The role of p66ShcA-TLR9 signaling in myocardial remodeling and innate immune responses

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To my family

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Sincerely,

Anton Baysa

Drammen, May 2020

List of included papers

Paper I

The p66ShcA adaptor protein regulates healing after myocardial infarction.

Anton Baysa, Julia Sagave, Andrea Carpi, Tatiana Zaglia, Marika Campesan, Christen Dahl, Dusan Bilbija, Maria Troitskaya, Lars Gullestad, Marco Giorgio, Marco Mongillo, Fabio Di Lisa, Jarle Vaage, Guro Valen.

Basic Research in Cardiology, 2015 Mar;110(2):13. doi: 10.1007/s00395-015-0470-0. Epub 2015 Feb 14.

Paper II

Toll-like receptor 9 signaling after myocardial infarction: Role of p66shcA adaptor protein.

Anton Baysa, Azzam Maghazachim, Kristin Larsen Sand, Marika Campesan, Tania Zaglia, Marco Mongillo, Marco Giorgio, Fabio Di Lisa, Lars H. Mariero, PhD Jarle Vaage, Guro Valen, Kåre-Olav Stensløkken.

Manuscript, to be submitted.

Paper III

Release of mitochondrial and nuclear DNA during on-pump heart surgery: Kinetics and relation to extracellular vesicles.

Anton Baysa, Anton Fedorov, Kirill Kondratov, Arno Ruusalepp, Sarkis Minasian, Michael Galagudza, Maxim Popov, Dmitry Kurapeev, Alexey Yakovlev, Guro Valen, Anna Kostareva, Jarle Vaage, Kåre-Olav Stensløkken.

J Cardiovasc Translational Res. 2018, Dec 12. doi: 10.1007/s12265-018-9848-3.

Selected abbreviations

AP1	Activator protein 1.
BTK	Bruton's tyrosine kinase
CABG	Coronary artery bypass grafting
CPB	Cardiopulmonary bypass
DAMP	Damage-associated (or danger-associated) molecular pattern
DHX	DExD-H box helicase
DNAse	Deoxyribonuclease
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HDAC2	Histone deacetylase 2
HEK293	Human embryonic kidney 293 cells
IFN-α1	Interferon-alpha 1
IFN-β1	Interferon-beta 1
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
MAVS	Mitochondrial antiviral-signaling protein
mtDNA	Mitochondrial deoxyribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
nDNA	Nuclear deoxyribonucleic acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nucleotide-binding oligomerization domain (NOD)-like receptor
NSTEMI	Non-ST-segment elevation myocardial infarction
qPCR	Quantitative polymerase chain reaction
RAGE	Receptor for advanced glycation end products
RNAseH1	Ribonuclease H1
ROS	Reactive oxygen species
Rpl 32	60S ribosomal protein L32

ShcA	Src-homology-2-domain-containing transforming adapter protein A
STEMI	ST-segment elevation myocardial infarction
TBK1	TANK-binding kinase 1
TLR	Toll-like receptor
Tmem173	Transmembrane protein 173
TNFα	Tumor necrosis factor-alpha
TREX1	Three prime repair exonuclease 1
Unc93b1	Unc-93 homolog B1 (C. elegans)

General introduction

Clinical perspectives

Ischemic heart disease: Global and national status

Ischemic heart disease is a leading mortality cause worldwide(1)(2). Moreover, disability due to ischemic heart disease has grown faster than the mortality in most regions since 1990(3). However, there are large variations in the mortality and morbidity between countries and regions(4). The developed Western countries show the least ischemic heart disease mortality whereas Eastern Europe and post-Soviet states demonstrate the highest mortality from ischemic heart disease(5). Ischemic heart disease mortality peaked in Western Europe in the 1970s followed by a steady decrease(4). However, the prevalence of ischemic heart disease has been increasing, partly due to an older population(3). The same trend has occurred in Norway, where mortality from ischemic heart disease has been reduced by 50 % since the peak in the 1970s(4).

Ischemic heart disease: Emerging trends and therapy limitations

Some factors may offset further decline or stabilization in ischemic heart disease burden. Despite the reduction in smoking and saturated fat consumption, risk factors such as obesity and diabetes mellitus type II are likely to rise in the Nordic region in the future(6). Patients with ischemic heart disease often suffer from concomitant diabetes mellitus and arterial hypertension which pose independent risks of myocardial injury and mitigate available treatment options(7). Consequently, the prevalence and burden of ischemic heart disease may rise in developed countries, including Norway.

Inadequate coronary blood supply (myocardial ischemia) leads to cardiomyocytes necrosis (myocardial infarction). The adverse cardiac remodeling occurs post-infarction and encompasses loss of functional myocardium, the formation of scar tissue, interstitial fibrosis, and reduced cardiac function, paving the road to heart failure(8,9). Management of ischemic heart disease is mainly supportive. Available pharmacological treatment may reduce the risk of complications, but not the progression of the disease(10). Myocardial revascularization is offset by reperfusion injury and later development of restenosis(11). The advanced prehospital patient care allows quick diagnosis and aggressive treatment but demands complex infrastructure and mainly available in the developed countries(12). Remote myocardial conditioning(8), gene therapy(13), cell and tissue engineering(14), and bio-compatible artificial heart(15) are still under development. Thus, neither progression of atherosclerosis nor progression of ischemic heart disease into ischemic heart failure can be efficiently terminated with available treatments. The search for new therapeutic targets is warranted.

Ischemic heart disease: Anti-inflammatory treatment

Inflammation is a crucial part of both myocardial ischemic damage and myocardial healing post-infarction, and thus a potential intervention target. Treatment of myocardial infarction with glucocorticoids is controversial due to the possible deterioration of myocardial healing in some patients(16)(17). Numerous classic non-steroidal antiphlogistic drugs demonstrated cardioprotective effects in animal models but failed in clinical studies(18). The broad-specter anti-inflammatory therapies cannot discriminate between the beneficial aspect of sterile inflammation during myocardial healing and maladaptive, damaging inflammation. Targeting specific inflammatory pathways at a specific stage of myocardial healing could be a better option to mitigate adverse cardiac remodeling (19).

Theoretical background

Myocardial healing post-infarction: role of inflammation

During an acute myocardial infarction, insufficient blood supply and oxygen delivery to cardiac tissue will lead to a dramatic drop in ATP production. Besides, the accumulation of waste products will further jeopardize the homeostatic environment leading to the death of cardiac cells. This is especially dangerous for cardiomyocytes, the contractile cell of the heart, which has close to no regenerating capacity. Depending on the time of the occlusion, a myocardial infarction will lead to an internal wound that needs to be healed.

Wound healing is an adaptive response to injury. Healing after myocardial infarction (myocardial healing) passes four stages: necrotic, inflammatory, proliferative, and scar remodeling(20). Adverse cardiac remodeling is thus the better term to define this process highlighting its maladaptive nature.

Necrotic cardiomyocytes release a plethora of cellular debris which triggers immune pathways and initiates recruitment of inflammatory cells into the infarcted area. Then immune cells infiltrate damaged tissue to remove the dead cells and to produce a microenvironment for the growth of granulation tissue(21). Clearance of dead cells and debris reduces pro-inflammatory signaling and stimulates fibroblast proliferation, angiogenesis, and production of granulation tissue(22). Granulation tissue maturates to form scar through collagen remodeling and myofibroblasts proliferation(23). Fibroblasts protect cardiomyocytes(24) by attenuating local inflammation(21). The extracellular matrix can also modulate cellular inflammatory responses(25). For instance, activated matrix metalloproteinases (MMPs) digest the collagen scaffold leading to chamber dilatation, in some cases to heart rupture, and contribute to cardiac fibrosis by deposition of denatured collagens.

Inflammation plays a crucial role during all stages of myocardial healing. Cardiomyocytes die upon inflammatory stimuli; immune cells degrade cellular debris and stimulate fibroblasts proliferation, while fibroblast and extracellular matrix modulate inflammatory responses and cardiomyocyte survival(26). This thesis is focused on upstream signaling events occurring

early during the inflammatory stage of myocardial infarction when the release of cellular debris triggers responses of the innate immune system.

Innate immunity: The danger theory

The innate immune system produces stereotypic and programmed responses to common molecular signatures of infectious agents, known as pathogen-associated molecular patterns (PAMPs). These molecules are recognized by pattern recognition receptors (PRRs) activating pro-inflammatory pathways and the recruitment of immune cells. The innate immune response is a first-line defense as it does not require pre-exposure to the pathogenic agent(27).

The innate immune system was first considered as a part of the defense against external threats by discriminating "self" from "non-self", the dominating theory of immune function(28). Later, it has been suggested that the immune system responds primarily to damage, which can be induced both by external and internal factors, the so-called "danger theory" (29),[32]. This theory postulates that endogenous and foreign cells and tissues can be tolerated if they are not damaging (fetal tissues, commensal bacteria) triggering the immune response only in case of damage (mechanical tissue injury, pathogenic microorganisms). Accordingly, the term "damage-associated molecular patterns" (DAMPs) was introduced(30). The danger theory is not universally accepted among immunologists as (i) not all immune responses are induced by damage and (ii) immune responses can cause damage per se, crediting the problem of causality(31). However, this theory is generally accepted when applied to innate immunity.

Innate immunity: TLR9 pathway

The Toll-like receptors (TLRs) are one of many PRR families within the innate immune system. Of 22 known TLR genes(32), 13 (TLR1-TLR13) are present in the mammalian genome(33,34). Each of these TLR genes encodes a receptor with affinity to the specific class of PAMPs or DAMPs(35). Five TLRs, namely TLR3, -7, -8, -9, and -13 recognize different types of nuclear acids(36,37). The DNA sequences with a high number of unmethylated CpG (cysteine and guanine) groups is a typical ligand for TLR9. CpG- rich motifs are hallmarks of bacterial DNA(38).

The TLR9-signaling pathway has two main adapter proteins: myeloid differentiation primary response 88 (MyD88), and Unc-93 homolog B1 (Unc93b1), the latter is an endoplasmic reticulum-resident chaperone protein and a key regulator of TLR9 trafficking(39). Under normal conditions, TLR9 is constantly transported from the endoplasmic reticulum to endolysosomes by Unc93b1(40). To avoid autoimmune responses TLR9 is excluded from the cell surface and silenced until the N-terminal half of ectodomain is cleaved off in the endolysosome(41). This step is dependent on proteolytic action of asparagine endopeptidase and cathepsins which sequentially cleaves and trims the N-terminal(42). Cleaved and ligand-bound TLR9 forms a homodimer(43). In immune cells, TLR9 conducts signals via MyD88-dependant (immediate response) and MyD88-independent (postponed response) pathways. TLR9 activates MyD88 and launches a phosphorylation cascade via interleukin-1 receptor-associated kinases 1 and 4 (IRAK1 and 4) to TNF receptor-associated factor 6 (TRAF6)(43).

TRAF6 activates mitogen-activated protein kinase 7 (MAP3K7) followed by activation of nuclear factor-kB (NF-kB) via inhibitors of nuclear factor kappa-B kinase (IKK) and activation of activator protein 1 (AP-1) via mitogen-activated protein kinase (MAPK) pathways(44). NF-kB and AP1 rapid-acting primary transcription factors initiate inflammation, cell stress response program, or cell proliferation, depending on cell type and pathophysiological situation(39). Alternatively, signals from activated TLR9 can be wired through the MyD88-independent pathway via activation of TIR-domain-containing adapter-inducing interferon-β (TRIF) TRAF3. TRAF3 activates IKK-ε followed by stimulation of interferon regulatory factors 3 and 7 (IRF3, 7) leading to interferon secretion and T-cell activation(45).

Innate immunity: Clinical significance of the TLR9 pathway

TLR9 is expressed in normal myocardium, in the infarction zone after myocardial ischemia, and in cardiac tissue during heart failure(46). Studies in animals and small patient groups suggest that the cardiac TLR9 pathway may be important for the development and outcome of myocardial infarction. TLR9-activation has been connected to atrial fibrillation after coronary artery bypass grafting[54]. Activation of the NF-kB – TLR9 axis has been reported in heart failure patients(47). Myocardial ischemia releases endogenous DNAs(48) which may be potential TLR9-ligands.

Innate immunity: TLR9 signaling and endogenous mitochondrial DNA in inflammation and ischemic heart disease

Self-DNA can be both immunogenic and immunosuppressive depending on the nucleotide sequence, methylation status, and protein association(49). Mitochondria play a crucial role when it comes to immunogenicity and autoimmunity(50). In basal conditions, mitochondria stay at the crossroads of energy production, inflammation, and reactive oxygen species generation(51). If damaged, mitochondria release multiple types of DAMPs triggering an innate immune response(52). Mitochondria inherit some bacterial features from an ancestral bacterial endosymbiont. Like bacterial DNA, mitochondrial DNA (mtDNA) is circular and enriched in CpG motifs(53). Our group demonstrated that circulating mtDNA increases in patients after myocardial infarction(54), extracellular mtDNA induced death of mouse cardiomyocytes via TLR9-dependent activation of NF-kB(55) and that inhibition of extracellular mtDNA uptake reduces inflammation in cardiomyocytes challenged with hypoxia/reperfusion(56). Accumulation of mtDNA in the heart promotes heart failure in a mouse model of pressure overload(53). In patients with ST-segment elevation, mtDNA is released into circulation after myocardial reperfusion(57). However, nuclear DNA (nDNA) is less immunogenic than mtDNA or even has anti-inflammatory properties, depending on sequence structure(58).

The ShcA gene: a regulator of heart development, metabolism, and structural integrity

Four different Shc genes encoding multiple splice isoforms are presented in the mammalian genome: ShcA(59), ShcB(60), ShcC(61), and ShcD(62,63) genes. ShcB and ShcC are predominantly expressed in brain tissue, while ShcD is expressed in brain and skeletal muscle. At the same time, ShcA is widely expressed throughout human and mouse tissues, and codes for three different proteins, p66ShcA, p46ShcA, and p52ShcA (the digit refers to

kDa size of the protein)(64). These isoforms are accumulated in endoplasmic reticulum wherefrom they are recruited to plasmalemma, mitochondria, or nucleus upon specific signaling events(65). This gene has been initially seen as a regulator of different tyrosine kinase receptors(65). The pro-mitotic p52ShcA is antagonized by pro-apoptotic p66ShcA, while the role of the p46ShcA isoform is unclear(66). Several lines of evidence indicate that p66ShcA adaptor protein and other ShcA isoforms are involved in normal heart development, metabolism, cardiac response to ischemia, myocardial healing and remodeling, and innate immunity. In the embryonic heart, the ShcA gene regulates the interaction between cardiac cells and the extracellular matrix and is essential for cardiogenesis(67) and normal heart function(68). ShcA-null mice die in utero due to severe underdevelopment of the heart(67). Adult mice with cardio-specific mutated ShcA expression develop fatal heart dilatation due to activation of matrix metalloproteinases and extracellular matrix disruption(68).

The p66ShcA adaptor protein: metabolism accelerator and a positive regulator of oxidative stress

The p66ShcA isoform works as ROS-sensor, amplifying ROS production via a positive feedback signaling loop and promoting ROS-induced apoptosis(69). Targeted deletion of p66ShcA in mice prevents systemic oxidative stress(70), decreases total energy expenditure, and increases glucose utilization(71). This isoform is crucial for mammalian metabolism as thermogenesis and fat accumulation decreased in p66ShcA knockout mice(72). Even aging was slightly slowed in p66ShcA knock out mice(73). Consequently, p66ShcA functions both as a nutrient sensor and a metabolism accelerator(72,74). The p66ShcA should be in mitochondria to perform these functions. Upon stress or nutritional stimuli, the p66ShcA isoform is phosphorylated by protein kinase C beta (PKCβ) on serine residue 36 (Ser36). This event induces p66ShcA translocation from the cytoplasm into the mitochondrial intermembrane space(75). At that point p66ShcA isoform couples with cytochrome c(76). During normal conditions, the p66ShcA coupled with cytochrome c captures electrons from the electron transport chain and donates them to the complex of retinol and PKCδ activating pyruvate dehydrogenase, stimulating Krebs cycle and oxidative phosphorylation(77). However, during cellular stress more p66ShcA is translocated into mitochondria where it shunts electrons from complex III leading to the collapse of membrane potential and production of reactive oxygen species(78).

As a potent activator of both mitochondrial respiration and oxidative stress, p66ShcA is involved in many pathological processes and may be relevant for ischemic heart disease. Accumulated data suggest an association of p66ShcA polymorphism and human longevity(79). However, the p66ShcA gene is highly conservative in humans and sequence variations of importance for heart pathology are rare(80). The severity of breast(81), colorectal(82), and prostate(83) cancers are associated with increased p66ShcA levels in tumor tissue. p66ShcA expression is increased in patients with diabetes(84) as well as in patients undergoing hemodialysis (85). Moreover, there is a positive correlation between body mass index and blood levels of p66ShcA protein(86) in obese patients. An increased blood level of this isoform was shown in patients with coronary artery disease(87) and acute coronary syndrome(88).

The p66shcA isoform: at the crossroads of myocardial ischemia, wound healing, and innate immunity

Animal and in vitro studies demonstrate that p66ShcA adaptor protein aggravates both acute and chronic ischemic tissue damage. Lack of p66ShcA increases tissue resistance to ischemia-reperfusion injury in skeletal muscle(89) and brain(90). Targeted deletion of p66ShcA was found to be cardioprotective in an ex vivo model of acute ischemia/reperfusion injury due to attenuated oxidative stress(91). Moreover, cutaneous wound healing in conditions of ischemia and diabetes mellitus was improved in p66ShcA knockout mice(92).

p66ShcA is involved in adaptive immunity as a negative regulator of lymphocyte activation contributing to immunotolerance and preventing systemic autoimmunity(93,94). p66ShcA may be instrumental for innate immunity and sterile inflammation as mice with targeted p66ShcA deletion have decreased lipopolysaccharide (LPS)-induced inflammation(95). p66ShcA can facilitate innate immune responses via ROS production. Indeed, p66ShcA deletion leads to less superoxide production in activated macrophages(96). Moreover, ShcA proteins interact with numerous protein kinases, including those relevant for innate immunity. For instance, the ShcA proteins can be activated by Btk(97), a kinase that also plays a central role in innate immunity(98).

Accordingly, the p66ShcA signaling might be involved in acute ischemic damage, wound healing, and innate immune responses in the context of myocardial infarction. In this work, we aim to establish a link between p66shcA and TLR9 signaling to demonstrate coupling with innate immune responses during the post-infarction remodeling of the heart.

Circulating extracellular DNA: origins, forms, and functions

Extracellular or free-circulating DNA (exDNA) of multiple biological origins has been shown in human blood. exDNA can be endogenous, originated from nuclear(99) and mitochondrial(100) genomes or exogenous, released from bacteria(101) (both symbionts and pathogenic), viruses(102), parasites(103), transplanted tissues(104), fetal tissues(105) and even from ingested food(106).

Endogenous exDNA reaches circulation by different mechanisms, ranging from passive release upon apoptosis or necrosis to active release from actively dividing or activated cells(107). Some authors consider bone marrow as the main source of exDNA under normal conditions(108). Production of exDNA is balanced by its active degradation by the set of intracellular, interstitial, and plasmatic DNAses, enzymes degrading exDNA. However, exDNA can be protected from degradation by vesicles, histones, and some other proteins(109). Additionally, released exDNA can be taken up by other cells. Circulating lymphocytes can actively absorb and accumulate exDNA to release it again upon specific stimuli(110). Equilibrium between release, reuptake, and degradation defines the concentration of circulating mtDNA under normal conditions and in the pathology.

The size of exDNA fragments varies from macromolecular complexes and parts of chromosomes to ultrashort degraded DNA fragments(111). Released endogenous exDNA can be associated with histones and circulate as nucleosomes (112). exDNA can be also particle-associated. It can be packaged into exosomes, vesicles, microparticles, ectosomes, and

apoptotic bodies. exDNA is also released in the form of the vesicles covered with DNA-histone complexes(113) or it can be bound on the outer cell surface(113). The exosomal and vesicular exDNA populations are not homogeneous. Different vesicle size and content have been demonstrated(114). Each tissue can produce exosomes containing the specific signature of exDNA, RNA, and proteins, named secretome(111).

Biological functions and effects of exDNA are unclear, but evidence suggests immunological surveillance, messenger function, horizontal transfer, and exchange of gene sequences between tissues(109).

Clinical significance of extracellular DNA

Increased levels of circulating exDNA have been observed during intensive physical activity, pregnancy, old age, different autoimmune conditions, orthopedic trauma, septic shock, transplant rejection, numerous cancers, degenerative diseases, and even psychiatric illnesses(108).

Circulating exDNA has been studied as a biomarker in ischemic heart disease(115) and heart transplant rejection(116). Extracellular vesicles isolated directly from porcine venous blood after myocardial ischemic preconditioning contained both exDNA of nuclear and mitochondrial origin(117). The severity of coronary atherosclerosis and susceptibility to thrombosis correlated with exDNA levels(118). The release of exDNA in the complex with nucleosomes and neutrophil extracellular traps causes hypercoagulation and inflammation. This process is instrumental for the development of ischemic heart disease as it positively correlates with myocardial injury and disease progression(119). Accordingly, in patients treated with percutaneous coronary intervention for STEMI, exDNA accumulation has been shown in coronary microcirculation associated with neutrophil activation and thrombosis(120).

Extracellular mtDNA as a DAMP

Despite structural similarities, the immunogenicity of bacterial, mitochondrial, and genomic exDNA is different(121). Mitochondrial exDNA (mtDNA) is alike bacterial DNA(122). However, mtDNA is adjusted to be immunologically “invisible” for the cell(123). In contrast to unmethylated and CpG-rich bacterial DNA, mtDNA is highly methylated and CpG content is comparatively reduced. However, mtDNA demonstrates high TLR9-dependent immunogenicity when coupled with mitochondrial transcription factor alfa (TFAM)(124) or high mobility group box 1 protein (HMGB1)(125). Immunogenicity of mtDNA can be further increased via oxidation(126), nitrosation(127), or fragmentation(128).

Extracellular mtDNA is a potent pro-coagulant and neutrophil activator. Neutrophils release extracellular traps upon stimulation with mtDNA(129). Prolonged tissue exposure to neutrophil extracellular traps damages other cells with a release of more mtDNA(130) Additionally, neutrophils actively release mtDNA upon activation(131) creating a vicious circle of tissue damage. Extracellular mtDNA can also induce apoptosis and inflammation via TLR9 signaling and mtDNA has a direct cytotoxic effect on cardiomyocytes(132). Moreover, extracellular mtDNA induces a pro-fibrotic phenotype in cultured fibroblasts suggesting involvement in extracellular matrix remodeling(133).

Role of DNA transporters in ischemic heart disease

Cardiomyocytes import extracellular DNA fragments, possibly via receptor-mediated endocytosis(134). However, the exact transportation mechanism is unknown. The receptor for advanced glycation end-product (AGER) may contribute to uptake of mtDNA from the extracellular space after myocardial infarction. AGER is a PPR itself with HMGB1 as a typical ligand(135). It has been demonstrated that DNA-containing immune complexes activate TLR9 cooperating with HMGB1 and AGER to get into the cell(136,137). AGER is directly involved in the development of both acute and chronic ischemic myocardial injury(138). Further, the cardiac release of mtDNA and HMGB1 was shown to aggravate myocardial injury via a TLR9- and AGER-dependant mechanism in a mouse model of ischemia-reperfusion injury(139). Interestingly, AGER is involved in p66ShcA-dependent oxidative stress under some pathological conditions(140). Another mechanism of mtDNA delivery into cardiomyocytes is by the nucleolin(56). Our group has recently shown that nucleolin is expressed on cardiomyocyte membrane(56) and has an affinity to extracellular mtDNA and nDNA(141). Nucleolin can be relevant for ischemic heart disease as its expression is positively correlated with the extent of myocardial damage(142). At the same time, nucleolin blockade attenuates inflammation in cardiomyocytes stimulated with mtDNA and hypoxia-reoxygenation(56). Although, there is no literature showing interactions between nucleolin and TLR9 or p66ShcA proteins, extracellular signal-regulated kinase (ERK) is known to induce nucleolin expression(143). TLR9 activates(144) while p66ShcA inhibits(145) ERK signaling pathway upon oxidative stress.

Aims of the study

We suggest that the p66ShcA and TLR9 signaling interplay during myocardial infarction and adverse cardiac remodeling. We also suggest that damaged myocardium triggers local innate immune response via the release of DAMPs leading to a maladaptive inflammatory cascade and damage amplification. We hypothesize that local DAMP release can promote systemic inflammation and that extracellular mitochondrial DNA is an important DAMP in ischemic heart disease.

Specifically, we aimed at:

1) Investigating the effects of p66ShcA on myocardial healing, fibrosis, heart rupture, inflammation, and oxidative stress after acute myocardial infarction (paper I).

2) To investigate the functional link between p66ShcA and TLR9 pathways in a model of chronic myocardial injury (paper II).

3) To investigate the release dynamics of mtDNA during open-heart surgery using extracorporeal circulation as a clinical model of systemic inflammation (paper III).

Methodological considerations

The present project combines *in vitro* and *in vivo* animal studies together with the use of patient material. For a detailed description of techniques, please refer to articles I-III.

Patients

The studies on human material were performed in agreement with the ethical standards stated in the 1964 Declaration of Helsinki. Different studies were approved by the Regional Ethics Committee in Oslo, Norway, Tartu University Hospital, Estonia, or the Institutional Ethics Committee at Almazov National Medical Research Centre, Russia. Written, informed consent was obtained from all patients. Five different clinical materials were used. Essential patient information is summarized in table 1:

Materials and methods table 1: Overview of patient material

Patients	Institution	Sample	Article	Male/Female	Clinical information
CABG (N=11)	Tampere University Hospital, Finland	Left ventricle (tru-cut)	I	3/8	Average age:68±8; Ejection fraction: >60; Preoperative myocardial infarction:4
CAD (N=9) CMP (N=15)	Oslo University Hospital, Norway	Left ventricle from explanted heart	I, III	CAD: 0/9 CMP: 3/12	CAD: Average age: 55±14; Ejection fraction: 23.1±6.2; Preoperative myocardial infarction:9 CMP: Average age: 40±18; Ejection fraction: 21.9±7.5; Preoperative myocardial infarction: 0
CABG (N=12)	Tartu University Hospital, Estonia	Plasma	III	9/3	Average age: 65±9 Preoperative myocardial infarction: 6.
CABG (N=12)	Almazov National Medical Research Center, Russian Federation	Plasma	III	8/4	Average age: 69±5 Preoperative myocardial infarction: 9

CABG – coronary artery bypass grafting; CAD –end-stage heart failure due to coronary artery disease; CMP – end-stage cardiomyopathy due to non-ischemic pathology; CI- cardiac index, CO- cardiac output.

Materials and methods table 2: Plasma sampling (Paper III)

Patients		Timepoint							
		1	2	3	4	5	6a	6b	7
Series 1	Peripheral artery	x	x	x	x	x	x		x
	Coronary sinus		x		x	x			
	Pulmonary artery		x			x			
	Extracorporeal circuit			x	x	x			
Series 2	Peripheral artery	x						x	
	Coronary sinus					x			

Time points: 1 - before surgery, 2 - after cannulation, immediately before the start of cardiopulmonary bypass (CBP), 3 - after 20 minutes of CBP, 4 - 5 min after declamping the aorta and start of reperfusion, 5 – 30 min after the start of reperfusion, 6a – 1 h after the end of the operation, 6b / 2 h after the end of the operation, 7 – the morning of 1st postoperative day. Series 1 (Tartu University Hospital, Estonia), Series 2 (Almazov National Medical Research Center, Russian Federation)

Clinical considerations limit studies on patients. Moreover, patients represent mixed populations regarding gender, age, co-morbidity, polypharmacy, clinical routines, and genetic background. Exclusion criteria reduce heterogeneity but decrease patient group size. Thus, it may be a problem to extrapolate findings obtained from a homogeneous group of patients to the whole patient population.

In papers I and III cardioplegia was used as a model of ischemia-reperfusion injury. However, hypothermia and hyperkalemia during cardioplegia have their separate damaging effects on cardiac tissue in addition to that of ischemia alone(146). Moreover, the duration of cardioplegic arrest varied from patient to patient, which can add to the variation in measured parameters.

It is problematic to obtain enough material from the myocardium during open-heart surgery. In paper I thru-cut biopsies from the left atrial wall were harvested in patients undergoing coronary artery bypass grafting with CPB and cardioplegic arrest. This provided enough material for mRNA extraction, but not for protein determination by immunoblotting. These patients had mild functional impairment (NYHA classes II-III with left ventricular ejection fraction more than 60%). In material for paper I larger biopsies were sampled from the left ventricular free wall of explanted hearts from patients with end-stage heart failure and used for both protein and RNA analysis. Samples from patients, with ischemic and non-ischemic end-stage heart failure, provided information about differences between these types of heart failure. Moreover, despite standardized tissue sampling (apex cordi) in the explanted hearts, the extent and scale of coronary atherosclerosis, myocardial ischemia, hibernated, fibrotic and inflamed myocardium can vary considerably from patient to patient.

For paper III blood samples were serially sampled in patients undergoing coronary artery bypass grafting (CABG) to measure circulating n- and mtDNA. The main caveat of CABG is multiple sources of exDNA release. Both surgical trauma, endothelial and platelet damage due to cardiopulmonary bypass (CPB) and myocardial ischemia-reperfusion injury after the cardioplegic arrest can increase exDNA in circulation. To differentiate the respective roles of CPB and the surgical trauma, a prospective randomized study comparing on- and off-pump surgery is needed. Furthermore, the release of exDNA during on-pump surgery is dependent on the length of CPB(147): Consequently, sicker patients with more complex surgery and longer time on CPB may have higher levels of exDNAs in the circulation. The study was not powered to allow valid correlation of circulating mtDNA with clinical outcome.

We used the method of differential centrifugation to segregate and isolate extracellular vesicles in plasma samples. The main limitation of the approach is the lack of specificity towards the cellular origin of the isolated extracellular vesicles and exosomes(147). Other limitations are the sensitivity of ultracentrifugation to different external factors that cannot be standardized(148), destruction of some particles during the procedure, and contamination of vesicular and exosomal fractions with cell debris, organelles and large aggregates of biomolecules(148). It is also reported a significant loss of ultra-short DNA fragments during ultracentrifugation(149). We employed electron microscopy to ensure the presence of right-size particles in corresponding fractions. Western blotting study of the exosomal marker (CD9) in the fractions was not conclusive due to the uniform expression of CD9 in all fractions, suggesting the use of more specific exosomal markers in future projects. Immunoaffinity chromatography can be also considered to increase both yield and selectivity of isolation(148). Another problem is high variation in exDNA concentrations between healthy and diseased individuals, while reliable internal control is absent(150). To cope with this problem spiking with external standard sequence (MW2060) was used.

Mice

All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals (United States National Institute of Health, NIH Publication no. 85-23, revised 1996), and ethical permission was obtained from The Norwegian Animal Research Authority. The following experimental series were performed:

1) Three-month-old male C57BL/6J mice were purchased from NOVA-SCB (Nittedal, Norway) (n=105 in total). Chronic myocardial infarction in vivo, sham operation, and non-treated mice. Hearts were sampled one day, one, two, four- and six-weeks post-infarction. (Article I)
2) Three-month-old male p66ShcA knockout mice of C57BL/6J background and their littermate siblings as wild types (WT) (generated by Marco Giorgio (Charles River Laboratories, Milan, Italy)) (n=52 in total). Chronic myocardial infarction in vivo. Hearts were sampled after three days or six weeks post-infarction (Article I)
3) Three-month-old male p66ShcA knockout mice of C57BL/6J background and their littermate siblings as wild types (WT) (generated by Marco Giorgio[1] (Charles River Laboratories, Milan, Italy)) (n=20 in total). Chronic myocardial infarction in vivo and

intact animals. Hearts and spleen were sampled before and three days post-infarction (Article I, II)

4) Wild type three-month-old male C57BL/6J mice from NOVA-SCB (Nittedal, Norway) (n=30 in total). Chronic myocardial infarction in vivo and sham operation in vivo. Hearts were sampled one day and one-week post-infarction. (Article II)
5) Additional three-month-old male p66ShcA knockout mice of C57BL/6J background and their littermate siblings as wild types (WT) (n=10 in total). Chronic myocardial infarction in vivo. Hearts were sampled one-week post-infarction (Article II)
6) Wild type three-month-old male C57BL/6J mice from NOVA-SCB (Nittedal, Norway) (n=30 in total). TLR9 stimulation in vivo. Hearts were sampled 30 minutes after stimulation with a TLR9 agonist (Article II).
7) Wild type three-month-old male C57BL/6J mice from NOVA-SCB (Nittedal, Norway) (n=46 in total). Heart harvesting for cardiac cell isolation and culturing and liver harvesting for mtDNA isolation (Article II).

Extrapolation from animal studies to human pathology is always complicated due to species differences and limitations of the animal models, for instance, disease models are introduced in healthy animals.

Mice are more resistant to ischemia and, in general, to stress than big mammalians(151). The mouse genome has 99% homology to the human genome(152). Despite the high genome homology, the difference in transcriptome is significant between humans and mice (153). More than 4500 genes were found to have divergent expression between the species. The transcriptome of the mouse heart has more common transcripts between mouse and human than other tissues. At the same time, the transcriptomes responsible for immunity, metabolism, and stress responses were most divergent between the species.

Human and mouse hearts are developmentally similar and share the general plan of structure(154). However, the mouse heart has much more collateral vessels and the separate coronary basin in the septum(151). Myocardial infarction develops from the central regions of the myocardial wall in mice, while it spreads from the endocardium to the epicardium in the human heart. The atrial compartment of the mouse heart is smaller than in humans due to the common orifice for pulmonary veins in the left atria and absence of sinus venous with preservation of left superior vena cava(154). Mouse heart has less cardiac fibroblasts and connective tissue than big mammalians(155). Moreover, mice have a hundred-time less cardiac weight and a hundred-time higher heart rate than humans do.

In this study we used inbred C57BL/6J mice; the sub-strain is known to have dysfunction of gene encoding nicotinamide nucleotide transhydrogenase. This mutation predisposes the mice to oxidative stress and accelerated tissue damage(156). It leads to mtDNA damage accumulation and potentially increasing p66ShcA signaling and immunogenicity of mtDNA.

In constituent knockout mice, the protein of interest is absent throughout all embryologic stages. Consequently, some adaptations may develop to compensate for the lacking function which may influence the results of experiments. No such compensation at the organ or tissue

level was observed in our study. We found no difference in baseline heart morphology and echocardiography parameters between p66ShcA knockout and wild type mice. Previous literature also reports similar hemodynamic and ventricular wall thickness, despite increased cardiomyocytes numbers in p66ShcA knockout mice compared to wild type littermates(157). Hypercellularity of p66Shca knockout hearts can be explained by decreased apoptosis during fetal heart development as p66ShcA is pro-apoptotic and has the highest expression in fetal cardiac tissue(158).

There are two known types of p66ShcA knockout mice, ShcP and ShcL, which have different phenotypes (159). ShcP type of p66shcA knockout mice has been used in most studies, including ours. Neomycin phosphotransferase gene (Neo) was used to knock out the p66ShcA part of the ShcA gene. ShcP mice have the Neo construct still present in the first intron of ShcA gene which alters expression of all three isoforms. It results in the efficient deletion of p66ShcA in all tissues and downregulation of p56ShcA isoform in all tissues. Expression of p46ShcA isoform is tissue-specific, being down-regulated in heart, skeletal muscle, and liver while being up-regulated in white adipose tissue, spleen, and macrophages(159). Another type of p66ShcA knockout mice, ShcL, was developed by Prof. Tomas Prolla, University of Wisconsin – Madison, USA. ShcL mice have p66ShcA deleted and Neo construct removed. ShcL mice have no p66ShcA expression across all tissues; expression of the other two isoforms is not changed(159).

This difference in ShcA gene activity between the knockout variants may influence the results of studies in the two types. ShcP mice have insulin insensitive fat tissue and high insulin sensitivity of the other tissues, making them resistant to obesity due to a high-fat diet. The ShcL variant is sensitive to insulin and they are susceptible to obesity induced by a high-lipid diet. So far, no other phenotype differences are known. The p46ShcA and p52ShcA isoforms may be cardioprotective and its downregulation in the heart can partly explain conflicting reports on cardioprotection in the ShcP variant of p66ShcA knockout mice(160). Upregulation of p46ShcA in macrophages and possibly other immune cells might modify innate immune responses, including responses to myocardial infarction(96).

In vivo model of myocardial infarction

The small size of the mouse makes any surgical procedure complicated. The amount of tissue available for analysis is also limited. The development and use of an in vivo mice model of myocardial infarction have a long learning curve and great variability among operators. We experienced a reduction of intra- and postoperative mortality from 45% to 5% and a considerable increase in throughput of the procedure within one year of intensive practice.

We employed permanent occlusion of the left descending coronary artery due to lower intraoperative mortality, infarct size variability, and higher reproducibility compared to the model with reperfusion. The main limitation of our model is the lack of reperfusion injury which reduces the relevance of this model to the modern situation in clinics. On the other hand, all three stages of myocardial infarction were observed on histology, which makes the model with permanent occlusion still suitable for the study of myocardial healing and adverse

cardiac remodeling post-infarction. To standardize infarct size, the artery was ligated as proximally as possible to block the collateral bypass. This leads to large transmural infarction with high postoperative mortality due to heart rupture, which is a feature of murine models of myocardial ischemia(161).

Anesthesia, surgical trauma, hypothermia, dehydration, and bleeding must be tightly controlled as these variables will influence myocardial resistance to ischemia. For anesthesia, we used isoflurane, which has cardioprotective properties(162). Buprenorphine (Tamgesic) was administrated for analgesia. This drug falls in the group of semi-synthetic opioids, which might have a cardioprotective effect as well(163). Another challenge is the temperature drop during anesthesia. It has been reported for a rabbit model that each 1°C decrease in body temperature results in a 10% reduction of infarct size(164). To cope with this problem heating pad, blankets, and constant core temperature monitoring with a rectal probe were implemented. Bleeding as big as 100 µL can be detrimental for a mouse(151). Surgical trauma per se can induce pro-inflammatory cascades separately from myocardial infarction. We standardized our technique to minimize surgical trauma and avoid any major bleeding. If profuse bleeding happened the mouse was excluded from the experiment. The mice were also supplemented with subcutaneous saline to compensate dehydration due to evaporation from the surgical wound.

Echocardiography

A mouse is a challenging object for echocardiography due to small body size, high heart rate, susceptibility to hypothermia, and narrow therapeutic windows for anesthetics. Sophisticated equipment and tightly controlled conditions are necessary. Mouse-designed echocardiography (Vevo system) was performed one day before and six weeks after myocardial infarction. Before every echocardiography procedure, the thoracic region was shaved, and isoflurane mixed with pure oxygen was administered for anesthesia via a mask. The position of the probe was fixed during the procedure; the body temperature was controlled and kept at 37°C. To ensure reproducibility, the electrocardiogram and body temperature was registered by electrodes integrated with the heating plate.

Cell experiments

In this study different cell types and in vitro systems were used for further follow-up of our in vivo findings. Isolation of primary cardiac cells (fibroblasts and cardiomyocytes) is a harsh procedure with a strong hypoxic challenge and inevitable death of some cells. The advantage of this model is that cell-specific expression of proteins and cell-specific effects of treatment, as well as loss-and-gain of function experiments, can be performed. At the same time, this approach is limited by the fact that (I) cardiomyocytes can be cultured not longer than 24 hours; (II) cardiac fibroblasts can be passaged many times, although we can expect that they will change phenotype from passage to passage. (III) Another issue with cultured fibroblasts is that fibroblasts growing in two-dimensional culture have very different extracellular proteins expression profiles than in three-dimensional culture or in vivo(165). Thus, cultured fibroblasts are not entirely optimal for modeling of the in vivo situation. We used this system for our gain-and-loss of function studies to demonstrate the functional link between the

proteins of interest in cell-specific context. (IV) Isolated primary cardiac cells can be also contaminated with immune, smooth muscle, and endothelial cells. However, the qPCR analysis confirmed the purity of the cells in our projects(56).

Gain-of-function experiments allow us to verify findings made in mice with constitutive p66ShcA knockout. Despite high homology, we cannot exclude that interaction between mouse proteins and human p66ShcA is modified.

We used commercially available Hek 293 (branded as Hek-Blue by Invivogen) cells co-transfected with mouse TLR9 and inducible secreted embryonic alkaline phosphatase reporter coupled to NFκB. In paper II these cells were also co-transfected with human p66ShcA. Hek 293 cell line is a transformed embryonic kidney cell line with signaling which can be distinct from what one can observe in cardiac cells. These are human cells overexpressing mouse TLR9 and obtained results should be interpreted carefully. We also employed Hek cells with inducible secreted embryonic alkaline phosphatase reporter coupled to NFκB, but without TLR9 expression to isolate the effects of transfection.

Molecular biology techniques

Histology and immunohistochemistry

Histology and immunohistochemistry are the classical and reliable methods to prove and quantify infarct size, cardiac fibrosis, and inflammatory infiltrate pathological changes. They are also central for quality assessment of our in vivo model of myocardial infarction. Blinded software-assisted quantification was used to avoid observer bias.

Western blotting

This technique is semi-quantitative, only considerable changes in expression can be detected. At the same time, this approach is useful for confirmation of results obtained from qPCR. For each antibody, protocol customization was performed, and negative/positive technical and biological controls were used. We demonstrated the specificity of ShcA antibody using material from p66ShcA knockout mice – a clear absence of the corresponding 66kDa band was detected. Material from TLR9 knockout mice developed by Akiras group was not suitable as negative biological control due to these mice still produce the non-functional, but detectable protein. Hek-Blue cells overexpressing TLR9 and control Hek-Blue cells stimulated with a TLR9 agonist were able to prove the specificity of our antibody since the corresponding band was the most detectable in the cells overexpressing TLR9 and stimulated with the agonist.

Co-immunoprecipitation and DuoLink assay

Co-immunoprecipitation is old, but a still useful method for initial proof of protein-protein interactions. It is a relatively simple and robust technique if done together with proven antibodies and negative technical controls. However, the positive result does not mean direct interaction between the proteins studied; it rather demonstrates the presence of the molecules in the same protein complex. DuoLink assay is an overly sensitive and specific technique for the detection of interaction between two proteins, which is superior to co-

immunoprecipitation. Positive and negative technical and biological controls were used to subtract the unspecific signal.

Summary of results

Paper I

The p66ShcA adaptor protein regulates healing after myocardial infarction.

Anton Baysa, Julia Sagave, Andrea Carpi, Tatiana Zaglia, Marika Campesan, Christen Dahl, Dusan Bilbija, Maria Troitskaya, Lars Gullestad, Marco Giorgio, Marco Mongillo, Fabio Di Lisa, Jarle Vaage, Guro Valen.

Basic Research in Cardiology, 2015 Mar;110(2):13. doi: 10.1007/s00395-015-0470-0. Epub 2015 Feb 14.

Aim: Investigate the role of p66ShcA adapter protein in myocardial remodeling post-infarction.

Key findings:

- Myocardial infarction induced expression of cardiac p66ShcA.
- p66ShcA knockout mice showed improved survival and decreased incidence of heart rupture 10 days post-infarction.
- Expression of cardiac MMP-2 was reduced; fibroblast activation and collagen accumulation were increased, while oxidative stress was attenuated in p66ShcA knockout mice post-infarction.
- p66ShcA was a positive regulator of MMP-2 expression in cultured cardiac fibroblasts.

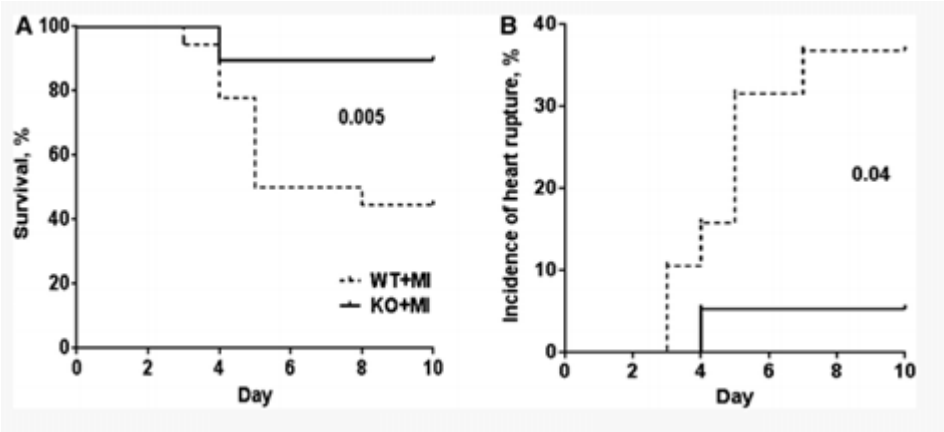

Figure 1 (Figure 2 in article 1): *Survival rates and heart rupture incidence in p66ShcA knockout (KO) and wild type mice (WT) after permanent myocardial ischemia (MI). The Kaplan–Meier method was used to analyze survival rates (**A**) and the incidence of heart rupture (**B**) during a 10-day observation period.*

Paper II

Toll-like receptor 9 signaling after myocardial infarction: Role of p66shcA adaptor protein.

Anton Baysa, Azzam Maghazachim, Kristin Larsen Sand, Marika Campesan, Tania Zaglia, Marco Mongillo, Marco Giorgio, Fabio Di Lisa, Lars H. Mariero, PhD Jarle Vaage, Guro Valen, Kåre-Olav Stensløkken.

Aim: Investigate if p66ShcA adaptor protein is functionally linked to the TLR9 pathway in the setting of myocardial infarction

Key findings:

- Myocardial ischemia induced cardiac TLR9 expression, but not in the hearts of p66ShcA knockout mice.
- TLR9 expression was increased and TLR9 signaling was enhanced by p66ShcA overexpression in vitro.
- TLR9 and p66ShcA may form a protein complex. The protein complex dissipated upon TLR9 activation in vivo and in vitro.

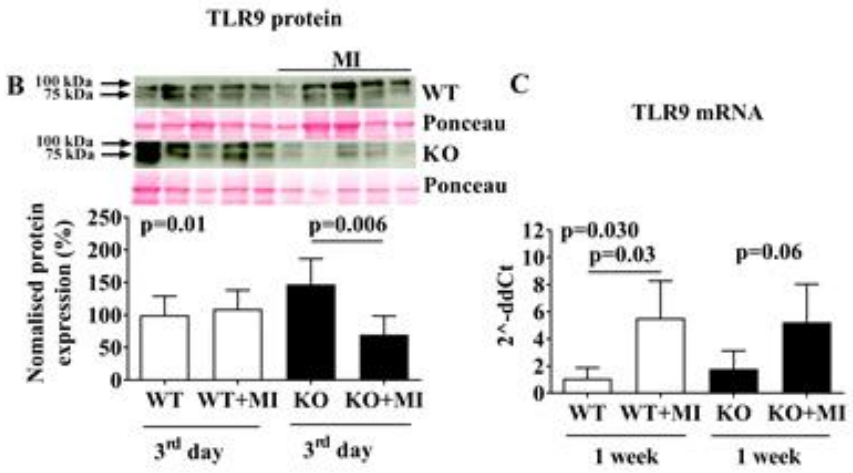

Figure 2 (Figure 4 in article 2). *Expression of TLR9 in hearts of p66ShcA knockout (KO) and wild type mice (WT) mice shown by immunoblots before as well as three days (B) and one week (C) after myocardial infarction (MI) (n=5 in each group). A one-way ANOVA with Sidak post-test was used to test significant differences. Data are shown as mean±SD.*

Paper III

Release of mitochondrial and nuclear DNA during on-pump heart surgery: Kinetics and relation to extracellular vesicles.

Anton Baysa, Anton Fedorov, Kirill Kondratov, Arno Ruusalepp, Sarkis Minasian, Michael Galagudza, Maxim Popov, Dmitry Kurapeev, Alexey Yakovlev, Guro Valen, Anna Kostareva, Jarle Vaage, Kåre-Olav Stensløkken.

J Cardiovasc Translational Res. 2018, Dec 12. doi: 10.1007/s12265-018-9848-3.

Aim: Investigate the release of mtDNA and nDNA during on-pump CABG and its distribution between extracellular vesicles and non-vesicular compartments in plasma.

Key findings:

- mtDNA and nDNA levels increased after the start of surgery before the start of cardio-pulmonary bypass (CPB) and increased further during CPB with mtDNA copy number 1000-fold higher than nDNA.
- mtDNA was predominantly localized to the vesicular fractions whereas nDNA was predominantly in supernatant before the intervention.
- mtDNA was primarily increased in the supernatant, while nDNA was primarily increased in vesicular fractions after surgery start and CPB activation.

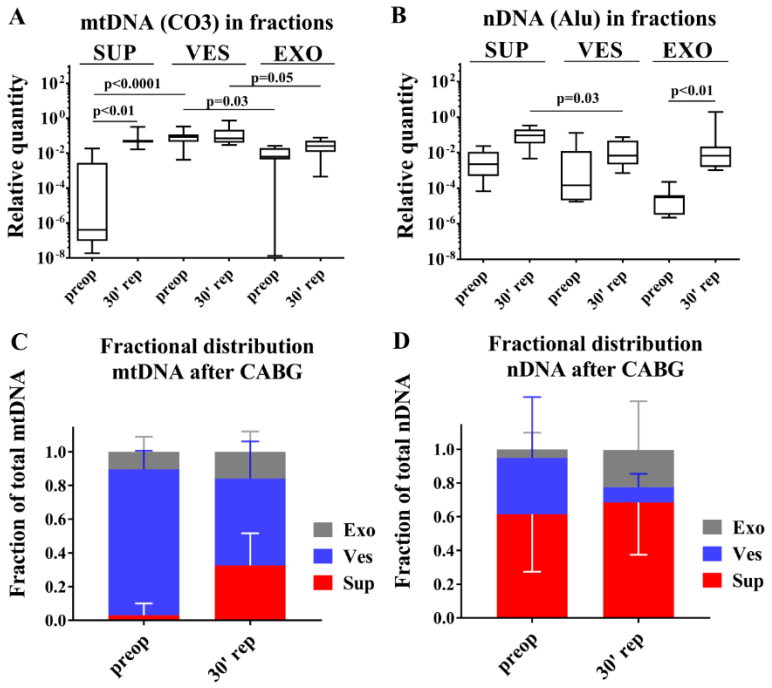

Figure 3 (Figure 3 in article 3) *Localization of mitochondrial (mtDNA) (A) and nuclear DNA (nDNA) (B) in different plasma fractions in patients undergoing coronary artery bypass*

grafting (n=12) with cardiopulmonary bypass and cardioplegia. Blood was sampled preoperatively (preop), and 30 minutes after the start reperfusion of the cardioplegic heart (30' rep). The plasma was separated into supernatant (SUP), microvesicles (VES), and exosomes (EXO). DNAs were measured by qPCR and expressed as a relative quantity. Data are presented as in figure in the article. Panels C and D show the fractional distribution of mtDNA and nDNA preoperatively and 30 minutes after the start reperfusion of the cardioplegic heart (30' rep).

General discussion

Our main findings are:

1. Myocardial infarction induced p66ShcA and TLR9 pathways in mice and patients with myocardial infarction.
2. p66ShcA knockout mice had attenuated adverse cardiac remodeling, reduced oxidative stress, and decreased activation of MMPs and the TLR9 pathway.
3. In vitro studies characterized p66ShcA as a positive regulator of the TLR9 pathway.
4. TLR9 was found in the same protein complex with ShcA proteins.
5. There was a massive, but a transient release of extracellular mtDNA and fraction shift in the bloodstream of patients undergoing CABG.

ShcA and TLR9 expression in the natural course of myocardial infarction

ShcA isoforms

p66ShcA is abundant in the fetal heart(166) but is downregulated shortly after birth(167). Its expression remains low throughout adulthood(168). We observed p66ShcA and p46ShcA isoforms upregulation by myocardial infarction, while expression of p52ShcA was not affected in our model. p66ShcA was induced in a biphasic manner. Its expression peaked in the first-week post-infarction corresponding to the inflammatory phase of myocardial healing. Then p66ShcA peaked again in the second week coinciding with the proliferative phase, followed by steadily increased expression during the six weeks of observation. Interestingly, p46ShcA isoform resides in the mitochondrial matrix(169) where it inhibits a lipid oxidation enzyme (3-ketoacyl CoA thiolase)(160). Thus, the p46ShcA isoform is a negative regulator of mitochondrial fatty acid beta-oxidation and crucial for heart metabolism. This may be beneficial in myocardial infarction as pharmacological inhibition of mitochondrial fatty acid beta-oxidation is cardioprotective(170). Upregulation of the p46ShcA isoform is also shown in regenerating liver suggesting its utility for wound healing(171). Consequently, the upregulation of the p46ShcA isoform may be beneficial for myocardial healing. Additionally, we observed transient phosphorylation of all three ShcA isoforms post-infarction on Y239/240, a conserved tyrosine residue. Phosphorylation on this residue regulates protein-protein interactions(172), apoptosis(173), and angiogenesis(174)

Upregulation of p46- and p66ShcA isoforms in the myocardium may be explained by a shift in the population of cardiac cells (infiltrating macrophages, proliferating myofibroblasts) and/or upregulation in residual cells. Indeed, the literature suggests that any type of cardiac cells can upregulate p66ShcA expression upon stress. For instance, p66ShcA upregulation due to obesity and aging is associated with fibrosis and an increased number of cardiac myofibroblasts (175). Ischemic myocardium is also populated by p66ShcA-rich macrophages derived from infiltrated monocytes (176) and endothelial cells(177) post-infarction. Cardiomyocytes can upregulate p66ShcA upon stress(178).

Our results suggest that the induction of p66ShcA and p45ShcA isoforms together with Y239/240 ShcA phosphorylation is a stress-induced event. These translational and posttranslational modifications of ShcA isoforms are instrumental for cellular responses to

oxidative stress(179,180), immune cell function(181), and thus may play a role in adverse heart remodeling. However, we can only speculate about the cellular and subcellular localization of p46- and p66ShcA isoforms as no in situ expression experiments were performed.

TLR9 pathway

This work is the first observation of TLR-9 pathway expression in the natural course of early myocardial remodeling after myocardial infarction. We found TLR9 protein upregulation one week after myocardial infarction coinciding with the inflammatory and early proliferative stages of myocardial healing. This observation possibly reflects the recruitment of TLR-9-rich immune cells into the ischemic myocardium. The TLR9 transporter, Unc93b1, and the main TLR9 adaptor protein, MyD88, were also upregulated at this time point suggesting induction of the whole TLR9 pathway post-infarction. This finding is in agreement with a previously reported ischemia-induced upregulation of the TLR9 pathway in skeletal muscle(182), intestine(183), liver(184), and brain(185). Furthermore, we found Btk activation and upregulation post-infarction. Btk is an important kinase for the TLR9-mediated dendric cell activation(186) and pathogen phagocytosis(187). Btk is instrumental in heart physiology since Btk-inhibitors promote atrial fibrillation(188). Reduced Btk activity and its association with arrhythmia align with the negative dromotropic effect of TLR9 activation. Btk expression may reflect the general TLR9 pathway upregulation and immune cell infiltration. Collectively, these data support the possible involvement of TLR9 signaling in myocardial remodeling post-infarction.

The literature on the role of TLR9 during myocardial ischemic injury is contradictory as TLR9 activation can be both cardiotoxic and cardioprotective. Early in myocardial ischemic injury, short-term TLR9 activation is beneficial as acute TLR9 stimulation enhances cellular survival via AMPK activation(189). Later works demonstrated that treatment with synthetic TLR9 agonists before(190), during(191), and after(192) myocardial ischemia is cardioprotective or have no effect at all(193). On the other hand, chronic TLR9 stimulation later in myocardial remodeling is damaging for heart function since prolonged TLR9 activation by bacterial DNA in vivo leads to decreased cardiomyocyte contractility and damaging inflammatory response via NF-kB activation(194). Moreover, chronic accumulation of undigested mtDNA promotes heart failure via the same mechanism(195). TLR9 signaling has pro-fibrotic action in the lungs(196) and can activate MMPs inducing heart rupture(197). Timing of ischemic myocardial damage, ligand type, and load can determine the net effect of TLR9 activation.

Our observations and existing literature highlight ShcA isoforms and the TLR9 pathway as potential therapeutic targets in the context of myocardial infarction and early phase of cardiac remodeling. This also raises a question about the interaction between the p66ShcA isoform and the TLR9 pathway. One can hypothesize that myocardial infarction leads to prolonged activation of p66ShcA and stimulation of the TLR9 pathway. Therefore, maladaptive, and pro-inflammatory TLR9 activation after myocardial infarction may overwhelm the short-term cardioprotective TLR9-dependent AMPK action. Collectively these data indicate that the

putative ShcA-TLR9 signaling axis may have an important, possibly detrimental, role in cardiac remodeling.

Myocardial remodeling and TLR9 pathway in p66ShcA knockout mice.

p66ShcA deletion improves myocardial healing post-infarction.

Cardiac remodeling after myocardial infarction is characterized by incomplete myocardial regeneration, activation of a fetal genetic program, and inflammation. Activation of p66ShcA might represent this maladaptive program. Our main hypothesis was that p66shcA deletion reduced ischemic damage as increased tissue resistance to ischemia-reperfusion injury was demonstrated in an ex vivo model of acute myocardial ischemia-reperfusion(91) as well as for skeletal muscle(198) and brain(90). Unexpectedly we found no reduction of myocardial infarction in the p66ShcA knockout mice. The size of the mature scar was also similar between groups. This can be partly explained by the pleiotropy of p66ShcA function in myocardial ischemia. There is evidence that p66ShcA deletion worsens mild myocardial ischemic injury in vivo(199). Paradoxically, the same study demonstrates a beneficial effect of p66ShcA deletion for more severe ischemia(199). However, protection was absent when the duration of ischemia was further increased(91) corroborating our results. This indicates that p66ShcA deletion has a cardioprotective effect only for moderate ischemia and can even increase susceptibility to myocardial injury during reperfusion(199). Consequently, p66ShcA may play different roles at different stages of ischemic myocardial injury. The use of ex vivo and in vivo experimental models can clarify the variation in the extent of cardioprotection observed in previously published works(200). Lack of infarct reduction in our work can partly be explained by extensive ischemia, partly by the fact that many wild type mice died of heart rupture. Our method of myocardial ischemia without reperfusion produced large hemorrhagic and transmural infarction. Consequently, the wild mice with the largest myocardial injury were negatively selected due to higher mortality as compared to p66ShcA knockout mice. As discussed in the chapter “Methodological Considerations”, baseline expression of p52- and p46ShcA isoforms was altered in the type of p66ShcA knockout mice (ShcP) we used in our study. We cannot exclude that modulation of these two isoforms might influence myocardial remodeling in our study. However, when p66ShcA was specifically overexpressed or knocked down in cultured fibroblasts, MMP-2 alterations were confirmed to be p66ShcA-specific. For future studies, it can be valuable to combine both types of p66ShcA knockout mice (ShcP and ShcL) to dissect the phenotypic effects of all three isoforms. Furthermore, hypothermia is cardioprotective and the p66ShcA knockout mice prone to quicker heat loss than wild type mice due to reduced thermogenesis(201,202). Consequently, during the surgery and echocardiography, we paid special attention to keep the mice's body temperature at the physiological level.

In our model, p66ShcA deletion improved myocardial healing rather than attenuated acute ischemic injury. Echocardiography demonstrated less left ventricle dilatation in the p66ShcA knockout mice post-infarction as compared to wild type mice suggesting reduced adverse remodeling. This agrees with previously reported findings of improved skin healing(203)

and skeletal muscle repair after ischemic injury(204) in p66ShcA knockout mice. Reduction in heart rupture incidence is the strongest evidence of improved myocardial healing in p66ShcA knockout mice. Decreased cardiac MMP-2 activation in p66ShcA knockout mice can partly explain this phenotype, as MMP-2 is an important factor causing heart rupture(205). MMP-2 is activated by ROS(206), while p66ShcA is a well-known promoter of intracellular oxidative stress. Diminished MMP-2 dimerization in p66ShcA knockout hearts supported this possibility because the dimerization depends on oxidative stress(207). Furthermore, we demonstrated positive regulation of MMP-2 by p66ShcA in our in vitro system. Previous reports support the association of p66ShcA activation with MMP2 upregulation during inflammation(157,208). Several MMPs are upregulated by oxidative stress(209). Interestingly, the targeted deletion of p66ShcA increased MMP-13 post-infarction. Unlike MMP-2 and MMP-9, MMP-13 is reported to reduce cardiac fibrosis(210) and thrombosis(210). MMP-13 is activated early and transitionally after myocardial infarction(211). Indeed, less interstitial fibrosis was observed in the septal wall distant to the infarct in our p66ShcA knockout mice compared to wild type littermates. Additionally, the knockout mice had more cardiac fibroblasts and increased production of collagen type I in the infarct area. These findings are corroborated by previous reports stated that p66ShcA deletion prevented cardiac upregulation of connective tissue growth factor (CTGF) and thrombospondin after infarction. CTGF facilitates tissue fibrosis and is expressed in different pathological conditions. In humans, both CTGF and thrombospondin is strongly upregulated during myocardial infarction and associated with adverse cardiac remodeling (212).

Our results suggest that p66ShcA promoted remote interstitial fibrosis and heart rupture post-infarction. Its deletion attenuated cardiac fibrosis in remote parts of the myocardium and improved mechanical properties of granulation tissue and fresh scar via altered MMPs expression profile. Interestingly, p66ShcA is required for the formation of focal adhesions and attachments between cells and the extracellular matrix(213,214). p66ShcA is also a pro-apoptotic protein(215). Therefore, better survival of cardiac cells along with modified interaction with extracellular matrix may also explain reduced remote fibrosis in our model.

p66ShcA regulates the production of reactive oxygen species and we demonstrated decreased cardiac lipid peroxidation one-week post-infarction in p66ShcA knockout mice. Furthermore, p66ShcA deletion reduces lipid peroxidation in an ex vivo model of cardiac ischemia-reperfusion injury(91) suggesting attenuated oxidative stress during all stages of ischemic cardiac damage and remodeling. The pro-inflammatory effect of p66ShcA is well documented(216) and indicate possible modification of cardiac innate immune responses in the knockout mice. Both lipid peroxidation and p66ShcA expression are increased in the blood of patients with the acute coronary syndrome(217) and patients on hemodialysis (218).

p66ShcA is necessary for TLR9 induction after myocardial ischemia.

Myocardial infarction led to the TLR9 pathway upregulation only if the p66ShcA isoform was present. At the same time, p66ShcA knockout mice had higher baseline TLR9-expression than wild type littermates. The p66ShcA-dependent TLR9 upregulation is in agreement with the known induction of general inflammation via p66ShcA-induced oxidative

stress(216). Oxidative stress may be instrumental for TLR9 signaling as oxidized DNA is much more potent immunogen than unmodified DNA(219). Mitochondrial DNA (mtDNA) might be an easy target of oxidation as mitochondrial damage is associated with both oxidative stress and mtDNA release(220). Damaged mitochondria loaded with oxidized mtDNA can directly fuse with TLR-9-expressing lysosomes during autophagy. Moreover, mtDNA stimulates the fusion of lysosomes and autophagosomes via TLR9(221), while TLR9 can be also expressed in mitochondria(222). It implies the co-localization of mtDNA with the source of oxidative damage and the pool of TLR9. Finally, a positive feedback loop exists as TLR9 activation in itself leads to oxidative stress(223). At the same time, oxidized DNA can quench TLR9 expression in some cells (224). We cannot exclude that TLR9 signaling is more active under oxidative stress in the presence of p66ShcA, explaining the downregulation of TLR9 signaling in p66ShcA knockout mice. Unexpectedly, p66ShcA knockout mice have increased baseline TLR9 expression. To explain this one can assume that p66ShcA knockout mice might have increased load of bacterial DNA due to reduced thermogenesis, impaired autophagy, and subclinical autoimmunity because of decreased lymphocyte apoptosis.

p66ShcA is a positive regulator of TLR9 signaling

Induction of TLR9 and p66ShcA coincides during myocardial ischemia and may have a complex net-effect. Demonstration of altered TLR9 signaling in p66ShcA knockout mice may indicate an interaction between these two proteins. This was supported by the demonstrated upregulation of the TLR9 pathway and TLR9-dependent NF-kB activation in p66ShcA overexpressing cells (Paper II). Accumulation of truncated TLR9 in wild type mouse heart confirmed the efficacy of the systemic TLR9 agonist administration. Our co-immunoprecipitation and co-localization experiments demonstrated that TLR9 and ShcA proteins exist in the same protein complex, which is dissociated upon TLR9 stimulation. Mitochondria or mitochondria-associated membranes may be the platform for interaction between these two signaling systems(225). To understand the function of the TLR9-p66ShcA signaling axis, it is important to characterize TLR9-specific DAMPs in the context of myocardial infarction. MtDNA is a TLR9 ligand and circulating mtDNA may play an important role in myocardial ischemia (226).

Extracellular mtDNA in the circulation during open heart surgery with extracorporeal circulation.

We chose to study the possible release of mtDNA using open-heart surgery with extracorporeal circulation as a model of whole-body inflammation and cold cardioplegic arrest as a model of myocardial ischemia in patients undergoing CABG. Our findings suggested a rapid, but transient release of mtDNA in cell- and particle-free form as well as the presence of clearance mechanisms. We demonstrated that mtDNA is released upon surgical trauma in a specific pattern: (1) preferably particle-free mtDNA was accumulated after systemic damage; (2) higher mtDNA copy number as compared to nDNA was released; and (3) mtDNA had higher turnover than nDNA. Our results suggest that surgical trauma and extracorporeal circulation were the main causes of mtDNA release. These findings are in the line with previously reported evidence of exDNA release from stressed endothelium,

damaged platelets, activated immune cells, neutrophil traps, and traumatized skeletal muscles(227,228). Tissue injury, endothelial, and blood cell activation and damage were the most likely sources of mtDNA (227).

Surgery before the start of cardiopulmonary bypass (CPB) caused a substantial release of exDNAs as both mtDNA and nDNA raised before the induction of CPB. The level of exDNAs increased further during CPB which can be explained by both CPB-related cell injuries in general, but particularly by blood cell activation and injury by the extracorporeal circulation. Then exDNAs level normalized shortly after the operation. The operation lasts around three hours, while the normal half-life of exDNA is 15 minutes(229) suggesting constant exDNA turnover during CPB with continuous release and degradation. The observed levels represent the net result of these processes.

In the clinical model, the pulmonary vascular bed might be important for exDNA metabolism during CABG. ExDNA might be degraded within the pulmonary vascular bed which acts as a chemical filter for a variety of bioactive substances(230,231). At the same time, the pulmonary vasculature can also release additional exDNA from trapped platelets upon reperfusion due to platelet sequestration in the lungs during extracorporeal circulation(232). It is well established that platelet activation induces microembolisation in the pulmonary circulation and releases a plethora of pro-inflammatory substances(233). We compared the levels exDNAs in the pulmonary artery and coronary venous blood with the level in peripheral arterial blood. No gradient of exDNA was found either across the pulmonary or the coronary circulation. exDNA in our clinical model may be released from 1. Surgery-damaged tissues; 2. The heart upon reperfusion after cardioplegic, ischemic arrest; 3. The lungs; 4. Blood cells and endothelial cells activated or damaged by the extracorporeal circulation. Our data shows that the massive release primarily or occurs in the peripheral circulation. The present clinical model is suitable to study the general release of exDNA, but not organ-specific release.

mtDNA and nDNA had different distribution in extracellular vesicles. We assume that the most extracellular vesicles originate from blood cells and endothelium during operation. mtDNA was mainly localized to microvesicles preoperatively but was released predominantly in particle-free form during the operation. In contrast, nDNA was observed mainly in supernatant before the operation but released in exosomal fraction during operation. The massive influx of particle-free mtDNA during tissue damage may contribute to the whole-body inflammation caused by surgery and CPB as mtDNA is more proinflammatory than DNA. The biological effects of vesicle-associated exDNAs may be complex. Extracellular vesicles are now recognized as an important part of cell-to-cell communication in health and disease(234). Moreover, cardiac-specific exosomes exist containing a specific molecular signature to convey biological messages to target cells(235). Some of these effects can be pro-inflammatory. As previously reported, increased mtDNA in circulation during heart surgery is associated with platelet activation, the release of inflammatory cytokines, and postoperative atrial fibrillation(236). Moreover, increased levels of circulating mtDNA are associated with mortality in critically ill patients(237), acute heart failure(238), and cardiovascular disease in general (226).

The p66ShcA isoform can potentially modify mtDNA immunogenicity via increased mtDNA oxidation. Immunogenicity of circulating mtDNA is also increased if mtDNA is bound to mitochondrial transcription factor A (TNFA)(239) or high mobility group protein B1 (HMGB1)(240). Interestingly, intracellular HMBGB1 is cardioprotective, but cardiotoxic when released extracellulary[292], possibly due to association with this DAMP.

Circulating mtDNA is endocytosed by cardiac and other cells to be stored or degraded. During this process, mtDNA can be transferred to TLR9-expressing lysosomes, ensuring high signal specificity(241). Cardiomyocytes can actively uptake mtDNA via the surface protein nucleolin. Additionally, AGER can transport protein-bound DNA into cells and is upregulated after myocardial infarction. Moreover, AGER can fine-tune TLR9 responses to specific ligands(242). Erythrocytes may scavenge circulating mtDNA to prevent the triggering of innate immunity (243). However, uptake of mtDNA by neutrophils can lead to their activation, netosis, coagulation, and tissue damage with the release of even more mtDNA (227).

Main conclusions and some clinical perspectives

The theoretical framework of this study is based on the danger theory of immunology and the concept of fetal and pro-inflammatory genetic reprogramming post-infarction. Induction of ShcA and TLR9 genes can be a part of such reprogramming. Our findings suggest an interplay between innate immunity, extracellular matrix turnover, and oxidative stress under the control of p66ShcA – TLR9 signaling axis. We have partial evidence of physical interaction between TLR9 and ShcA isoforms via protein complex formation, indicating that p52- and p46ShcA isoforms might be also involved in TLR9 signaling after myocardial infarction. These findings strengthen the importance of cardiac TLR9 pathway, and possibly other DNA-sensing pathways for the pathophysiology of ischemic heart disease. Circulating mtDNA might have an important position in p66ShcA-TLR9 interaction. Theoretically, measurement of circulating oxidized mtDNA, its association with TFAM and HMGB1, and the degree of mtDNA fragmentation might be biomarkers of ischemic myocardial injury. Coronary artery bypass grafting is a suitable clinical model to study the acute pathophysiology of circulating mtDNA.

This work contributes to the understanding of myocardial remodeling and sterile inflammation after myocardial infarction. The data suggest that p66ShcA promotes adverse heart remodeling after myocardial infarction via ROS-dependent regulation of inflammatory responses to endogenous mtDNA and extracellular matrix turnover. Our means to inhibit p66ShcA are still limited. However, some lifestyle interventions can reduce p66ShcA activation. Particularly, caloric restriction and exercise inhibits, while hypercaloric diet and cold exposure induce p66ShcA[80] expression. The p66ShcA isoform expression can be also manipulated. A novel heteroretinoid, SHetA2, has a direct inhibitory action on p66ShcA(244). Ascorbic acid quenches p66ShcA-induced generation of ROS and downregulates p66ShcA expression via increased ubiquitination(245). PKC inhibitors prevent Ser36 p66ShcA phosphorylation and mitochondrial translocation(246). At the same time, numerous TLR9 agonists and antagonists are being developed(247), some are currently under clinical studies(248). However, the unknown therapeutic window and organ-specific delivery are limitations for the clinical application of theseTLR9 ligands. Cleansing of circulating mtDNA by treatment with recombinant DNAses or by plasmaphereses might be a potential treatment strategy to reduce the load of immunogenic mtDNA after myocardial infarction.

Future research opportunities

Molecular biology of cardiac TLR9 and p66ShcA.

1. To study TLR9 isoforms and subcellular localization in cardiomyocytes and cardiac fibroblasts.
2. To study TLR9 and ShcA transcription and translation in the myocardium. Search for relevant transcriptions factors and networks of microRNA using bioinformatics and genomic\transcriptomic approaches.
3. To study localization (endolysosomes, mitochondria, and sarcoplasmic reticulum) of TLR9 and ShcA isoforms in cardiomyocytes and cardiac fibroblasts. Further co-localization study to confirm the putative TLR9-p66ShcA complex.
4. To study TLR9 and ShcA localization in different types of cardiac mitochondria (subsarcolemmal vs. intrafibrillar).
5. To study if the TLR9 pathway plays any role in cardiac contractility and conductivity, especially in the settings of ischemic heart disease. To study TLR9 expression in Purkinje fibers and nodal cells.

p66ShcA and TLR9 knockout mice

1. To study p66ShcA signaling and myocardial remodeling in the TLR9 knockout mice after myocardial infarction.
2. To study the effect of p66ShcA deletion on mtDNA oxidation, nitrosylation, and fragmentation.
3. To study cardiac remodeling postinfarction in mice with cardiac-specific deletion of p66ShcA gene.
4. Conditional myocardium-specific p66ShcA knockout mice to study p66ShcA role at different stages of myocardial infarction and cardiac remodeling. Alternatively, a specific p66ShcA inhibitor (SHetA2) can be used.
5. To study the effect of other retinoids on p66ShcA signaling and cardiac remodeling postinfarction.
6. To study other DNA-sensing pathways and other receptors affine to extracellular mtDNA in a mouse model of myocardial ischemia and remodeling.
7. To study possible subclinical immunodeficiency and pathogen susceptibility in p66ShcA knockout mice.
8. Role of p52- and p46ShcA isoforms in cardiac TLR9 signaling. Application of both ShcP and ShcL types of p66ShcA knockout mice to dissect functions of different ShcA isoforms in myocardial infarction and healing.

Clinical studies

1. To study detailed structure, content, and (patho)physiological meaning of mtDNA-containing particles from patients at different stages of ischemic heart disease.
2. To study detailed structure, content, and (patho)physiological meaning of mtDNA-containing particles in patients during surgery, trauma, and critical illness.

3. To study the biochemical modification of free circulating mtDNA (oxidation, fragmentation, methylation, nitrosylation, protein-association, and other possible modifications) released after myocardial infarction or surgical injury. Assessment of their immunogenicity and damage potential.
4. To investigate if variations in the natural history of ischemic heart disease depend on the polymorphism of TLR9 gene.
5. To study p66ShcA accumulation and phosphorylation as a marker of cardio-senescence in the aging healthy population. Risk stratification and alignment to mtDNA levels and TLR9 signaling.
6. To study p66ShcA expression and signaling in populations (1) treated with retinoids, (2) polymorphism of retinol processing genes, (3) with vitamin A deficiency, or (4) hypervitaminosis A.

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Papers I - III

I

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ORIGINAL CONTRIBUTION

The p66ShcA adaptor protein regulates healing after myocardial infarction

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Abstract Heart rupture and heart failure are deleterious complications of myocardial infarction. The ShcA gene encodes for three protein isoforms, p46-, p52- and p66ShcA. p66ShcA induces oxidative stress. We studied the role of p66ShcA post-infarction. Expression of p66ShcA was analyzed in myocardium of patients with stable angina ($n = 11$), in explanted hearts with end-stage ischemic heart failure ($n = 9$) and compared to non-failing hearts not suitable for donation ($n = 7$). p66ShcA was increased in the patients with stable angina, but not in the patients with end-stage heart failure. Mice ($n = 105$) were subjected to coronary artery ligation. p66ShcA expression and phosphorylation were evaluated over a 6-week period. p66ShcA expression increased transiently during the first weeks post-infarction. p66ShcA knockout mice (KO) were compared to wild type ($n = 82$ in total). KO had improved survival and reduced occurrence of heart rupture post-infarction. Expression of cardiac matrix metalloproteinase 2 (MMP-2) was reduced; fibroblast activation and collagen accumulation were facilitated, while oxidative stress was attenuated in KO early post-infarction. 6 weeks post-infarction, reactive fibrosis and left ventricular dilatation were diminished in KO. p66ShcA regulation of MMP-2 was demonstrated in cultured fibroblasts: lack or overexpression of p66ShcA in vitro altered expression of MMP-2. Myocardial infarction induced cardiac p66ShcA. Deletion of p66ShcA improved early survival, myocardial healing and reduced cardiac fibrosis. Upon myocardial infarction p66ShcA regulates MMP-2 activation. The role of p66ShcA in human cardiac disease deserves further study as a potential target for reducing adverse cardiac remodeling post-infarction.

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Introduction

Myocardial infarction reshapes cardiac extracellular matrix and may lead to heart failure and heart rupture [12]. The mortality of heart failure is as high as for many types of cancer [2]. Heart failure progression post-infarction correlates with myocardial fibrosis, an accumulation of collagens in infarcted and remote regions [31]. The current treatment of ischemic heart failure has limited anti-fibrotic effect [16]. Heart rupture, with a mortality rate of 75–90 %, is the most catastrophic complication of myocardial infarction. Despite a twofold reduction in heart rupture incidence due to thrombolytic therapy and percutaneous coronary intervention, it still remains a serious clinical concern. Heart rupture is responsible for 10–20 % of deaths after myocardial infarction where elderly females are especially susceptible [23].

Metalloproteinases (MMPs) are involved in development of both heart rupture and heart failure [29]. Upon activation, MMPs digest the collagen scaffold leading to chamber dilatation or in some cases to the rupture, and contribute to cardiac fibrosis via deposition of denatured collagens [17]. Interstitial collagenase MMP-13 and membrane-associated collagenase MT-MMP1 together with the gelatinases MMP-2 and 9 are crucial mediators of remodeling after myocardial infarction [21]. Matricellular proteins [e.g., thrombospondin 1 and connective tissue growth factor (CTGF)] are non-structural proteins of the extracellular matrix. They contribute to wound healing, fibrosis and may regulate MMPs [3]. Discovery of upstream factors regulating this network of MMPs and matricellular proteins in conditions of myocardial infarction is important for optimal therapy.

Members of the ShcA (src homology 2 domain containing transforming protein) family of adaptor proteins might regulate cardiac remodeling. ShcA encodes for three isoforms, p66ShcA, p52ShcA, and p46ShcA, which have different subcellular localization and function. ShcA proteins are highly expressed in fetal life where they are crucial for cardiogenesis [9]. The p66ShcA isoform is down-regulated in the adult heart, and once reactivated can be an important regulator of oxidative stress and cardiomyocyte proliferation [18]. ShcA-null mice die in utero due to abnormal heart development [15], while adult mice with cardio-specific ShcA knock-in develop fatal heart dilatation due to activation of MMPs [28]. By contrast, p66ShcA knockout (KO) mice have a 30 % increase in lifespan and show resistance to oxidative stress [18]. KO mice have attenuated oxidative stress leading to enhanced myocardial tissue survival after ex vivo ischemia/reperfusion injury [5] and protection against diabetic heart failure [22].

We hypothesized that p66ShcA is instrumental for remodeling after myocardial infarction through regulation of MMPs. To test this, we (i) investigated the expression of p66ShcA in hearts of patients with intermediate and end-stage coronary artery disease to verify the gene expression in clinical settings, (ii) evaluated if p66ShcA expression was induced by myocardial infarction in mice, (iii) studied the function of p66ShcA in cardiac remodeling using KO mice, and (iv) examined if p66ShcA regulated MMP expression.

Methods

Human myocardial tissue

The studies were in agreement with the 1964 Declaration of Helsinki, and were approved by the Regional Ethics Committee in Norway, and Tampere University Hospital, Finland. Written, informed consent was obtained from all patients or family members. Essential patient information is summarized in Supplemental Table 1. The patients were elective patients with stable angina pectoris undergoing open heart surgery with coronary artery bypass grafting (CABG, average age 55 ± 14 years), or patients with end-stage heart failure due to coronary artery disease undergoing cardiac transplantation (CAD, average age 69 ± 8 years). Tru-cut biopsies from the left ventricular free wall were sampled in the CABG patients immediately before cardioplegic arrest and provided enough material for mRNA extraction. Larger biopsies were sampled from the left ventricular free wall of explanted hearts from patients with end-stage heart failure due to ischemic heart disease ($n = 9$), and used for both protein and RNA analysis. Biopsies from hearts of patients dying suddenly of non-cardiac reasons were used as controls. These patients were sex- and age-matched individuals ($n = 7$) whose hearts were used for homograft preparation.

Animal studies

All experiments were performed according to the Position of the American Heart Association on Research Animal Use, and ethical permission was obtained from the Norwegian Animal Research Authority. Cardiac expression of p66ShcA was evaluated serially in C57BL/6J male 3-month-old mice

after permanent ligation of the left coronary artery, and compared with sham-operated and non-operated animals ($n = 105$ in total, see supplement for details). The role of p66ShcA in remodeling of the infarcted heart was studied using male C57BL/6J 3-month-old KO mice with wild type (WT) littermates [18]. Survival, heart function, and cardiac fibrosis were assessed 6 weeks after infarction ($n = 19$ in each group). Additional hearts were harvested three days post-infarction (i) to evaluate infarct size and inflammatory cell infiltration ($n = 7$ in each group) and (ii) to assess expression of MMPs, collagens and matricellular proteins compared to baseline expression in intact hearts ($n = 5$ in each group). For analysis of fibroblast activation, collagen expression and oxidative stress, WT and KO mice were operated and hearts were sampled 1 week post-infarction ($n = 5$ in each group).

Western blotting analysis

Proteins were extracted from human left ventricular biopsies and mouse hearts. The extracts were separated by electrophoresis and transferred to membranes (see supplement). Total or tyrosine-phosphorylated isoforms of ShcA proteins, MMP-2, MMP-13 or MT-MMP-1, were detected (supplementary Table 2).

Gelatine zymography

Samples were prepared as for Western blotting, but without irreversible inhibition of proteases and separated by electrophoresis in non-reducing conditions in gelatin-containing gels. After gel renaturation and Coomassie staining, proteolytic activity of cardiac MMP was visualized (see supplement).

Real-time quantitative PCR

Total cardiac RNA was extracted and reverse-transcribed to cDNA. Relative expression of target genes was assessed. Human HPRT and mouse Rpl32 were used as housekeeping genes (supplementary Table 3 and procedures in supplement).

Histology and immunohistochemistry

Hearts were harvested on the third day, 1 week and 6 weeks after myocardial infarction. They were formalin-fixed and OCT-embedded. Hearts were cut into 6 or 10 mm thick slices at three levels: basal, apical and the level between. Infarct size was blindly evaluated after staining with Masson's trichrome and inflammatory cell infiltration was assessed by counting the number of macrophages and neutrophil granulocytes (see supplement) 3 days post-infarction.

Immunofluorescence on cryosections

Expression of collagen I and smooth muscle actin was evaluated in heart cryosections 1 week post-infarction. The hearts were fixed in 1 % paraformaldehyde (PFA) (Sigma) for 15 min at room temperature, equilibrated in sucrose gradient at 4 °C, frozen in liquid nitrogen, sectioned and processed for histological and immunofluorescence analyses. 10-μ myocardial sections were obtained with a cryostat (Leica CM1850, Leica Microsystems GmbH, Wetzlar, Germany) and were stained with primary antibodies diluted in 1X PBS, supplemented with 1 % BSA and 0.5 % Triton-X100 (all from Sigma) over night at 4 °C. The following primary antibodies were used for this study: rabbit anti-collagen I (1:800, Acris); FITC-conjugated anti-Smooth Muscle Actin (1:2,000, Sigma, clone 1A4) and mouse anti-α-actinin (1:200, Sigma). Cy2-, and Cy3-conjugated secondary antibodies, all from Jackson lab (UK), were used to detect primary antibodies. Sections were analyzed at the fluorescence microscope (Leica DC130, Leica Microscopes, Germany).

4-hydroxynonenal (4HNEJ-2) immunostaining

Ventricular cryosections from both control and p66 KO hearts were incubated with blocking solution (10 % goat serum in 1X PBS) for 30 min at RT. Sections were incubated with anti-HNEJ-2 antibody (1:25, Abcam) diluted in 1X PBS, supplemented with 5 % goat serum (S2007 Sigma) and 10 % saponin, overnight at 4 °C. FITC-conjugated secondary antibody (1:250, Life technologies) was used to detect the primary antibody. Sections were analyzed at the fluorescence microscope (Leica DC130, Leica Microscopes, Germany).

Morphometric quantification of collagen-I deposition

To quantify collagen-I deposition, control and p66 knockout hearts were analyzed 7 days upon permanent left descending coronary artery ligation. A total of four cryosections from the mid-portion of the ventricles, for each heart, were analyzed. The area occupied by collagen-I was estimated in six randomly chosen fields of 69,000 μm^2, in the infarcted area, corresponding to a total of 24 fields/heart. Images were acquired at confocal microscope (Leica SP5, Leica Microsystems). Comparison between study groups was performed by Student t test. Results are presented as means ± s.e.m.

Quantification of ROS production

ROS production was quantified using 4HNE-J2 fluorescence imaging on ventricular cryosections, and both control and p66 knockout hearts were analyzed 7 days upon permanent left descending coronary artery ligation. A total of three cryosections from the mid-portion of the ventricles, for each heart, were analyzed. In each section, 4HNE-J2 fluorescence intensity was estimated in 30 randomly chosen fields, in three biological replicates, from both the border zone of myocardial infarction and the remote area in the interventricular septum and the right ventricle. Images were acquired at the fluorescence microscope (Leica DC130, Leica Microscopes, Germany). Comparison between study groups was performed by Student *t* test. Results are presented as means ± SD.

Functional evaluation

Left ventricular dimensions, volume, mass, and derived functional parameters were obtained using mouse echocardiography (Vevo 770) 1 day before the operation, and 6 weeks after infarction in KO and WT mice (see supplement).

Cell cultures

Primary mouse cardiac fibroblasts (CF) were isolated as described previously [20] and used to study the functional relationship between p66ShcA and MMP-2. p66ShcA was overexpressed with pBabe plasmid containing the p66ShcA gene [4] (see supplement). Cells were cultured in conditions of hypoxia (pO2 = 0.3 %) for 12 h and reoxygenated for 3 h. Proteins were extracted and expression of MMP-2 and p66ShcA was determined with Western blotting. Additionally, mouse embryonic fibroblasts with constituent p66ShcA knock-down were used to test if lack of p66ShcA influences MMP-2 expression.

Statistical analysis of data

Data with non-Gaussian distribution were evaluated with Mann–Whitney test for inter-group analysis of normalized expression (real-time PCR, Western blot, zymography) and histology (infarct size, area of fibrosis and inflammatory cells). Student's *t* test was used for continuous normally distributed data (echocardiography, cell culture experiments). Survival rate was evaluated by the Kaplan–Meier method (GraphPad Prism, San Diego, CA). All data are expressed as Mean ± SD, if not stated otherwise. $P < 0.05$ was considered as significant.

Results

Expression of p66ShcA in human and mouse heart

p66ShcA mRNA was increased in the left ventricle of patients with stable angina undergoing CABG as compared to control hearts. However, p66ShcA mRNA was not increased in the hearts of patients with ischemic heart failure (Fig. 1a). No significant differences were detected in p66ShcA protein expression between control hearts and failing hearts (data not shown). The small size prevented protein analyses in biopsies from CABG patients. Expression of mouse p66ShcA mRNA increased after permanent ligation of the left coronary artery, but not after sham operation. The highest level was seen after 2 weeks, returning to baseline after 6 weeks (Fig. 1b). Expression of p66ShcA protein peaked 1 week post-infarction, and remained elevated throughout observation (Fig. 1c, d). The expression of p52ShcA was not affected (Fig. 1e). p46ShcA isoform was upregulated the first day post-infarction, and was further increased one and 4 weeks later (Fig. 1f). Since phosphorylation of ShcA proteins on 239/240 and 317 tyrosine residues is reported to be paramount to the mechanical stress response [28], we examined it 1 day and 1 week post-infarction. ShcA proteins were phosphorylated only 24 h after infarction (Fig. 1g). The p66ShcA isoform was phosphorylated only on Y239/240 (Fig. 1 h, l). The p52ShcA (Fig. 1i, m) and p46ShcA (Fig. 1k, n) isoforms were phosphorylated at both tested tyrosine residues.

p66ShcA knockout mice had improved heart remodeling

During the first week after myocardial infarction, 53 % of WT mice died compared with a mortality of 10.5 % for KO mice (Fig. 2a). The main cause of death was heart rupture in the apical segment (37 %, 7/19), and this was reduced to 5 % in KO mice (Fig. 2b). KO and WT mice were observed for 40 days after left coronary artery ligation. Histology showed large hemorrhagic and transmural myocardial infarction of the anterior wall 3 days post-infarction. Neither the infarct size on the third day, nor the post-infarction scar (reparative fibrosis) 6 weeks later was reduced by p66ShcA deletion (Fig. 3a). Inflammatory cell infiltration was similar between the groups on the third day post-infarction (Fig. 3b). In contrast to reparative fibrosis of the anterior wall, reactive fibrosis in the septal wall remote to the post-infarction scar was attenuated in p66ShcA$^{-/-}$ mice compared to WT 6 weeks post-infarction (Fig. 3c). Pre-operative echocardiography showed no significant difference ($p = 0.06$) in posterior wall dimension between KO and WT

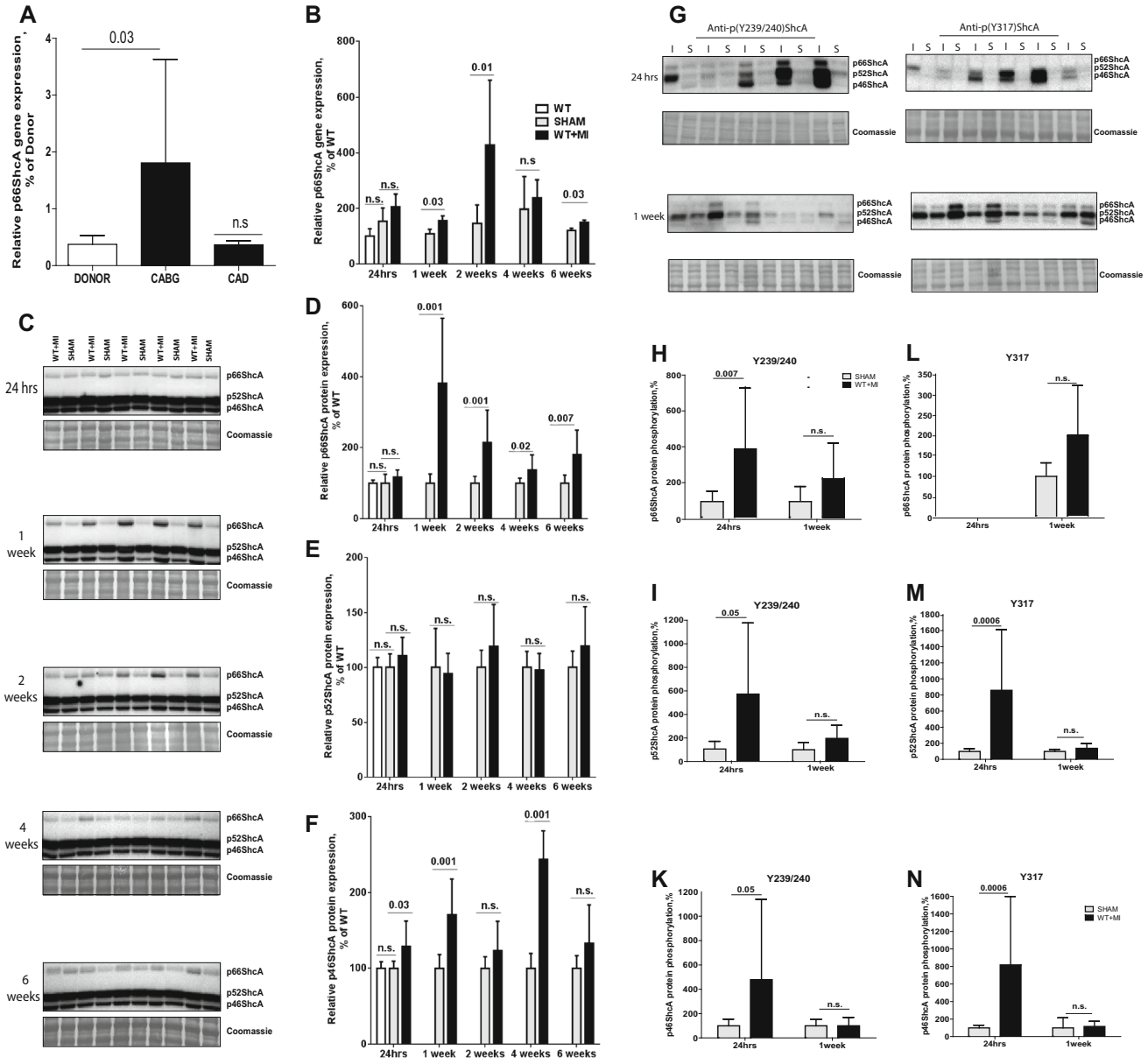


Fig. 1 Cardiac ischemia induced p66ShcA expression and phosphorylation. **a** Biopsies were sampled from the left ventricle of patients who died suddenly of non-cardiac causes and were scheduled for homograft donation (Donor, $n = 7$), from patients with stable angina undergoing coronary artery bypass grafting (CABG, $n = 11$), or from explanted hearts with end-stage heart failure due to coronary artery disease (CAD, $n = 9$). mRNA was extracted from the samples to measure relative expression of p66ShcA with real-time PCR (ddC_t method), using HPRT as a reference gene. C57BL/6 mice were subjected either to in vivo permanent occlusion of the left coronary artery (WT + MI), to sham operation (SHAM), or no intervention was performed (WT, $n = 10$ in each time point). Hearts were harvested serially thereafter. Total RNA was extracted from whole left ventricular tissue, and amplified with real-time PCR using Rpl32 as reference gene (ddC_t method) (**b**). Total left ventricular proteins were extracted, separated by electrophoresis, transferred to membranes and incubated with an anti-ShcA antibody or antibodies specific to ShcA protein phosphorylated on Y239/240 or Y317 residues. A representative Western blot showing all three ShcA isoforms for five time points, together with protein loading (by Coomassie). The optical density of the p66, p52, and p46ShcA bands is shown as *bar graphs* (mean ± SD) (**c**–**f**). A representative Western blot showing phosphorylated ShcA isoforms for two time points, together with protein loading (by Coomassie). The optical density of the phosphorylated p66, p52, and p46ShcA bands is shown as *bar graphs* (mean ± SD) (**g**–**n**)

mice. Other morphological and functional parameters were similar between the groups (Supplementary Table 4). 6 weeks after myocardial infarction, the changes in the heart performance were similar in the two groups. Although the absolute values of left ventricular diameter and relative wall thickness were not different between groups, left ventricular dilatation was decreased in KO mice after normalization to pre-operative value in each individual mouse (Fig. 3d).

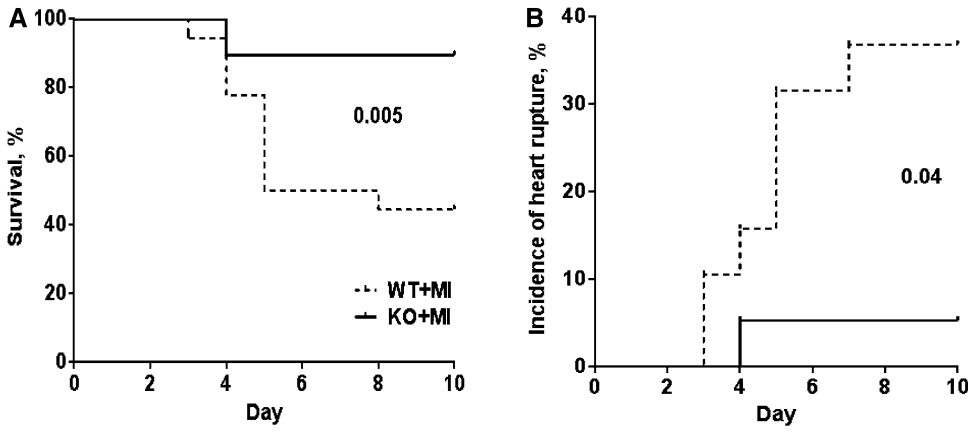


Fig. 2 Targeted deletion of p66ShcA improved survival and decreased heart rupture rate after myocardial infarction. The Kaplan–Meier method was used to analyze survival rates (**a**) and incidence of heart rupture (**b**) during a 10-day observation period

Targeted deletion of p66ShcA altered collagen expression and fibroblast activation post-infarction

Wild type and p66shcA knockout mice have similar baseline expression of collagen type I and III subunits at mRNA level. 3 days post-infarction, α1 subunit of collagen type I was upregulated in knockout mice, but did not change in WT mice post-infarction (Fig. 4a). 1 week after infarction immunohistochemistry analysis showed that collagen type I staining was higher in the infarcted area of knockout mice (Fig. 4b). Consistent with these data, activation of cardiac fibroblast into myofibroblasts was higher in hearts from p66ShcA knockout hearts, as compared to those from WT mice (Fig. 4c).

p66ShcA modified expression of MMP-2 and matricellular proteins

Expression of cardiac gelatinizes, MMP-2 and 9, was evaluated by gelatin zymography. Both enzymes were upregulated 3 days post-infarction in WT hearts. In KO mice, the increase of active MMP-2 expression post-infarction was attenuated, while MMP-9 expression was unchanged (Fig. 5a). Expression of membrane-associated and interstitial collagenases, MT-MMP-1 and MMP-13, was assessed by Western blotting 3 days post-infarction. MT-MMP-1 expression was induced by infarction in both groups. MMP-13 expression was not affected in infarcted WT hearts, but KO animals had increased expression of MMP-13 3 days after myocardial infarction (Fig. 5b). Gene expression of CTGF and thrombospondin-1 increased after myocardial infarction in WT mice, but not in KO animals (Fig. 5c, d).

Knockdown and overexpression of p66ShcA altered MMP-2 level in mouse fibroblasts

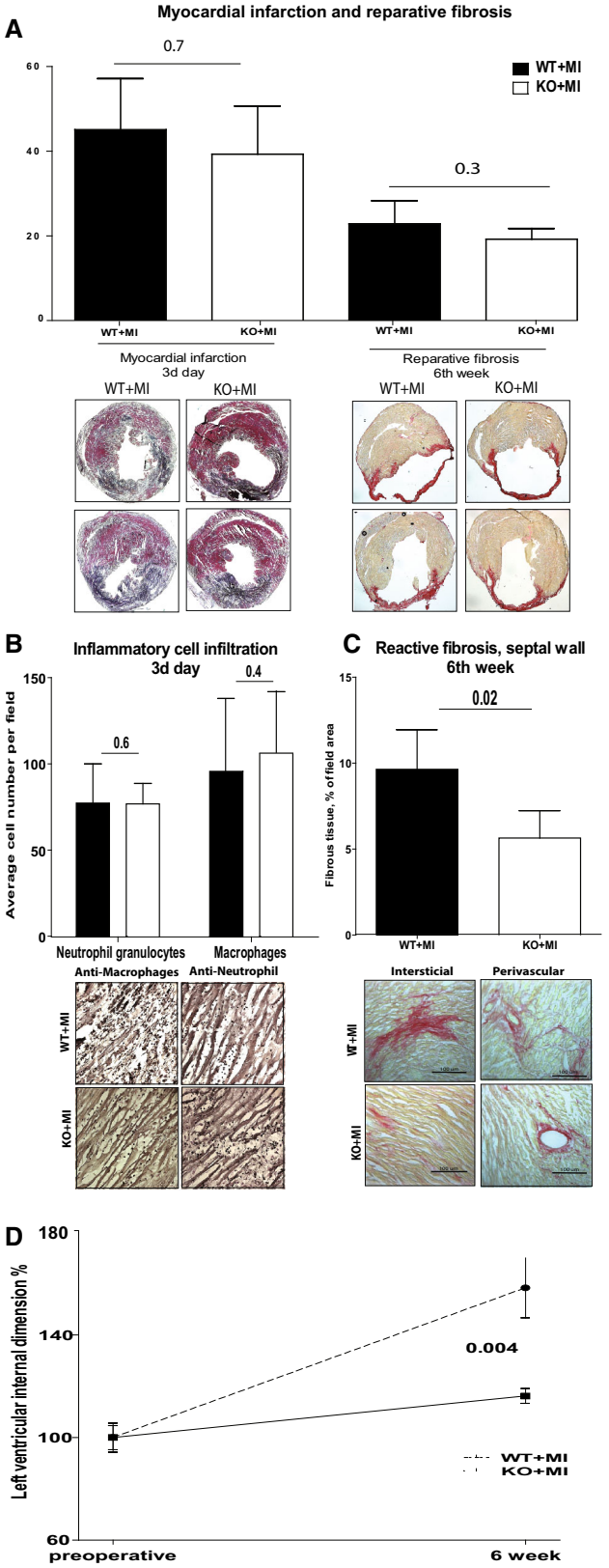
Regulation of MMP-2 expression by p66ShcA was studied in vitro. Since fibroblasts are the main source of MMPs in the heart [21], mouse embryonic fibroblasts (MEF) and primary cardiac mouse fibroblasts were used as model systems. The cells were stressed with hypoxia–reoxygenation to induce MMP-2 activation. MEFs with p66ShcA knockdown had p66ShcA expression reduced by 70 % as compared to wild type cells (Fig. 6a, b). Deletion of p66ShcA resulted in a decrease of MMP-2 active form paralleled by an increase in MMP-2 dimers as compared to wild type cells (Fig. 6c). Primary cardiac fibroblasts transfected with pBabe plasmid with cloned p66ShcA gene had sixfold increase of p66ShcA expression (Fig. 5d, e). In these cells, expression of total and dimeric MMP-2 was increased only after hypoxia–reoxygenation, but not in normoxic conditions (Fig. 5f).

Targeted deletion of p66ShcA attenuates oxidative stress in myocardium one week after infarction

Lipid peroxidation detected with 4-hydroxynonenal staining measured at infarcted area was significantly higher in WT than in KO mice 1 week after left coronary artery ligation (Fig. 7).

Discussion

The major findings of our study are: (i) p66ShcA was upregulated in hearts of patients with ischemic heart disease without heart failure, (ii) myocardial infarction transiently induced expression and phosphorylation of cardiac p66ShcA in mice, (iii) KO mice displayed improved survival after infarction due to reduced occurrence of heart rupture, (iv) which was associated with reduced expression of active MMP-2, facilitated fibroblast activation, reduced reactive fibrosis, collagen synthesis, reduction in oxidative stress and attenuated dilatation of the left ventricle, (v) studies in vitro revealed that p66ShcA have a crucial role in the regulation of MMP-2 expression in hypoxia–reoxygenation.



◀**Fig. 3** p66ShcA knockout mice had improved cardiac remodelling after myocardial infarction. The left coronary artery was permanently ligated to induce myocardial infarction (MI) in p66ShcA knockout (KO) and wild type (WT) mice. Hearts were harvested 3 days ($n = 5$ in each group) or 6 weeks later ($n = 19$ in each group). Sections (6 mm) were fixed in formalin, embedded in OCT and stained with Masson's trichrome (*blue*—infarct, *red*—normal myocardium) or Picrosirius red (*red*—fibrosis, *yellow*—normal myocardium) to evaluate infarct size or postinfarction scar, respectively. Digital photographs of entire slice (magnification ×4) from four sections of each heart were taken and analyzed with ImageJ software. Infarction or scar areas were blindly measured. Infarct size or reparative fibrosis was calculated as a ratio to the total slice area with exclusion of ventricular cavities. Data are Mean ± SD (**a**). Sections (6 mm) from hearts sampled at the third day post-infarction were incubated with anti-macrophage (F4/80) and anti-neutrophil primary antibodies (NIMP-14R). Digital photographs of four standard fields (magnification ×400) within the border zone of infarction from four sections of each heart were analyzed with ImageJ software to calculate the number of specifically stained inflammatory cells (*black*). The calculated estimation of cell infiltration is shown as *bar graphs* (mean ± SD) (**b**). Sections (6 mm) from hearts sampled 6 weeks post-infarction were stained with Picrosirius red for reactive fibrosis. Digital photographs from four standard fields within non-infarcted myocardium (magnification ×200) from four sections of the ventricular septum in each heart were used for quantification with ImageJ software. Positively stained fibrotic area was related to the total field area. Data are mean ± SD (**c**). Echocardiography (Vevo 770) was used to evaluate heart function 6 weeks post-infarction, and measurements were compared to the pre-operative value (=100 %). Left ventricular diameter in diastole is shown as mean ± SD (**d**)

Following a large body of evidence linking p66ShcA with both oxidative stress and ischemic injury, but also considering the lack of clinical data, initially we aimed at characterizing p66ShcA changes in diseased human hearts. We found that p66ShcA expression was increased in ventricular myocardium of patients with stable angina and preserved heart function, but not in the myocardium of patients with end-stage ischemic HF. The elucidation of a possible role of p66ShcA in myocardial adaptation to an ischemic insult would hardly be feasible at the clinical level. Therefore, we implemented experimental studies based on genetic approaches. The transient upregulation and phosphorylation of cardiac p46- and p66ShcA suggest active involvement of the molecules in early cardiac remodelling after myocardial infarction. In contrast to p52ShcA, which promotes cell proliferation, both p66ShcA and p46ShcA isoforms are involved in development of cell death [18]. The transient increase of tyrosine phosphorylation of all the ShcA isoforms on the first day post-infarction might be physiologically important, since mutations of these tyrosine residues lead to diminished cardiac tolerance to pressure overload [28]. ShcA phosphorylation on tyrosine residues 239/240 and 317 favors the left ventricular adaptive response to mechanical stress [28], and may indicate recruitment of the proteins to receptor tyrosine kinases in infarcted heart. The incidence of heart rupture in WT mice was similar to previous reports [23]. We observed dramatically improved early survival and fivefold decrease in the incidence of heart rupture in KO mice. Basic cardiac morphology and hemodynamics were similar to wild type littermates although left ventricular wall

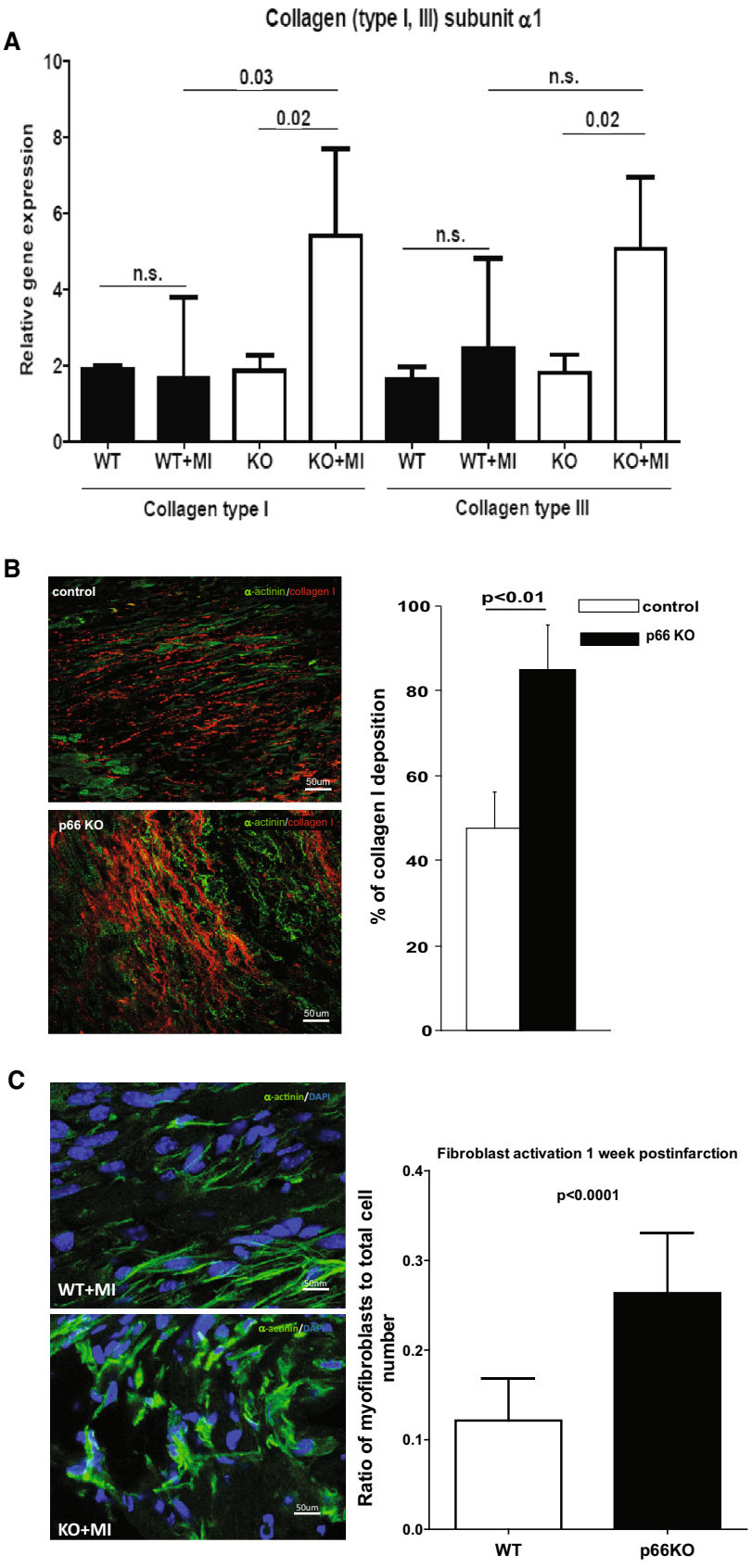


Fig. 4 p66ShcA deletion increased collagen I deposition and myofibroblast proliferation post-infarction. **a** The left coronary artery was ligated to induce myocardial infarction (MI) in p66ShcA knockout (KO + MI) and wild type mice (WT + MI). Non-treated p66ShcA knockout and wild type mice were used (KO and WT) ($n = 5$ in each group). Hearts were harvested 3 days post-infarction for qPCR. Total cardiac mRNA was extracted and amplified by real-time PCR using Rpl32 as housekeeping gene (standard curve method) to evaluate expression of collagen I and III subunits. **b** WT and p66ShcA hearts were harvested 1 week post-infarction, fixed in 1 % PFA, OCT embedded and sliced into 10 μm sections. Confocal immunofluorescence analysis on ventricular cryosections from wild type control and p66ShcA$^{-/-}$ (**p66KO**) co-stained with antibodies to sarcomeric actinin (*green signal* α-actinin) and collagen I (*red signal*) (*left panels*). The percentage of infracted myocardial area occupied by collagen I was evaluated in both control (*white bar*) and p66KO (*black bar*) mice (*right panel*). *Scale bar* 50 mm ($n = 4$ hearts for each group). Data are shown as mean ± SD. **c** Confocal immunofluorescence analysis on ventricular cryosections from control and p66KO mice, 1 week post-infarction, co-stained with antibodies to smooth muscle actin (*green signal*, α-actinin). Nuclei were counterstained with DAPI (blue signal) (*left panels*). The percentage of myofibroblast, on total cardiac interstitial cells, was estimated in both control (*white bar*) and p66KO (*black bar*) hearts (*right panel*). *Scale bar* 50 mm. ($n = 4$ hearts for each group). Data are shown as mean ± SD

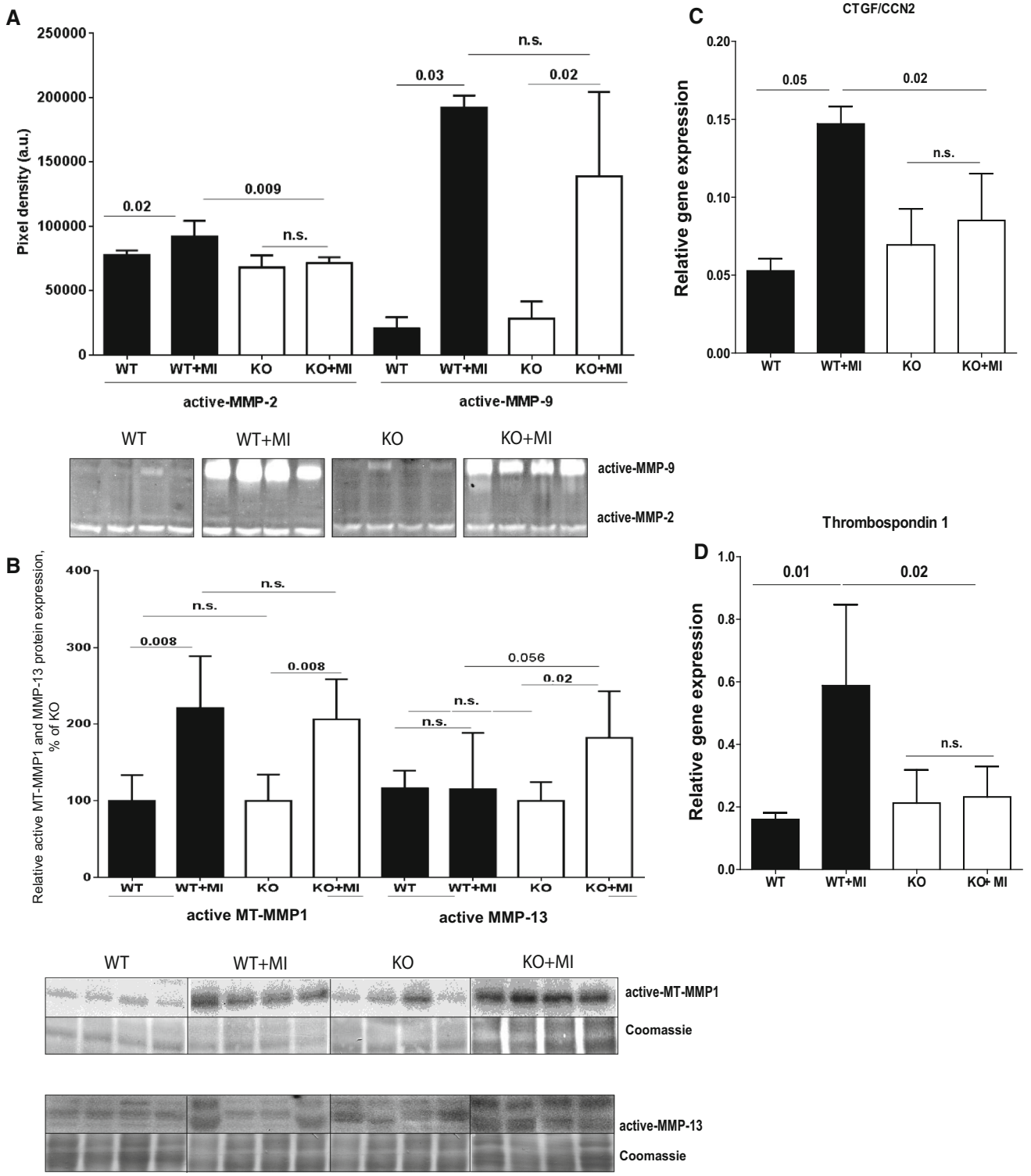


Fig. 5 p66ShcA knockout mice had altered expression of MMP-2 and matricellular proteins. The left coronary artery was ligated to induce myocardial infarction (MI) in p66ShcA knockout (KO + MI) and wild type mice (WT + MI). Additionally, non-treated p66ShcA knockout and wild type mice were used (KO and WT) ($n = 5$ in each group). Hearts were harvested 3 days post-infarction for Western blotting, zymography or qPCR. Total cardiac protein extracts were separated in native conditions on a gel copolymerized with gelatin. The gel was renaturated, and MMP-2 and -9 activities were estimated as the area of gel free of gelatin. A representative zymography and quantification of all results are shown (**a**). The same extracts were used for immunoblotting with anti-MMP13 and MT-MMP-1 antibodies. Representative immunoblot and quantification related to protein loading (Coomassie) are shown (**b**). Total cardiac mRNA was extracted and amplified by real-time PCR using Rpl32 as housekeeping gene (standard curve method) to evaluate expression of matricellular proteins: connective tissue growth factor (CTGF) (**c**), Thrombospondin 1 (**d**). Data are shown as mean ± SD

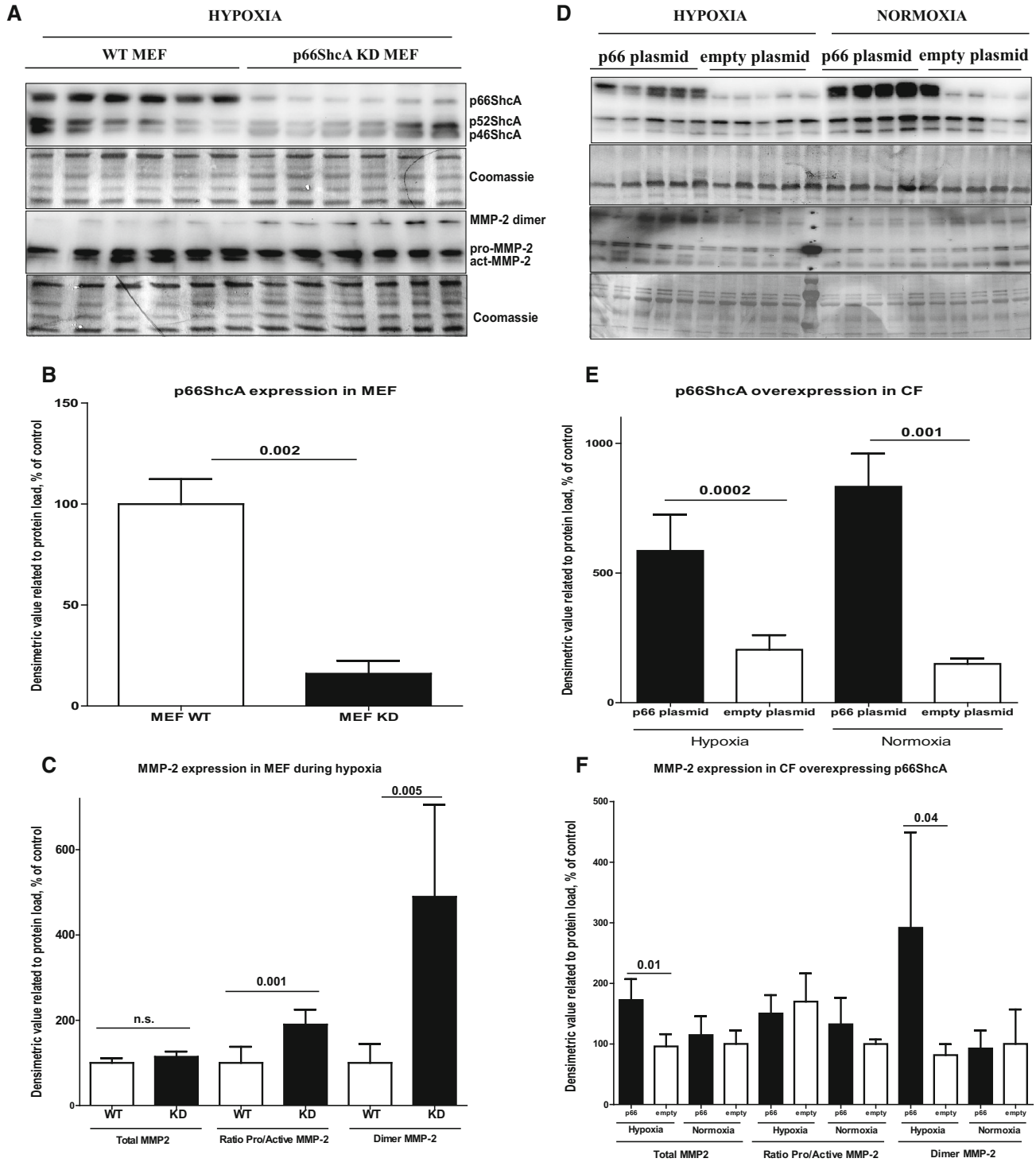


Fig. 6 p66ShcA overexpression or knockdown modified MMP-2 expression in mouse fibroblasts. Mouse embryonic fibroblasts (MEF) with constitutive p66ShcA knock-down (KD) or wild type cells (WT) were cultured for three passages. Primary cardiac fibroblasts (CF) were isolated from mouse hearts ($n = 5$) and cultured for three passages. p66ShcA overexpression was performed by retroviral infection with pBabe plasmid containing p66ShcA gene. Cells were subjected to hypoxia ($pO_2 = 0.3$ %, $pCO_2 = 5$ %) for 12 h followed by 3 h of reoxygenation, while the control cells were cultured in normoxic conditions for 15 h. The cells were sampled and proteins were extracted for Western blotting. Left panel shows the effect of p66ShcA knock-down (KD) in MEF cells after hypoxia/reoxygentaion on the expression of p66ShcA (**a**, **b**) and MMP-2 (**c**). *Middle panel* shows the effect of hypoxia/reoxygenation or normoxia on p66ShcA (**d**, **e**) and MMP-2 (**f**) expression in CF with p66ShcA overexpression (p66 plasmid/p66) or control cells (empty plasmid/empty). The calculated band densities are presented as *bar graphs* (mean ± SD). Representative western blotting and loading controls (Coomassie) are shown for each *panel*

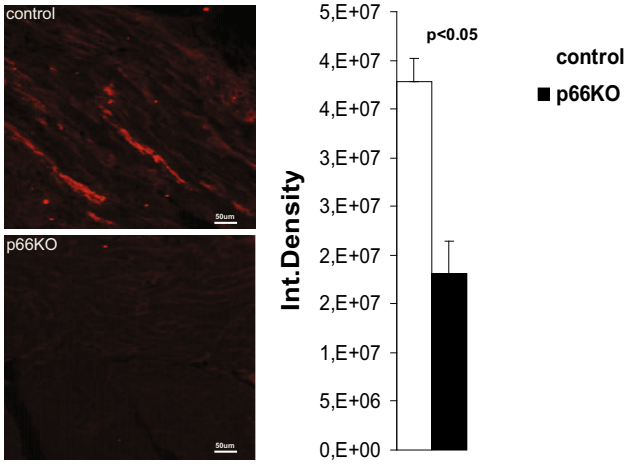


Fig. 7 Oxidative stress was attenuated in infarcted myocardium of p66ShcA knockout mice. WT and p66ShcA hearts were harvested 1 week post-infarction, fixed in 1 % PFA, OCT embedded and sliced into 10um sections. Immunofluorescence analysis on ventricular cryosections from control (WT) and p66ShcA$^{-/-}$ mice (p66 KO), 7 days upon left coronary artery ligation. The sections were stained with 4-hydroxynonenal (4HNE-J2) antibody (*red*). Data are shown as mean ± SD

thickness was increased in knockout mice. Relative wall thickness was, however, similar between groups before and after myocardial infarction suggesting that this phenotype difference is insufficient to explain protection against heart rupture. We also demonstrated that deletion of p66ShcA attenuates reactive fibrosis and left ventricular dilatation 6 weeks post-infarction. In accordance with the present findings, dilated cardiomyopathy can be attenuated by p66ShcA deletion [8]. The improved survival of KO mice indicates that p66ShcA is instrumental for early cardiac remodelling after myocardial infarction.

The improved survival in the present study was not accompanied by functional recovery. We found neither difference in infarct size, post-infarction scar size nor in inflammatory cell infiltration between the groups. Lack of p66ShcA increases tissue resistance to ischemia–reperfusion injury, as demonstrated in vivo for skeletal muscle [30] and brain [24]. Targeted deletion of p66ShcA was found to be cardioprotective in an ex vivo model of acute ischemia–reperfusion injury due to attenuated oxidative stress [5]. This cardioprotection appears to apply only to a limited window of ischemia duration. In fact, protection was no longer observed when ischemia continues longer than 60 min [5]. At the same time, more recent evidence suggests that the absence of p66ShcA exacerbates the mild myocardial injury induced by 30-min short period of ischemia in vivo. Paradoxically, a tendency for infarct size reduction was observed in case of 60-min long ischemia in the same study [1]. p66ShcA might, therefore, play different roles at different stages of ischemic myocardial injury. Infarct size and experimental model choice between ex vivo and in vivo can also explain the variation in the extent of cardioprotection observed in previously published works [11]. However, protection was reported upon reperfusion, while we used a model of chronic myocardial ischemia without reperfusion. An additional and relevant factor to explain the lack of infarct size reduction in KO mice is that many WT mice died of heart rupture. Therefore, the hearts with the largest injury were negatively selected. To elucidate the molecular mechanism explaining beneficial effects of p66ShcA deletion, cardiac collagens, fibroblast activation, expression of MMPs and matricellular proteins were evaluated. Fibroblast activation and reparative fibrosis are crucial for myocardial tissue integrity post-infarction [7]. In skeletal muscle [30] and skin [6], not only tissue survival, but also tissue repair *per se* can be improved after p66ShcA ablation. Large myocardial infarction without reperfusion induced in our model can explain lack of fibroblast activation and collagen deposition 3 days post-infarction. However, at this time point, we observed increased upregulation of collagen type I at mRNA level in KO mice as compared to wild type, which may also contribute to the improved myocardial healing later. Immunohistochemistry demonstrated facilitated fibroblast activation and collagen production in p66ShcA knockout mice as compared to WT. This can be seen as improved myocardial healing and can explain the reduction in heart rupture.

We demonstrated also that p66ShcA deletion attenuated expression of active MMP-2, as well as gene expression of matricellular proteins. MMPs are involved in heart rupture, dilatation and fibrosis. The downregulation of MMP-2 in the myocardium of KO mice might be beneficial. Indeed, MMP-2 knockout mice have been shown to be protected against heart rupture and fibrosis [10], although MMP inhibition did not elicit any effect in patients suffering for myocardial infarction [13]. MMP2 is also upregulated in non-infarcted tissue after myocardial infarction that may lead to accumulation of altered collagens and diffuse fibrosis [29]. As shown by the decrease in lipid peroxidation, p66ShcA deletion is likely to reduce the formation of ROS required for the increase in MMP-2 expression [19] and development of cardiac fibrosis [26]. The beneficial effect afforded by MMP-2 down-regulation might be reinforced by the increase in MMP-13. This protease which was increased in KO mice after MI, facilitates wound healing [25]. At variance from MMP-2 and -13 that are expressed in cardiac cells [25, 31], MMP-9 and MT-MMP-1 are predominantly expressed in infiltrating immune cells early post-infarction [31]. Targeted deletion of p66ShcA isoform had no effect on Inflammatory cell infiltration. This might explain why MMP-9 and MT-MMP-1 were not altered in KO mice.

The anti-fibrotic effects of p66ShcA deletion are likely to be contributed by down-regulation of matricellular proteins. In fact, in the early post-infarction phase, hearts from KO mice displayed a decrease in gene expression of thrombospondin-1 and CTGF, two pro-fibrotic proteins [27].

Supporting our in vivo findings, we demonstrated that p66ShcA overexpression in vitro resulted in upregulation of total MMP-2 expression, while p66ShcA knockdown was associated with lower expression of activated MMP-2. In both systems we observed accumulation of dimerized MMP-2. Although the function of MMP-2 homodimers is unknown, the dimerization of MMP-2 is redox-dependent [14], which may explain its dependence on p66ShcA expression. Expression of MMP-2 forms was altered only in the cells subjected to hypoxia-reoxygenation, while changes in p66ShcA expression under normoxic conditions had no effect on MMP-2 expression. Overall these data demonstrate that p66ShcA can regulate MMP-2 expression, activation and dimerization during hypoxia.

Conclusion

The p66ShcA isoform appears to promote heart rupture as a consequence of reduced myocardial healing along with increased reactive fibrosis. The adverse outcome is likely to be contributed by p66ShcA-induced formation of ROS that alter MMP expression. Indeed, under chronic ischemia p66ShcA deletion resulted in downregulation of MMP-2 associated with an increase in collagen content, expression of matricellular proteins and fibroblast activation. Based upon our results, the increase in p66ShcA expression observed in hearts of patients suffering from coronary artery disease appears to represent an adverse adaptation. Therefore, p66ShcA might be a target for pharmacological or genetic manipulation during early stages of coronary artery disease or myocardial ischemia to facilitate myocardial healing.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The human studies were in agreement with the 1964 Declaration of Helsinki, and were approved by the Regional Ethics Committee in Norway, and Tampere University Hospital, Finland. Written, informed consent was obtained from all patients or family members. All animal experiments were performed according to the Position of the American Heart Association on Research Animal Use, and ethical permission was obtained from the Norwegian Animal Research Authority.

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ORIGINAL ARTICLE

Release of Mitochondrial and Nuclear DNA During On-Pump Heart Surgery: Kinetics and Relation to Extracellular Vesicles

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Abstract

During heart surgery with cardiopulmonary bypass (CPB), the release of mitochondrial (mtDNA) and nuclear DNA (nDNA) and their association to extracellular vesicles were investigated. In patients undergoing elective coronary artery bypass grafting (CABG, $n = 12$), blood was sampled before, during, and after surgery from peripheral artery, pulmonary artery, and the coronary sinus. Plasma was separated in three fractions: microvesicles, exosomes, and supernatant. mtDNA and nDNA were measured by qPCR. mtDNA and nDNA levels increased after start of surgery, but before CPB, and increased further during CPB. mtDNA copy number was about 1000-fold higher than nDNA. mtDNA was predominantly localized to the vesicular fractions in plasma, whereas nDNA was predominantly in the supernatant. The amount of free mtDNA increased after surgery. There was no net release or disappearance of DNAs across the pulmonary, systemic, or coronary circulation. Extracellular DNAs, in particular mtDNA, may be important contributors to the whole-body inflammation during CPB.

Keywords Extracellular DNA · Cardiac surgery · Exosomes · Microvesicles

Introduction

Open heart surgery with cardiopulmonary bypass (CPB) triggers the immune system and initiates a whole-body inflammatory reaction [8]. This is caused by the extracorporeal circulation per se, by the surgical trauma, and by ischemia reperfusion such after cardioplegic arrest [10]. The mitochondria not only have multiple important roles in ischemia–

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reperfusion injury both as mediators of injury [21] but may also be cardioprotective [16]. Mitochondrial DNA (mtDNA) displays many features of bacterial DNA and has recently been shown to initiate inflammation [34] and acute cardiomyocyte death in mice [5]. Nuclear DNA (nDNA) has some immunogenic actions, but less than mtDNA [3]. Extracellular DNAs are present in the circulation in protein-associated, particle-bound, and cell-bound forms [25]. Furthermore, extracellular DNA is present in microvesicles and exosomes from a variety of cells and such extracellular vesicles have been suggested to play a role in inflammatory responses [14]. The compartmentalization of DNA in the circulation may be important for the physiological effects of extracellular DNA, for instance, their ability to reach potential immune receptors. Extracellular vesicles may also be a part of the crosstalk between cells and may convey biological messages to target cells [24].

mtDNA is elevated in plasma of patients with acute myocardial infarction 3 h after reperfusion by a percutaneous coronary intervention [4]. mtDNA is also found in the circulation during shock and trauma [34] and may be released by neutrophil extracellular traps [19]. Recently, increased levels of mtDNA have been shown in connection with heart surgery with CPB and the peak levels are associated with platelet activation [23] and with the occurrence of postoperative atrial fibrillation [26]. There may be release of DNA in the pulmonary circulation due to trapping of platelets and leukocytes during CPB. DNAs may also be taken up or degraded in the pulmonary microcirculation which serves as a chemical filter [1].

We hypothesize that (1) extracellular DNAs are released into the circulation during heart surgery; (2) DNAs may be markers of myocardial injury after cardioplegic arrest and reperfusion; (3) during heart surgery, there may be a gradient of DNA across the pulmonary vascular bed; and (4) release of DNA is associated with extracellular vesicles. Accordingly, the purpose of the present work was (1) to study the release kinetics of mtDNA and nDNA during on-pump CABG; (2) to study the net release or disappearance of extracellular DNAs across the systemic, the pulmonary, or the coronary vascular bed; and (3) to investigate the distribution of extracellular DNAs between extracellular vesicles and the supernatant in the circulation during surgery and CPB.

Materials and Methods

Coronary Artery Bypass Grafting

Series 1 Patients ($n = 12$, Tartu University Hospital, Estonia) with normal heart function undergoing elective on-pump coronary artery bypass grafting (CABG) were included. All patients had a pulmonary artery catheter and a catheter in the coronary sinus for infusion of cardioplegia and blood sampling. Male:female ratio was 9:3, age 65 ± 9 years (mean ± SD). Length of CPB and cross clamping was 57 ± 15 and 32 ± 9 min, respectively. CPB was conducted at a temperature of 34 °C. Warm, intermittent blood cardioplegia was given ante- and retrogradely. The number of distal anastomoses was 3.9 ± 0.8. Blood was sampled into EDTA tubes from peripheral artery, pulmonary artery, or coronary sinus before, during, and after surgery (Table 1).

Series 2 After series 1 was finished, the possibility to study the localization of DNAs in extracellular vesicles in the circulation became available. Patients ($n = 12$) with normal heart function undergoing elective CABG in Almazov National Medical Research Centre, Saint Petersburg, Russia, were included. Male:female ratio was 8:4, age 69 ± 5 years. Length of CPB and cross clamping was 118 ± 42 and 72 ± 24 min, respectively. CPB was conducted at a temperature of 34–35 °C. Warm, intermittent blood cardioplegia was given antegradely. The number of distal anastomoses was 3.3 ± 1.3. Arterial blood (EDTA anticoagulation) was sampled before, during, and after surgery (Table 1).

Plasma Isolation and Fractions

Within 30 min after blood sampling, the plasma was isolated using double centrifugation at 1600×*g* for 10 min at room temperature, aliquoted and frozen at − 80 °C.

Differential centrifugation was used to separate plasma into three fractions. Thawed aliquot of plasma was centrifuged at 1600×*g* for 10 min at 4 °C. One milliliter of 1600×*g* supernatant was transferred to clean tube and centrifuged at 16,000×*g* for 10 min at 4 °C using centrifuge 5415R (Eppendorf) and fixed angle rotor F-45-24-11.

Table 1 Time points for sampling of blood before, during, and after coronary artery bypass grafting with cardiopulmonary bypass in two different series of patients

Patients		Time point							
		1	2	3	4	5	6a	6b	7
Series 1	Peripheral artery	x	x	x	x	x	x		x
	Coronary sinus		x		x	x			
	Pulmonary artery		x			x			
	Extracorporeal circuit			x	x	x			
Series 2	Peripheral artery	x						x	
	Coronary sinus					x			

Time points: 1 = before start of surgery, 2 = after cannulation, immediately before start of cardiopulmonary bypass (CPB), 3 = after 20 min of CPB, 4 = 5 min after declamping the aorta and start of reperfusion, 5 = 30 min after start of reperfusion, 6a = 1 h after end of operation, 6b = 2 h after end of operation, 7 = morning of 1st postoperative day

The supernatant after centrifugation at 16,000×*g* was transferred to clean tube, mixed with 24 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, pH 7.4), and centrifuged at 100,000×*g* for 2 h at 4 °C using Avanti J-30I ultracentrifuge (Beckman Coulter) and swing bucket rotor JS-24.

Pellets obtained after centrifugation at 16,000×*g* (microvesicular fraction, VES) and 100,000×*g* (exosomal fraction, EXO) were washed and resuspended in 1 ml of PBS for qPCR analysis or in 50 μl of PBS for electron microscopy.

Supernatants after centrifugation at 100,000×*g* were concentrated to a volume of 1 ml (supernatant, SUP) using Vivaspin 20 centrifugal concentrators (VS2021; Sartorius).

Low-Voltage Scanning Electron Microscopy

For quality control, microvesicular and exosomal fractions were subjected to low-voltage scanning electron microscopy as described earlier [18].

Isolation of DNA

Total DNA from 240 μl of human whole plasma or 200 μl of plasma fractions was isolated using NucleoSpin Plasma XS DNA isolation kit according to the manufacturer recommendations. DNA samples were treated with heparinase before PCR [17]. For normalization of measured DNA levels, each plasma sample was spiked (0.4 pg/μl) with an external standard DNA sequence MW2060 [9].

qPCR and Western Blotting

Primers (Supplementary Table 1) were designed by Primer Express software (Applied Biosystems, Foster City, USA) and purchased from Eurofins MWG Operon (Ebersberg, Germany). Amplification was performed in 7500 Real-Time PCR system (Applied Biosystems). Relative levels of mtDNA marker cytochrome C oxidase subunit III (CO3) and the single copy nDNA marker beta-2-microglobulin (B2M) were determined by qPCR with hydrolysis probes and TaqMan® Universal PCR Master Mix (Applied Biosystems). Relative levels of external standard sequence (MW2060) and nDNA marker Alu, a high-copy repetitive sequence (> 100,000 copies in genome), were determined by qPCR with primers and qPCRmix-HS SYBR+ROX master mix (Evrogen). All data was normalized to the added external control gene (MW2060), and the relative quantity of both mtDNA and nDNA for each sample was calculated as follows: $2^{Cq\ MW2060}/2^{Cq\ target\ gene}$, where Cq is quantification cycle of the corresponding target. Analysis of DNA levels from series 1 was performed in Oslo, Norway and from series 2 in St. Petersburg, Russia.

Western blot analysis was performed in total and fractionated plasma samples using antibodies against a marker of extracellular vesicles, CD9, (ab65230, Abcam), or the mitochondria marker, Mitofusin2 (ab56889, Abcam).

Analysis of Troponin

In CABG patients from Tartu, Estonia, plasma levels of troponin T were measured using STAT (Roche) electrochemiluminescence immunoassay on ECLIA with analyzer Cobas-e-411. In CABG patients from Almazov National Medical Research Centre, levels of the cardiospecific isoform of troponin I were measured using chemiluminescent immunoassay ARCHITECT STAT troponin-I (Abbot) with ARCHITECT i2000 immunoassay analyzer.

Statistics

Statistical analyses of serial matched measurements were done with Friedman test with Dunn's post hoc correction for multiple comparisons. In the text, all values are given as mean ± SD unless otherwise stated. GraphPad Prism (San Diego, CA) was used for statistical analysis.

Results

Postoperative course was uneventful for all patients operated without any perioperative infarctions as evaluated by postoperative electrocardiograms and release of biomarkers (troponin T and I).

Kinetics of Extracellular DNA

Series 1 of CABG patients were used to study kinetics of extracellular DNA levels during surgery. Extracellular mtDNA and nDNA increased during surgery before start of CPB, reached maximum at 20'CPB, stayed high during reperfusion, and decreased 1 h postoperatively (Fig. 1). Maximal DNA release was not associated with length of CPB (data not shown). The increase of nDNA was relatively higher and more long-lasting compared to that of mtDNA. However, calculating the relative levels of circulating extracellular DNA showed that the copy number of mtDNA was 4500-fold higher than nDNA in the preoperative samples (median value 4564 (1493–13,281, 25 and 75 percentiles respectively)). The findings of a much more copies of mtDNA compared to nDNA were consistent in every vascular bed (Fig. 1). One hour postoperatively, the ratio mtDNA/nDNA was significantly reduced ($p = 0.02$, median value 520 (96–1066, 25 and 75 percentiles respectively)). No net degradation or release of extracellular DNA that occurred across the pulmonary, coronary, or systemic circulation was observed. However, gradients

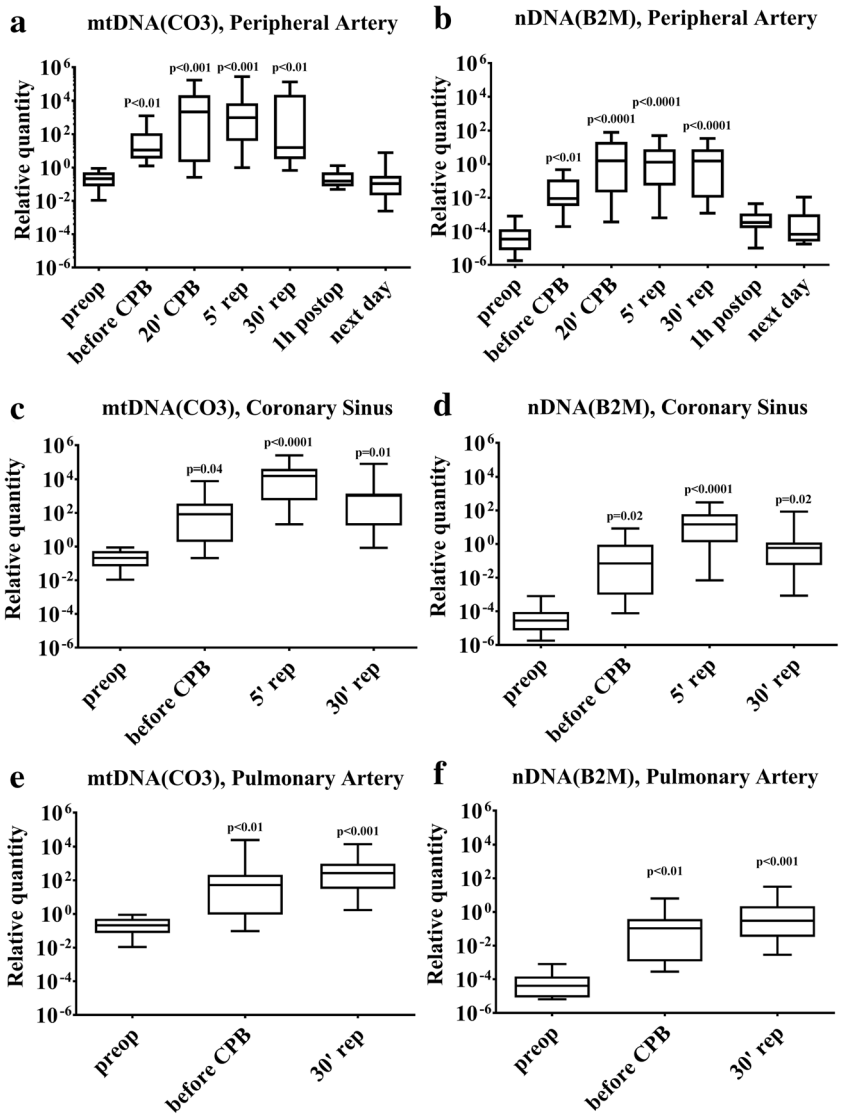

Fig. 1 Mitochondrial (mtDNA) and nuclear DNA (nDNA) measured in the radial artery, the coronary sinus, and the pulmonary artery in patients undergoing coronary artery bypass grafting (n = 12) with cardiopulmonary bypass (CPB) and cardioplegia. Blood was sampled preoperatively (preop), after surgery immediately before CPB (before CPB), 5 and 30 min after starting reperfusion of the cardioplegic heart (5′ rep and 30′ rep, respectively), 1 h postoperatively, and the next day (next day). DNAs were measured by qPCR and expressed as relative quantity. Data are presented as box plots with median values, 25 and 75% percentiles. Whiskers represent maximum and minimum values. All values are compared to baseline

observed in individual patients might indicate that either a release or degradation occurred in some patients (Fig. 2). These patients did not differ in any other way such as longer CPB time or longer cross-clamping.

Distribution of Extracellular DNA in Plasma Fractions

Series 2 of CABG patients were used to study the distribution of extracellular DNAs between extracellular vesicles and the supernatant of plasma during the operation. At baseline, mtDNA was highest in the vesicular fraction; however, after operation, the increase was in the supernatant (Fig. 3a, b). At baseline, most nDNA was in the supernatant fraction and the level in exosomal fraction was the lowest. But the only fraction with a significant increase of nDNA during surgery was the exosomes. Again, mtDNA was higher than nDNA (Fig. 3a, b). There were no significant correlations between time of CPB or time of aortic clamping and extracellular DNA. The relative distributions of extracellular DNA between the three fractions of plasma when their sum was set to 1 are shown in Fig. 3c, d.

Characterization of Plasma Fractions

Vesicle populations in plasma fractions were visualized by low-voltage scanning electron microscopy (Fig. 4a). The marker of the mitochondrial outer membrane (Mitofusin2) was present both in microvesicular and exosomal fraction but not detected in whole plasma or in the supernatant (Fig. 4b). The lack of Mitofusin2 signal in whole plasma can be explained by the dilution of protein extract from plasma to ensure that equal amount of proteins was loaded in each lane. Specific antigen of extracellular vesicles (CD9) was present in all fractions (Fig. 4b). Some extracellular CD9 may remain in the supernatant even after centrifugation at 150,000×g [30] which can explain CD9 in the supernatant fraction.

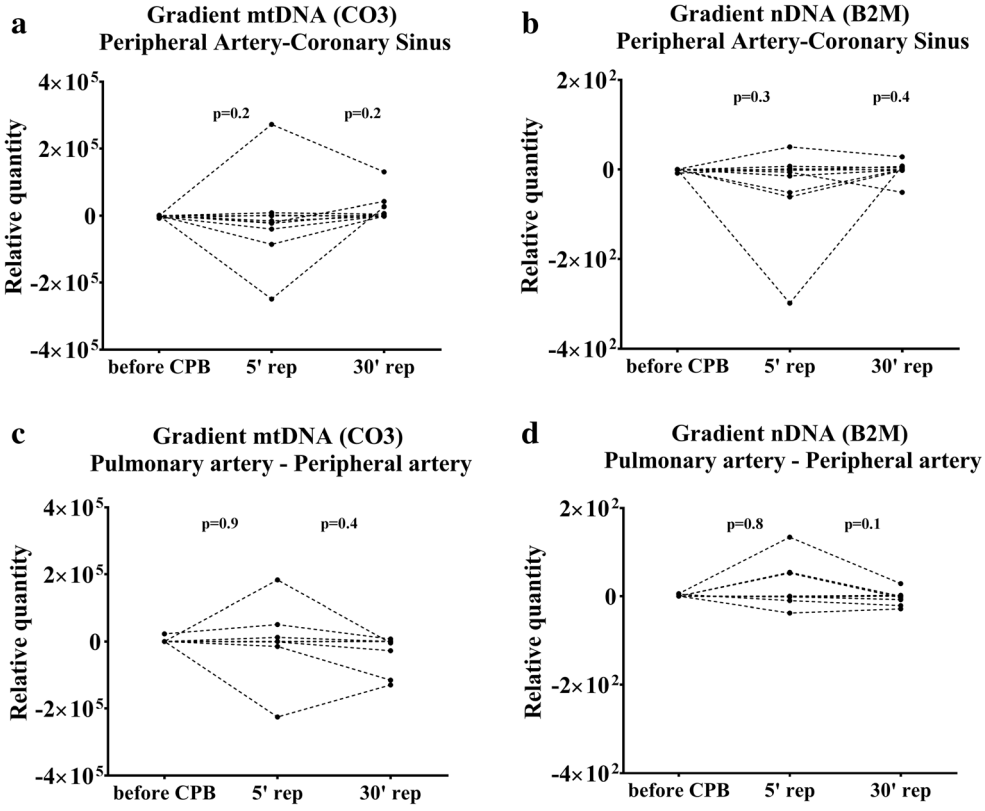

Fig. 2 Gradients of mitochondrial (mtDNA) and nuclear DNA (nDNA) showing no statistically significant disappearance or release across the pulmonary, systemic, or coronary vascular beds in patients undergoing coronary artery bypass grafting ($n = 12$) with cardiopulmonary bypass and cold cardioplegic arrest. Blood was sampled concomitantly from the pulmonary artery, the coronary sinus, and radial artery after surgery immediately before start of CPB (before CPB), as well as 5 and 30 min after start reperfusion of the cardioplegic heart (5′ rep and 30′ rep, respectively). DNAs were measured by qPCR and expressed as relative quantity

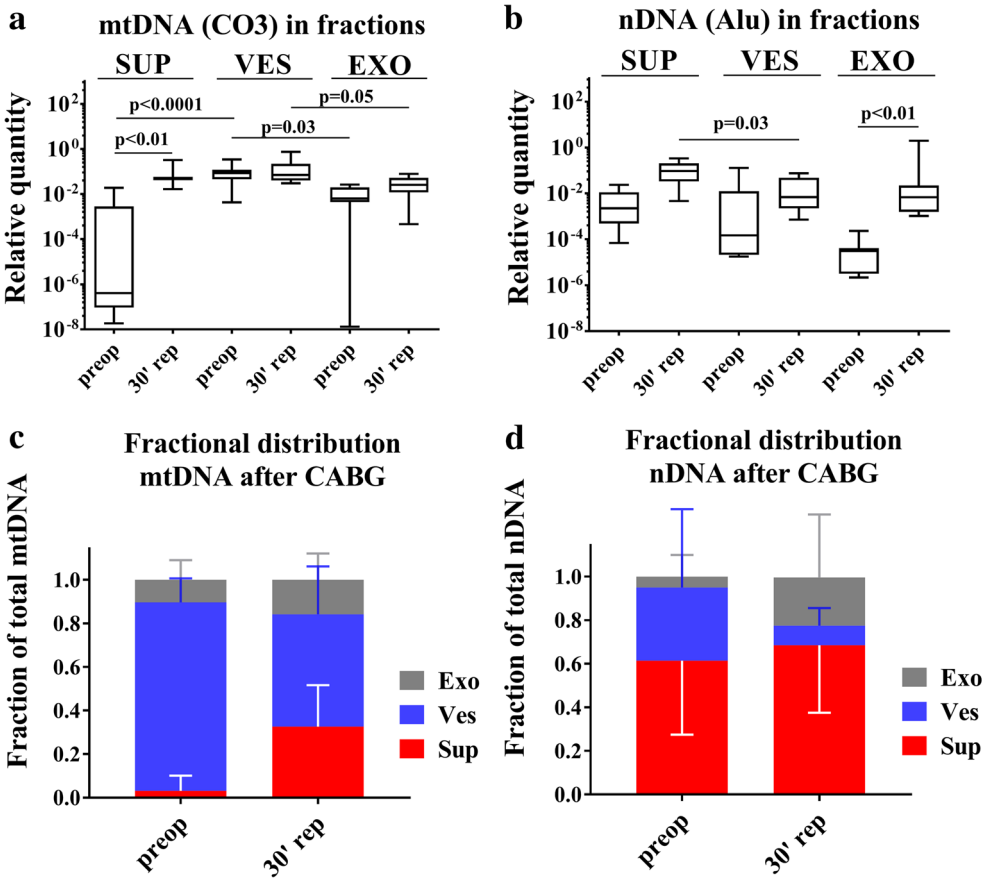

Fig. 3 Localization of mitochondrial (mtDNA) (**a**) and nuclear DNA (nDNA) (**b**) in different plasma fractions in patients undergoing coronary artery bypass grafting ($n = 12$) with cardiopulmonary bypass and cardioplegia. Blood was sampled preoperatively (preop) and 30 min after start reperfusion of the cardioplegic heart (30′ rep). The plasma was separated into supernatant (SUP), microvesicles (VES), and exosomes (EXO). DNAs were measured by qPCR and expressed as relative quantity. Data are presented as in Fig. 1. **c**, **d** The fractional distribution of mtDNA and nDNA preoperatively and 30 min after start reperfusion of the cardioplegic heart (30′ rep)

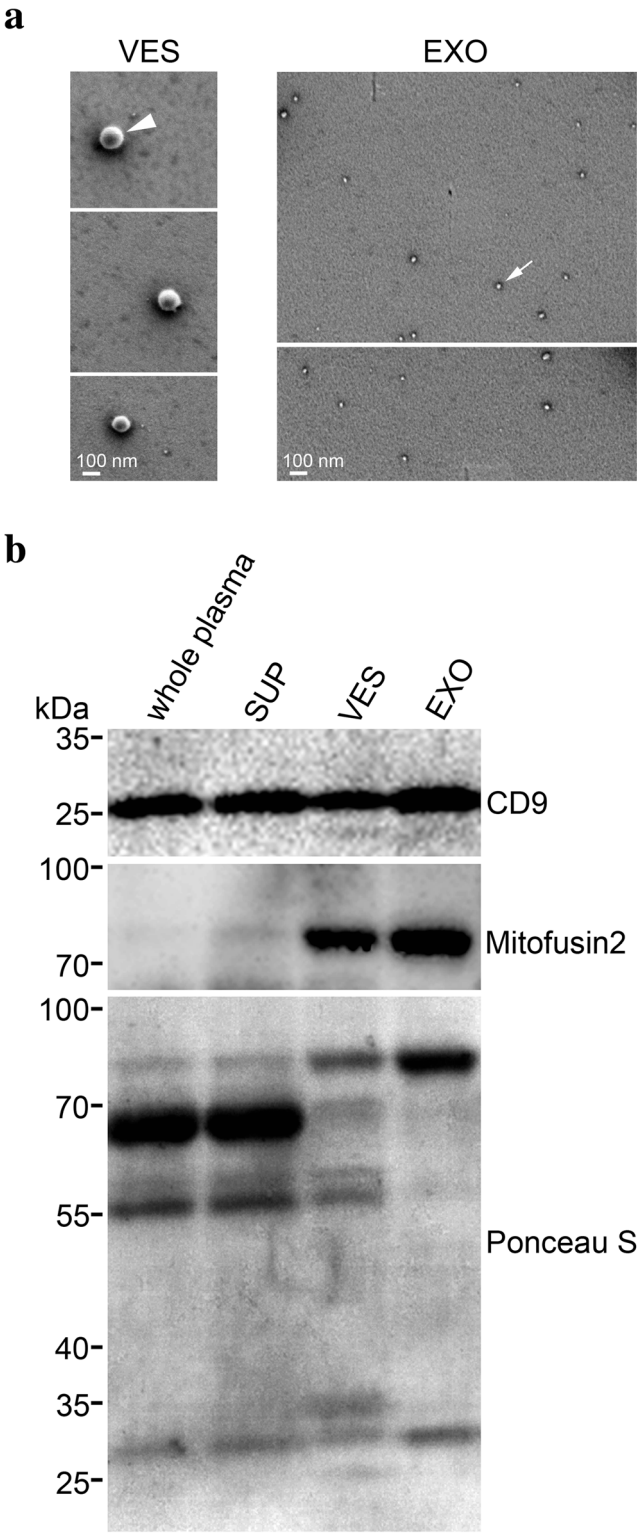

Fig. 4 **a** Microphotographs of objects in the microvesicular (pelleted at 16,000×*g*, VES) and exosomal (pelleted at 100,000×*g*, EXO) fractions of plasma, obtained by low-voltage scanning electron microscopy. **b** Detection of CD9 (marker of extracellular vesicles) and Mitofusin2 (mitochondrial outer membrane marker) in the protein extracts from whole plasma and its fractions using Western blotting. Ponceau S staining was used to demonstrate protein profiles of samples

Moreover, some markers of extracellular vesicles such as CD9 may not be entirely exosome-specific [27].

Discussion

Both vesicle-associated and non-vesicle-associated mtDNAs in the circulation of healthy individuals are known [7]. Less is known how this may change during surgery and trauma. The present study was not powered to make correlations with clinical outcome but concentrated on the basic mechanisms of release kinetics of DNAs during on-pump heart surgery, bringing to attention DNAs, in particular mtDNA, as a potential important contributor to the whole-body inflammation during surgery. This investigation also brings into attention extracellular vesicles as a part of surgical pathophysiology. The main findings of the present investigation were the following: (1) the mtDNA copy number in plasma was much higher than nDNA; (2) the surgical trauma released both nDNA and mtDNA; (3) increased levels of extracellular DNA were maintained during CPB; (4) mtDNA in the circulation was primarily in microvesicles/exosomes, while nDNA was mainly in the supernatant; and (5) during heart surgery, mtDNA increased in the supernatant while nDNA increased in the exosomal fraction of plasma. All taken together, there is a large release of the highly pro-inflammatory mtDNA during heart surgery with CPB.

Release and Kinetics of DNAs

During preparation for CPB, a major release of mtDNA and nDNA occurred. By spiking the plasma samples with a known amount of synthetic DNA fragment and using this as external control DNA, we can speculate about the ratio between mtDNA and nDNA before and after cardiac interventions. The preoperative ratio of mtDNA:nDNA was about 4500 in plasma. This is in accordance with the mtDNA:nDNA ratio from cardiac and skeletal cells [20]. The high amount of mtDNA lasted throughout the observation period. The rise in extracellular DNA before start of CPB showed that surgery itself caused a massive release of DNAs. This is in agreement with previous findings that tissue trauma releases DNA [33]. While on-pump and suturing anastomoses, the tissue trauma is minimal. Extracellular DNA increased further during CPB suggesting that CPB per se caused a significant release of DNA. The normal half-life of extracellular DNA is about 15 min [29], indicating a constant turnover during CPB. A continuous release and degradation or uptake of DNAs likely occurred during operation, and we observed the net result which was independent of length of CPB. The mtDNA/nDNA ratio in arterial blood decreased during surgery and

CPB. This might indicate that although the mtDNA release and amount was highest, the nDNA release was relatively higher, or the disappearance of mtDNA may be more pronounced.

The continuous release of DNAs during CPB is most likely caused by the extracorporeal circulation, suction, and blood–air interaction causing a whole-body inflammatory reaction. In previous studies, mtDNA was elevated for hours and even 1–2 days [23, 26], whereas in the present study, the level of mtDNA was back to normal 1 h postoperatively. To differentiate the respective roles of CPB and the surgical trauma, a prospective randomized study of on- and off-pump surgery is needed. That extracorporeal circulation releases DNAs is supported by the finding that hemodialysis induces DNA release [1]. Furthermore, release of DNA during on-pump surgery is dependent on the length of CPB [22].

Kinetics in Different Vascular Beds

No important perioperative net release or disappearance of mtDNA was found in the pulmonary or the coronary vascular bed, both of which have an ischemia–reperfusion injury during total CPB. Either the ischemic insult was too small or the observation time after declamping (30 min) was too short to observe any differences. Consequently, the present study suggested that extracellular DNAs may not be suitable as markers of myocardial injury during heart surgery using cardioplegic arrest.

A possible disappearance might occur across the pulmonary vascular bed which acts as a chemical filter taking up or degrading a variety of substances [2]. Furthermore, DNAs might be released from trapped blood cells in the pulmonary circulation upon reperfusion. However, no gradient was observed across the pulmonary circulation. Interestingly, 5 min after declamping, some big fluctuations in gradients across the different vascular beds were observed, but mean gradients were not significantly influenced.

Characteristics and Mechanisms of DNA Release

The present study is the first to investigate release of DNAs in extracellular vesicles induced by surgical trauma and CPB. DNAs may be released from dead or injured cells as well as by active release from living cells or through neutrophil extracellular traps [19]. Both ischemia reperfusion and CPB may activate and injure endothelial and blood cells and activate immune cells such as leukocytes and macrophages with subsequent release of DNAs. It has been shown that isolated neutrophils from CPB patients release less mtDNA compared to neutrophils isolated before surgery [26].

Extracellular DNAs from different organs and cells may be associated with microvesicles and exosomes in plasma [15, 28]. These organelles are loaded with highly bioactive materials and are released from cells by a variety of stimuli [13, 24]. During heart surgery, most extracellular vesicles probably originate from blood cells and endothelium. In the present study, mtDNA was at baseline mainly localized to microvesicles, but its release during operation was almost exclusively in the non-vesicular fraction of plasma. It is reasonable to suggest that the free mtDNA has the strongest effect as an initiator of inflammation. In contrast, nDNA was mainly in the supernatant. Some of the nDNA was associated with microvesicles before CPB, while after CPB, it was released in exosomal fraction. Extracellular vesicles were previously regarded as cell debris but are now recognized as an important part of signalling and cell-to-cell communication in health and in disease [15]. It has been found that exosomes from cardiomyocytes convey biological messages to target cells [31], and extracellular vesicles are able to initiate and cause inflammatory reactions [14]. They may be an important contributor to the whole-body inflammation caused by surgery and CPB.

Extracellular vesicles may act as transporters of bioactive substances between cells. We can only speculate about the relative importance of DNAs free in plasma or in extracellular vesicles. It is tempting to suggest that the free DNA, which can act directly with blood and immune cells and also with endothelium, might be the important fraction. Consequently, it may be very important that the release of mtDNA during operation was primarily as “free” mtDNA.

mtDNA and nDNA have some inflammatory effects in common as both activate coagulation and platelets [3]; however, mtDNA has much stronger effects on leukocytes and inflammation. Mitochondria are believed to be evolutionary endosymbionts derived from bacteria, and mtDNAs have similarities to bacterial DNA [6, 11]. This resemblance to bacterial DNA makes mtDNA able to trigger inflammatory responses. Moreover, mtDNA increased cell death in mice cardiomyocytes in vitro while no effect was found with nDNA [5].

mtDNA leaking from damaged tissue or actively being released from living cells may enter the circulation as (1) mtDNA anchored to damaged mitochondria membrane, (2) mtDNA in intact free mitochondria, (3) released from neutrophil extracellular traps, and (4) inside microvesicles or exosomes. However, the extracellular vesicle fractions may contain not only exosomes and microvesicles but also fragments of cell membranes, organelles and chromatin [32], and large aggregates of biomolecules [12]. The extracellular vesicle fractions may contain mitochondria or mitochondrial membranes, as Mitofusin2 was increased in these samples.

Limitations of Study

1. A clear differentiation between the effect of the surgical trauma and CPB on release of mtDNA and nDNA has not been investigated, although it is likely from the present findings that both induced DNA release.
2. No data are available showing the cellular origin of the extracellular vesicles.
3. The patients operated upon are simple, straightforward cases. The pattern of release may be different in sicker patients with more complex surgery and longer aortic clamping and time on CPB.
4. The study cohort is too small for making any important correlations with clinical outcome.

Conclusion and Clinical Relevance

Surgery and CPB induced a pronounced release of mtDNA and nDNA. A major part of the mtDNAs in the circulation is associated with extracellular vesicles. There was no early release from the heart after cardioplegic arrest. Extracellular DNAs, in particular mtDNA, may play an important role in whole-body inflammation during major surgery, in particular cardiac surgery using extracorporeal circulation. So far, we do not know the possible role of extracellular vesicles in this context. At the time being, there are no possibilities to block mtDNA; however, future inhibition of mtDNA or its effectors may attenuate sterile inflammation after trauma and major surgery.

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Compliance with Ethical Standards

The studies were performed in agreement with the ethical standards stated in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The studies were approved by the Regional Ethics Committee in Oslo, Norway; Tartu University Hospital, Estonia; or the Institutional Ethics Committee at Almazov National Medical Research Centre, Saint Petersburg, Russia. Written, informed consent was obtained from all patients.

Conflict of Interest The authors declare that they have no conflict of interest.

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