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Developmental Regulation of Endothelial-to-Hematopoietic Transition from iPSCs

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Abstract

Developmental Regulation of Endothelial-to-Hematopoietic Transition from iPSCs

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Genome Sciences

Hematopoietic stem cells (HSCs) arise in mid-gestation embryos from a specialized hemogenic endothelium (HE). In this process, HE cells undergo a unique fate change termed endothelial-to-hematopoietic transition, or EHT. While induced pluripotent stem cells (iPSCs) give rise to HE with robust hemogenic potential, generation of bona fide HSCs from iPSCs remains a challenge. Here, we map single cell dynamics of EHT during embryoid body differentiation from iPSCs and integrate it with human embryo datasets to identify key transcriptional differences between *in vitro* and *in vivo* cell states. We further map ligand-receptor interactions associated with differential expression of developmental programs in the iPSC system. We found that the expression of endothelial genes was incompletely repressed during iPSC EHT. Elevated FGF signaling by FGF23, an endothelial pathway ligand, was associated with differential gene expression between *in vitro* and *in vivo* EHT. Chemical inhibition of FGF signaling during EHT increased HSPC generation in the zebrafish system, while an FGF agonist had the opposite effect. Consistently, chemical inhibition of FGF

signaling increased hemogenic output from iPSCs. In summary, we map the dynamics of EHT from iPSCs at single cell resolution and identify ligand-receptor interactions that can be modulated to improve iPSC differentiation protocols. We show, as proof of principle, that chemical inhibition of FGF signaling during EHT improves hematopoietic output in zebrafish and the iPSC system.

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Statement of Previous Publication

Large portions of this dissertation are being simultaneously published on bioRxiv and will also eventually be published in a peer-reviewed journal. The primary author, Rachel Courtney Wellington, reserves the right to republish this work at any time.

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Data and Code Availability

All code used to generate figures and data for this manuscript can be found on GitHub. All sequencing files generated can be found on GEO. A web interface for the data is also provided by the Chan Zuckerberg Initiative CELLxGENE platform. The GitHub, GEO and CELLxGENE information can be found in the bioRxiv paper listed in [Statement of Previous Publication](#).

Chapter 1. Introduction

1.1 Hematopoietic stem cells (HSCs) as a therapy for blood disorders

Hematopoietic stem cells (HSCs) are defined by three main characteristics. First, they can give rise to all the various blood cell types, including all subsets of erythroid (erythrocytes, megakaryocytes, etc.) and lymphoid (T cells, etc.) cells. Second, they must be able to self-renew. Last, they must be able to engraft, meaning that they must be able to both self-renew and generate all blood cell types when transplanted into an individual. Due to these three characteristics, HSCs are a valuable treatment for various blood disorders as they can repopulate a patient's blood system^{1, 2}.

HSC transplantation (HSCT) has been a recognized therapeutic strategy for over 60 years for both hereditary and malignant disorders². The two most common types of HSCT are autologous, which involves transplantation of a patient's own HSCs, and allogeneic, which involves the transplantation of HSCs from an HLA-matched or partially matched donor. In 2017, the European Society for Blood and Marrow Transplantation (EBMT) reported that 59% of transplants they recorded in 2015 were autologous and 41% were allogeneic². Autologous HSCT is used for the treatment of a subset of malignancies and autoimmune disorders, with the advantage of avoiding graft-versus-host disease (GVHD). However, autologous treatment for disorders that stem from genetic conditions is contraindicated, as it does not correct inherited mutations found in the patient's HSCs, and it is also generally contraindicated in the treatment of acute leukemias, where the graft-versus-leukemia (GVL) effect of an allogeneic graft is critical to prevent disease relapse. Additionally, HSCs are an incredibly rare cell type in the human adult¹ and often many HSCs are needed for genetic editing, so discovering a

way to generate a large population of them is critical for improved HSCT. This issue has led to a widespread interest in being able to generate HSCs from patient-derived, corrected induced pluripotent stem cells (iPSCs), which can be induced to generate patient-specific HSCs through the recapitulation of human embryonic development *in vitro*.

1.2 Embryonic hematopoietic development

In the embryo (human, mouse and zebrafish), hematopoiesis occurs in multiple waves: the initial HSC-independent waves directly produce a variety of lineage-restricted hematopoietic progenitors (HPCs) and a second wave where HSCs are first generated and become a required precursor to sustained HPC production for the life of an organism³⁻⁸. During both HSC-independent and HSC-competent waves, hematopoietic cells are generated from specialized hemogenic endothelium (HE) precursors in a process called endothelial-to-hematopoietic transition (EHT), a unique fate change that involves concordant downregulation of endothelial (i.e. VEGF and FGF) and induction of hematopoietic expression (i.e. CD43 and lineage-specific markers) programs^{9, 10} (**Figure 1.4.1**).

In human embryos, the first waves of EHT start in the yolk sac around Carnegie stage (CS) 7 and involves the sequential production of lineage-restricted erythroid-myeloid precursors (EMPs), lymphoid/myeloid progenitors (LMP), and multipotent progenitors (MPP)^{6, 11}. The generation of HSCs capable of reconstituting hematopoiesis following transplantation begins in the aorta-gonad-mesonephros (AGM) region around CS14^{12, 13}. Nascent HSCs and HPCs egress into the bloodstream and colonize the fetal

liver around CS16-17 and then bone marrow at birth¹². It is generally recognized that between CS12-14 (corresponding to E9.5-10.5 and 24-48 hours post-fertilization in mouse and zebrafish embryogenesis, respectively) intra-embryonic HE undergoes developmental maturation to produce HSC-competent HE^{3, 14}.

The developmental maturation of HE involves a variety of well-known signaling mechanisms in the context of the embryo (i.e. TGF β , Notch, Wnt/ β -catenin, CXCL12, hemodynamic forces, etc.). For example, autocrine Transforming Growth Factor β (TGF β) signaling initiates Notch signaling via promotion of Jagged1 (JAG1) expression leading to HE programming while paracrine TGF β signaling helps drive EHT¹⁵. Notch1 is required for the emergence of HE from developing arterial vessels while both Notch 1 and Notch 2 are involved in subsequent embryonic HSC expansion¹⁶. Wnt/ β -catenin has been shown to be required for both HSC specification and function¹⁷. As a final example, CXC motif chemokine ligand 12 (CXCL12) signaling, predominantly through CXC motif chemokine receptor 4 (CXCR4), independently suppresses the endothelial cell program while hematopoietic programs are activated to establish the hemogenic endothelium state¹⁸.

Since EHT is a transient cell state, there has been considerable interest in capturing this developmental transition using high-depth single cell RNA sequencing (sc-RNA-seq). A number of reports have provided a single cell map of EHT during mouse embryogenesis¹⁹⁻²³. In human embryos, Zeng et al.³ first mapped EHT during early human embryogenesis (CS10-13), a developmental window preceding HSC emergence. Crosse et al.⁴, and more recently Calvanese et al.⁵, have generated single cell maps of EHT during the window of HSC formation (CS14-17) in human

embryogenesis. The development of these human embryo EHT transcriptomic maps provides a unique opportunity for the direct comparison of embryo hematopoiesis to iPSC hematopoiesis to address improvements to the current protocols for HSC development *in vitro*.

1.3 Hematopoietic development from iPSCs

In the iPSC system, temporal dynamics of EHT have also been well defined²⁴⁻²⁶. Mesoderm is established by day 3 of human iPSC differentiation and requires BMP signaling (i.e. BMP4) for its establishment. A subset of this mesoderm, Vascular Endothelial Growth Factor Receptor 2 / Kinase Insert Domain Receptor (VEGFR2/KDR)⁺ Glycophorin A (CD235a)⁺, is associated with the development of hematopoietic precursors that give rise to HSC-independent HE and requires Activin-nodal signaling for its establishment, whereas KDR⁺ CD235a^{neg} mesoderm is capable of giving rise to potentially Definitive CD34⁺CD43^{neg} HE first arises from mesodermal precursors around day 6 of human iPSC differentiation and peaks around day 8²⁵. This HE can be further isolated based on expression of CD76 and CD44, has recently been shown to express CD32, and can undergo EHT to give rise to multilineage hematopoietic progenitors²⁴⁻²⁶. Despite the difference in time scales, iPSC-derived hematopoiesis generally recapitulates the stages of embryo hematopoietic ontogeny, critically arterialized endothelium, a precursor to HSC-competent HE^{29, 30}, and definitive CD32⁺ HE capable of clonal multilineage hematopoiesis²⁶.

Although several single cell transcriptomic studies have captured iPSC-derived EHT^{3, 5, 24, 29, 31, 32}, we still lack a high-resolution map of stepwise gene expression changes during EHT in iPSC differentiation with direct comparison to hematopoiesis in

the embryo. Key regulators of hematopoietic ontogeny including transcription (e.g., RUNX1, GATA2, c-MYB), epigenetic (EZH1/2, SUV39), signaling (Notch, Wnt, TGF), inflammatory (NFκB, IFN-γ, IL-1β), and biophysical (stretch, blood flow) factors have been identified³³. For example, in the context of the iPSC system, Wnt/β-catenin inhibition leads to the production of HSC-independent HE while its activation leads to a more mature-like HE state²⁸. EZH1, but not EZH2, appears to inhibit the production of multi-lineage progenitors, such as HSCs³⁴. Notch activation with an immobilized Notch ligand has been shown to be sufficient to drive HSC expansion post-specification¹⁶. This is just a few examples of what is known about factors involved in HE and HSC specification. However, despite this knowledge, HSCs have still never been reproducibly generated from iPSCs. Therefore, a better understanding of the stage-specific regulation of these factors would be instrumental to improve iPSC differentiation protocols. Direct comparison between carefully staged *in vitro* and *in vivo* cell states spanning hematopoietic ontogeny would facilitate the identification of targetable pathways that can improve iPSC-derived hematopoiesis.

1.4 Figures and Legends

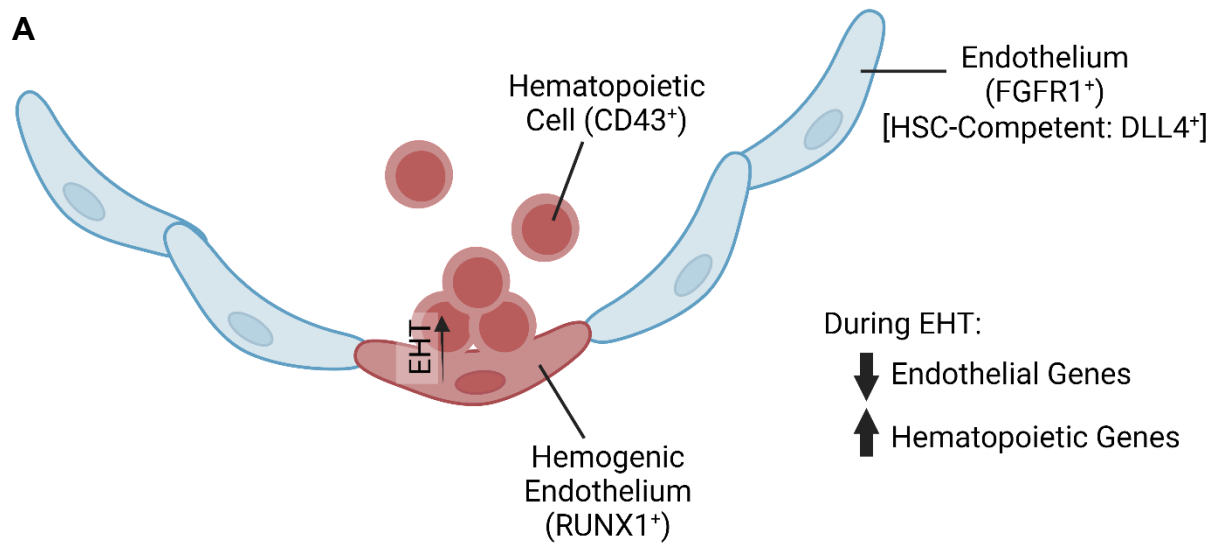


Figure 1.4.1. Hematopoietic Ontogeny. A. Simplified model of the endothelial-to-hematopoietic transition (EHT), its precursors and a few of the markers involved.

Chapter 2. Single-cell profiling of hematopoiesis from iPSCs reveals a limited *in vitro* endothelial-to-hematopoietic transition window

2.1 Introduction

Hematopoietic stem cells (HSCs) with the potential to sustain the hematopoietic system for life arise in mid-gestation from a specialized population of hemogenic endothelium (HE) lining the dorsal aorta and major embryonic arteries^{6, 33, 35}. In this process, HE cells undergo endothelial-to-hematopoietic transition (EHT), a unique fate change that involves concordant downregulation of endothelial and induction of hematopoietic expression programs^{9, 10}. Induced pluripotent stem cells (iPSCs) represent a therapeutically valuable source of human hematopoietic cells³⁶. When induced to differentiate into hematopoietic lineages, iPSCs give rise to definitive HE with multilineage hematopoietic potential³⁷. However, to date, the generation of bona fide HSCs remains an inefficient process. This broadly suggests that there are differences in hematopoietic ontogeny during iPSC differentiation compared to hematopoiesis in the embryo. While important progress has been made in mapping EHT in human embryogenesis at the single cell level³⁻⁵, we lack a high-resolution map of EHT during iPSC differentiation.

In the iPSC system, the temporal dynamics of EHT have been well defined²⁴⁻²⁶. Definitive CD34⁺CD43^{neg} HE first arises from mesodermal precursors around day 6 of human iPSC differentiation and peaks around day 8²⁵. This HE can be further isolated based on expression of CD76 and CD44, has recently been shown to express CD32, and can undergo EHT to give rise to multilineage hematopoietic progenitors²⁴⁻²⁶. While hematopoietic development occurs in the human embryo over the course of many

weeks, hematopoietic development in the iPS system typically occurs over only about 2 weeks. Despite a difference in time scales, iPSC-derived hematopoiesis generally recapitulates the stages of embryo hematopoietic ontogeny, critically arterialized endothelium, a precursor to HSC fate^{29, 30}, and definitive CD32⁺ HE capable of clonal multilineage hematopoiesis²⁶. However, despite all this knowledge, a comprehensive single cell map of all the cell types that arise *in vitro* during hematopoietic differentiation has not been generated as most studies focus on the mesodermal precursors and specific EHT-related populations. Therefore, we generated an unbiased single cell atlas for all cell types arising in the embryoid body throughout EHT from iPSCs.

2.2 Results

2.2.1 Single-cell temporal profiling of hematopoietic ontogeny *in vitro*

The endothelial-to-hematopoietic transition (EHT) is a key event in hematopoietic ontogeny; however, the dynamics of EHT during iPSC differentiation have not been deeply profiled in comparison to the embryo. To capture the dynamic transcriptional landscape of EHT, we first performed an unbiased single cell transcriptional profiling of embryoid bodies (EBs) cultured under hematopoietic differentiation conditions. We collected nuclei from whole EBs on days 7, 8, 11, 14, 18 and 21 of differentiation and performed well-based single cell combinatorial indexing RNAseq (sci-RNA-seq) enabling the capture of a large number of cells for assessment of hematopoietic development (**Figure 2.4.1A**). After standard filtration steps (see **Methods**), sequencing data for a total of 166,085 cells across all days of differentiation were acquired (**Figure 2.4.1B**).

Cell populations were identified based on the top relative expression score of previously identified CS12-16 whole embryo cell type gene marker sets³⁸ for

each individual cell cluster (**Figure 2.4.1B, Figure S2.4.1A-D**). We further refined this annotation using extended cell type marker scores (**Figure S2.4.1D**). This analysis confirmed that EBs are comprised of cell types from all germ layers: mesoderm (somite, blood, fibroblast, lateral plate mesoderm), endoderm, and ectoderm (neural, Schwann, epithelium). Hematopoietic cells were further characterized based on the expression of *RUNX1*, *SPN* (CD43), and *PTPRC* (CD45), while precursor endothelial populations were identified by the expression of *CDH5*, *KDR*, *TIE1*, and *CD34* (excluding *RUNX1/SPN/PTPRC* hematopoietic cells) (**Figure 2.4.1C-D, Figure S2.4.1E-F**).

Hematopoietic and endothelial populations were connected by a “bridge”, representing HE cells undergoing EHT to acquire hematopoietic cell fate prior to differentiating into various hematopoietic lineages. Endothelial cells proximal to the bridge acquired arterial markers, such as *DLL4*, consistent with arterial endothelial (AE) cells as the precursor of HE (**Figure 2.4.1C**). Notably, EHT was robust in early ontogeny peaking around day 8 and declining thereafter, with fewer cells transiting at day 11, and EHT virtually ceasing by day 18 of differentiation (**Figure 2.4.1D, Figure S2.4.1F**). At the same time, endothelial and hematopoietic populations became more separated over time, indicating their progressive transcriptional divergence later in ontogeny (**Figure 2.4.1D, Figure S2.4.1F**). These results show that, akin to embryogenesis, EHT is a transient cell state during iPSC differentiation with the peak in our system around day 8, consistent with the known timing of EHT across various differentiation protocols^{25, 30, 34, 39-41}.

2.2.2 Hemogenic Endothelium Receives Signals from Various Cell Populations Throughout Differentiation

Key signaling pathways that are regulators of hematopoietic ontogeny have been previously identified, such as those involving bone morphogenic protein (BMP), transforming growth factor β (TGF β), neurogenic locus notch homolog (NOTCH), WNT, fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF)³³. A better understanding of how these signals are transmitted *in vitro*, particularly at the level of HE as it undergoes EHT, would be instrumental to improving iPSC differentiation protocols. To investigate the signaling interactions of HE during *in vitro* differentiation, we utilized CellPhoneDB to identify cell-cell interactions on each day of differentiation based on the transcript levels of interaction partner pairs expressed in HE (cluster 18) and the cells of other clusters (**Figure 2.4.1B, Figure S2.4.1C**). We identified several signaling pathways that have been previously identified as influencing hematopoietic ontogeny such as BMP, VEGF, NOTCH, FGF and TGF β signaling. These signals persist at multiple days of differentiation and HE receives these signals from heterogeneous predicted cell sources, reflective of the secretion of many of the involved ligands allowing for both local and distant signaling.

This analysis led to several observations. First, BMP ligands are predicted to be both received and secreted by HE during the time points where EHT is most active (days 7-8) up until the time that endothelium and blood begin to separate into distinct trajectories and stop undergoing EHT (day 14) (**Figure 2.4.2A,B, Figure S2.4.2A-G**). Second, VEGF and FGF signaling persists throughout EHT (**Figure 2.4.3A,B, Figure S2.4.3A-H**). Third, HE receives NOTCH signaling via DLL4 throughout EHT, reflective of there being arterialized HE *in vitro* (**Figure 2.4.4A, Figure S2.4.4A-F**). Finally, HE TGF β

and WNT signaling involve a wide range of cell partners, reflective of the broad utility of these pathways in both hematopoietic ontogeny and other cellular functions (**Figure S2.4.5A-H, Figure S2.4.6A-G**).

We also identified cell-cell interactions between all clusters during iPSC hematopoietic differentiation spanning EHT (day 7: **Figure S2.4.7**, day 8: **Figure S2.4.8**, day 11: **Figure S2.4.9**, day 14: **Figure S2.4.10**). Surprisingly, significant cell-cell interactions were detected only for a subset of clusters. This is likely in part due to the limited set of interactions considered by CellPhoneDB in comparison to other methods, as CellPhoneDB prioritizes the comprehensiveness of the interactions in individual pathways in their database over the number of pathways⁴². However, CellPhoneDB does consider most of the pathways known to be involved in hematopoietic development in addition to others. The number of interactions detected is also reflective of the nature of the sequencing done, since we primarily detected actively produced transcripts given the use of nuclei over whole cells.

2.3 Discussion

EHT is a critical transient stage in hematopoietic ontogeny. Here, we interrogate the single cell kinetics of EHT during iPSC differentiation and the cellular signals that direct it. Although several single cell transcriptomic studies have captured iPSC-derived EHT^{3, 5, 24, 29, 31, 32}, we still lack a high-resolution map of gene expression changes during EHT in iPSC differentiation and the cell populations that arise during *in vitro* hematopoiesis that provide critical developmental signals. To this end, we performed an unbiased single cell transcriptional profiling of whole EBs using sci-RNA-seq, which showed that, akin to the embryo, EHT is a transient process that peaks around day 8 of

differentiation in our EB system. Additionally, we used this high-resolution map to develop a list of signaling interactions associated with key signaling pathways as well as predicted pathways not previously defined. This analysis will provide a framework for further investigation into the improvement of iPSC protocols.

2.4 Figures and legends

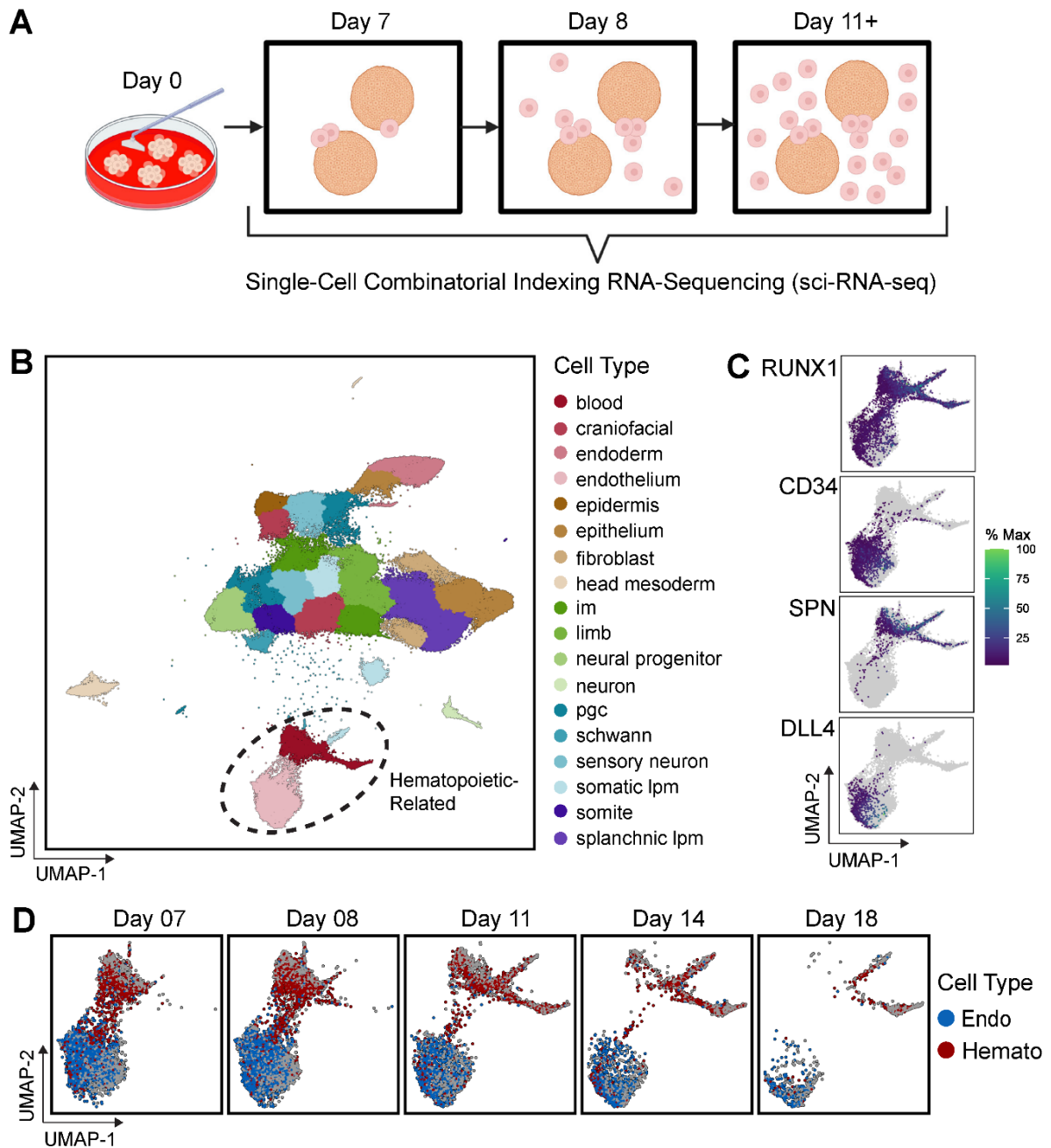
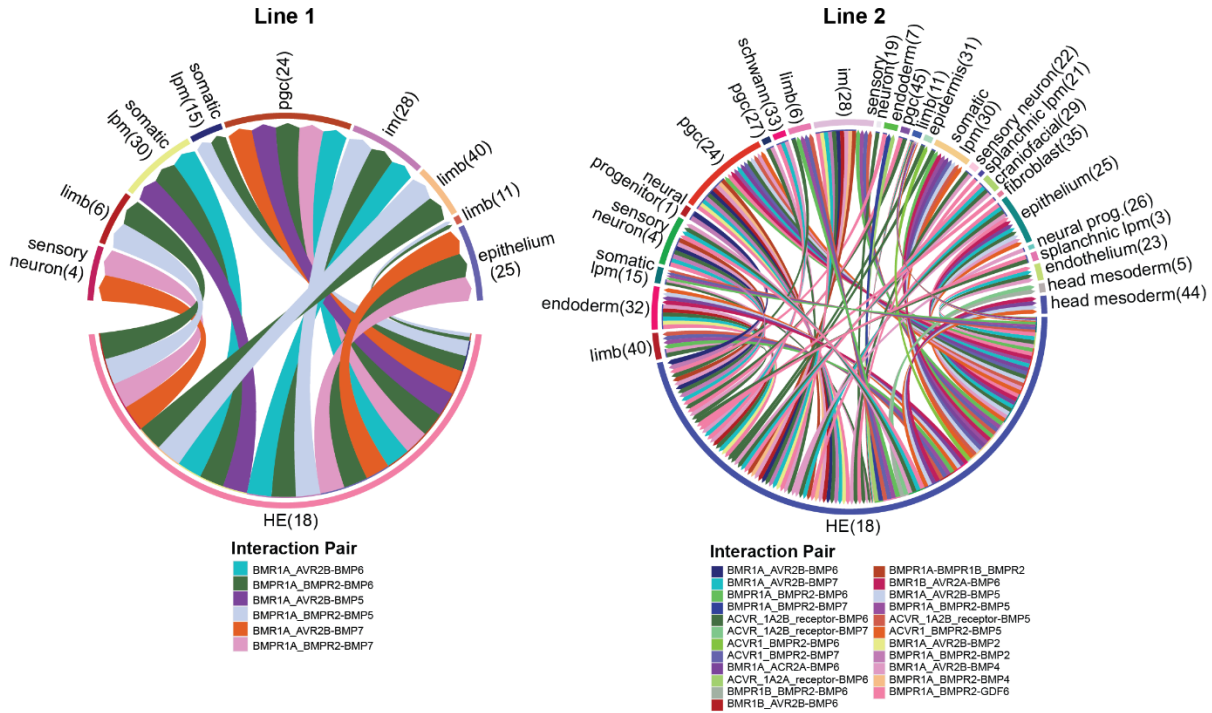


Figure 2.4.1. Single cell combinatorial indexing RNA-sequencing reveals limited EHT window in vitro. (A) Experimental scheme of single cell profiling during iPSC differentiation into HPCs. Whole EBs were dissociated on days 7, 8, 11, 14, 18 and 21 of EB differentiation and nuclei profiled by sci-RNA-seq. (B) UMAP dimensional reduction of combined sci-RNA-seq data from days 7, 8, 11, 14, 18 and 21 days of EB

differentiation. Cell types are labeled based on the maximal scores of gene signatures from Xu et al (ref. 34) shown in Figure S2.4.1A. The dashed circle contains the hematopoietic and endothelial populations. (C) Expression of RUNX1, CD34, SPN (CD43), and DLL4 transcripts in the hematopoietic and endothelial populations. (D) Hematopoietic (red) and endothelial (blue) populations shown by day of differentiation showing the temporal dynamics of hematopoiesis. Cell types were identified as hematopoietic (RUNX1, SPN, PTPRC aggregate gene score) or endothelial (CDH5, KDR, TIE1, CD34, and zero hematopoietic aggregate gene score). All data is the combination of samples from two iPSC lines: Line 1 is an iPS line derived from CD45+ hematopoietic cells and Line 2 is an iPS line derived from mesenchymal stem cells.

A *BMP Signaling, Day 7*



B BMP Signaling, Day 8

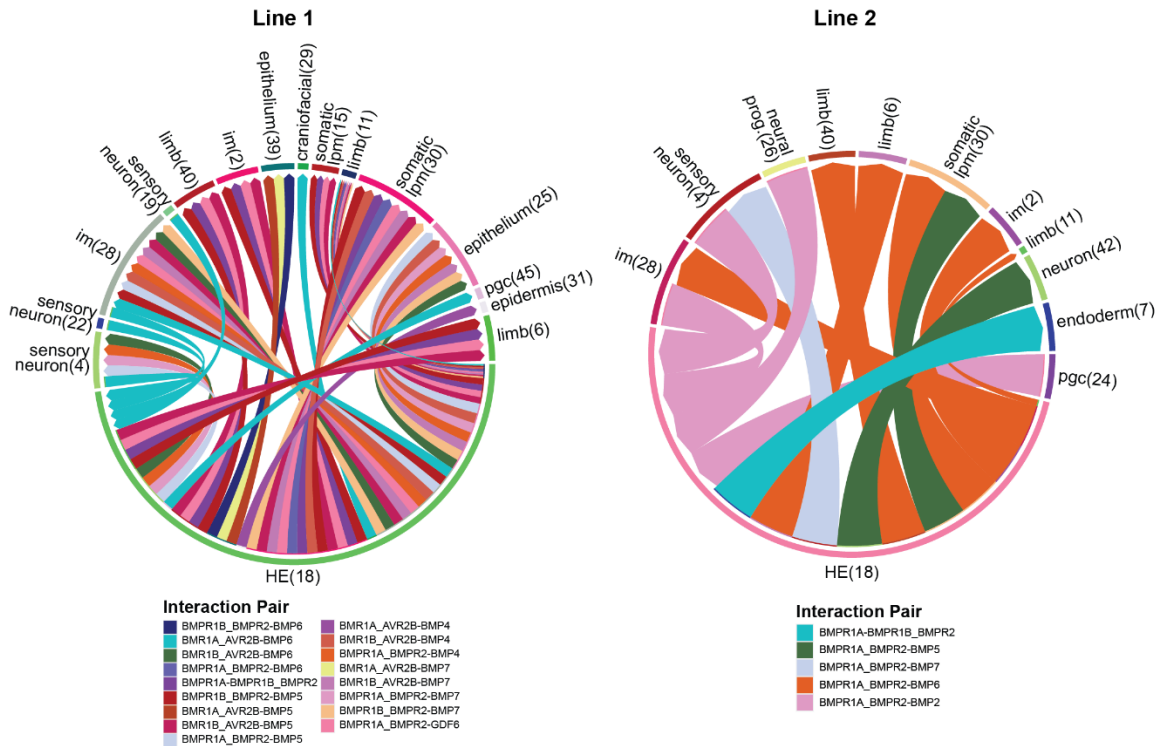
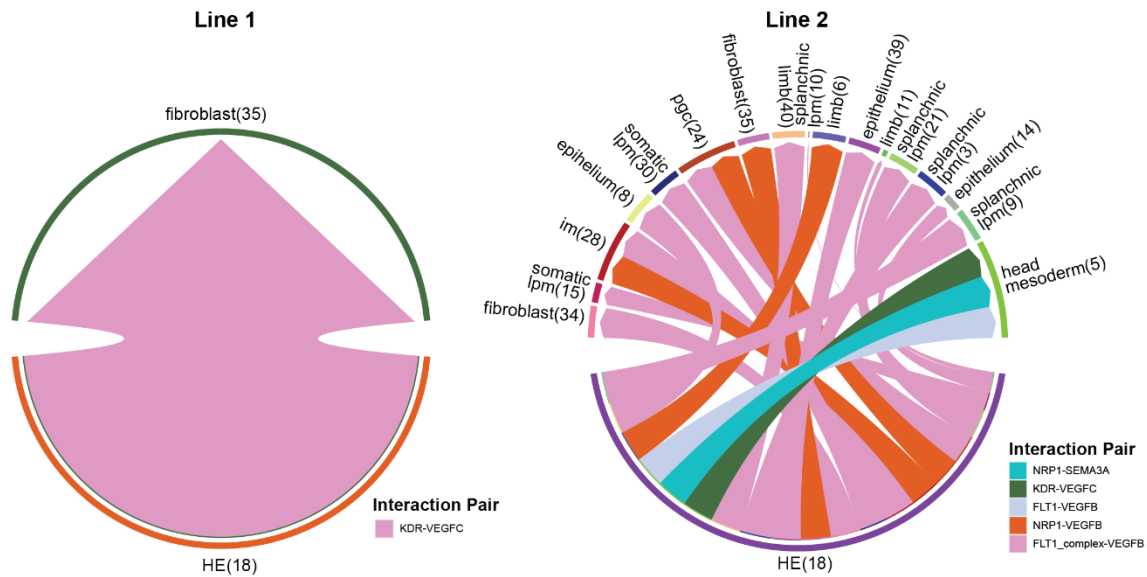


Figure 2.4.2. BMP Signaling Between HE and Other Populations. (A-B)
CellPhoneDB predicted bone morphogenic protein (BMP) signaling interactions on day 7 (A) and day 8 (B) of iPSC hematopoietic differentiation between HE (cluster 18) and

other cell clusters as identified in Figure S2.4.1C with cell type annotations based on Figure 2.4.1B. Arrow heads indicate the receiver cell type and the flat ends of connections represent the producer cell type. Color-coded interaction types are indicated in the legend as “receiver”-“producer”. Line 1 is an iPS line derived from CD45+ hematopoietic cells and Line 2 is an iPS line derived from mesenchymal stem cells.

A VEGF Signaling, Day 7



B VEGF Signaling, Day 14

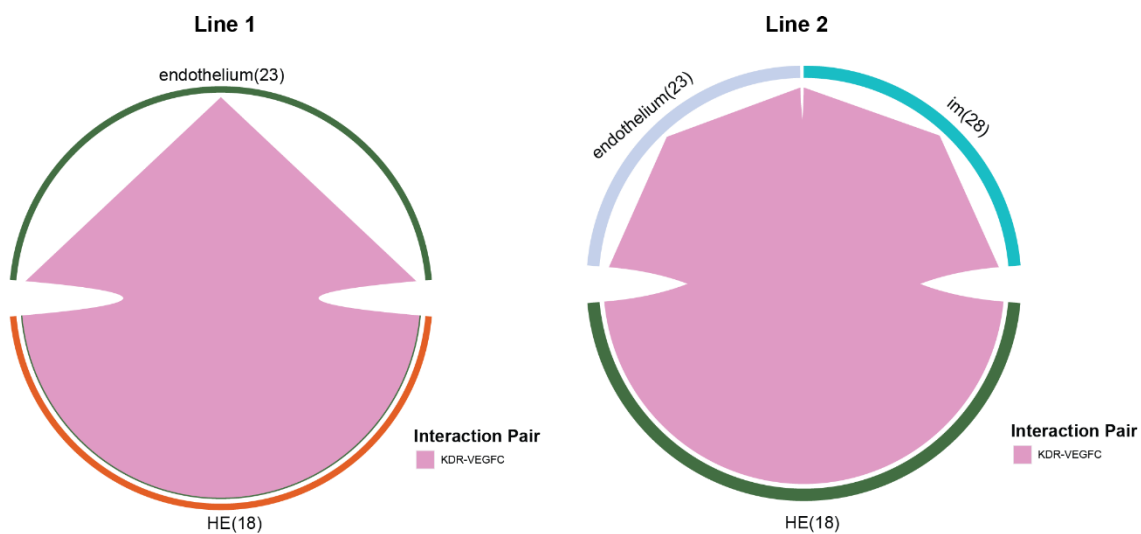


Figure 2.4.3. VEGF Signaling Between HE and Other Populations. (A-B)

CellPhoneDB predicted Vascular Endothelial Growth Factor (VEGF) signaling interactions on day 7 (**A**) and day 14 (**B**) of iPSC hematopoietic differentiation between HE (cluster 18) and other cell clusters as identified in Figure S2.4.1C with cell type annotations based on Figure 2.4.1B. Arrow heads indicate the receiver cell type and the flat ends of connections represent the producer cell type. Color-coded interaction types are indicated in the legend as “receiver”-“producer”. Line 1 is an iPS line derived from CD45⁺ hematopoietic cells and Line 2 is an iPS line derived from mesenchymal stem cells.

A *Notch Signaling, Day 7*

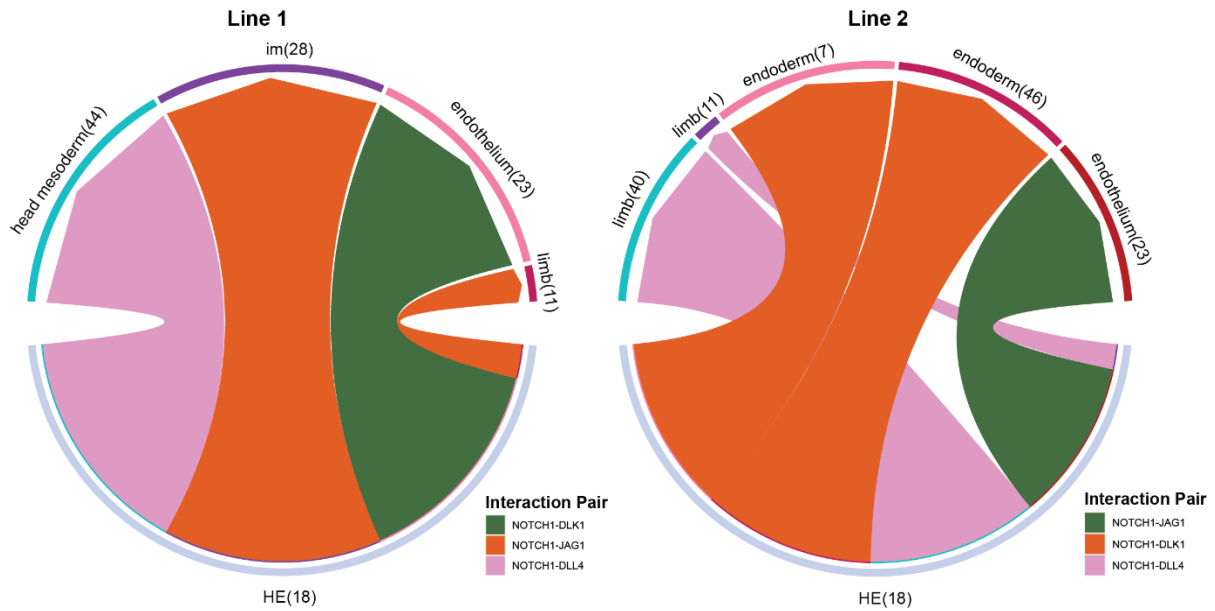


Figure 2.4.4. Notch Signaling Between HE and Other Populations. (A)

CellPhoneDB predicted NOTCH signaling interactions on day 7 of iPS cell hematopoietic differentiation between HE (cluster 18) and other cell clusters as identified in Figure S2.4.1C with cell type annotations based on Figure 2.4.1B. Arrow heads indicate the receiver cell type and the flat ends of connections represent the producer cell type. Color-coded interaction types are indicated in the legend as “receiver”-“producer”. Line 1 is an iPS line derived from CD45+ hematopoietic cells and Line 2 is an iPS line derived from mesenchymal stem cells.

34). B. Cell type scoring of sci-RNA-seq clusters based on Xu et al. table S1B (ref. 34). C. Clusters identified using Monocle 3 for the sci-RNA-seq data. D. Clusters labeled with the maximum cell type score from (B). E. Percentages of hematopoietic-related cells relative to all cells separated by day of differentiation and iPSC line. F. sci-RNA-seq populations separated by day of collection with identification of cell types as either hematopoietic (aggregate RUNX1+, CD43+, CD45+ gene score) or endothelial (CDH5+, KDR+, TIE1+, CD34+ and zero hematopoietic aggregate gene score). All data is the combination of samples from two iPSC lines: Line 1 is an iPS line derived from CD45+ hematopoietic cells and Line 2 is an iPS line derived from mesenchymal stem cells.

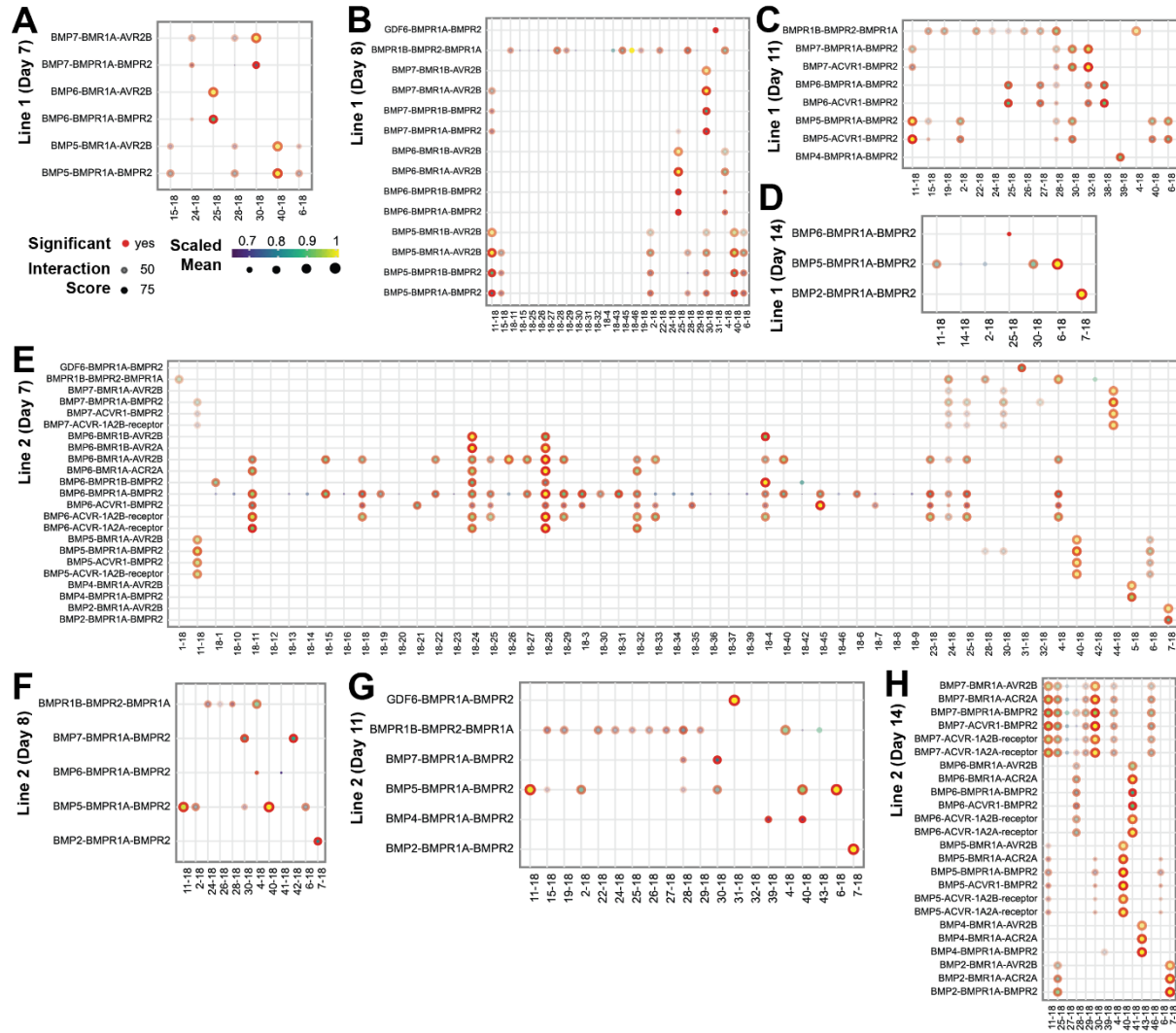
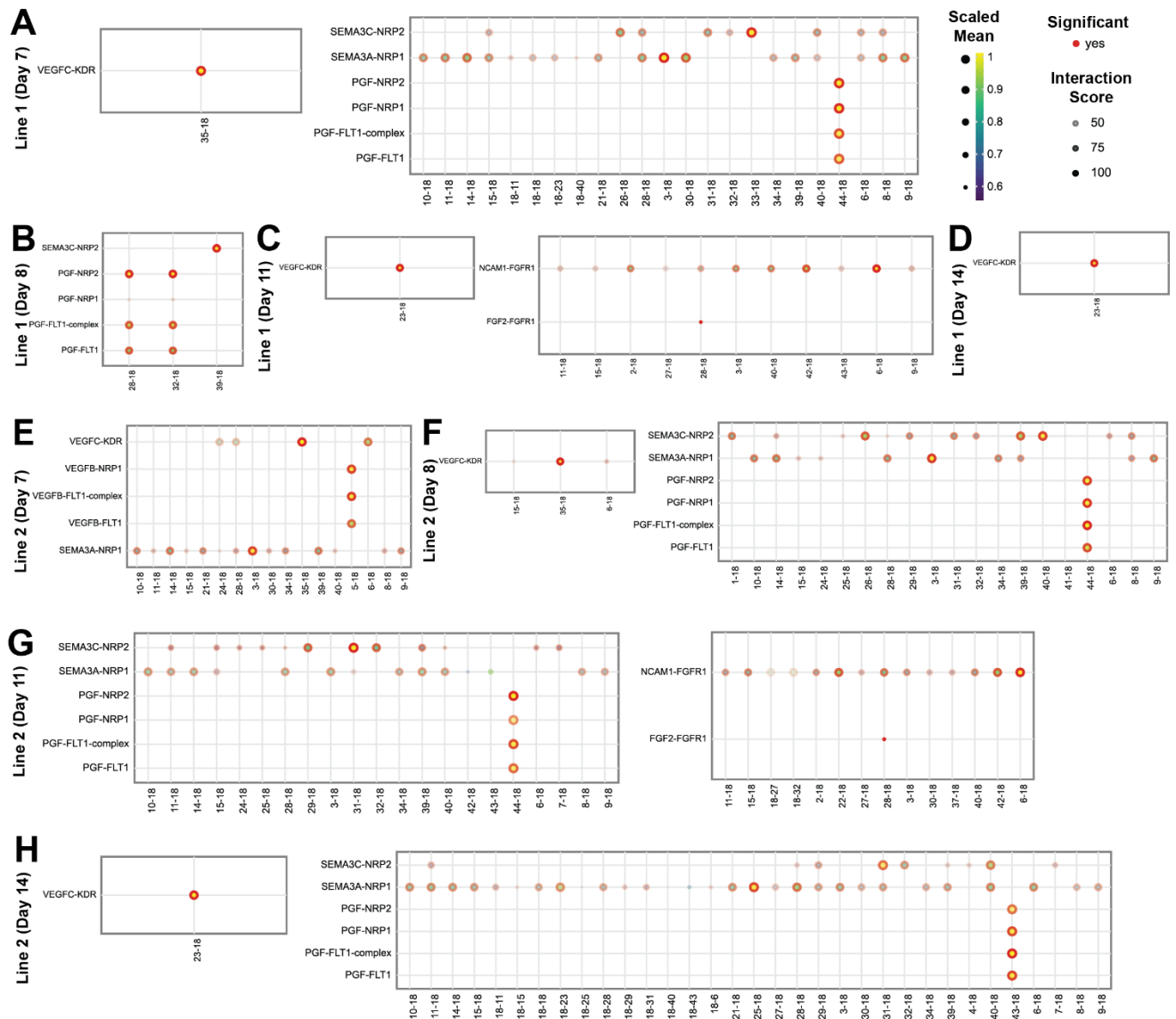


Figure S.2.4.2. Extended HE BMP Signaling Interactions. (A-D) CellPhoneDB predicted bone morphogenic protein (BMP) signaling interactions of iPSC hematopoietic differentiation between HE (cluster 18) and other cell clusters as identified in Figure S2.4.1C for day 7 (A), day 8 (B), day 11 (C) and day 14 (D) of iPSC hematopoietic differentiation. (E-H) CellPhoneDB predicted BMP signaling interactions of iPSC hematopoietic differentiation between HE (cluster 18) and other cell clusters as identified in Figure S2.4.1C for day 7 (E), day 8 (F), day 11 (G) and day 14 (H) of iPSC hematopoietic differentiation. The legend represented in (A) is also used for (B-H). The scaled mean represents the relative average ligand and receptor expression involved in the interactions and is denoted by both the color and size of the dots in the plots. Significance is denoted by a red outline of the dots. The interaction scores are denoted by the transparency of each dot. Line 1 is an iPS line derived from CD45+ hematopoietic cells and Line 2 is an iPS line derived from mesenchymal stem cells.



CD45+ hematopoietic cells and Line 2 is an iPS line derived from mesenchymal stem cells.

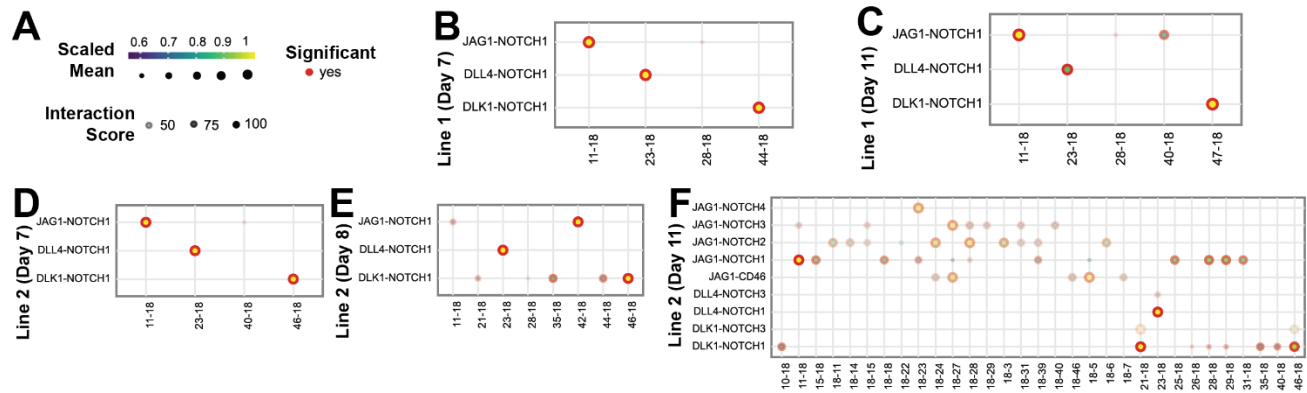


Figure S2.4.4. Extended HE Notch Signaling Interactions. (A) The legend for (B-F). The scaled mean represents the relative average ligand and receptor expression involved in the interactions and is denoted by both the color and size of the dots in the plots. Significance is denoted by a red outline of the dots. The interaction scores are denoted by the transparency of each dot. (B-C) CellPhoneDB predicted NOTCH signaling interactions of iPSC hematopoietic differentiation between HE (cluster 18) and other cell clusters as identified in Figure S2.4.1C for day 7 (B) and day 11 (C) of iPSC hematopoietic differentiation. (D-F) CellPhoneDB predicted NOTCH signaling interactions of iPSC hematopoietic differentiation between HE (cluster 18) and other cell clusters as identified in Figure S2.4.1C for day 7 (D), day 8 (E) and day 11 (F) of iPSC hematopoietic differentiation. Line 1 is an iPS line derived from CD45+ hematopoietic cells and Line 2 is an iPS line derived from mesenchymal stem cells.

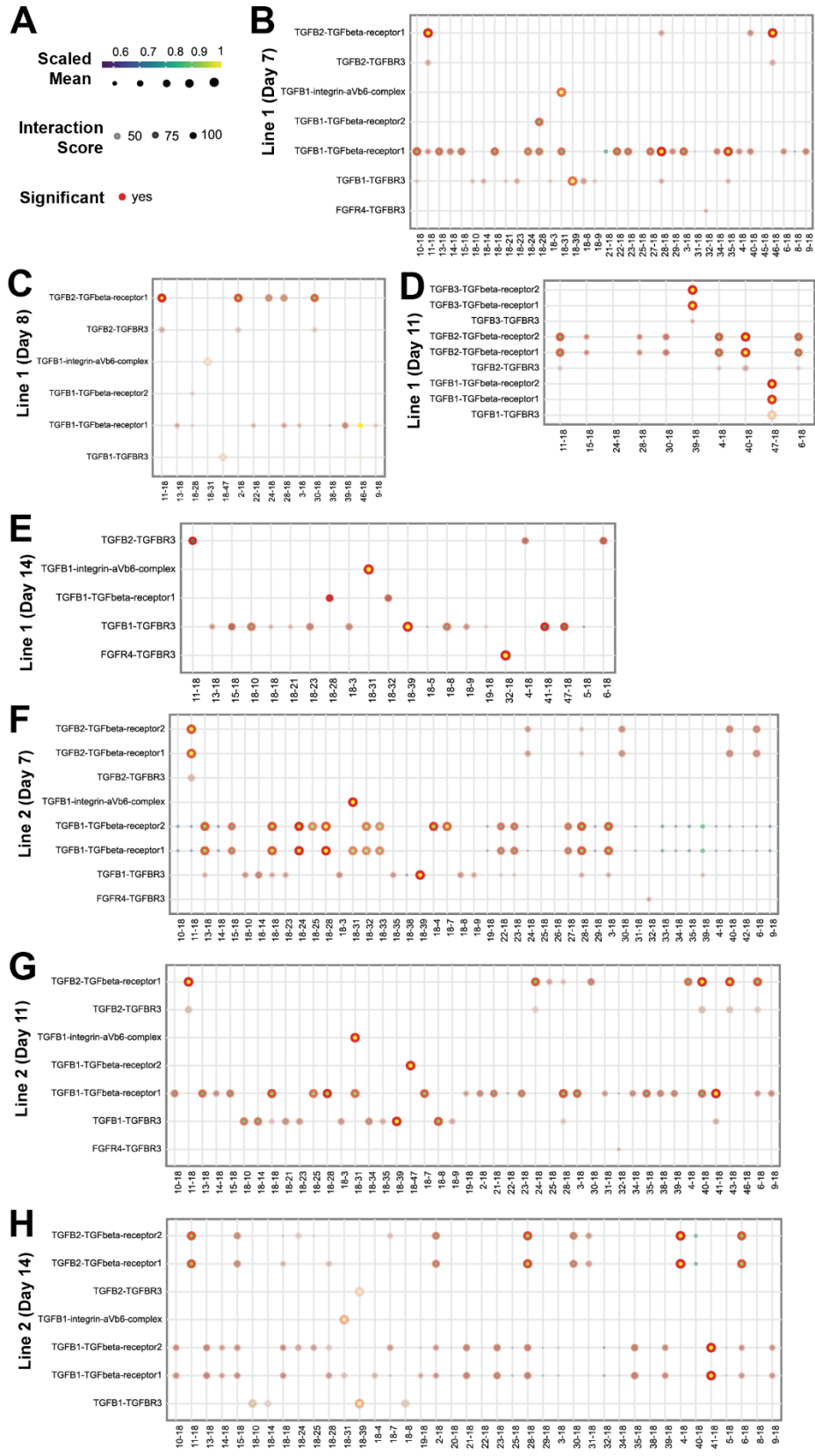


Figure S2.4.5. HE TGF β Signaling Interactions. (A) The legend for (B-H). The scaled mean represents the relative average ligand and receptor expression involved in the interactions and is denoted by both the color and size of the dots in the plots. Significance is denoted by a red outline of the dots. The interaction scores are denoted by the transparency of each dot. (B-E) CellPhoneDB predicted transforming growth factor β (TGF β) signaling interactions of iPSC hematopoietic differentiation between HE (cluster 18) and other cell clusters as identified in Figure S2.4.1C for day 7 (B), day 8 (C), day 11 (D) and day 14 (E) of iPSC hematopoietic differentiation. (F-H) CellPhoneDB predicted TGF β signaling interactions of iPSC hematopoietic differentiation between HE (cluster 18) and other cell clusters as identified in Figure S2.4.1C for day 7 (F), day 11 (G) and day 14 (H) of iPSC hematopoietic differentiation. Line 1 is an iPS line derived from CD45⁺ hematopoietic cells and Line 2 is an iPS line derived from mesenchymal stem cells.

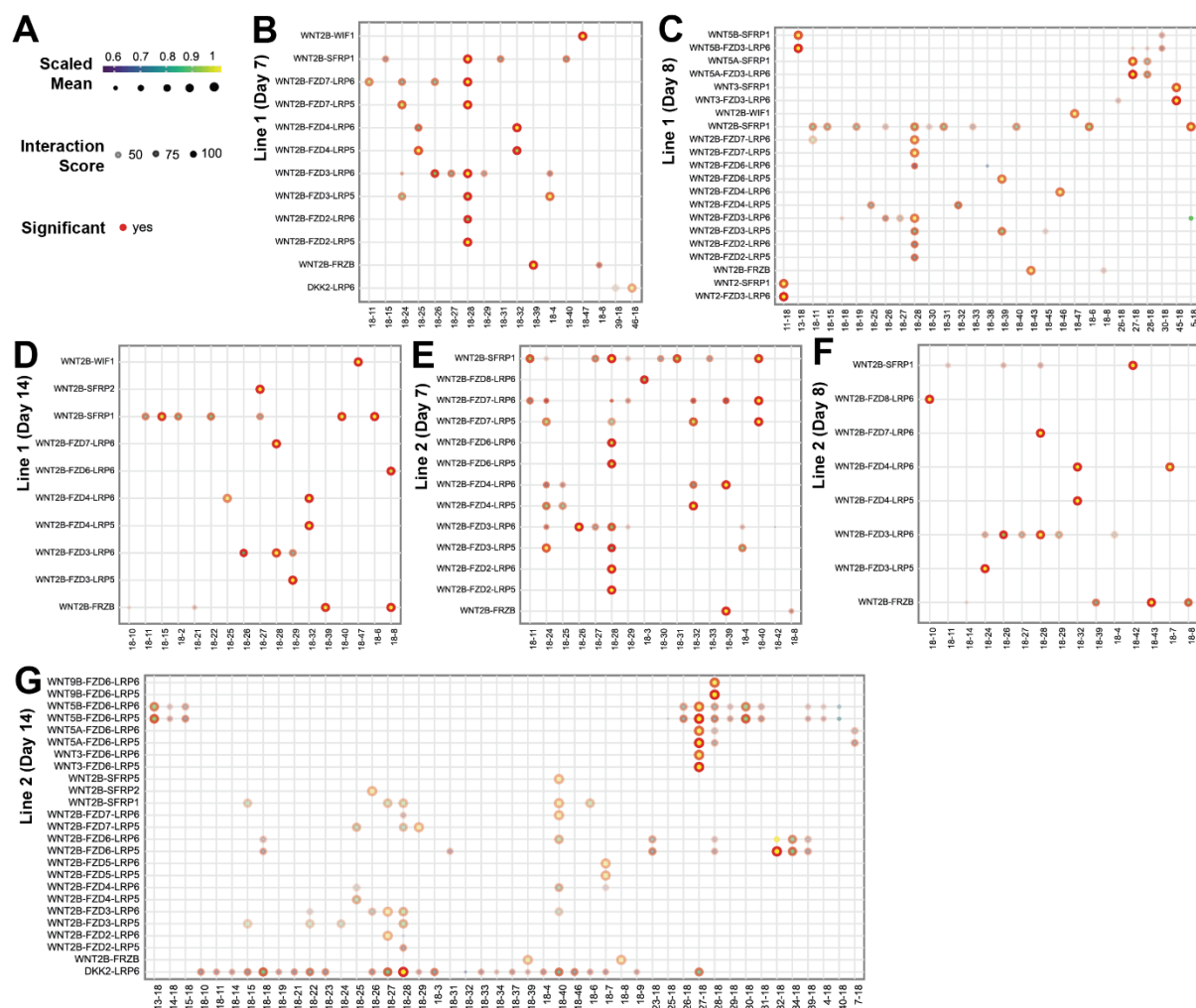


Figure S2.4.6. HE WNT Signaling Interactions. (A) The legend for (B-G). The scaled mean represents the relative average ligand and receptor expression involved in the interactions and is denoted by both the color and size of the dots in the plots. Significance is denoted by a red outline of the dots. The interaction scores are denoted by the transparency of each dot. (B-D) CellPhoneDB predicted transforming growth factor β (TGF β) signaling interactions of iPSC hematopoietic differentiation between HE (cluster 18) and other cell clusters as identified in Figure S2.4.1C for day 7 (B), day 8 (C), and day 14 (D) of iPSC hematopoietic differentiation. (E-G) CellPhoneDB predicted TGF β signaling interactions of iPSC hematopoietic differentiation between HE (cluster 18) and other cell clusters as identified in Figure S2.4.1C for day 7 (E), day 8 (F) and day 14 (G) of iPSC hematopoietic differentiation. Line 1 is an iPS line derived from CD45⁺ hematopoietic cells and Line 2 is an iPS line derived from mesenchymal stem cells.

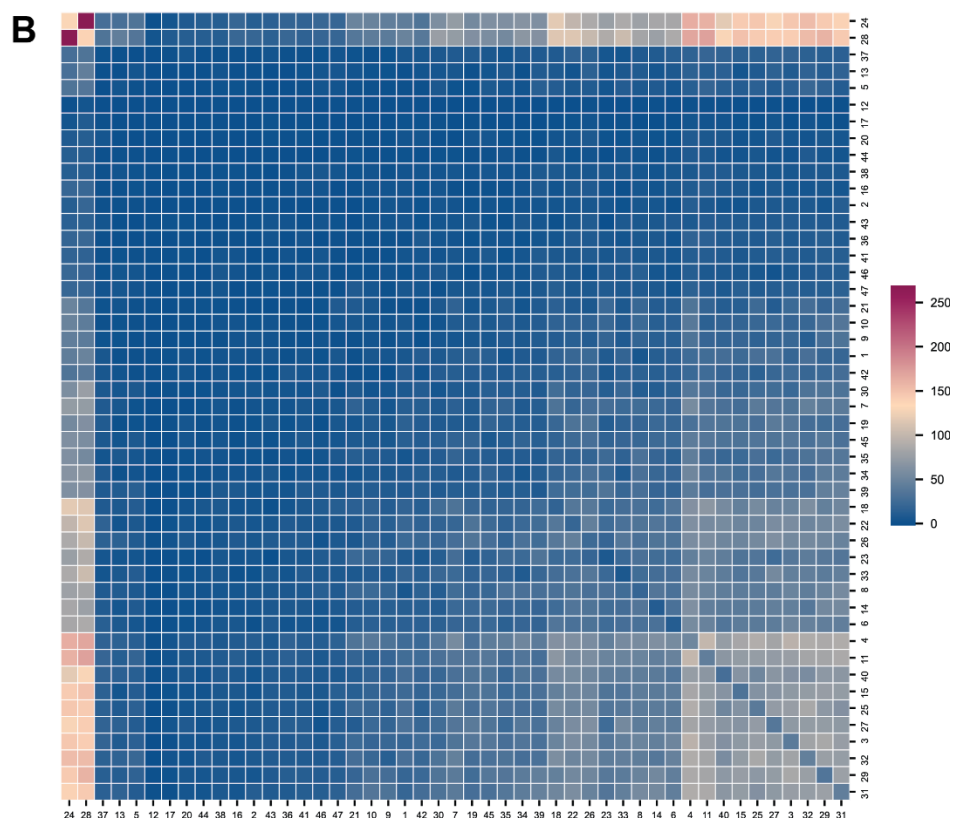
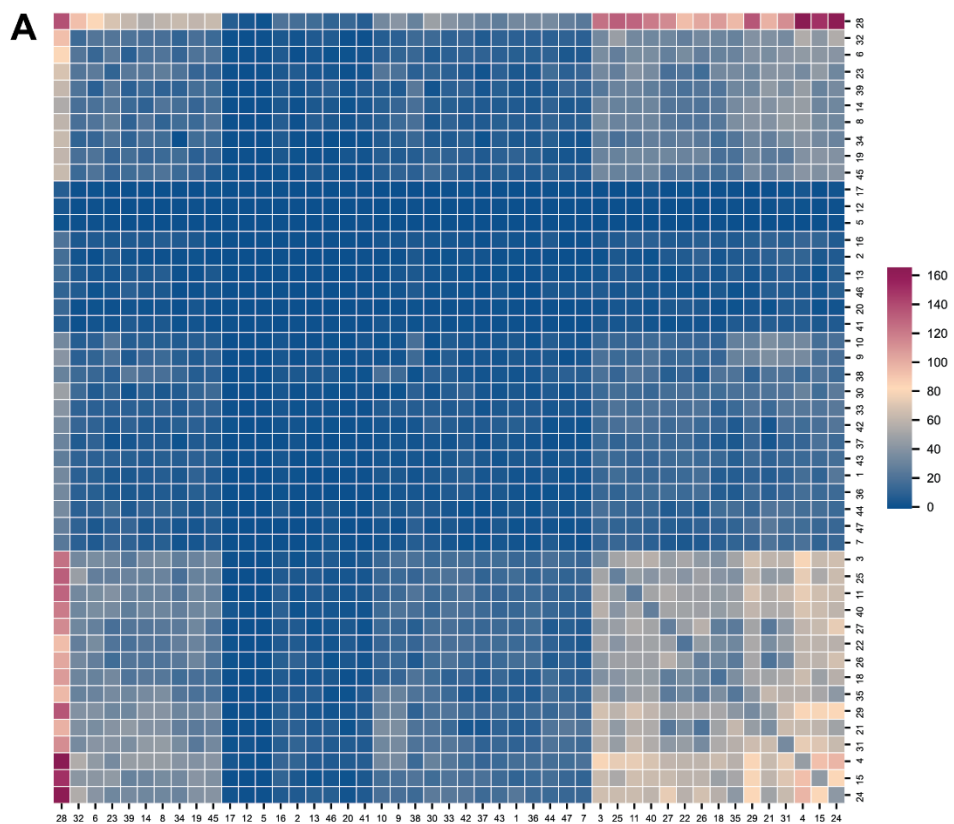


Figure S2.4.7. Cell-cell signaling interactions on day 7 of iPSC hematopoietic differentiation. (A-B) Heatmap of the number of significant interactions between any given two cell types on day 7 of iPSC hematopoietic differentiation numbered by the clusters found in **Figure S2.4.1C** for line 1 (an iPSC line derived from CD45⁺ cells) (**A**) and line 2 (an iPSC line derived from mesenchymal stem cells) (**B**).

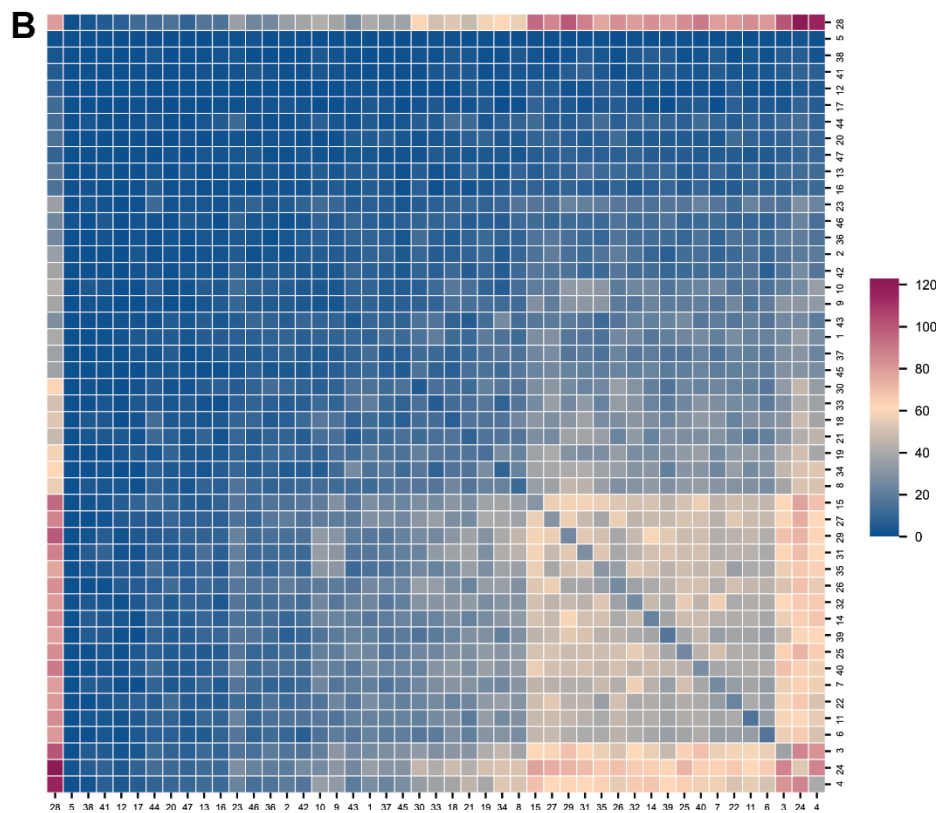
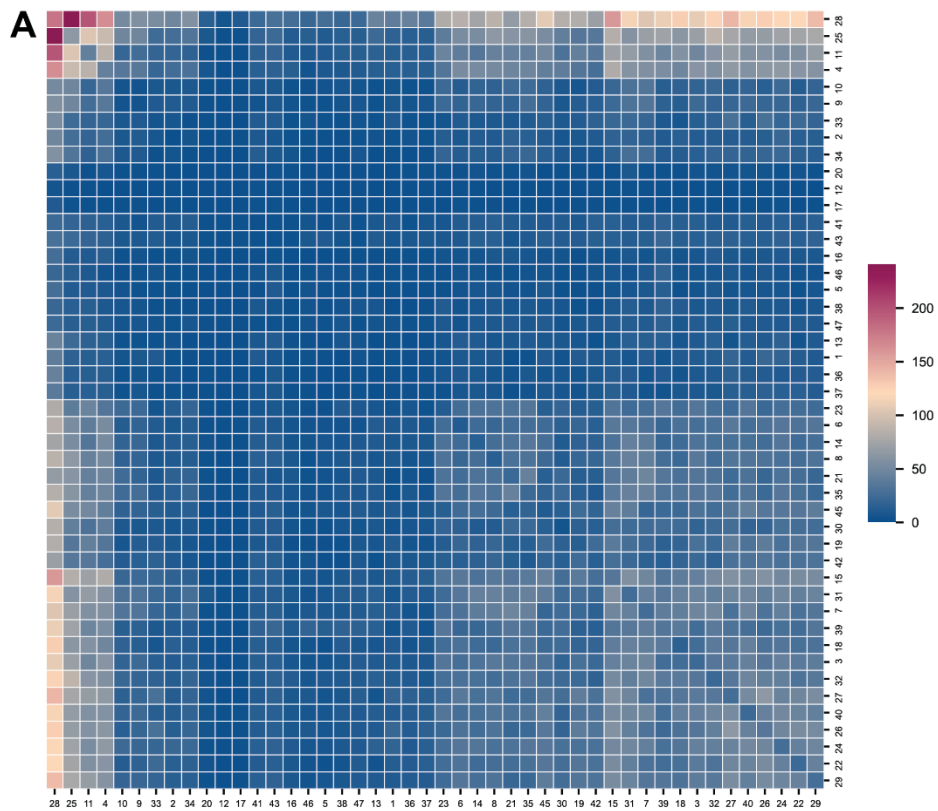


Figure S2.4.8. Cell-cell signaling interactions on day 8 of iPSC hematopoietic differentiation. (A-B) Heatmap of the number of significant interactions between any given two cell types on day 8 of iPSC hematopoietic differentiation numbered by the clusters found in **Figure S2.4.1C** for line 1 (an iPSC line derived from CD45⁺ cells) (**A**) and line 2 (an iPSC line derived from mesenchymal stem cells) (**B**).

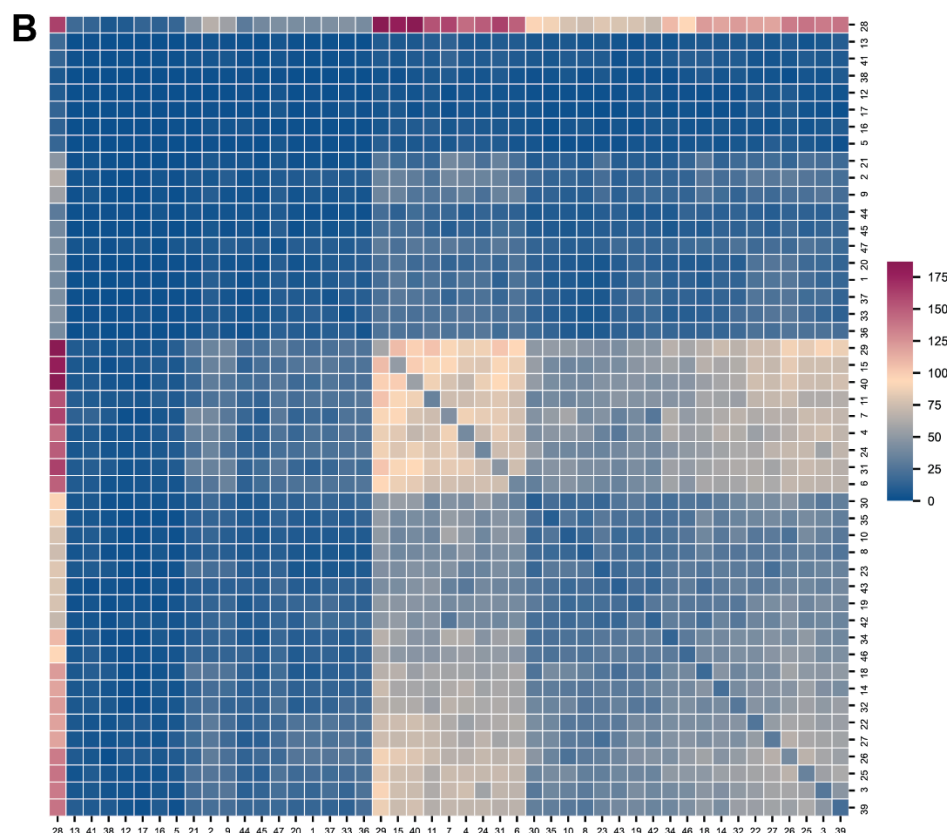
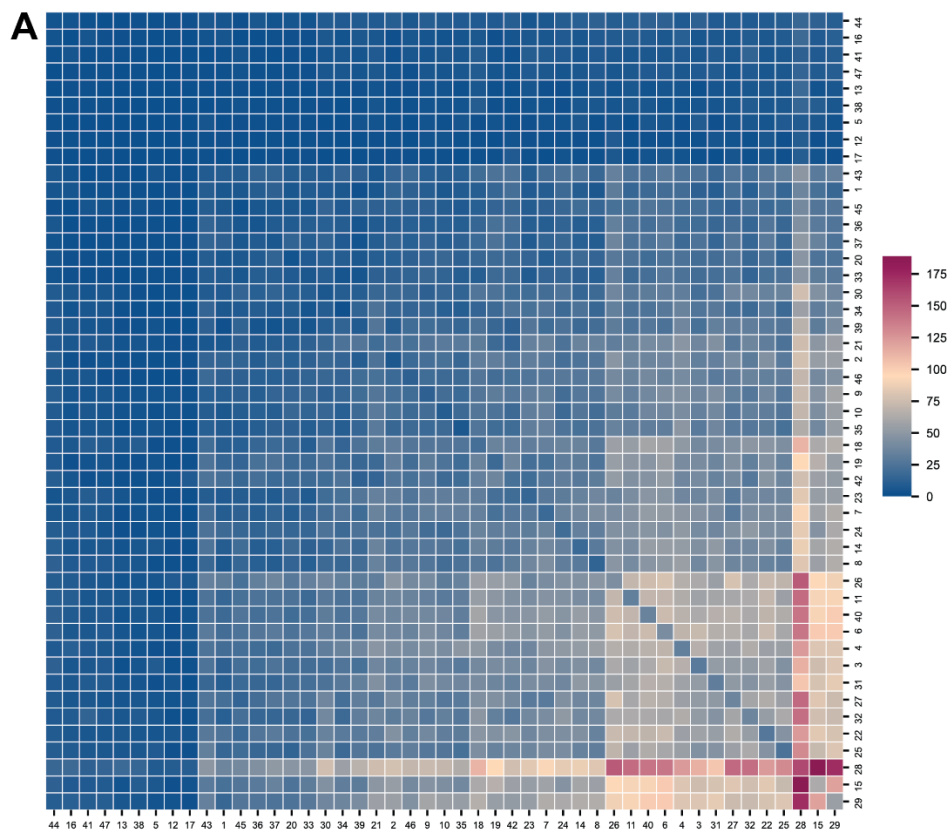


Figure S2.4.9. Cell-cell signaling interactions on day 11 of iPSC hematopoietic differentiation. (A-B) Heatmap of the number of significant interactions between any given two cell types on day 11 of iPSC hematopoietic differentiation numbered by the clusters found in **Figure S2.4.1C** for line 1 (an iPSC line derived from CD45⁺ cells) (**A**) and line 2 (an iPSC line derived from mesenchymal stem cells) (**B**).

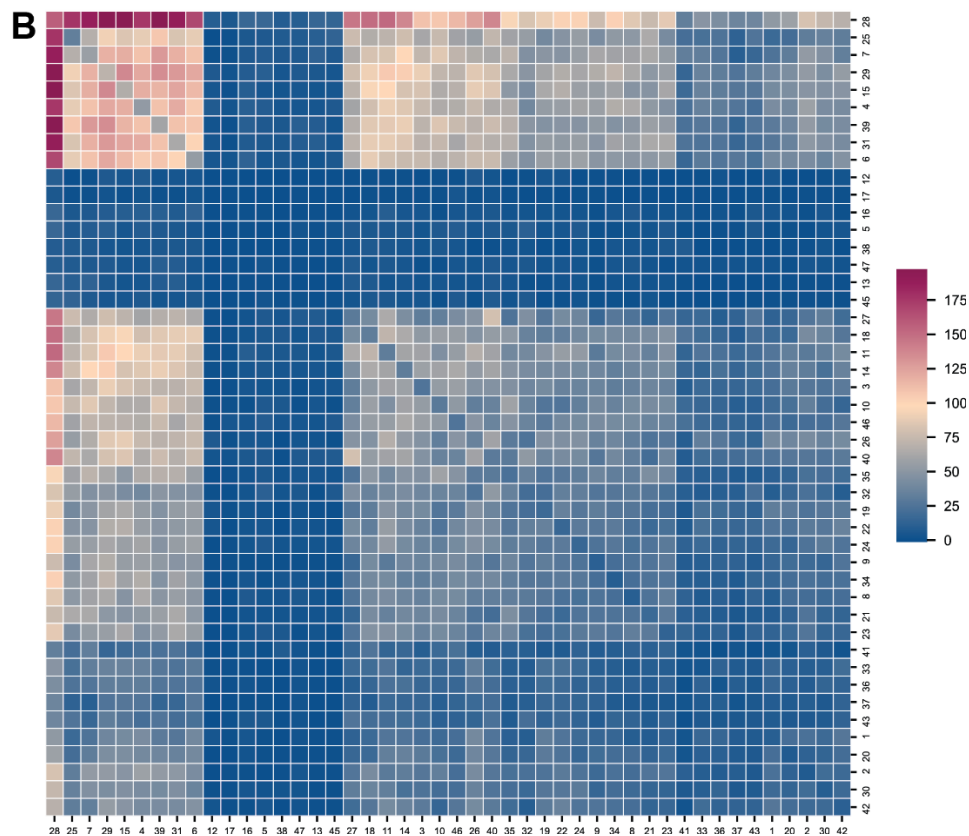
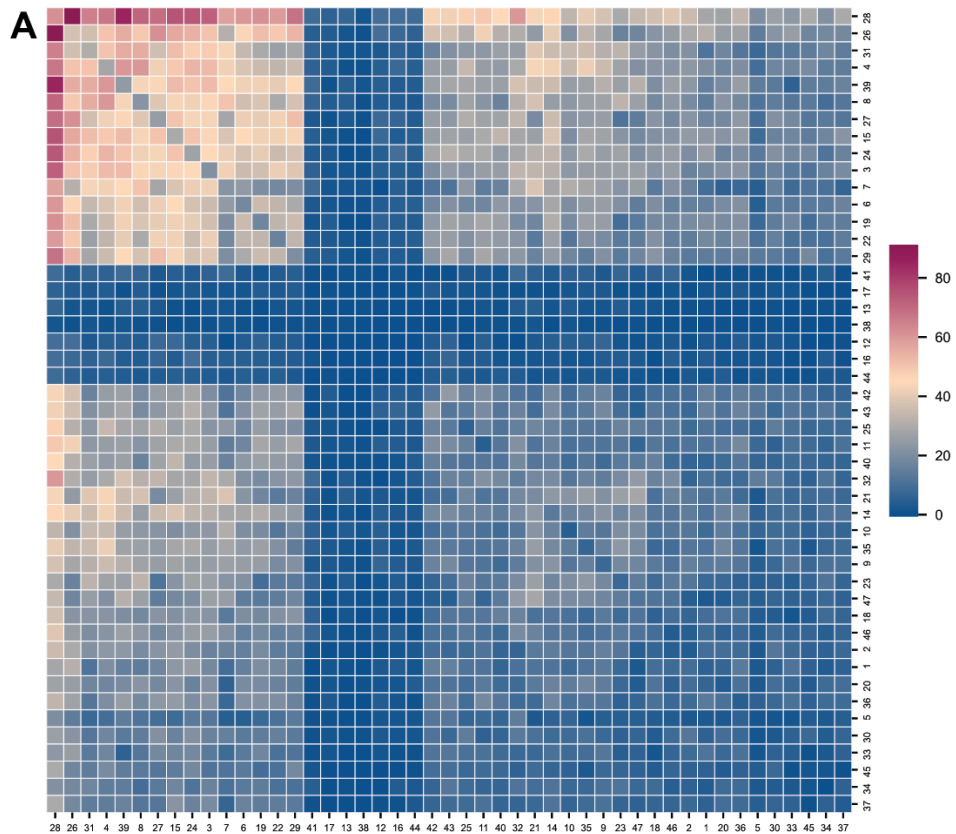


Figure S2.4.10. Cell-cell signaling interactions on day 14 of iPSC hematopoietic differentiation. (A-B) Heatmap of the number of significant interactions between any given two cell types on day 14 of iPSC hematopoietic differentiation numbered by the clusters found in **Figure S2.4.1C** for line 1 (an iPSC line derived from CD45⁺ cells) (**A**) and line 2 (an iPSC line derived from mesenchymal stem cells) (**B**).

2.5 Tables

General Materials		
Reagent	Company	Catalog Number
DMEM/F12	Thermo Fisher	11330-032
KOSR	Thermo Fisher	10828028
L-glutamine	Thermo Fisher	25030081
MEM-NEAA	Thermo Fisher	11140-050
2-mercaptoethanol	Thermo Fisher	21985-023
collagenase IV	Thermo Fisher	17104019
DMEM	Thermo Fisher	11960069
FBS (batch-tested)	Sigma	F0926, Lot: 201M111
Sodium pyruvate	Thermo Fisher	11360-070
KO DMEM	Thermo Fisher	10829-018
Ascorbic acid	Sigma	A4544-25G
Penicillin Streptomycin	Thermo Fisher	15140122
MilliporeSigma™ Transferrin, Holo, Human Plasma, Protein	Fisher Scientific	61-639
mitomycin C	Thermo Fisher	BP25312
mTESR Plus	Stem Cell Technologies	100-0276
collagenase B	Sigma-Aldrich	11088807001
IMDM	Thermo Fisher	12440061
gentle cell dissociation reagent	Stem Cell Technologies	100-0485
PBS	Thermo Fisher	14190250
Erdaftinib (JNJ-42756493) (used for zebrafish)	Cayman Chemicals	21813
Erdaftinib (JNJ-42756493) (used for iPS)	MedChemExpress	HY-18708
BCI (hydrochloride)	Cayman Chemicals	95130-23-7
pronase	Sigma	11459643001
tricaine methanesulfonate	Syncaine® Fish Anesthetics	MS-222
matrigel	Fisher Scientific	8774552

Table 2.5.1. Materials used in Experiments.

Antibodies		
Reagent	Company	Catalog Number
CD34-PE	BD	555822
CD43-PerCpCy5.5	BD	563521
CD45-APC	BD	555485
DAPI	ThermoFisher	D1306
CD34-PeCy7	BD	560710
CD144-FITC	BD	560411
CD184-APC	BD	560936
DLL4-PE	BD	564412
PE Mouse IgG1, κ Isotype Control	BD	555749
PerCP-Cy™5.5 Mouse IgG1 κ Isotype Control	BD	550795
APC Mouse IgG1, κ Isotype Control	BD	555751
IgG1 Mouse, FITC Isotype Control	BD	349041
PE-Cy7 Mouse IgG1 Isotype Control	BD	557872

Table 2.5.2. Antibodies used in Experiments.

Cytokines		
Reagent	Company	Catalog Number
bFGF	Peprotech	100-18C
hSCF	Peprotech	300-07
hFLT3-L	Peprotech	300-19
hBMP4	R&D Systems	314-BP
hG-CSF	Peprotech	300-23
hIL-6	Peprotech	200-06
hIL-3	Peprotech	200-03
hFGF-23	Peprotech	100-52

Table 2.5.3. Cytokines used in Experiments.

MEF Media Recipe	
Component	Concentration
DMEM	-
FBS	9.10%
L-Glutamine	1mM
MEM-NEAA	1mM
Sodium Pyruvate	1mM

Table 2.5.4. Media Recipe used in MEF Culture.

iPSC Media Recipe	
Component	Concentration
DMEM/F12	-
KOSR	20%
L-Glutamine	1mM
MEM-NEAA	1mM
2-ME	0.1mM
bFGF	10ng/mL

Table 2.5.5. Media Recipe used in iPSC Culture.

Embryoid Body Media Recipe	
Component	Concentration
KO DMEM	-
FBS	20%
L-Glutamine	1mM
MEM-NEAA	1mM
2-ME	0.1mM
Ascorbic Acid	50µg/mL
Pen-Strep	100U/mL
Holo-tranferrin	100µg/mL

Table 2.5.6. Media Recipe used in Embryoid Body Culture.

2.6 Methods

Cell Lines: hiPSC lines derived from mesenchymal stem cells (MSC-iPS)⁴³ and CD45+ blood cells (45-iPS)³⁴ were used for the generation of embryoid bodies. Mouse embryonic fibroblasts (MEFs) that were used for iPSC culture pre-embryoid body formation were acquired from the University of Washington Institute for Stem Cell and Regenerative Medicine (ISCRM) core, which were expanded and treated with mitomycin C prior to use.

Materials: All materials used and their catalog numbers can be found in **Table 2.5.1**, **Table 2.5.2** and **Table 2.5.3**.

hiPSC Culture: hiPSCs were maintained on hESC-Qualified Matrigel in mTESR Plus media. 6-7 days prior to EB formation, iPSCs were transferred onto MEFs and cultured in DMEM/F12 supplemented with knockout serum replacement (KOSR), L-glutamine, non-essential amino acids (MEM-NEAA), 2-mercaptoethanol, and basic fibroblast growth factor (bFGF). All catalog numbers and media concentrations can be found in **Table 2.5.1**, **Table 2.5.2**, **Table 2.5.3**, **Table 2.5.4**, **Table 2.5.5**, and **Table 2.5.6**.

Embryoid Body Differentiation and Dissociation: MEF-cultured hiPSCs were treated with 1mg/mL collagenase IV prior to scraping whole colonies. Scraped whole colonies were allowed to gravity settle, were transferred into EB culture media (**Table 2.5.6**) and put on a shaker at 37°C (Day 0 of differentiation, a.k.a. D0). After approximately 24hr (D1), EBs were collected into 15mL conical tubes and gravity settled. They were resuspended in EB culture media supplemented with final concentrations of 100ng/mL human stem cell factor (hSCF), 100ng/mL fms-like tyrosine kinase 3 ligand (hFLT3L), 50ng/mL bone morphogenic protein 4 (hBMP4), 10 ng/mL granulocyte colony-stimulating factor (hG-CSF), 25 ng/mL interleukin-6 (hIL-6) and 10 ng/mL interleukin-3 (hIL-3) and

again put on a shaker at 37°C. EB culture media with the listed cytokines was refreshed on D5 and D9. On days of collection for sc-RNA-seq or flow cytometry, EBs and all surrounding media were collected into 15mL conical tubes and spun at 1350rpm for 5 minutes to ensure the capture of both cells within the EBs as well as floating hematopoietic cells. The samples were then incubated in IMDM with 2mg/mL Collagenase B at 37°C for 2 hours while shaking with trituration approximately every 30 minutes. After 2 hours, 9mL of IMDM was added to the EB solution and the samples were spun at 1350rpm for 5 minutes. The EBs were then incubated in 2mL Gentle Cell dissociation reagent in a 37°C water bath for 15 minutes followed by vigorous trituration until they formed a single cell solution. 10mL of 3% fetal bovine serum (FBS) in PBS was added and the solution was filtered through a 70µM filter with a subsequent 2mL 3% FBS in PBS wash. The single cell solution was then spun down at 1350rpm for 5 minutes, resuspended in 3% FBS in PBS and the cells were counted using a Denovix CellDrop cell counter prior to subsequent assays.

Single Cell RNA-Sequencing: For single cell combinatorial indexing RNA-sequencing (sci-RNA-seq), EBs derived from both MSC-iPS and 45-iPS were dissociated on D7, D8, D11, D14, D18 and D21 per the protocol outlined above. Cells were then resuspended in a cell lysis buffer (10mM Tris-HCl pH 7.4, 10mM NaCl, 3mM MgCl₂, 0.1% IGEPAL CA-630) followed by gentle pipetting. The resulting nuclei were then filtered through a 70mM strainer and incubated in 4% paraformaldehyde in 1.25X PBS for 15 minutes with inversion every 5 minutes. The nuclei were then spun at 800g for 6 minutes and resuspended in nuclei suspension buffer (10mM Tris-HCl pH 7.4, 10mM NaCl, 3mM MgCl₂, 1% SUPERase In RNase Inhibitor). The nuclei were flash frozen and stored in

liquid nitrogen until processing. These nuclei samples were submitted to the Brotman Baty Institute (BBI) for sci-RNA-seq (3-level, RNA3-028-a), which was performed similarly to the published protocol (<http://dx.doi.org/10.17504/protocols.io.9yih7ue>) and sequenced using a NextSeq 2000.

FASTQ File Processing: FASTQ files for the sci-RNA-seq samples were used to generate barcode, feature and count matrix files internally by the BBI according to their processing pipeline (see published protocol above). Briefly, processing was done using Python 3.7.7, TrimGalore v.0.6.4, CutAdapt v.1.18, single-end trimming mode, a quality Phred score cutoff of 20 and the default maximum trimming error rate, followed by alignment to Homo sapiens GRCh38 using STAR v.2.6.1d, followed by filtering using samtools v.1.9, followed by removal of duplicates and gene assignment using bedtools v.2.27.1 and bamtools v.2.5.1, followed by the removal of doublets using scrublet v.0.2.1.

Sci-RNA-Seq Transcriptomic Analysis: The Monocle3 v1.3.1 R package was used for the analysis of sci-RNA-seq data. Cells with a low number of unique molecular identifiers (UMIs) were removed (see GitHub repository linked in [Data and Code Availability](#) for the code and cutoffs used per sample). The maximum number of UMIs detected across all samples was 62698, so no upper UMI limit was set. Cells with more than 10% of UMIs mapping to mitochondrial genes were also removed. All samples were combined into a single cell_data_set object for downstream analysis. Batch correction was not needed.

Cell Type Identification: Hematopoietic-related populations in the sci-RNA-seq data were identified by generating an aggregate score for sets of genes identified by Xu et al. for each cell type, which was then plotted in UMAP space. These scores were then used to qualitatively annotate Monocle3-determined clusters as specific cell types. See the

provided GitHub repository ([Data and Code Availability](#)) for code pertaining to the generation of the aggregate gene set scores and cluster resolution.

Cell-Cell Interaction Analysis: The code used for this analysis and the output files can be found in the GitHub repository ([Data and Code Availability](#)). Briefly, CellPhoneDB v5.0.0 was utilized for this analysis following CellPhoneDB method 2 in Python 3.8.19. Dot plots and heatmaps were generated using ktplotspy 0.2.4 in python. Circos plots were generated in R v4.3.2 using the circlize v4.3.3 package. Python packages associated with this analysis and their versions are: pandas v2.2.2, anndata v0.6.20, numpy v1.26.4, plotnine v0.13.6.post27+g6bf7fe7 and matplotlib v3.9.1.

2.7 Acknowledgments

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supported by the Institute for Stem Cell and Regenerative Medicine (ISCRM) fellows award. Additionally, we thank the Chan Zuckerberg Initiative for hosting our data on their CELLxGENE platform.

Chapter 3. Comparison of iPSC to human embryo endothelial-to-hematopoietic transition reveals modulable pathways

3.1 Introduction

In human embryos, the first waves of EHT starts in the yolk sac around Carnegie stage (CS) 7 and involves the sequential production of lineage-restricted erythroid-myeloid precursors (EMPs), lymphoid/myeloid progenitors (LMP), and multipotent progenitors (MPP)^{6, 11}. HSCs first arise in the human embryo from a developmentally matured HE around CS14^{12, 13} before they eventually colonize the fetal liver around CS16-17^{3, 12, 14}. In the iPSC system, HE first arises by around day 6 of human iPSC differentiation and expands by around day 8, during which time it undergoes EHT to generate multilineage hematopoietic progenitors²⁴⁻²⁶. Despite the difference in time scales, iPSC-derived hematopoiesis generally recapitulates the stages of embryo hematopoietic ontogeny,, critically arterialized endothelium, a precursor to HSC fate^{29, 30}, and definitive CD32⁺ HE capable of clonal multilineage hematopoiesis²⁶.

Since EHT is a transient cell state, there has been considerable interest in capturing this developmental transition using high-depth single cell RNA sequencing (sc-RNA-seq). A number of reports have provided a single cell map of EHT during mouse embryogenesis¹⁹⁻²³. In human embryos, Zeng et al.³ first mapped EHT during early human embryogenesis (CS10-13), a developmental window preceding HSC emergence. Crosse et al.⁴, and more recently Calvanese et al.⁵, have generated single cell maps of EHT during the window of HSC formation (CS14-17) in human embryogenesis. Although several single cell transcriptomic studies have captured iPSC-derived EHT^{3, 5, 24, 29, 31, 32}, we still lack a high-resolution map of stepwise gene expression changes during EHT in

iPSC differentiation with direct comparison to hematopoiesis in the embryo. Key regulators of hematopoietic ontogeny including transcription (e.g., RUNX1, GATA2, c-MYB), epigenetic (EZH1/2, SUV39), signaling (Notch, Wnt, TGF), inflammatory (NFκB, IFN-γ, IL-1β), and biophysical (stretch, blood flow) factors have been identified³³. A better understanding of signaling factor stage-specific roles would be instrumental to improve iPSC differentiation protocols. Direct comparison between carefully staged *in vitro* and *in vivo* cell states spanning hematopoietic ontogeny would facilitate the identification of targetable pathways that can improve iPSC-derived hematopoiesis.

To this end, we map EHT dynamics during embryoid body differentiation from iPSCs and identify key transcriptional differences between *in vitro* and *in vivo* cell states. By mapping ligand-receptor interactions associated with the differential expression of developmental programs, we identify elevated FGF signaling as a barrier to hematopoietic specification. These data identify a panel of ligands that may be modulated to improve iPSC differentiation protocols.

3.2 Results

3.2.1. Developmental dynamics of EHT *in vitro* and *in vivo*

To generate a high-resolution map of EHT from iPSCs, we next focused on the peak of EHT at day 8 of EB differentiation. To enrich for HE cells, we purified endothelial and hematopoietic cells positive for CD34 or CD43/CD45 (**Figure 3.4.1A**, **Figure S3.4.1A-B**). As expected, almost all cells in our analysis were endothelial (*CD34*, *CD43^{neg}*) expressing arterial marker *DLL4*, or hematopoietic (*RUNX1*, *CD43^{pos}*) (**Figure 3.4.1B**). We captured a robust population of HE cells (*RUNX1*, *CD34*, *DLL4*, *CD43^{neg}*) actively undergoing EHT (**Figure 3.4.1B-D**). We next identified endothelial and

hematopoietic subtypes based on published marker sets³⁻⁵ and assigned supervised cell type annotations to clusters identified in UMAP space using Monocle 3 (**Figure 3.4.1C-G**). This analysis revealed the following conclusions. First, endothelial cells expressing *DLL4* were proximal to HE. As expected, cluster 27 on the endothelial “side” of the bridge was identified as HE. Interestingly, cluster 26a was highly enriched for both an HE and HSC-like signature⁵, whereas a second trajectory stemming from HE (cluster 26b) did not exhibit the HE or HSC signature, but instead expressed a strong erythrocyte signature. Cluster 26a was further evaluated for the HSC signature by looking at 6 key HSC genes⁵, which revealed the acquisition of *RUNX1*, *MLLT3*, *MECOM*, some *HOXA9* and *HLF*, but not *SPINK2*. Both trajectories gave rise to hematopoietic precursors and lineages, including erythroid and myeloid. These data capture the transcriptome changes of HE undergoing EHT and suggest that iPSCs give rise to both an early (cluster 27) and more mature, late HE (cluster 26a), which is in line with what has been shown regarding diverging HE developmental trajectories *in vitro*³⁷.

To uncover molecular pathways essential for HSC generation, we next sought to compare the dynamics of EHT from iPSCs to HSC-competent human embryos. To this end, we first integrated our EB day 8 sc-RNA-seq data with published human embryo sc-RNA-seq datasets using Seurat canonical correlation analysis (see **Methods**). The published human embryo datasets include CS10-13 preceding HSC formation from Zeng et al.³, CS16 from Crosse et al.⁴, and CS14-17 from Calvanese et al.⁵, the latter studies spanning active HSC generation. iPSC-derived and human embryo datasets overlapped in common UMAP space (**Figure 3A-B**). We identified HE undergoing EHT (*RUNX1*, *CD34*, *DLL4*, *CD43*^{neg}) at CS10-CS13 and CS14 time points, with increasing separation

of the endothelial (*CD34*, *CD43*^{neg}) and hematopoietic (*RUNX1*, *CD43*^{pos}) components at CS15 and CS17 (**Figure 3.4.2A-C**), resembling what we observed in our initial *in vitro* time course (**Figure 2.4.1D**). As in the iPSC data, *DLL4*+ AE (cluster 36) preceded the *RUNX1*+ HE “bridge” (cluster 75) giving rise to all hematopoietic lineages (**Figure 3.4.2B-C**). We further rigorously validated that developmentally equivalent populations were correctly integrated by transferring cell labels from our day 8 EB data as well as each individual published dataset onto the common UMAP (**Figure 3.4.2D-M Figure S3.4.2A-R**). These data suggest that iPSC-derived HE undergoes similar developmental transitions to those in human embryo AGM enabling their direct molecular comparison.

3.2.2. Comparative molecular analysis of EHT *in vitro* and *in vivo*

To uncover molecular pathways essential for HSC generation, we next compared transcriptome changes during EHT between iPSC and HSC-competent human embryos (CS14-17). We focused on the AE preceding EHT (cluster 36), HE undergoing EHT on the endothelial side of the “bridge” (cluster 75), and emergent HSPCs exiting EHT (cluster 74) in our integrated dataset (**Figure 3.4.3A,D, Figure S3.4.3A,D**). We first identified differentially expressed genes (DEGs) between iPSC and HSC-competent human embryo HE undergoing EHT, EHT (HE+HSPC), or early HSPCs using a linear regression fit to a mixed-negative binomial model accounting for batch effects in Monocle 3. DEGs downregulated in the iPSC EHT were enriched for gene ontology (GO) terms related to cytokine signaling and hemopoiesis (**Figure S3.4.3A-C**). Consistent with HSC-incompetent EHT, significantly downregulated genes in iPSC EHT cells included transcription factors essential for HSC and lymphoid fate specification, including

HOXA5/7/10, *HLF*, *MLLT3*, *SPINK2*, *SPI1*, *GFI1*, *GATA2*, *TCF7*, and *BCL11A*, and signaling receptors including *CD37*, *CD40*, *CD44*, *CD52*, *CD68*, *CD74*, *CSF1R*, *IL6R*, *IL3RA*, and *IL11RA* (**Table S4A-EHT**). Downregulated genes were enriched for transcriptional targets of MYCN, PU.1, CEBPA, and NFκB by TRRUST analysis. Since most of these transcriptional changes likely reflect the HSPC output of EHT, we next separately analyzed HE and early HSPCs (**Figure 3.4.3A-C**, **Figure S3.4.3D-F**). Both HE and early HSPCs had fewer transcriptional changes, however genes downregulated in iPSC HE were still enriched for GO terms related to cytokine signaling and hematopoiesis, and additionally HSC homeostasis (**Figure 3.4.3C**). Key downregulated genes in iPSC HE included transcriptional regulators *SOX17*, *GATA2*, *REL*, *ID2*, and *NKX2-3* and signaling factors *ALDH1A1* (retinoic acid signaling), *CD74*, *IL11RA*, and *IL3RA*. Upregulated genes in iPSC HE were enriched for GO terms related to mitochondrion organization (**Figure 3.4.3B**), and surprisingly included endothelial genes *KDR* and *FLT1*. Downregulated genes in iPSC early HSPCs also remained enriched for GO terms related to cytokine signaling and hemopoiesis, but not HSC homeostasis (**Figure S3.4.3D-F**).

We next tracked gene expression dynamics along hematopoietic ontogeny independently in the iPSC system and human embryos. To this end, we identified DEGs in the transition from AE to HE, and the subsequent transition from HE to HSPC either *in vitro* or *in vivo* (**Figure 3.4.3D-I**). In the AE to HE transition, enriched GO terms related to ribosome biogenesis, cell activation, splicing, and translation, were induced in the embryo (**Figure 3.4.3E**, **Figure S3.4.3G**), reflecting a more activated cell state of HE. At the same time, GO terms related to blood vessel development, cell motility, endothelial development, and cell junctions, were downregulated in embryo HE (**Figure 3.4.3F**,

Figure S3.4.3H), reflecting a gradual loss of endothelial cell identity. These downregulated GO terms were similarly downregulated in the AE to HE transition in iPSCs (**Figure 3.4.3F, Figure S3.4.3H**), suggesting that the loss of endothelial cell identity occurs appropriately at this stage. By contrast, upregulated terms were less conserved in iPSCs (**Figure 3.4.3E, Figure S3.4.3G**). In the subsequent developmental transition from HE to HSPC, GO terms related to leukocyte biology and immune process were upregulated in the embryo (**Figure 3.4.3G, Figure S4I**), while terms related to blood vessel development, cell motility, and endothelium development were downregulated (**Figure 3.4.3H, Figure S3.4.3J**), reflecting acquisition of hematopoietic identity and loss of endothelial identity. Interestingly, endothelial cell identity was less strongly repressed in the HE to HSPC transition in iPSCs than the embryo (**Figure 3.4.3H, Figure S3.4.3J**). Among these, key endothelial cell fate genes *FLT1* (VEGFR1) and *KDR* (VEGFR2) were appropriately repressed in embryo-derived HSPCs but continued to be expressed in iPSC-derived HSPCs at significantly higher levels (**Figure 3.4.3K**). Additionally, several key HSC markers were appropriately activated in HSPCs from the embryo, but not from iPSCs (**Figure 3.4.3L**). Taken together these data suggest that iPSC-derived EHT is characterized by lower expression of HSC-specifying transcription factors and signaling pathways, and a surprising retention of endothelial cell gene expression.

3.2.3. A map of ligand-receptor interactions to inform iPSC differentiation

Since iPSC-derived EHT was characterized by the misexpression of signaling factors, we reasoned that the hemogenic potential can be augmented by modulation of specific ligand-receptor signaling pathways. To identify candidate signaling pathways, we

connected upstream signals with differential gene expression in iPSC-derived EHT using the NicheNetR package⁴⁴. We applied NicheNetR to our integrated dataset and identified ligand-receptor pairs predicted to account for differential gene expression between iPSCs and HSC-containing embryos. We required that receptors be expressed on HE cells, without defining the ligand source to allow for ligands produced by other sources than the cells in our dataset (e.g., hormones or cell culture media components). Top ligand-receptor interactions predicted to signal upstream of genes differentially expressed between *in vitro* and *in vivo* HE included signaling pathways previously implicated in regulating EHT, including TNF, interferon gamma, TGF α , EDN1, and HAS2 signaling via CD44^{33, 45} (**Figure 3.4.4A**). Extending the analysis to the entire EHT population (HE + HSPC) identified TGF β signaling, HAS2 signaling, and NOTCH signaling pathways, which are known regulators of EHT (**Figure S3.4.4B-D**)³³. Ranking the pathways by the activity score and number of target genes identified  NLGN3, APOE, TNF, ADAM17, CAMP, IL-13, HAS2, IFNG, TNFA, FGF23, EDN1, and AGT as the top modifiable pathways for iPSC-derived HE (**Figure 3.4.4A-B**). These data uncover the signaling pathways that may be mis-regulated in iPSC-derived EHT resulting in suboptimal hematopoietic output and serve as a resource to improve iPSC differentiation protocols.

Endothelial identity was not properly suppressed in iPSC-derived EHT resulting in persistent expression of endothelial genes, such as *KDR* and *FLT1* (**Figure 3.4.4K**). Interestingly, fibroblast growth factor FGF23 was identified in NicheNetR analysis as a potential upstream signal for 36 differentially expressed genes between *in vitro* and HSC-dependent *in vivo* HE (**Figure 3.4.4A-B**, **Figure S3.4.4A**). FGF signaling plays an important role in endothelial biology and is essential for generation of HSC-competent

HE⁴⁶. FGFR1 is the main FGF receptor expressed on HE (**Figure 3.4.4C**). Interestingly, endothelial cells expressed abundant FGF23 and the more canonical ligand FGF2 (**Figure 3.4.4D**). In addition, FGF2 was widely expressed in non-endothelial populations in our data (**Figure 3.4.4D**). Taken together, these data led us to hypothesize that FGF signaling is attenuated in HSC-competent HE during EHT *in vivo*, but remains activated during iPSC-derived EHT, and that inhibition of FGF signaling during EHT can augment HSPC generation.

3.3 Discussion

EHT is a critical transient stage in hematopoietic ontogeny. Here, we interrogate the single cell kinetics of EHT during iPSC differentiation and integrate it with human embryo datasets to generate a high-resolution map of EHT. Although iPSCs give rise to definitive HE with robust multilineage hematopoietic potential, they do not efficiently generate bona fide HSCs, suggesting broad differences compared to hematopoiesis in the embryo. By comparing the molecular changes at developmentally equivalent stages of EHT *in vitro* and *in vivo*, we uncover several insights. By connecting these expression changes with upstream signaling factors, we identify ligand-receptor pairs predicted to be dysregulated in iPSC differentiation.

Although several single cell transcriptomic studies have captured iPSC-derived EHT^{3, 5, 24, 29, 31, 32}, we still lack a high-resolution map of gene expression changes during EHT in iPSC differentiation and its direct comparison to the human embryo. We generated a high-resolution map of iPSC-derived EHT at day 8. By comparing the molecular changes at developmentally equivalent stages of EHT *in vitro* and *in vivo*, we uncover several insights. First, as in the embryo, AE clusters closest to HE, suggesting that

arterialized endothelium emerges as a precursor to definitive HE in iPSC differentiation, as previously shown^{12, 28, 29, 40}. Second, iPSC-derived EHT generates two populations of HE, one of which appears to be a precursor to both erythroid progenitors and a second HE population that acquires an HSC-like signature; however, the expression of key HSC fate genes and signaling factors is diminished overall. Moreover, we find that the developmental transition from AE to HE to HSPC is marked by progressive global silencing of endothelial cell fate and induction of hematopoietic fate. However, we observe a persistent high expression of endothelial fate genes in nascent iPSC-derived HSPCs. Our findings point to the mis-regulation of specific developmental programs in iPSC differentiation. Our integrated dataset is available to the community (see [Data and Code Availability](#)) and will be a resource for further interrogation of EHT dynamics.

EHT in the embryo occurs at specific anatomical sites within developing clusters, critically in the AGM. The nascent HSPCs in these clusters are exposed to defined developmental signals and biophysical forces. By contrast, hematopoiesis in EBs is modulated by signals provided in the culture media. We reasoned that inappropriate signaling pathways in developing EBs can be identified based on expression differences between cells undergoing EHT in iPSCs and human embryos. These may include factors that are present in the embryo and need to be included in the iPSC protocol, or conversely, factors present in iPSC media or secreted in the EBs that are normally absent at sites of hematopoiesis in the embryo. This analysis has uncovered several predicted signaling pathways, for instance $TGF\alpha/\beta$, Notch, interferon gamma, and EDN1, known to be essential regulators of EHT^{33, 45}. We also identified candidate pathways not currently known to regulate EHT or hematopoiesis, such as those involving the ligands NLGN3, a

target of WNT/ β -catenin that hasn't been explored in the context of EHT⁴⁷, APOE, a cholesterol transporter⁴⁸, ADAM17, a metalloproteinase typically associated with endothelium⁴⁹, CAMP, also known as LL-37 which is involved in innate immunity⁵⁰, and IL-13, an interleukin that has been linked to endothelial cell mobility⁵¹. Overall, our ligand-receptor analysis provides a resource for future interrogation of pathways to improve iPSC differentiation protocols.

3.4 Figures and legends

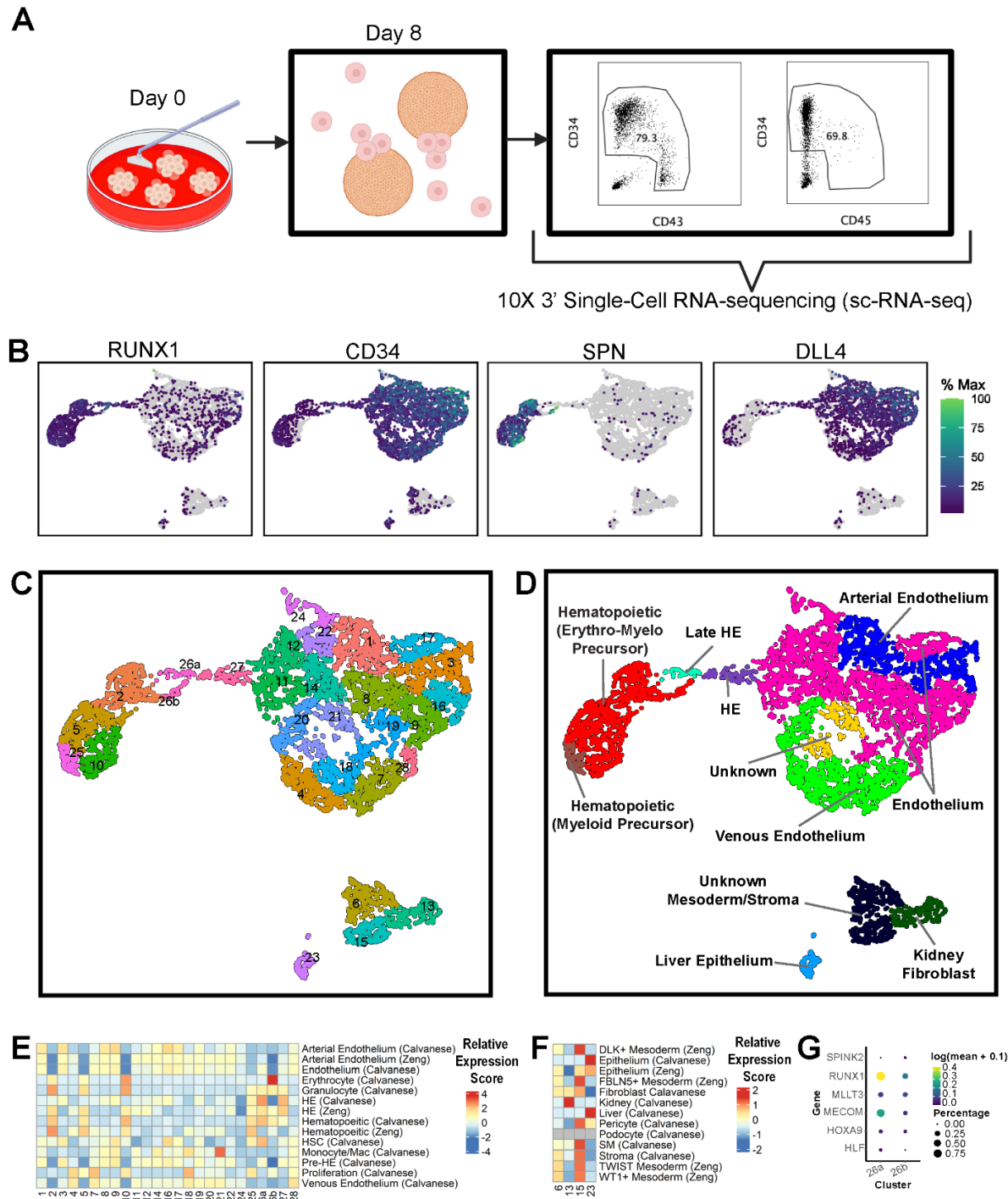


Figure 3.4.1. Developmental dynamics of EHT during iPSC differentiation. (A) Experimental scheme of 10X single cell RNA-sequencing of EHT on day 8 of differentiation. iPSCs were differentiated as EBs into HPCs, dissociated on day 8 and

hematopoietic and endothelial populations were flow sorted for profiling by 10X 3' sc-RNA-seq based on the expression of CD34, CD43, and CD45, as shown. **(B)** Expression of *RUNX1*, *CD34*, *SPN* (CD43), and *DLL4* transcripts. **(C)** UMAP showing unsupervised clustering. **(D)** Supervised clustering with cell types identified by top gene signatures in E-F. **(E,F)** Heatmap of relative expression (aggregate gene scores in a scaled matrix) of published gene signatures for each cluster in C. **(G)** Expression of HSC genes from Calvanese et al. (ref. 5) in HE clusters 26a and 26b.

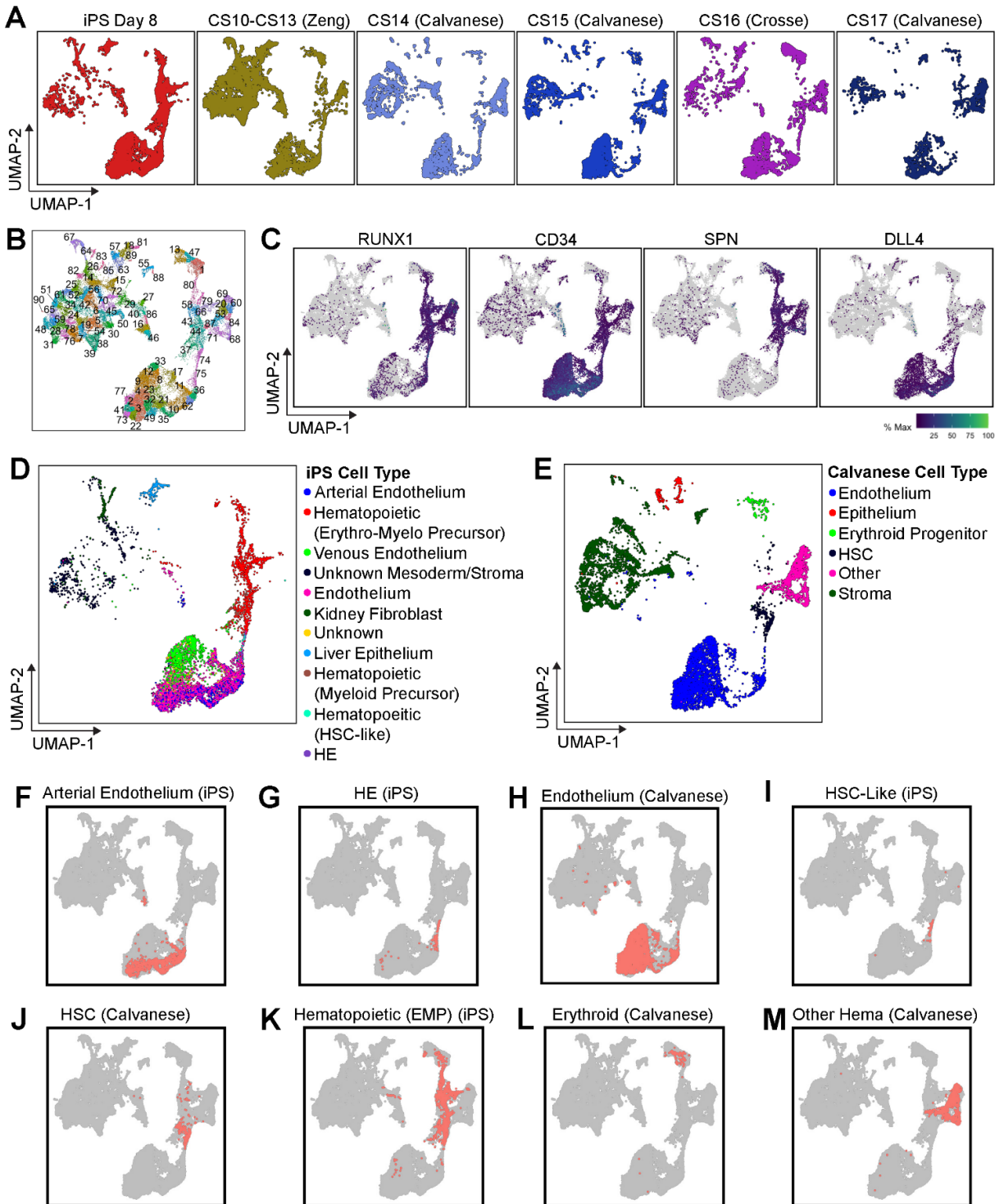


Figure 3.4.2. Developmental dynamics of EHT *in vitro* compared to *in vivo*. (A) Integration of day 8 iPSC-derived EB and human embryo 10X sc-RNA-seq data from Zeng et al. (ref. 3), Crosse et al. (ref. 4) and Calvanese et al. (ref. 5) into the same

UMAP dimensionally-reduced space using Seurat canonical correlation analysis, with individual datasets separated post-integration. **(B)** Integrated UMAP with unsupervised clustering in Monocle 3. **(C)** Expression of *RUNX1*, *CD34*, *SPN* (CD43), and *DLL4* transcripts. **(D,E)** Transfer of cell type labels from the day 8 EB dataset (D) and Calvanese et al. (E) onto the integrated space for verification of proper integration. **(F-M)** Label transfer of key developmental populations from the day 8 iPSC and the *in vivo* Calvanese et al. datasets onto the integrated UMAP: iPSC arterial endothelium (F), iPSC hemogenic endothelium (G), Calvanese endothelium (H), iPSC HE expressing HSC signature (cluster 26a in Figure 2) (I), Calvanese HSC (J), iPSC erythroid and myeloid hematopoietic (K), Calvanese erythroid cells (L), Calvanese other hematopoietic (M).

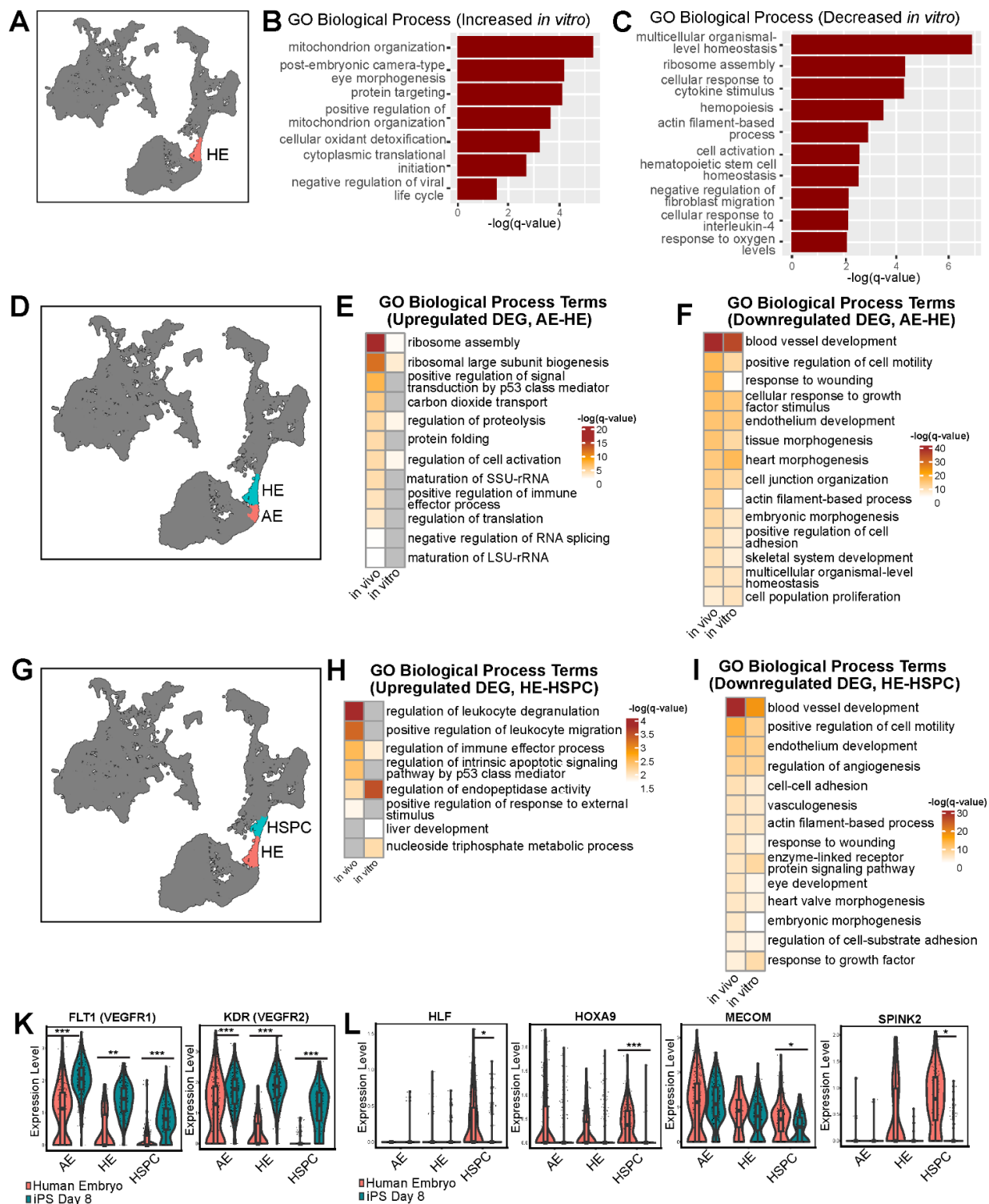


Figure 3.4.3. Comparative molecular analysis of EHT *in vitro* and *in vivo*. (A) HE population (cluster 75 in integrated dataset) used for differential gene expression analysis. (B,C) Gene ontology (GO) terms significantly upregulated (B) or downregulated (C) in iPSC-derived vs. HSC-competent human embryo HE (Metascope,

$q < 0.05$). **(D)** Arterial endothelium (AE; cluster 36, red) and HE (cluster 75, blue) populations used for differential expression analysis. **(E,F)** GO terms significantly upregulated (E) or downregulated (F) during AE-to-HE transition (Metascape batch mode, $q < 0.05$). **(G)** HE (cluster 75, red) and hematopoietic stem and progenitor cell (HSPC; cluster 74, blue) populations used for differential expression analysis. **(H,I)** GO terms significantly upregulated (H) or downregulated (I) during HE-to-HSPC transition (Metascape batch mode, $q < 0.05$). **(K)** Expression of representative endothelial identity genes *FLT1* and *KDR* in HSC-competent human embryos (CS14-17; red) and day 8 iPS (blue) AE (cluster 36), HE (cluster 75) and HSPCs (cluster 74). **(L)** Expression of HSC genes *HLF*, *HOXA9*, *MECOM*, and *SPINK2* in HSC-competent human embryos (CS14-17; red) and day 8 iPS (blue) AE (cluster 36), HE (cluster 75) and HSPCs (cluster 74). For (K, L) * $q < 0.05$, ** $q < 0.005$, and *** $q < 0.0005$ using a linear regression fit to a mixed-negative binomial model accounting for batch effects.

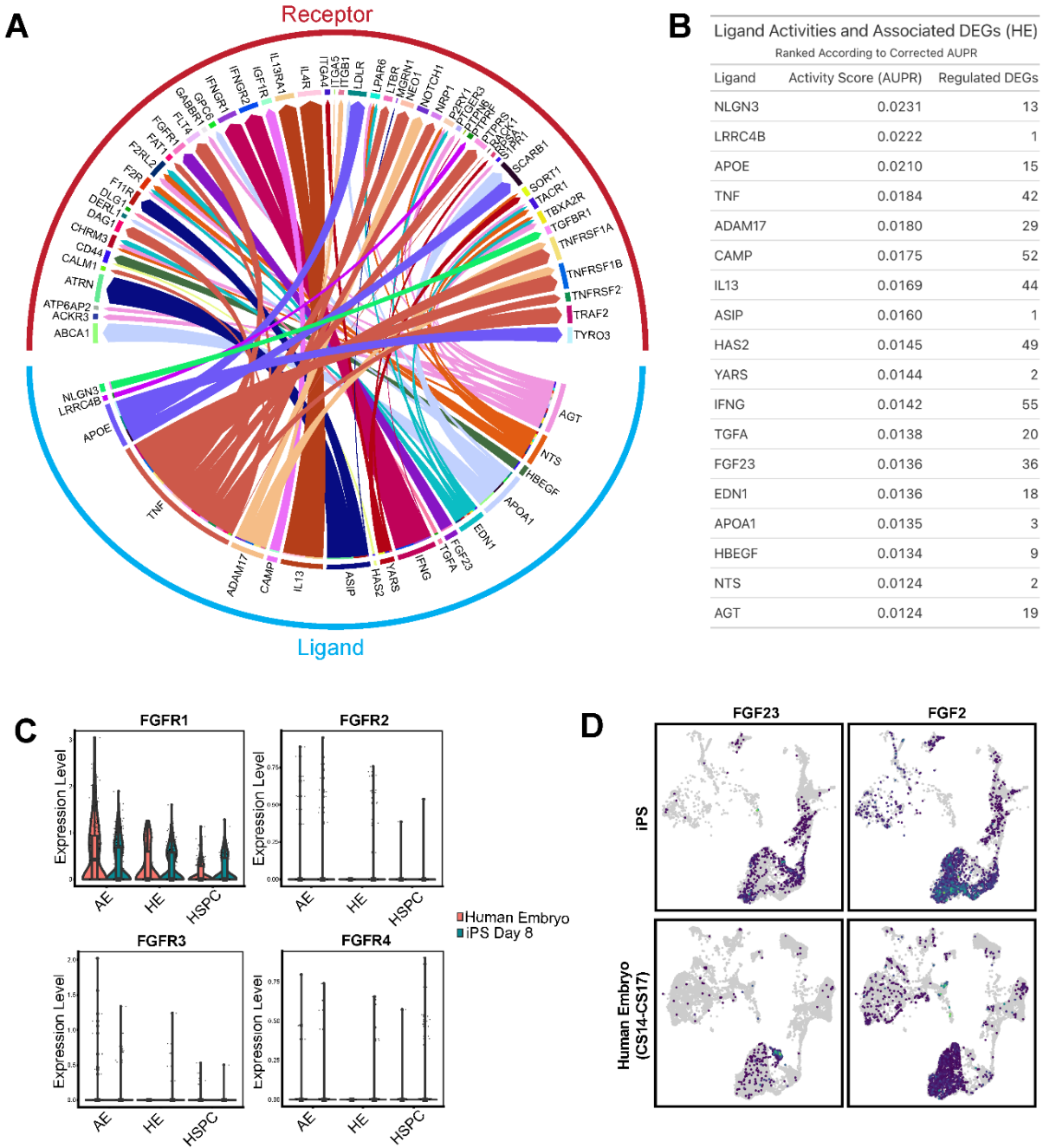


Figure 3.4.4. A map of ligand-receptor interactions to inform iPSC differentiation. (A) Top ligand-receptor interactions identified by NicheNetR v.1 associated with differentially expressed genes in day 8 iPSC EBs vs. *in vivo* (CS14-17 human embryo) hemogenic endothelium (cluster 75 of integrated dataset). Ligands (bottom) are connected to their respective receptors (top) expressed on HE cells. (B) Top ligands in (A) ranked by the number of DEGs and activity score defined as the area under the precision recall curve (AUPR). (C). Expression of FGF receptors (*FGFR1-4*) during *in vitro* (red) and *in vivo* (blue) EHT. (D). Expression of *FGF2* and *FGF23* ligands in the integrated dataset, split by day 8 iPS (top) or HSC-competent embryo (bottom).

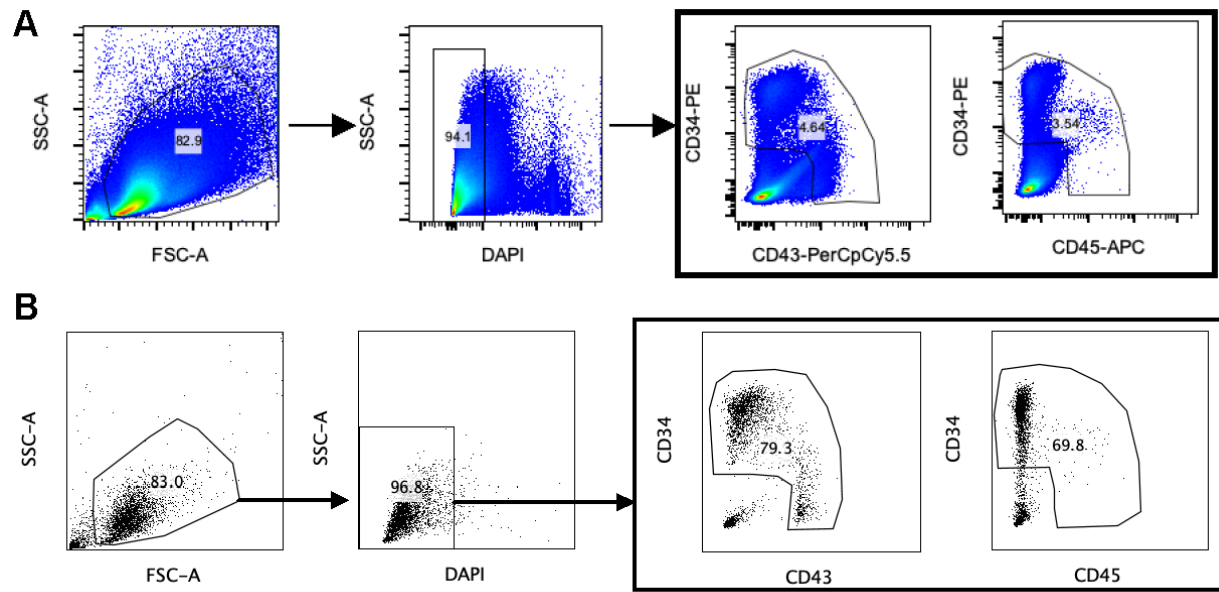


Figure S3.4.1. Isolation of iPSC-derived hematopoietic and endothelial cells for 10X sequencing. Related to Figure 3.4.1. **(A)** Flow sort gating for isolation of CD34⁺CD43⁺CD45⁺ hematopoietic and endothelial cells from day 8 of EB differentiation. **(B)** Flow sorted populations from (A) showing sorting purity of hematopoietic and endothelial populations.

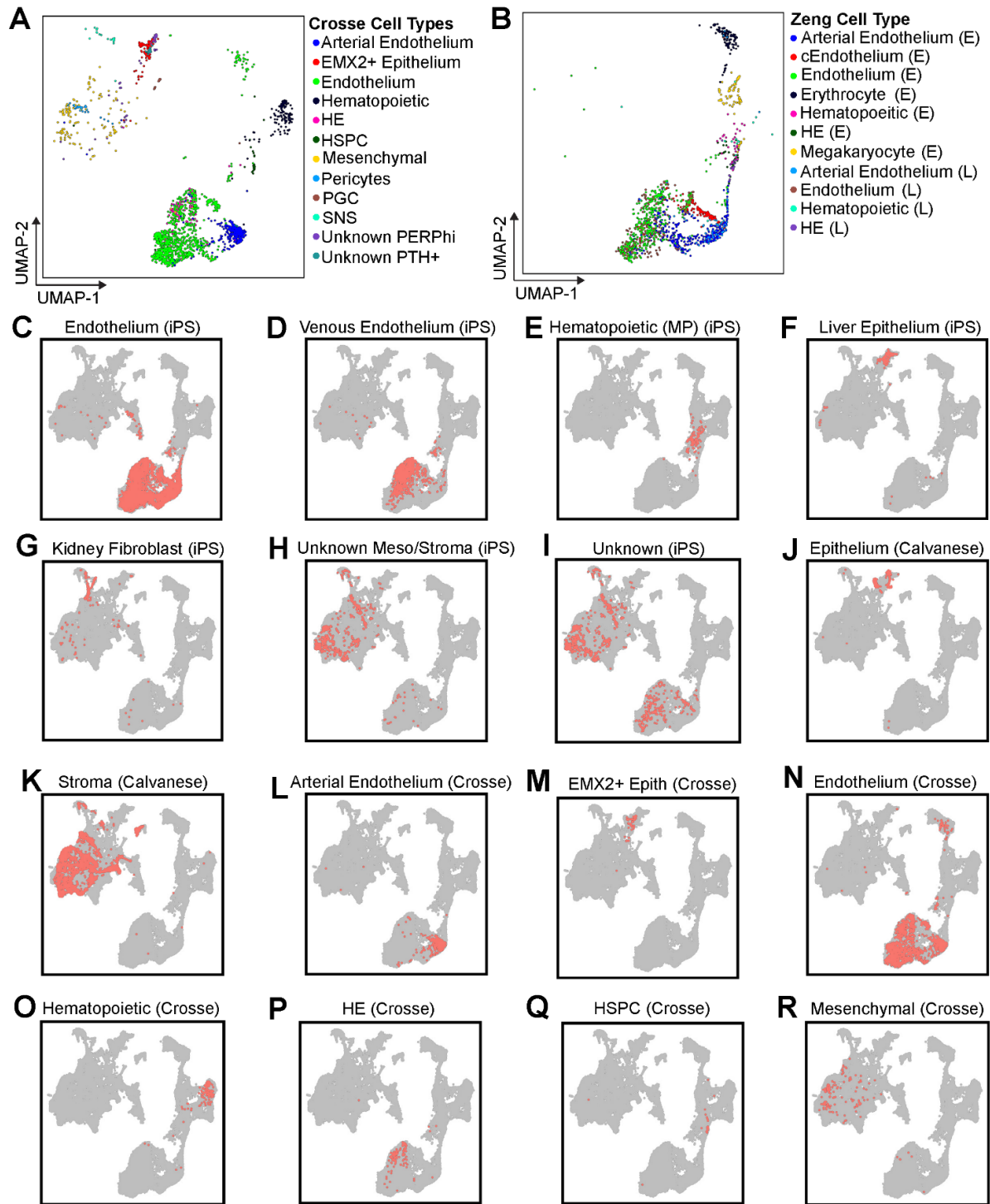


Figure S3.4.2. Cell type label transfers for validation of the integrated dataset. Related to Figure 3.4.2. **(A)** Cell type label transfer from Crosse et al. (ref. 4) onto the integrated scRNA-seq dataset. **(B)** Cell type label transfer from Zeng et al. (ref. 3) onto the integrated scRNA-seq dataset. **(C-R)**. Representative cell type label transfers from each original dataset: day 8 EB, Zeng et al. (ref. 3), Crosse et al. (ref. 4), and Calvanese

et al. (ref. 5) onto the integrated dataset to determine where previously identified cell types are in UMAP space.

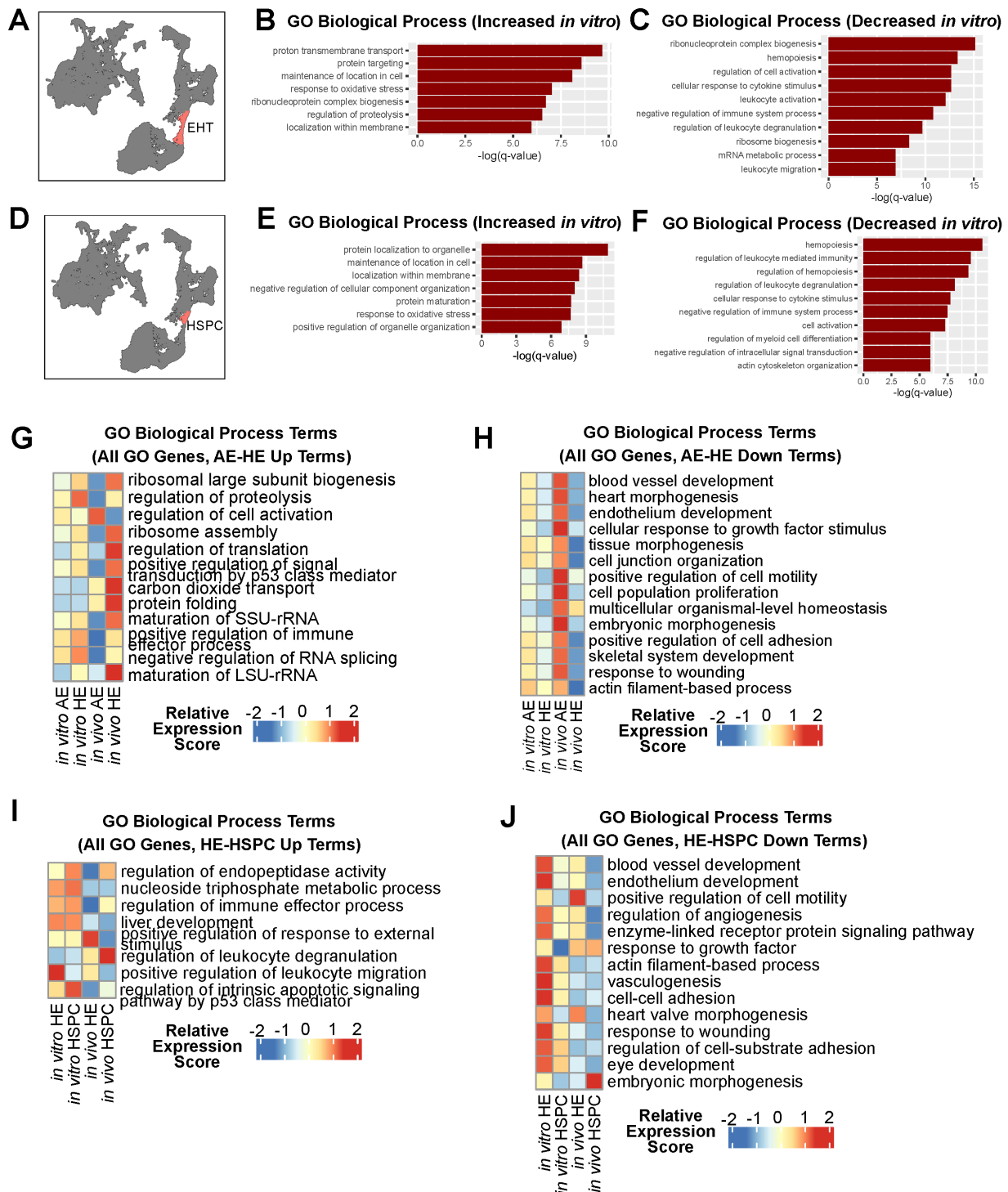


Figure S3.4.3. Expanded comparative molecular analysis of EHT *in vitro* and *in vivo*. Related to Figure 3.4.3. (A) Combined HE (cluster 75) and early HSPC (cluster 74) population undergoing EHT used for differential gene expression analysis. (B,C) Gene ontology (GO) terms significantly upregulated (B) or downregulated (C) in iPSC-

derived vs. HSC-competent human embryo EHT (Metascape, $q < 0.05$). **(D)** Early HSPC (cluster 74) population used for differential gene expression analysis. **(E,F)** GO terms significantly upregulated (E) or downregulated (F) in iPSC-derived vs. HSC-competent human embryo early HSPC (Metascape, $q < 0.05$). **(G)** Relative aggregate expression score of all genes associated with the GO terms upregulated in the AE to HE transition in Figure 4E. **(H)** Relative aggregate expression score of all genes associated with the GO terms downregulated in the AE to HE transition in Figure 3.4.3F. **(I)** Relative aggregate expression score of all genes associated with the GO terms upregulated in the HE to HSPC transition in Figure 4H. **(J)** Relative aggregate expression score of all genes associated with the GO terms downregulated in the HE to HSPC transition in Figure 4I. For (G-I), aggregate gene expression scores were calculated and combined in scaled matrices which were used for heatmap generation.

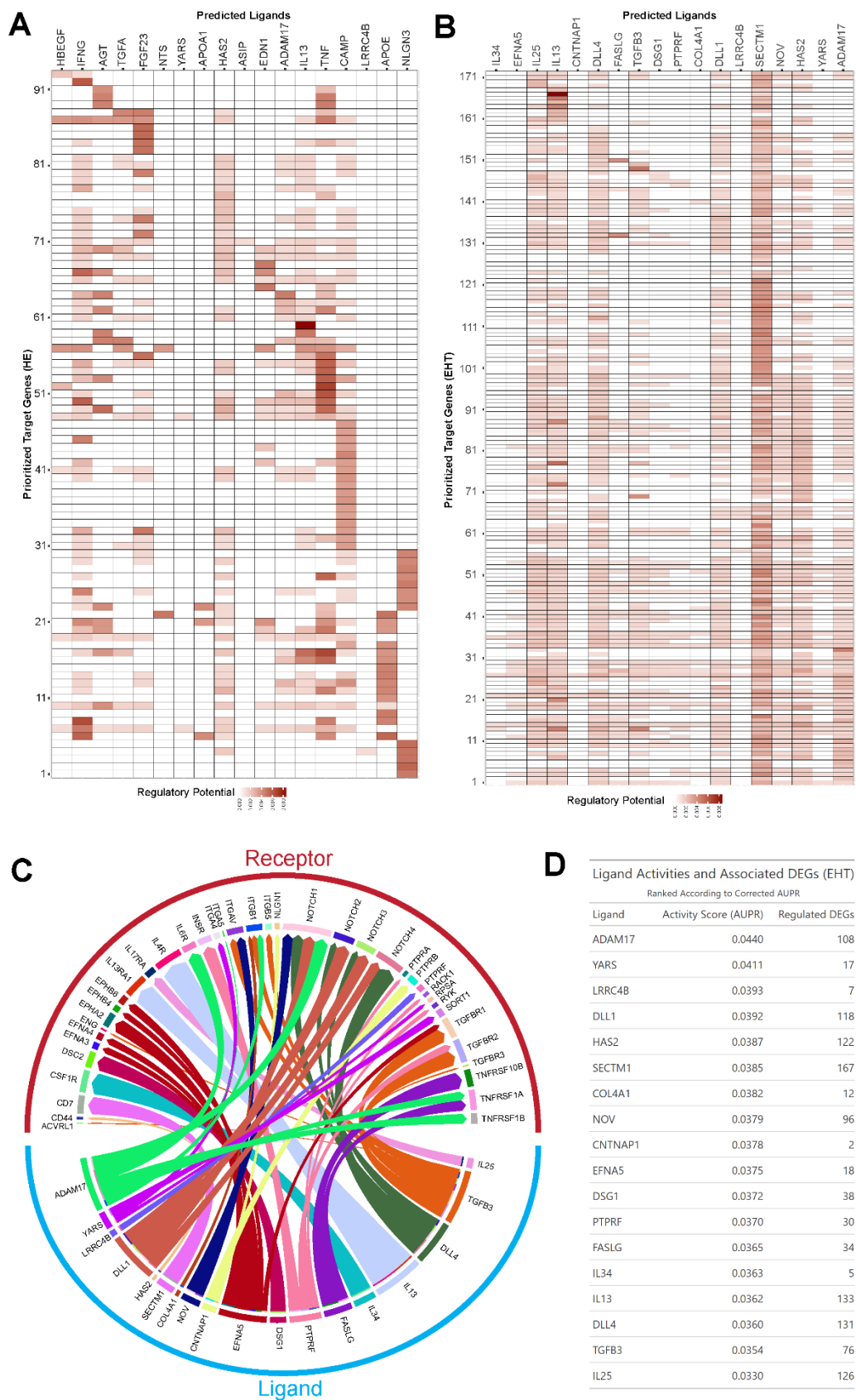


Figure S3.4.4. Expanded map of ligand-receptor interactions to inform iPSC differentiation. Related to Figure 3.4.4. **(A)** Regulatory potential of each predicted ligand for HE (columns) to regulate target DEGs (row); DEGs are listed in Table S5C. **(B)** Regulatory potential of each predicted ligand for EHT (HE + HSPC) to regulate target DEGs; DEGs are listed in Table S5D. **(C)** Top ligand-receptor interactions identified by NicheNetR v.1 associated with differentially expressed genes in day 8 iPSC EBs vs. HSC-competent human embryo EHT (HE cluster 75 + HSPC cluster 74 of integrated dataset). Ligands (bottom) are connected to their respective receptors (top) expressed on EHT cells. **(D)** Top ligands in (C) ranked by the number of DEGs and activity score defined as the area under the precision recall curve (AUPR).

3.5 Methods

Cell Lines: hiPSC lines derived from mesenchymal stem cells (MSC-iPS)⁴³ and CD45+ blood cells (45-iPS)³⁴ were used for the generation of embryoid bodies. Mouse embryonic fibroblasts (MEFs) that were used for iPSC culture pre-embryoid body formation were acquired from the University of Washington Institute for Stem Cell and Regenerative Medicine (ISCRM) core, which were expanded and treated with mitomycin C prior to use.

Materials: All materials used and their catalog numbers can be found in **Table 2.5.1**, **Table 2.5.2**, and **Table 2.5.3**.

hiPSC Culture: hiPSCs were maintained on hESC-Qualified Matrigel in mTESR Plus media. 6-7 days prior to EB formation, iPSCs were transferred onto MEFs and cultured in DMEM/F12 supplemented with knockout serum replacement (KOSR), L-glutamine, non-essential amino acids (MEM-NEAA), 2-mercaptoethanol, and basic fibroblast growth factor (bFGF). All catalog numbers and media concentrations can be found in **Table 2.5.1**, **Table 2.5.2**, **Table 2.5.3**, **Table 2.5.4**, **Table 2.5.5**, and **Table 2.5.6**.

Embryoid Body Differentiation and Dissociation: MEF-cultured hiPSCs were treated with 1mg/mL collagenase IV prior to scraping whole colonies. Scraped whole colonies were allowed to gravity settle, were transferred into EB culture media (**Table 2.5.6**) and put on a shaker at 37°C (Day 0 of differentiation, a.k.a. D0). After approximately 24hr (D1), EBs were collected into 15mL conical tubes and gravity settled. They were resuspended in EB culture media supplemented with final concentrations of 100ng/mL human stem cell factor (hSCF), 100ng/mL fms-like tyrosine kinase 3 ligand (hFLT3L), 50ng/mL bone morphogenic protein 4 (hBMP4), 10 ng/mL granulocyte colony-stimulating factor (hG-CSF), 25 ng/mL interleukin-6 (hIL-6) and 10 ng/mL interleukin-3 (hIL-3) and

again put on a shaker at 37°C. EB culture media with the listed cytokines was refreshed on D5 and D9. On days of collection for sc-RNA-seq or flow cytometry, EBs and all surrounding media were collected into 15mL conical tubes and spun at 1350rpm for 5 minutes to ensure the capture of both cells within the EBs as well as floating hematopoietic cells. The samples were then incubated in IMDM with 2mg/mL Collagenase B at 37°C for 2 hours while shaking with trituration approximately every 30 minutes. After 2 hours, 9mL of IMDM was added to the EB solution and the samples were spun at 1350rpm for 5 minutes. The EBs were then incubated in 2mL Gentle Cell dissociation reagent in a 37°C water bath for 15 minutes followed by vigorous trituration until they formed a single cell solution. 10mL of 3% fetal bovine serum (FBS) in PBS was added and the solution was filtered through a 70µM filter with a subsequent 2mL 3% FBS in PBS wash. The single cell solution was then spun down at 1350rpm for 5 minutes, resuspended in 3% FBS in PBS and the cells were counted using a Denovix CellDrop cell counter prior to subsequent assays.

Flow Cytometry: For the collection of cells used in the D8 10X sc-RNA-seq experiment, all cells from the dissociated EBs (45-iPS-derived) were stained with CD34-PE, CD43-PerCpCy5.5, CD45-APC and DAPI. The cells were then sorted on a BD FACSAria III into 50% FBS in PBS. DAPI was used to gate out dead cells. The gates shown in **Figure S3.4.1A-B** were set within the BD FACSDiva software during the sort. For cell type checks following general dissociation, 100,000 cells in 3% FBS in PBS were used per sample for staining and the flow cytometry was performed on a BD LSRII or BD FACSymphony A3 with subsequent analysis via FlowJo software. For each experiment, 100,000 cells of a single sample (the one with the most cells in any given experiment)

was used for isotype staining. Samples collected on D5-8 were stained with CD34-PeCy7, CD144-FITC, CD43-PerCpCy5.5, CD184-APC and DLL4-PE. Samples collected post-D8 were stained with the same panel, except CD45-APC instead of CXCR4-APC. DAPI was used for gating out dead cells in each experiment. All antibodies and their catalog numbers are listed in **Table 2.5.2**.

Single Cell RNA-Sequencing: For the 10X 3' Chromium Next GEM v. 3.1 sc-RNA-seq sample, 45-iPS-derived D8 EBs were dissociated according to the protocol outlined above and were spun down and resuspended in 0.04% ultrapure BSA post-sort, then loaded onto a Chromium single cell controller. All subsequent steps were performed according to the manufacturer's protocol. Sequencing was performed on a NovaSeq 6000 S1.

FASTQ File Processing: FASTQ files for the iPS-derived EB sample were processed using CellRanger v.4.0.0 to align reads to the GRCh38-2020-A reference genome. FASTQs from Zeng et al.³ were also processed using CellRanger v.4.0.0 to align reads to the GRCh38-2020-A reference genome. FASTQs from Crosse et al.⁴ were processed using CellRanger v.3.1.0 on the 10X Cloud to align to the GRCh38-3.0.0 reference genome. The count matrices and sample information submitted to the GEO database by Calvanese et al.⁵ were used for downstream analyses.

10X 3' sc-RNA-seq Transcriptomic Analysis: The Seurat v.4.0.1 R package was used for the initial analysis of 10X sc-RNA-seq data. Density plots were utilized to determine all UMI and percent mitochondrial read cutoffs. Cells with UMIs < 100, UMIs > 100,000 and more than 20% mitochondrial reads were removed from the D8 iPS-derived EB dataset. Cells with UMIs < 100, UMIs > 7000, and more than 20% mitochondrial reads

were removed from the Zeng et al. CS10 data. Cells with UMIs < 100, UMIs > 9000, and more than 10% mitochondrial reads were removed from the Zeng et al. CS11 data. Cells with UMIs < 100, UMIs > 7000, and more than 40% mitochondrial reads were removed from the Zeng et al. CS13 data. Cells with UMIs < 100, UMIs > 100,000, and more than 10% mitochondrial reads were removed from all Crosse et al. data. Cells with UMIs < 500 and more than 5% mitochondrial reads were removed from the Calvanese et al. data. Each dataset was normalized individually prior to integration. As the canonical correlation analysis (CCA) integration function in Seurat requires a selection of top variable features, the FindVariableFeatures function was set to the maximum number of features in each dataset to avoid filtering of relevant genes as setting the maximum number any lower than the maximum number of genes represented in the dataset resulted in improper alignment (data not shown). The datasets were then subsequently integrated using CCA. The integrated data was dimensionally reduced using 50 principal components and two-dimensional unique manifold approximation projection (UMAP). For downstream differential gene expression and pseudotime analysis, the Seurat integration object was converted into a Monocle cell_data_set object for use in Monocle3 pipelines. All coded used can be found as stated in [Data and Code Availability](#).

Differential Expression Analysis: All differential expression analysis (except that conducted internally by NicheNetR) was conducted using Monocle3. For the comparison of *in vitro* to *in vivo* HE or EHT populations, linear regression using a mixed-negative binomial model to remove batch effects associated with dataset and/or stage (categorized as iPS, Crosse et al. or Calvanese et al., or CS10-17 or iPS, respectively) was used. To identify genes that were differentially expressed between AE (Monocle3 cluster 36) and

HE (Monocle3 cluster 75), or HE (Monocle3 cluster 75) and early HSPCs (Monocle3 Cluster 74) for the D8 hiPS-derived EB *in vitro* data, a standard linear regression as implemented by Monocle3 was utilized since there were no batch effects associated with dataset to be removed. To identify genes that were differentially expressed between AE (Monocle3 cluster 36) and HE (Monocle3 cluster 75), or HE (Monocle3 cluster 75) and early HSPCs (Monocle3 Cluster 74) for the HSC-competent *in vivo* data (Crosse et al. and Calvanese et al.), linear regression using a mixed-negative binomial model to remove batch effects associated with dataset (publication) was used. Differentially expressed genes with q-value > 0.05 and estimates ($\log_2$ Fold Change) < 0.25 or estimates > -0.25 were filtered out.

Cell Type Identification: Hematopoietic-related populations in the 10X sc-RNA-seq data were identified by generating an aggregate score for sets of genes identified by Zeng et al. or Calvanese et al. for each cell type, which was then plotted in UMAP space. These scores were then used to qualitatively annotate Monocle3-determined clusters as specific cell types. See the provided GitHub repository for code pertaining to the generation of the aggregate gene set scores and cluster resolution.

Gene Ontology : The list of differentially expressed genes ($q < 0.05$, $0.25 < \log_2(FC) < -0.25$) from the comparison of *in vitro* to *in vivo* HE or EHT populations was uploaded to Metascape v.3.5⁵² for Gene Ontology (GO) analysis. Significant (q-value / FDR < 0.05) summary GO Biological Process Terms were filtered from the resulting output files. To compare pathways that are differentially regulated during the AE-HE or HE-HSPC transitions between *in vitro* and HSC-dependent *in vivo*, the differentially expressed gene lists for *in vitro* and *in vivo* were uploaded together to Metascape for batch analysis.

Significant (q-value / FDR < 0.05) summary GO biological process terms were then identified.

NicheNetR: For NicheNetR analysis, NicheNetR (12-01-2022 released version) functions were used that internally implemented the Wilcoxon-rank sum for differential expression analysis via Seurat v.4, which identified a similar, although more limited, set of differentially expressed genes (compared to the Monocle3 linear regression analyses) that was ultimately used for upstream ligand-receptor identification. Analysis was done following the NicheNetR Seurat vignette. Receiver cells were designated as either HE (Seurat cluster 61) or EHT populations (Seurat cluster 61 and Seurat cluster 59). Sender cells were set as undetermined to reflect the unknown nature of the source of the ligands that may be signaling to the HE or EHT populations in our data. See the GitHub repository ([Data and Code Availability](#)) associated with this paper for the complete code and associated tables.

3.6 Acknowledgements

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Chapter 4. FGF signaling inhibits the endothelial-to-hematopoietic transition

4.1 Introduction

In the human embryo, HSCs arise from arterialized hemogenic endothelium, a mature form of hemogenic endothelium that undergoes regulation by a series of factors involved in hematopoietic ontogeny, including, but not limited to signaling by several factors, such as fibroblast growth receptor (FGF)^{9, 10, 33}. In zebrafish, a commonly used hematopoietic development model organism, FGF signaling via fibroblast growth factor receptor 4 (FGFR4) expressed in the somites, a nearby cell population to the developing HSC-competent HE niche, is required for bridging the gap between integrating upstream Wnt and generating downstream Notch signals that are required for HSC development⁴⁶. Additionally, transgenic perturbation of FGF signaling via FGFR1 in zebrafish revealed that FGF signaling is also repressive of HSC emergence via inhibition of BMP signaling in the developing arterial regions⁵³. Therefore, FGF has a complex role in the regulation of HSC emergence through the integration of FGF signals via multiple FGFRs, FGFR1 and FGFR4.

In previous chapters, we have shown that the *in vitro* system recapitulates several key steps in HSC development, such as the development of arterialized hemogenic endothelium, but that several factors, such as downregulation of FGF signaling or upregulation of HSC specification markers, are not properly regulated. We see that FGFR4 is not particularly prevalent in HSC-competent human embryo or iPSC-derived HE by transcript (**Figure 3.4.4C**) and that instead FGFR1 appears to be the predominantly expressed receptor. Additionally, direct comparison between carefully staged *in vitro* and *in vivo* cell states spanning hematopoietic ontogeny and the mapping

of ligand-receptor interactions associated with the differential expression of developmental programs identified elevated FGF signaling via FGFR1 as a barrier to hematopoietic specification ([Chapter 3](#)). Here, we explore the role of FGF signaling in EHT, both in embryo and in iPSC systems, via chemical perturbation, which can be easily used in future iPSC protocols for the improvement of *in vitro* hematopoiesis.

4.2 Results

4.2.1. Chemical modulation of FGF signaling during EHT regulates hematopoiesis *in vivo*

To determine the role of FGF signaling during EHT *in vivo*, we first monitored FGF activity in the zebrafish dorsal aorta (DA) using the *dusp6*-d2EGFP transgenic reporter. Consistent with previous reports⁵³, FGF activity in the DA was attenuated following the onset of EHT at 30 hours post-fertilization (hpf) compared to 21 hpf preceding EHT (**Figure S4.4.1A**). We next modulated FGF signaling in zebrafish using a small-molecule FGFR inhibitor Erdafitinib⁵⁴ and an FGF signaling agonist, BCI, that acts by inhibiting a negative feedback loop through DUSP1/6 phosphatases⁵⁵. Erdafitinib and BCI concentrations were titrated using the *dusp6*-d2EGFP reporter to identify concentrations with highest specific activity that did not affect embryonic development (**Figure S4.4.1B-C**). Treatment with Erdafitinib at 16-24 hpf prior to the onset of EHT did not cause a significant change in the number of CD41⁺ HSPCs in the floor of the dorsal aorta (DA) at 52 hpf (**Figure S4.4.1D-E**). By contrast, treatment with BCI at 16-24 hpf led to a significant decrease in CD41⁺ HSPCs in the DA, suggesting that FGF signaling is tightly regulated in early HE specification. We next treated zebrafish with Erdafitinib and BCI starting at 24 hpf, coinciding with the onset of EHT, and assessed the output of CD41⁺ HSPCs at 52 hpf. Inhibition of FGF signaling with Erdafitinib led to a significant increase in CD41⁺

HSPCs in the DA, whereas activation of FGF signaling with BCI led to a significant reduction in CD41⁺ HSPCs in the DA (**Figure 4.4.1A-B**). These data demonstrate that FGF signaling is attenuated in HSC-competent HE and negatively regulates EHT, such that chemical inhibition of FGFR can augment HSPC generation *in vivo* during EHT.

4.2.2. Chemical modulation of FGF signaling during EHT regulates hematopoiesis *in vitro*

We next tested whether chemical inhibition of FGF signaling during iPSC-derived EHT, where FGF activity is predicted to be high, can augment HSPC generation from iPSCs. To this end, we treated EBs derived from two different iPSC lines with Erdafitinib and BCI starting on day 7 of differentiation, coinciding with the beginning of EHT, and removed the small molecules on day 8. In addition, we tested the addition of bFGF (a.k.a. FGF2) or FGF23 ligands on day 7, with removal on day 8 or continued culture. We then measured the generation of CD34⁺CD43⁺ HSPCs on day 10 of differentiation, 48 hours after the removal of the small molecules to allow for EHT to proceed prior to measuring hematopoietic output. Treatment with 0.1 μ M Erdafitinib led to a significant increase in the proportion of CD34⁺CD43⁺ HSPCs on day 10 (n = 9 independent experiments), whereas treatment with FGF agonist BCI, bFGF, or FGF23 ligands had no effect on CD34⁺CD43⁺ HSPC output (**Figure 4.4.1C-E**, **Figure S4.4.1F-I**). Taken together, we identify FGF signaling as one of the pathways upstream of EHT gene expression and demonstrate that it acts as a negative regulator of EHT both *in vivo* and in the iPSC system. Accordingly, chemical inhibition of FGF signaling during EHT augments iPSC-derived hematopoiesis.

4.3 Discussion

FGF signaling emerged from the analysis in Chapter 3 as a potential negative regulator of EHT. FGF signaling has previously been identified as essential for specification of HE from endothelial precursors and transgenic perturbation of FGF-FGFR1 signaling has previously revealed an inhibitory role of FGF signaling in HSC emergence^{46, 56}. However, FGF signaling is attenuated at the onset of EHT in the embryo but remains activated during iPSC-derived EHT⁵³. Our analysis has implicated the canonical FGF2 ligand, widely secreted in EBs and commonly supplemented in iPSC differentiation protocols, and a non-canonical FGF23 ligand, linked to adult phosphate and calcium homeostasis with known roles in erythropoiesis, iron regulation and inflammation⁵⁷⁻⁶¹. We show that FGF signaling is a negative regulator of EHT that can be chemically modulated to augment hematopoiesis *in vitro*.

In the zebrafish, treatment with pan-FGFR inhibitor at the onset of EHT augments HSPC generation, while treatment with a small molecule agonist drastically reduces HSPC production. Similarly, in EB differentiation, inhibition of FGFRs during the developmental window coinciding with the peak of EHT, boosts HSPC generation from iPSCs. Given that many iPSC differentiation protocols incorporate exogenous FGFs in the serum or as recombinant proteins^{27, 40}, the dose and time-dependent modulation of FGF signaling via removal of exogenous FGFs and/or treatment with small molecule inhibitors during the peak of EHT should be considered to optimize HSPC generation in iPSC differentiation protocols, and perhaps optimize rare HSC fate specification *in vitro*.

In summary, single cell comparative analysis of EHT and ligand-receptor predictions identify a negative role of FGF signaling in hematopoietic ontogeny. Our single

cell map of EHT and ligand-receptor analysis provide a resource for future interrogation of novel pathways to improve iPSC differentiation protocols.

4.4 Figures and legends

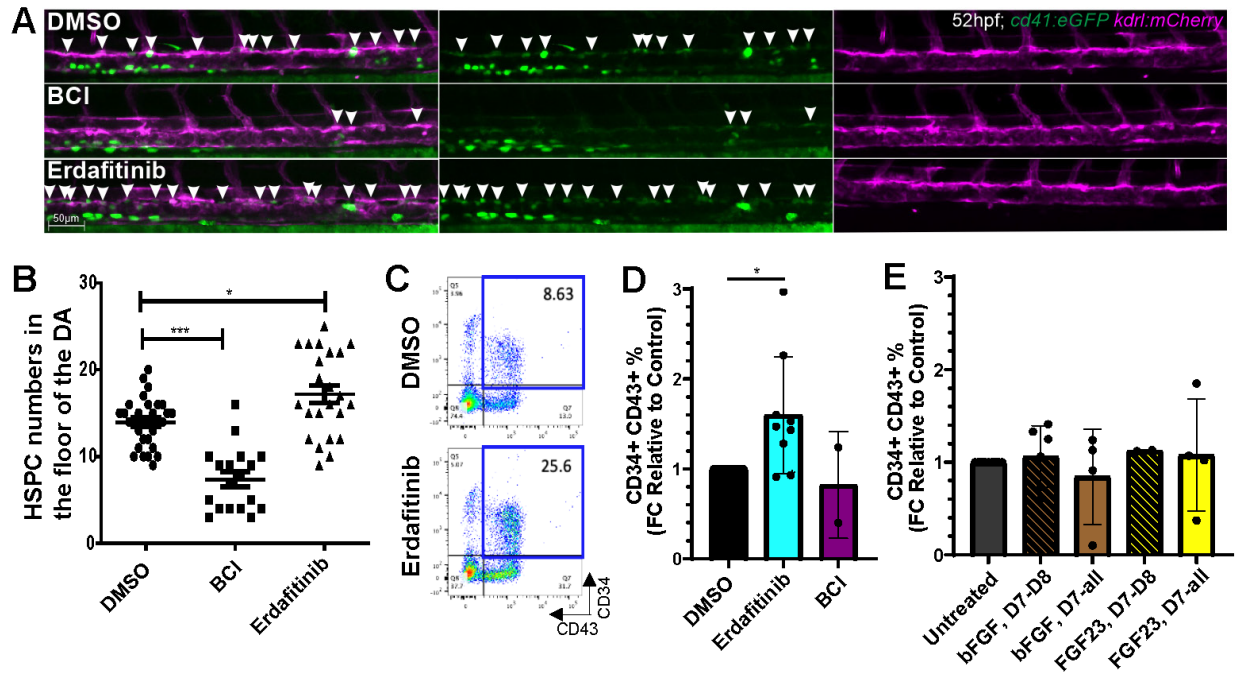


Figure 4.4.1. Chemical modulation of FGF signaling during EHT regulates hematopoiesis *in vivo* and *in vitro*. (A,B) Representative images (A) and quantification (B) of the number of HSPCs (white arrows) in the floor of the dorsal aorta (DA) following treatment with an FGF small molecule-inhibitor (erdafitinib) or agonist (BCI). Zebrafish were treated with 7 µM erdafitinib, 10 µM BCI, or vehicle (DMSO) during EHT from 24-52 hpf. *kdrl:mCherry*⁺ *cd41:eGFP*⁺ HSPCs were quantified at 52 hpf. Mean ± s.e.m. of 30 embryos in 2 independent experiments; significance determined by unpaired two-tailed Student's t-test (*p<0.05, ***p<0.001). (C-E) Representative flow plots (C) and quantification (D,E) of the number of CD34⁺CD43⁺ iPSC-derived HSPCs following treatment with an FGF small molecule-inhibitor (erdafitinib), agonist (BCI), or FGF ligands (bFGF, FGF23). (D) EBs were treated with 0.1 µM erdafitinib, 10 µM BCI, or vehicle (DMSO) from day 7-8 of differentiation and the proportion of CD34⁺CD43⁺ HSPCs was quantified at day 10. (E) EBs were treated with FGF ligands bFGF and FGF23 or vehicle (0.1% BSA) from days 7-8 (D7-8) or days 7-10 (D7-all) of differentiation and the proportion of CD34⁺CD43⁺ HSPCs quantified at day 10. Mean ± s.d. shown of n = 9 independent experiments with two different iPSC lines for erdafitinib (single outlier point removed), and n = 2 - 6 independent experiments with two different iPSC lines for other conditions; significance determined by paired two-tailed Student's t-test (*p<0.05).

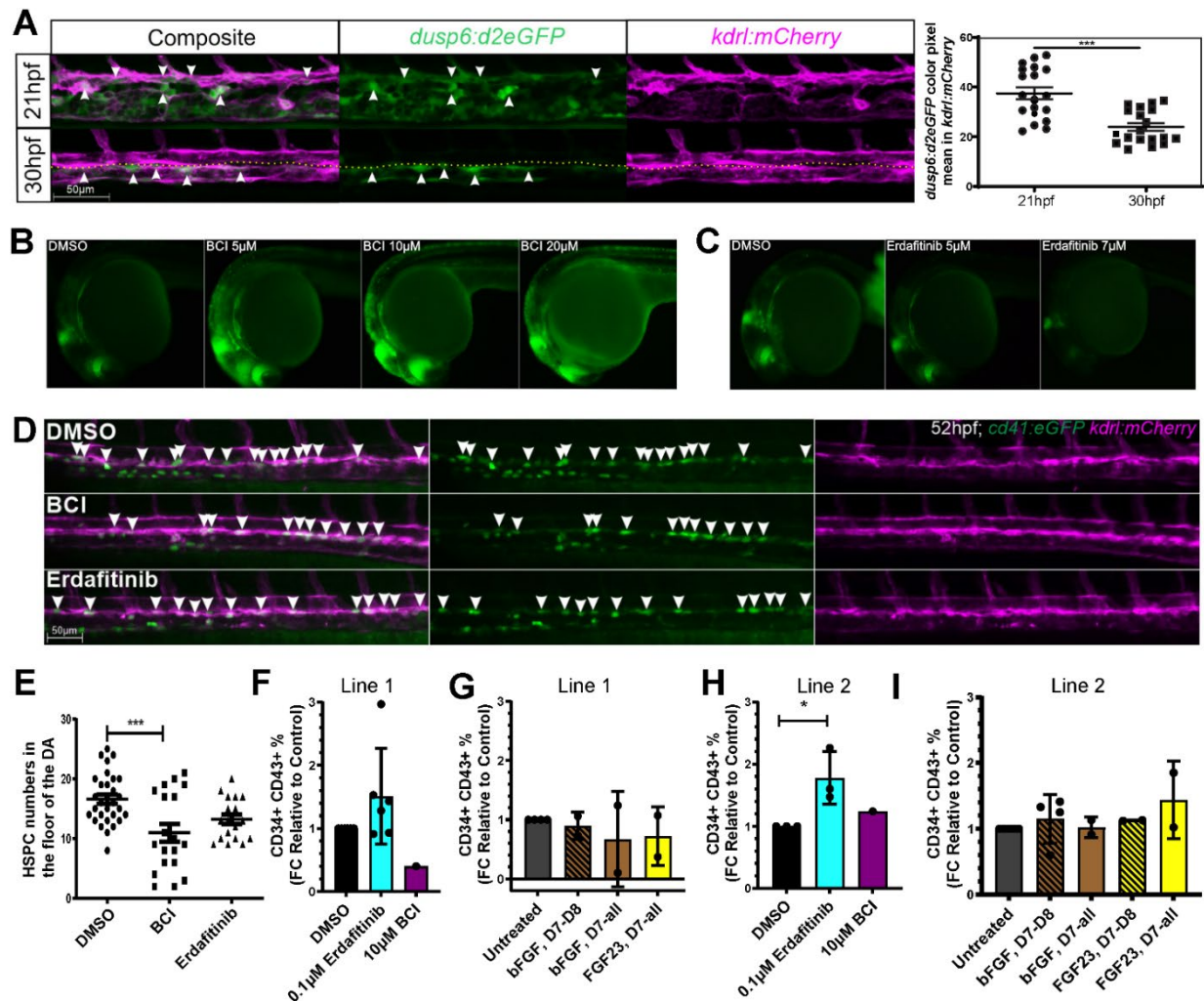


Figure S4.4.1. Expanded chemical modulation of FGF signaling during EHT regulates hematopoiesis *in vivo* and *in vitro*. Related to Figure 4.4.1. **(A)** Representative images (left) and quantification (right) of the number of hemato-endothelial cells with activated FGF signaling in the zebrafish DA prior to EHT (21 hpf) or post-EHT (30 hpf). Activated FGF signaling is quantitated using the *dusp6:d2eGFP* *kdrl:mCherry* double-positive cells. Mean $\pm$ s.e.m. of 30 embryos in 2 independent experiments, unpaired two-tailed Student's t-test (* $p < 0.05$, *** $p < 0.001$). **(B)** Titration of BCI in zebrafish using the *dusp6:d2eGFP* FGF reporter. 10 μ M was used in experiments in Figure 6. **(C)** Titration of erdafitinib in zebrafish using the *dusp6:d2eGFP* FGF reporter; 7 μ M was used in experiments in Figure 6. **(D-E)** Representative images (D) and quantification (E) of the number of HSPCs (white arrows) in the DA following treatment with an FGF small molecule-inhibitor (erdafitinib) or agonist (BCI) prior to EHT. Zebrafish were treated with 7 μ M erdafitinib, 10 μ M BCI, or vehicle (DMSO) 16 - 24 hpf and *kdrl:mCherry*⁺ *cd41:eGFP*⁺ HSPCs were quantified at 52 hpf. Mean $\pm$ s.e.m. of 30 embryos in 2 independent experiments, unpaired two-tailed t-test (* $p < 0.05$, *** $p < 0.001$). **(F-I)** Percent of CD34⁺CD43⁺ iPSC-derived HSPCs following treatment with erdafitinib, BCI, or FGF ligands (bFGF, FGF23) as shown in Figure 6C-D for the

two different iPSC lines (lines 1 and 2). Mean $\pm$ s.d. (single outlier point removed), paired two-tailed Student's t-test (* $p < 0.05$).

4.5 Methods

Ethics declarations and approval for animal experiments: All experiments in zebrafish were performed according to the Guidelines for Ethical Conduct in the Care and Use of Animals⁶², Iowa State University Animal Care and Use Committee IACUC-20-025, and IACUC-20-024 approved protocols, and in compliance with ARRIVE guidelines PMID: 32663219, and the American Veterinary Medical Association (2020) and NIH guidelines for the humane use of animals in research.

Zebrafish husbandry and strains: Tg(dusp6:d2eGFP)pt6 zebrafish were kindly donated by Michael Tsang (PMID:17553162). Other zebrafish lines used in this study were: wt AB* (ZIRC), Tg(kdrl:HsHRAS-mCherry)s896⁶³ (referred to as kdrl:mCherry throughout the manuscript), Tg(-6.0itga2b:eGFP)la2⁶⁴ (referred to as cd41:eGFP throughout manuscript), and various intercrosses of these lines were utilized. Zebrafish were mated, staged, raised, and processed as described (Westerfield, M. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish Danio* (Brachydanio) rerio. (University of Oregon Press., 2000) in a circulating aquarium system at 28 °C.

Zebrafish chemical treatments and microscopy visualization of Tg(dusp6:D2eGFP): 30 16 hpf heterozygous Tg(dusp6:d2eGFP) zebrafish embryos were placed per well on 12-well plates containing 3ml embryo water plus 5μM or 7μM Erdafitinib (JNJ-42756493, Item No. 21813, Cayman Chemical); or 5μM or 20μM BCI (hydrochloride) (Item No. 21945, Cayman Chemical). DMSO was added along with the chemicals to reach a final concentration of 1% to increase permeability. Chemicals were washed off with embryo water at 24hpf. Embryos were then dechorionated with pronase (11459643001, Sigma), anesthetized in 200 mg/ml tricaine (Syncaïne® Fish Anesthetics,

MS-222) and the anterior part of the embryo was imaged for *dusp6* activity in a Leica M205 FCA with Leica Application suite X software (v3.7.6.25997).

Live confocal imaging of HSPCs and *dusp6* activity in zebrafish embryos: To visualize HSPCs and *dusp6* activity, live confocal microscopy was performed at 52 hpf on Tg(cd41:eGFP; kdrl:mCherry), and Tg(*dusp6*:d2eGFP; kdrl:mCherry), double-transgenic embryos, respectively, at the indicated developmental times. Z sections of the DA region were imaged on a Zeiss LSM 700 Laser Scanning Confocal with Zen Black software (v14.0.27.201). *Dusp6* activity within the kdrl:mCherry⁺ cells of the aorta-gonad-pronephros region of the embryo was quantified from individual Z stacks by ImageJ (v1.53f51), by enclosing 3-4 kdrl⁺ endothelial ROI's limited by two contiguous intersegmental vessels using the "Freehand selection". Green channel pixel mean values were then analyzed using "Color Histogram". cd41⁺, kdrl⁺ HSPCs were manually counted throughout the confocal stack comprising the DA and denoted by arrows. Images were processed with ImageJ (v1.53f51). All animals were anesthetized in 200 mg/ml tricaine (Syncaïne® Fish Anesthetics, MS-222) before microscopic analysis.

***In vitro* FGF Experiments:** For Erdafitinib, BCI and FGF ligand treatments *in vitro*, EBs were generated following the same protocol outlined previously. On D7, EBs were pooled in a 50mL conical tube and split as evenly as possible into each of the different conditions. Erdafitinib was used at 0.1 μ M⁵⁴; 10 μ M Erdafitinib led to significant cell death in EBs (data not shown). 10 μ M BCI was used to be in line with the zebrafish data. Both Erdafitinib and BCI were removed approximately 1 day after addition. For treatment with the FGF ligands (either for D7-D8 or added on D7 and maintained through D10 (D7-all)), either 10ng/mL FGF2⁶⁵ or 100ng/mL FGF23⁶⁶ was added. Erdafitinib and BCI were both kept at 1000X

concentration in DMSO. bFGF and FGF23 were kept at 1000X concentration in 0.1% BSA in PBS. For the DMSO control, 1µl of DMSO was added per ml of media. For the untreated control, 1µl of 0.1% BSA in PBS was added per ml of media to ensure consistency. On D10, EBs were dissociated according to the previously discussed protocol and the percentage of live cells in the EB plus their surrounding media that were CD34+ CD43+ was determined.

Statistical Analysis: For *in vitro* FGF signaling experiments, the percentage of CD34+ CD43+ cells in the treatment condition was normalized to the percentage of CD34+ CD43+ cells in the control for each individual experiment, as hematopoietic output efficiency varied from experiment to experiment. Percentages for Erdafitinib and BCI treated cells were normalized to the DMSO control percentage and percentages for ligand (bFGF or FGF23) treated cells were normalized to the untreated condition. If multiple wells were collected for each condition in an experiment (technical replicates), the average percentage was calculated for each condition prior to normalization. Using GraphPad Prism 10, a paired, two-tailed t-test was used to determine significance between normalized control and treatment groups with a significance cut-off of $p < 0.05$. For zebrafish experiments, GraphPad Prism 5 was utilized to perform statistical analysis and represent data. Data were analyzed by unpaired two-tailed t-test and confidence intervals at 95%, or ordinary one-way ANOVA with Tukey's post-test and confidence intervals at 95%.

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