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The RUNX1 +24 enhancer and P1 promoter identify a unique subpopulation of hematopoietic progenitor cells derived from human pluripotent stem cells

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Abstract

Derivation of hematopoietic stem cells from human pluripotent stem cells remains a key goal for the fields of developmental biology and regenerative medicine. Here, we use a novel genetic reporter system to prospectively identify and isolate early hematopoietic cells derived from human embryonic stem cells (hESCs) and human induced pluripotent cells (iPSCs). Cloning the human *RUNX1c* P1 promoter and +24 enhancer to drive expression of tdTomato (tdTom) in hESCs and iPSCs, we demonstrate that tdTom expression faithfully enriches for *RUNX1c*-expressing hematopoietic progenitor cells. Time-lapse microscopy demonstrated the $tdTom^+$ hematopoietic cells to emerge from adherent cells. Furthermore, inhibition of primitive hematopoiesis by blocking Activin/Nodal signaling promoted the expansion and/or survival of $tdTom^+$ population. Notably, RUNX1c/$tdTom^+$ cells represent only a limited subpopuation of $CD34^+CD45^+$ and $CD34^+CD43^+$ cells with a unique genetic signature. Using gene array analysis, we find significantly lower expression of Let-7 and mir181a microRNAs in the RUNX1c/$tdTom^+$ cell population. These phenotypic and genetic analyses comparing the RUNX1c/$tdTom^+$ population to $CD34^+CD45^+$ umbilical cord blood and fetal liver demonstrate several key differences that likely impact the development of HSCs capable of long-term multilineage engraftment from hESCs and iPSCs.

Keywords

Embryonic stem cells; hematopoiesis; hematopoietic progenitor cells; induced pluripotent stem cells

Introduction

The ability of human pluripotent stem cells to differentiate into any cell lineage makes them an important resource for studies of developmental biology and regenerative medicine[1].

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Specifically, hematopoietic stem cells (HSCs) derived from pluripotent stem cells could be utilized for reconstitution of defective or diseased blood cell lineages. However, this goal has remained very challenging and elusive for several reasons[2]. For example, current culture conditions may not adequately support development or isolation of HSCs defined by capacity for long-term multilineage engraftment when transplantated into immunodeficient mice (typically termed SCID repopulating cells, SRC). Additionally, hematopoietic populations derived from human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) appear biased toward development of primitive hematopoietic cells, which likely do not to have the ability to reconstitute all blood cell lineages, especially in adult recipients[3–5]. To date, standard cell surface antigens have been used to identify hESC/iPSC-derived hematopoietic cells. However, none of these populations have demonstrated SRC potential, especially when compared to control cell populations isolated from human umbilical cord blood (UCB)[2, 3, 6–9]. Interestingly, two recent studies found that human hematopoietic cells isolated from hESC/iPSC-derived teratomas were able to engraft myeloid and lymphoid cells in both primary and secondary recipients, though still at a relatively low level[10, 11].

Here, we aimed to better identify human definitive hematopoietic populations by isolation of cells derived from both hESCs and iPSCs based on expression of the Runt-related transcription factor 1 (*RUNX1*). *Runx1* is a master regulator of hematopoiesis in vertebrates and has been shown to be necessary for emergence of definitive HSCs from hemogenic endothelium in the developing mouse embryo[12–16]. Runx1$^{-/-}$ mice die *in utero* due to a complete lack of a definitive blood system[12]. The *Runx1* locus in mouse and zebrafish contains two promoters, the proximal P2 and distal P1, which differentially drive expression of the Runx1b/a and Runx1c isoforms, respectively[17–19]. Transgenic reporter models have demonstrated the P2 promoter to be active throughout both primitive and definitive hematopoiesis, while P1 activity is largely restricted to the emergence of the definitive wave[20–22]. Likewise, isolation of hematopoietic populations in the developing mouse embryo based on expression from either promoter demonstrates that cells with P1 activity are enriched for definitive progenitors over the population with only P2 activity[22]. Both promoters rely on an intronic enhancer lying 24 kb downstream from the P1 promoter for expression specific to hematopoietic tissues[21, 23]. The +24 enhancer was also shown in a transgenic mouse model to be active in hemogenic endothelial cells directly prior to the emergence of HSCs, as well as the HSC clusters themselves[24]. Furthermore, this same study found the enhancer activity to be specific for HSCs within the mouse bone marrow. Interestingly, overexpression of Runx1a, a splice variant of Runx1b lacking the C-terminal transcriptional regulatory domain, has recently been shown to enhance stem cell expansion and engraftment from both mouse HSCs and hESC-derived populations[25]. While Runx1b$^{-/-}$ mice have severely impaired hematopoietic development, animals deficient for Runx1c$^{-/-}$ only show a modest decrease in definitive hematopoiesis[18].

Given the challenges to isolate functional HSCs from hESCs and iPSCs, and what appears to be a direct relationship between the onset of *Runx1c* expression and definitive hematopoiesis in the developing mouse embryo, this study sought to determine whether there exists a similar developmental relationship in human pluripotent stem cells. To do so, we developed

a transgenic reporter for RUNX1c in both hESCs and human iPSCs by cloning portions from the endogenous human *RUNX1* locus which correlate with important *Runx1c* regulatory elements in mouse. These studies demonstrate that RUNX1c expression in human development is restricted to a subpopulation of emerging hematopoietic cells with a unique genetic signature that offers important insight into differences between hESC/iPSC-derived hematopoietic cells and human cell populations isolated from UCB and fetal liver that have SRC potential.

Materials and Methods

Cloning and Plasmids

Transposon-encoding plasmids were constructed using standard molecular cloning techniques. Transposons were constructed using T2 inverted terminal repeat sequences separated by 1,800 base pairs (bp) of bacterial sequence consisting of the ColE1 bacterial origin of replication and kanamycin (Kan) resistance gene. pKT2/mCAG:GFPzeo (Figure 1A) encodes a fusion between the green fluorescent protein (GFP) reporter gene and the zeocin (Zeo) drug-selection marker (Invivogen) transcriptionally regulated by a CpG- free enhancer/elongation factor 1-α promoter/intron sequence (CLP) (Invivogen). pKT2/R1c:tdTomato was constructed by cloning the cDNA for tdTomato (provided by Roger Tsien, University of California San Diego, La Jolla, CA) attached to the Rabbit β-globin poly A flanked upstream by 957 base pairs of the Runx1c P1 distal promoter (Chr 21: 36,421,198-36,422,155) and downstream by 365 base pairs of the +24 intronic enhancer (Chr 21: 36,399,033-36,399,398) between the T2 elements of pKT2/mCAG:GFPzeo (Figure 1A). The RUNX1c P1 distal promoter and +24 enhancer were amplified from H9 genomic DNA using standard PCR. The Sleeping Beauty 100 transposase (Addgene) was designed as previously described[62]

Human ES Cell Culture and Gene Transfer

Human ES cells (H9) (University of Wisconsin, Madison, WI) were maintained as colonies on mitomycin C-treated MEFs or on Matrigel-coated plates in ES cell growth medium consisting of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (F12) (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 15% Knockout Serum Replacer (Invitrogen), 1 mM L-glutamine (Cellgro/Mediatech, Herndon, VA, http://www.cellgro.com), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), 1% minimum essential medium nonessen-tial amino acids (Invitrogen), 4 ng/ml basic fibroblast growth factor (R&D Systems Inc., Minneapolis, http://www.rndsystems.com), and 1% penicillin-streptomycin (Invitrogen) incubated at 37°C in a humidified atmosphere containing 5% CO_2. Five days before nucleofection, human ES cells were transferred to Matrigel-coated plates to remove MEFs. For 1 hour prior to nucleofection, cells were treated with 10 μM Y-27632 (Millipore, Billerica, MA, www.emdmillipore.com) to increase viability. Per sample, 2×10^6 cells were treated with 1 ml of 0.05% Trypsin supplemented with 2% chick serum, harvested, pelleted, and nucleofected in clumps with 6 μg of transposon DNA and 2 μg of transposase in 100 μl of nucleofector solution (Kit V; Amaxa Inc., Gaithersburg, MD, http://www.amaxa.com) using program setting B-16. Nucleofected cells were immediately resuspended in human ES cell

growth medium prewarmed to 37°C supplemented with 10 μM Y-27632 and seeded onto MEFs for continued culture. Successful transfectants were selected days 3–7 post nucleofection using 4 μM zeocin (Invivogen, San Diego, CA, USA), and clonal lines were derived by single cell passage of the selected, bulk population onto MEFs using 0.05% Trypsin + 2% chick serum. After 7 days of clonal growth, individual colonies were transferred to separate wells of MEFs for continued culture.

Hematopoietic Differentiation of hPSCs as Spin EBs

H9 hESC and UCB-derived iPSC transgenic RUNX1c reporter lines were adapted to passage as single cells using TrypLE (Invitrogen) as previously described[63]. Briefly, after harvest into single cells, the cells were resuspended in BPEL media[63] supplemented with Bmp4 (20 ng/mL), VEGF (20 ng/mL) and SCF (40 ng/mL) and plated at a density of 3,000 cells/well in a 96-well plate and spun in a centrifuge to create cell aggregates. For TGF-β signaling studies, the TGF-β inhibitor SB-431542 was applied days 2–5 at 6 μM. After 6 days of incubation, the EBs were transferred to gelatinized, 24-well adherent plates in BEL media[63] supplemented with VEGF (40 ng/mL), SCF (50 ng/mL), IL-3 (30 ng/mL), IL-6 (30 ng/mL), TPO (30 ng/mL) and EPO (3 U/mL). To harvest for analysis, the media containing the non-adherent fraction was removed from each well while the adherent cells were detached with 0.05% trypsin containing 2% chick serum for 4–8 minutes. Both fractions were combined and filtered to create a single-cell suspension and subjected to subsequent analysis.

In vitro generation of NK cells

Day 14 td+ hematopoietic progenitor cells were transferred to 24-well plates with a confluent monolayer of irradiated OP9-DL1 stromal cells in the NK medium designed to maximize NK cell growth. Briefly, cells were co-cultured in 2:1 mixture of Dulbecco modified Eagle medium/Ham's F12 (DMEM/Ham's F-12, Cellgro/Mediatech) basal media supplemented with 20% heat-inactivated human AB serum (Nabi), 5 ng/ml sodium selenite (Sigma), 50 μM ethanolamine (MP Biomedicals), 20 mg/L ascorbic acid (Sigma), 25 μM 2-ME, 2mM L-glutamine, 1% P/S (Invitrogen), 10 ng/ml Interleukin-15 (IL-15, PeproTech), 5 ng/ml Interleukin-3 (IL-3, PeproTech), 20 ng/ml Interleukin-7 (IL-7, PeproTech), 20 ng/ml SCF (PeproTech), and 10 ng/ml FMS-like tyrosine kinase 3 ligand (Flt3L, PeproTech). Medium containing fresh cytokines was changed twice a week with the exception of IL-3 which was only included for the first week of co-culture. Stromal cells were replaced with fresh cells once a week. Cells were harvested at appropriate time points during NK cell co-culture, counted for viable cells, and assayed for phenotypic analysis.

Maintenance of stromal cells for NK derivation

OP9-DL1 cells were cultured in appropriate medium, which was composed of 79% of α-MEM medium (Invitrogen), without ribonucleosides and dexyribonucleosides but with 2 mM L-glutamine, 20% of characterized FBS (ATLANTA BIO, Optima), 1% of P/S (Invitrogen) and 1.5 g/L sodium bicarbonate (Sigma). OP9-DL1 stromal cells were made and kindly provided by Juan Carlos Zúñiga-Pflücker lab from Sunnybrook Research Institute (SRI). OP9-DL1 cells were made from OP9 cells but ectopically express the Notch ligand Delta-like 1 (DL1).

Results

Generation of transgenic RUNX1c reporter hESCs and iPSCs

Tracking the emergence of early, definitive hematopoietic progenitor cell populations potentially containing HSCs from hESCs requires a reporter gene which distinguishes not only hematopoietic from non-hematopoietic cells, but also specifically identifies definitive cells within the hematopoietic compartment. The *RUNX1c* P1 distal promoter has been shown to be specifically associated with emerging HSCs in the aorta-gonad-mesonephros (AGM)[20, 24]. Likewise, hematopoietic cells from the mouse embryo displaying P1 and Runx1b proximal P2 promoter activity are enriched for definitive hematopoietic potential in the CFU assay as opposed to cells showing P2 activity alone[18, 20]. Both promoters require the +24 intronic enhancer for hematopoietic specific expression[21, 23]. Therefore, we constructed a reporter cassette using conserved regions of the human *RUNX1* locus showing homology to the mouse P1 promoter and +24 enhancer to track definitive hematopoietic differentiation from hESCs and iPSCs. A 957 bp fragment of the P1 promoter and 365 bp of a conserved intronic region representing the human +24 enhancer were cloned from H9 hESC genomic DNA (Figure. 1a). Both of these fragments were inserted flanking tdTomato (tdTom) cDNA to best represent the layout of the endogenous locus. Immediately downstream we included a constitutively driven GFPzeo fusion gene to identify and select for cells with a successfully integrated transgene (Figure 1a). Using the pKT2 transposon as a backbone, this entire construct was stably inserted into H9 hESCs as well as human iPSCs by co-nucleofection with a plasmid containing the *Sleeping Beauty (SB)* transposase. We have previously demonstrated *SB* to be highly efficient at stable gene modification in hESCs[26]. After selection for hESCs and iPSCs containing the stably inserted transgene, single cell clones were obtained. For both the hESC and iPSC reporters, two clones were carefully analyzed for the ability to undergo hematopoietic differentiation. Though each clone demonstrated various rates of hematopoietic differentiation under these conditions, patterns of hematoendothelial marker expression, including tdTom, were consistent and identical to non-transgenic hESCs, (Supplemental figure 1). This consistency among individually derived clones and non-transgenic hESCs indicated that hematopoietic differentiation was not affected by the presence or position of the transgene.

The human P1 promoter and +24 enhancer elements drive tdTomato expression mimicking that of endogenous Runx1c

To test whether the human *RUNX1c* cis-regulatory elements in our transgene would drive expression of the tdTomato (tdTom) reporter to faithfully represent expression of endogenous RUNX1c, we used the spin embryoid body (EB) method for hematopoietic differentiation of the hESC reporter cells (Supplemental figure 2). Under these conditions, $CD34^+$ and $CD31^+$ hematoendothelial cells develop by day 3 (Figure 1B). By day 7 an initial wave of early hematopoietic cells expressing CD41a or CD43 are produced, while more mature populations expressing CD45 typically did not emerge until around day 12 (Figure 1B). Similarly, the first $tdTom^+$ cells are first identified at approximately days 9–10, though the frequency was so low (<1%) and dim that it was hard to determine if they were real, while a more substantial (1–3%) tdTom+ population was apparent by days 12–13 in this system (Figure 1C). The emergence and proliferation of the $tdTom^+$ population directly

correlated with both an increase in endogenous RUNX1c and tdTom messenger RNA (mRNA) by qPCR (Figure 1D). Specificity of tdTom expression in RUNX1c$^+$ cells is further demonstrated by enrichment for endogenous RUNX1c transcript in the sorted tdTom$^+$ cells, as compared to unsorted and tdTom$^-$ cells, which had nearly undetectable levels of RUNX1c (Figure 1E). Because CD73$^+$ mesenchymal/stromal cells are also produced in these EB conditions, we analyzed our tdTom$^+$ cells for CD73. Notably, CD73$^+$ cells are all tdTom$^-$, indicating that tdTom expression was specific for the developing hematopoietic populations (Figure 1F). Finally, we observed by fluorescent microscopy that tdTom expression was first visible in the non-adherent, cobblestone-appearing hematopoietic cells which began proliferating days 11–13 (Figure 1G). Together, these results suggest that our reporter system faithfully reports the expression of *RUNX1c* in human cells and that *RUNX1c* expression is limited to hematopoietic populations.

tdTomato expression enriches for hematopoietic progenitors and is restricted to a sub-set population of hematoendothelial cells

We next wanted to determine which early endothelial and hematopoietic cell surface antigens were expressed on the tdTom$^+$ cells. All of the emerging populations expressing CD34, CD31, CD41a, CD43 and CD45 initially lacked tdTom expression, though each turned tdTom$^+$ at later time points (Figure 2A). By day 12, the first emerging (less than 10% of the total population) tdTom$^+$ cells were CD31$^+$, CD43$^+$, CD34$^+$, CD45$^-$ and showed mixed expression for CD41a (58 +/− 5%) (Figure 2A and B). As the tdTom$^+$ cells expanded past 20% of the total population after day 15, CD34 co-expression decreased to 14.7 +/− 2% of total tdTom$^+$ cells, while CD45 co-expression increased to 64.4 +/− 7%, indicating that the initial wave had differentiated into more mature lineages (Figure 2A and C). CD31 and CD43 co-expression continued to be expressed on the vast majority of tdTom$^+$ cells, with tdTom being restricted to the CD31^{low}, CD43^{hi} subsets (Figure 2A,C). CD41a co-expression showed no discernable change on the tdTom+ population at 50.7 +/− 7% after expansion of the initial wave (Figure 2C). CD235a, a marker for primitive erythrocytes[27] and CD45RA, which is absent on multi-potent HSCs with T-cell potential[28–30] showed limited to no expression on the emerging tdTom$^+$ population (Supplemental Figure 2).

We next analyzed the CD34$^+$CD45$^+$ and CD34$^+$CD43$^+$ populations for expression of tdTom since these phenotypes enrich for hematopoietic progenitor cells [31–34]. During initial tdTom$^+$ cell emergence and expansion, neither of these populations showed significant enrichment for tdTom over the bulk population (Figure 2D–F), indicating that these cells are heterogeneous for expression of RUNX1c, with a majority of these populations being tdTom/RUNX1c$^-$. Next, we used a standard colony-unit forming assay to demonstrate isolated tdTom/RUNX1c$^+$ cells were enriched for hematopoietic progenitor cells, demonstrated by increased ability to form typical hematopoietic colonies (430 +/− 143 per 10^5 cells) as compared to both tdTom$^-$ cells (131 +/− 15 per 10^5 cells) and unsorted cells (163 +/− 28 per 10^5 cells) (Fig 2G).

To further investigate the capability of the tdTom+ cells, we isolated the day 14 tdTom+ cells by flow cytometry, and induced them to differentiate into NK cells, as previously described. After three weeks, the differentiated cells expressed NK cell marker CD56 at a

level comparable to Nk cells produced from cord blood CD34+ cells (Supplemental figure 3). Notably, the tdTom expression decreased during the NK cell differentiation (Supplemental figure 3) suggesting loss of Runx1c expression in the mature cells.

Time-lapse imaging of emerging tdTomato+ cells

Fluorescent microscopy of the differentiating EBs showed tdTom expression to vary in intensity within the non-adherent, hematopoietic population. We noticed single tdTomato^{bright} cells that were often associated with the adherent tdTomato^{-} endothelial outgrowths and were surrounded by larger numbers tdTomato^{dim} cells, especially at earlier time points when the first tdTom^{+} cells were emerging, (Figure 3A,B). We were also able to identify occassional tdTom expression in adherent, likely endothelial cells derived in these cultures (Figure 3C–F). In some instances, the endothelial cell appeared to be giving rise to a tdTomato^{bright} hematopoietic cell through asymetrical division (Figure 3D–F). Isolation of the CD34^{+}CD31^{+} putative hematoendothlial population on day 9, prior to the emergence of tdTom^{+} cells, demonstrated these cells to be bipotential for generation of tdTom^{+} hematopoietic progenitors as well as VE-Cadherin^{+} mature endothelial cells (Supplemental figure 4).

In order to more definitively demonstrate where the emerging tdTom+ cells were being created, we conducted time-lapse confocal microscopy on the differentiating EBs from days 9–11. As expected based on our flow cytometric data showing the emergence of CD43^{+} and CD41a^{+} cells by day 6, there were already an abundance of tdTom^{-}, non-adherent hematopoietic cells at this time. Though flow cytometric analysis did not show tdTom^{+} cells until days 11–12, time-lapse imaging demonstrates these rare cells to exist as early as day 9, suggesting that the initial dim cells are indeed real (Supplemental movie 1–3). Here, a tdTom^{-} cell appearing to be of stromal origin divides, creating two cells which quickly turn tdTom^{+} with a round, hematopoietic morphology (Supplemental movie 1–2). In one instance, we demonstrate two of these events in the same field of view with the first lineage being tracked by red arrows and the second, later occurrence by black arrows (Supplemental movie 1). These daughter cells then became highly mobile, making numerous cell-cell contacts and going through 1–2 divisions themselves between days 9–11. In some cases, the larger, stromal precursor was tdTom^{+} before giving way to hematopoietic daughter cells (Supplemental movie 3), though it is hard to distinguish the exact lineage of the precursor cell. These results appear to recapitulate the early stages of *in vivo* hematopoiesis wherein the hemogenic endothelium of the AGM region begins to express Runx1 before giving rise to a Runx1^{+} definitive HSC[20, 35–37]

Inhibition of primitive hematopoiesis through the Activin/Nodal pathway increases the proliferation of tdTom^{+} cells

In order to test whether expression of our *RUNX1c* reporter could distinguish between primitive and definitive hematopoietic populations, we applied an inhibitor of Activin/Nodal signaling, SB-431542. Activin is required for the development of primitive erythrocytes in mouse embryonic stem cells[38, 39]. SB-431542 has been utilized in hESC differentiation cultures to restrict the development of primitive hematopoietic cells and promote definitive progenitor cells as assessed by T-cell developmental potential[40]. Our initial studies found

that application of SB-431542 on the first day of EB differentiation completely ablated hematopoietic development, while treatment with SB-431542 at days 3 and beyond showed little to no effect on the differentiation. In contrast, incubation of developing EBs with SB-431542 from days 2–5, corresponding to mesoderm specification, gave an optimal effect with substantial inhibition $CD31^+$, $CD41a^+$ and $CD43^+$ cells, especially at day 9 (Figure 4a). Day 9 EBs also demonstrated an almost complete loss of erythroid progenitors with addition of SB-431542, consistent with the previous study[40] (Fig 4B). Activin/Nodal inhibition did not appear to change the timing of RUNX1c/$tdTom^+$ cell formation, as these cells first emerge between days 11–13 in both SB-431542 and untreated samples. However, the $tdTom^+$ cells increased 2-fold in SB-431542 treated samples after their emergence, accompanied by a 2-fold increase in cell numbers (Figure 4C). Day 13 CFU analysis of the $tdTom^+$ population showed an increased colony forming potential with inclusion of SB-431542 (Supplemental figure 5). Furthermore, sub-gating on the $CD34^+CD43^+$ and $CD34^+CD45^+$ populations demonstrated each to be enriched 2 fold for percentage of tdTom expressing cells over the untreated group (Fig 4D,E). These results suggest that inhibition of primitive hematopoiesis in our differentiation cultures, while not affecting the timing of $RUNX1c^+$ cell emergence, does promote the expansion and/or survival of $RUNX1c^+$ hematopoietic cells.

iPSC RUNX1c reporter displays similar characteristics

To test whether the RUNX1c reporter construct used in our H9 hESCs would yield similar results in other human pluripotent cells, we inserted our RUNX1c reporter construct into an iPSC line derived in our lab from $CD34^+$ UCB cells, which we termed Dub7. Our group has previously demonstrated effective hematopoietic development from the Dub7 line as well as other human iPSCs[41]. We again derived individual clones which had successfully integrated the transgene. As with the hESC reporter, the emergence and expansion of $tdTom^+$ cells closely correlated with expression of endogenous *RUNX1c*. Sorted $tdTom^+$ cells showed a trend towards enrichment for endogenous *RUNX1c* transcripts compared to both $tdTom^-$ and unsorted populations (Figure 5a). As such, we determined that the reporter transgene accurately reflected expression of endogenous RUNX1c in the Dub7 iPSC line.

Flow cytometric analysis demonstrated $tdTom^+$ coexpression patterns with hematopoietic cell surface antigens very similar to the H9 hESC reporter line, with $tdTom^+$ cells being restricted to the $CD31^+$ and $CD43^+$ compartments, while the $CD45^+$ cells did not gain tdTom expression until later time points (Fig 5B). CFU analysis of the iPSC-derived $tdTom^+$ cells again demonstrated enrichment for hematopoietic progenitor cells, as compared to $tdTom^-$ and unsorted cells (Figure 5C). Additionally, we subjected the differentiating cells to the Activin/Nodal SB-431542 inhibitor. Similar to the hESCs, we found optimal inhibition for increased hematopoietic development between days 2–5 of differentiation. Though the increase in $tdTom^+$ cells was not as robust in the SB-431542 treated iPSC samples, we did consistently demonstrate greater tdTom expression in $CD31^+$, $CD41a^+$, $CD43^+$ and $CD45^+$ cells (Fig 5D). Taken together, these data demonstrate that RUNX1c has a similar expression during hematopoietic development in iPSCs and hESCs.

Genetic Analysis of tdTom+ cells

We next compared gene expression between our hESC and iPSC-derived hematopoietic populations and $CD34^+$ UCB or $CD34^+$ fetal liver (FL). Both UCB and FL contain HSCs and have robust SRC capability[42–44]. We conducted mRNA gene array analysis, as well as analysis of 800 miRNAs. Undifferentiated, $tdTom^+$, $tdTom^-$, $CD34^+CD45^+$ and $CD34^+CD43^+$ populations were sorted and analyzed from both the hESC and iPSC RUNX1c reporter cell lines as well as $CD34^+CD45^+$ cells isolated from UCB and FL. Clustering within the mRNA samples showed similarity between all hESC and iPSC-derived $CD34^+CD45^+$, $CD34^+CD43^+$ and $tdTom^+$, with the $tdTom^-$ and undifferentiated sources grouping separately (Figure 6A). The $CD34^+$ UCB clustered within the same tree as the hESC and iPSC-derived hematopoietic cells, but established an entirely separate branch. Thus, we were able to define clear differences in expression patters between these samples and the hESC/iPSC-derived cells. Interestingly, we noticed an upregulation of genes commonly associated with cell proliferation in hESC/iPSC-derived samples as compared to UCB and FL, including CCND1, CCND3 and BRCA1 (Figure 6B).

miRNA analysis was conducted using the same RNAs. To more closely examine only relevant miRNAs, total normalized expression for each miRNA was averaged across all hematopoietic samples (excluding $tdTom^-$ and undifferentiated cells) and the top 105 were used for cluster analysis. As with the mRNAs, the hESC/iPSC-derived hematopoietic samples and $CD34^+$ UCB/FL clustered separately from the $tdTom^-$ and undifferentiated cells (Figure 6C). Furthermore, $CD34^+$ UCB and FL were again grouped together, showing groups of miRNAs with distinct expression patterns when compared to the hPSC-derived samples. The most clear example of this was the Let-7 miRNA family, including Let-7a, Let-7b and Let-7g, which all showed increased expression in UCB and FL (Figure 6D). Interestingly, Let-7a has been shown to target CCND1 both directly and indirectly to suppress cell proliferation[45–49]. Our gene array data also demonstrates mir181a to be upregulated 10-fold in $CD34^+$ UCB as compared to any of the hESC or iPSC-derived samples (Figure 6D). miR181a was previously found to be upregulated in $CD34^+CD38^-$ UCB as compared to the slightly more committed $CD34^+CD38^+$ progenitors[50]. Furthermore, ectopic expression of mir181 in hematopoietic stem/progenitor cells has been shown to enhance B-cell development[51].

Finally, we conducted qRT-PCR analysis for individual genes within the HoxA cluster, as these have been reported to show reduced expression in hESC/iPSC-derived samples as compared to $CD34^+$ UCB. Notably, we found that the sorted $tdTom^+$ populations from both the hESC and iPSC reporter lines have reduced expression for all genes tested within the HoxA locus (Figure 6E). Taken together, this genetic analysis demonstrates that there are numerous key hematopoietic mRNAs and miRNAs which are deficient for expression in both hESC and iPSC-derived hematopoietic progenitors as compared to populations known to contain SRCs.

Discussion

In the present study, we have shown that the distal P1 promoter and +24 intronic enhancer that regulate *RUNX1c* expression have increased activity in a specific subpopulation of

hematopoietic cells derived from both hESCs and iPSCs. Isolation of the RUNX1c$^+$ (tdTom$^+$) cells derived from hESCs and iPSCs demonstrates this population has a distinct genetic signature based on both mRNA and miRNA expression compared to hematopoietic cells isolated by typical phenotypic surface antigens: CD34, CD41, CD43, and CD45. While other studies have previously examined the phenotypic qualities of Runx1c-expressing cells using reporter models in both zebrafish and mice, to our knowledge this is the first such report developed in human pluripotent stem cells. Previous *in vitro* and *in vivo* studies evaluating expression of the three Runx1 isoforms during mouse development have found the onset of Runx1c expression to be specific for emerging, definitive mESC-derived HSCs, as opposed to Runx1b and Runx1a, which were expressed throughout hematopoiesis[20]. Furthermore, the same report found increased Runx1c expression in the AGM region of E10.5-E11.5 mouse embryos, where the first definitive HSCs arise[20].

Use of this promoter/enhancer reporter system differs from a direct genetic knock-in reporter systems that have been used for other studies of hematopoiesis from hESCs[52, 53]. However, the construct used for these studies does contain the P1 promoter and +24 enhancer from hESC genomic DNA as dictated by homology to regions utilized for similar reporter systems which have been successful in mice[21, 24]. Our results show appropriate specificity of *RUNX1c* expression in the tdTom+ cells. Specifically, there is a complete lack of tdTom expression in non-hematopoietic tissues, such as the CD73$^+$ cells, and the timing of tdTom expression closely correlates with that of endogenous RUNX1c. Furthermore, there is an advantage to use of our reporter construct based on the efficiency to engineer multiple cell lines using the *Sleeping Beauty* system over a short period of time. Indeed, we are able to use both hESCs and iPSCs for these studies. From both hESCs and iPSCs, we were able to generate several transgenic clones and validate consistency in both overall hematopoietic differentiation and expression of the tdTom/RUNX1c reporter construct. This comparison between different starting cell populations provides greater confidence the expression patterns seen were not a consequence of transgene integration effects. Additionally, use of this transgene system can avoid problems associated with RUNX1 haploinsufficieny that has been seen with mouse direct knock-in approaches[14, 54]

These studies demonstrate that emerging populations of tdTom$^+$ cells appear to recapitulate mouse and zebrafish developmental systems[24, 37]. Lam *et al* used a Runx1-eGFP zebrafish reporter to demonstrate Runx1 expression in aortic endothelial cells (ECs) subsequent to emergence of definitive HSCs, while Ng *et al* found pronounced +24 enhancer activity specific to ECs lining the ventral wall of the dorsal aorta along with the associated HSC clusters. Though such *in vivo* models are not feasible in humans, Park *et al* used hESCs and hiPSCs to show large quantities of CD34$^+$CD45$^+$ hematopoietic progenitors arising directly from the adherent EC population, suggesting that a similar bipotential hematoendothelial subset exists in human hematopoietic ontogeny[55]. Our fluorescent microscopic studies similarily demonstrate the *RUNX1c*/tdTom reporter is expressed in rare adherent endothelial cells that appear to be undergoing asymmetrical cell division giving rise to a tdTom$^+$ hematopoietic cells. Furthermore, fluorescent time-lapse microscopy more clearly demonstrates the emergence of tdTom$^+$ cells from what appears to be an endothelial progenitor. The precise timing of this transition in relation to activation of

the reporter is difficult to conclude, however, due to its fluidity and the lack of markers to distinguish endothelial from hematopoietic phenotype other than morphology. Regardless, these studies appear to recapitulate the mouse and zebrafish systems and provide further evidence for the role of *RUNX1c* in the human endothelial to hematopoietic transition. The finding that all tdTom-expressing cells were all positive for the hematoendothelial marker CD31 also supports this conclusion.

Our mRNA and miRNA genetic analysis provides some potentially valuable insight regarding the engraftment deficiencies of hESC/iPSC-derived hematopoietic progenitors as compared to UCB and FL. While it seems probable that culture conditions are a cause of this discrepancy, determining variations in specific gene expression networks may help to explain exactly what these deficiencies are. We have identified several miRNAs, including the Let-7 family and mir181a, which are underexpressed in hESC/hPSC derived cells as compared to $CD34^+$ UCB/FL. This was then supported by an increase in cell cycle/proliferation-promoting genes *CCND1*, *CCND3* and *BRCA1*, highlighting potential miRNA-mRNA interactions involved with these networks. These pathways have implications regarding crucial HSC qualities such as quiescence and multilineage differentiation[45–61]. Furthermore, similar to studies showing a positive role for HoxB4 in mESC-derived progenitor engraftment[56, 57], we have demonstrated that expression from the HoxA locus appears to be deficient in our hESC/iPSC-derived progenitors.

Though RUNX1c delineates putative definitive HSCs in mice, additional characterization of these human cells, as well as selection on the basis of other key transcription factors is necessary. Regulatory elements for several other hematopoietic transcription factors such as Pu.1 and C/EBPα, both direct targets of RUNX1 and critical regulators of myeloid development, may also be utilized to create reporter systems[58–61]. Indeed, using the system described here to quickly and efficiently create l promoter/enhancer reporter cell lines from hESCs and iPSCs will allow us to readily accumulate profiles on other specific subpopulations of cells that express critical transcription factors (or multiple transcription factors). Finally, our genetic analysis of hESC/hiPSC-derived $tdTom^+$, $CD34^+CD45^+$ and $CD34^+CD43^+$ cells in comparison to $CD34^+$ UCB/FL provides numerous potential targets for gene modification of hPSCs which could enhance *in vitro* SRC output and quality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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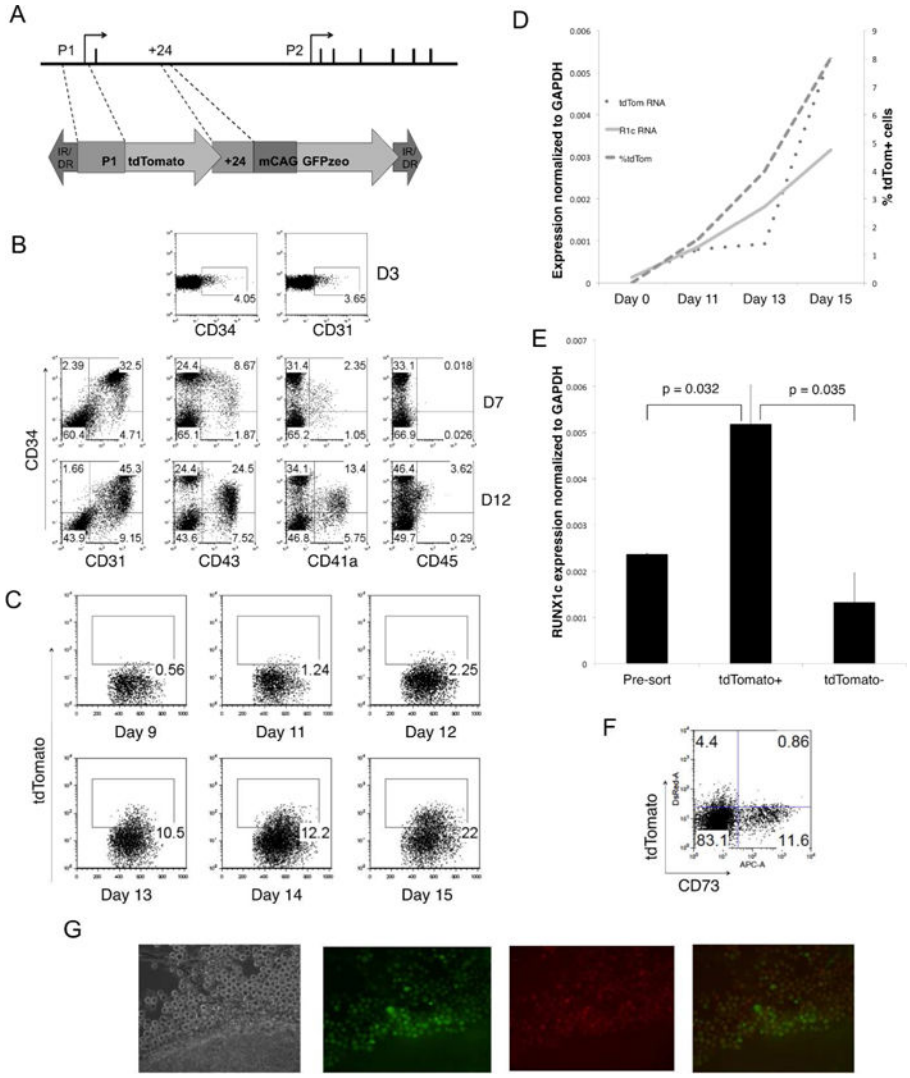

Figure 1. Stable integration of a reporter driven by *RUNX1c* regulatory elements into H9 hESCs allows for fluorescent labeling of emerging RUNX1c^{+} hematopoietic populations over a directed differentiation time course

A. The Runx1c reporter was constructed using 957 bp of the human endogenous RUNX1c promoter (P1), and 356 bp of the +24 enhancer directly flanking tdTomato. A constitutive GFPzeo fusion gene was included to allow for selection of cells obtaining the transgene. The entire cassette lies between the T2 IR/DR transposon elements for stable genomic insertion by the Sleeping Beauty transposase. **B.** Hematopoietic differentiation of the H9 hESC RUNX1c reporter as Spin EBs. EBs were disaggregated and analyzed for co-expression of hematopoietic extra-cellular markers on days 3, 7 and 12. **C**. Similar hematopoietic differentiation of the H9 hESC RUNX1c reporter as in 1B but with flow cytometric analysis for expression of the tdTom reporter. **D**. Time-course differentiation with analysis of disaggregated EBs on days 11–14 for the %tdTom+ proportion by flow cytometry as well as expression RUNX1c and tdTom mRNA by qPCR. Values are shown as the average from 3 independent experiments. **E.** Day 13 post-sort qPCR analysis. tdTomato+ cells show enrichment for endogenous RUNX1c transcript over both the tdTomato− and pre-sort

populations. Values are shown as the average of three biological replicates with error bars representing S.E.M. **F.** Differentiated reporter cells expressing the mesenchymal marker CD73 do not express tdTomato. **G**. Fluorescent microscopy of day 21 Spin EBs showing a large expansion of hematopoietic cells which are $tdTom^{+}$.

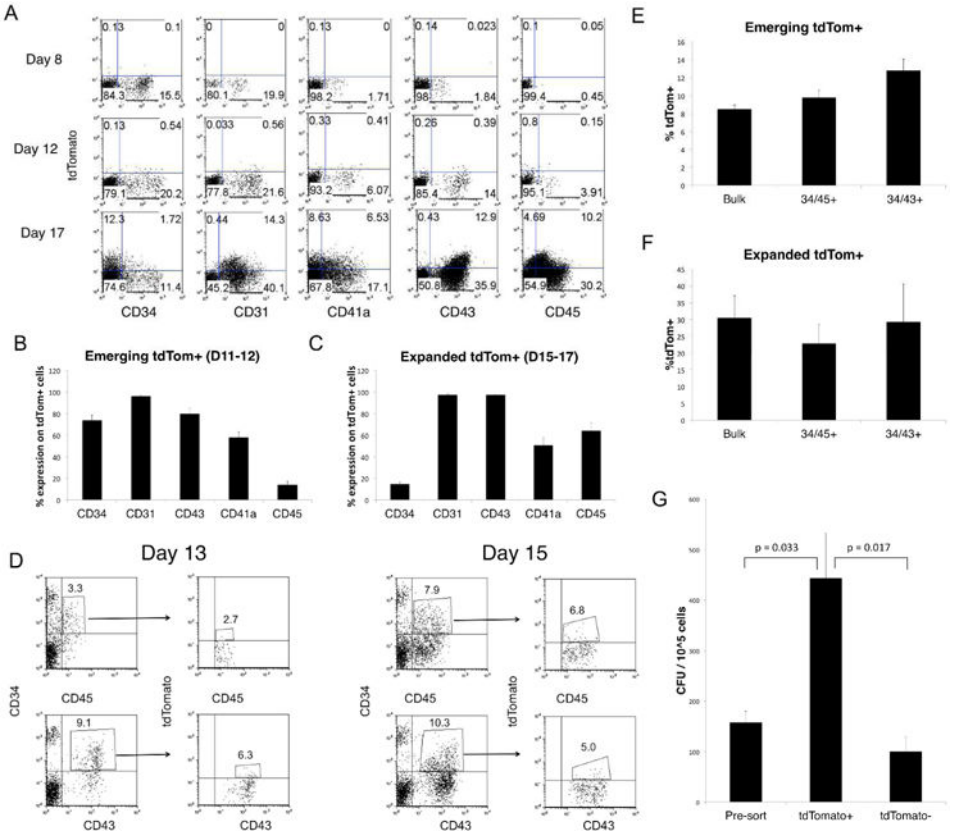

Figure 2. Hematopoietic progenitor potential and phenotypic analysis of the tdTom$^+$ fraction

A. Flow cytometric analysis of RUNX1c reporter over hematopoietic differentiation time course for tdTom and various hematoendothelial markers. **B**. Flow cytometric analysis of emerging tdTom+ cells (day 12–13). The tdTom+ cells were sub-gated and analyzed for expression of various hematoendothlial markers. Shown is the percent of the tdTom population expressing either CD34, CD31, CD43, CD41a or CD45. Values are the average of 10 biological replicates with error bars representing S.E.M. **C**. Similar analysis to Figure 2B but after tdTom+ cells expanded to over 20% of the population (day 15–17). Values are the average of 6 biological replicates with error bars representing S.E.M. **D**. CD34$^+$CD45$^+$ and CD34$^+$CD43+ cells were subgated and assessed for tdTom expression on days 13 and 15 of differentiation. **E,F**. Percent of CD34$^+$CD45$^+$ and CD34$^+$CD43+ cells expressing tdTom compared to the bulk population shows little to no tdTom enrichment in these hematopoietic progenitors at both the emerging (E) and expanded (F) tdTom time points. Values are the average of four biological replicates with error bars representing S.E.M. **G**. Hematopoietic progenitor potential of the tdTom+ population as assessed by ability to form hematopoietic colonies in methylcellulose. H9 reporter cells were differentiated for 13 days as spin EBs then FACS sorted for tdTomato expression and placed in CFU. Samples contained 1×10^5 cells at plating and colonies were enumerated after 6–7 days of incubation. Values are the average of four biological replicates with error bars representing S.E.M.

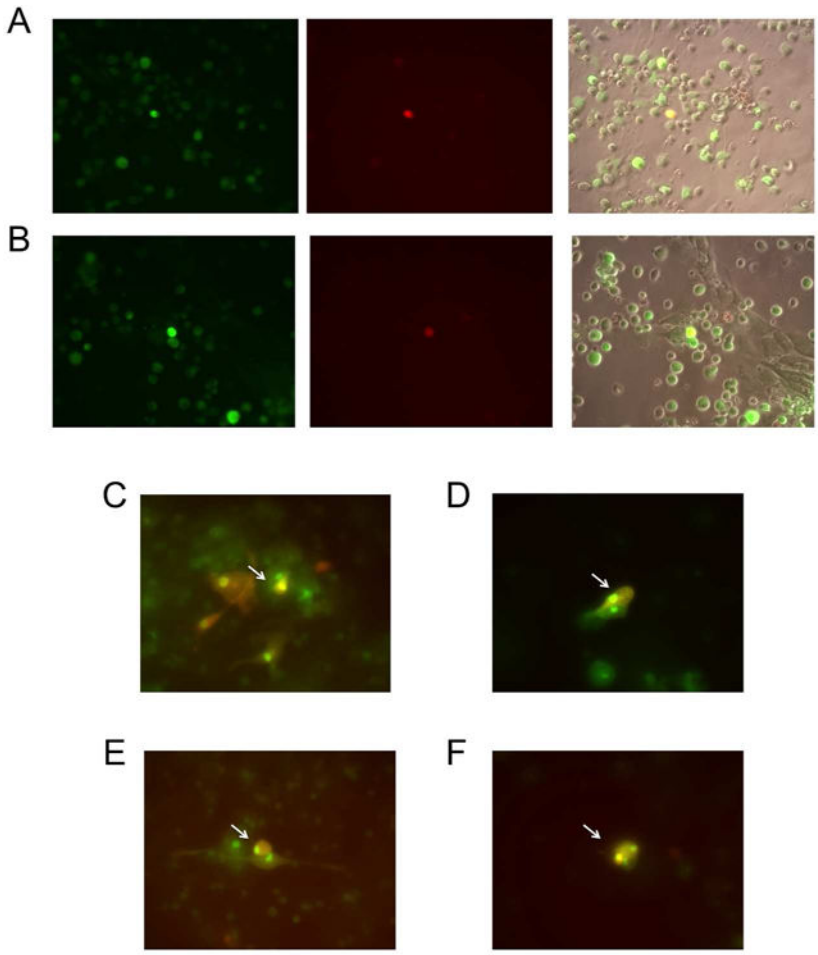

Figure 3. Fluorescent microscopy of H9 hESC RUNX1c reporter line differentiating under hematopoietic conditions

A,B. Day 13 cells viewed under 20× magnification. A single $GFP^{+}tdTomato^{bright}$, non-adherent hematopoietic cell can be seen among several $GFP^{+}tdTomato^{-}$ hematopoietic cells. **C–F**. Day 13 unsorted cells imaged under 40× magnification. tdTomato expression can be seen in cells with both endothelial and hematopoietic morphology. In some cases, a $GFP^{+}tdTomato^{-}$ endothelial cell can be seen giving rise to a $GFP^{+}tdTomato^{Bright}$ cell through asymmetrical division (D–F). There were also instances of the endothelial cells being $tdTomato^{Bright}$ themselves.

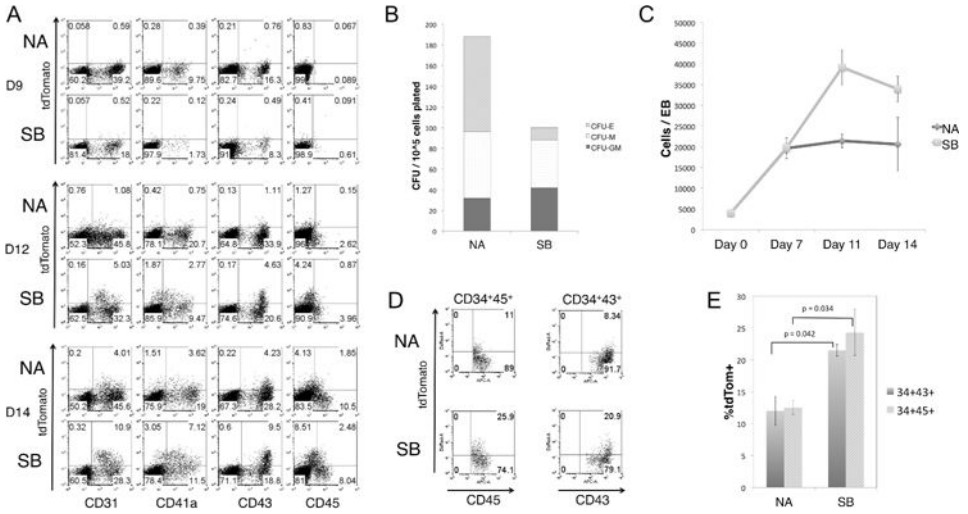

Figure 4. Inhibition of primitive hematopoiesis through Activin/Nodal signaling promotes proliferation and/or survival of tdTom$^+$ cells

A. Day 9, 12 and 14 flow cytometric analysis of the RUNX1c reporter hESCs for early hematopoietic markers and tdTom with (SB-431542) or without (NA) application of SB-431542. **B**. Colony forming unit assay for cells harvested on day 9 of differentiation showing proportions of CFU-M, CFU-GM and CFU-E. **C**. Cell numbers from dissociated EBs on day 0, 7, 11 and 14 with or without application of SB-431542. Numbers shown are cells per EB harvested. Values are the average of three biological replicates with error bars representing S.E.M. **D,E.** Assessment of tdTomato expression from the CD34$^+$CD45$^+$ and CD34$^+$CD43$^+$ populations for both untreated and SB-431542 treated samples. Values are the average of three biological replicates with error bars representing S.E.M.

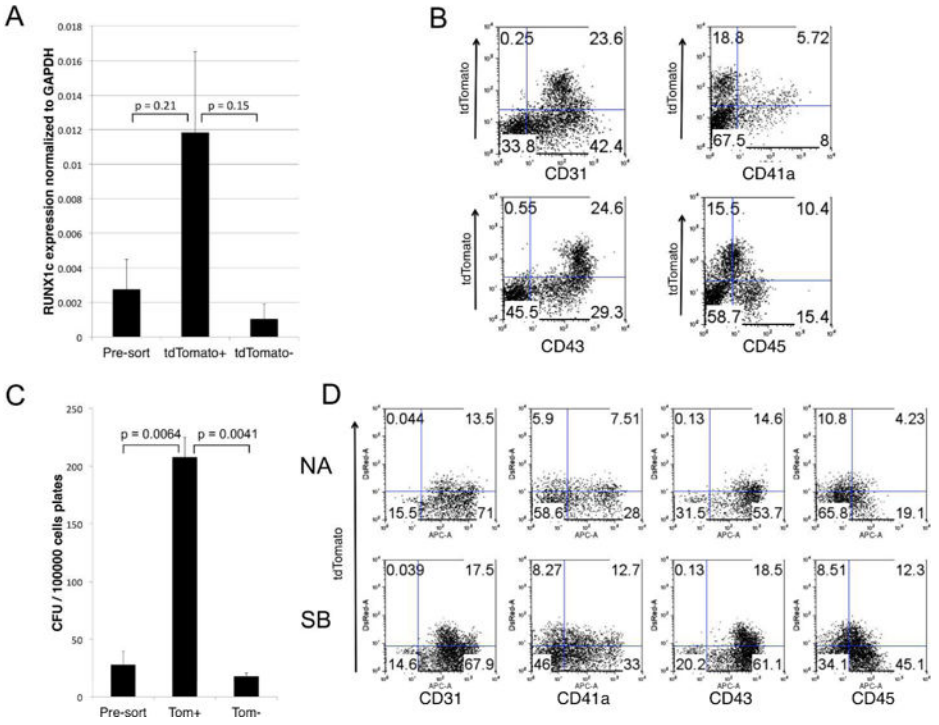

Figure 5. Hematopoietic differentiation of human cord blood-derived iPSC RUNX1c reporter

A. qPCR analysis for expression of endogenous RUNX1c mRNA in Spin-EB differentiated tdTomato^{+} cells vs. the unsorted and tdTomato^{-} population. Values are the average of two biological replicates with error bars representing S.E.M. **B.** Flow cytometry for co-expression of tdTomato with various hematoendothelial and hematopoietic extra-cellular markers in differentiating reporter cells. **C.** Colony forming unit assay enumerating hematopoietic progenitors from Spin-EB derived tdTomato^{+} cells vs. the unsorted and tdTomato− population. **D.** Flow cytometric analysis showing the effect of SB-431542 Activin/Nodal inhibitor on expression of tdTomato with various hematopoietic extra-cellular markers over hematopoietic differentiation. Cells were analyzed between days 12 and 14 depending on the timing of the expression of tdTomato and the extra-cellular marker.

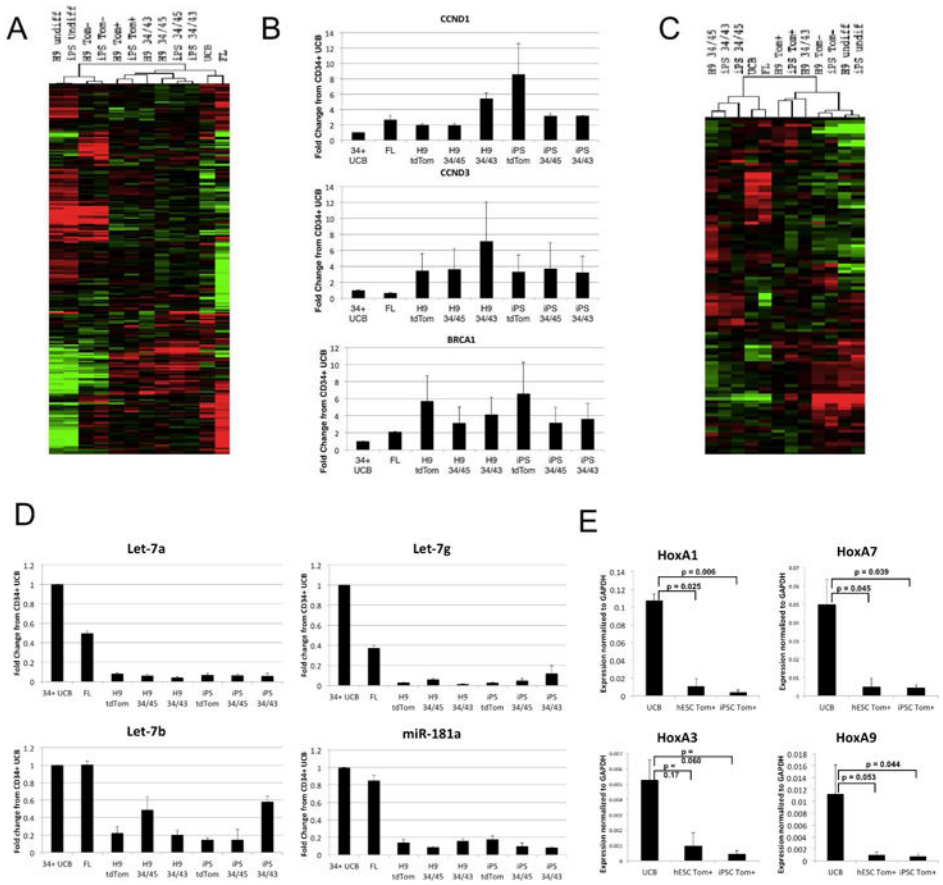

Figure 6. Gene expression analysis comparing hPSC-derived hematopoietic populations to CD34$^+$ human UCB and CD34$^+$ human fetal liver

Gene array analysis was conducted using two biological replicates for each sample. Sorted samples were collected on day 13 of differentiation. **A.** Dendrogram representation of gene array analysis from sorted hESC and iPSC populations as well as CD34$^+$ UCB cells. Similarity between samples is represented by positioning within the cluster tree. Normalization was conducted using 6 different housekeeping genes. **B.** Graphical representation of the numerical data used to make the dendogram in 6A for genes CCND1, CCND3 and BRCA1. Values are averaged and presented relative to CD34+ UCB. **C.** Dendogram analysis as in 6A but with the top 105 expressed miRNAs across all hematopoietic samples. **D.** Graphical representation of the numerical data from 6C. **E.** qPCR analysis for HOXA cluster genes in tdTomato$^+$ cells from hESC/iPSC Runx1c reporter lines as well as CD34$^+$ human UCB. Values are the average of 2–3 biological replicates with error bars representing S.E.M.