



RESEARCH PAPER



Age-related epigenetic drift deregulates *SIRT6* expression and affects its downstream genes in human peripheral blood mononuclear cells

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ABSTRACT

Sirtuin 6 (*SIRT6*) exerts a protective effect on health and extends the lives of model organisms. We, therefore, aimed to clarify whether age-related epigenetic drift is responsible for differences in *SIRT6* expression in peripheral blood mononuclear cells (PBMCs) of healthy young ($n = 55$, mean age 27.5 ± 4.4 years), middle-aged ($n = 51$, 65.4 ± 3.3 years), and long-lived ($n = 51$, 93.9 ± 3.6 years) humans. *In silico* analysis was performed using the STRING network. No age-related differences were observed in the percentage of *SIRT6* CpG island methylation. However, the age affected the expression of miR-34a-5p, miR-125a-5p, miR-186-5p, miR-342-5p and miR-766-3p (all $p < 0.0001$), miR-181-2-3p and Let-7c (both $p = 0.0003$), and miR-103a-3p ($p = 0.0069$). A negative association was observed between *SIRT6* mRNA and miR-186-5p ($r_s = -0.25$, $p = 0.026$), and a positive association was observed with miR-34a-5p ($r_s = 0.31$, $p = 0.0055$) and miR-181a-2-3p ($r_s = 0.39$, $p = 0.0002$). *SIRT6* mRNA also negatively correlated with the expression of *TP53* ($r_s = -0.41$, $p = 0.0126$) and *MYC* ($r_s = -0.35$, $p = 0.0448$). Notably, the expression of several miRNAs and genes was similar in young and long-lived groups but different from the middle-aged group. We conclude that age-related epigenetic changes can affect the expression of *SIRT6* in PBMCs and, in this way, possibly influence immunosenescence. Moreover, molecular events could differentiate ‘normal’ ageing from that of long-lived individuals.

ARTICLE HISTORY

Received 23 December 2019
Revised 9 April 2020
Accepted 21 April 2020

KEYWORDS

SIRT6; interaction network; miRNA; methylation; peripheral blood mononuclear cells (PBMCs); aging; longevity

Introduction


Sirtuins form a conserved family of nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylases. Invertebrates have a single sirtuin isoform, whereas mammals have seven sirtuins (*SIRT1-7*) that are expressed in different cell compartments [1]. *SIRT6*, which is present mainly in the nucleus, plays an essential role in the post-translational epigenetic modification of histone proteins, chromatin formation, and maintenance of genome integrity [2,3]. It decreases oxidative stress, increases resistance to DNA damage [4,5], and takes part in DNA repair [6] and metabolic homeostasis [7]. *SIRT6* also plays a role in acute and chronic inflammatory responses, where it exhibits both pro- and anti-inflammatory activities [8,9].

Overexpression or increased activity of *SIRT6* orthologs have beneficial effects on the lifespans of model organisms. For example, overexpression of *sir2* (silent information regulator 2) in *S. cerevisiae*

extended its life by 30 to 50% [10–12], the presence of additional copies of *dSir2* extended life of *D. melanogaster* by 57% [13], while *SIRT6* overexpression in male mice resulted in a 15% lifespan elongation [14]. Notably, *SIRT6* appears also to affect the length of life in humans. The C allele of the *SIRT6* rs350846 polymorphism is positively associated [15], while the T allele of rs117385980 is negatively associated with longevity [16]. Furthermore, individuals with the CC or CT genotype of rs107251 had a greater 5-year mean survival time compared to carriers of the TT genotype [17]. In addition to the ageing-delaying functions of *SIRT6* related to DNA repair and telomere homeostasis, *SIRT6* exerts protective effects on human health through various molecular mechanisms (e.g., by regulation of stem cell biology, cancerogenesis, and metabolic homeostasis), which may be the underlying causes for the extended lifespan [18,19].

Epigenetic modifications, such as DNA methylation, post-translational modifications of

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 Supplemental data for this article can be accessed here

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histone proteins, and the action of small non-coding RNA molecules, undergo ageing-related changes [20]. In the context of DNA methylation, this phenomenon was called an ‘epigenetic drift’ [21]. However, this term is currently extended to the other epigenetic changes (e.g., miRNA interference) that occur with age, since all of them affect age-related changes in the transcriptome [22–24].

In our previous work, we showed that *SIRT6* expression in human peripheral blood mononuclear cells (PBMCs) significantly decreases with age; however, such a decrease was not observed in PBMCs of long-lived, healthy study subjects (Supplementary Figure S1) [23]. In the current study, using unique biological material obtained from healthy middle-aged and long-lived individuals, we aimed to clarify whether two epigenetic regulatory mechanisms (i.e., DNA methylation and the action of miRNAs) could be responsible for the age-related differences in *SIRT6* expression in PBMCs and whether these *SIRT6* alterations correlated with the expression of interacting genes associated with vital cellular processes, including apoptosis and immunity.

Results

Analysis of the methylation status of the *SIRT6* promoter region

In silico analysis identified a 564-bp fragment within the *SIRT6* gene, located between –292 bp and +272 bp relative to the transcription start site (TSS). For methylation analysis, this CpG island was divided into two parts, and each fragment contained additional surrounding sequences. The first fragment was located between –412 and –10 bp (32 potential methylation sites), and the second fragment was located between –3 to +302 bp (29 potential methylation sites) relative to the TSS. Our method of methylation analysis revealed that the first fragment had a higher percentage of the methylated CG pairs than the second; however, no age-related differences in the percentage of methylation in both analysed regions were observed

(Supplementary Figure S2). There was also no correlation between the percentage of methylation and *SIRT6* mRNA levels, suggesting that methylation does not play an essential role in ageing-related *SIRT6* expression changes in PBMCs.

Expression of *in silico*-selected *SIRT6* mRNA-interacting miRNAs in PBMCs

miRNAs that could potentially interact with *SIRT6* mRNA, were selected using the MirWalk (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk/>), miRanda (<http://www.microrna.org/microrna/home.do>), and TargetScan (<http://www.targetscan.org/>) programs, as well as literature data [25]. Results of the *in silico* analyses are summarized in Supplementary Table S1. Two out of eight studied miRNAs, let-7c and miR-342-5p, appeared to interact with the coding sequence (CDS), while miR-181a-2-3p with the 5′UTR. Taking into account an increasing amount of data indicating the role of 5′UTR and CDS regions in the regulation of gene expression, we have decided to include these miRNAs into our experiments as they could have additional interaction sites in the 3′UTR [26,27].

miRNA expression analysis was performed using real-time PCR. No significant gender-related differences in miRNA levels were observed, and further analyses were performed for all study subjects combined. To normalize the data, we intended to use U6 small nuclear RNA (U6 snRNA) expression as a reference, but in our data set, its levels significantly differed between the studied groups. However, the age did not affect the mean value of the miR-125b-5p cross point (Y: 32.7, E: 32.8, LL: 32.2); therefore, its expression was used as a reference for other analyses.

Expression of the remaining miRNAs was affected by age: $p < 0.0001$ for miR-34a-5p, miR-125a-5p, miR-186-5p, miR-342-5p and miR-766-3p; $p = 0.0003$ for miR-181-2-3p and Let-7c; $p = 0.0069$ for miR-103a-3p. The multiple comparisons tests (post-hoc) showed significant differences between the age groups (Figure 1). Age-related alterations in miR-34a-5p expression have been previously described [23] and confirmed in the current study.

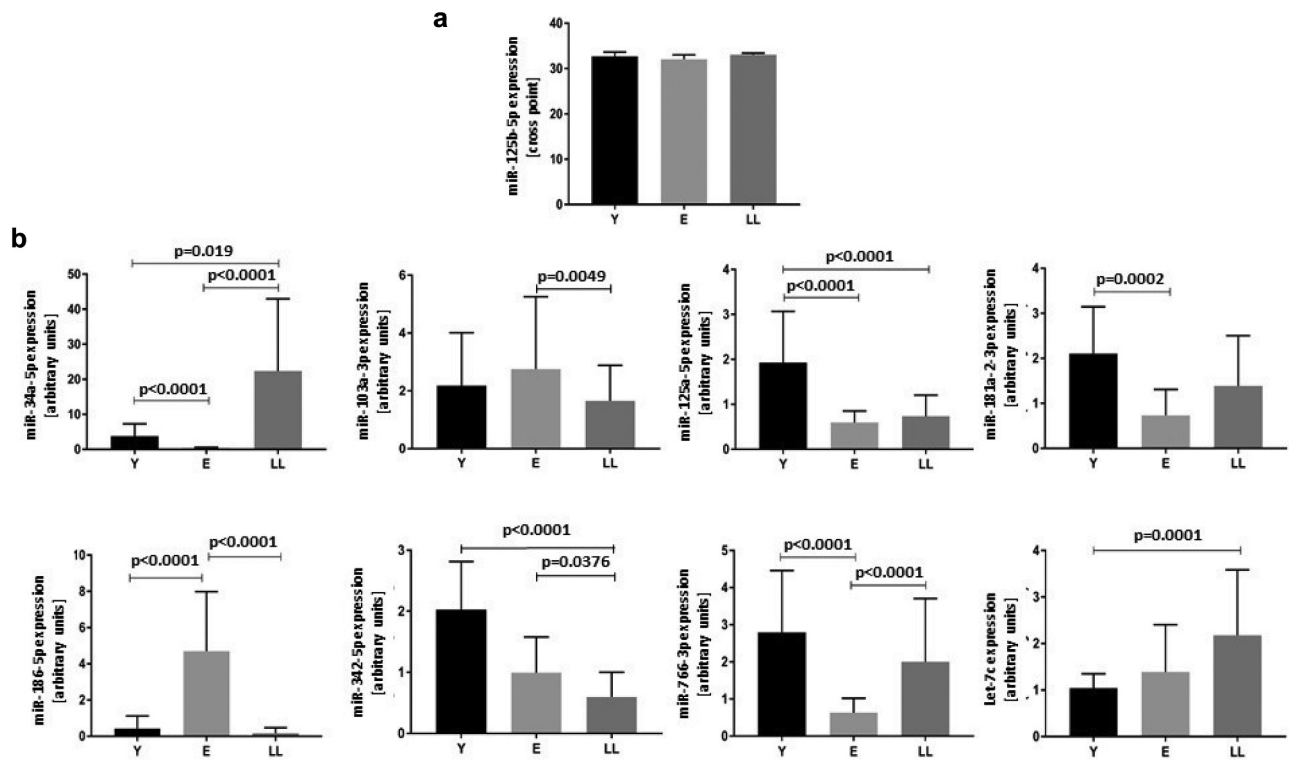


Figure 1. Age-related expression of miRNAs indicated *in silico* as interacting with *SIRT6* mRNA in PBMCs. (a) Levels of miR-125b-5p are not affected by age. (b) Levels of miR-34a-5p, miR-103-3p, miR-125a-5p, miR-181a-2-3p, miR-186-5p, miR-342-5p, miR-776-3p and Let-7c are significantly affected by age. Results are presented as median with interquartile range. Statistical analysis was performed with the Kruskal-Wallis test and Dunn's multiple comparisons test. Y: young (mean age 27.5 ± 4.4 years), E: middle-aged (65.4 ± 3.3 years), LL: long-lived (93.9 ± 3.6 years).

Analysis of the interaction between selected miRNAs and *SIRT6* mRNA

Interactions of miR-34a-5p and miR-766-3p with the *SIRT6* mRNA have been previously described [28,29]. Therefore, only miR-125a-5p, miR-181a-2-3p, miR-186-5p, and Let-7c were subjected to functional analysis. We performed co-transfection of HEK293 with synthetic miRNA precursors and pmirGLO_ *SIRT6* reporter vector containing the entire 3'UTR of *SIRT6* mRNA. There was no significant reduction in luminescence following co-transfection of the pmirGLO_ *SIRT6* vector with miR-125a-5p, pre-miR-181a-2-3p, or pre-let-7c. However, co-transfection of pmirGLO_ *SIRT6* and pre-miR-186-5p caused a 24% decrease ($p = 0.0006$) in luminescence (Figure 2(a)). Moreover, overexpression of miR-186-5p (80% transfection efficiency) resulted in a 54% decrease in endogenous *SIRT6* protein levels (Figure 2(b)). The Western blot analysis was performed once, and the results of densitometric scanning are provided in Supplementary Table S2.

Correlation of miRNAs expression with the expression of *SIRT6*

A lack of evidence confirming a direct interaction of a given miRNA with *SIRT6* mRNA does not mean that the miRNA does not affect *SIRT6* expression because such an effect can be the result of indirect interactions. Therefore, we performed correlation analyses between all miRNAs affected by age and *SIRT6* mRNA expression in all studied age groups. Detailed data regarding the number of individuals included in each correlation analysis are presented in Supplementary Table S3.

The Spearman's rank correlation showed a relationship between *SIRT6* expression and three miRNAs. A negative association was observed for miR-186-5p ($r_s = -0.25$, $p = 0.026$), and positive correlations were found for miR-34a-5p ($r_s = 0.31$, $p = 0.0055$) and miR-181a-2-3p ($r_s = 0.39$, $p = 0.0002$) (Table 1). Considering the values of the r_s coefficient, these correlations were weak; however, such a situation is common

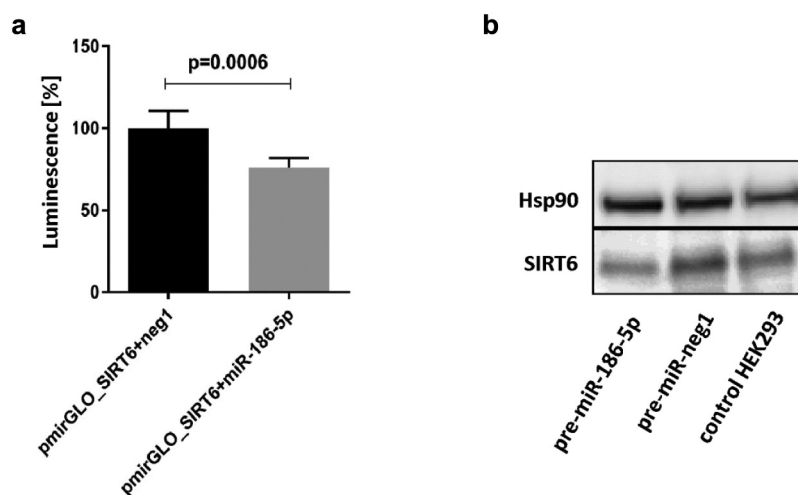


Figure 2. Effects of interaction of the 3'UTR of *SIRT6* mRNA with miR-186-5p. (a) Co-transfection of the pmirGLO_ *SIRT6* vector containing the full-length 3'UTR of *SIRT6* mRNA and the firefly luciferase reporter gene with pre-miR-186-5p results in a significant decrease in luminescence as compared to co-transfection with the pre-neg-miR negative control (neg1). Results are presented as mean with standard deviation. Statistical analysis was performed with a two-tailed Student *t*-test. (b) Transfection of HEK293 cells with pre-miR-186-5p results in a decrease in the expression of endogenous *SIRT6* protein. Hsp90 – heat shock protein 90, control protein expression of which is not affected by miR-186-5p. The Western blot analysis was performed once, and the results of densitometric scanning are provided in Supplementary Table S2.

Table 1. Correlation of miRNAs expression with the expression of *SIRT6* in human PBMCs.

miRNA	r_s	p value*
miR-34a-5p	0.3097	0.0055
miR-103a-3p	-0.1336	0.2094
miR-125a-5p	0.1212	0.2553
miR-181a-2-3p	0.3933	0.0002
miR-186-5p	-0.2525	0.0257
miR-342-5p	0.0097	0.9293
miR-766-3p	0.2054	0.0521
Let-7c	-0.1343	0.2068

*The Spearman's rank correlation was used for the analysis of the association between the expression of *SIRT6* and miRNAs.

if only selected elements of complicated networks and multiple interactions are analysed.

mRNA expression of *SIRT6*-interacting genes and proteins

Because we were interested in the ageing of immune cells, we evaluated genes and their protein products that are involved in the regulation of three cellular processes: immunity, apoptosis, and ageing, which are also direct or indirect *SIRT6* interactors. To study the connection network between *SIRT6* and proteins involved in the processes mentioned above, the STRING network version 10.5 (<https://string-db.org/>) was used with a minimum required interaction score of 0.4 (medium confidence).

Based on an extensive search of the literature, we selected 15 genes (*ICAM1*, *CD48*, *STAT2*, *RDH11*, *DISC1*, *TP53*, *BCL2*, *IGF1 R*, *MYC*, *BAP1*, *BAX*, *BAK*, *CASP8*, *CASP9*, *FAS*), whose products may be involved in the processes mentioned above. Next, using STRING, we verified whether these proteins form an interaction network with *SIRT6*, and allowing up to 5 interactors in the first shell to fill the 'gaps' in the resulting network. In our search, we marked only text mining, database, and experiments as data sources. We rejected proteins that were not *SIRT6* interactors (*DISC1*, *RDH11*, and *BAP1*) and added *MDM2*, which was indicated by STRING as a direct interactor of this sirtuin. The results of the analysis are presented in Figure 3 [30–47].

We also tested whether the mRNA expression of direct *SIRT6* interactors (*TP53*, *MYC*, and *MDM2*), as well as indirect *SIRT6* interactors (*BCL2*, *CD48*, and *ICAM1*), changed in PBMCs in an age-related manner (Figure 4(a,b), respectively). We confirmed previous findings that ageing affected the expression of multiple genes, but its effect was much less pronounced in long-lived, healthy individuals compared to average-ageing people, represented here by the E group [23,48–50].

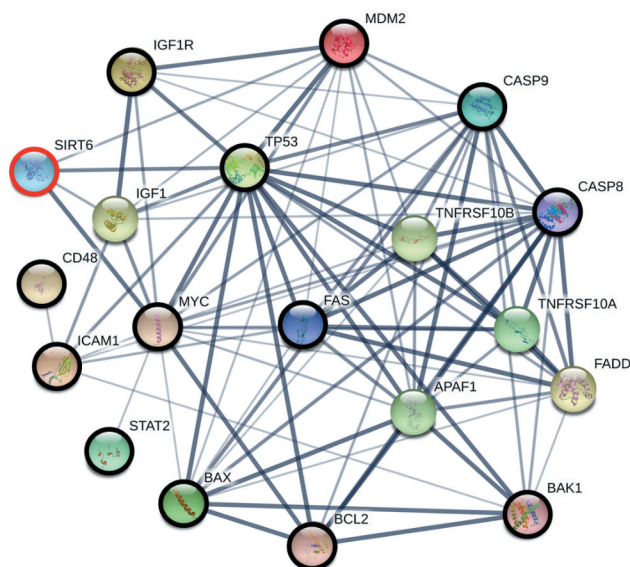


Figure 3. The interaction network between proteins, each of which is involved in the regulation of immunity, apoptosis, and ageing (bold circles), which are also direct or indirect SIRT6 interactors. The network was constructed using the STRING functional protein association network version 10.5 (<https://string-db.org/>) with the minimum required interaction score of 0.4 (medium confidence). To fill 'gaps' between SIRT6 and these proteins, the program was allowed to include up to 5 additional interactors in the first shell.

Notably, *SIRT6* expression was negatively correlated with both *TP53* ($r = -0.41$, $p = 0.0126$) and *MYC* ($r = -0.35$, $p = 0.0448$) mRNA levels. However, there was no correlation between *SIRT6* and *MDM2* mRNA levels ($r = -0.28$, $p = 0.1115$). We did not find any significant correlations between the expression of *SIRT6* and its indirect interactors. Detailed data regarding the number of individuals included in each correlation analysis has been presented in Supplementary Table S4.

Discussion

It is estimated that in the majority of people, genetic factors determine their lifespan in only approximately 25% [48,49]. A small percentage of the population can achieve longevity due to the favourable combination of polymorphisms in genes regulating the rate of ageing, which predisposes to an efficient metabolism or an efficient response to different stresses. Those who are not genetically predisposed to longevity can modify their lifespan by targeting the same pathways with a proper lifestyle or

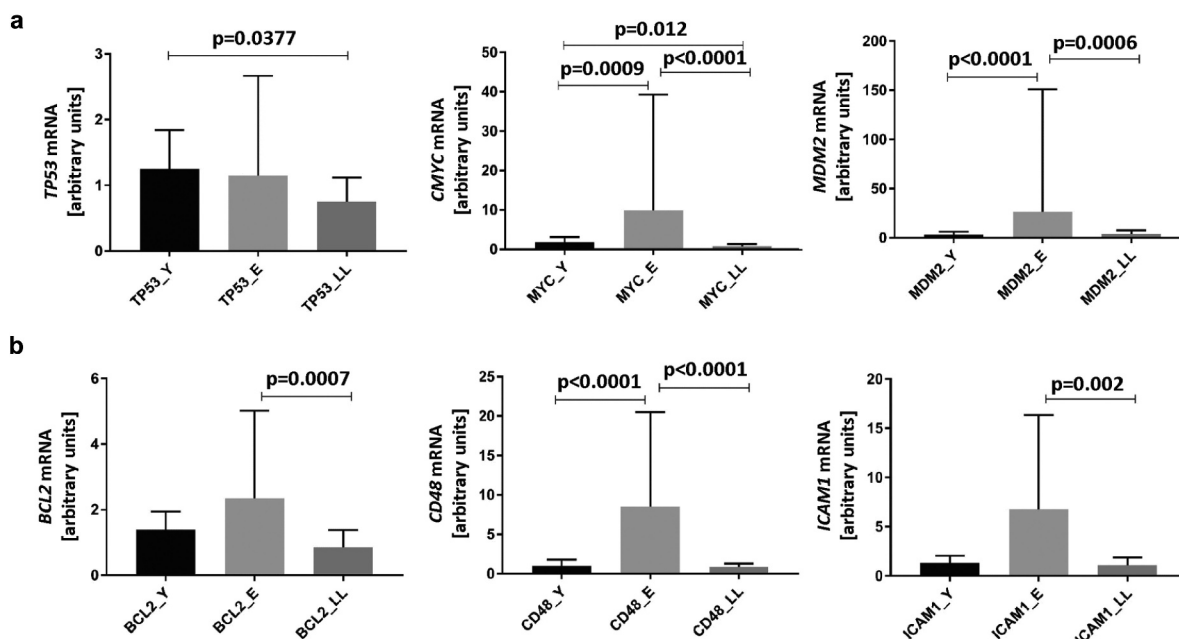


Figure 4. Age-related mRNA expression of proteins interacting with SIRT6 in PBMCs. Results, normalized against the expression of the β -actin gene (ACTB), are presented in arbitrary units as median mRNA levels with interquartile range. Statistical analysis was performed with the Kruskal-Wallis test and Dunn's multiple comparisons test. (a) Direct SIRT6 interactors TP53, MYC, and MDM2. (b) Indirect SIRT6 interactors BCL2, CD48, and ICAM1. Y: young (mean age 27.5 ± 4.4 years), E: middle-aged (65.4 ± 3.3 years), LL: long-lived (93.9 ± 3.6 years).

interventions. Epigenetic factors mediate this beneficial effect of lifestyle on genetic predisposition [50–52].

We previously reported that *SIRT6* expression significantly decreases with age, being the lowest in the PBMCs of middle-aged subjects and rising again in long-lived individuals, where it reaches a level similar to that in cells of young people [23]. Having in mind that ageing is associated with an epigenetic drift, we decided to investigate whether epigenetic mechanisms stand behind the age-related fluctuations in the *SIRT6* level in PBMCs. As hypermethylation is one of the most common mechanisms responsible for ageing-associated decreases in gene expression, we first analysed *SIRT6* promoter methylation. However, we found neither significant differences in the percentage of methylation between the different age groups nor significant correlations between methylation and *SIRT6* expression levels. These results are contrary to previously published data that showed higher methylation levels in the *SIRT6* promoter in 20 to 79-year-old study subjects compared to those aged 9 to 19 years. These inconsistent results are likely due to the many methodological differences between the two studies. In our study, groups were approximately 3-fold larger, and all participants were healthy. The age groups were defined differently, and the methods of analysis were also different [53]. Nevertheless, the age-related expression pattern of *SIRT6* in our study subjects seemed to match the methylation trend described by Sahin et al. [53]. Thus, the role of methylation in the age-related *SIRT6* expression changes remains to be clarified.

Another epigenetic mechanism that can affect gene expression is the activity of miRNAs. Analysis of the *SIRT6* mRNA using the *TargetScan*, *miRWalk*, and *miRanda* programs indicated that several miRNAs could potentially interact with this mRNA. Direct interactions between *SIRT6* mRNA and some of the candidates were previously demonstrated [28,29]. In this study, we showed that miR-186-5p bound to the 3'UTR of *SIRT6* mRNA and negatively affected endogenous *SIRT6* expression in HEK293 cells, which is consistent with data obtained in non-small cell lung carcinoma [54] and suggests that this interaction can be responsible for the negative correlation between *SIRT6* mRNA

and miR-186-5p also in PBMCs. However, an *in vitro* study with a reporter vector with cloned *SIRT6* 3'UTR sequence bearing mutations in the miR-186-5p binding sites would further support the notion that miR-186-5p interaction with *SIRT6* mRNA is of functional significance.

In contrary, under our experimental conditions, miR-125a-5p, miR-181a-2-3p, and Let-7c did not interact with *SIRT6* mRNA. The fact that not all *in silico*-selected miRNAs interacted with the target mRNA could reflect the lack of full compatibility between mathematical predictions and biological events. It should be taken into account, however, that our experimental model might not be optimal for the detection of weaker interactions.

For the *SIRT6* mRNA-interacting miRNAs, miR-34a-5p correlated positively, miR-186-5p negatively, and miR-766-3p did not correlate with *SIRT6* mRNA expression. A negative correlation between miR-186-5p and *SIRT6* expression is compatible with the assumption that while miRNA expression increases, its target gene expression decreases. However, we detected a positive correlation between *SIRT6* mRNA and miR-34a-5p. It has been previously indicated that gene regulatory networks often include feedback motifs (i.e., 'feed-forward loops' overrepresented in pure transcription regulatory networks or less abundant 'feedback loops'). As part of complex regulatory networks, miRNAs coordinate transcriptional regulation in signalling networks. Positive correlations could be explained by downstream effects, such as inhibited repression or disturbances in feedback loops. Moreover, miRNA-mRNA positive correlations have been previously reported [55–57]. An important limitation of our results is the fact that the observed correlations, measured by the r_s value, should be interpreted as weak. Nevertheless, ageing-associated changes in the expression of miRNAs, especially miR-186-5p, are most likely an important mechanism responsible for age-related differences in *SIRT6* expression.

Maintenance of *SIRT6* expression might be one of the mechanisms promoting stress resistance and longevity. Indeed, *Sirt6*-knockout mice have a significantly shortened lifespan accompanied by degenerative and metabolic defects reminiscent of

premature ageing [58]. SIRT6-deleted embryonic stem cells and mouse embryonic fibroblasts demonstrate impaired proliferation and increased sensitivity to DNA-damaging agents, resulting in chromosomal abnormalities [4]. The depletion of SIRT6 leads to telomere dysfunction with end-to-end chromosomal fusions and premature cellular senescence [59–61]. Therefore, the high expression of SIRT6 that we previously described in PBMCs of long-lived individuals could suggest a function for SIRT6 in the immune system to delay immunosenescence, which promotes longevity. Indeed, SIRT6 overexpression results in a significant decrease in TNF- α , IL-1 β , sRANKL, IL-6, and IL-17 levels in rheumatoid arthritis mouse serum and delays the replicative senescence of human fibroblast-like foetal lung cells by attenuating NF- κ B signalling [62,63]. In addition, old transgenic mice overexpressing SIRT6 have a lower level of adipose inflammation compared to wild-type mice [64]. In line with this hypothesis, we observed low SIRT6 expression in PBMCs of middle-aged study participants representing a population that is not predisposed to longevity.

The STRING analysis revealed that SIRT6, along with other proteins, is involved in many cellular processes, including immunity and apoptosis. Subsequently, we found that expression levels of the majority of the analysed downstream genes encoding proteins belonging to the SIRT6 network were age-dependent. Among them were MYC and p53 being not only direct SIRT6 targets but also transcription factors playing critical roles in many cellular events. Although the criteria we set may have affected the result of the STRING analysis, the mRNA levels of both MYC and p53 negatively correlated with SIRT6 expression, strongly suggesting that age-related differences in their expression in PBMCs, at least to some extent, depended on ageing-related changes in SIRT6 expression.

The mechanistic studies of Zhang et al. established that p53 directly activates the expression of SIRT6 [38], while Li and co-workers pointed out physical interactions between p53 and SIRT6 *in vitro* and *in vivo*, making the last one a co-activator for downstream processes [65]. TP53 induces cell cycle arrest, DNA repair, apoptosis, and senescence, and also affects other, non-canonical pathways [66]. It

inhibits inflammation by antagonizing the action of NF- κ B, suppressing inflammatory cytokines, and controlling T-cell differentiation [67,68]. Therefore, its decreased expression in PBMCs of long-lived individuals could contribute to inflammaging, the process of low-grade, chronic inflammation that accompanies ageing, which is a risk factor for the development of many diseases [69]. Moreover, in some individuals, it can be a risk factor for the development of lymphomas and chronic lymphocytic leukaemia, blood diseases most commonly diagnosed in people of advanced age.

In contrast, decreased expression of MYC could have beneficial effects. MYC accelerates proliferation, promotes cell growth and survival, enhances cellular metabolism, and triggers or sensitizes cells to apoptosis [70]. Its overexpression plays a crucial role in multiple cancers, including leukaemias and lymphomas [71]. It upregulates IL-8, IL-10, TNF- α , and TGF- β [72]. Therefore, its low expression in PBMCs of long-lived individuals lowers the risk of neogenesis and inflammaging, which can be one of the mechanisms underlying longevity. Our findings regarding the possible SIRT6 – MYC interactions are confirmed in functional studies where SIRT6 by deacetylation of acH3K56 residues at MYC target genes contributed to the inhibition of MYC transcriptional output [72,73].

Of note in this study, we showed that not only the expression of various miRNAs differed between middle-aged and long-lived individuals, but also SIRT6 expression was lower, and the expression levels of other genes (e.g., MYC, MDM2, BCL2, CD48, and ICAM1) were higher in middle-aged study subjects. These and other molecular differences could lead to different susceptibilities to age-related diseases and underlie differences in the rate of ageing and life expectancy.

In conclusion, this study demonstrated age-associated alterations in miRNA control that could lead to alterations in the expression of SIRT6 and some SIRT6 interactors. These effects, in turn, could affect the function of PBMCs. In addition, the differences in the expression of various miRNAs and genes in middle-aged and long-lived individuals and similarities between young and long-lived individuals described here emphasize that average ageing differs from that of individuals predisposed to longevity, who have already reached a very advanced age.

Materials and methods

Materials

PBMCs were obtained from non-obese individuals (BMI < 30 kg/m²) belonging to three age groups: young (Y, n = 55, 29 women and 26 men, age range 19 to 42 years, mean age 27.5 ± 4.4 years), middle-aged (E, n = 51, 28 women and 23 men, age range 60 to 73 years, mean age 65.4 ± 3.3 years), and long-lived (LL, n = 51, 31 women and 20 men, age range 90 to 102 years, mean age 93.9 ± 3.6 years). Members of the E group had only a 1:97 chance to reach the mean age of the LL group (Statistics Poland, <https://stat.gov.pl/obszary-tematyczne/ludnosc/ludnosc/ludnosc-piramida/>). Therefore, in this study, the E group represented people who were not predisposed to longevity and aged in a 'standard' way.

The study participants underwent a comprehensive medical examination, and those from the middle-aged (E) and long-lived (LL) groups were evaluated using the Activities of Daily Living (ADL) and Mini-Mental State Examination (MMSE) scales [74]. All study participants were non-obese, without a history of myocardial infarction, stroke, type 2 diabetes mellitus, cancer, or neurodegeneration. However, in the E and LL groups, moderate hypertension was allowed, and some study participants belonging to the LL group had a mild degree physical or cognitive disability (ADL score 5–6 and/or MMSE score 20–23) [75,76].

The study protocol was approved by the Bioethics Committee of the Medical University of Warsaw. All participants gave informed written consent for participation in the study. The anonymity of patients was preserved at all stages of this investigation.

Isolation of PBMCs and RNA

PBMCs and RNA isolation were performed as previously described [77].

DNA methylation

A 3-kb fragment of *SIRT6* located from 1.5 kb upstream to 1.5 kb downstream of the primary TSS (transcript ENST00000337491.6, 94 release of the ENSEMBL database) was analysed using the MethPrimer 2.0 online tool (<http://www.urogene.org/methprimer2/>) with the following settings: CpG island longer than 200 kb with a minimum of 50% GC content. Methylation status was assessed using the OneStep qMethyl™-Lite Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. The sequences of the primers and probes used for the 5' fragment of the CpG island were forward 5' 'GTA ACTCTGCGTGGCATTCA3', reverse 5' 'CCCCACGGGAACAATAAAGT3', and UPL probe #56 (Roche Diagnostics, Mannheim, Germany). For the 3' end of the CpG island, the primers and probe were forward 5' 'AGGATGTCGGTGAATTACGC3', reverse 5' 'CCTGGA ACTACATCCCCTCTC3', and UPL probe #81 (Roche Diagnostics). Methylation-sensitive digestion was carried out for 2 h at 37°C. Digestion was followed by an initial denaturation for 10 min at 95°C, then 45 cycles of 30 s at 95°C, 30 s at 59°C for the 5' fragment or 60°C for the 3' fragment, 30 s at 72°C, and one cooling cycle. Reactions were performed in the LightCycler 480 II (Roche Diagnostics).

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Reverse transcription and RT-PCR

The Universal cDNA Synthesis Kit and the miRCURY LNA™ Universal RT microRNA PCR system (EXIQON, Vedbaek, Denmark) were used for reverse transcription and evaluation of the expression of miRNAs, respectively. Reverse transcription was performed with 20 µg of total RNA as a template. miRNA expression analysis was performed with cDNA corresponding to 0.02 ng of input RNA in the LightCycler 480 II (Roche Diagnostics). Primers were designed using miRprimer software [78] and are listed in Supplementary Table S5. The reaction conditions were as follows: 10 min at 95°C, 45 cycles of 10 s at 95°C, 1 min at 60°C, and a melting curve cycle.

Reverse transcription for the analysis of gene expression was performed as previously described [23]. The PCR reaction was carried out as follows: 5 min at 95°C, 45 cycles of 10 s at 95°C, 10 s at 60°C, 10 s at 72°C. The primer sequences for the expression analysis are presented in Supplementary Table S6. The *CD48* primers were designed by our laboratory. All other primer

sequences were based on previous reports [77,79–83]. Results, normalized against the expression of the β -actin gene (*ACTB*) were presented in arbitrary units (AU) as mean mRNA levels.

Reporter vector for mRNA-miRNA interaction analysis

DNA corresponding to the full-length 3'UTR of *SIRT6* mRNA (514 bp) was amplified using Dream Taq polymerase (Thermo Scientific, Vilnius, Lithuania) from 100 ng of cDNA prepared from the RNA isolated from PBMCs. The forward (5' **ACTAGAGCTCCAGCTGACCAGGGTGCTT3'**, STOP codon is underlined) and reverse (5' **CTAACTCGAGGCAAGAAAGAAATTGTTTT-TATTG3'**) primers contained *SacI* or *XhoI* restriction sites (bolded sequences), respectively. The PCR conditions consisted of 4 min at 94°C, 39 cycles of 30 s at 94°C, 30 s at 59°C, 30 s at 72°C, and a final extension of 4 min at 72°C. The PCR product was cloned into the pmirGLO reporter vector (Promega, Madison, WI, USA) and sequenced (pmirGLO_*SIRT6*).

Cell culture and transfection

Human embryonic kidney cells 293 (HEK293; Sigma-Aldrich, St. Louis, MO, USA) were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum at 37°C in 5% CO₂. Twenty-four hours before transfection, cells were seeded in a 96-well plate for evaluation of miRNA interaction with the 3'UTR of *SIRT6* mRNA (3 x 10⁴ per well) or 12-well plate for the assessment of the effect of miRNA on endogenous *SIRT6* expression (2.2 x 10⁵ per well). Cells were transfected at 80% confluency using Lipofectamine 2000 according to the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA).

For miRNA interaction with the 3'UTR of *SIRT6* mRNA, 40 ng of the reporter plasmid and 5 pmol (30 nM final concentration) of the synthetic pre-miRNA (Life Technologies) were used. Pre-miR miRNA Precursor Negative Control #1 (Ambion, Life Technologies) served as a negative control. Cells were cultured for 24 h after transfection, washed with phosphate-buffered saline, and

lysed for 15 min with 30 μ l Passive Lysis Buffer (Promega) on a rocking platform. The luminescence was measured using the Centro XS3 LB 960 luminometer (Berthold Technologies, Bad Wilbad, Germany). The luminescence of the firefly luciferase substrate was normalized against that of the *Renilla* luciferase substrate. The mean luciferase activity in extracts from cells co-transfected with the pmirGLO_*SIRT6* vector and synthetic pre-neg-miRNA was normalized to 100%. All experiments were repeated six times.

For the assessment of the miRNA effect on endogenous *SIRT6* expression, 40 pmol (30 nM final concentration) of synthetic pre-miR-186-5p or pre-miR miRNA Precursor Negative Control #1 were used.

Western blot analysis

For the evaluation of endogenous *SIRT6*, cells were harvested 24 h after transfection using radioimmunoprecipitation assay (RIPA) buffer. Total protein (30 μ g) was resolved on a 4 to 15% Mini-PROTEAN[®] TGX[™] gel (Bio-Rad, Hercules, CA, USA) and transferred to a nitrocellulose membrane. The membrane was incubated with mouse anti-*SIRT6* primary antibody (1:200, sc-517196, Santa Cruz Biotechnology, Dallas, TX, USA) and anti-mouse IgG secondary antibody (1:2000, sc-516102, Santa Cruz Biotechnology). As a control, blots were incubated with rabbit anti-Hsp90 primary antibody (1:1000, sc-7947, Santa Cruz Biotechnology) and anti-rabbit IgG secondary antibody (1:10000, AP132P, Millipore, Darmstadt, Germany). Proteins were detected using the Clarity[™] ECL Western Blotting Substrate (Bio-Rad) and visualized with the GeneGnome XRQ bioimaging system (Syngene, Cambridge, MA, USA). Densitometric measurement was performed with Image Studio Lite software (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

The Shapiro-Wilk test was used to assess the normality of distributions. Whenever three age groups were analysed, and the distribution was not normal, the Kruskal-Wallis test and Dunn's multiple comparisons test were used. The two-

tailed Student *t*-test was used for the analysis of the interaction of miRNAs with *SIRT6* mRNA. The U Mann-Whitney test was used to examine the expression of genes in two age cohorts. The Spearman's rank correlation was used for the analysis of the association between the expression of *SIRT6* and other factors and the analysis of other associations. The strength of the correlation was interpreted as 'weak' when Spearman's correlation coefficient value (r_s , a statistical measure of the strength of a monotonic relationship between paired data) was between 0.2 and 0.39, 'moderate' when 0.4 to 0.59, 'strong' when 0.6 to 0.79 and 'very strong' when r_s was between 0.8 and 1.0 [84]. All calculations were performed with GraphPad Prism 7.0. Significance was indicated by $p < 0.05$.

Disclosure statement

The authors declare no conflict of interest.

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