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MicroRNA Regulation of DNA Repair Gene Expression in Hypoxic Stress

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Abstract

Genetic instability is a hallmark of cancer; the hypoxic tumor microenvironment has been implicated as a cause of this phenomenon. MicroRNAs (miR) are small nonprotein coding RNAs that can regulate various cellular pathways. We report here that two miRs, miR-210 and miR-373, are up-regulated in a hypoxia-inducible factor-1α–dependent manner in hypoxic cells. Bioinformatics analyses suggested that these miRs could regulate factors implicated in DNA repair pathways. Forced expression of miR-210 was found to suppress the levels of RAD52, which is a key factor in homology-dependent repair (HDR); the forced expression of miR-373 led to a reduction in the nucleotide excision repair (NER) protein, RAD23B, as well as in RAD52. Consistent with these results, both RAD52 and RAD23B were found to be down-regulated in hypoxia, but in both cases, the hypoxia-induced down-regulation could be partially reversed by antisense inhibition of miR-210 and miR-373. Importantly, luciferase reporter assays indicated that miR-210 is capable of interacting with the 3′ untranslated region (UTR) of *RAD52* and that miR-373 can act on the 3′ UTR of *RAD23B*. These results indicate that hypoxia-inducible miR-210 and miR-373 play roles in modulating the expression levels of key proteins involved in the HDR and NER pathways, providing new mechanistic insight into the effect of hypoxia on DNA repair and genetic instability in cancer. [Cancer Res 2009;69(3):1221–9]

Introduction

Tumor cells exhibit a higher mutational frequency than their normal counterparts (1), providing the potential to adapt to otherwise hostile microenvironments. The mechanisms underlying this capacity have not been fully elucidated. However, models of genomic instability have been well studied with respect to inherited mutations that lead to cancer-prone phenotypes, as in the case of hereditary nonpolyposis colon cancer, which is linked to deficient mismatch repair (MMR) activity (2). Additionally, data indicate that the relatively higher rates of mutational frequencies in tumors may be partially attributed to the effects of tumor microenvironmental stresses, themselves (3). In addition to low pH and nutrient deprivation, hypoxia is a key component of the tumor microenvironment (4). Hypoxia leads to increased angiogenesis, up-regulates glycolytic enzymes, and provides selective pressure for cells that are capable of evading apoptosis (5, 6).

Recent work has shown that hypoxia can also promote genetic instability by affecting the DNA repair capacity of tumor cells (7, 8). Due to the transcriptional down-regulation of the *MLH1* and *MSH2* genes and the *BRCA1* and *RAD51* genes, the MMR and the homology-dependent repair (HDR) pathways, respectively, are suppressed in hypoxic cells (9–11). In the case of MMR, *MSH2* and *MLH1* are down-regulated in a c-MYC–dependent manner due to the displacement of c-MYC by MAX binding to the *MLH1* promoter (8). The mechanism of this down-regulation also depends on histone deacetylation at the respective promoters (9). With respect to HDR, the down-regulation of two key factors, *RAD51* and *BRCA1*, contributes to a functional loss of HDR activity and is mediated by E2F4/p130 transcriptional repression via increased occupancy at the respective proximal promoter regions, which is in response to hypoxic stress (11, 12). Thus, there are several DNA repair-associated genes that are affected by hypoxia, but all DNA repair genes are not regulated in the same manner. For example, some DNA repair genes, such as *RAD51B* (involved in HDR) and *ERCC1* [involved in nucleotide excision repair (NER)], were found to be up-regulated in hypoxia (12). Functionally, NER was also found to be suppressed in hypoxic cells, as measured by a reduction in the removal of UV damage from reporter gene substrates, but the mechanism for the reduced DNA repair was not elucidated (13, 14). No changes in the expression levels of two key mediators in the pathway, XPA and XPD, were observed in hypoxia in one initial study (13).

Whereas classic transcriptional factors, such as E2F and c-MYC, have important roles in regulating genes implicated in cancer, recent work has identified a new level of genetic control invoking small nonprotein coding RNAs, or microRNAs (miR), which target mRNA destabilization (15), suppress target mRNA translation (15), or act through novel interactions with promoter sequences (16). In general, miRs bind to the 3′ untranslated regions (UTR) of target mRNAs via imperfect base-pairing complementarity leading to degradation (17) or translation inhibition (18). The regulation of mRNAs by miRs has been shown to influence multiple cellular processes, including apoptosis, differentiation, and cell survival (19).

Here, we have examined miR expression in response to hypoxic stress as another potential mechanism that might alter the factors involved in DNA repair. We report that two key miRs, miR-210 and miR-373, are up-regulated in hypoxia in a hypoxia-inducible factor-1α (HIF-1α)–dependent manner. Previous work indicated that miR-210 is regulated by hypoxia and that this phenomenon occurred in ovarian (20) and breast (21) cancer cells. We find that miR-210 targets RAD52, a member of the HDR pathway, whereas miR-373 can target both RAD52 and RAD23B. Because miRs have imperfect complementarity with target genes, these two factors are

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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unlikely to be the only ones regulated by these miRs. Mechanistically, levels of both RAD52 and RAD23B were found to be down-regulated in hypoxic cells. Bioinformatics revealed miR-210 and miR-373 binding sites in the 3′ UTRs of these genes. In normoxic cells, the forced expression of miR-210 decreased endogenous levels of RAD52; the forced expression of miR-373 suppressed both RAD52 and RAD23B levels. In hypoxic cells, the inhibition of miR-210 and miR-373 partially reversed the hypoxia-induced down-regulation of RAD52 and RAD23B, respectively. The suppression of RAD52 by miR-210 and miR-373 offers a new explanation for the reduced HDR activity in hypoxic cells, whereas the down-regulation of RAD23B by miR-373 provides a new mechanism for the previously unexplained reduction of NER in hypoxia. These findings also identify a new role for miRs in the regulation of DNA repair in response to hypoxia.

Materials and Methods

Bioinformatics. Sequence information was analyzed by using the University of California at Santa Cruz Genome Browser (22). miR sequence data were taken from miRBase Sequences[3] (23–25). Target predictions were performed using miRBase Targets[4] for miR-210 and *RAD52* interactions, which uses the miRanda algorithm (26). For the interactions between miR-373 and both *RAD23B* and *RAD52*, the TargetScan[5] algorithm was used. TargetScan predicts the biological targets of miRs by searching for the presence of conserved 8-mer and 7-mer sites matching the seed region of a miR (27–29).

Cell culture and treatment. HeLa cervical carcinoma cells and MCF-7 breast cancer cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). The HIF-1α nullizygous and parental mouse embryonic fibroblast (MEF) cell lines (30) were maintained in DMEM containing 10% FBS. Cells were exposed to hypoxia in culture, as described previously (3).

Microarray analysis of miRs. Total RNA was harvested from HeLa cells exposed to normoxia or hypoxia (0.01% O_2) for 24 h and sent for microarray analysis (LC Sciences, LLC). Total RNA (2–5 μg) was size fractionated using a YM-100 Microcon centrifugal filter (Millipore) and the small RNAs (<300 nucleotides) isolated were 3′-extended with a polyadenylate [poly(A)] tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent Cy3 and Cy5 dye labeling. The probes were complementary to target miRs (from miRBase), which contained a spacer segment of polyethylene glycol to extend the coding segment away from the substrate, which also enabled for homogenous hybridization melting temperatures. Hybridization images were collected using a GenePix 4000B laser scanner (Molecular Devices) and digitized using Array-Pro image analysis software (Media Cybernetics). Data were normalized by subtracting the background and using a LOWESS (locally weighted regression) filter, which reduces system-related variation. For two-color experiments, the ratio of the two sets of detected signals ($\log_2$ transformed, balanced) and P values of the t test were calculated. Differentially expressed signals having P values of <0.01 were considered to be significant.

Plasmids. The plasmid expressing miR-210 was prepared by amplifying genomic fragment encompassing precursor miR-210 and ~200-bp upstream and downstream flanking sequences using the following primers that contain the cleavage sequences for *Eco*RI: 5′-ATTGAATTCT-GAAGTTGGGCCGAGAGC-3′ (forward) and 5′-ATTGAATTCCGCA-CACTGGTACCCTGG-3′ (reverse). The 576-bp fragment was cloned into the pBABE-puro vector. The miR-373 expression plasmid was constructed by cloning the genomic sequence of miR-373 as well as a ~200-bp flanking sequence into the pBABE-puro vector. This 571-bp genomic region was PCR amplified (GoTaq Green Master Mix, Promega) using the following forward and reverse primers, which contain the respective cleavage sequences for *Bam*HI and *Sal*I: MIR373-F-5′-CATTGGGATCCCTGGGCGACAGAGC-AAGACT-3′ and MIR373-R-5′-GATACGTCGACCATGCATGCTGCCTAC-CAAA-3′. The short hairpin RNA (shRNA) against HIF-1α was targeted against the sequence 5′-GAAGGAACCTGATGCTTTA-3′, which is located 146 bp downstream of the start codon. Duplexes created to express the shRNA hairpin included the following oligonucleotides: 5′-GATCCCCGAAG-GAACCTGATGCTTTATTCAAGAGATAAAGCATCAGGTTCCTTCTTTTTG-GAAAT-3′ (forward) and 5′-CTAGATTTCCAAAAAGAAGGAACCTGATGC-TTTATCTCTTGAATAAAGCATCAGGTTCCTTCGGG-3′ (reverse).

The 3′ UTRs of *RAD52* and *RAD23B* were cloned into the pMIR-REPORT-*FLUC* miRNA Expression Reporter Vector (Applied Biosystems). PCR was used to amplify the 3′ UTR of *RAD52* (2.4 kbp) using the following forward and reverse primers containing the respective cleavage sequences for *Spe*I and *Mlu*I: RAD52NUF1-5′-GCATTGACTAGTTGGACCCACGCTCTG-AAATC-3′ and RAD52UTR-5′-CGATACACGCGTTGACGTGATGCCA-GAAGTG-3′. Similarly, PCR was used to amplify a 2.4-kbp region of the *RAD23B* 3′ UTR using the following forward and reverse primers containing the respective cleavage sequences for *Spe*I and *Mlu*I: RAD23NUTF-5′-GCATTGACTAGTGGGCTCATATCCACAAT-3′ and RAD23NUTR-5′-CGA-TACACGCGTAGAATCCTAGCCATCCAG-3′.

Real-time quantitative PCR. Total RNA was extracted from cells with the mirVana miRNA Isolation kit (Applied Biosystems). Synthesis of cDNA from total RNA was performed using reverse transcription primers that were specific to miR-210, miR-373, or an endogenous control miR, RNU19, and the Taqman miRNA Reverse Transcription kit (Applied Biosystems). As miR-373 is expressed at relatively low levels, the cDNA synthesized from the reverse transcription reaction was further amplified using the Taqman PreAmp Mix (Applied Biosystems). To analyze the mRNA expression of *DEC1, RAD52, RAD23B*, and *18S* rRNA, total RNA was isolated as above but was reverse transcribed into cDNA using the High-Capacity cDNA Archive kit (Applied Systems). To assess miR and mRNA expression, the cDNAs prepared from the specific reverse transcription reactions were used in PCRs containing Taqman Universal PCR Master Mix with No AmpErase UNG and premixed Taqman assays (Applied Biosystems). The Taqman assays for miRs only detect the mature miR species.

Reactions were carried out in a 96-well optical reaction plate with optical caps (Agilent Technologies) in a Mx3000p Real-Time PCR Detection System spectrofluorometric thermal cycler (Agilent Technologies). Reactions proceeded with an initial 10-min incubation at 95°C followed by 40 cycles of amplification: 95°C for 15 s and 60°C for 1 min. Fluorescence was measured in real time; the cycle threshold (C_t) values were calculated using the Mx3000p algorithm (Agilent Technologies). Comparative quantitation was performed by comparing the C_t value obtained from the amplification of a given target miRNA with that determined for the normalizer, RNU19. For mRNA, *18S* was used as a normalizer. Relative miRNA and mRNA abundance was calculated using the $-\Delta\Delta C_t$ method.

Validation of miRNA regulation determined by the LC Sciences platform was performed using a Taqman low-density array (Applied Biosystems) as a method for detecting miR expression. Total RNA was isolated as previously mentioned. RNA was reverse transcribed to produce cDNA using pooled reverse transcription primers (Applied Biosystems). After pooled cDNA synthesis, the samples were loaded into 384-well microfluidics cards and run on an ABI Prism 7900HT sequence detection system (Applied Biosystems). Internal controls on the card were used to normalize the data on each card, as well as between cards.

MiRNA forced expression and inhibition. To force the expression of miRs, pre-miRs were purchased as partially duplexed RNA molecules (Applied Biosystems). To inhibit miRs, single-stranded RNA oligonucleotides were purchased from Applied Biosystems. These were specific for miR-210 [pre-miR: product ID, PM10516; sequence, 5′-CUGUGCGUGUGACAGCGG-CUGA-3′ (sense) and 5′-UCAGCCGCUGUCACACGCACAG-3′ (antisense); anti-miR: product ID, AM10516; sequence, 5′-UCAGCCGCUGUCACACGCA-CAG-3′] and miR-373 [pre-miR: product ID, PM11024; sequence, 5′-GAAG-UGUGCUUCGAUUUUGGGGUGU-3′ (sense) and 5′-ACACCCCAAAAUCGA-AGCACACUUC-3′ (antisense); anti-miR: product ID, AM11024; sequence, 5′-ACACCCCAAAAUCGAAGCACUUC-3′] or a negative control [negative

[3] http://microrna.sanger.ac.uk/sequences/
[4] http://microrna.sanger.ac.uk/targets/v5/
[5] http://www.targetscan.org/

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control #1, pre-miR: sequence, 5′-AGUACUGCUUACGAUACGG-3′ (sense) and 5′-CCGUAUCGUAAGCAGUACU-3′ (antisense); anti-miR: sequence, 5′-AAGUGGAUAUUGUUGCCAUCA-3′]. The pre-miR molecules are each composed of a duplexed guide strand and a passenger strand and are processed by the RISC complex (31). The negative controls are derived from prokaryotic miRs, which are physiologically inert. Transfection of the respective miRs (pre-miRs) or miR inhibitors (anti-miRs) was performed using the reverse transfection technique recommended for use with the siPORT *NeoFX* transfection reagent (Applied Biosystems). Transfections were carried out according to the manufacturer's protocol using 3 μL of transfection reagent and a final concentration of 50 nmol/L of each oligonucleotide per well on a six-well plate. Medium was replaced at 24 h after the transfection.

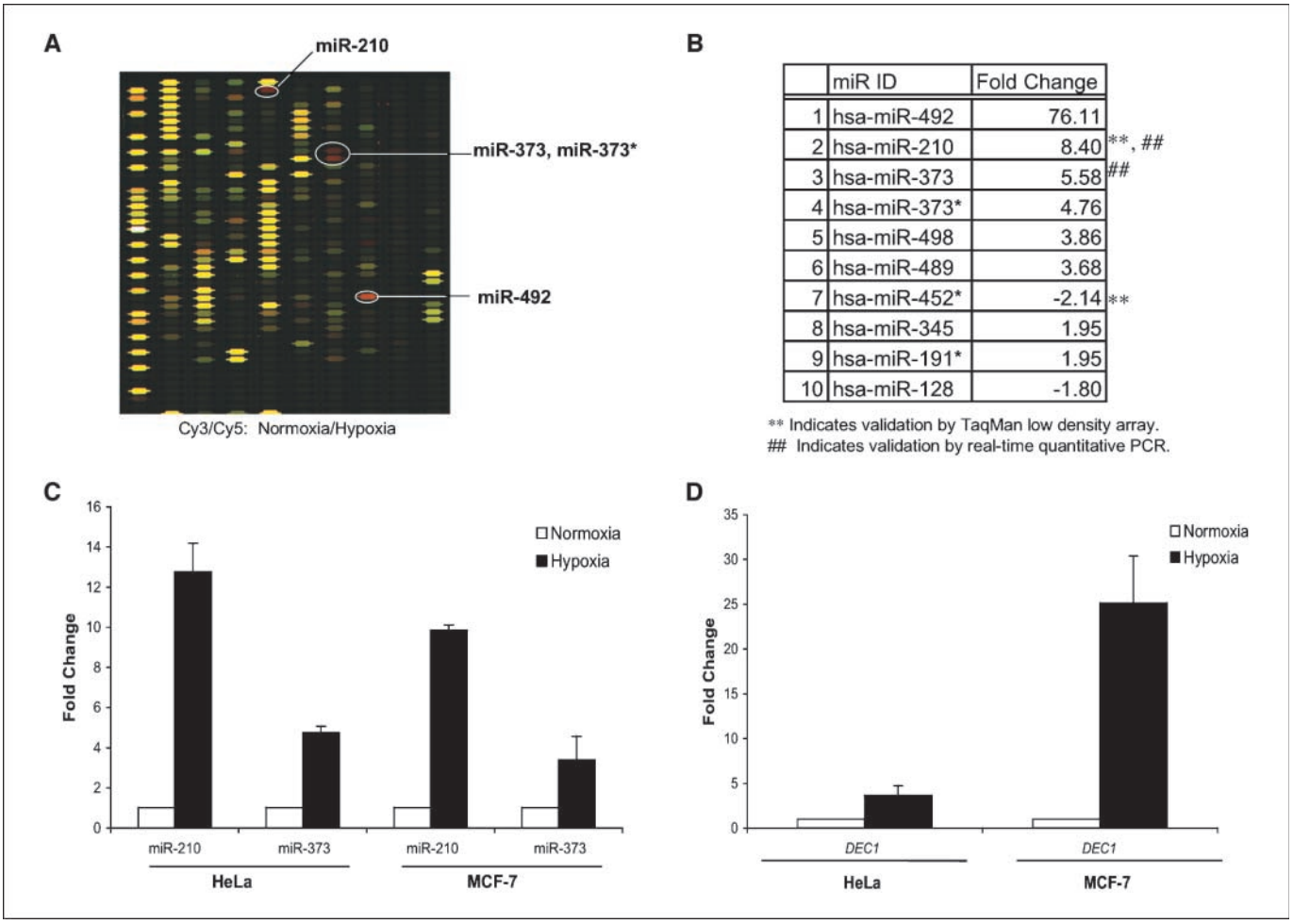
The FuGENE 6 Transfection Reagent (Roche Applied Sciences) was used to transfect the miR-210 and miR-373 pBABE constructs into MCF-7 cells. For each well on a six-well plate, 1 μg of plasmid DNA and 3 μL of FuGENE 6 reagent were transfected, as indicated in the manufacturer's protocol.

Western blot. Western analyses of whole-cell lysates were performed as previously described (32). Nitrocellulose membranes were probed for RAD52 (5H9, 1:500; GeneTex, Inc.), RAD23B (HHR23B, 1:250; Rockland Immunochemicals, Inc.), HIF-1α (clone 54, 1:250; BD Biosciences), MRE11 (12D7, 1:1,000; GeneTex), or XPA (1:500; ref. 33). β-Actin (1:1,000; Santa Cruz Biotechnology) was used as a loading control. An anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (HRP; 1:1,000; Santa Cruz Biotechnology) was used to detect RAD52, HIF-1α, and β-actin. An anti-rabbit IgG secondary antibody conjugated with HRP (1:1,000; Santa Cruz Biotechnology) was used to detect XPA. An anti-goat IgG secondary antibody conjugated with HRP was used to detect RAD23B (1:1,000; Rockland Immunochemicals). Immunodetection was performed using an enhanced chemiluminescence kit (GE HealthCare), according to the protocol supplied by the manufacturer.

Luciferase assays. As indicated above, reporter constructs containing the 3′ UTRs of both *RAD52* and *RAD23B* were designed to encompass the respective miR-210 and miR-373 binding sites. For transfection, 10^5 MCF-7 cells were seeded in triplicate into 12-well culture plates and transfected with 5 μg of each reporter construct by using the FuGENE 6 reagent. At the same time, 5 μg of either pBABE-miR-210 or pBABE-373 were also transfected into the cells. *Renilla* luciferase activity from a cotransfected pRL-SV40 control vector (2.5 ng/well) was used for normalization. Firefly and *Renilla* luciferase activities were measured by using the Dual-Luciferase Reporter Assay System kit (Promega), according to the manufacturer's instructions.

Results

Hypoxia up-regulates miR expression. As miR regulation has been implicated in carcinogenesis and tumor growth, we hypothesized that hypoxia might regulate miR expression. After



	miR ID	Fold Change	
1	hsa-miR-492	76.11	
2	hsa-miR-210	8.40	**, ##
3	hsa-miR-373	5.58	##
4	hsa-miR-373*	4.76	
5	hsa-miR-498	3.86	
6	hsa-miR-489	3.68	
7	hsa-miR-452*	-2.14	**
8	hsa-miR-345	1.95	
9	hsa-miR-191*	1.95	
10	hsa-miR-128	-1.80	

** Indicates validation by TaqMan low density array.
Indicates validation by real-time quantitative PCR.

Figure 1. Microarray analyses indicate dynamic regulation of miRs in hypoxia. *A,* total RNA was extracted from HeLa cells that were exposed for 24 h to hypoxia (0.01% O_2) or normoxia and processed by microarray analyses, where red (Cy5) and green (Cy3) indicate up-regulation and down-regulation, respectively. The top three miRs regulated in hypoxia, miR-492, miR-373, and miR-492, are highlighted on the composite (Cy3 and Cy5) array diagram. *B,* hypoxia induces changes in miR expression, as indicated by the fold changes in transcript levels detected in HeLa cells. *C,* miR-210 and miR-373 are up-regulated in HeLa and MCF-7 cells, as determined by RTQ-PCR. *D,* the up-regulation of *DEC1* in hypoxia in HeLa and MCF-7 cells serves as a positive control for confirming physiologically relevant hypoxia. *C* and *D, columns,* mean from triplicate experiments in all cases; *bars,* SE.

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exposing HeLa cells to normoxia or hypoxia (0.01% O_2) for 24 hours, total RNA was harvested; the expression-profiling experiments were performed using RNA that was enriched for miRs. In this experiment (accession number E-MEXP-1790), we compared the Cy3-labeled normoxia signal with the Cy5-labeled hypoxia signal (Fig. 1*A*). In addition to previous reports indicating that miR-210 is induced in hypoxia (21, 34), we determined that miR-492, miR-373, miR-373*, miR-498, miR-489, miR-345, and miR-191* were up-regulated >1.95-fold (Fig. 1*B*). The "star" annotation is similar to the passenger sequence found in small interfering RNAs. It is indicative of a miR strand sequence that is partially complementary to the "nonstar," or guide strand of the miR. Both the unstarred and starred mature miRs belong to the same precursor miR. In contrast and among significant changes in expression, miR-452* and miR-128 were down-regulated >1.80-fold. The findings with respect to both miR-210 and miR-452* were also validated by performing Taqman low-density array analyses in HeLa cells.

The most significantly up-regulated miRs, miR-492, miR-373, and miR-210, were further investigated using individual Taqman MicroRNA Assays. In these experiments, we analyzed the expression levels of these miRs in HeLa, as well as in MCF-7 cells, after 48 hours of hypoxia or normoxia, which is a time point that we have previously shown to be biologically relevant for changes in DNA repair activity (11). Both MCF-7 and HeLa cells had increased levels of miR-210; these were induced ~10- to 12-fold above basal levels in both cell lines (Fig. 1*C*). Additionally, the levels of miR-373 were increased ~3- to 4-fold in MCF-7 and HeLa cells in response to hypoxia. However, the expression level of miR-492 was below our detection threshold (data not shown); we were therefore not able to validate the initial results with respect to miR-492. We also measured the expression of *DEC1*; transcript levels were induced in hypoxia (Fig. 1*D*), serving as a positive control for the physiologic hypoxic response in both cell lines, which is in line with published reports (35, 36). In all cases, the levels of the respective miRs and mRNAs in normoxia and hypoxia were normalized to RNU19 and *18S* expression, respectively.

Based on the induction of miR-210 and miR-373 in hypoxia, we sought to identify potential transcription factor binding sites in the promoters of these miRs, which could be responsible for mediating induction in hypoxia. As a starting point, we used an *in silico* approach to search for binding sites for HIF-1 using consensus sequences of "cgtg" or "cgta" and the MatInspector tool from Genomatix Software GmbH (37). We found that the 5′ regulatory region upstream of miR-210 contained four such sites (Fig. 2*A*). Likewise, miR-373 contained three sites in the 1.5-kb region upstream of its transcriptional start site. To determine whether HIF-1α was involved in the regulation of miR-210 and miR-373, we used a matched set of MEFs that were either wild-type or knocked out for HIF-1α (30). After hypoxia treatment for 48 hours (0.01% O_2), total RNA was harvested from the cells and analyzed by real-time quantitative PCR (RTQ-PCR). The MEFs that were positive for HIF-1α were capable of inducing miR-210, which is in contrast to the MEFs that were deficient in HIF-1α (Fig. 2*B, top*). In parallel, the hypoxia-inducible gene *DEC1* was seen to be up-regulated only in the HIF-1α–expressing MEFs (Fig. 2*B, bottom*).

Because there is no mouse homologue of human miR-373, we could not use the MEFs to study miR-373 regulation. Therefore, we expressed a HIF-1α-shRNA vector in human cells. Expression of this vector in MCF-7 cells blocked the accumulation of HIF-1α in hypoxia (Fig. 2*C, inset*). With regard to miR-373 expression, the HIF-1α-shRNA vector suppressed the induction of miR-373 in hypoxic cells, indicating a role for HIF-1α in the up-regulation of miR-373 in response to hypoxia (Fig. 2*C*). HIF-1α-shRNA expression also reduced the induction of miR-210 in hypoxia (Fig. 2*D*), which is consistent with the findings based on the HIF-1α–proficient and HIF-1α–deficient MEFs.

Hypoxia induces the down-regulation of DNA repair factors. To determine the downstream effect of the miR up-regulation, we performed *in silico* analyses to identify putative targets of miR-210 and miR-373. As the processed mature miRs are the functional molecules, we investigated the capacity of the miRs to bind possible target genes (Fig. 3*A*). Because of our interest in understanding the regulation of DNA repair genes in hypoxia, we used miRBase (23–25) and TargetScan (27–29), which use different algorithms to examine the ability of miR-210 and miR-373 to interact with the 3′ UTRs of genes that are involved in DNA repair activity. We determined that miR-210 was predicted to be capable of binding to the 3′ UTR of the HDR gene, *RAD52*, at two different sites: site 1 and site 2 (Fig. 3*B*). miR-373 was predicted to bind to the 3′ UTRs of both *RAD52* and the NER gene, *RAD23B* (Fig. 3*B*).

To examine the potential *in vivo* regulation of RAD52 and RAD23B by miR-210 and miR-373, we used transient forced expression of synthetic pre-miR molecules. We detected the forced expression of the respective miRs by RTQ-PCR as early as 24 hours and as late as 72 hours after transfection (data not shown). After ensuring that the transfected miRs were expressed, we analyzed the effect on the protein expression of RAD52 and RAD23B by immunoblotting. We found that the forced expression of miR-210 suppressed the expression of RAD52, whereas miR-373 led to the decreased expression of both RAD52 and RAD23B (Fig. 3*C* and *D*). Forced expression of miR-210 and miR-373 from a vector-based system resulted in a similar pattern of regulation (Supplementary Fig. S1*A*). In addition, the expression of two other repair factors, XPA (involved in NER) and MRE11 (involved in HDR), was not altered by miR-210 or miR-373 (Supplementary Fig. S1*B*). We did not detect changes in the accumulation of either *RAD52* or *RAD23B* mRNA levels (data not shown).

To further establish the physiologic relevance of these observations, we next examined the expression of RAD52 and RAD23B in hypoxia. We performed immunoblotting experiments to analyze RAD52 and RAD23B levels in normoxic versus hypoxic (48 hours, 0.01% O_2) cells. In both HeLa and MCF-7 cells, RAD52 levels were decreased in response to hypoxia (Fig. 4*A*). Likewise, hypoxia also reduced the levels of RAD23B (Fig. 4*B*).

As both RAD52 and RAD23B were down-regulated in hypoxia, we next asked whether miR-210 or miR-373 had roles in the suppression of either RAD52 or RAD23B. To probe the influence of these miRs on RAD52 and RAD23B levels in hypoxia, we used synthetic oligonucleotides (anti-miRs) designed to target the mature forms of miR-210 or miR-373 and thereby inhibit their expression. The ability of such synthetic RNA-based anti-miRs to block miRNA activity has been established in prior studies (38). MCF-7 cells were transfected with the specific anti-miR molecules targeting miR-210, miR-373, or with negative control anti-miR molecules 24 hours before hypoxia or normoxia treatment. At 48 hours after transfection, the cells were harvested for the analysis of RAD52 and RAD23B expression by Western blot. In normoxic cells, the expression of RAD52 was unaffected by anti-miR-210 or anti-miR-373. However, in hypoxic cells, the suppression of RAD52 expression that occurs in hypoxia was somewhat alleviated by pretreatment with anti-miR-210 (Fig. 4*C*). In the case of RAD23B

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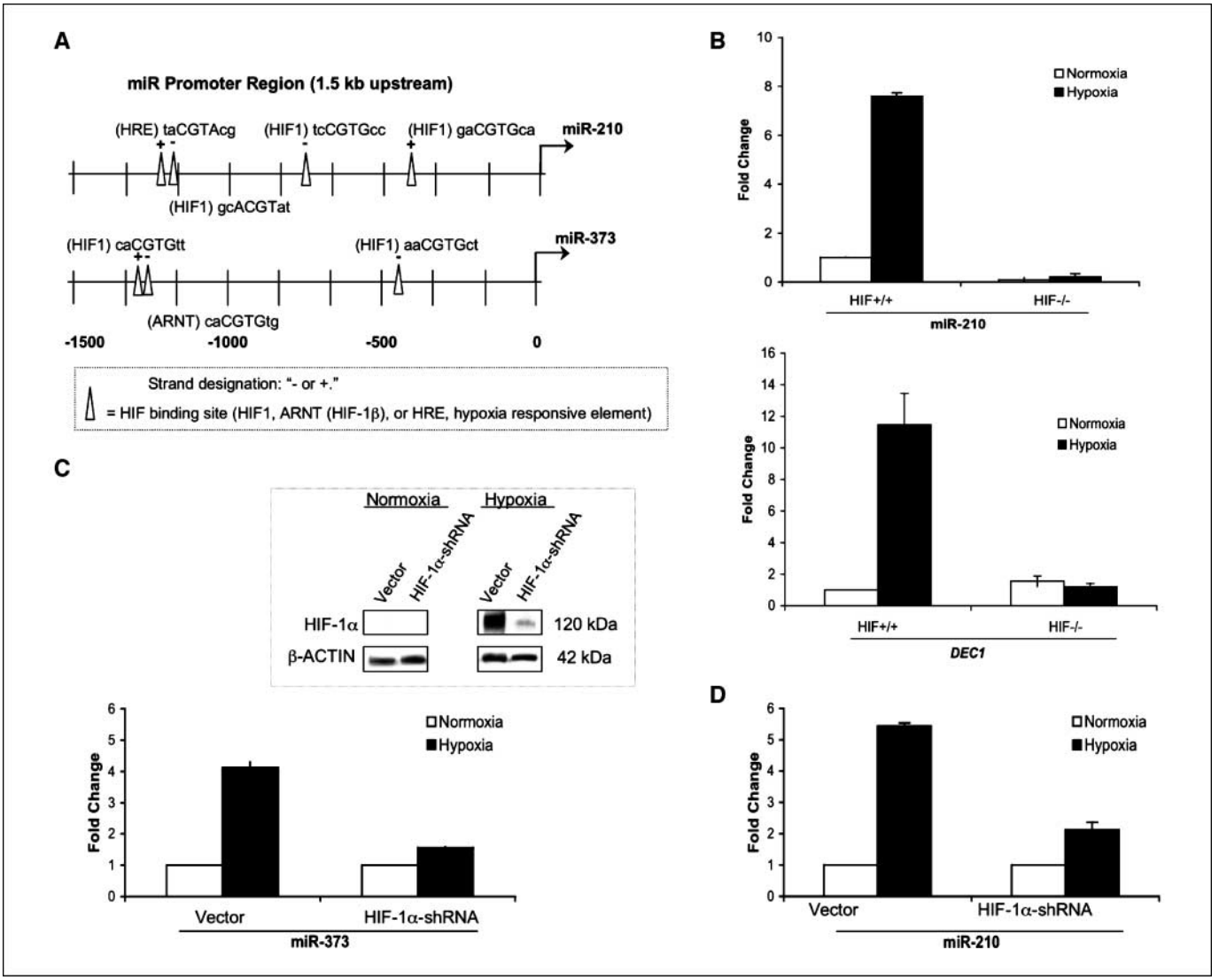

Figure 2. HIF-1α is a key transcriptional activator of miR-210 and miR-373. *A,* bioinformatics analyses using Genomatix MatInspector identifies putative HIF-1 binding sites in the 5′-upstream transcription factor binding regulatory sequences of miR-210 and miR-373. *B,* RTQ-PCR is used to analyze miR-210 regulation in MEFs with or without expression of HIF-1α. Hypoxia treatments were for 48 h at 0.01% O_2. *DEC1* induction in hypoxic MEFs serves as a control for confirming physiologically relevant hypoxia. *C,* induction of miR-373 in hypoxic cells is attenuated by the knockdown of HIF-1α via shRNA. MCF-7 cells infected with either an empty lentiviral vector or a lentiviral vector expressing HIF-1α-shRNA were exposed to normoxia or hypoxia and miR-373 levels were measured by RTQ-PCR. *Inset,* knockdown of HIF-1α is achieved through HIF-1α-shRNA expression in MCF-7 cells under normoxia or hypoxia. *D,* induction of miR-210 in hypoxic cells is reduced by shRNA-mediated knockdown of HIF-1α, as in *C. B* to *D, columns,* mean from three replicates in all cases; *bars,* SE.

expression, normoxic cells had increased RAD23B levels when treated with anti-miR-373 (Fig. 4*D*). In the hypoxic cells, pretreatment with anti-miR-373 slightly rescued the levels of RAD23B relative to the otherwise hypoxia-suppressed levels in the other samples.

To test whether miR-210 and/or miR-373 may be implicated in the regulation of RAD52 or RAD23B via binding to the respective 3′ UTR targeting sites, we designed constructs using the pMIR-REPORT-*FLUC* vector, which contains a multiple cloning site downstream of a luciferase reporter gene-coding region. The strategic cloning of the 3′ UTRs in these constructs allows the luciferase reporter to be subjected to regulation that mimics the putative miRNA target in the 3′ UTR of the respective gene. Thus, on induction, miR-210 or miR-373 should be capable of binding to the respective target site(s) in the cloned 3′ UTR and thereby inhibit the protein expression of luciferase. For these experiments, ~2-kb genomic fragments, each comprising regions of the *RAD52* and *RAD23B* 3′ UTRs, respectively, were inserted 3′ relative to the luciferase reporter gene (Fig. 5*A* and *B*). The resulting firefly luciferase reporter plasmids, pMIR-REPORT-*FLUC+RAD52* 3′ UTR or pMIR-REPORT-*FLUC+RAD23B* 3′ UTR, were transiently cotransfected with constructs expressing either miR-210 or miR-373. Forced expression of miR-210 and miR-373 was validated by RTQ-PCR (Fig. 5*C* and *D, insets*) and by assaying for the suppression of RAD52 levels in the context of forced miR-210 expression (Supplementary Fig. S1*A*). As controls, firefly luciferase constructs lacking the 3′ UTR targeting sequences were tested in separate samples. In each case, the transfection mixtures included a *Renilla* luciferase reporter vector, allowing a readout for normalizing the transfection efficiency. At 24 hours after transfection in MCF-7 cells, lysates were harvested for the analysis of firefly luciferase activity, which was normalized to *Renilla* luciferase activity. In comparison with the vector control, the miR-210

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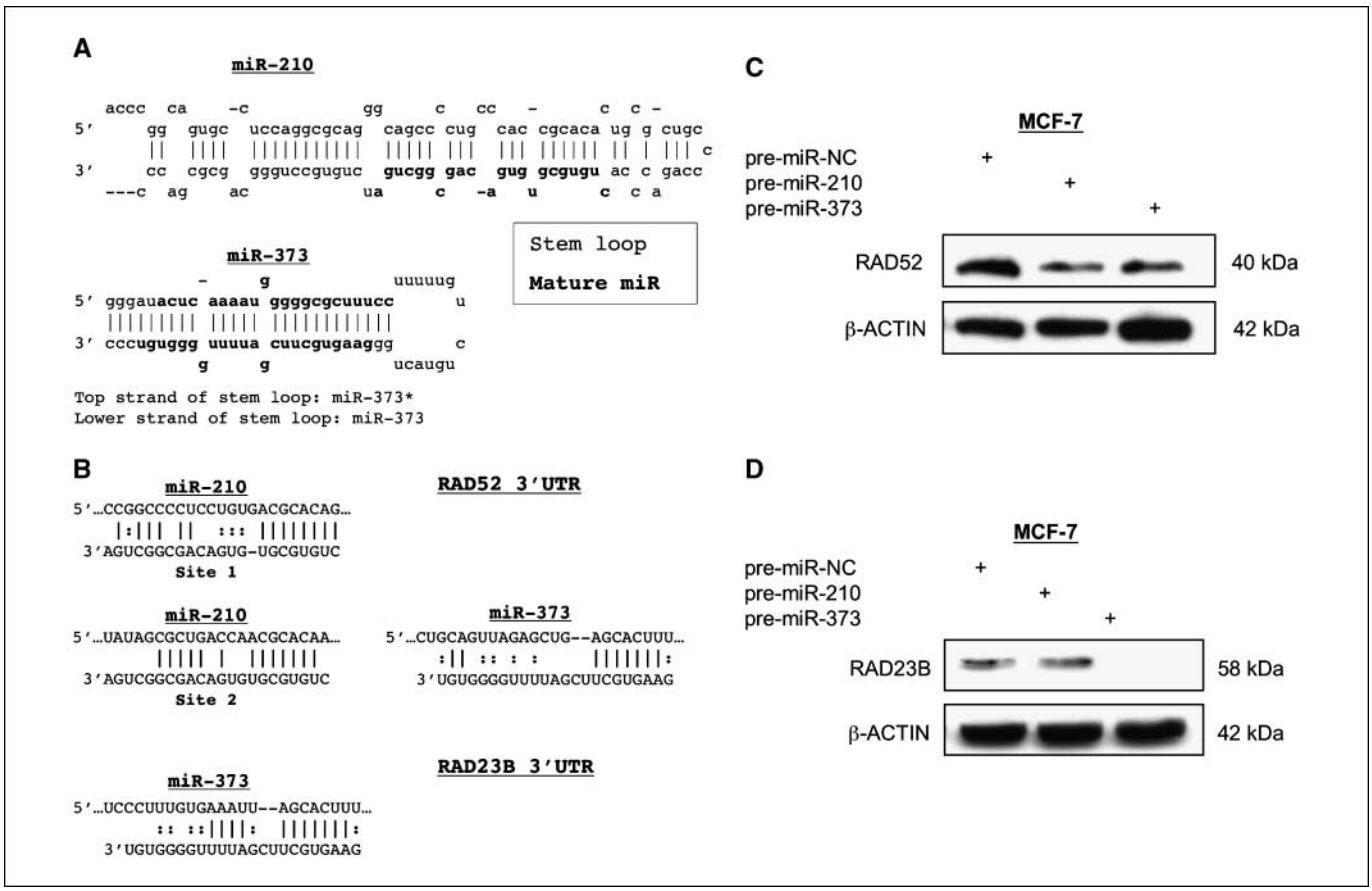

Figure 3. MiR-210 and miR-373 regulate the DNA repair genes *RAD52* and *RAD23B*. *A,* bioinformatics analyses of miR-210 and miR-373 predict binding to the 3′ UTRs of genes implicated in DNA repair. The mature miRs (*bold text*) are processed from the stem-loop structures of miR-210 and miR-373. MiR-373 is processed into two mature miRs, miR-373 and miR-373*, as indicated. *B, RAD52* contains two predicted binding sites for miR-210 in its 3′ UTR and one site for miR-373. *RAD23B* contains a single conserved site for miR-373 family binding, as indicated. The predicted pairing of mRNA target region (*top*) and miRNA (*bottom*) is as indicated, where a line ("|") indicates hydrogen base pairing and a colon (":") indicates Watson-Crick/wobble base pairing. *C,* forced expression of miR-210 and miR-373 can down-regulate RAD52 protein levels. MCF-7 cells were transfected with the indicated pre-miRs, as described in Materials and Methods. Whole-cell lysates were prepared from MCF-7 cells at 72 h after transfection; RAD52 protein levels were assayed by Western blot. *D,* forced expression of miR-373, but not of miR-210, suppresses RAD23B protein levels. MCF-7 cells were transfected with pre-miRs as above; the total cell lysates were analyzed for RAD23B expression.

expression vector reduced the luciferase expression from the vector containing the 3′ UTR of the *RAD52* gene (Fig. 5*C*). Likewise, forced miR-373 expression vector led to reduced luciferase expression when the vector contained the 3′ UTR of the *RAD23B* gene (Fig. 5*D*). Taken together, these findings further suggest that miR-210 and miR-373 forced expression each can cause the down-regulation of *RAD52* and *RAD23B* expression, respectively, via a mechanism that targets the 3′ UTR of these genes.

Discussion

Here, we have shown that hypoxic stress induces the expression of a series of miRs in human cells. One of these, miR-210, was predicted to target the mRNA of the DNA repair factor, RAD52; another, miR-373, was predicted to target both RAD52 and the NER damage recognition factor, RAD23B. Consistent with these *in silico* analyses, we found that the forced expression of either miR-210 or miR-373 reduced the levels of RAD52 and that miR-373 also suppressed RAD23B expression levels. This regulation is relatively specific, as the forced expression of miR-210 and miR-373 did not affect the levels of two other DNA repair factors: MRE11 and XPA. In experiments comparing hypoxic with normoxic cells, we found that hypoxia induces the down-regulation of both RAD52 and RAD23B. The pretreatment of cells with synthetic anti-miRs directed against miR-210 or miR-373 was found to reverse the hypoxia-mediated down-regulation of RAD52 or RAD23B, respectively. Finally, experiments in which the 3′ UTRs of the *RAD52* and *RAD23B* genes were inserted downstream of the firefly luciferase coding region in reporter vectors further showed the roles of miR-210 and miR-373 in the regulation of these genes.

The miR microarray data also suggested that miR-492 is strongly up-regulated in hypoxia. In the initial analysis, the apparent respective fold change for miR-492 was much greater than those of miR-210 and miR-373. However, we were unable to validate this preliminary result. On close inspection of the data, we noted that the absolute expression numbers were below the threshold that would allow for reliable determination of fold change and relative expression. Consequently, the normoxia levels for miR-492 may not be accurate and the fold change may be somewhat of an artifact of the array data. Thus, we did not further investigate miR-492 regulation.

Interestingly, even in normoxic cells, anti-miR-373 treatment resulted in an up-regulation of RAD23B, indicating a key role for miR-373 in modulating the basal expression of RAD23B. As the effect of anti-miR-373 activity in normoxia, where miR-373 levels are comparably low, is more profound than the effect of anti-miR-373 in hypoxia, there may be other mechanisms that regulate

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RAD23B expression. On the other hand, anti-miR-373 transfection had no detectable effect on the rescue of RAD52 suppression in hypoxic cells, indicating that although the forced miR-373 expression can inhibit RAD52, miR-373 may have a lesser role in the suppression of RAD52 in hypoxic cells.

Our results indicate that both miR-210 and miR-373 are induced in hypoxia in a HIF-1α–dependent manner. This was previously observed for miR-210 (34) and is newly reported here for miR-373. These observations add to the complex network of HIF-1α–responsive pathways and provide a novel link between HIF-1α and DNA repair activity via miR pathways. Downstream of this regulation, our data indicate that synthetic antisense oligonucleotides specifically designed to target miR-210 or miR-373 can rescue RAD52 and RAD23B regulation in hypoxia. However, we note that this reversal is not complete. Interestingly, miR-373, which has a low basal expression, seems to have a more profound effect in up-regulating RAD23B expression in normoxic than in hypoxic cells. This indicates that there may be other mechanisms that affect RAD23B expression. Indeed, several other groups have found that combinations of miRs can act together on the 3′ UTR of an individual gene (39, 40); moreover, no single miR may be sufficient to result in the complete inhibition of a protein (41). Hence, there may also be other miRs, as well as other factors, which may participate in the complex regulation of RAD52 and RAD23B. As such, RAD52 and RAD23B are certainly not the only targets of miR-210 and miR-373, respectively. Nonetheless, miR-210 and miR-373 clearly have key modulatory roles in regulating these factors.

RAD52 is an important factor in HDR. As a recombinational repair mediator, it is thought to assist in loading RAD51 onto DNA to form nucleoprotein filaments (42, 43). Functionally, our prior work has already established that HDR activity is reduced in hypoxic cells using both episomal and chromosomal assays for HDR of double-strand breaks (10, 11). Initially, we attributed the observed reduction in HDR activity in hypoxic cells specifically to the down-regulation of BRCA1 and RAD51 due to transcriptional repression involving E2F4/p130 (11, 12). We can now include the miR-mediated suppression of RAD52 levels as another mechanism by which HDR is suppressed in hypoxia.

RAD23B is a key component of the XPC/RAD23B complex that mediates damage recognition in the NER pathway (44). Interestingly, we had previously reported that NER activity is functionally reduced in hypoxic cells using two different assays for UV damage repair (13), but we were unable to determine the mechanism at that time. Our finding here that RAD23B is suppressed by miR-373 in hypoxic cells provides new mechanistic insight into this phenomenon.

Whereas previous reports have indicated that other DNA repair factors involved in HDR, specifically *BRCA1* and *RAD51*, can be down-regulated in hypoxia by transcriptional repression via E2F4/p130 heterodimeric complexes (11, 12), the data presented here indicate that DNA repair factors may also be regulated in hypoxia by miRs, further contributing to overall tumor genetic instability. Importantly, however, altered repair capacity may render hypoxic cells vulnerable to targeted strategies that exploit specific DNA repair deficiencies, potentially providing an opportunity for designing new cancer therapies. In addition, there may also be potential in using miRs as prognostic indicators of progression and metastasis (21, 45). In fact, miR-373 expression has been correlated with increased invasion and metastasis in one study (46). Hence, our finding that miR-373 is induced in response to hypoxia provides mechanistic links not only to hypoxia-induced genetic instability but also to hypoxia-induced metastatic potential.

As our data clearly show that DNA repair factors are suppressed via a miR-dependent pathway in hypoxia, why would a cell down-regulate DNA repair under such conditions? It is conceivable that by shutting down these pathways, ATP pools may be preserved under metabolic stress. Conceptually, the shutdown of certain DNA metabolic pathways may resemble features of the autophagy pathway, in which cellular components are sacrificed in the effort

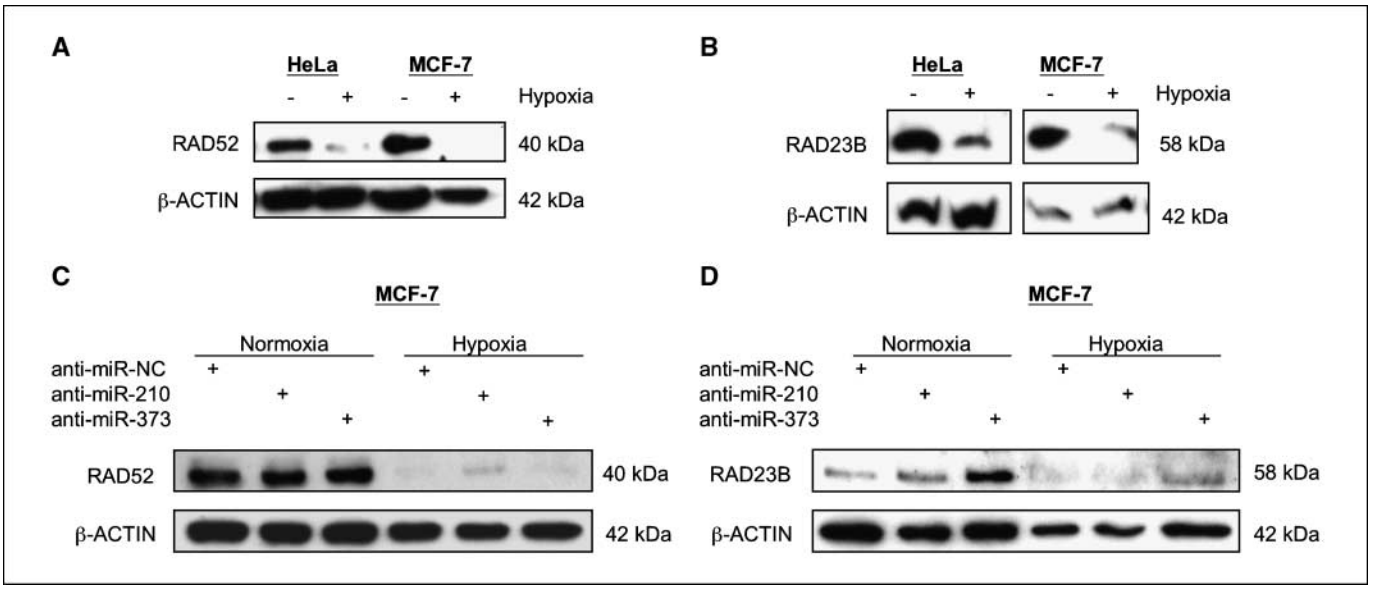

Figure 4. Hypoxia causes the down-regulation of RAD52 and RAD23B in pathways mediated by miR-210 and miR-373. *A,* Western blotting indicates down-regulation of RAD52 expression in hypoxia (48 h, 0.01% O_2) versus normoxia in HeLa and MCF-7 cells. *B,* Western blot analysis shows RAD23B down-regulation in hypoxia versus normoxia in HeLa and MCF-7 cells. *C,* Western blot analysis for RAD52 expression was performed on whole-cell lysates in MCF-7 cells following pretreatment (24 h) with anti-miR-210, anti-miR-373, or negative control #1 anti-miR (*anti-miR-NC*) and exposure to normoxia or hypoxia (48 h, 0.01% O_2), as described in Materials and Methods. *D,* RAD23B expression in MCF-7 cells after pretreatment with anti-miR-210, anti-miR-373, or anti-miR-NC and exposure to normoxia or hypoxia is detected by Western blot.

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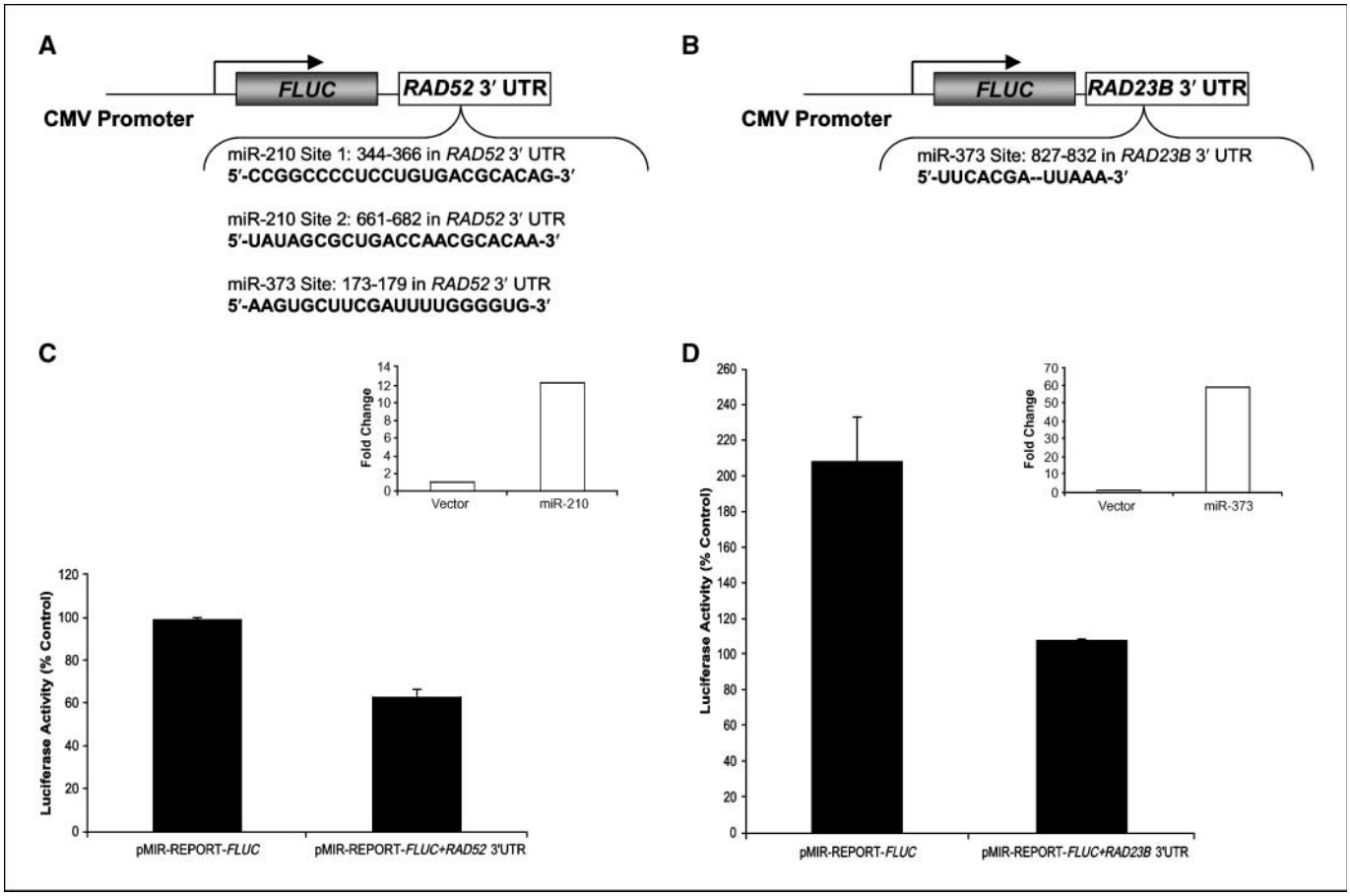

Figure 5. Luciferase reporter constructs indicate that miR-210 and miR-373 can act on the 3′ UTRs of the *RAD52* or *RAD23B* genes, respectively. *A,* diagram of the pMIR-REPORT-*FLUC*+*RAD52* 3′ UTR vector indicates the sequences of the putative target sites for miR-210 and miR-373. *B,* diagram of the pMIR-REPORT-*FLUC*+*RAD23B* 3′ UTR vector shows the predicted binding site for miR-373. *C,* miR-210 suppresses the expression of the pMIR-REPORT-*FLUC*+*RAD52* 3′ UTR vector. The pMIR-REPORT-*FLUC*+*RAD52* 3′ UTR or the pMIR-REPORT-*FLUC* vector containing only the luciferase coding region and no extra 3′ UTR was transfected into MCF-7 cells at the same time that a vector expressing miR-210 was introduced. The cotransfection of a vector expressing *Renilla* luciferase was used for normalization to control for the transfection efficiency. After 24 h, the cells were harvested and assayed for luciferase activity. Results are adjusted for transfection efficiency and normalized to the effect of an empty control pBABE vector. *Columns,* mean from duplicate experiments, which were each carried out in triplicate; *bars,* SE. *D,* miR-373 suppresses the expression of pMIR-REPORT-*FLUC*+*RAD23B* 3′ UTR. Experiments were carried out as in *C*, except that the forced expression of miR-373 was performed using pBABE-miR-373. *C* and *D, insets,* levels of miR-210 and miR-373 expression that were achieved via the pBABE-miR-210 and pBABE-miR-373 vectors, respectively, as quantitated by RTQ-PCR.

of self-preservation (47). Regardless of the reason, the consequences of decreased DNA repair include genomic instability, which confers a mutator phenotype on cancer cells in the adverse environment of a hypoxic tumor. This could be advantageous for the individual cancer cells but would be deleterious for the host.

Disclosure of Potential Conflicts of Interest

The authors have no conflicts of interest.

Acknowledgments

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Correction: Article on MicroRNAs and DNA Repair

In the article on microRNAs and DNA repair in the February 1, 2009 issue of *Cancer Research* (1), there is an error in the "Luciferase assays" section of Materials and Methods. The amount of construct transfected should be 0.5 μg.

1. Crosby ME, Kulshreshtha R, Ivan M, Glazer PM. MicroRNA regulation of DNA repair gene expression in hypoxic stress. Cancer Res 2009;69:1221–9.

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MicroRNA Regulation of DNA Repair Gene Expression in Hypoxic Stress

Meredith E. Crosby, Ritu Kulshreshtha, Mircea Ivan, et al.

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