Journal of
Proteomics & Bioinformatics

Sriharshan et al., J Proteomics Bioinform 2014, 7:10
http://dx.doi.org/10.4172/jpb.1000335

Research Article Open Access

Radiation-Induced Crosstalk between MicroRNAs and Proteins of the Endothelium: *In silico* Analysis

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Abstract

Ionising radiation causes damage at various levels in the exposed cell. The initial injury and the resulting cellular response to the damage involve complex crosstalk between the regulators of the DNA damage response recognition signalling and repair pathways. System-level research is required to gain more insight into these pathways. In this study we have used an *in silico* method to connect the altered proteome and miRNAome networks after radiation exposure by using Ingenuity Pathway Analysis tool and further verification for seed sequence matches by manually searching in microrna.org, mirDB, mirwalk, miRBase, and Targetscan databases. The endothelial cell was used as a model system as the endothelium is one of the main cellular systems damaged by ionising radiation. The interaction analysis revealed that changes at the miRNA level occur shortly after irradiation (4 and 12 hours) and thus often precede the alterations in the proteome that mostly take place later (24 hours). The two networks are closely intertwined emphasizing the regulatory role of miRNAs in the protein expression. Beside the well described pathways of the initial radiation response, such as oxidative stress and mitochondrial dysfunction, additional pathways such as Rho signalling (Rho family GTPases, Rho GDI and RhoA signalling) are involved in the endothelial response. In conclusion, the *in silico* analysis presented here is a valuable tool for identification of radiation targets and biomarkers for further validation. Furthermore, it can be used for any cellular or tissue model of interest.

Keywords: Proteomics; Ionising radiation; miRNA; miRNA targets; miRNA prediction; Endothelium; Rho signaling; Oxidative stress; Biomarker

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that play an important role in regulating most cellular processes, including those triggered by stress response [1,2]. They can regulate their target genes in a direct manner, where distinct miRNAs can target one messenger RNA (mRNA) or single miRNAs can target multiple mRNA transcripts, facilitating fine tuning and control over a wide range of cellular functions. miRNAs function by inhibiting protein synthesis, both by repressing translation and by facilitating deadenylation and subsequent degradation of mRNA targets [3]. Consequently, investigating the miRNAome-proteome interaction is essential for understanding both the process and extent of cell response to radiation injury.

Ionising radiation damages cells primarily by inducing ionisation and DNA damage that directly and indirectly trigger alterations in the expression levels of miRNAs [4-6] and proteins [7,8]. The regulatory network of miRNAs has been shown to be essential for cells to respond and repair the damage caused by radiation exposure [4,9]. Global suppression of miRNA expression leads to reduced cell survival, suggesting that their role is largely restorative [4]. Several studies have shown radiation-induced alterations in protein expression in tissues, primary cells, and cell lines [8,10-15]. Some of these occur in the immediate aftermath of exposure and are directed to damage repair and survival [8]. However, a number of other pathways less obviously involved are also rapidly activated by irradiation [8,14,16]. In order to quantify the contribution of translational repression by miRNAs in radiation response, the use of proteomics techniques is crucial, as miRNAs are able to regulate their targets at the translational level without affecting mRNA abundance, and are hence undetected by classical transcriptome analyses.

The endothelium is one of the main cellular systems damaged by ionising radiation [17-19]. It is responsible for normal tissue sensitivity to irradiation that limits the doses that can be applied to tumours [20,21]. We have previously shown altered expression levels of miRNAs [4,9] and proteins [8,14] in primary endothelial cells and cell lines exposed to gamma radiation. Even low doses of ionising radiation had a significant impact on miRNA expression that was directly related to protein expression alterations [14].

Several studies in the recent past have focussed on *in silico* analyses using bioinformatics tools to establish relationships and regulation patterns between miRNAs and proteins [14,22-24]. Prompted by our previous data we aimed in this current work to systematically investigate the *in silico* interactions between radiation-induced alterations in the miRNA and protein levels in the endothelial cell line EA.hy926 [4,8,9].

Methods

Cell culturing, harvesting, irradiation and microRNA expression analysis

Endothelial cell line EA.hy926 was used for the irradiation experiments as follows. The cells were irradiated with a dose of 2.5 Gy using a ^{137}Cs-γ source and harvested at 12 hours for miRNA analysis. Cell culturing for was done as described by Kraemer et al. [4]. The

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Received August 12, 2014; **Accepted** September 17, 2014; **Published** September 22, 2014

Citation: Sriharshan A, Kraemer A, Atkinson MJ, Moertl S, Tapio S (2014) Radiation-Induced Crosstalk between MicroRNAs and Proteins of the Endothelium: *In silico* Analysis. J Proteomics Bioinform 7: 327-331. doi:10.4172/jpb.1000335

miRNA expression level analysis was carried out using TaqMan® arrays and single primer assays [4]. The significance of the fold change in the miRNA (± 2.0) and protein expression (± 1.3) at 4 hours and 24 hours is expressed as p-values and calculated in our previous publications [4,8]. For the miRNA changes at the 12-hour time point p-values are shown in the Supplementary Figure 2. Data are based on three independent experiments with standard error of the mean (SEM). Significance of n-fold changes was calculated by using the one sample t-test.

Bio informatics analysis

Ingenuity Pathway Analysis (IPA), a knowledge database generated from peer-reviewed scientific publications, was used to obtain information about relationships, biological mechanisms, functions and pathways [25]. All differentially regulated miRNAs and proteins with their corresponding Swiss-Prot accession numbers were imported into the IPA including the fold changes of both [25,26].

Proteins and miRNAs found to be deregulated in our previous studies [4,8] were uploaded into the IPA software in the following order:

1) 4-, 12-, 24-hour miRNAs with 4- and 24-hour proteins
2) 4-hour miRNAs with 4-hour proteins
3) 4-hour miRNAs with 24-hour proteins
4) 12-hour miRNAs with 4-hour proteins
5) 12-hour miRNAs with 24-hour proteins
6) 24-hour miRNAs with 24-hour proteins

The proteins and miRNAs were connected using the build tool in the IPA software. Based on the databases TarBase, TargetScan and miRecords the IPA software predicted targets of the miRNAs. The connections were filtered to have only direct interactions and further sorted for only negative regulations. Interactions were only considered valid if and only if the analysis showed both a miRNA that was down-regulated and its target protein that was up-regulated or vice versa. The miRNA targets were chosen based on the total context score. A total context score below 0.4 was considered to be significant (Figure 2) (http://www.targetscan.org/fish_62/docs/context_score.html) [27]. These interactions were further verified for seed sequence matches by manually searching in microrna.org, mirDB, mirwalk, miRBase, and Targetscan databases (Figures 3-5) [28-39]. Supplementary Table 1 shows the miRNA and protein interactions verified by each database.

Results and Discussion

Proteomic and miRNA alterations

The proteomic alterations observed using the SILAC and 2D-DIGE methods in the EA.hy926 cells after 4 and 24 hours after the dose of 2.5 Gy have been published previously [8]. Fifty-eight proteins at 4 hours, and 136 proteins at 24 hours, were significantly deregulated.

The miRNA alterations were studied in the same cell line using the same radiation dose and time points as for the proteomics analysis. These data have been published previously by Kraemer et al. [4,9]. To further demonstrate the transient nature of miRNA regulation after irradiation we additionally measured altered miRNA expression levels 12 hours after irradiation. The numbers of altered miRNAs and shared miRNAs at the different time points are shown in the Venn diagram in Figure 1A. The numbers of deregulated and shared proteins are shown in Figure 1B. The list of deregulated miRNAs at 12 hours after irradiation is shown in Supplementary Table 2. The lists of miRNAs and proteins found to be differentially regulated at 4 hours and 24 hours are published in our previous studies [4,8].

The existence of distinct phases of the response is indicated by the fact that only one protein, desmoplakin, or two microRNAs, miR-323-3p and miR-517b, are shared among the significantly deregulated at the 4 h and 24 h time points [4,8].

Relationship between deregulated miRNAs and proteins-*in silico* analysis

All miRNAs that were found to be differentially expressed at the time points 4, 12, and 24 hours together with proteins found to be deregulated at 4 and 24 hours after irradiation were uploaded into the

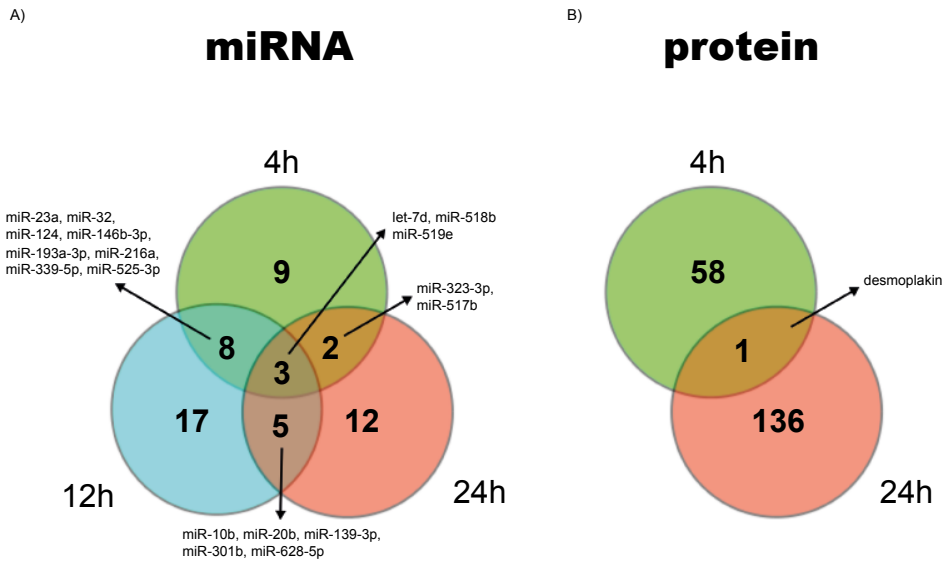

Figure 1: Venn diagrams showing the overlap between radiation-responsive miRNAs or proteins in EA.hy926 cells at different time points. The overlap between miRNAs at 4 hours, 12 hours and 24 hours after irradiation is shown in A and between proteins at 4 hours and 24 hours after irradiation in B.

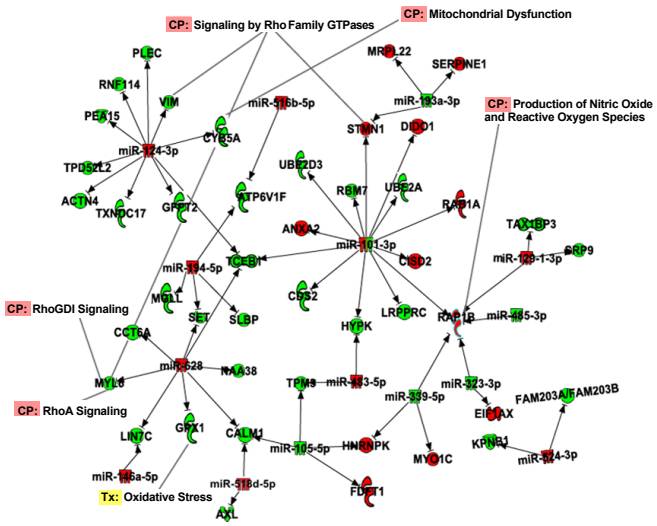

Figure 2: Predicted interactions of miRNAs deregulated at 4, 12 and 24 hours and their target proteins deregulated at 4 and 24 hours after radiation exposure of 2.5 Gy. Differentially up- or down-regulated molecules are marked in red and green, respectively. TX, toxic pathway; CP, canonical pathway.

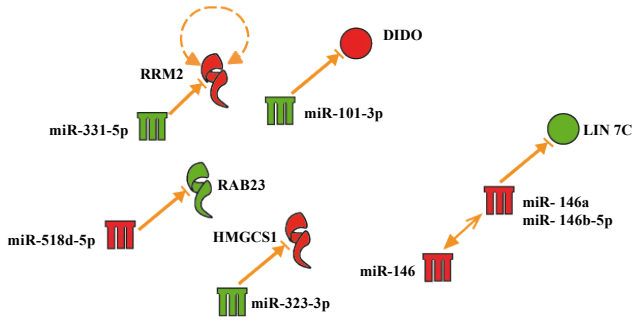

Figure 3: Predicted interactions of miRNAs and their target proteins 4 hours after radiation exposure of 2.5 Gy. Differentially up- or down-regulated molecules are marked in red and green, respectively.

IPA. Their interactions are shown in Figure 2.

For an initial less stringent analysis no further manual database verification of potential miRNA-target interactions was done as we wanted to create a general network of all possible interactions (Figure 2). The most significant radiation-induced toxic pathway (TX) was the oxidative stress pathway. Canonical pathways (CP) found to be affected by radiation exposure were mitochondrial dysfunction, nitric oxide and ROS production, Rho GDI signalling and Rho A signalling. The Rho signalling and oxidative stress pathways were also found to be affected in the proteomics analysis as shown in our previous study [8].

Of the 22 miRNAs and 59 proteins found to be deregulated at 4 hours a more stringent verification of potential miRNA-target interactions was done as described in Materials and Methods. This showed that five proteins were predicted to be the direct targets of five individual miRNAs (Figure 3). The predicted miRNA-protein interactions were as follows:

a) miR-146a and miR-146b-5p (↑) with LIN7C (↓)

b) miR-331-5p (↓) with RRM2 (↑)

c) miR-101-3p (↓) with DIDO1 (↑)

d) miR-518d-5p (↑) with RAB23 (↓)

e) miR-323-3p (↓) with HMGCS1 (↑)

(↓) (↑) represent down- and up-regulation, respectively.

As the expression level alteration of miRNAs is expected to precede the alteration in their target protein level, we combined the data from miRNAs found to be differentially regulated at 12 hours and proteins deregulated at 24 hours after irradiation. Direct regulation of ten proteins by seven miRNAs was observed (Figure 4).

a) miR-101-3p (↑) with HYPK (↓)

b) miR-124-3p (↓) with FAF2 (↑)

c) miR-193a-3p (↓) with MRPL22 (↑)

d) miR-194-5p (↑) with MGLL (↓), ATP6V1F (↓)

e) miR-339-5p (↓) with HNRNPK, MYO1C (both ↑)

f) miR-516b-5p (↑) with ATP6V1F (↓)

g) miR-628-5p (↑) with CCT6A, MYL6, CALM1 (all ↑)

(↓) (↑) represent down- and up-regulation, respectively

As expected, no predicted interactions were found between the

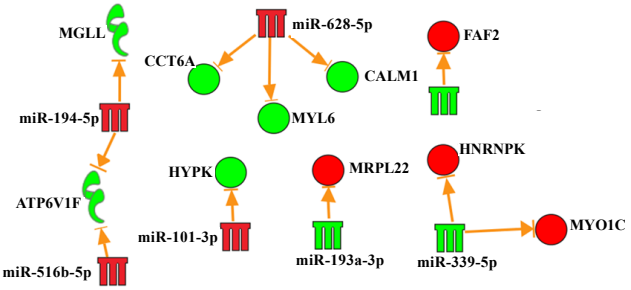

Figure 4: Predicted interactions of miRNAs found to be differentially regulated at 12 hours and proteins deregulated at 24 hours after irradiation (2.5 Gy). Differentially up- or down-regulated molecules are marked in red and green, respectively.

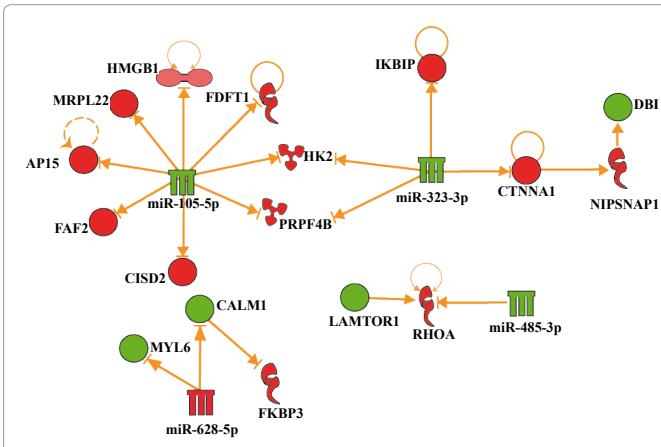

Figure 5: Predicted interactions of miRNAs and their target proteins 24 hours after radiation exposure of 2.5 Gy. Differentially up- or down-regulated molecules are marked in red and green, respectively.

deregulated miRNAs at 12 hours and of the deregulated proteins at 4 hours after irradiation.

For miRNAs and proteins found to be deregulated 24 hours after irradiation, the predicted network indicated direct interactions between four miRNAs and fifteen proteins that could be interpreted as evolving complexity. In Figure 5 (deregulated molecules of 24 hours after irradiation) the miR-105-5p and miR-323-3p formed central nodes for several deregulated target proteins. The predicted interactions were as follows:

a) miR-105-5p (↓) with FDFT1, HMGB1, MRLP22, AP15, FAF2, CISD2, HK2, PRPF4B (all↑)

b) miR-323-3p (↓) with PRPF4B, IKBIP, CTNNA1, HK2 (all↑)

c) miR-485-3p (↑) with LAMTOR1 and RHOA (both↓)

d) miR-628-5p (↑) with CALM1, MYL 6 (both↓)

(↓) (↑) represent down- and up-regulation respectively.

In general, it seems that the number of the deregulated target proteins compared to the number of deregulated miRNAs in the radiation response increases within the time frame of this study (24 hours). Considering the timing between miRNA and protein changes our study showed that in a certain number of cases miRNA and protein changes coincide, indicating a fast protein expression regulation by the miRNAs (Figures 3 and 5). However, for other proteins the miRNA regulation clearly precedes protein chances with a considerable time lap (Figures 2 and 4). Especially the proteins suggested to be regulated by the concerted action of several miRNAs belong to this category.

The predicted interactions from Figure 2 were as follows:

a) TCEB1 (24 h (↓)) with miR-101-3p (12 h (↑)), miR-124-3p (4 h, 12 h (↑)), miR-628-5p (12, 24 h (↑))

b) RAP1B (12 h (↑)) with miR-323-3p (4 h (↑)), miR-339-5p (4 h, 12 h (↑))

c) SET (24 h (↑)) with miR-194-5p (12 h (↑)) and miR-628-5p (12 h, 24 h (↑))

d) ATP6V1F (24 h (↓)) with miR-194-5p (12 h (↑)) and miR-516-5p (12 h (↑))

(↓) (↑) represent down- and up-regulation, respectively.

Furthermore, the proteins that may be regulated by one miRNA such as miR-105-5p (Figure 5) do not all belong to the same biological pathway. Thus, the miRNAs appear to form an umbrella network connecting the different signaling pathways and potentially orchestrating it.

The more stringent analysis highlighted the progression of miRNA-regulated processes somewhat differently than the general IPA analysis although some pathways were overlapping. At four hours, both apoptosis (miR-101-3p; DIDO) and autophagy (miR-518d-5p; RAB23) were present (Figure 3) whereas at 12 and 24 hours anti-apoptosis (miR-101-3p; HYPK) and protein folding (miR-628-5p; CCT6A; miR-101-3p; HYPK) seemed to dominate. In addition, pathways such as endothelial nitric oxide synthesis (miR-628-5p; CALM1), organisation of the cytoskeleton (miR-339-5p; MYO1C), RhoA signalling and RhoGDI signalling (miR-628-5p; MYL6) were present at 12 and 24 hours (Figure 4). All these pathways, and in addition glycolysis (miR-105-5p, miR-323-3p; HK2), were also affected at only 24 hours (Figure 5).

All the pathways found in this *in silico* analysis make biological sense, starting from the programmed cell death and pathways associated with early events (apoptosis, autophagy), progressing towards pathways characteristic to endothelial dysfunction (NO bioavailability, increased ROS production) [40]. Recent data indicate a close crosstalk between NO signalling and Rho signalling [41] in the endothelial dysfunction. Rho family proteins regulate several cellular functions including cytoskeletal organisation, membrane trafficking, cytokinesis, cell proliferation, cell motility and transcriptional regulation. Rho signaling pathways have been shown to be responsive even to low-dose radiation [16,42] and their activation is associated with the development of cardiovascular disease [43].

To date none of the protein–miRNA interactions predicted by our model is experimentally validated. This is not surprising knowing the multitude of putative interactions compared to the small portion of validated interactions within a cell [3]. However, we used highly stringent prerequisites for the identification of miRNA target interactions including the use of several prediction algorithms. This, together with the consideration of an inverse correlation of miRNA and protein expression, suggests a high plausibility for these predictions [44]. Especially the TargetScan algorithm used in this study proofed its high prediction capacity in analysing miRNA effects on the proteome level [45,46].

Thus, our *in silico* study provides interesting candidate miRNA-target protein interactions and biological pathways for experimental validation that is necessary for elucidating radiation effects on the endothelium. We conclude that the expression alterations of miRNAs and proteins shown here are time-dependent, closely related and intertwined. Rapid radiation-induced decrease or increase in the miRNA levels may trigger subsequent alterations in several target proteins belonging to distinct cellular pathways. This emphasises the essential role of miRNAs as central regulators of the cellular response.

Acknowledgement

We thank Stefanie Winkler for excellent technical assistance.

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