Characterizing the relationship between telomere length and DNA methylation age in induced pluripotent stem cells during neuronal differentiation

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Background

Induced pluripotent stem cells (iPSCs) and their resultant neurons are popular models for studying diseases of aging, such as Alzheimer's disease (AD). One hallmark of aging is the decreasing length of telomeres, the repetitive, protective DNA sequences at the end of each chromosome. Another method for assessing aging is through epigenetic age, as DNA methylation at certain loci is known to change robustly with time [1]. It has also been shown by others that telomere length in iPSC-derived neurons is shorter than the that of the stem cells from which they are derived [2]. However, to our knowledge no one has looked at both epigenetic age and telomere length in the same samples. Therefore, we have collected DNA from different stages of the differentiation of iPSCs into neurons in order to look at the relationship between epigenetic age and telomere length.

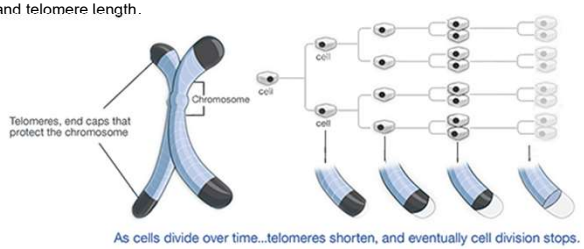

Figure 1. Diagram illustrating how telomere length decreases with cellular division [3].

Methods

iPSC generation and differentiation

Human fibroblast cells were reprogrammed using lentiviral transfection of six transcription factors (Klf4, Sox2, Oct4, c-Myc, Lin28 and Nanog). The resulting iPSC colonies were expanded and differentiated over a number of weeks [4] **(figure 2)**. Cells were collected at four different time points: day 0 (iPSCs), day 16 (neuronal progenitor cells, NPCs), day 37 (mature neurons) and day 58 (mature neurons). DNA was extracted using a standard phenol chloroform extraction protocol.

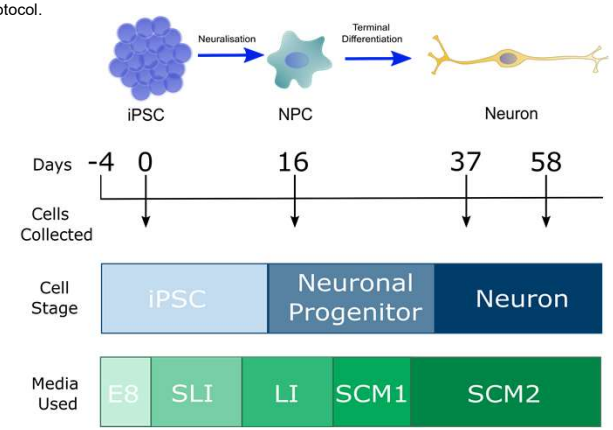

Figure 2. Neuronal differentiation timeline.
Diagrammatical representation of the important time points throughout neuronal differentiation. This includes the days when the samples were collected and the cell culture media used at each cellular stage. including when cells were collected and cellular stage. Where E8 = Essential 8 medium (Gibco), LI/SLI = neuronal induction media, SCM1/2 = synaptojuice 1/2 media, for more information on what each media contains please refer to [4].

Infinium Human Methylation EPIC BeadChip Array

After DNA extraction, the samples were bisulfite treated before being amplified and hybridised on the BeadChip. Intensities taken from the array are then extracted and methylation values calculated.

Calculation of Epigenetic Age

The coefficients and intercept for both the Horvath [1] and Steg [5] age calculators were downloaded and were applied using the using the 'agep' function of the wateRmelon package [6]. The epigenetic ages calculated by the Horvath clock were then converted from years to days post-conception to allow comparisons to be made. To test for differences in predicted epigenetic age between cell stages for each clock, we used an ANOVA followed by Tukey's honest significant difference (HSD) test to allow for multiple comparisons.

Quantitative Real Time PCR

Methodology used is an adaptation of the method developed by O'Callaghan and Fenech [7] and Cawthon [8]. In brief, we performed qRT-PCR in triplicate in 384 well optically clear plates in a total reaction volume of 5µl. On each plate we also included 8 standard DNA samples in triplicate of known copy number. Samples were run on a QuantStudio 6K qPCR machine.

Primer/Standard design

Telomere primers and standards were taken from Cawthon, R., 2002 [8] as here the primers had been specifically designed to prevent primer dimer derived amplification products. GAPDH primers were designed to map to the transcription start site and the GAPDH standard was used as this is the predicted PCR amplicon.

Primer sequences	GAPDH Forward	GCCCCCGGTTTCTATAAATT
	GAPDH Reverse	GAACAGGAGGAGCAGAGAG
	Telomere Forward	GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT
	Telomere Reverse	TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA
Standard sequences	GAPDH	GCCCCCGGTTTCTATAAATTGAGCCCGCAGCCTCCCGCTTCGCTCTCTGCTCCTCCTGTTC
	Telomere	(TTAGGG)[14]

Table 2. Primer and standard sequences.
Table showing the primer and standard sequences to determine telomere length, where GAPDH is used as the single copy gene to determine absolute telomere length. The telomere standard is compromised of the same hexanucleotide sequence repeated 14 times.

Results

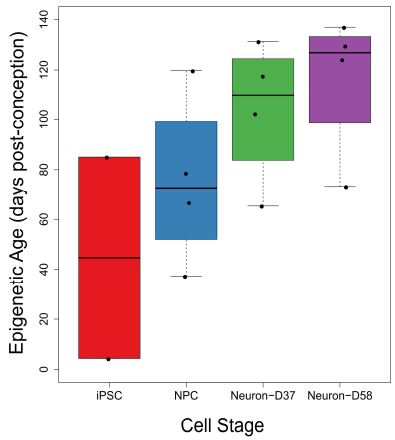

Figure 3. The predicted biological ages of iPSC-derived neurons through differentiation using the Horvath age clock.
As two iPSC samples did not pass the quality control checks, there are only two samples in the iPSC group on each graph. First we established the epigenetic ages using the Horvath skin and blood clock [1] and converted the output (of years) to days post-conception to make it easier to compare to the Steg clock [5]. Whilst there is a linear increase in epigenetic age with time/differentiation the "oldest" sample only has an age of ~140 days post conception. This is the equivalent of approximately 20 weeks and shows that all of the samples have a fetal epigenetic age. It is important to bear in mind that this age clock was not trained on fetal data, however it is still interesting they are predicted as being fetal despite this.

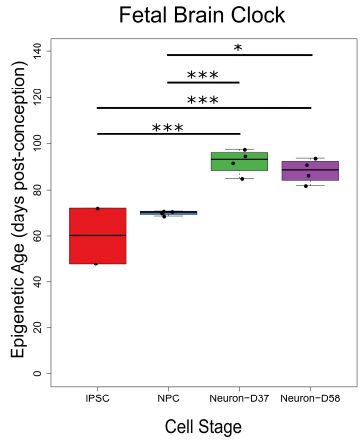

Figure 4. The predicted biological ages of iPSC-derived neurons through differentiation using the Steg age clock.
Following on from using the Horvath clock we used the Steg *et al* [5] clock as this has been trained on fetal brain sample data. From this we can see that the estimated epigenetic age of iPSCs and NPCs are significantly lower than the mature Day 37 and 58 neurons, but that there is no difference between iPSCs and NPCs or between Day 37 and 58 neurons. The lack of significance between the two terminally neuronal time points could suggest that after a certain time post terminal differentiation that epigenetic age no longer increases. This is in contrast with the ages from the Horvath clock.

The age of each sample is given in days post-conception. Key: $*P < 0.05$ and $***P < 0.005$.

Conclusions

In this study we have collected iPSCs at different time points as they differentiate into cortical neurons in order to evaluate the relationship between ageing in culture, epigenetic age and telomere length. In this first part of the study we use two different epigenetic age clocks to predict age. Whilst both clocks predict the all of the samples to be fetal, the trajectory of the ages through time is different. As the Steg clock was trained on fetal data and the resultant ages are less variable it appears more reliable for this dataset. In order to test this more thoroughly and to determine if the neuronal ages do plateau requires more time points beyond 58 days.

Optimisation of the telomere and single copy gene primers have now been completed and so work is under way to measure telomere length in all of the samples.

References

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