ARTICLE
Journal of Cellular Biochemistry 114:606–615 (2013)

Journal of Cellular Biochemistry

miR-200c Enhances Radiosensitivity of Human Breast Cancer Cells

Jing Lin,[1‡] Cong Liu,[1‡] Fu Gao,[1] R.E.J. Mitchel,[2] Luqian Zhao,[1] Yanyong Yang,[1] Jixiao Lei,[1] and Jianming Cai[1*]

[1]*Department of Radiation Medicine, Faculty of Naval Medicine, Second Military Medical University, Xiangyin Road, Shanghai 200433, PR China*

[2]*Radiological Protection Research and Instrumentation Branch, Atomic Energy of Canada Limited, Chalk River Laboratories, Chalk River, Ontario, Canada K0J 1J0*

ABSTRACT

Due to the intrinsic resistance of many tumors to radiotherapy, current methods to improve the survival of cancer patients largely depend on increasing tumor radiosensitivity. It is well-known that miR-200c inhibits epithelial–mesenchymal transition (EMT), and enhances cancer cell chemosensitivity. We sought to clarify the effects of miR-200c on the radiosensitization of human breast cancer cells. In this study, we found that low levels of miR-200c expression correlated with radiotolerance in breast cancer cells. miR-200c overexpression could increase radiosensitivity in breast cancer cells by inhibiting cell proliferation, and by increasing apoptosis and DNA double-strand breaks. Additionally, we found that miR-200c directly targeted TANK-binding kinase 1 (TBK1). However, overexpression of TBK1 partially rescued miR-200c mediated apoptosis induced by ionizing radiation. In summary, miR-200c can be a potential target for enhancing the effect of radiation treatment on breast cancer cells. J. Cell. Biochem. 114: 606–615, 2013.

KEY WORDS: miR-200c; RADIATION THERAPY; RADIOSENSITIVITY; BREAST CANCER; TBK1

Breast cancer is the major cause of cancer-related deaths and the most common malignancy of women in the world. It is estimated that more than 207,000 women in the U.S. will be newly diagnosed with breast cancer [Jemal et al., 2010]. Radiation therapy, one of the three primary modalities, is used in breast cancer treatment and its benefits have been studied extensively during the last decades. Clinical studies in women with breast cancer who undergo breast-conserving surgery, have demonstrated that whole-breast irradiation could reduce the risk of local recurrence and improve survival outcomes [Fisher et al., 2002; Ragaz et al., 2005; Bartelink et al., 2007]. However, for some malignant tumors, that are not sensitive to ionizing radiation, radiotherapy may not kill the tumor cells effectively. What is more, increased radiation doses for treating radioresistant malignant cells will increase toxic effects in the skin and subcutaneous tissues. Finding agents, which sensitize malignant cells to radiation, can lower effective therapeutic doses, thereby increasing tumor response while minimizing normal tissue toxicity.

MicroRNAs (miRNAs) are small non-coding RNAs, which regulate gene expression by targeting mRNAs through base pairing at partially or fully complementary sites for cleavage or translational repression. Several miRNAs have been found to play key roles in breast cancer progression [Quesne and Caldas, 2010]. Recent researches have shown that miR-200c, one member of the miR-200 family on chromosome 12, inhibited epithelial–mesenchymal transition (EMT) by directly targeting ZEB1, ZEB2, MSN, FN1, and ARHGAP19 [Hurteau et al., 2007; Burk et al., 2008; Gregory et al., 2008; Korpal et al., 2008; Hurteau et al., 2009; Howe et al., 2011]. EMT is a developmental process in which epithelial cells acquired the migratory and invasive mobility. Since then, it has been reported that miR-200c suppressed tumorigenicity of human breast cancer stem cells (BCSCs), suppressed cell growth and induced differentiation, and inhibited the colony formation of breast cancer cells by modulating expression of BMI1 [Shimono et al., 2009; Liopoulos et al., 2010]. In addition, miR-200c regulated induction of

‡Jing Lin and Cong Liu contributed equally to this work.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 31070761, 31100605; Grant sponsor: Institute of Modern Physics; Grant number: 2011–2013.

*Correspondence to: Prof. Jianming Cai, Department of Radiation Medicine, Second Military Medical University, Xiangyin Road, Shanghai 200433, P.R. China. E-mail: cjm882003@yahoo.com.cn

Manuscript Received: 21 February 2012; Manuscript Accepted: 7 September 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 18 September 2012

DOI 10.1002/jcb.24398 • © 2012 Wiley Periodicals, Inc.

apoptosis by targeting FAP-1 [Schickel et al., 2010] and restored sensitivity to chemotherapeutic agents by reducing TUBB3 [Cochrane et al., 2009, 2010]. Together, the data indicate that miR-200c could inhibit migration and invasion, stem cell growth and tumorigenicity, and chemoresistance.

Although miR-200c presents an attractive target for cancer therapy, its potential radiosensitizing effects is unclear. It is well-known that ionizing radiation (IR) can induce reactive oxygen species. Magenta et al. [2011] found that the miR-200 family was induced by oxidative stress and that the most upregulated miRNA was miR-200c. In the work presented here, we show that miR-200c was induced by IR. To test the hypothesis that miR-200c can enhance the radiosensitivity of breast cancer cells, we used different approaches to investigate the role of miR-200c in the radiation response of MCF-7 breast cancer cells (with high levels of miR-200c expression) and MDA-MB-231 breast cancer cells (with low levels of miR-200c expression) [Gregory et al., 2006].

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

The breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection. MCF-7 breast cancer cells were cultured in high-glucose DMEM (PAA Laboratories Ltd.) with 10% fetal bovine serum (FBS; PAA Laboratories Ltd.). MDA-MB-231 breast cancer cells were cultured in high-glucose DMEM (PAA Laboratories Ltd.) with 5% FBS. All cells were maintained in a 37°C incubator with an atmosphere of 5% CO_2. Transfection was performed with a Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Double-stranded hsa-miR-200c mimics (miR-200c), single-stranded has-miR-200c inhibitor (inh-200c), and their relative negative control RNA (miR-NC and inh-NC; GenePharma, Shanghai, China) at a final concentration of 50 nM was introduced into cells. SiRNA (siTBK1 and the negative control siRNA) were designed according to Korherr et al. [2006] and were synthesized by Biomics (Shanghai, China). Cells were transfected in DMEM with no FBS for 6 h, and then replaced in DMEM with 10% FBS.

REVERSE TRANSCRIPTION PCR

Total RNA was extracted with Trizol (Invitrogen) according to the manufacturers recommended procedures [Gregory et al., 2006]. RNA was suspended in DNase/RNase free water and then RNA quantity and quality were determined at 260/280 nm using a spectrophotometer (GeneQuant, Pharmacia). Following manufacturer's instructions, RNA was reverse transcribed into cDNA in a reaction containing reaction buffer, PrimeScript™ RT Enzyme Mix I (PrimeScript™ RT reagent Kit; Takara Biomedicals), reverse transcription primer for miR-200c and U6 (ROCHE, UK). The reaction proceeded at 42°C for 15 min, then at 85°C for 5 s. Real-time-PCR for miRNA was performed on the cDNA with a standard LNA miRNA Fluorescence Quantitative kit protocol (ROCHE) [Erika et al., 2007]. U6 RNA was used as a miRNA internal control. The relative miRNA levels were calculated using the comparative Ct method.

CONSTRUCTION OF VECTORS AND LUCIFERASE REPORTER ASSAY

The 3′-untranslated regions (3′-UTRs) of TBK1 containing an intact miR-200c recognition sequence were amplified by PCR from genomic DNA, and the PCR product was subcloned into a pGL3-promoter vector (Generay Biotechnology Corp., Shanghai, China) immediately downstream of the luciferase gene. A pGL3 construct containing the TBK1 3′-UTR wide type (WT) or mutation (Mut) in the seed sequence was directly synthesized (Generay Biotechnology Corp.). The human ZEB1 and ZEB2 overexpression plasmid were purchased (Proteintech Group, Inc., Shanghai, China). The human TBK1 overexpression plasmid was donated by Zhengfan Jiang from Peking University. All the plasmids were devoid of the 3′UTR sequence, and the vector was pcDNA 3.1.

NIH 3T3 cells were cotransfected with 500 ng of pGL3-TBK1-WT or pGL3-TBK1-Mut constructs with miR-200c mimics or the negative control miR-NC. Each sample was cotransfected with 50 ng of pRL-TK plasmid expressing renilla luciferase to monitor the transfection efficiency (Promega). A luciferase activity assay was performed at 48 h with the dual luciferase reporter assay system (Promega). The relative luciferase activity was normalized with renilla luciferase activity [Liu et al., 2011ab].

CLONOGENIC ASSAY

Different numbers of cells (100–1,000), were plated in 6-well plates, and then were exposed to increasing doses of radiation (0, 2, 4, and 8 Gy) as indicated. Twenty-four hours later, the medium were replaced and the cells incubated until they formed colonies with at least 50 cells. The colonies were rinsed with PBS and stained with methanol/crystal violet dye. Colonies with >50 cells were scored as a surviving colony. The data are presented as the mean ± SD. The curve $S = 1-(1-e^{-KD})^N$ was fitted to the experimental data using the program GraphPad.Prism.v5.0.

CELL VIABILITY AND APOPTOSIS ASSAY

Cell viability was detected by Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan), following manufacturer's instruction [Lou et al., 2010]. Twenty-four hours after irradiation, the cells were labeled with Annexin V-FITC and propidium iodide (PI) provided by BIPEC, following manufacturer's instructions [Chen et al., 2004].

IMMUNOFLUORESCENCE

A double-strand break (DSB) assay was done by counting the colocalized phospho-γH2AX foci after radiation. In short, 1×10^4 cells were seeded into 12-well culture plate containing a glass cover slip in each well and were exposed to total dose of 8 Gy IR. Half an hour later, cells were fixed in formaldehyde for 15 min, permeabilized in 0.5% Triton X-100 in PBS for 15 min, and blocked in 1% bovine serum albumin/PBS for 15 min twice at room temperature. Cells were then incubated with a mouse monoclonal anti-γH2AX antibody (1:500) overnight at 4°C, followed with FITC-conjugated goat-anti-mouse secondary antibody (1:500) for 1 h. To stain the nuclei, DAPI was added to the cells and incubated for another 15 min. The cover slip was then removed from the plate and mounted on to a glass slide, and observed [Daniel et al., 2004; Zhou et al., 2006]. The number of merged DAPI (green) and phospho-

γH2AX foci (red) was determined, and to prevent bias in selection of cells that display foci, all the cells were counted in the field of vision (at least 50 cells).

ANTIBODIES AND WESTERN BLOT ANALYSIS

MCF-7 breast cancer cells were seeded in 60 mm plates overnight and then transfected with inh-200c or inh-NC, whereas MDA-MB-231 cells were transfected with miR-200c or miR-NC. Twenty-four hours later, the proteins were obtained using ProteoJET™ Mammalian Cell Lysis Reagent (Fermentas) according to manufacturer's protocol, and then analyzed by Western blotting [Hao et al., 2011] to detect TBK1 (Cell Signaling Technology) and β-actin (Sigma). The secondary antibody was purchased from Sigma.

GAMMA IRRADIATION

Cells were exposed to different doses of radiation (^{60}Co-gamma rays, dose rate: 1 Gy/min) in the irradiation center (Faculty of Naval Medicine, Second Military Medical University, China), depending upon the requirement of the study [Qian et al., 2010ab].

STATISTICAL ANALYSIS

Quantitative data were expressed as means ± SD. The expressions of miR-200c and the effects of DSBs were compared by the Kruskal–Wallis signed-rank test. The others were assessed by one-way ANOVA using SPSS 13.0 software. Statistically significant differences were considered as $P < 0.05$.

RESULTS

LOW LEVELS OF miR-200c EXPRESSION CORRELATED WITH RADIOTOLERANCE IN BREAST CANCER CELLS

Previous observations that miR-200c was induced by oxidative stress [Magenta et al., 2011], prompted us to hypothesize that miR-200c expression may be related to irradiation. We determined the expression of miR-200c and the radiosensitivity of MCF-7 and MDA-MB-231 cells using real-time-PCR and clonogenic assay, respectively. In line with previous reports [Gregory et al., 2006; Cochrane et al., 2009], the level of miR-200c expression was nearly 19 times higher in MCF-7 cells than in MDA-MB-231 cells (Fig. 1A). In contrast, MCF-7 cells (SF2, 0.5 ± 0.03) were more radiosensitive than MDA-MB-231 cells (Fig. 1B, SF2, 0.8 ± 0.53).

Next, we determined whether miR-200c was expressed differently when the two breast cancer cell lines were irradiated with the increasing doses. The levels of miR-200c expression in both breast cancer cell lines were increased by IR and the induction was dose dependent (Fig. 1C,D). But the level of IR-induced miR-200c expression was higher in MCF-7 cells than in MDA-MB-231 cells. Collectively, these observations suggested that the higher level of miR-200c expression was associated with higher radiosensitivity.

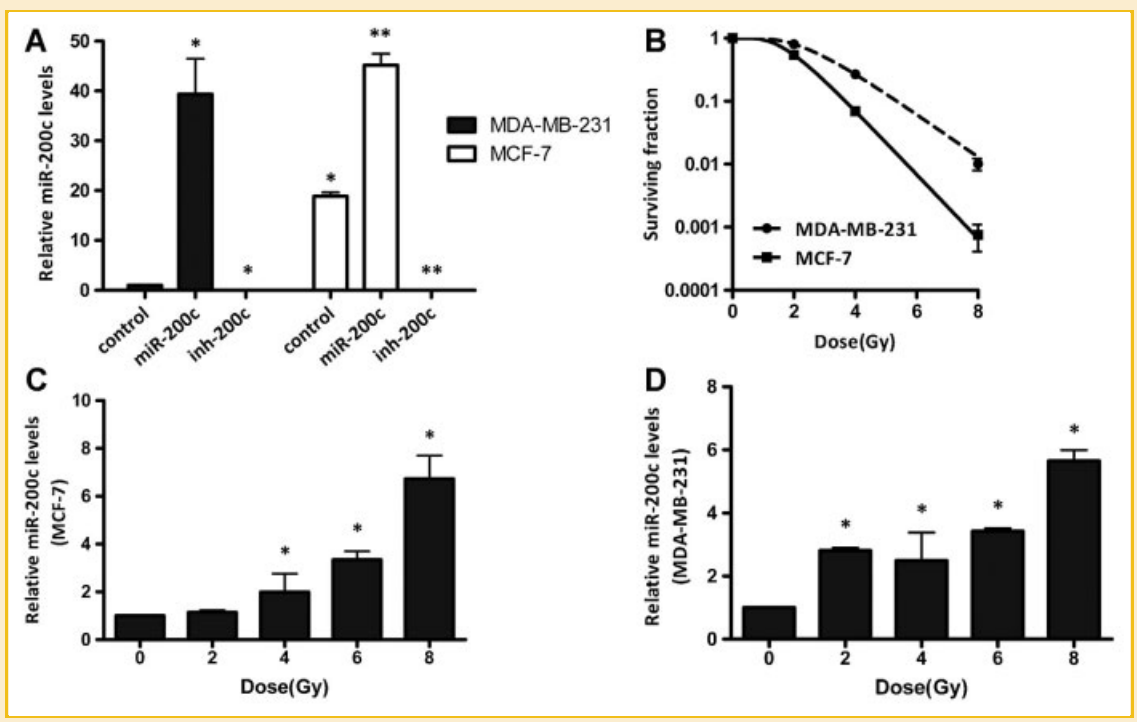

Fig. 1. Low levels of miR-200c expression correlate with radiotolerance in breast cancer cells. A: The relative expression of miR-200c in MCF-7 and MDA-MB-231 cells was detected using real-time PCR. The relative expression of miR-200c in breast cancer cells transfected with miR-200c mimics or miR-200c inhibitor were also shown ($^{*}P < 0.05$ vs. untreated MDA-MB-231 cells; $^{**}P < 0.05$ vs. untreated MCF-7 cells). B: Clonogenic assays were done in breast cancer cells and survival curves were constructed by fitting mean values from three independent experiments with a linear-quadratic model. MCF-7 cells showed higher radiosensitivity than MDA-MB-231 cells. C,D: miR-200c expression exhibited a dose-dependent increase in breast cancer cells after IR (4 Gy) for 6 h (n = 3; $^{*}P < 0.05$ vs. untreated cells, respectively). IR-induced miR-200c expression level was higher in MCF-7 cells than in MDA-MB-231 cells.

miR-200c OVEREXPRESSION ENHANCED IR-INDUCED DEATH AND REPRESSED PROLIFERATION IN BREAST CANCER CELLS

Whether miR-200c may have an effect on radiation response has not yet been addressed. The expression of miR-200c of cells treatment with miR-200c mimic was determined using real-time-PCR. A significant increase was shown in cells transfected with the miR-200c mimic versus the control (Fig. 1A). The level of miR-200c expression was nearly 2.4 times higher in MCF-7 cells and 36 times higher in MDA-MB-231 cells, compared with the respective control. IR-induced cell death was measured in breast cancer cells transfected with miR-200c or the negative control miR-NC. A significant decrease in cell viability with or without IR was detected after miR-200c treatment versus the negative control miR-NC. In response to IR (8 Gy) alone, miR-200c alone, and miR-200c + IR, the respective levels of dead cells at 24 h were 24.5%, 13.8%, and 38.3% in MCF-7 cells and 10.0%, 10.7%, and 26.5% in MDA-MB-231 cells (Fig. 2A,B). The data at 8 Gy were also measured at different time points (Fig. 2C,D). At 48 h, a significant decrease in cell viability was detected in MDA-MB-231 cells, but not in MCF-7 cells. To further analyze the radiosensitizing ability of miR-200c, we used a clonogenic assay to assess survival of breast cancer cells after IR. Our results showed that a significant decrease in clonogenic growth after IR was observed in breast cancer cells with miR-200c treatment versus the negative control miR-NC (Fig. 2E, at 8 Gy, control, 0.069 ± 0.003; miR-NC, 0.069 ± 0.002; miR-200c, 0.027 ± 0.003; Fig. 2F, control, 0.27 ± 0.023; miR-NC, 0.29 ± 0.035; miR-200c, 0.19 ± 0.048).

miR-200c OVEREXPRESSION ENHANCED IR-INDUCED DSBs IN BREAST CANCER CELLS

Enhanced DSB repair is an important mechanism by which cells may become resistant to IR [Nunez et al., 1996; Foray et al., 1997; Taneja

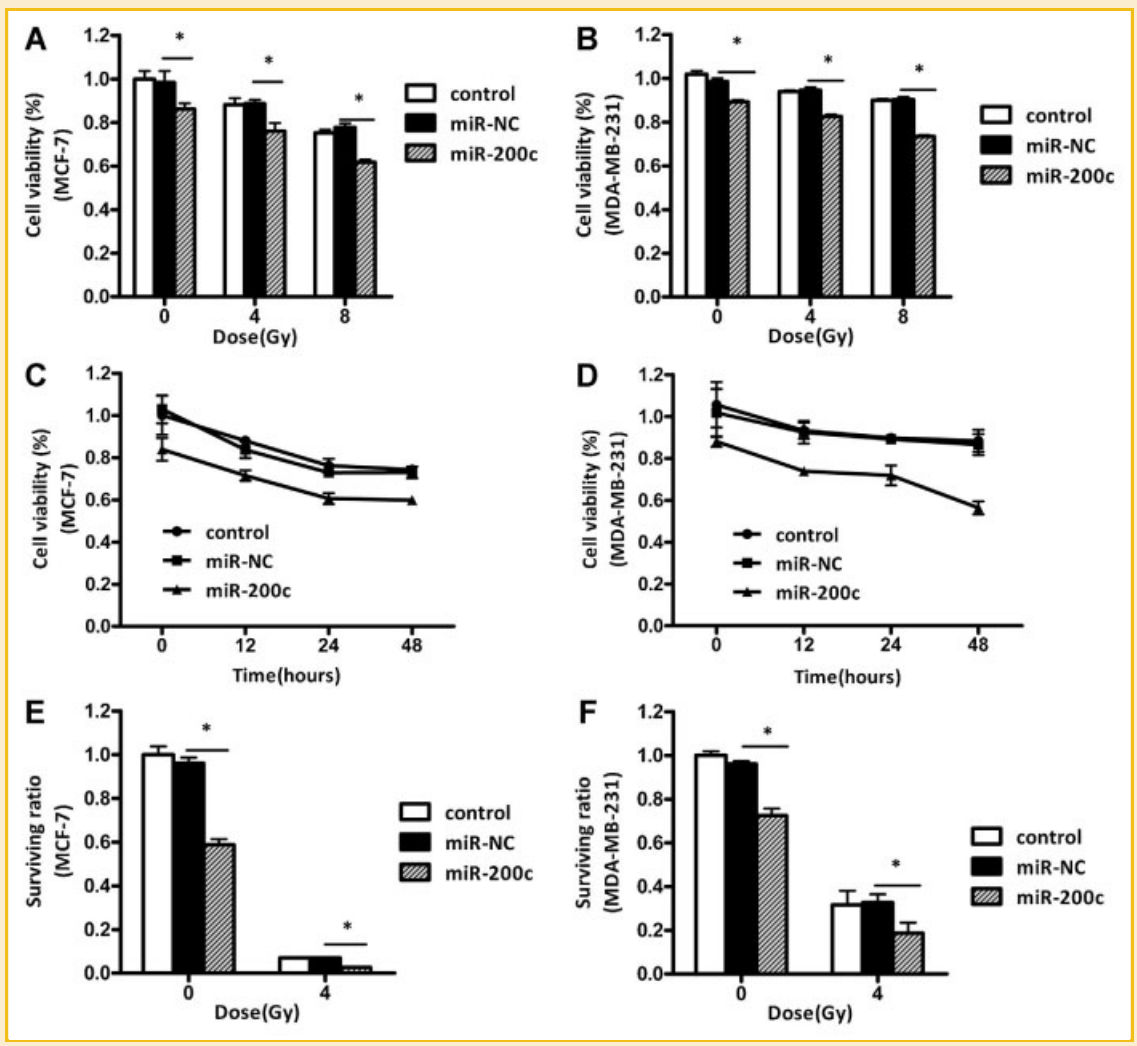

Fig. 2. miR-200c overexpression enhanced IR-induced death and repressed proliferation in breast cancer cells. A,B: Cell viability was determined at 24 h after different doses of radiation (0, 4, and 8 Gy). A significant decrease in cell viability with or without IR was detected with miR-200c treatment ($n = 6$, $^*P < 0.05$ vs. miR-NC). C,D: Cell viability was determined after 8 Gy at different time points ($n = 6$, $^*P < 0.05$ vs. miR-NC group at 0 h). E,F: Clonogenic assays after 0 and 4 Gy were done in breast cancer cells treated with miR-200c or miR-NC. A significant decrease in clonogenic growth after IR was observed in cells with miR-200c treatment versus the negative control with miR-NC ($n = 3$, $^*P < 0.05$ vs. miR-NC).

et al., 2004], and IR-induced DSBs were formed significantly 0.5 h after IR [Kong et al., 2010]. An early step in DSB repair involves the rapid phosphorylation of γH2AX that form foci at the damage sites. We observed that more foci formed in breast cancer cells with miR-200c treatment versus the negative control miR-NC. Detailed analyses in MCF-7 cells revealed that about 90% of the control cells did not contain any foci in cells transfected with miR-NC. In contrast, only about 45% did not contain any foci in cells transfected with miR-200c. After IR, about 43% of the control cells did not contain any foci in cells transfected with miR-NC, whereas about 17% in cells transfected with miR-200c (Fig. 3A). A uniform result in MDA-MB-231 cells was revealed (Fig. 3B).

miR-200c OVEREXPRESSION ENHANCED IR-INDUCED APOPTOSIS IN BREAST CANCER CELLS

Clearly, overexpression of miR-200c in cells was correlated with an increased sensitivity to IR (Figs. 2 and 3A,B). To assess the influence of apoptosis on this increased sensitivity, IR-induced apoptosis was measured in breast cancer cells transfected with miR-200c or the negative control miR-NC. A significant increase in apoptosis with or without IR was detected with miR-200c treatment versus the negative control miR-NC. In response to IR (8 Gy) alone, miR-200c alone, and miR-200c + IR, the respective levels of apoptotic cells at 24 h were 15.8%, 13.3%, and 30.9% in MCF-7 cells and 11.0%, 10.2%, and 33.2% in MDA-MB-231 cells (Fig. 3C,D).

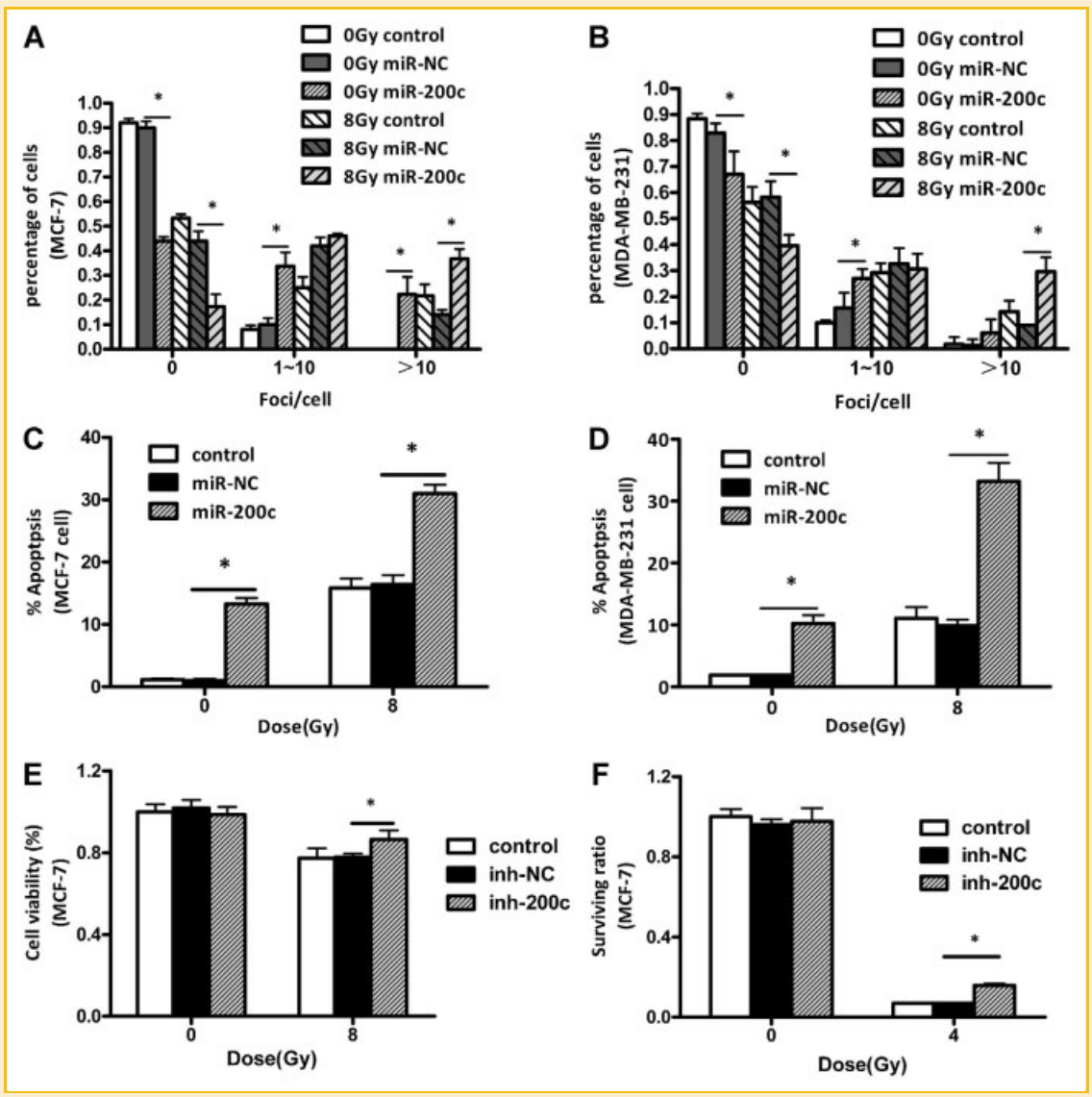

Fig. 3. miR-200c overexpression enhanced IR-induced DSBs and apoptosis in breast cancer cells. A,B: A double-strand break (DSB) assay after 0 and 8 Gy was done in breast cancer cells treated with miR-200c or miR-NC. Quantitative analyses were performed at 0.5 h post-radiation ($n = 3$, $^*P < 0.05$ vs. miR-NC). C,D: Cellular apoptosis was measured 24 h after 0 Gy or 8 Gy. A significant increase in apoptosis with or without IR was detected with miR-200c treatment versus the negative control with miR-NC ($n = 3$, $^*P < 0.05$ vs. miR-NC). E: Cell viability was determined in MCF-7 cells 24 h after irradiation (0 and 8 Gy). A significant decrease in radiosensitivity was detected with inh-200c treatment versus the negative control inh-NC ($n = 6$, $^*P < 0.05$ vs. inh-NC). F: Clonogenic assays after 0 or 4 Gy were done in MCF-7 cells treated with inh-200c or inh-NC. A significant increase in clonogenic growth after IR was observed with inh-200c treatment versus the negative control inh-NC ($n = 3$, $^*P < 0.05$ vs. inh-NC).

miR-200c INHIBITOR REDUCED IR-INDUCED DEATH AND ENHANCED PROLIFERATION IN MCF-7 CELLS

We have shown above (Figs. 2 and 3) that miR-200c overexpression increased radiosensitivity in breast cancer cells. Consequently, we tested the hypothesis that underexpression of miR-200c may therefore decrease radiosensitivity. The expression of miR-200c of cells treatment with miR-200c inhibitor was determined using real-time-PCR. A significant inhibition was shown in cells transfected with the miR-200c inhibitor (inh-200c) versus the negative control inh-NC (Fig. 1A). Due to too low a level of miR-200c expression in MDA-MB-231 cells, IR-induced death was measured in MCF-7 cells. A significant decrease in radiosensitivity was detected with inh-200c treatment versus the negative control inh-NC. In the IR (8 Gy) alone, inh-200c alone, and inh-200c + IR, the average levels of dead cells at 24 h were 22.7%, 1.5%, and 13.5% respectively (Fig. 3E). We also used clonogenic assay to assess survival of breast cancer cells after IR. A significant increase in clonogenic growth after IR was observed in MCF-7 cells with the inh-200c treatment versus the negative control inh-NC (Fig. 3F, control, 0.069 ± 0.002; inh-NC, 0.069 ± 0.001; inh-200c, 0.159 ± 0.009).

miR-200c DIRECTLY TARGETS TBK1

Whereas it has been shown previously that miR-200c regulates ZEB1 and ZEB2, many other putative miR-200c targets are predicted by bioinformatics based on complementarity, but remain to be validated. As predicted by several methods for target gene prediction (TargetScan, PicTar, and miRBase), TBK1 (TANK-binding kinase 1), an atypical IκB kinase family member, was identified as a candidate target of miR-200c that might be responsible for increasing radiosensitivity.

As shown in Figure 4A, the TBK1-encoded mRNA contained a 3′UTR element, which was partially complementary to miR-200c, indicating that miR-200c would directly target this site. To test whether TBK1 is a direct target of miR-200c, the TBK1 complementary sites, with or without mutations, were cloned into the firefly luciferase gene and cotransfected with miR-200c mimics or the negative control miR-NC in NIH 3T3 cells. As shown in Figure 4B, miR-200c significantly reduced the luciferase activity of the WT construct of the TBK1 3′UTR compared with the negative control miR-NC, whereas such a suppressive effect was not observed in the Mut construct of TBK1 3′UTR cells. The miR-200c mimics at final concentrations of 50 and 100 nM reduced the luciferase activity, but there were no significant differences between the two groups.

To determine whether miR-200c downregulated TBK1, miR-200c or miR-NC was transfected into MDA-MB-231 cells, and inh-200c or inh-NC was transfected into MCF-7 cells, respectively. The protein expression level of TBK1 was measured by western blotting assay. Our results showed that miR-200c inhibition increased the TBK1 expression in MCF-7 cells (Fig. 4C), and on the contrary, miR-200c overexpression reduced TBK1 expression in MDA-MB-231 cells (Fig. 4D).

TBK1 KNOCKDOWN ENHANCED IR-INDUCED DEATH AND REPRESSED PROLIFERATION IN MDA-MB-231 CELLS

We have determined that miR-200c directly targeted TBK1 (Fig. 4). We therefore tested the role of TBK1 knockdown on radiosensitivity by measuring IR-induced deaths in MDA-MB-231 cells treated with siTBK1 versus the controls. A significant increase in radiosensitivity was detected with siTBK1 treatment versus the negative control siRNA. In response to IR (8 Gy) alone, siTBK1 alone, and siTBK1 + IR, the respective levels of dead cells at 24 h were 11.9%, 11.2%, and 33.2% (Fig. 5A). We also used clonogenic assay to assess survival of

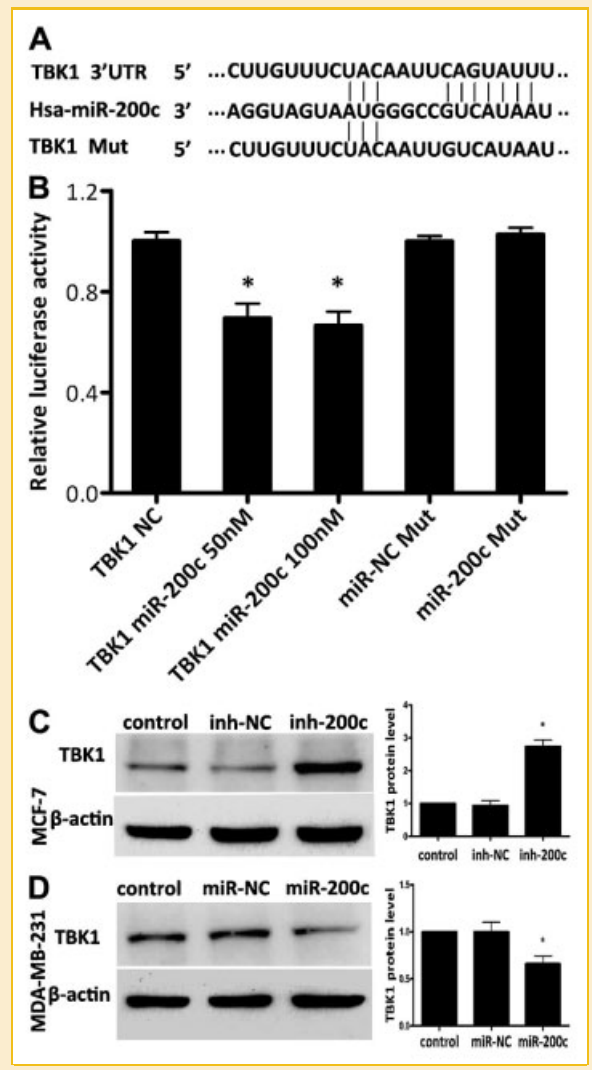

Fig. 4. miR-200c directly targets TBK1. A: Schematic representation showed the miR-200c target sequence within the 3′UTR of TBK1. The numbers indicated the positions of the nucleotides in the reference wild-type sequences (WT) and mutation sequences (Mut). B: A luciferase activity assay was performed at 48 h. The pGL3 firefly luciferase reporter plasmids with WT or Mut 3′UTR sequences of TBK1 were transfected into NIH 3T3 cells along with miR-200c (50 nM or 100 nM) or miR-NC and TK plasmids for normalization. The mean of the results from the cells transfected with miR-NC and WT 3′UTR sequences of TBK1 was set as 100% ($n = 3$, $^*P < 0.05$ vs. miR-NC + TBK1). C,D: miR-200c regulated endogenous TBK1 protein expression. Representative Western blot demonstrated miR-200c inhibitor upregulated TBK1 protein expression in MCF-7 cells, whereas miR-200c overexpression downregulated TBK1 protein expression in MDA-MB-231 cells (columns represent mean of three replicate samples and error bars represent SD, $^*P < 0.05$).

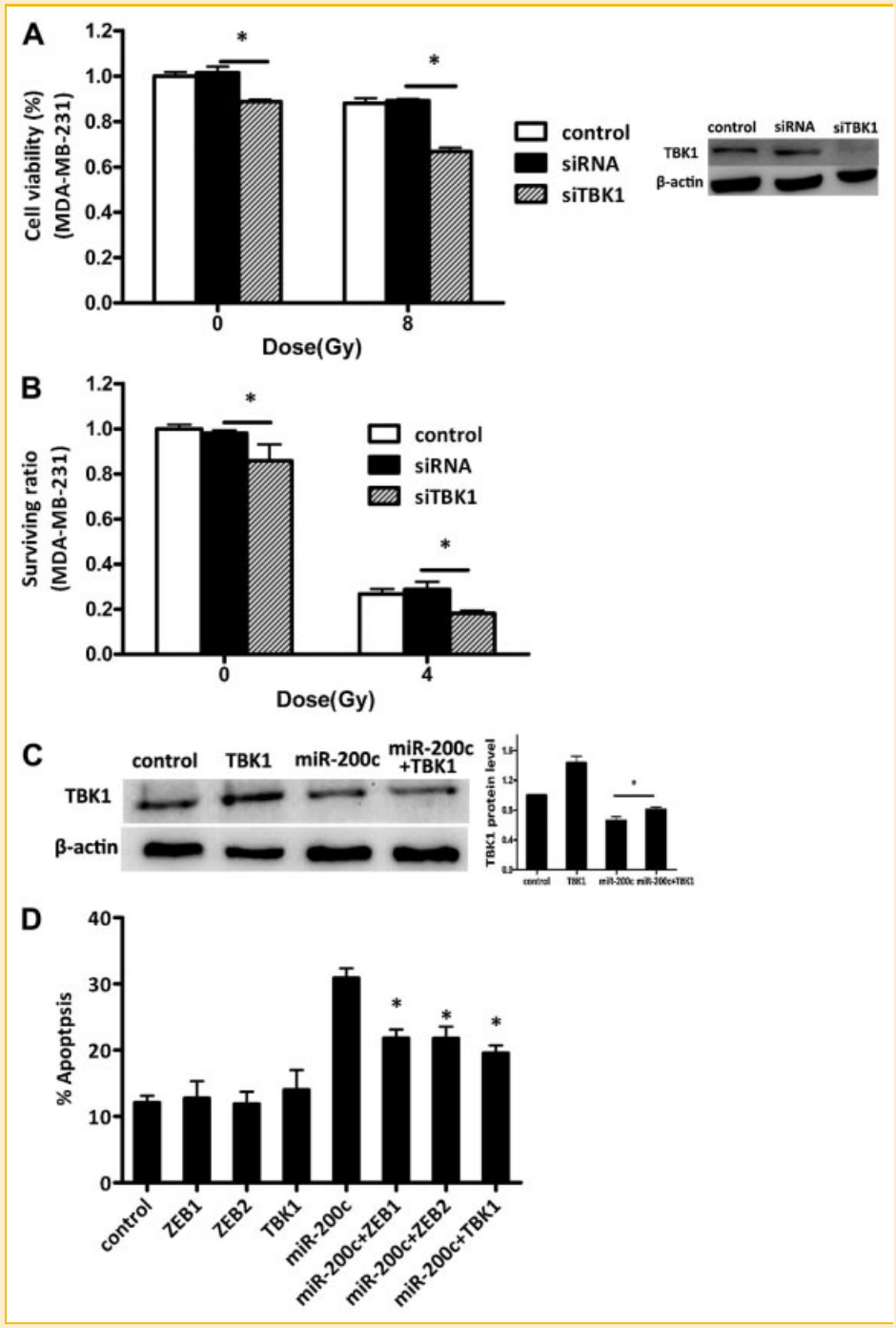

Fig. 5. TBK1 knockdown enhanced radiosensitivity in MDA-MB-231 cells and partially rescued miR-200c mediated apoptosis induced by IR. A: Cell viability was determined 24 h after different doses of radiation (0 and 8 Gy). A significant decrease in cell viability with or without IR was detected in MDA-MB-231 cells with siTBK1 treatment ($n = 6$, $^{*}P < 0.05$ vs. siRNA). B: Clonogenic assays were done after 0 and 4 Gy in MDA-MB-231 cells with siTBK1 treatment or siRNA. A significant decrease in clonogenic growth to IR was observed in cells with siTBK1 treatment versus the negative control with siRNA ($n = 3$, $^{*}P < 0.05$ vs. siRNA). C: Representative Western blot demonstrates that TBK1 overexpression can partially rescue downregulation of TBK1 protein expression induced by miR-200c (columns represents mean of three replicate samples and error bars represent SD, $^{*}P < 0.05$). D: TBK1 overexpression markedly inhibited miR-200c-induced apoptosis after 8 Gy in MDA-MB-231 cells as well as ZEB1 and ZEB2 (control represents empty vector pcDNA 3.1, $n = 3$, $^{*}P < 0.05$ vs. miR-200c).

breast cancer cells after IR. A significant decrease in clonogenic growth to IR was observed in MDA-MB-231 cells with siTBK1 treatment versus the negative control siRNA (Fig. 5B, control, 0.267 ± 0.022; siRNA, 0.287 ± 0.034; siTBK1, 0.181 ± 0.012).

TBK1 PARTIALLY RESCUED miR-200c MEDIATED APOPTOSIS INDUCED BY IR

To prove the functional relevance of TBK1 regulation by miR-200c, apoptosis was measured in MDA-MB-231 cells that were cotrans-

fected with miR-200c and TBK1, ZEB1 or ZEB2, respectively, and in parallel, cells single transfected with empty vector (control), miR-200c, TBK1, ZEB1 or ZEB2, respectively. The expression of TBK1 in cells transfected with TBK1 results from exogenous TBK1 (transfected TBK1, contains no 3′UTR) and endogenous TBK1 (natural TBK1 in cells, contains 3′UTR complementary sites targeted by miR-200c). miR-200c cannot target exogenous TBK1, but can target endogenous TBK1. Our results showed that TBK1 overexpression partially inhibited downregulation of TBK1 protein expression induced by miR-200c (Fig. 5C). TBK1 overexpression markedly inhibited miR-200c-induced apoptosis at 8 Gy in MDA-MB-231 cells (Fig. 5D). In response to negative control, TBK1 alone, miR-200c alone, and miR-200c + TBK1, the respective levels of apoptotic cells at 24 h were 12.0%, 13%, 31%, and 20%. ZEB1 or ZEB2 also prevented miR-200c-induced apoptosis induced by IR. These observations suggested that TBK1 partially rescued miR-200c mediated apoptosis induced by IR.

DISCUSSION

Due to the intrinsic resistance of many tumors to radiotherapy, current methods to improve the survival of cancer patients largely depend on increasing tumor radiosensitivity. Recently, miRNAs have been studied for the development of new targeted therapies in cancer therapy. miR-200c has been extensively studied in the epithelial-to-mesenchymal transition (EMT) [Hurteau et al., 2007; Burk et al., 2008; Gregory et al., 2008; Korpal et al., 2008]. Upregulation of miR-200c inhibits cancer aggressiveness and metastases, inhibits their growth and tumorigenicity, and chemoresistance in some tumors [Cochrane et al., 2009, 2010; Shimono et al., 2009; Liopoulos et al., 2010; Schickel et al., 2010]. However, the effect of miR-200c on radiosensitivity in breast cancer has not been investigated, and little is known about the molecular mechanisms. In the study presented here, we investigated whether miR-200c plays an important role in regulating radiation response in breast cancer cells.

Firstly, we found that breast cancer cells with a higher expression of miR-200c showed higher radiosensitivity. Furthermore, higher level of miR-200c expression was induced by IR in breast cancer cells displaying higher radiosensitivity. Those observations suggested that low level of miR-200c expression might be related to radiotolerance.

Secondly, to determine the effect of miR-200c on radiosensitivity, assays for cell viability, clonogenicity, apoptosis, and DSB were done in breast cancer cells transfected with miR-200c or the negative control miR-NC. The results showed that miR-200c overexpression increased radiosensitivity in breast cancer cells by inhibiting cell proliferation, increasing apoptosis and increasing DNA double-strand breaks. In line with several reports [Shimono et al., 2009; Schickel et al., 2010; Magenta et al., 2011], miR-200c overexpression inhibited cell proliferation, increased apoptosis and DNA double-strand breaks in unirradiated cells, via regulation of ZEB1, FAP-1, BMI1 et al. Based on the results of cell viability and apoptosis assays, we found that miR-200c overexpression showed more effect on radiosensitivity in cells expressing lower levels of miR-200c (MDA-MB-231 cells) than in cells higher levels miR-200c (MCF-7 cells). There appeared to be an additive effect in MCF-7 cells, and asynergic effect in MDA-MB-231 cells. We also found that miR-200c inhibitor decreased radiosensitivity in breast cancer cells as measured by increased proliferation.

Finally, to elucidate the molecular mechanisms of the effect of miR-200c on radiosensitivity in breast cancer cells, a new miR-200c target, TBK1, was detected for the first time and TBK1 protein was shown to be regulated by miR-200c. In addition, our preliminary studies showed that TBK1 knockdown increased radiosensitivity in MDA-MB-231 cells. TBK1 overexpression markedly inhibited miR-200c-induced apoptosis after 8 Gy in MDA-MB-231 cells. We cannot rule out that other miR-200c direct targets may have a role in the induction of apoptosis following IR, such as BMI1 [Hurteau et al., 2009; Shimono et al., 2009], FAP1 [Schickel et al., 2010] and TUBB3 [Cochrane et al., 2009, 2010].

TBK1 has been defined as a principle hub in cell regulatory networks responsive to inflammatory cytokines and pathogen surveillance receptors [Fitzgerald et al., 2003; Kawai and Akira, 2006, 2007]. In cancer cells, TBK1 was involved in oncogenic transformation by suppressing a programmed cell death response to oncogene activation [Bodemann and White, 2008], and Ou et al. [2011] found that TBK1 directly activated AKT. Furthermore, the PI3-K/AKT pathway was associated radiation resistance mechanisms, and direct inhibition of this pathway increased radiosensitivity by antagonizing the radiation induced cellular defense mechanisms [Choi et al., 2004; Schuurbiers et al., 2009]. Consequently, this study presented here indicates that miR-200c might enhance radiosensitivity of human breast cancer cells by downregulating TBK1 via inhibiting the AKT pathway. Prospective studies are needed to test this hypothesis.

In summary, the work present here shows for the first time that miR-200c increased radiosensitivity in breast cancer cells, especially in MDA-MB-231 cells. TBK1 knockdown was also involved in enhancing radiosensitivity. We found that not all of the miR-200c effect on radiosensitivity could be attributed to single targeting of TBK1. The possible underlying mechanisms appear to be multifaceted, because of the many targets of miR-200c. Our results suggest that miR-200c overexpression may represent a therapeutic target in radiotherapy.

ACKNOWLEDGMENTS

We would like to thank PhD Zhengfan Jiang (Peking University) for providing the human TBK1 overexpression plasmid. We wish to thank Dr. Meijia Zhao (department of nuclear medicine of Shanghai Pulmonary Disease Hospital Affiliated Tongji University) for technical assistance. This work was supported by a grant from National Natural Science Foundation of China (No. 31070761 and No. 31100605) and in part by an open grant from Institute of Modern Physics (No. 2011–2013).

REFERENCES

Bartelink H, Horiot JC, Poortmans PM, Struikmans H, Van den Bogaert W, Fourquet A, Jager JJ, Hoogenraad WJ, Oei SB, Warlam-Rodenhuis CC. 2007. Impact of a higher radiation dose on local control and survival in breast-

conserving therapy of early breast cancer: 10-year results of the randomized boost versus no boost EORTC22881-1088 2 trial. J Clin Oncol 25:3259–3265.

Bodemann BO, White MA. 2008. RalGTPases and cancer: Linchpin support of the tumorigenic platform. Nat Rev Cancer 8:133–140.

Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, Brabletz T. 2008. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep 9:582–589.

Chen T, Guo J, Yang M, Han C, Zhang M, Chen W, Liu Q, Wang J, Cao X. 2004. Cyclosporin A impairs dendritic cell migration by regulating chemokine receptor expression and inhibiting cyclooxygenase-2 expression. Blood 103:413–421.

Choi JA, Park MK, Kang CM, Um HD, Bae S, Lee KH, Kim KH, Kim JH, Cho CK, Lee YS, Chung HY, Lee SJ. 2004. Opposite effects of Ha-Ras and Ki-Ras on radiation-induced apoptosis via differential activation of PI3K/Akt and Rac/p38mitogen-activated protein kinase signaling pathways. Oncogene 23:9–20.

Cochrane DR, Spoelstra NS, Howe EN, Nordeen SK, Richer JK. 2009. MicroRNA-200c mitigates invasiveness and restores sensitivity to microtubule targeting chemotherapeutic agents. Mol Cancer Ther 8:1055–1066.

Cochrane DR, Howe EN, Spoelstra NS, Richer JK. 2010. Loss of miR-200c: A marker of aggressiveness and chemoresistance in female reproductive cancers. J Oncol 2010:821717.

Daniel R, Ramcharan J, Rogakou E, Taganov KD, Greger JG, Bonner W, Nussenzweig A, Katz RA, Skalka AM. 2004. Histone H2AX is phosphorylated at sites of retroviral DNA integration but is dispensable for post integration repair. J Biol Chem 279:45810–45814.

Erika VG, Rongmei W, Marion W, Eric FW, Roger PH. 2007. A highly sensitive RT-PCR method for detection and quantification of microRNAs. Plant Methods 3:12.

Fisher B, Anderson S, Bryant J, Margolese RG, Deutsh M, Fisher ER, Jeong JH, Wolmark N. 2002. Twenty-year follow-up of a randomized trial comparing total mastectomy, lumpectomy, and lumpectomy plus irradiation for the treatment of invasive breast cancer. N Engl J Med 347:1233–1241.

Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM, Maniatis T. 2003. IKK epsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 4:491–496.

Foray N, Arlett CF, Malaise EP. 1997. Radiation-induced DNA double strand breaks and the radiosensitivity of human cells: A closer look. Biochimie 79:567–575.

Gregory JH, Simon DS, Graham JB. 2006. Potential mRNA degradation targets of hsa-miR-200c identified using informatics and qRT-PCR. Cell Cycle 5:1951–1956.

Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ. 2008. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol 10:593–601.

Hao J, Zhang S, Zhou Y, Liu C, Hu X. 2011. MicroRNA 421 suppresses DPC4/Smad4 in pancreatic cancer. Biochem Biophys Res Commun 406: 552–557.

Howe EN, Cochrane DR, Richer JK. 2011. Targets of miR-200c mediate suppression of cell motility and anoikis resistance. Breast Cancer Res 13: R45.

Hurteau GJ, Carlson JA, Spivack SD, Brock GJ. 2007. Overexpression of the microRNA hsa-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin. Cancer Res 67:7972–7976.

Hurteau GJ, Carlson JA, Roos E, Brock GJ. 2009. Stable expression of miR-200c alone is sufficient to regulate TCF8 (ZEB1) and restore E-cadherin expression. Cell Cycle 8:2064–2069.

Jemal A, Siegel R, Xu J, Ward E. 2010. Cancer statistics, 2010. CA Cancer J Clin 60:277–300.

Kawai T, Akira S. 2006. Signaling to NF-kappa B by Toll-like receptors. Trends Mol Med 13:460–469.

Kawai T, Akira S. 2007. TLR signaling. Semin Immunol 19:24–32.

Kong ZL, Xie DX, Thomas B, Pavithra R, Sandeep B, Chen DJ, Amyn AH, Arup C, Hsieh JT, Saha D. 2010. Downregulation of human DAB2IP gene expression in prostate cancer cells results in resistance to ionizing radiation. Cancer Res 70(7):2829–2839.

Korherr C, Gille H, Schafer R, Koenig-Hoffmann K, Dixelius J, Egland KA, Pastan I, Brinkmann U. 2006. Identification of proangiogenic genes and pathways by high-throughput functional genomics: TBK1 and the IRF3 pathway. Proc Natl Acad Sci 103(11):4240–4245.

Korpal M, Lee ES, Hu G, Kang Y. 2008. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J Biol Chem 283:14910–14914.

Liopoulos D, Lindahl AM, Polytarchou C, Hirsch HA, Tsichlis PN, Struhl K. 2010. Loss of miR-200 inhibition of Suz12 leads to polycomb-mediated repression required for the formation and maintenance of cancer stem cells. Mol Cell 39:761–772.

Liu C, Gao F, Li B, Mitchel RE, Liu X, Lin J, Zhao L, Cai J. 2011a. TLR4 knockout protects mice from radiation-induced thymic lymphoma by down-regulation of IL6 and miR-21. Leukemia 25:1516–1519.

Liu C, Li B, Cheng Y, Lin J, Hao J, Zhang S, Mitchel RE, Sun D, Ni J, Zhao L, Gao F, Cai J. 2011b. miR-21 plays an important role in radiation induced carcinogenesis in BALB/c mice by directly targeting the tumor suppressor gene Big-h3. Int J Biol Sci 7:347–363.

Lou J, Chu G, Zhou G, Jiang J, Huang F, Xu J, Zheng S, Jiang W, Lu Y, Li X, Chen Z, He J. 2010. Comparison between two kinds of cigarette smoke condensates (CSCs) of the cytogenotoxicity and protein expression in a human B-cell lymphoblastoid cell line using CCK-8 assay, comet assay and protein microarray. Mutat Res 697:55–59.

Magenta A, Cencioni C, Fasanaro P, Zaccagnini G, Greco S, Sarra-Ferraris G, Antonini A, Martelli F, Capogrossi MC. 2011. miR-200c is upregulated by oxidative stress and induces endothelial cell apoptosis and senescence via ZEB1 inhibition. Cell Death Differ 18(10):1628–1639.

Nunez MI, McMillan TJ, Valenzuela MT, Ruiz de Almodovar JM, Pedraza V. 1996. Relationship between DNA damage, rejoining and cell killing by radiation in mammalian cells. Radiother Oncol 39:155–165.

Ou YH, Torres M, Ram R, Formstecher E, Roland C, Cheng T, Brekken R, Wurz R, Tasker A, Polvenrino T, Tan SL, White MA. 2011. TBK1 directly engages Akt/PKB survival signaling to support oncogenic transformation. Mol Cell 41:458–470.

Qian L, Cao F, Cui J, Huang Y, Zhou X, Liu S, Cai J. 2010a. Radioprotective effect of hydrogen in cultured cells and mice. Free Radic Res 44:275–282.

Qian L, Cao F, Cui JG, Wang Y, Huang Y, Zhao L, Jiang H, Cai J. 2010b. The potential cardioprotective effects of hydrogen in irradiated mice. J Radiat Res 51:741–747.

Quesne JL, Caldas C. 2010. Micro-RNAs and breast cancer. Mol Oncol 4: 230–241.

Ragaz J, Olivotto IA, Spinelli JJ, Phillips N, Jackson SM, Wilson KS, Knowling MA, Coppin CM, Weir L, Gelmon K. 2005. Locoregional radiation therapy in patients with high-risk breast cancer receiving adjuvant chemotherapy: 20-year results of the British Columbia randomized trial. J Natl Cancer Inst 97:116–126.

Schickel R, Park SM, Murmann AE, Peter ME. 2010. miR-200c regulates induction of apoptosis through CD95 by targeting FAP-1. Mol Cell 38:908–915.

Schuurbiers OC, Kaanders JH, van der Heijden HF, Dekhuijzen RP, Oyen WJ, Bussink J. 2009. The PI3-K/Akt-pathway and radiation resistance mechanisms in Non-small cell lung cancer. J Thorac Oncol 4:761–767.

Shimono Y, Zabala M, Cho RW, Lobo N, Dalerba P, Qian D, Diehn M, Liu H, Panula SP, Chiao E, Dirbas FM, Somlo G, Pera RA, Lao K, Clarke MF. 2009. Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. Cell 138:592–603.

Taneja N, Davis M, Choy JS, Beckett MA, Singh R, Kron SJ, Weichselbaum RR. 2004. Histone H2AX phosphorylation as a predictor of radiosensitivity and target for radiotherapy. J Biol Chem 279:2273–2280.

Zhou CX, Li ZX, Diao HL, Yu YK, Zhu W, Da YY, Chen FF, Yang J. 2006. DNA damage evaluated by γH2AX foci formation by a selective group of chemical/physical stressors. Mutat Res 604(1–2):8–18.