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RESEARCH HIGHLIGHT

Telomere dynamics in dyskeratosis congenita: the long and the short of iPS

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Seminal experiments by Hayflick in the 1960s demonstrated that normal human diploid cells have a finite replicative life span in culture [1]. The Hayflick "limit" is explained at least in part by the decay in telomeres, repeat sequences that cap the ends of chromosomes [2]. Telomeric repeats are only incompletely extended by DNA polymerase and thus erode with each cell division. When telomeres become critically short, cells stop dividing and senesce. The telomere length in somatic cells is an "endowment" inherited from the stem cells from which they derive. Stem cells contain telomerase, a multisubunit ribonucleoprotein polymerase that adds repeats to telomere ends and thereby counteracts telomere attrition and replicative senescence, allowing stem cells to self-renew and maintain adult tissues throughout life. Unlike most telomerase components, the telomerase reverse transcriptase (*TERT*) is absent in somatic cells, and therefore the gatekeeper of telomerase function. *TERT* is expressed in highly self-renewing or immortalized cells such as embryonic cells, germ cells, cancer cells, and some adult stem cells, and forced expression of *TERT* in somatic cells overcomes replicative senescence and contributes to malignant transformation. Reprogramming of somatic cells to an embryonic state by nuclear transfer or factor-mediated direct reprogramming activates *TERT*, and is associated with the acquisition of self-renewal capacity [3, 4].

Dramatic support for the theory that aging of cells and tissues and perhaps even the whole organism depends on telomere length comes from study of the human premature aging syndrome dyskeratosis congenita (DC). The skin and mucosal lesions for which DC is named are the outward manifestations of a systemic degenerative disorder characterized by a propensity for bone marrow failure, pulmonary fibrosis, hematologic and epithelial malignancy, and other pleiotropic defects. In the past 12 years, elegant studies have revealed mutations in seven genes as a cause of DC, all of which encode telomerase (*DKC1, TERT, TERC, NOP10, NHP2, TCAB1*) or telomere structural (*TINF2*) components. Moreover, mutations in some of these genes have been found in familial degenerative disorders without classical DC, including aplastic anemia, myelodysplastic syndrome, and idiopathic pulmonary fibrosis [5]. Primary somatic cells from DC patients display short telomeres and abridged replicative capacity *in vitro*, observations that link Hayflick's limit, telomere biology and human aging and disease.

DC and DC-like disorders show a wide variation in clinical severity, age of onset, and organ involvement, and genotype-phenotype correlations are poorly established. Most DC-associated mutations entail only partial loss of function, implying that severe or null germline mutations are incompatible with human life. Mouse modeling of DC is hindered by major interspecies differences in telomere biology. Given their markedly longer telomeres, even telomerase-null mice are viable and healthy for several generations. Somatic cells from DC patients are of limited utility due to their premature senescence *in vitro*. Faced with these challenges, our group and more recently the group of Batista *et al.* have attempted to tackle DC pathophysiology by directly reprogramming patient somatic cells into induced pluripotent stem (iPS) cells [6, 7]. A comparison of the data in these two reports illustrates the advantages and limitations of the iPS approach for modeling human diseases.

Both groups used viral transduction to introduce the typical cocktail of 4 transcription factors – *OCT4, SOX2, KLF4, c-MYC* – into primary cultures of patient skin fibroblasts, and both found decreased efficiency of reprogramming DC cells relative to normal cells. Stable overexpression of *TERT*

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and *TERC* prior to reprogramming, or co-expression of *TERT* during reprogramming improved the efficiency of iPS cell derivation. Batista *et al*. also improved reprogramming efficiency by using multiple rounds of viral infection and by deriving iPS cells under low oxygen (5%) conditions. These observations indicate a relative resistance of DC patient fibroblasts to reprogramming, which may be due to decreased replicative capacity and/or activation of cellular stress responses secondary to shortened telomeres.

In our study, we derived iPS cells from two patients with mutations in *DKC1*, which encodes dyskerin, and from one patient with a heterozygous null mutation in the telomerase RNA component (*TERC*). Dyskerin stabilizes *TERC* and mutations in *DKC1* are associated with lower steady-state levels of *TERC*, which is limiting for overall telomerase activity [8]. Whereas fibroblasts from *DKC1* mutant patients exhaust their telomeres and rapidly senesce when grown in culture, the two iPS clones we generated from fibroblasts carrying a deletion of leucine 37 in dyskerin (del37L mutation) unexpectedly displayed self-renewal for at least 20-30 passages (at the time of our report), and showed elongation of telomeres with passage. Based on experiments in *DKC1* del37L fibroblasts, *TERC* is expected to be limiting for telomere elongation, but examination of the *DKC1* del37L iPS cells showed 6-8 fold increased steady-state *TERC* levels relative to the parent fibroblasts. We found a similar upregulation of *TERC* in another *DKC1* mutant iPS cell line (A386T), in iPS cells from a patient with a heterozygous null *TERC* mutation, and in wild-type iPS cells. To explain the observed upregulation of *TERC* in iPS cells, we found increased steady-state levels of *DKC1*/dyskerin, as well as binding of pluripotency-associated transcription factors to the *TERC* and *DKC1* genetic loci, which may enhance expression of these genes in iPS cells. Collectively, these results showed that multiple telomerase components are activated after reprogramming to pluripotency – not only *TERT*, but also *TERC* and *DKC1*, the genes defective in the DC genotypes we reprogrammed – providing a plausible mechanism by which iPS cells maintain self-renewal despite telomerase lesions.

Batista *et al*. reprogrammed fibroblasts carrying several unique DC-associated genetic mutations. iPS cells from two different patients with heterozygous null mutations in *TERT* showed a 50% reduction of overall telomerase activity compared to wild-type cells, consistent with a haploinsufficient mechanism of disease. iPS cells from a patient with a compound heterozygous mutation in *TCAB1*, a factor the authors have previously defined as responsible for trafficking telomerase to the Cajal body [9], showed defective accumulation of *TCAB1*, dyskerin and *TERC* in the Cajal bodies and mislocalization of *TERC* to the nucleolus. Importantly, overall telomerase activity was not decreased in cell-free lysates of *TCAB1*-mutant cells, supporting a novel mechanism of disease in which *TCAB1*-mutant cells suffer from subcellular mislocalization of an otherwise functional telomerase holoenzyme. The authors also reprogrammed fibroblasts from 2 patients with *DKC1* mutations, del37L as well as L54V. In keeping with our results, Batista *et al*. found a decrease in steady-state *TERC* levels and telomerase activity in *DKC1*-mutant iPS cells relative to normal cells. Based on northern blot quantitation, they found no apparent deficit in other RNAs bound by dyskerin (H/ACA motif-carrying snoRNAs and scaRNAs). Consistent with a mechanism of defective telomerase holoenzyme assembly in *DKC1*-mutant iPS cells, the authors showed reduced association of *TERC* with dyskerin and TCAB1 by immunoprecipitation. Collectively, Batista *et al*. showed that iPS cells carrying a variety of DC-associated mutations recapitulate the predicted molecular defects in telomerase quantity, assembly, or trafficking.

Batista *et al*. next examined telomere length dynamics in DC-mutant iPS cells as a function of passage. Five independent clones of *TERT* mutant iPS cells from two patients exhibited self-renewal for at least 8 passages as well as some degree of telomere lengthening between passages 3 and 8, although less than the telomere elongation found in wild-type cells. In contrast, *TCAB1* mutant iPS cells showed telomere attrition during passage, demonstrating a significant functional telomerase defect in intact cells. In *DKC1* L54V iPS cells, the authors found self-renewal for at least 10 passages and stability of telomere length over this time span. However, in five iPS clones carrying the *DKC1* del37L iPS cells (and in contrast to our results using cells of the same *DKC1* genotype), the authors found telomere shortening during passage. 3 of 5 iPS clones maintained self-renewal until at least 19 passages, while the other two clones were propagated to 36 passages before exhaustion. At late passage, the latter iPS clones showed an increase in p53 and p21, and increased telomere signal-free ends, suggesting replicative senescence in these iPS cells due to telomere erosion. In Batista *et al*.'s hands, therefore, *DKC1*-mutant iPS cells exhibited senescence *in vitro*, which they argue may reflect the premature exhaustion of stem cells in patients *in vivo*.

Although both studies of iPS cells from DC patients reported the upregulation of multiple telomerase subunits following reprogramming, what is the basis of the apparent discrepancy in the two studies in the telomere dynamics of iPS cells from the same *DKC1* del37L genetic background? While Batista *et al*. found telomere attrition and abrupt loss of self-renewal at passage 36 in at least one of their *DKC1* del37L iPS clones, one of our *DKC1* del37L iPS clones continues to exhibit self-renewal at passage 66 in independent hands (unpublished observations). Several

possible and testable explanations exist, which reflect a number of concerns about uniformity in iPS cells as models of human disease. First, iPS cells show significant clonal variability, and recent studies show differences in telomerase activity and telomere elongation capacity even amongst wild-type iPS cells of the same genetic background [10, 11]. Second, due to the inefficiency of the reprogramming process as usual and of DC iPS cells in particular, it is possible that additional genetic variation may have been selected during the reprogramming process (e.g., somatic mutations [12], copy number variations [13, 14]), and that these underlie differences in net telomere elongation or attrition in the cells. In our study, we ruled out *TERC* locus amplification and demonstrated telomerase downregulation and telomere shortening when iPS cells were differentiated. However, it is possible that other genetic or epigenetic clonal factors (such as viral transgene integrations and/or their effects on neighboring genes) may be responsible for salient differences between the clones in the two studies. Indeed, although measured using different semi-quantitative methods, telomerase activity in our *DKC1* del37L iPS clones was approximately 40% of wild-type iPS cells, versus only 5-15% of wild-type in the iPS clones created by Batista *et al*. Third, iPS cell cultures are heterogeneous and difficult to maintain in a pristine state, and therefore culture conditions and differentiation could explain some degree of variability in self-renewal and telomere dynamics amongst clones. In the future, clonal variation may be mitigated by using transgene-free reprogramming methods or by selecting iPS clones based on transcriptional and epigenetic profiles [15].

Despite these issues, what insights might be gained and what hypotheses formed based on the study of DC iPS cells? First, both papers show that direct reprogramming can capture the effects of human telomerase mutations in stem cells, a cell type that is highly relevant to understanding DC but difficult to procure from the patient. Second, both studies demonstrate an unexpected upregulation of not only *TERT* but also multiple other telomerase components (*TERC, DKC1, TCAB1*) indicative of a novel set-point for telomerase activity in pluripotent stem cells. A similar coordinated upregulation of telomerase might better reflect the telomerase activity levels in DC patient stem cells *in vivo*, and may underlie the considerable capacity for self-renewal observed in DC iPS cells by both groups. Because human iPS cells cycle in less than 24 hours and are typically passaged every 5-6 days, DC iPS cells that have exhausted even after 36 passages have divided more than 150 times, vastly more than would be possible in primary somatic cell cultures from DC patients. In both studies, despite induction of multiple telomerase components, most patient-derived iPS cells still displayed compromised levels of *TERC* and/or telomerase activity compared to wild-type cells. The relative telomerase activity in DC iPS cells may determine net telomere elongation or attrition, and combined with telomere endowment in stem cells *in vivo*, may dictate age of onset and severity of disease in patients. Further analysis of additional DC mutant alleles including those underlying more severe disease variants (such as Hoyeraal-Hreidarsson syndrome and Revesz syndrome), plus knock-down or targeted deletion studies of telomerase components, may help define a threshold of telomerase activity required for self-renewal in human pluripotent stem cells. It will also be important to ascertain the contribution of non-telomerase dependent mechanisms (such as sister chromatid exchange) on telomere dynamics in the pluripotent state. Finally, these studies demonstrate the use of a variety of patient samples to model a genetically complex and heterogeneous disease. The availability of self-renewing iPS cells carrying patient alleles provides abundant material to define molecular mechanisms of disease in a variety of genotypes, which should facilitate the development of tailored therapies for DC.

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