CONFIDENTIAL

Regenerative Medicine Minnesota
Progress Report
Due: 3/31/2017

Grant Title: Identification of novel protein drivers of definitive hematopoiesis

Grant Number: RMM 11215 DS001

Principal Investigator: Bruce Blazar, MD

Project Timeline: 3/1/2016 - 2/28/2018

Progress to Date:
For each specific aim in your proposal, please describe progress and obstacles and/or achievements. Please address any deviations from estimated timelines and limit ***total*** response to 2000 words. Figures and tables are allowed.

Progress. Despite unavoidable obstacles that surfaced during the conduct of this award (and have been solved), substantial progress was made as described below.

Aim I: To utilize a high-throughput screening system with a novel transcriptional fluorescent reporter to identify extracellular proteins that promote the in vitro formation and/or expansion of definitive human HSPC from iPSC.

As we reported previously, we generated and validated a novel synthetic DNA construct that incorporates all known Runx1 regulatory elements [redacted], allowing the activity of each promoter to be non-invasively monitored by the expression [redacted] in mouse ESC/HSC cells (Figure 1). We similarly cloned human [redacted] elements [redacted].

After validating reporter expression in an human hematopoietic malignancy cell line under drug resistant conditions, we proceeded to testing in an optimized in vitro IPSC hematopoietic differentiation culture that involves embryoid body formation over 8 days and progression to CD34+ cells over the subsequent 9 days. [redacted] Under drug selection conditions, reporter gene expression was observed for both P1 and P2 downstream reporter elements. Now under conditions that support CD34 differentiation the definitive hematopoiesis [redacted] was seen, consistent with the desired expression pattern for definitive hematopoietic cell development [redacted].

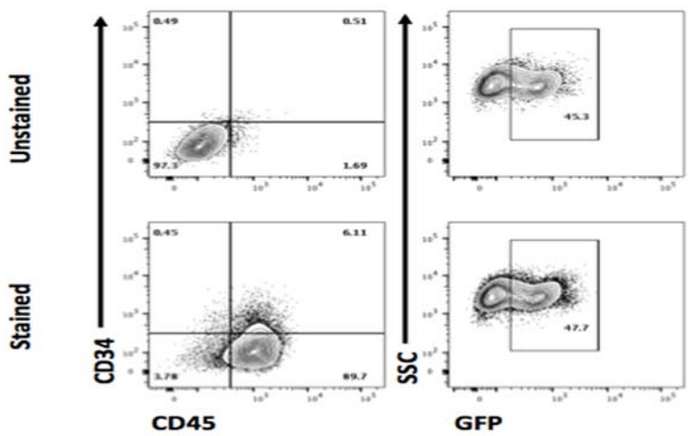
For high throughput screening, it is essential to be able to benchmark the assay with a positive control using protein(s) known to support definitive hematopoiesis differentiation with human cells. For this purpose, we tested a range of concentrations of [redacted] known to expand human CD34+ and stem/progenitor cells and [redacted] also reported to expand similar populations as positive controls for hematopoietic stem cell expansion/phenotype. In 384 well plate format to simulate high throughput conditions [redacted] had the desired effect. Further, toward optimizing the high throughput conditions used by our corporate collaborators, we determined in a series of experiments that the media typically used for such screening was compatible with our differentiation culture system as assessed by P1 reporter expression after 3 or 6 days of in vitro culture.

Over the course of this past year, we have generated ~30M hematopoietic stem cells in two batches of CD34+ differentiated from IPSCs containing AAVS1 integrated Runx1 reporters (per Figure 1) then further differentiated

over an additional 9 days into hematopoietic stem cells before freezing at various concentrations for shipping to the company (Table 1).

# cells	# Vials
1.1Million	16
225K	20
100K	36

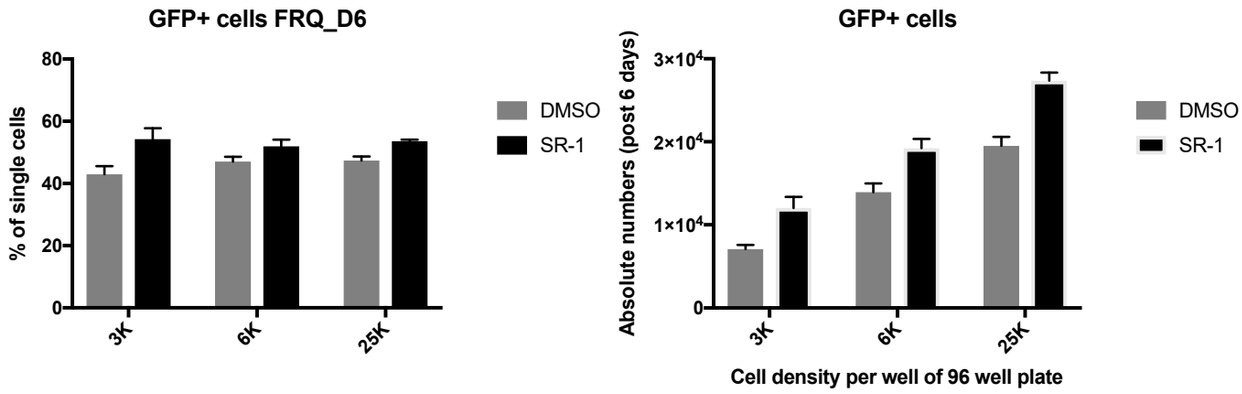
Phenotyping is shown below (Fig 2). Hematopoietic stem cells are CD45+ GFP+ cells.



In preparation for high throughput screening, screening conditions at UMN were optimized for 96 well plates using thawed samples containing ~300K purified CD45+ cells, that were seeded at 3,6, or 25K per well in the presence or absence of the optimal SR1 concentration then cultured for 3 vs 6 days as below.

Conditions	# cells/ well in 96 well plate						
media	3K	B2	B3	B4	B5	B6	B7
		C2	C3	C4	C5	C6	C7
	6K	D2	D3	D4	D5	D6	D7
		E2	E3	E4	E5	E6	E7
	25K	F2	F3	F4	F5	F6	F7

For 3K and 6K samples have cells from two wells per analysis (to meet the required number of events for flow analysis on Fortessa flow cytometer), whereas for 25K cells are from only single well of 96 well plate. A graphic representation is shown in Figure 3 below for the frequency and absolute numbers of P1 reporter expressing cells.



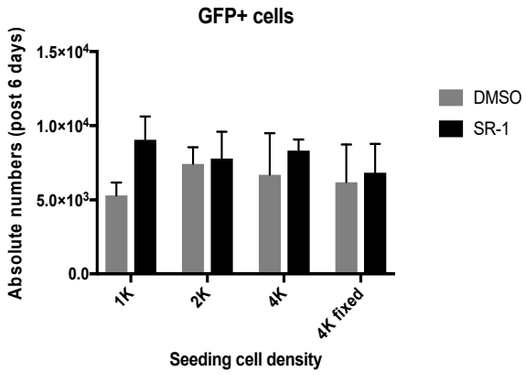
The profile of 3K/well initial seeding and 6 days of culture is shown below. One of 3 triplicate samples. Similar results were seen with 6K/well and 25K/well.

From these studies we concluded that: In vitro derived hematopoietic stem cells (at day 8+9) can be frozen and revive without viability (>95%) or recovery issues and have ability to proliferate and respond to [redacted] for expansion; hematopoietic stem cells maintain CD45 and RUNX-1 promoter [redacted] driven GFP expression; in a 96 well format plate we have successfully tested minimum 3K cells/well for screening conditions.

We next sought to answer 2 questions: At what stage of differentiation process (e.g first 8 days of embryoid body; second 9 days after further differentiation) can the cells be frozen down without significantly affecting assay performance? What is the optimal seeding density and assay format (optimal density for 96 well plate is 3K/well with ~50% GFP+ cells and 2 fold expansion after [redacted])?

FOR 96 well format		Estimated cells needed		
	# 96w cell plates	@1000 cells/well	@2000 cells/well	@3000 cells/well
Assay Development (@ n=2)	24	2.3E+06	4.6E+06	6.9E+06
Primary Screen (@ n=2)	168	1.6E+07	3.2E+07	4.8E+07
Retest (@ n=4)	24	2.3E+06	4.6E+06	6.9E+06
Confirmation with purified protein (DR @ n=4)	8	7.7E+05	1.5E+06	2.3E+06
TOTAL	**224**	**2.2E+07**	**4.3E+07**	**6.5E+07**

We next sought to determine the feasibility of generating large numbers of cells at UMN to ship to the company. Assuming we can maintain ~50% GFP+ and 2x expansion, it may be feasible to seed as little as 500 cells/well in 96 or 384 well plates and cut down total amount to ~11 x 10^6 cells. We proceeded to test batch to batch reproducibility from 2-3 independent runs with fixed variables of 3K cells/96 well plate, 6 days of [redacted] and to compare these results to 384 well optimization seeding 1, 2,4K/well for 6 days, combining wells as needed to acquire sufficient events (4 wells for 1K, 2 wells for 2K and 1 well for 4K). We were able to show that 1K cells/well, combining wells provided sufficient events for analysis as well as the required differentiation profile and with [redacted] facilitated expansion at 1K cells/well (Figure 6). We averaged 5K for control and ~9K GFP+ viable events in [redacted] treated samples with ~1300 to 2250 GFP+ cells respectively per well of 384 well plate seeded with 1K cells for six days screening conditions. Final cell viability ranged 50-60% in 384 well plates. The profile for 1K cells/well is below (Figure 6) with similar results for 2K and 4K cells/well.



We concluded that Seeding of 1K cells per well of 384 well plate seems sufficient to perform flow analysis (with manual handling we have more inconsistency of these results in two separate experiments). Manual pipetting errors may be rectified with robotics; 500-1000 cells per well in 96 well format plate is an alternative option. Further, Six days post treatment cells can be fixed using 1% paraformaldehyde without loss of GFP signal

With these data in hand, we shipped cells to our corporate partner who: Reproduced our manual hematopoietic stem cell assay, optimized friendly high throughput screening treatment, staining and acquisition of protocols/settings to maximize cell recovery and assay window; demonstrated that 500 cells/well may be feasible for screening with confirmation pending for fully automated treatment and processing runs and the need to develop automated screening protocols and data analysis workflows that will be finalized in March 2018. Corporate improvements in our approach including switching type of plate that reduced noise levels for pooled staining from more than 1 well, changing cell washing protocol that improved sample acquisition and cell recovery through a series of experiments with a range of cell concentrations per well as low as 500, and finalizing staining panel for high throughput screening.

Aim II: To validate candidate proteins and characterize resultant HSPC phenotypically by surface antigen and transcriptional profiling; and functionally by *in-vitro* colony forming-unit assays and in vivo engraftment assays in immunodeficient mice.

The final steps of validation and characterization require identification of hits from the high throughput screening. We are highly motivated to complete this aim and will do so once we have the needed information. The primary screen is estimated to be completed end of Q2, retest screen early Q3, prioritization mid-Q3, corporate confirmation late Q3/early Q4, and UMN confirmation of hits Q4. Once confirmed, in vitro and in vivo characterization will begin using hits that correspond to those with available reagents or that could have reagents synthesized for such testing beginning Q1, 2019 (as a best estimate).

Achievements. 1. We completed the initial step in developing a system for future high throughput screening by GFP detection along with CD45 expression.

2. We identified and validated a positive protein control for human hematopoietic stem cell expansion.

3. We generated 30M HSCs in two batches of CD34+ to HSC differentiation that have been frozen and sent to the company for testing.

4. Demonstrated that in vitro derived hematopoietic stem cells (at day 8+9) can be frozen and revive without viability (>95%) or recovery issues and have ability to proliferate and respond to SR-1 for expansion while maintaining CD45 and RUNX-1 promoter (P1) driven GFP expression in a 96 well format plate with a minimum 3K cells/well.

5. Demonstrated that paraformaldehyde fixation preserves GFP reporter expression in a 384 well format.

6. Exported cells and protocol to our corporate partner who successfully validated our findings and made improvements in assay conditions, now highly compatible with their high throughput screening protocol.

Obstacles and Deviations from timeline. Several factors precluded completion of the timeline, though we will finish the project as soon as possible without requesting new funds. On the corporate side, the company needed to buy new equipment specifically for this project with resources that they made available to do so. Most importantly the company moved facilities including all laboratories and offices to a new location in a different part of the city. In addition, my own laboratory moved buildings. This necessitated complete shut-down of all cultures, incubators, etc. On the other end, this required recalibrating and cleaning all equipment, replacement of an incubator gage that did not survive the move, and cytokines that upon testing in these differentiation assays proved to have lost efficacy and had to be replaced. Since not all the equipment we used could be moved, this also required purchasing of -150 C freezer for storage and other such items. Lastly, we have had to rescreen serum for this project. Despite these obstacles, substantial progress toward completion of our timeline was made as described above.

Please list any of the following that have resulted from the Minnesota Regenerative Medicine grant funding:
Publications and/or manuscripts submitted for publication:

Related to human induced pluripotent stem cells or human stem cell manipulation

Wagner JE, Brunstein CG, Boitano AE, DeFor T, McKenna D, Sumstad D, Blazar BR, Tolar J, Le C, Jones J, Cooke MP, Bleul CC. Phase I/II Trial of StemRegenin-1 Expanded Umbilical Cord Blood Hematopoietic Stem Cells Supports Further Testing as a Stand Alone Graft. *Cell Stem Cell* 18:144-55, 201

Webber BR, Osborn MJ, McElroy AN, Twaroski K, Lonetree CL, DeFeo AP, Xia L, Eide C, Lees CJ, McElmurry RT, Riddle MJ, Kim CJ, Patel DD, Blazar BR, Tolar J. CRISPR/Cas9-based genetic correction for recessive dystrophic epidermolysis bullosa. NPJ Regen Med. 2016;1. pii: 16014. doi: 10.1038/npjregenmed.2016.14

Osborn MJ, Lonetree C, Webber BR, DeFeo AP, McElroy AN, MacMillan ML, Wagner JE, Blazar BR, Tolar J. CRISPR/Cas9 Targeted Gene Editing and Cellular Engineering in Fanconi Anemia. *Stem Cells and Development* 25:1591-1603, 2016

Disclosures/patents: We plan to submit a patent on the hits identified by the screen.

Grant applications and/or awards: We plan to submit a grant on the hits identified by the screen.

Budget Update:
Please report the initial year's funding vs. spending and comment on any variance of >20% of estimated budget.

Reporting to all Minnesotans:

Briefly and using lay language, please describe your overall progress and how it is significant to the patients in need of regenerative medicine therapies in Minnesota. This will be used on the RMM website to demonstrate how funds are being used to advance the health of all Minnesotans.

We have established a novel assay system to find proteins produced by the body that cause hematopoietic stem cells to mature and divide. We are in the process of screen thousands of proteins with a company in California for their capacity to promote hematopoietic stem cell expansion. The company has a comprehensive and unique library of cell surface and secreted proteins to screen our reporter cells. After completing the identification of candidates, we will validate those candidates then strive to be able to bring those to use in the clinic either in vitro hematopoietic stem cell expansion approaches or for in vivo administration. The goal is to improve the hematopoietic system recovery after radiation, chemotherapy or with congenital disorders to decrease the morbidity and mortality of states in which the hematopoietic system is defective or in low abundance.