSK3β-UTR-3p-F | CTAGTACAGTATAAAGCTTGAGGAG  |
| mGSK3β-UTR-Mut-5p-F | AGACTGATGTTTTTTTTTACGAG |
| mGSK3β-UTR-Mut-3p-F | CCAAGAATTTTGTTTTTCTTTTACTAAA |
| hGSK3β-UTR-5p-F | GCGCGCTGCTGCTGCTGCTG     |
| hGSK3β-UTR-3p-F | CTAGTACAGTATAAAGCTTGAGGAG  |
| hGSK3β-UTR-Mut-5p-F | AGACTGATGTTTTTTTTTACGAG |
| hGSK3β-UTR-Mut-3p-F | CCAAGAATTTTGTTTTTCTTTTACTA AA |

the cloning plasmid of pSP72 GSK3B are listed in Table 1. The mouse GSK3B gene was verified with enzyme digestion (KpnI and XbaI) and sequencing. The DNA encoding partial 3’-UTR of GSK3B was PCR-amplified from mouse or human DNA; DNA fragments from partial 3’-UTR of GSK3B were cloned downstream of firefly luciferase reporter gene in pGL3-control plasmid (Promega). The predicted complementary sites of miR-21 or the mutated precursor sequences (300 bp in total) were purchased from GeneCopoeia Inc., and then inserted into pcDNA3.1 expression plasmids.

Mimic/Inhibitor of MiRNA Assay—Transfection—NiR-21 mimic or inhibitor and control siRNAs were purchased from Ambion Inc., and the control RNA and a pool of siRNA against GSK3B, CCND1 (cyclin D1), or CDC25A were purchased from Dharmacon Inc. Cells were transfected with the plasmid, mimic, or inhibitor siRNA for 24–48 h, and then collected for further experiments. Plasmid and siRNA transfections were performed using Lipofectamine 3000 (Invitrogen), according to the manufacturer’s protocol.

Cell Survival Assay—Cell sensitivity to radiation was evaluated for loss of colony-forming ability as described previously (28).

HRR or NHEJ Reporter Assay—The NHEJ assay reporter (pEGFP-pem1-Ad2) vector was obtained from Dr. Eric Hendrickson’s lab (50), and the human 293FT cells expressed an integrated NHEJ reporter were obtained from Dr. Pandita’s lab (51). The HRR reporter was obtained from Dr. Jasins’s lab (52, 53). Wild type MEF or human 293FT cells were treated with control RNA, miR-21 mimic, or miR-21 inhibitor and GSK3B-overexpressing plasmid, GSK3B, or CDC25A siRNA for 24 h and then transfected with the reporter (NHEJ or HRR) and I-Scel for an additional 48 h. NHEJ or HRR efficiency was measured by quantifying fluorescent signals in cells after transfection with an I-Scel plasmid using flow cytometry as described previously (54).

Antibodies and Immunoblotting—The whole cell lysates were prepared as described previously (Wang et al. (28)). The antibodies against human and mouse DNA-PKcs, RPA70, Ku70, ligase IV, XRCC4, XRCC2, XRCC3, CDC25A, HA, β-actin, and CRY2 were purchased from Santa Cruz Biotechnology Inc. The antibody against human cyclin D1 was purchased from Cell Signaling Technology Inc. The antibody against phosphorylated DNA-PKcs was purchased from Abcam Inc. The antibodies against human and mouse GSK3B, Rad51, and γ-H2AX were purchased from EMD Millipore Inc. The antibody against human CRY2 was purchased from Bethesda Laboratories Inc.

Negative Correlation between miR-21 and GSK3B in Human Tumors—TCGA data were downloaded from the Broad Institute FireBrowse Data Portal. TCGA mRNA-seq data (build 2015060100) and TCGA miRNA-seq data (build 2015110100) were downloaded from FireBrowse and used for the analysis.

Statistical Analysis—The statistical significance of comparisons between two groups was determined using Student’s t test. p values less than 0.05 were considered statistically significant.

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