Gsk3 Inhibition Rescues Growth And Telomere Dysfunction In Dyskeratosis Congenita Ipsc-Derived Type Ii Alveolar Epithelial Cells

Rafael Jesus Fernandez
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Gsk3 Inhibition Rescues Growth And Telomere Dysfunction In Dyskeratosis Congenita Ipsc-Derived Type Ii Alveolar Epithelial Cells

Abstract

Dyskeratosis congenita (DC) is a rare genetic disorder characterized by deficiencies in telomere maintenance leading to very short telomeres and the premature onset of certain age-related diseases, including pulmonary fibrosis (PF). PF is thought to derive from epithelial failure, particularly that of type II alveolar epithelial (AT2) cells, which are highly dependent on Wnt signaling during development and adult regeneration. We use human iPSC-derived AT2 (iAT2) cells to model how short telomeres affect AT2 cells. Cultured iAT2 cells with a mutation in DKC1, the most common cause of DC, accumulate shortened, uncapped telomeres and manifest defects in the growth of alveolospheres, hallmarks of senescence, and apparent defects in Wnt signaling. The GSK3 inhibitor, CHIR99021, which mimics the output of canonical Wnt signaling, enhances telomerase activity and rescues the defects. These findings support further investigation of Wnt agonists as potential therapies for DC related pathologies. Furthermore, this thesis describes the development of a transplantation of iAT2 cells into immunocompromised mice as well as the development of a novel iPS line with another DC mutation.

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GSK3 INHIBITION RESCUES GROWTH AND TELOMERE DYSFUNCTION IN DYSKERATOSIS CONGENITA IPSC-DERIVED TYPE II ALVEOLAR EPITHELIAL CELLS

Rafael Jesus Fernandez

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To view a copy of this license, visit: https://creativecommons.org/licenses/by-nc-sa/4.0/?ref=ccchooser
I dedicate this work to my daughter Anne.
May you never lose your sense of wonder for God’s world.
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Dyskeratosis congenita (DC) is a rare genetic disorder characterized by deficiencies in telomere maintenance leading to very short telomeres and the premature onset of certain age-related diseases, including pulmonary fibrosis (PF). PF is thought to derive from epithelial failure, particularly that of type II alveolar epithelial (AT2) cells, which are highly dependent on Wnt signaling during development and adult regeneration. We use human iPSC-derived AT2 (iAT2) cells to model how short telomeres affect AT2 cells. Cultured iAT2 cells with a mutation in DKC1, the most common cause of DC, accumulate shortened, uncapped telomeres and manifest defects in the growth of alveolospheres, hallmarks of senescence, and apparent defects in Wnt signaling. The GSK3 inhibitor, CHIR99021, which mimics the output of canonical Wnt signaling, enhances telomerase activity and rescues the defects. These findings support further investigation of Wnt agonists as potential therapies for DC related pathologies. Furthermore, this thesis describes the development of a transplantation of iAT2 cells into immunocompromised mice as well as the development of a novel iPS line with another DC mutation.
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CHAPTER 1 : INTRODUCTION

Portions of this section are adapted from the review article “A regulatory loop connecting Wnt signaling and telomere capping: possible therapeutic implications for dyskeratosis congenita” published in the Annals of the New York Academy of Science (2018).
AGING: WHY FIGHT THE END?

Why study aging? Other than for the noble pursuit of knowledge, what use might we have for learning more about why we age? Is it to live forever?

Elderly patients often complain that age leads to a loss of their capacity to do the tasks and activities that they enjoy. This pithy definition captures so much of the human experience of aging. Those tempted to study it in the search for a fountain of youth are quickly disillusioned, for as Jose Adolph so adequately noted in his short story "Nosotras No" those immortal “tadpoles” who took the injection, “would be forever miserable.”

If immortality is not our goal, then what is our goal in understanding aging? Aging is one of the most important risk factors for disease. Despite this extreme importance, we have few known medications to slow it and no known drugs to reverse it. There remains a medical ethical imperative to alleviate suffering, and since we all suffer often because of diseases related to our age, we are called to help soften this decline going forward. Our more appropriate goal is not to extend lifespan but rather to extend healthspan, the healthy years before we pass on. In other words, it’s not the age you live to, but how you get there.

Dr. Peter Attia outlines a plan for understanding how to extend healthspan in a lecture he gave at the Whitehead Institute. In it, he delineates some of the history behind how we came to understand which of these interventions and strategies help us to extend healthspan. One of these is studying centenarians, people who live past 100 years. Comparing these individuals who aged extremely well to the rest of the population has greatly aided our efforts to understand healthspan. Centenarians and their long lived children have significantly long telomeres, the molecular structures that shorten with age and have been associated with aging and pathology. Conversely, studying premature aging syndromes and telomeres, the molecular structures most often associated with these disorders, offers insights as well.

PREMATURE AGING SYNDROMES REVEAL TELOMERES AS IMPORTANT MEDIATORS OF AGING RELATED DISEASES

A premature aging syndrome is loosely defined as a genetic disorder, whose primary manifestation involves the cardinal features of aging in patients younger than expected in the general population. These diseases include but are not limited to aplastic anemia (the loss of the
bone marrow to produce blood cells), pulmonary and hepatic fibrosis (the destruction of lung and liver tissue architecture primarily by the excessive presence of collagen or other structural fibers), loss of skin turgor, and hair graying.

The principal of these diseases is dyskeratosis congenita (DC), but other syndromes with similar underlying genetic defects and overlapping signs and symptoms also exist, such as aplastic anemia (AA), Hoyeraal-Hreidarsson (HH) syndrome, Coats plus (CP) syndrome, and Revesz syndrome (RS). The clinical presentations and mutations underlying these syndromes have been well reviewed elsewhere (See Refs. 3,4), and so this summary only provides a brief overview of these syndromes. The classical signs of DC typically present in childhood and include abnormal skin pigmentation, nail dystrophy, and leukoplakia. Bone marrow failure is a major cause of death in DC patients, but it can be treated by transplantation of normal allogeneic bone marrow. However, other serious DC pathologies, including pulmonary fibrosis, cancer, vasculopathies, osteoporosis and other bone abnormalities, genitourinary malformations, liver cirrhosis, and gastrointestinal disorders, are currently not well treated and contribute to morbidity and mortality. The same mutations that cause DC can also lead to AA, which primarily results in bone marrow failure without other DC pathologies. In contrast, HH, CP and Revesz syndromes are generally more severe and display both defects characteristic of DC and additional pathologies. HH patients typically display intrauterine growth retardation, microcephaly, and cerebellar hypoplasia. CP patients often suffer from an exudative retinal vasculopathy (in isolation known as Coats’ disease) plus GI bleeding, bone fractures, poor wound healing, and brain abnormalities, including calcified cysts and loss of white matter. RS combines the pathologies in CP with HH-like features including cerebellar hypoplasia and intrauterine growth retardation.

These syndromes share overlapping but different features, leading many reviewers to refer to these diseases as being on the DC spectrum. Careful genetic studies of these rare premature aging syndromes have uncovered mutations in genes that function at telomeres. These findings support the idea that dysfunctional telomeres play a role in the pathogenesis of these rare syndromes. Telomeres functionally hide and protect, or “cap,” the ends of chromosomes. Capping is supported by sufficient telomere length and by the action of telomere-
associated proteins called shelterins. Capped telomeres prevent the DNA damage checkpoint machinery from recognizing chromosome ends as double strand DNA breaks (DSBs).

Uncapped telomeres activate multiple cell-cycle checkpoint responses that can lead to a host of potentially injurious molecular and cellular consequences, including degradation of chromosome ends by exonucleases, recombination of chromosome ends generating unstable chromosomes, of cell cycle arrest, elevated levels of apoptosis, and even the emergence of cancer cells.

Telomeres are made up of long stretches of tandemly repeated DNA sequences, which can vary between species. In vertebrates this repeated sequence is “TTAGGG.” These long stretches of repetitive DNA at the chromosome end are capped by shelterins (TERF1, TERF2, POT1, TINF2, ACD1 and TERF2IP in humans). The length of telomeric repeats shortens during chromosomal and cellular replication and can even shorten to the point of uncapping. Shortening typically occurs in a gradual fashion and was originally attributed to the “end replication problem,” where the lagging strand synthesis of the telomere leaves a short overhang through successive cell cycles. However, careful study of telomere ends through the cell cycle revealed that most of the progressive shortening seen as cells divide is due to exonucleolytic resection of replicated ends to generate the single stranded 3’ overhangs needed for telomere function. Yet, shortening can also be sudden and dramatic. For example, oxidative damage of telomere DNA or a DNA replication fork break during replication through a telomere can each cause rapid telomere shortening. Shortening can be countered by the action of the enzyme telomerase, which synthesizes new telomere repeat DNA onto existing ends. It is an enzyme complex made up of a catalytic subunit TERT, the RNA template component TERC, and several other factors.

However, in humans, telomerase levels are limited, and telomerase does not prevent telomere shortening with age. Thus, telomere uncapping is thought to contribute to several age related diseases, including cardiovascular diseases, diabetes mellitus, osteoporosis, cirrhosis, pulmonary fibrosis, and immunosenescence.

The mutations underlying these premature aging syndromes occur in genes encoding factors that support telomerase-mediated telomere extension (DKC1, TINF2, TERT, TERC, NOP10, NHP2, PARN, WRAP53, ACD, and POT1) and telomere replication (CTC1, STN1 and
Moreover, this latter set of factors may also impact telomerase-mediated telomere extension given the similarity between the pathologic consequences of mutations in these genes and the former set. This potential indirect impact is important because the DC spectrum of diseases is often called “the telomere syndrome.” However, it might be misleading to imply that these diseases reveal the full range of how telomere dysfunction can contribute to pathology.

A distinct characteristic of the pathologies associated with the DC spectrum is that all of the tissues affected express high levels of telomerase in their stem cell compartments. However, telomere defects can arise for reasons unrelated to telomerase dysfunction. For example, Werner syndrome (WS) is caused by the loss of a DNA helicase, WRN. WRN plays a variety of roles genome-wide that prevent mutations, which presumably contribute to some WS pathologies such as cancer. Loss of WRN leads to pathologies that are most pronounced in mesenchymal tissues (e.g. dermis, adipose tissue, bone), expressing little-to-no telomerase. However, there is strong evidence that telomere defects contribute to WS as well. In particular, WRN is critical for the normal replication of telomeres. Further, WS defects can be suppressed experimentally by telomerase overexpression in vitro and in vivo, indicating the importance of telomere defects in WS pathogenesis and explaining the natural restriction of WS pathologies to primarily mesenchymal tissues. Thus, the DC spectrum and WS provide insight into how telomere dysfunction impacts tissues with higher vs. lower levels of telomerase respectively. But neither is likely to fully reveal how telomere defects contribute to normal age-related pathologies. Importantly, all of these diseases currently suffer from a lack of curative therapies. Thus, information from basic studies will provide much needed insight into therapeutic approaches to ameliorate and one day cure these diseases.

UNCAPPED TELOMERES CONTRIBUTE TO DISEASES OF AGING IN NON-RARE INDIVIDUALS

Beyond the development of curative therapies, studying rare DC spectrum diseases has offered insight into the pathways underlying aging related pathologies in non-rare individuals. For example, uncapped telomeres play an important role in DC spectrum diseases. Interestingly, many patients with aging related pathologies also have uncapped telomeres (reviewed in detail in
This data comes from two broad categories of studies: 1) measuring telomere length, which affects capping, and its association with various age related diseases and 2) assessing uncapped telomeres, using Telomere dysfunction Induced Foci (TIFs) based assays, in the pathologic specimens of age related diseases.

Shorter telomeres are more likely to become uncapped and dysfunctional, driving pathology. However, short telomeres are not necessarily dysfunctional. While a variety of methods are used to assess telomere length, they fail to assess capping status or function of telomeres. Instead, these methods provide either an average telomere length or a distribution of telomere lengths, which may belie telomere functionality. With this caveat, these techniques are amenable to large scale studies, which allow epidemiologists to study the correlations between telomere length and various diseases.

Many investigators have established a strong correlation between short telomeres and multiple different age related pathologies, both in circulating leukocytes as well as the primary diseased tissues themselves. Moreover, advancements in associative studies, such as Mendelian randomization analysis, further support the idea that short telomeres play a causal role in disease pathogenesis, for example in the cases of cardiovascular diseases, Alzheimer’s disease, and pulmonary fibrosis.

Mendelian randomization leverages established genetic associations from GWAS studies to remove some of the confounding variables that might obscure how short telomeres cause specific diseases. To better understand the importance of these findings, the following paragraphs will explore the power these new studies bring to bear on our understanding of what causes PF. When asking a question such as whether short telomeres cause disease, epidemiologists would typically employ an observational cohort study. In this study design, researchers find patients with short telomeres and ask what diseases are more associated with short telomere patients than patients with normal telomere length. But association does not prove causation, and these studies are always bedeviled by confounding variables that could at least in part explain the association between short telomeres and specific diseases. For instance, in the
case of pulmonary fibrosis, people with short telomeres might be more likely to smoke, so maybe smoking is the true cause of PF rather than short telomeres.

To directly assess a causative relationship between short telomeres and disease, an investigator would need to take a randomized group of individuals, shorten their telomeres, and observe what disease(s) they would develop over their lifetimes. Aside from the impracticalities and the obvious ethical issues, this experiment would provide evidence for short telomeres driving certain diseases. But can we find a way to approximate this experiment? While current associative studies do not control for all potentially confounding variables, Mendelian randomization provides a new implementation of genome-wide association studies (GWAS) to control for alternative explanations. GWASs use genomic sequencing to find specific single-nucleotide polymorphisms (SNPs) that are associated with specific phenotypes, allowing for unbiased detection of genetic polymorphisms associated with a specific phenotype. By Mendel’s laws of genetic inheritance, alleles are randomly segregated and genetic variants are assigned at birth and do not change with development or aging, unlike telomere length. So, one could conceivably use the genetic information associated with a specific phenotype to ask whether specific SNPs associated with telomere length correlate more with a disease than short telomeres per se. Thus, Mendelian randomization leverages GWAS data under the principle of Mendelian inheritance to ask whether a SNP associated with short telomeres is more likely to explain the emergence of a particular disease. These studies argue strongly for a causal link between short telomeres and many different diseases.

It has become increasingly clear that it is not simply short but rather uncapped telomeres that directly contribute to disease (i.e. telomere length is only one contributor to telomere capping, and it is furthermore also difficult to estimate the length of each telomere within a cell using the most commonly used measurement techniques, which provide only estimates of the mean length of telomeres overall). TIFs, or telomere dysfunction induced foci, are a measure of uncapped telomeres in cells and rely on direct evidence of DNA damage responses localized to telomeres. This damage can be detected using a telomeric PNA FISH probe or another telomere specific protein such as a shelterin. TIFs have been found in aged hearts and the TIF-positive cells have
been shown to contribute to cardiac hypertrophy and fibrosis. During lung fibrosis in mice, TIF-positive cells impair appropriate regeneration and their removal improves disease resolution. Also, TIFs are found in aged melanocytes and can induce TIFs in surrounding cells, likely leading to worsening tissue pathology. In summary, the connections between short, uncapped telomeres and aged tissues from normal individuals argues that the telomere dysfunction seen in DC patients may be an early manifestation of the normal aging that occurs in the general population. These data support using studies of DC patients to understand normal human aging and its associated pathologies.

**MODEL SYSTEMS TO STUDY DC AND ITS RELATED PATHOLOGIES**

The rarity of DC, estimated at one in one million, has made studying how to treat it difficult. Genetic mouse models have proven useful when tackling other rare genetic diseases. Three strategies have been attempted to model DC in mice: 1) introduction of pathologic DC mutations, 2) functional inactivation of telomerase, and 3) tissue-specific ablation of telomere maintenance factors.

DKC1 was first associated with DC using a family registry where it was found to have significant homologies to known pseudouridine synthases from yeast and rats as well as other organisms. Pseudouridine synthases catalyze the conversion of uridines into pseudouridines. These modified uridines are important for the stability and processing of many different RNAs including snoRNAs, rRNAs, and most relevant to this work, the RNA component of telomerase: TERC.

Early strategies to model DC involved introducing DKC1 mutations into mice, and these studies discovered that the pathological effects recapitulated aspects of DC seen in humans. These studies also proposed multiple mechanisms for how DKC1 mutations result in DC, including telomere dysfunction and defects in rRNA processing. The current consensus points to telomere dysfunction as the main driver of DC pathologies.

Another DC model used deletion of Pot1b and a partial loss of the mouse telomerase RNA component (mTR) and demonstrated some other hallmarks of DC: principally a hyperpigmentation in the skin as well bone marrow failure. These results were likely due to the
severe telomere shortening defect caused by the deletion of Pot1b and exacerbated by reduced telomerase activity. However, this model has not yet been used to model other pathologies of DC.

Telomerase knockout mice have also been used to study DC. Both deletions in mTR and mTERT shorten telomeres with successive generations of mice. Highly replicative tissues, such as intestine and the testes, show the earliest signs of pathologies in these mice. These mice also exhibit higher rates of cancer. But the pathologies that manifest in these mice only partially mimic those found in patients with DC. This difference highlights that mouse tissues may have differing predilections to dysfunction when compared to their human counterparts.

Many researchers then turned to tissue specific deletion of shelterin components as a way to understand how telomere dysfunction affects specific tissues. Hepatocyte restricted knockout of Trf2, one of the shelterins involved in preventing a DDR at the telomere, showed increased telomere dysfunction which did not lead to a p53 response (which was presumably explained by the fact that it led to chromosome end-to-end fusions) and which allowed for endoreduplication in hepatocytes during regeneration. In contrast keratinocyte restricted genetic deletion of Trf1, another shelterin usually involved in telomere replication, yielded persistently uncapped telomeres, causing severe epidermal stem cell failure and perinatal mortality.

Similarly, in the bone marrow compartment, knockout of Trf1 resulted in pancytopenia and loss of hematopoietic stem cell function in serial transplantation assays, mimicking one of the main pathologies of DC. Turning to the lung, inducible deletion of Trf2 in type II alveolar epithelial (AT2) cells led to telomere dysfunction in these cells and failure in their progenitor cell function. Furthermore, and most interestingly, knockout of Trf1 in AT2 cells caused mice to develop a progressive fibrotic response similar to that seen in pulmonary fibrosis of DC patients. These studies provide evidence that many of the pathologies of DC can be modeled, at least in part, in the mouse.

However, mouse models of DC may miss critical human-mouse specific differences in disease pathogenesis and progression. Indeed, mouse and human telomere biology is fundamentally different. Laboratory mouse cells generally have significantly longer telomeres, upregulate telomerase more easily, and immortalize more readily than human cells, thereby
complicating the study of short telomere diseases in mice \(^{61-65}\). Furthermore, mounting evidence continues to delineate the differences between mice and humans in various tissues \(^{66-71}\). Given these caveats with mouse models, human iPS cells have been engineered to model DC pathologies. Two groups reported the ability to generate iPS cell models from patients with various DC mutations \(^{72-74}\). Another group reported the generation of a human ES cell line with complete knockout of TERT, leading to apoptosis and loss of the line \(^{75}\). These iPS/ES cell lines provide some of the first models that could be used to study DC mutations in human cells. The advent of many directed differentiation protocols offers the prospect of understanding the cause of various DC pathologies and provides opportunities to search for ways to treat DC patients.

**SUMMARY OF A Wnt-TELOMERE FEEDBACK LOOP**

While DC models have not always recapitulated the exact phenotype of the disease, they have proven useful to understand how uncapped telomeres play a role in the disease. For example, work by Qijun Chen and Ting Yang found a direct connection between short, uncapped telomeres and the Wnt pathway that could lead to a novel therapeutic paradigm for DC.

They chose a mouse model with critical telomere shortening due to homozygous deletion in the RNA template component of telomerase, mTerc \(^{76}\). Because mice have long telomeres, initial generations lacking telomerase are relatively normal. However, over several generations, their telomeres shorten to the point of uncapping, causing pathology primarily in tissues with high rates of cell turnover, including the gastrointestinal epithelium. Prior to my work, pilot studies that transplanted normal bone marrow into late generation mTerc-/- mutants rescued intestinal pathology (See Ref. \(^{77}\) and Q. Chen and F.B. Johnson, unpublished). This result was surprising because the rescue included improved telomere capping and reduced levels of apoptosis in intestinal epithelial cells, even though the wild type bone marrow-derived cells were present only in the stroma underlying the epithelium. These data suggested that signals coming from the wild type cells were impacting telomere capping in a non-cell autonomous fashion.

Given the requirement of Wnt in the support of the intestinal stem cell niche, they hypothesized that the rescue involved Wnt signaling. Direct tests of this idea revealed a remarkably broad downregulation in late generation mTerc-/- mutants of genes in the canonical
Wnt signaling pathway and of targets of Wnt signaling that mark and are required for normal function of crypt base columnar cells (CBCs), including Lgr5, Ascl2, and Sox9\textsuperscript{76}. CBCs are multipotent stem cells that cycle frequently and give rise to all cell types in the intestinal epithelium. Consistent with frequent cell turnover, they also express telomerase \textsuperscript{78}. The downregulation of Wnt signaling in mutant CBCs could not be explained by cell loss, and was accompanied by a similar downregulation in the stroma and by the upregulation of Wnt pathway inhibitors, indicating a regulatory response to uncapped telomeres. Importantly, pharmacological upregulation of the Wnt pathway using exogenous R-spondin1, or the GSK-3 inhibitors

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{A positive feedback loop connects WNT and telomere capping}

In normal healthy cells, telomeres are capped, bound by shelterins and protected from being recognized as DNA double strand breaks. Since many of the shelterins are Wnt target genes, the Wnt pathway helps to support the capping function at telomeres. As telomeres shorten, less shelterins are able to bind until they reach a critical point where the DNA double strand break repair machinery recognizes the critical telomere as a damaged end. At this point the telomeres become uncapped, upregulating TP53 and miR-34a. miR-34a has many targets including many factors of the Wnt Pathway. This downregulation of the Wnt pathway decreases shelterin expression. Decreased shelterin expression has been linked in many model systems to an increase in uncapped telomeres. This creates a positive feedback loop where uncapped telomeres further drive the creation of more uncapped telomeres through a Wnt-pathway based downregulation of shelterin genes, worsening telomere uncapping. Re-activating the Wnt pathway using agonists such as GSK-3 inhibitors upregulates shelterins and upregulates TERT which lengthens telomeres, thereby reducing the number of uncapped telomeres and allowing the Wnt pathway to maintain adequate shelterin expression and capped telomeres.
CHIR99021 or lithium, all restored expression of Wnt pathway factors and targets in the mutant epithelium just as normal bone marrow transplantation did (Q. Chen and F.B. Johnson, unpublished). Remarkably, both the pharmacologic treatments and transplants also restored telomere capping in the mutant epithelium. Thus a positive feedback loop exists in the intestine between telomere capping and Wnt pathway activity (Figure 1-1., shaded box).

Subsequent studies comparing cultured human intestinal organoids derived from iPS cells from normal and DC (DKC1 mutant) donors revealed a similar feedback loop. The evolutionary advantage of such a loop may be to remove stem cells that have incurred oncogenic mutations and are thus at risk for forming tumors. When a cell activates an oncogene, the drive to proliferate can cause replication stress, termed Oncogene induced replication stress. Since telomeres are fragile sites and difficult to replicate through, this stress would cause premature telomere breakage and uncapping, leading to withdrawal of Wnt support, and thus the death or permanent cell cycle arrest of these cells.

Mechanistically, how telomere uncapping leads to downregulation of multiple factors in the Wnt pathway is explained, at least in part, by the action of the miR-34a. miR-34a targets many components of the Wnt pathway. miR-34a is also the most upregulated microRNA in the intestines of late generation telomerase mutant mice. Additionally, miR-34a expression is p53-dependent, and thus the well-known activation of p53 by uncapped telomeres likely contributes to miR-34a upregulation. Inhibition or deletion of miR-34a rescued intestinal telomere capping, Wnt gene expression, and pathology in the mutants. While miR-34a targets Wnt pathway transcripts directly, miR-34a may also target Wnt signaling indirectly through targeting the transcripts of p53 and other genes involved in Wnt signaling, including SIRT1.

Moving to the other arm of the Wnt-telomere feedback loop, the mechanism by which Wnt signaling supports telomere capping does not involve telomere lengthening (as expected for mice fully lacking telomerase), but rather appears to ensure the proper level of shelterin proteins. Indeed, six shelterins (Trf1, Trf2, Tin2 and Pot1a/b) are encoded by Wnt target genes, which were selectively downregulated in mutant intestine and which had their expression restored by Wnt pathway agonists. Moreover, in cultured human DKC1 mutant intestinal organoids, TERF2
overexpression was sufficient to restore telomere capping and Wnt pathway gene expression. This result was consistent with the established capacity of TERF2 overexpression to enable replicatively aged human fibroblasts with telomeres that would be uncapped at normal levels of TERF2 to continue to maintain their capped state and thus shorten even further before activating checkpoint responses \(^{79,87}\). While additional mechanisms may contribute to the telomere capping-Wnt feedback loop, this loop provides potentially therapeutic insight into diseases related to premature telomere shortening or dysfunction.

Wnt has been connected to telomeres by other studies as well. First, the Wnt-to-telomere capping half of the feedback loop, in particular the regulation of TERF2, the human homolog of mouse Trf2 and an important shelterin, by β-catenin, was described by Diala et al., who also demonstrated its importance for telomere capping in cancer cells \(^{88}\). And the telomere capping-to-Wnt half of the loop was observed by Tao et al., who observed downregulation of Wnt pathway components and target gene expression in late generation \(mTerc^{-/-}\) mutants as well as in mice exposed to gamma irradiation \(^{89}\). Second, TERT is a well-established target of Wnt/β-catenin and the transcriptional activator encoded by one of their key upregulated target genes, MYC, and Wnt signaling can upregulate telomerase activity \(^{90,91}\). Third, TERT has been reported to complex with the β-catenin/TCF transcription complex and thereby enhance upregulation of Wnt target genes. It was proposed that this activity explains developmental abnormalities reminiscent of WNT3a deficiency that were manifest in first-generation Tert-deficient mice, prior to apparent telomere shortening \(^{92,93}\).

Some of the findings in this third case have been controversial, but this case is distinct from the positive feedback loop \(^{94,95}\). In particular, the proposed role of TERT in mediating Wnt signaling was revealed by TERT deficiency, whereas the mice used to uncover the telomere loop lacked the RNA component of telomerase and the human organoid experiments were carried out in the context of normal TERT and TERC. Moreover, the proposed role for Tert was apparent in first-generation Tert-/- mice prior to apparent telomere uncapping, whereas our feedback loop does not falter until later generations of Terc-/- mice when they display uncapped telomeres. Overall, there are several links between Wnt and telomeres, which may reflect their cooperation
in supporting pro-growth states during development and within adult tissues and in enhancing their mutual failure to block the progression of premalignant cells into cancer.

**PULMONARY FIBROSIS: A PERSISTENT PROBLEM FOR DC PATIENTS**

Significant progress has been made in treating the bone marrow failure of DC patients, particularly via transplantation, but PF remains a major life-limiting pathology. Pulmonary fibrosis (PF) is a subtype of interstitial pneumonia that is progressive and chronic, replacing the normal lace-like alveolar architecture with a patchy, hyperproliferative fibrous tissue. While there are known exposures and causes of PF (e.g. exposure to silica and radiation), the most insidious remains idiopathic pulmonary fibrosis. Genetic studies of rare, familial PF began to offer insights into the pathophysiology of PF.

The first class of mutations uncovered were those involved in making surfactant, a surface acting agent that prevents atelectasis, or collapse, in alveoli. More genes related to epithelial biology and surfactant processing emerged as key drivers in other families, lending credence to the underlying hypothesis that dysfunctional epithelial cells caused PF, originally proposed by Haschek and Witschi in 1979. Another set of mutations also emerged from families with higher propensity for PF, namely telomere related genes, TERT and TERC. More mutations in telomere related genes are uncovered with each passing year in PF patients. These two sets of mutations provide strong evidence for the importance of facultative progenitor cell dysfunction or stress in PF pathogenesis. However, the exact mechanisms of how these two sets of genes contribute to pulmonary fibrosis remains unknown.

These genetic studies, along with other cell biological findings, have helped make epithelial dysfunction the dominant paradigm for explaining the pathophysiology of pulmonary fibrosis. More specifically, AT2 cells have become the main cell of interest. AT2 cells are facultative progenitor cells that rapidly proliferate and differentiate to regenerate the lung after acute injury. Transgenic mouse studies found that acute damage and depletion of AT2 cells can yield a fibroproliferative response. Furthermore, as noted previously, knockout of Trf2 in mouse AT2 cells leads to a primary, progressive fibrotic response similar to that seen in patients with PF. Also, AT2 cell specific deletion of Sin3a, a transcription factor whose loss
leads to an upregulation of senescence like features \(^{119}\), leads to PF in mice \(^{118}\). Most convincingly, AT2 cell restricted expression of a dominant negative allele of \(Sftp\)c, the most specific surfactant for AT2 cells, generates a fibrotic response \(^{111,120–122}\). These studies argue strongly that dysfunctional AT2 cells are an important cell type that can drive PF.

While AT2 cells can initiate PF, dysfunctional AT2 cells interact with many other cells to drive fibrosis, particularly fibroblasts and immune cells \(^{113,121,123}\). The exact nature of these interactions is an active area of research and mouse models are helpful in obtaining insight into the essential signaling pathways involved. But as highlighted with telomeres, mouse-human differences continue to limit our full understanding of PF pathogenesis.

Given the importance of AT2 cells as initiators of PF and the various interspecies differences, there lies a great need for human models of AT2 cell dysfunction. Work by many groups has provided the opportunity to use iPSC-directed differentiation to generate AT2 cells \(^{124–128}\). This technology provides multiple benefits for unravelling how dysfunctional AT2 cells can drive PF. First, they allow us to study AT2 cells in isolation from the cellular crosstalk normally found in a fibrotic lung, providing a useful reductionist model to understand AT2 cell intrinsic responses to telomere dysfunction. These models also provide an almost unlimited source of human AT2 cells when compared to sourcing primary human AT2 cells from human lungs rejected for transplant. This unlimited supply could be theoretically used to transplant into damaged mouse lungs to study the human AT2 cells \emph{in vivo} and compare them to their mouse counterparts. Technically, these studies are challenging with primary human AT2 cells both due to limited access to cells and the current inability to cryopreserve or recover these cells.

Furthermore, these iPSC derived AT2 cells could be used to re-engineer the alveolus to study the complex interplay between them and the other tissue residents of the alveolus. In summary, developing this new model of telomere dysfunction in iPSC derived AT2 cells have the potential to unlock the capacity to ask targeted questions regarding the cell autonomous and cell non-autonomous effects of telomere dysfunction in human AT2 like cells.
CHAPTER 2 : GSK3 INHIBITION RESCUES GROWTH AND
TELOMERE DYSFUNCTION IN DYSKERATOSIS CONGENITA
IPSC-DERIVED TYPE II ALVEOLAR EPITHELIAL CELLS

Portions of this section are adapted from the article “GSK3 inhibition rescues growth and
telomere dysfunction in dyskeratosis congenita iPSC-derived type II alveolar epithelial cells”
posted on bioRxiv (2020).
INTRODUCTION

Dyskeratosis congenita (DC) is a rare genetic disorder characterized by bone marrow failure, skin abnormalities, elevated risk of certain cancers, and liver and pulmonary fibrosis (PF). These pathologies are caused by abnormally shortened and uncapped telomeres arising from deficiencies in telomere maintenance, typically due to defects in the action of telomerase. Significant progress has been made in treating the bone marrow failure of DC patients, particularly via transplantation, but PF remains a major life-limiting pathology.

PF is a subtype of interstitial pneumonia that is chronic and progressive, replacing the normal lace-like alveolar architecture with patchy, hyperproliferative fibrous tissue. Current therapies are only modestly effective and do not reverse the underlying fibrosis, and lung transplantation is not always an option. While much of the early work in PF pathogenesis focused on unraveling the contributions of fibroblasts, genetic studies of families with a predisposition to PF argue that defects in alveolar epithelial cells and telomeres are key drivers of disease. Recent work in mice shows that dysfunctional AT2 cells, the stem cells of alveoli, can lead to a progressive chronic fibrotic response similar to that seen in patients. Furthermore, many of the same genes which when mutated cause DC have also been linked to familial PF. In sporadic PF, both age and short telomeres are risk factors, and these risks are linked because age is associated with telomere shortening in the lung, particularly in AT2 cells. Consistent with a role for telomere dysfunction in driving PF, AT2 cells in sporadic PF express hallmarks of senescence and have shorter telomeres in fibrotic regions than those in non-fibrotic regions. Additionally, two human Mendelian randomization studies argue that short telomeres are a cause of PF. Murine studies also argue that telomere dysfunction and senescence in AT2 cells can drive PF. Although causality is thus evident, exactly how AT2 cell telomere dysfunction leads to PF is poorly understood.

Previous work in our lab using mouse and human iPSC-derived organoid models of DC intestinal defects uncovered a positive feedback loop by which telomere capping and canonical Wnt signaling support one another under normal conditions to maintain the intestinal stem cell.
niche. In the setting of telomere dysfunction, this virtuous cycle becomes vicious: the resulting suppression of Wnt signaling interferes with stem cell function directly and it also amplifies telomere dysfunction by diminishing Wnt-dependent expression of telomere maintenance factors, including the catalytic subunit of telomerase, TERT, and several of the telomere-protective shelterins. These studies demonstrated that Wnt pathway agonists can rescue these defects, raising the possibility that Wnt agonism could be of therapeutic benefit in DC. Given how telomere dysfunction in AT2 cells appears to drive PF, we wondered if Wnt agonism might be of benefit in pulmonary fibrosis. Wnt signaling is important for lung epithelial cell development and regeneration of the adult lung in response to injury.

There is evidence for enhanced Wnt/β-catenin signaling in both alveolar epithelium and in fibroblastic foci in the lungs of individuals with idiopathic pulmonary fibrosis (but without apparent DC). Furthermore, some findings from mouse studies argue that excess Wnt signaling promotes pulmonary fibrosis. However, as noted above, canonical Wnt signaling clearly plays important roles in normal alveolar homeostasis, and it is possible that a primary defect in Wnt signaling in alveolar epithelial cells, leading to failure to maintain alveolar integrity, could signal a secondary fibrotic response that is also Wnt-dependent. Re-establishing epithelial homeostasis might prevent activation of fibrosis entirely, thus also blocking any potential direct effects of Wnt on fibrosis. Furthermore, the mouse studies used bleomycin, a potent DNA double strand break inducer, to induce damage that is more acute and severe than the chronic and gradual accumulation of uncapped telomeres underlying pulmonary fibrosis in DC, and it is thus unclear how accurately the bleomycin model reflects pathogenic mechanisms in DC. Of note, there is evidence that lithium can be helpful in promoting lung repair in a model of emphysema induced by elastase, including a restoration of levels of nuclear β-catenin in alveolar epithelial cells, expression of Wnt target genes, and reduced collagen levels. Differential activation of canonical over non-canonical Wnt signaling might also be beneficial, given that enhanced non-canonical signaling contributes to TGF-β-induced pulmonary fibrosis in mice. Overall, more must be understood before it can be predicted what is the balance of potential
positive and negative effects of lithium or other Wnt agonists on the pathogenesis of pulmonary fibrosis in DC.

Wnt signaling is complex and context dependent\textsuperscript{157}, and the exact spatial, temporal and cell type-specificity of Wnt signaling in PF remains an area of intense investigation. It is difficult to extrapolate from observational pathologic studies of fully developed PF to the potential functional impact of Wnts at earlier stages of the disease, and the large number of interacting cell types in PF lungs also makes it challenging to identify primary drivers in such studies. Furthermore, differences between mouse and human telomere biology together with the generally lower susceptibility of mice to PF makes mouse modeling difficult. We therefore generated AT2 cell organoids by directed differentiation of human iPSCs (iAT2 cells) to explore how telomere dysfunction might impact their function\textsuperscript{124,158}.

By comparing iAT2s that are isogenic except for an introduced mutation in the gene most often mutated in DC, X-linked \textit{DKC1}, we show that mutant iAT2 cells become senescent in concert with telomere shortening and uncapping. iAT2 cells with short, uncapped telomeres exhibit gene expression changes consistent with decreased Wnt signaling, and treatment with GSK3 inhibitors, such as CHIR99021, rescues their growth and telomere dysfunction. These findings raise the possibility that Wnt agonists may be of benefit in rescuing the stem cell and telomere defects of AT2 cells associated with PF in DC patients.
METHODS
IPSC LINE GENERATION AND MAINTENANCE
The patient derived AG04646 DKC1 A386T line was obtained from our previous studies\textsuperscript{79}. The BU3 NKX2.1::GFP SFTPC::TdTomato line, an iPS line derived from BU3 where the NKX2.1 gene and the SFTPC gene have been edited to have fluorescent gene expression reporters in the endogenous locus, was a generous gift from Dr. Darrell Kotton at Boston University. iPS cells used for differentiation were maintained on growth factor reduced Matrigel (Corning) coated plates in StemMACS™ iPS-Brew XF medium (Miltenyi Biotec). Cells were cultured in clusters and passaged every 4-5 days using StemMACS Dissociation reagent (Miltenyi Biotec). All iPS lines were genotyped using an RFLP at the relevant important loci and the sequence was confirmed by Sanger sequencing. All cells were routinely screened for mycoplasma contamination using a PCR based assay\textsuperscript{159}.

CRISPR EDITING FOR GENERATING PAIRED DKC1 MUTANT CELL LINES IN BU3 NGST
To generate the introduced BU3 NGST line harboring the DKC1 mutation, we used the CRISPR track on the UCSC genome browser to select candidate guideRNAs (gRNAs) that targeted as close to the individual mutation as possible, had easily mutable PAM sites and would introduce a new restriction site to make screening easier (see the Key Resources Table for exact sequences and Figure 2.1. for details). gRNAs were ordered as oligos from IDT and cloned into pX458, a gift from Dr. Feng Zhang’s lab (Addgene # 48138). The candidate guides were tested for cutting efficiency by transfecting them into HEK293T cells and assaying cutting efficiency using T7E1 digestion of the PCR amplified locus. The most efficient guides were chosen and ssODN HDR templates were designed to eliminate the PAM. iPS cells were then nucleofected with the Amaxa Nucleofection system using the following program (P3, CA-137) (Lonza). The cells were allowed to recover for 36-48 hours at high density in the presence of ROCK inhibitor and then isolated by FACS for GFP\textsuperscript{+} cells. They were plated at low density (2500 cells/10 cm plate) and allowed to form single colonies. After seven to ten days, individual clones were selected and transferred to 96 well plates, and screened for introduction of the restriction site for each mutation. Restriction enzyme positive clones were expanded and then subjected to sanger sequencing for identification of correctly edited clones. Successfully edited clones were checked for normal
karyotype by G-banding (Cell Line Genetics), mycoplasma contamination, and pluripotency marker expression by immunofluorescence, and were subsequently passaged for at least 5 passages before being re-genotyped to ensure that the clones were not mixed. During differentiations, all iAT2 cells were genotyped by restriction digest to ensure that the mutation was not lost with passage.

**DIRECTED DIFFERENTIATION INTO NKX2.1+ LUNG PROGENITORS AND SFTPC+ IPS- DERIVED AT2 CELLS**

A modified version of the protocol described in Jacob et al. was used to generate SFTPC expressing iAT2 cells. In brief, iPS cells were seeded at 500,000 cells per well on a 6-well plate with ROCK inhibitor for 24 hours and incubated at 5% O2 | 5% CO2 | 90% N2. Definitive endoderm was induced using the StemDiff Definitive Endoderm kit for 3 days. Next, the cells were split at a ratio of 1:3 onto fresh Matrigel plates and anteriorized using dorsomorphin (2 µM) and SB431542 (10 µM) in complete Serum Free Differentiation Media (cSFDM) for three days. Cells were then differentiated into NKX2.1+ progenitors by incubating in CBRa media (cSFDM containing CHIR99021 (3 µM), BMP4 (10 ng/mL), and retinoic acid (100 nM)) for 7 days changing media every 2 days at first and then increasing to every day media changes when the media became more acidic. On day 15 or 16, NKX2.1+ progenitors were isolated using a FACSJazz sorter using the endogenous NKX2.1::GFP reporter.

NKX2.1+ sorted cells were replated at a density of 400,000 cells/mL in 90% Matrigel supplemented with 10% of CK+DCI+TZV media (cSFDM containing 3 µM CHIR99021, 10 ng/mL KGF, 100 nM dexamethasone, 100µM 8Br-cAMP and 100 µM IBMX and 2 µM TZV) (from now on referred to as 90/10 Matrigel). The Matrigel droplets were allowed to cure at 37 °C for 20-30 minutes and then overlaid with an appropriate amount of CK+DCI+TZV Media. These alveolosphere containing matrigel droplets were incubated at 37˚C room air incubated supplemented to 5% CO2 for 14 days changing with fresh media every other day. On Day 28, the iAT2 containing alveolospheres were sorted on a FACSJazz sorter for SFTPC+ cells using the endogenous SFTPC::TdTomato reporter. These sorted SFTPC+ cells were replated at a concentration of 65,000 cells / mL in 90/10 Matrigel drops and grown in K+DCI+TZV at 37˚C in an ambient air incubator supplemented to 5% CO2 for 3 weeks changing media every other day.
ALVEOLOSHERE COUNTING AND FORMATION EFFICIENCY CALCULATIONS
Alveolosphere images were taken on a Leica Thunder widefield microscope using a 1.25x objective. Z-stacks were maximum projected and then thresholded using ImageJ to create a binary file. Binary files were eroded and dilated to ensure maximum determination of the alveolosphere size. Finally the binary images were separated by watershedding and alveolospheres were counted using Analyze Particles in ImageJ.

IMMUNOFLUORESCENCE MICROSCOPY OF IAT2 ALVEOLOSHERES
Alveolospheres were washed with PBS and then fixed in place using 2% PFA at room temperature at room temperature for 30 minutes and then dehydrated and paraffin embedded and sectioned. Once cut, slides were deparaffinized, rehydrated, permeabilized, and antigens were retrieved by steaming for 15 minutes in a citrate buffer (Vector Labs). After blocking, each slide was incubated with a primary antibody using the concentrations listed in the Key Resource Table. Slides were incubated with primary antibody at 37 °C for two hours. After washing, slides were incubated with appropriate fluorochrome conjugated secondary antibodies (see Key Resources Table for antibody details). Slides were then washed, counterstained with DAPI, and mounted. Images were acquired using a Leica Thunder Widefield Microscope. TIFs were stained as described in Herbig et al. In brief, cut slides were deparaffinized, rehydrated, permeabilized and antigens were retrieved as for other immunofluorescence stains. Slides were blocked and stained for 53BP1 and then stained with an appropriate fluorochrome conjugated secondary antibody. Slides were then re-fixed with paraformaldehyde, quenched with glycine, re-dehydrated in an ethanol series, and air dried. The slides were then stained with the PNA probe. The slides were washed, rehydrated in an ethanol series, and stained with a tertiary fluorochrome conjugated antibody. Slides were then washed, counterstained with DAPI, and mounted. TIF images were acquired using a Leica SP8 Confocal microscope. Quantification of nuclei was carried out in ImageJ in a blinded fashion.

MEASUREMENT OF TELOMERASE ACTIVITY WITH TRAP
iPS cells or iAT2 cells were cultured as indicated in each figure legend. 100,000 cells were harvested using methods described and lysed using NP-40 lysis buffer and processed as described in Herbert et al. In brief, lysates were incubated with a telomerase substrate and
incubated at 30˚C for telomerase to add telomere repeats. The reactions were then PCR amplified. Telomere repeats were resolved on a 4-20% TBE polyacrylamide gel and visualized by staining with SYBR Green nucleic acid gel stain. Relative telomerase activity was quantified using ImageJ focusing on the first six amplicons averaged across the dilutions.

**MEASUREMENT OF TELOMERE LENGTHS BY TRF AND TESLA**
Telomere lengths were measured as described in Lai et al.\textsuperscript{161,162}. DNA was isolated from cells using a Gentra Puregene kit (Qiagen). DNA was quantified by fluorometry using QuBit 2.0 (Invitrogen). For TRF analysis in brief, 500 ng of DNA was digested with CviAII overnight followed by digestion with a mixture of BfaI, MseI, and NdeI overnight. For TeSLA in brief, 50 ng of DNA was ligated to telorette adapters, then digested with CviAII, then digested with a combination of BfaI, MseI and NdeI, dephosphorylated, and TeSLA adapters (AT/TA Adapters) were ligated on. These TeSLA libraries were PCR amplified using Lucigen’s FailSafe polymerase kit with Pre-Mix H.

Southern blotting was carried out using previously established protocols with some modification\textsuperscript{139,161,163}. TRFs and TeSLA PCR reactions were separated on a 0.7% agarose gel at 0.833 V/cm for 24 hours. The gel was depurinated and denatured and then transferred to a Hybond XL membrane (Cytiva) by capillary transfer using denaturation buffer. The Hybond membrane was hybridized using a DIG-labeled telomere probe overnight. The blot was then washed and exposed using CDP-Star on an LAS-4000 Image Quant imager (Cytiva). TRFs were analyzed using ImageQuant while TeSLAs were analyzed using the MatLab software developed previously\textsuperscript{161}.

**MEASUREMENT OF TELOMERE LENGTHS BY QPCR**
Average telomere length was measured by qPCR as described in\textsuperscript{164,165} with some modifications.

In brief, isolated genomic DNA was quantified by QuBit fluorometry (Invitrogen) and diluted to within the range of a standard curve constructed from a mixture of all samples analyzed. Triplicate qPCR reactions of the Telomeric (T) product and the Single copy gene (S) (\textit{HBB}) were amplified using a Roche LightCycler 480 II (Roche) using the following programs: T PCR Program 95˚C for 10 minutes, 40 cycles of 95˚C for 15 seconds, 56˚C for 1 minute; S PCR Program 95˚C for 10 minutes, 40 cycles of 95˚C for 15 seconds, 58˚C for 1 minute. Cq values
were computed using the second derivative method, and T/S ratios were calculated using the $2^{-\Delta\Delta Ct}$ method.

**RNA-SEQUENCING AND DATA ANALYSIS**

SFTPC+ sorted cells from the indicated times during, counted and harvested in TRIzol and stored at -80 °C until further processing. The RNA was isolated using a Direct-Zol kit (Zymo Research). RNA concentration was obtained by QuBit fluorometry (Invitrogen) and the integrity was checked by tape station analysis (Agilent Technologies). All samples had RINs >8, and the libraries were prepared by poly-A selection and sequenced by GeneWiz, LLC.

RNA sequencing libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina following manufacturer's instructions (NEB). Briefly, mRNAs were first enriched with Oligo(dT) beads. Enriched mRNAs were fragmented for 15 minutes at 94 °C. First strand and second strand cDNAs were subsequently synthesized. cDNA fragments were end repaired and adenylated at 3’ ends, and universal adapters were ligated to cDNA fragments, followed by index addition and library enrichment by limited-cycle PCR. The sequencing libraries were validated on the Agilent TapeStation (Agilent Technologies), and quantified using a Qubit 2.0 Fluorometer (Invitrogen) as well as by quantitative PCR (KAPA Biosystems).

The sequencing libraries were pooled and clustered on one lane of a flowcell. After clustering, the flowcell was loaded on the Illumina HiSeq instrument (4000 or equivalent) according to manufacturer’s instructions. The samples were sequenced using a 2x150bp paired end configuration. Image analysis and base calling were conducted by the HiSeq control software. Raw sequence data (.bcl files) generated from Illumina HiSeq was converted into fastq files and de-multiplexed using Illumina’s bcl2fastq 2.17 software. One mismatch was allowed for index sequence identification.

Fastq files were checked for quality using FastQC. Raw sequence files (fastq) for 22 samples were mapped using salmon (https://combine-lab.github.io/salmon/)\textsuperscript{166} against the human transcripts described in Gencode (version v33, built on the human genome GRCm38, https://www.gencodegenes.org), with a 70.5% average mapping rate yielding 30.4M average total
input reads per sample. Transcript counts were summarized to the gene level using tximport (https://bioconductor.org/packages/release/bioc/html/tximport.html), and normalized and tested for differential expression using DESeq2 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html). Subsets of time-matched samples were used to compute pairwise contrast statistics for mutant vs. wt at each time. Raw FASTQ files and analyzed counts are available on GEO under the accession number GSE160871.

Gene set enrichment analysis (GSEA) \(^{167}\) was carried out in R (v4.0.2) \(^{168}\) using RStudio (v1.3.1056) \(^{169}\), the tidyverse (v1.3.0) \(^{170}\), and the readxl package (v1.3.1) \(^{171}\). GSEA was run for contrasts of interest in pre-ranked mode using the DESeq2 statistic as the ranking metric \(^{172}\). Annotated molecular signatures from the Hallmark Collection (H), Curated Collection (C2), and Regulatory Target Molecular Collection (C3) maintained by the Molecular Signatures Database were accessed in RStudio using the msigdb package (v7.1.1) \(^{173–175}\). The clusterProfiler package (v3.17.1) was used to perform GSEA on the unfiltered, sorted gene lists \(^{176}\). GSEA results were viewed using the DT package (v0.15) \(^{177}\). GSEA plots were generated using the enrichplot package (v1.9.1) \(^{178}\).

Genes that differed in expression >2 fold and were associated with an adjusted p-value < 0.05 from the D70 time point were also analyzed through the use of Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com) \(^{179}\) through the University of Pennsylvania Molecular Profiling Facility.

**STATISTICAL METHODS**

Statistical methods are outlined in each of the figure legends. Each replicate "n" represents an entirely separate differentiation from the pluripotent stem cell stage. Quantitative data is represented as the mean with error bars representing the standard error of the mean. Student’s t-tests (unpaired and two-tailed) were used for determining statistical significance for all comparisons unless otherwise noted.
RESULTS
ENGINEERING THE BU3 NGST IPS CELL LINES WITH A DC MUTATION

To model the AT2 cells from DC patients, we engineered using CRISPR-Cas9 a well-characterized, causal DC mutation in **DKC1 (DKC1 A386T)** into the BU3 **NKX2.1::GFP SFTPC::TdTomato (NGST)** human iPS cell line. This NGST line allows for easy isolation by

Figure 2-1: Introduction of the **DKC1 A386T** mutation into the BU3 **NKX2.1::GFP, SFTPC::TdTomato** iPS Cell Line

(A) CRISPR based editing strategy for introducing the **DKC1 A386T** mutation into the BU3 **NKX2.1::GFP, SFTPC::TdTomato** iPS cell line.
(B) Genotyping by RFLP of **DKC1 A386T** clonal cell lines and their WT counterparts that also were exposed to the CRISPR reagents using the introduced NdeI site.
(C) Sanger sequence verification of the introduction of the mutation yielding 1156G>A. Note the WT sequence is marked in red.
(D) Paired iPS cell clones maintain high expression of **NANOG**, a marker of pluripotency (scale bars, 100 µm).
(E) Paired iPS cell clones show normal karyotypes after the introduction of the **DKC1 A386T** mutation.
FACS sorting NKX2.1+ lung progenitors as well as iAT2s that express SFTPC. We established an isogenic pair of cell lines: an introduced mutant line and a corresponding wild-type line (Figure 2-1). Both iPS lines maintained markers of pluripotency and normal karyotypes after the introduction of the DKC1 A386T mutation (Figure 2-1).

Figure 2-2: DKC1 A386T iPS cells show decreased telomerase activity and telomeres shorten with passage

(A) Quantitative telomeric repeat amplification protocol (qTRAP) assay for telomerase activity in iPS cells shows a reduction in relative telomerase activity in DKC1 A386T introduced line (n = 3). AG04646 is an iPSC line derived from a DC patient with the A386T mutation, and the WT line is an unedited clone derived along with the A386T-introduced clone from the BU3 parental iPSC line.

(B) Telomeric repeat amplification protocol (TRAP) assay for telomerase activity in iPS cells using a 5 fold dilution series of the cellular extracts show a reduction in relative telomerase activity in DKC1 A386T introduced lines.

(C) Terminal restriction fragment (TRF) telomere length analyses in the WT and DKC1 A386T paired iPS cells compared to the parental iPS Line (BU3 NGST) and the patient derived iPSC line (AG04646). Above each lane is the number of passages from the introduction of the mutation.
Previous work \cite{72,73,79} established that iPS cells with the \textit{DKC1 A386T} mutation exhibit decreased telomerase activity resulting in telomere shortening with passage. We confirmed that telomerase activity was reduced, and telomeres shortened with successive passages, in the BU3 NGST \textit{DKC1 A386T} iPS cell line when compared to its wild-type control (Figure 2-2 A-C).

**IAT2 CELLS WITH SHORT TELOMERES FAIL TO FORM ALVEOLOSPHERES AND GROW IN SIZE**

We next differentiated these paired iPS cell lines into iAT2 cells using the protocol developed by Jacob et al. (See Figure 2-3-A for differentiation strategy, Figure 2-4-A and B for representative sorting strategies). Using iPSCs 25 passages after the introduction of the mutation initially yielded iAT2s that grew in a similar fashion to wild type, but which developed a growth

![Figure 2-3: DC iAT2s fail to form alveolospheres with successive passaging](image)

\begin{itemize}
  \item [(A)] Differentiation protocol used to probe the effects of the \textit{DKC1 A386T} mutation on iAT2 cells. (D.E. - Definitive Endoderm Specification; Ant. - Anteriorization)
  \item [(B)] Representative images of differentiating WT and \textit{DKC1 A386T} mutant bearing iAT2 alveolospheres. (scale bars, 100 µm as indicated for iPS and NKK2.1+ progenitors; 1 mm for all alveolosphere images)
  \item [(C)] Quantifications of alveolosphere area and formation efficiency on D70. (n = 4; ** p < 0.01, **** p < 0.0001, Student’s t-test)
  \item [(D)] Quantification of the percentage SFTPC+ cells and the number of SFTPC+ cells produced with passage of the iAT2 cells shows \textit{DKC1 A386T} iAT2 alveolospheres accumulate fewer SFTPC+ cells. (n = 4; ** p < 0.01, Student’s t-test)
  \item [(E)] RNAseq of sorted SFTPC+ iAT2 cells at different passages show AT2 cell genes are not grossly affected by the \textit{DKC1 A386T} mutation. (n = 4). \textit{W} = wild type, \textit{M} = mutant.
\end{itemize}
defect characterized by lower alveolosphere formation efficiency as well as smaller alveolospheres. The phenotype became apparent by 50 days of culture (D50) and was dramatic by D70 (Figure 2-3-A-C). In contrast, using iPSCs only five passages after the introduction of the mutation yielded iAT2s without any defects in alveolosphere growth or size at D70 (Figure 2-4-C). These data indicate that the defects observed were due to progressive telomere shortening after...
introducing the \textit{DKC1} mutation, and not the immediate effects of telomerase deficiency (or other potential deficiencies) caused by the \textit{DKC1 A386T} mutation \textit{per se}.

Surfactant protein C (SFTPC) is a highly specific marker of AT2 cells \textsuperscript{180}, and the yield of SFTPC::TdTomato\textsuperscript{+} (SFTPC\textsuperscript{+}) cells was reduced significantly at D70 in \textit{DKC1} mutant cultures, while the percentage of SFTPC\textsuperscript{+} cells generated at each time point was not different, suggesting that there is a defect in AT2 cell proliferation or survival (Figure 2-4-D). Sorted SFTPC\textsuperscript{+} cells from iAT2 cell alveolospheres maintained expression of multiple AT2 specific genes suggesting that the introduced mutation did not affect lineage specification (Figure 2-4-E). Thus, DC iPS cells can generate iAT2 cell alveolospheres, however these alveolospheres lose the capacity to self-renew with successive passaging.

Figure 2-4: Representative sorting strategies and differentiation of early passage iPS cells yields no growth defect (See Page 29)

(A) Representative sorting strategy for NKX2.1\textsuperscript{+} progenitors.
(B) Representative sorting strategy for SFTPC\textsuperscript{+} cells from iAT2 alveolospheres.
(C) Differentiation protocol used to probe the effects of the \textit{DKC1 A386T} mutation on iAT2 cells. (D.E. - Definitive Endoderm Specification; Ant. - Anteriorization)
(D) Representative images of differentiating WT and \textit{DKC1 A386T} mutant bearing iAT2 alveolospheres derived from iPS cells that have undergone 5 passages after introduction of the mutation. (scale bars, 1 mm)
(E) Representative images and quantification of CC3 staining at D70 shows no significant difference between WT and \textit{DKC1 A386T} mutant iAT2s. (n = 4, no difference, p = 0.96, Student’s t-test)
(F) Average telomere length as measured by telomere qPCR of WT and DC iAT2 cells with passage (n = 2, p-values listed on the figure)

Figure 2-5: DC iAT2 cells at D70 show hallmarks of senescence (see Page 31)

(A) Gene expression profiling of iAT2 cells at D28 and D50 show no difference between WT and \textit{DKC1 A386T} in expression of markers of proliferation and a cell cycle inhibitor, while at D70 cells, there is a significant decrease in \textit{MCM2} and \textit{MKI67} as well as a significant increase in \textit{CDKN1A} (p21). (n = 4, **** p < 0.0001, DESeq2 pairwise contrast statistics)
(B) At D70, \textit{DKC1 A386T} mutant iAT2 cells have a higher fraction of cells with 53BP1 foci. (n = 4, * p < 0.05, Student’s t-Test; scale bars, 10 µm; Insets highlight cells with 53BP1 foci as noted by the white arrowheads)
(C) At D70, \textit{DKC1 A386T} mutant iAT2s have a higher fraction of cells positive for p21. (n = 4, * p < 0.05, Student’s t-Test; scale bars, 10 µm)
(D) At D70, \textit{DKC1 A386T} mutant iAT2s have a higher fraction of cells with TIFs. (n = 4, * p < 0.05, Student’s t-Test; scale bars, 10 µm; Insets highlight cells with TIFs, each one noted by a white arrowhead)
(E) Representative TeSLA of \textit{DKC1 A386T} iAT2 alveolospheres shows telomeres shorten with passage.
(F) Quantification of \textit{DKC1 A386T} iAT2 cell telomere lengths shows a preponderance of short telomeres appears as the iAT2 cells approach D70, red colored data points highlight telomeres under the 1.6kb threshold. (n = 2, “Shortest 20%” reports the 20th percentile of telomere length, in Kb)
DC IAT2 CELLS AT D70 SHOW HALLMARKS OF SENESCENCE

To better understand the AT2 cell defects, we further compared the wild type and mutant iAT2 alveolospheres at different passages. Gene expression over successive passages of sorted SFTP+C+ cells from iAT2 alveolospheres showed decreases in proliferation markers (MKI67 and MCM2) as well as an increase in expression of the cell cycle inhibitor CDKN1A (p21), and these changes were most pronounced in mutant alveolospheres (Figure 2-5-A). D70 mutant iAT2
alveolospheres showed an increase in DNA damage marked by 53BP1 foci (Figure 2-5-B), and an increased fraction of cells expressing p21 protein (Figure 2-5-C), but no increase in apoptosis (Figure 2-4-E).

Measuring telomere length using qPCR, DC and WT iAT2 cells showed no significant change in average telomere length with passage, although average telomere lengths in mutants trended shorter than in WT (Figure 2-4-F). However, I measured telomere lengths using TeSLA \(^{161}\), which is more sensitive for the detection of short telomeres. TeSLA uses a series of digestions and ligations of genomic DNA to eventually PCR amplify single telomeres and visualize them using a telomeric specific probe. This technique revealed that DC iAT2 cells had a preponderance of short telomeres at D70 (Figure 2-5-E & F). TeSLA on WT cell lines was not performed as the telomeres were not seen to shorten with passage as seen at the iPS stage (see Figure 2-2C). While this does not rule out that there may be telomere shortening in the WT iAT2s with passage, since they do not show a senescent phenotype and lack many of the markers of senescence, it is unlikely there would be short telomeres given the vast body of literature about telomere lengths and senescence.

Consistent with this, DC iAT2 alveolospheres showed an increased number of telomere dysfunction induced foci (TIFs), a hallmark of uncapped telomeres (i.e. telomeres that signal DNA damage responses and cell cycle checkpoint arrest \(^7\) (Figure 2-5-D). These findings indicate that the short and uncapped telomeres that accumulate with passage of DC iAT2 cells lead them to senesce.

**RNA-SEQ REVEALS DIFFERENTIALLY EXPRESSED PATHWAYS AND SHOWS THE WNT-TELOMERE FEEDBACK LOOP IS ACTIVE IN DC IAT2 CELLS**

To further understand changes in the DC iAT2 cells, we measured the gene expression of sorted SFTPC+ iAT2 cells using RNA-seq (Figure 2-6-A). We found very few significantly differentially expressed genes when comparing wild type and mutant cells at D28 and D50, but a large number of differentially expressed genes at D70 (Figure 2-6-B), arguing that the gene expression changes seen at D70 are likely driven by uncapped telomeres. Gene Set Enrichment Analyses (GSEA) and Ingenuity Pathway Analysis (IPA) revealed an upregulation of the DNA damage response, the unfolded protein response (UPR), mitochondrial related functions.
(oxidative phosphorylation, the respiratory electron chain) and a downregulation of hypoxia related signaling, and hedgehog signaling along with other changes (See Table 2-2 for a full list). IPA analysis revealed similar changes as well as defects in multiple pathways controlled by inflammatory cytokines like IL1β, IL6, IL17 and others (See Table 2-3 for the full lists). We found a marked upregulation in DC iAT2 cells of many pathways associated with pulmonary fibrosis (Table 2-1 for curated list of IPF related pathways, see Table 2-2 & 2-3 for unedited analysis). These included the UPR \(^{112,181}\), thyroid hormone metabolism \(^{182}\), p53 signaling \(^{183}\), mitochondrial dysfunction and mitophagy \(^{182,184}\), and caveolin function \(^{185}\). This analysis also showed an upregulation of non-canonical Wnt signaling (Figure 2-6-C), which correlated with a significant upregulation in WNT5A and WNT11, known non-canonical Wnt ligands (Figure 2.6.D).

Furthermore, almost every FZD gene, encoding co-receptors for canonical Wnt signaling, was down regulated in DC iAT2 cells (Figure 2-6-E). Also, GSEA analysis found genes with TCF7 targets in their promoters are downregulated in DC iAT2 cells at D70 (Figure 2-6-F). IPA of master regulators at D70 revealed a decrease in genes controlled by lithium chloride (which can potentiate Wnt signaling) and TCF7 along with an upregulation of genes usually stimulated by Wnt pathway inhibitors (Figure 2-6-G). GSEA also revealed a significant downregulation of targets of miR34a, a miRNA that we previously demonstrated negatively regulates many

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**Figure 2-6**: RNA-seq along the differentiation of iAT2 cells reveals a time dependent change in Wnt response (See Page 34)

(A) A schematic to show how cells were prepared for RNA-seq.
(B) Volcano plots at D28, D50, and D70 show how the number of differentially expressed genes increases at D70.
(C) GSEA Analysis at D70 comparing DKC1 A386T iAT2 cells reveals an upregulation of non-canonical Wnt Signaling and the PCP (Planar Cell Polarity) pathway.
(D) RNA-seq analysis shows upregulation of WNT5A and WNT11, non-canonical WNTs associated with pulmonary fibrosis. (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, DEseq2 pairwise contrast statistics)
(E) RNA-seq analysis shows broad downregulation of many FZD receptors. (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, DEseq2 pairwise contrast statistics)
(F) GSEA Analysis at D70 comparing DKC1 A386T iAT2 cells reveals an downregulation of genes with TCF7 bindings sites in their promoters.
(G) Ingenuity Pathway Analysis reveals master regulators at D70 including downregulation of Lithium chloride, TCF7, and upregulation of multiple Wnt inhibitors.
(H) GSEA Analysis at D70 comparing DKC1 A386T iAT2 cells reveals a downregulation of genes with miR34A binding sites.
(I) TINF2 and POT1 are downregulated in DKC1 A386T iAT2 cells at D70. (* p < 0.05, ** p < 0.01, *** p < 0.001, DEseq2 pairwise contrast statistics)
components of the Wnt pathway in response to uncapped telomeres (Figure 2-6-H). The genes encoding four of the six shelterins, proteins that bind and help maintain normal telomere function, are direct targets of the canonical Wnt transcriptional effector β-catenin, and two of these, TINF2 and POT1, were downregulated in D70 DC iAT2 cells, which may contribute to telomere uncapping beyond simple telomere shortening (Figure 2-6-I). These data indicate that Wnt signaling in AT2 cells is greatly affected by shortened telomeres and that non-canonical Wnt signaling may be elevated while canonical (β-catenin-dependent) signaling may be diminished.
While there are some descriptions of Wnt target genes that are specific to AT2 cells, given our previous results in the intestine, we sought to understand what genes were controlled by canonical Wnt signaling using a natural experiment embedded in how iAT2 cells are generated. Since the differentiation of iAT2 cells requires CHIR99021, a canonical Wnt signaling...
agonist, and we culture the cells without CHIR99021 after their specification, D28 and D50 provide two timepoints with which to compare the effect of Wnt on iAT2 cells. Clustering analysis of the transcriptomes of D28 and D50 WT iAT2 cells uncovered two groups of genes (Figure 2-7-A) which when used to filter differentially expressed genes between mutant and wildtype iAT2 cells at D70 revealed four new clusters (Figure 2-7-B). GO analysis of a subset that goes up with the removal of CHIR99021 and goes up in mutant D70 iAT2 cells reveals an enrichment for genes involved in IL1 signaling, one of the principal signaling pathways involved in AT2 to AT1 cell conversion. Finally, GSEA analyses at D70 of mutant and wildtype iAT2 cells uncovers many of the other pathways involved in AT1 cell differentiation (Inflammatory response being linked to IL1β and Hypoxia and Glycolysis both being import for generating the transitional cell that yields an AT1 cell all having negative normalized enrichment scores at D70), arguing that telomere dysfunction may affect the ability of AT2 cells to differentiate into AT1 cells (Figure 2-7-D).

Figure 2-8: CHIR99021 rescues growth and telomere defects in DC iAT2 cell alveolospheres (See Page 37)

(A) Differentiation protocol used to test how CHIR99021 affects growth of DC iAT2s.
(B) Representative images of differentiating WT and DKC1 A386T mutant bearing cells with increasing amounts of CHIR99021. (scale bars, 1 mm for all alveolosphere images)
(C) Quantifications of alveolosphere formation efficiency after treatment with differing concentrations of CHIR99021. (n = 4, * p < 0.05, **** p < 0.0001, Student’s t-test)
(D) When D70 alveolospheres are cultured with 3 µM CHIR99021, DKC1 A386T mutant iAT2 cells have a lower fraction of cells with S3BP1 foci. Note, data for no CHIR99021 bars are from Figure 2. (n = 3, * p < 0.05, Student’s t-test; scale bars, 10 µm)
(E) When D70 alveolospheres are cultured with 3 µM CHIR99021, DKC1 A386T mutant iAT2 cells have a lower fraction of p21 positive cells. Note, data for no CHIR99021 bars are from Figure 2. (n = 3, * p < 0.05, Student’s t-test; scale bars, 10 µm)
(F) When D70 alveolospheres are cultured with 3 µM CHIR99021, DKC1 A386T mutant iAT2 cells have a lower fraction of TIF positive cells. Note, data for no CHIR99021 bars are from Figure 2. (n = 3, * p < 0.05, Student’s t-test; scale bars, 10 µm; Insets highlight cells with TIFs, each one noted by the white arrowheads)
(G) Telomeric repeat amplification protocol (TRAP) assay for telomerase activity in iAT2 cells using 5-fold extract dilutions. (n = 2)
CHIR99021 DOWNREGULATES SENESCENCE MARKERS AND HEALS TIFS IN DC iAT2 ALVEOLOSPHERES

To understand how CHIR99021 affects DC iAT2s at D70 we characterized the status of telomeres. CHIR treated iAT2 cells showed fewer cells with 53BP1 foci, fewer p21+ cells and fewer TIF+ cells (Figure 2-8-D-F). Single telomere analysis showed no change in average telomere length and no change in the number of shortest telomeres (Figure 2-9-C-D). iAT2 cell telomerase activity was restored in mutant cells treated with CHIR99021 to levels similar to those seen in WT cells (Figure 2-8-G). Together, these data argue that GSK3 inhibition not only rescues the growth of DC iAT2 cells, but it also rescues telomere defects, most likely through upregulation of telomerase activity that could extend telomeres that are shorter than those that can be detected by TeSLA; it is also possible that telomerase is contributing to telomere capping via lengthening-independent mechanisms (Perera et al., 2019). Of note, withdrawal of CHIR99021 during only the 14 days prior to D70 was sufficient to elicit the growth defect (Figure 2-9-A-B), consistent with an only modest increase in telomere length associated with the increased telomerase activity. Together, these data argue that Wnt agonism not only rescues the growth of DC iAT2 cells, but it also rescues telomere defects, likely through upregulation of telomerase activity.

Figure 2-9: Long-term treatment of CHIR99021 does not prevent the growth defect in DC iAT2 cells upon subsequent withdrawal of CHIR99021 and CHIR99021 lengthens telomeres modestly in DC iAT2 cells but does not decrease shortest telomeres (See Page 39)

(A) Differentiation protocol used to test how pre-treatment culture with CHIR99021 affects growth of DC iAT2s.
(B) Representative images of iAT2 alveolospheres at D70 with and without CHIR99021 shows that pre-treatment does not prevent the defect when CHIR99021 is removed. (scale bars, 1 mm)
(C) Rescue of DC iAT2 alveolosphere growth with CHIR98014. These alveolospheres grew from cells plated at 400 cells/µL. (scale bars, 1 mm)
(D) TeSLA blot of DKC1 A386T iAT2 alveolospheres treated with 3 µM CHIR99021.
(E) Quantification of TeSLA blot of DKC1 A386T iAT2 alveolospheres shows slight increase in average telomere length or in fraction of shortest telomeres. (n = 1, "Shortest 20%" reports the 20th percentile of telomere length, in Kb)
SUPPLEMENTARY TABLES
All of the supplementary tables are available at the following url:
https://www.biorxiv.org/content/10.1101/2020.10.28.358887v1.

TABLE 2-1 DIFFERENTIALLY EXPRESSED PATHWAYS FROM D70 DC IAT2 CELLS THAT ARE SIMILAR TO CHANGES SEEN IN IPF
These tables display selected results from GSEA and IPA analyses that highlight pathways found to be differentially regulated in mutant iAT2 cells at D70 when compared to wildtype cells. The first table displays GSEA results along with the pathway name, normalized enrichment score (NES) and adjusted p-value (D70 p-adj). The second table displays IPA results from the Canonical Pathways analysis. These are gene sets that are differentially regulated in mutant iAT2 cells at D70 when compared to wildtype cells. The p-value reports the significance of enrichment of the molecules in that gene set, and the activation score reports how concordant the gene expression changes are with what is predicted from the literature embedded in IPA (a negative z-score argues that the gene set is down regulated in the mutant iAT2 cells, whereas a positive z-score argues that the gene set is upregulated in mutant iAT2 cells; the lack of a z-score is indicative there was insufficient evidence to provide a z-score.) The "molecules" column lists the genes that were in that gene set that were also found in our differentially expressed gene list when comparing mutant iAT2 cells to wildtype cells.

TABLE 2-2 GSEA RESULTS COMPARING D70 DC TO WT IAT2 CELLS
These tables provide the unedited output of the GSEA analysis using the C2 curated gene sets, H hallmark gene sets, and C3 regulatory target gene sets when comparing D70 mutant to wild type iAT2 cells. The table reports the name of the gene set (ID), the size of the gene set (setSize), the raw enrichment score (enrichmentScore), the normalized enrichment score (NES), along with the p-value, the adjusted p-value (p.adjust) and false discovery rate q-value (q-values). The "coreenrichment" column displays the genes in the gene set.

TABLE 2-3 IPA RESULTS COMPARING D70 DC TO WT IAT2 CELLS
These tables provide the unedited output of the IPA analysis. The summary tab lists metadata associated with the analysis. The "Analysis Ready Molecules" lists the differentially expressed genes that differed in expression >2 fold and were associated with an adjusted p-value < 0.05 from D70 mutant iAT2 cells (DEG list). The “Canonical Pathways tab” lists pathways curated from the literature and the p-value for enrichment using the DEG list used in the IPA.
analysis. The “Upstream Regulators” tab lists the transcription factors, cytokines and other genetic regulators whose target genes are in the DEG list. “Causal Networks” seeks to build a regulatory network based off of the “Upstream Regulators” to identify master regulators of the DEG list. For more information on interpreting ingenuity analysis results see 179.
DISCUSSION
We used isogenic human iPS cell lines to generate DC mutant iAT2 cells with shortened telomeres to interrogate how telomere dysfunction can affect AT2 cell function. We found that shortened and uncapped telomeres are associated with a defect in alveolosphere formation by iAT2 cells. This defect is characterized by senescent iAT2 cells that upregulate many pathways associated with pulmonary fibrosis including the UPR, mitochondrial biogenesis and function, thyroid hormone signaling, and p53 signaling. DC mutant iAT2 cells also suppress canonical Wnt signaling, and consistent with this, GSK3 inhibition rescues telomerase activity, telomere capping, and alveolosphere formation. This system provides a new preclinical model to better understand PF pathogenesis and how potential new PF therapeutics affect AT2 cell function in the context of telomere dysfunction.

Wnt signaling is complex, and can be broken down into two major categories: β-catenin dependent (canonical) signaling and β-catenin independent (non-canonical) signaling. These distinctions can also be blurred as evidenced by studies that show how non-canonical ligands, such as WNT5A, can activate both arms of Wnt signaling. These complexities therefore make the conflicting reports about whether β-catenin dependent Wnt signaling is of benefit or of harm in pulmonary fibrosis unsurprising. Given the heterogeneity of the disease both in space and time and the context dependence of Wnt signaling, these studies can often only capture a snapshot of the fibrotic response. Furthermore, there are clear differences between mice and humans with regard to telomere and lung biology. These limitations highlight the need for human models capable of assessing the spatial, temporal, and cell type specific properties of Wnt signaling in PF pathogenesis.

Our study also provides evidence of upregulation of β-catenin-independent signaling in DC iAT2 cells and that re-activating β-catenin dependent Wnt signaling using GSK3 inhibitors might provide support for AT2 cell proliferation in the context of telomere dysfunction. It is tempting to extrapolate from other models of lung disease to understand how the activity of the β-catenin dependent Wnt pathway might be of benefit in pulmonary fibrosis. β-catenin dependent Wnt signaling improved regeneration and survival in a model of emphysema, and inhibition of
WNT5A, and thus presumably some component of β-catenin independent Wnt signaling, improved repair in a model of COPD. Our work, consistent with previous studies, argues that β-catenin dependent Wnt signaling supports AT2 cell telomere capping and proliferation, which may be of benefit during regeneration and repair.

Our previous work uncovered a positive feedback loop between Wnt signaling and telomeres in the intestine. Here we show that aspects of the Wnt-telomere feedback loop appear to be at play in AT2 cells, arguing that this connection between Wnt and telomeres is present not just in proliferative tissues such as the intestine, but also in lung cells, cells from a tissue that are normally quiescent but proliferate in response to injury. Previous work highlighted the importance of telomerase during alveolar regeneration. Furthermore, given previous demonstrations that TERT expression and telomerase activity can be stimulated by Wnt, we expected an upregulation of telomerase activity in iAT2 cells when treated with GSK3 inhibitors.

This Wnt-telomere feedback loop might not be the only regulatory loop at play in AT2 cells. Given our evidence of upregulation of mitochondrial processes in DC iAT2 cells and how telomere dysfunction can drive mitochondrial dysfunction and how mitochondrial dysfunction can drive telomere dysfunction, we hypothesize that telomeres might be an integrator of multiple cellular stress responses. Furthermore, our finding of the upregulation of genes associated with the UPR argues that telomere dysfunction could drive the UPR. Many reports have described connections between senescence, a usual consequence of telomere dysfunction, and the UPR, and we hypothesize that the UPR might in turn drive telomere dysfunction. These multiple integrated loops might help explain how these various vital cellular processes combine to cause dysfunction in AT2 cells in PF.

Recent work, using mouse AT2 cell organoids, has elucidated a developmental trajectory by which AT2 cells can differentiate via an intermediate state into type I alveolar epithelial (AT1) cells in response to bleomycin injury. Of note, the intermediate cells are characterized by high levels of p53 signaling and DNA damage which resolves with their final transition to an AT1 cell fate, arguing that repairing DNA damage, potentially at telomeres, is an important step in
the transition to an AT1 cell. Furthermore, many of the pathways that promote the differentiation of AT2 cells into AT1 cells are downregulated in DC iAT2 cells, including those involving IL1β, glycolysis, and HIF1α. We speculate that DC AT2 cells with short uncapped telomeres may have trouble suppressing DNA damage at telomeres and therefore in differentiating into AT1 cells during regeneration, perhaps thus contributing to fibrosis. Testing this idea in the human iPSC-derived alveolosphere model will require technical advances to enable the generation of AT1 cells. Regardless, our DC iAT2 cell model recapitulates many hallmarks of PF AT2 cells and offers a new system to probe the underlying biology of PF.
CHAPTER 3 : Preliminary success in transplanting iAT2s into mice and generation of new DC iPS cell lines

Portions of this section are adapted from the article “Orthotopic Transplantation and Engraftment of Human Induced Pluripotent Stem Cell-Derived Alveolar Progenitor Cells into Murine Lungs” posted on bioRxiv (2020).
INTRODUCTION

The generation of the DC iAT2 cell lines allow for modeling of telomere dysfunction in human AT2 cells. However, limitations remain with extrapolating these findings to the pathophysiology of PF. Chief among these is the lack of a true fibrotic response in vitro. This is expected given that these cultures are a pure population of AT2 cells and lack the other cell types that may play a role in PF, specifically fibroblasts. Given the significant evidence that argues that damaged AT2 cells can drive a fibrotic response, there exists a significant need to develop a system to understand how short telomeres in AT2 cells signal and contribute to the development of fibrosis.

One satisfying possibility to explore this would be to rebuild the lung from iPS derived human cells, co-culturing these iAT2 cells with iPS derived counterparts of fibroblasts, endothelial cells, and immune cells. While the current technologies allow for the derivation of fibroblast, endothelial cells, and some immune cells, co-culture experiments would be difficult given the complex medias and growth factors needed to support these individual cell types. Furthermore, these experiments would come with the caveat that directed differentiations usually promote expansion of these cell types, something that is more akin to development or regeneration, not the homeostatic state of a normal tissue. This may limit the generalizability of these models.

Another approach that may bear more fruit would be to transplant human iPS derived AT2 cells into damaged mouse lungs. This would provide a way to look at how dysfunctional human AT2 cells affect a regenerating lung, providing a model system with which to test which pathways dysfunctional AT2 lead to PF. Since these mice would necessarily need to be immunocompromised to prevent rejection of the human cells, they would allow for studying the interactions of AT2 cells with fibroblasts, endothelial cells, and other epithelial cell types to unlock which signals human AT2 cells send to their environment to generate a fibrotic response. It also may provide a supportive environment to coax these iAT2 cells into AT1 like cells, a process that to date has been difficult to reliably model in vitro.

These new models help understand the subsequent effects short telomeres have on the surrounding cell types of the lung and may help uncover how short telomeres contribute to fibrotic
response through sick AT2 cells. But while understanding the downstream consequences of
dysfunctional AT2 cells is important, finding ways to prevent or ameliorate the dysfunction in AT2
cells may provide a way to prevent PF from ever emerging.

This work has focused on how Wnt pathway agonists may be used to ameliorate the
underlying telomere dysfunction and likely the underlying dysfunction of the AT2 cells. Other
groups focused on DC caused by PARN mutations and showed that the deficiency of TERC, the
RNA component of telomerase, leads to telomere shortening in these cells\textsuperscript{204--206}. When TERC is
initially transcribed, a poly(A)polymerase, PAPD5, adds an A-tail to TERC and other non-coding
RNAs. This tail needs to be trimmed by PARN before the TERC can complex with TERT and
form an active telomerase complex. Inhibitors of PAPD5 have been shown to restore telomerase
activity in PARN mutant iPS cells as well as in cells with DKC1 mutations\textsuperscript{207}. This raises the
possibility of using a combination of therapies to help ameliorate DC pathologies across a wide
variety of mutations. To test these therapies, more tractable models of these different DC
mutations are needed.

One of the most severe manifestations of DC like telomere disorders is HH, and a
common mutations found in these patients are in the gene TINF2. TINF2 mutations were first
uncovered in DC patients\textsuperscript{208} and eventually also discovered in families with a predisposition for
pulmonary fibrosis\textsuperscript{106,209}. Cellular studies have argued that one way these mutations cause DC
defects if by affecting the ability of telomerase to associate with telomeres\textsuperscript{210,211} or may affect the
processivity of telomerase\textsuperscript{212}.

To address these issues, this chapter provides preliminary evidence that human iAT2
cells can be transplanted into immunocompromised mice and describes the establishment of a
new iPSC line with a well characterized TINF2 mutation.
METHODS

MICE
8- to 10-week-old mice were used for all experiments with males and females in roughly equal proportions. Experimenters were not blinded to mouse age or sexes. NOD.Cg-Prkdcsid Il2rgtm1Wjl/SzJ (referred to as NSG) mice were utilized as recipients for all transplantation experiments. All animal experiments were carried out under the guidelines set by the University of Pennsylvania’s Institutional Animal Care and Use Committees and followed all NIH Office of Laboratory Animal Welfare regulations.

BLEOMYCIN INJURY MODEL AND CELL TRANSPLANTATION
Mice were first anesthetized using 3.5% isoflurane in 100% O2 via an anesthesia vaporizer system. Mice were intranasally administered 4mg/kg body weight bleomycin sulfate (13877-10, Cayman Chemicals) in a total volume of 30 μL PBS. Only injured mice that lost ≥10% of their starting body weight by day 4 post-injury and survived to the time of transplant were considered to be adequately injured and used for all experiments involving bleomycin injury. Cells were transplanted at day 10 post-bleomycin administration.

IPS CELL LINE MAINTENANCE
iPS cells used for differentiation were maintained on growth factor reduced Matrigel (Corning) coated plates in StemMACS™ iPS-Brew XF medium (Miltenyi Biotec). Cells were cultured in clusters and passaged every 4-5 days using StemMACS Dissociation reagent (Miltenyi Biotec). All iPS lines were genotyped using an RFLP at the relevant important loci and the sequence was confirmed by Sanger sequencing. All cells were routinely screened for mycoplasma contamination using a PCR based assay.

DIRECTED DIFFERENTIATION INTO NKX2.1+ LUNG PROGENITORS AND SFTPC+ IPS-DERIVED AT2 CELLS
NKX2.1+ sorted cells were replated at a density of 400,000 cells/mL in 90% Matrigel supplemented with 10% of CK+DCI+TZV media (cSFDM containing 3 μM CHIR99021, 10 ng/mL KGF, 100 nM dexamethasone, 100μM 8Br-cAMP and 100 μM IBMX and 2 μM TZV) (from now on referred to as 90/10 Matrigel). The Matrigel droplets were allowed to cure at 37 °C for 20-30 minutes and then overlaid with an appropriate amount of CK+DCI+TZV Media. These alveolosphere containing matrigel droplets were incubated at 37°C room air incubated.
supplemented to 5% CO₂ for 14 days changing with fresh media every other day. On Day 28, the iAT2 containing alveolospheres were sorted on a FACSJazz sorter for SFTPC+ cells using the endogenous SFTPC::TdTomato reporter. These sorted SFTPC+ cells were replated at a concentration of 65,000 cells / mL in 90/10 Matrigel drops and grown in K+DCI+TZV at 37˚C in an ambient air incubator supplemented to 5% CO₂ for 3 weeks changing media every other day.

**CRISPR EDITING FOR GENERATING PAIRED TINF2 R282H MUTANT CELL LINES IN BU3 NGST**

To generate introduced BU3 NGST lines harboring TINF2 mutation, we used the CRISPR track on the UCSC genome browser to select candidate guideRNAs (gRNAs) that targeted as close to each individual mutation as possible, had easily mutable PAM sites and when silently mutating certain bases would introduce a new restriction site to make screening easier (see Figure 3.2. for details). gRNAs were ordered as modified RNAs from Synthego. iPS cells were nucleofected using the Amaxa Nucleofection system using the following program (P3, CA-137). They were allowed to recover for 36-48 hours at high density in the presence of rock inhibitor. They were plated at low density (2500 cells/10 cm plate) and allowed to form single colonies. After seven to ten days, individual clones were handpicked and transferred to 96 well plates and screened for introduction of the restriction site for each mutation. Restriction enzyme positive clones were expanded and then subjected to sanger sequencing for identification of correctly edited clones. Successfully edited clones were checked for mycoplasma contamination, and passaged for at least 5 passages and then re-genotyped to ensure that the clones were not mixed clones.
RESULTS

IAT2 AND PROGENITOR CELLS SUCCESSFULLY ENGRAFT IN NSG MICE AND MAY DIFFERENTIATE INTO AT1 CELLS

To prepare cells for engraftment, WT late passage iPS cells were differentiated into iAT2 as described above. NSG mice were given bleomycin, and ten days after bleomycin injury, the mice received 600,000 to 2 million NKX2.1+ progenitor cells or SFTPC+ iAT2 cells. Transplants engrafted in all recipients that were examined. Human iAT2 cells maintained expression of SFTPC and were easily identifiable both by the NKX2.1::GFP transgenic reporter and the human mitochondrial antibody stain. Furthermore, some of these transplanted cells stretched out, taking on an AT1-like cell morphology, suggesting that these cells may differentiate into AT1 cells.

Figure 3-1: iAT2 cells engraft in mouse lungs and can differentiate into cells with an AT1 like morphology

(A) Differentiation protocol used to test generate iAT2 cells, a representative flow plot and when they were transplanted into bleomycin injured NSG mice.

(B) Representative images of iAT2 cell engraftment into a mouse lung. (scale bars, 25 μm for all images)

(C) Representative images of engrafted iAT2 cells. Dotted white encirclement highlights a human cell with an AT1 like morphology. (scale bars, 25 μm)
These preliminary results offer opportunities to further investigate how AT2 cells, and possibly their progeny, can affect an intact lung architecture.

**GENERATION OF A NEW DC TINF2 R282H IPS CELL LINE**

Our investigation of DC iAT2s has been limited to the *DKC1* A386T mutation. An open question remains whether using Wnt agonism to treat the defects of DC stem cells will be of use in cells with other mutations. To further model other DC mutations, we introduced the *TINF2* R282H mutation into the BU3 NGST line. Given its autosomal dominant inheritance pattern, this heterozygous mutation has been shown to be associated with HH, a severe form of the DC syndrome.

**Figure 3-2: Introduction of the TINF2 R282H mutation into the BU3 NGST IPS Cell Line (see Page 51)**

(A) CRISPR based editing strategy for introducing the *TINF2* R282H mutation into the BU3 *NKKX2.1::GFP, SFTPC::TdTomato* IPS cell line.

(B) Sanger sequence verification of the introduction of the mutation yielding. Note the WT sequence is marked in red. The mismatched C is a synonymous mutation that impairs binding of the CRISPR gRNA back to the same sequence.

(C) TeSLA of *TINF2* R282H IPS cells shows telomeres shorten with increasing allele frequency.
spectrum of disorders\textsuperscript{208}. Sanger sequencing confirmed the establishment of a WT control line and heterozygous and homozygous clone (See Figure 3-2-B). By measuring the shortest telomeres 5 passages after the introduction of the mutation, homozygous clones have a greater preponderance of short telomeres compared to their heterozygous or WT counterparts (Figure 3-2-C). This line provides a new genetic background to test how telomere dysfunction affects different stem cells.
DISCUSSION

This chapter describes preliminary results that help extend our capacity to model DC pathologies using iAT2 cells. Transplanting iAT2 cells reveals the capacity of AT2 cells to both engraft, maintain their specification, and possibly differentiate into AT1 like progeny. By using similar transplantations studies, we can further investigate how dysfunctional AT2 cells drive pulmonary fibrosis and may offer an in vivo model to test novel therapeutics.

More specifically, this new method would help uncover insights into how telomere dysfunctional AT2 cells can alter the regeneration response to bleomycin injury by comparing the histological consequence of engraftment of AT2 cells with and without such dysfunction, as well as areas that were damaged in the same lung but did not engraft any transplanted AT2 cells. This histological comparison during the regeneration process after bleomycin injury would provide experimental evidence that an AT2 cell with telomere dysfunction could drive PF by altering the regeneration response.

Furthermore, given this preliminary evidence of differentiation of the human iAT2 cells transforming into AT1-like shaped cells, this new model would help understand more carefully the process of differentiation of AT2 cells into AT1 cells. Multiple groups have started to uncover the pathways involved in mouse AT2 to AT1 cell differentiation, and thus this humanized mouse model might allow for the first time the study of how human AT2 cells differentiate into AT1 cells in a more realistic environment than has previously been possible. Studying how dysfunctional telomeres affect this differentiation process may help further elucidate how the transitioning AT2/AT1 cells fit in the pathogenesis of PF.

Another advantage of this new model is that it may provide a permissive niche to further mature the human iAT2 cells. Many iPS derived cell types suffer from having transcriptional programs more similar to developmental progenitors than to adult cell types. By allowing the iAT2s to grow in a more physiologic environment, this model could help elucidate how short-telomeres affect AT2 cells at homeostasis should they be allowed to engraft for sometime and allow for the regeneration post bleomycin to complete. This new model would provide a way to understand the mouse and human differences that are specific to human AT2 cells in a relatively
physiologic tissue microenvironment, with the caveat that these recipient mice are severely immunocompromised.

This chapter also describes the establishment of a new DC iPS line with the TINF2 R282H mutation. While all described patients with this mutation are heterozygotes because of the autosomal dominant inheritance pattern seen in many of the families with these mutations, I describe here an allelic series where we have iPS cells with homozygosity of the TINF2 R282H mutation. While this does not model a genotype found in patients, it provides a tool that may manifest telomeric dysfunction at an earlier stage, which may be of benefit to future investigators using this line. It could also be useful for understanding the sub-cellular mechanism by which this TINF2 mutation causes telomere shortening. Additionally, this new line allows us to test whether Wnt agonism might be more broadly applied to patients with other DC mutations. We expect Wnt agonism will be of benefit in these cells by driving shelterin expression improving capping. But as has been shown earlier, some of the benefit may be mediated by increasing telomerase activity. While it remains unclear exactly how this mutation leads to telomere shortening, this new model may provide a way to test which of these arms of the Wnt-telomere feedback loop is most important for patients with this specific mutation. To summarize, this work also establishes a novel allelic series to understand the mechanism by which the R282H mutation shortens telomeres.
CHAPTER 4 : SUMMARY AND FUTURE DIRECTIONS
SUMMARY
This thesis established a novel approach for testing how telomere dysfunction induced by a causative human DC mutation affects iPSC derived human AT2 cells. Using isogenic pairs of human iPSC lines with or without a DC mutation followed by directed differentiation into AT2-containing alveolospheres, we found that telomere shortening and uncapping led to senescence of these cells. The senescent cells display changes in gene expression that connect with several known aspects of PF pathology. Furthermore, the gene expression changes indicated suppression of canonical Wnt pathway activity, suggesting that the normal support of AT2 cells by Wnt/β-catenin activity is perturbed. Supporting this, we find that pharmacological Wnt stimulation via GSK3 inhibition can rescue the telomere defects and senescent phenotypes. Therefore, this novel approach adds to recent evidence that Wnt pathway agonists should be further investigated as potential therapeutics for telomere-driven pathologies, and provides a new system for exploring mechanisms underlying pathologies driven by telomere dysfunction in AT2 cells. Furthermore, this work highlights preliminary evidence for successful transplantation of these iAT2 cells into mouse lungs and the establishment of a novel DC iPS line that will aid in future studies of the involvement of senescent AT2 cells in PF.

FUTURE DIRECTIONS
LIMITATIONS OF THE MODEL
Models are never perfect representations of the pathologies they seek to represent, and this model is no different. There are many caveats that color the conclusions from this model. First, these iAT2s are most similar in their expression patterns to 2nd trimester lung, meaning that the gene expression profile of these iAT2s is not a perfect replica of the adult cells this system aims to model. This is a persistent issue for iPSC-derived models of disease and highlights the need to develop more accurate culture conditions that might provide for the maturation of such model cells into states that more fully reflect those found in adult tissues. Furthermore, this model only utilizes AT2 cells, in a basement membrane substrate that is different from that experienced by human AT2 cells. Further work developing new extra-cellular matrix attachments that more closely mimic the membranes from lung may provide a way to further mature these iAT2s and uncover new insights into how telomere dysfunction affects AT2 cells.
DEVELOPMENT OF NEW MODELS TO STUDY TELOMERE DYSFUNCTION IN THE LUNG

While this thesis describes a variety of new models to study how AT2 cells affect PF, the requirement of using passaged iPS with short telomeres to generate senescent iAT2s is especially burdensome. This requires significant time to passage the pathologic mutation at the iPS stage and could conceivably cause issues with the differentiation of the iAT2s themselves. And furthermore, it is difficult to predict exactly at what passage sufficient telomeres will uncap to generate the senescent phenotype.

To avoid this, new iPS models that employ an inducible transgene that causes telomeric damage could be constructed. There are multiple different transgenes that could accomplish this. The first is the dominant negative TERF2 allele described originally by the de Lange lab. This truncated TERF2 protein causes telomeric fusions in MEFs, but a human version expressed for a limited amount of time could conceivably induce telomeric damage without fusions. This could create a system that causes graded amounts of telomere dysfunction allowing the study of both dysfunctional telomeres as well as situations where the telomeres fuse, as in cancer cells. Another possible transgene is the TRF1-FOK1 endonuclease fusion developed by the Greenberg lab. Using this targeted endonuclease would cause significant telomere shortening and uncapping that may reliably induce senescence in iAT2s. Finally, another option would be to use CRISPR to target the telomeric repeat, accomplishing something similar to the TRF1-FOK1 construct. These new transgenic models would simplify the process of making senescent iAT2s and allow for quicker experiments to study how telomere dysfunction affects AT2 cell biology.

TELOMERES AS AN INTEGRATOR OF MULTIPLE STRESS RESPONSES

Given that we have found many pathways that have previously been associated with PF are enriched in the transcriptomes of senescent iAT2s, we hypothesized above that telomeres might be a central node linking some of these pathways. Given the previous connections our lab uncovered between Wnt and telomeres, we have provided literature that supports connections between telomeres and many of these other pathways. This may help explain how dysfunction of any specific pathway, e.g. mitochondrial dysfunction or UPR stress, may lead to telomere dysfunction that causes AT2 senescence, thereby losing their vital role in proliferating repairing damaged tissues.
To explore this idea further, TIF assays could be used to ascertain the capping state of telomeres in the lungs of mice with ER stress or mitochondrial stress that have generated PF like phenotypes\textsuperscript{111,120,122,184}. Even though there are significant mouse and human differences in telomere biology, these studies would provide early evidence that telomeres may sit at the center of these multiple pathways that are associated with PF. Another experiment would be to overexpress shelterins, like TERF2, in these damaged AT2 cells to see if it could rescue the damage caused by the ER or mitochondrial stresses. Further characterization of how these pathways are interconnected at the telomere might offer other ideas for therapeutics to ameliorate the stress these AT2 cells experience and thereby reverse or prevent PF.

**UNDERSTANDING EXTRACELLULAR SIGNALING BY DYSFUNCTIONAL AT2 CELLS**

Understanding how AT2 cells signal to their surrounding environment to drive PF remains incompletely explored. Anjali Jacob, one of the original authors of the differentiation protocol from the Kotton lab, observed that the fraction of cells that express the SFTPC::tdTomato reporter varies depending on the density of the organoids in Matrigel\textsuperscript{215}. This observation implies that the density at which the iAT2 cells are cultured may influence their gene expression and behavior. iAT2s are cultured in manner more akin to how AT2 cells proliferate in close proximity during lung regeneration\textsuperscript{147,216} than how AT2 cells are found at homeostasis, dotting the lung. Therefore, telomere dysfunction in homeostatic AT2s may behave differently than those from our model. Furthermore, this raises the possibility that there may be a secreted factor that builds up in a dense culture of AT2 cells that may change their behavior in a significant way. This iAT2 model system could be used to uncover this unknown factor and it may play a role in PF pathogenesis given the hyperproliferation of AT2 islands that are a characteristic of the fibrotic lung.

While many models have delineated the AT2 cell’s role in initiating fibrosis, these are manipulated model systems, and do not recapitulate the insidious nature by which PF presents in humans. Since telomeres are causally linked to PF, this raises the questions as to whether the initiation of PF pathology requires a primary insult (such as an infection or inhalation of a damaging agent) that initiates a regenerative response that is derailed by telomere dysfunction or rather is the initiated by a senescent AT2 cells experiencing only telomere dysfunction. Studying
this question in the human context would be difficult, but currently available mouse models could be used to probe this question by turning on the senescent state either at homeostasis (as has been done in most of the papers describing these models) or at specific times post injury during repair and regeneration of the lung.

This thesis has also helped develop new models to study how senescent iAT2 cells signal to their surrounding environment to drive PF. One area of investigation is how the senescence associated secretory phenotype (SASP), which has been described in cell lines, might play a role in driving the underlying fibrotic response. There is evidence from other cell models, but careful characterization of the SASP of each cell type is important as there can be significant differences depending on the method of senescence induction and the specific cell type involved. Given how AT2 cells are normally secretory cells, using mass-spectrometry techniques or antibody arrays to probe the conditioned media of senescent iAT2s would be an important next step to understand how the SASP of non-cancerous AT2 cells may contribute to PF. Furthermore, using CRISPR knockouts of specific genes in the pathways identified by these unbiased secretome studies, coupled with transplantation into NSG mice could provide a tractable system to test the importance of these secreted signaling for generating a PF response.

Another avenue ripe for study using this model would be to use the conditioned media to understand the changes in gene expression in other primary cells such as human macrophages, lung endothelial/vascular cells and lung fibroblasts. Furthermore, these iPS lines would offer insights to test how different DC mutations cause changes in the behavior and gene expression of different cell types and allow for mixing and co-culture of these different cell types. Even though these cells would be immature and likely replicate fetal tissues, these types of cultures may provide a reductionist model to test ideas of how uncapped telomeres drive a fibrotic response in the lung.

UNRAVELLING B-CATENIN-INDEPENDENT SIGNALING IN AT2 CELLS

While this thesis has focused on using the canonical Wnt-β-catenin signaling pathway to promote repair and survival of AT2 cells, this model has also shown the importance of the non-canonical β-catenin independent signaling pathway. The upregulation of β-catenin-independent
signaling in DC iAT2 cells has been similarly seen in epithelia of patients with IPF. 

Often called the Planar Cell Polarity pathway, these proteins (e.g. VANGL, CELSR, FAT and DCHS1) interact with a wide variety of different pathways and help to integrate signals that control proliferation, organ/tissue size, migration and interconnection. It is tempting to speculate that telomere dysfunction might alter the capacity of AT2 cells to sense and integrate these signals and this may be a way that telomere dysfunction mediates an abnormal tissue response that leads to the fibrotic phenotype.

**USING MOUSE MODELS TO UNRAVEL WHEN TELOMERE DYSFUNCTION CAUSES FAILURE OF REPAIR**

While short telomeres are causally linked to fibrosis, one outstanding question remains as to whether the fibrotic response is due to homeostatic telomere dysfunction, or rather, telomere dysfunction that occurs in the setting of a response to an injury that derails regeneration towards a fibrotic response. One way to understand this would be to induce telomere dysfunction in a mouse lung at homeostasis and compare it with the response of a mouse lung that experiences telomere dysfunction during regeneration post injury.

Another way to approach this question might be to use the various inducible models of PF in the mouse in a new way. Since bleomycin injury is a well characterized injury model and the regenerative window is well known, one can imagine giving these mice bleomycin to damage their lungs and inducing the various fibrotic drivers during this regenerative window. Checking for dysfunctional telomeres may help unravel exactly when telomere function is important for normal regeneration of the lung and may give an early window into what happens in PF patients.

**UNDERSTANDING HOW DYSFUNCTIONAL TELOMERES AFFECT THE AT2-AT1 TRANSITIONAL CELL AND HOW THIS CORRELATES TO PF**

Three recent studies helped elucidate a developmental trajectory for AT2 cells to become AT1 cells involving inflammatory signaling, p53 signaling, hypoxia and glycolytic metabolism. One of these studies showed an abundance of transitional cell markers in PF lungs and hypothesized that the accumulation of these transitional cells, which have large amounts of DNA damage, may contribute to the pathology of PF. While it remains to be seen that the DNA damage seen in these transitional cells localizes to telomeres, it is tempting to imagine how short
telomere models of PF could be used to better understand the AT2 to AT1 transition and possibly how this may correlate with PF.

One way to probe this would be to first look at PF lungs, and see if TIFs are found in the transitional cells. Another extension of this line of work would be to ask how the DNA damage seen in these transition cells disappears by the time the cells become AT1 cells. If the damage is throughout the genome, then it is likely repaired by the underlying DNA damage machinery. However, if the damage seen in these transitional cells is localized to telomeres, then this may imply that telomerase may be necessary for the repair and appropriate differentiation of AT1 cells. A way to test this would be use the bleomycin model in G1 telomerase knockout mice and count the number of AT1 cells post repair, along with other markers of intermediate cells at different stages of repair. If the DNA damage occurring during the transition to an AT1 cell arrests the cells in a senescent like state that drives a fibrotic response, these experiments could begin to uncover an important mechanism of how short telomeres cause PF.

An underappreciated aspect of AT2 cell biology is that they experience geometrical stretch forces. As AT2 cell differentiate into AT1 cells, they undergo a process of cellular transformation that stretches the once cuboidal cell into thin paper-like cells. These forces are very likely transmitted to the nucleus. Telomeres are often associated with the nuclear periphery and stretching forces can cause telomere attrition. This raises the possibility that telomere dysfunction is part of the normal differentiation of AT1 cells from AT2 cell in the adult lung. Looking carefully as to whether stretch during differentiation causes telomere dysfunction may add to our understanding if this fundamental regenerative process and offer a possible explanation for how it becomes deranged in PF.

Finally, our work has been limited to just AT2 cells, but they are not the only cells implicated in PF pathogenesis. Future studies could reconstruct the alveolus on a chip, similar to those described by Huh and colleagues. Given the multitude of iPS directed differentiation protocols, re-engineering the alveolus could provide novel insights and a reductionist model to understand the cell-cell interactions that are important for PF pathogenesis.
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