The Role of TGF Beta and PPAR Alpha Signalling Pathways in Radiation Response of Locally Exposed Heart: Integrated Global Transcriptomics and Proteomics Analysis

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Abbreviation: PPAR alpha, peroxisome proliferator activated receptor alpha; ; TGF beta 1, transforming growth factor beta 1; IPA, Ingenuity Pathway Analysis; LC-ESI-MS/MS, liquid chromatography-electron spray ionisation- tandem mass spectrometry; Gy, gray;

ABSTRACT

Epidemiological data from patients undergoing radiotherapy for thoracic tumours clearly show the damaging effect of ionising radiation on cardiovascular system. The long-term impairment of heart function and structure after local high-dose irradiation is associated with systemic inflammatory response, contraction impairment, microvascular damage and cardiac fibrosis. The goal of the present study was to investigate molecular mechanisms involved in this process. C57BL/6J mice received a single X-ray dose of 16 Gy given locally to the heart at the age of 8 weeks. Radiation-induced changes in the heart transcriptome and proteome were investigated 40 weeks after the exposure. The omics data were analysed by bioinformatics tools and validated by immunoblotting. Integrated network analysis of transcriptomics and proteomics data elucidated the signalling pathways that were similarly affected at gene and protein level. Analysis showed induction of transforming growth factor (TGF) beta signalling but inactivation of peroxisome proliferator-activated receptor (PPAR) alpha signalling in irradiated heart. The putative mediator role of mitogen-activated protein kinase (MAPK) cascade linking PPAR alpha and TGF beta signalling was supported by data from immunoblotting and ELISA. This study indicates that both signalling pathways are involved in radiation-induced heart fibrosis, metabolic disordering and impaired contractility, a pathophysiological condition that is often observed in patients that received high radiation doses in thorax.

INTRODUCTION

Epidemiological studies show an increased risk of cardiovascular disease (CVD) associated with high local doses of ionizing radiation to the heart. This is observed in patients after thoracic radiotherapy treatment for breast cancer, Hodgkin´s disease or childhood cancers.[1-4] A significant increase in the mortality from CVD has been reported for patients treated by radiotherapy for left-sided breast cancer compared to those with right-sided cancer.[1,5-7] The recent development of radiation therapy practice and equipment has decreased the heart dose from left-tangential radiotherapy considerably over the past 40 years, however certain parts of the heart still receive more than 20 Gy in approximately half of left-sided breast cancer patients.[8] High-dose radiation leads to late adverse cardiac side effects in including damage to pericardium, myocardium, valves and coronary vessels, as well as cardiomyopathy and myocardial fibrosis.[9, 10] Since breast cancer is by far the most common cancer in women worldwide and most patients are treated by radiotherapy, an estimated increased risk for CVD induced by radiation (2.5%/Gy) concerns a large number of people[11] and thus remains one of the important health concerns.[12] Therefore a deep investigation of the molecular mechanisms of CVD following irradiation of the heart is urgently needed.

We have previously shown using a mouse model (C57Bl/6J) that local cardiac high-dose radiation caused cardiac metabolic impairment that was coupled to mitochondrial dysfunction and reduction in the activity of PPAR alpha complex. [13] It also induced a systemic inflammatory response and increased the level of free fatty acids in blood.[13, 14] A similar study showed morphological disorders of the heart such as increased left ventricle (LV) wall thickening weeks and increased interstitial collagen accumulation in

LV myocardium after 40 weeks.[15] Moreover, increased structural and microvascular damage, inflammation, diffuse amyloidosis, late fibrosis and even sudden death of some mice was observed between 30-40 weeks after irradiation at 16 Gy.[15]

PPAR alpha, a key regulator of the lipid metabolism in the heart, is involved in the development of CVD.[16, 17] Impairment of lipid metabolism has been described as a consequence of altered transcriptional activity of PPAR alpha.[18]Inhibition of PPAR alpha is accompanied by increased levels of circulating cytokines and enhanced local production of profibrotic factors such as TGF beta.[16, 19]

TGF beta family represents cytokines that are involved in the regulation of inflammation, and cellular growth.[20] The increased expression of TGF beta has been found to correlate with induced myocardial hypertrophy and fibrosis in mice and humans.[21, 22] Radiation-induced tissue toxicity has also been associated with TGF beta activation.[23-25] Binding of TGF beta ligands to corresponding receptors activates this canonical SMAD-dependent signalling pathway that leads to translocation of cytoplasmic SMAD proteins into the nucleus to regulate the transcription of the target genes.[26] Activated TGF beta receptors also induce non-SMAD signalling that includes participation of different members of the MAPK pathway.[27]

It has been shown that activated PPAR alpha complex interferes with TGF beta signalling[28, 29] but the molecular mechanism is not well understood. MAPK cascade plays a regulatory role in both PPAR alpha and TGF beta signalling.[20, 30] The importance of MAPK components in the control of radiation-induced cellular stress is well known.[13, 14, 31]

The goal of the present study was to investigate the mechanism involved in long-term radiation-induced cardiac damage, especially the role of PPAR alpha and TGF beta signalling pathways in this process. C57BL/6J mice were irradiated with single X-ray doses of 16 Gy and the cardiac tissue was studied 40 weeks post-irradiation by transcriptomics and proteomics analyses. Both omics data sets predicted the activation of TGF beta and inactivation of PPAR alpha signalling pathways. This study strongly suggests a crosstalk between the two pathways via MAPK signalling. The combined analysis on proteome and transcriptome was performed to provide a comprehensive and in depth analysis of alterations associated with radiation-induced cardiac damage including fibrosis and amyloidosis observed in the same animals by Seemann et al.[15]

EXPERIMENTAL SECTION

Animals

Male C57BL/6J mice aged 8 weeks were purchased from Charles River Laboratories, France. Animals were randomly allocated to different treatment groups of 10-15 animals and housed in temperature controlled room with 12 hour light- dark cycle. Standard mouse chow and water was provided *ad libitum*. Irradiation was carried out as previously described by Seemann et al.[15] Briefly, 4-7 animals were irradiated with a single dose of 16 Gy locally to heart using 250 kV X-rays, operating at 12 mA and filtered with 0.6 mm of copper. Mice were not anesthetised during irradiation procedure but were held in a prone position in restraining jigs with thorax fixed using adjustable hinges. The same number of age-matched controls (sham irradiated with 0 Gy) were always included providing the appropriate comparison for irradiated groups at that time

point. The dose rate was calculated as 0.94 Gy/min. The position and field size (10.6 ×15.0 mm) was determined by pilot studies using soft X-rays (25 kV, 85 mA) to visualise the heart. Up to 30 % of lung volume was included in the field and the rest of the body was shielded with a 3 mm thick lead plate. The animals were sacrificed 40 weeks after irradiation. Heart samples were prepared as described as before.[15] Experiments were in agreement with the Dutch law on animal experiments and welfare and in line with the international Guide for the Care and Use of Laboratory Animals (8th edition).

Materials

Beta-octylglucoside, SDS, and ammonium bicarbonate were obtained from Sigma (St. Louis, MO); acetone, acetonitrile (ACN), formic acid (FA), and trifluoroacetic acid (TFA) were obtained from Roth (Karlsuhe, Germany); dithiothreitol (DTT), iodoacetamide, tris-(hydroxymethyl) aminomethane (Tris), and sequencing grade trypsin were obtained from Promega (Madison, WI); was obtained from Bruker Daltonik (Bremen, Germany). All solutions were prepared using HPLC grade water from Roth (Karlsuhe, Germany).

Gene expression profiling

Gene expression analysis was performed as described before.[32] Total RNA was isolated from frozen sections (30 sections of 30 µm per mouse using 4–7 mice per group) of the mid part of the horizontally cut heart using Trizol® Reagent (Invitrogen Corporation, Carlsbad, USA), according to the manufacturer's protocol. The quantity of total RNA was determined spectrophotometrically (NanoDrop, Thermo scientific, Wilmington, USA) followed by a quality check measured by a Agilent 2100 Bioanalyzer

with the RNA Integrity Number (RIN) (Agilent technologies, Santa Clara, USA). Samples with a RIN above 7 were used for DNAse treatment and amplified (350 ng per sample) using Illumina Totalprep RNA Amplification kit (Ambion, Grand Island, USA). Before hybridisation individual RNA was pooled for each treatment group. Hybridisation of a RNA to Illumina Expression Bead Chips Mouse Whole Genome (WG-6 vs. 2.0) and subsequent washing, blocking and detecting were performed according to the manufacturer's protocol (Illumina, San Diego, USA). Samples were scanned on the IlluminaR BeadArray™ 500GX Reader using IlluminaR BeadScan image data acquisition software (version 2.3.0.13). MouseWG-6 vs. 2.0 BeadChip contains the full set of MouseRef-8 BeadChip probes with additional 11,603 probes from RIKEN FANTOM2, NCBI REfSeq as well from the MEEBO database.

Before analysing, the database was normalised using robust spline normalisation method within the microarray facility of the Netherlands Cancer Institute. Log2 ratio between expression of genes from control mice and expression of genes from irradiated mice were calculated, as well as the sum of the expression of genes from both control and irradiated mice. Genes with sums below 6 were discarded. The threshold for standard deviation (SD) was set to 3 and mean ± nSD was calculated to identify genes that are above an expression-value of 6 and above threshold 3 of SD. Among these genes, the genes with fold changes greater than 1.5-fold or less than 0.64-fold were defined as significantly differentially expressed.

Proteome profiling

Protein extraction and quantification

Frozen heart samples obtained form 5 mice per group were lysed as described previously. Cardiac tissue was ground to a fine powder with a cold (-20°C) mortar and pestle before being suspended in lysis buffer (SERVA).[13] Same animals were used for transcriptomics and proteomics. Protein concentration was determined by the Bradford assay following the manufacturer's instructions (Thermo Fisher).

Protein purification and mass spectrometry

Protein lysates (10 µg) from each animal were digested using a modified filter-aided sample preparation (FASP) protocol.[33] Briefly, the samples were reduced with 10 mM DTT at 60°C for 30 min, followed by alkylation with 15 mM iodoacetamide for 30 min at room temperature in the dark.[33] Samples were diluted using 8 M urea in 0.1 M Tris/HCl, pH 8.5, and centrifuged using a 30 kDa cut-off filter (Pall Corporation). After washing with 8 M urea in 0.1 M Tris/HCl, pH 8.5, and with 50 mM ammonium bicarbonate (ABC), the proteins were initially digested on the filter with 1 µg Lys-C (Wako Chemicals GmbH) in 50 mM ABC at room temperature, followed by addition of 2 µg trypsin (Promega) and digestion overnight at 37°C. Tryptic peptides were collected by centrifugation and acidified with trifluoric acid (TFA) to a pH of 2.0. Samples were stored at -20°C.

Prior to LC-MS/MS analysis the samples were centrifuged (16,000 g) for 5 min at 4°C. Each sample (approx. 0.5 µg) was analysed separately on a LTQ OrbitrapXL (Thermo Fisher Scientific) coupled to Ultimate 3000 nano-HPLC (Dionex) as described previously.[14]

Label-free quantification

The raw files of the individual measurements were loaded to the Progenesis QI software and analysed as described previously.[34, 35] Peptide features in the individual runs were aligned in order to reach a maximum overlay of at least 87%. After feature detection, the singly charged features and features with charges higher than +7 were excluded. Protein identification was performed using the Mascot search engine (Matrix Science, version 2.5.1) in the Ensembl mouse database (release 75, 23354020 residues, 51771 sequences).

The following search parameters were used: 10 ppm peptide mass tolerance and 0.6 Da fragment mass tolerance, one missed cleavage was allowed, carbamidomethylation (C) was set as fixed modification, and oxidation (M) and deamidation (N, Q) were allowed as variable modifications. Search results were reimported into the Progenesis QI software and the resulting summed normalised abundances of the unique peptides for every single protein were used for the calculation of abundance ratios and statistical analysis. For final quantifications, proteins with ratios greater than 1.30-fold or less than 0.77-fold (t-test; $q \leq 0.05$) were defined as being significantly differentially expressed. The FDR (q value) calculation was used to adjust p-values.[36, 37]

Interaction and signalling network analysis

For deregulated proteins, protein-protein interaction and signalling networks were analysed by the software tool INGENUITY Pathway Analysis (IPA) (http://www.INGENUITY.com)[38] and the search tool STRING version 10 (http://string-db.org), coupled to the Reactome database (http://www.reactome.org).[39]

Sandwich ELISA assay

The alteration in the phosphorylation status of SMAD 2/3 was assessed using PathScan® phospho- SMAD 2 (Ser465/467) / SMAD 3 (Ser423/425) Sandwich ELISA Kits (#120001). The data were compared to the level of total SMAD 2 / 3 sandwich ELISA kit (Cell Signaling) (#12000C). The assays react with mouse. The measurement was performed using three biological replicates.

Immunoblotting analysis

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (GE Healthcare) using a TE 77 semidry blotting system (GE Healthcare) at 1 mA/cm for 2 h. The membranes were blocked with either 5% non-fat dry milk powder or 3% BSA in TBST for 2 h at room temperature, washed four times in TBST for 5 min and incubated overnight at 4°C with primary antibodies using the dilutions recommended by the manufacturer. Immunoblotting analysis of heart lysates were performed using anti-ERK 44/42 (# 9202), anti-phospho ERK 44/42 (Thr 202/Tyr 204) (# 9101), anti-p38 (# 9212), anti-phospho p38 (Thr 180/Tyr 182) (# 9211), anti-PPAR-alpha (sc-1982), anti-phospho PPAR alpha (Ser 12) (ab3484), anti-JNK1/JNK2 (ab179461), anti-phospho JNK1/JNK2 (Thr 183 / Tyr 185) (ab4821), anti-TAK-1 (ab109526), anti-phospho TAK-1 (Thr 187) (ab192443), anti-SMAD 4 (sc-7966), anti-MYH6 (sc-168676), anti- MYH7 (sc-53089), anti-ATPB (ab14730). Blots were washed four times with TBST after primary antibody incubation and incubated with secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) or alkaline phosphatase for 2 h at room

temperature and developed using the ECL system (GE Healthcare) or 1-step NBT/BCIP method (ThermoFisher) following standard procedures. ATPB5 were not changed in the proteomic profile after 8 and 16 Gy and used as loading control. All antibodies react with mouse. Quantification of digitised images of immunoblot bands from three biological replicates were quantified using ImageJ software (http://rsbweb.nih.gov/ij/).

2.8 MicroRNA analysis

Total RNA was isolated from frozen heart of sham and irradiated animals and purified using the mirVana™ Isolation Kit (Thermo Fisher Germany) according to the manufacturer's protocol. For microRNA studies the OD ratio of 260 nm / 280 nm from RNA lysates was estimated using a Nanodrop spectrophotometer. This ratio reflecting the RNA quality ranged between 2.0 and 2.1. Samples were stored at −20°C until further analysis. Expression of single microRNA using TaqMan Single MicroRNA Assay (Thermo Fisher, Germany) was performed according to manufacturer's protocol on a StepOnePlus device (Applied Biosystems, Germany) using Taqman primers. For single microRNA analysis following Taqman primers were used: SnoRNA 202 (ID: PN4427975), miR-21-5p (ID: #477975). All primers were purchased from Thermo Fisher, Germany. Expression levels of microRNA 21 were calculated based on the 2-ΔΔCt method with normalisation to control SnoRNA 202.

2.9 Statistical analysis

The student´s t-test (unpaired) was used as statistical test. Group difference was considered as statistically significant with p values of $p^* < 0.05$, $p^{**} < 0.01$, and $p^{***} < 0.001$. The error bars were calculated as standard deviation (SD). All experiments were done with at least three biological replicates.

2.10 Data availability

The raw MS data can be accessed from the RBstore (https://www.storedb.org/store_v3/study.jsp?studyId=1040).[13, 14]

RESULTS

Irradiation alters genes involved in the inflammatory response

Increased levels of inflammation, diffuse amyloidosis, and fibrosis have been observed previously as late effects in locally irradiated murine heart.[15] We performed gene expression analysis to identify genes involved in these pathways.

The full list of 16,892 identified genes is provided in the supporting information (SI) Table S1. The analysis showed that 185 genes were significantly differentially expressed in irradiated samples compared to controls (±1.5-fold) using the statistical criteria described in Methods (Table S2 in the SI).

The analysis showed that chemokines formed the main cluster of significantly deregulated genes. Many of these genes were associated with tissue inflammation and metabolic diseases (Figure A1 and Table S2 and S3 in the SI).The most significantly altered networks were “metabolic disease”, “inflammatory disease”, and “cardiovascular system development and function”.

“Tissue fibrosis”, “complement system” and “acute phase response signalling” were the most important deregulated functional pathways (Figure 1B and Table S3 in the SI). Differently expressed genes were associated with toxic pathways including cardiac fibrosis, hypertrophy, necrosis, and stenosis (Table S3 in the SI).

Irradiation changes the cardiac proteome

To further elucidate the mechanisms of gene alterations, the cardiac proteome was analysed after irradiation. The complete list of all identified and quantified peptides and proteins with fold changes and *p* and *q* values are shown in the supporting information (Tables S4-S7in SI). Global proteomics analysis of the heart tissue identified 1,038 proteins in total (Table S5 in the SI). Of 940 quantified proteins, 87 proteins were significantly changed in expression (2 unique peptides; fold change $\geq$ 1.30 or $\leq$ 0.77; $q \leq 0.05$) after radiation exposure. The expression of 31 proteins was down-regulated and that of 56 proteins up-regulated (Table S7 in SI).

A detailed analysis of functional interactions and biological pathways was performed using IPA software. Many of the altered proteins were associated with tissue skeletal disorders, and metabolic and inflammatory diseases (Figure 2A and Table S8 in the SI). The analysis showed that “organismal injury and abnormalities”, “free radical scavenging” and “metabolic disease” were the main networks altered in the cardiac proteome after irradiation (Table S8 in the SI).

The pathways “actin cytoskeleton signalling”, “mitochondrial dysfunction” and “acute phase response signalling” as well as “nuclear factor (erythroid-derived 2)-like 2,

NFE2L2-mediated oxidative stress response" were significantly affected in irradiated hearts (Figure 2B and Table S8 in the SI).

The majority of significantly altered proteins were associated with different heart diseases including heart hypertrophy, inflammation, heart failure and heart fibrosis (Table S8 in the SI).

Common transcription regulatory pathways are affected in both gene and protein data sets

Based on IPA upstream regulator analysis, TGF beta 1 was predicted activated after irradiation in both data sets (Figure 3A and 3B, and Tables S3 and S8 in the SI) whereas PPAR alpha was deactivated (Figure 3C and 3D, and Tables S3 and S8 in the SI). Furthermore, the analysis of both data sets predicted induction of different interleukins including IL6, and IL1A in irradiated hearts (Figure S1A and S1B).

MAPK-p 38 was also predicted to be activated after irradiation (Figure S2A and S2B in the SI). Other components of TGF beta signalling including SMAD 3 and MAPK-ERK, and JNK were predicted to be induced only at the gene expression level after radiation exposure (Figure S3 A-C in the SI).

The analysis also showed that several significantly deregulated genes and proteins formed an amyloid precursor protein (APP)-related protein cluster supporting cardiac amyloidosis observed previously in these animals [15] (Figure S4 in the SI).

TGF beta induces SMAD-dependent and SMAD-independent pathways after irradiation

As SMAD proteins mediate the TGF beta signalling, the levels of SMAD 2 and 3 proteins were measured. The analysis showed a significant increase in the ratio of phosphorylated / total level of SMAD 2 and 3 after exposure (Figure 4A, $p < 0.05$), suggesting activation of SMAD 2/3. Active SMAD 2 and 3 are known to interact with SMAD 4 to assemble a complex that is required for efficient TGF-beta SMAD-dependent signal transduction [26]. In good agreement with this, enhanced protein level of SMAD 4 was detected in the irradiated heart (Figure 4B, $p < 0.05$).

In addition, the protein components of SMAD-independent pathway, TGF beta associated kinase 1 (TAK1), and JNK1/2, and their phosphorylated forms, were analysed (Figure 4C and 4D). Significant increase in the ratio of phosphorylated / total level of TAK 1 and JNK 2 was found in the irradiated heart (Figure 4C and 4D, $p < 0.05$). Active TGF beta is known to induce MAPK signalling.[40] Therefore, the protein expression of components of the MAPK pathway, ERK 44/42 and p 38 and their phosphorylated forms, was measured (Figure 4C and 4D). The ratio of phosphorylated / total ERK 42 and p 38 was significantly increased (Figure 4C and 4D, $p < 0.05$) at irradiated hearts compared to control whereas there was no significant effect on the ratio of phosphorylated / total level of ERK 44 (Figure 4C and 4D).

Transcription factor PPAR alpha is inactivated by irradiation

The predicted inhibition of PPAR-alpha was analysed by measuring the level of phosphorylated (inactive) and total protein. The ratio of phosphorylated / total PPAR alpha was significantly increased in irradiated hearts compared to control (Figure 4C and 4D, $p < 0.05$) suggesting reduced PPAR alpha transcriptional activity.

miR-21 is induced in irradiated heart

As miR-21 is known to regulate the activity of PPAR alpha[41] and TGF beta[42], the expression level of miR-21 was compared in irradiated to control hearts. The analysis showed that the expression of miR-21 was significantly increased after irradiation compared to the control (Figure 5A, $p < 0.01$).

The protein expression of myosin heavy chain isoforms is altered after irradiation

To validate the changes in the structural proteins indicated by the proteomics analysis the levels of cardiac myosin heavy chain isoforms 6 (MYH 6) and 7 (MYH 7) were measured using immunoblotting. The level of MYH 6 was significantly decreased and MYH 7 significantly increased in irradiated hearts compared to control (Figure 5B and 5C, $p < 0.05$). The shift of MYH 6 to MYH 7is a known human heart pathology associated with.[43]

Integration of all omics data indicates PPAR alpha and TGF beta as common regulators of radiation response

Among the significantly deregulated genes and proteins, annexin A1, fibronectin and serine (or cysteine) peptidase inhibitor 3 were found in both data sets up-regulated suggesting a similar response to radiation exposure at gene and protein level (Table S2 and S7).

A reconstruction of integrative networks of all deregulated genes and proteins indicated a high degree of interconnectivity between the two data sets with PPAR alpha and TGF beta as common regulators (Figure 6).

DISCUSSION

In this study, radiation-induced transcriptome and proteome changes were examined in mouse heart tissue 40 weeks after local radiation exposure (16 Gy). A similar study done by Seemann et al. showed functional disorders in the irradiated heart including microvascular damage, inflammation, diffuse amyloidosis, and late fibrosis.[15] The goal of the present study was to investigate molecular mechanism involved in such long-term radiation-induced cardiac damage using exactly similar experimental design (age-at-exposure, dose and time point) as in the previous study. [15]

The novelty of the present study is to correlate radiation-induced proteome and transcriptome changes to the functional impairments observed before.[15] We noticed only modest direct correlation between gene and protein expression. This is in agreement with previous data showing poor or even negative correlation between gene and protein expression levels.[44, 45] One reason for this is the different regulatory mechanisms for gene and protein expression [46, 47], such as gene expression regulation by non-coding RNAs or protein expression regulation by post-translational modifications.[47]

In spite of expression differences between individual genes and proteins, both profiles revealed same biological processes affected by irradiation. The integrated analysis elucidated the signalling pathways that are commonly deregulated in proteome and

transcriptome level. These processes were closely related to heart pathologies such as cardiac remodelling, hypertrophy, metabolic perturbation, and fibrosis.

The activation of TGF beta [22, 48] was predicted in both transcriptomics and proteomics data sets. TGF beta signalling is able to initiate both canonical SMAD-dependent and non-canonical SMAD-independent transduction pathways.[40] Here, we show enhanced phosphorylation of SMAD 2 and 3 and increased level of SMAD 4 after radiation exposure suggesting an activation of the SMAD-dependent pathway.

The so-called SMAD-independent pathway initiates with activation of TAK1, followed by a series of phosphorylation events of MAPK members such as ERK, p 38, and JNK.[40] The transcriptomic analysis of this study predicted radiation-induced activation of these factors that was confirmed by immunoblot analysis showing increased phosphorylation of TAK1, p 38, ERK 42 and JNK 2. These data suggest an alteration in both canonical and non-canonical TGF beta signalling. Cross-talk between the two signalling pathways has been suggested previously as TGF beta-induced activation of the ERK and JNK kinases results in phosphorylation of SMAD proteins and thus regulates their activation.[40]

Enhanced levels of TGF beta are involved in radiation-induced cardiac fibrosis that is characterised by excess fibroblast proliferation and deposition of collagen fibres.[15, 49] In line with this, our study showed a marked alteration in the expression of extracellular matrix proteins such as biglycan, decorin, and collagens 6 and 10 in the irradiated heart. Excessive accumulation of collagen fibres leading to cardiac fibrosis and dysfunction has previously been reported in different cardiac diseases[50, 51] including radiation-induced heart disease.[52] In agreement with this, similar mice as used in this study

showed diffused amyloidosis caused by extracellular deposition of insoluble, abnormal fibrils that derived from aggregation of misfolded proteins in the myocardium.[15] It was suggested that the sudden death of nearly half of the irradiated mice was caused by this cardiac amyloidosis.[15] In good agreement with this finding, transcriptomics and proteomics data of the present study show that significantly altered extracellular matrix genes and proteins form a cluster of APP-related factors.

Consistent with our previous data[13, 14, 53], this study indicated a persistent alteration of cardiac metabolism due to decreased PPAR alpha activity related to its increased phosphorylation.[30] PPAR alpha regulates lipid metabolism in the heart and reduced PPAR alpha activity is associated with the development of CVD.[16, 17] The activity of PPAR alpha transcription factor is known to be regulated at the phosphorylation level by MAPK-ERK and p38 pathways.[54] The enhanced phosphorylation of ERK found in this study strongly indicates that increased phosphorylation and subsequent inactivation of PPAR alpha results from the activation of MAPK pathway.[30]

Besides metabolism, the expression of genes involved in the inflammatory and acute phase response is regulated by PPAR alpha activation.[55] The gene and protein expression analysis of this study showed that the inflammation-related factors including acute phase proteins and different chemokines were markedly and permanently affected in irradiated hearts. The gene expression profiling predicted activation of different interleukins. This is supported by previous studies showing enhanced levels of inflammatory markers in serum, cardiac endothelial cells and heart tissue in mice after local heart irradiation.[15, 56] Consistent with this finding, the persistent inhibition of the

PPAR alpha signalling pathway is suggested to lead to pro-inflammatory homeostasis in irradiated heart.[53]

PPAR alpha and TGF beta signalling pathways are contributing to the cardiac homeostasis. The regulatory cross-talk between PPAR alpha and TGF beta was reported previously.[57, 58] TGF beta-treated cardiac myocytes showed significantly supressed fatty acid oxidation due to impaired PPAR alpha activity.[57] In line with this, PPAR alpha agonist significantly inhibited fibrotic LV remodelling[59] that is extremely prevalent in PPAR alpha knockout mice.[28] Agonist-activated PPAR alpha has been shown to inhibit TGF beta signal transduction by suppression of c-JUN expression, a downstream target of JNK.[58, 60] On the other hand, JNK activation inhibited PPAR alpha gene expression and fatty acid oxidation in mouse hearts and in a human cardiomyocyte-derived cell line.[61] Furthermore, the interaction between PPAR alpha and SMAD pathways has been previously suggested in mouse heart.[57]

PPAR alpha and TGF beta are known to be regulated by miR-21.[41, 42] This study showed significant upregulation of miR-21 expression in the irradiated heart. Increased levels of mir-21 have been reported in heart failure and radiation-induced cardiac ischemia.[53, 62, 63]

Both cardiac fibrosis and cardiac energy metabolism impairment are known to affect the heart contractile function.[64, 65] Proteomics data of this study showed significant changes in the cardiac structural proteins. This correlates well with previous data showing progressive structural damage in locally irradiated murine hearts.[15] The myosin isoform switching between MYH 6 and MYH 7 shown in the present study is a well-known

human heart pathology[43, 66] that was also detected in rats irradiated with a total body high-dose exposure.[67]

CONCLUSION

This study provides evidence for long-term alterations in the cardiac transcriptome and proteome after local irradiation. The integrated analysis of transcriptome and proteome shows a complex and complimentary network of genes and proteins involved in the radiation-induced heart pathology. A putative model for the role of PPAR alpha and TGF beta signalling pathways is presented in Figure 7. It proposes that radiation-induced activation of MAPK cascade connects these pathways and modulates them, resulting in metabolic disordering, fibrosis, and inflammation.

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Notes: The authors declare no competing financial interest.

Supporting Information

The Role of TGF Beta and PPAR Alpha Signalling Pathways in Radiation Response of Locally Exposed Heart: Integrated Global Transcriptomics and Proteomics Analysis

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Table S-7. The list of significantly deregulated proteins in irradiated heart

Table S-8. IPA Analysis summary of significantly deregulated proteins in irradiated heart

REFERENCES

1. Darby, S. C.; Ewertz, M.; McGale, P.; Bennet, A. M.; Blom-Goldman, U.; Bronnum, D.; Correa, C.; Cutter, D.; Gagliardi, G.; Gigante, B.; Jensen, M. B.; Nisbet, A.; Peto, R.; Rahimi, K.; Taylor, C.; Hall, P., Risk of ischemic heart disease in women after radiotherapy for breast cancer. *N Engl J Med* **2013,** 368, (11), 987-98.
2. Swerdlow, A. J.; Higgins, C. D.; Smith, P.; Cunningham, D.; Hancock, B. W.; Horwich, A.; Hoskin, P. J.; Lister, A.; Radford, J. A.; Rohatiner, A. Z.; Linch, D. C., Myocardial infarction mortality risk after treatment for Hodgkin disease: a collaborative British cohort study. *J Natl Cancer Inst* **2007,** 99, (3), 206-14.
3. Carr, Z. A.; Land, C. E.; Kleinerman, R. A.; Weinstock, R. W.; Stovall, M.; Griem, M. L.; Mabuchi, K., Coronary heart disease after radiotherapy for peptic ulcer disease. *Int J Radiat Oncol Biol Phys* **2005,** 61, (3), 842-50.
4. Tukenova, M.; Guibout, C.; Oberlin, O.; Doyon, F.; Mousannif, A.; Haddy, N.; Guerin, S.; Pacquement, H.; Aouba, A.; Hawkins, M.; Winter, D.; Bourhis, J.; Lefkopoulos, D.; Diallo, I.; de Vathaire, F., Role of cancer treatment in long-term overall and cardiovascular mortality after childhood cancer. *J Clin Oncol* **2010,** 28, (8), 1308-15.
5. Darby, S.; McGale, P.; Peto, R.; Granath, F.; Hall, P.; Ekbom, A., Mortality from cardiovascular disease more than 10 years after radiotherapy for breast cancer: nationwide cohort study of 90 000 Swedish women. *Bmj* **2003,** 326, (7383), 256-7.
6. Darby, S. C.; McGale, P.; Taylor, C. W.; Peto, R., Long-term mortality from heart disease and lung cancer after radiotherapy for early breast cancer: prospective cohort study of about 300,000 women in US SEER cancer registries. *Lancet Oncol* **2005,** 6, (8), 557-65.
7. Guven, M.; Guven, G. S.; Oz, E.; Ozaydin, A.; Batar, B.; Ulutin, T.; Hacihanefioglu, S.; Domanic, N., DNA repair gene XRCC1 and XPD polymorphisms and their association with coronary artery disease risks and micronucleus frequency. *Heart Vessels* **2007,** 22, (6), 355-60.
8. Taylor, C. W.; Povall, J. M.; McGale, P.; Nisbet, A.; Dodwell, D.; Smith, J. T.; Darby, S. C., Cardiac dose from tangential breast cancer radiotherapy in the year 2006. *Int J Radiat Oncol Biol Phys* **2008,** 72, (2), 501-7.
9. Cuzick, J.; Stewart, H.; Rutqvist, L.; Houghton, J.; Edwards, R.; Redmond, C.; Peto, R.; Baum, M.; Fisher, B.; Host, H., Cause-specific mortality in long-term survivors of breast cancer who participated in trials of radiotherapy. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **1994,** 12, (3), 447–453.
10. Shimizu, Y.; Pierce, D. A.; Preston, D. L.; Mabuchi, K., Studies of the mortality of atomic bomb survivors. Report 12, part II. Noncancer mortality: 1950-1990. *Radiation research* **1999,** 152, (4), 374–389.
11. Little, M. P.; Azizova, T. V.; Bazyka, D.; Bouffler, S. D.; Cardis, E.; Chekin, S.; Chumak, V. V.; Cucinotta, F. A.; de Vathaire, F.; Hall, P.; Harrison, J. D.; Hildebrandt, G.; Ivanov, V.; Kashcheev, V. V.; Klymenko, S. V.; Kreuzer, M.; Laurent, O.; Ozasa, K.; Schneider, T.; Tapio, S.; Taylor, A. M.; Tzoulaki, I.; Vandoolaeghe, W. L.; Wakeford, R.; Zablotska, L. B.; Zhang, W.; Lipshultz, S. E., Systematic review and meta-analysis of circulatory disease from exposure to low-level ionizing radiation and estimates of potential population mortality risks. *Environ Health Perspect* **2012,** 120, (11), 1503-11.
12. Baker, J. E.; Moulder, J. E.; Hopewell, J. W., Radiation as a risk factor for cardiovascular disease. *Antioxid Redox Signal* **2011,** 15, (7), 1945-56.
13. Azimzadeh, O.; Sievert, W.; Sarioglu, H.; Yentrapalli, R.; Barjaktarovic, Z.; Sriharshan, A.; Ueffing, M.; Janik, D.; Aichler, M.; Atkinson, M. J.; Multhoff, G.; Tapio, S., PPAR Alpha: A Novel Radiation Target in

Locally Exposed Mus musculus Heart Revealed by Quantitative Proteomics. *Journal of Proteome Research* **2013,** 12, (6), 2700-2714.
14. Azimzadeh, O.; Sievert, W.; Sarioglu, H.; Merl-Pham, J.; Yentrapalli, R.; Bakshi, M. V.; Janik, D.; Ueffing, M.; Atkinson, M. J.; Multhoff, G.; Tapio, S., Integrative proteomics and targeted transcriptomics analyses in cardiac endothelial cells unravel mechanisms of long-term radiation-induced vascular dysfunction. *J Proteome Res* **2015,** 14, (2), 1203-19.
15. Seemann, I.; Gabriels, K.; Visser, N. L.; Hoving, S.; te Poele, J. A.; Pol, J. F.; Gijbels, M. J.; Janssen, B. J.; van Leeuwen, F. W.; Daemen, M. J.; Heeneman, S.; Stewart, F. A., Irradiation induced modest changes in murine cardiac function despite progressive structural damage to the myocardium and microvasculature. *Radiother Oncol* **2012,** 103, (2), 143-50.
16. Smeets, P. J.; Planavila, A.; van der Vusse, G. J.; van Bilsen, M., Peroxisome proliferator-activated receptors and inflammation: take it to heart. *Acta Physiol (Oxf)* **2007,** 191, (3), 171-88.
17. Fruchart, J. C., Peroxisome proliferator-activated receptor-alpha (PPARalpha): at the crossroads of obesity, diabetes and cardiovascular disease. *Atherosclerosis* **2009,** 205, (1), 1-8.
18. Finck, B. N., The PPAR regulatory system in cardiac physiology and disease. *Cardiovasc Res* **2007,** 73, (2), 269-77.
19. Diep, Q. N.; Benkirane, K.; Amiri, F.; Cohn, J. S.; Endemann, D.; Schiffrin, E. L., PPAR alpha activator fenofibrate inhibits myocardial inflammation and fibrosis in angiotensin II-infused rats. *J Mol Cell Cardiol* **2004,** 36, (2), 295-304.
20. Derynck, R.; Zhang, Y. E., Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **2003,** 425, (6958), 577–584.
21. Hein, S.; Arnon, E.; Kostin, S.; Schönburg, M.; Elsässer, A.; Polyakova, V.; Bauer, E. P.; Klövekorn, W.-P.; Schaper, J., Progression from compensated hypertrophy to failure in the pressure-overloaded human heart: structural deterioration and compensatory mechanisms. *Circulation* **2003,** 107, (7), 984–991.
22. Rosenkranz, S.; Flesch, M.; Amann, K.; Haeuseler, C.; Kilter, H.; Seeland, U.; Schlüter, K.-D.; Böhm, M., Alterations of beta-adrenergic signaling and cardiac hypertrophy in transgenic mice overexpressing TGF-beta(1). *American journal of physiology. Heart and circulatory physiology* **2002,** 283, (3), H1253-62.
23. Anscher, M. S., Targeting the TGF-beta1 pathway to prevent normal tissue injury after cancer therapy. *Oncologist* **2010,** 15, (4), 350-9.
24. Barcellos-Hoff, M. H.; Derynck, R.; Tsang, M. L.; Weatherbee, J. A., Transforming growth factor-beta activation in irradiated murine mammary gland. *J Clin Invest* **1994,** 93, (2), 892-9.
25. Scharpfenecker, M.; Kruse, J. J.; Sprong, D.; Russell, N. S.; Ten Dijke, P.; Stewart, F. A., Ionizing radiation shifts the PAI-1/ID-1 balance and activates notch signaling in endothelial cells. *Int J Radiat Oncol Biol Phys* **2009,** 73, (2), 506-13.
26. Shi, Y.; Massague, J., Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **2003,** 113, (6), 685-700.
27. Zhang, Y. E., Non-Smad pathways in TGF-beta signaling. *Cell Res* **2009,** 19, (1), 128-39.
28. Lakatos, H. F.; Thatcher, T. H.; Kottmann, R. M.; Garcia, T. M.; Phipps, R. P.; Sime, P. J., The Role of PPARs in Lung Fibrosis. *PPAR Res* **2007,** 2007, 71323.
29. Lee, C. H.; Kim, H. D.; Shin, S. M.; Kim, S. G., A Novel Mechanism of PPARgamma Regulation of TGFbeta1: Implication in Cancer Biology. *PPAR Res* **2008,** 2008, 762398.
30. Barger, P. M.; Brandt, J. M.; Leone, T. C.; Weinheimer, C. J.; Kelly, D. P., Deactivation of peroxisome proliferator-activated receptor-alpha during cardiac hypertrophic growth. *J Clin Invest* **2000,** 105, (12), 1723-30.
31. Munshi, A.; Ramesh, R., Mitogen-activated protein kinases and their role in radiation response. *Genes Cancer* **2013,** 4, (9-10), 401-8.

32. Seemann, I.; Te Poele, J. A.; Luikinga, S. J.; Hoving, S.; Stewart, F. A., Endoglin haplo-insufficiency modifies the inflammatory response in irradiated mouse hearts without affecting structural and mircovascular changes. *PLoS One* **2013,** 8, (7), e68922.
33. Wisniewski, J. R.; Zougman, A.; Nagaraj, N.; Mann, M., Universal sample preparation method for proteome analysis. *Nat Methods* **2009,** 6, (5), 359-62.
34. Hauck, S. M.; Dietter, J.; Kramer, R. L.; Hofmaier, F.; Zipplies, J. K.; Amann, B.; Feuchtinger, A.; Deeg, C. A.; Ueffing, M., Deciphering membrane-associated molecular processes in target tissue of autoimmune uveitis by label-free quantitative mass spectrometry. *Mol Cell Proteomics* **2010,** 9, (10), 2292-305.
35. Merl, J.; Ueffing, M.; Hauck, S. M.; von Toerne, C., Direct comparison of MS-based label-free and SILAC quantitative proteome profiling strategies in primary retinal Muller cells. *Proteomics* **2012,** 12, (12), 1902-11.
36. Chen, J. J.; Wang, S. J.; Tsai, C. A.; Lin, C. J., Selection of differentially expressed genes in microarray data analysis. *Pharmacogenomics J* **2007,** 7, (3), 212-20.
37. Storey, J. D.; Tibshirani, R., Statistical significance for genomewide studies. *Proc Natl Acad Sci U S A* **2003,** 100, (16), 9440-5.
38. Wu, J.; Liu, W.; Bemis, A.; Wang, E.; Qiu, Y.; Morris, E. A.; Flannery, C. R.; Yang, Z., Comparative proteomic characterization of articular cartilage tissue from normal donors and patients with osteoarthritis. *Arthritis Rheum* **2007,** 56, (11), 3675-84.
39. D'Eustachio, P., Reactome knowledgebase of human biological pathways and processes. *Methods Mol Biol* **2011,** 694, 49-61.
40. Derynck, R.; Zhang, Y. E., Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **2003,** 425, (6958), 577-84.
41. Zhou, J.; Wang, K. C.; Wu, W.; Subramaniam, S.; Shyy, J. Y.; Chiu, J. J.; Li, J. Y.; Chien, S., MicroRNA-21 targets peroxisome proliferators-activated receptor-alpha in an autoregulatory loop to modulate flow-induced endothelial inflammation. *Proc Natl Acad Sci U S A* **2011,** 108, (25), 10355-60.
42. Wang, T.; Zhang, L.; Shi, C.; Sun, H.; Wang, J.; Li, R.; Zou, Z.; Ran, X.; Su, Y., TGF-beta-induced miR-21 negatively regulates the antiproliferative activity but has no effect on EMT of TGF-beta in HaCaT cells. *Int J Biochem Cell Biol* **2012,** 44, (2), 366-76.
43. Miyata, S.; Minobe, W.; Bristow, M. R.; Leinwand, L. A., Myosin heavy chain isoform expression in the failing and nonfailing human heart. *Circulation research* **2000,** 86, (4), 386–390.
44. Koussounadis, A.; Langdon, S. P.; Um, I. H.; Harrison, D. J.; Smith, V. A., Relationship between differentially expressed mRNA and mRNA-protein correlations in a xenograft model system. *Sci Rep* **2015,** 5, 10775.
45. Vogel, C.; Marcotte, E. M., Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet* **2012,** 13, (4), 227-32.
46. Olivares-Hernandez, R.; Bordel, S.; Nielsen, J., Codon usage variability determines the correlation between proteome and transcriptome fold changes. *BMC Syst Biol* **2011,** 5, 33.
47. Wu, G.; Nie, L.; Zhang, W., Integrative analyses of posttranscriptional regulation in the yeast Saccharomyces cerevisiae using transcriptomic and proteomic data. *Curr Microbiol* **2008,** 57, (1), 18-22.
48. Dobaczewski, M.; Chen, W.; Frangogiannis, N. G., Transforming growth factor (TGF)-beta signaling in cardiac remodeling. *J Mol Cell Cardiol* **2011,** 51, (4), 600-6.
49. Sun, W.; Ni, X.; Sun, S.; Cai, L.; Yu, J.; Wang, J.; Nie, B.; Sun, Z.; Ni, X.; Cao, X., Adipose-Derived Stem Cells Alleviate Radiation-Induced Muscular Fibrosis by Suppressing the Expression of TGF-beta1. *Stem Cells Int* **2016,** 2016, 5638204.
50. Diez, J., Altered degradation of extracellular matrix in myocardial remodelling: the growing role of cathepsins and cystatins. *Cardiovasc Res* **2010,** 87, (4), 591-2.

51. Westermann, D.; Mersmann, J.; Melchior, A.; Freudenberger, T.; Petrik, C.; Schaefer, L.; Lullmann-Rauch, R.; Lettau, O.; Jacoby, C.; Schrader, J.; Brand-Herrmann, S. M.; Young, M. F.; Schultheiss, H. P.; Levkau, B.; Baba, H. A.; Unger, T.; Zacharowski, K.; Tschope, C.; Fischer, J. W., Biglycan is required for adaptive remodeling after myocardial infarction. *Circulation* **2008,** 117, (10), 1269-76.
52. Taunk, N. K.; Haffty, B. G.; Kostis, J. B.; Goyal, S., Radiation-induced heart disease: pathologic abnormalities and putative mechanisms. *Front Oncol* **2015,** 5, 39.
53. Azimzadeh, O.; Azizova, T.; Merl-Pham, J.; Subramanian, V.; Bakshi, M. V.; Moseeva, M.; Zubkova, O.; Hauck, S. M.; Anastasov, N.; Atkinson, M. J.; Tapio, S., A dose-dependent perturbation in cardiac energy metabolism is linked to radiation-induced ischemic heart disease in Mayak nuclear workers. *Oncotarget* **2016**. doi: 10.18632/oncotarget.10424
54. Gervois, P.; Torra, I. P.; Chinetti, G.; Grotzinger, T.; Dubois, G.; Fruchart, J. C.; Fruchart-Najib, J.; Leitersdorf, E.; Staels, B., A truncated human peroxisome proliferator-activated receptor alpha splice variant with dominant negative activity. *Mol Endocrinol* **1999,** 13, (9), 1535-49.
55. Marx, N.; Kehrle, B.; Kohlhammer, K.; Grub, M.; Koenig, W.; Hombach, V.; Libby, P.; Plutzky, J., PPAR activators as antiinflammatory mediators in human T lymphocytes: implications for atherosclerosis and transplantation-associated arteriosclerosis. *Circ Res* **2002,** 90, (6), 703-10.
56. Sievert, W.; Trott, K. R.; Azimzadeh, O.; Tapio, S.; Zitzelsberger, H.; Multhoff, G., Late proliferating and inflammatory effects on murine microvascular heart and lung endothelial cells after irradiation. *Radiother Oncol* **2015,** 117, (2), 376-81.
57. Sekiguchi, K.; Tian, Q.; Ishiyama, M.; Burchfield, J.; Gao, F.; Mann, D. L.; Barger, P. M., Inhibition of PPAR-alpha activity in mice with cardiac-restricted expression of tumor necrosis factor: potential role of TGF-beta/Smad3. *Am J Physiol Heart Circ Physiol* **2007,** 292, (3), H1443-51.
58. Kintscher, U.; Lyon, C.; Wakino, S.; Bruemmer, D.; Feng, X.; Goetze, S.; Graf, K.; Moustakas, A.; Staels, B.; Fleck, E.; Hsueh, W. A.; Law, R. E., PPARalpha inhibits TGF-beta-induced beta5 integrin transcription in vascular smooth muscle cells by interacting with Smad4. *Circ Res* **2002,** 91, (11), e35-44.
59. Iglarz, M.; Touyz, R. M.; Viel, E. C.; Paradis, P.; Amiri, F.; Diep, Q. N.; Schiffrin, E. L., Peroxisome proliferator-activated receptor-alpha and receptor-gamma activators prevent cardiac fibrosis in mineralocorticoid-dependent hypertension. *Hypertension* **2003,** 42, (4), 737-43.
60. Ogata, T.; Miyauchi, T.; Irukayama-Tomobe, Y.; Takanashi, M.; Goto, K.; Yamaguchi, I., The peroxisome proliferator-activated receptor alpha activator fenofibrate inhibits endothelin-1-induced cardiac fibroblast proliferation. *J Cardiovasc Pharmacol* **2004,** 44 Suppl 1, S279-82.
61. Drosatos, K.; Drosatos-Tampakaki, Z.; Khan, R.; Homma, S.; Schulze, P. C.; Zannis, V. I.; Goldberg, I. J., Inhibition of c-Jun-N-terminal kinase increases cardiac peroxisome proliferator-activated receptor alpha expression and fatty acid oxidation and prevents lipopolysaccharide-induced heart dysfunction. *J Biol Chem* **2011,** 286, (42), 36331-9.
62. Cheng, Y.; Zhang, C., MicroRNA-21 in cardiovascular disease. *J Cardiovasc Transl Res* **2010,** 3, (3), 251-5.
63. Thum, T.; Gross, C.; Fiedler, J.; Fischer, T.; Kissler, S.; Bussen, M.; Galuppo, P.; Just, S.; Rottbauer, W.; Frantz, S.; Castoldi, M.; Soutschek, J.; Koteliansky, V.; Rosenwald, A.; Basson, M. A.; Licht, J. D.; Pena, J. T.; Rouhanifard, S. H.; Muckenthaler, M. U.; Tuschl, T.; Martin, G. R.; Bauersachs, J.; Engelhardt, S., MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. *Nature* **2008,** 456, (7224), 980-4.
64. Doenst, T.; Nguyen Td Fau - Abel, E. D.; Abel, E. D., Cardiac metabolism in heart failure: implications beyond ATP production. *Circ Res.* **2013,** 113(6):709-24, (1524-4571 (Electronic)).
65. Khan, R.; Sheppard, R., Fibrosis in heart disease: understanding the role of transforming growth factor-beta in cardiomyopathy, valvular disease and arrhythmia. *Immunology* **2006,** 118, (1), 10-24.
66. Lowes, B. D.; Minobe, W.; Abraham, W. T.; Rizeq, M. N.; Bohlmeyer, T. J.; Quaife, R. A.; Roden, R. L.; Dutcher, D. L.; Robertson, A. D.; Voelkel, N. F.; Badesch, D. B.; Groves, B. M.; Gilbert, E. M.; Bristow,

M. R., Changes in gene expression in the intact human heart. Downregulation of alpha-myosin heavy chain in hypertrophied, failing ventricular myocardium. *The Journal of clinical investigation* **1997,** 100, (9), 2315–2324.
67. Litten, R. Z.; Fein, H. G.; Gainey, G. T.; Walden, T. L.; Smallridge, R. C., Alterations in rat cardiac myosin isozymes induced by whole-body irradiation are prevented by 3,5,3'-L-triiodothyronine. *Metabolism: clinical and experimental* **1990,** 39, (1), 64–68.

Figure legends

Figure 1. Pathway and network analysis of significantly differentially expressed genes in irradiated heart. Bars indicate top networks (A) and canonical pathways (B) and y-axis displays the – (log) significance. Taller bars are more significant than shorter bars. A positive z-score implies potential activation (orange) and a negative z-score indicates potential inhibition (blue) of the pathway (http://www.INGENUITY.com).

Figure 2. Pathway and network analysis of significantly differentially expressed proteins in irradiated heart. Bars indicate top networks (A) and canonical pathways (B) and y-axis displays the – (log) significance. Taller bars are more significant than shorter bars. A positive z-score implies potential activation (orange) and a negative z-score indicates potential inhibition (blue) of the pathway (http://www.INGENUITY.com).

Figure 3. The analysis of transcriptomics and proteomics upstream regulators. Graphical representation of the deregulated genes and protein networks with their upstream transcriptional regulators at 16 Gy is shown (http://www.INGENUITY.com). The up-regulated proteins are marked in red and the down-regulated in green. The nodes in blue and orange represent transcription factors. The genes and protein IDs are available in Tables S2, S3, S7 and S8 in SI.

Figure 4. Validation of TGF beta and PPAR alpha signalling pathways. Immunoblot analysis of the levels of TAK-1, phospho TAK-1, JNK1 / JNK2, phospho JNK1 / JNK2,

ERK 44/42, phospho ERK 44/42 ,p 38, phospho p 38, PPAR-alpha, and phospho PPAR alpha is shown (A). The columns represent the average ratios of relative protein expression in sham- and irradiated samples after background correction and normalisation to ATPB5 expression (B) (t test; * $p < 0.05$, ** $p < 0.01$; n = 3).

Figure 5. Analysis of radiation-induced changes in miR-21 and the myosin heavy chain isoforms. Analysis of miR-21 showed significant upregulation of miR-21 in the irradiated heart compared to controls (A) (t test; *$p < 0.05$, **$p < 0.01$; n = 3). Immunoblot analysis of the levels of- myosin heavy chain 6 (MYH 6), and myosin heavy chain 7 (MYH 7) is shown (B). The columns represent the average ratios of relative protein expression in sham- and irradiated samples after background correction and normalisation to ATPB5 expression (C) (t test; *$p < 0.05$, **$p < 0.01$; n = 3).

Figure 6. Combined analysis for common regulatory networks of all significantly deregulated cardiac genes and proteins. The TGF beta (A) and PPAR alpha (B) networks were generated by introducing all proteins and genes found significantly up- or downregulated in this study to the STRING software. Proteins are shown in brown and genes in blue balls. The protein and gene IDs are available in Tables S2 and S7 in SI. Mapk3: ERK1; Mapk1: ERK2; Mapk8: JNK; map3k7: TAK1; and Mapk14: p 38.

Figure 7. Proposed model for the role of TGF beta and PPAR alpha pathways in radiation-induced cardiac damage.

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