A reverse genetics cell-based evaluation of genes linked to healthy human tissue age

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ABSTRACT: We recently developed a binary (i.e., young vs. old) classifier using human muscle RNA profiles that accurately distinguished the age of multiple tissue types. Pathway analysis did not reveal regulators of these 150 genes, so we used reverse genetics and pharmacologic methods to explore regulation of gene expression. Using small interfering RNA, well-studied age-related factors (i.e., rapamycin, resveratrol, TNF-α, and staurosporine), quantitative real-time PCR and clustering analysis, we studied gene–gene interactions in human skeletal muscle and renal epithelial cells. Individual knockdown of 10 different age genes yielded a consistent pattern of gene expression in muscle and renal cells, similar to in vivo. Potential epigenetic interactions included HIST1H3E knockdown, leading to decreased PHF19 and PCDH9, and increased ICAM5 in muscle and renal cells, while ICAMS knockdown reduced HIST1H3E expression. Resveratrol, staurosporine, and TNF-α significantly regulated the in vivo aging genes, while only rapamycin perturbed the healthy-age gene expression signature in a manner consistent with in vivo. In vitro coordination of gene expression for this in vivo tissue age signature indicates a degree of direct coordination, and the observed link with mTOR activity suggests a direct link between a robust biomarker of healthy neuromuscular age and a major axis of life span in model systems.—Crossland, H., Atherton, P. J., Strömberg, A., Gustafsson, T., Timmons, J. A. A reverse genetics cell-based evaluation of genes linked to healthy human tissue age. FASEB J. 31, 000–000 (2017). www.fasebj.org

KEY WORDS: epigenetic ‧ neuromuscular ‧ rapamycin ‧ siRNA

Chronological age is a major correlate for many diseases, including Alzheimer disease, cancer, and cardiovascular diseases. It has been hypothesized that differences in the rate of biologic aging represent an important reason why, along with key environmental factors, each human can develop age-correlated disease at different chronological ages (1). For this reason, it is important that robust biomarkers of biologic age are developed to facilitate a more personalized approach to health care. For example, such a biomarker could be applied to middle-aged subjects for the purpose of offering disease prevention advice. The analysis of gene expression changes with aging using microarray technology represents one important technology to realize this aim. Indeed, several meta-analyses studying different tissues and organisms have been used in an attempt to identify common signatures of aging (2–6).

The majority of studies have used linear regression to build models that span <20 to >100 yr (6–8). However, when studying humans, this type of molecular profiling incorporates not only markers of age but also clinical disease and complex interactions with drug treatment that are currently impossible to deconvolute. Further, as a result of population stratification and changes in the genetic contribution to survival beyond 80 yr (9), these types of linear correlative approaches are problematic. We recently took a different approach: we used machine learning to distinguish muscle tissue obtained from healthy older subjects with functional capacity matched to younger subjects (i.e., a binary classifier and not a linear correlative model). Using thousands of samples, we validated a 150-gene RNA tissue diagnostic able to accurately (>90% performance in independent samples) classify young from older human muscle, brain, and skin tissue (5). Gene expression changes with age were hypothesized to be adaptive, and hence regulation of the gene expression pattern is potentially beneficial to health. Notably, given that the signature was discovered in human muscle, exercise training does not regulate the healthy aging profile.

This article includes supplemental data. Please visit http://www.fasebj.org to obtain this information.

ABBREVIATIONS: BSA, bovine serum albumin; mTOR, mechanistic target of rapamycin; qPCR, quantitative PCR; ROS, reactive oxygen species; siRNA, small interfering RNA

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cases and controls, this reproducible signature of tissue age was diagnostic for cognitive status in 2 independent cohorts as well as being related to renal function over a 12-yr follow-up period in older men belonging to a birth cohort and hence the same chronological age (10). In each study, the more the gene expression pattern matched the pattern observed in healthy older muscle tissue (5), the better the health status the older person had compared to someone with a similar chronological age but lower gene score.

Surprisingly, pathway and gene ontology analysis failed to identify any dominant pathway in the 150 genes (5). Individually, some of the 150 genes did show links to published age-related phenotypes, such as LMNA, a gene encoding a nuclear lamina protein that is linked to Hutchinson-Gilford progeria syndrome (11). However, for the majority of the genes, little was known about their biologic function, particularly in relation to aging. Nevertheless, our analysis in multiple human cohorts suggests that regulation of these genes may contribute to healthy aging, and investigation of how these genes are regulated or interact is therefore required to provide greater understanding into mechanisms of human aging.

The main aim of the present study was to determine whether interactions existed between individual members of the gene set, and in particular if we could replicate the pattern of regulation in vivo. To achieve this aim, we used small interfering RNA (siRNA)-mediated gene knockdown and putative pro- (staurosporine and TNF-α) or anti- (resveratrol and rapamycin) age-related molecules. We found that the age classifier genes demonstrated coordinated changes in vitro that paralleled the up-regulation of gene changes with age in vivo. Further, we found evidence that rapamycin induced expression changes reflecting the same directional changes observed in vivo in healthy human muscle with age.

MATERIALS AND METHODS

Cell culture, siRNA, and drug treatments

Human primary skeletal muscle cells were established from muscle biopsy samples from healthy young adult men as previously described (12). This procedure was approved by the ethics committee of the Karolinska Institutet (Stockholm, Sweden). Isolated myoblasts (passages 4 and 5) were cultured on uncoated 12-well plates in DMEM/Nutrient Mixture F-12 (Thermo Fisher Scientific, Waltham, MA, USA) or Silencer Select from Thermo Fisher Scientific, and 4-well plates in DMEM/F-12 (Thermo Fisher Scientific). Cells were cultured at 37°C with 5% CO2. Hence cells were treated for 24 h with the following chemicals: 100 nM rapamycin (Sigma-Aldrich), 50 μM resveratrol (Sigma-Aldrich), 10 ng/ml TNF-α (Sigma-Aldrich), and 10 nM staurosporine (Sigma-Aldrich). Bovine serum albumin [BSA; final concentration 0.0001% (w/v)] was used as a vehicle control for TNF-α, and DMSO [final concentration 0.03% (v/v)] was used as a vehicle control for rapamycin, resveratrol, and staurosporine. Cells were collected in Trizol 48 h after transfection.

For drug treatment studies, experiments were performed on human primary muscle cells 6 days after switching to differentiation medium and 24 h after a medium change. Myotubes were treated for 24 h with the following chemicals: 100 nM rapamycin (Sigma-Aldrich), 50 μM resveratrol (Sigma-Aldrich), 10 ng/ml TNF-α (Sigma-Aldrich), and 10 nM staurosporine (Sigma-Aldrich). Bovine serum albumin [BSA; final concentration 0.0001% (w/v)] was used as a vehicle control for TNF-α, and DMSO [final concentration 0.03% (v/v)] was used as a vehicle control for rapamycin, resveratrol, and staurosporine. Cells were collected in Trizol, and each experiment was repeated over 3 passages (n = 4 well replicates for each experiment).

Annexin V/propidium iodide staining of human renal epithelial cells

Human renal epithelial cells were cultured in 6-well plates and transfected with 20 nM siRNA at ~50% confluence. After 48 h, the medium was removed, and cells were washed in warm PBS. Cells were incubated in a solution containing annexin V–FITC conjugate and propidium iodide using an annexin V–FITC apoptosis detection kit (Sigma-Aldrich). After 10 min incubation at room temperature, cells were fixed using ice-cold methanol: acetone, washed in PBS, mounted, and stained with DAPI using Fluoroshield mounting medium with DAPI.

RNA extraction, cDNA synthesis, and real-time quantitative PCR

RNA was extracted using Trizol according to the manufacturer’s protocol. RNA was resuspended in 20 μl of RNase-free water and quantified using a NanoDrop (Thermo Fisher Scientific). RNA (500 ng) was reverse transcribed using the High Capacity cDNA synthesis kit (Thermo Fisher Scientific). cDNA was amplified using 1:5, and real-time quantitative PCR (qPCR) was performed using 1 μl cDNA in triplicate with 10 μl master mix containing the following: SYBR Select Master Mix (Thermo Fisher Scientific) and
shown). The forward AGTTCCCTGCCTCGGAGATA, reverse CFLAR, MBNL1, CACC; ACTCCTCAGCGTATGCCTCA; SIRT5, forward CTATGATAACTTTGGCGTGCTG, reverse CALR, TCCACAGGCGGT, reverse TAGCGATGGGGCTTCTTCAC; Statistical analyses...multiple testing. For drug treatment experiments, 2-way ANOVA between control and siRNA-treated groups, correcting for multiple testing. For drug treatment experiments, 2-way ANOVA...effect on mRNA expression. Our second aim with this analysis was initially used to test whether each compound had an overall effect on gene expression with aging. Overall, Fisher’s exact test allowed us to assess consistency (i.e., whether the drug response was similar or distinct from the in vivo pattern of changes with muscle tissue aging).

Distance-based cluster analysis was used assess the relationship between gene expression responses across all experiments. The analysis was done in the R statistical environment (https://cran.r-project.org) using the library package Library(gplots) and Euclidean-based distance, with the setting Complete. Gene expression was calculated as a percentage of control, and row- and column-based clustering was implemented. This approach represents a robust method for assessing how groups of conditions may or may not relate to each other (in this case using Euclidean distance) based on the accumulated influence of each phenotype measured (in this case gene expression). Principal component analysis was carried out on the published muscle gene expression data using the 150 probe sets. In R, the following command was implemented: pcaData <- prcomp(data(,)), center = T, scale = T, and the first 2 principal components were plotted and genes selected that fulfilled 2 criteria: 1) demonstration of distinct and measurable location in the principal component analysis plot and 2) detectable by real-time qPCR in the cell culture system.

RESULTS

Twenty genes were selected after root–mean–square normalized principal component analysis of the original biologic age model that distinguished young from old healthy human skeletal muscle with ~93% success (5). These 20 were chosen because they demonstrated a high degree of variance and because they were robustly expressed in both cell types used for the in vitro studies. We plotted the expression of these 20 genes in skeletal muscle profiles obtained from young (mean age 26 yr; n = 10), middle-aged (mean age 49 yr; n = 19), and old (mean age 70 yr; n = 16) healthy subjects using our previously published microarray data (4) to examine the changes across 4 decades (Fig. 1). This analysis demonstrated that there were several categories of patterns of gene expression change. Nine demonstrated a potentially linear pattern of change, while the remainder demonstrated a dramatic step either at young to middle age or middle age to old age. [This pattern is expected, as the selection process for the 150 genes relied on a nonlinear selection process (5).] As with the ontology/pathway analysis for the full 150 genes (5), this subset of 20 genes did not fall into any specific ontologic pathway. Furthermore, a manual search of the GenAge database revealed that only one of the 20 genes (LMNA) was found to be associated with aging in other model organisms. However, using a simple literature analysis, the known biologic functions attributed to the 20 genes was evaluated, with some biochemical or molecular properties relating to proposed mechanisms for aging (Table 1), including epigenetic regulation (CARM1, HIST1H3E), lipid metabolism (CD36), and stem cell function (RUNX1).

siRNA-mediated knockdown of age classifier genes in human skeletal muscle and kidney cells

Ten genes, down-regulated in vivo with muscle age (Fig 1), were selected for individual siRNA-mediated knockdown...
(2 independent siRNAs and a total of 60 independent cell cultures) to examine the interrelationship between each gene and the remaining 19 from the age classifier (5). Because the classifier was diagnostic for age across different tissue types, we wished to establish if any individual gene knockdown mediated responses that were consistent across more than one human cell type. We chose to use human primary skeletal muscle cells because the age gene classifier was built on skeletal muscle tissue and renal epithelial cells, as the age genes were shown to be correlated with renal function over a 12-yr span (5). We verified that the siRNAs did not cause any obvious adverse effects related to cell health/viability using renal cells used for immunofluorescence staining against annexin V and propidium iodide to assess apoptosis status and evidence for necrosis, respectively (Supplemental Fig. 1). Initial real-time qPCR analysis revealed no effect of the transfection reagent on any of the genes analyzed (data not shown) in either cell type.

Individual knockdown of the 10 selected age classifier genes induced multiple expression changes in the remaining 19 genes. To determine the global relationship across all siRNA experiments, we used distance-based clustering (Euclidean) and grouped the entire data set across all siRNA experiments, we used distance-based clustering (Euclidean) and grouped the entire data set.

Overall, the consistent response in both renal and muscle cells supports our original in vivo analysis (5).

Clustering by gene expression rather than by siRNA treatment revealed an intriguing result, where many of the genes (CD36, MBNL1, PCDH9, RBMS9, and TGFBR3) that are up-regulated in vivo with age in healthy muscle were up-regulated across all experiments (regardless of which gene was being down-regulated). This indicates that these genes may be up-regulated in vivo as a secondary response to down-regulation of key members of the 150 genes. We also noted, as expected, that genes down-regulated in vivo clustered together (Fig. 2B), partly reflecting the fact that we experimentally knocked down half of those genes while the remaining genes that were down-regulated in vivo nevertheless clustered with these down-regulated genes and not with the distinct group of in vivo up-regulated genes.

There were some cell-type-specific patterns; for example, in human primary skeletal muscle cells, knockdown of ICAM5, HIST1H3E, SHISA4, RUNX1 AIMP2, LMNA, and SLC38A10 had a significant impact on the other age genes overall (P < 0.05, P < 0.05, P < 0.05, P < 0.01, P < 0.01, P < 0.001 vs. control, respectively; 2-way ANOVA main effect; Fig. 3A, C), whereas siRNA-mediated knockdown of NPEPL1, CARM1, and PHF19 mRNA had minimal impact on expression of the other age genes in these cells (Fig. 4). In human primary renal epithelial cells, ICAM5, HIST1H3E, and AIMP2 had an overall effect on classifier gene expression (P < 0.001, P < 0.05, P < 0.001 vs. control, respectively; 2-way ANOVA main effect; Fig. 3B, D), whereas the remaining genes had little impact (Fig. 5).

In muscle cells, knockdown of ICAM5 caused a significant change in expression of 4 of the other age genes (HIST1H3E was decreased and RUNX1, CARM1, and SIRT5 were increased (Fig. 3A). ICAM5 knockdown in kidney cells also down-regulated HIST1H3E (along with PHF19), while SHISA4, TGFBR3, CD36, and PCDH9 were up-regulated (Fig. 3B).

**Figure 1.** Expression intensity values for 20 age classifier genes (5) using previously published microarray data from human skeletal muscle across different age groups (4). Bars represent means ± SEM, and data are normalized to average of young group. Red/blue bars represent down-/up-regulated genes with aging in vivo.
affected several age genes with a relatively consistent effect across muscle and renal cells (Fig. 3C, D). Thus, the response of \textit{HIST1H3E} appeared consistent across cell types, and \textit{HIST1H3E} was the most regulated gene \textit{in vivo}. Knockdown of the remaining 8 genes had varying effects on expression of the other age classifier genes (Figs. 4 and 5). Overall, the degree of statistical significance for individual genes will be reflective of sample size, and in general, the more powerful methodology—cluster analysis—revealed coordinated gene expression changes.

\textbf{Effects of selected age-associated chemical mediators on expression of age classifier genes}

Another key facet warranting exploration is the impact of putative positive/negative regulators of tissue aging on healthy tissue age genes. Thus, skeletal muscle cells were treated with selected age-related factors in an effort to assess if each tissue age gene might be regulated by these aging-related chemicals. For each individual treatment, control genes known to be associated with that compound were measured as confirmation of activity in our cells (Supplemental Fig. 2). Initial experiments also verified that the vehicle controls DMSO and BSA had no effect on expression of the 20 age genes (Supplemental Fig. 3).

Resveratrol is a naturally occurring polyphenolic compound with antioxidant activity that activates \textit{SIRT1}, an NAD\textsuperscript{+}-dependent histone deacetylase, and possibly \textit{AMPK} (14, 15). Resveratrol has reported antiaging effects in yeast (14), flies (16), and, in some studies, mice (17, 18). Treatment of differentiated skeletal muscle cells with resveratrol had an overall effect on expression of the age classifier genes as assessed by ANOVA ($P < 0.05$; Fig. 6).

\begin{table}[h]
\centering
\caption{Selected age classifier genes and their known biologic functions}
\begin{tabular}{llll}
\hline
No. & Gene symbol & Gene name & Direction with age & Biology \\
\hline
1 & \textit{RUNX1} & Runt-related transcription factor 1 & Down & Transcription factor; differentiation of hemopoietic stem cells \\
2 & \textit{PHF19} & PHD finger protein 19 & Down & Binds methylated histone H3 and recruits polycomb repressive complex 2 \\
3 & \textit{ICAM5} & Intercellular adhesion molecule 5, telencephalon & Down & Transmembrane glycoprotein involved in adhesion \\
4 & \textit{SLC38A10} & Solute carrier family 38, member 10 & Down & Putative neutral amino acid transporter \\
5 & \textit{SIRT5} & Sirtuin 5 & Down & NAD-dependent protein deacetylase; mitochondrial replication-dependent histone; core component of nucleosome \\
6 & \textit{HIST1H3E} & Histone cluster 1, H3e & Down & Calcium binding protein in endoplasmic reticulum; protein folding; possible nuclear receptor modulation \\
7 & \textit{CALR} & Calreticulin & Down & Calcium binding protein in endoplasmic reticulum; protein folding; possible nuclear receptor modulation \\
8 & \textit{NPEPL1} & Aminopeptidase-like 1 & Down & May catalyze removal of unsubstituted N-terminal AA from various peptides \\
9 & \textit{CARM1} & Coactivator-associated arginine methyltransferase 1 & Down & May catalyze removal of unsubstituted N-terminal AA from various peptides \\
10 & \textit{LMNA} & Lamin A/C & Down & Mutation linked to Hutchinson-Gilford progeria syndrome; nuclear membrane structural component; DNA replication; chromatin organization \\
11 & \textit{SHISA4} & Shisa homolog 4 (\textit{Xenopus laevis}) & Down & Transmembrane protein; may inhibit Wnt and FGF signaling \\
12 & \textit{AIMP2} & Aminoacyl tRNA synthetase complex-interacting multifunctional protein 2 & Down & Required for aminoacyl-tRNA synthetase complex assembly; proapoptotic \\
13 & \textit{TGFBR3} & TGF, $\beta$ receptor III ($\beta$-glycan, 300 kDa) & Up & Membrane proteoglycan that acts as coreceptor with other TGF-$\beta$ receptor superfamily members \\
14 & \textit{CD36} & CD36 molecule (thrombospondin receptor) & Up & Receptor for oxidized lipids \\
15 & \textit{MBNL1} & Muscle blind-like 1 & Up & RNA binding; regulates splicing \\
16 & \textit{SKAP2} & Src kinase-associated phosphoprotein 2 & Up & RNA binding; regulates splicing \\
17 & \textit{CFLAR} & CASP8- and FADD-like apoptosis regulator & Up & Apoptosis regulator; lacks caspase activity; overexpression impacts on muscle satellite cell proliferation \\
18 & \textit{PCDH9} & Protocadherin 9 & Up & Ca\textsuperscript{2+}-dependent transmembrane protein important in cell adhesion in neural tissues \\
19 & \textit{RBMS3} & RNA binding motif, single-stranded interacting protein 3 & Up & RNA binding protein \\
20 & \textit{ALDH6A1} & Aldehyde dehydrogenase 6 family, member A1 & Up & Mitochondrial; valine and pyrimidine catabolic pathways \\
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pattern of in vivo changes seen between old and young tissue ($P = 0.0033$ with Fisher’s exact test). Induction of SIRT1 and AMPK gene expression by resveratrol confirmed expected features of resveratrol treatment (Supplemental Fig. 2).

Rapamycin is an antibiotic that binds to and inhibits mTOR, a nutrient-responsive kinase that influences metabolism and cellular growth via the PI3K/AKT pathway (19). Genetic or pharmacologic inhibition of mTOR extends the life span of Caenorhabditis elegans (20), Saccharomyces cerevisiae (21), and mice (18, 22). Rapamycin treatment reduced EIF4E and S6K, as expected (Supplemental Fig. 2). Rapamycin also had an effect on expression of the age classifier genes ($P < 0.05$ by 2-way ANOVA) and moreover induced a pattern of change that was not different from the pattern of changes noted in vivo with tissue age (Fig. 6B; $P = 0.2273$ with Fisher’s exact test). Thus, rapamycin treatment altered gene expression in our cell studies in a manner similar to that observed with tissue age in healthy old muscle.

There are also compounds that actively regulate detrimental age-related phenotypes. It has been proposed that reduced autophagic capacity with aging may be responsible for increased expression of proinflammatory mediators that result from mitochondrial dysfunction and increased reactive oxygen species (ROS) production (23). TNF-α was also chosen as a proaging factor because aging in older humans has been correlated with a proinflammatory phenotype (24). Staurosporine is a potent inducer of oxidative stress, and excessive ROS can impair oxidative phosphorylation and promote accumulation of mitochondrial DNA mutations, leading to widespread cellular damage and numerous features of aging (25, 26). Muscle cells were treated for 24 h with TNF-α and staurosporine, and changes in expression of positive control (Supplemental

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**Figure 2.** Heat maps of siRNA experiment data. Distance-based clustering (Euclidean) was used to determine global relationship across all siRNA experiments, grouping data set according to either siRNA target (A) or by age gene response (B). Each column represents mRNA expression data from single siRNA experiment, with every row being 1 of 20 measured genes. Letters “k” and “m” preceding each gene symbol represents data from kidney and muscle cells, respectively. Seventy percent of siRNA-targeted genes produced relatively consistent pattern of gene expression responses in both muscle and renal cells. Colors represent magnitude of change in expression for each gene (red, down-regulated; blue, up-regulated) with siRNA treatment vs. controls.
Fig. 2) and age classifier genes were examined. There was a main effect of both TNF-α and staurosporine treatment on gene expression (both $P < 0.05$ vs. vehicle controls; Fig. 6C, D), but again these changes were significantly different from the pattern of changes observed in vivo with aging ($P = 0.0004$ for staurosporine, $P < 0.0001$ for TNF-α with Fisher’s exact test). Thus, only rapamycin demonstrated an in vitro response that was consistent with what was observed in vivo.

DISCUSSION

Extensive studies in animal models and human tissues have been undertaken to gain insights into the process of aging and have identified many genes that show age-dependent changes in expression (4, 27, 28). Nonetheless, in most cases, each individual study identified a different set of genes or did not apply rigorous classification methods to verify consistency. Further, for all of the epidemiologically derived molecular signatures of aging, the identified patterns of gene expression incorporate the additional influences of clinical disease and/or drug treatment (6, 29) and thus do not represent aging per se. The present study was undertaken to examine the regulation of our recently identified gene expression signature derived from healthy older muscle tissue (5). This set of 150 genes was able to reproducibly classify young vs. older human muscle, brain, and skin tissue across 7 independent cohorts. However, pathway analysis showed no enrichment for any particular biologic process. Thus, analyses into the regulation of these genes could help our understanding of healthy aging processes in humans if we could regulate the signature in human cells with drugs or if one or more genes in the list regulated the other members of the signature.

Our first set of experiments aimed to address whether there were interactions between the age classifier genes that could be driving the changes in expression with aging. In doing so, we found that none of the individual age genes, when down-regulated using siRNA, exactly mimicked alterations in mRNA seen in vivo, indicating it to be unlikely that a single gene is driving our RNA signature. Nonetheless, when the entire pattern of in vitro gene expression was examined, those genes up-regulated in vivo clustered together as a distinct group in the in vitro data, indicating a coordinated response to siRNA-mediated loss of expression of genes seen to be down-regulated in healthier older tissue in vivo. Whereas some genes analyzed had little impact on expression of other age genes (PHF19, CARM1, NPEPL1), clear interactions between other genes were observed, motivating the need for future investigation of the entire 150-gene set to identify the
nature of the gene networks comprising the healthy aging signature.

One gene that influenced several of the age genes was *HIST1H3E*. There is accumulating evidence that widespread epigenetic changes occur throughout life, including changes to histone posttranslational modifications and DNA methylation patterns (30–32). For instance, deletion of histone methylation complex components extends life span in worms and flies (33, 34). The *HIST1H3E* gene encodes the H3.1, a core component of

**Figure 4.** Impact of classifier gene knockdown on expression of age classifier genes in human skeletal muscle cells. Data are normalized to β-actin and expressed relative to controls (transfection reagent only). Each bar represents mean ± SEM from 2 to 3 independent experiments and for 2 different siRNAs targeting *RUNX1* (A), *SHISA4* (B), *AIMP2* (C), *LMNA* (D), *SLC38A10* (E), *NPEPL1* (F), *CARM1* (G), or *PHF19* (H) (n = 9–12 cell culture well replicates). Red/blue bars represent down-/up-regulated genes with aging *in vivo*. *P < 0.05, **P < 0.01, ***P < 0.001 vs. transfection reagent controls.
the nucleosome. Knockdown of HIST1H3E induced similar changes in both kidney epithelial and skeletal muscle cells, causing a reciprocal increase in the expression of ICAM5 and SHISA4, and down-regulation of PHF19 and PCDH9. PHF19 is a polycomb-encoding gene that functionally interacts with methylated histone H3 (35). SHISA4, PCDH9, and ICAM5 all encode cell surface proteins, but their potential links to

Figure 5. Impact of classifier gene knockdown on expression of age classifier genes in human kidney cells. Data are normalized to β-actin and expressed relative to controls (transfection reagent only). Bars represent means ± SEM from 2 to 3 independent experiments and for 2 different siRNAs targeting RUNX1 (A), SHISA4 (B), AIMP2 (C), LMNA (D), SLC38A10 (E), NPEPL1 (F), CARM1 (G), or PHF19 (H) (n = 9–12 cell culture well replicates). Red/blue bars represent down-/up-regulated genes with aging in vivo. *P < 0.05, **P < 0.01, ***P < 0.001 vs. transfection reagent controls.
epigenetic processes remain unclear. We analyzed microarray expression patterns of additional histone encoding genes within the same histone cluster as HIST1H3E across age in a skeletal muscle data set (Supplemental Fig. 4) to determine whether this age-dependent change was unique to the HIST1H3E gene. Although some genes within the cluster were down-regulated with age, changes in HIST1H3E were the most marked.

ICAM5 also regulated age gene expression, but it appeared to affect different genes in kidney and skeletal muscle cells. ICAM5 knockdown caused a reduction in HIST1H3E expression in both cell types, further supporting a regulatory link between this adhesion molecule and histone expression. In skeletal muscle cells, ICAM5 influenced expression of RUNX1, a transcription factor linked to histone deacetylases; CARM1, a methyl-transferase that can methylate histones; and SIRT5, a protein deacetylase. This indicates that ICAM5 may have an important role regulating gene expression and histone modification processes. Thus, further work will be required to explore the potential role of ICAM5 in relation to aging and the possible link between alterations in intercellular communication and epigenetic regulation.

Figure 6. Impact of resveratrol (A), rapamycin (B), TNF-α (C), and staurosporine (D) treatment on expression of age classifier genes in skeletal muscle cells. Cells were treated for 24 h with 100 nM rapamycin, 50 μM resveratrol, 10 ng/ml TNF-α, or 10 nM staurosporine (n = 3), and experiments were performed over 3 passages. Data are normalized to β-actin and expressed relative to DMSO or BSA controls. Blue/red bars represent down-/up-regulated genes with aging in vivo.

Thus, eventhought the gene with INT RNA signal are able to accurately classify age of multiple human tissues in vivo, the relative functional importance of these genes could vary in different cell types.

Although RUNX1 had little impact on overall expression of the age classifier genes, knockdown did cause a significant increase in CD36 expression in muscle cells. CD36 is a transmembrane glycoprotein that has previously been reported to increase with aging and may be important for clearance of circulating oxidized lipids (36). CD36 may also contribute to accumulation of lipids within cardiac myocytes and hence cardiomyopathy in aged mice (37). CD36 has been shown to contain RUNX-binding elements, and RUNX3 can repress CD36 expression in myeloid cells (38). Little is understood about the potential link of RUNX1 to age-related pathways, although it is a genomic mediator of adaptation to exercise (39, 40) and thus may be an example of a key determinant of age–gene–environmental interactions. Whether increased CD36 expression with aging is protective or damaging is unclear, but this change may be the result of reduced expression of RUNX transcription factors.

Lamins form a major component of the nuclear lamina, which is crucial for genomic stability because it provides a scaffold for chromatin and protein complexes (41). Mutation of the lamin genes cause accelerated aging syndromes such as Hutchinson-Gilford progeria syndrome. Thus, it represented an ideal target to study in relation to our age genes. It has been reported that normal aging is associated with accumulation of progerin (the aberrant prelamin A isoform), which may be linked to histone modification and DNA damage (42), a feature that accompanies aging. LMNA knockdown in kidney cells had little impact on age
gene expression, but in muscle, there appeared to be a general increase in mRNA abundance. This was also observed for AIMP2, a gene required for assembly of the aminoacyl-tRNA synthase complex (43), and SLC38A10, a neutral amino acid transporter. These observations suggest that reduced expression of these genes may have influenced general mRNA translation, resulting in general accumulation of mRNA transcripts. It would seem critical that a future analysis examines the posttranscriptional regulation of these markers of healthy older tissue using, for example analysis of the mRNA engaged with the translational machinery.

Our second major aim was to determine links between positive/negative aging compounds and our age genes. Each of the 4 age-related compounds screened caused significant changes in gene expression for positive control genes as assessed by ANOVA (Supplemental Fig. 2) and when compared to vehicle control, but only rapamycin yielded gene expression changes in a pattern that was consistent with what we observed in vivo with age (Fig 1) (5). Indeed, Fisher’s exact test revealed that the RNA profile of rapamycin was not significantly distinguishable from in vivo muscle aging (i.e., genes that were up-regulated in vivo were more frequently up-regulated by rapamycin, and vice versa). Although the directional changes and consistency with the in vivo pattern were established using unbiased statistical analysis, the directional overlap was not 100%. However, because we were comparing gene expression changes in a primary cell model system measured over periods of days to in vivo changes with aging over decades, one might not expect complete concordance. The protein kinase mTOR responds to a variety of signals including growth factors, energy status, stress, and amino acids to regulate cell growth and metabolism (19). mTOR forms part of 2 distinct complexes, mTORC1 and mTORC2; mTORC1 is a critical regulator of protein synthesis and autophagy, whereas mTORC2 may have a predominant role in glucose metabolism and cell survival. Rapamycin has been shown to specifically target mTORC1, but chronic treatment can also inhibit mTORC2 (44). With a growing number of studies reporting beneficial effects of rapamycin on extension of life span and health span (18, 20, 22), this indicates that these gene expression markers for healthy tissue age warrant further detailed investigation in relation to their potential regulation by or link with mTOR.

The effect of resveratrol on expression of the healthy age genes was assessed. Previous studies on resveratrol demonstrated its ability to extend life span in C. elegans, Drosophila melanogaster, and S. cerevisiae (14, 16). It was reported that the beneficial effects of resveratrol may be mediated through activation of sirtuins, though resveratrol can also activate AMPK signaling (14, 15). In rodents, reduced risk of death due to high-calorie feeding was observed in mice with resveratrol treatment (17). In humans, resveratrol has some reported benefits on metabolic parameters (insulin sensitivity, fat metabolism), mimicking a caloric restriction phenotype (45). In this study, changes in expression of the age genes did not significantly replicate the directional changes with healthy aging in vivo, despite seeing an effect of rapamycin, which is also viewed as a calorie-restriction mimetic. This implies that the age genes were not directly regulated by SIRT1- or AMPK-activated pathways.

We also examined whether our healthy aging RNA signature might be linked to any inflammatory or oxidative stress–related pathways. Inflammation-induced tissue damage has been proposed to accumulate with aging, potentially as a result of reduced autophagic capacity or buildup of senescent cells (23, 24). Accumulation of ROS is thought to lead to widespread cellular damage with aging, although conflicting evidence regarding the role of ROS in aging has been reported (26, 46, 47). Expression of many of the age classifier genes responded to treatment with TNF-α and staurosporine; however, the changes were inconsistent with the in vivo directional age changes, and it was clear that both treatments caused cell stress responses that would have mostly likely influenced thousands of other genes. This suggests that in vivo, these age genes are not being influenced by inflammatory or oxidative stress–related pathways.

In summary, we have demonstrated in vitro coordination of gene expression within our in vivo validated tissue age signature, as well as potential epigenetic interactions. Further analysis of the interactions between the entire 150 genes and their potential link to mTOR signaling is merited.
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