Cell Biology

Evidence for a role of human blood-borne factors in mediating age-associated changes in molecular circadian rhythms

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Abstract

Aging is associated with a number of physiologic changes including perturbed circadian rhythms; however, mechanisms by which rhythms are altered remain unknown. To test the idea that circulating factors mediate age-dependent changes in peripheral rhythms, we compared the ability of human serum from young and old individuals to synchronize circadian rhythms in culture. We collected blood from apparently healthy young (age 25-30) and old (age 70-76) individuals and used the serum to synchronize cultured fibroblasts. We found that young and old sera are equally competent at driving robust ~24h oscillations of a luciferase reporter driven by clock gene promoter. However, cyclic gene expression is affected, such that young and old sera drive cycling of different genes. While genes involved in the cell cycle and transcription/translation remain rhythmic in both conditions, genes identified by STRING and IPA analyses as associated with oxidative phosphorylation and Alzheimer's Disease lose rhythmicity in the aged condition. Also, the expression of cycling genes associated with cholesterol biosynthesis increases in the cells entrained with old serum. We did not observe a global difference in the distribution of phase between groups, but find that peak expression of several clock controlled genes (*PER3*, *NR1D1*, *NR1D2*, *CRY1*, *CRY2*, and *TEF*) lags in the cells synchronized with old serum. Taken together, these findings demonstrate that age dependent blood-borne factors affect peripheral circadian rhythms in cells and have the potential to impact health and disease via maintaining or disrupting rhythms respectively.

eLife assessment

The authors tested the hypothesis that age-dependent factors in human sera affect the core circadian clock or its outputs in cultured fibroblasts. While the paper does not provide molecular mechanistic insight how sera from young versus old donors lead to differences in cyclic gene expression, it does provide **fundamental** insights and a possible explanation for previous observations showing that circadian gene expression in peripheral tissues tend to dampened or phase-shift with age. As presented, the strength of the evidence is, however, **incomplete**.

Introduction

Circadian rhythms are known to regulate homeostatic physiology including sleep:wake, hormone production and body temperature, and their dysregulation with aging is accompanied by adverse health consequences[1–3], raising the possibility that health decline with age is caused in part by circadian dysfunction. Although the mechanisms responsible for age effects on

circadian rhythms are unknown, signals from the central clock in the suprachiasmatic nucleus (SCN) dampen with age[3–5] and rhythms change in peripheral tissues in different ways[5, 6]. Here we aimed to develop a culture model to study the effect of aging on human rhythms of peripheral tissues. Given that serum can reset the clock in peripheral fibroblasts[7], we questioned the extent to which serum factors normally contribute to peripheral rhythms of gene expression and how they might affect rhythms with age, given that blood-borne factors can influence other aspects of aging[8].

We collected blood samples from young (age 25-30) and old (age 70-76) apparently healthy individuals following observations of their behavior and physiology, and tested the hypothesis that age-dependent factors in the sera affect the core clock or outputs of the core clock in cultured fibroblasts. In support of this theory, here we show that genes associated with oxidative phosphorylation and mitochondrial functions lose rhythmicity in the presence of aged serum factors. We also find that the expression of several molecular clock genes (*PER3*, *NR1D1*, *NR1D2*, *CRY1*, *CRY2* and *TEF*) is phase delayed when synchronized with aged serum. These novel findings suggest that it may be possible to treat impaired circadian physiology and the associated disease risks by targeting blood borne factors.

Results

We enrolled 8 old and 7 young human subjects (**Fig.1A**), whose demographics are in **Table S1**. Midline Estimating Statistic of Rhythm: a rhythm-adjusted mean (MESOR) data obtained from wearable EKG demonstrated that heart rate in old subjects trended lower with increased variability (**Fig. 1B**&**C**). Young subjects displayed a trend of higher activity in the sympathetic nervous system (SNS), and lower activity in the parasympathetic nervous system (PNS) (**Fig S1A**), with no significant difference in cortisol levels (**Fig S1B**).

To monitor rhythms in culture, *BMAL1-luciferase* transfected BJ-5TA fibroblasts[9] were synchronized with old/young serum[7, 10, 11], and circadian effects were assessed by luciferase assay[9] over 4 days (**Fig S2A**). No significant differences were observed in the period, amplitude, and phase of the *BMAL1-luciferase* rhythm with young versus old serum treatment (**Fig S2B**).

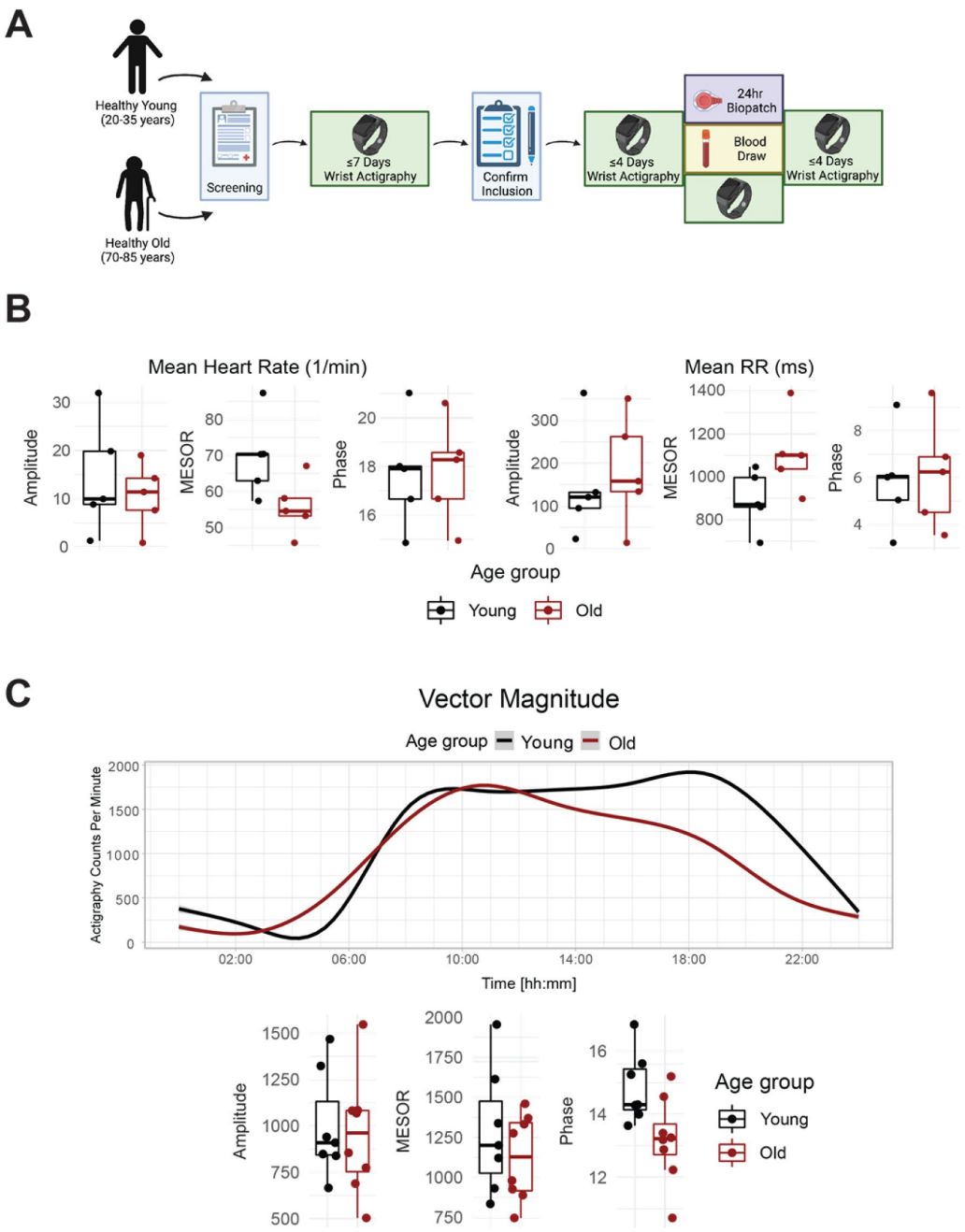

Fig 1.

Healthy elderly individuals tend to have lower heart rate, increased heart rate variability, and phase advanced activity patterns relative to young subjects.

Experimental protocol for enrolment and monitoring of human subjects **(A)**. As assessed by Zyphyr BioPatch, EKG measurements suggest that MESOR of average heart rate decreases with age (p=0.056) and that MESOR of heart rate variability increases with age (p=0.056). N=5 per group. MESOR and amplitude were tested using two-sided Wilcoxon rank sum exact test, while phase was tested by Kuiper's two-sample test **(B)**. Average activity counts across three axes (vector magnitude), as recorded by the actigraph device plotted throughout the day (top) and analyzed for circadian rhythm (bottom) **(C)**. While the amplitudes of activity did not differ between the age groups, the older individuals trended towards an early phase (p~0.055, Kupier's two-sample test) compared to young individuals. N=7 for young and N=8 for old. Lines in the top panel of C are smoothed means (fit with penalized cubic regression splines) for data from each age group. Dots in the bottom panel of C are subject-level cosinor parameter estimates derived from cosinor fits to the actigaraphy data. Summary Figure (A) was made with BioRender.com. Boxplot midlines correspond to median values, while the lower and upper hinges correspond to the first and their quartiles, respectively. Boxplot whiskers extend to the smallest/largest points within 1.5 * IQR (Inter Quartile Range) of the lower/upper hinge.

To assess serum effects on circadian gene expression, we first performed RNA-seq around-the-clock on fibroblasts synchronized with serum from a single old or single young individual and found that, compared to the first day of synchronization (CT12-34), the second day (CT 36-58) showed greater differences in MESORs between young and old serum-treated groups (**Fig. S3**). This is not surprising because the first day includes acute responses of the fibroblasts to serum, which can mask circadian rhythms, and so the second day is expected to reveal differences in endogenous rhythms between samples. Day 2 also revealed different phases of cyclic expression between young and old groups for a larger number of genes. We proceeded to collect fibroblasts synchronized by sera from eight different subjects (four old, four young with two male and two female per group) at two-hour intervals, from 32 to 58 hours post serum addition. This added an extra two timepoints to the second day to facilitate the calculation of rhythmicity. Using CircaCompare[12] and a weighted BIC>0.75 cutoff, a significant number of genes were found to lose (1519 genes) or gain (637 genes) rhythmicity with age, underscoring the impact of age on the ability of serum to synchronize circadian rhythms, while 1209 genes were rhythmic in both groups (**Fig 2A**). Additionally, we used CircaCompare to estimate MESORs, amplitudes, and phases of gene oscillatory patterns in young and old groups, and to compare these cosinor parameters between groups (**Fig 2B**). Of the genes that were rhythmic in both groups, many also showed changes with age. For instance, 568 cyclically expressed genes showed a change in MESOR with age ($q < 0.05$ for MESOR differences), with MESOR values increasing for 163 genes and decreasing for 405 genes in the old serum-treated samples. Using p-values provided by CircaCompare we were able to detect changes in amplitude (**Fig 2C**) for only a small number of genes (39 genes had decreased amplitude in old and 2 had increased amplitude). Using CircaCompare and a $q < 0.05$ cutoff for phase differences in genes rhythmic in young and old, we detected 20 genes with advanced phase, and 34 with delayed phase (**Fig 2D**).

Search Tool for the Retrieval of Interacting Genes (STRING)[13] and Ingenuity Pathway Analysis (IPA)[14] were used for functional genomics, and both approaches indicate a maintenance of cycling of cell cycle genes, and a loss of rhythmicity of genes associated with oxidative phosphorylation in the aged serum (**Fig 3** and S4). STRING analysis revealed that the dominant pathways that are rhythmic in both young and old conditions, cell cycle and DNA replication demonstrate a decrease in MESOR with old serum. Checkpoint control and chromosomal replication pathways were expressed cyclically in both young and old conditions; however, several chromosomal replication pathway genes exhibit decreased MESORs in the aged sera (**Fig S4 D,E**, **Table S2**). MESORs of steroid biosynthesis genes, particularly those, relating to cholesterol biosynthesis, were also increased in the old sera condition (**Fig 3**, **Table S3**).

As noted, the pathways that lost rhythmicity in the old sera included oxidative phosphorylation, implicating mitochondrial dysfunction. By STRING analysis, 24 out of the 26 genes associated with oxidative phosphorylation are also among the Alzheimer's Disease STRING network highlighting the disease relevance of this class of genes that loses cycling in older individuals. Given that Alzheimer's pathology is closely associated with the accumulation of oxidative stress[15], it is possible that loss of cycling contributes to oxidative damage. An additional 31 genes in the Alzheimer's Disease STRING network lose rhythmicity with aged serum; these include amyloid precursor protein (APP) and apolipoprotein E (APOE). APP is by definition the precursor of amyloid beta, which accumulates in AD[16]. While APOE plays an important role in lipid transport, specific variants of this gene are strongly associated with AD risk as APOE interacts with amyloid beta in amyloid plaques, a hallmark of the disease [17].

Lastly, several clock genes showed differences in expression with the aged serum, most notably genes in the Circadian Rhythm KEGG pathway (**Fig 4**). In particular, expression of *CRY1*, *CRY2*, *NR1D1*, *NR1D2*, *PER3*, and *TEF* was significantly phase delayed after synchronization with old serum (**Fig 4**). Importantly, the RNA-seq did not reveal a difference in the phase or amplitude of *BMAL1* expression with age, supporting our *BMAL1-luciferase* findings, although the MESOR significantly increased with age.

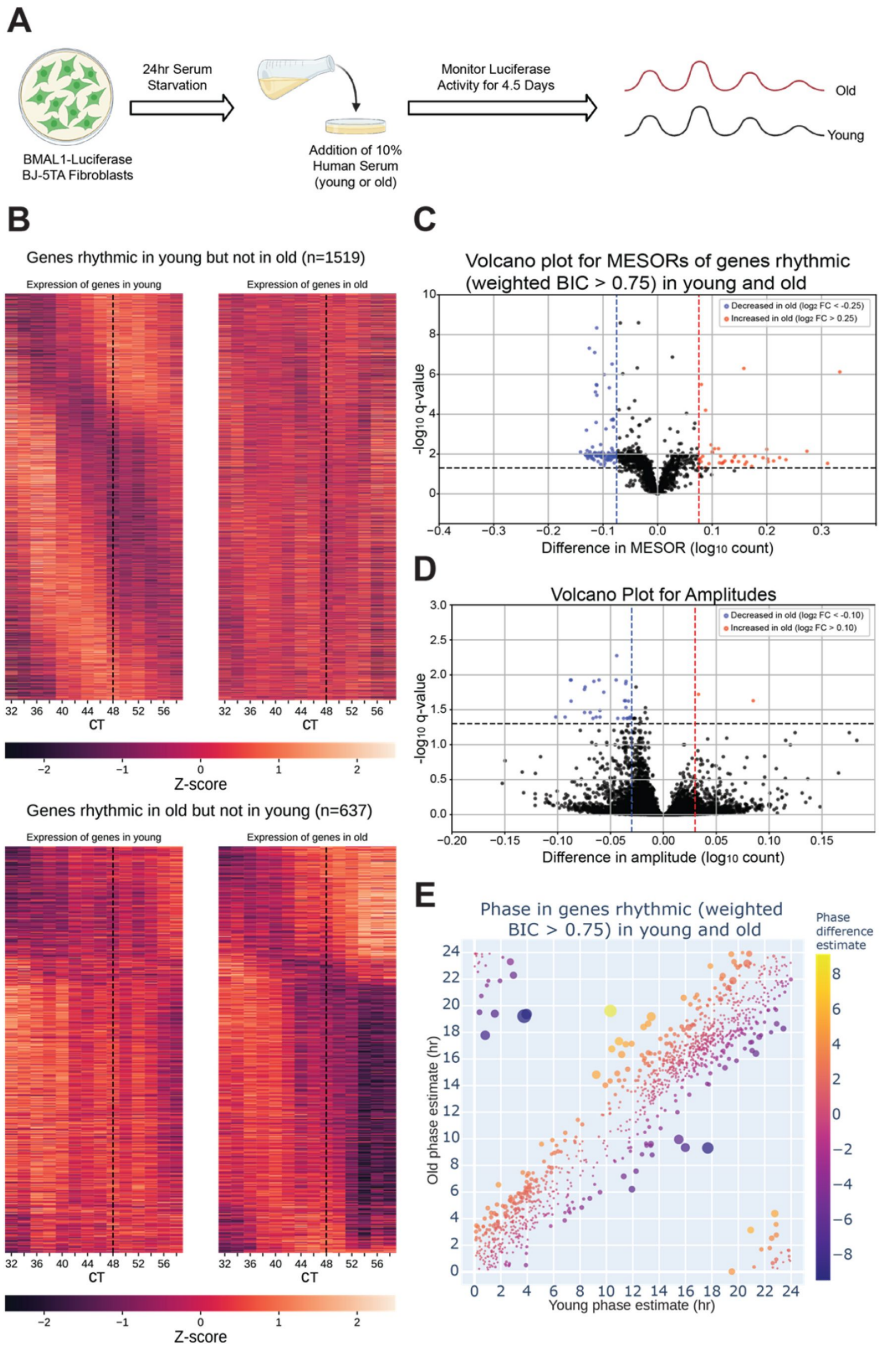

Fig 2.

The circadian transcriptome is differentially affected by entrainment with sera from young and old human subjects.

A visual representation of the serum starvation-serum addition protocol to synchronize the *BMAL1-luciferase* BJ-5TA fibroblasts **(A**, made with BioRender.com**)**. When comparing the circadian transcriptome entrained by either young or old sera (n=4 sera per group), 1519 genes lost rhythmicity with age (B, top) while only 637 genes gained rhythmicity with age (B, bottom). Weighted BIC criterion with a threshold of 0.75 was used to assess rhythmicity **(B)**. The number of genes rhythmic in young and old (according to weighted BIC > 0.75 criterion) that show a detectable change in MESOR (q < 0.05 criterion for MESOR difference) is 568, with MESOR increasing in 163 and decreasing in 405 genes. Out of these, 40 genes with increased MESOR (red) and 98 genes with decreased MESOR (blue) also satisfy the condition $|\log_2 FC| > 0.25$ **(C)**. We were only able to detect change in amplitude for a small number of genes, 39 genes had decreased amplitude in old and 2 had increased amplitude using CircaCompare. Only 30 genes with decreased amplitude also satisfy the condition $|\log_2 FC| > 0.1$ (blue) **(D)**. For phase, using a test provided by CircaCompare, under q < 0.05 cutoff for age-related phase differences in genes rhythmic in young and old (with BIC>0.75 cycling criteria), we detected 20 genes with advanced phase, and 34 with delayed phase **(E)**.

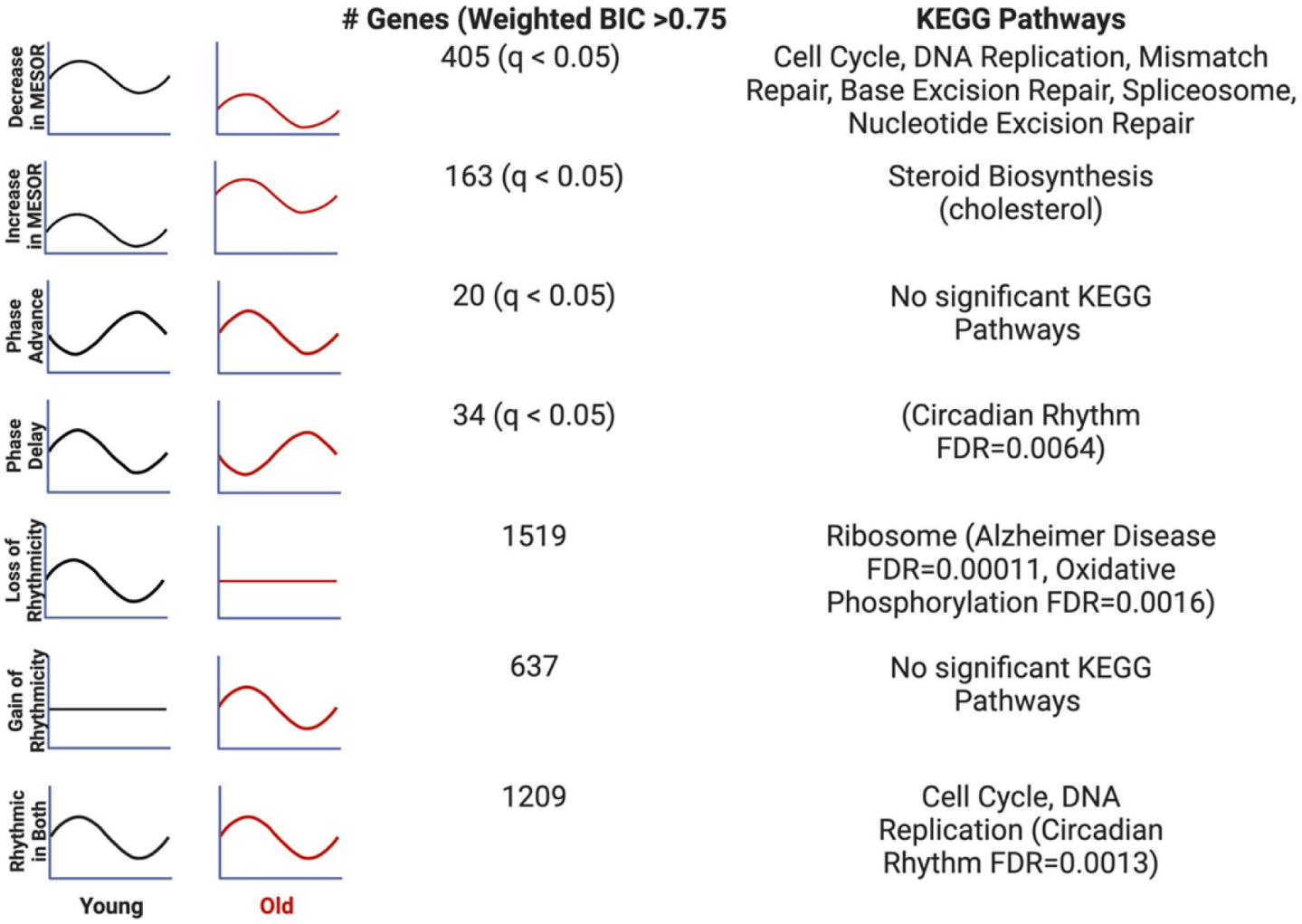

Fig 3.

Each type of circadian change is associated with different KEGG pathways by STRING analysis

Entrainment of the fibroblasts in culture with old serum significantly altered the circadian transcriptome despite use of the same cells in both young and old conditions, suggesting the aged serum itself affects the regulation of specific pathways in the cell. Significant KEGG Pathways (FDR <1x10^{-4} unless specified) are indicated next to each category of genes. Age related pathways such as Alzheimer's Disease/oxidative phosphorylation are associated with a loss of rhythms in the old condition. Cell cycle and DNA replication pathways remain rhythmic in the old serum condition, but cycle with a decreased MESOR. Rhythmic genes were determined using CircaCompare. Rhythmicity was determined using the weighted BIC > 0.75 criterion while p-values for difference in MESOR and phase were determined using CircaCompare.

Discussion

Although studies of the aged SCN have revealed persistent cycling of clock gene expression but a breakdown of output signals[4, 5], whether age-related changes in systemic signaling impact organismal clocks has yet to be elucidated. We demonstrate here that while the core clock continues to cycle in cultured fibroblasts synchronized with serum from old volunteers, the circadian transcriptome is different from that seen in cells treated with serum from young individuals. Through this analysis of the role of serum in age-induced changes in circadian rhythms, we suggest a potential mechanism for other studies, which showed that specific genes lose, gain, or maintain rhythmicity with age[18–22]. This phenomenon, known as circadian reprogramming, may illuminate which pathways are affected by or become more impactful for cellular maintenance through aging in a tissue specific manner[23]. Interestingly, many of the genes in the circadian transcriptome that exhibit age-related changes are independently implicated in aging. For instance, some of the genes that lose rhythmicity in the aged condition are involved in oxidative phosphorylation and mitochondrial function, both of which decrease with age[24, 25]. We suggest that the age-related inefficiency of oxidative phosphorylation derives at least in part from loss of cycling due to changes in serum signals.

In order to study the effect of circulating factors on age-related changes of the circadian transcriptome, we utilized the well-established serum starvation-serum addition protocol[7] to synchronize cells in culture. Fibroblasts from old and young subjects have robust clocks and respond similarly to synchronization with dexamethasone[10]; however, when synchronized with dexamethasone in a media containing old serum, they exhibited shortened circadian periods[10]. This effect was reversed by heat inactivating old serum. We did not observe a significant difference in period length between young and old serum synchronization in our *BMAL1-luciferase* experiment, perhaps because of the absence of dexamethasone. Our use of serum to synchronize allowed us to more closely simulate *in vivo* conditions where blood is an important mode by which central clocks can entrain peripheral clocks to maintain synchrony with the day:night cycle. Previously, serum factors were shown to activate the immediate early transcription factor, SRF, in a diurnal manner to confer time of day signals to both cells in culture and to mouse liver[7]. We show here that effects of age are also mediated by serum. We find that while the number of rhythmic transcripts in the young serum condition (~10%) is higher than the old serum condition (~4%), both conditions demonstrate a much larger number of cycling transcripts in these cultured fibroblasts than in other cell culture studies[26–29]. However, in mammals, up to 20% of transcripts can cycle in a given tissue[30, 31], and the low number of cycling transcripts in culture has been cited as a major limitation of the culture model[28]. A major contributing factor to robust in vivo cycling might be human serum as the synchronization signal. In this way, our model of mimicking signaling to peripheral tissues by using serum directly from humans may more accurately recapitulate the human condition.

Together, these findings indicate that at least some of the age-related changes in the circadian clock and circadian transcriptome are derived from signals circulating in the serum and not the age of the tissue. This has profound implications for understanding and treating circadian disruption with age, and could also be relevant for other age-related pathology, given established links between circadian disruption and diseases of aging. Notably, many of the genes whose cycling is affected by old serum contribute to age-associated disorders.

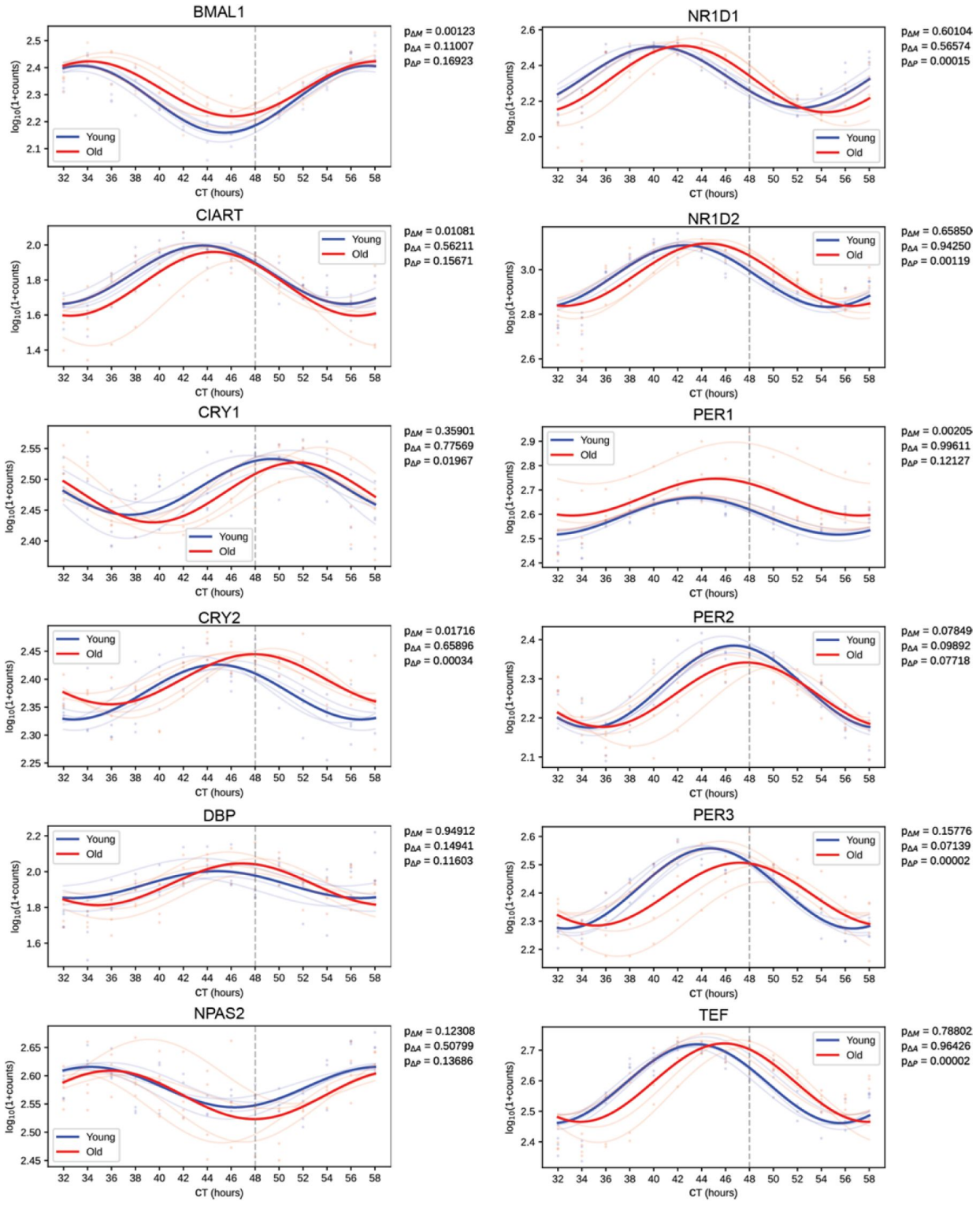

Fig 4.

Synchronizing with old serum phase delays the expression profile of several core clock genes

Traces of molecular clock mRNA transcripts, the average curve (bold) of results of individual sera (faded). P-values for the difference in MESOR (P_#x1D759;M), amplitude (P_#x1D759;A), and phase (P_#x1D759;P) are shown for the comparison of young and old conditions. Several clock genes are significantly phase delayed (*CRY1, CRY2, NR1D1, NR1D2, PER3, TEF*) in response to synchronization with old serum. While *BMAL1* is not phase delayed, the MESOR significantly increases with age. N=4 subjects per timepoint for both young and old groups.

Materials and methods

Clinical research study

This clinical research study enrolled apparent healthy participants from the volunteer pool maintained by the Institute for Translational Medicine and Therapeutics (ITMAT), University of Pennsylvania. The Institutional Review Board of the University of Pennsylvania (Federal wide Assurance FWA00004028; IRB Registration: IORG0000029) approved the clinical study protocol (Penn IRB#832866). The study was registered on *ClinicalTrials.gov (http://clinicaltrials.gov/)* with identifier NCT04086589. After obtaining informed consent from all volunteers, study assessments were conducted in the Center for Human Phenomic Science (Penn CHPS#3002) in accordance with relevant GCP guidelines and regulations. We originally intended on recruiting n=20 per group; however, patient recruitment was halted due to the COVID-19 pandemic. Participants met criteria for inclusion (in general good health, either 70-85 years of age for the elderly cohort or 20-35 years of age for the young cohort, and a wrist-actigraphy-based average TST (total sleep time) ≥ 6 hours per night occurring between 22:00 – 08:00). Participants were excluded due to pregnancy or nursing, shift work (defined as recurring work between 22:00-05:00), a history of clinically significant obstructive sleep apnea, transmeridian travel across ≥2 time zones in the two weeks prior to study assessments and one week after, > 2 drinks of alcohol per day, and use of illicit drugs. Blood collections were done from the median cubital vein via venipuncture using a 22 G butterfly needle (BD, Franklin, Lakes, NJ, USA). All blood draws occurred at 14:00, the same time as Pagani et al.[32]. One subject returned for a repeat clinical assessment including biosampling to provide additional sample.

Acquisition of accelerometry data streams

Participants wore a triaxial actigraph device (wGT3X-BT, ActiGraph, Pensacola, FL) on the non-dominant wrist. The devices were initialized using the following parameters: start date and time were synchronized with atomic server time without pre-defined stop date/time, at 60 Hz sampling rate for the three accelerometer axes, enabled for delay modus, steps, lux, inclinometer, and sleep while active. Raw data were downloaded from the device in AGD and GT3X file format in one second epochs using ActiLife software (version 6, ActiGraph, Pensacola, FL) and submitted for further analyses. For visualization and cosinor analysis, actigraphy data were aggregated into 1-minute intervals by summing ActiGraph counts across each minute. Cosinor analyses of the data were adapted from the single component cosinor analysis reviewed by Cornelissen[33], as well as the cosine fit described by Refinetti et al. [34].

Briefly, the measurement times for the actigraphy data were recalculated as hours since midnight on the first day of measurement, within each participant's data. For each actigraphy variable and each participant, the lm() function in R (v4.2.0) was used to perform a cosinor fit with a fixed 24-hour period. The two cosinor coefficients from these fits were used to calculate participant-level amplitudes and circadian phases, while the intercepts provided MESOR estimates. The two-sided Wilcoxon rank sum exact test, as implemented by R's wilcox.test() function, was used to test for significant differences in amplitude and MESOR between the age groups. A two-sample Kuiper's test, as implemented by the kuiper_test(nboots = 10000) function from the twosamples R package (v2.0.0), was used to test for significant differences in circadian phase between the age groups.

Acquisition of EKG data

The Zephyr BioPatch devices (Zephyr Technology, Annapolis, MD) were deployed as previously established[35]. All subjects included in this analysis wore the BioPatch for at least 24hrs. EKG recordings were analyzed by Kubios HRV Premium (ver. 3.5.0, Kubios Team, Kuopio, Finland) to report time-of-day-specific measures of cardiovascular function consisting of heart rate, RR intervals, sympathetic and parasympathetic nervous activity (SNS and PNS).

Preprocessing in Kubios was set to automatic beat correction to remove artifacts and Smoothn priors for detrending. Cosinor fits and tests for differences in circadian parameters between age groups were performed as described for the actigraphy data. The circadian parameters MESOR and amplitude were tested for significant differences between the age groups by Wilcoxon rank sum exact test (two-sided), while phase was tested by Kuiper's two-sample test to account for the circular measurement.

Cortisol Measurements

Cortisol was measured in human serum by coated tube RIA (MP Bio, Solon OH) in duplicate. Tubes were counted on Perkin Elmer gamma counter and data reduced by STATLia software.

Generation of stable BJ-5TA cell line expressing *BMAL1-dLuc-GFP*[9]

Virus was generated and cells were infected as we've previously described[36]. Briefly, LentiX 293T cells (Clonetech) were transfected with Lipofectamine 3000 PLUS (Life Tech) using manufacturer instructions. The transfection included 18ug of DNA per reaction and the plasmid (BMAL1-dLuc-GFP) to packaging vector (DVPR, Addgene) to envelope (VSV-G, Addgene) ratio was 10:1:0.5. Media was changed 24hrs post-transfection after the cells were checked using a fluorescence scope to make sure cells were >50% GFP positive. Supernatant with the virus was collected at 48hrs and 72hrs post transfection and spun down at 3000 RPM for five minutes (to eliminate any cells/debris). BJ-5TA cells were infected with fresh virus upon virus collection.

Polybrene (Sigma-Aldrich, 10 mg/mL) was also added to aid with infection. Transduced BJ-5TA cells (>2000 cells) were sorted (FACSMelody, BD Biosciences) for high GFP expression. Once the cell line was established, blasticidin was added to the culture at 2ug/ml. Due to fragility of the cell line in the presence of antibiotic, BJ-5TA *Bmal1-luciferase* cells with differing expression levels of GFP were sorted. The line with highest stable luminescence oscillation was used for all experiments reported here.

Serum entrainment and bioluminescent recording

BJ-5TA *BMAL1-dLuc-GFP* cells in 24-well plates (~confluent) were washed (2x) with DPBS and given serum free media for 24hrs. After the starvation, cells were given media with 10% human serum and 200uM beetle luciferin potassium salt. Each well of cells was given media with the serum from a single patient. The cells were continuously monitored by LumiCycle luminometer (Actimetrics) for 4.5 days from the point of serum addition. LumiCycle raw data was exported using LumiCycle software (Actimentrics). The data were analyzed by BioDare2 (biodare2.ed.ac.uk[37]) using FFT NLLS with baseline detrending. Any replicates that were not cycling or had a period outside of the 20-28hr range was excluded from analysis. Both the serum free media and serum added media used the recipe from[9] at pH 7.4; however, the serum free media had no serum and the serum media had 10% human serum instead of FBS.

Sample preparation, RNA extraction, and RNA sequencing

BJ-5TA *BMAL1-dLuc-GFP* cells in 24-well plates (~confluent) were washed (2x) with DPBS and given serum free media (DMEM with Penn Strep) for 24hrs. After 24hrs cells were given media with human serum (DMEM, Penn Strep, 10% human serum). Serum starvation was staggered every 12 hours over two days to allow for samples to be collected on the same day.

Upon sample collection wells were place on ice and rinsed with cold DPBS and then put in cold RLT buffer with 2-Mercaptoethanol (10uL/mL). Samples were frozen at -80 overnight and sent to Admera for RNA extraction and sequencing. Total RNA was extracted with RNeay mini kit

to isolate poly(A)+ transcripts based on NEBNext® Poly(A) mRNA Magnetic Isolation Module manual (New England BioLabs Inc., Massachusetts, USA). Prior to first strand synthesis, samples are randomly primed (5' d(N6) 3' [N=A,C,G,T]) and fragmented based on manufacturer's recommendations. The first strand is synthesized with the Protoscript II Reverse Transcriptase with a longer extension period, approximately 40 minutes at 42°C. All remaining steps for library construction were used according to the NEBNext® UltraTM II Non Directional RNA Library Prep Kit for Illumina® (New England BioLabs Inc., Massachusetts, USA). Final libraries quantity was assessed by Qubit 2.0 (ThermoFisher, Massachusetts, USA) and quality was assessed by TapeStation D1000 ScreenTape (Agilent Technologies Inc., California, USA). Final library size was about 430bp with an insert size of about 300bp. Illumina® 8-nt dual-indices were used. Equimolar pooling of libraries was performed based on QC values and sequenced on an Illumina® NovaSeq S4 Illumina, California, USA) with a read length configuration of 150 PE for 40 M PE reads per sample (20 M in each direction).

RNA-seq and statistical analysis

Raw RNA-seq reads were aligned to the GRCh38 build of the human genome by STAR version 2.7.10a[38]. The dataset contained an average of 19,265,220 paired-end non-stranded 150 bp reads mapping uniquely to genes, per sample. Data were normalized and quantified at both gene and exon-intron level, using a downsampling strategy implemented in PORT (Pipeline Of RNA-seq Transformations, available at *https://github.com/itmat/Normalization*), version 0.8.5f-beta_hotfix1. Both STAR and PORT were provided with gene models from release 106 of the Ensembl annotation[39].

MESOR, amplitude, and phase estimates, as well as p-values for the difference in MESOR, amplitude, and phase, were calculated with CircaCompare[12], version 0.1.1. Only rhythmic genes were taken into consideration in pathway and other analyses involving MESORs and phases. The criterion for rhythmicity was either based on (BH adjusted) p-values reported by CircaCompare, or weighted BIC values, obtained by an approach similar to dryR[40]. In the latter approach we fitted four models of rhythmicity, one modeling gene expression that is rhythmic in both cells treated with young or old sera, another modeling gene expression rhythmic in neither cells treated with young nor old sera, and two modeling gene expression rhythmic in either young or old sera treated cells respectively. The BIC values were calculated for the four models and were weighted to obtain numbers between 0 and 1. All methods were provided with $\log_{10}(1 + \text{PORT normalized count})$ values and were run on R, version 4.1.2, accessed through Python, version 3.9.9, via rpy2, an interface to R running embedded in a Python process, *https://rpy2.github.io/*, version 3.4.5. Additionally, we used Nitecap[41] to visualize and explore circadian profiles of gene expression.

STRING pathway analysis

We performed STRING[13] pathway analyses using STRING API version 11.5. Enrichment analyses were performed on the sets of genes rhythmic in both groups (weighted BIC > 0.75) with observed decrease in MESOR, increase in MESOR, advance in phase, and delay in phase according to CircaCompare q < 0.05 criterion. We also performed analyses on the sets consisting of genes rhythmic only in young group (weighted BIC > 0.75), only in old group (weighted BIC > 0.75), and the set of genes rhythmic in both groups (weighted BIC > 0.75). Finally, two additional analyses were performed, on the sets of genes with observed decrease and increase of amplitude (CircaCompare q < 0.05).

Ingenuity Pathway Analysis

QIAGEN IPA[14] was used to identify pathways enriched in various subsets of cycling genes. The following subsets of genes were identified using a combination of CircaCompare stats and BIC cutoffs: (1) Decreased MESOR in old sera (929 genes) – CircaCompare rhythmic q < 0.05 in old, CircaCompare rhythmic q < 0.05 in young, CircaCompare MESOR difference q < 0.05, MESOR

difference (old – young) < 0. (2) Increased MESOR in old sera (515 genes) – same selection criteria as 'Decreased MESOR in old sera,' except MESOR difference (old –young) > 0. (3) Phase advance in old sera (148 genes) - CircaCompare rhythmic q < 0.05 in old, CircaCompare rhythmic q < 0.05 in young, CircaCompare Phase difference q < 0.05, phase difference (old – young) < 0. (4) Phase delay in old sera (156 genes) – same selection criteria as 'Phase advance in old sera,' except phase difference (old – young) > 0. (5) Loss of rhythmicity in old sera (1519 genes) – weighted BIC > 0.75 for 'Rhythmic in Young but not Old' model. (6) Gain of rhythmicity in old sera (637 genes) – weighted BIC > 0.75 for 'Rhythmic in Old but not Young' model. (7) Rhythmic in old and young sera (1209 genes) – weighted BIC > 0.75 for 'Rhythmic in both Old and Young' model. Each of these gene subsets were processed separately with IPA's core analysis, using default parameters.

For visualization via heatmap, we perform three rounds of normalization within each gene. First, we mean-normalize the read counts within each age group and serum treatment group (A, B, C, D). This is to account for baseline differences between sera collected from the different subjects. Second, we collapse replicates at each timepoint by calculating their means. Third, we calculate Z-Scores across all timepoints, within each age group. Note, these normalization procedures are to aid with visualization of the data and were not used as part of the statistical analyses.

Data and materials availability

Sequencing data are deposited in Gene Expression Omnibus (NCBI) under accession number TBD. All additional data files are available upon request.

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Author Contributions

Conceptualization, J.E.S. and A.S.; Methodology, J.E.S, A.M., N.F.L., Y.L., C.T.H., G.G., C.S., S.L.Z, and A.S.; Formal Analysis, J.E.S, S.L.Z, A.M., N.F.L., Y.L., and C.T.H; Investigation, J.E.S, S.L.Z., and C.S.; Writing – Original Draft Preparation, J.E.S. and A.S.; Writing – Review & Editing, J.E.S, A.M., N.F.L., Y.L., C.T.H., G.G., C.S., S.L.Z, and A.S.; Visualization, J.E.S., A.M, N.F.L.; Supervision, S.L.Z and A.S.

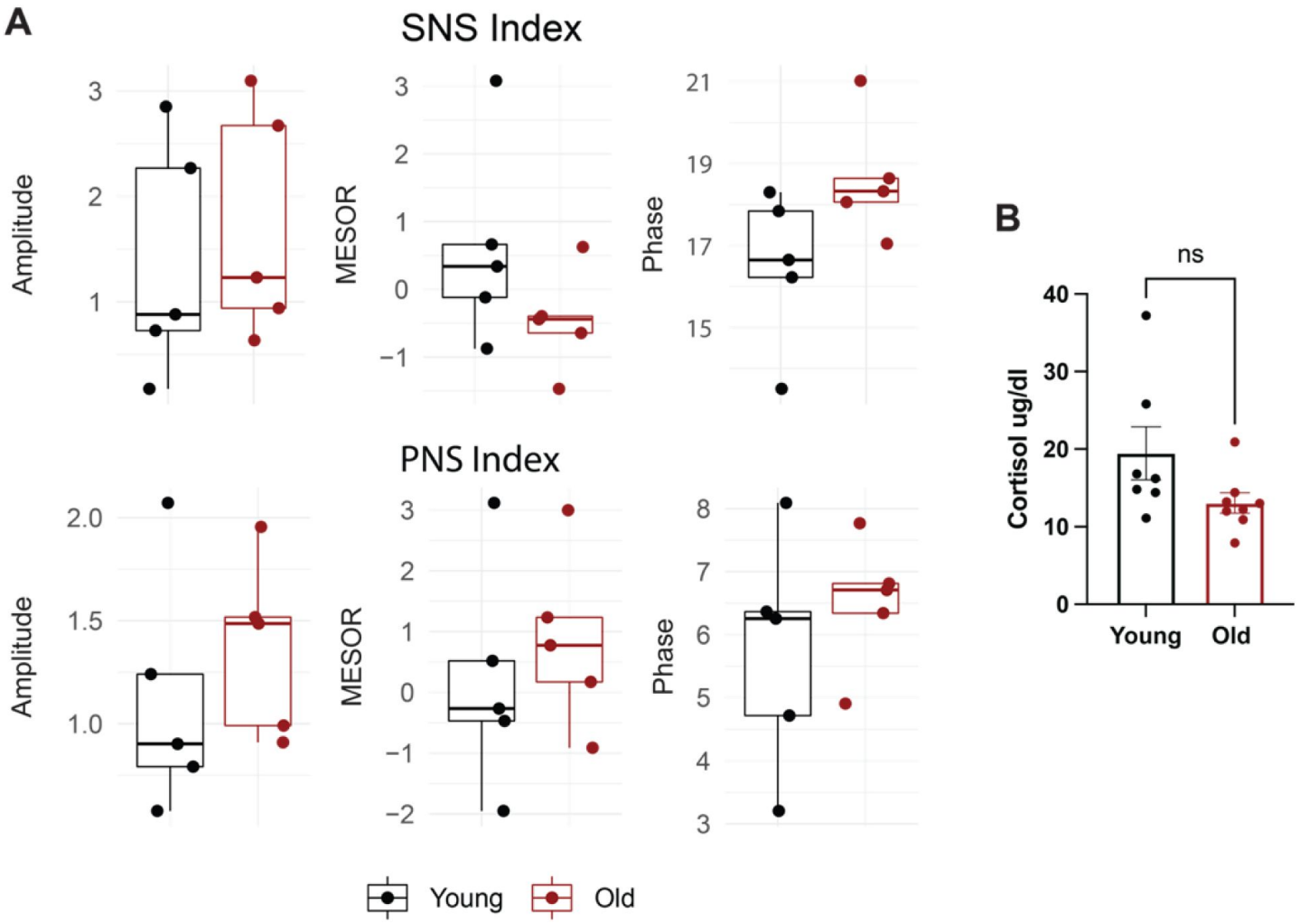

Fig S1.

There are no significant differences in grip strength, sympathetic, parapsympathetic nervous system indices, or cortisol levels between young and old individuals

Sympathetic nervous system (SNS, A, top) and parasympathetic nervous system (PNS, A, bottom) indices were measured using EKG. Amplitude, MESOR, and Phase were calculated from the resulting oscillations and the Median, quartiles, and SEMs are shown. Dots represent individuals. P>0.05. N=5 per group. MESOR and amplitude were tested using Wilcoxon rank sum exact test, while phase was tested by Kuiper's two-sample test. Boxplot midlines correspond to median values, while the lower and upper hinges correspond to the first and third quartiles, respectively. Boxplot whiskers extend to the smallest/largest points within 1.5 * IQR (inter Quartile Range) of the lower/upper hinge **(A)**. Serum cortisol levels are not statistically different by unpaired t-test (**B**, N=7 young, 8 old).

Age Group	Sex	Age (y)	BMI (kg/m^2)	Total Sleep Time (TST) [h]
20-35	Male	30	25.9	6.4
	Female	30	28.7	7.4
	Female	25	23.7	6.6
	Male	28	25.0	9.7
	Female	28	21.2	6.9
	Male	28	25.2	7.4
	Male	28	25.9	9.3
Young	***Mean***	***28.1***	***25.1***	***7.7***
	SD	***1.7***	***2.3***	***1.3***
70-85	Male	76	22.3	5.8
	Male	75	28.8	7.8
	Female	73	32.4	7.7
	Female	74	26.4	7.5
	Male	73	21.5	6.6
	Male	71	26.7	11.3
	Male	74	27.2	7.0
	Male	70	26.8	6.7
Old	***Mean***	***73.3***	***26.5***	***7.6***
	SD	***2.0***	***3.4***	***1.7***

Total sleep time (TST) was calculated from ActiLife 6 determined in-bed and out-of-bed times averaged across nights with available actigraphy data (≥7 nights). Old (N=8), Y-young (N=7).

Table S1.

Demographic information for subjects in the study.

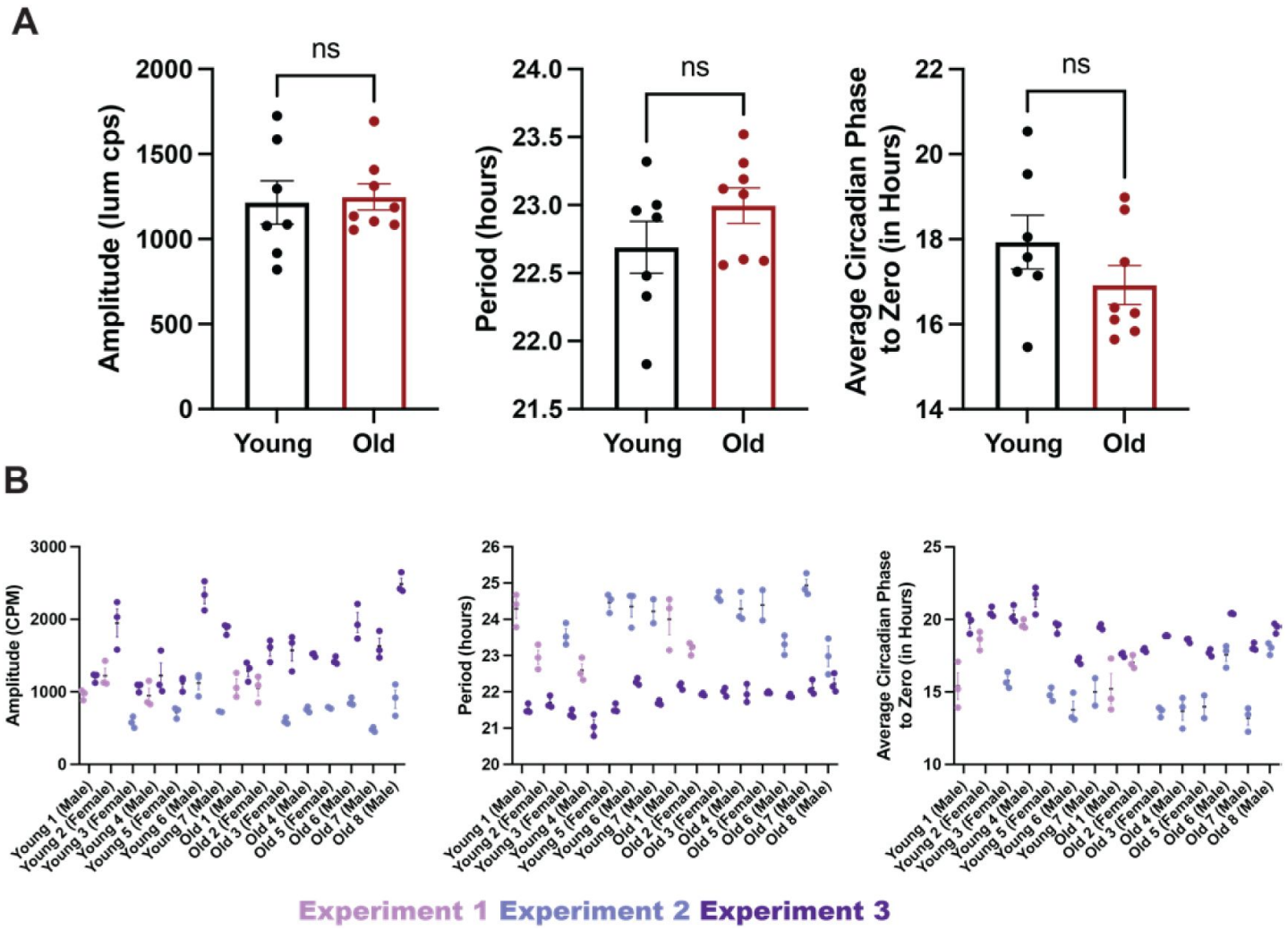

Fig S2

Young and old serum contain equivalent levels of cortisol and are equally effective at entraining cells in culture

(A) Cells synchronized with human serum from either young (N=7 subjects) or old (N=8 subjects) individuals did not show differences in amplitude (as measured in luminescence counts per second, bottom, left), period (bottom, middle), or phase (bottom, right). Data points represent average values for a specific patient's serum over 2 experiments with 2-3 replicates per experiment. A visual representation of the individual replicates averaged in A **(B)** Summary statistics are displayed as mean +/- SEM. Means compared by unpaired t-test. Despite individual and run to run variability, cells synchronized with human serum from either young (N=7 subjects) or old (N=8 subjects) individuals showed similar amplitude (top, left), period (top, right), and phase (bottom) of BMAL1-luciferase rhythms. Data points represent individual replicates within an experiment. Wells run in the same experiment are displayed in the same color. Each subject's sample was run in 2-3 replicates.

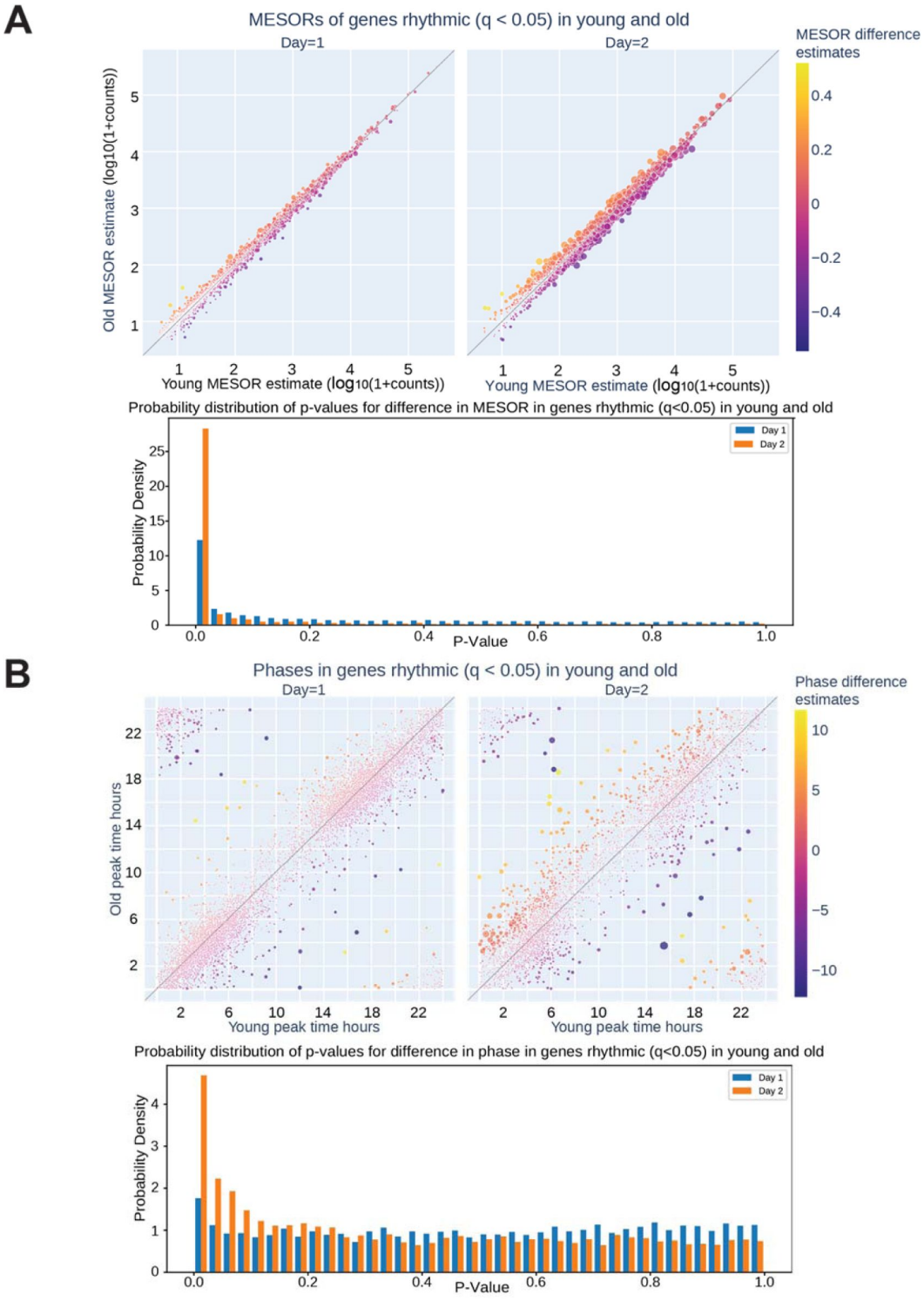

Fig S3.

The circadian transcriptomes of cells synchronized with young or old sera deviate significantly on day two.

On day two of serum entrainment, young and old transcriptional rhythms differentiated. CircaCompare analysis of RNA sequencing revealed that on Day 2 of serum entrainment analysis (CT 36-58) the MESOR differences of cycling genes are larger between the cells entrained with young or old serum **(A**, top**)**. Additionally, more genes were phase shifted in the old serum condition compared to young serum on Day 2 **(B**, top**)**. The distribution of p-values shows an enrichment of low p-values on Day 2 for both MESOR and phase differences (A, bottom, B, bottom). The size of each circle in the scatter plots is proportional to $-\log_{10}q$, so the bigger the circle, the smaller the q-value for the difference between young and old for each metric.

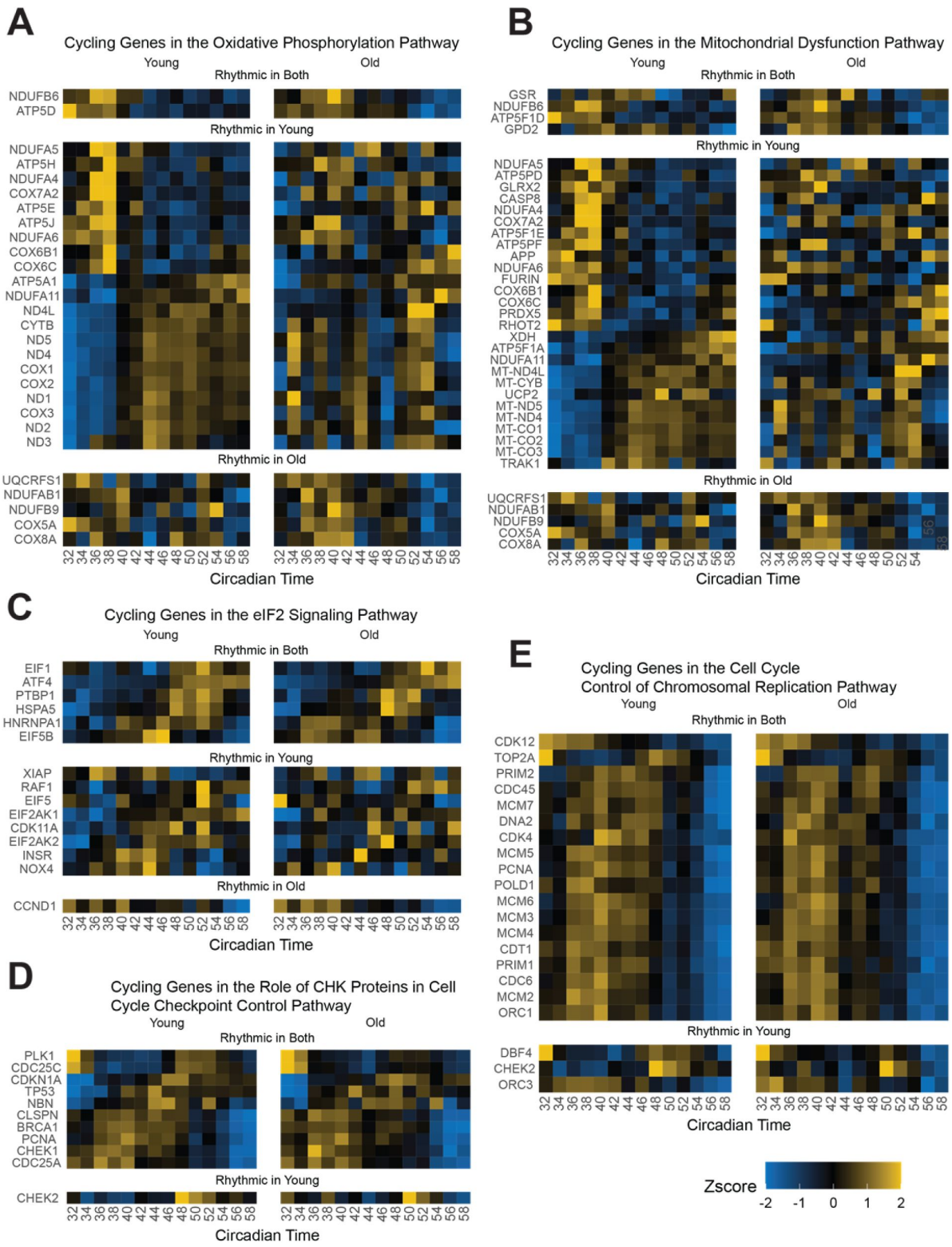

Fig S4

Oxidative phosphorylation genes and chromosomal replication genes are affected by entrainment with old serum

Additional pathway analysis employing IPA further emphasizes finding with STRING analysis. Genes identified by IPA analysis to be associated with oxidative phosphorylation/mitochondrial dysfunction and eIF2 genes lose rhythmicity with age (BIC >0.75) **(A, B, C)**. Cell cycle checkpoint control (CHK proteins) **(D)** and chromosomal replication genes **(E)** maintain their cycling in the aged condition, which is consistent with the continued division of cells.

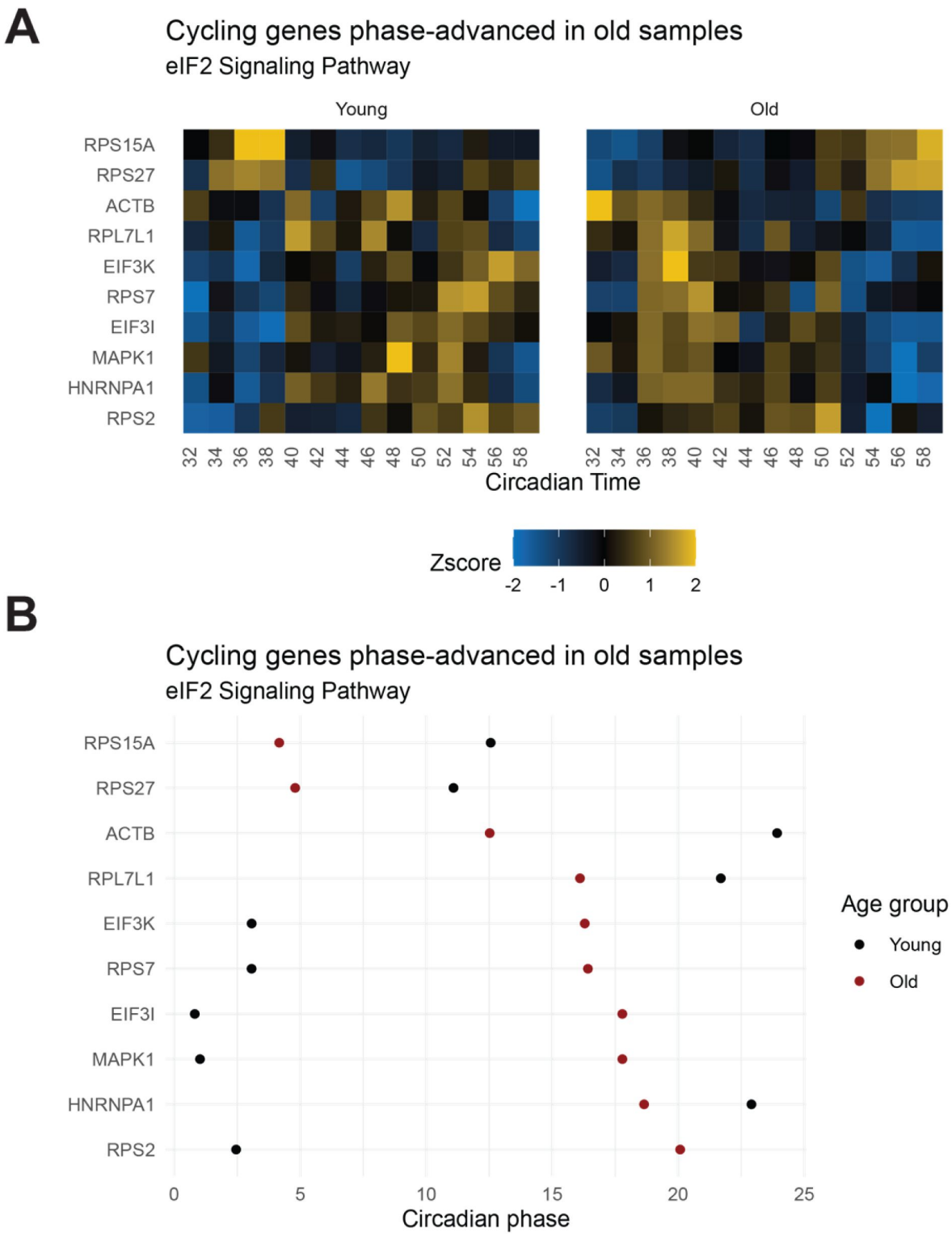

Fig S5

eIF2 signaling pathway genes that maintain cycling with age phase advance in the old serum condition

From the IPA analyses, the eIF2 signaling pathway was enriched (BIC >0.75) in two datasets: 1) genes cycling in the young sera and not the old and 2) cycling genes phase-advanced in the old sera ($q<0.05$). A significant number of genes that maintain rhythmicity in the old sera condition are phase advanced as visualized in the heat map **(A)** or plot of peak phase **(B)**. As the eIF2 pathway is involved in protein translation, this suggests that the timing of protein translation is phase advanced in the older serum and/or less rhythmic.

Gene	Symbol	Old_mesor_estimate	Young_mesor_estimate
ENSG00000014138	POLA2	1.942220257	2.030676546
ENSG00000062822	POLD1	2.727523056	2.828026817
ENSG00000073111	MCM2	3.003984606	3.115331295
ENSG00000076003	MCM6	3.124116273	3.203138512
ENSG00000085840	ORC1	2.177440998	2.305154417
ENSG00000091651	ORC6	2.415062674	2.513336383
ENSG00000093009	CDC45	2.428834807	2.540049538
ENSG00000094804	CDC6	2.566892644	2.679401971
ENSG00000097046	CDC7	2.279003902	2.377425914
ENSG00000100297	MCM5	3.17577594	3.288560637
ENSG00000101868	POLA1	2.640718445	2.728951631
ENSG00000104738	MCM4	3.310670684	3.413600036
ENSG00000112118	MCM3	3.168296215	3.269321473
ENSG00000117748	RPA2	2.754645539	2.798914718
ENSG00000131747	TOP2A	3.722304752	3.798890616
ENSG00000132383	RPA1	3.238584015	3.287556646
ENSG00000132646	PCNA	3.232760966	3.321439626
ENSG00000135446	CDK4	3.143303063	3.172936739
ENSG00000138346	DNA2	2.113453067	2.238476228
ENSG00000146143	PRIM2	2.620994795	2.675363852
ENSG00000164815	ORC5	2.361538047	2.414675015
ENSG00000166508	MCM7	3.381394362	3.482444492
ENSG00000167258	CDK12	3.10241713	3.129181459
ENSG00000167513	CDT1	2.474002764	2.590319547
ENSG00000198056	PRIM1	2.116803844	2.231962095

Table S2

Genes with decreased MESOR in the IPA cell cycle control of chromosomal replication pathway

Table of genes that are rhythmic in both conditions with a decreased MESOR with old serum treatment compared to young serum treatment by CircaCompare. All genes are involved in the cell cycle/DNA replication pathway.

Gene	Symbol	Old_mesor_estimate	Young_mesor_estimate
ENSG00000001630	CYP51A1	2.040377336	1.871207095
ENSG00000079459	FDFT1	3.330291993	3.19001773
ENSG00000104549	SQLE	3.116822033	2.950386727
ENSG00000110921	MVK	2.530172998	2.4121956
ENSG00000112972	HMGCS1	2.93111747	2.696086286
ENSG00000113161	HMGCR	3.011982868	2.833316048
ENSG00000116133	DHCR24	3.506294106	3.343492054
ENSG00000120437	ACAT2	2.71791125	2.58109012
ENSG00000132196	HSD17B7	1.940436315	1.782521965
ENSG00000147383	NSDHL	2.540302415	2.468852126
ENSG00000160285	LSS	3.486802994	3.411431601
ENSG00000160752	FDPS	3.143592562	3.081359846
ENSG00000167508	MVD	2.819963909	2.68386468
ENSG00000172893	DHCR7	2.990620862	2.797567922

Table S3

Genes with increased MESOR in the IPA cholesterol biosynthesis pathway

Table of genes that are rhythmic in both conditions with a decreased MESOR with old serum treatment compared to young serum treatment by CircaCompare. All genes are involved in the cholesterol biosynthesis pathway.

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Reviewer #1 (Public Review):

Aging is associated with a number of physiologic changes including perturbed circadian rhythms. However, mechanisms by which rhythms are altered remain unknown. Here authors tested the hypothesis that age-dependent factors in the sera affect the core clock or outputs of the core clock in cultured fibroblasts. They find that both sera from young and old donors are equally potent at driving robust ~24h oscillations in gene expression, and report the surprising finding that the cyclic transcriptome after stimulation by young or old sera differs markedly. In particular, genes involved in the cell cycle and transcription/translation remain rhythmic in both conditions, while genes associated with oxidative phosphorylation and Alzheimer's Disease lose rhythmicity in the aged condition. Also, the expression of cycling genes associated with cholesterol biosynthesis increases in the cells entrained with old serum. Together, the findings suggest that age-dependent blood-borne factors, yet to be

identified, affect circadian rhythms in the periphery. The most interesting aspect of the paper is that the data suggest that the same system (BJ-5TA), may significantly change its rhythmic transcriptome depending on how the cells are synchronized. While there is a succinct discussion point on this, it should be expanded and described whether there are parallels with previous works, as well as what would be possible mechanisms for such an effect.

Major points:
Fig 1 and Table S1. Serum composition and levels of relevant blood-borne factors probably change in function of time. At what time of the day were the serum samples from the old and young groups collected? This important information should be provided in the text and added to Table S1.

Fig 2A. Luminescence traces: the manuscript would greatly benefit from inclusion of raw luminescence traces.

Fig 2. Of the many genes that change their rhythms after stimulation with young and old sera, what are the typical fold changes? For example, it would be useful to show histograms for the two groups. Does one group tend to have transcript rhythms of higher or lower fold changes?

Fig. 2 Gene expression. Also here, the presentation would benefit from showing a few key examples for different types of responses.

What was the rationale to use these cells over the more common U2OS cells? Are there similarities between the rhythmic transcriptomes of the BJ-5TA cells and that of U2OS cells or other human cells? This could easily be assessed using published datasets.

For the rhythmic cell cycle genes, could this be the consequence of the serum which synchronizes also the cell cycle, or is it rather an effect of the circadian oscillator driving rhythms of cell cycle genes?

While the reduction of rhythmicity in the old serum for oxidative phosphorylation transcripts is very interesting and fits with the general theme that metabolic function decreases with age, it is puzzling that the recipient cells are the same, but it is only the synchronization by the old and young serum that changes. Are the authors thus suggesting that decrease of metabolic rhythms is primarily a non cell-autonomous and systemic phenomenon? What would be a potential mechanism?

The delayed shifts after aged serum for clock transcripts (but not for Bmal1) are interesting and indicate that there may be a decoupling of Bmal1 transcript levels from the other clock gene phases. How do the authors interpret this? could it be related to altered chronotypes in the elderly?

Reviewer #2 (Public Review):

Schwarz et al. have presented a study aiming to investigate whether circulating factors in sera of subjects are able to synchronize depending on age, circadian rhythms of fibroblast. The authors used human serum taken from either old (age 70-76) or young (age 25-30) individuals to synchronise cultured fibroblasts containing a clock gene promoter driven luciferase reporter, followed by RNA sequencing to investigate whole gene expression.

This study has the potential to be very interesting, as evidence of circulating factors in sera that mediate peripheral rhythms has long been sought after. Moreover, the possibility that those factors are affected by age which could contribute to the weaken circadian rhythmicity observed with aging.

Here, the authors concluded that both old and young sera are equally competent at driving robust 24 hour oscillations, in particular for clock genes, although the cycling behaviour and

nature of different genes is altered between the two groups, which is attributed to the age of the individuals. This conclusion could however be influenced by individual variabilities within and between the two age groups. The groups are relatively small, only four individual two females and two males, per group. And in addition, factors such as food intake and exercise prior to blood drawn, or/and chronotype, known to affect systemic signals, are not taken into consideration. As seen in figure 4, traces from different individuals vary heavily in terms of their patterns, which is not addressed in the text. Only analysing the summary average curve of the entire group may be masking the true data. More focus should be attributed to investigating the effects of serum from each individual and observing common patterns. Additionally, there are many potential causes of variability, instead or in addition to age, that may be contributing to the variation both, between the groups and between individuals within groups. All of this should be addressed by the authors and commented appropriately in the text.

The authors also note in the introduction that rhythms in different peripheral tissues vary in different ways with age, however the entire study is performed on only fibroblast, classified as peripheral tissue by the authors. It would be very interesting to investigate if the observed changes in fibroblast are extended or not to other cell lines from diverse organ origin. This could provide information about whether circulating circadian synchronising factors could exert their function systemically or on specific tissues. At the very least, this hypothesis should be addressed within the discussion.

In addition to the limitations indicated above I consider that the data of the study is an insufficiently analysis beyond the rhythmicity analysis. Results from the STRING and IPA analysis were merely descriptive and a more comprehensive bioinformatic analysis would provide additional information about potential molecular mechanism explaining the differential gene expression. For example, enrichment of transcription factors binding sites in those genes with different patters to pinpoint chromatin regulatory pathways.

Author Response

We thank the editors and the reviewers for their comments. In response, we plan to revise the manuscript in order to provide the details requested and include additional bioinformatic analysis of the data, along the lines suggested by the reviewers. We will also take into account individual variations among the subjects investigated in this study, and discuss the extent to which factors other than age might contribute to the results. And we will expand the discussion to consider how our results may apply to other cells/tissues and how they relate to other findings in the field.