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Implications of mitochondrial DNA mutations in human induced pluripotent stem cells

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Single-cell analyses in recent years have shown major differences in the transcriptome between individual cells in the same induced pluripotent stem cell-derived clones. Although these differences are in part attributable to genetic and epigenetic modifications of the nuclear genome, emerging evidence suggests that variants in mitochondrial DNA also play a pivotal role.

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Induced pluripotent stem cells (iPSCs) have become a key technology for biomedical research in academia and industry. They underpin large-scale international efforts dissecting human disease mechanisms, are used as model systems for drug screening and therapeutic development and are being tested in regenerative medicine clinical trials. Harnessing this technology is not without its challenges, one of which is to understand why different clonal lineages derived from a single donor behave differently in different contexts. Recent studies[1–7] have shown that iPSCs derived from somatic cells contain mitochondrial single-nucleotide variants (mtSNVs) that affect only a proportion of the total mitochondrial DNA (mtDNA) content (that is, they are heteroplasmic). Interestingly, the proportion of mutant mtDNA (heteroplasmy level or fraction) can differ markedly in the derived iPSC cell lines. These findings highlight the need for routine screening of iPSCs for mtDNA variants.

mtDNA mutations accumulate with age
Mitochondria are the primary source of intracellular energy in the form of adenosine triphosphate (ATP)[8]. Mitochondrial proteins form critical components of the oxidative phosphorylation (OXPHOS) system, which is essential for aerobic ATP production, but they can be disrupted by mutations of mtDNA. For over two decades it has been known that fibroblasts isolated from aged humans contain more mtDNA mutations than cells taken from younger people, but the extent of this mosaicism has only just come to light. The introduction of next-generation sequencing with high-depth coverage of mtDNA has shown that heteroplasmic mtSNVs are a near ubiquitous finding in multiple tissues, including derived primary fibroblast cell lines. This background of low-level heteroplasmy includes a mixture of germline inherited mtSNVs and an age-dependent accumulation of somatic variants reflecting the ongoing replication of mtDNA throughout life[8].

Cell reprogramming affects the mtDNA landscape
Dramatic shifts in the heteroplasmy level during cell reprogramming resemble events that occur in the germ line of several species, where a restriction in the amount of mtDNA leads to a statistical sampling effect or 'genetic bottleneck', and different proportions of mutant mtDNA in the daughter cells. A similar mechanism is plausible during cell reprogramming, although it is not clear whether this occurs at the cellular, organellar or mtDNA level — or all of these. A purely stochastic genetic bottleneck mechanism accelerates the rate of genetic drift, with heteroplasmy levels increasing or decreasing with equal probability. However, there is now evidence of selection for and against variants in different parts of the mitochondrial genome during cell reprogramming, driving heteroplasmy levels up or down[1–7]. This phenomenon is intriguing because the selection is apparent for heteroplasmic mutations present at very low levels (<2.5%), indicating that even low-frequency mutations can have functional consequences. Pluripotent cells are largely dependent on glycolysis to generate ATP, which is thought to explain why they can tolerate even very high levels of known pathogenic mtDNA mutations. However, the recovery of oxidized NADH required to maintain the glycolytic flux can be achieved either by lactic fermentation or through the G3PDH shuttle and OXPHOS complexes III and IV, bypassing complex I, enabling cells to tolerate mtDNA mutations in complex I genes. Overall, these findings point towards the actual process of reprogramming driving the selection of low-level heteroplasmic variants, and not the bioenergetic requirements of iPSCs per se. mtDNA variants have also arisen in human embryonic stem cell (ESC) lines[9], suggesting generality of the process in pluripotent cells.

Some iPSC lines contain mtDNA mutations that are not detectable in the parental cell line[1–7]. The major trinucleotide mutational signature of these variants is distinct from those previously described for the nuclear genome[1] and differs from errors introduced by the mtDNA polymerase-γ (POLG), implicating a novel mechanism

> **mtDNA mutations are pervasive in iPSC clones**

yet to be discovered. Although many of the mtSNVs present in iPSC clones are silent or have no detectable effect, a substantial proportion affects cell function, contributing to differences in the expression of genes involved in cell proliferation, telomere maintenance and OXPHOS functions. The de novo mutations define subpopulations within individual iPSC clones, with an extraordinary level of heterogeneity, creating hitherto 'invisible' sub-lineages that differ between sub-clones.

A major shift in cell metabolism occurs when cells differentiate and become more dependent on aerobic ATP synthesis. This requires a burst in biosynthetic capacity to establish new complex cellular architectures. Differentiating cells might, therefore, be expected to lose mutations that compromise OXPHOS. However, this is not supported by in vitro studies, where cells differentiated into mesoderm, endoderm, neuronal precursors and mature neurons show similar heteroplasmy levels to the parental iPSC lines[1,2]. It is perhaps surprising that fibroblasts, cardiomyocytes and neuronal precursor cells derived from iPSCs containing high levels of mtDNA mutations still retain high heteroplasmy levels that produced OXPHOS deficits[2,5,6]. This finding implies a diversification of the mitochondrial anabolic and catabolic functions during differentiation involving different fuels for ATP generation, the provision of substrates for anabolic roles and, possibly, tissue-specific redox balances. Mutations affecting respiratory complexes in different ways could be tolerated or selected against in different contexts.

Implications for the use of iPSCs

It will be interesting to see whether the same patterns occur in vivo, and whether somatic genetic bottlenecks also shape heteroplasmy levels in specific cell types. Is the segregation purely random or is there selection for and/or against specific mtDNA variants? Could positive selection for certain mtDNA variants contribute to the relatively high frequency of certain pathogenic mtDNA variants across diverse populations, such as the m.3243 A > G and m.11778 G > A mutations? And is this relevant for common diseases, such as Parkinson disease, that present over half a century later with a higher burden of mtDNA mutations in affected tissues? Some of the answers to the above questions may come by following the fate of heteroplasmic mtSNVs in developing organoids at the single-cell level. If the change in gene expression during differentiation drives selection of specific mtDNA heteroplasmic variants, or subcategories of variants, this should emerge systematically. Detecting these patterns will likely require very large studies because specific mutations may behave differently, and the effects may be subtle when superimposed on the major changes due to an underlying bottleneck effect. We already know, from murine models, that purifying selection occurs through the germline bottleneck, affecting differently mtDNA variants in protein-coding genes or tRNAs. Tissue-specific preferential selection of mitochondrial genomes also occurs in a mouse model heteroplasmic for different mtDNA haplotypes, with different tissue-dependent directionality. Interestingly, the background mtDNA haplotype can also influence iPSC generation from mouse embryonic fibroblast (MEFs)[10].

> **clinical outcome could depend on somatic mutations of mtDNA**

This could be due to excess reactive oxygen species generation during the 'burst' of biosynthesis because it is ameliorated by the addition of N-acetylcysteine (NAC) during reprogramming[10]. In keeping with this explanation, there was strong selection favouring one haplotype during iPSC generation, an effect that was eliminated if NAC is added during reprogramming[10]. These findings show that both random and selective mechanisms can operate in concert, shaping the fate of the mtDNA heteroplasmy during iPSC reprogramming and differentiation. Human iPSCs-derived organoids provide a new opportunity to deepen our understanding of mtDNA heteroplasmy segregation during tissue differentiation, with implications for the tissue specificity of mtDNA diseases.

Overall, mtDNA mutations are pervasive in iPSC clones, and, importantly, they can have functional consequences. There is therefore a strong argument to screen iPSC lines for deleterious mutations and at least incorporate this information into the interpretation of any further work. Given the dynamic state, it will be important to screen for mutations before and after any experiments. Understanding the mtDNA landscape is even more important for regenerative medicine studies, where the clinical outcome could depend on somatic mutations of mtDNA. It would be a tragic irony to deliver cells containing deleterious mtDNA mutations into the substantia nigra of patients with Parkinson disease, in whom somatic mtDNA mutations are thought to contribute to the primary pathology. If we are to avoid these pitfalls, screening iPSCs for mtDNA variants should become routine, as is already the case for nuclear chromosomal rearrangements.

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