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DIFFERENTIAL REGULATION OF microRNA EXPRESSION IN IRRADIATED AND BYSTANDER CELLS

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The ionizing radiation (IR) induces a variety of biological effects in irradiated cells. Additionally, the irradiated cells communicate with unirradiated cells and induce changes in them through a phenomenon termed as the bystander effect. The nature of the bystander effect signal and how it impacts unirradiated cells remains to be discovered. Examination of molecular changes in bystander cells due to signals from irradiated cells could lead to the identification of the pathways underlying the bystander effect. To gain insight into the molecular pathways affected by the transmission of signal from irradiated cells to bystander cells, we monitored the microRNA (miRNA) transcriptional changes. miRNAs control gene expression at the posttranscriptional level. In previous studies from our laboratory the modulation of miRNA in irradiated human cells were identified. In the present work human lymphoblasts TK6 cells in a medium exchanged bystander effect model system were used to analyze miRNA expression alterations by employing the real time RT-PCR technology. The relative expression of several miRNAs involved in *RAS*, *c-MYC* and *BCL2* gene regulation were examined. The let-7 family of miRNAs was upregulated in irradiated cells but most of these miRNAs remained repressed in bystander cells. The miR-17-3p, miR-19b, and miR-18a were upregulated in irradiated cells but were repressed in the bystander cells. The miR-17-5p, miR-142-3p, miR-142-5p, and miR-19a were induced only for a short time in bystander cells. The miR-15a, miR-16, miR-143, miR-145, miR-155, and miR21 were upregulated in irradiated TK6 cells. While the expression of miR-15a, miR-16, miR-155, and miR-21 was repressed, the miR-143 and miR-145 expression was induced in bystander cells. These results indicate the involvement of miRNA modulation in irradiated and bystander cells.

Keywords: **micro-RNA, non-protein coding RNA, TK6 cells, radiation-induced bystander effect.**

The traditional paradigm of radiation-induced effects is based on cells damaged directly by the radiation. Current evidence suggests that in cell populations exposed to ionizing radiation (IR), biological effects occur in a much larger proportion of cells than those are estimated to be traversed by radiation [1–3]. The irradiated cells are capable of providing signals to the neighboring cells, and that signal results in biological effects to nearby unirradiated cells. This phenomenon was termed as bystander effect. Evidence for a bystander effect in irradiated cell populations was first reported in 1992 [4]. Direct evidence of the bystander effect was provided in studies where the transfer of medium from irradiated cells was shown to induce cell killing of unirradiated bystander cells [5]. Non-targeted effects of IR have significant implications for understanding mechanisms of radiation action (reviewed in [2]).

Biological responses in bystander cells were investigated in many studies. An increase in the frequency of micronuclei formation, sister chromatid exchanges, gene mutations and chromosomal instability were reported to take place in bystander cells [4, 6–9]. Apart from these, an increase in the frequency of random mitochondrial mutations was found in bystander cells exposed to conditioned media from irradiated tumor explants, and this was associated with a reduction in mitochondrial membrane potential [10]. Changes in gene expression were observed in bystander cells [11]. Expression levels of *p53*, *p21* and *MDM2* genes in bystander cells were modulated [12]. The expression changes of 18 genes, involved in mitogen-activated protein kinase (MAPK) pathway, following exposure to IR were shown in bystander cells [13]. The α-particle-induced metabolic reactive oxygen species (ROS) production activates signaling pathways in bystander cells [14]. It is suggested that many factors influence a bystander response including the linear energy transfer (LET) of the radiation. The existence of bystander effect after high LET radiation, such as α-particles is

Abbreviations: IR – ionizing radiation; ROS – reactive oxygen species.

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very well documented [15]. A low LET X-irradiation was shown to be capable of inducing medium-mediated bystander effects in fibroblasts [16].

The mechanism(s) underlying the bystander effect has not been completely understood. It was suggested that the production of ROS, direct cell-to-cell signaling via gap junctional intercellular communication or soluble factor(s) released into the medium surrounding irradiated cells could be responsible [4, 5, 11, 17–19]. The effect may be mediated by signaling pathways responsive to oxidative stress. The activation of stress-related kinases and their downstream transcription factors JNK, ERK1/2, p90RSK, Elk-1 and ATF2 was shown to take place in bystander cells [12]. Free radicals originating in cellular membranes and mitochondria are believed to play an important role in the bystander response [20], and protection effect of ROS-scavenging catechins on bystander cells from radiation effects was also reported [21].

This study was undertaken to understand molecular processes by which the IR exposure may elicit bystander responses. Our goal was to examine microRNA (miRNA) expression in irradiated and bystander cells. Changes in miRNA levels were shown to be associated with many human diseases. miRNAs are small non-protein-coding single-stranded RNAs of ~22 nucleotides that function as negative gene regulators. miRNAs control a wide range of biological functions by regulating hundreds of mRNA targets [22]. miRNAs negatively regulate their targets by binding with perfect or nearly perfect complementarity to mRNAs to induce the RNA-mediated interference pathway [23]. miRNAs are bound by Argonaute (Ago) proteins in RNA-induced silencing complexes (RISCs) [24]. Most miRNAs do not degrade their mRNA targets as a mechanism of gene regulation. These miRNAs bind to imperfect complementary sites within the 3'-untranslated regions of their mRNA targets. In this case the target-gene repression occurs post-transcriptionally at the level of translation [25] resulting in reduced protein levels but the mRNA levels remain unaffected [26]. The degree of complementarity between a miRNA and its target mRNA determines the mechanism of post-transcriptional regulation [27]. Nearly perfect pairing induces cleavage of the target mRNA, whereas partial pairing results in translational repression and mRNA decay. A single miRNA has the capability to bind to as many as 200 diverse mRNA targets ranging from transcription factors, secreted factors, receptors and transporters [26, 28], thus potentially controlling the expression of about one-third of human mRNAs.

X-irradiation can induce a medium-mediated bystander response in unirradiated human TK6 cells co-cultured with irradiated cells in a transwell insert culture dish [9, 16]. In the present study, we asked whether medium-mediated bystander effect in TK6 are accompanied by alterations in the miRNA expression levels. To our knowledge, the modulation of miRNAs in bystander cells co-cultured with irradiated cell conditioned medium has not been reported. Here, different miRNA expression in irradiated and bystander cells was detected. Irradiated cells displayed an ability to induce changes in the expression level of miRNAs in bystander cells.

EXPERIMENTAL

Cell culture and maintenance. The human lymphoblast cell line TK6 was obtained from Dr. Howard Liber (Colorado State University, Fort Collins, CO, USA). The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum ("Invitrogen", USA), 100 mg/mL of streptomycin and 100 U/mL of penicillin. The cell culture was maintained at a density of 2.5×10^5 cells/mL in a 37°C-incubator with 5% CO_2 and 100% humidity.

Ionizing radiation treatment and cell co-culture. Irradiation of 3×10^6 cells was performed with a RAD Source 2000 X-ray Biological irradiator ("Alpharetta", USA) available at the University of Vermont. The cells were seeded at a density of 3×10^6 per mL and irradiated at room temperature with a dose of 2 Gy at a dose rate of 1.7 Gy/min.

The cell culture transwell inserts ("Becton Dickinson Biosciences", USA) were used to study the bystander effect [16]. The bottom of the insert is a membrane with 1 μm pores at a density of $1.6 \times 10^6/cm^2$. The polyethylene terephthalate membranes are highly permeable and allow increased rate of basolateral diffusion of nutrients and other molecules for transport, secretion or binding studies. The transwell culture insert dish has a growth area of 4.2 cm^2, and the companion well of a six-well plate has a growth area of 9.6 cm^2. The distance from the membrane of the insert dish to the bottom of the well of the companion plate is 0.9 mm.

The culture media of irradiated cells was changed immediately after irradiation with fresh unirradiated media. The unirradiated cells were plated into six-well dishes at a density of 2×10^5 cells/mL. The inserts containing 2×10^5 cells/mL of irradiated cells were put into the wells of dishes (fig. 1), and the six-well plates along with inserts were returned to the incubator. The irradiated and bystander cells were co-cultured to allow the induction of X-ray-induced bystander effect until the time at which cells were harvested. The control samples were treated in the same way, except for irradiation. The treated and bystander cells were incubated at 37°C and harvested at 0, 0.5, 4, 8, 12, and 24 h before RNA isolation. There was one well for each time-point, and the experiment was repeated three times independently. All the timencourse samples were compared to zero-time point control.

Cell viability assay. The viability of cells was measured by a trypan blue exclusion assay. Cell suspension (80 μL) was put into a well of a 24-well plate (1×10^5 cells/well)

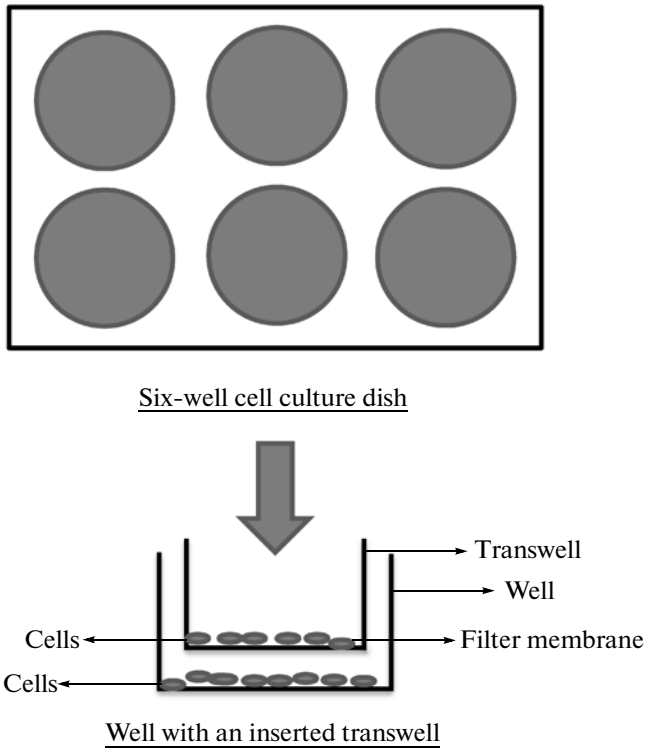

Fig. 1. The co-culture system using transwell to study bystander effect. The unirradiated cells were plated in six-well dishes, as described in the experimental section. Transwell inserts were placed in each well of the dish, and irradiated cells were put in the insert to initiate co-culture of irradiated and unirradiated cells.

and incubated at 37°C for 0–24 h after irradiation. Twenty microliters of trypan blue solution (0.05% in phosphate-buffered saline, PBS) were added to the cell suspension. After 10 min at room temperature, cell number was measured with the use of hemocytometer ("Millipore", USA).

RNA isolation. The miRNAs were isolated from the harvested cells. The control (mock irradiated) and irradiated cells were counted with a hemocytometer. Approximately 5×10^6 cells were pelleted by centrifugation at 1 500 rpm for 5 min, and washed with 1 mL of the (Mg^{2+} and Ca^{2+})-free Dulbecco in PBS ("Invitrogen"). Small RNAs, less than 200 nucleotides, were isolated from cells using the "mirVana™ miRNA" isolation kit following the enrichment procedure for small RNA recovery ("Applied Biosystems", USA). The quantity and quality of miRNAs were evaluated with the use of the BioPhotometer ("Eppendorf", USA) and electrophoresis in 2% agarose gel with ethidium bromide detection, respectively.

miRNA. Here, miRNA targets *hsa-let-7a, hsa-let-7b, hsa-let-7c, hsa-let-7d, hsa-let-7e, hsa-let-7f, hsa-let-7g, hsa-let-7i, hsa-miR-142-3p, hsa-miR-142-5p, hsa-miR143, hsa-miR-145, hsa-miR-155, hsa-miR-15a, hsa-miR-16, hsa-miR-17-3p, hsa-miR-17-5p, hsamiR-18a, hsa-miR-19a, hsa-miR-19b, and hsa-miR-21* were investigated. All miRNAs selected for this study are expressed in TK6 cells and are modulated in TK6 cells after irradiation with X-rays [29].

These miRNA genes were selected from the Sanger Center miRNA Registry at http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml. All TaqMan assays and endogenous controls for miRNA analysis and assays on demand for gene expression analysis were purchased from "Applied Biosystems", USA. Standard TaqMan assays were designed using PrimerExpress software ("Applied Biosystems"). Since most of the miRNAs from let-7 family differ from each other only by 1–3 nucleotides, the TaqMan probes for different members of let-7 family could bind to several miRNAs. Although there is no information available on TaqMan probes for these miRNAs at the Applied Biosystem's website, the specificity of these probes was assured by the manufacturer. All RNA samples for miRNA analysis were normalized based on the TaqMan Gene Expression Assays for human endogenous RNU44 control.

cDNA synthesis reactions. Reverse transcriptase (RT) reactions were set up using a commercial kit from "Applied Biosystems", which contained RNA samples, 50 nM stem-loop RT-primer, RT-buffer, 0.25 mM each of dNTPs, 3.33 U/μL of MultiScribe™ reverse transcriptase and 0.25 U/μL of RNase inhibitor. The 15 μL reaction mixtures were incubated in Techne TC-312 thermocycler ("Burlington", USA) for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. All RT-reactions, including no-template controls and RT-minus controls, were run in duplicate.

Quantitative real-time polymerase chain reaction (QPCR) and data analysis. QPCR was performed on a 7900HT Sequence Detection System ("Applied Biosystems") by using a standard TaqMan PCR kit protocol. A 10 μL sample for the PCR contained 0.67 μL of RT-product, TaqMan Universal PCR Master Mix, 0.2 μM TaqMan probe, 1.5 μM forward primer and 0.7 μM reverse primer. The reactions were incubated in a 384-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative expression values of cycle thresholds were calculated by using the comparative delta delta cycle threshold, $\Delta\Delta C_T$, method [30] by normalization to the control miRNA RNU44. The threshold cycle (C_T) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Statistical significance was determined using analysis of variance (ANOVA). The statistics and data analysis were performed with ABI prism and GraphPad Prism 5 software, both licensed to the University of Vermont. C_T values for the 21-targets of miRNAs in each cell line were statistically evaluated using a one-way T-test ($p < 0.05$). All experiments were repeated three times and differences in miRNA levels were statistically significant between the irradiated and bystander cells.

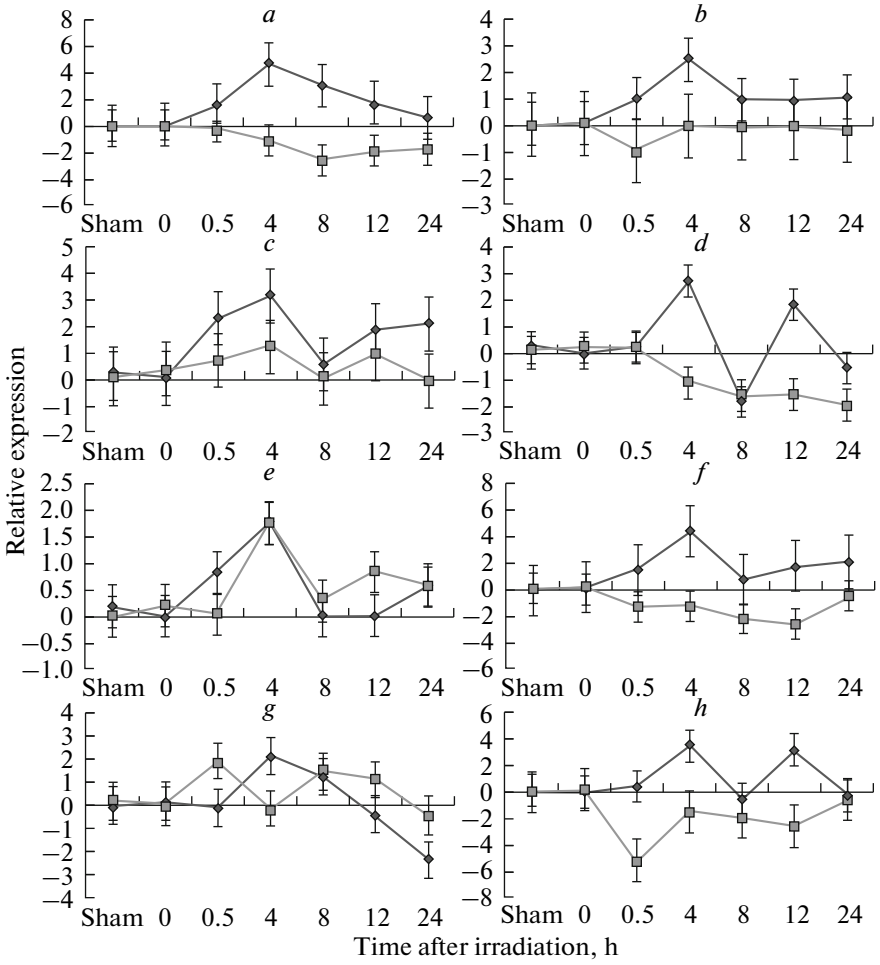

Fig. 2. The expression of let-7 miRNA family members relative to unirradiated controls in 2-Gy irradiated TK6 and bystander cells. The miRNA expression was assessed at 0, 0.5, 4, 8, 12 and 24 h post irradiation. The relative expression is shown as Log_2 transformed values for: *a* – let-7a, *b* – let-7b, *c* – let-7c, *d* – let-7d, *e* – let-7e, *f* – let-7f, *g* – let-7g, *h* – let-7i. ◆ – Irradiated cells, ■ – bystander cells. Here and further, error bars indicate the standard error of the mean (SEM) for three independent experiments.

RESULTS

TK6 cells were irradiated with 2-Gy dose of X-rays and co-cultured with unirradiated cells using a transwell system [9, 16] to study miRNA responses in both irradiated and bystander cells (fig. 1). At 0, 0.5, 4, 8, 12 and 24 h time points the irradiated and bystander cells were harvested. The miRNA isolated from the harvested cells was subjected to QRT-PCR expression analysis. The expression level of several miRNAs including eight let-7 family members, and miRNAs involved in *c-MYC* and *BCL2* gene regulation was evaluated. Different expression levels of miRNAs relative to sham controls were observed in the irradiated and bystander cells.

The expression analysis of miR-let-7a, miR-let-7b, miR-let-7c, miR-let-7d, miR-let-7e, miR-let-7f, miR-let-7g, and miR-let-7i in irradiated and bystander cells during a 24-h time period is shown in figure 2. The expression of miR-let-7a, miR-let-7b, miR-let-7c, miR-let-7e, miR-let-7f, and miR-let-7g miRNAs was upregulated and reached a maximum level at 4 h in irradiated cells (fig. 2). There was a decline in the expression of these miRNAs at the 8-h time point. There were two peaks of induction, namely: one at 4 h and the other at 12 h – in the expression levels of miR-let-7d, and miR-let-7i (fig. 2*d*,*h*).

The expression of most of eight let-7 miRNAs in bystander cells remained downregulated throughout the 24 h time period (fig. 2). The miR-let-7c showed two peaks of expression upregulation: one at 4 h and another at 12 h (fig. 2*c*). The miRlet-7e was induced at 4 h and then its expression level was declined (fig. 2*e*). The miR-let-7g was induced at 0.5- and 8-h time points (fig. 2*g*). There were significant differences in the miR-let-7a ($p = 0.04$) and miR-let-7f ($p = 0.03$) expression levels in the bystander cells in comparison with unirradiated control cells. There were significant differences in the relative expression of miRlet-7a ($p = 0.02$), miR-let-7b ($p = 0.01$), miR-let-7c ($p = 0.02$),

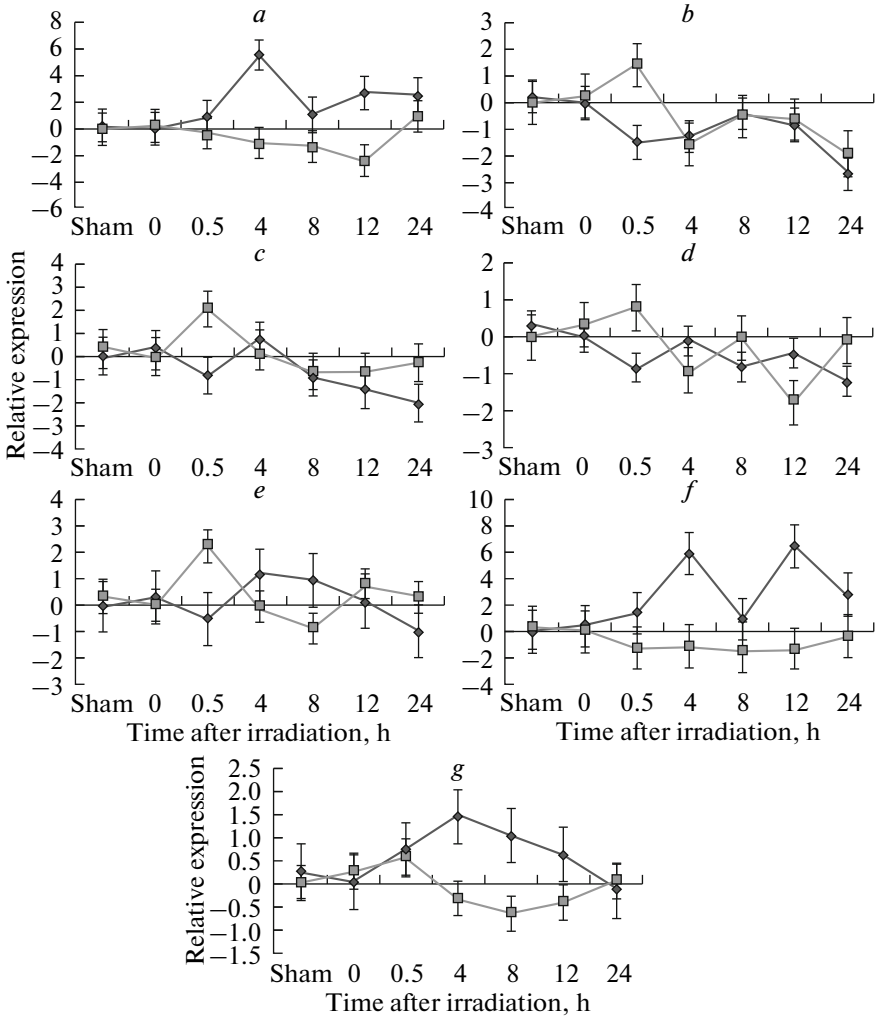

Fig. 3. The expression of *c-MYC*-related miRNA relative to sham-irradiated controls in 2-Gy irradiated TK6 and bystander cells. The miRNA expression was assessed at 0, 0.5, 4, 8, 12 and 24 h post irradiation. The relative expression is shown as Log_2 transformed values for: *a* – miR-17-3p, *b* – miR-17-5p, *c* – miR-142-3p, *d* – miR-142-5p, *e* – miR-19a, *f* – miR-19b, *g* – miR-18a. ◆ – Irradiated cells, ■ – bystander cells.

miR-let-7f ($p = 0.01$), and miR-let-7i ($p = 0.04$) between irradiated and bystander cells.

The modulation of miR-17-3p, miR-17-5p, miR-142-3p, miR-142-5p, miR-19a, miR-19b, and miR-18a in irradiated TK6 and bystander cells was evaluated (fig. 3). These miRNAs have shown to be involved in the regulation of *c-MYC* gene. In irradiated TK6 cells, miR-17-3p, miR-19a, miR-19b, and miR-18a were upregulated until 4 h following the IR treatment and downregulated after that time. An induction in the expression level of miR-19b was seen at the 12-h time point (fig. 3). The miR-17-5p, miR-142-3p, and miR-142-5p remained repressed after IR-treatment when monitored during the 24-h time course experiment (fig. 3). In bystander cells miR-17-5p, miR-142-3p, miR142-5p, miR-18a, and miR-19a were induced at the early time point of 0.5 h and were repressed at later time points. Only miR-19a expression was upregulated later, at 12 h (fig. 3*e*). The expression of miR-17-3p was repressed at 12-h time point and miR-19b remained repressed throughout the time of the experiment. There was significant difference in the miR-19b ($p = 0.026$) expression levels in the bystander cells in comparison with unirradiated control. There was significant difference in the relative expression of miR-17-3p ($p = 0.04$) and miR-19b ($p = 0.02$) in irradiated and bystander cells.

Results obtained for miR-15a, miR-16, miR-143, miR-145, miR-155, and miR-21 expression in irradiated and bystander cells are shown in figure 4. All of these miRNAs were upregulated in irradiated TK6 cells. The highest level of expression was reached at 4 h in the case of miR-155 (fig. 4*c*) and miR-21 (fig. 4*f*). The maximum expression of miR-15a was observed at 12 h (fig. 4*a*). Two distinct peaks of induction were seen for miR-16, miR-143, and miR-145. These miRNAs were upregulated at 4 h and 24 h post irradiation (fig. 4*b*,*d*,*e*).

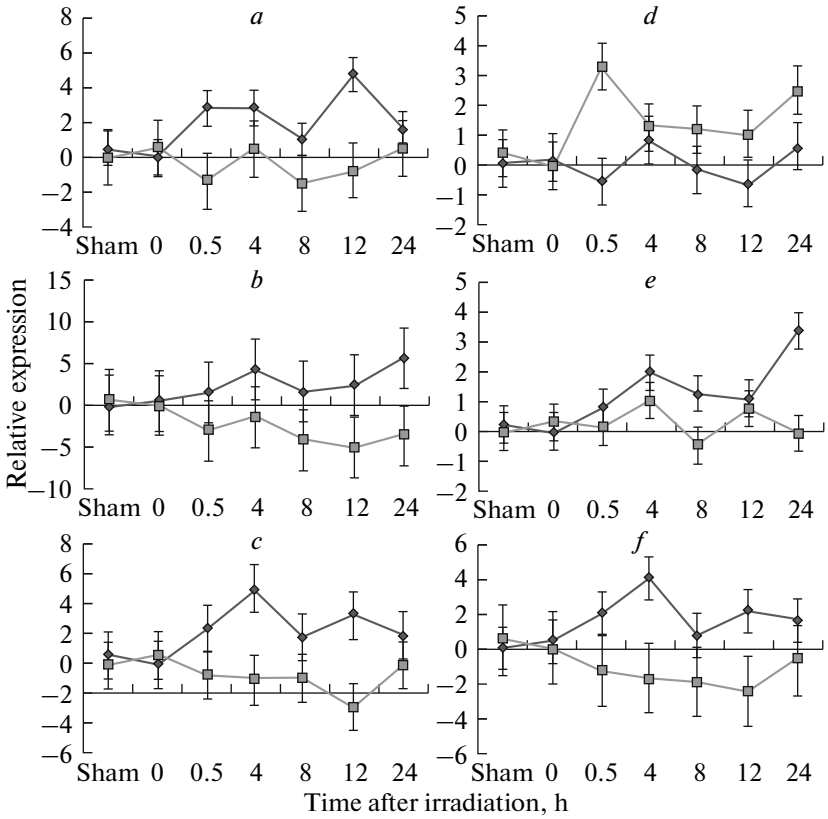

Fig. 4. Modulation of various miRNAs in irradiated TK6 and bystander cells. The cells were treated with a 2-Gy X-ray dose and co-cultured with unirradiated cells. The expression relative to sham-irradiated controls, shown as Log_2 transformed values, was computed at 0, 0.5, 4, 8, 12, and 24 h time points for: *a* – miR-15a, *b* – miR-16, *c* – miR-155, *d* – miR-143, *e* – miR145, *f* – miR-21. ◆ – Irradiated cells, ■ – bystander cells.

The expression of miR-15a, miR-16, miR-155, and miR-21 was repressed in bystander cells (fig. 4). The miR-143 expression in bystander cells had two peaks of induction. It was first induced at 4 h and again at 24 h after a downregulation (fig. 4*d*).The miR-145 also had two peaks of upregulation; one as seen was at the 4-h time point and another was observed at 12 h (fig. 4*e*). There was significant difference in the miR-16 ($p = 0.027$), miR-143 ($p = 0.02$), and miR-21 ($p = 0.03$) expression levels in the bystander cells in comparison with the unirradiated control cells.There was significant difference in the relative expression of miR-15a ($p = 0.02$), miR-16 ($p = 0.008$), miR-155 ($p = 0.02$), miR-143 ($p = 0.04$), and miR-21 ($p = 0.01$) between irradiated and bystander cells.

Finally, we investigated, if the increasing levels of miRNAs in bystander cells were due to contamination of these cells by the medium with accumulated miRNAs resulted from irradiated cell destruction. At first, viability of cells after IR at various time points was monitored. During the 24-h time course no evidence of cell destruction as assessed by trypan blue exclusion assay was obtained (fig. 5). Furthermore, the expression levels of several miRNAs in the culture medium were evaluated. The results indicated that these miRNAs were absent in the culture medium (fig. 6).

DISCUSSION

Understanding the effects of IR is important for evaluating its use in therapy along with assessing risk to human health from its exposure. Various biological effects are induced after exposure to IR [31]. Modern radiobiology documents the phenomenon of bystander effect, whereby cells that are not traversed by IR ex-

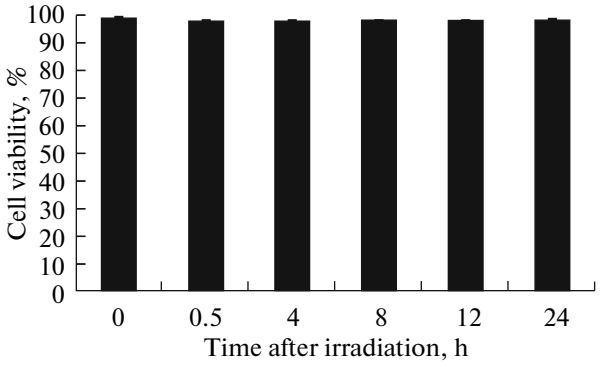

Fig. 5. Cell viability in irradiated TK6 cells. The cells were treated with a 2-Gy X-ray dose and sampled at 0, 0.5, 4, 8, 12 and 24 h time points. The cell viability expressed as percentage values was determined by a trypan blue exclusion assay.

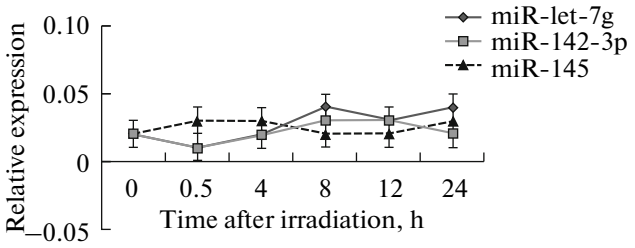

Fig. 6. Investigation of various miRNAs in the culture medium from irradiated TK6. The cells were treated with a 2-Gy X-ray dose and the culture medium was collected at 0, 0.5, 4, 8, 12 and 24 h time points. The miRNA expression levels relative to sham-irradiated controls are shown as Log_2 transformed values.

hibit responses like irradiated cells. The existence of a radiation bystander effect is now well-established. It complicates the interpretation of risks arising from abscopal effects in radiotherapy, occurrence of secondary tumors or cancers as a result of radiation accidents. The need for better understanding the bystander effect was realized [3]. The regulatory mechanisms involved in the bystander response have not been completely elucidated. To provide insight into the pathways responding in bystanders, we set out to measure miRNA expression levels. Here, we used X-ray irradiated human lymphoblasts, TK6 cells, in a transwell cell coculture system that precludes contact between targeted and bystander cells as a model system to examine miRNA expression alterations. A dose of 2 Gy was used to mimic the radiation therapy treatment and to avoid high toxic effects. TK6 cells for this study were selected because bystander effect was known to occur in these cells after treatment with 2-Gy dose of X-rays. It was shown that the X-ray-induced bystander signals produced mutagenic responses in TK6 cells [9]. We compared the miRNA expression patterns in directly irradiated and bystander cells after such single treatment. Several miRNAs examined in this study control the expression of *RAS*, *c-MYC* and *BCL-2* genes that are implicated in the IR-induced cellular response. In our previous studies it was shown that these miRNAs are modulated in human cells that were IR-treated [29, 32, 33]. The focus of the current study was to determine, if the miRNA expression changes are involved in bystander cells.

The monitoring of miRNA expression in X-ray-treated cells revealed differences in the relative expression of let-7 family of miRNA between the directly irradiated and bystander cells. The let-7 miRNA family is a negative regulator of the *RAS* oncogene [34]. Downregulation of *RAS* gene is associated with a poor prognosis of lung, pancreatic and colon cancers [35]. The let-7 miRNA family exhibited altered expression in response to IR-treatment [36]. It was shown that expression levels of the let-7 miRNA family members were upregulated in irradiated TK6 cells [29]. Here, we show that let-7 family miRNAs are upregulated in irradiated cells, but most of them are repressed in bystander cells (fig. 2). The miR-let-7c, miR-let-7e, and miR-let-7g were induced in both bystander and irradiated cells. The modulation of let-7 miRNAs might have a role in the response of bystander cells after IR-treatment.

In previous studies it was shown that the expression of the miRNA associated with *c-MYC* translocation was upregulated in cells exposed to gamma-radiation [32]. The investigation of these miRNAs in irradiated TK6 cells indicated that the miR-17-3p, miR-19a, miR-19b, and miR18a were upregulated (fig. 3). In contrast, the expression of miR-17-3p, miR-19b, and miR-18a remained repressed in bystander cells. The miR-17-5p, miR-142-3, and miR-142-5p were not induced in irradiated TK6 cells (fig. 3). In bystander cells the miR-19a was upregulated and miR-17-5p, miR-142-3p, miR-142-5p, and miR-19a were induced although only for a short time. These results suggest that miRNAs regulating *c-MYC* show differential expression in bystander cells. The proto-oncogene *c-MYC* regulates the expression of genes involved in cell division, cell growth, and apoptosis [37]. The *c-MYC* induces a cluster of miRNAs known as miR17-92. This cluster includes miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92-1 and is amplified in B-cell lymphomas [38]. Induced expression of the *c-MYC* correlated with tumor development and miR-17-92 cluster was designated as a potential oncogene [38]. The miR-17-5p targets *p21* and *BIM* genes [39]. Overexpressed miR-17-3p, miR18a, and miR-19a were found in lung cancers and B-cell lymphomas, and these are involved in angiogenesis [37]. Using microbeams, Iwakawa et al. [40] found different gene expression profiles in carbon ions-irradiated and bystander cells. Genes related to the cell cycle or death (*CDKN1A*, *GADD45A*, *NOTCH1* and *BCL2L1*), and cell communication *(IL1B*, *TCF7* and *ID1*) were upregulated in irradiated cells, but not in bystander cells. We previously reported that the radiation-induced gene expression profile in irradiated cells differed from unirradiated bystander cells [1]. Data on miRNA expression suggest that some pathways leading to biological effects in bystander cells may differ from those in directly irradiated cells.

Negative regulators of B-cell lymphoma miR-15a and miR-16-1 are downregulated in chronic lymphocytic leukemia [41]. These miRNAs target *MYB*, a gene that is involved in myeloid, lymphoid, or mixed-lineage leukemias [42]. miR-15a and miR-16 were upregulated in X-ray irradiated TK6 cells. Two distinct peaks of induction at 4-h and 24-h time points post irradiation were seen for miR-16. The miR-15a and miR-16 were not induced in bystander cells (fig. 4). These results suggest different responses to IR exposure in TK6 and bystander cells. The miR-15a and miR-16 also negatively regulate *BCL2* [40], and *BCL2* was implicated in the radiation response of TK6 cells [43].

The miR-143, miR-145, miR-155, and miR-21 were upregulated in X-ray irradiated TK6 cells (fig. 4). The miR-143 and miR-145 were also induced in bystander cells. The miR-143 regulates connective tissue growth factor and its expression correlates with adipocyte differentiation and carcinogenesis [44]. Altered expression of miR-145 and miR-155 was detected in lung cancers [45]. Similar expression patterns observed for certain miRNAs in directly irradiated and bystander cells indicate common functions in the IR-induced pathways. In many studies the gene expression in directly irradiated and bystander cells was compared. Global gene expression analyses of bystander and irradiated cells showed that *CDKN1A* level increased in directly exposed cells but was not changed in the bystander cells [46]. NF-κB expression regulated *PTGS2* (cyclooxygenase-2), *IL8* and *BCL2A1* genes, responded similarly in bystander and irradiated cells [46]. No differences in the transcriptomes of cells grown in medium from X-irradiated cells or directly irradiated were seen [47]. From transcript patterns of cells grown in culture medium from irradiated cells it was concluded that X-irradiated and bystander cells had similar changes in most functional pathways [48].

Cells exposed to IR release factors that induce DNA damage, chromosomal instability, apoptosis, alterations in gene expression, and changes in the proliferation rate of neighboring unexposed cells. Our findings suggest that miRNA levels are disturbed in bystander cells and may have a role in regulating the radiation response. There could be several possible reasons of miRNA expression alterations in bystander cells. The induction by ROS from medium, or an increase in the activity of transcription factors could be contributors to this effect. The modulation of miRNAs could be responsible for large-scale gene expression alterations in bystander cells leading to induction of non-targeted cellular effects. These observations provide a link of miRNA modulation after radiation exposure to the subsequent multiple gene regulation. The alteration in the balance of signaling is likely to lead to different outcomes in irradiated cells and their bystanders. These results imply that intercellular signaling between irradiated and bystander cells activate intracellular signaling, leading to the miRNA transcriptional stress response in bystander cells.

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REFERENCES

1. Chaudhry M.A. 2006. Bystander effect: biological endpoints and microarray analysis. *Mutat. Res.* **597**, 98–112.
2. Wright E.G. 2010. Manifestations and mechanisms of non-targeted effects of ionizing radiation. *Mutat. Res.* **687**, 28–33.
3. Goodhead D.T. 2010. New radiobiological, radiation risk and radiation protection paradigms. *Mutat. Res.* **687**, 13–16.
4. Nagasawa H., Little J.B. 1992. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. *Cancer Res.* **52**, 6394–6396.
5. Mothersill C., Seymour C. 1997. Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *Int. J. Radiat. Biol.* **71**, 421–427.
6. Nagasawa H., Little J.B. 1999. Unexpected sensitivity to the induction of mutations by very low doses of alpha-particle radiation: evidence for a bystander effect. *Radiat. Res.* **152**, 552–557.
7. Watson G.E., Lorimore S.A., Macdonald D.A., Wright E.G. 2000. Chromosomal instability in unirradiated cells induced *in vivo* by a bystander effect of ionizing radiation. *Cancer Res.* **60**, 5608–5611.
8. Nagasawa H., Little J. 2002. Bystander effect for chromosomal aberrations induced in wild-type and repair deficient CHO cells by low fluences of alpha particles. *Mutat. Res.* **508**, 121.
9. Zhang Y., Zhou J., Held K.D., Redmond R.W., Prise K.M., Liber H.L. 2008. Deficiencies of double-strand break repair factors and effects on mutagenesis in directly gamma-irradiated and medium-mediated bystander human lymphoblastoid cells. *Radiat. Res.* **169**, 197–206.
10. Gorman S., Fox E., O'donoghue D., Sheahan K., Hyland J., Mulcahy H., Loeb L.A., O'sullivan J. 2010. Mitochondrial mutagenesis induced by tumor-specific radiation bystander effects. *J. Mol. Med.* **88**, 701–708.
11. Azzam E.I., de Toledo S.M., Gooding T., Little J.B. 1998. Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. *Radiat. Res.* **150**, 497–504.
12. Little J.B., Azzam E.I., de Toledo S.M., Nagasawa H. 2002. Bystander effects: intercellular transmission of radiation damage signals. *Radiat. Prot. Dosimetry.* **99**, 159–162.
13. Asur R., Balasubramaniam M., Marples B., Thomas R.A., Tucker J.D. 2010. Bystander effects induced by chemicals and ionizing radiation: evaluation of changes in gene expression of downstream MAPK targets. *Mutagenesis.* **25**, 271–279.
14. Azzam E.I., de Toledo S.M., Spitz D.R., Little J.B. 2002. Oxidative metabolism modulates signal transduction and micronucleus formation in bystander cells

from alpha-particleirradiated normal human fibroblast cultures. *Cancer Res.* **62**, 5436–5442.

15. Sowa M.B., Goetz W., Baulch J.E., Pyles D.N., Dziegielewski J., Yovino S., Snyder A.R., de Toledo S.M., Azzam E.I., Morgan W.F. 2010. Lack of evidence for low-LET radiation induced bystander response in normal human fibroblasts and colon carcinoma cells. *Int. J. Radiat. Biol.* **86**, 102–113.
16. Yang H., Asaad N., Held K.D. 2005. Medium-mediated intercellular communication is involved in bystander responses of X-ray-irradiated normal human fibroblasts. *Oncogene.* **24**, 2096–2103.
17. Azzam E.I., de Toledo S.M., Little J.B. 2001. Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha -particle irradiated to nonirradiated cells. *Proc. Natl. Acad. Sci. USA.* **98**, 473–478.
18. Seymour C.B., Mothersill C. 2000. Relative contribution of bystander and targeted cell killing to the low-dose region of the radiation dose-response curve. *Radiat. Res.* **153**, 508–511.
19. Prise K.M., Belyakov O.V., Folkard M., Michael B.D. 1998. Studies of bystander effects in human fibroblasts using a charged particle microbeam. *Int. J. Radiat. Biol.* **74**, 793–798.
20. Hanot M., Hoarau J., Carriere M., Angulo J.F., Khodja H. 2009. Membrane-dependent bystander effect contributes to amplification of the response to alpha-particle irradiation in targeted and nontargeted cells. *Int. J. Radiat. Oncol. Biol. Phys.* **75**, 1247–1253.
21. Law Y.L., Wong T.P., Yu K.N. 2010. Influence of catechins on bystander responses in CHO cells induced by alpha-particle irradiation. *Appl. Radiat. Isot.* **68**, 726–729.
22. Fabian M.R., Sonenberg N., Filipowicz W. 2010. Regulation of mRNA translation and stability by microRNAs. *Annu. Rev. Biochem.* **79**, 351–379.
23. Yekta S., Shih I.H., Bartel D.P. 2004. MicroRNA-directed cleavage of HOXB8 mRNA. *Science.* **304**, 594–596.
24. Kai Z.S., Pasquinelli A.E. 2010. MicroRNA assassins: factors that regulate the disappearance of miRNAs. *Nat. Struct. Mol. Biol.* **17**, 510.
25. Pillai R.S., Bhattacharyya S.N., Artus C.G., Zoller T., Cougot N., Basyuk E., Bertrand E., Filipowicz W. 2005. Inhibition of translational initiation by Let-7 microRNA in human cells. *Science.* **309**, 1573–1576.
26. Lim L.P., Lau N. C., Garrett-Engele P., Grimson A., Schelter J.M., Castle J., Bartel D.P., Linsley P.S., Johnson J.M. 2005. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature.* **433**, 769–773.
27. Chekulaeva M., Filipowicz W. 2009. Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Curr. Opin. Cell Biol.* **21**, 452–460.
28. John B., Enright A.J., Aravin A., Tuschl T., Sander C., Marks D.S. 2004. Human microRNA targets. *PLoS Biol.* **2**, e363.
29. Chaudhry M.A., Kreger B., Omaruddin R.A. 2010. Transcriptional modulation of micro-RNA in human cells differing in radiation sensitivity. *Int. J. Radiat. Biol.* **86**, 569–583.
30. Livak K.J., Schmittgen T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods.* **25**, 402–408.
31. Abdullaev S.A., Antipova V.N., Gaziev A.I. 2009. Extracellular mutant mitochondrial DNA content is sharply elevated in the blood plasma of irradiated mice. *Mol. Biol. (Mosk.).* **43**, 990–996.
32. Chaudhry M.A. 2009. Real-time PCR analysis of micro-RNA expression in ionizing radiation-treated cells. *Cancer Biother. Radiopharm.* **24**, 49–56.
33. Chaudhry M.A., Sachdeva H., Omaruddin R.A. 2010. Radiation-induced micro-RNA modulation in glioblastoma cells differing in dna-repair pathways. *DNA Cell Biol.* **29**, 553–561.
34. Johnson S.M., Grosshans H., Shingara J., Byrom M., Jarvis R., Cheng A., Labourier E., Reinert K.L., Brown D., Slack F.J. 2005. RAS is regulated by the let-7 microRNA family. *Cell.* **120**, 635–647.
35. Goodsell D.S. 1999. The molecular perspective: the ras oncogene. *Oncologist.* **4**, 263–264.
36. Weidhaas J.B., Babar I., Nallur S.M., Trang P., Roush S., Boehm M., Gillespie E., Slack F.J. 2007. MicroRNAs as potential agents to alter resistance to cytotoxic anticancer therapy. *Cancer Res.* **67**, 11111–11116.
37. Xi Y., Edwards J.R., Ju J. 2007. Investigation of miRNA biology by bioinformatic tools and impact of miRNAs in colorectal cancer–regulatory relationship of c-Myc and p53 with miRNAs. *Cancer Inform.* **3**, 245–253.
38. He L., Thomson J.M., Hemann M.T., Hernando-Monge E., Mu D., Goodson S., Powers S., Cordon-Cardo C., Lowe S.W., Hannon G.J., Hammond S.M. 2005. A microRNA polycistron as a potential human oncogene. *Nature.* **435**, 828–833.
39. Fontana L., Fiori M.E., Albini S., Cifaldi L., Giovinazzi S., Forloni M., Boldrini R., Donfrancesco A., Federici V., Giacomini P., Peschle C., Fruci D. 2008. Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM. *PLoS One.* **3**, e22–36.
40. Iwakawa M., Hamada N., Imadome K., Funayama T., Sakashita T., Kobayashi Y., Imai T. 2008. Expression profiles are different in carbon ion-irradiated normal human fibroblasts and their bystander cells. *Mutant. Res.* **642**, 57–67.
41. Cimmino A., Calin G.A., Fabbri M., Iorio M.V., Ferracin M., Shimizu M., Wojcik S.E., Aqeilan R. I., Zupo S., Dono M., Rassenti L., Alder H., Volinia S., Liu C.G., Kipps T.J., Negrini M., Croce C.M. 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc. Natl. Acad. Sci. USA.* **102**, 13944–13949.
42. Chung E.Y., Dews M., Cozma D., Yu D., Wentzel E.A., Chang T.C., Schelter J.M., Cleary M.A., Mendell J.T., Thomas-Tikhonenko A. 2008. c-Myb oncoprotein is an

essential target of the dleu2 tumor suppressor microRNA cluster. *Cancer Biol. Ther.* **7,** 1758–1764.

43. Akerman G.S., Rosenzweig B.A., Domon O.E., Tsai C.A., Bishop M.E., Mcgarrity L.J., Macgregor J.T., Sistare F.D., Chen J.J., Morris S.M. 2005. Alterations in gene expression profiles and the DNA-damage response in ionizing radiation-exposed TK6 cells. *Environ. Mol. Mutagen.* **45**, 188–205.
44. Shahi P., Loukianiouk S., Bohne-Lang A., Kenzelmann M., Kuffer S., Maertens S., Eils R., Grone H.J., Gretz N., Brors B. 2006. Argonaute a database for gene regulation by mammalian microRNAs. *Nucl. Acids Res.* **34**, D115–118.
45. Yanaihara N., Caplen N., Bowman E., Seike M., Kumamoto K., Yi M., Stephens R.M., Okamoto A., Yokota J., Tanaka T., Calin G.A., Liu C.G., Croce C.M., Harris C.C. 2006. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell.* **9**, 189–198.
46. Ghandhi S.A., Yaghoubian B., Amundson S.A. 2008. Global gene expression analyses of bystander and alpha particle irradiated normal human lung fibroblasts: synchronous and differential responses. *BMC Med. Genomics.* **1**, 63.
47. Herok R., Konopacka M., Polanska J., Swierniak A., Rogolinski J., Jaksik R., Hancock R., Rzeszowska-Wolny J. 2010. Bystander effects induced by medium from irradiated cells: similar transcriptome responses in irradiated and bystander K562 cells. *Int. J. Radiat. Oncol. Biol. Phys.* **77**, 244–252.
48. Rzeszowska-Wolny J., Herok R., Widel M., Hancock R. 2009. X-irradiation and bystander effects induce similar changes of transcript profiles in most functional pathways in human melanoma cells. *DNA Repair (Amst).* **8**, 732–738.