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The *VEGFA*₁₅₆*b* isoform is dysregulated in senescent endothelial cells and may be associated with prevalent and incident coronary heart disease

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Abstract

Coronary heart disease (CHD) is a leading cause of morbidity in people over 65 years of age; >40% of all deaths are due to this condition. The association between increasing age and CHD is well documented; the accumulation of senescent cells in cardiac and vascular tissues may represent one factor underpinning this observation. We aimed to identify senescence-related expression changes in primary human senescent cardiomyocytes and endothelial cells and to relate transcript expression in peripheral blood leucocytes to prevalent and incident CHD in the InCHIANTI study of aging. We quantified splicing factor expression and splicing patterns of candidate transcripts in proliferative and senescent later passage endothelial cells and cardiomyocytes using qRT-PCR. Senescence-associated isoforms also expressed in peripheral blood leucocytes were then examined for associations with CHD status in 134 pairs of age, sex and BMI-matched CHD cases and controls. Splicing factor expression was dysregulated in senescent cardiomyocytes, as previously reported for endothelial cells, as was the expression of alternatively expressed cardiac and vascular candidate genes in both cell types. We found nominal associations between the expression of *VEGFA*₁₅₆*b* and *FNI-E111A* isoforms in peripheral blood mRNA and CHD status. Dysregulated splicing factor expression is a key feature of senescent

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Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Author contribution

E.L. carried out the experiments, analysed the data and contributed to the manuscript. L.C.P. contributed to data analysis and reviewed the manuscript. B.P.L. contributed to data analysis and reviewed the manuscript. S.B. is the curator of the InCHIANTI study and reviewed the manuscript. D.M. contributed to discussion, data analysis and reviewed the manuscript. L.F. organised the sample cohort and contributed to the manuscript. L.W.H. designed and managed the study, interpreted the data and wrote the manuscript.

cardiomyocytes and endothelial cells. Altered splicing of key cardiac or endothelial genes may contribute to the risk of CHD in the human population.

Introduction

Cardiovascular disease is the major cause of mortality and morbidity in the developed world [1,2]. Age is one of the predominant risk factors for this group of disorders, and produces structural, molecular and functional changes in cardiac and vascular tissues [3–5]. The accumulation of senescent cells may be a contributor to cardiovascular pathology, since cells in the myocardium and vasculature are subject to cellular aging [6,7] and have known roles in cardiovascular dysfunction [8]. Senescent cells are one of the major drivers of aging phenotypes; selective removal of such cells from transgenic mice results in rejuvenation and amelioration of multiple aging phenotypes [9,10]. Perfusion of aged myocardium with neonatal cells was able to restore more youthful patterns of gene expression to human cardiomyocyte progenitors or senescent rat myocytes, and these molecular changes were also associated with significant improvements in cardiovascular outcomes [11].

Senescent cells display altered gene expression patterns compared with non-senescent cells [12]. In particular, senescent cells and older organisms display reductions in cellular plasticity and adaptive capacity [13,14], which is in part determined by patterns of alternative mRNA processing. Over 95% of genes express more than one gene product (isoform) under different physiological conditions [15]. Splice-site usage and patterns of alternative splicing are driven by a battery of splicing regulatory proteins termed as splicing factors [16,17]. Some studies have shown that genes encoding splicing factors are strongly dysregulated with aging in population studies and in senescent cells cultured *in vitro*, and are associated with lifespan in mammalian and invertebrate model systems [17–19]. Furthermore, small molecule restoration of splicing factor expression was recently demonstrated to be associated with rescue from multiple senescence phenotypes in aged fibroblasts [20].

In the present study, we aimed to examine senescence-related gene expression changes in human primary cardiomyocytes and endothelial cells, and to relate these changes to cardiovascular risk in a human population. As previously reported for endothelial cells and fibroblasts [17], we found that senescent cardiomyocytes demonstrate some dysregulation of splicing factor expression. We noted changes to the expression of genes with roles in cardiac or endothelial cell function in senescent cells. Both cell types demonstrated different alterations in the expression of senescence-associated secretory phenotype (SASP) proteins. Some deregulated isoforms expression in senescent cardiomyocytes or endothelial cells were also detected in mRNA extracted from human peripheral blood. The expression of the anti-angiogenic vascular endothelial growth factor 165b isoform (*VEGFA_{165b}*) isoform and the alternatively expressed *FN-IEIII*A isoform showed some evidence of a nominal association with prevalent and incident coronary heart disease (CHD) in participants from the InCHIANTI study of aging [21]. This work provides evidence that dysregulation of alternative splicing may alter the transcriptomic output of aged cardiomyocytes or

endothelial cells, and that these changes may be associated with the development of cardiovascular diseases.

Methods

Culture of early passage and senescent cardiomyocytes and endothelial cells

Cultures of non-senescent and senescent endothelial cells and cardiomyocytes were used in the present study. Although the cultures were not from multiple isolates, each culture underwent senescence independently in three biological replicates to produce cultures that were not absolutely identical. Human aortic endothelial cells (HAoEC) and human cardiac myocytes (HCM) were seeded at a density of 6×10^3 cell/cm² and were cultured in specific growth medium (C-22022 for HAoEC and C-22070 for HCM, PromoCell). Earlier passage proliferative cultures were at population doubling (PD) = 24 for endothelial cells and PD = 28 for cardiomyocytes. For the production of senescent cultures, cells were counted and equal numbers of cells seeded at each passage in continuous culture until the growth of the culture slowed to less than 0.5 PD/week. This occurred at PD = 65 and PD = 75 for endothelial cells and cardiomyocytes respectively. Cells were maintained at 37°C and 5% CO₂ and used when not confluent to ensure that cessation of growth was not due to contact inhibition. Cell senescence was assessed in three biological replicates by the biochemical senescence marker senescence-associated β -galactosidase (SA β -Gal), tested in triplicate using a commercial kit (Sigma–Aldrich, U.K.) according to manufacturer's instructions, with a minimum of 300 cells assessed per replicate. Senescence was also quantified in molecular terms by assessing the expression of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene (a known molecular marker of cell senescence) and by changes in cell morphology typical of senescence as in our previous work [17,20]. Total RNA (100 ng) was reverse transcribed in 20 μ l reactions using the Superscript III VILO kit (Life Technologies, Foster City, U.S.A.). *CDKN2A* expression was measured by qRT-PCR relative to three empirically determined endogenous control genes (*GUSB*, *PPIA* and *GADPH*) on the QuantStudio 12K Flex platform (Applied Biosystems, Foster City, U.S.A.). PCR reactions contained 2.5 μ l Taq-Man Universal Mastermix (no AMPerase) (Applied Biosystems, Foster City, U.S.A.), 900 nM each primer, 250 nM probe and 0.5 μ l cDNA in a total volume of 5 μ l. PCR conditions were a single cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Quantification of secretion of SASP cytokines in early passage and senescent cardiomyocytes and endothelial cells

Cells were seeded at 6×10^4 cells per well in a six-well plate and cultured until 95% confluence. Cell supernatants were then harvested and stored at -80°C. Levels of SASP proteins GM-CSF, IFN γ , IL1 β , IL2, IL6, IL8, IL10, IL-12p70, and TNF α were measured in cell supernatants using the K15007B MesoScale Discovery multiplex ELISA immunoassay (MSD, Rockville, U.S.A.) in six biological replicates. Proteins were quantified relative to a standard curve using a Sector Imager SI-6000 according to the manufacturer's instructions. Statistical analysis was carried out with the computer-assisted Prism GraphPad Program (Prism version 5.00, GraphPad Software, San Diego, CA).

Quantification of splicing factor expression in early passage and senescent cardiomyocytes

An *a priori* list of splicing factor candidate genes were chosen on the basis that they were associated with human aging in populations and had been characterised in primary human fibroblasts and endothelial cells in our previous work [17,18]. The list of genes included the positive regulatory splicing factors *SRSF1*, *SRSF2*, *SRSF3*, *SRSF6*, *SRSF7*, *PNSIR* and *TRA2B*, the negative regulatory splicing inhibitors *HNRNPA0*, *HNPNA1*, *HNPNA2B1*, *HNPND*, *HNPNH3*, *HNPMPK*, *HNPMPM*, *HNPMPUL2* and the core spliceosomal factors *AKAP17A*, *LSM2*, *LSM14*, *IMP3* and *SF3B1*. Gene expression was measured as described above, using a custom TaqMan Low Density Array (TLDA) format from Life Technologies (Foster City, U.S.A.) as previously described [17]. Transcript expression was assessed by the Comparative C_t approach, relative to the *IDH3B*, *GUSB* and *PPIA* endogenous control genes and normalised to their expression in RNA from early passage cells.

Quantification of candidate gene expression in early passage and senescent cardiomyocytes and endothelial cells

A panel of candidate genes were selected for analysis based on biological relevance, documented roles in cardiac or endothelial dysfunction and insight into the differential functionality of alternatively expressed isoforms. The identity of genes tested in each cell type and the rationale for their inclusion is given in Table 1. Probes specific to particular isoforms were designed to unique regions of the transcripts in question where possible (assay sequences are available upon request). Assays were validated by standard curve analysis of seven serial 1:2 dilutions of pooled cDNA from cardiomyocytes and endothelial cells. Reverse transcription and qRT-PCR conditions are described above.

Associations between candidate gene expression and CHD in the InCHIANTI population

The InCHIANTI study of aging is a population study of aging [21]. Participants undertook detailed assessment of health and lifestyle parameters at baseline, and again at four subsequent follow-ups (FU2; 2004–2006, FU3; 2007–2009 and FU4; 2012–2014). We selected 268 participants for study for whom we had both an RNA sample at FU3 and clinical information at FU3 and FU4. Participants were categorised according to the presence of CHD as either: (i) no CHD (mean age: 75.9 years, S.D.: 13.4 years, $n=134$), (ii) CHD at least 3 years prior to FU3 (mean age: 78.8 years, S.D.: 7.4 years, $n=45$), (iii) CHD within 3 years before FU3 (mean age: 79.8 years, S.D.: 8.9 years, $n=80$) and (iv) no overt disease at the time of RNA sampling but CHD onset between FU3 and FU4 (mean age: 80.3 years, S.D.: 9.2 years, $n=9$). CHD was defined as the presence of myocardial infarction or angina. Each CHD case was paired with a control participant matched for age (5 year age bins), sex and BMI (four categories; underweight (<18.5), normal (18.5–25), overweight (25–30) and obese (30+)). The mean age of controls was 75.9 years (S.D. = 13.4 years). All human studies have been carried out in accordance with the ethical standards laid out in the Declaration of Helsinki.

Blood was collected into PAXgene tubes (BD Biosciences) at FU3, and extracted using the PAXgene blood RNA kit (Qiagen, Paisley, U.K.). One hundred nanograms RNA reverse

transcribed using the High-Capacity cDNA RT kit (Thermo Fisher, U.K.) and transcript expression levels were quantified as described above. Gene expression data were normalised where necessary prior to analysis, according to the most appropriate transformation metric indicated by the Statistics software (STATA-SE 14).

We used paired logistic regression models to investigate the association between the gene expression levels of the senescence-related alternative isoforms and both prevalent and incident CHD (STATA-SE 14, Statacorp LLC, U.K.). Models were adjusted for smoking (pack-years), study-site and cell counts (% neutrophils, monocytes, basophils, eosinophils). Associations with age were assessed by fully adjusted linear regression. Analyses were performed on the entire cohort, but also stratified by sex. We investigated the effect of multiple testing (Bonferroni correction), but also report suggestive associations with nominal P -value <0.05 .

Results

Senescent cardiomyocytes and endothelial cells demonstrate different senescence characteristics

Senescence reached at PD = 75 and 65 for cardiomyocyte and endothelial cell cultures respectively. Earlier passage proliferative cardiomyocytes and endothelial cell PD times were 4 and 5 days compared with 10 and 12 days in the senescent cultures. Despite the similarities in cell kinetics, the senescent cell percentage for late passage cardiomyocyte cultures was consistently lower than seen in later passage endothelial cells. SA- β -Gal staining in the cultures rose from 12 to 52% for the endothelial cells but from 9 to 30% in the cardiomyocyte cultures (Figure 1A). Differences were also evident in molecular markers of senescence, where we noted a five-fold increase in the expression of *CDKN2A* in senescent endothelial cells but less than one-fold increase in late passage cardiomyocytes (Figure 1B). The SASP of late passage endothelial cells was characterised by significantly more of the signature SASP cytokine IL-6 at senescence, whereas levels of GM-CSF and IL-8 were reduced compared with early passage cells. In contrast, senescent cardiomyocytes unexpectedly displayed significantly reduced expression of many SASP components (Table 2).

Changes in splicing factor expression in senescent cardiomyocytes

We previously reported changes in splicing factor expression in senescent endothelial cells [17]. Here, we demonstrate that similar changes were present in senescent cardiomyocytes (Table 3), although these were less marked than those previously identified in endothelial cells; 7 of the 20 splicing factor transcripts analysed were senescence-associated in cardiomyocytes compared with 9 out of 20 transcripts in senescent endothelial cells [17]. Different splicing factors were affected in late passage cardiomyocytes compared with late passage endothelial cells [17], with only *TRA2B* showing changes in both cell types.

Changes in patterns of alternative splicing are evident in both senescent cardiomyocytes and endothelial cells

We also assessed expression of a panel of *a priori* candidate genes, selected on the basis of key roles in cardiomyocyte or endothelial cell function or dysfunction (Table 1). We saw total expression changes for Catenin β 1 (*CTNNB1*), intracellular cell adhesion molecule (*ICAM*) and vascular cell adhesion molecule 1 (*VCAM*) genes, and also changes for the fibronectin (*FNI*), *ENG*, *IDI*, *VEGFA* and *TJPI* isoforms in senescent endothelial cells. In senescent cardiomyocytes, the expression of *GJA5*, cardiac muscle troponin T (*TNNT2*) and *ATP2A2* isoforms was altered (Table 4). In some cases, the dysregulated expression was limited to one alternatively expressed isoform (e.g. *TNNT2*, *IDI*), while in other cases multiple alternatively expressed isoform was affected (e.g. *VEGFA*, *ENG*, *GJA5*, *ATP2A2*).

Expression of alternative isoforms of the *FN1* and *VEGFA* genes, and changes in *ICAM* expression in peripheral blood may be correlated with CHD status

We first assessed whether genes demonstrating senescence-related expression changes in endothelial cells and cardiomyocytes were expressed in peripheral blood. Of the 11 transcripts that were significantly dysregulated in senescent endothelial cells, 10 (*CTNNB1*, endothelin receptor type B (*EDNRB*), *FN1-EIIIA*, *FN1-EIIIB*, endoglin long form (*ENG(L)*), *ICAM*, inhibitor of DNA binding 1, HLH protein transcript 1 (*IDI(1)*), *VCAM*, vascular endothelial growth factor 121 isoform (*VEGFA₁₂₁*) and *VEGFA_{165b}*) were also expressed in peripheral blood. Of the five transcripts showing dysregulated expression in senescent cardiomyocytes, only two (*ATP2A2-A* and *ATP2A2-B*) were detected in peripheral blood.

We next assessed whether senescence-related changes in the expression or processing of these isoforms could impact on cardiovascular phenotypes by exploring associations between their expression and prevalent or incident CHD (defined as angina or MI) in participants from the InCHIANTI study of aging using paired logistic regression analysis. In 125 paired cases and controls matched for age, sex and BMI, we noted associations between prevalent CHD and expression of the *FN1-EIIIA* isoform and the *VEGFA_{165b}* isoform, although these associations did not make the multiple testing threshold (coefficients: 0.98 and -0.65, S.E.M.: 0.45 and 0.31, $P=0.024$ and 0.039 for *VEGFA_{165b}* and *FN1-EIIIA* respectively; Table 5). Although both these associations were nominal, the positive association between CHD and expression of the *VEGFA_{165b}* isoform was most persuasive (Figure 2A). Only *ATP2A2(B)* was associated with age in peripheral blood (β coefficient: -0.007, SE: 0.003, $P=0.022$).

We then assessed associations between transcript expression and incident CHD. Cases were designated as (i) CHD diagnosed more than 3 years from time of sampling ($n=45$), (ii) incident CHD diagnosed within 3 years from time of sampling ($n=80$) and (iii) future CHD (i.e. no CHD at time of sampling, but CHD incident up to 3 years following sampling) ($n=9$). Controls in each analysis were participants without CHD who did not develop CHD by the final follow-up ($n=134$). Again, we observed associations between *FN1-EIIIA* and *VEGFA_{165b}* expression and CHD status. We also observed a nominal inverse correlation between CHD status and the expression of the *ICAM* gene (coefficient: -0.63, S.E.M.: 0.31,

$P=0.052$) (Table 5). While again not surviving adjustment for multiple testing, the data were most persuasive for an association between *VEGFA*_{165b} expression and CHD (Figure 2B–D), where the expression level of *VEGFA*_{165b} was elevated in 7/8 of the samples from participants who had not yet developed CHD compared with their paired controls, although the numbers in this group ($n=9$) were too small for definitive conclusion (Figure 2D). We also noted sex differences, with *VEGFA*_{165b} and *ICAM* associations being driven by the men (coefficients 1.96 and -1.07 , S.E.M.: 0.75 and 0.49, $P=0.009$ and 0.028 respectively; Table 5) and the *FNI-EIIIA* effect being driven by women (coefficient: -1.02 , S.E.M.: 0.47, $P=0.030$; Table 5).

Discussion

There is rising evidence that some aging phenotypes are driven by the accumulation of senescent cells in multiple tissues [9,10]. Recent data suggests that dysregulated alternative splicing may contribute to the development of senescence [17,18,20,22] and here, we report changes in splicing factor expression and alternative splicing in senescent endothelial cells and cardiomyocytes. Furthermore, the expression of the anti-angiogenic *VEGFA*_{165b} isoform and the embryonic *FNI-EIIIA* isoform in peripheral blood demonstrate nominal associations with CHD in participants from the InCHIANTI study of aging.

The characteristics of senescent cells differ from tissue to tissue. Despite similarities in growth kinetics, late passage endothelial cells cultures show higher senescent cell loads, different expression of SASP proteins and more dysregulation of splicing factors and alternative splicing patterns than do late passage cardiomyocytes. Some of our observations may point to the fraction of growth arrested, but not senescent, cells in the cardiomyocyte cultures being higher, which may partially explain the attenuated senescence characteristics we observe. The endothelial cell SASP is also known to be attenuated in response to DNA damaging agents [23], and more generally, the SASP response in other cell models demonstrates differences in composition depending on the stimulus for senescence [24]. The differences between cell types we identify are perhaps unsurprising, since patterns of gene expression and alternative splicing are understandably highly tissue specific. It is also known that SASP differs from cell type to cell type. Our data suggest that the changes occurring in senescent endothelial cells may have more impact on the aging cardiovascular system than do changes in senescent cardiomyocytes. One caveat of our study is that although our tissue culture work was carried out in three biological replicates, we did not have data from independent isolates of cardiomyocytes or endothelial cells. This is because of the scarcity of well-characterised primary isolates. However, since each of our biological replicates has undergone independent replicative senescence in parallel they are not identical cultures as each will have accrued different mutations and epigenetic changes during the senescence process, although they do of course share a genetic background.

The portfolio of splicing factor changes in senescent endothelial cells and cardiomyocytes is interesting. In our previous work, we found the most dysregulated splicing factors to belong to the HNRP class of regulators, which typically inhibit splice-site usage. In contrast, only a single SR splice activator, *SRSF7*, demonstrated dysregulation at the level of mRNA transcript in senescent endothelial cells [17]. In contrast, senescent cardiomyocytes

demonstrated dysregulation of both *SR* and *HNRNP* transcripts. This may indicate that a higher proportion of aberrant splice-site usage in endothelial cells than it does in cardiomyocytes, although it is difficult however to predict the precise consequences of these changes at the level of individual genes, since each individual splice site is controlled by a unique balance of activators and inhibitors [25].

Our data are consistent with splicing patterns in senescent cardiomyocytes and endothelial cells contributing to age-related cardiac and endothelial dysfunction. We observed dysregulated expression of both *GJA5* isoforms, both *ATP2A2* isoforms and the short isoform of *TNNT2* in senescent cardiomyocytes. *GJA5* encodes a gap junction protein, polymorphisms in this gene have been associated with atrial fibrillation [26,27]. The *ATP2A2* gene encodes a sarcoplasmic/endoplasmic reticulum calcium transporter, which has previously been implicated in chronic heart failure [28]. The *TNNT2* gene encodes cardiac troponin T2, mutations which have been associated with familial hypertrophic cardiomyopathy and stroke [29,30]. In senescent endothelial cells, we noted dysregulated expression of multiple genes, including *FNI*, *VCAM*, *ICAM*, *ENG*, *IDI* and *VEGFA*. *FNI* encodes fibronectin, an important component of the extracellular matrix, isoforms of which have been implicated in thrombosis [31]. *VCAM* and *ICAM* encode cellular adhesion molecules, and have been implicated in vascular inflammation, coronary flow and stroke [32–34]. *ENG* encodes endoglin, a molecule with roles in cardiac remodelling, which has been implicated in myocardial fibrosis [35]. *IDI* encodes an inhibitor of DNA binding, with roles in cardiac cell growth, senescence and differentiation [36]. Finally, *VEGFA* is a key mediator of angiogenesis, which has been implicated in multiple pathologies including CHD [37].

We observed nominal ($P<0.05$) associations between the peripheral blood expression of specific *FNI* and *VEGFA* isoforms and CHD in InCHIANTI study participants. Our data are most supportive of a nominal association of elevated *VEGFA*_{165b} levels in people with CHD, driven by the men in the cohort. *VEGFA* is a secreted protein which undergoes extensive alternative splicing, and encodes isoforms with both angiogenic and anti-angiogenic potential through the differential use of an alternative splice site within exon 8 [38]. Increased expression of the consensus, angiogenesis promoting *VEGFA* isoforms following cardiac ischemia would serve to promote the formation of new blood vessels and allow reperfusion. The anti-angiogenic *VEGFA*_{165b} isoform however, may act to inhibit neovascularisation and impede cardiac recovery. Accordingly, *VEGFA*_{165b} has previously been associated with several cardiovascular phenotypes including coronary artery disease, hypertension, myocardial infarction and peripheral artery disease in humans [39,40]. Elevated levels of *VEGFA*_{165b} have also been documented in ischemic heart tissue from patients who have undergone surgical reperfusion following myocardial infarction [40]. Although the number of patients in our cohort with incident CHD between follow-up 3 and 4 was too small ($n=9$) to be able to draw definitive conclusions, there is observationally a trend towards elevated *VEGFA*_{165b} expression in participants who did not have overt CHD at the time of collection, but who went on to exhibit symptoms. Should this observation be confirmed in a larger study, this would raise the exciting possibility that the expression levels of this isoform in the peripheral circulation could represent a potential biomarker for future CHD. Although less convincing, we also noted a nominal association with the expression of

the *FN1-E111A* isoform with CHD which was predominantly driven by effects in women. This isoform is predominantly embryonic, but has been suggested to play a role in cardiac wound healing responses after MI [41]. If this tenuous association is later confirmed in larger studies, it is possible that the lower levels of expression we note in CHD patients may reflect poorer capacity for repair.

In conclusion, our data are supportive of a model whereby senescent endothelial cells and cardiomyocytes *in situ* may express a different portfolio of alternatively expressed isoforms leading to gradual impairment of cardiac and endothelial function and increased probability of cardiovascular events. Increased expression of alternatively expressed transcripts such as the *VEGFA_{165b}* isoform, may therefore lead to not only progressive cardiovascular remodelling, but also impaired neovascularisation following ischemic events in both the heart and the periphery. Strategies to specifically target splicing events such as those that lead of the production of *VEGFA_{165b}* are now being explored; such as Translarna™, one such splicing therapeutic, was recently licenced for the treatment of Duchenne Muscular Dystrophy. Characterisation of senescence-related splicing events in aging heart and vascular tissues may therefore identify potential new therapeutic targets for future cardiovascular protection.

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Abbreviations

BMI	body mass index
CDKN2A	cyclin-dependent kinase inhibitor 2A
CHD	coronary heart disease
HAoEC	human aortic endothelial cell
HCM	human cardiac myocyte
MI	myocardial infarction
PD	population doubling
SASP	senescence-associated secretory phenotype
VEGFA_{165b}	vascular endothelial growth factor 165b isoform

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Clinical perspectives

- CHD is a leading cause of morbidity and mortality in the Western world. Like many other common chronic diseases, age is a major risk factor for the onset of CHD.
- Cellular senescence is a major driving force for organismal aging. Selective removal of such cells is sufficient to ameliorate multiple aging phenotypes in animal models. Cell senescence is in turn partly driven by dysregulated mRNA processing.
- We identified alterations to splicing regulation and alternative splicing in senescent cardiomyocytes and endothelial cells. The anti-angiogenic *VEGFA_{156b}* isoform of the *VEGFA* gene was seen to be associated with both prevalent and incident CHD. These data suggest that changes to the regulation of key cardiovascular genes may contribute to age-associated risk of CHD.

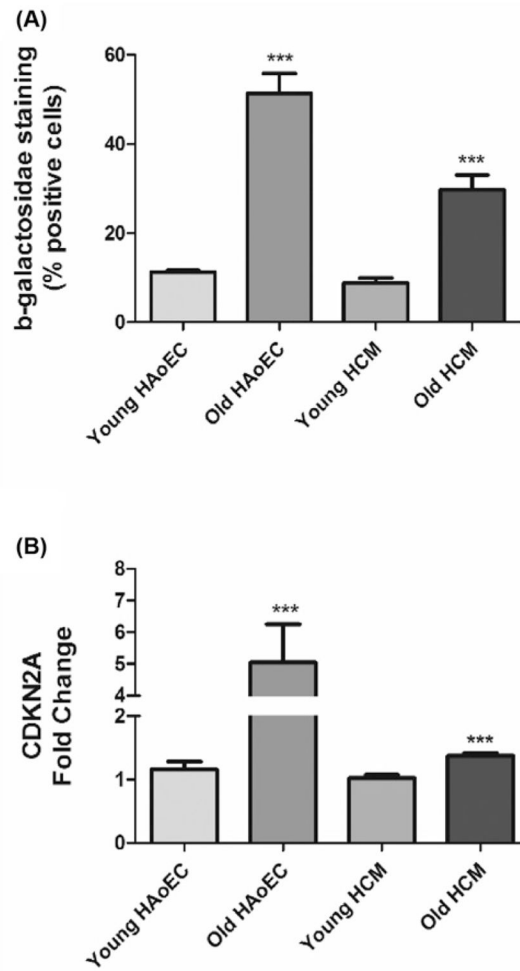


Figure 1. Senescence parameters in cardiomyocytes and endothelial cells

(A) Senescent cell load was assessed by SA β -Gal staining in early and late passage endothelial cells and cardiomyocytes. (B) Senescent cell load was assessed by mRNA expression of the senescence associated transcript of the *CDKN2A* gene. Statistical significance is indicated by stars, ***, $P=0.0001$. Error bars represent \pm S.E.M. of six independent experiments.

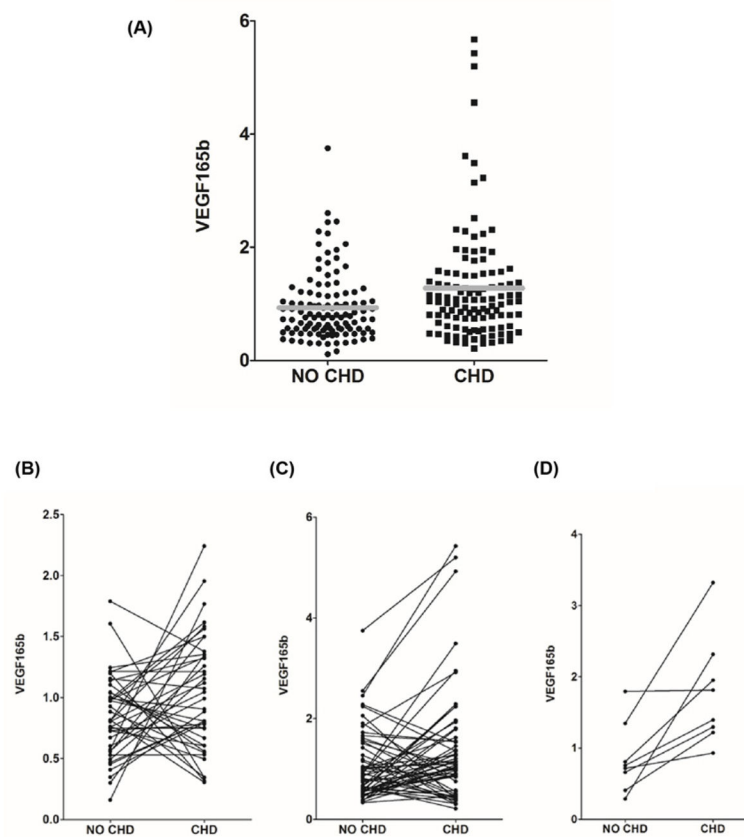


Figure 2. Association of peripheral blood mRNA levels of the anti-angiogenic VEGFA_{165b} isoform and presence of CHD

(A) The relationship between VEGFA_{165b} expression and prevalent CHD. The expression levels of the VEGFA_{165b} isoform of the *VEGFA* gene calculated relative to the level of three endogenous control genes and expressed relative to the median level in the sample cohort is given on the Y-axis. The presence or absence of CHD in the assayed participants at the time of assessment is given on the X-axis. Each black circle represents levels in a single participant without CHD, each black square represents levels in a single participant with CHD. The mean level in participants with and without CHD are indicated by grey bars. (B–D) The relationship between the expression levels of the VEGFA_{165b} isoform and incident CHD. The expression levels of the VEGFA_{165b} isoform calculated relative to the level of three endogenous control genes and expressed relative to the median level in the sample cohort is given on the Y-axis in samples paired for age, sex and BMI. Each pair of linked circles represents a single case and paired control. Category 1: cases with CHD diagnosed more than 3 years prior to the time of sampling (B). Category 2: cases with CHD diagnosed less than 3 years prior to the time of sampling (C). Category 3: individuals who did not have CHD at the time of sampling but who subsequently developed CHD >3 years after the time of sampling (D).

Table 1

Genes and transcripts selected for expression analysis

	Gene name	Transcript accession	Reason for selection
ENDOTHELIAL CELS			
<i>Total expression</i>			
<i>CTNNB1</i>	Catenin β 1	NM 001098209	Key component of Wnt signalling, implicated in several aspects of cardiovascular differentiation and disease
<i>EDNRB</i>	Endothelin receptor type B	NM 000115.3	Involved in cardiac remodelling following myocardial infarction
<i>ICAM-1</i>	Intracellular cell adhesion molecule 1	NM 000201	Key regulators of atherosclerotic plaque development
<i>VCAM-1</i>	Vascular cell adhesion molecule 1	NM 001078	Key regulators of atherosclerotic plaque development
<i>FNI</i>	Fibronectin	NM 001306129	Extracellular matrix protein with roles in atherosclerosis
<i>Alternative isoforms</i>			
<i>FNI(EIIIA)</i>	Fibronectin EIIIA isoform	NM 212482	Mouse isoform specific knockout exhibit cardiovascular defects
<i>FNI(EIIIB)</i>	Fibronectin EIIIB isoform	NM 001306130	Mouse isoform specific knockout exhibit cardiovascular defects
<i>ENG(L)</i>	Endoglin long form	NM 001114753	Negatively regulates angiogenesis and plays a role in heart development
<i>ENG(S)</i>	Endoglin short form	NM 001278138	Positively regulates angiogenesis and plays a role in heart development role
<i>IDI(1)</i>	Inhibitor of DNA binding 1, HLH protein transcript 1	NM 002165.3	Involved in endothelial cell differentiation
<i>IDI(2)</i>	Inhibitor of DNA binding 1, HLH protein transcript 2	NM 181353.2	Involved in endothelial cell differentiation and associated with senescence
<i>VEGFA₁₂₁</i>	Vascular endothelial growth factor 121 isoform	NM 001171628	Pro-angiogenic isoform of VEGFA
<i>VEGFA_{165b}</i>	Vascular endothelial growth factor 165b isoform	NM 001171626	Anti-angiogenic isoform of VEGFA
<i>TJPI(A)</i>	Tight junction protein 1 A isoform	NM 175610.3	Controls angiogenesis and endothelial barrier formation to stabilise cardiomyocyte integrity
<i>TJPI(B)</i>	Tight junction protein 1 B isoform	NM 003257.4	Controls angiogenesis and endothelial barrier formation to stabilise cardiomyocyte integrity
CARDIOMYOCYTES			
<i>Total expression</i>			
<i>GATA4</i>	GATA-binding protein 4	NM 001308093	Cardiogenic transcription factor
<i>Alternative isoforms</i>			
<i>ANKI(L)</i>	Ankyrin-B long isoform	NM 001127493.1	Localisation and membrane stabilisation of ion transporters and ion channels in cardiomyocytes, involved in cardiac arrhythmias
<i>ANKI(S)</i>	Ankyrin-B short isoform	NM 001148.4	Localisation and membrane stabilisation of ion transporters and ion channels in cardiomyocytes, involved in cardiac arrhythmias
<i>GJA5(L)</i>	Gap junction α -5 long isoform	NM 005266	Associated with cardiac arrhythmias
<i>GJA5(S)</i>	Gap junction α -5 short isoform	NM 181703	Associated with cardiac arrhythmias
<i>TNNT2</i>	Cardiac muscle troponin T	NM 001276346	Associated with cardiomyopathy
<i>TNNT2(S)</i>	Cardiac muscle troponin T short form	NM 001001431	Associated with cardiomyopathy
<i>NPPA</i>	Natriuretic peptide A	NM 006172	Low levels associated with atrial fibrillation
<i>NPPA(AS)</i>	Natriuretic peptide A – antisense1	ENST00000446542.5	Negative regulator of NPPA

	Gene name	Transcript accession	Reason for selection
<i>NPPA(S)</i>	Natriuretic peptide A short form	ENST00000376476.1	Alternative isoform of NPPA involved in cardiac development
<i>ATP2A2(A)</i>	Sarco/endoplasmic reticulum Ca ²⁺ -ATPase A isoformy	NM 170665	Involved in cardiac muscle contraction
<i>ATP2A2(B)</i>	Sarco/endoplasmic reticulum Ca ²⁺ -ATPase B isoform	NM 001681	Involved in cardiac muscle contraction

The gene acronym, gene name, transcript accession number(s) and known function of a panel of candidate genes selected for analysis are given above.

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Table 2

Expression of SASP proteins in senescent endothelial cells and cardiomyocytes

	Young endothelial cells	Old endothelial cells	Young cardiomyocytes	Old cardiomyocytes
GM-CSF	10.55 (0.33)	4.55 (0.15)***	3.76 (0.14)	1.60 (0.08)***
IFNγ	3.51 (0.42)	4.19 (0.34)	1.44 (0.19)	0.05 (0.01)***
IL-12	0.51 (0.12)	0.56 (0.13)	0.12 (0.07)	0.08 (0.02)
IL-1β	0.39 (0.14)	0.35 (0.03)	0.41 (0.04)	0.39 (0.05)
IL-2	2.35 (0.42)	1.49 (0.15)	0.44 (0.05)	0.30 (0.06)
IL-6	710.30 (27.1)	1238.58 (46.8)***	2567.7 (275.6)	1682.5 (174.1)*
IL-8	17756.78 (937.3)	3477.4 (355.3)***	334.3 (12.7)	281.8 (12.1)**
IL-10	0.29 (0.04)	0.31 (0.04)	0.47 (0.08)	0.10 (0.02)***
TNFα	0.44 (0.09)	0.55 (0.14)	0.15 (0.03)	0.14 (0.05)

The expression of SASP proteins in early passage cardiomyocytes and endothelial cells compared with those in late passage cells, as determined by multiplex ELISA is given above. The results are expressed as pg/ml and are the mean \pm S.E.M. of six independent experiments. Statistical significance by two-way ANOVA is indicated by stars, with * = 0.05, ** = 0.001 and *** = <0.0001 compared with the young value.

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Table 3

Disrupted splicing factor expression in senescent cardiomyocytes

	Early passage cardiomyocytes	Late passage cardiomyocytes
<i>AKAP17A</i>	1.011 (0.067)	0.970 (0.039)
<i>HNRNPA0</i>	1.025 (0.101)	0.917 (0.030)
<i>HNRNPA1</i>	1.021 (0.092)	1.063 (0.037)
<i>HNRNPA2B1</i>	1.026 (0.105)	1.254 (0.04)
<i>HNRNPD</i>	1.035 (0.143)	1.732*** (0.271)
<i>HNRNPH3</i>	1.033 (0.119)	1.579* (0.170)
<i>HNRNPK</i>	1.025 (0.108)	0.745* (0.037)
<i>HNRNPM</i>	1.015 (0.079)	0.951 (0.028)
<i>HNRNPUL2</i>	1.041 (0.136)	1.031 (0.064)
<i>IMP3</i>	1.016 (0.081)	0.983 (0.035)
<i>LSM14A</i>	1.025 (0.102)	0.817 (0.025)
<i>LSM2</i>	1.009 (0.057)	0.927 (0.163)
<i>PNISR</i>	1.046 (0.143)	1.197 (0.052)
<i>SF3B1</i>	0.996 (0.083)	1.201* (0.028)
<i>SRSF1</i>	1.012 (0.071)	1.054 (0.098)
<i>SRSF2</i>	1.006 (0.047)	0.851* (0.022)
<i>SRSF3</i>	1.015 (0.079)	1.181 (0.155)
<i>SRSF6</i>	1.014 (0.075)	1.265 (0.242)
<i>TRA2B</i>	1.049 (0.146)	0.634* (0.086)
<i>SRSF7</i>	1.015 (0.083)	0.668* (0.089)

The expression profiles of transcripts encoding splicing regulatory factors in early and late passage cardiomyocytes are given below.

Quantifications refer to the level of each splicing factor for each gene, normalised to the geometric mean of the level of each gene in early passage cells. The results are expressed as the mean of six independent experiments. Statistical significance is indicated by stars, with * = 0.05, ** = 0.001 and *** = <0.0001. Figures in parentheses refer to the S.E.M. Transcripts significantly altered in senescent cardiomyocytes are indicated in bold italic type.

Table 4

Disrupted gene expression and patterns of alternative splicing in senescent cardiomyocytes and endothelial cells

	Early passage cells	Late passage cells
ENDOTHELIAL CELLS		
Total expression		
<i>CTNNB1</i>	<i>1.187 (0.074)</i>	<i>2.134 (0.151)***</i>
<i>ENDRB</i>	1.240 (0.132)	1.364 (0.224)
<i>FNI</i>	<i>1.191 (0.083)</i>	<i>2.312 (0.212)***</i>
<i>VCAMI</i>	<i>1.135 (0.089)</i>	<i>6.063 (0.513)***</i>
<i>ICAMI</i>	<i>1.248 (0.205)</i>	<i>3.811 (0.384)***</i>
Alternative isoforms		
<i>FNI(EIHA)</i>	<i>1.186 (0.068)</i>	<i>2.193 (0.180)***</i>
<i>FNI(EIHB)</i>	<i>1.255 (0.155)</i>	<i>2.335 (0.196)***</i>
<i>ENG(L)</i>	<i>1.183 (0.064)</i>	<i>2.471 (0.271)***</i>
<i>ENG(S)</i>	<i>1.139 (0.065)</i>	<i>1.556 (0.156)*</i>
<i>IDI(1)</i>	<i>1.450 (0.133)</i>	<i>1.911 (0.167)*</i>
<i>IDI(2)</i>	1.270 (0.162)	1.044 (0.110)
<i>VEGFA₁₂₁</i>	<i>1.287 (0.199)</i>	<i>2.760 (0.532)*</i>
<i>VEGFA_{165b}</i>	<i>1.172 (0.122)</i>	<i>2.438 (0.369)**</i>
<i>TJPI(A)</i>	1.025 (0.102)	0.921 (0.102)
<i>TJPI(B)</i>	1.270 (0.176)	1.309 (0.158)
CARDIOMYOCYTES		
Total expression		
<i>GATA4</i>	1.584 (0.291)	1.332 (0.157)
Alternative isoforms		
<i>ANKI(L)</i>	1.277 (0.191)	1.477 (0.102)
<i>ANKI(S)</i>	1.463 (0.228)	2.368 (0.393)
<i>GJA5(L)</i>	<i>1.614 (0.346)</i>	<i>2.789 (0.414)*</i>
<i>GJA5(S)</i>	<i>1.687 (0.398)</i>	<i>4.324 (0.810)***</i>
<i>TNNT2(S)</i>	<i>1.464 (0.217)</i>	<i>0.589 (0.074)***</i>
<i>TNNT2</i>	1.930 (0.500)	1.923 (0.225)
<i>NPPA</i>	1.474 (0.196)	1.420 (0.324)
<i>NPPA(AS)</i>	1.343 (0.158)	1.541 (0.223)
<i>NPPA(S)</i>	1.642 (0.348)	1.450 (0.211)
<i>ATP2A2(A)</i>	<i>1.382 (0.064)</i>	<i>2.013 (0.457)*</i>
<i>ATP2A2(B)</i>	<i>1.375 (0.036)</i>	<i>2.113 (0.158)**</i>

The expression profiles of transcripts encoding splicing regulatory factors in early and late passage cardiomyocytes and endothelial cells are given above. Quantifications refer to the level of each transcript for each gene, normalised to the geometric mean of the level of each gene in early passage cells. The results are expressed as the mean of six independent experiments. Statistical significance is indicated by stars, with * = 0.05, ** = 0.001 and *** = < 0.0001. Figures in parentheses refer to the S.E.M. Transcripts showing differential expression in late passage cells are indicated in bold italic type.

Table 5

Associations between the presence of CHD and expression of senescence-related endothelial and cardiomyocyte transcripts in peripheral blood

Transcript	β coefficient	S.E.M.	95% CI	P-value
<i>Prevalent CHD at the time of assessment (125 cases compared with 125 controls)</i>				
<i>CTNNB1</i>	-0.07	0.26	-0.59 to 0.44	0.786
<i>EDNRB</i>	0.26	0.16	-0.04 to 0.56	0.092
<i>FN1(EIIIA)</i>	-0.65	0.31	-1.27 to -0.03	0.039
<i>FN1(EIIIB)</i>	-0.16	0.29	-0.72 to 0.40	0.580
<i>ICAM1</i>	-0.52	0.33	-1.17 to 0.14	0.125
<i>IDV(1)</i>	-0.09	0.20	-0.48 to 0.30	0.655
<i>IDV(2)</i>	-0.16	0.15	-0.45 to 0.14	0.312
<i>ATP2A2(A)</i>	-0.16	0.26	-0.64 to 0.38	0.616
<i>ATP2A2(B)</i>	-0.06	0.25	-0.55 to 0.43	0.810
<i>VCAM1</i>	0.09	0.10	-0.12 to 0.29	0.394
<i>VEGFA₁₂₁</i>	-0.05	0.23	-0.50 to 0.40	0.832
<i>VEGFA_{165b}</i>	0.98	0.44	0.13 to 1.83	0.024
<i>Incident CHD: Controls (n=134), Cases: category 1 (n=45), category 2 (n=80), category 3 (n=9)</i>				
<i>CTNNB1</i>	-0.08	0.24	-0.55 to 0.40	0.758
<i>EDNRB</i>	0.25	0.15	-0.04 to 0.54	0.090
<i>FN1(EIIIA)</i>	-0.55	0.28	-1.10 to -0.01	0.049
<i>FN1(EIIIB)</i>	-0.21	0.28	-0.76 to 0.35	0.465
<i>ICAM1</i>	-0.63	0.32	-1.25 to -0.01	0.046
<i>IDV(1)</i>	-0.06	0.19	-0.44 to 0.32	0.770
<i>IDV(2)</i>	-0.12	0.14	-0.39 to 0.15	0.392
<i>ATP2A2(A)</i>	-0.12	0.26	-0.62 to 0.39	0.647
<i>ATP2A2(B)</i>	-0.08	0.25	-0.57 to 0.41	0.743
<i>VCAM1</i>	0.15	0.10	-0.04 to 0.34	0.131
<i>VEGFA₁₂₁</i>	0.03	0.23	-0.41 to 0.41	0.892
<i>VEGFA_{165b}</i>	0.91	0.41	0.11 to 1.70	0.025
<i>Incident CHD (men only): Controls (n=65), Cases: category 1 (n=21), category 2 (n=41), category 3 (n=3)</i>				
<i>CTNNB1</i>	-1.05	0.54	-2.12 to 0.01	0.052
<i>EDNRB</i>	0.35	0.27	-0.18 to 0.87	0.200
<i>FN1(EIIIA)</i>	-3.91	5.20	-14.11 to 6.28	0.452
<i>FN1(EIIIB)</i>	1.83	1.47	-4.72 to 1.05	0.213
<i>ICAM1</i>	-1.07	0.49	-2.03 to -0.12	0.028
<i>IDV(1)</i>	-0.03	0.29	-0.59 to 0.54	0.927
<i>IDV(2)</i>	-0.44	0.23	-0.90 to 0.02	0.062
<i>ATP2A2(A)</i>	-0.18	0.39	-0.94 to 0.58	0.648
<i>ATP2A2(B)</i>	-0.12	0.40	-0.90 to 0.65	0.757
<i>VCAM1</i>	0.31	0.17	-0.04 to 0.65	0.080
<i>VEGFA₁₂₁</i>	-0.08	0.35	-0.77 to 0.62	0.827

Transcript	β coefficient	S.E.M.	95% CI	P-value
<i>VEGFA_{163b}</i>	1.97	0.75	0.50 to 3.44	0.01
<i>Incident CHD (women only): Controls (n=69), Cases: category 1 (n=24), category 2 (n=39), category 3 (n=6)</i>				
<i>CTNNB1</i>	0.53	0.36	-0.18 to 1.24	0.142
<i>EDNRB</i>	0.23	0.24	-0.23 to 0.70	0.325
<i>FN1(EIIIA)</i>	-1.03	0.47	-1.96 to -0.10	0.030
<i>FN1(EIIIB)</i>	0.20	0.37	-0.51 to 0.92	0.578
<i>ICAM1</i>	-0.30	0.48	-1.25 to 0.65	0.534
<i>IDV(1)</i>	-0.12	0.30	-0.71 to 0.48	0.701
<i>IDV(2)</i>	0.07	0.20	-0.31 to 0.45	0.726
<i>ATP2A2 (A)</i>	-0.17	0.38	-0.92 to 0.57	0.648
<i>ATP2A2 (B)</i>	-0.12	0.33	-0.77 to 0.53	0.724
<i>VCAM1</i>	0.10	0.14	-0.18 to 0.38	0.489
<i>VEGFA₁₂₁</i>	0.09	0.31	-0.53 to 0.70	0.777
<i>VEGFA_{163b}</i>	0.19	0.55	-0.89 to 1.26	0.735

Associations between transcript expression and CHD assessed by adjusted paired logistic regression analysis are given above. CHD is defined as the presence of angina or myocardial infarction. Cases are divided into category 1 (individuals with CHD diagnosed more than 3 years prior to the time of sampling), category 2 (individuals with CHD diagnosed less than 3 years prior to the time of sampling) and category 3 (individuals who did not have CHD at the time of sampling but who subsequently developed CHD more than 3 years after the time of sampling). Significant associations are given in bold italic type. Abbreviations: 95% CI, 95% confidence interval.