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ORIGINAL ARTICLE

Old blood from heterochronic parabionts accelerates vascular aging in young mice: transcriptomic signature of pathologic smooth muscle remodeling

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Abstract Vascular aging has a central role in the pathogenesis of cardiovascular diseases contributing to increased mortality of older adults. There is increasing evidence that, in addition to the documented role of cell-autonomous mechanisms of aging, cell-nonautonomous mechanisms also play a critical role in the regulation of vascular aging processes. Our recent transcriptomic studies (Kiss T. et al. *Geroscience*. 2020;42(2):727–748) demonstrated that circulating anti-geronic factors from young blood promote vascular rejuvenation in aged mice. The present study was designed to expand upon the results of this study by testing the hypothesis that circulating pro-geronic factors also contribute to the genesis of vascular aging phenotypes. To test this hypothesis, through heterochronic parabiosis, we determined the extent to which shifts in the vascular transcriptome (RNA-seq) are modulated by the old systemic environment. We reanalyzed existing RNA-seq data, comparing the transcriptome in the aorta arch samples isolated from isochronic parabiont aged (20-month-old) C57BL/6 mice [A–(A); parabiosis for 8 weeks] and young isochronic parabiont (6-month-old) mice [Y–(Y)] and also assessing transcriptomic changes in the aortic arch in young (6-month-old) parabiont mice [Y–(A); heterochronic parabiosis for 8 weeks] induced by the presence of old blood derived from aged (20-month-old) parabionts. We identified 528 concordant genes whose expression levels differed in the aged phenotype and were shifted towards the aged phenotype by the presence of old blood in

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young Y–(A) animals. Among them, the expression of 221 concordant genes was unaffected by the presence of young blood in A–(Y) mice. GO enrichment analysis suggests that old blood-regulated genes may contribute to pathologic vascular remodeling. IPA Upstream Regulator analysis (performed to identify upstream transcriptional regulators that may contribute to the observed transcriptomic changes) suggests that the mechanism of action of pro-geronic factors present in old blood may include inhibition of pathways mediated by SRF (serum response factor), insulin-like growth factor-1 (IGF-1) and VEGF-A. In conclusion, relatively short-term exposure to old blood can accelerate vascular aging processes. Our findings provide additional evidence supporting the significant plasticity of vascular aging and the existence of circulating pro-geronic factors mediating pathological remodeling of the vascular smooth muscle cells and the extracellular matrix.

Keywords Heterochronic parabiosis · Aging · Vascular aging · Transcriptome · Aneurysm · Atherosclerosis · Aorta

Introduction

Diseases of the large arteries (including aorta aneurysm and large vessel atherosclerosis and its complications [e.g., ischemic stroke, ischemic heart disease]) are among the most common causes of serious long-term disability and cardiovascular mortality among older adults in the developed world [1]. These diseases account for approximately one-third of all deaths in the United States in those over 65 and nearly two-thirds of all deaths in those over the age of 85 [2].

Epidemiological studies demonstrate that the impact of traditional risk factors (including high blood pressure, hypercholesterolemia, tobacco smoking, obesity, diabetes mellitus, etc.) on the incidence of the aforementioned large vessel diseases are dwarfed by the single most important risk factor for these vascular pathologies: advanced aging [1]. In order to develop novel approaches for the prevention and treatment of age-related large vessel pathologies, it is essential to elucidate the contribution of shared biological mechanisms of aging to the functional and phenotypic changes that are manifested in the old vasculature [3, 4]. Previous studies have characterized several important cell-autonomous mechanisms that drive functional decline in the aging vasculature [1], including mitochondrial dysfunction [5, 6], increased oxidative stress [1, 7–17], impaired production and bioavailability of NO [11, 18–20], cellular NAD+depletion, and energetic dysfunction [5, 21–24] and dysregulation of sirtuin pathways [8, 25].

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Over the past decade, major advances in the field of geroscience, coupled with advances in animal model development and multiomics technologies, have led to explosive growth in biological information that implicates cell-nonautonomous mechanisms as also playing critical roles in driving organismal aging processes and pathogenesis of age-related diseases [26–31]. It was also recognized that aging is characterized by complex alterations of inter-organ communication, which also modulate cell-autonomous processes of aging in the cardiovascular system, exacerbating the genesis of vascular pathologies [1]. Accordingly, there is growing evidence that there are factors present in the circulation, which are derived from other organ systems (including the adipose tissue, the endocrine system, the gastrointestinal tract, the central nervous system, the immune system) and regulate cellular aging processes in the vasculature. The view has emerged that there are multiple circulating anti-geronic factors extant, which can reverse or prevent the genesis of cardiovascular aging phenotypes and whose production declines with age [4].

Parabiosis is a surgical procedure for joining two animals to allow for blood exchange via their shared circulatory systems [32–35]. By comparing heterochronic (young–old) and isochronic (young–young and old–old) parabiont pairs of animals [29, 31, 34–55], it can be investigated how circulating (humoral and/or cellular) factors derived from one animal contribute to the genesis of aging phenotypes in various organ systems in the other parabiont. Using heterochronic parabiosis in mice as an experimental tool, our recent studies [54] provided critical evidence that the presence of young blood derived from young parabionts significantly improves endothelium-dependent vasorelaxation and attenuates the production of reactive oxygen species in vessels of heterochronic parabiont aged mice. Using RNA-seq, we also assessed transcriptomic changes in the aortic arch associated with aging and heterochronic parabiosis [54]. We have identified 212 discordant genes whose expression levels differed in the aged phenotype but have shifted back toward the young phenotype by the presence of young blood in aged heterochronic parabiont animals [54]. Pathway analysis suggested that vasoprotective effects conferred by young blood include mitochondrial rejuvenation. On the basis of the transcriptomic changes, insulin-like growth factor-1 (IGF-1) has been identified as a putative circulating anti-geronic factor that contributes to the vasoprotective effects mediated by exposure to young blood [54]. This finding accords with the results of earlier studies, which demonstrated that circulating levels of IGF-1 decline in older adults [56] and aged mice [27]. Importantly, IGF-1 exerts multifaceted vasoprotective effects, maintaining a youthful vascular phenotype and function [27, 56–63].

Experiments using heterochronic parabiosis also suggested that in addition to anti-geronic factors, there are also pro-geronic factors present in the circulation that can accelerate cellular aging processes. The production of circulating pro-geronic factors increases with age and they mediate deleterious cell-nonautonomous effects in multiple organs, including the skeletal muscle, central nervous system, and the heart [29, 39, 47, 48, 50, 52, 64].

The present study was designed as a follow-up investigation to test the hypothesis that age-related changes in circulating pro-geronic factors also contribute to vascular aging. To test this hypothesis, we reanalyzed our previously published transcriptomic dataset [54] assayed from the aortas of heterochronic (young–old) and isochronic (young–young and old–old) parabiont pairs of experimental mice. We determined to what extent, if any, transposition of aging phenotypes could be observed in the young aorta by exposure to an old systemic environment.

Methods

Animals and parabiosis surgery

To elucidate the effects of old blood on the vascular transcriptome, in the present study we reanalyzed our previously published transcriptomic dataset [54] assayed from the aortas of heterochronic (young–old) and isochronic (young–young and old–old) parabiont pairs of experimental mice. Here we provide a short description of the experimental procedures based on the description of the methods in our original publication [54]. As previously described [54], young (4-month-old) and aged (18-month-old) male C57BL/6 mice were obtained from the aging rodent colony maintained by the National Institute on Aging at Charles River Laboratories (Wilmington, MA). Mice were housed under specific pathogen-free conditions at the rodent barrier facility at Albert Einstein

College of Medicine under a controlled photoperiod (12 h light; 12 h dark) with unlimited access to water and were fed a standard chow diet (ad libitum). Parabiosis surgery in young and aged animals was carried out by the Einstein Health Span Core [65], according to published protocols [66, 67] as reported previously [30]. Surgical unions were performed between young animals (isochronic; young Y–(Y); $n=4$ pairs), aged animals (isochronic old; A–(A); $n=4$ pairs), and young and aged mice (heterochronic Y–(A) and A–(Y); $n=5$ pairs) as described [30]. Following surgery, animals were kept on a partial heating pad overnight. Pairs were then intensively monitored and received subcutaneous (s.c) injections of Banamine (2 mg/kg each) immediately post-op and twice a day for three days and then once daily for four days. Animals also received 1 mL of Ringer's lactate (s.c.) immediately after, daily for three days post-op to prevent dehydration. Animals remained joined for ~ 8 weeks prior to sacrifice. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Albert Einstein College of Medicine and the University of Oklahoma Health Sciences Center. All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

RNA isolation, cDNA synthesis, library construction, and next-generation sequencing

RNA was isolated from the aortic arch samples using AllPrep DNA/RNA Mini Kit (Qiagen) as previously described [68, 69]. Prior to 3′-tag RNA-seq analysis quality control measures were implemented. The concentration of RNA was ascertained via fluorometric analysis on a Thermo Fisher Qubit fluorometer. The overall quality of RNA was verified using an Agilent Tapestation instrument. Following initial QC steps sequencing libraries were generated using the Lexogen Quantseq FWD library prep kit according to the manufacturers' protocol by the Clinical Genomics Core of the Oklahoma Medical Research Foundation [70]. Briefly, the first strand of cDNA was generated using 5′-tagged poly-T oligomer primers. Following RNase digestion, the second strand of cDNA was generated using 5′-tagged random primers. A subsequent PCR step with additional primers added the complete adapter sequence to the initial 5′ tags, added unique indices for demultiplexing of samples, and amplified the library. Final libraries for each sample were assayed on the Agilent Tapestation for appropriate size and quantity. These libraries were then pooled in equimolar amounts as ascertained via fluorometric analyses. Final pools were absolutely quantified using qPCR on a Roche LightCycler 480 instrument with Kapa Biosystems Illumina Library Quantification reagents. Sequencing was performed using custom primers on an Illumina NextSeq 500 instrument with High Output chemistry and 75 bp single-ended reads.

RNA-seq data analysis and visualization

Raw sequencing reads were trimmed of their Illumina TruSeq adapter sequences using *Trimmomatic v0.35* [71], filtered for contaminants of ribosomal, mitochondrial, and hemoglobin transcripts, then aligned to the mouse genome version GRCm38 using *Kallisto v0.43.03* [72]. Samples were checked for outliers and separation by principle components analysis (PCA) with the R function *prcomp*. Raw expression counts were summarized at the gene level to transcript-length adjusted, library-size scaled counts per million (CPM) with the R/Bioconductor package *tximport* [73]. Differential expression analysis was performed using the empirical Bayes approach implemented in the R/Bioconductor package *DESeq2* [74]. Significantly differentially expressed (DE) genes had an absolute log2 fold change ≥ 0.585 (corresponding to a change of 50% or more in the expression) and the False Discovery Rate FDR-adjusted p-value ≤ 0.05. Gene annotation was done using *biomaRt* [75] in the R/Bioconductor package. The R package *pheatmap v1.0.12* was used to perform hierarchical clustering and to generate the heat maps. The *org.Mm.eg.db v3.8.2* R/Bioconductor package was used to collect Gene Ontology, KEGG, and Reactome terms associated with the DE (differentially expressed) genes. The same package was used to translate Ensemble IDs to Entrez IDs when it was required by the statistical packages.

Functional annotation

To collect gene annotation data the g:Profiler service version e102_eg49_p15_e7ff1c9 was used via the R interface and with fdr correction method applying significance threshold of 0.05 [76]. The results were plotted using the ggplot2 v3.3.5 package [https://ggplot2.tidyverse.org] in the R v4.1.1 environment and the EnrichmentMap v3.3.3 tool [77] in the Cytoscape v3.8.2 environment [78].

To identify upstream regulators that potentially explain the observed gene expression changes in our samples we used upstream regulator analysis (URA) algorithm [79] implemented in the ingenuity pathway analysis (IPA; QIAGEN) software [79]. IPA is a commercial software package most commonly utilized to intersect DE genes with known biological functions and pathways maintained in the ingenuity knowledge base, a collection of nearly 5 million experimental findings manually curated from either literature or third-party databases. Detailed information can be found at http://qiagen.force.com/KnowledgeBase/.

Gene set enrichment analysis

Gene set enrichment analysis was performed using the fgsea v1.18.0 package implemented in R [80]. Results were plotted in ggplot2.

Results

Exposure to old blood mimics age-related changes in vascular mRNA expression profile

By re-analyzing the previously collected transcriptomic data [81] data we assessed transcriptomic changes in the aorta arch associated with aging and exposure to aged blood via heterochronic parabiosis. Biological replicates from the isochronic parabiosis mice [Y–(Y) and A–(A)] exhibited distinct clustering by PCA, suggesting distinct aortic transcriptomes (Fig. 1A). Transcriptomic profiles of aortas of young mice exposed to aged blood [Y–(A)] clustered between the isochronic pairs in the PCA, suggesting old blood shifted the aortic transcriptome of young mice toward an aged phenotype. In our original publication of the transcriptomic data [54] we documented the presence of an outlier (Y–(A) sample #3 in the original dataset). This sample was excluded from the present re-analysis of the dataset.

In Fig. 1B a heat map is shown as a graphical representation of normalized expression values of genes that are differentially expressed both in aorta samples derived from isochronic parabiont aged mice [A-(A)] and heterochronic parabiont young mice [Y–(A)] as compared to those in aorta samples obtained from isochronic parabiont young mice [Y–(Y)]. Hierarchical clustering analysis reveals groups of genes whose expression is similarly up- or down-regulated in aortas of both A–(A) mice and Y–(A) mice relative to Y–(Y) mice but is unaffected by exposure to young blood in aged mice [A–(Y)].

We then determined the number of genes that were significantly upregulated or downregulated ("differentially expressed"; DE; fold-change ≥ 1.5 or ≤ 0.67; $p < 0.05$ adjusted for multiple comparisons) in the aorta by aging or by heterochronic parabiosis. We then filtered for genes that are significantly altered (adjusted $p < 0.05$), expressed at an appreciable level (fragments per kilobase of transcript per million mapped reads > 1). There were 347 DE genes in A–(A) animals compared to Y–(Y) controls [81]. We further identified 39 DE genes in Y–(A) mice compared to A–(A) controls. In Fig. 1C a volcano plot shows statistical significance (*p*-value) versus magnitude of age-related change in gene expression. Red symbols denote genes, whose expression levels significantly differed in Y–(A) mice.

We realized that significance cut-offs to identify DE genes shared between the age-effect [A–(A) vs. Y–(Y)] and old blood-effect [Y–(A) vs. Y–(Y)] datasets might be too stringent with the result that the analysis in Fig. 1C might miss concordant patterns of gene expression with important biological relevance for old blood-induced accelerated vascular aging and age-related vascular pathologies. Thus, we also used a slightly relaxed approach to detect concordant transcriptional patterns (here termed "pro-geronic shifts") by comparing the age-effect and old blood-effect gene expression datasets using combination criteria that took into account the effect direction (Fig. 2). Specifically, genes were ranked by their effect size direction and ranked lists were compared to identify overlapping genes across a continuous significance gradient. Genes with "pro-geronic shifts" were defined as concordant genes that (1) were DE based on both *p*-value and fold-change criteria either in the age-effect or the old-blood-effect comparison, (2) satisfied a fold-change criterion with a cutoff of ≥ 1.5 or ≤ 0.67.

in the comparison in which the statistical significance criterion was not met, and (3) satisfied the criterion that the effect directions of the age-effect

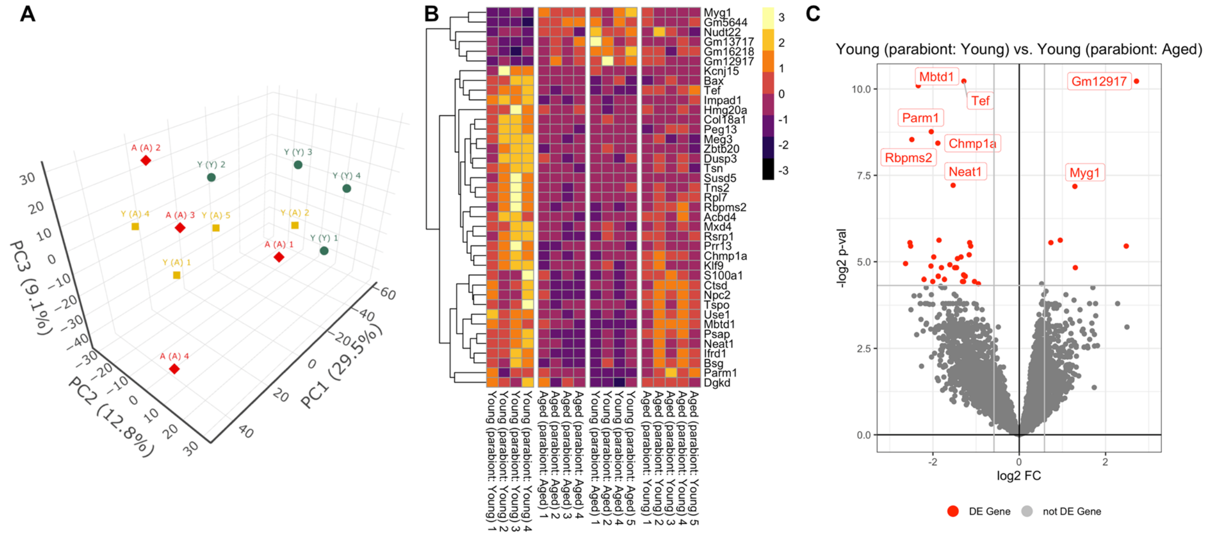


Fig. 1 Old blood induces aging-like changes in vascular mRNA expression profile Panel A: Principal component analysis (PCA) plot of mRNA expression profiles in aorta samples derived from isochronic parabiont young mice [Y–(Y)], isochronic parabiont aged mice [A–(A)] and heterochronic parabiont young mice [Y–(A)*]*. The profiles from Y–(Y) mice (green) cluster separately from clusters representing A–(A) mice (red) and Y–(A) mice (yellow) in the space of the first three principal components. The A–(A) and Y–(A) expression profiles were more similar and clustered less discriminately in the PCA, indicating the impact of old blood on the aorta transcriptome in young mice. PC1, PC2, and PC3: Principal components 1, 2, and 3, respectively. Panel B: The heat map is a graphic representation of normalized expression values of genes that are differentially expressed both in aorta samples derived from isochronic parabiont aged mice [A–(A)] and heterochronic parabiont young mice [Y–(A)] as compared to those in aorta samples obtained from isochronic parabiont young mice [Y–(Y)]. The expression values for these genes in aorta samples obtained from heterochronic parabiont aged mice [A–(Y)] are also shown. Hierarchical clustering analysis reveals groups of genes whose expression is similarly up- or down-regulated in aortas of both A–(A) mice and Y–(A) mice but is unaffected by the presence of young blood in A–(Y) mice. Panel C: Volcano plot depicting differentially expressed genes comparing aortic samples derived from Y–(Y) and Y–(A) mice. Stratified *p*-values are plotted against expression fold changes for results obtained in Y–(A) samples normalized to Y–(Y) samples. Colored points refer to genes whose expression is significantly altered in Y–(A) mice

and old blood-effect are the same. We found that these combination criteria found more biologically meaningful sets of genes showing pro-geronic shift ($n = 528$) than the analysis requiring concordant genes meet both fold change and statistical significance criteria (Fig. 2A, blue dots; Fig. 2B, three internal intersections in gray). These data suggest that changes in circulating pro-geronic factor(s) contribute to age-related dysregulation of vascular gene expression.

Old blood induces aging-like transcriptomic changes by regulating genes expressed in endothelial cells and smooth muscle cells in the mouse aorta

Next, we aimed to determine whether old blood factors promote aging-like gene expression changes in endothelial cells (EC) or vascular smooth muscle cells (SMC). To identify genes that are enriched in endothelial cells and SMCs, we reanalyzed single-cell data RNA-seq data obtained from the intima and madia layers of wild-type mouse aortas, published by Deng et al [82].

Unbiased Louvain clustering of cells resolved 2 robust, transcriptionally distinct clusters of aorta-derived cells (Fig. 3A–C). Clusters of endothelial cells and vascular smooth muscle cells (SMCs) were identified by the significant, cluster-specific markers calculated by the MAST method as described [83]. Then, we determined which concordant genes from our dataset showing pro-geronic shifts are expressed in the endothelial cells and/or the SMCs (Fig. 3D). We found that old blood induces aging-like transcriptomic changes in young mouse aortas by regulating genes expressed both in endothelial cells and SMCs.

Old blood-induced vascular transcriptomic changes in young mice predict pathologic vascular

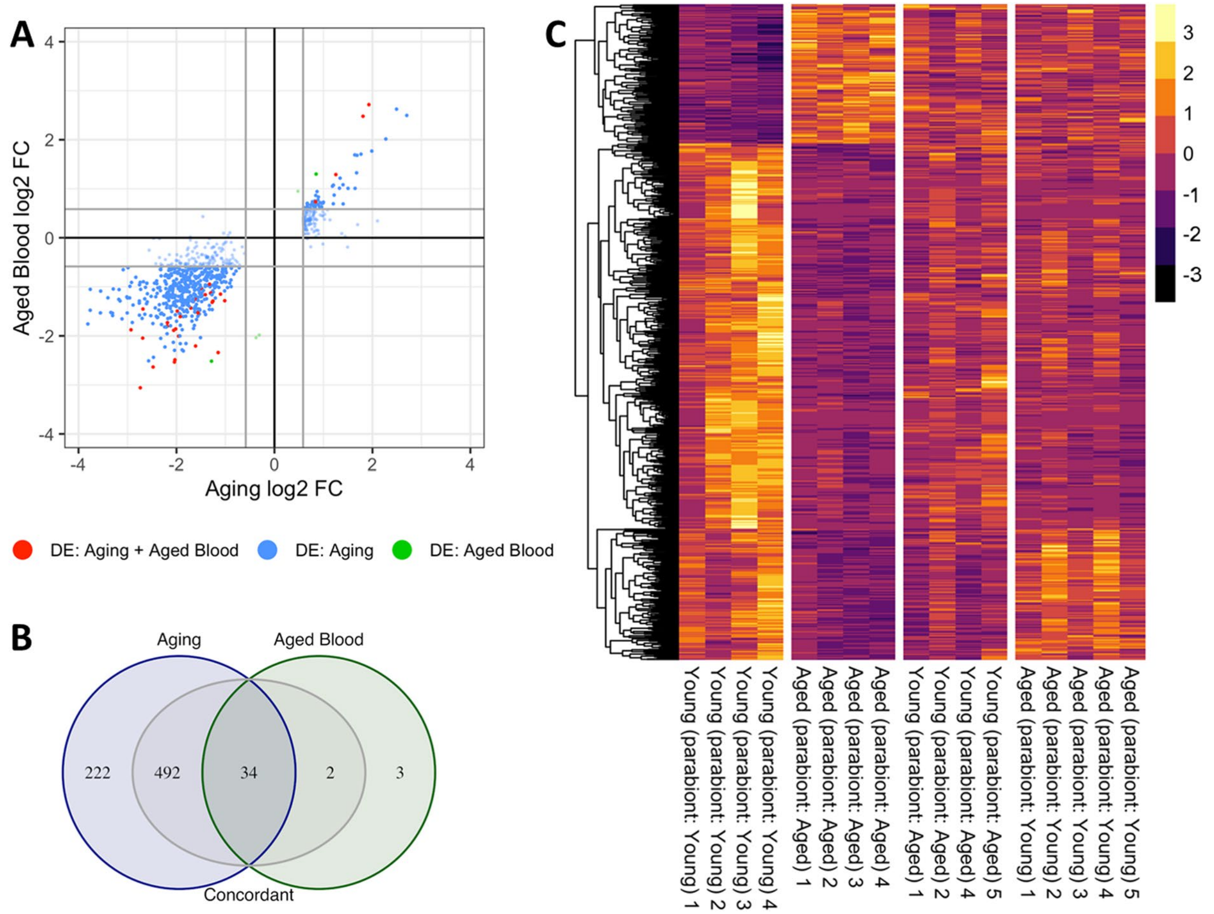


Fig. 2 Old blood promotes aging-like changes in vascular mRNA expression profile: identification of concordant genes Panel A: Old blood-induced changes in gene expression ($\log_2$ fold changes; heterochronic parabiont young [Y–(A)] mice vs. isochronic parabiont young [Y–(Y)] mice) plotted against age-related changes ($\log_2$ fold changes; isochronic parabiont aged [A-–(A)] mice vs. isochronic parabiont young [Y–(Y)] mice) in the aortic transcriptome. Red symbols indicate concordant differentially expressed genes, whose expression significantly changes with age, and the direction of this effect is mimicked by exposure to old blood. Blue and green symbols denote concordant genes, whose expression similarly changes in aging and by old blood exposure, but only the aging (blue) or the old blood effect (green) reaches the cutoff for statistical significance. Panel B: Venn diagrams showing the numbers of differentially expressed mRNAs in each group. The blue circle represents genes, which are significantly up- or down-regulated in aged mice as compared to young mice. The green circle represents genes, which are significantly up- or down-regulated by the presence of old blood in young mice. The intersection of the two circles represents concordant differentially expressed genes. Grey areas represent concordant genes, whose expression similarly changes in aging and by old blood exposure, but only the aging or the old blood effect reaches the cutoff for statistical significance. Panel C: The heat map is a graphic representation of normalized expression values of concordant genes in aorta samples derived from Y–(Y), A–(A), and Y–(A) mice. Data for aorta samples derived from A–(Y) mice are shown for comparison. Note that the expression of the majority of concordant genes, which are down-regulated by old blood in young mice, is unaffected by the presence of young blood in old mice. These data indicate that many of the effects of progeronic factors, which induce accelerated aging phenotypes in young mice, cannot be rescued by the presence of young blood in old mice

remodeling, suggesting a possible role for circulating factors present in old blood in the pathogenesis of aorta aneurysms and atherosclerosis.

We performed GO (gene ontology) enrichment analysis to explore potential biological functions of the old blood-induced concordant genes with

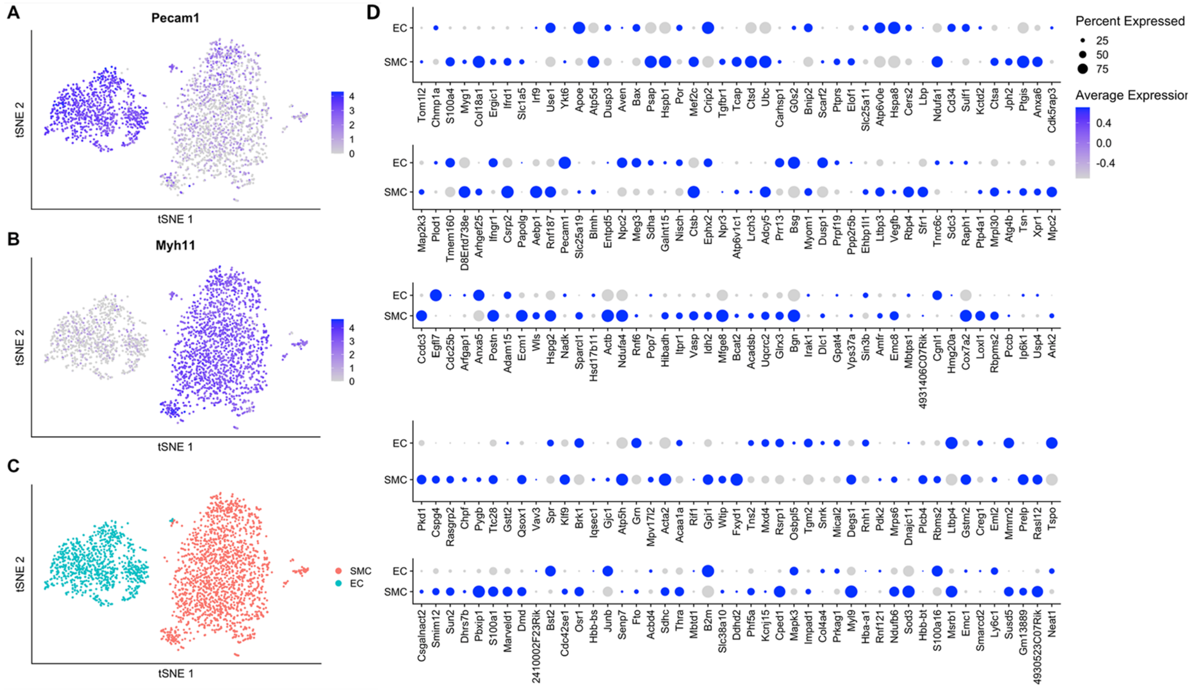


Fig. 3 Old blood induces aging-like transcriptomic changes by regulating genes expressed in endothelial cells and smooth muscle cells in the mouse aorta To determine the expression pattern of concordant genes in endothelial cells (ECs) and vascular smooth muscle cells (SMCs), single-cell RNA-seq data were obtained in ECs, and SMCs derived from the mouse aorta and published by Deng et al [82] were reanalyzed. Panel A–C: Identification of ECs and SMCs based on differentially expressed marker genes. Panel C depicts two-dimensional tSNE plots based on differentially expressed marker genes, colored by cluster. Panels A and B depict the expression of a canonical endothelial cell marker (*Pecam1*) and a canonical smooth muscle cell marker (*Myh11*), respectively. Relative expression values for each cell in each cluster identified in the two-dimensional tSNE plots are shown. Panel D: Bubble plot shows the relative expression of the concordant genes in mouse aorta ECs and SMCs. Bubble size is proportional to the percentage of cells expressing a gene, and color intensity are proportional to average scaled gene expression within a cluster. Note that old blood induces aging-like transcriptomic changes in young mouse aortas by regulating genes expressed both in endothelial cells and smooth muscle cells

pro-geronic shifts. GO enrichment analysis of concordant genes identified functions in vascular SMC and extracellular matrix remodeling (Fig. 4A; Table S1).

Overrepresentation analysis of GO terms revealed that genes regulating vascular SMC function and processes involved in vascular remodeling (including the GO terms "regulation of cell migration" [GO:0030334] and "blood vessel development" [GO:0001568]), genes regulating oxidative stress responses (including the GO terms "response to oxidative stress" [GO:0006979] and "reactive oxygen species biosynthetic process" [GO:1903409]), extracellular matrix related genes (including the GO terms "connective tissue development" [GO:0061448]; "extracellular matrix organization" [GO:0030198] and "collagen metabolic process" [GO:0032963]), and genes regulating apoptosis (including the GO terms "regulation of cell death" [GO:0010941] and "apoptotic process" [GO:0006915] and "positive regulation of cell death" [GO:0010942]) were overrepresented among the concordant genes.

For functional enrichment visualization, we used the EnrichmentMap Cytoscape App [77]. Figure 4B–D shows the enrichment maps in which significantly enriched GO terms associated with concordant genes are organized into a network. Nodes represent gene-sets (GO terms), edges represent mutual overlap, and, in this way, mutually overlapping GO terms cluster together. Note that the pro-geronic shift in gene expression profile in aortas of Y–(A) animals are associated with transcriptional

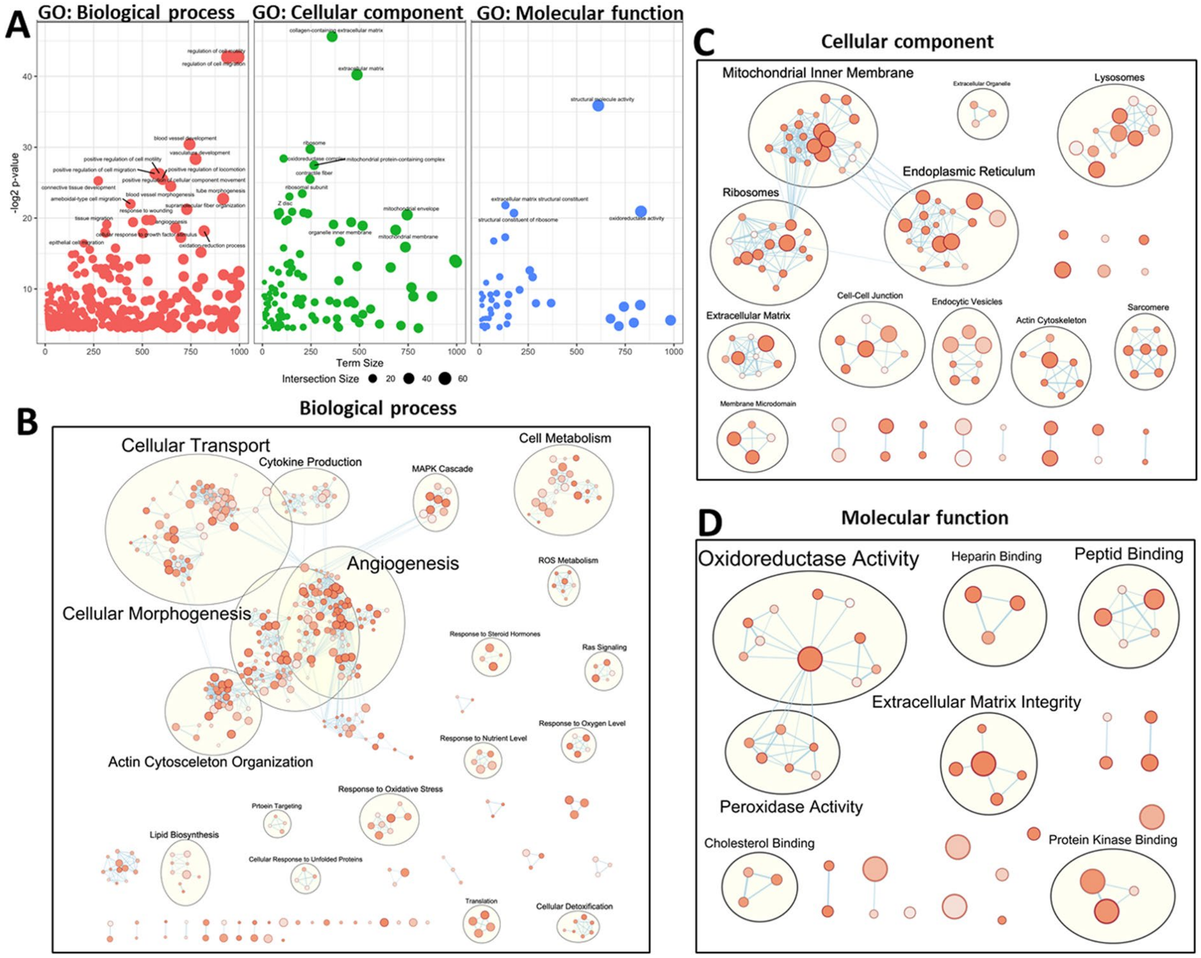
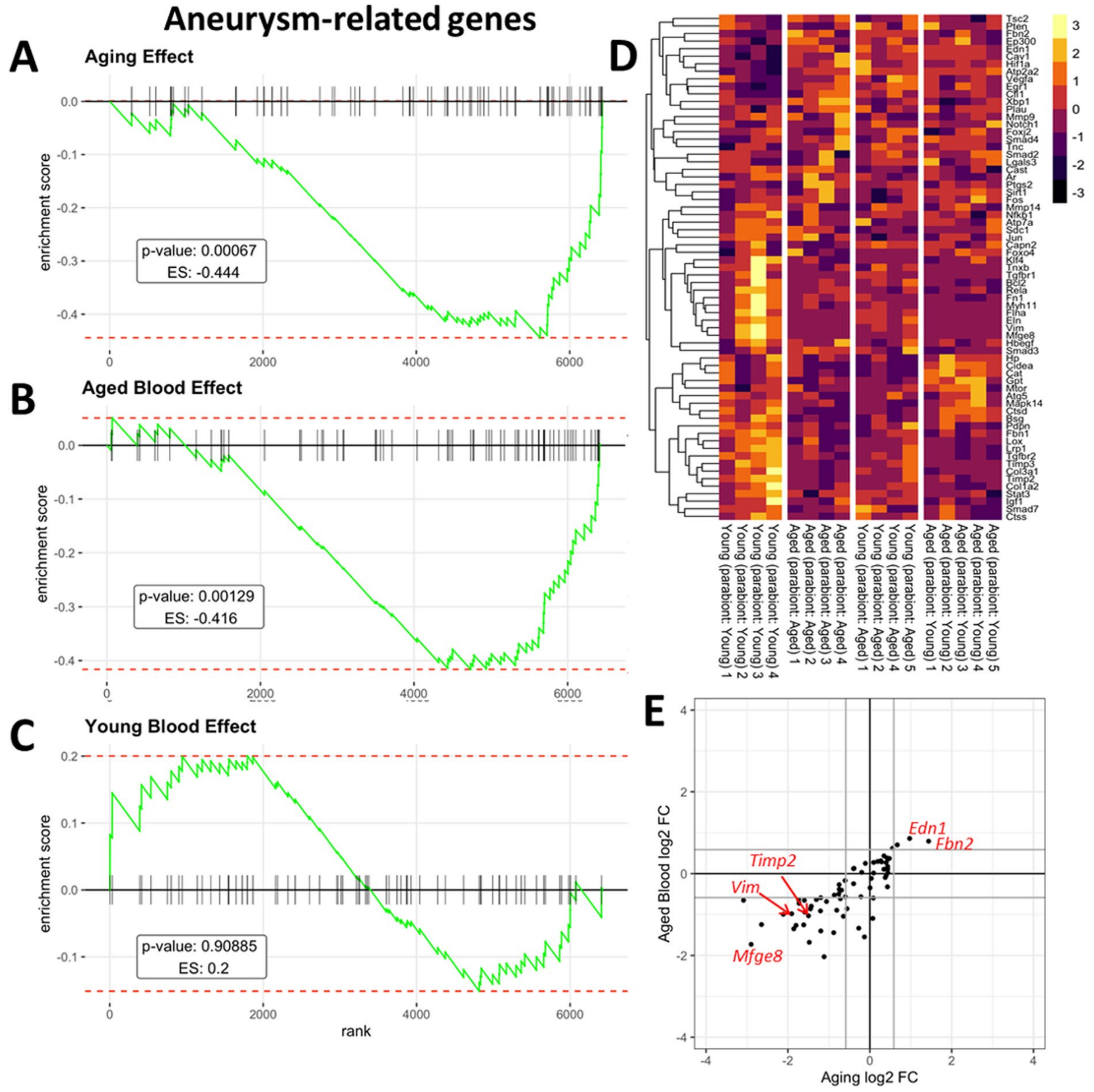


Fig. 4 GO enrichment analysis results: old blood induces a gene expression signature indicating pathological remodeling of the aorta Panel A: Bubble plots visualizing gene ontology (GO) representatives of biological processes (BP, left panel), cellular components (CC, middle panel), and molecular functions (MF, right panel), which are over-represented (enriched) in the concordant genes induced by old blood. Terms are shown at –log2 (adjusted *p*-value; cut off > 0.05) vs. term size. Bubble size indicates the number of genes annotated with a term in the list of concordant genes. Note that the presence of old blood in young animals is associated with transcriptional changes indicating multifaceted biological processes related to vascular remodeling. Panels B, C: Enrichment maps visualizing shared, significantly enriched GO terms (Panels B, C: Biological processes, cellular components, and molecular functions, respectively) for concordant genes organized into a network. Note that mutually overlapping GO terms cluster together

changes indicating pathological vascular remodeling and oxidative stress-related responses.

In humans, aging is known to promote the pathogenesis of aorta aneurysms and atherosclerosis [1]. In the present study, we aimed to elucidate how aorta aneurysm- and atherosclerosis-related gene expression is altered by circulating factors present in old blood. To compile a list of genes with known functions related to the pathogenesis of aorta aneurysms and atherosclerosis we identified genes associated with aorta aneurysms and atherosclerosis using the IRIDESCENT text mining package [84]. IRIDESCENT's database contains over 730,000 recognized terms and phrases, representing over 162,000 unique concepts obtained from public databases (OMIM, Entrez Gene, Gene Ontology, ChemID, FDA approved drugs, and disease ontology terms). Over 22 million MEDLINE abstracts were processed to identify co-occurring terms. This creates a network of concepts, weighted by their frequency of



co-occurrence. Concepts such as "aorta aneurysm" and "atherosclerosis" were then queried for frequently co-occurring gene names. We used Gene Set Enrichment Analysis (GSEA) for interpreting the expression of aorta aneurysm- and atherosclerosis-related genes [85] in all of our comparisons of mouse pairs. GSEA of these aorta aneurysms- and atherosclerosis-related genes was performed using a ranked gene list for each comparison of mouse pairs based on the gene-level signed statistics (Figs. 5 and 6, respectively). Figure 5A–C depicts a running-sum statistic (enrichment score) based on Fig. 5D, increasing when a gene is a member of the aorta aneurysm-related gene set and decreasing when it is not. Note that in aged mice, enrichment scores increased predominantly on the right indicating age-related down-regulation of genes related to extracellular matrix homeostasis (Fig. 5A). In response to the presence of old blood in young mice enrichment scores showed similar increases on the right, indicating that presence of old blood in young mice mimics the effects of aging on extracellular matrix homeostasis (Fig. 5B). In contrast, young

◂**Fig. 5** Presence of old blood in young mice mimics age-related changes in vascular expression of genes associated with the pathogenesis of aorta aneurysms Panel A: Gene set enrichment analysis (GSEA) to test the effect of aging on the enrichment of the set of genes associated with the pathogenesis of aorta aneurysms by comparing aorta samples derived from isochronic parabiont young mice [parabiont: young; Y–(Y)] and isochronic parabiont aged mice [parabiont: aged; A–(A)]. Aging-induced gene expression changes were ranked from most up-regulated (left) to most down-regulated (right). Ticks represent genes encoding aneurysm-related factors. Shown is a running-sum statistic (enrichment score) based on panel D, increasing when a gene is a member of the aneurysm-related gene set and decreasing when it is not. Panel B: GSEA showing the effect of exposure to old blood on the enrichment of aneurysm-related genes. Aorta samples derived from isochronic parabiont young mice [parabiont: young; Y–(Y)] and heterochronic parabiont young mice [parabiont: aged; Y–(A)] were compared. Note that in aged mice, enrichment scores increased predominantly on the right indicating age-related down-regulation of genes related to extracellular matrix homeostasis. In response to the presence of old blood in young mice, enrichment scores showed similar increases on the right, indicating that the presence of old blood in young mice mimics the effects of aging on aneurysm-related genes. Panel C: Youngblood had no consistent effect on aneurysm-related genes (comparison: A–(Y) vs. A–(A)). Panel D: The heat maps are graphical representations of normalized expression values of aneurysm-related genes. Hierarchical clustering analysis revealed the similarities on aortic expression profiles of aneurysm-related genes in aged mice and old blood exposed young mice. Panel E: Old blood-induced changes in aneurysm-related gene expression ($\log_2$ fold changes; Y–(A) vs. Y–(Y)) plotted against age-related changes ($\log_2$ fold changes; A–(A) vs. Y–(Y)) in aneurysm-related gene expression. Note that many aneurysm-related genes are dysregulated both in aging and by the presence of old blood in young animals as well

blood had no consistent effect on aneurysm-related genes (Fig. 5C). Figure 5E shows that many aneurysm-related genes are dysregulated both in aging and by the presence of old blood in young animals as well. GSEA of atherosclerosis-related genes yielded similar results (Fig. 6), showing that many genes involved in the regulation of atherogenesis are similarly dysregulated both in aging and by the presence of old blood in young animals.

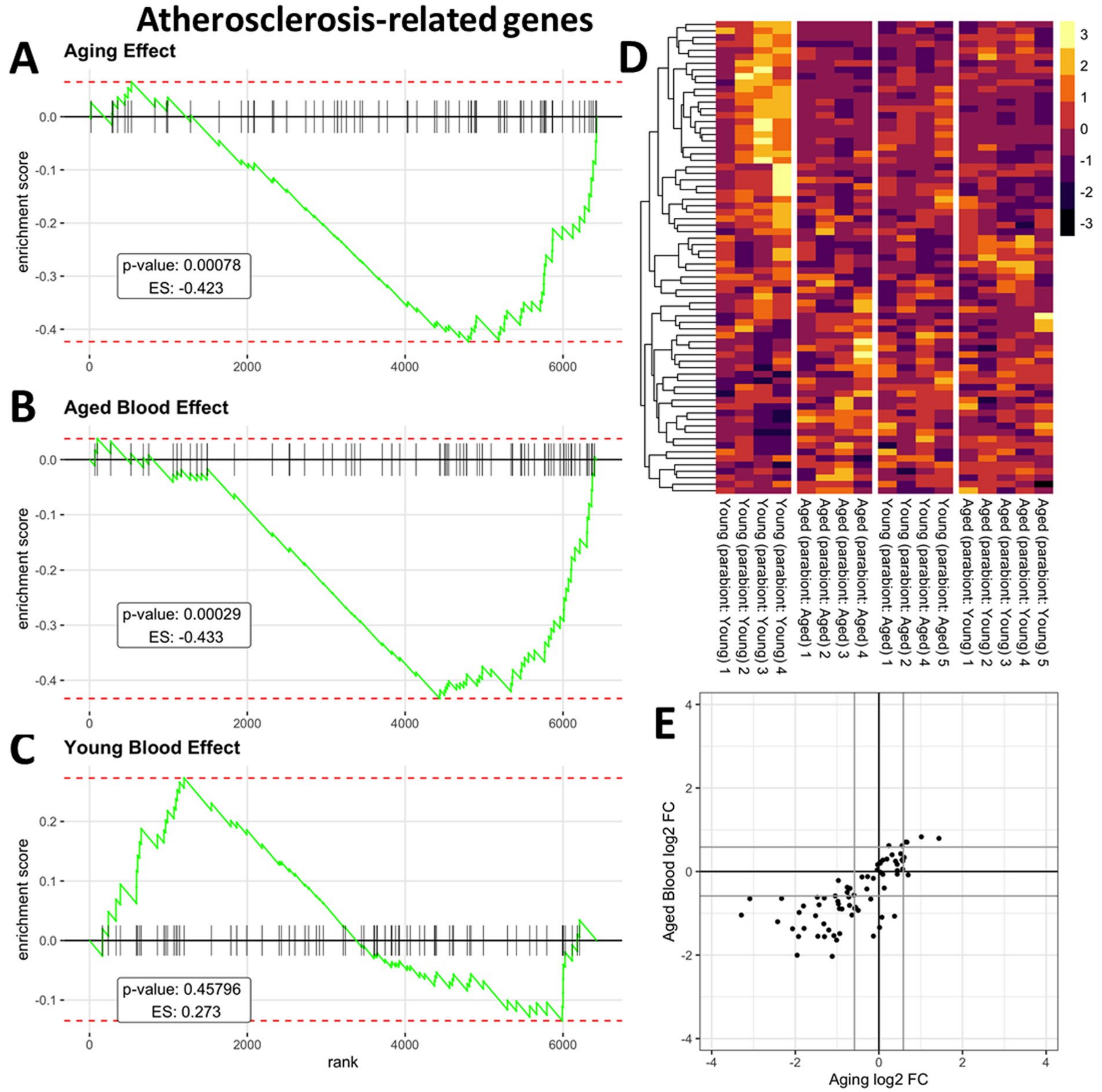
Ingenuity upstream regulator analysis

We have performed IPA upstream regulator analysis [54] to identify upstream transcriptional regulators that may contribute to the observed transcriptomic changes in our dataset, which can help to identify the mechanism of action of pro-geronic factors present in the old blood. The upstream regulator analysis is based on information in the ingenuity knowledge base (a curated relational database of the available biomedical literature) on the expected effects between transcriptional regulators and their target genes. Using the IPA Upstream Regulator analysis, it was examined how many known targets of each transcriptional regulator were differentially expressed in our samples and the direction of these gene expression changes were compared to what is expected from the literature. On the basis of the observed direction of change, a prediction of the activation state of the predicted transcriptional regulators ("activated" or "inhibited") was made. For each potential transcriptional regulator two statistical measures, an overlap p-value and an activation z-score were computed. The overlap p-value calls likely upstream regulators based on the significant overlap between the DE genes and known targets regulated by that particular transcriptional regulator. The activation z-score is used to infer the activation state of the predicted transcriptional regulators ("activated" or "inhibited") based on comparison with a model that assigns random regulation directions. The results of the IPA upstream regulator analysis are shown in Table 1. In particular, the IPA upstream regulator analysis predicts that old blood-induced accelerated vascular aging is associated with inhibition of pathways mediated by SRF (serum response factor), insulin-like growth factor-1 (IGF-1) and VEGF-A (Table 1).

Effect of old blood on SRF-regulated pathways

To investigate the contribution of SRF-regulated pathways to the effects induced by old blood we analyzed the expression of known SRF-regulated genes (Table S2). SRF-regulated genes were identified using the MotifMap database [86], and a literature search [87]. A list of SRF-regulated genes was also retrieved from the ingenuity knowledge base.

We used GSEA for interpreting the expression of SRF-driven genes. Figure 7A,B depicts a running-sum statistic (enrichment score) based on Fig. 7C. Note that in aged mice, enrichment scores increased predominantly on the right indicating age-related down-regulation of SRF-driven genes (Fig. 7A). In response to the presence of old blood in young mice, enrichment scores showed similar increases on the right, indicating that the presence of old



blood in young mice mimics the effects of aging on SRF-driven genes (Fig. 7B). We also performed comparison analysis using the Ingenuity Pathway Analysis tool to depict aging- and old-blood-effect changes in the SRF-driven genes (Fig. 7D and E, respectively). Note that many SRF-driven genes are similarly dysregulated both in aging and by the presence of old blood in young animals as well.

Discussion

The key finding of this study is that relatively short-term exposure to an old humoral environment in young mice can promote the acquisition of accelerated vascular aging phenotypes, including dysregulation of the expression of genes related to pathological vascular remodeling.

Although significant progress has been made in recent years to understand the genesis of cardiovascular aging phenotypes that arise as the consequence

◂**Fig. 6** Presence of old blood in young mice mimics age-related changes in vascular expression of genes associated with the pathogenesis of atherosclerosis Panel A: Gene set enrichment analysis (GSEA) to test the effect of aging on the enrichment of the set of genes associated with the pathogenesis of atherosclerosis by comparing aorta samples derived from isochronic parabiont young mice [parabiont: young; Y–(Y)] and isochronic parabiont aged mice [parabiont: aged; A–(A)]. Aging-induced gene expression changes were ranked from most up-regulated (left) to most down-regulated (right). Ticks represent genes encoding atherosclerosis-related factors. Shown is a running-sum statistic (enrichment score) based on panel D, increasing when a gene is a member of the atherosclerosis-related gene set and decreasing when it is not. Panel B: GSEA showing the effect of exposure to old blood on enrichment of atherosclerosis-related genes. Aorta samples derived from isochronic parabiont young mice [parabiont: young; Y–(Y)] and heterochronic parabiont young mice [parabiont: aged; Y–(A)] were compared. Note that in aged mice enrichment scores increased predominantly on the right indicating age-related dysregulation of genes related to atheroprotection. In response to the presence of old blood in young mice enrichment scores showed similar increases on the right, indicating that the presence of old blood in young mice mimics the effects of aging. Panel C: Youngblood had no consistent effect on atherosclerosis-related genes (comparison: A–(Y) vs. A–(A)). Panel D: The heatmaps are graphic representations of normalized expression values of atherosclerosis-related genes. Hierarchical clustering analysis revealed the similarities on aortic expression profiles of atherosclerosis-related genes in aged mice and old blood exposed young mice. Panel E: Old blood-induced changes in atherosclerosis-related gene expression ($\log_2$ fold changes; Y–(A) vs. Y–(Y)) plotted against age-related changes ($\log_2$ fold changes; A–(A) vs. Y–(Y)) in atherosclerosis-related gene expression. Note that many genes are similarly dysregulated both in aging and by the presence of old blood in young animals as well

of spontaneous, stochastic damage [4, 16, 18, 21, 70, 88–97], the relative contribution of cell-autonomous and non-autonomous mechanisms to vascular aging remained unclear. Here we show for the first time that circulating factors present in the blood of old mice promote accelerated vascular aging mimicking select transcriptional changes associated with old age in the aorta. Our findings support the concept that cell-non-autonomous mechanisms play important roles in driving vascular aging processes and thereby likely promote the pathogenesis of age-related cardiovascular diseases [1]. Previous studies also demonstrated that the presence of old blood in the circulation of young heterochronic parabionts also promotes aging-like phenotypic changes in the liver, heart, and brain [31, 47, 49, 52, 55, 98]. Interestingly, circulating noncellular factors present in the old blood were also shown to confer pro-geronic effects on the central nervous system [99, 100], suggesting that key circulating anti-geronic factor(s) may penetrate the blood–brain barrier and/or exert their deleterious effects on the brain by promoting accelerated aging in the cerebral microcirculation. The remarkable level of the malleability of vascular aging phenotypes in response to both anti-geronic [7, 101–103] and pro-geronic circulating factors highlight the potential for therapeutic interventions to reverse the deleterious effects of aging in the circulatory system via targeting the systemic milieu directly or indirectly.

Aging is associated with structural remodeling of the aorta both in humans [104, 105] and laboratory rodents [106–108]. These aging-induced structural and cellular changes alter the mechanical properties of the aorta and contribute to the genesis of age-related large vessel diseases, including atherosclerosis and aneurysm formation. Here we report that circulating factors present in the blood of old mice induce aging-like changes in vascular remodeling-related gene expression in the aorta of young parabionts. On the basis of previous findings [108–112] we posit that old blood-induced dysregulation of genes driving production, assembly, and deposition of extracellular matrix components, SMC proliferation, and apoptosis contribute to the genesis of accelerated vascular aging phenotypes. In addition, dysregulation of genes regulating ROS metabolism may also contribute to old blood-mediated accelerated vascular aging.

Our GSEA results provide additional transcriptomic insight into the contribution of circulating factors to the pathogenesis of specific age-related vascular diseases. Specifically, we predict that circulating factors present in old blood may exacerbate biological processes involved in the pathogenesis of aorta aneurysm and atherosclerosis. Further studies are evidently needed to test these predictions experimentally.

Importantly, the vasculature comes in contact with each plasma constituent derived from the old blood (including circulating hormones, cytokines, other proteins, peptides, lipid mediators, micropeptides, metabolites, and circulating exosomes) as well as circulating cellular factors, all of which may confer important pro-geronic effects. The exact nature and the cellular origins of circulating pro-geronic factors responsible for the induction of accelerated vascular aging in our studies remain obscure. In the present study, we performed upstream regulator analysis to gain insight into the cellular pathways induced by

Table 1 Predicted upstream transcriptional regulators that may mediate the observed aging-like vascular effects of circulating pro-geronic factors present in old blood Results of the IPA Upstream Regulator analysis. Shown are predicted upstream transcriptional regulators that may contribute to the observed old blood-induced transcriptomic changes in our dataset. The overlap *p*-value calls likely upstream regulators based on the significant overlap between dataset genes and known targets regulated by a transcriptional regulator

Upstream regulator	***Name/alias***	***Remark***	***Molecule type***	***Predicted activation state***	***Activation z-score***	***P-value of overlap***
TGFB1	Transforming growth factor-β	Regulates VSMC differentiation and vascular remodeling; regulates VSMC aging [146]; TGF-β1 increases in aged humans and mice [147]	Growth factor	Inhibited	−3.8	4.35E-14
TP53	Tumor protein P53	tumor suppressor; atheroprotection in aorta [148], prevents pathological remodeling [149]	Transcription regulator	Inhibited	−3.55	3.87E-13
PRL	Prolactin	Serum prolactin increases with age in men [150]	Cytokine	Inhibited	−3.665	9.96E-11
Alpha catenin		Links cadherins to the actin cytoskeleton; promotes cell–cell adhesions; α-catenin regulates Wnt/β-catenin signaling [151]; Wnt/beta-catenin pathway is activated during advanced arterial aging in humans [152]	Group	Activated	3.266	1.19E-08
AGT	Angiotensinogen	angiotensin signaling regulates VSMC remodeling and arterial aging [153, 154]	Growth factor	Inhibited	−3.927	0.000000045
ROR1	Receptor tyrosine kinase-like orphan receptor 1	Ror1/2 knockout mice exhibit cardiovascular hyperinflammation [155]	Kinase	Inhibited	−2.414	0.000000352
SEMA7A	Semaphorin 7A	SEMA7A regulates atherogenesis [156]; TGBβ regulates SEMA7A	Transmembrane receptor	Inhibited	−2.236	0.0000016
UBA1	Ubiquitin-like modifier activating enzyme 1	Plays central role in ubiquitination; regulates cell cycle, endocytosis, signal transduction, apoptosis, DNA damage repair, and transcription; pro-atherogenic [157]	Enzyme	Activated	2.236	0.000002
CD44	Homing cell adhesion molecule	Regulates cell–cell interactions, cell adhesion and migration; regulates atherogenesis [158]	Cell-surface glycoprotein	Inhibited	−2.165	0.00000315

Table 1 (continued)

Upstream regulator	*Name/alias*	*Remark*	*Molecule type*	*Predicted activation state*	*Activation z-score*	*P-value of overlap*
CCR2	C–C chemokine receptor type 2	Receptor for MCP-1; regulates aneurysm formation [159]; circulating MCP-1 increases with age [160]	G-protein coupled receptor	Inhibited	−2.138	0.0000081
LARP1	La-related protein 1	Novel target of the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway. In response to cellular mTOR activity, LARP1 serves as a phosphorylation-sensitive molecular switch regulating ribosome biogenesis [161]	Translation regulator	Activated	2.646	0.0000104
CR1L	Complement C3b/C4b receptor 1 like	Cofactor for complement factor I	Other	Activated	2.2	0.0000105
IKZF1	Ikaros	Up-regulated in aorta aneurysm [162]	Transcription regulator	Activated	2.177	0.0000177
MRTFB	MKL/myocardin-like protein 2	Binds to SRF and act as coactivators; regulates VSMC differentiation; regulated in vascular injury	Transcription regulator	Inhibited	−2.043	0.0000254
IL10RA	Interleukin 10 receptor, alpha subunit	Mediate the immunosuppressive signal of IL-10, and thus inhibits the synthesis of proinflammatory cytokines; IL-10 attenuates neointimal proliferation; IL-10 levels positively associate with CVD risk [163]	Transmembrane receptor	Inhibited	−2.887	0.0000266
SNAI1		Transcription factor that promotes the repression of E-cadherin to regulate epithelial to mesenchymal transition	Transcription regulator	Inhibited	−2.608	0.0000946

Table 1 (continued)

Upstream regulator	*Name/alias*	*Remark*	*Molecule type*	*Predicted activation state*	*Activation z-score*	*P-value of overlap*
SNAI2		Transcription factor that regulates cell differentiation and migration; regulates epithelial to mesenchymal transition; mediates anti-apoptotic effects	Transcription regulator	Inhibited	−2.387	0.0000275
MKNK1	MAP kinase-interacting serine/threonine-protein kinase 1	Regulates cardiovascular remodeling [164]	Kinase	Inhibited	−2.646	0.0000369
ITGA11	Integrin alpha 11	Binds to type I collagen; regulates tissue fibrosis [165];	Integrin	Inhibited	−2	0.0000399
CCN2	CTGF/connective tissue growth factor	Regulates cell adhesion, migration and proliferation, angiogenesis, and vascular remodeling [166–169]	growth factor	Inhibited	−2.611	0.0000599
SRF	Serum response factor	Regulates VSMC growth, migration and proliferation [170]; binds to a serum response element (SRE) associated with a variety of genes including immediate early genes such as c-fos, fosB, junB, egr-1, and -2, and muscle genes such as actins and myosins. Regulates cell cycle, apoptosis, cell growth, and cell differentiation [170]. It is a downstream target of mitogen-activated protein kinase pathway (MAPK). SRF is down-regulated in aging skeletal muscle in mice and humans [121]. Muscle specific loss of SKF promotes muscle atrophy [121]. Endothelium-specific knockdown of SRF promotes aorta aneurysm formation [171]	Transcription regulator	Inhibited	−2.613	0.0000761

Table 1 (continued)

Upstream regulator	*Name/alias*	*Remark*	*Molecule type*	*Predicted activation state*	*Activation z-score*	*P-value of overlap*
SMAD7	Mothers against decapentaplegic homolog 7	Inhibits TGFβ1 signaling	Transcription regulator	Activated	2.759	0.0000791
VEGFA	Vascular endothelial growth factor A	Induces angiogenesis, endothelial cell growth, promotes cell migration, and inhibits apoptosis; VEGF signaling decreases with age; an increase in circulatory VEGF in aged mice exerts rejuvenating effects on the aged vasculature and alleviates multiple adverse age-related processes [122]	Growth factor	Inhibited	−2.764	0.000103
PRKG1	cGMP-dependent protein kinase 1, alpha isozyme	Humans heterozygous for an activating mutation in PRKG1 develop thoracic aortic aneurysms and dissections as young adults. Mice heterozygous for this activating mutation also develop age-dependent aortic dilation with increased smooth muscle cell apoptosis, elastin fiber breaks, and oxidative stress [172]	Kinase	Activated	2	0.000183
HIF1A	Hypoxia-inducible factor 1-alpha	Transcription factor; regulates cellular responses to systemic oxygen levels; regulates tissue regeneration and repair	Transcription regulator	Inhibited	−2.032	0.000219
AR	Androgen receptor	Activated by testosterone and dihydrotestosterone; plasma testosterone varies considerably in aging C57BL/6 J mice [173]; testosterone protects against aortic aneurysms [174, 175]	Ligand-dependent nuclear receptor	Inhibited	−2.843	0.000528

Table 1 (continued)

Upstream regulator	*Name/alias*	*Remark*	*Molecule type*	*Predicted activation state*	*Activation z-score*	*P-value of overlap*
ERG	ETS-related gene	Regulates cell proliferation, differentiation, angiogenesis, inflammation, and apoptosis; genetic variant associates with aorta aneurysm [176]	Transcription regulator	Inhibited	−2.183	0.000704
ANGPT2	Angiopoietin-2	Antagonist for both ANGPT1 and TIE2; vascular remodeling; inhibits development of aortic aneurysm and atherosclerosis [177]	Growth factor	Inhibited	−2.085	0.000771
FAS	FasR, tumor necrosis factor receptor superfamily member 6 (TNFRSF6)	Death receptor; activation by its ligand (Fas ligand, FasL) leads to apoptosis; apoptosis of VSMCs through the Fas-FasL pathway contribute to the pathogenesis of aorta aneurysms [178]; circulating FasL increases with age in humans [179]	Transmembrane receptor	Activated	2.226	0.00217
AHR	Aryl hydrocarbon receptor	AhR promotes aging phenotypes across species [180]; compounds produced by the microbiota can activate AhR	Ligand-dependent nuclear receptor	Activated	2.657	0.0025
IGF1	Insulin-like growth factor-1	In aging circulating levels of IGF-1 decline and circulating levels of IGF-1 binding proteins increase; IGF-1 is critical for maintenance of a youthful vascular phenotype [56–62, 181, 182]	Growth factor	Inhibited	−2.075	0.00299

pro-geronic factors present in old blood. This analysis highlighted the possible role of TGFβ signaling and other pathways involved in vascular remodeling. The possibility indicated by this analysis that pro-geronic factors present in old blood may inhibit the tumor suppressor and vasculoprotective p53 pathway is also highly intriguing.

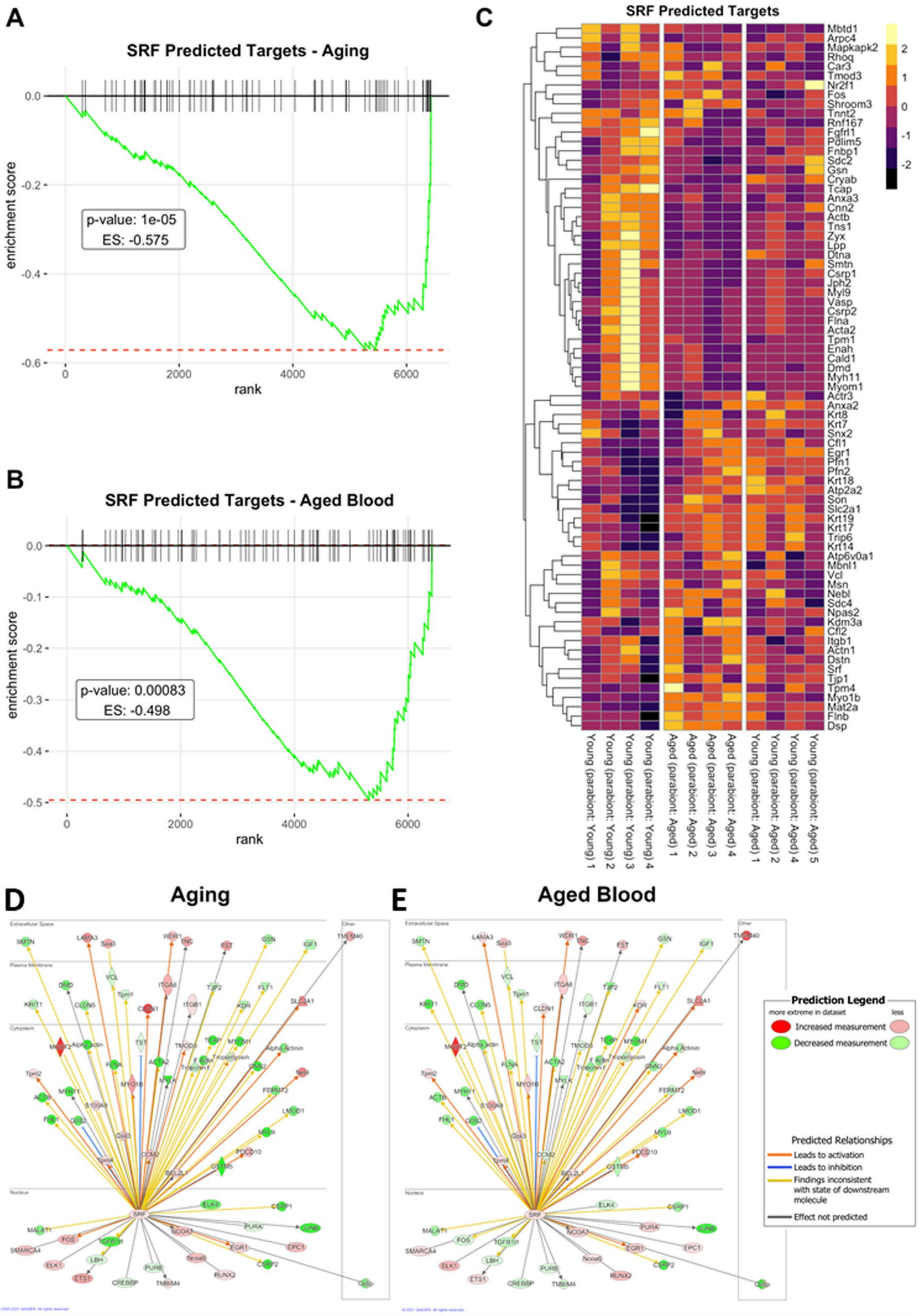
It is also potentially interesting for the discovery of novel cell-nonautonomous mechanisms of aging that the upstream regulator analysis identified inhibition of SRF-driven pathways as a putative mediator of the effects of old blood on the aorta. Greenberg discovered in 1984 that the addition of young serum to quiescent cells in culture rapidly stimulates *c-fos* and thereby promotes cell growth [113]. Subsequently, it was demonstrated that several growth factors can have synergistic effects and a short DNA sequence was identified in the promoter region of *c-fos* and other serum-responsive genes (termed serum response element or SRE), which renders the cells responsive to stimulation by young serum samples [114]. Treisman identified the transcription factor that binds to SRE and named it Serum Response Factor or SRF. [115, 116]. Interestingly, cells of the cardiovascular system, including VSMCs, were found to be particularly sensitive to stimulation by young serum, and SRF was shown to regulate several VSMC-specific genes [87, 117–120]. SRF is down-regulated in aging skeletal muscle in mice and humans [121]. Importantly, muscle-specific knockdown of SRF was shown to promote muscle atrophy [121]. Future studies should determine the roles of dilution of young blood factors by old blood [99, 100] and the effects of specific pro-geronic factors in old blood, which interfere with the young blood factors activating SRF-driven pathways.

Interestingly, our analysis also suggests that the presence of old blood inhibits VEGF-A and IGF-1 signaling in young vessels. These findings are potentially significant as VEGF-A signaling in the vasculature is known to decrease with age, and recent studies show that an increase in circulatory VEGF-A in aged mice exerts rejuvenating effects on the aged vasculature [122]. Similarly, circulating free IGF-1 also declines in aging [123–126], in part, due to an increased presence of IGF-1 binding proteins in the circulation [127–129]. Previous studies provided ample evidence that decreases in circulating levels of free IGF-1 [59] contribute to the genesis of vascular aging phenotypes, including endothelial dysfunction [60], impaired autoregulation of cerebral blood flow [61], pathological remodeling of the extracellular matrix and the media [57], increased atherogenesis [130], impaired vascular oxidative stress resistance [131, 132], impaired angiogenesis, and capillary rarefaction [133]. Follow-up investigations should further interrogate experimentally the role of disrupted VEGF-A and IGF-1 signaling in the deleterious vascular effects of old blood transfer.

Other circulating factors, whose levels are altered in young mice by heterochronic parabiosis or systemic administration of old plasma or blood and which may confer aging-like effects in multiple organs (e.g., brain, heart, and skeletal muscle) include β2-microglobulin and TGFβ family member cytokines [31, 52]. There is also evidence that TNFα [134] and other inflammatory cytokines (e.g., CCL11) may serve as circulating pro-geronic factors. However, transcriptomic analysis of the aortas did not indicate that these cytokines play a central role in old blood-mediated accelerated aging in the mouse aorta. Factors secreted by senescent cells in various organs of aged mice (the senescence-associated secretory phenotype [SASP] [135, 136]), which also are present in the systemic circulation, have also been proposed to drive age-related dysfunction [4].

Perspectives

The present results support the remarkable plasticity of vascular aging in preclinical models and its amenability to modulation by circulating pro-geronic/old blood factors. Follow-up investigations are warranted to define the exact nature of the circulating old blood factors that regulate critical pathways involved in vascular aging. Studies using blood exchange paradigms, in which pro-geronic factors are diluted by replacing half of the plasma in mice with saline containing 5% albumin, suggest that noncellular factors play a key role in the mediation of the old blood effects in many organs [99]. Nevertheless, future studies are warranted to determine the potential roles of aged leukocytes that exhibit a pro-inflammatory phenotype in the pathogenesis of age-related vascular diseases [137]. Interesting in that regard is that 10 to 20% of adults aged 70 or older exhibit clonal hematopoiesis of indeterminate potential (CHIP), characterized by somatic mutations in leukocytes, which associates with a pro-inflammatory status and increased risk for


A
SRF Predicted Targets - Aging
enrichment score
0.0
-0.2
-0.4
-0.6
p-value: 1e-05
ES: -0.575
rank
0
2000
4000
6000
B
SRF Predicted Targets - Aged Blood
enrichment score
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
p-value: 0.00083
ES: -0.498
rank
0
2000
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6000
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SRF Predicted Targets
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Rhoq
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Tmod3
Nr2f1
Fos
Shroom3
Tnnt2
Rnf167
Fgfrl1
Pdlim5
Fnbp1
Sdc2
Gsn
Cryab
Tcap
Anxa3
Cnn2
Actb
Tns1
Zyx
Lpp
Dtna
Smtn
Csrp1
Jph2
Myl9
Vasp
Csrp2
Flna
Acta2
Tpm1
Enah
Cald1
Dmd
Myh11
Myom1
Actr3
Anxa2
Krt8
Krt7
Snx2
Cfl1
Egr1
Pfn1
Pfn2
Krt18
Atp2a2
Son
Slc2a1
Krt19
Krt17
Trip6
Krt14
Atp6v0a1
Mbnl1
Vcl
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Sdc4
Npas2
Kdm3a
Cfl2
Itgb1
Actn1
Dstn
Srf
Tjp1
Tpm4
Myo1b
Mat2a
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Young (parabiont: Young) 1
Young (parabiont: Young) 2
Young (parabiont: Young) 3
Young (parabiont: Young) 4
Aged (parabiont: Aged) 1
Aged (parabiont: Aged) 2
Aged (parabiont: Aged) 3
Aged (parabiont: Aged) 4
Young (parabiont: Aged) 1
Young (parabiont: Aged) 2
Young (parabiont: Aged) 4
Young (parabiont: Aged) 5
D
Aging
E
Aged Blood
Extracellular Space
Plasma Membrane
Cytoplasm
Nucleus
Other
SMTN
LAMA3
Saa3
WDR1
TNC
FST
GSN
IGF1
TMEM40
VCL
DMD
ITGA6
TJP2
FLT1
KRT1
CLDN5
Tpm1
CLDN1
ITGB1
KDR
SLC2A1
TST
TMOD3
MYOM1
Alpha Actinin
Tropomyosin
Tpm2
ACTA2
FLNA
MYO1B
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CNN2
Nebl
FERMT2
ACTB
MYH11
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LMOD1
FHL1
Gsk3
MYL9
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PDCD10
Tpm4
BCL2L1
GSTM5
SRF
ELK4
CSRP1
MALAT1
FOS
TGFB1I1
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JUNB
SMARCA4
NCOA2
EGR1
EPC1
ELK1
LBH
Ncoa6
CSRP2
ETS1
CREBBP
PURB
TMEM4
RUNX2
Dsp
Prediction Legend
more extreme in dataset
less
Increased measurement
Decreased measurement
Predicted Relationships
Leads to activation
Leads to inhibition
Findings inconsistent with state of downstream molecule
Effect not predicted
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◀**Fig. 7** Old blood induces aging-like transcriptomic changes, in part, by dysregulating SRF-driven gene expression Panel A: Gene set enrichment analysis (GSEA) to test the effect of aging on the enrichment of the set of genes whose expression is regulated by SRF (serum response factor) by comparing aorta samples derived from isochronic parabiont young mice [parabiont: young; Y–(Y)] and isochronic parabiont aged mice [parabiont: aged; A–(A)]. Aging-induced gene expression changes were ranked from most up-regulated (left) to most down-regulated (right). Ticks represent genes encoding SRF-regulated proteins. Shown is a running-sum statistic (enrichment score) based on panel C, increasing when a gene is a member of the SRF-driven gene set and decreasing when it is not. Panel B: GSEA showing the effect of exposure to old blood on the enrichment of SRF-regulated genes. Aorta samples derived from isochronic parabiont young mice [parabiont: young; Y–(Y)] and heterochronic parabiont young mice [parabiont: aged; Y–(A)] were compared. Note that in aged mice, enrichment scores increased predominantly on the right indicating age-related down-regulation of SRF-driven genes. In response to the presence of old blood in young mice, enrichment scores showed similar increases on the right, indicating that the presence of old blood in young mice mimics the effects of aging on SRF-driven genes. Panel C: The heat maps are graphical representations of normalized expression values of SRF-driven genes. Hierarchical clustering analysis revealed the similarities on aortic expression profiles of SRF-driven genes in aged mice and old blood exposed young mice. Panel D-E: Comparison analysis using the ingenuity pathway analysis (IPA) tool. The upstream regulator heat maps depict age- and old blood-related changes in the expression of SRF-driven genes. Each symbol on the map represents a gene product in the SRF pathway. The symbols are set to color by age-related (panel D) and old blood exposure-induced (panel E) changes in gene expression (fold change). Red color indicates up-regulation, green color indicates down-regulation

atherogenesis [138–141]. Future studies should also determine whether the observed cell non-autonomous mechanisms of vascular aging are not strain- or species-dependent and elucidate their role in humans. We used an 8-week parabiosis protocol, which represents ~ 6% of the maximum lifespan potential of the mouse strain used. This is biologically equivalent to ~ 6 years for human life. Studies (e.g., using a serum transfer paradigm) are warranted to determine how shorter exposures to circulating pro-geronic and/or anti-geronic factors affect vascular phenotypes. Subsequent studies are also warranted to identify the critical organ(s), tissues, and cell types, which contribute to the synthesis/release of the circulating pro-geronic factors that drive vascular aging processes. A potentially highly promising area of research includes the investigation of the role of factors secreted by senescent cells in distant organs (e.g., the adipose tissue [142–145]). Finally, studies targeting cell non-autonomous mechanisms of vascular aging identified using the aforementioned approaches may lead to the development of new treatments for the prevention of age-related vascular diseases.

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Declarations

Competing interest Dr. Anna Csiszar serves as Associate Editor for The Journal of Gerontology, Series A: Biological Sciences and Medical Sciences, and GeroScience. Dr. Andriy Yabluchanskiy serves as Guest Editor for The American Journal of Physiology-Heart and Circulatory Physiology. Dr. Derek Huffman serves as Associate Editor for GeroScience. Dr. Zoltan Ungvari serves as Editor-in-Chief for GeroScience and as Consulting Editor for The American Journal of Physiology-Heart and Circulatory Physiology.

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