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Real-time imaging of human endothelial-to-hematopoietic transition *in vitro* using pluripotent stem cell derived hemogenic endothelium

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During embryogenesis, human hematopoietic stem cells (HSCs) first emerge in the aorta-gonad-mesonephros (AGM) region via transformation of specialized hemogenic endothelial (HE) cells into premature HSC precursors. This process is termed endothelial-to-hematopoietic transition (EHT), in which the HE cells undergo drastic functional and morphological changes from flat, anchorage-dependent endothelial cells to free-floating round hematopoietic cells. Despite its essential role in human HSC development, molecular mechanisms underlying the EHT are largely unknown. This is due to lack of methods to visualize the emergence of human HSC precursors in real time in contrast to mouse and other model organisms. In this study, by inducing HE from human pluripotent stem cells in feeder-free monolayer cultures, we achieved real-time observation of the human EHT *in vitro*. By continuous observation and single-cell tracking in the culture, it was possible to visualize a process that a single endothelial cell gives rise to a hematopoietic cell and subsequently form a hematopoietic-cell cluster. The EHT was also confirmed by a drastic HE-to-HSC switching in molecular marker expressions. Notably, HSC precursor emergence was not linked to asymmetric cell division, whereas the hematopoietic cell cluster was formed through proliferation and assembling of the floating cells after the EHT. These results reveal unappreciated dynamics in the human EHT, and we anticipate that our human EHT model *in vitro* will provide an opportunity to improve our understanding of the human HSC development.

Key words: hematopoietic stem and progenitor cell, aorta-gonad-mesonephros region, live cell imaging

Significance

Hematopoietic stem cells (HSCs) are first emerged through drastic transition of anchorage-dependent hemogenic endothelial (HE) cells to round and floating hematopoietic precursors. In this study, by inducing efficient differentiation of human pluripotent cells into HE, the authors provide a novel model that enables real-time observation of the human endothelial-to-hematopoietic transition (EHT) *in vitro*. Live-cell imaging of the culture clarifies how an endothelial cell gives rise to hematopoietic cells and reveals unappreciated dynamics in the human EHT.

Introduction

Hematopoietic stem cells (HSCs) are a rare cell population at the top of the hematopoietic hierarchy. HSCs possess self-renewal capacity for a lifetime and can give rise to all blood cell lineages. Among multiple waves of blood cell production

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during vertebrate embryogenesis, precursors of HSCs first emerge in a specific region of mesoderm called aorta-gonad-mesonephros (AGM). In human, between 4- and 6 weeks after gestation [1], immature HSC precursors emerge from specialized embryonic aorta tissue called hemogenic endothelium (HE) in the AGM region [2-5] . Once emerged, HSC precursors remain immature, transiently attaching to the inner side of the aorta vessels to form cell clusters, termed intra-aortic hematopoietic clusters (IAHCs) [2]. Transplantable HSCs are rare at this stage (one per AGM [6]) and the precursors need further maturation to acquire robust bone marrow engraftability after migrating from the AGM to the fetal liver. Nonetheless, the generation of HSC precursors by the HE, a process termed the endothelial-to-hematopoietic transition (EHT), is a crucial event for the HSC development. During EHT, the flat and anchorage-dependent HE cells undergo dramatic morphological and phenotypic changes to round and floating hematopoietic cells.

Some mechanisms underlying HSC emergence have been revealed using mouse, chicken, and zebrafish as models and found to be highly conserved between these vertebrates. Particularly, the sparse distribution of EHT locations within the HE has been observed across the model organisms [7], which indicates that a rare fraction of the HE cells undergo the EHT, existing as singularities. Nevertheless, certain species-dependent differences have been observed. For example, EHT location is restricted to the ventral side of the embryonic aorta in human, in contrast to mouse EHT that takes place in both dorsal and ventral sides [8-10]. These and substantial species-dependent differences in HSC phenotypes imply potential differences in EHT mechanisms between human and other model organisms. Recent single-cell- and spatial transcriptome studies on human embryos revealed molecular landmarks of the human HSC precursors, HEs, and other arterial endothelium cells and further identifies landmark genes for EHT that potentially coordinate the signaling switched during the human EHT [11]. However, there have been no reports describing an *in vitro* model for real-time visualization of human EHT, which would enable us to investigate its molecular mechanisms as in mouse [12,13] and other model organisms [14,15].

In this study, to uncover the human EHT mechanisms, we visualize the human EHT *in vitro* by inducing HE from human pluripotent stem cells in monolayer cultures. Live-cell imaging of the induced HE unveils how an endothelial cell gives rise to hematopoietic cell clusters and reveal unappreciated dynamics in the human EHT.

Materials and Methods

Human Induced Pluripotent Stem Cell (iPSC) Culture

Human induced pluripotent stem cell (iPSC) line NB4-1 was derived from CD34 positive mononuclear cells isolated from peripheral blood of 54-year-old male and then reprogrammed using Sendai Virus vectors carrying POU5F1, SOX2, KLF4, and MYC [16]. The iPSCs were maintained in StemFit SK02N Medium (Ajinomoto) as described by Nakagawa et al. [17] on six-well culture plates coated with 1 mL per well 2% Cultrex (R&D Systems) diluted with pre-chilled DMEM/F12 overnight at 4ºC. Our work was conducted with oversight by institutional review board.

Human iPSC derived Hemogenic Endothelium

Differentiation of the iPSCs into hemogenic endothelium was induced by a protocol modified from Uenishi et al[18]. NB4-1 cells seven or eight days after last passaging were dissociated into single cells and then seeded on a Cultrex-coated six-well plate as described above at 1 X 10^4 cells per cm^2. One day after seeding (day 0), the cells were washed with STEMdiff APEL2 medium (StemCell Technologies) and then cultured in the medium supplemented with 2 mmol l^{-1} Lithium Chloride (Sigma), 15 ng ml^{-1} Activin A, 50 ng ml^{-1} BMP4, 50 ng ml^{-1} basic FGF, and 0.5 µmol l^{-1} Y-27632 (Wako) in hypoxia (5% O_2, 5% CO_2) at 37ºC. On day 2, the culture medium was changed to APEL2 supplemented with 50 ng ml^{-1} basic FGF, 50 ng ml^{-1} VEGF, and 2.5 µmol l^{-1} SB431542 (Sigma). On day 4, the culture medium was changed to APEL2 supplemented with 50 ng ml^{-1} basic FGF, 50 ng ml^{-1} VEGF. On day 5, the cells were washed with PBS, singularized with TrypLE, re-seeded on a Cultrex-coated plate at 7.5 X 10^4 cells per cm^2, and then cultured in APEL2 supplemented with 50 ng ml^{-1} basic FGF, 50 ng ml^{-1} VEGF, 50 ng ml^{-1} IGF1, 50 ng ml^{-1} IGF2, 50 ng ml^{-1} TPO, 50 ng ml^{-1} IL6, 20 ng ml^{-1} SCF, 10 ng ml^{-1} IL3, 10 ng ml^{-1} FLT3 Ligand, 50 ng ml^{-1} EGF, and 5 µmol l^{-1} Y-27632 in normoxia (20% O_2, 5% CO_2) at 37ºC. On day 6 (for live-cell imaging) or days 8 and 10 (for flow cytometry), the medium was replaced with the medium without Y-27632 with gentle handling to keep the transiently attached blood-like cells as possible. For some experiments indicated, the cells were treated with different combination of cytokines and chemicals based on a protocol described by Shu et al [19]. Briefly, the cells were cultured in the APEL2 supplemented with 3 µmol l^{-1} CHIR99021, 2 ng ml^{-1} Activin A, and 10 ng ml^{-1} BMP4. On day 2, the medium was changed to APEL2 supplemented with 40 ng ml^{-1} VEGF and then 40 ng ml^{-1} basic FGF was added to the cultures on day 3. On day 4, the medium was changed to APEL2 supplemented with 40 ng ml^{-1} basic FGF and 40 ng ml^{-1} VEGF. All growth factors used were purchased from BioLegend.

Flow Cytometry

For flow cytometric analysis, floating or transiently attached induced hematopoietic cells in the culture were collected

with the culture medium after gentle pipetting. In some experiments, the induced endothelial cells remaining after collection of the hematopoietic cells were also harvested by washing with PBS and dissociation by using TrypLE. After washing with flow buffer (2% Fetal Bovine Serum and 1 mmol l^{-1} EDTA in PBS) and filtration through 40 µm cell strainer, the cells were counted by using Countess 3 (Thermo) and then 1 X 10^5 cells per sample were stained with antibody cocktail diluted in the flow buffer at 4ºC dark for 30 minutes. After washing, the cells were re-suspended in the buffer supplemented with 7-AAD (BioLegend) and analyzed by using FACS Aria III or FACS Verse (BD Biosciences). All flow cytometry data was analyzed with FlowJo software (version 10.8.1). The antibodies and dilutions used were: BV421 anti-CD31 (BioLegend #303123, 1:100) PE anti-CD31 (BioLegend #303105, 1:100), PE/Cy7 anti-CD34 (BioLegend #343526, 1:100), APC/Cy7 anti-CD34 (BioLegend #343513, 1:200), FITC anti-CD43 (BD Biosciences #555475, 1:200), PE anti-CD43 (BD Biosciences #560199, 1:40), BV421 anti-CD45 (BioLegend #304032, 1:40), FITC anti-CD45 (BioLegend #304006, 1:20), and APC anti-CD184 (BioLegend #306509, 1:50).

Live-Cell Imaging

Time-lapse imaging of the live cells with incubation was performed using All-in-one Fluorescence Microscope (BZ-X800, KEYENCE, Osaka, Japan) equipped with a Plan Fluor 10x PH objective lens (NA0.30, BZ-PF10P, KEYENCE) and a stage-top chamber and temperature controller with built-in CO2 gas mixer (INUG2-KIW, Tokai hit, Shizuoka, Japan) at 37°C and 5% CO2. Using the 10X objective lens, phase contrast images of the cells maintained at 37ºC and 5% CO2 were taken every 10 minutes for 36 hours. The data was analyzed with BZ-X analyzer software.

Results and Discussion

Phenotypic Identification of the Pluripotent-Stem-Cell (PSC) Derived Human HE

To observe human EHT *in vitro*, we first tested a feeder-free monolayer culture system to induce differentiation of human iPSCs into the HE based on those in recent reports [18,19] (Figure 1A). Given the HEs in the human embryos were characterized by co-expression of hematopoietic marker CD34, endothelial marker CD31 (also called PECAM-1), and arterial marker CD184 (CXCR4) and lack of CD43 (SPN) expression as validated by a recent single-cell transcriptome study [11], the expression of these cell-surface antigens in the cultures was assessed through flow cytometric analyses. As a result, the cells treated with a combination of growth factors and chemical compounds including an inhibitor of the Activin/BMP/TGFβ pathway, SB431542 [18] showed markedly higher frequencies of the $CD43^-CD34^+CD31^+CD184^+$ cells (Figure 1B). This result is consistent with inhibitory mediators of Activin/BMP/TGFβ signaling (*SMAD6* and *SMAD7*) peaked in the HEs in the human embryos [11] and suggests a key role of the suppression of Activin/BMP/TGFβ signaling in HE specification.

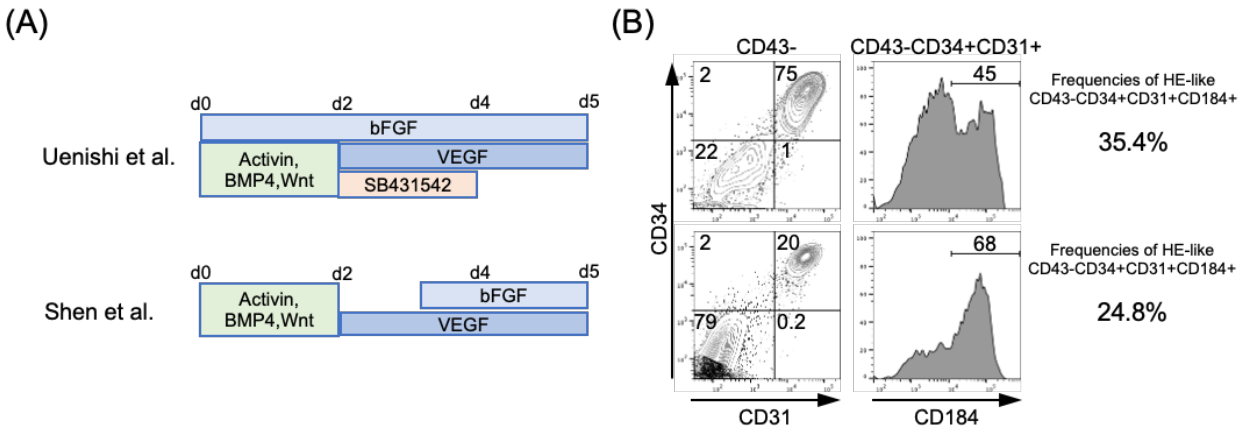

Figure 1 (A) Schematic representation of the two protocols to induce differentiation of human PSCs into HE based on two previous studies by Uenishi et al. and Shen et al. Wnt represents chemical Wnt activators. (B) Flow cytometric analysis of the cultures containing CD43-CD34+CD31+CD184+ HE-like cells induced from human PSCs with the protocols based on Uenishi et al. (upper panels) and Shen et al. (lower panels) represented in (A).

Emergence of the Phenotypic HSC Precursors from the PSC Derived Human HE

Next, we sought to validate the capacity of the PSC-derived human phenotypic HE to generate the HSC precursors. By re-seeding the PSC-derived HE cells at day 5 and continuing culture with a combination of cytokines, round and floating blood-like cells appeared within 3 days (day 8, Figure 2A). A part of these blood-like cells formed cell clusters (Figure

2A), which may reflect the nature of the HSC precursors forming IAHC after emerging from the HE in the human embryonic aorta [11]. Since nascent HSC precursors in the human AGM acquire expression of hematopoietic markers CD43 and CD45 (*PTPRC*) in addition to CD34 while losing expression of endothelial marker CD31 and arterial marker CD184 [11], we performed flow cytometric analyses for the round and floating blood-like cells in the culture. As a result, 70% of the emerged blood-like cells displayed the HSC-precursor phenotype ($CD31^{-}CD184^{-}$ $CD34^{+}CD43^{+}CD45^{+}$, Figure 2B) within 7 days after re-seeding the induced HE (day 12), indicating dramatic HE-to-HSC switching in molecular marker expressions similar to what occurs during the human EHT. These results indicate that the PSC-derived human HE has a capacity to give rise to the phenotypic HSC precursors and thus can be an effective *in vitro* model of human EHT.

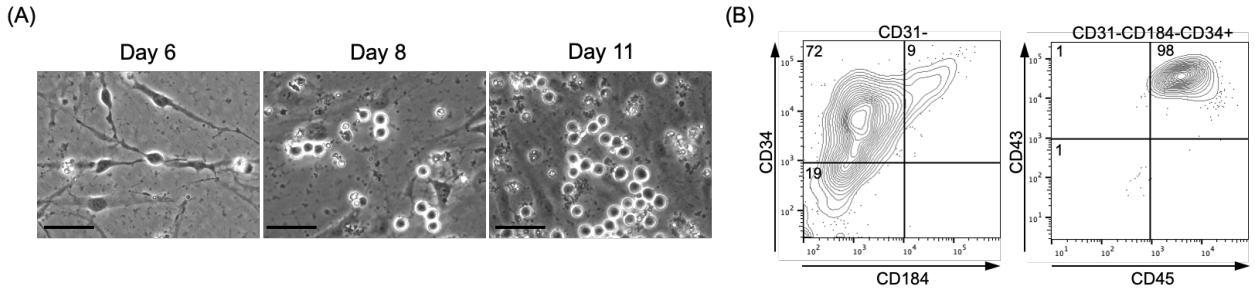

Figure 2 (A) Phase contrast images of the PSC-derived HE cultures on day 6 (1 day after re-seeding the induced HE), and on days 8 and 11 (3- and 6 days after re-seeding, respectively) with nascent blood-like cells. Scale bars = 50 μm. (B) Flow cytometric analysis of the blood-like cells emerged from the PSC-derived HE harvested on day 12. Of note, CD31+ cell was not found among the 20,000 blood-like cells analyzed in the experiment.

In Vitro Visualization of Human EHT

Finally, to investigate dynamics in human EHT, we performed live-cell imaging for the PSC-derived HE cells between day 6 and day 7.5. By capturing the images of the same fields in the culture every 10 minutes and then retrospectively tracking the individual blood-like cells at day 7.5, we could visualize the process in which the adherent epithelial cell transitioned to a floating blood-like cell (Figure 3). Within this process, the adherent epithelial cell gradually transformed into round blood-like cells, started to float, and then proliferated as suspension cells to form the cell clusters. Notably, emergence of the blood-like cells was dependent on the direct transformation and not linked to asymmetric cell division. Additionally, the cluster of blood-like cells was formed through proliferation and assembly of the nascent blood-like cells after the EHT.

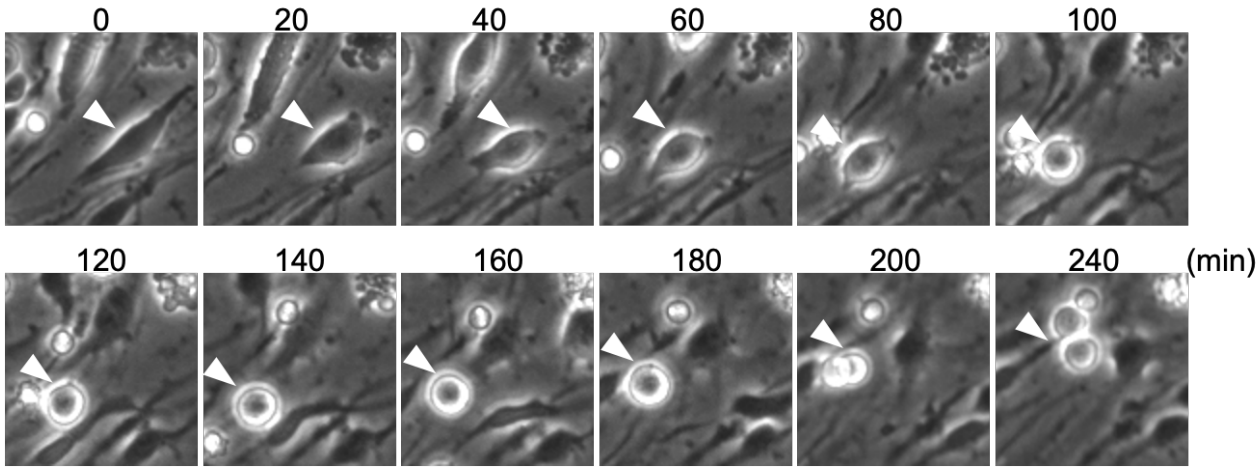

Figure 3 Live-cell imaging of emergence of the blood-like cells from the PSC-derived HE starting at day 6 (1 day after re-seeding the induced HE). A single adherent epithelial cell indicated with arrowheads gradually transformed into a round blood-like cell (min 0 - min 100), started to float (min 120 - min 180), and then proliferated (min 200 - min 240). Scale bar = 10 μm.

The direct epithelial-to-hematopoietic transformation, not depending on asymmetric cell division, was observed in mouse PSCs differentiated on OP9 feeder cells [13], zebrafish embryos *in vivo* [14], and isolated quail HE cultured *ex vivo* [15], suggesting that it is conserved features among vertebrate EHT. More recent EHT models using human pluripotent stem cells rely on conventional methods that depend on cell-aggregation [20]. The EHT occurs in the outgrowth from the large cell-aggregations that rapidly spread across the culture surface in these cultures, which might not be suitable for real-time visualization. Compared to those models used in the previous studies [12-15,20], our *in vitro* model described here enabled real-time and high-resolution observation of human EHT without the difficulty in isolating of a limited number of HE cells from the embryos and/or the requirement of feeder-cell-dependent culture, which can introduce inconsistencies between culture batches and the risk of contamination of the feeder cells in molecular analyses. In our EHT model, the cells underwent a drastic change in cell surface marker expressions as observed in human EHT [11], which enhances validity of our model for visualizing emergence of the HSC precursors *in vitro*. Future efforts will be needed to clarify whether the emerged morphologically- and phenotypically identified HSC precursors harbor the capability to give rise to transplantable HSCs.

Given these advances and species-dependent differences found in HSC development between human and other model organisms, we anticipate that our model will provide an invaluable opportunity to improve our understanding of the human EHT, which may be orchestrated by unappreciated molecular and mechanistic events and multicellular interactions within the AGM region.

Conclusion

In conclusion, directed differentiation of human PSCs into phenotypically identified human HE can be induced in feeder-free monolayer cultures, which is enhanced by suppression of Activin/BMP/TGFβ signaling in the specification period. Hematopoietic cells efficiently emerge from the induced human HE, accompanied by drastic HE-to-HSC-precursor switching in molecular marker expressions. High-resolution and real-time visualization of hematopoietic-cell emergence can be achieved using the induced human HE. This reveals dynamics of the human EHT, which is dependent on direct transformation and not linked to asymmetric cell division. Meanwhile, the hematopoietic cell cluster formation occurs through proliferation and assembly after the EHT.

Conflict of Interest

The authors declare no competing interests.

Author Contributions

M.N. designed the experiments and interpreted the data. Y.Y. and M.N. performed the experiments and wrote the manuscript. H.K., Y.M., and K.Y. generated and provided the NB4-1 cell line.

Data Availability

The evidence data generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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