Wnt signaling controls the specification of definitive and primitive hematopoiesis from human pluripotent stem cells

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Efforts to derive hematopoietic stem cells (HSCs) from human pluripotent stem cells (hPSCs) are complicated by the fact that embryonic hematopoiesis consists of two programs, primitive and definitive, that differ in developmental potential. As only definitive hematopoiesis generates HSCs, understanding how this program develops is essential for being able to produce this cell population in vitro. Here we show that both hematopoietic programs transition through hemogenic endothelial intermediates and develop from KDR+CD34−CD144− progenitors that are distinguished by CD235a expression. Generation of primitive progenitors (KDR+CD235a−) depends on stage-specific activin-nodal signaling and inhibition of the Wnt–β-catenin pathway, whereas specification of definitive progenitors (KDR+CD235a+) requires Wnt–β-catenin signaling during the same time frame. Together, these findings establish simple selective differentiation strategies for the generation of primitive or definitive hematopoietic progenitors by Wnt–β-catenin manipulation, and in doing so provide access to enriched populations for future studies on hPSC-derived hematopoietic development.

Most evidence suggests that primitive and definitive hematopoiesis in the mouse and human develop from distinct progenitors that are specified at different sites and times in the early embryo (reviewed in ref. 1). Primitive hematopoiesis is thought to derive from a multipotent progenitor called the hemangioblast, which is characterized by co-expression of the receptor tyrosine kinase KDR (also known as FLK1 or VEGFR2) and the primitive streak transcription factor brachyury, and by its ability to generate both vascular and hematopoietic progeny2. This program rapidly transitions through a specialized population of endothelial cells with hemogenic potential, known as hemogenic endothelium3. In contrast, definitive hematopoiesis is best defined by development from hemogenic endothelium that is specified at different sites throughout the embryonic vasculature (reviewed in refs. 1,4).

Studies with mouse and human embryonic stem cells (hESCs) have provided compelling evidence that hematopoietic development in differentiation cultures recapitulates key stages of embryonic hematopoiesis5–8. In both models, the onset of the primitive hematopoietic program is marked by upregulation of the KDR receptor and the development of hemangioblasts7,8, followed by a transient wave of primitive hematopoiesis that gives rise to a limited spectrum of lineages, including primitive erythroid, macrophage and megakaryocyte (reviewed in ref. 9). Definitive hematopoiesis, as measured by T-lymphoid potential, emerges after the establishment of the primitive hematopoietic program and develops from a progenitor population that displays characteristics of hemogenic endothelium10–12. Analyses of the signaling pathways that regulate the development of the two hPSC-derived human hematopoietic programs revealed that they differ in their requirement for activin-nodal signaling at the early stages of differentiation. Whereas specification of primitive hematopoiesis is temporally dependent on the activin-nodal pathway, definitive hematopoietic development is not12. These observations suggest that primitive and definitive hematopoiesis are specified early within hPSC differentiation cultures and raise the possibility that, with appropriate markers, it might be possible to physically separate the progenitors of these two programs during the stage of activin-nodal dependence.

RESULTS

CD235a is expressed on primitive hematopoietic progenitors

To identify surface antigens that define primitive and definitive progenitors, we screened hESC-derived mesoderm populations at different stages on an anti-CD antibody array containing ~370 known antibodies13 (http://www.ocigc.ca/antibody/). From this screen, glycophorin A (CD235a) was the only antigen to show an interesting pattern as it was expressed on a subpopulation of cells within embryoid bodies during early mesoderm differentiation (data not shown). This was an unexpected finding as glycophorin A is considered to be an erythroid-specific antigen. It is, however, in line with the observation that this marker is expressed on hPSC-derived CD34+ cells14, suggesting that it is likely to be present on nonerythroid cells early in human development.

To determine whether CD235a marks cells with hematopoietic potential at this early time, we analyzed its expression and that of KDR on embryoid body populations of different stages generated from H1 hESCs using our previously described step-wise, serum-free
approach (Fig. 1a). Kinetic analyses revealed that CD235a was first expressed at day 3 of differentiation on a subset of KDR+ mesoderm, 24 h after the emergence of the T+ (also known as T brachyury) primitive streak–like population (Fig. 1b–d). At this stage, the mesodermal cells do not express CD34 or CD144, markers found on hemogenic endothelium, hematopoietic or endothelial lineage cells (reviewed in refs. 4,15). Its co-expression with KDR at day 3 suggests that CD235a marks a subpopulation of hematopoietic mesoderm, possibly fated to the primitive lineage. To investigate this possibility, we analyzed the expression of CD235a after manipulation of the activin-nodal signaling pathway, given that it is required for primitive hematopoietic development.16,16. Inhibition of activin-nodal signaling by addition of the small-molecule antagonist SB-431542 (SB) between days 2 and 3 of differentiation prevented the development of the KDR+CD235a+ population (Fig. 1e). In contrast, activation of the pathway by the addition of activin A during the same period of time led to an increase in its size. These changes in response to manipulation of the activin-nodal pathway support the hypothesis that expression of CD235a marks primitive hematopoietic progenitors.

To formally test this hypothesis, we next isolated the KDR+CD235a+ and KDR+CD235a− fractions from day 3 activin-induced embryoid bodies and the KDR+CD235a− fraction from day 3 SB-treated embryoid bodies (Fig. 1f) and assayed them for hemangioblasts as a first measure of primitive hematopoietic potential. The activin A–derived KDR+CD235a+ population contained a tenfold higher frequency of hemangioblasts than the corresponding KDR+CD235a− population (Fig. 1g). No hemangioblasts were detected in the KDR−CD235a− fraction (data not shown) or in the SB-treated KDR+CD235a− fraction (Fig. 1g). These results demonstrate that CD235a is expressed on the hemangioblast and confirms that its expression marks an early stage of primitive hematopoietic development.

To further characterize the potential of the different KDR+ mesoderm populations, we isolated them by fluorescence-activated cell sorting (FACS), aggregated the cells and cultured the aggregates (as in Fig. 1f) for up to 6 d (total of 9 d of culture) to replicate the time frame used in our previous study to identify primitive and definitive hematopoiesis in hPSC-derived populations. The derivative populations were analyzed for cell surface markers, including CD34 to monitor the emergence of hemogenic endothelium or hemogenic endothelial progenitors, CD43 as a measure of primitive hematopoietic potential, and CD43 as an indication of hematopoietic specification. CD43 is a hematopoietic-specific marker that is expressed on progenitors of both the primitive and definitive lineages.12,14,17 All three KDR+ progenitors gave rise to CD34+ cells within 1 d of culture, likely reflecting specification of the hematopoietic and vascular lineages. The KDR+CD235a+ mesoderm generated the largest CD34+ population and the only one that co-expressed CD235a (Fig. 1h; top row). After 6 d of aggregate differentiation, the CD34+ KDR+ CD235a+ population contained a tenfold higher frequency of hemangioblasts (Fig. 1i).
In addition to the CD43+ cells, both the KDR+CD235a+ and KDR+CD235a− progenitors gave rise to a distinct CD34+CD43− population by day 9 of culture (Fig. 1h). This was of interest, as until recently, this cell surface expression pattern was associated only with hPSC-derived progenitors of the definitive hematopoietic program. Given this, we assayed these populations for T-lymphoid and erythroid progenitors to assess their hematopoietic potential (as in Fig. 1f). For the erythroid analyses, CD34+CD43− populations generated following the reaggregation of either day 3 CD235a+ or CD235a− cells were isolated by FACS and co-cultured with OP9-DL1 stromal cells for 7 d, as we have shown that this co-culture step is necessary to promote the development of erythroid progenitors from the embryoid body–derived CD34+CD43− population12. Following co-culture, the cells were harvested and assayed for colony-forming potential in methylcellulose. Notably, both CD34+CD43− populations generated similar numbers of medium- to large-sized, burst-like erythroid colonies (Fig. 2a, b) that were morphologically distinct from the smaller EryP-CFC–derived colonies (Figs. 1i and 2b). Analyses of the embryonic (HBE1) and fetal (HBG1,2) globin gene expression patterns (reviewed in ref. 18) in these erythroid colonies suggested that the CD34+CD43− progenitors they were generated from were different. The burst-like large colonies obtained from the CD34+CD43− population derived from the KDR+CD235a− mesoderm expressed CD43, whereas those generated from either the activin A–induced or SB-treated KDR+CD235a− mesoderm (Fig. 1i). Taken together with the hemangioblast and flow cytometric analyses, these findings demonstrate that the earliest identified progenitor of the human primitive hematopoietic program is marked by the co-expression of KDR and CD235a.

**Figure 2** KDR+CD235a− mesoderm–derived CD34+CD43− cells possess definitive hematopoietic potential, but both CD34+ populations possess hemogenic endothelium-like potential. (a) Large burst-like erythroid colony–forming potential per 10,000 cells from CD34+CD43− populations, derived as in Fig. 1f. CD34+CD43− cells were analyzed for erythroid colony–forming potential after 7 d of co-culture with OP9-DL1. n = 3; mean ± s.e.m. Student’s t-test, P = 0.16. (b) Representative erythroid colony morphology obtained following CD34+CD43− isolation (upper panels), as in a, or of EryP-CFC (lower panel), as in Fig. 1i. Scale bars, 100 µm. (c) qRT-PCR of erythroid colonies for globins HBE and HBG. n = 3; mean ± s.d. ANOVA **P = 0.002. (c, i) Ratio of HBG/HBE expression. n = 3; mean ± s.e.m. ANOVA *P = 0.04. (d) CD11b− and CD14− cell potential of CD34+CD43− populations, derived as in Fig. 1f. CD34+CD43− cells were analyzed for NK cell potential after 21 d OP9-DL4 co-culture. (e) CD34+CD43− cells were analyzed for T cell potential after 21 d CD7− OP9-DL4 co-culture. (f), (j) Flow cytometry analysis of expression of cell surface markers on CD34+CD43− populations, as in Fig. 1f. Blue, KDR+CD235a− derived; red, KDR+CD235a+ derived. (g) qRT-PCR of transcription factors on isolated CD34+CD43− populations, as in Fig. 1f. n = 3; mean ± s.e.m. Student’s t-test, P > 0.05. (h, i) Hemogenic endothelium potential of CD34+CD43− cells. KDR+CD235a− (top) and KDR+CD235a+ (bottom) mesoderm–derived CD34+CD43− cells were isolated and plated onto thin-layer, Matrigel-coated plasticware for 7 d. Adherent endothelium and nonadherent hematopoietic cells were visible (h) and examined by flow cytometry analysis for CD144 and CD45 expression after 7 d (i).
revealed that the cells in the KDR\(^+\)CD235a\(^-\) mesoderm–derived colonies expressed more embryonic than fetal globin and in this regard were similar to the EryP-CFC–derived colonies. Roughly equal levels of \(HBBG1,2\) and \(HBE1\) were detected in the large colonies generated from the KDR\(^+\)CD235a\(^-\) mesoderm–derived CD34\(^+\)CD43\(^-\) progenitors (Fig. 2c, ii). These patterns indicate that the large colonies obtained from the CD34\(^+\)CD43\(^-\) population generated from the KDR\(^+\)CD235a\(^-\) mesoderm contain primitive erythroblasts and develop from a progenitor that arises late in the culture after the emergence of the EryP-CFCs.

As lymphoid potential is a distinguishing feature of definitive hematopoiesis\(^7\), we next analyzed each of the two aggregate-derived CD34\(^+\)CD43\(^-\) populations for T-lymphoid and natural killer (NK) cell potential using the OP9-DL4 co-culture assay\(^12,19\). Both CD34\(^+\) populations efficiently gave rise to a CD56\(^+\)CD11blow population, indicating that both possess NK cell potential (Fig. 2d). In striking contrast, T cell potential was restricted to the KDR\(^+\)CD235a\(^-\) mesoderm–derived CD34\(^+\)CD43\(^-\) population (Fig. 2e). Taken together with the above erythroid analyses, these results provide strong evidence that the KDR\(^+\)CD235a\(^-\) and KDR\(^+\)CD235a\(^+\) mesoderm–derived CD34\(^+\) populations contain progenitors of definitive and primitive hematopoiesis, respectively.

Both programs transition through CD34\(^+\) hemogenic endothelium

Further characterization of the respective day 6 CD34\(^+\)CD43\(^-\) populations revealed that both express the set of surface markers (CD144, KDR and CD117, but not CD45; Fig. 2f) and transcription factors (RUNX1, GATA2, SCL and LMO2; Fig. 2g) commonly used to identify hemogenic endothelium (reviewed in refs. 4, 15). When plated under appropriate conditions in vitro, hemogenic endothelium undergoes an endothelial-to-hematopoietic transition that can be measured by the emergence of round cells that express the pan-hematopoietic marker CD45 (ref. 14). Both CD34\(^+\)CD43\(^-\) populations gave rise to adherent monolayers within 3 d of hemogenic endothelium culture, and by day 7, nonadherent CD45\(^+\) populations were readily detected (Fig. 2h, i). Collectively, these observations demonstrate that both the KDR\(^+\)CD235a\(^-\) and KDR\(^+\)CD235a\(^+\) progenitors transition through a CD34\(^+\)CD43\(^-\) hemogenic endothelium stage that cannot be distinguished based on the markers currently used to identify this population\(^13\).

Wnt-β-catenin signaling regulates specification of either program

Next we investigated the role of specific signaling pathways in regulating primitive and definitive hematopoiesis with the goal of identifying strategies to promote one program or the other. We previously demonstrated that activin-nodal signaling is required for specification of primitive hematopoiesis and that stage-specific inhibition of this pathway generates populations enriched in definitive progenitors\(^12\). However, modulation of this pathway did not negatively affect definitive hematopoietic development, indicating that other factors may regulate this program. In the initial studies, we chose to investigate...
Wnt–β-catenin given its role in the regulation of hematopoiesis in the mouse ESC differentiation model. For these analyses, we focused on the mesoderm specification stage (days 2–3) immediately following T induction (Fig. 1a). Inhibition of the pathway by the addition of the small-molecule IWP2 (ref. 24) led to a twofold increase in the size of the CD235a+ population compared to the DMSO-treated control. In contrast, addition of the GSK-3 inhibitor CHIR99021 (CHIR), a Wnt agonist,25, during the same time frame induced inhibition of the CD235a+ population (Fig. 3a,b). The effect of Wnt–β-catenin signaling on the emergence of the KDR+CD235a+ population was observed with two hESC lines (H1 and HES2) and one human induced pluripotent stem cell (hiPSC) line (MSC-iPS1; Fig. 3b), suggesting that it is a conserved mechanism for human hematopoietic specification. Analyses of hemangioblast potential showed that the IWP2-treated KDR+CD235a+ fraction was enriched for these progenitors, indicating that under these conditions, as in the manipulated cultures, CD235a expression marks the onset of primitive hematopoiesis (Fig. 3c). When isolated and cultured as aggregates, the three different KDR+ progenitors gave rise to CD34+ cells within 24 h of culture (Fig. 3d). As expected, only the IWP2-treated KDR+CD235a+ progenitors generated a CD34+CD235a+ population. At day 6 of aggregate culture, more than 90% of the IWP2-treated KDR+CD235a+ mesoderm–derived population and almost 40% of the corresponding KDR+CD235a+ mesoderm–derived population expressed CD43 (Fig. 3e). Very few CD34+ cells were detected in the culture generated from the CHIR-treated KDR+CD235a+ progenitors (Fig. 3e), suggesting that CHIR treatment, similar to SB treatment (Fig. 1i), inhibited primitive hematopoiesis. Primitive erythroid potential (EryP-CFC) correlated with the proportion of CD43+ cells and was found to be highly enriched in the population generated from the IWP2-derived KDR+CD235a+ progenitors (Fig. 3f), indicating that inhibition of Wnt did not affect primitive hematopoiesis.

Although Wnt–β-catenin inhibition did not alter the balance of primitive hematopoiesis between the KDR+CD235a+ and KDR+CD235a− mesoderm–derived populations, it did affect their definitive hematopoietic potential. As expected, the KDR+CD235a+ mesoderm–derived CD34+CD43+ progenitors lacked T cell potential, as demonstrated by the absence of CD45+ cells in the co-culture (Fig. 3c, bottom row). Surprisingly, when Wnt–β-catenin was inhibited between days 2 and 3, the KDR+CD235a− mesoderm–derived CD34+CD43− population was also devoid of T cell potential, suggesting...
we investigated the earliest stage of hematopoietic commitment in vitro and gained the following insights that now enable us to derive populations consisting of either primitive or definitive progenitors. First, we found that expression of CD235a at day 3 of differentiation marks the KDR+ mesoderm fated to the primitive hematopoietic lineage and distinguishes it from the KDR-CD235a- mesoderm that gives rise to definitive hematopoiesis. Second, we showed that specification of the definitive program depends on Wnt-β-catenin signaling and that stage-specific manipulation of this pathway promotes definitive hematopoiesis while inhibiting primitive hematopoiesis. Third, we demonstrated that both programs transit through a hemogenic endothelial intermediate that undergoes an endothelial-to-hematopoietic transition and gives rise to CD45+ hematopoietic cells.

Together, these findings support a model (Fig. 5) of human hematopoietic development in which the primitive hematopoietic program, defined by the emergence of KDR+CD235a- mesoderm, is specified by the combination of activin-nodal signaling and inhibition of the Wnt-β-catenin pathway, whereas specification of definitive KDR-CD235a- mesoderm is dependent on Wnt-β-catenin signaling. Within 24 h of differentiation, both mesoderm populations give rise to CD43+ cells that may represent the earliest stage of commitment to hemogenic endothelium. Following 6 d of culture (total of 9 d), the KDR+CD235a- mesoderm generates a large CD43+ population that represents the emergence and expansion of primitive hematopoiesis, as demonstrated by the presence of EryP-CFC. Although we identified CD34+CD43- hemogenic endothelium in these reaggregation cultures after 6 d, it was clearly specified much earlier, likely within 24 h with the emergence of the CD34+ population, as EryP-CFC are present by day 6. In this model, primitive hemogenic endothelium would generate hematopoietic progeny over a period of several days, possibly generating different progenitors at different times. Hematopoietic commitment of the CHIR-treated KDR+CD235a- mesoderm did not occur in the 6-day aggregate cultures as indicated by the low number of CD43+ cells and lack of hematopoietic progenitors (Fig. 3e,f). Hematopoietic potential was only revealed after culture of the day 6 CD34+ cells in hemogenic endothelial conditions (Fig. 2h,i) or with Notch ligand expressing stromal cells (OP9-DL1 or OP9-DL4; Fig. 2a,d,e), indicating that specification of these definitive progenitors may be Notch dependent, an interpretation consistent with previous studies showing that definitive hematopoietic development is dependent on Notch signaling (reviewed in ref. 28). Whether or
not earlier-stage CD34+ definitive populations would display similar potential remains to be determined.

Hematopoietic development in various model organisms is typically thought to occur in distinct, successive ‘waves’ (reviewed in ref. 1). The observations that the primitive KDR+CD235a+ mesoderm and the definitive KDR+CD235a− mesoderm are specified at the same time within 72 h of the onset of differentiation are not consistent with an exclusively temporal model of human hematopoietic development. Rather, they are more in line with findings from Runx1-Cre lineage tracing studies in the mouse that demonstrated HSC contribution from yolk sac progenitors specified as early as embryonic stage E7.5, a stage overlapping with the emergence of the primitive hematopoietic program29,30. The ability to separate the primitive and definitive hematopoietic lineages at the KDR+ mesoderm stage of development has enabled us to monitor the progression of these two programs and demonstrate that they both transition through hemogenic endothelium intermediates that can be distinguished only on the basis of T-lymphoid potential, as both generate erythroid, myeloid (data not shown) and NK lineage cells, and both display surface marker phenotypes and gene expression patterns currently used to define hemogenic endothelium. The observation that both KDR+ progenitors generated cells with an NK phenotype provides evidence for the existence of distinct primitive and definitive NK cells, a concept supported by previous studies31–32. The erythroid colonies generated from the two populations expressed different ratios of HBE1 and HBG1,2 globin, suggesting that the colony-forming cells they derived from represented different stages of human erythroid development. Although the expression pattern of the KDR+CD235a+ mesoderm–derived colonies is indicative of primitive erythroblasts, the pattern of the colonies generated from the KDR+CD235a− mesoderm is not consistent with the next stage of erythroid development, the fetal stage, as they retain high levels of HBE1. Although unexpected, this pattern may be indicative of a transition stage in human embryonic and fetal erythropoiesis as colonies generated from human 6-week-old fetal liver also co-express HBE1 and HBG1,2 globin33. The lack of markers to distinguish the primitive and definitive CD34+CD43− populations demonstrates that the identification of a hemogenic endothelium population without documentation of T-lymphoid potential can no longer be used as an indication of definitive hematopoiesis. It also raises the distinct possibility that hemogenic endothelium populations isolated from hPSC-differentiation cultures on the basis of existing markers such as CD34, CD144 and CD45 are mixtures of primitive and definitive hematopoietic cells.

The findings that Wnt–β-catenin signaling inhibits primitive hematopoiesis are consistent with recent observations that activation of the pathway at early stages in hPSC differentiation cultures led to a reduction in hemangioblast potential and inhibition of primitive erythroid potential, respectively34,35. This effect of Wnt–β-catenin signaling in the human system appears to be opposite to that described in the mouse ESC model, in which activation of the pathway during early mesoderm specification is required for establishment of the primitive program32. However, these differences may reflect subtle differences in the stage at which the populations were analyzed, as Liu et al.36 recently demonstrated that transient Wnt inhibition via Er71 expression is required for specification of murine primitive hemangiogenic mesoderm at a stage comparable to that evaluated in our study in the human cultures. In addition to a role at the early mesoderm specification stage, several recent studies have demonstrated that Wnt signaling functions at the level of HSC development in the aorta-gonad-mesonephros region of the developing mouse embryo. We found that inhibition of the Wnt–β-catenin pathway by retinoic acid signaling was necessary for HSC development from hemogenic endothelium37, whereas the study of Ruiz-Herguido et al.38 provided evidence that Wnt signaling was required at a stage before HSC specification, possibly at the level of hemogenic endothelium development. Together, these observations establish a role for Wnt signaling at multiple stages of definitive hematopoietic development, ranging from induction of the program to specification and/or maturation of the HSC.

Endogenous levels of Wnt–β-catenin signaling in some hPSC lines such as H1 hESCs appear to be sufficient for specification of the definitive hematopoietic program, whereas in others they may be too low to establish this fate. Given the variability of endogenous Wnt signaling across different hPSC lines39, these findings highlight the importance of establishing appropriate levels of Wnt–β-catenin signaling during the mesoderm specification step for generating the definitive hematopoietic program. Through stage-specific manipulation of the Wnt–β-catenin pathway, it is now possible to routinely generate hPSC-derived populations containing definitive but not primitive hematopoietic progenitors. Attempts to repopulate recipient animals with enriched hPSC-derived CD34+ definitive progenitors have not yet been successful, suggesting that these cells may represent pre-HSCs that require additional maturation signals to engraft an animal. Progenitors with T cell potential have been identified in the mouse embryo at stages before the emergence of HSCs40,41. Although the origin of these T-lymphoid cells has not yet been established, it is possible they derive from progenitors comparable to the hPSC-derived CD34+ hemogenic endothelium and similarly require additional signals to mature. hPSC-derived definitive hematopoietic progenitors, generated through the strategy described in this study, represent ideal targets for defining the pathways that regulate the generation of HSCs from the CD34+ hemogenic endothelium stage cells.

METHODS

Methods and any associated references are available in the online version of the paper.

ACKNOWLEDGMENTS

We would like to thank the SickKids–UHN Flow Cytometry Facility for their expert assistance with cell sorting, in particular A. Khandani. This work was supported by National Institutes of Health grants U01 HL100395 and CIHR MOP93569, HOP83070 and MOP12927. C.M.S. and A.D. were supported by the McMurrich Post-Doctoral Fellowship and the Magna-Golftown Post-Doctoral Fellowship, respectively.

AUTHOR CONTRIBUTIONS

C.M.S., A.D., G.A., M.K. and G.K. all participated in the design of the experiments. C.M.S., A.D., G.A. and M.K. performed the experiments. C.M.S. and G.K. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Medwinsky, A., Rybtsov, S. & Toudou, S. Embryonic origin of the adult hematopoietic system: advances and questions. Development 138, 1017–1031 (2011).
2. Huber, T.L., Kouskoff, V., Fehling, H.J., Palis, J. & Keller, G. Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. Nature 432, 625–630 (2004).
3. Lancrin, C. et al. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. Nature 457, 892–895 (2009).
4. Antas, V.I., Al-Drees, M.A., Prudence, A.J., Sugiyama, D. & Fraser, S.T. Hemogenic endothelium: a vessel for blood production. Int. J. Biochem. Cell Biol. 45, 692–695 (2013).
ONLINE METHODS

Maintenance and differentiation of hESCs and hiPSCs. The hESC lines H1 (ref. 42) and HEK2, and hiPSC line MSc-iPS1 (ref. 27) were maintained on irradiated mouse embryonic fibroblasts in hESC media, as described previously 8. For differentiation, hiPSCs were cultured on Matrigel-coated plasticware (BD Biosciences, Bedford, MA) for 24 h, followed by embryoid body generation, as described previously 12. Briefly, hiPSCs dissociated with sequential collagenase B (1 mg/ml) and trypsin-EDTA (0.05%) treatment, followed by scraping. Aggregates were resuspended in SFD 43 supplemented with t-glutamine (2 mM), ascorbic acid (1 mM), monothioglycerol (MTG, 2 mM), Sigma), transferrin (150 µg/ml), and BMP-4 (10 ng/ml), bFGF (5 ng/ml), activin A, SB-431542 (6 µM), CHIR99021 (3 µM), and/or IWP2 (3 µM). On the third day of differentiation, embryoid bodies were cultured to StemPro-34 media supplemented as above, and VEGF (15 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), OP9-DL1 co-culture for burst-like erythroid colonies–like analysis. Isolated CD34+CD43− cells were added to individual wells of a 6-well plate containing OP9-DL4 cells, and cultured with: T cells: rhFlt-3L (5 ng/ml) and rhIL-7 (5 ng/ml); NK cells: rhFlt-3L (5 ng/ml) and rhIL-15 (10 ng/ml) in 24-well plates for 7 d. Cells were harvested and plated in 1% methylcellulose as described below.

Hemangioblast colony assay. Analysis of hemangioblast colony forming potential was performed by plating 1 × 10 4 cells in 1% methylcellulose supplemented with 10% FCS (Atlas), murine GM-CSF (25 ng/ml), IL-7 (5 ng/ml), EPO (2 U/ml), IL-6 (5 ng/ml), IL-3 (40 ng/ml), Flt3L (10 ng/ml), and rhSCF (100 ng/ml) as described in detail previously 9. Colonies were quantified after 6 d of culture.

Hematopoietic colony assay. Analysis of hematopoietic colony potential was performed by plating 2 × 10 4 cells in 1% methylcellulose supplemented with 10% plasma-derived serum (Animal Technologies), 5% protein-free hybridoma media (Invitrogen), t-glutamine (2 mM), SCF (100 ng/ml), EPO (2 U/ml), IL-6 (5 ng/ml), IL-3 (40 ng/ml), TPO (40 ng/ml), IL-11 (5 ng/ml), IGF-1 (25 ng/ml) and GM-CSF (1 ng/ml), as described in detail previously 9. Colonies were quantified after 10–14 d.

OP9-DL4 co-culture for NK/T-lineage differentiation. OP9 cells expressing Delta-like 4 (OP9-DL4) were generated and described previously 19, 44. 5–20 × 10 4 isolated CD34 CD43− cells were added to individual wells of a 6-well plate containing OP9-DL4 cells, and cultured with: T cells: rhFlt-3L (5 ng/ml) and rhIL-7 (5 ng/ml); NK cells: rhFlt-3L (5 ng/ml) and rhIL-15 (10 ng/ml) (Peprotech, Rocky Hill, NJ). rhSCF (100 ng/ml) was added to both cultures for the first 6 d. Every 5 d co-cultures were transferred onto fresh OP9-DL4 cells by vigorous pipetting and passing through a 40-µm cell strainer. Cells were analyzed by flow cytometry on the days indicated. Populations were gated on CD34+ events.

Flow cytometry and cell sorting. The following antibodies were used for these studies: KDR (clone 89106) (15:100 dilution), CD235α-APC (clone HIR2) (1:100 dilution), CD34-PE-Cy7 (clone RPA-T4) (1:50 dilution), CD35-PECy7 (clone L17F12) (1:20 dilution), CD7-FITC (clone M-T701) (15:100 dilution), CD34-APC (clone 8G12) (1:100 dilution), CD34-PE-CY7 (clone 4H11) (1:100 dilution), CD43-PE (1:20 dilution), CD4-PE-Cy7 (clone RPA-T4) (1:50 dilution), CD56-PE or APC (clone B159) (1:20 dilution), CD117-APC or FITC (15:100 dilution), CD8-PE (clone RPA-T8) (1:10 dilution), CD34-APC (clone 8G12) (1:100 dilution), CD34-PE-CY7 (clone 4H11) (1:100 dilution), CD43-PE (1:20 dilution) or FITC (15:100 dilution) (clone 1G10), CD45-APC-eFluor750 (clone 2D1) (1:20 dilution), CD56-PE or APC (clone B159) (1:20 dilution), CD117-APC (clone 104D2) (1:20 dilution), CD144-PE (clone 123413) (1:20 dilution). All antibodies were purchased from BD Biosciences (San Diego, CA) except CD34-PE-CY7 which was purchased from eBioscience (San Diego, CA), and KDR was purchased from R&D Systems. Cells were sorted with FACSAriaII (BD) cell sorter at the Sick Kids/UHN Flow Cytometry Facility.

Hemogenic endothelium analysis. 2 × 10 4 CD34+CD43− cells were cultured for 7 d on thin-layer Matrigel-coated plasticware in StemPro-34 media supplemented as described above, with an additional 10 ng/ml BMP4.

OP9-DL1 co-culture for burst-like erythroid colonies–like analysis. Isolated CD34+CD43− 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 d. Cells were harvested and plated in 1% methylcellulose as described below.

410–417 (2004).
42. Thomson, J.A. et al. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145–1147 (1998).
43. Sturgeon, C.M. et al. Primitive erythropoiesis is regulated by mitR-126 via nonhematopoietic Vcam-1+ cells. Dev. Cell 23, 45–57 (2012).
44. Schmitt, T.M. et al. Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. Nat. Immunol. 5, 410–417 (2004).