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14. ABSTRACT

Dyskeratosis congenita is a disorder that arises due to prematurely shortened telomeres and characterized by a classical clinical triad of leukoplakia, skin dyspigmentation and nail dystrophy with concomitant marrow failure. DC symptomology, to a degree, corresponds to critically shortened telomeres that limits cellular replicative potential and thus prematurely exhausts stem cell pools. Our previous findings support a hypothesis whereby shortened telomeres increase DNA damage responses within the cell leading to heightened reactive oxygen species (ROS). Recent work supported under this research grant have uncovered a suppressed steady-state RNA expression and protein levels of the NRF2 and NRF3 pathways within DC skin fibroblasts. This suppression is also found in cells exposed to an environment with experimentally-elevated oxidative stress. Furthermore, preliminary RNA-Seq experiments suggest that senescence associated secretory pathway (SASP) is altered in DC cells and suppressed upon mobilization of telomerase. Finally, decreased oxidative stress levels could be found in DC cells upon forced expression of an NRF2 heterodimerizing protein, MAFG, suggesting that suppression of this pathway yields to an unmitigated increase in ROS. Together, these findings provide support for a mechanism whereby shortened telomeres mobilize a DNA damage response that in turn suppresses antioxidant proteins leading to an increase in oxidative stress.

15. SUBJECT TERMS

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Introduction

Most cells rely on telomere attrition to undergo a limited number of cell divisions as a means to circumvent uncontrolled cell growth. Telomere attrition is one well-characterized means by which replication potential is limited, as illustrated by the dramatic phenotype presented by patients with Dyskeratosis Congenita (DC). DC arises due to mutations found in telomere/telomerase related genes. The classical clinical triad of DC is leukoplakia, skin dyspigmentation and nail dystrophy with concomitant bone marrow failure (BMF). DC symptomology, to a degree, corresponds to critically shortened telomeres that limits cellular replicative potential and thus prematurely exhausts stem cell pools. We have previously characterized DC cells and demonstrated inherent defects in multiple cell types and have uncovered evidence that elevated reactive oxygen species (ROS), due to shortened telomeres, may be causative in limiting cellular potential. Other labs have indicated that hematopoietic stem cells (HSC) are acutely sensitive to oxidative stress, which could be a contributing factor to BMF in DC. This increase in ROS appears to be related to a signal transduction pathway initiated by shortened telomeres and transduced by the DNA damage response (DDR) via p53. **Together, this evidence suggests that ROS may be causative towards establishing a senescent state in a short-telomere background.** Little is known about relevant factors lying downstream of p53 that ultimately lead to telomere-dependent elevated ROS. Preliminary evidence generated by our lab has found expression of the antioxidant-related genes *NRF2* and *TXN* are repressed in DC fibroblasts and rescued upon exogenous expression of *TERT*. DC fibroblasts expressing *NRF2* and *NRF3* also acquire a phenotype similar to normal cells while NRF2 shRNA expressing controls appear senescent. Furthermore, individual overexpression of SOD genes indicated *EcSOD* expression significantly decreased ROS. Data gathered as part of this research grant continues to support the outlined hypothesis. This includes further verification of NRF2 and NRF3 suppression at the RNA and protein levels. Potential deregulation of NRF2 heterodimerizing partners like MAFG could also be implicated by data gathered here. Finally, evidence of an altered senescence-associated secretory phenotype has been found in DC fibroblasts. Together, our accumulating data continue to provide evidence how telomere attrition facilitates entry into senescence.

Keywords (limit to 20 words)
Dyskeratosis congenita, bone marrow failure, aging, telomere, oxidative stress, reactive oxygen species, p53, stem cells

Accomplishments

Accomplishments: Major Goals

Our timeline suggested the following approach to this study:

Year 1 – Make iPS cells, evaluate antioxidants, assess pathways
Year 2 – Study HSCs and modulation of ROS
Year 3 – Fully evaluate antioxidants on multiple cell types/genotypes

Although our research funding began in October of 2015, the vast majority of goals proposed for Year 1 have been accomplished during this nine-month period. The following Table was submitted as part of our Statement of Work and has been modified slightly to highlight and update the work completed within each Aim.

Specific Aim 1: Mechanism of oxidative stress	**Proposed Timeline**	**% Completion**
Major Task 1 Overexpress/knockdown genes of interest	Months	
Vector construction	3-6	Finished
Cell infections	6-12	Nearly Finished
Milestone(s) Achieved: Assessment of effect	2	In progress
Local IRB/IACUC Approval	3	Finished
Milestone Achieved: HRPO/ACURO Approval	6	Finished
Major Task 2 CRISPR knockout/RNAseq		
Viral infection/prep	3-6	CRISPR KO virus library prep: finished; RNA-Seq: ~20%
Cell manipulation	3-6	CRISPR KO virus infection: 0%; Single cDNA infections: nearly finished
Bioinformatics	1	CRISPR KO library: 0%; RNA-Seq: ~20%
Milestone(s) Achieved: Assessment of effect	4	N/A
Specific Aim 2: Anti-oxidant screening	**Timeline**	**% Completion**
Major Task 1 library/drug acquisition		
Modify/optimize treatment conditions	1	5% (began candidate drug screen; have not begun large library screen)
DHE analyses in fibroblasts first, then other cells	5	5% (began candidate drug screen; have not begun large library screen)
Milestone(s) Achieved: Create pool of molecules that decrease DHE and increase cell proliferation	9	N/A
Specific Aim 3: Production of HSC from iPS cells	**Timeline**	**% Completion**
Major Task 1 Create iPSC	Months	
Production of iPSC clones	1-3	Finished*

Clone characterization	1-3	Finished*
Milestone(s) Achieved Acquisition of stable pluripotent cells	3-6	Finished*
Major Task 2 Create HSC		
HSC characterization/differentiation	3-6	0%
Milestone(s) Achieved: Achieve true HSC	12-18	0%
Major Task 3 Evaluate ROS in HSC		
Subset analysis of HSC	3-6	0%
Examine pathways	3-6	0%
Milestone(s) Achieved: Determine factors effecting ROS in HSC	9	0%

Accomplishments: Major activities

The first nine months of our research focused primarily on the initial stages of Aim 1 and Aim 3. **Aim 1, Major Task 1**: all cDNA have been cloned into a lentiviral backbone for the purpose of overexpression within DC and control fibroblasts. This will permit the assessment of whether restoration of antioxidant gene expression, found suppressed in DC cells, can ameliorate deficits found in within these cells. These cDNAs include the NRF2 family members (NRF1, NRF2, NRF3), NRF2 heterodimerizing proteins (MAFF, MAFG, MAFK, ATF4, cJun) and related metabolic genes of interest (Pgc1α and Pgc1β). We have also acquired a number of shRNAs from OpenBiosystems that will be used throughout this study that include those that target p53, p21 (CDKN1A), NRF2, the NRF2 ubiquitination complex (KEAP1) and the NRF2 transcriptional competitor (BACH1). Given the extent of this pathway we may need to clone more cDNA or acquire further shRNAs to better characterize and/or verify certain findings. Cell infections and antibiotic selections have been performed on 90% of these cDNAs/shRNAs and cells frozen on each of these conditions. Preliminary oxidative studies have been performed in a subset of these infections (data presented below). **Aim 1, Major Task 2**: RNA-Seq has been performed on a number of cell conditions, including skin fibroblasts and skin fibroblasts overexpressing TERT (Control, Control-TERT, DC, DC-TERT) in addition to control lymphocytes and DC lymphocytes. Bioinformatics have also been performed (DESeq package, DE analysis) on these RNA-Seq experiments to elucidate key differences among these subsets of cells. To bolster the RNA-Seq gene discovery approach we also proposed using chromatin immunoprecipitation (ChIP). At this time, no ChIP experiments have been performed. Major Task 2 also proposed the preparation of a CRISPR knockout (KO) viral library as an additional gene discovery approach. This library relies on CRISPR targeting to each gene within the human genome and the byproduct of CRISPR targeting, non-homologous end joining (NHEJ), to create a frameshift knockout. This library has been acquired, cloned in bacteria to maintain the library diversity, transfected into 293T cells for viral preparation and titered to ensure accurate multiplicity of infection (MOI) when ready for infection. Cells will be infected and selected in the near future.

Specific Aim 2 was designed to test candidate drugs and a library of FDA approved drugs for their ability to decrease ROS within DC cells an improve DC cellular functions (i.e. proliferation, decreased senescence). At this time, only a handful of candidate drugs have been

tested for decreases in ROS (DHE staining). Initial experiments testing of all candidate drugs and the use of the FDA approved drug library will start within Year 2.

Specific Aim 3 was designed to provide access to hematopoietic progenitor cells (HPCs) from DC cells which are difficult to acquire from patients due to their propensity for bone marrow failure and anemia. At this time induced pluripotent stem cells (iPSCs) have been manufactured from DC patients' cells that harbor mutations in the TERT and DKC1 loci. Greater than 10 clones have been produced from each mutation and have been characterized for pluripotency and frozen for further analyses at a later date. At this time, attempts to generate iPSCs from our patient's cells with a TINF2 mutation have been unsuccessful after two attempts (highlighted in the above Table by asterisks) however further attempts will be performed in the near future. We have proposed within Specific Aim 3 to differentiate these iPSCs (and controls) into hematopoietic progenitor cells for further analyses. Initial experiments to derive and characterize DC HPCs will take place within Year 2.

Accomplishments: Specific Objectives

Within the first year our objective was to finish as much of Aim 1 as possible (create all lentiviral vectors and subsequent infections, perform gene discovery experiments [RNA-Seq, CRISPR KO library, ChIP] and initiate experiments within Aim 2 and Aim 3. Since our research funding began in October of 2015, we are on track to accomplish the vast majority of Aim 1 within a full calendar year and are on schedule to pursue the major portions of Aim 2 and 3 with early experiments having been completed.

Accomplishments: Significant results or key outcomes

To date, we have accumulated further evidence supporting the modulation of NRF2 at the protein level (Western) by short telomere signaling that is in agreement with preliminary evidence provided for the application of this grant (RNA expression changes). It appears as telomere shortening takes place, NRF2 RNA and protein levels undergo a concomitant decrease. Upon activation of telomerase (exogenous TERT expression) and subsequent telomere elongation, NRF2 protein (Figure 1) and NRF3 RNA levels (Figure 2 & 3) are restored and oxidative stress is decreased (preliminary data). Consistent with our hypothesis, antioxidant responses are also suppressed during periods of heightened oxidative stress compared to controls (exposure to increasing concentrations of peroxide; data not shown). We hypothesize that p53 may be actively suppressing antioxidant responses as part of the DNA damage response. We have previously found that expression of p53 shRNA decreases ROS and have now found evidence that p53 shRNA also increase NRF2 and NRF3 RNA expression and protein levels (NRF2 data not presented; Figure 4).

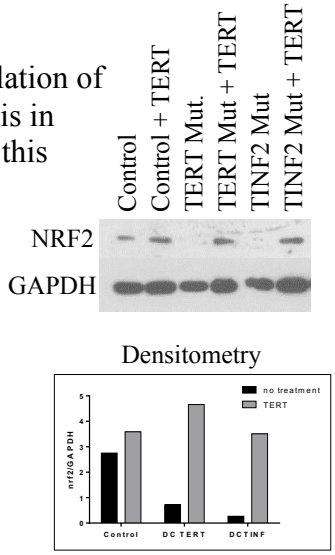

Figure 1 Increased NRF2 Protein in DC Cells Upon Exogenous TERT Express

RNA-Seq has provided substantial insight into the changes that take place between controls and DC cells (fibroblasts [Figure 5] and lymphocytes) as well as changes that take place in DC cells 'corrected' by expression of TERT. For example, when looking for gene expression profile differences between DC and control cells that reflect changes found in DC cells compared to 'rescued' DC-TERT expressing cells we found 74 genes that were in elevated in both controls and DC-TERT cells and 54 that were decreased in DC cells. Interestingly, NRF3 was found increased in control and DC-TERT expressing cells while the NRF2 heterodimerizing partner FOS was elevated in DC cells suggesting a potential inhibitory element. Furthermore, when performing a gene ontology enrichment analysis, there is an overrepresentation of CXCR chemokine receptor binding genes (p=0.0007) suggesting a modified autocrine/paracrine effect within the secretory pathway. Other potentially interesting genes were also uncovered relating to telomere biology and transcriptional regulation that may be of interest for further study. Regarding RNA-Seq performed on DC and control lymphocytes (three controls, three DC patients), the gene most significantly decreased in DC cells was GSTM1 (glutathione S-transferase mu 1; p=0.0005, 600-fold decrease in DC cells). In DC lymphocytes, suppression of two other glutathione-S-transferase (GST) genes, GSTM2 and GSTT1 was also found (4-6-fold, p<0.0001).

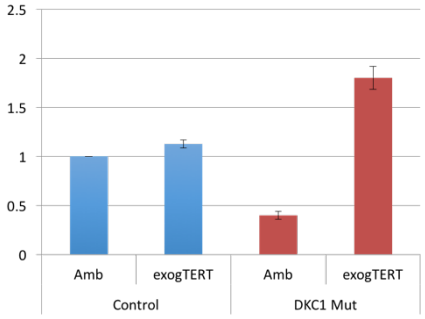

Figure 2 Rescue of NRF3 Expression in DKC1-Mutated Cells Upon Exogenous TERT Expression (QRTPCR)

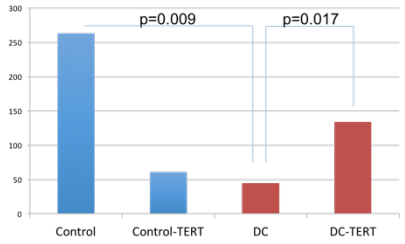

Figure 4 Rescue of NRF3 Expression in TINF-Mutated Cells Upon Exogenous TERT Expression (RNA-Seq)

Importantly, GSTM1 and GSTM2 are NRF2 transcriptional targets and GSTM1 and GSTT1 have been previously implicated in the acquisition of aplastic anemia.
Candidate genes within the NRF2 family and pathway have been cloned into lentiviral vectors for the purpose of circumventing the suppression of the endogenous gene by overexpressing the exogenous cDNA in DC cells. If an increase in oxidative stress is causal towards premature entry into senescence then restoration of key antioxidant genes may afford improved parameters to DC cells. One particular example of an unexpected candidate gene that decreased ROS (DHE staining) upon lentiviral overexpression was MAFG (Figure 5). Further research will be required to assess the nature of the relationship between MAFG and the NRF2 family to decrease ROS in this context.

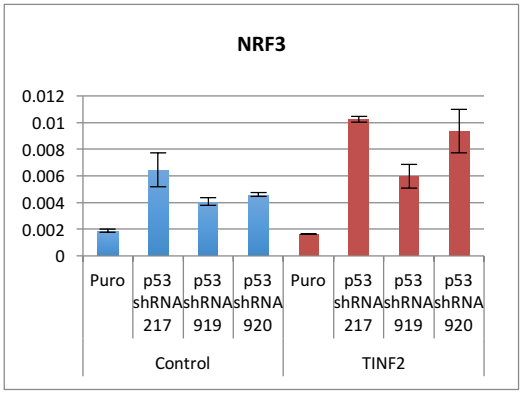

Figure 3 De-repression of NRF3 Expression by p53 shRNA

Accomplishments: Results Dissemination

Data from this project has been presented publicly at the American Society of Hematology (ASH) in December of 2015 as a poster presentation by the postdoc, Erik Westin. Further dissemination in the near future regarding data accumulated under this grant will take place at the 2016 ASH Meeting and manuscript preparation will likely be underway prior to that meeting.

For the next reporting period, we anticipate that we will have accumulated data regarding a number of key experiments. Namely,

1. Oxidative stress and proliferation data regarding cDNA and shRNA expression studies
2. Gene discovery approaches (RNA-Seq, CRISPR-KO, ChIP) initiated
3. Drug Screen: the drug screen will begin in Year 2 and likely completed initial analysis of all compounds
4. DC iPSCs: initial experiments will be underway to evaluate hematopoietic progenitors and their ability to differentiate into multiple lineages (myeloid/erythroid/lymphoid)

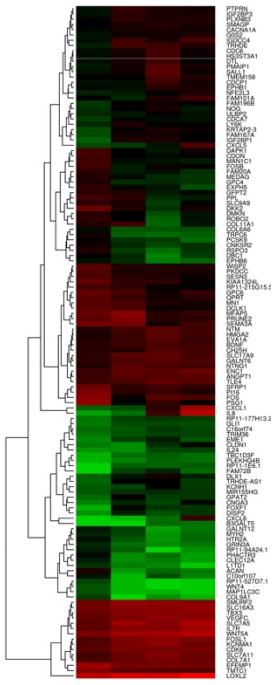

Figure 5 Hierarchical Clustering of Genes Differentially Expressed in Control and DC Cells and TERT-Expressing Cells

Impact

The impact of this project is multifaceted given the nature of key elements found within this project. We are studying a bone marrow failure disorder that is caused by prematurely shortened telomeres. Telomere attrition and maintenance also have significant biological implications in aging as well as cancer. Furthermore, we have found that telomere shortening within these cells leads to a concomitant increase in reactive oxygen species which have also been implicated in aging and cancer. Through characterization of the signaling pathway by which telomere shortening leads to premature cell death, we can shed light on potential targets within the pathway that can be disrupted/augmented to prevent bone marrow failure. These same findings may also have implications for aging and cancer research.

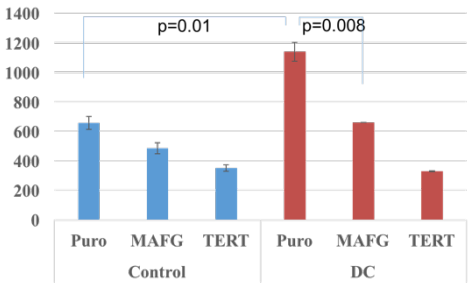

Figure 6 Decreased Reactive Oxygen Species in DC Cells Expressing NRF2-Heterodimerizing Partner MAFG

Impact on other disciplines: Nothing to report

Impact on tech transfer: Nothing to report

Impact beyond science/technology: Nothing to report

Changes/Problems

No significant changes have been made to the research. At this time the only significant, unanticipated problem is the inability to manufacture TINF2 iPSCs. We have a number of different methodologies (viral vs episomal; feeder vs feeder-free; addition of inhibitors or additional genes to derivation cocktail) to derive iPSCs and will modify our current strategies to produce these iPSCs.

Products: Nothing to report

Participants and Other Collaborating Organizations:

Name:	*Erik Westin*
Project Role:	*Postdoc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12 months
Contribution to Project:	*Performed all major experiments*
Funding Support:	*DoD W81XWH-15-1-0099*

Name:	*Tim Townes*
Project Role:	*Collaborator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2 months
Contribution to Project:	*Provided direction and project oversight*
Funding Support:	*DoD W81XWH-15-1-0099,* Hyundai Hope on Wheels

Name:	*Frederick Goldman*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.6 month
Contribution to Project:	*Provided direction and project oversight*
Funding Support:	NIH, *DoD W81XWH-15-1-0099*

Special Reporting Requirements: Nothing to report

ASH Abstract, December 2015

Suppression of Antioxidant Responses in Dyskeratosis Congenita Cells

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Dyskeratosis Congenita (DC) is a bone marrow failure disorder characterized by a triad of leukoplakia, skin dyspigmentation and nail dystrophy. Pathologies found in these patients arise due to mutations found within a number of genes (*DKC1, TERT, TERC, TINF2, TCAB1*, *CTC1, NOP10, C16orf57, NHP2* and *PARN*) that limit telomere maintenance/elongation, resulting in severely shortened telomeres. Previous studies in our lab have demonstrated impaired proliferation, limited lifespan and aberrant DNA damage response pathways in DC cells. These studies have also uncovered a significant reactive oxygen species (ROS) increase within every cell type investigated thus far. This ROS increase correlates with telomere dysfunction and the subsequent activation of the p53 DNA damage response pathway, which can be rescued by exogenous *TERT* or*p53*-shRNA expression. We have acquired skin punch biopsies from two patients with DC carrying either a *TERT* or *DKC1* mutation. Here, we have investigated a potential candidate pathway largely characterized as a key antioxidant regulator in hematopoietic cells, NRF2 (NFE2L2). NRF2 is a redox-sensitive basic leucine zipper transcription factor that, together with its heterologous partners (small MAF proteins, cJun, ATF, etc), binds to antioxidant response elements (AREs) within gene promoters in a pro-oxidant environment. We compared the RNA expression via QRTPCR of *NRF2* in control and DC skin fibroblasts and found a significant reduction in DC cells (*TERT* mutation: 1.5 fold; *DKC1* mutation: 2.6 fold). Protein levels of NRF2 were also decreased in DC fibroblasts compared to controls. *TXN* is a gene whose expression is increased by NRF2 in a pro-oxidant environment. *TXN* expression was also significantly reduced (*TERT* mutation: 2.1 fold; *DKC1* mutation: 2.2 fold). To test whether *NRF2* suppression in DC cells is due to telomere dysfunction, we exogenously expressed *TERT* via retrovirus in DC and control fibroblasts. *TERT* expression led to dramatic increases in *NRF2* (*TERT* mutation: 3.4 fold, *DKC1* mutation: 3.7 fold) and *TXN* (*TERT* mutation: 3.7 fold, *DKC1* mutation: 1.6 fold). In contrast, *TERT* expression in control cells increased *NRF2* only 1.3 fold while *TXN* decreased 1.4 fold. Finally, we wanted to compare the expression of NRF2/TXN in low and elevated oxidative environments (4% vs 21% O_2). Control cells *increased* the *TXN* expression in 21% O_2 (*NRF2*: no change, *TXN*: 2.8 fold) while DC cells *suppressed NRF2* (*TERT* mutation: no change, *DKC1*mutation: 3 fold decrease) and *TXN* expression (*TERT* mutation: 1.4 fold decrease, *DKC* mutation: 2.3 fold decrease). Functional studies have found DC cells grown in low oxygen increase their proliferative capacity perhaps due to, in part, the NRF2 pathway. Together, these data support a hypothesis whereby shortened/dysfunctional telomeres suppress NRF2 activity and an antioxidant response to a pro-oxidant environment. Based upon previous research, this pathway is likely dependent on the activation of p53 as an intermediary between dysfunctional telomere signaling and the subsequent suppression of NRF2 activity. An abrogated antioxidant response in shortened telomere cells may promote entry into senescence

and pathologies related to aging. Systemic pharmacological intervention that reduces ROS could reverse this process and form the basis to alleviate DC and related symptomology associated with this multi-organ disorder.

List of Personnel Receiving Pay for Research Effort

1. Erik Westin
2. Tim Townes
3. Frederick Goldman