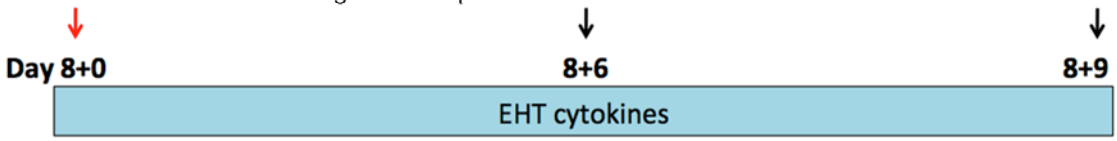
Progress to Date:

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.

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.

Earlier we generated and validated a novel synthetic DNA construct that incorporates Runx1 regulatory elements, allowing the activity of to be non-invasively monitored in mouse ESC/HSC cells. We similarly cloned promoter elements to assess hematopoietic stem cells/precursor cells. Expression in K562 cells showed high level of Runx1 reporter expression after puromycin drug selection vs puromycin plasmid

In order to achieve that we targeted this synthetic construct into human iPSC cells derived from T stem cell memory cells (TSM cells). We established our own protocol with optimized culture conditions for epithelial to hematopoietic transition (EHT) to generate hematopoietic stem cells (HSCs) from Tsm-iPSC derived CD34+ Hemogenic endothelia cells in *in-vitro* conditions. This included IPSC differentiation into embyroid bodies (EB) with Bhematopoietic cytokines. In phase II, CD34+ cells are isolated and seeded into matrigel coated plates.



We confirmed the level of RUNX1 mRNA expression with our new HSC differentiation protocol with high levels seen in Runx1-day 9 HSCs derived from IPSCs >K562 cells and no expression seen in Runx1- day 9 HSCs without HSC differentiation (not shown). After a total of 17 days, RUNX1 promoter element driven reporter were silenced upon differentiation of iPSCs into HSCs (not shown).

Therefore, we decided to try another approach as below. We have targeted RUNX1 promoter reporter construct into a safe haven locus previously reported to remain open and accessible to the transcription factors after directed differentiation. We screened stable reporter containing iPSCs and sequence confirm for the site-specific integration into that locus. We performed transient transfection of RUNX1 promoter reporter construct along with a puromycin plasmid to permit drug resistance in K562 cells. Fluorescent reporter protein expression is seen.

EB differentiation assays from IPSCs followed by EHT were established to generate HSCs and assess reporter expression by flow analysis. All our HSCs in day 17 total cultures express RUNX1 element driven GFP.

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.

We established the optimized culture conditions for EHT to generate HSCs from CD34 progenitors and characterized using panel of surface markers for human HSCs that includes CD34, CD45, CD90, CD45RA, CD38. In comparison with human cord blood HSCs we have seen quite a similar pattern of surface markers based HSCs phenotype. For proteome HTS platform we optimized conditions for stem cell differentiation. Following 8 days of EB differentiation from IPSC cultures and 9 days of EHT, day 17 cells expressing Runx1-GFP, cells were placed in 394 well plates to simulate high throughput conditions. Cells were cultured an additional 3 or 6 days in the presence of FP is media from our collaborator FivePrime,that is required for use in high throughput screening studies. The latter was tested at various ratios. Both support CD34+, GFP+ cells after the addition 6 days of culture (below) with higher GFP expression levels seen on day 6 vs day 3 (not shown).
We selected SR1 as a positive control and further have tittered SR1 from the optimal concentration of 0.75 μM used for human clinical studies and found that this concentration is biologically optimal in

expanding IPSC-derived CD34+ cells after EHT and culture in 384 well plates. We are now poised to begin screening with Five Prime after optimizing cell yields and detection methods as discussed on several conference calls and an in person visit by the PI (not funded by RMM).

Publications and/or manuscripts submitted for publication: None to date until screen is completed and hits identified.

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

Grant applications and/or awards: None

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 collaborating with a company in California that has a comprehensive and unique library of cell surface and secreted proteins to screen our reporter cells. After identifying 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.