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TITLE: Functional Rescue of Definitive Hematopoietic Potential in Stem Cells Harboring
Telomerase Mutations Associated with Bone Marrow Failure

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14. ABSTRACT The experiments performed during this first year of funding are in line with the timeline proposed in our initial application. In brief: Aim 1) We have successfully created a novel human pluripotent stem cell model, where we can control the expression and activity of telomerase during any stage of blood development. This system allows us to understand the specific consequences of telomere dysfunction during human hematopoietic development. In addition, we have used a similar system to understand the role of DNA damage signaling in the induction of bone marrow failure in settings of eroded telomeres. We have also developed different cellular tools to investigate the role of TERC stability during blood development in dyskeratosis congenita cells. We determined that we can significantly increase blood output in DC settings by altering the endogenous processing of TERC in these cells. Aim 2) We have developed a methodology to obtain, from human pluripotent stem cells, an entirely new population of definitive hematopoietic progenitors which recapitulate the signal requirements for hematopoietic stem cell specification during embryogenesis. This now allows us to decipher the specific requirements for specification of physiologically relevant definitive hematopoietic progenitors, in settings of dyskeratosis congenita and aplastic anemia. We now will comprehensively characterize the engraftment capacity of these definitive hematopoietic progenitors, in healthy and disease settings, in order to determine their capacity to treat bone marrow failure patients.								
15. SUBJECT TERMS - Telomerase; telomeres; definitive hematopoiesis; bone marrow failure; dyskerin, RNA decay, miRNA, human pluripotent stem cells, development, signal pathways								
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1. INTRODUCTION

Telomeres, the physical ends of eukaryotic chromosomes, are progressively shortened upon each cellular division. Telomere shortening can be prevented by telomerase, a reverse transcriptase that synthesizes telomeres from an RNA template, *TERC*. As hematopoietic stem cells and their downstream progenitors are required to generate functional blood cells throughout life, telomerase activity is essential for blood cell homeostasis. The consequences of impaired telomerase in the circulatory system are well exemplified in patients suffering with aplastic anemia (AA) and dyskeratosis congenita (DC), two bone marrow failure syndromes (BMFs) that are associated with mutations in different telomerase components. The goal of this proposal is to use the *in vitro* hematopoietic differentiation of genetically engineered human pluripotent stem cells (hPSCs), harboring clinically relevant mutations in telomerase, as a tractable system to decipher the molecular pathways able to revert BMF in patients (Aim 1), and as a source of immunologically matched HSCs for transplantation therapy (Aim 2). The experiments proposed here will significantly improve hematopoietic output in cells with mutations in telomerase, generating novel alternatives that can be pursued for the clinical management of DC and AA patients.

2. KEYWORDS

Telomerase; telomeres; hematopoiesis; bone marrow failure; dyskerin, RNA decay, miRNA

3. ACCOMPLISHMENTS

3.1 Major goals of project (as stated in the approved SOW)

- 1) Modulation of miRNA-34a and miRNA-145 levels during hematopoietic differentiation of telomerase mutant cells (part of Specific Aim 1).
Target date for completion: 18 months
Current Percentage of completion: 25%
- 2) Inhibition of *TERC* degradation during hematopoietic differentiation of DKC1_A353V cells (part of Specific Aim 1).
Target date for completion: 18 months
Current Percentage of completion: 90%
- 3) To understand the signal requirements for HSC specification from hPSCs (part of Specific Aim 2).
Target date for completion: 18 months
Current Percentage of completion: 90%

Overall, the project has developed according to the described original plan. While subtask 1 is being performed slower than expected, subtasks 2 and 3 were developed faster than initially planned and are very close to completion (see sub-section 3.4 for a detailed description of activities).

3.2 Major Activities

Over this first reporting period we have been actively working on all the original goals proposed in our application. We currently have 4 trainees whose projects are directly related to this grant. Wilson Fok and Alexandre Vessoni, postdoctoral fellows in the Batista Lab, are actively working on the experiments proposed on Specific Aim 1 of this grant, while John Creamer (PhD candidate) and Stephanie Luff (postdoctoral fellow) from the Sturgeon Lab are working on the experiments described on Specific Aim 2.

3.3 Specific Objectives

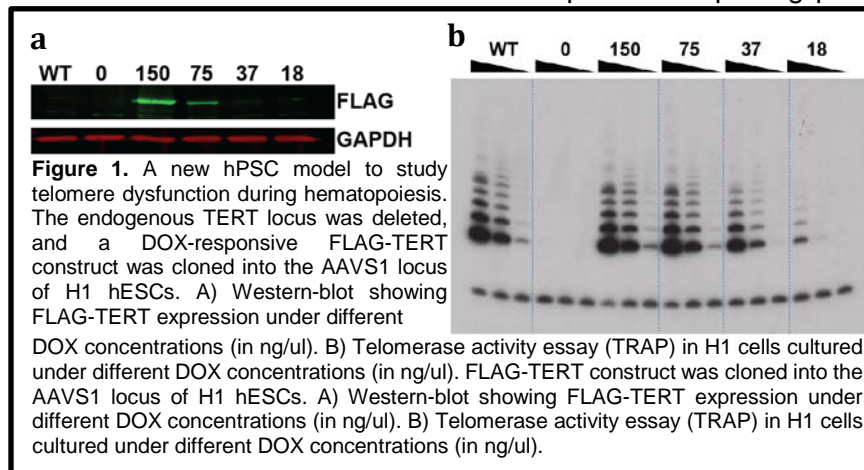
The main objectives of this first reporting period followed the proposed timeline specified in our funded application. Those included depicting in detail the molecular consequences of p53 pathway stabilization (in particular the role of miRNA-34a and miRNA-145 activation) in bone marrow failure in DC settings (Aim 1 of the proposal), mediating *TERC* decay during hematopoiesis of DKC1_A353V mutant cells (Aim 1 of the proposal)

and finally, increasing the efficiency of HSC specification in *in vitro* differentiation cultures by manipulation of different relevant signaling pathways, including sonic hedgehog (SHH), aryl hydrocarbon receptor (AhR), and retinoic acid (RA; Aim 2 of the proposal). As will be described in detail in the “Significant Results” section, we have made significant progress on all of our objectives and the original timeline proposed in our approved SOW is being followed. We are already preparing a manuscript for publication in this upcoming reporting period and, in addition, we have created many cellular and molecular tools that will allow us to investigate in detail the consequences of telomerase abnormalities in the development of BMF in DC and AA patients.

3.4 Significant Results

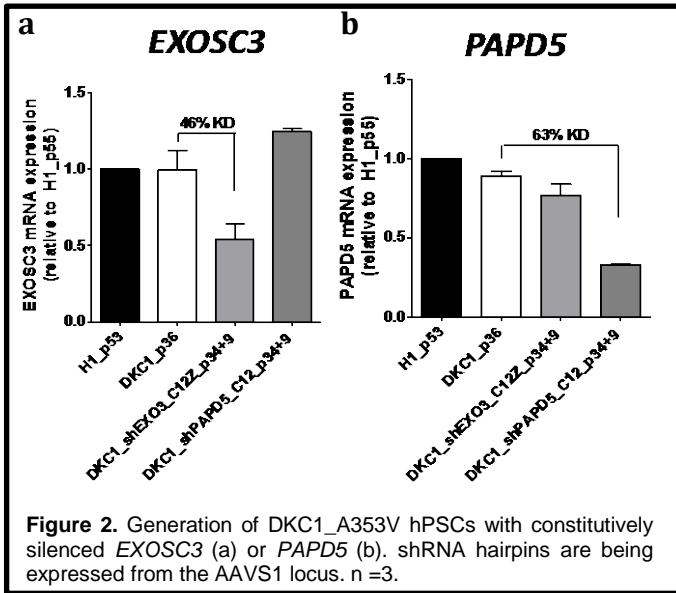
Specific Aim 1: The major objectives of this Aim are to understand the role that miRNA-34a and miRNA-145 play in the development of hematopoietic failure in patients with dysfunctional telomerase (Aim 1A), as well as the modulation of RNA decay as a strategy to rescue telomerase activity and hematopoietic differentiation in patients DC and AA harboring mutations in *DKC1* (Aim 1B).

To start answering the questions proposed in Aim 1A, during this first reporting period we successfully created a novel hPSC model, where we can turn the expression of *TERT* on or off at any given point during blood development, through a doxycycline-inducible system (Figure 1A). These cells were engineered using zinc finger nucleases (ZFN), by introducing the two vectors that comprise the TET-ON system (CAG_rTA and TRE-TERT) into the adeno-associated virus site 1 (AAVS1) “safe harbor” locus. Our preliminary data shows that these cells work as expected, and we can easily control telomerase activity (Figure 1B) and telomere length (not shown) by addition of DOX to the culture media. These cells will allow us to perform, during the next reporting period, hematopoietic differentiations at defined and precise telomere lengths. This will provide a significant improvement in our ability to understand, and modulate, the molecular mechanisms responsible for hematopoietic failure in patients with progressive telomere shortening. In addition, we have previously shown (preliminary data in our original application) that miRNA-145 and miRNA-34a were up-regulated after p53 activation in DC mutant hPSCs. Over the previous reporting period we started working on developing a new



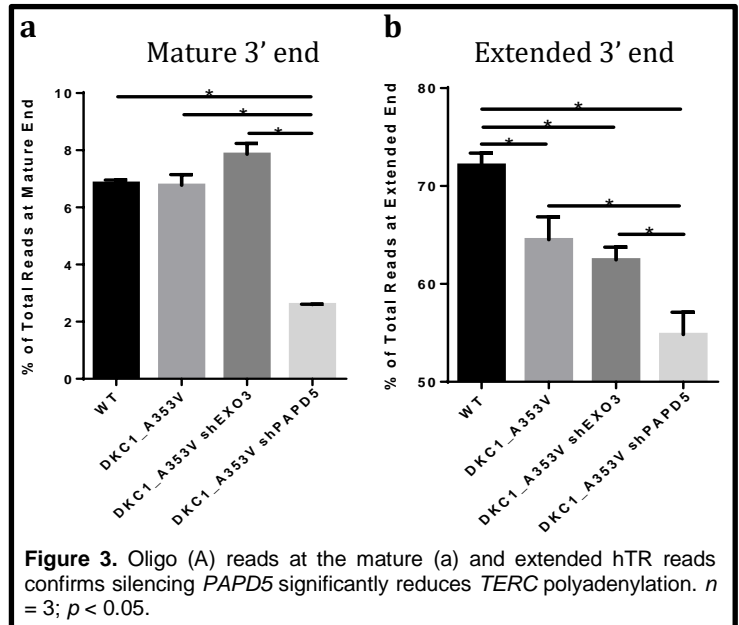
cellular system, where telomeres are progressively shorter, but these miRNAs are not activated. This system will allow us to understand the contribution of these non-coding RNAs to the hematopoietic failure observed in AA and DC patients harboring mutations in telomerase. We have successfully tested and cloned specific antagomir sequences against both miRNA-34a and miRNA-145 that efficiently reduce their activation after DNA damage (data not shown). We are currently engineering *DKC1_A353V* and *TERT_P704S* mutant hPSCs to harbor these antagomirs within the AAVS1 locus, under doxycycline-inducible conditions, so as to perform *in vitro* hematopoietic differentiation experiments in cells that retain short telomeres, efficient p53 pathway stabilization, but have an impaired miRNA-34a and miRNA-145 response. These experiments will allow us to directly interrogate the role of these miRNAs in hematopoietic failure in cells harboring mutations commonly found in DC and AA. At the same time, we have actively been working on generating the opposite model, wild-type cells (normal telomere maintenance) with forced miRNA-34a and miRNA-145 expression. We have already cloned these sequences into targeting vectors and will transduce them into wild-type hPSCs in the near future. Combined, these new cellular models will allow us to describe in detail the role of miRNA-34a and miRNA-145 and their targets, in blood development.

In Aim 1B our objective is to understand if the modulation of *TERC* degradation can improve the hematopoietic output of cells harboring clinically relevant *DKC1* mutations. Thus, we sought to determine if telomerase function can be increased by inhibition of *TERC* decay in *DKC1_A353V* hPSCs, and similarly determine if stabilization of

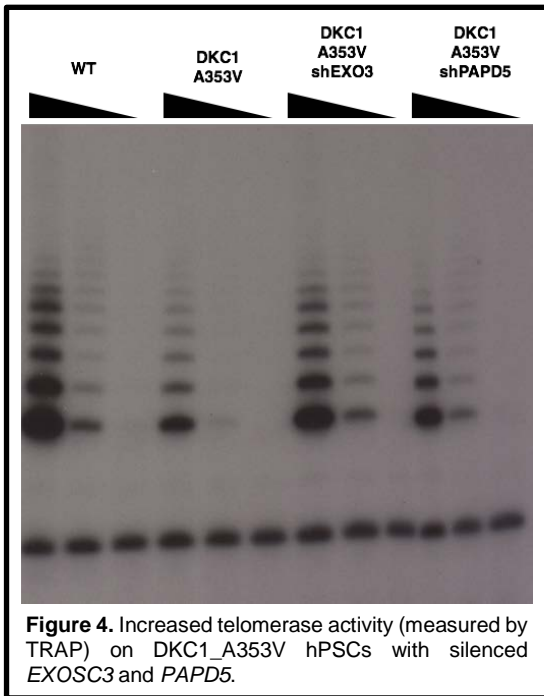


condition exhibiting a significant difference in relation to WT is the mutant DKC1_A353V sample (number of A's: 4 vs 3.6). Silencing *PAPD5* increased the mean poly-A tail in DKC1_A353V hPSCs to levels similar to WT hPSCs, again confirming that modulation of *PAPD5* controls *TERC* 3' end processing. Our preliminary data with these cells also indicates that there is increased telomerase activity (Figure 4) and lengthening of telomeres (not shown) in DKC1_A353V hPSCs with silenced *EXOSC3* or *PAPD5*. These positive results prompted us to perform our hematopoietic differentiation experiments in these cells. Our preliminary data indicates that reducing *TERC* decay significantly rescues definitive hematopoietic output in our DKC1_A353V hPSCs, back to WT levels (Figure 5A). Moreover, we also observe a significant reduction in primitive hematopoietic output (Figure 5B), which we have recently demonstrated to be a characteristic of cells with dysfunctional telomeres (Fok W, *et al* 2017). Combined, our data clearly establishes that modulation of *TERC* decay is a viable strategy to rescue telomerase function and hematopoietic output in cells harboring mutations in *DKC1*.

Specific Aim 2: In Aim 2, our goal is to improve the efficiency of HSC specification from hPSCs, via additional signal pathway manipulation during their *in vitro* differentiation. This will be essential to demonstrate that autologous hematopoietic stem cell transplantation from hPSCs is a viable therapeutic approach for BMF patients, including AA and DC. This Aim is therefore divided into two parts: 1) understanding the specific requirements for specification of definitive hematopoiesis in AA and DC cells (Aim 2A), and 2) to efficiently generate gene-corrected DC HSCs *in vitro* (Aim 2B). As it was described in our approved SOW, experiments for Aim 2B will only be performed between months 12 and 24 of funding and therefore are not going to be described in this initial Progress Report, as no murine xenographs have been performed.



We have recently identified 5 candidate signal pathways that may contribute to definitive hematopoietic specification from hPSCs, via RNA-Seq and subsequent Gene Set Enrichment Analysis (GSEA) of program-restricted mesodermal populations (Creamer, Dege *et al.* 2017). Specifically, this approach identified that the mTOR, sonic hedgehog (SHH), prostaglandin E2, aryl hydrocarbon receptor (AhR), and retinoic acid (RA) signal pathways were active in KDR+CD235a- definitive hematopoietic mesoderm. In our original proposal, we presented preliminary data confirming the roles of SHH and prostaglandin E2 as enhancing definitive hematopoietic specification from hPSCs.

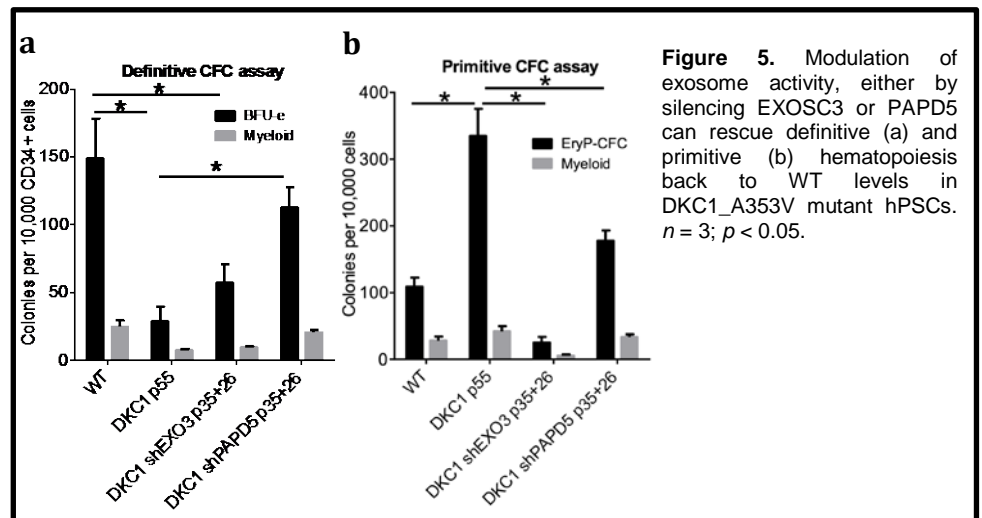


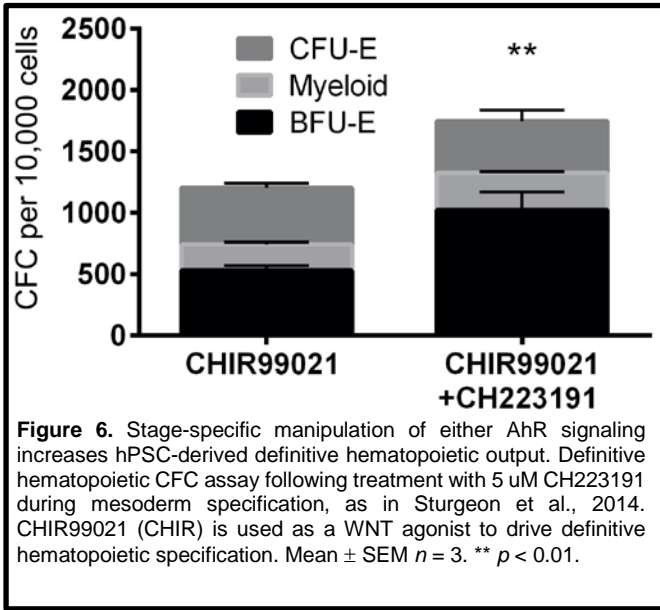
During our studies, the Zandstra group identified that mTORC1 inhibition enhances hematopoietic output from hPSCs, via global enhancement of BMP- and Activin-mediated mesendoderm specification (Nazareth, Rahman et al. 2016). As Activin signaling specifically drives primitive hematopoietic specification (Kennedy, Awong et al. 2012, Sturgeon, Ditadi et al. 2014), this indicated that our GSEA analyses flagged mTOR signaling as differentially active because it acted as a regulator of primitive, but not definitive, hematopoietic specification. In contrast, when we manipulated AhR signaling during mesoderm specification, we identified a clear role in regulating definitive hematopoietic specification, as treatment with the AhR antagonist CH223191 lead to significant improvement in definitive hematopoietic specification (Figure 6).

Our final candidate pathway, RA signaling, was identified by GSEA as differentially active between primitive and definitive hematopoietic mesoderm. However, as we utilize chemically defined, serum-free and stroma-free differentiation conditions that do not contain any vitamin A or retinol-derivatives, both primitive and definitive hematopoietic mesoderm lacked any active RA signaling. To better understand why this pathway was being flagged by GSEA, we first asked whether our WNT-derived KDR+CD235a-

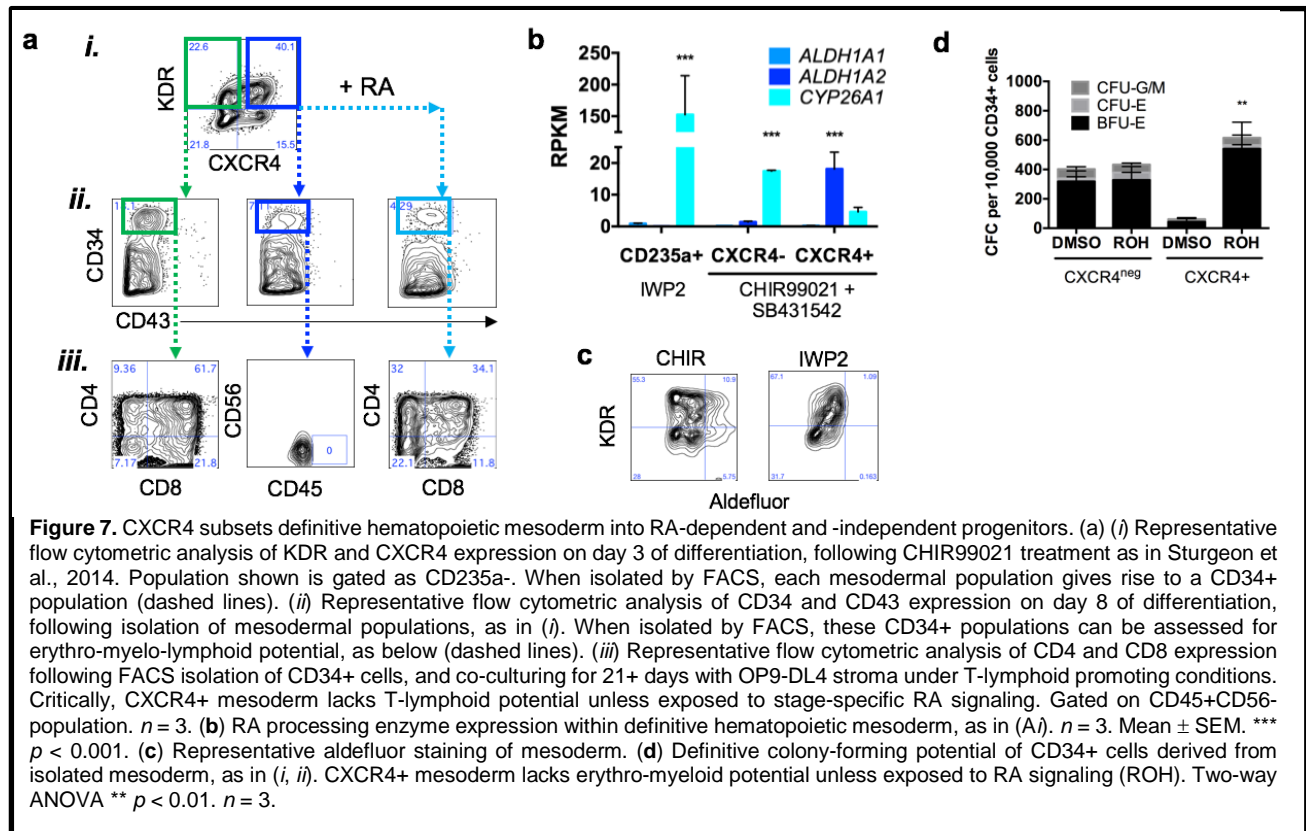
mesodermal population that harbors definitive hematopoietic potential was heterogeneous for other populations. Our RNAseq analyses of day 3 mesoderm identified a novel WNT-dependent cell surface antigen, CXCR4, that subdivides KDR+ mesoderm (Figure 7Ai). We isolated each subset of cells by FACS, and cultured them for an additional 5 days, as in (Sturgeon, Ditadi et al. 2014). Both populations gave rise to a CD34+CD43- population (Figure 7Aii). However, only the CD34+ cells derived from KDR+CXCR4^{neg} mesoderm harbored T-lymphoid and erythro-myeloid potential (Figure 7Aiii, and not shown). Therefore, the definitive hematopoietic progenitors we obtain from hPSCs via WNT signaling originates from a KDR+CD235a-CXCR4^{neg} mesodermal population.

While a novel observation, this did not yet explain our identification of RA signaling as a candidate pathway for hematopoietic specification. Therefore, we then performed additional RNA-Seq analyses on these novel mesodermal subsets, and focused our attention on the expression of the genes related to RA signaling. CD235a+ mesoderm, which harbors primitive hematopoietic potential, had very high *CYP26A1* expression, which negatively regulates the availability of all-trans retinoic acid (ATRA). This is consistent with a repressive role of ATRA on the primitive hematopoietic program (Goldie, Lucitti et al. 2008, de Jong, Davidson et al. 2010). Interestingly, CXCR4^{neg} mesoderm also exhibited strong *CYP26A1* expression, suggesting it was similarly unresponsive to RA signaling. However, CXCR4+ mesoderm instead showed significant expression of *ALDH1A2*, which is responsible for synthesis of ATRA from retinol (Figure 7B). Similarly, aldefluor (ALDF), which will fluoresce in cells with active ALDH activity (Jones, Barber et al. 1995, Kumar, Sandell et al. 2012), was detectable only in a subset of CHIR-derived mesoderm (Figure 7C), suggesting this population is poised to respond to RA signaling.





Exogenous signal pathway manipulation must be performed in the correct temporal order, on the appropriate progenitor population(s), to faithfully recapitulate definitive hematopoietic specification. We therefore isolated each mesodermal population by FACS based on CXCR4 expression and then cultured them in the presence or absence of retinol (ROH) during Stage 2 of differentiation. We found that, following RA signaling, CXCR4⁺-derived CD34⁺ cells now possessed definitive erythro-myeloid and T-lymphoid potential (Figure 7D). Collectively, these observations suggest that we have established the ontological origin of an hPSC-derived RA-dependent HE population, an essential hallmark of physiologically relevant definitive hematopoietic potential and represents a major development in the field of hPSC-derived hematopoietic specification. During the next reporting period we will apply these insights specifically to DC hPSCs, and assess the engraftment capacity of RA-dependent CD34⁺ hematopoietic stem/progenitor cells.



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3.5 Opportunities for training and professional development

During this first reporting period we had 4 trainees who work exclusively on the experiments proposed in this project:

Wilson Fok: 4th year Postdoctoral Fellow.
Alexandre Vessoni: 2nd year Postdoctoral Fellow
Stephanie Luff: 1st year Postdoctoral Fellow
John Creamer: 3rd year PhD candidate

These trainees have participated in several opportunities for training and professional development. They actively participate and have the opportunity to present their data at the following seminar series:

- Division of Hematology Seminar Series: Weekly, (Thursday's at 12pm), students and postdocs from the Divisions of Hematology and Oncology present their recent data to the faculty from those same Divisions.
- "hPSC Work-in-Progress Group": Monthly, members from labs at Washington University who work on human embryonic stem cell biology differentiation meet and present their most recent data in a very informal setting.

Trainees also actively participate in different "journal clubs" where faculty, students and postdocs present papers of interest that were recently published in top tier journals. These include:

- Hematology Division Journal Club: Meets every Tuesday at 12pm and discusses papers on a variety of subjects related to hematopoiesis, stem cell biology, telomere maintenance, and general hematology.
- Hematopoietic Development and Malignancy Program: Meets every Wednesday at 12pm and discusses papers related to hematopoiesis and leukemia.

Trainees are also exposed to the latest research developments around the world, through a series of seminar series at Washington University that bring renowned world-experts to present their recent findings on our campus. These include our own Division of Hematology Seminar series, the Department of Genetics and the Department of Developmental Biology Seminar Series, which run from October through June. Wilson, Alexandre, Stephanie and John have also had the chance to meet personally with various visiting speakers, in an informal setting over lunch.

Finally, both the Batista and Sturgeon groups have their own lab meetings (on Wednesday afternoon and Friday mornings), where trainees present their data and discuss how to improve results and the direction of their projects. In addition, our trainees also have additional training on Responsible Conduct of Research by attending the year-long series provided by our Division, where different faculty members discuss specific topics of relevance, including "Human Experimentation", "Collection, Recording, and Analysis of Data", "Accusation of Fraud", "Student-Mentor Relationships" and "Authorship, Peer Review, and Confidentiality".

3.6 Dissemination to communities of interest

Results originated from this grant have been disseminated to communities of interest by:

- **Invited talks at different research institutions:**
 - Cincinnati Children's Hospital (Batista)
 - Children's Hospital of Philadelphia (Batista and Sturgeon)
 - University of Colorado Boulder (Batista)
 - Universidade de Sao Paulo (Batista)
 - Stowers Institute (Sturgeon)
- **Presentations at different scientific meetings/conferences:**
 - Mutagen Meeting 2017 (Sao Paulo, Brazil; Batista)
 - International Society for Stem Cell Research (Fok and Sturgeon)

3.7 Plan of work to accomplish goals during next reporting period

The experiments presented in this progress report are in line with our approved SOW. The research in the next year will continue to follow the plan outlined in that section. Specifically, we propose to:

Aim 1) We will use our iTERT-hPSCs (DOX-controlled) to analyze the role of miRNA-34a and miRNA-145 activation on hematopoietic progenitors with different telomere lengths, as proposed in our original application. Additionally, we will perform hematopoietic differentiation experiments in WT hPSCs constitutively expressing miRNA-34a and miRNA-145, as well as in telomerase mutant hPSCs with constitutively silenced miRNA-34a and miRNA-145. In both settings we will specifically analyze the role of these microRNAs in hematopoietic failure in AA and DC patients. Over the next reporting period we will also analyze if inhibition of *TERC* decay can restore *TERC* localization in DKC1_A353V hPSCs by performing immunofluorescence coupled to RNA *in situ*-hybridization. Our goal is to submit a manuscript with data gathered in this Aim within the upcoming funding year.

Aim 2) These studies have identified an entirely novel hPSC-derived CD34+ population that harbors RA-dependent definitive hematopoietic potential, a hallmark of HSC development. We will begin our 4th Major Task (described in our original SOW), assessing the engraftment potential of this population, using both healthy control, and DKC_A353V hPSCs with long and short telomeres. We anticipate that healthy control hPSCs will yield a stably engrafting population, representative of an HSC-like population. Similarly, we anticipate that DKC_A353V hPSCs with long telomeres will generate a similar population. However, as the engrafted cells proliferate, telomere length will decay, due to compromised telomere maintenance. The successful completion of this Aim will have created a *bona fide* model of bone marrow failure from hPSCs, which we can then use to screen prospective therapeutic strategies, such as those described in Aim 1. This would be a major advance in the field. Our goal is to generate two manuscripts within the next 2 years, describing the *in vitro* generation of HSC-like CD34+ cells from hPSCs, and their use to model DC in a xenograft model.

4. IMPACT: nothing to report

5. CHANGES/PROBLEMS: nothing to report

6. PRODUCTS

1) **Journal publications:** *nothing to report*

2) **Books or other non-periodical, one-time publications:** *nothing to report*

3) **Presentations**

- Cincinnati Children's Hospital (National)
- Children's Hospital of Philadelphia (National)
- Universidade de Sao Paulo (International)

- Stowers Institute (National)

7. PARTICIPANTS

7.1 What individuals have worked on the project

- a. Luis Batista, PI, 1.80 calendar months
- b. Christopher Sturgeon, PI, 1.80 calendar months
- c. Kendra Sturgeon, Sr. Research Technician, 1.09 calendar months
- d. Alexandre Vessoni, Post Doc Associate, 1.00 calendar months
- e. Ho-Chang Jeong, Post Doc Associate, 0.30 calendar months

7.2 Change in active support of the PI

a. Batista

- a. **New:** American Federation for Aging Research, PI, 07/01/17 – 06/30/19, \$50,000/yr., no overlap
- b. **New:** NIH R01, PI, 08/01/17 – 05/31/18, \$250,000/yr., no overlap
- c. **New:** Washington University Micro CDI, PI, 01/01/18 – 12/31/18, supplies only, no overlap

b. Sturgeon

- a. **New:** NIH R01, Co-Investigator, 08/01/17 – 05/31/18, \$250,000/yr., no overlap
- b. **New:** NIH R21, Co-Investigator, 12/22/17 – 11/30/19, salary only, no overlap
- c. **New:** Washington University CRM Seed Grant, Co-PI, 02/01/18 – 1/31/19, supplies only, no overlap

7.3 Other organizations involved as partners: nothing to report

8. SPECIAL REPORTING REQUIRMENTS: nothing to report

9. APPENDICES: Not Applicable