

# Ionizing radiation regulates long non-coding RNAs in human peripheral blood mononuclear cells

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## ABSTRACT

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts that modulate mRNA and microRNA (miRNA) expression, thereby controlling multiple cellular processes, including transcriptional regulation of gene expression, cell differentiation and apoptosis. Ionizing radiation (IR), a strong cellular stressor, is known to influence gene expression of irradiated cells, mainly by activation of oxidative processes. Whether and how IR also affects lncRNA expression in human peripheral blood mononuclear cells (PBMCs) is still poorly understood. Exposure of PBMCs to IR dose-dependently activated p53 and its downstream target p21, ultimately leading to cell-cycle arrest and/or apoptosis. Cleavage of caspase-3, a specific process during apoptotic cell death, was detectable at doses as low as 30 Gy. Transcriptome analysis of 60 Gy-irradiated PBMCs revealed a strong time-dependent regulation of a variety of lncRNAs. Among many unknown lncRNAs we also identified a significant upregulation of Trp53cor1, MEG3 and TUG1, which have been shown to be involved in the regulation of cell cycle and apoptotic processes mediated by p53. In addition, we found 177 miRNAs regulated in the same samples, including several miRNAs that are known targets of upregulated lncRNAs. Our data show that IR dose-dependently regulates the expression of a wide spectrum of lncRNAs in PBMCs, suggesting a crucial role for lncRNAs in the complex regulatory machinery activated in response to IR.

**KEYWORDS:** lncRNA, PBMC, paracrine, radiation, p53, apoptosis

## INTRODUCTION

Protein coding genes of the mammalian genome are flanked by non-transcribed sequences whose biological functions are still largely unknown [1]. During the last decade, such sequences have been shown to give rise to biologically active transcripts, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) [2]. lncRNAs are larger than 200 nucleotides, and can be further classified as RNAs deriving from introns of protein-coding genes (intronic lncRNAs), as antisense strands of protein coding genes (natural antisense

transcripts), or as RNAs deriving from areas of the genome that neither overlap exons, nor other lncRNAs, nor protein coding genes (long intervening non-coding RNAs (lincRNA)). Although the number of lncRNAs identified has risen rapidly in recent years, only few studies are available that identify specific functions of selected lncRNAs, such as the regulation of gene expression by interaction with chromatin-modifying proteins [3]. Mammalian lncRNAs have been most intensively investigated in pathophysiological conditions, including cancer development and progression [4]. While some lncRNAs are involved

in metastasis of breast [5] and colorectal cancer [6], others sensitize tumor cells to radiotherapy and to enhanced cell death in response to radiation [7]. One of the best investigated lncRNAs is Trp53cor1 (lincRNA-p21), which has been shown to be upregulated in response to IR [7] and which downregulates several p53 target genes, thereby inhibiting cell proliferation and inducing apoptosis [8].

PBMCs are frequently used to investigate the biological effect of IR because these cells are particularly sensitive in response to IR [9, 10]. Amundson *et al.* performed whole-genome microarray expression analysis of *ex vivo* irradiated PBMCs (0.5, 2, 5 and 8 Gy) and established a core set of 74 genes that allows a correct discrimination between different types of treatment at 6 h or 24 h after irradiation [10]. Notably, more than one-third of these genes regulated in response to IR were downstream targets of p53. Furthermore, irradiated PBMCs have been increasingly investigated in the field of regenerative medicine, as it has been shown that infusion of apoptotic PBMCs or their secreted paracrine factors [11–17] leads to tissue regeneration and beneficial immunomodulation in various experimental diseases [18].

Although high-dose radiation (up to 60 Gy) is used to induce apoptosis of PBMCs, thereby enhancing their capacity to induce tissue regeneration [12–18], it is currently completely unknown how high-dose radiation affects lncRNA expression. Recently, we established miRNA–mRNA–transcription factors networks after 60 Gy IR [19]. Based on these observations, we speculated that in addition to miRNAs and transcription factors, a subset of lncRNAs might also be crucial for gene regulation in response to IR.

The aim of this study was to investigate the dose-dependent regulation of lncRNAs in PBMCs exposed to IR.

## MATERIALS AND METHODS

### Cell separation, radiation and RNA extraction

This study was approved by the Ethics Committee of the Medical University of Vienna (Ethics Committee vote number: 1236; 2013) and conducted according to the principles of the Declaration of Helsinki and Good Clinical Practice. Written, informed consent was obtained from all participants. PBMCs from healthy donors were gamma-irradiated *ex vivo* with dosages ranging from 0.9 to 60 Gy from a <sup>137</sup>Caesium source, as published previously [19]. Non-irradiated PBMCs from the same donor served as controls. Protein immunoblots and quantitative PCR (qPCR) were performed as biological triplicates and technical duplicates. One representative experiment of three is shown.

### Quantitative PCR

qPCR for human lncRNA-p21 (forward primer 5'-CCCGGGCTTGTCTTTTGT-3', reverse primer 5'-GAGTGGGTGGCTCACTCTTCTG-3'), KGFLP1 (forward primer 5'-TCATCTGTACGGTTGGGACA-3', reverse primer 5'-GCCACTGTCTGATTTCCAT-3'), FLJ46906 (forward primer 5'-GAAATGGATCACCGGAGAGA-3', reverse primer 5'-TGGCGCAGAATTTTCTTTCT-3'), DNMI1P46 (forward primer 5'-ATGCTTCAGTTGGCTGGAGT-3', reverse primer 5'-CACGGTCTTGTGACAATGG-3'), NKAPP1 (forward primer 5'-GGATACTGCTGAGGCTCCTG-3', reverse primer 5'-CCATTCCCCAGTCAGAGAAC-3'), PRKCQ-AS1

(forward primer 5'-AGCAGATTGGGTGGAAAGAG-3', reverse primer 5'-CTTTGCAGCTTCAAGCACAG-3'), NKAPP1 (forward primer 5'-GGATACTGCTGAGGCTCCTG-3', reverse primer 5'-CCATTCCCCAGTCAGAGAAC-3'), TUG1 (forward primer 5'-TAGCAGTTCCTCAATCCTTG-3', reverse primer 5'-CACAAATTCCTCATTTCC-3'), MEG3 (forward primer 5'-CTGCCCCTACACCTCACG-3', reverse primer 5'-CTCTCCGCCGTCTGCGCTAGGGGCT-3'), XLOC\_010725 (forward primer 5'-AGGACCTGCCAGGCTATTTT-3', reverse primer 5'-CACACTGCAGGCTCATCTGT-3'), XLOC\_006035 (forward primer 5'-CACTGCCTGGCACATAGTTG-3', reverse primer 5'-AAACA TCCCCGGAAGTCTTAA-3') and the house-keeping gene beta-2-microglobulin (B2M; forward primer 5'-GATGAGTATGCCTGCCGTGTG-3', reverse primer 5'-CAATCCAAATGCGGCATCT-3') were performed using Light Cycler Fast Start DNA Master SYBR Green I (Roche Applied Science, Penzberg, Germany) according to a published protocol [20].

### Microarray analysis

PBMCs from four donors were irradiated with 60 Gy. At 2, 4 and 20 h after irradiation, total RNA was extracted using Trizol<sup>®</sup> reagent according to the manufacturer's instructions. In addition, RNA was extracted from PBMCs immediately after drawing blood. RNA integrity was verified by an Agilent 2100 Bioanalyzer (Agilent, Böblingen, Germany). Raw data of gene expression are available at the Gene Expression Omnibus website using the accession number GSE55955 [19]. In total, 28 samples from the four volunteers were used for microarray analysis using Agilent Human Oligo Microarray (8×60K; G4851A; #028004; Agilent Technologies). This array detects 7419 lncRNAs and 27 958 Entrez Gene RNAs. The composition of target transcripts was based on Unigene Build 216, Ensemble Release 53 and RefSeq Build 36.3. Agilent's Feature Extraction software was used for image analysis.

### Immunoblot analysis

PBMCs were irradiated with different dosages (0.9 Gy, 1.9 Gy, 3.75 Gy, 7.5 Gy, 15 Gy, 30 Gy, 60 Gy) and cultivated for 4 and 24 h. Cells were washed, lysed in SDS–PAGE loading buffer, sonicated, centrifuged and denatured before loading. Protein (50 µg) was subjected to SDS–PAGE electrophoresis (GE Amersham Pharmacia Biotech, Uppsala, Sweden). The proteins were then electrotransferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) and immunodetected using primary antibodies against p53 (1 µg/ml; Abcam, Cambridge, UK), p21 (1 µg/ml; Abcam, USA) and cleaved caspase 3 (0.5 µg/ml, Asp1, R&D Systems, Minneapolis, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the housekeeping protein (0.2 µg/ml; Biogenesis, Poole, UK). Reaction products were detected by chemiluminescence with the Chemi Glow reagent (Biozyme Laboratories Limited, South Wales, UK) according to the manufacturer's instructions.

### Cell cycle analysis

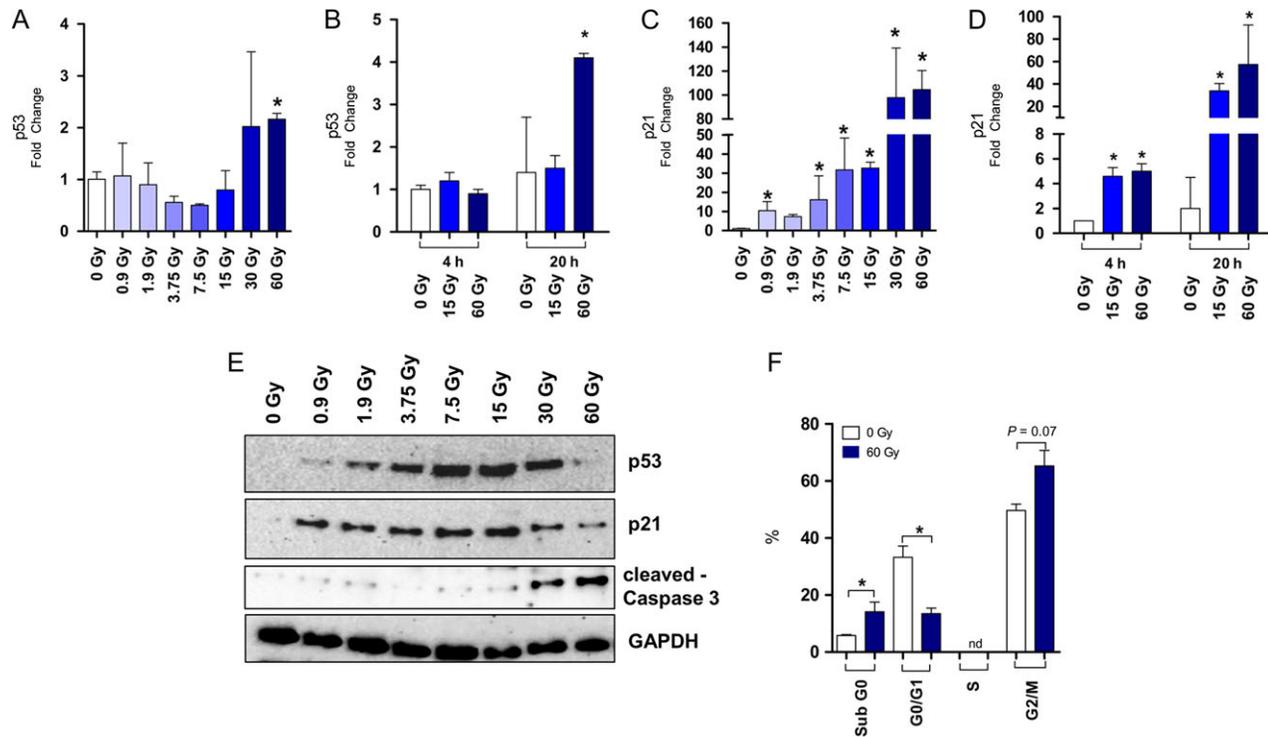
PBMCs were irradiated with 60 Gy and cultivated at a concentration of 25 × 10<sup>6</sup> cells for 24 h. Cell cycle analysis was performed

using the BrdU cell-cycle kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Briefly, 20 h after irradiation, cells were incubated with BrdU (10  $\mu$ M, kit component) for 4 h. After fixation (BD Cytfix/Cytoperm™, kit component) cells were stained with a fluorescein isothiocyanate (FITC) conjugated anti-BrdU antibody (BD Biosciences, kit component) for 30 min. After washing, cells were stained with 7-AAD (BD Biosciences, kit component) and immediately analyzed on a FACS-Calibur (BD Biosciences). Gates were set according to the manufacturer's instructions, and data were evaluated using the FlowJo software (Tree Star, Ashland, OR, USA).

### Statistical analysis of lncRNA expression data

Data pre-processing was based on single-experiment normalized data supplied by the Miltenyi company. The data from each of the three time-points were separately both log<sub>2</sub> transformed and then

normalized by quantile-normalization. Expression values for identical replicate probes were summarized by calculating their mean, resulting in values for 42 405 probe sets, and a filtering step was applied to reduce the number of multiple hypotheses. Only those genes whose sum of squared pairwise differences was equal to or above a threshold of 1, were included in the final analysis. The threshold of 1 was chosen to include at least all genes with a fold change in expression of 0.5 or above. Differential expression was analyzed by paired *t*-tests in the statistical computing environment R 2.14.2. The resulting *P*-values were corrected for multiplicity by applying the Benjamini–Hochberg adjustment over all *P*-values calculated for a time-point. Genes with an adjusted *P*-value < 0.05 and a fold change (FC) > 2.0 between irradiated and non-irradiated samples were considered significantly different. LncRNAs were defined as genes annotated with NR-numbers, XR-numbers or lincRNA. Overlaps of lncRNAs between the different time-points were visualized using the program Venny (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>).



**Fig. 1.** Time- and dose-dependent induction of p53 and its downstream events in human peripheral blood mononuclear cells (PBMCs). qPCR analysis revealed a dose-dependent induction of (A) p53 and its downstream target (C) p21 after exposure to IR. The highest fold change was detectable for both genes after irradiation with 60 Gy. (B) Whereas at 4 h p53 was not significantly altered at the gene level, a 5-fold increase in p53 mRNA level was detectable 24 h after irradiation, (D) p21 was significantly upregulated at both 4 and 24 h after irradiation with either 15 or 60 Gy. (E) p53, p21 and active caspase-3 were evaluated using immunoblot analysis 24 h after irradiation. IR induced a dose-dependent accumulation of p53 and upregulation of p21. Active caspase-3 was only detectable after high-dose radiation with 30 and 60 Gy. GAPDH served as an internal loading control. (F) Cell cycle analysis of 60 Gy-irradiated PBMCs showed an increase in dead cells (Sub G0) and G0/G1 as well as G2/M cell cycle arrest. Neither non-irradiated nor irradiated cells showed proliferation after 24 h in culture (S). One experiment of three carried out in triplicate is shown. Data are presented as mean + SEM; \**P* < 0.05, paired *t*-test; *n* = 3.

### Hierarchic clustering

NetWalk open-source software was used for hierarchic clustering of lncRNA log<sub>2</sub> expression data. A Euclidean distance metric and weighted pair group method with Arithmetic mean was used for clustering.

### lncRNA-miRNA and lncRNA-mRNA target prediction

miRcode [21], DIANA lncRNA target prediction [22] and starBase v2.0 [23] in combination with literature research of validated RNA-RNA interactions were used for generating lncRNA-miRNA-mRNA interaction networks. These tools identify RNA-RNA and RNA-protein interactions based on cross-linking immune precipitation (CLIP)-sequencing data or on identified miRNA recognition elements. Only those lncRNAs, miRNAs and mRNAs that were significantly regulated in response to irradiation were included in the analyses. Selected interactions were displayed graphically.

## RESULTS

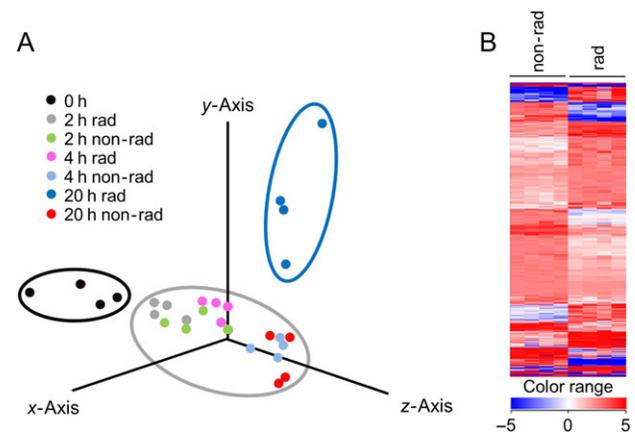
### High-dose IR induces cell cycle arrest and apoptosis in PBMCs

To investigate dose-dependent alterations to the induction of cell cycle progression and apoptosis in human PBMCs, we  $\gamma$ -irradiated the cells with increasing dosages (0.9, 1.9, 3.75, 7.5, 15, 30, 60 Gy). mRNA expression of p53 and its downstream target p21 showed a time- and dose-dependent increase in response to IR. Whereas we detected a significant upregulation of p53 mRNA levels exclusively with high-dose radiation 20 h after irradiation (60 Gy, Fig. 1A and B), its downstream target gene *p21* was significantly upregulated on the mRNA level at much lower dosages (0.9 Gy; Fig. 1C) and at earlier time-points (4 h; Fig. 1D). Although p53 expression was only weakly regulated by IR on the mRNA level, immunoblot analysis of p53 at 20 h after IR already showed a strong accumulation of p53 protein after 0.9 Gy irradiation with maximum protein levels detectable after 15 Gy (Fig. 1E). In accordance with the strong p53 protein accumulation, we also detected a strong upregulation of p21 protein at all radiation dosages (Fig. 1E). Active caspase-3, a marker for the execution phase of apoptosis, was only detectable after high-dose irradiation with 30 and 60 Gy. Interestingly, high-dose IR induced less p53 accumulation than lower dosages, which was accompanied by a weaker p21 upregulation and enhanced caspase-3 cleavage.

To investigate whether the lower p53 accumulation levels 20 h after IR were due to different time kinetics (faster activation of p53 could lead to faster proteasomal degradation), we also determined the amount of accumulated p53 at 4 h after IR. As shown in the supplementary Figure 1, the levels of accumulated p53 4 h after irradiation with different dosages was comparable with those after 20 h, suggesting that different mechanisms account for p53 activation after low- and high-dose IR. Since high-dose IR is known to induce cell death in PBMCs, we next investigated cell death and cell cycle progression in the irradiated cells. Our cell cycle analyses revealed that only 15–20% of the cells died 20 h after irradiation (Sub G<sub>0</sub>; Fig. 1F). All other cells were arrested in the cell cycle, indicated by a complete lack of dividing cells (S Phase; Fig. 1F). This cell cycle arrest was also detectable in the untreated cells, suggesting that our culture conditions did not favor PBMC proliferation.

### High-dose IR regulates lncRNA expression

To investigate the effect of high-dose IR on lncRNA expression, we  $\gamma$ -irradiated PBMCs with 60 Gy and analyzed lncRNAs using Agilent microarray chips, detecting 7419 lncRNAs and 27 958 mRNAs. The unselected lncRNA expression values were displayed in a principal component analysis (PCA). As shown in Fig. 2A, PCA allowed a clear discrimination of non-irradiated and  $\gamma$ -irradiated cells. We detected a time-dependent increase in differentially expressed lncRNAs in response to IR. Whereas only 21 lncRNAs were significantly regulated 2 h after irradiation (supplementary Table S1), we detected 210 (supplementary Table S2) and 724 (supplementary Table S3) differentially expressed lncRNAs at 4 and 20 h after exposure to IR, respectively. Only three lncRNAs were significantly regulated at each time-point (one upregulated; two downregulated). However, more than one-third of the regulated lncRNAs 4 h after irradiation were also regulated 20 h after exposure to IR. The overlap of the differentially expressed lncRNAs at the three time-points is visualized in supplementary Fig. 2. In Fig. 2B all regulated lncRNAs at 20 h after irradiation are displayed in a heatmap.

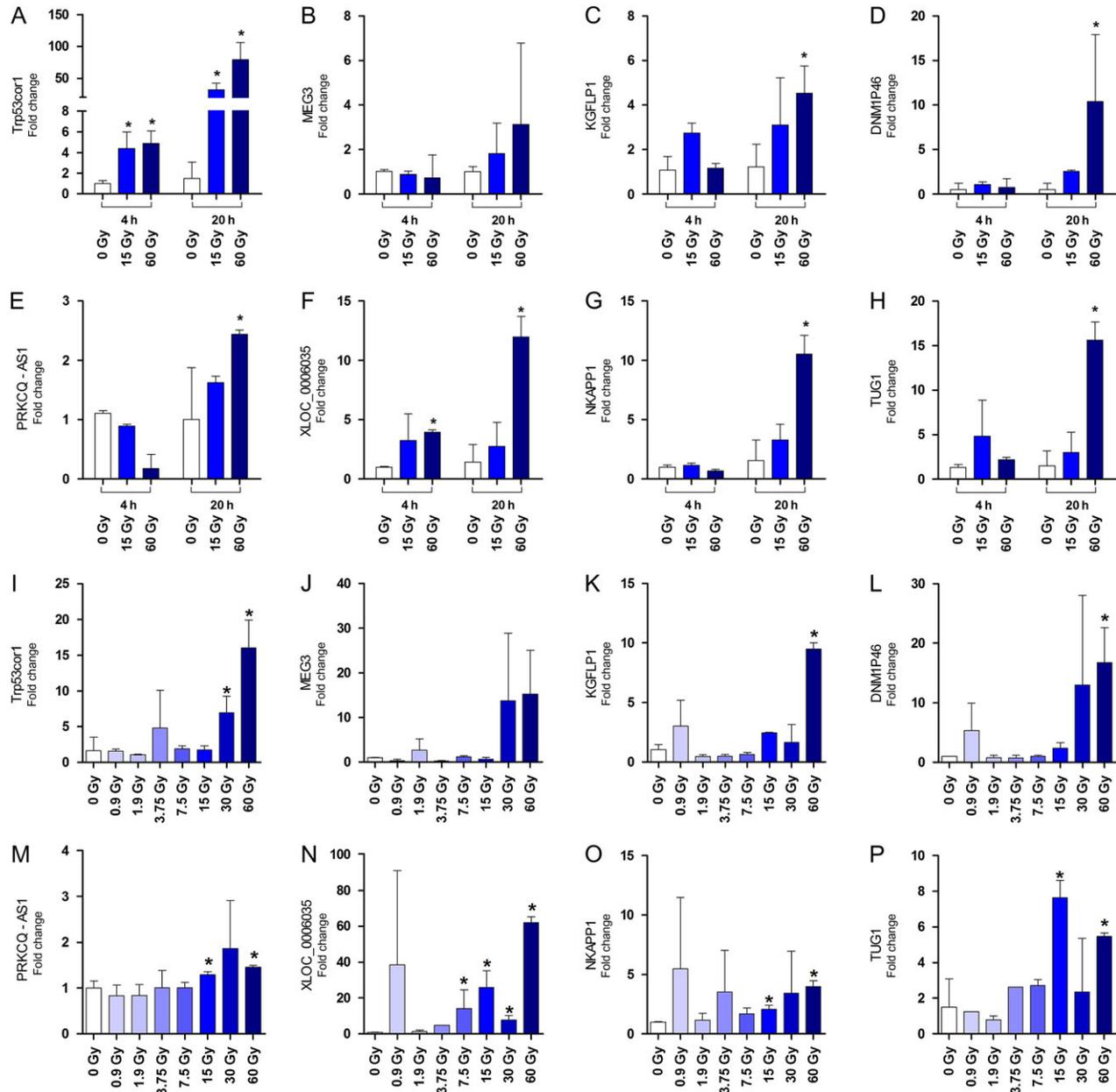


**Fig. 2.** Principle component analysis (PCA) and heat map of significantly regulated lncRNAs in response to IR. (A) This figure depicts lncRNA expression changes in response to cell cultivation and irradiation. All processed samples are shown with respect to the first three components and are colored according to treatment and time-point. In total, 28 independent experiments were performed. All samples built visible clusters according to treatment and cell culture period, with highest expression changes between irradiated (blue oval) and non-irradiated cells (black oval) at 20 h after treatment. All cultured samples differed significantly from non-cultured samples (blue and grey ovals). 0 h = peripheral blood mononuclear cells (PBMCs) processed immediately after blood draw; rad = irradiated samples; non-rad = non-irradiated samples. (B) A heat map showing the expression values of 724 significantly regulated lncRNAs 20 h after irradiation. The range of expression was from  $-\log 5.0$  (blue, downregulation) to  $\log 5$  (red, upregulation).

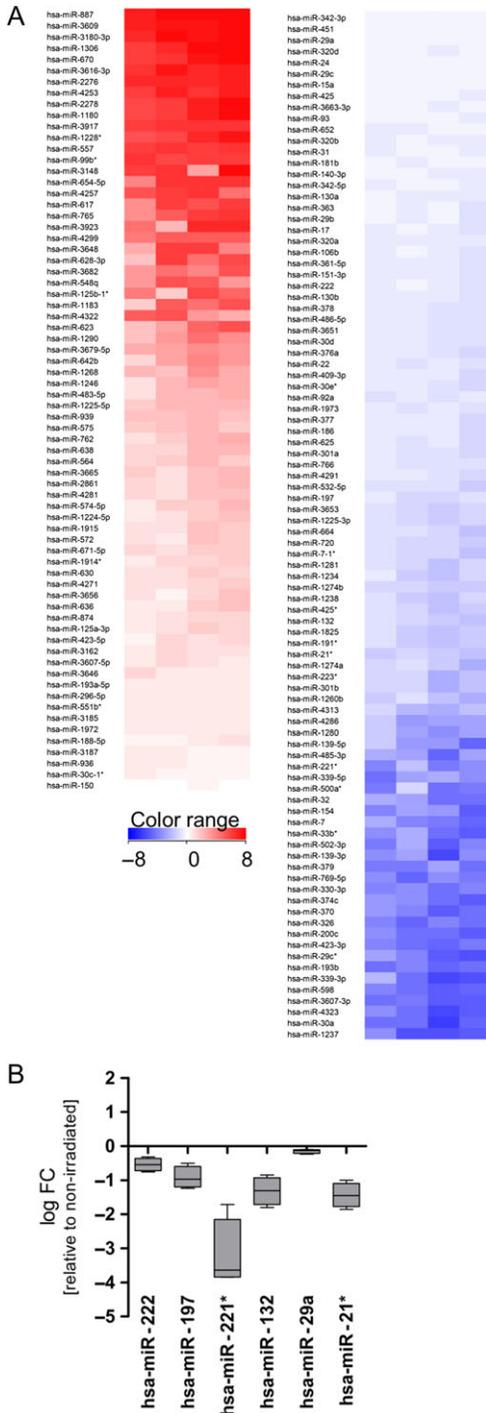
Selected lncRNAs were further subjected to qPCR validation (Fig. 3). Trp53cor1 was induced at both time-points after irradiation (Fig. 3A) and showed a dose-dependent increase with a 16-fold induction at 20 h after irradiation with 60 Gy (Fig. 3I). Similarly, the expression values of other lncRNAs also peaked at 20

h after exposure to high-dose IR, and could be well correlated to microarray data.

To further corroborate the role of p53 in the regulation of these lncRNAs, we used an alternative strategy to activate p53, namely by incubating PBMCs with nutlin-3. This specific chemical inhibitor of



**Fig. 3. Quantification of lncRNAs by qPCR.** Irradiated (ranging from 0.9 to 60 Gy) and non-irradiated human peripheral blood mononuclear cells (PBMCs) were cultured for 4 or 20 h prior to RNA isolation. Expression values were normalized relative to values in non-irradiated PBMCs. Relative gene expression levels were calculated based on the mean value from four samples using the comparative Ct method. Samples were normalized to the housekeeping gene B2M. Data are presented as mean + SD. In panels A–H, time- and dose-dependent changes of eight selected lncRNAs are given. In panels I–P, dose-dependent changes at 20 h after irradiation are shown of the same eight lncRNAs. One experiment of three carried out in duplicates is shown. Data are presented as mean + SEM \* $P < 0.05$ , paired  $t$ -test.



**Fig. 4.** High-dose radiation regulated miRNA expression. Differentially expressed miRNAs in 60 Gy irradiated versus non-irradiated peripheral blood mononuclear cells (PBMCs) are shown. (A) The heat map shows log fold expression change of 177 miRNAs differentially expressed 20 h after irradiation. The range of expression was from  $-8$  (blue,

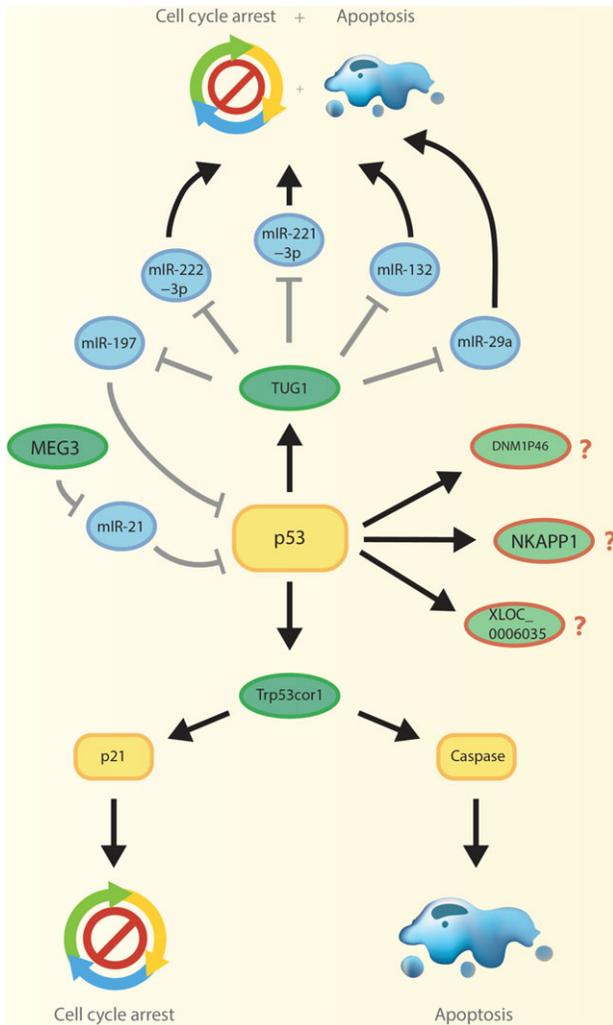
MDM2 that inhibits the degradation of p53, thereby leading to p53 accumulation. Addition of nutlin-3 to PBMC cultures did not induce p53 mRNA, but led to a strong upregulation of p21. In addition we found a significant upregulation of several lncRNAs, including Trp53cor1, MEG-3, TUG1, XLOC\_0066035, NKAPP1 and DNMI46, suggesting that at least these lncRNAs are indeed regulated via a p53-dependent mechanism (supplementary Fig. 3A–K).

Since the tumor suppressor gene p53 is one of the major regulators of cellular processes after IR, we performed *in silico* analysis to establish interaction networks between p53 and selected lncRNAs regulated after exposure to IR. In addition, we also included mRNA–miRNA–lncRNA interactions. We therefore also analyzed miRNAs regulated 20 h after high-dose IR from the same samples. In total, 177 miRNAs were differentially expressed in the irradiated PBMCs at 20 h after irradiation (Fig. 4A). We further investigated several miRNAs that are known targets of lncRNAs. We indeed found significant downregulation of these miRNAs (miR-197, miR-222-3p, miR-221-3p, miR-132, miR-29a and miR-21; Fig. 4B) and upregulation of the corresponding lncRNAs targeting these miRNAs (TUG1 and MEG3; Fig. 3). Figure 5 shows, based on our data and on previous publications, an *in silico* network analysis including p53, regulated lncRNAs and miRNAs. The network depicts interactions between p53 and the lncRNAs Trp53cor1, MEG-3, TUG1 and DNMI46 (C15orf51) that have been shown to be either targets of p53 [24] or themselves target p53 via miRNAs [25]. Trp53cor1 downstream either activates p21 leading to cell cycle arrest [26] and/or caspas-3 cleavage leading to execution of apoptosis [27]. TUG1 leads to inhibition of several miRNAs (miR-197, miR-222-3p, miR-221-3p, miR-132, miR-29a), which could counteract the p53-dependent induction of cell cycle arrest and apoptosis. The network also shows lncRNAs with so far unknown functions (DNMI46, NKAPP1 and XLOC\_0066035) that are upregulated via p53.

**DISCUSSION**

In this study we evaluated the effects of high-dose IR on the regulation of lncRNAs and cellular processes, such as cell cycle progression and cell death, in human PBMCs. We showed that irradiation with a dose of  $\geq 30$  Gy induced cleavage of the executor caspase-3, suggesting that high-dose IR is necessary for caspase-3-dependent induction of apoptosis in our experimental setting. Our analysis further revealed that  $\sim 10\%$  of all detectable lncRNAs were regulated in PBMCs 20 h after high-dose IR, and among these several were linked to p53 signaling. For example, the lncRNA Trp53cor1, a p53 downstream target, was one of the most highly regulated lncRNAs. To confirm an involvement of p53 accumulation in the regulation of lncRNAs, we also activated p53 with nutlin-3. Together these data suggest that a specific set of lncRNAs are involved in the

downregulation) to 8 (red, upregulation). The names of the genes are given on the right border of the heat map. (B) Expression values of six selected miRNAs known to be regulated by p53 are given as box plots (10–90 percentile) ( $n = 4$ ).



**Fig. 5. lncRNA–miRNA–mRNA interaction network.** p53 displayed a central role in executing apoptotic pathways *via* modulating lncRNA activity. lncRNAs are highlighted in green, miRNAs in blue and mRNAs in yellow. Six lncRNAs induced in response to high-dose radiation were linked with p53 activity either as an upstream molecule (MEG3) or downstream target (TUG1, Trp53cor1, DNMI1P46, NKAPP1 and XLOC\_0006035). Trp53cor1 downstream activated both—p21 leading to cell cycle arrest and activation of caspase-3 eventually resulting in apoptosis. The lncRNA TUG1 counteracted the induction of cell cycle arrest and apoptosis by inhibiting miRNAs (miR-197, miR-222-3p, miR-221-3p, miR-132 and miR-29a). p53 also activated three other lncRNAs with unknown functions (encircled in red; DNMI1P46, NKAPP1 and XLOC\_0006035).

regulation of cell-cycle arrest and apoptosis in response to high-dose irradiation via a p53-dependent mechanism.

Human PBMCs react particularly sensitively to IR, and are therefore commonly used in radiation research to study the effects of IR

*in vivo* and/or *in vitro* [9, 10]. More recently, exposure of PBMCs to high-dose IR has been shown to induce cell signaling pathways that enable the use of the irradiated cells or their paracrine factors (secretome) as therapeutics in regenerative medicine [11–18, 28]. Whereas the effects of radiation doses below 10 Gy are very well studied, the effects of high-dose IR on PBMCs are largely unknown.

In this study we were able to show in our experimental set-up that IR time- and dose-dependently induced the accumulation of p53 and the expression of its downstream target p21. Interestingly, p53 accumulation and p21 expression were less pronounced after high-dose IR. However, high-dose IR led to the activation of caspase-3, a marker for execution of apoptosis. This observation could suggest that after high-dose irradiation different biological pathways that are also regulated by the accumulation p53 are activated. More sophisticated experiments would be needed to test this hypothesis, but our results provide a basis for future studies on this topic. It was striking that only high-dose IR induced apoptosis in our experimental setting. As the induction of apoptosis also depends on the cell-cycle phase of the cells [29], it is conceivable that the relative low radiosensitivity of PBMCs is due to the lack of cycling cells.

The biological effects of selected lncRNAs on cell proliferation, differentiation and apoptosis, highlighting the role of p53 in these processes, have already been explored [8, 26, 27]. p53 is both a transcriptional activator of lncRNA expression and a target for lncRNA-mediated modulation of its function [30]. UVB- [27] as well as  $\gamma$ -irradiation [7] are known to induce p53 and its downstream lncRNA, Trp53cor1, leading to apoptosis. As demonstrated by Wang *et al.*, overexpression of the lncRNA Trp53cor1 directly induced caspase-3 cleavage and apoptosis [31]. Our results are in line with this study, showing that Trp53cor1 was dose-dependently upregulated in response to IR, and expression levels of Trp53cor1 correlated with the amount of cleaved caspase-3. However, Trp53cor1 is not only responsible for p53-dependent cell death, but also modulates p21-mediated cell cycle inhibition, which is thought to be independent of the p53–Trp53cor1 apoptotic pathway [26]. Therefore it might be conceivable that, depending on the trigger (e.g. radiation dose), Trp53cor1 initially promotes p21-dependent cell cycle arrest and (after exceeding a cell-specific threshold) caspase-3-dependent cell death.

To further evaluate the role of p53 in the regulation of lncRNAs, we exposed PBMCs to Nutlin-3, which leads to concentration-dependent p53 stabilization. As expected, Nutlin-3 induced gene expression of p21 and Trp53cor1. Moreover, several lncRNAs that were upregulated in response to high-dose IR were also induced in Nutlin-3-treated cells, supporting our hypothesis that p53 is indeed involved in the regulation of several radiosensitive lncRNAs.

Beside the interaction of lncRNAs with p53, it has been proposed that lncRNAs also interact with other non-coding RNAs such as miRNAs [1]. lncRNAs might bind to miRNAs, thereby inhibiting miRNA binding to its target mRNAs [32]. Bioinformatics tools such as starBase [23] or DIANA-LncBase [22], in combination with cross-linking immune precipitation–high throughput sequencing data, enable researchers to predict interactions between small and large non-coding RNAs and to better describe regulative biological networks. This bioinformatics approach revealed an interaction network that involves p53-controlled lncRNAs and regulated radioreponsive miRNAs. All miRNAs shown in our network were

downregulated in response to IR. Two of these miRNAs (miR-197 and miR-21), directly interacting with and inhibiting p53 [33, 34], are targets of two lncRNAs (TUG1 and MEG3) that were induced by IR [25]. Thus an IR-induced upregulation of these lncRNAs would repress the action of the miRNAs, leading to a de-repression of p53. In addition, several miRNAs (miR-222-3p, miR-221-3p, miR-132 and miR-29a) that are also targets of the lncRNA TUG1, have been shown to promote cell cycle arrest and apoptosis by targeting other mRNAs, including KIT, AKT and BCL2 [35, 36]. It is tempting to speculate that the inhibition of these miRNAs could counteract the induction of excessive cell death after high-dose IR. Our study also identified several so far uninvestigated lncRNAs (DNM1P46, NKAPP1 and XLOC\_0006035), which might be important for the response to IR.

Our study has several implications for radiation research. Since high-dose IR is increasingly used in cell-based and cell-free (secretome) therapies to trigger beneficial signaling pathways [11, 18], our study adds new information on the underlying regulatory mechanisms, by taking into account that lncRNAs also contribute to gene regulation in response to IR. These mechanisms might be important for the induction of the regenerative capacities of cells observed by several groups [12–18, 28]. However, the exact functions of most of the identified lncRNAs are still not known. Hence, knock-out or overexpression experiments will be necessary in the future to fully understand their contributions to the IR-mediated cellular responses. In addition, by demonstrating a close relationship between p53, the lncRNA Trp53cor1 and cleaved caspase-3 expression, our data further corroborate clinical data showing a p53-dependent deregulated expression of the lncRNA Trp53cor1 in certain malignancies such as chronic lymphocytic leukemia [37] and colorectal cancer [38].

Taken together, our study proposes p53–lncRNA–miRNA interactions that orchestrate the processes of cell cycle and programmed cell death in response to high-dose IR. Our study is the first to show that, together with mRNAs and miRNAs, lncRNAs are also involved in the cellular stress reactions in PBMCs in response to high-dose IR. Understanding the whole spectrum of the complex regulatory processes involved in IR-induced cell cycle arrest and apoptosis, including lncRNAs, might build the basis for future studies on the development of novel therapeutic avenues for cancer treatment.

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#### SUPPLEMENTARY DATA

Supplementary data is available at the *Journal of Radiation Research* online.

#### CONFLICT OF INTEREST

The Medical University of Vienna has claimed financial interest. Aposcience AG holds patents related to this work (EP20080450198

and EP20080450199). HJA is a shareholder of Aposcience AG. All other authors declare no potential conflicts of interest.

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