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BRIEF COMMUNICATION

Reprogramming ageing and longevity genes restores paracrine angiogenic properties of early outgrowth cells

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Aims Impaired tissue vascularization is a major determinant of cardiovascular disease (CVD) in the elderly. Accumulation of reactive oxygen species (ROS) may interfere with vascular repair, but the underlying mechanisms remain unknown. Early outgrowth cells (EOCs) play an important role in endothelial repair. We investigated whether key lifespan genes involved in ROS, i.e. the mitochondrial adaptor $p66^{Shc}$ and the AP-1 transcription factor JunD, contribute to age-related EOCs dysfunction in humans.

Methods and results Early outgrowth cells were isolated and cultured from peripheral blood mononuclear cells of young and old healthy volunteers. Early outgrowth cells isolated from aged individuals displayed $p66^{Shc}$ gene up-regulation and reduced JunD expression. Deregulation of $p66^{Shc}$ and JunD in aged EOCs led to up-regulation of NADPH oxidase, reduced expression of manganese superoxide dismutase (MnSOD) and increased O_2^- generation. This was associated with an impairment of EOCs-induced migration of mature endothelial cells. Secretome profiling revealed that angiogenic chemokines such as stromal-derived factor-1 and monocyte chemoattractant protein-1 were deregulated in conditioned medium collected from aged EOCs. Interestingly, $p66^{Shc}$ silencing or JunD overexpression blunted age-related O_2^- production via the NADPH/MnSOD axis, and restored paracrine angiogenic potential of aged EOCs.

Conclusion Reprogramming ageing and longevity genes preserves EOCs functionality by affecting their paracrine properties. These findings set the basis for novel therapeutic strategies to improve for vascular repair after injury and in CVD in the elderly.

Keywords Endothelial progenitor cells • Ageing • Regenerative medicine • Cardiovascular disease • Vascular repair • Angiogenesis

Introduction

Ageing is the main risk factor for cardiovascular disease (CVD) and results in a progressive functional decline of organs and the vasculature.[1] This may explain at least in part why most individuals develop CVD at age 65 and older.[2,3] Particularly, CVDs that depend on tissue neovascularization represent a major medical problem. The incidence of stroke, claudication, and myocardial infarction all increase in elderly patients, and they have worse outcomes when ischaemia and infarction occurs.[4] These clinical observations are mostly explained by the notion that elderly patients have reduced capillary density and impaired angiogenesis in response to ischaemia.[5]

Thus, understanding vascular repair process may unravel novel therapeutic targets to reduce age-related CVD. Angiogenic early outgrowth cells (EOCs) contribute to endothelial regeneration and limit neointima formation after vascular injury.[6,7] Such circulating angiogenic cells—which exhibit phenotypic features of myeloid and endothelial cells—do not themselves cover vascular lesions, but

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facilitate vascular repair in a paracrine manner through the release of angiogenic factors.[8–10] Accumulation of reactive oxygen species (ROS) may affect the functionality of bone-marrow-derived cells with angiogenic potential.[11–13] However, the molecular mechanisms remain poorly understood. We have previously characterized two important modulators of ROS affecting lifespan and CVD,[14,15] i.e. the mammalian adaptor p66^{Shc} and the AP-1 transcription factor JunD. While the former is involved in mitochondrial generation of ROS, vascular senescence, and reduced lifespan in mice,[14,16] the latter protects against vascular ageing by orchestrating the expression of key enzymes involved in redox balance.[15] The present study was designed to investigate whether p66^{Shc} and JunD contribute to age-dependent impairment of EOCs functionality. Specifically, we postulate that reprogramming the expression of such ageing and longevity genes may rescue EOCs-related angiogenic properties in this setting.

Methods

All the experimental procedures are described in detail in Supplementary material online.

Subjects

Young ($n = 10$, 25 ± 5 years) and aged ($n = 10$, 65 ± 6 years) healthy volunteers free of overt CVD were recruited at the University Hospital Zürich, Switzerland and Karolinska University Hospital, Stockholm, Sweden. The study was approved by the local ethics committee and complies with the Helsinki Declaration of 1975, as revised in 2008. Written informed consent was obtained from each participant before inclusion.

Statistical analysis

All data are presented as means $\pm$ SD. Statistical comparisons were made by using the Student's *t*-test for unpaired data and one-way ANOVA followed by Bonferroni's *post hoc* test when appropriate. Probability values of <0.05 were considered statistically significant. All analyses were performed with GraphPad Prism Software (version 6.03).

Results

p66^{Shc} and JunD in early outgrowth cells of aged individuals

p66^{Shc} and JunD play a key role in age-dependent oxidative burst.[14,15] We found that gene expression of p66^{Shc} and JunD was significantly affected by ageing, i.e. the pro-oxidant mitochondrial adaptor p66^{Shc} was up-regulated in aged EOCs, whereas expression of the AP-1 transcription factor JunD was reduced (*Figure 1A*). Altered p66^{Shc}/JunD expression was associated with increased O_2^- generation, as assessed by electron spin resonance spectroscopy (*Figure 1B*). Moreover, aged EOCs were unable to promote migration of mature endothelial cells contrary to young cells (*Figure 1C*).

Genetic reprogramming blunts oxidative signatures in aged early outgrowth cells

To investigate whether p66^{Shc} and JunD contribute to EOCs dysfunction, these genes were reprogrammed in EOCs isolated from aged individuals. Both silencing of p66^{Shc} or JunD overexpression blunted age-driven O_2^- generation by restoring the balance between oxidant and antioxidant enzymes (*Figure 1D–F*). Modulation of p66^{Shc} and JunD increased the expression of manganese superoxide dismutase (MnSOD), while blunting NADPH subunit Nox2 (*Figure 1E* and *F*). This latter finding was confirmed by reduced NADPH activity (*Figure 1G*).

p66^{Shc} and JunD drive age-dependent loss of angiogenic paracrine activity

We next asked whether deregulation of p66^{Shc} and JunD in aged EOCs may affect their angiogenic paracrine properties. Secretome profiling of relevant angiogenic cytokines/chemokines showed a profound alteration of stromal-derived factor-1 (SDF-1), monocyte chemoattractant protein-1 (MCP-1), and interferon-γ (IFN-γ) in conditioned medium collected from old when compared with young EOCs (*Figure 1H*). Noteworthy, reprogramming p66^{Shc} and JunD expression restored SDF-1 levels, while reducing MCP-1 in conditioned medium of aged EOCs (*Figure 1I* and *J*). In contrast, IFN-γ was not affected by p66^{Shc}/JunD reprogramming (see Supplementary material online, *Figure S1*). Interestingly enough, we found that conditioned medium collected from reprogrammed EOCs enabled migration of mature endothelial cells (*Figure 1K*).

Discussion

Here we for the first time show that deregulation of genes involved in longevity and oxidative stress, namely p66^{Shc} and JunD, drive age-dependent impairment of EOCs functionality in humans. Several lines of evidence support our conclusions. (i) The adaptor p66^{Shc} is up-regulated, whereas JunD expression is reduced in EOCs isolated from aged when compared with young individuals; (ii) perturbation of such ageing and longevity genes is coupled to ROS generation and impaired endothelial cell migration; (iii) ROS resulting from p66^{Shc}/JunD deregulation impair endothelial cell migration by altering EOCs-dependent secretion of key angiogenic/inflammatory mediators such as SDF-1 and MCP-1; and (iv) reprogramming p66^{Shc} and JunD significantly suppresses ROS generation and restores paracrine angiogenic properties, thus favouring endothelial cell migration.

Recently, several studies have focused on approaches to regenerate the endothelium and induce vessel formation.[17] Of note, infusion of EOCs reduces neointimal hyperplasia after arterial injury.[6,7] Moreover, EOCs contribute to neovascularization in both ischaemic hind limbs and acute myocardial infarction models.[18] Importantly, low numbers and impaired function of circulating EOCs are associated with endothelial dysfunction and poor cardiovascular outcome.[19] Although the role of EOCs in re-endothelialization and endothelial function is undisputed, the underlying molecular mechanisms remain elusive. Generation of ROS is a key event altering functionality of bone-marrow-derived cells.[12] Thus, we have hypothesized that lifespan determinants involved in oxidative stress might affect vascular healing by altering EOCs angiogenic potential. The adaptor p66^{Shc} is a pro-oxidant enzyme involved in mitochondrial disruption and cellular death.[20] Mice lacking p66^{Shc} gene display prolonged lifespan[16] and are protected against oxidative stress and endothelial dysfunction.[14] In contrast, the AP-1 transcription factor

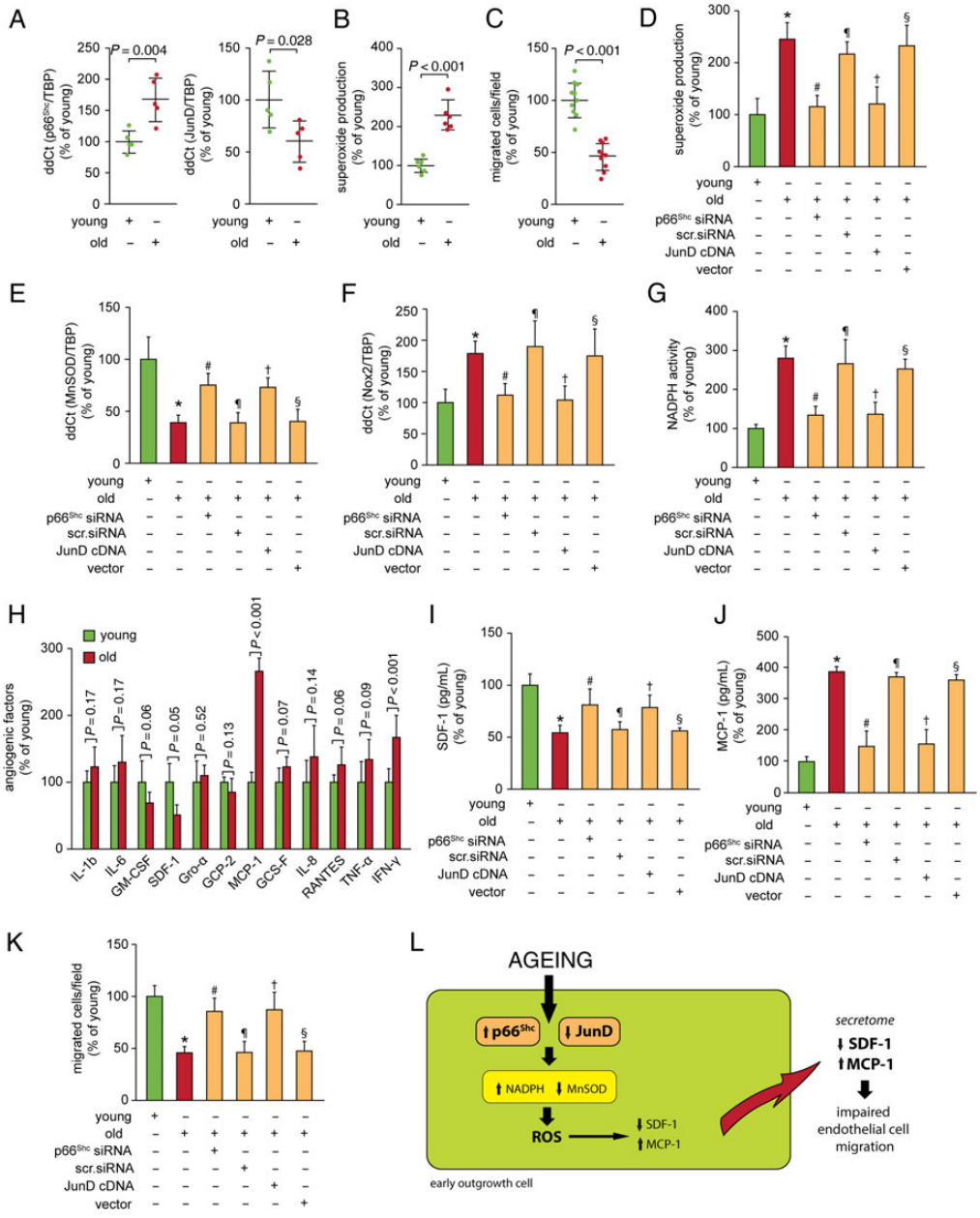

Figure 1 (*A*) Gene expression of the adaptor p66^{Shc} and AP-1 transcription factor JunD in early outgrowth cells isolated from young and aged subjects ($n = 5$ per group). (*B*) Electron spin resonance spectroscopy analysis of superoxide anion generation in young and old early outgrowth cells ($n = 6$ per group). (*C*) Early outgrowth cells-dependent migration of human aortic endothelial cells ($n = 10$ per group). (*D*) Electron spin resonance spectroscopy analysis of superoxide anion generation in young and old early outgrowth cells, in the presence or in the absence of p66^{Shc} knockdown or JunD overexpression. Scrambled siRNA and cDNA vector were used as controls, respectively ($n = 4–6$ per group). *$P < 0.001$ vs. young; $^{\#}P < 0.001$ vs. old; $^{¶}P = 0.003$ vs. p66^{Shc} siRNA; $^{†}P < 0.001$ vs. old; $^{§}P = 0.001$ vs. JunD cDNA. (*E*) Gene expression of manganese superoxide dismutase in aged early outgrowth cells after reprogramming of p66^{Shc} and JunD ($n = 5$ per group). *$P < 0.001$ vs. young; $^{\#}P = 0.004$ vs. old; $^{¶}P = 0.004$ vs. p66^{Shc} siRNA; $^{†}P = 0.008$ vs. old; $^{§}P = 0.012$ vs. JunD cDNA. (*F*) Gene expression of NADPH oxidase subunit Nox2 ($n = 5$ per group). *$P = 0.009$ vs. young; $^{\#}P = 0.040$ vs. old; $^{¶}P = 0.010$ vs. p66^{Shc} siRNA; $^{†}P = 0.015$ vs. old; $^{§}P = 0.024$ vs. JunD cDNA. (*G*) NADPH oxidase activity in the different experimental groups ($n = 5$ per group). *$P < 0.001$ vs. young; $^{\#}P < 0.001$ vs. old; $^{¶}P < 0.001$ vs. p66^{Shc} siRNA; $^{†}P < 0.001$ vs. old; $^{§}P < 0.001$ vs. JunD cDNA. (*H*) Secretome profiling in conditioned medium from young and old early outgrowth cells ($n = 6$). (*I*) Effect of p66^{Shc}/JunD reprogramming on SDF-1 levels in the conditioned medium from young and aged early outgrowth cells ($n = 5$ per group). *$P < 0.001$ vs. young; $^{\#}P = 0.005$ vs. old; $^{¶}P = 0.016$ vs. p66^{Shc} siRNA; $^{†}P = 0.012$ vs. old; $^{§}P = 0.025$ vs. JunD cDNA. (*J*) Monocyte chemoattractant protein-1 levels in the different experimental groups ($n = 5$ per group). *$P < 0.001$ vs. young; $^{\#}P < 0.001$ vs. old; $^{¶}P < 0.001$ vs. p66^{Shc} siRNA; $^{†}P < 0.001$ vs. old; $^{§}P < 0.001$ vs. JunD cDNA. (*K*) Migration of human aortic endothelial cells exposed to conditioned medium from young and old early outgrowth cells , in the presence or in the absence of p66^{Shc}/JunD reprogramming ($n = 10$ per group). *$P < 0.001$ vs. young; $^{\#}P < 0.001$ vs. old; $^{¶}P < 0.001$ vs. p66^{Shc} siRNA; $^{†}P < 0.001$ vs. old; $^{§}P < 0.001$ vs. JunD cDNA. All results are presented as mean ± SD. Symbols refer to Bonferroni-adjusted *P*-values. (*L*) Schematic representing main study findings.

JunD is a longevity gene involved in vascular homeostasis.[15] Indeed, young *JunD*$^{-/-}$ mice display early endothelial dysfunction and vascular senescence.[15] Here we have demonstrated that human ageing is associated with altered expression of p66^{Shc} and JunD, thus favouring O_2^- generation and EOCs dysfunction (a schematic is provided in *Figure 1L*). Importantly, gene silencing of p66^{Shc} or JunD overexpression blunted ROS generation by restoring the balance between oxidant and scavenger enzymes. Mechanistically, ROS induced by deregulation of p66^{Shc}/JunD reduced SDF-1, while increasing MCP-1 in the secretome of aged EOCs. Stromal-derived factor-1 and its major receptor CXCR4 regulate stem cell motility and development. Accordingly, *in vivo* stem cell homing to the bone marrow, their retention, engraftment, and egress to the circulation, all require SDF-1/CXCR4 interactions.[21] Here we show that genes regulating lifespan modulate SDF-1 expression in EOCs, thus affecting endothelial cell migration, a pivotal determinant of vascular healing. On the other hand, we also found that reprogramming of p66^{Shc}/JunD suppressed the levels of MCP-1, which plays a central role in age-related inflammation and mediates an array of events including apoptosis.[22] A possible interpretation of our findings is that high MCP-1 levels in the EOCs secretome may affect viability of mature endothelial cells in a paracrine manner, thus impeding their migration to the site of vascular injury. Furthermore, our study strengthens the notion that inflammation plays a critical role in the etiologic pathway linking ageing and CVD. In line with our results, seminal work has previously demonstrated that accumulation of mitochondrial ROS drive pro-inflammatory transcriptional programmes, thus activating an array of detrimental pathways including the NLRP3 inflammasome, an important activator of cellular death programmes.[23] Ongoing large-scale Phase III trials are now underway with agents that lead to marked reductions in circulating cytokines such as IL-6 and C-reactive protein as well as other inflammatory pathways.[23] Undoubtedly, future mechanistic and translational studies are needed to further explore the role of redox genes p66^{Shc} and JunD in inflammation-driven vascular ageing.

Taken together, our novel findings show that ageing and longevity genes may play an important role in the regulation of EOCs-induced cell migration by modulating oxidative stress during life. An interesting perspective is that deregulation of p66^{Shc} and JunD may represent a common molecular signature linking different cardiovascular risk factors (i.e. diabetes, hypertension, and smoking) to EOCs dysfunction. Hence, targeting ageing and longevity genes may represent a future approach to boost endothelial healing in patients with CVD, regardless of age.

In conclusion, the present study—with potential clinical relevance for regenerative medicine—provides mechanistic insight into the regulation of EOC functionality and identifies novel molecular targets to improve neovascularization in elderly patients with CVD disease.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Authors' contributions

F.P. and S.C. performed statistical analysis and acquired the data. T.F.L. handled funding and supervision. F.P., S.C., and T.F.L. conceived and designed the research and drafted the manuscript. N.K. involved in the cell preparation. N.K. and F.C. made critical revision of the manuscript for key intellectual content.

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Conflict of interest: none declared.

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CARDIOVASCULAR FLASHLIGHT

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Acute myocardial infarction with cardiogenic shock from a left coronary cusp thrombus

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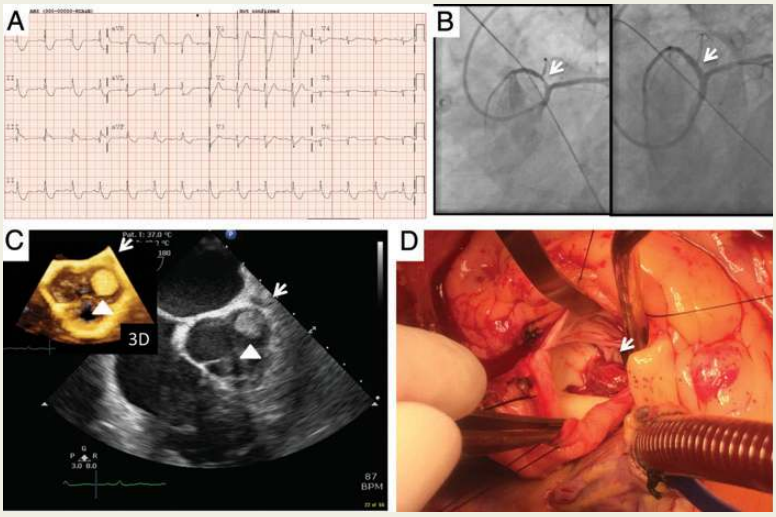

A 36-year-old Chinese male without significant cardiac risk factors, presented with 5 hours of chest pain following an overnight alcoholic binge. Electrocardiogram (ECG) showed widespread ischaemic changes (*Panel A*) and he was sent for primary percutaneous coronary intervention. Clinically he was in cardiogenic shock and initial right femoral artery pressure was 60/40 mmHg. Diagnostic angiography showed a hazy lesion with Thrombolysis In Myocardial Infarction (TIMI) grade 2 flow in the ostial to proximal left anterior descending artery (LAD) with distal embolization to the obtuse marginal branch. Both lesions were wired and intravascular ultrasound showed a thrombotic LAD lesion (*Panel B*) with red thrombus seen on thromboaspiration. A drug-eluting stent was successfully implanted over the LAD lesion. Blood pressure and ECG changes recovered post-procedure. An hour later, the patient developed severe chest pain, hypotension, and similar ECG changes again. Repeat coronary angiography showed a widely patent stent and an intra-aortic balloon pump was inserted. Transthoracic followed by transoesophageal echocardiography demonstrated a 1.2 × 1.2 cm mobile mass attached to the root of the left aortic coronary cusp, close to the left main coronary artery ostium (*Panel C*). He underwent successful surgical excision of the mass (*Panel D*). Subsequent histology demonstrated thrombus without the presence of fibroblasts.

We hypothesize that the mobile thrombotic mass caused intermittent occlusion of the left main coronary artery resulting in intermittent ischaemia with cardiogenic shock. The thrombus observed in the LAD may have been an extension from the thrombotic mass. This case illustrates a very unusual non-atherosclerotic aetiology of myocardial infarction with a favourable outcome.

Panel A: Twelve-lead electrocardiogram demonstrating diffuse ischaemic changes. *Panel B*: A left anterior oblique caudal (spider) view showing hazy proximal left anterior descending artery before (left arrow) and after (right arrow) stent implantation. *Panel C*: 1.2 × 1.2 mobile mass (arrow head) demonstrated with transoesophageal echocardiography with close proximity to the left main coronary artery (arrow). *Panel D*: Surgical excision of thrombus (arrow).