Published in final edited form as:

Cancer Res. 2012 August 15; 72(16): 4037–4046. doi:10.1158/0008-5472.CAN-12-0103.

MiR-96 downregulates REV1 and RAD51 to promote cellular sensitivity to cisplatin and PARP inhibition

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Abstract

Cell survival after DNA damage relies on DNA repair, the abrogation of which causes genomic instability. The DNA repair protein RAD51 and the trans-lesion synthesis DNA polymerase REV1 are required for resistance to DNA interstrand crosslinking agents such as cisplatin. In this study, we show that overexpression of miR-96 in human cancer cells reduces the levels of RAD51 and REV1 and impacts the cellular response to agents that cause DNA damage. miR-96 directly targeted the coding region of RAD51 and the 3'-untranslated region of REV1. Overexpression of miR-96 decreased the efficiency of homologous recombination and enhanced sensitivity to the poly(ADP-ribose) polymerase (PARP) inhibitor AZD2281 in vitro and to cisplatin both in vitro and in vivo. Taken together, our findings indicate that miR-96 regulates DNA repair and chemosensitivity by repressing RAD51 and REV1. As a candidate therapeutic, miR-96 may improve chemotherapeutic efficacy by increasing the sensitivity of cancer cells to DNA damage.

Keywords

miR-96; DNA repair; drug resistance; cisplatin; PARP inhibitor

Introduction

To preserve genomic stability, cells have developed an elaborate DNA damage response and repair network to fix DNA lesions that continuously arise due to exposure to endogenous or exogenous genotoxins (1). DNA repair plays a critical role in preventing the development of cancer, while defective DNA repair in cancer cells can be exploited for cancer therapy using DNA damaging agents.

Interstrand DNA crosslink (ICL)-inducing agents, such as cisplatin, carboplatin, melphalan, cyclophosphamide and mitomycin C, are widely used for the treatment of cancer. Repair of ICLs requires the coordination of multiple DNA repair pathways including the Fanconi

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Potential conflicts of interest: none

anemia pathway (2), translesion synthesis (TLS), homologous recombination (HR) and endonuclease-mediated DNA processing (3).

HR is the critical pathway for the repair of DNA double strand breaks (DSBs) in S and G2-phases of the cell cycle (4), and requires numerous factors including the recombinase RAD51 and the breast/ovarian cancer susceptibility gene products, BRCA1 and BRCA2. Tumors defective in HR, such as breast/ovarian cancers with BRCA1 or BRCA2 deficiency, are responsive to treatment with ICL-inducing agents as well as poly(ADP-ribose) polymerase (PARP) inhibitors (5). TLS, carried out by a multitude of mutagenic DNA polymerases such as REV1 (6), protects the genome from large deletions by replicating across ICLs and other occluding lesions (3). Thus, targeting these DNA repair pathways involved in ICL repair is a logical strategy for overcoming cellular resistance to ICL-inducing agents (5, 7, 8). In addition, REV1-mediated TLS is an error prone process, which contributes to the mutagenic effects of many anti-tumor DNA damaging agents and may play a critical role in the development of acquired chemoresistance (9). Therefore, inhibiting REV1-mediated TLS may prevent the emergence of chemoresistance.

MicroRNAs (miRNAs) are small non-coding RNAs that act as important regulators of gene expression. Aberrant expression of miRNAs is often seen in cancer (10). These cancer-related miRNAs can function as tumor suppressors or oncogenes and modulate many aspects of carcinogenesis, such as cell proliferation, cell cycle control, apoptosis, metastasis and angiogenesis (11). Some of these miRNAs can play important roles in the regulation of DNA repair and cellular sensitivity to DNA damaging chemotherapeutics. For example, miR-210 and miR-373 target RAD52 and RAD23B, respectively, and may regulate nucleotide excision repair and HR in hypoxia (12). MiR-24 and miR-138 downregulate histone H2AX (13, 14) and miR-421 targets ATM kinase (15) to modulate cellular response to multiple DNA damaging agents. Thus, these miRNAs may potentially be used as putative therapeutic agents that benefit cancer treatment.

The miR-183-96-182 polycistronic miRNA cluster is located at chromosome 7q32.2. Expression of miRNAs in this cluster is often increased in many common cancers, such as colon (16), lung (17), melanoma (18), breast (19), ovarian (20, 21), glioblastoma (22), prostate (23), liver (24), endometrial (25), bladder (26, 27) and germ cell (28) cancers, and may serve as potential tumor markers in multiple cancers (22, 23, 27). They are involved in the regulation of a wide range of cellular processes including cell proliferation (18, 19, 25, 29), senescence (30), cell migration (31, 32) and metastasis (18).

By performing a cell-based miRNA library screen that uses ionizing radiation (IR)-induced RAD 51 foci formation as a readout in U2OS cells (Huang et al, manuscript in preparation), we have recently identified several miRNAs, including miR-96, as putative negative regulators of RAD51-mediated HR repair. Here, we show the functional roles of miR-96 in the regulation of DNA repair and chemosensitivity.

Materials and methods

Cell lines

U2OS, HeLa, HCC1937 and MDA-MB-231 were purchased from the American Type Culture Collections. HCT116 was obtained from Clurman Lab (Fred Hutchinson Cancer Research Center). BRCA2-deficient ovarian cancer cell line PEO1 and its BRCA2-proficient revertant PEO1 C4-2 were previously described (33). These cell lines have been tested and authenticated by STR DNA profiling (Bio-Synthesis, Inc.) in May 2012. HCC1937 cells were cultured in RPMI supplemented with 15% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. All other cell lines were grown in

DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were all maintained in a humidified 5% CO₂-containing atmosphere at 37° C.

Plasmids, siRNAs, miRNA mimics, miRNA inhibitors and lentivirus production

3'-UTR of human RAD51 (position 1 to 980 bp) and human REV1 (position 14–783 bp) was amplified by PCR and cloned into pGL3-control (Promega) to obtain pGL3-RAD51 3'UTR and pGL3-REV1 3'UTR plasmids. RAD51 MRE2 of miR-96 (position 1259–1346 bp of RAD51 mRNA) was amplified by PCR and cloned into pGL3-control vector. Putative binding sites of miR-96 in RAD51 MRE2 and REV1 3'-UTR were mutated using the QuikChange site-direct mutagenesis kit (Stratagene). Plasmids were delivered into cells using TransIT-LT-1 reagent (Mirus). The coding sequence of RAD51 was PCR amplified and cloned into pMMPpuro retroviral vector with a N-terminus DsRed signal to obtain pMMP-DsRed-RAD51 plasmid. The pLLEV-EGFP-REV1 lentiviral plasmid was kindly provided by Dr. Canman (University of Michigan Medical School) (7). MiR-96 and the spanning sequences (150 bp on each end) were amplified by PCR and inserted into pLemiR lentiviral vector (Open Biosystems) for generating miR-96 precursor. The plko1-shREV1 (5'-CCGGGCTGTTCTGATGGAAATCAAACCTCGAGTTTGATTTCATACGAACAGCTTTT TG-3') and shBRCA1 (5'-CCGGTATAAGACCTCTGGCATGAATCTCGAGATTCATGCCAGAGGTCTTATATTT T TG-3') lentiviral vectors were purchased from Sigma. Retrovirus and lentivirus were produced as described (14).

Specific siRNAs were used to knock down REV1 (5'-ATCGGTGGAATCGGTTTGGAA-3') (7) and BRCA2 (5'-AACACAATTACGAACCAAAC-3') (34) (Qiagen). Transfection of siRNAs, miRNA mimics (Dharmacon) was done at 10 nM using HiPerFect reagent (Qiagen). Transfection of miR-96 power inhibitor (Exiqon) was done at 25 nM using Lipofectamine RNAiMax (Invitrogen). MiRNA mimic negative 2 control (miR-neg, Dharmacon), miRNA negative power inhibitor B (miR-neg inhibitor, Exiqon) or luciferase siRNA (siLuc, 5'-AACGTACGCGGAATACTTCGA-3', Qiagen) served as negative controls.

Immunofluorescence microscopy

Cells treated with IR were fixed for immunofluorescent staining as described (14). Mouse anti- BRCA1 (D-9, 1:200; Santa Cruz) and RPA2 (NA18, 1:1000; Calbiochem) and rabbit anti- RAD51 (H-92, 1:200; Santa Cruz) and FANCD2 (NB100-182, 1:2000; Novus) were used as primary antibodies. Images were acquired with a microscope (TE2000, Nikon) and analyzed using MetaVue (Universal Imaging). About 200 cells per experimental point were scored for presence of foci.

Western blot analysis

SDS-PAGE electrophoresis was done with whole-cell extracts as described (14). Primary antibodies included mouse anti-BRCA1 (D-9, 1:200; Santa Cruz), Vinculin (V9131, 1:20000; Sigma) and rabbit anti-RAD51 (H-92, 1:2000; Santa Cruz), REV1 (sc-48806, 1:400; Santa Cruz), RAD51D (NB100-166, 1:2000; Novus), RAD51C (NB100-177C2, 1:2000; Novus) and Actin (Sc-1616-R, 1:10000; Santa Cruz).

Real-time PCR

Total RNAs were extracted using Trizol reagent (Invitrogen) and reverse-transcribed using the Taqman microRNA Reverse Transcription Kit or the Taqman cDNA Reverse

transcription Kit (Applied Biosystem). The Taqman MiRNA Assay Kit or Gene Expression Kit was used for quantitative PCR reaction. The comparative Ct value was employed for quantification of transcripts. RNU24 and 18srRNA served as controls for miRNA and gene expression, respectively.

Cell cycle analysis

Cells were pulse labeled with 30 $\mu\text{mol/L}$ 5-bromo-2'-deoxyuridine (BrdU, Sigma) for 15 minutes and then fixed with 70% ice-cold ethanol. Cells were then stained for DNA content (propidium iodide) and BrdU incorporation with anti-BrdU rat monoclonal antibody (MAS250, Harlan Sera-Lab) followed by FITC-conjugated goat anti-rat antibody (Jackson ImmunoResearch). Flow cytometry analysis was then performed to determine the distribution of cell cycle.

Homologous recombination (HR) assay

A U2OS cell clone stably expressing HR reporter direct repeat of GFP (U2OS DR-GFP) was a gift from Drs. Maria Jasin and Koji Nakanishi (35). U2OS DR-GFP cells were sequentially transfected with siRNAs or miRNA mimics and pCBASce vector. Two days later, cells were harvested and fixed for flow cytometry analysis as previously described (14). The percentages of GFP-positive cells in HA-positive population were resulted from HR repair induced by DSBs.

Crystal violet assay

Cells were seeded onto 12-well plates at 1×10^4 cells/well and treated with cisplatin, paclitaxel (Sigma) or AZD2281 (Axon Medchem). After incubation for 5–7 days, monolayers were fixed and stained for determination of chemosensitivity as previously described (14). Cell survival was calculated by normalizing the absorbance to that of non-treated controls.

Clonogenic survival assay

Chemosensitivity was also determined by a standard clonogenic survival assay (36). Briefly, HeLa cells transfected with microRNA mimics were seeded in 6-well plates, treated with cisplatin for 24 hours and then allowed to recover for 11 days. Cells were fixed and stained with Crystal Violet. Colonies with 50 cells or more were counted.

Luciferase assay

U2OS cells were co-transfected with miRNA mimics/inhibitors, pRL-TK Renilla plasmid and pGL3-control firefly luciferase vectors containing empty, wild-type or mutant RAD51 or REV1 3'-UTR sequence. Two days post-transfection, cells were lysed for measurement of luciferase activities using the Dual-Luciferase Assay kit (Promega). Relative luciferase activity was calculated by normalizing the ratio of Firefly/Renilla luciferase to that of negative control-transfected cells.

Xenograft mice study

MDA-MB-231 cells (1×10^6) stably infected with lentivirus producing control or pLemiR-96 were subcutaneously injected into the flank of 6–7 week old female NOD SCID mice (NOD.Cg-Prkdc^{scid}il2rg^{tm1Wjl}/SzJ, FHCRC CCEMH). Mice were randomized into two groups (9 mice each) and injected with 20 mg/Kg of cisplatin or PBS once the tumor was established (about 40 mm³). Tumor sizes were measured every 3 or 4 days after cisplatin treatment and the volume was calculated using the formula: Volume=length*width²*0.52. TTV₂₀₀ was the time of tumor volume reaching 200 mm³. The tumor growth delay was defined as: median of TTV₂₀₀(treatment) – median of

TTV₂₀₀(PBS). All the animal work was approved by the FHCRC Institutional Animal Care and Use Committee.

Statistical analysis

All the statistic analyses were performed with student t-test (paired, 2-tail). All results were expressed as mean $\pm$ standard deviation except for survival results (mean $\pm$ standard error). P -value < 0.05 was considered significant.

Results

MiR-96 is a negative regulator of RAD51 foci formation

The three miRNAs of miR-183-96-182 cluster have similar but slightly different seed sequences (Supporting information (SI) Fig. S1A). We first evaluated the effect of these miRNAs on RAD51 foci formation following DNA damage individually. Ectopic expression of the miRNAs was confirmed by real-time RT-PCR in U2OS cells (Fig. S1B). Overexpression of miR-96, but not miR-182 or miR-183, significantly reduced the percentage of cells with at least 10 RAD51 foci (Fig. 1A and 1B, 47% reduction, $P < 0.05$) and the average number of RAD51 foci per cell (Fig. 1C, $P < 0.01$) after treatment with IR. In contrast, none of the three miRNAs significantly affected IR-induced FANCD2, BRCA1 or RPA2 foci formation (Fig. 1B). Overexpression of miR-96 also modestly but significantly inhibited cisplatin-induced RAD51 foci formation (Fig. S2). Overexpression of miR-96 had no significant effect on cell cycle distribution (Fig. 1D), indicating that inhibition of RAD51 foci formation by miR-96 is not due to block of cell cycle progression. Furthermore, overexpression of miR-96 significantly reduced the expression of RAD51 both at protein and mRNA levels in U2OS cells, while it had no effect on protein expression of BRCA1, BRCA2, RAD51C and RAD51D (Fig. 1E and 1F). These findings suggest that inhibition of RAD51 foci by miR-96 is due to repression of RAD51 itself.

MiR-96 inhibits HR and enhances cellular sensitivity to cisplatin and a PARP inhibitor

RAD51 plays a critical role in HR (37). Therefore, we examined the effect of miR-96 on HR by monitoring the population of GFP-positive cells in U2OS DR-GFP cells (35). As expected, depletion of BRCA2, a critical protein for loading RAD51 onto single stranded DNA (ssDNA) at DSB sites (37), significantly reduced HR efficiency by 3.2 fold (Fig. 2A and 2B, 15.3% vs. 4.7%, $P < 0.001$). Overexpression of miR-96 led to a 2-fold reduction of HR activity (Fig. 2A and 2B, 15.7% vs. 7.4%, $P < 0.001$), indicating that miR-96 is a negative regulator of HR repair.

HR-deficient cells are sensitive to cisplatin and PARP inhibitors (5, 38, 39). Consistent with the notion that miR-96 acts to suppress HR, miR-96-transfected U2OS cells were more sensitive to cisplatin (Fig. 2C) and a PARP inhibitor, AZD2281 (Fig 2D), than control cells. Overexpression of miR-96 also reduced the expression of RAD51 in HeLa (cervical cancer cell) and BRCA2-proficient ovarian cancer cell line, PEO1 C4-2 (33) (Fig. S3C), and sensitized them to both cisplatin and AZD2281 (Fig. S3A and S3B). Sensitization of HeLa cells to cisplatin by miR-96 overexpression was also confirmed in clonogenic survival assays (Fig. S3D). Furthermore, delivery of miR-96 primary sequence using lentivirus (pLemiR-96) efficiently produced miR-96 in U2OS cells (Fig. S3E) and rendered cells more sensitive to both cisplatin and AZD2281 compared to cells infected with control virus (pLemiR-NSC) (Fig. S3F and S3G). In contrast, overexpression of miR-96 did not sensitize U2OS cells to paclitaxel (Fig. S3H), a widely used chemotherapeutic agent that disrupts microtubule dynamics (40). Therefore, miR-96-mediated sensitization to cisplatin and a PARP inhibitor was not a result of general cellular toxicity.

Downregulation of RAD51 is critical for miR-96-mediated sensitization to a PARP inhibitor, but not to cisplatin

Next, we examined whether reduction of RAD51 expression by miR-96 is critical for cellular sensitivity to cisplatin and AZD2281 in U2OS cells. Overexpression of DsRed-tagged RAD51 (Fig. 3A) prevented miR-96-mediated sensitization to AZD2281 (Fig. 3B), suggesting that miR-96-mediated AZD2281 sensitivity is primarily a result of RAD51 reduction. However, to our surprise, overexpression of RAD51 had only a mild effect on cisplatin sensitivity in miR-96-overexpressing cells (Fig. 3C). This observation led us to hypothesize that miR-96-mediated cisplatin sensitivity is not simply due to defective HR repair mediated by RAD51 reduction. To test this hypothesis, we evaluated the effect of miR-96 on chemosensitivity in HR-deficient cells. ShRNA-mediated depletion of BRCA1, an important regulator of HR (41), significantly sensitized U2OS cells to both AZD2281 and cisplatin (Fig. 3D–3F). Overexpression of miR-96 further sensitized BRCA1-depleted U2OS cells to cisplatin (Fig. 3F), but not to AZD2281 (Fig. 3E). Furthermore, overexpression of miR-96 sensitized BRCA1-deficient breast cancer cell line HCC1937 (42) (Fig. S4A) and BRCA2-deficient ovarian cancer cell line PEO1 (33) (Fig. S4D) to cisplatin, but not to AZD2281 (Fig. S4B and S4E). These findings support the notion that miR-96 overexpression sensitizes cells to cisplatin by inhibiting another mechanism of chemoresistance in addition to HR.

Downregulation of REV1 is critical for miR-96-mediated cisplatin sensitization

To further explore the molecular mechanism responsible for miR-96-mediated cisplatin sensitization, we analyzed the predicted targets of miR-96 from three algorithms (MiRanda, TargetScan and PicTar) (SI Table S1). Among the top 100 predicted targets by each algorithm, 55 genes were predicted by at least two algorithms (Table S2). We focused on REV1, an error-prone Y-family DNA polymerase that is required for cellular resistance to cisplatin (7, 8). During ICL repair, REV1 initiates TLS across the unhooked ICLs followed by either HR-mediated repair or HR-independent repair (7, 8). As expected, REV1 expression was significantly reduced in miR-96-overexpressing U2OS cells as shown by both western blot and quantitative RT-PCR (Fig. 4A, 4B, 4D, 3A and 3D). Overexpression of miR-96 also reduced the expression of REV1 in several other cancer cell lines (HeLa, PEO1 C4-2 (Fig. S3C), HCC1937 and PEO1 cells (Fig. S4C and S4F)).

Depletion of REV1 significantly sensitized HR-proficient U2OS cells to cisplatin (Fig. 4C). It also increased cisplatin sensitivity in HR-deficient HCC1937 and PEO1 cells (Fig. S4A and S4D), implying that downregulation of REV1 may contribute to miR-96-mediated cisplatin sensitivity in HR-deficient background. Indeed, miR-96 had only very mild effect on cisplatin sensitivity in U2OS cells when both RAD51 and REV1 were ectopically overexpressed (Fig. 4D and 4E), suggesting that miR-96 regulates cisplatin sensitivity mainly by repressing RAD51 and REV1.

RAD51 and REV1 are direct targets of miR-96

The 3'UTR of RAD51 transcript contains a putative miR-96 binding site (RAD51 MRE1, Fig. 5A) predicted by prediction algorithms MiRanda and TargetScan (Table S1), while the 3'UTR of REV1 transcript contains a putative miR-96 binding site (REV1 MRE, Fig. 5A) predicted by all three prediction algorithms (Table S1). In addition, the coding region of RAD51 contains another putative miR-96 binding site (RAD51 MRE2, Fig. 5A). To demonstrate whether they are direct targets of miR-96, we cloned the 3'-UTR of either RAD51 or REV1 mRNA and the RAD51 MRE2 site with the surrounding sequences downstream of the open-reading frame of the luciferase gene of pGL3 vector (RAD51 3'UTR, REV1 3'UTR and RAD51 MRE2, Fig. 5A and Fig. 5B) and co-transfected either of them with miR-96 or negative mimics into U2OS cells. Overexpression of miR-96

significantly downregulated luciferase activity of the construct fused with REV1 3'UTR without affecting that of the empty vector (Fig. 5C). Mutation of the potential miR-96 binding site in REV1 3'UTR (REV1 3'UTR mutant, Fig. 5B) completely abolished the inhibitory effect of miR-96 on luciferase activity (Fig. 5C), implying that REV1 mRNA is a direct target of miR-96.

Interestingly, miR-96 failed to affect luciferase activity of the construct containing RAD51 3'UTR (Fig. 5C), but significantly reduced that of the construct containing MRE2 (a potential miR-96 binding site in the coding region of RAD51) (Fig. 5A, C). Mutation of the MRE2 abrogated the inhibitory effect of miR-96 on luciferase activity (Fig. 5C). Accordingly, overexpression of miR-96 caused a mild reduction of the ectopically expressed DsRed-tagged RAD51 (Fig. 3A, lane 3 vs lane 4 and Fig. 4D, lane 1 vs lane 2), which does not carry the 3'-UTR. These data suggest that RAD51 mRNA is also a direct target of miR-96.

MiR-96 is broadly conserved across many species (Fig. S5) as are the miR-96 binding sites in RAD51 and REV1 transcripts (Fig. S6), suggesting conservation of the trans-regulatory interactions between miR-96 and its targets, RAD51 and REV1.

Lastly, we examined whether REV1 and RAD51 are regulated by miR-96 at physiological levels of miR-96 expression. Transfection of a miR-96 specific inhibitor eliminated miR-96-mediated reduction of luciferase activity of REV1-3'UTR reporter (Fig. 5D), suggesting that miR-96 was efficiently suppressed by this inhibitor. However, transfection of the miR-96 inhibitor only mildly upregulated the expression of REV1 and RAD51 in HCT116 cells (Fig. 5E). These results suggest that endogenous levels of miR-96 downregulate REV1 and RAD51 very mildly and that miR-96-mediated regulation is only one of the mechanisms that regulate REV1 and RAD51 expression.

MiR-96 enhances chemosensitivity in a xenograft model

To further demonstrate the role of miR-96 in chemosensitization, we tested the role of miR-96 on cisplatin sensitivity in a mice xenograft model. Tumorigenic MDA-MB-231 human breast cancer cells (43) were stably infected with lentivirus encoding either non-targeting control or miR-96 (Fig. 6A). Overexpression of miR-96 reduced the expression of REV1 and RAD51 in MDA-MB-231 cells (Fig. 6B), and mildly, but significantly, sensitized MDA-MB-231 cells to cisplatin *in vitro* (Fig. 6C). While cisplatin treatment or overexpression of miR-96 alone only modestly prevented the tumor growth of xenografted MDA-MB-231 cells ($p > 0.05$), overexpression of miR-96 strongly reduced tumor growth after cisplatin treatment during the 3-week observation period (Fig. 6D). Furthermore, combinational treatment with cisplatin and miR-96 also caused a 7-day growth delay of tumor volume reaching 200 mm³ (about 4 times of the volume before cisplatin treatment), while cisplatin or miR-96 alone elicited no and 3-day delay, respectively (Fig. S7). These data support the notion that miR-96 is a potent cisplatin sensitizer *in vivo*.

Discussion

DNA damaging agents are important therapeutic interventions for cancer therapy. However, their clinical use is sometimes limited due to acquired chemoresistance. In this study, we demonstrated that miR-96 regulates DNA repair and chemosensitivity by suppressing the expression of two important DNA repair genes, RAD51 and REV1. The RAD51 recombinase promotes HR repair of DSBs and ICLs (37, 44). REV1-mediated TLS plays a critical role both in cellular resistance to ICL-inducing agents and in the development of acquired chemoresistance (9). Therefore, simultaneous inhibition of RAD51 and REV1 is a theoretically valid strategy to sensitize tumor cells to DNA damaging agents and to prevent

the development of chemoresistance, although there is a concern that this combination may also lead to toxicity in some normal tissues. Thus, miR-96, which downregulates both RAD51 and REV1, can be a potentially powerful therapeutic agent for improving the efficacy of conventional chemotherapy.

The miRNAs of the miR-183-96-182 cluster share similar seed sequences and have been reported to share the same targets such as Foxo1 (25, 29). However, we did not observe significant effects of miR-182 or miR-183 on either RAD51 foci formation (Fig. 1B and Supplementary Fig. S2) or cellular sensitivity to cisplatin or AZD2281 (Fig. S8). These observations suggest that miR-96 is the critical miRNA of this cluster responsible for inhibition of DNA repair and chemosensitivity. During the preparation of this manuscript, Moskwa et al reported that the miR-183-96-182 cluster miRNAs are rapidly reduced after IR treatment in HL-60 cells and MCF-7 cells and that overexpression of miR-182 inhibits HR by reducing BRCA1 expression (43). Consistent with this, we also found that expression of miR-96 was rapidly, but modestly, reduced in U2OS cells after IR (Fig. S9). This in turn may allow efficient recruitment of DNA repair proteins (such as RAD51) to DNA damage sites. However, we did not see a significant effect of miR-182 on BRCA1 expression in U2OS cells (Fig. S10). This discrepancy may be due to different cell lines used in these studies, as function and targets of miRNAs can be context dependent (45). Nevertheless, both studies highlight the important roles of the miR-183-96-182 cluster in DNA repair and chemosensitivity.

The miR-183-96-182 cluster is located at chromosome 7q32, a region surrounded by multiple important oncogenes such as Met, CDK6 and BRAF (18). This region is often amplified in cancer and the miR-183-96-182 cluster miRNAs are upregulated in many cancers and can promote cell proliferation, migration and metastasis by targeting multiple transcriptional factors (18, 19, 25, 29, 31, 32). Cooperation between miR-96-mediated DNA repair deficiency and the oncogenic properties of Met, CDK6 and BRAF may promote tumorigenesis. Inhibition of miR-96 mildly upregulated the expression of RAD51 and REV1 in some cancer cell lines (Fig. 5E), but the physiological role of miR-96 in the regulation of DNA repair remains unclear and will require further investigation. Whether miR-96 expression levels can serve as a prognostic marker to predict the chemosensitivity of tumor is another important question to be addressed in the near future.

Importantly, multiple studies suggest that ectopic expression of the miR-183-96-182 cluster may have therapeutic potential, despite their oncogenic potential. Overexpression of miR-96 inhibits pancreatic cancer tumorigenesis by decreasing the expression of KRAS (46). Overexpression of miR-183 inhibits migration of breast cancer cells (31, 32), whereas overexpression of miR-182 suppresses the proliferation, migration and invasion of lung cancer cells (47, 48). Our current study showed that overexpression of miR-96 caused a mild growth defect in multiple cell lines, such as U2OS (Fig. S11) and in a xenograft model (Fig. 6D). Importantly, overexpression of miR-96 strongly potentiated the ability of cisplatin to inhibit tumor growth *in vivo* (Fig. 6D). All these studies support the idea that overexpression of miR-183-96-182 can shift their oncogenic roles towards tumor suppressive functions, and that combining their expression with DNA damaging agents may lead to substantial benefit for tumor management.

Taken together, miR-96 is an important regulator of DNA repair and a potential therapeutic agent (chemosensitizer) for cancer. Future studies will address whether miR-96 mimic may be useful as a chemosensitizer in therapy for certain types of cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support: This work was supported by Howard Hughes Medical Institute, the National Institutes of Health (NIH)/NHLBI [R21 HL092978 to T.T.], the NIH/NCI [R01 CA125636 to T.T.], Fanconi Anemia Research Fund (to T.T.) and NIH [P30 DK56465 to Y.W. and T.T.]. Y.W. is a research fellow supported by Canadian Institute of Health Research. J.W. and P.C. are supported by PHS NRSA 2T32 GM007270 from NIGMS.

We thank Drs. Maria Jasin, Koji Nakanishi, Christine Canman and Muneesh Tewari for reagents and Dr. Dipanjan Chowdhury for sharing the data. We thank Drs. Muneesh Tewari and Ronald Cheung for critical reading of the manuscript. We also thank members of FHCRC animal facility, Kemp Lab, Porter lab and Swisher lab for materials and/or technical assistance, and all the members of Taniguchi lab for technical support and discussions.

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Figure 1. MiR-96 inhibits DNA damage-induced RAD51 foci formation

(A–C) U2OS cells were transfected with negative control (miR-neg) or miR-96 mimics (10 nM), treated with ionizing radiation (IR) (10 Gy) and then fixed for immunofluorescent staining of RAD51, RPA2, FANCD2 or BRCA1 six hours after IR. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). The representative images of RAD51 immunostaining were shown in (A). Quantitation of relative percentage of cells with at least 10 foci (normalized to miR-neg-transfected cells) and average number of foci per cell were shown in (B) and (C), respectively. (Scale bar = 20 μ m) (mean $\pm$ SD, n=3). (D) U2OS cells were transfected with miR-neg, miR-96 or miR-16, a known cell cycle regulator (49). After 48 hours, cells were processed for FACS analysis of cell cycle profile. (E–F) U2OS cells were transfected with miR-neg or miR-96 and cells were harvested 48 hours following transfection for western blot analysis (E) or real-time RT-PCR.

Figure 2. MiR-96 inhibits homologous recombination and sensitizes U2OS cells to cisplatin and AZD2281

(A–B) U2OS DR-GFP cells were sequentially transfected with miRNA mimics or siRNAs and HA-tagged pBASceI plasmid. Cells were incubated for 48 hours and then processed for FACS analysis of GFP-positive cells in HA-positive population (mean $\pm$ SD, $n=5$). *** $P < 0.001$ (C–D) U2OS cells were transfected with miRNA mimics and reseeded 48 hours after transfection for cisplatin (C), and AZD2281 (D) sensitivity assays (mean $\pm$ SEM, $n=3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 3. Downregulation of RAD51 is critical for miR-96-mediated AZD2281 sensitivity, but not for cisplatin sensitivity

(A–C) U2OS cells were infected with retrovirus expressing control (ctrl) or RAD51. After 72 hours, cells were reseeded and transfected with miR-neg or miR-96 mimics. After another 48 hours, cells were either reseeded for AZD2281 (B) and cisplatin (C) sensitivity assays (mean $\pm$ SEM, $n=3$) or harvested for western blot analysis (A). (D–F) U2OS cells were infected with retrovirus producing shRNAs against control (ctrl) or BRCA1. After 72 hours, cells were reseeded and transfected with miR-neg or miR-96 mimics. After another 48 hours, cells were plated for AZD2281 (E) and cisplatin (F) sensitivity assays (mean $\pm$ SEM, $n=3$) or harvested for western blot analysis (D).

Figure 4. Downregulation of REV1 is critical for miR-96-mediated cisplatin sensitivity (A–B) U2OS cells were transfected with miRNA mimics or infected with retrovirus producing shRNAs as indicated. After 72 hours, cells were harvested for western blot analysis (A) and real time RT-PCR (B). (C) U2OS cells expressing control or REV1 shRNAs were plated for cisplatin sensitivity assay (mean $\pm$ SEM, $n=3$). (D–E) U2OS cells were infected with lentivirus expressing control (ctrl) or RAD51 and REV1. After 72 hours, cells were reseeded and transfected with miR-neg or miR-96 mimics. After another 48 hours, cells were plated for cisplatin sensitivity assays (E) (mean $\pm$ SEM, $n=3$) or harvested for western blot analysis (D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 5. RAD51 and REV1 are direct targets of miR-96

(A, B) Putative miR-96 binding sites (wildtype and mutants) in the transcripts of RAD51 or REV1. (C) Wildtype or mutant RAD51 3'-UTR, RAD51 MRE2 or REV1 3'-UTR were cloned into the pGL3 vector, as 3' fusions to the luciferase gene. U2OS were co-transfected with the indicated miRNA mimics and luciferase vectors. Luciferase activity was assayed 48 h later and normalized to that of negative control-transfected cells (mean $\pm$ SD, n=4). (D) U2OS cells were co-transfected with plasmids and miRNA inhibitors (25 nM) as indicated. Cells were lysed for luciferase assay 48 h later. (E) HCT116 cells were transfected with miRNA mimics or inhibitors. Cells were harvested for western blot 72 h after transfection. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 6. MiR-96 enhances cisplatin sensitivity in vivo

(A) Overexpression of miR-96 in MDA-MB-231 cells by lentiviral delivery of pLemiR-96. (B) Reduction of REV1 and RAD51 expression in MDA-MB-231 cells stably expressing miR-96. (C) Overexpression of miR-96 mildly sensitizes MDA-MB-231 cells to cisplatin during the 5-day in vitro survival assay (mean $\pm$ SEM, $n=3$). (D) MDA-MB-231 cells stably expressing miR-96 were injected into mice subcutaneously. After the tumor was established, mice were i.p. injected with 20 mg/kg cisplatin followed by monitoring of tumor size for 3 weeks (mean $\pm$ SEM, $n=9$). * $P < 0.05$, ** $P < 0.01$