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microRNA-21 Negatively Regulates Cdc25A and Cell Cycle Progression in Colon Cancer Cells

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Abstract

microRNAs (miRNA) are small noncoding RNAs that participate in diverse biological processes by suppressing target gene expression. Altered expression of *miR-21* has been reported in cancer. To gain insights into its potential role in tumorigenesis, we generated *miR-21* knockout colon cancer cells through gene targeting. Unbiased microarray analysis combined with bioinformatics identified cell cycle regulator Cdc25A as a *miR-21* target. *miR-21* suppressed Cdc25A expression through a defined sequence in its 3′-untranslated region. We found that *miR-21* is induced by serum starvation and DNA damage, negatively regulates G_1-S transition, and participates in DNA damage–induced G_2-M checkpoint through down-regulation of Cdc25A. In contrast, *miR-21* deficiency did not affect apoptosis induced by a variety of commonly used anticancer agents or cell proliferation under normal cell culture conditions. Furthermore, *miR-21* was found to be underexpressed in a subset of *Cdc25A*-overexpressing colon cancers. Our data show a role of *miR-21* in modulating cell cycle progression following stress, providing a novel mechanism of Cdc25A regulation and a potential explanation of *miR-21* in tumorigenesis. [Cancer Res 2009; 69(20):8157–65]

Introduction

microRNAs (miRNA) are evolutionarily conserved, 20- to 25-nucleotide-long, noncoding RNAs that bind to their targets through partial complementary sequence recognition. This results in either degradation of mRNA or inhibition of translation, thus modulating expression of miRNA targets (1). Several hundred miRNAs have been identified in human cells (2). It is estimated that a single miRNA can regulate hundreds of targets, and ≥30% of human mRNAs are regulated by miRNAs (1, 2). Therefore, it is not surprising that miRNAs are involved in diverse biological processes, including cell differentiation, proliferation, and apoptosis, presumably through a myriad of targets (2).

Deregulation of miRNAs contributes to human pathogenesis including cancer (2). For example, aberrant expression of miRNAs, including *miR-21, miR-17-92, miR-15, miR-16*, and *let-7*, has been reported in cancer (3). Furthermore, a substantial number of miRNA genes are located in the fragile sites in the genomic regions that are frequently amplified, deleted, or rearranged in cancer, providing plausible mechanisms of deregulated expression (4, 5). A theme is emerging that a miRNA can be considered either a tumor suppressor or an oncogene depending on its targets in different tissues and cell types (6–8). Identification of relevant targets or pathways controlled by miRNAs will ultimately provide insights into their biological functions.

Altered expression of *miR-21* has been reported in cancer. For example, *miR-21* was reported to have substantially higher expression in normal tissues than in colon cancers or in NCI-60 tumor cell lines (8). On the other hand, *miR-21* is overexpressed in cancers of the breast, lung, pancreas, prostate, stomach, and brain (9, 10). Higher expression of *miR-21* was found in colon adenocarcinomas than in the normal mucosa and was associated with decreased overall survival (11). A limited number of genes, including *PTEN, TPM1, Pdcd4, Spry1*, and *Spry2*, have been reported to be targets of *miR-21*, suggesting potential functions in regulating cell proliferation, apoptosis, and invasion (12–16). However, the precise role of *miR-21* in cancer remains to be defined.

The cell division cycle 25 (Cdc25) family of proteins are highly conserved dual-specificity phosphatases that dephosphorylate and activate cyclin-dependent kinase complexes. Three isoforms have been identified in mammalian cells, Cdc25A, Cdc25B, and Cdc25C (17). Overexpression of Cdc25 family proteins, mostly Cdc25A and Cdc25B, correlates with more aggressive disease and poor prognosis in some cancers and leads to genetic instability in mice (18, 19). Cdc25A positively regulates G_1-S and G_2-M transitions by activating distinct cyclin/cyclin-dependent kinase complexes (18, 19). Moreover, timely inactivation of Cdc25A facilitates checkpoint activation on DNA damage. Cdc25A activities are tightly regulated by multiple mechanisms during the cell cycle, and ubiquitin-mediated proteolysis is the major mechanism of Cdc25A turnover (17). For example, hyperphosphorylation of Cdc25A by the ATR-Chk1 signaling leads to its degradation and contributes to a delay in the cell cycle, which allows either DNA repair or apoptosis, depending on the extent of DNA damage (17, 19, 20).

In the current study, we reported a novel role of *miR-21* in modulating cell cycle progression and DNA damage checkpoint activation via Cdc25A. *Cdc25A* was identified and validated as a *miR-21* target using *miR-21* knockout colon cancer cell lines. *miR-21* was found to be induced by serum starvation, negatively regulates G_1-S transition, and participates in DNA damage checkpoint activation in response to γ-irradiation.

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

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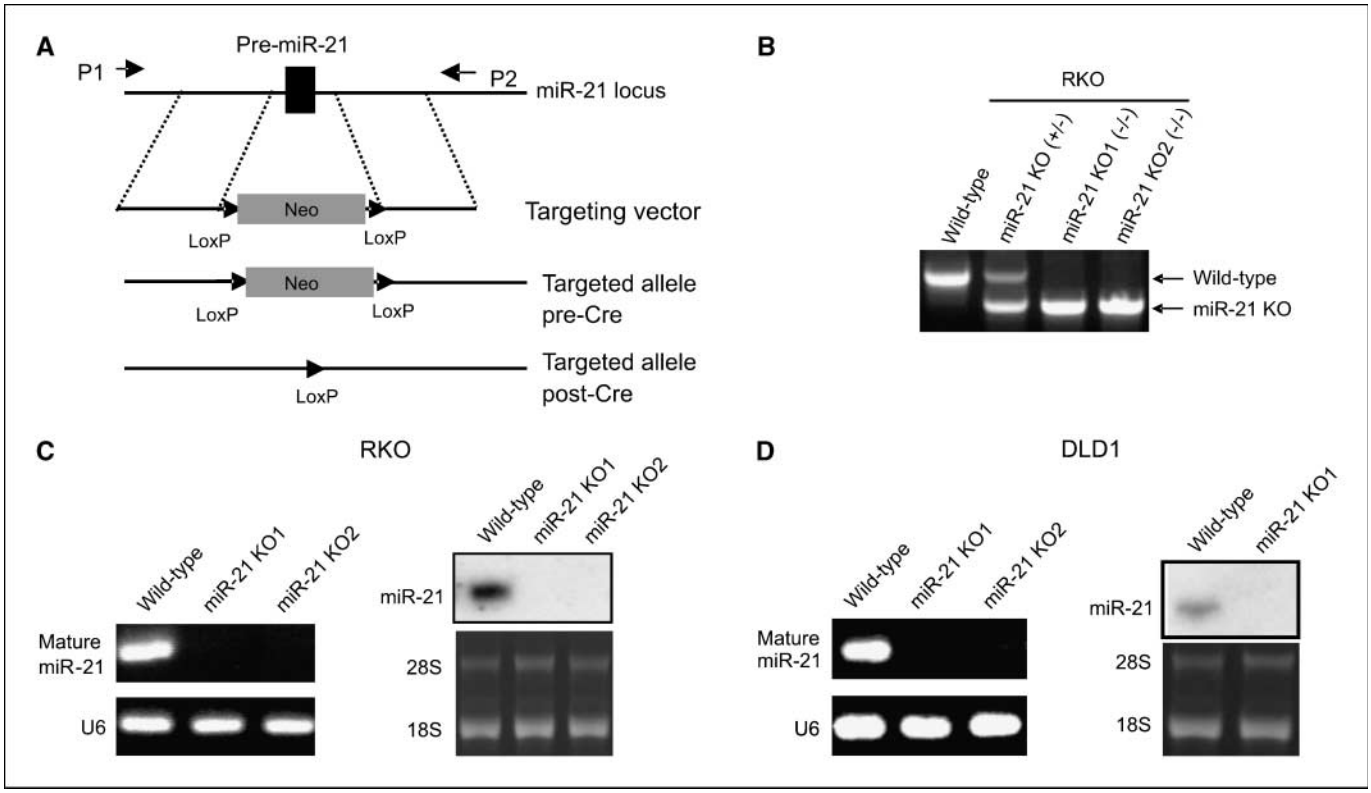

Figure 1. Targeted deletion of the primary *miR-21* locus in RKO and DLD1 colon cancer cells. *A,* schematic diagram of *miR-21* targeting strategy. The targeting construct consists of two homologous arms and the neomycin-resistant gene (*Neo*) flanked by two LoxP sites. Homologous recombination resulted in a deletion of 750 bp, including the sequence encoding mature *miR-21*. The same construct was used in the second round of gene targeting after the excision of *Neo* gene by Cre recombinase. The positions of the primers (P1 and P2) for PCR screening are indicated. *B,* identification of *miR-21* knockout (*KO*) clones by genomic PCR. *C,* mature *miR-21* expression was measured by RT-PCR (*left*) or Northern blot (*right*) in the indicated RKO cell lines. *D,* mature *miR-21* expression was measured as in *C* in the indicated DLD1 cell lines.

Our data provide a novel mechanism of *Cdc25A* mRNA turnover and a potential role of *miR-21* deregulation in tumorigenesis.

Materials and Methods

Targeting the *miR-21* gene. Gene targeting vectors were constructed using a recombinant adeno-associated virus (AAV) system as described (21–23) with minor modifications. Briefly, two homologous arms flanking the *miR-21* locus, which are 1.17 and 1.15 kb, respectively, along with the neomycin-resistant gene cassette (*Neo*), were inserted between two *Not*I sites in the AAV shuttle vector pAAV-MCS (Stratagene) by a four-way ligation reaction. Packaging of recombinant AAV was done by using the AAV Helper-Free System (Stratagene) according to the manufacturer's instructions. RKO and DLD1 cells were infected with recombinant AAV and selected by G418 (0.4 mg/mL) for 3 weeks. G418-resistant clones were screened by PCR for targeting events with primer pairs listed in Supplementary Table S1 using pooled genomic DNA (24). The same targeting construct was used in the second round of gene targeting following the excision of *Neo* gene flanked by LoxP sites in a heterozygous clone with an adenovirus expressing Cre recombinase (24). After the second round of gene targeting, *Neo* was excised by adenovirus expressing Cre recombinase infection again, and gene targeting was verified by genomic PCR, reverse transcription-PCR (RT-PCR), and Northern blot. The detailed procedures of gene targeting and PCR screening are available upon request, and the primers used are listed in Supplementary Table S1.

Microarray analysis. Total RNA was isolated 48 h following transfection from cells cultured in T25 flasks. Microarray analysis was done and relative gene expression was analyzed as described previously by the Core Facility at the University of Pittsburgh School of Medicine (25).

Cell culture. Human colorectal cancer cell lines RKO and DLD1 were obtained from the American Type Culture Collection and cultured in McCoy's 5A modified medium (Invitrogen) supplemented with 10% defined fetal bovine serum (Hyclone) and 100 units/mL penicillin and 1% streptomycin (Invitrogen). Cells were maintained at 37°C with 5% CO_2. In some experiments, cells were grown in medium containing 0.5% serum. Details on serum-stimulated G_1-S transition, radiation-induced transient G_2-M checkpoint, and clonogenic survival are described in Supplementary Material.

miRNA target prediction. The miRNA targets were predicted using the algorithms TargetScan[3] and PicTar.[4]

Isolation of miRNAs, real-time PCR assays, and Northern blotting for mature miRNAs. The expression of mature miRNAs was determined by real-time PCR (26) and Northern blot. The expression of protein coding mRNAs was quantitated by real-time PCR. Details are described in Supplementary Material.

Transfections. Transfection with 100 nmol/L pre-*miR-21*, 100 nmol/L anti-*miR-21* (Ambion), or 200 nmol/L *Cdc25A* small interfering RNA (siRNA; Dharmacon) was done with Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. The target sequence for *Cdc25A* is GGAAAAUGAAGCCUUUGAG (27). Cells plated at 20% to 30% confluence in 6-well plates were transfected twice in 48 h and split into T25 flask 10 h after the second transfection. The next day, the cells in T25 were either subjected to serum starvation and stimulation or irradiated as described above.

[3] http://genes.mit.edu/tscan/targetscanS2005.html
[4] http://pictar.bio.nyu.edu/cgi-bin/PicTar_vertebrate.cgi

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Luciferase reporter constructs. The reporter constructs containing the 3′-untranslated region (3′-UTR) of *Cdc25A* were cloned into the pMIR-REPORT vector (Ambion) using PCR-generated fragment. Site-directed mutagenesis with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) was used to introduce mutations in the *miR-21* binding site. Reporter assays were carried out as described previously with a transfection control (28). All the experiments were done in triplicate and repeated at least three times on different days. Details were described in Supplementary Material (Supplementary Table S2).

Bromodeoxyuridine incorporation and mitotic index. Bromodeoxyuridine (BrdUrd) incorporation was analyzed by microscopy or flow cytometry following staining with anti-BrdUrd, Alexa Fluor 488–conjugated antibody. Mitotic index was measured by phosphorylated histone 3 staining. Detailed methods are described in Supplementary Material.

Western blotting. Western blotting was done as described previously (29). The antibodies used for Western blotting included those against Cdc25A, Cdc25C, Cdc2, cyclin B1, Chk1 (Santa Cruz Biotechnology), phosphorylated histone H3 (Millipore), α-tubulin (EMD Biosciences), phosphorylated Cdc2 (Cell Signaling), Cdc25B (BD Biosciences), and β-Trcp (Invitrogen). Quantification of relative expression was determined by densitometry as described (30).

Statistical analysis. Statistical analysis was done using GraphPad Prism IV software. *P* values were calculated by Student's *t* test. *P* values <0.05 were considered significant. Mean ± SD is displayed in the figures.

Results

Targeted deletion of *miR-21* in colon cancer cells. Aberrant expression of *miR-21* has been reported in colon cancer (8). We were interested in determining its potential role in tumorigenesis by identifying *miR-21* targets. To avoid the limitations of down-regulating miRNA expression with antisense oligos (31), we knocked out the *miR-21* precursor sequence in RKO and DLD1 colorectal cancer cells using the recombinant AAV system (Fig. 1*A*; refs. 21, 22). Both of these lines express relatively high levels of *miR*-21 (8, 32). After two rounds of homologous recombination, *miR-21* knockout clones were identified by PCR amplification of the corresponding genomic regions (Fig. 1*B*; Supplementary Fig. S1*A*). RT-PCR and Northern blot confirmed that the mature *miR-21* was not expressed in these knockout clones (Fig. 1*C* and *D*).

Identification of potential *miR-21* targets through microarray analysis. miRNAs regulate their target genes via mRNA degradation and/or inhibition of translation (33). Their potential targets can be identified using high-throughput methods such as microarray analysis (34). To identify potential *miR-21* targets predicted to have elevated expression in *miR-21*

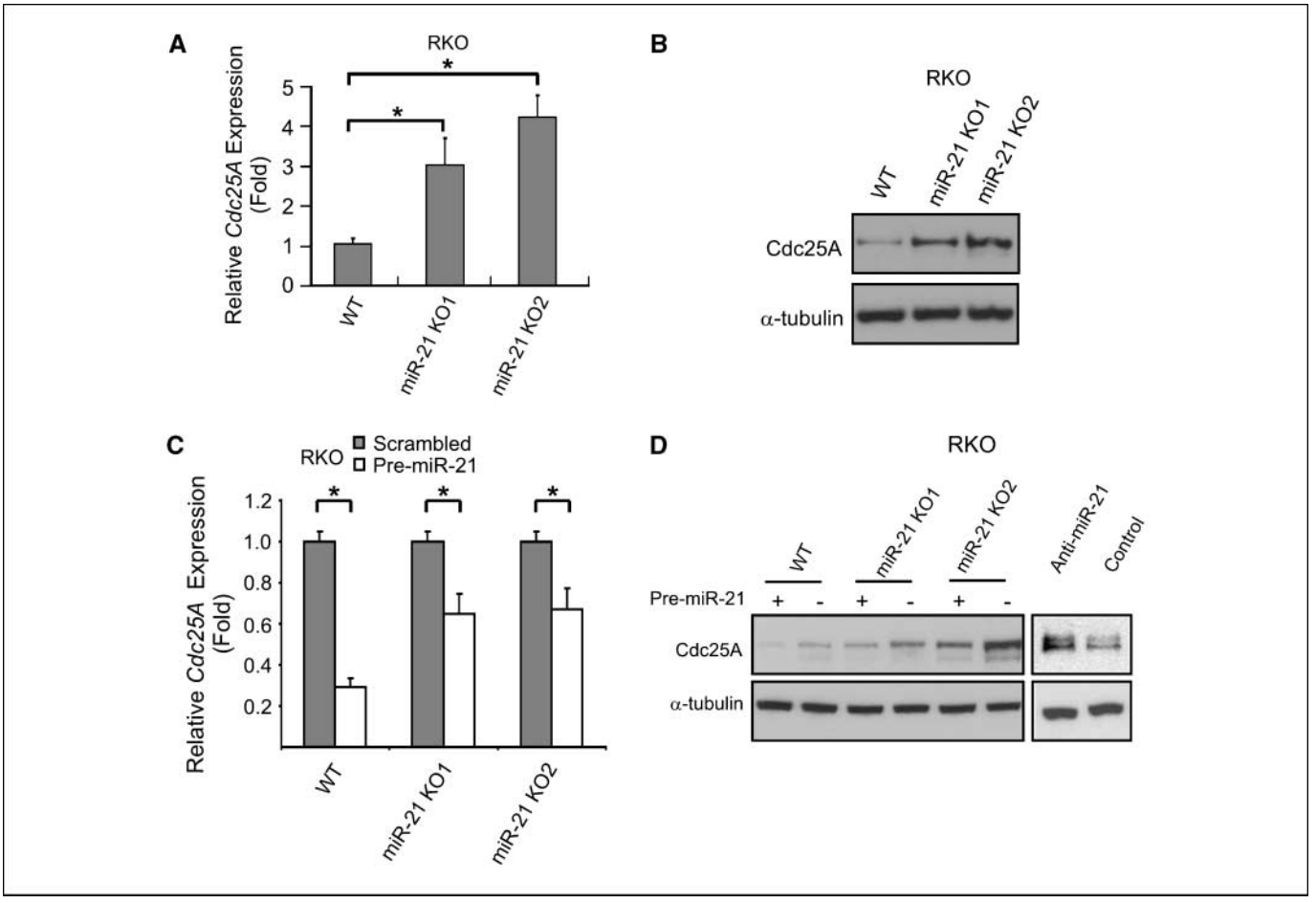

Figure 2. *Cdc25A* is regulated by *miR-21* in colon cancer cells. *A,* relative *Cdc25A* mRNA expression was measured by real-time RT-PCR in RKO wild-type (*WT*) and *miR-21* knockout cells. Levels were standardized to *Cdc25A* mRNA in the wild-type cells normalized to *GAPDH*. Mean ± SD ($n = 3$). *, $P < 0.02$. *B,* expression of Cdc25A protein was determined by Western blotting. *C,* effect of pre-*miR-21* on *CDC25A* mRNA levels. Cells were transfected with pre-*miR-21* or control siRNA for 48 h and analyzed for *CDC25A* expression by RT-PCR. Mean ± SD ($n = 3$). *, $P < 0.04$. Expression levels were normalized to those in control siRNA-transfected cells. Mean ± SD of three experiments. *D,* effects of anti-*miR-21* or pre-*miR-21* on Cdc25A expression. RKO cells were transfected with control siRNA, anti-*miR-21*, and pre-*miR-21*. Cdc25A levels were analyzed by Western blotting at 48 h after transfection.

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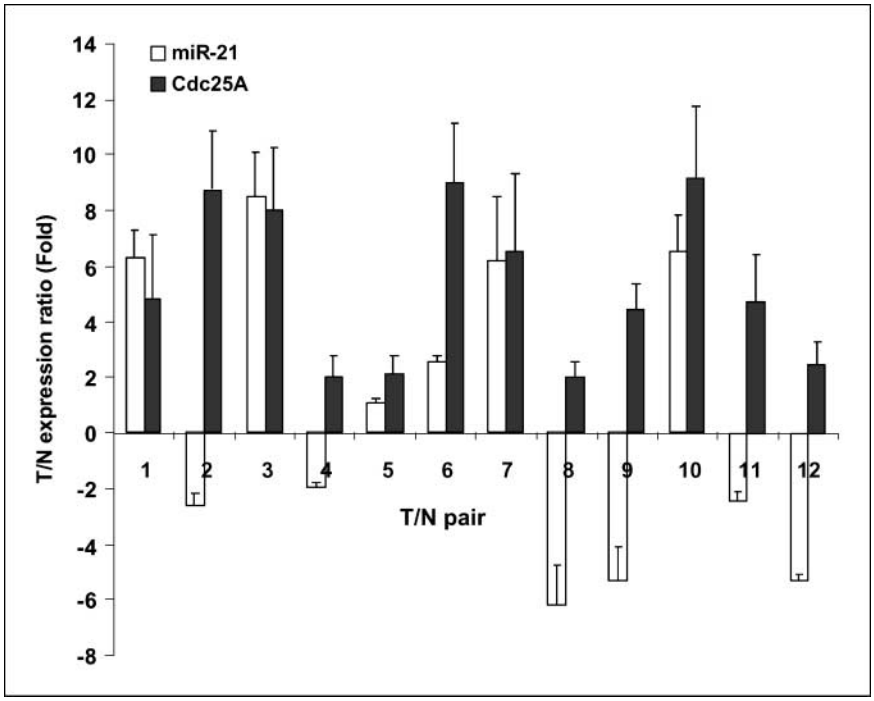

Figure 3. *miR-21* levels in *Cdc25A*-overexpressing colon cancers. Levels of *miR-21* were analyzed by real-time RT-PCR in 12 pairs of matched adjacent normal (*N*) and tumor (*T*) tissues that overexpress *Cdc25A*. *miR-21* levels were normalized to that of U6. *Cdc25A* levels were normalized to that of *GAPDH*. Mean ± SD ($n = 3$).

knockout cells, we performed microarray analysis on RKO parental cells transfected with pre-*miR-21* and *miR-21* knockout cells transfected with a control siRNA for 48 h. Over 100 candidates showed at least a 2-fold increase in their expression in *miR-21* knockout cells (Supplementary Table S3; $P \leq 0.02$). Interestingly, several proteins involved in cell cycle and DNA damage responses were among them. We then chose 12 candidates and validated 7 of them by quantitative RT-PCR analysis (Supplementary Fig. S2). Cdc25A was chosen for further analysis due to its well-established role in cell cycle regulation and cancer.

Cdc25A expression is regulated by *miR-21* in colon cancer cells. To validate Cdc25A as a *miR-21* target, its mRNA and protein levels were compared in parental and *miR-21* knockout RKO cells. Consistent with the results obtained in microarray analysis, Cdc25A levels were significantly up-regulated in *miR-21* knockout cells (Fig. 2*A* and *B*). Transfection of pre-*miR-21* decreased the levels of Cdc25A transcript and protein (Fig. 2*C* and *D*; Supplementary Figs. S1*B* and S3). These findings were confirmed in DLD1 parental and *miR-21* knockout cells (Supplementary Fig. S3*A-D*). *miR-21* deletion did not affect the expression of the other two Cdc25 family members, Cdc25B and Cdc25C, or its established regulators Chk1 or β-TrCP (Supplementary Fig. S4*A*). Transient transfection of anti-*miR-21* also elevated Cdc25A expression in RKO cells as did *miR-21* targeting (Fig. 2*D, right*). To examine the expression of *miR-21* in relation to Cdc25A in cancer, we analyzed the expression of *miR-21* in 12 colon cancers that overexpress *Cdc25A* using matched normal and tumor tissues (35). *miR-21* was found to be underexpressed in 6 of 12 (50%) tumors (range, 2- to 7-fold; Fig. 3).

Cdc25A is a *miR-21* target. On a closer inspection, a putative *miR-21* binding site located in the 3′-UTR of *Cdc25A* gene was predicted by two algorithms (TargetScan and PicTar; Fig. 4*A*). Importantly, this putative miR-21 binding site is 100% conserved in five species in the region that pairs with the seed sequence (Fig. 4*A*). The 3′-UTR of *Cdc25A* containing this site was cloned into pMIR-REPORT miRNA reporter vector. The luciferase activities of this reporter in *miR-21* knockout cells were ~60% higher than that in parental RKO cells but were suppressed by pre-*miR-21* transfection (Fig. 4*B*), suggesting a regulatory element in its 3′-UTR. We then mutated the *miR-21* binding site in the reporter construct Luc-*Cdc25A*-Mut-UTR and found its activities were similar in parental and *miR-21* knockout RKO cells (Fig. 4). Transfection of pre-*miR-21* did not decrease the activities of the mutant reporter in either parental or *miR-21* knockout cells (Fig. 4*B*), suggesting specificity of this sequence. We also examined the expression of several reported *miR-21* targets in the microarray data or by Western blotting, including PTEN, Pdcd4, Bcl-2, TMP1, Spry1, and Spry2 (Supplementary Table S4; Supplementary Fig. S4*B*). Only *Spry1* and *Spry2* appear to be significantly up-regulated (1.8- and 1.44-fold) in *miR-21* knockout cells but not the other three genes (Supplementary Table S4; Supplementary Fig. S4*B*). Together, these results indicate that *miR-21* regulates Cdc25A through the *miR-21* binding site in its 3′-UTR and establish Cdc25A as a direct target of *miR-21*.

***miR-21* inhibits cell proliferation following serum starvation and delays G_1-S transition through Cdc25A.** Cdc25A is an important regulator of cell cycle progression during G_1-S transition (36, 37). To evaluate whether *miR-21* affects cell cycle progression, we compared the growth rate of parental and *miR-21* knockout cells under normal serum (10%) and low-serum condition (0.5%) over a course of 7 days. The growth rate of parental and *miR-21* knockout cells was indistinguishable under the normal serum condition in the entire 7 days (Supplementary Fig. S4*C*). However, RKO *miR-21* knockout cells exhibited enhanced proliferation over wild-type cells in the low-serum condition (Fig. 5*A, top*). Under these conditions, no significant levels of apoptosis were detected in either wild-type or *miR-21* knockout cells (data not shown). Using quantitative RT-PCR, we found that *miR-21* levels were induced 2- to 10-fold by serum starvation in wild-type cells starting at 24 h (Fig. 5*A, middle*). Serum starvation also caused an apparent reduction in Cdc25A levels, which was significantly blunted in *miR-21* knockout cells (Fig. 5*A, bottom*).

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The induction of *miR-21* in parental cells and elevated Cdc25A and enhanced proliferation in *miR-21* knockout cells were also observed following complete serum starvation (0% serum; data not shown).

Because serum has been well documented to stimulate the G_1-S transition, we therefore specifically evaluated a potential role of *miR-21* in this process (17, 27, 38). Parental and *miR-21* knockout RKO cells were serum starved for 48 h and subsequently stimulated with 10% FCS. The cell cycle profiles were followed by flow cytometry in a time-course experiment. Serum addition induced a higher degree of S-phase entry in *miR-21* knockout cells compared with parental RKO cells. The effects were most pronounced at 15 h (17% versus 7%) and 16 h (16% versus 10%) and gradually diminished (Fig. 5*B*). BrdUrd staining indicated increased DNA synthesis in *miR-21* knockout cells compared with parental cells (Fig. 5*C*; Supplementary Fig. S5*A*). Consistent with an accelerated entry into S phase, high levels of Cdc25A were detected in *miR-21* knockout cells as early as 15 h (Supplementary Fig. S5*B*). Transfection of pre-*miR-21* significantly reduced DNA synthesis and Cdc25A levels in *miR-21* knockout cells (Fig. 5*D*). No significant difference in the cell cycle distribution was found between wild-type and *miR-21* knockout RKO or DLD1 cells growing in log phase with 10% serum (Supplementary Fig. S5*C*). Taken together, these results indicate that *miR-21* induction inhibits the G_1-S transition by suppressing Cdc25A expression.

***miR-21* modulates DNA damage–induced G_2-M checkpoint through Cdc25A.** Cdc25A has been shown to regulate the G_2-M transition and its inactivation is critically involved in establishing a G_2-M checkpoint following γ-irradiation (17, 27, 38). We tested whether *miR-21* is involved in establishing a transient G_2-M checkpoint 1 h following irradiation by analyzing phosphorylated histone H3–positive cells (39). As expected, radiation (12 Gy) induced a >90% drop in mitotic cells, which was significantly inhibited in *miR-21* knockout RKO cells (Fig. 6*A*; Supplementary Fig. S6*A*). Using quantitative RT-PCR, we found that *miR-21* levels were induced in parental RKO cells within 1 h of γ-irradiation (Fig. 6*A, right*). *miR-21* knockout DLD1 cells also exhibited a defective G_2-M checkpoint associated with elevated levels of Cdc25A (Supplementary Fig. S6*B* and *C*). Transfection of pre-*miR-21* or *Cdc25A* siRNA significantly suppressed the fraction of mitotic cells in both wild-type and *miR-21* knockout cells, but the inhibition was more pronounced in *miR-21* knockout cells (Fig. 6*B*). Transfection of anti-*miR-21* only elevated the fraction of mitotic cells in wild-type cells but did not further elevate that in *miR-21* knockout cells (Fig. 6*B*). Cdc25A levels rapidly decreased following radiation in both parental and knockout cells but were substantially higher in *miR-21* knockout cells (Fig. 6*C*). As expected, transfection of pre-*miR-21* or *Cdc25A* siRNA decreased the levels of Cdc25A, whereas anti-*miR-21* elevated those in wild-type cells (Fig. 6*C*).

To determine whether *miR-21* affects radiosensitivity, we evaluated the clonogenic survival of parental and *miR-21* knockout RKO cells. *miR-21* knockout RKO cells were found to have increased clonogenic survival following several doses of irradiation (Fig. 6*D*). *miR-21* has been reported previously to

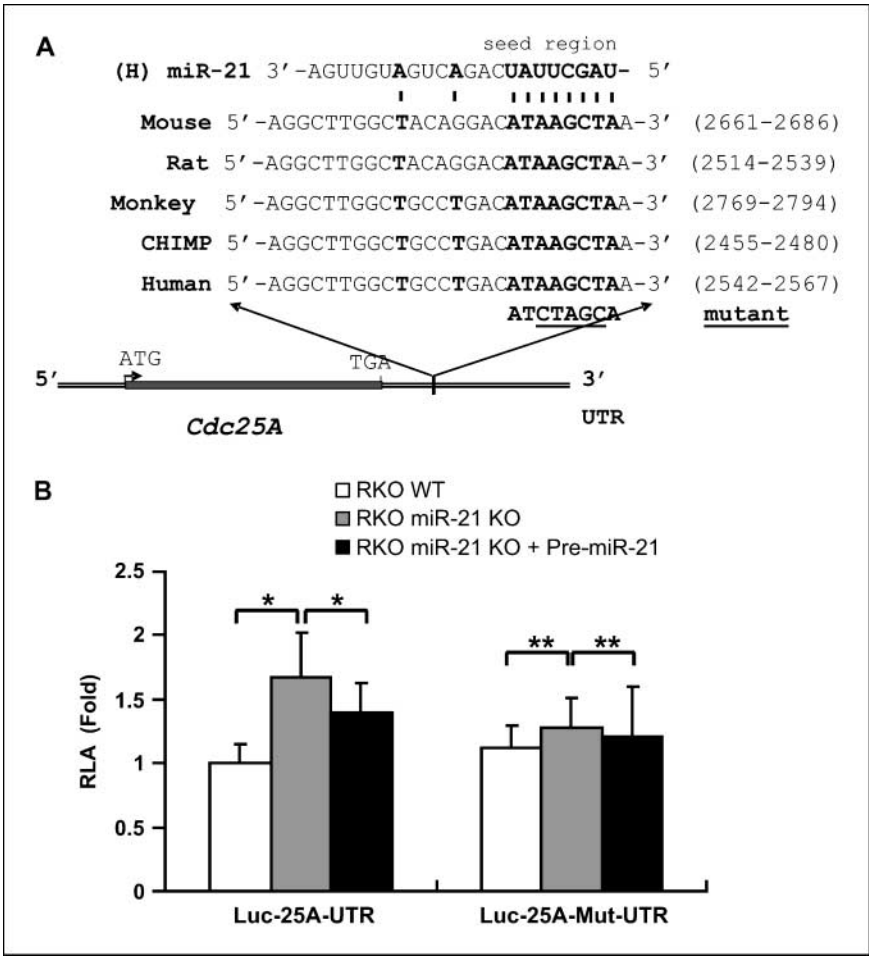

Figure 4. Conserved *miR-21* binding site in the 3′-UTR of *Cdc25A*. *A,* schematic representation of *CDC25A* transcript with its 3′-UTR. The predicted *miR-21* binding sites in the *Cdc25A* gene of five species are shown with *miR-21* targeting sequences aligned (GenBank accession numbers NM_007658, NM_133571, XR_014086, XM_001155610, and NM_201567, respectively). The base pairing nucleotides are in bold. *B,* activities of the Luc-*Cdc25A*-UTR reporter or *miR-21* binding site mutated Luc-*Cdc25A*-MUT-UTR reporter in RKO wild-type and *miR-21* knockout cells with or without pre-*miR-21* transfection. Mean ± SD ($n = 3$). *, $P < 0.05$; **, $P > 0.1$. The mutated nucleotides in the Luc-*Cdc25A*-MUT-UTR reporter are underlined in *A*.

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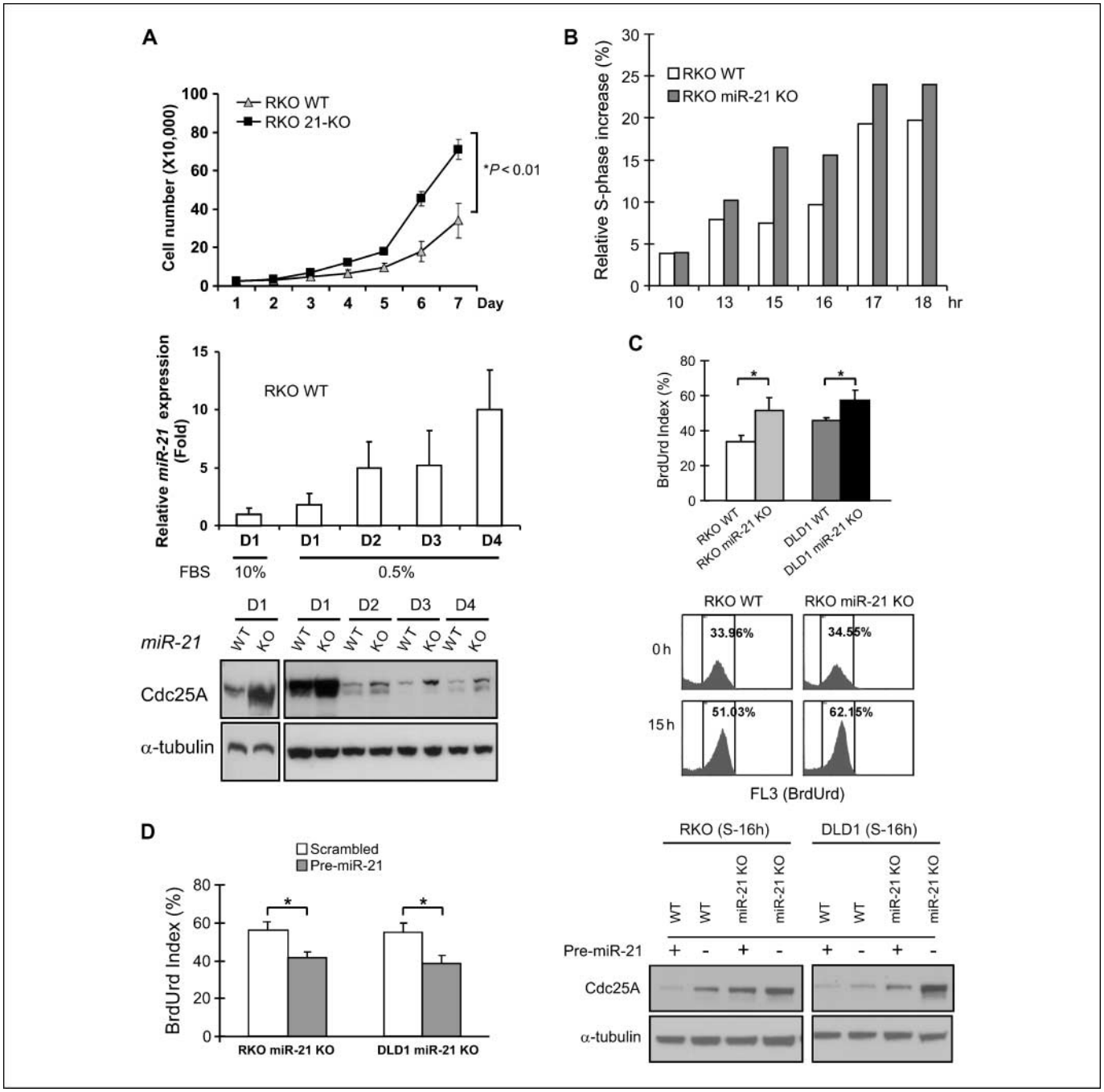

Figure 5. *miR-21* modulates cell proliferation under low-serum conditions and G_1-S transition through Cdc25A. *A,* cells were cultured in medium containing 0.5% serum for 7 d. Cell numbers were determined by counting. Levels of *miR-21* and Cdc25A were determined by real-time RT-PCR and Western blotting, respectively. Levels of mature *miR-21* expression were normalized to those of *U6*. Cells cultured in 10% serum for 1 d were used as controls. Mean ± SD ($n = 3$). *B,* RKO cells were cultured in serum-free medium for 48 h and then stimulated with 10% FCS. Cell cycle analysis was done using flow cytometry. Increases in the fraction of S-phase cells at the indicated time points compared with 0 h were quantified. *C,* BrdUrd incorporation in parental and *miR-21* knockout cells with or without pre-*miR-21* transfection. Cells were subjected to treatment as in *B* and pulse-labeled with BrdUrd for 15 min. BrdUrd/4′,6-diamidino-2-phenylindole staining was done at 15 h following 10% serum stimulation. The percentage of BrdUrd-positive cells was scored by fluorescence microscopy (*top*). Mean ± SD ($n = 3$). *, $P < 0.05$. BrdUrd incorporation was also determined by flow cytometry (*bottom*). *D,* effects of pre-*miR-21* on BrdUrd incorporation were analyzed as in *C*. Mean ± SD ($n = 3$). *, $P < 0.05$. Effects of pre-*miR-21* on Cdc25A levels at 16 h following serum stimulation were analyzed by Western blotting in the indicated cell lines.

regulate cell proliferation and apoptosis in glioblastoma and breast cancer cells (9, 13, 40). However, *miR-21* did not appear to affect apoptosis induced by a variety of anticancer agents in either RKO or DLD1 cells (Supplementary Fig. S4*D*; data not shown). The regulation of Cdc25A by miR-21 appears to be independent of the tumor suppressor p53, as it occurs in both *p53* wild-type RKO cells and *p53* mutant DLD1 cells. The above results suggest that miR-2-mediated down-regulation of Cdc25A contributes to the activation of the G_2-M checkpoint following radiation.

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Discussion

Our study provides a novel function of *miR-21* in regulating cell cycle progression and checkpoint activation through Cdc25A in colon cancer cells. This conclusion is supported by several lines of evidence: increased expression of Cdc25A in *miR-21* knockout RKO and DLD1 cells, which is suppressed by expression of pre-*miR-21*; a putative *miR-21* binding site in the 3′-UTR that is subject to *miR-21* regulation; the induction of *miR-21* by serum starvation and DNA damage, accelerated G_1-S transition in *miR-21* knockout cells; and compromised G_2-M checkpoint in response to γ-irradiation, all of which were partially rescued by pre-*miR-21* or *Cdc25A* knockdown.

The major mechanism of rapid turnover of Cdc25 family proteins is regulated by ubiquitin-mediated proteolysis (19). Our findings suggest that the full extent of Cdc25A inactivation requires *miR-21* in colon cancer cells, which represents a novel mechanism of *Cdc25A* mRNA turnover. An involvement of *miR-21* in cell cycle progression following stress is supported by several recent studies, as it was induced by the chemotherapeutic drug 5-fluorouracil in colon cancer cells (41) and by UV irradiation in primary fibroblasts (42) or in colon cancer cells (Supplementary Fig. S6*D*). Cdc25A contains a large number of phosphorylation sites recognized by cyclin-dependent kinase 1, Chk1/Chk2, and p38 (17, 19). However, extensive effort in the mapping of phosphorylation sites in Cdc25A and the use of cells deficient in Chk2 or ATM indicate that many such sites are not required for Cdc25A-mediated G_2-M checkpoint following DNA damage (20, 43–45). Our data suggest that *miR-21*-mediated Cdc25A down-regulation facilitates the rapid establishment of the G_2-M checkpoint following DNA damage.

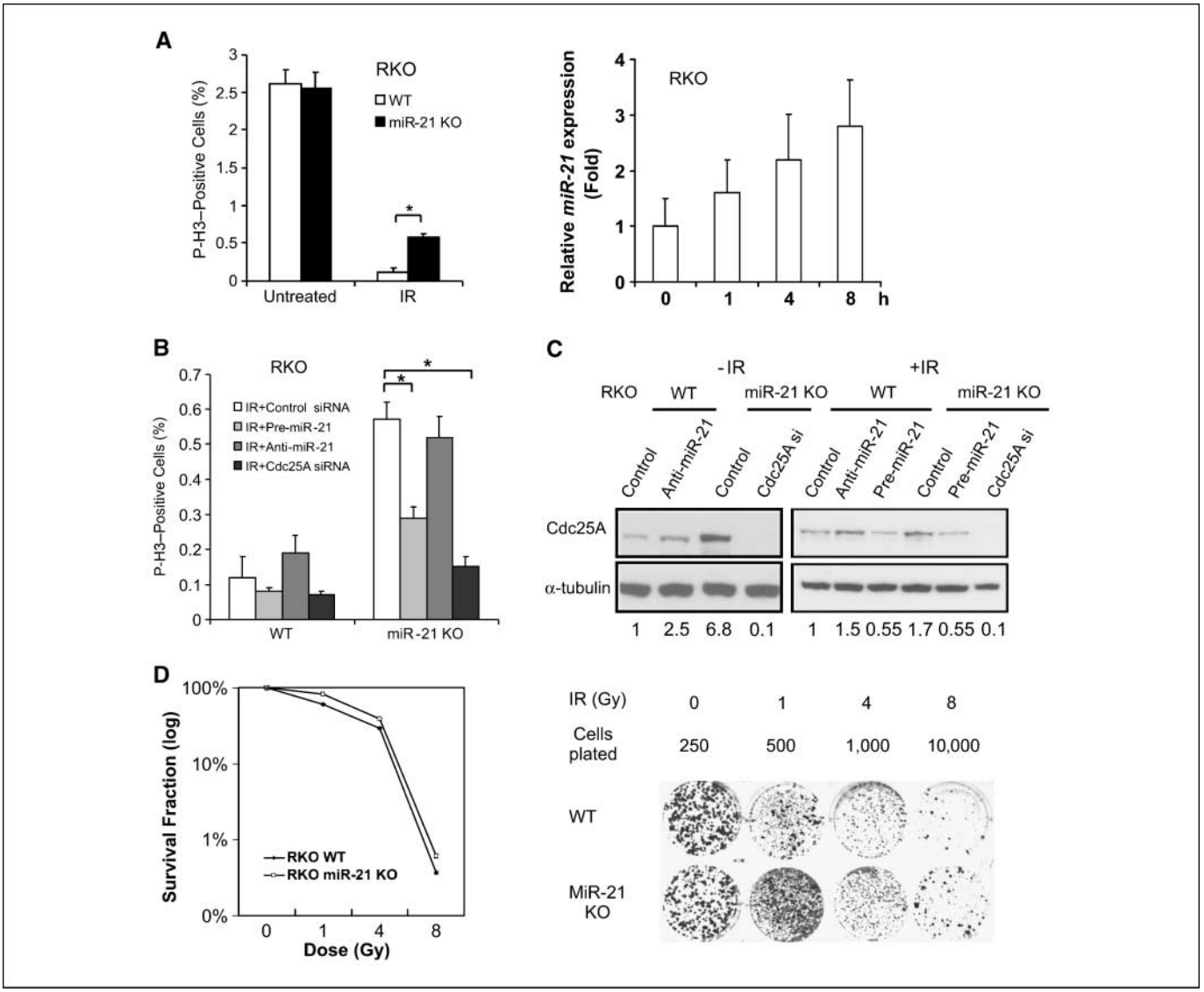

Figure 6. *miR-21* modulates irradiation-induced G_2-M checkpoint through Cdc25A. *A,* RKO cell lines were harvested at 1 h after 12 Gy irradiation (*IR*). Cells were stained with phosphorylated histone H3 (*P-H3*; Ser^{10}) antibody and the nuclei were counterstained by propidium iodide. The fractions of phosphorylated histone H3–positive cells were analyzed by flow cytometry and plotted. Mean ± SD ($n = 3$). *, $P < 0.05$. Levels of *miR-21* normalized to those of *U6* at indicated time points after irradiation were determined by real-time RT-PCR. Mean ± SD ($n = 3$). *B,* RKO cells were transfected with control, pre-*miR-21*, anti-*miR-21*, or *Cdc25A* siRNA twice in 48 h. Cells were replated overnight before irradiation at 12 Gy and analyzed as in *A*. Mean ± SD ($n = 3$). *, $P < 0.05$. *C,* levels of Cdc25A were analyzed in indicated cells following indicated treatments as in *B* by Western blotting and quantitated by densitometry. *D,* cells were irradiated at three doses of irradiation. Clonogenic survival was quantified using colony formation assay (*left*). Mean ± SD ($n = 3$). Representative pictures for colony enumeration are shown with the number of cells plated (*right*).

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Interestingly, the elevated Cdc25A levels in unstressed *miR-21* knockout cells do not appear to affect proliferation but profoundly affect cell cycle checkpoint and progression following stress (DNA damage or serum starvation), suggesting the importance of fully inactivating Cdc25A under these conditions. These conditional phenotypes associated with *miR-21* might be particularly relevant, as growth factor deprivation and DNA damage have been shown to play important roles in tumorigenesis (46). In addition, *Cdc25A* was recently found to be a target of *miR-16* that participates in UV-induced DNA damage response (42). Taken together, these observations suggest that critical cell cycle regulators such as Cdc25A are subject to modulation by miRNAs.

Our data provide a novel mechanism of how *miR-21* could potentially contribute to tumorigenesis by compromising cell cycle progression and DNA damage–induced checkpoint function under those conditions, which can lead to chromosomal instability that promotes tumorigenesis (47). The cell cycle is composed of highly regulated machinery; the precise coordination of a timely entry into and exit from various stages during normal cell cycle is crucial for maintaining normal cell division that entails faithful DNA replication and segregation. In addition, most, if not all, of the cells in the human body are constantly encountering endogenous or exogenous insults that can damage DNA, and proper activation of checkpoints and recovery from them is probably just as important in ensuring genome integrity. Altered expression of *miR-21* can conceivably cause genomic instability and lead to oncogenesis by relaxing or tightening this engine driving cell cycle through Cdc25A-dependent activation of cyclin/cyclin-dependent kinase complexes and may also affect therapeutic responses. Similar to *Chk2*-deficient cells (48), *miR-21* knockout cells exhibit compromised checkpoint and radioresistance. Given the complexity of the regulation of miRNA targets, much work remains to define and characterize *miR-21* targets to better understand its biology in different tissues and cancer. Therefore, future work will determine whether *miR-21* affects chromosomal stability following DNA damage and other aspects of tumor biology through novel targets.

Overexpression of Cdc25A and Cdc25B is correlated with more aggressive disease and poor prognosis in some cancer patients (19). The reasons for Cdc25A overexpression are still not clear. Our data offer reduced *miR-21* expression as a plausible explanation of Cdc25A overexpression in perhaps a subset of colon cancers. Other factors such as overexpression of c-Myc and E2F or inactivation of glycogen synthase kinase-3β are likely to be involved (19, 49). It is established that Cdc25A activities are tightly regulated by multiple mechanisms during cell cycle, including inhibitory and activating phosphorylation, changes in intracellular localization, and interactions with other proteins (17). Given a central role of Cdc25A in regulating cell cycle progression, it is perhaps not surprising that additional mechanisms such as miR-16 (42) can fine-tune its activity or levels.

Lastly, *miR-21* appears to regulate a distinct set of genes and have a limited role in regulating anticancer drug–induced apoptosis in colon cancer cells. The discrepancies in targets identified by different groups are perhaps not surprising, as miRNAs are known to regulate targets in a tissue-specific and cell type–specific manner (6). It is also possible that some of these targets are primarily regulated by miR-21 at the level of translation. The *miR-21* targeted cells and the targeting vector established in this study should be very useful for further dissecting *miR-21* biology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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