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Mitochondria: a matter of life and death after irradiation?

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List of abbreviations

53BP1	p53 binding protein 1	MTT	methyl-thiazolyl-tetrazolium
ADP	adenosine diphosphate	NAC	N-acetylcysteine
ApoE^{-/-}	apolipoprotein E knock-out	NaCl	sodium chloride
ATM	ataxia telangiectasia mutated	NAD(P)H	nicotinamide adenine dinucleotide (phosphate) hydrate
ATP	adenosine triphosphate	ND	NADH-ubiquinone oxidoreductase
BCA	bicinchoninic acid assay	NEAA	non-essential amino acids
BSA	bovine serum albumin	NFDM	non-fat dry milk
cDNA	complementary DNA	NHEJ	non-homologous end-joining
CM-	chloromethyl derivat of 2',7'-	O₂[•]	superoxide
H₂DCFDA	dichlorodihydrofluorescein	OH[•]	hydroxyl radical
COX	cytochrome C oxidase	ORI	origin of replication
cPPT	central polypurine tract	PBS	phosphate buffered saline
CT	computerized tomography	Pen/strep	penicillin/streptomycin
CuZnSOD	copper zinc superoxide dismutase	PFA	paraformaldehyde
CVD	cardiovascular disease	PIG	pre-immunized goat serum
DAPI	4',6-diamidino-2-phenylindole	PPARγ	Peroxisome proliferator-activated receptor γ
Dil-Ac-LDL	Dil-labeled acetylated LDL	Puro	puromycin
DMEM	Dulbecco's modified Eagle medium	PVDF	polyvinylidene difluoride
DSB	double strand breaks	qPCR	quantitative polymerase chain reaction
ECL	enhanced chemiluminescence	ROS	reactive oxygen species
EF1	elongation factor 1	ROSI	rosiglitazone
EtBr	ethidium bromide	RSV	respiratory syncytial virus
ETC	electron transport chain	SEM	standard error of mean
FBS	fetal bovine serum	shRNA	short hairpin RNA
Gy	gray	suCMV	super strong cytomegalovirus
H₂O₂	hydrogen peroxide	Sv	sievert
HBSS	Hank's balanced salt solution	SV40	simian virus 40
HCAEC	human coronary artery endothelial cells	Tamra	6-carboxytetramethylrhodamine
HEK	human embryonic kidney	tBHP	tert-butyl hydroperoxide
HIV-1	human immunodeficiency virus 1	TBST	tris-buffered saline with tween20
HR	homologous recombination	Tet	tetrachloro-6-carboxy-fluorescein
HRP	horseradish peroxidase	TFAM	mitochondrial transcription factor A
HSP	heavy strand promotor	TICAE	telomerase immortalized human coronary artery endothelial cells
ICRP	International Commission on Radiological Protection	TMRM	tetramethylrhodamine methyl ester
LDL	low density lipoproteins	TNB	tris-NaCl-blocking buffer
LSP	light strand promotor	tRNA	transfer RNA
LTR	long terminal repeat	TU	transducing units
MDC1	mediator of DNA damage checkpoint protein 1	VE	vascular endothelial
MgCl₂	magnesium chloride	VSV-G	Vesicular stomatitis virus glycoprotein
Mito-GFP	mitochondrial green fluorescent protein	WPRE	woodhuck hepatitis post-transcriptional regulatory element
MnSOD	manganese superoxide dismutase	W_R	radiation weighting factor
MOI	multiplicity of infection	W_t	tissue weighting factor
MRN	Mre11, Rad52 and Nbs1	γH2AX	phosphorylated histone H2A variant X
mtDNA	mitochondrial DNA		

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Samenvatting

Recente epidemiologische studies hebben aangetoond dat blootstelling aan ioniserende straling een risicofactor kan zijn voor de ontwikkeling van atherosclerose. Endotheelcellen, die de binnenkant van het hart en de bloedvaten bekleden, worden beschouwd als het belangrijkste doelwit in stralingsgeïnduceerde cardiovasculaire schade. Tot op heden, is het nog niet duidelijk welke mechanismen verantwoordelijk zijn voor het effect van straling op endotheelcellen. Het is geweten dat atherosclerose gekenmerkt wordt door oxidatieve stress, wat kan leiden tot DNA-schade, inflammatie, cel senescentie en celdood. Onze hypothese is dat mitochondriën de stralingsgevoeligheid van endotheelcellen kan vergroten door meer reactieve zuurstofsoorten (ROS) te produceren na blootstelling aan X-stralen, wat resulteert in een verhoogde kans op DNA-schade.

Menselijke telomerase geïmmortalizeerde endotheelcellen van de coronaire bloedvaten, of kortweg TICAE-cellen, werden gemodificeerd met lentivirale transductie of farmacologische additie. Voor het laatstgenoemde werden twee geneesmiddelen, namelijk ethidium bromide en rosiglitazone, toegevoegd aan de TICAE-cellen om mitochondriale transcriptie, respectievelijk, te verminderen of te vermeerderen. Na lentivirale transductie, waren de TICAE-cellen niet meer levensvatbaar genoeg om in cultuur te houden. Hierdoor werden enkel de farmacologisch gemodificeerde TICAE-cellen verder gekarakteriseerd voor mitochondriale DNA (mtDNA) inhoud, mitochondriale proteïne hoeveelheid, metabolische toestand, NAD(P)H reductieve capaciteit en functionaliteit om te valideren of modificatie succesvol was. Ethidium bromide zorgde ervoor dat het mtDNA vrijwel volledig geëlimineerd werd, wat resulteerde in een verminderde genexpressie van de elektronen transport keten. Het effect van rosiglitazone op de mtDNA inhoud werd niet volledig opgehelderd. Toch waren de genen die coderen voor de elektronen transport keten in de rosiglitazone gemodificeerde cellen aangetast. Na blootstelling aan X-stralen werd de hoeveelheid cellulaire en mitochondriale ROS bepaald samen met de mitochondriale membraanpotentiaal, het ATP-gehalte en de DNA-dubbelstrengbreuken (DSB's). In de ethidium bromide gemodificeerde TICAE-cellen, gekenmerkt door een verlaagde mitochondriale membraanpotentiaal, werd minder ROS geproduceerd na blootstelling aan hoge dosis X-stralen. Bovendien waren de hoeveelheid DSB's ook sterk gedaald. Voor de rosiglitazone gewijzigde TICAE-cellen kon een gewijzigde elektronen transport keten niet bevestigd worden. Maar ook was, na een X-stralen blootstelling van 2 Gy, de cellulaire ROS hoeveelheid niet verlaagd.

We kunnen daarom suggereren dat mitochondriën een effect hebben op de stralingsgevoeligheid van endotheelcellen. Het onderliggende mechanisme werd echter niet volledig opgehelderd. Een gewijzigde elektronen transport keten zou verantwoordelijk kunnen zijn voor de verminderde productie van ROS, wat uiteindelijk zal leiden tot een verlaagde hoeveelheid DSB's. We kunnen concluderen dat verder onderzoek nodig is. Het vinden van een mechanisme om uit te leggen op welke manier endotheelcellen reageren op ioniserende straling is zeer relevant voor het verbeteren van de stralingsbescherming, maar ook voor het betere inschatten van het effect van radiotherapie. Daarom heeft dit onderzoek een belangrijke maatschappelijke en economische impact.

Summary

Recent epidemiological studies suggest ionizing radiation exposure as a risk factor of atherosclerosis. The key targets in radiation-induced cardiovascular damage are considered to be the endothelial cells, lining the cardiovascular system. To date, the mechanisms that clarify the endothelial cell response towards ionizing radiation are not fully identified. In general, it is known that atherosclerosis is characterized by oxidative stress leading to DNA damage, inflammation, cell senescence and cell death. We hypothesized that mitochondria drive the radiosensitivity of endothelial cells due to increased production of reactive oxygen species (ROS) after X-ray exposure, resulting in a higher risk of DNA damage.

Telomerase immortalized human coronary artery endothelial (TICAE) cells were modified with lentiviral transduction or pharmacological incubation. In the latter, ethidium bromide or rosiglitazone were supposed to, respectively, diminish or enhance mitochondrial transcription. After lentiviral transduction, the TICAE cells were not viable enough to remain in culture. Therefore, only the pharmacologic incubated TICAE cells were characterized for mitochondrial DNA (mtDNA) content, mitochondrial protein levels, metabolic state, NAD(P)H reductive capacity and functionality to validate if pharmacological incubation succeeded. Ethidium bromide almost completely eliminated mtDNA content, which resulted in a diminished gene expression of the electron transport chain. The effect of rosiglitazone on mtDNA content was not completely elucidated. However, the genes encoding for the electron transport chain of the rosiglitazone modified TICAE cells were also impaired. After X-ray exposure, the amount of cellular and mitochondrial ROS was determined together with the mitochondrial membrane potential, the ATP levels and the DNA double strand breaks (DSBs). It was suggested that due to the impaired oxidative phosphorylation, confirmed by a lowered mitochondrial membrane potential, less ROS was produced after high dose ionizing radiation exposure in the ethidium bromide modified TICAE cells, compared to the control TICAE cells. Furthermore, after 2 Gy X-ray dose a lowered amount of DSBs were observed in the ethidium bromide modified TICAE cells, which could result from the lowered ROS production. For the rosiglitazone modified TICAE cells, an impairment of the oxidative phosphorylation could not be confirmed. As such, no reduction in the cellular ROS levels was observed after 2 Gy X-ray dose.

Thus, it was suggested that mitochondria actually have an effect on the radiosensitivity of endothelial cells. However, the underlying mechanism is not completely elucidated. We suggested that an impairment of the oxidative phosphorylation is involved in reducing the ROS production, which will eventually result in lowered DSBs. However, further research is necessary. This experimental work has important societal and economic implications. Finding a mechanism to explain the endothelial cells response towards ionizing radiation is highly relevant to further improve the current radiation protection system and to develop future countermeasures for e.g. radiotherapy patients.

1. Introduction

The predominant cause of death worldwide is cardiovascular disease (CVD). In 2012, CVD accounted for 17.5 million deaths, which is about 31% of all the deaths that occurred worldwide (3, 4). The term CVD covers diseases that involve the heart, such as cardiomyopathy and heart failure, or the blood vessels. In the latter blood supply to the heart, brain and extremities is impaired, respectively leading to coronary artery disease, carotid artery disease and peripheral arterial disease. In the majority of the cases, the vascular diseases are caused by atherosclerosis which is the narrowing or complete obstruction of blood vessels by plaque formation. Hypertension, high blood cholesterol level, smoking, diabetes, gender, age, genetic predisposition and dietary factors are the best-known risk factors contributing to the development of atherosclerosis (5-7). However, epidemiological evidence demonstrates that exposure to ionizing radiation is also associated with an elevated risk of CVD (7).

1.1. Ionizing radiation as a risk factor for cardiovascular disease

For a long time, it was assumed that the heart and blood vessels were radioresistant because of their limited proliferative capacity (8, 9). However, from the late 1960s onwards epidemiological evidence demonstrates that exposure to ionizing radiation due to accidents (e.g. the atomic bomb), occupation, diagnostics or radiotherapy is associated with an elevated risk of CVD (7, 10). The disease progression of CVD after ionizing radiation exposure is very slow. It can take 10 to 15 years before radiation-related cardiovascular morbidity or mortality is observed. Therefore, the radiation sensitivity of the heart and blood vessels has been underestimated (8).

1.1.1. What is ionizing radiation?

From natural to man-made sources, ionizing radiation is a widespread feature for life on earth. Ionizing radiation can be defined as an electromagnetic wave (e.g. γ - and X-rays) or particle (e.g. α - and β -particles, protons and neutrons) with enough energy to remove tightly bound electrons from an atom, causing the atom to become ionized. The naturally occurring decay of radioactive isotopes results in the release of γ -rays, α - or β -particles, which can penetrate or pass through the human body (11). Humans are continuously exposed to naturally occurring ionizing radiation by cosmic rays or other natural background radiation. In rare cases, nuclear accidents can lead to the exposure of humans to man-made radioactivity. During the nuclear accident in Chernobyl, radioactive elements (iodine, cesium and strontium) formed by the nuclear energy production were released, which emitted β -particles and γ -rays (12). Furthermore, in the formation of nuclear energy, neutrons with no electrical charge are emitted as a byproduct (11). These neutrons, together with γ -rays, are released during an atomic bomb accident (12). The electromagnetic γ -rays are identical in properties with X-rays, however they differ in their origin. In comparison to γ -rays, X-rays are artificially made by an X-ray generator. When high energy electrons interact with a specific material such as gold or tungsten, the metal atom loses electrons from the inner shell. An electron from a higher energy level, replaces the loss at the lower energy level while emitting X-rays (11). X-rays are extensively used for medical applications such as radiotherapy and diagnostic imaging techniques (X-ray radiography and X-ray computerized tomographic (CT) scan) (12). Table 1 indicates the effective doses of some ionizing radiation sources (13-16).

Table 1: The effective doses (mSv) of some ionizing radiation sources.

Source	Effective dose (mSv)	Time span of natural background exposure (2.5 mSv/y) to reach this dose
Commercial flight (per hour)	0.001 – 0.010	9 minutes – 1.5 days (depending on height)
Regular dental X-ray	0.09	13 days
Chest x-ray	0.1	15 days
Mammography	3	15 months
CT-scan head	5 – 50	2 – 20 years
Annual occupational dose limit	20	8 years

The average dose of ionizing radiation absorbed by a tissue or organ is quantified by the gray (Gy), which is the most common unit of measurement. The Gy unit is defined as 1 joule of initial energy absorbed per kilogram of tissue. Different types of ionizing radiation do not exert the same biological effect. Therefore, ionizing radiation can also be expressed in Sievert (Sv), which measures the equivalent dose to tissues or organs (17). To calculate the equivalent dose, the absorbed dose is multiplied by the radiation weighting factor (W_R) which is dependent on the type of ionizing radiation. For X- or γ -rays, 1 Gy is equivalent to 1 Sv. However for higher energy radiations, the conversion is more complex (e.g. 1 Gy of α -particles equals 20 Sv and 1 Gy of neutrons equals 10 Sv) (11). This also implies that α -particles and neutrons have a greater biological effectiveness than X- or γ -rays (12). Furthermore, not every tissue or organ has the same sensitivity to ionizing radiation. Therefore, the tissue weighting factor (W_t) is established to determine the effective dose for the whole human body. The effective dose is the sum of the equivalent doses to all tissues and organs multiplied by their W_t (18).

In this master's thesis, the focus will be on exploring the effect of X-rays on endothelial cells. Therefore, the W_t is not applicable. A distinction will be made between low, moderate and high dose ionizing radiation. Based on literature, following cut-off values were defined: < 0.5 Gy is low dose, $0.5 \text{ Gy} \leq 1 \text{ Gy}$ is medium dose and $> 1 \text{ Gy}$ is high dose ionizing radiation (7).

1.1.2. Epidemiological evidence

Several epidemiological studies have already demonstrated a relationship between ionizing radiation from medical, accidental and occupational exposure and late occurring CVD (7-9, 19). In the late 1960s, Fajardo and Stewart were the first to describe myocardial infarctions and premature atherosclerosis after treatment with radiotherapy (10). After radiotherapy for the treatment of Hodgkin's disease, the risk of developing CVD, especially myocardial infarction, increased with time (Figure 1.1) (20). In 2013, a study of breast cancer patients treated with radiotherapy indicated that per gray increase in ionizing radiation exposure the excess risk of major coronary events increased with 7.4% (Figure 1.2). Radiotherapy is commonly administered in 2 Gy dose-fractions of X-rays in order for the tumor to receive an accumulated dose of ≥ 40 Gy. However, the heart will also receive part of the ionizing radiation dose. Darby *et al.* estimated that the overall mean dose to the heart was 2.9 Gy for right- and 6.6 Gy for left-sided breast cancer. Coherently, the rates for coronary events were significantly higher for women with left-sided breast cancer (21). A larger scale epidemiological study of 23 500 women receiving radiotherapy showed similar conclusions. Women

treated for cancer at the left breast, had 44% more risk of death from heart disease (22). In a study of peptic ulcer patients treated with radiotherapy, a 24% increased risk of coronary heart disease mortality due to ionizing radiation exposure was observed. The part of the heart directly in the radiation field (5%) received a dose ranging between 7.6 and 18.4 Gy. Scattered radiation with doses of 1.6-3.9 Gy was received by the rest of the heart (95%). It remained unclear whether the high ionizing radiation doses or the smaller doses were responsible for the described health effects (23). Several studies have further indicated that also lower doses of ionizing radiation exposure were associated with possible CVD risks (9, 23). A study of Darby *et al.* (1987) showed an increased risk on CVD in patients, receiving X-ray doses ranging from 1.87 to 2.04 Gy (24). A 14% increase in CVD risk per gray in γ -ray and neutron exposure, was demonstrated by a study of the atomic bomb survivors in Hiroshima and Nagasaki (2010). The increase in risk already started from doses as low as 0.5 Gy to 3 Gy (Figure 1.3). However, no statistically significant result was seen for ionizing radiation doses lower than 0.5 Gy (25, 26).

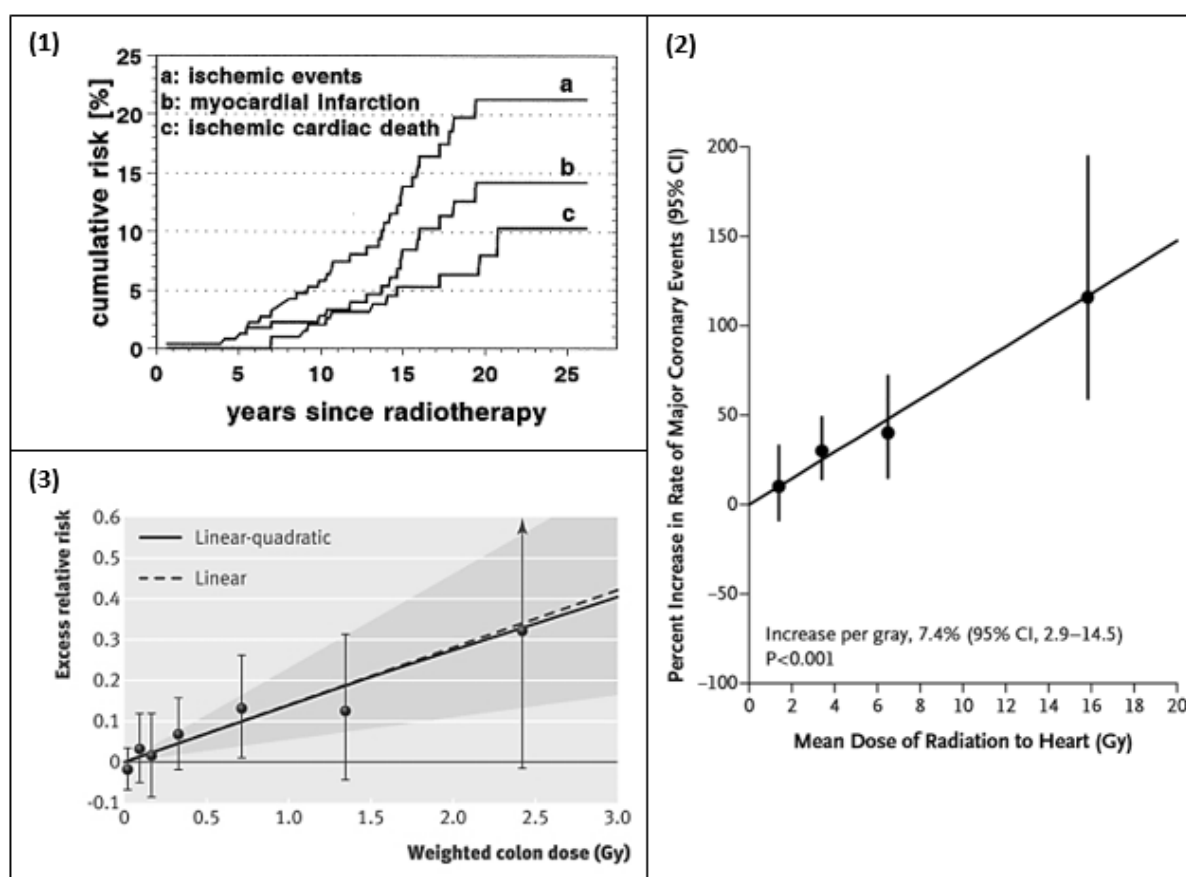


Figure 1: Increase in cardiovascular disease risk according to (1) Reinders *et al.*, (2) Darby *et al.* and Shimizu *et al.* (3). Reinders *et al.* (1999) followed 258 Hodgkin's disease patients receiving a mean total X-ray dose of 36.6 Gy. An increased risk of ischemic events (from 6.4% at 10 years to 21.2% at 20 years), myocardial infarction (3.4% at 10 years to 14.2% at 20 years) and ischemic cardiac death (from 2.6% at 10 years to 10.2% at 20 years) was indicated. Darby *et al.* (2013) included 2168 breast cancer patients of which 963 had major coronary events. The IR dose was different for right and left breast cancer, respectively 2.9 and 6.6 Gy (21). The life span study consisted out of 86 611 atomic bomb survivors who were exposed to doses ranging from 0 to 3 Gy. From 0.5 Gy until 3 Gy, a 14% increase in CVD risk per gray increase in ionizing radiation exposure was seen. Lower than 0.5 Gy, the increase was not statistically significant (25, 26).

An interesting population cohort to study the effect of low dose ionizing radiation exposure on the cardiovascular system, are the radiation workers. The national dose registry of Canada showed an increased CVD risk due to occupational ionizing radiation exposure with respectively 2.3% and 12.1%, for males and females per 10 mSv of ionizing radiation exposure (27). Furthermore, Hauptmann *et al.* (2003) indicated that radiologic technologists, exposed to a chronic X-ray dose of 0.3 Sv per year, had an increased CVD mortality risk (28). A study by Ivanov *et al.* (2006) of 61 017 Chernobyl emergency workers, exposed to a cumulative dose between 0.15 Gy and 0.25 Gy, showed an excess risk of ischemic heart disease morbidity (29). Although the significant correlation between low dose ionizing radiation and CVD described in aforementioned articles, Vrijheid *et al.* (2007) found that the increasing trend for circulatory disease mortality with dose was not significant. Vrijheid *et al.* (2007) performed the largest epidemiological study of nuclear industry workers, consisting out of 275 000 participants from 15 countries who received an average cumulative dose of 20.7 mSv (30). Azizova *et al.* (2015) investigated the Mayak nuclear facility workers, receiving a mean cumulative dose of 0.78 Gy. A significant increase in CVD incidence was seen, however the mortality rate was not significantly increased after low dose ionizing radiation exposure (31). Therefore, for doses lower than 0.5 Gy a positive association between dose and excess risk of CVD can only be suggested.

Furthermore, other risk factors of CVD, such as gender, age, genetic predisposition, dietary factors, hypertension, high blood cholesterol level, smoking and diabetes may have a confounding effect on the study results. Therefore, the data should be adjusted for these confounding factors in order to find a correct association (8, 25). The studies performed by Darby *et al.* (2013) (21), Shimizu *et al.* (2010) (25), Ozasa *et al.* (2011) (26), Carr *et al.* (2005) (23), Reinders *et al.* (1999) (20) and Azizova *et al.* (2015) (31) have corrected their results for the confounding factors. Thus, it can be assured that the association between ionizing radiation dose and CVD risk is no artifact from confounding factors. However, the results of Ashmore *et al.* (1998) (27), Ivanov *et al.* (2006) (29) and Vrijheid *et al.* (2007) (30) need to be interpreted with caution because no correction for confounding factors was performed.

1.1.3. Experimental animal studies

With the help of animal models, the development and progression of atherosclerosis after ionizing radiation exposure is investigated. Stewart *et al.* (2006) explored the plaque formation in apolipoprotein E knock-out (ApoE^{-/-}) mice, which are more prone to develop atherosclerotic plaques. After exposure to 14 Gy of ionizing radiation, no increased amount of plaques was observed in the irradiated carotid arteries. Still, the plaques that had developed after irradiation showed more inflammatory characteristics; such as abundant macrophages, low collagen content and intraplaque hemorrhage resulting in a plaque more prone to rupture. Moreover, atypically swollen endothelial cells were present after irradiation, which indicates a changed endothelial cell function after ionizing radiation exposure (32). Inflammatory plaque development was also observed after 20 times exposure to a 2 Gy X-ray dose, which is similar to the irradiation scheme used for radiotherapy (33). Furthermore, increased atherogenesis was seen in ApoE^{-/-} mice, that were chronically irradiated for 300 days until an accumulated dose of 0.3 Gy or 6 Gy was achieved (34).

Thus, it is evidenced by epidemiological and animal studies that ionizing radiation exposure has a negative impact on the cardiovascular system. However, it is difficult to draw a conclusion from the epidemiological data concerning doses lower than 0.5 Gy. In order to be better aware of the possible

radiation-induced cardiovascular risks, it is crucial to integrate biological and molecular knowledge from *in vitro* and *in vivo* studies into epidemiological studies (2, 35).

1.2. Radiation-induced cardiovascular disease

The International Commission on Radiological Protection (ICRP) estimated that 1 % of the individuals exposed to a dose of 0.5 Sv will develop CVD more than 10 years after exposure. Due to improvements in radiotherapy and diagnostic imaging techniques, lower X-ray doses can be used. Therefore, it is expected that the risk of CVD is going to decline. However, the frequent occurrence of CVD without ionizing radiation exposure, the contribution of very common risk factors for CVD and the increasing use of ionizing radiation for diagnostic medical purposes contribute to the fact that radiation-induced CVD is still a major societal concern.

1.2.1. Atherosclerosis underlying cardiovascular disease

The key targets in CVD are believed to be the endothelial cells, lining the blood vessels (1, 36). To maintain a normal function of the vascular system, endothelial cells are continuously sensing and responding to changes in the extracellular environment. Dysfunction of the endothelium is important in the development and progression of CVD (37). Endothelial injury, for example induced after irradiation, can initiate a chronic inflammatory response in which leukocytes and monocytes adhere to the arterial wall. Due to increased permeability of the endothelium and the increased expression of adhesion molecules, the monocytes migrate from the blood into the subendothelial intima where a transformation to macrophages will occur. By engulfing low density lipoproteins (LDL), the macrophages will turn into foam cells. The lipid accumulation within macrophages causes the release of inflammatory cytokines, which leads to migration and proliferation of smooth muscle cells and deposition of extracellular matrix molecules (e.g. elastin, collagen and proteoglycans). By accumulation of the elastin, collagen and proteoglycans, a fibrous cap is formed. Lipids from the macrophages, foam cells and smooth muscle cells, will accumulate underneath the fibrous cap, where a lipid or necrotic core finds its origin. The lipid core covered with the fibrous cap, forms the atherosclerotic plaque. Three different clinical outcomes can emerge after atherosclerotic plaque formation. Firstly, progressive growth of the plaques can lead to chronic occlusion of the blood vessels, which will severely limit blood flow. Secondly, a vascular thrombus can be formed after plaque erosion or rupture, which will partially or completely obstruct blood vessels. This can lead to myocardial or cerebral infarction, better known as heart attack or stroke. Thirdly, the vessel wall underlying the atherosclerotic plaque can rupture, leading to blood-filled swelling of the vessel wall called aneurysm (5, 38, 39).

1.2.2. Cellular mechanism of radiation-induced atherosclerosis

Dysfunction of the endothelium after ionizing radiation exposure is suggested to result from induction of pro-inflammatory signaling, procoagulant alterations, oxidative stress and genetic instability (Figure 2). The damaged endothelium can eventually lead to premature vascular ageing, which will result in atherosclerotic plaque formation. Several studies have evidenced that high and low dose of ionizing radiation does not exert the same effects (9, 40-43). The effect of the two best-known mechanisms of ionizing radiation, namely oxidative stress and genetic instability on endothelial cell function is further elaborated in the following paragraphs.

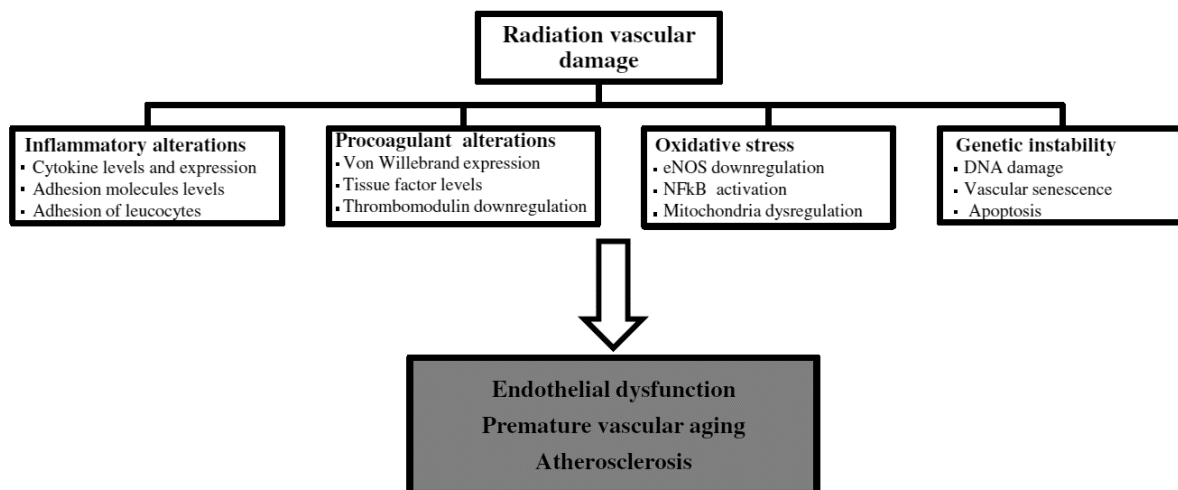


Figure 2: Schematic overview of the biological and molecular mechanisms in radiation-induced endothelial dysfunction (41). eNOS: endothelial NOS, NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells.

DNA damage, e.g. base modifications, DNA cross links, single strand breaks and double strand breaks (DSB), is the most important consequence of ionizing radiation (44). Rombouts *et al.* (2013) indicated a significant increase in DSBs in endothelial cell lines after ionizing radiation exposure down to 0.05 Gy (36). To repair DNA damage, cell cycle check points are activated and a DNA damage response is initiated. The cell cycle check points at G₁ or G₂/M phase can delay or stop cell cycle progression in order for the cells to repair the DNA damage. The most lethal form of radiation-induced DNA damage are the DSBs. A signal transduction pathway, involving ataxia telangiectasia mutated (ATM) kinase, is activated at the checkpoints as a DSB detector. The ATM kinase will phosphorylate the histone H2A variant X (H2AX), which results in the formation of γH2AX foci (44). The presence of the γH2AX foci will further recruit DNA repair proteins, such as the mediator of DNA damage checkpoint protein 1 (MDC1) and the p53 binding protein 1 (53BP1), to the DSBs (Supplementary figure 1) (45). The MDC1 and 53BP1 protein play a role in selecting one of the repair pathways (46). The two major repair pathways; non-homologous end-joining (NHEJ) and homologous recombination (HR) are responsible for repair of the DSBs. While NHEJ can occur throughout the entire cell cycle, HR only occurs at S and early G₂-phase. Even though the DNA damage response is well-organized, wrongly or unrepaired DNA damage can sometimes emerge. Wrongly repaired DNA damage will cause genomic instability, which will eventually lead to cytotoxic, mutagenic and carcinogenic effects. When the DNA damage remains unrepaired, cell death will pursue by induction of apoptosis or the cell proliferation is halted by triggering cell senescence (47). Rombouts *et al.* (2013) demonstrated that after ionizing radiation exposure the amount of apoptotic cells increased with dose, even after doses as low as 0.1 or 0.5 Gy depending on endothelial cell type (36). However, Pluder *et al.* (2011) indicated an increase in apoptotic endothelial cells only after exposure to 5 Gy, not after 0.2 Gy (48). So, after low dose ionizing radiation exposure the apoptotic cell death response is not fully elucidated. Furthermore, the induction of endothelial cell senescence after high dose ionizing radiation exposure is confirmed by several *in vitro* studies (49-51).

Besides directly ionizing the cellular biomolecules, ionizing radiation can also exert a damaging effect on the DNA, RNA, lipid and protein content by formation of reactive oxygen species (ROS) originating

from ionization of the cellular water content (indirect effect). Examples of the formed ROS are hydrogen peroxide (H_2O_2), superoxide ($\text{O}_2^\bullet$) and hydroxyl radical ($\text{OH}^\bullet$). An imbalance between ROS production and the antioxidant (e.g. catalase) activity leading to ROS scavenging, is defined as oxidative stress (52). Except for the more stable H_2O_2 , most ROS disappears within 10^{-3} seconds after formation (53). However, an increase in endogenous cellular ROS production was observed after longer time periods following irradiation exposure. This indicated that ionizing radiation stimulates the production of secondary ROS derived from sources within the cell (54). *In this master's thesis, it is elucidated if mitochondria are the endogenous source inducing ROS production in endothelial cells after ionizing radiation exposure.*

1.3. Mitochondria as key players in radiation-induced atherosclerosis

Mitochondria are crucial for all eukaryotic cells, because they supply most of the cellular energy by oxidative phosphorylation (Supplementary figure 2). The four electron transfer complexes (I until IV) of the electron transport chain produce a proton gradient over the inner mitochondrial membrane by several oxidation-reduction reactions. Complex V, an adenosine triphosphate (ATP) synthase, uses this proton gradient to produce the necessary ATP (55). The mitochondrial genome encodes for 13 polypeptides of the electron transport chain (NADH dehydrogenase, cytochrome c oxidase, ATPase and cytochrome b subunits), 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (Supplementary figure 3) (56). The remaining 79 polypeptides of the electron transport chain are nuclear-encoded (57). The extra-nuclear DNA of the mitochondria is organized in circular double stranded manner, consisting out of 16 600 base pairs and is present in the cells at 100 - 10 000 copies per cell depending on the cell type. It is evidenced that endothelial cells have a lower mitochondrial content, indicating that mitochondrial-dependent oxidative phosphorylation is not such an important energy source for endothelial cells. Besides functioning as the powerhouses of the cell, mitochondria also have a role in the generation of ROS, the regulation of calcium and the activation of cell death (52).

Under normal circumstances, a baseline level of mtROS is produced as a byproduct of the oxidative phosphorylation (58). These baseline levels of ROS play important physiological roles; the best-known are the defense against infectious pathogens and signal transduction for cellular proliferation, migration and survival. In this way, mtROS keeps the cells healthy and in check. However, during conditions of homeostatic disturbance oxidative stress can arise (52). Such a condition of homeostatic imbalance can arise when cells are exposed to ionizing radiation. Through the generation of radicals, ROS can induce DNA damage which can lead to accelerated apoptosis and senescence of endothelial cells. Eventually, this damaged endothelium will lead to the formation of atherosclerotic plaques (59).

1.4. Scientific or societal relevance

Finding a mechanism to explain the effect of irradiation on endothelial cell lines is of high relevance (1). In order to be better aware of the possible radiation-induced cardiovascular risks, it is crucial to integrate biological knowledge into epidemiological studies. Both epidemiological and biological knowledge are necessary to better understand the possible risks and effects of radiation on the cardiovascular system (2). This better understanding can improve the current radiation protection standards, which aim to protect individuals from the deterministic effects of radiation and minimize

the risk of developing radiation-induced diseases (60). Furthermore, this improved understanding can help doctors to better inform and evaluate patients who need radiotherapy of the chest region.

When the mechanism behind the radiosensitivity of endothelial cells is known, diagnostic parameters can be developed to rapidly identify radiation-induced CVD. Moreover, research can be performed to develop radioprotective agents, focusing on the mitochondrial pathways. Breast cancer or lymphoma patients treated with radiotherapy may benefit from these radioprotective agents causing less harm to the cardiovascular system (61).

To conclude on the long term, this research may contribute to implications for public health and society. Besides better defined radiation protection standards, public health can also be improved with efficient diagnostic parameters and radioprotective agents. In this way more radiation-induced CVD can be avoided, which decreases the expenses of society for CVD treatment and improves life expectancies and quality of life.

1.5. The research plan

The main goal of this master's thesis was to verify if mitochondria influence endothelial cell dysfunction after ionizing radiation exposure. We hypothesized that mitochondria drive the radiosensitivity of endothelial cells by increasing the production of ROS after exposure to X-rays, resulting in a higher amount of DNA damage.

To verify the hypothesis, three objectives were defined. In the first objective, TICAIE cells were modified to obtain cell lines with either an excess or a limited amount of mitochondria. The modification was performed with lentiviral transfection and pharmacological incubation. The lentiviral constructs either contained a complementary DNA (cDNA) or multiple short hairpin RNA (shRNA) sequences of the mitochondrial transcription factor A (TFAM) gene, a key activator of mitochondrial transcription. By incorporation of the TFAM gene in the cellular genome, the cDNA lentiviral particles were expected to increase TFAM protein expression, resulting in increased activation of the mitochondrial transcription. Contrary to the cDNA sequence, the incorporation of the shRNA lentiviral sequence in the TICAIE cells were expected to knock-down the TFAM gene, leading to subsequent suppression of the mitochondrial transcription. Eventually, this was expected to result in TICAIE cells depleted of mtDNA, called p^0 -cells (62, 63). To easily detect the in- or decrease in mitochondrial content, the TICAIE cells were first transfected with a lentiviral particle containing mitochondrial green fluorescent protein (mito-GFP).

For the pharmacological approach, the TICAIE cells were incubated with the drug, rosiglitazone, which is a peroxisome proliferator-activated receptor gamma (PPAR γ)-agonist. Several studies with adipocytes have indicated that treatment with rosiglitazone increases mitochondrial biogenesis, resulting in a higher mitochondrial mass (64-66). Until 2010, rosiglitazone was used by diabetes type II patients as an insulin sensitizer. However, due to associated cardiac toxicity, the drug was withdrawn from the market. Furthermore, p^0 -cells were achieved by cultivation with ethidium bromide, an intercalating agent especially toxic to the mitochondria leading to decreased mtDNA

content (55, 67-69). Moreover, ethidium bromide is a well-known inhibitor of mtDNA replication (69).

The effect of ethidium bromide and rosiglitazone is already verified for multiple cell lines, but until now it was not tested on endothelial cells. Therefore, a second objective was defined to characterize the endothelial cell lines exposed to ethidium bromide and rosiglitazone. By assessing mitochondrial DNA content, TFAM protein expression and metabolic state, it could be validated if the mitochondrial content was modified. Moreover, the nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) reductive capacity, the amount of cell death, the doubling time and endothelial cell function was examined.

A third objective was to determine the radiosensitivity of the modified TICAE cell lines after X-ray exposure. The cellular and mitochondrial ROS levels, the mitochondrial membrane potential and the ATP levels were tested. Finally, the DNA DSB after X-ray exposure were examined to verify the amount of damage exerted after ionizing radiation exposure.

2. Material and methods

2.1. Production of modified endothelial cell lines

2.1.1. Cell culture

As part of the ProCardio FP7 study human coronary artery endothelial cells (HCAEC) (ECACC, Salisbury, UK), telomerase immortalized by Lowe et al (70), were distributed to the SCK•CEN Radiobiology Unit laboratory. In this master's thesis, the immortalized HCAEC cells are referred to as telomerase immortalized human coronary artery endothelial (TICAE) cells. MesoEndo cell growth medium (Sigma-Aldrich Co. LLC, Diegem, Belgium) was used to cultivate the TICAE cells. According to a closed recipe, the MesoEndo was supplemented with fetal bovine serum (FBS), growth factors, trace elements and antibiotics. Human embryonic kidney (HEK) 293T/17 cells (ATCC, Molsheim Cedex, France) were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco™ Thermo Fisher Scientific, Aalst, Belgium) supplemented with 10% FBS (Gibco™ Thermo Fisher Scientific, Aalst, Belgium), 1% penicillin/streptomycin (pen/strep), 1% non-essential amino acids (NEAA) (Gibco™ Thermo Fisher Scientific, Aalst, Belgium), 1% L-glutamine (Sigma-Aldrich Co. LLC, Diegem, Belgium) and 0.1% sodium pyruvate (Gibco™ Thermo Fisher Scientific, Aalst, Belgium). The TICAE cells were kept at 37°C and 5% CO₂ in a humidified incubator and split every two-three days.

The MesoEndo medium used to cultivate the ethidium bromide modified TICAE cells, was supplemented with 50 µg/ml uridine (Applichem, Darmstadt, Germany), 2 mM L-glutamine, 100 µg/ml sodium pyruvate and 25 mM HEPES (Gibco™ Thermo Fisher Scientific, Aalst, Belgium). The MesoEndo medium used to cultivate the TICAE cells modified with TFAM cDNA lentiviral constructs, was supplemented with 50 µg/ml uridine, 2 mM L-glutamine, 100 µg/ml sodium pyruvate and 5 mM N-acetylcysteine (NAC) (Sigma-Aldrich Co. LLC, Diegem, Belgium).

2.1.2. Cultivation of TICAE cells with ethidium bromide or rosiglitazone

As a back-up plan for the lentiviral transduction of respectively TFAM cDNA and shRNA, TICAE cells were cultivated either with rosiglitazone (Sigma-Aldrich Co. LLC, Diegem, Belgium) or ethidium bromide (Sigma-Aldrich Co. LLC, Diegem, Belgium). A concentration of 50 µM rosiglitazone was continuously administered to the TICAE cells. Three different concentrations of ethidium bromide, 12.5, 25 and 50 ng/ml, were added to the growth medium of the TICAE cells during seven weeks of cell culture. After seven weeks, the ethidium bromide addition was stopped.

2.1.3. Lentiviral transduction with premade lentiviral particles

Mito-GFP lentiviral transduction

A pre-made LocLight™ sub-cellular labeling lentivirus, containing a green fluorescent protein sequence targeted to the mitochondria (mito-GFP) (LVP452-G) (AMS Biotechnology, Frankfurt, Germany), was added to the TICAE cells (Supplementary figure 4). At the time of transduction, the

TICAE cells were 50% confluent. Several multiplicities of infection¹ (MOIs) (1, 10, 12.5 and 20) were used. To enhance transduction efficiency, 60 ng/μl polybrene (Santa Cruz Biotechnology, Heidelberg, Germany) was added. The transduction rate was checked with fluorescence microscopy, 72 hours after transduction. The lentiviral particles contained a blasticidin selection marker. In order to retain correctly transduced cells and avoid false positives, the cells were exposed to 7.5 μg/ml blasticidin (Gibco™ Thermo Fisher Scientific, Aalst, Belgium).

TFAM shRNA lentiviral transduction

Ready to use TFAM shRNA lentiviral particles (sc-38053-V) (composition is confidential), consisting of three to five expression constructs encoding for target-specific shRNAs, were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The TICAE cells at a confluence of 50% were incubated with the lentiviral particles at a MOI of 50, together with 8 μg/ml polybrene. The positively transduced TICAE cells were selected by addition of 1 μg/ml puromycin (Santa Cruz Biotechnology, Heidelberg, Germany).

2.1.4. Lentiviral transduction with self-assembled lentiviral particles

Mito-GFP lentiviral transduction

The mito-GFP lentiviral particles were self-assembled with the LentiStarter 2.0 kit (System Biosciences, California, USA). The pCT-Mito-GFP-pCMV lentiviral construct (CYTO102-PA-1) (Supplementary figure 5) was mixed with pPACKH1-plasmid mix and PureFection reagent. Together with 8 μg/ml polybrene, the HEK 293T/17 cells (50% confluency) were incubated with this mixture overnight at 37°C. The culture medium was replaced 12 to 18 hours after incubation. Next, the supernatant, collected 48 and 72 hour after transduction, were combined and filtered through a 0.45-micron filter to remove cells and debris. The virus-containing supernatant was added to the culture medium of the TICAE cells. At the time of transduction, the cells were 50% confluent. After transduction of the TICAE cells, puromycin selection (1 μg/ml) was performed to select for positively transduced cells.

TFAM shRNA lentiviral transduction

The TFAM shRNA lentiviral particles were packaged with HEK 293T/17 cells, following the Lenti-vpak packaging kit protocol (Origene, Herford, Germany). The HEK 293T/17 cells were 50% confluent at the time of incubation with an expression construct (composition is confidential) and packaging vector. The incubation of the HEK 293T/17 cells was performed overnight at 37°C, 12 to 18 hours later the culture medium was replaced. The second and the third day, the viral supernatant from 293T/17 producer cells was harvested. Cells and debris in the supernatant was removed by centrifugation and by passing it through a 0.45-micron filter. Together with 8 μg/ml polybrene, the viral supernatant was added to the TICAE cell culture medium. The TICAE cells were 50% confluent at the time of transduction. The positively transduced TICAE cells were selected with 1 μg/ml puromycin.

¹ Multiplicity of infections (MOI): the number of viral particles added per cell. An MOI of 1 means that 1 cell is transduced with 1 lentiviral particle. The lentiviral titer expresses the amount of lentiviral particles present in a certain volume (transducing units (TU)/ml). The total amount of transducing units can be calculated by multiplying the number of cells that needed transduction, with the desired MOI.

TFAM cDNA lentiviral transduction

Self-assembled lentiviral particles were produced by 293T/17-cells, that were 50% confluent at the day of transduction. A pLenti-ORF expression construct of the human TFAM cDNA (RC215488L1) (Origene, Herford, Germany) (Supplementary figure 6), a pLenti-C-Myc-DDK vector (PS100064) (Origene, Herford, Germany) and the packaging kit (TR30022) (Origene, Herford, Germany) were combined according to Lenti-vpak packaging kit protocol (See description above). Together with 8 µg/ml polybrene, the viral supernatant was added to the TICA cells with a confluency of 50%.

2.1.5. Confirming lentiviral presence

After packaging of the lentiviral particles by the HEK 293T/17 producer cell line, the cell medium was tested for lentiviral presence with the Lenti-X GoStix™ (Clontech Laboratories Inc., Leusden, The Netherlands), used according to manufacturer's instruction (Supplementary figure 7). When a clear band was generated, a lentiviral titer¹ of more than 5.10⁵ IFU/ml was produced by the HEK 293T/17 producer cells.

2.2. Characterization of the modified endothelial cell lines

2.2.1. DNA extraction and quantitative polymerase chain reaction (qPCR)

With the QIAamp DNA mini kit (Qiagen, Antwerp, Belgium), DNA was extracted and purified from the cultured cells following manufacturer's instructions. The DNA concentration was measured with the Nanodrop™ spectrophotometer (Thermo Fischer Scientific, Aalst, Belgium).

In comparison to a genomic reference gene (RNase P), the amount of mitochondrial DNA (mtDNA) present in the cell lines was measured by quantitative PCR. The PCR mastermix was composed of 1x Taqman® Universal Master Mix II (Applied Biosystems™ Thermo Fischer Scientific, Aalst, Belgium), 1x Taqman® RNase P copy number reference assay kit (Applied Biosystems™ Thermo Fischer Scientific, Aalst, Belgium), 8 µM probe (Eurogentec, Seraing, Belgium) (Table 2) and 20 µM primer pair (Eurogentec, Seraing, Belgium) (Table 2). Twelve microliter of the PCR mastermix was loaded in a 96-well PCR plate, to which 8 µl nuclease free water (Bio-Rad, Temse, Belgium) containing 70 ng purified DNA was added. The qPCR was performed with four replicates of each DNA sample. The well plate was measured with ABI 7500 Fast PCR systems (Applied Biosystems™ Thermo Fischer Scientific, Aalst, Belgium). The following cycle was completed: incubation for 2 minutes at 50°C, polymerase activation for 10 minutes at 95°C, followed by 40 amplification cycles of denaturing (15 seconds at 95°C) and annealing and extending (1 minute at 60°C). The DNA products were quantified with the new mathematical method of Pfaffl (71)

Table 2: The probe and primer pair used to analyze the total amount of mtDNA (72).

Name	Sequence
Probe mtDNA ^{Total}	5'-tet-CCCATGAGGTGGCAAGAAAT-tamra-3'
Forward primer HSSN1307	5'-GTACCCACGTAAAGACGTTAGG-3'
Reverse primer HSAS8542	5'-TACTGCTAAATCCACCTTCG-3'

The probe for total mtDNA was labeled at the 5'-end with a tetrachloro-6-carboxy-fluorescein reporter dye (TET) and at the 3'-end with a 6-carboxytetramethylrhodamine quencher dye (TAMRA).

Ramos et al. (2009 and 2011) described a set of nine primers to amplify the entire human mtDNA without the amplification of nuclear encoded DNA sequences (73, 74). The primer pairs are described in Table 3. The PCR reaction mix consisted of 30 ng DNA diluted in nuclease free water, 50 pmol of the forward and reverse primer (Eurogentec, Seraing, Belgium), 0.5 mM magnesium chloride (MgCl₂) (Sigma-Aldrich Co. LLC, Diegem, Belgium) and Taq&Load PCR master mix (Applied Biosystems™ Thermo Fischer Scientific, Aalst, Belgium). Four replicates were used for each DNA sample. The PCR experiments were performed in a PCR machine (Applied Biosystems™ Thermo Fischer Scientific, Aalst, Belgium) following a protocol consisting out of an initial denaturation step at 94°C for 5 minutes, 35 cycles of amplification (1 minutes at 94°C, 40 seconds at annealing temperature and 2.5 minutes at 72°C) and a final extension step at 72°C for 5 minutes. After gel electrophoresis in a 2% agarose gel (Eurogentec, Seraing, Belgium), the DNA amplification products were visualized with GelRed coloring (Biotium, Brussels, Belgium) using Fusion FX imaging device. The intensity and volume of the bands was quantified using the BIO1D software.

Table 3: The nine primer pairs covering the entire human mtDNA (73, 74).

Fragment	Fragment length	Name	Sequence (5'–3')	Primers pair length (bp)	T _a	T _m
1	1822	14898for 151rev	tagccatgcactactaccagaga ggatgaggcaggaatcaagac	22	60	60.3
2	1758	16488for ^{a)} 1677rev ^{a)}	ctgtatccgacatctggttct gttagctcagagcggtcaagt	22	60	60.3
3	2543	1404for ^{a)} 3947rev	acttaagggtcgaagggtgatt tcgatgtgaagcctgagacta	22	57	58.4
4	3005	3734for 6739rev	aagtcacccctagccatcattcta gatatcatagctcagaccatacc	23	61	58.9
5	2664	6520for 9184rev	tcactatactactaacagaccgc gtagaggcttactagaagtgtg	23 22	58 58.4	58.9 58.4
6	1738	8910for ^{a)} 10648rev	cttaccacaaggcacacctaca ggcacaatatggctaagaggg	22	61	60.3
7	1866	10360for 12226rev	gtctggcctatgagtgaactaca cagttctgtgagcttctcgg	22	61	60.3
8	1853	11977for 13830rev	ctccctctacatatattaccacaac aagtcctaggaagtgcagcga	24 23	63	59.3 60.6
9	1872	13477for ^{a)} 15349rev ^{a)}	gcagggaataccttcctcacag gtgcaagaataggaggtggagt	22	63	60.3

The length, annealing temperature (T_a) and melting temperature (T_m) of the forward and reverse primer are described.

The PCR products (10 ng/ml), together with their primers (10 μM), were send to Eurofins Genomics (Ebersberg, Germany) for Sanger sequencing. The sequences were aligned according to the reference human mtDNA sequence (Refseq NC 012920) with the Basic Local Alignment Tool (BLAST).

2.2.2. Protein extraction and western blot

A volume of 200 μl RIPA lysis buffer (Roche, Brussels, Belgium), consisting out of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40/IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS and a protease and phosphatase inhibitor tablet, was added to 1.10⁶ cells. After further homogenization of the cells with a tissue lyser II device (Qiagen, Antwerp, Belgium), the cell lysates were centrifuged at 14 000 g for 10 minutes to remove cell debris. The protein concentration was measured with the bicinchoninic acid (BCA) assay kit (Sigma-Aldrich Co. LLC, Diegem, Belgium), following manufacturer's instructions. The absorbance of the proteins treated with BCA was compared with a bovine serum albumin (BSA) standard curve.

Seven micrograms of protein samples in triplicate, together with 4x Laemmli buffer (Bio-Rad, Temse, Belgium) and β -mercaptoethanol (Sigma-Aldrich Co. LLC, Diegem, Belgium), were separated on a 4-15% Criterion™ TGX stain free gel (Bio-Rad, Temse, Belgium) using an electrophoresis system (Bio-Rad, Temse, Belgium). The precision plus protein standards kaleidoscope (Bio-Rad, Temse, Belgium) was used as ladder to determine molecular weight. The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Temse, Belgium) with a blotting device (Bio-Rad, Temse, Belgium). The blots were blocked in 5% non-fat dry milk (NFDM) (Bio-Rad, Temse, Belgium) diluted in 1x tris-buffered saline with Tween20 (TBST) (Sigma-Aldrich Co. LLC, Diegem, Belgium) for 90 minutes at room temperature. Afterwards, the proteins were incubated overnight at 4°C with the primary antibody (1/250 dilution of mouse monoclonal anti-TFAM (C-9) (sc-376672); 1/250 dilution of rabbit polyclonal anti-TOM20 (FL-145) (sc-11415) (Santa Cruz Biotechnology, Heidelberg, Germany); 1/200 dilution of mouse monoclonal anti-DDK (OTI4C5) (Origene, Herford, Germany)). After washing the blots with 1x TBST, the proteins were incubated with secondary antibody for 45 minutes at room temperature (1/10 000 goat anti-mouse horseradish peroxidase (HRP) (62-6520); 1/10 000 goat anti-rabbit HRP (65-6120) (Life Technologies, Merelbeke, Belgium)). The protein bands were visualized using the enhanced chemiluminescence (ECL) detection kit (Bio-Rad, Temse, Belgium) and scanned with Fusion FX imaging device (Vilber Lourmat, Eberhardzell, Germany). The western blot data were normalized against total protein levels. The total protein staining with Serva Purple (Serva, Heidelberg, Germany) was performed as follows. After imaging, the blots were kept overnight in 1x TBST at 4°C. The previously used antibodies were removed with five minutes incubation in Restore™ PLUS Western Blot Stripping Buffer (Thermo Fischer Scientific, Aalst, Belgium). Next, the blots were washed two times with 1x TBST for five minutes. With staining buffer containing 0.62% boric acid and 0.385% sodium hydroxide diluted in MilliQ, the blots were basified. The blots were stained with Serva Purple® (1/200) for 30 minutes at room temperature in the dark and rinsed with fixation solution (1% citric acid and 15% ethanol diluted in MilliQ) for five minutes at room temperature in the dark. Afterwards, the blots were washed three times with 100% ethanol for two minutes. Finally, the blots were dried and imaged using the Fusion FX imaging device. The volume and intensity of the protein bands was analyzed using the BIO1D software (Vilber Lourmat, Eberhardzell, Germany).

2.2.3. Glucose deprivation assay

The control and modified TICAE cells were plated in a 96-well plate to reach 70 to 90% confluency. After washing with PBS, six replicates were incubated in 100 μ l of glucose-free DMEM medium (Gibco™ Thermo Fisher Scientific, Aalst, Belgium) containing 10% FBS, 2 mM Glutamine, 1 mM Sodium-pyruvate and 10 mM HEPES. The six control replicates were treated in the same way, although 100 μ l glucose-rich MesoEndo medium was added. The replicates were fixated twice with 10% paraformaldehyde (PFA) (Sigma-Aldrich Co. LLC, Diegem, Belgium) for 15 minutes at room temperature, first diluted in 100 μ l cell medium then undiluted. After washing two times with PBS, the cells were stained with 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich Co. LLC, Diegem, Belgium), diluted in tris-NaCl-blocking buffer (TNB), for one hour at 37°C. The nuclei were visualized with the Eclipse Ti automated inverted wide-field epifluorescence microscope (Nikon, Brussel, Belgium) equipped with a 20 $\times$ magnification (Plan Fluor, numerical aperture 0.6) dry

objective and a Nikon TE2000-E camera controlled by the NIS Elements software. The amount of DAPI-colored nuclei were counted with FIJI software (75).

2.2.4. NAD(P)H reductive capacity measured with MTT assay

To measure the NAD(P)H reductive capacity of the modified endothelial cells compared to the control TICAE cells, a Methyl-Thiazolyl-Tetrazolium (MTT) assay was performed. For each cell line, 70 to 90% confluence in a 96-well plate was obtained for 12 replicates. A concentration of 5 mg/ml MTT (Sigma-Aldrich Co. LLC, Diegem, Belgium) diluted in phosphate buffered saline (PBS) (Gibco™ Thermo Fisher Scientific, Aalst, Belgium) was added to the cell medium. The MTT solution was incubated for four hours at 37°C and 5% CO₂. After carefully removing cell medium, 175 µl MTT solvent consisting of 1/7 Sorenson's glycine buffer (0.1 M glycine buffer (Bio-Rad, Temse, Belgium), 0.1 M sodium chloride (NaCl) (Merck, Overijse, Belgium), pH 10.5) and 6/7 DMSO, was added per well. When the solvent was properly mixed, the absorbance was measured at 570 nm with the CLARIOstar microplate reader (BMG Labtech, Temse, Belgium). The MTT absorbance was normalized for cell number. Another eight replicates were fixated, stained with DAPI, visualized and counted as described above (see glucose deprivation assay). This test was repeated two times.

2.2.5. Doubling time determination

To define the doubling time of the modified and control TICAE cells, the amount of cells was counted for four days after seeding. For each cell line and time point, four replicates were seeded in a 6-well plate with a confluence of 50%. At five different time points after seeding (baseline, day 1, day 2, day 3 and day 4), the cells were fixed, stained with DAPI, visualized and counted as described above (see glucose deprivation assay).

2.2.6. VE-Cadherin immunocytochemical staining

To visualize the vascular endothelial (VE) cadherin on the modified and control TICAE cells, an immunocytochemical staining was performed. Six replicates of each cell line, with 70 to 90% confluence, were seeded on an 8-well Labtek Chamber Slide (Thermo Fischer Scientific, Aalst, Belgium). After fixating the cells for 15 minutes with 2% formaldehyde, the cells were permeabilized with 0.25% Triton X-100 (Sigma-Aldrich Co. LLC, Diegem, Belgium) diluted in PBS. Afterwards, the cells were blocked in 5% BSA (Sigma-Aldrich Co. LLC, Diegem, Belgium) and 0.1% Tween 20 (Sigma-Aldrich Co. LLC, Diegem, Belgium) diluted in PBS. For the primary antibody, the cells were incubated with mouse monoclonal VE-cadherin antibody (sc-9989) (1/300 dilution) (Santa Cruz Biotechnology, Heidelberg, Germany) for one hour at 37°C. The secondary antibody goat anti-mouse Alexa fluor 568 (A-11011) (1/300 dilution) (Life Technologies, Merelbeke, Belgium) was added to the cells together with Alexa fluor TRITC phalloidin (1/2 000 dilution) (Sigma-Aldrich Co. LLC, Diegem, Belgium) and 1 µg/ml DAPI for one hour at 37°C. Finally, the cells were washed two times with 1x PBS for ten minutes. After removal of the labtek chamber, the slide was mounted with mowiol (Sigma-Aldrich Co. LLC, Diegem, Belgium) and covered with a big coverslip. The staining was visualized with the Nikon Eclipse Ti automated inverted wide-field epifluorescence microscope equipped with a 20× magnification (Plan Fluor, numerical aperture 0.6) dry objective and a Nikon TE2000-E camera controlled by the NIS Elements software. The intensity of the VE-cadherin and phalloidin staining was determined with the FIJI software (75).

2.2.7. Dil-Ac-LDL immunocytochemical staining

Another immunocytochemical staining was performed to visualize the uptake of low-density lipoprotein (LDL) by the control and modified TICAEC cells. In a 96-well plate, twelve replicates per cell line were seeded with a confluency of 70 to 90%. To the cell culture medium, 10 µg/ml Dil-labeled acetylated LDL (Dil-Ac-LDL) (Bioquote Limited, York, UK) was added. The cells were incubated for four hours at 37°C and 5% CO₂. After washing, the cells were stained with 1µg/ml DAPI, diluted in TNB, for one hour at 37°C. The staining was visualized with the Nikon Eclipse Ti automated inverted wide-field epifluorescence microscope equipped with a 20× magnification (Plan Fluor, numerical aperture 0.6) dry objective and a Nikon TE2000-E camera controlled by the NIS Elements software. With the FIJI software, the intensity of the Dil-Ac-LDL signal was measured. The intensity was normalized for amount of DAPI-stained nuclei, counted with the FIJI software (75).

2.3. **Testing radiosensitivity of the endothelial cells after exposure to X-rays**

2.3.1. Irradiation of the endothelial cell lines

In advance to the ROS, DNA damage, apoptosis and ATP assays, the control and modified TICAEC cells were irradiated with a Xstrahl RX generator (250 kV, 12 mA, 3.8 mm Al and 1.4 mm Cu) (Camberley, UK). With a dose rate of 0.5 Gy/min, multiple X-ray doses (0.05, 0.1, 0.5 and 2 Gy) were generated to irradiate the cell lines. For the ATP assay, only an X-ray dose of 2 Gy was used.

2.3.2. The measurement of ROS levels with CM-H₂DCFDA or MitoSOX fluorogenic dye and mitochondrial membrane potential with TMRM

The total cellular ROS levels of the modified and control endothelial cell lines formed 24 hours after X-ray exposure, were indicated by staining with the fluorogenic chloromethyl derivate of 2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) dye. In combination with the CM-H₂DCFDA, a tetramethylrhodamine methyl ester (TMRM) staining was performed to determine the mitochondrial membrane potential. The second staining, performed 24 hours after X-ray exposure, consisted of MitoSOX, a mitochondrial superoxide indicator, in combination with Mitotracker Green which passes through the plasma membrane to stain the mitochondria.

For each cell line and radiation dose, 12 replicates were seeded in a clear bottom and black rim 96-well plate. After 48 hours, when a confluence of 70-90% was reached, the 96-well plates were irradiated as described above. Twenty-four hours after irradiation, the cells were washed twice with 20 mM HEPES diluted in Hank's balanced salt solution (HBSS) (Sigma-Aldrich Co. LLC, Diegem, Belgium). Next, the cells were incubated for 25 minutes in the dark at room temperature either with 100 nM TMRM (Life Technologies, Merelbeke, Belgium) and 2 µM CM-H₂DCFDA (Life Technologies, Merelbeke, Belgium) or 200 nM Mitotracker green (Invitrogen™ Thermo Fisher Scientific, Ninove, Belgium) and 5 µM MitoSOX (Invitrogen™ Thermo Fisher Scientific, Ninove, Belgium). After incubation, the cells were washed with 20 mM HEPES diluted in HBSS. The sham irradiated samples were treated in the same way. The stains were visualized with the Nikon Eclipse Ti automated inverted wide-field epifluorescence microscope equipped with a 40× magnification (Plan Fluor, numerical aperture 0.6) dry objective and a Nikon TE2000-E camera controlled by the NIS Elements software. After imaging, the cells were incubated for three minutes with 0.04 mM tert-butyl hydroxypoxide (tBHP) (Sigma-Aldrich Co. LLC, Diegem, Belgium), as a positive control for CM-H₂DCFDA. The positive control for MitoSOX was 50 µM Rotenone A (Sigma-Aldrich Co. LLC, Diegem,

Belgium). Again, both stains were measured with fluorescence microscopy. Quantification of the staining intensity was performed with FIJI and R Studio software (75). The macro for R Studio was kindly provided by Prof. dr. Winnok De Vos and Tom Sieprath, PhD-student, from the department of molecular biotechnology at Gent University (76).

2.3.3. Visualization of DNA damage with γ H2AX/53BP1 immunocytochemical staining

To respectively mark DNA double strand breaks and the early repair response after the DNA breaks, the control and modified TICAE cells were stained for γ H2AX and 53BP1. Per cell line and radiation dose, six replicates were seeded in an 8-well Labtek at 70 to 90% confluence. Forty-eight hour after cell seeding the labteks were irradiated. At several time points after X-ray exposure (0.5, 1, 4 and 24 hour), cells were fixated with 2% PFA diluted in PBS. After permeabilization in 0.25% Triton X-100 in PBS, cells were blocked in pre-immunized goat (PIG) serum (Invitrogen™ Thermo Fisher Scientific, Ninove, Belgium) diluted in TNB. The TICAE cells were incubated for one hour at 37°C with mouse monoclonal γ H2AX primary antibodies (1/300 dilution) (05-636) (Merck, Overijse, Belgium) and rabbit polyclonal antibody 53BP1 (1/1000 dilution) (NB100-304) (Novus Biologicals, Littleton, USA). Together with 1 μ g/ml DAPI, the cells were incubated with secondary antibodies Alexa fluor 488 (1/300 dilution) (A-11001) (Life Technologies, Merelbeke, Belgium) and 568 (1/1000 dilution) for one hour at 37°C in dark conditions. The sham irradiated samples were treated in the same way. After removal of the labtek chamber, the slide was mounted with Prolong Diamond Antifade mountant (Life Technologies, Merelbeke, Belgium) and covered with a big coverslip. The staining was visualized with the Eclipse Ti (automated inverted wide-field epifluorescence microscope) equipped with a 40 $\times$ magnification (Plan Fluor, numerical aperture 0.6) dry objective and a Nikon TE2000-E camera controlled by the NIS Elements software. Per condition a mosaic of 12 fields was acquired with a lateral spacing of 500 μ m. Each field was acquired as a z-stack of nine planes axially separated by 1 μ m. Images were analyzed with Fiji software (75) using the Cellblocks toolbox. The software allowed to analyze each nucleus based on the DAPI signal. Within each nucleus, pixel size and intensity emitted from the Alexa 488 (γ H2AX) and Alexa 568 (53BP1) fluorochrome were analyzed after which the foci number per nucleus is determined in a fully automatic manner. Per cell line, each dose-time point (e.g. 2 Gy 30 minutes) is represented by 6 biological replicates of which at least 200 nuclei were analyzed. The macro for FIJI was provided by Prof. dr. Winnok De Vos from the department of molecular biotechnology at Gent University.

2.3.4. ATP assay

After (sham-)irradiation, the amount of ATP produced by the control and modified TICAE cells was measured with a Luminescence ATP detection assay (Perkin Elmer, Brussels, Belgium), according to the manufacturer's instructions. In a 96-well plate, ten replicates per cell line were seeded with a confluence of 70-90%. The ATPlite solution (100 μ l) was added to the 96-well plate 0.5, 1, 4 and 24 hours after X-ray exposure. The 96-well plate was mixed for two minutes at 700 RPM on a microplate shaker to allow total lysis of the cells. Afterwards, the luminescence was measured with the CLARIOstar microplate reader.

2.4. Statistical analysis

The qPCR, the western blot, the MTT, the VE-cadherin and the Dil-Ac-LDL data were analyzed with a 1-way ANOVA with Tukey's Multiple comparison test. The glucose deprivation assay had two variables, namely days and cell line. Therefore, a 2-way ANOVA was used for analysis. For the CM-H₂DCFDA, the TMRM and the MitoSOX assay, the three cell lines were compared for each radiation dose. Therefore, a 2-way ANOVA with Bonferroni Posttests was used. The γ H2AX assay was analyzed separately for each cell line with time and irradiation dose as the independent variables. Thus, for the γ H2AX assay also a 2-way ANOVA with Bonferroni Posttests was performed. For all the statistical analysis, the GraphPad Prism 5.01 program (GraphPad Software Inc., California, USA) was used.

3. Results

3.1. Endothelial cell lines transduced with ready-to-use lentiviral particles

3.1.1. Mito-GFP lentiviral transduction

Different multiplicities of infection (MOIs) of the pre-made mito-GFP Loclight™ lentivirus were used to transduce the TICAEC cells with a green fluorescent protein labeling the mitochondria. The MOI defines the number of viral particles per cell that is added to the cell population. First a MOI of 1 was used, which means that per cell in culture 1 lentiviral particle was added. However, with fluorescence microscopy no green fluorescent light was detected, indicating that a MOI of 1 did not lead to the presence of transduced TICAEC cells. (Figure 3.1). Moreover, selection with blasticidin resulted in death of the remaining living cells after one day. This indicated that the lentivirus, containing a blasticidin selection marker, was not inserted in the TICAEC cells. Next, a higher MOI of 10 and 20 was used to transduce the TICAEC cells. However, with fluorescence microscopy still no green fluorescent TICAEC cells were visualized and after blasticidin selection no living TICAEC cells remained. Finally, a MOI of 12.5 together with 60 ng/ml polybrene resulted in a low transduction rate. Fluorescence microscopy showed a few cells emitting green fluorescent light (Figure 3.2). However, the transduction rate was too low to keep the cells in culture after blasticidin selection.

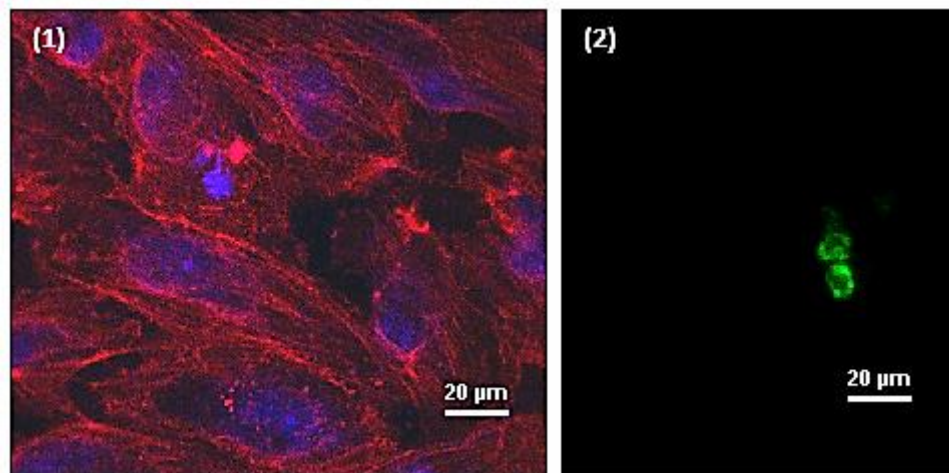


Figure 3: Fluorescence microscopy imaging of the TICAEC cells transduced with premade mito-GFP lentiviral particles. (1) Fixed TICAEC cells were stained with fluorescent DAPI and phalloidin-TRITC, which respectively visualizes the nucleus and the cytoskeleton. With a MOI of 1, 10 and 20, no green fluorescent mitochondria were detected. (2) Due to adequate polybrene addition, a MOI of 12.5 resulted in a few green fluorescent cells, visualized with live cell imaging.

3.1.2. TFAM shRNA lentiviral transduction

Another ready-to-use lentiviral particle was used to transduce TICAEC cells with a TFAM shRNA construct. One day after transduction the cell medium acidified. An improvement in cell growth was seen after addition of the buffering agent, HEPES. However, after puromycin selection no living cells remained, indicating the absence of transduced TICAEC cells.

3.2. Endothelial cell lines transduced with self-assembled lentiviral particles

The low transduction efficiency of the ready-to-use lentiviral particles was not sufficient to insert a mito-GFP or TFAM shRNA sequence in the TICAEC cells. To overcome this problem, a higher lentiviral MOI was necessary. This was achieved by assembling the expression vector, the envelop vector and the packaging constructs in 293T/17 producer cells, resulting in the production and release of the lentiviral particles in the cell medium (Supplementary figure 8). In this master's thesis, these lentiviral particles are referred to as self-assembled lentiviral particles. With the Lenti-X Gostix™, the supernatant derived from the 293T/17 cells producing the mito-GFP, TFAM shRNA and TFAM cDNA lentiviruses was tested for the presence of lentiviral particles. A lentiviral titer of more than 5.10^5 IFU/ml was detected in the supernatant of the 293T/17 producer cells for all the self-assembled lentiviruses. As such, a MOI of at least 1.4 could be calculated.

3.2.1. Mito-GFP lentiviral transduction

In comparison to the premade, the self-assembled lentiviral particles containing mito-GFP provided a much better transduction efficiency. Live cell imaging by fluorescence microscopy demonstrated that approximately 80% of the TICAEC cells contained green fluorescent mitochondria (Figure 4). After puromycin selection, the entire cell population contained mito-GFP. Heterogeneity of the green fluorescent signal indicated that not every cell has inserted the same amount of lentiviral particles.

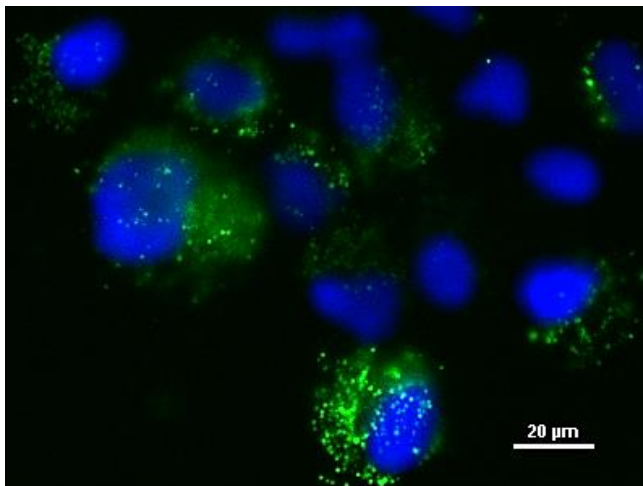


Figure 4: Live cell imaging with fluorescence microscopy of TICAEC cells transduced with self-assembled mito-GFP lentiviral particles. DAPI staining was performed to visualize the cell nucleus. The image is a projection of Z-stacks.

3.2.2. TFAM shRNA lentiviral transduction

After transduction with self-assembled TFAM shRNA lentiviral particles, the TICAEC cells were selected for transduced cells with puromycin addition. Little cell death was detected, which indicates an efficient transduction. However, just as with the premade lentiviral particles the cell medium acidified after transduction. The addition of HEPES and NAC could not save the cells.

3.2.3. TFAM cDNA lentiviral transduction

The TICAEC cells transduced with TFAM cDNA lentiviral particles were validated with western blot, because the lentiviral construct contained no selection marker. To the TFAM cDNA sequence in the lentiviral construct, a DDK-tag was attached. Since the DDK-tag adds molecular weight to the inserted TFAM protein, the native TFAM present in the cells could be distinguished from the TFAM encoded by the inserted TFAM cDNA (Figure 5.1). The cells transduced with TFAM cDNA showed double bands, representing the presence of native TFAM and inserted TFAM with DDK-tag. In this way, an

efficient transduction of the TICAIE cells with the TFAM cDNA sequence was observed. Determining the presence of the DDK protein with western blot, was a second validation for efficient transduction. However, in the control cell lines some native DDK was observed (Figure 5.2).

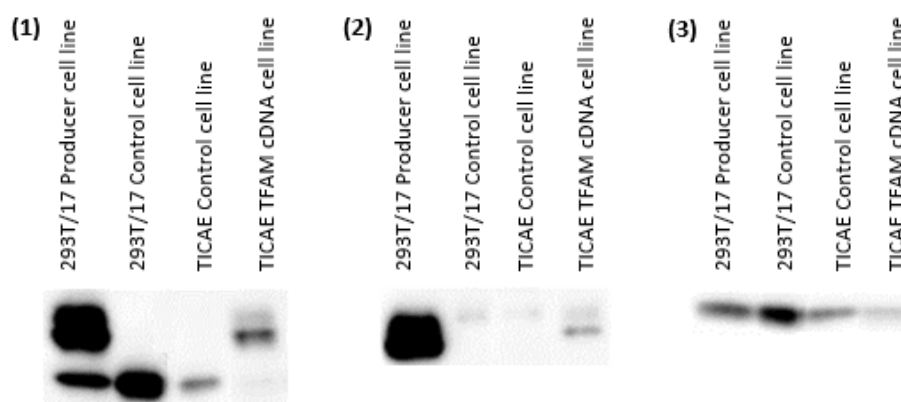


Figure 5: Western blot results of (1) TFAM, (2) DDK and (3) TOM20 protein after TFAM cDNA transduction. The 293T/17 cell lines were used to produce the lentiviral particles, which were later used to transduce the TICAIE cells. Molecular weight of TFAM: 25 kDa, DDK: 23kDa and TOM20: 20 kDa.

For the quantitative analysis of TFAM, the mitochondrial TOM20 protein present on the outer membrane was used as normalization because it gives an indication of the amount of mitochondria present in the cells (Figure 5.3). Figure 6 shows that both the lentiviral transduced 293T/17 and TICAIE cells contained the TFAM cDNA construct. The 293T/17 producer cell line contained much more inserted TFAM than the transduced TICAIE cells. In the TICAIE cells, 13 times more inserted TFAM was present than native TFAM. After two weeks of cultivation, the antioxidant NAC was added to the TICAIE cells transduced with TFAM cDNA to keep them viable. However, after two more weeks in culture the transduced cells died.

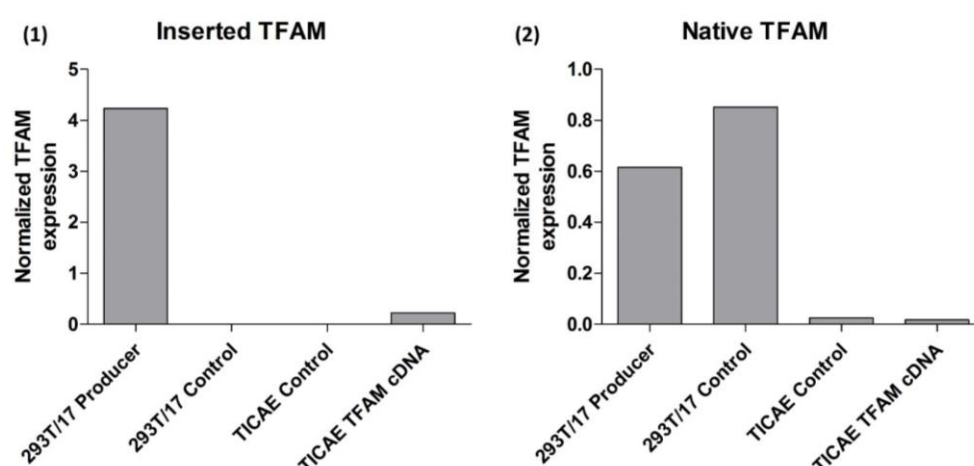


Figure 6: The amount of (1) inserted and (2) native TFAM present in the modified cell lines. The amount of TFAM was normalized for total protein staining and the amount of mitochondria (TOM20) present in the cells.

3.3. Endothelial cell lines modified with ethidium bromide and rosiglitazone

Due to the low efficiency of the lentiviral particles to modify the TICAIE cells and the difficulty to keep the transduced cells viable, TICAIE cells were cultured with ethidium bromide and rosiglitazone in

order to respectively diminish and enhance mitochondrial content. The TICA cells were cultivated for seven weeks with three different concentrations of ethidium bromide: 12.5, 25 and 50 ng/ml. After seven weeks, the addition of ethidium bromide was stopped. The cell medium of the TICA cells cultivated with ethidium bromide acidified as well. A pH of 6.17 was measured instead of 7.2 for the control cells. After HEPES addition to buffer the medium, cells started to grow better. In parallel, TICA cells were cultivated with a continuous supply of 50 μ M rosiglitazone.

3.3.1. Analysis of the mitochondrial DNA

In comparison to the amount of genomic DNA, the relative amount of mitochondrial DNA in the modified cell lines was measured. After seven weeks of ethidium bromide cultivation, the mtDNA copy number of the TICA cells cultivated with a concentration of 50 ng/ml was significantly decreased with 99.7% (Figure 7). The mtDNA copy number of the TICA cells cultivated with 12.5 and 25 ng/ml ethidium bromide, was declined with respectively 71.9% and 91.4%. However, this decline was not significant. Subsequently, after 7 weeks the TICA cells modified with 50 ng/ml ethidium bromide were further cultivated two weeks without ethidium bromide addition. This resulted in a slightly increased mtDNA. Still, it remained significantly declined (98.5%) compared to the control TICA cells. A significant increase of 305.6% in mtDNA copy number was observed in the TICA cells cultivated 7 weeks with rosiglitazone.

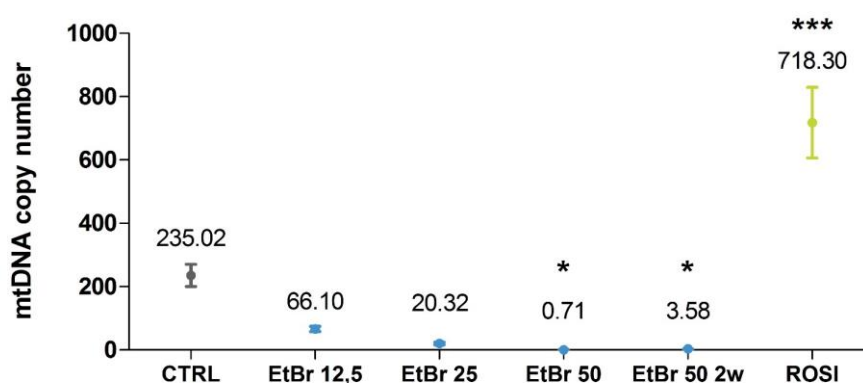


Figure 7: The amount of mitochondrial DNA present in the modified TICA cells. The cells were cultivated for seven weeks in the presence of 12.5, 25 and 50 ng/ml ethidium bromide (EtBr) or 50 μ M rosiglitazone (ROSI). After this 7 weeks, the ethidium bromide (50 ng/ml) modified TICA cells were cultivated two weeks without ethidium bromide addition (EtBr 50 2w). The error bars represent the standard error of mean (SEM). *: 0.01 < p < 0.05. ***: p < 0.0001.

Furthermore, to give an idea of how ethidium bromide and rosiglitazone have modified the mtDNA, the entire mtDNA of the control, ethidium bromide and rosiglitazone modified TICA cells was amplified by PCR after seven weeks of culture, using the nine primer pairs designed by Ramos et al. (2009 and 2011) (73, 74). The nine different overlapping parts of mtDNA (Supplementary figure 3) assessed with these nine primer pairs, had a differential augmentation or decline following the modification by ethidium bromide or rosiglitazone compared to the control mtDNA (Figure 8). Except for cytochrome C oxidase subunit III (COXIII), a diminishment was observed in the genes of complex I, III, IV and V for the ethidium bromide as the rosiglitazone modified TICA cells. The only difference in the rosiglitazone modified TICA cells was an increase of COXI, part of complex IV, and NADH-ubiquinone oxidoreductase chain 2 (ND2), part of complex I. Furthermore, ND5 was decreased more (73%) after rosiglitazone modification, compared to the ethidium bromide modified TICA cells (23%).

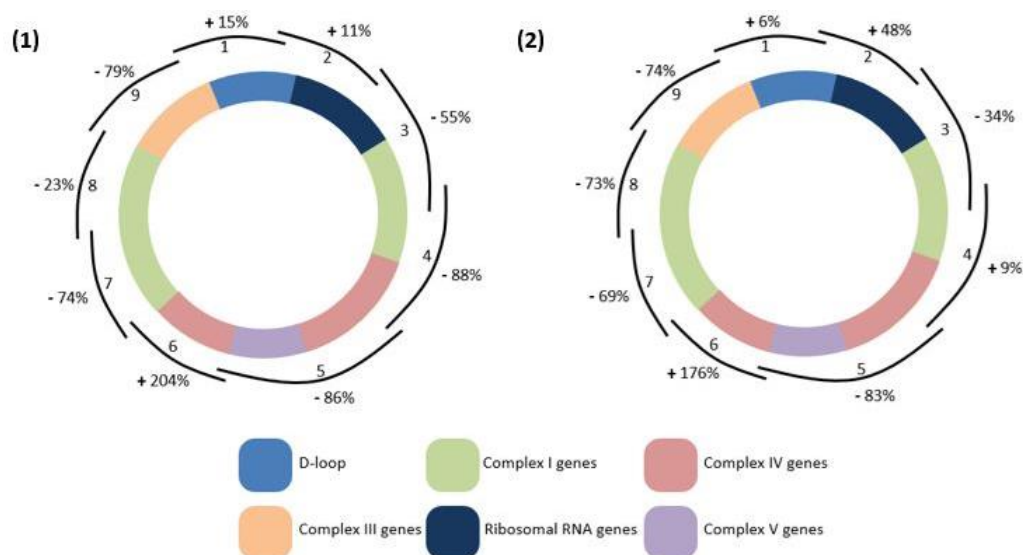


Figure 8: Schematic representation of the differences in the mitochondrial DNA expression of the (1) ethidium bromide and (2) rosiglitazone TICA cells, related to the control TICA cells. The nine mitochondrial DNA parts, amplified by the nine primer pairs (black lines), are indicated together with the percentage augmentation or decline after ethidium bromide or rosiglitazone modification. In colors the different coding regions of the mitochondrial DNA were indicated.

With the Sanger sequencing, following percentages of misidentities and gaps were identified for the control and ethidium bromide and rosiglitazone modified TICA cells (Table 4).

Table 4: The percentage of misidentities and gaps for the control and modified TICA cells

	Control TICA cells	Ethidium bromide TICA cells	Rosiglitazone TICA cells
% Misidentities	0.63	3.91	0.84
% Gaps	0.28	0.38	0.36

3.3.2. Quantification of the TFAM protein

During the ethidium bromide and rosiglitazone cultivation period of seven weeks, the amount of TFAM protein present in the TICA cells was weekly determined with western blot (Figure 9.1). A gradual decline was observed for each ethidium bromide concentration, while, for rosiglitazone no difference was seen (Figure 9.2).

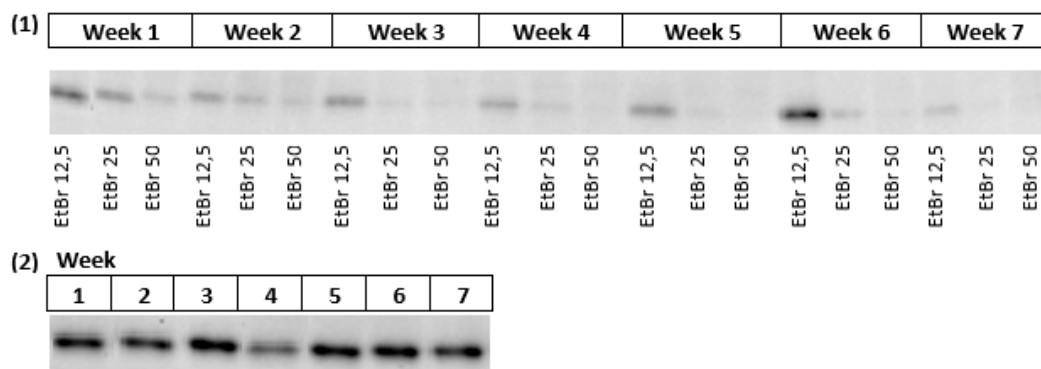


Figure 9: Western blot results of the TFAM protein in the TICA cells after (1) ethidium bromide and (2) rosiglitazone cultivation. Every week during ethidium bromide (EtBr) (12.5, 25 and 50 ng/ml) or rosiglitazone (50 μM) cultivation, the level of TFAM proteins was measured.

For a proper quantification, the amount of TFAM protein was normalized for the mitochondrial content present in the cells. The latter one was quantified with western blot of the TOM20 protein (results not shown). An ethidium bromide concentration of 50 ng/ml eliminated the TFAM proteins in the TICA cells the most; the TFAM amount was almost diminished to zero (Figure 10.3). Therefore, this cell line was selected for further use. The higher level of TFAM present in week 6 of ethidium bromide cultivation could be explained by a technical error (Figure 10.1). A continuous supply of 50 μ M rosiglitazone did not significantly change the amount of TFAM present in the mitochondria (Figure 10.4). The TFAM protein level of the control TICA cells was only measured for one replicate because too less protein was extracted, therefore no error bars were visible.

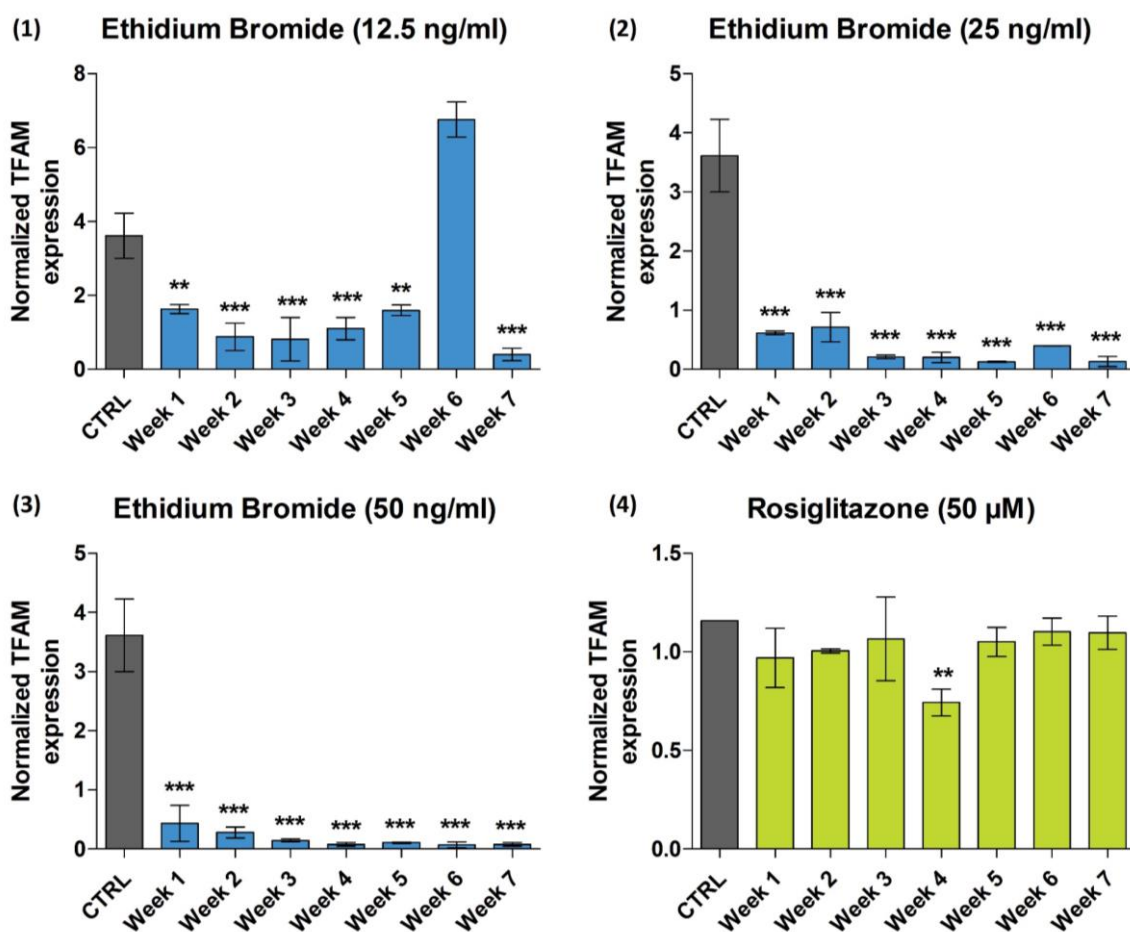


Figure 10: Relative TFAM protein quantification of the TICA cells cultivated with (1) 12.5, (2) 25 and (3) 50 ng/ml ethidium bromide or (4) 50 μ M rosiglitazone. (1, 2, 3) During seven weeks of ethidium bromide or (4) rosiglitazone cultivation, the amount of TFAM was weekly determined. The amount of TFAM was normalized for total protein staining and the amount of mitochondria present in the cells. The error bars represent the standard error of mean (SEM). **: $0.001 < p < 0.01$. ***: $p < 0.0001$.

3.3.3. Determination of metabolic state with glucose deprivation assay

The control and modified TICA cells were cultivated in cell medium deprived of glucose, however glutamine and fatty acids were still present. For each cell line, the starvation of glucose led to the induction of cell death (Figure 11.1). When little glucose is present, cells cannot produce the necessary ATP by glycolysis and need to fall back on oxidative phosphorylation. One day after glucose deprivation, the ethidium bromide and rosiglitazone modified cells contained less cells compared to

the control TICAE cells (Figure 11.2). However, after two and three days this difference is decreasing. The rosiglitazone modified TICAE cells have even more cells left.

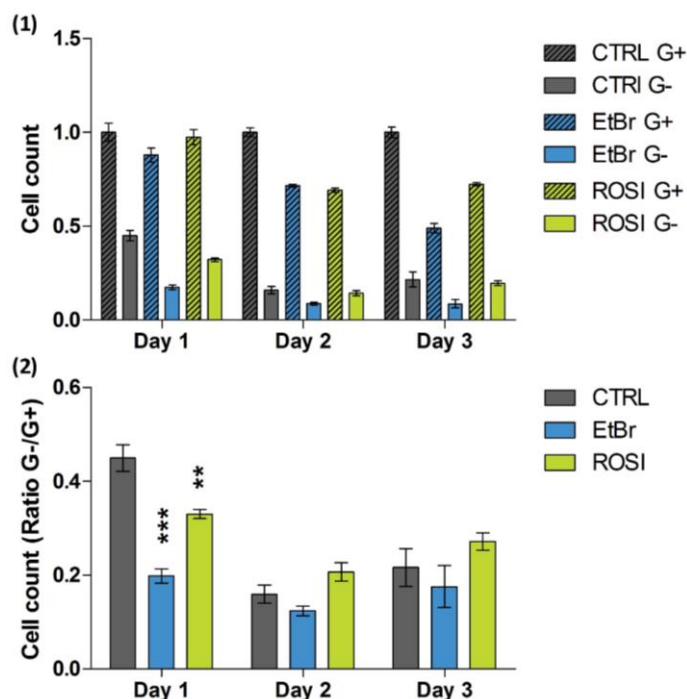


Figure 11: The amount of cells after glucose deprivation. (1) The cell count of the control, ethidium bromide (EtBr) and rosiglitazone (ROSI) cells in glucose-rich (G+) and glucose-free (G-) medium was followed for three days. (2) The amount of cells in glucose-free (G-) medium was normalized for the amount of cells present in glucose-rich (G+) medium. The error bars represent the standard error of mean (SEM). **: $0.001 < p < 0.01$. ***: $p < 0.0001$.

3.3.4. NAD(P)H reductive capacity measured with MTT assay

Yellow colored MTT is reduced to purple colored formazan by NAD(P)H dehydrogenases present in the electron transport chain of mitochondria. Therefore, the absorbance of formazan at 570 nm gives an indication of the dehydrogenase reductive capacity (77). In the ethidium bromide and rosiglitazone modified TICAE cells the reductive capacity was significantly decreased (Figure 12).

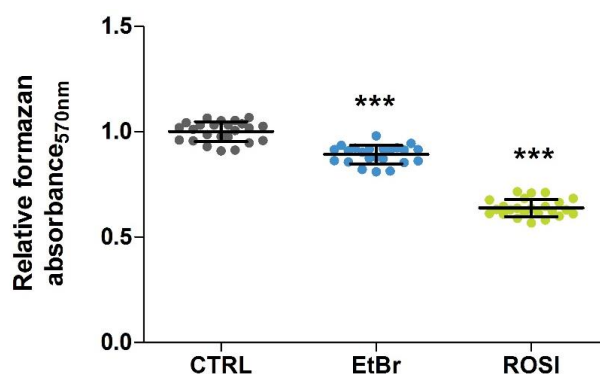


Figure 12 : The relative NAD(P)H reductive capacity of the three cell lines: control, rosiglitazone and ethidium bromide modified cells. The absorbance of formazan, measured at a wavelength of 570 nm, gives an indication of the NAD(P)H reductive capacity normalized to cell number. The error bars represent the standard error of mean (SEM). ***: $p < 0.0001$.

3.3.5. Measurement of the doubling time

For the three cell lines, the amount of cells was counted one, two and four days after seeding. Due to technical errors, the measurement of day three was dropped. The doubling time of the control TICAE

and ethidium bromide TICAEC cells did not differ much (Figure 13). The TICAEC cells modified with rosiglitazone need less time to double their cell amount, in comparison to the control TICAEC cells.

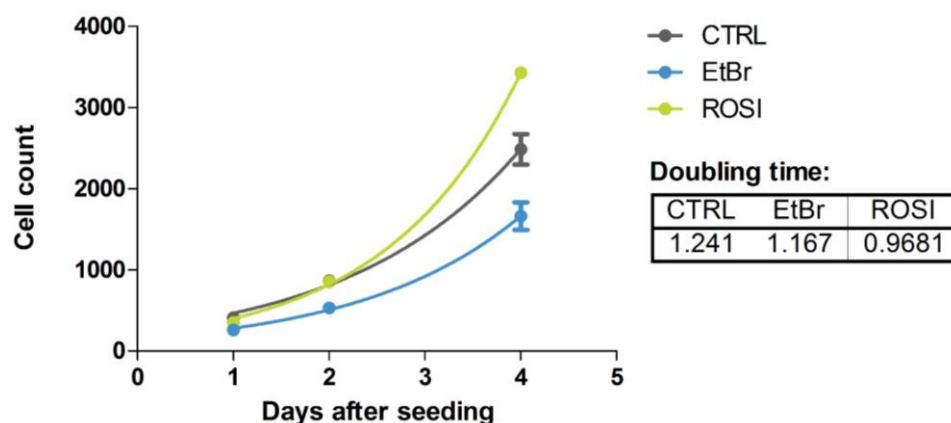


Figure 13: The cell count and the doubling time of the control and ethidium bromide and rosiglitazone modified TICAEC cells. An exponential growth curve emerged from the cell counts at the three different time points. From this exponential curve, the growing constant K and the doubling time ($\ln(2)/K$) was calculated. The error bars represent the standard error of mean (SEM).

3.3.6. VE-Cadherin immunocytochemical staining

The VE-cadherin immunocytochemical staining was performed to evaluate the endothelial cell function after modification. The specific endothelial adhesion molecule, VE-cadherin, is located between the endothelial cell junctions where it has an important role in maintenance and control of endothelial cell function (78). No significant difference was found in VE-cadherin presence of the ethidium bromide modified TICAEC cells in comparison with the control TICAEC cells (Figure 14). However, the VE-cadherine signal was significantly reduced in the rosiglitazone modified TICAEC cells.

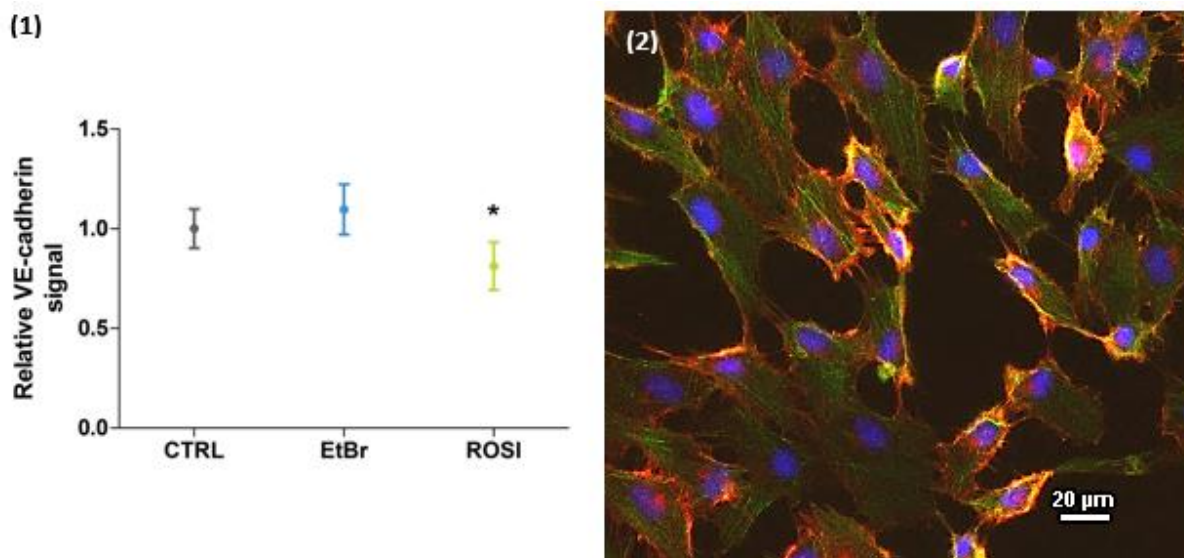


Figure 14: The relative VE-cadherin signal in the control, ethidium bromide and rosiglitazone TICAEC cells. (1) In order to normalize the intensity of the VE-cadherin signal for cell size and cell amount, the VE-cadherin intensity is corrected for phalloidin staining the cell cytoskeleton. The error bars represent the standard error of mean (SEM). (2) An immunocytochemical staining with DAPI, phalloidin and VE-Cadherin of the control TICAEC cells.

3.3.7. Dil-Ac-LDL immunocytochemical staining

An important function of endothelial cells is the intake of low-density lipoproteins (LDL) by receptor-mediated endocytosis. By analyzing the intake of acetylated LDL, labeled with a Dil-fluorescent probe that is specifically targeted to the endothelial cells, an impression of endothelial cell function can be made (79). As well for the ethidium bromide as for the rosiglitazone supplemented cell lines, the LDL-intake was diminished. However, a significant decrease was only observed in the ethidium bromide modified cell lines (Figure 15).

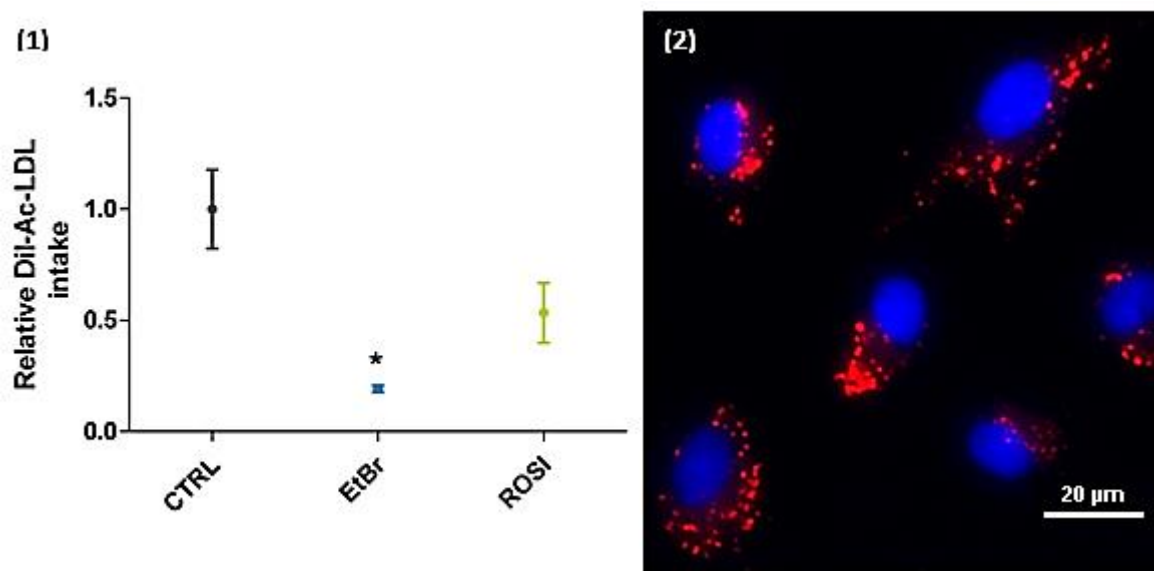


Figure 15: The relative Dil-Ac-LDL intake by the control, rosiglitazone and ethidium bromide modified TICA cells. (1) The intensity of the Dil-Ac-LDL signal was normalized for the amount of cells. The error bars represent the standard error of mean (SEM). (2) An immunocytochemical staining of the control TICA cells with Dil-Ac-LDL and DAPI. The image is a projection of Z-stacks. *: $0.01 < p < 0.05$.

3.4. Testing the radiosensitivity of the endothelial cells after X-ray exposure

3.4.1. Determination of mitochondrial membrane potential with TMRM

TMRM is a cell-permeable, positively charged, fluorescent dye which accumulates in the negatively charged matrix of mitochondria. This negative charge is a consequence of the electron transport chain; H^+ -ions are pumped from the matrix to the intermembrane space of the mitochondria when ATP is produced. The more negatively charged the mitochondria are, the more ATP is produced and the more TMRM will accumulate inside the matrix (Figure 18) (80, 81). Figure 16 indicates that the ethidium bromide TICA cells already had a significantly lower basal mitochondrial membrane potential compared to the control TICA cells. Moreover, after X-ray exposure the mitochondrial potential remained significantly lower for the TICA cells cultivated with ethidium bromide. Only after 0.1 Gy X-ray exposure the mitochondrial potential is significantly reduced for the rosiglitazone modified TICA cells.

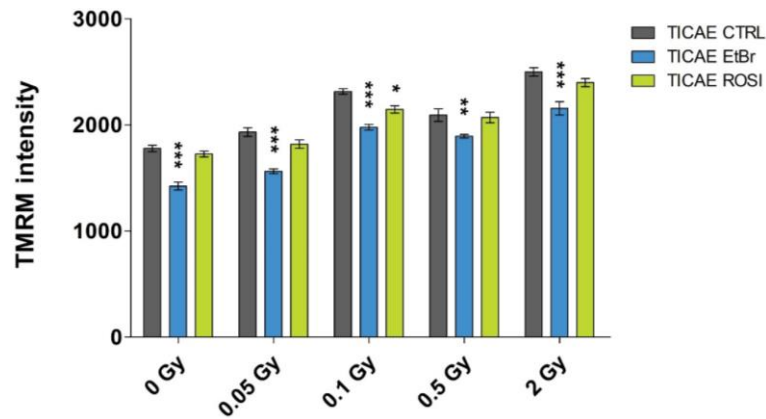


Figure 16: The mitochondrial membrane potential after X-ray exposure. The staining intensities of the red fluorescent TMRM, indicating the mitochondrial membrane potential, were represented. The error bars represent the standard error of mean (SEM). *: $0.01 < p < 0.05$. **: $0.001 < p < 0.01$. ***: $p < 0.0001$.

3.4.2. Measurement of cellular ROS levels with CM-H₂DCFDA

After X-ray exposure, the total cellular amount of reactive oxygen species (ROS) formed in the modified and control TICAE cells was measured by addition of CM-H₂DCFDA (Figure 17). Due to its positive charge, CM-H₂DCFDA passively diffuses into the cells where it is cleaved by esterase to DCFDA anion which is retained in the cell. When ROS are present DCFDA is oxidized, which results in the fluorescent DCF (81). Therefore, the amount of fluorescence can be related to the cellular amount of ROS. In response to X-ray doses of 2 Gy, the ethidium bromide modified TICAE cells produced a significantly lower amount of ROS compared to the control TICAE cells (Figure 18.1). While for the rosiglitazone modified TICAE cells, no significant difference was observed. For each X-ray dose, another 96-well plate with the three cell lines was used. Moreover, no inter-plate control could be obtained because it was only possible to irradiate an entire 96-well plate. Therefore, no comparison could be made between the X-ray doses.

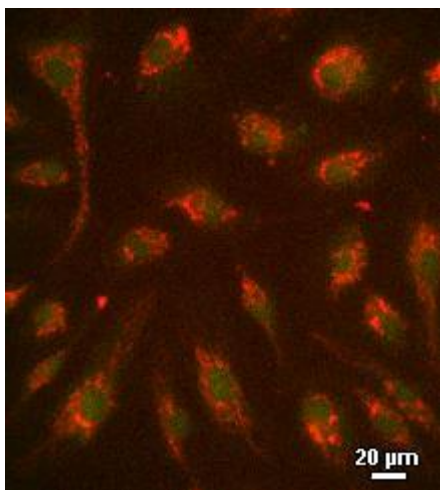


Figure 17: The CM-H₂DCFDA and TMRM staining of control TICAE cells fort BHP addition. TMRM is a red dye, while CM-H₂DCFDA is green.

After measurement of the fluorescent DCF, tBHP was added to the irradiated control and modified TICAE cells leading to an increase in ROS production (Figure 18.2). The CM-H₂DCFDA signal after tBHP addition for the X-ray dose of 0.1 Gy is not shown due to technical problems with the software. Only, after a 2 Gy X-ray exposure, the increase in ROS levels was significantly lower for the ethidium bromide as the rosiglitazone modified TICAE cells compared to the control TICAE cells (Figure 18.3).

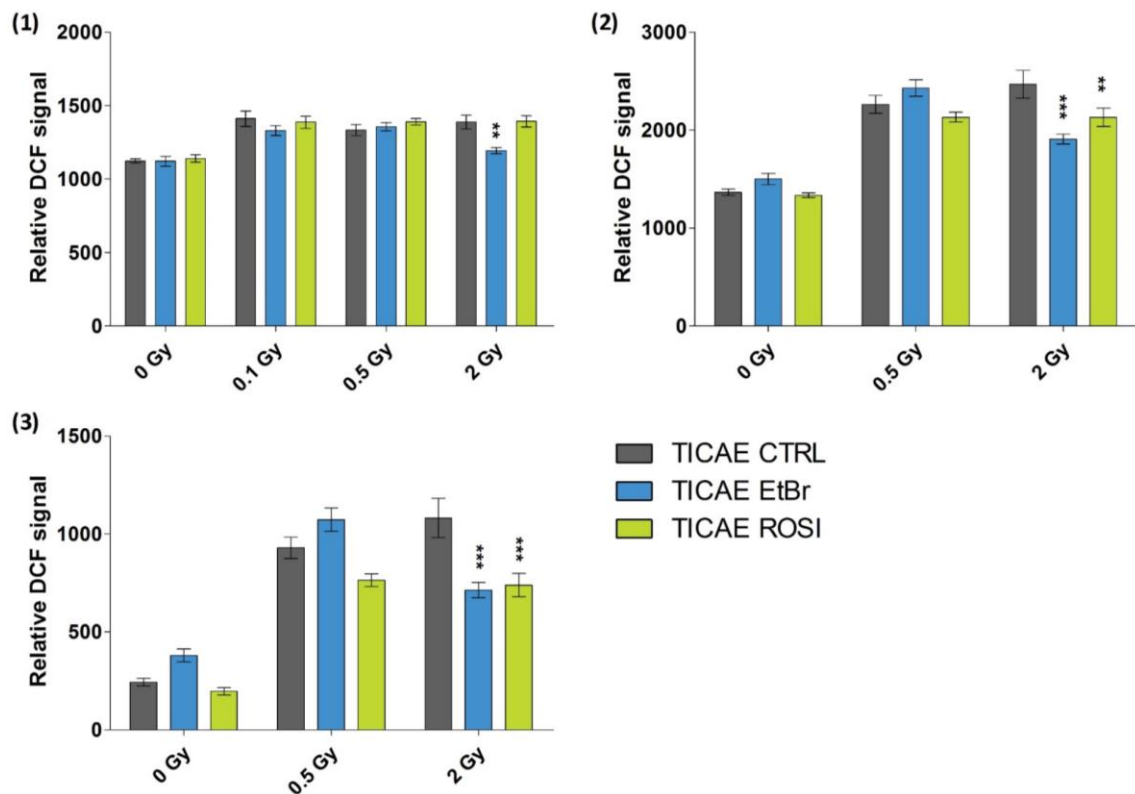


Figure 18: The cellular ROS levels after X-ray exposure and subsequent addition of tBHP for the control and ethidium bromide and rosiglitazone modified TICAE cells. The amount of DCF fluorescent signal, indicating the cellular ROS formation, was visualized for the control and ethidium bromide (EtBr) or rosiglitazone (ROSI) modified TICAE cells (1) before and (2) after addition of tBHP. (3) The gain in ROS level after addition of tBHP was also visualized. The error bars represent the standard error of mean (SEM). **: 0.001 < p < 0.01. ***: p < 0.0001.

3.4.3. Measurement of mitochondrial ROS levels with MitoSOX

MitoSOX, was added to the endothelial cell lines after X-ray exposure to measure the predominant form of mitochondrial ROS, superoxide. Mitochondrial targeted hydro-ethidine (MitoSOX) can passively diffuse into the endothelial cells, where it can be oxidized by the present superoxide and form the red fluorescent hydroxymitoethidium (82). The ethidium bromide and rosiglitazone modified TICAE cells have a significantly lower basal level of mitochondrial superoxide (Figure 19.1). The superoxide level of the ethidium bromide modified TICAE cells was significantly reduced after 2 Gy X-ray exposure. However, for the rosiglitazone modified TICAE cells the superoxide level was significantly reduced after 0.1, 0.5 and 2 Gy X-ray exposure. Also for the MitoSOX, no comparison could be made between the X-ray doses (As mentioned in 3.4.1).

After measurement of MitoSOX, rotenone was added to obtain an increase in superoxide formation, as it inhibits the electron transfer from complex I to ubiquinone. As such, the free electrons are reducing cellular oxygen to the superoxide radical. However, no major difference in ROS levels was observed before and after rotenone addition (Figure 19.2). This implicated that the added concentration of rotenone did not serve as a good positive control (gain not shown).

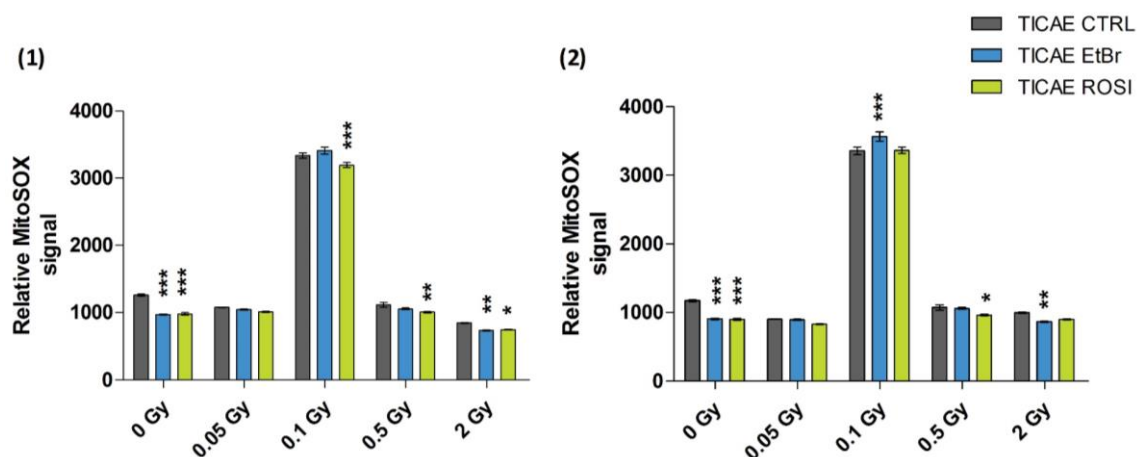


Figure 19: The mitochondrial ROS levels after X-ray exposure and subsequent addition of rotenone for the control and ethidium bromide and rosiglitazone modified TICAe cells. The amount of MitoSOX signal, indicating the mitochondrial ROS formation, was visualized for the control and ethidium bromide (EtBr) or rosiglitazone (ROSI) modified TICAe cells (1) before and (2) after rotenone addition. The error bars represent the standard error of mean (SEM). *: $0.01 < p < 0.05$. **: $0.001 < p < 0.01$. ***: $p < 0.0001$.

3.4.4. ATP assay

Luciferase and D-luciferin was added to the lysates of the control and modified TICAe cells. Luciferase will consume the present ATP to convert D-luciferin into oxyluciferin, resulting in the production of light. As such the emitted light is proportional to the cellular ATP concentration. Compared to the control TICAe cells, the ethidium bromide modified TICAe cells produced significantly less ATP measured 0.5, 1, 4 and 24 hours after exposure to an X-ray dose of 2 Gy (Figure 20). For the rosiglitazone modified TICAe cells, a small but significant decrease was visible 30 minutes after an X-ray dose of 2 Gy. Furthermore, 24 hours after a 2 Gy X-ray dose a significant increase in ATP production was observed.

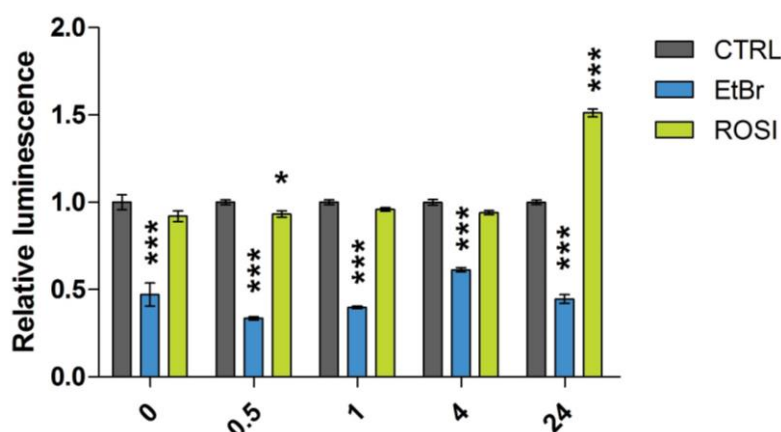


Figure 20: The ATP level measured after 2 Gy X-ray exposure for the control and ethidium bromide (EtBr) and rosiglitazone (ROSI) modified cell lines. The emitted luminescence, normalized for the amount of cells, is proportional to the ATP concentrations. The error bars represent the standard error of mean (SEM). *: $0.01 < p < 0.05$. ***: $p < 0.0001$.

3.4.5. Visualization of DNA damage with γ H2AX/53BP1 immunocytochemical staining

The double strand breaks (DSBs) induced by ionizing radiation were visualized with immunofluorescent staining of γ H2AX. These γ H2AX foci specifically localize around the DSB (36, 83). By immunofluorescent staining of the 53BP1 protein, which is part of the DSB repair mechanism (Supplementary figure 1), the repair response after ionizing radiation was visualized. After X-ray exposure to 0.05, 0.1, 0.5 and 2 Gy, the amount of γ H2AX and 53BP1 foci were measured at several time points (0.5, 1, 4 and 24 hours). In general, the amount of γ H2AX foci increased with X-ray dose (Figure 21). Furthermore, 24 hours after X-ray exposure the γ H2AX foci were already decreased to basal level. Compared to the control TICA cells, the ethidium bromide and rosiglitazone modified TICA cells had a decreased amount of γ H2AX foci after an X-ray dose of 2 Gy. However, four and 24 hours after irradiation more γ H2AX foci remained, which indicates a decreased γ H2AX response for the ethidium bromide and rosiglitazone modified TICA cells. Compared to the γ H2AX foci, less 53BP1 foci were visualized after ionizing radiation exposure. The amount of 53BP1 foci, which increased with X-ray dose, were also decreased to basal level 24 hours after ionizing radiation exposure (Figure 22). However, for the rosiglitazone modified TICA cells, a higher amount of 53BP1 foci remained 24 hours after irradiation. Compared to the control TICA cells, the rosiglitazone modified TICA cells had a slower 53BP1 response after exposure with a 2 Gy X-ray dose. The 53BP1 response peaked at four hours instead of one hour after ionizing radiation exposure. The ethidium bromide modified TICA cells had a decreased amount of 53BP1 foci formed 30 minutes and one hour after 0.1, 0.5 and 2 Gy X-ray exposure.

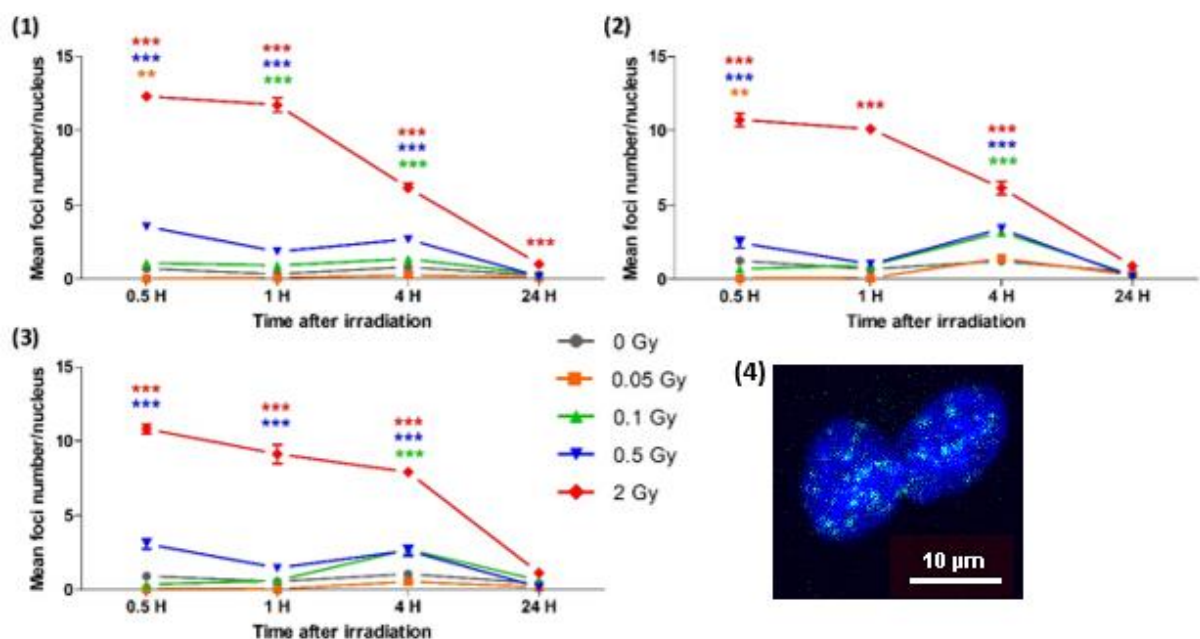


Figure 21: The amount of γ H2AX foci per cell nucleus after X-ray exposure for the (1) control and (2) ethidium bromide and (3) rosiglitazone modified TICA cells. The γ H2AX foci were measured 0.5, 1, 4 and 24 hours after X-ray exposure. The error bars represent the standard error of mean (SEM). **: 0.001 < p < 0.01. ***: p < 0.0001. (4) The γ H2AX staining with DAPI.

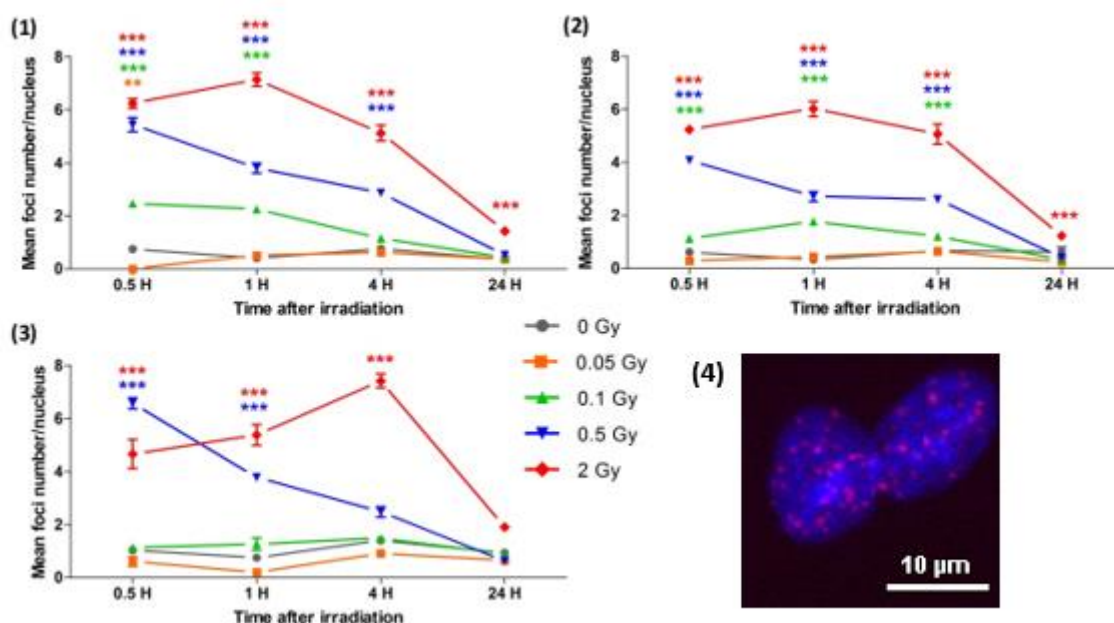


Figure 22: The amount of 53BP1 foci per cell nucleus after X-ray exposure for the (1) control and (2) ethidium bromide and (3) rosiglitazone modified TICAEC cells. The 53BP1 foci were measured 0.5, 1, 4 and 24 hours after X-ray exposure. The error bars represent the standard error of mean (SEM). **: 0.001 < p < 0.01. ***: p < 0.0001. (4) The 53BP1 staining with DAPI.

3.5. Determining mtDNA state during the radiosensitivity measurements

Before the ROS, the mitochondrial potential, the ATP and the DNA damage assay, the mtDNA content was determined with qPCR. In this way, it could be verified if the ethidium bromide and rosiglitazone modified TICAEC cells retained their, respectively, diminished and enhanced mtDNA content throughout the experiments. Ethidium bromide cultivation was stopped after seven weeks. Thus, during the experiments no ethidium bromide was added to the modified TICAEC cells. At week 14 and 17, respectively, seven and ten weeks without ethidium bromide addition, the mtDNA content was still declined (Figure 23.1). The mtDNA levels were compared with the mtDNA levels in figure 5. For the rosiglitazone modified TICAEC cells, 50 µM of rosiglitazone was continuously added to the cell medium throughout all the experiments. At week 14 and 17, the mtDNA content was also declined (Figure 23.2).

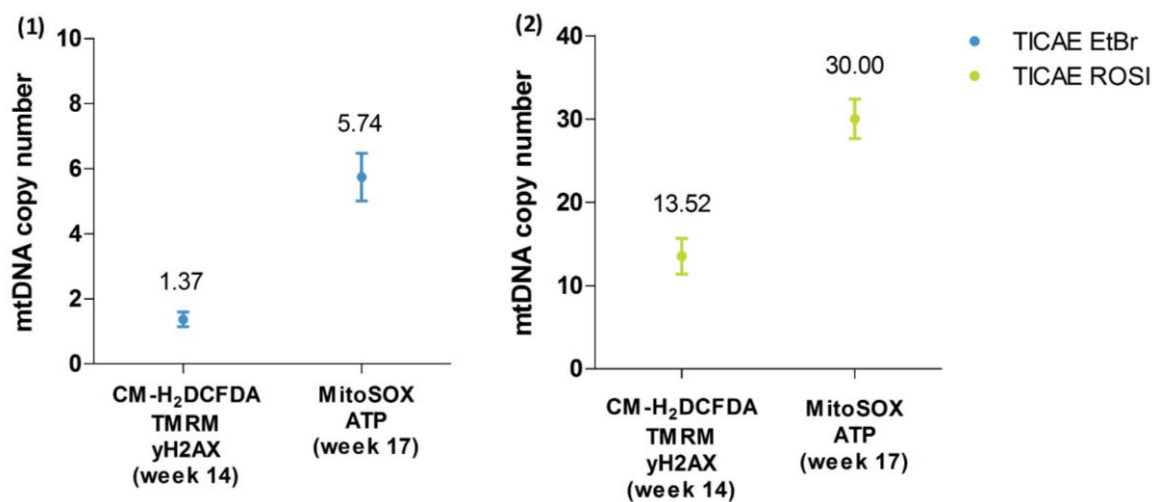


Figure 23: The mtDNA amount of the (1) ethidium bromide and (2) rosiglitazone modified TICAE cells. The mtDNA content was measured throughout the CM-H₂DCFDA and TMRM, MitoSOX, ATP and γH2AX assays. The error bars represent the standard error of mean (SEM).

4. Discussion

By enhancing or diminishing the mitochondrial content in telomerase immortalized coronary artery endothelial (TICAE) cells, the correlation between mitochondrial presence and cellular sensitivity after X-ray exposure could be examined. The modification of the endothelial cells was performed with lentiviral transduction and pharmacological incubation.

The use of this immortalized TICAE cell line entailed many advantages. The endothelial cells grew fast, were easy to handle and had no limited life span. Due to the immortalization, the endothelial cell characteristics were not changed during long-term culture (36). Furthermore, for all the experiments the same batch of cells was used, which limited the variability. However, a major disadvantage of using an *in vitro* one-cell model is that physiology of the coronary artery cannot be mimicked.

4.1. Endothelial cell lines transduced with lentiviral particles

For all the transductions, a human immunodeficiency virus type 1 (HIV-1) was used, containing enveloped single stranded linear RNA. The HIV vectors are all replication incompetent and self-inactivating to improve safety. The envelope gene which is naturally present in HIV-1 to enable infection of different cell types was replaced with a vesicular stomatitis virus glycoprotein (VSV-G) gene to broaden cellular tropism. A major advantage of the lentiviral particles is that they can efficiently infect dividing and non-dividing cells by actively entering the nucleus (84). Several studies have already indicated that endothelial cells are efficient targets for lentiviral transduction. The transduction of bovine aortic endothelial cells with lentiviral vectors, resulted in high expression of the inserted genes (85). Also a study of human coronary artery endothelial cells (HCAEC) indicated that lentiviruses are an efficient transduction tool (86).

In the first trial, the TICAE cells were transduced with mito-GFP lentiviral particles in order to stain the mitochondria fluorescent green. It was intended to co-transduce the, as such generated, mito-GFP TICAE cells with cDNA or shRNA lentiviral particles containing the TFAM gene. Unfortunately, it took too long to obtain efficiently mito-GFP transduced TICAE cells. Therefore, simultaneously other TICAE cells were transduced with cDNA or shRNA lentiviral particles containing the TFAM gene. Due to the ability to bind upstream of the mtDNA promoters, the nucleus-encoded TFAM gene can activate mitochondrial transcription (69, 87). Besides the role of TFAM in transcription initiation, it also has a structural role by binding to the mtDNA as a scaffold. TFAM is part of the nucleoid, the region inside the mitochondria containing most of the genetic material, where it functions as the histones of the mitochondrial genome (88). Thus by overexpression or silencing of the TFAM gene with respectively cDNA or shRNA lentiviral constructs, we aimed to make endothelial cells containing more or less mitochondria.

4.1.1. Efficient transduction with self-assembled mito-GFP lentiviral particles

The pre-made mito-GFP Loclight™ lentiviral particles did not lead to an efficient transduction. Even a MOI of 20 did not result in efficiently transduced TICAE cells, probably because no polybrene was

added. Polybrene is a positively charged polycation which could enhance transduction efficiency by reducing the electrostatic repulsion forces between negatively charged cells (89). When during lentiviral transduction a 60 ng/ml polybrene was added to the TICAE cells, a MOI of 12.5 already resulted in a few cells emitting green fluorescent light. Therefore, we could determine that an appropriate concentration of polybrene is necessary for lentiviral transduction. Even a higher MOI could not lead to efficient transduction, if the appropriate concentration of polybrene was not present.

The self-assembled lentiviral particles containing mito-GFP induced a better transduction than the pre-made mito-GFP Loclight™ lentiviral particles. With the self-assembled lentiviral particles, a MOI of at least 1.4 was estimated. However, based on the efficient lentiviral transduction, the MOI was probably much higher. As such, approximately 80% of the TICAE cells were efficiently transduced. Still, an appropriate concentration of polybrene (8 µg/ml) was necessary for efficient transduction. Another study, has already indicated that HCAEC can be transduced with lentiviral particles containing GFP (86), however targeting the mitochondria was not yet performed in previous studies.

4.1.2. Efficient transduction with self-assembled TFAM shRNA lentiviral particles

The self-assembled lentiviral particles containing the TFAM shRNA (MOI > 1.4), together with 8 µg/ml polybrene, resulted in efficiently transduced TICAE cells, while the ready-to-use lentiviral particles did not lead to transduction of the TICAE cells. The cell medium of the transduced TICAE cells needed supplementation with HEPES and NAC in order to overcome poor cell growth and even cell death. This could be explained by an impairment of the oxidative phosphorylation, caused by silencing the TFAM gene and subsequent loss of mtDNA (69). Therefore, the transduced TICAE cells completely rely on anaerobic glycolysis to produce the necessary ATP. A consequence of the anaerobic glycolysis is an excessive production of lactic acid (63), which resulted in premature acidification of the cell medium. HEPES, an organic zwitterionic buffering agent, was added to help maintain the physiological pH. The cell medium was also supplemented with NAC, an antioxidant leading to increased glutathione levels to keep cells viable. However, the TICAE cells did not receive pyruvate and uridine, substrates of anaerobic glycolysis, in the culture medium probably leading to exhaustion of the cellular energy levels. This can be the reason why the cells died a few weeks after lentiviral transduction.

4.1.3. Efficient transduction with self-assembled TFAM cDNA lentiviral particles

With western blot, it was verified that the TICAE cells were efficiently transduced with a cDNA construct containing the TFAM gene (MOI > 1.4) and 8 µg/ml polybrene. For producing the lentivirus, the 293T/17 producer cells assembled the lentiviral constructs. The high amount of TFAM protein present in the producer cells can be explained by the high amount of lentiviral construct plasmids added to the 293T/17 producer cells. However, the cDNA lentiviral construct did not contain a selection marker. Therefore, no selection of the transduced TICAE cells was possible. In this way, a cell population which only contains the modified TICAE cells could not be obtained. A few weeks after lentiviral transduction, the TICAE cells died. Uridine and pyruvate were added as substrates to the cell medium to produce the necessary ATP. Still, this could not save them. This may be explained by a too high concentration of TFAM due to the constitutively expressed TFAM cDNA. As said before, TFAM can provide stabilization by covering the entire mtDNA as a scaffold. However, Kanki *et al.*

(2004) indicated that a too high amount of TFAM can result in a decreased mtDNA amount, which will eventually lead to an impaired oxidative phosphorylation (90).

4.2. Endothelial cell lines modified with ethidium bromide and rosiglitazone

The optimization of the lentiviral transduction protocols took longer than expected. Due to this reason, we decided to set up the pharmacological modification of the TICAE cells in parallel. Ethidium bromide and rosiglitazone were expected to respectively, diminish or enhance mitochondrial content.

4.2.1. Mitochondrial modification with ethidium bromide

Several human cell lines (e.g. osteosarcoma (63), promyelocytic leukemia (91), thyroid cells (67), neuroblastoma (92) and human neuronal cells (68)) were already efficiently modified with ethidium bromide to establish a cell line lacking mtDNA, the so called ρ^0 cells. Moreover, bovine aortic endothelial cells were also modified with ethidium bromide (93, 94). However, no publications were found that dealt with HCAEC. Ethidium bromide is a DNA intercalating agent damaging the DNA. When low concentrations are used, ethidium bromide preferentially damages the mtDNA which is more vulnerable than the nuclear DNA, owing to the poor repair mechanisms. Hereby, ethidium bromide interferes with enzymes of the DNA replication machinery leading to eventual disappearance of the mtDNA after a couple of cell divisions, whereas nuclear DNA is maintained (62). The mtDNA copy number per cell is dependent on cell type, approximately a cell contains between 1 000 and 2 000 copies of mtDNA (68). After a 7-week cultivation (21 rounds of cell division) of the TICAE cells with 50 ng/ml ethidium bromide, less than 1 copy of mtDNA per cell was left. However, no complete removal of mtDNA was achieved. Therefore, a rise in mtDNA was observed after two weeks cultivation of the TICAE cells without ethidium bromide. As reported in literature the amount of mitochondrial DNA in the human VA2-B cell line was decreased with 90% after three days of 20 ng/ml ethidium bromide addition. Upon removal of ethidium bromide from the cell culture medium, the amount of mtDNA was restored in 5 days (95). Also, in a human neuronal cell line treated with 50 μ M of ethidium bromide, an increase in mtDNA was detected after withdrawal of ethidium bromide (68). However, throughout all the experiments we performed for testing the radiosensitivity of the modified TICAE cells (week 14 and 17), the mtDNA copy number remained lower than 10. The other ethidium bromide concentrations, e.g. 12.5 and 25 ng/ml, did not diminish mtDNA copy number to less than one copy per cell. However, it is still not completely elucidated, if these low concentrations of ethidium bromide are not damaging the nuclear DNA (63).

The mtDNA partly encodes for the electron transport chain, which is crucial for the efficient production of necessary ATP via oxidative phosphorylation in order for the cells to survive (63). Since the loss of mtDNA leads to impairment of the oxidative phosphorylation, the cell medium needed to be supplemented with nutrients, such as L-glutamine and sodium pyruvate to keep producing the necessary energy via anaerobic glycolysis, the cellular back-up of the oxidative phosphorylation. Due to mitochondrial aberration dihydroorotate dehydrogenase, responsible for the pyrimidine biosynthesis, is also impaired. Therefore, uridine was added to retain pyrimidine biosynthesis (62, 63). So when the necessary supplements were added, the ethidium bromide modified TICAE cells remained viable in cell culture. However, the energy production of the modified cells is completely

dependent on anaerobic glycolysis, which resulted in increased lactic acid release in the culture medium. HEPES was added to the cell culture to help buffering the medium. Kukat *et al.* (2008) also showed increased lactate production in a human osteosarcoma cell line treated with ethidium bromide (63). The damaged electron transport chain, upon ethidium bromide addition, was also suggested by the PCR of the remaining mtDNA. It was indicated that the genes encoding for complex I, III, IV and V of the electron transport chain were diminished, except the COX III genes encoding for a part of complex IV was enhanced. Complex I, III, IV and V were impaired because these complexes were partly encoded with mitochondrial DNA (55). The several coding regions of the mitochondrial DNA were influenced differently by ethidium bromide, which can be explained by heteroplasmy in mtDNA. Protein levels of the several electron transport chain complexes were not measured. Therefore, an impairment of the oxidative phosphorylation could only be suggested. After deprivation of glucose, the ethidium bromide cells did not remain in culture, which is another indicating that the oxidative phosphorylation can be impaired. Finally, by measuring the mitochondrial membrane potential and the ATP level without ionizing radiation exposure, it was functionally confirmed that the oxidative phosphorylation was indeed impaired. A significantly decreased membrane potential and significantly lowered ATP levels were observed.

The depletion of mtDNA after ethidium bromide treatment resulted in a significant decline in the TFAM protein amount. As said before, TFAM is packaging the entire mtDNA. When less mtDNA is present, also less TFAM protein is necessary to cover the mtDNA (88, 90). In literature, a HeLa cell line which was depleted of mtDNA by ethidium bromide, also contained low amounts of the TFAM protein (96). Furthermore, Seidel-Rogel *et al.* (2002) showed that TFAM was reduced to the same extent as the mtDNA after ethidium bromide cultivation of HeLa cells (69). Moreover, ethidium bromide cultivation resulted in an increase of misidentities, meaning that more point mutations could be observed. This is confirming the DNA damaging effect of ethidium bromide.

The reductive capacity of NAD(P)H in the modified cells was examined with the MTT assay. Hereby, the effect of ethidium bromide on the NAD(P)H dehydrogenases (complex I) of the electron transport chain was further elucidated. The ethidium bromide modified cells, show a decreased reductive capacity probably due to the impairment of complex I. However, besides the presence in the mitochondria, other NAD(P)H enzymes are generated in the endoplasmic reticulum, endosomes, lysosomes and plasma membrane (77). So, this decrease could not solely be attributed to complex I of the electron transport chain.

Finally, we also evaluated whether the functionality of the endothelial cells was preserved after ethidium bromide cultivation. The amount of VE-cadherin adhesion proteins and the doubling time remained the same. However, after addition of ethidium bromide the LDL uptake was significantly diminished. This indicated that ethidium bromide can have an influence on endothelial cell functioning.

4.2.2. Mitochondrial modification with rosiglitazone

Rosiglitazone, a thiazolidinedione, is an activator of PPAR γ which regulates gene expression by binding to DNA response elements (97, 98). Several studies have indicated that rosiglitazone induces mitochondrial biogenesis in human adipocytes, regardless of their PPAR γ activity. However, the exact

underlying mechanism is not fully elucidated (65, 99, 100). After 7 weeks, the continuous supply of 50 μ M rosiglitazone to the TICA cells caused a 305.6% increase in mtDNA. However, at week 14 and 17, when the experiments were performed for testing radiosensitivity, the mtDNA copy number was decreased a lot. Rosiglitazone may first enhance mitochondrial biogenesis, and after a longer cultivation period rosiglitazone may induce the same phenotype as observed after ethidium bromide cultivation.

The several coding regions of the mtDNA were differently affected by rosiglitazone, in almost the same manner as described with ethidium bromide. Except for the COX I gene (involved in complex IV) and ND2 gene (involved in complex I), which were slightly increased after rosiglitazone cultivation compared to the control TICA cells, while in ethidium bromide modified TICA cells these genes were diminished. Furthermore, the ND5 gene encoding for complex I showed a larger decrease compared with ethidium bromide. These results suggested an impairment of the electron transport chain, leading to a diminished oxidative phosphorylation. This was in line with the glucose deprivation assay. At day 1, a decreased amount of rosiglitazone modified TICA cells remained in culture, indicating that the oxidative phosphorylation was not capable of producing the necessary ATP. However, at day 2 and 3, a slight increase in the amount of cells was observed compared to the control TICA cells. Probably, the cells have adapted to the induced stress. A study investigating the rat mitochondria from the liver after thiazolidinedione treatment, also showed a reduced aerobic respiration, because of an inhibition of complex I (101). The mitochondrial membrane potential and the ATP production were slightly decreased, however not significant. Moreover, the cell medium of the rosiglitazone TICA cells showed no signs of acidification. Probably, this might indicate that impairment of the oxidative phosphorylation is present, however not explicit enough or that the seriously damaged mitochondria are removed from the cell by mitophagy, leading to increased activation of the remaining mitochondria to keep producing the necessary ATP (102).

The nuclear-encoded TFAM protein in the TICA cells was not affected by rosiglitazone cultivation. In contrast to the reported response in adipocytes, where a small increase in the TFAM protein was observed after 6 days of rosiglitazone treatment (65). Nevertheless, Pardo *et al.* (2011) demonstrated that the expression of TFAM in adipocytes remained unchanged after 15 days of rosiglitazone treatment given to mice (99). Maybe no difference in TFAM levels was observed, because rosiglitazone was chronically administered to the TICA cells and therefore, had the time to adapt to the stress response.

As said before it is uncertain if the MTT assay can be used to make conclusions about the reductive capacity of complex I in the electron transport chain. However, after treatment with rosiglitazone the NAD(P)H reductive capacity is decreased after ethidium bromide treatment. This is in line with the diminished gene expression of the electron transport chain and the decreased amount of cells in culture after glucose deprivation, indicating an impairment of the oxidative phosphorylation.

Finally, it was elucidated if the rosiglitazone treatment influenced the endothelial cell functioning. The rosiglitazone modified TICA showed no difference in the uptake of LDL. Furthermore, a shorter doubling time was observed. However, the presence of VE-cadherin adhesion proteins was significantly reduced. This made it not entirely clear if rosiglitazone was negatively influencing the TICA cells on functional level.

4.3. Testing the radiosensitivity of the endothelial cells after X-ray exposure

Ionizing radiation causes the formation of unstable water radiolysis products, including O_2^* and OH^* which disappear in less than 10^{-3} seconds. Another water radiolysis product, H_2O_2 is more stable (53). However, it has been demonstrated that ionizing radiation increases the ROS levels several hours after exposure. Hereby, it was indicated that ionizing radiation stimulates the production of secondary ROS derived from endogenous sources (54). In this master's thesis, we hypothesized that mitochondria can be responsible for the production of secondary ROS after ionizing radiation exposure. In the previous paragraphs, it was suggested that ethidium bromide as well as rosiglitazone induced a dysfunction of the mitochondrial oxidative phosphorylation. The aim was to investigate if these modified TICA cells, had a differential response towards ionizing radiation exposure.

4.3.1. The mitochondrial membrane potential after X-ray exposure

The mitochondrial membrane potential is formed by the electrical gradient between the mitochondrial intermembrane space and matrix, and is determined by the activity of complex I until IV of the electron transport chain (103, 104). By measuring the mitochondrial membrane potential, the activity of the entire electron transport chain is measured, not the activity of the separate complexes. This gives a general view on electron transport chain functionality (105). The mitochondrial membrane potential is the driving force of, amongst others, the ATP and ROS production (103, 104). A lower mitochondrial membrane potential is due to a lower activity of the electron transport chain, which also results in lower amount of ATP production and ROS formation (104). Without ionizing radiation exposure, as well as after an X-ray dose of 0.05, 0.1, 0.5 and 2 Gy, the mitochondrial membrane potential is significantly decreased in the ethidium bromide modified TICA cells. This represents a lower activity of complex I until IV, which is in line with the diminished gene expression of complex I, III and IV due to ethidium bromide addition. Thus, the finding that the mitochondrial membrane potential was lowered, can confirm on functional level that the oxidative phosphorylation was impaired in ethidium bromide modified TICA cells. For the rosiglitazone modified TICA cells, it remained a suggestion that the oxidative phosphorylation was impaired because there is a reduction in mitochondrial membrane potential however not significant.

4.3.2. Cellular and mitochondrial ROS production after X-ray exposure

With CM- H_2DCFDA , the amount of cellular ROS after X-ray exposure was determined in the control and modified TICA cells. Since CM- H_2DCFDA is oxidized by several types of ROS (such as H_2O_2 and OH^*), the results give an indication of the general amount of ROS inside the cell (106). When performing this assay, we encountered a couple of difficulties: the CM- H_2DCFDA dye was very sensitive to light and focusing of the fluorescence microscope for an entire 96-well plate was not possible. As explained in paragraph 3.4.1, no comparison could be made between irradiation doses, therefore we could only compare the different cell lines within a radiation dose. After a 2 Gy dose of ionizing radiation exposure, a significantly decreased amount of ROS was produced in the ethidium bromide modified TICA cells, which were characterized by the decreased mtDNA content and an impaired oxidative phosphorylation. Therefore, we propose that mitochondria play a role in secondary ROS formation after X-ray exposure and that the underlying mechanism involves a dysfunctional oxidative phosphorylation. The decreased ROS production in the ethidium bromide

modified TICA cells after ionizing radiation exposure, can be explained by a diminished oxidative phosphorylation. The rosiglitazone modified TICA cells, showed no diminished ROS production after 2 Gy X-ray exposure. At the time point of the CM-H₂DCFDA assay, the mtDNA level in the rosiglitazone modified TICA cells was also decreased and an impaired oxidative phosphorylation was suggested. However, no decrease in cellular ROS production after ionizing radiation exposure can probably be explained by the fact that the oxidative phosphorylation was not dysfunctional enough or that the most dysfunctional mitochondria were removed, leading to increased activity of the remaining mitochondria. In literature, it has already been indicated that without a functional electron transport chain, mitochondria are incapable of generating ROS (107). The amount of cellular ROS was again measured after the addition of tBHP, which lead to an increase in cellular ROS production. For the ethidium bromide and the rosiglitazone modified cell lines a significantly diminished increase in ROS formation was demonstrated after a 2 Gy X-ray dose. This indicated that the ethidium bromide and rosiglitazone modified TICA cells had a higher antioxidant capacity to encounter the formed ROS after ionizing radiation exposure. Through the ethidium bromide and rosiglitazone treatment, the TICA cells have already been exposed to a stress factor. Therefore, the cells might have adapted to this damaging agent by increasing antioxidant capacity.

Furthermore, the amount of mitochondrial produced superoxide (O₂[•]) in the control and modified TICA cells was examined. In the ethidium bromide and rosiglitazone modified TICA cells, the basal level of ROS was significantly less compared to the control TICA cells. After a 2 Gy X-ray dose, the amount of O₂[•] produced by the ethidium bromide modified TICA cells remained significantly lower. This decrease may be the result of less O₂[•] production after ionizing radiation exposure or a diminished mitochondrial activity. Also for the MitoSOX assay, no comparison could be made between irradiation doses. In contrast to the cellular ROS levels, the O₂[•] levels in the rosiglitazone modified TICA cells were significantly decreased after an X-ray dose of 0.1, 0.5 and 2 Gy. However, the fluorescent signal detected with fluorescence microscopy was very low. As such, a lot of background was present in the pictures. This can result in an improper analysis with the FIJI and R Studio software. Therefore, these MitoSOX data were not so reliable. After MitoSOX detection rotenone was added, which normally leads to an increase in O₂[•] production. Probably, a too low rotenone concentration was added because no difference was observed, neither for the control as the modified TICA cells. There is also another reason why the MitoSOX results should be interpreted with caution. Due to the reaction of MitoSOX with O₂[•], fluorescent hydroxymitoethidium is formed. However, also nonspecific oxidation products are released, which have an overlapping fluorescence spectra with the specific hydroxymitoethidium. In conclusion, the fluorescence formed in the mitochondria is probably not a reliable indicator (106).

4.3.3. The ATP production after X-ray exposure

Without ionizing radiation exposure, the ATP level in ethidium bromide modified TICA cells was significantly diminished. Due to the impairment of the oxidative phosphorylation by ethidium bromide, no sufficient amount of ATP could be produced. This was already reflected in the decreased doubling time of the ethidium bromide modified TICA cells. In literature, an osteosarcoma cell line of which the mtDNA was depleted with 86% also showed a decreased mitochondrial membrane potential and the amount of ATP was decreased with 90% (105). After a 2 Gy X-ray dose, the ATP production in the control TICA cells was not changed, while, in the ethidium bromide modified TICA cells the ATP production was significantly lowered. This observation is in line with a decrease in

the mitochondrial membrane potential in the ethidium bromide modified TICA cells 24 hours after a 2 Gy X-ray dose. For the rosiglitazone modified TICA cells, an increased ATP production was only observed 24 hours after exposure to a 2 Gy X-ray dose. However, the mitochondrial membrane potential was not significantly changed 24 hours after a 2 Gy X-ray dose.

4.3.4. The amount of DNA double strand breaks after X-ray exposure

A consequence of ionizing radiation exposure is the formation of DNA double strand breaks (DSBs). One of the first steps in the repair mechanism is the phosphorylation of histone H2AX, resulting in the formation of γ H2AX foci (108). With fluorescence microscopy, the γ H2AX foci were visualized. The ethidium bromide and rosiglitazone modified TICA cells had a lower amount of DSBs. For the ethidium bromide modified TICA cells the lower amount of damage can be explained by less production of cellular ROS or a decreased energy state. A lower amount of ATP in the ethidium bromide modified TICA cells was already indicated (see 4.3.3.). For the rosiglitazone modified TICA cells, no decreased ATP production was visible, so the decrease in DSBs in the rosiglitazone modified TICA cells could not be explained by diminished ATP production. The γ H2AX foci in the irradiated cell lines disappear with time, this represents an effective DSB repair response. However, as well for the ethidium bromide as the rosiglitazone modified TICA cells, the repair response was slowed down compared to the control TICA cells. This was indicated by the fact that after 4 and 24 hours following radiation exposure more γ H2AX foci remained. This suggests that due to the pharmacological interference, the repair mechanism has been damaged or stalled. Amongst others, 53BP1 is recruited in one of the following steps of the DSB repair mechanism (108). This explains why less 53BP1 foci were visualized compared to the γ H2AX foci. The decrease in 53BP1 foci with time, also indicates an efficient DSB repair response. However, for rosiglitazone modified TICA cells the repair response was not so efficient, because more 53BP1 foci remained after 24 hours compared to the control. After 24 hours following ionizing radiation exposure, the DSB are not completely repaired. This gives us an explanation why the ATP levels are still increased after 24 hours. Moreover, 30 minutes after an X-ray dose of 2 Gy, the repair response in the rosiglitazone modified TICA cells was not initiated, which is in line with a significant decrease of the ATP levels at 30 minutes. The peak in 53BP1 foci occurred after 4 hours instead of 1 hour following ionizing radiation exposure, indicating that the repair response was initiated with delay.

5. Conclusion

To date, no mechanism is found to fully clarify the endothelial response towards ionizing radiation. A couple of research groups focus on different aspects of the endothelial cells trying to resolve the mechanism, e.g. genomic instability, inflammation, cell senescence and cell death. Not one of the aforementioned aspects gave a decisive answer. Our research group in the SCK•CEN focused on a new point of view, namely the mitochondria. We consider them as a new target in the radiation-induced endothelial cell response.

The main goal of this research project was to verify if mitochondria influence endothelial cell dysfunction after ionizing radiation exposure. The mitochondrial content of telomerase immortalized coronary artery endothelial (TICAE) cells was modified with lentiviral transduction and pharmacological incubation. In order to efficiently transduce TICAE cells with lentiviral particles, it was necessary to use a sufficient MOI together with an appropriate concentration of polybrene. A diminished and enhanced amount of mitochondria were supposed to result from transduction of the mitochondrial transcription factor (TFAM) gene in, respectively, a shRNA and cDNA lentiviral construct. It seemed that both the diminishment, as well as the enhancement of the TFAM gene, resulted in an impairment of the oxidative phosphorylation. Therefore, supplementation with HEPES, NAC, uridine, L-glutamine and sodium pyruvate was necessary to keep the cells viable. However, no efficiently transduced TICAE cells remained viable in culture. In future experiments it is better to work with inducible instead of constitutive lentiviral constructs to overcome cell death after multiple cell divisions. With a QuickTiter™ Lentivirus Titer p24 ELISA, the lentiviral titer of the self-assembled lentivirus can be determined more precisely. Furthermore, the lentiviral particles need further characterization to define if the enhancement or diminishment of the TFAM gene is actually in- or decreasing the mitochondrial content. This can be done by the same experiments as performed during the characterization of the ethidium bromide and rosiglitazone modified TICAE cells. However, the focus should be more on defining the amount of mitochondria and the functionality of the electron transport chain. Moreover, it is important that the lentiviral constructs contain a selection marker to efficiently select for transduced cells.

In parallel, the TICAE cells were modified either with ethidium bromide, that is known to preferentially intercalate in the mitochondrial DNA (mtDNA), or rosiglitazone, that would increase mitochondrial biogenesis. After seven weeks of cultivation, the amount of mtDNA in the ethidium bromide modified TICAE cells was reduced to less than one copy number per cell. However, because mtDNA was not completely eliminated we could not speak of p^0 cells in the strict sense. In the ethidium bromide modified TICAE cells, an impairment of the oxidative phosphorylation could be confirmed. However, for the rosiglitazone modified TICAE cells this impairment was only a suggestion. Moreover, a clear effect of rosiglitazone on the mtDNA was not determined. After seven weeks, a significant decrease in mtDNA was observed, while, after 14 and 17 weeks the mtDNA was again decreased to lower levels as the control TICAE cells. Therefore, we proposed that both ethidium bromide and rosiglitazone could have resulted in the same phenotype with a decreased amount of mtDNA and an impaired oxidative phosphorylation. Further experiments are needed to confirm this suggestion. Firstly, it is important to verify on protein level, by western blot or functional, by inhibition of the several complexes (e.g. rotenone an inhibitor of complex I), if the

mitochondrial electron transport chain is actually impaired after treatment with ethidium bromide or rosiglitazone. Furthermore, the influence of rosiglitazone on the mtDNA must be followed up in function of time to verify if rosiglitazone is indeed diminishing mtDNA after longer incubation times. To optimally verify the effects of ethidium bromide and rosiglitazone cultivation on the TICA cells, deep genomic sequencing can be performed to identify all genomic (mitochondrial and nuclear) modifications, even the smallest deletions and insertions. Moreover, the morphological changes in the modified mitochondria can be determined with transmission electron microscopy.

By exposing the modified TICA cells to X-rays, a correlation between mitochondrial presence and radiosensitivity could be examined. After an X-ray dose of 2 Gy, the ethidium bromide modified TICA cells produced a lower amount of secondary cellular ROS. The lower ROS production can result from a decreased mitochondrial membrane potential after ethidium bromide modification, indicating an impairment of the oxidative phosphorylation. However, no decrease in cellular ROS production after ionizing radiation exposure was observed for the rosiglitazone modified TICA cells, indicating that the oxidative phosphorylation was not dysfunctional enough or that the most dysfunctional mitochondria were removed, leading to increased activity of the remaining mitochondria. The CM-H₂DCFDA and MitoSOX assay had a considerable amount of limitations, e.g. difficult to perform and depended on many interfering factors. Therefore, in future experiments the amount of cellular ROS will be properly determined with another assay, namely ROS-Glo H₂O₂ assay (Promega, Leiden, The Netherlands). The antioxidant capacity will further be investigated after X-ray exposure with western blot for antioxidant proteins and with kits, e.g. GSH/GSSG-Glo assay.

Due to the lower level of ROS formed or due to lower energy state, less DNA double strand breaks (DSBs) were formed. Therefore, we could suggest that by diminishing the activity of the mitochondrial electron transport chain, less ROS are produced which will eventually lead to lower levels of DSBs. In future experiments, the ATP assay will be performed for every radiation dose, with appropriate normalization of the amount of cells. In literature, it has been indicated that through the generation of free radicals, ROS can induce DNA damage which can eventually lead to accelerated apoptosis and senescence (59). Therefore, after X-ray exposure the CellTox Green assay will determine at which time points cell death will occur. When the specific time points are determined, the type of cell death will be verified with dedicated kits to examine necrosis and early and late apoptosis. In this way, it can be determined if the amount of cell death is also decreased when oxidative phosphorylation is impaired. In this way, the ROS formation can be related to the other characteristics of atherosclerosis.

In conclusion, it was suggested that mitochondria actually have an effect on the radiosensitivity of endothelial cells. However, the underlying mechanism is not completely elucidated. We suggested that an impairment of the oxidative phosphorylation is involved in reducing the ROS production, which will eventually result in lowered DSBs. However, further research is necessary. Although ethidium bromide addition results in an efficient decline of mitochondrial content, not a complete representative population is formed because the mutagenic effects of ethidium bromide on the nuclear DNA cannot be excluded. Moreover, it was found that ethidium bromide, as well as rosiglitazone, caused a functional impairment of the endothelial cells. Therefore, in future experiments it is better to use lentiviral constructs to transfect the TICA cells with TFAM shRNA and cDNA to get a more representative endothelial cell line with a, respectively, diminished and

enhanced, mitochondrial content. The already made TICAE cells transduced with mito-GFP can be further transduced with TFAM shRNA or cDNA lentiviral particles. In this way, the modified TICAE cells can rapidly be characterized for mitochondrial content.

To end, on the long term this experimental research can have implications for the radiation protection of healthy tissue. By pharmaceutical inhibition of the electron transport chain in the mitochondria for only a short period of time, e.g. during radiotherapy, the amount of ROS can be limited resulting in less cell damage. However, due to the important role of mitochondria in energy production and other physiological processes, this effect needs to be reversible. On the other hand, pharmaceutical enhancement of the electron transport chain, e.g. in tumor cells, can increase the amount of ROS production resulting in a more damaging effect during radiotherapy.

7. References

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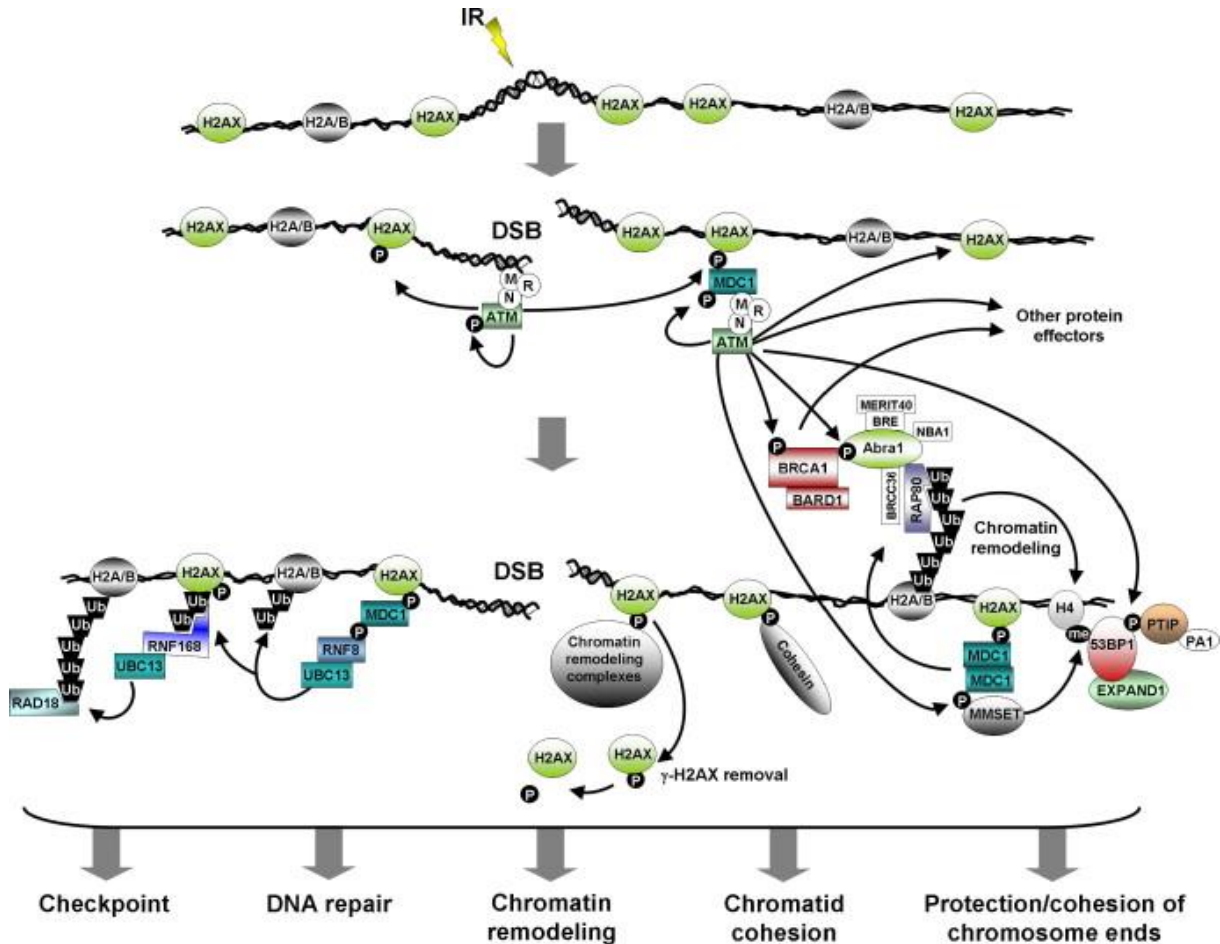
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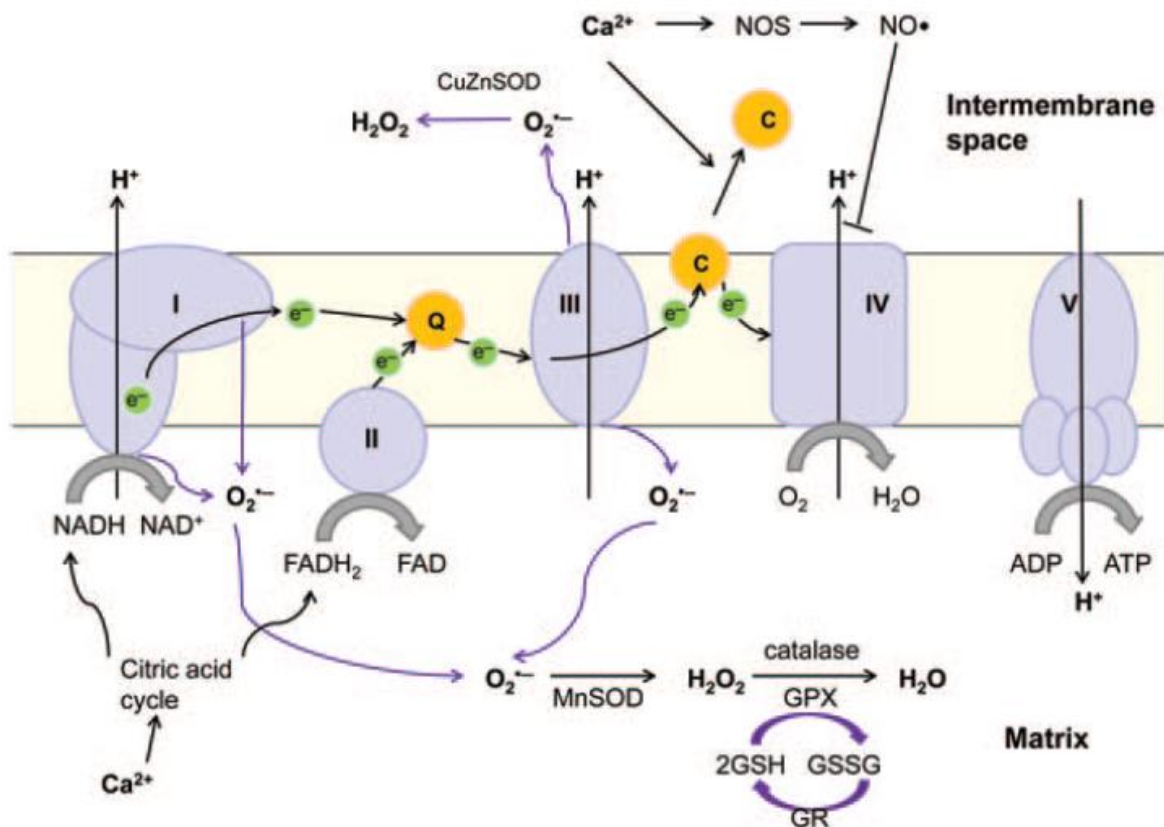
8. Supplements

Supplementary figure 1: The DNA damage response after ionizing radiation exposure (108).



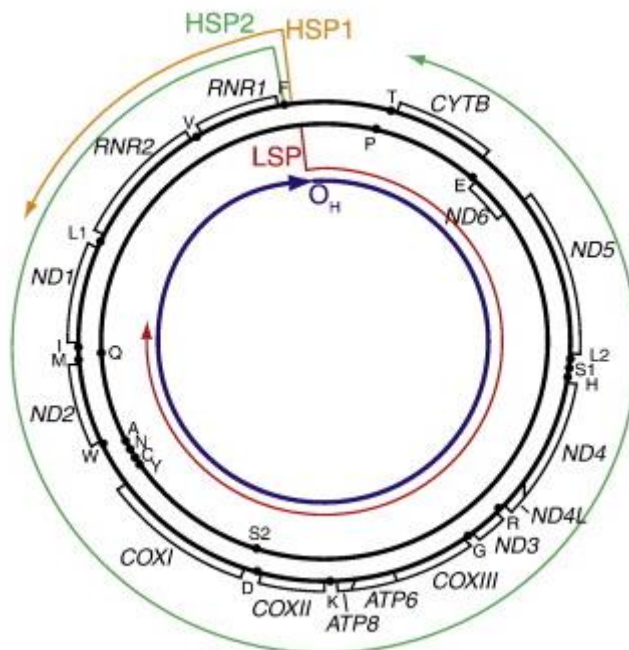
The DNA damage response signaling pathway is activated after a double strand DNA break (DSB) induced by ionizing radiation exposure. The Mre11, Rad22 and Nbs1 protein (MRN) complex binds to the DSB, which leads to the recruitment of ataxia telangiectasia mutated (ATM) kinase. The histone H2A variant X (H2AX) is phosphorylated by ATM, which allows binding of the mediator of DNA damage checkpoint protein 1 (MDC1). This results in the enrollment of other proteins, including the MRN complex and ATM kinases. By increased phosphorylation of H2AX, other DNA repair proteins concentrate at the DSB, amongst others the p53 binding protein 1 (53BP1). MDC1 can also recruit RNF8, which initiated poly-ubiquitylation of histone H2 at the site of the DSB. A second wave of protein accumulation will follow. The formation of foci is stimulating DNA repair pathways, such as non-homologous end-joining (NHEJ) or homologous recombination (HR). Also checkpoint activation can be activated (108, 109). (P: phosphate, Ub: ubiquitin, me: methyl, M: MRE11, N: NBS1, R: RAD50. To simplify, single histones are shown)

Supplementary figure 2: The oxidative phosphorylation in mitochondria (52).



On the inner mitochondrial membrane, complex I until IV form the electron transport chain (ETC). At this site, oxidative phosphorylation takes place. Electrons are supplied by nicotinamide adenine dinucleotide hydrate (NADH) at complex I (NADH dehydrogenase) and flavine adenine dinucleotide hydrate (FADH) at complex II (succinate dehydrogenase). The electrons are transferred to ubiquinone (Q), which will transfer them to complex III (ubiquinone cytochrome C oxidoreductase). Via cytochrome C, the electrons are delivered to complex IV (cytochrome c oxidase) leading to formation of H₂O from O₂. As electrons are transferred, protons are pumped to the intermembrane space to create a gradient, more specifically the mitochondrial membrane potential. Due to positive potential created in the intermembrane space, protons (H⁺) flow through complex V (ATP synthase) leading to the formation of adenosine triphosphate (ATP) from adenosine diphosphate (ADP). Through the leakage of electrons from complex I and III superoxide (O₂^{•-}) is formed and dismutated to hydrogen peroxide (H₂O₂) by manganese (MnSOD) or copper zinc superoxide dismutase (CuZnSOD). Catalase or glutathione peroxidase (GPX) can lead to reduction of H₂O₂ to H₂O. GPX catalyzes the oxidation of reduced glutathione (GSH) to form H₂O. If H₂O₂ is not reduced, it can form the hydroxyl radical (OH[•]) (52).

Supplementary figure 3: Diagram of the mitochondrial genome organization (88).



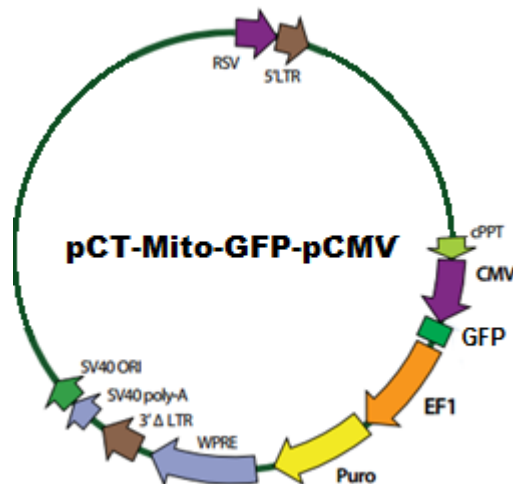
Mitochondrial DNA contains three promoters; the light strand promoter (LSP), the heavy strand promoter 1 (HSP1) and the heavy strand promoter 2 (HSP2). The pre-mRNA generated from LSP is represented in red, from HSP1 in orange and from HSP2 in green. The location where the initiation for the first-strand DNA replication takes place is indicated with O_H. The LSP controls the RNA synthesis of the light strand encoding for one protein-coding gene, namely NADH-ubiquinone oxidoreductase chain 6 (ND6), eight tRNAs and generates the primer for the first strand mtDNA replication at O_H. The HSP1, which is located 150 base pairs upstream of the LSP, controls the expression of the first heavy strand containing mRNA for two tRNAs and two ribosomal RNAs (RNR1 and RNR2). The HSP2 is situated another 60 base pairs upstream of the HSP1. It encodes for the remaining 12 tRNAs and 12 protein coding transcripts; ND1, ND2, cytochrome C oxidase subunit I (COXI), COXII, ATP synthase subunit 6 (ATP6), ATP8, COXIII, ND3, ND4L, ND4, ND5 and cytochrome B (CYTB) (88).

Supplementary figure 4: The premade mito-GFP lentiviral construct.



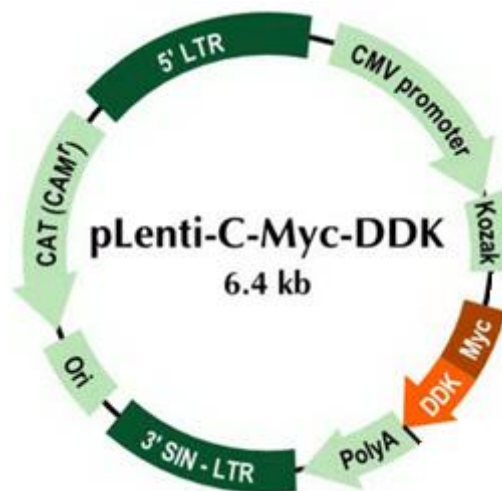
The expression construct of the LocLight™ sub-cellular labeling lentivirus contains the mito-GFP together with its super strong cytomegalovirus (suCMV) promoter and a blasticidin selection marker, with its respiratory syncytial virus (RSV) promoter.

Supplementary figure 5: The expression vector for assembling the self-made mito-GFP lentiviruses.



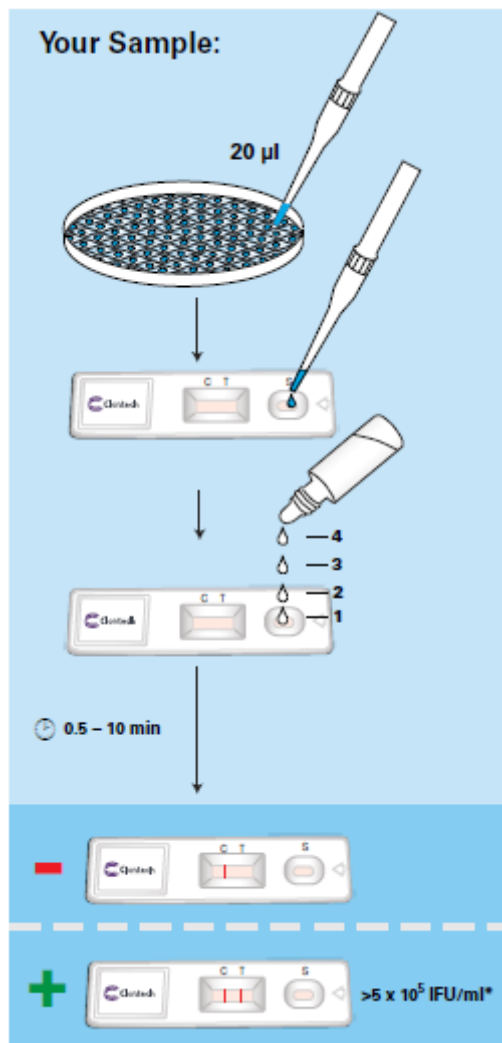
The mito-GFP construct will be integrated next to its CMV promoter region. Furthermore, the construct contains: a puromycin selection marker (Puro) together with its elongation-factor 1 (EF1) promoter, a woodhuck hepatitis post-transcriptional regulatory element (WPRE) to enhance stability of the vector, a central polypurine tract (cPPT) which allows the virus to penetrate the nuclear membrane, 5' and 3' long terminal repeats (LTR) that flank the insert to facilitate integration in the host genome, simian virus 40 (SV40) poly-A signal to terminate transcription and the SV40 origin of replication (ORI) (110, 111).

Supplementary figure 6: The pLenti-C-Myc-DDK expression vector for assembling the TFAM cDNA lentivirus.



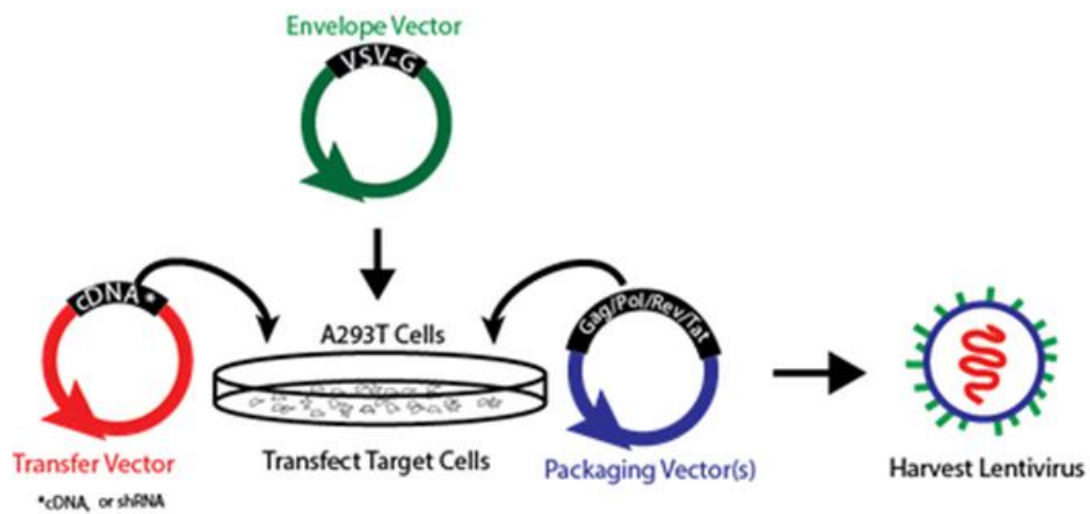
The TFAM cDNA construct can be integrated at the Kozak sequence, next to its CMV promoter region. When expressed, the TFAM protein is tagged with a Myc-DDK tag. The origin of replication (Ori) is an integrated place where the replication will start and the poly(A)-tail is required for termination of transcription. The 3' and 5' LTRs, flank the expression construct to facilitate integration in the host genome (111).

Supplementary figure 7: The Lenti-X GoStix™ protocol of Clontech Laboratories Inc. (Leusden, The Netherlands).



To the sample well (S) of the GoStix cassette, 20 µl lentiviral supernatant is added. Next, 4 drops of the Chase buffer are added to the sample well. It can take between 0.5 and 10 minutes for a band to develop. A control band will always appear (C), and a test band (T) will appear if your sample contains sufficient lentivirus.

Supplementary figure 8: Schematic overview of the lentiviral assembling by 293T/17 cells.



Three plasmids; a transfer vector containing the expression construct of interest, a packaging vector and an envelope vector were added to the 293T/17 producer cells. After a brief incubation period, the supernatant containing the virus is used to transduce the TICA cells.

