

Myocardial connexin-43 and PKC signalling are involved in adaptation of the heart to irradiation-induced injury: Implication of miR-1 and miR-21

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Abstract. Intercellular connexin-43 (Cx43) channels are essential for electrical coupling and direct cardiac cell to cell communication to ensure heart function. Expression of Cx43 is altered due to stressful conditions and also affected by the alterations in extracellular matrix. We aimed to explore the effect of chest irradiation on myocardial expression of Cx43 and miR-1 which regulates GJA1 gene transcription for Cx43. Implication of miR-21 that regulates expression of extracellular matrix proteins and PKC signalling that may affect Cx43-mediated coupling was examined as well. Western blot and real-time PCR analyses revealed that six weeks after the exposure of healthy Wistar rats chest to single irradiation of 25 Gy significant myocardial alterations were observed: 1) increase of total Cx43 protein expression and its functional phosphorylated forms; 2) suppressed levels of miR-1; 3) enhanced expression of PKC ϵ which phosphorylates Cx43; 4) increase of miR-21 levels; 5) increase of PKC δ expression. These results suggest that irradiation causes post-transcriptional regulation of myocardial Cx43 expression by miR-1 possibly through miR-21 and PKC signalling. We conclude that single dose of irradiation has the potential to enhance myocardial intercellular communication that might be beneficial for the heart that needs to be investigated in details in further studies.

Key words: Irradiation injury — Rat heart — Connexin-43 — miR-1 — miR-21 — PKC

Introduction

Myocardial synchronization and a proper heart function depend on gap junction connexin-43 (Cx43) channels that are essential for electrical coupling between cardiomyocytes and direct molecular signal propagation (Severs et al. 2008). Cardiac cell-cell communication is also important for the regulation of cell growth, differentiation, apoptosis and adaptation of differentiated cardiac cells. Numerous endogenous and exogenous factors can modulate Cx43 channels function to enhance or inhibit intercellular communication in both physiological and pathological conditions (Salameh and

Dhein 2005; Tribulova et al. 2015). Importantly, tumor promoters and oncogenes inhibit coupling, which activate proliferation while anti-tumor compounds and anti-oncogene drugs can reverse this process (Salameh and Dhein 2005).

Radiotherapy using heavy-ions is frequently applied for treating cancer. The expression of Cx43 is highly sensitive to ionizing radiation. Even low dose of irradiation induces a significant elevation of Cx43 in a variety of cell types (Azam et al. 2003). Radiation up-regulates Cx43 in both intact and infarcted rabbit heart ventricle (Amino et al. 2006). Consequently, enhanced coupling is associated with long-lasting protection of the heart from malignant arrhythmias (Amino et al. 2010). Nevertheless, the mechanisms responsible for the radiation-induced Cx43 up-regulation are not known. To this context, it is interesting to note that microRNA-1 (miR-1) is target *GJA1* gene that encodes Cx43 (Yang et al. 2007) and elevated miR-1 represses Cx43 expression in

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viral myocarditis (Xu 2012). In addition, miR-1 regulates cardiac cell death by targeting Bcl-2 and over-expression of miR-1 induces apoptosis (Tang et al. 2009). Irradiation affects distinctive microRNAs in various tissues (Khan et al. 2013); however, it is not known if it may affect miR-1 levels in the heart. We hypothesize that down-regulation of miR-1 might result in up-regulation of myocardial Cx43 in response to irradiation.

MicroRNAs fundamentally influence the pathogenesis of various events (Kukreja et al. 2011), including adverse cardiac remodelling such as hypertrophy and fibrosis (van Rooij et al. 2012). These myocardial alterations compromise cell-to-cell contacts and Cx43 channels mediated communication in diseased or aged heart and promote the occurrence of malignant arrhythmias (Tribulova et al. 2008; Jansen et al. 2012; Radosinska et al. 2013). Fibrotic remodelling is attributed to miR-21 that is specifically enriched in cardiac fibroblasts promoting their activation and induction of extracellular signal-regulated signalling pathway (Thum et al. 2008). Protein sprouty homolog 1 (Sprouty1) as well as phosphatase and tensin homolog (PTEN) are identified as direct targets of miR-21. They regulate the expression of extracellular matrix metalloproteinase-2 (MMP-2) (Bauersachs 2012). In contrast with the detrimental effects of over-expression of miR-21 in cardiac fibroblasts there is evidence that up-regulation of myocardial miR-21 early after myocardial infarction confers the cardioprotection by suppressing apoptosis, inflammation and necrosis hence promoting survival (Yin et al. 2009; Bauersachs 2012; Quin et al. 2012; Toldo et al. 2014). Nevertheless, it remains unknown whether the expression of miR-21 in the heart is altered following irradiation.

Numerous reports include ours suggest that one of the important pathways implicated in the modulation of heart function is PKC signalling (Steinberg 2004; Lin et al. 2008). It can modulate Cx43-mediated intercellular communication, cardiac remodelling and viability. Therefore, it is interesting to understand whether PKC would be altered in response to radiation.

Based on this background information, we designed this study to elucidate the impact of irradiation on the heart and to relate it with potential alterations in the expression of Cx43, miR-1, miR-21 and protein kinase C (PKC).

Materials and Methods

All procedures were approved by the Institutional Animal Care Committee. Male Wistar rats were obtained from Velaz Praha (Czech Republic) and maintained on a 12:12-h light/dark cycle with free access to food and water. Three-month-old animals ($n = 12$) were anesthetized with thiopental (65 mg/kg b.w.) followed by irradiation with single dose of 25

Gy given locally on mediastinal area at the level of heart using 5 MeV/1 kW electron linear accelerator UELR 5-1S with tungsten converter to X-rays (Producer NIEFA St. Petersburg, RF) as previously described (Mézešová et al. 2013). Control animals ($n = 12$) were shielded with lead plates. A single dose of 25 Gy to the rat chest corresponds to the therapeutic dose of irradiation commonly used in patients. Six weeks later the hearts were excised from anesthetized animals, rinsed with ice-cold physiological saline, weighed, frozen in liquid nitrogen, and stored in freezer until analysis. Left ventricular tissue was taken for analysis of Cx43 and PKC expression using Western blotting.

MicroRNA reverse transcription and TaqMan-based qRT-PCR analysis for estimation of miR-1 and miR-21

Total RNA including small RNA was isolated from frozen left ventricular tissue of control and irradiated Wistar rats using miRNA mini kit according to the manufacturer's protocol (QIAGEN Sciences, MD, USA). Concentration and purity of the isolated RNA was checked using Nanodrop ND-1000 spectrophotometer (Agilent technologies, CA, USA). Briefly 10 ng of total RNA was subjected for reverse transcription reaction with miRNA specific RT primers using microRNA reverse transcription kit (Applied Biosystems, CA, USA) in accordance to manufacturer's instructions. Real time PCR was performed using Roche Light cycler 480 II (Roche Applied Science, IN, USA). Taqman miRNA assay probe (Applied Biosystems, CA, USA) was applied to determine the expression level of miR-1 and miR-21. To normalized RNA content, endogenous U6 small RNA was used. Reverse transcription was performed using stem loop specific microRT primers under following condition: 16°C for 30 min; 42°C for 30 min and 85°C for 5 min. The obtained cDNA was diluted in 1:3 ratios and subjected to real-time PCR using Taqman amplicon specific assay probe under the following PCR cycle condition: 95°C for 10 min; 95°C for 15 s and 60°C for 60 s.

Western blotting for Cx43, PKC ϵ and PKC δ expression

Isolated left ventricular samples ($n = 6$ per group) were powdered and solubilized in SB20 (20% SDS, 10 mmol/l EDTA, 0.1 mol/l tris(hydroxymethyl)aminomethane (TRIS), pH 6.8) by sonicator UP 100H (Hielscher, Teltow, Germany). Total proteins (10–40 μ g) from each sample were separated by SDS-PAGE on 10% acrylamide gels, transferred onto a nitrocellulose membrane and blocked with 5% non-fat dry milk in Tris-buffered saline. For determination of Cx43, the membrane was incubated with primary rabbit polyclonal antibody (diluted 1:2000; Anti-Connexin 43C 6219; Sigma-Aldrich, St Louis, Missouri, USA). For determination of PKC ϵ the membrane was incubated with primary rabbit

polyclonal antibody (C-15, sc-214) and anti-PKC δ (diluted 1:1000; C-17, sc-213; Santa Cruz Biotechnology Inc., Dallas, Texas, USA) overnight at 4°C followed by further incubation for 1 h at room temperature with the secondary donkey antibody (1:2000 peroxidase labeled anti-rabbit immunoglobulin; Amersham Biosciences, Piscataway, New Jersey, USA). The densitometric analysis for the corresponding band was done using Carestream Molecular Imaging Software (USA).

In-situ myocardial immunolabeling of Cx43

Cryostat sections from the left ventricle were used for in-situ immunodetection of Cx43 using mouse monoclonal anti-Cx43 antibody (Chemicon International, Inc.) and secondary FITC-conjugated goat anti-mouse antibody. Immunostained sections were examined using fluorescence microscope (Axiostar; Carl Zeiss, Jena, Germany) and images were acquired digitally for subsequent quantitative image analysis (Soft Imaging System, GmBh, Germany). Twenty randomly selected test areas were investigated *per* heart. From each test field a histogram of Cx43 fluorescence intensity was obtained and used for the calculation of Cx43 signal. The area of positive Cx43 labelling was defined as the number of pixels with the Cx43 signal intensity exceeding a threshold of 30 on the 0–255 gray scale. Total number of Cx43 positive pixels was expressed as integral optical density (IOD) *per* area. This parameter, expressed in arbitrary units, was compared between groups.

Statistical analysis

All values of experiments were expressed as means \pm SD. Statistical analysis was performed by using one way two-tailed ANOVA test. A probability value less than 0.05 was considered to reflect statistically significant differences.

Results

Biometrical parameters of experimental rats are summarized in Table 1. Six weeks after the exposure of healthy rats to 25 Gy irradiation, the body weight was significantly lower

Table 1. Biometric parameters of experimental rats

	Wistar controls (<i>n</i> = 12)	Wistar irradiated (<i>n</i> = 12)
Body weight (g)	405 \pm 7	310 \pm 12**
Heart weight (g)	1.05 \pm 0.01	1.25 \pm 0.25*
Left ventricular weight (g)	0.75 \pm 0.07	0.76 \pm 0.1

* $p < 0.05$, ** $p < 0.01$ vs. Wistar controls.

as compared to the non-irradiated controls. Furthermore, a small but significant increase of heart weight (with no change in left ventricular weight) was registered when compared to non-irradiated rats.

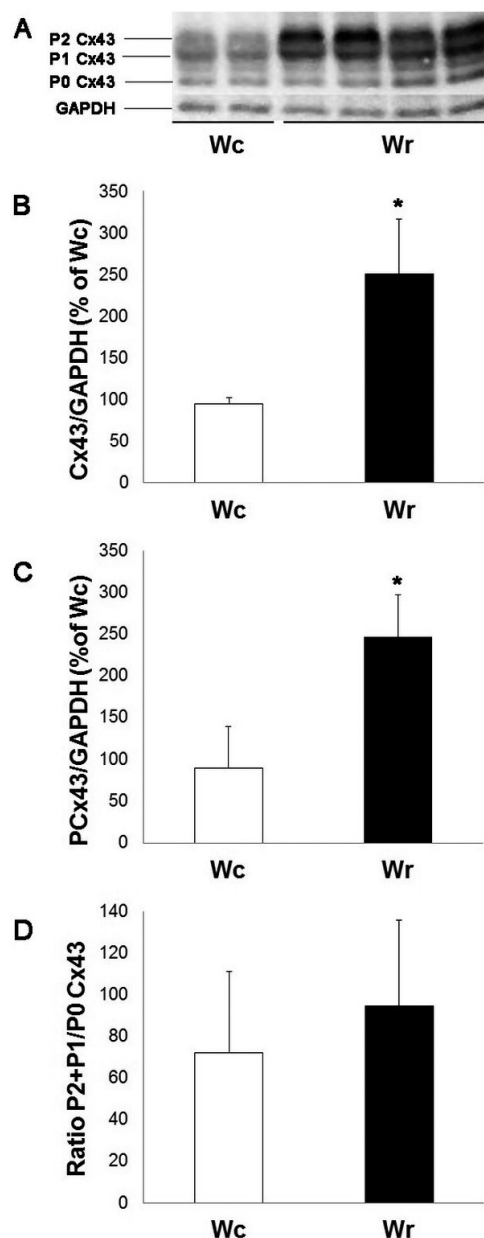


Figure 1. Representative immunoblots (A) and quantitative evaluation of myocardial expression of total Cx43 protein (B), phosphorylated forms of Cx43 (C) and ratio of phosphorylated to non-phosphorylated forms of Cx43 (D) normalized to GAPDH in control (Wc, *n* = 6) and irradiated Wistar rat hearts (Wr, *n* = 6). Note that Cx43 is expressed in two functional phosphorylated (P1, P2) and one non-phosphorylated (P0) forms. Data are means \pm SD, * $p < 0.05$ vs. Wc.

Irradiation caused significant increase in the expression of total myocardial Cx43, its functional phosphorylated forms as well as ratio of phosphorylated to non-phosphorylated forms of Cx43 in left ventricles (Fig. 1). In addition,

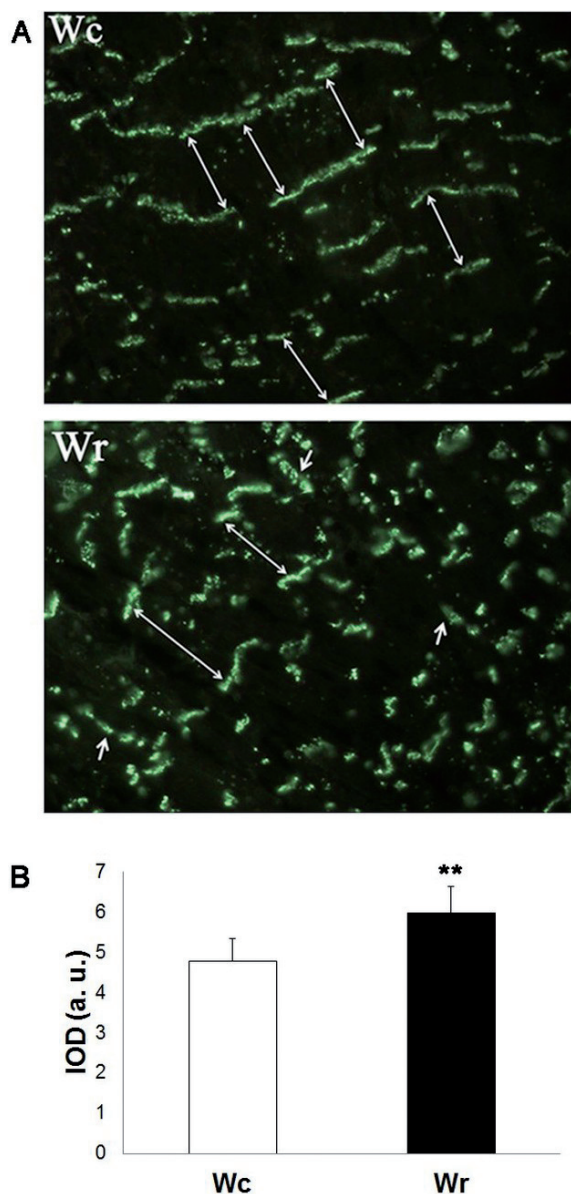


Figure 2. A. Cx43 immunolabeling in the left ventricles of non-irradiated (Wc) and irradiated Wistar rat hearts (Wr). Note the conventional distribution of Cx43-positive gap junctions predominantly at the intercalated discs (double-head arrows) in Wc. In addition, Cx43 is distributed on lateral surfaces of the cardiomyocytes (short arrows) of Wr hearts. Objective $\times 40$. B. Quantitative image analysis of the Cx43 immunofluorescence signal revealed increased integral optical density (IOD) in irradiated compared to non-irradiated rat hearts. Results are the mean \pm SD of 20 images per heart (6 hearts per group). ** $p < 0.05$ vs. Wc.

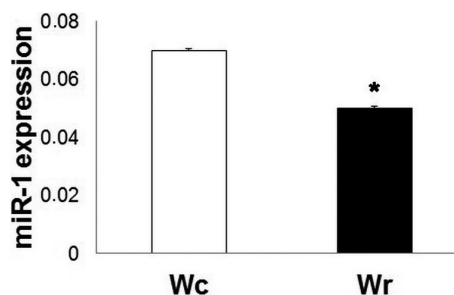


Figure 3. Myocardial expression of miR-1 in control (Wc, $n = 6$) and irradiated Wistar rat hearts (Wr, $n = 6$). Endogenous U6 small nuclear RNA (U6snRNA) was used as control for miR-1. Data are means \pm SD, * $p < 0.05$ vs. Wc.

moderate abnormalities in cardiomyocyte Cx43 distribution were also detected by *in situ* immunolabeling in the irradiated rats. Accordingly, besides the normal pattern of Cx43-immunopositivity confined to the intercalated discs, some cardiomyocytes exhibited enhanced immunopositive staining on lateral cardiac cell membranes as well (Fig. 2). Quantitative image analysis revealed increased immunopositive signal reflecting Cx43 expression in irradiated rat heart compared to control rats (Fig. 2).

In parallel to post-irradiation related up-regulation of myocardial Cx43 protein, the expression level of miR-1 was significantly reduced in left ventricles compared to non-irradiated controls (Fig. 3). On the other hand, myocardial expression of miR-21 was significantly increased six weeks after the irradiation (Fig. 4).

Expression of both isoforms of PKC, i.e. PKC ϵ and PKC δ was significantly increased in the left ventricular tissue as determined by Western blotting and shown on Fig. 5 and Fig. 6 six weeks after the exposure of rats to chest irradiation.

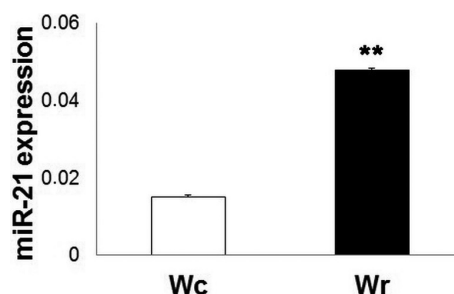


Figure 4. Myocardial expression of miR-21 in control (Wc, $n = 6$) and irradiated Wistar rat hearts (Wr, $n = 6$). Endogenous U6 small nuclear RNA (U6snRNA) was used as control for miR-21. Data are means \pm SD, * $p < 0.05$ vs. Wc.

Discussion

The present study demonstrates the possible implication of myocardial Cx43, miR-1 and miR-21 as well as PKC signaling in post-irradiation response of the rat heart. We showed that in addition to previously reported radiation-induced up-regulation of Cx43 protein and mRNA in intact as well as infarcted rabbit heart ventricle (Amino et al. 2006), there is an increase of functional phosphorylated forms of Cx43. Enhanced phosphorylation of Cx43 may be in part attributed to increased expression of PKC ϵ . Our previous studies suggest that enhanced Cx43 phosphorylation due to activation of PKC ϵ was linked with slowing of myocardial conduction and decreasing susceptibility of diabetic or hypothyroid rat hearts to malignant arrhythmias (Lin et al. 2006, 2008; Bacova et al. 2013). In contrast, reduced expression of PKC ϵ accompanied by decreased Cx43 phosphorylation in hyperthyroid rat hearts was highly proarrhythmic (Bacova et al. 2013). Enhanced Cx43 expression in post-irradiated rat heart was also detected by quantitative image analysis of *in situ* immunolabeling of Cx43. Moderate alterations in topology of Cx43 likely indicate early stage of myocardial remodelling, as previously shown in experimental hypertension (Radosinska et al. 2011, 2013). Lateralization of Cx43 in chronic models of cardiac injury is often associated with myocardial hypertrophy (Lin et al. 2008; Radosinska et al. 2011). It is likely that to compensate irradiation-related adverse effects, the heart activates processes resulting in hypertrophy of individual cardiomyocytes. Nevertheless, left ventricular weight was not changed in irradiated rats suggesting that myocardial hypertrophy was not pronounced

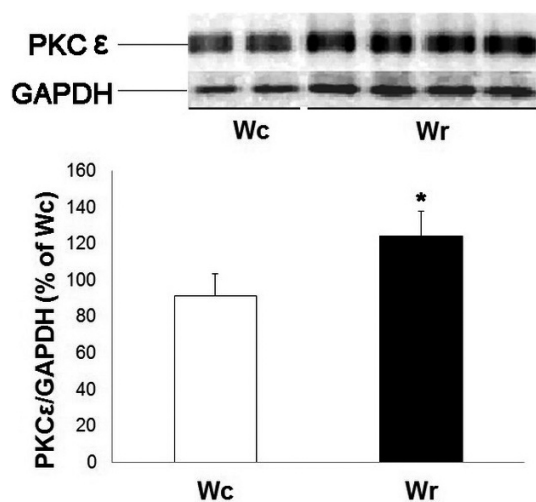


Figure 5. Representative immunoblots (upper panel) and quantitative evaluation of myocardial expression of PKC ϵ normalized to GAPDH in control (Wc, $n = 6$) and irradiated Wistar rat hearts (Wr, $n = 6$). Data are means \pm SD, * $p < 0.05$ vs. Wc.

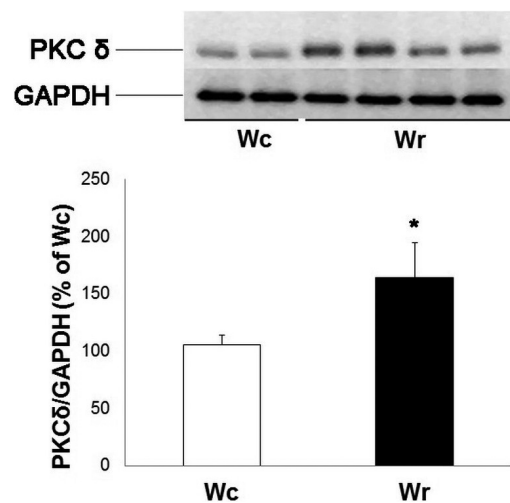


Figure 6. Representative immunoblots (upper panel) and quantitative evaluation of myocardial expression of PKC δ normalized to GAPDH in control (Wc, $n = 6$) and irradiated Wistar rat hearts (Wr, $n = 6$). Data are means \pm SD, * $p < 0.05$ vs. Wc.

likewise observed alterations in topology of Cx43. On the other hand, heart weight was moderately elevated likely on the account to right ventricle of the heart. This assumption is supported by the fact that the ratio of right to left ventricles was increased in this model of rat irradiation (Mezesova et al. 2014). The issue needs to be addressed more in details in further studies.

In the present study, we demonstrated that up-regulation of Cx43 was associated with suppression of miR-1. Previous studies have shown that over-expression of miR-1 was correlated with reduced Cx43 protein expression in viral myocarditis (Xu et al. 2012) while down-regulation of miR-1 by Ang II stimulation was associated with increased Cx43 expression (Curio et al. 2013). Interestingly, irradiation of the heart resulted in protection from malignant arrhythmias (Amino et al. 2006) and hence suppression of miR-1 exerted antiarrhythmic potential most likely by targeting GJA1 (which encodes Cx43) and KCNJ2 (which encodes the K⁺ channel subunit Kir2.1) (Yang et al. 2007). Thus, it appears that irradiation-induced antiarrhythmic effects can be in part attributed to down-regulation of miR-1 which may be linked with up-regulation of Cx43 and PKC ϵ related phosphorylation. In contrast, miR-1 overexpression inhibits electrical coupling and depolarized cardiac cell membrane by post-transcriptional repression of GJA1 and KCNJ2 which likely accounts for its arrhythmogenic potential (Yang et al. 2007). Pro-arrhythmogenic effect of elevated miR-1 in infarcted heart was attenuated by its down-regulation induced with propranolol (Lu et al. 2009). Over-expression of miR-1 also exacerbated cardiac ischemia-reperfusion injury (Pan et al. 2012) and impaired cardiac contractile function in

mouse models (Ai et al. 2012). Taken together, it appears that irradiation-induced suppression of miR-1 might be beneficial for the heart. Noteworthy, miR-1 is released to blood circulation thus it has a potential to be a novel biomarker of cardiac injury (Ai et al. 2010) and possibly proarrhythmic state.

Compared to suppression of miR-1, ionizing radiation has been reported to stimulate expression of miR-21 in various types of mammalian cells (Shi et al. 2012), similar to our results in the heart tissue. Interestingly, miR-21 is over-expressed in almost all types of human cancers and it has been suggested that miR-21 underlies radioresistance of cancer because its inhibition increases radiosensitivity *via* activation of PTEN promoting the apoptosis in tumor cells (Liu et al. 2014). In parallel to the increased levels of miR-21, there was an increase of both expression and activity of MMP-2 in left ventricles of irradiated rat hearts compared to non-irradiated (Barancik et al. 2013). These activation of cardiac remodelling in response to irradiation which may accelerate over time since miR-21 activates fibroblasts (Thum et al. 2008; Bauersachs 2012; van Rooij et al. 2012) and enhanced proteolytic activity of MMP-2 promotes fibroblast migration (Bauersachs 2012). Moreover, it has been recently identified that fibroblast exosomal-derived miR-21 is like a potent paracrine acting RNA molecule that induces cardiomyocyte hypertrophy (Bang et al. 2014). In response to stress, the heart undergoes an extensive cardiac remodelling resulting in cardiac hypertrophy and fibrosis that over time contribute to heart failure. Delayed inhibition of miR-21 after myocardial infarction attenuates reactive fibrosis and heart failure development (Bauersachs 2012). However, the anti-apoptotic and proliferation promoting effects of miR-21 may be pro-survival to cardiac tissue in early adaptation period in response to radiation-induced injury.

In the context of alterations of miR-21 it should be noted that we have found significant increase of PKC δ expression six weeks after irradiation. PKC δ is a serine/threonine kinase, which plays a key role in growth regulation, tissue remodelling and cell apoptosis (Steinberg 2004). PKC δ was up-regulated in spontaneously hypertensive rat hearts (Radosinska et al. 2013) and in pressure-overloaded myocardium (reviewed in Steinberg 2004) suggesting its involvement in the induction of compensatory hypertrophy. Moreover, PKC δ acts as a switch between cardiomyocyte hypertrophy and apoptosis and chronic PKC δ over-expression facilitated cardiomyocyte apoptosis and contractile dysfunction (Steinberg 2004). Increased expression of both miR-21 and PKC δ in response of the heart to irradiation injury suggests their implication in processes of myocardial remodelling.

In summary, our results show that a single dose of chest irradiation triggers myocardial alterations six weeks later which most likely reflect adaptation of the heart to radiation

injury (Slezak et al. 2015). These myocardial adaptive changes could have beneficial as well as adverse consequences. Importantly, single dose of irradiation exhibits potential to enhance cardiac Cx43-mediated cell-cell coupling and communication *via* down-regulation by miR-1 rendering protection of the heart against malignant arrhythmias. Further investigations are needed to determine the cause and effect relationship of these observations.

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Conflict of interest. The authors declare no conflict of interest.

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