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MicroRNA-21 negatively regulates Cdc25A and cell cycle progression in colon cancer cells

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Abstract

MicroRNAs are small non-coding 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 in 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′UTR. We found that *miR-21* is induced by serum starvation and DNA damage, negatively regulates G1-S transition, and participates in DNA damage-induced G2-M checkpoint through downregulation 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 demonstrated 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.

Keywords

MicroRNA-21; Cdc25A; cell cycle; DNA damage; colon cancer

Introduction

MicroRNAs(miRNAs) are evolutionarily conserved, 20–25 nucleotide long, non-coding RNAs which 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% or more 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).

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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 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*, *Spry 1* and *Spry 2* 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 (CDK) 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 G1-S and G2-M transitions by activating distinct cyclin/Cdk complexes (18,19). Moreover, timely inactivation of Cdc25A facilitates checkpoint activation upon 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 regulate G1-S transition, and participate in DNA damage checkpoint activation in response to γ-irradiation. 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 rAAV system as described (21–23) with minor modifications. Briefly, two homologous arms flanking the *miR-21* locus, which are 1.17 kb and 1.15 kb, respectively, along with the neomycin-resistant gene cassette (*Neo*), were inserted between two *NotI* sites in the AAV shuttle vector pAAV-MCS (Stratagene, La Jolla, CA) by a 4-way ligation reaction. Packaging of rAAV was performed by using the AAV Helper-Free System (Stratagene) according to the manufacturer's instructions. RKO and DLD1 cells were infected with rAAV 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 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 Lox P sites in a heterozygous clone with an adenovirus expressing Cre recombinase (Ad-Cre) (24). After the second round of gene

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targeting, *Neo* was excised by Ad-Cre infection again, and gene targeting was verified by genomic 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 Table S1.

Microarray analysis

Total RNA was isolated 48 h following transfection from cells cultured in T25 flasks. Microarray analysis was performed 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 American Type Cell Collection (ATCC, Manassas, VA) and cultured in McCoy's 5A modified media (Invitrogen, Carlsbad, CA) supplemented with 10% defined FBS (HyClone, Logan, UT), 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 G1-S transition, radiation-induced transient G2/M checkpoint and clonogenic survival are described in the supplemental material.

miRNA target prediction

The miRNA targets were predicted using the algorithms TargetScan (http://genes.mit.edu/tscan/targetscanS2005.html) and PicTar (http://pictar.bio.nyu.edu/cgi-bin/PicTar_vertebrate.cgi).

Isolation of microRNAs, Real-Time Polymerase Chain Reaction (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 the supplemental material.

Transfections

Transfection with 100 nM pre-*miR-21*, 100 nM anti-*miR-21* (Ambion) or 200 nM *Cdc25A* siRNA (Dharmacon, Lafayette, CO) was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. The target sequence for *Cdc25A* is GGAAAAUGAAGCCUUUGAG (27). Cells plated at 20–30% 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.

Luciferase reporter constructs

The reporter constructs containing the 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 previously described with a transfection control (28). All the experiments were performed in triplicate and repeated at least three times on different days. Details were described in the supplemental material (Table S2).

BrdU incorporation and mitotic index

BrdU incorporation was analyzed by microscopy or flow cytometry following staining with anti-BrdU, Alexa Fluor 488 conjugated antibody. Mitotic index was measured by

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phosphorylated histone 3 (H3) staining. Detailed methods are described in supplemental material.

Western blotting

Western blotting was performed as previously described (29). The antibodies used for Western blotting included those against Cdc25A, Cdc25C, Cdc2, Cyclin B1, Chk1 (Santa Cruz, Santa Cruz, CA), phospho-histone H3 (Millipore, Bellerica, MA), α-tubulin (EMD Biosciences, San Diego, CA), phospho-Cdc2 (Cell Signaling, Danvers, MA), Cdc25B (BD Biosciences, Sparks, MD), and β-Trcp (Invitrogen). Quantification of relative expression was determined by densitometry as described (30).

Statistical analysis

Statistical analysis was performed using GraphPad Prism IV software. P values were calculated by student's (t) test. P-values less than 0.05 were considered significant. The means +/− one standard deviation (SD) were 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 downregulating miRNA expression with antisense oligos (31), we knocked out the *miR-21* precursor sequence in RKO and DLD1 colorectal cancer cells, using the recombinant adeno-associated virus (rAAV) system (Fig. 1A) (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. 1B, S1A). RT-PCR and Northern blot confirmed that the mature *miR-21* was not expressed in these knockout clones (Figs. 1C 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* KO cells, we performed microarray analysis on RKO parental cells transfected with pre-*miR-21* and *miR-21* KO cells transfected with a control siRNA for 48 hours. Over a hundred candidates showed at least a 2-fold increase in their expression in *miR-21* KO cells (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 (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* KO RKO cells. Consistent with the results obtained in microarray analysis, Cdc25A levels were significantly up-regulated in *miR-21* KO cells (Fig. 2A and B). Transfection of precursor (pre)-*miR-21* decreased the levels of Cdc25A transcript and protein (Figs. 2C, D, and S1B and S3). These findings were confirmed in DLD1 parental and *miR-21* KO cells (Fig. S3A–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 Beta-TrCP (Fig. S4A). Transient transfection of anti-*miR-21* also elevated Cdc25A expression in RKO cells as did *miR-21* targeting (Fig. 2D, right panel). To examine the expression of *miR-21* in

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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 from 2–7 fold) (Fig. 3).

Cdc25A is a *miR-21* target

Upon a closer inspection, a putative *miR-21* binding site located in the 3′ untranslated region (3′UTR) of *Cdc25A* gene was predicted by two algorithms (TargetScan and PicTar) (Fig. 4A). Importantly, this putative miR-21 binding site is 100% conserved in five species in the region that pairs with the seed sequence (Fig. 4A). The 3′-UTR region of *Cdc25A* containing this site was cloned into pMIR-REPORT™ miRNA reporter vector. The luciferase activities of this reporter in *miR-21* KO cells were about 60% higher than that in parental RKO cells, but were suppressed by pre-*miR-21* transfection (Fig. 4B), 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* KO RKO cells (Fig. 4). Transfection of pre-*miR-21* did not decrease the activities of the mutant reporter in either parental or *miR-21* KO cells (Fig. 4B), 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, Spry 1 and Spry 2 (Table S4 and Fig. S4B). Only *Spry 1* and *Spry 2* appear to be significantly upregulated (1.8- and 1.44-fold) in *miR-21* KO cells, but not the other 3 genes (Table S4 and Fig. S4B). 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 G1-S transition through Cdc25A

Cdc25A is an important regulator of cell cycle progression during G1–S transition (36,37). To evaluate whether *miR-21* affects cell cycle progression, we compared the growth rate of parental and *miR-21* KO 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* KO cells was indistinguishable under the normal serum condition in the entire 7 days (Fig. S4C). However, RKO *miR-21* KO cells exhibited enhanced proliferation over WT cells in the low serum condition (Fig. 5A, top panel). Under these conditions, no significant levels of apoptosis were detected in either WT or *miR-21* KO cells (data not shown). Using quantitative RT-PCR, we found that *miR-21* levels were induced 2–10 fold by serum starvation in WT cells within 24 hours (Fig. 5A, middle panel). Serum starvation also caused an apparent reduction in Cdc25A levels, which was significantly blunted in *miR-21 KO* cells (Fig. 5A, bottom panel). The induction of *miR-21* in parental cells, and elevated Cdc25A and enhanced proliferation in *miR-21* KO cells were also observed following complete serum starvation (0% serum) (data now shown).

Since serum has been well documented to stimulate the G1-S transition, we therefore specifically evaluated a potential role of *miR-21* in this process (17,27,38). Parental and *miR-21* KO RKO cells were serum starved for 48 hours and subsequently stimulated with 10% fetal calf serum. 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* KO cells compared with parental RKO cells. The effects were most pronounced at 15 (17 % *vs*. 7%) and 16 (16% *vs*. 10%) hours, and gradually diminished (Fig. 5B). BrdU staining indicated increased DNA synthesis in *miR-21* KO cells compared to parental cells (Figs. 5C and S5A). Consistent with an accelerated entry into S phase, high levels of Cdc25A were detected in *miR-21* KO cells as early as 15 hours (Fig. S5B). Transfection of pre-*miR-21* significantly reduced DNA synthesis and Cdc25A levels in *miR-21* KO cells (Fig. 5D). No significant difference in the cell cycle distribution was found between WT or *miR-21* KO RKO or DLD1 cells growing in

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log phase with 10% serum (Fig. S5C). Taken together, these results indicate that *miR-21* induction inhibits the G1-S transition by suppressing Cdc25A expression.

miR-21 modulates DNA damage-induced G2/M checkpoint through Cdc25A

Cdc25A has been shown to regulate the G2-M transition and its inactivation is critically involved in establishing a G2/M checkpoint following γ-irradiation (17,27,38). We tested whether *miR-21* is involved in establishing a transient G2/M checkpoint 1 hour following irradiation by analyzing phospho-histone-H3 (p-H3) positive cells (39). As expected, radiation (12 Gy) induced an over 90% drop in mitotic cells, which was significantly inhibited in *miR-21* knockout RKO cells (Fig 6A, S6A). Using quantitative RT-PCR, we found that *miR-21* levels were induced in parental RKO cells within one hour of γ-irradiation (Fig. 6A, right panel). *miR-21* KO DLD1 cells also exhibited a defective G2/M checkpoint associated with elevated levels of Cdc25A (Fig. S6B and S6C). Transfection of pre-*miR-21* or *Cdc25A* siRNA significantly suppressed the fraction of mitotic cells in both WT and *miR-21* KO cells, but the inhibition was more pronounced in *miR-21* KO cells (Fig. 6B). Transfection of anti-*miR-21* only elevated the fraction of mitotic cells in WT cells but did not further elevated that in *miR-21* KO cells (Fig. 6B). Cdc25A levels rapidly decreased following radiation in both parental and KO cells, but were substantially higher in *miR-21* KO cells (Fig. 6C). As expected, transfection of pre-*miR-21* or *Cdc25A* siRNA decreased the levels of Cdc25A while *anti-miR-21* elevated those in WT cells (Fig. 6C).

To determine whether *miR-21* affects radiosensitivity, we evaluated the clonogenic survival of parental and *miR-21* KO RKO cells. *miR-21* KO RKO cells were found to have increased clonogenic survival following several doses of irradiation (Fig. 6D). *miR-21* has previously been reported to 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 (Fig. S4D and 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 downregulation *of* Cdc25A contributes to the activation of the G2/M checkpoint following radiation.

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* KO RKO and DLD1 cells that 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 G1-S transition in *miR-21* KO cells; and compromised G2/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 (5-FU) in colon cancer cells (41), and by UV irradiation in primary fibroblasts (42) or in colon cancer cells (Fig. S6D). Cdc25A contains a large number of phosphorylation sites recognized by CDK1, Chk1/Chk2, and p38 (17,19). However, extensive effort in the mapping of phosphorylation sites in Cdc25Aand the use of cells deficient in Chk2 or ATM indicate that many such sites are not required for Cdc25A-mediated G2/M checkpoint following DNA

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damage (20,43–45). Our data suggest that *miR-21*-mediated Cdc25A downregulation facilitates the rapid establishment of the G2/M checkpoint following DNA damage. Interestingly, the elevated Cdc25A levels in unstressed *miR-21* KO cells do not appear to affect proliferation, but profoundly affect cell cycle checkpoint and progression following stress (ie. DNA damage or serum starvation), suggesting the importance of fully inactivating Cdc25Aunder 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 microRNAs.

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/CDK complexes, and may also impact therapeutic responses. Similar to *Chk2*-deficient cells (48), miR-21 KO 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-3 beta (GSK-3 beta) are likey 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- 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.

Supplementary Material

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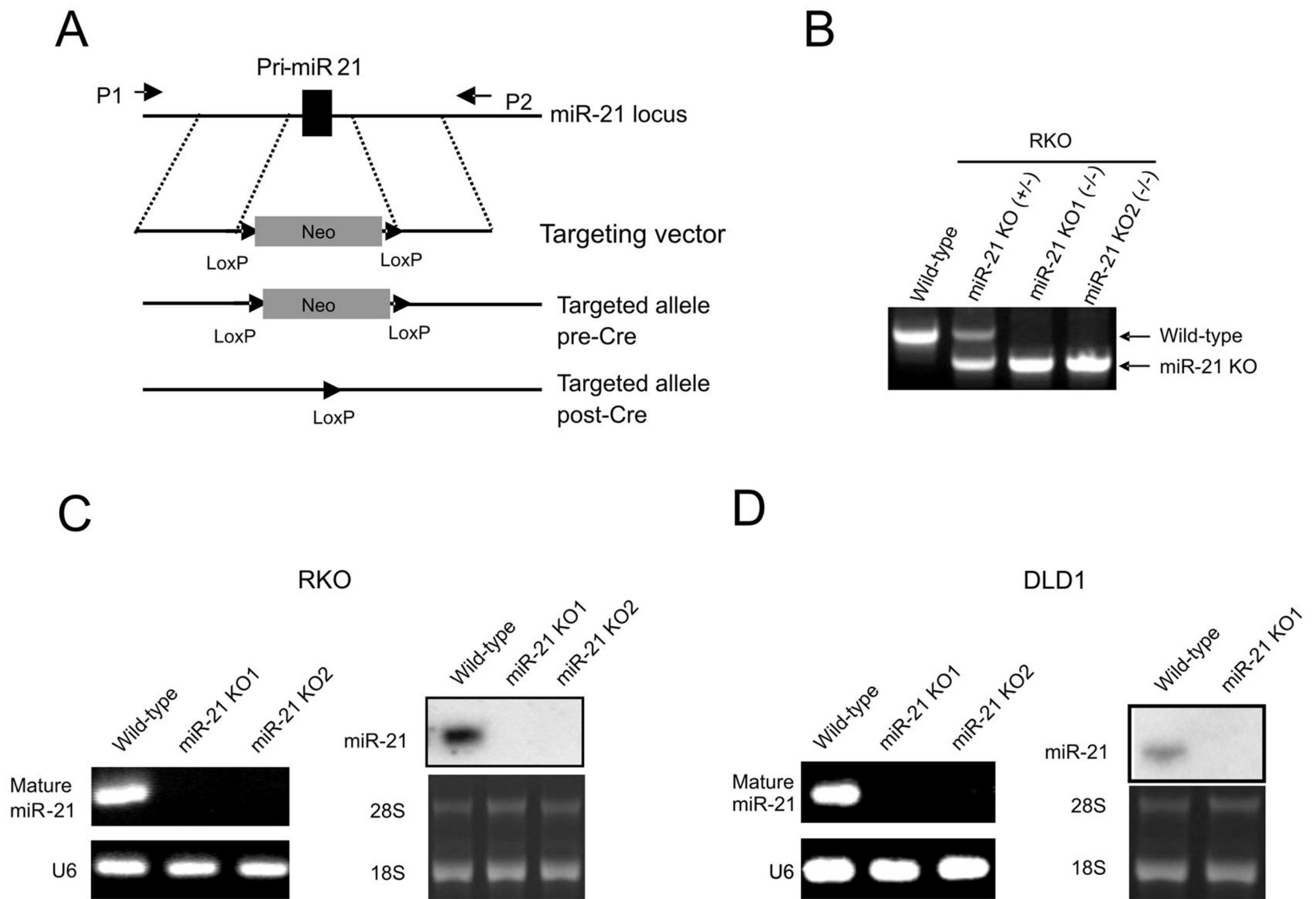

Figure 1. Targeted deletion of the primary *miR-21* locus in RKO and DLD1 colon cancer cells
A. Schematic diagram of *miR-21* targeting strategy. The targeting construct consists of two homologous arms and the neomycin-resistance gene (*Neo*) flanked by 2 LoxP sites. Homologous recombination resulted in a deletion of 750 base pairs, 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 were indicated. **B.** Identification of *miR-21*-KO clones by genomic PCR. **C.** Mature *miR-21* expression was measured by RT-PCR (left panel) or northern blot (right panel) in indicated RKO cell lines. **D.** Mature *miR-21* expression was measured as in **C** in indicated DLD1 cell lines.

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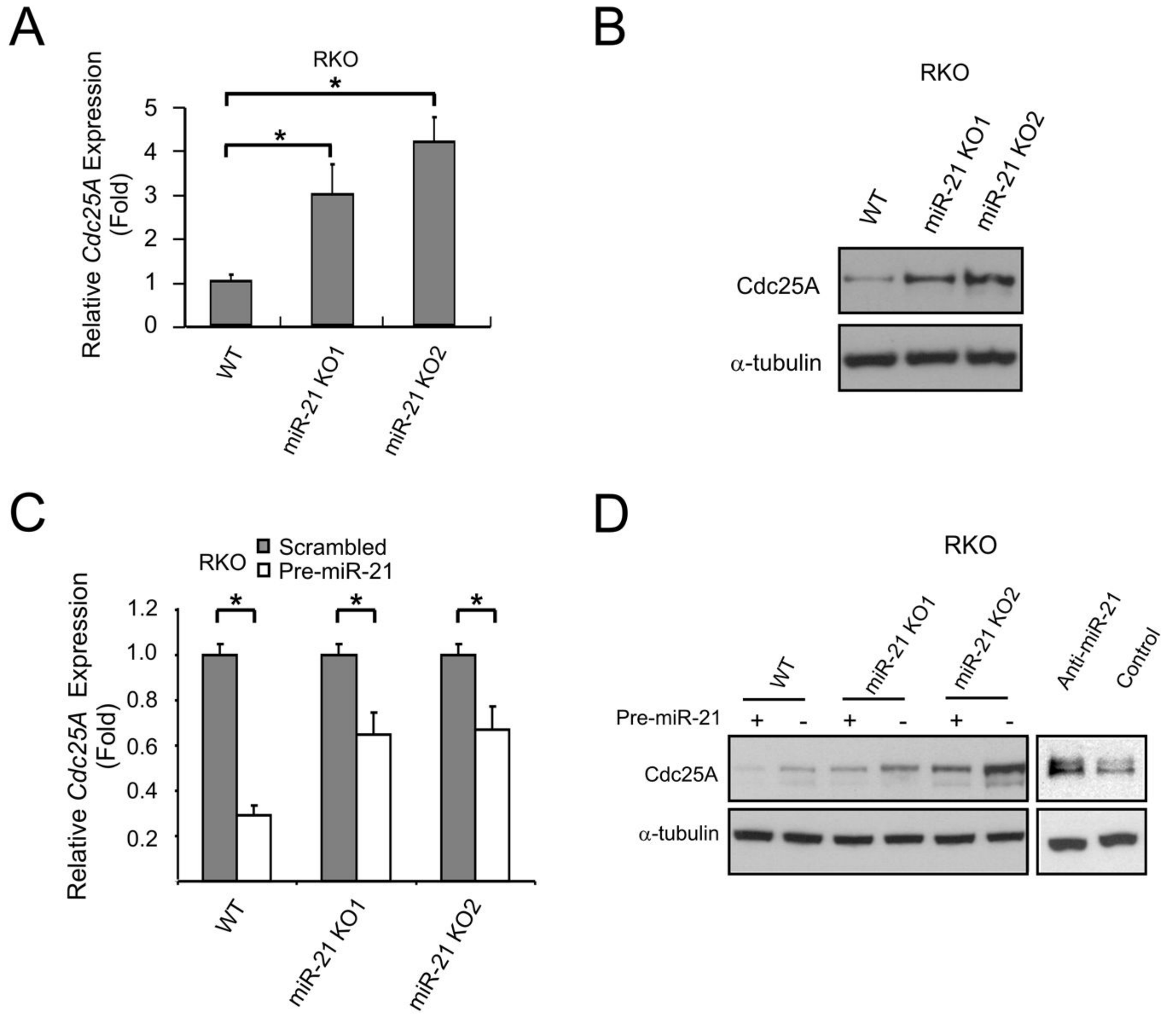

Figure 2. *Cdc25A* is regulated by *miR-21* in colon cancer cells

A. Relative *Cdc25A* mRNA expression was measured by real-time RT-PCR in RKO wild-type (WT) and *miR-21* knockout (KO) cells. Levels were standardized to *Cdc25A* mRNA in the WT cells normalized to *GAPDH*. Values are means ± SD, n=3. *P<0.02. **B.** Expression of Cdc25A protein was determined by Western blotting. **C**. The effect of precursor *miR-21* on *CDC25A* mRNA levels. Cells were transfected with precursor *miR-21* or control siRNA for 48 hrs and analyzed for *CDC25A* expression by RT-PCR. Values are means ± SD, n=3. *P<0.04. Expression levels were normalized to those in control siRNA transfected cells. The values are mean of three experiments +/− SD. **D.** The effects of anti-*miR-21,* or pre-*miR-21* on Cdc25A expression. RKO cells were transfected with control siRNA, anti-*miR-21*, pre-*miR-21*. Cdc25A levels were analyzed by Western blotting at 48 h after transfection.

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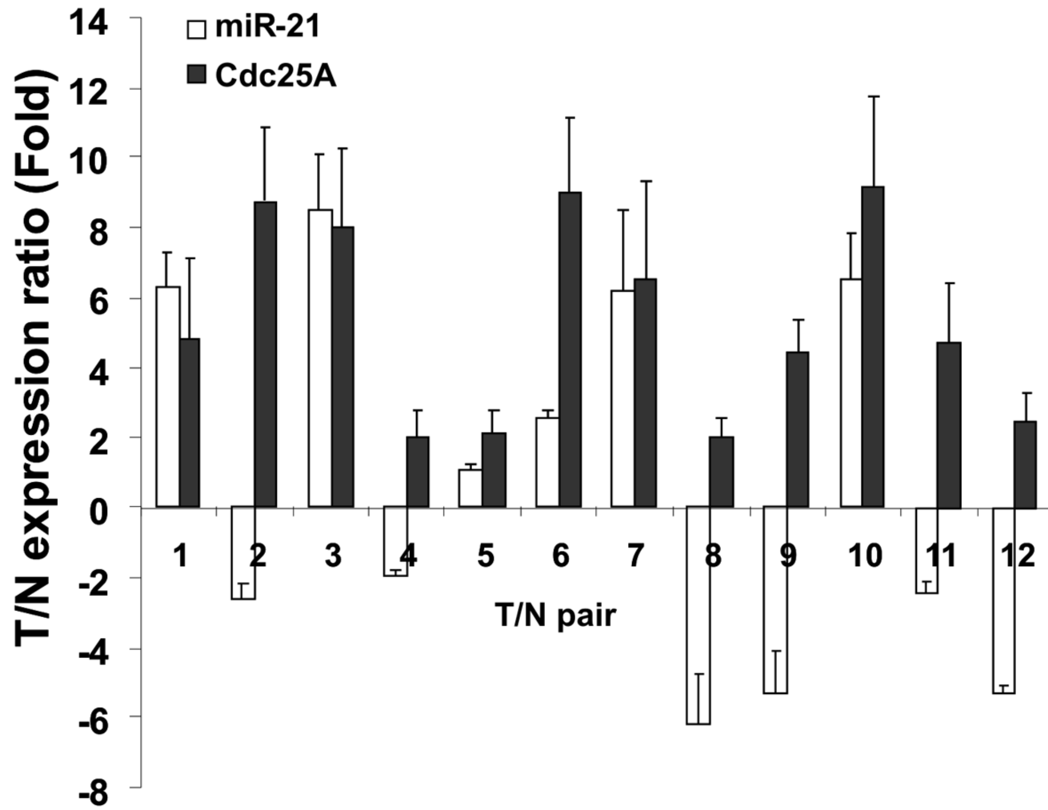

Figure 3. *The miR-21* levels in *Cdc25A* overexpressing colon cancers

The levels of *miR-21* were analyzed by Real-Time RT-PCR in 12 pairs of matched adjacent normal and tumor tissues that overexpress *Cdc25A*. The *miR-21* levels were normalized to that of U6. The *Cdc25A* levels were normalized to that of *GAPDH*. Values are means ± SD, n=3.

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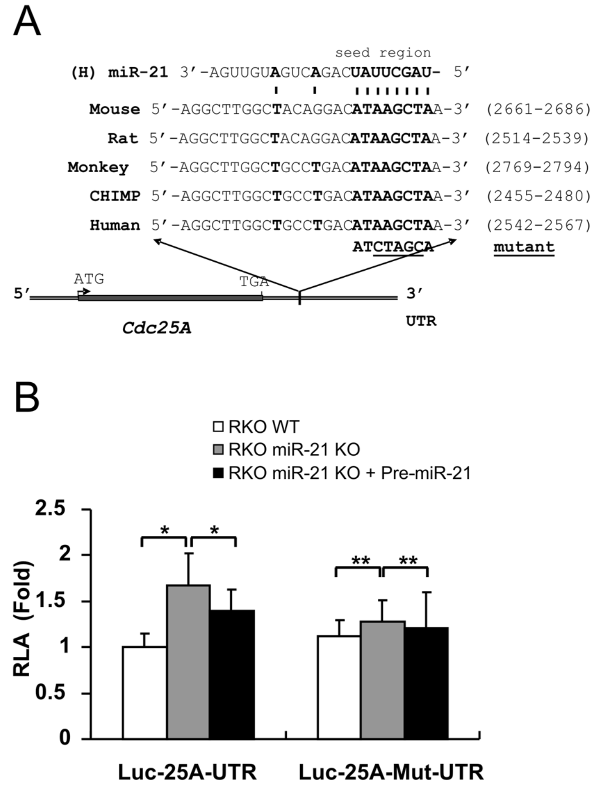

Figure 4. The conserved *miR-21* binding site in the 3′UTR of *Cdc25A*.

A. Schematic representation of *CDC25A* transcript with its 3′ UTR. The predicted *miR-21* binding sites in the *Cdc25A* gene of 5 species are shown with *miR-21* targeting sequences aligned (GenBank accession numbers are 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* KO cells with or without precursor *miR-21* transfection. Values are means ± 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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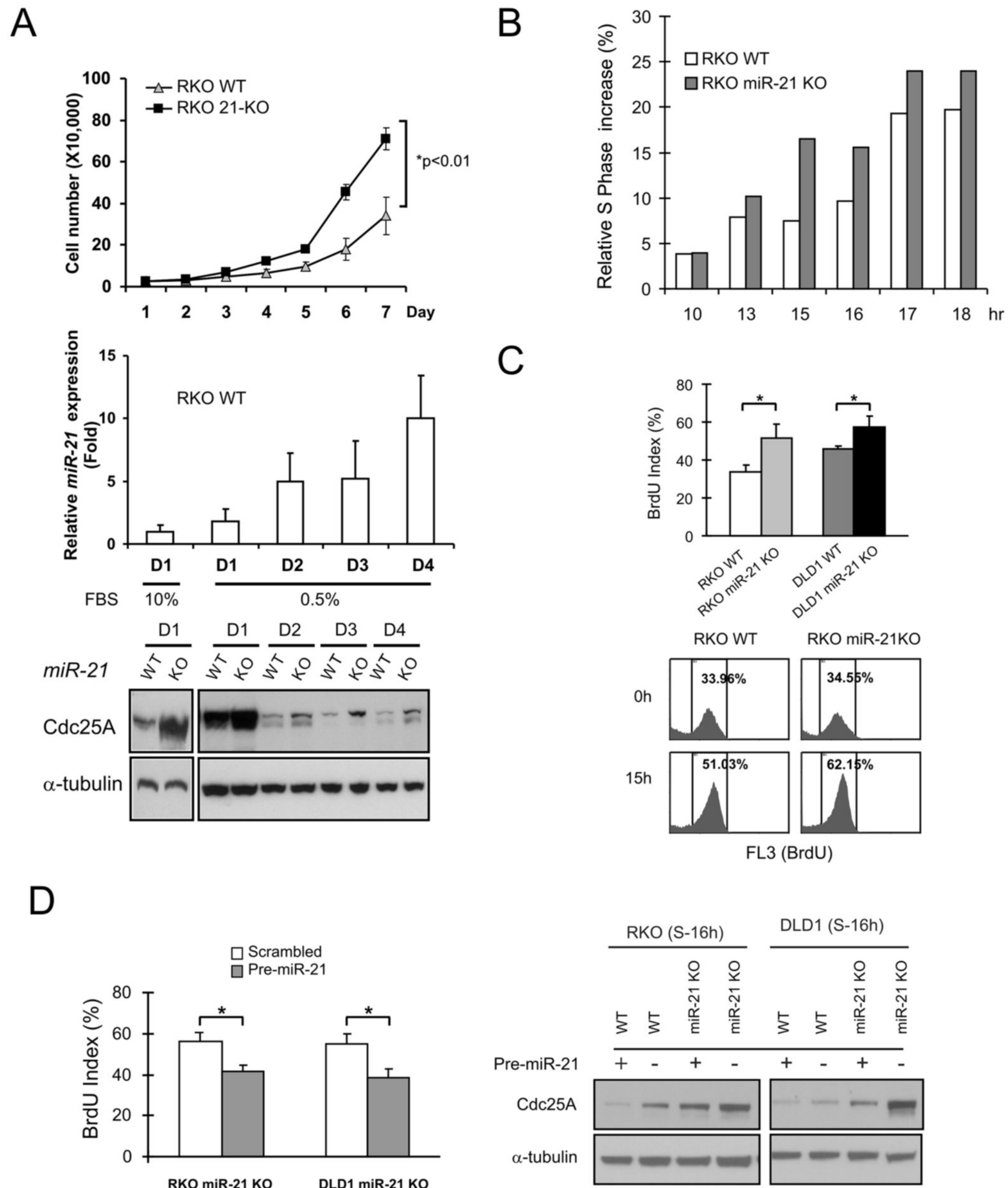

Figure 5. *miR-21* modulates cell proliferation under low serum conditions and G1-S transition through Cdc25A

A. The indicated cells were cultured in medium containing 0.5% serum medium for 7 days. The cell numbers were determined by counting. The levels of *miR-21* and Cdc25A were determined by realtime RT-PCR and Western blotting, respectively. The levels of mature *miR-21* expression were normalized to those of *U6*. The cells cultured in 10% serum for one day were used as controls. Values are means ± SD, n=3. **B**. The indicated RKO cells were cultured in serum free medium for 48 h and then stimulated with 10% fetal calf serum. Cell cycle analysis was performed using flow cytometry. The increases in the fraction of S-phase cells at the indicated time points compared with 0 h were quantified. **C.** BrdU incorporation in parental and *miR-21* KO cells with or without precursor *miR-21* transfection. Cells were subjected to treatment as in **B** and pulse labeled with BrdU for 15 minutes. BrdU/DAPI staining was performed at 15 h following 10% serum stimulation. The percentage of BrdU positive cells were scored by fluorescence microscopy (top panel). Values are means ± SD, n=3.

*P<0.05. BrdU incorporation was also determined by flow cytometry (low panel). **D.** The effects of pre-*miR-21* on BrdU incorporation were analyzed as in **C**. Values are means ± SD, n=3. *P<0.05. The effects of Pre-*miR-21* on Cdc25A levels at 16 h following serum stimulation were analyzed by Western blotting in the indicated cell lines.

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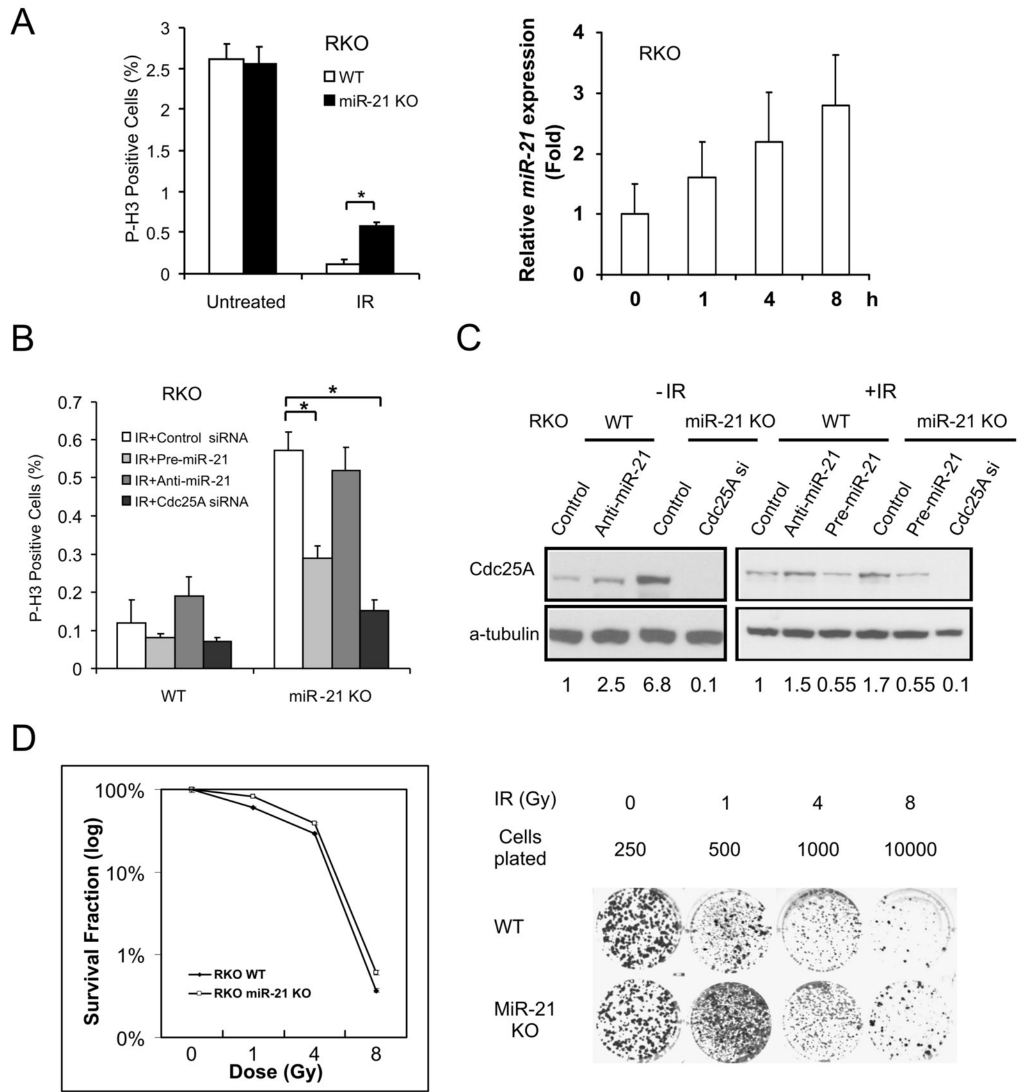

Figure 6. *miR-21* modulates irradiation-induced G2/M checkpoint through Cdc25A

A. The RKO cell lines were harvested at 1 h after 12 Gy irradiation (IR). The cells were stained with phospho-histone H3 (Ser10) antibody and the nuclei were counterstained by propidium iodide (PI). The fractions of p-H3 positive cells were analyzed by flow cytometry and plotted. Values are means ± SD, n=3. *P<0.05. The levels of *miR-21* normalized to those of *U6* at indicated time points after IR were determined by realtime RT-PCR. Values are means ± SD, n=3. **B.** The indicated RKO cells were transfected with control, pre-*miR-21*, anti-*miR-21* or *Cdc25A* siRNA twice in 48 h. The cells were replated overnight prior to irradiation at 12Gy, and analyzed as in **A.** Values are means ± SD, n=3. *P<0.05. **C.** The levels of Cdc25A were analyzed in indicated cells following indicated treatments as in **B** by Western blotting, and quantitated by densitometry. **D.** The indicated cells were irradiated at three doses of IR. The clonogenic survival was quantified using colony formation assay (left panel). Values are means ± SD, n=3. Representative pictures for colony enumeration are shown in the right panel with the number of cells plated noted.