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MicroRNA-Mediated Processes are Essential for the Cellular Radiation Response

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A detailed understanding of the mechanisms that determine the variable cellular sensitivity to radiation is needed for improved radiation therapy as well as for the identification of individuals with innate radiation hypersensitivity. MicroRNAs (miRNAs) are a class of small non-coding RNAs that post-transcriptionally regulate protein expression. Alterations in miRNA expression patterns in response to ionizing radiation have been shown, but there are almost no data describing the functional impact of these miRNA changes. We report here the results of studies on the functional roles of miRNAs in the radiation response in immortalized and primary endothelial cells. Global suppression of miRNA expression was achieved through downregulation of Argonaut e-2 (AGO2) or DICER proteins using RNAi. The reductions in either DICER or AGO2 led to increased cell death after irradiation, indicating a prosurvival function of miRNAs. Furthermore, while cell cycle checkpoint activation and apoptosis were compromised, DNA double-strand break repair was not affected by the lack of miRNAs. The differential sensitivity of these pathways implies the independent activation of the two response pathways rather than a concerted DNA damage response. The miRNAs that were changed after 2.5 Gy irradiation were identified by TaqMan-based low-density array technology. Of the miRNAs showing an upregulation 4 h or 24 h after radiation exposure, we were able to establish prosurvival and antiapoptotic functions for three miRNAs. Taken together, our data indicate a general prosurvival role for miRNA-mediated gene regulation during the radiation response. We show a functional association between miRNAs, apoptosis and cell cycle checkpoint activation in irradiated cells.

INTRODUCTION

MicroRNAs (miRNAs) are important components in the post-transcriptional regulation of gene expression. Their actions include repression of protein synthesis and induction of targeted mRNA degradation. MiRNA-mediated regulation is implicated in various processes, including cellular homeostasis and stress responses (*1, 2*). Functionally, it has been shown that one miRNA may regulate the levels of up to 100–200 target transcripts, mostly by imperfect miRNA binding to the 3′-UTR of target mRNAs. It has been suggested that most human genes are regulated by at least one miRNA (*3, 4*). Aberrantly expressed miRNAs have been connected with diverse diseases, including cancer (*5, 6*).

MiRNAs are small non-coding RNA molecules (21–23 nt in length) that are transcribed by RNA polymerase II initially as longer primary transcripts (pri-miRNA). They are subsequently processed in a series of endonucleolytic reactions into mature miRNAs that are capable of selectively binding their target mRNAs. Proteins involved in the processes of miRNA-induced gene silencing include DICER and AGO2 (Argonaute-2). DICER is an RNase III endonuclease containing a helicase domain and an RNA binding domain. DICER acts in the endonucleolytic processing of miRNAs by cleaving their loop to release a duplex RNA stem (*7*). This duplex RNA is incorporated into the RNA-induced silencing complex (RISC), a multi-protein complex that also contains the Argonaute proteins (AGO1 to 4 in humans). Here one strand of the duplex RNA becomes the mature miRNA, while the other one is degraded by the endonucleolytic activity of the AGO2 protein. The RISC complex is guided to the target mRNA molecules by the mature miRNA and causes gene silencing (*8*).

Ionizing radiation induces a wide variety of damage to DNA, proteins and lipids (*9*). To counteract this damage, cells activate a complex response network comprising multiple pathways (*10, 11*). Therefore, the most effective molecules in regulating the DNA damage response are those that can rapidly regulate a large network of genes. MiRNA activity is also implicated in the cellular response to ionizing radiation (*12, 13*), and alteration of cellular radiation sensitivity can be triggered through the manipulation of a

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single miRNA species (*14, 15*). However, the processes affected by miRNA in the radiation response remain to be elucidated. Therefore, the present study was performed to investigate the influence of miRNA-mediated gene regulation on radiation response mechanisms. We tested the broad importance of miRNAs for radiation response by targeted knockdown of AGO2 and DICER proteins. Functional consequences of the loss of miRNA processing on the radiation response were analyzed, including cell cycle checkpoint activation, DNA double-strand break repair and apoptosis. A specific set of miRNAs showing altered expression levels after irradiation were identified and studied individually to determine their importance for radiation resistance.

MATERIALS AND METHODS

Cell Culture, Transfection and Irradiation

The endothelium-derived cell line EA.hy926, initially described by Edgell *et al*., was used as a model system (*16*). The cells were cultivated in DMEM (Dulbecco's modified Eagle's medium), which was supplemented with 10% fetal calf serum and HAT supplement (5 m*M* hypoxanthine, 20 μ*M* aminopterin and 0.8 m*M* thymidine) as described previously (*16*). Cells were incubated at 37°C in a humidified atmosphere containing 95% air/5% CO_2. Pooled human umbilical vein endothelial cells (HUVECs) were purchased from Provitro (Germany). The cells were cultivated in DMEM-F12 medium supplemented with 2% fetal calf serum, 0.4% ECGS/H, 0.1 ng/ml epidermal growth factor, 1 ng/ml basic fibroblast growth factor, and 1 μg/ml hydrocortisone. Cells were used up to passage 10.

For transfection with small RNA molecules, 2×10^5 cells were seeded into 60-mm culture plates containing 3 ml medium (full DMEM for EA.hy926 cells and full DMEM-F12 medium for HUVECs). Twenty-four hours later, 10 n*M* of either siDICER, siAGO2 (Qiagen) or a scrambled control siRNA (siControl) (Qiagen) was transfected per well using RNAi Max Transfection reagent (Invitrogen) according to the manufacturer's instructions. The sequences of siRNAs used were: siAGO-2: ACGGGUCUGUGGUGAUAAATT; siDICER: UUUAUCACCACAGACCCGUAT. To transfect an miRNA inhibitor (inh-146a, inh-146b-5p, inh-301b, inh-518b, inh-518d-5p, inh-525-3p and inh-628-5p) (Exiqon) or an unspecific control inhibitor (inh-control) (Exiqon) cells were prepared as above. For each transfection, 100 pmol miRNA inhibitor or control inhibitor and 5 μl RNAi Max Transfection reagent were diluted with 250 μl serum-free medium and preincubated at room temperature for 5 min. The diluted miRNA inhibitors and diluted RNAi Max Transfection reagent were then combined, incubated at room temperature for 20 min, and added to the transfection plates. After 6 h the medium was changed, and measurements were performed 48 h after transfection.

Gamma irradiation at the indicated doses was performed on exponentially growing cells using a ^{137}Cs source (HWM-D 2000, Waelischmüller, Germany) at a dose rate of 0.53 Gy/min.

Protein Analysis

Cells were lysed in RIPA buffer (150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% desoxycholate, 5 m*M* EDTA) supplemented with the 1 m*M* protease inhibitors sodium orthovanadate and phenylmethanesulfonyl fluoride (Roche) for 10 min on ice. After centrifugation the collected supernatants were subjected to SDS polyacrylamide gel electrophoresis, blotted on nitrocellulose membranes, and incubated with primary antibodies directed against either AGO2 (Cell Signaling Technology), DICER1 (Cell Signaling Technology), p53 (Santa Cruz), p21 (Cell Signaling Technology) or phospho-CHK-2 (Thr68) (Cell Signaling Technology). To monitor protein loading, the membranes were stripped and reincubated with anti-actin (Santa Cruz). HRP (horseradish peroxidase)-conjugated anti-mouse or anti-rabbit antibodies (Santa Cruz) were used to detect binding of primary antibodies. The chemoluminescence reaction and detection of the signals were performed using an ECL detection kit (Amersham). Quantification of digitized signals was performed using TotalLab TL 100 software.

Cell Proliferation and Survival

Cell viability was determined with a colorimetric cell proliferation kit I (Roche). Twenty-four hours before treatment, 1000 to 2000 cells per well were seeded into 24-well plates. Five days after irradiation, 200 μl fresh growth medium and 20 μl MTT labeling reagent were added and the cells were incubated for another 4 h. After the addition of 200 μl prewarmed solubilization solution, the samples were incubated for another 24 h at 37°C, and absorbance was determined at 570 nm using a spectrophotometer (TECAN). For the measurement of clonogenic survival, transfected cells were reseeded at a range of densities (100-1000 cells per plate) and 24 h later γ irradiation was performed. After 7–10 days, the colony formation capacity was assayed after ethanol fixation and Giemsa staining. Cell proliferation was determined by viable cell counting 24, 48 and 72 h after seeding (using Trypan blue exclusion).

Cell Cycle Profiling

DNA staining of isolated nuclei for cell cycle analysis was performed using a modification of the method of Nüsse *et al*. (*17*). At each indicated time, the treated cells were trypsinized and collected by centrifugation at 300*g* for 5 min, and the supernatant was carefully removed. The cell pellet was gently resuspended in 300 μl of a solution containing 10 m*M* NaCl, 4 m*M* Na-citrate, 10 μg/ml RNase, 0.3% Nonidet P-40, and 50 μg/ml propidiumiodide (PI) and gently vortexed. The cell suspensions were incubated for 30 min at room temperature followed by the addition of 300 μl of solution containing 70 m*M* citric acid, 250 m*M* sucrose and 50 μg/ml PI. The cell suspensions were mixed and stored at 4°C before flow cytometry. Cell cycle distributions were analyzed on a FACScan LSR II (Becton-Dickinson) (excitation wavelength: 488 nm; emission wavelength: >610 nm, LSR II, Becton Dickinson/FACS DIVA Software).

Apoptosis

For the detection of apoptotic cells, cultures of exponentially growing cells were irradiated over a dose range of 0–10 Gy as described above. Cells were harvested 48 h after irradiation by trypsinization and washed in phosphate-buffered saline (PBS). The cells were stained as described for the cell cycle analysis and investigated by FACS analysis as above. Cells with a DNA content less than that of cells in the G_1 phase of the cell cycle were assigned to the sub-G_1 fraction and are considered to be apoptotic. For analyses of caspase-3 activity, the cells were washed with PBS and collected by trypsinization 24 h after γ irradiation. Cells were resuspended in 38 μl lysis buffer [25 m*M* Tris (pH 7.5), 120 m*M* $NaCl_2$ and 0.3% Triton X-100]. After 10 min incubation on ice, the cell lysates were clarified by centrifugation at 16,000*g* for 5 min at 4°C. For each sample, 80 μl of the assay buffer (2 m*M* in DMSO, Calbiochem) was incubated with 2 μl caspase-3 substrate Ac-DEVD-pNA (12.5 m*M* in DMSO, Calbiochem). After transfer of the buffer/substrate solution to a 96-well plate, 18-μl aliquots of cell lysate were added. Blank samples were prepared as a negative control. Absorbance at 405 nm was monitored at 37°C every 15 min for 5 h using a TECAN spectrophotometer.

Immunostaining and Confocal Microscopy

For γ-H2AX focus analysis, 3×10^5 cells were seeded on slides in 4-well chambers and transfected with siRNA. After treatment, the cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS for 5 min followed by blocking with 1% BSA for 60 min. The cells were probed with anti-γ-H2AX (Ser139) (dilution 1:250, Millipore) antibody overnight at 4°C followed by a 1-h incubation at room temperature in the dark with fluorescence-labeled secondary antibody [1:500, Cy-3 goat anti mouse IgG (Jackson ImmunoResearchLab)]. Nuclear counterstaining was performed with Hoechst 33342, and cover slips were mounted with Vectashield (Vector Laboratories). Images were obtained using an LSM510 and processed using LSM5 image software (Zeiss).

Profiling miRNA Expression

MiRNA profiling of exponentially growing adherent EA.hy926 cells or HUVECs was carried out 4 h and 24 h after a single irradiation with 2.5 Gy. The cells were trypsinized and pelleted by centrifugation at 300*g* for 5 min and washed with 1 ml PBS. The miRNAs were purified with a mirVana™ miRNA Isolation Kit (Ambion), according to the manufacturer's protocol. For the microarray studies, the quality and the concentration of the RNA samples were determined with an Infinite 200 NanoQuant (TECAN). The RNA quality for the microarray analysis ranged between 1.9 and 2.05 using an OD ratio of 260/280. Total RNA (450 ng per pool) was reverse transcribed using a Human Multiplex RT Set and TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). The 7.5-μl reaction volumes were incubated in a thermocycler for 40 cycles of 2 min at 16°C, 1 min at 42°C, and 1 min at 85°C followed by a 5-min step at 85°C and were then held at 4°C. Global profiling of miRNA expression of sham-irradiated and γ-irradiated EA.hy926 cells and HUVECs was performed using the TaqMan® Low Density Array A v.2 (TLDA) based on an Applied Biosystems 7900HT Micro Fluidic Card (Applied Biosystems). The TLDA card contained 384 lyophilized human miRNA sequences plus three small nucleolar RNAs (snoRNAs), *RNU6B*, *RNU48* and *RNU44*, used as endogenous controls. MiRNA analysis was done in triplicate at 4 h and 24 h after irradiation with 2.5 Gy. For real-time polymerase chain reaction (PCR), a standard TaqMan PCR kit protocol on a 7900HT Fast Real-Time PCR System (Applied Biosystems) was used. The reactions were performed at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were analyzed by using Sequence Detection System software (v. 2.3) (Applied Biosystems). The relative expression of each miRNA was normalized against the endogenous controls using the equation $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{irradiated} - \Delta Ct_{control}$ and $\Delta Ct = (Ct_{miRNA} - Ct_{snoRNAs})$ (*18*). To identify the miRNAs with robust changes in expression after exposure to ionizing radiation, we used relative change analysis and *P* values calculated by a Student's *t* test as significance criteria. As cutoffs, a deregulation of a factor ≥ 2 and *P* values < 0.01 were employed.

miRNA Target Transcript Prediction

We obtained miRNA target transcripts by using a consensus method of four different miRNA target prediction tools. Besides TargetScanS (*19*) and PicTar (*20*), we used predictions from Pita (*21*) and RNA22 (*22*). From all predictions, we considered miRNA-transcript relationships that were predicted by a minimum of two different tools. In addition, we performed a Gene Ontology (GO) (*23*) term enrichment analysis, which was carried out with the R package GOstats. To correct for multiple testing, we used the Benjamini-Hochberg procedure (*24*) and called an association significant if the *P* value was less than 0.05.

RESULTS

DICER and AGO2 are Both Required for the Cellular Radiation Response

To analyze the functional impact of miRNAs on the radiation response, we first inhibited global miRNA processing by downregulating the expression of DICER and AGO2. EA.hy926 cells were transfected with chemically synthesized siRNA oligonucleotides directed against either DICER or AGO2. As a control, the EA.hy926 cells were transfected with a scrambled siRNA. Western blot analysis showed that DICER and AGO2 were downregulated to less than 2% and 25% of their respective control levels (Fig. 1A).

DICER is critical for pre-miRNA processing, and AGO2 contributes to subsequent miRNA processing and stability. Thus, to validate the effect of the DICER and AGO2 knockdown on miRNAs, we quantified the amounts of miR-10b, miR-101, miR-518b and miR-523-5p, which are all abundantly expressed in untransfected EA.hy926 cells. Quantification of these miRNAs by real-time PCR using specific primers for the mature miRNAs revealed a strong reduction of all four miRNAs (Fig. 1B), indicating a global reduction of the cellular content of mature miRNAs after DICER and AGO2 depletion.

Then DICER and AGO2 knockdown cells were analyzed for their radiation sensitivity. Cells were irradiated with up to 5 Gy and cellular proliferation activity was determined as an indicator of survival 5 days after irradiation (Fig. 2B). Without irradiation the proliferation rates of DICER- or AGO2-depleted cells were similar to that of the controls (Fig. 2A), indicating that DICER or AGO2 knockdown had no effect on the overall proliferation capacity or on cellular viability. However, after irradiation, both AGO2 and DICER knockdown cells showed a reduced proliferation activity compared to that of the irradiated control transfected cells. To confirm the increased radiation sensitivity of AGO2- or DICER-depleted cells, we measured clonogenic survival. Here we also observed reduced survival capacities after irradiation in cells having downregulation of DICER or AGO2 but again not in irradiated control transfected cells (Fig. 2C). Thus our results showed that unimpaired miRNA expression is required for maintaining cellular survival after exposure to ionizing radiation.

Impaired Cell Cycle Checkpoint Activation and Increased Apoptosis in DICER- or AGO2-Depleted EA.hy926 Cells after Exposure to Ionizing Radiation

The role of miRNAs in radiation-induced cell cycle checkpoint activation, DNA double-strand break repair and apoptosis, which are critical processes in radiation response, was analyzed. Cell cycle distribution was monitored by propidium iodide staining of DNA content 48 h after irradiation (Fig. 3A, B). In the nonirradiated state, no differences in cell cycle distribution between the DICER

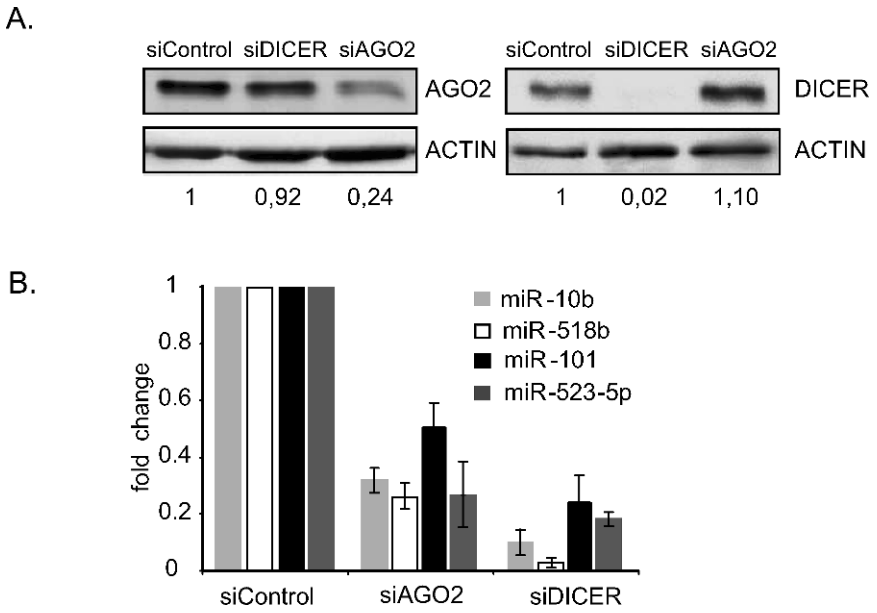

FIG. 1. SiRNA-mediated knockdown of DICER and AGO2 in EA.hy926 cells. Cells were harvested 48 h after transfection to determine the efficiency of siRNA-mediated downregulation of DICER and AGO2 expression. Panel A: DICER and AGO2 protein amounts were estimated by Western blotting of protein extracts derived from control siRNA (siControl)-, siDICER- and siAGO2-transfected cells. Detection of actin served as an internal loading control. The numbers below the images indicate the relative intensity of the corresponding band relative to actin. Panel B: Expression analysis of single miRNAs by real-time PCR in EA.hy926 cells transfected with control siRNA (siControl), siDICER or siAGO2. Results are mean values of three independent experiments ± SD. Fold change = relative change.

and AGO2 knockdown cells and the control transfected cells could be detected. With increasing radiation dose both AGO2 and DICER knockdown cells displayed an accumulation of cells in G_2/M phase accompanied by a reduction of cells in G_1. This suggested either an impairment of the G_1/S cell cycle checkpoint activation or a pronounced G_2/M block after DNA damage induction in the absence of miRNA processing. That the deficiency lies in the activation of the G_1/S checkpoint was established by the failure of the cells to accumulate the CDK inhibitor protein p21. Surprisingly, this was not due to impaired phosphorylation of CHK-2 at Thr68 or failure of the stabilization of p53 (Fig. 3D). Additionally, the cell cycle distribution after irradiation showed the appearance of significant sub-G_1 peaks in both irradiated AGO2 and DICER knockdown cells, reflecting ongoing radiation-induced apoptotic cell death. In control cells, 11% (5 Gy) and 18% (10 Gy) of the cells were present in the sub-G_1 phase, whereas in DICER or AGO2 knockdown cells 20–22% (5 Gy) and 32–36% (10 Gy) were in sub-G_1 phase, respectively (Fig. 4A). To confirm this result, we determined apoptotic activity by quantifying caspase-3 activity (Fig. 4B). Nonirradiated control cells and AGO2- or DICER-depleted cells showed similar basal caspase-3 activities. Twenty-four hours after irradiation, caspase-3 activity was increased in a dose-dependent manner in all cells, but a significantly stronger increase was evident in both AGO2 and DICER knockdown cells compared to controls (Fig. 4B). The possibility that the increased sensitivity to radiation in the absence of miRNA processing was due to differences in the initial DNA damage was excluded by monitoring DNA double-strand break induction and repair using the γ-H2AX focus assay (Fig. 3C). We did not observe any differences in the kinetics of γ-H2AX focus appearance and disappearance between controls and cells depleted for DICER or AGO2. Thus neither the induction nor subsequent repair of DNA double-strand breaks is inhibited by a lack of miRNAs. Together these results consistently demonstrate a prominent role of the miRNA processing machinery for normal cell cycle regulation and suppression of apoptosis after exposure to ionizing radiation.

MiRNA Profiling after Exposure to Ionizing Radiation

To identify which individual miRNAs are involved in the survival response after irradiation, we performed miRNA profiling in EA.hy926 cells. Out of 384 miRNAs studied, around 200 were detectable (Supplementary Fig. 1; http://dx.doi.org/10.4667/RR2638.1.S1). MiRNAs with altered expression were detected at both 4 h and 24 h after irradiation. Four hours after irradiation, 22 miRNAs showed a significant deregulation, whereby the expression of 14 miRNAs was decreased while 8 miRNAs were significantly increased (Fig. 5A). Twenty-four hours after irradiation, 18 miRNAs were downregulated, whereas 4 miRNAs were upregulated (Fig. 5B). The number of miRNAs downregulated after irradiation suggested that the

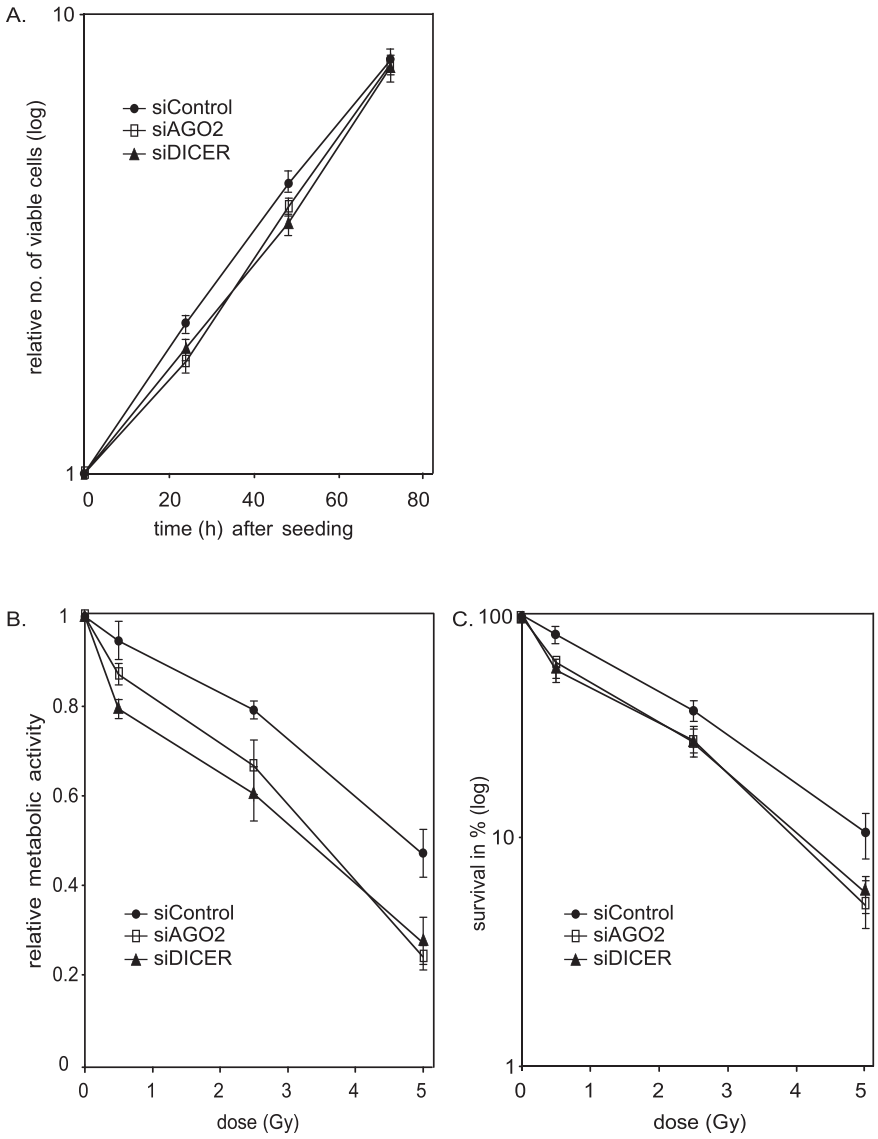

FIG. 2. Survival after irradiation is dependent on DICER and AGO2. Panel A: Growth characteristics of EA.hy926 cells after transfection with control siRNA (siControl), siDICER or siAGO2. The diagram represents the mean of two independent experiments. Proliferation activity (panel B) determined by the MTT assay 5 days after irradiation and clonogenic survival (panel C) at the indicated doses of EA.hy926 cells transfected with siControl, siDICER or siAGO2. The results are mean values of three independent experiments ± SD.

rapid upregulation of gene expression known to accompany irradiation (*25, 26*) may be achieved by downregulation of miRNAs and may represent an important mechanism in the cellular radiation response. Only 4 downregulated miRNAs (let-7d, miR-519e, miR-323-3p, miR-517b) and 1 upregulated miRNA (miR-518b) were changed at both 4 h and 24 h after irradiation, whereas all other identified miRNA changes were transient, appearing at either 4 h or 24 h. The noticeable differences between miRNAs that were altered after 4 h and 24 h suggested different time-dependent regulatory functions for miRNAs in the radiation response.

miR-216a, miR-518d-5p and miR-525-3p Contribute to Radiation Resistance

After identification of radiation-inducible miRNAs, our aim was to test the individual impact of each upregulated miRNA for the radiation response. Because of their high levels of induction after irradiation, miR-146a, miR-146b-5p, miR-216a, miR-518b, miR-518d-5p, miR-525-3p, miR-628-5p and miR-301b were chosen for further analysis. EA.hy926 cells were transiently transfected with specific chemically modified single-stranded miRNA inhibitors (anti-miRs) and their radiation sensitivity was tested. Control EA.hy926 cells were transfected with a nonspecific

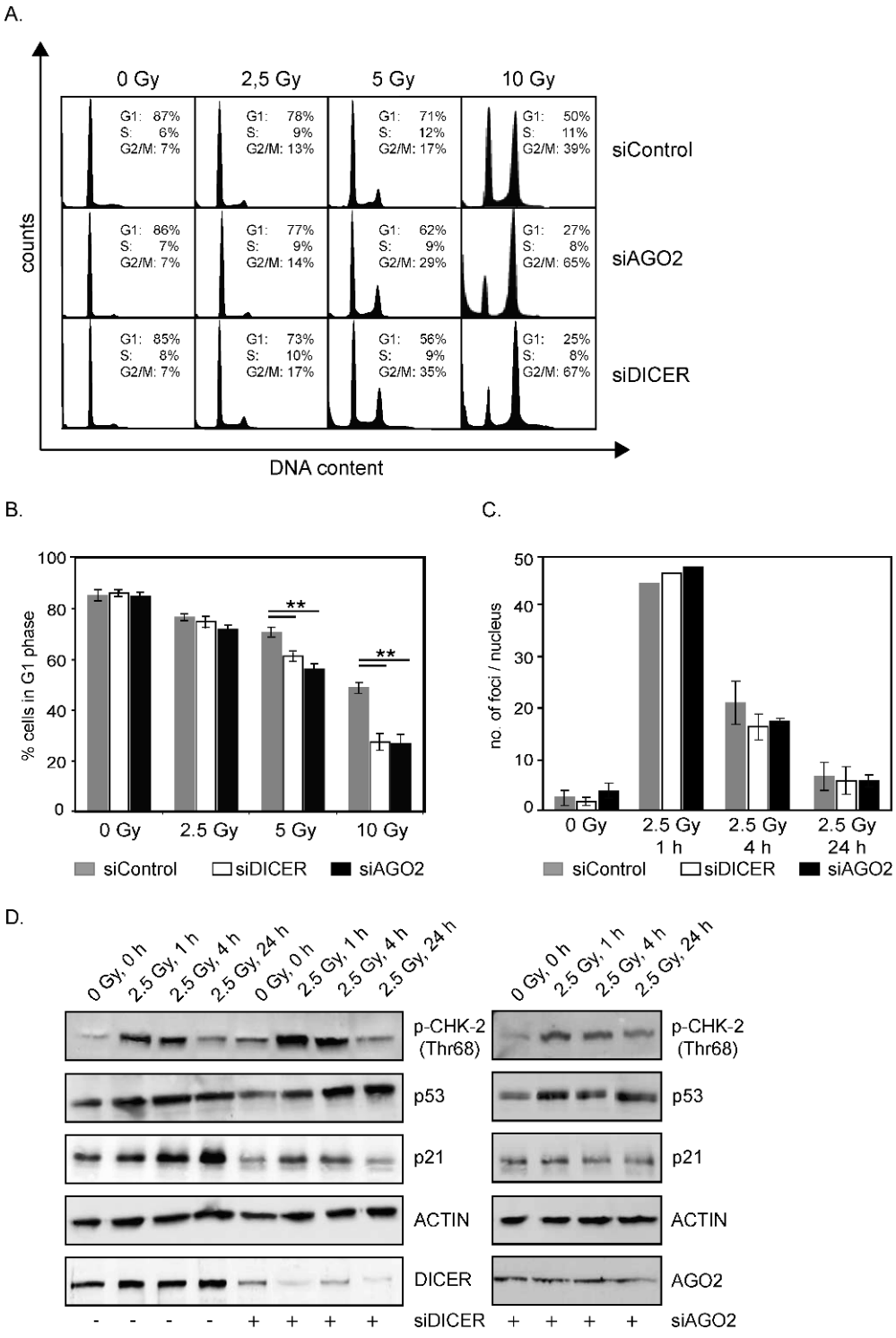

FIG. 3. Cell cycle progression and DNA double-strand break repair in DICER or AGO2 knockdown cells after irradiation. Panel A: Cell cycle distribution was evaluated in cells transfected with control siRNA (siControl), siDICER or siAGO2 by PI staining and flow cytometry 24 h after irradiation. Panel B: Quantification of cells in G_1 phase 24 h after irradiation with the indicated doses. Error bars show SD of three independent experiments; significance was determined by Student's *t* test: $*P < 0.01$, $**P < 0.005$. Panel C: Monitoring of DNA double-strand break repair by γ-H2AX foci counted at the indicated times after irradiation with 2.5 Gy. Panel D: Analysis of cell cycle relevant proteins p-CHK-2 (Thr68), p53 and p21 by Western blotting at the indicated times. Detection of ACTIN served as loading control. One representative blot out of three is shown.

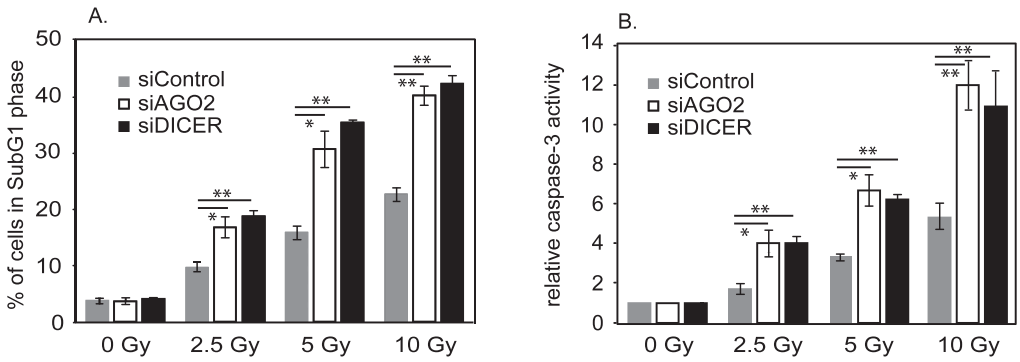

FIG. 4. Determination of radiation-induced apoptosis in DICER or AGO2 knockdown cells. Panel A: Forty-eight hours after irradiation, the apoptotic cell fraction was quantified by sub-G_1 analysis in control siRNA (siControl)-, siDICER- or siAGO2-transfected cells. Panel B: Increased apoptosis in siDICER- or siAGO2-transfected cells is accompanied by elevated caspase-3 activity. Caspase-3 activity was determined 24 h after irradiation. Means of three independent experiments ± SD are shown.

miRNA inhibitor (inh-control). The results for the inhibition of the individual miRNAs that were upregulated 4 h after irradiation are shown in Fig. 6A, while Fig. 6B shows the results of inhibiting those miRNAs deregulated at 24 h. For the five analyzed miRNAs from 4 h, the inhibition of miR-216a, miR-518d-5p and miR-525-3p led to an increased radiation sensitivity, while inhibition of miR-146a and miR-146b-5p did not significantly affect radiation sensitivity. The inhibition of the three miRNAs upregulated after 24 h had no effect on survival after irradiation. Cell cycle distribution and apoptosis were quantified 48 h after irradiation in cells with a repression of each candidate miRNA. Quantification of G_1- and G_2/M-phase populations in nonirradiated and irradiated cells resembled the results for control siRNA transfected EA.hy926 cells (Fig. 3A, B) and showed no differences between controls and inhibitor-transfected cell lines (data not shown). The sub-G_1 population was significantly increased after irradiation after inhibition of miR-216a, miR-518d-5p or miR-525-3p, indicating a higher amount of apoptotic cells after irradiation compared to irradiated control cells (scramble) (Fig. 6C). Increased radiation-induced apoptosis in the miRNA-blocked cells was confirmed by detection of caspase-3 activity (Fig. 6D). These results show a requirement for each of the investigated miRNAs, miR-216a, miR-518d-5p and miR-525-3p, for cell survival after irradiation by influencing radiation-induced apoptosis, while the cell cycle checkpoint activation was not affected. Consistent with these experimental data, miR-216a, miR-518d-5p and miR-525-3p were predicted to target several transcripts in apoptosis and stress response (Table 1).

MicroRNAs Mediate Radiation Response in Primary Cells

To validate our findings showing a prosurvival role of miRNAs in the radiation response, we repeated the global miRNA knockdown in primary human umbilical vein endothelial cells (HUVECs). Transfection of HUVECs with siRNA directed against DICER led to a reduction of the DICER protein to 38% of control transfected cells (Fig. 7A, inset). An efficient knockdown of AGO2 could not be achieved by the investigated siRNA (data not shown). Subsequently, DICER knockdown cells were analyzed for their radiation sensitivity. Cells were irradiated with 5 Gy, and cellular proliferation activity was determined as an indicator of survival 5 days after irradiation (Fig. 7A). Knockdown and control cells showed a similar proliferation activity in the absence of irradiation (data not shown). However, as demonstrated in the immortalized endothelial cell line EA.hy926, irradiated DICER knockdown HUVECs

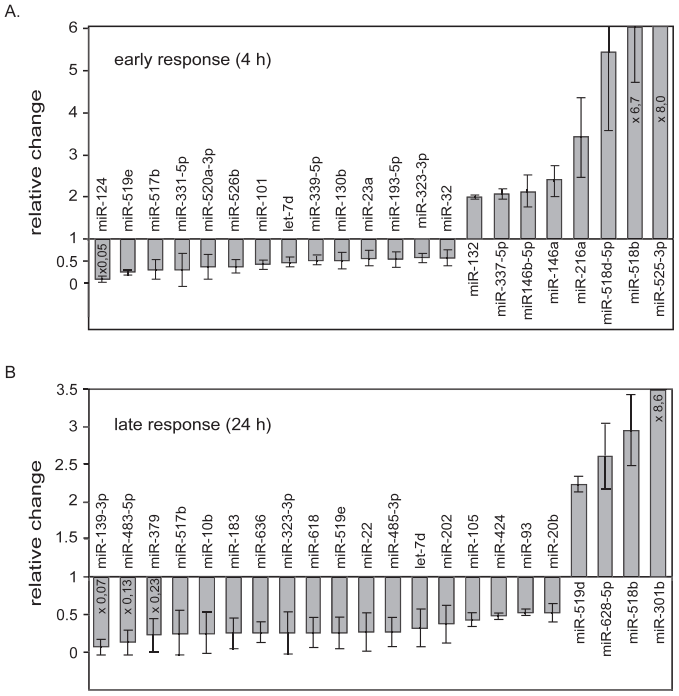

FIG. 5. MiRNA expression profiling in EA.hy926 cells after exposure to ionizing radiation. Differentially expressed miRNAs 4 h (panel A) and 24 h (panel B) after irradiation with 2.5 Gy ($P < 0.005$, $0.5 >$ relative change > 2.0).

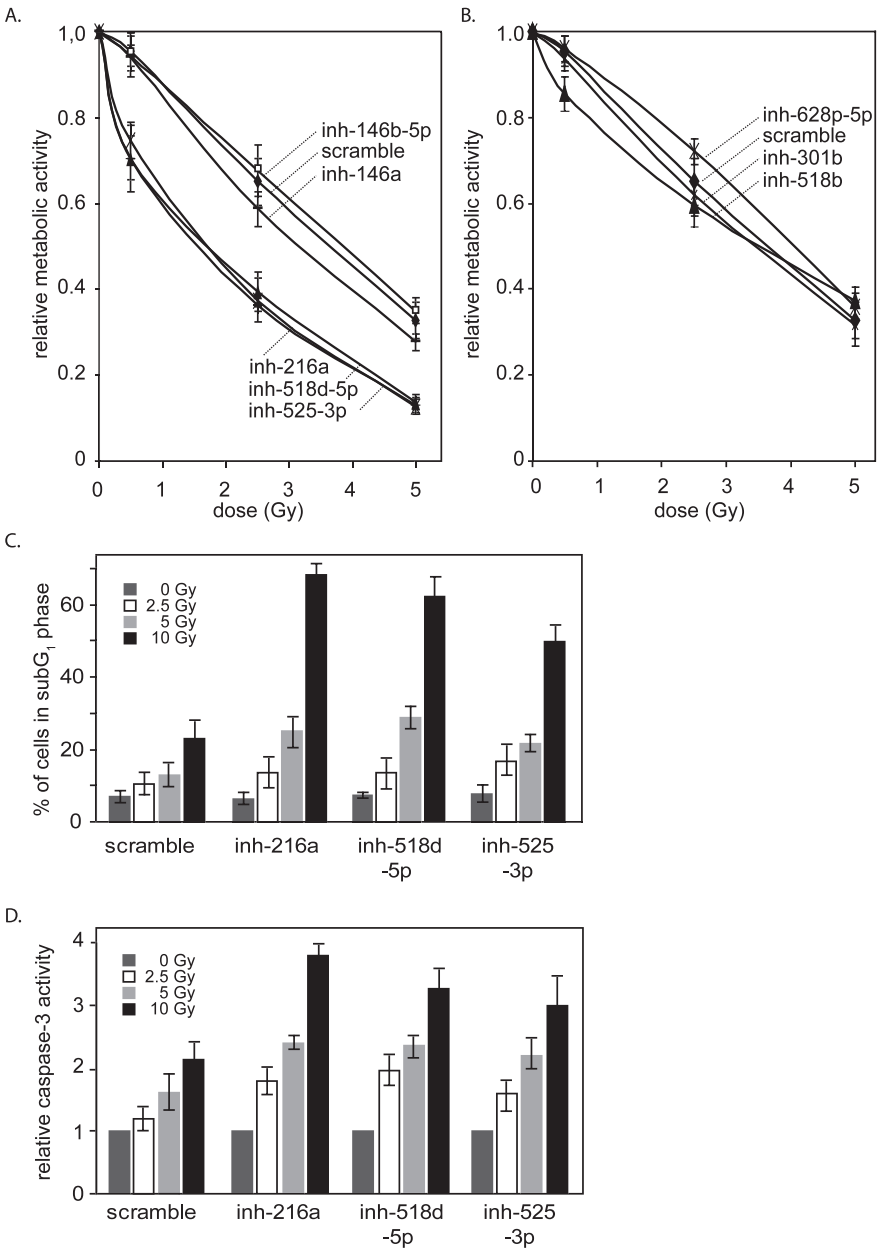

FIG. 6. Impact of single miRNAs on survival and apoptosis after irradiation. Panel A: miR-146a, miR-146b-5p, miR-216a, miR-518d-5p and miR-525-3p were inhibited by transfection of specific anti-miRs, and metabolic activity was quantified by the MTT assay 5 days after irradiation. Panel B: As in panel A for miR-301b, miR-518b-5p and miR-628-5p. Panel C: Inhibition of miR-216a, miR-518d-5d and miR-525-3p led to increased radiation-induced apoptosis quantified by sub-G_1 analysis 48 h after irradiation and caspase-3 activity 24 h after irradiation (panel D). All diagrams represent the means of three independent experiments, and error bars show SD.

showed a reduced proliferation activity compared to that of the irradiated control transfected HUVECs. Measurement of radiation-induced apoptosis by quantification of sub-G_1 fractions demonstrated a significantly higher increase in apoptotic cells in irradiated DICER knockdown cells compared to the controls (Fig. 7B).

Next, radiation-induced expression changes of individual miRNAs were identified 4 h and 24 h after irradiation by low-density arrays (Supplementary Fig. 1; http://dx.doi.org/10.1667/RR2638.1.S1). Only three miRNAs (miR-337-5p, miR-10b and miR-332-3p) were deregulated at 4 h and 24 h in HUVECs. More overlap was found for miRNAs deregulated in HUVECs and EA.hy926 cells at identical times (Fig. 7E).

For individual analysis of miRNAs, we transfected HUVECs with miRNA inhibitors (anti-miRs) against the radiation-upregulated miR-146a, miR-216a, miR-301b, miR-525-3p and miR-628-5p and examined survival and

TABLE 1
Predicted Target Transcripts of miR-216a, miR-518d-5p and miR-525-5p Related to Cell Death/Apoptosis, Stress Response, DNA Repair or Cell Cycle Arrest

miR-216a						
Cell death/apoptosis	AKT1S1	AQP2	BCL11B	BLOC1S2	CEBPG	CUL3
	GRIN2A	HTT	MEF2D	PDE5A	PRKCA	PRKCE
	RARB	RFFL	SERBP1	SMAD7	UACA	XIAP
Stress response	AQP2	BMPR2	CEBPG	GRIN2A	HTT	PTPLAD1
	SLC30A9	UACA	UBE2V2	XIAP	XRRA1	
DNA repair	CEBPG	SLC30A9	UBE2V2			
miR-518d-5p						
Cell death/apoptosis	BDKRB2	CEBPG	COL18A1	CUL3	EDAR	EYA2
	FGF2	LIG4	NIPL	NOTCH2	PDE1B	PLAGL1
	ROBO1	SFRP5	TBX5	TMBIM6	UBQLN1	VAV2
Stress response	CEBPG	CUL4B	EYA2	LIG4	MB	MORF4L1
	MRPS35	PDE1B	SCARA3	SMC1A	VAPB	
DNA repair	CEBPG	CUL4B	EYA2	LIG4	MORF4L1	SMC1A
miR-525-3p						
Cell death/apoptosis	ADIPOQ	AKT1	AQP2	ARHGEF12	BCL2	BCL2L2
	BDKRB2	BID	BIRC2	CDH1	CEBPG	DAPK2
	DYRK2	EGFR	EGLN3	EIF2AK3	ESR1	FASLG
	FGF2	GJA1	HIP1	HIPK1	KAT2A	LIG4
	LRRK2	NELL1	NET1	NOTCH2	PDE3A	PRKCE
	RAF1	SIRT1	SLC11A2	TBRG4	TGFB3	TIA1
	TMBIM6	TNFRSF19	TNFRSF8	TP63	TWIST1	TXNDC5
Stress response	AKT1	AQP2	ARNT	ATP1A2	AVPR1A	BCL2
	CEBPG	CETN1	DNMT3B	DTX3L	DUSP3	DYRK2
	EGFR	EIF2AK3	FASLG	GUCA1B	HIPK1	LRRK2
	MAP2K4	MRE11A	NBN	NCOR1	PEBP1	PLEKHB1
	POLD3	PXDN	RHO	SDR16C5	SIRT1	SLC11A2
	SSRP1	TNFRSF19	TP63	XRRA1		
DNA repair	CEBPG	MRE11A	NBN	POLD3	SIRT1	SSRP1
Cell cycle arrest	BTG4	MACF1	NBN	NOTCH2	RASSF1	TBRG4
	VASH1					

apoptosis after irradiation. In agreement with the results for the immortalized cell line, the inhibition of miR-216a and miR-525-3p in HUVECs resulted in lower survival and higher induction of apoptosis after irradiation, while inhibition of miR-146a and miR-301 had no effect (Fig. 7C, D). Additionally, blocking of miR-628-5p (found to be upregulated 24 h after irradiation) radiosensitized the HUVECs. Thus these results consistently indicate the importance of miRNAs in primary endothelial cells in the radiation response and cell survival.

DISCUSSION

Endothelial cells are highly sensitive to ionizing radiation (*27, 28*). As a consequence, therapeutic anti-tumor radiation doses are limited by the increased killing of endothelial cells in adjacent normal tissue. Therefore, a better understanding of the radiosensitivity in endothelial cells will contribute to strategies for improved radiation therapy. In this study we have identified an important role of miRNAs in the cellular response to ionizing radiation in the endothelial cell line EA.hy926. Reductions in the miRNA processing proteins DICER or AGO2 led to increased radiation-induced cell killing, indicating a prosurvival function for miRNAs. The miRNA effects were mediated by actions on cell cycle arrest and suppression of apoptosis after irradiation. Furthermore, we found that the expression of individual miRNAs showing upregulation after irradiation, namely miR-216a, miR-518d-5p and miR-525-3p, had prosurvival and anti-apoptotic effects after exposure of endothelial EA.hy926 cells to ionizing radiation. Similar effects after manipulating miRNAs in the primary human umbilical vein endothelial cell system strengthen the general importance of miRNAs for cellular resistance to radiation.

Cells demonstrate a complex network of responses to counteract radiation-induced damages, including post-transcriptional protein modification and transcriptional induction of genes (*11, 25, 26, 29*). Silencing of DICER or AGO2 led to increased radiation sensitivity. This suggests that miRNA-mediated gene regulation is a further cellular pathway for the response to radiation. Analysis of specific cellular radiation response pathways indicated that silencing of either DICER or AGO2 resulted in an increased apoptosis after exposure. These observations are in agreement with recent reports that showed that DICER depletion induces an increased stress-induced apoptosis provoked by serum withdrawal in endothelial cells and other model systems (*30–32*). In addition to the increase in apoptosis, we also observed that the cell cycle distribution was altered in response to radiation after knockdown of

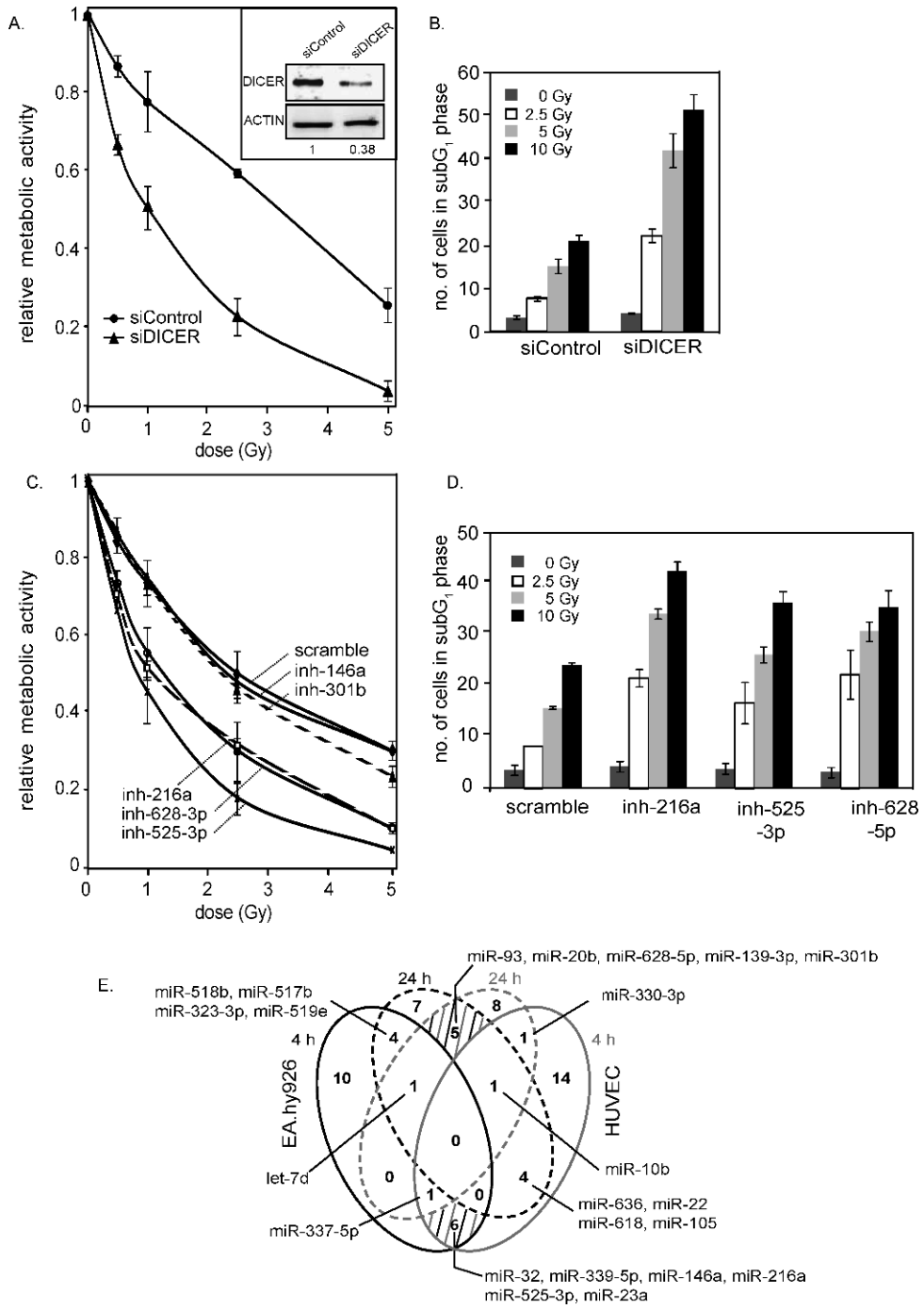

FIG. 7. Impact of miRNAs on survival and apoptosis after irradiation in primary HUVECs. Panel A: Proliferation activity of DICER knockdown cells determined by the MTT assay 5 days after irradiation. Inset: Western lot analysis of DICER protein in extracts derived from control siRNA (siControl)- and siDICER-transfected cells. Detection of actin served as an internal loading control. The numbers below the images indicate the relative intensity of the corresponding band relative to actin. Panel B: Forty-eight hours after irradiation, the apoptotic cell fraction was quantified by sub-G_1 analysis in control siRNA (siControl)- and siDICER-transfected cells. Panel C: miR-146a, miR-146b-5p, miR-216a, and miR-525-3p were inhibited by transfection of specific anti-miRs, and proliferative activity was quantified by the MTT assay 5 days after irradiation. Panel D: Inhibition of miR-216a, miR-525-3p and miR-628-5p led to increased radiation-induced apoptosis quantified by sub-G_1 analysis 48 h after irradiation. All data represent the means of three independent experiments, and error bars show SD. Panel E: Venn diagram showing the overlap between radiation-regulated miRNAs in EA.hy926 cells and in HUVECs at 4 h and 24 h after irradiation.

DICER or AGO2. These results suggest that DICER- and AGO2-mediated miRNA processing is required for the recovery of cells after stress conditions. As expected, p21 is upregulated in control cells after exposure to ionizing radiation. The lack of p21 accumulation in DICER or AGO2 knockdown cells despite proficient p53 stabilization suggests the involvement of miRNA-mediated processes in p21 regulation downstream of p53. This result is in accordance with the recent observation that the stabilization of p21 in stress response depends on miRNA overexpression (*33*). Both miRNA processing proteins appear to have negligible importance for sustaining normal proliferation, at least for the limited time scale covered by our study. Indeed, our results are supported by studies pointing to the central role of miRNAs in adapting cell cycle regulation and apoptosis to physiological needs (*34–36*).

Changes in the miRNA expression profile in response to ionizing radiation have been described in various cellular systems (*12–14*). In our hands specific changes were also identified, with a number of individual miRNAs being either increased or reduced in response to ionizing radiation. Comparison of miRNAs regulated at 4 h and 24 h suggests that different subsets of miRNAs are required for the regulation of early and late radiation response proteins. Since miRNAs are primarily negative regulators of gene expression (unless they target a repressor), this result suggests that both repression of specific target genes by miRNA upregulation as well as upregulation of some genes by miRNA downregulation may occur simultaneously due to changes in miRNA expression during the radiation response.

We identified miR-216a, miR-518d-5p and miR-525-3p as being essential for survival and suppression of apoptosis in the endothelial cell line EA.hy926 after irradiation. For primary HUVECs, the same radiosensitizing effect was found for the miRNAs miR-216a, miR-525-3p and miR-628-3p. An inhibition of any of these miRNAs sensitized the irradiated cells. Computational target prediction suggested various putative target proteins in the field of apoptosis and stress response, but so far only the regulatory function of miR-216a on the apoptosis-inducing protein PTEN has been proven experimentally (*37–39*). Furthermore, all of these miRNAs have been shown to be deregulated in cancer, including breast and ovarian cancer (http://mips.helmholtz-muenchen.de/phenomir). Despite the experimentally verified involvement of miR-146b-5p and miR-301 in radiation-relevant processes like EGFR activation (*40*) and ERK/CREB signaling (*41*), their inhibition had no impact on cellular radiation sensitivity in endothelial cells. However, proteomic approaches analyzing the impact of blocking or introducing of individual miRNAs in cultured cells revealed that the effect of miRNA regulation on an individual gene transcript is moderate, with changes of around twofold, but transcript levels of hundreds of genes are affected (*3, 42*). Therefore, miRNAs potentially modulate whole processes through a modest regulation of many genes than strong repression of a small subset of targets. Consistent with such a model, our computational target prediction produced an enrichment of target genes in the GO terms of cell death/apoptosis and stress response for miRNA-216a, miR-518d-5p and miR-525-3p.

In conclusion, our results suggest that in endothelial cells the expression of miRNAs in response to cellular stress such as ionizing radiation play an important role in the response of cells. We directly demonstrate that miRNAs regulate cell cycle checkpoint activation and apoptosis, while DNA double-strand break repair was unaffected. Because deregulation of specific miRNAs or a change of the complete miRNA pool has the consequence of changing cellular radiation sensitivity, the modulation of specific miRNAs or their targets may thus be an important tool for radiotherapy in the future.

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