Potential Role for Transforming Growth Factor Beta (TGFβ) 1 in Improving Megakaryopoiesis in the Inherited Platelet Disorder of Familial Platelet Disorder with Predisposition to Acute Myelogenous Leukemia.

Progress Report

Familial Platelet Disorder with Predisposition to Acute Myelogenous Leukemia (FPD/AML) is an autosomal dominant disorder associated with heterogenous manifestations of qualitative and quantitative platelet defects in addition to an increased risk of developing AML. FPD/AML is caused by monoallelic germline mutations to the transcription factor RUNX1, which is critical for the initiation of definitive hematopoiesis and promoting megakaryopoiesis. There is mounting evidence that hematopoietic stem and progenitor cells and megakaryocytes share several genes, cell surface markers, and signaling pathways that regulate both cell types. We hypothesize that drugs that correct RUNX1 expression and/or activity in the megakaryocyte lineage would not only correct the platelet defect, but also the hematopoietic progenitor cell (HPC) defect and prevent malignant transformation. This summer we studied the effect of a TGFβR1 inhibitor, RepSox, which was previously described to enhance megakaryopoiesis in human $CD34^+$ cells and increase RUNX1 levels. We investigated its effect on human megakaryopoiesis on four induced pluripotent stem cell (iPSC) lines: 1) FPD/AML (F/A), derived from a FPD/AML patient with a previously characterized splice acceptor mutation in exon 4 of *RUNX1* resulting in anomalous splicing and early RUNX1 truncation due to a frameshift, 2) FPD/AML-cor, an isogenic CRISPR/Cas9-corrected line derived from F/A, 3) CHOPWT6, a wild-type iPSC line previously characterized, and 4) CHOPWT6-mut, an isogenic WT-derived with the FPD/AML patient splice acceptor mutation introduced via CRISPR/Cas9.

F/A and CHOPWT6-mut iPSC-derived megakaryocytes and platelets reiterated several features seen in FPD/AML patients consistent with a role of RUNX1 in HPC proliferation and megakaryopoiesis, but not thrombopoiesis. Both F/A and CHOPWT6-mut lines have decreased megakaryocyte yields per HPC compared to their respective isogenic control (42% and 29%, respectively). Low-dose (50-100 nM) RepSox treatment during megakaryocyte differentiation ameliorated yield defects in both cell lines with a return to levels not significantly less than untreated isogenic controls. However, response to RepSox by the mutant lines could be enhanced to RepSox-treated control levels if on Day 3 of the 5-day differentiation, the culture media was replaced, suggesting that the conditioned media was preventing a full response to RepSox.

To test whether TGFβ1 released during megakaryopoiesis was responsible for the decreased yield in the mutant lines, we added polyclonal pan-TGFβ-specific blocking antibody to the culture media on Day 0 and saw improvements in megakaryocyte yields, which were near identical to RepSox pretreatment. Assaying for TGFβ1 levels in the conditioned media on Day 5 showed no increased in levels in the mutant lines relative to their respective isogenic controls.

Both RepSox and the polyclonal pan-TGFβ-specific blocking antibody do not directly reduce TGFβ1 levels within megakaryocytes. In order to investigate the effect of reduced intracellular TGFβ1, we conducted a siRNA mediated knockdown of *TGFB1* using lipofection. A literature search for protocols transfecting HPCs with siRNA using Lipofectamine (Invitrogen) family reagents yielded no hits. Thus we adapted a protocol that double transfected hematopoietic stem cells (HSC) and achieved high knockdown efficiencies (upwards of 90%). Due to the inevitable effect of inherent differences between HSCs and HPCs, we conducted several titrations to obtain the ideal conditions for our system. After differentiation of the seeded HPCs, we used FACS to obtain the percentage of cells that displayed megakaryocyte markers ($CD41a^+$, $CD42b^+$). We used an ELISA assay (DuoSet, R&D) to quantify TGFβ1 concentration in conditioned media samples, collected from cultures on Day 5. We found that the ideal conditions for our system to be a 250,000 HPC seed population, 15 minute incubation time with the siRNA-lipofectamine complexes prior to addition of differentiation media, and 100 pmol of si-TGFβ1. Moving forward we plan to transfect patient iPSC-derived HPCs with the necessary isogenic controls using these optimum conditions. We will also verify, independent of ELISA, whether cells transfected with si-TGFβ1 do have decreased TGFβ1 mRNA transcript levels and decreased total TGFβ1 protein. TGFβ1 signaling is complex and widespread, and as a result, observing alterations in phenotype may be more subtle and nuanced than previously expected. Moving forward we plan further understand the intricacies of TGFβ1 signaling and evaluate the leukemic transformation risk that has not yet been evaluated in our studies thus far.

Acknowledgements

This work would not have been possible without the support of Dr. Brian Estevez, Dr. Danuta Jarocha, and Dr. Mortimer Poncz who all continue to provide scientific guidance and mentorship. A special thank you to the University Scholars program, specifically Drs. Harriet Joseph and Alain Plante, for providing funding and guidance without which my involvement in this project would not have been possible.