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Understanding telomere diseases through analysis of disease-specific iPS cells

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Abstract

A unique characteristic of tissue stem cells is the ability to self-renew, a process that enables the life-long maintenance of many organs. Stem cell self-renewal is dependent in part on the synthesis of telomere repeats by the enzyme telomerase. Defects in telomerase and in genes in the telomere maintenance pathway result in diverse disease states, including dyskeratosis congenita, pulmonary fibrosis, aplastic anemia, liver cirrhosis and cancer. Many of these disease states share a tissue failure phenotype, such as loss of bone marrow cells or failure of pulmonary epithelium, suggesting that stem cell dysfunction is a common pathophysiological mechanism underlying these telomere diseases. Studies of telomere diseases in undifferentiated iPS cells have provided a quantitative relationship between the magnitude of biochemical defects in the telomerase pathway and disease severity in patients, thereby establishing a clear correlation between genotype and phenotype in telomere disease states. Modeling telomere diseases in iPS cells has also revealed diverse underlying disease mechanisms, including reduced telomerase catalytic activity, diminished assembly of the telomerase holoenzyme and impaired trafficking of the enzyme within the nucleus. These studies highlight the need for therapies tailored to the underlying biochemical defect in each class of patients.

Telomere dysfunction causes replicative senescence and compromises human health

Normal human health and lifespan require efficient maintenance of telomeres, the nucleoprotein structures that protect chromosome ends. Telomeres are comprised of TTAGGG repeats extending up to 15kb length in humans and ending with a short single-stranded overhang. A six-member protein complex – shelterin – coats the telomere sequences, protects the chromosome terminus from recognition as damaged DNA, and suppresses activation of cell cycle checkpoints, inappropriate chromosome ligation, homologous recombination and end-resection that would otherwise occur in the absence of these protective structures [1,2]. In dividing cells, telomeres encounter the end-replication

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problem, the inability of DNA polymerase to fully replicate the lagging strand, a process that is offset by telomerase, the enzyme that adds telomere repeats to chromosome ends. Telomere shortening in primary human cells lacking telomerase is the cause of replicative senescence in culture, as a subset of telomeres are recognized as damaged DNA, leading to activation of cell cycle checkpoints, including the tumor suppressor proteins p53 and Rb [3,4]. Overexpression of TERT, the protein catalytic subunit of telomerase, in primary fibroblasts is sufficient to reconstitute telomerase, elongate telomeres and render these cells immortal in culture [5].

Dyskeratosis congenita and related telomere diseases

Telomerase has been broadly linked to human aging and to cancer. Telomeres shorten with advancing age in diverse human tissues [6], and telomerase knockout mice exhibit a premature aging syndrome [7,8], which together support a model in which telomere shortening contributes to certain aspects of human aging. Telomerase is expressed in 90% of human tumors where it appears critical for endowing cancers with unlimited growth potential. However, whether telomerase is upregulated during carcinogenesis or is expressed in the cell of origin for cancers remains unresolved. Numerous genes in the telomere homeostasis pathway have been implicated in these diseases and short telomeres are an invariant feature in patients. These findings suggest that impaired stem cell function caused by telomere dysfunction represents a common pathophysiology in telomere disease states. Dyskeratosis congenita (DC) is a rare genetic disease most often seen in children and characterized by an epidermal triad of nail dystrophy, oral leukoplakia – pre-neoplastic dysplasia of the oral mucosa – and skin hypo- and hyperpigmentation (Box 1). DC patients often present with aplastic anemia - decreased peripheral blood counts and a bone marrow biopsy that shows severe depletion of blood forming elements in the bone marrow [9,10]. Patients with DC also develop lung disease, including pulmonary fibrosis, a scarring of the lung characterized by loss of lung epithelium, prominent fibrosis, and impaired gas exchange. Many other organs are affected in DC patients and additional features include hair loss, hair graying, intestinal dysfunction, infertility and liver disease. Cirrhosis, or scarring of the liver, is also seen in some DC patients and has been linked to germline mutations in telomerase independent of DC [11,12] (Box 1). Despite the fact that telomerase is important for human tumorigenesis, DC patients are highly cancer-prone, rather than cancer resistant. DC patients develop diverse cancers at a rate estimated to be up to 1000-fold greater than age-matched controls, including myelodysplastic syndrome, acute myeloid leukemia and epithelial cancers of the head and neck [13,14]. The mechanisms underlying this strong cancer predisposition may relate to cycles of chromosome fusion-bridge-breakage and chromosomal rearrangements driven by telomere dysfunction [15-18] and the ability of uncapped telomeres to promote tetraploidization and aneuploidy [19,20].

In some individuals, germline mutations in the telomerase pathway manifest in a more tissue restricted manner without yielding all the diverse features of DC. Ten percent of families with a hereditary form of pulmonary fibrosis, and at least one percent of patients with sporadic IPF harbor deleterious mutations in TERT or in the telomerase RNA component, TERC [21,22] (Fig. 2a). Similarly, approximately 5% of patients with sporadic aplastic anemia possess point mutations in TERT or TERC [23-25]. It remains incompletely understood how telomerase mutations cause the multi-systemic syndrome of DC as opposed to more restricted disease states of pulmonary fibrosis or aplastic anemia, but possibilities include the inciting mutations themselves, modifier genes, incomplete phenotyping of patients, environmental exposures and differences in inherited telomere length [9].

Many tissue stem cells and progenitors cells *in vivo* require intact telomere function

Telomerase expression patterns *in vivo* are not yet well understood, but evidence suggests that telomerase activity is detectable in certain progenitor cells, including embryonic stem cells, hematopoietic progenitor cells [26,27], gastrointestinal progenitor cells [28], and in hair follicle progenitor cells [29]. A definitive link between telomeres and stem cell function has been established using telomerase knockout mice. Deletion of telomerase in mice is initially well tolerated due to the very long telomeres of laboratory mice. However, as telomerase knockout mice are intercrossed, telomeres shorten resulting in dysfunctional telomeres beginning in the fourth generation of telomerase deficiency. These mice exhibit multi-systemic defects particularly in renewing tissues including male germ cells, lymphocytes, and gastrointestinal crypts [7,30]. Hematopoietic stem cells from telomerase-deficient strains exhibit impaired self-renewal through transplantation [31,32] and undergo apoptosis when stimulated in culture [33]. Muscle stem cells are also impaired when a mutation in the Duchenne's gene is studied on a telomerase-mutant background [34]. These experiments showed that certain stem cells and progenitor cells require intact telomere function for efficient self-renewal or survival. Direct studies of stem cell function in patients with telomere diseases have not yet been possible owing to the difficulty in isolating, propagating and studying primary stem cells from humans, particularly in contexts such as bone marrow failure where stem cells are likely reduced in number.

The reprogrammed state: primed for efficient maintenance of telomeres

Mouse and human ES cells express high levels of telomerase relative to many other cell types, which results in effective maintenance of telomere lengths with passage in culture and immortal growth, bypassing the telomere shortening and replicative senescence responses seen in human fibroblasts [35]. When human fibroblasts are reprogrammed to iPS cells, the TERT gene is switched on through unknown means, reconstituting telomerase activity. This process of reactivating telomerase during reprogramming leads to significant elongation of telomeres to a new set point [36-38]. How such a new equilibrium of telomere lengths is achieved or maintained in the iPS-state is not understood. In addition to the catalytic core of the enzyme, TERT and TERC, telomerase requires the dyskerin core complex, comprised of dyskerin, NHP2 and NOP10, as well as TCAB1, to function in living cells. Dyskerin is an RNA-binding protein and a pseudouridine synthase, involved in modification of splicing RNAs and ribosomal RNAs. Dyskerin binds the H/ACA element in TERC and is essential for telomerase biogenesis and stability [39](Fig. 2a). TCAB1 binds the CAB box element in TERC and is required for telomerase trafficking to Cajal bodies and to telomeres and is essential for telomere maintenance [40-42](Fig. 2a). In addition to switching on expression of TERT, the levels of each of the principal components of telomerase are elevated in iPS cells and ES cells compared with fibroblasts. TERC levels are substantially increased after reprogramming, which may reflect the presence of TERT and a net increase in assembling transcribed TERC into a stable ribonucleoprotein complex [37]. In addition, the TERC promoter was shown to be a transcriptional target of nanog, suggesting that in the pluripotent state, TERC is transcribed at higher levels [36]. Dyskerin and TCAB1 proteins are also increased in iPS cells compared to fibroblasts, which likely contribute to the increased telomerase enzymatic activity and efficiency of telomere maintenance after reprogramming [36,37]. These findings are consistent with a model in which the iPS cell state is primed for increased expression of telomerase and efficient maintenance of telomeres.

Mutations in the telomerase pathway impair reprogramming efficiency

Reactivation of telomerase is an important determinant of reprogramming efficiency. Despite the presence of shorter telomeres, fibroblasts from aged individuals and centenarians are reprogrammed with an efficiency comparable to that of fibroblasts from younger donors because telomerase upregulation is part of the reprogramming process. Even fibroblasts that had been passaged in culture to reach replicative senescence were efficiently reprogrammed to iPS cells [43]. In each of these cases, telomerase upregulation leads to telomere elongation with reprogramming, circumventing the activation of cellular checkpoints, such as p53, that would otherwise inhibit reprogramming efficiency. In contrast, mutations in telomerase significantly inhibit reprogramming as short, dysfunctional telomeres cannot be efficiently elongated because the telomerase enzyme is inactivated or functionally impaired. Complete inactivation of telomerase in mouse embryo fibroblasts from late generation TERC knockout mice impaired reprogramming efficiency compared with telomerase WT cells [44]. Partial inhibition of telomerase due to genetic mutations in telomerase components in cells from patients with DC also significantly reduced reprogramming efficiency. This telomere barrier to reprogramming was overcome either by overexpression of TERT in DC fibroblasts [36] or by culturing cells under conditions of low atmospheric oxygen, which mitigates cellular stress [37]. Even under conditions of low oxygen, some fibroblast cultures from DC patients remain resistant to reprogramming. This resistance may be explained in part by the fact that, at least in mouse embryo fibroblasts, the TERT promoter is upregulated approximately two weeks after the reprogramming process is initiated, making it likely that telomerase reactivation occurs too late to repair damaged telomeres and enable full reprogramming for many DC cultures [45]. Thus, DC fibroblasts with particularly short telomeres, coupled with a mutation that severely impairs the function of the enzyme, may result in failure of reprogramming even in low oxygen.

Revealing disease mechanisms through analysis of iPS cells

Because of their short telomeres, many fibroblast cultures from DC patients grow poorly and become senescent after very few cell divisions in culture. It is therefore striking that, when successfully reprogrammed, these DC iPS cells exhibit a renewed vigor in culture and divide a much larger number of times than the corresponding fibroblasts from which they were derived. The simple explanation for this phenomenon relates to the switching on of telomerase, which is able to ensure improved telomere maintenance. Thus, even a hypomorphic telomerase enzyme impaired by mutation is superior to having no telomerase at all. The generation of these iPS cells from patients with DC or aplastic anemia has allowed the creation of tissue culture stem cell models for detailed biochemical study of these diseases, circumventing a major roadblock in analyzing these conditions from rare tissue stem cell populations.

DC is caused by mutations in diverse components of the telomere maintenance pathway, including each of the defined components of the telomerase enzyme. Mutations in the catalytic core of the enzyme – TERT and TERC – result in an autosomal dominant (AD) form of the disease. Typically, the disease-causing allele is impaired in catalytic function, leaving a reduced level of telomerase activity, which derives from the remaining wild-type allele [46]. Telomerase activity in TERT-mutant or TERC-mutant iPS cells from DC patients or aplastic anemia patients is reduced approximately two-fold, consistent with these mutations acting through haploinsufficiency [37,47]. An X-linked form of DC is caused by mutations in dyskerin and iPS cells from these patients show a marked reduction in telomerase activity, leaving a residual activity between 10-40% of controls [36,37]. The reason for this much greater reduction of telomerase activity in the X-linked form of DC compared with the AD form is due to the essential role of dyskerin in assembling the

telomerase RNP [39,48]. The dyskerin point mutations in X-linked DC iPS cells markedly compromise the overall levels of TERC and the amount of TERC incorporated into active telomerase holoenzyme [37]. In an autosomal recessive form of DC, compound heterozygous mutations in TCAB1 prevent telomerase from properly localizing to Cajal bodies and to telomeres [40,49]. In iPS cells from TCAB1-mutant DC patients, telomerase is no longer detectable in Cajal bodies, but instead accumulates in nucleoli. Telomerase activity from TCAB1-mutant iPS cells is not diminished, indicating that this form of disease is due to a severe trafficking defect in which the otherwise active enzyme is mislocalized to a nuclear compartment from which it cannot act on telomeres [37]. Interestingly, certain IPF-derived mutations in TERT compromise the ability of telomerase to be physically recruited to telomeres through interaction with the telomere binding protein TPP1, indicating that trafficking and recruitment defects may be common in telomere diseases [50,51]. Other forms of DC remain to be modeled in iPS cells, including: a rare autosomal recessive form due to mutations in NHP2 or NOP10, two components of the dyskerin core complex [52,53](Fig. 1a); a common AD form caused by mutations in TIN2, a core protein in the shelterin complex at telomeres [54](Fig. 1b); a common AD form caused by mutations in RTEL, a helicase involved in unwinding higher order DNA conformations at telomeres [55,56] (Fig. 1c); and a related disease, Coats plus syndrome, a disorder that phenotypically overlaps with DC and is due to mutations in CTC1, a cofactor for DNA polymerase alpha required for efficient telomere replication [57] (Fig. 1d).

Quantitative analysis of genotype-phenotype relationships in DC using iPS cells

As is the case for many genetic diseases, DC presents with a range of severity, from mild to very severe. In the most severe form, Hoyeraal-Hreidersson syndrome, patients exhibit defects at birth including small size, cerebellar hypoplasia and failure to thrive [9]. In the more conventional forms of DC, there also exists a range of severities. The most severe forms of DC are typically caused by mutations in dyskerin, TIN2, TCAB1 and RTEL. Patients with mutations in these genes present at birth or in early childhood, tend to have a larger number of involved organ systems, exhibit the epidermal triad and have a shorter lifespan. In contrast, patients with heterozygous mutations in TERT or TERC commonly present in young adulthood or later, lack the epidermal triad, have fewer involved organs systems and live longer on average. Patient-derived iPS cells have shed light on the mechanisms underlying this wide phenotypic variation. The ability of telomeres to be elongated with reprogramming of cells from patients with different forms of DC serves as a robust and quantitative readout of the functional integrity of the telomerase pathway. Heterozygous mutations in TERT or TERC, in DC or aplastic anemia, compromise telomerase function by at most 50%, which impairs telomere elongation during reprogramming. Telomere lengths in these iPS cells increase significantly, but reach an equilibrium below that of iPS cells generated from donors with an intact telomerase pathway [37,47]. In contrast, telomeres shorten substantially during reprogramming of cells isolated from patients with severe forms of DC, including those caused by mutations in dyskerin or TCAB1. In some cases, iPS cells harboring dyskerin mutations have not shown telomere shortening and these differences may be attributable to epigenetic variation in iPS clones or to variations in culture conditions [36]. Compromise of telomerase function in iPS cells from the severe forms of DC can result in continued telomere shortening during extended passage of these cells, eventually compromising self-renewal and leading to differentiation accompanied by activation of the p53 tumor suppressor pathway [37]. This impairment of self-renewal may occur in vivo in tissue specific stem cells and underlie the loss of bone marrow cells or pulmonary epithelial cells seen in patients with telomere diseases (Fig. 2).

These findings show that the wide variation in clinical phenotypes in DC correlates closely with the severity of the biochemical defect in telomere maintenance in iPS cultures. Telomere shortening during the reprogramming process predicts a severe clinical manifestation of DC, whereas modest lengthening during reprogramming indicates a mild form of the disease. These data fit closely with recent analyses of blood leukocyte telomeres from DC patients, which similarly showed that patients with the most severe forms of the disease harbored shorter telomeres than those with mild clinical manifestations [58]. Genetic anticipation also contributes to disease severity, with clinical phenotypes worsening in advancing generations within a kindred, particularly for the TERT-mutant and TERC-mutant forms [59,60]. Undoubtedly, other variables also contribute to disease severity including unknown modifier genes, starting telomere lengths and environmental factors, among others. One major goal of iPS cell related research on DC and related telomere diseases is to discover new therapies that could mitigate or reverse the telomere shortening caused by the inciting mutations. To date, these findings from iPS cells have revealed that such therapies would need to be tailored to the specific underlying biochemical defect, for example, enhancing telomerase assembly in patients with dyskerin mutations, boosting catalytic function in patients with mutations in TERT or mitigating the deleterious effects of telomerase mutations that impair trafficking and recruitment.

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Box 1

Dyskeratosis congenita: a multisystemic syndrome characterized by bone marrow failure, pulmonary fibrosis, defects in many other tissues and an increased cancer risk. Typically associated with an epidermal triad characterized by nail dystrophy, oral leukoplakia, and abnormal skin pigmentation. The most severe variant is Hoyeraal-Hreidarrson syndrome, which in addition includes cerebellar hypoplasia, growth retardation and impaired mental development. Short telomeres are an invariant feature of the syndrome. Mutations in the following genes in the telomere homeostasis pathway have been identified in these patients: DKC1, TERT, TERC, NHP2, NOP10, TIN2, TCAB1, CTC1 and RTEL1.

Pulmonary Fibrosis: characterized by loss of pulmonary epithelium and by the progressive scarring of the lung, represents perhaps the most common manifestation associated with telomere dysfunction. Patients typically present in adulthood and develop shortness of breath and hypoxia. Autosomal dominant mutations in TERT and TERC have been found in at least 10% of inherited cases and short telomeres are also present in idiopathic patients with intact telomerase genes.

Aplastic Anemia: reduced peripheral blood counts and hypocellularity of the bone marrow cellularity. Treated with immune suppression or with bone marrow transplantation, although patients with telomerase mutations do not respond to the former. Mutations in TERT and TERC have been found in 10% of cases.

Liver Cirrhosis: telomere shortening has been associated with liver disease and a small number of patients with DC present with liver cirrhosis, replacement of liver tissue by fibrosis and scar tissue. Some patients with liver cirrhosis without other aspects of DC also harbor germline telomerase mutations.

Highlights

- DC is a genetic disease with features of stem cell dysfunction in diverse tissues
- Mutations in the telomere homeostasis genes underlie DC
- Diverse biochemical defects lead to impaired telomere maintenance in DC iPS cells
- DC iPS cells allow genotype-phenotype comparisons
- Severity of biochemical defects in DC iPS cells predict disease severity

Figure 1.

Germline mutations in DC and other telomere disease states. DC has been linked to germline mutations in 9 genes that impinge on telomere homeostasis: TERT, TERC, DKC1, NHP2, NOP10, TCAB1, TIN2, CTC1 and RTEL1 (each in red). These can be divided into four groups based on current understanding of how these proteins function: (a) telomerase components, (b) shelterin proteins, (c) the RTEL helicase and (d) the CST complex proteins.

a) Normal telomerase

b) Mutant telomerase

Figure 2.

Telomere shortening impairs stem cell self-renewal in telomere disease states. Stem cells possess the ability to self-renew or to differentiate to give rise to more committed progenitors. The presence of telomerase and intact telomere function favor continued tissue stem cell self-renewal and efficient tissue homeostasis during life (a). In contrast, mutations in the telomerase pathway impair the ability of mouse hematopoietic stem cells to self-renew *in vivo* and impair self-renewal in human iPS cells from patients with severe forms of DC (b). These self-renewal defects likely contribute to the tissue failure phenotypes seen *in vivo*, including aplastic anemia and pulmonary fibrosis.