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T Lymphocyte Potential Marks the Emergence of Definitive Hematopoietic Progenitors in Human Pluripotent Stem Cell Differentiation Cultures

Marion Kennedy,[1,3] Geneve Awong,[1,2,3] Christopher M. Sturgeon,[1] Andrea Ditadi,[1] Ross LaMotte-Mohs,[2,4] Juan Carlos Zúñiga-Pflücker,[2] and Gordon Keller[1,*]
[1]McEwen Centre for Regenerative Medicine, University Health Network, Toronto, Ontario M5G 1L7, Canada
[2]Department of Immunology, University of Toronto and Sunnybrook Research Institute, Toronto, Ontario M4N 3M5, Canada
[3]These authors contributed equally to this work
[4]Present address: Wellstat Therapeutics, 930 Clopper Road, Gaithersburg, MD 20878, USA
*Correspondence: gkeller@uhnresearch.ca
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SUMMARY

The efficient generation of hematopoietic stem cells from human pluripotent stem cells is dependent on the appropriate specification of the definitive hematopoietic program during differentiation. In this study, we used T lymphocyte potential to track the onset of definitive hematopoiesis from human embryonic and induced pluripotent stem cells differentiated with specific morphogens in serum- and stromal-free cultures. We show that this program develops from a progenitor population with characteristics of hemogenic endothelium, including the expression of CD34, VE-cadherin, *GATA2*, *LMO2*, and *RUNX1*. Along with T cells, these progenitors display the capacity to generate myeloid and erythroid cells. Manipulation of Activin/Nodal signaling during early stages of differentiation revealed that development of the definitive hematopoietic progenitor population is not dependent on this pathway, distinguishing it from primitive hematopoiesis. Collectively, these findings demonstrate that it is possible to generate T lymphoid progenitors from pluripotent stem cells and that this lineage develops from a population whose emergence marks the onset of human definitive hematopoiesis.

INTRODUCTION

The ability to generate hematopoietic stem cells (HSCs) from human pluripotent stem cells (PSCs; embryonic stem cells [hESCs] and induced pluripotent stem cells [hiPSCs]) would enable the production of unlimited numbers of patient-matched stem cells for transplantation and the derivation of novel in vitro models for studying human hematopoietic development and disease. Numerous studies have shown that it is possible to derive hematopoietic lineage cells from hPSCs, either by coculturing them with stromal cells in serum-based media or by directing their differentiation with specific morphogens in defined serum-free media (Chadwick et al., 2003; Davis et al., 2008; Kaufman et al., 2001; Kennedy et al., 2007; Ledran et al., 2008; Ng et al., 2005; Pick et al., 2007; Vodyanik et al., 2006; Yu et al., 2010; Zambidis et al., 2005). Although these approaches yield a broad spectrum of hematopoietic progenitors, transplantation of the progeny from such cultures into immunocompromised mice has typically resulted in low levels of engraftment often restricted to the myeloid lineages (Lu et al., 2009; Tian et al., 2006; Wang et al., 2005). These findings suggest that the conditions used for hematopoietic differentiation do not support the development of HSCs. A major factor contributing to the failure in generating HSCs from hPSCs is the complexity of the embryonic hematopoietic system, which consists of at least two distinct programs, only one of which gives rise to HSCs.

HSCs are generated from the definitive hematopoietic program and develop from a specialized population of endothelial cells, known as hemogenic endothelium (HE; Dzierzak and Speck, 2008). In the mouse, HE is specified at different sites within the developing vasculature, of which the best characterized is the para-aortic splanchnopleura (P-Sp)/aorta-gonad-mesonephros (AGM) region found in the caudal portion of the embryo. Mouse HE is characterized by expression of a panel of hematopoietic and endothelial markers, including VE-cadherin (VE-cad), Sca-1, c-Kit, CD34, *Runx1*, *Scl*, *Gata2*, and *Lmo2* (reviewed in Dzierzak and Speck, 2008). HSCs are first detectable in the AGM region at E10.5 and are characterized by the acquisition of low CD45 expression in addition to the aforementioned set of markers (Bertrand et al., 2005; Taoudi and Medvinsky, 2007; Yokomizo and Dzierzak, 2010). The human P-Sp/AGM region is also a site of definitive hematopoiesis because it contains progenitors that express markers indicative of HE and hematopoietic development including CD31, CD34, CD45, C-KIT, *SCL*, *C-MYB*, *GATA2*, and *GATA3* (Labastie et al., 1998; Marshall et al., 1999; Oberlin et al., 2002; Tavian et al., 2001) and by gestational day 32 has in vivo multilineage repopulating capacity (Ivanovs et al., 2011).

Definitive hematopoiesis is preceded by an earlier, yolk sac (YS)-restricted program, known as primitive hematopoiesis, that is characterized by the production of primitive erythroblasts, macrophages, and megakaryocytes (reviewed in Palis et al.,

2010). Although most evidence indicates that primitive hematopoiesis is restricted in potential and does not have the capacity to generate HSCs or lymphoid cells, recent studies have shown that the YS can generate lymphoid progenitors prior to or in the absence of circulation (Rhodes et al., 2008; Yoshimoto et al., 2011, 2012). Further characterization of these YS populations, however, revealed that the lymphoid cells developed from a VE-cad$^+$CD41$^-$ HE-like progenitor, distinct from the VE-cad$^-$CD41$^+$ primitive hematopoietic progenitors (Yoshimoto et al., 2011, 2012). These findings indicate that the YS displays both primitive and definitive hematopoietic potential and that the two populations develop from distinct progenitors.

Most studies to date support the interpretation that lineage development from PSCs recapitulates lineage commitment in the embryo (Murry and Keller, 2008). Thus, the generation of HSCs from PSCs will depend on establishing culture conditions that not only promote HE development but also on methods to identify these progenitors as they are specified. In an earlier study, we used T cell potential to map the onset of definitive hematopoiesis in mouse ESC (mESC) differentiation cultures and demonstrated that this program initiates from a Flk-1$^+$ *Sox17*$^+$ progenitor that emerged 48 hr following the onset of primitive hematopoiesis (Irion et al., 2010). Because several studies have demonstrated that it is possible to generate T lymphocytes from hESCs (Galic et al., 2006; Timmermans et al., 2009), it should be possible to use a similar strategy to map the emergence of the human definitive hematopoietic program in the hPSC differentiation cultures.

In this study, we used T cell potential to monitor definitive hematopoietic development in hESC and hiPSC serum-free differentiation cultures. With this approach, we identified a hematopoietic progenitor population that emerges between days 6 and 9 of differentiation, expresses markers indicative of HE as well as early definitive hematopoietic progenitors, and displays erythroid, myeloid, and T cell potential. This progenitor can be distinguished from primitive hematopoiesis based on surface markers and by the fact that its development from mesoderm is not dependent on Activin/Nodal signaling. The characteristics of this population suggest that it represents the in vitro equivalent of the human definitive hematopoietic program and, as such, progenitors of human HSCs.

RESULTS

Serum-free and Stroma-free Hematopoietic Differentiation of hESCs

To generate progenitors of the definitive hematopoietic program under serum- and stroma-free conditions, we induced the differentiation of H1 hESCs as embryoid bodies (EBs) in chemically defined media with an optimized, stage-specific combination of BMP-4, Activin A, bFGF, and VEGF together with hematopoietic cytokines (Figure 1A). With this induction scheme, colony-forming cells were detected as early as day 6 of differentiation. Their number increased modestly over the next 3 days and then dramatically by day 11 of differentiation before declining to low levels by day 15 (Figure 1B). Most of the progenitors detected at these stages were erythroid restricted, although low numbers of both multipotential erythroid-myeloid and myeloid colony-forming cells were also present. The predominance of erythroid progenitors and their transient pattern of development suggest that this early hematopoietic population may represent human primitive hematopoiesis.

Because Activin/Nodal signaling is required for primitive hematopoietic development in mESC cultures (Nostro et al., 2008; Pearson et al., 2008), we next varied the Activin A concentration to determine if this pathway impacts hESC-derived hematopoiesis (Figure 1C). Increasing concentrations of Activin A led to a reduction in myeloid progenitors and an increase in erythroid progenitors detected in day 13 EBs. In contrast, inhibition of the pathway by addition of the Activin/Nodal inhibitor SB-431542 (SB; Inman et al., 2002) eliminated almost all erythroid progenitors (Figure 1C). These observations indicate that the development of this early erythroid progenitor population is influenced by the levels of Activin/Nodal signaling between days 2 and 4 of differentiation. Given that 0.3 ng/ml Activin A effectively induced both myeloid and erythroid progenitors, we used this concentration for the subsequent studies, unless otherwise indicated.

EBs were assayed at defined time points for the expression of CD34, CD43, CD41, and CD45, cell surface markers previously shown to be expressed on the earliest hematopoietic cells that develop in hESC-differentiation cultures (Vodyanik et al., 2006). A substantial population of CD34$^+$ cells was detected by day 6 of differentiation. This population steadily declined in size over the following 9 days and was no longer detectable by day 15 (Figure 1D). CD43$^+$ cells emerged by day 9, at which time the CD34 and CD43 expression pattern was similar to that reported by others (Timmermans et al., 2009; Vodyanik et al., 2006). The CD34$^+$CD43$^+$ population declined over time, whereas the CD34$^-$CD43$^+$ population increased. CD41$^+$ cells were present by day 9 of differentiation, whereas CD45$^+$ cells were not detected at significant levels until day 13.

Hematopoietic Potential of the CD34/CD43 Populations

Because the profile observed at day 9 of differentiation (Figure 2A) most closely resembled the stage at which Timmermans et al. (2009) identified T cell progenitors, we next analyzed the different CD34/CD43 fractions from this stage for hematopoietic potential by colony assays and surface marker expression. All erythroid, myeloid, and erythroid/myeloid progenitors segregated to the CD43$^+$ fractions (Figure 2B), confirming findings from earlier studies (Timmermans et al., 2009; Vodyanik et al., 2006). Neither P1 (CD34$^+$CD43$^-$) nor P5 (CD34$^-$CD43$^-$) contained any colony-forming cells. The majority of the erythroid colonies generated from the CD43$^+$ progenitors were small with a tight morphology and contained large nucleated cells that expressed high levels of ε-globin and very low levels of β-globin (Figures 6D and 6E), indicating that they are primitive erythroblasts.

Previous studies have shown that the coexpression of CD41a and CD235a identifies an early-developing population in hESC cultures that contains primitive erythroid and megakaryocyte progenitors (Klimchenko et al., 2009; Vodyanik et al., 2006). Flow cytometric analyses showed that CD41a and CD235a were broadly expressed on the CD43$^+$ P3 and P4 populations that also exclusively contained the erythroid progenitors. CD45 expression was restricted to small subsets of the P2 and P3

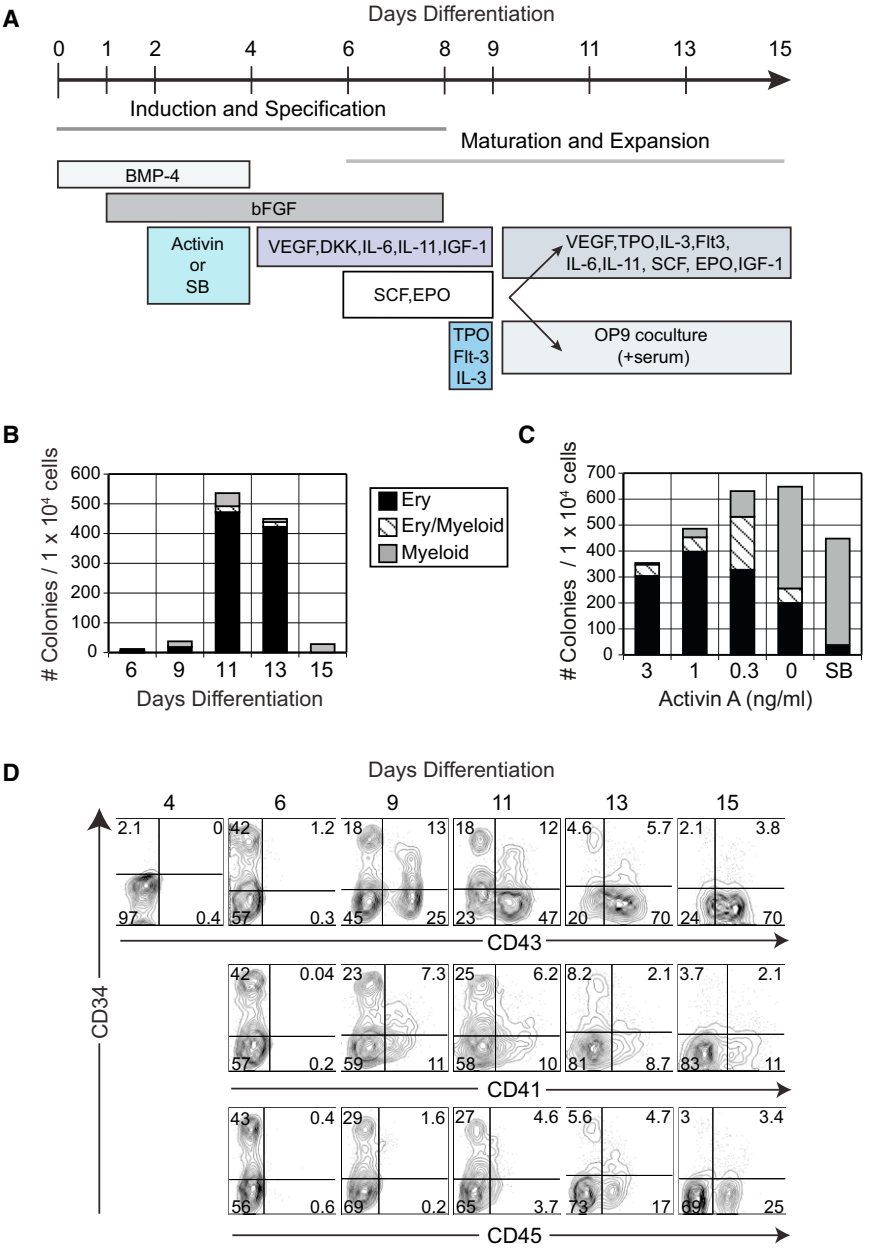

Figure 1. Hematopoietic Induction of hESCs with Activin A and BMP-4

(A) Differentiation scheme used for hematopoietic induction of human PSCs. EBs were generated during the first 24 hr of culture in the presence of BMP-4, subsequently cultured in the presence of BMP-4 and bFGF for the next 24 hr, and then in the presence of BMP-4, bFGF, and Activin A for the following 48 hr (days 2–4). For some experiments, SB was added in place of Activin A. At day 4, BMP-4 and Activin A (or SB) were removed and replaced with VEGF, IL-6, IL-11, IGF-1, SCF, EPO, TPO, Flt-3, IL-3, and DKK1 as indicated.

(B) Frequency of hematopoietic progenitors detected in EBs over time. Ery, erythroid colonies; Ery/Myeloid, colonies consisting erythrocytes and low numbers of myeloid cells; Myeloid, colonies consisting of either macrophages or mast cells.

(C) Effect of Activin A stimulation or inhibition on hematopoietic progenitor development in day 13 EBs.

(D) Flow cytometric analyses of CD34, CD43, CD41, and CD45 expression on the indicated days in EBs treated with 0.3 ng/ml Activin A plus the cytokines shown in (A).

fractions and was never coexpressed with CD235a (Figure 2C; data not shown). Cells in the P1 and P5 fractions did not express any of these markers. Taken together, these observations are consistent with those described previously and support the interpretation that the early expression of CD41a and CD235a marks the emergence of human primitive hematopoiesis.

The CD43$^+$CD41a$^+$CD235a$^+$ Populations Develop from CD34$^+$ Progenitors

We were next interested in determining if the primitive erythroid population generated under defined conditions is derived from a CD34$^+$ intermediate because a previous study has shown that the earliest hESC-derived hematopoietic cells generated in serum-induced cultures develop from a CD34$^+$ progenitor (Vodyanik et al., 2006). To address this question, we analyzed progenitors at day 6 of differentiation, a stage prior to the expansion of the CD43$^+$CD41a$^+$CD235a$^+$ primitive population. Both the CD34$^+$CD43$^-$ and the smaller CD34$^+$CD43$^+$ populations detected at this stage (Figure 2D) were sorted, reaggregated, cultured for an additional 3 days (9 days total), and then analyzed. The entire CD34$^+$CD43$^+$-derived population expressed CD43, and the majority of these cells coexpressed CD41a and CD235a (Figure 2D, lower panel). In contrast, only 50% of the CD34$^+$CD43$^-$-derived population expressed CD43, and of these, approximately 60% coexpressed CD41a and CD235a (Figure 2D, middle panel). Consistent with these differences, the CD34$^+$CD43$^+$ population generated 13-fold more progenitors than the CD34$^+$CD43$^-$ population (Figure 2E), the majority of which were primitive erythroid. The CD34$^+$CD43$^-$ population gave rise to predominantly myeloid progenitors. Taken together, these data clearly demonstrate that human primitive hematopoiesis develops from a CD34$^+$ progenitor that emerges as early as day 6 in the differentiation cultures and can be identified by coexpression of CD43.

Definitive Hematopoietic Potential of the CD34/CD43 Fractions

Quantitative RT-PCR (qRT-PCR) analyses revealed that *SOX17*, which defines the emergence of definitive hematopoiesis in the

mESC differentiation model (Irion et al., 2010), was expressed at highest levels in the P1 cells, to a lesser extent in the P2 cells, and not at all in the P3 and P4 primitive populations (Figure 2F). *LMO2* and *GATA2* were expressed in all populations, whereas *GATA1* expression was highest in the P4-derived population that contained the highest frequency of erythroid progenitors.

To further assess the definitive potential of the day 9 $CD34^+$/$CD43^+$ populations, each was assayed for T cell potential by coculturing them on OP9-DL4 (Schmitt et al., 2004). T cell progenitors were only detected in P1 (Figure 3A), consistent with the observation that these cells express the highest levels of *SOX17*. The P3 and P4 fractions failed to generate any $CD45^+$ cells following 2 weeks of culture on OP9-DL4 stroma, whereas the P2 cells gave rise to a transient $CD45^+$ population that was detectable at 2 weeks but failed to undergo T lymphopoiesis (Figure 3A). The P1 cells generated $CD5^+CD7^+$ T cell progenitors as early as day 14 of culture, $CD4^+CD8^+$ T cells by day 28 and $CD3^+$ T cells expressing either T cell receptor $\alpha\beta$ ($TCR\alpha\beta$) or $TCR\gamma\delta$ at day 42 (Figures 3B and 3C). To further characterize the extent of T cell development, we analyzed genomic DNA from the cocultured cells for the presence of TCR $D\beta2$-$J\beta2$ rearrangements. As shown in Figure S1, the hESC-derived T cells contained multiple PCR products, indicative of polyclonal $D\beta2$-$J\beta2$ rearrangements. The expression patterns of early and late T lineage differentiation markers from hESC-derived $CD34^+$/OP9-DL4 cultures described here are similar to what is typically observed from cord blood-derived HSCs (Awong et al., 2009).

Temporal analyses of EB development revealed that the $CD34^+$ population at day 6 of differentiation also contained progenitors with T cell potential (Figure 3D), as did both the $CD34^+CD43^-$ and $CD34^+CD43^{lo}$ populations at day 11 (Figure 3E). The $CD34^+CD43^{lo}$ population identified at this stage may be similar to the T progenitor population described previously by Timmermans et al. (2009). In contrast, the day 3 KDR^+ hemangioblast population did not give rise to T cells, indicating that progenitors with lymphoid potential develop sometime between days 3 and 6 of differentiation (data not shown). Together, the findings from these analyses demonstrate that at day 9 of differentiation, definitive hematopoietic progenitors, as defined by T cell potential, are restricted to a $CD34^+CD43^-$ population and distinct from the $CD43^+$ primitive hematopoietic population.

The Requirement for Activin/Nodal Signaling Distinguishes Primitive and Definitive Hematopoiesis

Because Activin/Nodal signaling is known to play a role in primitive hematopoiesis in mESC differentiation cultures (Nostro et al., 2008; Pearson et al., 2008) and our earlier findings here showed that it is required for the early wave of erythroid progenitors (Figure 1C), we were next interested in determining if we could selectively block the development of the entire $CD43^+$/$CD41a^+$/$CD235a^+$ population through appropriately staged inhibition of the pathway. When added within the first 24 hr of differentiation, the Activin/Nodal inhibitor SB completely blocked the induction of the KDR^+CD34^+ hematopoietic mesoderm population detected at day 5 of differentiation (Figure 4A), consistent with the known requirement of this pathway for primitive streak/mesoderm formation (Conlon et al., 1994). However, if addition of SB was delayed and added between days 2 and 4 of differentiation, KDR^+ and $CD34^+$ populations developed normally and were similar in size to those in the Activin A-induced population (Figure 4C). Western blot analyses showed the presence of phospho-SMAD2 in the day 2 EBs, indicating that endogenous Activin/Nodal signaling is active at this stage (Figure 4B). Densitometry confirmed that the levels of phospho-SMAD2 were significantly reduced following SB treatment, indicating that SB blocked Activin/Nodal signaling. Analyses of day 9 EBs revealed that inhibition of the pathway between days 2 and 4 completely blocked the development of the $CD43^+$ population but did not appear to impact the $CD34^+$ cells (Figure 4C). Complete inhibition of the $CD43^+$ population at day 9 was dependent on the addition of SB at day 2 of differentiation because delay until day 3 resulted in the development of some $CD43^+$ cells (Figure S2). A large $CD43^+$ population developed from the SB-treated EBs by day 12 of culture. However, in contrast to those derived from Activin A-induced EBs, these cells did not express CD41a or CD235a but did express CD45 (Figure 4C). The marker profile of the SB-treated $CD43^+$ population did not change between days 12 and 16 of culture. As expected, at day 9, the majority of the Activin A-induced population coexpressed CD41a and CD235a.

Consistent with the absence of the $CD43^+CD41a^+CD235^+$ population and with our earlier observations, erythroid progenitors were not detected in the SB-treated EBs at any of the time points assayed here (Figure 4D). qRT-PCR analyses revealed that the $CD34^+$ population isolated from the SB-treated EBs expressed higher levels of *SOX17* and *AML1C* than the $CD34^+CD43^-$ population generated in the Activin A-induced EBs (Figure 4E). Levels of expression of *LMO2*, *GATA1*, *GATA2*, and *HOXB4* were comparable in the two populations.

Similar to Activin A-induced $CD34^+$ cells, the SB-treated day 9 $CD34^+$ progenitors generated T cells when cultured on OP9-DL4 cells (Figure 5A). Interestingly, limiting dilution analyses revealed that the frequency of T cell progenitors in the SB-treated $CD34^+$ population was more than 3-fold higher than in the Activin A-induced $CD34^+CD43^-$ population (Table 1), indicating that inhibition of the primitive hematopoietic program early in the differentiation cultures coincides with an enrichment of T cell progenitors in the day 9 $CD34^+$ population. As observed with the T cells generated from the Activin A-induced progenitors, T cells derived from the SB-treated $CD34^+$ cells also displayed polyclonal $D\beta2$-$J\beta2$ rearrangements (Figure S1). By days 36–43 of coculture, the majority of cells exhibited $TCR\beta$ rearrangements as shown by the loss of the germline band.

To determine if these T cells were functional, cells cultured for 35–40 days were stimulated for 4 days with soluble α-CD3 and α-CD28 antibodies. As shown in Figure 5B, the stimulated $CD3^+$ T cells showed an increase in forward scatter compared to the control cells, reflecting an increase in size indicative of the early stages of activation (June et al., 1990). The α-CD3/α-CD28-induced cells also expressed significantly higher levels of CD25 and CD38, two markers classically upregulated on activated human T cells ($p \leq 0.01$) (Funaro et al., 1990; Schuh et al., 1998). Also, consistent with normal human T cell activation, an increase in the CD45RO isoform was observed on stimulated hESC-derived T cells (Figure 5B). These changes in the

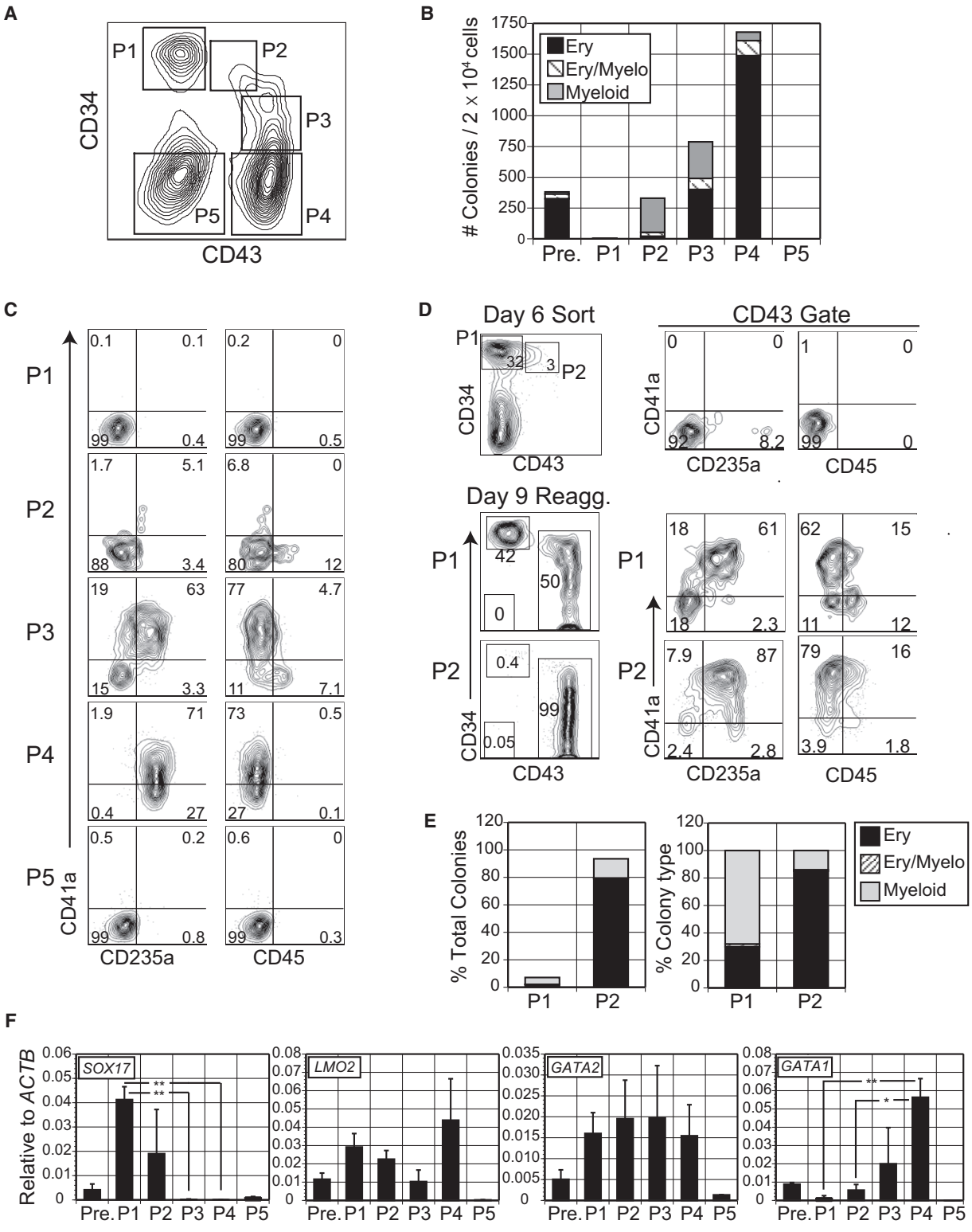

Figure 2. Primitive Hematopoietic Potential of Day 9 CD34/CD43 Populations

(A) Flow cytometric analyses showing the CD34 and CD43 fractions isolated from day 9 EBs. P1, 10% ± 3%; P2, 1.5% ± 0.7%; P3, 12.5% ± 4%; P4, 21% ± 8%; P5, 35% ± 9% (±SD, n = 5).

(B) Erythroid and myeloid progenitor potential of the CD34/CD43 fractions.

(C) Flow cytometric analyses showing expression of CD41a, CD235a, and CD45 on the fractions indicated in (A).

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expression of activation markers show that hESC-derived T cells bear a functional TCR capable of sensing and responding to stimuli. Together with the demonstration of extensive TCR rearrangements, the presence of functional TCRs suggests that hESC-derived T cells are undergoing normal maturation.

Collectively, the findings from these studies demonstrate that early-stage inhibition of the Activin/Nodal pathway blocks primitive hematopoiesis while enhancing the T cell potential of the definitive $CD34^+$ population. They also show that the $CD43^+$ population that develops in the SB-treated EBs differs from the day 9 Activin A-induced population with respect to CD41a and CD235a expression and, as such, allow us to define distinct primitive and definitive stages of CD43 development.

Hematopoietic Potential of the $CD34^+$ Population

The Activin A-induced (data not shown) and SB-treated definitive $CD34^+$ population coexpresses CD31, KDR, and VE-CAD, markers found on HE (reviewed in Tavian et al., 2010), and CD90 and CD117, markers found on $CD34^+$ cord blood-derived HSCs (Figure 6A; Doulatov et al., 2010; Notta et al., 2011). These populations did not, however, express CD45. To determine whether the $CD34^+$ populations display myeloid and erythroid potential in addition to T cell potential, the cells were cultured on OP9-DL1 stromal cells in the presence of hematopoietic cytokines. OP9-DL1 cells were used rather than the wild-type OP9 cells that are normally used for expansion of hematopoietic cells (Feugier et al., 2005) because we found that they supported the development of higher numbers of erythroid progenitors (data not shown). Following 7 days of coculture, the Activin A-induced $CD34^+$ cells generated a $CD43^+CD45^+$ population, as well as a small $CD41a^+$ population. Coexpression of CD42b on some of these $CD41a^+$ cells suggests that they represent developing megakaryocytes. The SB-treated $CD34^+$ cells gave rise to a $CD43^+CD45^+$ population (Figure 6B) that did not express significant levels of CD41a or CD235a.

The $CD34^+CD43^-$ populations also acquired erythroid and myeloid progenitor potential following 7 days of coculture (Figure 6C). Interestingly, the SB-treated cells generated significantly higher numbers of erythroid and erythroid-myeloid progenitors than the Activin A-induced progenitors, suggesting that, in addition to T cell progenitors, this population is also enriched in erythroid/myeloid potential. The erythroid progenitors generated from the coculture gave rise to colonies (Figure 6D) that were substantially larger than the day 9 $CD43^+$-derived primitive erythroid colonies. Although both colony types contained nucleated red cells (Figure 6D, lower panels), the $CD34^+$-OP9-DL1 coculture-derived colonies expressed significantly higher amounts of β-globin than the CD43-derived primitive colonies (Figure 6E). The reverse pattern was observed for ε-globin expression, although the large colonies still express considerable levels of this globin. The $CD34^+$-derived myeloid population consisted of progenitors of the macrophage, mast cell, and neutrophil lineages (Figure S3). Collectively, the findings from these coculture studies clearly demonstrate that the $CD34^+$ definitive population displays erythroid and myeloid in addition to T cell potential.

Definitive Hematopoietic Development from iPSCs

To determine if the directed differentiation approach described above can be applied to other human PSC lines, we differentiated the iPSC line MSC-iPS1 (Park et al., 2008) as in Figure 1A. As shown in Figures 7A and 7B, differentiation led to the development of expected $CD34^+/CD43^+$ and $CD34^+/CD41^+$ populations, as well as the spectrum of erythroid, erythroid/myeloid, and myeloid progenitors. The addition of SB between days 2 and 4 inhibited the development of the $CD41^+/CD43^+$ populations and the erythroid and erythroid-myeloid progenitors, as observed with the hESC line. Furthermore, the $CD34^+$ cells produced by either Activin A or SB differentiation conditions generated $CD4^+CD8^+$ T cells that coexpressed CD3 (Figure 7C) and displayed Dβ2-Jβ2 TCR rearrangement (Figure S1). Taken together, these observations demonstrate that the combination of T cell development and staged manipulation of Activin/Nodal signaling can be used to identify and enrich for definitive hematopoietic progenitors in hiPSC cultures and distinguish them from primitive hematopoietic progenitors.

DISCUSSION

The derivation of HSCs from hPSCs will require strategies that establish the developmental program that gives rise to this population in the early embryo. Insights from studies using different model organisms outline a developmental progression leading to the generation of HSCs that includes the induction of a definitive hematopoietic progenitor population known as HE and the subsequent specification of this population to a hematopoietic fate, giving rise to multipotent progenitors and engraftable cells. A major challenge in recapitulating embryonic hematopoiesis in PSC differentiation cultures is that the two hematopoietic programs are not spatially separate, and as a consequence, the predominance of primitive hematopoiesis at early stages makes it difficult to identify the definitive hematopoietic progenitors as they develop. In this study, we used T cell potential to track the onset of definitive hematopoiesis from hPSCs and, in doing so, identified a definitive hematopoietic program that can be distinguished from the primitive hematopoietic program based on developmental potential, cell surface markers, and dependency on Activin/Nodal signaling.

(D) Hematopoietic potential of day 6 $CD34^+CD43^-$ and $CD34^+CD43^+$ populations. Day 6 Sort (upper panels), flow cytometric analyses showing populations isolated for hematopoietic studies. Cells were isolated, cultured for 3 days as aggregates, and analyzed. Day 9 Reagg. (middle and lower panels), flow cytometric analyses showing expression of CD34, CD43, CD41a, CD235a, and CD45 on populations generated from indicated sorted fractions following 3 days of culture.
(E) Primitive erythroid/myeloid potential of the day 9 aggregate populations generated from the P1 and P2 day 6 fractions. Left graph indicates the proportion of total colonies generated from the entire $CD34^+$ population that were derived from the P1 and P2 fractions. Right graph shows the proportion of different types of colonies generated from the two different fractions.
(F) qRT-PCR expression analyses of the different CD34/CD43 fractions for the indicated genes. Error bars represent mean ± SD of the mean of samples from three independent experiments. Asterisks indicate statistical significance as determined by t test: $^*p \leq 0.05$ and $^{**}p \leq 0.01$.

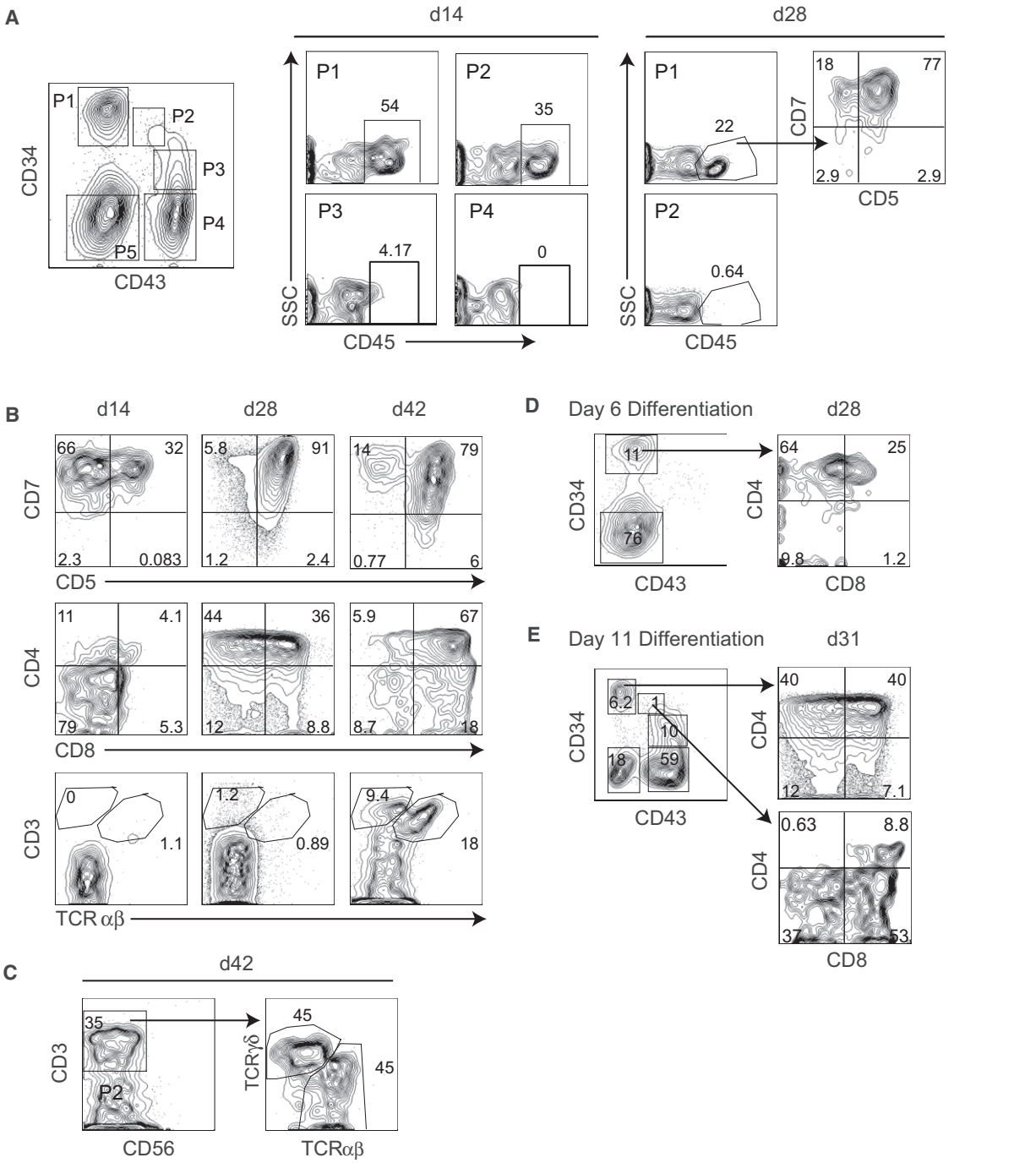

Figure 3. T Cell Potential of the CD34/CD43 Populations

(A) Flow cytometric analyses showing the proportion of CD45$^+$ cells generated from fractions P1–P4 following 14 days of coculture on OP9-DL4 and of CD7$^+$CD5$^+$ T lymphoid progenitors generated from fraction P1 at day 28 (d28) of coculture.

(B) Kinetics of T cell development from the CD34$^+$CD43$^-$ fraction. Cultures were harvested as indicated and analyzed by flow cytometry.

(C) Development of TCRαβ and TCRγδ T cells from CD34$^+$CD43$^-$ progenitors at day 42 of coculture.

(D) T cell potential of day 6 CD34$^+$CD43$^-$ progenitors. Cultures were harvested and analyzed for CD4 and CD8 expression on day 28.

(E) T cell potential of CD34$^+$CD43$^-$ and CD43$^+$CD43^{lo} progenitors isolated from day 11 EBs. Cultures were harvested and the cells analyzed on day 31.

Related to Figure S1.

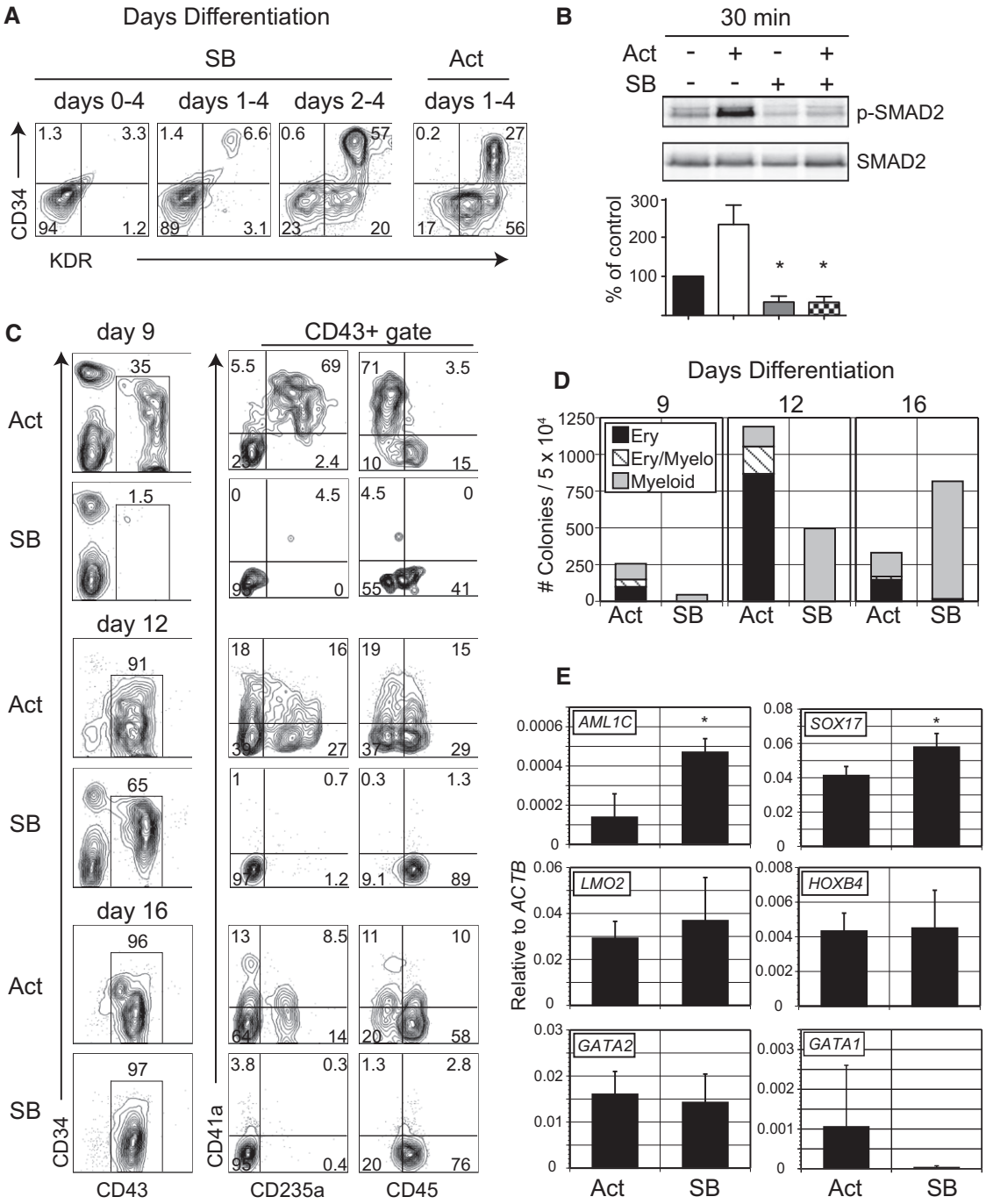

Figure 4. Potential of CD34$^+$ Progenitors Isolated from SB-Treated EBs

(A) Flow cytometric analysis showing development of CD34$^+$KDR$^+$ populations at day 5 of differentiation, following addition of SB or Activin A (Act) during the indicated times.

(B) Immunoblot analysis for the expression of Smad2 and phospho-Smad2 from cell lysates of EBs treated for 30 min at day 2 of differentiation with DMSO, Activin-A, and/or SB; densitometry was performed and depicted as a graph of phospho-SMAD2 levels as percentage (%) of control expression (DMSO).

(C) Flow cytometric analyses showing coexpression of CD41a, CD235a, and CD45 on CD43$^+$ populations in Activin A-induced and SB-treated EBs on days 9, 12, and 16 of differentiation.

(D) Progenitor potential of Activin A-induced and SB-treated EBs at the indicated days of differentiation.

(E) qRT-PCR-based expression analyses of CD34$^+$ fractions isolated from day 9 Activin A-induced and SB-treated EBs. Error bars represent mean ± SD of the mean of samples from three independent experiments. Asterisks indicate statistical significance as determined by t test: *$p \leq 0.05$.

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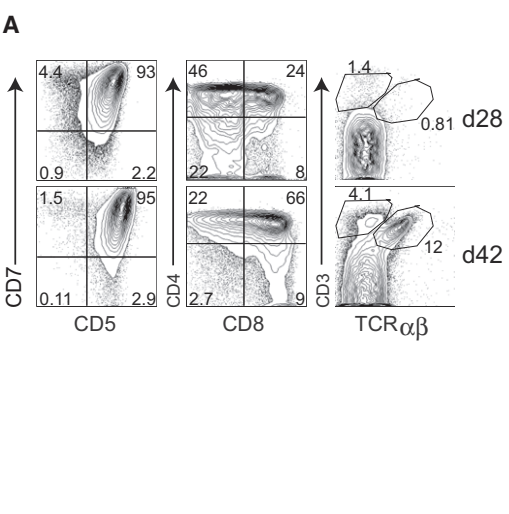

Figure 5. Lymphoid Potential of the SB-Treated CD34$^+$ Population

(A) Flow cytometric analyses of SB-treated EBs showing development of CD7$^+$CD5$^+$, CD4$^+$CD8$^+$, and CD3$^+$TCR$\alpha\beta^+$ populations at days 28 and 42 of coculture.

(B) Functional analyses of hESC-derived T cells. Flow cytometric analyses showing forward (FSC) and side (SSC) scatter parameters and expression of CD25, CD38, and CD45RO on hESC-derived T cells following 4 days of stimulation (Stim, black lines) with α-CD3 and α-CD28 antibodies. Control cells were treated with cytokines alone (Non-Stim, shaded histogram).

Related to Figure S1.

The expression profile of the definitive CD34$^+$ population, which includes the transcription factors *GATA2*, *LMO2*, *AML1C*, as well as endothelial (KDR, CD31, VE-CAD), but not hematopoietic (CD45 or CD43), surface markers, suggests that it represents the equivalent of human HE. The generation of a population with these characteristics that possesses T cell potential is unique and represents a critical first step in generating HSCs. Several other studies have described hESC-derived endothelial progenitor populations that display hematopoietic potential (Choi et al., 2012; Hong et al., 2011; Wang et al., 2004; Zambidis et al., 2005). In the most recent of these reports, Choi et al. (2012) identified a hemogenic endothelial progenitor (HEP) that appears to be distinct from the BL-CFC (hemangioblast), the progenitor of the primitive hematopoietic program. However, because lymphoid potential was not evaluated in this or any of the other studies, it is unclear if these populations represent progenitors of the definitive hematopoietic program.

Following coculture for 7 days on OP9-DL1, the CD34$^+$ population upregulates expression of CD43 and CD45 and acquires erythroid and myeloid progenitor potential, a transition that may represent the equivalent of the specification of HE to a hematopoietic fate. Our preliminary studies have shown that OP9-DL1 stroma is more efficient at promoting erythroid progenitor development than OP9 stroma, suggesting that Notch signaling may be required for this specification step. Current studies are aimed at defining the role of the Notch pathway at this stage. The CD34$^+$-derived erythroid progenitors generate large erythroid colonies that are morphologically distinct from and express significantly higher levels of β-globin than the Activin-induced CD43$^+$-derived primitive erythroid colonies. These observations combined with the fact that they develop from phenotypically and temporally different populations clearly demonstrate that these erythroid progenitors are not the same. Previous studies have described the emergence of different erythroid progenitors in serum-induced EBs over time and suggested that they represented progeny of both primitive and definitive hematopoiesis (Chadwick et al., 2003; Zambidis et al., 2005). Although the CD34$^+$-derived erythroid progenitors described here are distinct from the CD43$^+$-derived primitive progenitors, it is not clear what stage of development they represent because the cells they generate still express high levels of ε-globin. Because little is known about the different stages of human erythropoiesis in the early embryo, it is possible that the CD34$^+$-derived progenitors represent one step beyond the primitive program, a transition between primitive and definitive erythropoiesis.

With the identification of the CD34$^+$ definitive progenitors in day 9 EBs, we were able to define distinct human definitive and primitive hematopoietic populations (model; Figure 7D) that display the following characteristics. First, the primitive hematopoietic population that emerges at day 9 expresses CD43 together with CD41a and CD235a, whereas the definitive population that develops after day 9 expresses CD43 together with CD45, but not CD41a or CD235a. CD41a and CD235a are expressed at later stages in the definitive program where they are restricted to the megakaryocyte and erythroid lineages, respectively (Andersson et al., 1981; Phillips et al., 1988). Second, development of the human primitive hematopoietic program is dependent on Activin/Nodal signaling beyond day 2 of differentiation. Definitive hematopoiesis, in contrast, does not require this pathway between days 2 and 4 of differentiation. Third, both primitive hematopoiesis and definitive hematopoiesis develop from a CD34$^+$ progenitor. Coexpression of CD43$^+$ with CD34 at day 6 appears to define the onset of the primitive program because the majority of the primitive erythroid progenitors

Table 1. Progenitor Frequency Analysis of Activin A- or SB-Treated CD34$^+$ Cells

Treatment[a]	Progenitor Frequency^{-1} (95% Confidence Limits)[b]
Activin A	2,594 (2,045–3,289)
SB	739 (603–905)

[a]Sorted CD34$^+$ CD43$^-$ cells obtained from EB cultures treated with either Activin A or SB-431542 (SB) were placed in limiting numbers in wells of a 96-well plate containing OP9-DL4 cells and cultured for 16 days before harvesting for flow cytometric analysis.

[b]Individual wells were scored for the presence of T cells based on CD45$^+$CD43$^+$CD7$^+$CD5$^+$ staining. Statistical analysis was performed via the method of maximum likelihood applied to the Poisson model.

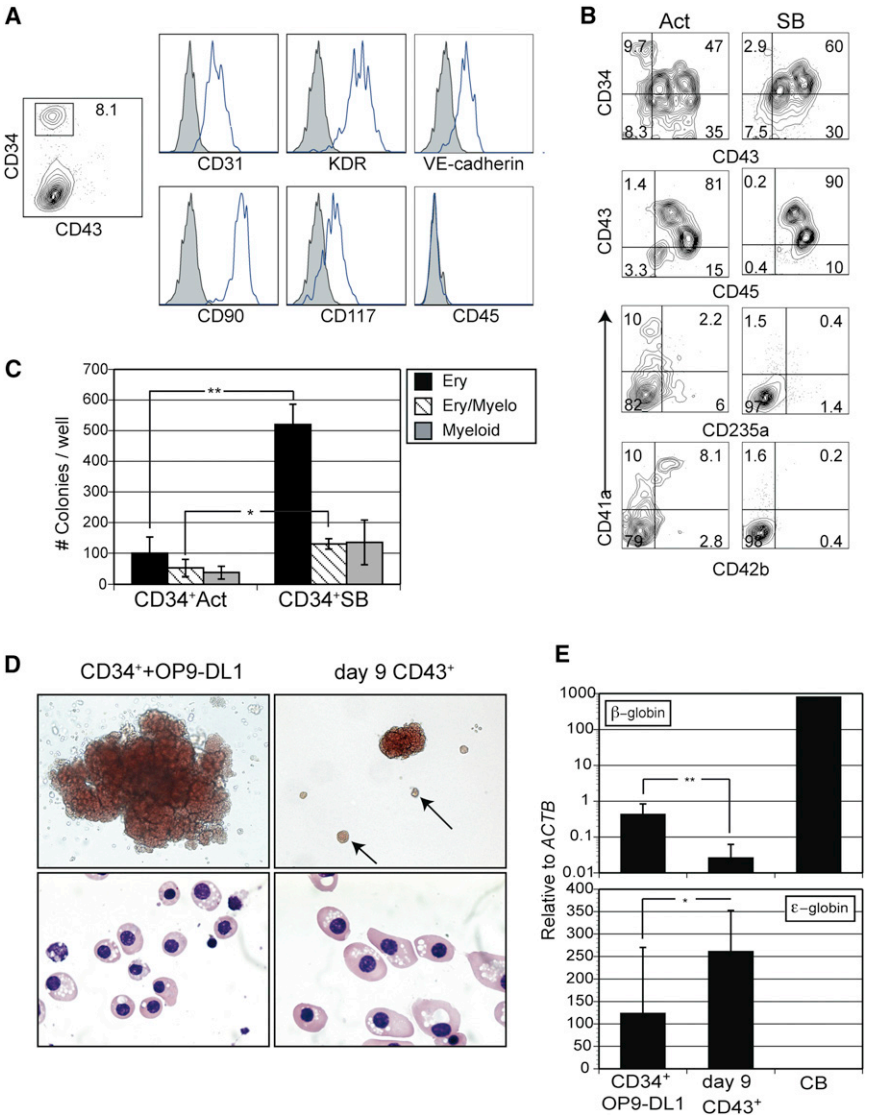

Figure 6. Hematopoietic Potential of CD34$^+$ Progenitors

(A) Flow cytometric analyses of the CD34$^+$ population from day 8 SB-treated EBs.

(B) Flow cytometric analyses of SB-treated and Activin A-induced CD34$^+$ populations following 7 days of coculture on OP9-DL1 stromal cells. Cultures were initiated with 20,000 cells of each population in a well of a 24-well plate.

(C) Progenitor potential of the two CD34$^+$ populations following 7 days of coculture. Colony numbers are calculated per well. Error bars represent SD of the mean of culture of a single experiment, representative of five independent experiments. Asterisks indicate statistical significance as determined by t test: *p $\leq$ 0.05 and **p $\leq$ 0.001.

(D) Photographs showing morphology and relative size of the colonies (and the cells from them) generated from SB-treated CD34$^+$ progenitors following 7 days of coculture and from CD43$^+$ primitive progenitors (CD43$^+$) plated directly following sorting from day 9 EB populations. Arrows indicate small, primitive erythroid-like colonies. Original magnification: colonies, ×100; cells, ×1,000.

(E) qRT-PCR analyses of β- and ε-globin expression in pools of CD34$^+$- and CD34$^-$CD43$^+$-derived erythroid colonies (CB: cord blood-derived HSC). Error bars represent SD of the mean of samples from seven or more individually isolated colonies. Asterisks indicate statistical significance as determined by t test: *p $\leq$ 0.05 and **p $\leq$ 0.01. Related to Figure S3.

are derived from this population. The fact that both programs develop from a CD34$^+$ progenitor clearly indicates that this marker is not sufficient for monitoring the development of hematopoiesis in the PSC differentiation cultures and highlights the need for identifying new surface markers that distinguish primitive and definitive progenitors at the earliest stages of development.

Manipulation of the Activin/Nodal signaling pathway with the small molecule SB in this study has provided insights into its role in the establishment of the hematopoietic system in hESC cultures, as well as into the origin of human definitive hematopoiesis. SB has been identified as a highly potent and selective inhibitor of the Activin/Nodal pathway, with no observed inhibition of other pathways or kinases, including BMP-4, ERK, JNK, or p38 MAPK (Inman et al., 2002). Our demonstration that SB reduces phospho-SMAD2 levels in day 2 EBs (Figure 4B) and that it has opposing effects to that of Activin A on erythroid colony formation (Figure 1B) provide strong evidence that its effect on the primitive erythroid lineage is mediated through inhibition of the Activin/Nodal pathway and not an unknown target. Previous studies have demonstrated that the Activin/Nodal in addition to BMP-4 and Wnt pathways all play a role in the early induction steps in hPSC cultures, including primitive streak formation, mesoderm induction, and hematopoietic specification (Davis et al., 2008; Jackson et al., 2010; Kennedy et al., 2007; Kroon et al., 2008; Sumi et al., 2008; Vijayaragavan et al., 2009). The staged addition of the Activin/Nodal pathway inhibitor in this report reinforces a role for this pathway in the early stages of development because addition between days 1 and 2 of differentiation prevented the development of any KDR$^+$ cells, indicating a lack of mesoderm formation. Additionally, our study documents a previously unidentified requirement for Activin/Nodal signaling at the earliest stages of human primitive hematopoietic development. In this regard, mouse and human primitive hematopoiesis appear to be regulated similarly because previous studies have indicated that this pathway is also required for mouse primitive hematopoietic development (Nostro et al., 2008; Pearson et al., 2008). Although our western analyses demonstrate the presence of phospho-SMAD 2 indicative of

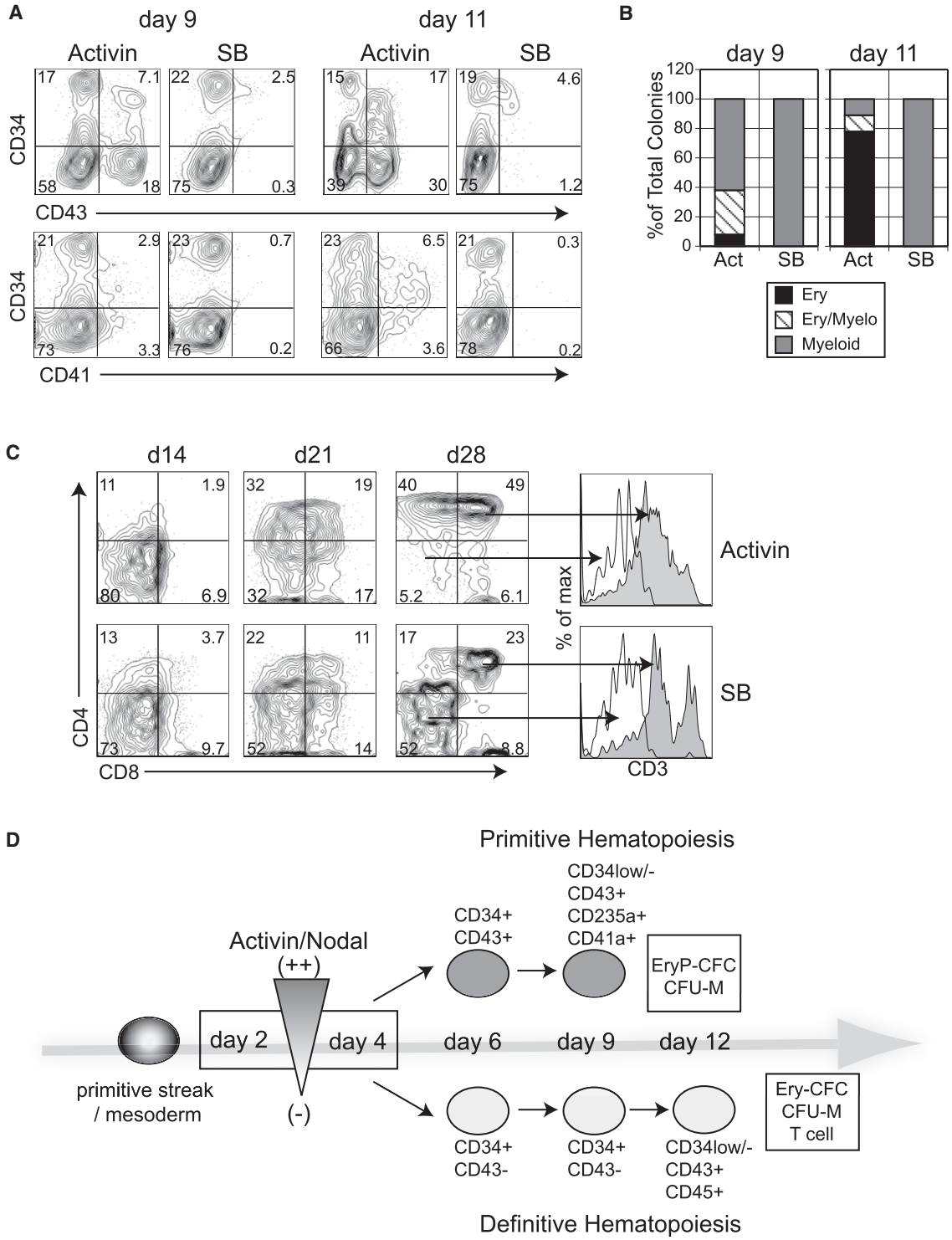

Figure 7. Hematopoietic Potential of hiPSC-Derived CD34/CD43 Populations

(A) Flow cytometric analyses of hiPSC-derived SB-treated and Activin A-induced $CD34^+$ populations.

(B) Progenitor potential of the two hiPSC $CD34^+$ populations at days 9 and 11 of differentiation.

(C) Flow cytometric analyses of SB-treated and Activin A-induced populations showing emergence of $CD4^+$ and $CD8^+$ cells at indicated times. At day 28, the $CD8^+CD4^+$ populations coexpressed CD3.

(D) Model showing emergence of primitive and definitive hematopoiesis in hESC differentiation cultures. Primitive hematopoiesis is dependent on Activin/Nodal signaling between days 2 and 4 of differentiation and develops from a $CD34^+CD43^+$ progenitor at day 6 of differentiation. The primitive hematopoietic program

(legend continued on next page)

Activin/Nodal signaling in the day 2 EBs, the interacting and/or downstream pathways mediating this effect are currently unknown. Addition of agonists and/or antagonists of other pathways including Wnt, Notch, and SHH together with SB between days 2 and 4 of differentiation had little impact on the inhibition, suggesting that they are not directly involved in mediating this effect (data not shown).

In addition to blocking primitive hematopoiesis, the addition of SB at early stages of differentiation also appeared to impact the potential of the $CD34^+$ population. The SB-treated $CD34^+$ population expressed higher levels of *SOX17* and *AML1C* and contained a higher frequency of erythroid and T cell progenitors than the corresponding Activin A-induced population. These observations clearly demonstrate that manipulation of signaling pathways at early stages of differentiation can impact the potential of later-stage cells and, as such, highlight the importance of using defined induction conditions and precise stage-specific protocols for such studies.

In summary, the findings reported here have identified a definitive hematopoietic progenitor population that displays T lymphoid, myeloid, and erythroid potential as well as surface markers and gene expression patterns indicative of a pre-HSC population. We hypothesize that the definitive progenitors identified here represent the first step in the generation of HSCs from hPSCs and, as such, provide a readily accessible target population for defining the regulatory pathways that control its specification to the earliest hematopoietic progenitors and maturation to transplantable stem cells. In addition to providing a marker for definitive hematopoiesis, the ability to generate T cells from hPSCs under defined induction conditions offers unique opportunities to investigate the developmental origins of this lineage, as well as the functional potential of the cells in in vitro and in vivo models.

EXPERIMENTAL PROCEDURES

Maintenance and Differentiation of hESCs and hiPSCs

The hESC line H1 (Thomson et al., 1998) and the reprogrammed hiPSC line (MSC-iPS1; Park et al., 2008) were used in this study. They were maintained on irradiated mouse embryonic fibroblasts in hESC media as described previously (Kennedy et al., 2007). Prior to differentiation, the cells were feeder depleted by culturing on Matrigel (BD Biosciences, Bedford, MA, USA) in hESC media for 24–48 hr. To generate EBs, hPSCs were treated with collagenase B (1 mg/ml; Roche, Indianapolis, IN, USA) for 20 min followed by a short trypsin-EDTA (0.05%) step. Cells were gently scraped with a cell scraper to form small aggregates (10–20 cells). Aggregates were resuspended in StemPro-34 (Invitrogen), supplemented with penicillin/streptomycin (10 ng/ml), L-glutamine (2 mM), ascorbic acid (1 mM), monothioglycerol (MTG, 4×10^{-4} M; Sigma-Aldrich), and transferrin (150 μg/ml). BMP-4 (10 ng/ml), bFGF (5 ng/ml), Activin A, 6 μM SB, VEGF (15 ng/ml), Dkk (150 ng/ml), IL-6 (10 ng/ml), IGF-1 (25 ng/ml), IL-11 (5 ng/ml), SCF (50 ng/ml), EPO (2 U/ml final), TPO (30 ng/ml), IL-3 (30 ng/ml), and Flt-3L (10 ng/ml) were added as indicated. Cultures were maintained in a 5% CO_2/5% O_2/90% N_2 environment for the first 8 days and then transferred to a 5% CO_2/air environment. All recombinant factors are human and were purchased from R&D Systems (Minneapolis).

OP9-DL4 Coculture for T Lineage Differentiation

OP9 cells retrovirally transduced to express Delta-like 4 (OP9-DL4) were generated and maintained in α-MEM supplemented with penicillin/streptomycin and 20% FBS (OP9 media) as previously described (La Motte-Mohs et al., 2005; Schmitt et al., 2004). A total of 5–10 × 10^4 sorted human EB-derived subsets was added to individual wells of a 6-well plate containing OP9-DL4 cells and cultured in OP9 media supplemented with rhFlt-3L (5 ng/ml) and rhIL-7 (5 ng/ml) (PeproTech, Rocky Hill, NJ, USA). rhSCF (100 ng/ml) was added for the first 8 days only. Every 5 days, cocultures were transferred onto fresh OP9-DL4 cells by vigorous pipetting and passaging through a 40 μm cell strainer to remove stromal cells.

OP9-DL1 Coculture for Erythroid/Myeloid Differentiation

Sorted cells were cultured at a concentration of 2 × 10^4 cells per well on irradiated OP9-DL1 monolayers in OP9 media with VEGF (5 ng/ml), TPO (30 ng/ml), SCF (50 ng/ml), Flt3 (10 ng/ml), IL-11 (5 ng/ml), and BMP-4 (10 ng/ml) in 24-well plates for 7 days. Cells were harvested as above.

Reaggregation Assay

Sorted populations were resuspended in day 6 media (Figure 1A) at 25 × 10^4 cells/ml, and 50 μl was added to a well in a low-cluster 96-well plate. The following day, two wells/condition were pooled and transferred into a low-cluster 24-well plate with the addition of 1 ml of media. Twenty-four hours later, day 8 media were added to the wells and moved to normoxic conditions.

T Cell Activation

SB-treated $CD34^+CD43^-$ cells were cocultured on OP9-DL4 cells for 37–40 days. At the time of stimulation, cocultures were seeded onto fresh OP9-DL4 cells in individual wells of a 12-well plate. All wells received OP9 media supplemented with 2 ng/ml rhIL-7 and rhIL-2, and stimulated wells received the addition of 5 μg/ml α-CD3 (clone *HIT3a*) and 1 μg/ml α-CD28 (clone *28.2*) mAbs. After 4 days, flow cytometry was performed.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and Extended Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.11.003.

LICENSING INFORMATION

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develops as a $CD43^+CD41a^+CD235a^+$ population that can be detected by day 9 of differentiation. Definitive hematopoiesis is not dependent on Activin/Nodal signaling between days 2 and 4 of differentiation, is specified as early as day 6 but does not expand until day 12, at which stage it is detected as a $CD43^+CD45^+$ population that does not express CD41a and CD235a. CFU-M, myeloid progenitors; EryP-CFC, primitive erythroid progenitors; Ery-CFC, erythroid progenitors. Related to Figure S1.

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