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Prog-Tg mice and endothelial cells“

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Table of content

1 INTRODUCTION	4
1.1 Lamins.....	4
1.2 The vascular system	5
1.3 Aging	6
1.3.1 Aging in the vascular system	7
1.3.2 Aging and vascular diseases	9
1.4 Atherosclerosis.....	10
1.5 Hutchinson Gilford Progeria Syndrome (HGPS).....	12
1.5.1 Genetic abnormalities in HGPS.....	14
1.5.2 HGPS versus normal aging.....	15
1.5.3 Atherosclerosis in Hutchinson-Gilford Progeria Syndrome	16
1.5.4 Therapeutic approaches	17
1.5.5 Other laminopathies	19
1.6 Myocardin-Related Transcription Factor (MRTF) mediated regulation of the vascular system.....	19
2 MATERIAL AND METHODS	23
2.1 Material for tissue isolation and cell culture	23
2.2 Generation of bi-transgenic mice expressing laminA or progerin	23
2.3 Mice dissection.....	25
2.4 Isolation of endothelial cells from tissue	25
2. Tissue homogenization for RT qPCR	26
2.6 Histological examinations of heart and aortic tissue.....	27
2.6.1 Hematoxylin-Eosin staining.....	27
2.6.2 Masson's Trichrome staining	28
2.7 RNA isolation from endothelial cells	28
2.8 Quantitation of mRNA expression levels in tissue and endothelial cells.....	29
2.8.1 cDNA synthesis.....	29
2.8.2 Determination of primer specificity	30
2.8.3 Validation of the performance with a standard curve	31
2.8.4 Primer sequences used for the determination of gene expression levels .	32
2.8.5 RT qPCR.....	33
2.8.6 RT qPCR analysis.....	34
2.9 Shear stress application on subconfluent endothelial cultures seeded on a 6- channel slide	34

2.10 Immunofluorescence	35
2.10.1 Primary antibodies	35
2.10.2 Antigen retrieval and staining procedure	36
2.10.3 ImageJ analysis and intensity evaluations	36
3 RESULTS	37
4 DISCUSSION	47
4.1 Phenotypic changes in transgenic mice	47
4.2 The role of progerin expressing endothelial cells in cardiac hypertrophy	47
4.3 MRTF-A changes correlate with changes in expression levels of pro- atherogenic genes.....	49
6 REFERENCES	53
7 APPENDIX	58
7.1 List of figures	58
7.2 List of tables	58
7.3 List of abbreviations	59
7.4 Abstract	60

1 INTRODUCTION

1.1 Lamins

One characteristic feature of the cell's nucleus is the nuclear envelope consisting of an outer and an inner membrane with perinuclear space containing nuclear pores continuous with the outer membrane. Nuclear pores form a connection between the nucleus and the cytoplasm. The inner side of the nuclear membrane is lined with a protein-rich fibrous layer. It is 30-100 nm thick and connects the inner membrane to the chromatin. This layer consists mainly of three membrane proteins, lamin A, B and C. Lamin A and C have a mostly identical amino acid sequence. However, lamin A contains an additional 133 amino acid long C-terminal sequence. Lamin A and C are encoded by the same gene by alternative splicing. All types of lamins contain an NLS (nuclear localization signal) sequence. [1] [2] [3]

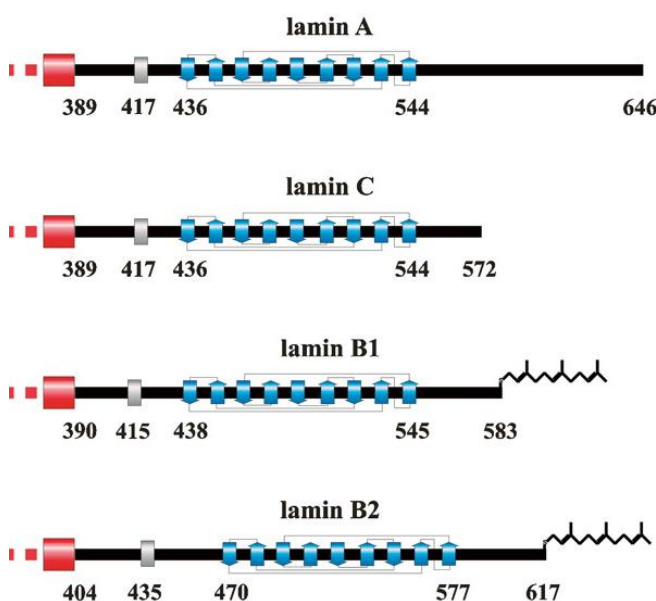


Fig. 1: a schematic representation of all types of human lamins consisting of an alpha helical rod (red), a nuclear localization signal (grey) and an Ig-fold (blue); lamin B1 and B2 are farnesylated as well as carboxymethylated at their C-terminal while lamin A and C are not. [4]

Nuclear lamins build a thin mesh-like network which interacts with various proteins as well as with chromatin and it is necessary for maintenance of the nuclear envelope's structure. Furthermore, lamins form a protective barrier between the cytoplasm and the endoplasmic reticulum. They are a special class of type V intermediate filaments forming a three-dimensional network. These proteins are cable-like fibres with a diameter of approximately 10 nm involved in maintaining the typical nuclear architecture. They strengthen the shape and stability of the nuclear envelope,

anchoring nuclear pore complexes and chromosomes. Lamins interact with various membrane proteins of the inner membrane and thus with the chromatin. [5] [7]

The membrane proteins and lamins are connection points of DNA and the nuclear envelope. During mitosis, the nuclear envelope is disassembled. This disassembly is regulated by phosphorylation and results in the breakdown of the protective barrier. [8]

The *LMNA* gene codes for two types of lamins, lamin A and lamin C. Two other genes called *LMNB1* and *LMNB2* encode B-type lamins, lamin B1 and lamin B2/3. *LMNA* is 25 kb long and lamin A is formed from exon 1 to exon 12. Exon 1 to exon 10 are responsible for building up lamin C. B-type lamins are essential in embryonal development and present in all cells at birth. However, A-type lamins are missing during embryonal development and are expressed at later stages. Furthermore, B-type lamins possess a farnesylated and carboxymethylated C-terminal ends which anchors those lamins into the inner nuclear membrane. Hence unlike B-type lamins, A-type lamins appear mobile in the nucleus. (see figure 1) [6] [8]

Inside every nucleus there are millions of binding sites for lamin A. Various proteins bind to this lamin network and perform different functions. Interferences in this complex network through mutations in *LMNA* can lead to the development of laminopathies. Two hypothesis were established in order to explain the origin of such laminopathies. The “structure hypothesis” assumes that mutation in lamins increase the nuclear fragility and that disturbed response to mechanical stress leads to early apoptosis. The “gene-regulation hypothesis” says that lamins play an important role in DNA transcription regulation and therefore influencing cellular processes. Today’s knowledge proposes a combination of both theories and their tight interaction. [9] [10]

1.2 The vascular system

The circulatory system also called vascular system is built up of vessels which transport blood and also lymph through the body. The system is necessary to deliver oxygen and nutrients to all body organs and tissues and to remove waste matter. Lymphatic fluid is a clear and colourless liquid including water and blood. The lymphatic system supports the protection and maintenance of the whole organism by filtering and draining the lymph. [11] [12]

Components of the vascular system are arteries that transport oxygenated blood away from the heart, veins that carry the blood back to the heart and capillaries which are small blood vessels forming a connection between arteries and veins. [11] [12] [13]

By pumping of the heart, blood circulates through the body within the vascular system. Through the arteries, oxygen-saturated blood leaves the heart in order to supply the body with oxygen. Using veins, blood is returned to the heart and re-saturated by pulmonary activity. The vessels consist of three main layers called tunica externa, media and intima. The latter one including the endothelium. [11] [12] [13]

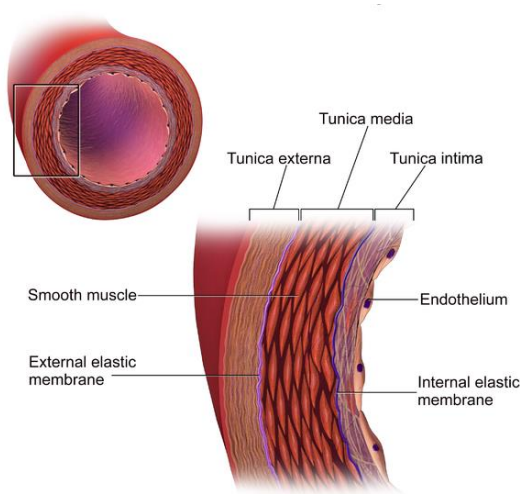


Fig. 2: The structure of a mammalian artery indicating the tunica intima including the endothelium and the internal elastic membrane, the tunica media consisting out of a smooth muscle layer and the external elastic membrane and the tunica externa. [14]

1.3 Aging

There are several theories regarding aging and its cause. However, most genes, molecules and signalling pathways appearing in all of these theories are linked and influencing each other. Originally, aging was understood as an accumulation of damages in the most important cellular biomolecules. Aging and the associated physical decline were described as a attrition process. After decades of intense molecular research, it is known that the aging of cells and organisms is also influenced and modulated by genes and an evolutionary conserved mechanisms. Examples for these mechanisms are the mTOR signalling pathway, oxidative stress or mitochondrial activity. The manipulation of such single genes and mechanisms can lead to a prolonged lifespan. [15] [16] [17]

These different aging theories are related to each other, an example is the amount of energy production in mitochondria linked to the generation of oxidative stress and many redox-regulating enzymes and oxidative stress-responding genes. Those

connected protein- and signalling pathways form a molecular matrix of aging. It seems like there are almost indefinite interlocking. [15] [16]

The journal *Cell* published an article concerning aging and its “key hallmarks of aging”. During this study, nine characteristics are presented which are described as the “common denominator of aging”. These hallmarks contributing to the complex and multifactorial process of aging are: telomere attrition, genomic instability, epigenetic alterations and chromatin remodelling, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, defects in the nuclear architecture, stem cell exhaustion and altered intercellular communication and cellular senescence. [17] [18]

There is also a relation between aging and evolution. Usually, aging is defined as the increasing loss of function accompanied by decreased fertility and rising mortality. [19] During the study of different species, evolutionary aging theories were developed which explain why aging principally happens. These theories can predict that: it is unlikely that there are specific genes promoting the aging process and that aging is not programmed but a result of the accumulation of somatic damages emerging from the limited expenditure for maintenance and repair. Hence, prolonged lifespan is controlled by genes regulating activities like DNA repair or anti-oxidative defence. There are a variety of genes influencing resistance against geriatric diseases. Furthermore, there could be disadvantageous gene activity. Those could have escaped from the forces of natural selection or they are pleiotropic genes which give an advantage in younger age against a disadvantage in the aging organism. However, there is no pressure of selection against aging because selection happens on a reproductive level. [20] [21]

Based on plenty of data, the influence of the interaction between genes, environment and their complex cooperation determine the aging process and the resulting aging phenotype. [21] [12]

1.3.1 Aging in the vascular system

The entire vascular system is known to undergo many changes during aging, including coronary and peripheral arteries, capillaries and the heart itself. One special hallmark is the hypertrophy of heart tissue due to a thickened heart wall in an aging heart. Cardiac valves regulate the unidirectional blood flow and become stiff and

porous. Furthermore, there is a loss of cardiomyocytes in the sinoatrial node. In order to compensate this loss, an increase in cardiomyocyte size occurs and causes a progressive decline in the physiological cardiac functions. This leads to a structural change in blood vessels as well, like the increase of aorta's thickness and stiffness. Smaller vessels undergo similar alterations. The heart tries to overcome these obstructions by enhancing its pumping capacity. All of these changes lead to hypertension due to increased blood pressure, hypertrophy of the cardiac tissue and heart failure. [22] [23] [24]

The inner layer of all vessels is composed of a layer of endothelial cells, the so-called vascular endothelium. It is in direct contact with the circulating blood, serving as a barrier between blood and underlying cell layers in the vessel. Furthermore, it is a very important player in the maintenance of physiological functions, for example in the regulation of blood pressure by vasoconstrictive and vasodilatory mechanism, vascular homeostasis and promotion of angiogenesis. During the aging of the endothelium, endothelial cells become flat, enlarged and have a polypoid nucleus. These factors promote cellular senescence. Furthermore, alterations in the cytoskeleton, proliferation, angiogenesis and cell migration occur frequently. [23] [24]

An important protein family expressed by endothelial cells is VEGF (vascular endothelial growth factor). VEGF-signalling is crucial for vasculogenesis and angiogenesis by the de novo production of endothelial cells. Loss of VEGF genes is linked to vascular abnormalities and embryonic lethality. Furthermore, a very important molecule called eNOS is produced by endothelial cells. eNOS (endothelial nitric oxide synthase) generates nitric oxides which promote vasodilation and smooth muscle cell proliferation. As shown in previous studies, such anti-atherosclerotic functions mediated by endothelial cells decrease with age; meaning, old endothelium produces less eNOS, but its exact mechanism is still unknown. Theories also propose that eNOS activity can be influenced by shear stress, hormones and growth factors. [25] [26] [27] [35]

Furthermore, aging causes several alterations in VSMC's (vascular smooth muscle cells) as well. VSMC's are also regulators of the vascular wall by vasoconstrictive and vasodilatory mechanisms. They migrate from the media to the intima and accumulate there. These misplaced muscle cells are less functional and do not

respond to growth factors, leading to endothelial alterations and impaired vascular performances. [28]

The formation of new blood vessels called angiogenesis is a very important physiological process. Hypoxia activates the transcription of HIF-1 (hypoxia-inducible factor 1) which increases the generation of VEGF and other growth factors. Based on several studies, VEGF levels seem attenuated and angiogenesis is reduced during aging. Also wound healing is linked to angiogenesis and markedly decreased in old individuals. Decreased levels in eNOS resulting from reduced VEGF cause impaired angiogenesis and disturbed wound healing. [29] [30]

In addition, in old organisms changes in cell-cycle regulating molecules which affect the proliferation of endothelial cells were observed. This results in the increased senescence of endothelial cells which limits neovascularization. NO prevents this endothelial specific senescence, which is linked to decreased eNOS levels. However, TERT (telomerase reverse transcriptase) is also active in endothelial cells which prevents senescence by counteracting telomere shortening. After several passages, EC's show reduced NO and a loss of TERT activity. Hence, VEGF is a potent anti-senescence agent, suppressing p16 and p21 activity in endothelial cells. [30] [31]

Another agent causing endothelial aging and dysfunction is oxidative stress. Aged endothelium generates free radicals which are suspected to accelerate aging. Oxidative stress markers were found in arteries of aged animals, leading to an increased production of reactive oxygen species (ROS). One of the main free radicals is super oxide anion (O_2^-). Its formation takes place in aged mitochondria due to mitochondrial DNA damage. The presence of NO and O_2^- leads to the formation of peroxynitrite, an aggressive free radical. It was found out that this peroxynitrite can inactivate eNOS in the endothelium. The enhanced production of O_2^- can be explained by the switch of eNOS from an NO producing enzyme to an O_2^- producing enzyme. [32] [33] [34]

1.3.2 Aging and vascular diseases

Aged vascular endothelium is tightly linked to the development of several vascular diseases including CVD (cardiovascular disease) linked to atherosclerosis. The identification of molecular changes that arise during aging of the endothelium gives insights into the development as well as into novel therapies of CVD's. [36]

CVD's are the number one cause of death worldwide. Coronary artery disease (CAD) which means blocking of coronary arteries due to atherosclerosis is the major pathology of CVD resulting in reduced blood and oxygen supply of the heart. A complete vascular blockage leads to heart attack. [36]

Endothelin, a vascular derived growth factor was found to be significantly increased in aged endothelial cells. EC's as well as VSMC's express endothelin specific receptors called ET-A and ET-B. ET-A activation results in vasoconstriction and proliferation of VSMC's, however, ET-B activation leads to an enhanced generation of NO, causing vasodilation. Previous studies indicated that ET-A receptors mainly contribute to the development of atherosclerosis. Moreover, endothelin acts also as an suppressor of eNOS in the endothelium by ET-A activation. [37] [38]

With increasing age, the expression of various adhesion molecules such as ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule 1) involved in the development of atherosclerosis, is increased. ICAM-1 and VCAM-1 facilitate the binding of immune cells to endothelial cells and support the migration of these cells into the arterial wall. Monocytes differentiate into macrophages and become foam cells by taking up oxLDL. Furthermore, enhanced ICAM-1 levels are an important marker for inflammation which accelerates the process of atherosclerosis. Activated T-cells secrete cytokines and VSMC's begin to proliferate. [35] [39]

1.4 Atherosclerosis

Arteriosclerosis is derived from the Greek "arteria", meaning artery, and "sclerosis" meaning hardening. It is a general term for chronic degenerative diseases of arteries and it is used as a broader term for vessel changes caused by atherosclerosis. Other rare reasons are inflammatory processes or relapsing embolisms. [40] [41]

However, atherosclerosis or calcification of arteries means a damage of the blood vessel's inner layer (intima) and lipid depositions in middle and big arteries. These injuries in the inner and middle vessel's layer (media) emerge clearly more often nowadays than in former times. This fact can be attributed to changed lifestyle habits. In general, atherosclerosis is accelerated by the aging process. In western society, atherosclerosis is responsible for approximately 50% of deaths, with men more affected than women. [40] [42]

Atherosclerosis is a multifactorial disease with not yet entirely clear initial origins. Based on several studies it is possible to identify potential reasons, factors and its biochemical course of action. Different risk factors determine the manner and the extent of the disease pattern. Common risk factors are hypertonia, nicotine consumption, overweight, physical inactivity, diabetes mellitus, fat metabolic disorders, gout or hormonal disorders, resulting in impairment of blood circulation and liver disorders. Furthermore, toxins like alcohol and drugs massively stress the metabolisms and the whole organism. High blood pressure is developed as a compensation mechanism and leads to injuries of the blood vessels. [41]

Usually, atherosclerosis takes a long and silent course. It starts with degenerative changes in the arteries like vessel wall hardening, thickened and constricted vessels with lost elasticity and circulatory disorders. This results in the disruption of the intima and the damage of the endothelial layer causing the incorporation of proteins, lipids and minerals (like calcium) into the tunica intima. This state is called the atheroma and is still curable using appropriate medication and change in life style. If untreated these debris can be inflamed and can cause ulceration. Scar tissue is formed which builds an inelastic and sclerotic layer. Together with necrotic cells, so-called plaques arise. During this stage, an increase in smooth muscle cell proliferation and collagen production is monitored. This fibrosis occurs during chronic inflammations or the damage during the aging-process. Overall, this leads to an increased formation of connective tissue which is composed of collagens to a large extent and replaces damaged tissue. Thrombi are built on the already rough and cracked artery wall, leading to a partial or total stenosis which can be the origin of embolisms. The vessel gets stiff and sclerotic. [40] [41] [42]

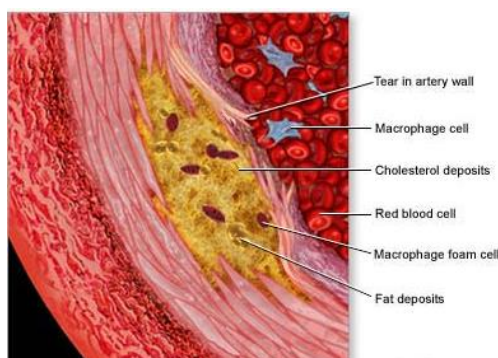


Fig. 3: An atherosclerotic plaque consisting of a necrotic core containing lipids, foam cells and other debris surrounded by a fibrous cap enriched in vascular smooth muscle cells (VSMCs) and collagens. Plaque formation is initiated by an endothelial dysfunction, infiltration of lipids and inflammatory cells, VSMC

proliferation and migration followed by their senescence and apoptosis and ending with the plaque rupture resulting in thrombosis. [42]

During the last decades, two main hypotheses concerning the development of atherosclerosis arose. The response-to-injury hypothesis was established by Russel Ross in 1976 and is based on the injury of the inner artery wall layer starting the process of atherosclerosis. Those injuries can originate from traumas evoked from high blood pressure, mechanical injuries or biochemical damage by bacterial toxins. Two different phenomena occur as a response. Growth factors trigger cellular proliferation and from the middle vessel layer smooth muscle cells migrate into the intima. Furthermore, lipoproteins invade the damaged intima and are oxidized. Thus, macrophages are attracted and killer cells bind to the oxLDL. Foam cells develop and lead to focal plaques. ^[43]

The lipoprotein-induced-atherosclerosis hypothesis established by Joseph Goldstein focuses on the negative influence of LDL on the emergence of atherosclerosis. The onward course of the disease is similarly described in both hypothesis. The inflammation caused by these foam cells reaches lower vessel layers and leads to a gradual tissue alteration. These alterations cause stiffness and fragility of the vessels. In the artery, a lipid clot consisting of foam cells arises. If these plaques begin to break, blood clotting reactions happen which reduce the vessel's diameter. ^[43]

Myocardial hypertrophy arises in most patients suffering from coronary atherosclerosis and hypertension. This pathogenesis seems related to dyssynergy and dilation of the cardiac chambers. These factors increase the mean of contractile stress and the energy per beat of the non-ischemic myocardium, which undergoes hypertrophy. Hypertrophy is defined as vascular enlargement resulting in increased muscular tissue. ^{[42] [43]}

1.5 Hutchinson Gilford Progeria Syndrome (HGPS)

The term Progeria comes from the Greek and means premature aging. In clinic, two different forms of HGPS are known: the Progeria type I in children and Progeria type II (Werner syndrome) exclusively arising in adult people. Initially, the Hutchinson Gilford Progeria Syndrome was described by Jonathan Hutchinson and Hastings Gilford in 1886. HGPS is a very rare autosomal dominant genetic disorder affecting 200-250 children worldwide. It is caused by a mutation concerning the gene coding for lamin A which is involved in the stabilization of the cell nuclei inner membrane. It is assumed that this mutation occurs spontaneously, meaning parents of suffering children show no abnormalities. How this mutation arises is still unclear. ^{[44] [45] [47] [48]}

Visible symptoms of HGPS arise after the first year of life, including restricted growth, alopecia, scaly skin, reduced subcutaneous fat, arthrosis, osteoporosis, malpositioning of joints and atherosclerosis. All of these symptoms arise also in elderly people. Even the appearance of these kids is evocative to old wrinkled people. Hence, HGPS is best known as the acceleration of aging. ^{[44] [47]}

Currently, there is no cure for HGPS and therapy is limited to the treatment of symptoms. In order to improve the cardiac circulation and muscular strength, physiotherapeutical activities are needed. Lotions and bath supplements are used to treat their dry and sensible skin, somehow improving life quality. Furthermore, research on medications is ongoing, but farnesyl-transferase inhibitors or acetylsalicylic acid were used to prevent vascular occlusions. ^{[44] [48]}

On average, children suffering from HGPS reach an age of only 13 years. Mainly, they die due to CVD's (chronic vascular diseases) like myocardial infarction or stroke. ^[46]

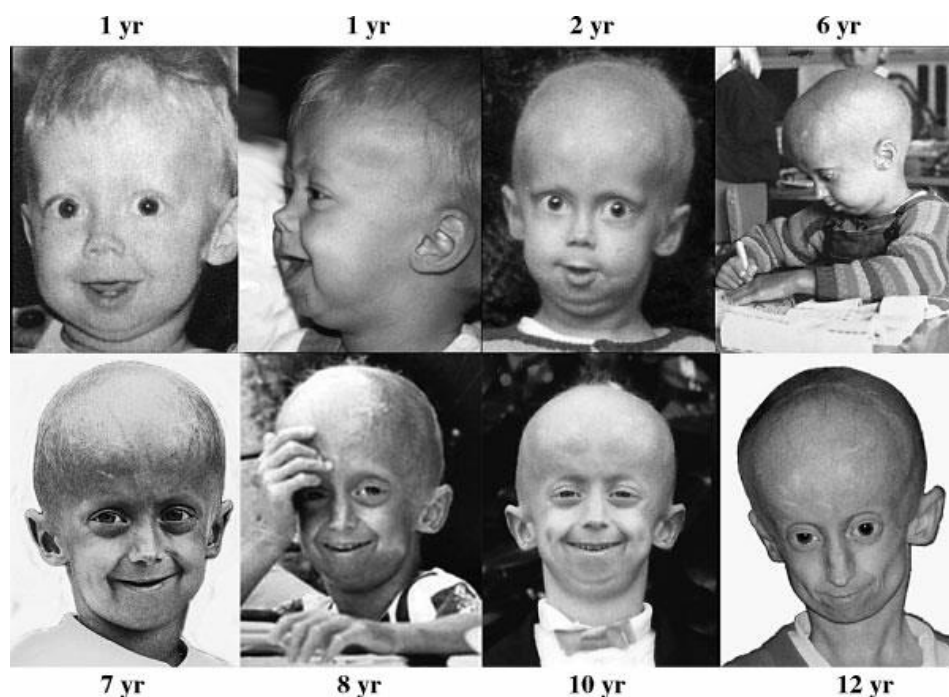


Fig.4: An HGPS patient at the age of 1 year to 12 years and his typical appearances like alopecia, scaly skin, prominent scalp veins and absent subcutaneous fat. ^[48]

1.5.1 Genetic abnormalities in HGPS

Mammalian lamin A is a member of the polypeptide family of lamins. Alternative splicing in exon 11 of prelamin A synthesizes prelamin A and C. Prelamin A contains a carboxyl-terminal CAAX box which is modified by farnesylation. Next, the last 3 amino acids are cleaved and the carboxy-terminal cysteine is methylated. Another proteolytic cleavage step occurs, removing the last 15 coding amino acids to produce mature lamin A. [45] [46]

However, progerin is generated by an incompletely processed lamin A which stays permanently farnesylated. A 50 amino acid deletion in prelamin A caused by a mutation-mediated missplicing event, removes the site of the second proteolytic cleavage. This farnesylated protein accumulates in the cell's nuclei, leading to loss of mechanical responsiveness of the nuclear envelope. The typical causing mutation is a point mutation in exon 11 in the LMNA gene (G608G). Immunofluorescence microscopy revealed that 40-50% of investigated fibroblasts show abnormal morphologies of the nuclear envelope. Blebbing and lobulation of cell nuclei are typical characteristics of HGPS. [45] [47]

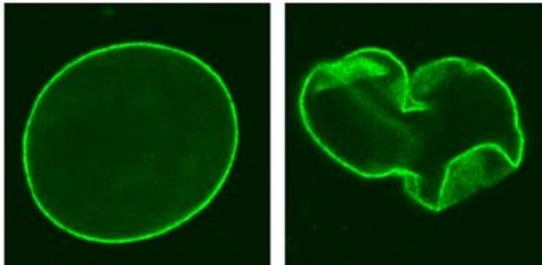


Fig.5: cell nucleus; a regular uniformly formed wildtype nucleus (left) and an HGPS nucleus showing dramatically aberrant morphology [49]

ZMPSTE-24 codes for a metalloproteinase which contributes to the post-translational proteolytic processing of pre-lamin A. HGPS can also be caused by a recessive mutation in the ZMPSTE-24 gene. Based on new research, HGPS is thought to have paternal origin. Progeria amongst siblings is very rare. Until today, only 3 cases of brotherly HGPS were reported. [45] [50]

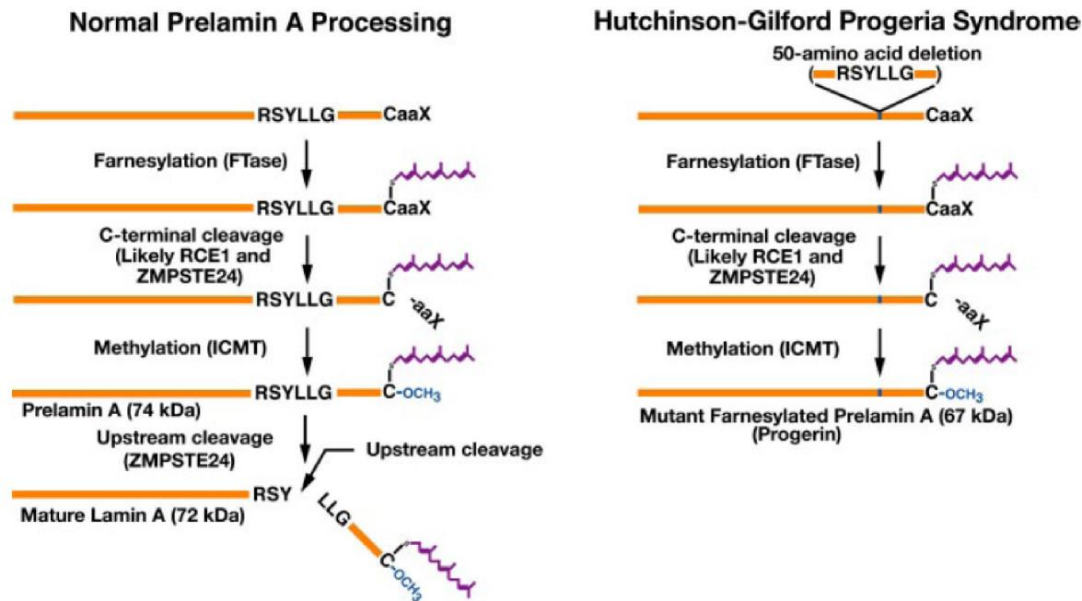


Fig. 6: The formation of lamin A from pre-lamin A in a healthy patient (left) due to a missing cryptic splicing site, ZMPSTE24 cannot work properly, consequently no mature lamin A can be formed and the farnesylated pre-lamin A, so-called Progerin accumulates in the nucleus (right) [50]

1.5.2 HGPS versus normal aging

Based on previous studies by researchers from Howard University using microarray gene expression analysis of fibroblasts, symptoms appearing in HGPS can be attributed to altered cellular signalling pathways similar to normal aging process. This is also confirmed by increased progerin accumulation in healthy old cell nuclei. 65 main signalling pathways from young HGPS and middle-aged to old healthy patients were examined and they showed very similar activation states. For example HIF-1, SMAD, TNF and TGF-beta signalling pathways were all upregulated. Furthermore, the MAPK pathway was upregulated in young HGPS and older healthy patients. Also, NFkB is hyper-activated in HGPS cells which acts as a response to damage, stress and inflammation. Overall progerin accumulations represent organismal aging and cellular senescence. This insight established completely new potential targets for drug development. [51] [54]

Regarding the nine hallmarks of aging mentioned above, several common features in HGPS and old patients occur. As A-type lamins are directly interacting with DNA and histones, progerin-expressing nuclei show relevant alterations in chromatin organization. Loss of peripheral heterochromatin and decreased number of

repressive histone marks were detected, changing epigenetic regulation. This results in a change in gene expression and genome stability. Furthermore, genome stability is attenuated due to the impairment of DNA damage repair pathways such as p53-binding protein-1 and Rad50/51. Accelerated telomere shortening was also detected in HGPS cells grown in culture. [18] [51] [54]

Since A-type lamins are involved in the differentiation and proliferation of mesenchymal stem cells (MSC's), self-renewal and differentiation are affected in HGPS patients likely due to impaired signalling and chromatin organization. [51]

Since progerin is mostly expressed in mesenchymal-derived tissue, HGPS patients suffer from decreased bone and skin density, problems with joints and skeletal muscles, reduced adipose tissue and poor large and small arteries. All of these symptoms arise in healthy old-aged people as well. [18] [19]

1.5.3 Atherosclerosis in Hutchinson-Gilford Progeria Syndrome

Since cardiovascular disease is the number one cause of death in children suffering from HGPS, atherosclerosis treatment constitutes a promising field in finding new therapies for HGPS. Almost all HGPS patients report from fatty plaques, calcified lesions or myocardial changes like diffuse fibrosis and ventricular hypertrophy and dilation resulting in myocardial infarction (MI). [50]

In HGPS patients, atherosclerosis can also affect the cerebrovasculature system. Using angiographic and MRI studies, cerebral infarction in the carotid and vertebral regions were documented in HGPS children. Even mild head injury can lead to epidural hematomas, probably triggered by advanced atherosclerosis of the intracranial vessels. [55]

Stehbens et al confirmed that these vascular changes in HGPS are comparable to those found in the general aging process. However, normal serum lipoprotein levels are found in HGPS patients. Furthermore, smoking or poor diet are unlikely to influence the atherogenic progress in HGPS children. A resistance to insulin was found in some patients but this alone is not expected to cause atherosclerosis in the first two decades of life. A more probable theory is the accelerated aging displaying telomere shortening, replicative senescence or impaired DNA repair mechanisms which also affects the vascular wall components. Another important point is the defective regeneration of intimal injuries by mutated endothelial cells. [51] [56]

Olive et al reported about atherosclerosis in two HGPS patients and compared it to conventional CVD of aging. They showed atherosclerotic lesions which were largely fibrotic and chronic inflamed plaques. Furthermore, flow limiting stenosis was observed. These plaques were composed of densely packed collagen I fibres with regions of loosely packed type III collagens. Most of the lesions also displayed regions of calcifications. In VSMC's, a co-localization of progerin and SMA (smooth muscle actin) was detected. In addition, progerin-positive endothelial cells were found in HGPS patients, but showed lower expression levels than VSMC's. Thus, progerin is present in all layers of the vessel. Furthermore, Olive et al showed an increase of progerin levels averaging 3.34% per year. They reported on thickened intima and a loss of SMC's in a stiff and less compliant aorta. Also, degenerative alterations, calcification and expansion of fibrosis were found in cardiac valves of HGPS patients. Furthermore, progerin-expressing cells seem to be more susceptible to oxidative stress. Olive et al also found changes in collagen depositions and response to mechanical stress or inflammation. [46] [47]

1.5.4 Therapeutic approaches

There are several approaches to treat HGPS. First of all, correcting the mutant protein by blocking the generation of the toxic farnesyl group. Farnesyltransferase inhibitors (FTI's) were shown to correct nuclear shape and cell proliferation. During a clinical trial from 2007 to 2009, lonafanib (FTI) improved bone structure, body weight gain and vascular stiffness. [51] [57]

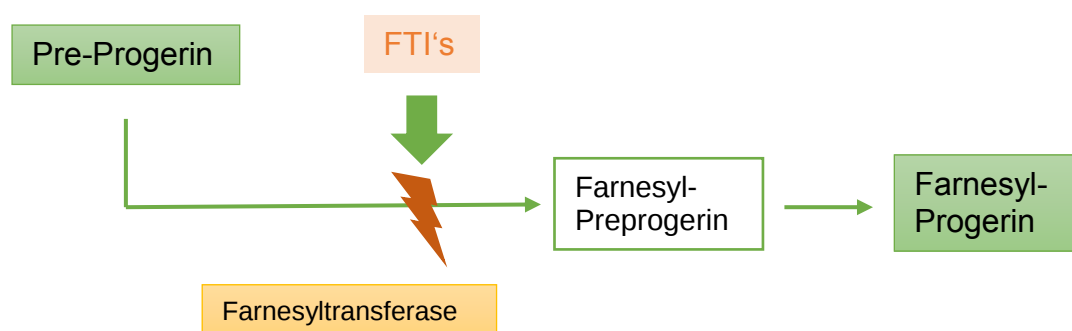


Fig.7: A farnesyltransferase inhibitor is used to inhibit the farnesylation of pre-lamin A and to compensate the impossible action of ZMPSTE24 [57]

Another treatment aims at the decrease of cellular Progerin levels using proteasomal degradation and autophagy. Macro-autophagy is activated in times of stress like starvation. Rapamycin, an inhibitor of the mTOR pathway enhances autophagy and

was shown to improve some cellular phenotypes of HGPS. Furthermore, an antioxidant called sulforaphane improves proteasome activity and autophagy, decreasing progerin levels. [51] [57]

By the introduction of a short antisense oligonucleotide, the cryptic splicing site in exon 11 of HGPS pre-mRNA can be blocked. This results in a decreased concentration of Progerin mRNA. This was tested in several mutant mouse models which showed enhanced body weight and a prolonged life span. A new approach targeting HGPS is Resveratrol treatment. It is an activator of SIRT-1 and acts beneficial on life span and general health in worms and fruit flies. The exact mechanism remains unclear, but there seems to be a connection between laminA and SIRT-1. SIRT-1 influences many different body compartments, all affected during the aging process as well as during HGPS (see figure 8). [9] [18] [58]

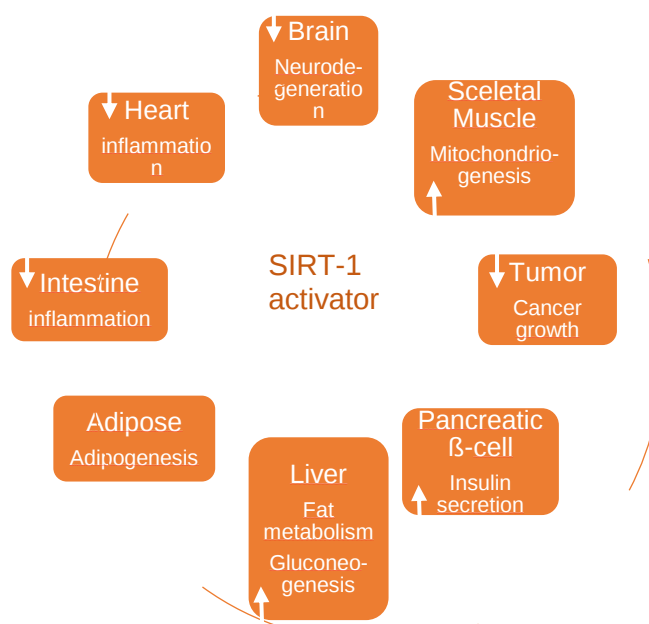


Fig.8: Various organs are targeted by SIRT-1 activators which have effects on aging and its impact like inflammation, mitochondriogenesis or the metabolism. This activation is supposed to improve cardiac functions by positively influencing vasorelaxation and vasoconstriction, anti-inflammatory effects by macrophages and foam cell formation. Furthermore, SIRT-1 activators can improve eNOS, angiogenesis and anti-apoptotic activity. [58]

1.5.5 Other laminopathies

The Charcot-Marie-Tooth neuropathy is a laminA-linked autosomal dominant genetic disorder starting in childhood, characterized by a chronic progressive neuronal muscular dystrophy due to a degeneration of peripheral nerves with a hypertrophy of the Schwann layer. This results in symmetric paralysation of the foot and lower leg, a clubfoot and the reduction of tendon reflex.

The Emery-Dreifuss muscular dystrophy is an X-chromosomal hereditary disorder of progressive muscular dystrophy resulting from a lack of Emerin (a protein localized in the inner nuclear membrane). The autosomal form of the Emery-Dreifuss muscular dystrophy is linked to laminA mutation. It starts with a prodromal-progressive muscular weakness between 6th and 19th year of age. Other symptoms are early cardiomyopathy, hyperventilation and reduced lifespan.

Mandibuloacral dysplasia is a laminA-linked laminopathy goes along with mandibular- and clavicle hyperplasia and missing closure of the cranial openings.

Further laminopathies are familial lipodystrophy, limb-girdle muscular dystrophy and dilative cardiomyopathy. ^{[10] [47]}

1.6 Myocardin-Related Transcription Factor (MRTF) mediated regulation of the vascular system

An important feature of the mammalian cardiovascular system is the capability to respond to different kinds of stress in order to maintain cardiovascular homeostasis. Transcriptional co-activators and repressors function in response to vascular injury and mechanical stress by the transduction of extracellular signal to the nucleus. MRTF's interact with the serum response factor (SRF), a MADS box transcription factor, which activates a variety of genes involved in cytoskeletal organization and cell migration. SRF binds to so-called CArG boxes, regulatory elements controlling the expression of smooth muscle α actin. However, SRF is also crucial in the regulation of vascular smooth muscle cells (SMC's). Schratt et al reported about SRF-deficient embryonic stem cells which are not able to differentiate into cells of mesoderm origin. Furthermore, SRF-null mice suffer from embryonic lethality before gastrulation, whereas SRF mutant cardiomyocytes show disorganized stress fiber formation. Reduced SRF gene expression causes dilated cardiomyopathy resulting in heart failure. ^{[60] [62]}

In mammals, two different types of MRTF's are known, MRTF-A and MRTF-B. They are derived from an ancestral gene in *Drosophila melanogaster* (DMRTF), which also interacts with SRF and is a transcriptional co-activator. Mutants show severe failure in cytoskeleton organization and cell migration. Mice with a loss of MRTF-B in the myocardium suffer from cardiac outflow tract defects whereas MRTF-B null mice already die at mid-gastrulation. The schematic representations of MRTF-A and B in figure 9 show an RPEL, a basic (+), a glutamine-rich (Q), an SAP, LZ and a TAD domain. Importantly, the RPEL domain is involved in binding MRTF to monomeric G-actin. The basic and glutamine-rich domains are necessary for proper SRF-binding activity, whereas the leucine-zipper is required for the homo- and heterodimerization with other MRTF's. Furthermore, the SAP domain is involved in the organization of the nucleus, in chromosomal dynamics and apoptosis. [62]

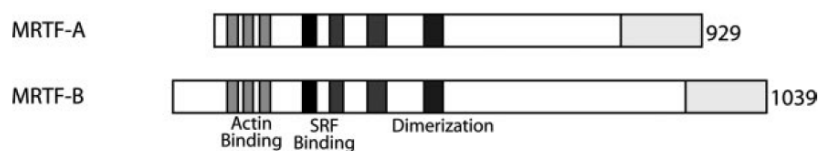


Fig. 9: schematic representation of human MRTF's containing a RPEL motif (grey) which binds to actin, a basic and a glutamine-rich domain which bind to SRF and a SAP and a leucine zipper motif used for the dimerization with other MRTF family members [62]

In humans, MRTF-A is located at chromosome 22q13.2 and is expressed in various cell lineages. However, during the embryonic state, it is increased in mesenchymal cells, muscle cells and epithelial cells. MRTF's are mainly localized in the cytoplasm but during stress, they translocate into the nucleus. [62]

RhoA signaling influences actin dynamics by the promotion of F-actin assembly. Furthermore, free monomeric G-actin prevents the translocation of MRTF into the nucleus. Therefore, the concentration of free G-actin can influence the interaction of MRTF's and SRF. [59] [62]

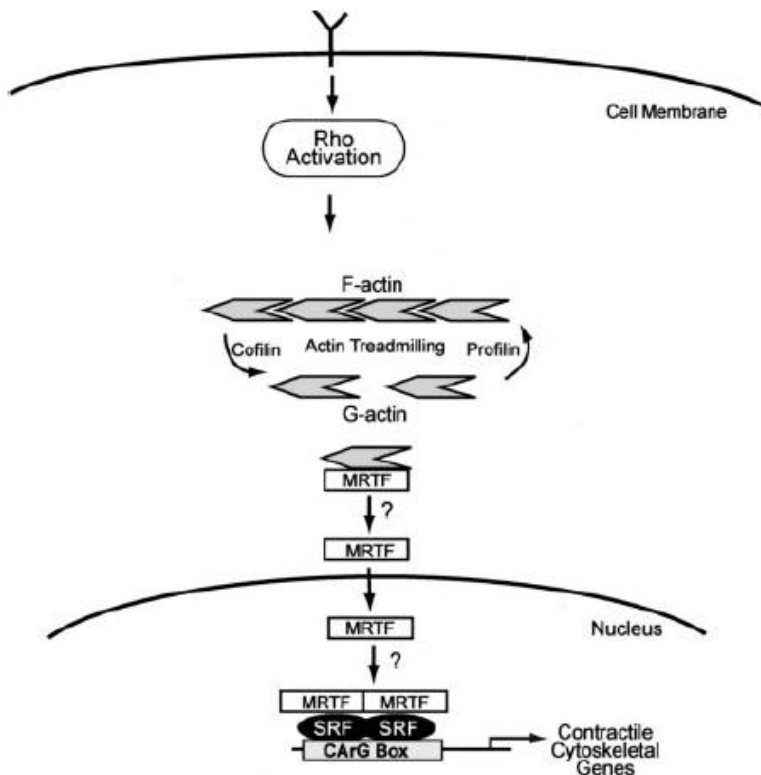


Fig.10: Rho activation via ROCK/LIM kinase promotes the formation of F-actin out of monomeric G-actin resulting in the release of MRTF traveling into the nucleus meaning reduced G-actin results in increased MRTF accumulation. MRTF mediates the transcription of SRF-dependent genes which encode contractile and cytoskeletal proteins like alpha actin, myosin heavy chain alpha and beta as well as BNP or ANP. Via MRTFs it is possible to establish a transducing cascade from the cell membrane to the nucleus triggered by extra-nuclear signals like mechanical stress or serum stimulation [60] [62] [63]

Unpublished data from our lab (Osmanagic-Myers et.al) showed altered F/G-actin levels in bi-transgenic endothelial cells during serum stimulation. Vice versa, inhibition of actin polymerization can repress the transcription of genes coding for SMC-contractile proteins. Previous studies addressing MRTF-null mice show dilated cardiomyopathy and heart failure in 35% of all cases, though, they were viable, fertile and born in a Mendelian male to female ratio. [62]

Lammerding et al reported a disturbed nucleo-cytoplasmic transport of MRTF-A caused by changed actin dynamics in LMNA^{-/-} mutant cells. Now, the question arises how progerin influences this shuttling of MRTF-A and how these alterations influence stress responses in endothelial cells resulting in cardiomyopathy. [66]

MRTF-A plays an important role in endothelin-1 activation due to hypoxic stress. Weng et al showed MRTF-A and endothelin expression in endothelial cells. Overexpression of MRTF-A was evoked by angiotensin II and strong connected to Ang-II-induced cardiac fibrosis and hypertrophy. In accordance with this, hypertrophic marker genes like BNP and ANP were significantly up-regulated. Kuwahara et al reported a MRTF-A mediated activation of BNP and identified SRF-binding sites within the BNP promoter. Additionally, MRTF-A null mice show reduced ET-1 protein

levels and an increase in the size of cardiomyocytes. Furthermore, MRTF-A^{-/-} mice displayed decreased hypertrophic responses to chronic Ang-II treatment, whereas wild type mice show increases in heart/body weight ratios. [61] [63] [64] [65]

Lauriol et al proved that loss of RhoA (controlling actin polymerization and MRTF-A localization) in cardiomyocytes leads to decreased fibrosis as well as attenuated contractility and heart failure. In response to extracellular stimuli, it also contributes to the induction of fibrogenic cytokines and the development of fibrosis. Furthermore Small et al reported a direct relation between collagen I/III and MRTF-A levels and described therapeutic approaches using ROCK-inhibitors to improve pathological fibrosis. [60] [63]

The aim of this study was an assessment of the implications on atherosclerosis caused by endothelial-specific progerin expression in mice regarding hypertrophy, fibrosis and vasoconstriction. Altogether, we propose a model as depicted in figure 11.

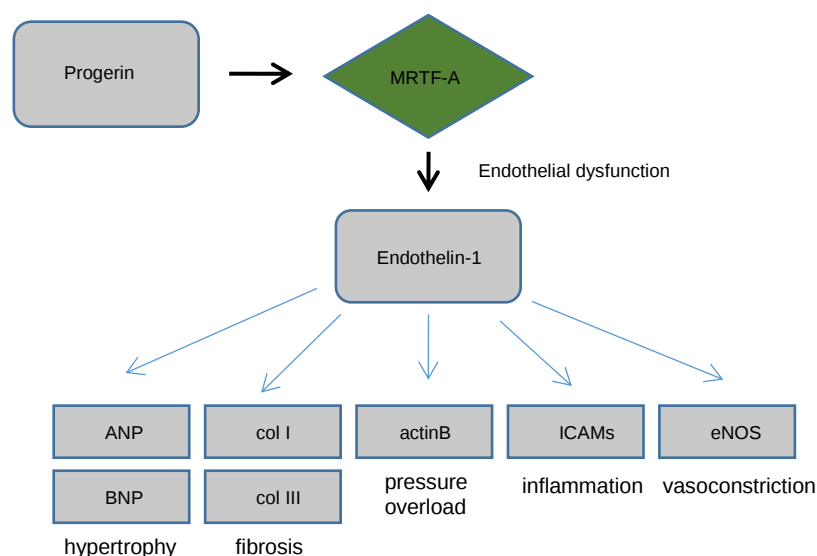


Fig. 11: model depicting endothelial-MRTF-A mediated pro-atherosclerotic changes through trans-activating the endothelin-1 gene expression. MRTF-A might be a response to extracellular angiotensin II signaling which is normally conveyed to nucleus through the lamina network, but now, MRTF-A might be dysregulated by an accumulation of progerin in the cell. [65]

2 MATERIAL AND METHODS

2.1 Material for tissue isolation and cell culture

Base medium	400 ml DMEM
	100 ml FCS
	12.5 ml 1M HEPES
	Penicillin/Streptomycin 1:100
	L-Glutamine 1:100
Complete culture medium	48 ml base medium
	500 µl non-essential amino acids
	500 µl sodium pyruvate
	1 ml ECGS (endothelial culture growth supplement)
	200 µl Heparin

2.2 Generation of bi-transgenic mice expressing laminA or progerin

Minigenes of human wt and mutant laminA (LA^{wt} and LA^{G608G} fig.12) were generated and ligated into a tetop vector, containing a downstream IRES (internal ribosomal entry site), the coding region of eGFP and a SV40 polyA site, were introduced in the mouse genome and founders were born and bred in order to generate F1 lines. [67]

Initially, those F1 lines were intercrossed with K5tTA transgenic mice expressing the transactivator under the control of the keratin5 (K5) promoter and minigene expression was checked by protein detection. Minigene expression was limited to keratin 5- expressing cells like teeth, hair follicle or interfollicular epidermis. All tests showed very specific minigene expression in keratin 5 expressing cells. In the presence of DOX (doxycycline) the transcription is turned off. [67]

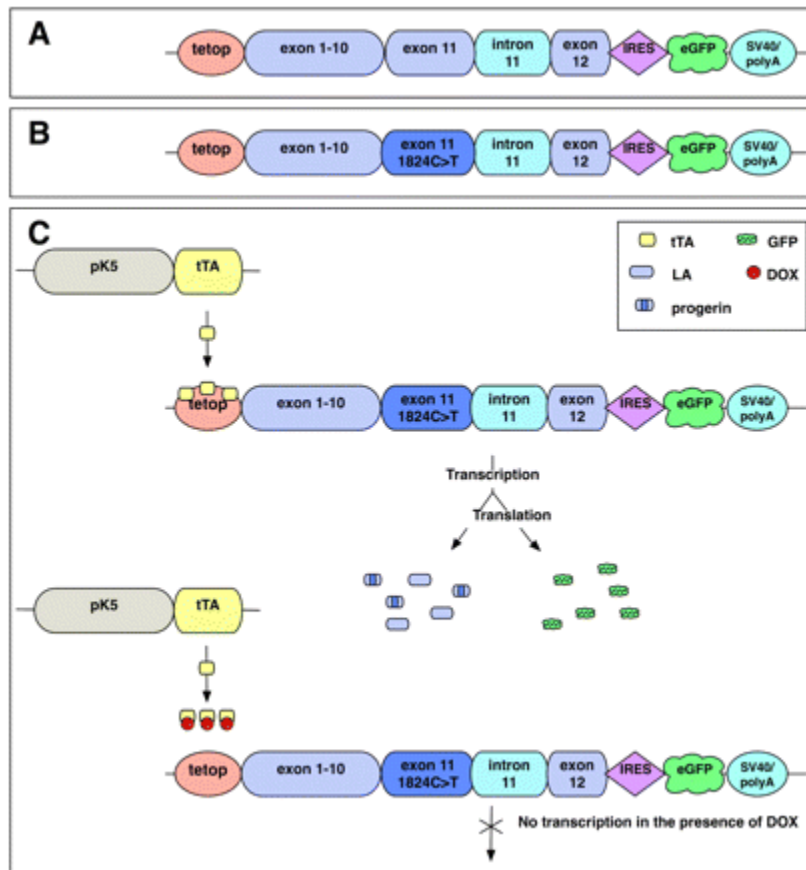


Fig.12: A, B: minigenes of human lamin A, wt (A) and transgenic G608G (B) differing only in nucleotide 1824 in exon 11, the most common HGPS mutation. Furthermore, minigenes carry an IRES, eGFP and a SV40 polyA site. C: the tet-off system used for the generation of bi-transgenic mice; transcription is turned off during the presence of doxycycline (DOX). [67]

We crossed mice containing the LA and progerin minigene with transgenic mice expressing the transactivator under the control of the endothelial-specific VE-Cadherin promoter. Immunofluorescence microscopy confirmed endothelial cell specific expression as seen in figure 13.

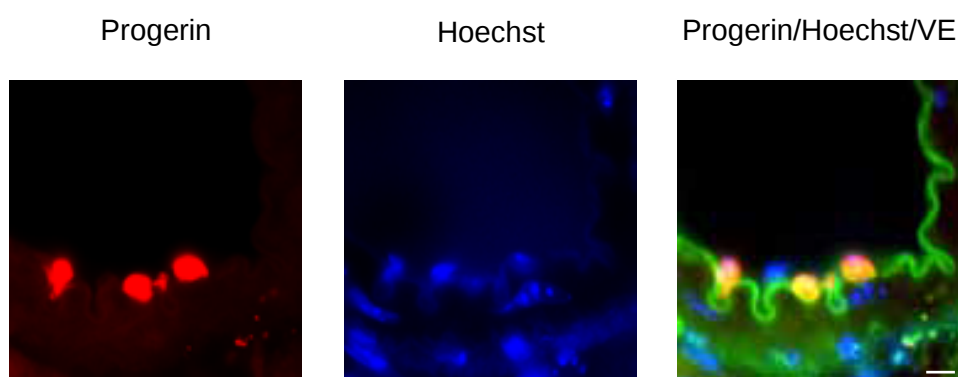


Fig.13: immunofluorescent imagination of transgenic coronary artery depicting a strong, distinct signal in the nucleus only in Progerin-expressing endothelial cells (evidenced by Hoechst and VE staining), scale bar 10µm

2.3 Mice dissection

Mice were anesthetized by isoflurane inhalation, the body weight was determined on micro scales and mice were sacrificed by decapitation. Their body was transferred and pinned onto a dissection table using needles. Isoflurane is a very common vasodilator in veterinary medicine and leads to decreased blood pressure, followed by an attenuated cardiac output per minute and respiratory depression. The bodies of the mice were covered with ordinary ethanol and skin and subcutaneous fat was cut from bottom to the top of the abdomen and the thorax. The sternum was broken with scissors and ribs were pinned to the dissection table. Furthermore, heart and lung were carefully removed. The heart weight was determined on micro scales in order to calculate the heart-body weight ratio. The aorta was extracted using micro scalpels. Organs were washed with 1xPBS and processed depending on further investigations.

2.4 Isolation of endothelial cells from tissue

Mostly, lung tissue of young mice (approximately 8-12 days) was used in order to isolate endothelial cells. It was much more difficult to isolate pure endothelial cultures out of older mice. The older the animal the more fibroblasts contaminated the preparations. We also tried to isolate endothelial cells from heart tissue, but the yield was extremely limited.

After washing with PBS, lungs were preserved in ice cold base medium and transferred to tissue culture plate in sterile conditions. The tissue was minced with scalpels in a Petri dish and incubated in 5ml 1x collagenase for 45 minutes at 37°C. Collagenase cleaves peptide bridges between proline and other amino acids in order to degrade collagens. The enzyme was inactivated by adding 10ml complete culture medium and the mixture was sheared with a 20ml syringe and an 18G needle approximately 10 times. The cell suspension was filtered using a 43µm diameter strainer and transferred into a new 20ml falcon. For harvesting the cell mixture, the suspension was centrifuged at 1000rcf for 3 minutes. After aspiration of the supernatant, the pellet was resuspended in 5ml complete culture medium. Cells were plated onto a pre-coated Petri dish. For coating, a 2% gelatin solution in 1xPBS was used and incubated for several hours before seeding at 37°C. On the next day, cells were washed with 1xPBS and fresh medium was added. In order to enrich for endothelial cells, sheep anti-rat IgG magnetic dynabeads® were coated with a rat

anti-ICAM-2 antibody (1:10) over night at 4°C. These beads specifically sort for endothelial cells expressing the ICAM-2 protein. When the mixed cell culture appears confluent, mostly on the next day, cells were washed, trypsinized and incubated with these ICAM-2 beads for 45 minutes at 4°C. Incubation at a low temperature avoids clotting and ensures proper separation of the cells. Overgrowing fibroblasts are very prone to stick on all other types of cells.

After incubation, magnetic hulks were used to separate endothelial cells sticking to magnetic beads from the rest of the cell suspension. Therefore, the cell suspension was transferred into an 1.5ml Eppi and placed into the magnetic hulk. The medium was aspirated and beads were washed with an ice cold 1xwashing solution in 1xPBS without magnesium and calcium. The missing ions avoid clotting of cells as well. This procedure was repeated for at least 5 times. After the last washing, beads were suspended in complete culture medium and plated on 2cm coated Petri dishes. If there are still cellular contaminations in the culture after a few days, sorting was repeated.

2. Tissue homogenization for RT qPCR

In order to prepare mRNA from lung and heart tissue, a special procedure was introduced. Organs were put into 1ml Falcons filled with ceramic beads and 600µl of cold lysis buffer (RLT buffer) taken from RNeasy Plus Mini Kit® by Quiagen were added. Depending on the type of tissue, different sizes of ceramic beads were used. Due to its robust structure, heart tissue required much bigger beads (1.5mm diameter) than spongiform lung tissue (0.5mm diameter).

In order to homogenize the tissue, the tubes were balanced and clamped into the Precellyse® homogenizer. The tissue was disrupted for 20 seconds. Afterwards, tissues lysates were cooled on ice in order to avoid destruction of mRNA. The homogenized emulsion was then transferred into an Eppi and centrifuged at maximum speed for 5 minutes and frozen in liquid nitrogen at -80°C.

For further processing, Quiagen RNeasy Plus Mini Kit® was used. The suspension was transferred onto a gDNA eliminator column placed in a 2ml collection tube and centrifuged at 8000g for 30s. The column was discarded and the flow-through was mixed well by pipetting with 700µl 70% RNase-free ethanol. This mixture was transferred to an RNeasy Spin column and centrifuged at 8000g for 15s. 700µl RW1

washing buffer was added to the column and centrifuged at 8000g for 15s. Next, 500µl RPE buffer was added and the centrifugation step was repeated for 2 minutes at 8000g. To ensure a proper elution of the RNA, the column has to be complete dry, therefore another centrifugation step at full speed for 1 minute was implemented. As a final step, the pellet was eluted with 30µl RNase free water at 8000g for 1 minute. For measuring RNA concentration and its purity (A260/280), Nanodrop® was used.

2.6 Histological examinations of heart and aortic tissue

Histological analysis was carried out on heart and aortic tissue samples. Whereas cardiac tissue was embedded in paraffin blocks, aortas were prepared for cryosectioning. For paraffin embedding, tissue was fixed in a 4% paraformaldehyde solution in 1xPBS overnight. On the next day, the tissue was washed with PBS and conserved in 70% ethanol for a couple of hours. Next, the tissue was deposited into a plastic cassette and loaded to an automatic embedding machine overnight. These paraffin blocks were cut into 5µm thick sections using a microtome, drawn onto a glass slide and stored in cataloged cardboard boxes. Before further processing, samples had to be liberated from paraffin using a heating plate at 80°C and xylene and rehydrated in a descending alcohol series.

Aortic tissue was also fixed in 4% PFA overnight, washed in PBS but then, incubated in a 20% sucrose solution in water overnight. On the next day, samples were deposited into plastic wells, covered with a gel like medium called OCT consisting of poly ethylene glycol and polyvinyl alcohol and rapidly frozen in liquid nitrogen. This is a very crucial step because the faster the sample is frozen, the less chance of ice crystal artifacts will occur. The ice blocks were sectioned in 1µm thick slices using a cryostat and drawn on special slides suitable for cryosections. These slides were stored in a -80°C freezer. Before further processing, the samples were thawed at room temperature for at least 15 minutes.

2.6.1 Hematoxylin-Eosin staining

This kind of histological examination consists of two single stains and is one of the most common routine techniques for morphological investigations. Hematoxylin colorizes all acidic respectively basophilic structures in blue, particularly cell nuclei containing DNA and the rough endoplasmic reticulum. Eosin is a synthetic acidic colouring agent which stain all basic cellular structures like cell plasma proteins, mitochondria, the smooth endoplasmic reticulum or collagens.

Tissue samples were incubated in hematoxylin solution for 8 minutes. They were differentiated in an 0.5% HCl-alcohol solution. In order to achieve a more precise staining result, blueing up was performed by rinsing the sample with common tap water for 10 minutes. Next, slides were incubated in eosin solution for 2 minutes and rinsed with tap water before they were dehydrated in an ascending alcohol series and cleared in xylene. Samples were mounted in Eukit® and dried overnight at room temperature.

2.6.2 Masson's Trichrome staining

Another more complex histological technique is the Masson's Trichrome stain. It is a three-step coloring protocol by Claude Masson staining cytoplasm in pink, cell nuclei in black and collagens in blue or green, depending on the used chemicals.

For colorizing cell nuclei, samples were stained with Mayer Hemalum for 10 minutes and rinsed with water. Next, 5 minutes in Ponceau Fuchsin was used to stain the cytoplasm pinkish. In order to "blue up" and fix the stain 2 baths in 1% acetic acid and 1 bath in phosphomolybdic acid were performed. In our case, a bath of 5 minutes in anilin blue was used to stain connective tissue, especially collagens. Finally, the sample was rinsed with 1% acetic acid. Ascending alcohol series and N-butyl acetat were used to dehydrate the tissue again and the sample was mounted with Eukitt®.

2.7 RNA isolation from endothelial cells

For isolating RNA out of cellular material, Promega® ReliaPrep RNA Cell Miniprep system was applied. Before treating Cell culture dishes with 250µl BL+TG lysis buffer, they were washed with ice cold PBS. Using the lysis buffer, the plate was "washed", the tough sheared lysate was transferred into an Eppi and 85µl isopropanol was added. It was vortexed for 5 minutes in order to bind DNA and RNA and the mixture was transferred into the Minispin column placed in a collection tube. It was centrifuged at 12 000-14 000g for 30s, the flow-through was discarded and 500µl Wash solution was added to the column. Again, the Minispin column was centrifuged at 12 000- 14 000g for 30s and the collection tube was emptied. Meanwhile, the DNase I incubation mix was prepared using 24µl of yellow core buffer, 3µl MnCl₂ and 3µl DNase I per prep. This step is especially important to get rid of genomic DNA which could contaminate the RNA sample. This mix was incubated on the column for 15 minutes at room temperature. Several washing steps with 200µl Column Wash Solution and 500µl RNA Wash Solution with intermediate

centrifugation steps were accomplished. In order to dry the isolated RNA, the Minispin Column was centrifuged at maximum speed for 2 minutes. Finally, the RNA was eluted with 30µl of nuclease-free water at 12 000- 14 000g for 1 minute. Again, Nanodrop® was used to determine the concentration and purity of the RNA. The isolated RNA was stored at -80°C.

2.8 Quantitation of mRNA expression levels in tissue and endothelial cells

One of the most valuable techniques in biological research today is the amplification of nucleic acids and their quantitative detection. Conventionally, common PCRs are conducted in order to create amplicons which are then checked by running on an agarose gel. However, RT qPCR is able to measure the accumulation of the amplified product by including a fluorescent molecule into the reaction. It serves as a reporter of the increase in the quantity of cDNA by measuring the increase in the fluorescent signal. These DNA-binding dyes only stick to double stranded nucleic acids. Special equipped thermal cyclers with fluorescent detectors are required for this procedure. At the beginning, the fluorescent signal remains in the background not detectable for the cycler. But after some cycles of exponential amplification, the product accumulates to a detectable signal. This is called the threshold cycle or C_T value which is mainly depended on the amount of template present at the start of the reaction.

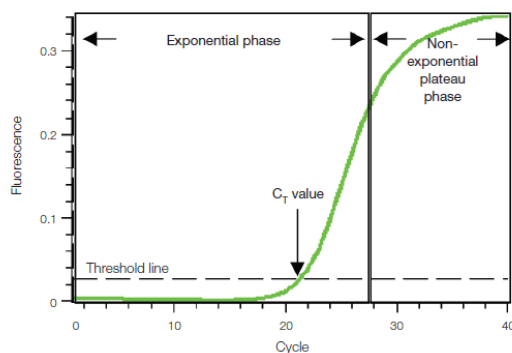


Fig.14: amplification plot, also showing the baseline-subtracted fluorescence (below C_T value) [67]

2.8.1 cDNA synthesis

There are two different possible techniques used in qPCR application, one- or two-step. With the multiplex variant it is possible to combine the reverse transcription of the RNA and the whole amplification reaction in one Eppi. We decided to use the one-step technique because of less experimental variations and fewer pipetting steps necessary. Another important point is the decision between the use of total RNA or

purified mRNA as a template. When mRNA is used, more purification steps which could influence the final results are necessary. However, we decided to generate cDNA out of total RNA. Furthermore, we used oligo d(T)s as reverse transcriptase primers which contain a stretch of thymine residues that bind to the RNA's poly-A tail. The reaction mix was composed of:

100 ng RNA

1.25 µl oligo d(T) (100 µM)

4 µl buffer (5x, supplied by Thermo scientific®)

0.5 µl RNase inhibitor (10 000 u)

2 µl dNTP's (10 mM)

1 µl Reverse transcriptase (10 000 u)

nuclease-free water

20 µl in total

This mixture was incubated at 42°C for one hour. The enzyme was inactivated at 70°C for 10 minutes.

2.8.2 Determination of primer specificity

We used SYBR Green® which is an unspecific binding dye that will detect any double-stranded DNA. However, we had to verify that our PCR assay only generates the desired product. Therefore, we used a melting curve analysis. Ideally, high specificity of the assay is shown in a single sharp peak whereas negative controls (with no template) should not show any peaks in order to exclude primer dimer formation. Non-specific products seen as additional shoulders can often be erased by lowering the primer concentrations. If there are still non-specific products detected, redesigning of the primers was necessary. Finally, to check the right size of the product, it was loaded onto an agarose gel and electrophoresis was performed. Only one sharp band will prove a specific desired product. In order to check the best fitting and most specific annealing temperature, a temperature gradient of 55 – 65°C was tested.

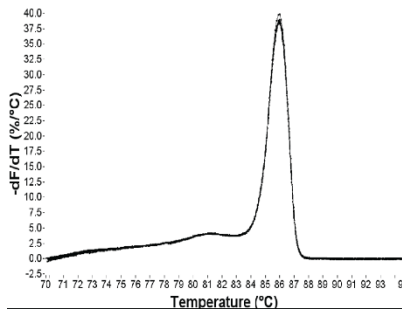


Fig. 15: melting or dissociation curve depicting a sharp peak of a specific product at >80°C with a very low unspecific product at lower temperature; product fractionated on a DNA SYBR stained 2% agarose gel showing a specific product [68]

2.8.3 Validation of the performance with a standard curve

The amplification plot (figure 16) shows an exponential phase followed by a non-exponential plateau. Ideally during exponential phase, the perfect doubling of each amplicon occurs with each amplification cycle. Therefore, serial diluted duplicons of the amplicon will generate evenly spaced amplification curves as seen in Fig 16.

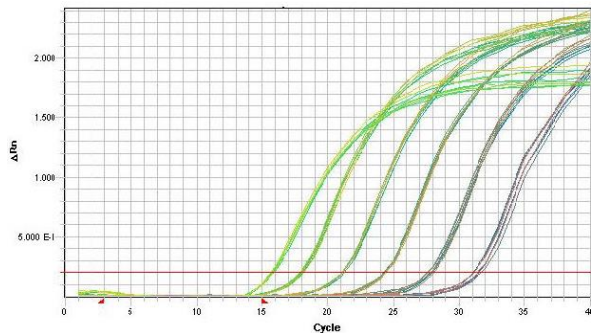


Fig.16: to assess reaction optimization, a standard curve was generated using serial dilutions. [70]

In order to test primer efficiency, CT values are plotted versus the logarithm of nucleic acid input and a linear regression is performed. To calculate the efficiency, the following formula was used:

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1$$

For the determination of how well the data fit on a straight line, the correlation coefficient R^2 was calculated. For example, this coefficient is influenced by pipetting accuracy. If R^2 is ≤ 0.985 results may not be reliable. At an efficiency of 100%, the amplicon will double with each cycle and the slope of the standard curve will be -3.33 ($100 = 10^{(-1/-3.33)} - 1$). Generally, an efficiency of 80 – 110% meaning a slope between -3.9 and -3.0 is acceptable (see figure 17).

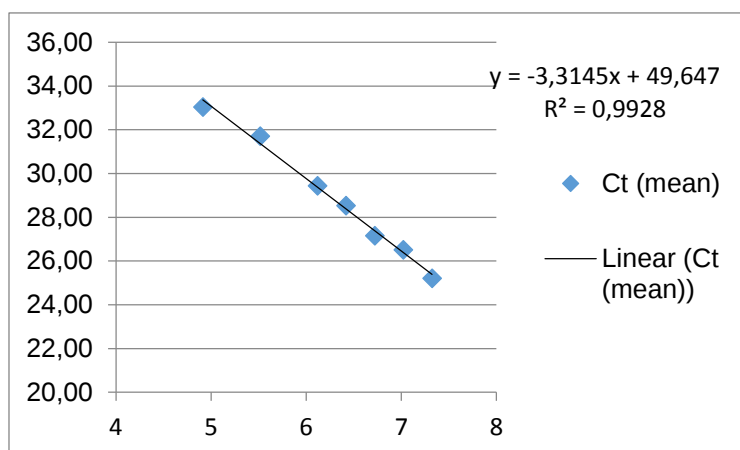


Fig. 17: An example of a performed primer validation plotted on a linear diagram with calculated functional equation and regression coefficient.

2.8.4 Primer sequences used for the determination of gene expression levels

Tbl.1: primer sequences used in RT qPCR for the determination of pro-atherosclerotic markers

Oligo name	Sequence (5'-3')
VE cadherin	CTGCTCACGGACAAGATCAGC CTCTTTTGGCGATGGTGGGC
HPRT	GCAGTCCCAGCGTCGTGATTA TGATGGCCTCCCATCTCCTTCA
ActA2	GTACCACCATGTACCCAGGC GAAGGTAGACAGCGAAGCCA
eNOS	GCATGGGCAACTTGAAGAGTG GCTGCCCACTTCCCAATTCT
Col4	CCCAAGTGCACCAGCATAAC AGAAGAACACCCATGGCAGG
Myh7	CACCAACCTGTCCAAGTTCC AGAGCTACTCCTCATTGAGGC
ActB	ACAGCTTCTTTGCAGCTCCT TTGTGACGACCAGCGCA
MRTF-A Transcript 2	AAGAACGACCGTCCCACCC CGCGTCGTCATTTTCGCCAG
MRTF-A Transcript 1	TTGACTTGCCTGAAGGGTGGG CATGATCCCTTGGCTCACCA
Edn-1	ATCTGGGTCAACACTCCCGA ACTTTGGGCCCTGAGTTCTT

BNP	GGGCACAAGATAGACCGGAT GCCAGGAGGTCTTCCTACAA
ANP	TTGGAGCCCAGAGTGGACTA GCTCAAGCAGAATCGACTGC
Col1a1	CTGACGCATGGCCAAGAAGA ATACCTCGGGTTTCCACGTC
Col1a2	TGGATACGCGGACTCTGTTG GGCCCTTTCGTA CTGATCCC
Col3a1	TCCGGGAATAACGTCAGTC GGAAGCCCATTTCACCCAGG
ICAM-1	CAGATGCCGACCCAGGAGAG CCGCTAGCTCCAAAACGCAG
ICAM-2	GCTCACCGGCACAGAGGAGA TATGGGCTTCAGGGGCACAG

2.8.5 RT qPCR

For testing pulmonary tissue, 100 ng of RNA was used. Unfortunately, cardiac tissue and endothelial cells did not gain such a big yield and only 50 resp. 25 ng could be utilized. The used master mix was composed of:

10 µl SYBR Green® master mix by Thermo Scientific

4.5 µl forward primer

4.5 µl reverse primer

25-100 ng template

nuclease – free water

20 µl in total

The master mix with the desired primer pair but without the template was pipetted on ice and transferred into a 96 well plate. For running the final assay, triplicons were prepared in order to calculate a mean value out of three similar C_T values. Next, the template was added and the plate was sealed with a foil. After spinning down the plate, it was placed into the thermal cycler and the program was designed with a special software. The following cycling program was started:

95°C	3 min	} 40X
95°C	3 sec	
Adjacent annealing temperature	20 sec	
72°C	20 sec	

In order to avoid any non-specific product, after every assay a melting curve analysis was started. Furthermore, the amplicons were checked on an agarose gel.

2.8.6 RT qPCR analysis

First, CT triplicon values were summed up and a mean value was calculated. Outliers were neglected. It is crucial to test for a house keeping gene during each assay as well as for each template. During the analysis of the assay, this house keeping gene serves as normalizer. In our case HPRT (Hypoxanthine-guanine-phosphoribosyl transferase), an enzyme which recycles purine in cells, was used.

For the evaluation of the RT qPCR results, a comparative method was used:

1. $C_T(\text{gene of interest}) - C_T(\text{house-keeping gene}) = dC_T$
2. $C_T(\text{wildtype}) - C_T(\text{wildtype}) \text{ or } C_T(\text{transgene}) - C_T(\text{wildtype}) = ddC_T$
3. $2^{ddC_T} = \text{normalized relative quantity}$

2.9 Shear stress application on subconfluent endothelial cultures seeded on a 6-channel slide

Unidirectional laminar flow is encountered in most small healthy vessels such as small veins and arteries. In order to mimic such kind of flow, a special pump was used. It was calibrated and adjusted to a force of 12 dyne which is according to the physiological blood pressure in healthy organisms. 1 dyne corresponds to the acceleration of 1g with 1cm/s² or a force of 10⁻⁵N.

Channels were coated with a 0.2% gelatine solution and 10⁴ endothelial cells were seeded onto the plate per channel. Hoses with 1mm diameter were connected to the channels and complete culture medium was used to generate unidirectional laminar flow of 12 dyne at 37°C for 6 hours. It is very crucial to avoid air bubbles or foaming

in order to prevent endothelial cell detachment. Channels used for negative controls were sealed with parafilm. Subsequently, cells were fixed with a 4% paraformaldehyde solution in 1xPBS, 0.1M glycine and 0.1% Triton-X 100 solution and stored in a 1:500 NaN₃ solution at 4°C.

2.10 Immunofluorescence

IF is a biochemical and medical analysis method using antibodies coupled to a fluorochrome. Antigens can be detected and quantitatively examined.

We used indirect immunofluorescence. The epitope of the first antibody binds to the epitope of the desired protein. A second antibody coupled to the fluorochrome binds specifically to the first antibody. For the negative control, the first antibody was omitted in order to check for signals resulting from unspecific binding of the second antibody.

As a secondary antibody system, we used biotin and streptavidin, one of the strongest non-covalent bondings found in biology.

As we used material gained from mouse tissue as well as primary antibodies acquired from mice, a MOM-Kit had to be used in order to avoid unspecific signals. Advantages are a significant reduction of endogenous mouse Ig staining and simple procedures.

2.10.1 Primary antibodies

Tbl.2: antibodies used for immunofluorescence and their adapted dilution factor

Antibody	Dilution factor
goat α MRTF-A	1: 50
rb α collagen I	1:50
rb α collagen IV	1:50
mouse α Progerin	1:30
goat α VE cadherin	1:50
mouse α smooth muscle actin	1:100
rb α eNOS	1:50

All primary antibodies were incubated overnight at 4°C.

2.10.2 Antigen retrieval and staining procedure

Before starting the staining procedure, epitopes of tissue samples which were embedded in paraffin had to be revealed. Therefore slides were rehydrated in alcohol covered with TE buffer and treated in a steamer at 850W for 60 minutes. When the slides were cooled down, samples were washed in PBS-T and incubated with 20% normal donkey serum in PBS-T for 20 minutes as a pre-blocking step. Next blocking was performed using the MOM blocking solution, diluted as described in the manual in PBS-T. Primary antibodies were diluted as seen in table 2 in the provided protein concentrate and incubated at 4°C overnight. After several washing steps in PBS, specific biotin was diluted to a concentration given in the manual and incubated for 15-20 minutes. Streptavidin was diluted in PBS-T and incubated for one hour. Hoechst staining was performed for 5 minutes and slides were washed in PBS and water in order to remove all salt residuals. When slides were dry, they were mounted in Mowiol, a polyvinyl alcohol, and dried overnight. Samples were observed using a Zeiss LSM 700 confocal microscope and its according software.

2.10.3 ImageJ analysis and intensity evaluations

Images were analysed using ImageJ. Brightness and contrast were equally adjusted. For all samples equally sized areas of interest were encircled using the tool freehand selection and raw intensity values were measured. Equally sized areas in the region surrounding the sample area were also measured that represented unspecific background intensity. These values were subsequently subtracted from fluorescence intensity of the sample fluorescence intensity. Average intensity was calculated from all images obtained from different animals of the same genotype. Statistical evaluations were performed using Windows Excel software.

3 RESULTS

We first assessed phenotypic differences between wildtype and transgenic mice. Prog-Tg but not LMNA-Tg mice appeared to be leaner compared to Wt littermates with reduced abdominal and subcutaneous fat deposits (fig. 18A, B). We observed significantly decreased body weights of Prog-Tg but not LMNA-Tg mice in comparison to Wt littermates (fig. 18C, D).

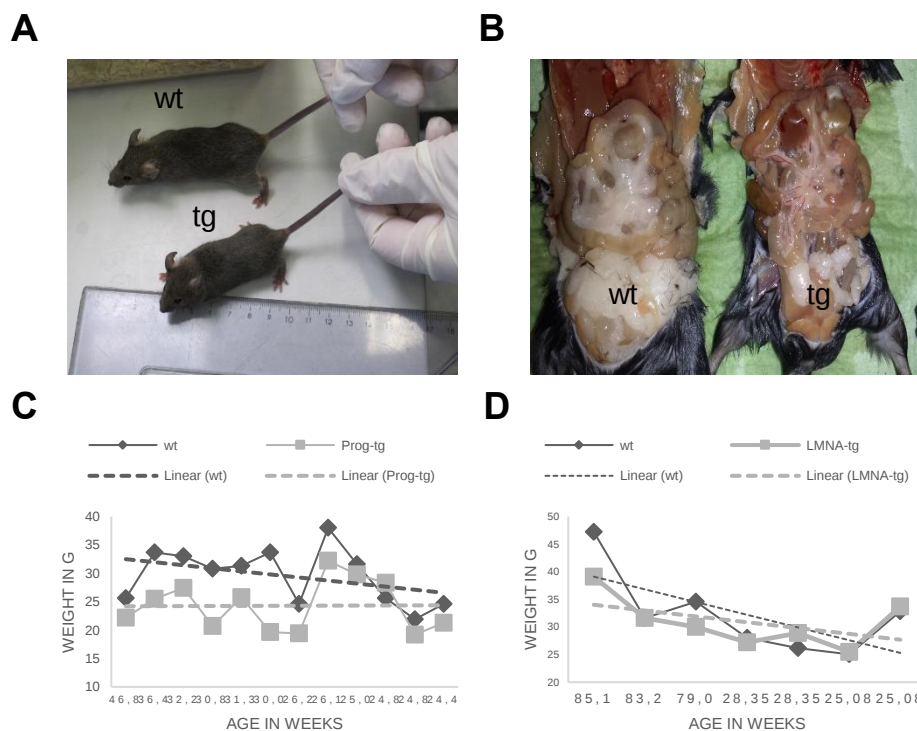


Fig. 18: **A,B:** obvious differences in body size and amount of abdominal fat between wildtype and progerin-transgenic mice; **C,D:** body weight in grams plotted against life span (time point of sacrifice) of wildtype, Progerin and LMNA

hypertrophy in transgenic mice with evident narrowing of the cardiac lumen (fig. 19A). It must be noted that cardiac histological examinations were always embedded in paraffin and slices at the same cardiac section level were compared (hearts were sliced along the vertical axis from top to the bottom).

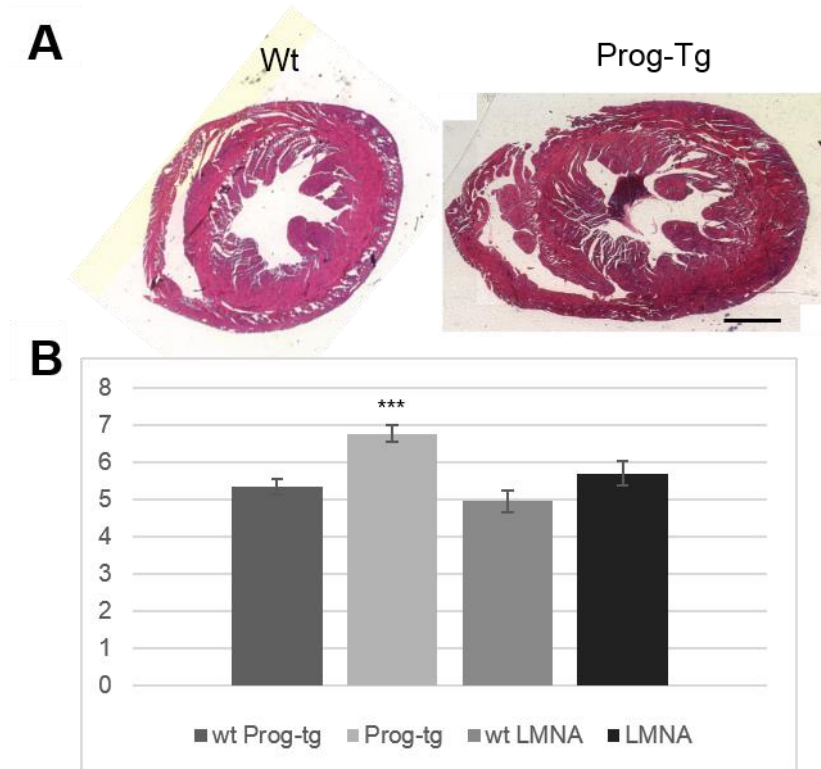


Fig 19: A: HE stainings of wildtype and transgenic hearts showing severe hypertrophy and lumen narrowing, scale bar 1000 μ m B: Calculated HBW ratios were plotted on a graph, Prog: n=15 p=0.0006; LMNA: n=7, p=0.07, statistical analysis was done using the paired student's t-test

In order to investigate cardiac fibrosis, more histological examinations were performed using HE staining and Masson's Trichrome staining that is used for the detection of collagens in blue colour. In figure 20 a 20x and a 63x cardiac magnification of blood vessel sample is shown. Cardiac vessels of Prog-Tg mice showed a thickened collagen layer at the intimal layer however also at the adventitial layer of blood vessels in comparison to their Wt littermates (fig. 20, lower panels). Statistical evaluations were not performed. In order to confirm the increased cardiac collagen levels, gene expression levels of different types of collagens were investigated via RT qPCR. This revealed significantly increased collagen III but not I α I, I α II, II and IV levels in cardiac tissue (fig. 21B). Thus, these data indicated fibrosis related to first stages of atherosclerosis development in 25 week old transgenic mice.

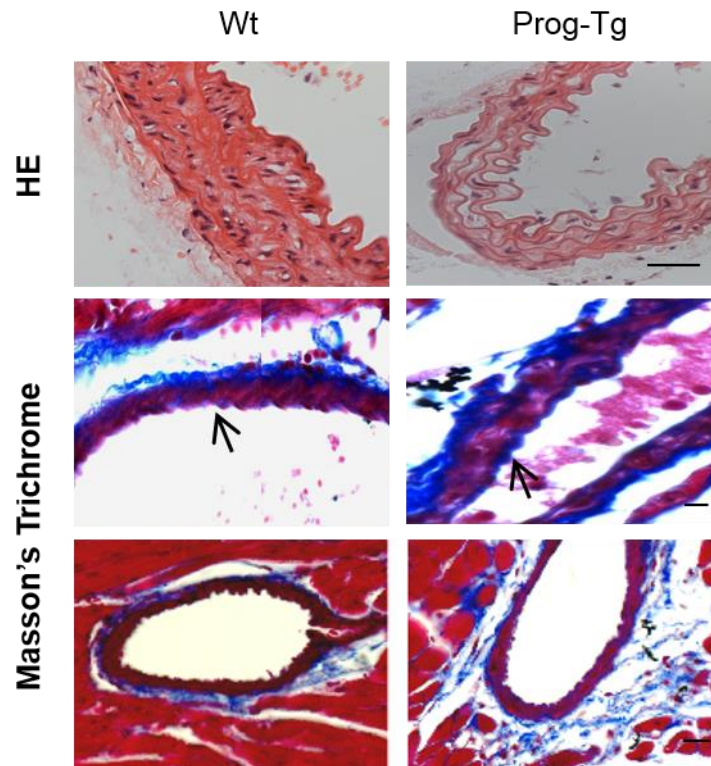


Fig. 20:HE: HE staining of aorta, scale bar 10µm, Masson's Trichrome staining of cardiac vessels in 25 week old mice Note: increased cardiac collagen accumulation revealed by Masson's Trichrome staining (right side) in 20 weeks old transgenic mice compared to wildtype mice (left side), scale bar 10µm respectively 20µm

In order to search for more cardio-vascular abnormalities, aortic HE stains were performed and 63x magnification images were taken (fig. 20, upper panel). Only in some of the transgenic aortas structural alterations were observed. Wholes a porous or riddle structure were observed in Prog-Tg aortas. First, we assumed that these wholes were caused by mineral depositions, but this was excluded after an ordinary aortic Alizarin Red stain (data not shown). Aortic histological examinations were performed using cryo-sectioning. Aortas could not be tested for fat depositions, because alcoholic solutions had to be used during histological examinations. A loss of SMC's could be an explanation although we found increased SMA expression in transgenic aortas. Importantly, the more brittle appearance of the aorta in Prog-Tg animals in comparison to Wt animals could be caused by the altered extracellular matrix deposition.

After showing fibrosis, we wanted to check for other pro-atherogenic symptoms in tissue. Therefore, we started to investigate typical hypertrophy and inflammation markers using RT qPCR and specific primer sequences (fig. 21).

We first examined expression levels of several candidate genes in pulmonary tissue (fig. 21A). Hypertrophy markers also appearing in ordinary atherosclerosis like BNP showed increased expression levels in pulmonary tissue (fig. 21A). However, ANP and endothelin-1 were not altered. Fibrotic markers like several kinds of collagens, myosin heavy chain 7 were not affected. Endothelial-specific markers like VE-cadherin and ICAMs were also checked in order to check for an enhance rate of apoptosis. ICAMs which are also a marker for inflammation as well as VE were unchanged. Importantly, eNOS, a diagnostic marker for atherosclerosis susceptibility, was significantly altered in Prog-Tg animals.

We next assessed several other genes in cardiac tissue (fig. 21B). Interestingly, cytoskeletal genes were found deregulated in cardiac tissue, such as significantly increased actin B and decreased myosin heavy chain 7 indicating cardiac hypertrophy and increased vascular stiffness in Prog-Tg mice. However, typical hypertrophy markers like ANP or endothelin were unchanged. Again, unaltered levels of VE cadherin and ICAM1 and 2 indicate similar endothelial cell numbers excluding effects of increased apoptosis or inflammation in Prog-Tg mice. Major fibroblast-derived collagen III marker of fibrosis was significantly increased but not collagen I or endothelium-derived collagen IV as mentioned above. Collagens showed no significant alterations in isolated endothelial cells from lung (fig. 25A and data not shown). Importantly, similar to pulmonary tissue, there was a significant downregulation of eNOS mRNA in Prog-Tg animals.

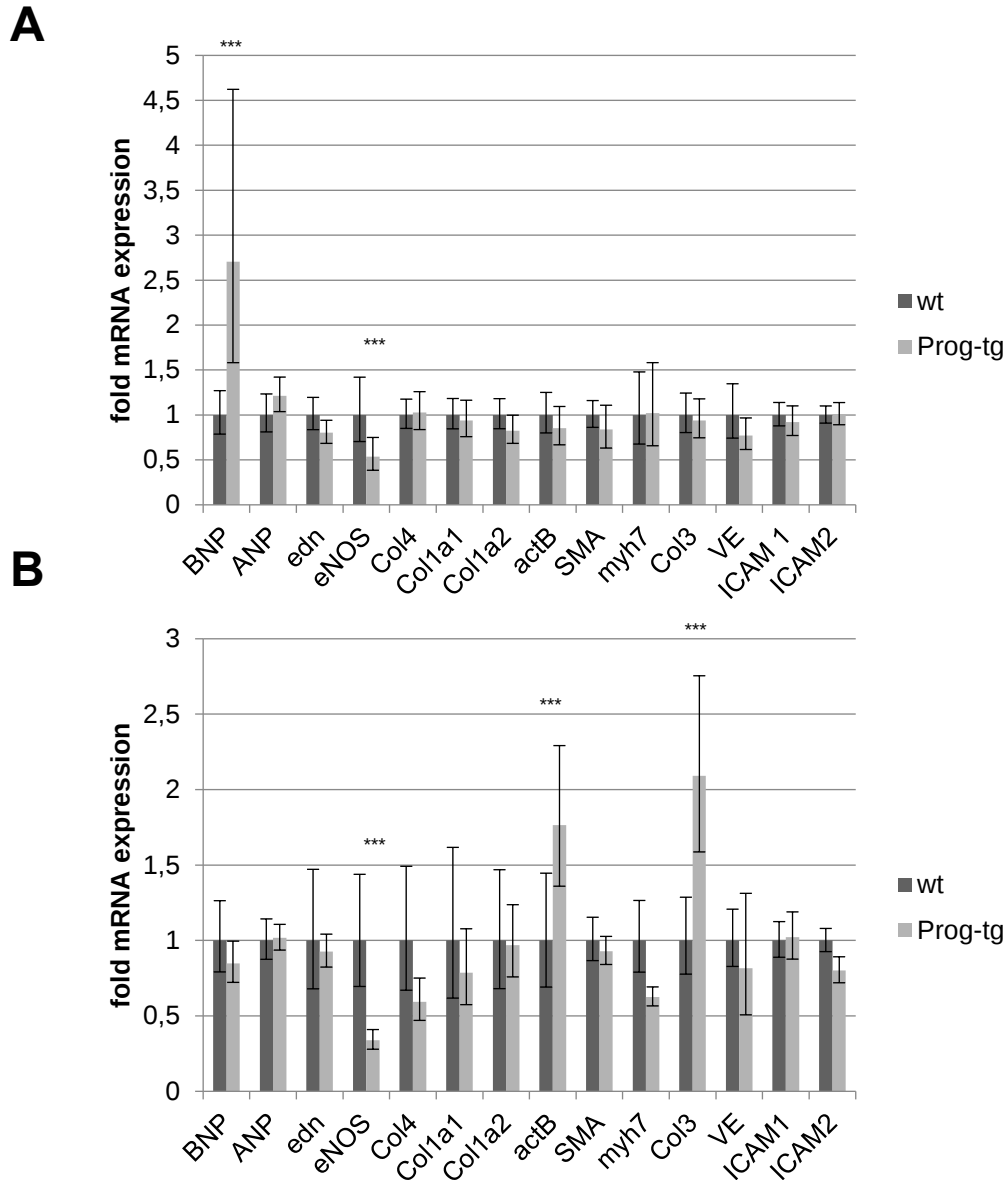


Fig. 21: A: Pulmonary gene expression levels of different atherogenic markers: BNP (n=7, p=0.05), ANP (n=5), edn (n=5), eNOS (n=6, p=0.03), Col4 (n=5), Col1a1 (n=5), Col1a2 (n=5), actB (n=6), SMA (n=6), myh7 (n=5), Col3 (n=3), VE (n=6), ICAM1 (n=5), ICAM2 (n=5) B: Cardiac gene expression levels of different atherogenic markers: BNP (n=5), ANP (n=5), edn (n=5), eNOS (n=5, p=0.02), Col4 (n=8, p=0.08), Col1a1 (n=5), Col1a2 (n=5), actB (n=5, p=0.01), SMA (n=7), myh7 (n=5, p=0.07), Col3 (n=5), VE (n=5), ICAM1 (n=2), ICAM2 (n=2), statistical analysis was done using paired student's t-test

The above results indicated pro-atherogenic changes in bi-transgenic animals. We next searched for upstream acting transcriptional regulators of these genes particularly the deregulated eNOS gene. According to our hypothesis, MRTF-A appeared as a good candidate. Therefore, we assessed MRTF-A localization in Wt and Prog-Tg endothelial cells under no flow and flow (12 dyn/cm² for 6h) conditions. We found increased accumulation of MRTF-A at the nuclear periphery of Prog-Tg compared to Wt endothelial cells (fig. 22, left panels). Shear stress induced slight

increase of MRTF-A in the nuclear interior but this was not further statistically evaluated. Quantitation of MRTF-A 1 and 2 gene expression levels using RT qPCR revealed reduction in MRTF-A 1 and 2 in Prog-Tg endothelial cells but no changes in pulmonary or cardiac tissue (fig. 22, right panel). These results need however further evaluation and optimization of qPCR conditions since there was a dramatic variation within cell samples particularly regarding MRTF-A gene expression in endothelial cells.

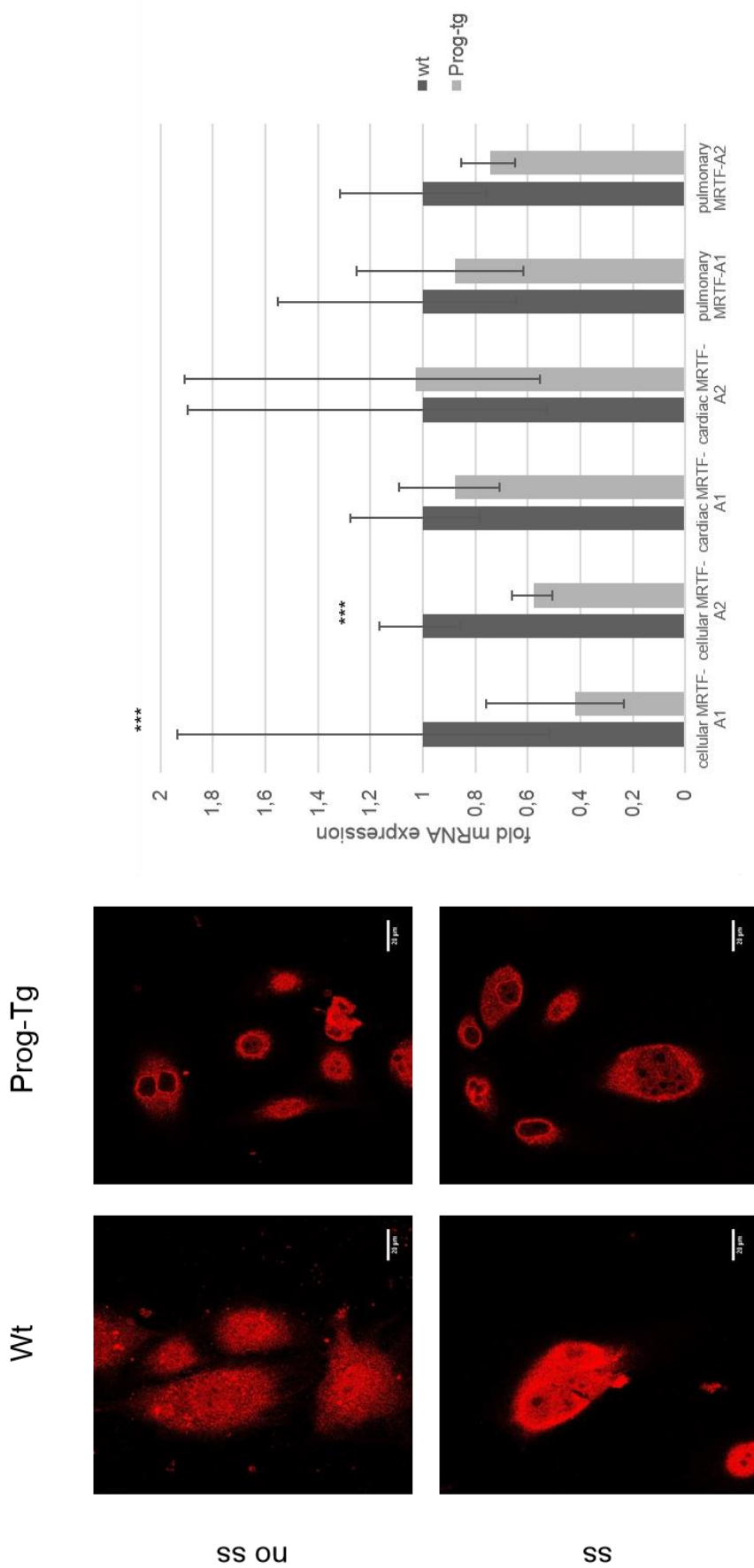


Fig. 22: MRTF-A localization in Wt and Prog-Tg endothelial cells under no flow and flow shear stress (ss) expose (6h, 12 dyn/cm²) Immunofluorescence using goat anti-MRTF-A1 antibody (1:50). MRTF-A1 and 2 gene expression levels in isolated endothelial cells (1: n=3, p=0.0004; 2: n=4, p=0.004) cardiac (1: n=6, p=0.2; n=7, p=0.8) tissues, scale bar 20µm, statistical analysis done with paired student's t-test

We next assessed MRTF-A localization in vivo in aortic tissue. Nuclear accumulation of MRTF-A appeared in vivo in Prog-Tg compared to Wt as well as in endothelial cells (fig. 23A, left panels in red). To quantify MRTF-A nuclear accumulation, confocal stack images were taken with inverse confocal Zeiss 700 microscope using different magnifications (20x, 63x, 100x) and average intensity profiles calculated using ImageJ software. Endothelial but also smooth muscle cell nuclei found in intimal and medial aortic layers were assessed. 100x magnified images revealed increased MRTF-A levels in nuclei of Prog-Tg endothelial cells compared to Wt cells but no changes in smooth muscle nuclei (fig. 23A). Nuclear MRTF-A increase in Prog-Tg nuclei was however not significant with a p-value of 0.07. A similar approach was used in order to evaluate smooth muscle actin (SMA) and eNOS fluorescence intensity in aortic sections Figure 24A and B show increased SMA and decreased eNOS specific aortic immunofluorescence staining in Prog-Tg compared to Wt aortas. Importantly, negative controls using only Biotin and Streptavidin were performed and showed only weak background signals. All other immunofluorescence examinations were checked by negative controls (not shown).

Statistical evaluation of the corresponding average intensities revealed significantly increased SMA levels detected only in smooth muscle but not endothelial cells (fig. 24, diagrams on the left). Due to this increased SMA levels, a loss of smooth muscle cells as reported in previous studies regarding HGPS atherosclerosis could not be confirmed.

To correlate our findings directly with endothelial dysfunction, we next assessed expression levels of genes tested in figure 21 in isolated endothelial cells. Again we observed similar alterations as in pulmonary tissue regarding increased BNP as well as decreased eNOS gene expression levels in cultured endothelial cells (fig. 25B). Furthermore, endothelin known to be regulated by MRTF-A was tendentially upregulated in Prog-Tg endothelial cells compared to Wt cells. Altogether, these results confirm pro-atherogenic alterations in endothelial cells caused by endothelial-specific progerin expression.

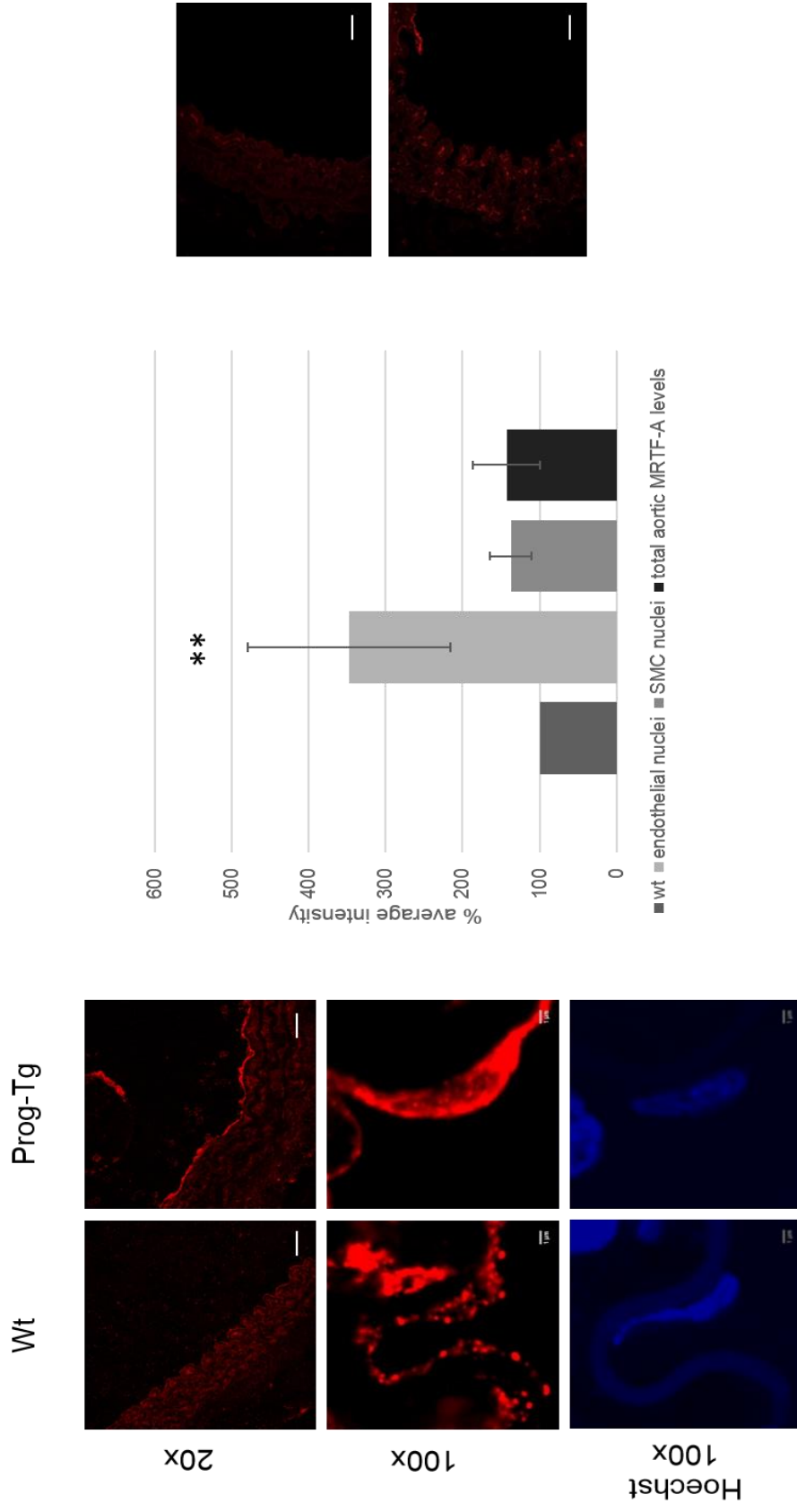


Fig.23: Evaluation of MRTF-A localization and levels in aorta A: immunofluorescence microscopy using goat anti-MRTF-A1 antibody (1:50). average fluorescence intensity evaluated in aortas from Wt and Prog-Tg mice (n=8) in nuclei of endothelial cells and smooth muscle cells. Wt versus Prog-tg endothelial nuclei (p=0.07) and in smooth muscle cell nuclei (p=0.5) as well as in the whole aortic tissue (p=0.1) was measured using ImageJ software, scale bar 10µm respectively 1µm **B** Negative controls without primary antibodies but only with the previous used secondary system (Biotin-Streptavidin), 1 scale bar 10µm

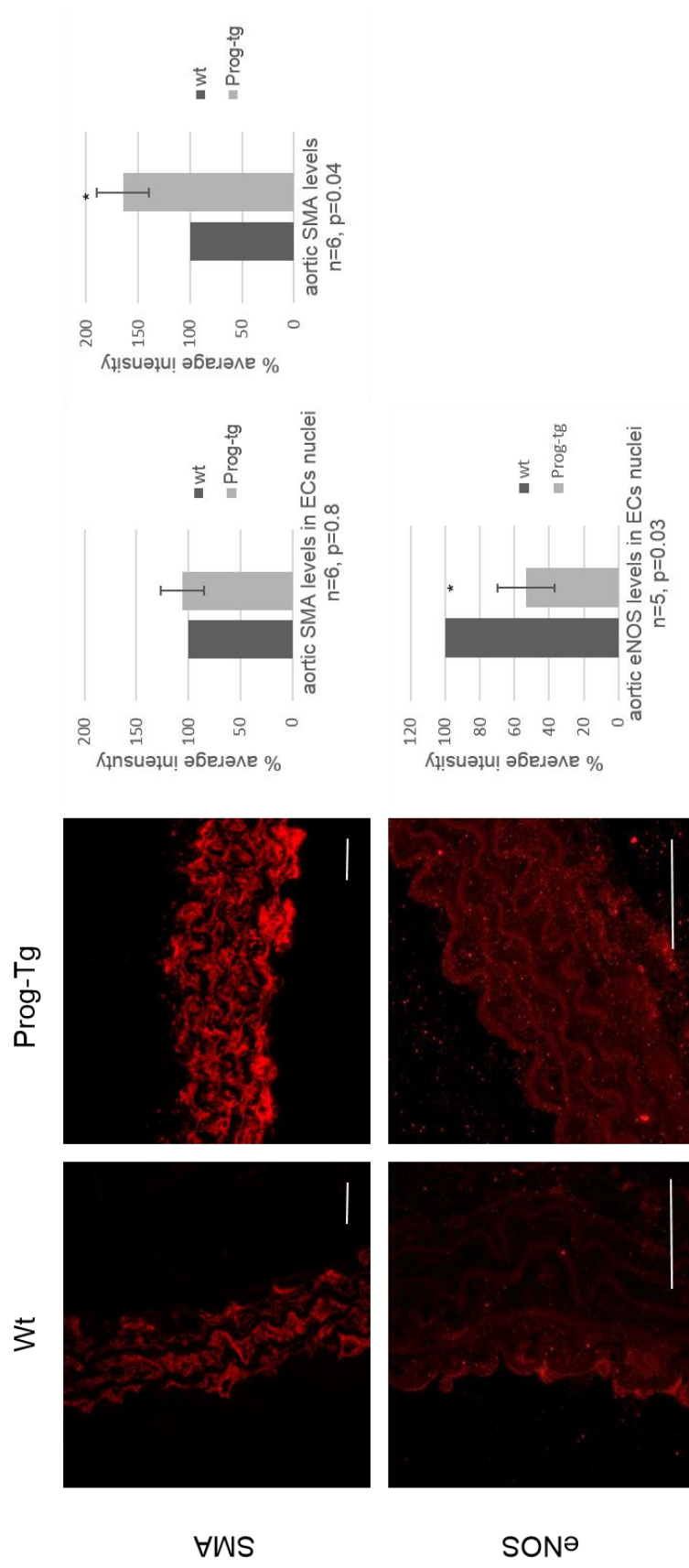


Fig. 24: Evaluation of SMA and eNOS fluorescence intensities in aortas from Wt and Prog-Tg mice. Immunofluorescence using mouse anti-SMA antibodies (1:100) in aortas of wildtype (left) and Prog-Tg (right) mice with their corresponding average SMA fluorescence intensities (%) in endothelial cell nuclei ($p=0.04$) and in total aortic tissue ($p=0.04$); (n=6), scale bar 10 μ m. Immunofluorescence using rabbit anti-eNOS antibodies (1:50) of Wt (left) and Prog-Tg (right) mice with their corresponding average fluorescence intensities in endothelial cell nuclei (n=5, $p=0.03$)

4 DISCUSSION

4.1 Phenotypic changes in transgenic mice

Examining transgenic mice, several severe phenotypic changes attracted our attention. These changes included obvious reduction in body size and body fat percentage.

Reasons for these changes could be altered metabolic function (causing a need for a higher calorie consumption), changes in adipogenesis or malnutrition due to poor physical condition. Moreover, endothelial cells might influence these phenotypic alternations. However, it is difficult to distinguish whether progerin has a primary or secondary effect on body, weight and body fat percentage.

It would be interesting in further studies to examine blood pressure, pulse rate and stress.

4.2 The role of progerin expressing endothelial cells in cardiac hypertrophy

During the dissection of mice, heart hypertrophy was another phenotypic feature observed. For statistical evaluation, heart and body weight were measured and plotted on a graph (fig.19) The heart-body weight ratio (HBW) showed a statistically significant increase in the Prog-Tg. Fibrosis or amount and size of cardiomyocytes could be responsible for the cardiac hypertrophy, which can lead to a stenosis. In order to examine cardiomyocytes we conducted histological examinations on cardiac tissue. After counting the cardiomyocytes a statistical evaluation showed no significant difference in the amount of cardiomyocytes. However, unpublished data by Osmanagic-Myers et al showed differences regarding cell size as well as nuclei elongation. The nuclei elongation is probably due to a higher proliferation rate. [71] Therefore proliferation markers should be checked. Studies by Cupesi et al support this hypothesis as they found significantly reduced cardiac hypertrophic response to mechanical stress due to decreased cardiomyocytes size in *Lmna*^{+/-} mice. [72]

The fibrotic phenotype might be caused by heart insufficiency, ventricular stiffness, increased mechanical load or scarred tissue. For our examination heart and aortic tissue sections were stained with Masson's Trichrome to examine collagen in the samples. Increased collagen depositions in cardiac blood vessels of Prog-Tg mice

were similar to findings in HGPS human patients reported by Olive et. al. The question that arises is which cell type is the source of excessive collagen production, either endothelial cells, cardiomyocytes or others. Different types of collagens were examined since typical fibroblast-derived collagen type I and III are increased in atherosclerotic patients and collagen IV is produced in endothelial cells. Collagen I is mostly found in scarred tissue and it also co-localizes with collagen III. [22] [46] [73] [74] [75]

In cardiac tissue, significantly increased collagen III levels were found using RT-PCR. These results could not be reproduced in endothelial cells, although further repetitions are necessary in order to generate significant values. These data strongly support the notion that fibroblasts are the predominant source of excessive collagen production. Collagen I and IV were also tested with the same method, but no significant changes in the heart tissues were found. For further examination immunofluorescence microscopy of aortic tissue was used. However, no change in collagen I and IV levels were detected (data not shown). The aortic tissue showed a trend to increased collagen XII production, but this is not statistically relevant and further repetitions are necessary. Vidak et al showed a massive influence of progerin on the regulation of ECM components including collagen III and XII.^[80]

We hypothesised that a combination of both – fibrosis and cardiomyocyte elongation – cause the hypertrophy. Another important observation concerning reasons for hypertrophy is mentioned by Nelson et al. Their study suggests that hypertrophy could be caused by an overexpression of SRF. Based on these findings we propose that MRTF-A may be impaired by progerin. In accordance, Small et al. showed that MRTF-A ^{-/-} mice have reduced cardiac fibrosis after myocardial infarct. [60] [76]

In order to gain additional information, aortic tissue was histologically examined. We detected a brittle structure in the muscular layer of the aorta, which was not visible in immunofluorescence studies. Also calcifications could be eliminated as possible reason since Alizarin Red S staining showed no changes in aortas from Wt and Prog-Tg animals. The fragility caused by the elongated nuclei of the cardiomyocytes might lead to a porous structure and therefore to a rupture of the aorta during sectioning. However, we could not observe such an effect in immunofluorescence examination. Such a brittle appearance might be a consequence of either increased collagen accumulation or changes in collagen cross-linking and network formation as already reported in other cell types of progerin expressing cells.

4.3 MRTF-A changes correlate with changes in expression levels of pro-atherogenic genes

As we already found some symptoms indicating increased susceptibility to atherosclerosis development, which is the primary cause of death in children suffering from HGPS, we decided to investigate some specific atherogenic gene expression markers with quantitative RT-PCR. We decided on investigating pulmonary tissue as the lung contains more endothelial cells due to micro-vascularisation for a sufficient oxygen exchange. We tested downstream targets of MRTF-A like pro-atherogenic, hypertrophic markers ANP and BNP and showed a significant increase in BNP. [77]

The anti-atherogenic eNOS was decreased, which is a major prognostic marker of atherosclerosis. Endothelial markers VE and ICAM 1 and 2 were used as a control for endothelial numbers. Surprisingly, we noticed a slight decrease in VE expression levels which might be due to higher apoptotic activity in progerin-transgenic endothelial cells. A very interesting patented explanation is reported by Jansen et al. They suggest a role of endothelial microparticles (EMP) as an attenuator of ICAM-1 and a reflector of endothelial dysfunction. Hayashi et al. proposed another model according to which NFkappaB plays a major role in ICAM-1 mRNA expression and they suggested that MRTF-A inhibits ICAM-1 gene transcription by forming a nuclear complex with NFkappaB p65. This shows an important role of MRTF-A in the homeostasis of vascular endothelium. [39]

Another direct downstream target of MRTF-A, smooth muscle actin alpha (SMA) did not reveal any significant changes in gene expression. Unfortunately, no conclusive results on collagen expression in ECM were found. Matrix metalloproteinases could have been checked as well as potential modulators of ECM are involved in the development of atherosclerotic plaques.

Consistent with heart tissue defects, eNOS levels were also significantly reduced in heart tissue indicating it was a general phenomenon. SMA also remained unchanged. Unexpectedly, hypertrophic marker ANP was unaltered in wildtype and transgenic mice. Another interesting finding was the significantly increased ActB in cardiac tissue as well as in endothelial cells, implicating stiffer endothelial tissue. We hypothesized that increased ActB levels are a consequence of deregulated MRTF-A signalling in Prog-Tg cells, since MRTF-A is a major transcriptional regulator of

cytoskeletal gene expression. Myosin heavy chain 7 (myh7) was also found tendentiously downregulated in Prog-Tg mice that could be a further potential cause of the brittle structure in aortas of Prog-Tg mice.

ICAM1 and ICAM2 as well as VE gene expression levels were unaltered. This result does not support the hypothesis of increased apoptotic activity of progerin-expressing endothelial cells.

Our model is based on the hypothesis that MRTF-A is the key connecting regulator of all these altered gene expressions. Therefore a closer investigation of the cellular localization and gene expression levels in endothelial cells, lung and heart tissue was conducted. The immunofluorescence examination of endothelial cells of MRTF-A revealed a conspicuous accumulation of MRTF-A at nuclear periphery of Prog-Tg cells. A study of Ho et al. with lamin A/C deficient and LMNA mutant cells showed affected MRTF-A complexes as well. Based on our finding we concluded that progerin either directly or indirectly attracts MRTF-A at the nuclear periphery. Whether MRTF-A resides outside the nucleus or inside at the lamina directly trapped by progerin is to be further investigated using digitonin based procedure. Ho et al. used an ERK1/2 inhibitor to reduce MRTF-A's nuclear export and achieved a reduced cardiac phenotype. The investigation showed a decrease on the gene expression levels of MRTF-A (see figure 21) but also a delocalization, which might cause the changes on MRTF-A activity mentioned beforehand. Also the missing stress in in vitro cultures might cause a decrease of MRTF-A levels. In figure 22 we see significantly elevated MRTF-A levels in vivo in aortic tissue. Also from literature it is known that MRTF-A levels increase when mechanical stress is applied. Neither heart nor lung tissue exhibited changed MRTF-A mRNA levels presumably due to a smaller amount of endothelial cells compared to muscular and other types of cells. [66]

Since we found changes in MRTF-A, we investigated the aorta and statistically evaluated the data. In nuclei of endothelial but not smooth muscle cells MRTF-A was significantly increased in Prog-Tg compared to Wt cells. There is an overall trend of elevated MRTF-A in the entire aorta detectable but this increase is not significant. These results do not correspond with the data found in the MRTF-A gene expression levels. However, the export rate has to be investigated eventually as the export rate might also influences the MRTF-A downstream target activity.

The MRTF-A specific immunofluorescence was also performed on the heart tissue and showed an increased signal for MRTF-A in the entire tissue. No difference between endothelial and muscular cells was found. This does not correlate with the before mentioned results in the aortic tissue. Nevertheless, according to negative control MRTF-A signal was highly specific, and it remains unclear why the increased signal is detectable in the entire tissue.

The more stress the cell is exposed to, the more G-actin is polymerized to F-actin. MRTF-A can move to the cell nucleus upon an increase in F/G actin ratio, where it affects the SRF dependent genes. Increased actin levels correlate directly with MRTF-A nuclear accumulation in Prog-Tg cells. The heart tissue showed increased actin-B while the aortic tissue exhibited increased SMA. This might also be a possible explanation for the increased stiffness of the vascular tissue in Prog-Tg animals. The increased stiffness might cause a higher risk of rupturing. As shown in figure 24A besides affecting SMA also eNOS is decreased in the aortic tissue, which again hints to a pro-atherogenic phenotype. The study of Fang et al also found suppression of eNOS as well as a significant upregulation of SRF expression after stimulating MRTF-A by applying oxidative LDL to endothelial cells. Although Fang et al used a different method for cell stimulation, our study is consistent with these findings, as two studies proved that oxidative LDL as well as mechanical stress both lead to an upregulation of type IV collagen synthesis in endothelial cells. Furthermore, they showed that increased eNOS also upregulates the type IV collagen synthesis. In our study the mechanical stress was increased while eNOS levels were decreased. However, we did not detect collagen IV upregulation in heart tissue using qPCR. [32] [78] [79]

Following examination of heart and lung tissue we also test the relevant genes in endothelial cells by qPCR. The fact that we observed decreased eNOS and increased BNP expression levels in isolated endothelial cells indicated that the above observed effects in heart and lung tissue were a direct consequence of progerin induced endothelial dysfunction.

In conclusion our results show for the first time that progerin expression in endothelial tissue causes pro-atherogenic changes leading to endothelial dysfunction and cardiovascular pathology. Furthermore, by identification of pro-atherogenic targets

affected in progerin expressing endothelial cells it provides novel mechanical insights in progeric cardiovascular disease.

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“Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.“

7 APPENDIX

7.1 List of figures

Fig. 1	human lamins
Fig. 2	artery wall
Fig. 3	atherosclerotic plaque
Fig. 4	HGPS patient
Fig. 5	cell nucleus' deformation
Fig. 6	formation of lamin A resp. Progerin
Fig. 7	farnesyltransferase inhibitor
Fig. 8	organs targeted by SIRT-1 activators
Fig. 9	human MRTF-A
Fig. 10	MRTF-A activation via Rho-A
Fig. 11	proposed working model
Fig. 12	used minigenes and tet-off system
Fig. 13	Progerin signal only in endothelial cells in vivo
Fig. 14	qPCR amplification plot
Fig. 15	qPCR melting curve
Fig. 16	qPCR standard curve
Fig. 17	primer validation
Fig. 18	phenotypical changes in bi-transgenic mice
Fig. 19	cardiac alterations in bi-transgenic mice
Fig. 20	HE and Trichrome Masson stains of cardiac material
Fig. 21	pulmonary and cardiac mRNA expression levels
Fig. 22	cellular MRTF-A localization and expression levels
Fig. 23	aortic MRTF-A localization and average intensities
Fig. 24	aortic SMA and eNOS localization and average intensities
Fig. 25	cellular gene expression levels with and without an MRTF-A inhibitor

7.2 List of tables

Tbl. 1	qPCR primer sequences
Tbl. 2	antibodies used for immunofluorescence

7.3 List of abbreviations

NLS	nuclear localization signal
VEGF	vascular endothelial growth factor
eNOS	endothelial nitric oxide synthetase
VSMC	vascular smooth muscle cell
HIF-1	hypoxia inducible factor 1
NO	nitric oxide
TERT	telomerase reverse transcriptase
EC	endothelial cell
ROS	reactive oxygen species
CVD	chronic vascular disease
ET	endothelin
CAD	coronary artery disease
ICAM	intercellular adhesion molecule
VCAM	vascular cell adherent molecule
oxLDL	oxidative low density lipoprotein
HGPS	Hutchinson Gilford progeria syndrome
MSC	mesenchymal stem cell
MI	myocardial infarction
FTI	farnesyltransferase inhibitor
SRF	serum response factor
MRFT	myocardin related transcription factor
LZ	leucine zipper
BNP	brain natriuretic peptide
ANP	artery natriuretic peptide
ECGS	endothelial culture growth supplement
FCS	fetal calf serum
DOX	doxycycline
PFA	paraformaldehyde

7.4 Abstract

Hutchinson Gilford Progeria syndrome (HGPS) is a rare degenerative disease caused by a single point mutation in *LMNA* gene, encoding nuclear proteins lamin A and C. The disease-causing mutation induces mis-splicing of the *LMNA* transcript and results in the generation of a truncated prelamin A variant that cannot be correctly processed. Unlike wild-type lamin A, the disease variant called progerin, is stably farnesylated and carboxymethylated at the C-terminus and tightly bound to the nuclear membrane. HGPS patients show many signs of premature aging, including growth retardations, alopecia, osteoporosis, and loss of subcutaneous fat and develop cardiovascular disease and atherosclerosis leading to death before the age of 20 years.

The goal of this study was to investigate the contribution of the vascular endothelium to cardiovascular disease in progeria patients, because an impaired endothelial function is known as a major risk factor for atherosclerosis development during normal aging. We developed a new transgenic mouse model, expressing progerin selectively in the vascular endothelium. Using quantitative RT-PCR, histology and immunofluorescence microscopy of cardiovascular tissue, including heart, aorta and lung as well as in isolated primary endothelial cells we found cardiovascular impairments like cardiac hypertrophy with increased heart to body weight ratios, increased expression of various extracellular matrix components (collagen, smooth muscle actin) and fibrosis and activation of pro-atherogenic signaling, such as an increase in endothelin expression and a reduction in endothelial nitric oxide synthase (eNOS) levels.

Mechanosensitive myocardin-related transcription factor A (MRTF-A) accumulated at the nuclear periphery, suggesting that MRTF-A-mediated athero-protective signalling was affected in endothelium-specific progerin mice. Importantly all these effects are specific for progerin expression in the endothelium, as control mice expressing wild-type lamin A in the endothelium or wild type littermates did not show this phenotype.

Overall, our study showed for the first time that cardiovascular abnormalities in HGPS can at least in part be rooted in an impaired endothelium mediated by the expression of progerin.

Das Hutchinson Gilford Progerie Syndrom (HGPS) ist eine seltene, degenerative Krankheit. Sie wird durch eine Einzelpunktmutation im *LMNA* Gen verursacht, das für die Kernproteine Lamin A und C kodiert. Die Krankheit wird durch inkorrektes Spleißen des *LMNA* Transkripts und der folglich verkürzten Bildung von Prelamin A ausgelöst. Dieses kann nicht richtig weiterverarbeitet werden, woraus eine fehlerhafte Lamin A Variante namens Progerin entsteht. Anders als das Wildtyp-Lamin A besitzt Progerin eine Farnesyl- und Carboxymethyl-Gruppe am C-Terminus und ist dadurch an die nukleare Membran gebunden. Patienten die an HGPS leiden zeigen viele Zeichen des vorzeitigen Alterns, wie zum Beispiel Wachstumshemmung, Haarausfall, Osteoporose und Schwund an subkutanen Fett. Außerdem leiden sie vorzeitig an kardiovaskulären Krankheiten und Atherosklerose, die zum frühen Tod vor einem Alter von 20 Jahren führen.

Das Ziel dieser Arbeit war die Erforschung eines Progerin-exprimierenden vaskulären Endothels und dessen Beteiligung am Entstehen von kardiovaskulären Krankheiten. Auch während des normalen Alterungsprozesses gilt gestörtes Endothelgewebe als ein wesentlicher Risikofaktor für die Entstehung von Atherosklerose. Wir haben ein neues transgenes Mausmodell entwickelt, das Progerin ausschließlich im vaskulären Endothelgewebe exprimiert. Mithilfe von RT-PCR, histologischen Untersuchungen und Immunofluoreszenz-Mikroskopie wurde kardiovaskuläres Gewebe wie Herz, Aorta, Lunge und isolierte primäre Endothelzellen untersucht. Wir fanden verschiedene kardiovaskuläre Beeinträchtigungen wie Herzhypertrophie und ein erhöhtes Verhältnis von Herz- zu Körpergewicht, eine erhöhte Expression von extrazellulären Matrixkomponenten (Kollagen, smooth muscle actin) und Fibrose. Außerdem fanden wir eine Aktivierung von pro-atherogenen Signalen wie erhöhte Expression von Endothelin und eine Verringerung von eNOS (endothelial nitric oxide synthase).

In der Kernperipherie fanden wir eine erhöhte Ansammlung des mechanosensitiven MRTF-A (myocardin-related transcription factor A). Daraus folgerten wir, dass das athero-protective Verhalten von MRTF-A in transgenen Mäusen gestört wird. Zudem wurden all diese Symptome nur in Progerin-transgenen Mäusen gezeigt, nicht aber in Kontrollmäusen die wt LaminA exprimieren oder Wildtypgeschwistern.

Zusammenfassend zeigt unsere Arbeit, dass kardiovaskuläre Krankheiten die in HGPS Patienten auftreten zumindest teilweise durch gestörtes Endothelgewebe, hervorgerufen durch die Bildung von Progerin, verursacht werden.