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CONTRASTING CONSERVED AND TISSUE-SPECIFIC RESPONSES TO AGEING IN VERTEBRATES

DANIELA ESSER*, LENA BEST*, and CHRISTOPH KALETA

Research Group Medical Systems Biology, Institute for Experimental Medicine, University of Kiel, Kiel, Germany

SUMMARY

Ageing is the most important risk factor for diseases accounting for more than 90% of mortality in western countries. While genomic damage as the root cause of cancer is well established, the primary causes of most degenerative diseases of ageing such as cardiovascular diseases, neurodegenerative diseases and type 2 diabetes are still unknown. In a previous work, we have shown that there is a common core of ageing-associated gene regulation, conserved across vertebrate species and tissues.

In this work, we explored the association between this conserved core signature of ageing and the actual manifestation of ageing in individual tissues in more detail. To this end, we characterized transcriptomic signatures of ageing in individual tissues and investigated the contribution of the microbiome as a potential driver of ageing-associated inflammation. While core versus tissue-specific signatures showed only small differences in phylogenetic conservation or network connectivity, a considerable percentage of genes was found exclusively in tissue-specific signatures. However, on the functional level, tissue-specific signatures showed a very high overlap with the core signature of ageing. Moreover, cancer-regulated genes were enriched among genes of the core signature while they were depleted among the tissue-specific signatures. In contrast, for genes deregulated in degenerative diseases, we observed the tendency of an enrichment amongst genes of the tissue-specific signatures. Through comparisons of ageing signatures to genes regulated in response to microbial colonization, we observed a significant overlap with genes of the core signature of ageing.

Overall these results indicate that tissue ageing on the transcriptomic level is mostly driven by the core signature of ageing and that the microbiome is a potential modulator of this signature.

INTRODUCTION

Ageing is associated with a continuous functional decline and is recognized as a dominant factor driving the pathology of diseases contributing to the majority of mortality in western countries (*Lopez* et al., 2006; *López-Otín* et al., 2013). There is a remarkable conservation of ageing-associated pathologies such as cancer, cardiovascular disease and cognitive decline across vertebrates (*Dean* et al., 1981; *Genade* et al., 2005; *Pettan-Brewer* and *Treuting*, 2011).

* Both authors contributed equally

However, despite a large number of studies investigating ageing from the molecular (*López-Otín* et al., 2013) to the population level (*Jones* et al., 2014), a coherent picture of the processes that are driving ageing and their individual contribution to the pathogenesis of ageing diseases is just emerging (*López-Otín* et al., 2013). An often-observed feature of ageing is a low-grade inflammatory phenotype ("inflammaging") (*Chung* et al., 2009) that is characterized by a minor activation of the immune system even in absence of a detectable pathogen (*Franceschi* and *Campisi*, 2014). Inflammaging is an important risk factor for morbidity and mortality in the elderly (*Franceschi* et al., 2000).

A key problem in the study of the ageing phenotype is its remarkable complexity which makes a clear distinction between causal and consequential pathological changes a challenging task (*López-Otín* et al., 2013; *Bousquet* et al., 2014; *Franceschi* and *Campisi*, 2014; *Castellani* et al., 2016). In particular, while genomic mutations as a key event in cancer are well established (*Valko* et al., 2004), the root causes of degenerative ageing diseases such as cardiovascular diseases or neurodegenerative diseases have yet to be identified (*Carretero* and *Oparil*, 2000; *Galkina* and *Ley*, 2009; *Dexter* and *Jenner*, 2013; *Reitz* and *Mayeux*, 2014). One contributing factor is that ageing diseases are most often studied in isolation in the particular organ system they affect (*Castellani* et al., 2016), while extensive comorbidities between ageing diseases (*Fried* et al., 1999) hint at general underlying mechanisms not restricted to individual organ systems (*Masoro*, 2005; *Bousquet* et al., 2014).

In order to elucidate common pathomechanisms underlying ageing diseases, we recently have analysed a comprehensive next-generation sequencing data set of vertebrate ageing covering four organisms (human, mouse, zebrafish and the killifish *Nothobranchius furzeri*) across up to four tissues (brain, liver, skin, blood) (*Aramillo Irizar* et al., 2018). Using a novel method assessing differential activity directly on a functional level, we identified a highly conserved core of ageing-associated transcriptional regulation including an induction of inflammatory processes and a down-regulation of cell-cycle as well as developmental processes. While many of these processes have been implicated in ageing pathologies in individual species before (*Lee* et al., 2000; *Teta* et al., 2005; *Chung* et al., 2009), we found that they are part of a conserved core of ageing-associated regulation in the studied vertebrates.

By investigating the association between transcriptional signatures of ageing with differential gene expression observed in ageing related diseases, we found that ageing opposes transcriptional changes of cancer but is aligned with alterations in degenerative disorders. Intriguingly, these results are in line with epidemiological data showing a peak in cancer incidence between 70 to 80 years of age (*Harding* et al., 2012), while the incidence of degenerative diseases increases up to the oldest age groups (*Berzlanovich* et al., 2005; *Harding* et al., 2012). Thus, ageing moves the transcriptome away from cancer but brings it closer to that of degenerative diseases. On the functional level, we found that immune- and cell cycle-related processes most strongly contributed to this antagonism, which is supported by their central role in ageing pathologies (*Campisi*, 2013; *Jurk* et al., 2014). We observed a similar antagonism on the genetic level whereby for the majority of shared risk loci between cancer and degenerative diseases the allele that predisposed to

cancer protected against degenerative diseases and vice versa (*Aramillo Irizar* et al., 2018). We found the strongest signals close to central regulators of immune function and cellular senescence such as the P16/Ink4A locus, a master regulator of cellular senescence (*Congrains* et al., 2013), the Lnk/SH2B3 protein, a key regulator of cytokine signalling (*Devallière* and *Charreau*, 2011), and VAMP8 a central regulator of autophagy (*Diao* and *Liu*, 2016).

Besides ageing-associated changes in the host, ageing and inflammation might also be triggered by changes in microbiome composition in the elderly. Bacterial composition changes continuously throughout lifetime (*O'Toole* and *Jeffery*, 2015) and microbial diversity continuously becomes richer in humans with increasing age (*Lozupone* et al., 2012). Diseases associated with ageing often involve or are preceded by inflammaging, which can be aggravated by changes in the microbial composition (*Fransen* et al., 2017). A microbiome transfer experiment from old to young germ-free mice led to the upregulation of TNF-α and a dysregulation of pathways involved in the immune response. Inflammaging supporting bacteria were enriched after that treatment, including a higher amount of *Akkermansia*, TM7 bacteria, and *Proteobacteria* (*Thevaranjan* et al., 2018). A similar outcome can be observed when germ-free and old mice are co-housed (*Fransen* et al., 2017). Common inflammaging biomarkers, such as circulating pro-inflammatory cytokines, cannot be detected in germ-free mice and treating old mice with anti-TNF could alleviate microbiome-driven inflammation (*Thevaranjan* et al., 2018). In addition, in the ageing microbiome, less bacteria are able to synthesize β-glucuronidases, which are key modulators of epithelial cell toxicity caused by drugs (*Langille* et al., 2014). Moreover, the amount of monosaccharide-utilizing compared to polysaccharide-using bacteria positively correlates with age in mice (*Langille* et al., 2014).

The immune system of the host can be altered by a modified microbial composition caused by environmental factors including lifestyle and diet. As consequence, a disturbed gut homeostasis can increase the risk for diseases (*Langille* et al., 2014; *Fulde* et al., 2018). This is especially the case in elderly humans, in which poor health is associated with imbalances in the microbiome (*Claesson* et al., 2012). Thus, species distribution in the microbial flora is strongly associated with phenotypes of the host such as inflammation, the ability of independent living, sarcopenia as well as geriatric depression. For instance, independently living human individuals are characterized by a higher number of bacteria, which are able to perform biosynthesis of short chain fatty acids (*Claesson* et al., 2012). Taken together, host-microbiome interactions are potential key modulators of health and contribute to the physical conditions of the host.

While we have characterized the conserved transcriptomic signature of ageing in great detail in our previous work, we did not explore the actual manifestation of this signature in individual tissues (*Aramillo Irizar* et al., 2018). Thus, the aim of this work is to investigate conserved tissue-specific ageing in more detail. We aim to investigate two central hypotheses in ageing research in more detail, 1) whether there is a common mechanism underlying ageing in all tissues, which would point to a common driver of ageing (for instance the microbiome) or 2) whether ageing on the organismal level is basically the sum of functional deterioration in individual tissues. In the first

case, we would expect that tissue-specific ageing presents just a tissue-specific manifestation of the changes we observe in the core ageing signature. In the second case, we would expect strong differences in ageing between tissues and the core signature representing the least common denominator. Moreover, we explored the potential role of the microbiome as an important trigger for ageing-associated inflammation and thereby as a potential key driver of the observed transcriptomic signature of ageing.

MATERIAL AND METHODS

Data

To identify sets of conserved ageing-associated genes, we used a similar approach as described in a previous study, in which we searched for commonly regulated processes across species (*Aramillo Irizar* et al., 2018). More specifically, we used expression values of genes that were orthologue across all the considered four species. We determined orthologues across all four species (zebrafish, killifish, human and mouse) using the R-package orthology. We considered only genes with an orthologue in all four species which had detectable expression across all studied tissues and species (RPKM>0). After removing genes that did not pass the ANOVA-based testing procedure 3748 genes remained [see the Methods section of (*Aramillo Irizar* et al., 2018) for details]. In order to reduce bias due to different reference gene sets, all analyses described in this work were performed using these 3748 genes as basis. The core ageing signature comprised genes, which were conserved over all tissues and species. Genes with a conserved pattern for all species but only one tissue were assigned to the tissue ageing signatures. The specific ageing signatures contained all genes, which were associated with ageing only for one tissue in one species.

Differentially expressed genes were calculated for 13 cancer types investigated in the consortia 'International Cancer Genome Consortium' (ICGC) (Hudson et al., 2010) and 'The Cancer Genome Atlas' (TCGA). The following tissues were considered: lung (LUAD-US), breast (BRCA-US), prostate (PRAD-US), uterus (UCEC-US), kidney (KIRC-US), head and neck (HNSC-US), colorectal (COAD-US), liver (LIHC-US), bladder (BLCA-US), skin (SKCM-US), cervix (CESC-US), pancreas (PAAD-US), and ovary (OV-US). Read counts per gene were downloaded from the ICGC data portal (https://dcc.icgc.org/) (*International Cancer Genome Consortium* et al., 2010). Sample groups were compared with DeSeq2 v1.8.2. Thereby, gene outliers were replaced with trimmed mean value. All other disease data sets were collected from published studies and processed as described by *Aramillo Irizar* et al. (2018). Genes differentially expressed between germ-free and conventionally raised mice were taken from *Pan* et al. (2018). Only transcription data of adult mice (12 to 16 weeks of age) were considered in our study.

Enrichment analyses

Gene set enrichment analyses for KEGG pathways and transcription factor binding sites (TFBS) were performed with the online tool innateDB (*Lynn* et al., 2008). To test for significance, a hypergeometric test was applied. Gene set enrichment analyses for Gene Ontology (GO) terms were conducted with the online tool g:profiler (*Reimand* et al., 2016). Exclusively the GO class "Biological processes" was considered. All p-values were corrected

for multiple testing using the Benjamini Hochberg approach (*Benjamini* and *Hochberg*, 1995). Protein-protein interaction networks were created with the R package igraph v1.2.1 based on the String database v10 using medium confidence for connection predictions.

Enrichment of ageing signature genes in disease-associated genes

The enrichment score was calculated as [(<number of genes in ageing signature> / <number of disease associated genes>) / (<number of genes in ageing signature> / <number of all investigated genes>)]. Thereby, the regulation direction was not considered.

Calculation of evolutionary conservation per gene

The latest Version (May 8th, 2015) of phylogenetic p-values (phyloP) from the PHAST software (*Pollard* et al., 2010) for multiple alignments of 99 vertebrate genomes against the human genome (*Siepel* et al., 2005) were obtained from the University of California, Santa Cruz's webserver under the address: "http://hgdownload.soe.ucsc.edu/goldenPath/hg38/phyloP100way/". We evaluated phyloP-100-way genetic conservation scores for every single base position of the signatures' genes. The phyloP scores of genes found in any of our ageing signature were averaged over all base-pairs of that gene, leaving us with one mean phyloP score per gene. Chromosome, gene and exon structures were based on the latest version of the human genome (hg38). The mean scores per gene were then compared across signatures.

RESULTS

While we determined commonly differentially regulated processes in our previous work (*Aramillo Irizar* et al., 2018), we applied this approach directly on the gene expression level to identify genes that show a conserved regulation across four vertebrate species (*Homo sapiens*, *Mus musculus*, *Danio rerio* and *Nothobranchius furzeri*) and across tissues (blood, brain, liver and skin). Thus, we obtained a core signature of ageing comprising 985 genes and tissue specific signatures comprising 789 genes for blood, 1423 genes for brain, 826 genes for liver and 1281 genes for skin (referred to as tissue signatures). Moreover, we compared those signatures to differentially expressed genes from ageing tissues in mice and humans (referred to as specific signatures) (*Aramillo Irizar* et al., 2018). Following the two central hypotheses, we investigated the overlap between the core and the tissue signatures of ageing (Figure 1). We found that the signatures of blood and liver were smaller in size than the core signature, while the brain and skin signatures were larger. Tissue signatures showed an average overlap of 38 % with the core genes. Thus, there is a large overlap of the tissue signatures with the overall core signature of ageing. Considering the specific signatures, we found that they showed an average overlap of 61 % with either core or tissue signatures demonstrating that the tissue-specific manifestation of ageing in individual species shows a high concordance with conserved ageing in the corresponding tissue across species (Figure 1).

Conservation and connectivity of the individual ageing signatures

In a first step, we investigated the phylogenetic conservation of genes belonging to the individual signatures. Please

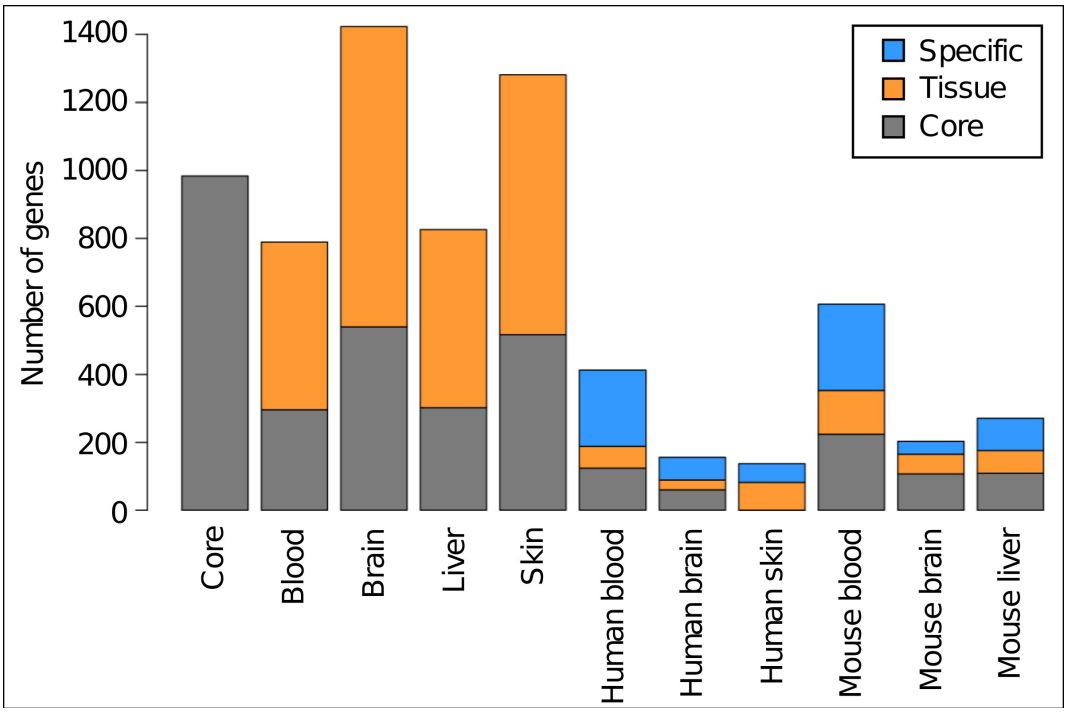

Figure 1: Number of genes involved in each ageing signature. A large proportion of the tissue ageing signature and the specific ageing signature is already enclosed in a more general ageing signature.

note that when considering tissue signatures, we excluded genes also belonging to the core signature. We found no significant differences regarding the phylogenetic conservation between the core signature and the tissue signatures (Figure 2A). Subsequently, we investigated the network context of the genes belonging to the ageing signatures based on their connectivity in the String database (*Szklarczyk* et al., 2015). Only interactions between genes belonging to one of the ageing signatures were considered. We found that genes belonging to the core signature tended to have fewer connections to other genes than the tissue signatures (Figure 2B-D). This was especially reflected in a higher number of nodes (= genes) with at least 50 different interaction partners, the so-called hub genes, in the tissue ageing signatures (Figure 2C). Also, from the ten proteins with highest connectivity, only one belonged to the core ageing signature (108 interaction partners). However, with 87 % the vast majority of proteins of the core signature remained unconnected.

Functional characterization of core vs. tissue-specific ageing

Next, we functionally characterized the genes belonging to individual tissues. We determined significantly enriched processes as well as enriched transcription factor binding sites in the individual ageing signatures.

Following the results from our previous work, particularly immune related pathways and cell cycle processes were enriched among the different ageing signatures. This is also in agreement with the central role of these

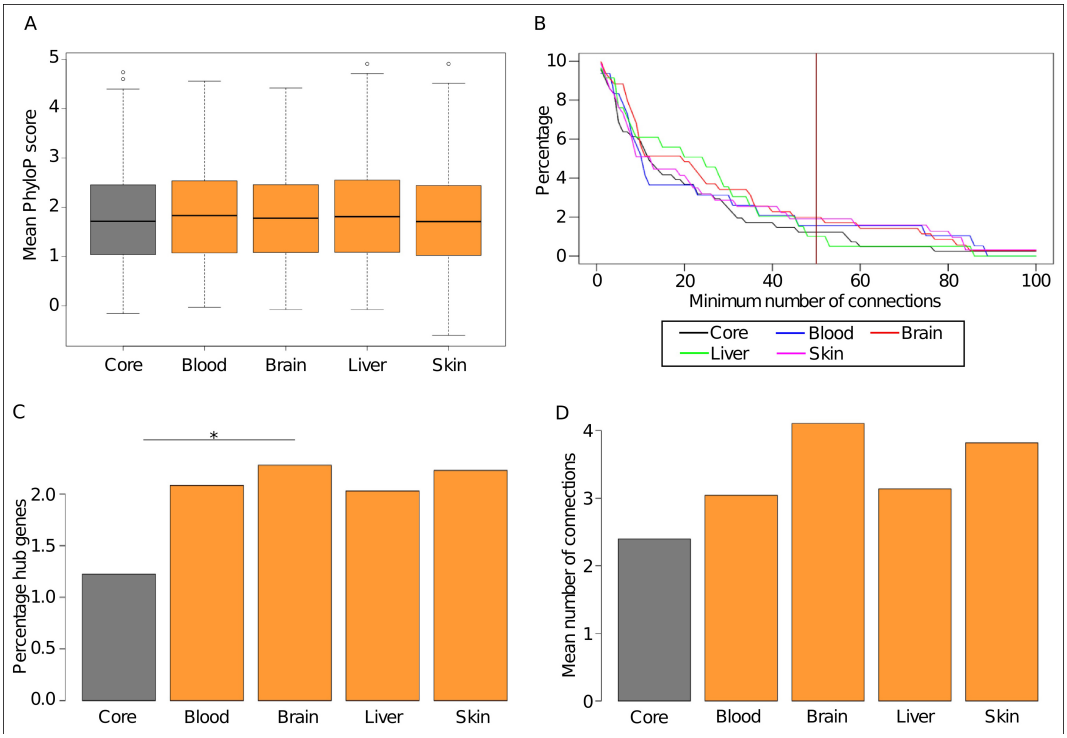

Figure 2: Characteristics of ageing signatures. (A) Genetic conservation of age-linked genes across species. The phyloP scores of genes found in the ageing signatures were averaged over all base-pairs of a gene, based on the human reference (hg38), and compared across signatures. (B) Number of connections within the interaction network based on all ageing-associated genes (core, tissue, and specific signature). Number of connections to all other nodes (independent of the signature) were counted. For better clarity, the y-axis is constrained to a plotting range of 0 % to 10 % and the x-axis to a maximal number of 100 connections (maximal number of connections is 309, three genes had more than 100 connections across all signatures). (C) Number of hub genes (genes with more than 50 connections) within the network described in (B). (D) Average number of connections within the network described in (B).

processes in ageing pathologies (*Chung* et al., 2009; *Campisi*, 2013). We performed gene set enrichment analyses based on gene ontology (GO) terms using once the complete tissue signatures and once the tissue signatures excluding genes of the core signature. We found the highest number of enriched processes for the core signature even in comparison to the brain and skin signatures which contained more genes (Figure 3A). Importantly, we found that most of the enriched processes in the tissue signatures were also enriched in the core signature. This was true both, when considering tissue signature genes not belonging to the core signature only, and when considering the full gene sets. Thus, a mean of 65.2 % of the functional groups (GO terms) enriched in the tissue signature were also enriched in the core signature when considering only genes specific to the tissue signature and 70.6 % when considering the entire gene set.

Testing for the enrichment of transcription factor binding sites (TFBS), we found an enrichment of transcription factors in the core signature, known to play a central role in the pathogenesis of ageing diseases, such as HIF-1 (involved in ischemic disease,

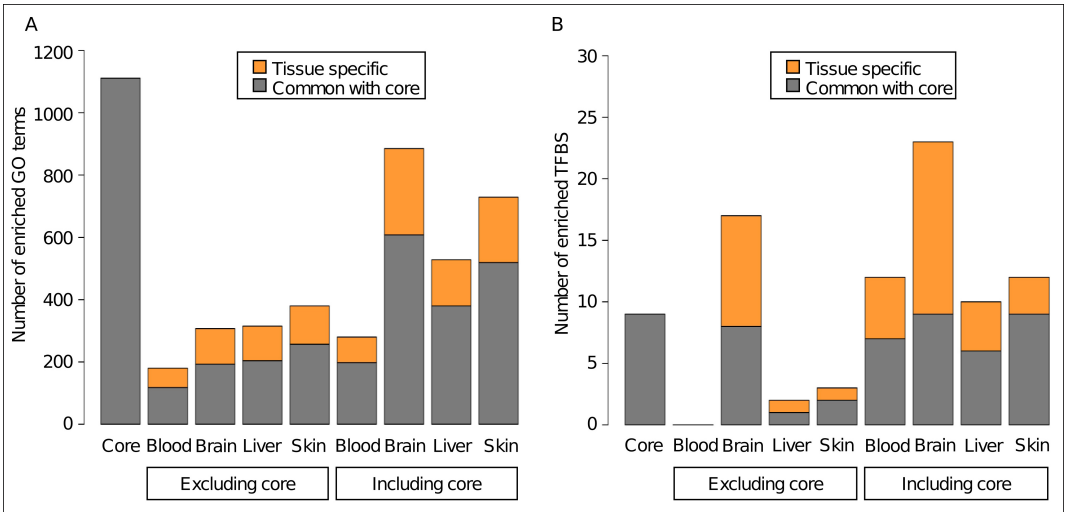

Figure 3: (A) Functional enrichment analysis using gene ontology (GO) cellular function terms. Number of GO terms enriched for each ageing signature. (B) Transcription factor enrichment analysis. Number of TFBS enriched for each ageing signature.

tumour angiogenesis), E2F-4 DP-2 (involved in tumour suppression, colorectal cancer), and E3F1 DP-2 (involved in tumour suppression).We observed a larger number of enriched transcription factors for the complete versus the reduced tissue signatures (Figure 3B). However, for all tissues except brain the enriched transcription factors showed a strong overlap with the core ageing signature: on average 54.6 % of the transcription factors enriched in the reduced tissue signatures were also enriched in the core signature and on average 58.1 % of transcription factors were enriched in the full gene sets.

Disease-specific characterization of ageing signatures

Since ageing is the most important risk factor for human disease (*Lopez* et al., 2006; *López-Otín* et al., 2013), we determined the enrichment of disease-regulated gene sets with genes belonging to the individual signatures of ageing. Specifically, we investigated to which extent genes, which are differentially expressed in cancer or ageing-associated degenerative diseases, showed an enrichment among genes belonging to the core or the tissue-specific signatures (Figure 4). While cancer-associated genes showed a significant enrichment among genes forming the core signature of ageing (Fisher's exact test p-value = 0.0461), they showed a significant depletion among the tissue-specific signatures (Fisher's exact test p-value = 0.0187). In contrast, for degenerative diseases the enrichment among genes belonging to the core ageing signature was less pronounced, while we observed a larger number of cases of enrichment among gene sets belonging to the tissue-specific ageing signature. In this context it is also important to emphasize that cancer-associated genes exclusively originated from the affected tissue while deregulated genes from degenerative diseases originated mostly from blood expression data [cf. (*Aramillo Irizar* et al., 2018) for details]. Thus, the comparison to the blood signature is likely most representative for the degenerative disease signatures.

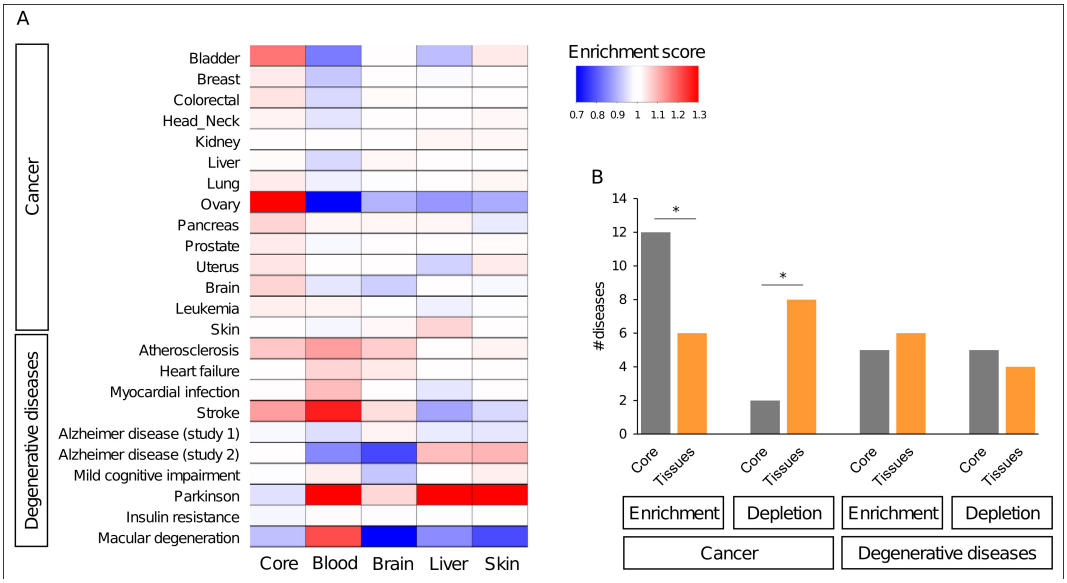

Figure 4: (A) Enrichment of genes belonging to the individual signatures among genes deregulated in different ageing diseases. (B) Frequency of enrichment of disease-deregulated genes in core and tissue signatures. For the three tissue signatures, average values across the four tissues are shown.

The microbiome is a modulator of the core signature of ageing

Next, we investigated to which extent the different ageing signatures were associated with the host response to microbial colonization. Hence, we compared the signatures to gene expression differences in colon of adult mice possessing a microbiome compared to germ-free mice (*Pan* et al., 2018) (Figure 5). Of the 3748 genes considered for the ageing signatures, 355 showed a differential expression in germ-free versus conventionally raised mice. We found that in particular the core signature of ageing showed an enrichment of genes differentially expressed during microbial colonization (Fisher's exact test p-value = 0.022) while there was no enrichment of the other signatures. In contrast, the core signature of ageing showed a significant enrichment of microbiome-regulated genes in comparison to brain and liver signatures. Intriguingly, comparing the direction of changes between colonized mice versus germ-free mice with the direction of changes observed in the ageing signature, 68 % of the genes belonging to the core signature showed an opposing direction of regulation during colonization.

DISCUSSION

While we identified a common signature of ageing in our previous work (*Aramillo Irizar* et al., 2018), we here explored the tissue-specific representation of this signature. Specifically, we investigated two partially competing hypotheses in ageing research that 1) ageing is driven by a common underlying process across all tissues or 2) that ageing on the organismal level is

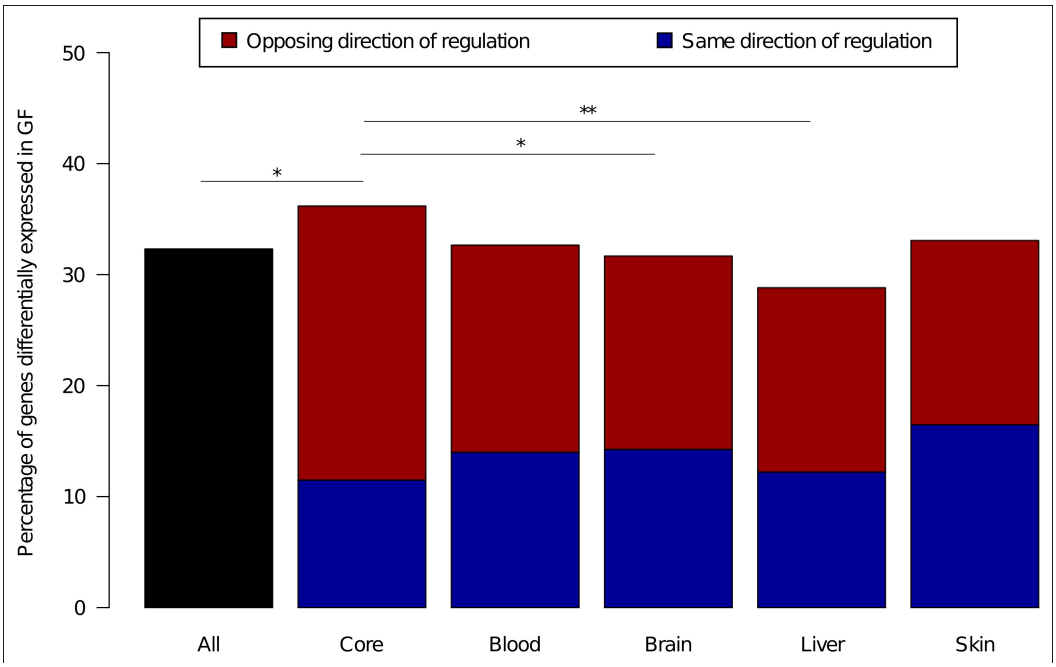

Figure 5: Comparison between ageing signatures and differentially expressed genes in response to microbial colonization. "All" represents the total percentage of all considered genes differentially expressed upon microbial colonization. For each signature the fraction of genes showing the same or opposing direction of regulation are indicated. "Same direction of regulation" corresponds to genes that pointed in the same direction of response in germfree versus colonized mice like in young vs. old mice.

mostly a consequence of functional deteriorations in individual organs.

While we found mostly no differences in phylogenetic conservation between the core and tissue-specific signatures of ageing, the tissue-specific signatures tended to show a stronger degree of interactions. On the functional and the regulatory level tissue-specific signatures showed a very strong overlap to the core signature of ageing: Most processes or transcription factor binding sites enriched for genes belonging to the tissue ageing signatures showed also an enrichment for the genes of the core ageing signature. This supports the hypothesis that ageing in individual tissues probably just represents different tissue-level manifestations of the same underlying processes driving the core ageing signature. On the molecular level, the observed differences in the tissue-specific signatures compared to the core ageing signature represent probably just tissue-specific downstream consequences of a process' deregulations by the core ageing signature. These downstream consequences could arise, for instance, due to the induction of tissue-specific compensatory processes or due to a reallocation of cellular resources away from tissue-specific processes to processes that need to be induced as part of the core ageing signature.

Testing for enrichment of disease-regulated genes among the individual signatures revealed that cancer-regulated genes tend to show a stronger enrichment among genes belonging to the core signature of ageing compared to the tissue-specific signatures of ageing. In contrast, degenerative diseases of ageing did not show this tendency.

In our previous work we hypothesized that the antagonism between cancer and degenerative diseases might be driven by an accumulation of genomic damage with age (*Aramillo Irizar* et al., 2018). This accumulation of damage leads to an induction of processes aimed at the suppression of potentially malignant cells which, while to some extent preventing the proliferation of potential cancer cells, drives tissue degeneration and thereby degenerative diseases as a side effect. This hypothesis is well in line with our observation of the enrichment of disease-specific gene sets in the individual ageing signatures: While cancer-deregulated genes as a potential causative factor for the ageing signature are enriched among the core signature of ageing, genes deregulated in degenerative diseases do not show this tendency.

We moreover tested the influence of the microbiome as a potential driver of the core ageing signature. The core ageing signature was significantly enriched for genes differentially expressed between germ-free and conventionally raised mice, whereas no enrichment was found with the tissue-specific signatures of ageing. Importantly, microbial colonization was associated with an expression signature opposing the direction of regulation observed in the core signature of ageing. Microbial colonization seems to lead to a rejuvenation of the colon which is likely explained by a larger colon mass in conventional versus germ-free animals (*Wostmann*, 1981) thus requiring a stronger proliferation while the core signature of ageing is associated with an inhibition of cellular proliferation. These observations are in some contrast to previous observations about an extended lifespan of germ-free animals (*Thevaranjan* et al., 2018). However, the lifespan-effect is context-dependent. Thus, under caloric restriction germ-free mice show a shortened lifespan (*Tazume* et al., 1991). Moreover, to our knowledge lifespan effects of microbial colonization (i.e. after maturation) have not been tested yet.

Overall, these analyses tend to favour the first hypothesis postulating that ageing is driven by a common process across organs. The tissue-specific manifestation of disease-processes potentially represent downstream consequences of a deregulation of cellular processes in response to the core signature of ageing. Specifically, on a functional and regulatory level the tissue-specific signatures of ageing showed a very high degree of similarity to the core signature of ageing. Moreover, the enrichment of cancer-deregulated genes in the core signature of ageing and a depletion among tissue-specific signatures strongly supports our hypothesis that processes that are actually geared to suppress the proliferation of potential cancer cells are one of the main drivers of ageing-associated regulation conserved across tissues. This is also supported by the enrichment of genes deregulated in degenerative diseases in the tissue-specific signatures, which we suppose arose as a manifestation of the downstream consequences of the core signature in the individual tissues.

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