ROLE OF NR4A TRANSCRIPTION FACTORS IN HUMAN LYMPHATIC AND HEMATOPOIETIC LINEAGE SPECIFICATION

by

YUJIE CHEN

(Under the Direction of Natalia B. Ivanova)

ABSTRACT

The members of the NR4A family of nuclear hormone receptors, NR4A1-3, play important albeit redundant roles in maintaining adult hematopoietic stem cell quiescence and vascular homeostasis. However, the roles of these receptors in the development of hematopoietic and endothelial cell lineages remain largely unexplored, due to embryonic lethality observed in knockout mice and limited human data. In this study, we utilize human embryonic stem cell (hESC)-based in vitro models to investigate the roles of the NR4A family in hematopoietic progenitor cell (HPC) formation via endothelial-to-hematopoietic transition in single, double, and triple NR4A knockout hESC cultures. We observed the formation of CD31+ cells expressing lymphatic endothelial cell markers in NR4A-deleted cultures, suggesting a novel progenitor role for NR4A receptors in lymphatic-like endothelial cell differentiation. Additionally, NR4A deletion increases HPC population accompanied by sustained expression of HPC markers, indicating enhanced HPC generation and/or maintenance in knockout cultures.

INDEX WORDS: NR4A1, NR4A2, NR4A3, Hematopoietic Stem and Progenitor Cells, Lymphatic Endothelial Cells, Nuclear Hormone Receptors

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The University of Georgia
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DEDICATION

To Dr. Benjamin Edward Mulligan, who guided me as a mentor, supported me as a friend, and cherished me as a grandfather. His memories and teachings continue to inspire me.

To stem cell and hematopoietic stem cell research community, whose relentless pursuit of knowledge drives us all forward.

ACKNOWLEDGEMENTS

I am grateful to Dr. Natalia Ivanova and Dr. Fabio Santori for welcoming me into their lab and providing me with the opportunity to engage in groundbreaking work. Sincere thanks to my current and past committee members, including Dr. Ivanova, Dr. Lance Wells, Dr. Nadja Zeltner, and Dr. Takahiro Ito for their invaluable feedback in refining my project. I also want to thank the Biochemistry and Molecular Biology Department, especially Dr. Zachary Wood and Angie Stockton, for their dedicated time and support.

Special thanks to Dr. Jinglian Tao for teaching me how to grow as a researcher, and her friendship that provided me with comfort and camaraderie. I also extend my gratitude to Dr. Siddharth Savadatti for countless hours of enriching discussions during undergraduate and graduate studies that broadened my personal and professional perspectives. And thank you to Dr. Tania Rozario for always believing in my potential and what I can achieve.

I am deeply grateful for the enduring influence of Dr. Benjamin Edward Mulligan, whose support and guidance echo through my work even after his passing. His mentorship not only shaped my academic journey but also provided a foundation of moral and emotional support that remains with me. Equally, I owe immense thanks to my family—my mother, father, younger sister, and my cousins—whose support and love have created an environment that enabled me to pursue my career. Their sacrifices and belief in me have been fundamental and I am forever indebted to them and to Dr. Mulligan for their significant contributions to my life.

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CHAPTER 1

INTRODUCTION

Chen, Yujie, et al. "Nuclear Receptors and the Hidden Language of the Metabolome." Cells 13, no. 15: 1284. (2024)

Nuclear Hormone Receptors Literature Review Contribution

Nuclear Hormone Receptors (NHRs) function as transcription factors that play a pivotal role in integrating metabolic processes with cellular functions. The activity of NHRs is modulated by small molecules, which are either products of endogenous metabolism or derived from dietary sources (Tao et al. 2020). This property makes them excellent pharmacological targets. Indeed, 16% of all existing drugs are NHR agonists/antagonists (Santos et al. 2017). The human genome encodes 48 NHRs, each play an important role in human biology and disease. Although extensive research has uncovered many details about the biology of NHRs and their ligands, numerous aspects remain unquestioned. Recently, we published a review article explores ligand-NHR specificity, the evolutionary transition of ligands from hormones to metabolic sensors, and the intricate feedback loops between ligand biosynthesis and NHR regulation (Chen et al. 2024). My specific contribution to this work included designing and creating all the visual figures. Each figure is illustrating key concepts. Figure 1: Presents a detailed taxonomy of NHRs, categorizing them into four classes based on their functional and structural characteristics. Figure 2: Clarifies the distinctions between orthostatic and allosteric ligands—orthostatic ligands bind within the NHR ligand-binding pocket, whereas allosteric ligands attach at alternative sites on the ligand-binding domain. Figure 3: Traces the evolutionary trajectory of NHRs, illustrating their development and diversification across the evolutionary tree. Figure 4: Demonstrates how ligands exert selective pressure on NHRs, facilitating the structural evolution of ligands to optimize their fit and function with specific receptors. Figure 5: Describes the feedback mechanisms involving vitamin receptors, detailing how these receptors regulate the synthesis and degradation of vitamins, thereby maintaining a balanced internal environment through tightly controlled feedback loops. Figure 6: Outlines the conventional methodologies employed in ligand identification for NHRs, by

experimental approaches used to discover and characterize ligands. Additionally, I contributed to comprehensive revisions of the drafts to ensure the content's accuracy and readability.

Chapter 2

PROJECT DESCRIPTIONS

2.1 Hematopoietic and Vascular Systems

Hematopoiesis, or blood development, is fundamentally interconnected with the vasculature systems. Endothelial cells (ECs) and hematopoietic cells originate from the shared mesodermal progenitor, the hemangioblast, during embryogenesis (Vogeli et al. 2006). This shared origin enables endothelial and hematopoietic cells to maintain a close spatial relationship, creating a specialized microenvironment where their ongoing interaction that supports hematopoietic maintenance and differentiation from development (Heck, Ishida, and Hadland 2020) into adulthood (Pinho and Frenette 2019).

Normal hematopoiesis occurs in several distinct waves across different anatomical sites in developing vertebrate embryo. It is depended on the regulation and formation of multipotent hematopoietic stem cells (HSCs), orchestrated through intrinsic transcription factor networks that control gene expression and the cell cycle, coupled with extrinsic factors secreted by the vascular niche in the bone marrow (Heck, Ishida, and Hadland 2020; Butler, Kobayashi, and Rafii 2010; Yancopoulos et al. 2000; Yuan et al. 2004). The first transient or primitive wave occurs at E7.0 in extraembryonic yolk sac to give rise to unipotent erythroid and myeloid progenitors (Ditadi, Sturgeon, and Keller 2017). The second wave occurs at E8.5-E9 in both yolk sac and intra-embryonic para-aortic splanchnopleure to generate lymphoid-primed multipotent progenitors as well as hematopoietic stem cell (HSC) precursors. The final or definitive wave occurs at E10.5-E11.5 in the dorsal aorta of aorta-gonad mesonephros to generate multipotent HSCs through the

process of Endothelial-to-Hematopoietic Transition (EHT). During EHT, the specialized ECs within the embryonic vasculature gradually reprograms and transform into HSCs (Sugden and North 2021; Calvanese et al. 2022). Once established, these HSCs undergo expansion and self-renewal in the fetal liver between E12.5-E14.5, and eventually migrate to the bone marrow, where they home into the HSC niche. This vasculature and stromal niche in the bone marrow support the lifelong self-renewal of HSCs and the production of all mature blood cell lineages within an organism lifespan (Dzierzak and Bigas 2018). This includes the formation of red blood cell-producing erythrocytes, immune-responsive macrophages, antibody-secreting B cells, and tumor-killing T cells (Dzierzak and Philipsen 2013; Boada-Romero et al. 2020; Nutt et al. 2015; Oh and Fong 2021). Mutations in genes that regulate HSC formation and proliferation, as well as disruptions in the hematopoietic niche, are known to cause various blood disorders and hematological malignancies. (Daver et al. 2019; Sood, Kamikubo, and Liu 2017; Calvi et al. 2003).

2.2 NR4A1-NR4A3 and Their Impact in Hematopoietic and Vascular Systems

The Nuclear Hormone Receptor NR4A family included three highly homologous transcription factors: NR4A1, NR4A2, NR4A3. Each receptor contains a unique activation function AF-1 domain located in the N-terminus, which interacts with co-activators/co-repressors and functions independently of ligands. In additionally, these receptors also have DNA-binding domain with two zinc-fingers that will bind to hormone response elements. The C-terminus have ligand-binding domain that includes a ligand-dependent transactivation function AF-2 (Herring, Elison, and Tessem 2019; Boulet et al. 2022). The endogenous ligands for NR4A1-NR4A3 remain unidentified, and these receptors have traditionally been considered as ligand-independent due to crystallographic studies showing that their ligand-binding pockets are occupied by hydrophobic

residues (Flaig et al. 2005; Wang et al. 2003). Instead, NR4A receptors are considered as immediate-early genes, functioning primarily through rapid and transient induction of their expression in response to extracellular signals such as ERK and JNK (Maxwell and Muscat 2006). However, recent research has shown that NR4A ligand-binding domains are highly dynamic (de Vera et al. 2019). Additionally, separate studies have shown that NR4A transcriptional activities can be modulated directly by prostaglandin E1 and A1 binding to the ligand-binding domain (Rajan et al. 2020).

NR4A receptors are expressed in various tissues and are influenced by a diverse signaling pathways, enabling them to impact biological process such as cellular proliferation, apoptosis, inflammation, and angiogenesis (Herring, Elison, and Tessem 2019). NR4A receptors have gained prominence in hematopoietic research as mutations in these receptors have been identified in patients with hematological malignancies, including Acute Myeloid Leukemia and Non-Hodgkin's Follicular Lymphoma (Boulet et al. 2022; Safe and Karki 2021).

NR4A receptors contribute to normal hematopoiesis through both cell-intrinsic and cell-extrinsic roles, influencing not only the genetic regulation within HSCs but also modulating the microenvironment that supports their development and function. NR4A receptors extrinsically regulate adult HSCs quiescence and differentiation by orchestrating endothelial cell proliferation (Liu et al. 2003) and maintain vascular homeostasis. Overexpression of NR4A1 in Human Umbilical Vein Endothelial Cells (HUVEC) disrupts endothelial cell cycle progression by enhancing the expression of the G1 cell cycle inhibitor $p27^{kip1}$ and reducing the levels of cyclin A (Arkenbout et al. 2003). Moreover, NR4A1 plays a critical role in dampening endothelial cell activation thereby regulate leukocyte adhesion via the NF-kB signaling pathway by binding to the

promoter of IκBα. Overexpression of NR4A1 significantly enhanced the IκBα promoter's activity for increased inhibition of NF-kB to prevent its translocation into the nucleus (You et al. 2009).

In addition to extrinsic regulation, studies in mice showed that Nr4a receptors also exert an intrinsic transcriptional regulation of adult HSCs quiescence. Overexpression of Nr4a2 have shown to promotes HSCs quiescence by activating early G1 cell cycle inhibitor p18 INK4c, while deletion of a single Nr4a2 allele induces HSC proliferation (Sirin et al. 2010). In a separate study, mouse Nr4a1 and Nr4a3 limit adult HSCs proliferation through direct interaction with enhancer element C/EBPα and NF-kB. Additionally, deletion of Nr4a1 and Nr4a3 in adult HSCs downregulated C/EBPα-driven antiproliferative network while concurrently promote NF-kB-driven inflammatory responses (Freire and Conneely 2018). Deletion of Nr4a1 and Nr4a3 also shown to downregulate AP-1 transcription factors c-Jun and JunB as well as apoptosis regulators Fas-L and TRAIL, leading to abnormal proliferation of HSCs and myeloid progenitor cells (Mullican et al. 2007). While deletion of Nr4a1 and Nr4a3 leads acute myeloid leukemia, reduced expression results in mixed myelodysplastic/myeloproliferative neoplasms in mice (Ramirez-Herrick et al. 2011). However, individual deletion of Nr4a1 or Nr4a3 exhibit minimal impact, indicating functional redundancy among these homologous receptors (Mullican et al. 2007). Furthermore, studies with engineered CAR-T cells in mice, where all three NR4A receptors were deleted, have demonstrated effectiveness in targeting solid tumors (Chen et al. 2019). Therefore, investigation into NR4A receptors is crucial, given their potential as targets for therapeutic interventions in cancer treatment.

2.3 Scientific Premise and Impact

While the importance of Nr4a in regulating adult HSCs is well-established using murine models, the distinct roles of each receptor in embryonic development, particularly how they influence HSC development through the EHT, remain largely unexplored. The embryonic lethality observed in Nr4a knockout mice (DeYoung et al. 2003; Zetterström et al. 1997) precluded analyses of developmental roles of the Nr4a family within hematopoietic and endothelial lineages. Although NR4A receptors are highly conserved across species, the specific differences in gene expression patterns, signaling pathways, and regulatory networks between species may significantly influence how they function in human hematopoiesis (Balkenhol et al. 2020; Breschi, Gingeras, and Guigó 2017; Ingersoll et al. 2010). Likewise, the role of this NHR family in human blood and endothelial lineage specification have not been studied.

To dissect the roles of NR4A receptors in human hematopoietic and endothelial development, we chose to model the specification of human hematopoietic and endothelial cells with human embryonic stem cells (hESC) in vitro. We utilized CRISPR/Cas9 genome editing to generate single, double, and triple NR4A knockout (KO) hESC lines. Analyses of hematopoietic development in these KO lines revealed two striking phenotypes.

We found that while wild-type (WT) cultures formed hematopoietic progenitor cells (HPCs) via EHT, dKO and tKO cultures also formed CD31+ cells that expressed vein EC markers NR2F2 and NRP2 (You et al. 2005; Soker et al. 1998). Furthermore, the key markers of lymphatic EC lineage PROX1, FLT4, and LYVE1 (Wigle and Oliver 1999; Kaipainen et al. 1995; Banerji et al. 1999) were induced in dKO and tKO cultures but were not detected in WT cultures. Based on these data, we hypothesize that during hematopoietic specification of hESCs, there is a NR4A-

dependent progenitor cell that, in the absence of NR4A receptors, gives rise to lymphatic-like ECs (LECs).

Our preliminary studies also demonstrated that while HPCs that form in WT cultures failed to maintain the expression of HPC markers RUNX1, MYB, and GATA2, beyond day 3 of the EHT, the NR4A-KO cultures exhibited elevated and sustained expression of these markers. (North et al. 2002; Greig, Carotta, and Nutt 2008; Tsai et al. 1994). Furthermore, the fraction of CD34+CD45+ HPCs in NR4A-KO cultures was increased and these cells exhibited enhanced myeloid colony forming capacity. We will test the hypothesis that NR4A receptors regulate the maintenance of HPCs via two distinct albeit not mutually exclusive mechanisms. First, NR4A receptors could limit proliferation of HPCs in a cell autonomous manner. This hypothesis aligns with previous findings in mice where the deletion of Nr4a2 (Sirin et al. 2010) or simultaneous deletion of Nr4a1/3 enhance HSC proliferation and result in acute myeloid leukemia (Mullican et al. 2007). The second mechanism is non-cell autonomous and is mediated by LEC-like cells that are present in NR4A-KO cultures and could serve as a HPC niche. Prior reports have shown that, in the bone marrow, ECs establish a vascular niche that play a vital role in maintaining hematopoiesis through the release of angiocrine factors (Kobayashi et al. 2010), including EC-derived growth factors ANGPT2 and adhesion molecule VCAM-1 (Yancopoulos et al. 2000; Jacobsen et al. 1996). Moreover, it has been reported that proliferating LECs in human and mouse bone marrow secrete CXCL12 that supports the regeneration of bone and hematopoietic tissues after injury (Biswas et al. 2023). Thus, research proposed will elucidate NR4A-regulated intrinsic and paracrine mechanisms that support the developing human HPCs.

NR4A receptors are members of the nuclear hormone receptor superfamily, which can be regulated by endogenous or dietary small molecules, positioning them as appealing

pharmacological targets. The association between increased angiogenesis in leukemia underscores the critical role of the vascular and lymphatic system in cancer. By examining how NR4A receptors affect vascular and lymphatic endothelial cell behavior, this project could provide a new strategy to manipulate vascular and lymphatic growth thereby impacting tumor metastasis and treatment efficacy. Furthermore, this project is crucial for understanding the underlying mechanisms of NR4A in blood cell development and the maintenance of blood system homeostasis, offering insights into fundamental biological processes. The reduction or silencing of NR4A expression frequently observed in hematological malignancies highlights the critical role of the NR4A receptors in cancer biology (Ramirez-Herrick et al. 2011; Mullican et al. 2007). (Mullican et al. 2007)Thus, understanding how the loss of NR4A receptors affects human hematopoiesis is critical for developing new treatments for hematopoietic malignancies.

2.4 Results: Generation of NR4A KO hESC Lines

To investigate the roles of NR4A receptors in human hematopoiesis, I utilized CRISPR/Cas9 gene editing technology to create seven NR4A-KO lines from H1 hESCs (Sanjana, Shalem, and Zhang 2014; Shalem et al. 2014). This set included single knockouts (sKOs) for each of the three NR4A receptors, double knockouts (dKOs) for every possible combination, and a triple knockout (tKO) encompassing all three NR4A receptors. For gene editing, I utilized transient transfection with LentiCrisprV2 vectors harboring gRNAs targeting specific NR4A genes (Santos et al. 2016) **(Fig.1A)**. To enhance the efficiency of clone screening and ensure targeted disruption of gene function, pairs of gRNAs were meticulously designed to induce genomic deletions just upstream of the DNA-binding domain for each NR4A gene. The location of these deletions disrupts critical regulatory elements that are essential for gene expression and protein functionality,

while also minimizing potential off-target effects **(Fig. 1B)**. This targeting ensures that observed phenotypes are directly linked to specific alterations in NR4A function. KO clones with frame-shift deletions were selected based on PCR results and further confirmed through genomic sequencing **(Fig. 1C, Fig. 2)**. To enhance the robustness of subsequent experimental analyses and reduce the risk of off-target effects, two clones from each NR4A KO cell line were established for further studies.

2.5 Results: Deletion of NR4A receptors in hESCs upregulated EC markers *in vitro*.

To investigate the impact of NR4A deletion on the development of hESCs-derived HPCs, I adopted a published protocol (Sugimura et al. 2017) which utilizes specific cytokine cocktails to induce HPCs differentiation. Single cell suspension of hESCs were plated in AggreWell400 tissue-culture plates and cultured in mTeSR+ medium for 2 days to promote uniform embryoid body formation. This was followed by a 7-day differentiation protocol towards mesoderm, hemangioblast, and hematopoietic progenitor cells. At day 7, when expression of RUNX1 peaked, CD34+ cells were isolated using anti-CD34 magnetic beads and seeded onto Matrigel-coated tissue-culture dishes to undergo EHT for 7 days **(Fig. 3A)**. During differentiation, the NR4A receptors are induced as early as day 4 and upregulated during EHT **(Fig. 3B)**. This early expression and upregulation highlight the critical role of NR4A receptors in initiating and supporting the complex process of EHT, where ECs are transformed into HPCs. The timing of NR4A activation suggests that these receptors may act as pivotal regulators in the early stages of hematopoietic differentiation, potentially influencing cell fate decisions and the efficiency of hematopoiesis. Deletion of both NR4A2 and NR4A3 (dKO) and all three NR4A (tKO) did not

alter the expression patterns of ESC marker OCT4 or mesoderm marker MIXL1, suggesting that the observed NR4A phenotypes are post-mesodermal and likely EC and HPC specific **(Fig. 3C)**.

During the first 7 days of differentiation, the dKO and tKO cultures showed a reduction in the KDR+ hemangioblast population at day 4 **(Fig. 3D)** and an increase in expression of pan-EC marker CD31, arterial markers SOX17 and CXCR4, and venous markers NR2F2 and NRP2 **(Fig. 4A-E)**. The decrease in KDR+ hemangioblast suggests a potential alteration in the developmental pathways within these NR4A KO cultures, and that NR4A receptors might normally act to sustain a reservoir of these progenitors for proper vascular and hematopoietic development. In dKO/tKO cultures the expression of venous EC markers NR2F2 increased throughout the EHT whereas in WT cultures this marker was rapidly downregulated - in agreement with previous studies (Sugimura et al. 2017) **(Fig. 4F).** A notable increase in CD31+ cells in dKO/tKO cultures also observed by FACS **(Fig. 4G)**. CD31+CD73+ venous ECs were also detected by FACS in dKO/tKO but low in WT cultures **(Fig. 4H).** These results could indicate that NR4A receptors play a critical role in modulating the dynamics of EC phenotypes during EHT, with their deletion skewing cell fate decisions towards maintaining or even augmenting venous endothelial characteristics.

Furthermore, key markers of lymphatic EC (LEC) commitment and differentiation PROX1, FLT4, and LYVE1 were also upregulated in dKO/tKO cultures while they are minimally expressed in WT cultures **(Fig. 5A-D).** Developmental origins of LECs are still being debated, with evidence suggesting that they could arise from venous ECs (Srinivasan et al. 2007) or from non-venous hemangioblast (Stanczuk et al. 2015) which are traditionally associated with hematopoietic cell development. LECs can also emerge from paraxial mesoderm (Stone and Stainier 2019) as opposed to the dorsal lateral mesoderm origin of other ECs. My preliminary data

indicates that in the absence of NR4A receptors, the pathway of hematopoietic specification branches out to generate LEC-like cells in addition to HPCs. This observation suggests the existence of a LEC-competent progenitor cell whose fate is regulated by the NR4A-controlled gene network.

2.6 Results: Deletion of NR4A receptors in hESCs alters HPCs development *in vitro*.

Consistent with previous reports, expression of HPC marker RUNX1 in WT cultures did not persist beyond day 7+3 of differentiation (Sugimura et al. 2017). In contrast, dKO/tKO cultures exhibited continuous increase in the expression of HPC markers in RUNX1, MYB and GATA2 **(Fig. 6A-C)**. Alongside the increased in CD31+ ECs population, dKO/tKO cultures also exhibited an increase in the CD34+CD45+ HPCs population **(Fig. 6D)**. This finding suggests that in the absence of NR4A receptors, HPCs population could be capable of *in vitro* expansion. This aligns with previous finding in mice showing that deletion of Nr4a receptors promotes hematopoietic cell proliferation (Mullican et al. 2007; Freire and Conneely 2018; Sirin et al. 2010). This observation was supported by the morphological evidence of abundant hematopoietic clusters and underlying ECs in both dKO/tKO cultures during EHT **(Fig. 6E)**.

Prior research also highlighted the importance of vascular niche formed by ECs which secrete angiocrine factors to support HSCs (Kobayashi et al. 2010). Notably, LECs have been shown to produce CXCL12 in the bone marrow, aiding bone and hematopoietic regeneration after tissue damage (Biswas et al. 2023). The NR4A dKO and tKO cultures also exhibited highly elevated CXCL12 mRNA level compared to WT or hESC derived pure vein population **(Fig. 6F).** These observations suggest that NR4A receptors act to modulate the proliferation of both ECs and HPCs, thereby maintaining a stable hematopoietic environment. In the dKO/tKO cultures, the absence of

NR4A receptors eliminates this critical regulatory control, leading to an unchecked expansion of both endothelial and hematopoietic progenitor populations. The elevated CXCL12 chemokine in the NR4A deleted culture suggests that NR4A deletions not only endothelial cell dynamics, but also potentially enhance the environment or signaling that supports the expansion of HPCs.

Studies in mice have shown that embryonic deletion of Nr4a also augments downstream myeloid progenitor cell proliferation and reduces the efficiency of lymphoid differentiation (Mullican et al. 2007; Sekiya et al. 2013). To explore the effects of NR4A receptors on human HPC function, I conducted colony-forming unit (CFU) assays to assess the myeloid differentiation potential of NR4A tKO HPCs. Due to the heterogeneity in the initial 3D HPC protocol, I adopted a monolayer stage-specific differentiation protocol that achieved over 90% HPC purity (Fowler et al. 2024) for downstream functional analysis. hESCs were differentiated into arterial endothelium, then re-seeded on Notch agonist-coated plates to induce hemogenic endothelium and eventually HPC formation **(Fig. 7A)**. These HPCs were disassociated into a single-cell suspension and plated in methylcellulose-based media for CFU assays for 14 days. The colonies were examined under inverted microscope and quantified based on cell morphologies **(Fig. 7B)**. NR4A dKO/tKO HPCs demonstrated significantly higher myeloid colony formation compared to WT HPCs, with a notable increase in progenitors for GM (Granulocyte-Macrophage) **(Fig. 7C).** This enhanced colony-forming capacity suggests that NR4A receptors may play a suppressive role in myeloid proliferation and differentiation, thereby, help maintain hematopoietic balance and prevent the potential exhaustion of progenitor pools.

2.7 Figures

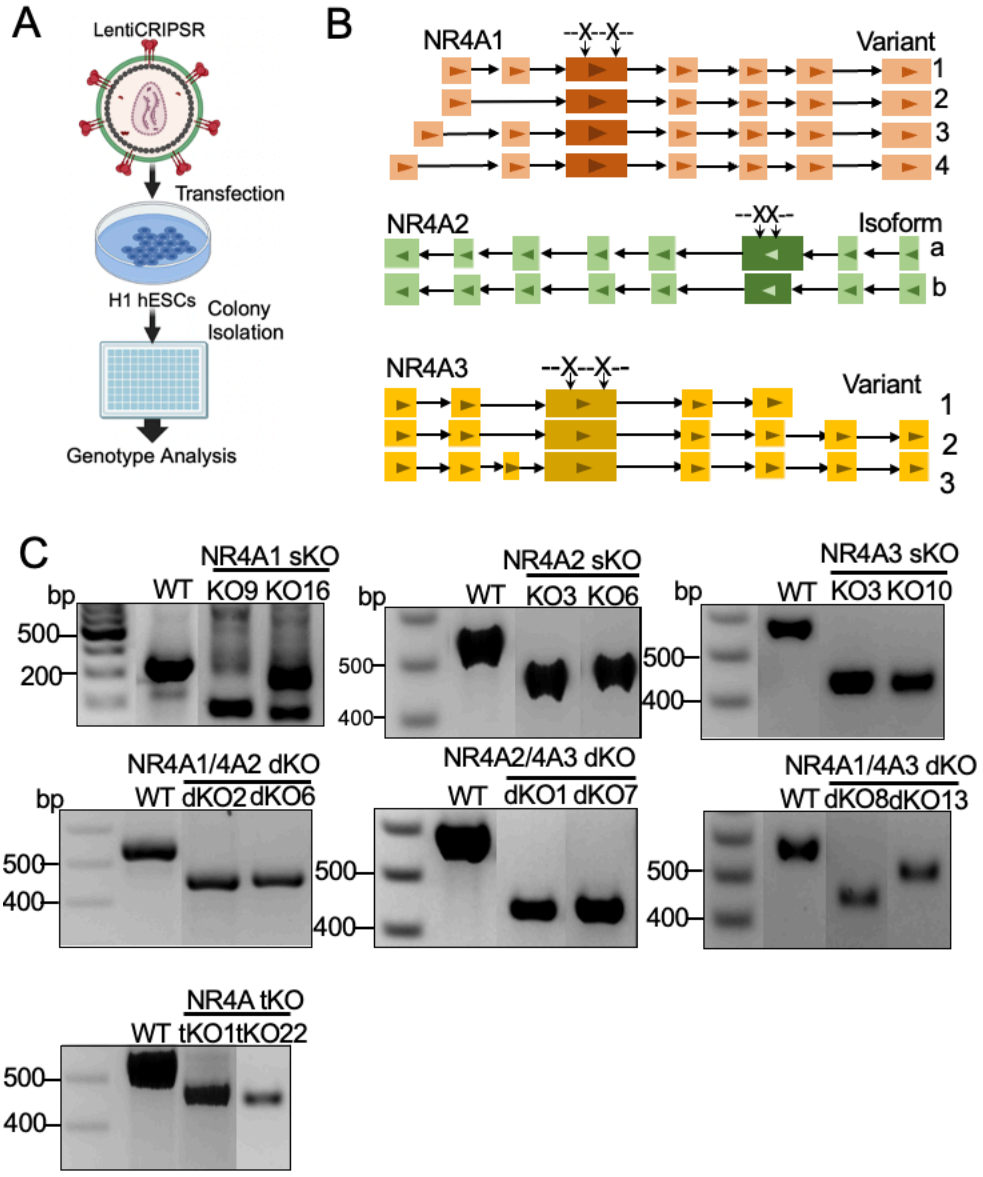

Figure 1: Generation of seven NR4A-KOs from WT hESCs. **(A)** Schematic for deletion of each NR4A gene using CRISPR/Cas9 editing. Transfection of two gRNA vectors was used to create NR4A-KOs. Clones were selected using puromycin, collected and genotyped using PCR and genomic sequencing. **(B)** Two gRNAs were designed based prior to DNA-binding domain for each NR4A genes. **(C)** PCR results of each NR4A-KO cell lines.

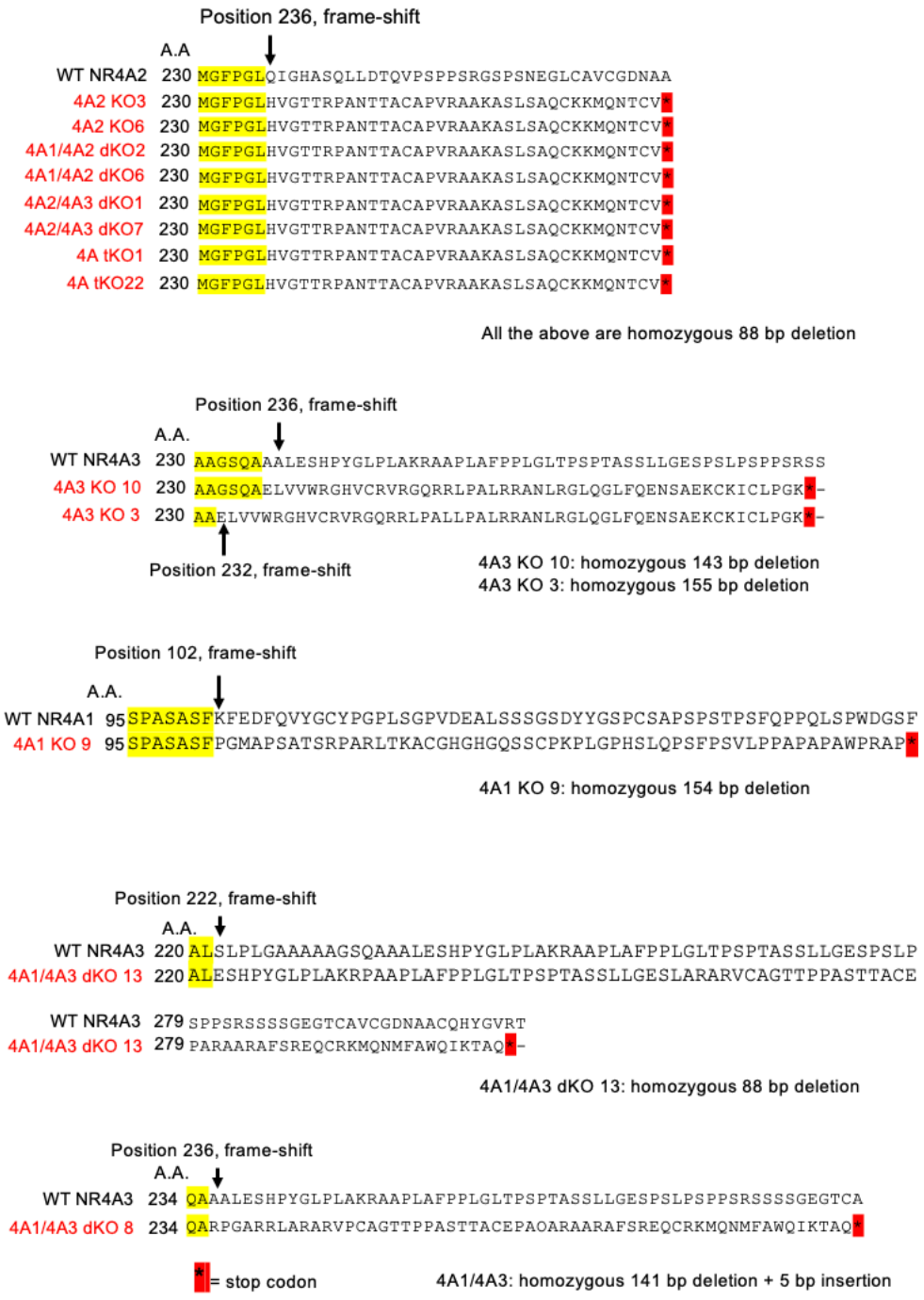

Figure 2: Genomic sequencing results for each NR4A-KO cell lines. Clones of each NR4A-KOs cell lines are selected based on frame-shift mutation and presence of pre-mature stop codon; this is to ensure inactivation of the NR4A protein. Two or more clones are selected for each cell line ensure phenotype is NR4A dependent instead of off-target effect.

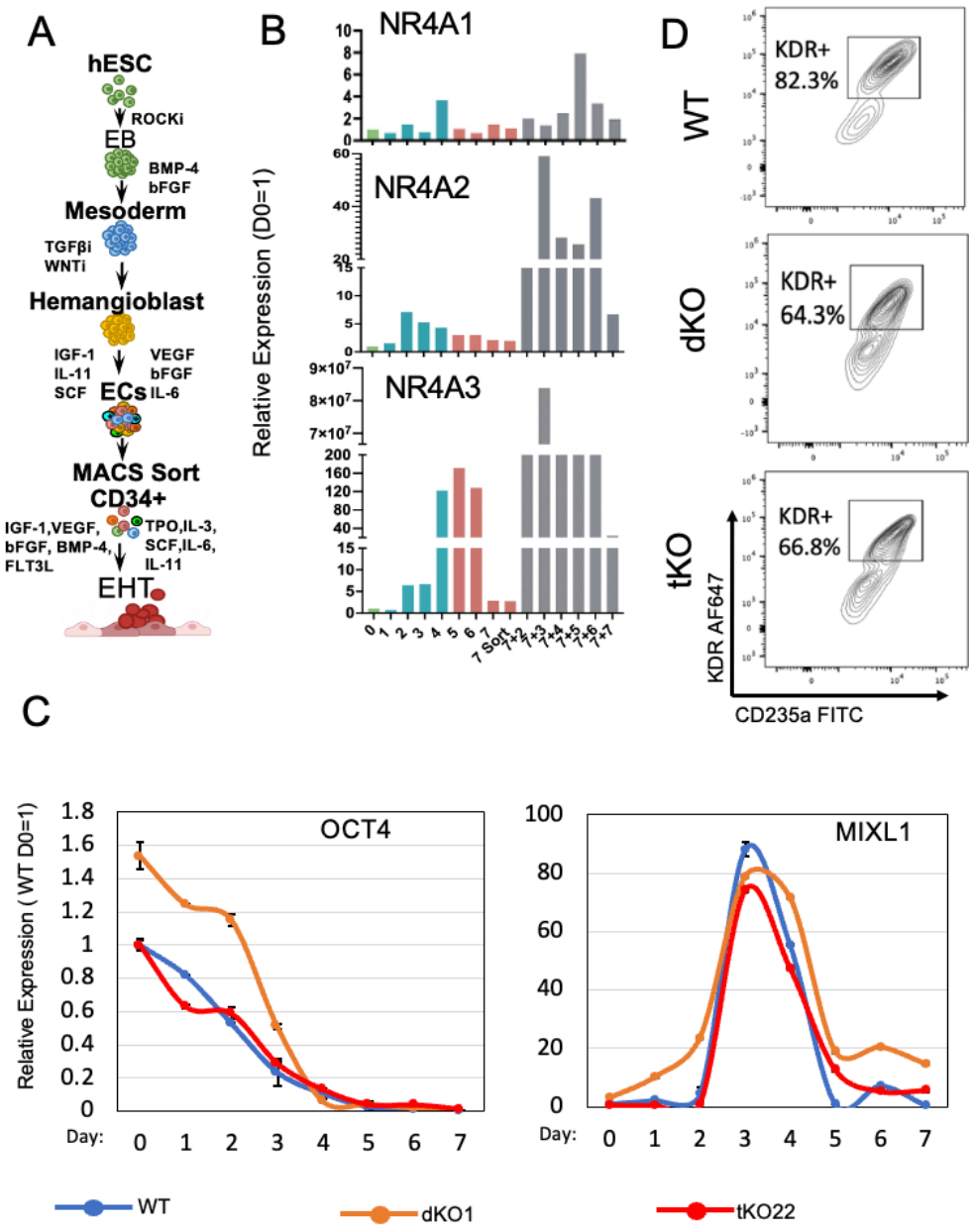

Figure 3: Deletion of NR4A did not alter pluripotency and mesoderm induction but change hemangioblast population. (A) Timeline for in vitro HPCs specification. **(B)** NR4A1, NR4A2 and NR4A3 RNA expression during HPCs specification. Blue: mesoderm induction and formation of hemangioblast; pink: ECs and hematopoietic specification; gray: EHT. **(C)** RNA expressions for first 7 days. OCT4: pluripotency; MIXL1: lateral mesoderm **(D)** FACS analysis of KDR+CD235- hemangioblast at Day 4 of HPCs development for WT and NR4A KO.

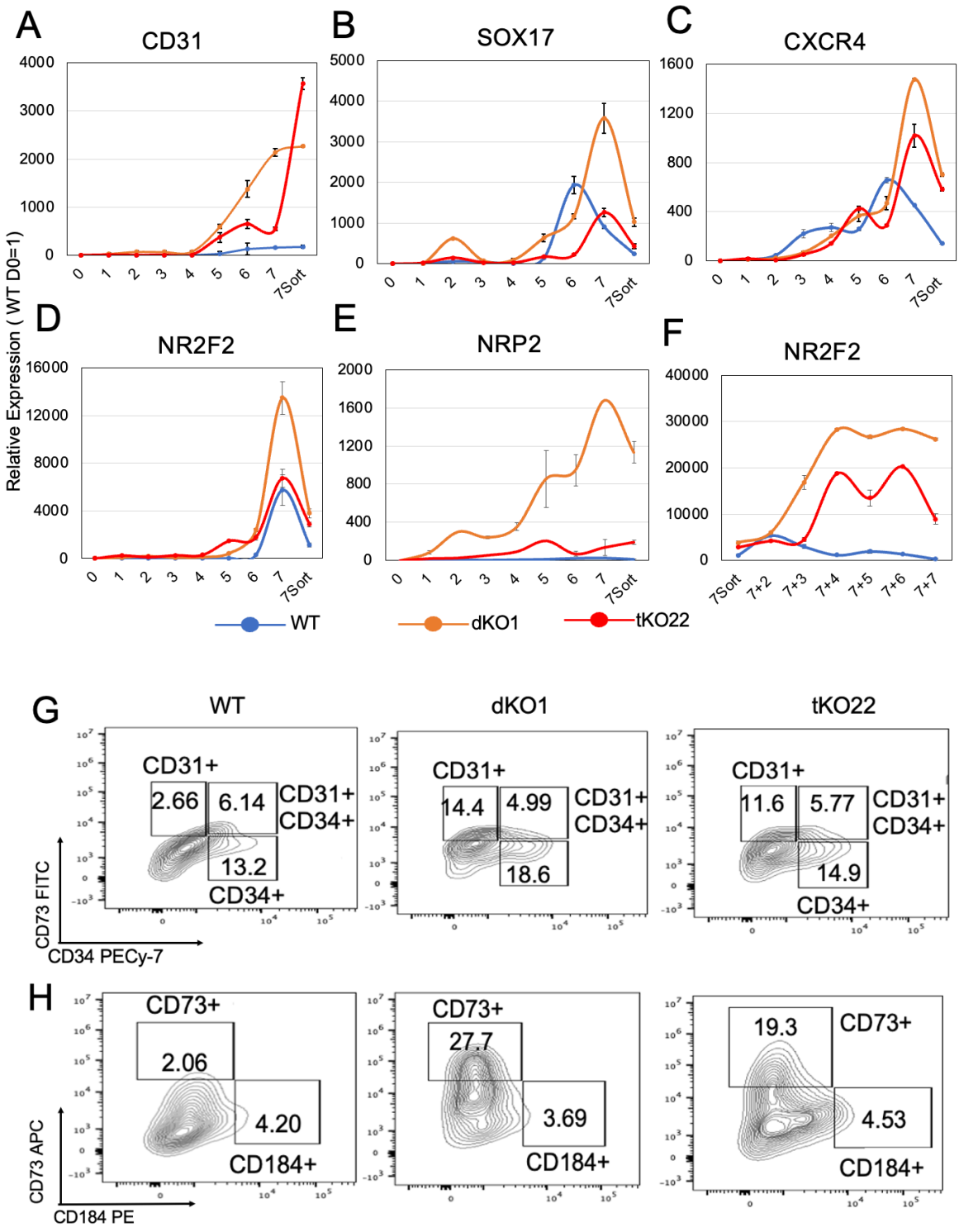

Figure 4: Deletion of NR4A increases ECs population and markers. (A-E) qPCR RNA expressions for ECs prior to EHT. Pan-EC marker: CD31; arterial EC makers: SOX17, CXCR4;

vein EC marker: NF2F2, NRP2. (F) qPCR RNA expression for vein ECs during EHT. **(G-H)** FACS analysis of CD31+ EC population. CD34+ represent hematopoietic and endothelial progenitors. CD73, CD184 represent vein and arterial ECs, respectively.

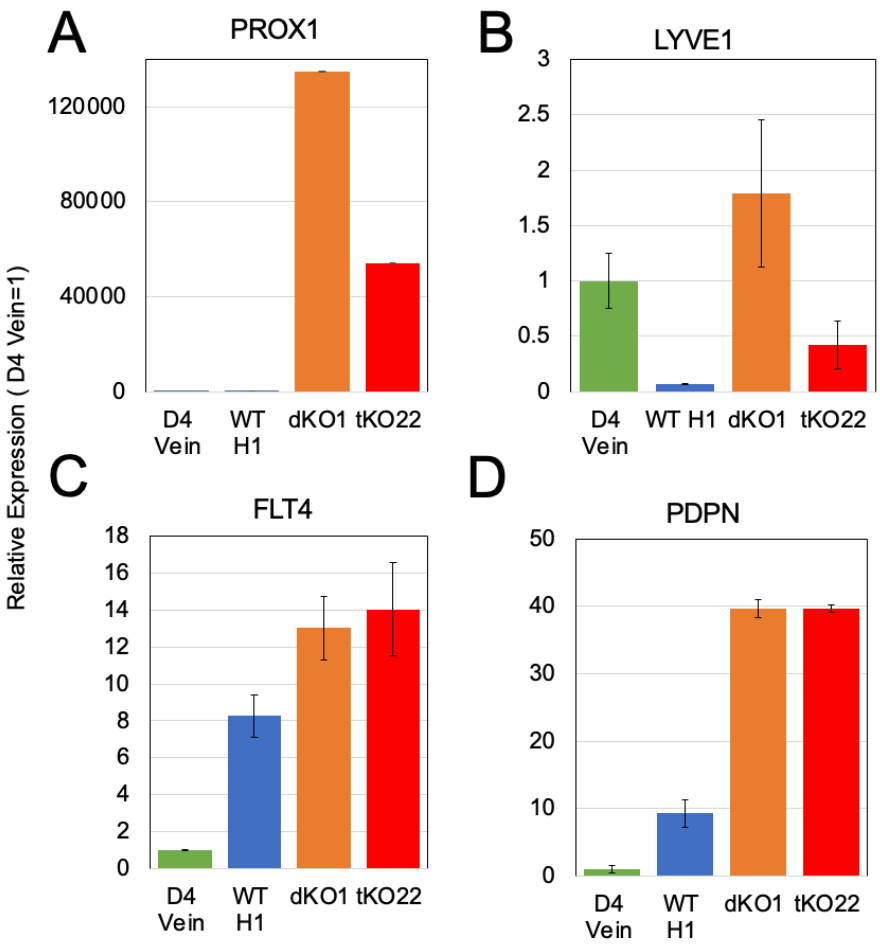

Figure 5: Deletion of NR4A increases LECs markers. (A-D) qPCR RNA expressions of lymphatic EC markers and CXCL12 chemokine of WT and NR4A KO at Day 7+6 relative to vein-like EC population derived using protocol (Fowler et al. 2024).

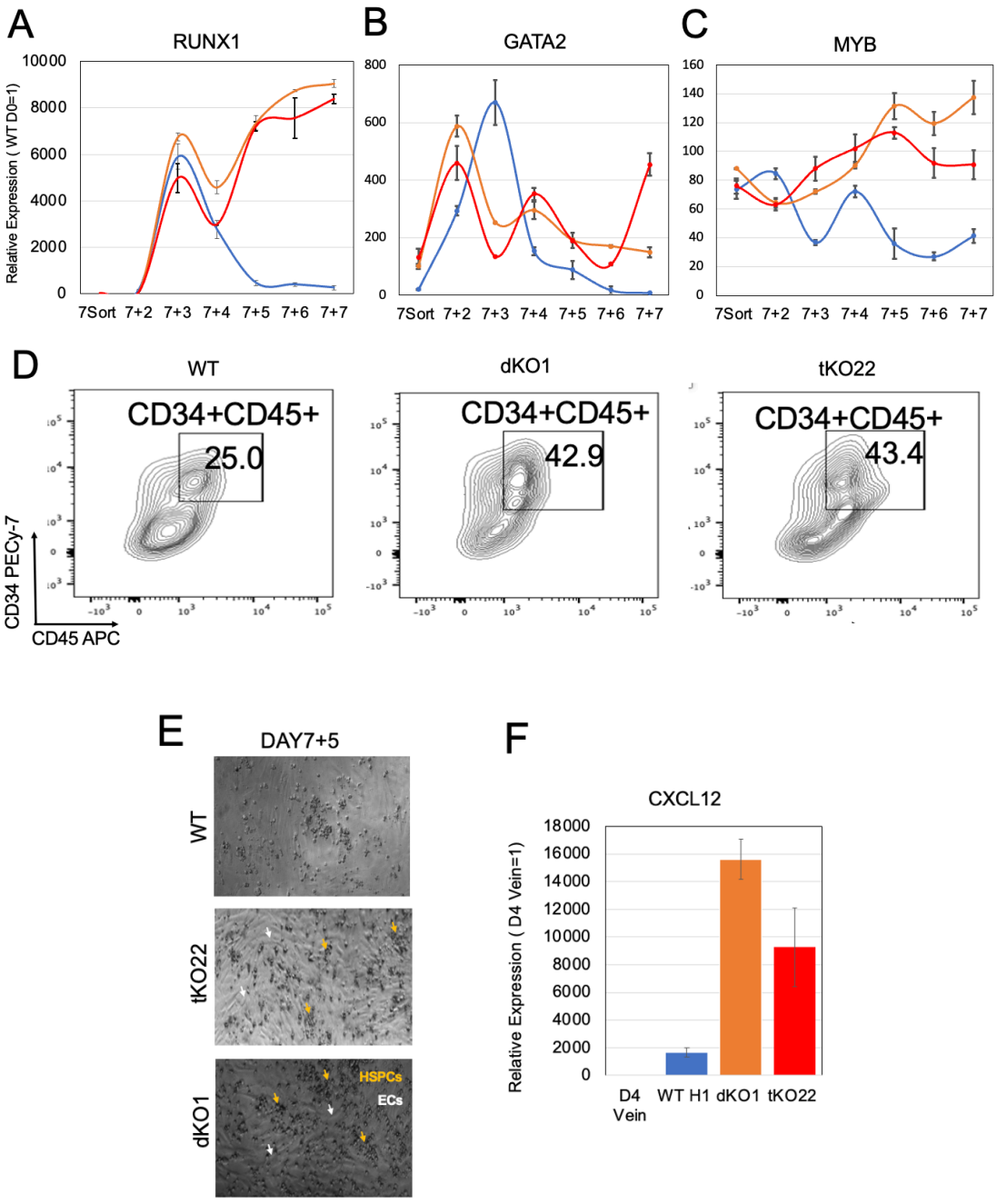

Figure 6: Deletion of NR4A increases HPCs population and CXCL12 chemokine marker. **(A-C)** qPCR RNA expressions of HPC markers during EHT. **(D)** FACS analysis of CD34+CD45+ HPC population at EHT Day 7+7. **(E)** Light microscopy of EC and HPCs at EHT Day 7+5. **(F)** qPCR RNA expressions of CXCL12 chemokine of WT and NR4A KO at Day 7+6 relative to vein-like EC population de-rived using protocol (Fowler et al. 2024).

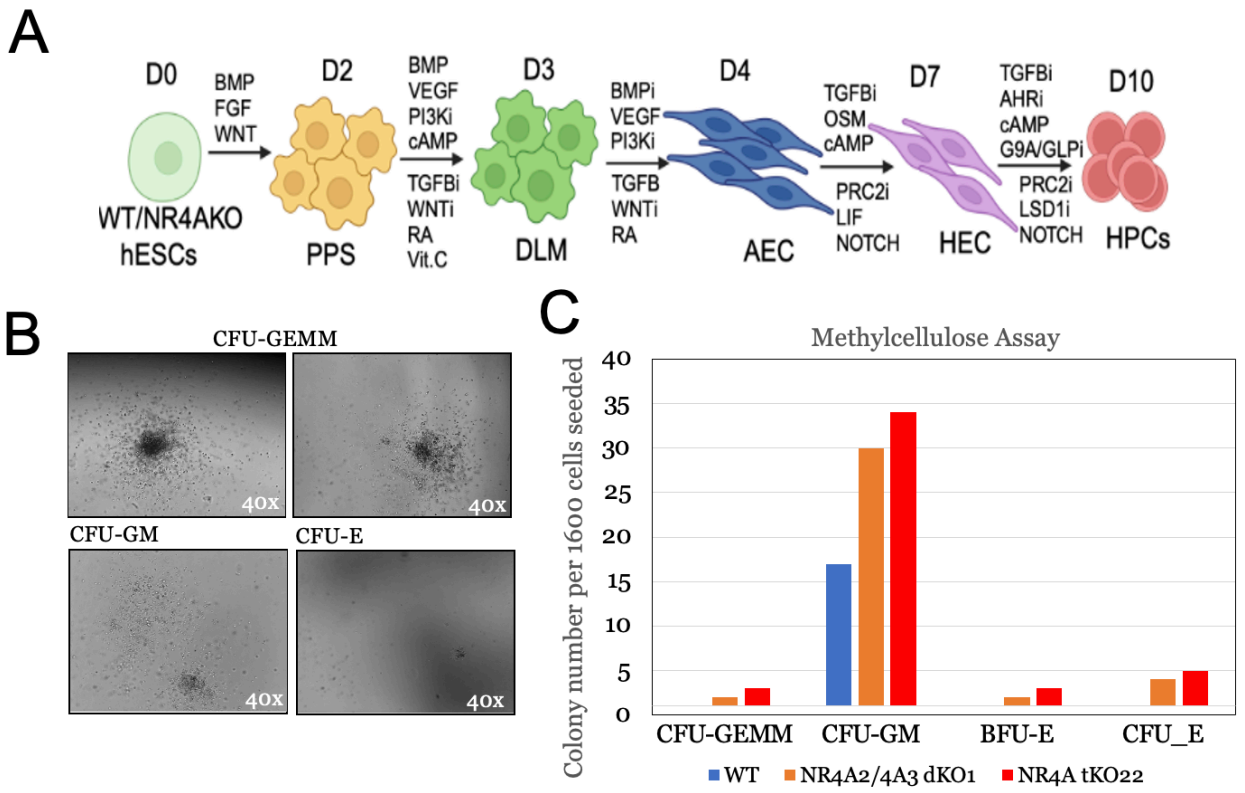

Figure 7: Deletion of NR4A increases myeloid forming potential of HPCs. (A) Timeline for in vitro HPCs specification using monolayer method of differentiation. **(B)** Light microscopy of myeloid colonies after 14 days of culture. **(C)** Myeloid colony counts after 14 days of culture in methylcellulose. GEMM (Granulocyte, Erythrocyte, Monocyte, Megakaryocyte), GM (Granulocyte-Macrophage), BFU-E (Burst Forming Unit-Erythroid), and CFU-E (Colony Forming Unit-Erythroid) colonies.

2.8 Methods

Maintaining hESCs

H1 hESCs were cultured on tissue culture plates that had been pre-coated with Matrigel (BD Biosciences, 354277) and maintained in mTeSR Plus medium (Stem Cell Technologies, 100-0276). The medium was refreshed every other day. Every 4-5 days, the cells were passaged at a 1:6 ratio using standard clump passaging techniques with a detachment solution composed of 25% Dispase (Stem Cell Technologies, 07913) and 75% mTeSR Plus. Cells were incubated at 37°C for 1 hour, then collected and briefly washed with DMEM/F12 (Gibco, 11320033) twice before being re-seeded onto fresh Matrigel-coated plates. For quality control, only dishes exhibiting over 70% of characteristic ESC colonies were selected for experiments. The Matrigel base membrane was diluted in 1:180 using DMEM/F12 and used to coat tissue culture plasticwares for at least 1 hour at 37°C. After incubation, the Matrigel-DMEM/F12 mixture was aspirated and washed with DMEM/F12, leaving behind a thin film for cell plating. To prepare the plates, Matrigel was diluted 1:180 in DMEM/F12 and used to coat the tissue culture plastics for at least 1 hour at 37°C. After the incubation, the Matrigel-DMEM/F12 mixture was aspirated, and the plates were washed gently with DMEM/F12 to remove Matrigel residue and leave behind a thin film suitable for cell seeding. Cell morphology was monitored daily under a microscope to ensure optimal growth and health. For quality control, only dishes displaying more than 70% characteristic hESC colonies were selected for further experiments.

Generation of NR4A sKO, NR4A dKO, and NR4A tKO hESC lines

CRISPR/Cas9-mediated deletion of the NR4A gene involved using two gRNAs targeting common variant of each NR4A gene, designed to delete approximately 150 bp upstream of the

gene's DNA-binding region. These gRNAs **(Table 1)** were synthesized as custom oligonucleotides by Eurofins Genomics, annealed, and cloned into the LentiCRISPR V2 vector (Addgene, 52961). Day 0: Undifferentiated and confluent H1 hESCs were dissociated into single cells using Accutase (Stem Cell Tehnologies, 07920). Cells were pelleted by centrifugation of 1,000rpm for 5 minutes at room temperature. 1x10^6 cells were resuspended in 100 µL of cold R buffer from the Neon Transfection System Kit (Invitrogen, MPK10025) with 4 µg of each gRNA mixture (8 µg total for both gRNAs). Electroporation was performed using the Neon Transfection System (Invitrogen, NEON1) at 1200 V, 20 ms pulses twice. Cells were then plated on Matrigel-coated plates in StemFlex™ medium (Gibco, A3349401) supplemented with 10 mM Y27632 ROCK inhibitor (Stem Cell Technologies, 72302) for first 24 hours. Day 1: Cells were transitioned to StemFlex™ medium without the ROCK inhibitor for an additional 24 hours. Day 2 - Day 4: Cells were treated using puromycin (Sigma-Aldrich, P8833) at 0.5 µg/mL for 48 hours select for successfully edited cells. Following puromycin selection, individual colonies were isolated, picked, and expanded in StemFlex™ medium. Genomic DNA from each colony was extracted using the KAPA Express Extract Kit (KAPA Biosystems, 07961804001) according to manufacturer instructions. To verify the genomic deletions, PCR was conducted with primers positioned outside the expected deletion sites **(Table 2).** The PCR products were then purified using the GeneJET PCR Purification Kit (Thermo Fisher, K0701), according to the manufacturer's protocol, and subjected to Sanger sequencing. Specific sequencing primers were used to accurately identify and confirm the deletion sequences **(Table 2).** NR4A1/4A2 dKO and NR4A1/4A3 dKO cell lines were generated using NR4A1 clones, with gRNAs specifically targeting the NR4A2 and NR4A3 genes, respectively, and validated using the above procedures. Similarly, the NR4A2/4A3 dKO was produced from

NR4A2 clones with gRNAs aimed at the NR4A3 gene. The NR4A tKO was developed by further targeting the NR4A2 gene in NR4A1/4A3 dKO cells with corresponding gRNAs.

Embryoid Body Hematopoietic Differentiation of hESCs

Embryoid body (EB) differentiation was performed as previously described (Sugimura et al. 2017) with minor modifications. Initially, AggreWell™ 400 plates (Stem Cell Technologies, 34415) were prepared using an anti-adherence rinsing solution (Stem Cell Technologies, 07010) according to the manufacturer's guidelines.

- Day -2: Largely confluent WT and NR4A KO hESCs were dissociated into single cells using Accutase, then collected, centrifuged, and resuspended in mTeSR Plus medium supplemented with 10 mM Y27632. Approximately $6x10^5$ cells were seeded per well in the AggreWell™ 400 24-well plate, with each microwell receiving about 500 cells to consistently form one EB per microwell. To ensure uniform cell distribution and consistent EB formation, the AggreWell™ 400 plates containing the hESCs were centrifuged at 100xg for 3 minutes. The hESCs were then cultured for 48 hours at 37°C with culture conditions of 20% O_2 and 5% CO_2.
- Day 0: The mTeSR Plus supplemented with ROCK inhibitor medium from WT and NR4A KO hESCs, was carefully and completely removed using p1000 and p200 pipette tips to prevent withdrawing EBs. The medium was then replaced with StemPro-34 (Gibco, 10639011) supplemented with 2 mM GlutaMAx (Gibco, 35050061), 10 ng/mL penicillin/streptomycin (Gibco, 15140-122), 1 mM ascorbic acid (Sigma Aldrich, A4403), 150 µg/mL human holo-transferrin (Sigma Aldrich, T0665), 0.4 mM monothioglycerol (Nacalai, 33709-62) [hereafter referred to as ‘Supplemented

StemPro-34], and 10 ng/mL BMP4 (Pepro Tech, 120-05), and the cultures were incubated in 5% CO_2, 5% O_2 , and 90% N_2 condition for 24 hours. Media was added slowly against the wall of AggreWell™ 400 to ensuring the EBs remained undisturbed in their respective microwells.

- Day 1: bFGF (Peprp Tech, 100-18B) was added to achieve the final concentration of 5ng/mL. Culture was incubated in 5% CO_2, 5% O_2, and 90% N_2 for additional 24 hours to further facilitate mesoderm patterning.
- Day 2: The media added using Supplemented StemPro-34 with final concentrations of 6 μM SB421542 (Stem Cell Technologies, 72234), 3 μM CHIR99021(Tocris, 4423), 5 ng/mL bFGF and 10 ng/mL BMP-4. Culture was incubated in 5% CO_2, 5% O_2, and 90% N_2 for additional 48 hours for formation of hemangioblast population.
- Day 4: Media from WT and NR4A KO hESC cultures was completely removed using p1000 and p200 pipette tips, ensuring that embryoid bodies (EBs) remained undisturbed. The medium was then replaced with StemPro-34 supplemented with 15 ng/mL VEGF (PeproTech, 100-20) and 10 ng/mL bFGF. Culture was incubated in 5% CO_2, 5% O_2, and 90% N_2 for 48 hours to promote differentiation toward an endothelial cell population and hematopoietic specification.
- Day 6: The media added using Supplemented StemPro-34 with final concentrations of 15ng/mL VEGF, 10ng/mL bFGF, 10ng/mL IL-6 (Pepro Tech, 200-06), 25ng/mL IGF-1 (Pepro Tech,100-11), 5ng/mL IL-11 (Pepro Tech 200-11), and 50ng/mL SCF (Pepro Tech 300-07). The culture was then incubated under conditions of 5% CO_2, 5% O_2, and 90% N_2 for 24 hours for the development of the hematopoietic progenitor population.

- Day 7: EBs were harvested from AggreWell™ 400 plates by forceful dispensing of DMEM/F12 with a p1000 pipette to detach the EBs from their microwells. The detached EBs were collected, washed with PBS (Gibco, 14190144), and dissociated into single cells using the Embyroid Body Dissociation Kit (Miltenyi Biotec, 130-096-348), following the manufacturer's instructions. The dissociated cells were filtered through 70um cell strainer and then incubated in CD34 microbeads with Fc buffer from CD34 Microbead Kit (Miltenyi Biotec, 130-046-702) for magnetic-activated cell sorting (MACS) to selectively enrich CD34+ hematopoietic progenitor cells as per the manufacturer's guidelines. Subsequently, $2.5x10^5$ CD34+ cells were seeded onto one well of Matrigel-coated 24-well plates for monolayer differentiation in Supplemented StemPro-34 medium with the following cytokines: 30 ng/mL TPO (Pepro Tech, 300-18), 10 ng/mL IL-3 (Pepro Tech, 200-03), 50 ng/mL SCF, 10 ng/mL IL-6, 5 ng/mL IL-11, 25 ng/mL IGF-1, 5 ng/mL VEGF, 5 ng/mL bFGF, 10 ng/mL BMP-4, 10 ng/mL FLT3L [here after referred to as 'Supplemented StemPro-34 EHT media'] and 10 mM Y27632. The cultures were maintained in 5% CO_2, 5% O_2, and 90% N_2 for 24 hours.
- Day 7+1: The attached monolayer of CD34+ hematopoietic progenitor cells was briefly washed with DMEM/F12 before adding fresh Supplemented StemPro-34 EHT media. The culture was maintained for 48 hours n 5% CO_2, 5% O_2, and 90% N_2 conditions to facilitate the endothelial-to-hematopoietic transition, with media changes every 24 hours.
- Day 7+4 to Day 7+7: Supplemented StemPro-34 EHT media was added directly to the cultures without changing the existing media to preserve the hematopoietic clusters that were detached and in suspension.

Monolayer Stage-Specific Differentiation of Hematopoietic Progenitors of hESCs

Prior to differentiation, CDM2 and CDM3 differentiation media were prepared according to the methods described previously (Fowler et al. 2024). To prepare CDM2 and CDM3, polyvinyl alcohol was first suspended in either IMDM or F12 by gentle warming and magnetic stirring. After polyvinyl alcohol was dissolved, the remaining media components were mixed, and the complete media was sterilely filtered through a 0.22 mm filter prior to use.

- CDM2, is used for initial differentiation into posterior primitive streak, lateral mesoderm, and artery endothelium, and the composition is consists of: 50% IMDM + GlutaMAX (Thermo Fisher, 31980-097), 50% F12 + GlutaMAX (Thermo Fisher, 31765-092), supplemented with 1 mg/mL polyvinyl alcohol (Sigma, P8136-250G), 1% v/v chemically defined lipid concentrate (Thermo Fisher, 11905-031), 450 mM 1-thioglycerol, 0.7 mg/mL recombinant human insulin (Sigma, 11376497001), 15 mg/mL human transferrin (Sigma, 10652202001), and 1% v/v penicillin/streptomycin.
- CDM3, is specifically used for the differentiation into hemogenic endothelium and hematopoietic progenitor cell, and the composition is consisting of: 45% IMDM + GlutaMAX, 45% F12 + GlutaMAX, 10% KnockOut Serum Replacement (Thermo Fisher, 10828028), 1 mg/mL polyvinyl alcohol, 1% v/v chemically defined lipid concentrate (Thermo Fisher, 11905-031) and 1% v/v penicillin/streptomycin.

Hematopoietic progenitor differentiation is performed as previously described in a stage-specific manner using cytokines designed to enhance the formation of a pure population of progenitor cells while inhibiting the development of unwanted cell types (Fowler et al. 2024). All differentiation conditions were maintained in 20% O_2, 5% CO_2, and 37°C condition.

- Day 0 (Seeding hESCs for differentiation): Confluent hESCs were dissociated into single cells using Accutase and then counted. Subsequently, these cells were seeded at a density of 30,000-50,000 hPSCs/cm^2 (i.e., 1.2-2x10^5 hESCs per well of 24 well) into Matrigel-precoated tissue culture plates in mTeSR Plus medium was supplemented with 10 mM Y27632, a ROCK inhibitor, to enhance hPSC survival following passaging, for 24 hours.
- Day 1-2 (Posterior primitive streak induction): Day 0 hPSCs were briefly washed using DMEM/F12 to remove all traces of mTeSR Plus + Y27632. Then, they were differentiated towards posterior primitive streak in CDM2 media supplemented with 40 ng/mL BMP4, 6 mM CHIR99021, and 20 ng/mL FGF2 for 48 hours. Posterior primitive streak induction media was refreshed every 24 hours.
- Day 3 (Lateral mesoderm induction): Cells were washed briefly and gently using DMEM/F12 and differentiated towards lateral mesoderm in CDM2 media supplemented with 40 ng/mL BMP4, 2.5 mM GDC-0941 (Sigma-Aldrich 509226), 10 mM Forskolin (Tocris, 1099), 2 mM SB505124 (Selleckchem, S2186), 100 ng/mL VEGF, 1 mM XAV939 (Biogems, 2848932), 200 mg/mL AA2P (Sigma, 49752-10G), and 0.5 nM TTNPB (Tocris, 0761) for 24 hours.
- Day 4 (Arterial endothelium induction): Following a brief wash using DMEM/F12, the lateral mesoderm cells from Day 3 were differentiated into artery endothelial cells using CDM2 media supplemented with the following: 15 ng/mL Activin A (Pepro Tech, 120-14P), DMH1 at 250 nM (Tocris, 4126), GDC-0941 at 2.5 mM, VEGF at 100 ng/mL, XAV939 at 1 mM, AA2P at 200 mg/mL, and TTNPB at 0.5 nM. The differentiation was carried out over 24 hours.

- Day 5 to Day 7 (Hemogenic endothelium induction): Prior to further differentiation, 24-well tissue culture plate was prepared by coating with a solution composed of recombinant human truncated vitronectin at a concentration of 10 mg/mL in PBS, combined with 100 nM of the high-affinity engineered NOTCH agonist DeltaMAX [gift of Vincent Luca's laboratory (Gonzalez-Perez et al. 2023)]. The plates were coated for at least 1 hour at 37°C. Then, DeltaMAX/vitronectin solution was aspirated, and the plates were gently washed three times with PBS before cell seeding. Day 4 Artery endothelial cells were dissociated into a single-cell suspension using Accutase and re-seeded densely at 500,000 cells/cm^2 onto the plate precoated using DeltaMAX/Vitronectin. These cells were then further differentiated towards hemogenic endothelium in CDM3 media supplemented with 10 mM Forskolin, 20 ng/mL LIF (R&D Systems, 7734-LF-025), 10 ng/mL OSM (R&D Systems, 295-OM-010), 2 mM SB505124, and 1 mM UNC1999 (Tocris, 4904) and maintained for 72 hours. Media was refreshed every 24 hours, ensuring a complete change to support optimal differentiation conditions.
- Day 8 to Day 10 (hematopoietic progenitor induction): Hemogenic endothelium cells were transitioned to hematopoietic progenitors using CDM3 media supplemented with 10 mM Forskolin, 2 mM SB505124, 750 nM SR1 (Selleckchem, S2858), 75 nM UM171 (ApexBio, A8950), 500 nM UNC0638 (Tocris, 4343), and 1 mM UNC1999, and the culture was continued for 72 hours. To preserve the integrity of emerging semi-adherent cells, the medium was added gently without performing a wash. Media changes were conducted every 24 hours on maintain optimal conditions. On last day, rather than a full media change, media were added to the existing culture.

Differentiation of hPSC-derived monolayer hematopoietic progenitors into myeloid and erythroid cells in methylcellulose culture

Day 10 hESC-derived hematopoietic progenitors were dissociated using TrypLE (Thermo Fisher, 12604013) for 3-5 minutes at 37 °C, followed by gentle trituration. Cells were collected and centrifuged at 1,000rpm for 5 minutes. Cells were resuspended in Iscove's MDM with 2% FBS, counted, and cultured in methylcellulose using MethoCult H4435 Enriched (STEMCELL Technologies, 04435) with $1.6x10^3$ cells per 35mm culture dish (Stem Cell Technologies, 27100) prepared in triplicate. Each culture dish was placed inside a 10cm dish containing sterile H_2O to maintain humidity. Cultures were incubated for 14 days in 20% O_2, 5% CO_2, and 37°C condition. Colonies were then manually counted to identify each colony forming unit (CFU) containing granulocyte, erythroid, monocyte, and megakaryocytes (CFU-GEMM), granulocyte and monocyte cells (CFU-GM), erythroid cells (CFU-E), as well as burst forming unit-erythroid (BFU-E).

Dissociation, Antibody Staining, And Flow Cytometry Analysis of Cultured Cells

Different methods were used to dissociate distinct types of cultured cells for flow cytometric analysis. First, Day 4 Hemangioblast EB was collected and washed using PBS before it is dissociated using Embryoid Body Dissociation Kit into single cells according to manufacture instruction. Second, Day 7+7 Hematopoietic Progenitors and Endothelial cells were dissociated by incubation in TrypLE Express for 3-5 minutes at 37°C, followed by gentle trituration. After dissociation, cells were diluted 1:10 in DMEM/F12 and centrifuged at 1,00rpm for 5 minutes.

Post-dissociation, cell pellets were resuspended in a blocking buffer containing 5% v/v donkey serum in PBS and incubated for 15 minutes on ice. This was followed by staining with fluorophore-conjugated antibodies for 30 minutes in the dark at 4°C. After staining, cells were washed twice

with FACS buffer made with PBS with 5% FBS (Gibco, A5256701) and resuspended in FACS buffer supplemented with DAPI in 1:10,000 dilution (Thermo Fisher, D1306) to differentiate live from dead cells. Cells were filtered through a 45 μm cell strainer prior to analysis on a Beckman Coulter CytoFlex Analyzer. Data analysis was performed in FlowJo, starting with gating based on forward and side scatter properties, then height and width to exclude doublets. Live cells were identified by their lack of DAPI staining. Flow cytometry analysis was conducted at specified days during the EB method of hematopoietic differentiation using designated antibody panels:

- Day 4 Hemangioblast panel: CD235a-FITC (Coutler, IM2212U), CD309/KDR-AF648 (BD Pharmingen, 560495)
- Day 7+7 Hematopoietic Progenitors panel: CD34-PECy7 (BD Pharmingen, 560710), CD45-APC (BD Biosciences, 340942)
- Day 7+7 Endothelial panels: CD31-AF488 (BD Bioscience 558068), CD73-APC (Invitrogen, 17-0739-42), CD184-PE (Invitrogen, 12-9991-82)

Quantitative PCR

Different methods were employed to prepare various types of cultured cells for quantitative PCR (qPCR). For 3D cultures, EBs were detached from their microwells by forcefully dispensing DMEM/F12 into each well, collected, and washed with PBS before being lysed directly in the collection tube using TRIzol Reagent (Thermo Fisher, 15596026). In contrast, cells from monolayer cultures were lysed directly in their culture plates. For RNA extraction, RNA was first isolated using chloroform followed by purification via phenol-chloroform extraction.1ug of total RNA was then reverse transcribed using random hexamers and SuperScript II Reverse Transcriptase (Invitrogen, 18090010) mixed. The resulting cDNA was diluted in water, with 20

ng used per RT-qPCR reaction. The qPCR reactions were conducted on a CFX384 Touch Real-Time PCR Detection System (BioRad, 1855484) using iTaq Universal SYBR Green Supermix (Bio-Rad, M7128), with standard curves generated for each primer set to calculate Ct values. The expression threshold was set at 100 RFU, and to normalize expression levels across samples, all values were scaled to GAPDH as an internal control.

2.9 Tables

Table 1: gRNA Sequences Used to Generate NR4A KO hESCs

Gene	gRNA sequence (5'-3')
NR4A1	caccgTCTGCCTCCTTCAAGTTCGA
	aaacCGAGCAGGGGCTGCCATAGTc
NR4A2	caccGGGGCTGTGCGCTGTGTGTG
	aaacGCAGATCGGCCACGCGTCTC
NR4A3	caccGGCTCTCAAGCGCGGCGGCC
	caccGCCAGACGACGAGCTCCTGC

Table 2: Primers and Sequencing Primers Used to Confirm NR4A KO hESCs

Name	Sequence (5'-3')
NR4A1-Forward	CTCTACCAGCTGCCAGGAAC
NR4A1-Reverse	GCAGGCCTTCGTAAGTCTGG
NR4A1-Sequencing	AGTCCAGCCATGCTCCTCAG
NR4A2-Forward	CGCCTCTCCCTCTTCTCCTT
NR4A2-Reverse	TCTCAGACACCCGGAAGTCC
NR4A2-Sequencing	CCCAGTCTTTCTGCTTCCCTT
NR4A3-Forward	GCTTCCCGCTCTTCCACTT
NR4A3-Reverse	GCTGCACATGGACCTCAGTA
NR4A3-Sequencing	TCAAACCGGGAAGAACAAGG

Chapter 3

FUTURE DIRECTIONS

The members of the NR4A family of NHRs, NR4A1-3, play important albeit redundant roles in maintaining adult HSC quiescence and vascular homeostasis. In this study, we chose to investigate the developmental roles of the NR4A receptors using hESC-based in vitro models that mimic developmental ontogeny. By analyzing hematopoietic development in single, double, and triple NR4A KO hESCs, our preliminary data showed that they play a crucial role in regulating the balance between different cell fates, specifically by influencing the endothelial-to-hematopoietic transition (EHT) and the maintenance of HPCs. This finding provided novel insights into the developmental role of NR4A receptors that was previously unexplored due to the absence of human data. Future studies should focus on clarifying the roles of NR4A receptors in the development of hematopoietic and endothelial lineages by identifying an NR4A-dependent progenitor cell that, in the absence of these receptors, differentiates into LEC-like cells. Additionally, further research is needed to uncover the intrinsic and paracrine mechanisms regulated by NR4A receptors that support the development and maintenance of human HPCs.

3.1 Characterize the LEC-Competent Differentiation Intermediate

Our initial preliminary data suggests that in the absence of NR4A receptors, the pathway of hematopoietic specification diverges to also generate LEC-like cells alongside HPCs (**Fig. 5A-D**) This finding points to the existence of a LEC-competent progenitor cell, whose fate appears to be regulated by an NR4A-controlled gene network. However, the specific point at which lineage

divergence occurs remains to be elucidated. To dissect cellular heterogeneity and pinpoint the lineage branchpoint regulated by NR4A receptors, scRNA-seq is proposed as the next step. This analysis should be conducted on both WT and NR4A-tKO cultures at critical stages of lineage specification, including hESCs, mesoderm, hemangioblast, and the EHT. Examining the developmental trajectories of WT and NR4A dKO/tKO HPCs from hESCs will identify differentiation intermediates and gene sets responsive to NR4A deletion. Comparing these clusters will clarify the relationships among different cell populations forming during differentiation. The scRNA-seq data will also help identify markers for each lineage and stage, facilitating cell isolation by FACS. Gene set enrichment analysis (GSEA) on progenitors that define HPC and LEC-like clusters will unravel the molecular networks controlled by NR4A receptors. Experimentally manipulating these networks in WT cultures—through Dox-inducible overexpression or CRISPRi knockdowns using promoter-tethered gRNAs (Wang et al. 2018) —will help determine if WT cultures can be induced to form LEC-like cells similar to those observed in NR4A tKO cultures. Given our preliminary data that showed reduction in KDR+ hemangioblast in NR4A tKO cultures at Day 4 of differentiation, this early hemangioblast is therefore hypothesized as a likely candidate for the LEC-competent progenitor **(Fig. 3D).**

Once the regulatory gene network influenced by NR4A is delineated, NR4A-responsive genomic targets and global transcriptional programs that control this cell fate decision will need to be identified. ChIP-seq should be performed on WT (experiment) and NR4A-KO (negative control) cultures at the critical timepoint when the LEC-like lineage diverges from the hematopoietic path. This analysis will confirm NR4A receptor binding in the progenitor cells. Subsequent cross-referencing of scRNA-seq and ChIP-seq data will pinpoint direct NR4A targets within these progenitors. Targets will be categorized based on their expression in NR4A-KO

versus WT cells—activated, repressed, or non-responsive. Previous work has suggested that NR4A binding might be enriched at enhancers and promoters of genes that promote hematopoietic fate, such as c-MYC, this aligns with our preliminary findings that indicate a potential role of NR4A as a positive regulator of hematopoiesis (Boudreaux et al. 2019). Alternatively, NR4A binding to the regulatory elements of EC/LEC genes would suggest that NR4A receptors repress LEC development.

The NHR family is characterized by its ability to interact with both co-activators and co-repressors, facilitating diverse transcriptional outcomes. Previous research has shown that NR4A receptors bind to co-activators such as SRC and p300 to promote transcriptional activation, enhancing processes like cellular differentiation and growth (Bending and Zikherman 2023). Conversely, they interact with co-repressors like CoREST and SMRT, suppressing gene expression linked to cellular quiescence and apoptosis (Ye et al. 2022). These findings suggest that NR4A receptors can form complexes with cofactor proteins that either activate or repress transcription. These cofactors, potentially acting as pioneer factors, may facilitate NR4A binding to specific target genes, thereby determining their regulatory roles.

To explore NR4A cofactors in LEC-competent progenitor cells, proximity labeling will be employed using a Dox-inducible lentiviral miniTurbo-NR4A transgene, with a miniTurbo-GFP transgene tagged with a nuclear localization signal serving as a control (Branon et al. 2018) Mass spectrometry analysis will follow, with comparative analyses between GFP and NR4A samples excluding overlapping proteins and focusing on those significantly enriched in NR4A samples for detailed examination. Validation of co-binding between NR4A receptors and their cofactors at select genomic targets will involve ChIP-qPCR. Additionally, knocking down key cofactors using a Dox-inducible lentiviral CRISPRi system will further clarify the roles of these cofactors by

analyzing NR4A binding and target gene expression changes via ChIP-qPCR and RT-qPCR. Knocking down pioneer factors is expected to disrupt NR4A recruitment and its regulatory effects, whereas targeting cofactors that modulate NR4A activity may affect target regulation without altering NR4A binding. The TurboID method will not only identify both novel and established regulators of LEC fate but also highlight interactions such as those between NR4A1 and ETS factors ERG and FLI1, which suppress acute myeloid leukemia (Duren, Boudreaux, and Conneely 2016) or NR2F2 which activates PROX1 for lymphatic differentiation(Srinivasan et al. 2010) and its known to heterodimerize with NR4A1(Wu et al. 1997).

Our preliminary data showed that NR4A deletion HPC cultures form LEC-like cells expressing key lymphatic markers, the functionality of these cells remains to be validated (**Fig. 5A-D**). To test this, LEC-like cells will first be isolated from NR4A-tKO cultures by FACS, specifically selecting for LIVE1+PODOPLANIN+ cells, which will then be embedded in Matrigel. In this environment, the LEC-like cells are anticipated to form vessel-like structures (Lee et al. 2015). These LEC-like cells will be injected into ear wounds of immunodeficient NSG mice, with the expectation that functional cells will integrate into the host lymphatic system.

3.2 Characterize cell autonomous and non-cell autonomous mechanisms responsible for the enhanced HPC maintenance in NR4A KO hematopoietic cultures.

Our preliminary data underscore the crucial role of NR4A receptors in the development of HPCs from hESCs, particularly in enhancing the formation of HPCs as well as myeloid progenitors and enhancing myeloid colony formation capabilities **(Fig. 6A-E, Fig 7B-C)**. Previous research has demonstrated that embryonic deletion of NR4A receptors significantly impacts the formation of CD4+ regulatory T cells in the mouse thymus, leading to a markedly reduced population (Sekiya

et al. 2013). Building on these findings, the next phase of experimentation will be to investigate whether NR4A deletion in hESC-derived HPCs similarly affects the differentiation of lymphoid lineages. The influence of NR4A deletion on lymphoid lineage differentiation will be examined using a co-culture system where HPCs are co-cultured with OP9-DLL1 stromal cells. This setup provides the essential NOTCH signaling required for proper T cell development (Holmes and Zúñiga-Pflücker 2009).

HPCs not only differentiate into myeloid and lymphoid lineages but also have self-renewal capabilities crucial for maintaining the hematopoietic progenitor pool **(Fig. 6E)**. (Dzierzak and Bigas 2018). This self-renewal capacity is often assessed through in vivo engraftment tests using NSG immunodeficient mice. Research indicates that HPCs derived from AML iPSCs show enhanced engraftment and deletion of Nr4a in mice leads to an AML-like phenotype (Fowler et al. 2024; Mullican et al. 2007). Consequently, the self-renewal ability of NR4A-deleted HPCs should be evaluated by intrafemorally injection into sub-lethally irradiated 8–12-week-old NSG mice. To further probe self-renewal potential, secondary transplantation is performed: human CD34+ HPCs, isolated from the primary transplants, are re-transplanted into new recipients.

Preliminary data indicate the co-development of LEC-like cells and HPCs in NR4A dKO/tKO cultures, raising the question of whether LEC-like cells from NR4A tKO cultures could serve as a maintenance niche to support HPCs non-cell autonomously (**Fig. 5A-D, Fig 6F**). To investigate this, HPCs will be cocultured with three types of endothelial cells (ECs): NR4A tKO LEC-like cells and vein-like and artery-like ECs derived from hESCs following a published protocol cultured in EHT media (Ang et al. 2022). Arterial ECs, known for their ability to support hematopoiesis, will serve as the control. Lentiviral vector expressing GFP under the control of a constitutive pUbC promoter will be used to track WT HPCs in coculture experiments (Wang et al.

2012). Additionally, transwell cocultures will be set up where HPCs are physically separated from EC monolayers but can still receive soluble factors from ECs. If LEC-like cells act as the HPC niche, it is anticipated that WT HPCs will exhibit increased proliferation and sustained marker expression in direct coculture with LEC-like cells, a response that may not be mirrored in cocultures with vein-like or artery-like ECs. Furthermore, if HPC maintenance is driven by secreted factors, similar increases in HPC numbers should be observed in both direct and trans-well cocultures. Conversely, if maintenance relies on membrane-bound proteins, only direct cocultures are expected to show an increase in HPC numbers. Once the nature of the maintenance signal is established, RNA-seq analyses of LEC-like, vein-like, and artery-like ECs will identify candidate factors selectively expressed in supportive versus non-supportive ECs. Modulating the expression of these candidate signaling molecules—either through overexpression in non-supportive ECs or CRISPRi knockdown in supportive ECs—will further delineate their roles. Specifically, enhancing expression of known HPC maintenance factors such as ANGPT1, IL7, or WNT3A in non-supportive ECs is expected to promote HPC maintenance, while their knockdown in supportive ECs should inhibit it (Biswas et al. 2023).

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