Published in final edited form as:

Cancer Lett. 2015 December 1; 369(1): 184–191. doi:10.1016/j.canlet.2015.08.011.

NRF2/miR-140 signaling confers radioprotection to human lung fibroblasts

Nadire Duru, Ramkishore Gernapudi, Yongshu Zhang, Yuan Yao, Pang-Kuo Lo, Benjamin Wolfson, and Qun Zhou*

Department of Biochemistry and Molecular Biology, Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD 21201, USA

Abstract

Breast and lung cancer patients who are treated with radiotherapy often have severe side effects, including radiation-induced lung damage and secondary cancers. Activation of the NRF2 pathway is a well-known mechanism that protects cells against radiation induced oxidative stress, but its role in radiation-induced lung damage is not well understood. Using human lung fibroblasts (HLFs) we found that ionizing radiation (IR) leads to BRCA1-dependent activation of NRF2 through the inhibition of KEAP1 function, promoting the nuclear accumulation of NRF2, and activating critical radioprotective mechanisms. We discovered that NRF2 directly binds to the miR-140 promoter and increases its expression in response to IR treatment. Gain and loss of function studies further showed the ability of miR-140 to regulate lung fibroblast self-renewal upon irradiation, a potential mechanism to contribute to the regulation of DNA repair. We verified our *in vitro* findings using primary lung fibroblast cultures from wild type and Nrf2 (KO) mice. Using these models we showed that IR induces overexpression of Brca1, Nrf2 and miR-140 in lung tissue after irradiation. These data reveal a novel radioprotective mechanism in which IR promotes NRF2 nuclear translocation and subsequent activation of miR-140 transcription in HLFs.

Keywords

Radiation-induced injury; Radioprotection; BRCA1; NRF2; miR-140

Introduction

Radiation therapy is a commonly used treatment for both lung and breast cancer patients. Ionizing radiation (IR) introduces overwhelming genetic lesions in the DNA of irradiated cells, causing cell death. Radiotherapy is frequently used as a primary or an adjuvant therapy and can prevent cancer recurrence by killing cells that remain after surgery [1,2]. While shaped IR beams are used to specifically irradiate cancerous areas, off-target effects remain a serious burden on patients receiving radiation therapy. The lungs are one of the most

*Corresponding author. Tel.: +1 410 706 1615; fax: +1 410 706 8297. ; Email: qzhou@som.umaryland.edu (Q. Zhou)

Conflict of interest

All authors declare that they have no conflict of interest.

sensitive organs to radiation, and chest radiation has a high chance of leading to radiation-induced lung injury [3].

The patients at highest risk for radiation-induced lung injury are those receiving higher, more frequent doses targeting larger areas. Radiation-induced lung injury usually occurs 1–6 months after completion of radiation therapy and begins with pneumonitis, an early-stage inflammation-dependent lung disease characterized by loss of epithelial cells, edema, inflammation, and occlusion of the airways, air sacs and blood vessels. Within 6 months to a year, pneumonitis progresses to lung fibrosis, the chronic scarring of lung tissue. Lung fibrosis permanently impairs lung function, greatly reducing patient quality of life and in some cases leading to death [4].

A crucial cellular mechanism for radioprotection is the activation of nuclear factor–erythroid2–related factor 2 (NRF2), a transcription factor that activates the expression of antioxidant and anti-inflammation genes [5]. Under normal conditions, NRF2 is bound by cytoplasmic protein Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1 (KEAP1), which facilitates NRF2 ubiquitination and lysosomal degradation. IR creates free radicals within the cell, which disrupt the NRF2–KEAP1 complex, stabilizing NRF2 and promoting its translocation to the nucleus. Nuclear NRF2 binds to antioxidant response elements (ARE) and activates the transcription of antioxidant and cytoprotective genes including NQO1, GSTs and HO-1 [6]. The tumor suppressor BRCA1 is critical for double-strand DNA break repair and maintaining genomic integrity. Recent investigations have demonstrated a new role of BRCA1 in the NRF2 signaling pathway. BRCA1 is able to bind to cytoplasmic NRF2 where it prevents NRF2 degradation by promoting dissociation of the KEAP1–NRF2 complex [7]. This may be a novel mechanism by which BRCA1 is involved in cellular radioprotection, and a crucial signaling pathway for lung and breast cancer patients who receive radiotherapy.

NRF2 activates the expression of several noncoding RNAs, including both microRNAs (miRNA) and long-noncoding RNAs (lncRNA). miRNAs are short (18–24 nucleotides) non-coding RNAs that bind specific sequences in the 3'UTR of target mRNAs, leading to mRNA degradation or translational inhibition. miRNAs regulate several signaling networks, including those involved in the cellular radiation response, maintenance and differentiation of stem cells, cellular stress responses, cell proliferation and apoptosis [8,9]. NRF2 has been found to either activate or repress the expression of several miRNAs [10–14]. NRF2 activates transcription of miR-125b1 and inhibits transcription of miR-29b1, leading to apoptotic resistance in AML cell lines [13]. NRF2 also activates miR-125b expression in epithelial kidney cells, which provides cellular protection during chemotherapy [12]. Finally, NRF2 activation of miR-1 and miR-206 can help drive tumorigenesis through metabolic regulation in human lung cell carcinoma cell lines [14]. We have recently demonstrated that NRF2 inhibits lncRNA-Regulator of Reprogramming (ROR), and is important for maintenance of a normal stem cell subpopulation in the mammary epithelium [15]. These studies illustrate the important role of NRF2 in regulation of noncoding RNAs.

In this study we examined the BRCA1/NRF2 response to IR in human lung fibroblast cells. We identified nuclear export of BRCA1 as a key step in the activation of the NRF2 signaling

pathway. We found that miR-140 is a new NRF2 target gene in response to radiation. Finally, we characterized the role of the NRF2/miR-140 pathway in radioprotection of lung fibroblasts.

Materials and methods

Cell culture and siRNA and shRNA-mediated target gene inhibition

Human normal lung fibroblast cells (CCD-19Lu) were purchased from American Type Culture Collection (ATCC). They were cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Rockford, IL). MCF10A immortalized human mammary epithelial cells were obtained from ATCC (Rockville, MD) and were grown as described previously [16]. The cells were incubated in 5% CO₂ at 37 °C. The siRNA targeting BRCA1 was purchased from Santa Cruz (Santa Cruz Biotechnology; Dallas, TX). Cell transfections with siRNA and shRNA were conducted at 70% cell confluence using Lipofectamine 3000 reagent (Invitrogen; Carlsbad, CA) according to the manufacturer's protocol.

Isolation and culture of mice lung fibroblast

The protocol to isolate fibroblast cells from the mouse lungs was adapted from Seluanov et al. [17]. Nrf2 knockout mice were obtained from Dr. Thomas Kensler (University of Pittsburgh School of Medicine). The care and use of all mice were approved by the Animal Care and Use Committee at the University of Maryland School of Medicine. Briefly, wild-type, Nrf2(KO) [15] and miR-140(KO) C57BL/6 mice were sacrificed and their lungs were harvested. Small pieces of lungs were incubated in dissociation buffer containing 10× Collagenase/Hyaluronidase and P/S. The tissue pieces were washed with PBS and were incubated in the attachment/growth medium (DMEM, 1% P/S, 15% FBS). Once cells attached and started to grow the medium was changed to EMEM to support the growth of fibroblasts.

Irradiation of the cells and mice

Radiation was delivered to cultured cells using a Gammacell 3000 gamma irradiator (dose rate: 3.75 Gy/min; Nordion International Inc). C57BL/6 mice were irradiated with 10 Gy and 13 Gy single doses and their lungs were harvested at 6 months and 1 year post-irradiation. These lung tissues are kindly provided by Dr. Isabel L. Jackson (Department of Radiation Oncology at University of Maryland School of Medicine).

Western blotting and subcellular fractionation

Total cell lysates (15–50 µg) were separated by SDS–PAGE and blotted onto polyvinylidene difluoride membrane. The membrane was incubated with specific primary antibody overnight followed by the horseradish peroxidase (HRP)-conjugated secondary antibody, and visualized by the ECL Western blotting detection system (Thermo Scientific; Rockford, IL). β-actin (Sigma; St Louis, MO, USA) was used as the loading control. Antibodies against BRCA1, NRF2 and KEAP1 were purchased from Santa Cruz (Santa Cruz Biotechnology; Dallas, TX). To determine the subcellular expression of proteins, cells were subjected to subcellular fractionation using the cell fractionation kit (Cell Signaling;

Beverly, MA) according to the manufacturer's protocol. PCNA (Santa Cruz Biotechnology; Dallas, TX) was used as a loading control for the nuclear fraction.

Immunofluorescence staining and *in situ* hybridization of miR-140

Immunofluorescence assay was conducted with fixing cells in 8-well chamber slides with 4% ice-cold paraformaldehyde and permeabilizing them with PBS containing 0.2% Triton-X. After the cells were blocked in PBS containing 10% FBS and 1% BSA, they were incubated with primary antibody overnight. The cells were incubated with fluorochrome-conjugated secondary antibody and they were stained with DAPI and mounted for analysis. *In situ* hybridization assay was conducted with seeding cells into 8-well chamber slides. After 24 h, cells were fixed with 4% formaldehyde and were permeabilized with 0.1% triton-x-100 in PBS. *In situ* hybridization for miR-140 was performed as previously described using 5'-digoxigenin-tagged probe [18]. Colorimetric detection reaction was performed using NBT/BCIP (Roche; Indianapolis, IN, USA) for 48 h. The slides were stained with Nuclear Fast Red (Sigma; St Louis, MO, USA) and images were captured using Nikon Eclipse Ti (Nikon Instruments Inc.; Melville, NY, USA).

Chromatin immunoprecipitation (ChIP) assay and quantitative real-time PCR (qRT-PCR)

The transcription factor binding sites were predicated using the Consite program (consite.genereg.net/cgi-bin/consite). One NRF2 binding site (–7844 to –7835; ccaggaagc) in the miR-140 promoter region was identified and confirmed by the ChIP assay as described previously [19]. Immunoprecipitated chromatin was analyzed by real-time qPCR using one set of primers for the miR-140 promoter (5'-ggtaa acgaggcccaaat-3' and 5'-gagcagtagatttagagccagca-3'). For qRT-PCR, total RNA was extracted with TRIzol reagent (Invitrogen; Carlsbad, CA) and analysis of mRNA/miRNA expression was performed as described previously with normalization to either GAPDH or β -actin for mRNAs and to U6 small nuclear RNA for miRNAs [19].

Scratch assay and collagen contraction assay

Migration capacity of the cells was assessed with scratch assay that was conducted as previously described [20] with 3×10^5 cells grown in each of 6-well plates to 100% confluence followed by culture in medium without serum for 24 h for cell starvation. The cells were scraped diagonally with a sterile pipette's tip to create the gap and the filling capacity was monitored at different times, as specified in results. Collagen gel contraction assay was set up according to manufacturer's instructions (Cell Biolabs; CBA-201; San Diego, CA). In brief, a cell suspension of 2.0×10^6 cells/mL was obtained. 100 μ L of the cell suspension was mixed with 400 μ L of neutralized collagen solution and added to one well of a 24-well cell culture plate and allowed to solidify for one hour at 37 °C. After polymerization, 1 mL of complete media was added to each well and the cells were incubated for 48 h. The stress was released by running a sterile pipette tip along the sides of the well. The culture dish was then scanned immediately after the stress was released (time 0) and at the other time points that were determined. The area of the collagen gel was then measured using ImageJ software (NIH).

Sphere formation assay

CCD-19Lu single cells were obtained by filtrating trypsinized cells through 40- μ m cell strainers (Fisher Scientific; Pittsburgh, PA) and counted. For sphere formation assays, 2000 cells/mL were seeded in six-well plates coated with 2% polyhema (Sigma; St Louis, MO) in DMEM/F12 containing 2% B27, 20 ng/mL EGF, 4 μ g/mL insulin, and 0.4% BSA. After 7 days of culture, spheres were quantified by light microscopy.

Statistical analysis

Statistical analysis was performed using the Graph Pad Prism software and data were assessed by the 2-tailed Student t test. A difference was considered significant when $P < 0.05$ (*) or $P < 0.01$ (**). Data are presented as mean $\pm$ S.D.

Results

Ionizing radiation activates the BRCA1-NRF2 pathway in human lung fibroblasts

We first investigated whether radiation impacts the expression of BRCA1 and NRF2 in human lung fibroblasts (HLFs). Our preliminary data showed that IR treatment (5 Gray (Gy), 10 Gy, 15 Gy and 20 Gy) increased both BRCA1 and NRF2 protein expression levels in a dose dependent manner at 24 h (data not shown). Based on these results, we used the 24-hour time point and 20 Gy dose for future experiments. We treated CCD-19Lu HLFs with 20 Gy of IR and determined the protein level of BRCA1 and NRF2 using western blotting. As shown in Fig. 1A, radiation treatment increased protein levels of BRCA1 and NRF2 compared to non-irradiated cells. Due to the increases in both BRCA1 and NRF2 protein levels in irradiated cells, as well as the previously reported findings showing BRCA1 binds NRF2 to prevent KEAP1 mediated degradation, we hypothesized that radiation-induced activation of BRCA1 increases NRF2 protein expression. To test this hypothesis, we used siRNA to knock down BRCA1 in HLFs. BRCA1 knockdown decreased NRF2 protein expression in both non-irradiated (Fig. 1B) and irradiated (Fig. 1C) cells, indicating that NRF2 expression in HLFs is BRCA1 dependent.

Ionizing radiation regulates the nuclear translocation of NRF2 in a BRCA1-dependent mechanism in human lung fibroblasts

Previous studies have shown that IR induces export of nuclear BRCA1 to the cytoplasm in breast cancer cell lines [21,22]. Studies also demonstrated that IR could activate nuclear translocation of Nrf2 in epithelial cells and mouse macrophages [23,24]. To confirm these in HLFs, we irradiated CCD-19Lu cells with 20 Gy IR and examined BRCA1 and NRF2 protein expressions in the nucleus and cytoplasm using western blot analysis following subcellular fractionation. Consistent with previous findings, in irradiated cells we observed higher BRCA1 protein expression in the cytoplasmic fraction and higher NRF2 protein levels in the nuclear fraction of irradiated cells (Fig. 1D and E). To further confirm these findings we used immunofluorescence staining of BRCA1 and NRF2 to visualize their localization within the cell. In non-irradiated cells BRCA1 and NRF2 were predominantly located in the nucleus and cytoplasm, respectively, whereas in irradiated cells BRCA1 was mainly located in the cytoplasm and NRF2 was mainly expressed in the nucleus (Fig. 2C).

These data demonstrate that nuclear export of BRCA1 and nuclear translocation of NRF2 are induced by IR in HLFs. We next wanted to determine if BRCA1 was necessary for the IR-induced nuclear translocation of NRF2. Interestingly, BRCA1 knockdown led to a dramatic decrease in both cytoplasmic and nuclear NRF2 protein levels in non-irradiated cells. When BRCA1 knockdown cells were irradiated, nuclear expression of NRF2 was even more substantially diminished compared to wild-type irradiated cells (Fig. 1E). These results demonstrate that radiation increases BRCA1 expression and promotes its translocation from the nucleus to the cytoplasm leading to BRCA1-dependent nuclear translocation of NRF2.

Radiation activation of BRCA1 inhibits KEAP1 function

KEAP1 is a negative regulator of NRF2 that promotes NRF2 ubiquitination and degradation. BRCA1 is a positive regulator of NRF2 that binds to NRF2 and prevents its KEAP1-mediated degradation [6,7]. While radiation activation of BRCA1 increases NRF2 protein levels, it is unknown whether this is due to increased stability or activation of transcription. Therefore, we investigated the impact of BRCA1 on KEAP1. We isolated nuclear and cytoplasmic fractions from non-irradiated and 20 Gy-irradiated CCD-19Lu cells. In non-irradiated cells, BRCA1 is expressed in both the nucleus and cytoplasm but its expression is much higher in the nucleus. KEAP1 is also expressed in both the nucleus and cytoplasm, but in non-irradiated cells is predominantly expressed in the cytoplasm. To determine if BRCA1 interacts with and negatively regulates KEAP1 in the cytoplasm, we knocked down BRCA1 with siRNA. BRCA1 knockdown led to increased KEAP1 protein levels in both the cytoplasm and nucleus of non-irradiated cells (Fig. 2A). We also observed BRCA1 regulation of KEAP1 in irradiated cells. When cells were irradiated with 20 Gy, BRCA1 translocated from the nucleus to the cytoplasm and nuclear KEAP1 expression increased 3-fold. Knockdown of BRCA1 in irradiated cells further increased KEAP1 nuclear expression. Interestingly, KEAP1 expression level is elevated in cytoplasm with radiation despite the upregulation of BRCA1. However, when BRCA1 was inhibited with siRNA in irradiated cells, KEAP1 expression level increased 1.5-fold compared to irradiated cells that were not targeted with siBRCA1. This suggested that irradiation might increase KEAP1 expression in cytoplasm through a mechanism that is not BRCA1 dependent. To verify these findings we performed immunofluorescence staining of BRCA1, NRF2 and KEAP1 in both non-irradiated and irradiated CCD-19Lu cells. BRCA1 was predominantly nuclear while NRF2 and KEAP1 were largely cytoplasmic in non-irradiated cells. Irradiated cells had high levels of cytoplasmic BRCA1 co-localized with KEAP1 and NRF2 in the areas surrounding the nucleus. Additionally, while irradiated cells still showed cytoplasmic NRF2, there was a dramatic increase in nuclear NRF2 staining (Fig. 2B). These results demonstrate that BRCA1 has a negative effect on KEAP1 protein levels. Altogether, these data suggest that radiation induced activation and nuclear export of BRCA1 disrupts the KEAP1–NRF2 complex, stabilizing NRF2 and promoting its nuclear translocation and activation of downstream targets.

miR-140 is a new NRF2 target gene in human lung fibroblasts

miRNAs are short non-coding RNA molecules that function by targeting untranslated regions of protein coding mRNAs and have been shown to regulate several signaling pathways involved in the cellular radiation response [9,10]. Given that radiation is able to

alter the expression of several miRNAs, it is likely that the radiation-induced NRF2 transcriptome includes miRNAs in HLFs. Using *in situ* hybridization (ISH) analysis with a miR-140-specific probe [18], we detected a dramatic elevation of miR-140 expression levels in irradiated HLFs. This was verified by qPCR analysis, which also showed increased expression of miR-140 in irradiated HLFs (Fig. 3A). We selected miR-140 as one of the most promising candidates for further analysis because: (1) we performed *in silico* analysis (<https://www.genomatix.de/>) and identified a putative NRF2 binding site in the miR-140 promoter region (−7844/−7835, the transcription start site set as default +1) (Fig. 3B, top panel); (2) miR-140 is significantly upregulated in lung cancer cells in response to irradiation [25]; and (3) miR-140 inhibits proliferation and stem cell self-renewal [26]. q-PCR analysis showed an increased expression level of miR-140 in irradiated HLFs.

To determine if NRF2 directly binds to the miR-140 promoter and subsequently activates miR-140 transcription, we performed ChIP followed by qPCR in a MCF10A cell model [15]. As seen in Fig. 3B, increased NRF2 protein expression leads to enhanced binding to the miR-140 promoter. Consistent with these observations, overexpression of NRF2 increased miR-140 expression (Fig. 3B). To confirm that NRF2 is important for regulation of miR-140 levels in HLFs, we used siRNA or shRNA, respectively, to knock-down BRCA1 or NRF2 and found that inhibition of the NRF2 signaling by BRCA1 siRNA or NRF2 shRNA substantially decreased miR-140 expression, indicating that miR-140 is a target gene of NRF2 (Fig. 3C and D).

miR-140 inhibits self-renewal of lung fibroblasts

Self-renewal is the ability of stem cells to go through cycles of cell division while maintaining their undifferentiated state. This is necessary to maintain a constantly replenishing pool of progenitor cells, which are able to generate new daughter cell populations. IR causes DNA damage, and in stem cells this may induce tumorigenic mutations, leading to secondary cancers. We have previously demonstrated that miR-140 is an important regulator of breast cancer stem cell self-renewal [26]. We next wanted to determine if miR-140 has similar effects in HLFs. We overexpressed miR-140 in CCD-19Lu cultured cells and determined their self-renewal ability using the sphere formation assay. miR-140 overexpressing CCD-19Lu cells were plated on non-adherent, polyhema coated plates. Under these conditions, cells capable of self-renewal form spheroid colonies, while others die from anoikis. The number of spheres formed is indicative of the self-renewal capabilities of the plated cells. miR-140 overexpression decreased sphere formation significantly in both non-irradiated and 20 Gy-irradiated cells (Fig. 4A). In contrast, when miR-140 expression was suppressed by a miR-140 sponge plasmid, both non-irradiated and irradiated cells exhibited considerably higher sphere formation (Fig. 4A). These data suggest that NRF2-activated miR-140 inhibits the self-renewal of HLFs.

Ionizing radiation activates the BRCA1/NRF2/miR-140 radioprotective pathway *in vitro*

To reveal protective roles of BRCA1 and NRF2 in radiation-exposed lung fibroblasts, we examined cellular migration and contraction. To investigate cellular migration, we used an *in vitro* scratch assay. Irradiated cells had slower migration rate compared to non-irradiated cells, which was decreased even more dramatically when either BRCA1 or NRF2 was

knocked down (Fig. 4B and C). Interestingly, non-irradiated cells with NRF2 knockdown also had decreased migration compared to control cells (Fig. 4B). The ability of cells to contract their extracellular matrix is an essential function for tissue repair. Using collagen gel contraction assays, we showed that treatment with 20 Gy IR significantly increased the contractile ability of CCD-19Lu cells, as the size of the gel formed by non-irradiated cells was approximately 2-fold larger than that of 20 Gy-irradiated cells (Fig. 4C). Knockdown of either BRCA1 or NRF2 led to defective contractile ability of irradiated cells compared to control cells, demonstrating that the BRCA1/NRF2 signaling pathway is required for the contraction of HLFs. These data demonstrate that NRF2 is necessary for both cell migration and contraction in response to radiation.

Ionizing radiation activates the Brca1/Nrf2/miR-140 radioprotective pathway in mouse lung tissue

To validate whether the Brca1/Nrf2/miR-140 signaling pathway is activated *in vivo* lung tissue, C57BL/6 mice were irradiated with 13 Gy radiation to their thoracic areas. The mice were monitored and sacrificed one year after radiation treatment, and lung tissues from irradiated and non-irradiated mice were harvested. We found that the expression levels of Brca1, Nrf2 and miR-140 were dramatically upregulated in lung tissues isolated from the mice even a year after irradiation (Fig. 5A). To further examine the radioprotective role of Nrf2 in mouse lung fibroblasts, we isolated lung fibroblasts from wild-type (WT) and Nrf2 knockout (KO) C57BL/6 mice and then performed a scratch assay. Consistent with the results from HLFs (Fig. 4C), the migration ability of both non-irradiated and irradiated primary Nrf2(KO) mouse lung fibroblasts was significantly impaired compared to wild-type counterparts (Fig. 5B). We next assessed the sphere forming capacity of non-irradiated and irradiated Nrf2(KO) primary lung fibroblasts. Nrf2(KO) cells had higher sphere formation compared to WT regardless of radiation treatment (Fig. 5C). Furthermore, we isolated lung fibroblasts from Brca1(KO) and Brca1/Nrf2 double-KO C57BL/6 mice to assess their sphere formation capacity. As expected, both Brca1(KO) and Brca1/Nrf2 double KO lung fibroblasts formed more spheres compared to their WT counterparts (Fig. 5D). To validate the radioprotective role of miR-140 in mouse fibroblasts, we isolated lung fibroblasts from WT and miR-140 knockout (KO) C57BL/6 mice and then performed sphere formation assay. Consistent with the results from HLFs (Fig. 4C), the sphere formation capacity of both non-irradiated and irradiated primary miR-140 (KO) mouse fibroblasts was significantly higher compared to wild-type counterparts (Fig. 5E). Overall, these data indicate that the Brca1/Nrf2/-140 radioprotective pathway may have long-term persistence after initial stimulus, and could be a protective mechanism against future cellular insults.

Discussion

Side effects of radiotherapy, including radiation-induced lung injury and secondary cancers, are likely inevitable when breast and lung cancer patients are treated with chest radiotherapy [3]. By deciphering the molecular mechanisms of cellular radioprotection, we will be able to better prevent normal tissue damage. Identification of key signaling pathways will aid clinicians in creating more personalized radiotherapy regimens, and help identify cellular injury and potentially tumorigenic mutations in their early stages so they can be targeted for

preventive treatment. Herein, we report a novel molecular mechanism for protection against IR in HLFs. Our findings show that IR leads to BRCA1-dependent increase in NRF2 protein levels, which in turn activates miR-140 expression (Fig. 6). In the classic NRF2 pathway, oxidative stress disrupts the association of NRF2 with its negative regulator KEAP1 [27], thus leading to its accumulation in the nucleus. Consistently, we observed that radiation-induced activation of BRCA1 stabilized NRF2 and promoted its translocation to and accumulation in the nucleus where it can bind to gene promoters and activate NRF2-dependent gene transcription. Our data indicate that BRCA1 is exported from the nucleus to the cytoplasm in response to IR. Once in the cytoplasm, BRCA1 binds to NRF2, promoting its nuclear translocation and allowing it to activate NRF2 target genes. Interestingly, we also observed an overall increase in KEAP1 protein levels in BRCA1-knockdown cells, indicating a potential novel role for BRCA1 in the negative regulation of KEAP1. Further studies will interrogate the characteristics of this relationship, which would help identify novel mechanisms controlling NRF2 activation.

We identified miR-140 as a novel downstream target of NRF2 and found that it is involved in the self-renewal of HLFs. To our knowledge, this is the first study showing the involvement of a miRNA in HLF radioprotection. Radiation treatment of HLFs significantly increased expression of miR-140 through a BRCA1/NRF2-dependent mechanism. Overexpression of miR-140 significantly suppressed the sphere formation of irradiated HLFs. This decrease in self-renewal may give radiation-damaged cells enough time to repair via restriction of cell proliferation. Additionally, it could be a protective mechanism against the formation of secondary cancers. Numerous proteins are involved in DNA damage response (DDR) and miRNAs have been found to be associated with many of these proteins [28,29]. Therefore, miRNAs could be used as potential markers to predict the radiosensitivity of a tissue. The considerable length of time miR-140 remains upregulated in mouse lung tissue (at least 1 year) and its role in the radioprotective pathway of HLFs make it a potentially useful biomarker for radiation response.

Using mouse primary lung fibroblast cultures and *in vivo* lung tissue from C57BL/6 WT, Brcal(KO) and Nrf2(KO) mice we verified our *in vitro* findings. Moreover, we found that Brcal, Nrf2 and miR-140 expression levels were still significantly upregulated in lung tissue even after a year mice were irradiated. This finding reveals the long-term effect and persistence of the Brcal/Nrf2/miR-140 radioprotective pathway after radiation treatment. By maintaining high expression of BRCA1, NRF2 and miR-140, cells that have been exposed to radiation may be protected from future cellular insults.

The BRCA1/NRF2/miR-140 pathway ultimately leads to a shield against radiation damage, which protects cells both in short and long terms. One of the major issues in current radiation treatment of lung and breast cancers is that high doses of IR can effectively kill tumor cells but also lead to lung tissue damage including pneumonitis and fibrosis. Significant work has been done to characterize the signaling pathways and cells involved in radiation-induced lung fibrosis [30], but emphasis should also be on the signaling pathways that might prevent radiation induced damage. To better protect lung tissue from significant damage during IR treatment, new therapeutics need to be developed that can shield normal cells from IR-induced damage. Activation of the BRCA1/NRF2/miR-140 pathway may

present a promising radioprotection strategy to protect lung fibroblasts from radiation-induced damage, which might further prevent radiation-induced lung injury for patients who are treated with radiotherapy.

Acknowledgments

Funding

This work was supported by Grants from the NCI R01 (CA163820A1 and CA157779A1) (Q.Z) and the American Cancer Society (RSG-12-006-01-CNE) (Q.Z.).

References

1. McCloskey SA, Lee SP, Steinberg ML. Roles and types of radiation in breast cancer treatment: early breast cancer, locoregionally advanced, and metastatic disease. *Curr. Opin. Obstet. Gynecol.* 2011; 23:51–57. [PubMed: 21150600]
2. Benveniste MF, Welsh J, Godoy MC, Betancourt SL, Mawlawi OR, Mundena RF. Newera of radiotherapy: an update in radiation-induced lung disease. *Clin. Radiol.* 2013; 68:e275–e290. [PubMed: 23473474]
3. Ghafoori P, Marks LB, Vujaskovic Z, Kelsey CR. Radiation-induced lung injury. Assessment, management, and prevention. *Oncology.* 2008; 22:37–47. [PubMed: 18251282]
4. Provatopoulou X, Athanasiou E, Gounaris A. Predictive markers of radiation pneumonitis. *Anticancer Res.* 2008; 28:2421–2432. [PubMed: 18751429]
5. Ohtsuiji M, Katsuoka F, Kobayashi A, Aburatani H, Hayes JD, Yamamoto M. Nrf1 and NRF2 play distinct roles in activation of antioxidant response element-dependent genes. *J. Biol. Chem.* 2008; 283:33554–33562. [PubMed: 18826952]
6. Ray PD, Huang BW, Tsuiji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell. Signal.* 2012; 5:981–990. [PubMed: 22286106]
7. Gorrini C, Baniasadi PS, Harris IS, Silvester J, Inoue S, Snow B, et al. BRCA1 interacts with Nrf2 to regulate antioxidant signaling and cell survival. *J. Exp. Med.* 2013; 210:1529–1544. [PubMed: 23857982]
8. Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat. Cell Biol.* 2009; 11:228–234. [PubMed: 19255566]
9. Chaudhry MA. Radiation-induced microRNA: discovery, functional analysis, and cancer radiotherapy. *J. Cell. Biochem.* 2014; 115:436–449. [PubMed: 24129925]
10. Chorley BN, Campbell MR, Wang X, Karaca M, Sambandan D, Bangura F, et al. Identification of novel NRF2-regulated genes by ChIP-Seq: influence on retinoid X receptor alpha. *Nucleic Acids Res.* 2012; 40:7416–7429. [PubMed: 22581777]
11. Shah NM, Rushworth SA, Murray MY, Bowles KM, MacEwan DJ. Understanding the role of NRF2-regulated miRNAs in human malignancies. *Oncotarget.* 2013; 8:1130–1142. [PubMed: 24029073]
12. Joo MS, Lee CG, Koo JH, Kim SG. miR-125b transcriptionally increased by Nrf2 inhibits AhR repressor, which protects kidney from cisplatin-induced injury. *Cell Death Dis.* 2013; 4:e899. [PubMed: 24176857]
13. Shah NM, Zaitseva L, Bowles KM, MacEwan DJ, Rushworth SA. NRF2-driven miR-125B1 and miR-29B1 transcriptional regulation controls a novel anti-apoptotic miRNA regulatory network for AML survival. *Cell Death Differ.* 2015; 22:654–664. [PubMed: 25323587]
14. Singh A, Happel C, Manna SK, Acquah-Mensah G, Carrerero J, Kumar S, et al. Transcription factor NRF2 regulates miR-1 and miR-206 to drive tumorigenesis. *J. Clin. Invest.* 2013; 123:2921–2934. [PubMed: 23921124]
15. Zhang Y, Xia J, Li Q, Yao Y, Eades G, Gernapudi R, et al. NRF2/long noncoding RNA ROR signaling regulates mammary stem cell expansion and protects against estrogen genotoxicity. *J. Biol. Chem.* 2014; 289:31310–31318. [PubMed: 25231996]

16. Zhang Y, Eades G, Yao Y, Li Q, Zhou Q. Estrogen receptor α signaling regulates breast tumor-initiating cells by down-regulating miR-140 which targets the transcription factor SOX2. *J. Biol. Chem.* 2012; 287:41514–41522. [PubMed: 23060440]
17. Seluanov A, Vaidya A, Gorbunova V. Establishing primary adult fibroblast cultures from rodents. *J. Vis. Exp.* 2010; 44:2033. [PubMed: 20972406]
18. Yamashita S, Miyaki S, Kato Y, Yokoyama S, Sato T, Barrionuevo F, et al. L-Sox5 and Sox6 proteins enhance chondrogenic miR-140 microRNA expression by strengthening dimeric Sox9 activity. *J. Biol. Chem.* 2012; 287:22206–22215. [PubMed: 22547066]
19. Eades G, Yao Y, Yang M, Zhang Y, Chumsri S, Zhou Q. miR-200a regulates SIRT1 expression and epithelial to mesenchymal transition (EMT)-like transformation in mammary epithelial cells. *J. Biol. Chem.* 2011; 286:25992–26002. [PubMed: 21596753]
20. Song G, Zhang Y, Wang L. MicroRNA-206 targets notch3, activates apoptosis, and inhibits tumor cell migration and focus formation. *J. Biol. Chem.* 2009; 284:31921–31927. [PubMed: 19723635]
21. Feng Z, Kachnic L, Zhang J, Powell SN, Xia F. DNA damage induces p53-dependent BRCA1 nuclear export. *J. Biol. Chem.* 2004; 279:28574–28584. [PubMed: 15087457]
22. Wang H, Yang ES, Jiang J, Nowsheen S, Xia F. DNA damage-induced cytotoxicity is dissociated from BRCA1's DNA repair function but is dependent on its cytosolic accumulation. *Cancer Res.* 2010; 70:6258–6267. [PubMed: 20631074]
23. Kim SB, Pandita RK, Eskiocak U, Ly P, Kaisani A, Kumar R, et al. Targeting of Nrf2 induces DNA damage signaling and protects colonic epithelial cells from ionizing radiation. *Proc. Natl. Acad. Sci. U.S.A.* 2012; 109:E2949–E2955. [PubMed: 23045680]
24. Tsukimoto M, Tamaishi N, Homma T, Kojima S. Low-dose gamma-ray irradiation induces translocation of Nrf2 into nuclear in mouse macrophage RAW264.7 cells. *J. Radiat. Res.* 2010; 51:349–353. [PubMed: 20410673]
25. Liu Z, Huang S. Inhibition of miR-191 contributes to radiation-resistance of two lung cancer cell lines by altering autophagy activity. *Cancer Cell Int.* 2015; 15:16. [PubMed: 25685068]
26. Li Q, Yao Y, Eades G, Liu Z, Zhang Y, Zhou Q. Downregulation of miR-140 promotes cancer stem cell formation in basal-like early stage breast cancer. *Oncogene.* 2014; 33:2589–2600. [PubMed: 23752191]
27. Yi YW, Kang HJ, Bae I. BRCA1 and oxidative stress. *Cancers (Basel).* 2014; 6:771–795. [PubMed: 24704793]
28. Czochoz JR, Glazer PM. MicroRNAs in cancer cell response to ionizing radiation. *Antioxid. Redox Signal.* 2014; 21:293–312. [PubMed: 24206455]
29. Hu H, Gatti RA. MicroRNAs: new players in the DNA damage response. *J. Mol. Cell Biol.* 2011; 3:151–158. [PubMed: 21183529]
30. Ding H, Li JJ, Sun LQ. Molecular mechanisms and treatment of radiation-induced lung fibrosis. *Curr. Drug Targets.* 2013; 14:1247–1356.

Fig. 1.

Radiation induces BRCA1-dependent activation of NRF2 in human lung fibroblasts. Non-irradiated and irradiated (20 Gy) cells were harvested 24 h after treatment and western blot analysis was used to examine the expression of BRCA1 and NRF2. (A) IR induced the upregulation of BRCA1 and NRF2 in CCD-19Lu HLFs. (B) NRF2 expression in HLFs is BRCA1-dependent. Cells with or without siRNA (siBRCA1) transfection were used to examine the expression of BRCA1 and NRF2. (C) IR induced the BRCA1-dependent activation of NRF2. BRCA1 siRNA was used to knock down BRCA1. (D) IR treatment altered cytoplasmic levels of BRCA1 and NRF2. Non-irradiated and irradiated cells were transfected with siRNA against BRCA1 in irradiation experiments to examine the impact of BRCA1 on levels of NRF2. β-actin was used as the loading control for the cytoplasmic fraction. (E) Nuclear protein levels of BRCA1 and NRF2 were altered by IR treatment. PCNA was used as the loading control for the nuclear fraction. A representative immunoblot from three independent experiments giving similar results is shown for each western blot experiment. Densitometry for western blot was performed using UN-SCAN IT Gel program.

Fig. 2. BRCA1 negatively regulates KEAP1. (A) BRCA1 knockdown led to elevated levels of KEAP1 in non-irradiated and irradiated lung fibroblasts. Non-irradiated and irradiated (20 Gy) cells in the presence or absence of siRNA BRCA1 were harvested 24 h after treatment and cytoplasmic and nuclear fractions were isolated for assessing the expression of KEAP1. β-actin and PCNA were used as the loading controls for the cytoplasmic and nuclear fractions, respectively. (B) Immunofluorescent staining of BRCA1, NRF2 and KEAP1 in HLFs in response to radiation treatment. Cells were fixed 24 h after irradiation and then stained with Cy3-conjugated antibody against KEAP1 and FITC-conjugated antibody against BRCA1 or NRF2. (C) The translocation of BRCA1 and NRF2 was altered in response to IR. Immunofluorescence staining was performed with Cy3-conjugated antibody against BRCA1 and FITC-conjugated antibody against NRF2. A representative image from two independent experiments is shown for immunofluorescence staining.

Fig. 3.

Expression of miR-140, a downstream target of NRF2, is activated upon IR. (A) IR increased miR-140 expression in HLFs. Non-irradiated and irradiated (20 Gy) cells were fixed 24 h after irradiation and then subjected to *in situ* hybridization for detecting miR-140 expression. (B) miR-140 promoter region contains a binding site for NRF2 and miR-140 is a transcription target of NRF2. ChIP assays were performed on control and NRF2-overexpressing (OE) MCF10A cells to examine the *in vivo* binding of NRF2 to the DNA region containing its putative binding site. (C) Expression of miR-140 in HLFs is BRCA1-dependent. The qRT-PCR analysis of miR-140 expression was performed on CCD-19Lu cells transfected with the siRNA against BRCA1. (D) Expression of miR-140 in HLFs is NRF2-dependent. The qRT-PCR analysis of miR-140 expression was performed on CCD-19Lu cells transfected with the shRNA against NRF2. Data represent the mean $\pm$ S.D (n = 3).

Fig. 4. BRCA1/NRF2/miR-140 is a radioprotective signaling pathway in human lung fibroblasts. (A) Evaluation of the role of miR-140 in radioprotection with sphere formation assays. Non-irradiated and 20 Gy-irradiated cells transfected with or without miR-140 overexpressing (OE) plasmids or miR-140 inhibition (KD) plasmids were subjected to the sphere formation assay. (B, C) Evaluation of the importance of BRCA1 for radioprotection via scratch assay. Non-irradiated and irradiated cells (20 Gy) transfected with or without siRNA BRCA1 as well as shRNA NRF2 were synchronized with serum starvation for 24 h before the assay. The migration capacity of the cells was calculated as the ratio of filled gaps at 24 h to the unfilled gap at 0 hour. (D) Evaluation of the importance of BRCA1 and NRF2 for radioprotection with collagen gel contraction assays. Non-irradiated and 20 Gy-irradiated cells transfected with or without siRNA BRCA1 or shRNA NRF2 were subjected to the collagen gel contraction assay. The contraction capacity was calculated by measuring the gel area with ImageJ 24 h after the stress was lifted. Data represent the mean $\pm$ S.D. (n = 3). *p < 0.05; **p < 0.01.

Fig. 5.

The Brca1/Nrf2/miR-140 signaling axis is a radioprotective pathway in mouse primary cultures and *in vivo* lung tissue. (A) Expression analysis of the Brca1, Nrf2 and miR-140 in mouse lung tissue with or without IR treatment. C57BL/6 mice were left unirradiated or they were irradiated with a single dose of IR (13 Gy). The lung tissues were harvested one year post-irradiation and the expression levels of Brca1, Nrf2 and miR-140 were detected with immunohistochemistry analysis. A representative image from three independent experiments is shown. (B) Evaluation of the importance of Nrf2 for radioprotection in primary lung fibroblast cultures via scratch assay. Primary lung fibroblast cultures isolated from wild-type (WT) and Nrf2 (KO) C57BL/6 mice were used in scratch assay. (C, D) Evaluation of the importance of Nrf2 and miR-140 for radioprotection in primary cultures with sphere formation assay. Primary lung fibroblast cultures isolated from wild-type (WT), Nrf2 (KO) C57BL/6 and miR-140 (KO) mice were used in sphere formation assay. Data represent the mean $\pm$ S.D (n = 3). *p < 0.05; **p < 0.01.

Fig. 6.

IR-induced activation of BRCA1/NRF2/miR-140 signaling pathway in human lung fibroblasts. In the absence of irradiation BRCA1 and NRF2 are mainly located in the nucleus and cytoplasm, respectively. KEAP1 is expressed more in cytoplasm and it functions as a negative regulator of NRF2. IR causes the translocation of BRCA1 from nucleus to cytoplasm, where it inhibits KEAP1 and leads the dissociation of KEAP1–NRF2 complex. NRF2 translocates to and accumulates in nucleus where it can induce the expression of antioxidant genes as well as miRNAs including miR-140. IR activated BRCA1/NRF2/miR-140 signaling axis then decreased the self-renewal of lung fibroblasts while increasing their migration and contraction.