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Abstract

Aims: Cardiovascular ageing is a key determinant of life expectancy. Cellular senescence, a state of irreversible cell cycle arrest, is an important contributor to ageing due to the accumulation of damaged cells. Targeting cellular senescence could prevent age-related cardiovascular diseases. In this study, we investigated the effects of neuregulin-1 (NRG-1), an epidermal growth factor with cardioprotective and anti-atherosclerotic effects, on cellular senescence.

Methods & results: Senescence was induced in cultured rat aortic endothelial cells (ECs) and aortic smooth muscle cells (SMCs) by 2 hours exposure to 30 μM hydrogen peroxide (H_2O_2). Cellular senescence was confirmed after 72 hours using senescence associated-β-galactosidase staining (SA-β-gal), cell surface area, and Western blot analyses of senescence-associated pathways (acetyl-p53, p21). Recombinant human NRG-1 (rhNRG-1, 20 ng/ml) significantly reduced H_2O_2-induced senescence, as shown by a lower number of SA-β-gal positive cells, smaller surface area and lower expression of acetyl-p53. In C57BL/6 male mice rendered diabetic with streptozotocin (STZ), rhNRG-1 attenuated cellular senescence in aortic ECs and SMCs.

Next, we created mice with SMC-specific knock-down of the NRG-1 receptor ErbB4. Aortic SMCs isolated from SMC-specific ErbB4 deficient mice (ErbB4$^{f/+}$ SM22α-Cre+) showed earlier cellular senescence *in vitro* compared to wild type (ErbB4$^{+/+}$ SM22α-Cre+) SMCs. Furthermore, when rendered diabetic with STZ, ErbB4$^{f/+}$ SM22α-Cre+ male mice showed significantly more vascular senescence than their diabetic wild type littermates and had increased mortality.

Conclusions: This study is the first to explore the role of NRG-1 in vascular senescence. Our data demonstrate that NRG-1 markedly inhibits stress-induced premature senescence in vascular cells *in vitro* and in the aorta of diabetic mice *in vivo*. Consistently, deficiency in the NRG-1 receptor ErbB4 provokes cellular senescence *in vitro* as well as *in vivo*.

1. Introduction

Ageing is associated with alterations in cardiovascular structure and function that are partly driven by chronic inflammation and oxidative stress.[1] At a cellular level, ageing is characterized by a process known as cellular senescence, a state of irreversible growth arrest.

Normally, cellular senescence is a protective process, keeping the body safe from tumour growth by inhibiting uncontrolled proliferation. At the same time it is deemed responsible for ageing-associated changes in several organs including the cardiovascular system.[2,3] Although senescent cells undergo a permanent growth arrest, they remain viable and actively secrete inflammatory mediators until removed by the immune system. As such, these cells can negatively influence the neighbouring microenvironment by a phenomenon known as the senescence-associated secretory phenotype (SASP).[4,5] Cellular senescence and SASP play an important role in cardiovascular ageing and disease.[6–8]

Cells can enter the senescent state by DNA damage caused by intrinsic and extrinsic stress factors, known as "stress induced premature senescence" (SIPS).[9] Oxidative stress and hyperglycaemia are two major factors causing SIPS.[10,11] Stress-induced DNA-damage provokes a DNA-damage response, which subsequently leads to activation of two important cell cycle regulatory pathways, p53 and p16.[12–14] The exact nature of activation of either of these two pathways in the context of cellular senescence depends on species and cell type.[15–18] The p53 protein contains several functional domains that can be post-translationally modified (via phosphorylation and acetylation), resulting in different cellular responses such as apoptosis, senescence and tumour suppressor activity.[19] Acetylation of p53 on six C-terminal lysine (k) residues (k317, k370, k372, k373, k381, k382 and k386) in response to oxidative stress leads to its activation.[14,17,20] Subsequently, p21 is activated, leading to cell cycle arrest.[15] Therefore, activation of both the p53 or p16 pathway is frequently used together with SA-β-gal staining to confirm cellular senescence.[21–27]

Neuregulins belong to the epidermal growth factor family and are known to induce growth and differentiation of several cell types. Neuregulin-1 (NRG-1) is produced by cardiac and vascular endothelial cells (ECs) and acts through ErbB2, ErbB3 and ErbB4 tyrosine kinase receptors expressed on ECs and SMCs in a paracrine and autocrine fashion.[28–30]

In the past two decades, the cardioprotective effects of NRG-1 have been extensively studied.[31–36] NRG-1 has been shown to attenuate development of cardiac dysfunction in several animal models of heart failure and recombinant human NRG-1 (rhNRG-1) is currently tested in clinical trials as a novel treatment for heart failure.[37] In addition, NRG-1 is known to influence apoptosis and cell death pathways.[29,38] However, the effects of NRG-1 on cellular senescence in cardiovascular diseases are not known. Therefore, we studied the effects of NRG-1 on stress-induced cardiovascular senescence, both *in vitro* and *in vivo* in a mouse model of type 1 diabetes. To study vascular senescence in diabetic mice, a model of type 1 diabetes induced by administration of streptozotocin (STZ) was selected. In comparison to models of type 2 diabetes, this model leads to cellular senescence in the vasculature within a relatively short period of time.

2. Material & methods

2.1 Cell culture

Rat aortic ECs and SMCs were isolated from male Sprague Dawley rats. Freshly isolated aortas were incubated in 1 mg/ml collagenase type II CLS, 1 mg/ml soybean trypsin inhibitor and 0.74 µl/ml elastase (all from Worthington) for 15 min at 37°C to remove the adventitia.

Thereafter, the aorta was cut longitudinally and placed in a fresh enzyme solution for 20 min at 37°C to isolate ECs, then the remaining aortic tissue was incubated in fresh enzyme solution for 1 h at 37°C to obtain SMCs. ECs were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS, Sigma). SMCs were grown in DMEM/Nutrient Mixture F-12 (MDEM-F12), supplemented with 10% FBS. All media contained 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Experiments were conducted on cells from passage number 3 to 7. Cultures with ≥95% purity were used for each cell type. For SMCs, alpha smooth muscle actin antibody (Sigma A2547, 1:1000) staining was used to measure the purity of cell cultures. The purity of ECs was measured using CD31 (BD PharmingenTM 557355 for mouse and BD PharmingenTM 550300 for rat, 1:1000) labeled with Alexa Flour 488 (Molecular Probes by Life Technologies) and visualized using an automated cell counter (countess II FL, ThermoFisher Scientific).

To induce cellular senescence, $1x10^4$ cells per well (24 well plate) were treated with hydrogen peroxide (30 μM, H_2O_2, Fisher BioReagents) for 2 h. It is well-established that short exposure to H_2O_2 leads to senescence whereas longer exposure leads to apoptosis.[39] To render cells quiescent, medium was switched to low FBS (1%) conditions 24 h before the experiments. After exposure to H_2O_2, medium was replaced with 10% FBS supplemented medium for the remainder of the study. Half of the wells were pre-treated with rhNRG-1 (20 ng/ml, Peprotech) 30 min before exposure to stress stimulus and during the entire experiment.

We isolated 13 batches of SMCs from 13 animals and 7 batches of ECs from 7 animals. For each experiment, 4 to 6 batches of cells were used in almost the same cell serial passaging (3-7). Within each batch of cells, treatments were done in four fold simultaneously and an average was used in the statistical analysis. 1x104 cells/well were plated initially and 100 total cells per well were randomly counted towards of senescence-associated β-galactosidase (SA-β-gal) positive cells, using Image J software in a blinded manner. ≥95% of cells used for this experiment was viable. Cell viability was monitored using propidium iodide (PI). A comparable well of cells was subjected to PI to label death cells. Briefly, cells were labelled with 1μg/ml PI (Molecular Probes) and 5μg/ml Hoechst (Life Technologies) and immediately visualized using fluorescent microscopy (EVOS® FL Cell Imaging System, ThermoFischer Scientific).

2.2 Animal models

All animal experiments were approved by the institutional ethics committee of the University of Antwerp and conform to the guide for the care and use of laboratory animals published by the US National Institute of Health (update version 2011).

SMC specific ErbB4 deficient mice were created by breeding mice homozygous for the ErbB4 flox allele ($ErbB4^{f/f}$, MMRRC Repository) with transgenic mice expressing Cre recombinase under control of the SMC-specific SM22α promoter (SM22α-Cre+, Jackson laboratory, 004746). No pups were born with complete knock out (KO) of the ErbB4 gene in SMCs, indicating complete ErbB4 KO in SMCs might be lethal; only heterozygote offspring could be obtained (58.3% $ErbB4^{f/+}$ SM22α-Cre+ and 41.7% $ErbB4^{+/+}$ SM22α-Cre+). These $ErbB4^{f/+}$ SM22α-Cre+ heterozygote mice (n=20 survival) were used as ErbB4 deficient mice for further experiments. $ErbB4^{+/+}$ SM22α-Cre+ (n=20 survival) served as wild-type control. C57Bl/6 mice (n=27 survival) were obtained from Charles River.

Diabetes mellitus type 1 was induced in all described mouse strains at the age of 13 weeks by low-dose streptozotocin (STZ) treatment (60 mg/kg in 0.05 M sodium citrate, 5 consecutive days, intraperitoneal). Age-matched control mice were sham-treated with citrate buffer. Blood glucose levels were determined (glucometer Elite XL) and mice with blood glucose levels below 300 mg/dl two weeks after the STZ injections were excluded from the experiment. Mice were randomized to receive either daily rhNRG-1 injections (20 μg/kg, intraperitoneal) or vehicle for 9 weeks. At the end of follow-up, mice and rats used to isolate aortic ECs and SMCs were euthanized with an overdose of pentobarbital (100 mg/kg intraperitoneal, CEVA Santé Animale). Tissues, including aorta and heart were embedded in Neg-50 frozen section medium (ThermoFisher).

Male Sprague Dawley rats used to isolate aortic ECs and SMCs were euthanized with an overdose intraperitoneal injection of 100 mg/kg pentobarbital.

2.3 Senescence associated β-galactosidase staining

Senescence was determined by using the Senescence Cells Histochemical Staining Kit (Sigma CS0030) following manufacturer's instructions. Briefly, $1x10^4$ cells were incubated with fixation buffer for 10 min at room temperature. After washing steps, cells were incubated with staining mixture (containing X-gal) at 37°C for 24-30 h and then counterstained with Nuclear Fast Red. Light microscopic pictures were acquired with Universal Grab 6.1 software using an Olympus BX40 microscope. The percentage of senescence-associated β-galactosidase (SA-β-gal) positive cells was quantified using Image J software in a blinded manner. The same staining kit was used for isolated aortic tissue of the *in vivo* experiments. Frozen sections (5 μm) were incubated for 48 h at 37°C without CO_2, followed by counterstaining with Nuclear Fast Red. Total

area of positive staining was calculated using Image J software and normalized to total surface area of each aorta.

2.4 Histology

Fluorescent staining was performed on frozen sections of the aorta with specific antibodies against acetyl-p53 (rabbit anti-acetyl-p53 (lys 317), Sigma SAB4503014) as a senescence marker and against CD31 (rat anti-CD31, BD Pharmingen 550274) as a marker for ECs. Furthermore, ErbB4 and ErbB3 receptors were stained with specific antibodies against ErbB4 (rabbit anti-ErbB4 antibody (C-18), Santa Cruz, sc-283) and ErbB3 (rabbit anti HER3/ErbB3, cell Signaling, D22C5). Nuclei were counterstained using DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride, Sigma). Images were taken using the EVOS® FL Cell Imaging System (ThermoFischer Scientific). Total number of cells and number of ac-p53 positive cells were calculated to make a percentage of positive cells in ECs and SMCs.

2.5 Western blotting

Cultured cells were harvested in RIPA lysis buffer (Sigma) supplemented with protease and phosphatase inhibitors (complete Mini and PhosSTOP, Roche) on ice for 5 min. Aorta segments were mixed in RIPA lysis buffer using a Precellys 24 tissue homogenizer (Bertin Instruments). After centrifugation to pellet non-dissolved material, protein concentration of the supernatant of each sample was determined using the bicinchoninic acid method (BCA, ThermoFischer). Equal amounts of protein were separated on Bolt 4-12% bis-tris gels (Invitrogen) and blotted onto polyvinylidene fluoride membranes. Membranes were blocked in Odyssey® Blocking Buffer (Li-Cor Biosciences) and probed with primary antibody (overnight, 4°C). Following primary antibodies were used: rabbit anti-acetyl-p53 antibody (Lys317, Sigma SAB4503014, 55 kDa), rabbit anti-p21 antibody (Abcam, ab109199, 18 kDa), mouse anti-β-actin (Sigma, A5441, clone AC-15, 42 kDa), rabbit anti-Akt (Cell Signaling 9272, 60 kDa), rabbit anti-phospho-Akt (S473, Cell Signaling 9271, 60 kDa), and rabbit anti-ErbB4 (ErbB-4 Antibody (C-18), Santa Cruz, sc-283, 150 kDa). Subsequently, infrared (IR)-conjugated secondary antibodies (anti-rabbit: IgG926-32211 and anti-mouse: IgG926-68070; Li-COR Biosciences) were used for IR fluorescence detection using an Odyssey SA infrared imaging system (Li-COR Biosciences). Each Western blot panel pictured in this paper was derived from one single blot.

2.6 Cell cycle analysis

Cell cycle analysis was performed using the Vindelov method.[40] Briefly, cells were permeabilised with trypsin, followed by ribonuclease treatment to ensure DNA staining only. Finally, nuclei were stained with propidium iodide (Molecular Probes) and analysed by flow cytometry (BD FACScan) to quantify DNA content. Results were analysed with FCS Express 4 Flow software (De Novo software).

2.7 Statistical analysis

All data were analysed using Graph-pad Prism software version 6.0 and presented as Mean±SD. When two groups were compared, an unpaired two-tailed t-test was used. When four groups were compared, two-way ANOVA was used with genotype and cell passage number as factors. When three groups were compared, group data was checked for equality of variances (Brown-Forsythe test) and analysed with one-way ANOVA followed by Bonferroni correction for multiple comparisons. When variances were significantly different, Kruskal-Wallis test was used instead. Results were considered significant at $p < 0.05$.

3. Results

3.1 NRG-1 attenuated stress-induced cellular senescence *in vitro*

We determined the effects of NRG-1 on senescence induced by oxidative stress in cultured vascular SMCs and ECs (fig. 1A). In both cell types, rhNRG-1 significantly decreased the number of SA-β-gal positive cells induced by H_2O_2 (fig. 1B-C). Moreover, NRG-1 also attenuated the increase in cell surface area – another marker of senescence – in the same cells (fig. 1D-E).

Figure 1J-K shows representative Western blot analyses of the expression of cell cycle regulators acetyl-p53 and p21 in both cultured cell types. Densitometric analysis confirmed significant higher expression of both acetyl-p53 and p21 in H_2O_2-treated conditions. This was attenuated by treatment with rhNRG-1 in all conditions, with the exception of p21 in SMCs (fig. 1F-I).

3.2 NRG-1 attenuated vascular senescence in a mouse model of type 1 diabetes

The role of NRG-1 in cellular senescence was further explored *in vivo* using a mouse model of STZ-induced type 1 diabetes. Diabetic C57Bl/6 mice (STZ) showed extensive SA-β-gal positive staining compared to vehicle, which was attenuated when diabetic animals were treated with rhNRG-1 (fig. 2A-B).

To define the cell type(s) evoking this cellular senescence signal, immunofluorescence staining was performed (fig. 2C). Significantly higher expression of acetyl-p53 was observed in both ECs and SMCs of STZ animals compared to vehicles, an increase that was prevented by rhNRG-1 treatment (fig. 2D-E).

Next, we confirmed these immunohistochemistry results with Western blotting of aortic tissues using antibodies against acetyl-p53 and p21. Similar to the immunostaining, NRG-1 prevented the upregulation of acetyl-p53 and p21 protein levels in aortic tissue of diabetic animals (fig. 2F-G). Together, these data indicate that treatment with rhNRG-1 prevents cellular senescence in aortic ECs and SMCs of diabetic mice.

3.3 NRG-1 activates Akt signalling pathway

Figure 2H shows Western blot analysis of expression and phosphorylation of Akt, an important downstream pathway of the ErbB receptors.[41,42] There was significantly higher phosphorylation of Akt in STZ mice treated with rhNRG-1 (fig. 2H).

3.4 NRG-1 has no impact on the diabetic phenotype

Four weeks after STZ treatment, blood glucose levels amounted to 524±63 mg/dl in STZ-treated C57Bl/6 animals and 164±29 mg/dl in the non-diabetic vehicle group. rhNRG-1 treatment did not reduce the blood glucose concentration (481±46 mg/dl) in this experimental setting. Diabetic C57Bl/6 mice showed a reduced body weight at the end of the study (22.42±1.90 g) compared to the non-diabetic animals (28.45±1.90 g). rhNRG-1 treatment did not further reduce the body weight (22.27±1.80 g).

3.5 ErbB4 deficient SMCs show earlier signs of senescence *in vitro*

NRG-1 is a ligand to both ErbB3 and ErbB4 receptors. To locate these in the aorta, we performed immunofluorescence staining (fig. 3A). ErbB4 staining was most pronounced in SMCs whereas ErbB3 staining was most pronounced in ECs (fig. 3A). Because the most prominent NRG-1 receptor in the aorta appeared to be ErbB4 in SMCs, we developed a SMC-specific ErbB4 KO mouse. Mice with a floxed ErbB4 receptor were crossed with transgenic mice expressing Cre-enzyme under a SMC-specific SM22α promotor. We were unable to generate mice homozygous for the floxed ErbB4 in combination with the SM22α-Cre+, indicating complete KO of the ErbB4 gene in SMCs is lethal at the embryonal stage.

We isolated aortic SMCs from ErbB4$^{f/+}$ SM22α-Cre+ mouse, i.e. mice in which one copy of the ErbB4 gene is deleted in SMCs. Western blot analysis confirmed decreased expression of ErbB4 in aortic SMCs of ErbB4$^{f/+}$ SM22α-Cre+ mice (fig. 3B). Immunofluorescence staining also confirmed that ErbB4 expression is significantly reduced in ErbB4$^{f/+}$ SM22α-Cre+ mice (fig. 3C-D).

Next, we isolated and cultured aortic SMCs from ErbB4$^{f/+}$ SM22α-Cre+ mice and ErbB4$^{+/+}$ SM22α-Cre+ control mice. ErbB4$^{f/+}$ SM22α-Cre+ SMCs showed significantly more SA-β-gal positivity at the fifth passage *in vitro*, compared to ErbB4$^{+/+}$ SM22α-Cre+ SMCs at the same passage (fig. 3E-F). In addition, using the Vindelov method we performed cell cycle analysis at passage 1 and passage 5. ErbB4$^{f/+}$ SMCs showed a higher fraction of cells with nuclei in G_1-phase at passage 5 compared to ErbB4$^{+/+}$ SMCs at the same passage (fig. 3G-I), suggestive for a G_1-cell cycle arrest and senescence. No difference in the fraction of cells in G1-phase was seen in the first passage (fig. 3G). An increase in fraction of cells in G1-phase in the ErbB4$^{+/+}$ SMCs was only seen after 14 passages. But, the amount of SA-β-gal positive SMCs in response to H_2O_2 was similar between cells in passage 3 and passage 14. (Data not shown)

Consistent with the *in vivo* results, rat SMCs expressed ErbB4 but not ErbB3 receptors. ErbB4 expression in culture rat SMCs increased in response to NRG-1 treatment but not in response to H_2O_2. (data not shown)

3.6 ErbB4 deficiency in SMCs accelerates vascular senescence in the diabetic mice

Using STZ, we induced diabetes in ErbB4$^{f/+}$ SM22α-Cre+ mice and ErbB4$^{+/+}$ SM22α-Cre+ control littermates. Diabetic ErbB4$^{f/+}$ SM22α-Cre+ mice showed significantly higher cellular senescence in the aorta in comparison to their diabetic wild type littermates (fig. 4A-B).

Immunofluorescence staining of acetyl-p53 (fig. 4C) not only showed significantly more cellular senescence in diabetic ErbB4$^{f/+}$ SM22α-Cre+ mice in comparison with their normal diabetic littermates, but even a significant increase in cellular senescence in non-diabetic ErbB4$^{f/+}$ SM22α-Cre+ in comparison with their normal non-diabetic littermates (fig. 4D).

In addition, mortality was higher in diabetic ErbB$^{f/+}$ SM22α-Cre+ mice compared to diabetic ErbB$^{+/+}$ SM22α-Cre+ mice (fig. 4E). Also, more cardiac hypertrophy was observed in the diabetic ErbB$^{f/+}$ SM22α-Cre+ mice compared to diabetic wild type mice. (Supplementary fig. 1)

In order to rule out other mechanisms of cell death, markers of autophagy and apoptosis in vitro and in vivo were measured (Supplementary fig. 2). Neither autophagy nor apoptosis were induced in these experiments.

3.7 ErbB4 deficiency in SMCs has no impact on diabetic phenotype

Blood glucose levels in type 1 diabetic (STZ) ErbB4$^{f/+}$ SM22α-Cre+ mice were 529±47 mg/dL and 499±38 mg/dL in their diabetic wild type littermates. Baseline blood glucose levels in non-diabetic (Vehicle) mice showed no differences between transgenic and wild type mice (175±5 mg/dL in ErbB4f/+ SM22α-Cre+ mice vs. 172±5 mg/dL in wild type animals). (Supplementary fig.5) Also, diabetes did not change ErbB3 and ErbB4 expression in ECs and SMCs in ErbB4$^{f/+}$ SM22α-Cre+ mice and their wild type littermates (ErbB4$^{+/+}$ SM22α-Cre+). (Supplementary fig. 3)

However, capillary density was increased in hearts of diabetic mice (Supplementary fig. 4) and this increase was attenuated in hearts of ErbB4 deficient mice.

4. Discussion

This study provides evidence for the protective role of NRG-1 in vascular senescence. We demonstrated that rhNRG-1 inhibits oxidative stress-induced cellular senescence in cultured aortic ECs and SMCs. Moreover, aortic SMCs isolated from mice with a SMC-specific deficiency in the NRG-1 receptor ErbB4 (ErbB4$^{f/+}$ SM22α-Cre+) become prematurely senescent as compared to SMCs from wild type littermates. *In vivo*, daily treatment of type 1 diabetic mice with rhNRG-1 for 9 weeks attenuates diabetes-induced vascular senescence. Consistently, diabetes induces significantly more vascular senescence in mice deficient for the ErbB4 receptor in SMCs compared to their wild type littermates.

The NRG-1/ErbB signalling system is important in cardiac development and pathophysiology.[36,43] NRG-1 attenuates development of heart failure in several animal models, including diabetic cardiomyopathy.[37,44–47] Recombinant human NRG-1 (rhNRG-1) is currently being tested in clinical trials as a novel treatment for heart failure.[48,49]

NRG-1 is produced by ECs and exerts its effects through ErbB tyrosin kinase receptors.[50] Although NRG-1 is most abundant in ECs, its ErbB receptors are widely expressed throughout the body, ranging from gastro-intestinal, neuronal, respiratory and cardiovascular system.[51–54] In the vasculature, ErbB receptors are located on ECs as well as on the underlying SMCs. Therefore; endothelium-derived NRG-1 can play its role via a paracrine or autocrine signalling in both cell types. The NRG-1/ErbB signalling system is crucial for vascular responses to stress.[50] ECs produce NRG-1 in response to inflammation, hypoxia and oxidative stress.[55–57] In addition, there are data indicating that NRG-1 has anti-oxidant effects[38,58,59], which could partly explain the protective effects against hydrogen peroxide in our cell culture experiments. NRG-1 is also a proangiogenic factor: it plays an important role in angiogenesis and arteriogenesis after ischaemic injury.[60] NRG-1 regulates endothelial progenitor cell biology by affecting their survival and differentiation capacity.[60,61] Xu and colleagues also demonstrated an anti-atherosclerotic effect of NRG-1 in ApoE−/− mice and showed NRG-1 prevents foam cell formation in primary cultured human monocyte-derived macrophages.[62] Furthermore, NRG-1 reduces neointimal formation after vascular injury in rat, through a decreased vascular SMC proliferation and migration.[63] We add to this evidence demonstrating NRG-1 attenuates EC and SMC senescence, importantly influencing mortality. Two other important cell death mechanisms, autophagy and apoptosis, were not observed during this study in the aorta of diabetic animals *in vivo*, neither *in vitro* experiments.

Effects of NRG-1 on cellular proliferation and apoptosis have been well described and are mainly ascribed to the activation of Akt and MAPK signalling proteins.[29,38] However, no information is available on a potential role in cellular senescence. Interestingly, recent evidence suggests an effect of NRG-1 on ageing.[64] It has been shown that NRG-1 levels and its receptor ErbB4 are sustained throughout the entire life of the long-life naked mole rat, which could be the reason of its continued normal brain function and its concomitant normal activity.[64] Our data adds to this evidence by showing that NRG-1 prevents premature senescence, an important ageing mechanism, both *in vivo* and *in vitro*. However, we were not able to detect significant levels of NRG-1 expression in aortic segments (data not shown), which might indicate that local paracrine signalling might be less important that signalling by circulating NRG-1. The effect of NRG-1/ErbB4 signalling on replicative senescence remains to be elucidated.

In our NRG-1 receptor knockdown model, we chose to knock down the ErbB4 receptor. NRG-1 exerts its effect via three different ErbB tyrosine kinase receptors: ErbB2, ErBb3 and ErbB4. Among these, ErbB4 is the only receptor with both ligand binding domain and tyrosine kinase activity.[65,66] The ErbB2 receptor lacks a ligand-binding domain, while ErbB3 lacks tyrosine kinase activity. Therefore, ErbB2 and ErbB3 need heterodimerisation with other ErbB receptors to exert their effect. In addition, on immunofluorescence staining ErbB4 expression in aorta was more pronounced than ErbB3 expression. Although we did not observe any evidence of tumour formation in this study, one of the earliest findings regarding the important role of NRG-1/ErbB signalling system in the adult human heart, was made in cancer patients treated with an anti-ErbB2 drug (trastuzamab).[67] This finding supports the notion that ErbB2 might have a role in both tumorigenesis and cardioprotection. The exact role of NRG-1 in balancing protective effects and tumour growth enhancing effects have still to be sorted out, but recent evidence indicates that activation of ErbB4 by NRG-1 has cardioprotective effects while activation of ErbB3 by NRG-1 has growth enhancing effects.[68]

Recent evidence supports the idea that cellular senescence plays a role in different cardiovascular diseases, including atherosclerosis, hypertension and heart failure.[8,69–72] Atherosclerosis as a dominant contributor to cardiovascular diseases, can be caused by senescence of ECs, vascular SMCs and their progenitor cells.[70,73–75] Human coronary ECs found on the surface of atherosclerotic plaques and vascular SMCs isolated from atherosclerotic vessels and advanced atherosclerotic plaques display features associated with cellular senescence, such as morphological features of premature senescence, increased SA-β-gal activity, upregulation of cell cycle regulators (p16, p21), decreased telomerase activity and telomere shortening.[7,76–78] As such, attenuation of vascular senescence by NRG-1 might be an important new therapeutic strategy to prevent cardiovascular disease. Here, we studied vascular senescence in larger arteries but not in resistance sized vessels. Therefore, although our findings are relevant for pathophysiological processes such as atherosclerosis, it is unclear if they are also relevant for physiological processes such as blood pressure regulation.

In this study, we did not perform an in-depth investigation on the effect of NRG-1 on cardiac function, but consistent with the literature diabetes led to an increased cardiac hypertrophy as well as increased capillary density.[79–81] The mechanisms by which partial ErbB4 deletion in SMCs could lead to decreased capillary density in the heart is unclear and remains to be elucidated.

Although the animal models used in this manuscript do not show features of atherosclerosis, the present study shows that NRG-1 prevents senescence in vascular ECs and SMCs *in vitro* and also in the vasculature of diabetic animals. Diabetes is the most common age-associated chronic disease with high morbidity and mortality. About half of diabetic patients die due to cardiovascular diseases.[82] The NRG-1/ErbB signalling pathway has been reported to reverse cardiac remodelling observed in diabetic rats.[37,44–46] Hyperglycaemia in type 2 diabetes

leads to p53 activation and myocyte cell death.[83] Another study shows the effect of advanced glycation end-products on the onset of premature epithelial cell senescence in diabetic nephropathy.[84] However, we did not observe significant amounts of cellular senescence in 28 weeks old db/db mice (BKS.Cg-+Lepr^{db}/+Lepr^{db} /OlaHsd), a mouse model of type 2 diabetes (data not shown). In our experimental setting, the induction of diabetes mellitus by STZ is used as a source of endogenous oxidative stress that can induce senescence. The role of oxidative stress in the pathogenesis of vascular abnormalities in diabetes is well-established and oxidative stress is known to induce premature senescence through DNA damage.[85–87] In this study, we did not use the same stimulus for induction of senescence in the *in vitro* and *in vivo* experiments. For the *in vivo* experiments, STZ-injection caused a diabetes model with extremely high circulation glucose levels, independent of insulin secretion.[88] This STZ-model allowed for induction of vascular senescence in a short period of time. Surprisingly, high glucose levels *in vitro* did not induce reproducible cellular senescence in freshly isolated rat aortic SMCs and ECs, however hydrogen peroxide (H_2O_2) consistently did induce premature senescence *in vitro*. Using H_2O_2 to induce senescence in cell culture may have some limitations, as it can cause multiple effects. However, sub lethal doses of H_2O_2 (20-200 μM) are generally used to induce cellular senescence without causing cell death.[10,89–91] In this study, a low dose (30 μm) is used to avoid other forms of cell death such as apoptosis and necrosis.

Indeed our results show premature senescence in STZ-treated mice regardless of genotype. Moreover, partial deletion of the ErbB4 receptor in SMCs led to an increased senescence and mortality in a model of type-1 diabetes. Therefore, by deduction, we propose that inhibition of senescence provides a new window of opportunity to prevent atherosclerosis and its pathophysiology.

5. Conclusion

This study is the first to provide insight into the role of the NRG-1/ErbB pathway in cellular senescence. Activation of NRG-1/ErbB signalling attenuates stress-induced cellular senescence *in vitro* and *in vivo*, whereas inhibition of this pathway causes the opposite. As such our data provide a novel mechanism by which NRG-1 exerts its protective effects. In addition, abating the process of cellular senescence may be an important therapeutic strategy to tackle age-related cardiovascular diseases.

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8. ‘Conflict of Interest: None declared.’

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Figure Legends

Figure 1. NRG-1 attenuated oxidative stress-induced senescence in vascular smooth muscle cells and endothelial cells *in vitro*. A) Rat aortic SMCs and ECs were treated with vehicle, H_2O_2, or H_2O_2 + rhNRG-1. After 72 hours, SA-β-gal staining was performed; SA-β-gal= blue, representative image. Scale bar= 50 μm. B) Quantification of number of SA-β-gal positive SMCs and C) quantification of number of SA-β-gal positive ECs. Cell surface area of D) SMCs and E) ECs was measured as a marker of cellular senescence (Four batches of cells were used). F-K) Western blot analysis for cell cycle regulatory protein ac-p53 and p21, as markers of cellular senescence (n=6, representative images). Mean±SD. One-way ANOVA followed by Bonferroni correction for multiple comparisons; with the exception of Western blot analysis for p21 in SMCs (Kruskal-Wallis test). *= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$. H_2O_2= hydrogen peroxide, SA-β-gal= senescence associated beta-galactosidase, SMCs= smooth muscle cells, ECs= Endothelial cells, rhNRG-1= recombinant human neuregulin-1, ac-p53= acetyl-p53 protein, p21= cyclin-dependent kinase inhibitor 1, p21 protein.

Figure 2. NRG-1 attenuated diabetes-induced senescence in vascular smooth muscle cells and endothelial cells *in vivo*. C57/Bl6 mice were treated with STZ to induce diabetes type I (STZ), and randomized to vehicle (- NRG-1), or hrNRG-1 treatment (+ NRG-1). A) SA-β-gal staining of aortic tissues; SA-β-gal positive= blue, representative image. Scale bar= 10 μm. (n=10) B) Surface area of SA-β-gal normalised to total area. C) Immunofluorescence staining for acetyl-p53 (red), CD31 (ECs, green) on aortic tissue (representative image). Scale bar= 100 μm. D) Percentage of ac-p53 positive SMCs and E) percentage of ac-p53 positive ECs, determined by manual counting. (n=9) Western blot analysis of F) acetyl-p53 and G) p21 H) Akt and phosphorylated Akt (P-Akt) in aortic tissues. Mean±SD. Data of D and E were analysed with one-way ANOVA followed by Bonferroni post hoc test. All other data were analysed using non-parametric Kruskal-Wallis test. *= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$. Ac-p53= acetyl-p53 protein, p21= cyclin-dependent kinase inhibitor 1, p21 protein, SMCs= smooth muscle cells, ECs= Endothelial cells, STZ= streptozotocin, rhNRG-1= recombinant human Neuregulin-1, SA-β-gal= senescence associated beta galactosidases.

Figure 3. Vascular smooth muscle cells with decreased ErbB4 expression showed increased senescence *in vitro*. A) Immunofluorescence staining for CD31 (ECs, green) and ErbB3/ErbB4 (red) in C57/BL6 mice. Scale bar= 50 μm. B) Western blot analysis of ErbB4 isolated aortic SMCs of heterozygote $ErbB4^{f/+}$ SM22α-Cre+ and $ErbB4^{+/+}$SM22α-Cre+ (littermate control) animals (representative image of 3 experiments). C) Immunofluorescence staining for CD31 (ECs, green) and ErbB4 (red) on the aorta of $ErbB4^{f/+}$ SM22α-Cre+ and $ErbB4^{+/+}$ SM22α-Cre+ animals. Scale bar= 50 μm. D) % of ErbB4 positive SMCs in $ErbB4^{+/+}$ SM22α-Cre+ and $ErbB4^{f/+}$ SM22α-Cre+ animals (n=4, two-tailed T-test). E) SA-β-gal staining (blue) of vascular SMCs isolated from $ErbB4^{+/+}$ SM22α-Cre+ and $ErbB4^{f/+}$ SM22α-Cre+ littermates. Scale bar= 50 μm. F) % of SA-β-gal positive SMCs after 5 passage (n=6, two-tailed T-test). G-I) Vindelov method to determine the cell cycle phase in vascular SMCs isolated from $ErbB4^{f/+}$ SM22α-Cre+ compared to $ErbB4^{+/+}$ SM22α-Cre+ littermates. The first peak shows G0/G1 phase of cell cycle and the second peak shows G2 phase of cell cycle. G) SMCs from culture passage 1 of $ErbB4^{+/+}$ SM22α-Cre+ (black line) and $ErbB4^{f/+}$ SM22α-Cre+ (red line). H) SMCs from culture passage 5 of $ErbB4^{+/+}$ SM22α-Cre+ (orange line) and $ErbB4^{f/+}$ SM22α-Cre+ (blue line). I) Graph of data from panel G and H: percentage cells arrested in G0/G1 (n=3). *= $p < 0.05$; **= $p < 0.01$. VSMCs= vascular smooth muscle cells, p1= first passage, p5= fifth passage, SA-β-gal= Senescence associated β-galactosidases, CD31= Platelet endothelial cell adhesion molecule (PECAM-1), β-act= Beta-actin.

Figure 4. Smooth muscle cell specific ErbB4 receptor knockdown mice showed increased vascular senescence during diabetes. A and B) SA-β-gal staining (blue) on aortic tissue from ErbB4$^{+/+}$ SM22α-Cre+ and ErbB4$^{f/+}$ SM22α-Cre+ mice; non-treated (vehicle) and diabetic (STZ) (n=10). Scale bar= 10 μm. Two-way ANOVA was used. C and D) Immunofluorescence staining for CD31 (ECs, green) and ac-p53 protein (red). Comparison of ac-p53 positive SMCs in aorta of ErbB4$^{+/+}$ SM22α-Cre+ animals (control littermates) and ErbB4$^{f/+}$ SM22α-Cre+ animals (n=10). Scale bar= 50 μm. Two-way ANOVA was used. E) ErbB4$^{+/+}$ SM22α-Cre+ diabetic animals (littermate controls, n=22) have a significant higher survival rate compared to diabetic ErbB4$^{f/+}$ SM22α-Cre+ animals (n=30). Log-rank test was used. Mean±SE. *= $p < 0.05$;***= $p < 0.001$.
SA-β-gal= Senescence associated β-galactosidases, ac-p53= acetyl-p53 protein, CD31= Platelet endothelial cell adhesion molecule (PECAM-1), β-act= Beta-actin, SMCs= smooth muscle cells, ECs= Endothelial cells, STZ= streptozotocin, rhNRG-1= recombinant human neuregulin-1.

Figure 1

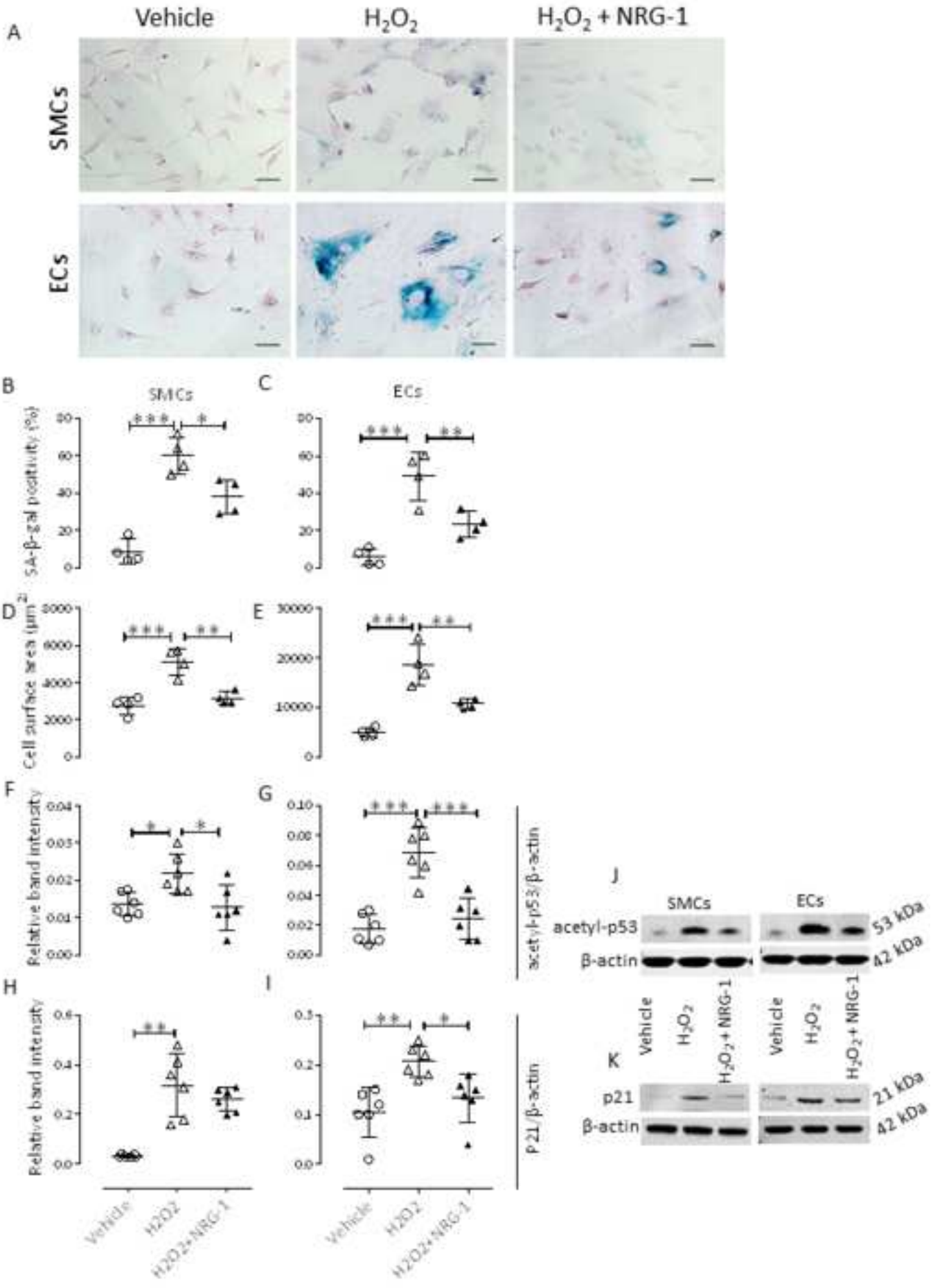

Figure 2

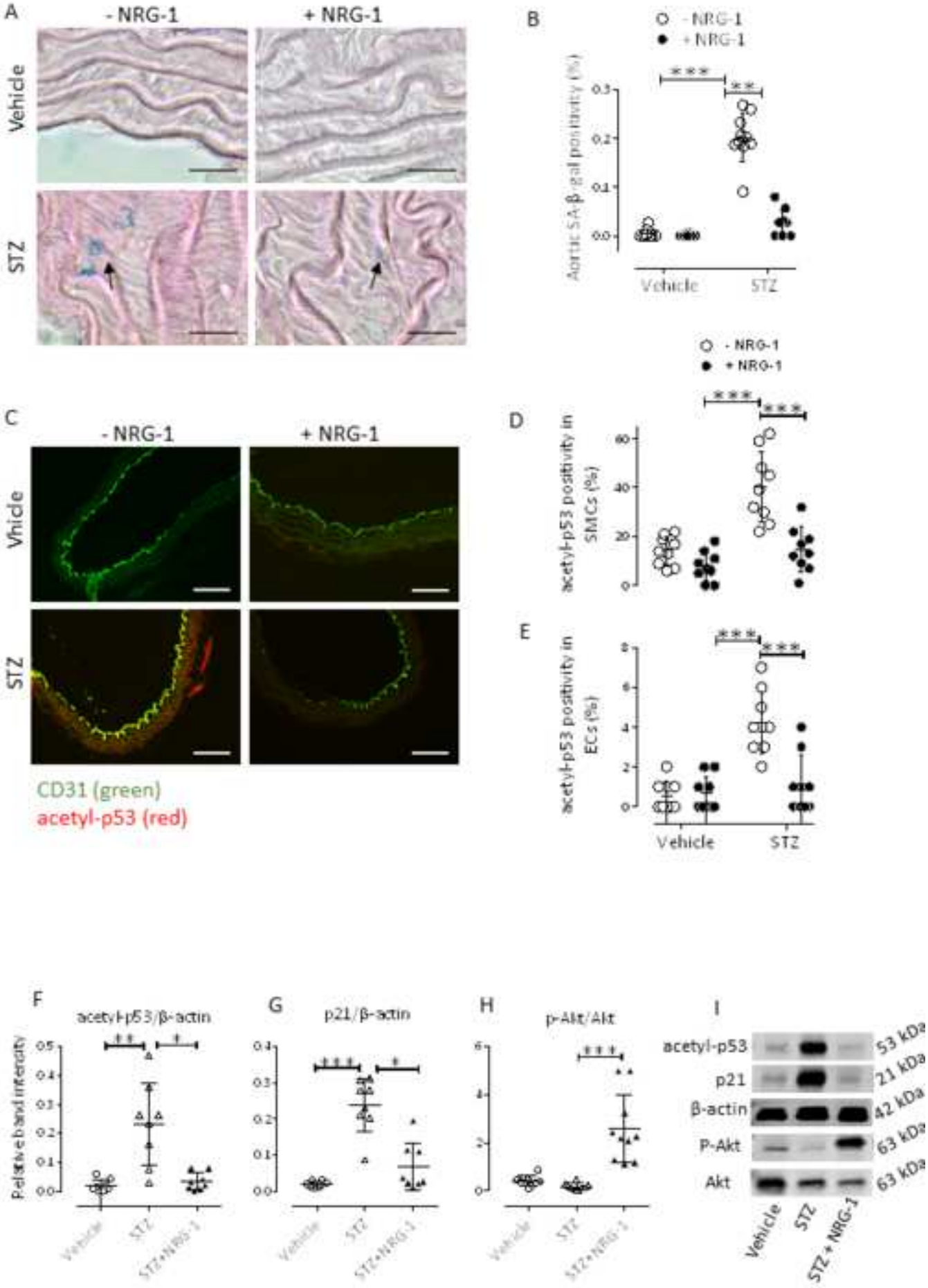

Figure 3

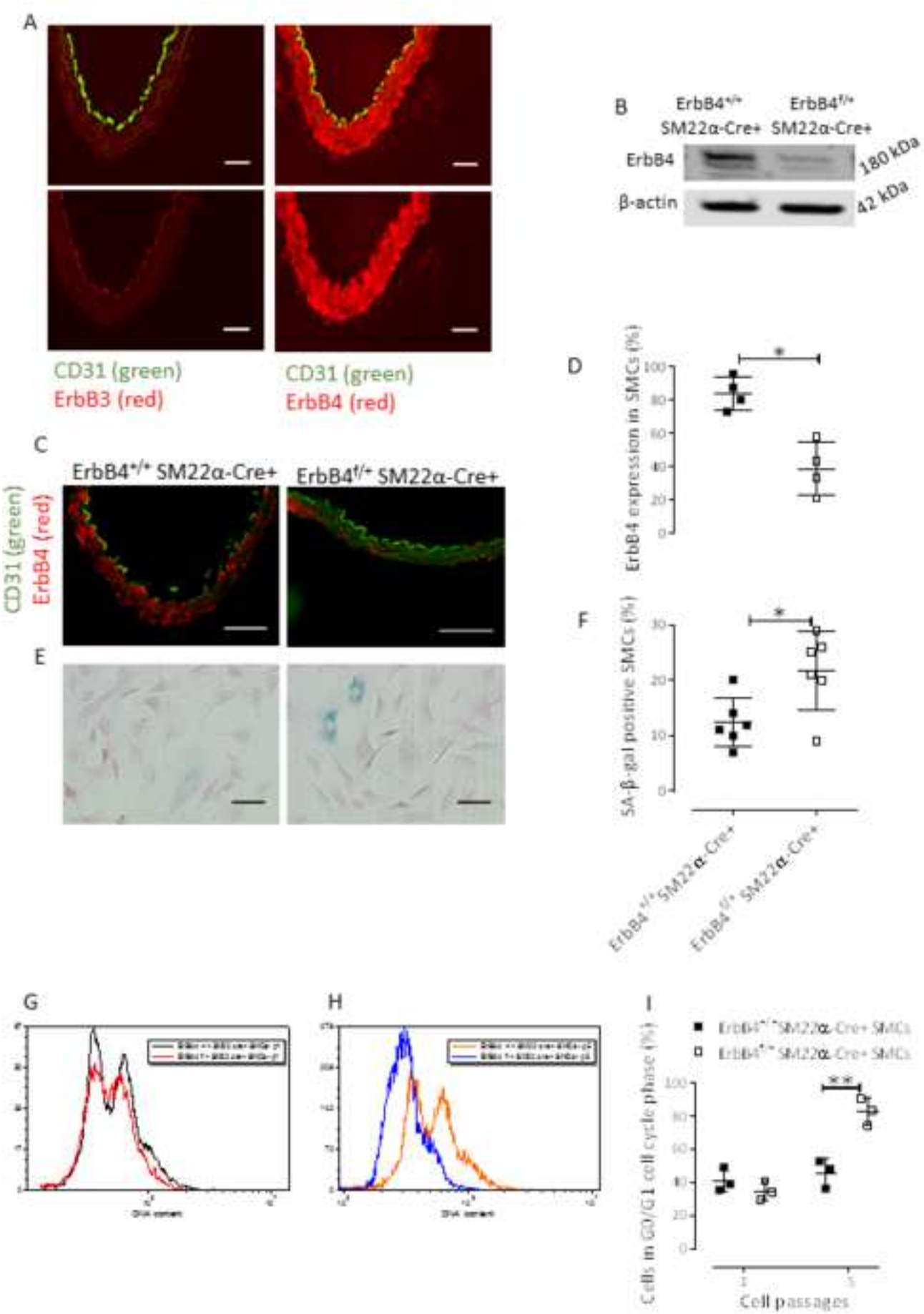

Figure 4