Radiation-induced cardiopulmonary dysfunction
van der Veen, Sjoerdjje Johanna

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EARLY ENDOTHELIAL CELL LOSS IN RADIATION-INDUCED LUNG DYSFUNCTION

Sonja J. van der Veen, M.D., 1,2 Francois Paris, Ph.D., 3 Nolwenn Dubois, 3 Ghazaleh Ghobadi, Ph.D., 1,2# Reza Fardid, Ph.D., 1* Hette Faber,1,2 Sytze Brandenburg, Ph.D.,4 Johannes A. Langendijk, M.D., Ph.D.,2 Peter van Luijk, Ph.D.,2 Robert P. Coppes, Ph.D.,1,2

1Department of Cell Biology, 2Department of Radiation Oncology, 3KVI-Center for Advanced Radiation Research, University of Groningen, Groningen, the Netherlands.
3Cancer Research Center, INSERM UMR892, Nantes, France.

# Current address: Department of Radiation Oncology, the Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital, Amsterdam, the Netherlands.
* Current address: Department of Radiology, Faculty of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran.
Recent studies suggested that radiation-induced lung dysfunction is initiated by acute endothelial cell loss subsequently leading to vascular remodelling and pulmonary hypertension in a pre-clinical rat model. Here we show that endothelial cell loss seems to occur at 8 hours and 2 weeks after irradiation and that acute apoptosis might play an initiating role leading to a cascade of further endothelial cell loss. Preliminary results indicate that transplantation with endothelial progenitor cells at these early time-points after irradiation may repair/replenish damaged endothelial cells possibly reducing the development of radiation-induced lung dysfunction. Successful prevention of radiation-induced lung dysfunction may open avenues for further clinical use to improve quality of life of the patient and allowing tumour-dose escalation and potentially increasing survival.

The efficacy of radiation treatment for thoracic tumours is limited by potentially life-threatening radiation-induced lung dysfunction (RILD). In stage III NSCLC patients even the most advanced radiotherapy techniques still result in a poor survival with 20% of the patients developing RILD. Traditionally, RILD is divided into an early inflammatory phase known as "radiation pneumonitis" occurring around 1 to 6 months after irradiation and a subsequent fibroproductive phase often referred to as "lung fibrosis" occurring after 6 months. Irradiation of a limited sub-volume of the lung generally does not lead to clinically relevant pulmonary complications. This allows for dose escalation for patients with less advanced tumours improving cure rates, such as in early stage lung cancer. However, treatment of more advanced tumours is more challenging and due to the larger irradiated lung volumes still prohibits dose escalation in many cases.

Recently, we showed in a preclinical rat model that vascular damage could initiate pulmonary complications after irradiation of larger lung volumes. Pulmonary endothelial cell (EC) loss and consequential perivascular edema induced volume-dependent vascular remodelling. Subsequently, this caused pulmonary hypertension and right ventricle hypertrophy of the heart. Vascular damage preceded alveolar inflammation and occurred at lower doses. Moreover, the consequential increase in mean pulmonary artery pressure correlated strongly with respiratory rate. This indicates that early RILD after larger volume irradiation in this rat model may be determined by pulmonary vascular remodelling resulting from acute pulmonary EC loss.

The importance of acute EC loss in the development of radiation-induced normal tissue damage has been established for other tissues such as intestine, spinal cord and rectum. It was also demonstrated that irradiated ECs induce migration and proliferation of vascular smooth muscle cells, one of the most prominent features of vascular remodelling in our rat model for lung toxicity. Since ECs are important targets of radiation in different organs, they may have a pivotal role in the development of early radiation-induced lung morbidity too.

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Summary

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Introduction

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The importance of acute EC loss in the development of radiation-induced normal tissue damage has been established for other tissues such as intestine, spinal cord and rectum. It was also demonstrated that irradiated ECs induce migration and proliferation of vascular smooth muscle cells, one of the most prominent features of vascular remodelling in our rat model for lung toxicity. Since ECs are important targets of radiation in different organs, they may have a pivotal role in the development of early radiation-induced lung morbidity too.

As such, stem cell therapy for RILD could be considered. Under homeostatic conditions bone marrow derived non-hematopoietic stem cells, such as endothelial progenitor cells (EPCs), fibrocytes and mesenchymal stem cells (MSCs) circulate in the blood and contribute...
to repair of tissues such as lung in response to damage. Circulating EPCS are especially considered to be involved in the repair and regeneration of damaged blood vessels throughout the body. Considering the pivotal role for EC loss, a cell-based therapy using EPCs may be an attractive option in the treatment of RILD. In the present study, first we studied radiation-induced pulmonary EC loss in more detail by investigating EC loss at early time-points after irradiation and studying the role of apoptosis in initiating EC loss. Next, we investigated the potential of EPC transplantation to ameliorate early RILD.

Material and Methods

Animals
Adult male albino Wistar rats (n=3-7 per group, 270-320 g) of the Hsd/Cpb:WU strain, bread in a specific pathogen free colony (Harlan-CPB, Rijswijk, The Netherlands) were used in the experiments. They were housed five to a cage under a 12 h light - 12 h dark cycle and fed rodent chow (RMH-B, Hope Farms, Woerden, The Netherlands) and water ad libitum. The experiments were performed in agreement with the Netherlands Experiments on Animals Act (1977) and the European Convention for the Protection of Vertebrate Animals Used for Experimental Purposes (Strasbourg, 18.III.1986).

Irradiation procedure
The animals used in the experiments were either irradiated with protons or photons. We started performing our experiments with proton irradiation, which enabled us to irradiate different subvolumes of the lung in a more accurate way than with photon irradiation. Later on we switched to photon irradiation due to practical and logistic reasons but also since the goal of our experiments changed. In our previous experiments using protons we studied the response after irradiation of different subvolumes of rat lungs, in the experiments using photons we studied lung morphology and performed intervention studies needing less accurate irradiation.

Proton irradiation: The rats were anesthetized with an i.p. injection of xylazine (Rompun; Bayer, Leverkusen, Germany) plus S-ketamine (Ketalar; Pfizer, Capelle aan de IJssel, The Netherlands) and placed in a holder hanging on a positioning rod by their upper incisors for irradiation. The use of 150-MeV protons in a fixed beam line facilitated the irradiation of subvolumes of the lung with sharply demarcated (20–80% penumbra of approximately 1 mm) radiation fields. Radiation portals were designed using planning CT images of 5 age-matched rats, as described previously. Using protons, 50% of the lung was irradiated to 20 Gy with a single fraction (Fig. 1). Control animals were anaesthetized and sham irradiated.

Photon irradiation: Using an orthovoltage X-ray machine operated at 200 kV and 15 mA the lungs of the animals were irradiated from two parallel opposing anterior/posterior-anterior irradiation fields. Two separate positioning holders were needed for the anterior-posterior and subsequent posterior-anterior irradiations in a single treatment session. Rat
lungs were irradiated with a volume of 50% and 100% to respectively 22 Gy and 13 Gy with a single fraction of photons (Fig. 1).

Figure 1: Overview of irradiation portals used. Rat lungs were irradiated to a volume of 50% with protons (20 Gy) or photons (22 Gy) or to a volume of 100% using photons (13 Gy).

Histologic examinations
Histologic examination was performed at 4, 6, 8, 12, 24 hours, 3 days, 1 week, 2 weeks and 8 weeks after radiation. The tissue was collected as published previously. Briefly, the animals were heparinized while the heart was still beating. After opening the thoracic cavity both pulmonary and systemic circulation were perfused in situ via the right ventricle and liver incision with PBS (pH 7.3). Subsequently the lungs were removed, of the proton irradiated animals the right cranial lobe was tied and 1 mm³ sections of irradiated (lateral part) and non-irradiated (medial part) were collected and frozen in liquid nitrogen for mRNA analysis. See supplementary figure S1 for an overview of how the lung parts were collected. The rest of the lung was intratracheally infused with 4% formaldehyde in PBS (pH 7.3). The trachea was tied and the entire specimen was immersed in 4% buffered formaldehyde for overnight fixation. A clear margin between the irradiated and shielded tissues was visible macroscopically, conforming the shape of the irradiation portal. Lung tissue samples were taken from both inside and outside the radiation field, with sufficient margins to ensure that the tissue was either irradiated (“in-field”; i.e., tissue receives more than 97% of the prescribed dose) or shielded (“out-of-field”; tissue receives less than 7% of the prescribed dose), and embedded in paraffin.

Of the rats irradiated with photons the right cranial lobe was tied and frozen sections of 1 mm³ of the irradiated (lateral part) and the non-irradiated part (medial) were collected for mRNA analysis (see Supplementary Figure S1). The rest of the lung was inflated with TissueTec and frozen in liquid nitrogen. Paraffin sections of 5 µm containing standardized samples of irradiated or non-irradiated lung tissue were stained and examined by light microscopy. A specific rat endothelial cell staining (HIS52) was used to analyse endothelial cell loss. A TUNEL (TdT-mediated dUTP Nick-End Labelling) assay (Promega) was performed to assess apoptotic ECs. Frozen lung sections (5-25 µm) of irradiated (lateral part of left lung and lateral parts of the right median and caudal lobe, Supplementary figure S1) and non-irradiated (accessory lobe, Fig. S1) were stained and examined by fluorescence microscopy. A TUNEL assay was used to assess and quantify EC apoptosis in the lungs.
Chapter 05 | Early EC loss in RILD

Quantitative polymerase chain reaction (QPCR)
Total RNA was extracted from the lung tissue using the Stratagene Absolutely RNA Miniprep kit and cDNA was generated subsequently using the M-MLV Reverse Transcriptase kit (Invitrogen) according to the manufacturers’ protocols. Biorad IQTM SYBR@ Green Supermix was used to perform amplifications in a Bio-Rad IQ iCycler machine as described by the manufacturer. Based on the protocol, the following conditions of QPCR were used: 2 minutes at 95 ºC for the initial denaturation, 40 cycles of denaturation for 30 seconds at 95 ºC, annealing for 30 seconds at 60 ºC, extension for 30 seconds at 72 ºC. The following primers were used to amplify SM DP1: 5' -GAACACCATCACAGACCTC and 5' - ATTAGAAACCGGATCTGGAGTT. Target genes were quantified relative to the reference gene using the mathematical model described by Pfaffl et al. 18.

Ceramide quantification
Lipids were extracted from frozen lung tissue samples with chloroform/methanol as described by Van der Vusse et al. 19 . The samples were pulverized and immediately transferred to tubes containing methanol at −21 ºC. Methanol contained butyalted hydroxytoluene as an antioxidant (30 mg/100 mL, Sigma, St Louis, MO, USA). Ceramide was isolated by means of thin-layer chromatography as described previously 20. To isolate ceramide, the plates were first developed to one-third of the total length with a solvent composed of chloroform–methanol-25% NH₃ (20 : 5 : 0.2, v/v/v) dried and rechromatographed using a solvent composed of heptane–isopropyl ether–acetic acid (60 : 40 : 3, v/v/v). Standards of ceramide (Sigma) were run along with the appropriate samples. The lipid bands were visualized under ultraviolet light after being sprayed with a 0.5% solution of 3,3'-dichlorofluorescein in absolute methanol. The gel bands containing ceramide were scrapped off the plate and transferred into screw tubes containing methylpentadecanoic acid (Sigma) as an internal standard. Fatty acids were transmethylated along with the gel in the presence of 14% boron fluoride in methanol at 100 ºC for 90 min 21. The resulting methyl esters were analysed by gas-liquid chromatography using a Hewlett-Packard 5890 Series II and a fused Hp-INNOWax (50 m) capillary column (Aligent Technologies, Palo Alto, CA, USA). The individual fatty acid methyl esters were quantified using the area corresponding to the internal standards (Sigma). The recovery rate of fatty acids was 91.5 ± 7.2%.

EPC culturing and characterization
EPCs were isolated from rat bone marrow 22. After flushing the tibia and femur of inbred male Wistar Kyoto rats mononuclear cells were isolated by density gradient centrifugation at 400 g for 30 minutes. Isolated cells were seeded on fibronectin-coated culture flasks and maintained in Dulbecco modified Eagle medium (DMEM) containing 20% fetal bovine serum. After 4 days in culture, non-adherent cells were removed by washing with PBS, and new medium was applied thereafter. At day 7 of culture, the adherent cells were harvested by trypsinization for analysis or transplantation. Early EPCs were recognized as attached spindle-shaped cells (Fig. 4A). Characterization was performed by examining endocytosis of acetyl-LDL and surface expression of isolectin and examined using fluorescence confocal...
microscopy with absorption wavelengths at 555 nm (acetyl-LDL) and 495 nm (isoelectin) (Fig. 4B).

**EPC labelling**
To allow detection of donor cells, cells were stained with PKH-26, a fluorescent dye that incorporates in the cell membrane. The experimental procedures were conducted according to the protocol included in the dye kit (Sigma Aldrich). The presence of donor EPCs in the pulmonary vasculature was detected by fluorescence microscopy (Fig. 4C).

**EPC transplantation**
The rats were transplanted twice, at 8 hours after irradiation and at 2 weeks after irradiation. The rats were anesthetized and orally intubated with a sterile plastic catheter. The sham group was given 0.2 ml PBS at both time points by slowly infusion via the right jugular vein. The EPC transplantation group was given 0.6-1.0 x 10^6 cells in 0.2 ml PBS/BSA 0.2% at 8 hours after irradiation and 0.5 x 10^6 cells in 0.2 ml PBS/BSA 0.2% at 2 weeks after irradiation by slowly infusion via the right jugular vein. At 8 weeks after irradiation, all rats were sacrificed and lung tissues were collected.

**BR measurements**
To assess response of pulmonary function to radiation, the breathing rate (BR) was measured before and every week after the irradiations up to week 8, as previously described. After two training sessions, a breathing rate (BR) at rest was recorded for each rat, which took place less than a week before irradiation. An unrestrained animal was placed in a 1500 ml airtight but transparent tube of a whole-body plethysmograph connected to a pressure transducer. The frequency of pressure changes inside the tube was recorded and displayed on a calibrated chart as breaths per minute (bpm). A mean BR of an animal was then calculated from minimum of 4 steady regions of the recording lasting ≥ 15 seconds. If the measurement required more than 5 minutes to obtain, the animal was let out of the tube and rested to prevent anxiety as well as drop of oxygen inside the tube. A mean BR of a dose group (bpm) with its standard error (SEM) was calculated from the means of individual animals at each time point. The increase of BR, relative to the mean BR in weeks 0–2 after irradiation, was used as an indicator of loss of pulmonary function. The animals were sacrificed at the end of the experiment at 8 weeks or earlier when they suffered severe pulmonary toxicity.

**Statistical analysis**
The one-way ANOVA with the post hoc Bonferroni test was used to analyse differences in EC apoptosis, mRNA levels of the SMDP-1 gene and ceramide levels at different time-points after irradiation. The students T-test was used to analyse differences in breathing rate increase of EPC transplanted rats after irradiation. The values of p<0.05 were considered significant.
Results

Early endothelial cell loss
To investigate early radiation-induced EC loss, proton-irradiated rat lung tissues were analysed at early time points after irradiation of 50% lung volume to a dose of 20 Gy. A specific rat EC staining (HIS52) was used to study EC loss at early time-points after irradiation. Figure 2 shows representative pictures of pulmonary vessels at the different time-points after irradiation. Figure 2A shows a vessel of an unirradiated animal containing a continuous brown staining of ECs indicating that the unirradiated animals have a continuous layer of pulmonary ECs. No change was observed at 4 hours after irradiation (Fig. 2B), but at 8 hours discontinuity of the EC layer appears (Fig. 2C). Interestingly, at 12 hours (Fig. 2D), 24 hours (Fig. 2E), 3 days (Fig. 2F) and 1 week after irradiation (Fig. 2G) a continuous layer of ECs was observed possibly indicating EC repair. However, at 2 weeks after irradiation (Fig. 2H) again a discontinuity/disruption of the EC lining is present possibly indicating a second phase of EC loss. At 8 weeks after irradiation (Fig. 2I) the vessels have a continuous layer of ECs but clearly show features of vascular remodelling as published previously 1.

![Figure 2: Early pulmonary EC loss after 50% lung irradiation.](image)

A specific rat EC staining (HIS52) was used to study EC loss at early time-points after irradiation. Figure 2A shows that the unirradiated animals have a continuous layer of pulmonary ECs. Four hours after irradiation (B) the EC layer still has a continuous appearance while 8 hours after irradiation a discontinuity appears. Twelve hours (D), 24 hours (E) 3 days (D), and 1 week after irradiation (G) the EC layer is continuous while 2 weeks after irradiation (H) again a discontinuity/disruption of the EC lining is present. At eight weeks after irradiation (I) the vessels have a continuous layer of ECs but clearly show features of vascular remodelling.
Early endothelial cell apoptosis

Next, we investigated if this early EC loss was due to acute radiation-induced apoptosis. First, we performed a TUNEL staining on the lung tissue to detect apoptotic cells in situ (Fig. 3A). Quantification of EC apoptosis showed an increase of apoptotic ECs in the irradiated lung tissue compared to non-irradiated rat lungs (Fig. 3B). At all early time-points after irradiation, i.e., 4 to 12 hours, around 1-1.5% apoptotic ECs were observed while the non-irradiated lung tissue showed around 0.1% apoptotic ECs. The slight increase of apoptotic ECs in the irradiated field was statistically significant compared to the increase observed in non-irradiated lungs. The percentage apoptotic cells outside of the irradiated field was not significantly increased compared to control (Fig. 3B).

![Early radiation-induced pulmonary EC apoptosis](image)

**Figure 3: Early radiation-induced pulmonary EC apoptosis.** A) A TUNEL staining shows apoptotic cells after irradiation. B) Quantification of EC apoptosis showed a significant increase of around 1-1.5% apoptotic ECs in the irradiated lung tissue. C) A significant increase of SMDP-1 mRNA was observed at 4 hours, 24 hours, 3 days, 2 weeks and 8 weeks after irradiation in the out-of-field lung tissue. One week and 2 weeks after irradiation a significant increase was observed in the irradiated lung tissue. D) Quantification of the levels of the proapoptotic lipid ceramide did not show differences between tissues of irradiated animals versus non-irradiated control animals. *** p < 0.001, ** p < 0.01 compared with control.

To get more insight into the cellular pathway leading to apoptosis we measured mRNA levels of the pro-apoptotic SMDP-1 gene both in the irradiated lung tissue (InField), and in the out-of-field lung tissue (OutField) (Fig. 3C). From 4 hours until 2 weeks after irradiation gradually increasing mRNA levels of SMDP-1 were observed both in- and out-of-field. In the irradiated field SMDP-1 mRNA expression was significantly elevated at 1 and 2
weeks after irradiation. In the non-irradiated field a significant increase was observed at 4 hours, 24 hours, 3 days, 2 weeks and 8 weeks after irradiation. At 8 weeks post-irradiation the mRNA levels were lower compared to the earlier time-points, but still higher compared to non-irradiated lung tissue which may indicate that apoptotic ECs are lost at that time-point.

The pro-apoptotic gene SMDP-1 codes for acid sphingomyelinase which hydrolyses sphingomyelin to ceramide, a proapoptotic lipid. As such, to further investigate the cellular pathway leading to apoptosis, we studied the level of ceramide in the lung tissue as shown in Figure 3C. In contrast to the increase of SMDP-1 levels, no differences between levels of ceramide were observed in lung tissue of non-irradiated animals versus irradiated animals.

In summary, significant increases in apoptosis related parameters, but not ceramide, were observed in pulmonary ECs in the irradiated lungs at early time-points after irradiation.

**EPC culturing, characterization and visualization**

So far, our data showed early EC loss after irradiation. Next, we investigated if early transplantation with endothelial progenitor cells (EPCs) could replenish the EC loss and consequently ameliorate RILD. EPCs could be cultured in endothelial growth medium (Fig. 4A). Early EPCs were recognized as attached spindle-shaped cells. Characterization was performed by examining endocytosis of acetyl-LDL and surface expression of isolectin and examined using fluorescence confocal microscopy with absorption wavelengths at 555 nm (acetyl-LDL) and 495 nm (isolectin) (Fig. 4B). To test whether these cells engraft in the lung, we intravenously injected cultured rat EPCs labelled with the membrane dye PKH26 after irradiation. Thirty minutes after injection the rat was sacrificed and the lung tissue was analysed for presence of labelled EPCs by using fluorescence microscopy. Figure 4C shows that these cells are visible in the lung endothelium indicating engraftment to the lung after transplantation. As such, EPC transplantation was shown to be practically feasible and warrants further investigation in the use of amelioration of RILD.

**Effect of EPC transplantation on cardiopulmonary function**

To assess the effect of EPC transplantation on cardiopulmonary function, breathing rates of EPC- transplanted and control-animals (PBS-infused) were weekly analysed after irradiation (Fig. 5A). The animals were photon-irradiated with a lung volume of 50% and 100% (including the heart) to a dose of respectively 22 Gy and 13 Gy. EPC transplantation was performed at two time-points, 8 hours and 2 weeks after irradiation, corresponding with the time-points showing EC loss (Fig. 2). Breathing rates were analysed at 4, 5 and 6 weeks after irradiation. In the 50% lung irradiated group, no significant differences were observed between the breathing rates of EPC or PBS injected animals (Fig. 5B). In the 100% irradiated group, at 4 and 6 weeks post-irradiation the breathing rate increases of EPC transplanted animals were significantly lower compared to PBS-injected animals (Fig. 5C). These preliminary results indicate that EPC transplantation may be a potential therapy to reduce RILD.
sphingomyelin to ceramide, a proapoptotic lipid. As such, to further investigate the reduction of RILD.

These preliminary results indicate that EPC transplantation may be a potential therapy to transplanted animals were significantly lower compared to PBS-injected animals (Fig. 5C). In the 100% irradiated group, at 4 and 6 weeks post-irradiation the breathing rate increases of EPC-transplanted animals were higher compared to PBS-injected animals (Fig. 5B). In the 50% lung irradiated group, no significant differences were observed between the breathing rates of EPC or PBS injected animals (Fig. 5B). The animals were photon-irradiated with a lung volume of 50% and 100% after irradiation. In the non-irradiated field a significant increase was observed at 4 weeks after irradiation. The pro-apoptotic gene SMDP-1 codes for acid sphingomyelinase which hydrolyses ceramide, and we observed significant increases in apoptosis related parameters, but not ceramide, were shown in Figure 3C. In contrast to the increase of SMDP-1 levels, no differences between the mRNA levels were lower compared to the earlier time-points, but still higher compared to non-irradiated lung tissue which may indicate that apoptotic ECs are lost at that time-point.

So far, our data showed early EC loss after irradiation. Next, we investigated if early transplantation with endothelial progenitor cells (EPCs) could replenish the EC loss and consequently ameliorate RILD. EPCs could be cultured in endothelial growth medium (Fig. 4A). Early EPCs were recognized as attached spindle-shaped cells. After 10-14 days colony forming appears. B) Characterization was performed by examining endocytosis of acetyl-LDL and surface expression of isolectin and examined using fluorescence microscopy. C) To test whether these cells engraft in the lung we intravenously injected cultured rat EPCs labelled with the membrane dye PKH26. Thirty minutes after irradiation these cells were clearly visible in the lung endothelium indicating rapid engraftment to the lung after transplantation.
Firradiation, at least in part via apoptosis. This acute EC loss might play an initiating role in the present study we observed that pulmonary EC loss occurs hours after thoracic irradiation, leading to a cascade of further EC loss. Preliminary results indicate that early SMDP-1 and apoptosis prone ECs occurred, indicating that apoptosis might be triggered very early after irradiation. SMDP-1 codes for acid sphingomyelinase which hydrolyses sphingomyelin to ceramide, an important second messenger of the apoptosis pathway. An imbalance between these factors due to EC retraction results in an increased permeability to low molecular weight solutes. Early disruption of the endothelial lining with increased permeability and perivascular edema found in our and other studies may decrease the blood flow to the heart. To assess the effect of EPC transplantation on cardiopulmonary function breathing rate measurements were performed weekly after transplantation of 50% lung irradiated animals with 22 Gy and 100% lung irradiated (including the heart) with 13 Gy (Fig. 5A). No significant differences were observed between the breathing rates of EPC or PBS injected animals in the 50% irradiated groups (Fig. 5B). In the 100% irradiated group, at 4 and 6 weeks post-irradiation the breathing rate increases of EPC transplanted animals were significantly lower compared to PBS-injected animals (Fig. 5C). The grey line in figure A depicts the breathing rate of un-irradiated animals. * p < 0.05 comparison between PBS and EPC injected animals. Animals injected with PBS: n=2 per group. Animals injected with EPCs: n=3 per group.

**Figure 5: Pulmonary function after EPC transplantation in thoracic irradiated rats.** To assess the effect of EPC transplantation on cardiopulmonary function breathing rate measurements were performed weekly after transplantation of 50% lung irradiated animals with 22 Gy and 100% lung irradiated (including the heart) with 13 Gy (Fig. 5A). No significant differences were observed between the breathing rates of EPC or PBS injected animals in the 50% irradiated groups (Fig. 5B). In the 100% irradiated group, at 4 and 6 weeks post-irradiation the breathing rate increases of EPC transplanted animals were significantly lower compared to PBS-injected animals (Fig. 5C). The grey line in figure A depicts the breathing rate of un-irradiated animals. * p < 0.05 comparison between PBS and EPC injected animals. Animals injected with PBS: n=2 per group. Animals injected with EPCs: n=3 per group.

**Discussion**

In the present study we observed that pulmonary EC loss occurs hours after thoracic irradiation, at least in part via apoptosis. This acute EC loss might play an initiating role leading to a cascade of further EC loss. Preliminary results indicate that early transplantation with EPCs after irradiation may possibly reduce the development of RILD. Our study shows that pulmonary EC loss already occurs at 8 hours after lung irradiation. Even earlier, as soon as 4 hours after irradiation, an increase of the pro-apoptotic gene SMDP-1 and apoptosis prone ECs occurred, indicating that apoptosis might be triggered very early after irradiation. SMDP-1 codes for acid sphingomyelinase which hydrolyses...
sphingomyelin to ceramide, an important second messenger of the apoptosis pathway. However, assessment of the level of ceramide in the irradiated lung tissue showed that the increased level of SMDP-1 did not correlate with increases of ceramide. An explanation for these findings may be the low level of apoptosis and/or the quick hydrolysis of ceramide to one of its metabolites (sphingosine, ceramide-1 phosphate, glucosyl ceramide).

Since our results showed that the amount of apoptotic ECs only reaches around 1.5% of the total amount of the pulmonary ECs, apoptosis seems not to be the only cause of EC loss. Hypothetically, apoptotic ECs may lead to changes in vascular integrity. As previously shown to occur hours to days after radiation in pulmonary ECs, the loss of endothelium integrity due to EC retraction results in an increased permeability to low molecular weight solutes. Early disruption of the endothelial lining with increased permeability and perivascular edema found in our and other studies may decrease the blood flow in the irradiated vasculature. As a compensatory effect, the pressure, blood flow and thereby shear stress would increase in the vasculature damaging more ECs in both the irradiated parts of the lung as in the non-irradiated parts. This may also explain the elevation of the pro-apoptotic gene SMDP-1 in the out-of-field lung tissue.

Moreover, radiation-induced EC damage/dysfunction may lead to changes in the pulmonary vascular pressure since ECs modulate the vascular tone by the release of constricting and relaxing factors, e.g. endothelin-1, nitric oxide, prostacyclin, and putative endothelium-derived hyperpolarizing factors. An imbalance between these factors due to EC damage/dysfunction may contribute to alterations in the vascular tone. This has been demonstrated in cardiopulmonary diseases, such as primary and secondary pulmonary hypertension, chronic obstructive lung disease, cardiopulmonary bypass, and congestive heart failure. Interestingly, an early increase of the pulmonary vascular pressure has been observed previously in sheep at 2 hours after thoracic irradiation possibly due to before mentioned processes.

The increased pressure and shear stress may induce a second phase of EC loss as observed at 2 weeks. Between these 2 phases a repair of the EC lining may have occurred e.g. by endogenous endothelial progenitor cells explaining the continuous layer between 8 hours and 2 weeks. The further loss of ECs due to pressure changes, blood flow and thereby shear stress may lead to vascular remodelling, increased pulmonary pressure and subsequent right ventricle hypertrophy. This very closely resembles pulmonary arterial hypertension (PAH), a disease where the elevated pulmonary vascular resistance leads to high right ventricle systolic pressure and subsequent right ventricular hypertrophy ultimately causing heart failure. It is known that EC loss and dysfunction plays a central role in the initiation of the pathogenesis of vascular remodelling and progression of PAH. Different factors are described leading to EC loss/dysfunction in this disease e.g. reactive oxygen species (ROS), genetic mutations, shear stress, autoimmunity, defects in Ang 1- Tie 2- BMPR 1a-BMPR 2 signalling pathway and/or inflammation. Production of ROS and inflammation are also known pathologies after irradiation. EC dysfunction may then lead to EC proliferation, increased coagulability, production of cytokines/growth factors, a humeral imbalance and/or exposure of subendothelium to soluble growth factors. EC proliferation and increased coagulability may lead to vascular luminal obliteration. Altogether these phenomena lead to vasoconstriction, smooth muscle and adventitial hypertrophy and proliferation, the features of vascular remodelling and PH.
Acute vascular remodelling and increased mean pulmonary artery pressure have been observed after thoracic irradiation in rats, dogs and sheep. Moreover, patients undergoing lung irradiation that are suffering from pre-existing pulmonary vascular disease, manifesting as sub-clinical increases in pulmonary arterial pressure, are known to have an increased risk for developing RILD. Altering apoptosis and proliferation rates of EC and smooth muscle cells may affect vascular remodelling, resulting in changes in blood flow that lead to shear stress, perpetuating the problem.

Our preliminary results indicate EPC transplantation after thoracic irradiation may decrease RILD. Bone marrow-derived EPCs were shown to have a positive effect on pulmonary vascular hemodynamics in PAH. Therefore, the cascade of vascular remodelling and pulmonary hypertension may be reduced or could be compensated by EC replacement post-thoracic irradiation. Additionally, previous studies showed that EC formation in the irradiated lung could also be induced by mobilization of bone marrow derived EPCs.

Further optimization of the transplantation may lead to better results. Next to that, two distinct subsets of EPCs could be transplanted: the early outgrowth EPCs and the late outgrowth EPCs, of which the former mostly promotes angiogenesis through paracrine effects and the latter forms vessels. Furthermore, a different amount of transplanted cells, different time points of transplantation or a more selected group of cells could lead to a better response.

Currently, there is no consensus on the initial lesion in the radiation response of the lung, but based on more recent studies, it results from disruption of the balance and communication between various cell populations of the pulmonary parenchyma. As such, independently or secondary to the vascular remodelling all other cell types are also affected by irradiation. This makes it conceivable that a combination of transplanted cells could be capable of regenerating the tissue after a radiation insult. Successful recovery of RILD will open avenues for further clinical use, improvement of quality of life, allowing for tumour-dose escalation and potentially increasing survival.

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have an increased risk for developing RILD 41. Altering apoptosis and proliferation rates of disease, manifesting as sub-clinical increases in pulmonary arterial pressure, are known to effects and the latter forms vessels 47,48. Furthermore, a different amount of transplanted distinct subsets of EPCs could be transplanted: the early outgrowth EPCs and the late.

Bone marrow-derived EPCs were shown to have a positive effect on pulmonary EC and smooth muscle cells may affect vascular remodelling, resulting in changes in blood flow. Our preliminary results indicate EPC transplantation after thoracic irradiation may decrease pulmonary hypertension may be reduced or could be compensated by EC replacement with potentially operable stage I non-small cell lung cancer.

EC and smooth muscle cells, different time points of transplantation or a more selected group of cells could lead to a better response. Additionally, previous studies showed that EC formation in the post-thoracic irradiation. Furthermore, a different amount of transplanted distinct subsets of EPCs could be transplanted: the early outgrowth EPCs and the late. Bone marrow-derived EPCs were shown to have a positive effect on pulmonary EC and smooth muscle cells may affect vascular remodelling, resulting in changes in blood flow. Our preliminary results indicate EPC transplantation after thoracic irradiation may decrease pulmonary hypertension may be reduced or could be compensated by EC replacement with potentially operable stage I non-small cell lung cancer.

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50% lung lateral - 22 Gy

Supplementary figure S1: Overview of lung parts collected for in-field and out-of-field analysis after 50% irradiation.