Tenascin C Promotes Hematoendothelial Development and T Lymphoid Commitment from Human Pluripotent Stem Cells in Chemically Defined Conditions

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SUMMARY

The recent identification of hemogenic endothelium (HE) in human pluripotent stem cell (hPSC) cultures presents opportunities to investigate signaling pathways that are essential for blood development from endothelium and provides an exploratory platform for de novo generation of hematopoietic stem cells (HSCs). However, the use of poorly defined human or animal components limits the utility of the current differentiation systems for studying specific growth factors required for HE induction and manufacturing clinical-grade therapeutic blood cells. Here, we identified chemically defined conditions required to produce HE from hPSCs growing in Essential 8 (E8) medium and showed that Tenascin C (TenC), an extracellular matrix protein associated with HSC niches, strongly promotes HE and definitive hematopoiesis in this system. hPSCs differentiated in chemically defined conditions undergo stages of development similar to those previously described in hPSCs cocultured on OP9 feeders, including the formation of VE-Cadherin⁺CD73⁻CD235a/CD43⁻HE and hematopoietic progenitors with myeloid and T lymphoid potential.

INTRODUCTION

In the embryo, hemogenic endothelium (HE) has been identified as an immediate direct precursor of hematopoietic progenitors and hematopoietic stem cells (HSCs) (Bertrand et al., 2010; Boisset et al., 2010; Jaffredo et al., 2000; Kissa and Herbomel, 2010; Zovein et al., 2008). Thus, the ability to produce HE from human pluripotent stem cells (hPSCs) is considered a critical step toward the de novo generation of blood progenitors and stem cells. The recent identification and characterization of HE in hPSC cultures by our lab and others have provided a platform for investigating pathways that control HE formation and subsequent HSC specification (Choi et al., 2012; Kennedy et al., 2012; Rafii et al., 2013). However, the use of xenogeneic or allogeneic feeder cells, poorly defined serum and matrix proteins, or proprietary medium and supplements of undisclosed chemical composition limits the utility of the current differentiation systems for studying factors that are essential for HE development and specification. Here, after plating hPSCs from a single-cell suspension in a completely chemically defined medium that was free of serum components and xenogeneic proteins, we identified a set of factors and matrix proteins that are capable of supporting hematopoietic differentiation. Importantly, we showed the critical role of the HSC niche matrix component Tenascin C (TenC) in supporting the development of hematoendothelial and T lymphoid cells from hPSCs.

In our previous studies (Choi et al., 2012; Vodyanik et al., 2006, 2010), we identified distinct stages of hematopoietic development following hPSC differentiation in coculture with OP9 (Figure 1). Plating hPSCs onto OP9 stromal cells induces the formation of primitive streak and mesodermal cells that can be detected based on the expression of apelin receptor (APLNR) and the absence of endothelial (CD31 and VE-cadherin [VEC]), endothelial/mesenchymal (CD73 and CD105), and hematopoietic (CD43 and CD45) cell-surface markers, i.e., by the EMH⁻lin⁻ phenotype (Choi et al., 2012; Vodyanik et al., 2010). The early EMH⁻lin⁻APLNR⁺ cells that appear in OP9 coculture on day 2 of differentiation express primitive posterior mesoderm (PM) genes (T, MIXLI, FOXF1, and GATA2) and display the APLNR⁺PDGFR⁺KDR⁺ phenotype (hereafter referred to as A⁺P⁺ cells). These cells possess mesenchymoangioblast (MB) potential, i.e., the potential to form colonies with the capacity to differentiate into mesenchymal stem cells (MSC) and endothelial cells. On day 3 of differentiation, A⁺P⁺ cells acquire blast (BL)-CFU or hemangioblast (HB) potential (Vodyanik et al., 2010). With advanced maturation, EMH⁻lin⁻APLNR⁺ mesodermal cells lose BL-CFC activity, upregulate KDR, and downregulate PDGFRα, i.e., they acquire the hematovascular mesodermal precursor (HVMP) phenotype,
EMHlin−KDRhiAPLNR+PDGRαlo− (hereafter referred to as Khi cells). Khi HVMP cells downregulate the primitive streak genes T and MIXL1, and upregulate genes associated with lateral plate and hematovascular mesoderm development (FOXF1, ETV2, and GATA2). Khi HVMPs are highly enriched in cells with the potential to form hematopoietic clusters on OP9 (Choi et al., 2012). The first endothelial cells that coexpress VEC and CD31 emerge from Khi mesodermal cells by day 4 of differentiation. The emerging VEC+ cells represent a heterogeneous population that includes CD235a/CD43/CD73+ nonhemogenic endothelial progenitors (non-HEPs) and CD235a/CD43/CD73+ hemogenic endothelial progenitors (HEPs) (Choi et al., 2012). HEPs lack hematopoietic CFC potential, but acquire it after coculture with stromal cells. The first hematopoietic cells that express CD43 emerge within the VEC+ cells on day 4–5 of differentiation. These cells express low levels of CD43 (CD43lo) and coexpress CD235a, but lack CD41a expression, i.e., they have the phenotype VEC+CD43loCD235a+. Because these cells have the capacity to form hematopoietic colonies in the presence of FGF2 and hematopoietic cytokines, as well as to form a monolayer of endothelial cells on fibronectin, we designated them as angiogenic hematopoietic progenitors (AHPs). The CD41a+ cells emerge within the CD235a+ population. These CD235a+CD41a+ cells are highly enriched in erythro-megakaryocytic progenitors and lack endothelial potential. The progenitors with broad myelolymphoid potential and the lin− CD34−CD43−CD45− phenotype can be detected in hPSC cultures shortly after the emergence of CD235a+CD41a+ cells. Acquisition of CD45 expression by lin− cells is associated with progressive myeloid commitment (Vodyanik et al., 2006). In the present work, we demonstrated that a TenC-based, chemically defined system is able to generate all mesodermal and endothelial transitional stages and myelolymphoid progenitors that we observed using the serum- and OP9 feeder-based differentiation system described above. Because our differentiation system utilizes hPSCs growing in chemically defined xenogene-free Essential 8 (E8) medium on vitronectin (VTN) (Chen et al., 2011), it provides the opportunity to produce clinical-grade endothelial and myelolymphoid progenitors from hPSCs for therapeutic purposes.

RESULTS

IMDM/F12-Based Medium Is Essential for Efficient Differentiation of hPSCs into Hematopoietic Lineages from a Single-Cell Suspension in 2D Culture

Previously, our lab developed an hPSC differentiation protocol for the efficient generation of hematopoietic progenitors using a coculture method on the mouse stromal cell
line OP9 (Vodyanik et al., 2005; Vodyanik and Slukvin, 2007). Although the OP9 system supports efficient generation of HE and multilineage hematopoietic progenitors (Figure 1), this system is very sensitive to variations in serum quality, stromal cell maintenance, and the size of the hPSC colonies and clumps used for differentiation (Choi et al., 2011; Vodyanik and Slukvin, 2007). Forming embryoid bodies (EBs) is another commonly used approach for inducing HE and hematopoietic progenitors from hPSCs (Kennedy et al., 2012; Ng et al., 2005, 2008; Wang et al., 2004). However, EB methods often rely on serum or undefined media and supplements, and also have significant drawbacks, such as asynchronous differentiation, high variability, and dependence on the initial clump size. Additionally, inconsistency in the quality of hPSCs caused by variations in the albumin batches used for hPSC maintenance may lead to variations in the efficiency of blood development.

To overcome these limitations, we set out to characterize chemically defined media and matrix proteins capable of supporting hematopoietic differentiation without serum from a single-cell suspension of H1 human embryonic stem cells (hESCs) maintained in a completely defined xenograft-free system using E8 medium on VTN (Chen et al., 2011). First, we plated hESCs as single cells and allowed them to attach for 24 hr in E8 medium supplemented with 10 μM Rho kinase inhibitor on Matrigel (MTG), VTN, or Collagen IV (ColIV) in normoxia. Then, the medium was changed to either basal growth factor-free mTeSR1, E8 (DF4S), E8 with an IMDM base (I4S), or E8 with an IMDM/F12 base (IF4S) supplemented with human recombinant BMP4, FGF2, and VEGF factors, which are commonly used to induce blood formation from hPSCs (Pick et al., 2007; Salvagiotto et al., 2011). After 4 days of differentiation, the cell cultures were evaluated for the presence of CD31+ cells, which coexpress KDR and VEC and are highly enriched in hematopoietic progenitors (Choi et al., 2012). Flow-cytometric analysis showed that the cells that differentiated on CollV-coated plates in IF4S differentiated most efficiently into CD31+ hematopoietic precursors (Figure S1 available online). Later, we found that the addition of polyvinylalcohol, nonessential amino acids (NEAA), GlutaMAX, chemically defined lipid concentrate, and monothioglycerol increased cell viability and differentiation efficiency (data not shown). The basal medium thus obtained is referred to as IF9S (IMDM/F12 plus nine supplements; see Table S1 for the complete composition of the medium). These results demonstrated that the selected medium and supplements made it possible to obtain hematopoietic cells in a chemically defined, xeno-gene-free condition on CollIV matrix from hPSCs maintained in E8 medium.

Analysis of the Molecular Signatures of Hematopoiesis-Supporting Stromal Cell Lines Identified TenC as an Extracellular Matrix that Is Uniquely Expressed in OP9 Feeders with High Hematopoiesis-Inducing Potential

Previously, we showed that OP9 is superior to S17 and MS5 stromal cell lines for inducing hematopoietic differentiation (Vodyanik et al., 2005). We also found that day 8 overgrown OP9 cultures are superior to day 4 semiconfluent OP9 cultures for inducing hematopoietic CFCs, including multipotential GEMM-CFCs. The observation that the confluence of the stromal cells has an effect on differentiation efficiency suggested that an extracellular matrix influences hematopoietic differentiation. In order to find the matrix protein(s) that is critical for the hematopoiesis-supporting activity of OP9, we performed molecular profiling of S17 and MS5 stromal cell lines with low hematopoiesis-inducing potential. In addition, we compared overgrown OP9 (day 8) with semiconfluent OP9 (day 4) monolayers. Transcriptome analysis revealed 21 genes that showed at least 3-fold higher expression in day 8 overgrown OP9 cells as compared with all other stromal cells (Figure 2A). These included genes encoding Ptn (pleiotrophin), a secreted regulator of HSC expansion and regeneration (Himburg et al., 2010); Rspa3 (R-spondin 3), an important regulator of Wnt signaling and angioblast development (Kazanskaya et al., 2008); and the extracellular matrix protein Postn (periostin), which is required for B lymphopoiesis (Siewe et al., 2011). Interestingly, one the most highly upregulated genes in overconfluent OP9 was Tnc (TenC) (Figure 2B). TenC is expressed by mesenchymal cells underlying hematopoietic clusters in the aorta-gonado-mesonephros (AGM) region and is required for intraembryonic and postnatal hematopoiesis (Marshall et al., 1999; Nakamura-Ishizu et al., 2012; Ohta et al., 1998). It is also expressed in the bone marrow stem cell niche (Nakamura-Ishizu et al., 2012). Because of these unique properties, we tested whether TenC could support hematopoietic differentiation more effectively than CollIV.

TenC Facilitates the Development of Mesoderm and Hematopoietic Precursors in Chemically Defined Cultures following Stage-Specific Treatment of FGF2, BMP4, Activin A, LiCl, VEGF, and Hematopoietic Cytokines

In previous studies, we identified the major stages of hematopoietic development from hPSCs using the OP9 coculture system (Figure 1; Choi et al., 2012; Slukvin, 2013; Vodyanik et al., 2005, 2006, 2010). In order to reproduce the hematopoietic development observed in OP9 coculture, we searched for the optimal combinations of morphogens, growth factors, and extracellular matrices to facilitate the stepwise progression of hPSC differentiation
toward mesoderm, HE, and blood cells in chemically defined conditions.

During embryonic development, BMP4, Wnt, and TGFβ/Nodal/Activin A signaling pathways are critical for initiating primitive streak formation and subsequent mesoderm development (Gadue et al., 2005; Keller, 2005). It has been shown that the activation of these signaling pathways is essential to induce the expression of brachyury (T) and KDR (Flk-1, VEGFR2), and initiate mesodermal commitment of mouse PSCs and hPSCs (Cerdan et al., 2012; Kennedy et al., 2007; Nostro et al., 2008; Pearson et al., 2008; Pick et al., 2007; Salvagiotto et al., 2011). We found that high concentrations of BMP4 (50 ng/ml) combined with low concentrations of Activin A (15 ng/ml) and a supplement of LiCl (2 mM) consistently induced expression of the mesodermal surface markers APLNR, and PDGFRβ expression on day 4 of differentiation (Figures 3A and 3C), similar to what was observed for A+P+ mesodermal cells obtained from day 2 hPSCs differentiated in OP9 coculture (Vodyanik et al., 2010). After 2 days of differentiation, we found that only FGF2 and VEGF were sufficient for induction of endothelial progenitors, we supplemented our cultures with SCF, TPO, IL-6, and IL-3 hematopoietic cytokines in addition to VEGF and FGF2, starting from day 4 of differentiation. Although we noticed that the continuous treatment of cultures with FGF2 and VEGF was sufficient for induction of endothelial progenitors and hematopoietic specification, the addition of hematopoietic cytokines was essential to increase the output of these cells in chemically defined cultures. On day 5 of differentiation in these conditions, we observed the three major subsets of the VEC+ population as
identified in a previous study (Choi et al., 2012): VEC^+CD235a^+CD43^-CD73^+ (non-HEPs), VEC^+CD235a^-CD43^-CD73^- (HEPs), and VEC^+CD43^-CD235a^- (AHPs) (Figures 4A and 4B). When these subsets were sorted and plated in endothelial conditions, they all formed a monolayer of VEC-expressing cells with the capacity to uptake AclDL and form vascular tubes in the tube formation assay, consistent with OP9 coculture (Figure 4C). However, hematopoietic CFC potential was mostly restricted to the VEC^+CD43^-CD235a^- cells (Figures 4A and 4B). When these subsets were sorted and cultured on OP9 as previously described (Choi et al., 2012). In these conditions, hematopoietic CFC potential was significantly greater in cultures on TenC compared with those on ColIV (Figure 5D).

Tenascin C is superior to ColIV for supporting hematopoietic differentiation from a variety of hPSC lines

Although we developed the differentiation protocol using H1 hESCs, we found that the chemically defined conditions described here also supported the formation of HE and blood progenitors from another hESC line (H9) and human induced pluripotent stem cells (hiPSCs) generated from fibroblasts or bone marrow mononuclear cells (Figure S5). Previously, we demonstrated that hiPSCs obtained through reprogramming of bone marrow mononuclear cell (BM) hiPSCs differentiated less efficiently into blood cells on OP9 feeders compared with fibroblast-derived (FB) hiPSCs (Hu et al., 2011). We reproduced that finding when we differentiated BM and FB iPSCs on ColIV. However, differentiation on TenC restored the hematopoietic differentiation potential of BM hiPSCs to the level seen with hESCs and FB hiPSCs (Figure S5), thereby confirming that TenC is superior to ColIV for promoting hematopoietic differentiation from hPSCs.

TenC uniquely supports specification of T lymphoid progenitors from hPSCs

To find out whether our culture system supports the establishment of the definitive hematopoietic program from hPSCs, we analyzed the T cell potential of blood cells generated in our system as an indicator of definitive hematopoiesis (Kennedy et al., 2012). When we collected CD43^+ floating cells from day 9 differentiated cultures and plated them onto OP9 expressing DLL4 (OP9-DLL4), CD7^+CD5^+ lymphoid progenitors began to emerge by week 2 of coculture. By week 3, CD4^+CD8^+ double-positive T cells arose (Figure 6A). Interestingly, CD43^+ cells generated on both ColIV and TenC had the capacity to generate CD5^-CD7^- lymphoid progenitors, although CD43^+ cells generated on ColIV had a significantly lower potential. However, progression toward CD4^-CD8^- T lymphoid cells was consistently observed only from CD43^+ cells generated on TenC, and not from ColIV cultures (Figure 6B). To confirm T cell development, we analyzed the genomic DNA of the hematopoietic cells from OP9-DLL4 cultures for the presence of T cell receptor (TCR) rearrangements. This analysis demonstrated the presence of multiple PCR products of random V-J and D-J rearrangements at the β locus and V-J rearrangements at the γ locus, indicative of a polyclonal T lineage repertoire (Figures 6C and 6D). Overall, these findings signify that the extracellular matrix protein TenC is essential for supporting the generation of hematopoietic cells with myeloid and lymphoid potential from hPSCs in chemically defined conditions. However, we failed to obtain engraftment following transplantation of TenC differentiated cells in immunocompromised mice (data not shown), which suggests that additional
Figure 3. Mesodermal Development from H1 hESCs in Chemically Defined Conditions on ColIV and TenC

Cultures differentiated on ColIV versus TenC for 2, 3, and 4 days in chemically defined conditions.

(A and B) Flow-cytometry plots (A) and graphs (B) comparing the percentage of A+P+ primitive mesodermal population on days 2 and 3.

(C) Expression of mesoderm lineage genes measured by quantitative PCR and normalized to RPL13A, comparing day 3 P+ cells and day 4 Khi cells.

(D) Comparison of the MB/HB colony-forming potential of day 2, day 3, and day 4 cultures.

(E and F) Flow-cytometry plots (E) and graphs (F) comparing the percentage of KDRhiCD31+ (Khi) HVMP, CD31+, and KDRloCD31+ (Klo) populations on day 4 of differentiation.

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maturation signals are required to activate the self-renewal program in hematoendothelial progenitors generated in our system.

**DISCUSSION**

During the last decade, significant progress has been made in achieving hematopoietic differentiation from hPSCs. Multiple protocols for hematopoietic differentiation have been developed and have made it possible to routinely produce blood cells for experimentation. However, generating HSCs with long-term reconstitution potential from hPSCs remains a significant challenge. Hematopoietic cells and HSCs arise from a specific subset of endothelium (HE) in the embryo (Bertrand et al., 2010; Boisset et al., 2010; Jaffredo et al., 2000; Kissa and Herbomel, 2010; Zovein et al., 2008). Therefore, the ability to interrogate the signaling pathways that induce HE specification and the endothelial-to-hematopoietic transition in a completely chemically defined environment is essential in order to identify the factors required for HSC specification. Although the original protocols for hematopoietic differentiation employed xenogeneic feeder cells and/or serum, several serum- and feeder-free systems for hematopoietic differentiation have been described recently (Ng et al., 2008; Salvagiotto et al., 2011; Smith et al., 2013; Wang et al., 2012). However, these protocols still require serum components (albumin) and it remains unclear whether these protocols reproduce the distinct waves of...
hematopoiesis, including the generation of HE with definitive lymphomyeloid potential, observed in the original differentiation systems. Recently, Kennedy et al. (2012) developed a feeder- and stroma-free condition for EB-based hematopoietic differentiation in a proprietary medium with undisclosed nutrient supplements from hPSCs expanded on mouse embryonic fibroblasts. These conditions reproduced primitive and definitive waves of hematopoiesis and generated HE with T lymphoid potential. Here, we developed a protocol that enables the efficient production of blood cells in completely chemically defined conditions, free of serum and xenogeneic proteins, from a single-cell suspension of hPSCs maintained in chemically defined E8 medium (Chen et al., 2011). Our protocol eliminates the variability associated with animal- or human-sourced albumins, xenogenic matrices, clump size variation, and asynchronous differentiation observed in EB systems. It also reproduces the typical waves of hematopoiesis, including the formation of HE and definitive hematopoietic progenitors, observed in hPSCs differentiated on OP9. Importantly, based on molecular profiling of OP9 and stromal cell lines with different hematopoiesis-inducing activity, we found that the TenC matrix protein, which is uniquely expressed in OP9 with robust hematopoietic potential, strongly promotes hematopoietoendothelial and T lymphoid development from hPSCs. TenC is a disulfide-linked hexameric glycoprotein that is mainly expressed during embryonic development. Although TenC mostly disappears in adult organisms, its expression is upregulated during wound repair, neovascularization, neoplasia (Hsia and Schwarzbauer, 2005), and limb regeneration (Stewart et al., 2013). TenC is found in adult bone marrow, where it is expressed predominantly in the bone marrow region (Klein et al., 1993; Soini et al., 1993). TenC supports the proliferation of bone marrow hematopoietic cells (Seiffert et al., 1998) and erythropoiesis (Seki et al., 2006). TenC-deficient mice were shown to have lower bone marrow CFC potential (Ohta et al., 1998), failed to reconstitute hematopoiesis after bone marrow ablation, and showed a reduced ability to support engraftment of wild-type HSCs (Nakamura-Ishizu et al., 2012). In addition, TenC is expressed in the thymus (Hemesath and Stefansson, 1994) and plays an important role in T cell development, as evidenced by decreased T lymphoid progenitors in the

**Figure 5. Major Subsets of CD43+ Cells Generated after 8 Days of Differentiation of H1 hESCs in Chemically Defined Conditions on ColIV and TenC**

(A) Flow-cytometry analysis shows major subsets of CD43+ cells generated in cultures on ColIV and TenC. Lower dot plots show CD43+ gated cells.

(B) Cultures on TenC produce more CD43+ cells.

(C) Hematopoietic CFC potential is limited to the CD43+ subpopulations.

(D) Cultures differentiated on TenC produce more CFCs than cultures differentiated on ColIV.

In (B)–(D), error bars are mean ± SE from at least three experiments (*p < 0.01). See also Figure S5.
thymus and an increased proportion of T cells in the bone marrow of TenC-deficient mice (Ellis et al., 2013). Interestingly, high levels of TenC expression were also detected in the human and chicken AGM region (Anstrom and Tucker, 1996; Marshall et al., 1999), the site where the first HSCs emerge, and in hematopoietic sites of human fetal liver (Papadopoulos et al., 2004). Because TenC expression is highly enriched in the subaortic mesenchyme directly underneath hematopoietic clusters, it was suggested that TenC plays a pivotal role in HSC development during embryogenesis (Marshall et al., 1999). TenC is also involved in the regulation of angiogenesis and cardiac endothelial progenitors (Ballard et al., 2006). Our studies demonstrated the superior properties of TenC for promoting hematopoietical development from hPSCs. The positive effect of TenC was obvious at all stages of differentiation, including the enhancement of hematopoietic mesoderm, HE, and CD43* hematopoietic progenitors. Importantly, TenC was able to support the development of definitive hematopoietic cells with T lymphoid potential, whereas we were not able to obtain such cells in cultures on ColIV. The TenC molecule is composed of an amino-terminal oligomerization region followed by heptad repeats, EGF-like and fibronectin type III repeats, and a fibrinogen globule (Hsia and Schwarzbauer, 2005). Each of these domains interacts with different surface receptors, including integrins α9β1, αvβ3, and αvβ6, and toll-like receptor 4 (TLR-4) (Midwood et al., 2011). It is believed that the effect and interaction of TenC with cells requires the integrated action of multiple domains (Fischer et al., 1997), although several unique mitogenic domains capable of inducing the proliferation of hematopoietic cells were identified within this molecule (Seifert et al., 1998). The interaction of TenC with α9β1 integrin plays a central role in TenC-mediated expansion of hematopoietic stem and progenitor cells (Nakamura-Ishizu et al., 2012) and may be required for normal T cell development (Ellis et al., 2013). Several signaling mechanisms implicated in cell interaction with TenC have been identified, including the suppression of fibronectin-activated focal adhesion kinase signaling, Rho-mediated kinase signaling, and stimulation of Wnt signaling pathways (reviewed in Orend, 2005). Further studies to identify the mechanism of TenC signaling on hPSCs and their hematopoietic derivatives would help elucidate the role of this matrix protein during development. It is also important to determine the developmental stages that are most affected by TenC and clarify whether TenC simply enhances the hematopoietical commitment.

In summary, the findings presented here identify the TenC matrix protein, as well as completely chemically defined conditions that are free of serum/serum components and animal proteins and are capable of supporting the scalable production of HE and definitive blood cells from hPSCs. This differentiation system will enable the precise interrogation of signaling molecules implicated in hematopoietic differentiation and provides a platform for producing cGMP-grade blood cells for clinical application.

Figure 6. T Cell Potential of Hematopoietic Cells Collected from H1 hESC Cultures Differentiated for 9 Days in Chemically Defined Conditions on either ColIV or TenC (A and B) Flow-cytometry analysis (A) and percentages (B) of cells collected under ColIV or TenC conditions after culture on OP9-DLL4 for 3 weeks. Error bars are mean + SE from at least three experiments (*p < 0.01). (C and D) Analysis for TCR rearrangement by genomic PCR. H1 T cells are T cells derived from differentiating H1 hESCs on TenC. PB control is peripheral blood (positive control), and H1 hESCs are undifferentiated H1 hESCs (negative control).
EXPERIMENTAL PROCEDURES

hPSC Maintenance
hPSCs, WA01 (H1) and WA09 (H9) hESCs, the DF19-9-7T human fibroblast iPSC line, and the IISH2i-BM9 bone marrow-derived iPSC line (WiCell, Madison, WI) were maintained on VTN or MTG in E8 medium supplemented with FG2 and TGFβ (Peprotech). Cells were passaged when they reached 80% confluency using 0.5 mM EDTA in PBS. The cells were maintained in normoxic conditions with 5% CO2.

hPSC Differentiation
Single-cell suspensions of hPSCs were obtained by treating the hPSC cultures at 80% confluency with 1× TrypLE (Life Technologies). Single cells were plated at an optimized density ranging from 5,000 cells/cm² to 15,000 cells/cm² (depending on the cell line) onto six-well plates coated with 0.5 µg/cm² of CollIV (Sigma-Aldrich) or 0.5 µg/cm² TenC (Millipore) in E8 medium supplemented with 10 µM Rho kinase inhibitor (Tocris Y-27632). After 24 hr (day 0), the medium was changed to IF9S medium (Table S1 for the complete composition of the medium) supplemented with 50 ng/ml BMP4 (Peprotech), 15 ng/ml Activin A (Peprotech), 50 ng/ml FGF2 (Milenyi Biotech), 2 mM LiCl (Sigma), and, on occasion, 1 µM Rho kinase inhibitor to increase cell viability. On day 2, the medium was changed to IF9S medium supplemented with 50 ng/ml FGF2 and 50 ng/ml VEGF. On day 4, the medium was changed to IF9S medium supplemented with 50 ng/ml FGF2, VEGF, TPO, SCF, IL-6, and 10 ng/ml IL-3. On day 6, additional IF9S medium supplemented with the same six factors were added to the cultures without aspirating the old medium. IF9S (IMDM/F12 with nine supplements) was made in-house with the following components: 50% IMDM and 50% F12 (Life Technologies) supplemented with 64 µg/l L-ascorbic acid 2-phosphate Mg2+ salt (Sigma-Aldrich), 40 µl/l monothioglycerol (Sigma-Aldrich), 8.4 µg/l additional sodium selenite (Sigma-Aldrich), 10 g/l polyvinyl alcohol (Sigma-Aldrich), 1× Glutamax (Life Technologies), 1× nonessential amino acids (Life Technologies), 0.1× chemically defined lipid concentrate (Life Technologies), 10.6 mg/l Holo-Transferrin (Sigma-Aldrich), and 20 mg/l insulin (Sigma-Aldrich). Differentiation was conducted in a hypoxic condition from day 0 to day 5, and then in a normoxic condition from day 6 to day 9 (Figure 1). The 1× TrypLE was used to dissociate and collect cells for analysis.

MB, HB, and Hematopoietic CFC Assays
MB and HB were detected as described previously (Vodyanik et al., 2010). Hematopoietic CFCs were detected using serum-containing H4436 Methocult (Stem Cell Technologies) or serum-free H4236 Methocult with added FGF2 (20 ng/ml), SCF (20 ng/ml), IL-3 (10 ng/ml), IL-6 (10 ng/ml), and EPO (2 U/ml) as described previously (Choi et al., 2012).

Assessment of the Hematoendothelial Potential of Differentiated hPSCs
Sorted day 4 or day 5 cultures were plated on a confluent layer of OP9 cells in α-MEM (GIBCO) supplemented with 10% FBS (HyClone) supplemented with 100 µM monothioglycerol, 50 µg/ml ascorbic acid, 50 ng/ml SCF, TPO, IL-6, and 10 ng/ml IL-3 at a density of 5,000 cells/well of a six-well plate as described previously (Choi et al., 2012). Cultures were analyzed 4–7 days later by immunofluorescent staining or by flow cytometry (Choi et al., 2012).

T Cell Differentiation of Day 9 Cultures
The OP9 cell line expressing human DLL4 (OP9-DLL4) was established by using lentivirus expressing human DLL4 under the EF1α promoter. After hPSC differentiation for 9 days, the floating CD43+ cells were collected; strained through a 70 µm cell strainer (BD Biosciences); resuspended in T cell differentiation medium consisting of α-MEM (GIBCO) supplemented with 20% FBS (HyClone), IL7 (5 ng/ml), FLT3L (5 ng/ml), and SCF (10 ng/ml); and cultured on OP9-DLL4. After 4 days, the cells were harvested using a collagenase IV (GIBCO) solution (1 mg/ml in DMEM/F12; GIBCO) and 1× TrypLE (Life Technologies), and passed onto a fresh layer of OP9-DLL4. After 3 days, the cells were passaged again. Subsequent passages were conducted every 7 days for up to 4 weeks, and then floating cells were collected for flow analysis and genomic DNA extraction for TCR rearrangement assay.

Statistical Analysis
Statistical analysis was performed using Microsoft Excel. Data obtained from multiple experiments were reported as the mean ± SE. A two-tailed Student’s t test was used to compare two groups. Differences were considered significant when p < 0.01.

ACCESSION NUMBERS
The microarray data reported in this work have been deposited in the Gene Expression Omnibus under accession number GSE61580.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.09.014.

AUTHOR CONTRIBUTIONS
G.U. designed, conducted, and analyzed experiments; interpreted experimental data; made figures; and contributed to writing of the paper. D.T. conducted and analyzed experiments. J.L. generated experimental data; made figures; and contributed to writing of the paper. A.K. performed T cell differentiation. J.T. contributed to concept development and directed molecular profiling studies. I.S. developed the concept, led and supervised all aspects of the studies, analyzed and interpreted data, and wrote the paper.

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REFERENCES

Anstrom, K.K., and Tucker, R.P. (1996). Tenascin-C lines the migratory pathways of avian primordial germ cells and hematopoietic progenitor cells. Dev. Dyn. 206, 437–446.

Ballard, V.L., Sharma, A., Duignan, I., Holm, J.M., Chin, A., Choi, R., Hajjar, K.A., Wong, S.C., and Edelberg, J.M. (2006). Vascular tenascin-C regulates cardiac endothelial phenotype and neovascularization. FASEB J. 20, 717–719.

Bertrand, J.Y., Chi, N.C., Santos, B., Teng, S., Stainier, D.Y., and Traver, D. (2010). Haematopoietic stem cells derive directly from aortic endothelium during development. Nature 464, 108–111.

Boisset, J.C., van Cappellen, W., Andrieu-Soler, C., Galjart, N., Dzierzak, E., and Roblin, C. (2010). In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature 464, 116–120.

Cerdan, C., McIntyre, B.A., Mechael, R., Levadoux-Martin, M., Yang, J., Lee, J.B., and Bhatia, M. (2012). Activin A promotes hematopoietic fate development through upregulation of brachyury in human embryonic stem cells. Stem Cells Dev. 21, 2866–2877.

Chen, G., Gulbranson, D.R., Hou, Z., Bolin, J.M., Ruotti, V., Probasco, M.D., Smuga-Otto, K., Howden, S.E., Diol, N.R., Propson, N.E., et al. (2011). Chemically defined conditions for human iPSC derivation and culture. Nat. Methods 8, 424–429.

Choi, K.D., Vodyanik, M., and Slukvin, I.I. (2011). Hematopoietic differentiation and production of mature myeloid cells from human pluripotent stem cells. Nat. Protoc. 6, 296–313.

Choi, K.D., Vodyanik, M.A., Togaratti, P.P., Suknuntha, K., Kumar, A., Samarjeet, F., Probasco, M.D., Tian, S., Stewart, R., Thomson, J.A., and Slukvin, I.I. (2012). Identification of the hemogenic endothelial progenitor and its direct precursor in human pluripotent stem cell differentiation cultures. Cell Rep 2, 553–567.

Ellis, S.L., Heazlewood, S.Y., Williams, B., Reitsma, A.J., Grassinger, J., Bour, J., Heazlewood, C.K., Chidgey, A.P., and Nilsson, S.K. (2013). The role of Tenasin C in the lymphoid progenitor cell niche. Exp. Hematol. 41, 1050–1061.

Fischer, D., Brown-Lüdi, M., Schulltess, T., and Chiqut-Ehrismann, R. (1997). Concerted action of tenasin-C domains in cell adhesion, anti-adhesion and promotion of neurite outgrowth. J. Cell Sci. 110, 1513–1522.

Gadue, P., Huber, T.L., Nostro, M.C., Kattman, S., and Keller, G.M. (2005). Germ layer induction from embryonic stem cells. Exp. Hematol. 33, 955–964.

Hemesath, T.J., and Stefansson, K. (1994). Expression of tenascin in thymus and thymic nonlymphoid cells. J. Immunol. 152, 422–428.

Himburg, H.A., Muramoto, G.G., Daher, P., Meadows, S.K., Russell, J.L., Doan, P., Chi, J.T., Salt, A.B., Lento, W.E., Reyta, T., et al. (2010). Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. Nat. Med. 16, 475–482.

Hsia, H.C., and Schwarzauer, J.E. (2005). Meet the tenascins: multifunctional and mysterious. J. Biol. Chem. 280, 26641–26644.

Hu, K., Yu, J., Suknuntha, K., Tian, S., Montgomery, K., Choi, K.D., Stewart, R., Thomson, J.A., and Slukvin, I.I. (2011). Efficient generation of transgene-free induced pluripotent stem cells from normal and neoplastic bone marrow and cord blood mononuclear cells. Blood 117, e109–e119.

Jaffredo, T., Gautier, R., Brajeul, V., and Dieterlen-Liévre, F. (2000). Tracing the progeny of the aortic hemangioblast in the avian embryo. Dev. Biol. 224, 204–214.

Kazanskaya, O., Ohkawara, B., Herout, M., Wu, W., Maltry, N., Augustin, H.G., and Niehrs, C. (2008). The Wnt signaling regulator R-spondin 3 promotes angioblast and vascular development. Development 135, 3655–3664.

Keller, G. (2005). Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes Dev. 19, 1129–1155.

Kennedy, M., D’Souza, S.L., Lynch-Kattman, M., Schwartz, S., and Keller, G. (2007). Development of the hemangioblast defines the onset of hematopoiesis in human ES cell differentiation cultures. Blood 109, 2679–2687.

Kennedy, M., Awong, G., Sturgeon, C.M., Ditadi, A., LaMotte-Mohs, R., Zúñiga-Pflücker, J.C., and Keller, G. (2012). T lymphocyte potential marks the emergence of definitive hematopoietic progenitors in human pluripotent stem cell differentiation cultures. Cell Rep 2, 1722–1735.

Kissa, K., and Herbold, P. (2010). Blood stem cells emerge from aortic endothelium by a novel type of cell transition. Nature 464, 112–115.

Klein, G., Beck, S., and Müller, C.A. (1993). Tenasin is a cytoadhesive extracellular matrix component of the human hematopoietic microenvironment. J. Cell Biol. 123, 1027–1035.

Marshall, C.J., Moore, R.L., Thorogood, P., Brickell, P.M., Kinnon, C., and Thrasher, A.J. (1999). Detailed characterization of the human aorta-gonad-mesonephros region reveals morphological polarity resembling a hematopoietic stromal layer. Dev. Dyn. 215, 139–147.

Midwood, K.S., Hussenet, T., Langlois, B., and Orend, G. (2011). Advances in tenasin-C biology. Cell. Mol. Life Sci. 68, 3175–3199.

Nakamura-Ishizu, A., Okuno, Y., Omatsu, Y., Okabe, K., Morimoto, J., Uede, T., Nagasawa, T., Suda, T., and Kubota, Y. (2012). Extracellular matrix protein tenasin-C is required in the bone marrow microenvironment primed for hematopoietic regeneration. Blood 119, 5429–5437.

Ng, E.S., Davis, R.P., Azzola, L., Stanley, E.G., and Elefanty, A.G. (2005). Forced aggregation of defined numbers of human...
embryonic stem cells into embryoid bodies fosters robust, reproducible hematopoietic differentiation. Blood 106, 1601–1603.

Ng, E.S., Davis, R., Stanley, E.G., and Elefanty, A.G. (2008). A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. Nat. Protoc. 3, 768–776.

Nostro, M.C., Cheng, X., Keller, G.M., and Gadue, P. (2008). Wnt, activin, and BMP signaling regulate distinct stages in the developmental pathway from embryonic stem cells to blood. Cell Stem Cell 2, 60–71.

Ohta, M., Sakai, T., Saga, Y., Aizawa, S., and Saito, M. (1998). Suppression of hematopoietic activity in tenasin-C-deficient mice. Blood 91, 4074–4083.

Orend, G. (2005). Potential oncogenic action of tenasin-C in tumorigenesis. Int. J. Biochem. Cell Biol. 37, 1066–1083.

Papadopoulos, N., Simopoulos, C., Sigalas, J., Kotini, A., Cheva, A., and Tamiolakas, D. (2004). Induction of hepatic hematopoiesis with tenasin-C expression during the second trimester of development. Eur. J. Obstet. Gynecol. Reprod. Biol. 113, 56–60.

Pearson, S., Sroczyńska, P., Lacaud, G., and Kouskoff, V. (2008). The stepwise specification of embryonic stem cells to hematopoietic fate is driven by sequential exposure to Bmp4, activin A, bFGF and VEGF. Development 135, 1525–1535.

Pick, M., Azzola, L., Mossman, A., Stanley, E.G., and Elefanty, A.G. (2007). Differentiation of human embryonic stem cells in serum-free medium reveals distinct roles for bone morphogenetic protein 4, vascular endothelial growth factor, stem cell factor, and fibroblast growth factor 2 in hematopoietic differentiation. Stem Cells 25, 2206–2214.

Rafii, S., Kloss, C.C., Butler, J.M., Ginsberg, M., and Klein, G. (1998). Mitogenic and adhesive effects of tenasin-C on human hematopoietic cells are mediated by various functional domains. Matrix Biol. 17, 47–63.

Sekine, M., Kameoka, J., Takahashi, S., Harigae, H., Yanai, N., Obinata, M., and Sasaki, T. (2006). Identification of tenasin-C as a key molecule determining stromal cell-dependent erythropoiesis. Exp. Hematol. 34, 519–527.

Siewe, B.T., Kalis, S.L., Le, P.T., Witte, P.L., Choi, S., Conway, S.J., Druschitz, L., and Knight, K.L. (2011). In vitro requirement for peristin in B lymphopoiesis. Blood 117, 3770–3779.

Slukvin, I.I. (2013). Deciphering the hierarchy of angiohematopoietic progenitors from human pluripotent stem cells. Cell Cycle 12, 720–727.

Smith, B.W., Rozelle, S.S., Leung, A., Ubellacker, J., Parks, A., Nah, S.K., French, D., Gadue, P., Monti, S., Chui, D.H., et al. (2013). The aryl hydrocarbon receptor directs hematopoietic progenitor cell expansion and differentiation. Blood 122, 376–385.

Soini, Y., Kamel, D., Apaja-Sarkkinen, M., Virtanen, I., and Lehto, V.P. (1993). Tenasin immunoreactivity in normal and pathological bone marrow. J. Clin. Pathol. 46, 218–221.

Stewart, R., Rascón, C.A., Tian, S., Nie, J., Barry, C., Chu, L.F., Ardalani, H., Wagner, R.J., Probasco, M.D., Bolin, J.M., et al. (2013). Comparative RNA-seq analysis in the unsequenced axolotl: the oncogene burst highlights early gene expression in the blastema. PLoS Comput. Biol. 9, e1002936.

Vodyanik, M.A., and Slukvin, I.I. (2007). Hematopoietendothelial differentiation of human embryonic stem cells. Curr. Protoc. Cell Biol. Chapter 23, 6.

Vodyanik, M.A., Bork, J.A., Thomson, J.A., and Slukvin, I.I. (2005). Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lympho-hematopoietic potential. Blood 105, 617–626.

Vodyanik, M.A., Thomson, J.A., and Slukvin, I.I. (2006). Leukosialin (CD43) defines hematopoietic progenitors in human embryonic stem cell differentiation cultures. Blood 108, 2095–2105.

Vodyanik, M.A., Yu, J., Zhang, X., Tian, S., Stewart, R., Thomson, J.A., and Slukvin, I.I. (2010). A mesoderm-derived precursor for mesenchymal stem and endothelial cells. Cell Stem Cell 7, 718–729.

Wang, L., Li, L., Shojaei, F., Levac, K., Cerdan, C., Menendez, P., Martin, T., Rouleau, A., and Bhatia, M. (2004). Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. Immunity 21, 31–41.

Wang, C., Tang, X., Sun, X., Miao, Z., Lv, Y., Yang, Y., Zhang, H., Zhang, P., Liu, Y., Du, L., et al. (2012). TGFβ inhibition enhances the generation of hematopoietic progenitors from human ES cell-derived hematogenic endothelial cells using a stepwise strategy. Cell Res. 22, 194–207.

Zovein, A.C., Hofmann, J.J., Lynch, M., French, W.J., Turlo, K.A., Yang, Y., Becker, M.S., Zanetta, L., Dejana, E., Gasson, J.C., et al. (2008). Fate tracing reveals the endothelial origin of hematopoietic stem cells. Cell Stem Cell 3, 625–636.