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From development toward therapeutics, a collaborative effort on blood progenitors

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The National Heart, Lung, and Blood Institute Progenitor Cell Translational Consortium Blood Progenitor Meeting was hosted virtually on November 5, 2020, with 93 attendees across 20 research groups. The purpose of this meeting was to exchange recent findings, discuss current efforts, and identify prospective opportunities in the field of hematopoietic stem and progenitor cell research and therapeutic discovery.

Understanding the niche microenvironment for hematopoietic stem and progenitor cells

The niche microenvironment for hematopoietic stem and progenitor cells (HSPCs) contributes to key functions of HSPCs, such as self-renewal, differentiation, and engraftment. The molecular signals involved in niche stimulation remain to be found, and the niche cells could have therapeutic potential. Several groups reported their recent discoveries in the HSPC niche microenvironment in this meeting.

Shahin Rafii (Weill Cornell Medicine) presented the recent work by his group on adaptable and hemodynamic vascular niche. The goal of his project is developing a system to identify the signatures of vascular niche for hematopoiesis and to elucidate the pathways that determine such vascular heterogeneity in different organs. His group discovered that *Fli1* is required for the proper residence of HSPCs in the hematopoietic niche, and *Fli1*-deficient hematopoietic stem cells (HSCs) display precocious stem cell exhaustion and failure in engraftment (Badwe et al., 2017; Itkin et al., 2019). The mechanism that *Fli-1* enables HSPCs to sense and interact with their vascular niche is Notch signaling, and enforced Notch signaling successfully rescued the sensing of niche microenvironmental cues in *Fli-1*-deficient HSPCs and their functionality. Dr. Rafii also discussed their recent achievement in engineering human perfusable and adaptive endothelium for uncovering hematopoietic vascular niche-specific functions. This system would also allow the investigation of the vascular heterogeneity of different organs. His group found that transient ETV2 reactivation in the adult endothelial cells resets their adaptability and enables them to vascularize tissues in an organ-specific manner. These "reset" vascular endothelial cells (R-VECs) are also able to self-assemble into vascular networks, transport human blood, and physiologically support organ-specific functions including insulin-producing cells and cardiomyocytes, as well as HSPCs (Palikuqi et al., 2020). For example, insulin-producing cells vascularized with R-VECs show improved metabolic profiles in insulin production. Cardiomyocytes vascularized with R-VECs started to synchronize their contraction and pump blood efficiently. HSPCs co-cultured with the vascular endothelium generated by R-VECs are able to undergo rapid expansion in a large microfluidic device. In addition to normal physiological vascularization, R-VECs are also able to vascularize tumor organoids such as human colon carcinoma tissue. Therefore, the R-VECs are a powerful tool to study vascular endothelial heterogeneity. A current phase 1 open-label dose-escalation trial of such engineered endothelial cell infusion therapy is targeted to improve hematopoietic niche damage (Scordo et al., 2020). This suggests that repairing the vascular niche might be able to rescue organ damage and improve organ regeneration. R-VECs might possess the potential of providing organ-specific endothelium regeneration, for example, R-VECs activated with GATA4 for liver endothelium regeneration and GATA6 for heart endothelium regeneration.

Paul Frenette (Albert Einstein College of Medicine) and his group examined the presence and function of sensory nerves in the HSPC niche. Prior work of the Frenette lab reported the function of sympathetic nerves in the HSPC niche, in particular how granulocyte colony-stimulating factor (G-CSF) in the bone marrow signals the bone marrow peripheral nerves, which regulate the mobilization and retention of HSPCs from the bone marrow vascular niche (Katayama et al., 2006; Méndez-Ferrer et al., 2008). Recent work from the Frenette lab focuses on how the bone marrow sensory nerves relay external environmental cues and their interplay with the sympathetic nervous system. HSPC maintenance requires collaboration of sympathetic

and somatosensory nerves in the hematopoietic niche. Depletion of either sympathetic or somatosensory neurons would perturb the normal function of HSPCs and induce HSPC maintenance and migration defects (Gao et al., 2020). Denervation of sensory neurons by either resiniferatoxin treatment or Nav1.8-Cre-induced genetic ablation severely suppressed G-CSF-induced HSPC mobilization. The Frenette group identified calcitonin gene-related peptide (CGRP) as the crucial factor produced by the sensory nerves that regulate HSPC mobilization, and showed that the administration of CGRP is sufficient in restoring HSPC mobilization in their denervation models. In contrast, another neuropeptide, substance P, does not have an impact on HSPC mobilization. Furthermore, they reported that CGRP acts directly on the CALCRL-RAMP1 heterodimer expressed on the HSPCs. Deletion of either *Ramp1* or *Calcrl* in the hematopoietic compartment, but not stroma, would cause defective HSPC mobilization. Reciprocal transplantation experiments confirmed that RAMP1 and CALCRL heterodimer expressed on HSPC itself are downstream of CGRP signals for HSPC mobilization. Direct activation of peripheral nerves would also induce HSPC mobilization. For example, spicy food ingestion, or capsaicin administration, enhanced HSPC mobilization upon G-CSF injection. These results indicate that peripheral nerves in the bone marrow can exert long-range cues that trigger a physiological response in the hematopoietic system. These findings may contribute to our current understanding and the therapeutic potential of HSPC mobilization, particularly in the context of HSPC transplants.

Understanding the niche microenvironment helps us gain better knowledge of HSPCs and develop potential novel therapies. The findings by the Rafii group and the Frenette group demonstrated that the niche microenvironment could be engineered and stimulated to manipulate HSPC maintenance and functions. For example, recent literature has suggested that vascular endothelial cells in the hematopoietic niche maintains HSPC functions during steady state, aging, and injury (Barbier et al., 2020; Chen et al, 2019, 2021; Guo et al., 2017; Saçma et al., 2019). Endothelial progenitor cell infusion therapy is an emerging field that may help treat multiple hematologic disorders (Kim et al., 2019; Poulos et al., 2017). Further studies of the molecular signals contributed by each niche cell type could provide additional insights into the HSPC niche microenvironment.

Lineage tracing and clonal dynamics of hematopoiesis

Recent advancement in the genetic barcoding system has enabled the community to investigate the clonality and lineages of HSPCs in detail. The Zon lab (Boston Children's Hospital and Harvard University) reported our recent progress in developing the technological tools for performing lineage tracing and reported our findings regarding the clonal dynamics in the hematopoietic system. Using a single-cell GESTALT-based barcoding system (McKenna et al., 2016), whereby an adult barcode fish was crossed to the guide fish expressing the Cas9 protein under heat shock, it was possible to label embryonic cells during embryogenesis as well as the nascent HSCs as they are born in the aorta. This allowed the tracking of clones of blood cells in the adult kidney marrow and thymus. Stem and progenitor cells were barcoded at 28 h (developmental stage when HSC are born in fish embryos). Whereas lineage-biased clones were derived from embryonic clones, the balanced clones were later produced at 28 h post fertilization. Additionally, within the thymus, most of the adult thymic T cells share a common origin with kidney marrow T cells, and some clones in the thymus are unique. In future, by coupling the barcoding technique with single-cell RNA sequencing (scRNA-seq), it would be interesting to identify gene expression patterns of each clone that may pinpoint marker genes associated with one progeny versus the other during the clonal transition.

Fernando Camargo (Boston Children's Hospital and Harvard University) described his group's recent developments in the Sleeping Beauty lineage-tracing model and TARIS, an improved Tn-integration sequencing technique. The development of such tools enabled researchers to perform lineage tracing and clonal analysis *in situ*, and the Camargo group was able to identify a population of long-lasting multi-potent progenitors (MPPs) as one of the major drivers of adult hematopoiesis (Rodriguez-Fraticelli et al., 2018). With the *in situ* barcoding system, the Camargo group found that these long-lasting MPPs potentially arise from a developmentally distinct population from the HSPCs. Such long-lasting MPPs diverged from HSCs in the fetal liver between i9.5 and i13.5 stages of development, but these long-lasting MPPs continued to contribute to adult hematopoiesis. The Camargo group was also able to selectively label these long-lasting MPPs using an Flt3-CreER system, which show minimal labeling in the HSCs. They found that while most HSCs remain quiescent in adults, the long-lasting MPPs display a long-term lymphoid-biased contribution. The findings reshaped our current understanding of hematopoietic lineage landscape and highlighted the unique presence and functionality of fetal-derived long-lasting MPPs in adult hematopoiesis.

Clonal hematopoiesis has been described to contribute to multiple hematologic disorders. Developing lineage-tracing tools to study clonal dynamics of hematopoiesis created opportunities to thoroughly inspect the fate decisions in blood lineages. The methods presented at this meeting and other previously published methods demonstrated the ability to study clonality at single-cell resolution in many biological systems (Henninger et al., 2017; Kalhor

et al., 2018; Ludwig et al., 2019). These tools can help us better understand developmental processes and investigate the clonal origin of diseases. Future studies using these lineage-tracing tools could help us better understand the dynamic processes of normal and malignant hematopoiesis.

Modeling human hematopoiesis with pluripotent stem cells

Human pluripotent stem cells (hPSCs) hold great promise in helping us better understand human hematopoiesis and hematologic disorders. These cells could have therapeutic potential. Multiple approaches were described for the better modeling of human hematopoiesis using PSCs during the meeting.

One fundamental approach to better human hematopoiesis using hPSCs is to engineer better hPSC lines. Elias Zambidis (Johns Hopkins School of Medicine) presented a chemical reprogramming method using tankyrase/PARP-inhibition-based small-molecule methods to obtain naive hPSCs. The goal of his research is to improve the functional utility of PSCs by decreasing the lineage-primed bias, increase of proliferation, survival, and genomic integrity of differentiated progenitors, and enhancing gene-targeting efficiencies. These advantages would ultimately allow the derivation of adult-stage patient-specific transplantable progenitors. However, the current hPSCs are often lineage-primed and display interline variability and epigenetic memory. Therefore, it is crucial to develop naive hPSCs with resolution of these epigenetic barriers. The Zambidis lab has reported a chemical reprogramming method for deriving naive hPSCs. They found that a small-molecule cocktail of LIF, XAV939 (a tankyrase/PARP inhibitor), and classical 2i (MEK/GSKβ inhibition) stably supports naive hPSCs with a robust functional pluripotent state. These reprogrammed tankyrase/PARP-inhibited naive hPSCs show normal epigenetic imprints and have improved multi-lineage differentiation (Park et al., 2018; Zimmerlin and Zambidis, 2020). The application of such patient-derived naive PSCs in vascular progenitor therapies demonstrated improved efficacy in rescuing ischemic retina (Park et al., 2019, 2020). Furthermore, the Zambidis group also proposed that these naive hPSCs can be potentially used to create optimized human-animal chimera systems for better disease modeling and, potentially, cell transplantation. Blastocyst complementation in murine embryos confirmed that these naive hPSCs can successfully reconstitute human hematopoietic cells in the fetal liver. One important future prospect of this technique is to create adult hemato-vascular progenitors within mice or other model organisms.

In addition to adult human hematopoiesis, researchers recognized the importance of modeling developmental hematopoiesis. Gordon Keller (McEwen Stem Cell Institute) presented their strategies to model human yolk sac hematopoiesis with hPSCs. Prior evidence showed that primitive yolk sac-derived cells are present in adult organisms, such as microglia cells and Kupffer cells, which suggests the importance of understanding human yolk sac hematopoiesis. The Keller group was able to identify and separate different hematopoietic programs that occur early in development through the activin-BMP gradient (Sturgeon et al., 2014). The early common hemato-vascular progenitors express KDR and CD34, which then give rise to different daughter lineages that are responsible for either primitive or erythroid-myeloid progenitor/lymphoid-myeloid progenitor (EMP/LMP) hematopoiesis. They identified that CD235a/b^{+} mesoderm was able to generate both primitive hematopoiesis and multi-lineage hematopoiesis, suggesting that the equivalent of the human yolk sac hematopoietic program is as diverse as that of the mouse. These novel embryonically derived hematopoietic cells might uncover previously uncharacterized implications in human health.

Igor Slukvin (University of Wisconsin—Madison) also presented strategies to generate aorta-gonad-mesonephros (AGM)-like hemogenic endothelium with arterial characteristics. The two strategies that the Slukvin group identified were either reprogramming through mesoderm differentiation or direct conversion using transcription factor inductions. The first strategy was to differentiate hPSCs in a two-dimensional chemically defined culture to obtain hemogenic endothelium. These immature hemogenic endothelia would undergo arterialization upon Notch signaling upregulation and then enter endothelial-to-hematopoietic transition (EHT). His group discovered that SOX17 regulates such a process. Utilizing SOX17 doxycycline-inducible overexpression and SOX17 knockout lines, the Slukvin group found that SOX17 overexpression enhances Notch signaling and also directly binds and activates CDX2 promoter, which regulates the expression of HOXA genes. They further characterized the arterial hemogenic endothelium, which expresses DLL4, CXCR4, and relatively high levels of SOX17 (Jung et al., 2021; Uenishi et al., 2018). They also presented the direct induction of hemogenic endothelium using the combination of ETV2 and GATA2, or SCL and GATA2 transcription factors (Elcheva et al., 2014). They further elucidated the function of these transcription factors. ETV2 alone was required to program hPSCs into endothelium. GATA2 is dispensable for hemogenic endothelium specification but is essential for EHT (Kang et al., 2018). They then found that ETV2 directly upregulates GATA2 expression. Hence, they were able to use transient expression of ETV2 in hPSCs using modified mRNA to generate hemogenic endothelium, induce EHT, and obtain neutrophils in a serum-free and xenogenic-free condition (Brok-Volchanskaya et al., 2019).

Modeling hematologic disorders with patient-derived induced PSCs (iPSCs) presents a unique opportunity for us to recapitulate disease progress and identify potential druggable candidates. Sergei Doulatov (University of Washington) presented the recent work from his lab on iPSC models of myelodysplastic syndrome (MDS). MDS is a known genetically heterogeneous disease driven by clonal disorders of HSPCs. The Doulatov group successfully reprogrammed patient donor samples to iPSCs and reconstructed the clonal evolution underlying MDS, which allows them to study the temporal order of mutations that cause the clonal disorder of HSPCs. With their iPSC model, they revealed that pre-leukemic clonal hematopoiesis occurred with DNMT3A R882H mutation, which promotes iPSCs to hematopoietic progenitor cell expansion. They also identified SF3B1 mutation as the underlying cause of MDS with ring sideroblasts (MDS-RS). In SF3B1-mutant iPSCs, coordinated mis-splicing of iron metabolism genes caused MDS-RS during erythroid differentiation, and overexpression of iron metabolism genes was able to rescue the ring sideroblast disease phenotype (Hsu et al., 2019). Their study elucidated the mechanism of potential genetic predisposition of malignant clones of HSPCs that may cause MDS, which could be therapeutic targets in the treatment. Furthermore, their study demonstrated the potential of using patient-specific iPSCs to study hematopoietic malignancies, in particular those driven by clonal disorders of HSPCs.

hPSCs allow us to complement findings from *in vivo* model organisms. The above findings included optimization of hPSC lines and directed differentiation of hPSCs into desired lineages, as well as modeling hematopoietic disorders using patient-derived iPSCs. Along with the findings presented at this meeting, multiple efforts have been made to acquire naive hPSCs (Chang et al., 2021; Giulitti et al., 2019), and patient-derived iPSCs have been created to model many hematologic disorders such as MDS and other myeloid leukemias (Chao et al., 2017; Wang et al., 2021; Wesely et al., 2020). These approaches will allow us to model and discover human biology and provide valuable insights for potential translational therapies in human cells and patients.

Uncovering developmental hematopoiesis

Another much discussed subject at this meeting concerned deciphering the mechanisms that control developmental hematopoiesis. Under this topic, Trista North (Boston Children's Hospital) discussed the EZH1-mediated regulation of HSPC emergence from hemogenic endothelium during EHT. EZH1 is part of the PRC2 chromatin-repressive complex and canonically represses gene transcription. However, EZH1 may act as activator of gene transcription in a context-specific manner. A previous study from George Daley's lab (Boston Children's Hospital) showed that heterozygous mice with EZH1 mutation have an increase in HSC population (Vo et al., 2018). To investigate the cellular basis of this observation, the North lab extended this study in zebrafish by knocking down EHZH1 using morpholinos. EZH1 morphants showed an increase in RUNX1/CMYB expression as well as CD41 expression, showing an increase in HSPC potential. In fact, there is an increase in lymphoid potential as $RAG1^+$ lymphocytes were increased in thymus. Expression of GATA2b, which is enriched in hemogenic endothelium, was increased, without any significant change in the other waves of hematopoiesis, e.g., red blood cell (RBC) or EMP population. Further investigation suggested that under EZH1 knockdown, arterial endothelial markers, e.g., EPHB2, were decreased, suggesting that loss of EZH1 promotes HSPC state at the expense of arterial endothelial fate (Soto et al., 2019a, 2019b).

Along this line, Brandon Hadland (Fred Hutchinson Cancer Research Center) discussed the origin of multi-potency and self-renewal properties in mouse embryos during EHT. Among the many waves of EHT, the Hadland lab investigated (1) whether the earlier LMP wave and the subsequent HSC wave in arteries arise from same population and (2) whether these multi-potent and self-renewal properties are uncoupled and independent of each other. Using an AGM endothelial cell-derived *in vitro* vascular system that supports EHT from hemogenic precursors derived from murine embryos, the group identified a minor subset of endothelial population of developing mouse embryos with robust HSC activity at around embryonic day 9. This population is enriched in surface markers, e.g., EPCR and CD61 (Hadland et al., 2018, 2019). Furthermore, using an index sorting co-culture-based strategy that enables observation of one cell at a time, heterogeneity in engraftment potential was observed in early stages of EHT that is biased toward lymphoid fate. However, this was eventually replaced by the predominance of HSC colony-forming cells with robust self-renewal potential. In addition, the group was able to identify markers, e.g., CXCR4, enriched in the rare subset of HSC colony-forming cells compared with the predominant progenitor colony-forming subset. Overall, these data supported a model in which an initial multipotent potential of HSC is required during early arterial differentiation. However, in order to generate the true long-term potential of HSCs, further maturation of the arterial endothelial precursors is required before EHT so as to give rise to a population of cells with increasing and more robust multi-lineage potential. Interestingly, EZH1-EZH2-mediated gene-regulatory networks might play a key role in patterning such key cell-fate transition events.

Hanna Mikkola (University of California, Los Angeles) talked about EHT during early human embryogenesis, specifically looking into the following key questions. (1) What

is the identity of early human HSCs? (2) Other than AGM, what could be the other tissues that harbor HSC development during early human embryogenesis? (3) How do nascent HSCs transition to fully functional engraftable HSCs in humans? (4) What are the key distinguishing features of hemogenic endothelium that produces HSCs compared with the endothelium that does not make HSCs? Using scRNA-seq technique in human embryos starting from the early first trimester stage, the Mikkola lab was able to identify a very early nascent HSC cluster in AGM that expresses RUNX1, HOXA9, MLLT3, and its target HLF. Comparison of these nascent HSCs with the other RUNX1$^+$ clusters indicated that these other hematopoietic cells are lineage-biased, presumably progenitors with immune cell fate. Such a comparative approach generated a list of 30 genes that could serve as a nascent HSC score card that includes both known and novel nascent HSC genes, e.g., SPINK2. Using SPINK2 as a marker, the group was able to identify other hematopoietic tissues besides AGM at later time-points of human embryogenesis, including placenta, yolk sac, liver, head, and heart. Interestingly, however, while yolk sac and placental SPINK2 population showed similar HSC properties as the AGM cells based on the HSC signature (RUNX1$^+$HOXA9$^+$MLLT3$^+$HLF$^+$SPINK2) and nascent HSC scorecard, those in the liver, head, and heart lacked HSC features. Rather, it appeared that they are progenitor in nature. Using the scRNA-seq approach to further compare the gene expression signature of AGM with fetal liver at later stages of embryogenesis (at around 6 weeks when the HSC program shifts from AGM to fetal liver), the Mikkola lab observed a downregulation of endothelial program and upregulation of self-renewal signature marked with enriched expression of MLLT3 and its target genes (Calvanese et al., 2019) along with increased expression of major histocompatibility complex (MHC) class II genes. The group further investigated the origin of human HSCs and found that the immediate endothelial precursor for HSCs is largely arterial and not venous in nature. Only the HSCs originating in the AGM, and not the progenitors, expressed HOXA cluster genes. Further investigation led to the identification of key molecular landmarks and precise duration of activity of crucial signaling pathways (e.g., Notch, transforming growth factor β, WNT) that enabled mapping of the transition of a specific subtype of arterial endothelium into definitive HSCs during human embryogenesis. Altogether, this study showed promise in modeling the generation of various cell types from embryonic stem cells in general that could help us better understand the pathogenesis of various diseases that originate *in utero*.

Uncovering the molecular signals and cellular events during developmental stages provides new insights into HSPC biology. The key events and molecular signatures reported here and in many other studies summarize the generation of HSPCs from AGM endothelial cells through EHT using animal models and early human embryonic samples (Böiers et al., 2013; Bonkhofer et al., 2019). These findings could help us better understand the normal developmental processes of definitive HSPC origin and target potential pathways for congenital hematologic disorders.

Enhancing the therapeutic potential of hematopoietic stem cells

Several speakers discussed the emerging strategies to better utilize HSCs to increase their therapeutic potential. James Palis (University of Rochester Medical Center) reported on this topic as part of the Philadelphia hub. He detailed on how to improve the production red cells from iPSCs that can be used to produce cultured red cells for patients having difficulty in finding compatible blood for chronic transfusion programs. Murine erythroid progenitors with distinct spatiotemporal origin possess distinct self-renewal and differentiation capability. To increase the output of mature red cells in an *in vitro* culture system by increasing the number of self-renewing precursors, which could also be terminally matured further using a directed differentiation approach, the Palis lab compared the gene expression profiles of *ex vivo* self-renewing erythroblasts with *in vivo* differentiating pro-erythroblasts. They identified several members of PRC1 chromatin complex, e.g., Bmi-1, that are overexpressed in the self-renewing cells. Overexpression of Bmi-1 in bone marrow-derived progenitor cells increases the self-renewing potential of these cells in *in vitro* culture medium containing erythropoietin/stem cell factor (EPO/SCF) and dexamethasone. Exogenously injected Bmi-1 overexpressing progenitors engrafted successfully in immune-compromised mice and remained within the recipient animals for a limited amount of time, suggesting that they are not immortalized. Importantly, human peripheral blood mononuclear cells (PBMCs) transduced with Bmi-1 can lead to cultures of erythroblasts that expand and continue to self-renew for around 3 months following weekly purification using fluorescence-activated cell sorting. They show completely normal karyotype unlike other available immortalized erythroid cell lines. These cells represent cell surface phenotypes of immature erythroid cells (CD71$^+$, CD235a$^+$) and can be differentiated to mature red cells with 50% enucleation efficiency in a cytokine-dependent manner. Moreover, overexpression of Bmi-1 in human iPSCs led to self-renewing blood progenitors, suggesting that overexpression of Bmi-1 could be a promising strategy to improve the yield of functional red cells in *in vitro* culture systems (Kim et al., 2015; Olsen et al., 2018).

Christopher Thom (Children's Hospital of Philadelphia) addressed Tropomyosin 1 (TPM1)-related mechanisms

that impact *in vitro* hematopoiesis. With interest in improving strategies to expand megakaryocyte and platelet production *in vitro*, he followed a human genetic approach to identify polymorphisms in TPM1 that lead to altered platelet traits. Using a primitive hematopoiesis system, wherein human iPSCs could be differentiated into primitive streak to the mesoderm stage, he interrogated the effect of loss of TPM1 compared with wild-type cultures (Thom et al., 2019, 2020). The mesodermal cells normally acquire an endothelial fate, and the hemogenic population within this endothelium specifically undergo EMT to produce HPCs. Loss of TPM1 did not alter the iPSC stage, but at the endothelial phase there was an increased number of $KDR^+/CD31^+$ endothelial cells that led to a 2- to 3-fold increased production of $CD43^+$ HPCs compared with wild-type cells. Comparison of gene expression programs between the wild-type and TPM1 knockout cultures at the iPSC stage, $KDR^+/CD31^+$ endothelial stage, and non-adherent $CD43^+$ HPC stage did not reveal any significantly altered pathways in the iPSC phase. However, altered expression of around 1,000 genes at the endothelial phase and around 3,000 genes at the HPC stage distinguished the TPM1 knockdown cells from those of the wild type. In the endothelial stage, TPM1 knockout cells showed upregulation of genes related to tumor necrosis factor α signaling, EMT, and guanosine triphosphatase signaling, e.g., KRAS. Such a signature along with downregulation of the p53 pathways may provide better survival potential to TPM1 knockout cells. At the HPC stage, TPM1 loss of function showed upregulation of cell-cycle genes, supporting the reliance of cell-cycle dependence of hemogenic endothelium that was recently observed in the field. Interestingly, TPM1 knockout HPCs showed a decrease of platelet gene expression signatures related to coagulation pathways, suggesting how TPM1 could alter human platelet traits. Whether TPM1 solely controls the hemogenic endothelium in a cell-intrinsic fashion and/or it influences interaction between hemogenic and stromal cells within the endothelium remains to be explored in future.

Following similar interests to improve the yield of platelet production *in vitro* that could be used for transfusion in patients, Thorsten Schlaeger (Boston Children's Hospital) talked about a strategy that could produce 100 billion functional platelets in a relatively small bioreactor. His group used hiPSC-derived megakaryocytes that were conditionally immortalized by doxycycline-dependent expression of cMYC, BMI1, and BCL-XL (Nakamura et al., 2014). Through a high-content chemical-genetics screen, the group identified microtubule-destabilizing vinca alkaloids (vinblastine and vincristine) as promoters of megakaryocyte maturation. Compound-treated megakaryocytes produced larger and more elaborate pro-platelet-like extensions. Platelet production was even more efficient when vincristine was added after the cells were treated with an Aurora kinase inhibitor to promote polyploidization (Wen et al., 2012), another hallmark of terminally differentiating megakaryocytes. With this dual-compound strategy, each megakaryocyte produced more than 100 platelets on average, leading to more than 100 billion mature platelets in a 10-L bioreactor system. These bioreactor-produced platelets are functional because they represent key features, such as ring-shaped microtubule patterning and agonist-induced activation and aggregation *in vitro*, and they participate in thrombus formation *in vivo* and rescue hemostasis in thrombocytopenic mice.

Identifying strategies to generate cytotoxic immune cells with superior anti-tumor activity was another exciting topic at the meeting. Ran Jing, a postdoctoral fellow in George Daley's lab (Boston Children's Hospital), described their approach to produce human iPSC-derived T cells for chimeric antigen receptor (CAR) T cell therapy. Previously, following a screening approach to find epigenetic factors, the Daley lab had identified EZH1 as a major repressor of lymphoid differentiation from ($CD34^+$, $CD45^+$) myelo-erythroid progenitors derived from human iPSCs (Vo et al., 2018). Hence, using genetic knockdown of EZH1 in iPSC-derived myelo-erythroid progenitors coupled with a stromal-free culture system, the group has successfully generated a robust T cell population (EZ-T cells) that can largely recapitulate normal T cell maturation. EZ-T cells first develop as $CD5^+/CD7^+$ cells and gradually transition into a $CD4^+/CD8^+$ double-positive population. The majority of these cells then undergo positive selection to ultimately become $CD8^+$ single positive mature T cells. At this time, they predominantly express T cell receptor α and β and lack expression of CD1a, the marker for immature T cells. As further evidence for maturation, CD8 exists as an α/β heterodimer similar to PBMC-derived mature T cells. EZ-T cells can be loaded with anti-CD19-CARs, expand efficiently, and show robust CD69 activation compared with control iPSC-derived T cells. Finally, EZ-T cells show anti-tumor activity by killing the Nalm6 B cell acute lymphoid leukemia cell line in co-cultures.

Dan Kaufman (University of California, San Diego) described three strategies to engineer human iPSCs that can generate natural killer (NK) cells with enhanced *in vivo* anti-tumor activity. The first strategy involves overexpression of CARs with NK-cell-specific signaling and transmembrane domains in iPSC-derived NK cells using transposon-mediated gene modification. These engineered NK cells maintain typical surface markers, e.g., CD56, and showed improved anti-tumor activity in an *in vivo* ovarian cancer xenograft tumor model compared with human peripheral blood-derived NK cells, unmodified iPSC-derived NK cells, or iPSC-derived NK cells that overexpress T cell-specific CARs (Li et al., 2018). In the second strategy,

Kaufman's group genetically engineered iPSC-derived NK cells to express a stabilized version of CD16 that is normally cleaved when NK cells are activated by ADAM17, which is normally expressed by NK cells as well as tumor cells (Jing et al., 2015; Romee et al., 2013). Using the cleavage-resistant CD16 in iPSC-derived NK cells, they were able to maintain expression of CD16 in 99% of NK cells even after they became activated with either exposure to tumor or by pharmacological activation with ionomycin. Moreover, combined with anti-CD20 antibody, these iPSC-derived NK cells with stabilized CD16 showed improved cytotoxic activity toward Raji cells using an *in vivo* lymphoma model (Zhu et al., 2020a). As the third strategy, the group carried out CRISPR/Cas9-mediated deletion of *CIS*, a downstream regulator of interleukin-15 (IL-15) signaling, via ubiquitination and degradation JAK, leading to inhibition of the JAK/STAT pathway that is required normally for NK cell function (Bernareggi et al., 2020; Delconte et al., 2016; Kershaw et al., 2013). Knockdown of CIS-induced overexpression of genes related to lymphocyte activation and these iPSC-derived NK cells showed increased sensitivity to IL-15 (Zhu et al, 2020a, 2020b). Finally, in an *in vivo* leukemia model, the engineered NK cells showed improved anti-tumor activity compared with wild-type iPSC-derived NK cells. These iPSC-derived NK cell strategies have successfully transitioned to the clinic in collaboration with Fate Therapeutics as anti-cancer therapies. The NK cell product FT500 has been used as the very first iPSC-derived therapy in the United States. FT516 is the engineered NK cells that express non-cleavable IL-16, whereas FT596 represents the iPSC-derived NK cells engineered to express an anti-CD19 CAR, non-cleavable CD16, and recombinant IL-15.

Irving Weissman (Stanford Institute of Stem Cell Biology and Regenerative Medicine) discussed targeting CD47 to treat malignant clonal expansion. His lab had previously shown that Hoxb5^{+} long-term (LT)-HSCs are attached to bone marrow venous sinusoidal endothelial albuminal surface and are fairly constant in number, around 100 in a mouse femur. These LT-HSCs could migrate from one niche to the other at a constant rate (Chen et al., 2016). The studies by Weissman's group led them to hypothesize that the mutations that are pre-AML (acute myeloid leukemia), which can occur in any cell of the hematopoietic series, only start oncogenesis when they initiate HSC clones. With this hypothesis, he discussed the concepts of cell of origin and progression of leukemia. Acute leukemia progression occurs in HSCs to make a clone and to generate leukemic stem cells where pre-leukemic clones of HSCs compete with non-leukemic HSCs. In this context, precancerous cells express calreticulin, which acts as the "eat me" signal, and the emergent cancer clones overcome this with the "don't eat me" signal CD47 that facilitates evasion of phagocytosis (Feng et al., 2018). This knowledge provided a means to use anti-CD47 antibody (magrolimab) as a therapeutic agent. Inclusion of anti-CD47 antibody with azacytidine in high-risk MDS and AML patients resulted in almost complete long-term remission (Chao et al., 2020). Similarly, a combination of anti-CD47 with rituximab led to remission of 50% of diffuse large B cell lymphoma/follicular leukemia patients who were otherwise refractory to only rituximab treatment (Advani et al., 2018). In addition, CD47 blocking antibodies could restore phagocytosis and prevent atherosclerosis, and a combination of anti-CD47 and anti-IL-6 could prevent idiopathic pulmonary fibrosis (Wernig et al., 2017). These results highlight that the lessons learned from hematopoiesis form a commonality with virtually all other organs, where tissue stem cells undergo clonal expansion to acquire a pathogenic/cancerous fate by expressing CD47. This opens up anti-CD47 antibody-mediated strategies as a promising therapeutic avenue for clonal hematopoietic diseases.

Finally, to conclude the therapeutic session, Irwin Bernstein (Fred Hutchinson Cancer Research Center) elaborated on approaches to improve anti-tumor activity of immune cells by activating Notch signaling. A major blockade in tumor immunotherapy is the immunosuppressive tumor microenvironment, much of which is associated with the presence of anti-inflammatory immunesuppressive M1 macrophages. Hence the idea of the Bernstein group was to change the fate of M1 macrophages to pro-inflammatory M2 macrophages with enhanced antitumor activity. To this end, they have generated a bispecific Notch-activating agent whereby a Notch binding domain (e.g., HA-Delta4) is linked to a cell-targeting domain (e.g., CD33) that is expressed on tumor cells. Using this strategy in an *in vivo* mouse model where melanoma cells expressed CD33, Notch activation within M1 macrophages led to increased expression of MHC class II and inducible nitric oxide synthase expression, suggesting overall enhancement of antigen-presenting and pro-inflammatory activities of these macrophages. In other studies, development of CAR T cells in the presence of Notch ligand delta-like 1 (DLL1) also resulted in improved tumor-killing activity using an *in vivo* Raji tumor model. Following an alternative approach to activate naive T cells with CD3/CD28 dynabeads in the presence of DLL1, the Bernstein lab was able to alter the memory potential and effector function. In comparison with the control immunoglobulin G treatment, T cells exposed to DLL1 showed enrichment of IL-10 and depletion of IL-4 transcripts by scRNA-seq, which was associated with increased and decreased levels of extracellular secretion of these cytokines, respectively. Overall, these results suggested alteration of immune cell fate by changing the transcriptional and metabolomics states by stimulation with Notch signaling.

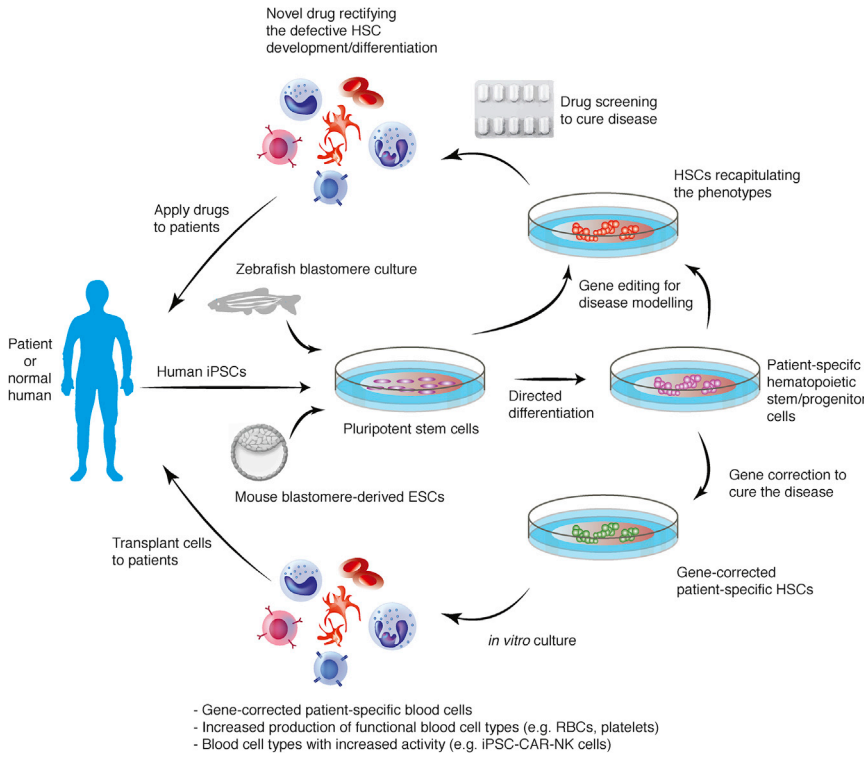

Figure 1. Schematic summarization of the ongoing research regarding different biological systems to benefit HSC-based cellular regenerative therapies presented at the meeting

HSCs provide valuable sources of potential cell-based therapies. The above studies describe the multiple strategies to optimize and scale up *in vitro* production of erythrocytes and platelets for potential transfusion-based therapies. Furthermore, they present the methods to engineer more potent CAR T cells and CAR NK cells and discuss the stimulation of signaling pathways to improve the efficacy of tumor immunotherapies. These reports, along with several studies from other groups (An et al., 2018; Bachanova and Miller, 2014), together could benefit the current and potential future therapies used in the blood transfusion and cancer immunotherapy fields.

Future perspectives

The high quality and immensely informative talks presented at this meeting generated tremendous excitement among the audiences. This meeting clearly highlighted the pace with which the HSC field is moving forward. Continued flow of knowledge about cell-intrinsic biology of HSCs and novel findings about the hematopoietic niche that alters HSC development in a non-cell-autonomous manner would help in discovering new models for hematopoietic disorders and identifying innovative therapeutic strategies (summarized in Figure 1). For example, downregulating WNT/TGFβ pathways could be an exciting new therapeutic direction to increase production of HSCs. Another important direction would be to explore the heterogeneity of endothelium and the factors that define the developmental origin of different endothelial tissues to make them similar or distinct from each other. Apart from endothelium, the downstream complexity of HSC differentiation in distinct blood lineages (e.g., continuous versus specific waves of hematopoiesis through MPP and lymphoid progenitors) makes this process more intriguing. Several talks implicate that EHT and downstream HSC differentiation are highly dynamic and need sequential functioning of several signaling pathways. This highlights the importance of extracellular signaling pathways to maintain the heterogeneity of HSC and its microenvironment. Specific physical/chemical cues within the HSC niche, such as shear stress, electrical current, magnetic field, responsiveness to initial heartbeat, and local concentration of SCF shape the epithelial heterogeneity important for HSC development in AGM that needs further investigation. Relying only on single-cell analysis (e.g., scRNA-seq) in this regard may not be sufficient; rather, it requires development of additional experimental systems that can analyze the extrinsic regulation of hematopoietic precursors.

The meeting participants discussed the translational aspects of basic developmental hematopoiesis for generating *in vitro* blood products in the foreseeable future. Ultimate goals would be to produce "off-the-shelf" RBC/platelets/immune cells from iPSCs to cure hematopoietic disorders.

Future progress in a few key directions would be vital: (1) improvement of bioreactor technology to produce blood products more efficiently; (2) generation of hematopoietic developmental-stage appropriate cellular blood products to treat patients with RBC and platelet disorders; (3) investigation to produce and deliver myeloid progenitors for patients with fungal infections or individuals who are undergoing radiation therapies. Along these lines, delivery of *in vitro* generated neutrophils from iPSCs, which might have reduced net formation to avoid lung blockades, has substantial future potential. Utilization of neutrophil heterogeneity to engineer specific neutrophils that would avoid lung capillaries and target appropriate organs could also be an innovative strategy in this regard.

Finally, the group acknowledged the critical involvement of the National Institutes of Health (NIH)/National Heart, Lung, and Blood Institute (NHLBI) that is crucial for the HSC research and summarized the need for additional NIH-mediated implications of research policies that could benefit the field. The current NIH funding for cellular therapies for regenerative medicine compared with other research topics, e.g., cancer, is scarce. The cellular therapies involving various organs, including HSC-related regenerative medicine, such as stem cell transplant, vascular therapy, and *in vitro* production of blood-related products, are immensely impactful in treating a large repertoire of human diseases. A future review of an extensive list of such potential therapeutic avenues could help NIH further appreciate the impact of regenerative medicine and initiate "moonshot"-like programs that already exist to support cancer research. This is important in the context of the current COVID-19 pandemic that has resulted in a severe blood shortage and has also caused lung and kidney damage even in moderate to mild cases of COVID-19 disease. Future research supporting the *in vitro* generation of these tissues could help these patients. Another NIH policy-related topic that was considered in length was to develop a platform that would appeal against the current restrictions about the use of human fetal tissues to better understand hematopoiesis during early embryogenesis. Although other model systems such as mouse, zebrafish, and human iPSCs have been used in this context, there still exists extensive knowledge gaps regarding how exactly these events happen in early human embryos. This is largely due to the difference in stem and progenitor cell markers, the precise timeline of HSC development, and the vastly distinct anatomy of HSC niche among organisms. The real implications of the proposed HSC-based therapies identified in various systems eventually need to be tested in the human context. Hence, allowance of human fetal research will bridge the cross-species knowledge gaps, promote further collaboration among the groups, and significantly decrease the transition time from initial discovery to patient treatment.

AUTHOR CONTRIBUTIONS

A.C. and T.H. contributed equally to writing the meeting report. L.I.Z. organized the meeting and supervised the meeting report.

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CONFLICTS OF INTEREST

L.I.Z. is a founder and stockholder of Fate Therapeutics, CAMP4 Therapeutics, Amagma Therapeutics, and Scholar Rock. He is a consultant for Celularity and Cellarity outside the submitted work. All other authors declare no conflicts of interest.

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