Unraveling the Global microRNAome Responses to Ionizing Radiation in Human Embryonic Stem Cells

Mkytya V. Sokolov*, Irina V. Panyutin, Ronald D. Neumann

Nuclear Medicine Division, Department of Radiology and Imaging Sciences, Clinical Center, National Institutes of Health, Bethesda, Maryland, United States of America

Abstract

MicroRNAs (miRNA) comprise a group of short ribonucleic acid molecules implicated in regulation of key biological processes and functions at the post-transcriptional level. Ionizing radiation (IR) causes DNA damage and generally triggers cellular stress response. However, the role of miRNAs in IR-induced response in human embryonic stem cells (hESC) has not been defined yet. Here, by using system biology approaches, we show for the first time, that miRNAome undergoes global alterations in hESC (H1 and H9 lines) after IR. Interrogation of expression levels of 1,090 miRNA species in irradiated hESC showed statistically significant changes in 54 genes following 1 Gy of X-ray exposures; global miRNAome alterations were found to be highly temporally and cell line - dependent in hESC. Time-course studies showed that the 16 hr miRNAome radiation response of hESC is much more robust compared to 2 hr-response signature (only eight genes), and may be involved in regulating the cell cycle. Quantitative real-time PCR performed on some miRNA species confirms the robustness of our miRNA microarray platform. Positive regulation of differentiation-, cell cycle-, ion transport- and endomembrane system-related processes were predicted to be negatively affected by miRNAome changes in irradiated hESC. Our findings reveal a fundamental role of miRNAome in modulating the radiation response, and identify novel molecular targets of radiation in hESC.

Introduction

MicroRNAs (miRNAs) are considered to constitute a class of small noncoding RNAs vitally involved in regulation of gene expression and signal transduction [1]. Published data suggest miRNAs act as post-transcriptional regulators that may control the expression of about 60% of human genes [2] by means of messenger RNA (mRNA) decay and/or translational repression [3]. Deregulated expression of miRNAs was shown to underlie many diseased states; and miRNAome patterns prove to be highly specific, in many cases accurately reflecting the stage and prognosis of disease [4,5]. Increasing body of evidence implies changes in miRNA expression profiles after genotoxic stress exposures, including ionizing radiation (IR) [6,7,8]. Previous studies analyzed miRNA expression following IR either in fully differentiated human normal somatic cells/artificial 3D tissues [9,10,11,12,13,14] or in cancerous cells [15,16,17,18,19,20]. Human embryonic stem cells (hESC) represent pluripotent human cells with unique capabilities to differentiate into virtually all cell types of a human body. Human stem cells are believed to be endowed with mechanisms to ensure the superior genome fidelity that are just begun to be explored systematically [21,22]. Exposures to medical, environmental or accidental sources of radiation can challenge the human well-being in the exposed populations, including pregnant women. We, and others, recently studied the IR response of hESC extensively [23,24,25,26,27,28,29]. However, none of the published papers focused on elucidating the miRNA signatures in hESC after IR exposures. Moreover, since the human genome is estimated to encode more than 1,000 of distinct miRNA species, it is imperative to employ high-throughput methodologies to fully analyze the role of miRNA species in biological processes. To the best of our knowledge, only one recent report analyzed miRNAome changes in irradiated human cells at a whole genome-wide level [20].

In the present work, we aimed to study the effects of IR on global miRNAome in hESC, to perform prediction of the biological processes/themes affected by miRNAome changes, and to develop a miRNA-based gene expression signature specific for irradiated, but not for non-irradiated, hESC. For the first time, we found that miRNAome undergoes genome-wide alterations in hESC after IR. All miRNAs published in the Sanger miRBase release version 15.0 (http://microrna.sanger.ac.uk/sequences/index.shtml) were interrogated. The expression levels of 1,090 miRNA species in irradiated hESC showed statistically significant changes in 54 genes following 1 Gy of X-ray exposures (p<0.05). We found that many miRNA species were modulated in a cell line-specific manner in hESC after IR, with H1 cell line being more radiation responsive than H9. Hierarchical clustering, class prediction and Gene Ontology analysis were performed to characterize hESC miRNAome response to radiation in more detail. Positive regulation of differentiation-, cell cycle-, ion transport- and endomembrane system-related processes were predicted as being negatively affected by alterations in miRNAome in irradiated hESC.
Materials and Methods

1. Cell culture and treatments

Human ESCs (H1 and H9 cell lines, WiCell, Madison, WI, passage 35-48; BG01V line, ATCC, Manassas, VA) were routinely cultured in mTeSR-1 medium (Stemcell Technologies, Vancouver, Canada) using cell culture vessels coated with BD Matrigel hESC-qualified Matrix (BD Biosciences, San Jose, CA) at 37°C and 5% CO2. Cells were grown following supplier’s protocol and as described in [23,24]. Cell cultures were exposed to 1 Gy of X-ray irradiation using X-RAD 320 Biological Irradiator unit (Precision X-Ray, Inc., North Branford, CT; dose rate about 1 Gy/min; 320 kV, 12.5 mA) then the cells were returned to CO2 incubator and harvested at 2 h and 16 h post-irradiation for analysis. The mock-irradiated cells for each time-point were used as a control. For functional analysis of hsa-mir-575, both gain- and loss-of-function studies were carried out. For overexpression studies, specific hsa-mir-575 mirVana miRNA mimic transfection experiments were performed with H1 hESC cultures according to manufacturer’s protocol (Applied Biosystems, Carlsbad, CA). MirVana miRNA inhibitor (Applied Biosystems, Carlsbad, CA) was used to knock-down hsa-mir-575 expression in a separate set of experiments. Transfection studies, in parallel with negative control miRNA inhibitor experiments, were carried out with Stemfect RNA Transfection kit per vendor’s protocol (Stemgent, Cambridge, MA). To examine the viability of hESC in colonies upon hsa-mir-575 overexpression, cells were incubated at 37°C for 1 h with Hoechst 33342 (8 μg/ml; Molecular Probes, Eugene, OR) and propidium iodide (PI, 20 μg/ml; Sigma, St. Louis, MO) as in [25]. Cell colonies were visualized using a fluorescence microscope (Axioplan 2, Zeiss, Thornwood, NY) equipped with a fluorescent light source.

2. RNA sample preparation, probe labeling and DNA microarray procedure

The extraction of total RNA was performed with miRNeasy kit (Qiagen, Valencia, CA) per manufacturers’ instructions. The amount and quality of RNA samples were assessed on the Agilent 2100 Bioanalyzer with RNA 6000 Nano Reagents and Supplies (Agilent, Santa Clara, CA). Subsequently, 0.5 μg of total RNA was used in each reaction to generate labeled samples with miRCURY LNA microRNA Hy5 Power labeling kit (Exiqon, Woburn, MA). MiRNA spike control was added to the RNA samples prior to the labeling reactions following the manufacturer’s protocol. The labeled targets corresponding either to experimental or control samples were separately hybridized to “3D-Gene” oligo microarrays provided by the manufacturer’s (Toray Industries Inc., Tokyo, Japan; miRBase release 15.0) containing 1,090-elements spotted in duplicate using Takara Hybridization chambers (Takara Bio, Inc., Japan). Protocols for microarray hybridization and washing were as provided by manufacturer. Hybridized DNA microarrays were scanned on an Axon GenePix DNA microarray scanner (Molecular Devices, Inc., Sunnyvale, CA), and TIFF images were subsequently generated for further analysis. The sample labeling, hybridization, “3D-Gene” array washing and scanning were conducted by Toray Industries Inc. (Tokyo, Japan).

3. Microarray data analysis

Analyses were performed using BRB-ArrayTools Version 4.2.0 developed by Dr. Richard Simon and BRB-ArrayTools Development Team (Biometric Research Branch, National Cancer Institute, NIH). Time-matched irradiated versus sham-irradiated samples were used to determine the radiation-responsive miRNA species for each data point. The data from the microarray was collected and analyzed in accordance to the Minimum Information About a Microarray Experiment (MIAME) guidelines. MIAME-compliant raw data for this series of experiments have been deposited in the ArrayExpress database maintained by the European Bioinformatics Institute (accession no. E-MEXP-3366). Differentially expressed miRNA genes were identified using random-variance t-test [30] and as in [31,32]. Changes in gene expression were considered statistically significant if the p values for corresponding genes were less than 0.05.

4. Prediction of miRNA targets

Candidate miRNA species with a microarray intensity signal ≥100 and p-value≤0.05, identified as being differentially expressed after IR exposures with BRB-ArrayTools, were chosen for target prediction analysis. Miranda database was used to predict miRNA targets [33]. Targets were input to the Database for Annotation, Visualization and Integrated Discovery (DAVID) [34,35] themes analysis. Biological themes/processes were determined using the Functional Annotation Clustering feature using p-value less than 0.05.

5. Quantitative real-time PCR

Total RNA samples used for the microarray analysis were reverse transcribed to cDNA template using RT-specific primers (Applied Biosystems, Carlsbad, CA). Then, TaqMan miRNA assays were performed in triplicate using miRNA Cells-to-Ct kit reagents (Applied Biosystems) per manufacturer’s instructions. The U6 snRNA was used as an internal control. RT-PCR was performed on iCycler iQ (Bio-Rad, Inc., Hercules, CA) in 20-μl reactions by using TaqMan Assay-on-Demand primers/probe sets (Applied Biosystems) for the following miRNA genes: hsa-miR-302b, hsa-miR-575, hsa-miR-1274b, hsa-miR-1915 and hsa-miR-1973. In a separate set of experiments, H1 hESC cultures transfected either with hsa-mir-575 mirVana miRNA mimic or miRNA inhibitor were subjected to lysis 5 days post-transfection. TaqMan gene expression studies for POU5F1 and SOX2 were carried out in triplicate with Cells-to-Ct; kit reagents (Applied Biosystems, and 18S RNA expression was used as a reference. Quantitative RT-PCR data were analyzed as in [32].

Results and Discussion

To identify radiation inducible miRNA species and characterize changes in miRNAome after IR in hESC, we exposed both H1 and H9 cell lines to 1 Gy of X-rays. These cell lines are most extensively studied among hESC. Our miRNA microarray data analysis revealed that 53 and six miRNAs, were differentially expressed in irradiated H1 and H9 cells respectively, compared to corresponding sham-irradiated hESC cultures (Figure S1). These results indicate that miRNA expression profiles of hESC after IR exposures are largely cell line-dependent.

Further bioinformatics analyses revealed that only seven miRNA genes were differentially expressed in H1 at the “early” (2 hr) time point post-IR (p<0.05) (Figure 1); among them, only four miRNA species showed more than 1.5 – fold induction compared to sham-irradiated cells (hsa-miR-15b, hsa-miR-1274b, hsa-miR-302b and hsa-miR-1973) (Table 1). Gene expression studies showed that IR-induced alterations in miRNAome in H9 cell line (2 hr post-IR) involve the up-regulation of only two miRNA genes, namely, hsa-miR-1973 and hsa-miR-92a (Table 2). In both cell lines, the level of upregulation of miRNA species at this time point was less or equal to 2-fold over corresponding non-irradiated baseline values. No significant down-regulation of miRNA was observed at 2 hr following IR exposures. Interestingly, lack of statistically
Comparison of the dynamics of IR response between H1 and H9 cell lines.

proven repression of any miRNA species at relatively early 4 hr post 1.25 Gy IR exposure of human blood was reported recently [13].

On the contrary, at 16 hr timepoint after IR, we observed both statistically significant up-regulation (Table 3) and repression (Table 4) of select miRNA species in H1 hESC. Irradiated H1 cells showed up-regulation of 42 miRNA genes, and down-regulation of only eight miRNAs at this time post 1 Gy IR exposure (p<0.05). A much higher magnitude of changes in the level of expression of IR-modulated miRNAs was observed as part of the “late” hESC response to IR (Table 5); 33 miRNA species were induced more than 2 – fold in irradiated H1 compared to control sham-exposed cell cultures. In contrast, we found that only five miRNAs were significantly overexpressed in H9 cell line, with a single miRNA species, hsa-miR-575, being overexpressed more than 2 fold (p<0.05) (Table 5).

To identify the factors that affect the patterns of microRNAome changes after IR exposures, all experimental samples were subjected to hierarchical clustering (Figure 2). This analysis grouped hESC samples in accordance to IR exposure conditions, as a result of dynamic of cellular response to radiation. The timing of post-IR exposure response, and not a cell type – specific changes, was the most prominent determinant of the clustering of samples analyzed.

Our recent study demonstrated no statistically significant downregulation of mRNA gene transcripts after 1 Gy of IR exposure in H9 cells [24]. Intriguingly, in the present study we observed a few miRNAs that were upregulated in H9 under these same conditions (Table 2 and Table 5). Since miRNAs are considered to be one of the powerful epigenetic means to silence gene expression, it may seem puzzling at first glance. However, it is thought that in mammals the primary mode of miRNA-mediated gene repression is through inhibition of translation, not direct mRNA target degradation [36]. Therefore, it could explain the lack of downregulation of any protein-coding transcripts observed in our previous work [24]. The repression of genes involved in hESC differentiation after IR exposures may explain the maintenance of pluripotentiality in surviving irradiated hESC that we reported before [25]. Changes in cell cycle profile of IR-exposed hESC [24] may also be the consequence of miRNAome alterations. For example, one of the key genes responsible for cell cycle progression and about 26% of all phosphorylation events in hESC is CDK1/2 [37], which we found to be the major predicted target of the most highly upregulated miRNA (hsa-miR-575) in our present study. Interestingly, hsa-miR-575 is known to be overexpressed in some aggressive human cancers [38], and is downregulated upon human leukemic HL-60 cell differentiation induced by 4-hydroxynonenal, a product of lipid peroxidation [39]. We sought to investigate the role of hsa-miR-575 in hESC in more detail. Our studies aimed to mimic the overexpression of this miRNA species showed no evidence for an increase in cell killing in H1 hESC cultures (Figure S2). Moreover, we did not observe signs of differentiation in these cultures since continuous expression of markers of pluripotency, such as POU5F1 and SOX2, was evident at least 5 days post-transfection (Table S1). Interestingly, upon treatment of irradiated H1 cells with hsa-miR-575 inhibitor as part of our loss-of-function studies, we observed substantial down-regulation of POU5F1 and upregulation of SOX2 (Table S1). Both genes are known to play an important role in maintenance of pluripotency in undifferentiated hESC, and major alterations in their expression levels may result in differentiation [40,41]. Our results may support the assumption that upregulation of hsa-miR-575 in irradiated hESC serves to prevent differentiation of these cells, but additional studies into the exact function of hsa-miR-575 are needed to be performed in a future.

In general, the majority of differentially expressed miRNAs that we identified in our global screen of irradiated hESC are not well-

![Figure 1. Venn diagrams of significantly upregulated miRNA species in human embryonic stem cells after IR exposures (p<0.05). Comparison of the dynamics of IR response between H1 and H9 cell lines. doi:10.1371/journal.pone.0031028.g001](image)

Table 1. Differentially expressed miRNA species in H1 hESC at 2 hr post 1 Gy of X-ray exposures (p<0.05).

| Name       | p-value | Fold change 2 hr |
|------------|---------|------------------|
| hsa-miR-15b| 0.0136  | 2.018            |
| hsa-miR-1274b| 0.0161 | 1.789            |
| hsa-miR-302b| 0.0107 | 1.623            |
| hsa-miR-1973| 0.0126 | 1.504            |
| hsa-miR-720| 0.0151  | 1.484            |
| hsa-miR-1274a| 0.0385 | 1.480            |
| hsa-miR-20a| 0.0007  | 1.475            |

doi:10.1371/journal.pone.0031028.t001

Table 2. Differentially expressed miRNA species in H9 hESC at 2 hr post 1 Gy of X-ray exposures (p<0.05).

| Name       | p-value | Fold change 2 hr |
|------------|---------|------------------|
| hsa-miR-1973| 0.0309  | 1.732            |
| hsa-miR-92a| 0.0193  | 1.548            |

doi:10.1371/journal.pone.0031028.t002
characterized yet. However, the involvement of some IR-responsive miRNAs in biological processes was already reported. For example, the most highly induced miRNA in H1 cells at 2 hr post-1 Gy of IR exposures, hsa-miR-15b, was shown to sensitize human cancer cells to apoptosis [42]. This might be relevant for cell fate choice in hESCs as well, since massive apoptosis was observed in irradiated hESCs after 1 Gy of X-ray exposures [25]. In addition, the up-regulation of this miRNA species could influence many other aspects of cellular homeostasis: miR-15b is overexpressed in bronchioalveolar lung stem cells [43], is a direct transcriptional target of E2F1 modulating cell cycle progression [44], and is known to affect cell metabolism through decreasing ATP levels [45].

Hsa-mir-302b was shown to be highly expressed in hESCs [46] and is directly responsible for “stemness” characteristics in human cells being part of stem cell-enriched mir-302-367 cluster [47,48]. Mir-302 species were determined to be highly integrated with Oct4/Sox2 transcriptional machinery in hESCs being partly responsible for unique abbreviated cell cycle profile in hESCs [49]. The modest upregulation of this miRNA species following IR exposures may prevent hESCs from undergoing spontaneous differentiation. Other IR-modulated miRNA species, such as hsa-mir-20a, is a component of yet another stem cell-enriched family, namely, mir-17-92, which is implicated in modulating E2F activity on cell cycle progression [50] and in the repression of execution of cellular senescence program [51,52]. Hsa-mir-149* also regulates E2F1 activity and exerts its function through induction of apoptosis [53]. One more hESC highly expressed miRNA, hsa-mir-1909, was implicated in hESC physiology by targeting Notch [54].

A large number of overexpressed IR-responsive miRNAs that we identified in our work were found to be deregulated in human cancers, such as hsa-mir-513 [55], hsa-mir-744 [56], hsa-mir-92a [57,58], hsa-mir-1228* [59], hsa-mir-671-5p [60], hsa-mir-638 [38], hsa-mir-370 [61], and hsa-mir-675 [62]. Hsa-mir-663 is involved in inflammatory conditions and suppression of cell proliferation [63,64] which can underlie hESC response to IR exposures. Interestingly, two of significantly upregulated miRNAs after IR exposures in hESCs, such as hsa-mir-1275 and hsa-mir-302b, were

Table 3. Up-regulated miRNA species in H1 hESC at 16 hr post 1 Gy of IR exposures (p<0.05).

| Name     | p-value | Fold change 16 hr |
|----------|---------|-------------------|
| hsa-miR-575 | 0       | 3.817             |
| hsa-miR-1915 | 0.003  | 3.322             |
| hsa-miR-3195 | 0.004  | 3.105             |
| hsa-miR-3196 | 0.002  | 2.845             |
| hsa-miR-744  | 0.018  | 2.843             |
| hsa-miR-1908 | 0.002  | 2.804             |
| hsa-miR-1975 | 0.009  | 2.780             |
| hsa-miR-663  | 0.005  | 2.762             |
| hsa-miR-3178 | 0       | 2.744             |
| hsa-miR-149* | 0.002  | 2.728             |
| hsa-miR-4257 | 0.002  | 2.726             |
| hsa-miR-762  | 0.005  | 2.671             |
| hsa-miR-1469 | 0.003  | 2.668             |
| hsa-miR-4281 | 0       | 2.663             |
| hsa-miR-614  | 0.003  | 2.646             |
| hsa-miR-4327 | 0.004  | 2.609             |
| hsa-miR-2861 | 0.022  | 2.581             |
| hsa-miR-1268 | 0.014  | 2.555             |
| hsa-miR-24   | 0.013  | 2.492             |
| hsa-miR-1228* | 0.002  | 2.461             |
| hsa-miR-638  | 0.012  | 2.401             |
| hsa-miR-1275 | 0.0034 | 2.396             |
| hsa-miR-711  | 0.001  | 2.385             |
| hsa-miR-671-5p | 0.005  | 2.340             |
| hsa-miR-3197 | 0.004  | 2.312             |
| hsa-miR-1909 | 0       | 2.205             |
| hsa-miR-3141 | 0.0001 | 2.167             |
| hsa-miR-3180-3p | 0     | 2.130             |
| hsa-miR-1308 | 0.0009 | 2.112             |
| hsa-miR-92b* | 0.005  | 2.105             |
| hsa-miR-187* | 0.009  | 2.055             |
| hsa-miR-513a-5p | 0.0081 | 2.032             |
| hsa-miR-1973 | 0.0126 | 2.028             |
| hsa-miR-370  | 0.0015 | 1.943             |
| hsa-miR-494  | 0.0063 | 1.866             |
| hsa-miR-1914* | 0.004  | 1.758             |
| hsa-miR-940  | 0.0189 | 1.725             |
| hsa-miR-1274b | 0.0161 | 1.631             |
| hsa-miR-1260b | 0.0007 | 1.547             |
| hsa-miR-675  | 0.0311 | 1.497             |
| hsa-miR-874  | 0.0159 | 1.418             |
| hsa-miR-612  | 0.0219 | 1.404             |

doi:10.1371/journal.pone.0031028.t003

Table 4. Down-regulated miRNA species in H1 hESC at 16 hr post 1 Gy of IR exposures (p<0.05).

| Name     | p-value | Fold change 16 hr |
|----------|---------|-------------------|
| hsa-miR-886-3p | 0.0448 | 0.712             |
| hsa-miR-93   | 0.0013 | 0.706             |
| hsa-miR-302d | 0.003  | 0.681             |
| hsa-miR-17   | 0.028  | 0.639             |
| hsa-miR-20a  | 0.0007 | 0.626             |
| hsa-miR-302a | 0.0004 | 0.586             |
| hsa-miR-20b  | 0.0008 | 0.567             |
| hsa-miR-302b | 0.0107 | 0.449             |

doi:10.1371/journal.pone.0031028.t004

Table 5. Differentially expressed miRNA species in H9 hESC at 16 hr post 1 Gy of X-ray exposures (p<0.05).

| Name     | p-value | Fold change 16 hr |
|----------|---------|-------------------|
| hsa-miR-575 | 0.0227 | 2.171             |
| hsa-miR-513a-5p | 0.0383 | 1.796             |
| hsa-miR-711  | 0.0283 | 1.686             |
| hsa-miR-1973 | 0.0309 | 1.535             |
| hsa-miR-1275 | 0.0387 | 1.501             |

doi:10.1371/journal.pone.0031028.t005
recently found to localize to mitochondria [65]. Given the importance of mitochondrial compartment in the maintenance of stem cell homeostasis and responses to different stress factors [66], the functional validation of targets of these miRNA species might prove to be instrumental in unraveling the underpinnings of hESC unique biological capabilities.

We observed several miRNA species that were downregulated following irradiation (Table 4). Repression of hsa-mir-93 could block cell differentiation after IR exposures in part by relieving STAT3 [67]. Other biological effect of decreased expression of hsa-mir-93 at 16 hr post-IR in H1 could be a decreased cell survival [68], probably as a result of modulation of its targets such as E2F1 and CDKN1A [69]. Interestingly, CDKN1A was found to be a target for other down-regulated miRNA species we identified in our present study, namely, hsa-mir-302a, hsa-mir-302b, and hsa-mir-302d [70]. With CDKN1A being one of the key radiation-responsive genes in human cells, the possible involvement of miRNAome alterations in its regulation after IR definitely merits further investigations.

We attempted to compare our data on global miRNAome alterations in irradiated hESC with published datasets. However, since the knowledge about changes in miRNA levels in human cells following irradiation is very limited to date, we were able to identify only a few miRNA species in our subset of IR-modulated miRNAs which were reported before as responsive to IR exposures. Among them, we found hsa-mir-20a, shown to be IR-responsive in fully differentiated human endothelial cells [10] and implicated in regulation of IR-induced premature senescence [52]; hsa-mir-24, a member of the miR-23b cluster that interferes with TGF-β expression and shown to be up-regulated after IR [20]. Mir-24 is implicated in halting cell cycle progression and inhibition of apoptosis [71], and impairment of DDR and senescence program execution [72,73]. In addition, hsa-mir-744 and hsa-mir-17 were also found to be deregulated by IR exposures by others [20]. Our studies with BG01V line of hESC, considered being karyotypically abnormal, identified hsa-mir-1915 and hsa-mir-1274b as differentially expressed at 2 hr and 16 hr post-IR, respectively (Table S2). We believe that further experiments will examine the relative contribution of cell type specificity to a repertoire of IR-modulated miRNAs at a whole-genome level in various human cells.

We analyzed the possible targets of differentially expressed miRNAs by running MiRanda algorithm search. The results are presented in Tables S3, S4, S5, S6, S7. At least some of the highly scored targets may represent an integrated network with miRNAome and act in concert in irradiated hESC; for example, LIN9, a putative target of hsa-mir-1973, regulates MYB which is by itself is predicted to be a target of hsa-mir-15b (Table S3), and crucially affects the cell cycle machinery through the cyclins and CDK1 [74]. Hence, modulation of regulators of key biological processes by alterations in miRNAome may represent a powerful strategy used by IR-exposed hESC to cope with genotoxic stress.

Despite numerous recent advances, the human miRNAome still remains mainly unexplored regarding the physiological function of specific miRNA species within cells. It should be noted that deciphering the function of individual miRNAs is challenging. There are many families comprising microRNAs differing only in one-two nucleotides which make a functional assignment to these...
redundant genes a complicated task. Each miRNA is thought to target numerous putative transcripts that have non-related functions, thereby the experimental validation of specific targets for miRNAs is still in its infancy. Therefore, determining which biological processes might be prime candidates for miRNA-mediated regulation of gene expression after IR exposures might

### Table 6. Gene Ontology analysis of affected biological themes based on predicted miRNA targets in H1 cell line.

| Exposures     | Overrepresented categories                        | EASE score |
|---------------|--------------------------------------------------|------------|
| 1 Gy, 2 hrs   | Positive regulation of cell differentiation      | 2.0E-4     |
|               | Cell projection                                  | 6.3E-4     |
|               | Ion binding                                      | 0.004      |
|               | Transcription activator activity                 | 0.0041     |
|               | Alternative splicing                             | 0.0067     |
|               | Cell cycle                                       | 0.01       |
|               | Response to endogenous stimulus                  | 0.013      |
|               | Pore complex                                     | 0.015      |
|               | Response to hormone stimulus                     | 0.019      |
|               | Endomembrane system                              | 0.026      |
|               | Regulation of transcription                      | 0.045      |
| 1 Gy, 16 hrs  | Protocadherin gamma                              | 5.0E-19    |
|               | Alternative splicing                             | 1.6E-16    |
|               | Membrane-enclosed lumen                          | 2.3E-5     |
|               | Regulation of mRNA stability                     | 2.9E-5     |
|               | Cell projection                                  | 6.2E-5     |
|               | Positive regulation of cell differentiation      | 2.2E-4     |
|               | Positive regulation of transcription from RNAP2 promoter | 5.1E-4 |
|               | mRNA stabilization                               | 7.6E-4     |
|               | Muscle tissue development                        | 0.0027     |
|               | Positive regulation of cell proliferation        | 0.0032     |
|               | Heart development                                | 0.0091     |

doi:10.1371/journal.pone.0031028.t006

### Table 7. Gene Ontology analysis of affected biological themes based on predicted miRNA targets in H9 cell line.

| Exposures     | Overrepresented categories                        | EASE score |
|---------------|--------------------------------------------------|------------|
| 1 Gy, 2 hrs   | Ion transport                                     | 0.0027     |
|               | Ion binding                                       | 0.0071     |
|               | Membrane-bounded vesicle                         | 0.012      |
|               | Heart development                                | 0.018      |
|               | Cell projection part                             | 0.019      |
|               | Endomembrane system                              | 0.02       |
|               | Positive regulation of cell differentiation      | 0.022      |
|               | Cytoskeleton                                     | 0.035      |
|               | Cell death                                       | 0.049      |
| 1 Gy, 16 hrs  | Mesenchyme development                            | 4.6E-4     |
|               | Cell cycle control                               | 5.2E-4     |
|               | Negative regulation of nucleic acid metabolic process | 6.9E-4 |
|               | Negative regulation of macromolecule biosynthesis | 0.0014     |
|               | Negative regulation of transcription             | 0.0019     |
|               | Heart development                                | 0.0056     |
|               | Cell morphogenesis involved in differentiation   | 0.011      |
|               | Cytoskeleton                                     | 0.048      |

doi:10.1371/journal.pone.0031028.t007
alterations in miRNAome affect the proteome of hESC of inhibition. Therefore, future studies will focus on how human proteome presumably remain effective over this degree [75]. Given these considerations, most proteins comprising the majority of IR-modulated miRNA showed a time-dependent ionizing radiation exposure s. We showed that the gene and cell cycle regulation.

projection, transcription activation, alternative splicing, cell death hESCs could result in an increased miRNA control of genes Table 7. Our data indicate that IR exposures of H1 and H9 predicted to be targeted by miRNAs are shown in Table 6 and biological themes overrepresented in the pools of the genes component categories. The results of the GO analysis of the biological themes overrepresented in the pools of the genes predicted to be targeted by miRNAs are shown in Table 6 and Table 7. Our data indicate that IR exposures of H1 and H9 hESCs could result in an increased miRNA control of genes involved in positive regulation of cell differentiation, cell projection, transcription activation, alternative splicing, cell death and cell cycle regulation.

To partially validate miRNAome changes we obtained in our study, we compared data on miRNAs expression with two independent techniques, that is, miRNA microarray platform and quantitative RT-PCR (Table 8). In general, we observed a good concordance between these datasets, thus confirming the robustness of our approach.

In summary, we found that microRNAome of human embryonic stem cells undergoes global alterations following ionizing radiation exposures. We showed that the gene expression signature for global miRNAome alterations at a “late”, 16 hr time point is significantly different in comparison to that of “early” response. The changes that we observed in the levels of miRNA species constituting the whole human microRNAome are substantially cell line-dependent, with H1 cell line of hESC being more responsive to IR than H9 line. Surprisingly, there were just a few H9-specific IR-modulated miRNA species compared to H1. Since these two cell lines are most thoroughly analyzed to date among all published hESC lines, it might be of interest to probe miRNAome of these lines under more various experimental settings in a future. We observed consistent patterns of miRNA response to IR exposures, including several miRNA species that were systematically differentially expressed at both time points. But the majority of IR-modulated miRNA showed a time-dependent response in both H1 and H9 hESCs, with a more robust response occurring at a late time point. It should be noted that the degree of target repression imposed by miRNAs is probably quantitatively modest since most of specific miRNA species endogenous targets is usually downregulated by less than 50% [75]. Given these considerations, most proteins comprising the human proteome presumably remain effective over this degree of inhibition. Therefore, future studies will focus on how alterations in miRNAome affect the proteome of hESC following IR-exposures, and how it translates to the ultimate cell fate of these unique human cells that show great promise in both disease modeling and cell regenerative therapy approaches.

The strength of our study is based upon the comprehensive coverage of the whole human miRNAome in an experimental platform that we employed, in profiling the changes in miRNAs in two different hESC lines, and in analyzing the dynamics of response of hESC to IR exposures at the level of miRNAome. We identified a number of radiation-responsive miRNA species with as yet unknown functions that provide a broad avenue to future research in this area. We attempted to outline some of hypothesis that can foster the investigation of functional relevance of miRNAome alterations in hESC. Our study reveals new insights into how hESC respond to genotoxic stress, in particular, to IR exposures resulting in global alterations in microRNAome in these cells. The findings may contribute to improved understanding of the biology of hESC, and the mechanisms underlying enhanced genomic maintenance in hESC.

Supporting Information

**Figure S1** Venn diagram of a total number of differentially expressed miRNA species in human embryonic stem cells after IR exposures (p<0.05).

(FIG)

**Figure S2** H1 hESC culture staining for viability upon hsa-mir-575 changes in gene expression studies. Cell cultures were stained with Hoechst 33342 (shown in blue) and propidium iodide (in red).

A – control, 0 Gy; B – mock transfection; C – hsa-mir-575 mimic transfection; D – 1 Gy, 24 hrs post- IR.

(FIG)

**Table S1** Taqman qRT-PCR on cultured H1 hESCs. Shown are means and standard errors for gene expression changes obtained following hsa-mir-575 expression modulation in comparison to mock-treated cell cultures.

(DOC)

**Table S2** Taqman qRT-PCR on cultured BG01V hESCs. Shown are means and standard errors for miRNA expression changes obtained for indicated timepoints after irradiation in comparison to mock-treated cell cultures.

(DOC)

**Table S3** Up-regulated (>1.5 - fold) miRNA genes (1 Gy, 2 hr) in H1 as determined by microarray analysis (p<0.05).

(DOC)

**Table S4** Selection of top 10 up-regulated miRNA genes (1 Gy, 16 hr) in H1 as determined by microarray analysis (p<0.05).

(DOC)
Table S5  Down-regulated (>1.5 - fold) miRNA genes (1 Gy, 16 hr) in H1 (p<0.05).  

(DOC)

Table S6  Up-regulated (>1.5 - fold) miRNA genes (1 Gy, 2 hr) in H9 (p<0.05).  

(DOC)

Table S7  List of up-regulated (>1.5 - fold) miRNA genes (1 Gy, 16 hr) in H9 (p<0.05).  

(DOC)

References

1. Inui M, Martello G, Piccolo S (2010) MicroRNA control of signal transduction. Nat Rev Mol Cell Biol 11: 252–263.
2. Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19: 92–105.
3. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136: 215–233.
4. Croce CM (2009) Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 10: 704–714.
5. Latronico MV, Condorelli G (2009) MicroRNAs and cardiac pathology. Nat Rev Cardiol 6: 149–159.
6. Jasinski S, Song SY, Lao K, Chung LW, Johnstone PA (2008) Radiation modulation of microRNA in prostate cancer cell lines. Prostate 68: 1599–1606.
7. Mars OC, An J, Sarojini H, Wu H, Wang E (2008) Changes in MicroRNA expression patterns in human fibroblasts after low-LET radiation. J Cell Physiol 210: 822–828.
8. Chaudhry MA (2009) Real-time PCR analysis of microRNA expression in ionization-radiation-treated cells. Cancer Biol Ther 24: 49–56.
9. Simone NL, Soule BP, Ly D, Saleh AD, Savage JE, et al. (2009) Ionizing radiation-induced oxidative stress alters miRNA expression. PLoS One 4: e6377.
10. Wagner-Ecker E, Schwager C, Wirscher U, Abdulahali A, Huber P (2010) MicroRNA expression after ionizing radiation in human endothelial cells. Radiat Oncol 5: 23.
11. Kovalchuk O, Zemel EJ, Filkowski JN, Ahamarino AM, Dickey JS, et al. (2010) microRNAome changes in bystander three-dimensional human tissue models suggest priming of apoptotic pathways. Carcinogenesis 31: 1882–1888.
12. Nikiforova MN, Gandi M, Kelly L, Nikiforov YE (2010) MicroRNA dysregulation in human thyroid cells following exposure to ionizing radiation. Thyroid 21: 261–266.
13. Flemt P, Paul S, Amundson SA, Young EF, Barker CA, et al. (2011) Radiation-Induced Micro-RNA Expression Changes in Peripheral Blood Cells of Radiotherapy Patients. Int J Radiat Oncol Biol Phys.
14. Vincenti S, Wilson M, Gabow A, Marks DS, Sander C (2008) The microRNA.org resource: targets and expression. Nucleic Acids Res 36: D149–153.
15. Ji AR, Ku SY, Cho MS, Kim YY, Kim YJ, et al. (2010) Reactive oxygen species modulate multidrug resistance by targeting BCL2 in human gastric cancer cells. Mini Rev Med Chem 46: 282–288.
16. Cha HJ, Seong KM, Bae S, Jung JH, Kim CS, et al. (2009) Identification of specific microRNAs responding to low and high dose gamma-irradiation in the human lymphoblastoid line IM9. Int J Radiat Biol 34: 1645–1652.
17. Cha HJ, Seong KM, Bae S, Jung JH, Kim CS, et al. (2009) Identification of specific microRNAs responding to low and high dose gamma-irradiation in the human lymphoblastoid line IM9. Int J Oncol 34: 1661–1668.
18. Yan X, Chai HJ, Shin Y, Lee EM, Bae S, et al. (2009) Identification of ionizing radiation-responsive microRNAs in the IM-9 human B lymphoblastic cell line. Int J Oncol 34: 1655–1659.
19. Yan X, Chai HJ, Shin Y, Lee EM, Bae S, et al. (2009) Identification of ionizing radiation-responsive microRNAs in the IM-9 human B lymphoblastic cell line. Int J Oncol 34: 1655–1659.
20. Wright GW, Simon RM (2003) A random variance model for detection of differential gene expression in small microarray experiments. Bioinformatics 19: 244–255.
21. Sokolov MV, Neumann RD, Panyutin IG (2007) Effects of DNA-targeted ionizing radiation produced by 5-125Iodo-2′-deoxyuridine on global gene expression in primary human cells. BMC Genomics 8: 192.
22. Sokolov MV, Smirnova NA, Camerini-Otero RD, Neumann RD, Panyutin IG (2006) Microarray analysis of differentially expressed genes after exposure of normal human fibroblasts to ionizing radiation from an external source and from DNA-incorporated iodine-125 radiouclide. Gene 382: 47–56.
23. Betel D, Wilson M, Gabow A, Marks DS, Sander C (2008) The microRNA.org resource: targets and expression. Nucleic Acids Res 36: D149–153.
24. Dennis GJ, Sherman BT, Lempicki RA (2009) DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol 10: R003.
25. Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4: 44–57.
26. Williams AE (2008) Functional aspects of animal microRNAs. Cell Mol Life Sci 65: 545–562.
27. Van Hoof D, Munoz J, Beaum SR, Pinkse MW, Linding R, et al. (2009) Phosphorylation dynamics during early differentiation of human embryonic stem cells. Cell Stem Cell 5: 214–226.
28. Yao Y, Suo AL, Li ZF, Liu LY, Tian T, et al. (2009) MicroRNA profiling of human gastric cancer. Mol Med Rep 2: 963–970.
29. Pizzimenti S, Ferracin M, Sabbioni S, Tadolli C, Pettazzoni P, et al. (2009) MicroRNA expression changes during human leukemia HL-60 cell differentiation induced by 4-hydroxynonenal, a product of lipid peroxidation. Free Radic Biol Med 46: 282–288.
30. Adachi K, Suemori H, Yasuda SY, Nakatsui NJ, Kawase E (2010) Role of SOX2 in maintaining pluripotency of human embryonic stem cells. Genes Cells 15: 455–470.
31. Ji AR, Ku SY, Cho MS, Kim YY, Kim YJ, et al. (2010) Reactive oxygen species enhance differentiation of human embryonic stem cells into mesendodermal lineage. Exp Mol Med 42: 175–186.
32. Xia L, Zhang D, Du R, Pan Y, Zhao L, et al. (2008) miR-15b and miR-16 modulate multidrug resistance by targeting BCL2 in human gastric cancer cells. Int J Cancer 123: 372–379.
33. Qian S, Ding YQ, Xu R, An JH, Ao XJ, et al. (2008) MicroRNA expression profile of bronchiolaeveral stem cells from mouse lung. Biochem Biophys Res Commun 377: 668–673.
34. Olif M, Haochen D, Gimbarg D (2011) miR-15 and miR-16 Are direct transcriptional targets of E2F1 that limit E2F-induced proliferation by targeting cyclin E. Mol Cancer Res 9: 440–447.
35. Nishi H, Ono K, Iwagawa Y, Hiro T, Nagao K, et al. (2010) MicroRNA-15b modulates cellular ATP levels and degrades mitochondria via Arf6 in mouse neonatal rat cardiac myocytes. J Biol Chem 285: 4920–4930.
36. Cao H, Yang CS, Rana TM (2009) Evolutionary emergence of microRNAs in human cancer. Nat Rev Mol Cell Biol 10: 704–714.
37. Brubaramyam D, Lamouille S, Judson RL, Liu JY, Bucay N, et al. (2011) Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. Nat Biotechnol 29: 445–446.
38. Barroso-del Jesus A, Lucena-Aguilar G, Menendez P (2009) The miR-302-367 cluster as a potential stemness regulator in ES cells. Cell Cycle 8: 394–398.

Acknowledgments

We wish to thank William DeGraff, Radiation Biology Branch, CCR, National Cancer Institute, for his invaluable help with cell culture X-ray irradiations.

Author Contributions

Conceived and designed the experiments: MVS. Performed the experiments: MVS. Analyzed the data: MVS. Wrote the paper: MVS IVP RDN. Approved of the final manuscript: RDN.

Human Embryonic Stem Cell miRNAome and Radiation
49. Card DA, Hebbel PR, Li L, Troetter KW, Komatsu Y, et al. (2008) Oct4/Sox2-regulated miR-302 targets cyclin D1 in human embryonic stem cells. Mol Cell Biol 28: 6426–6438.

50. Trompeter H, Abbad H, Iwaniuk KM, Hafner M, Remnick N, et al. (2011) MicroRNAs MiR-17, MiR-20a, and MiR-106b act in concert to modulate E2F activity on cell cycle arrest during neuronal lineage differentiation of U87GC. PLoS One 6: e16138.

51. Rizzo M, Mariani L, Pito L, Rainaldi G, Simili M (2010) miR-20a and miR-290, multi-faceted players with a role in tumourigenesis and senescence. J Cell Mol Med 14: 2633–2640.

52. Wang Y, Scheiber MN, Neumann C, Calin GA, Zhou D (2010) MicroRNA Regulation of Ionizing Radiation-Induced Premature Senescence. Int J Radiat Oncol Biol Phys.

53. Lin RJ, Lin YC, Yu AL (2010) miR-149* induces apoptosis by inhibiting Akt1 and E2F1 in human cancer cells. Mol Carcinog 49: 719–727.

54. Li Y, Mine T, Ioannides CG (2010) Short GC-rich RNA similar to miR-1909 and 1915 fold in silico with the 5’-UTR and ORF of Notch and responders: potential for the elimination of cancer stem cells. Oncol Rep 24: 1443–1453.

55. Zhao JJ, Yang J, Lin J, Yao N, Zhu Y, et al. (2009) Identification of miRNAs associated with tumorigenesis of retinoblastoma by miRNA microarray analysis. Childs Nerv Syst 25: 13–20.

56. Nour-Syakama AM, Yoke-Kqueen C, Sabariah AR, Shiraz MS, Singh A, et al. (2011) Differential microRNA expression and identification of putative miRNA targets and pathways in head and neck cancers. Int J Mol Med.

57. Shigoka M, Tsuchida A, Matsudo T, Nagakawa Y, Saito H, et al. (2010) MicroRNA miR-302 targets cyclin D1 in human embryonic stem cells. Mol Cell.

58. Chen ZL, Zhao XH, Wang JW, Li BZ, Wang Z, et al. (2011) microRNA-92a regulates miR-302 targets and pathways in head and neck cancers. Oncol Rep 24: 105–112.

59. Guled M, Lahti L, Lindholm PM, Salmenkivi K, Bagwan I, et al. (2009) Deregulation of miR-92a expression is implicated in hepatocellular carcinoma development. Pathol Int 60: 351–357.

60. Schmitz KJ, Helwig J, Bertram S, Sheu SY, Suttorp AC, et al. (2011) Differential expression of microRNA-675, microRNA-139-3p and microRNA-335 in benign and malignant adenocarcinoma tumours. J Clin Pathol 64: 529–535.

61. Nit G, Qiu H, Jo H (2011) MicroRNA-663 upregulated by oscillatory shear stress plays a role in inflammatory response of endothelial cells. Am J Physiol Heart Circ Physiol 300: H1762–1769.

62. Pilkinton M, Sandoval R, Song J, Ness SA, Colamouni OR (2007) MiP/LIN-9 binds to ''seedless'' 3’-UTR microRNA recognition elements. Mol Cell 35: 610–625.

63. Ni CW, Qiu H, Zun L, Peng L, et al. (2010) Tumor-suppressive mir-663 gene induces mitotic catastrophe growth arrest in human gastric cancer cells. Oncol Rep 24: 105–112.

64. Schmitz KJ, Helwig J, Bertram S, Sheu SY, Suttorp AC, et al. (2011) Differential expression of microRNA-675, microRNA-139-3p and microRNA-335 in benign and malignant adenocarcinoma tumours. J Clin Pathol 64: 529–535.

65. Lar A, Navarro F, Maher CA, Maliszewski LE, Yan N, et al. (2009) miR-24-5p induces apoptosis by inhibiting Akt1 and E2F1 in human cancer cells. Mol Carcinog 49: 719–727.

66. Borgdorff V, Lleonart ME, Bishop CL, Fessart D, Bergin AH, et al. (2010) Multiple microRNAs rescue from Ras-induced senescence by inhibiting p21(Waf1/Cip1). Oncogene 29: 2262–2271.

67. Pan J, Hu H, Zhou Z, Sun L, Peng L, et al. (2010) Tumor-suppressive mir-663 gene induces mitotic catastrophe growth arrest in human gastric cancer cells. Oncol Rep 24: 105–112.

68. Bandiera S, Ruberg S, Cagnard N, Hanein S, et al. (2011) Nuclear outsourcing of RNA interference components to human gastric cancer cells. Stem Cells 26: 455–464.

69. Petrocca F, Visone R, Onelli MR, Shah MH, Nicoloso MS, et al. (2008) E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer. Cancer Cell 13: 272–286.

70. Lal A, Navarro F, Maher CA, Maliszewski LE, Yan N, et al. (2009) miR-24 inhibits cell proliferation by targeting E2F2, MYC, and other cell-cycle genes via p21(Waf1/Cip1). Oncogene 28: 2195–2206.

71. Foshay KM, Gallicano GI (2008) miR-17 family miRNAs are expressed during early mammalian development and regulate stem cell differentiation. Dev Biol 326: 413–443.

72. Lal A, Kim HH, Abdelmohsen K, Kusano Y, Pullmann R Jr., et al. (2008) p16(INK4a) translation suppressed by miR-24. PLoS One 3: e1864.

73. Lal A, Pan Y, Navarro F, Dykhoom DM, Moreau L, et al. (2009) miR-24-mediated downregulation of H2AX suppresses DNA repair in terminally differentiated blood cells. Nat Struct Mol Biol 16: 492–498.

74. Shigoka M, Tsuchida A, Matsudo T, Nagakawa Y, Saito H, et al. (2010) E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer. Cancer Cell 13: 272–286.

75. Bandiera S, Ruberg S, Cagnard N, Hanein S, et al. (2011) Nuclear outsourcing of RNA interference components to human gastric cancer cells. Stem Cells 26: 455–464.